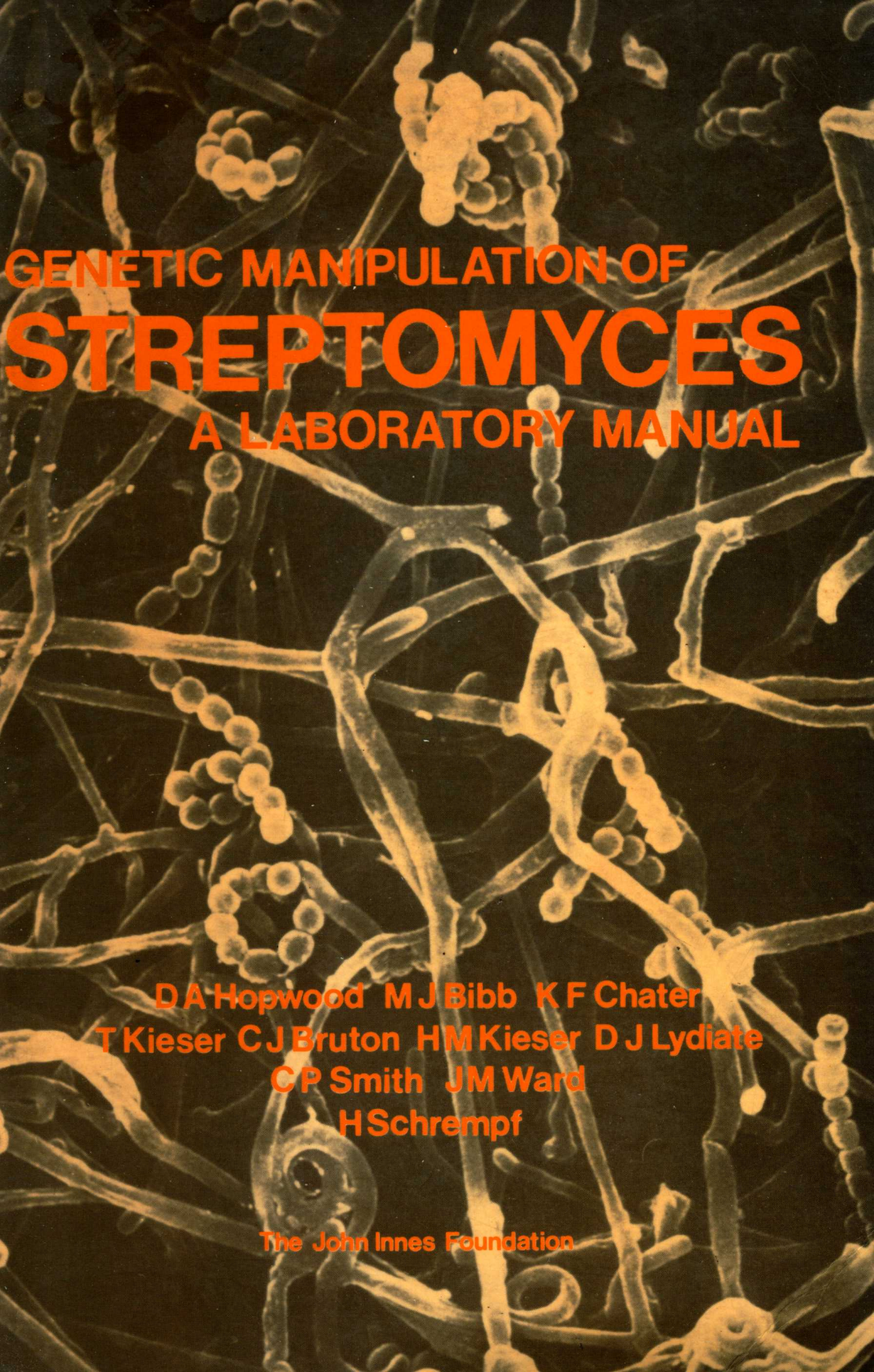

GENETIC MANIPULATION OF STREPTOMYCES

A LABORATORY MANUAL

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Interest in the genetics and molecular biology of Streptomyces has recently increased quite significantly from the modest level shown during the first twenty years or so after the discovery of genetic recombination in Streptomyces in the mid-1950's. The main stimulus for this wider awareness has undoubtedly been the development of efficient systems for gene cloning in streptomycetes. Added to the already existing possibilities for in vivo genetic analysis via plasmid-mediated conjugation, and later protoplast fusion, in vitro techniques have opened the way to penetrating analyses of the special features of these Gram-positive prokaryotes, notably their complex morphological differentiation and their enormous capacity to synthesise antibiotics.

Those now developing a first-time interest in Streptomyces genetic manipulation are coming from several types of background. On the one hand, scientists already experienced in some aspect of the streptomycetes, such as taxonomy, ecology, metabolism or antibiotic production, wish to use the techniques of genetics and molecular biology to analyse a system or to develop new strains. They have a store of knowledge about Streptomyces, but may lack a familiarity with the concepts or practical techniques of molecular biology. On the other hand, molecular biologists who had worked with another organism, usually Escherichia coli, are now turning to Streptomyces; they are already experienced in the techniques of DNA manipulation, but not in the special tricks required for Streptomyces. This manual aims to help both these kinds of people. In our own laboratories, when introducing students and other visitors to the art of Streptomyces genetics we have found it useful to have available in one manual most of the protocols that we use in genetically manipulating these organisms, even if some of the methods concerned with their DNA are essentially the same as, and have been derived from, techniques already developed by E. coli molecular biologists. We recognise that this has inevitably led to a rather sketchy treatment of some important topics. We recommend the excellent Cold Spring Harbor Laboratory Manual on Molecular Cloning (Maniatis et al., 1982) for a much more comprehensive coverage of the theory and practice of DNA manipulation and for experiments with E. coli and its plasmids and phages, some of which are directly relevant to Streptomyces workers for the construction of lambda or cosmid libraries or for the propagation of shuttle vectors.

With few exceptions, the protocols included in this manual are those in routine use at the John Innes Institute. Undoubtedly, there are many good alternative procedures, but we deemed it safer to stick to familiar methods rather than relaying information taken from the literature without personal experience. The procedures are mostly optimised for the strains of Streptomyces coelicolor and Streptomyces lividans that we mostly use. However, a feature which distinguishes Streptomyces molecular biology from comparable work with E. coli, Bacillus or yeast is the very wide range

of independent strains in which people are interested; this results particularly from the need to analyse the genetic control of the production of a wide variety of antibiotics and enzymes. The procedures described here will undoubtedly work successfully with many of these organisms but, for others, will need modification. This applies especially, but not exclusively, to conditions for protoplast formation, transformation and regeneration. We have included some references to alternative procedures, but it will still be necessary to make individual modifications to the protocols for particular strains.

The manual contains a small amount of background information at the beginning of some of the sections, and some literature references; however, since the manual is meant to be mainly a compilation of practical procedures, this material has been kept rather brief. Those unfamiliar with the literature on Streptomyces genetics might find it useful to read one of the recent reviews of the field (Chater and Hopwood, 1983; Hopwood and Chater, 1984). Three reviews on gene cloning in Streptomyces are by Chater et al. (1982); Hopwood and Chater (1982), and Bibb et al. (1983).

Most of the manual consists of protocols for specific procedures and we have tried to be reasonably consistent in presenting them, although there are some variations in lay-out that reflect their authorship by different individuals. The subdivisions of a typical protocol are: introductory remarks (where appropriate); a list of materials (sub-divided, slightly arbitrarily, into: biological; solutions, chemicals, etc.; small equipment; and equipment); the procedure itself; and notes on the procedure. Successive steps in the procedure are numbered; where an asterisk appears against the number of a step, a remark with a corresponding number will be found in the "notes" section. Many of the protocols call for specific reagents or buffers and the composition of these is included in the materials section of that protocol. Where a buffer or medium is required for several protocols, its composition will usually be found in the Appendix (but it may also be included in particular protocols).

The production of this manual was stimulated by practical courses sponsored by the European Molecular Biology Organisation at the John Innes Institute in September 1983 and July 1985. We are grateful to EMBO for the financial support of these courses, and to the participants on the first course for consumer-testing the first draft of the manual and for making valuable suggestions for its improvement. We thank Anne Williams for her painstaking and good-humoured production of the camera-ready typescript. We hope that the manual will be useful. We shall be grateful to hear from satisfied or dissatisfied users so that we may benefit from their experience in revising the manual if the occasion arises.

1 PREPARATION OF ORGANISMS AND PHAGES

STREPTOMYCES CULTURES ON AGAR

To obtain uniform cultures on an agar surface (a slant or Petri plate), which will usually be incubated to yield well sporulating growth, the organisms have to be inoculated over the entire surface of the medium since the colonies (in contrast to those of most moulds) will spread only over a limited distance within a reasonable time; point inoculation will not yield a good culture. It is best to use a suspension of inoculum in liquid as starting material. This need not be a carefully prepared spore suspension (although this is very suitable) - a loopful of spores and/or mycelial fragments made by rubbing a loop carrying a drop of water over a small area of an existing culture is quite satisfactory. Fresh slants, often with a drop of liquid at the bottom, are easiest to inoculate, but sporulation tends to occur best under rather dry conditions. Therefore the slants are often incubated with the agar surface horizontal for the first 24h or so in order that the liquid soaks into the surface of the agar early in the life of the culture. Otherwise, the bottom section of the cultures may sporulate very late.

It is undesirable to propagate cultures by successive rounds of mass culture. Instead they should be plated out and a single colony taken to start the next slant culture. This precaution reduces the possibility of mass reversion of a marker, loss of an unselected plasmid, etc., which is otherwise an ever-present possibility when the "revertant" form has a growth or sporulation advantage over the desired genotype.

MAKING A *STREPTOMYCES* SPORE SUSPENSION

Most streptomycetes produce copious haploid "uninucleate" spores. These arise in chains in the aerial mycelium; the individual spores can readily be separated by suspending and shaking in water (for some strains, a wetting agent is needed, e.g. 0.1% Tween 80 or 0.001% Triton X100). The resulting suspensions are used for many purposes, such as inoculating liquid medium to produce mycelium for isolating plasmid or chromosomal DNA, RNA or enzymes or for preparing protoplasts, for the isolation of mutants; and for the analysis of recombination or plasmid transfer in crosses. Suspensions of spores in 20% glycerol, kept frozen at -20°, will usually remain viable for years, even if they are repeatedly thawed and re-frozen for sampling purposes (but there are exceptions - e.g. non-sporulating strains such as bald mutants and some streptomycin-sensitive strains of *S. glaucescens*).

Steps in the simple procedure of preparing a spore suspension are: scraping the surface of a sporulating agar culture to suspend the spores in water; filtering the crude suspension through cotton wool to remove mycelial fragments and pieces of agar medium; pelletting the spores by centrifugation and re-suspension, in order to remove compounds dissolved from the growth medium (these may include growth factors which could interfere with the selective use of auxotrophic markers, or "staling" materials which may reduce the longevity of the spores or inhibit germination).

MATERIALS

Biological: fresh slant or plate cultures of the strains.
Solutions, chemicals, etc: sterile water; 20% glycerol (sterilised by autoclaving).
Small equipment: inoculating loop; pipettes; filter tubes containing non-absorbent cotton wool (see diagram, page 5); centrifuge tubes; screw cap containers, e.g. 7.5ml ("Bijou" bottles) and 20ml (wide-necked McCartney bottles or "Universal" containers).
Equipment: vortex mixer; bench centrifuge.

PROCEDURE

*1. Add c. 9ml of sterile water to the slant or plate.

2. Scrape the surface of the culture with an inoculating loop, first with gentle pressure and then gradually more vigorously, so as to suspend the spores.

3. Pour the crude suspension back into the container that held the sterile water and agitate the liquid as violently as possible on a vortex mixer for a minute or so.

4. Filter the suspension through non-absorbent cotton wool, using a filter tube (as illustrated).

5. Pour the filtered suspension into a centrifuge tube and spin for 5-10min at c. 3000rpm to pellet the spores.

*6. As soon as the centrifuge stops, pour off the supernatant.

*7. Agitate the tube on the vortex mixer for a few seconds to disperse the pellet in the drop of water remaining in the tube.

8. Add sterile 20% glycerol (usually 1-2ml for the spores from a well-sporulating slant or plate) and briefly agitate again.

*9. Transfer the suspension to a screw cap bottle (7.5ml "Bijou" bottles are convenient) for freezing.

NOTES

1. It is convenient to keep a supply of 20ml screw-cap bottles (wide-necked McCartney bottles or "Universals") containing 9ml amounts of sterile water ready for making spore suspensions.

6. If the spore pellet is left in the tube, even for a few minutes after the centrifuge has stopped, it will often become detached from the wall of the tube.

7. It is easiest to separate the spores in a minimum volume of liquid.

9. If the spores are for immediate use only, they can be suspended in water; if you then decide to keep them, add a roughly equal volume of 40% glycerol and freeze.