

A detailed scanning electron micrograph of Streptomyces bacteria. The image shows a complex network of long, thin, filamentous structures (mycelia) that are intertwined. Numerous small, spherical spores are visible, some clustered in dense, rounded groups and others more isolated. The overall appearance is that of a highly branched and spore-producing microorganism.

GENETIC MANIPULATION OF STREPTOMYCES

A LABORATORY MANUAL

**DA Hopwood M J Bibb K F Chater
T Kieser C J Bruton H M Kieser D J Lydiate
C P Smith J M Ward
H Schrempf**

The John Innes Foundation

GENETIC MANIPULATION OF STREPTOMYCES

A LABORATORY MANUAL

D.A. Hopwood, M. J. Bibb, K.F. Chater, T. Kieser,
C.J. Bruton, H.M. Kieser, D.J. Lydiate, C.P. Smith, J.M. Ward
(John Innes Institute, Norwich)

H. Schrempf
(Institut für Genetik und Mikrobiologie, University of Munich)



Y076438

1985

The John Innes Foundation

Norwich

THE JOHN INNES FOUNDATION
John Innes Institute,
Colney Lane,
Norwich NR4 7UH,
England

Copyright © 1985 by
The John Innes Foundation

ISBN 0 - 7084 - 0336 - 0

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 layout that reflect their authorship by different individuals. The subdivisions of a typical protocol are: introductory remarks (where appropriate); a list of materials (subdivided, 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.

I	PREPARATION OF ORGANISMS AND PHAGES	1
	<u>Streptomyces</u> Cultures on Agar 2	
	Making a <u>Streptomyces</u> Spore Suspension 3	
	Plating out a <u>Streptomyces</u> Spore Suspension 6	
	Growth of <u>Streptomyces</u> Mycelium 7	
	Pre-germination of <u>Streptomyces</u> Spores 8	
	Growth of <u>Streptomyces</u> Mycelium for Isolation of "Total" DNA 10	
	Preparation of Protoplasts from <u>Streptomyces</u> <u>lividans</u> 66 and <u>S. coelicolor</u> A(2) 12	
	Purification of <u>Streptomyces</u> Phages 15	
	Divalent Cations for Phage Propagation 17	
	Single Plaque Isolation of <u>Streptomyces</u> Phages 18	
	Preparation of High Titre <u>Streptomyces</u> Phage Stocks 19	
	Isolation of New <u>Streptomyces</u> Phages 21	
	Preparation of ϕ C31 Lysogens 23	
	Preservation of <u>Streptomyces</u> Strains and Phages 26	
	Preparation of lyophils 26	
	Growing a culture from the lyophil 28	
	Growth of <u>E. coli</u> for Plasmid Isolation 29	
	Preparation of Competent <u>E. coli</u> Cells 31	
II	IN VIVO <u>STREPTOMYCES</u> GENETICS	33
	Mutagenesis of <u>Streptomyces</u> 34	
	Mutagenesis of <u>Streptomyces</u> Spores by Ultraviolet Light (UV) 37	
	Mutagenesis of <u>Streptomyces</u> Spores by Near Ultra- violet Light in the Presence of 8-meth- oxypsoralen (NUV+8-MOP) 39	
	Mutagenesis of <u>Streptomyces</u> Spores by N-methyl- N'-nitro-N-nitrosoguanidine (NTG) 40	
	Genetic Analysis by Conjugation and Protoplast Fusion 42	
	Making, Harvesting and Plating Crosses 44	
	Characterising Recombinant Genotypes 46	

Grids for Preparing Master Plates	49
Calculations of the Results of Matings	50
Recombination frequency	50
Frequency of plasmid transfer	50
Genetic mapping	51
Establishing a Genetic Map in a New Streptomycete by the "Four-on-Four" Procedure	52
Mapping a New Marker by a Single Selection	58
Plate-Crosses	60
Detection of Conjugative Plasmids by Pock Formation after Crossing with <u>Streptomyces lividans</u>	61
Protoplast Fusion	64
Genetic Analysis by Protoplast Fusion	68
III PREPARATION OF CHROMOSOMAL, PLASMID AND PHAGE DNA	69
Notes on Phenol	70
Note on SDS	70
Methods for Isolating <u>Streptomyces</u> "Total" DNA	71
Isolation of <u>Streptomyces</u> "Total" DNA: Procedure 1	72
Isolation of <u>Streptomyces</u> "Total" DNA by Caesium Chloride Gradient Centrifugation: Procedure 2	75
Isolation of <u>Streptomyces</u> "Total" DNA: Procedure 3	77
Rapid Small Scale Isolation of <u>Streptomyces</u> "Total" DNA: Procedure 4	79
Procedures for the Isolation of Plasmid DNA	81
Large Scale Preparation of Plasmid DNA from <u>Streptomyces</u> by Neutral Lysis: Procedure 1	82
Plasmid Isolation by Alkaline Lysis (<u>Streptomyces</u> or <u>E. coli</u>): Procedure 2	85
Small Scale Preparation of DNA of SCP2* Derivatives: Procedure 3	93
Large Scale Preparation of <u>Streptomyces</u> Phage DNA	95
Small Scale Preparation of <u>Streptomyces</u> Phage DNA	99

IV TRANSFORMATION AND TRANSFECTION 103Transformation and Transfection in Streptomyces 104Transformation of Streptomyces Protoplasts with
Plasmid DNA 107Transformation of Streptomyces Protoplasts with
Plasmid DNA: "Original Procedure" 108Rapid Small Scale Procedure for the Transformation of
Streptomyces Protoplasts with Plasmid DNA
110Recognition and Selection of Plasmid-Containing
Streptomyces Strains after Transformation
112

Lethal zygotis reaction (pocks) 112

Fertility 112

Resistance markers 112

Detection of melanin-producing colonies 113

Physical screening for plasmids 113

Soft agar overlays to select antibiotic-
resistant transformants 113

Antibiotic concentrations for overlays 114

Transfection of Streptomyces Protoplasts 115

Procedure I: with DNA-free liposomes 115

Procedure II: without DNA-free liposomes 116

Preparation of Positively-Charged DNA-Free Liposomes
(Liposome Supernatant Fluid) for
Transfection 118Transformation of Competent E. coli Cells with
Plasmid DNA 120**V IN VITRO MANIPULATION OF DNA 123**

Precipitation of DNA 124

Purification of Nucleic Acids by Extraction with
Phenol/Chloroform 125Removal of Ethidium Bromide and Caesium Chloride
from DNA Prepared by Dye-Buoyant Density
Centrifugation 127

Quantitation of DNA and RNA 129

Spectrophotometric determination of DNA or RNA	129
Quantitation of double-stranded DNA by ethidium bromide fluorescence	129
Endonuclease Digestion of DNA	131
Agarose Gel Electrophoresis of DNA	136
Frequently used gel conditions	136
Influence of gel conditions	138
DNA Size Standards for Agarose Gel Electrophoresis	139
DNA size standards I: 1-30 kb	140
DNA size standards II: 0.1-4 kb	141
Preparation of a 20 x 20 cm Agarose Gel	142
Preparation of a Minigel on a 5.1 x 7.6 cm Glass Slide	143
Preparation of Samples for Gel Electrophoresis	144
Photography of Gels	145
Alternative and Related Procedures	146
Acrylamide Gel Electrophoresis of DNA	147
Recovery of DNA Fragments from Gels	149
Electroelution into dialysis tubing	149
Isolation from low melting point agarose gels	150
Size Fractionation of DNA by Sucrose Gradient Centrifugation	152
Ligation of DNA	154
Theory	154
Ligation conditions	155
Removal of 5' Phosphate Groups from DNA by Treatment with Alkaline Phosphatase	158
VI CLONING OF DNA: CHOICE OF VECTORS AND STRATEGIES	161
General Points about Cloning	162
Cloning with Plasmid Vectors	164
Choice of vector	164
Preparation of vector DNA	164
Preparation of target ("donor") DNA	165
Ligation conditions	166
Transformation and selection of transformants	166

Analysis of clones	167
Promoter-probe plasmids	167
Cloning with ϕ C31 Phage Vectors	169
General features	169
Choice of ϕ C31 vectors for plaque hybridisation	171
Choice of ϕ C31 vectors for mutational cloning	171
Choice of ϕ C31 vectors for screening by "complementation" of mutants or acquisition of new capabilities	172
Choice of cloning site: a caveat	172
Ligation conditions	172
Transfection	173
Maximising and estimating insert frequency	173
Construction and stability of lysogens	174
Homogenotisation	175
Application of ϕ C31 to gene fusions	176
Statistical Considerations in Making Clone Libraries	178

VII TECHNIQUES USING RADIOLABELLED DNA 181

Introduction	182
General Points about Handling ^{32}P -Labelled Materials	182
Labelling of DNA by Nick Translation	183
Transfer of DNA from Agarose Gels to Nitrocellulose Filters (Southern Transfer)	187
Transfer of DNA from <u>Streptomyces</u> Colonies to Nitrocellulose Filters (for "Colony Hybridisation")	191
Transfer of <u>Streptomyces</u> Phage DNA to Nitrocellulose Filters (for "Plaque Hybridisation")	193
DNA-DNA Hybridisation on Nitrocellulose Filters	194
Autoradiography of Labelled DNA on Nitrocellulose Filters	200
5' End-labelling of DNA with T4 Polynucleotide Kinase	202
Labelling of Double-stranded DNA by Filling in Recessed 3' Termini	205

Advantages over treatment with kinase for DNA labelling	207
Labelling of 3' Termini with Terminal Transferase	208
Hints for Sequencing <u>Streptomyces</u> DNA	210
General comments	210
Alternative and additional base-specific reactions for sequencing end-labelled DNA	211
VIII TECHNIQUES FOR HANDLING RNA	213
Isolation of RNA from <u>Streptomyces</u>	214
Note on RNA purification by caesium chloride centrifugation	219
Transfer of Denatured RNA to Nitrocellulose and Hybridisation with ³² P-labelled DNA ("Northern Blotting")	221
The Dot-Blot Assay	225
S ₁ Nuclease Mapping of Transcribed DNA sequences	226
Addendum: High resolution S ₁ mapping	230
APPENDIX: MEDIA AND MAPS	231
Agar Media	232
Minimal medium (MM)	233
Complete medium (CM)	234
R2 medium	235
R2YE medium	236
MMT	237
Difco nutrient agar (DNA)	238
Oxoid nutrient agar (ONA)	238
Soft nutrient agar (SNA)	238
L agar	238
Liquid Media	239
Yeast extract-malt extract medium (YEME)	239
Tryptone soya broth (TSB)	239
Difco nutrient broth (DNB)	239
L broth	239
Minimal liquid medium (NMMP)	240
Labelling medium	241

Antibiotic Concentrations for Selection of Resistant
Strains 242

Growth Factor Supplements 244

Buffers 245

P (protoplast) buffer 245

L (lysis) buffer 245

T (transformation) buffer 246

TNE buffer 246

TE buffer 246

SM buffer 247

20 X SSC 247

G buffer 247

Buffers recommended for enzymes used on DNA 248

Strains of Streptomyces coelicolor, S. lividans and
S. parvulus 256

Strains of S. coelicolor A3(2) 257

List of Markers of S. coelicolor A3(2) 258

Linkage Map of S. coelicolor A3(2) 265

Strains of S. lividans 66 266

Linkage Map of S. lividans for Ten Markers 266

Strains of S. parvulus ATCC 12434 267

Information on and Maps of Plasmids and ϕ C31 Phage,
and Vectors Derived from Them 268

Physical and Functional Map of SLP1.2 269

Physical and Functional Map of pIJ101 270

Physical and Functional Map of SCP2* 271

Partial Restriction Maps of DNA Fragments Carrying
Markers Used in Vector Construction 272

Tabulations of Distances for Restriction Maps of
Marker Fragments 274

aph 275

hyg 276

mel 277

mls 278

tsr 279

vph 280

Sequences of Marker Genes 281

aph 281tsr 282vph 283Table of Streptomyces Plasmid Vectors 284

Restriction Maps of Plasmid Vectors 286

pIJ61 286

pSLP181 288

Conjugative pIJ101 derivatives 290

pIJ702 292

pIJ385 294

pIJ364 296

pIJ680 298

Promoter-probe vectors pIJ424 and pIJ425 300

Map of Tn5 304

SCP2* derivatives 305

pIJ916 306

pIJ922 308

pIJ940 310

pIJ941 312

pIJ943 314

Restriction Map of ϕ C31 316Genealogies and Maps of ϕ C31 Vectors 317

KC505 and KC518 318

KC515 319

Table of ϕ C31 Vectors 320

REFERENCES

321

INDEX

335

I 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; pelleting 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.