DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH

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RECOMBINANT DNA ADVISORY COMMITTEE

MINUTES OF MEETING

June 3, 1988

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DEPARTMENT OF HEALTH AND HUMAN SERVICES
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RECOMBINANT DNA ADVISORY COMMITTEE

MINUTES OF MEETING[1]

June 3 1988

The Recombinant DNA Advisory Committee (RAC) was convened for its thirty-eighth meeting at 9:00 a.m. on June 3, 1988, in Building 31C, Conference Room 6, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892. Dr. Gerard J. McGarrity (Chair), presided. In accordance with Public Law 92-463, the meeting was open to the public. The following were present for all or part of the meeting:

Committee members:

Ronald M. Atlas
Michael F. Brewer
Donald C. Carner
James F. Childress
Mitchell L. Cohen
Bernard D. Davis
Robert P. Erickson
Martin F. Gellert
Irving S. Johnson
Edward L. Korwek
Brian F. Mannix
Robert D. McCreery
Gerard J. McGarrity
R. Scott McIvor
Robert F. Murray
Paul E. Neiman
Joseph S. Pagano
David Pramer
Monica Riley
Jeffrey W. Roberts
Anne K. Vidaver
William J. Gartland, Jr. (Executive Secretary)

A committee roster is attached (Attachment).

Ad hoc consultants:

Susan Gottesman, National Institutes of Health
Robert Lanman, National Institutes of Health
Robert McKinney, National Institutes of Health

Liaison representative:

Daniel P. Jones, National Endowment for the Humanities

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[1]The RAC is advisory to the National Institutes of Health (NIH), and its recommendations should not be considered as final or accepted. The Office of Recombinant DNA Activities should be consulted for NIH policy on specific issues.

Non-voting agency representatives:

Joel M. Dalrymple, Department of Defense
George D. Duda, Department of Energy
Bernard Greifer, Department of Commerce
Morris A. Levin, Environmental Protection Agency
Henry Miller, Food and Drug Administration
George P. Shibley, Department of Agriculture
Sue A. Tolin, Department of Agriculture

National Institutes of Health staff:

Stanley Barban, NIAID
Irving Delappe, NIAID
Rosalind Gray, OD
John Irwin, OD
Becky Lawson, NIAID
Suzanne Varimbi, OD

Others:

Elizabeth Anderson, Environmental Protection Agency
Jarl Anderson, Nova Laboratories, Inc.
Brian Becker, Blue Sheet
Carter Blakey, Federation of American Societies for Experimental Biology
Arindam Bose, Pfizer, Inc.
Kim Brell, Hill and Knowlton
Theodore J. DeLoggio, Pennwalt Corporation
Charles J. Eby, Hill and Knowlton
Carol Ezzell, Nature
Peter Farnham, American Society for Biochemistry and Molecular Biology
Malcolm Finkelman, Genex Corporation
Mark A. Foglesong, Eli Lilly and Company
Joseph R. Fordham, Nova Laboratories, Inc.
Jeffrey L. Fox
David E. Giamporcaro, Nash, Railsback, & Plesser
Richard H. Glover, Food and Drug Administration
Robert Goldberg, Merck Research Laboratories
Alan R. Goldhammer, Industrial Biotechnology Association
Ann Ruth Herkes, German Embassy
Michael Hyer, StenoTech, Inc.
John E. Jangstetter, Genentech, Inc.
Bent F. Jensen, Nova Industri A/S
Dorothy Jessup, Department of Agriculture
Chris Joyce, New Scientist Magazine
Peggy R. Kearney, Biometric Research Institute, Inc.
A. S. Lubiniecki, Genentech, Inc.
Jack J. Manis, Upjohn Company
William L. Muth, Eli Lilly and Company

Michael Olexa, Department of Agriculture
John H. Payne, Department of Agriculture
H. Graham Purchase, Department of Agriculture
Stephen Queener, Eli Lilly and Company
Rex Rhein, McGraw-Hill World News
Jeremy Rifkin, Foundation on Economic Trends
Noel Riley, Mackler, Cooper, and Gibbs
Keith Schneider, New York Times
Mark Segal, Environmental Protection Agency
Philip Shapiro, Integrated Genetics
Alan Shipp, Association of American Medical Colleges
Janet Shoemaker, American Society for Microbiology
Wes Skidmore, Food and Drug Administration
Clarence E. Styron, Monsanto Company
William Szkrybalo, Pharmaceutical Manufacturers Association
Joseph Van Houten, Schering-Plough Corporation
William J. Walsh, III, Currents International, Inc.
Rick Weiss, Science News

I. CALL TO ORDER AND INTRODUCTORY REMARKS.

Dr. McGarrity, Chair, called the meeting of the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health (NIH) to order at 9:00 a.m., June 3, 1988. He said the meeting was called pursuant to a Federal Register notice which, being 30 or more days prior to today's date, met requirements of the NIH Guidelines for Research Involving Recombinant DNA Molecules. He stated that the meeting would remain open to the public for its entirety, and that he expected the meeting to conclude within one day.

Dr. McGarrity asked Dr. Gartland if a quorum was present and Dr. Gartland assured the Chair that a quorum was in attendance.

Dr. McGarrity noted that he intended to make every effort to abide by the distributed agenda with respect to time estimates for each item of business. He reminded the committee that in recognizing persons for comments he would use the following order: primary and secondary reviewers on each item as set forth in the agenda; other members of RAC; ad hoc consultants to the RAC; NIH staff members; members of the public who had submitted written comments; and finally, other members of the public. He underlined that RAC was advisory to the Director of NIH; and in light of this, persons with minority opinions should voice them so as to provide Dr. Wyngaarden with the entire spectrum of opinions on a given topic. Dr. McGarrity then told the committee that in all voting he would call first for the affirmative, then for the negative, and finally for abstentions. He underlined that if any voting member felt compelled to abstain due to conflict of interest, that such member should notify the Chair so that the record could duly reflect such.

Dr. McGarrity then made note of Mailings I and II which were sent to members prior to the meeting. He also noted that some recently received materials were supplied at the table for each member. Further, he said because of a scheduling conflict Item Number IX would be taken out of order and discussed immediately following the luncheon recess.

Dr. McGarrity welcomed the following new members present at their first meeting: Dr. Ronald Atlas, Mr. Michael Brewer, Dr. James Childress, Dr. Martin Gellert, Mr. Brian Mannix, Mr. Robert McCreery, Dr. Scott McIvor, Dr. Richard Mulligan, Dr. Robert Murray and Dr. Monica Riley.

As Dr. McGarrity was technically a new member, he introduced himself to the committee and thanked his predecessors, in particular Ray Thornton, Kenneth Burns, and Robert Mitchell for their advice since his recent appointment as Chair. He said he was honored and privileged to serve as Chairman of such a distinguished group. He said he had served as both a member and

ad hoc consultant in the past and he underlined the "blending" brought about by Dr. Fredrickson's decision to appoint lay members to the committee, which has added broad-based viewpoints and perspectives to the workings of the committee and added greatly to the success of the group.

Dr. McGarrity then introduced Dr. Susan Gottesman, Mr. Robert Lanman, and Dr. Robert McKinney, who were the ad hoc consultants to the committee for this meeting.

Dr. McGarrity called upon Mr. Carner to present the minutes of the meeting of September 21, 1987.

II. APPROVAL OF THE MINUTES OF THE FEBRUARY 2, 1987, MEETING.

Mr. Carner said he had no additions or corrections to the minutes. However, Dr. Johnson said he felt the wording on page 16 of the minutes should be clarified to meet the intention of the comment which was that the NIH counsel be a part of the working group, rather than any request by Dr. Johnson that he himself be on the working group.

Dr. Korwek said portions of the minutes on page 9 needed to be reworded concerning the NIH Guidelines and the National Environmental Policy Act (NEPA) and that he would speak with staff about such changes to simply clarify his intent.

There being no other additions, deletions or alterations, Dr. McGarrity called for approval of the minutes by voice vote which was unanimous.

Dr. McGarrity called on Dr. Cohen as primary reviewer to discuss Item Number III, the proposed amendments of the NIH Guidelines regarding transgenic animals.

III. PROPOSED AMENDMENTS OF NIH GUIDELINES REGARDING TRANSGENIC ANIMALS (tabs 1310/IV, 1321, and 1322).

Dr. Cohen said the Working Group on Transgenic Animals had met on March 28, 1988, and discussed two basic issues:

1. whether the NIH Guidelines for Research Involving Recombinant DNA Molecules should be revised regarding coverage of transgenic animals; and,

2. containment guidelines for experiments involving the introduction of recombinant DNA into the genome of animals, particularly introduction of DNA from human pathogens.

Dr. Cohen said the working group defined "transgenic" as an animal in which the germ line had been altered by stable introduction of DNA. He said the working group had been cognizant that such experiments could be conducted using either recombinant or non-recombinant DNA and that the NIH Guidelines should cover certain types of recombinant transgenic animal experiments. However, the consensus of the group was that individual sections of the NIH Guidelines be modified regarding transgenics, rather than to attempt to modify the definition of "recombinant DNA." Such changes were presented in tab 1321, items A through F. Within these suggested amendments to the NIH Guidelines were cautionary statements that proper containment must be adhered to when utilizing transgenic animals in which increased transmission of a recombinant pathogen or production of undesirable traits in a host animal could occur.

Dr. Gottesman said there were complications in that the Recombinant DNA Advisory Committee (RAC) had recommended to the Director, NIH, that certain changes be made to the NIH Guidelines at the RAC meeting of September 21, 1987, and in fact, some of the working group recommendations are to amend the same sections awaiting the Director's action.

Dr. Gottesman noted the NIH IBC had initiated thinking on broadening coverage of transgenic animals by requesting guidance from the Office of Recombinant DNA Activities (ORDA). The IBC had understood that if a transgenic animal is made using recombinant DNA technology that it is covered by the NIH Guidelines because the molecule which is the source of the new DNA is recombinant. However, they sought an answer to a case in which a gene is cloned and cut away from its vector and then the gene, apart from the vector, is introduced in the germ line to make a transgenic animal. The question is whether such experiments should fall under the purview of the NIH Guidelines.

Dr. Gottesman said the human gene therapy situation is the model on which the working group chose to base its recommendations. The NIH Guidelines state that recombinant DNA or DNA or RNA derived therefrom, if used in human gene therapy, will be covered. However, this approach would not cover experiments in which transgenic animals were made without ever going through a recombinant DNA stage. Dr. Gottesman said she felt there was no way to close all loopholes without changing definitions within the NIH Guidelines dramatically and that the working group had made the clear decision they did not wish to change the basic definition of recombinant DNA which has been in use for over ten years.

Dr. McGarrity said he felt it was an important point that the Director of NIH had not acted on previous relevant amendments to the NIH Guidelines and that it complicated this discussion.

Dr. Gottesman asked Dr. Gartland for an update on the situation of the amendments from the September 21, 1987, meeting.

Dr. Gartland said tab 1322 was basically the proposed revisions as they emerged from the September 21, 1987, meeting. On advice of legal counsel an environmental assessment is being prepared with the assistance of the Department of Agriculture and this is the reason the Director has not taken final action on this issue.

Dr. Neiman said he thought the issues raised by the NIH IBC were appropriate and the proposed amendments cover the loopholes that exist, but he felt Dr. Gottesman's point was also valid that future changes may be necessary as technology progresses and direct introduction of DNA may become possible. However, the issue is speculative and not a practical issue at this point in time. He fully supported the limited changes to the NIH Guidelines proposed.

Dr. Johnson noted he was not a member of the working group but he felt there was a certain amount of ambiguity and complexity of language in tabs 1310, 1321, and 1322 which seemed to bring into question whether natural mammalian reproduction would be brought under scrutiny in that breeding colonies of animals which sexually transmit the genome of interest is a basic facet of transgenic animal work. He said the language may be ambiguous enough to impede such work.

Dr. Korwek said he was also confused and asked for a definition of the term "transgenic animal." Dr. Gottesman said that a specific definition had not been developed by the group. Dr. Korwek asked if rearrangements would be considered "transgenics," to which Dr. Johnson replied that crossovers and rearrangements occur naturally. Dr. Gottesman said the phrase "stable introduction of DNA into the germ line," defines "transgenic" but the word "introduction" may be the key to determine if something should fall under the NIH Guidelines.

Dr. Cohen reiterated the decision of the working group to not change the definition of "recombinant DNA" in the NIH Guidelines while at the same time having concern over containment of animals in which human pathogens have been inserted into the germ line. He said the focus was on alerting IBCs and researchers that such experiments may need special containment.

Dr. Korwek said he had no problem with such a goal, but he expressed concern about the implementation of the language and whether it imposes a restriction that otherwise would not be there. He said he would like rearrangements to be specifically discussed in terms of being categorically included or excluded.

Dr. Gottesman asked for opinions of persons more involved in the field as to whether rearrangement could take place using

recombinant DNA but without the recombinant DNA being present in the final transgenic animal. Dr. Mulligan cited a scenario of a deletion introduced into a mouse in which recombinant DNA as defined by the NIH Guidelines would not be present in the final construct.

Dr. Gottesman replied by saying there had been an amendment that the RAC had contemplated some time ago which would have exempted deletions from the NIH Guidelines. Dr. Gartland said the amendment referred to by Dr. Gottesman was also currently awaiting environmental assessment but said that deletions, in the context of contained research, were exempt.

Dr. Erickson said the discussion of deletions was not important in regard to transgenic animals because of the importance of having a DNA marker in such animals for research purposes. However, he felt Dr. Johnson's comment was an issue which was of importance and he felt the working group meant the wording to address sexual transmission of the modified genome outside the laboratory.

Dr. Gellert said there were ways he could think of whereby rearrangements into the germ line of an animal would not contain recombinant DNA. He noted it is possible to turn on the immunoglobulin recombination system with a transient plasmid, then have the plasmid remove itself from the germ line, producing an animal with rearranged immunoglobulin genes.

Mr. Mannix asked if the word "foreign" should not be added to "DNA" to make it clear that deletions, rearrangements and duplicate gene copies were not intended to be covered. Dr. Gottesman said she felt the word "foreign" itself would have to be defined and would only make the situation more complex.

Dr. Gellert said current technology in introducing viral genomes in transgenic animals was to clone a retroviral genome, cut it back out of the plasmid and then introduce it into the germ line of animals. He could envision a similar experiment being done by cultivating a retrovirus in a suitable cell line, letting the RNA become reverse transcribed into double-stranded DNA in the viral cores, reisolating the material and using that to generate a transgenic animal. Thus, none of these steps would qualify as "recombinant DNA," but the same product would result as from the recombinant method.

Dr. Neiman replied the working group was aware of just such an experiment and discussed it at some length. He was not sure the experiment could be done at present because of the low efficiency of introduction of DNA and the numbers of transgenics successfully produced would be small in terms of the number of injections. The result of such viral infections are populations of DNA of unknown content and Dr. Neiman didn't feel many

scientists would be willing to start an experiment in which most of the successful insertions would be non-functional. In light of this, the working group felt the only way to cover such an experiment would be by redefining "recombinant DNA."

Dr. Gellert said he could also envision an experiment in which viral cores could be inserted directly into an animal embryo.

Dr. Neiman said such experimentation has not yet been accomplished but conceded it was not unlikely to happen in the future. He said it may be premature to attempt to develop guidelines for a technology which is as yet undeveloped.

Dr. Riley said she viewed the term "recombinant DNA" in two ways: one, in which recombinant DNA is introduced into the germ line of an animal, and another in which a non-recombinant DNA molecule, foreign to the animal's genome, is introduced in the animal, thus producing a recombinant sequence stably integrated into the germ line. She thought this was not a burning issue at present.

Dr. Davis replied such a definition would make the terms "recombinant" and "transgenic" synonymous. Dr. Atlas added he felt the working group was avoiding coming to grips with the definition of "transgenic" and said that there would be ambiguity without a clear definition of what is to be covered by the NIH Guidelines.

Dr. Gottesman said the NIH Guidelines only dealt with transgenic animals in the context of recombinant DNA and that as such they would not cover transgenics made without the use of recombinant DNA. She added that if members of the RAC felt all transgenic animals should be included that perhaps a redefinition of "transgenic" or "recombinant DNA" may be in order and that this is a complex task and may broaden the entire scope of the NIH Guidelines.

Dr. Johnson said he still felt that natural mammalian reproduction and hybridization experiments could be construed to fall under the NIH Guidelines by the current definitions and he suggested the word "foreign" be inserted before "DNA" to clarify this issue.

Dr. Neiman said that in a mammalian cell or higher animal cell it could be possible to insert non-foreign DNA but in a totally novel context, and that perhaps "foreign" is not a solution to the problem of defining "transgenic."

Dr. Cohen said he felt the decision should be made as to whether it is appropriate for the NIH Guidelines to be extended to cover all transgenic animals. This might require redefining "recombinant DNA" or renaming the NIH Guidelines.

Dr. Gottesman suggested that the proposed introductory paragraph to Section III-B-4 of the NIH Guidelines be amended to read:

> "This section covers experiments involving whole animals, both those in which the animal's genome has been altered by stable introduction of recombinant DNA or RNA, or DNA or RNA derived therefrom, into the germ line (transgenic animals) and experiments involving viable recombinant-DNA-modified microorganisms tested on whole animals. For the latter, other than viruses which are only vertically transmitted, the experiments may not be carried out at BL1-N containment; a minimum containment of BL1 or BL2-N is required."

Dr. Davis said he was reluctant to introduce the word "recombinant" because historically the RAC has tried to emphasize the principle that what is of concern is the nature of the organism and not the technique by which it was formed and he said this suggested wording added emphasis to the concept that process was more important than product.

Dr. Childress said, as a newcomer, he wanted to know what the background of the issue was and whether it was going to be addressed in any other forum. Dr. Gartland reiterated the history of the matter. He said the NIH Biosafety Committee had proposed that all transgenics should be covered by the NIH Guidelines, but they did not define "transgenic." A working group had been set up by the RAC to look into the matter and tab 1321 is the report of that working group. He said that no other group, at this point, is constituted to look at this issue. Dr. Gartland said the committee had two options: one, to restrict the proposed revision of the NIH Guidelines to covering "recombinant DNA" and advise the Director of NIH that there may be a broader problem; or two, take the position that most of the concerns will be resolved by this limited modification of the NIH Guidelines.

Dr. Gottesman suggested she go through the working group recommendations one at a time to indicate where they fit into the NIH Guidelines.

Recommendation A refers to the incorporation of the following language in Section II of the NIH Guidelines:

> "Biosafety Level 1 for animals (BL1-N) describes containment which is used for animals in which the germ line has been modified through recombinant DNA techniques (transgenic animals) and is designed to eliminate the possibility of sexual transmission of the modified genome or transmission of recombinant-DNA-derived viruses known to be transmitted only vertically (i.e., transmitted from animal parent to offspring only

by sexual reproduction). Procedures, practices, and facilities follow classical methods of avoiding genetic exchange between animals."

The working group recommended that "(transgenic animals)" be deleted from this paragraph, but to define "transgenic animal" later as "the stable introduction of DNA into the germ line."

In recommendation B, the working group recommended adding the following new sentence at the beginning of Section III-B-3, before the paragraph entitled "Caution":

> "Experiments involving the introduction of eukaryotic viral genomes into the germ line of animals are covered in Section III-B-4."

Dr. Gottesman said this was introductory information designed to aid IBCs in reviewing experimental protocols in which there is possibility of transmission.

In recommendation C, the working group recommended changing the title of Section III-B-4 from "Recombinant DNA Experiments Involving Whole Animals," to "Experiments Involving Whole Animals, Including Transgenic Animals." Dr. Gottesman said this was merely a change in title to aid IBCs in finding information and did not affect the substance of the NIH Guidelines.

In recommendation D, the working group recommended the following wording be incorporated into Section III-B-4 of the NIH Guidelines:

> "This section covers experiments involving whole animals, both those in which the animal's genome has been altered by stable introduction of DNA into the germ line (transgenic animals) and experiments involving viable recombinant-DNA-modified microorganisms tested on whole animals. For the latter, other than viruses which are only vertically transmitted, the experiments may not be carried out at BL1-N containment; a minimum containment of BL1 or BL2-N is required.
>
> "<u>Caution:</u> Special care should be used in the evaluation of containment conditions for some experiments with transgenic animals. For example, such experiments might lead to the creation of novel mechanisms or increased transmission of a recombinant pathogen or production of undesirable traits in the host animal. In such cases, serious consideration should be given to increasing the containment conditions."

Dr. Gottesman said this is the change which has been the subject of much discussion today and that suggestions have included altering the first sentence by some means to suggest that the introduction of DNA is performed by artificial means rather than natural breeding techniques. She added the cautionary paragraph is providing further guidance to IBCs and investigators.

In recommendation E, the working group recommended a modification to Section III-B-4-a of the NIH Guidelines, to read as follows:

> "Recombinant DNA, or DNA or RNA molecules derived therefrom, from any source except for a eukaryotic viral genome may be transferred to any non-human vertebrate or any invertebrate organism and propagated under conditions of physical containment comparable to BL1 or BL1-N and appropriate to the organism under study [2]. Animals containing sequences from viral vectors, if the sequences do not lead to transmissible infection either directly or indirectly as a result of complementation or recombination in animals, may be propagated under conditions of physical containment comparable to BL1 or BL1-N and appropriate to the organism under study. Experiments involving the introduction of other sequences from eukaryotic viral genomes into animals are covered under III-B-4-b. For experiments involving recombinant DNA modified Class 2, 3, 4, or 5 organisms (1) using whole animals, see Section III-B-1."

Dr. Gottesman said this sets containment for a whole class of experiments and supersedes a change made last September to this same section which was an attempt to avoid the use of the phrase "greater than two-thirds of the eukaryotic viral genome."

In recommendation F, the working group recommended Section III-B-4-b of the NIH Guidelines be modified to read as follows:

> "For experiments involving recombinant DNA, or DNA or RNA derived therefrom, involving whole animals, including transgenic animals, and not covered by Section III-B-1 or Section III-B-4-a, the appropriate containment will be determined by the IBC (22)."

Dr. Gottesman said this closed a loophole whereby an investigator could cut the DNA out of the vector and argue the experiment did not involve recombinant DNA.

Dr. Davis said he would accept that there is no other body in a better position than the RAC to offer guidelines for work on transgenic animals, that work is currently taking place on transgenic animals and guidelines are necessary. He suggested

two words "in solution" be added in the first sentence of Recommendation D:

> "This section covers experiments involving whole animals, both those in which the animal's genome has been altered by stable introduction of DNA **in solution** into the germ line (transgenic animals) and experiments involving viable recombinant-DNA-modified microorganisms tested on whole animals.

Dr. McGarrity noted there was no motion on the table and asked whether the committee felt that final details of language could be worked out during the recess or whether, if closure were at hand, the issue could be dealt with before the recess.

Dr. Neiman moved that the modifications to the NIH Guidelines, as stated by Dr. Gottesman be adopted. Dr. Erickson seconded the motion.

Dr. Gartland asked for clarification of the wording of the first sentence of Recommendation D. Dr. Gottesman suggested the wording as presented on page 365, having been already moved for adoption, be discussed either before or after the recess and an amendment proffered. She reiterated the points previously made regarding changing the definition of "recombinant DNA" versus covering transgenic animals in a broader sense.

Dr. McGarrity said the recess was in order and the committee adjourned for a brief recess.

Dr. McGarrity called the committee back to order at 10:53 a.m. to continue the morning's discussion. He said there were two ways to proceed: (1) change the definition of "recombinant DNA" to make it more encompassing or inclusive of transgenic animals which would probably require Federal Register publication for public comment; or (2) adopting the recommendations of the working group with some modifications with the understanding that further changes could be made as the technology moves forward in the coming years. He noted Dr. Wyngaarden had expressed an interest that some body should be looking at the issue of transgenic animals and, as Dr. Davis said earlier, the RAC at present is the most likely place for such discussions to take place.

Dr. McGarrity called for a "sense of the committee" as to whether it is comfortable with the motion currently before it or if it would be inclined to technically overstep the bounds of the NIH Guidelines, possibly with the support of the Director, to say there may be some areas that it or IBCs should look at that technically do not come under the current definition of "recombinant DNA." The committee voted by show of hands to continue its discussion of the current motion before it regarding the recommendations of the Working Group on Transgenic Animals.

Dr. Korwek said he still had a problem with expansion of the applicability of the NIH Guidelines beyond recombinant DNA but because of this being a new area he felt it needed further discussion.

Dr. Gottesman suggested a sense of the committee be taken on the issue of pursuing a change in the definition of "recombinant DNA," or the title of the NIH Guidelines. Dr. Korwek said he preferred to leave the definition alone because it is used throughout the NIH Guidelines and further problems could be created by any change in definition. Further, he said he was not in favor of the language as currently exists in Recommendation D because it covers non-recombinant DNA transgenics.

Dr. Atlas moved that Recommendation D be amended to include the phrase "recombinant DNA, or DNA or RNA derived therefrom" in place of the term "DNA" in the first sentence. It would therefore read:

> "This section covers experiments involving whole animals, both those in which the animal's genome has been altered by stable introduction of **recombinant DNA, or DNA or RNA derived therefrom,** into the germ line (transgenic animals) and experiments involving viable recombinant-DNA-modified microorganisms tested on whole animals."

Dr. Mannix seconded the motion. Dr. Neiman accepted this language as a "friendly amendment," and Dr. Erickson concurred.

Dr. Gartland suggested the use of the word "molecules" to standardize this phrase with its use in Recommendation F. Dr. Davis noted that RNA cannot be incorporated into the genome of an animal and therefore it should be dropped. Dr. Gellert said if "RNA" were removed then the word "molecules" was unnecessary because that referred to the RNA.

The final wording agreed upon for this sentence then became:

> "This section covers experiments involving whole animals, both those in which the animal's genome has been altered by stable introduction of **recombinant DNA, or DNA derived therefrom,** into the germ line (transgenic animals) and experiments involving viable recombinant-DNA-modified microorganisms tested on whole animals."

There being no other amendments to the motion, Dr. McGarrity put the motion to a vote. The motion passed by a vote of 20 in favor, none opposed, and no abstentions.

IV. PROPOSED AMENDMENT OF SECTION I-C OF THE NIH GUIDELINES (tabs 1310/I, 1317, 1318, 1319, 1320, 1327, and 1328).

Dr. McGarrity called on Dr. Cohen to begin discussion of this agenda item. Dr. Cohen delineated the history of the proposal from the Foundation on Economic Trends to amend Section I-C of the NIH Guidelines to cover projects conducted abroad if the NIH had contributed any developmental funding. He said there was general dissatisfaction with the proposal but also with the lack of a procedure to prevent recurrences of such things as the Wistar rabies vaccine episode. He said a working group had convened on February 1, 1988, to address this situation and the working group had grappled with several issues involved in this proposal.

The first issue was there are reasons to conduct recombinant DNA projects abroad, namely that diseases can be best studied in endemic areas and the working group did not believe they could propose anything which would stifle such responsible research.

The working group recognized that the NIH has limited jurisdiction over international projects, that foreign governments are autonomous, and that NIH jurisdiction pertains primarily to institutions and investigators receiving financial support from the NIH. Further, the working group recognized many problems associated with international research were a result of incomplete or inadequate communication.

Therefore, the purpose of the final proposal (tab 1317) was to attempt to facilitate proper communications and ensure appropriate approvals for international projects which are concerned with deliberate release and human gene therapy. The proposal instructs researchers whose projects are a direct extension of NIH-funded research to provide NIH with evidence that the project is in compliance with the NIH Guidelines, the host country's guidelines, or is acceptable by an appropriate Government office of the host country. The aim is to recognize the autonomy of foreign governments while assuring proper communication occurs and at the same time not hindering responsible research.

Dr. Gottesman said Section I-C of the current NIH Guidelines covers all recombinant DNA research within the United States and was applicable to projects done abroad if supported by NIH funds. The proposal from the Foundation on Economic Trends was in response to an experiment which had taken place in Argentina that was determined as not falling under the NIH Guidelines because no NIH funds were used for the testing abroad. However, the development of the recombinant molecules used in the field test was performed at an U.S. institution and was funded by the NIH.

Dr. Gottesman said the problem was in definition of the term "project." The NIH defines a project in terms of what appears in a grant application. The working group felt some discomfort that a "project" could be defined to suit the purposes of an investigator and bypass applicability of the NIH Guidelines by moving portions of an experiment to a foreign country. On the other hand, the working group did not want to dictate to foreign governments what appropriate safety conditions were required.

Therefore, the final proposal defined a project as research done using "materials containing recombinant DNA developed with NIH funds and the research is a direct extension of the development process." This definition has been criticized by some as too vague and by others as being too narrow. There has also been criticism of allowing for written acceptance by a foreign government in lieu of compliance with the NIH Guidelines. Dr. Gottesman said the working group's major concern was that the foreign government be aware of the research and to allow them to make an appropriate decision on safety issues.

Dr. Korwek said that criticisms by both the Food and Drug Administration and the Foundation on Economic Trends were valid but that by closing some loopholes others may be opened and therefore he welcomed suggestions for amending the proposed language for Section I-C.

Mr. Lanman said he was interested in how this provision was to be enforced as currently the only mechanism for enforcement was through curtailment of NIH funding to violating institutions and investigators. He suggested the following wording to tie in NIH funding of recombinant DNA research to the NIH Guidelines:

> "The NIH Guidelines are also applicable: (1) to projects done abroad if they are supported by NIH funds or (2) to research done abroad if it involves deliberate release into the environment or testing in human beings of materials containing recombinant DNA if the research **is a direct extension of research conducted at or sponsored by an institution that receives any support for recombinant DNA research from the NIH."**

Mr. Lanman proposed a major change in the working group's proposed wording to make foreign applicability rules the same as domestic applicability rules. That is, he proposed replacing the entire first sentence of the proposed paragraph with the following:

> **"The NIH Guidelines are also applicable to projects done abroad if they are supported by NIH funds or conducted at or sponsored by an institution that**

receives any support for recombinant DNA research from the NIH."

He said this would go beyond the working group's limitation to testing in humans or deliberate release into the environment, but he said it had the advantage of simplifying matters and applying the same rules to foreign research as are applied to domestic research.

Dr. McGarrity asked for clarification of the phrase "direct extension" in Mr. Lanman's first suggestion. He asked if this was meant to apply to domestic work in which an investigator turned his research over to a commercial company for development. Mr. Lanman replied he envisioned this only applying to work done abroad.

Dr. McGarrity then asked how his second suggested change would deal with the host country which has established rules. Mr. Lanman said his suggested wording only applied to the first sentence and that he would retain the remainder of the wording from the working group's recommendation.

Dr. McGarrity asked if organizations and institutions such as the American Type Culture Collection and his own institution, the Coriell Institute for Medical Research, which are repositories for biological materials and receive NIH funding, would be held responsible for eventual uses of materials which they have supplied. Investigators might then use them in an experiment which those organizations took no more part in than to supply the original cell culture, virus, bacterium, or plasmid. He questioned whether this was really enforceable.

Dr. Johnson said there was another issue that the language didn't address and that was putting the original NIH grantee in a position of, "trying to guarantee all future downstream research." He said the grantees probably could not guarantee that and as such this could lead to an unwillingness to share materials for research with others unless some guarantee could be given to them that they would not be penalized. Additionally, he said researchers in other countries should be free to improve on research materials developed in this country and should be able to look for better applications to human, animal or other types of problems using them in any way that they see fit. Furthermore, the proposed wording of Section I-C was also putting foreign governments in a position of having to accept the NIH Guidelines or develop their own, and this may be unenforceable.

Dr. Atlas agreed and said the only way an investigator could then protect himself would be to not share biological materials with his colleagues.

Dr. Cohen said the working group tried to deal with this issue. The proposal was not designed to impede responsible research, but to make sure that appropriate people know the research is going on so that any potential problems can be dealt with. He said any suggestions to this end would be most welcome.

Dr. Gottesman said she saw the proposal as making applicability more specific, limited to projects only, rather than the overall work being undertaken by an institution, when dealing with a foreign institution. She questioned whether this proposed amendment to Section I-C would have helped in the Argentinean incident had it been in place. She said it was really addressing a different issue.

Dr. McGarrity said that the collaboration was with a commercial organization, not an academic institution, and in his opinion unless commercial organizations voluntarily followed the NIH Guidelines such experimentation would not be covered.

Dr. Gartland said he understood that Wistar turned the vaccine over to a French company who then collaborated with PAHO which conducted the field trial in Argentina. Mr. Jeremy Rifkin said his understanding was that the NIH supplied funds specifically to Wistar to conduct the research and the only portion of the research that was not funded by NIH was the small portion of funds needed to do the experiment in Argentina.

Dr. Gottesman asked Mr. Lanman if his suggested rewording of Section I-C would cover the Wistar incident. Mr. Lanman said it was directly pertinent because his issue was to what extent domestic applicability should differ from foreign applicability. He asked how we would deal with the same situation if it occurred domestically. He said the phrase "sponsored by" might be a more ambiguous term and subject to more interpretation.

Mr. Rifkin commended the working group for their recommendations. He said the key word was "responsibility," and the reason the issue was raised at all was the nature of the experiment in Argentina. He said Wistar had received millions of dollars from NIH to develop a rabies vaccine and then took money from other sources to conduct the field experiment in Argentina in connection with PAHO and a French company. He said the working group did a good job at closing the loopholes but that the phrase "direct extension" still left a loophole for interpretation.

Mr. Rifkin said there was legal precedent in definitions found in regulatory language promulgated by the Council on Environmental Quality (CEQ) for the use of the word "connected" rather than "direct." He said that actions are connected if they, "cannot or will not proceed unless other actions are taken previously or simultaneously and are inter-dependent parts of a larger action and depend on the larger action for their justification."

Mr. Rifkin agreed there was still some room for interpretation and said, "There is no way that you can develop any kind of legal standard that cannot in some way be misinterpreted or abused." However he felt the CEQ definition was a "federal standard," whereas "direct" had no standard definition used by any agency in similar matters.

Mr. Rifkin said he did not agree with the proposal to allow foreign countries not having their own guidelines to issue written acceptance in lieu of compliance with the NIH Guidelines. He said this would weaken the existing NIH Guidelines. He said the point was that the NIH had set international standards and that other governments had looked to the RAC and the NIH in adopting their own standards. He said the NIH had a responsibility for ensuring no double standards are set for research and that it has a responsibility for any kind of international application of research which it funds. Not to do so would send a clear message to other nations that they could use third world countries, or other countries, as dumping grounds for deliberate release experiments. He said to prevent this he would like the wording suggested by the Foundation on Economic Trends to be substituted for the proposed wording.

Upon questioning by Dr. McGarrity, Mr. Rifkin said he did not suggest that Wistar was involved directly in the Argentinean release of the rabies vaccine.

Dr. Johnson said he did not wish to comment on the CEQ definitions but that he was concerned with the concept of an NIH grantee trying to ensure appropriate control of downstream research.

Dr. Neiman said he questioned the legal precedent of the use of the term "foreseeable" as to whether it was based on an understanding of connectiveness in a scientific sense. He said he preferred the use of the word "direct," even if undefined.

Dr. Neiman asked how the working group intended to cover the issue raised by the Foundation of countries who did not have their own guidelines as addressed in the final sentence of the proposed amendment to Section I-C. Dr. Cohen said it was considered as an oversight mechanism to cover those instances where no guidelines are in place and approval comes from a country to do research on a disease which is endemic to its geographic area or population.

Dr. Neiman said Mr. Lanman's suggested wording used sponsorship by an institution, as opposed to the project, as the basis for potential enforcement and that seemed consistent with what is done generally for human experimentation in this country today. He endorsed this approach.

Dr. McGarrity asked if NIH supported development of a non-recombinant vaccine, and it goes to a foreign country for field testing, what human subjects regulations would be used.

Mr. Lanman said that Department of Health and Human Services funding was tied to DHHS Human Subjects Regulations. Dr. Miller said it would also require FDA approval because interstate commerce regulations require FDA approval of shipment for use in a foreign country.

Dr. Pagano said he liked the phrasing as proposed. He said if the host country did not have guidelines they would need to accept whatever is to be tested and this is bolstered by the statement whereby the NIH reserved the right to withhold funding. He said, "They can review, they should review," but "I don't think we can rigidly hold other countries to our standards because their problem may be different and there is a risk versus benefit to consider here." In other words the host country is allowed the leeway to make its own guidelines but if a U.S. investigator or institution is introducing a product to a country which does not have guidelines, the NIH still has a right and a means to prevent it.

Dr. Childress said he agreed but the phrase "in lieu of compliance with the NIH Guidelines" should be worded to indicate what was expected was conduct reasonably consistent with the NIH Guidelines.

Mr. Brell, Attorney and Officer for the Foundation on Economic Trends said he felt acceptance by an appropriate Government agency in the host country after proper scientific consideration of risk was necessary. He said he felt the word "connected" should be used along with the word "direct," to show that if something is part of a larger cohesive whole it must fall under the NIH Guidelines as well as if it is predicated on previous or simultaneous actions. This language had been defined by the CEQ and is used in assessing environmental impact.

Dr. Gottesman asked for an interpretation of the word "sponsored" by the lawyers in the group. She asked if it were too broad a term and whether it would be interpreted to include all "direct extensions." She said a possible solution to this may be to use the term "sponsored by." Dr. Gottesman said that the term "connected" may have a legal meaning but that all research is predicated on research in the past and tomorrow's research is reasonably foreseeable from research going on today.

Dr. Murray asked how developing or non-industrialized nations process requests coming to them for research and what issues come up in such a setting, and what obligation a researcher has to control the use of materials and results of research. He said he believed in the Argentinean field trial the investigators were

trying to circumvent mechanisms to inform the Government and protect people in order to move the project ahead more rapidly than they should have.

Mr. Lanman said, in response to Dr. Gottesman's suggestion to use the term "sponsored by," that it could be interpreted broadly and the toughest part of interpretation would be whether provision of research materials alone constitutes sponsorship. He suggested an alternative to the use of "direct extension." He said the following phrase should be substituted:

> "The research is subject to control through funding, sponsorship, or the direct provision of research materials by an institution within the United States or its territories that receives any support for recombinant DNA research from the NIH."

Dr. McGarrity reiterated his question as to whether this would apply to repositories who supply viruses, bacteria, tissue samples and cell cultures. Mr. Lanman said another way of doing it may be to tie provision of research materials to a formal collaboration to prevent a company or organization providing research material to an external source to do research in order to bypass applicability of the NIH Guidelines.

Mr. Brewer said he liked Mr. Lanman's suggestion in that it would solve virtually all of the problems with the exception of people who were deliberately trying to get around the NIH Guidelines, and that perhaps there were other agencies and institutions of the Federal Government more appropriate to handle those situations. He said he thought the best NIH could do is try to create a situation in which it ties the money it provides to researchers and institutions to an understanding that the duty falls upon those researchers and institutions to conduct their work and oversee it in accordance with the NIH Guidelines.

Dr. Pramer drew the parallel with human subjects research and said that local IRBs are not concerned with where the research is taking place, but rather the standard of work, the quality of research, and how well confidentiality and protection of health and welfare of the human subjects is assured. Furthermore, the IRBs are charged by the institution to review all projects which are using human subjects, irrespective of funding.

Mr. Rifkin asked Mr. Lanman if, using his proposed terminology, the Wistar Institute gave a vaccine to a private French company who in turn gave it to PAHO for a field trial, whether this would be a direct provision.

Mr. Lanman said it would not be direct.

Mr. Rifkin said this then defeated the whole purpose of the working group's proposal and for that reason the word "connected," rather than "directed," needed to be used, because surely this work was connected and would therefore require NIH oversight. Mr. Rifkin said if Mr. Lanman's proposal were to be accepted it would show that the NIH and the Federal Government were not willing to take responsibility for research it funds and that there would be further incidents in the future.

Dr. Davis said he was surprised by Mr. Lanman's interpretation that this would not be a direct provision and agreed with Mr. Rifkin that there was a problem. He said, "if there is a general agreement that 'direct' would not cover that, [the Wistar experiment] then we have not done our job adequately."

Dr. Cohen said some of the problem is in talking about a researcher developing something and someone else using it, and this could be resolved by the use of the word "foreseeable," but the original researcher might not foresee an eventual use for his research. However, in the case of a researcher who develops a vaccine, as in the Wistar case, it becomes obvious that a field test is a foreseeable consequence of the initial research.

Dr. McGarrity pointed out that the NIH ruled in that case the field test was not a direct extension of the research because no field test was mentioned in the original grant application or in progress reports. Mr. Lanman said this was part of what was considered as well as the fact that the field test was not funded by the NIH.

Mr. Mannix suggested a different approach, to require that progress reports include disclosure of any planned testing, whether abroad or domestic.

Dr. Gottesman said her interpretation of Mr. Lanman's response to Mr. Rifkin was that if "direct extension" did not cover such an experiment, that "sponsored by" might. She said "connected" and "foreseeable" would meet with the same problems in terms of who is the person who is responsible to foresee a use for research materials.

Mr. Brell asked what problems were seen with using both "connected" and "reasonably foreseeable," since these were both terms defined by the Council on Environmental Quality. Dr. Gottesman reiterated that science is based on all knowledge that has come before and therefore it would be hard to convince any court that any particular experiment is not "connected" to any other research. She said, "the point is that these definitions have to be written for scientists as well as lawyers if they are going to be of any use to any of us that are doing research."

Mr. Rifkin asked to put aside definitions and to look at principle. He once again cited the Wistar experiment and asked Dr. Gottesman if she felt the research in Argentina was a "direct extension."

Dr. Gottesman replied she was on the working group and that the wording "direct extension" was put into the recommendation because it was the consensus of that group that the field trial was a direct extension, that it was clearly what had to be done next in development of the rabies vaccine.

Mr. Rifkin said it was important to him to have the principle established rather than argue semantics so that in the future there would be no problem in interpreting the NIH Guidelines.

After the luncheon recess, Dr. McGarrity called the committee to order and asked for pertinent discussion and comments with an aim toward a closure on the subject.

Dr. Gottesman said since she was an ad hoc member of the RAC and had no voting privileges she would proffer some text for the group to work on. She proposed a revision of Section I-C of the NIH Guidelines to read as follows:

> "The NIH Guidelines are also applicable to recombinant DNA projects done abroad: (1) if they are supported by NIH funds or conducted at, or sponsored by, an institution that receives any support for recombinant DNA research from NIH; and (2) if it involves deliberate release into the environment or testing in humans of materials containing recombinant DNA developed with NIH funds and the research is a direct extension of an NIH-supported project.
>
> "If the host country, however, has established rules of conduct of recombinant DNA projects, then a written assurance of compliance with those rules may be submitted to NIH in lieu of compliance with the NIH Guidelines. Alternatively, if the host country does not have such rules, written acceptance of the proposed project by an appropriate Government office of the host country is necessary. The NIH reserves the right to withhold funding if the safety practices to be employed abroad are not reasonably consistent with the NIH Guidelines."

Dr. Gottesman said she knew of an alternatively worded proposal which was somewhat simpler and asked if, after having it read, a straw vote could be taken on which alternative to discuss further.

Mr. Brewer then suggested the following alternative revision to Section I-C of the NIH Guidelines:

> "The Guidelines are applicable to all recombinant DNA research conducted at or sponsored by an institution that receives any support for recombinant DNA research from the National Institutes of Health (NIH). This includes research performed by NIH directly.
>
> "An individual receiving support for research involving recombinant DNA must be associated with or sponsored by an institution that can and does assume the responsibilities assigned in these Guidelines.
>
> "For any recombinant DNA research done in another country, if that country has established rules for the conduct of recombinant DNA projects, then a certificate of compliance with those rules may be submitted to the NIH as evidence of compliance with the NIH Guidelines. Alternatively, if the host country does not have such rules, written acceptance of the proposed project by an appropriate Government office of the host country is necessary. The NIH reserves the right to withhold funding if the safety practices are not reasonably consistent with the NIH Guidelines."

Mr. Brewer said this puts the burden on every institution to adhere to the NIH Guidelines and take them seriously and that additionally letters could be written to all institutions to say that the NIH expects people to live not only by the written NIH Guidelines but the spirit of these Guidelines. He said this could be done easily and would not discourage the normal exchange of scientific research material and ideas. This letter could be made a permanent part of the file for each institution and investigator. If this kind of rule had been in effect there would have been strong ground for the NIH to withhold further funding from Wistar or any other institution which violated its intent and spirit.

Dr. Korwek asked if the NIH ruling that the Wistar project was not encompassed within the NIH Guidelines was because of the definition of "project," which the NIH interpreted to be synonymous with "research grant." Dr. Gartland said the history of the third paragraph of Section I-C was it was written to cover research grants being awarded to foreign institutions.

Mr. Mannix asked which funds were being sought to be withheld, funds for a particular project or institution-wide funding. Dr. Gottesman said she believed it to be institutional funding which is a lever NIH has used with institutions to make sure everyone within an institution abides by the rules. Therefore a violation

on a single project could jeopardize continued funding on any or all recombinant DNA work at the institution.

Dr. McKinney said he believed this could be interpreted also to cover a third party action in which an institution funded by NIH could be held responsible for research initiated by them and turned over to a second party who, in turn, allow a third party to continue further research without the consent or knowledge of the originating institution.

Dr. Atlas said he would prefer the phrase "or done in collaboration with," to be put in the language of the proposal to obviate the innocent third party liability.

Mr. Brewer said he would accept "in collaboration with" as an additional phrase.

Dr. McGarrity asked whether he could get a feeling of the committee as to which proposal they were more comfortable with. Dr. Riley asked Dr. Gottesman what was in her proposed language that was not in the Brewer proposal. She replied the major difference was the reiteration of the "direct extension" terminology. She said she was not sure that "collaboration" would fit in the proposed language but said the phrase "sponsored by" could be footnoted to include "collaboration."

Dr. Cohen said the concept of "collaboration" would eliminate the "direct extension" phraseology because it involves active participation and he said it dealt with the problem nicely.

Mr. Brewer then reiterated his proposal in the form of a motion to read:

> "The Guidelines are applicable to all recombinant DNA research conducted at or sponsored by or done in collaboration with an institution that receives any support for recombinant DNA research from the National Institutes of Health (NIH). This includes research performed by NIH directly.
>
> "An individual receiving support for research involving recombinant DNA must be associated with or sponsored by an institution that can and does assume the responsibilities assigned in these Guidelines.
>
> "For any recombinant DNA research done in another country, if that country has established rules for the conduct of recombinant DNA projects, then a certificate of compliance with those rules may be submitted to the NIH as evidence of compliance with the NIH Guidelines. Alternatively, if the host country does not have such rules, written acceptance of the proposed project by an

appropriate national government office of the host country is necessary. The NIH reserves the right to withhold funding if the safety practices employed abroad are not reasonably consistent with the NIH Guidelines."

Dr. Vidaver seconded the motion.

Dr. Cohen asked if "in lieu of" could be substituted for "as evidence of" in the third paragraph. Mr. Brewer said he would accept this as a friendly amendment. Dr. Miller had the concern the term "collaboration" could be interpreted too broadly. He said scientists know what is meant by "collaboration," but thought some qualifying language needed to be added such as "active continual collaboration," to clarify the intent of the language. Mr. Brewer said he would drop the phrase "in collaboration with," if anyone had any wording that could better be substituted without raising ambiguities.

Mr. Brell said an institution should bear the responsibility for monitoring to ensure the products of their research are not being used in a way that would contravene the provisions of the NIH Guidelines and the RAC was being too timid in their use of such words as "collaboration." He said he wanted the term "appropriate government agency" defined. Mr. Brewer said he would be willing to accept expanded language similar to "appropriate national scientific or health official," if Mr. Brell thought that would clarify the issue.

Dr. Riley asked if, in the case of a foreign country that did not have regulations in place, the only safeguard would be the signing-off by that country's government official. Dr. McGarrity explained the final sentence of I-C would still be in effect, that, "the NIH reserves the right to withhold funding" if the safety practices are not proper.

Dr. Gottesman asked for clarification as to whether the current two sets of proposals differed so much from what appeared in the Federal Register notice that a vote would not be in order to approve either, and that it may have to be put out for public comment again. Mr. Lanman said he believed the proposal was now so broad that this was going to be necessary. Dr. Gottesman asked whether a vote should be taken to have one or the other proposals perfected and published for public comment. Dr. McKinney suggested the motions currently on the table be withdrawn.

Mr. Brewer withdrew his motion from consideration at this time by the RAC.

Dr. Tolin said she was in favor of the language being proposed. She cautioned that in adding requirements for compliance the

committee keep in mind that we cannot force foreign governments to comply, and that perhaps acceptance of these guidelines would be a more favorable target.

Dr. McGarrity asked Mr. Lanman if Mr. Brewer's withdrawn motion was a reasonable starting point for the next round of publication in the Federal Register, and still allow for limited modification at the next meeting of the RAC. Mr. Lanman said there were several options:

1. A decision could be made on one version to recommend to the Director of NIH;

2. The Director, NIH, could be asked to publish alternate versions for comment; or

3. It could be left to the Director's discretion to publish a version which would meet certain criteria, and allow the Director to provide the specific language.

Dr. Gartland asked whether the sense of the committee was that what was voted on today go out for further comment and brought back to the October RAC meeting or whether the whole issue should go back to a working group for even further work and brought back in October. Dr. Korwek said there was enough dispute over what specific words meant and how they should be construed, and that there were many diverse viewpoints being dealt with, and he felt it should go back to the working group for eventual republication for comment. General agreement was voiced around the table for sending the proposals back to the working group.

Dr. McGarrity thanked Dr. Cohen and the working group for their efforts on these topics.

V. PROPOSED AMENDMENT OF SECTION III-B-5 OF THE NIH GUIDELINES: (tabs 1310/III, 1313, 1328, 1329, 1331).

Dr. Cohen said the proposal had come from Dr. Joseph Fordham of Novo Laboratories requesting Section III-B-5 of the NIH Guidelines be changed to read:

> "For large-scale (LS) fermentation experiments, involving non-pathogenic and non-toxicogenic recombinant strains of host organisms having an extended history of safe industrial use, the appropriate physical containment conditions need to be no greater than those for the host organism unmodified by recombinant DNA techniques; the IBC can specify higher containment if it deems necessary."

Dr. Cohen said he had no major objection to the request, but he was slightly concerned because it implies that interpretation is left to the principal investigator and that perhaps continued use of exchanger lists or definitions of what organisms are covered under such amendments may be preferable.

Dr. McKinney said he was troubled by the lack of definition of such terms as "extended history" and "safe industrial use." He said "extended history" could be interpreted to mean almost any time frame, and that due to lack of recorded fermentation accidents and lack of monitoring, there was no way to judge what "safe industrial use" was. He said it was important that risk assessments be done so that such terms could be eliminated and experiments could be judged on the facts at hand, not history which may or may not be available for a given material.

Dr. Pramer said he was not uncomfortable with this broad proposal based on the proposals being received from industry, particularly if the one from Eli Lilly to be discussed next is taken as an example. He said with the past experience with Saccharomyces and E. coli, he was willing to accept such a proposal, especially since it was still subject to the system of checks and balances built into the NIH Guidelines, i.e., IBC review and recommendations.

Dr. Riley said the terms "non-pathogenic" and "non-toxicogenic" were generic and needed more definition, and she believed a case-by-case review was still necessary until a group could establish universally acceptable definitions of organisms that could be exempted.

Dr. Johnson said, in reference to Dr. McKinney's comments, that people had been looking for adverse reactions in large-scale fermentation and the only one was allergenicity to products such as penicillin, but that workers were avidly monitored. Furthermore, if a general approval of organisms widely used in large-scale fermentation is not made available, the RAC could expect a long list of single case proposals in the coming years which could hinder U.S. competitiveness in the field.

Dr. Gottesman questioned whether the flexibility is already there and this just allows for the recognition of the flexibility and the problem of specifying containment could be left to the local IBCs on a case-by-case basis.

Dr. McGarrity said he believed local IBCs could recommend lower than BL1-LS. Dr. Gartland said the next sentence of the current Section III-B-5 recommends following Appendix K, where appropriate, and "where appropriate" is the language which provides flexibility for the IBC. However, he said, IBCs are reluctant to use this.

Dr. McKinney said this was the underlying issue, the reluctance on the part of a number of IBCs to take on the responsibility for determining what containment levels will be used. He agreed with Dr. Gottesman that the proposed recommendation does nothing that the current III-B-5 does not already do. He said a narrative could be added to describe good industrial practices but these vary according to the process involved and the group performing the fermentation.

Dr. Gartland said he had spent time with representatives from Eli Lilly discussing the way the NIH Guidelines are currently constructed. In essence, if a small-scale experiment requires BL1, then the same experiment done on a large-scale basis would require BL1-LS, but the wording "where appropriate" added a certain amount of flexibility.

Dr. Miller said the FDA had proposed over a year and a half ago that going to large-scale with exempt organisms such as E. coli, B. subtilis, or Saccharomyces required only very low containment, and that BL1-LS was unwieldy, expensive and unnecessary for academia and for industry. Experience since then has shown that utilization of lower than BL1-LS containment for these organisms had produced no demonstrable problems and had met with a great deal of acceptance and enthusiasm from industry. He said FDA urged that some generic criteria be adopted for exactly the same reasons, because many of these organisms require only very low containment, that BL1-LS is unwieldy, expensive and unnecessary, and that this will allow industry to plan for future production facilities which will need to be built as the industry expands.

Dr. Miller said the FDA would prefer criteria closer to the OECD proposed generic criteria mainly because these have been adopted and accepted throughout the world.

Dr. Cohen suggested expanding the list of exempt organisms. Dr. McKinney suggested deleting the second sentence of the current III-B-5 and substituting the statement, "Where appropriate, Appendix K shall be used," and allow the IBCs to decide appropriate containment on the basis of such data as they deem necessary to make their decisions. Dr. Gottesman said she believed there could be changes made to Appendix K to add flexibility.

Dr. McKinney said he agreed with Dr. Riley that the NIH Guidelines were developed with the greatest concern being addressed to risk to humans performing recombinant DNA research. This concern has now shifted, as the technology has grown, from the laboratory to the environment outside the laboratory and the impact of recombinant DNA on the total world. He stressed more attention needed to be paid to the total environment but that any language needed to be flexible enough to move at the pace of the technology.

Dr. Gottesman suggested no changes be made to Section III-B-5, but to revise the wording of Appendix K-I of the NIH Guidelines to read:

> "The selection of the physical containment level requirement is based on the containment guidelines as established in Part III of the Guidelines. For purposes of large-scale research or production, three physical containment levels are established. These are referred to as BL1-LS, BL2-LS, and BL3-LS. The BL1-LS level of physical containment is recommended for large-scale research or production of viable organisms containing recombinant DNA molecules which require BL1 containment at the laboratory scale. For large-scale fermentation experiments involving non-pathogenic and non-toxicogenic recombinant strains of host organisms having an extended history of safe industrial use, the IBC may consider suggesting containment consistent with good industrial practice rather than using BL1-LS."

Dr. Gottesman said this would add flexibility. It would remove the requirement for BL1-LS and make it a recommendation, thereby putting more flexibility in the hands of the IBC.

Dr. Gellert moved Dr. Gottesman's proposed revision to Appendix K-I of the NIH Guidelines be adopted. Dr. Pramer seconded the motion.

Dr. Miller asked what BL1-LS was being recommended for. Dr. Gottesman replied it would be recommended for experiments requiring BL1 containment at the laboratory scale. Dr. Miller suggested deleting the sentence recommending BL1-LS for experiments requiring BL1 containment at the laboratory scale because he felt it was negated in the next sentence. Further, he said he would incorporate some of the language of the Federal Register notice of June 26, 1986, to say, "For recombinant DNA-containing organisms meeting certain criteria, appropriate large-scale containment will be no greater than that for the host organism unmodified by recombinant DNA techniques."

Mr. Mannix suggested breaking Appendix K-I into K-I-a and K-I-b. Appendix K-I-a would be prefaced by "Except as in K-I-b...." and would read as the current K-I, but changing the word "required" to "recommended." Appendix K-I-b would consist of the proposed language before the committee for large-scale fermentation and read, "For large-scale fermentation experiments involving non-pathogenic and non-toxicogenic recombinant strains of host organisms having an extended history of safe industrial use the appropriate physical containment conditions need be no greater than those for the host organism unmodified by recombinant DNA

techniques; the IBC can specify higher containment if it deems it necessary."

Dr. Van Houten, the Chairman of the IBC at Schering-Plough Corporation said the IBC there felt they had the power to lower containment, but they did not feel comfortable with the possible associated environmental release. He said IBCs would appreciate studies being conducted to reassure IBC members that if such decisions to lower containment are made there are sound scientific facts upon which to base those decisions.

Dr. McKinney replied saying establishment of the biosafety levels was based on assessment of risk associated with working with the recombinant organisms and principally the risk to humans. He suggested possibly a "Points to Consider" document should be developed for guidance to IBCs in considering whether they should lower containment below BL1. Further, he suggested industry could come up with good industrial large-scale practices criteria which could be used for IBCs to measure their decisions.

Dr. Johnson reiterated that if industry were to be forced into case-by-case review of large-scale fermentation production, it would place the American industry at a distinct economic disadvantage, because the rest of the world is operating under generic exclusion criteria. Dr. Miller said there were requirements for an entirely closed system in use for low-risk, well understood and often used industrial organisms.

Dr. Foglesong from Eli Lilly said another consideration, aside from processing in the fermentor, is processing outside. Under BL1-LS standards you are required to inactivate the organism before removing it from the fermentor.

Dr. Gottesman restated the amended wording for Appendix K-I as follows:

> "The BL1-LS level of physical containment is recommended for large-scale research or production of viable organisms containing recombinant DNA molecules which require BL1 containment at the laboratory scale. For large-scale fermentation experiments involving non-pathogenic and non-toxicogenic recombinant strains of host organisms having an extended history of safe industrial use, the IBC may set large-scale containment conditions at those appropriate for the host organism unmodified by recombinant DNA techniques and consistent with good industrial large-scale practices."

Dr. McGarrity ruled that this wording was substantially different from that of the motion on the floor, whereupon Dr. Gellert withdrew his motion and Dr. Pramer his second. Mr. Mannix moved

adoption of the superseded wording for Appendix K-1 as presented by Dr. Gottesman and the motion was duly seconded.

There being no further discussion on the motion, Dr. McGarrity put the motion to a vote and the motion passed by a vote of 15 in favor, none opposed, and Dr. Johnson abstained from voting.

VI. PROPOSAL TO INCLUDE PSEUDOMONAS MENDOCINA IN SUBLIST A, APPENDIX A, (tabs 1310/V, 1323).

Dr. Roberts said the proposal was a simple case of including an organism in sublist A of Appendix A. He said it merited little discussion, was well documented and clear. Dr. Erickson said he was of the same view.

Dr. Gottesman said organisms on the list are supposed to exchange information between each other and therefore have already seen each other's genetic complement. Therefore, if it is made by recombinant DNA, it is not novel. Plasmid mobilization of chromosomal DNA has been taken as evidence for chromosomal gene exchange. A separate issue is does this exchange take place in both directions.

Dr. Roberts moved that the proposal from Dr. Burt Ensley of AMGen (tab 1323) be approved. Mr. Brewer seconded the motion. There being no further discussion, Dr. McGarrity put the motion to a vote. The motion passed by a vote of 14 in favor, none opposed, and no abstentions.

VII. LARGE-SCALE PRODUCTION INVOLVING CEPHALOSPORIUM ACREMONIUM STRAIN LU-79-6 (tab 1310/II, 1311, 1313, 1324, 1329).

Dr. McKinney said he was in agreement with the proposal by Eli Lilly to approve this at less than BL1-LS containment, but he had some concerns from the documentation which he felt should be brought to the committee's attention.

He said there seemed to be some disagreement between the request, the heading of the letter, and the closing paragraph of the letter from Eli Lilly in how they intended to handle the committee's decision. In the request they had asked that the RAC approve conditions for experiments and products involving Cephalosporium acremonium. However, the closing of the letter said that the Lilly IBC would determine what conditions would be used depending on the outcome of the experiments. He asked for clarification of what role the Lilly IBC was going to have and whether RAC was being asked to approve production methodology beyond the experimental stage.

Dr. Foglesong said Lilly was asking for RAC approval but that the IBC was maintaining the option to require a containment level higher than that approved by the RAC, and that approval was being sought for both experimental and production methodology.

Dr. McKinney suggested that the IBC should compare their good industrial practices at least against BL1-LS so judgments can be made as to what exactly will be done. The focus should not be limited to infectivity or pathogenicity of the organism being fermented and environmental concerns should be taken into account. He said Dr. Johnson had already alluded to concern over the issue of antigenicity of the culture.

Dr. McKinney noted the proposal stated, "It is intended that the effluents from the process, including the biomass will be inactivated prior to removal from the facility." Lilly believes the strain to be safe insofar as any viable cells of this organism which would reach the environment would be at low concentration and not hazardous to man or the environment.

Dr. McKinney said he wanted to know if in fact it was intended that the biomass and the waste from the process would be discharged into the environment, and if so, how this would be done. However, he stressed he was in basic agreement with the process and the intended method of operation.

Dr. McGarrity said Dr. Pramer and he had discussed this proposal and that Dr. Pramer had implied he viewed this proposal favorably. Dr. Miller said the FDA was essentially in agreement that the organism is wholely appropriate to be grown at a lower containment level and the recombinant DNA modification was not significant to raise the level of containment.

Dr. McKinney reiterated his concern about potential environmental impact because he felt there was a lack of data addressing introduction of this organism into the environment.

Dr. McGarrity asked if this same concern would hold true on the discussion of the revision of Appendix K-1 where the IBCs were allowed, where appropriate, to lower containment from BL1-LS. Dr. McKinney replied this would apply, however the institution would then assume the responsibility, rather than NIH supplying approval.

Dr. Fordham from Novo Scientific said all such products would undergo an environmental assessment before approval by other agencies such as EPA or FDA.

Dr. Foglesong said with more than ten years' experience with recombinant DNA technology and several decades experience in fermentation technology it is the belief of Lilly that the LU-4 strain constructed by Lilly, like its non-recombinant

progenitors, is non-pathogenic and that experiments and production under conditions less restrictive than BL1-LS were consistent with the recently approved amendment to the NIH Guidelines.

Dr. Foglesong cited the following:

1. The non-recombinant progenitors of this have derived from a common ancestral strain, ATCC 11550, and have been used safely in industry without any special containment for over 25 years.

2. The difference between the recombinant strain and its non-recombinant progenitor has been well characterized and consists of the insertion of an extra copy of a key C. acremonium antibiotic biosynthetic gene along with well characterized heterologous vector DNA back into the non-recombinant derivative of ATCC 11550.

3. Several factors exist to address Dr. McKinney's concerns about biological containment: the gene and heterologous vector DNA are stably integrated into the host chromosome; C. acremonium is an asexual fungus lacking any system for genetic change; C. acremonium contains no transducing viruses; and, the host organisms are at a significant competitive disadvantage with respect to the original wide type organism, or for that matter Streptomyces present in soil, and in experiments performed could not survive in soil after 4 days.

Dr. Foglesong said investigators at Lilly have experimented with the recombinant strain for 12 months under BL1 conditions in the laboratory and for 8 months at BL1-LS in a 150 liter pilot plant study. During this time no observation was made of any unexpected changes in the fundamental characteristics of the C. acremonium host. He said it is believed that the recombinant strain can be characterized as a product of self-cloning and although the vector contains heterologous DNA it is Lilly's belief that the DNA does not change the non-pathogenic character of the host and does not present a significant risk to human health or to the environment.

Dr. McKinney said he appreciated the information on the non-recovery from soil after 4 days and suggested the RAC consider approving this petition pending Lilly's satisfactory explanation

of how disposal would occur, the concentration of live organisms in the material being disposed, and the method of disposal.

Dr. Riley said the RAC was acting as an IBC and that it seemed wrong for the RAC to be dealing with such detailed questions. Dr. McKinney said that the revision of Section III-B-5 which was voted on today is merely a recommendation to the Director of NIH and that there was no guarantee that he would accept it. Therefore, the RAC must act because the current accepted version of the NIH Guidelines requires RAC approval for such experiments.

Dr. Johnson said he sensed some inconsistency in such an approach because the NIH in the past has tried to avoid getting involved in the review process for industry. Liability eventually resides with the manufacturer. Dr. McKinney said the RAC had made a decision years ago to look at recombinant organisms being considered for large-scale production only from a scientific perspective without consideration of the engineering issues. In being asked to review and approve a process at less than BL1-LS containment, the RAC was perhaps in this instance being asked to act as an IBC insofar as this is the kind of information an IBC should consider.

Dr. Johnson said if the Gottesman proposal were accepted that would give the IBC the clear authority which it would be happy to exercise in this case.

Mr. Mannix said, "in the spirit of the recommendation that was just made on the previous agenda item, I move that we look at this as a specific case and recommend exactly what Lilly proposed." Dr. Pagano seconded the motion.

Dr. Atlas asked if this meant it would be sent to the IBC and they would be allowed to make the decision. Mr. Mannix said it was essentially to remove the requirement for BL1-LS and leave it to the discretion of the IBC.

The motion, having been duly made and seconded, was put to a vote by Dr. McGarrity and was passed by a vote of 12 in favor, none opposed, and three abstentions. Dr. Johnson asked that his abstention be noted on the record.

VIII. PROPOSED CLARIFYING STATEMENTS REGARDING TRANSPOSONS (tab 1316).

Dr. Vidaver said the background for this request for consideration was an incident in which there was a question of introduction into the environment of a recombinant DNA-containing microorganism. The experiment involved a plasmid transfer in a mating experiment which resulted in a mutant by use of a suicide vector containing a transposon, leaving open the question of whether or not this was recombinant DNA. The issue is for the

RAC to make clear whether or not the source of a transposon should be considered. In this case the donor plasmid was generated by recombinant DNA technology but the microbial derivative or transconjugant did not contain any DNA sequences other than those of the unmodified transposon as evidenced by genetic means and DNA hybridization. The data were examined by two independent groups including a committee of the NIH.

Dr. Vidaver asked the committee to consider alternative one of tab 1316, that is:

> "Unmodified transposons (wild-type) that become inserted into the chromosome, even if carried by a recombinant plasmid, are not subject to these guidelines. For example, it is common to use vectors that either are naturally unstable (suicide vector) in a desired host or that can be rendered unstable by manipulating physiological conditions. In the process of suicide (inability of the vector to replicate), transposon transfer may occur. this process is not considered recombinant DNA."

Dr. Vidaver said this could be an addendum to Section I-V, or inserted as a footnote as it is simply a clarification of what does and does not constitute recombinant DNA.

Dr. McGarrity said final action could not be taken on this issue because it had not been published in the Federal Register for public comment. However, it was on the agenda in an attempt to gain some sense of the committee for future action.

Dr. Roberts said he agreed that the alternative presented by Dr. Vidaver was completely clear as to what a transposon does and that it does not constitute recombinant DNA as considered by the RAC. Dr. Pagano agreed. Dr. Erickson said he was not quite so sanguine about it. He said looking at it from the mammalian point of view and looking at retroviruses and retrotransposons there were manipulations that could be imagined in a non-modified retrotransposon such as injection into an early embryo causing integrations that would not naturally occur and could potentially be harmful. He said he had no other alternative to propose but he did not feel completely satisfied with this alternative.

Dr. Gellert asked what impact this would have on experiments involving inserting a transposon carrying a drug-resistance element into a bacterial species where that drug resistance is not normally found. Dr. Gellert asked if this were done, not by mating, but by construction of a plasmid in vitro, which carries the transposon into a strain where the plasmid will disappear but the transposon will insert, would this be an approved experiment or not. Dr. Vidaver replied it had been done and that antibiotic

resistance is the most commonly used way that transposons are manipulated.

Dr. McGarrity advised, since there could be no resolution of this issue, that Dr. Vidaver and other interested committee members correspond with Dr. Gartland's office to attempt to prepare a proposal for the next meeting of the RAC.

IX. PROPOSED AMENDMENT OF APPENDIX C-IV TO INCLUDE BACILLUS LICHENIFORMIS (tab 1310/VI, 1312, 1330).

Dr. Riley said the proposal had been made to add Bacillus licheniformis to Appendix C-IV of the NIH Guidelines because of similarity between it and Bacillus subtilis, which is presently on the list. She said she found no reason to believe that B. licheniformis is unsafe, and it could be left to local IBCs as to what containment should be for large-scale application, and that further they would have the option to deal with it at less than BL1-LS, in light of the previous discussions of the day.

Dr. Roberts agreed. He said it would be inconsistent with the spirit of the NIH Guidelines not to include it because of its similarity to an organism already on the list.

Dr. Gellert said he agreed in general, but he asked about the spore-forming potential of B. licheniformis. He also raised the issue that in the submission from Novo, it stated that if the proposed amendment was not agreed to, that each such non-exempt large-scale experiment would require special RAC review, and he questioned whether this would be the case.

Dr. McGarrity said he believed that would depend on the fate of the amendments voted at today's meeting, but that in the worst possible case they would indeed have to come in with each individual application.

Dr. Gellert added that the proposal is not unique to B. licheniformis, and this may set a precedent for other strains similar to B. subtilis.

Dr. Fordham said as far as the organism being asporogenic, they assumed it would have to meet the criteria of having equal frequency of reversion with B. subtilis. Dr. Gellert asked that something denoting this be added to the language of the proposal.

Dr. Riley then moved to approve the following:

> "In Appendix C-IV, add Bacillus licheniformis to the title and to the first sentence such that it would read:

"Appendix C-IV - Experiments Involving *Bacillus subtilis* or *Bacillus licheniformis* Host-Vector Systems.

"Any asporogenic *Bacillus subtilis* or asporogenic *Bacillus licheniformis* strain...."

Mr. Brewer seconded the motion. Whereupon, there being no further discussion, the motion was put to a vote by Dr. McGarrity and passed by a vote of 14 in favor, none opposed, and one abstention.

X. FUTURE MEETING DATES (tab 1325, 1326).

Dr. McGarrity reminded the committee of the next meeting of the Recombinant DNA Advisory Committee which will be October 3, 1988 in Bethesda, Maryland, and advised members interested that the Human Gene Therapy Subcommittee would meet on July 29, 1988 in Bethesda, Maryland.

XI. RETIRING MEMBERS OF RAC.

Dr. McGarrity presented certificates of appreciation from the U.S. Department of Health and Human Services, the Public Health Service and the National Institutes of Health to the following retiring members of the committee and thanked them for their wise counsel and participation over their terms on the committee: Dr. Mitchell Cohen, Dr. Irving Johnson, Dr. Edward Korwek, Dr. Ann Vidaver, and Dr. David Pramer.

Having concluded the agenda and there being no further business to be discussed, Dr. McGarrity adjourned the committee at 3:31 p.m., on June 3, 1988.

William J. Gartland, Jr., Ph.D.
Executive Secretary

I hereby acknowledge that, to the best of my knowledge, the foregoing Minutes and Attachments are accurate and complete.

Date: 10/3/88

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April 20, 1988

M E M O R A N D U M

To: Members
Recombinant DNA Advisory Committee

From: Executive Secretary

Subject: June 3, 1988, Meeting - Mailing I

The next meeting of the committee will be on June 3, 1988, at the National Institutes of Health, Building 31C, Conference Room 6, 9000 Rockville Pike, Bethesda, Maryland 20892. The meeting will begin at 9 a.m. This will be a one day meeting.

Room reservations have been made for the evening of June 2, 1988, at the Marriott Hotel in Bethesda (301-897-9400) for those of you who will need accommodations. If you wish to change or cancel these reservations, please contact Ms. Becky Lawson in my office at 301-770-0131. For arrival after 6 p.m., a deposit in the amount of one night's stay is required by either a check in the amount of $71 or a major credit card authorization. The hotel will not hold the room past 6 p.m. without a deposit.

Dr. Gottesman, Mr. Lanman, and Dr. McKinney will be attending the meeting as consultants.

A preliminary list of primary reviewers is included in this mailing.

Enclosed for your consideration are the following documents:

Proposed major actions published in Federal Register.....1310

Large-scale production involving Cephalosporium acremonium strain LU4-79-6.....1311

Proposed amendment of Appendix C-IV to include Bacillus licheniformis.....1312

Proposed amendment of Section III-B-5.....1313

Please bring all these materials with you to the meeting.

William J. Gartland, Jr., Ph.D.

Enclosures

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Primary

Dr. Cohen.....1310/II, 1310/III, 1310/VI, 1311, 1312, 1313

Dr. Davis.....1310/II, 1311

Dr. Gellert.....1310/VI, 1312

Dr. McKinney.....1310/II, 1310/III, 1311, 1313

Dr. Pramer.....1310/II, 1310/III, 1311 1313

Dr. Riley.....1310/III, 1310/VI, 1312, 1313

Dr. Roberts.....1310/VI, 1312

[Billing Code 4140-01]

1310

DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes Of Health

Recombinant DNA Research: Proposed Actions Under Guidelines

Agency:

National Institutes of Health, PHS, DHHS.

Action:

Notice of Proposed Actions Under NIH Guidelines for Research Involving Recombinant DNA Molecules.

Summary:

This notice sets forth proposed actions to be taken under the National Institutes of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules. Interested parties are invited to submit comments concerning these proposals. These proposals will be considered by the Recombinant DNA Advisory Committee (RAC) at its meeting on June 3, 1988. After consideration of these proposals and comments by the RAC, the Director of the National Institutes of Health will issue a decision on these proposals in accord with the NIH Guidelines.

Date:

Comments received by May 24, 1988, will be reproduced and distributed to the RAC for consideration at its June 3, 1988, meeting.

Address:

Written comments and recommendations should be submitted to the Director, Office of Recombinant DNA Activities, 12441 Parklawn Drive, Suite 58, Rockville, MD 20852. All comments received in timely response to this notice will be considered and will be available for public inspection in the above office on weekdays between the hours of 8:30 a.m. and 5:00 p.m.

For Further Information:

Background documentation and additional information can be obtained from the Office of Recombinant DNA Activities, 12441 Parklawn Drive, Suite 58, Rockville, Maryland 20852, (301) 770-0131.

Supplementary Information:

The NIH will consider the following actions under the NIH Guidelines for Research Involving Recombinant DNA Molecules:

I. Proposed Amendment of Section I-C.

Section I-C of the NIH Guidelines currently reads as follows:

"I-C General Applicability.

"The Guidelines are applicable to all recombinant DNA research within the United States or its territories which is conducted at or sponsored by an institution that

receives any support for recombinant DNA research from the National Institutes of Health (NIH). This includes research performed by the NIH directly.

"An individual receiving support for research involving recombinant DNA must be associated with or sponsored by an institution that can and does assume the responsibilities assigned in these Guidelines.

"The Guidelines are also applicable to projects done abroad if they are supported by NIH funds. If the host country, however, has established rules for the conduct of recombinant DNA projects, then a certificate of compliance with those rules may be submitted to NIH in lieu of compliance with the NIH Guidelines. The NIH reserves the right to withhold funding if the safety practices to be employed abroad are not reasonably consistent with the NIH Guidelines."

In a letter dated January 9, 1987, Mr. Edward Lee Rogers, Counsel for the Foundation on Economic Trends, and Mr. Jeremy Rifkin, Foundation on Economic Trends, Washington, D.C., proposed that the following text be inserted after the first sentence of the third paragraph of Section I-C:

"For purposes of the preceding sentence, the term 'project' includes any research or development of the recombinant organism or other product or process in question, including all such work that is reasonably forseeable when the NIH support is received. NIH support includes both money grants and any type of in-kind support, including research conducted directly by NIH, supplies, equipment, the use of facilities, and biological research materials. NIH support has been given where the source of funds or in-kind support is, directly or indirectly, the NIH."

This proposed amendment of Section I-C was initially published for comment in the Federal Register of March 11, 1987 (52 FR 7525), prior to a scheduled RAC meeting on June 15, 1987. The June 15, 1987, meeting was postponed and rescheduled on September 21, 1987. Accordingly, this proposed amendment was published again for comment in the Federal Register of August 11, 1987 (52 FR 29800).

After extensive discussion at its meeting on September 21, 1987, the RAC voted to establish a working group to make recommendations regarding international projects and to report back to the full RAC.

A Working Group on International Projects met at the NIH on February 1, 1988. After extensive discussion, the

working group voted seven in favor, none opposed, and no abstentions that the following proposed revision of the last paragraph of Section I-C be published for comment:

"The NIH Guidelines are also applicable: (1) to projects done abroad if they are supported by NIH funds or (2) to research done abroad if it involves deliberate release into the environment or testing in humans of materials containing recombinant DNA developed with NIH funds and the research is a direct extension of the development process. If the host country, however, has established rules for the conduct of recombinant DNA projects then a written assurance of compliance with those rules may be submitted to NIH in lieu of compliance with the NIH Guidelines. Alternatively, if the host country does not have such rules, written acceptance by an appropriate government office of the host country is necessary in lieu of compliance with the NIH Guidelines. The NIH reserves the right to withhold funding if the safety practices to be employed abroad are not reasonably consistent with the NIH Guidelines."

II. Large-Scale Production Involving Cephalosporium Acremonium Strain LU4-79-6.

In a letter dated December 4, 1987, Dr. Mark A. Fogelsong of Eli Lilly and Company, Indianapolis, Indiana, requests

approval to conduct large-scale experiments and production involving Cephalosporium acremonium strain LU4-79-6 under less than Biosafety Level 1 - Large Scale (BL1-LS) conditions. Information on C. acremonium and construction of strain LU4-79-6 are provided in the submission.

III. Proposed Amendment of Section III-B-5.

In a letter dated March 24, 1988, Dr. Joseph R. Fordham of Novo Laboratories, Inc., Danbury, Connecticut, requests that Section III-B-5, Experiments Involving More Than 10 Liters of Culture, be amended by adding the following paragraph:

"For large-scale (LS) fermentation experiments, involving non-pathogenic and non-toxicogenic recombinant strains of host organisms having an extended history of safe industrial use the appropriate physical containment conditions need be no greater than those for the host organism unmodified by recombinant DNA techniques; the IBC can specify higher containment if it deems it necessary."

Justification for the proposed change is included in the submission.

IV. Proposed Amendment of NIH Guidelines Regarding Transgenic Animals.

The RAC was asked by the NIH Institutional Biosafety Committee to consider guidelines for experiments involving transgenic animals that do not technically fall under the NIH Guidelines because the DNA introduced into the animal is not "recombinant DNA" as defined by the Guidelines. The issue is whether these types of animals should be covered by the NIH Guidelines for Research Involving Recombinant DNA Molecules or other guidelines.

The Working Group on Transgenic Animals met at NIH on March 28, 1988, and recommended a series of amendments to the NIH Guidelines.

Background. On June 26, 1986, the U.S. Department of Agriculture (USDA) issued an "Advanced Notice of Proposed USDA Guidelines for Biotechnology Research (51 FR 23367). A notice in the December 9, 1986, Federal Register (51 FR 44397) stated that USDA would be proposing new provisions relating to agricultural research for inclusion in the NIH Guidelines in lieu of the Advanced Notice of Proposed USDA Guidelines for Biotechnology Research published on June 26, 1986. USDA organized a workshop in Arlington, Virginia, on December 13-15, 1986, in part to develop proposed guidelines for plants and animals. Proposed changes to the NIH Guidelines developed by USDA were

reviewed and revised by an NIH RAC Working Group on Revision of the Guidelines which met on June 22 and July 16, 1987.

In this proposal, language was developed to modify Sections II and III of the NIH Guidelines to refer specifically to containment for research with whole plants and animals. Containment is described in the proposed amendments Appendix P for plants and Appendix Q for animals. The suggested modifications and additions will provide guidance to the Institutional Biosafety Committees (IBCs) and Principal Investigators (PIs) to assure safe conduct of such experiments.

The proposed changes were published in the Federal Register of August 11, 1987 (52 FR 29800), for public comment.

The proposed modifications were discussed extensively by the RAC at its September 21, 1987, meeting. During the discussion, the RAC recommended a number of changes in the language as published for comment. The NIH is in the process of promulgating these proposed changes. Paragraph numbers cited below refer to the paragraphs as numbered in the August 11, 1987, Federal Register announcement (52 FR 29800).

A. On September 21, 1987, the RAC recommended that paragraph 7 as published for comment in the Federal Register of August 11, 1987, be incorporated into Section II of the NIH Guidelines as follows:

"Biosafety Level 1 for animals (BL1-N) describes containment which is used for animals in which the germ line has been modified through recombinant DNA techniques (transgenic animals) and is designed to eliminate the possibility of sexual transmission of the modified genome or transmission of recombinant-DNA-derived viruses known to be transmitted only vertically (i.e., transmitted from animal parent to offspring only by sexual reproduction). Procedures, practices, and facilities follow classical methods of avoiding genetic exchange between animals."

The Working Group on Transgenic Animals on March 28, 1988, recommended that "(transgenic animals)" be deleted from this paragraph.

B. The Working Group on Transgenic Animals on March 28, 1988, recommended that the following new sentence be added at the beginning of Section III-B-3 before the paragraph entitled "Caution":

"Experiments involving the introduction of eukaryotic viral genomes into the germ line of animals are covered in Section III-B-4."

C. On September 21, 1987, the RAC recommended that the title of Section III-B-4 as published for comment in paragraph 32 in the Federal Register of August 11, 1987, be changed to read as follows:

"Recombinant DNA Experiments Involving Whole Animals."

The Working Group on Transgenic Animals on March 28, 1988, recommended that the title of Section III-B-4 be amended to read as follows:

"Experiments Involving Whole Animals, Including Transgenic Animals."

D. On September 21, 1987, the RAC recommended that paragraph 34 as published for comment in the Federal Register of August 11, 1987, be incorporated into Section III-B-4 of the NIH Guidelines as follows:

"This section covers experiments involving whole animals, both those in which the animal's genome has been altered by recombinant DNA techniques and experiments involving viable recombinant-DNA-modified microorganisms tested on

whole animals. For the latter, other than viruses which are only vertically transmitted, the experiments may not be carried out at BL1-N containment; a minimum containment of BL1 or BL2-N is required."

The Working Group on Transgenic Animals on March 28, 1988, recommended that this section be amended to read as follows:

"This section covers experiments involving whole animals, both those in which the animal's genome has been altered by stable introduction of DNA into the germ line (transgenic animals) and experiments involving viable recombinant-DNA-modified microorganisms tested on whole animals. For the latter, other than viruses which are only vertically transmitted, the experiments may not be carried out at BL1-N containment; a minimum containment of BL1 or BL2-N is required.

"Caution: Special care should be used in the evaluation of containment conditions for some experiments with transgenic animals. For example, such experiments might lead to the creation of novel mechanisms or increased transmission of a recombinant pathogen or production of undesirable traits in the host animal. In such cases,

serious consideration should be given to increasing the containment conditions."

E. On September 21, 1987, the RAC recommended that a modified paragraph 37 as published for comment in the Federal Register of August 11, 1987, be incorporated as Section III-B-4-a of the NIH Guidelines as follows:

"Recombinant DNA, or RNA molecules derived therefrom, from any source except for a eukaryotic viral genome may be transferred to any non-human vertebrate or any invertebrate organism and propagated under conditions of physical containment comparable to BL1 or BL1-N and appropriate to the organism under study [2]. Animals containing sequences from viral vectors, if the sequences do not lead to transmissible infection either directly or indirectly as a result of complementation or recombination in animals, may be propagated under conditions of physical containment comparable to BL1 or BL1-N and appropriate to the organism under study. For experiments involving recombinant DNA modified Class 2, 3, 4, or 5 organisms (1) using whole animals, see Section III-B-1."

The Working Group on Transgenic Animals on March 28, 1988, recommended that this section be amended to read as follows:

"Recombinant DNA, or DNA or RNA molecules derived therefrom, from any source except for a eukaryotic viral genome may be transferred to any non-human vertebrate or any invertebrate organism and propagated under conditions of physical containment comparable to BL1 or BL1-N and appropriate to the organism under study [2]. Animals containing sequences from viral vectors, if the sequences do not lead to transmissible infection either directly or indirectly as a result of complementation or recombination in animals, may be propagated under conditions of physical containment comparable to BL1 or BL1-N and appropriate to the organism under study. Experiments involving the introduction of other sequences from eukaryotic viral genomes into animals are covered under III-B-4-b. For experiments involving recombinant DNA modified Class 2, 3, 4, or 5 organisms (1) using whole animals, see Section III-B-1."

F. On September 21, 1987, the RAC recommended that paragraph 41 as published for comment in the Federal Register of August 11, 1987, be incorporated as Section III-B-4-b of the NIH Guidelines, as follows:

"For experiments involving whole animals and not covered by Section III-B-1 or Section III-B-4-a, the appropriate containment will be determined by the IBC (22,23)."

The Working Group on Transgenic Animals on March 28, 1988, recommended that this section be amended to read as follows:

"For experiments involving recombinant DNA, or DNA or RNA derived therefrom, involving whole animals, including transgenic animals, and not covered by Section III-B-1 or Section III-B-4-a, the appropriate containment will be determined by the IBC (22)."

V. Proposed Amendment of Appendix A to Include Pseudomonas Mendocina.

In a letter dated March 28, 1988, Dr. Burt D. Ensley of AMGen, Thousand Oaks, California, requests that *Pseudomonas mendocina* be included in Sublist A of Appendix A of the NIH Guidelines. Data in support of the request are included in the submission.

VI. Proposed Amendment of Appendix C-IV to Include Bacillus licheniformis.

In a letter dated March 30, 1988, Dr. Joseph R. Fordham of Nova Laboratories, Inc., Danbury, Connecticut, request that *Bacillus licheniformis* be included to the title and to the first sentence of Appendix C-IV of the NIH Guidelines. This section would be amended to read as follows:

"Appendix C-IV. Experiments Involving Bacillus subtilis or Bacillus licheniformis Host-Vector Systems.

"Any asporogenic Bacillus subtilis or Bacillus licheniformis strain...."

Data in support of the request are included in the submission.

OMB's "Mandatory Information Requirements for Federal Assistance Program Announcements" (45 FR 39592) requires a statement concerning the official government programs contained in the Catalog of Federal Domestic Assistance. Normally NIH lists in its announcements the number and title of affected individual programs for the guidance of the public. Because the guidance in this notice covers not only virtually every NIH program but also essentially every Federal research program in which DNA recombinant molecule techniques could be used, it has been determined to be not cost effective or in the public interest to attempt to list these programs. Such a list would likely require several additional pages. In addition, NIH could not be certain that every Federal program would be included as many Federal agencies, as well as private organizations, both national and international, have elected to follow the NIH Guidelines. In lieu of the individual program listing, NIH invites readers to direct questions to the information address above about whether individual programs listed in the Catalog of Federal Domestic Assistance are affected.

Dated: April 5, 1988

/s/ James C. Hill

James C. Hill, Ph.D.
Acting Director
National Institute of Allergy and
Infectious Diseases

Lilly

Eli Lilly and Company

Lilly Corporate Center
Indianapolis, Indiana 46285
(317) 276-2000

1311

December 4, 1987

Dr. William J. Gartland, Jr.
Director
Office of Recombinant DNA Activities
Building 31, Room 3B10
National Institutes of Health
Bethesda, Maryland 20892

Re: Petition to conduct Large Scale experiments and production involving Cephalosporium acremonium strain LU4-79-6 with less than BL1-LS containment conditions under the "Guidelines for Research Involving Recombinant DNA Molecules" (Guidelines)

Dear Dr. Gartland:

Eli Lilly and Company (Lilly) hereby petitions to conduct Large Scale (LS) experiments and production with recombinant Cephalosporium acremonium strain LU4-79-6 ([r]DNA strain) under less than BL1-LS conditions. This specific strain and

the physical containment conditions are more fully described in the accompanying attachment.

BACKGROUND:

Lilly is preparing to evaluate the [r]DNA strain in a production scale fermenter to determine if favorable yields of a cephalosporin antibiotic can be obtained under industrial conditions and, if so, to subsequently engage in LS production.

Lilly believes that the [r]DNA strain constructed by Lilly scientists, like its non-recombinant progenitors, is non-pathogenic and that LS-experiments and production with the [r]DNA strain in production scale fermenters under conditions less restrictive than BL1-LS would pose no danger to humans or the environment. The basis for this belief is more fully described in the accompanying attachment in addition to being summarized below as follows:

Non-[r]DNA strains of C. acremonium (all derived from a common ancestral strain ATCC 11550, see attachment for a discussion regarding importance of the ATTC designation for this lineage of strains) have been used safely without containment to a closed system at industrial scale for over 25 years; we believe this experience has clearly established the non-pathogenicity of the host

fungus. The difference between the [r]DNA strain and its immediate non-recombinant progenitor, which is currently used at an industrial scale, has been well-characterized in laboratory experiments. The [r]DNA strain contains an extra copy of a key C. acremonium antibiotic biosynthetic gene that results in increased production of the cephalosporin antibiotic. This gene and heterologous vector DNA, present in the [r]DNA strain in a stably integrated form, are described and discussed in detail in the accompanying attachment. Genetic stability of the [r]DNA strain and host characteristics (no sexual system, no transducing virus, attenuated nature of host) that provide biological containment of recombinant DNA in the [r]DNA strain are also described. Based on the above-noted experience with the non-[r]DNA C. acremonium strains, and the well-characterized nature of the [r]DNA strain and its immediate non-[r]DNA parent strain, Lilly believes that the [r]DNA strain is non-pathogenic and safe for Large Scale experiments and production under conditions less restrictive than BL1-LS without the need for experiments beyond

those described herein. Investigators at Lilly have experimented with the [r]DNA strain for eight months under BL1 conditions in the laboratory and for four months under BL1-LS conditions in 150-liter pilot plant vessels to establish the strain's yield superiority and to observe its general characteristics. Favorable yields were observed and the fundamental non-pathogenic characteristics of the C. acremonium host were not changed by the presence of the recombinant DNA.

Lilly believes that the [r]DNA strain may be considered or classified as a product of self-cloning experiments because the construction of the [r]DNA strain involved insertion of DNA from C. acremonium strain ATCC 11550 back into a non-recombinant derivative of ATCC 11550 using a vector. Although the vector contains heterologous DNA, that DNA in the [r]DNA strain does not change the non-pathogenic character of the host and does not present a significant risk to health or the environment.

If this petition is granted, Lilly intends to conduct LS-experiments and, if LS-experiments justify, subsequent production with the [r]DNA strain in industrial scale fermenters under conditions less restrictive than BL1-LS, if its IBC concurs.

Sincerely,

ELI LILLY AND COMPANY

Mark A. Foglesong

Dr. Mark A. Foglesong
Secretary, Institutional Biosafety Committee

MAF:pkp

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NATURE OF *CEPHALOSPORIUM ACREMONIUM* STRAIN LU4-79-6

Parent of strain LU4-79-6 : *C. acremonium* strain 394-4 is the immediate parent of strain LU4-79-6. *C. acremonium* strain 394-4 was derived from *C. acremonium* strain ATCC 11550 by many rounds of mutation/screening and mutation/selection for improved production of cephalosporin C. Recombinant DNA technology was not used. *C. acremonium* strain 394-4 has been used more than 5 years safely at production scale in the manufacture of cephalosporin antibiotics without evidence of any pathogenicity.

General description of ATCC 11550 and its non-recombinant derivatives

NOMENCLATURE: Parenteral cephalosporin antibiotics are manufactured from cephalosporin C, a natural product of a non-pathogenic lineage of filamentous fungal strains, which consists of the original ancestral strain, ATCC 11550, and its non-recombinant derivatives such as strain 394-4. ATCC 11550 and its derivatives represent one of many species of morphologically similar filamentous fungi that have been grouped together under the name, hyphomycetes, by W. Gams [Cephalosporium-like Schimmelpilze (Hyphomycetes), Gustav Fisher Verlag, Stuttgart, 1971]. It is important to distinguish ATCC 11550 and its derivatives from other hyphomycetes which have been designated with the following species names: *Cephalosporium acremonium*, *Cephalosporium chrysogenum*, *Acremonium chrysogenum*, and *Acremonium strictum*. Various investigators have referred to ATCC 11550 and its derivatives by each of these species names and other investigators have referred to some pathogenic fungi with the same species names; many different fungi bear or have borne these same species designations. Biochemical traits that give rise to pathogenicity have not been used as the basis for assigning these species designations. Thus pathogenicity or non-pathogenicity cannot be assumed on the basis of the species designations. However, association of traits with a particular culture type [such as ATCC 11550] which is preserved in a recognized culture collection [such as the American Type Culture Collection (ATCC)] under a unique collection number, is unambiguous. Therefore to refer unambigously to the fungal strains that served as the progenitors of the recombinant strain LU4-79-6 one should always use the unique designation "ATCC 11550 and derivatives". The species designation associated with the ATCC designation is of less importance; continued use of the original species name has the advantage of constancy.

NATURAL HABITAT. Hyphomycetes have been isolated from soil, decaying plant material, and in the case of ATCC 11550, from sea water near a sewage outfall off the Sardinian coast [G. Brotzu, Richerche su di un nuovo antibiotico. Lav. Ist. Ig. Cagliari, 1948; Abraham; Pharmacol. Rev. 14, 473, 1962].

GROWTH REQUIREMENTS. ATCC 11550 and most high-yielding derivatives used industrially to produce cephalosporin C are able to grow on minimal media and require no special nutrients for survival. In fact, mutations which introduce a nutrient requirement usually lower production of cephalosporin C.

TEMPERATURE RANGE FOR GROWTH. ATCC 11550 and its derivatives grow best at about 27 C and grows poorly at temperatures above 30 C and virtually not all at 37 C and above. This characteristic makes it highly unlikely that ATCC 11550 and its derivatives could ever cause an opportunistic infection in mammals. Not surprisingly no opportunistic infection with ATCC 11550 or its derivatives has ever been reported among employees that work with this fungus at industrial scale. This use covers a period of approximately 25 years. Again the specific nature of ATCC 11550 and its derivatives must be emphasized as distinct from other fungi that have been classified as varieties of the *Cephalosporium acremonium*, *Cephalosporium chrysogenum*, *Acremonium chrysogenum*, or *Acremonium strictum*. The range of temperatures allowing growth of ATCC 11550 and its derivatives is not characteristic of all fungi bearing these species designations. Some fungi classified as varieties of *Acremonium strictum* may be able to grow at 37 C.

SPORULATION. As indicated by W. Gams, ATCC 11550 is notable for its poor conidiation. Conidial spores are produced when the strain grows on solid medium so that spore-bearing aerial mycelia, conidiophores, can be formed. High-yielding derivatives of ATCC 11550 form even fewer conidia than ATCC 11550. Vegetative hyphae of ATCC 11550 and high-yielding derivatives of ATCC 11550 fragment after exponential growth in liquid medium. Both conidia and fragmented hyphae are similar to vegetative hyphae in their sensitivity to adverse conditions and appear to serve merely as a means for dispersal.

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GROWTH RATE: The growth rate of ATCC 11550 is slower than most soil organisms, e.g. bacteria such as Streptomyces and Pseudomonas species and fungi such as Aspergillus species. The high-yielding derivatives of ATCC 11550 grow several times slower than the wild type ATCC 11550. Co-inoculation of equal numbers of spores of a typical high-yielding strain and ATCC 11550 onto enriched agar medium results, after a week's growth at 25 C, in a confluent lawn of ATCC 11550. Less than one per cent of cells are represented by the slower growing high-yielding strain.

MECHANISMS BY WHICH ATCC 11550 EXCHANGES GENETIC INFORMATION. Exchange of genetic information is rare in ATCC 11550 and its derivatives. ATCC 11550 has no sexual system and reproduces solely by mitotic division. Rare heterokaryon formation can be demonstrated by inoculating ca. 10^6 conidia of each of two different stable auxotrophs of ATCC 11550 or its derivatives onto supplemented medium, allowing growth to confluency (e.g. 10^9 viable count). Removal of the mycelial mat, wash of that mat, fragmentation of the mycelia, and plating on minimal medium can yield ca. 10 heterokaryons. Under optimal conditions one heterokaryon may be formed per 10^8 colony forming units after such a process. Induced mitotic segregation may yield ca. 50 recombinant progeny from the heterokaryons. The frequency of genetic recombination can be increased 100 fold if cell walls are removed and protoplast fusion is brought about by special laboratory manipulations. Attempts have been made to form stable heterokaryons of ATCC 11550 with other fungi, e.g. *Penicillium chrysogenum*, using protoplast fusion without success. It may be safely surmised from laboratory experience that ATCC 11550 and its derivatives when present, diluted in sewage or soil, would recombine at a negligible rate with itself and even more rarely, if at all, with other fungi or bacteria. Natural genetic exchange of ATCC 11550 with other organisms has never been documented.

General Safety of *C. acremonium*: Strains of *C. acremonium* derived from ATCC 11550 have been used for over twenty-five years in the production of cephalosporin C for manufacture of parenteral cephalosporin antibiotics, e. g. cephalothin, cefazolin, cefamandole. The production is world-wide by more than 10 major pharmaceutical companies. No indication of pathogenicity of these *C. acremonium* strains has ever been observed. In addition, *C. acremonium* ATCC 11550 and derivatives of this strain have been studied in universities throughout the world and no indication of pathogenicity of those strains have been observed. The literature [e.g. see below] on the biosynthesis and manufacture of cephalosporins by *C. acremonium* [ATCC 11550 and derivatives] has been reviewed many times; no indication of pathogenicity for these strains has ever been cited **[Attachment #1]**.

Many experts have extensive experience working with strains of *C. acremonium* [derived from ATCC 11550] in industry (e.g. Dr. Stephen W. Queener at Lilly) and/or university and are qualified observers of the non-pathogenic nature of *C. acremonium* at the production level and/or laboratory level. If desired, a partial list of experts from other pharmaceutical companies and from universities can be provided. For the most part, those names can be derived from the list of authors of reviews given in Attachment #1.

The British counterpart of ORDA--the Health and Safety Executive--acting on the recommendation of the Genetic Manipulation Advisory Group at Medical Research Council--the British counterpart for RAC--formally recognized the non-pathogenic nature of *Cephalosporium acremonium* , ATCC 11550 and derivatives, [also designated *Acremonium chrysogenum*] in 1979 by exempting the organisms from statutory notice for self-cloning experiments **[Attachment #2]**.

Transforming DNA used to produce *C. acremonium* strain LU4-79-6:

C. acremonium strain LU4-79-6 was obtained by transforming *C. acremonium* strain 394-4 with plasmid pPS56 **[Attachment #3]**. Transformation of *C. acremonium* has been described previously **[Attachment #4 and #6]**.

pPS56 contains the following genetic elements: (1) *E. coli* origin of replication [ColE1 ori, source pBR322 via intermediate plasmid pMLC12 **[Attachment #5]**. (2)

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M13 ori, source plasmid pMLC12 [see **Attachment #5**] (3) chloramphenicol acetyl transferase [CAT] gene [source pBR328 via intermediate plasmid pMLC12 [see **Attachment #5**]. (4) hybrid hygromycin B phosphotransferase [HPT] gene [**Attachment #6**]. This gene was constructed by splicing the promoter sequence from the *C. acremonium* isopenicillin N synthetase gene [**Attachment #7**] to the open reading frame of an HPT gene from *E. coli* [**Attachment #8**]. The *E. coli* strain was isolated from swine intestine. (5) the *C. acremonium* expandase/hydroxylase gene [**Attachment #9**] from *C. acremonium* strain ATCC11550.

All other DNA present in pPS56 represents either *C. acremonium* sequences which were adjacent to the expandase/hydroxylase gene in the *C. acremonium* genome or represent short , non-coding, heterologous DNA sequences. The latter sequences serve to connect the genetic elements in pPS56. Their presence reflects the mechanics of constructing pPS56.

Location and stability of transforming DNA in *C. acremonium* strain LU4-79-6

The transforming DNA in *C. acremonium* strain LU4-79-6 is stably integrated into the high molecular weight DNA of the transformant [**Attachment #10**]. The insertion is stable and addition of hygromycin B to fermentation media is not required for maintenance of the inserted transforming DNA or for maintenance of the transformed phenotype: (1) resistance to hygromycin B (2) increased intracellular expandase activity (3) increased conversion of penicillins to cephalosporin C. The high molecular weight DNA of *C. acremonium* strain LU4-79-6 is believed to be a poor transforming agent; attempts to transform *E. coli* to chloramphenicol resistance with total *C. acremonium* LU4-9-6 DNA have failed (see below).

Character of genetic elements in transforming DNA of *C. acremonium* strain LU4-79-6

Genetic elements that are not expressed in C. acremonium LU4-79-6:

ColE1 origin of replication. The ColE1 origin of replication in pPS56 does not function in *C. acremonium* strain LU4-79-6. No freely replicating forms of pPS56 can be detected in the *C. acremonium* transformant. This origin allowed large amounts of pPS56 to be made readily (from *E. coli*) to aid transformation of fungus with pPS56.

M13 origin of replication. This origin encodes no proteins. It does not function without other genes present in helper or intact phage [see **Attachment #5**] and therefore could not function in *C. acremonium*. This DNA serves to connect the CAT gene and ColE1 ori in pPS56.

CAT gene. pPS56 transforms *E. coli* to chloramphenicol resistance efficiently however, there is no free pPS56 in strain LU4-79-6. The CAT gene is not expressed in *C. acremonium* strain LU4-79-6 [**Attachment #12**]. The CAT gene allowed large amounts of pPS56 to be made (in *E. coli*) to aid transformation of fungus with pPS56.

Genetic elements that are expressed in C. acremonium LU4-79-6:

Hybrid HPT gene. The HPT gene of pPS56 is a hybrid, dominant, selectable gene that codes for hygromycin B phosphotransferase [HPT], an enzyme that catalyzes the phosphorylation of hygromycin B. Hygromycin B is an aminoglycoside antibiotic. ATCC 11550, strain 394-4, and intermediate strains of this *C. acremonium* lineage are sensitive to hygromycin B. Expression of the hybrid HPT gene in *C. acremonium* transformants [**Attachments #11 & 4**] imparts resistance to hygromycin B; the phosphorylated form of hygromycin B is not toxic to *C. acremonium*.

Expandase/hydroxylase gene. This natural *C. acremonium* gene codes for a bifunctional enzyme, expandase/hydroxylase, which catalyzes the conversion of penicillin N to deacetoxycephalosporin C and catalyzes the conversion of the latter compound to deacetylcephalosporin C. These steps occur in the biosynthesis of

cephalosporin C. The presence of an extra copy of the expandase/hydroxylase gene in *C. acremonium* strain LU4-79-6 results in increased production of expandase/hydroxylase relative to parent strain 394-4 [**Attachment #13**]. Increased intracellular concentration of expandase/hydroxylase correlates to increased conversion of penicillin N (less penicillin excreted) into cephalosporins (increased production of cephalosporin C) relative to parent strain 394-4 [**Attachment #13**].

Safety of Strain LU4-79-6

Recombinant strain is non-pathogenic. Strains of *C. acremonium* that contain the above genetic elements stably integrated into their genomes could not reasonably be expected to be rendered pathogenic by virtue of integration of the DNA in pPS56. *C. acremonium* has been subjected to mutagen treatment and selection for over 25 years and no pathogenic strain has arisen. The likelihood of inducing pathogenicity due to the physical presence of inserted pPS56 DNA into a genetic element of the *C. acremonium* genome is therefore not reasonable. The expression of the HPT gene in *C. acremonium* and presence of this enzyme in the cytoplasm [**Attachments 4&11**] could not reasonably to expected to render *C. acremonium* pathogenic. The ColE1 ori, M13 ori, and CAT gene are not expressed in *C. acremonium* LU4-79-6 and presence of these elements in *C. acremonium* could not reasonably be expected to render *C. acremonium* pathogenic. Eight months of observation in the laboratory under BL1 conditions and four months observation in the pilot plant under BL1-LS conditions confirm the expected non-pathogenic character of strain LU4-79-6.

Presence of resistance genes in recombinant strain cannot adversely affect treatment of infectious bacteria. The chloramphenicol acetyl transferase [CAT] gene and the open reading frame of the hygromycin B phosphotransferase [HPT] gene already exist in nature and in bacterial pathogens. Chloramphenicol is used in clinical medicine to treat a few bacterial pathogens. The use is narrow because of the toxicity of chloramphenicol. *C. acremonium* and fungi in general are not sensitive to chloramphenicol at clinically useful concentrations used to kill bacteria. Chloramphenicol is therefore not used to treat fungal infections. Hygromycin B is used to treat bacterial dysentery in swine. Hygromycin B is relatively toxic and is not approved for use in human medicine; it is also not approved for treatment of fungal infections in animals or plants, and approval is not being sought or considered.

C. acremonium is an asexual fungus, i.e. it lacks a system for genetic exchange. *C. acremonium* has an endogenous dsRNA mycophage which is transferred only through spores; no phage-mediated gene transfer has ever been observed. The genetic isolation of *C. acremonium* is familiar to all who have worked with this fungus in the laboratory. The probability of transfer of the CAT and/or HPT gene from integrated forms in the genome of *C. acremonium* to a bacterium is virtually nonexistent and certainly less than transfers among bacteria that certainly do occur frequently. Attempts have been made to transfer HPT gene from *C. acremonium* strain LU4-79-6 to *C. acremonium* strain ATCC 11550 during mixed growth of these strains [**Attachment #14**]. No transfers were observed. Attempts have been made to transfer pPS56-derived CAT gene from *C. acremonium* strain LU4-79-6 to *E. coli* by cell to cell interaction and by exposure of total DNA from LU4-79-6 to *E. coli* [**Attachment #14**]. No transfers were observed. The high molecular weight DNA of *C. acremonium* LU4-79-6 is believed to be a poor transforming agent. Even if a rare transfer of the CAT or HPT gene from a *C. acremonium* strain to a bacterium occurred, this would represent a negligible addition to the pool of bacterial chloramphenicol resistance genes and hygromycin B resistance genes already present in nature. Little or no expression of the HPT open reading frame via fungal

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promoter in the hybrid gene would be expected upon transfer of that gene to a bacterium.

C. acremonium strains used for production of cephalosporin C at production scale are at a great competitive disadvantage with respect to the original wild type fungal strain or streptomycetes abundantly present in the soil. Strain 394-4 and LU4-79-6 grow much more slowly and form far fewer conidia than the wild type fungus and soil streptomyces. The competitive disadvantage has been demonstrated in the laboratory by inoculating equal number of wild type and production strain cells to agar medium. Confluent growth recovered from the medium was predominantly (>99%) wild type.

PHYSICAL CONTAINMENT CONDITIONS INTENDED FOR LS EXPERIMENTS AND PRODUCTION WITH LU4-79-6

LS experiments in industrial scale fermenters are required to determine if superior cephalosporin C yields, previously observed for LU4-79-6 in the laboratory and pilot plant, can be obtained under production-like conditions. LS experiments and, later, production with LU4-79-6 would be carried out in 68,000 liter stainless steel fermenters, each equipped with cooling coils and flat-bladed turbine-driven agitators. Stacked Clipper Oil Seals (lip seals) at the opening where an agitator shaft enters a fermenter would restrict egress of the LU4-79-6 cells from a fermenter and would minimize entry of organisms into the fermenter. Exhaust gas from a fermenter would not be filtered, but a tangential-entry cyclonic separator would restrict loss of LU4-79-6 cells via the exhaust gas. A fed-batch fermentation with forced aeration would be carried out. After fermentation, the culture of LU4-79-6 cells would be pumped from a fermenter to a belt filter and LU4-79-6 cells would be separated from broth by the belt filter. Any cells remaining in the belt-filtered broth would be removed by a resin-mediated acidification step and a press filter. Cells removed by the belt filter would be sent to a holding tank. The above process will be run in accordance with good industrial large scale practice.

Effluents containing significant quantities of LU4-79-6 cells, e.g. rinse water from the belt filter and contents of the holding tank, would be inactivated prior to removal from the facilities used to manufacture cephalosporin antibiotics. These physical conditions would limit introduction of viable LU4-79-6 cells into the environment and would prevent the release of high concentrations of viable LU4-79-6 cells. Lilly believes that strain LU4-79-6 is safe and that viable cells of this organism, which would reach the environment in low concentration using the above process, are not hazardous to man or his environment.

A schematic of the process for preparing cephalosporin C with LU4-79-6 and for isolating cephalosporin C from the broth produced by LU4-79-6 is shown below.

Dr. Stephen W. Queener, Senior Research Scientist, Eli Lilly and Company, Indianapolis, IN 46285

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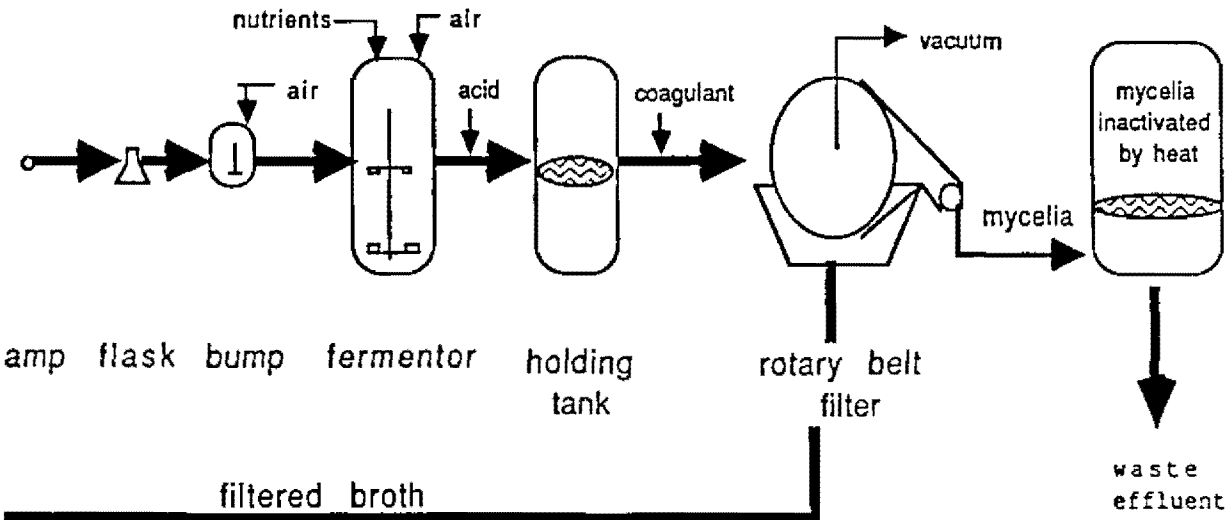
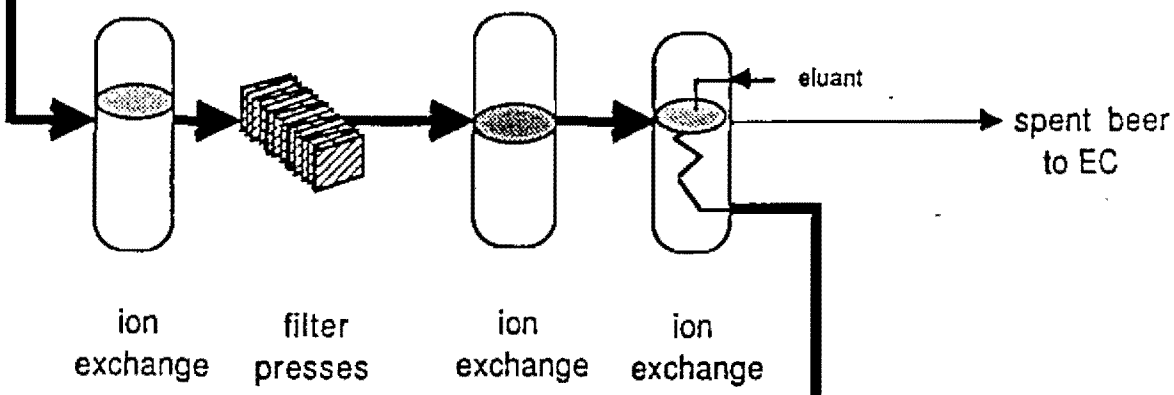
Ceph C Production Flowsheet:

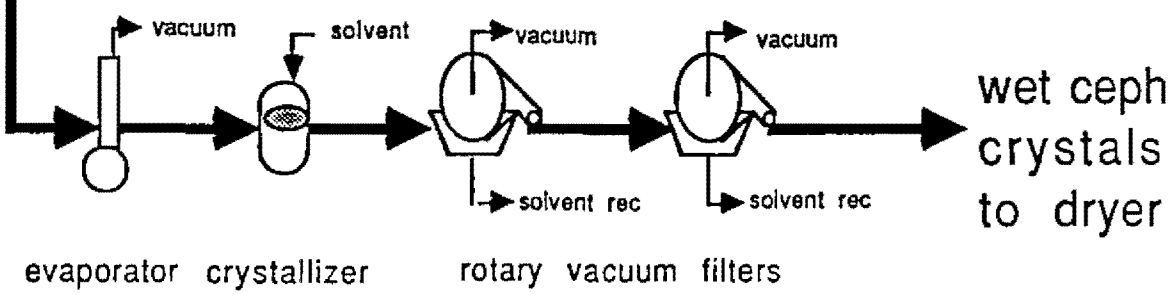
A. Fermentation:



B. Isolation:



C. Crystallization:



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The extensive literature on biosynthesis and manufacture of cephalsporins by *C. acremonium* cite no evidence for pathogenicity, see partial list of reviews below:

1. Bunnell, C. A., Wayne D. Luke, and Fred M. Perry, Jr. "Industrial Manufacture of Cephalosporins." in Beta-lactam Antibiotics for Clinical Use. Ed. S. F. Queener, J. A. Webber, S.W. Queener. Marcel Dekker, Inc., New York (1986) pp. 255-283.
2. Ball, Christopher. "Filamentous Fungi." in Genetics and Breeding of Industrial Microorganisms. Ed. C. Ball. CRC Press, Inc. Boca Raton, Florida. (1984) pp. 159-188.
3. Elander, Richard P., and Hatsuo Aoki. "Beta-lactam-producing Microorganisms: Their Biology and Fermentation Behavior." in Chemistry and Biology of Beta-lactam Antibiotics. Volume 3. Ed. R. B. Morin and M. Gorman. Academic Press. New York. (1982), pp. 83-153.
4. Queener, S. W. and N. Neuss. "The Biosynthesis of Beta-lactam Antibiotics." in Chemistry and Biology of Beta-lactam Antibiotics. Volume 3. Ed. R. B. Morin and M. Gorman. Academic Press. New York. (1982), pp. 83-153.
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7. Demain, Arnold L. "Biochemistry of Penicillin and Cephalosporin Fermentations." Llyodia 37,(1974) pp 147-167.
8. Abraham, E. P. and G.G.F. Newton "Penicillins and Cephalosporins." in Antibiotics Vol. 2 Ed. D. Gottlieb and P.D. Shaw. Springer-Verlag, New York, pp. 1-16.
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GMAG NOTE 8 - SUPPLEMENT 1

This Note should be read in conjunction with GMAG Note 8 (March 1979)

'Self-cloning' experiments

The Health and Safety Executive has accepted the Group's advice that self-cloning experiments using the following organisms should be added to those exempted from statutory notifications:

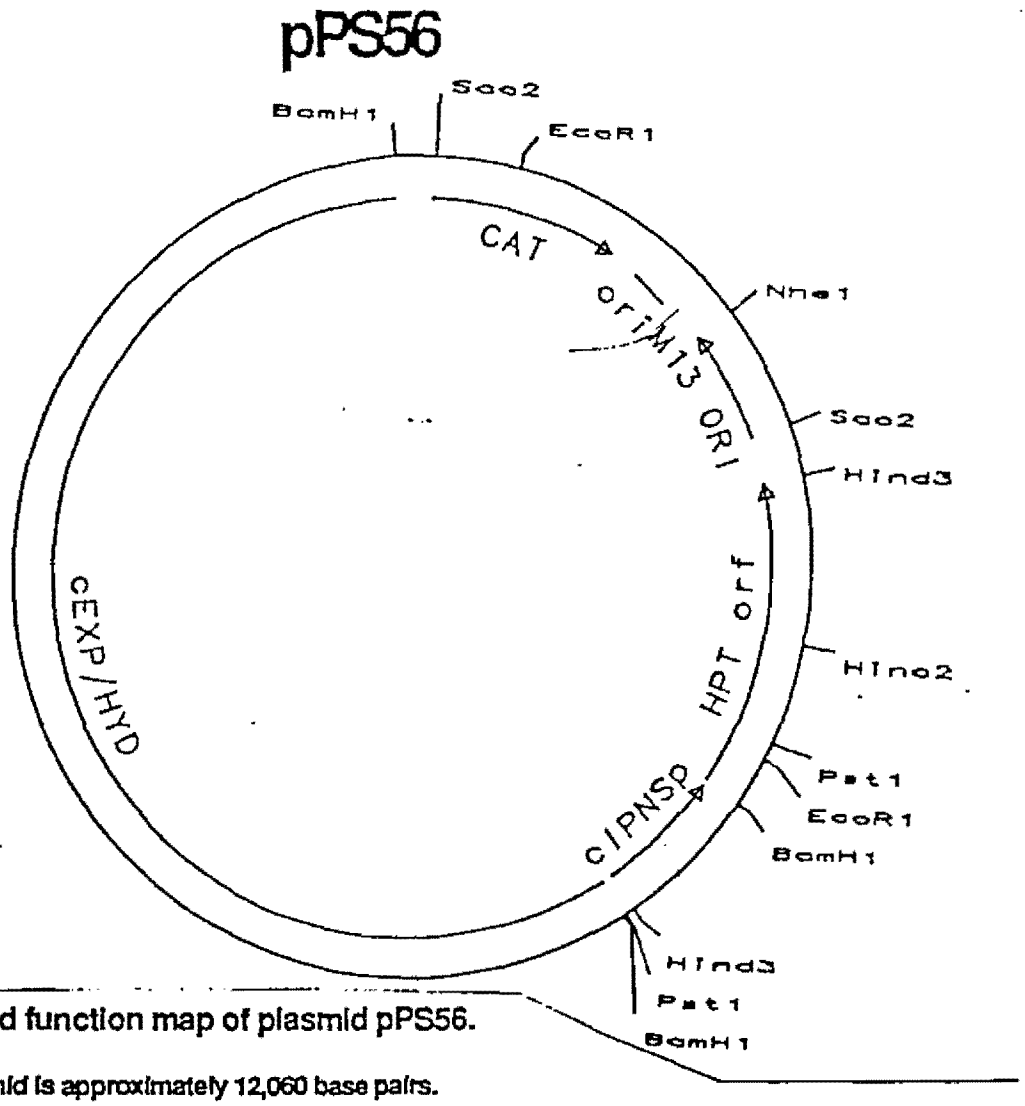
a) Non-pathogenic strains of *Streptomyces*
b) Non-pathogenic strains of *Micromonospora*
c) Strains of *Nocardia mediterranei*
d) *Klebsiella pneumoniae* strain M 5al
e) *Acremonium chrysogenum*
f) *Penicillium chrysogenum*
g) *Bacillus thuringiensis*
h) Mouse cells with polyoma virus (ie cloning mouse DNA back into mouse cells)

Use of the products of genetic manipulation

The Group advise that the products of any of the 'self-cloning' experiments listed above and also clones which are constructed by the insertion of *Xenopus laevis* vitellogenin mRNA sequences may be used under conditions of good microbiological practice.

December 1979

Plasmid pPS56 was used to transform production strain 394-4 to produce transformant LU4-79-6. Strain LU4-79-6 contains the natural expandase/hydroxylase gene plus an additional copy of that gene in the form of pPS56-derived DNA inserted stably into the *C. acremonium* genome.



Restriction site and function map of plasmid pPS56.

Total size of this plasmid is approximately 12,060 base pairs.

<u>DNA for delivery back into *C. acremonium*</u>:
cEXP/HYD = a 7.0 kb fragment of *C. acremonium* DNA which carries the expandase/hydroxylase gene (note: there are at least 3 more Hind3 sites within this fragment, unmapped at this time]

<u>hybrid HPT gene (for selection in *C. acremonium*)</u>:
cIPNSp = promoter from the isopenicillin N synthetase gene of *C. acremonium*
HPT orf = open reading frame from the hygromycin B phosphotransferase gene of *E. coli*.

<u>genetic elements to allow preparation of pPS56 in *E. coli*</u>:
M13 ori = M13 origin of DNA replication (no function, present due to construction mechanics)
ori = ColE1 origin of replication (for replication of pPS56 in *E. coli*)
CAT = chloramphenicol acetyl transferase gene from *E. coli*. (for selection in *E. coli*)

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A System for Genetic Transformation of *Cephalosporium acremonium*

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Eli Lilly and Company, Indianapolis, Indiana 46285

Industrial strains of the fungus *Cephalosporium acremonium* produce large amounts of a beta-lactam antibiotic, cephalosporin C, which is chemically modified to manufacture a number of clinically important parenteral cephalosporin antibiotics, e.g., cefamandole and cephalothin (2). The growth of the wild type or industrial strains of *C. acremonium* is not inhibited by a variety of beta-lactam antibiotics, including cephalosporin C. Several bacteria produce beta-lactam antibiotics, but because of the bacterial peptidoglycan cell wall structure, their growth can be inhibited by these antibiotics if the antibiotics reach and react with sensitive membrane-bound "penicillin-binding proteins." By applying recombinant DNA technology to *C. acremonium*, it might be possible to ferment large amounts of beta-lactam compounds that are more useful than cephalosporin C as starting materials for the manufacture of cephalosporin antibiotics. To apply recombinant DNA methods to *C. acremonium*, we have constructed plasmid pIT221 (Fig. 1) and have devised conditions which allow this plasmid and related constructs to genetically transform *C. acremonium*. The following genetic elements were included in pIT221 to make it a useful vector for cloning DNA into *C. acremonium*.

(i) The origin of DNA replication and the ampicillin resistance marker of pBR322 were included on a 2.8-kilobase (kb) fragment (Fig. 1, region bounded by the 8 o'clock *Pst*I site and the 12 o'clock *Sal*I/*Xho*I junction). These elements allow pIT221 to transform *Escherichia coli*. Large amounts of pIT221 are easily obtained from ampicillin-resistant *E. coli* transformants.

(ii) An autonomous replication sequence was included in pIT221 on a 1.9-kb fragment of *C. acremonium* mitochondrial DNA (Fig. 1, region bounded by the 5:30 and 8 o'clock *Pst*I sites) to increase the frequency of transformation of *C. acremonium* by the plasmid vector. Per microgram of transforming DNA, *Saccharomyces cerevisiae* (yeast) replicative plasmids (YRp), e.g., YRp7, produce 50- to 5,000-fold more yeast transformants than *S. cerevisiae* integrative plasmids (YIp; e.g., YIp5) (8). The 1.9-kb *C. acremonium* mitochondrial DNA fragment, when inserted into YIp5, produced plasmid pPS1 (5), which replicates in *S. cerevisiae*. Thus, by definition (7), the fragment is an autonomous replication sequence. Esser and Tudzynski (9) have suggested that this fragment contains a *C. acremonium* origin of replication.

(iii) A hybrid gene, known to be dominant and selectable in *S. cerevisiae* (3), was included in pIT221. In lieu of a *C. acremonium* promoter, which was not available, a promoter from the 5' end of the yeast phosphoglycerol kinase gene was spliced, in correct translational phase, to the protein-coding region of a bacterial gene to obtain the hybrid gene. When the bacterial protein-coding region is transcribed and its mRNA is translated, a hygromycin phosphotransferase (HPT) is formed, and this enzyme phosphorylates the broad-spectrum aminocyclitol antibiotic hygromycin B (4). Hygromycin B at low concentration, e.g., 10 μg/ml, in the absence of magnesium ion and other antagonists, kills *C. acremonium*. However, the phosphorylated product formed from ATP and hygromycin B by HPT catalysis is not toxic to *C. acremonium*, even when present at 1 mg/ml. The hybrid HPT gene in pIT221 is bounded (Fig. 1) by the 5 o'clock *Bam*HI site and a 2:30 *Bam*HI/*Bgl*II junction (end of arrow point, Fig. 1).

(iv) A unique *Sph*I site for cloning was included in a nonessential fragment of pBR322 present in the 0.275-kb region of pIT221 bounded by the *C. acremonium* autonomous replication sequence and the hybrid HPT gene. A nonessential, nonfunctional *Sma*I fragment of bacterial DNA (Fig. 1, smaller region bounded by *Sma*I sites at 11:58 and approximately 1 o'clock) was also included in pIT221 and may be replaced by cloned *Sma*I fragments.

Before attempting to transform *C. acremonium*, the biological activities of the *C. acremonium* autonomous replication sequence and the hybrid HPT gene, now present in pIT221, were retested in *S. cerevisiae*. These elements were active in the parent plasmids used to construct pIT221, and restriction mapping demonstrated the presence of the elements in pIT221 (Fig. 1), but mapping could not rule out minute inactivating alterations during construction. Therefore, pIT221 was used to transform the hygromycin B-susceptible (HgmB^s) *S. cerevisiae* strain DBY-746. DBY-746 contains no pBR322-hybridizing DNA sequences. Covalently closed circular and

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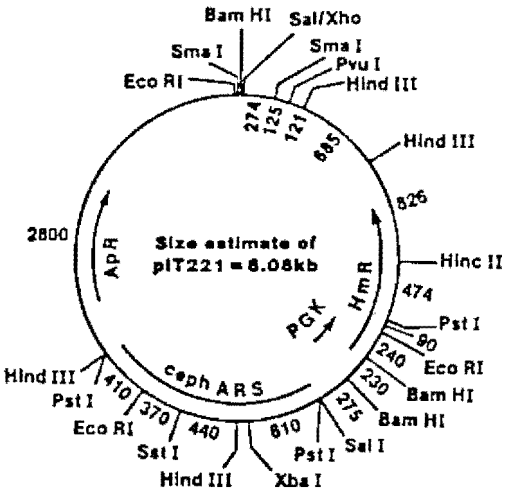

FIG. 1. Restriction map of plasmid vector pIT221. See text for description of genetic elements and cloning sites in plasmid.

open-circle forms of pIT221 were observed as pBR322-hybridizing DNAs in the hygromycin-resistant (HgmB^r) transformant pIT221-DBY-746 (Fig. 2). Thus, both the replicating activity of the autonomous replication sequence and the hygromycin B-detoxifying activity of the hybrid HPT gene in pIT221 were demonstrated. HgmB^s segregants were observed to arise spontaneously from the HgmB^r transformant, pIT221-DBY-746, at high frequency, as expected for spontaneous loss of the plasmid bearing the autonomous replication sequence and HPT, pIT221. A systematic examination of many conditions led to the discovery of parameters that allowed pIT221 to transform *C. acremonium*. To our knowledge, a system for the genetic transformation of this fungus has not been published in a scientific journal or proceedings and, therefore, the conditions are outlined below.

To obtain *C. acremonium* protoplasts which are transformable, hyphae in the correct physiological state can be prepared reproducibly by starting from a frozen conidial suspension. The hyphae are obtained from this seed by growth in a minimal-base medium containing (in grams per 100 ml): sucrose, 3.16; glucose, 0.22; L-asparagine, 0.75; ammonium acetate, 0.022; potassium dihydrogen phosphate, 1.5; dipotassium monohydrogen phosphate, 2.1; sodium sulfate, 0.075; magnesium sulfate heptahydrate, 0.018; calcium carbonate, 0.2; calcium chloride, 0.006; and (in milliliters) corn steep liquor (4% nitrogen), 0.05; salts solution, 0.1; and water. Salts solution contained (grams per liter): $Fe(NH_4)(SO_4)_2 \cdot 6H_2O$, 15; $MnSO_4 \cdot 4H_2O$, 3; $ZnSO_4 \cdot 7H_2O$, 3; and $CuSO_4 \cdot 5H_2O$, 0.8. Duration of culture should be sufficient to supply 1 g of cells in log-phase growth at 29°C. One gram of hyphae is removed and treated for 90 min at 29°C with 10 mM dithiothreitol to reduce disulfide bonds in cell walls. Dithiothreitol-treated cell walls are effectively removed without loss of transformability of cells by digestion with β-glucuronidase at 25 mg/ml in the presence of McIlvaine's buffer (neutral pH) containing osmotic stabilizer, 0.8 M NaCl, and physiological stabilizer, 0.02 M $MgSO_4$, for 3 h at 29°C. Protoplasts are separated from any

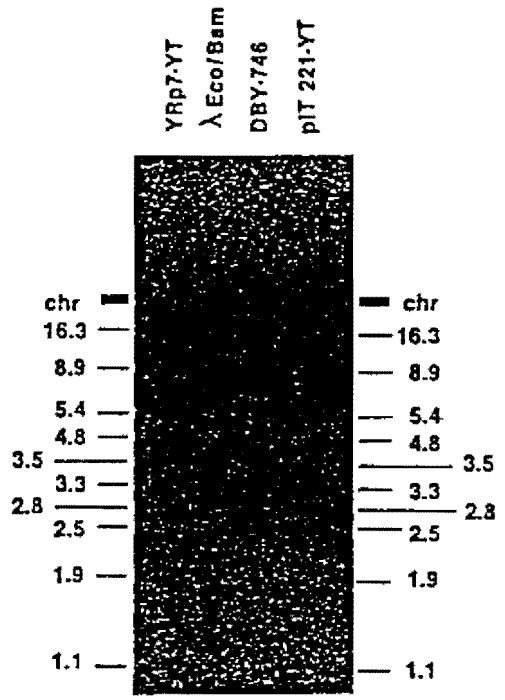

FIG. 2. Biological activities of the *C. acremonium* autonomous replication sequence and the hybrid HPT gene in pIT221 demonstrated by the acquisition of the Hgm^r phenotype concurrent with the physical acquisition of the covalently closed circular and multimeric form of pIT221 in yeast transformant (YT) pIT221-DBY746. Total DNA was extracted from parent *S. cerevisiae* strain DBY-746 and from two transformants of DBY-746 prepared by Ca^{2+}/polyethylene glycol-mediated transformation (method of Beggs [1]) by plasmids YRp7 (5.7 kb) and pIT221 (8.1 kb), respectively. Selections were for tryptophan independence (transformant YRp7-DBY-746) and hygromycin resistance (pIT221-DBY-746). DNAs from each strain were separated by agarose gel electrophoresis, and separated DNAs were probed with ^{32}P-labeled pBR322. Source of DNA (3 μg each lane) was as follows: lane 1, YRp7-DBY-746; lane 2, lambda (DNA applied after digestion with *Eco*RI and *Bam*HI); lane 3, DBY-746; lane 4, pIT221-DBY-746. Specific activity of probe is 1.45×10^8 cpm/μg.

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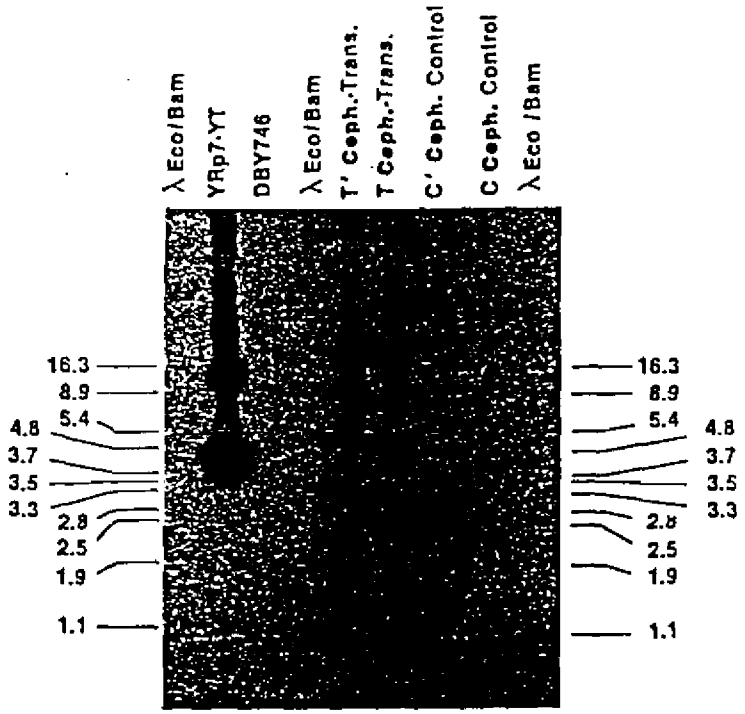

FIG. 3. Presence of pIT221 DNA in the high-molecular-weight DNA of total DNA from HgmB^r pIT221-*C. acremonium* transformant T1. Total DNA was extracted from T1 and from the untransformed HgmB^s *C. acremonium* parental strain, ATCC 11550. DNA molecules in each extract were separated by agarose gel electrophoresis and probed (6) with ^{32}P-labeled pBR322. As a positive control, total DNA was extracted from a yeast transformant, YRp7-DBY-746, known to carry a freely replicating plasmid (YRp7) composed, in part, of pBR322 sequences. As a negative control, total DNA was extracted from the untransformed parental yeast strain, DBY-746, which is known not to contain any DNA with pBR322 sequences. Source of DNA (3 μg, each lane) is as follows: lanes 1, 4, and 9, lambda (DNA applied after digestion with *Eco*RI and *Bam*HI); lane 2, YRp7-DBY-746; lane 3, DBY-746; lanes 5 and 6, HgmB^r pIT221-*C. acremonium* transformant T1; lanes 7 and 8, HgmB^s ATCC 11550. Specific activity of ^{32}P-labeled pBR322 is 3.9 × 10^8 cpm/μg.

undigested hyphae by filtration through porous commercial toweling, collected by centrifugation, washed three times in osmotic-physiological stabilizer to remove nucleases, and resuspended in 0.8 M NaCl. (Magnesium, which alleviates the toxicity of hygromycin B to *C. acremonium*, must be absent from the selection step after transformation.) Transformation is carried out in a mixture containing, per milliliter: 30 × 10^6 protoplasts, 80 μmol of NaCl, 0.5 μl of freshly distilled dimethyl sulfoxide, 53 μmol of $CaCl_2$, 180 mg of polyethylene glycol (Baker 4000), and 2 μg of transforming DNA at neutral pH. After 10 min at room temperature, the protoplasts are removed by centrifugation and are resuspended in 0.8 M NaCl. Cell walls are regenerated, and colonies are recovered on Trypticase soy agar osmotically stabilized with 10.3% sucrose. Low temperature, for example, 15°C, is critical for the early stage of cell wall regeneration. After 24 h, hygromycin B can be added in an overlay to a final medium concentration of 100 μg/ml, and incubation is continued at a higher temperature, e.g., 25°C, to allow transformant cells to grow to colonies of 0.3- to 1-cm diameter in 12 to 60 days. Only colonies which can be subcultured to fresh selective medium are scored as transformants. In 11 of 14 experiments using these conditions, a total of 147 HgmB^r, pIT221-*C. acremonium* transformants were recovered. All untransformed cells are killed under these conditions; a "dummy transformation" in which transforming DNA is omitted was always run as a control in each experiment, and no colonies were ever observed on the selective medium seeded with samples from a "dummy" transformation mixture.

Three *C. acremonium* transformants exhibiting different levels of hygromycin B resistance were chosen for study at the molecular level. Transforming DNA could be demonstrated to have integrated into the high-molecular-weight

DNA of these *C. acremonium* isolates. For example, in the total DNA of transformant T1, high-molecular-weight DNA that hybridized to ^{32}P-labeled pBR322 was observed in Southern hybridization experiments and no ^{32}P-labeled pBR322-hybridizing DNA was observed in the region of agarose gels that would correspond to electrophoretic migration of low-molecular-weight, freely replicating covalently closed circular or open-circle plasmid molecules. No DNA in the total DNA from the untransformed parent strain, ATCC 11550, hybridized to ^{32}P-labeled pBR322 (Fig. 3).

To further characterize the nature of the transformants, total DNA from each transformant was digested to completion with restriction enzymes that did not cut pIT221, or the DNA was digested to completion with enzymes that cut pIT221 once. Each sample of digested total DNA was separated by agarose gel electrophoresis, and separated DNA molecules were probed with ^{32}P-labeled pBR322 as before. Since more than one species of DNA in the total DNA of the transformants was observed to hybridize to ^{32}P-labeled pBR322 under these conditions, one must conclude that multiple insertions of pIT221 DNA into the high-molecular-weight DNA of *C. acremonium* occurred in the formation of the transformants that were examined. For example, when total DNA of transformant T1 was cut with *Kpn*I, which does not cut pIT221, two bands of DNA that hybridized to ^{32}P-labeled pBR322 were observed (lane 9, Fig. 4). Thus, a minimum of two insertions of pIT221 DNA was indicated. When total DNA from T1 was cut with *Sac*I, which cuts pIT221 once, eight bands of DNA that hybridized to ^{32}P-labeled pBR322 were observed in the digested total DNA from T1 (lane 2, Fig. 4). Thus, the estimate of the minimum number of pIT221 insertions in the high-molecular-weight DNA of T1 was raised to at least four. The same results were obtained from T1 cells derived from a single spore of T1 obtained by micromanipulation. No DNA that hybridized to ^{32}P-labeled pBR322 was present in digested total DNA from the untransformed ATCC 11550.

To prove that the basis of the HgmB^r phenotype characterizing the transformants was the expression of the HPT gene in pIT221, two experiments were carried out. In the first experiment ^{32}P-labeled pJC10, a plasmid probe containing the HPT gene, was used to demonstrate the presence of HPT DNA sequences in the high-molecular-weight DNA of transformant T1. For the DNAs involved, the probe, ^{32}P-labeled pJC10, could specifically detect the presence of HPT gene sequences in *C. acremonium* transformants: ^{32}P-labeled pJC10 did not hybridize to total DNA from ATCC 11550, nor to pBR322; and ^{32}P-labeled pJC10 hybridized to restriction fragments of pIT221 only if those fragments contained HPT DNA as judged from the known structure and restriction map of pIT221. ^{32}P-labeled pJC10 hybridized to high-molecular-weight DNA in the total DNA from T1, but did not hybridize to any DNA in the total DNA from ATCC 11550. In the second experiment, the HPT enzyme was shown to be present in cell-free extracts of T1 (assay of Rao et al. [4]), and the enzyme was demonstrated to be absent in extracts of ATCC 11550.

The stability of transformant T1 was examined by growing the transformant for 25 generations in the absence of hygromycin B, diluting

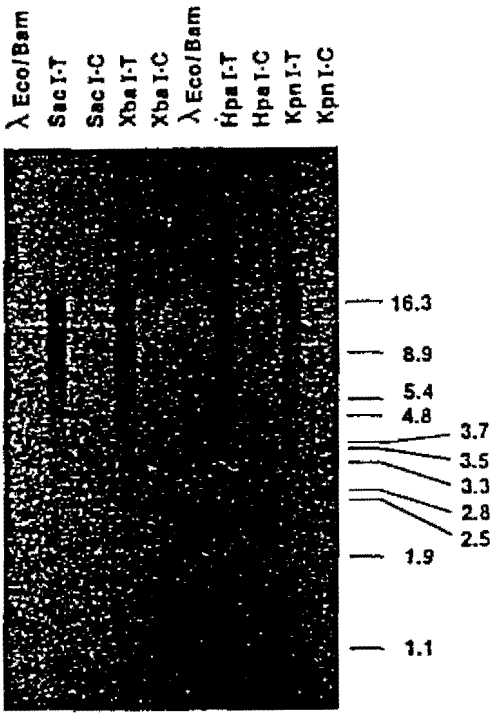

FIG. 4. Multiple inserts of pIT221 DNA in the high-molecular-weight DNA of HgmB^r pIT221-*C. acremonium* transformant T1, demonstrated by multiple ^{32}P-labeled pBR322-hybridizing DNAs in total DNA of T1 that has been completely digested with *Hpn*I, *Kpn*I, *Sac*I, or *Xba*I. Protocol is the same as for Fig. 3 except that total DNAs from T1 and from the untransformed *C. acremonium* parental strain, ATCC 11550, were digested with one of four restriction enzymes, listed above, prior to separation by agarose gel electrophoresis. Source of DNA (3 μg each lane) is as follows: lanes 1 and 6, lambda (DNA digested with *Eco*RI and *Bam*HI prior to electrophoresis); lanes 2, 4, 7, and 9, transformant T1; lanes 3, 5, 8, and 10, untransformed parent ATCC 11550. Specific activity of probe is 1.79×10^8 cpm/μg.

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the resulting culture, and plating equal amounts of the diluted suspension on two media: the same base medium with and without the selective agent (100 μg of hygromycin B per ml). Within experimental error, equal numbers of colonies arose. T1 was also used as seed in a cephalosporin C fermentation employing complex production medium. At the end of the fermentation all harvested cells were observed to be HgmB^r by the same criteria: equal samples of washed cells seeded to selective or nonselective medium produced the same number of colonies. Under the same conditions, strain ATCC 11550 was killed by the hygromycin B.

In summary, a system has been devised that allows low-frequency transformation of *C. acremonium* by integration that produces highly stable transformants. Transforming DNA and its associated phenotype, HgmB^r, were maintained in transformant T1 throughout a cephalosporin C fermentation in the absence of hygromycin B. The system should be useful in producing *C. acremonium* transformants with useful characteristics, particularly transformants which carry extra copies of *C. acremonium* genes to increase cephalosporin C production and transformants which carry hybrid genes to allow in situ modification of cephalosporin C.

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A) DNA Sequence of pMLC12 (source of *E. coli* origin of replication, M13 origin of replication, and chloramphenicol acetyl transferase [CAT] gene in pPS56)

B) *J. Mol. Appl. Genet. 2: 507-517 (1984)*
Note: pMLC12 is same as pSDC12 described in *J. Mol. Appl. Genet. 2: 507-517 (1984)* except *Hind* III site at end of the M13 ori in pSDC12 has been changed to *Nhe* I site in pMLC12.

C) *Gene 20: 305-316 (1982)*
Note: traces origin of chloramphenicol acetyl transferase gene back to pBR328

```
-ymbols 1696 to 1851 come from supF gene (inactivated by CAT segment)
-ymbols 1852 to 2630 come from chloromphenicol acetyltransferase gene
  (CAT gene from Tn9)
-ymbols 2631 to 2671 come from supF gene remnant

P  mlc12.        Checksum:  3986    From     1 to  2671

It     ymes: *

                    MAP  30-OCT-84  15:22          ..

                                S       S         S
                                A  M    AX        AX
                                U  B    UH        UH          B
                                3  O    3O        3O          B
                                A  2    A2        A2          V
                                        /         /
     GAATTTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTC
   1 ----------+---------+---------+---------+---------+---------+ 60
     CTTAAACTCGCAGTCTGGGGCATCTTTTCTAGTTTCCTAGAAGAACTCTAGGAAAAAAAG

        F        F
        NHT      N                      T     T              H
        UHT      U                      T     T              P
        DAH      4                      H     H              A
        212      H                      2     2              2
         //
     TGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGC
  61 ----------+---------+---------+---------+---------+---------+ 120
     ACGCGCATTAGACGACGAACGTTTGTTTTTTTGGTGGCGATGGTCGCCACCAAACAAACG

      S
      A        A                                          H
      U        L                                          H
      3        U                                          A
      A        1                                          1

     CGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATAC
 121 ----------+---------+---------+---------+---------+---------+ 180
     GCCTAGTTCTCGATGGTTGAGAAAAAGGCTTCCATTGACCGAAGTCGTCTCGCGTCTATG

                                      H
                                      A
                                      E
                                      3

     CAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCAC
 181 ----------+---------+---------+---------+---------+---------+ 240
     GTTTATGACAGGAAGATCACATCGGCATCAATCCGGTGGTGAAGTTCTTGAGACATCGTG

                                            F  F
                        M                   N  N
                        N    B  B           U  U
                        L    B  B           4  4
                        1    V  V           H  H

     CGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGT
 241 ----------+---------+---------+---------+---------+---------+ 300
     GCGGATGTATGGAGCGAGACGATTAGGACAATGGTCACCGACGACGGTCACCGCTATTCA

              S     H                              F
            HNC     I                  H          HN
            PCR     N                  P          HU          B
            AIF     F                  A          A4          B
            211     1                  2          1H          V
              /
     CGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCT
 301 ----------+---------+---------+---------+---------+---------+ 360
     GCACAGAATGGCCCAACCTGAGTTCTGCTATCAATGGCCTATTCCGCGTCGCCAGCCCGA

                      BH
                      SG        A                          D
                      PI        L                          D
                      1A        U                          E
                      21        1                          1
                       /
     GAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGAT
 361 ----------+---------+---------+---------+---------+---------+ 420
     CTTGCCCCCCAAGCACGTGTGTCGGGTCGAACCTCGCTTGCTGGATGTGGCTTGACTCTA
```

37

```
                                   HA
                                   AC
                                   12

     ACCTACAGCGTCAGCATTGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGT
   1 ————————————————————————————————————————————————————————————→ 480
     TGGATGTCGCACTCGTAACTCTTTCGCCGTGCGAAGGGCTTCCCTCTTTCCGCCTGTCCA

   1          F                                          BS
     H        N                   M   H         A        SC
     P        U                   N   H         L        TR
     A        4                   L   A         U        NF
     2        H                   1   1         1        11
                                                          /
     ATCCGGTAAGCCGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACG
 481 ————————————————————————————————————————————————————————————→ 540
     TAGGCCATTCGCCGTCCCAGCCTTGTCCTCTCGCGTGCTCCCTCGAAGGTCCCCCTTTGC

     BS                                                      S
     SC                                  H         M         TF
     TR                                  G         N         AA
     NF                                  A         L         ON
     11                                  1         1         11
     /
     CCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGT
 541 ————————————————————————————————————————————————————————————→ 600
     GGACCATAGAAATATCAGGACAGCCCAAAGCGGTGGAGACTGAACTCGCAGCTAAAAACA

                                        0-649 pBR322 ori  650-1248 M13 ori
                                                                  A         A
                N                                   AN        A         A
                L                                   LH        L         L
                A                                   LE        U         U
                4                                   11        1         1
                                                          /
     GATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCAAGCTAGCTTCTAG
 601 ————————————————————————————————————————————————————————————→ 660
     CTACGAGCAGTCCCCCCGCCTCGGATACCTTTTTGCGGTCGTTGCGTTCGATCGAAGATC

                                F
                                N                         A
                S               U                         L
                S               D                         U
                F               2                         1

     CTAGAAATTGTAAACGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGC
 661 ————————————————————————————————————————————————————————————→ 720
     GATCTTTAACATTTGCAATTATAAAACAATTTTAAGCGCAATTTAAAAACAATTTAGTCG

                    H                                     A
                    A                                     V
                    E                                     A
                    3                                     1

     TCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGCCC
 721 ————————————————————————————————————————————————————————————→ 780
     AGTAAAAAATTGGTTATCCGGCTTTAGCCGTTTTAGGGAATATTTAGTTTTCTTATCGGG

                                        H                     H
                                        I                     I
                                        N                     N
                                        F                     F
                                        1                     1

     GAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGAC
 781 ————————————————————————————————————————————————————————————→ 840
     CTCTATCCCAACTCACAACAAGGTCAAACCTTGTTCTCAGGTGATAATTTCTTGCACCTG

                                            F
                                            HN            H
                                            AU            P
                                            E4            H
                                            3H            1

     TCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCGCCCACTAGGTGAACCA
  41 ————————————————————————————————————————————————————————————→ 900
     AGGTTGCAGTTTCCCGCTTTTTGGCAGATAGTCCCGCTACCGGCGGGTGATGCACTTGGT
```

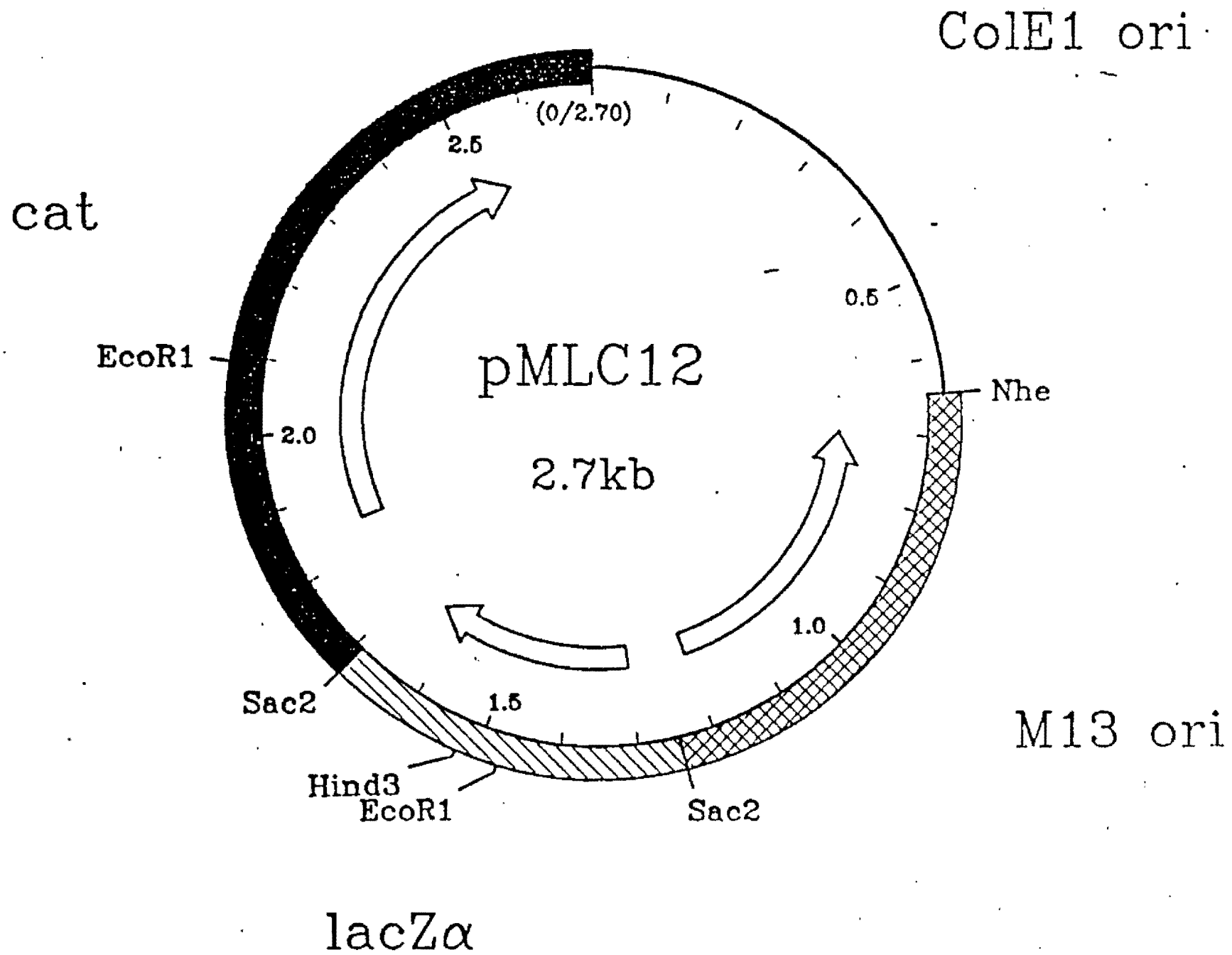
```
                    N    A   AIL                    L
                    L    O   NCA                    A
                    1    1   114                    4
                                /
     TCACCCAAATCAAGTTTTTTGCGGTCGAGGTGCCGTAAAGCACTAAATCCGAACCCTAAA
 901 ------------------------------------------------------------ 960
     AGTGGGTTTAGTTCAAAAAACCCCAGCTCCACGGCATTTCGTGATTTAGCCTTGGGATTT

          B
     N  BSG          A              HN
     L  APV          L              PA
     A  N1U          U              AE
     4  221          1              21
          //
     GGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGG
 961 ------------------------------------------------------------ 1020
     CCCTCGGGGGCTAAATCTCGAACTGCCCCTTTCGGCCGCTTGCACCGCTCTTTCCTTCCC

                                                        F    F
                M          HH         HH                N    NH
                B          HA         HA        B       U    UH
                O          AE         AE        B       4    DA
                2          12         12        V       H    21
                                                                /
     AAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTA
1021 ------------------------------------------------------------ 1080
     TTCTTTCGCTTTCCTCGCCCGCGATCCCGCGACCGTTCACATCGCCAGTGCGACGCGCAT

                F F            F            F
                N N H          HN           NH R
                U U H          HU           UH S
                4 D A          A4           DA A
                H 2 1          1H           21 1
                                                /
     ACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTACTATGGTTGCTTTGAC
1081 ------------------------------------------------------------ 1140
     TGGTGGTGTGGGCGGCGCGAATTACGCGGCGATGTCCCGCGCATGATACCAACGAAACTG

     BH                         H
     SG                         I M            AM          H
     PI                         N N            LN          A
     1A                         F L            UL          E
     21                         1 1            11          3
     /
     GAGCACGTATAACGTGCTTTCCTCGTTGGAATCAGAGCGGGAGCTAAACAGGAGGCCGAT
1141 ------------------------------------------------------------ 1200
     CTCGTGCATATTGCACGAAAGGAGCAACCTTAGTCTCGCCCTCGATTTGTCCTCCGGCTA
                                                650-1248 | 1249 - 1095 ∝
                                 H    BBSS      NUBeA F |  S
                R                I H  SSCA            NSAAN
                S                N P  TTRU            USVUL
                A                F H  XNF3            DTA9A
                1                1 1  111A            22264
                                          /           /
     TAAAGGGATTTTAGACAGGAACGGTACGCCAGAATCCTGGATCACCGCGGTCCCAATACG
1201 ------------------------------------------------------------ 1260
     ATTTCCCTAAAATCTGTCCTTGCCATGCGGTCTTAGGACCTAGTGGCGCCAGGGTTATGC

                F F          H            F
                NNNH     H   I            N AP
                UNUH     A   N            U LV          B
                DLDA     E   F            4 UU          B
                2121     3   1            H 12          V
                    /                         /
     CAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCC
1261 ------------------------------------------------------------ 1320
     GTTTGGCGGAGAGGGGCGCGCAACCGGCTAAGTAATTACGTCGACCGTGCTGTCCAAAGG

                                                            H
                H                          A               BGN
                H                          L               AIL
                A                          U               NCA
                1                          1               114
                                                            /
     CGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTCGGC
1321 ------------------------------------------------------------ 1380
     GCTGACCTTTCGCCCGTCACTCGCGTTGCGTTAATTACACTCAATCGAGTGAGTAAGCCG
```

```
      TR                  P
      NF                  A
      11                  2
ACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATA
1381 ----------+---------+---------+---------+---------+---------+ 1440
TGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACCTTAACACTCGCCTAT

                          E                   B  H   BS SS
                A         C   T   A BSBAGHNNAANCCSSX
                L         O   A   L APVVIPCCMLRRMSH
                U         R   G   U N1UAAA1H3AFFATO
                1         1   1   1 2211121 1A411112
                                      // ////// /////
ACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGCCCGGGGATC
1441 ----------+---------+---------+---------+---------+---------+ 1500
TGTTAAAGTGTGTCCTTTGTCGATACTGGTACTAATGCTTAAGCTCGAGCGGGCCCCTAG

*   H X H       F*  H
X   I SA1MT     NP  I A         H
B   N ACNNA     US  N L   B     A
A   F LCCLQ     4T  D U   B     E
1   1 11211     H1  3 1   V     3
      //
CTCTAGAGTCGACCTGCAGCCCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACT
1501 ----------+---------+---------+---------+---------+---------+ 1560
GAGATCTCAGCTGGACGTCGGGTTCGAACCGTGACCGGCAGCAAAATGTTGCAGCACTGA

        BS                  F
        SC                  N                       AP
        TR          F       U           B           LV
        NF          O       4           B           UU
        11          K       H           V           12
        /                                           /
GGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCT
1561 ----------+---------+---------+---------+---------+---------+ 1620
CCCTTTTGGGACCGCAATGGGTTGAATTAGCGGAACGTCGTGTAGGGGGAAAGCGGTCGA

                F       S
    M           HN      A   P
    N           AU      U   V
    L           E4      3   U
    1           3H      A   1

GGCGTAATAGCGAAGAGGCCGCCCGCACCGATCGCCCTTCCCAACAGTTGCGTAGCCTGA
1621 ----------+---------+---------+---------+---------+---------+ 1680
CCGCATTATCGCTTCTCCGGCGGGCGTGGCTAGCGGGAAGGGTTGTCAACGCATCGGACT
    1249-1695 F | 1696-1857  SupF inactivated
    lacZ
B       NS                          H       F   F   S
G       US                          G       N   NH  F
L       DT                          A       U   UH  A
1       22                          1       4   DA  N
                                            H   21  1
                                                /
ATGGCGAATGACCGCGGTAATTCTTTCTCAACGTAACACTTTACAGCGGCGCGTCATTTG
1681 ----------+---------+---------+---------+---------+---------+ 1740
TACCGCTTACTGGCGCCATTAAGAAAGAGTTGCATTGTGAAATGTCGCCGCGCAGTAAAC

        H                   H
        H                   A
        A                   E
        1                   3

ATATGATGCGCCCCGCTTCCCGATAAGGGAGCAGGCCAGTAAAAGCATTACCCGTGGTGG
1741 ----------+---------+---------+---------+---------+---------+ 1800
TATACTACGCGGGGCGAAGGGCTATTCCCTCGTCCGGTCATTTTCGTAATGGGCACCACC
                                                1856-1857 1857-2830
        F               H       F                       CAT
N A     N H             I       AN      T       T
L V     U A     B       N       LU      A       A
A A     4 E     B       F       U4      Q       Q
4 1     H 3     V       1       1H      1       1

GGTTCCCGAGCGGCCAAAGGGAGCAGACTCTAAAGCTGCCGTCATCGACTTCGACGAGAT
1 ----------+---------+---------+---------+---------+---------+ 1860
CCAAGGGCTCGCCGGTTTCCCTCGTCTGAGATTTCGACGGCAGTAGCTGAAGCTGCTCTA
```

34 bp

```
          LD      L
          UE      U
          11      1

     TTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATA
161  ---------+---------+---------+---------+---------+---------+ 1920
     AAAGTCCTCGATTCCTTCGATTTTACCTCTTTTTTTAGTGACCTATATGGTGGCAACTAT

                        S
                        F M                                 R
                        A N                                 S
                        N L                                 A
                        1 1                                 1

     TATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCT
1921 ---------+---------+---------+---------+---------+---------+ 1980
     ATAGGGTTACCGTAGCATTTCTTGTAAAACTCCGTAAAGTCAGTCAACGAGTTACATGGA

                     AP          H     A
                     LV          A     H
                     UU          E     A
                     12          3     3
                      /
     ATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGC
1981 ---------+---------+---------+---------+---------+---------+ 2040
     TATTGGTCTGGCAAGTCGACCTATAATGCCGGAAAAATTTCTGGCATTTCTTTTTATTCG

                                                            E
                H  H                   F                H  C
                P  A                   O                P  O
                A  E                   K                A  R
                2  3                   1                2  1

     ACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGAAT
2041 ---------+---------+---------+---------+---------+---------+ 2100
     TGTTCAAAATAGGCCGGAAATAAGTGTAAGAACGGGCGGACTACTTACGAGTAGGCCTTA

                              A     H     H H
                              L     P     P P
                              U     H     H H
                              1     1     1 1

     TCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCACCCTTGTTACA
2101 ---------+---------+---------+---------+---------+---------+ 2160
     AGGCATACCGTTACTTTCTGCCACTCGACCACTATACCCTATCACAAGTGGGAACAATGT

                N
                L
                A
                3

     CCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGAATACCACGACGATT
2161 ---------+---------+---------+---------+---------+---------+ 2220
     GGCAAAAGGTACTCGTTTGACTTTGCAAAAGTAGCGAGACCTCACTTATGGTGCTGCTAA

                                                        BS
     H                                                  SC HH
     P                                                  TR AP
     A                                                  NF EH
     2                                                  11 31
                                                         /  /
     TCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCT
2221 ---------+---------+---------+---------+---------+---------+ 2280
     AGGCCGTCAAAGATGTGTATATAAGCGTTCTACACCGCACAATGCCACTTTTGGACCGGA

                                                   BS
                                         D         SC H
                                         D         TR P
                                         E         NF H
                                         1         11 1
                                                    /
     ATTTCCCTAAAGGGTTTATTGAGAATATGTTTTTCGTCTCAGCCAATCCCTGGGTGAGTT
     ---------+---------+---------+---------+---------+---------+ 2340
     TAAAGGGATTTCCCAAATAACTCTTATACAAAAAGCAGAGTCGGTTAGGGACCCACTCAA
```

```
     P         H        AA      B              P         C
     H         A        LE      O              H         Q
     1         3        13      2              1         1
                         /
TCACCAGTTTTGATTTAAACGTGGCCAATATGGACAACTTCTTCGCCCCCGTTTTCACCA
-----------+-----------+-----------+-----------+-----------+ 2400
AGTGGTCAAAACTAAATTTGCACCGGTTATACCTGTTGAAGAAGCGGGGGCAAAAGTGGT

                         S             F        H
 N                       F             N        I
 L      S                A             U        N
 A      S                N             4        F
 3      P                1             H        1

TGGGCAAATATTATACGCAAGGCGACAAGGTGCTGATGCCGCTGGCGATTCAGGTTCATC
2401 -----------+-----------+-----------+-----------+-----------+ 2460
ACCCGTTTATAATATGCGTTCCGCTGTTCCACGACTACGGCGACCGCTAAGTCCAAGTAG

 N                      N                                RS
 L                      L                                SC
 A                      A                                AA
 3                      3                                11
                                                          /
ATGCCGTCTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGCG
2461 -----------+-----------+-----------+-----------+-----------+ 2520
TACGGCAGACACTACCGAAGGTACAGCCGTCTTACGAATTACTTAATGTTGTCATGACGC

                                                H    B          BS
                                               BGN   S          SC
                                               AIL   P          TR
                                               NCA   1          NF
                                               114   2          11
                                                /               /
ATGAGTGGCAGGGCGGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTAAACGCCTG
2521 -----------+-----------+-----------+-----------+-----------+ 2580
TACTCACCGTCCCGCCCCGCATTAAAAAAATTCCGTCAATAACCACGGGAATTTGCGGAC
                                          1852-2630 | 2631-2671
                                           cat      | H rest of SupF
                                           F        T I
                                           O        A N
                                           K        Q F
                                           1        1 1

GTGCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGTCGAATCCTTC
2581 -----------+-----------+-----------+-----------+-----------+ 2640
CACGATGCGGACTTATTCACTATTATTCGCCTACTTACCGTCTTTAAGCAGCTTAGGAAG

                                  H
                                  G
                                  A
                                  1

CCCCACCACCATCACTTTCAAAAGTCCGAAA
2641 -----------+-----------+-----------+ 2671
GGGGTGGTGGTAGTGAAAGTTTTCAGGCTTT
```


ColE1 ori
(0/2.70)
2.5
cat
0.5
EcoR1
pMLC12
Nhe
2.0
2.7kb
1.0
Sac2
1.5
M13 ori
Hind3
EcoR1
Sac2
lacZα

Minimal Size Plasmids Containing an M13 Origin for Production of Single-Strand Transducing Particles

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Summary: We have studied the requirements for efficient production of single-strand transducing particles from minimal size plasmids containing the phage M13 origin of replication. The most favorable origin fragment for production of transducing particles was found to extend from nucleotides 5372 to 5943 of the phage sequence, which includes a segment of the phage gene IV coding region and eliminates part of the gene II promoter. Minimizing vector size for M13 origin plasmids appears to be beneficial since transducing particle titer decreases with increasing plasmid size. A mutant helper phage was isolated and shown to improve the production of transducing particles from small plasmids, but to outcompete plasmids bearing large inserts. Several plasmid vectors which produce high yields of single-strand transducing particles and good ratios of transducing particles to helper phage have been constructed. Key Words: Plasmids—M13 phage—Single strand transduction.

The indispensable cis-acting elements for filamentous phage viral strand synthesis, conversion of viral strand to duplex, and extrusion of viral strands as packaged particles all appear to be located in or near the 508 bp viral intergenic region, which encodes no proteins (1–6). Because these elements are highly localized in the phage chromosome, it is possible to prepare artificial defective interfering particles which incorporate the phage origin sequences in plasmid cloning vehicles (3,7,8). Cells bearing these plasmids extrude packaged single-stranded forms of the plasmid DNAs when infected with wild-type or mutant helper phage (3,7,8). DNA prepared from the extruded particles is essentially uncontaminated by complementary strands. The pure single strands can be used for DNA sequencing, as templates for oligonucleotide-directed mutagenesis, to facilitate the preparation of very highly labeled single-strand DNA probes, to analyze the location of mutational lesions by methods not requiring sequencing, to prepare strand-specific cDNA libraries, and to examine the consequences of topological underwinding on DNA structure.

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In this article we describe plasmid cloning vehicles containing M13 origin fragments which can be used for the stable propagation of large eukaryotic DNA fragments. Upon infection with appropriate M13 helper phage, single-stranded particles are made in high yield, and in approximately equal abundance with the helper phage. Although the yield of single strands has been found to decline with increasing overall plasmid size, the vectors are sufficiently small that plasmids bearing 10 kb fragments can be efficiently prepared in single-stranded form. The small size of the vectors also facilitates the *in vitro* preparation of full-length duplex circles for oligonucleotide-directed mutagenesis.

Optimal function of the M13 origin vectors was found to require an origin fragment lacking part of the phage gene II promoter and containing gene IV coding sequences flanking the intergenic region. The gene II promoter was found to destabilize small plasmids, and the inclusion of the gene IV sequences was found to increase single-strand particle yields. An M13 mutant containing a sequence alteration in the gene II coding sequence was isolated, and shown to increase the yield of single strands from low molecular weight plasmids when used as a helper phage. However, wild-type M13 was found to allow a higher ratio of single-strand particles to helper phage to be produced from cells containing high molecular weight plasmids.

MATERIALS AND METHODS

Phage and Bacterial Strains

Strain XS127 was constructed by the sequential transfer into NK5449 (Δlac pro XIII gyrA thi rpoB argE, from N. Kleckner) of the F′ resident in JM103 (F′lacI^q lacZ ΔM15 proAB traD36) (9) and the p3 plasmid [RP1 bla(am) tet(am) tra] (10). Strain XS101 was constructed by mating the episome "F Km" [also identified as RSF2001,(23)] from MV12 (C600 ΔtrpE5 recA56 /RSF2001, from R. Hull) into HMS174 (hsdR recA1 rpoB331). Wild-type M13 was the gift of D. Ray. Phage f1 interference resistant mutants 1R-1 and 1R-2 were generously supplied by Kensuke Horiuchi.

Titers of Phage and Transducing Particles

To prepare transducing particles, a fresh overnight culture of male *Escherichia coli* containing M13 origin plasmid was preadsorbed for 10 min at 37°C with an aliquot of helper phage sufficient to give a multiplicity of infection of at least 10. The cells were diluted 1:40 to 1:100 in LB medium (11) and grown for at least 8 h at 37°C (overnight). Saturated cultures were centrifuged for 30 sec at 12,000 × *g* and the supernatants diluted in phage suspension medium. Aliquots of diluted supernatant were preadsorbed for 10 min at 37°C with 0.1 ml of a fresh overnight culture of the appropriate male *E. coli* strain. The cells were then plated in 3 ml soft agar (without drug) on either selective or nonselective plates. Nonselective plates contained LB agar (11) and selective plates contained, in addition, either ampicillin at 50 μg/ml for ampicillin resistance transducing particles, or ampicillin plus 15 μg/ml tetracycline for suppressor transducing particles. After 24 h incubation at 37°C, the transducing particle titers were scored as the number of colony forming units per ml. Controls for the spontaneous acquisition of drug resistance were performed in the same way, but diluted supernatant was not added. The frequency of appearance of particles produced by infection of vector plasmids lacking M13 origin fragments was not detectable in any experiment, although the spontaneous appearance of colonies simultaneously resistant to ampicillin and tetracycline was occasionally observed, and attributed to the appearance of chromosomal suppressor tRNA genes (typically at a frequency of $\sim 10^{-8}$/cell or less).

Plasmid Manipulations

Plasmid constructions were performed by standard methods (10,12). Both small- and

large-scale plasmid preparations were performed by the method of Birnboim and Doly (13). Where possible, selection for insertion of the M13 origin fragment was carried out by superinfection of the population of transformed cells with M13 helper phage. Suitable male transduction hosts were then infected with dilutions of the culture supernatant and the resulting colonies on selective agar were worked up for intracellular plasmid DNA in the usual manner. For the preparation of large amounts of extracellular single strand DNA, culture supernatants were concentrated by precipitation with 2% polyethylene glycol in 0.5 *M* NaCl. The precipitate resulting from a liter of infected culture was resuspended in 10 ml of phage suspension medium (SM Buffer) (11) and spun to remove residual bacteria. The supernatant was then brought to a solution density of 1.3 g/ml by the addition of solid CsCl, and centrifuged 4–6 h at 70,000 rpm in a Beckman VTi80 rotor. Phage/particle bands were collected, dialyzed briefly, and phenol extracted to liberate the single-stranded DNA.

RESULTS

A Plasmid Containing the Phage M13 Intergenic Region (a 514 bp Rsa1 Fragment) Interferes with Wild-Type Phage Growth

The M13 intergenic region falls between nucleotides 5498 and 6005 (14) and is largely contained in the 514 bp Rsa1 fragment spanning nucleotides 5487 to 6001 (14). This fragment was cloned via the addition of Hind3 linkers into the Hind3 site of a derivative of pBR322 in which the EcoRI to SalI segment was replaced by a polylinker. Candidate plasmids were introduced into XS101, a RecA1 strain harboring an F episome marked with a kanamycin resistance determinant, and tested for their ability to sustain or resist infection by M13 phage. Cells containing plasmids bearing the 514 bp origin fragment did not allow plaque formation by wild-type M13, although cells containing plasmids bearing a 522 bp fragment from the phage gene 1 region gave no reduction in plating efficiency relative to cells containing no plasmid. When cells containing the origin plasmid were infected with M13 in liquid culture at midlog phase (~2 × 10^8 cells/ml) and grown to saturation, the titer of particles capable of transducing male *E. coli* to ampicillin resistance was less than 10^9/ml in the broth supernatant.

Examination of the extrachromosomal DNA present in cells recovered from these cultures revealed abundant plasmid supercoil, slight amounts of M13 supercoil, and no apparent single strands arising either from M13 or from the M13 origin plasmid. The culture supernatants were concentrated by polyethylene glycol precipitation and analyzed for extracellular DNA, but the levels of phage and plasmid DNA were too low to be detected. Because there appeared to be very little extracellular or intracellular single-strand M13 DNA present in the infected cultures, mutants that could escape the interference of the origin plasmid were sought, on the supposition that some of the mutant phage might confer the ability to produce single strands on both phage and interfering plasmid.

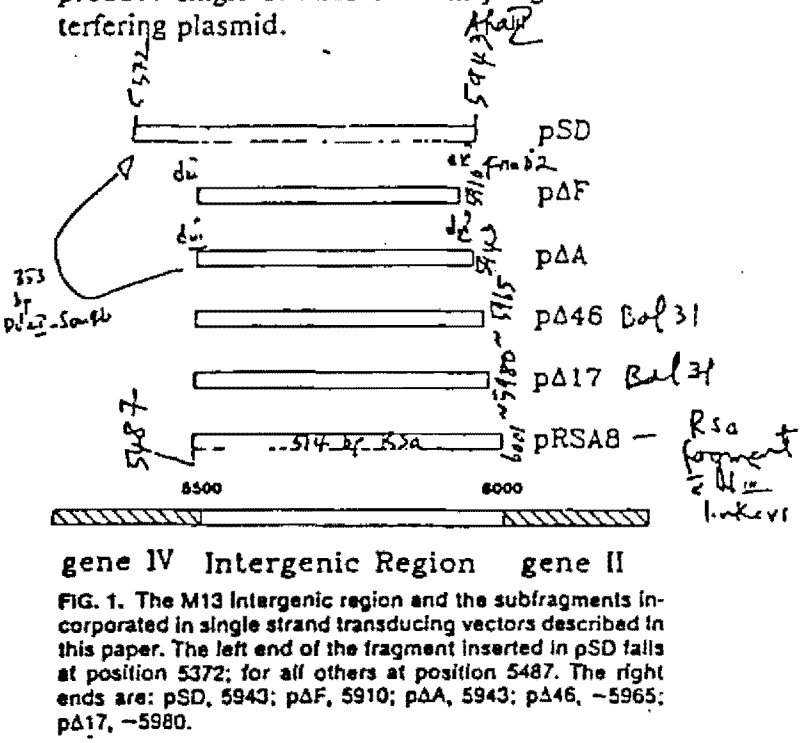

FIG. 1. The M13 intergenic region and the subfragments incorporated in single strand transducing vectors described in this paper. The left end of the fragment inserted in pSD falls at position 5372; for all others at position 5487. The right ends are: pSD, 5943; pΔF, 5910; pΔA, 5943; pΔ46, ~5965; pΔ17, ~5980.

Isolation of Phage M13 Mutants Resistant to Interference by Plasmids Containing the 514 bp Rsa1 Fragment

Cells containing the origin plasmid were grown to saturation in broth, infected with wild-type M13 at a multiplicity of 20 phage per cell, diluted 1:1000, and grown to saturation in liquid culture. A portion of the culture was diluted 1:1000 and regrown to saturation. The dilution and subculture was repeated three additional times. The supernatant of the resulting culture contained a titer of ~5 × 10^{10}/ml phage which were capable of forming plaques on the origin plasmid cells, as well as an equal titer of ampicillin resistance transducing particles. After a preliminary characterization, a single phage, designated rv1, was selected for further study. Analysis by digestion with a variety of restriction enzymes revealed the appearance of a new Dde1 site in the gene II coding sequence of this phage. A G to T transversion at position 517 is the only single base alteration which gives rise to the observed restriction fragment pattern. This alteration would result in the substitution of a serine residue for an alanine residue at about a quarter of the length from the carboxyl terminus of the gene II polypeptide product.

Derivation of an M13 Origin Fragment *Compatible with Suppressor Miniplasmids*

The 514 bp Rsa1 fragment was inserted in the Hind3 site of the suppressor miniplasmid πVX (10) and the resulting plasmid, pRSA101, was found to have a copy number approximately 20 times the copy number of pBR322. Only one orientation of the M13 origin could be recovered, and despite extensive efforts, no further fragments could be cloned into pRSA101 (data not shown). Since strong promoters have been shown to interfere with Col E1-like plasmid replication, and since the Rsa1 fragment contains the phage gene II promoter, we constructed four deletion mutants lacking various extents of the gene II proximal end of the fragment. Two of the mutants were generated by nuclease Bal 31 resection (terminating at positions ~5965 (pΔ46) and ~5980 (pΔ17) of the nucleotide sequence), and two by restriction enzyme digestion with the nucleases Aha3 and FnuD2 [positions 5943 (pΔA) and 5910 (pΔF), respectively]. The first two deleted origins were inserted as Hind3 fragments (after addition of a Hind3 linker to the deleted end) into the Hind3 site of the suppressor miniplasmid πAN7 (constructed by Henry Huang, personal communication; πAN7 was chosen over πVX for these experiments because, unlike πVX, it contains an intact copy of the promoter for the replication origin primer RNA (10) and has a higher copy number.) The second two origins were subsequently constructed by partial (FnuD2) or complete (Aha3) digestion of pΔ17 followed by complete EcoR1 digestion, isolation of the appropriate origin fragment, and insertion of the fragment into a πAN7 vector segment bearing one EcoR1 end and one flush end created by filling in an Xba site. This construction gave origin fragments bearing Hind3 sites at either end. Unlike the Bal 31-generated fragments, the more extensive deletion fragments could be successfully propagated in πAN7 following inversion of the fragment orientation relative to the vector. Inversion of orientation was carried out by liberating the insert fragment with Hind3 and relegating the insert to vector cut with Hind3.

Plasmids containing the deleted M13 origin fragments were tested for production of plasmid transducing particles. For this purpose it was necessary to construct a male host strain bearing the p3 plasmid (strain XS127, see Materials and Methods). The p3 plasmid bears amber mutated ampicillin and tetracycline resistance determinants, as well as an intact kanamycin resistance determinant. Plasmids containing suppressor tRNA genes thus confer simultaneous ampicillin and tetracycline resistance on the XS127 strain. The relative titers of the helper phage and transducing particles are shown in Table 1.

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TABLE 1. *Phage and transducing particle titers with different helper phage*

	rv1		Wild type		IR-1		IR-2	
	cfu	pfu	cfu	pfu	cfu	pfu	cfu	pfu
pΔ17	3.3	1.4	0.2	0.2	20	17.1	6.7	21.3
pΔ46	6.7	4.8	2.4	1.4	1.6	19.3	12.5	50
pΔA	4.1	2.4	0.6	0.3	1.6	20.3	11.0	30
pΔF	2.1	10.4	2.7	6.4	0.4	23.2	0.5	23.5

Cells bearing the plasmids listed in column 1 were infected with the indicated mutants and grown for 16 h at 37°C. Titers are given in colony-forming units (cfu), measuring transducing particle titer, or plaque-forming units (pfu), measuring helper phage titer, both in units of 10^{10}/ml.

Optimal Growth Conditions for Transducing Particle Production

Experiments were also carried out at this point to assess the influence of broth composition, temperature and duration of growth, and nature of the helper phage on the yield of transducing particles. The results, shown in Tables 2 and 3, demonstrate that transducing particles may be favorably produced in rich broth at 37°C, with rv1 as a helper phage. To evaluate the suitability of the deleted origin miniplasmids for propagation of large fragments, a 5.0 kb Bg12 fragment of human genomic DNA containing the β-globin gene (15) was inserted in the BamHI site of plasmids containing the two smallest origin fragments in both orientations. A 10.2 kb Pvu2 to XbaI fragment from the same locus (15) was also inserted in these plasmids, between the SmaI and XbaI sites. A

TABLE 2. *Effect of growth temperature on titer*

	rv1		Wild type	
	cfu	pfu	cfu	pfu
32°C	1.2	10.3	0.6	1.8
37°C	2.1	10.4	2.7	6.4
42°C	2.6	6.0	0.5	4.1

Cells bearing pΔF were infected with rv1 or wild-type phage and grown for 16 h at the indicated temperature in LB broth. Titers are given in units of 10^{10}/ml. cfu, Colony-forming units; pfu, plaque-forming units (see Table 1).

TABLE 3. *Effect of broth composition on titer*

	rv1		Wild type		IR-1		IR-2	
	cfu	pfu	cfu	pfu	cfu	pfu	cfu	pfu
LB	2.1	10.4	2.7	6.4	0.4	23.2	0.5	23.5
M9CA	1.6	7.1	1.0	6.4	0.4	9.5	0.5	20.2

Cells bearing pΔF were infected with the indicated helper phage and grown overnight at 37°C in the media listed in the first column. Titers are given in units of 10^{10}/ml. cfu, Colony-forming units; pfu, plaque-forming units (see Table 1).

schematic diagram of the plasmid structures is shown in Fig. 2. Although reasonable yields of transducing particles and acceptable ratios of these particles to helper phage could be found with plasmids bearing 5 kb inserts, plasmids bearing 10 kb inserts appeared to suffer severe competition from all types of helper phage, including wild type M13 (Tables 4 and 5). Some improvement in titers and ratios can be seen following serial subculture of the infected cultures, although the improvement is unpredictable and confined to the lower molecular weight plasmids (Tables 4 and 5 and unpublished results). The phage f1 interference resistance mutants IR-1 and IR-2 appear to strongly outcompete M13 origin plasmids.

Good yields of phage and transducing particles could also be recovered from plate stocks prepared by infection of the appropriate plasmid-bearing host with sufficient helper phage to give confluent plaque formation on the host lawn (Table 6). However, a poor yield of large transducing particles was again observed.

Both 5 and 10 kb insert plasmids were examined for stability to deletion and rearrangement under the helper phage cocultivation conditions. Analysis of the intracellular plasmid DNA revealed no alterations detectable by restriction enzyme analysis (not shown). Surprisingly, the predominant intracellular DNA species was the M13 origin plasmid. This result seemed at variance with the limited transducing particle titer. However, direct measurement of the amount of extracellular DNA

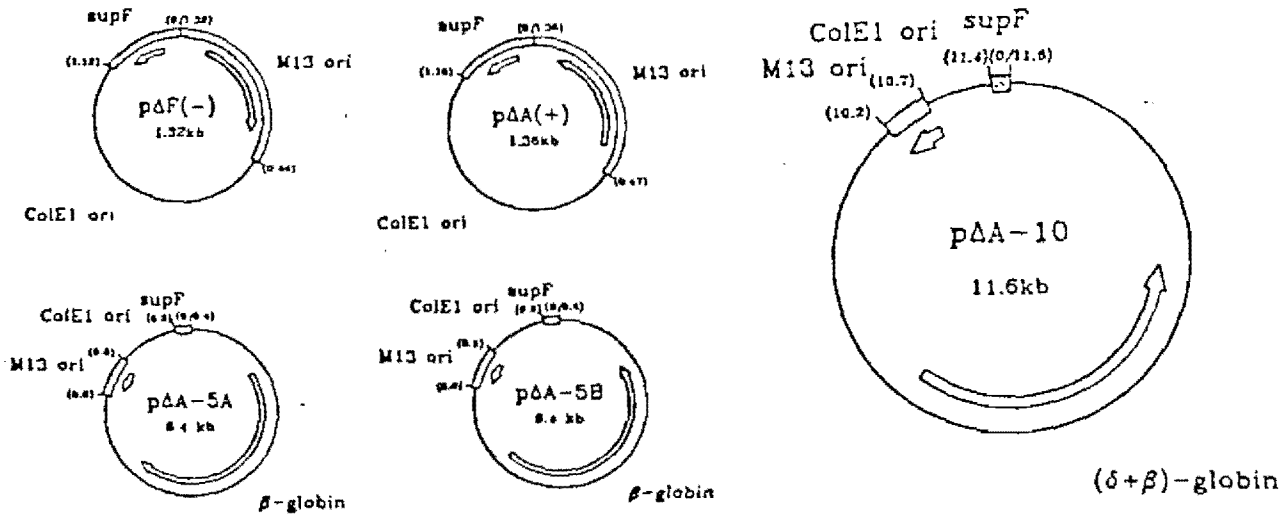

FIG. 2. The pΔ series of plasmids, used to evaluate the dependence of transducing particle titer on plasmid length and other variables described in the text. The plasmids incorporate the ΔF and ΔA origin fragments shown in Fig. 1. Their construction is described in the text. (+) and (−) denote the orientation of the M13 origin fragment relative to the direction of transcription of the *supF* gene, while A and B denote the orientation of the globin insert fragments relative to the M13 origin. Arrows show the direction of transcription of the fragments.

showed no major discrepancy with the titer, ruling out the possibility that the larger transducing particles were somehow incapable of establishing themselves as plasmids in the transduction host. The same high levels of intracellular DNA were observed whether the DNA was prepared by alkaline lysis of the cells or by the boiling method (16). The simplest explanation for these results appeared to be that the larger plasmids convert intracellular single strands back to duplex at a particularly high rate, leaving little single strand available for extrusion. If the phage packaging signals were incompletely represented in the origin fragment, the resulting tendency for single strands to accumulate in the cell might give rise to this type of behavior. A larger form of the origin fragment was therefore sought.

An M13 Origin Fragment Containing Gene IV Coding Sequences Increases the Yield of Transducing Particles

Cleavage of M13 DNA with the enzymes DdeI and Sau96 produced a 353 bp fragment which included 128 bp of gene IV coding sequence flanking the intergenic region on the gene IV side. (The fragment is 112 bp shorter

TABLE 4. *Titer as a function of duration of growth*

	First culture		Second culture		Third culture	
	cfu	pfu	cfu	pfu	cfu	pfu
pΔF-5A	1.2	15.0	2.7	16.2	ND	ND
pΔF-5B	0.3	7.3	4.3	18.0	5.8	2.9
pΔA-5A	1.1	13.3	1.9	4.7	0.1	18.0
pΔA-5B	0.9	4.0	3.1	10.8	ND	ND
pΔA-10	0.3	10.4	1.2	16.0	ND	ND

Cells bearing the plasmids listed in the first column (and shown in Fig. 2) were infected with *rv1*, diluted 1:40, and grown to saturation (first culture), then subcultured by 1:40 dilution one (second culture) or two (third culture) additional times. Titers are given in units of 10^{10}/ml. cfu, Colony-forming units; pfu, plaque-forming units (see Table 1).

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TABLE 5. *Helper phage effect on a 12 kb plasmid*

	rv1		Wild type		IR-2	
	cfu	pfu	cfu	pfu	cfu	pfu
First culture	0.3	10.4	1.4	4.9	1.1	16.0
Second culture	1.1	16.0	1.5	6.2	1.3	50

Cells containing the plasmid pΔA-10 were infected with the indicated phage, diluted 1:40 and grown to saturation in LB broth at 37°C (First culture, row 1), then rediluted the same amount and regrown (Second culture, row 2). Titers are given in units of 10^{10}/ml. cfu, Colony-forming units; pfu, plaque-forming units (see Table 1).

than that predicted from the nucleotide sequence because of the appearance of a polymorphic DdeI site at nucleotide position 5370.) The Dde to Sau fragment was ligated into the miniplasmid pΔA, replacing the resident Rsa to Sau fragment and extending the gene IV side of the origin by 116 bp. The Sau96 site was destroyed in the course of this step, and was shown by DNA sequencing to have been inadvertently filled in by the action of DNA polymerase. Cells bearing the resulting plasmid, pSD, gave markedly better titers of transducing particles following cocultivation with rv1 helper phage (Table 7). The 10 kb Pvu2 to Xba globin fragment was inserted in this plasmid and the relative titers of transducing particles and helper phage were determined as before. Good yields of transducing particles (~6 × 10^{10}/ml) and acceptable ratios of helper phage to particles (~2:1) were observed with wild-type helper phage (Table 7). However, all mutant helper phage gave high ratios of helper phage to transducing particles. The stability of the plasmid to cocultivation with wild-type helper phage was examined by restriction enzyme digestion of the intracellular DNA, and no macroscopic alterations were found (Fig. 3).

A variety of different fragments were cloned into the polylinker segment of pSD, and DNA was prepared from extracellular particles recovered by polyethylene glycol concentration of supernatants of liter cultures, followed by CsCl buoyant density centrifugation and phenol extraction (Materials and Methods). The ratio of plasmid to phage single strands appeared in most cases to be underestimated by measurement of plaque and transducing particle titer (Table 8); in almost every case more plasmid DNA than helper virus DNA was recovered from the culture supernatant (Fig. 4).

Construction of Cloning Vehicles for Production of DNA Single Strands

Several plasmid cloning vehicles were created to exploit the origin fragment in pSD and to facilitate the insertion of foreign sequences. Two vehicles, pSDL12 and pSDL13, contain the lac Z α-complementation fragment found in the plasmids pUC12 and pUC13 (17) inserted in the opposite orientation (relative to the M13 origin) to that found in the M13 mp series of phage vectors (Fig. 5). Chloramphenicol resistance versions of the pSDL plasmids were created by inserting a 780 bp chloramphenicol acetyltransferase resistance element derived from the plasmid pCM1 (18) into a Taq site in the suppressor tRNA gene (Fig. 5).

To facilitate the preparation of other plasmids containing the M13 origin, the plasmid pSDX2, which contains the origin fragment of pSD flanked by Xba linkers, was digested with Xba, the ends filled in with *E. coli* DNA polymerase large fragment, and the resulting fragments ligated to a variety of linkers in the presence of polyethylene glycol (19) at the optimum temperature previously determined for linker ligation (32°C). The reaction mix was diluted four-fold, digested with EcoRI, recircularized

TABLE 6. *Titers of plate stocks prepared with rv1*

	cfu	pfu
pΔF-5B	2.7	3.0
pΔA-5B	2.1	4.0
pΔA-10	0.6	16.0

Plate stocks were prepared by infecting 100 μl of saturated culture containing the plasmids in column 1 with 10^4 rv1 phage, and plating the infected cells in soft agar. Plate stocks were harvested after 16 h by scraping the top agar into phage suspension medium, vortexing, and sedimenting out the agar. Each plate gave approximately 5 ml of plate stock. Titers are given in units of 10^{10}/ml. cfu, Colony-forming units; pfu, plaque-forming units.

TABLE 7. *Titers observed with expanded origin plasmids*

	rv1		Wild type		IR-1		IR-2	
	cfu	pfu	cfu	pfu	cfu	pfu	cfu	pfu
pSD	13.3	7.6	4.9	4.7	ND	ND	ND	ND
pSD-10	1.4	31.2	6.1	13.2	1.6	23.7	3.0	38.8

Cells were infected with the indicated phage and grown for 12 h at 37°C in LB broth. ND, Not done. Titers are given in units of 10^{10}/ml.

at low concentration, and introduced into XS127. Transducing particles were prepared by rv1 infection of the transformants and used to transduce XS127 to ampicillin and tetracycline resistance. The resistant colonies were analyzed for the presence of plasmids bearing the M13 origin fragment flanked by the linkers of interest. This approach proved very convenient, with about 50% of the resulting colonies containing the desired plasmids; origin fragments flanked by Bgl2, Xho, Sac2, Cla, and Nco linkers were prepared in this manner (Fig. 5).

Recovery of Genomic DNA Sequences from a Bacteriophage λ Library by Recombination with a Suppressor Miniplasmid Containing the Phage M13 Origin of Replication

An origin fragment flanked by Bgl2 sites was excised with Bgl2 and ligated to a Bgl2 linearized miniplasmid containing a segment of the human β-globin 5′ flanking region (plasmid πBΔP) (10). The desired plasmid was isolated from transductant colonies and verified by restriction enzyme mapping. An aliquot of a secondary amplification of the Lawn et al. (15) human genomic library was plated on cells bearing this plasmid, and phage recombinants containing an integrated copy of the miniplasmid were isolated as described previously (10). Only one phage species was recovered in this screening, corresponding to the isolate "E" previously described (10). The absence of other phage species may be a consequence of the second round of amplification to which the library was subjected; in experiments with a related miniplasmid the secondary library also gave only phage "E" recombinants while the primary library gave additional species (20).

DISCUSSION

The M13 phage intergenic region appears to contain all of the cis-acting elements required for replication and extrusion of viral strands as filamentous particles (3). A derivative of plasmid pBR322 containing the phage intergenic sequences found on a 514 bp Rsa1 fragment was shown to strongly interfere with wild type M13 growth. An interference-resistant M13 mutant was isolated, and proved to allow reasonable titers of transducing particles (~5 × 10^{10}/ml) when used as a helper phage in conjunction with this plasmid. These observations are strikingly different from the recent results of Zagursky and Berman (8), who found that the same fragment inserted in the Pvu2 site of pBR322 showed no interference with wild type M13. It appears that the location of the M13 origin in the plasmid vector can strongly influence the relationship between interfering plasmid and helper phage.

Sequences at the gene II proximal end of the intergenic region were found to destabilize small plasmids. Deletion of sequences at the gene II proximal end of the intergenic region

FIG. 3. Stability of pSD10 transducing particles following prolonged cocultivation with M13 helper phage. Lane a is the BamHI fragment pattern produced by plasmid cocultivated with M13 rv1, lane b the pattern produced by pSD10 without cocultivation, lane c the pattern produced by cocultivation with wild-type M13, and d and e the fragment pattern of rv1 and wild-type phage without pSD10. The marker lane is phage λ DNA cut with BstE2.

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1-617-726-2000
515
MB = 5944

TABLE 8. *Titers from liter cultures of globin plasmids with wild-type helper*

	cfu	pfu
pSD-P29	5.6	4.8
pSD-G	8.9	7.0
pSD-10	6.3	13.4

pSD derivatives containing 3.7 kb (pSD-P29 and pSD-G) or ~10 kb (pSD-10) inserts were infected with wild-type helper and grown at 37°C overnight in LB broth. Titers are given in units of 10^{10}/ml. cfu, Colony-forming units; pfu, plaque-forming units.

FIG. 4. Single-strand DNA yields of various pSD derivatives. Agarose gel electrophoresis of the DNAs present in the supernatant of cells infected with M13 helper phage shows the relative abundance of the plasmid transducing particle DNA and the helper DNA. The length of the fragment inserted in pSD varies from lane to lane: a, 0.5 kb; b, 3.7 kb; c, 4.1 kb; d, 10.2 kb. The marker lane is phage λ DNA cut with *BstE2*, and the location of the single strand helper DNA is labeled "M13" in the right margin. The amount of helper phage DNA loaded in each lane is approximately constant.

allowed the origin fragment to be cloned in both orientations in various miniplasmids, but derivatives of these plasmids bearing large inserts competed poorly with helper phage.

Sequences lying within the gene IV coding region appeared to enhance the ability of the plasmid-borne M13 origin to compete with superinfecting helper virus. This conclusion must be considered provisional because the construction that gave rise to the extended M13 origin also resulted in the disappearance of a restriction site in the origin fragment, and the effects of this mutational event have not been isolated. Dotto et al. (3) have reported that the cis-acting sequences for extrusion of single strands are located in the phage f1 HaeIII F fragment, which spans nucleotides 5415 to 5725. If their results extend to M13, it would appear that the region between 5414 and 5487 encodes signals necessary for efficient maturation. However, the extended origin we have isolated also contains a sequence which is closely related to the single strand origin site and which has been observed to be an endpoint for the reiteration unit in the tandemly duplicated origin fragments found in naturally occurring f1 defective interfering particles (21,22). This sequence may play a role in the natural physiology of the virus. The appear-

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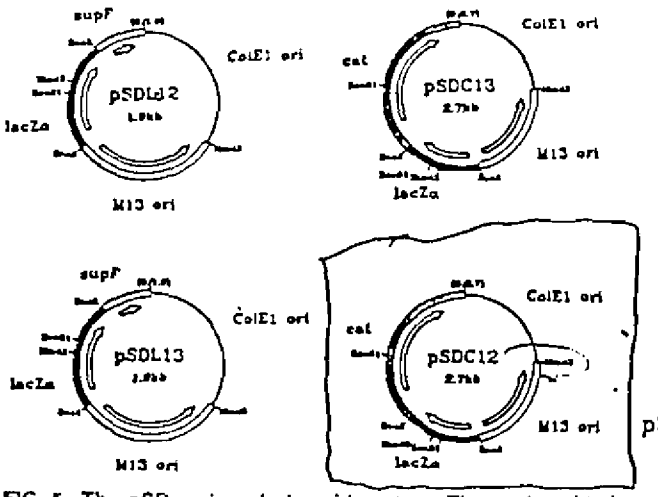

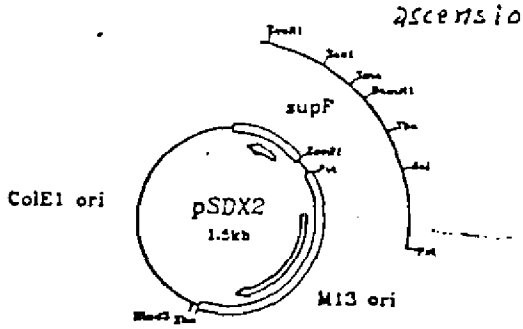

pSD-Cla, pSD-Bgl2, pSD-Nco, pSD-Sac2, pSD-Xho

FIG. 5. The pSD series of plasmid vectors. These plasmids incorporate the SD origin fragment shown in Fig. 1. The direction of transcription of the lac and cat segments is indicated with arrows. The names of various M13 "cassette" plasmids constructed in this study are listed beneath the map of pSDX2, which contains an Xba cassette.

ance of tandem duplications in the extended origin plasmids has not been observed in this study, although we anticipate that prolonged cocultivation would be necessary to select for such species.

The type of helper virus necessary for optimal production of plasmid transducing particles was found to vary with the type of origin and the size of the origin plasmid. The interference resistant mutant helper virus isolated in this work was found to have a nucleotide sequence alteration in the coding sequence of gene II, which directs the synthesis of an essential replication protein. This mutant was found to be the best helper phage for small plasmids. The best helper virus for large plasmids, however, was found to be wild-type M13. In general, increasing plasmid size correlated with decreasing efficiency of transducing particle production. The small size of the M13 origin plasmids presented here compensates in part for the tendency of plasmids bearing large inserts to perform poorly in competition with wild-type or mutant M13 phage. The growth conditions for optimal production of transducing particles were examined and found to be the same as those conventionally used for the production of M13 phage particles. The stability of plasmids bearing large inserts to rearrangement on cocultivation with helper phage was found to be very good. A variety of minimal length cloning vehicles have been constructed from the most efficient origin fragment and put to use in the isolation of large amounts of unrearranged plasmid single strands.

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Construction and characterization of the chloramphenicol-resistance gene cartridge: A new approach to the transcriptional mapping of extrachromosomal elements

(*Tn9* CAT gene; pBR327 promoters; readthrough transcription)

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SUMMARY

A system has been developed to transcriptionally map plasmid DNA molecules in vivo. This system is based upon the activity of the Tn*9* Cm^r gene whose product, chloramphenicol acetyltransferase (CAT), can be easily assayed enzymatically and identified phenotypically in *Escherichia coli*. The Cm^r gene was excised as a single DNA fragment from the plasmid cloning vector, pBR328, and its ends were converted to hexamer-specific restriction enzyme recognition sites. These modified Cm^r gene fragments were designated "CAT cartridges". The CAT cartridges were expressed at high levels only when inserted in the proper orientation, downstream of a plasmid promoter. Using DNA restriction endonuclease cleavage sites as insertion points, the CAT cartridges were inserted into plasmid pBR327, and the levels of CAT activity produced in cells containing the various plasmids were determined. The CAT activities at each position and orientation were used to construct a transcriptional map of plasmid pBR327. The positions and strengths of promoters demonstrated in vivo using the CAT cartridge coincided with those predicted by in vitro studies. Using the CAT cartridges it should be possible to study gene expression on any extrachromosomal element in the appropriate host.

INTRODUCTION

Two general methods have been developed to facilitate the in vivo analysis of gene regulation by fusing regulatory regions to structural genes whose products can be measured enzymatically. In one system, promoters on the enteric bacterial genome can be studied by utilizing the integration properties of bacteriophage Mu. Mu derivatives containing a β-galactosidase (*lacZ*) gene of *E. coli* can transpose into the bacterial chromosome to put β-galactosidase production under the control of a chromosomal promoter (Casadaban and Cohen, 1979). Alternatively, recombinant DNA techniques have been used to fuse promoters to promoter-less structural genes carried on plasmids. Recombinant plasmids are then introduced by transformation into host cells. This second general method has been used to study a range of promo-

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Abbreviations: a-Tc^r, anti-tetracycline resistance gene; Ap, ampicillin; Ap^r, ampicillin resistance; bp, base pairs; CAT, chloramphenicol acetyltransferase; Cm, chloramphenicol; Cm^r, chloramphenicol resistance; kb, kilobase pairs; SDS, sodium dodecyl sulfate; Tc^r, tetracycline resistance.

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ters in prokaryotic and eukaryotic hosts (West et al., 1979; An and Friesen, 1979; Casadaban and Cohen, 1980; Guarente and Ptashne, 1981; Rose et al., 1981; Goldfarb et al., 1981; McKenney et al., 1981).

We report a new in vivo method of analysis that combines features of both approaches. For our studies, several derivatives of the Cm^r gene of the bacterial transposon Tn*9* were constructed. Each derivative lacks the terminal insertion sequences and the Cm^r gene promoter that are present on the intact transposon, but contains the complete CAT polypeptide coding sequence and an *E. coli* ribosome binding site (Alton and Vapnek, 1979). These short DNA segments (about 780 bp), designated "CAT cartridges", are flanked by the hexamer-specific restriction sites (*Hin*dIII, *Bam*HI and *Sal*I). Using recombinant DNA techniques, the CAT cartridges were inserted around the plasmid pBR327, which is a 1089 bp deletion derivative of pBR322 (Soberon et al., 1980). *E. coli* cells were then transformed with each cartridge insertion derivative, and the direction of transcription and level of Cm^r gene expression were determined for each position. The results of these experiments enabled a transcriptional map of pBR327 to be constructed. The in vitro transcription studies of pBR322 by West and Rodriguez (1980, 1982) and by Stüber and Bujard (1981) have mapped the location and determined the relative strengths of a number of promoters on pBR322. Our findings using the CAT cartridges in vivo are in close accord with these in vitro studies.

The Cm^r gene was chosen for this system for the following reasons: (i) the nucleotide (Alton and Vapnek, 1979) and amino acid (Shaw et al., 1979) sequences have been determined for CAT, the product of the Cm^r gene; (ii) CAT activity can be easily assayed spectrophotometrically (Shaw, 1975); (iii) CAT enzyme can be purified to homogeneity from crude extracts in one step by affinity chromatography (Zaidenzaig and Shaw, 1976); (iv) few organisms produce CAT, eliminating the problem of background enzymatic activities of the host (Shaw et al., 1979); and (v) the Cm^r gene has been shown to be expressed and functional in the eukaryote, *Saccharomyces cerevisiae* (Cohen et al., 1980).

Based on the simplicity of the CAT cartridge system and the fidelity with which promoters on pBR327 were identified in vivo, the CAT cartridges can serve as a useful tool for studying promoters in a variety of prokaryotic and eukaryotic extrachromosomal elements.

MATERIALS AND METHODS

(a) Bacterial strains and plasmids

The *E. coli* strain RR1 and the plasmids pBR327 and pBR328 were described by West and Rodriguez (1982). For the maxicell analysis the ultraviolet sensitive *E. coli* strain CSR603 was used (Sancar et al., 1979).

(b) Media

Luria broth (LB) and M9 minimal medium plus casamino acids (M9, CAA) have been described for liquid and solid media (Miller, 1972). Sulfate-free Hershey salts and medium were prepared as described by Worcel and Burgi (1974).

(c) DNA preparation

Microgram quantities of plasmid DNA were prepared using an SDS-alkali denaturation method. A 1 ml stationary phase culture of plasmid-containing cells grown in LB medium was harvested in an eppendorf microcentrifuge. The cell pellet was washed with 1 ml of SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris, pH 8.0) and resuspended in 0.15 ml of the same buffer. To this mixture 5 µl of 10 mg/ml bovine pancreatic ribonuclease (Calbiochem-Behring) and 0.35 ml of a lysis solution (0.2 N NaOH and 1% SDS) were added. The tube was vortexed briefly, held on ice for 10 min and then mixed with 0.25 ml of cold 3.0 M Na · acetate buffer, pH 4.8 (Na^+ salt). After inverting several times, the tube was incubated on ice for another 20 min. The SDS and chromosomal precipitate was pelleted by centrifugation at 4°C for 5 min in the microcentrifuge. The supernatant was transferred to a clean 1.5 ml eppendorf tube to which an equal volume of −20°C isopropanol was added. The tube was inverted several times

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and centrifuged immediately at room temperature for 5 min. The supernatant was poured off, and the plasmid DNA pellet was washed once with 1 ml of −20°C 70% ethanol. After another centrifugation the supernatant was discarded, the pellet dried under vacuum and resuspended in 20 μl of de-ionized water. At this stage the DNA was suitable for digestion with restriction enzymes and transformation.

Milligram quantities of plasmid DNA were prepared using the Cm or spectinomycin amplification method of Soberon et al. (1980) followed by phenyl-neutral-red binding resin (Boehringer-Mannheim) column chromatography. A mixture of chromosomal and plasmid DNA was passed over the column resin, and non-supercoiled DNA was washed through, with 0.18 M $NaClO_4$ (Fisher) in a buffer containing 10 mM potassium phosphate and 10 mM EDTA, pH 6.0. Supercoiled plasmid DNA was eluted with 0.60 M $NaClO_4$ in the same buffer.

Purified DNA restriction fragments were extracted from 1% low melt agarose (Seakem) gels according to the procedure of Langridge et al. (1980).

(d) Transformation

E. coli cells were made competent for transformation by a modification of the method of Morrison (1979). Log phase cells were harvested from a 20 ml LB plus 0.2% glucose culture grown at 37°C. The cell pellet was resuspended in 10 ml of ice-cold 30 mM $CaCl_2$, incubated 20 min on ice and resuspended in 2 ml of ice-cold 30 mM $CaCl_2$ plus 15% glycerol. Transformations were performed by thawing on ice 0.2 ml aliquots stored at −68°C, adding plasmid DNA and incubating on ice for 60 min. After a 90-s heat pulse at 42°C, the cells were spread immediately on selective media to minimize the production of sibling colonies.

(e) CAT assay

Plasmid-containing cells were grown to a cell density of 5×10^8/ml in 1.5 ml cultures of LB plus 0.2% glucose at 37°C with shaking. Cells from this culture were pelleted and resuspended in 0.5 ml of extraction buffer (50 mM Trizma base, 30 μM dithiothreitol, pH 7.8). The cells were broken using a Branson sonifier and the extract was cleared of cell debris by centrifugation for 15 min at 4°C in a microcentrifuge. The supernatant was assayed for CAT activity in a 600 μl reaction volume by the spectrophotometric method of Shaw (1975). Substrates and reagents for the assay were purchased from Sigma. The rate of increase in absorbance at 412 nm was measured at 37°C to determine enzyme units in nmol of dithiobis-nitrobenzoic acid reduced per min per mg of total protein. Protein was assayed by the method of Bradford (1976) using a dye reagent concentrate (Bio-Rad) and bovine serum albumin (Sigma) as a standard.

(f) Maxicell protein labeling

Plasmid-encoded proteins were identified using the maxicell method of Sancar et al. (1979) with the following modifications. Cultures of plasmid-containing CSR603 cells were grown in 3 ml of M9, CAA medium at 37°C with shaking until a density of 3×10^8 cells/ml was reached. A 2.5 ml portion of this culture was transferred to a sterile 100-mm diameter petri dish and irradiated for 15 s with 254 nm ultraviolet light at an intensity of 1.25 $J/M^2/s$. 2 ml of irradiated cells was transferred to a 15 × 130 mm tube. D-cycloserine was added to a final concentration of 150 μg/ml, and the cells were incubated for 16 h with shaking at 37°C. The cells from 1.5 ml of this culture were then pelleted, washed twice with 1 ml of sulfate-free Hershey salts, and resuspended in 0.8 ml of sulfate-free Hershey medium. The culture was incubated at 37°C for 1 h without shaking, after which 0.2 ml of Hershey medium containing 5 μCi of [^{35}S]methionine (1380 Ci/mmol, Amersham) was added. After another hour of incubation at 37°C, the cells were pelleted, washed twice with 1.0 ml of 100 mM NaCl and resuspended in 60 μl of SDS sample buffer (Sancar et al., 1979). The mixture was heated to 95°C for 3 min and 20 μl aliquots were run on 12% polyacrylamide (Bio-Rad) denaturing gels using the buffer system of Laemmli (1970). Gels were saturated with a solution of 18% Omnifluor (New England Nuclear) in dimethylsulfoxide, rinsed thoroughly with de-ionized water and dried under vacuum. The dried gels were

autofluorographed at −68°C for 6 h using Kodak X-Omat AR5 film.

(g) Enzymatic digestions and ligations

The restriction endonucleases *Eco*RI and *Sal*I were gifts from R. Tait, *Pst*I was a gift from T. Okita, while *Hae*III, *Hae*II and *Bam*HI were prepared according to the procedure of Greene et al. (1978). Other restriction endonucleases were purchased from Bethesda Research Laboratories (BRL) and used according to their specifications. Cohesive and blunt-end ligation conditions, and the preparation of T4 DNA ligase were carried out according to the method of Tait et al. (1980). Fill-in reactions were performed in a volume of 100 μl containing 10 μg of the linear DNA and 2 units of *E. coli* DNA polymerase I "Klenow" fragment (PL Biochemicals). The fill-in buffer contained 40 mM phosphate, pH 7.4, 50 μM each deoxynucleotide triphosphate (Sigma), 6.2 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. Reactions were terminated after 30 min at 37°C.

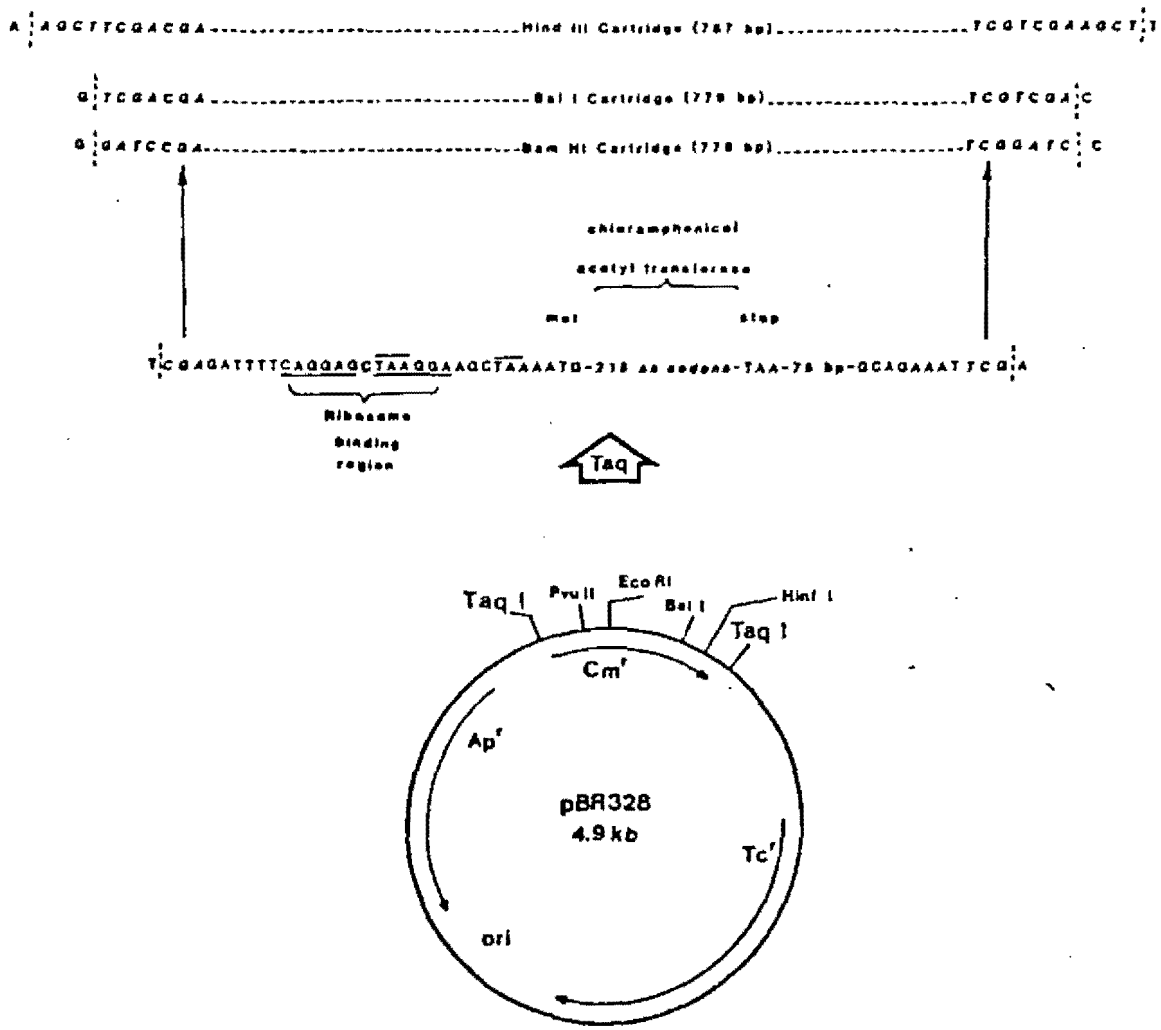

Fig. 1. Construction of the CAT cartridges. A partial restriction map of pBR328 is drawn approximately to scale. The *Pvu*II, *Eco*RI and *Bal*I cleavage sites are unique to the plasmid. Also shown are the salient features of the 773-bp *Taq*I fragment (see RESULTS). The two vertical arrows indicate the nucleotide sequences (5′ → 3′) at the ends of the *Bam*HI, *Sal*I and *Hin*dIII CAT cartridges. In addition to the 3 bp of the *Taq*I site, the *Bam*HI and *Sal*I cartridges possess 4 bp derived by fill-in reactions and blunt-end ligation to their respective sites in pBR327. The additional 8 bp at the end of the *Hin*dIII cartridge were derived by fill-in reactions and blunt-end ligation of the *Sal*I cartridge to the *Hin*dIII cleaved pBR327. The thick vertical arrow indicates a *Taq*I cleavage and the broken vertical lines separate cartridge sequences from plasmid sequences.

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RESULTS

(a) Construction of chloramphenicol-resistance-gene cartridges

The nucleotide sequence of the transposon, Tn*9*, indicates that the entire CAT polypeptide (plus 29 bp at the 5′ end and 84 bp at the 3′ end of the gene) is encoded on a 773-bp *Taq*I DNA fragment (Alton and Vapnek, 1979). As shown in Fig. 1, this 773-bp fragment contains single cleavage sites for *Eco*RI, *Hin*fI, *Bal*I and *Pvu*II, but no sites for *Sau*3A, *Hha*I, *Tha*I or any other known hexamer-specific restriction enzyme. The 29 bp preceding the CAT initiation codon include two TAA stop codons which are in the same translational reading frame with each other, but not with the CAT polypeptide. Also present in this region are two short sequences that share homology with the canonical *E. coli* ribosomal binding site. The 773-bp *Taq*I fragment does not contain the native Cm^r gene messenger RNA initiation point, RNA polymerase binding site, or cyclic AMP receptor protein binding site (LeGrice et al., 1982).

To change the *Taq*I restriction sites at the ends of the 773-bp fragment, the fragment was cloned in pBR327 by blunt-end ligation. The plasmid pBR327 was digested with either *Sal*I or *Bam*HI, the cohesive ends made blunt by a fill-in reaction, and the products blunt-end ligated to the treated *Taq*I fragment (see MATERIALS AND METHODS). Inserting the *Taq*I fragment into either the *Sal*I or *Bam*HI site restored the respective hexamer restriction sites on each end of the original *Taq*I fragment. Recombinant plasmids containing a *Sal*I or *Bam*HI CAT cartridge were isolated by selecting for Ap^r transformants on LB agar containing 20 μg/ml Ap and screening for the Cm^r pheno-

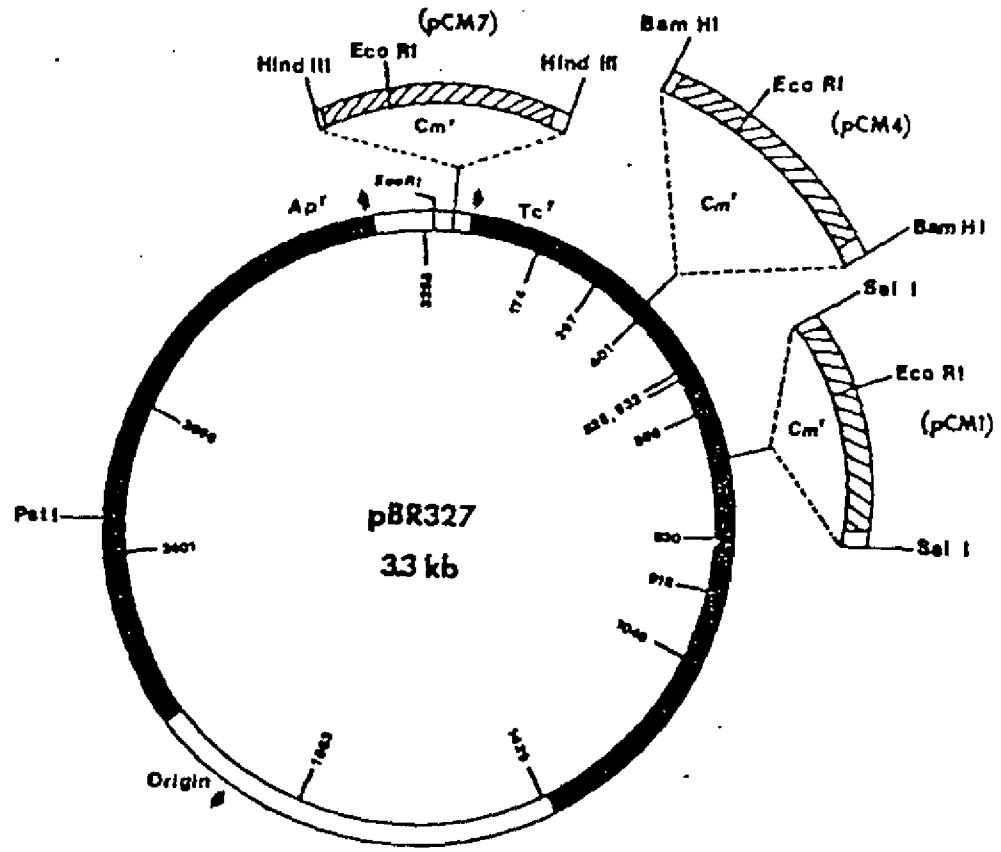

Fig. 2. A circular map of pBR327 showing the positions of various CAT cartridge inserts. The *Hin*dIII, *Sal*I and *Bam*HI cartridge inserts are shown in the R orientation (CAT coding sequence reads in the clockwise direction) outside the circle and corresponds to bp coordinates 29, 375 and 650, respectively. The numbers inside the circle are bp coordinates indicating those *Hae*III sites into which the *Sal*I cartridge was inserted. The size of all cartridge-containing plasmid derivatives is 4.1 kb.

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TABLE I

Effect of orientation on the expression of Cm^r from the CAT cartridges *

Plasmid	Cartridge position	Upstream promoter	Orientation	Resistance (μg/ml)	CAT specact. (nmol/min/mg prot.)
pBR328	–	Cm^r	–	600	4238
pCM1	*Sal*I	Tc^r	R	200	521
pCM1B	*Sal*I	Tc^r	L	10	8.5
pCM4	*Bam*HI	Tc^r	R	275	822
pCM4B	*Bam*HI	Tc^r	L	5	2.1
pCM7	*Hin*dIII	α-Tc^r	R	10	10.2
pCM7B	*Hin*dIII	α-Tc^r	L	275	826
pPV501	*Sal*I	ΔTc^r	R	5	4.0
none	–	–	–	2	< 0.2

* The designations R and L indicate a rightward or leftward orientation of the cartridge in pBR327 (Fig. 4). The resistance levels were determined by streaking liquid cultures on LB medium containing 0.2% glucose plus various concentrations of chloramphenicol. Plates were incubated 18 h at 30°C, then scored for the presence or absence of visible single colonies. The limit of resolution of the spectrophotometric assay for CAT was 0.2 specific activity units (nmol/min/mg protein). Standard units according to Shaw (1975) are measured in μmol/min/mg protein. All values are the averages of measurements made at least in triplicate.

type by replica plating to LB agar containing 30 μg/ml Cm. The *Sal*I CAT cartridge was transferred to the *Hin*dIII site of pBR327 in the same manner, regenerating *Hin*dIII sites on both ends of the DNA fragment. As indicated in Fig. 2, plasmids pCM1, pCM4 and pCM7 contain the *Sal*I, *Bam*HI and *Hin*dIII cartridges, respectively.

(b) Promoter-dependent expression of the CAT cartridge

As shown in Table I, the CAT cartridges were expressed at a level 12–19% that of the wild-type CAT activity level when placed downstream of either the Tc^r gene promoter (pCM1 and pCM4) or the α-Tc^r promoter (pCM7B). At the same positions but in the reverse orientations, the CAT cartridges were expressed at dramatically reduced levels. However, in every case a basal level of expression was detectable in terms of both the Cm^r phenotype and CAT activity. When the *Sal*I CAT cartridge was inserted into the *Sal*I site of pPV33, a Tc^r promoter deletion plasmid derived from pBR327 (West and Rodriguez, 1982), only basal levels of Cm^r and CAT activity were detected (pPV501). That high levels of Cm^r gene expression were observed only when the cartridges were inserted downstream of a plasmid promoter implies that each cartridge was missing its native promoter. This interpretation was verified by inserting the *Sal*I CAT cartridge into an M13 cloning vector M13mp7 (Messing et al., 1981) in the direction opposite to that of phage transcription. CAT activity was not detected in *E. coli* cells infected with this recombinant phage derivative (data not shown). These results are consistent with the in vitro studies on the Cm^r gene promoter by LeGrice et al. (1982).

(c) A map of transcriptionally active regions of pBR327

To determine the level and direction of transcription around the pBR327 genome, the *Sal*I CAT cartridge was inserted into various *Hae*III sites on the plasmid (Fig. 2). Plasmid pBR327 was partially digested with *Hae*III (approximately one cut per 20 plasmid molecules) and blunt-end ligated to purified *Sal*I cartridge DNA that had been made blunt-ended as described earlier. This treatment regenerated *Sal*I sites on each end of the inserted cartridge, facilitating subsequent restriction mapping of insertion sites in each derivative plasmid. Transformants were selected on LB, 0.2% glucose medium containing 10 μg/ml Cm. Selection on LB agar containing 10 μg/ml Cm limited our analysis to only those *Hae*III insertion sites where the CAT cartridge could be

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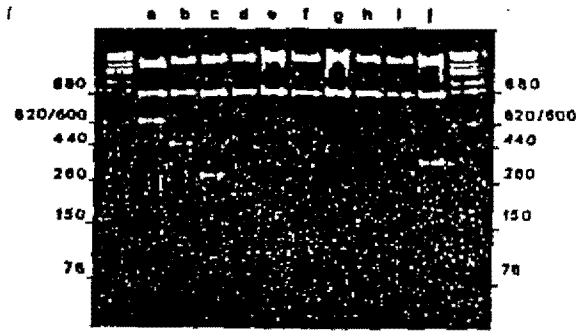

Fig. 3. The *Sal*I digestion patterns for pBR327 derivatives containing the *Sal*I cartridge in or near the Tc^r gene. All recombinants contain the 779-bp *Sal*I cartridge plus two other *Sal*I fragments whose sizes are used to estimate the particular *Hae*III insertion site in pBR327. The bp coordinates (see Fig. 2) for the various cartridges are as follows: Lane a, 3255; lane b, 174; lane c, 297; lane d, 401; lane e, 525; lane f, 532; lane g, 596; lane h, 830; lane i, 919; lane j, 1048. The original *Sal*I site is located at position 650 (out of a total of 3273 bp). The outer lanes contain ϕ29 DNA digested with *Hin*dIII to yield DNA fragments with the indicated sizes.

expressed at a detectable level. The selection for Cm^r clones on media containing less than 10 μg/ml Cm failed to discriminate chromosomal mutants for Cm^r from plasmid-mediated resistant cells. Plasmid DNAs were screened for unit length pBR327 joined to a single *Sal*I cartridge. The exact position of each insertion was determined using a combination of *Sal*I, *Pst*I, *Hin*fI, *Hha*I, *Taq*I, *Tha*I, *Hae*II and *Hae*III digestions. Of 121 Cm^r transformants examined in this manner, 34 were shown to be unit length pBR327 plus a single *Sal*I cartridge insertion. Of the 17 possible *Hae*III

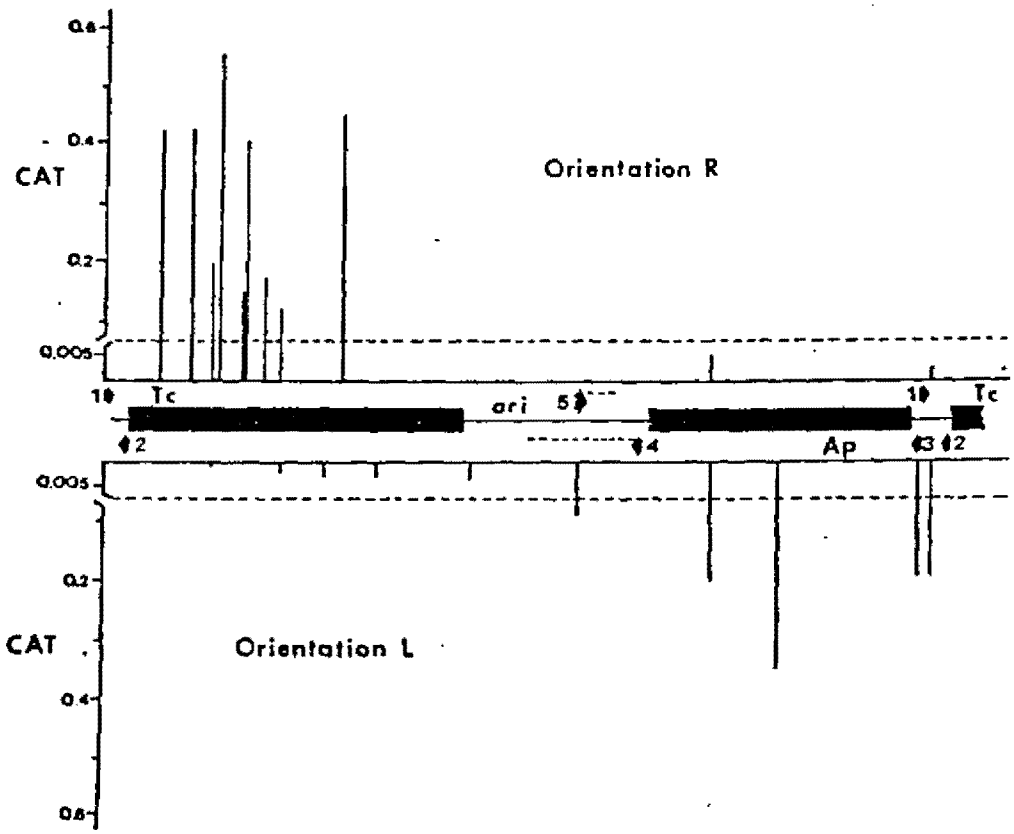

Fig. 4. CAT activities vs. the position and orientation of cartridge insertions. CAT activities are expressed as a fraction of the activity in cells containing pBR328, grown and assayed under the same conditions. The vertical bars indicate the level of CAT activity at various positions within plasmid pBR327. The dashed lines above and below the map of pBR327 (horizontal axis) denote boundary of an expansion of the vertical scale so that low levels of CAT activity can be visualized. The positions and orientations of previously identified promoters (see RESULTS) are indicated by numbers 1 through 5. Promoter 1 is the Tc^r gene promoter; promoter 2 is the "anti-tet" promoter; promoter 3 is the native Ap^r gene promoter; promoter 4 was identified by Itoh and Tomizawa (1980); promoter 5 is responsible for the production of a 104-base transcript (Chan et al., 1979). The upper half of the graph contains CAT activity values for cartridges inserted in the R orientation, and the lower half contains values for cartridges inserted in the L orientation.

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insertion sites, 14 sites received a cartridge. Only one site (position 2401 within the Ap^r gene) received a cartridge in each orientation. The *Sal*I digestion patterns for plasmids with a cartridge inserted in the vicinity of the Tc^r gene are shown in Fig. 3.

Cells containing each of the cartridge insertion plasmids were assayed for CAT activity, and the results are summarized in Fig. 4. Two major points are clear from these data. First, plasmid pBR327 has regions of high and low transcriptional activity and second, the cartridges are not expressed equally at different positions within a transcriptionally active region.

As expected from in vitro studies by West and Rodriguez (1980) and Stüber and Bujard (1981), inserting the CAT cartridge into any of several positions activates Cm^r gene expression. High levels of CAT activity (12–57% of wild-type) were observed when a CAT cartridge was inserted in the rightward orientation (R) downstream of the Tc^r gene promoter (promoter 1, Fig. 4) through the Tc^r gene, and in the leftward orientation (L) downstream of the α-Tc^r and Ap^r gene promoters (promoters 2 and 3, respectively) through the Ap^r gene. A slightly lower level of CAT activity (8.3%) was observed when the cartridge was inserted in the L orientation between the Ap^r and Tc^r genes at position 1863, downstream of promoter 4. This CAT activity value is likely to be an underestimate of transcriptional activity in the L orientation through position 1863 since inserting a cartridge at this position resulted in roughly an 8-fold reduction of plasmid copy number (copy number data not shown). Another strong promoter (promoter 5) is known to be responsible for a 104-base transcript in vitro and in vivo reading in the R orientation between the Ap^r and Tc^r genes. This promoter was not identified using the Cm^r gene cartridges. However, the only *Hae*III site in this area lies within the promoter sequence for the 104-base transcript (Chan et al., 1979). Inserting the cartridge into this *Hae*III site presumably disrupts the promoter, preventing CAT expression at detectable levels in the R orientation.

Low levels of CAT activity (0.05–0.48% of wild-type) were observed when the cartridge was inserted backwards in either the Ap^r or Tc^r gene, and in the R orientation at the *Hin*dIII site, disrupting the Tc^r gene promoter. When the CAT

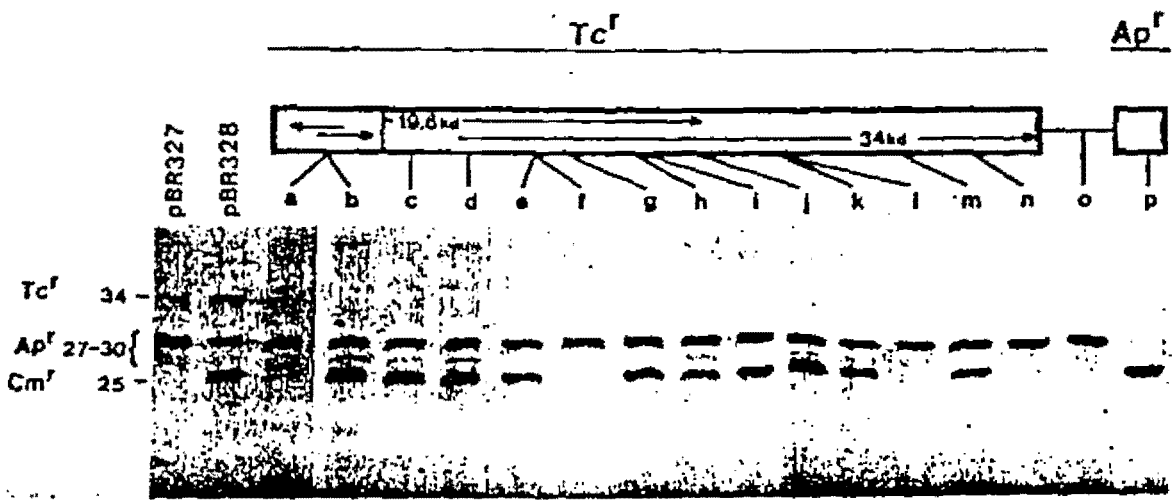

Fig. 5. Proteins expressed from plasmid genes using *E. coli* maxicells. The two lanes at the left of the figure show the pattern of the Tc^r, Ap^r and Cm^r polypeptides from plasmids pBR327 and pBR328. Other lanes contain polypeptides from cartridge-insertion plasmids, with the bp position and orientation of the cartridge indicated in parentheses: lane a, pCM7 (29R); lane b, pCM7B (29L); lane c, pCM101 (174R); lane d, pCM104 (297R); lane e, pCM4 (375R); lane f, pCM4B (375L); lane g, pCM105 (401R); lane h, pCM106 (525R); lane i, pCM108 (532R); lane j, pCM109 (596R); lane k, pCM1 (650R); lane l, pCM1B (650L); lane m, pCM113 (919R); lane n, pCM114 (1048L); lane o, pCM115 (1429L); lane p, pCM127 (2668L). Cartridge insertion sites relative to the Ap^r and Tc^r genes are indicated above the autofluorogram. The Tc^r polypeptides predicted from the nucleotide sequence (Sutcliffe, 1979) are also shown. The putative points of translational initiation for the M_r 19.6×10^3 polypeptide and the M_r 34×10^3 polypeptide are at nucleotide positions 86 and 259, respectively.

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cartridge was inserted into the Ap^r gene in the anti-sense orientation, CAT activity was produced at about 1/40 of the level in the forward or sense orientation. In comparison, expression of the CAT cartridge in the antisense orientation within the Tc^r gene ranged from about 1/600 to 1/200 of the level in the forward or sense orientation. The source of this low level of transcription in either case is not clear.

Downstream of the Tc^r gene promoter there is a discontinuous pattern of Cm^r gene expression versus the position of the inserted cartridge. This discontinuity cannot be attributed to differences in plasmid copy number since the copy number of each plasmid with a CAT cartridge inserted in the Tc^r gene was found to be nearly equal (data not shown). The level of Cm^r gene expression does not steadily decrease as the distance of the cartridge from the Tc^r promoter increases (see Fig. 4, R orientation in the Tc^r gene). Therefore, the length of the messenger RNA preceding the Cm^r gene does not appear to play an important role in causing this irregularity. Since the *Sal*I cartridge contains stop codons in only one reading frame, one might propose a competition between the production of fusion or truncated missense polypeptides and native CAT polypeptide to account for the discontinuity. This possibility was investigated by using the *E. coli* maxicell system (Sancar et al., 1979) to examine the sizes of the proteins produced by the various plasmids containing CAT cartridge insertions. When the CAT cartridges were inserted in the R orientation within the structural portion of the Tc^r gene, native size CAT polypeptide was observed (Fig. 5, lanes c, d, e, g, h, i, j, k, m). Cartridges inserted within the Tc^r gene in the L orientation resulted in the production of little or no CAT polypeptide (lanes f, l, and n). It should also be noted that all cartridge insertions into the Tc^r gene resulted in the disappearance of the M_r 34 × 10^3 Tc^r protein (Fig. 5, lanes pBR327 and pBR328). Although this protein is required for the expression of Tc^r (Tait et al., 1978), it appears to be preceded by and overlaps with an M_r 19.6 × 10^3 polypeptide of unknown function (Sutcliffe, 1979). A cartridge insertion in the non-overlapping region of the M_r 19.6 × 10^3 polypeptide coding sequence at position 174 (Fig. 5, lane c) would not be expected to affect the M_r 34 × 10^3 protein. Furthermore, no fusion products between CAT and the M_r 19.6 × 10^3 or 34 × 10^3 proteins were observed in the electrophoretic patterns shown in Fig. 5. This was unexpected since cartridge insertions at positions 533 or 297, 375, and 525, are in the same translational reading frames as the M_r 19.6 × 10^3 or 34 × 10^3 proteins, respectively.

When the CAT cartridges were inserted in the L orientation, abundant expression of the CAT enzyme was observed in several instances. Two of these cases involving insertions at positions 29 and 2668 are shown in Fig. 5. Inserting the CAT cartridge at position 29 (the *Hin*dIII site) abolished expression of the M_r 34 × 10^3 protein by disrupting the promoter for the Tc^r gene (Fig. 5a and b). The L orientation placed the Cm^r gene under the control of the α-Tc^r promoter (West and Rodriguez, 1982). Similarly, inserting the cartridge at position 2668 abolished production of β-lactamase, the Ap^r gene product (Fig. 5, lane p), but enabled the CAT gene to be expressed by readthrough transcription from the Ap^r gene promoter. In this case, the absence of a β-lactamase-CAT fusion polypeptide can be explained by the presence of two translational stop codons on the CAT cartridge (Fig. 1) in frame with translation of the Ap^r gene.

DISCUSSION

Using the CAT cartridge system of analysis, a map of the transcriptionally active regions of pBR327 was constructed. As shown in Fig. 4, low levels of transcription were observed in intergenic regions on the plasmid as well as in the anti-sense direction through both the Ap^r and Tc^r genes. Assuming that all plasmid transcription initiates at defined promoters (West and Rodriguez, 1980; Stüber and Bujard, 1981), intergenic and anti-sense transcription could be due to either the loss of termination signals during the construction of pBR327 (and pBR322) or occasional readthrough transcription past preferred termination sites. For example, readthrough transcription from promoter 5 (Fig. 4) is probably responsible for the expression of CAT from the cartridge inserted at position 2401 or 2668 (R orientation). This notion is

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consistent with earlier studies that reported the expression of promoter-deficient genes cloned in the anti-sense direction of the Ap^r gene (Chang et al., 1978). As these insertions illustrate, expression of a cloned gene in both orientations cannot be used as the sole criterion for determining whether a gene possesses its own promoter.

In transcriptional mapping studies the CAT cartridges can be used to determine the location and direction of transcription, but quantitative comparisons of promoter strength should not be attempted. To emphasize this point one need only consider the difference in the levels of expression between an insertion at position 525, which results in only 15% of the wild-type level of CAT activity, and an insertion at position 532, which results in 40% of the wild-type level (Fig. 2 and Fig. 4). Since these two insertions are downstream of the same promoter, and only 7 bp apart, the 3-fold difference in Cm^r gene expression must reflect some important difference between the two insertion sites rather than differences in promoter strength. For example, there may be regions within the Tc^r gene that affect RNA polymerase pausing and premature termination of transcription.

The maxicell analysis revealed that native size CAT polypeptide was always produced when the promoter-deficient CAT cartridge was inserted downstream of a plasmid promoter. This demonstrates that the CAT cartridge carries the ribosome binding site for the Cm^r gene. However, as mentioned above, the levels of Cm^r and CAT activities were not equal for different insertions within the Tc^r gene. Although Goldfarb et al. (1982) have demonstrated a competition between the production of native and fusion CAT polypeptides in *E. coli* when *Bacillus subtilis* DNA fragments are fused to the CAT cartridge, the lack of detectable polypeptide fusions in these studies suggests that this discontinuity in expression is not due to a competition between the CAT ribosome binding site and other ribosome binding sites preceding it.

Insertion of the CAT cartridge at any position within the Tc^r gene resulted in the inactivation of the Tc^r phenotype and the disappearance of the M_r 34×10^3 protein. Other studies using the CAT cartridges have shown that there is no transcriptional terminator at the 3′ end of the cartridges (data not included). Therefore, the absence of the M_r 34×10^3 protein resulting from a cartridge inserted nearly 100 bp upstream of its putative translational start point suggests a regulatory mechanism for the Tc^r gene similar to the attenuation control mechanism of amino acid biosynthetic genes (Yanofsky, 1980). The expression of the CAT cartridges within the Tc^r gene may represent a special case with respect to the way they are expressed in other genes.

The use of the CAT cartridges to map transcriptionally active regions on pBR327 may serve as a general method for the transcriptional mapping of a variety of extrachromosomal elements. As in this study, expression of the Cm^r gene at various points of insertion can be scored phenotypically, and CAT activity measured enzymatically. Additionally, production of the CAT polypeptide can be detected immunologically by protein blotting techniques using antibodies to purified CAT protein. Cm^r gene-specific RNA can also be examined by blotting and hybridization techniques using probes such as the plasmid pCM1 or purified CAT cartridge DNA fragments. The expression of Cm^r by a particular host is the only factor which limits the use of the CAT cartridges for transcriptional mapping. Since the Cm^r gene is expressed in *S. cerevisiae*, we are using the CAT cartridges and derivative plasmid vectors in yeast to examine promoters from yeast and other eukaryotic organisms.

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Efficient integrative transformation of *Cephalosporium acremonium*

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Summary. A hybrid gene, $IPNS_p/HPT_{orf}$, was constructed by placing an 850 bp sequence of *Cephalosporium acremonium* DNA next to the 5′ end of a bacterial open reading frame, HPT_{orf}. The sequence was obtained as an 850 bp *Nco*I restriction fragment from the 5′ noncoding region of the *C. acremonium* isopenicillin N synthetase (IPNS) gene. The HPT_{orf} was obtained from a bacterial gene that coded for a hygromycin B phosphotransferase (HPT). Plasmids that contained $IPNS_p/HPT_{orf}$ transformed *C. acremonium* to a stably maintained hygromycin B resistant phenotype. Southern analysis of total DNA from transformants demonstrated multiple integrations of the transforming DNA in the high molecular weight DNA of most transformants, but single integrations were observed in a few transformants. The number of transformants per μg of DNA was about 100 times greater than for plasmids that contained the HPT_{orf} without any juxtaposed eucaryotic promoter sequence. Plasmids with the promoterless HPT_{orf} and plasmids with a truncated *S. cerevisiae* phosphoglycerate kinase promoter juxtaposed to the HPT_{orf} transformed *C. acremonium* at equivalent low frequencies.

Transformation of *C. acremonium* with linearized plasmid DNA produced at least 2–3 fold more transformants than the corresponding circular molecule. Several observations were made concerning protoplast formation and handling which made the transformation procedure more efficient and allowed a greater proportion of protoplasts to regenerate to viable walled cells.

Plasmids were constructed that contained both the $IPNS_p/HPT_{orf}$ and additional elements: fragments of *C. acremonium* ribosomal DNA (rDNA), or a fragment of *C. acremonium* mitochondrial DNA possessing activity as an autonomous replication sequence (ARS) in *S. cerevisiae*, or putative transcriptional termination/polyadenylation signals from the IPNS gene. These plasmids transformed *C. acremonium* at frequencies experimentally equivalent to those containing $IPNS_p/HPT_{orf}$ without any of these additional elements.

Key words: *Cephalosporium acremonium* – Transformation – Hygromycin B phosphotransferase – Filamentous fungus – Beta-lactam antibiotics

Introduction

C. acremonium is a medically and industrially important filamentous fungus that produces the beta-lactam antibiotic cephalosporin C. Cephalosporin C is chemically modified to produce many clinically significant antibiotics (Bunnell et al. 1986). The asexual nature of *C. acremonium* impedes study and manipulation by "classical" genetic methods. Nevertheless, over the past three decades natural selection and mutagenesis/screening (Elander and Aoki 1982), biochemical selections (Queener et al. 1984) and protoplast fusion (Hamlyn et al. 1985) have been used to study the genetic characteristics of this organism and to dramatically improve the fungal production of cephalosporin C.

Compared to extensive studies of *Saccharomyces cerevisiae*, molecular biology in *C. acremonium* is at an early stage. Recently low frequency integrative genetic transformation of *C. acremonium* was demonstrated (Queener et al. 1985; Chapman et al. 1986). Transforming DNA consisted of plasmids carrying a hybrid antibiotic resistance gene, PGK_p/HPT_{orf}. The hybrid gene had an open reading frame, HPT_{orf}, obtained from a plasmid-borne gene that encoded a hygromycin B phos-

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photransferase (HPT) in *E. coli* (Rao et al. 1983). To form the hybrid gene the HPT_{orf} was juxtaposed to a fragment, PGK_p, obtained from the 5′ noncoding region of the phosphoglycerate kinase (PGK) gene of *S. cerevisiae* (Kaster et al. 1984). PGK_p exhibits promoter function in yeast as judged by its ability to promote expression of open reading frames in yeast when juxtaposed in correct orientation to those orfs (Kaster et al. 1984; Queener et al. 1985). In the native yeast gene this fragment is immediately adjacent to the 5′ end of the open reading frame, PGK_{orf}, that codes for PGK. PGK_p does not contain the two upstream activating sequences [demonstrated by Ogden et al. (1986)] that are present ca. 400 bp 5′ to the ATG of the PGK_{orf} in yeast. *C. acremonium* transformants obtained by exposing protoplasts to PGK_p/HPT_{orf}-containing plasmids were stable and exhibited a hygromycin B resistant phenotype not observed in the untransformed recipient strain. The transformed phenotype was associated with presence of an intracellular HPT that was not present in the untransformed recipient strain. HPT in *C. acremonium* transformants and HPT in the *E. coli* strain that harbored the natural HPT gene both exhibited the same substrate specificity in vitro. Transformation of *C. acremonium* occurred by integration and multiple integration events were observed in all transformants analyzed (Queener et al. 1985).

Application of recombinant DNA technology offered for the first time an ability to alter specifically and predictably characteristics relevant to antibiotic production in *C. acremonium*. For example, the gene which produces isopenicillin N synthetase (IPNS), a key enzyme in cepahlosporin C biosynthesis (Queener and Neuss 1982), was recently cloned (Samson et al. 1985). Returning this gene to *C. acremonium* in multiple copy fashion may increase antibiotic production. Although our original transformation system for *C. acremonium* made the latter manipulation possible, transformation frequency was only ca. 0.2 transformants per μg of DNA (Queener et al. 1985). Inefficiency of transformation slowed our gene-dosage studies and precluded practical application of many molecular biological techiniques: screening a library of *C. acremonium* DNA by complementation of *C. acremonium* mutants, disrupting a gene as described in *N. crassa* (Paietta and Marzluf 1985), or doing gene replacements like those reported in *Aspergillus nidulans* (Miller et al. 1985). In addition to these limitations, we observed considerable variation in transformation frequency, particularly when transforming strains other than the wild type strain of *C. acremonium*. To solve these problems, we have constructed a new hybrid HPT gene, $IPNS_p/HPT_{orf}$. Here we describe that construction and report that plasmids containing $IPNS_p/HPT_{orf}$ transform *C. acremonium* at frequencies 100-fold higher than those observed with our previous vectors. Observations that improved previously reported conditions for preparing and exposing protoplasts to transforming DNA (Queener et al. 1985) are also described.

Materials and methods

Strains and media. Wild type *C. acremonium* strain ATCC11550 was used for all transformation experiments. The media used for the routine growth and selection of *C. acremonium* transformants have been described (Queener et al. 1985). *E. coli* strain JM109 was used for all *E. coli* transformations and propagation of recombinant plasmids. 2 x YT medium (Miller 1972) supplemented with 100 μg/ml ampicillin, 40 μg/ml X-GAL and 40 μg/ml IPTG, all from Sigma Chemical Co., was used for selection of ampicillin resistant transformants.

Transformation. *E. coli* were transformed according to the method of Cohen et al. (1972). *C. acremonium* was transformed according to the method of Queener et al. (1985). In several experiments modifications of the published procedure were incorporated; these modifications are noted in the text.

DNA Isolation. DNA was isolated from *C. acremonium* by the method of Minuth et al. (1982). For small scale isolation of DNA from *C. acremonium* transformants, the mycelium of a single colony or a confluent lawn of growth was harvested from agar medium containing hygromycin B. For strain ATCC11550, mycelium was from the same medium without antibiotic. Mycelia were processed as described in the method above except the DNA was not purified by cesium chloride equilibrium centrifugation.

Recombinant plasmids were isolated from *E. coli* according to the method of Clewell (1972). Rapid small scale *E. coli* plasmid preparations were made as described by Klein et al. (1980). A modification of the rapid method of Eckhardt (1978) was used to identify plasmids carrying insertions or deletions when there was no phenotypic marker available to indicate the presence or absence of such events.

Preparation of ^{32}P-labeled DNA for use as hybridization probes. The method of Rigby et al. (1977) was used for labeling plasmid DNA for use as a probe in hybridization experiments. dATP, dGTP, and dTTP were purchased from Calbiochem. Deoxycytidine 5′-[alpha-^{32}P] triphosphate, triethylammonium salt was purchased from New England Biolabs. Specific activity of probes produced by this procedure were approximately 1×10^8 cpm/μg of DNA.

Transfer and hybridization of DNA. The method of Southern (1975) was used to transfer denatured DNA from agarose gels to nitrocellulose paper (BA85, Schleicher and Schuell). The DNA was fixed to the filters by baking at 80 °C for at least 2 h. The method presented in Maniatis et al. (1982) was employed for the hybridization of radio-labeled probes to DNA immobilized on the filters. Nitrocellulose filters exposed to probe DNA were washed, air dried, and exposed to Kodak X-Omat AR film with intensifying screens on both sides at −70 °C for an appropriate period of time.

Restriction endonuclease digestion, DNA fragment isolation, ligations and agarose gel electrophoresis. All of these procedures were as described previously (Skatrud and Queener 1984).

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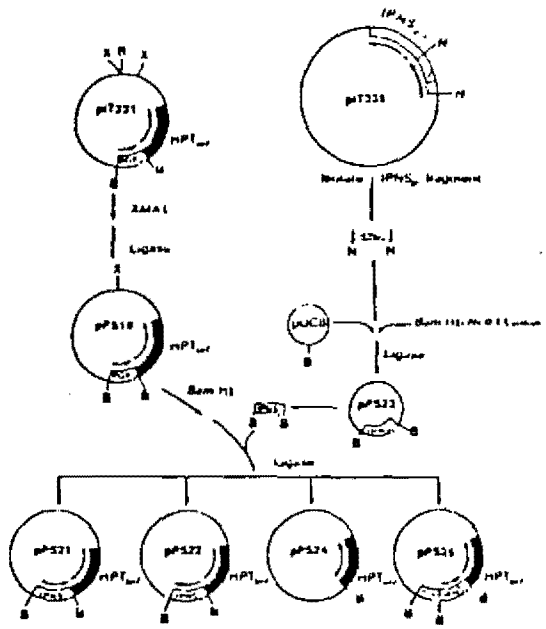

Fig. 1. Construction of plasmids pPS21, pPS22, pPS24, and pPS25. *Abbreviations:* PGK_p a 230 bp *Bam*HI fragment that exhibits promoter activity in *S. cerevisiae* – taken from the 5' noncoding region of the phosphoglycerate kinase gene of *S. cerevisiae*; HPT_{orf} the open reading frame of the hygromycin B phosphotransferase gene from *E. coli*; $IPNS_{orf}$ the open reading frame of the isopenicillin N synthetase gene from *C. acremonium*; $IPNS_p$ sequence of DNA that exhibits promoter activity in *C. acremonium* – taken from the 5' noncoding region of the isopenicillin N synthetase gene of *C. acremonium* on an 850 bp *Nco*I fragment (see Materials and methods). *Restriction enzymes:* X *Xma*I; B *Bam*HI; N *Nco*I. Only those restriction sites and functional elements critical to constructions are included in this figure and subsequent figures

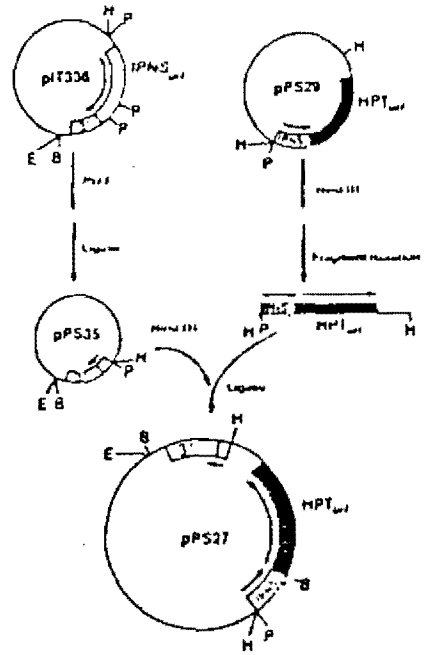

Fig. 2. Construction of plasmid pPS27. *Abbreviations:* $IPNS_{orf}$, $IPNS_p$, and HPT_{orf} are defined in the legend to Fig. 1 and in the text. *Restriction enzymes:* H *Hin*dIII; P *Pst*I; E *Eco*RI; B *Bam*HI

Preparation of synthetic oligonucleotides. Synthetic oligonucleotides used for the construction of linker molecules were prepared by Dr. R. Belagaje of Eli Lilly and Co. using an Applied Biosystem DNA synthesizer (Model 380-A) according to the manufacturer's recommendations.

Fractionation of protoplast mixtures. To separate large and small protoplasts by size, suspensions of freshly prepared protoplasts were fractionated by a low speed centrifugation (2 min, International Clinical Centrifuge Model CL, setting of 2). After the initial low speed centrifugation the residual enzyme was removed as previously described (Queener et al. 1985). Protoplasts obtained from the pellet of the initial low speed centrifugation (fraction #2) were larger than protoplasts from the initial supernatant (fraction #1).

Construction of plasmids. Plasmids pPS21, pPS25, pPS31, and pPS46 contain a new hybrid hygromycin B phosphotransferase gene, $IPNS_p/HPT_{orf}$. Plasmids pPS27, pPS29, and pXL6 also contain $IPNS_p/HPT_{orf}$ but lack 150 bp of *C. acremonium* DNA upstream of the hybrid gene that is present in the first four plasmids (described for pPS29 below). The starting materials for the construction of $IPNS_p/HPT_{orf}$ and plasmid pPS21 were plasmid pIT221 (Queener et al. 1985) and plasmid pIT335 (Samson et al. 1985). The *Bam*HI site at the 12 o'clock position in pIT221 was removed by digestion with *Xma*I and religation under dilute conditions to form pPS19. The 230 bp *Bam*HI fragment immediately adjacent to the HPT_{orf} was then removed from pPS19 by *Bam*HI digestion. To form plasmid pPS21, the fragment, PGK_p, in pPS19 was replaced with an 850 bp *Nco*I fragment from the 5' noncoding region of the isopenicillin N synthetase (IPNS) gene of *C. acremonium*. The sequence comprising the latter fragment, obtained by digesting pIT335 with *Nco*I, starts at the initiation codon of the IPNS gene and extends 850 bp in the 5' direction. Two synthetic oligonucleotides 5'-CATGAACAAC-3' and 5'-GATCCTTCTT-3' were phosphorylated with T4 DNA kinase. The phosphorylated oligonucleotides were annealed to form a linker with *Bam*HI and *Nco*I compatible ends. A mixture of the linker, the 850 bp *Nco*I fragment and *Bam*HI-digested pUC8 (Vieira and Messing 1982) was ligated. The ligation mixture was used to transform *E. coli* to ampicillin resistance. Plasmid pPS23 was isolated from a transformant and its structure verified by restriction analysis. A fragment containing the 850 bp sequence – now bounded by *Bam*HI restriction sites – was isolated. When this fragment was ligated to the *Bam*HI-digested pPS19, plasmids pPS21, pPS22, pPS24 and pPS25 were formed. The *Bam*HI ligation that formed pPS21 placed the initiation codon present on the linker in the proper reading frame with the HPT_{orf} and two lysine residues were added to the coding sequence just after the initiation codon. During the construction of pIT221 these two lysine residues had been removed from the native HPT_{orf}. Both the native HPT_{orf} (Rao et al. 1983) and the short HPT_{orf} (Kaster et al. 1984) code for active hygromycin B phosphotransferase enzymes that exhibit the same substrate specificity (Queener et al. 1985). All plasmids used in this study contain the native HPT_{orf} except plasmids pIT221 and pPS19 which contain the short HPT_{orf}. A flow diagram for the construction of pPS21 and related plasmids is presented in Fig. 1.

The construction of other plasmids is outlined below and/or is described schematically in appropriate figures. Plasmid pPS29

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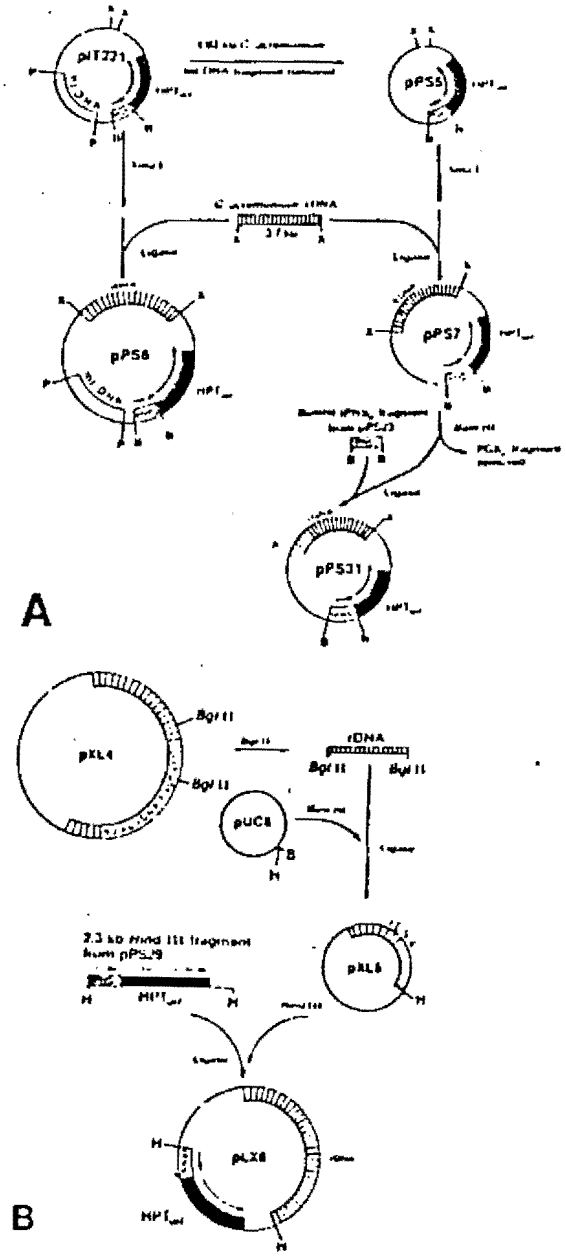

Fig. 3A, B. Construction of plasmids with ribosomal DNA inserts. A Construction of plasmids pPS5, pPS6, pPS7, and pPS31 containing a 3.7 kb *Xma*I restriction fragment of *C. acremonium* rDNA. B Construction of plasmid pXL6 containing a 2.9 kb *Bgl*II restriction fragment of *C. acremonium* rDNA. *Abbreviations:* HPT_{orf}, PGK_p, $IPNS_p$, and *mtDNA* are defined in the legends to Fig. 1 and Table 1; *rDNA* ribosomal DNA. *Restriction sites: B Bam*HI; *H Hin*dIII; *X Xma*I; *P Pst*I

was derived from pPS21 by deleting two restriction fragments. In pPS29 approximately 150 bp have been removed (on a *Pst*I fragment (from the 5' end of the 850 bp *Nco*I fragment present in pPS21)). The 1.93 kb *C. acremonium* mitochondrial DNA fragment (Tudzinski and Esser 1982; Skatrud and Queener 1984) was also removed (as a *Pst*I fragment) in forming pPS29. An abbreviated structure of pPS29 is presented in Fig. 2.

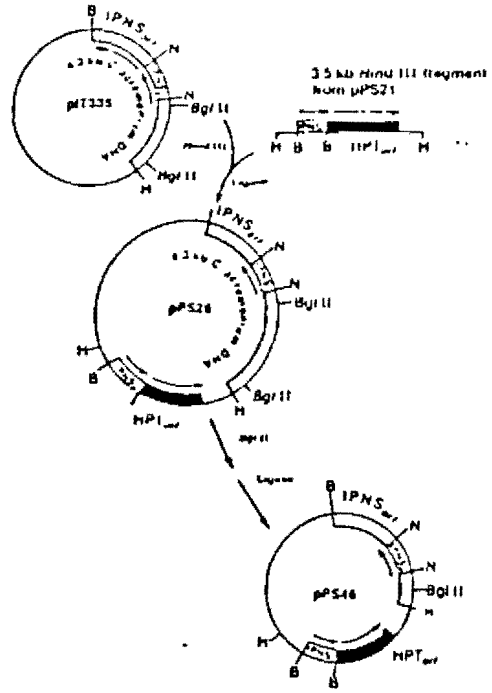

Fig. 4. Construction of plasmids pPS26 and pPS46. *Abbreviations:* $IPNS_{orf}$, $IPNS_p$, and HPT_{orf} are defined in Fig. 1 and in text; *Restriction sites: B Bam*HI; *H Hin*dIII; *N Nco*I

Plasmid pPS27 incorporates a *C. acremonium* sequence that comprises a portion (44 bp) of the 3' end of $IPNS_{orf}$, the open reading frame of the isopenicillin N synthetase gene, plus ca. 460 bp of the contiguous DNA immediately downstream from the translational stop codon of that gene. The ca. 500 bp sequence was incorporated approximately 600 bp from the 3' end of the HPT_{orf} (for construction details and orientation see Fig. 2).

Plasmids pPS6, pPS7, and pPS31 contain a fragment of *C. acremonium* DNA that encodes at least a portion of the 16-18S ribosomal RNA (rRNA) gene (Carr et al. 1987) on a *Xma*I fragment. These plasmids were constructed according to the flow diagram presented in Fig. 3A.

Subsequent to the construction of plasmids pPS6 and pPS7, it was discovered that the 3.7 kb *Xma*I insert in these plasmids contained a large region of lambda DNA juxtaposed to the DNA from the *C. acremonium* 16-18S rRNA gene. To obtain a plasmid that contained the hybrid HPT gene and a large tract of *C. acremonium* rRNA-encoding DNA without any lambda DNA present, plasmid pXL6 was constructed from pXL4, pUC8, and pPS29 as illustrated in Fig. 3B. Plasmid pXL6 carries a *Bgl*II fragment of *C. acremonium* DNA that contains DNA from the 16-18S rRNA gene, all of the 5.8S rRNA gene, and DNA from the 23-28S rRNA genes (Carr et al. 1987).

Plasmid pPS46 contains a hybrid HPT gene [$IPNS_p/HPT_{orf}$] and a complete IPNS gene. Plasmid pPS46 was constructed by ligating the approximately 3.5 kb *Hin*dIII restriction fragment of plasmid pPS21 into the single *Hin*dIII site of pIT335 to form intermediate plasmid pPS26. Plasmid pPS26 was then modified by deletion of the approximately 2.3 kb *Bgl*II restriction fragment within the *C. acremonium* DNA. This deletion produced plasmid pPS46 which possessed a unique *Bgl*II site within the *C. acremonium* DNA. The construction of pPS46 and intermediate plasmid pPS26 is illustrated in Fig. 4.

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Table 1. Transformation of *Cephalosporium acremonium* with plasmids containing the hybrid $IPNS_p/HPT_{orf}$ gene increased transformation frequency

Plasmid	Promoter	mtDNA[a]	rDNA	# Transformants per experiment (# of exp. trials)
$IPNS_p$ vs PGK_p promoter				
pIT221	PGK_p	+	–	4 (34)
pPS19	PGK_p	+	–	1 (5)
pPS24	no promoter	+	–	1 (5)
pPS22 (wrong orientation)	$IPNS_p$	+	–	2 (5)
pPS21	$IPNS_p$	+	–	493 (5)
pPS25	$IPNS_p$ + PGK_p	+	–	477 (5)
3' sequences of IPNS gene added to HPT gene				
pPS27 (compare to pPS29)	$IPNS_p$	–	–	239 (6)
mtDNA fragment removed, no 3' IPNS sequenced added				
pPS5 (compare to pIT221 and pPS19)	PGK_p	–	–	5 (5)
pPS29 (compare to pPS21)	$IPNS_p$	–	–	356 (5)
Ribosomal DNA added				
pPS6 (compare to pIT221 and pPS19)	PGK_p	+	+[b]	1 (6)
pPS7 (compare to pPS5)	PGK_p	–	+[b]	1 (7)
pPS31 (compare to pPS29)	$IPNS_p$	–	+[b]	128 (2)
pXL6 (compare to pPS29)	$IPNS_p$	–	+[c]	232 (4)

[a] mtDNA; 1.9 kb *Pst*I *C. acremonium* mitochondrial DNA fragment which confers ARS function to plasmids in *S. cerevisiae*
[b] 3.7 kb *Xma*I fragment containing *C. acremonium* ribosomal DNA (rDNA)
[c] 2.9 kb *Bgl*II fragment containing *C. acremonium* ribosomal DNA

In addition to the above mentioned functional elements, all plasmids used in this study contained an *E. coli* ampicillinase gene and the origin of DNA replication from pBR322 to permit transformation of *E. coli*.

Results

Plasmids containing a hybrid hygromycin B phosphotransferase gene expressed via a promoter from the isopenicillin N synthetase gene from C. acremonium transform C. acremonium at a high frequency

Plasmids pIT221 and pPS19 (Fig. 1) contain the hybrid antibiotic resistance gene, PGK_p/HPT_{orf}. These plasmids transformed *C. acremonium* at extremely low frequencies (Table 1 and Queener et al. 1985). Transformation of *C. acremonium* with plasmid pPS24, which contains no eucaryotic promoter directly upstream of HPT_{orf}, produced roughly the same low level of transformation as plasmids pIT221 and pPS19. In contrast, we previously observed that pIT221 transformed *S. cerevisiae* to hygromycin B resistance at moderately high frequency (Queener et al. 1985). These contrasting results suggested that the *S. cerevisiae* PGK_p functioned sufficiently to allow transcription of the HPT_{orf} in *S. cerevisiae* but did not exhibit significant promoter activity in *C. acremonium*. To overcome this problem a new hybrid antibiotic resistance gene, $IPNS_p/HPT_{orf}$, was constructed. The *S. cerevisiae* PGK_p in PGK_p/HPT_{orf} was replaced by 850 bp of DNA taken from the 5' non-coding region of the IPNS gene of *C. acremonium*. This fragment was presumed to carry a sequence, $IPNS_p$, that encoded information necessary for proper promoter function. A number of plasmids containing $IPNS_p/HPT_{orf}$ were constructed: pPS21, pPS25, pPS27, pPS29, pPS31, pPS46, and pXL6. These plasmids transformed *C. acremonium* at a frequency approximately 100-fold greater than pIT221 and pPS19 under the same conditions of transformation (Table 1). Plasmid pPS25 is analogous to pPS21 except the PGK_p was retained at the 5' end of the $IPNS_p$ fragment. pPS25 transformed *C. acremonium* at a frequency essentially equivalent to other plasmids that contained $IPNS_p/HPT_{orf}$. Plasmid pPS22 contains $IPNS_p$ adjacent to the 5' end of HPT_{orf} but in this plasmid the $IPNS_p$ is oriented opposite to the orientation in $IPNS_p/HPT_{orf}$. Transformation of *C. acremonium* with pPS22 was inefficient and quantitatively similar in frequency to the transformation observed for plasmid pPS24 that contained HPT_{orf} without any adjacent promoter (Table 1).

Transformation occurs by integration

We have previously demonstrated that, in each of a small number of representative transformants analyzed, transformation of *C. acremonium* by plasmid pIT221 occurred by integration into high molecular weight DNA (Queener et al. 1985). Multiple integration events were observed in each transformant. It was assumed that in a subset of the integration events the HPT_{orf} in the transforming DNA was positioned so that it might be efficiently expressed via an endogenous promoter.

Hybridization analyses of total uncut DNA from *C. acremonium* transformants obtained with plasmid pPS25, which contains $IPNS_p/HPT_{orf}$, are presented in Fig. 5. The transforming DNA was once again found associated with the high molecular weight DNA of transformants. This particular analysis cannot rule out

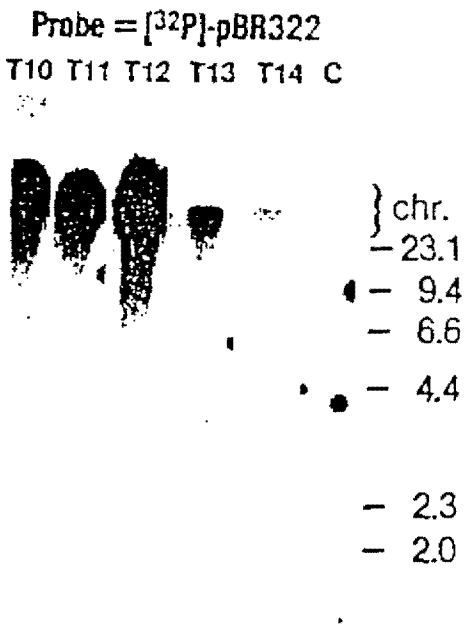

Fig. 5. Southern hybridization analysis demonstrating presence of transforming DNA in the high molecular weight DNA of *C. acremonium* cells transformed with plasmid pPS25. Total DNA was extracted from five independently isolated *C. acremonium* transformants (i.e., T10–T14) and the untransformed recipient strain ATCC11550 (C). DNA molecules (not cut with a restriction enzyme) from these isolates (approximately 2 μg DNA per sample) were separated by agarose gel electrophoresis. The DNA was transferred from the agarose gel to nitrocellulose filters by the method of Southern (1975) and hybridized as described by Maniatis et al. (1982). [^{32}P]-labeled pBR322 plasmid DNA was used as the hybridization probe to detect transforming DNA sequences. The autoradiogram was exposed to the nitrocellulose filter for 20 h. Extended exposure revealed no additional significant bands of hybridization. Notations at right of photograph denote position and size, in kilobases, of DNA standards and show position expected for unresolved *C. acremonium* chromosomes

the possibility of the presence of large multimers of the transforming plasmid. The difference in the intensity of the hybridization signal from isolates 10–12 as compared to isolates 13 and 14 suggested that the number of copies of the transforming DNA may vary considerably from one transformant to another. This suggestion was confirmed by hybridization analyses of the same DNA after restriction enzyme digestion (data not shown). A few isolates transformed with plasmids containing $IPNS_p/HPT_{orf}$ appeared to have a single insert of transforming DNA.

Presence of putative termination and polyadenylation sequences from the IPNS gene located 3′ to $IPNS_p/HPT_{orf}$ did not enhance transformation

The presence of homologous signals for proper transcriptional termination and messenger RNA polyadenylation should, in theory, enhance the expression of the hygromycin B phosphotransferase gene. Better expression of the selectable marker may, in turn, permit higher frequency of transformation which was the goal in this study. To subject this hypothesis to a preliminary test, plasmid pPS27 was constructed and transformation of *C. acremonium* with pPS27 and pPS29 was compared. Both pPS27 and pPS29 contain $IPNS_p/HPT_{orf}$ but pPS27 contains a putative termination/polyadenylation element not present in pPS29. The element in pPS27 is approximately 600 bp downstream of the 3′ end of the HPT_{orf}. The element comprises the terminal 44 bp of the *C. acremonium* $IPNS_{orf}$ plus ca. 460 bp of the *C. acremonium* sequences contiguous with and 3′ to the translational termination codon of that orf. Because of its position in native *C. acremonium* DNA, this fragment was presumed to contain signals for proper transcriptional termination and mRNA polyadenylation in *C. acremonium*. At equivalent DNA concentration both plasmids produced roughly the same number of transformants (Table 1). There were no convenient restriction sites nearer the 3′ end of the hygromycin B phosphotransferase gene. Rather than immediately pursuing possible improvement of transformation via constructions that would place the putative termination/polyadenylation element closer to the 3′ end of the HPT_{orf}, we chose to investigate how the form of our existing plasmids affected transformation.

Transformation of C. acremonium with linear DNA produces more transformants than the corresponding circular molecule

Hicks et al. (1979) and Orr-Weaver et al. (1981) have demonstrated 5- to 1000-fold increases in the integrative transformation frequency in *S. cerevisiae* when the transforming plasmid was first cut with a restriction enzyme in a region homologous with the recipient chromosomal DNA. Dhawale and Marzluf (1985) also reported an increase in transformation frequency when linearized plasmid DNA was used to transform *Neurospora crassa*. For *N. crassa* the increase in transformation frequency was only 3- to 4-fold.

Plasmid pPS46 (structure illustrated in Fig. 4) was digested with the restriction endonuclease *Bgl*II and the linearized molecule was used to transform *C. acremonium* to hygromycin B resistance. This enzyme cuts

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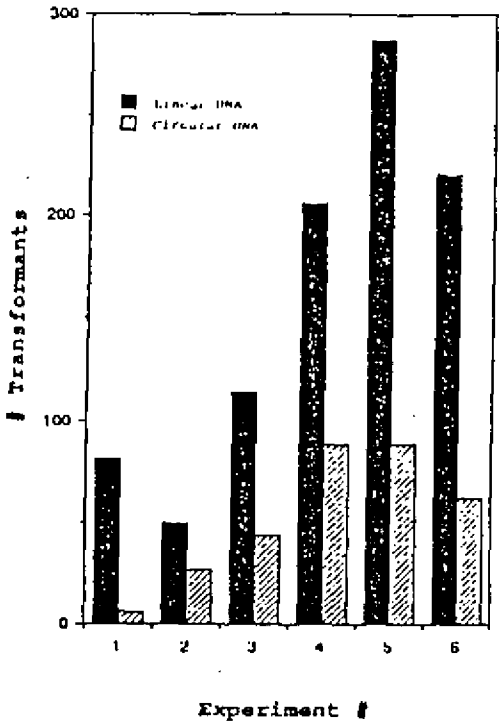

Fig. 6. Transformation of *C. acremonium* with linear DNA is more efficient than transformation with circular DNA. *C. acremonium* strain ATCC11550 was transformed to hygromycin B resistance by a modification of the method described by Queener et al. (1985). Twenty μg of pPS46 plasmid DNA were used in each transformation reaction, either circular or linearized by digestion with *Bgl*II

pPS46 plasmid DNA in a region homologous with the *C. acremonium* genome approximately 1,200 bp upstream from the 5′ end of the $IPNS_{orf}$. The results of six separate experiments are presented in Fig. 6. Several modifications of the transformation protocol were incorporated in these experiments which may account to some extent for the variability in the total number of transformants observed. However, regardless of alterations in conditions, we always observed at least a 2 to 3-fold increase in the frequency of transformation with linearized pPS46 plasmid DNA as compared to the same molecule in the circular form.

High frequency transformation of C. acremonium by plasmids with $IPNS_p/HPT_{orf}$ is not dependent on the presence of a C. acremonium mitochondrial DNA fragment that exhibits ARS function in S. cerevisiae or on 150 bp of DNA at the 5′ end of the 850 bp sequence that contains $IPNS_p$

Previous work demonstrated presence of one 1.93 kb *Pst*I fragment in the mitochondrial DNA of *C. acremonium* and showed that this fragment possessed ARS activity in *S. cerevisiae* (Tudzynski and Esser 1982; Skatrud and Queener 1984, Oeser et al. 1986). A plasmid with an ARS that functions in *S. cerevisiae* has the capacity to transform *S. cerevisiae* at high frequency. To test the capacity of the 1.93 kb mitochondrial DNA fragment to affect transformation in *C. acremonium*, we transformed *C. acremonium* with plasmids pPS29 and pPS21. Both plasmids contain a hybrid HPT gene composed of the HPT_{orf} and a 5′ non-coding region from the *C. acremonium* IPNS gene. Plasmid pPS21 contains the 1.93 kb fragment and pPS29 does not. Plasmids pPS21 and pPS29 were observed to transform *C. acremonium* at frequencies that were equivalent within experimental error and high relative to those observed for plasmids containing PGK_p/HPT_{orf} (Table 1).

High frequency transformation of *S. cerevisiae* by an ARS-containing plasmid occurs because the plasmid can replicate autonomously in *S. cerevisiae* and therefore does not have to integrate into the *S. cerevisiae* chromosomal DNA to be maintained. As described above, hybridization analysis of DNA isolated from *C. acremonium* transformed by plasmid pPS25, which contains $IPNS_p/HPT_{orf}$ and the 1.93 kb mitochondrial DNA fragment, indicated that transformation by this plasmid occurred by integration into high molecular weight DNA. No evidence of replicating plasmids was observed.

Thus, transformation and hybridization data suggested that presence of the mitochondrial DNA fragment did not contribute significantly to the higher frequency of transformation of *C. acremonium* that is associated with vectors containing the $IPNS_p/HPT_{orf}$ hybrid gene. Likewise, transformation and hybridization data suggested that presence of the mitochondrial DNA fragment did not contribute to transformation frequencies observed for plasmids containing PGK_p/HPT_{orf}. pIT221 and pPSo contain PGK_p/HPT_{orf} and the 1.93 kb *C. acremonium* mitochondrial DNA fragment. pPS5 and pPS7 were derived from these plasmids by deletion of the 1.93 kb mitochondrial DNA fragment (Fig. 3A). All four plasmids transformed *C. acremonium* at experimentally equivalent low frequencies (Table 1). The transforming DNA present in pIT221-*C. acremonium* transformants is integrated in the high molecular weight DNA of the transformants and does not exist in a freely replicating form (Queener et al. 1985).

In removing the 1.93 kb mitochondrial DNA fragment from pPS21 to form pPS29, 150 bp was also removed from the 5′ end of the 850 bp *Bam*HI fragment that contained $IPNS_p$. Since pPS21 and pPS29 transform *C. acremonium* at experimentally equivalent transformation frequencies, the high frequency transformation of *C. acremonium* by plasmids with $IPNS_p/HPT_{orf}$ is not dependent on that 150 bp.

The presence of ribosomal DNA sequences in vectors containing the hybrid gene, $IPNS_p/HPT_{orf}$, does not have a positive effect on integrative transformation frequency of C. acremonium

Szostak and Wu (1979) added *S. cerevisiae* DNA from ribosomal RNA genes (rDNA) to several *S. cerevisiae* integrative plasmids. They observed that derivatives which were unable to replicate autonomously in *S. cerevisiae* nevertheless transformed *S. cerevisiae* at an increased frequency. Transformation was by integration and the increase in frequency was as much as a hundred-fold.

In order to test the effect of rDNA sequences on integrative transformation in *C. acremonium*, fragments containing *C. acremonium* rDNA were cloned and partially characterized (Carr et al. 1987). One fragment included DNA from the 16–18S rRNA gene and a second contained DNA from at least three rRNA genes. DNA from the 16–18S rRNA gene and adjacent sequences were incorporated into two *C. acremonium* integrative vectors, pIT221 and pPS5, that contain PGK_p/HPT_{orf} (see Fig. 3). The derivatives – pPS6 and pPS7, respectively – and the original plasmids were used to transform *C. acremonium* (Table 1). Presence of the rDNA did not enhance the frequency of transformation.

Absence of a functional *C. acremonium* promoter at the 5′ end of the HPT gene in pPS6 and pPS7 could, in theory, negate a beneficial effect of the rDNA sequences. To test this concept, plasmids pPS31 and pXL6 were constructed (Fig. 3). Plasmids pPS29, pPS31, and pXL6 all contain the hybrid $IPNS_p/HPT_{orf}$ gene. Plasmid pPS29 is analogous to pPS31 and pXL6 except that pPS29 contains no rDNA sequences. pPS31 contains the same fragment of rDNA as do pPS6 and pPS7 analyzed above. Plasmid pXL6 contains a different fragment of *C. acremonium* rDNA (i.e. an approximately 2.7 kb *Bgl*II fragment that carrys DNA from the 16–18S rRNA gene, all of the 5.8S rRNA gene, and DNA from the 23–28S rRNA gene). None of these plasmids contain the mitochondrial fragment found in pIT221. The rRNA gene sequences present in pPS31 and pXL6 failed to produce transformation frequencies superior to those obtained with pPS29.

Alterations in transformation conditions allow improved transformation

The transformation system used thus far has been that described by Queener et al. (1985). Recently several modifications have been made to that system which have resulted in more predictable results from one experiment to another. We have found the use of DMSO is not required to obtain high frequency integrative transformation. It is also not necessary to remove the polyethylene glycol (PEG) from the transformation reaction prior to plating. The dithiothreitol treatment has been reduced from one hr. to 10 min without adversely affecting protoplast formation or transformation.

One to three percent (avg. 1.7% in 13 trials) of the protoplasts of *C. acremonium* produced by the cell wall active enzyme beta-glucuronidase (20 mg/ml, Sigma Chemical Co.) were capable of regeneration (formation of viable walled cells). In comparison when Novozym 234 (10 mg/ml, Novo Biolabs) was employed to produce protoplasts the regeneration frequency approached 10% (avg. 8.3% in 7 trials), however, there was not necessarily a corresponding increase in the transformation frequency. When using Novozym 234 at 10 mg/ml the time of digestion has been reduced from 90 min to 15 min.

The original description of the *C. acremonium* transformation system (Queener et al. 1985) utilized a one ml transformation mixture to which 9 ml of 20% PEG were added. Twenty μg of plasmid DNA were used per experiment, thus after addition of the PEG, the DNA concentration was 2 μg/ml. With these reaction conditions we have observed considerable variability in the number of transformants obtained with any particular plasmid (i.e., in some experiments as many as 500 transformants have been observed whereas in others no transformants were obtained). When the amount of plasmid DNA was increased to as much as 300 μg/ml), there was an increase in the number of transformants recovered. In these experiments only plasmids that contained $IPNS_p/HPT_{orf}$ were used for transformation. We found that equivalent numbers of transformants could be obtained by keeping the number of protoplasts constant at 1×10^8 and reducing the volume of the original transformation mixture to 0.1 ml. This was followed by the addition of 0.9 ml of 40% PEG rather than 20% PEG. The use of twenty μg of DNA per experiment then resulted in a final DNA concentration of 20 μg/ml after the addition of PEG. When the 0.1 ml reaction mixture has been used for transformation of *C. acremonium* ATCC11550 we have always obtained transformants. However, there was still a large degree of variability in the number of transformants obtained, for example, in a set of 19 experiments a range of approximately 75 to 1,400 transformants were produced per transformation with plasmids containing $IPNS_p/HPT_{orf}$. The variability suggested that perhaps there was a subpopulation of the protoplasts which were transformation competent and that the size of that subpopulation delivered to the transformation mixture varied from one transformation to another for some reason. Since growth conditions prior to protoplast formation were kept constant, it was deduced that something in the process of protoplast formation/handling must affect the number of transfor-

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Table 2. A fraction enriched for large *C. acremonium* protoplasts is also enriched for protoplasts that regenerate cell walls and transform by exposure to vectors that contain the hybrid $IPNS_p/HPT_{orf}$ gene

Experiment	Fraction 1 (Supernatant) enriched for small protoplasts		Fraction 2 (Pellet) enriched for large protoplasts		Unfractionated mixture of large and small protoplasts	
	rf[a]	# trans.[b]	rf	# trans.	rf	# trans.
1	2.9%	41	61.6%	265	(data from	
2	3.5%	125	14.5%	1,026	21 protoplast mixtures)	
3	4.9%	113	8.3%	256		
4	3.7%	74	15.0%	210		
Average	3.8%	88	25.0%	439	2.5%	231

[a] *rf* regeneration frequency. The rf is the number of osmotically fragile units that form a colony divided by the total number of protoplasts [haemocytometer count]. In experiment # 1, 1×10^7 protoplasts were used in the transformation; 1×10^8 protoplasts were used in each of the other transformation experiments

[b] *# trans.* number of hygromycin B resistant transformants

mation competent protoplasts delivered to the transformation mixture.

We observed a very heterogeneous population of protoplasts with regard to size when either of the above enzymes were used for protoplast formation. For example, size varied as much as 10-fold among protoplasts within a digestion reaction. Qualitative observation of regenerating protoplasts under the light microscope revealed large protoplasts producing new hyphae whereas the smaller ones remained inert. A method was devised to enrich for the larger protoplasts (see Methods). The percentage of protoplasts that regenerated cell walls was always larger for the protoplasts enriched for large size (fraction #2, Table 2; 8.3–61.6%) than the percentage for protoplasts enriched for small size (fraction #1, Table 2; 2.9–4.9%). Furthermore, a larger number of transformants was always observed when protoplasts from fraction #2 were used in transformation experiments.

Discussion

Transformation of filamentous fungi has been reported previously; e.g., *Neurospora crassa* (Case et al. 1979), *Aspergillus nidulans* (Tilburn et al. 1983; Yelton et al. 1984), *Podospora anserina* (Tudzynski et al. 1980; Brygoo and Debuchy 1985), *Cochliobolus heterostrophus* (Turgeon et al. 1985), and *C. acremonium* (Queener et al. 1985). Generally, transformation in these organisms has been observed to occur at low frequency by integration. We observed and first reported transformation of *C. acremonium* at a very low frequency by integration of a dominant selectable marker that imparted resistance to hygromycin B; approximately 0.2 transformants were produced per μg of transforming DNA (Queener et al. 1985; Chapman et al. 1986). In the current study, we have demonstrated that a new plasmid-borne hybrid HPT gene $IPNS_p/HPT_{orf}$ transforms *C. acremonium* at a frequency 100 times that observed for the hybrid gene, PGK_p/HPT_{orf}, which was used to transform this fungus in our earlier studies. Transformation of *C. acremonium* by both hybrid HPT genes occurred by integration.

C. acremonium RNA polymerase II probably interacts effectively with a *C. acremonium* sequence, $IPNS_p$, present in the 5′ noncoding region of the hybrid gene $IPNS_p/HPT_{orf}$ to allow transcription of the HPT_{orf}. The *C. acremonium* RNA polymerase probably interacts poorly, if at all, with a truncated *S. cerevisiae* promoter PGK_p present in the hybrid gene PGK_p/HPT_{orf}. The low frequency transformation of *C. acremonium* observed with PGK_p/HPT_{orf} could have occurred by the integration of that hybrid gene into the *C. acremonium* genome at positions adjacent to and in phase with natural *C. acremonium* promoters or could have occurred by multiple integrations of a poorly expressed PGK_p/HPT_{orf} gene. A plasmid, pPS24, that contains the HPT open reading frame and no eucaryotic promoter was observed to transform *C. acremonium* at the same low frequency as plasmids that contained PGK_p/HPT_{orf} (Table 1). This observation suggests that the truncated *S. cerevisiae* promoter PGK_p has negligible promoter activity in *C. acremonium*. Orientation of promoters, DNA sequences that function to allow binding of RNA polymerase so that this enzyme transcribes an adjacent open reading frame, is important for their function (Von Hippel et al. 1982). In the correct orientation to an adjacent *orf* promoter activity is high; in the opposite orientation the activity is virtually non-existent. If the

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higher frequency of transformation observed with $IPNS_p/HPT_{orf}$ was dependent on the promoter activity of the sequences in the 850 bp fragment juxtaposed to HPT_{orf}, then the orientation of the fragment would be expected to strongly effect transformation. This was indeed the case. All plasmids containing $IPNS_p/HPT_{orf}$ transformed *C. acremonium* efficiently. However, plasmid pPS22 carries a hybrid HPT gene in which the 850 bp *C. acremonium* sequence is oriented opposite the orientation of that sequence in $IPNS_p/HPT_{orf}$. pPS22 transformed *C. acremonium* at a low frequency. The frequency was no higher than that observed for plasmid pPS24 which contains the HPT_{orf} without any eucaryotic promoter.

Provided that promoters in *C. acremonium* are similar in size to those in *S. cerevisiae* (Struhl 1986), 700 bp should be large enough to include all the sequences necessary to promote transcription of orfs in *C. acremonium*. The 5-prime 150 bp of the 850 bp sequence juxtaposed to the HPT_{orf} did not appear to enable plasmids pPS21, pPS25, pPS31 and pPS46 to transform *C. acremonium* at frequencies significantly superior to those obtained with plasmids pPS29, pPS27 and pXL6 which lack that 150 bp in their $IPNS_p/HPT_{orf}$ gene.

If transformation by PGK_p/HPT_{orf} requires integration of that hybrid gene into the *C. acremonium* genome at positions adjacent to and in phase with natural *C. acremonium* promoters and transformation with $IPNS_p/HPT_{orf}$ has no such requirement because the integration event supplies an active promoter, then on average fewer integration events would be observed in a sampling of transformants obtained with $IPNS_p/HPT_{orf}$ than from an equivalent number of transformants obtained with PGK_p/HPT_{orf}. Southern analyses of restriction enzyme digested total DNA from transformants [data not shown] was consistent with this prediction.

Plasmid pPS24 may be useful as a promoter cloning vector. Recovery and examination of the DNA sequence directly 5′ of the HPT coding sequence from pPS24-derived *C. acremonium* transformants may reveal cellular sequences which permit expression of the HPT_{orf}. Puhn and Knippers (1985) have transformed mammalian cells with an intact thymidine kinase (TK) gene and a truncated version lacking the promoter region. The promoterless gene produced about a hundred-fold less transformants than the intact gene. In cells transformed by plasmids containing the promoterless gene, there were generally more integrations of transforming DNA than in cells transformed by plasmids carrying the intact gene. Yet the amount of TK produced by both types of transformants was very similar. Examination of transformants which were derived from the promoterless gene revealed that the reading frame of the TK gene was located just downstream of cellular sequences. The suggestion was made that these cellular sequences contained active promoter regions.

Extrapolating heterologous ARS function in *S. cerevisiae* to an analogous function in the original host organism may not be justified in many cases. Although the 1.93 kb *Pst*1 restriction fragment of *C. acremonium* mitochondrial DNA functions quite adequately as an ARS in *S. cerevisiae* (Skatrud and Queener 1984; Tudzynski and Esser 1982; Oeser et al. 1986), that fragment did not provide typical ARS function in plasmids used to transform *C. acremonium* in the current study and presence or absence of the fragment in plasmids had no significant effect on the ability of plasmids to transform *C. acremonium*. Maundrell et al. (1985) evaluated heterologous ARS activity in *S. cerevisiae* using cloned DNA from *Schizosaccharomyces pombe*. They found that *S. cerevisiae* failed to recognize several cloned *S. pombe* DNAs which functionated as an ARS in *S. pombe*; they identified other cloned *S. pombe* DNAs that possessed ARS activity in *S. cerevisiae* but these DNAs showed no ARS activity in *S. pombe*.

Addition of *C. acremonium* ribosomal DNA to the vector containing $IPNS_p/HPT_{orf}$ had no effect on transformation of *C. acremonium* whereas addition of *S. cerevisiae* ribosomal DNA to *S. cerevisiae* integrative vectors has been reported to dramatically increase transformation of *S. cerevisiae*. Tilburn et al. (1983) also observed no increase in the rate of integrative transformation in *Aspergillus nidulans* upon inclusion of a fragment of rDNA from *A. nidulans* into integrative plasmids. Mechanisms for homologous recombination in *S. cerevisiae* are probably much more developed than in filamentous fungi; non-homologous recombination appears to occur more readily than homologous recombination in filamentous fungi (Hynes 1986).

Although the addition of the 3′ region of a *C. acremonium* gene downstream from $IPNS_p/HPT_{orf}$ did not improve transformation of *C. acremonium* (pPS27 vs. pPS21, Table 1), a placement of the putative termination sequence closer to the 3′ end of the HPT_{orf} is required before utility of this approach is discounted. Homologous transcriptional termination and polyadenylation sequences present after foreign genes in other systems have increased the expression of the foreign gene product (Hitzeman et al. 1983). Gritz and Davies (1983) placed a hygromycin B phosphotransferase of from *Streptomyces hygroscopicus* between the promoter and termination sequences of the *S. cerevisiae CYC1* gene. High frequency transformation in *S. cerevisiae* was achieved with their vectors (equivalent to auxotrophic selection). In contrast, when Kaster et al. (1984) placed the hygromycin B phosphotransferase or from *E. coli* under the control of PGK_p from *S. cerevisiae* in the absence of PGK termination sequences the transforma-

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tion frequency in *S. cerevisiae* was approximately 10% of the frequency observed with auxotrophic selection.

The use of the HPT gene isolated by Rao et al. (1983) as a dominant selectable marker may be *generally* useful for the development of transformation systems in fungi sensitive to hygromycin B. The present study clearly demonstrates the utility of a hybrid HPT gene constructed by attaching the HPT_{orf} to a properly oriented and positoned promoter taken from the fungus to be transformed. This approach has been used successfully by others to develop transformation systems for *Ustilago maydis* (personal communication from Sally Leong, University of Wisconsin) and for *Chochliobolus heterostrophus* (personal communication from Olen Yoder, Cornell University). The dominance of hybrid HPT selectable markers takes on particular significance when transforming industrial organisms. Dominance permits transformation of organisms sensitive to hygromycin B, a broadly effective antibiotic, without prior mutagenesis to induce the appearance of a selectable trait. Detrimental pleiotropic mutations are often associated with introduction of a selectable trait into a highly developed industrial microorganism after treatment with a mutagen. These mutations frequently cause a decline in the capacity to produce the desired metabolite (MacDonald et al. 1963).

There are several reasons why larger protoplasts from a mixture of large and small protoplasts should, as observed in this study, regenerate cell walls better than the small protoplasts. It is doubtful that the smallest protoplasts received all the cytoplasmic elements necessary for survival and growth, thus they would be unable to regenerate or produce a transformant; however, they might sequester DNA and reduce the chance of a competent protoplast from being transformed. Variability in the size-distribution of protoplasts delivered to transformation reactions might explain some of the varibility in efficiency of transformation. Another concept which might be considered is the possibility of regional variation in cytoplasmic membrane composition in a differentiated microorganism such as *C. acremonium*. It may be necessary to include a specific area of the membrane in the protoplast in order to produce a transformation competent protoplast. For example, specialized structures near the septa may function in transport between individual cells within the mycelium. These same structures, if present on a protoplast, may enhance its competence for transformation. Large protoplasts would have a better chance of incorporating such an area.

The initial development of HPT vectors for transfromation of *C. acremonium* (Queener et al. 1985) and the improvement of those vectors described in this report was carried out with a wild type strain, ATCC11550. In general, highly mutagenized industrial strains of *C. acremonium* have been observed to regenerate cell walls and transform less efficiently than ATCC11550. However, the improvements in the transformation conditions described in this report and our new HPT vectors have facilitated transformation of a wider range of strains of *C. acremonium* including industrially important strains.

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Sequence of the *C. acremonium* IPNS promoter juxtaposed to the HPT open reading frame in plasmids pPS56 and pPS47 is double underscored.

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351  GCCGGGCCAA TCCTGAGCTT CGATAGACTG TTCCGGGCCT CATGTGGGTG
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451  GCCATGCAGT CTTCAGGGAC CACACTCCAT CGCCGCTGCT GGACCGTATG
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Isolation, sequence determination and expression in *Escherichia coli* of the isopenicillin N synthetase gene from *Cephalosporium acremonium*

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The enzyme isopenicillin N synthetase (IPS) catalyses the oxidative condensation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) to isopenicillin N, which is a central reaction in the pathway to clinically important penicillins and cephalosporins. Here we report the cloning, characterization and expression in *Escherichia coli* of the gene encoding the IPS protein in *Cephalosporium acremonium*. The IPS gene was identified by purifying IPS protein, determining the first 23 amino-terminal amino acids, preparing a set of synthetic oligonucleotides encoding a portion of the determined amino-acid sequence, and probing a cosmid genome library with the mixed oligonucleotides. A cosmid hybridizing with the probe was isolated and the IPS gene was localized and sequenced. The IPS gene encodes a polypeptide of relative molecular mass (M_r) 38,416. When this open reading frame was cloned into an *E. coli* expression vector and inserted into *E. coli*, the recombinant *E. coli* produced a new protein co-migrating with authentic IPS as the major protein of the cell (~20% of cell protein). Crude cell extracts condensed LLD-ACV to a penicillinase-sensitive molecule whose antibacterial activity indicated that it was isopenicillin N.

The conversion of LLD-ACV to isopenicillin N by IPS or cyclase is carried out by a variety of organisms, including *Penicillium chrysogenum*, *C. acremonium*, *Streptomyces clavuligerus* and other Actinomycetes. Penicillins and cephalosporins are isolated from these organisms for the manufacture of important β-lactam antibiotics. The cyclase enzyme, in the presence of Fe^{2+} and ascorbate, removes four hydrogens from LLD-ACV and consumes one oxygen, resulting in the formation of the two rings found in isopenicillin N[1]. Most of the studies of the IPS reaction have examined enzymes from *C. acremonium* and *S. clavuligerus*. The *C. acremonium* enzyme has been purified[2,3], and used to condense not only LLD-ACV to isopenicillin N, but also variants of LLD-ACV to different β-lactam structures[4-7]. In order to extend our knowledge of the properties and potential of the IPS enzyme, we have cloned the IPS gene and expressed the activity in *E. coli*.

IPS protein from *C. acremonium* was purified using modified published procedures[2] with additional reverse-phase HPLC steps. The purified protein was then subjected to amino-terminal sequence analysis, and 23 amino-acid residues were determined (see Fig. 1). Sixty-four synthetic oligonucleotides were prepared, one of which must be the perfect match of the coding information for amino acids 18-23 (Phe-Gly-Asp-Asp-Lys-Glu) encoded by the IPS gene. The 64 possible 17-mer oligonucleotides were synthesized in two pools of 32 (Fig. 1). The pool of 32 that hybridized most efficiently to restriction enzyme-cleaved *C. acremonium* genomic DNA immobilized on nitrocellulose was used for further experiments.

The synthetic 17-mer oligonucleotides were used to screen a cosmid genome bank for the IPS gene. The region of one cosmid, pIT328, which hybridized to the oligonucleotide probes was determined to be adjacent to the *E. coli* selectable markers by restriction mapping and Southern hybridizations, so the cosmid was truncated by restriction enzyme *Hin*dIII digestion and re-ligation to yield the 11-kilobase (kb) plasmid pIT335 (Fig. 2). The DNA sequence of a portion of pIT335 was determined by the Maxam-Gilbert technique[8] and the predicted amino-acid sequence of one region matched the experimentally determined amino-acid sequence of the IPS protein (Fig. 3). The open reading frame containing this information (Fig. 3) can encode a polypeptide of M_r 38,416. The reported M_r of the IPS protein, as determined by SDS-polyacrylamide gel electrophoresis, is 40,000 (ref. 3) (see also Fig. 4).

The predicted amino-acid sequence of the IPS protein from pIT337 (Fig. 3) begins with methionine and glycine residues which are not found in the protein isolated from *Cephalosporium* cells. These residues are evidently cleaved post-translationally in *Cephalosporium*.

The protein-coding region of the IPS gene consisted of 63% G·C base pairs. Because of the high G+C content, we used an algorithm described by Bibb *et al.*[9] to examine the G+C content of the DNA sequence as a function of position in the triplet codons. Bibb *et al.* have shown that the G+C content of the positions in the triplet codons is biased within protein-coding regions but relatively random outside protein-coding regions for organisms with DNA of high G+C content. In particular, Bibb *et al.* found that the G+C content of the first position in the codon was about average, while that of the second position was much lower, and that of the third position much higher

§ To whom correspondence should be addressed.

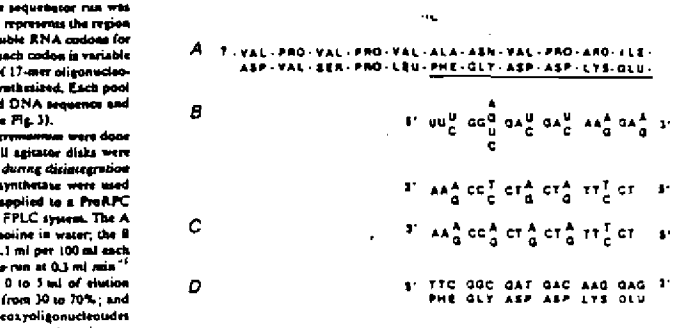

Fig. 1 Amino-terminal sequence analysis of IPS and synthesis of oligomeric DNA probes. *A*, The first 23 amino-terminal amino-acid residues of IPS. The question mark indicates that the first cycle of the sequenator run was ambiguous. The underlined sequence (residues 18-23) represents the region encoded by the synthetic oligonucleotides. *B*, The possible RNA codons for the underlined sequence in *A*. At the third position of each codon in variable in this region, all possibilities are shown. *C*, Two sets of 17-mer oligonucleotides incorporating the possibilities shown in *B* were synthesized. Each pool has a complexity of 32. *D*, The experimentally derived DNA sequence and the corresponding predicted amino-acid sequence (see Fig. 3).

Methods. Extraction and purification of IPS from *C. acremonium* were done as described by Fang *et al.*[2] except that the Dyno-Mill agitator disks were run at 2,000 r.p.m. to keep the temperature below 5° C during disintegration of the mycelium. The peak fractions containing the synthetase were used for further processing. Nearly pure IPS protein was applied to a Pro RPC HR 5/10 C_8 reverse-phase column using a Pharmacia FPLC system. The A solvent was 0.1% trifluoroacetic acid and 0.1% morpholine in water; the B solvent was 80% isopropanol, 20% acetonitrile with 0.1 ml per 100 ml each of trifluoroacetic acid and morpholine. The column was run at 0.3 ml min^{-1} with a series of the following linear gradients: from 0 to 5 ml of elution buffer, B went from 0 to 30%; from 5 to 23 ml B went from 30 to 70%; and from 23 to 25 ml B went from 70 to 100%. The 64 deoxyoligonucleotides were chemically synthesized in two pools of 32 17-mers each, using an Applied Biosystem DNA Synthesizer (Model 380-A) according to the manufacturer's recommended protocols. Oligomers were purified by 20% polyacrylamide/7 M urea gel electrophoresis.

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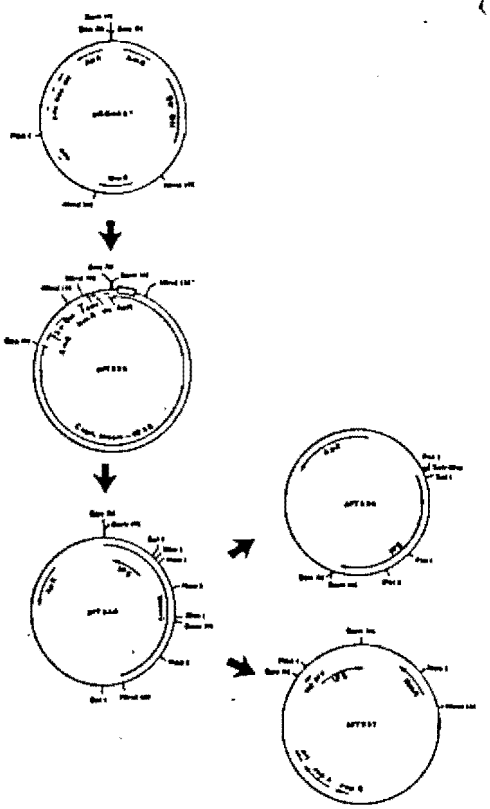

Fig. 2 Schematic diagrams of cosmids and plasmids used in the present study. The size of the circle is not proportional to the size of the plasmid. Except where indicated below, if a restriction enzyme cut site is indicated on the drawing, all restriction sites for that enzyme are included in the diagram. pKC462*, a 12.4-kb cosmid constructed by R. N. Rao and R. Stanzak (personal communication) which contains a *Streptomyces* origin of replication (Str Ori) from pFJ103 (ref. 10), and an *E. coli* origin of replication (ori) from pBR322. Antibiotic resistance markers on this plasmid are the ampicillin resistance gene (ApR) from pBR322, the apramycin resistance gene (AmR) from pKC222 (ref. 11), and the neomycin resistance gene (NmR) from Tn*5*. The three *cos* sites (cos) are from bacteriophage λ. pKC462* contains a unique *Bam*HI restriction site (used in this study for cloning) flanked by unique *Eco*RI restriction sites. pIT328, an ~50-kb plasmid created by ligating *C. acremonium* genomic DNA partially cleaved with restriction enzyme *Mbo*I into the *Bam*HI site of pKC462*. pIT328 contains ~40 kb of *C. acremonium* DNA (Ceph. Insert). The cross-hatched box indicates the protein-coding region of the IPS gene. *Hin*dIII* is one of at least 10 *Hin*dIII sites in the insert, the rest of which are not shown. pIT335, an 8.2-kb plasmid, was constructed by digesting pIT328 with *Hin*dIII and re-ligating, thus eliminating half the vector and all but ~4.5 kb of the *Cephalosporium* DNA insert. The IPS gene is transcribed in the direction of the arrow. Only the relevant restriction sites are shown, and one more unmapped *Nco*I restriction site exists outside the IPS gene in pIT335. pIT336, a 4.1-kb plasmid, was created by cloning a 1,400-bp *Eco*RI-*Xho*I fragment from pIT335 into the *Eco*RI-*Sal*I sites of pUC8 (ref. 12). The *Cephalosporium* DNA insert (IPS) contains most of the protein-coding region of the IPS gene. pIT337 is an 11.6-kb expression plasmid containing the complete coding region of the IPS gene. The 1,460-bp *Nco*I-*Bam*HI fragment from pIT333 was ligated with two fragments (an 8.7-kb *Nco*I-*Nco*I fragment and a 1.5-kb *Nco*I-*Bam*HI fragment) from pCZ106. pCZ106 is a derivative of pCZ115 (ref. 13) which contains the bovine growth hormone gene transcribed from the *E. coli* tryptophan promoter and a temperature-sensitive copy control (runaway replicon) system. The *E. coli* tryptophan promoter (trp pro) drives transcription of the IPS gene. The location and direction of transcription of the IPS gene and of the neomycin resistance gene (NmR) are also indicated.

Methods. *C. acremonium* genomic DNA was isolated from protoplasts prepared according to Queener *et al.*[14]. The protoplasts were lysed by incubating them in 3% *N*-lauroyl sarkosyl, 0.5 M Tris-HCl pH 9.0, 0.2 M EDTA at 65 °C for 15 min. The mixture was cooled and then extracted with buffered phenol (10 min) and chloroform (10 min). The DNA layer was mixed with a 1:10 volume of 5 M KOAc and spooled with 2 vol. of cold 100% ethanol. DNA was dissolved in 2 ml TE buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA) then treated with 50 μg ml^{-1} proteinase K (Sigma; Type XI) at 65 °C for 1 h in 0.01 M Tris-HCl pH 7.8, 5 mM EDTA, 0.5% SDS. The solution was then extracted for 10 min each with buffered phenol and chloroform and the DNA respooled and suspended in 0.5 ml TE buffer. Partial enzymatic digestions with *Mbo*I were done to achieve an average size of 30–40 kb. pKC462* was digested with *Hpa*I, treated with alkaline phosphatase, then cut with *Bam*HI. The partially digested genomic DNA was ligated into the *Bam*HI site of pKC462* and the recombinant DNA molecules were packaged into phage λ heads using Promega Biotec packaging extracts according to the recommendations of the manufacturer. *E. coli* SF8 cells grown in the presence of 0.2% maltose were transduced with the recombinant λ particles and cells containing cosmids were selected on plates containing 100 μg ml^{-1} ampicillin and then grown in liquid culture in microtitre dishes in the presence of 100 μg ml^{-1} apramycin. The library was screened according to the method of Grunstein and Hogness[15]. Oligonucleotide probes (described in Fig. 1 legend) were kinased under conditions described previously[16]. The nitrocellulose filters containing the recombinant *E. coli* were hybridized at 42 °C for 2 h in 10 × Denhardt's, 6 × SSC, 0.1% sodium pyrophosphate[17]. Washes were at room temperature in 6 × SSC for 30 min, followed by another 5-min wash in 6 × SSC at 42–58 °C.

than average. As shown in Fig. 3, the same pattern of G+C content bias was observed for the open reading frame encoding the IPS protein. This provides further evidence that the open reading frame identified by sequencing is correct for its entire length, since a shift in reading frame would have also shifted the G+C content bias.

To further prove that the open reading frame encodes the IPS protein, we fused the open reading frame to an *E. coli* expression system. The open reading frame from pIT335 was isolated on an *Nco*I-*Bam*HI fragment. The recognition sequence for the restriction enzyme *Nco*I, 5'-CCATGG, surrounds the methionine codon that initiates translation of the open reading frame (Fig. 3). The restriction enzyme *Bam*HI cleaves ~450 base pairs (bp) downstream from the proposed translation termination site. The *Nco*I-*Bam*HI fragment was ligated into an *E. coli* expression vector to create pIT337. The *E. coli* expression vector used the promoter and ribosome binding site from the *E. coli* tryptophan operon and a temperature-sensitive copy control system ('runaway replicon'). *E. coli* cells transformed with pIT337 produce a new protein which co-migrates with authentic IPS protein on SDS-polyacrylamide gels. At low temperatures the cells produce low amounts of the new protein, but at high temperatures they produce about 20% of total cell protein as the putative IPS polypeptide (Fig. 4). The other new major protein band produced by the transformed *E. coli* cells is kanamycin phosphotransferase, which is also encoded on the *E. coli* expression vector.

Crude cell-free extracts from cells containing pIT337 also contain IPS activity. Extracts were incubated with LLD-ACV and the reaction mixture tested for antimicrobial activity on agar plates impregnated with *Micrococcus luteus*. As shown in Fig. 4, generation of antimicrobial activity in the assay was dependent on the presence of the IPS gene in the *E. coli* expression vector and on exogenous addition of LLD-ACV. These results indicate that the open reading frame from pIT335 encodes a polypeptide with IPS activity, and that the polypeptide is expressed in an active form in *E. coli*.

The cloning and expression of the IPS gene from *C. acremonium* will facilitate further analysis of the penicillin-cephalosporin biosynthetic pathway. The enzymatic properties and structure/function relationships of IPS can now be tested in more detail because of the greater availability of the synthetase. The properties of the *Cephalosporium* gene, especially with respect to the control of transcription, can also be studied.

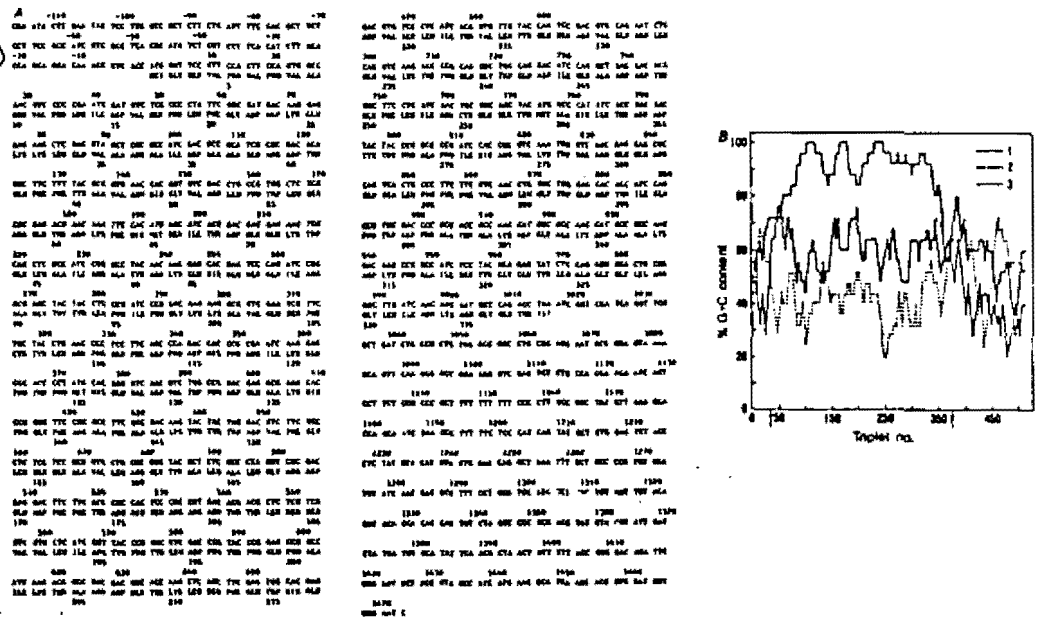

Fig. 3 *A*, Complete nucleotide and predicted amino-acid sequence of isopenicillin N synthetase gene from pIT337. The DNA sequence was determined by the chemical method of Maxam and Gilbert[8] as described previously[19]. Both strands were sequenced for over 95% of the protein-coding region, and the remaining areas were sequenced at least twice from different sites on the same strand. The primary sequence of the IPS gene, together with 118 bp preceding the initiation codon and 435 bp after the termination codon, is shown with the predicted amino-acid sequence of a 1,017-bp open reading frame. Numbers above the nucleotide sequence refer to base-pair location. Amino-acid residues are numbered below the protein sequence. The proposed translation initiation site is amino-acid 1. *B*, Analysis of G+C content as a function of position in the codon. Each curve represents the G+C content of one of the three codon positions. The per cent G+C at each base is calculated as a forward average of 20 codons according to the algorithm of Bibb *et al.*[9]. For clarity, the curves presented are smoothed slightly relative to the original computer print-out. The heavy line corresponds to the first base of a codon, the dotted line refers to the second base, and the thin line refers to the third position. The arrows along the axis indicate the limits of the IPS protein-coding region (see *A*).

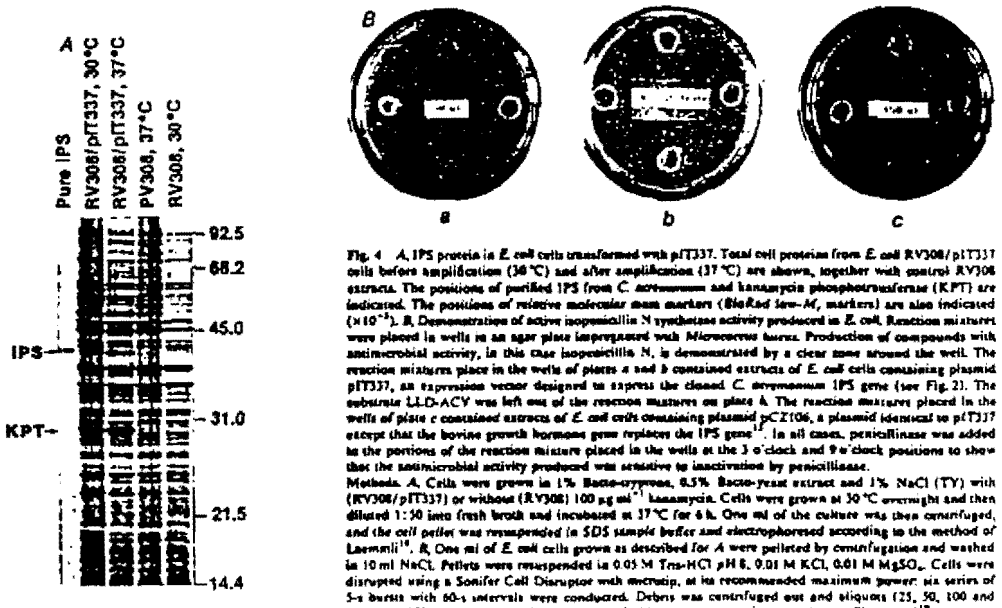

Fig. 4 *A*, IPS protein in *E. coli* cells transformed with pIT337. Total cell proteins from *E. coli* RV308/pIT337 cells before amplification (30 °C) and after amplification (37 °C) are shown, together with control RV308 extracts. The positions of purified IPS from *C. acremonium* and kanamycin phosphotransferase (KPT) are indicated. The positions of relative molecular mass markers (BioRad low-*M*$_r$ markers) are also indicated ($\times 10^{-3}$). *B*, Demonstration of active isopenicillin N synthetase activity produced in *E. coli*. Reaction mixtures were placed in wells in an agar plate impregnated with *Micrococcus luteus*. Production of compounds with antimicrobial activity, in this case isopenicillin N, is demonstrated by a clear zone around the well. The reaction mixtures place in the wells of plates *a* and *b* contained extracts of *E. coli* cells containing plasmid pIT337, an expression vector designed to express the cloned *C. acremonium* IPS gene (see Fig. 2). The substrate LLD-ACV was left out of the reaction mixtures on plate *b*. The reaction mixtures placed in the wells of plate *c* contained extracts of *E. coli* cells containing plasmid pCZ106, a plasmid identical to pIT337 except that the bovine growth hormone gene replaces the IPS gene[17]. In all cases, penicillinase was added to the portions of the reaction mixture placed in the wells at the 3 o'clock and 9 o'clock positions to show that the antimicrobial activity produced was sensitive to inactivation by penicillinase.

Methods. *A*, Cells were grown in 1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1% NaCl (TY) with (RV308/pIT337) or without (RV308) 100 μg ml^{-1} kanamycin. Cells were grown at 30 °C overnight and then diluted 1:50 into fresh broth and incubated at 37 °C for 6 h. One ml of the culture was then centrifuged, and the cell pellet was resuspended in SDS sample buffer and electrophoresed according to the method of Laemmli[10]. *B*, One ml of *E. coli* cells grown as described for *A* were pelleted by centrifugation and washed in 10 ml NaCl. Pellets were resuspended in 0.05 M Tris-HCl pH 8, 0.01 M KCl, 0.01 M $MgSO_4$. Cells were disrupted using a Sonifer Cell Disruptor with microtip, at its recommended maximum power: six series of 5-s bursts with 60-s intervals were conducted. Debris was centrifuged out and aliquots (25, 50, 100 and 150 μl) were assayed for isopenicillin N synthetase activity according to Shen *et al.*[16].

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Control of expression of the IPS gene is particularly interesting because β-lactam antibiotics are usually produced late in the growth cycle, suggesting the possibility of control of expression of the biosynthetic genes themselves. These aspects are presently under investigation.

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Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing

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ABSTRACT

We have characterized hygromycin B and apramycin resistance genes from an E. coli plasmid. We have localized the coding and control regions of these genes by deletion of DNA fragments from plasmids containing the genes. It was found that polypeptides with apparent molecular weights of 33,000 and 31,500 daltons are encoded by the apramycin resistance gene and polypeptides with apparent molecular weights of 42,500 and 41,500 daltons are encoded by the hygromycin B resistance gene. DNA sequence analysis identified a typical promoter sequence upstream of the genes. Deletion of this promoter eliminated both resistance phenotypes, and hygromycin B resistance could be restored by substitution of a promoter from a foreign gene. The region known to be necessary for hygromycin B resistance contained an open reading frame large enough to encode the hygromycin B resistance gene product. This open reading frame was fused with the amino terminus of B-galactosidase. This hybrid gene conferred hygromycin resistance to E. coli, and expression of resistance was under IPTG control.

INTRODUCTION

A dominant, selectable genetic marker functional in heterologous systems would be valuable for a variety of reasons. For example, it could be used on shuttle vectors to select for cells containing foreign DNA or as a vehicle for isolating promoters and control sequences from complex DNA. Ideally, the gene encoding the dominant marker would still be active after fusion with sequences from the host organism which promote expression.

One candidate for a widely useful dominant marker is the hygromycin B resistance gene from E. coli. Hygromycin B is an aminoglycoside antibiotic that inhibits protein synthesis and kills bacteria, fungi, and higher eukaryotes (1,2). A gene was recently isolated from E. coli which confers hygromycin resistance to this organism by phosphorylating the antibiotic (Rao, R.N., N.E. Allen, J.N. Hobbs, Jr., W.E. Alborn, Jr., H.A. Kirst and J.W. Paschal. Submitted to Antimicrobial Agents and Chemotherapy). During the course of this earlier work, the gene was moved to a multicopy plasmid derived from pBR322. We wanted to characterize the drug resistance genes on

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this hyorid plasmid so that we could test the utility of the genes or derivatives of the genes as dominant selectable markers.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The strains used were E. coli K12 derivatives RR1 (3), JA221, CSR603 (4, 5), DH1 (6), and BE904 (a lac Δ mutant; Kuhstoss, S., H. Hsiung, R. Belagaje, S.G. Burgett and R.N. Rao, manuscript in preparation). All plasmids were derivatives of pBR322 (7).

Bacterial Culture Conditions

Drug-resistant bacteria were selected on TY plates (8) containing the appropriate antibiotics at the following concentrations: Ampicillin (Ap), 50 μg/ml; apramycin (Am), 100 μg/ml; hygromycin B (Hm), 200 μg/ml; and tetracycline (Tc), 25 μg/ml. Assessment of Hm resistance was made by patch tests from cells grown on a different antibiotic except in the case of cells containing pTI105, which could be plated directly after transformation on hygromycin B-containing plates. β-galactosidase assays were performed according to Miller (8).

Maxicell Labelling

CSR603 cells containing plasmids were labelled with ^{35}S-methionine according to Sancar, et al. (4,5) except that after UV irradiation the cells were grown overnight in the presence of 100 μg/ml cycloserine.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Ames (9).

Formaldehyde-agarose gels were run as described by Zaret and Sherman (10). RNA blots were performed as outlined by Thomas (11).

DNA Sequencing

DNA sequence analysis was performed using the chemical cleavage method of Maxam and Gilbert (12). DNA labelling was carried out as described previously (13).

Conversion of Sac I site to Bam HI site.

After digestion with SacI, 4μg of pKC222 was incubated for 15 minutes at 37°C in 5 μl of 50 mM Tris (pH7.5), 10mM $MgCl_2$, 20μM each of dCTP, dATP, TTP and dGTP with 3 units of DNA polymerase I large fragment. After heat inactivation of the polymerase, BamHI linkers were ligated onto the blunt-ended DNA molecules, digested with Bam HI, and religated. After digestion with SacI to lower the number of parental plasmids, the DNA was

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used to transform strain RR1 to ampicillin resistance. Sequence analysis of one of the resulting plasmids, pTI104, showed that the treatment with DNA polymerase I large fragment had removed the 3'-extension generated by SacI.

Removal of 3' Extensions with T4 DNA Polymerase.

The 3' extension left by restriction enzyme KphI was removed with T4 DNA polymerase. About 1 μg of restricted DNA was incubated in 10 μl of 33 mM Tris.HCl, pH 7.8, 67 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and all four dXTP's at 150 μM at 37 degrees C for 5 minutes with 3 units of T4 DNA polymerase. The reaction was quenched at 65 degrees C for 5 minutes after adding 1 μl of 50 mM EDTA.

Materials

All enzymes were obtained from either Boehringer-Mannheim, Bethesda Research Labs or New England Biolabs. Apramycin and hygromycin B were obtained from Eli Lilly and Company. Ampicillin, tetracycline, cycloserine, and agarose were from Sigma Chemical Company. [^{35}-S]-methionine was from Amersham. 5-bromo-4-chloro-3-indolyl-B-D-galactoside (XG) was purchased from Bachem. BamHI linkers were from Collaborative Research.

RESULTS

Genetic Analysis of the Plasmid-Borne Genes

Resistance genes for hygromycin B (Hm) and apramycin (Am) are contained on pKC222, shown in figure 1a. This plasmid is derived from pBR322 (via pKC7) and includes a BglII-SalI fragment from a naturally occurring plasmid cloned into the BglII and SalI sites of pKC7 (14, Rao, R.N., N.E. Allen, J.N. Hobbs, Jr., W.E. Alborn, Jr., H.A. Kirst and J.W. Paschal. Submitted to Antimicrobial Agents and Chemotherapy). Hygromycin B resistance is mediated by an aminocyclitol phosphotransferase that inactivates by covalent addition of a phosphate to the 4-position of hygromycin B (Rao, R.N., N.E. Allen, J.N. Hobbs, Jr., W.E. Alborn, Jr., H.A. Kirst and J.W. Paschal. Submitted to Antimicrobial Agents and Chemotherapy). The gene is abbreviated as aph(4). Apramycin resistance is mediated by an aminocyclitol acetyltransferase that acetylates the nitrogen at the 3-position of apramycin (14). The gene is abbreviated as aac(3)IV.

Deletion experiments showed that information encoding Hm^r is on the left (BglII) side and information for Am^r is on the right (SalI) side of the insert in pKC222 (figure 1a). Deletion of a SacI piece (fragment 1) from the left side of the insert eliminates Am^r but not Hm^r. Deletion

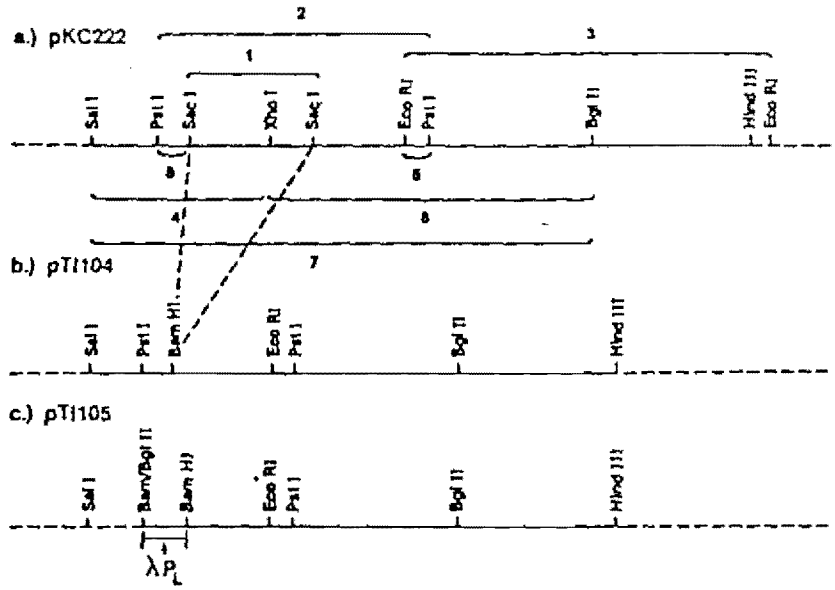

Figure 1. Plasmids encoding Hm^r and/or Am^r

A) pKC222 is derived from pBR322 via pKC7 and contains a SalI-BglII fragment isolated from a naturally occurring plasmid, pKC203, that confers resistance to apramycin and hygromycin B. (Rao, R.N., N.E. Allen, J.N. Hobbs, Jr., W.E. Alborn, Jr., H.A. Kirst and J.W. Paschal. Submitted to Antimicrobial Agents and Chemotherapy).

B) The BamHI site in pTI104 is derived from a 10 base pair long BamHI linker inserted into pKC222 after cutting with SacI and removal of the 3' extension with DNA polymerase.

C) The SalI-BamHI fragment of pTI104 is replaced by a Sal-BamHI fragment containing the λ leftward promoter. The BamHI site is inserted into λp_L at the HaeIII site upstream of the translational initiation site for the λ "N" protein and downstream from the transcriptional start site for "N" in RNA (16).

of an EcoRI piece (fragment 3) from the right side of the insert eliminates Hm^r but not Am^r. Subcloning the PstI piece (fragment 2) of pKC222 into pBR322 allows expression of Am^r but not Hm^r, which is consistent with the results of the deletion experiments.

Further subcloning experiments from pKC222 suggested that expression of Hm^r by the aph(4) gene requires information from the aac(3)IV gene. A SalI-XhoI subclone (fragment 4) did not express resistance to either Am or Hm, and neither did an Xho-BglII subclone (fragment 8), yet the sum of the two subclones (fragment 7) encoded resistance to both Hm and Am. Since

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deletion of the SacI piece (fragment 1) still permits Hm^r, yet the XhoI-BglII piece (fragment 8) is not sufficient for Hm^r, it appears that information to the left of the SacI site (including fragment 6 in figure 1a) is required for expression of Hm^r.

Physical Characterization of the Am^r and Hm^r Gene

The two most likely explanations for the importance of information to the left of the leftmost SacI site (figure 1a) for expression of Hm^r are: 1) the two resistances are conferred by one polypeptide with distinct domains; or 2) the two resistances are on separate proteins translated from a single mRNA.

The first hypothesis was tested by examining the polypeptide products of several plasmids using E. coli maxicells (4,5), which allow identification of plasmid encoded polypeptides. Figure 2 shows that the aph(4) and aac(3)IV genes encode distinct polypeptides. The hygromycin resistance gene encodes a polypeptide of about 42,000 daltons, and the apramycin resistance gene encodes a polypeptide of about 32,000 daltons. In E. coli cells containing the plasmid pKC222, which confers resistance to Hm, Am, and Ap, the predominant plasmid-encoded polypeptides are a doublet at about 32,000 daltons and another doublet at about 42,000 daltons. In cells containing pKC237, which is derived from pKC222 by deletion of an EcoRI piece (fragment 3 of figure 1a) and confers Am^r and Ap^r but not Hm^r, the doublet at 42,000 daltons is absent. In cells containing pKC241, which is derived from pKC222 by deletion of a SacI piece (fragment 1 of figure 1a) and confers Ap^r and Hm^r but not Am^r, the doublet at 32,000 is completely missing. (The remaining band near 32,000 comigrates with the Ap^r gene products of pBR322).

The second hypothesis to explain the requirement for aac(3)IV gene DNA in order to express the aph(4) gene and obtain Hm^r is that the genes are part of a polycistron with the aac(3)IV gene upstream from the aph(4) gene. This hypothesis predicts that a large mRNA species complementary to both the aac(3)IV and aph(4) genes should be found in cells containing pKC222.

Consistent with this idea is the finding that probes from both the aph(4) and aac(3)IV genes hybridize to large RNA molecules from E. coli cells containing pKC222 but not from cells without plasmid (data not shown).

Analysis of the Transcriptional Activator Region

The region containing the aac(3)IV and aph(4) genes should contain a promoter within the PstI-SacI piece (fragment 6 of figure 1a) of pKC222 based on the results of genetic experiments described earlier. Since the

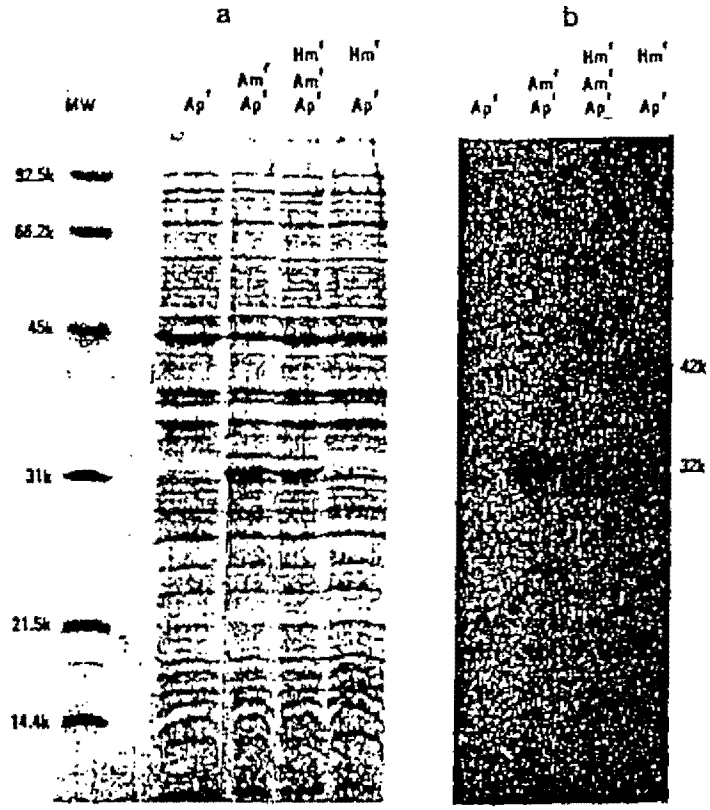

Figure 2. Identification of Plasmid Encoded Polypeptides Using E. coli Maxicells

Plasmids were transformed into the E. coli strain CSR603, a uvrA mutant. Plasmid encoded polypeptides were examined by labelling cells with [^{35}S]-methionine after UV inactivation and overnight growth in cycloserine. Panel a:Coomassie Blue stained 12.5 percent SDS-polyacrylamide gel. The first lane shows molecular weight markers of 92.5K, 66.2K, 45K, 31K, 21.5K and 14.4K daltons. The following lanes show total cellular protein from CSR603 containing these drug resistances: Ap^r) cells contain pBR322; Am^r Ap^r) cells contain pKC237, a derivative of pKC222 missing an EcoRI fragment (fragment 3 of figure 1a); Hm^r Am^r Ap^r) cells contain pKC222 (figure 1a); Hm^r Ap^r) cells contain pTI105 (figure 1c).
Panel b: the autoradiogram of the gel shown in panel a, which should identify the plasmid encoded polypeptides (4,5). The film (XAR-5, Kodak) was exposed for 24 hours at room temperature.

subcloned PstI piece (fragment 2) conferred Am^r, it most likely contains a promoter. Also, since deletion of a SacI piece (fragment 1) still permitted expression of Hm^r, the promoter must not be within the SacI piece.

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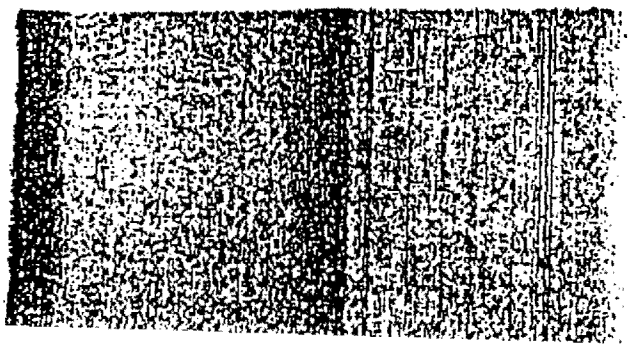
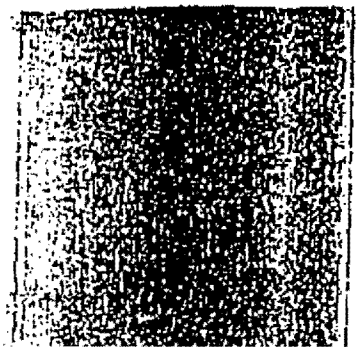

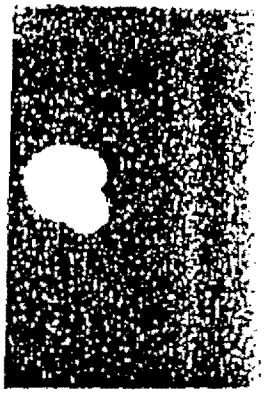

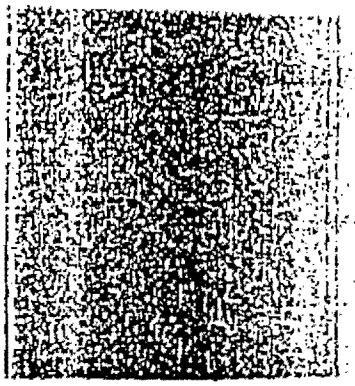

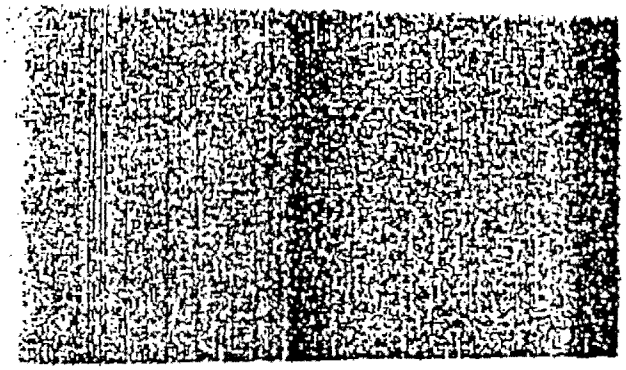

Table 1. Sequence of the Apramycin Resistance Gene Promoter

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....GACTATTTGCAACAGTGCCGTTGATCGTGCTATGATCGACTGATGTCATCAGCGGTGGAGTGCAATGTCGTGCAATACGAATGGCGAAAAGCCGAGCTC
        ***  *  *          ** *** **                          shine-   met                              SacI
    tgttgaca-ttt        attgttataatg    cat                    dalgarno
      -35       6-9 b.p.     -10      4-7
                                      b.p.
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The upper case letter are sequences obtained by Maxam-Gilbert sequencing (12) from pKC222 (figure 1a). The sequence is derived from fragment 6, figure 1a, and reads left to right (note the SacI site, which delineates the right end of fragment 6).
The lower case letters underneath the pKC222 sequence are consensus sequences for bacterial promoters (22).
Asterisks indicate matches between the apramycin resistance gene promoter and the E. coli consensus sequence.

Therefore, since the SacI fragment is contained within the PstI fragment, the promoter must be between the PstI and SacI sites. This prediction was tested by primary sequence analysis and by promoter exchange using known promoters.

The primary sequence analysis of the PstI-SacI fragment demonstrated typical E. coli promoters and translational initiation signals upstream of the SacI sites, as shown in Table I. These sequences would act in a left to right direction based on the orientation shown in figure 1a, as expected from the genetic studies.

In order to test the function of this proposed promoter, it was necessary to change the SacI site to a BamHI site. This was accomplished by removing the 3'-extension generated by SacI digestion and ligating 10-mer BamHI linkers to the flush ends. The resulting plasmid, pIT104, is shown in figure 1B.

The proposed promoter sequence, now contained on a PstI-BamHI fragment of pIT104, was able to promote β-galactosidase expression when fused to a fragment of the lacZ gene lacking activator sequences. The PstI-BamHI fragment was ligated to pMC1403, a plasmid containing the lacZ gene with a BamHI site at the codon for amino acid 8 (15). As shown in table 2, the BamHI site of pMC1403 should have the same translational reading frame as that proposed for the Pst-BamHI fragment from pIT104. In fact, cells missing the genomic copy of the lacZ gene but harboring this hybrid plasmid produced dark blue colonies on X-G ampicillin plates (8), indicating that the PstI-BamHI fragment could indeed drive transcription and translation. In a complementary experiment, it was found that a characterized promoter could drive expression of Km^r when spliced into the BamHI site of pIT104. A promoter from bacteriophage λ, λP_L, was ligated into pIT104 at the SalI-BamHI sites. The BamHI site in λP_L was artificially inserted at a HaeIII site in the untranslated leader region (115 bases downstream from the

Table 2. Fusion of the Apramycin Resistance Gene with β-galactosidase

pTI104	ATG/TCG/TGC/AAT/ACG/AAT/GGC/GAA/AAG/CCG/ccg/gat/ccg
β-gal	CCG/GAT/CCG

The upper line represents the sequence obtained from pTI104 near the BamHI site (figure 1b). This sequence is the same as the 3' end of the sequence shown in Table 1 except that the SacI site 3'-extension has been eliminated and a 10 base pair long BamHI linker has been inserted. The lower case letters represent nucleotides from the linker. The lower line represents the reading frame at the BamHI site of β-galactosidase in pMC1403 (15).

mRNA start site) and would not bring translational activator sequences with it. (16; Kuhstoss, S., H. Hsiung, R. Belagaje, S. G. Burgett and R. N. Rao, manuscript in preparation.) This hybrid plasmid, pTI105, shown in figure 1c, efficiently conferred Hm^r to E. coli.

DNA Sequence Analysis of the Hygromycin B Resistance Gene.

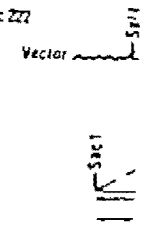
Previous experiments showed that the hygromycin B phosphotransferase was encoded within a 1.5 kb DNA fragment bounded by a SacI site and a BglII site (figure 1). In order to localize the gene more precisely, the primary sequence of this region was determined.

The sequencing strategy is shown in figure 3, and the primary sequence in figure 4. Computer analysis of the primary sequence identified a large open reading frame bounded by the SacI site on the 5' end and the BglII site on the 3' end. The amino acid sequence predicted for the polypeptide encoded by this open reading frame is shown in figure 4.

The open reading frame postulated to encode the hygromycin B phosphotransferase is preceded by a GGGGG sequence 9 bases upstream from the ATG which is postulated to initiate translation of the polypeptide. This sequence and the spacing are appropriate for a ribosome binding site in E. coli (17).

Fusion of the Hygromycin B Resistance Gene with β-Galactosidase

We fused the protein coding region of β-galactosidase with the putative coding region of the hygromycin B resistance gene. We utilized the unique HphI restriction site in the hygromycin B gene, which cuts 10 bp downstream of the initiating ATG (as shown in figure 5), as the junction site for the fusion. The 3' overhang of the HphI site was eliminated with T4 DNA polymerase (see Materials and Methods), and the blunt ended molecules were ligated to the β-galactosidase coding sequence on pUC8 (18). This β-galactosidase sequence has multiple restriction sites, including a unique



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DISCUSSION

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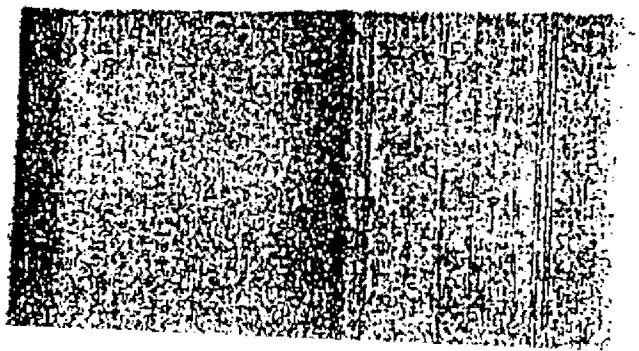
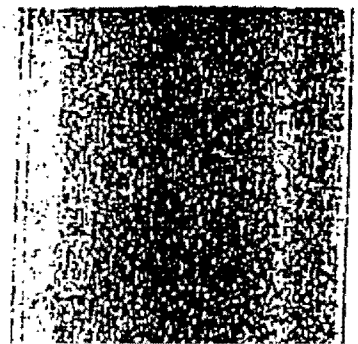

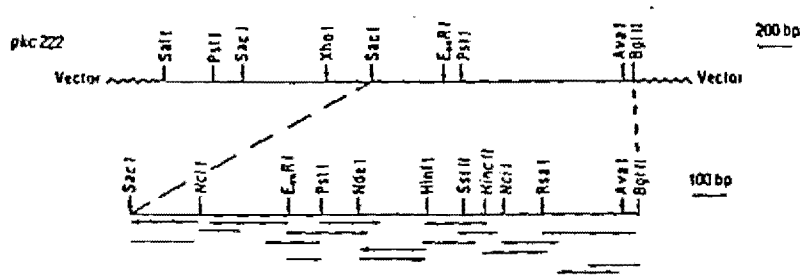

Figure 3. Sequencing Strategy Used for the Hygromycin B Resistance Gene
The primary sequence was determined using the chemical cleavage method of Maxam and Gilbert (12). The length of the arrows indicates the distance sequenced from the labelled end.

HincII site, downstream of the translation initiating ATG sequence. The reading frame postulated to encode the hygromycin B phosphotransferase polypeptide would be maintained by ligating together these blunt-ended molecules, as shown in figure 5. The resulting plasmid, named pKC307, was subjected to DNA sequence analysis and the sequence predicted in figure 5 was verified.

This hybrid gene conferred hygromycin B resistance to E. coli. The hygromycin B resistance was more efficiently conferred in the presence of IPTG, an inducer of β-galactosidase activity, than in the absence of IPTG, as shown in table 3. It should be noted that while the efficiency of plating on hygromycin B plates is low in both cases, efficiency of plating increased about 100 fold in the presence of IPTG.

DISCUSSION

Our analysis of a hygromycin B resistance gene from an E. coli plasmid shows it to be linked to an apramycin resistance gene as part of a polycistron. The apramycin resistance gene (aac(3)IV) precedes the hygromycin B resistance gene (aph(4)) on the primary transcript. The evidence for this configuration is threefold. First, the expression of the aph(4) gene requires information in front of the aac(3)IV gene. That is, the absence of a SacI-PstI piece of DNA, fragment 6 of figure 1a, eliminates expression of both genes, the absence of DNA downstream (fragment 1) eliminates expression of Am^r, and the absence of a piece of DNA even farther downstream (fragment 3) eliminates expression of Hm^r. Second, the two resistance genes encode separate polypeptides as shown by maxicell

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GAGCTCATGAGCGGAGAACGAGATGACGTTGGAGGGGCAAGGTCGCGCTGATTGCTGGGGC
         10        20        30        40        50        60

AACACGTGGAGCGGATCGGGGATTGTCTTTCTTCAGCTCGCTGATGATATGCTGACGCTCA
        70        80        90       100       110       120

ATGCCGTTTGGCCTCCGACTAACGAAAATCCCGCATTTGGACGGCTGATCCGATTGGCACGG
      130       140       150       160       170       180

                                                   MET LYS LYS
CGGACGGCGAATGGCGGAGCAGACGCTCGTCCGGGGGCAATGAGAT     ATG AAA AAG
    190       200       210       220       230

    5                   10                  15
PRO GLU LEU THR ALA THR SER VAL GLU LYS PHE LEU ILE GLU LYS PHE
CCT GAA CTC ACC GCG ACG TCT GTC GAG AAG TTT CTG ATC GAA AAG TTC
240         250         260         270         280

20                  25                  30                  35
ASP SER VAL SER ASP LEU MET GLN LEU SER GLU GLY GLU GLU SER ARG
GAC AGC GTC TCC GAC CTG ATG CAG CTC TCG GAG GGC GAA GAA TCT CGT
  290         300         310         320         330

                40                  45                  50
ALA PHE SER PHE ASP VAL GLY GLY ARG GLY TYR VAL LEU ARG VAL ASN
GCT TTC AGC TTC GAT GTA GGA GGG CGT GGA TAT GTC CTG CGG GTA AAT
     340         350         360         370         380

            55                  60                  65
SER CYS ALA ASP GLY PHE TYR LYS ASP ARG TYR VAL TYR ARG HIS PHE
AGC TGC GCC GAT GGT TTC TAC AAA GAT CGT TAT GTT TAT CGG CAC TTT
        390         400         410         420         430

        70                  75                  80
ALA SER ALA ALA LEU PRO ILE PRO GLU VAL LEU ASP ILE GLY GLU PHE
GCA TCG GCC GCG CTC CCG ATT CCG GAA GTG CTT GAC ATT GGG GAA TTC
          440         450         460         470

    85                  90                  95
SER GLU SER LEU THR TYR CYS ILE SER ARG ARG ALA GLN GLY VAL THR
AGC GAG AGC CTG ACC TAT TGC ATC TCC CGC CGT GCA CAG GGT GTC ACG
480         490         500         510         520

100                 105                 110                 115
LEU GLN ASP LEU PRO GLU THR GLU LEU PRO ALA VAL LEU GLN PRO VAL
TTG CAA GAC CTG CCT GAA ACC GAA CTG CCC GCT GTT CTG CAG CCG GTC
  530         540         550         560         570

                120                 125                 130
ALA GLU ALA MET ASP ALA ILE ALA ALA ALA ASP LEU SER GLN THR SER
GCG GAG GCC ATG GAT GCG ATC GCT GCG GCC GAT CTT AGC CAG ACG AGC
     580         590         600         610         620

            135                 140                 145
GLY PHE GLY PRO PHE GLY PRO GLN GLY ILE GLY GLN TYR THR THR TRP
GGG TTC GGC CCA TTC GGA CCG CAA GGA ATC GGT CAA TAC ACT ACA TGG
        630         640         650         660         670

        150                 155                 160
ARG ASP PHE ILE CYS ALA ILE ALA ASP PRO HIS VAL TYR HIS TRP GLN
CGT GAT TTC ATA TGC GCG ATT GCT GAT CCC CAT GTG TAT CAC TGG CAA
          680         690         700         710

    165                 170                 175
THR VAL MET ASP ASP THR VAL SER ALA SER VAL ALA GLN ALA LEU ASP
ACT GTG ATG GAC GAC ACC GTC AGT GCG TCC GTC GCG CAG GCT CTC GAT
720         730         740         750         760

180                 185                 190                 195
GLU LEU MET LEU TRP ALA GLU ASP CYS PRO GLU VAL ARG HIS LEU VAL
GAG CTG ATG CTT TGG GCC GAG GAC TGC CCC GAA GTC CGG CAC CTC GTG
  770         780         790         800         810
```

```
HIS ALA
CAC GCG
     820

ILE THR
ATA ACA

TYR GLU
TAC GAG
 910

GLU GLN
GAG CAG
     960

     260
SER PRO
TCG CCG
      10

275
TYR GLN
TAT CAG

GLY ARG
GGT CGA

THR GLN
ACA CAA
 1150

GLU VAL
GAA GTA
    1200

     340
ALA LYS
GCA AAG
      1

CAACTCGC
     131

TCGTCCAC
 1370

CCCTTCTG

CTGATCCC
      15
```

Figure 4. Primary
The sequence is
the BglII site in a
sequence encoded by
is also included.

93

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                    200                 205                 210
HIS ALA ASP PHE GLY SER ASN ASN VAL LEU THR ASP ASN GLY ARG
CAC GCG GAT TTC GGC TCC AAC AAT GTC CTG ACG GAC AAT GGC CGC
     820         830         840         850         860

                    215                 220                 225
ILE THR ALA VAL ILE ASP TRP SER GLU ALA MET PHE GLY ASP SER GLN
ATA ACA GCG GTC ATT GAC TGG AGC GAG GCG ATG TTC GGG GAT TCC CAA
            870         880         890         900

            230                 235                 240
TYR GLU VAL ALA ASN ILE PHE PHE TRP ARG PRO TRP LEU ALA CYS MET
TAC GAG GTC GCC AAC ATC TTC TTC TGG AGG CCG TGG TTG GCT TGT ATG
 910         920         930         940         950

        245                 250                 255
GLU GLN GLN THR ARG TYR PHE GLU ARG ARG HIS PRO GLU LEU ALA GLY
GAG CAG CAG ACG CGC TAC TTC GAG CGG AGG CAT CCG GAG CTT GCA GGA
    960         970         980         990         1000

    260                 265                 270
SER PRO ARG LEU ARG ALA TYR MET LEU ARG ILE GLY LEU ASP GLN LEU
TCG CCG CGG CTC CGG GCG TAT ATG CTC CGC ATT GGT CTT GAC CAA CTC
     1010        1020        1030        1040        1050

275                 280                 285                 290
TYR GLN SER LEU VAL ASP GLY ASN PHE ASP ASP ALA ALA TRP ALA GLN
TAT CAG AGC TTG GTT GAC GGC AAT TTC GAT GAT GCA GCT TGG GCG CAG
         1060        1070        1080        1090        1100

                295                 300                 305
GLY ARG CYS ASP ALA ILE VAL ARG SER GLY ALA GLY THR VAL GLY ARG
GGT CGA TGC GAC GCA ATC GTC CGA TCC GGA GCC GGG ACT GTC GGG CGT
            1110        1120        1130        1140

            310                 315                 320
THR GLN ILE ALA ARG ARG SER ALA ALA VAL TRP THR ASP GLY CYS VAL
ACA CAA ATC GCC CGC AGA AGC GCG GCC GTC TGG ACC GAT GGC TGT GTA
 1150        1160        1170        1180        1190

        325                 330                 335
GLU VAL LEU ALA ASP SER GLY ASN ARG ARG PRO SER THR ARG PRO ARG
GAA GTA CTC GCC GAT AGT GGA AAC CGA CGC CCC AGC ACT CGT CCG AGG
    1200        1210        1220        1230        1240

    340   1254
ALA LYS GLU |
GCA AAG GAA TAGAGTAGATGCCGACCGAACAAGAGCTGATTTCGAGAACGCCTCAGCCAG
     1250        1260        1270        1280        1290        1300

CAACTCGCGCGAGCCTAGCAAGGCAAATGCGAGAGAACGGCCTTACGCTTGGTGGCACAGTTC
     1310        1320        1330        1340        1350        1360

TCGTCCACAGTTCGCTAAGCTCGCTCGGCTGGGTCGCGGGAGGGCCGGTCGCAGTGATTCAGG
  1370        1380        1390        1400        1410        1420        1430

CCCTTCTGGATTGTGTTGGTCCCCAGGGCACGATTGTCATGCCCACGCACTCGGGTGATCTGA
          1440        1450        1460        1470        1480        1490

CTGATCCCGCAGATTGGAGATCGCCGCCCGTGCCTGCCGATTGGGTGCAGATCT
     1500        1510        1520        1530        1540
```

BglII

1546
1254
292

Figure 4. Primary sequence of the Hygromycin B Resistance Gene.
The sequence is reported from the rightmost SacI site in figure 4 to the BglII site in a 5' to 3' direction. The predicted amino acid sequence encoded by the large open reading frame found within this region is also included.

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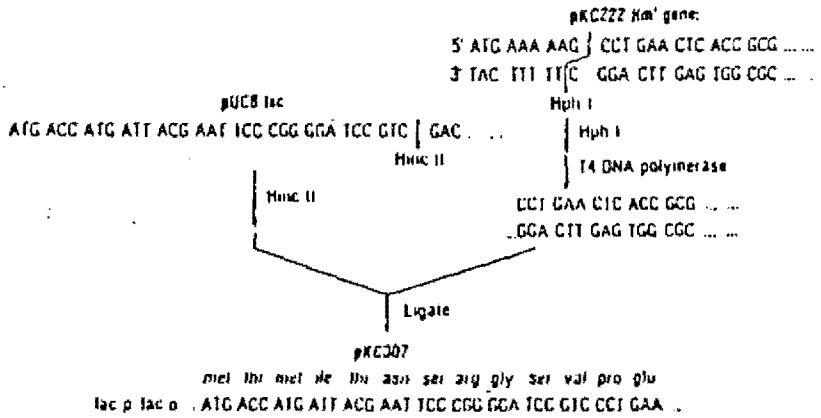

Figure 5. *Fusion of the Hygromycin B Resistance Gene with lacZ Coding Sequence*

The hygromycin B resistance gene was cut at a HphI restriction site, which cuts the upper strand after base 236 (figure 5). After removing the 3' extension with T4 DNA polymerase, the blunt ended molecules were ligated to the lac Z gene on pUC8 (18) cut at a HincII site. The predicted sequence of the fused construct, shown as pKC307, was verified by DNA sequence analysis.

analysis (figure 2) of plasmid-encoded proteins. Third, a large RNA species exists in E. coli cells harboring the relevant plasmid which hybridizes to probes from both genes. The transcriptional activator sequence responsible for the expression of the polycistron has been identified and characterized. Primary sequence analysis of the SacI-PstI fragment (fragment 6 of figure 1a) demonstrates a typical E. coli promoter sequence (table 1). When the SacI-PstI fragment is fused in frame with a lacZ gene lacking its promoter and first 8 amino acids, active β-galactosidase protein is produced (table 2). Also, if this SacI-PstI sequence is excised and replaced by a known promoter sequence, the leftward promoter from bacteriophage lambda, the resistance gene is once again expressed. Thus, physical and functional evidence support the conclusion that the SacI-PstI fragment contains the promoter for the aac(3)IV and aph(4) genes.

The separate polypeptides encoded by the resistance genes both appear as doublets on an SDS-polyacrylamide gel (figure 2). The polypeptide products of the aac(3)IV gene correspond to apparent molecular weights of 31,500 and 33,000 daltons, and the polypeptides made by the aph(4) gene correspond to apparent molecular weights of 41,500 and 42,500. This molecular weight estimate for the aph (IV) gene product agrees with the estimation (42,000

Table 3. The Influe

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Cells Grown with 1mM IPTG	no IP 1mM I

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Table 3. The Influence of IPTG on Expression of Hm Resistance from pKC307.

		Titer on TY	Titer on Ty plus ampicillin plus hygromycin B*	Efficiency of Plating on Drug Plates
Cells Grown	no IPTG in plates	5.6×10^9	1.9×10^4	3.4×10^{-6}
Without IPTG	1mM IPTG	5.6×10^9	3.2×10^5	5.7×10^{-5}
Cells Grown	no IPTG in plates	6.4×10^9	3.7×10^4	5.8×10^{-6}
with 1mM IPTG	1mM IPTG in plates	6.4×10^9	4.5×10^6	7.0×10^{-4}

Bacteria were grown overnight in TY broth (8), with or without 1 mM IPTG, then diluted and plated on plates with or without 1 mM IPTG. Plates contained TY plus 1.5 percent agar. When added IPTG was present at 1 mM, ampicillin was present at 100 ug/ml, and hygromycin B was present at 200 ug/ml.

*Control cells without plasmid acquire spontaneous hygromycin B resistance under these conditions at a frequency of less than 1 in 10^9 cells.

daltons) based on molecular sieving (Santerre, R.F., N.E. Allen, J.N. Hobbs, Jr., R.N. Rao and R.J. Schmidt. Manuscript in preparation), which implies that the enzyme exists in a monomeric state. The presence of doublets suggests that the polypeptide may be excreted into the periplasmic space, with the larger polypeptides corresponding to the molecules still inside the cell and retaining a signal polypeptide, and the smaller polypeptides corresponding to processed molecules lacking the signal polypeptide. This interpretation is consistent with the finding that the enzymatic activities associated with Km^r and Am^r can be osmotically shocked from the cells (Rao, R.N., N.E. Allen, J.N. Hobbs, Jr., W.E. Alborn, Jr., H.A. Kirst, and J.W. Paschal. Antimicrobial Agents and Chemotherapy, in press).

DNA Sequence of the Hygromycin Resistance Gene.

Since it was known that the hygromycin resistance gene was contained within the second SacI and BglII sites of pKC222 (figure 1), the primary sequence of this region was determined. The sequencing strategy is shown in figure 3, and the primary sequence itself in figure 4. One large open reading frame was found, and the amino acid sequence encoded within this

open reading frame is also shown in figure 4. No other open reading frame is within 40 percent of the size of this large open reading frame, which is large enough to encode a polypeptide with a molecular weight of 38,236 daltons. This predicted weight is about 10 percent lower than the value of about 42,000 daltons determined by gel electrophoresis for the hygromycin phosphotransferase polypeptide (figure 2). The reason for the discrepancy is not known, although it could be due to anomolous migration on SDS-polyacrylamide gels or posttranslational modification.

There are several noteworthy features of the sequence reported in figure 4. First, the predicted site of initiation of translation is preceded by a GGGGG sequence ending 9 bp before the ATG. This sequence and the spacing are typical of ribosome binding sites in E. coli (17). Another feature of the sequence is that there is a large open reading frame in the opposite strand, bounded by stop codons at bases 1348 and 143 in figure 5. However, there are no ATG codons preceded by ribosome binding sites in this reading frame. The significance of this open reading frame is not known.

The predicted amino acid sequence of the Hygromycin B phosphotransferase polypeptide encoded on the aph(4) gene was compared with that of the neomycin phosphotransferase polypeptides encoded on Tn5 (19) and Tn903 (20). No obvious similarities were found, even in the regions where the resistance genes from Tn5 and Tn903 show extensive homology. This result is not surprising since neomycin and related compounds are not substrates for the hygromycin B phosphotransferase polypeptide encoded on the aph(4) gene (Rao, R.N., N.E. Allen, J.N. Hobbs, Jr., W.E. Alborn, Jr., H.A. Kirst and J.W. Paschal. Antimicrobial Agents and Chemotherapy, in press).

Fusing the hygromycin B resistance gene to lac Z

We constructed protein fusions in order to test the functionality of the open reading frame predicted to be responsible for hygromycin B resistance. The putative hygromycin B resistance gene was cut at the unique HphI site which excises the DNA encoding the first three amino acids. After removing the 3' overhang, the DNA was joined to the amino terminus of a modified β-galactosidase gene carried on pUC8 (18). The resulting hybrid plasmid, outlined in figure 5, substitutes 11 amino acids for the three removed from the hygromycin resistance gene.

This hybrid gene, present in pKC307, is active in E. coli and the activity is stimulated by addition of IPTG, a gratuitous inducer of the β-galactosidase promoter. As shown in table III, cells containing pKC307 are partially resistant to 200 μg/ml hygromycin B. That is, when cells are

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plated on plates containing hygromycin B, about 1 cell in 10^5 grew into a visible colony. Since cells containing intact hygromycin B resistance gene on pIT105 or pKC222 give a 100 percent efficiency of plating on hygromycin B plates, the hybrid construct seems less efficient than the parental. The reduced efficiency could be due to lower specific activity and/or reduced stability of the hybrid polypeptide, or other problems with transcription or translation.

The reduced plating efficiency on hygromycin B plates can be partially overcome by addition of IPTG, a gratuitous inducer of transcription of the β-galactosidase gene. As shown in table III, the efficiency of plating on hygromycin B-containing plates is increased 100 fold by inclusion of IPTG.

These results support our prediction that the open reading frame depicted in figure 4 encodes the hygromycin B phosphotransferase polypeptide. It could also be argued that the β-galactosidase gene is simply providing a transcriptional activator, and that the correct open reading frame lies elsewhere downstream. This is not likely because construction of hybrid hygromycin B resistance genes that delete the same three amino acid codons but shift the reading frame do not confer resistance whereas similar constructs that maintain the reading frame do allow resistance (data not shown).

It was noted earlier that the hygromycin B phosphotransferase polypeptide might be a secreted protein, since the activity can be osmotically shocked from the periplasma and a doublet is observed on SDS-polyacrylamide gels (see figure 2). If this is the case one would expect to find a signal sequence at the beginning of the predicted amino acid sequence. The anticipated characteristics of a signal sequence are 1) a positively charged region at the start of the signal sequence and 2) a region of hydrophobic amino acid (21). The amino acid sequence shown in figure 5 meets the first requirement nicely, since the initiating methionine is followed by two lysines. However, the following amino acid sequence is not as hydrophobic as that found in other signal sequences in E. coli (21). It should be noted, however, that cells containing the hybrid hygromycin B resistance gene which have had the two lysine residues replaced by eleven other amino acids do not seem to have hygromycin B phosphotransferase activity in their periplasm. No phosphotransferase activity can be detected in extracts made by osmotically shocking the cells, but phosphotransferase activity can be detected in cell lysates (data not shown). Thus, the ability of the cells to secrete the polypeptide into the periplasm may be

abolished in the hybrid genes. The relationship, if any, between secretion of the polypeptide and conferral of hygromycin B resistance is not known.

The ability to form fusion polypeptides with the hygromycin B phosphotransferase protein has important implications for its possible use as a dominant selectable marker in heterologous systems. Ligation of appropriate promoters and translational initiation sequences from other organisms in front of the hygromycin resistance gene might allow the gene to be used as a dominant selectable marker in that organism, or even to isolate these activator sequences in the first place.

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CLONING AND EXPRESSION OF THE FUNGAL EXPANDASE/HYDROXYLASE GENE INVOLVED IN CEPHALOSPORIN BIOSYNTHESIS

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ABSTRACT

Cephalosporins are antibiotics characterized by a cephem ring system in which a beta-lactam ring is fused to a dihydrothiazine ring. At industrial scale, cephalosporin C is produced by the fungus Cephalosporium acremonium, and cephamycin C, a 7-alpha-methoxy-cephalosporin, is produced by Nocardia lactamdurans. These two cephalosporins and other beta-lactam compounds are modified chemically to form clinically important cephalosporins that effectively treat infections caused by many different bacteria. The utility of cephalosporins in clinical medicine is due, in part, to the relative resistance of the cephem ring system against hydrolysis by beta lactamases produced by many bacterial pathogens. The cephem ring system is synthesized in the conversion of penicillin N to deacetoxycephalosporin C (DAOC) by expansion of the five-membered thiazolidine ring of the penicillin to the six-membered dihydrothiazine ring. The enzyme that catalyzes this reaction, deacetoxycephalosporin C synthetase - often called expandase - has been purified from C. acremonium. During purification DAOCS activity never separated from and remained in constant ratio to deacetylcephalosporin C (DAC) synthetase activity. Deacetylcephalosporin C synthetase (DACS) activity functions in the biosynthetic pathway to cephalosporin C and cephamycin by catalyzing hydroxylation of

DAOC to form DAC. A partial amino acid sequence of the C. acremonium DAOCS/DACS protein has been obtained and this information used to clone a corresponding gene. The open reading frame observed upon sequencing this gene was expressed in E. coli and both DAOCS and DACS activities were found in extracts of these E. coli cells, providing unambiguous proof of the bifunctional nature of the DAOCS/DACS polypeptide from C. acremonium. Some regions of the predicted amino acid sequence of the DAOCS/DACS protein were similar to regions in the predicted amino acid sequence of another key enzyme involved in the biosynthesis of penicillins and cephalosporins - isopenicillin N synthetase. The cloned DAOCS/DACS gene represents an important tool for enhancing production of known cephalosporins and for synthesis of novel cephalosporins.

TEXT

The deacetoxycephalosporin C synthetase (DAOCS), or expandase, enzyme occupies a central position in the biosynthesis of cephalosporin antibiotics. As shown in Figure 1, the biosynthesis of penicillins and cephalosporins (e.g. penicillin G by Penicillium chrysogenum and cephalosporin C by Cephalosporium acremonium) occurs via the common intermediate delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV). Both organisms use a similar isopenicillin N synthetase enzyme (1,2) to condense LLD-ACV to isopenicillin N. After this reaction the pathways diverge. In P. chrysogenum, the alpha-aminoadipyl side chain can be exchanged for another acyl moiety (e.g. substitution of phenylacetyl moiety exchanged from phenylacetyl coenzyme A produces penicillin G) whereas in C. acremonium the L-alpha-aminoadipyl side chain is epimerized to the D configuration to produce penicillin N. Penicillin N is then used as a substrate for the deacetoxycephalosporin C synthetase (DAOCS), which catalyzes the expansion of the 5-membered thiazolidine ring to the 6-membered dihydrothiazone ring characteristic of cephalosporins. The remaining biosynthetic steps in the pathway to cephalosporin C in C. acremonium are modifications of the methyl group attached to the 6-membered ring (3'-position); first the methyl group is hydroxylated via the action of the

deacetylcephalosporin C synthetase (DACS), then the hydroxyl group is acetylated to form cephalosporin C.

The importance of the cephem ring system in clinical medicine created an interest in cloning the gene for DAOCS. A cloned, sequenced gene would provide useful structural information for understanding the catalytic mechanism of ring-expansion and structural studies would be aided by an abundant source of DAOCS made available through high expression of the DAOCS open reading frame in E. coli. In addition, DAOCS had been purified from C. acremonium and was found to also exhibit DACS activity (3). Cloning the gene(s) encoding these activities offered a definitive means of proving (or refuting) the existence of a bifunctional DAOCS/DACS polypeptide in C. acremonium.

The DAOCS gene was cloned using probes based on amino acid sequence of fragments of the purified protein. Direct amino terminal sequencing of the protein was not possible because of negligible yields on the sequenator, presumably due to amino terminal blockage. Since the amino terminal blockage was observed with separate batches of purified protein prepared under different conditions, it seems likely that the amino terminal blockage occurred in vivo (although we have not excluded the

possibility that the blockage was an artifact of the protein purification procedure). Sequences of fragments from the polypeptide were obtained using several different proteolysis methods and the results are summarized in the Table. Included within the sequence information for Fragment #1 was the sequence Trp-Gln-Asp-Tyr-Phe-Asp. The redundancy in the genetic code for this peptide is relatively low; 16 different 17-mer oligonucleotides account for all coding possibilities. Another sequence (amino acids 136 to 155 in the open reading frame of Figure 3, contained in Fragment #2) was used to derive a single long probe ("guessmer") of 60 bases. Codon preference for this probe was assumed to be equivalent to that of the C. acremonium isopenicillin N synthetase gene (1). The mixed probes and the "guessmer" probe were used separately to screen a cosmid bank of C. acremonium genomic DNA (1) and two cosmids were isolated which hybridized to both types of probes. A 7.2 kb BamHI fragment common to both cosmids which contained the region of hybridization was subcloned into pUC8 (5) to obtain pIT503, shown in Figure 2. The region of hybridization was sequenced using a combination of dideoxy and chemical cleavage sequencing methods as described previously (1). A single open reading frame was discovered which, when translated, contained all of the amino acid sequences generated by direct amino acid sequencing of peptide fragments. This open reading frame, along with flanking

noncoding sequences, is shown in Figure 3. Almost half of the predicted amino acid sequence has been verified by direct amino acid sequencing of peptide fragments.

The open reading frame shown in Figure 3 was expressed in E. coli and both DAOCS and DACS activities were unambiguously identified in crude cell extracts. The first expression vector used was pIT507, a derivative of pCZR336 (Figure 2). The relevant features of pCZR336 and pIT507 are a two cistron translation-enhancing element (6), the lambda pL promoter, the lambda cI857 temperature sensitive repressor of the pL promoter and a modified tetracycline resistance gene from pBR322 (7). Cells containing pIT507 were grown under conditions where the open reading frame from pIT503 should be expressed. Crude cell extracts were tested for DAOCS and DACS activities using an HPLC assay (3). Penicillin N was clearly shown to be converted to deacetoxycephalosporin C and then to deacetylcephalosporin C in the presence of the purified protein from E. coli. In addition, deacetoxycephalosporin C could be directly converted to deacetylcephalosporin C under similar conditions (see Figure 1 for details of the pathway to cephalosporin C), proving that both DAOCS and DACS activities are encoded by the insert in pIT507. Since the insert in pIT507 contains about 1.2 kb of DNA downstream of the open reading frame shown in Figure 3, it was

still possible that one of the two activities was encoded by this extra DNA. The 3' noncoding DNA was therefore shortened to about 120 bp in the expression vector pIT511 (Figure 2). Once again both DAOCS and DACS activities were clearly observed in cell extracts of E. coli cells containing pIT511, further substantiating the assignment of both activities to the single open reading frame. It is interesting to note that in all cases the E. coli derived DAOCS and DACS activities were found in a 9:1 ratio, which is about the same ratio observed for the DAOCS/DACS obtained from C. acremonium (3).

The DAOCS/DACS polypeptide accumulated to about 15% of total cell protein under appropriate conditions (Figure 4). E. coli cells expressing high levels of the DAOCS/DAC contained granules (inclusion bodies) when observed under phase contrast. It remains to be determined whether these granules are composed principally of DAOCS/DACS polypeptide. E. coli-derived DAOCS/DACS was purified to near homogeneity using a procedure described previously for the native protein (3). No attempt was made to solubilize granular DAOCS/DACS polypeptide during this purification procedure, and the purified material may therefore represent a small fraction of total DAOCS/DACS protein. The DAOCS/DACS enzymatic activities from E. coli were lost gradually during the course of the purification, even under conditions

which stabilize the native enzyme. However, the purified polypeptide from E. coli was indistinguishable from the native enzyme when examined by SDS-polyacrylamide gel electrophoresis or by tryptic cleavage patterns (data not shown).

Some regions of the predicted amino acid sequence of the DAOCS/DACS gene show intriguing similarities with those of the C. acremonium isopenicillin N synthetase gene. The predicted amino acid sequences of the two proteins were optimally aligned using the method of Needleman and Wunsch (8). Although the overall homology was only slightly greater than 11%, certain areas showed higher homologies. In particular, a ten amino acid region containing the cysteine residue at position 100 of the DAOCS/DACS was 50% homologous to the corresponding region containing the cysteine residue at position 106 of the isopenicillin N synthetase. The relevant sequences around these cysteine residues, with identities capitalized, are:

DAOCS/DACS: GLY-LYS-tyr-ser-asp-tyr-SER-thr-CYS-TYR
IPNS: GLY-LYS-lys-ala-val-glu-SER-phe-CYS-TYR

This region is of particular interest because we have previously shown that Cys106 of the isopenicillin N synthetase is important for substrate binding since replacement of the cysteine with a

serine caused a 5 fold increase in the Km of the enzyme for substrate and a 20 fold reduction in the specific activity (9). Perhaps Cys100 of the DAOCS/DACS is also involved in substrate binding, either directly or indirectly. This hypothesis can be tested by site-directed mutagenesis of the Cys100 codon of the DAOCS/DACS gene.

The bifunctional nature of the DAOCS/DACS polypeptide and the cofactor/substrate requirements shared by DAOCS and DACS (Fe^{+2}, alpha-ketoglutarate, and dioxygen) raise a question as to whether a single site or two active sites are responsible for the C. acremonium DAOCS and DACS activities. Prokaryotic organisms such as Streptomyces clavuligerus and Streptomyces lipmanii also synthesize cephalosporins and contain DAOCS and DACS activities. However, in contrast with the C. acremonium activities, the S. clavuligerus DAOCS and DACS activities are readily separable, strongly suggesting that the activities are determined by separate polypeptides, and thus separate active sites, in this prokaryote. The C. acremonium DAOCS/DACS polypeptide may have evolved by fusion of contiguous, separate genes coding for DAOCS and DACS and separate domains within the protein-coding region of the C. acremonium DAOCS/DACS gene may be discernible by creating a functional map of the gene via in vitro mutagenesis experiments. There are several examples where biosynthetic genes

are separate in one organism but fused in another (12). If separate DAOCS and DACS genes account for the separable DAOCS and DACS activities in Streptomyces clavuligerus, comparison of the protein coding regions of those two genes to the protein coding region of C. acremonium DAOCS/DACS gene should be helpful in identifying the two functional domains within the C. acremonium gene. At present, however, one cannot unambiguously rule out the possibility that separable DAOCS and DACS activities in S. clavuligerus result from post-translational modification of a single polypeptide.

The concept that the C. acremonium DAOCS/DACS polypeptide evolved from two separate contiguous prokaryotic DAOCS and DACS genes is consistent with a previous proposal that cephalosporin biosynthesis evolved first in prokaryotic organisms and that truncated forms of more complex prokaryotic pathways subsequently spread to eukaryotes (2). The reactions leading to cephalosporin C in C. acremonium may be thought of as a subset of the reactions leading to cephamycin C (7-alpha-methoxycephalosporin C) in S. clavuligerus. The pathway to penicillin G in P. chrysogenum can be thought of as a further truncation of a cephalosporin pathway (truncated after formation of isopenicillin N) followed by evolution of a side reaction allowing exchange of the L-alpha-aminoadipyl side chain of isopenicillin N with the phenylacetyl

moiety of phenylacetylCoA. If evolution occurred in this manner, eukaryotes would be expected to modify the genes acquired from prokaryotes to facilitate expression, since eukaryotic and prokaryotic transcriptional and translational activation sequences differ. For example, if the DAOCS and DACS activities in the prokaryotic species were expressed from a polycistron, the eukaryotic species would have been ill-equipped to directly express the prokaryotic genes because of the absence of polycistronic messages in eukaryotes. One solution would be to fuse the genes so that one polypeptide would contain both activities. In vivo fusion of genes leading to production of a single polypeptide has been observed in other systems (for example, fusion of the _his_D and _his_C genes of _Salmonella typhimurium_ (11)). It will be of interest to determine if separate, contiguous genes for DAOCS and DACS occur in _S. clavuligerus_ and to compare the protein coding regions for the DAOCS and DACS genes from _S. clavuligerus_ to that reported here for the _C. acremonium_ DAOCS/DACS polypeptide.

Table. Amino acid sequences of fragments of the DAOCS/DACS polypeptide.

Fragment	Corresponding Sequence in Figure 3	Type of cleavage
1	114-125	Tryptic
2	126-155	CNBr
3	136-157	Tryptic
4	281-287	Tryptic
5	65-71	Tryptic (mass spec)
6	173-180	Tryptic (mass spec)
7	81-105	Spontaneous degrad.
8	120-127	Trp oxidation
9	256-262	Lys C
10	103-125	CNBr
11	306-313	CNBr
12	5-14	Lys C
13	223-242	CNBr
14	234-256	Spontaneous degrad.

DAOCS/DACS protein used for these experiments was purified as described previously (3). Trypsin cleavage and isolation of the peptides was carried out as described (13). The tryptic peptides were sequenced using a Beckman 890D Spinning Cup Sequencer. Fragments 5 and 6 were isolated as an equimolar mixture and

required the aid of fast atom bombardment mass spectrometry and tandem mass spectrometry (14) to determine their sequences. Cyanogen bromide cleavage (15), tryptophan oxidative cleavage (16) and lys C cleavage (17) were also used to obtain fragments of the protein for sequencing. Direct sequencing of the intact polypeptide did not produce significant yields, indicating amino terminal blockage, but two different sequences were obtained directly from purified protein (fragments 7 and 14). Since these sequences matched predicted sequences from the DNA sequence (Figure 3), these sequences evidently arose from spontaneous degradation products of the DAOCS/DACS polypeptide. Intentional cleavage products were purified by reverse phase HPLC or size fractionation chromatography. The sequence of the peptides was determined as described previously (1).

Figure 1. Biosynthetic pathways to cephalosporin C and penicillin G in C. acremonium and P. chrysogenum, respectively. The pcb gene designation stands for penicillin/cephalosporin biosynthesis. The DAOCS/DACS gene reported here is encoded by the pcbEF gene of C. acremonium. pcbA and pcbB are reserved for proposed LLD-ACV biosynthetic genes.

Figure 2. Relationships of plasmids used to clone and express the DAOCS/DACS gene. The thick solid lines refer to C. acremonium DNA; the thin solid lines refer to vector DNA; the open boxes refer to relevant genes or controlling elements. Abbreviations used for these genes include DAOCS for the DAOCS/DACS gene from C. acremonium, AMP and TET represent the beta lactamase and tetracycline resistance genes, respectively, from pBR322 (18), lambda pL represents the leftward promoter from bacteriophage lambda, cI857 represents a temperature sensitive mutant of the cI repressor from bacteriophage lambda, and HGH represents the human growth hormone gene. pIT503 was constructed by cloning the approximately 7.2 kb BamHI restriction fragment which contained the DAOCS/DACS gene from the original cosmid isolate (pIT501, not shown) into pUC8 (5). The gene was expressed in E. coli using a derivative of pCZR336, a vector designed to express human growth hormone in E. coli (7). This plasmid contains the lambda pL transcriptional activator sequence followed by a two cistron

translational enhancer sequence (6), the cI857 gene, the human growth hormone gene and a modified form of the tetracycline resistance gene from pBR322. In order to facilitate the expression constructions, the amino terminal coding region of the DAOCS/DACS open reading frame was reconstructed through use of a synthetic linker. This linker extended to the SstI site encoding amino acids 21 and 22 of the open reading frame, and contained three changes relative to the wild type sequence: 1) an NdeI site (5'-CATATG) was incorporated at the translation initiation site, 2) the C residue at the third position of codon 5 was changed to a T, and 3) the C at the third position of codon 10 was changed to an A, thereby creating an XbaI restriction site. This linker was ligated to a SstI-BamHI fragment from pIT503 (containing the DAOCS/DACS gene) and replaced the human growth hormone gene in pCZR336, creating the expression vector pIT507. Plasmid pIT511 was constructed by deleting 1.2 kb of 3'-noncoding information from pIT507. pIT507 was cut with XbaI (120 bp downstream of DAOCS/DACS translational stop site) and BamHI, the ends were made blunt with the large fragment of *E. coli* DNA polymerase I, and the blunt ends were ligated together. This operation reconstructed the BamHI site but destroyed the XbaI site.

Figure 3. Sequence of the DAOCS/DACS open reading frame and flanking regions.

Figure 4. Expression of the DAOCS/DACS polypeptide in *E. coli*. E. coli K12 strain JM109 cells (19) were transformed with pIT507 or pIT511 (Figure 2) and grown at 30°C overnight in TY broth containing 10 micrograms/ml tetracycline. The cells were diluted ten fold into fresh broth and grown at 30°C for 1.5 hours. Then the temperature was increased to 42°C and growth was continued for 6.5 hours with vigorous shaking. The cells were collected by centrifugation and resuspended in 0.1 M Tris-HCl, pH6.8, 5% sodium dodecyl sulfate, 25% glycerol, 1 M beta-mercaptoethanol, .005% bromphenol blue and 7 M urea. Samples were heated at 100°C for 5 minutes, and electrophoresed into a denaturing 12.5% acrylamide gel. Lanes 1 and 2: JM109 cells containing the human growth hormone expression vector pCZR336, incubated at 30 degrees C (lane 1) or induced at 42 degrees C (lane 2); Lanes 3 and 4: JM109 cells containing pIT511, grown at 30 degrees C (lane 3) or induced at 42 degrees C (Lane 4); Lane 5, purified *C. acremonium* DAOCS/DACS polypeptide; Lanes 6 and 7: JM109 cells containing pIT507 grown at 30 degrees C (lane 7) or induced at 42 degrees C (lane 6); Lane 8: JM109 cells without any plasmid, grown at 37 degrees C.

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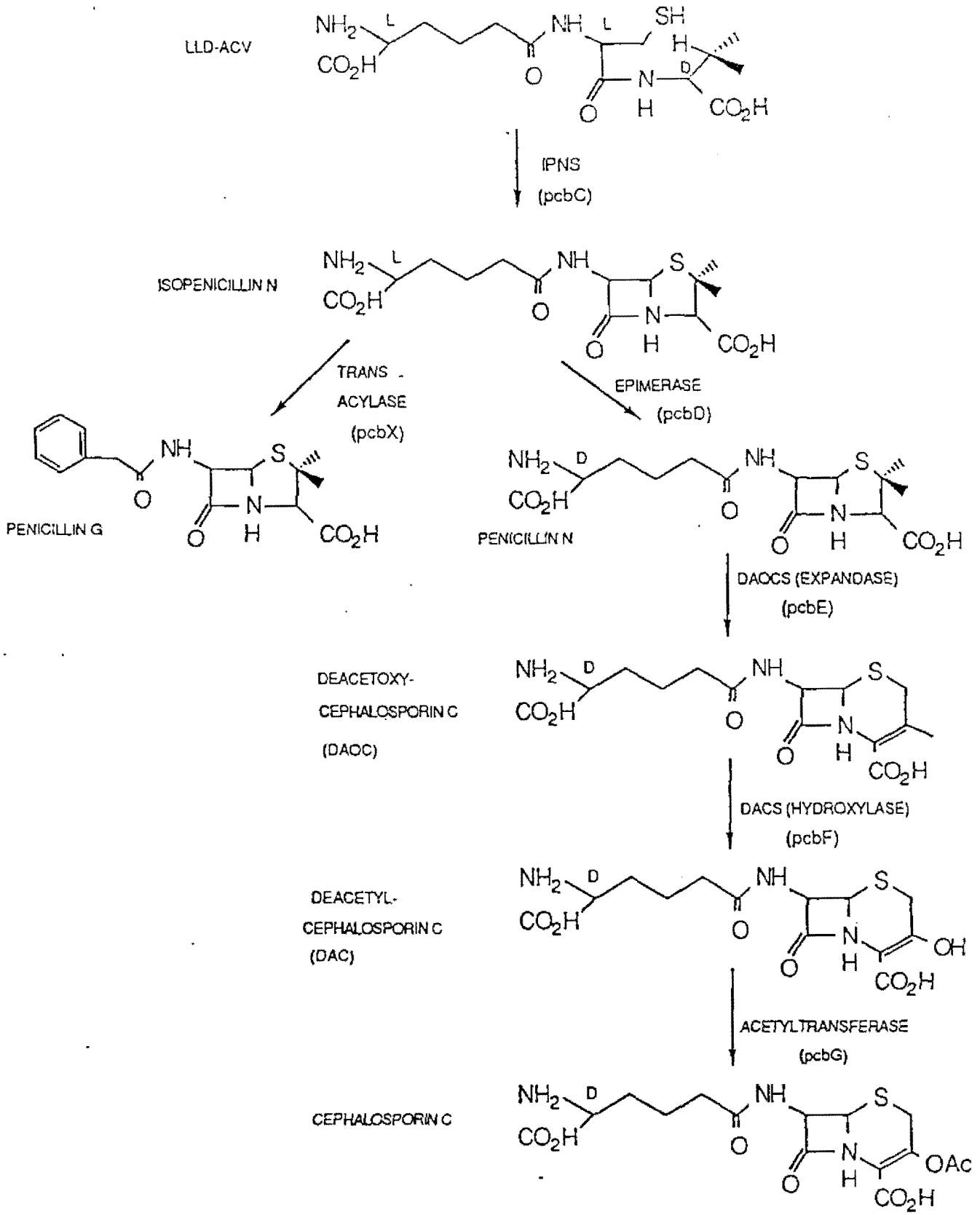
20. We thank Lilly Research Laboratories for support of this work, and R. Belagaje, S. Burgett, R. Chance, B. Glover, H. Hsiung, M. Johnson, J. Miller, R. Schoner, D. Smith, and P. Tackitt for assistance and helpful advice.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IPNS
(pcbC)
ISOPENICILLIN N
TRANS -
ACYLASE
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EPIMERASE
(pcbD)
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PENICILLIN N
DAOCS (EXPANDASE)
(pcbE)
DEACETOXY-
CEPHALOSPORIN C
(DAOC)
DACS (HYDROXYLASE)
(pcbF)
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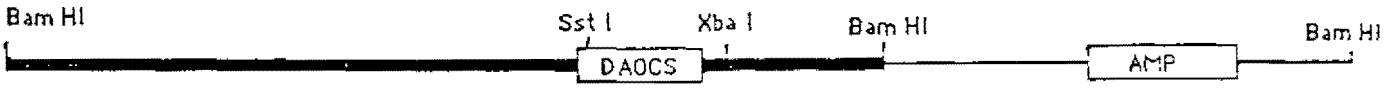
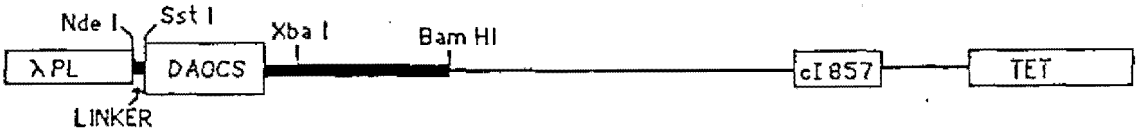
(DAC)
ACETYLTRANSFERASE
(pcbG)
CEPHALOSPORIN C

pIT503



pCZR336

pIT507



pIT511

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                -90                                                -60
  T TTC GTT CTC ACT GGG ATC TTG TGA ATC CTT AAA TTC CTC TTG CAG

                                -30
AAC TTT CCT CCA CGC TAC TCC TCT CAA GTC ATC GCT CAA AAC CAC AGC

                     10          20          30          40
ATC AAC ATG ACT TCC AAG GTC CCC GTC TTT CGT CTC GAC GAC CTC AAG
        MET THR SER LYS VAL PRO VAL PHE ARG LEU ASP ASP LEU LYS
                         5                  10
         50          60          70          80          90
AGC GGC AAG GTC CTC ACC GAG CTC GCC GAG GCC GTC ACC ACC AAG GGT
SER GLY LYS VAL LEU THR GLU LEU ALA GLU ALA VAL THR THR LYS GLY
15                  20                  25                  30
            100         110         120         130
ATC TTC TAC TTG ACC GAG AGC GGC CTG GTC GAC GAC GAC CAC ACC TCG
ILE PHE TYR LEU THR GLU SER GLY LEU VAL ASP ASP ASP HIS THR SER
                35                  40                  45
 140         150         160         170         180
GCG CGT GAG ACG TGC GTT GAC TTT TTC AAG AAC GGA AGC GAG GAG GAG
ALA ARG GLU THR CYS VAL ASP PHE PHE LYS ASN GLY SER GLU GLU GLU
            50                  55                  60
    190         200         210         220         230
AAG AGG GCC GTG ACG CTC GCC GAC CGT AAC GCC CGC CGC GGC TTC TCT
LYS ARG ALA VAL THR LEU ALA ASP ARG ASN ALA ARG ARG GLY PHE SER
        65                  70                  75
      240         250         260         270         280
GCC CTC GAG TGG GAG AGC ACC GCC GTC GTC ACC GAG ACG GGC AAG TAC
ALA LEU GLU TRP GLU SER THR ALA VAL VAL THR GLU THR GLY LYS TYR
    80                  85                  90
         290         300         310         320         330
TCG GAC TAC TCG ACG TGC TAC TCC ATG GGC ATC GGC GGC AAC CTG TTC
SER ASP TYR SER THR CYS TYR SER MET GLY ILE GLY GLY ASN LEU PHE
95                  100                 105                 110
            340         350         360         370
CCG AAC CGG GGC TTC GAG GAC GTC TGG CAG GAC TAC TTC GAC CGC ATG
PRO ASN ARG GLY PHE GLU ASP VAL TRP GLN ASP TYR PHE ASP ARG MET
                115                 120                 125
 380         390         400         410         420
TAC GGC GCA GCC AAG GAT GTC GCG CGC GCC GTT CTC AAC TCT GTG GGC
TYR GLY ALA ALA LYS ASP VAL ALA ARG ALA VAL LEU ASN SER VAL GLY
            130                 135                 140
    430         440         450         460         470
GCC CCG CTC GCC GGG GAG GAC ATT GAT GAC TTC GTC GAG TGC GAT CCC
ALA PRO LEU ALA GLY GLU ASP ILE ASP ASP PHE VAL GLU CYS ASP PRO
        145                 150                 155
      480         490         500         510         520
CTC CTC CGC CTA CGG TAC TTC CCC GAA GTG CCG GAG GAC CGC GTC GCC
LEU LEU ARG LEU ARG TYR PHE PRO GLU VAL PRO GLU ASP ARG VAL ALA
    160                 165                 170
         530         540         550         560         570
GAA GAG GAA CCC CTC CGC ATG GGA CCC CAC TAC GAC CTA TCG ACC ATC
GLU GLU GLU PRO LEU ARG MET GLY PRO HIS TYR ASP LEU SER THR ILE
175                 180                 185                 190
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            580         590         600         610
ACG CTC GTG CAC CAG ACA GCC TGC GCC AAC GGC TTC GTG AGC CTG CAG
THR LEU VAL HIS GLN THR ALA CYS ALA ASN GLY PHE VAL SER LEU GLN
                195                 200                 205
 620         630         640         650         660
TGC GAG GTG GAC GGA GAA TTC GTC GAC CTC CCG ACG CTC CCC GGC GCC
CYS GLU VAL ASP GLY GLU PHE VAL ASP LEU PRO THR LEU PRO GLY ALA
            210                 215                 220
    670         680         690         700         710
ATG GTC GTC TTC TGC GGC GCG GTC GGC ACC CTG GCC ACG GGC GGC AAG
MET VAL VAL PHE CYS GLY ALA VAL GLY THR LEU ALA THR GLY GLY LYS
        225                 230                 235
      720         730         740         750         760
GTC AAG GCG CCC AAG CAC CGG GTC AAG TCT CCC GGG CGC GAC CAG CGC
VAL LYS ALA PRO LYS HIS ARG VAL LYS SER PRO GLY ARG ASP GLN ARG
    240                 245                 250
         770         780         790         800         810
GTC GGC AGC AGC CGC ACG TCG AGC GTC TTC TTC CTG CGG CCG AAG CCC
VAL GLY SER SER ARG THR SER SER VAL PHE PHE LEU ARG PRO LYS PRO
255                 260                 265                 270
            820         830         840         850
GAC TTC AGC TTC AAC GTG CAG CAG TCG AGG GAG TGG GGT TTC AAC GTC
ASP PHE SER PHE ASN VAL GLN GLN SER ARG GLU TRP GLY PHE ASN VAL
                275                 280                 285
 860         870         880         890         900
CGC ATC CCG TCG GAG CGC ACG ACG TTC AGG GAG TGG CTT GGC GGG AAC
ARG ILE PRO SER GLU ARG THR THR PHE ARG GLU TRP LEU GLY GLY ASN
            290                 295                 300
    910         920         930         940         950
TAT GTC AAC ATG CGG AGG GAT AAG CCG GCG GCA GCG GAG GCG GCT GTC
TYR VAL ASN MET ARG ARG ASP LYS PRO ALA ALA ALA GLU ALA ALA VAL
        305                 310                 315
      960         970         980         990         1000
CCC GCG GCT GCC CCT GTC TCT ACC GCA GCT CCT ATA GCC ACT TAG GGA
PRO ALA ALA ALA PRO VAL SER THR ALA ALA PRO ILE ALA THR ???
    320                 325                 330
        1010        1020        1030        1040        1050
ACC CGC CGA TCG AGT AAT AAA TCT ACG GGA GTT TAA GAA GAA AAA TTG

            1060        1070        1080        1090
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 1100        1110
TTT CAA GTT TCA A
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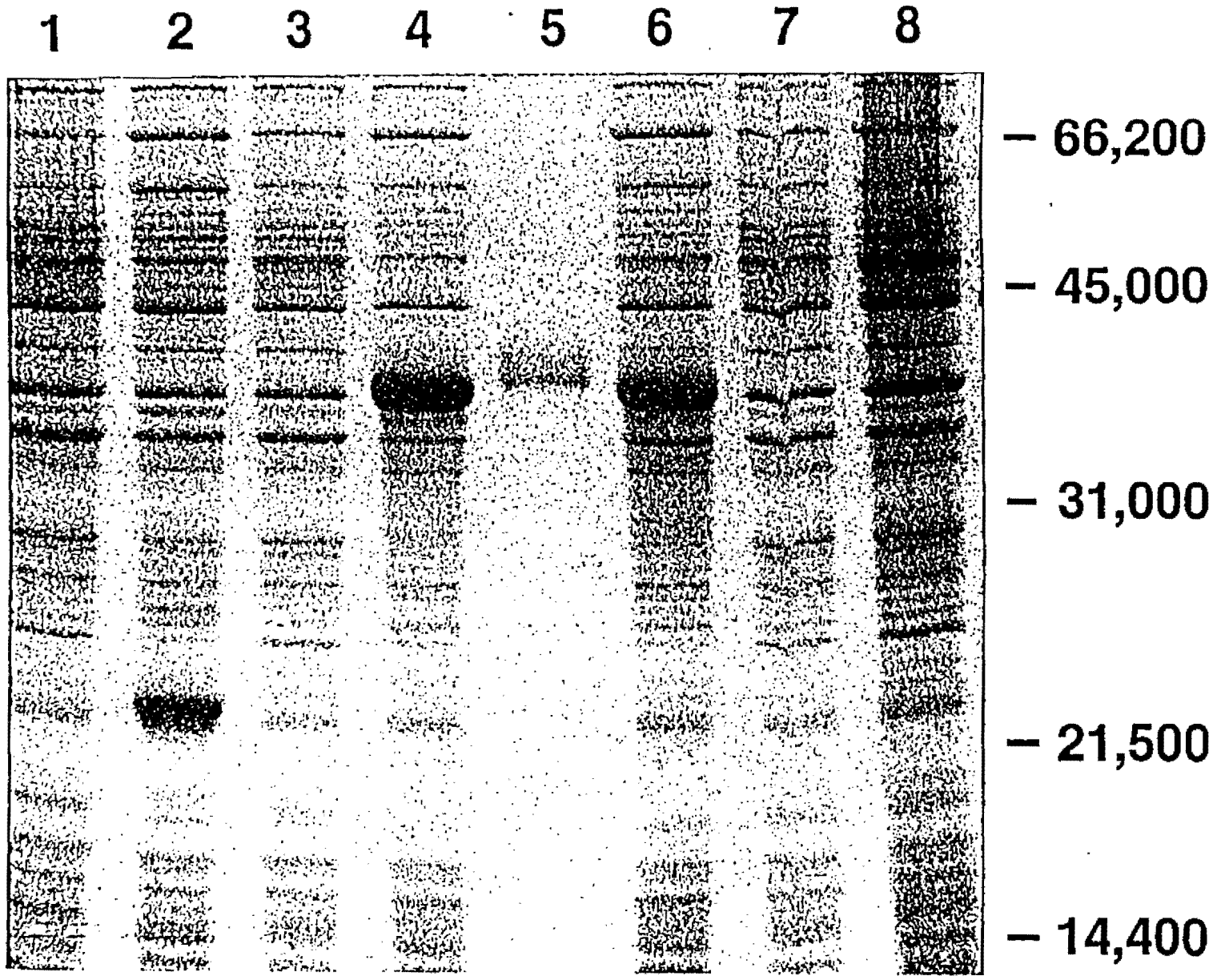
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7
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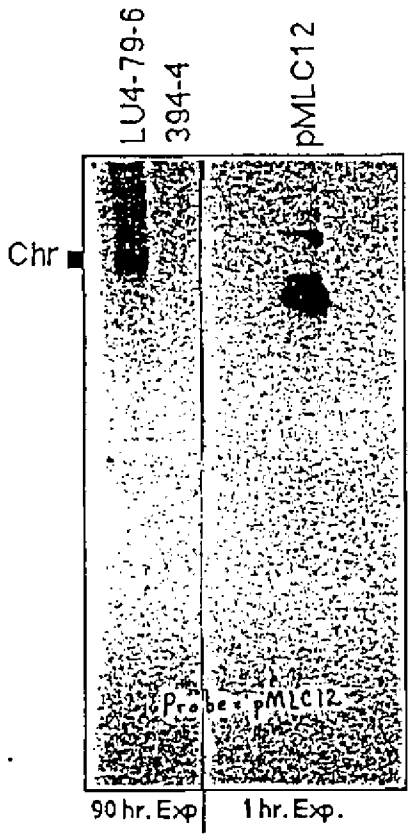

Transforming DNA is present in the high molecular weight DNA of hygromycin B resistant *C. acremonium* transformant LU4-79-6. Total DNA was extracted from LU4-79-6 and from the hygromycin B sensitive untransformed parent strain, 394-4. These DNAs and control DNAs (lamda DNA digested with HindIII and plasmid pMLC12) were each subjected to agarose gel electrophoresis. Agarose gels containing separated DNAs were analyzed by Southern hybridization with pMLC12 as the radioactive probe. The position corresponding to chromosomal DNA [detected by ehidium staining prior to Southern procedure] is marked ("chr"). pMLC12 contains no *C. acremonium* DNA but elements of pMLC12 are present in pPS56. Plasmid pPS56 was used to transform strain 394-4 to obtain strain LU4-79-6.

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17. Recombinant DNA studies in *Cephalosporium acremonium*

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The fungus *Cephalosporium acremonium* has been used for many years to produce the β-lactam antibiotic cephalosporin C, which is chemically modified to produce a number of clinically important parenteral cephalosporin antibiotics [6]. Until recently, all genetic manipulations performed on industrial strains of *C. acremonium* have been classical or random in nature, including mutation/screening and mutation/selection programs [16] and protoplast fusion, performed mostly for the purpose of improving cephalosporin C yield [5]. Another approach to strain improvement would require the application of recombinant DNA technology to *C. acremonium*. Recombinant techniques could be used to construct strains with multiple copies of antibiotic biosynthetic genes. Here we review the development of a system for the genetic transformation of *C. acremonium* [14] and the first cloning of a biosynthetic gene in the cephalosporin C pathway [18]. We also review the construction of vectors that contain the IPS gene and a hybrid gene that employs the IPS promoter, and transformation of *C. acremonium* with those vectors (Skatrud et al., manuscript in preparation).

To apply recombinant DNA methods to *C. acremonium*, we first constructed the plasmid pIT221 (Fig. 1). The following genetic elements were included in this construction to make it a useful vector for cloning DNA into *C. acremonium*:

(i) a 2.8 kilobase (kb) *Pst*I-*Eco*RI fragment that contains the origin of replication and ampicillin resistance gene from pBR322 to allow transformation and selection in *Escherichia coli*.

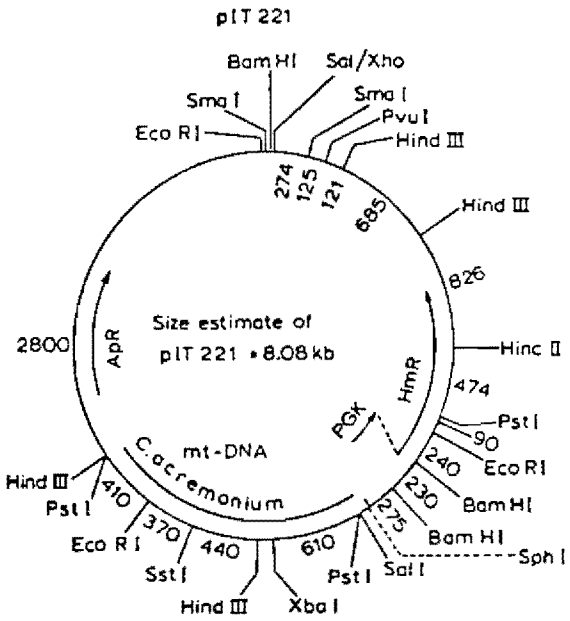

Fig. 1. Restriction map of plasmid vector pIT221. See text for the description of genetic elements and cloning sites in this plasmid. Distances (in base pairs) between restriction sites are indicated by number adjacent to the circle. The unique *Sph*I site, position indicated by dotted line, is approximately 90 bp from the *Sal*I site and approximately 175 bp from the *Bam*HI in the 275 bp *Sal*I-*Bam*HI-defined sequence immediately before the PGK promotor.

* Speaker at the Annual Meeting.
** To whom correspondence should be addressed.

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(ii) a 1.9 kb *Pst*I fragment of mitochondrial DNA that contains an autonomous replication sequence (ars) from *C. acremonium* to increase the transformation frequency in *C. acremonium*. This fragment was isolated in our laboratory and was confirmed as an ars in *Saccharomyces cerevisiae* by inserting the mitochondrial fragment into YIp5, a yeast integrative plasmid. The resulting plasmid, pPS1, replicates autonomously in *S. cerevisiae* [22]. Thus, by definition [24], the fragment is an ars. Tudzynski and Esser [25] had suggested that a similar 1.9 kb *Pst*I fragment of *C. acremonium* mitochondrial DNA (isolated from a different strain) might contain a *C. acremonium* origin of replication.

(iii) a hybrid gene, known to be dominant and selectable in yeast [8] that was constructed by splicing a promotor from the 5′ end of the yeast phosphoglycerate kinase (PGK) gene in correct translational phase to the protein-coding region of a hygromycin B phosphotransferase (HPT) gene isolated from a natural plasmid pKC203 found in *E. coli* W677(JR225) [17]. HPT phosphorylates the broad-spectrum aminocyclitol antibiotic hygromycin B [17]. Hygromycin B is toxic to *C. acremonium* at levels as low as 10 μg per ml, in the absence of divalent cation antagonists, whereas the phosphorylated product is not toxic to *C. acremonium* at concentrations as high as 1 mg/ml [14]. At the time of the construction of pIT221, there was no cloned *C. acremonium* gene promoter available.

(iv) two non-essential, non-functional fragments of bacterial DNA that contain a *Sph*I site suitable for introducing cloned DNA and a 275 bp *Sma*I fragment that can be replaced by cloned DNA. DNA has been introduced by insertion at the *Sph*I site and by replacement of the *Sma*I fragment without loss of the ability of the plasmid to transform *E. coli* (selection for ampicillin resistance) and without loss of the ability of the plasmid to transform *S. cerevisiae* and *C. acremonium* (selections for hygromycin B resistance).

Before attempting to transform *C. acremonium*, we tested the biological activities of the *C. acremonium* ars sequence and the hybrid HPT gene in pIT221 by transforming *S. cerevisiae* with pIT221. The recipient yeast strain used was DBY746, which is hygromycin B sensitive ($HgmB^s$) and contains no pBR322-hybridizing DNA sequences. Isolation of hygromycin B resistant ($HgmB^r$) yeast transformants such as pIT221-YT suggested that the transforming plasmid carried a functional HPT gene. Southern hybridization of uncut DNA from the yeast transformant, pIT221-YT, and the untransformed recipient strain using pBR322 as a probe clearly showed the presence of covalently closed circular and open circle forms of pIT221 in the transformant and confirmed the expected absence of pBR322 sequences in DNA of the untransformed recipient strain. $HgmB^s$ segregants were observed to arise spontaneously at high frequency from the transformant, pIT221-YT, as expected from the loss of the autonomously replicating pIT221 plasmid by segregation.

Having confirmed the biological activities of the vector in yeast, we systematically examined various parameters to discover conditions that would allow pIT221 to transform *C. acremonium*. The following conditions allowed transformation: cells growing exponentially in an enriched minimal medium were harvested, washed and treated with dithiothreitol. Cell walls were removed by exposure to β-glucuronidase in 0.8 M NaCl and 0.02 M $MgSO_4$. Spheroplasts produced were washed, suspended in 0.8 M NaCl, and then exposed to vector DNA, e.g., plasmid pIT221, as outlined in Table 1. Following the

Table 1

Conditions for transformation: exposure of DNA to *C. acremonium* spheroplasts

A. Reaction mixture:
1. 2–3 · 10^8 spheroplasts in 1.0 ml of 0.8 M NaCl.
2. Add 0.005 ml freshly distilled dimethylsulfoxide.
3. Add $CaCl_2$ to concentration of 80 mM.
4. Add 20 μg vector DNA (e.g., pIT221).
5. Add poly(ethylene glycol) (4000 Baker: 20% w/v in 50 mM solution of $CaCl_2$) to volume of 10 ml.

B. Incubation:
1. Time = 10 min.
2. Temperature = 25°C.
3. Harvest: centrifuge and resuspend in 3.0 ml of 0.8 M NaCl.

transformation procedure, aliquots of washed spheroplasts resuspended in 0.8 M NaCl were plated on trypticase-soy agar osmotically stabilized with 10.3% sucrose. Spheroplasts were then incubated at 15°C for 24 h to allow cell wall regeneration. A 4 ml overlay containing sufficient hygromycin B to give a plate concentration of 100 μg/ml was added. Plates were then incubated at 25°C to allow HgmB^r transformants to form visible colonies.

HgmB^r colonies obtained at low frequency using these conditions were analyzed in plating experiments for their ability to survive upon subculture to fresh selective medium, and for their level of resistance to HgmB. Only colonies which could be subcultured to fresh selective medium were scored as transformants. In 14 experiments using these conditions, a total of 147 HgmB^r, pIT221-*C. acremonium* transformants, with various levels of resistance to HgmB, were isolated. In all experiments, parallel transformation controls were run in which the vector was omitted from the transforming mixture; no colonies were observed on these plates. Thus, HgmB selection killed all untransformed cells.

To demonstrate the association of the hygromycin B resistance phenotype with transforming DNA, three *C. acremonium* colonies exhibiting various levels of resistance to HgmB were selected for DNA analysis. Total DNA from each of the three transformants was isolated by grinding mycelium manually under liquid nitrogen [10], and each sample of total undigested DNA was analyzed by the Southern hybridization procedure [23] using ^{32}P-labeled pBR322 DNA as probe. Each analysis demonstrated the presence of pBR322 sequences in the region of the gel corresponding to the location of high molecular weight *C. acremonium* DNA, and no hybridization was noted in the region of the gel that would correspond to either covalently closed or open circle forms of pIT221 (Fig. 2). These results indicated that transformation occurred by integration of the transforming DNA into high molecular weight *C. acremonium* DNA. As expected, no hybridization was observed to DNA from the untransformed recipient strain, ATCC 11550.

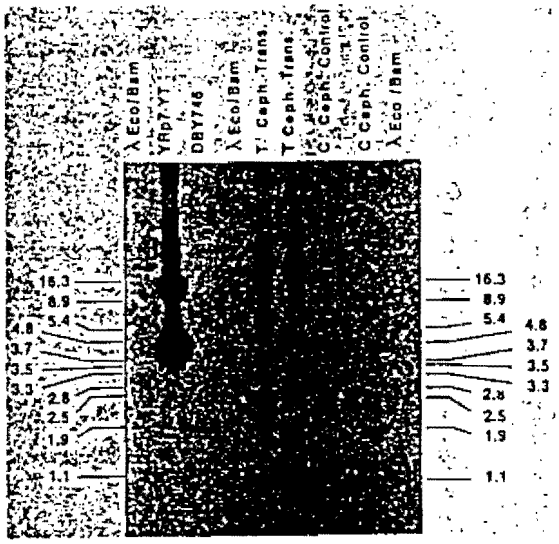

Fig. 2. pIT221 DNA is present as high-molecular-weight DNA in the total DNA from HgmB^r pIT221-*C. acremonium* transformant T1. Total DNA was extracted from T1 and from the untransformed HgmB^s *C. acremonium* recipient strain, ATCC 11550. DNA molecules in each extract were separated by agarose gel electrophoresis and probed [23] with ^{32}P-labeled pBR322. As a positive control, total DNA was extracted from a yeast transformant, YRp7-DBY746, known to carry a freely replicating plasmid (YRp7) composed, in part, of pBR322 sequences. As a negative control, total DNA was extracted from the untransformed recipient yeast strain, DBY746, which is known not to contain any DNA from pBR322. Source of DNA (3 μg, each lane) is as follows: lanes 1, 4 and 9, lambda (DNA applied after digestion with *Eco*R1 and *Bam*H1); lane 2, YRp7-DBY746; lane 3, DBY746; lanes 5 and 6, HgmB^r pIT221-*C. acremonium* transformant T1; lanes 7 and 8, HgmB^s *C. acremonium* recipient strain, ATCC 11550. Specific activity of ^{32}P-labeled pBR322 is $3.9 \cdot 10^8$ cpm/μg.

To characterize further the nature of the transformants, total DNA from the representative transformants was digested to completion with a variety of restriction enzymes that cut pIT221 either once or twice. Southern hybridization analysis using ^{32}P-labeled pBR322 as a probe demonstrated multiple hybridization bands. This suggested several integrations of DNA from the vector were present in the *C. acremonium* DNA. For example, when total DNA from transformant T1 was cut with *Kpn*I, which does not cut pIT221, two hybridization bands were observed, when a single insertion of

pIT221 DNA would have produced only 1 band. One of the two *Kpn*I fragments that hybridized to ^{32}P-pBR322 had a size of approximately 10 kb, consistent with *Kpn*I sites in *C. acremonium* DNA just outside a single insert of pIT221. The second of the two *Kpn*I fragments that hybridized to ^{32}P-pBR322 was not resolved from high molecular weight DNA and was more intense than the smaller band, suggesting a very large fragment containing more than one insert of pIT221. When the total DNA from the same transformant was cut with *Sac*I, which cuts pIT221 twice, eight bands of hybridization were observed. These results suggest as many as four separate integrations of pIT221 DNA are present in the T1 transformant.

To insure that transformant T1 was a pure isolate, a single spore of T1 was isolated by micromanipulation. The isolated spore was cultured, and total DNA was isolated. Samples of total DNA digested completely with either *Kpn*I or *Sac*I were analyzed by Southern hybridization as before. The bands of DNA that hybridized to ^{32}P-labeled pBR322 appeared identical to those observed in the experiment with the original T1 colony. As before, no hybridization to DNA from the untransformed recipient strain ATCC 11550 was observed.

Next, hybridization experiments were carried out to confirm the presence of an HPT gene derived from pIT221 in the DNA of HgmB^r *C. acremonium* transformants. Plasmid pJC10 [14] contains the natural HPT gene used to construct the hybrid HPT gene in pIT221; natural plasmid pKC203 [17] is the original source of the open-reading frame used to construct the hybrid HPT gene in pIT221. pJC10 was formed by circularizing the largest of the three *Bgl*II fragments that comprise plasmid pKC203. ^{32}P-labeled pJC10 did not hybridize to either pBR322 or total DNA from the untransformed wild type *C. acremonium* strain, ATCC 11550. When used as a probe against restriction digests of pIT221, ^{32}P-labeled pJC10 DNA hybridized only to fragments containing DNA comprising the protein-coding region of the hybrid HPT gene. Analysis of the structure of hybridizing and non-hybridizing fragments showed that the protein-coding region of the hybrid HPT gene was the sole basis for the hybridization of pJC10 to pIT221 (Fig. 3). Thus, pJC10 served as a specific and convenient probe for detecting a pIT221-derived HPT gene in the total DNA of each HgmB^r *C. acremonium* transformant. Used in this way, plasmid pJC10 would detect the presence of DNA derived originally from the open-reading frame of the HPT gene of pKC203. When total DNA from the T1 transformant was probed with ^{32}P-labeled pJC10, hybridization to the high molecular weight DNA of the transformant was observed.

For additional confirmation of transformation, a cell-free extract of T1 was assayed for the presence of the HPT enzyme. In the assay, HPT catalyzes the transfer of a [^{32}P]PO_4 moiety from [γ-^{32}P]ATP to form hygromycin B-[^{32}P]PO_4. HgmB-PO_4 binds to a phosphocellulose disc, but, under the conditions employed, HgmB and ATP do not. Radioactivity remaining on the phosphocellulose disc after washing is counted. HPT activity was present in the extract of transformant T1; the activity increased linearly with extract concentration, and the activity was lost upon boiling the cell-free extract (Fig. 4). HPT activity was absent in the extract from the untransformed *C. acremonium* recipient ATCC 11550: the amount of phosphocellulose-bound ^{32}P observed in assays of this extract was equivalent to the small amount of phosphocellulose-bound ^{32}P observed when reaction mixtures contained extract of transformant T1 but no substrate (Fig. 5). Rao et al. [17] previously demonstrated that extracts of *E. coli* K-12 bearing the natural HPT gene efficiently phosphorylated hygromycin B but did not phosphorylate the aminocyclitol antibiotic G418. Therefore, G418 was tested as a substrate for HPT in the extract from the *C. acremonium* transformant T1. The extract from the *C. acremonium* transformant T1 did not catalyze phosphorylation of G418 (Fig. 5).

To characterize further the nature of the transformants, the stability of the HgmB^r phenotype in several representative transformants was tested. Integration of the transforming DNA into the genome of a cell would be expected to produce a stable transformant that would retain a HgmB^r phenotype even after passage on non-selective medium. This

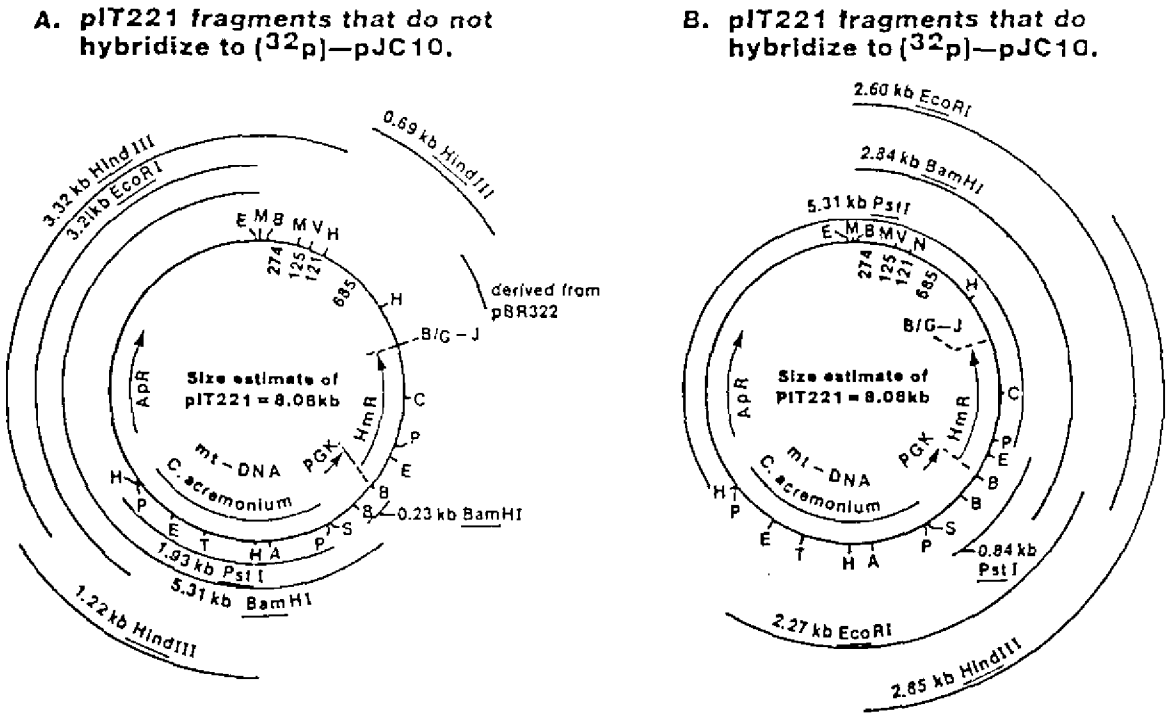

Fig. 3. The HPT DNA sequence in pIT221 is the basis of hybridization of ^{32}P-labeled pJC10. Total *C. acremonium* DNA, pBR322 DNA, and pIT221 DNA were probed [23] with ^{32}P-pJC10. Hybridization occurred only with pIT221. Four pIT221 DNA samples were then digested separately with *Pst*I, *Bam*HI, *Eco*RI, and *Hind*III. The DNA fragments in each digest were separated by agarose gel electrophoresis. The separated DNA fragments were identified by staining with ethidium bromide and were subsequently probed with ^{32}P-pJC10. Seven fragments from these digests did not hybridize to the ^{32}P-pJC10 (see panel A), and six fragments did hybridize to the ^{32}P-pJC10 (see panel B). *The fragments that did not hybridize comprised all of the non-HPT DNA sequences plus a small region* derived from pBR322. Hence all the non-HPT DNA did not have the capacity to hybridize to the probe. The only DNA common to all six fragments that hybridized to ^{32}P-pJC10 comprised the HPT DNA sequence and the same small region derived from pBR322. Thus, the basis of hybridization of ^{32}P-pJC10 to pIT221 is the HPT sequence which exists in the known structures of pIT221 and pJC10. Abbreviations: E, *Eco*RI; M, *Sma*I; B, *Bam*HI; V, *Pvu*II; H, *Hind*III; G, *Bgl*II; C, *Hinc*II; B/G-J, *Bam*HI-*Bgl*II junction.

stability was, in fact, observed. For example, transformant T1 was grown on non-selective agar for 25 generations; the resulting population of cells was diluted, and approximately 25–100 cells were spread onto two different agar media. The base ingredients in each medium were identical, but one medium contained a supplement of 100 μg/ml HgmB. Within experimental error, the number of colonies (21 and 101 in two separate experiments) that arose on the selective medium was equivalent to the number (21 and 106 in two separate experiments) that arose on the non-selective medium. Recipient strain ATCC 11550 was also grown on non-selective agar for 25 generations, and the resulting population of cells were analyzed on the two different agar media; no colonies of strain ATCC 11550 were observed on the selective medium seeded with approximately $4 \cdot 10^5$ cells of that strain. A total of 418 colonies were observed on a plate of non-selective agar medium seeded with ca. $4 \cdot 10^2$ cells.

The high stability of HgmB^r phenotype of transformant T1 was also observed following growth in a non-selective liquid medium. T1 was used as seed in a standard cephalosporin C fermentation [16] employing complex medium. At the end of the fermentation, aliquots of harvested cells were again plated on medium with and without HgmB. Again,

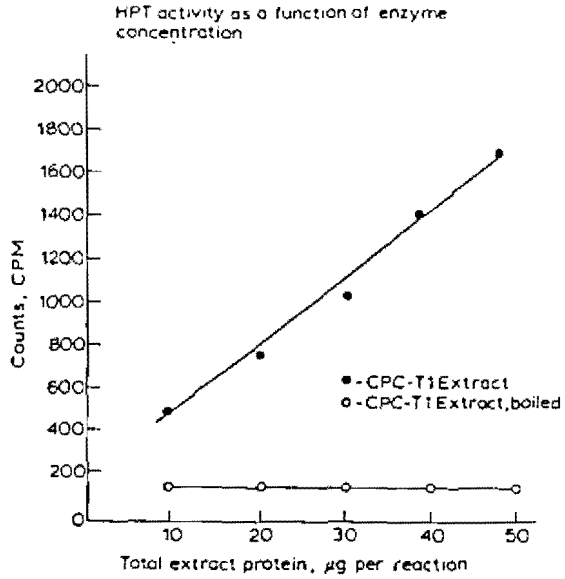

Fig. 4. Hygromycin phosphotransferase (HPT) activity is present in extracts of *C. acremonium* transformant T1 and the activity is sensitive to heat and is linear with the amount of extract added to the assay reaction mixture. Preparation of extracts was as follows: 96 h cells from complex, CF-5 medium [17] were washed twice with 10 mM Tris (pH 8.0) and washed cells were resuspended in same buffer supplemented with 0.5 mM $MgCl_2$ and 0.1 mM dithiothreitol (DTT). Resuspended cells were broken by one passage through a French Pressure Cell (1 inch piston, 20000 lb/in^2 4°C). The ruptured-cell mixture was centrifuged twice (Sorvall RC-5B: SS34 rotor, 16000 rpm, 30 min, 4°C and Beckman L3-50: type 675 rotor, 50000 rpm, 2 h, 4°C). The assay for HPT activity was that described by Rao et al. [17]: HPT catalyzes the transfer of a [^{32}P]PO_4 moiety from [γ-^{32}P]ATP to hygromycin B to form hygromycin B-[^{32}P]-phosphate. Hygromycin B-phosphate binds to a phosphocellulose disc, but hygromycin B and ATP do not under the conditions employed. Radioactivity remaining on a phosphocellulose disc after washing is counted. Various amounts of untreated (●) or boiled (○) cell-free extract protein (10, 20, 30, 40, 50 μg) were added to the assay reaction mixture to start the reaction. The reaction is stopped after 15 min by heating at 80–90°C for 5 min.

within experimental error, the number of colonies that arose on selective medium was equivalent to the number that arose on non-selective medium. An aliquot of cells obtained from a parallel control fermentation seeded with the untransformed ATCC 11550 recipient did not produce colonies when plated on medium containing HgmB, but an identical aliquot plated on medium without HgmB produced a confluent lawn of growth.

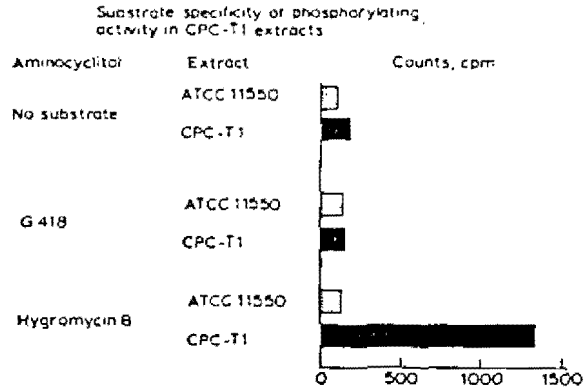

Fig. 5. Hygromycin phosphotransferase (HPT) is present in extracts of *C. acremonium* transformant T1, and the enzyme cannot utilize the aminocyclitol antibiotic, G418, as a substrate; there is no HPT activity in extracts of untransformed *C. acremonium* recipient strain ATCC 11550. Each reaction contained the following base ingredients: 40 μg of cell-extract protein, [γ-^{32}P]ATP (2 mM with respect to ATP, and specific activity of 10 μCi/mmol), 13 mM Tris (pH 8.0), 8 mM $MgCl_2$, 80 mM NH_4Cl, 2 mM DTT. Complete reaction mixtures used to compare the amount of HPT activity in the two extracts contained 4 mM hygromycin B in addition to the base ingredients indicated above. Reaction mixtures used to test the substrate specificity of the HPT in extracts contained 4 mM G418 in addition to the base ingredients. Reaction mixtures used to determine background radioactivity contained base ingredients but no hygromycin B or G418.

Having developed a system for introducing, maintaining, and expressing exogenously added DNA in *C. acremonium*, we shifted effort to identification and isolation of a *C. acremonium* gene involved directly in cephalosporin C biosynthesis. By isolating biosynthetic genes and reinserting several copies of these genes back into the genome of *C. acremonium*, one might hope to acheive a favorable gene-dosage effect that would increase cephalosporin C production. The gene for isopenicillin N synthetase was targeted for this purpose. Isopenicillin N synthetase (IPS), a key enzyme in the cephalosporin C biosynthetic pathway, had been isolated [7,13], and the enzyme has been studied in some detail (e.g., Refs. 1, 3, 4, 11 and 15).

The conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) to isopenicillin N by IPS of *C. acremonium* occurs in the presence of oxygen,

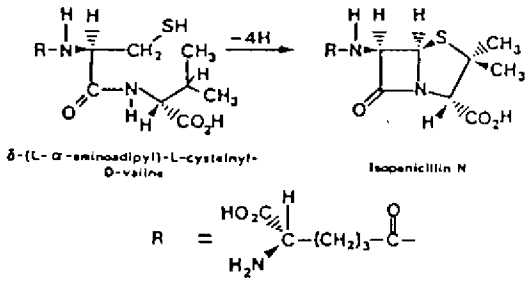

Fig. 6. Isopenicillin N synthetase catalyzes the conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, which has no antibacterial activity, to form isopenicillin N, which has significant antibacterial activity.

Fe^{2+}, ascorbate, and thiol (such as dithiothreitol) [2,9,11,12,19]. In the process, four hydrogens from LLD-ACV are removed and one dioxygen is consumed [26], resulting in the formation of the two rings found in isopenicillin N (Fig. 6).

The cloning of the gene for *C. acremonium* IPS was accomplished by purifying IPS from *C. acremonium*, determining its N-terminal sequence, constructing 17-mer oligonucleotide probes that included the coding information for amino acids 18–23 (Fig. 7), screening a cosmid library of *C. acremonium* DNA with the oligonucleotide probes, and mapping and sequencing the cosmid region that hybridized to the probes. The open reading frame containing the hybridizing region had a predicted amino acid sequence that matched the experimentally determined sequence. When this open reading frame was inserted into an *E. coli* expression vector and transformed into *E. coli*, it allowed the production of active IPS in *E. coli* (Fig. 8). The cloning and sequencing of the *C. acremonium* IPS gene, and the expressing of that *C. acremonium* gene in *E. coli* has been previously described in detail [18].

a. Experimentally determined amino acid sequence

? - VAL - PRO - VAL - PRO - VAL - ALA - ASN - VAL - PRO - ARG - ILE -
ASP - VAL - SER - PRO - LEU - PHE - GLY - ASP - ASP - LYS - GLU -

b. Possible codons

5' UU$^{U}_{C}$ GG$^{A}_{G}$ GA$^{U}_{C}$ GA$^{U}_{C}$ AA$^{A}_{G}$ GA$^{A}_{C}$ 3'

c. DNA probes

3' AA$^{A}_{G}$ CC$^{T}_{C}$ CT$^{A}_{G}$ CT$^{A}_{G}$ TT$^{T}_{C}$ CT 5'

3' AA$^{A}_{G}$ CC$^{A}_{G}$ CT$^{A}_{G}$ CT$^{A}_{G}$ TT$^{T}_{C}$ CT 5'

d. Experimentally determined DNA sequence and predicted amino acid sequence

5' TTC GGC GAT GAC AAG GAG 3'
PHE GLY ASP ASP LYS GLU

Fig. 7. Amino acid residues 18–23 in the N-terminus of IPS from *C. acremonium* were suitable for design of oligonucleotides which served as a probe for screening a cosmid bank of *C. acremonium* DNA and allowed location of the IPS gene in cosmid pIT328. (a) The first 23 amino-terminal amino acid residues of IPS were determined and are shown above. The question mark indicates that the first cycle of the sequenator run was ambiguous. The underlined sequence at amino acids 18–23 represents the region encoded by the synthetic oligonucleotides. (b) The possible RNA codons for the underlined sequence (in part a above) are given. Since the third position of each codon is variable in this region, all possibilities are shown. (c) Two sets of 17-mer oligonucleotides comprising the possibilities (shown in part b above) were synthesized. Each pool had a complexity of 32. (d) The experimentally derived DNA sequence that hybridized to the oligonucleotide probe and the corresponding predicted amino acid sequence are shown. For the complete DNA sequence and the predicted amino acid sequence of the open reading frame located by the oligonucleotide probe, see Samson et al. [18].

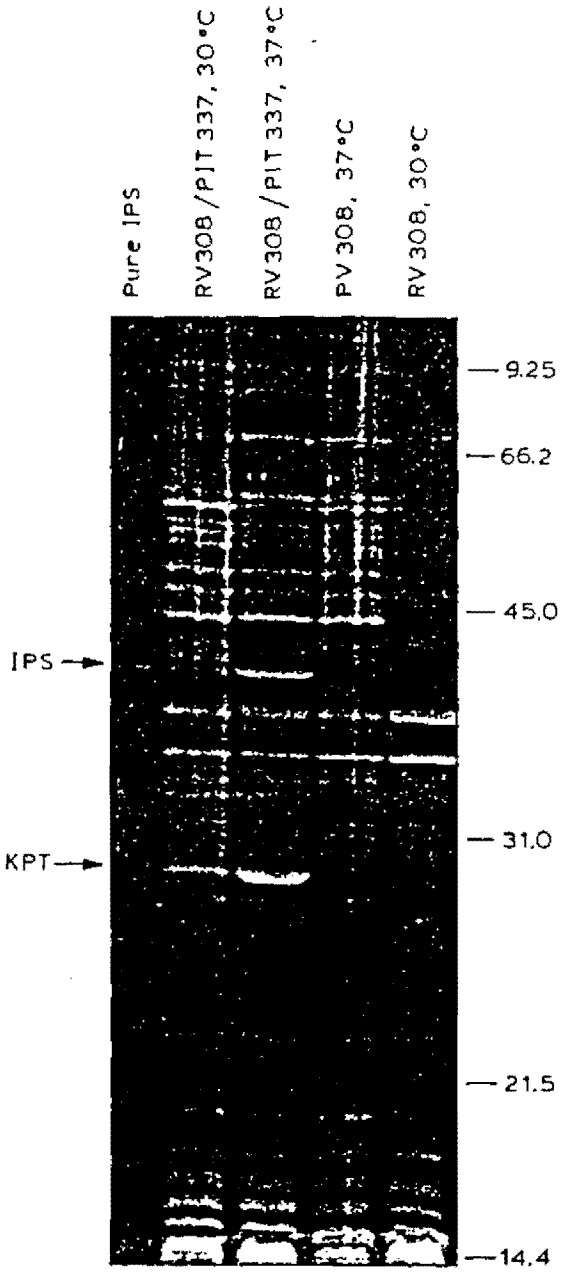

Fig. 8. 12.5% SDS protein gel shows IPS protein in *E. coli* cells transformed with pIT337. Total cell protein from *E. coli* RV308/pIT337 cells prior to amplification (30°C) and after amplification (37°C) are shown along with control RV308 extracts. Purified IPS from *C. acremonium* (IPS) and the kanamycin phosphotransferase (KPT) are indicated by arrows, as are the positions of molecular weight markers. Extracts of *E. coli* RV308/pIT337 cells catalyzed the conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N but extracts of RV308 containing a plasmid that differed from pIT337 by substitution of a bovine growth hormone gene in place of the IPS gene did not convert the tripeptide to isopenicillin N (bioassay of Shen et al. [21]).

The successful development of a transformation system that can produce *C. acremonium* transformants with stable multiple inserts of transforming DNA and the successful cloning of the *C. acremonium* IPS gene made possible the construction of strains of *C. acremonium* that contain multiple copies of IPS. To produce vectors capable of inserting multiple copies of the IPS gene into *C. acremonium*, the IPS gene has been placed in derivatives of vector pIT221. Some of these plasmids, e.g., pPS26, were modified to contain a hybrid gene made by splicing the promotor region of the *C. acremonium* IPS gene to the open reading frame of the bacterial hygromycin phosphotransferase gene. These vectors were compared to vectors containing a hybrid gene constructed by splicing the promotor from the yeast phosphoglycerate kinase gene to the bacterial HPT open reading frame. All other genetic elements on the compared plasmids were equivalent. The former plasmids transform *C. acremonium* at ca. 100-fold greater frequency (Skatrud et al., manuscript in preparation).

C. acremonium mutant N2 is deficient in the conversion of LLD-ACV to isopenicillin N as judged by its inability to form β-lactam antibiotics on agar medium and by its excretion of LLD-ACV in liquid medium [20]. We have observed the absence of isopenicillin N synthetase activity in an extract of N2 and – in the same experiment for comparison – the presence of IPS activity in an extract of the wild type strain ATCC 11550 (Note: when strains were propagated on agar medium in Petri plates, we observed no β-lactam antibiotics for N2; we observed only a penicillinase-sensitive antibiotic for the parent of N2, strain C-52 [a higher producing (cephalosporin C) derivative of ATCC 11550], and we observed a larger amount of penicillinase-sensitive antibiotic and a small amount of penicillinase-resistant antibiotic for strain 394-4 [a very high producing (cephalosporin C) strain]. When strains N2 and C-52 were propagated in liquid complex medium under conditions of high aeration, we observed small amounts of penicillin N and cephalosporin C for N2 and much larger amounts of these antibiotics for C-52. Thus, the conditions of growth for colonies on the agar medium appeared to support only

poor conversion of penicillins to cephalosporins and the completeness of the block in N2 appeared to depend on the method of propagating N2.)

Plasmid pPS26 has been used to transform N2 to hygromycin B resistance. Eleven hygromycin B-resistant, pPS26-transformants of strain N2 were chosen for analysis of antibiotic activity. When grown on agar medium, ten of the eleven transformants produced an antibiotic (bioassay with *Micrococcus luteus*) that was destroyed by penicillinase, but N2 did not produce detectable amounts of any antibiotic active against *M. luteus*. When grown in liquid complex medium under conditions of high aeration (conditions capable of supporting efficient cephalosporin C synthesis), two of the ten penicillin$^+$ isolates produced amounts of cephalosporin C which were clearly superior to the other isolates and N2; the amounts of cephalosporin C approached (80%) that observed for strain C-52. Hybridization analysis with an IPS gene probe indicated the presence of transforming DNA inserted in the genome of both of these isolates. The penicillin$^+$/HgmB^r phenotype exhibited by these two transformants on agar medium was probably due to a stable insertion of the transforming DNA into the genome of the recipient strain N2 (phenotype on agar medium: penicillin$^-$/HgmB^s). Likewise, the cephalosporin C titres of these two transformants propagated in liquid complex medium with high aeration suggested that the IPS gene present in the transforming DNA was functioning in these two HgmB^r *C. acremonium* transformants.

To investigate further the effects of inserting multiple copies of the IPS gene (and other DNA present in our vectors) into the *C. acremonium* genome, plasmid pPS26 was used to transform wild type *C. acremonium* (ATCC 11550) to hygromycin B resistance. The cephalosporin C titres of 100 HgmB^r transformants were compared to the titres of 100 natural selectants. Some transformants exhibited titres significantly higher than ATCC 11550. None of the transformants had titres superior to the best titres obtained with natural selectants of ATCC 11550. We have produced and analyzed 60 pPS26-transformants of a high-producing strain, 394-4, and – on preliminary analysis – some of these transformants appear superior to 394-4. An equal number of natural selectants of strain 394-4 will be selected and evaluated for comparison. It will probably be necessary to produce and screen a relatively large number of HgmB^r transformants to find a strain that carries multiple inserts of the IPS gene all positioned so that there are no deleterious mutations in the transformant genome.

If the amount of isopenicillin N synthetase is rate limiting in the biosynthesis of cephalosporin C in *C. acremonium* strain ATCC 11550 or strain 394-4 and if multiple copies of the IPS gene allow synthesis of more IPS in transformants of those strains, then improved antibiotic formation should be observable in transformants, provided that a sufficiently large number of transformants is screened to discard those with DNA inserts that produce deleterious mutations. If another biosynthetic step is rate limiting then the gene corresponding to that step must be cloned and introduced into *C. acremonium* in multiple copy to construct the desired improved strains.

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Extracts of *C. acremonium* strains LU4-79-6 (transformed with pPS56) and strain 394-4 (control: untransformed production strain) do not exhibit chloramphenicol acetyl transferase activity.

Method: 88 microliters of extract from the control (strain 394-4) or the transformant (strain LU4-79-6) were mixed with 10 microliters of fresh acetylCoA and 2 microliters of ^{14}C-Chloramphenicol (Spec. act 47.3 mmol/mCi) [2 microcuries per reaction]. Reactions were incubated at 37 degrees C for 90 minutes, extracted twice with 100 microliters of ethyl acetate, dried down and reconstituted in 15 ml ethyl acetate. Reconstituted samples were spotted onto silica TLC plates and run in chloroform: methanol (95:5 ascending). The plate was dried and exposed to XAR2 X-ray film for 64 hours at room temperature. To determine the sensitivity of the assay, various amounts of active chloramphenicol acetyl transferase (0.25, 0.025, and 0.0025 units) were spiked into the extract from strain 394-4.
Protein concentrations for the extracts were 8.6 mg/ml (strain 394-4) and 11.6 mg/ml (strain LU4-79-6). Acetylated forms of chloramphenicol were observed, as radioactive spots, for all reactions spiked with active CAT. No acetylated chloramphenicol was observed in reactions that contained only *C. acremonium* extract as enzyme source.

Assayed by Dr. Gretchen Kirchner
7/16/87

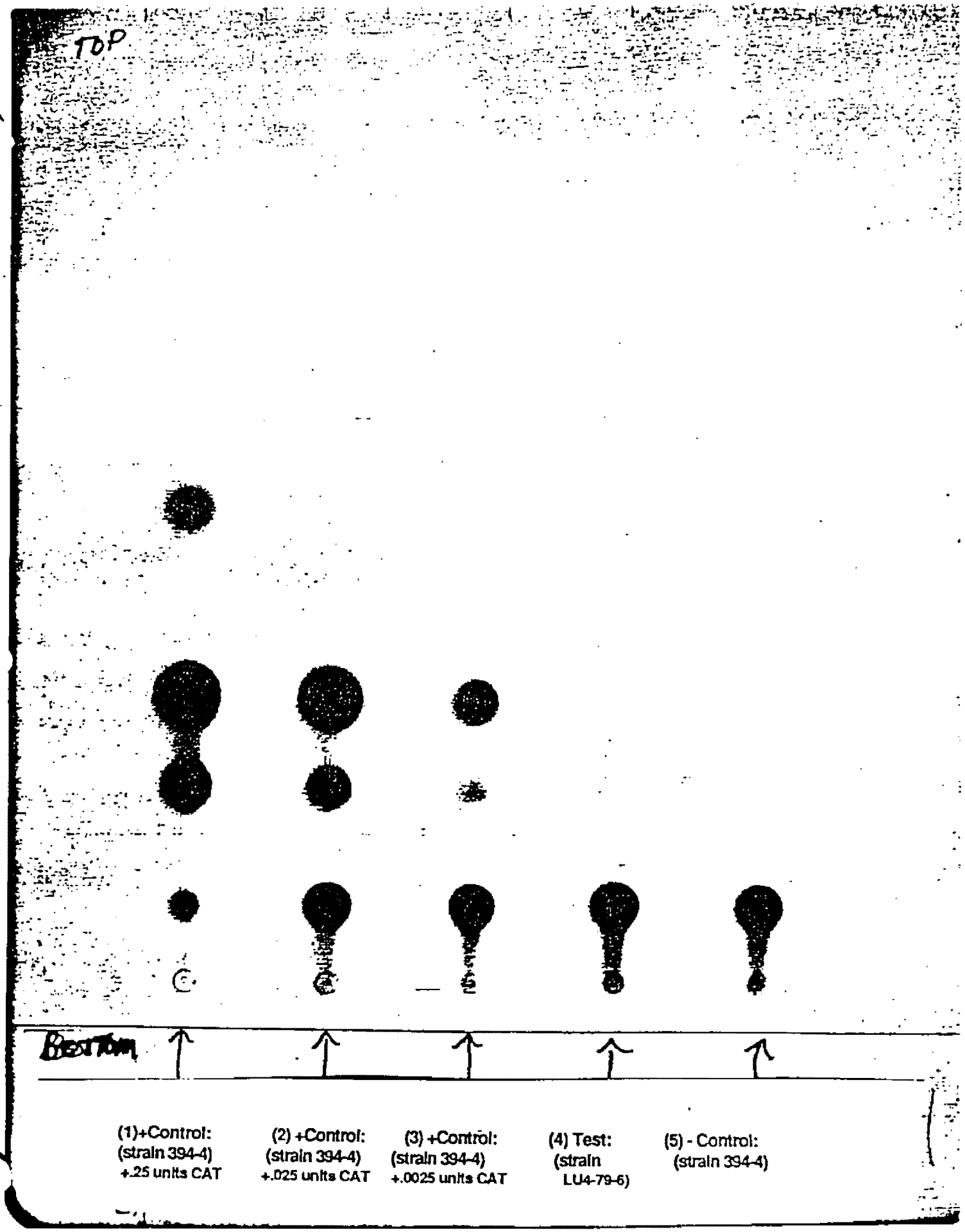

TOP
BOTTOM
(1)+Control:
(strain 394-4)
+.25 units CAT
(2) +Control:
(strain 394-4)
+.025 units CAT
(3) +Control:
(strain 394-4)
+.0025 units CAT
(4) Test:
(strain
LU4-79-6)
(5) - Control:
(strain 394-4)

Table 1. Summary of Original Shake Flask Fermentation Analyses for Strain LU 4-79-6: Transformant produces significantly more cephalosporin C and less penicillin than control strain 394-4**

Inoculum	Experiment	Cephalosporin C, %of control strain*	Penicillin , %of control strain*
LU4-79-6 original slant	#1	126%	79%
LU479-6, original slant	#2	115%	87%
LU4-79-6, original slant	#3	123%	87%
LU4-79-6, preserved lot	Post-preservation	152%	33%
LU4-79-6, original slant	Post-preservation	156%	42%

* Control strain is current production strain 394-4.
**Assay does not differentiate between isopenicillin N and penicillin N.
For experimental detail, see legend to Table 3.

Table 2. Summary Expandase Assay Data: Transformant LU4-79-6 exhibits significantly more expandase activity than control strain 394-4

EXPERIMENT	CELL AGE (hours)	EXPANDASE (% control)
#3[pre-preservation]	96	182
#4[post-preservation]	72	146
#4[post-preservation]	96	148
#4[post-preservation]	120	130

Experiment #3 - prepreservation, cephalosporin C at end of fermentation was 123% of control level.
Experiment #4 - postpreservation, cephalosporin C at end of fermentation was 152% of control level.
The production strain 394-4 and the hygromycin B resistant strain transformant LU4-79-6 were grown for the hours indicated in standard shake flask medium used to evaluate production of cephalosporin C. Cells were harvested by centrifugation, washed in buffer, sonicated to break cells, and cell-debris removed by centrifugation. Cell-free extracts were assayed for expandase activity as described by Dotzlaf et al., 1987 (J. Bacteriol., 1987). Enzyme data supplied by Mr. J. Dotzlaf.

Table 3. Data of Fermentation Analysis, Experiment #3: Transformant LU4-79-6 produces significantly more cephalosporin C and less penicillin** than production strain, 403.18, in standard shake flask fermentation

Sample Number	Strain I.D.	Cephalosporin Potency(%Control*)	Penicillin Potency(%Control*)
1	#3	74%	154%
2	#3	71%	154%
3	#4	70%	170%
4	#4	69%	177%
5	#5	79%	138%
6	#5	93%	133%
7	#6	121%	88%
8	#6	124%	87%
9	#7	85%	149%
10	#7	86%	143%
11	#8	89%	141%
12	#8	83%	135%
13	394-4	103%	100%
14	394-4	96%	101%
15	394-4	97%	106%
16	394-4	103%	92%

The production strain 394-4 and six hygromycin B-resistant transformants, LU4-79-3, -4, -5, -6, -7, and -8, were grown 7 days in standard shake flask medium used to evaluate production of cephalosporin C. Inocula for these fermentations were identical to those used for enzyme analysis and fermentations were carried out under identical conditions. Cells were removed by filtration and assayed for cephalosporin C and penicillin [assay for cephalosporin C is the "nicotinamide assay" which is specific for cephalosporin C and does not measure penicillins, or deacetoxycephalosporin C or deacetylcephalosporin C; the assay for penicillin is the "hydroxamate assay" is specific for penicillins and does not measure cephalosporins. Each transformant was prepared by transformantion of strain 394-4 with pPS56.

**The stereochemistry of the alpha-aminoadipyl side-chain of the penicillin in the current production strain has not been determined; however, the stereochemistry was determined earlier for strain 403.6 and was the D-configuration, i.e. the penicillin was penicillin N.

*Strain 394-4, the current production strain, is the control strain.

J. Chapman
March 20, 1987

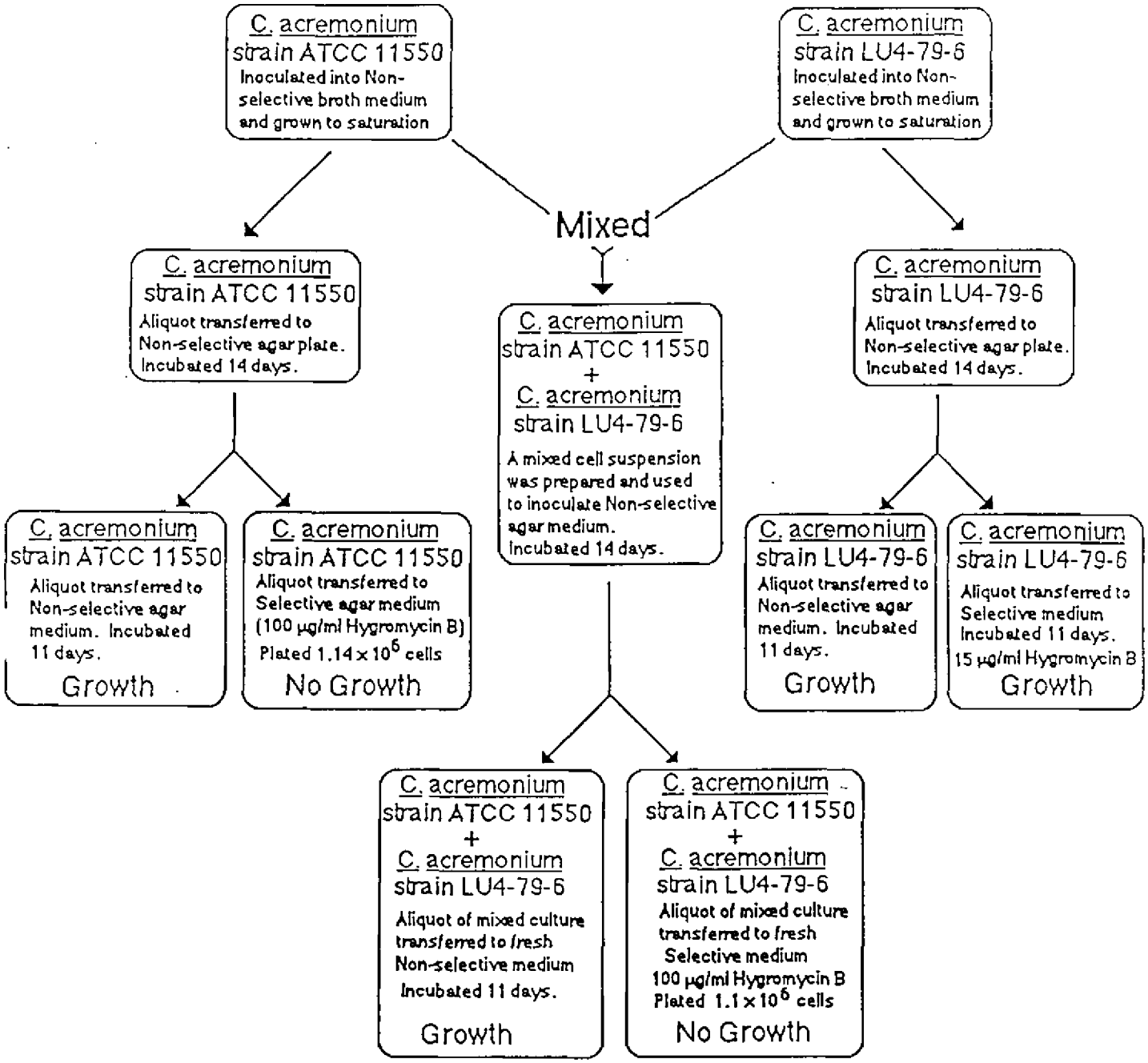
Table 4. Comparison of expandase specific activities in six transformants and control strain, 394-4, Experiment #3: 96-hour cells of transformant LU4-79-6 contains significantly more expandase activity than the production strain, 394-4

Strain name	Specific Activity (milliUnits/mg protein)	%Control
LU4-79-3	11.58	85
LU4-79-4	8.33	59
LU4-79-5	10.05	71
LU4-79-6	25.85	182
LU4-79-7	13.93	97
LU4-79-8	9.68	68
Control, 394-4	14.22	100

The production strain 394-4 and six hygromycin B resistant transformants, LU4-79-3, LU4-79-4, LU4-79-5, LU4-79-6, LU4-79-7, and LU4-79-8 were grown for 96 hours in the standard shake flask medium used to evaluate production of cephalosporin C. Ninety-six hours was chosen because previous studies have shown that expandase activity peaks at this time in the shake flask fermentation. Cells of each strain were harvested by centrifugation, washed in buffer, sonicated to break cells, and cell debris removed by centrifugation. Cell-free extracts were assayed for expandase activity as described by Dotzlaf and Yeh (J. Bacteriol. 169: 1611-1618 [1987]).

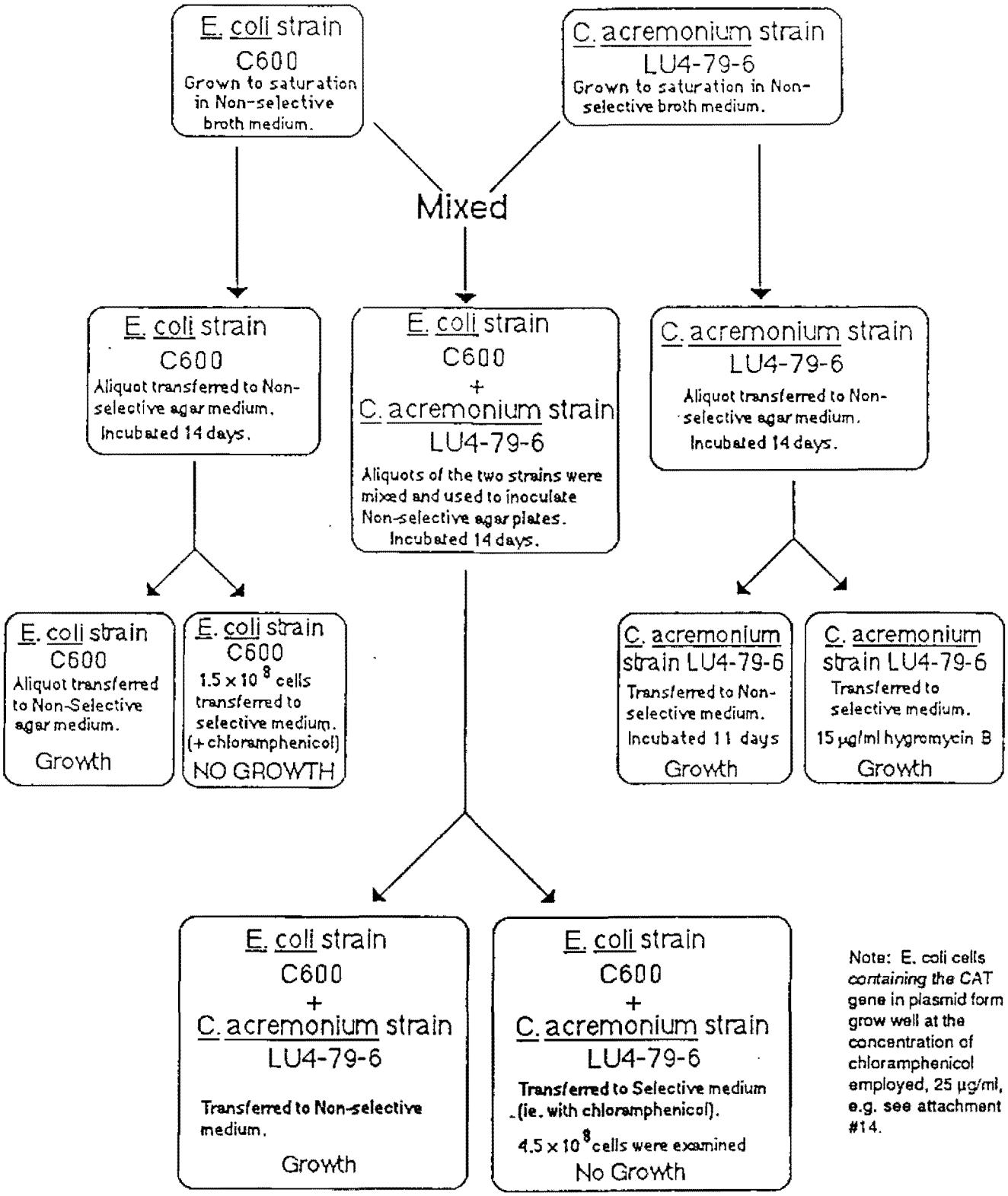
J. Dotzlaf
J. Chapman
3/20/87

Transfer of Hygromycin B resistance to another strain of C. acremonium not detected.



Note: Strain ATCC 11550 is vigorous and fast-growing; transformants of ATCC 11550 carrying the HPT gene are resistant to at least 100 µg/ml hygromycin B; as a control in the experiment above, transformant T1 which was obtained by transformation of ATCC 11550 to hygromycin B resistance, was grown successfully at 100 µg/ml hygromycin B. In contrast, LU4-79-6 and strain 394-4 , the untransformed parent of LU4-79-6, are less vigorous and slow-growing [strain 394-4 is a product of many rounds of mutation/screening and mutation/selection]. Transformants of 394-4 carrying the HPT gene are usually resistant to only 5 to 15 µg/ml hygromycin B.

Chloramphenicol resistance is not transferred to E. coli from C. acremonium strain LU4-79-6.



Note: E. coli cells *containing the* CAT gene in plasmid form grow well at the concentration of chloramphenicol employed, 25 µg/ml, e.g. see attachment #14.

DNA isolated from *Cephalosporium acremonium* strain LU4-79-6 is not able to transform competent *Escherichia coli* (C600) cells to chloramphenicol resistance under laboratory conditions.

DNA	Trial #1	Trial #2
Controls		
No DNA	0	0
pMLC12	9.2×10^5 trans./µg	6.2×10^6 trans./µg
394-4	0	0
LU4-79-6 (5µL) + pMLC12	1,028 cfu	3,728 cfu
LU4-79-6 (10µL) + pMLC12	334 cfu	2,152 cfu
LU4-79-6 (15µL) + pMLC12	418 cfu	568 cfu
Experimental		
LU4-79-6 (5µL)	0	0
LU4-79-6 (10µL)	0	0
LU4-79-6 (15µL)	0	0

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NOVO

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March 30, 1988

Dr. William J. Gartland, Jr.
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Dear Dr. Gartland:

Novo Laboratories, Inc. is the United States subsidiary of Novo Industri A/S, Denmark, a pharmaceutical and enzyme manufacturing company which utilizes rDNA technology.

Novo proposes the following amendment to the NIH "Guidelines for Research Involving Recombinant DNA Molecules" (Guidelines):

In appendix C-IV add "Bacillus licheniformis" to the title and to the first sentence such that it would read:

> *Appendix C-IV - Experiments Involving Bacillus subtilis or Bacillus licheniformis Host-Vector Systems*
>
> *Any asporogenic Bacillus subtilis or Bacillus licheniformis strain ...*

The proposed amendment is supported by the attachment describing the close taxonomic relationship between Bacillus subtilis and Bacillus licheniformis, the non-pathogenicity of Bacillus licheniformis and the extended history of safe industrial use of Bacillus licheniformis as a source of food-grade enzymes.

The reason for requesting this amendment is that it is possible to construct recombinant strains of Bacillus licheniformis, utilizing NIH certified host-vector systems which would not fall under exemption III-D-2, III-D-3 or III-D-4 of the guidelines although if the identical experiments had been carried out with Bacillus subtilis they would have been exempt. In the case of the Bacillus subtilis recombinant strain an Institutional Biosafety Committee could elect to set containment conditions to a level no greater than those for the host organism unmodified by recombinant DNA techniques (Appendix C-IV of guidelines, paragraph 3). In the case of the Bacillus licheniformis strain this option may not be possible under the existing guidelines if the experiments are not exempt.

Adding Bacillus licheniformis to Appendix C-IV of the Guidelines would remove this obstacle since the experiments in question would then be exempt. If the proposed amendment is not implemented each such non-exempt large-scale experiment would require special RAC review. In Novo's opinion this would be unnecessarily wasteful of resources considering the close taxonomic relationship between Bacillus subtilis and Bacillus licheniformis, the non-pathogenicity of Bacillus licheniformis and its extended history of safe industrial use.

NOVO

If this proposed amendment is implemented, Novo intends, if its Institutional Biosafety Committee concurs, to conduct large-scale experiments, under containment conditions less restrictive than BL1-LS for recombinant strains of Bacillus licheniformis which would then be exempt under the Guidelines.

Sincerely Yours,

Joseph R. Fordham

Joseph R. Fordham, Ph.D.
Manager
Regulatory Affairs

JRF/cjs

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ATTACHMENTS:

	NUMBER
Bergey's Manual - *B. licheniformis*	1
Literature survey: pathogenic potential of *B. licheniformis*	2
GRAS affirmation regulation for carbohydrases and proteases from *B. licheniformis*	3
Transformation of *B. licheniformis*	4
Extracellular penicillinases expressed in *B. licheniformis*	5
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I. TAXONOMY OF BACILLUS LICHENIFORMIS

Bacillus licheniformis was known between 1916 and 1944 as the Ford strain of *B. subtilis*. Based on a study by T. Gibson (1944) it was separately classified and the Seventh Edition of Bergey's Manual listed it as a distinct species. The descriptions of *B. subtilis* and *B. licheniformis* found in the second volume of Bergey's Manual (1986) are attached. Table 1 compares the growth characteristics of the two organisms. *B. subtilis* and *B. licheniformis* are also similar in that both have been used extensively as sources for amylases and proteases used in food processing and in the detergent industry. It should be noted that strains of *B licheniformis* used for industrial production of enzymes do not produce antibiotics.

II. EVALUATION OF THE PATHOGENIC POTENTIAL OF BACILLUS LICHENIFORMIS

1. Introduction

B. licheniformis has been used at Novo in the production of protease and starch decomposing enzymes for several decades. The pathogenic potential of the different strains used has been evaluated in *in vivo* animal models. Increasing interest in using *B. licheniformis* as a donor or recipient in recombinant DNA constructions has made this literature based survey necessary. The pathogenic potential is interpreted as an ability to cause infection in man.

2. Source Material

The evaluation of the pathogenicity of *B. licheniformis* to humans was made by searching in four databases using one or more of the queries *Bacillus licheniformis*, pathogenicity, disease and toxicity. In addition, literature on *Bacillus* species pathogenicity collected during the past ten years and commonly used handbooks in standard medical microbiology were consulted.

3. Occurrence

Bacillus species are ubiquitous in the environment and are common contaminants in soil and soil contaminated material. As with other *Bacillus* species growth of *B. licheniformis* is observed in many foods (Gibson, 1974).

4. Human Pathogenicity

a. Intoxication

The most common syndrome associated with *Bacillus* species is gastrointestinal intoxication. *Bacillus cereus* is known to produce both a diarrheal and emetic type of intoxication (Turnbull, 1981). The ability of certain *B. licheniformis* strains to cause a similar intoxication has not been confirmed. This search has not revealed any reports on gastrointestinal intoxication caused by *B. licheniformis*.

b. Infection

Four references citing infection with *B. licheniformis* were found. Young et al. (1982) describe a case of post neurosurgical infection with *B. licheniformis*. The infection developed after removal of an intraventricular meningioma. Tabbara et al. (1979) report on an eye infection with *B. licheniformis* following a corneal injury. Sugar et al. (1977) describe an incident of sepsis and peritonitis probably developed from a perforated infarcted jejunum from which *B. licheniformis* was released into the peritoneal cavity and the blood stream.

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Table 1
GROWTH CHARACTERISTICS

Test	*B. subtilis*	*B. licheniformis*
Tyrosine Agar Slants	Growth same as on agar.	Same as agar slants.
NaCl Broth:	Good growth up to a concentration of 7 percent NaCl; growth in a few cases in 10 or 12 percent.	Good growth in 5 to 8 percent NaCl, inhibition with higher concentrations. In a few cases, growth in 10 percent.
Milk	Slowly peptonized, usually becoming alcaline.	Not listed.
Milk Agar Streak Plate	Usually there is a wide zone of hydrolysis of the casein.	Usually narrow zone of hydrolysis of the casein.
Potato	Growth heavy, wrinkled to coarsely folded, spreading. Off white, yellow, pink, or brown. Variations: slimy, soft, thin, warty.	Growth heavy, spreading, wrinkled, warty, with extruded droplets. Pink. Submerged portion of potato red to violet. Variations: soft, off white.
Arabinose, Xylose, Glucose, Sucrose and Mannitol(with ammonium salts as source of nitrogen)	Acid but no gas. Usually no acid produced from lactose.	Acid but no gas. Acid production variable from lactose.
Starch	Hydrolyzed	Hydrolyzed
Acetylmethylcarbinol	Produced (37°C better incubation temperature than 32°C)	Produced (37°C better incubation temperature than 32°C)
pH of Glucose Broth	5.0 to 8.6 in 7 days.	5.2 to 8.2 in 7 days.
Citrates	Utilized	Utilized

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GROWTH CHARACTERISTICS

Test	B. subtilis	B. licheniformis
Nitrites	Produced from nitrates. No gas produced from nitrate broth under anaerobic conditions.	Produced from nitrates. No gas produced from nitrate under anaerobic conditions.
Lecithinase	Not produced	Not produced
	Aerobic, certain strains facultatively anaerobic. Growth scant, if any, in glucose broth under anaerobic conditions; pH of 14-day cultures in 5.5 or higher.	Aerobic, facultatively anaerobic. Growth in glucose broth under anaerobic conditions; pH is 5.2 or lower at 14 days. Usually a small amount of gas is produced.
Temperature Relations	Optimum growth temperatures lie between 28° and 40°C. The maximum temperature for growth is usually 50°C., but some cultures find 40° too warm for growth while still others will grow even up to 55°C.	Optimum, between 32° and 45°C. Maximum, between 50° and 56°C. (in water bath).
Growth Factors	Not essential.	Not essential.
Antibodies	Subtilin, subtenolin, bacillomycin and others.	Bacitracin, licheniformin
Habitat	Widely disturbed in soil and decomposing organic matter; also common as a laboratory contaminant.	Eidely distributed in soil and food; also common as a laboratory contaminant.

Finally Peloux et al. (1975) report on a case of bacteriemia caused by *B. licheniformis* in a patient given intravenous blood transfusions and catherization. *B. licheniformis* was isolated from eight of the ten blood portions given. Thus the common feature of these four quite different case histories is the introduction of *B. licheniformis* to the body through damaged tissue which normally should protect the organ or body fluids against microorganisms.

Through the past 25 years several reviews on infections with *Bacillus* species have been published.

Sliman et al. (1987) reviewed the records of five Cleveland hospitals and discovered thirty-eight patients with infections caused by *Bacillus* species. Fifteen of the isolates were identified as to species and none of them were classified as *B. licheniformis*. Cotton et al. (1987) retrospectively examined episodes of *Bacillus* bacteriemias at a hospital with a large proportion of immunocompromised patients. Seventeen cases were found and of these twelve were identified to the species level: six *B. cereus*, three *B. licheniformis*, two *B. pumilis* and one *B. sphaericus*. The patients were all in poor health. Fifteen patients had advanced malignancies and fourteen had intravenous catheters through which the bacteria might have been introduced.

Shamsuddin et al. (1982) report a panophthalmitis caused by *Bacillus* species. The three case histories described were all intravenous drug abusers and *B. cereus* was the infective organism. In the paper the microbial flora of forty-nine heroin samples and injection paraphernalia is described. *Bacillus* species were the most common isolates and four species were identified, ie. *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. licheniformis*. Shamsuddin et al. do not report on any case of ocular infection caused by *B. licheniformis* either from their own experience or earlier reports on eye infections caused by *Bacillus* species.

The review by Tuazon et al. (1979) deals with disseminated infections caused by *Bacillus* species. Tuazon et al. report on their experience with seven patients and furthermore they review the published reports on disseminated infections with *Bacillus* species, the earliest from 1913. *B. licheniformis* is isolated in none of the total of thirty-two cases reported. Pearson (1969) describing organisms of the *Bacillus* species associated with infections in forty-eight patients does not mention *B. licheniformis* as the cause of any infection.

Finally, in the textbooks consulted, *B. licheniformis* is not mentioned as a potential pathogen to man. (Jawetz, 1980; Wilson, 1984; Doyle, 1985).

5. Conclusion

This survey of the literature has revealed a total of seven cases of human infections caused by *B. licheniformis*. In three of the cases the patients were severely debilitated by advanced malignancies and in the other four the bacteria were introduced to the body through damage of the natural barriers, ie. surgical infection, wound infection, devitalized intestinal tissue or directly infused into the blood stream.

No reports have described *B. licheniformis* infections, which indicate that *B. licheniformis* causes any health hazard to humans apart from immunocompromized patients or patients exposed in a way that circumvent the normal biological defense mechanisms. Therefore, on the basis of this survey *B. licheniformis* can be regarded as a nonpathogenic microorganism.

III. HISTORY OF USE OF B. LICHENIFORMIS IN FOOD PROCESSING

Alpha-amylases and proteases produced by *Bacillus* species have a long history of use in food processing. Beckhorn, et al.(1965) in a review of the production and uses of microbial enzymes traced the common use of the alpha-amylases and proteases from *B. subtilis* back to 1929 and 1940 respectively. However, these two groups of enzymes came into more common use in the 1940s and 1950s. These uses include the production of nutritive substances (corn

syrup), liquefaction of adjuncts or mash in beer and distilled liquor, viscosity control of chocolate syrups, production of protein hydrolyzates, modification of cracker dough, and hydrolysis of protein in brewing mashes. *B. licheniformis* alpha-amylase has been used in the corn syrup industry since 1972 and proteases from this organism have been used to produce protein hydrolases since 1971.

The U.S. Food and Drug Administration (FDA) (1983) affirmed that carbohydrase and proteinase enzyme products derived from *B. licheniformis* are generally recognized as safe (GRAS) when used to hydrolyze starches and proteins in the production of certain foods. This GRAS affirmation was based on the evaluation of a petition submitted by Novo Laboratories, Inc. in 1973. The evaluation was based on scientific procedures. The petition established that *B. licheniformis* is widely recognized as a common contaminant found in many foods and that no toxicity or pathogenicity was associated with the presence of this organism in food. The scientific literature search contained in the petition disclosed no evidence that *B. licheniformis* produces disease in man.

The petition also contained unpublished pathogenicity studies and animal feeding studies to support the safety of the enzyme preparation. The pathogenicity studies confirmed that the production strain was neither pathogenic nor toxicogenic to rats, mice, guinea pigs, and rabbits. The animal feeding studies, including LD_{50} studies in both sexes of mice and rats, a twenty-eight-day study in the rat, two ninety-day studies in the rat and dog, respectively, a teratology study in the rat, and two dominant lethal studies in the male mouse and male rat, respectively provided corroboration that the anticipated levels of the enzyme preparations in food are safe for human consumption.

IV. PUBLISHED CLONING EXPERIMENTS WITH B. LICHENIFORMIS

The commercially important strains of *B. licheniformis* have been reported to be resistant to DNA-medicated transformation by standard methods. However, Grosch and Wollweber (1982) successfully introduced the plasmid pUB110 into a strain of *B. licheniformis* by a protoplast transformation procedure originally described for *B. subtilis*. Imanaka, et al. (1981,1983) using vector plasmids were able to clone the structural genes for penicillanase, *penP* from *B. licheniformis* ATCC 9945A into *B. subtilis* and *B. licheniformis*. Sonders, et al. (1985) in a patent application disclosed a method whereby genes expressing polypeptides of interest could be cloned in a number of industrially important *Bacillus* species including *B. licheniformis*.

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TAB 1