National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Canada

DNA SEQUENCING AND CHARACTERIZATION OF A CHLORAMPHENICOL RESISTANCE GENE FROM *STREPTOMYCES VENEZUELAE*

by

Roy Henry Mosher

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
July, 1993

ISBN 0-315-93711-4

Canada

Name ROY HENRY MOSHER

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four digit code in the spaces provided.

MICROBIOLOGY

SUBJECT TERM

0 4 7 0

SUBJECT CODE

U·M·I

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES

American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

A chloramphenicol resistance determinant from *S. venezuelae* ISP5230 genomic DNA, originally cloned as a 6.5-kb *Sst*I-*Sst*I fragment in the recombinant plasmid pJV3, was localized by *in vitro* deletion to a 2.4-kb *Kpn*I-*Sst*I DNA fragment in the recombinant plasmid pJV4. When subcloned in the phagemid vectors pTZ18R and pTZ19R, neither the 2.4-kb nor 6.5-kb fragment conferred chloramphenicol resistance on *Escherichia coli*.

DNA sequencing and nucleotide sequence analysis of the 2.4-kb fragment, predicted the presence of three complete and one incomplete open reading frames. The largest open reading frame, *orf-4*, encoded a hydrophobic polypeptide that showed significant sequence similarity to the putative chloramphenicol-efflux proteins of *Streptomyces lividans* and *Rhodococcus fascians*. Located immediately downstream of *orf-4* was an open reading frame (*orf-3*) encoding a polypeptide (Orf3) that showed limited but significant sequence similarity to a number of proteins that required nucleotide co-factors. The discovery of a conserved ATP/GTP-binding site motif near the amino-terminus of Orf3, suggested a biological process such as a phosphotransferase reaction requiring a high-energy co-factor. A comparison of the derived amino acid sequences for *orf-1* and *orf-2* with those in current databases showed no significant similarities.

Streptomyces lividans transformants RM3 and RM4, carrying plasmids pJV3 and pJV4 respectively, rapidly metabolized chloramphenicol to one predominant product. Structure analysis based on ^{1}H-NMR and ^{13}C-NMR spectra of the purified product showed the substance to be related to chloramphenicol but, with an electron-withdrawing group attached to carbon-1'. ^{31}P-NMR and mass spectroscopy established that the compound was chloramphenicol-1'-phosphate. The results strongly suggest that *orf-3* encodes a chloramphenicol kinase activity that detoxifies chloramphenicol by O-phosphorylation at carbon-1'. In a bioassay using the chloramphenicol-hypersensitive *Micrococcus luteus*, chloramphenicol-1'-phosphate showed negligible antibiotic activity when compared to chloramphenicol.

LIST OF ABBREVIATIONS

7-deaza-dGTP	7-deaza-2'-deoxyguanosine-5'-triphosphate
ATP	adenosine-5'-triphosphate
bp	base pair
CAH	chloramphenicol hydrolase
CAT	chloramphenicol acetyltransferase
cml^r	chloramphenicol resistance
cmpd.X	compound X
cpm	counts per minute
δ	chemical shift
D_2O	deuterium oxide
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine-5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-thiogalactopyranoside
kb	kilobase
M_r	relative molecular weight (dimensionless)
mer	oligomer
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	messenger RNA
NMR	nuclear magnetic resonance
nt	nucleotide
Orf	open reading frame (deduced amino acid sequence)

orf	open reading frame (nucleotide sequence)
p.p.m.	parts per million
p.s.i.	pounds per square inch
PABA	*p*-aminobenzoic acid
PAPPA	*p*-aminophenylpyruvic acid
PAPS	*p*-aminophenylserine
PEG	polyethylene glycol
PNBA	*p*-nitrobenzyl alcohol
PNPS	*p*-nitrophenylserinol
rbs	ribosome binding site
RNA	ribonucleic acid
RT	retention time
SDS	sodium dodecyl sulfate
ssDNA	single-stranded DNA
TES	*N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid
TMS	transmembrane segment or tetramethylsilane
tris	tris-(hydroxymethyl) aminoethane
tsr	thiostrepton resistance
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

ACKNOWLEDGMENTS

I would like to express my deepest thanks to Dr. Leo Vining for being not only an excellent research supervisor, but also a mentor. You have taught me that imagination, perseverance and compassion are the most important facets of a good researcher and teacher.

I thank Dr. Vining, Dalhousie University, and the Patrick Lett Bursary Committee for their financial support.

I would like to thank all the great people I have had the privilege of knowing and working with over the past 10 years in the *Streptomyces* group. I thank in particular: Dr. Janice Doull for first introducing me to the ever-fascinating world of *Streptomyces*; Dr. Kwamena Aidoo for his unique point of view on almost everything; Dr. Ashish Paradkar for always seeing the brighter side of things; Dr. Kofi Arhin for teaching me how to relax and take my time (I'm still working on this!); Yang Keqian for our long discussions about almost everything; and finally, Pete Brown for being a great honours student and an all round great guy! I would also like to thank Neelima Ranade for her friendship and good times; your sudden death saddened me greatly.

I thank Dr. Bill Shaw and Dominic Camp (University of Leicester, UK) for the oligonucleotide primers and for generously sharing unpublished information, and Dr Don Hooper, (Chemistry Dept., Dalhousie University) for the NMR spectra and for helping with their interpretation. I also thank Dr. Pierre Thibault (Institute for Marine Biosciences, National Research Council of Canada) for the mass spectrum and its interpretation.

I would like to thank Nancy McAllister-Irwin for being a great friend and for helping me to see that there is life outside the lab! And finally I would like to extend my sincerest love and gratitude to my family. I especially thank Dad, for first inspiring and encouraging my interest in science, you were my first mentor; and Mum, whose unending optimism and support really pulled me through the hard times.

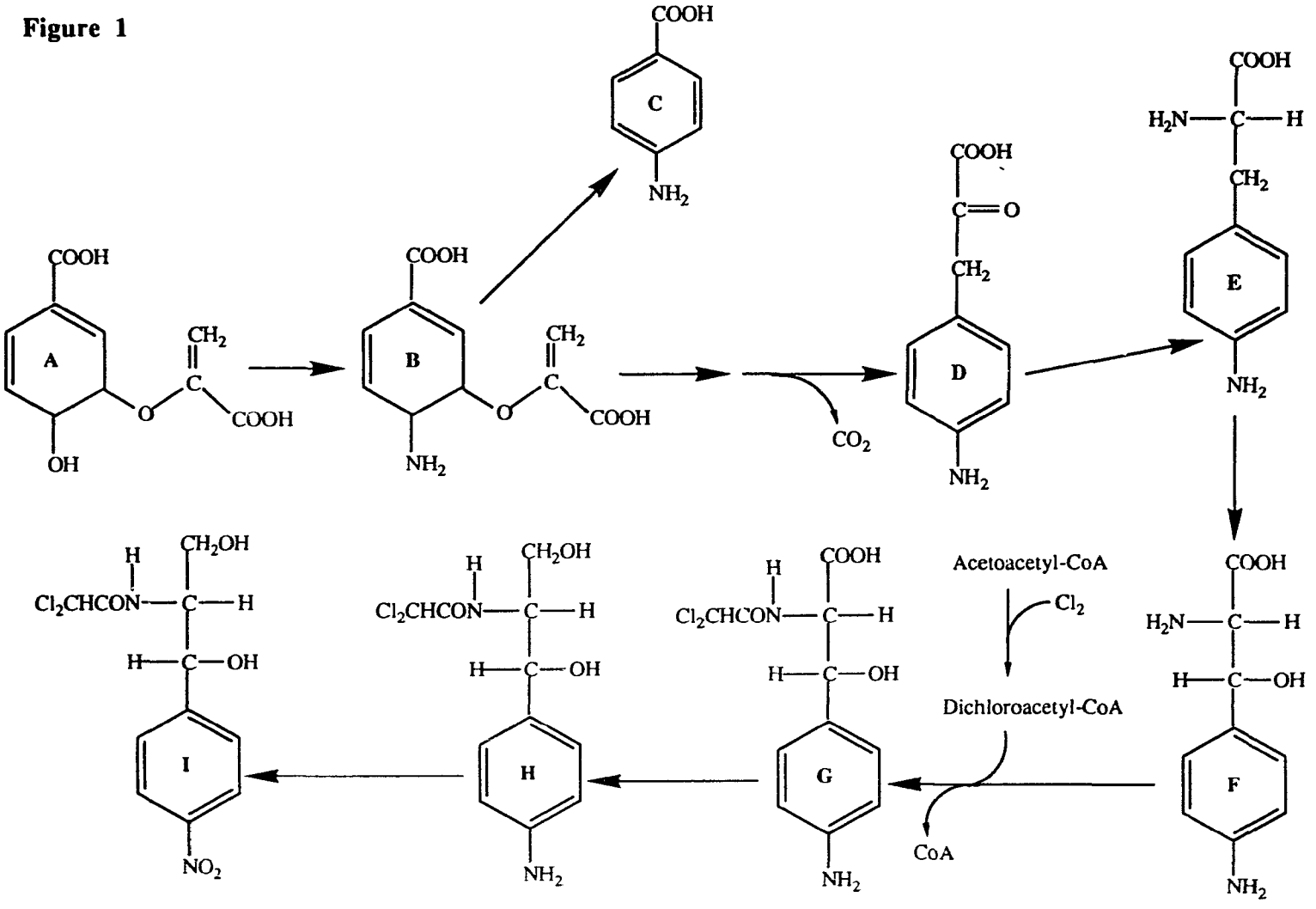
INTRODUCTION

Chloramphenicol is an antibiotic that possesses potent bacteriostatic activity and yet has a relatively simple molecular structure (Fig. 1). It was first isolated from cultures of *Streptomyces venezuelae* in 1947, but the production of chloramphenicol by fermentation was quickly supplanted by its chemical synthesis in 1949 (Malik, 1983). Chloramphenicol consists of three functional components: (i) a *p*-nitrophenyl moiety; (ii) a 1,3-propanediol moiety, and (iii) a dichloroacetamide group. Structure-function studies with chloramphenicol and its over 1000 derivatives indicate that removal of the dichloroacetyl side chain reduces bioactivity *in vivo*, primarily by reducing the rate of uptake through the cell membrane. However, the hydroxyl groups of the propanediol moiety are of utmost importance in the function of chloramphencol *in vitro* and *in vivo*; any modification of either hydroxyls results in a complete loss of bioactivity. The 1'-hydroxyl group can be replaced by an electronegative element such as fluorine without eliminating antibacterial activity, but fluoro-derivatives of chloramphenicol are highly toxic to mammals and are thus not of clinical value (Pongs, 1979; Shaw, 1983; and Yunis, 1988)

The specific pathway for biosynthesis of chloramphenicol (see Fig. 1) begins with chorismic acid. Chorismic acid is a branch-point metabolite in the shikimic acid pathway, leading to the aromatic protein amino acids tryptophan, phenylalanine, and tyrosine, and to *p*-aminobenzoic acid (PABA) and *p*-hydroxybenzoic acid. The first intermediate of the chloramphenicol biosynthetic pathway, 4-amino-4-deoxychorismic acid, is also the direct precursor of PABA. Recent evidence suggests that *S. venezuelae* ISP5230 may possess two genes for PABA synthase (P. Brown, personal communication); one of these may be clustered with the genes for chloramphenicol biosynthesis. The first truly unique step of the chloramphenicol biosynthetic pathway is probably the conversion of 4-amino-4-deoxychorismic acid to *p*-aminophenylpyruvic acid (PAPPA) via group translocation and oxidative decarboxylation. PAPPA can then be transaminated to form *p*-aminophenylalanine which is, in turn, hydroxylated to form *p*-aminophenylserine (PAPS) (Vining and

Figure 1 The chloramphenicol biosynthesis pathway **A** chorismic acid, **B** 4-amino-4-deoxychorismic acid, **C** *p*-aminobenzoic acid, **D** *p*-aminophenylpyruvic acid, **E** *p*-aminophenylalanine, **F** *p*-aminophenylserine, **G** α-*N*-dichloroacetyl-*p*-aminophenylserine, **H** α-*N*-dichloroacetyl-*p*-aminophenylserinol, **I** chloramphenicol

Figure 1


COOH
C
NH2
A
B
D
E
F
G
H
I
CH2
O
COOH
OH
CO2
Acetoacetyl-CoA
Cl2
Dichloroacetyl-CoA
CoA
H2N
CH2OH
Cl2CHCON
NO2

Westlake, 1984). PAPS is the most likely substrate for the incorporation of the dichloroacetyl side chain into the structure of chloramphenicol. Introduction of the side chain is thought to begin with the dichlorination and scission of acetoacetate to form dichloroacetoacetyl-CoA; the dichloroacetyl group is then transferred to the α-amino group of PAPS. The dichlorination step is probably catalyzed by one of two chloroperoxidase enzymes recently purified from cell extracts of *S. venezuelae* ISP5230 (Knoch *et al.*, 1989). The resulting α-N-dichloroacetyl-*p*-aminophenylserine is then reduced to the corresponding serinol, which is finally converted to chloramphenicol by oxidation of the *p*-amino group to a *p*-nitro group. A series of *S. venezuelae* mutants blocked in different steps of the chloramphenicol biosynthetic pathway has been isolated (Doull *et al.*, 1985). All of the mutations were mapped by conjugation and phage SV1-mediated transduction, to a 45-kb segment of the *S. venezuelae* chromosome between *cys* and *pdx* markers (Vats *et al.*, 1987). Many of the *S. venezuelae* mutants blocked in chloramphenicol production were more sensitive to chloramphenicol than the wild-type *S. venezuelae* (Doull, 1984).

Chloramphenicol resistance in *S. venezuelae* is not mediated by chloramphenicol acetyltransferase; instead an inducible permeability barrier and a constitutively expressed chloramphenicol hydrolase activity have been proposed as the primary mechanisms of resistance (Westlake and Vining, 1984). When *S. venezuelae* is grown under chloramphenicol-nonproducing conditions, exogenous chloramphenicol supplements are rapidly metabolized. However, under producing-conditions, the metabolism of exogenous chloramphenicol by *S. venezuelae* ceases when endogenous chloramphenicol production begins. Thus, as in many other antibiotic-producing streptomycetes, antibiotic resistance and biosynthesis in *S. venezuelae* are coordinately regulated.

Although chloramphenicol resistance in *S. venezuelae* has been studied in some detail, a number of unanswered questions still remain. For example, in *S. venezuelae* chloramphenicol biosynthesis occurs in the cytoplasm, and yet *S. venezuelae*, produces a constitutively expressed intracellular chloramphenicol hydrolase activity How does *S.*

venezuelae prevent the destruction of intracellular chloramphenicol? Furthermore, what is the nature of the inducible permeability barrier? Is it mediated by a chloramphenicol-efflux mechanism or by modification of the cell envelope? Antibiotic resistance genes are often clustered with antibiotic production genes on the chromosomes of streptomycetes Does coordinate expression of the proposed permeability barrier and chloramphenicol biosynthesis indicate that resistance and production genes are clustered in *S. venezuelae*?

To answer these questions and to fully understand chloramphenicol production, the chloramphenicol biosynthesis and resistance genes of *S. venezuelae* must be cloned and isolated. As a first step towards this goal, Mosher (1986) isolated a chloramphenicol resistance determinant by shotgun cloning *S. venezuelae* genomic DNA fragments in a streptomycete plasmid vector, and then using the genomic library to transform the chloramphenicol-hypersensitive strain *Streptomyces lividans* M252 By appropriate selection a chloramphenicol-resistant transformant, RM3, was isolated, it possessed a recombinant plasmid, pJV3, that contained a 6 5-kb insert of *S. venezuelae* DNA Preliminary biochemical studies suggested that pJV3 encoded the chloramphenicol-inactivating enzyme, chloramphenicol hydrolase.

The present study was designed to extend the results of Mosher (1986) by identifying the gene for chloramphenicol resistance cloned in pJV3, and by determining the mechanism of chloramphenicol resistance in the *S. lividans* transformant RM3 To accomplish this, the chloramphenicol resistance determinant in the 6 5-kb insert of pJV3 was localized by subcloning to a 2 4-kb DNA fragment The nucleotide sequence of the 2 4-kb fragment was determined and the putative chloramphenicol resistance gene was identified The mechanism of chloramphenicol resistance in *S. lividans* RM3 cultures was explored by using high performance liquid chromatography to follow the metabolism of exogenous supplements of chloramphenicol. The major product of chloramphenicol metabolism in RM3 was purified by preparative chromatography, and its identity was established from its spectroscopic properties.

LITERATURE REVIEW

The discovery of penicillinase-producing, antibiotic-resistant bacteria quickly followed the introduction of penicillin as the first clinically useful antibiotic (Waley, 1988). As new antibiotics were introduced into therapy and came into wide-spread use, a growing proportion of strains with multiple antibiotic resistance was encountered in the population of bacterial pathogens (Neu, 1992; Silver and Bostian, 1993). These antibiotic resistant bacteria evade the toxic effects of antibiotics by using at least one of the following general mechanisms: (i) antibiotic inactivation by chemical modification; (ii) cellular exclusion of the antibiotic; or (iii) modification of the antibiotic's target site (Davies, 1986).

The Gram-positive streptomycetes produce over 70% of the clinically useful antibiotics, many of which are autotoxic. In *Streptomyces*, the genes for antibiotic production and resistance are often clustered on the streptomycete chromosome; this close association facilitates the sequential expression of resistance and then production genes. Interestingly, the resistance mechanisms used by pathogenic bacteria are often analogous to those used by the antibiotic-producing streptomycetes. Significant similarities have been revealed by the direct comparison of amino acid sequences derived from various streptomycete antibiotic resistance genes with those of comparable resistance genes from pathogenic bacteria (Kirby, 1990). These and other studies have suggested that streptomycetes are the evolutionary progenitors of antibiotic resistance genes.

Many resistance genes of pathogenic enteric bacteria are found on R-plasmids. The hypothesis that they originated elsewhere and have been transferred intergenically was bolstered by the recent demonstration of conjugation between *Escherichia coli* and *Streptomyces lividans* (Mazodier and Davies, 1991). However, since many streptomycete genes are not expressed in *E. coli*, the chances of a direct transfer of antibiotic resistance genes from a streptomycete to an enteric pathogen are small. A more plausible hypothesis is that the genetic material was directly transferred to a closely related Gram-positive

bacterium; the genes would be altered slightly to accommodate the genetic machinery of the new host and then perhaps be transferred to another bacterium with a compatible but slightly different genetic environment. Successive rounds of transfer and adaptation would lead to the gradual dissemination of antibiotic resistance genes to bacteria distantly related to the original streptomycete host (Davies, 1992).

A variety of antibiotic resistance mechanisms have evolved in the antibiotic-producing streptomycetes. Many streptomycetes inactivate their autotoxic metabolites by direct covalent modification, and still others use cellular exclusion mechanisms or target-site modification (Cundliffe, 1989). However, this review will be limited to a discussion of the first two mechanisms only. It will compare the roles and functions of antibiotic resistance in both antibiotic-producing and antibiotic-nonproducing bacteria.

I. Antibiotic inactivation

A. Beta-lactams

The β-lactams are a family of clinically important antibiotics first discovered in the fungi (e.g. *Penicillium* sp., *Cephalosporium* sp., etc.) but also produced by a number of streptomycetes (e.g. *Streptomyces clavuligerus*, *Streptomyces jumonjinensis*, *Streptomyces lipmanii*), by other actinomycetes (e.g. *Nocardia lactamdurans*), and by some eubacteria. In the best known β-lactams the cyclic amide (β-lactam ring) is fused to either a thiazolidine or dihydrothiazine ring. The antibiotic spectrum of β-lactams is largely determined by the N-acyl-substituent attached elsewhere to the β-lactam ring. A large number of broad-spectrum penicillins and cephalosporins has been produced by either altering the fermentation conditions during antibiotic production, or by semi-synthesis, during which the acyl-group of penicillin G or cephalosporin C is enzymatically removed and then chemically replaced with one or other of a wide variety of acyl substituents (Gale *et al.*, 1981; Jensen, 1986).

Beta-lactams inhibit bacterial cell wall synthesis by irreversibly binding to the active site serine residue of transpeptidase, an enzyme responsible for the crosslinking of the peptidoglycan pentapeptide side-chains during cell wall synthesis. Most bacteria possess up to seven penicillin binding proteins (PBPs) located on the outer face of their cytoplasmic membranes. The larger PBPs are usually bifunctional enzymes catalyzing both transpeptidation and transglycosylation, while the smaller PBPs are usually DD-carboxypeptidases that catalyze the removal of a terminal D-alanine from the uncrosslinked pentapeptide side-chain (Gale *et al.*, 1981; Russell and Chopra, 1990).

The production of a β-lactamase is perhaps the most common cause of β-lactam resistance in Gram-negative bacteria. However, β-lactamase production has been observed in almost every bacterial strain tested; Gram-positive bacteria tend to excrete β-lactamase into their surrounding environment, while Gram-negative bacteria usually produce periplasmic enzymes (Waley, 1988). All β-lactamases catalyze the hydrolytic cleavage of the β-lactam ring, and range in molecular size from 12-50 kDa. A comparative analysis of the isoelectric points, amino acid sequences, and substrate profiles has resulted in the grouping of all known β-lactamases into four classes: **Class A** consists primarily of a group of plasmid-encoded enzymes from both Gram-positive and Gram-negative bacteria; **Class B** consists of a small group of metalloenzymes that require Zn^{2+} as a cofactor; **Class C** consists of a group of chromosomally-encoded enzymes found in most Gram-negative bacteria; and **Class D** consists of a small group of oxacillin-hydrolyzing enzymes. Classes A,C and D enzymes possess a serine residue at their active sites and are believed to catalyze hydrolysis of the β-lactam ring by essentially the same mechanism (Medeiros, 1989; Ghuysen, 1991).

In the serine-based enzymes, the catalytic process begins when the hydroxyl group of the active-site serine attacks the nucleophilic center at the β-lactam ring's amide bond. This generates an ester-linkage between the enzyme and antibiotic, and results in the formation of an acyl-enzyme intermediate. The ester-linkage is then hydrolyzed, the serine

group is regenerated, and a biologically inactive penicilloic acid is produced (Ghuysen, 1991).

A study of several aspects of β-lactamase structure and function has revealed that although the primary amino acid sequences of Class A and C enzymes are similar within the classes, there is very little similarity between classes. However, all serine-based β-lactamases do possess a similar active site structure, and x-ray crystallographic analysis has shown that even in enzymes that have little or no primary sequence similarity, there is an overall conservation of tertiary or three-dimensional structure. Interestingly, some of the low molecular weight PBPs, especially the extracellular DD-carboxypeptidase of *Streptomyces* R61, possess not only active site similarity to Class C β-lactamases but an overall similarity at the tertiary structure level. The extracellular DD-carboxypeptidase of *Streptomyces* R61 and other low molecular weight PBPs may be precursors of the periplasmic Class C β-lactamases (Ghuysen, 1991).

The mechanism by which the metalloenzymes catalyze hydrolysis of the β-lactam ring is poorly characterized. The zinc ion is tightly coordinated at the active site to three essential histidine residues and noncovalent interactions between the zinc ion and the β-lactam are extremely important to the reaction mechanism (Waley, 1988).

Several extracellular β-lactamases have been isolated from a number of β-lactam-nonproducing streptomycetes. Most of these enzymes appear to belong to Class A, as do most other β-lactamases from Gram-positive bacteria (Ogawara, 1993) All of these enzymes are similar to each other and to the Class A β-lactamases of other Gram-positive and Gram-negative bacteria, especially at the amino acid sequence level. However, many of the β-lactamases from Gram-positive bacteria such as *Bacillus* sp. and *Staphylococcus* sp. possess greater amino acid sequence similarity to streptomycete β-lactamases than to those from Gram-negative bacteria. *Streptomyces* enzymes may, therefore, represent a superfamily of β-lactamases from which the Class A enzymes of other Gram-positive and negative bacteria evolved (Forsman *et al.*, 1990).

Although nearly all species of *Streptomyces* produce low levels of β-lactamase, the function or role of this enzyme is uncertain. The PBPs of all streptomycetes tested possess an exceptionally low affinity for β-lactam antibiotics and this reduced affinity may provide the primary mechanism of resistance, even in those streptomycetes that produce high levels of β-lactamase (Ogawara, 1981).

Surprisingly, cultures of the cephamycin-producing *S. clavuligerus* possess no detectable β-lactamase activity (Ogawara and Horikawa, 1980). Instead *S. clavuligerus* produces clavulanic acid, a potent inhibitor of many β-lactamases, and also BIP, a proteinaceous β-lactamase inhibitor (Doran *et al.*, 1990). However, Coque *et al.* (1993) have recently reported the cloning of a β-lactamase gene from the cephamycin-producing *N. lactamdurans*. The β-lactamase is very similar (>50% identical amino acids) to the Class A β-lactamases from β-lactam nonproducing streptomycetes. The enzyme does differ from other streptomycete β-lactamases in being primarily cell bound. Interestingly, when protoplasts were formed from the *N. lactamdurans* mycelium, nearly all of the cell bound β-lactamase was released into the surrounding medium. The high level of mycolic acids characteristically present in nocardial cell walls may have blocked release of the β-lactamase and effectively created a periplasmic space.

Coque *et al.* (1993) noted that the nocardial β-lactamase has no significant effect on cephamycin C, however, it does attack penicillin N and isopenicillin N, known intermediates in the cephamycin biosynthetic pathway Perhaps the most interesting result of this study was the discovery that the β-lactamase gene is clustered with the cephamycin production genes. Coque *et al.* suggested that this finding supports the hypothesis that the Class A superfamily of β-lactamases had its origin in the β-lactam-producing actinomycetes. It is not known whether *S. clavuligerus* or any of the other β-lactam-producing streptomycetes possess β-lactamase genes. However, a number of β-lactamase genes cloned from various β-lactam nonproducing streptomycetes, gave strong signals when

hybridized with the genomic DNA of *S. clavuligerus* and other β-lactam producers (Jaurin *et al.*, 1988).

B. Aminoglycosides

The aminoglycosides are a large group of naturally occurring or semisynthetic bactericidal antibiotics which, with the exception of spectinomycin, consist of one or more aminosugars covalently linked to an aminocyclitol. Spectinomycin although normally classified as an aminoglycoside, does not possess an aminosugar, only an aminocyclitol; it is more accurately classified as an actinamine antibiotic. The aminoglycosides can be subdivided into two groups based on the aminocyclitol they possess. The largest and most clinically important group possesses the aminocyclitol 2-deoxystreptamine; this group can be further subdivided into 4,5-disubstituted deoxystreptamines (e.g neomycin B) and 4,6-disubstituted deoxystreptamines (e.g. kanamycin A, amikacin, tobramycin, and the gentamicins). The smaller group consists of antibiotics with the aminocyclitol streptamine (e.g. streptomycin, hydroxystreptomycin, and bluensomycin) none of which are used clinically (Davies and Yagisawa, 1983; Russell and Chopra, 1990; Lambert and O'Grady, 1992).

The aminoglycosides have only limited usefulness against Gram-positive bacteria but are extremely effective against a number of Gram-negative pathogens (Lambert and O'Grady, 1992). All aminoglycosides inhibit bacterial protein synthesis; the mechanism of action depends on the aminocyclitol portion of the antibiotic. Streptomycin binds irreversibly to the 30S subunit of the prokaryotic ribosome. If streptomycin binds before the 30S subunit joins with the 50S subunit, the initiation complexes subsequently formed are largely ineffective. If the antibiotic binds after the initiation complex has formed, protein synthesis is slowed and misreading of the mRNA occurs. However, the bactericidal effect of the antibiotic is primarily due to the ability of streptomycin to inhibit the formation of competent initiation complexes. The deoxystreptamine-based antibiotics are also bacteri-

cidal but bind to both subunits of the prokaryotic ribosome. Unlike the streptamine antibiotics, they inhibit protein synthesis primarily by blocking the binding of elongation factor G (Russell and Chopra, 1990).

The wide-spread and indiscriminate use of aminoglycoside antibiotics has resulted in the occurrence of a number of resistant pathogenic strains. Resistance in these bacteria is usually acquired by way of extrachromosomal R-plasmids or transposons which typically encode antibiotic inactivating enzymes. There are three major mechanisms of enzymatic inactivation mediated by three well characterized enzyme families (i) the N-acetyltransferases (AAC); (ii) the O-phosphotransferases (APH); and (iii) the O-nucleotidyltransferases (ANT) (Lambert and O'Grady, 1992). Interestingly, these enzymes can be grouped into classes, based on the aminocyclitol present in the target antibiotic. For example, none of the enzymes that act upon the streptamine antibiotics act on the deoxystreptamine antibiotics, and vice versa. However, many enzymes are cross-reactive within their respective classes (Cundliffe, 1989).

Aminoglycosides are produced primarily by streptomycetes and other actinomycetes (Davies and Yagisawa, 1983). Many of these producers possess aminoglycoside modifying activities which are similar in mechanism and specificity to those encoded by the R-plasmids. Furthermore, many producing organisms possess multiple aminoglycoside modifying enzymes. The best characterized example of this is in the streptomycin-producing *Streptomyces griseus* that produces at least two distinct aminoglycoside phosphotransferases, APH(6) and APH(3"), both of which readily phosphorylate streptomycin but at different sites (Heinzel *et al.*, 1988).

The *aphD* gene, encoding APH(6), is clustered with the streptomycin biosynthesis genes in *S. griseus* (Distler *et al.*, 1987). The *aphD* gene is located immediately downstream of *strR*, which encodes a positive regulatory protein that activates at least some of the biosynthesis genes. Northern analysis has shown that *aphD* and *strR* are cotranscribed; this operon-like arrangement is of vital importance in permitting the initial pro-

duction of the streptomycin-modifying enzyme encoded by *aphD* immediately before the onset of antibiotic synthesis. The *strR* gene is positively regulated by A-factor, a hormone-like substance produced by *S. griseus* just before the onset of streptomycin biosynthesis. Recent evidence has suggested that activation of *strR* by A-factor, involves at least two proteins, one of which binds directly to A-factor; the other is a repressor-like protein that binds to the promoter region of *strR*. The A-factor binding protein somehow causes the repressor protein to release the *strR* promoter, allowing transcription of both *strR* and *aphD* (Horinouchi and Beppu, 1992).

APH(6) phosphorylates not only streptomycin but many of the early intermediates in the streptomycin biosynthetic pathway; apparently APH(6) ensures that all of the metabolites starting with the formation of the streptidine moiety are phosphorylated (Cundliffe, 1992). *Streptomyces griseus* produces an extracellular streptomycin-6-phosphate phosphatase, which may activate the phosphorylated antibiotic after it is exported from the cell. The *strK* gene, encoding the phosphatase, is clustered with the streptomycin biosynthetic genes (Distler *et al.*, 1992).

The function or role of APH(3") is at present unknown and the *aphE* gene, encoding APH(3"), is apparently not clustered with the streptomycin biosynthetic genes. However, the extracellular phosphatase produced by *S. griseus* does possess some activity towards streptomycin 3"-phosphate, albeit at much lower level than with streptomycin 6-phosphate (Heinzel *et al.*, 1988; Mansouri and Piepersberg, 1991).

Several attempts have been made to identify the evolutionary origins of the R-factor encoded aminoglycoside-modifying enzymes. Many of these studies have pointed toward the aminoglycoside-producing actinomycetes (Trieu-Cuot *et al.*, 1987; Kirby, 1990; Shaw *et al.*, 1993). However, there is evidence that some of the enzymes may have derived from primary metabolic pathway enzymes. The substrate specificity of some enzymes might have been altered as a consequence of the strong selective pressure of intense antibiotic therapy (Shaw *et al.*, 1993).

C. Chloramphenicol

Chloramphenicol was first isolated from the cultures of *Streptomyces venezuelae* in 1947 by Parke Davis and Company. It possessed a broad spectrum of bacteriostatic activity and was quickly adopted as a potent antibiotic. Furthermore, because of its relatively simple chemical structure, the production of chloramphenicol by fermentation was quickly supplanted by its chemical synthesis on an industrial scale (Malik, 1983; Shaw, 1983).

The excellent pharmokinetic properties of chloramphenicol are largely offset by its apparent hemotoxicity. The most common effect of prolonged chloramphenicol treatment is a reversible, dose-related suppression of bone marrow erythropoiesis, which usually occurs when the concentration of the antibiotic exceeds 25 $\mu g.mL^{-1}$. Rarely (1 out of 20,000 to 600,000 cases) a fatal and irreversible aplastic anemia occurs. This effect is not dose-related, and no standard method for predicting the resulting pancytopenia and aplasia has ever been developed. As a result the clinical use of chloramphenicol is severely restricted; it is still recommended for the treatment of typhoid fever, various salmonella infections, anaerobic lung infections caused by *Bacteroides fragilis*, rickettsial infections, and bacterial meningitis caused by *Haemophilus influenzae*. Chloramphenicol is especially indicated for bacterial meningitis since it is bactericidal for *H. influenzae* and because it can reach high concentrations in the cerebro-spinal fluid (Lambert and O'Grady, 1992; Pongs, 1979; Yunis, 1988).

The bacteriostatic action of chloramphenicol is largely attributable to its inhibition of bacterial protein synthesis. Chloramphenicol binds primarily and reversibly to the 50S ribosomal subunit and inhibits protein synthesis by inhibiting peptidyltransferase activity. It is believed that chloramphenicol prevents or blocks peptidyltransferase from recognizing aminoacyl tRNAs within the acceptor (A) site of the ribosome (Cundliffe, 1990). Recent evidence suggests that chloramphenicol interacts directly with the 23S rRNA of the 50S

subunit by binding to nucleotides 2497-2505 (Marconi *et al.*, 1990). Chloramphenicol may bind weakly to another site on the 30S subunit.

Chloramphenicol resistance is most commonly mediated by chloramphenicol acetyltransferase (CAT) activity. CAT-producing strains of multiply antibiotic resistant *Shigella* were first observed in Japan in 1955. Since then, CAT genes have been found in nearly all bacterial species examined. In the enteric bacteria CAT genes are usually present on plasmids, transposons, or phage. The ubiquity of CAT in enteric bacteria has led to the establishment of three classes of the enzyme, primarily based on molecular weight, immunological cross reactivity, and response to specific enzyme inhibitors. CAT has been isolated from various non-enteric Gram-negative and Gram-positive bacteria including staphylococci, streptococci, *Bacillus* sp., and *Streptomyces* sp. (Shaw, 1983).

The best characterized enzyme is the Class III CAT (CAT_{III}) encoded by the conjugative plasmid R387. This enzyme is a trimeric protein with a subunit M_r of 24,965 Da (Shaw and Leslie, 1991). CAT catalyzes the transfer of an acetate group from its co-factor acetyl CoA to the C-1' hydroxyl of chloramphenicol. The acetate group is then transferred to the C-3' hydroxyl group by a nonenzymatic intra-molecular rearrangement. CAT then reacylates the C-1' hydroxyl group. Steady state kinetic studies have shown that the initial acetylation reaction occurs rapidly ($K_{CAT} = 600s^{-1}$) while the subsequent rearrangement and reacylation of 1-acetoxychloramphenicol are relatively slow (Shaw and Leslie, 1989).

A number of studies, culminating with the elucidation of the three-dimensional structure of CAT_{III} by x-ray crystallography, have identified a conserved histidine residue as essential for catalytic activity. X-ray crystallography has confirmed the structure and configuration of the enzyme's active site. These data, combined with the kinetic studies, indicate that the acetylation of chloramphenicol proceeds sequentially, without the formation of an acyl-enzyme intermediate. A structural model of the enzyme has revealed that the enzyme's active site is located within a tunnel, created at the interface between the

three subunits and extending from one side of the enzyme to the other (Shaw and Leslie, 1991).

X-ray crystallography of chloramphenicol-CAT_{III} and acetyl CoA-CAT_{III} complexes has suggested that the propanediol portion of the chloramphenicol molecule is directed into one side of the active site tunnel, while the acetate-carrying pantetheine chain of acetyl CoA is guided into the opposite side of the active site tunnel. The proposed catalytic mechanism begins with the C-1' hydroxyl group of chloramphenicol positioned within 2.8 Å of the N-3 atom of the imidazole side chain of histidine 195. The C-1' hydroxyl group of chloramphenicol is deprotonated by the imidazole nitrogen, which acts as a general base, and the resulting nucleophilic oxyanion attacks the adjacent (3.3 Å from the imidazole N-3 atom) acetate carbonyl group of acetyl CoA. The resulting tetrahedral transition state intermediate is probably stabilized by hydrogen bond formation with the hydroxyl group of serine 148 and ultimately resolves itself, generating 1-acetoxychloramphenicol and CoA (Shaw and Leslie, 1991). The catalytic mechanism of CAT_{III} is notable not only for its lack of an acyl-enzyme intermediate but also because of its extremely high efficiency.

II. Cellular exclusion

A. Outer membrane impermeability

The cells of Gram-negative bacteria are surrounded by two lipid bilayer membranes. The inner or cytoplasmic membrane is separated from the outer membrane by the periplasmic space. The outer membrane of many Gram-negative bacteria is highly asymmetric; the outer face of the membrane is composed of an extremely rigid layer of lipopolysaccharide and lipoprotein, while the inner face consists primarily of phospholipid. The outer membrane is impermeable to lipophilic and hydrophobic solutes, and therefore functions as an efficient sieving barrier for many of the large and hydrophobic antibiotics

(e.g. actinomycin D; thiostrepton, novobiocin, rifamycin and the macrolides) (Nikaido, 1989).

However, the effectiveness of the outer membrane as a sieving agent against small and relatively hydrophilic antibiotics is largely compromised by the presence of porins. The porins are a family of proteins that form water-filled channels through the outer membrane. A number of antibiotics including chloramphenicol, hydrophilic β-lactams, aminoglycosides, and tetracyclines gain entry to the periplasm through these channels. Alterations in the type and number of porin proteins in the outer membrane have frequently been linked to the antibiotic resistance of a number of clinically important Gram-negative bacteria. Nevertheless, changes in porin content have not been effective in conferring enhanced resistance, except when such changes are linked to the presence of a periplasmic β-lactamase (Hancock, 1987; Nikaido, 1989).

B. Active efflux of antibiotics

The passage of most molecules through the cytoplasmic membrane is usually mediated by facilitated or active transport systems. In bacteria, most solutes are taken up (influx) or removed from the cell (efflux) by active transport. Active transport is usually controlled by integral membrane proteins which form "gated" channels across the cytoplasmic membrane. Typically, these proteins couple the transport of solutes against a concentration gradient to the proton motive force (pmf) or to the direct hydrolysis of ATP. Perhaps the best studied system in bacteria is the active transport of sugars Sugars are usually taken up by cells using a H^+/symport mechanism during which the transport of a sugar molecule is coupled to the transport of a proton into the cell. The proton moves down a voltage gradient as well as a concentration gradient, and in doing so is believed to release sufficient free energy to drive the transport of a sugar molecule up a concentration gradient. Interestingly, the proteins that mediate H^+/symports in bacteria are similar in many respects to the sugar transport proteins in mammalian systems. Both groups consist

of integral membrane proteins that possess alternating hydrophobic and hydrophilic regions that typically form 12 hydrophobic or amphipathic α-helical transmembrane segments. A comparison of the amino acid sequences of many of these proteins reveals a significant level of similarity between the mammalian and bacterial proteins. However, the two groups differ in that the bacterial proteins are H^+/symporters whereas the mammalian proteins are passive or facilitated transport proteins. This difference is thought to reflect the disparity between the cellular environments of the two cell types. Animal cells are usually exposed to relatively constant levels of carbohydrate in the bloodstream (5-10 m*M*) whereas a bacterial cell normally operates in conditions of much lower sugar concentration (Nikaido and Saier, 1992; Baldwin, 1992).

The best characterized example of antibiotic efflux is the active transport of tetracyclines by the TET family of proteins in the Gram-negative bacteria. These proteins, like those of sugar transport, are integral membrane proteins and may form 12 α-helical transmembrane segments. The TET proteins are not significantly similar to the sugar transport proteins at the amino acid sequence level but they do promote the efflux of tetracycline using a H+/antiport mechanism. The antiport mechanism is similar to the symport mechanism except that the transport of a proton across the membrane is coupled to the efflux of a solute rather than to its influx. A smaller family of tetracycline efflux proteins (TETL and TETK) has been identified in the Gram-positive staphylococci and bacilli. These TET proteins possess some amino-terminal amino acid sequence similarity to the Gram-negative TET proteins but differ in that they probably form 14 instead of 12 α-helical transmembrane segments (Levy, 1992).

In an effort to understand the mechanism of action and evolution of these transport proteins, a number of investigations have been carried out during which sequence comparisons, site-directed mutagenesis and affinity labeling have been used to identify some of the important functional amino acid residues within the proteins. Initial comparisons of the sugar transport proteins at the amino acid level showed a set of conserved sequence

motifs that occurred at specific points within each of the proteins. A detailed comparison of these motifs revealed that some were repeated more than once within the sequence of the protein. For example, the motif (N/D)(R/K)XGR(K/R) was found between helices 2 and 3; a similar motif (E/D)(R/K)XGR(R/K) was identified between helices 8 and 9. The motif PESPR, following just after helix 6, is partially repeated as the motif PETKG after helix 12. This and other evidence has suggested that many of the present day 12-helix transport proteins evolved by gene duplication of an ancestral 6-helix protein gene (Baldwin, 1992).

Alignment of the tetracycline resistance proteins from Gram-negative bacteria has revealed a number of conserved motifs present in the mammalian and bacterial sugar transport proteins; the (N/D)(R/K)XGR(K/R) motif between helices 2 and 3 is especially well conserved. Likewise, all of the TET proteins possessed a large cytoplasmic loop of hydrophilic amino acids separating the first 6 α-helical segments from the 6 carboxy terminal helices a structural feature shared by nearly all of the sugar transport proteins. Significantly, mutations in either half of a TET protein eliminated antibiotic transport. However, when two genes for the same TET protein were introduced into one cell, each possessing a single mutation in either the amino-terminal helix cluster or the carboxy-terminal cluster, partial complementation and restoration of tetracycline efflux was observed. The results of this experiment together with those of other studies have suggested that, like the sugar transport proteins, the TET proteins probably arose by duplication of an ancestral 6-helix protein gene (Levy, 1992).

Subsequently, several putative antibiotic efflux genes from a variety of bacteria, both Gram-negative and positive, have been cloned. All of the proteins encoded by these genes can be grouped into two large categories: those with 12 transmembrane segments (TMS), and those with 14 TMS. Interestingly, almost all of the 12 TMS proteins possess a large hydrophilic, cytoplasmic loop between helices 6 and 7, a distinctive feature not shared by any of the 14 TMS proteins. Alignment of all proteins from both categories re-

vealed that each possesses conserved sequence motifs; however, these occur within the first 6 amino-terminal TMS. Within the two categories, each protein shares conserved motifs throughout its amino acid sequence. Alignments of the amino-terminal and carboxy-terminal TMS of the 12 TMS proteins have revealed considerable sequence similarity within each protein. However this was not true for analogous alignments of the 14 TMS proteins. These studies have suggested that the amino-terminal 6 TMS of both 12 and 14 TMS proteins evolved from a common 6 TMS ancestral protein. In the case of the 12 TMS proteins, the ancestral 6 TMS protein gene probably underwent gene duplication, generating a new gene encoding a 12 TMS protein. In the 14 TMS proteins the ancestral 6 TMS gene probably fused with a completely different 8 TMS gene, yielding a gene encoding a 14 TMS protein. The identity of the ancestral 8 TMS gene is still speculative (Paulsen and Skurray, 1993).

Until recently very few examples of antibiotic efflux proteins coupled to the hydrolysis of ATP were known in bacteria. The best characterized example of an ATP-driven antibiotic efflux system is the P-glycoprotein from mammalian cells. The P-glycoprotein is a large membrane-associated protein that promotes the active efflux of a number of chemotherapeutic drugs, including antitumor agents such as daunorubicin and doxorubicin (Endicott and Ling, 1989). The P-glycoprotein possess two almost identical ATPase domains, each of which is similar to a number of bacterial ATPase proteins, including many of the proteins involved in the efflux of toxic ions from bacterial cells (Silver *et al.*, 1989, Silver and Walderhaug, 1992).

Two antibiotic resistance genes, *drrA* and *drrB*, were recently cloned from the daunorubicin/doxorubicin producer *Streptomyces peucetius*. The genes are translationally coupled, and the presence of both is required to confer daunorubicin/doxorubicin resistance on the sensitive host *Streptomyces lividans*. Furthermore, both genes appear to be clustered closely with the antibiotic biosynthetic genes of *Streptomyces peucetius*. The *drrA* gene encodes a protein (DrrA) with a number of similarities to the ATPase domains

of the P-glycoprotein. The *drrB* gene encodes an extremely hydrophobic protein, DrrB, that is most likely an integral membrane protein. The DrrB amino acid sequence shows no similarity to that of any other known protein, and it has been suggested that DrrA and DrrB act in concert to actively export daunorubicin/doxorubicin from the cytoplasm of the producing organism (Guilfoile and Hutchinson, 1991).

Resistance genes from three different macrolide-producing streptomycetes have recently been cloned. All of these genes encode proteins with sequence similarity to the ATPase domains of the P-glycoprotein; however, it is not known whether these genes are closely associated with other genes involved in the actual transport of the antibiotics (Schoner *et al.*, 1992). A cluster of bleomycin resistance genes has been cloned from the bleomycin-producer *Streptomyces verticillus*. One of the cloned genes, *bleT*, encodes a protein that is similar to the ATPase domains of the P-glycoprotein. The *bleT* gene appears to be translationally coupled with an upstream open reading frame called *bleX*; it is not known whether *bleX* encodes another component of the putative bleomycin efflux system (Calcutt and Schmidt, 1991).

Nearly all of the well characterized and putative ATPase proteins possess two ATP-binding domains, one of which is located at the amino terminus and the other at the carboxy terminus of the proteins. Alignment of the amino acid sequences of the these two domains from a variety of different proteins has revealed a number of very significant similarities. It has been suggested that like the 12 TMS H^+/antiport proteins, many of the present day ATPase proteins evolved from an ancestral ATPase gene, encoding a protein with only one ATPase domain. A duplication of the ancestral gene most likely generated a fusion gene, encoding a protein with two ATPase domains (Silver *et al.*, 1989).

III. Antibiotic resistance in antibiotic producing bacteria

As the primary producers of antibiotics, streptomycetes invariably possess one or more mechanisms to protect themselves from their own autotoxic products. Many of these mechanisms involve direct covalent modification of the antibiotic. In some cases, however, the modifying enzymes are actually biosynthetic enzymes, the genes for which are clustered and co-regulated with other biosynthetic genes. The best examples of this are the *aphD* gene from *S. griseus*, the *pac* gene (encoding puromycin acetyltransferase) from the puromycin-producing *Streptomyces alboniger*, and the *bar* gene (encoding bialaphos acetyltransferase) from the bialaphos-producer *Streptomyces hygroscopicus*. Each of these genes plays a vital role in the biosynthetic pathway, ensuring that potentially toxic intermediates are inactive within the cell (Cundliffe, 1989; Distler *et al.*, 1992; Lacalle *et al.*, 1992; Kumada *et al.*, 1988).

Many streptomycetes are thought to excrete their antibiotic products in an inactive form. The antibiotics are then activated outside of the cell. *Streptomyces griseus* produces the extracellular enzyme streptomycin phosphate phosphatase, and likewise *S. alboniger* and *S. hygroscopicus* produce puromycin N-acetyl hydrolase and bialaphos N-acetyl hydrolase, respectively. The genes for each of these "activating" enzymes are clustered with their respective antibiotic biosynthetic genes. For example, the *pac* gene is located immediately downstream of the *dmpm* gene which encodes an enzyme catalyzing the last biosynthetic step in the puromycin pathway. Both genes are cotranscribed from the same promoter and, therefore, may be co-regulated (Raibaud *et al.*, 1991; Distler *et al.*, 1992; Lacalle *et al.*, 1992; Cundliffe, 1992).

A number of putative antibiotic transport genes are clustered with the corresponding production genes (Neal and Chater, 1987; Caballero *et al.*, 1991; Raibaud *et al.*, 1991; Guilfoile and Hutchinson, 1992; Butler *et al.*, 1989; Coque *et al.*, 1993). Many of these genes encode proteins with multiple hydrophobic segments, and some are significantly similar to each other and to the TET proteins of Gram-negative and Gram-positive bacte-

ria. The majority of these genes confer antibiotic resistance when introduced into sensitive hosts; however, most antibiotic producers appear to possess antibiotic resistance mechanisms in addition to the putative transport proteins. Thus, the primary role of antibiotic efflux proteins in some producing organisms may be antibiotic export, rather than antibiotic resistance (Butler *et al.*, 1989; Zhang *et al.*, 1992).

IV. Chloramphenicol resistance in *Streptomyces*

Chloramphenicol resistance in many streptomycetes is probably due to the presence of CAT activity (Shaw and Hopwood, 1976, Gil *et al.*, 1985, Mosher *et al.*, 1990). However, some streptomycetes exhibit an amplifiable, nonenzymatic chloramphenicol resistance mechanism. Recently, a chloramphenicol resistance gene was cloned from a chloramphenicol hyper-resistant strain of *Streptomyces lividans* Although *S. lividans* does not produce chloramphenicol, resistant variants possessing high-level antibiotic resistance can be selected by culturing the organism on increasing concentrations of chloramphenicol; Southern hybridization of the cloned gene to the genomic DNA of the hyper-resistant strain showed that the DNA carrying the resistance gene had been amplified many times within the *S. lividans* genome. Sequence analysis of the cloned DNA revealed one large open reading frame that encoded an extremely hydrophobic protein (CmlG) with significant similarity to the TET proteins of Gram-negative and Gram-positive bacteria. It was suggested that sufficiently high cellular levels of this protein may confer chloramphenicol resistance via active efflux of the antibiotic (Dittrich *et al.*, 1991).

An inducible chloramphenicol resistance gene has been cloned from the actinomycete *Rhodococcus fascians*. This gene is normally located on a large plasmid, native to *R. fascians*. Sequence analysis of the cloned resistance gene revealed one large open reading frame encoding a hydrophobic protein (CmlR) 52% identical to the CmlG protein of *S. lividans*. Analysis of the DNA upstream of the *cmlR* gene showed a number of inverted repeat sequences. When a fragment of this upstream region was subcloned in front

of a *xylE* reporter gene, it conferred chloramphenicol inducible expression of the *xylE* encoded catechol oxidase (Desomer *et al.*, 1992).

V. Chloramphenicol resistance in chloramphenicol producers

The chloramphenicol producer *Streptomyces venezuelae* 13s possesses chloramphenicol-sensitive ribosomes throughout its life-cycle (Malik, 1970). *In vitro* protein synthesis, with ribosomes extracted from either producing or nonproducing mycelium, was equally sensitive to chloramphenicol. *In vivo* protein synthesis, in antibiotic nonproducing-cultures was initially sensitive to exogenously supplied chloramphenicol, but synthesis in mycelium exposed to the antibiotic resumed after a short lag. When *S. venezuelae* was grown under chloramphenicol producing conditions, *in vivo* protein synthesis was also inhibited if cultures were exposed to exogenous antibiotic during early growth, before endogenous antibiotic production had begun; however, as endogenous production increased, the ability of exogenous chloramphenicol to inhibit *in vivo* protein synthesis decreased. Malik (1970) suggested that the apparent decrease in sensitivity of *in vivo* protein synthesis was caused by a decrease in cellular permeability towards antibiotic supplements associated with production of the antibiotic by the mycelium

Nonproducing cultures of *S. venezuelae* rapidly metabolized exogenously supplied chloramphenicol to *p*-nitrophenylserinol and then subsequently to N-acetyl-*p*-nitrophenylserinol; neither CAT activity nor O-acetylated derivatives of chloramphenicol were ever detected in *S. venezuelae* cultures (Malik and Vining, 1970; Malik and Vining, 1971; Shaw and Hopwood, 1976). Cell extracts of *S. venezuelae* 13s did possess a constitutively expressed, intracellular chloramphenicol hydrolase (CAH) activity. The specific activity of CAH in mycelial extracts was unaffected by increasing levels of exogenously supplied antibiotic. However, the metabolic decomposition of chloramphenicol decreased sharply in chloramphenicol-producing cultures as endogenous antibiotic production increased. At titres of endogenously produced antibiotic in the 25-30 $\mu g.mL^{-1}$ range, meta-

bolism of exogenously supplied chloramphenicol ceased. Since incorporation into the mycelium of radioactivity from exogenously supplied [6-^{3}H]glucose decreased drastically at this time, it was suggested that a change in overall cell permeability in the chloramphenicol-producing cultures might be responsible.

Although the extent of chloramphenicol decomposition in the milieu of nonproducing cultures supplemented with the antibiotic was not sufficient to reduce the concentration below the minimum inhibitory level, the mycelium became resistant and growth occurred. Malik and Vining (1970, 1971, 1972) concluded that the primary mechanism of chloramphenicol resistance in *S. venezuelae* was an inducible change in membrane permeability, perhaps mediated by a change in the rate of antibiotic uptake or by a chloramphenicol inducible efflux protein. An efflux system of the kind postulated was later found in *R. fascians* (Desomer *et al.*, 1992). It was considered possible that an enhanced level of antibiotic efflux coordinated with a reduced rate of antibiotic uptake represented the actual resistance mechanism.

Mosher *et al.* (1990) isolated a genomic DNA fragment from the chloramphenicol producer, *S. venezuelae* ISP5230 that conferred chloramphenicol resistance when introduced into a chloramphenicol-sensitive strain of *S. lividans*. Genomic DNA digested with the restriction enzyme *Sst*I was shotgun cloned into the cloning vector pIJ702 and used to transform the chloramphenicol-sensitive host *S. lividans* M252. The chloramphenicol-resistant transformant, RM3, contained a recombinant plasmid, pJV3, that carried a 6.5-kb piece of *S. venezuelae* DNA. When the ^{32}P-labeled 6.5-kb DNA fragment was used to probe *Sst*I-digested genomic DNA from various sources, only *S. venezuelae* 13s showed a hybridizing region. Because *Streptomyces phaeochromogenes* NRRLB 3559, another chloramphenicol producer, showed no hybridization, the cloned resistance gene was either not associated with chloramphenicol biosynthesis or was unable to hybridize with DNA from *S. phaeochromogenes* due to sequence divergence in the two species.

Initial experiments with *S. lividans* RM3, suggested that the cloned DNA fragment encoded a chloramphenicol-inactivating enzyme (Mosher, 1986). When cultures of *S. lividans* RM3 were supplemented with chloramphenicol, they underwent an initial lag in growth that was directly proportional to the amount of antibiotic added. This growth pattern was very similar to the one observed in cultures of chloramphenicol-resistant, CAT-producing strains of *E. coli* (Shaw, 1975).

Interestingly, nonproducing cultures of *S. venezuelae* 13s and *S. venezuelae* ISP5230, both showed a lag in growth proportional to increasing amounts of exogenously added antibiotic. However, by the end of their lag periods, both chloramphenicol producers had adapted to the level of antibiotic that initially inhibited their growth and showed no apparent growth lag when subcultured in a medium containing the same amount of chloramphenicol (Malik, 1970; Doull, 1984). The adaptive behavior of the *S. venezuelae* strains was presumed to result from an inducible permeability barrier, since one subculture of the adapted mycelium through antibiotic-free medium returned both organisms to their initial sensitive states. In contrast to *S. venezuelae*, *S. lividans* RM3 did not adapt to increasing levels of chloramphenicol; it always exhibited a lag in growth, even when subcultured from a high concentration of antibiotic to a lower concentration. These results suggested that RM3 possessed the chloramphenicol inactivating activity of *S. venezuelae* but lacked its inducible permeability barrier.

In support of the first possibility, a culture of *S. lividans* RM3 incubated for 4 days with [U-^{14}C]chloramphenicol, yielded by ethyl acetate extraction only 10% of the radioactivity added to the culture (Mosher, 1986). Subsequently, Mosher *et al.* (1990) identified labeled N-acetyl-*p*-nitrophenylserinol and *p*-nitrobenzyl alcohol in the ethyl acetate extract. These results were consistent with the presence, on the cloned fragment of *S. venezuelae* DNA, of a chloramphenicol hydrolase gene that conferred resistance on *S. lividans* M252 by antibiotic inactivation.

MATERIALS AND METHODS

I. Organisms and plasmids

All bacteria and plasmids used in this study are described in Table 1 Helper phage VCSM13 (Stratagene) was a gift from Dr R Lee, Biology Dept , Dalhousie University

II. Chemicals and biochemicals

Chemicals and solvents were of reagent grade unless otherwise stated Lysozyme was purchased from Boehringer-Mannheim, Montreal, PQ Phage lambda DNA, restriction endonucleases, T4-DNA ligase, exonuclease III, mungbean nuclease and Klenow fragment (*Escherichia coli* DNA polymerase I large fragment) were purchased from Bethesda Research Laboratories, Gaithersburg, MD, or Pharmacia Biotech Inc , Baie D'Urfé, PQ Polyethylene glycol 8000, chloramphenicol, 1,3-diacetoxychloramphenicol, ampicillin (sodium salt), kanamycin sulphate and *N,N,N',N'*-tetramethylenediamine (TEMED) were purchased from the Sigma Chemical Co , St Louis, MO 3-(*N*-Morpholino)propanesulfonate (MOPS), tris-(hydroxymethyl)aminomethane (Tris) in technical and ultrapure grades, N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonate (TES), agarose (electrophoresis grade), ultra-pure urea, Liqui-gel acrylamide solution (19 1), and [α-^{35}S]dCTP were purchased from ICN Biochemicals, Cleveland, OH Polyethylene glycol 1000 was purchased from Koch-Light, Haverhill, Suffolk, UK. Isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were purchased from Diagnostic Chemicals Ltd , Charlottetown, P E I Ultrapure agarose and ammonium persulfate were purchased from Bio-Rad Laboratories, Richmond, CA Yeast extract, malt extract, Bactopeptone, Casamino acids, tryptic soy broth, nutrient broth and Bacto-agar were from Difco Laboratories, Detroit, MI Thiostrepton was a gift from S J Lucania, E.R Squibb and Sons, New Brunswick, NJ *p*-Nitrobenzoic acid, *p*-nitrobenzaldehyde and *p*-nitrobenzyl alcohol were purchased from Kodak, Rochester, NY *p*-Nitrophenylserinol and *N*-acetyl-*p*-nitrophenylserinol were gifts from Parke, Davis and Co

Table 1: Bacteria and plasmids used.

Strain or plasmid	Characteristics	Source or reference
Streptomyces lividans M252	Cml^S	John Innes Institute, Norwich, UK.
S. lividans RM3	Cml^r (pJV3)	Mosher *et al.* (1990)
S. lividans RM4	Cml^r (pJV4)	This study; Mosher *et al.* (1990)
Escherichia coli TG1	Δ(*lac-pro*) *supE thi hsdD5*/F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15	Dr. Jonathan Wright, Biology Dept., Dalhousie, University.
Micrococcus luteus	Cml^S	Doull *et al.* (1985)
pJV3	pIJ702 carrying a 6.5-kb fragment of *S. venezuelae* ISP5230 DNA conferring Cml^r on *S. lividans* M252.	Mosher *et al.* (1990)
pJV4	pJV3 with a deletion in the *mel* promoter and 6.5-kb insert; it carries a 2.4-kb DNA fragment of *S. venezuelae* ISP5230, conferring Cml^r on *S. lividans* M252.	This study; Mosher *et al.* (1990).
pTZ18/19R	*E. coli* phagemid vector derived from pUC18/19; contains the T7 promoter and the f1 IG origin of replication.	Dr. Jonathan Wright, Biology Dept. Dalhousie University.
pJV5/6	pTZ18R with a 6.5-kb DNA insert of *S. venezuelae* ISP5230 DNA subcloned from pJV3; inserts in pJV5/6 are in opposite orientations.	This study; Mosher *et al.* (1990)
pJV7/8	pTZ18/19R with a 2.4-kb fragment of *S. venezuelae* ISP5230 DNA, containing the Cml^r determinant of pJV4.	This study; Mosher *et al.* (1990)
M13PB100	M13mp18 with a 2.4-kb insert of *S. venezuelae* ISP5230 DNA subcloned from pJV7.	Brown (1991)
M13PB101	M13PB100 with a deletion in the 2.4-kb insert; it carries a 0.2-kb fragment of *S. venezuelae* ISP5230 DNA.	Brown (1991)
pJV7Δ2.2	pJV7 with a deletion in the 2.4-kb insert; it carries a 0.2-kb fragment of *S. venezuelae* ISP5230 DNA.	This study
pBluescriptII SK+	A phagemid derivative of pUC18/19, containing an expanded polylinker, an f1 IG origin of replication, T3 and T7 promoters.	Dr. R. Lee, Biology Dept., Dalhousie University.
pJV9/10	pBluescriptII SK+ with a 2.2-kb insert of *S. venezuelae* DNA subcloned from pJV7; inserts in pJV9/10 are oppositely oriented.	This study

Inc., Ann Arbor, MI. *p*-Nitrophenylserine was a gift from Dr. L.C. Vining, Biology Dept., Dalhousie University. All oligonucleotides used were gifts from Dr. W.V. Shaw, University of Leicester, UK. Solid-phase extraction tubes, 1.35 cm (i.d.) x 1.6 cm (C_{18} reversed phase Sep-pak; 1000 mg) were purchased from Chromatographic Specialties, Brockville, Ont.

III. Media

Streptomyces lividans protoplasts were regenerated on R5 medium (Hopwood *et al.*, 1985) consisting of:

Sucrose	103 g
K_2SO_4	0.25 g
Glucose	10.0 g
Casamino acids	0.10 g
Yeast extract	5.0 g
TES	5.73 g
Distilled water to	1.0 L
Agar	22.0 g

Before addition of agar the solution was adjusted to pH 7.6 with 40% (w/v) NaOH. After autoclaving the following sterile solutions were added:

10X Trace element solution	0.2 mL
0.5% (w/v) KH_2PO_4	10.0 mL
5.0 *M* $CaCl_2$	4.0 mL
20% (w/v) Proline	15.0 mL
1.0 *M* $MgCl_2$	49.0 µL

The 10X trace element solution consisted of:

$ZnCl_2$	0.4 g
$FeCl_3.6H_2O$	2.0 g

$CuCl_2.2H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$Na_2B_4O_7.10H_2O$	0.1 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.1 g
Distilled water to	1.0 L

For maintaining *S. lividans* strains, R5 medium was modified to give R5(-S) medium. The R5 ingredients without sucrose or agar were adjusted to pH 7.6 and divided into 200-mL portions; each received 4.4 g agar and was autoclaved. Just before use the solidified medium was remelted in a microwave oven, briefly cooled, and supplemented with antibiotics when appropriate.

Cultures of *S. lividans* were grown in YEME medium (Hopwood *et al.*, 1985) for a number of purposes. This medium contained:

Yeast extract	3.0 g
Bacto-Peptone	5.0 g
Malt extract	3.0 g
Glucose	10.0 g
Distilled water	1.0 L

Before autoclaving the medium was adjusted to pH 7.7 with 1 *M* NaOH.

Streptomyces lividans strains grown for plasmid extraction were cultured in YEME medium supplemented to 10% (w/v) sucrose. After autoclaving, sterile 1 *M* $MgCl_2$ (2.0 $mL.L^{-1}$) and 20% (w/v) glycine (12.5 $mL.L^{-1}$) were added.

For preparing protoplasts of *S. lividans*, YEME medium was supplemented to 34% (w/v) with sucrose. YEME medium was prepared by first mixing the following components:

Yeast extract	0.3	g
Bacto-Peptone	0.5	g
Malt extract	0.3	g
Glucose	1.0	g
Sucrose	34.0	g
Distilled water to	80.0	mL

The solution was dispensed in four 20-mL portions into 250-mL Erlenmeyer flasks, and then autoclaved. Just before use the following sterile solutions were added:

20% (w/v) Glycine	625	µL
1.0 *M* $MgCl_2$	125	µL
Distilled water	4.25	mL

Complex (GNY) liquid medium, used to grow *S. lividans* for chloramphenicol metabolism studies, consisted of:

Glycerol	20.0	mL
Nutrient broth	8.0	g
Yeast extract	3.0	g
K_2HPO_4	5.0	g
Distilled water	1000	mL

The solution was dispensed in 50- or 100-mL portions into 500-mL Erlenmeyer flasks and autoclaved.

Soft nutrient (SN) agar, used to overlay protoplasts during transformation experiments (Hopwood *et al.*, 1985), consisted of:

Nutrient broth	8.0 g
Agar	3.0 g
Distilled water	1.0 L

The mixture was heated to boiling in a microwave oven to dissolve the agar, and divided into 50-mL portions before autoclaving.

Escherichia coli cultures were grown in L-broth (Hopwood *et al.*, 1985), which consisted of:

Bacto-Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Glucose	1.0 g
Distilled water	1.0 L

The components were dissolved and the medium was dispensed in 50-mL portions into 250-mL Erlenmeyer flasks and autoclaved. For semi-solid media (L-agar), 15 $g.L^{-1}$ agar was included.

For subcloning experiments in which X-gal and IPTG were used for blue/white screening of *E. coli* transformants possessing recombinant plasmids, LB-broth and LB agar (Sambrook *et al.*, 1989) were used to prepare competent cells and for the selection of transformed colonies. LB-broth consisted of:

Bacto-Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water to	1000 mL

The components were dissolved in 800 mL of water and adjusted to pH 7.0 with 1 *M* NaOH. The mixture was made up to 1000 mL with water, dispensed in 50-mL or 250-

mL portions, and autoclaved. For LB-agar, 15 $g.L^{-1}$ of agar was included in the medium before autoclaving. For blue/white screening of *E. coli* transformants, 250 mL of LB-agar was briefly cooled after autoclaving and supplemented with:

2% (w/v) X-gal in DMF*	625	µL
IPTG (25 $mg.mL^{-1}$)	500	µL
Ampicillin (100 $mg.mL^{-1}$)	250	µL

* DMF = dimethylformamide

Phagemid-carrying strains of *E. coli* were infected with helper phage VCSM13 and grown in Terrific broth (Sambrook *et al.*, 1989) supplemented with glucose (TBG) to generate single stranded copies of the phagemid. Terrific broth consisted of:

Bacto-Tryptone	1.2	g
Yeast extract	2.4	g
Glycerol	0.4	mL
Distilled water	90	mL

The components were dissolved and autoclaved. Upon cooling to ambient temperature, Terrific broth was supplemented with 1.8 mL of 20% (w/v) glucose (aseptically filtered through a cellulose acetate membrane; 0.2 µm pore size) and 10 mL of 0.89 *M* $KHPO_4$ (0.17 *M* KH_2PO_4, 0.72 *M* K_2HPO_4; sterilized by autoclaving).

M9 (minimal) medium (Sambrook *et al.*, 1989) used to detect chloramphenicol metabolism in recombinant strains of *E. coli* consisted of:

$Na_2HPO_4.7H_2O$	64	g
KH_2PO_4	15	g
NaCl	2.5	g
NH_4Cl	5.0	g

Distilled water to	1 0 L

The solution was divided into 200-mL portions and autoclaved After cooling to room temperature the following solutions were aseptically added to each portion

20% (w/v) Glucose	20 0 mL
1 *M* $MgSO_4$	2 0 mL
1 *M* $CaCl_2$	0 1 mL
Sterile distilled water to	1000 mL

IV. Buffers and solutions

TE buffer was used to dissolve DNA It consisted of 10 m*M* Tris (pH 8 0) and 1 m*M* disodium ethylenediamine tetraacetate (Na_2EDTA)

Buffered phenol was prepared by shaking liquefied aqueous phenol (90% w/v) containing 0 5 mg mL^{-1} 8-hydroxyquinoline with 1 *M* Tris (pH 8 0), and then with 0 1 *M* Tris (pH 8 0) until the pH of the phenol phase was above pH 7 8 The phenol phase was then transferred to a dark-glass bottle, thinly overlaid with of 0 1 *M* Tris (pH 8 0) containing 0 2% β-mercaptoethanol, and stored at 4°C for not longer than 1 to 2 months

Acid phenol/chloroform was prepared by mixing equal volumes of liquefied phenol and chloroform and then supplementing with 0 5 mg mL^{-1} 8-hydroxyquinoline The mixture was stored in a dark-glass bottle at room temperature for not longer than 2-3 months

Neutral phenol/chloroform was prepared by shaking phenol/chloroform (1 1) supplemented with 0 5 mg mL^{-1} 8-hydroxyquinoline, first with 1 *M* Tris (pH 8 0), and then with 0 1 *M* Tris (pH 8 8), until the pH of the phenol/chloroform phase was above pH 7 8 The mixture was then transferred to a dark-glass bottle, thinly overlaid with of 0 1 *M* Tris (pH 8 8), and stored at room temperature for not longer than 2-3 months

Agarose gel electrophoresis was carried out in TAE buffer TAE buffer was usually prepared as a 50X concentrate that consisted of

Tris-base	242	g
Glacial acetic acid	57.1	mL
0.5 *M* Na_2EDTA (pH 8.0)	200	mL
Distilled water to	1000	mL

The components were dissolved, passed through a coarse sintered-glass funnel, and autoclaved.

DNA samples to be analyzed by agarose gel electrophoresis were mixed prior to loading with 5X final stop buffer (FSB), which contained 50% (w/v) sucrose, 100 m*M* Tris-HCl (pH 8.0), 100 m*M* Na_2EDTA (pH 8.0), and 0.1% (w/v) bromphenol blue (sodium salt).

Polyacrylamide gel electrophoresis to resolve the products of sequencing reactions, was carried out in TBE buffer. TBE buffer was usually prepared as a 5X concentrate that consisted of:

Tris-base	108	g
Boric acid	55	g
0 5 *M* Na_2EDTA (pH 8.0)	40	mL
Distilled water to	2000	mL

The components were dissolved, passed through a coarse sintered-glass funnel, and autoclaved.

Gel-stock solution (5% (w/v) acrylamide, 7 *M* urea, and 0.5X TBE), used in the preparation of denaturing polyacrylamide sequencing gels, consisted of:

Distilled water	134	mL
5X TBE	50	mL
Liqui-gel solution*	62 5	mL
Ultrapure urea	210	g

* Liqui-gel solution consisted of a stabilized 40% (w/v) aqueous solution of acrylamide and bis-acrylamide (19:1).

The urea was slowly combined with the first three components of the mixture and dissolved by gently heating for 15 min with constant stirring The resulting solution was made up to a final volume of 500 mL with distilled water, clarified by vacuum filtration through a coarse sintered glass funnel, and degassed under high vacuum for 20 to 30 min The solution was then stored in a dark-glass bottle at 4°C for not longer than one to two months

Lysis (L) buffer in which the *Streptomyces* mycelium was incubated with lysozyme to form protoplasts for transformation (Hopwood *et al.*, 1985) consisted of 10 3% (w/v) sucrose (100 mL), 5 73% (w/v) TES buffer, pH 7 2, (10 mL), 2 5% (w/v) K_2SO_4 (1 0 mL), 10X trace element solution (0 02 mL), 0 5% (w/v) KH_2PO_4 (1 0 mL), 2 5 *M* $MgCl_2$ (0 1 mL) and 3 68% (w/v) $CaCl_2$ $2H_2O$ (1 0 mL) Sterile solutions of each component were mixed and kept as a stock solution at room temperature

Protoplast (P) buffer used to osmotically stabilize protoplasts during protoplast formation and transformation (Hopwood *et al.*, 1985) contained sucrose (103 g), K_2SO_4 (0 25 g), $MgCl_2$ $6H_2O$ (2 02 g), 10X trace elements solution (0 2 mL) and distilled water to 800 mL The solution was dispensed in 80-mL aliquots and autoclaved Just before use the following sterile solutions were added to each aliquot in the order given 0 5% (w/v) KH_2PO_4 (1 0 mL), 3 68% (w/v) $CaCl_2$ $2H_2O$ (10 0 mL), and 5 73% (w/v) TES buffer, pH 7 2, (10 0 mL)

The TES buffer used in L and P buffers was prepared by dissolving 5 73 g of TES in 80 mL distilled water, adjusting the pH to 7 2 with 1 *M* NaOH and making the volume up to 100 mL The solution was then autoclaved for 20 min

Transformation (T) buffer, which was mixed with polyethylene glycol 1000 to allow DNA transformation of protoplasts (Hopwood *et al.*, 1985) consisted of 10 3% (w/v) sucrose (25 mL), 10X trace element solution (0 02 mL), 2 5% (w/v) K_2SO_4 (1 0 mL) and distilled water (75 mL) Sterile solutions of each component were mixed and

kept as a stock solution at room temperature. Just before use sterile solutions of 5 *M* $CaCl_2$ (0.2 mL), and 1 *M* Tris-maleic acid buffer, pH 8.0, (0.5 mL) were added to 9.3 mL of the stock solution. Tris-maleic acid buffer was prepared by adjusting a 1 *M* Tris solution to pH 8.0 with solid maleic acid. For use in transformations, three parts (by volume) of T-buffer were added to one part (by weight) of sterile PEG 1000.

V. Sterilization

Solutions sterilized by autoclaving were heated to 121°C and 15 p.s i. for 20 min. Solutions sterilized by filtration were passed through sterile 0.2-μm pore-size cellulose acetate membranes.

VI. Culture conditions

A. *Streptomyces*

1. Spore suspensions

Spores from a single colony were spread on R5(-S) agar (supplemented with the appropriate antibiotic, as required) and allowed to incubate at 30°C until confluent sporulation had occurred. Sterile water (5-10 mL) was added to the plate culture and the spores were dislodged by scraping the culture surface with a sterile inoculating loop. The resulting suspension of spores and vegetative mycelium was transferred to a sterile culture tube, agitated briefly on a vortex mixer, and filtered aseptically through nonabsorbent cotton wool. The filtrate was then centrifuged at 3000xg; the resulting pellet was resuspended in sterile 20% (v/v) aqueous glycerol and stored at -20°C. For long-term storage, spore suspensions were kept at -70°C.

2. Plasmid isolation

Spores were spread onto R5(-S) agar medium and allowed to incubate at 30°C until the substrate mycelium had just begun to appear (24-48 h depending on the strain). A small amount of the mycelium was scraped from the agar surface, using a sterile tooth-

pick, and aseptically transferred to a microfuge tube, after alkaline lysis the mycelium was extracted for plasmid DNA The agar medium was usually supplemented with thiostrepton at 30 μg mL^{-1} to select for colonies carrying plasmids derived from pIJ702 To select for pJV3 and pJV4, the medium was supplemented with 12 5 μg mL^{-1} chloramphenicol instead

For isolating plasmid DNA of greater purity, spores, from a confluently sporulating Petri dish culture were harvested and filtered aseptically through nonabsorbent cotton wool into 50 mL of YEME medium containing 10% (w/v) sucrose (supplemented with either 5 μg mL^{-1} thiostrepton or 12 5 μg mL^{-1} chloramphenicol, as required) The culture was grown at 30°C for 24-48 h with shaking (250 rpm), dispensed into sterile 1 5-mL microfuge tubes, and centrifuged at 12,000xg for 5 min The supernatant was discarded, and the pellet was immediately extracted for plasmid DNA or frozen at -20°C

3 Preparing *S. lividans* protoplasts

Spores from a confluently sporulating *S. lividans* Petri dish culture grown on R5(-S) agar were dispersed in 4 25 mL of water The suspension was filtered aseptically through nonabsorbent cotton wool directly into a 250-mL Erlenmeyer flask containing 20 75 mL of YEME medium supplemented to give final concentrations of 34% (w/v) sucrose, 5 m*M* $MgCl_2$, and 0 5% (w/v) glycine The culture was then incubated with shaking at 30°C for 40 h

4. Chloramphenicol metabolism studies

Vegetative cultures of *S. lividans* RM3 or RM4 were grown in GNY medium supplemented with 12 5 μg mL^{-1} chloramphenicol These cultures were started with an inoculum of frozen mycelium or with the spores from a confluently sporulating plate culture and were grown at 28°C or 30°C with shaking (250 rpm) A 5% (v/v) sample was taken from each vegetative culture to inoculate fresh GNY medium every 3 to 4 days Cultures were visually inspected for purity at every subculture, and every second subculture was inspected by microscopy Vegetative cultures of *S. lividans* M252 were main-

tained in a similar fashion except that no chloramphenicol was added to the medium before inoculation.

5. Large scale chloramphenicol conversion cultures

A 500-mL vegetative culture of *S. lividans* RM4 was grown for 48 h at 30°C in GNY medium supplemented with 12.5 $\mu g.mL^{-1}$ chloramphenicol. The culture was divided into equal portions and the mycelium was pelleted by centrifugation at 35,000xg for 10 min at 4°C. Each pellet was then washed in 250 mL of sterile ddH_2O. The pellets were resuspended in 250 mL of sterile ddH_2O and aseptically transferred to two 1-L Erlenmeyer flasks. The cultures were then supplemented with chloramphenicol at 100-200 $\mu g.mL^{-1}$ and incubated at 30°C for 16-23 h.

B. *Escherichia coli*

1. Cell suspensions in glycerol

Escherichia coli strains were maintained as cell suspensions in glycerol at -70°C. Cells from a single colony were used to inoculate 2 mL of L-broth (supplemented with ampicillin to 100 $\mu g.mL^{-1}$ as required). The culture was grown overnight at 37°C with shaking (250 rpm). It was then mixed with an equal volume of sterile 50% (v/v) aqueous glycerol and stored indefinitely at -70°C. Cultures were revived by scraping the surface of the frozen suspension with a sterile inoculating loop and transferring the cells to L-broth or L-agar (supplemented with ampicillin as required).

2. Plasmid extraction

Plasmid-containing strains of *E. coli* were grown overnight with shaking at 37°C in 2 mL of L-broth supplemented with 100 $\mu g.mL^{-1}$ ampicillin. The culture was then divided equally and the cells were pelleted at 12,000xg for 5 min in a microfuge. The supernatant was discarded and the cells were either directly extracted for plasmid DNA or stored at -20°C until needed.

3. Single-stranded (ss) DNA extraction

As in the procedure of Karger and Jessee (1990), *E. coli* TG1 carrying recombinant phagemids were used to inoculate 2-mL portions of TBG-broth supplemented with 100 $\mu g.mL^{-1}$ ampicillin and 20 µL of helper phage VCSM13 stock solution (ca. 10^9 $pfu.mL^{-1}$); the cultures were grown at 37°C for 1.5 h with shaking, supplemented with kanamycin to 75 $\mu g.mL^{-1}$, and incubated as before for another 14-20 h. The culture broths were dispensed into sterile 1.5-mL tubes and centrifuged at 12,000xg for 5 min at 4°C. The supernatant solutions (1.2-mL) were transferred to sterile 1.5-mL tubes and either immediately extracted for ssDNA or stored frozen at -70°C.

VII. DNA extraction

A. *Streptomyces* plasmid DNA

Plasmid DNA was purified from *Streptomyces* using the alkaline extraction method of Kieser (1984). Briefly, the mycelium from 1.5 mL of a liquid culture was pelleted in a microfuge, resuspended in 500 µL of lysozyme solution (0.3 *M* sucrose, 25 m*M* Tris (pH 8.0), and 25 m*M* Na_2EDTA containing 2 $mg.mL^{-1}$ lysozyme) and incubated for 30 min at 37°C Next 250 µL of 0.2% SDS in 0.3 *M* NaOH was added and the mixture was vortexed immediately. The cleared lysate was incubated at 70°C for 15 min, cooled to room temperature in a water bath, and extracted with 160 µL of acid phenol/chloroform. The resulting aqueous phase (ca. 650 µL) was mixed with 70 µL of unbuffered 3 *M* sodium acetate and 700 µL of isopropanol and incubated at room temperature for 5 min. The precipitate was pelleted at 12,000xg for 3 min in a microfuge. The pellet was resuspended in 50 µL TE buffer at 37°C by gentle agitation every 5 min for 20 min.

To this solution, 5 µL of unbuffered sodium acetate followed by 25 µL of neutral phenol/chloroform were added. The mixture was vortexed and then centrifuged at 12,000xg for 3 min. The aqueous phase was removed and extracted successively with another 25 µL of neutral phenol/chloroform and 20 µL of chloroform/isoamyl alcohol

(24:1). The DNA in the aqueous phase was then precipitated with 50 µL of isopropanol and pelleted at 12,000xg. The pellet was resuspended in 100 µL of TE buffer and supplemented with 1/9 volume of 3 *M* sodium acetate (pH 5.2). The DNA was precipitated by adding 2.5 volumes of cold absolute ethanol and cooling at -20°C for 10 min. The precipitate was pelleted in a microfuge, washed with cold 70% (v/v) ethanol and vacuum dried.

B. *Escherichia coli plasmid DNA*

1. Magic Miniprep method

For the purification of high quality plasmid DNA from strains of *E. coli*, a Magic Minipreps™ kit (Promega, Madison WI) was used as recommended by the supplier. Briefly, 1 mL of an overnight culture of *E. coli* in L-broth was centrifuged at 12,000xg for 5 min and the supernatant was discarded. The pellet was then dispersed in 200 µL of Cell Resuspension Solution, and 200 µL of Lysis Solution was mixed in by inverting several times until the solution had cleared. The cleared lysate was mixed with 200 µL of Neutralization Solution by inverting several times. The heavy precipitate that formed was pelleted by centrifugation at 12,000xg for 5 min. The supernatant was then carefully decanted into a fresh sterile microfuge tube and combined with 1 mL of Magic Minipreps DNA Purification Resin. This mixture was then applied to a Magic minipreps column using a 3-mL syringe, and the immobilized resin was washed with 2 mL of Column Wash Solution. The column was inserted into a 1.5-mL tube and centrifuged at 12,000xg for 20 s to remove the last traces of Column Wash Solution, and then transferred to a fresh 1.5-mL tube. The plasmid DNA was eluted from the resin by applying 50 µL of prewarmed (65-70°C) TE buffer to the column and then centrifuging for 20 s at 12,000xg. The resulting solution usually contained 2-3 µg of plasmid DNA.

2. The Miniprep Kit Plus method

To quickly isolate small quantities of plasmid DNA for the screening of subclones and deletion clones, a Miniprep Kit Plus purification system (Pharmacia) was used as rec-

ommended by the supplier. Briefly, 1.5 mL of an overnight *E. coli* culture in TBG medium was centrifuged at 12,000xg for 5 min. After the last traces of the supernatant had been removed using a micropipette, the pellet was resuspended in 100 µL of cold Solution I by vortexing vigorously. The suspension was incubated on ice for 5 min and 200 µL of Solution II were added. This was mixed by inverting several times until cleared, and incubated for 5 min on ice. To the cleared lysate, 150 µL of Solution III were added, and the mixture was inverted several times and incubated on ice for 5 min. The resulting precipitate was pelleted by centrifugation at 12,000xg for 5 min and the supernatant was carefully transferred to a fresh microfuge tube. The DNA was precipitated from the supernatant by adding one volume of isopropanol and incubating at room temperature for 5-10 min. The precipitate was pelleted in a microfuge for 5 min and the supernatant was carefully pipetted off. The pellet was then washed by adding 250 µL of isopropanol to the tube and carefully pipetting off the isopropanol. The pellet was vacuum dried and resuspended in 50 µL of TE buffer yielding ca. 2 µg of DNA.

3. Single-stranded (ss) DNA

The procedure of Sambrook *et al.* (1989) was used. To precipitate phage particles, 200 µL of 20% (w/v) PEG 8000 in 2.5 *M* NaCl was mixed gently with 1.2 mL culture supernatant; the suspension was incubated at room temperature for 15 min. The phage particles were pelleted by centrifuging at 12,000xg for 5 min at 4°C. The supernatant was carefully discarded, and the microfuge tube was recentrifuged for 30 s at 12,000xg. The remaining supernatant was then removed with a micropipette.

The pellet was resuspended in 100 µL of TE buffer by vortexing vigorously. The suspension was extracted with 50 µL of buffered phenol by vortexing for 30 s, pausing for 1 min, and then vortexing again for 30 s. The emulsion was centrifuged at 12,000xg for 1 min, giving a cloudy aqueous phase that was transferred to a fresh microfuge tube and extracted with another 50 µL of buffered phenol. After the still turbid aqueous phase had been transferred to a new microfuge tube, it was extracted with one volume of chloro-

form:isoamyl alcohol (24:1). To precipitate the ssDNA from the clarified aqueous phase, 1/9 volume of 3 *M* potassium acetate (pH 5.2) and 2.5 volumes of absolute ethanol (room temperature) were added, and the mixture was incubated at room temperature for 15 min. The precipitate was pelleted by centrifugation at 12,000xg for 10 min at 4°C. The pellet was washed by adding 200 µL of cold 70% ethanol, vortexing briefly, and centrifuging at 12,000xg for 10 min at 4°C. The pellet was vacuum dried and resuspended in 20 µL of TE buffer. The typical yield was 2-3 µg of ssDNA.

VIII. Gel Electrophoresis

a. Agarose gel electrophoresis

Agarose gel electrophoresis of DNA was carried out using submarine horizontal agarose-slab minigels. Usually 0.21g of agarose in 30 mL of TAE buffer (0.7%) was dissolved by heating to boiling for 2 min in a microwave oven. The solution was allowed to cool for 10 min, then poured into a 7 x 8 cm gel tray with an 8 or 12 well comb at one end, and allowed to set (25 min) before the comb was removed. The gel was then placed in the electrophoresis chamber and covered to a depth of 2 to 3 mm with TAE buffer. Samples (8-12 µL) in FSB buffer were loaded into the sample wells and electrophoresed at 80 V (50 mA) for 1.5 h.

Gels were stained for at least 5 min in a solution of ethidium bromide (0.5-1.0 $\mu g.mL^{-1}$) at room temperature. They were rinsed with distilled water, observed by trans-illuminated ultraviolet light (300 nm) and photographed using a red filter and Kodak Ekta-pan film with a Polaroid M-P4 Land camera.

b. Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis of DNA sequencing reactions was carried out using a SEQUI-GEN® apparatus (Bio-Rad) with a 21 x 40 cm integral

plate/chamber (IPC) unit, 0 4-mm spacers, and a programmable power supply (Bio-Rad Model 3000 Xi)

To 10 mL of gel-stock solution in a 50-mL beaker, 50 µL of freshly prepared 25% (w/v) ammonium persulfate and 50 µL of TEMED were added, and quickly mixed in by gently swirling This mixture was poured across the entire length of a sealing strip (ca 3 x 25 cm) of Schleicher and Schull blotting paper, against which the bottom edge of the pre-assembled IPC unit was quickly pressed This caused rapid wicking of the gel-stock solution into the 0 4-mm space between the inner and outer plates of the IPC unit Pressure was applied to the IPC unit until (after about 2 min) the bottom opening of the IPC unit was completely sealed with a ca 2 cm-high plug of solidified polyacrylamide

To 40 mL of gel-stock solution in a 250-mL beaker, 40 µL of freshly prepared 25% (w/v) ammonium persulfate and 40 µL TEMED were added and quickly mixed in by gentle swirling This mixture was taken up in a 60-mL syringe with a 16 gauge (wide-bore) hypodermic needle, and used to slowly fill the space between the inner and outer plates of the IPC unit During this procedure the IPC unit was tilted at an ca 45° angle and rocked gently to release air-bubbles before the polyacrylamide gel had set Once the IPC unit was filled, the flat bottom edge of a sharkstooth comb was inserted ca 0 5 cm into the space between the IPC plates The top of the IPC unit was then covered with Saran wrap, and the whole unit was left overnight, tilted at a 10° angle, to allow the gel to polymerize

The bottom sealing strip was then removed, and the IPC unit was placed in the lower buffer chamber (universal base unit) of the SEQUI-GEN apparatus The upper buffer chamber (attached to back of the IPC unit) was filled with 0 5X TBE buffer and the comb was carefully removed The resulting flat surface, formed in the polyacrylamide by the comb, was immediately rinsed with 0 5X TBE buffer to remove any unpolymerized gel-stock mixture The lower buffer chamber was then filled with 400 mL of 0 5X TBE and the gel was usually pre-warmed to 55°C by supplying power at a constant 2900 V for

25-30 min. The power was then turned off, and the top of the gel was rinsed with 0 5X TBE to remove any accumulated urea. The sharkstooth comb (teeth downwards) was then inserted into the IPC unit. Once the samples were loaded, power was applied at 2900 V for 5 min, or until the temperature of the gel had risen to 55°C; the power was then re-set to 2200 V and the samples were usually electrophoresed for 1 to 2 h, maintaining a gel-temperature of 50-55°C.

The IPC unit was then disassembled, and the gel, still attached to the outer plate of the IPC unit was immersed in a fixing solution of 10% acetic acid and 10% methanol in water for 20 min. The fixing solution was carefully siphoned off, and the gel was quickly blotted on a piece of Whatman 3MM paper, slightly larger than the gel. This was placed on another piece of Whatman 3MM paper, gel-side up, and covered with Saran wrap. The Saran wrap was then carefully adjusted to remove any creases or bubbles between it and the gel. The 3MM paper and Saran wrap were trimmed to the size of the gel and placed in a Bio-Rad gel dryer. The gel was vacuum dried at 80°C for 1 h. After drying the Saran wrap was removed and the gel was exposed to a sheet of Kodak X-Omat AR film for 24 h at room temperature.

IX. Restriction endonuclease digestions and ligations

Conditions and procedures for carrying out restriction endonuclease digestions were generally those recommended by the enzyme supplier. DNA (0.5-1.0 µg) was mixed with 2 µL of 10X reaction buffer (supplied with the enzyme) and 1 to 2 µL (5-10 U) of restriction enzyme and made up to a final volume of 20 µL with TE buffer or distilled water. The reaction mixture was then incubated overnight at 37°C (or as recommended by the enzyme supplier). Digests were usually stopped by adding 4 µL of 5X FSB buffer and analyzed directly by agarose gel electrophoresis. If the digested DNA was to be used for other procedures, the reaction mixture was made up to a final volume of 400 µL with distilled water (or TE buffer) and extracted successively with one volume of neutral phe-

nol/chloroform and one volume of chloroform/isoamyl alcohol (24:1). The DNA was precipitated with 1/9 volume 3 *M* potassium acetate (pH 5.2) and 2.5 volumes of cold absolute ethanol for 15 min at -20°C, and then pelleted by centrifugation at 12,000xg. The pellet was washed with 70% ethanol, and dried under vacuum.

Conditions and procedures for ligations were usually those recommended by the supplier of the enzyme. In subcloning procedures, the insert and vector DNA, each dissolved in distilled water or TE buffer, were mixed in a 2:1 molar ratio, heated to 65°C for 5 min and then quickly cooled on ice for 10 min. Concentrated ligation buffer (supplied with the enzyme) and water were added to bring the total DNA concentration to 40 $\mu g.mL^{-1}$ The DNA was then incubated overnight at 12°C with 1 U of T4 DNA ligase (BRL) or 6-10 U of T4 DNA ligase (Pharmacia). The reaction mixture was made up to a final volume of 400 µL with distilled water, and the DNA was precipitated with 1/9 volume of 3 *M* potassium acetate (pH 5.2) and 2 5 volumes of cold absolute ethanol for 30 min at -70°C. The precipitated DNA was pelleted at 12,000xg for 10 min at 4°C, washed with 70% ethanol, and dried under vacuum.

X. Elution of DNA from agarose gels

A GENECLEAN® kit (Bio/Can Scientific) was used to elute DNA from agarose gels The procedure followed was that recommended by the supplier. DNA fractionated by agarose gel electrophoresis was stained with ethidium bromide and observed by U V transillumination (300 nm). The smallest piece of agarose gel carrying the desired DNA fragment was excised using the sharp edge of a sterile spatula. If the agarose gel slice weighed less than 0.4 g it was transferred to a sterile microfuge tube containing three volumes (by weight) of a saturated sodium iodide solution. The gel-slice was then melted by incubation at 55°C for 5 to 6 min. To the melt, 5 µL of Glass Milk suspension was added. The Glass Milk was dispersed by inverting the tube three times and the mixture was incubated at room temperature for 5 min. The Glass Milk was then pelleted by centrifugation

at 12,000xg for 5 s. The pellet was resuspended in 400 µL of New Wash solution and centrifuged at 12,000xg for 5 s. The supernatant was then carefully removed, and the process was repeated four more times. After the fifth wash, the pellet was briefly centrifuged and all of the excess New Wash solution was carefully removed. To elute the DNA from the Glass Milk, the pellet was resuspended in 10 µL of TE buffer and incubated at 55°C for 3 min. The Glass Milk was then pelleted at 12,000xg for 30 s and the supernatant was carefully removed to a fresh microfuge tube. This procedure was repeated, and the supernatants were pooled and stored at 4°C until required.

XI. Generating nested, overlapping deletions in the 2.2-kb insert of pJV9

(Adapted from Sambrook *et al.*, 1989)

To create a target site for exonuclease III, pJV9 DNA (2.5 µg) was digested with *Cla*I at a site immediately adjacent to the *S. venezuelae* DNA insert, and within the plasmid's polylinker sequence. To protect the upstream T7 primer-binding site, the *Cla*I-digested pJV9 DNA was digested with *Kpn*I, creating a four base 3'-overhang within the plasmid's polylinker. The digested DNA was precipitated with ethanol, washed with 70% ethanol, dried under vacuum, and resuspended in 5 µL of distilled water To the suspension, 2 µL of 10X ExoIII buffer (0.66 *M* Tris, pH 8.0, 66 m*M* $MgCl_2$), 11 µL of distilled water, and 2 µL of diluted exonuclease III (32.5 $U.\mu L^{-1}$) were added. The reaction mixture was triturated gently at 37°C, and 2-µL samples were removed every 30 s. The samples were pooled in a microfuge tube containing 6 µL of 5X mungbean nuclease buffer (250 m*M* sodium acetate (pH 5.0), 250 m*M* NaCl, 0.5 m*M* zinc acetate, 0.005% (v/v) Triton X-100, and 25% (v/v) glycerol). To the pooled exonuclease III products, 2 µL of distilled water and 2 µL of diluted mungbean nuclease (10 $U.\mu L^{-1}$) were added. The mixture was incubated at 37°C for 10 min, after which, 1.5 µL of 1 *M* Tris (pH 8.0) were added. The mungbean nuclease was then inactivated by heating the reaction mixture to 70°C for 5 min. When the mixture had cooled to room temperature, the following were

added: 2.5 μL of 100 m*M* $MgCl_2$, 5 μL dNTP's (dATP, dTTP, dGTP, and dCTP, each at 1.25 m*M*), 15 μL of distilled water, and 2 μL of Klenow fragment (1 $U.\mu L^{-1}$). The reaction mixture was allowed to incubate at room temperature for 20 min. A 15-μL sample of the "polished" products was combined with 5 μL of 10X One-Phor-All-buffer Plus (Pharmacia), 0.5 μL of 100 m*M* ATP, 26.5 μL distilled water, 3 μL of T4 DNA ligase (5 $U.\mu L^{-1}$; Pharmacia), and incubated overnight at 10°C. A 20-μL sample of the ligated DNA was used to transform competent cells of *E. coli* TG1 to ampicillin resistance. Cells from the resulting transformants were extracted for plasmid DNA and analyzed by agarose gel electrophoresis. Clones possessing plasmids with sizes ranging between that of pBluescriptII SK+ and pJV9, and differing by approximately 200 bp were selected, arranged in descending order of size and used for DNA sequencing.

XII. DNA sequencing reactions

A Sequenase® Version 2.0 DNA Sequencing Kit (United States Biochemical), and a Nucleotide Kit for Sequencing with Sequenase® T7 polymerase and 7-deaza-dGTP (United States Biochemical), were used for DNA sequencing reactions. The procedure used for carrying out sequencing reactions was as described by the supplier of the kits. Briefly, 1 μL of a synthetic (17-mer) oligonucleotide primer (3 to 18 ng) was mixed with 2 μL of 5X Sequenase Reaction buffer, and 7 μL of single stranded template DNA (ca. 1 μg) To anneal the primer to the template DNA, the mixture was heated to 65°C for 2 min in a dry-bath heating block. The block was then removed from the dry-bath heater and cooled slowly to room temperature over 30 min; the annealed primer/template solution was placed on ice until needed.

To start the labeling reaction, 10 μL of annealed primer/template solution was mixed with 1 μL of 0.1 *M* dithiothreitol, 2 μL of diluted labeling mix, 0.5 μL of [α-^{35}S]dCTP (1000 $Ci.mmol^{-1}$, 10 $\mu Ci.\mu L^{-1}$), and 2 μL of diluted Sequenase. After the labeling mixture had been incubated at room temperature for 2 to 3 min, 3.5-μL aliquots

were transferred to the inside walls of four microfuge tubes, pre-warmed to 48°C, and labeled A, G, C, and T; each tube contained 2 5-µL of the dideoxy-termination mixes for the respective nucleotides. The termination reactions were initiated by briefly centrifuging the microfuge tubes at 12,000xg. The microfuge tubes were transferred to a heating block and incubated at 48°C for 5 min. The tubes were then removed to a microfuge, and 4 µL of Stop solution were placed on the inside wall of each tube The termination reactions were stopped by centrifuging briefly at 12,000xg. The reactions could be stored at -20°C for one week If the reactions were to be used immediately, they were transferred to a boiling water bath for 2 min, placed on ice for 5 min and immediately loaded on a sequencing gel. All reactions were carried out in pairs using dGTP and 7-deaza-dGTP

To determine the sequence of the template DNA to within 10-15 nucleotides of the primer, 1 µL of Mn-buffer (supplied with kit) was added to the labeling reaction To read DNA sequences at greater than 200 nucleotides from the primer, undiluted labeling mix was used in the labeling reaction, the labeling reaction was incubated for 5 min, the termination reactions were allowed to proceed for 10-15 min, and the amount of labeled dCTP was doubled in the labeling reaction.

XIII. Computer-assisted DNA sequence analysis

The DNA sequences generated during this study were compiled and analyzed using the DNA STRIDER program (Marck, 1988) on a Macintosh Plus microcomputer. Sequence was also analyzed with the GCG Sequence Analysis Software Package (Genetics Computer Group Inc , Madison, Wisconsin), on a VAX 4500 mainframe computer. For multiple sequence alignments the MULTALIN program (Corpet, 1988) was used on a Tandy 2500SX microcomputer Similarity searches of the SWISSPROT and PIR databases used the on-line FASTA (Pearson and Lipman, 1988) and BLITZ sequence analysis programs of the European Molecular Biology Laboratory (EMBL) Netserver, at Heidelberg, Germany (Internet address Netserver@EMBL-Heidelberg DE).

XIV. Transformation

A. *Streptomyces*

1. Formation of protoplasts

Protoplasts were prepared as described by Hopwood *et al.* (1985). Mycelium harvested by centrifugation from 40 h cultures of *S. lividans* M252 in 25 mL of YEME was washed with twice the original volume of 10.3% (w/v) sucrose. The mycelial pellet was then either stored at -20°C until needed or incubated at 30°C for 30 min in 4 mL of filter sterilized P-buffer supplemented with 1 $mg.mL^{-1}$ of lysozyme. The extent of protoplast formation was determined by examining the reaction mixture under the 40X-objective of a phase-contrast microscope. If adequate, the suspension was mixed by pipetting three times, incubated at 30°C for an additional 15 min and diluted with 5 mL of P-buffer. The protoplast suspension was filtered aseptically through nonabsorbent cotton wool in a syringe and collected in a sterile polypropylene tube. Sometimes a sample was removed at this point and diluted 1/100 with P-buffer. The protoplasts present were counted on a hemocytometer under the 40X-objective of a phase-contrast microscope. Yields from a 25-mL culture of *S. lividans* M252 were usually in the range of 4×10^9 to 8×10^9. The protoplasts were pelleted (full speed in a clinical centrifuge) and resuspended in 4 mL of P-buffer. The centrifugation was repeated.

2. Transformation of protoplasts

The pelleted protoplasts were resuspended in the small amount of P-buffer left after discarding the supernatant, and DNA in TE buffer (10-20 µL) was added. Usually 1-2 µg of ligated open circular DNA was used in subcloning experiments. The mixture was gently shaken and as soon as possible, 0.5 mL of T-medium was quickly mixed in by pipetting three to four times. After about 30 s the sample was diluted with 5 mL of P-buffer and centrifuged. The protoplast pellet was resuspended in 1 mL of P-buffer and 100-µL samples were gently spread on R5 agar medium pre-dried for 6-7 h in a laminar airflow

hood until each plate had lost greater than 20% of its weight When the protoplasts had been incubated at 30°C for 18-20 h, regeneration could be detected by the faint haze on the agar when the plates were held up to the light For selecting transformants carrying pIJ702, pJV3, and pJV4, the regenerating colonies were overlaid at this time with SN agar supplemented with thiostrepton to give a final plate concentration of about 30 μg mL^{-1} (assuming a partially dried agar volume of 22 mL) Transformants were usually observed within 1 to 2 days. Melanin production by those containing pIJ702 was detected as a brown/black halo around colonies by the third day

B *Escherichia coli*

1. Preparation of competent cells

The preparation of competent cells and their transformation with plasmid DNA were carried out as described by Hopwood *et al.* (1985) Cells grown for 1.5 h in LB-broth supplemented with 20 m*M* $MgCl_2$ were cooled on ice for at least 10 min, and then aseptically transferred, in 10-mL aliquots, to sterile pre-chilled polypropylene culture tubes The cells were pelleted at 3000 rpm for 10 min in a clinical centrifuge at 5°C. By gently tapping the tubes, each of the pellets was resuspended in 1 mL of ice-cold, sterile 0 1 *M* $CaCl_2$, made up to 10 mL with ice-cold 0 1 *M* $CaCl_2$, and cooled on ice for 20 min The cells were then pelleted, resuspended in 1 0 mL of ice-cold 0 1 *M* $CaCl_2$, and incubated overnight on ice

2. Transformation of competent cells

Competent cells (100-μL) aliquots were transferred to pre-chilled sterile microfuge tubes and mixed with 0 1-0 2 μg supercoiled plasmid DNA or 1-2 μg of ligated open circular plasmid DNA in 5-10 μL TE buffer or distilled water The mixture was cooled on ice for 20 min, and then heat shocked at 45°C for 90 s The heat-shocked cells were cooled on ice for 2-3 min, mixed with 0 9 mL of LB-broth, and incubated at 37°C for 90 min without shaking. They were spread, in 100-μL aliquots, on LB-agar supplemented

with 100 μg mL^{-1} ampicillin and incubated overnight at 37°C For blue/white colony screening during subcloning experiments, LB-agar was also supplemented with 0 005% (w/v) X-gal and 0 05 mg mL^{-1} IPTG

XV. Chromatography

A Thin-layer chromatography

Thin layers (0 25 mm) of silica gel F254 on glass plates (5 x 20 cm, E Merck, Darmstadt) were used The chromatograms were developed with either chloroform-methanol (9 1, v/v) or ethyl acetate-acetic acid-water (14 7 4, v/v/v), then air dried and viewed under illumination with ultra-violet light (254 nm) to identify fluorescence-quenching zones

B High performance liquid chromatography

A Beckman System Gold series high performance liquid chromatograph (HPLC) with a dual-pump programmable solvent module 126, and programmable detector module 166 was used System Gold software, installed on a Jemini 286 (20 MHz) microcomputer was used to program the solvent and detector modules, and to collect, store, and analyze data in real-time

Initially, 20-μL samples of culture supernatant were applied directly to a Phenomenex Ultracarb 5 ODS (30) column (5 μm particle size, 4 6 x 50 mm) Beckman Ultrasphere Octyl and ODS columns (5 μm, 4 6 mm x 25 cm) were also used A 4 6 x 45 mm guard column preceded the main column The columns were developed with programmed linear gradients of water and methanol, or 0 03 *M* KH_2PO_4 (pH 3 3) and methanol, in one of the following four patterns

Method A The detector was set at 273 nm and the column was initially equilibrated with water The concentration of methanol was increased from 0 to 100% over 10

min at a flow rate of 1 mL/min. After 1 min at 100% the concentration of methanol was reduced over 2 min to 0%. The column was then re-equilibrated for 5 to 6 min in water.

Method B: The detector wavelength and flow rate were as in method A but the slope of the water/methanol gradient was increased; the concentration of methanol rose from 0 to 75% in the first 5 min. This was followed by a more gradual increase from 75 to 100% over the next 4 min. The 100% methanol concentration was maintained for 2 min, after which it decreased to 0% over 2 min. The column was then re-equilibrated in water for 5 to 6 min.

Method C: This was identical to method B except that phosphate buffer was used as the aqueous solvent instead of water. The method was later modified by setting the detector to 254 nm instead of 273 nm.

Method D: The detector was at 273 nm, and the column was initially equilibrated with 25% methanol in phosphate buffer. The methanol concentration was maintained at 25% for 2 min and then increased to 100% over the next 5 min. This concentration was maintained for 3 min, and then reduced to 25% over 1 min. The column was re-equilibrated with 25% methanol for 5 to 6 min.

C. Ion exchange chromatography

1. Anion exchange chromatography

A glass chromatography column (1 x 30 cm, Bio-Rad), with a porous polyethylene bed support and a Luer-lock fitting, was packed by gravity with the anion exchanger QAE-Sephadex A-25 (Pharmacia Fine Chemicals). The exchanger (3.8 g) was swollen in 50 mL of distilled deionized (dd) H_2O, overnight at 4°C. The ddH_2O was removed by vacuum filtration and the gel was washed twice with 50-mL portions of ddH_2O. The quaternary aminoethyl groups were converted to the hydroxide form by treating the gel successively by vacuum filtration with three 50-mL portions of 0.1 *M* NaOH. The gel was then washed with three 50-mL portions of ddH_2O, and converted to the acetate form with

50 mL of 2 *M* acetic acid. The gel was equilibrated for 1 h with constant stirring at room temperature in 100 mL of 2 *M* acetic acid, after which the acetic acid was removed by vacuum filtration and the gel was washed with 50 mL of ddH_2O. The gel was then resuspended in a small amount of ddH_2O and used to pack a 1 x 25 cm column.

Approximately 250 mL of supernatant (containing 80 $\mu g.mL^{-1}$ cmpd.X and 20 $\mu g.mL^{-1}$ chloramphenicol) from a 16-h incubation of chloramphenicol (100 $\mu g.mL^{-1}$) with an RM4 mycelial suspension in H_2O, were filtered through a 0.2-µm pore-size cellulose acetate membrane, and applied to the column in 10-mL aliquots; 5-mL fractions were collected using a Gilson Microfractionator and monitored at 273 nm. After the column had been washed with an equal volume of ddH_2O it was developed with 1 *M* ammonium formate (pH 2.5).

2. Cation exchange chromatography

A 10-mL pipette (Kimble, disposable) with a plug of nonabsorbent cotton wool as a bed support was packed with the cation exchanger SP-Sephadex C-25 (Pharmacia Fine Chemicals). The gel was swollen in distilled water and equilibrated to pH 2.0 with 0.1 *M* ammonium formate (pH 2 0). A total packed volume of ca. 9 mL was used A 5-mL sample of culture supernatant (adjusted to pH 2.0) was applied to the column, which was then washed with 5 mL of distilled water. Two 5-mL fractions were collected and analyzed by HPLC

D Preparative reversed-phase chromatography

A glass chromatography column (1 x 30 cm; Bio-Rad), with a porous polyethylene bed support and a Luer-lock fitting, was packed to height of 23 cm with C_{18} reversed-phase silica particles (Bakerbond, wide-pore) in 100% methanol. The methanol was replaced by washing the column with a linear methanol:ddH_2O gradient, after which the column was thoroughly washed with ddH_2O. Approximately 250 mL of supernatant (containing 140 $\mu g.mL^{-1}$ cmpd.X, 60 $\mu g\ mL^{-1}$ chloramphenicol) from a 23-h RM4 myce-

lial suspension in ddH_2O, were filtered through two thicknesses of Whatman #5 filter paper and evaporated to dryness at 40°C *in vacuo*. The residual amber film was redissolved in 1 mL of ddH_2O to form a yellowish turbid solution. This was acidified to pH 3.4 by adding 100 µL of 1 M ammonium formate (pH 2.5), giving an immediate stringy precipitate. The cmpd.X solution was clarified by filtering through a plug of nonabsorbent cotton wool tightly fitted into a Pasteur pipette, and applied directly to the column. The sample was successively washed into the column with 1 mL of 0.1 *M* ammonium formate (pH 2.5) and three 1-mL portions of ddH_2O. The column was then washed with ddH_2O; the effluent was collected in 1.8-mL volumes for the first ten fractions and 2.7-mL volumes for the next 49 fractions using a Gilson Microfractionator. All fractions were monitored at 273 nm; those with elevated absorbance were analyzed directly by HPLC.

XVI. Nuclear magnetic resonance (NMR) spectroscopy

A. ^{1}H-NMR

The ^{1}H-NMR spectra of compound X (9.5 mg) and chloramphenicol in 5-mm sample tubes were recorded at 250.1 MHz in methanol, using tetramethylsilane (TMS) as an internal reference. A Bruker AC-250 F spectrometer was used. Data was accumulated using 90° pulses (9.6 µs) with delays of 1 or 2 s; the data size was 16 K zero-filled to 32 K.

B. ^{13}C-NMR

The ^{13}C-NMR spectra for compound X were recorded at 62.5 MHz in D_2O, locked to solvent deuterium; the spectra for chloramphenicol were recorded in acetone using solvent ^{13}C as a reference. Data was accumulated using 90° pulses (6 µs) with delays of 1 or 2 s. The data size was 32 K.

C ^{31}P-NMR

The ^{31}P-NMR spectra for compound X were recorded at 101 2 MHz in D_2O, using 85% H_3PO_4 as an external reference to set 0 p p m. Data was accumulated using 90° pulses (6 μs) with delays of 1 or 2 s The data size was 32 K

XVII. Mass spectroscopy

The mass spectrum of compound X was obtained on an API/III triple quadrupole mass spectrometer (Perkin-Elmer SCIEX Instruments, Thornhill, Ontario) using nebuliser-assisted electrospray ionization (ionspray) The mass spectrum was acquired in the negative ion mode by injecting a 2-μL sample of a 1 5 mg/200 μL solution of compound X into a stream of 50% acetonitrile The flow rate to the mass spectrometer was 10 μL min^{-1} The mass spectrometer scanned over a mass range of 100-500 in steps of 0 1 Da with a dwell time of 2 ms step^{-1} The mass spectrometer was calibrated by injecting a solution of cesium nitrate, mass assignment was reliable to 0 5 Da

XVIII. Cell extracts

The mycelium from a 50-mL, 48-h culture of either *S. lividans* RM3 or RM4, grown in GNY supplemented with 12 5 μg mL^{-1} chloramphenicol, was pelleted at 35,000xg for 10 min at 4°C The supernatant was discarded and the mycelium was washed with 1 volume of 50 m*M* MOPS buffer (pH 6 8) The pellet was then resuspended in a final volume of 40 mL of MOPS buffer, transferred to a sterile 50-mL glass tube, and chilled in an ice/salt bath for 5-10 min The mycelium was sonicated at full power for 15 s and cooled for 1 min This was repeated twice, after which the sonicate was transferred into a sterile, pre-chilled centrifuge tube and centrifuged at 35,000xg for 20 min at 4°C The supernatant and pellet were then separated and each was made to a final volume of 50 mL with MOPS buffer

XIX. Bioassay for antibiotic activity

Rehydrated nutrient agar medium (Difco) was sterilized by autoclaving and then cooled to 50°C. The still-liquid medium was seeded (2% v/v) with a 48-h culture of *Micrococcus luteus* grown in nutrient broth, and 10-mL portions of the agar medium were transferred to sterile Petri-plates (9-cm diameter). Filter disks (1.3-cm diameter) impregnated with aqueous solutions of either compound X or chloramphenicol were placed on the surface of the gelled nutrient agar and the *M. luteus* cultures were incubated overnight at 30°C. The relative antibiotic activity of the test samples was determined by measuring the diameters of the inhibition zones surrounding the disks. Compound X was compared with known amounts of chloramphenicol.

RESULTS

I. Localizing the chloramphenicol resistance determinant of pJV3

The recombinant plasmid pJV3 contains a 6.5-kb *Sst*I-fragment of *S. venezuelae* ISP5230 genomic DNA cloned in the *Sst*I site of pIJ702; it complements the Cml^S phenotypes of *S. lividans* M252 and M417 (Mosher *et al.*, 1990). Restriction mapping of pJV3 showed two *Kpn*I sites separated by 5.2 kb (Fig. 2); one site was located within the vector portion of the plasmid and the other in the *S. venezuelae* insert. To localize the Cml^r determinant, pJV3 was digested with *Kpn*I; the mixture of fragments was ligated and used to transform *S. lividans* M252 (Fig. 2).

All of the resulting transformants were thiostrepton and chloramphenicol resistant, and all possessed a 7.1-kb plasmid, pJV4. Restriction mapping of pJV4 showed that the 5.2-kb *Kpn*I-*Kpn*I fragment, containing 1.1 kb of vector DNA and 4.1 kb of *S. venezuelae* DNA, had been deleted from pJV3 (see Fig. 2). Since pJV4 still conferred chloramphenicol resistance on *S. lividans* M252, it was concluded that the Cml^r determinant was located on the remaining 2.4 kb of *S. venezuelae* DNA.

II. Subcloning the chloramphenicol resistance determinant in *Escherichia coli*

To investigate the possibility that the Cml^r determinant of pJV3 might be expressed in *E. coli*, the plasmid was digested with *Sst*I and ligated with an *Sst*I digest of pTZ18R (Fig 3); the mixture was used to transform *E. coli* TG1. Two different recombinant plasmids of 9.4 kb, designated pJV5 and pJV6, were isolated from the ampicillin-resistant transformants. Digestion of both plasmids with *Sst*I yielded DNA fragments of 2.9 kb and 6.5 kb.

Digestion of the plasmids with *Kpn*I, *Pst*I, and *Sst*I both alone and in pairs, established that the 6.5-kb *Sst*I insert of pJV3 was present in opposite orientations with respect to the *lac*-promoter of pTZ18R (see Fig. 3). Single colonies of TG1 carrying either pJV5

Figure 2: Construction of pJV4 from pJV3 by deletion of a 5.2-kb *Kpn* I-*Kpn* I fragment. Distances shown are in kb with reference to the *Bam* HI site. The *S. venezuelae* DNA insert is shown as an unfilled segment.

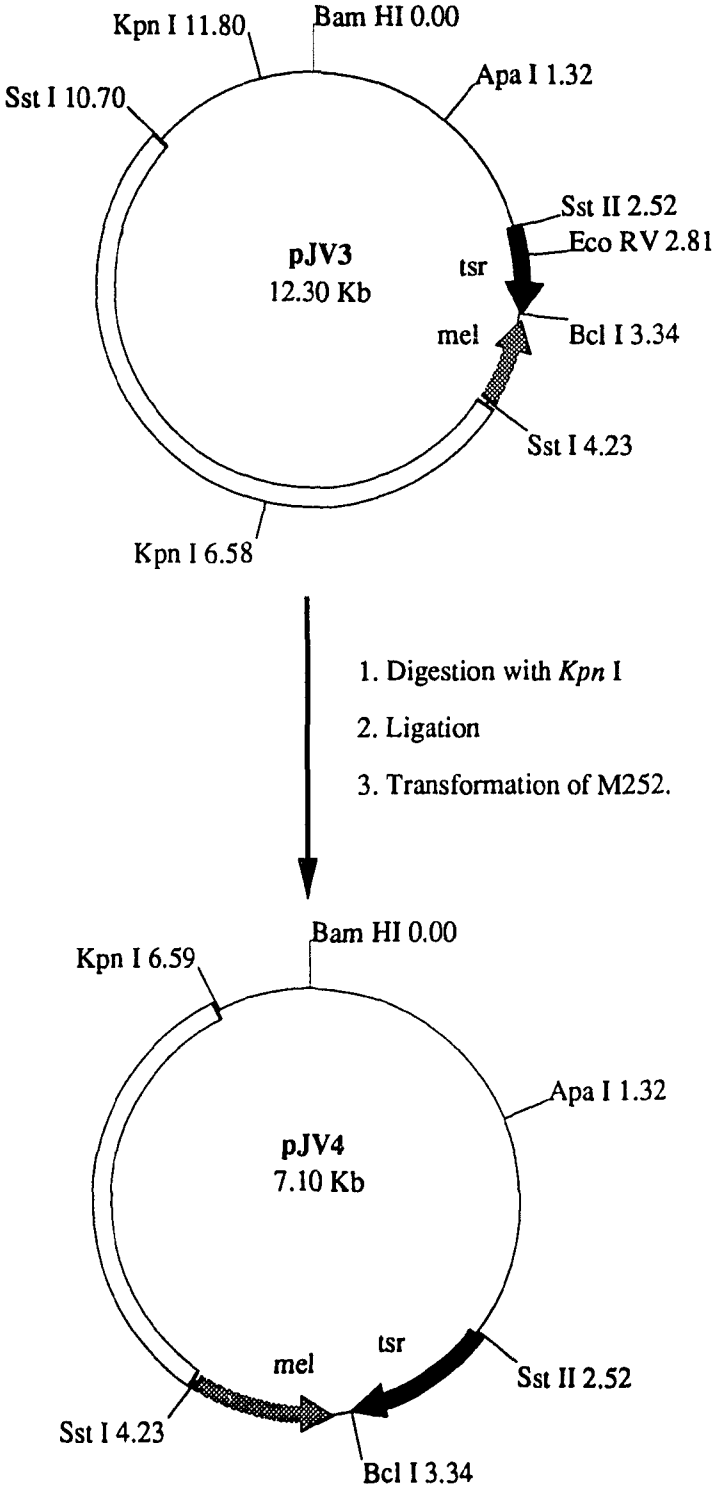
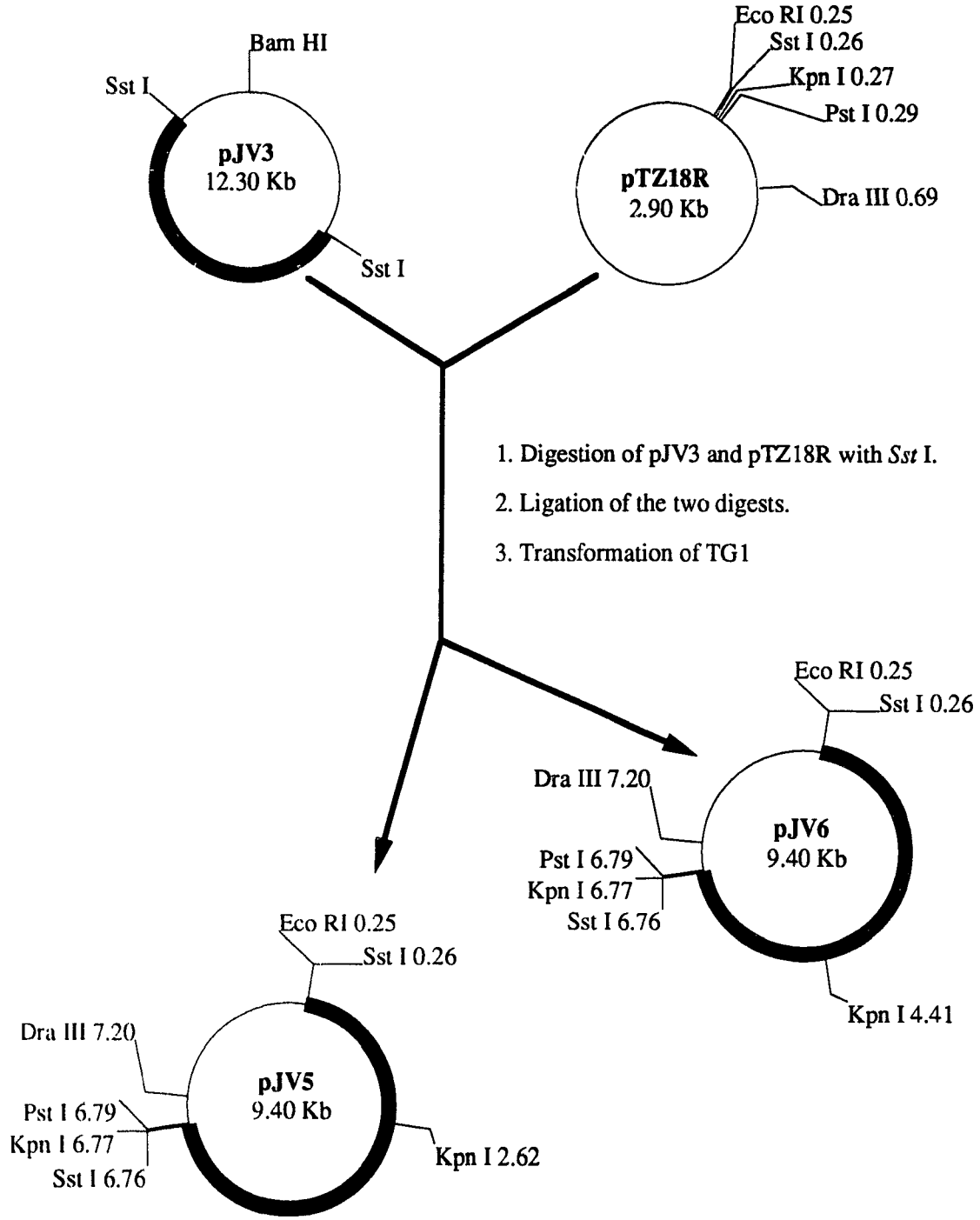


Figure 3: Construction of pJV5 and pJV6 by subcloning a 6.5-kb *Sst* I-*Sst* I fragment from pJV3 into pTZ18R. Thick regions represent *S. venezuelae* DNA and thin regions, vector DNA.



or pJV6 were then plated on L-agar supplemented with chloramphenicol at 10 $\mu g.mL^{-1}$. No growth was seen after 48h at 37°C, even when IPTG was included in the medium as an inducer.

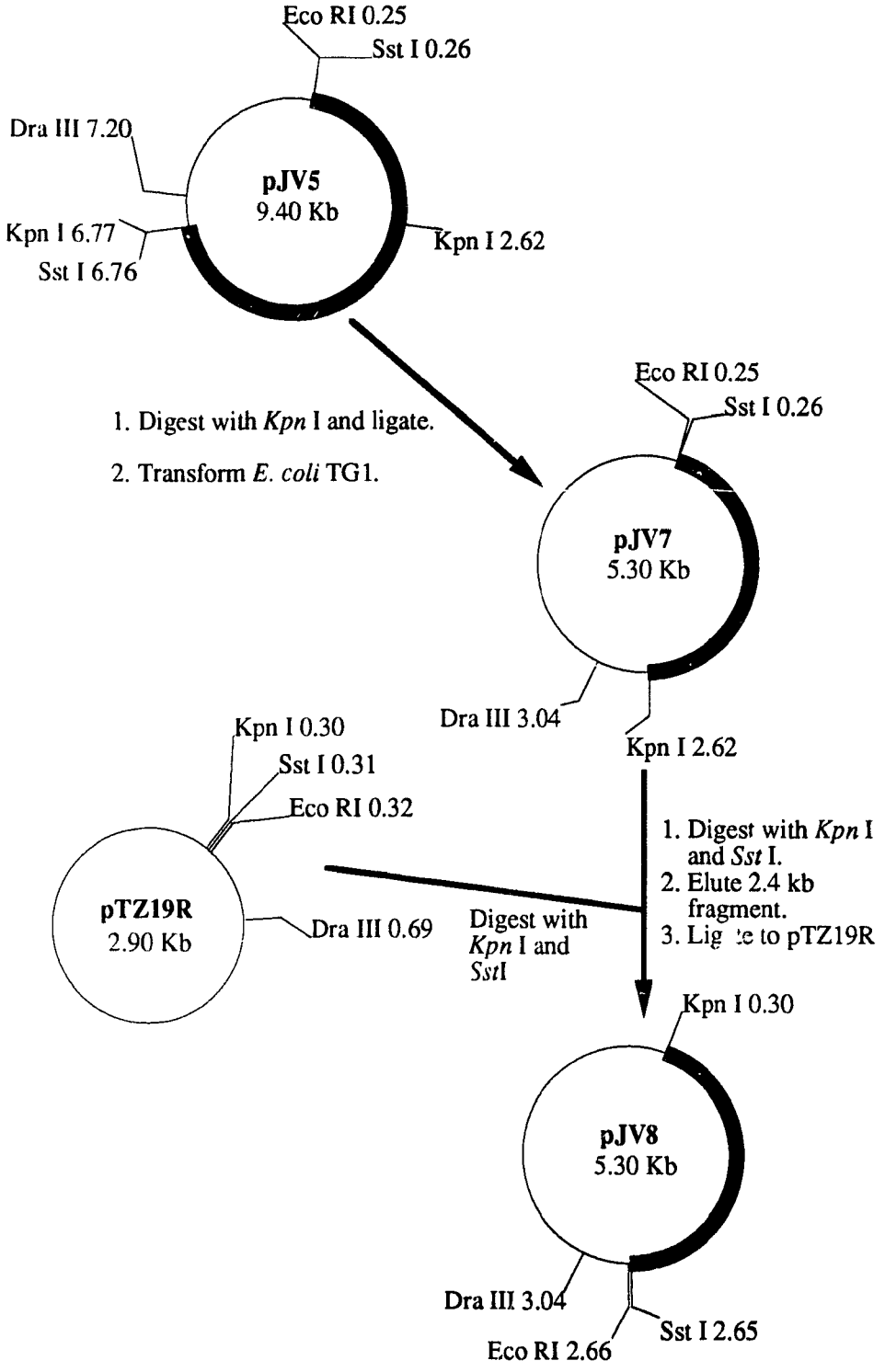
The failure of pJV5 and pJV6 to confer chloramphenicol resistance on *E. coli* TG1 suggested that the native streptomycete promoter(s) presumably associated with the resistance gene, if present on the insert, was incompatible with the transcription system of *E. coli*. An attempt was, therefore, made to place the Cml^r-determinant under the control of the *lac*-promoter of pTZ18R or pTZ19R. Both of the latter plasmids possess a multiple cloning site (polylinker) downstream from the *lac*-promoter and differ only in the orientation of this multiple cloning site. The inverse order of the restriction sites within the polylinker facilitates expression of open reading frames on either strand of the insert DNA.

An *in vitro* deletion strategy similar to that used to construct pJV4 was used to delete a 4.1-kb *Kpn*I-*Kpn*I DNA fragment from pJV5. This brought the remaining 2.4-kb *Kpn*I-*Sst*I segment of the insert into closer proximity to the *lac*-promoter. The polylinker of pTZ18R possesses a *Kpn*I site immediately adjacent to its *Sst*I site. By digesting pJV5 with *Kpn*I, ligating the fragments, and using the mixture to transform *E. coli* TG1, a plasmid (pJV7) possessing only the 2.4-kb *Kpn*I-*Sst*I DNA fragment of pJV4 was isolated (Fig. 4).

The *S. venezuelae* DNA insert in pJV7 was isolated by digesting the plasmid with *Kpn*I and *Sst*I, fractionating the digest by agarose gel electrophoresis, and eluting the 2.4-kb DNA fragment. This was ligated to pTZ19R that had been digested with *Kpn*I and *Sst*I, and the ligation mixture was used to transform *E. coli* TG1. Transformants were screened for plasmid DNA that, when digested with *Kpn*I and *Sst*I, produced restriction fragments of 2.9 kb and 2.4 kb (Fig. 4). The plasmid so isolated was designated pJV8.

When strains of TG1 containing pJV7 and pJV8 were cultured in L-broth, supplemented with 10 $\mu g.mL^{-1}$ chloramphenicol, with or without IPTG, no growth was ob-

Figure 4: Construction of pJV7 and pJV8. Deletion of a 4.1-kb *Kpn* I-*Kpn* I fragment from pJV5 yielded pJV7. To obtain pJV8 a 2.4-kb *Kpn* I-*Sst* I fragment from pJV7 was subcloned in pTZ19R. Thick regions represent *S. venezuelae* DNA and thin regions vector DNA.



served. To determine whether the Cml^r-determinant was being expressed but not conferring resistance, both strains were grown, in the presence or absence of IPTG, in M9 (minimal) medium supplemented with appropriate growth factors, ampicillin, and a sublethal dose (0.2 $\mu g.mL^{-1}$) of chloramphenicol. After 24 h incubation, culture supernatants were extracted with ethyl acetate. The extracts were concentrated to dryness and the residue was taken up in methanol, and examined by thin-layer chromatography with either a chloroform-methanol (9:1) or ethyl acetate-acetic acid-water (14:7:4) solvent system. As reference compounds, chloramphenicol, *p*-nitrophenylserinol, and *N*-acetyl-*p*-nitrophenylserinol were chromatographed with the samples when appropriate. However, no loss of the chloramphenicol supplement was detected and none of the breakdown products expected from the presence of chloramphenicol inactivating genes were observed. These results were consistent with a lack of expression of the Cml^r-determinant on the recombinant plasmids in these strains.

III. Subcloning of the 2.4-kb *Kpn*I-*Sst*I fragment of pJV7 for DNA sequencing

The dideoxy chain-terminating procedure usually requires that the candidate DNA be inserted downstream of a primer-binding site in a vector that, under specific conditions, can generate single-stranded copies of itself. The phagemid vector pBluescriptII SK+ provides a convenient system for subcloning and generating nested overlapping deletions. However, because the first and last restriction sites in the pBluescriptII SK+ polylinker are *Sst*I and *Kpn*I respectively, the direct subcloning of the 2.4-kb *Kpn*I-*Sst*I insert of pJV7 into pBluescriptII SK+ would have eliminated the entire polylinker from the vector. Brown (1991) detected the presence within the insert of a previously unmapped *Eco*RI restriction site. This was located approximately 0.2 kb from the *Kpn*I junction site, and 2.2 kb from the *Eco*RI site within the vector's polylinker. To avoid loss of the polylinker a 2.2-kb *Eco*RI-*Eco*RI subfragment of the pJV7 insert was subcloned into the *Eco*RI site of the phagemid's polylinker. One strand of the remaining 0.2-kb *Kpn*I-*Eco*RI subfragment of

the pJV7 insert was sequenced in the phage vector M13mp18. The vector M13mp18 replicates as a double-stranded (RF) molecule within the bacterial cell and produces single-stranded copies that are released from the cell as infectious phage particles. It possesses a polylinker identical to that found in pTZ18R, thus simplifying the movement of fragments between vectors.

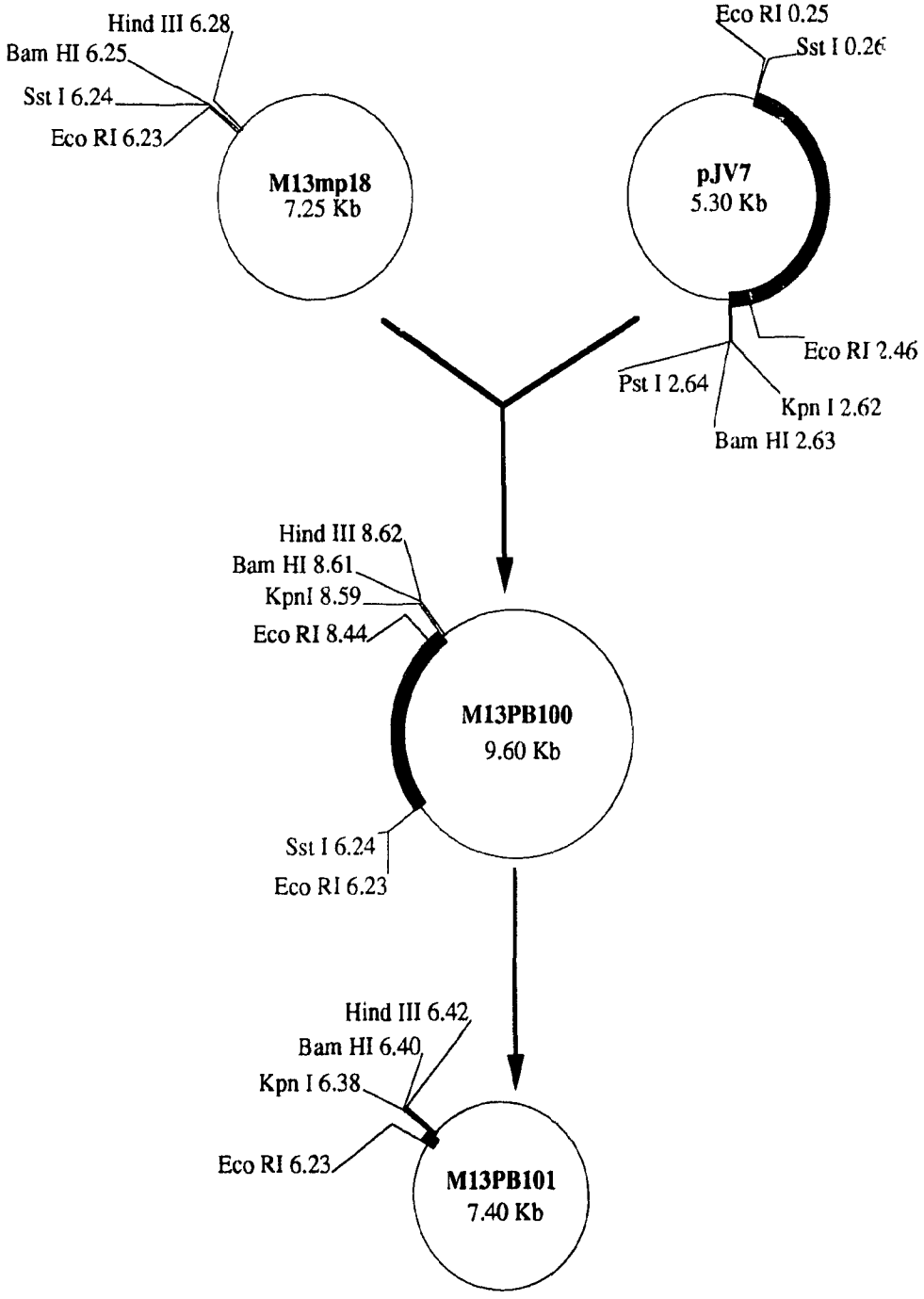
The 2.4-kb *Kpn*I-*Sst*I DNA fragment from pJV7 was subcloned into M13mp18 by Brown (1991), who carried out initial sequencing of the DNA fragment using M13PB100 and M13PB101 (see below). However, the data generated by Brown (1991) contained several sequencing ambiguities that were caused primarily by band compressions in sequencing reactions electrophoresed on denaturing polyacrylamide gels. To resolve the sequencing errors both M13PB100 and M13PB101 were resequenced in the present study.

A. Subcloning of the 2.4-kb insert of pJV7 in M13mp18.

In pJV7, the polylinker contained a *Bam*HI site immediately adjacent to its *Kpn*I site. The 2.4-kb insert was recovered from a *Bam*HI/*Sst*I double digest of the plasmid by agarose gel electrophoresis and elution of the appropriately sized fragment. It was ligated to M13mp18 RF-DNA, also digested with *Bam*HI and *Sst*I (Brown, 1991). Use of the ligation mixture to transform *E. coli* TG1 yielded the recombinant phage M13PB100 (Fig. 5). This was isolated and digested with *Bam*HI, *Kpn*I and *Sst*I to demonstrate that an insert identical to that of pJV7 was present (Brown, 1991).

By digesting M13PB100 with *Eco*RI, ligating the digest and using it to transform *E. coli* TG1, Brown (1991) isolated M13PB101, a deletion-clone possessing the 0.2-kb *Kpn*I-*Eco*RI segment from the M13PB100 insert (see Fig. 5). This permitted one strand of the 0.2-kb fragment to be sequenced from the universal priming site immediately upstream of the polylinker site of M13mp18.

Figure 5: Construction of M13PB100 and M13PB101. A 2.4-kb *Bam* HI-*Sst* I fragment from pJV7 was subcloned in M13mp18 to give M13PB100; deletion of a 2.2-kb *Eco* RI-*Eco* RI fragment from M13PB100 gave M13PB101. Thick regions represent *S. venezuelae* DNA and thin regions vector DNA. Adapted from Brown (1991).



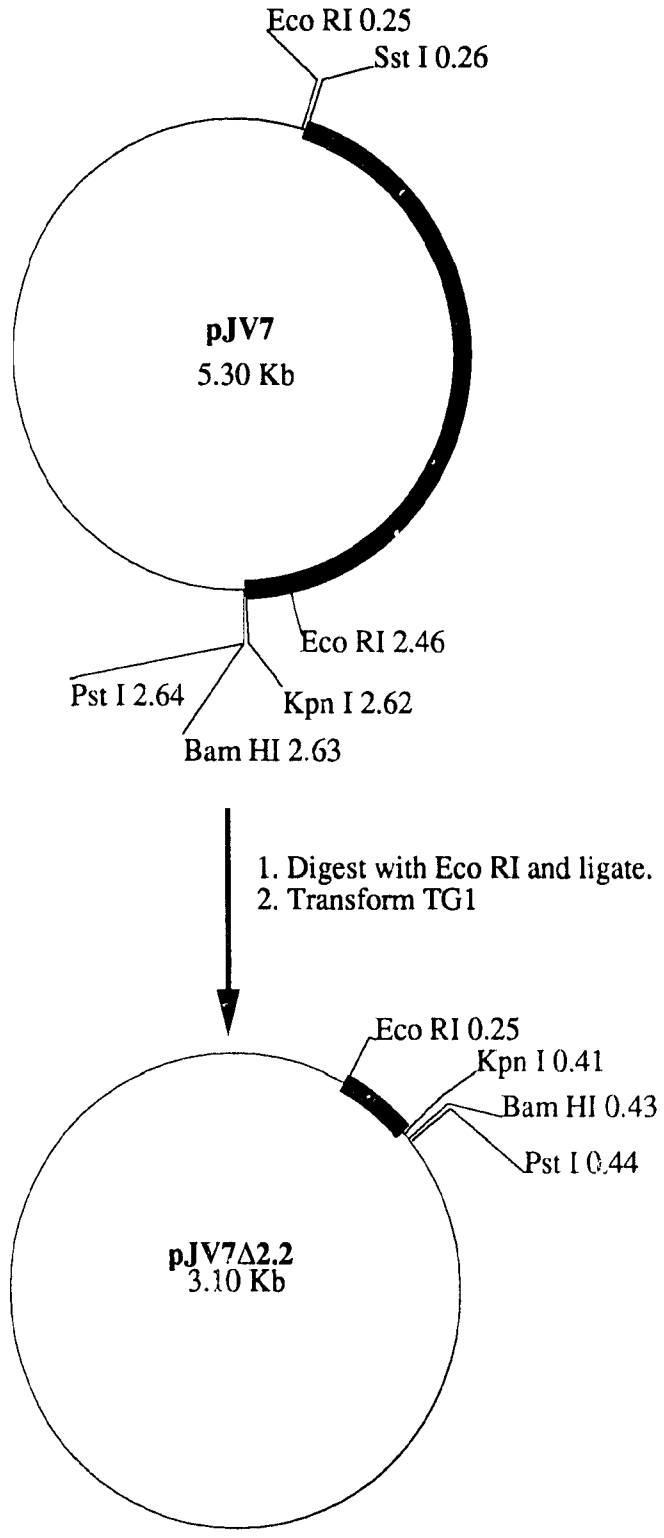
B. The *in vitro* deletion generating pJV7Δ2.2

To sequence the complementary strand of the 0.2-kb *Kpn*I-*Eco*RI fragment, pJV7 was digested with *Eco*RI, and the fragments, after ligation, were used to transform *E. coli* TG1. Plasmid pJV7Δ2.2 was isolated from one of the transformants; when digested with *Eco*RI, this plasmid gave a single 3.1-kb linear fragment. Double digestion with *Eco*RI and *Kpn*I produced a fragment of 2.9 kb; the 0.2-kb fragment presumed to accompany this was not visible by agarose gel electrophoresis and ethidium bromide staining. The results were consistent with the *in vitro* deletion of a 2.2-kb *Eco*RI-*Eco*RI fragment from pJV7 (Fig. 6). Since pJV7 is a derivative of plasmid pTZ18R a phagemid vector possessing an f1-origin of replication, it could be used to obtain a single-strand copy of the insert DNA for nucleotide sequencing. When *E. coli* TG1 was transformed with pJV7Δ2.2 and infected with helper phage VCSM13, single-stranded copies of the plasmid were generated and packaged into phage particles; these were then exported from the cell. Recovery of the phage particles from the culture supernatants and purification of the ssDNA provided the template for dideoxy sequencing reactions. A synthetic oligonucleotide (the reverse primer) binds to a complementary sequence immediately upstream of the *Eco*RI site of the polylinker region in pTZ18R. The 0.2-kb *Kpn*I-*Eco*RI DNA insert fragment retained in pJV7Δ2.2 was thus within range of the vector's reverse priming site, so the sequence of the DNA strand complementary to the one sequenced using M13PB101 could be determined.

C. Subcloning the 2.2-kb *Eco*RI-*Eco*RI fragment of pJV7 in pBluescriptII SK+

To facilitate sequencing of the remaining 2.2-kb *Eco*RI-*Eco*RI segment of DNA in M13PB100, this fragment was recovered from pJV7. The 2.2-kb fragment obtained by digestion with *Eco*RI was separated by agarose gel electrophoresis, eluted and ligated to the *Eco*RI-digested phagemid, pBluescriptII SK+. The ligation mixture was used to transform *E. coli* TG1. Plasmid DNA from ampicillin-resistant transformants was di-

Figure 6: The construction of pJV7Δ2.2 by deletion of a 2.2-kb *Eco* RI-*Eco* RI fragment from pJV7. Thick regions represent *S. venezuelae* DNA and thin areas vector DNA.



gested with *Sst*I and/or *Eco*RI. Electrophoretic analysis identified two recombinant plasmids, pJV9 and pJV10, containing the desired insert in opposite orientations with respect to the T7 priming site (Fig. 7).

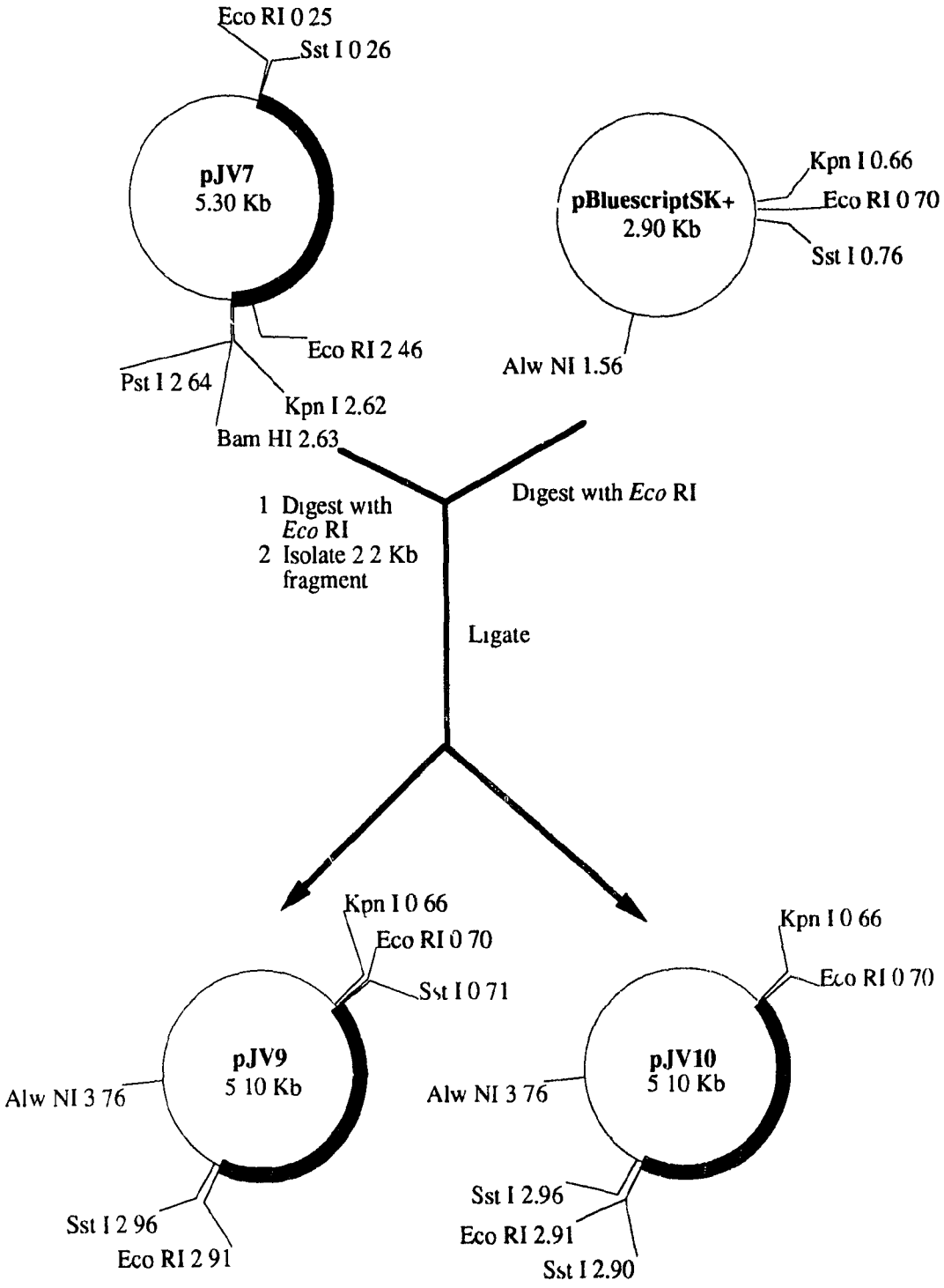
IV. DNA sequencing of the 0.2-kb *Kpn*I-*Eco*RI fragment of pJV7

The DNA sequence of one strand in the 0.2-kb *Kpn*I-*Eco*RI fragment was determined by using the universal primer (a synthetic 17-mer oligonucleotide that binds to the single-stranded DNA of M13mp18 at a site just upstream of the polylinker's *Hin*dIII restriction site) to sequence single-stranded DNA from M13PB100 and M13PB101. When necessary, 7-deaza-dGTP was used in place of dGTP in the labeling reaction, primarily to reduce the band compression common with G+C-rich streptomycete DNA in denaturing polyacrylamide sequencing gels. Sequence data from M13PB100 were extended across the *Eco*RI site and overlapped with DNA sequence obtained from pJV9 and pJV10 (see below). To sequence the complementary strand, helper phage VCSM13 was used to infect *E. coli* TG1 carrying pJV7Δ2.2. The single-stranded DNA copy of the plasmid thus produced was sequenced using the reverse primer, and yielded sequence data consistent with that produced from M13PB100 and M13PB101.

V. DNA sequencing of the 2.2-kb *Eco*RI-*Eco*RI fragment of pJV7

The products of a typical sequencing reaction can be fractionated by polyacrylamide gel electrophoresis to yield sequence data for approximately 170-200 nucleotides (nt) Therefore, to sequence a DNA molecule of 2200 nt it was necessary to create a set of nested, overlapping deletions extending across the entire length of the 2.2-kb insert in pJV9. In preparation for this, pJV9 was first digested with a mixture of *Cla*I and *Kpn*I. Digesting with *Cla*I created a four base, 5'-overhang of single-stranded DNA immediately adjacent to the 2.2-kb insert. The digested DNA was then treated with exonuclease III for increasing lengths of time; exonuclease III recognizes 5'-overhangs as substrates to cata-

Figure 7: The construction of pJV9 and pJV10 by subcloning a 2.2-kb *Eco* RI-*Eco* RI fragment from pJV7 into pBluescriptII SK+. Thick regions represent *S venezuelae* DNA and thin regions vector DNA.



lyze the progressive removal of successive nucleotides on one strand of a DNA molecule. In contrast, 3'-overhangs are not substrates for exonuclease III attack; digesting pJV9 with *Kpn*I created a 3'-overhang of single-stranded DNA just downstream of the T7-primer binding site. This site was thus protected for subsequent sequencing reactions. The products of exonuclease digestion were treated with mungbean nuclease to remove the single stranded portions of the DNA molecules, and with Klenow fragment to fill in any overhangs left after mungbean nuclease treatment. The blunt-ended products were then ligated and used to transform *E coli* TG1. Plasmid DNA from the transformants was fractionated by agarose gel electrophoresis using pBluescriptII SK+ and pJV9 as size references Approximately 25 clones possessing plasmids that migrated between the two reference samples, were selected for further study. The deletion plasmids were then arranged in descending order of size, and their hosts were infected with helper phage VCSM13 The resulting single-stranded DNA from each clone was used in dideoxy-sequencing reactions using the T7-primer, with and without 7-deaza-dGTP

As expected, the DNA sequences obtained from many of the clones overlapped, allowing the assembly of contiguous stretches of DNA sequence However, some sequences failed to overlap, producing gaps in the compiled DNA sequence. These were filled by synthesizing a series of 17-mer oligonucleotides designed to bind to regions adjacent to gaps in previously sequenced areas. Sequencing reactions extending from these synthetic primers allowed the construction of a contiguous 2200 nt DNA sequence (Fig 8)

To confirm the sequence generated from pJV9 the sequence of the complementary DNA strand was determined. A set of oligonucleotide primers based on the DNA sequence obtained from pJV9 was synthesized and used to sequence the single-stranded DNA generated from pJV10 The sequence of the complementary strand agreed with that produced from pJV9 (Fig. 9).

Figure 8: A schematic of the overall strategy used to sequence the 2.4-kb *Kpn*I-*Sst*I insert of *S. venezuelae* DNA in pJV7. The bar represents the DNA fragment (100 bp.cm^{-1}). The thick arrows below the bar represent predicted open reading frames. Each thin arrow represents the length of DNA sequence obtained from an individual deletion clone or from sequencing reactions primed with synthetic oligonucleotides. The thin arrows labeled (1), (2), and (3) represent DNA sequence obtained from clones pJV7Δ2.2, M13PB100, and M13PB101, respectively.

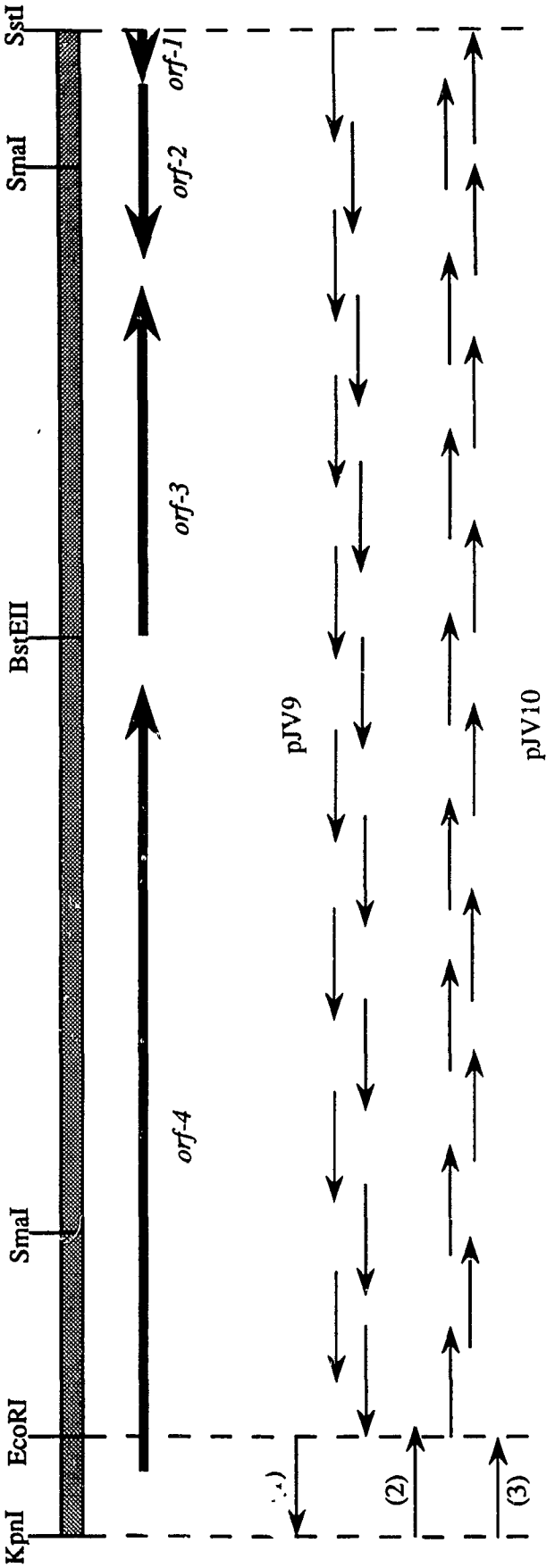

SstI
SmaI
BstEII
SmaI
EcoRI
KpnI
orf-1
orf-2
orf-3
orf-4
pJV9
pJV10
(2)
(3)

Figure 8

Figure 9: The complete nucleotide sequence (2355 bp) of the 2.4-kb *Sst*I-*Kpn*I DNA fragment subcloned from pJV3. The highlighted *Sst*I and *Kpn*I recognition sequences are located at nucleotides 1-6 and 2350-2355, respectively. A unique *Eco*RI recognition site at nucleotides 2197-2202 is also highlighted. The upper (5'→3') strand of the sequence is designated strand A, and the lower (3'→5') strand B.


5'
GAGCTCGGCCCGGACCCGCACTGGGTCTGCGGCAAGCAGGGGGTCGTGGTCGCGCCGGTGGGCGGCCTGA
1
CTCGAGCCGGGCCTGGGCGTGACCCAGACGCCGTTCGTCCCCCAGCACCAGCGCGGCCACCCGCCGGACT
3'
AGTGAGGAGCCCGCGCAGGTGACGCTCTACATCGACCCGCCGACCTGGCCGGGGCACGGCCGCATGTGGT
71
TCACTCCTCGGGCGCGTCCACTGCGAGATGTAGCTGGGCGGCTGGACCGGCCCCGTGCCGGCGTACACCA
CGCACCTCGTCAGCGACGTCTCCTTCGACGAGCTGCACGCCTTCGCCGCGTCGATCGGCGCGCCGCCCCG
141
GCGTGGAGCAGTCGCTGCAGAGGAAGCTGCTCGACGTGCGGAAGCGGCGCAGCTAGCCGCGCGGCGGGGC
GGCCTTCGAGCGGGACCACTACGACATCCCGTCCGACCGGTACGCCGACGCGGTGGCGGGCGGCGCGGTC
211
CCGGAAGCTCGCCCTGGTGATGCTGTAGGGCAGGCTGGCCATGCGGCTGCGCCACCGCCCGCCGCGCCAG
GAGGTCGGCTCGAAGGAGCTGCTGCGCCGCCTGACCGAGGCGGGCCTGCGCCGGCCGAAGGGCCGCCCGG
281
CTCCAGCCGAGCTTCCTCGACGACGCGGCGGACTGGCTCCGCCCGGACGCGGCCGGCTTCCCGGCGGGCC
CGTCCTGAGGGCCCGCGTCGAGGGCCGCCCGGCGTCCTGAGGACTCGCGTCGAGGACCTACGGGACGACG
351
GCAGGACTCCCGGGCGCAGCTCCCGGCGGGCCGCAGGACTCCTGAGCGCAGCTCCTGGATGCCCTGCTGC
TGGGCGGCGATCGCCCAGGCGCACTCGATCGACTCCTTGTGCGTGGTGTCGACCTCCACGTCGTACTCCA
421
ACCCGCCGCTAGCGGGTCCGCGTGAGCTAGCTGAGGAACACGCACCACAGCTGGAGGTGCAGCATGAGGT

Figure 9

```
    CGCCCTCGTGCACGACGTACGCCTGCTTCGCCGCCATGCCCCGCGACGCGGTCGCCGCGCGCGGTCTCCCG
491 ---------+---------+---------+---------+---------+---------+---------+
    GCGGGAGCACGTGCTGCATGCGGACGAAGCGGCGGTACGGGCGCTGCGCCAGCGGCGCGCGCCAGAGGGC

    GCCCTCGGCGACGGCGCCGTCGCACCGGACGCCGACCCAGAGCACGTCCAGGTCCCCGACGAAGCTCCGC
561 ---------+---------+---------+---------+---------+---------+---------+
    CGGGAGCCGCTGCCGCGGCAGCGTGGCCTGCGGCTGGGTCTCGTGCAGGTCCAGGGGCTGCTTCGAGGCG

    CAGCGCTCCTGGGCGGCGGCACCGCCGAGGAAGACGTCGTCGATGATGATCCGGGCGCCCGCGCGGGCCA
631 ---------+---------+---------+---------+---------+---------+---------+
    GTCGCGAGGACCCGCCGCCGTGGCGGCTCCTTCTGCAGCAGCTACTACTAGGCCCGCGGGCGCGCCCGGT

    TCGCGACGACGCCCTCGGCCCAGGCGCCCTCAAGGGCGCGGAACTCGGGCCCGATGCTCACCCCGCCGTC
701 ---------+---------+---------+---------+---------+---------+---------+
    AGCGCTGCTGCGGGAGCCGGGTCCGCGGGAGTTCCCGCGCCTTGAGCCCGGGCTACGAGTGGGGCGGCAG

    GGCGTCGAACTCGATGCCGCCTTCGGCGCTCTGCATCTTCAGGGGCATCGCCTCGATGAGGGAGTCGACG
771 ---------+---------+---------+---------+---------+---------+---------+
    CCGCAGCTTGAGCTACGGCGGAAGCCGCGAGACGTAGAAGTCCCCGTAGCGGAGCTACTCCCTCAGCTGC

    CCGAAGGCCAGCCAGGGCTCGGGAAGGACGGACTGGAGGCACCGTACGATGCCGGACTTCCCCGCGCTGG
841 ---------+---------+---------+---------+---------+---------+---------+
    GGCTTCCGGTCGGTCCCGAGCCCTTCCTGCCTGACCTCCGTGGCATGCTACGGCCTGAAGGGGCGCGACC

    AACCGCCGTTGAGGATGATCATCCGAGTGGTCACCCCGTCACCGTACGGCGCTGCCGGGACGGTGCGGAA
911 ---------+---------+---------+---------+---------+---------+---------+
    TTGGCGGCAACTCCTACTAGTAGGCTCACCAGTGGGGCAGTGGCATGCCGCGACGGCCCTGCCACGCCTT
```

Figure 9


ACGGATTTCACCCGGCGCGCACCAGTGCGGACGCGCCTCAGCAGCTCGACGTGGCCTTCCCCCTGGCCGG
981
TGCCTAAAGTGGGCCGCGCGTGGTCACGCCTGCGCGGAGTCGTCGAGCTGCACCGGAAGGGGGACCGGCC
TTCGGACGGGGTGGTCCCGCCCGCCCCTCTGCTCCGGGCGACCACGTGGCCGGGGGCCGGGCGCTTGGTC
1051
AAGCCTGCCCCACCAGGGCGGGCGGGGAGACGAGGCCCGCTGGTGCACCGGCCCCCGGCCCGCGAACCAG
AGGCGGAGGGCCAGGGCCGCCGTTCCCAGGCCCAGGACCGTCATCGCCGCGCCCGCCCAGGCCGTCGAGG
1121
TCCGCCTCCCGGTCCCGGCGGCAAGGGTCCGGGTCCTGGCAGTAGCGGCGCGGGCGGGTCCGGCAGCTCC
CGAAGCCGAGGTTCGCGTCGATGACCGTGCCGCCGAGCCAGGGGCCGCCCGTGTTGCCGAGGTTGAAGGC
1191
GCTTCGGCTCCAAGCGCAGCTACTGGCACGGCGGCTCGGTCCCCGGCGGGCACAACGGCTCCAACTTCCG
GGCGGTGGTGGTGGCGCCCGCGAGGGTGGGGGCGGCGCCGGCGACGTTGAACATGCGGGCGTTGAGGGCC
1261
CCGCCACCACCACCGCGGGCGCTCCCACCCCCGCCGCGGCCGCTGCAACTTGTACGCCCGCAACTCCCGG
GGGGCCGTGTAGAAGGCCGAGACGCCGAGGAGGAAGGTCAGCACGATCGTGGCGGCCGGGTTCGAGGCGA
1331
CCCCGGCACATCTTCCGGCTCTGCGGCTCCTCCTTCCAGTCGTGCTAGCACCGCCGGCCCAAGCTCCGCT
ACAGGGCCAGGGCCACGAGGAAGACGGTGGAGGCGGAGATGCCGGTGAGCAGCACGCCGAAGAGGTGCGC
1401
TGTCCCGGTCCCGGTGCTCCTTCTGCCACCTCCGCCTCTACGGCCACTCGTCGTGCGGCTTCTCCACGCG

Figure 9

```
      GTCGGCGACCCGGCCGCCGATCGTCGTACCGACGACGGCGCCGATGCCGAAGAGGCCGAGGACGCCGGAG
1471  ---------+---------+---------+---------+---------+---------+---------+
      CAGCCGCTGGGCCCGGCGGCTAGCAGCATGGCTGCTGCCGCGGCTACGGCTTCTCCGGCTCCTGCGGCCTC

      ACCCAGGCCTCGTCGAGGCCGGAGACGTCGGTGAGCAGCGGCGCGAGGTACGAGAAGGCGCAGAAGACGC
1541  ---------+---------+---------+---------+---------+---------+---------+
      TGGGTCCGGAGCAGCTCCGGCCTCTGCAGCCACTCGTCGCCGCGCTCCATGCTCTTCCGCGTCTTCTGCG

      CGCCCGCCGCGAGGGCCGTGACCGCGATCGACAGGAGGACCTGGCGGTCGCGGTAGATGGCGACCTCGTT
1611  ---------+---------+---------+---------+---------+---------+---------+
      GCGGGCGGCGCTCCCGGCACTGGCGCTAGCTGTCCTCCTGGACCGCCAGCGCCATCTACCGCTGGAGCAA

      CTTGAGAGGGCGGGGCCTGGTCTCGGGGAGCGGGATGCGGGGGATGCGGGTGACGACGCCGACGAGCGCG
1681  ---------+---------+---------+---------+---------+---------+---------+
      GAACTCTCCCGCCCCGGACCAGAGCCCCTCGCCCTACGCCCCCTACGCCCACTGCTGCGGCTGCTCGCGC

      ATGGCGGAGGCGAGGCCGACGGCCCAGAAGGCGGAGGCCCAGCCGAGGTGCTCGCCGAGGAAGGCGCCGG
1751  ---------+---------+---------+---------+---------+---------+---------+
      TACCGCCTCCGCTCCGGCTGCCGGGTCTTCCGCCTCCGGGTCGGCTCCACGAGCGGCTCCTTCCGCGGCC

      CGGGGACGCGCAGGACGTTGGCGATGGAGAGGCCGCCGATCATGACCGCCAGCGCCCGGGCCCGTGAGCC
1821  ---------+---------+---------+---------+---------+---------+---------+
      GCCCCTGCGCGTCCTGCAACCGCTACCTCTCCGGCGGCTAGTACTGGCGGTCGCGGGCCCGGGCACTCGG

      GACCGGGACCATCGCGAGGCCACCGCCGCGCCGACCGCCCAGAAGCCCGCGCAGGGCAGGGCGCTGATC
1891  ---------+---------+---------+---------+---------+---------+---------+
      CTGGCCCTGGTAGCGCTACCGGTGGCGGCGCGGCTGGCGGGTCTTCGGGCGCGTCCCGTCCCGCGACTAG
```

Figure 9


ACGCGGGAGGCGAAGAGGACCGCGTAGTTGGGGGCGAGGGCGCCGGCCATCTGGCGCAGGCCGAAGACGG
1961
TGCGCCCTCCGCTTCTCCTGGCGCATCAACCCCCGCTCCCGCGGCCGGTAGACCGCGTCCGGCTTCTGCC
TGATGAGGGCGATGAGGGTGGTCTTGCGGGGGAGCCGGAGGGTGGCGACGGCGAGGAGCGGTGCGCCGAC
2031
ACTACTCCCGCTACTCCCACCAGAACGCCCCCTCGGCCTCCCACCGCTGCCGCTCCTCGCCACGCGGCTG
GACCATGCCGATCGCGAACGCCGAGATGAGGAGTCCGGCGCGGGGGATGGAGACGTTCATGTCCTCCGCG
2101
CTGGTACGGCTAGCGCTTGCGGCTCTACTCCTCAGGCCGCGCCCCCTACCTCTGCAAGTACAGGAGGCGC
ATGGGCGGCACGAGGCCGGAGAGCATGAATTCGCTCGTCCCGAGCGCGAACGCGGACAGTCCGAGGATGT
2171
TACCCGCCGTGCTCCGGCCTCTCGTACTTAAGCGAGCAGGGCTCGCGCTTGCGCCTGTCAGGCTCCTACA
AGACGGCCAGGGGCATCCGGGGGGATGCGGCGGGCCCGGCGTCGGGGGTCGGGGTGGATGTCGTGGGCTC
2241
TCTGCCGGTCCCCGTAGGCCCCCCTACGCCGCCCGGGCCGCAGCCCCCAGCCCCACCTACAGCACCCGAG
3'
GGCGGAGGGAGACGGCATGACATGCGTCAACCTGTGGTTGGTACC
2311
CCGCCTCCCTCTGCCGTACTGTACGCAGTTGGACACCAACCATGG
5'

Figure 9

VI. Sequence analysis of the 2.4-kb *Kpn*I-*Sst*I fragment of pJV7

Streptomycete DNA possesses a high proportion of guanine and cytosine nucleotides (68-72 mol%). Consequently, codon usage by these organisms is biased in favour of those with G or C nucleotides in the third or degenerate position (Bibb *et al.*, 1984, Wright and Bibb, 1992). The CODONPREFERENCE program of the GCG sequence analysis software package analyzes a DNA sequence in all six possible reading frames for codon usage; from a pre-determined codon usage table, the program can also detect codon bias and display it graphically. The data generated can then be combined with the location of translational start and stop codons to predict the most likely position of an open reading frame (orf). CODONPREFERENCE analysis of strands A and B of the compiled DNA sequence (see Fig. 9) obtained from M13PB100, M13PB101, pJV7Δ2.2, pJV9, and pJV10 predicted three complete orfs and one incomplete orf within the 2.4-kb *Kpn*I-*Sst*I fragment of *S. venezuelae* DNA (Fig. 10 and 11).

A. Identifying open reading frames

The first region of codon bias (*orf*-1) was detected in the first reading frame of strand A; it extended from the *Sst*I junction site and ended approximately 80 nucleotides into the insert (Fig. 10). Inspection of the DNA sequence indicated that the start codon for *orf*-1 was outside the cloned DNA fragment. A TGA codon at nucleotides 73-75 (Fig. 12), was the first in-frame stop codon, and its location corresponded well with the decreased codon bias detected by the CODONPREFERENCE analysis.

A second region of codon bias (*orf*-2) was detected in the second reading frame of strand A, and extended from nucleotides 90 to 400 (Fig. 10). The DNA sequence in this region showed only one likely translational start point, a GTG codon at nucleotides 89-91 (Fig. 12). The first translational stop for *orf*-2 was a TGA codon at nucleotides 356-358; this agreed well with the drop in codon bias observed at this point in reading frame two.

Further examination of the DNA sequence in this region revealed a possible ribo-

Figure 10. A CODONPREFERENCE analysis of strand A for codon bias, third position GC bias, rare codon usage and possible open reading frames. The diagram shows three windows representing the three possible reading frames of strand A. The upper (– -- –) of the two continuous straight lines in each window represents the average GC content of the entire sequence; the lower line (– – – –) represents the random frequency of codon usage (rfcu), calculated using the -codon usage table of Wright and Bibb (1992) and the algorithm of Gribskov *et al.* (1984). The upper plotted line (----) represents the average GC usage at every third position in a sliding window of 25 triplets. Regions where this line lies above the upper straight line contain a biased GC content in the third position of each triplet. Similarly, the lower solid plotted line represents the codon preference statistic (P) for each codon over a sliding window of 25 triplets. Where it rises above the rfcu-line a biased codon usage is indicated. Possible open reading frames based on the presence of a GTG start codon and any of the three possible stop codons, are displayed as horizontal bars in the lower region of each window; a vertical line raised slightly above the horizontal at the end of a bar represents a start codon, while a vertical line flush with the horizontal represents a stop codon. Rare codons, calculated from a codon frequency table based on that of Wright and Bibb (1992), are displayed as short vertical lines just below the open reading frame display

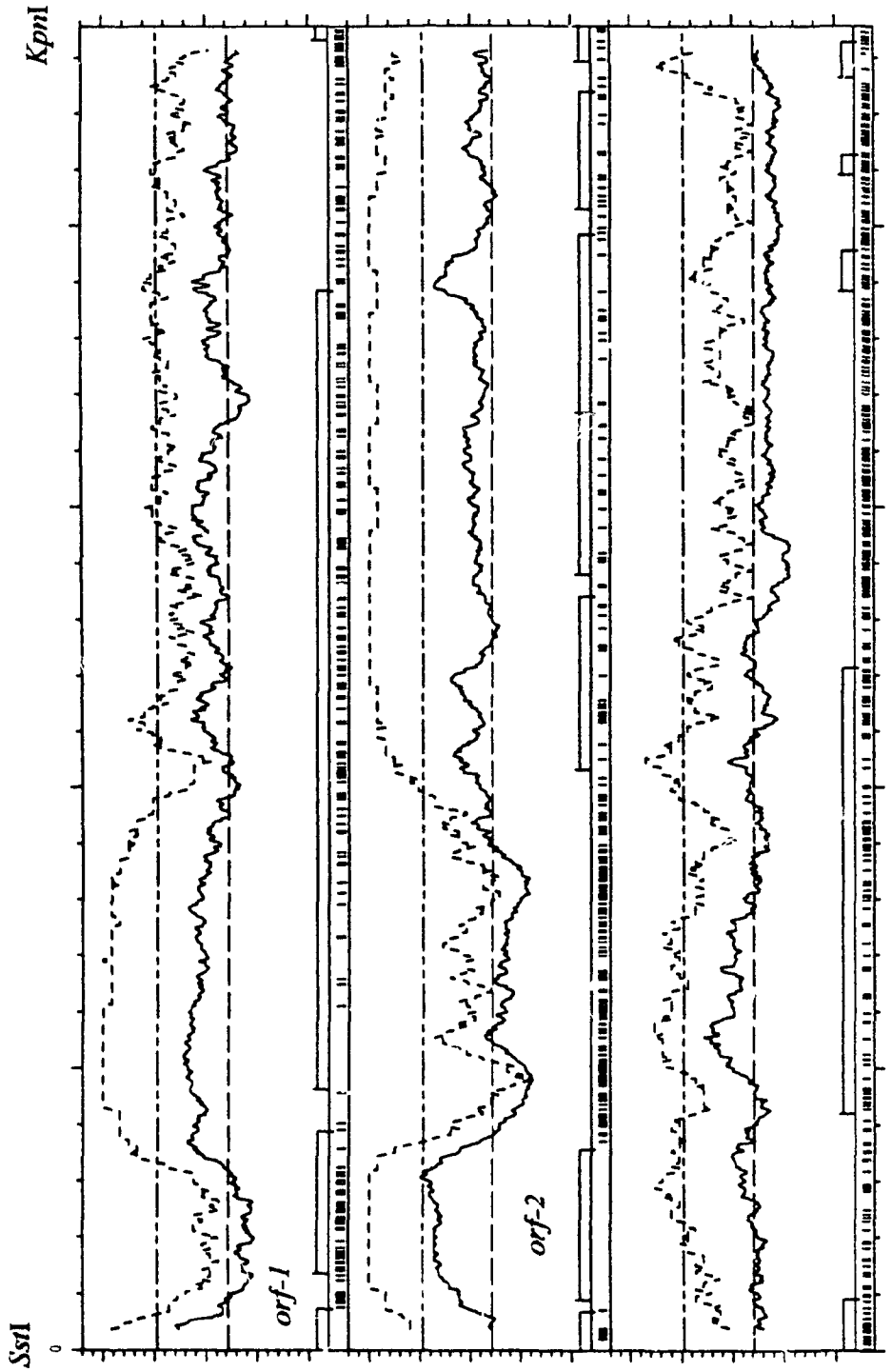

Third position GC bias
Codon Preference
SstI
KpnI
0
orf-1
orf-2

Figure 10

Figure 11: A CODONPREFERENCE analysis of strand B (reading 5'→3') for codon bias, third position GC bias, rare codon usage and possible open reading frames. (See Fig.10 for full description)

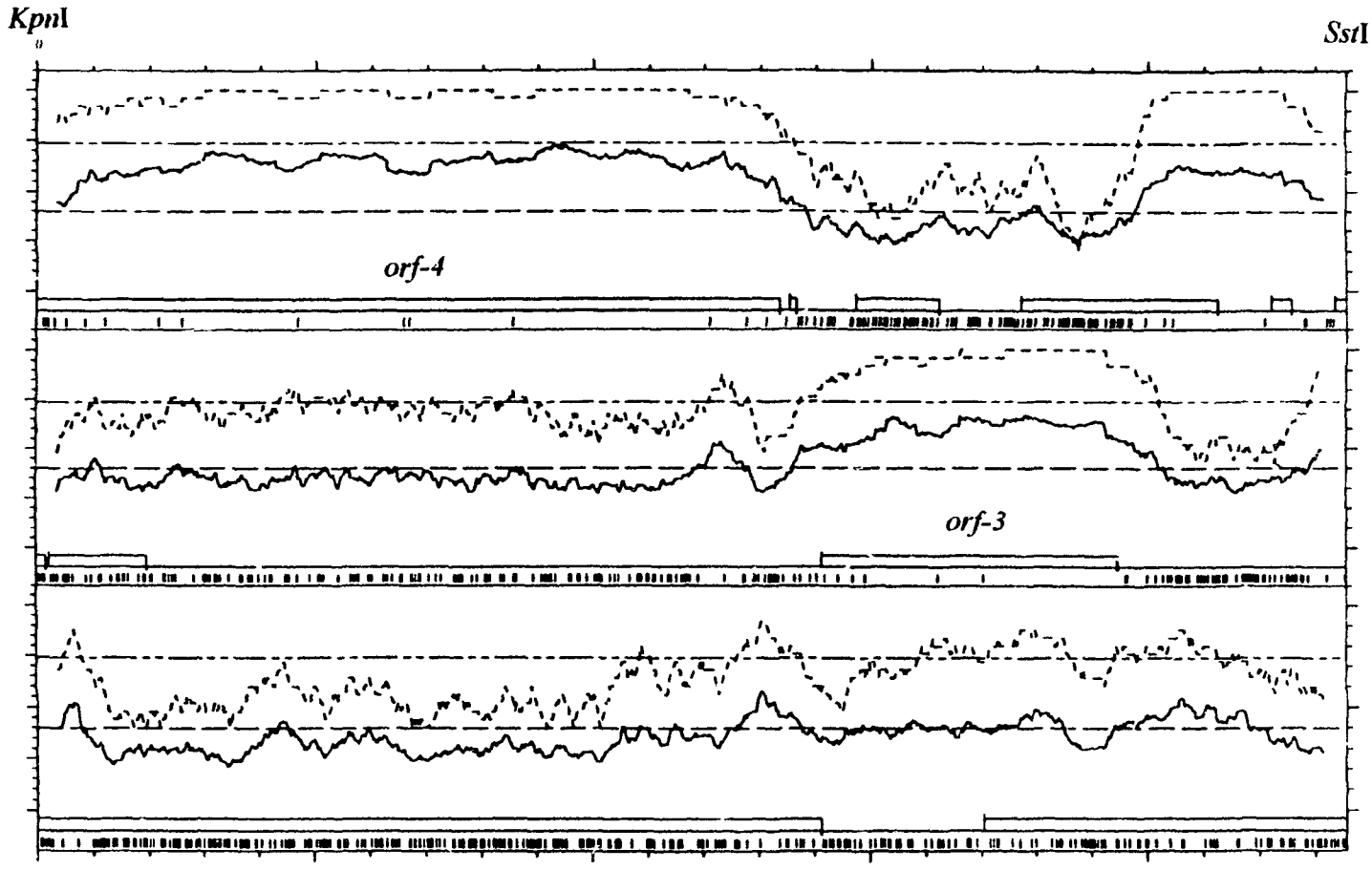

KpnI
SstI
orf-4
orf-3
Codon Preference
Third Position GC Bias

Figure 11

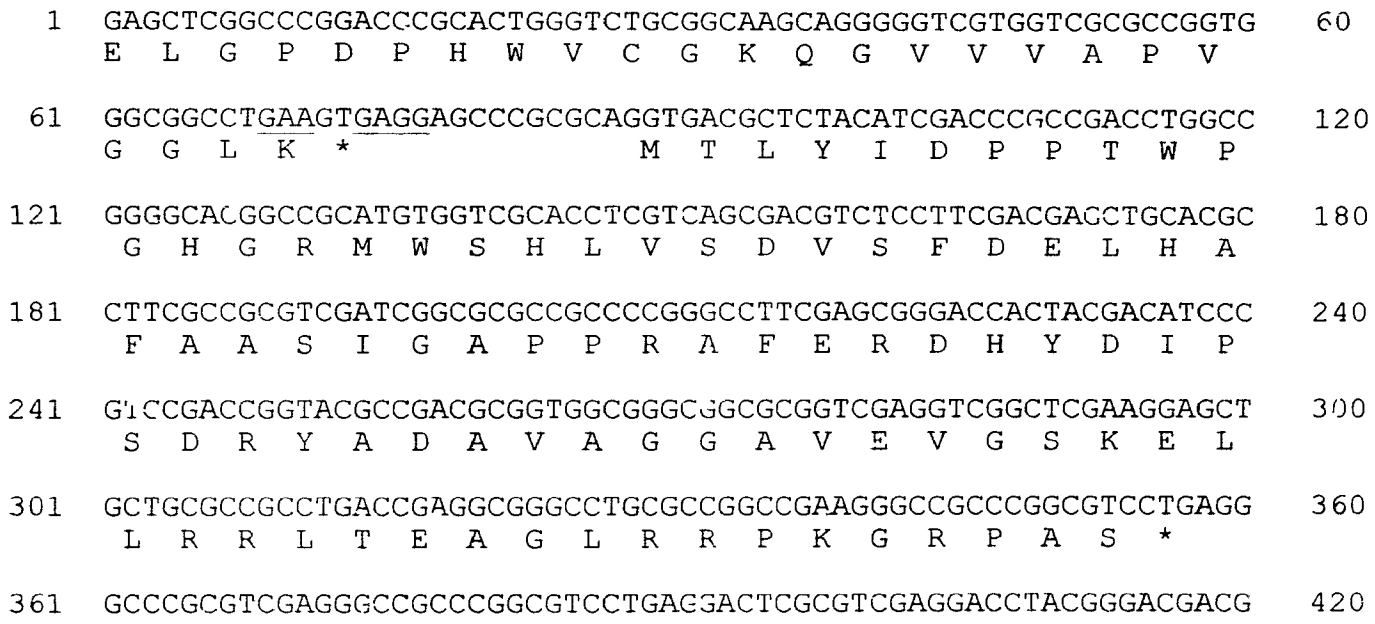


Figure 12: The deduced amino acid sequences of Orf1 and Orf2 on strand A (5'→3'). Translational stop codons are marked with a star. A potential ribosome binding site is underlined.

some binding site (rbs) at 11 base-pairs (bp) upstream of the translational start site for *orf*-2 (nucleotides 69-77). This site, 5'-GAAGTGAGG-3', showed good sequence complementarity (ΔG = -10 8 kcal.mol^{-1} using the rules of Tinoco *et al.* (1973) to calculate the free energy of binding between ribonucleotide sequences) to the 3'-end of the 16S rRNA of *S. lividans* (Strohl, 1992) Since the *orf*-2 rbs overlaps the translational stop codon for *orf*-1, the two open reading frames may be translationally coupled. However, a possible RNA polymerase binding site, consisting of -35 and -10 hexamers, can be identified at nucleotides 22-27 and 45-50, respectively. These hexamers, 5'-TGGGTC-3' and 5'-CGTGGT-3', are separated by 17 bp and are similar to the consensus sequences for Eσ^{70}-like streptomycete promoters (Strohl, 1992). Since the transcriptional start point for streptomycete genes is on average six bp downstream from the -10 hexamer (Seno and Baltz, 1989), transcription should begin at nucleotide 55 .

CODONPREFERENCE analysis of strand B showed a large region of codon bias (*orf*-4) in the first reading frame, extending from the *Kpn*I site at nucleotide 50 to nucleotide 1400 (Fig. 11). Two possible ATG start codons were present, the first at nucleotides 28-30 and the second at nucleotides 100-102 (Fig. 13) A putative rbs, 5'-AGGT-3', was located 11 bp upstream of the first start codon (nucleotides 13-16); it displayed limited but significant complementarity to the 3' end of the *S. lividans* 16S rRNA (ΔG = -9 4 kcal.mol^{-1}). However, no likely rbs was observed adjacent to the second start codon. No sequences similar to the streptomycete Eσ^{70}-like promoters were found at the -10 regions upstream of the first or second start codons. The first in-frame translational stop codon was a TGA located at nucleotides 1336-1338 (Fig. 13). The DNA sequence immediately adjacent to the translational stop codon contained two inverted repeat sequences, the first at nucleotides 1339-1362 and the second at nucleotides 1367-1378. When transcribed into mRNA the first repeat could potentially fold into an imperfect stem-loop structure with a ΔG = -12.1 kcal.mol^{-1}; the second repeat might form a small but perfect stem-loop structure with a ΔG = -4.8 kcal.mol^{-1} Free-energy values for stem-

Figure 13: The deduced amino acid sequences for Orf3 and Orf4 on strand B (5'→3'). Possible ribosome binding sites are underlined; active site motifs are double underlined; inverted or direct repeats are denoted by half arrows above or below the appropriate sequences; unmatched bases in inverted repeats are marked with a dot (·). Translational stop codons are marked with a star (*).

```
  1  GGTACCAACCACAGGTTGACGCATGTCATGCCGTCTCCCTCCGCCGAGCCCACGACATCCACCCCGACCC
  1                                M  P  S  P  S  A  E  P  T  T  S  T  P  T  P

 71  CCGACGCCGGGCCCGCCGCATCCCCCCGGATGCCCCTGGCCGTCTACATCCTCGGACTGTCCGCGTTCGC
 16    D  A  G  P  A  A  S  P  R  M  P  L  A  V  Y  I  L  G  L  S  A  F  A

141  GCTCGGGACGAGCGAATTCATGCTCTCCGGCCTCGTGCCGCCCATCGCGGAGGACATGAACGTCTCCATC
 39   L  G  T  S  E  F  M  L  S  G  L  V  P  P  I  A  E  D  M  N  V  S  I

211  CCCCGCGCCGGACTCCTCATCTCGGCGTTCGCGATCGGCATGGTCGTCGGCGCACCGCTCCTCGCCGTCG
 62  P  R  A  G  L  L  I  S  A  F  A  I  G  M  V  V  G  A  P  L  L  A  V  A

281  CCACCCTCCGGCTCCCCCGCAAGACCACCCTCATCGCCCTCATCACCGTCTTCGGCCTGCGCCAGATGGC
 86    T  L  R  L  P  R  K  T  T  L  I  A  L  I  T  V  F  G  L  R  Q  M  A

351  CGGCGCCCTCGCCCCCAACTACGCGGTCCTCTTCGCCTCCCGCGTGATCAGCGCCCTGCCCTGCGCGGGC
109   G  A  L  A  P  N  Y  A  V  L  F  A  S  R  V  I  S  A  L  P  C  A  G

421  TTCTGGGCGGTCGGCGCGGCGGTGGCCATCGCGATGGTCCCGGTCGGCTCACGGGCCCGGGCGCTGGCGG
132  F  W  A  V  G  A  A  V  A  I  A  M  V  P  V  G  S  R  A  R  A  L  A  V

491  TCATGATCGGCGGCCTCTCCATCGCCAACGTCCTGCGCGTCCCCGCCGGCGCCTTCCTCGGCGAGCACCT
156   M  I  G  G  L  S  I  A  N  V  L  R  V  P  A  G  A  F  L  G  E  H  L

561  CGGCTGGGCCTCCGCCTTCTGGGCCGTCGGCCTCGCCTCCGCCATCGCGCTCGTCGGCGTCGTCACCCGC
179   G  W  A  S  A  F  W  A  V  G  L  A  S  A  I  A  L  V  G  V  V  T  R
```

Figure 13

```
631  ATCCCCCGCATCCCGCTCCCCGAGACCAGGCCCCGCCCTCTCAAGAACGAGGTCGCCATCTACCGCGACC
202  I  P  R  I  P  L  P  E  T  R  P  R  P  L  K  N  E  V  A  I  Y  R  D  R

701  GCCAGGTCCTCCTGTCGATCGCGGTCACGGCCCTCGCGGCGGGCGGCGTCTTCTGCGCCTTCTCGTACCT
226    Q  V  L  L  S  I  A  V  T  A  L  A  A  G  G  V  F  C  A  F  S  Y  L

771  CGCGCCGCTGCTCACCGACGTCTCCGGCCTCGACGAGGCCTGGGTCTCCGGCGTCCTCGGCCTCTTCGGC
249   A  P  L  L  T  D  V  S  G  L  D  E  A  W  V  S  G  V  L  G  L  F  G

841  ATCGGCGCCGTCGTCGGTACGACGATCGGCGGCCGGGTCGCCGACGCGCACCTCTTCGGCGTGCTGCTCA
272  I  G  A  V  V  G  T  T  I  G  G  R  V  A  D  A  H  L  F  G  V  L  L  T

911  CCGGCATCTCCGCCTCCACCGTCTTCCTCGTGGCCCTGGCCCTGTTCGCCTCGAACCCGGCCGCCACGAT
296    G  I  S  A  S  T  V  F  L  V  A  L  A  L  F  A  S  N  P  A  A  T  I

981  CGTGCTGACCTTCCTCCTCGGCGTCTCGGCCTTCTACACGGCCCCGGCCCTCAACGCCCGCATGTTCAAC
319   V  L  T  F  L  L  G  V  S  A  F  Y  T  A  P  A  L  N  A  R  M  F  N

1051 GTCGCCGGCGCCGCCCCCACCCTCGCGGGCGCCACCACCACCGCCGCCTTCAACCTCGGCAACACGGGCG
342  V  A  G  A  A  P  T  L  A  G  A  T  T  T  A  A  F  N  L  G  N  T  G  G

1121 GCCCCTGGCTCGGCGGCACGGTCATCGACGCGAACCTCGCCTTCGCCTCGACGGCCTGGGCGGGCCCGGC
366    P  W  L  G  G  T  V  I  D  A  N  L  G  F  A  S  T  A  W  A  G  A  A

1191 GATGACGGTCCTGGGCCTGGGAACGGCGGCCCTGGCCCTCCGCCTGACCAAGCGCCCGGCCCCCGGCCAC
389   M  T  V  L  G  L  G  T  A  A  L  A  L  R  L  T  K  R  P  A  P  G  H
```

Figure 13

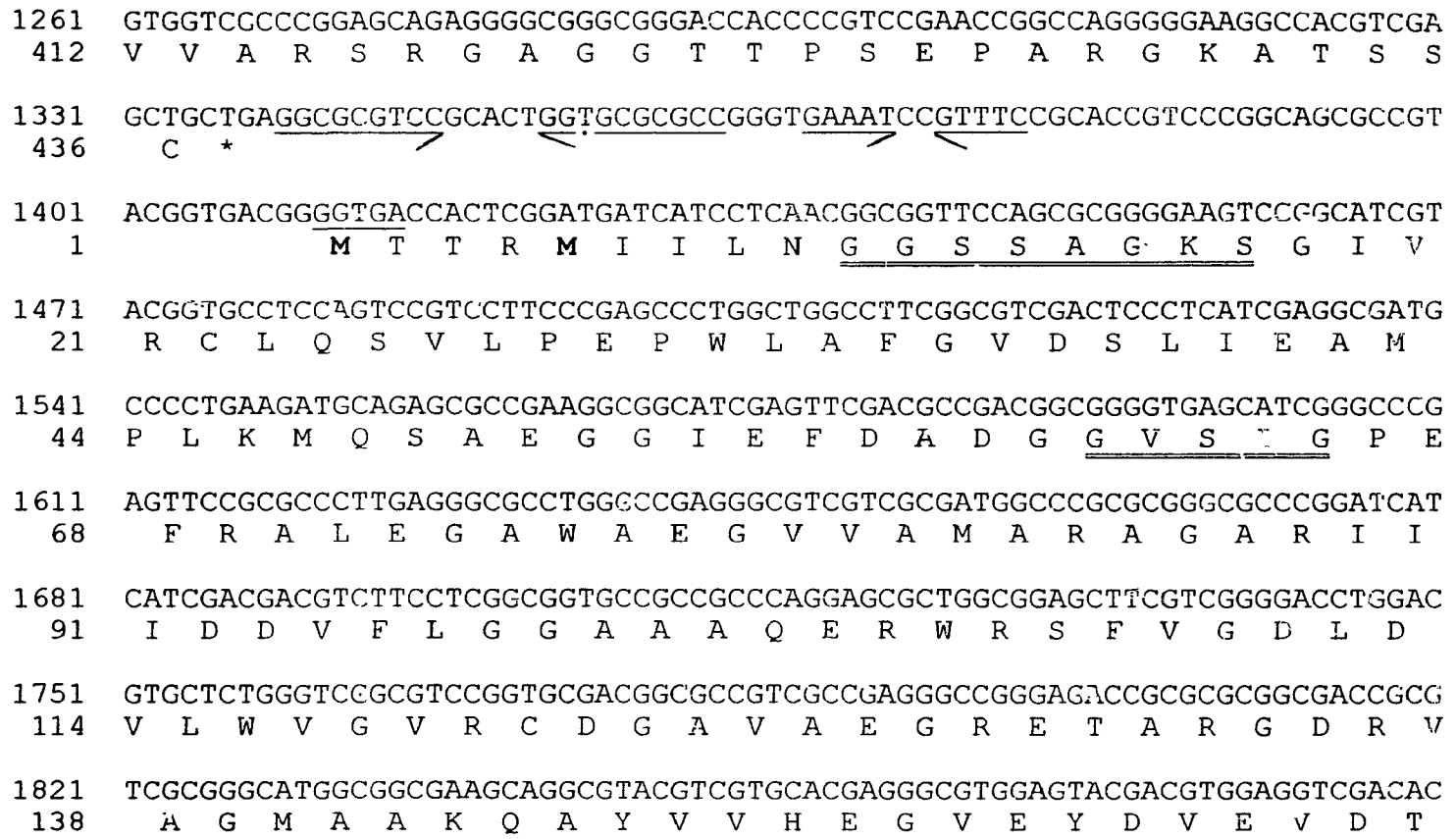

1261 GTGGTCCCCCGGAGCAGAGGGGCGGGCGGGACCACCCCGTCCGAACCGGCCAGGGGGAAGGCCACGTCGA
412 V V A R S R G A G G T T P S E P A R G K A T S S
1331 GCTGCTGAGGCGCGTCCGCACTGGTGCGCGCCGGGTGAAATCCGTTTCCGCACCGTCCCGGCAGCGCCGT
436 C *
1401 ACGGTGACGGGGTGACCACTCGGATGATCATCCTCAACGGCGGTTCCAGCGCGGGGAAGTCCGGCATCGT
1 M T T R M I I L N G G S S A G K S G I V
1471 ACGGTGCCTCCAGTCCGTCCTTCCCGAGCCCTGGCTGGCCTTCGGCGTCGACTCCCTCATCGAGGCGATG
21 R C L Q S V L P E P W L A F G V D S L I E A M
1541 CCCCTGAAGATGCAGAGCGCCGAAGGCGGCATCGAGTTCGACGCCGACGGCGGGGTGAGCATCGGGCCCG
44 P L K M Q S A E G G I E F D A D G G V S I G P E
1611 AGTTCCGCGCCCTTGAGGGCGCCTGGGCCGAGGGCGTCGTCGCGATGGCCCGCGCGGGCGCCCGGATCAT
68 F R A L E G A W A E G V V A M A R A G A R I I
1681 CATCGACGACGTCTTCCTCGGCGGTGCCGCCGCCCAGGAGCGCTGGCGGAGCTTCGTCGGGGACCTGGAC
91 I D D V F L G G A A A Q E R W R S F V G D L D
1751 GTGCTCTGGGTCCGCGTCCGGTGCGACGGCGCCGTCGCCGAGGGCCGGGAGACCGCGCGCGGCGACCGCG
114 V L W V G V R C D G A V A E G R E T A R G D R V
1821 TCGCGGGCATGGCGGCGAAGCAGGCGTACGTCGTGCACGAGGGCGTGGAGTACGACGTGGAGGTCGACAC
138 A G M A A K Q A Y V V H E G V E Y D V E V D T

Figure 13


1891 CACGCACAAGGAGTCGATCGAGTGCGCCTGGGCGATCGCCGCCCACGTCGTCCCGTAGGTCCTCGACGCG
161 T H K E S I E C A W A I A A H V V P *
1961 AGTCCTCAGGACGCCGGGCGGCCCTCGACGCGGGCCCTCAGGACGCCGGGCGGCCCTTCGGCCGGCGCAG
2031 GCCCGCCTCGGTCAGGCGGCGCAGCAGCTCCTTCGAGCCGACCTCGACCGCGCCGCCCGCCACCGCGTCG
2101 GCGTACCGGTCGGACGGGATGTCGTAGTGGTCCCGCTCGAAGGCCCGGGGCGGCGCGCCGATCGACGCGG
2171 CGAAGGCGTGCAGCTCGTCGAAGGAGACGTCGCTGACGAGGTGCGACCACATGCGGCCGTGCCCCGGCCA
2241 GGTCGGCGGGTCGATGTAGAGCGTCACCTGCGCGGGCTCCTCACTTCAGGCCGCCCACCGGCGCGACCAC
2311 GACCCCCTGCTTGCCGCAGACCCAGTGCGGGTCCGGGCCGAGCTC 2355

Figure 13

loop structures were calculated using the rules of Turner *et al.* (1987). The structures may signal rho-independent transcriptional termination, although their free-energies of formation are significantly higher than observed for putative transcriptional stop signals from other streptomycetes (Pulido and Jiménez, 1987; Guilfoile and Hutchinson, 1992).

The second reading frame in strand B showed a region of codon bias (*orf*-3) between nucleotides 1350 and 2100 (Fig. 11). This was associated with two possible start codons, GTG at nucleotides 1412-1414 and ATG at nucleotides 1424-1426 (Fig. 13). At 8 bp upstream of the ATG codon (i.e. at nucleotides 1411-1415) was a possible rbs, 5'-GGTGA-3', with good complementarity to the 3' end of *S. lividans* 16S rRNA (ΔG = -11.6 kcal.mol^{-1}). At 16 bp upstream of this putative rbs there was a possible -10 hexamer, 5'-CACCGT-3' (nucleotides 1381-1386). Examination of the sequence upstream of the GTG potential start codon uncovered no good candidates for a rbs but did locate a -10 hexamer (5'-TACGGT-3', nucleotides 1400-1405) that was similar to the -10 consensus sequence for Eσ^{70}-like promoters (Strohl, 1992). Transcription promoted from this hexamer should initiate at the first nucleotide of the GTG translational start codon; the concurrence of translational and transcriptional start sites has been observed with a number of streptomycete genes, especially those involved in antibiotic resistance and development (Strohl, 1992). No good candidates for -35 hexamer sequences were observed upstream of either of the possible -10 hexamers of *orf*-3.

The putative translational stop site for *orf*-3 was a TAG codon at nucleotides 1946-1948. Two perfect direct repeats were found downstream of it. The first began three base-pairs downstream of the TAG and consisted of the ten base-pair sequence 5'-CCTCGACGCG-3' (nucleotides 1951-1960) repeated exactly at nucleotides 1983-1992. The second repeat was 17 bp downstream of the TAG, and consisted of the 22 bp sequence 5'-CCTCAGGACGCCGGGCGGCCCT-3' (nucleotides 1964-1985) precisely repeated 10 bp downstream at nucleotides 1996-2017. The two repeats partially overlapped each other at nucleotides 1983-1985.

B. Comparative analysis of the derived amino acid sequences

1. **Orf4**

The analysis of *orf*-4 and its derived amino acid sequence was complicated by the presence of two possible translational start codons. If the first ATG at nucleotides 28-30 were to be the translational start codon, the DNA sequence of *orf*-4 would encode a polypeptide (Orf4) of 436 amino acids, with a deduced M_r of 43,783 (Fig. 13) When the FASTA sequence alignment program of Pearson and Lipman (1988) was used to compare this amino acid sequence with those in the Genbank and EMBL databases, Orf4 showed marked similarity (Fig. 14 and 15) to the chloramphenicol resistance proteins CmlR of *Rhodococcus fascians* (Desomer *et al.*, 1992) and CmlG of *Streptomyces lividans* (Dittrich *et al.*, 1991).

FASTA analysis also showed that the amino termini of CmlR and CmlG aligned optimally with methionine-25 of Orf4, instead of with methionine-1 Moreover, the amino-termini of two other related proteins (AraJ and NorA) preferentially aligned with methionine-25. These observations suggested that the actual translational start for *orf*-4 was the second in-frame ATG codon (at nucleotides 100-102; see Fig 13), and that *orf*-4 encoded a polypeptide of 412 amino acids, with a deduced M_r of 41,479. The codon usage was typical of streptomycete genes (Table 2). The FASTA alignments of Orf4 and the two chloramphenicol resistance proteins showed not only a high level of sequence identity but an overall preservation of functionally related amino acids (see Fig 14 and 15) A COMPARE-DOTPLOT analysis at high stringency between Orf4: CmlG (Fig 16), and Orf4::CmlR (Fig. 17) displayed a direct, positional conservation of similar amino acids throughout the sequences of the three proteins.

These results suggested that the relationship between Orf4, CmlR, and CmlG might be functionally significant. Supporting this was the bias in the physical characteristics of their amino acids, each possessing in excess of 60% hydrophobic (A, I, L, M, P,

Figure 14: A FASTA alignment of the amino acid sequences deduced for Orf4 and for CmlG from *Streptomyces lividans*. Identical amino acids are denoted with a solid line (|) and similar amino acids with a colon (:).

```
SCORES      Init1: 446 Initn: 766 Opt: 770
            39.8% identity in 372 aa overlap

                10        20        30        40        50        60
Orf4    MPSPSAEPTTSTPTPDAGPAASPRMPLAVYILGLSAFALGTSEFMLSGLVPPIAEDMNVS
                                    |||::|:|:::: |:||||||||:|||| ||:|::|:
CmlG                                MPLPLYLLAVAVCAMGTSEFMLAGLVPDIASDLGVT
                                            10        20        30

                70        80        90       100       110       120
Orf4    IPRAGLLISAFAIGMVVGAPLLAVATLRLPRKTTLIALITVFGLRQMAGALAPNYAVLFA
        :  || |:||||:||:|||||:|: :   ||:::|:::| :|:  : :|| :::::|| |
CmlG    VGTAGTLTSAFATGMIVGAPLVAALARTWPRRSSLLGFILAFAAAHAVGAGTTSFPVLVA
          40        50        60        70        80        90

              130       140       150       160       170       180
Orf4    SRVISALPCAGFWAVGAAVAIAMVPVGSRARALAVMIGGLSIANVLRVPAGAFLGEHLGW
        :||::||: ||| ||: ::| |:|||:::::|||||:::| ::|:|  ||:|::||: |||
CmlG    CRVVAALANAGFLAVALTTAAALVPADKQGRALAVLLSGTTVATVAGVPGGSLLGTWLGW
         100       110       120       130       140       150

              190       200       210       220       230
Orf4    ASAFWAVGLASAIALVGVVTRIP--RIPLPETRPRPLKNEVAIYRDRQVLLSIAVTALAA
         ::||||:: :  |  ||:: ||  | : ::|  :||: |:|  :::::||:: ::||::
CmlG    RATFWAVAVCCLPAAFGVLKAIPAGRATAAATGGPPLRVELAALKTPRLLLAMLLGALVN
         160       170       180       190       200       210
```

Figure 14

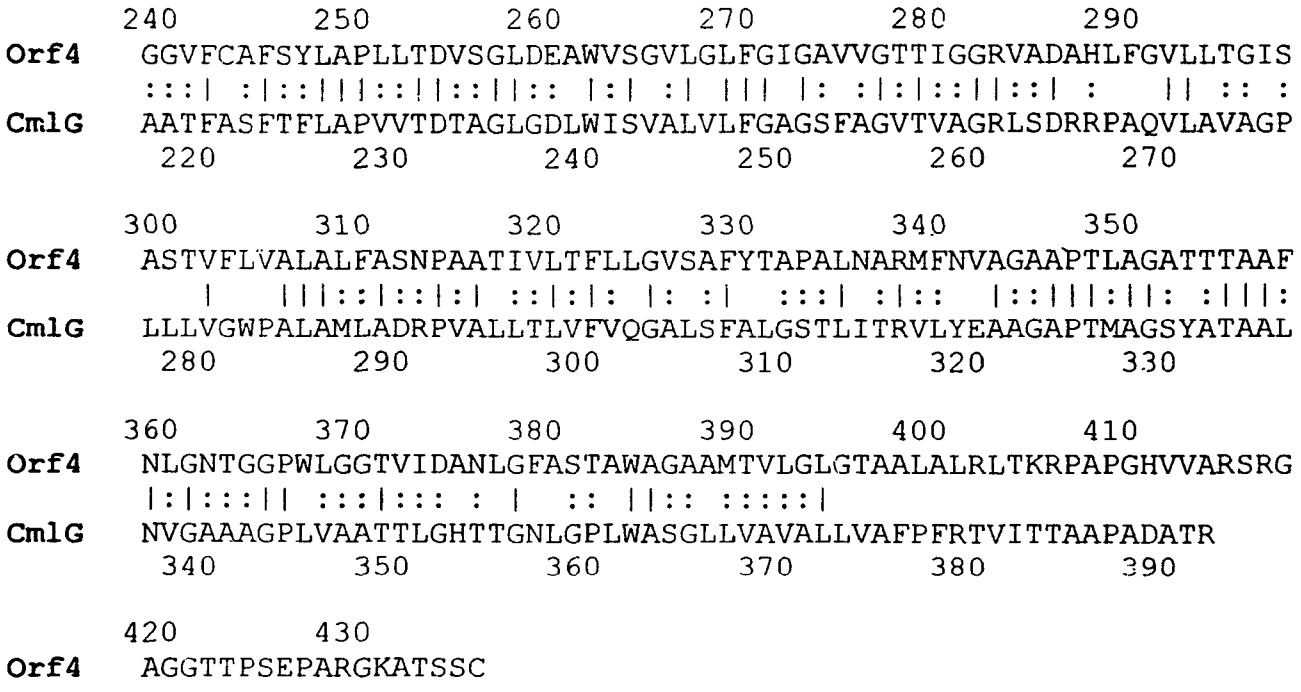

240 250 260 270 280 290
Orf4 GGVFCAFSYLAPLLTDVSGLDEAWVSGVLGLFGIGAVVGTTIGGRVADAHLFGVLLTGIS
CmlG AATFASFTFLAPVVTDTAGLGDLWISVALVLFGAGSFAGVTVAGRLSDRRPAQVLAVAGP
220 230 240 250 260 270
300 310 320 330 340 350
Orf4 ASTVFLVALALFASNPAATIVLTFLLGVSAFYTAPALNARMFNVAGAAPTLAGATTTAAF
CmlG LLLVGWPALAMLADRPVALLTLVFVQGALSFALGSTLITRVLYEAAGAPTMAGSYATAAL
280 290 300 310 320 330
360 370 380 390 400 410
Orf4 NLGNTGGPWLGGTVIDANLGFASTAWAGAAMTVLGLGTAALALRLTKRPAPGHVVARSRG
CmlG NVGAAAGPLVAATTLGHTTGNLGPLWASGLLVAVALLVAFPFRTVITTAAPADATR
340 350 360 370 380 390
420 430
Orf4 AGGTTPSEPARGKATSSC

Figure 14

Figure 15: A FASTA alignment of the amino acid sequences deduced for Orf4 and for CmlR from *Rhodococcus fascians*.

```
SCORES      Init1: 522 Initn: 522 Opt: 937
            42.2% identity in 386 aa overlap

                10        20        30        40        50        60
Orf4    MPSPSAEPTTSTPTPDAGPAASPRMPLAVYILGLSAFALGTSEFMLSGLVPPIAEDMNVS
                                    ||:|:|:||:::|| |||||||||||:| :|:|::||
CmlR                                MPFAIYVLGIAVFAQGTSEFMLSGLIPDMAQDLQVS
                                            10        20        30

                70        80        90        100       110       120
Orf4    IPRAGLLISAFAIGMVVGAPLLAVATLRLPRKTTLIALITVFGLRQMAGALAPNYAVLFA
        :| ||||:|||||||::||||:|::::| :|: :|:::::::| : :: |||: :::||::
CmlR    VPTAGLLTSAFAIGMIIGAPLMAIVSMRWQRRRALLTFLITFMVVHVIGALTDSFGVLLV
          40        50        60        70        80        90

               130       140       150       160       170       180
Orf4    SRVISALPCAGFWAVGAAVAIAMVPVGSRARALAVMIGGLSIANVLRVPAGAFLGEHLGW
        :|:::||: ||| ||: ::|::|||:: ::|| :|::||::|| |: ||:||:||| ||
CmlR    TRIVGALANAGFLAVALGAAMSMVPADMKGRATSVLLGGVTIACVVGVPGGALLGELWGW
         100       110       120       130       140       150

               190       200       210       220       230       240
Orf4    ASAFWAVGLASAIALVGVVTRIPRIPLPETRPRPLKNEVAIYRDRQVLLSIAVTALAAGG
         ::||:| | || |:::::::::|  : ::: |:: : |::  |:|:: | ::::|| :|:
CmlR    RASFWEVVLISAPAVAAIMASTPADSPTDSVPNATR-ELSSLRQRKLQLILVLGALINGA
         160       170       180       190       200       210
```

Figure 15

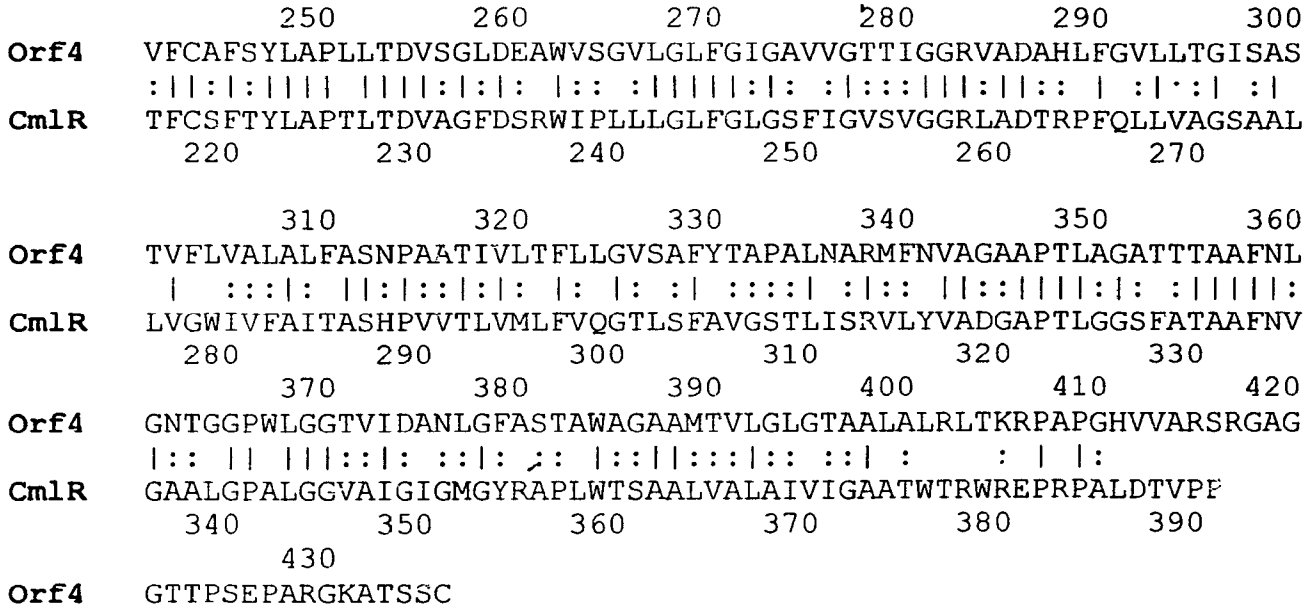

250 260 270 280 290 300
Orf4 VFCAFSYLAPLLTDVSGLDEAWVSGVLGLFGIGAVVGTTIGGRVADAHLFGVLLTGISAS
CmlR TFCSFTYLAPTLTDVAGFDSRWIPLLLGLFGLGSFIGVSVGGRLADTRPFQLLVAGSAAL
220 230 240 250 260 270
310 320 330 340 350 360
Orf4 TVFLVALALFASNPAATIVLTFLLGVSAFYTAPALNARMFNVAGAAPTLAGATTTAAFNL
CmlR LVGWIVFAITASHPVVTLVMLFVQGTLSFAVGSTLISRVLYVADGAPTLGGSFATAAFNV
280 290 300 310 320 330
370 380 390 400 410 420
Orf4 GNTGGPWLGGTVIDANLGFASTAWAGAAMTVLGLGTAALALRLTKRPAPGHVVARSRGAG
CmlR GAALGPALGGVAIGIGMGYRAPLWTSAALVALAIVIGAATWTRWREPRPALDTVPP
340 350 360 370 380 390
430
Orf4 GTTPSEPARGKATSSC

Figure 15

Table 2: A codon usage table for Orf4, showing the number of times each codon is used in Orf4.

TTT	phe	F	-	TCT	ser	S	-	TAT	tyr	Y	-	TGT	cys	C	-
TTC	phe	F	19	TCC	ser	S	12	TAC	tyr	Y	5	TGC	cys	C	3
TTA	leu	L	-	TCA	ser	S	1	TAA	ter	*	-	TGA	ter	*	1
TTG	leu	L	-	TCG	ser	S	7	TAG	ter	*	-	TGG	trp	W	6
CTT	leu	L	-	CCT	pro	P	1	CAT	his	H	-	CGT	arg	R	-
CTC	leu	L	39	CCC	pro	P	13	CAC	his	H	3	CGC	arg	R	13
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	-	CGA	arg	R	-
CTG	leu	L	16	CCG	pro	P	10	CAG	gln	Q	2	CGG	arg	R	5
ATT	ile	I	-	ACT	thr	T	-	AAT	asn	N	-	AGT	ser	S	-
ATC	ile	I	21	ACC	thr	T	17	AAC	asn	N	10	AGC	ser	S	4
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	-	AGA	arg	S	1
ATG	met	M	9	ACG	thr	T	12	AAG	lys	K	4	AGG	arg	S	2
GTT	val	V	-	GCT	ala	A	-	GAT	asp	D	-	GGT	gly	G	1
GTC	val	V	33	GCC	ala	A	50	GAC	asp	D	6	GGC	gly	G	39
GTA	val	V	-	GCA	ala	A	1	GAA	glu	E	2	GGA	gly	G	3
GTG	val	V	7	GCG	ala	A	26	GAG	glu	E	5	GGG	gly	G	4

Figure 16: A COMPARE-DOTPLOT analysis of the amino acid sequences deduced for CmlG and Orf4, using a window of 30 and a stringency of 18.

Figure 16

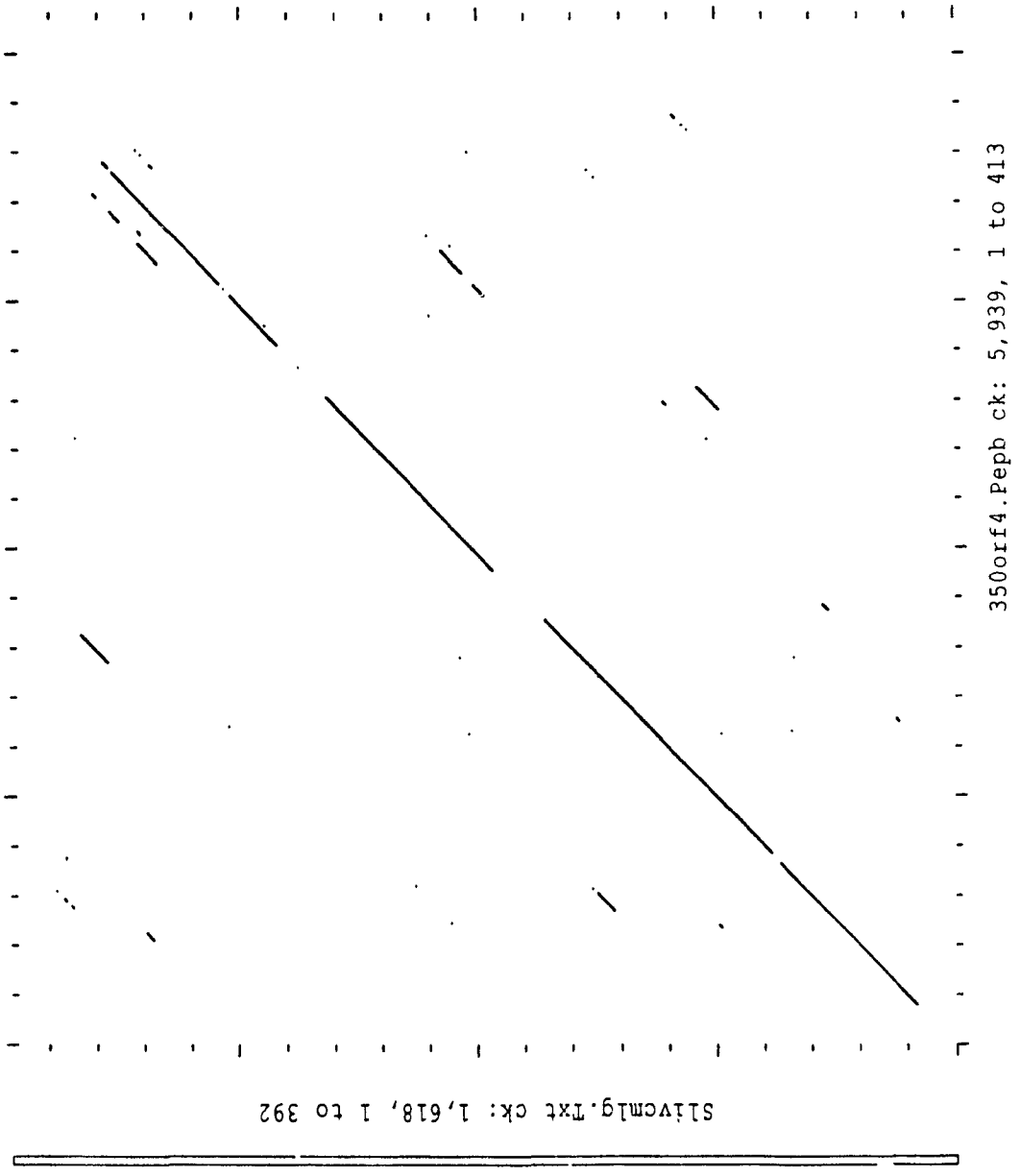
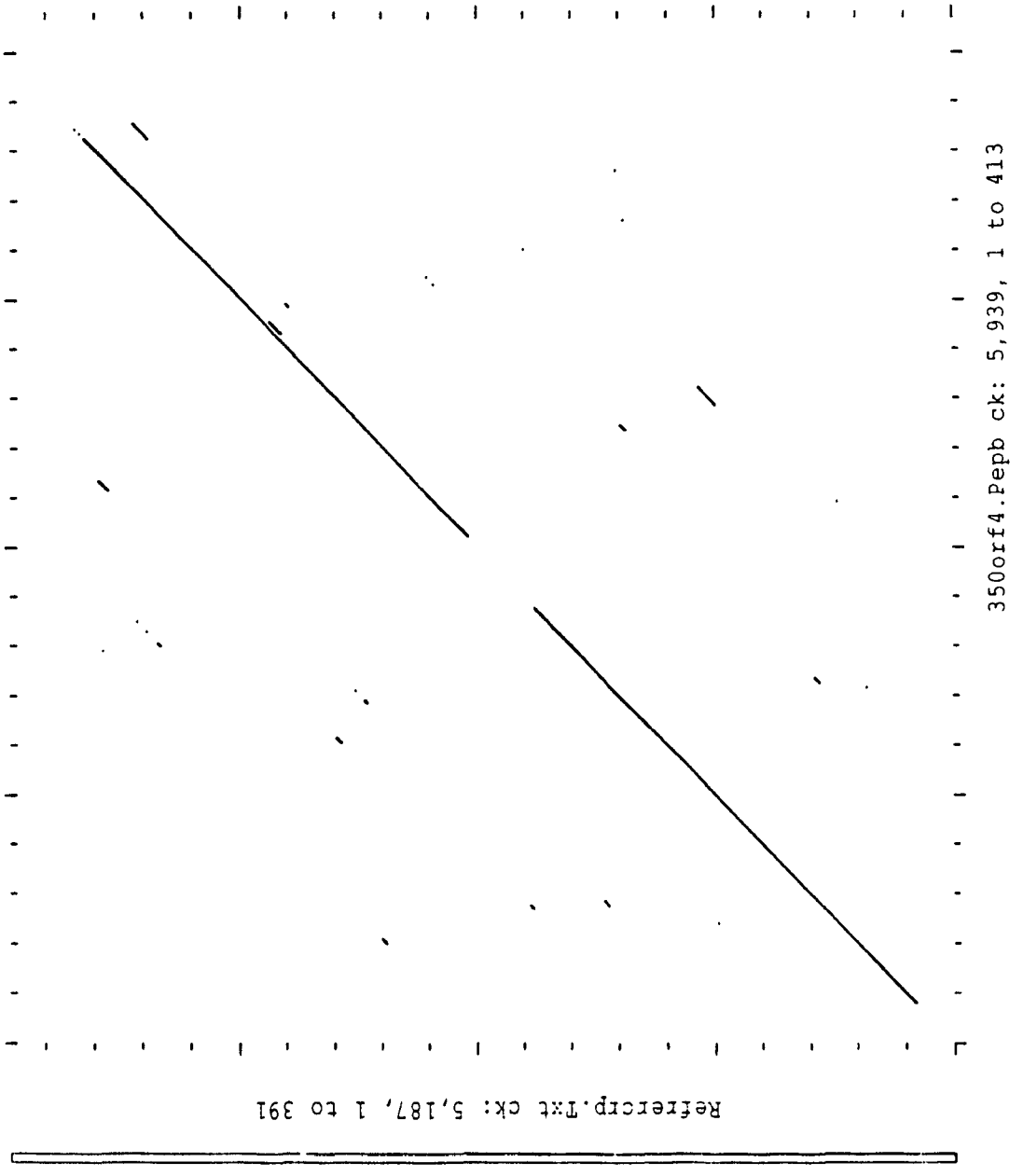


Figure 17: A COMPARE-DOTPLOT analysis of the amino acid sequences deduced for CmlR and Orf4, using a window of 30 and a stringency of 18.

Figure 17



F, W, V) and 27% polar uncharged amino acids (N, C, Q, G, S, T, Y) (Table 3). Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982) of Orf4, CmlR, and CmlG (Fig. 18, 19, 20) predicted an alternating arrangement of 12 hydrophobic and/or amphiphilic, membrane-spanning segments; each transmembrane segment (TMS) consisted primarily of 21 hydrophobic or uncharged polar amino acids (Fig. 21). The two-dimensional model of Orf4 constructed in Fig. 21 assumed each peak in the hydropathy plot (see Fig. 18) that equaled or exceeded an average hydrophobicity of 1.6, using a sliding window of 19 amino acids, represented a hydrophobic TMS; likewise a peak with an average hydrophobicity of less than 1.6 represented an amphiphilic TMS, possessing both hydrophobic and hydrophilic residues (Jähnig, 1990; Reithmeier and Deber, 1992; Saier, 1992). Most transport proteins have one or more amphiphilic TMS; these may be involved in forming the hydrophilic channel through which the substrate is transported. Many of the hydrophobic amino acids are strong β-sheet formers, but within the hydrophobic environment of the lipid membrane they are thought usually to assume a more energetically favourable α-helical conformation (Reithmeier and Deber, 1992). Thus, although the Kyte-Doolittle hydropathy plot does not predict conformation, it is likely that most of the hydrophobic TMS form α-helices, and that amphiphilic TMS form α-helices in which a majority of their hydrophilic residues are oriented away from the axis of the helix and towards the water-filled lumen of the transmembrane channel. In Fig. 21, the putative TMS 1, 3, 5, and 11 may constitute amphiphilic TMS since their average hydrophobicities are below 1.6 (see Fig 18). It is noteworthy that Orf4 was significantly similar in its amino acid sequence to several other antibiotic resistance proteins, each of which is a presumed integral membrane protein capable of catalyzing active antibiotic efflux (Table 4). A sequence alignment of Orf4 and related proteins is shown in Fig. 22.

2. **Orf3**

Two possible translational start codons, separated by 9 bp, were observed for *orf-3* (see Fig. 13). If translation were to begin at the GTG start codon (nucleotides 1412-

Table 3: A comparison of amino acid usage in Orf4, CmlG, and CmlR

		% N		
Amino Acid (N)	Type*	Orf4	CmlG	CmlR
Alanine	HYPHOB	18.6	21.9	14.8
Cysteine	PUN	0.7	1.0	0.5
Aspartic acid	NEG	1.5	2.0	2.8
Glutamic acid	NEG	1.7	0.8	1.3
Phenylalanine	HYPHOB	4.6	4.3	5.1
Glycine	PUN	11.4	10.5	11.0
Histidine	POS	0.7	0.5	0.5
Isoleucine	HYPHOB	5.1	1.8	6.1
Lysine	POS	1.0	0.8	0.5
Leucine	HYPHOB	13.3	16.1	13.6
Methionine	HYPHOB	2.2	1.8	3.3
Asparagine	PUN	2.4	1.0	1.0
Proline	HYPHOB	5.8	5.9	5.4
Glutamine	PUN	0.5	0.8	2.0
Arginine	POS	5.1	4.1	4.6
Serine	PUN	5.8	4.1	6.6
Threonine	PUN	7.0	9.4	6.9
Valine	HYPHOB	9.7	10.7	10.5
Tryptophan	HYPHOB	1.5	1.8	2.3
Tyrosine	PUN	1.2	0.8	1.0

* HYPHOB: hydrophobic
PUN: polar uncharged
POS: positively charged
NEG: negatively charged

Figure 18: A Kyte-Doolittle hydropathy plot of the deduced amino acid sequence of Orf4 using a window of 19. The vertical axis represents average hydrophobicity and the horizontal axis the number of amino acid residues.

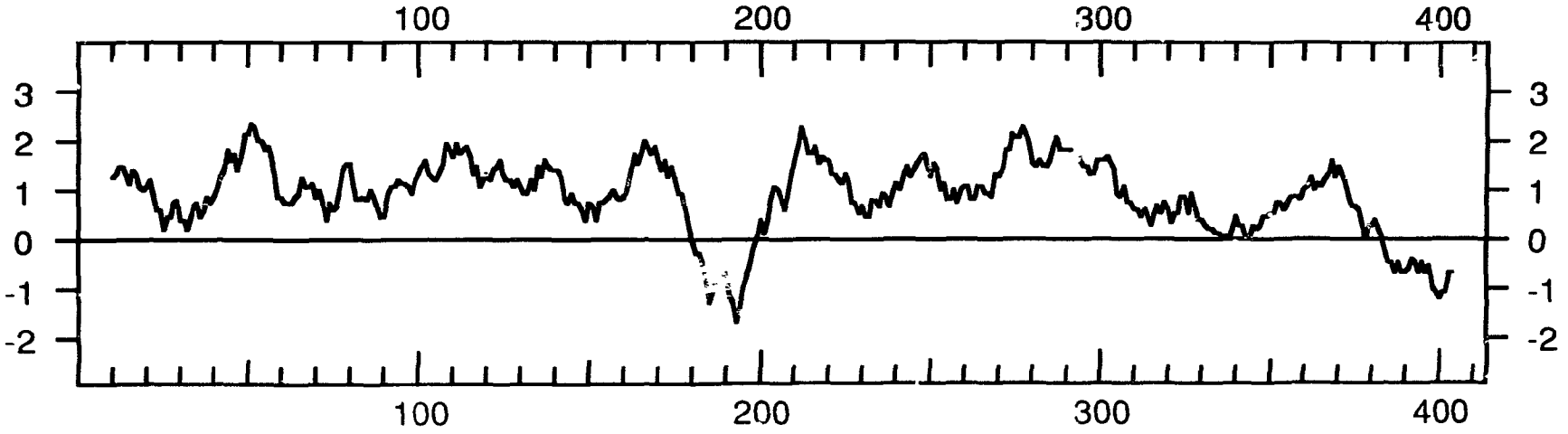


Figure 19: A Kyte-Doolittle hydropathy plot of the deduced amino acid sequence of CmlR (Adapted from Desomer *et al.* (1992) using a window of 19). The vertical axis represents average hydrophobicity and the horizontal axis the number of amino acids.

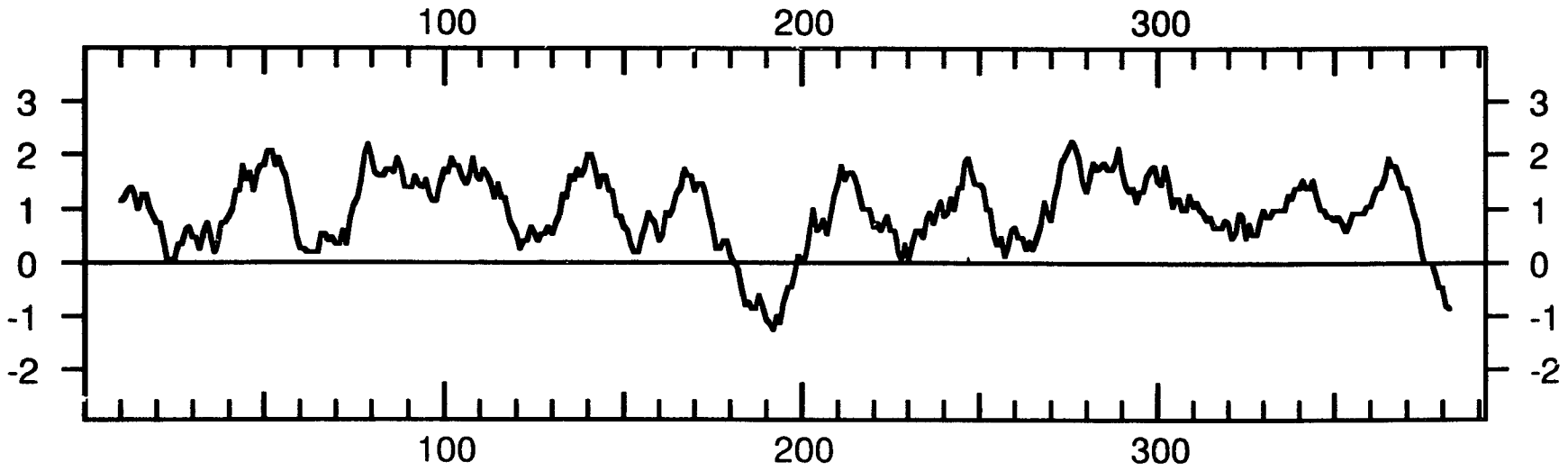


Figure 20: A Kyte-Doolittle hydropathy plot of the deduced amino acid sequence of CmlG (Adapted from Dittrich *et al.* (1991) using a window of 19). The vertical axis represents average hydrophobicity and the horizontal axis the number of amino acids.

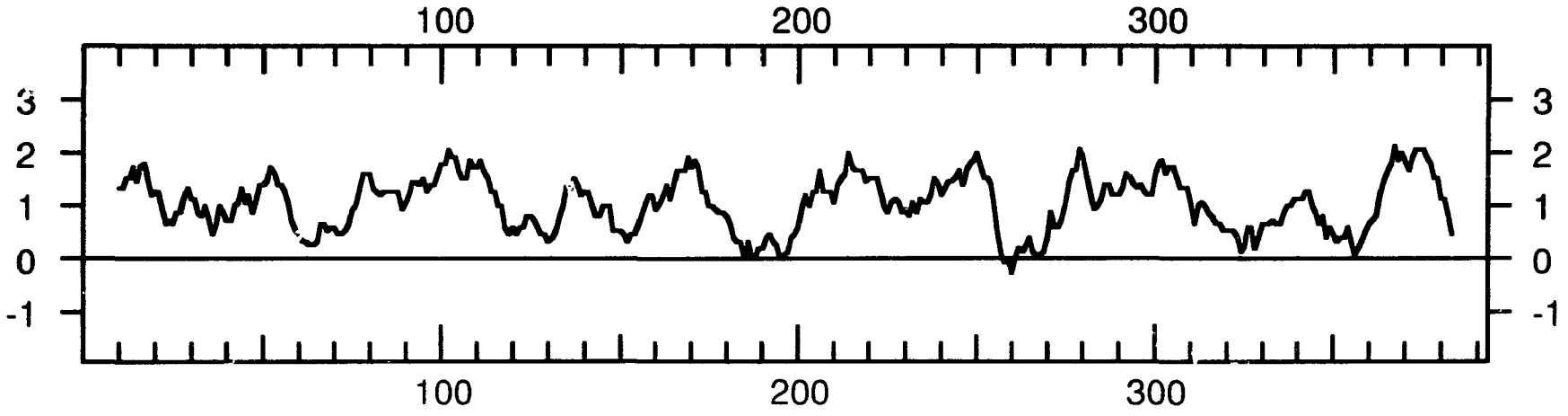


Figure 21 A two-dimensional model for the transmembrane distribution of the Orf4 polypeptide Each of the 12 α-helical, transmembrane domains consists of 21 amino acids Open circles represent hydrophobic residues, and filled circles are polar uncharged residues Charged residues are marked with the appropriate positive or negative sign The assignment of transmembrane domains is tentative and is based on a Kyte-Doolittle hydropathy analysis of Orf4, and also a comparison with the domains assigned in CmlR by Desomer *et al.* (1992)

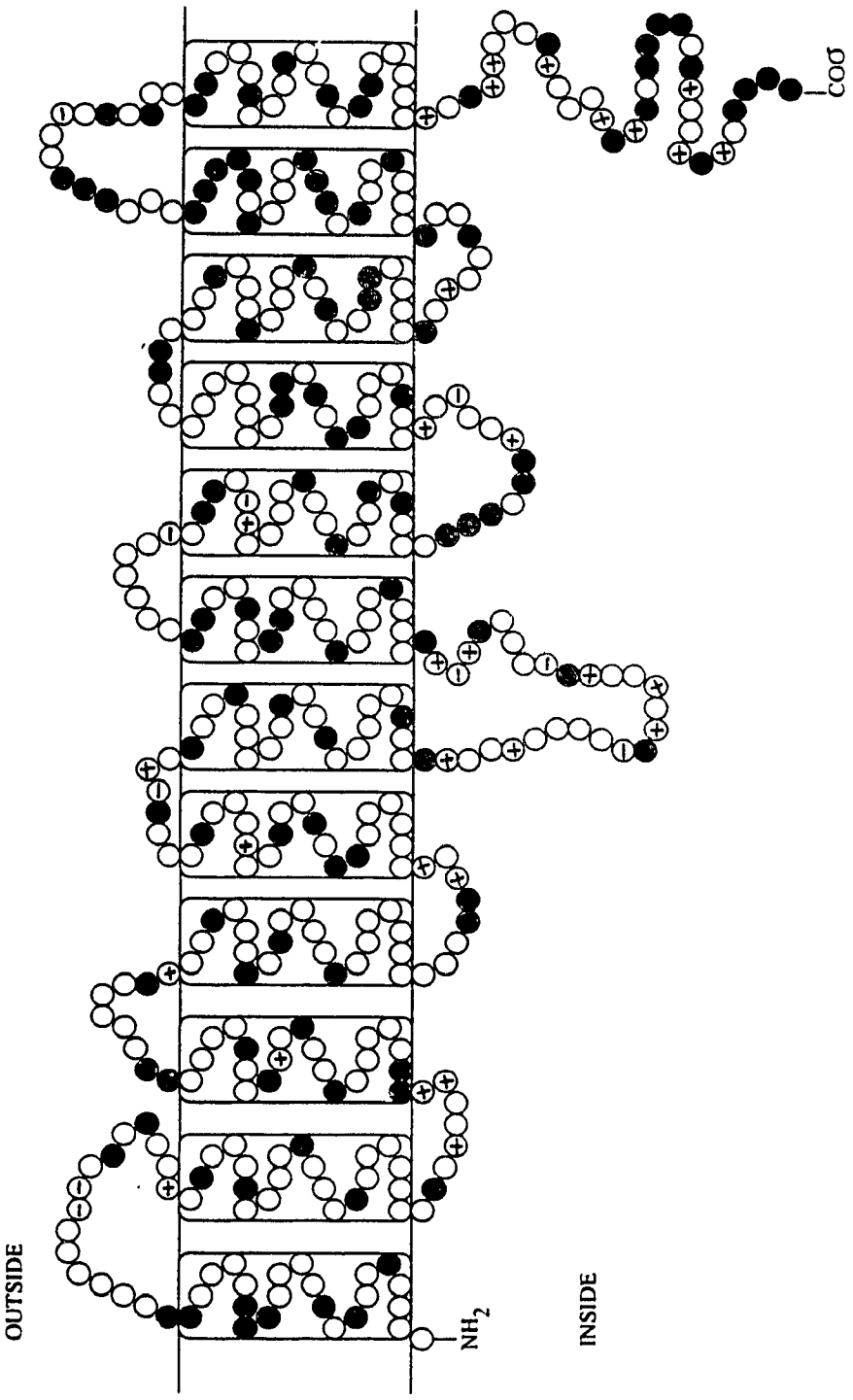


Figure 21

Table 4: A summary of the results from a FASTA comparison between the amino acid sequence deduced from *orf*-4 (Orf4) and the SWISS-PROT and PIR databases.

Organism	Protein*	Accession No.	Overlap	Identity	Similarity
Rhodococcus fascians	CmlR	S21395	386 a.a.	42.2%	83.2%
Streptomyces lividans	CmlG	S18593	372 a.a.	39.8%	80.1%
Pseudomonas aeruginosa	OpdE	S23860	377 a.a.	29.7%	70.3%
Escherichia coli	AraJ	P23910	391 a.a.	26.6%	71.1%
Bacillus subtilis	Bmr	A40137	365 a.a.	20.8%	66.0%
Streptomyces coelicolor	Mmr	P11545	371 a.a.	20.5%	63.3%
Staphylococcus aureus	NorA	P21191	358 a.a.	18.7%	64.5%
Escherichia coli	BicA	P28246	345 a.a.	17.7%	64.1%
Bacillus subtilis	MetA	S22742	317 a.a.	20.2%	61.5%
Bacillus stearothermophilus	TetL	P07561	352 a.a.	16.2%	62.2%

* **CmlR:** a chloramphenicol resistance protein; **CmlG**: a chloramphenicol resistance protein; **OpdE**: an integral membrane protein involved in imipenem uptake; **AraJ**: an integral membrane protein of unknown function; **Bmr**: a *Bacillus* multidrug resistance protein; **Mmr**: a methylenomycin resistance protein; **NorA**: a quinolone resistance protein; **BicA**: a bicyclomycin resistance protein; **MetA**: a methylenomycin resistance protein; **TetL**: a tetracycline resistance protein.

Figure 22: Alignment of Orf4 with the deduced amino acid sequences of 11 significantly similar transmembrane proteins*. The sequences were aligned using the FASTP program of Lipman and Pearce (1985) with a gap penalty of 8, and hierarchically clustered with the MULTALIN program of Corpet (1988). The consensus sequence row uses the following code: ! = I or V; $ = L or M; % = F or Y; + = B, D, E, N, Q or Z.

* The amino acid sequence deduced for CmlA a putative transmembrane chloramphenicol efflux protein from *Pseudomonas aeruginosa* (Bissonnette *et al.*, 1991) is included in the alignment. A direct alignment of CmlA and Orf4 showed 21.2% identity in a 433 amino acid overlap (data not shown).

```
CMLR      MPF..............AI...Y...VLGIAVFAQGTSEFMLSGLIPDMAQDLQVSV
CMLG      MPL..............PL...Y...LLAVAVCAMGTSEFMLAGLVPDIASDLGVTV
Orf4      MPL..............AV...Y...ILGLSAFALGTSEFMLSGLVPPIAEDMNVSI
OPDE   MTTRALDTANENPEQSGSWSGV...L...AIAVCAFALVASEFLPVSLLTPIANDLGTTE
ARAJ      MKK..............VI...L...SLALGTFGLGMAEFGIMGVLTELAHNVGISI
BMRP      MEK..............KN...ITLTILLTNLFIAFLGIGLVIPVTPTIMNELHLSG
NORA      MNK..............QI...F...VLYFNIFLIFLGIGLVIPVLPVYLKDLGLTG
BICA                                                MLMPLSIDMYLPALPVISAQFGVPA
CMLA        MSSKNFSWRYSLAATVL.......LLSPFDLLASLGMDMYLPAVPFMPNALGTTA
BMETA     MKNSGSIQESTSSTGISVL......IVLALGFLMATLDVTVVNVAMADMKNTLSMSL
MMR    MTTVRTGGAQTAEVPAGGRRDVPSGVKITALATGFVMATLDVTVVNVAGATIQESLDTTL
TETL      MNTSYSQSNLRHN...QIL......IWLCILSFFSVLNEMVLNVSLPDIANDFNKPP
consens   M                               L    F   L   $      P I ++L

CMLR   PTAGLLTSAFAIGMIIGAPLMAIVSMRWQRRRALLTFLITFMVVHVIGALTDSF.GVLLV
CMLG   GTAGTLTSAFATGMIVGAPLVAALARTWPRRSSLLGFILAFAAAHAVGAGTTSF.PVLVA
Orf4   PRAGLLISAFAIGMVVGAPLLAVATLRLPRKTTLIALITVFGLRQMAGALAPNY.AVLFA
OPDE   GMAGQGIAISGAFAVLTSLFISSVAGSLNRKTLLLGLTAAMGMSGAIVALAPNY.FVYML
ARAJ   PAAGHMISYYALGVVVGAPIIALFSSRYSLKHILLFLVALCVIGNAMFTLSSSY.LMLAI
BMRP   TAVGYMVACFAITQLIVSPIAGRWVDRFGRKIMIVIGLLFFSVSEFLFGIGKTV.EMLFI
NORA   SDLGLLVAAFALSQMIISPFGGTLADKLGKKLIICIGLILFSVSEFMFAVGHNF.SVLML
BICA   GSTQMTLSTYILGFALGQLIYGPMADSFGRKPVVLGGTLVFAAAAVACALANTI.VQLIV
CMLA   STIQLTLTTYLVMIGAGQLLFGPLSDRLGRRPVLLGGGLAYVVASMGLALTSSA.EVFLG
BMETA  SGVTWVVDGYILTFASLLLAGGALADRFGSKTIYILGLAVFVMASCLCAASING.QMLIA
MMR    TQLTWIVDGYVLTFASLLMLAGGLANRIGAKTVYLWGMGVFFLASLACALAPTA.ETLIA
TETL   ASTNWVNTAFMLTFSIGTAVYGKLSDQLGIKRLLLFGIIINCFGSVIGFVGHSFFSLLIM
consens   G  !  %     !G    G $ +R GRK  LL G   F       AL   %   L
```

Figure 22

```
   CMLR  TRIVGALANAGFLAVALGAAMSM.VPADMKGRATSVLLGGVTIACVVGVPGGALLGELWG
   CMLG  CRVVAALANAGFLAVALTTAAAL.VPADKQGRALAVLLSGTTVATVAGVPGGSLLGTWLG
   Orf4  SRVISALPCAGFWAVGAAVAIAM.VPVGSRARALAVMIGGLSIANVLRVPAGAFLGEHLG
   OPDE  GRALIGIVIGGFWSMSAATAMRL.VPANDVPRALALVNGGNALATVVAAPLGAWLGTLIG
   ARAJ  GRLVSGFPHGAFFGVGAIVLSKI.IKPGKVTAAVAGMVSGMTVANLLGIPLGTYLSQEFS
   BMRP  SRMLGGISAPFIMPGVTAFIADI.TTIKTRPKALGYMSAAISTGFIIGPGIGGFLAEVHS
   NORA  SRVIGGMSAGMVMPGVTGLIADI.SPSHQKAKNFGYMSAIINSGFILGPGIGGFMAEVSH
   BICA  MRFFHGLAAAAASVVINALMRDIYP.KEEFSRMMSFVMLVTTIAPLMAPIVGGWVLVWLS
   CMLA  LRILQACGASACLVSTFATVRDIYAGREESNVIYGILGSMLAMVPAVGPLLGALVDMWLG
  BMETA  GRLIQGIGAALFMPSSLSLLAASYLDERARARMFGLWAALVSAASALGPFIGGVLVQLAG
    MMR  ARLVQGAGAALFMPSSLSLLVFSFPEKRQRTRMLGLWSAIVATSSGLGPTVGGLMVSAFG
   TETL  ARFIQGAGAAAF.PALVMVVVARYIPKENRGKAFGLIGSIVAMGEGVGPAIGGMIAHYIH
consens   R ! G  AA F$                    RA   $      A   GP  G  L   G

   CMLR  WRASFWEVVLISAPAVAAIMASTPADSPTDSVP....NATR...ELSSLRQRKLQLILV.
   CMLG  WRATFWAVAVCCLPAAFGVLKAIPAGRATAAAT....GGPPLRVELAALKTPRLLLAML.
   Orf4  WASAFWAVGLASAIALVGVVTRIP..RIPLPET....RPRPLKNEVAIYRDRQVLLSIA.
   OPDE  WRGAFLCLVPVALVALAWQWTTLPSMRAGARAP....GPGNVFTVFALLKRPGVMLGML.
   ARAJ  WRYTFLLIAVFNIAVMASVYFWVPDIRDEAKG.........NLREQFHFLRSPAPWLIFA.
   BMRP  RLPFFFAAAFALLAAILSILT....LREPERNP....ENQEIKGQKTGFKRIFAPMYFI.
   NORA  RMPFYFAGALGILAFIMSIVL....IHDPKKST....TSGFQKLEPQLLTKINWKVFIT.
   BICA  WHYIFWILALAAILASAMIFFLIKETLPPERRQPFHIRTTIGNFAALFRHKRVLSY.ML.
   CMLA  WRAIFAFLGLGMIAASAAAWRF....WPETRVQ....RVAGLQWSQLLLPVKCLNFWLY.
  BMETA  WQSIFLINVPIGAAALISAYRILS..RVPGKSS....RVNIIGHLLGMMALGFLSYALIQ
    MMR  WESIFLLNLPIGAIGMAMTYRYIA..ATESRAT....RLAVPGHLLWIVALAAVSFALIE
   TETL  WSYLLLIPM.ITIITVPFLMKLLK..KEVRIKG....HFDIKGIIL..MSVGIVFFMLF.
consens  W   %         A   !                              $        $
```

Figure 22

```
CMLR  .........LGALINGATFCSFTYLAPTLTDVAGFDSRWIPLLLGLFGLGSFIGVSVGGR
CMLG  .........LGALVNAATFASFTFLAPVVTDTAGLGDLWISVALVLFGAGSFAGVTVAGR
Orf4  .........VTALAAGGVFCAFSYLAPLLTDVSGLDEAWVSGVLGLFGIGAVVGTTIGGR
OPDE  .........ASSLFFMGQFSLFTYVRPFLETVTGVHGAHVSLVLLVIGAAGFIGTLLIDR
ARAJ  .........ATMFGNAGVFAWFSYVKPYMMFISGFSETAMTFIMMLVGLGMVLGNMLSGR
BMRP  .........AFLIILISSFGLASFESLFALFVDHKFGFTASDIAIMITGGAIVGAITQVV
NORA  .........PVILTLVLSFGLSAFETLYSLYTADKVNYSPKDISIAITGGGIFGALFQIY
BICA  ..ASGFS..FAGMFSFLSAGPFVYIEINHIAPENFGYYFALNIVFLFVMTIFNSRFVRRI
CMLA  ..TLCYAAGMGSFFVFFSIAPGLMMGRQGVSQLGFSLLFATVAIAMVFTARFMGRVIPKW
BMETA GPSAGWRSPVILVAFTAAVLAFVLFLLREISAKTPILPASLYKNGRFSAAQFIGFLLNFA
MMR   GPQLGWTAGPVLTAYAVAVTAAALLALREHRVTNPVMPWQLFRGPGFTGANLVGFLFNFA
TETL  ......TTSYSISFLIVSVLSFLIFVKHIRKVTDPFVDPGLGKNIPFMIGVLCGGIIFGT
consens                     F  F %      !             $F  G   G

CMLR  LADT..RPFQLLVAGSAALLVGWIVFAITA.SHPVVTLVMLFVQGTLSFAV.GSTLISR.
CMLG  LSDR..RPAQVLAVAGPLLLVGWPALAMLA.DRPVALLTLVFVQGALSFAL.GSTLITR.
Orf4  VADA..HLFGVLLTGISASTVFLVALALFA.SNPAATIVLTFLLGVSAFYT.APALNAR.
OPDE  VLQR..RFFQTLVAIPLLMALIALVLTVLG.GWPAIVVVLLGLWGLTGTSA.PVGWWAW.
ARAJ  ISGRY.SPLRIAAVTDFIIVLALLMLFFCG.GMKTTSLIFAFICCAGLFAL.SAPLQIL.
BMRP  LFDRFTRWFGEIHLIRYSLILSTSLVFLLTTVHSYVAILLVTVTVFVGFDLMRPAVTTY.
NORA  FFDKFMKYFSELTFIAWSLLYSVVVLILLVFANGYWSIMLISFVVFIGFDMIRPAITNY.
BICA  GALNMFRSGLWIQFIMA.AWMVISALLGL.GFWSLVVGVAAFVGCVSMVSSNAMAVILDE
CMLA  GSPSVLRMGMGCLIAGA.VLLAITEIWALQSVLGFIAPMWLVGIGVATAVSVAPNGALRG
BMETA LFGGMFMLSLFLQEAGG.ASSFMAGVELLPMMAVFVIGNLLFARLANRFEAGQLMFVSMA
MMR   LFGSTFMLGLYFQHARG.ATPFQAGLELLPMTIFFPVANIVYARISARFSNGTLLTAFLL
TETL  VAGFVSMVPYMMKDVHQLSTAEIGSVIIFPGTMSVIIFGYIGGILVDR..RGPLYVLNIG
consens           $             L  L      !   $ %      F
```

Figure 22

```
   CMLR  VLYVADGAPTLGGSFATAAFNVGAALGPALGGVAIGIGMGYRAPLWTSAALVALAI.VIG
   CMLG  VLYEAAGAPTMAGSYATAALNVGAAAGPLVAATTLGHTTGNLGPLWASGLLVAVAL.LVA
   Orf4  MFNVAGAAPTLAGATTTAAFNLGNTGGPWLGGTVIDANLGFASTAWAGAAMTVLGLGTAA
   OPDE  IARVFPEDAEAGGGLFVAVVQLSIALGSTLGGLLFDRT.GYQATFFASAAMLLIAAFLTI
   ARAJ  LLQNAKGGELLGAAGGQIAFNLGSAVGAYCGGMMLTLGLAYNYVALPAALLSFAAMSSLL
   BMRP  LSKIAGNEQGFAGGMNIMFTSIGNVFGPIIGGMLFDIDVNYP.FYFATVTLAIGIALTIA
   NORA  FSNIAGERQGFAGGLNSTFTSMGNFIGPLIAGALFDVHIEAPIYMAIGVSLAGVVIVLIE
   BICA  FPHMAGTASSLAGTFRFG...IGAIVGALL..SLATFNSAWPMI.WSIAFCATSSILFCL
   CMLA  FDHVAGTVTAV..YFCLGGVLLGSIGTLII..SLLPRNTAWPVVVYCLTLATVVLGLSCV
  BMETA  VSCIIALLLFVLISPDFPYWQLAVLMSVMNLCTGITVPAMTTVIMQAAGQRHTNIAGAAL
    MMR  LAGAASLSM.VTITASTPYWVVAVAVGVANIGAGIISPGMTAALVDAAGPENANVAGSVL
   TETL  VTFLSVSFLTASFLLETTSWFMTIIIVFVLGGLLFTKTVISTIVSSSLKQQEAGAGMSLL
consens     !A       G        $G   G    G $                  $

   CMLR  AATW.TRWREP.............RPALDTVPP
   CMLG  FPFR.TVITTA.............APADATR
   Orf4  LALRLTKRPAPGHVVARSRGAGGTTPSEPARGKATSSC
   OPDE  LTAR.SKAPAG
   ARAJ  LYGRYKRQQAAD............TPVLAKPLG
   BMRP  WKAPAHLKAST
   NORA  KQHRAKLKEQNM
   BICA  YASRPKK.....................R
   CMLA  SRVKGSRGQGEHDVVALQSAGSTSNPNR
  BMETA  NANRQIGALV
    MMR  NANRQIGSLVGIAAMGVVLHSTSDWDHGAAISFLAVGLAYLLGGLSAWRLIARPERRSAV
   TETL  NFTSFLSEGTGIAIVGGLLSI
consens     R
```

Figure 22

1414), *orf*-3 would encode a 178 amino acid polypeptide (Orf3) with a typical streptomycete codon usage (Table 5) and a deduced M_r of 18,804. However, if translation began at the ATG start codon (nucleotides 1424-1426), *orf*-3 would encode a 174 amino acid polypeptide with a deduced M_r of 18,315.

A comparison of the deduced amino acid sequence of *orf*-3 with those in the GenBank database using the BLAST sequence alignment program, and with those in the EMBL database using the FASTA program, revealed no significant similarities. However, a search using the sequence alignment program BLITZ, showed that the amino-terminus of Orf3 was similar to the putative nucleotide binding sites of several ATP-requiring proteins (Fig. 23). The sequence GGSSAGKS (amino acids 10-17; see Fig. 13) in Orf3 closely fitted the "motif A" consensus sequence [A/G]XXXXGK[S/T] for an ATP/GTP binding site, also known as a "P-loop" (Walker *et al.*, 1982; Saraste *et al.*, 1990). These results suggested that Orf3 might be involved in a process such as a phosphotransferase activity requiring a high energy cofactor. Interestingly, Orf3 possessed the sequence GVSIG (amino acids 61-65; see Fig. 13) which conforms to the active site consensus motif GXSXG of esterases, lipases, transacylases and serine proteases; it is also similar to the active site motif GXCXG found in a number of glutaminyl amidotransferases (Brenner, 1988). Although Orf3 displayed no significant similarity to any of these enzyme families, the presence of the GXSXG motif suggested that it might be involved in a transferase reaction of some kind. An analysis of amino acid usage revealed that Orf3 was only moderately hydrophobic, and a Kyte-Doolittle hydropathy plot revealed no likely transmembrane domains.

3. **Orf2**

Translation of *orf*-2 from its GTG start codon at nucleotides 89-91 (see Fig. 12) would generate a polypeptide (Orf2) with typical streptomycete codon usage (Table 6) and a deduced M_r of 9,762. A comparison of the derived amino acid sequence of Orf2 with those in the GenBank and EMBL databases revealed no significant similarities. From

Table 5: A codon usage table for Orf3.

TTT	phe	F	-	TCT	ser	S	-	TAT	tyr	Y	-	TGT	cys	C	-
TTC	phe	F	5	TCC	ser	S	4	TAC	tyr	Y	2	TGC	cys	C	3
TTA	leu	L	-	TCA	ser	S	-	TAA	ter	*	-	TGA	ter	*	-
TTG	leu	L	-	TCG	ser	S	1	TAG	ter	*	1	TGG	trp	W	5
CTT	leu	L	2	CCT	pro	P	-	CAT	his	H	-	CGT	arg	R	-
CTC	leu	L	5	CCC	pro	P	4	CAC	his	H	3	CGC	arg	R	5
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	-	CGA	arg	R	-
CTG	leu	L	3	CCG	pro	P	1	CAG	gln	Q	4	CGG	arg	R	5
ATT	ile	I	-	ACT	thr	T	-	AAT	asn	N	-	AGT	ser	S	-
ATC	ile	I	11	ACC	thr	T	2	AAC	asn	N	1	AGC	ser	S	4
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	-	AGA	arg	S	-
ATG	met	M	5	ACG	thr	T	1	AAG	lys	K	4	AGG	arg	S	-
GTT	val	V	-	GCT	ala	A	-	GAT	asp	D	-	GGT	gly	G	2
GTC	val	V	14	GCC	ala	A	16	GAC	asp	D	11	GGC	gly	G	16
GTA	val	V	1	GCA	ala	A	-	GAA	glu	E	1	GGA	gly	G	-
GTG	val	V	5	GCG	ala	A	10	GAG	glu	E	14	GGG	gly	G	4

Figure 23: A BLITZ alignment of the amino-terminal amino acid sequence deduced for Orf3, using the ATG start codon, with the amino acid sequences deduced for various ATP-requiring proteins. Exact matches between amino acids are designated with an asterisk and conservative replacements with a dot. Putative ATP binding sites are highlighted in boldface. The "Predicted No." is the number of results expected by chance to have a score greater than or equal to the score of the result displayed, and is derived by analysis of the total score distribution.

CHLOROPLAST PROTEIN CS PRECURSOR. 424 AA.
Organism: *Arabidopsis thaliana*
Function: putative chloroplast transit peptide.
Accession No. PIR S08654
Predicted No. 1.090162e+02

Matches 10; Mismatches 8; Partials 6; Indels 0; Gaps 0;

```
           ... *  ..***  ** *  .***
Db     115 VMIMGDRGTGKSTTVRSLVDLLPE 138
Orf3     2 IILNGGSSAGKSGIVRCLQSVLPE 25
```

GLUTAMINE PERMEASE OPERON PROTEIN GLNQ. 242 AA.
Organism: *Bacillus stearothermophilus*
Function: involved in glutamine transport.
Accession No. PIR A42478
Predicted No. 1.232733e+02

Matches 8; Mismatches 3; Partials 8; Indels 0; Gaps 0;

```
           .... * *..*** .***.
Db      29 VVVIIGPSGSGKSTLVRCI 47
Orf3     1 MIILNGGSSAGKSGIVRCL 19
```

MAGNESIUM-CHELATASE 38 KD SUBUNIT. 350 AA.
Organism: *Rhodobacter capsulatus*
Function: involved in chlorophyll biosynthesis
Accession No. P26239
Predicted No. 1.393142e+02

Matches 10; Mismatches 7; Partials 7; Indels 0; Gaps 0;

```
           ... *  ..***  ** * ..***
Db      48 VLVFGDRGTGKSTAVRALAALLPE 71
Orf3     2 IILNGGSSAGKSGIVRCLQSVLPE 25
```

Figure 23

PANTOTHENATE KINASE (EC 2.7.1.33) (RTS PROTEIN). 316 AA.
Organism: *Escherichia coli*
Function: Involved in coenzyme A biosynthesis.
Accession No. PIR JV0016
Predicted No. 2.003563e+02

Matches 10; Mismatches 9; Partials 7; Indels 1; Gaps 1;

```
           .* . *. . ***   * **..*.  *
Db      90 IISIAGSVAVGKSTTARVLQALLS-RW 11
Orf3     1 MIILNGGSSAGKSGIVRCLQSVLPEPW 27
```

PHNL PROTEIN. 226 AA.
Organism: *Escherichia coli*
Function: involved in alkylphosphonate uptake.
Accession No. PIR D35719
Predicted No. 2.258708e+02

Matches 10; Mismatches 5; Partials 9; Indels 1; Gaps 1;

```
           ..*.* *..*** ..* *  . **.
Db      37 VVLHGHSGSGKSTLLRSLYANYLPD 61
Orf3     2 IILNGGSSAGKSGIVRCL-QSVLPE 25
```

Figure 23

Table 6: A codon usage table for Orf2.

TTT	phe	F	-	TCT	ser	S	-	TAT	tyr	Y	-	TGT	cys	C	-
TTC	phe	F	3	TCC	ser	S	3	TAC	tyr	Y	3	TGC	cys	C	-
TTA	leu	L	-	TCA	ser	S	-	TAA	ter	*	-	TGA	ter	*	1
TTG	leu	L	-	TCG	ser	S	3	TAG	ter	*	-	TGG	trp	W	2
CTT	leu	L	-	CCT	pro	P	-	CAT	his	H	-	CGT	arg	R	-
CTC	leu	L	2	CCC	pro	P	1	CAC	his	H	4	CGC	arg	R	5
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	-	CGA	arg	R	-
CTG	leu	L	5	CCG	pro	P	7	CAG	gln	Q	-	CGG	arg	R	4
ATT	ile	I	-	ACT	thr	T	-	AAT	asn	N	-	AGT	ser	S	-
ATC	ile	I	3	ACC	thr	T	2	AAC	asn	N	-	AGC	ser	S	1
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	-	AGA	arg	S	-
ATG	met	M	1	ACG	thr	T	1	AAG	lys	K	2	AGG	arg	S	-
GTT	val	V	-	GCT	ala	A	-	GAT	asp	D	-	GGT	gly	G	-
GTC	val	V	4	GCC	ala	A	4	GAC	asp	D	7	GGC	gly	G	7
GTA	val	V	-	GCA	ala	A	-	GAA	glu	E	-	GGA	gly	G	-
GTG	val	V	2	GCG	ala	A	7	GAG	glu	E	5	GGG	gly	G	1

analysis of the amino acid usage, Orf2 was only moderately hydrophobic and contained a relatively high proportion of charged amino acids. No potentially hydrophobic domains were detected in a Kyte-Doolittle hydropathy plot.

4. **Orf1**

The incomplete sequence of *orf*-1 (see Fig. 12) encoded a small polypeptide segment (Orf1) of 24 amino acids with a deduced M_r of approximately 2600. A search of the Genbank and EMBL databases revealed no significant similarity to known proteins. It is likely that Orf1 is the carboxy terminus of a larger protein encoded by a gene that was incomplete on the cloned 6.5-kb fragment of *S. venezuelae* DNA.

VII. Chloramphenicol metabolism

A. Compound X

In preliminary experiments, cultures of *S. lividans* transformants RM3 and RM4 were grown in GNY medium supplemented with 12.5 $\mu g.mL^{-1}$ chloramphenicol. Measurements by HPLC of the chloramphenicol concentration in culture supernatants indicated that the antibiotic was probably metabolized, but detection of possible metabolites was complicated by the presence of components in the GNY medium that absorbed at 273 nm and were eluted with retention times overlapping those of expected chloramphenicol metabolites. To reduce interference, the water/methanol gradient was adjusted to allow most of the medium components to be eluted more rapidly from the HPLC column. Under these conditions, chloramphenicol was eluted 2-3 minutes after the slowest of the GNY medium components. Analyses then showed conclusively that chloramphenicol disappeared from the broths of *S. lividans* RM3 and RM4 cultures during the first 24 h of incubation. Furthermore, no new peaks corresponding in retention times to either *N*-acetyl-*p*-nitrophenylserinol or *p*-nitrobenzyl alcohol could be detected.

Although these potential metabolites would have been eluted after the medium components, more polar or charged metabolites might have been present and have been

eluted with the GNY-associated peaks. If so they would not have been detected under the HPLC conditions used. To investigate whether chloramphenicol would be metabolized under nongrowing conditions, i.e. in the absence of GNY medium, mycelium from both RM3 and RM4 cultures grown in GNY medium for 48 h was washed with one volume of 50 m*M* MOPS buffer (pH 6.8) and resuspended in the same buffer supplemented with 50 $\mu g.mL^{-1}$ chloramphenicol. At time zero, samples from both cultures showed only one strong peak (RT 8.3 min.) for chloramphenicol. The area of this peak in both RM3 and RM4 cultures decreased by approximately 80% in the first 2 h of incubation, and a new peak (RT 3.4 min.) appeared. After 4 h incubation, almost 96% of the chloramphenicol in both cultures had been metabolized. The rate at which the peak area for chloramphenicol decreased indicated that both RM3 and RM4 cultures decomposed nearly 600 µg chloramphenicol.h^{-1}. Since the product at RT 3.4 min., provisionally named compound X (cmpd.X), increased in peak area in inverse proportion to the decreased area of the chloramphenicol peak, cmpd.X was probably a direct product of chloramphenicol metabolism.

When RM3 or RM4 culture supernatants were filter-sterilized and supplemented with chloramphenicol, no breakdown of the antibiotic was detected during 24 h incubation at 37°C. Therefore, intracellular or membrane-bound enzyme(s) were responsible for chloramphenicol breakdown. To confirm this, RM3 and RM4 mycelium was washed in 50 m*M* MOPS buffer (pH 6.8) and then resuspended in the same buffer. The resuspended mycelium from both cultures degraded approximately 95% of the antibiotic. When this mycelium was disrupted by sonication and the supernatants and pellets were separately incubated in MOPS buffer (pH 6.8) supplemented with chloramphenicol to 50 $\mu g.mL^{-1}$, the incubation mixtures showed no breakdown of the chloramphenicol supplement during 4 h. The results suggested that cell membrane integrity or the availability of one or more cofactors was important in chloramphenicol metabolism by RM3 and RM4.

In an effort to determine the identity of cmpd.X, various reference compounds were examined by HPLC. One of these, *p*-nitrophenylserinol, could not be eluted from

the reversed phase column with a water/methanol gradient. However, a gradient of methanol in 30 m*M* KH_2PO_4 (pH 3.3) eluted *p*-nitrophenylserinol as a sharp peak at RT 6.38 min. Under these conditions, cmpd.X eluted at RT 7.69 min. This retention time established that cmpd.X was not *p*-nitrophenylserinol and indicated that it was probably an acidic metabolite. The retention times for cmpd.X and various reference compounds using the buffer/methanol gradient are compared in Table 7.

No intermediate products were ever observed by HPLC in the culture supernatants of RM3 or RM4. Nevertheless, it was possible that *p*-nitrophenylserinol might be an intracellular intermediate, and therefore not detectable by HPLC analysis of culture supernatants. To test this hypothesis, GNY cultures of RM3, RM4, and M252 supplemented with 50 $\mu g.mL^{-1}$ of either *p*-nitrophenylserinol or chloramphenicol were incubated for 3 days. The M252 culture was completely inhibited by chloramphenicol, and HPLC analysis showed no change in the concentration of chloramphenicol. In the culture supplemented with *p*-nitrophenylserinol, M252 showed little growth during its first 24 h; however, some growth was observed after 48 h, and by 72 h, good growth had occurred. Interestingly, this culture accumulated a large amount of dark red pigment, possibly actinorhodin (Kieser and Hopwood, 1991). An HPLC analysis of the culture supernatant showed that the area of the *p*-nitrophenylserinol peak had decreased 67% by 72 h. Furthermore, a new peak was observed with a retention time identical to that of *N*-acetyl-*p*-nitrophenylserinol; the peak area was directly proportional to the decrease in area of the *p*-nitrophenylserinol peak.

In contrast to *S. lividans* M252, *S. lividans* RM3 and RM4 cultures grown in GNY medium supplemented with chloramphenicol or *p*-nitrophenylserinol showed strong growth after 24 h. After 72 h, HPLC analysis of clarified broths from the chloramphenicol-supplemented cultures showed that 97% of the chloramphenicol had been metabolized to cmpd.X. However, in the *p*-nitrophenylserinol-supplemented cultures, the peak area for *p*-nitrophenylserinol at this time had decreased by only 24%. HPLC analysis of both of

Table 7: HPLC retention times (RT) for chloramphenicol and related compounds.

Compounds	RT (min)
p-Nitrophenylserine	5.89
p-Nitrophenylserinol	6.38
N-Acetyl-*p*-nitrophenylserinol	7.19
Cmpd.X	7.69
p-Nitrobenzyl alcohol	8.15
p-Nitrobenzoic acid	8.22
Chloramphenicol	8.28
p-Nitrobenzaldehyde	8.50
1,3-Diacetoxychloramphenicol	8.94

* Samples were chromatographed on a Beckman Ultrasphere-Octyl column (5 µm; 4.6 mm x 25 cm) with a gradient of methanol in 30 m*M* KH_2PO_4 (pH 3 3) using method C.

these RM3 and RM4 cultures at 72 h showed three new peaks, RT 8.10, 8.43, and 8.57 min., none of which coincided in retention time with that of *N*-acetyl-*p*-nitrophenylserinol or any of the reference compounds tested (Table 8). The sum of the areas for the three new peaks equaled the total decrease in area of the *p*-nitrophenylserinol peak.

B. Purifying compound X

The evidence from HPLC analysis that cmpd.X was an organic acid was supported by a preliminary indication that the product was partially extractable with ethyl acetate from an aqueous solution only at or below pH 2.5. On the assumption that cmpd.X was an organic acid and would be fully ionized and negatively charged at neutral pH, the anion exchanger QAE-Sephadex A-25 was used to remove cmpd.X from incubation mixtures. This exchanger was chosen because it has quaternary aminoethyl functional groups that exhibit a relatively high exchange capacity over a wide pH range. In addition, the Sephadex matrix did not irreversibly adsorb chloramphenicol-related compounds, as did polystyrene-divinylbenzene ion exchange resins. Because of the difficulty anticipated in removing nonvolatile buffers from the final product, conversion of chloramphenicol by RM4 mycelium suspended in water instead of in MOPS buffer was investigated. Although the efficiency of the conversion was only 70-80% of that observed for RM4 mycelium suspended in MOPS buffer, cmpd.X was formed at a similar initial rate. The yield in the conversion solution was considered adequate for chromatographic purification on QAE-Sephadex A-25. Although various parameters were explored, the most successful procedure used the anion exchanger in the acetate form packed into a column (1 x 25 cm). A clarified sample (250 mL) of supernatant (containing 80 $\mu g.mL^{-1}$ cmpd.X and 20 $\mu g.mL^{-1}$ chloramphenicol by HPLC analysis) from a 16-h RM4 mycelial suspension culture in H_2O was applied to the column. Monitoring of the absorbance of the column effluent at 273 nm showed that both cmpd.X and chloramphenicol were retained by the exchanger. When the column was washed with 250 mL of H_2O, chloramphenicol but not cmpd.X

Table 8: HPLC retention times (RT) for chloramphenicol and related compounds.

Compound	RT (min)
p-Nitrophenylserine	4.10
p-Nitrophenylserinol	5.48
N-Acetyl-*p*-nitrophenylserinol	7.48
Cmpd.X	8.45
p-Nitrobenzyl alcohol	9.05
Chloramphenicol	9.26
p-Nitrobenzoic acid	9.66

* Samples were chromatographed on a Beckman Ultrasphere-Octyl column (5 µm; 4.6 mm x 25 cm) with a gradient of methanol in 30 m*M* KH_2PO_4 (pH 3.3) using method D.

was immediately eluted. To elute cmpd.X, the column was washed with 1 *M* ammonium formate (pH 2.5). The absorbance at 273 nm of the 5-mL fractions collected rose sharply after the fifth fraction and remained elevated ($A_{273} > 4.0$) for another six fractions; HPLC analysis of these fractions showed that both ammonium formate and cmpd.X were present, at high concentration. The ammonium formate was removed by evaporating the aqueous solutions and subliming the residue under high vacuum. By this procedure cmpd.X was obtained in a relatively pure form.

Concurrent with these efforts to purify cmpd.X by anion exchange chromatography, the use of reversed-phase chromatography was explored. A solution of cmpd.X from the incubation of chloramphenicol with RM4 mycelium in 50 m*M* MOPS buffer (pH 6.8) was acidified to pH 3.3 with dilute HCl, and successive 5-mL samples were applied to a reversed-phase, C18 Sep-pak column. HPLC analysis of the column effluent showed that while all cmpd.X in the first sample had been retained on the column, only 66% in the second sample, and only 23% in the third sample were retained. Washing the column with 4 mL of water eluted cmpd.X almost completely.

When the procedure was modified by preequilibrating the C18 Sep-pak column with 50 m*M* HCl (pH 2.0), and adjusting the conversion sample to pH 2.0 with dilute HCl before it was applied, cmpd.X was completely retained by the column. Washing with 5 mL of 50 m*M* HCl did not elute cmpd.X, but a subsequent wash with 5 mL of water eluted most of it. Attempts to repeat the procedure with a larger sample of cmpd.X showed that the effective capacity of the column could not be substantially increased, and only small amounts of cmpd.X could be recovered directly from the conversion solution at any one time with a Sep-pak C18 column. However, the procedure appeared to have potential, and was therefore modified in two ways: (1) by applying a more concentrated solution of cmpd.X to the column and (2) by using a larger column. To achieve the first, 250 mL of supernatant (containing 140 $\mu g.mL^{-1}$ cmpd.X, and 60 $\mu g.mL^{-1}$ chloramphenicol) from a 23 h incubation of chloramphenicol with an RM4 mycelial suspension in water,

was evaporated *in vacuo* at 40°C to dryness. The residual amber film was redissolved in 1 mL of water and verified by HPLC to contain unchanged cmpd.X at the desired (250X) concentration. This solution was acidified to pH 3.4 by adding 100 µL of 1 *M* ammonium formate (pH 2.5). The sample was successively washed into a column (1 x 23 cm) of C18 resin with 1 mL of 0.1 *M* ammonium formate (pH 2.5) and three, 1 mL portions of H_2O. The column was then irrigated with H_2O and eluate fractions were monitored at 273 nm. Cmpd.X began to elute from the column after 72 mL of effluent had been collected, and HPLC analysis showed that it was at highest concentration in the effluent collected between 77.4 and 93.6 mL. Furthermore, HPLC analysis showed that except for a minor, more polar impurity, cmpd.X was the only component present in these fractions. The fractions containing Cmpd.X were pooled, and evaporated to dryness at 40°C *in vacuo*.

C. Identifying compound X

Aromatic nitro-compounds absorb maximally at about 273 nm and aromatic amines at about 254 nm. To test the intensity of absorbance by cmpd.X at these two wavelengths, samples of a 2 hour RM4 culture supernatant containing approximately 75% cmpd.X and 25% chloramphenicol, were analyzed by HPLC with the detector at 254 nm and then at 273 nm. The peak areas of both cmpd.X and chloramphenicol were 50% lower at 254 nm than those recorded at 273 nm, indicating that both were aromatic nitro-compounds. As a reference aromatic nitro-compound, *p*-nitrophenylserinol showed a 50% drop in peak area when monitored at 254 nm instead of 273 nm.

At pH 2.0, most amino compounds are positively charged and will readily exchange on to sulfopropyl (SP) Sephadex. To test whether cmpd.X possessed a free amino-group, a 5-mL sample of cmpd.X (50 $\mu g.mL^{-1}$) was adjusted to pH 2.0 with dilute HCl and applied to a column containing SP Sephadex C25 cation exchanger, pre-equilibrated at pH 2.0; the column was then washed with 5 mL of water. Although *p*-aminobenzoic acid, *p*-nitrophenylserine and several other amine test compounds were retained

on this column, HPLC analysis of the effluent showed that cmpd.X was not retained by the cation exchange resin. The result suggested that cmpd.X did not possess a free amino-group.

To obtain additional information about the chemical structure of cmpd.X a sample (9.5 mg) of the material purified by reversed-phase chromatography was examined by ^{1}H-NMR (nuclear magnetic resonance) spectroscopy. The proton spectrum was very similar to that of chloramphenicol (Fig. 24 and 25), and gave signals with chemical shifts indicative of a dichloroacetyl group and a 1,4-disubstituted aromatic ring system (Tables 9 and 10). Nevertheless, the spectrum of cmpd.X did differ in the spin-spin splitting pattern of the proton signals associated with the propanediol moiety. In the chloramphenicol spectrum, the H-1a' and H-1b' resonances were split, as expected, into quartets by coupling with each other and with the vicinal proton H-2'. However, in cmpd.X, both of these protons exhibited complex spin-spin coupling, and were displaced downfield with respect to those in the chloramphenicol spectrum. Together, these data suggested that the protons of the propanediol moiety in cmpd.X, especially H-1a', H-1b', and H-2', were being deshielded by a fairly intense electron-withdrawing functional group, attached most likely to C-1'.

Examination of the same sample of cmpd.X by ^{13}C-NMR spectroscopy also gave a spectrum very similar to that of chloramphenicol (Fig. 26 and 27), but with the chemical shifts of most signals slightly downfield of those for chloramphenicol (Table 11). The signal associated with C-1' was further downfield than the corresponding signal in the chloramphenicol spectrum (62.2 p.p.m. vs 66.6 p.p.m.), supporting the presence of an electron-withdrawing group associated with C-1'.

D. Chloramphenicol-1'-phosphate

The discovery of a nucleotide-binding site in Orf3, the putative product of a gene within the *S. venezuelae* DNA inserts of pJV3 and pJV4, suggested that the electron-

Figure 24: A ^{1}H-NMR spectrum of chloramphenicol.

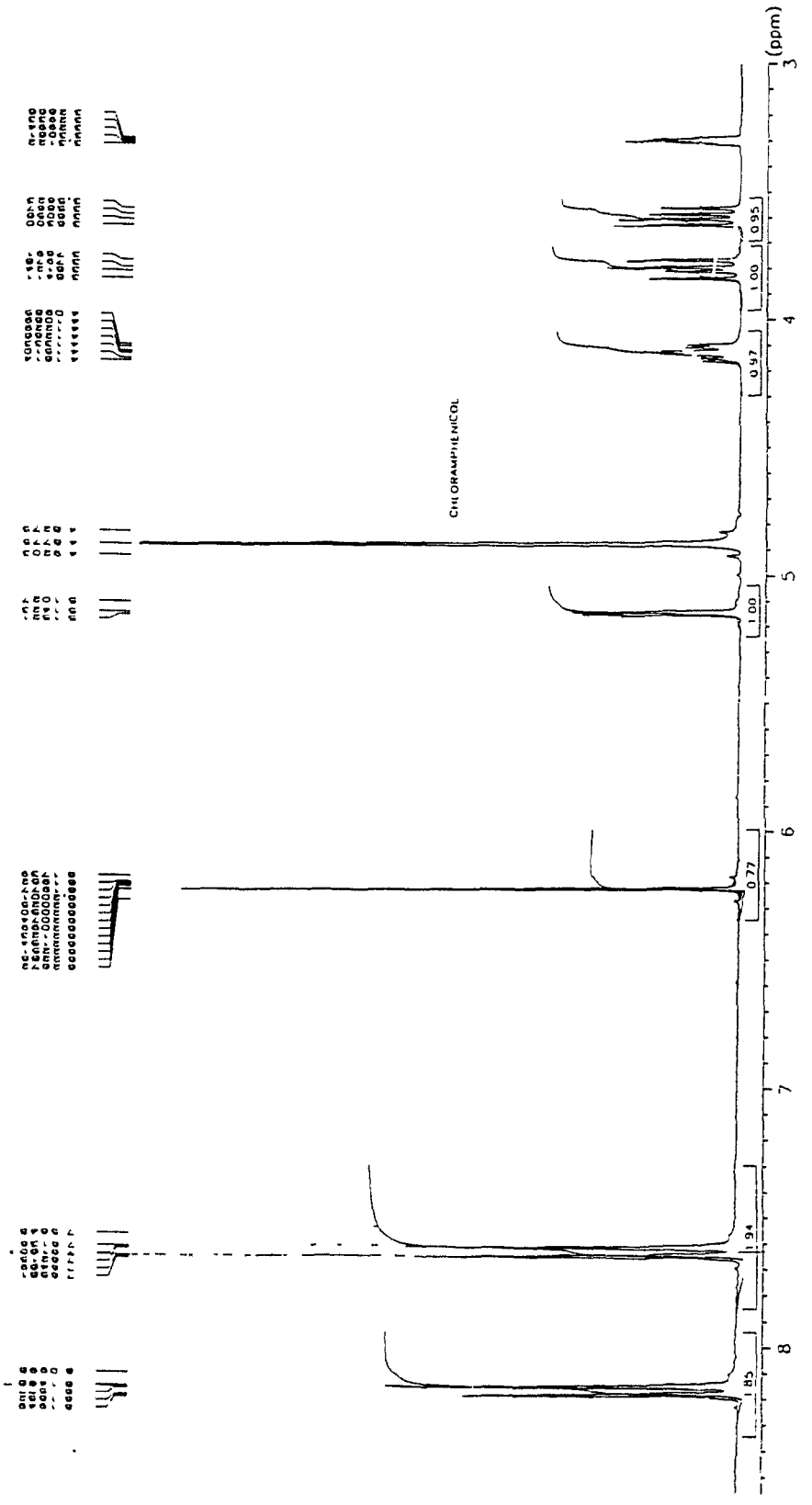


Figure 24

Figure 25: A ^{1}H-NMR spectrum of compound X.

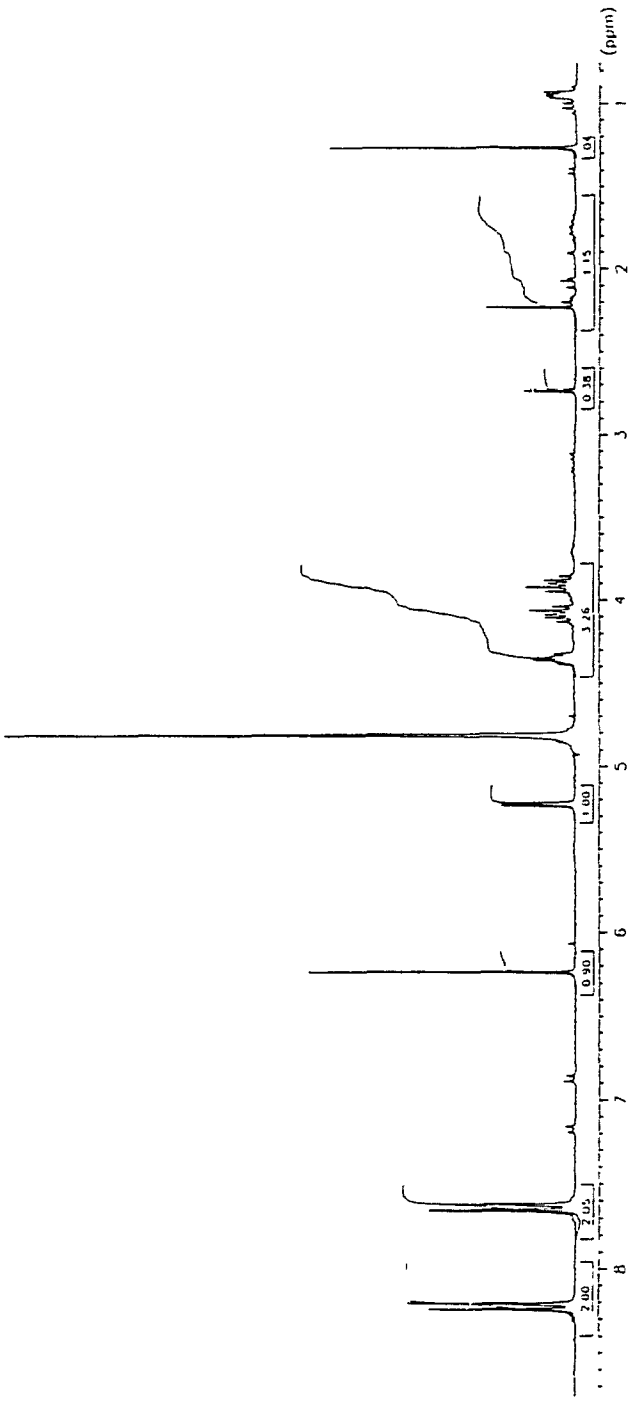


Figure 25

Table 9: ^{1}H-NMR chemical shifts (p.p.m.) of chloramphenicol*.

δ ^{1}H	Assignments
3.59	q, 1H, H-1a', $J_{1a',1b'}$ 6.1 Hz, $J_{1a',2'}$ 6.1 Hz
3.81	q, 1H, H-1b', $J_{1b',1a'}$ 7.2 Hz, $J_{1b',2'}$ 7.2 Hz
4.13	ct, 1H, H-2', $J_{2',1a'}$ 2.6 Hz, $J_{2',1b'}$ 2.6 Hz, $J_{2',3'}$ 2.6 Hz
5.15	d, 1H, H-3', $J_{3',2'}$ 2.5 Hz
6.22	s, 1H, C<u>H</u>Cl_2
7.89	AA'BB', 4H, H-2, H-3, H-5, H-6, consisting of doublets at 7.62 and 8.17 p.p.m., $J_{2,3} = J_{5,6} = 8.9$ Hz

* Recorded in methanol at 250.1 MHz using tetramethylsilane as an internal standard.

** q, quartet; ct, complex triplet; d, doublet; s, singlet; AA'BB', coupled 4-proton system.

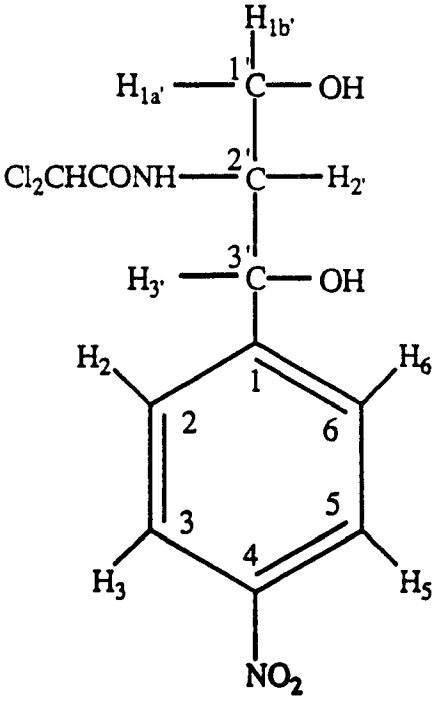


Table 10 ^{1}H-NMR chemical shifts (p p m) of compound X*

δ ^{1}H	Assignments
3 89	m, 1H, H-1a'
4 07	m, 1H, H-1b'
4 35	m, 1H, H-2'
5 22	d, 1H, H-3', $J_{3',2'}$ 3 5 Hz
6 23	s, 1H, C<u>H</u>Cl$_2$
7 92	AA'BB', 4H, H-2, H-3, H-5, H-6 consisting of doublets at 7 63 and 8 22 p p m , $J_{2,3}$ = 8 8 Hz, $J_{5,6}$ = 8 7 Hz

* Recorded in methanol at 250 1 MHz using tetramethylsilane as an internal standard

** Chemical shifts were also observed at 1 25 p p m and 2 22 p p m , these are believed to be due to a minor impurity in the cmpd X sample which was also observed by ^{13}C-NMR spectroscopy, mass spectroscopy, and HPLC analysis

*** m, multiplet, d, doublet, s, singlet, AA'BB', coupled 4-proton system

Figure 26: A ^{13}C-NMR spectrum of chloramphenicol.

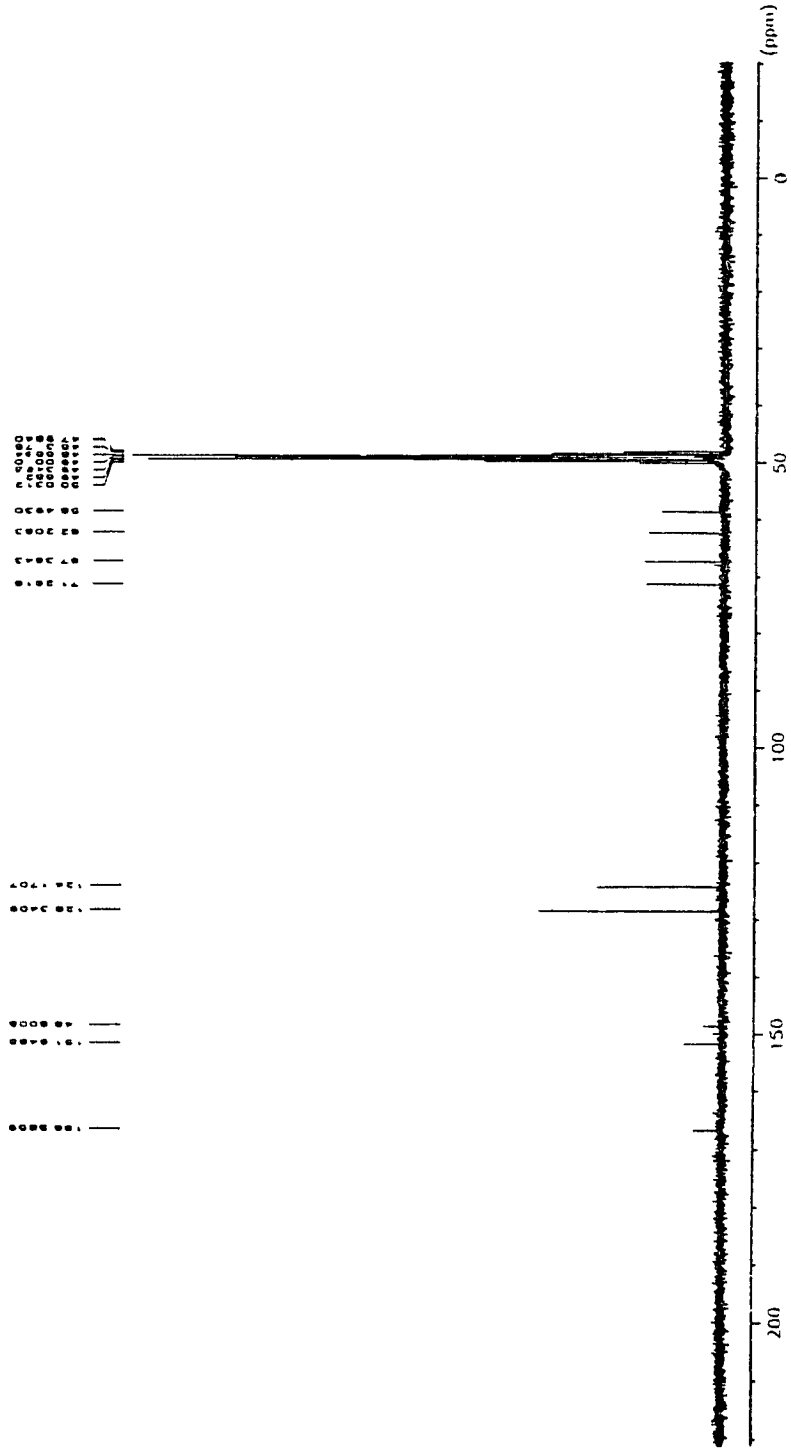


Figure 26

Figure 27: A ^{13}C-NMR spectrum of compound X.

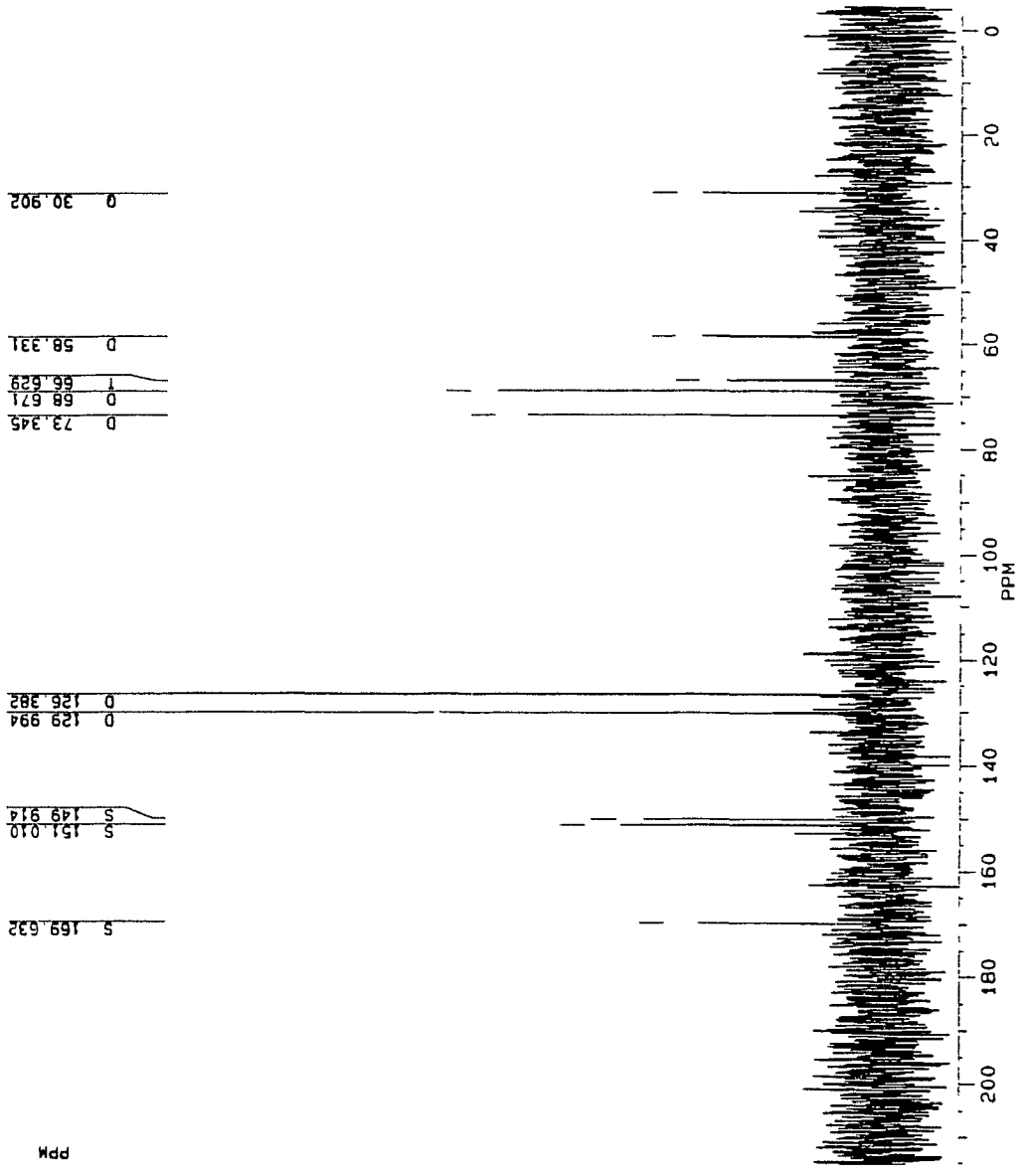

PPM
0
20
40
60
80
100
120
140
160
180
200
D 30.902
D 58.331
T 66.629
D 68.671
D 73.345
D 126.382
D 129.994
S 149.914
S 151.010
S 169.632

Figure 27

Table 11: ^{13}C-NMR chemical shifts (p.p.m.) of chloramphenicol and compound X.

	$\delta\ ^{13}C$	
Assignments	Chloramphenicol	Cmpd.X
s, <u>C</u>O	166.6	169.6
s, C-4	151.6	151.0
s, C-1	148.6	149.9
d, C-3, C-5	128.3	129.9
d, C-2, C-6	124.2	126.4
d, C-3'	71.3	73.3
d, <u>C</u>HCl_2	67.4	68.7
t, C-1'	62.2	66.6
d, C-2'	58.5	58.3

* The spectrum for chloramphenicol was recorded at 62.5 MHz in acetone, using solvent ^{13}C as an internal standard. The spectrum for cmpd.X was recorded in D_2O; locked to solvent deuterium.

** A chemical shift was also observed at 30.9 p.p.m. in the cmpd.X spectrum, and is believed to be due to a minor impurity in the sample. It was also observed by ^{1}H-NMR spectroscopy, mass spectroscopy and HPLC analysis.

*** s, singlet; d, doublet; t, triplet.

withdrawing moiety in cmpd.X might be a phosphate group. The ^{31}P-NMR spectrum of cmpd.X (Fig. 28) strengthened this evidence. The chemical shift of the single strong signal was consistent with the presence of an organo-phosphate compound. To confirm this, cmpd.X was analyzed by low-resolution negative ion-spray mass spectroscopy. The molecular-ions generated supported the presence of one phosphate group covalently bonded to chloramphenicol; the deduced M_r of the compound was 401.97, the value predicted for a monophosphate ester of the antibiotic (Fig. 29).

Early studies of the relationship between chloramphenicol structure and function showed that any alteration of the hydroxyl group at C-1' of chloramphenicol resulted in a complete loss of bioactivity (Pongs, 1979). Subsequent work has shown that acetylation of C-1' prevents chloramphenicol from binding to the 50S subunit of the prokaryotic ribosome (Shaw, 1983). To test whether phosphorylation of the C-1' hydroxyl would eliminate bioactivity, separate 1.3-cm filter disks were impregnated with two samples of purified cmpd.X (365 µg and 730 µg) dissolved in H_2O and used in a bioassay against a chloramphenicol-hypersensitive strain of *Micrococcus luteus*. As references, two disks impregnated with 5 µg and 10 µg samples of chloramphenicol were placed on the same bioassay plate. The results showed that the specific activity of cmpd.X against *M. luteus* was 0.04% that of chloramphenicol.

Figure 28: A ^{31}P-NMR spectrum of compound X, recorded at 101.2 MHz in D_2O using 85%(w/v) H_3PO_4 as an external standard.

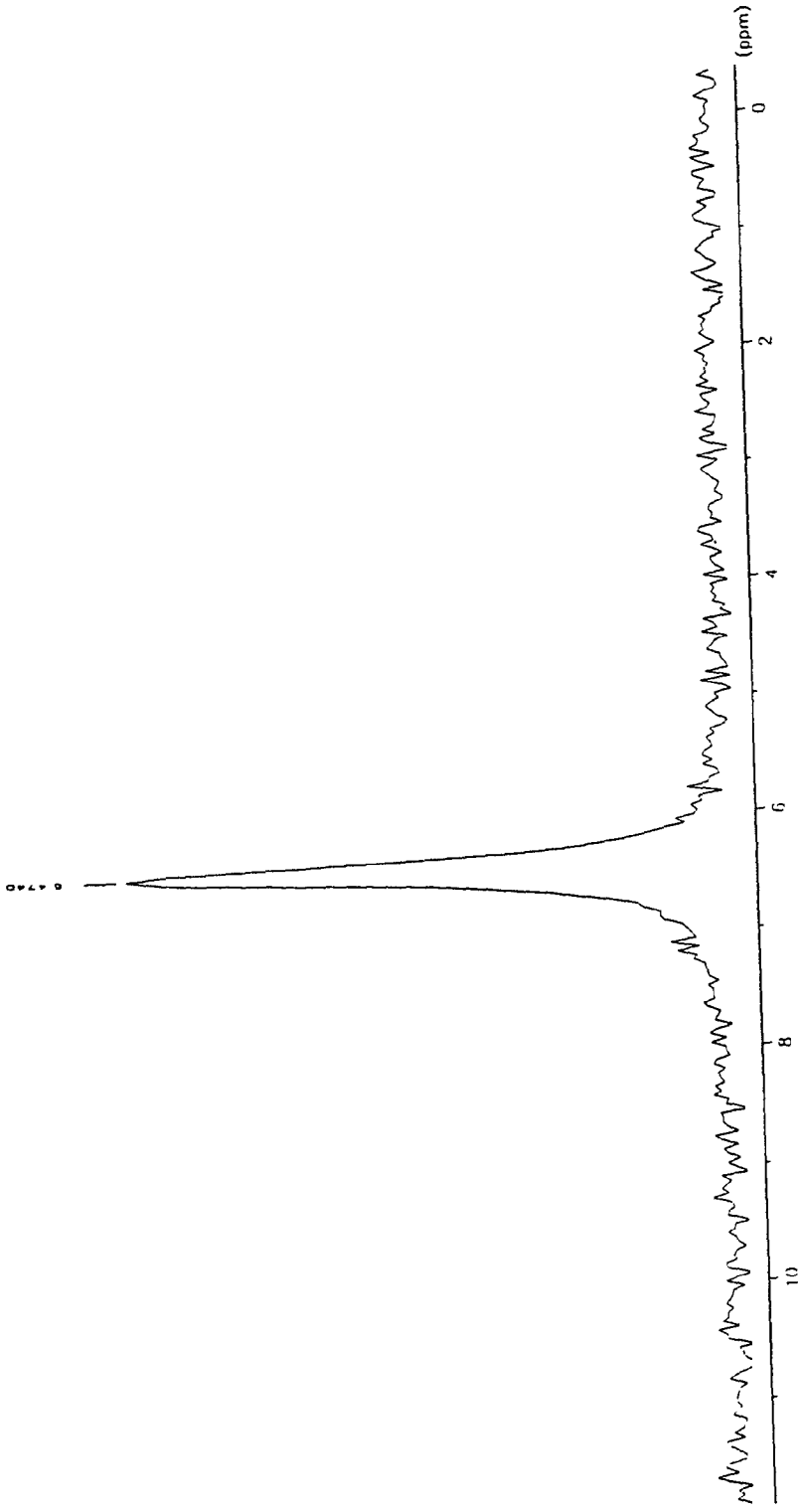


Figure 28

Figure 29: A low resolution mass spectrum of compound X.

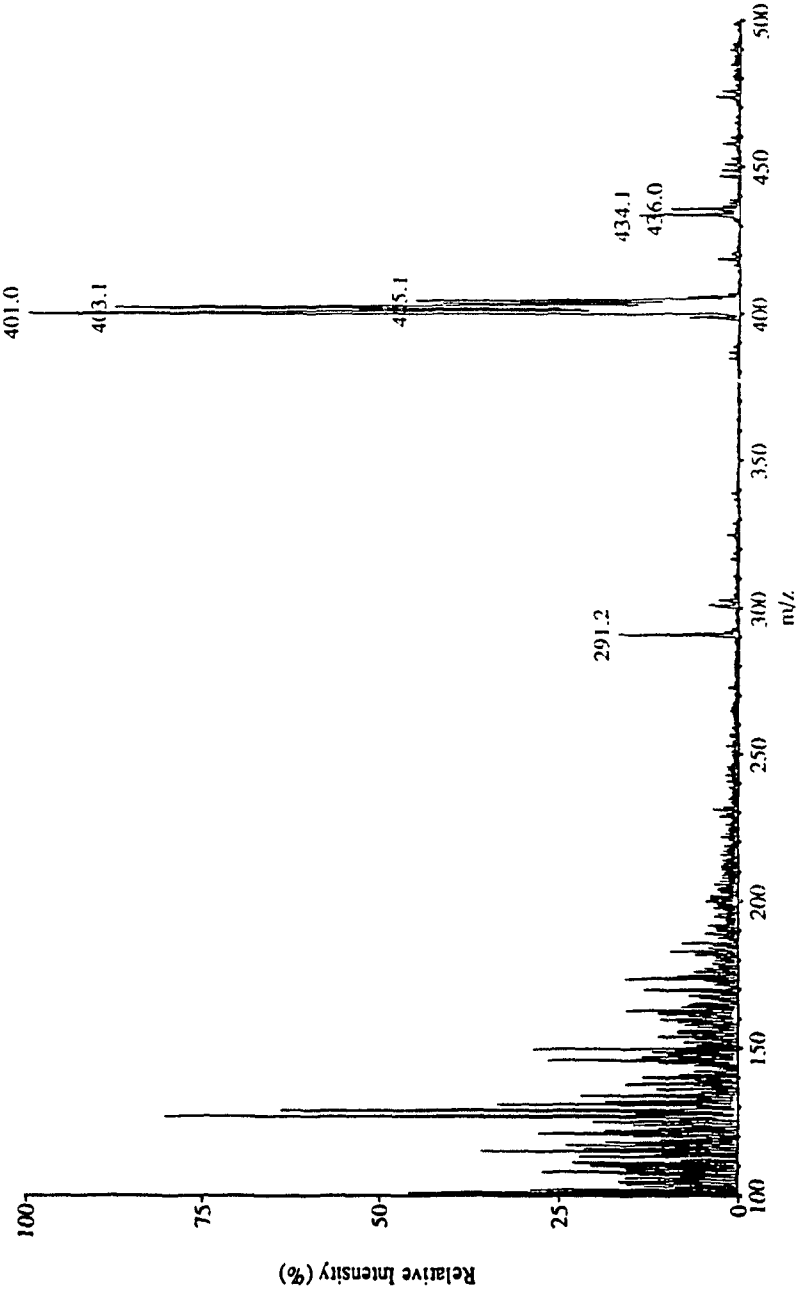

Relative Intensity (%)
100
75
50
25
0
100
150
200
250
300
350
400
450
500
m/z
401.0
403.1
435.1
434.1
436.0
291.2

Figure 29

DISCUSSION

Most antibiotic-producing actinomycetes possess multiple lines of defense against their autotoxic secondary metabolites. *Streptomyces venezuelae* possesses at least two mechanisms that protect its antibiotic-sensitive ribosomes from chloramphenicol (Vining and Westlake, 1984). The primary mechanism of resistance was postulated to be a chloramphenicol-inducible permeability barrier that is coordinately regulated with antibiotic biosynthesis. The second mechanism proposed (Malik and Vining, 1972) was an intracellular, constitutively expressed chloramphenicol hydrolase activity that inactivates any residual chloramphenicol that has leaked back into the cytoplasm after being exported. Malik (1970) found that nonproducing cultures of *S. venezuelae* 13s, supplemented with chloramphenicol initially converted the antibiotic to *p*-nitrophenylserinol, most of which was then rapidly metabolized to N-acetyl-*p*-nitrophenylserinol; small amounts of *p*-nitrophenylserinol were metabolized by an alternative pathway to form *p*-nitrobenzyl alcohol and *p*-nitrobenzoic acid.

In subsequent research, aimed at defining more precisely the molecular basis for these resistance mechanisms, genomic DNA from *S. venezuelae* ISP5230 was shotgun cloned in pIJ702 and introduced into the chloramphenicol-sensitive strain *S. lividans* M252. The chloramphenicol-resistant transformant, RM3, isolated by selection for growth on chloramphenicol-supplemented media possessed a recombinant plasmid, pJV3, carrying a 6.5-kb *Sst*I-*Sst*I fragment of *S. venezuelae* DNA. The pattern of chloramphenicol resistance expressed by this transformant was examined in cultures exposed to subinhibitory concentrations of the antibiotic during growth (Mosher, 1986). After 4 days in a medium supplemented with [U-^{14}C]chloramphenicol, *S. lividans* RM3 cultures had modified the antibiotic to the extent that only 10% of the radioactivity added could be recovered by extracting the culture supernatant under conditions that readily removed the antibiotic. Thin-layer chromatography (TLC) of the recovered material (extracted with ethyl acetate at alkaline pH) showed that the radioactivity was indeed mainly present in unmeta-

bolized chloramphenicol An additional 28% of the total added radioactivity was extracted into ethyl acetate when the residual culture supernatant was adjusted to an acidic pH, leaving 61% of the added radioactivity unaccounted for Thin-layer chromatography of the material recovered at acidic pH showed a single zone that traveled more slowly than chloramphenicol in chloroform/methanol (97 5 2 5), but the product was not examined further The results suggested that chloramphenicol had been metabolized to a single acidic product that may have partitioned poorly from the culture fluid into ethyl acetate, and thus have been partially extracted from the acidified supernatant solution In retrospect, these results may mean that the unidentified product was chloramphenicol-1'-phosphate

In a second experiment where clarified broths of *S. lividans* RM3 cultures supplemented with [U-^{14}C]chloramphenicol were extracted with ethyl acetate under alkaline (pH 8 5) and then acidic (pH 2 5) conditions, 20% of the total radioactivity added was in the alkaline extract, and only a negligible amount in the subsequent acidic extract, approximately 19% of the administered radioactivity was associated with the mycelium (Mosher *et al.*, 1990) Again, however, 61% of the radioactivity was not extracted at either pH When the alkaline extract was fractionated by preparative TLC, only two metabolites, *p*-nitrobenzyl alcohol and *N*-acetyl-*p*-nitrophenylserinol, representing 3 9% and 5 6%, respectively, of the total radioactivity administered to the culture were found The identification of these products, both labelled with radioactivity, in RM3 cultures supplemented with [^{14}C]chloramphenicol suggested that CAH activity was present, although the failure to detect *p*-nitrophenylserinol was surprising That *S. lividans* RM3 rapidly catabolized the metabolite, was demonstrated by supplementing cultures with *p*-nitrophenyl[hydroxymethylene-^{14}C]serinol After 24 h growth, only 14% of the administered radioactivity was extractable with ethyl acetate at alkaline pH, TLC analysis of this fraction identified only one labeled product, *N*-acetyl-*p*-nitrophenylserinol, and one unlabelled product, *p*-nitrobenzyl alcohol No unmetabolized *p*-nitrophenylserinol could be detected

(Mosher *et al.*, 1990). The combined results of these experiments suggested that in *S. lividans* RM3, chloramphenicol was first hydrolyzed to *p*-nitrophenylserinol by CAH, and then either rapidly *N*-acetylated or metabolized further to *p*-nitrobenzyl alcohol. Evidence from an experiment in which unlabelled *p*-nitrophenylserinol was used to supplement *S. lividans* M252 cultures, and both *N*-acetyl-*p*-nitrophenylserinol and *p*-nitrobenzyl alcohol were detected in culture extracts, indicated that enzymes indigenous to *S. lividans* M252 could metabolize the initial product of a putative chloramphenicol hydrolase (Fig. 30).

Although the results obtained with *S. lividans* cultures suggested that a gene encoding CAH activity had been cloned, the failure to recover over 60% of the metabolites generated from [U-^{14}C]chloramphenicol by ethyl acetate extraction, and the inability to detect *p*-nitrophenylserinol, indicated that a more complete understanding was required. In the present study, the identity of the products formed in cultures of *S. lividans* transformant RM3 was investigated by using HPLC as an initial means of analyzing culture filtrates. With a reversed-phase column and a water/methanol solvent gradient, clarified samples of cultures could be directly examined for polar and nonpolar metabolites. Preparative chromatographic procedures were then developed to isolate the principle product. In a parallel approach the genetic determinants responsible for chloramphenicol resistance were investigated. These were primarily focused on subcloning and sequencing of the cloned *S. venezuelae* fragment to establish whether a gene encoding CAH was present. Once the CAH gene was localized and sequenced, its deduced amino acid sequence could be compared to the amino-terminal sequence of purified CAH protein, the isolation and characterization of which were considered to be ultimate objectives.

I. Subcloning the chloramphenicol resistance determinant of pJV3

The *in vitro* deletion of a 5.2-kb *Kpn*I-*Kpn*I DNA fragment from pJV3 showed that all the gene(s) for chloramphenicol resistance cloned in that plasmid were located on the 2.4-kb *Kpn*I-*Sst*I insert subconed in pJV4. HPLC analysis showed that mycelial sus-

Figure 30: Two possible pathways for chloramphenicol metabolism. CAH: chloramphenicol hydrolase; CAT: chloramphenicol acetyltransferase; A: chloramphenicol; B: 1,3-diacetoxychloramphenicol; C: *p*-nitrophenylserinol; D: N-acetyl-*p*-nitrophenylserinol; E: *p*-nitrobenzoic acid; F: *p*-nitrobenzyl alcohol.

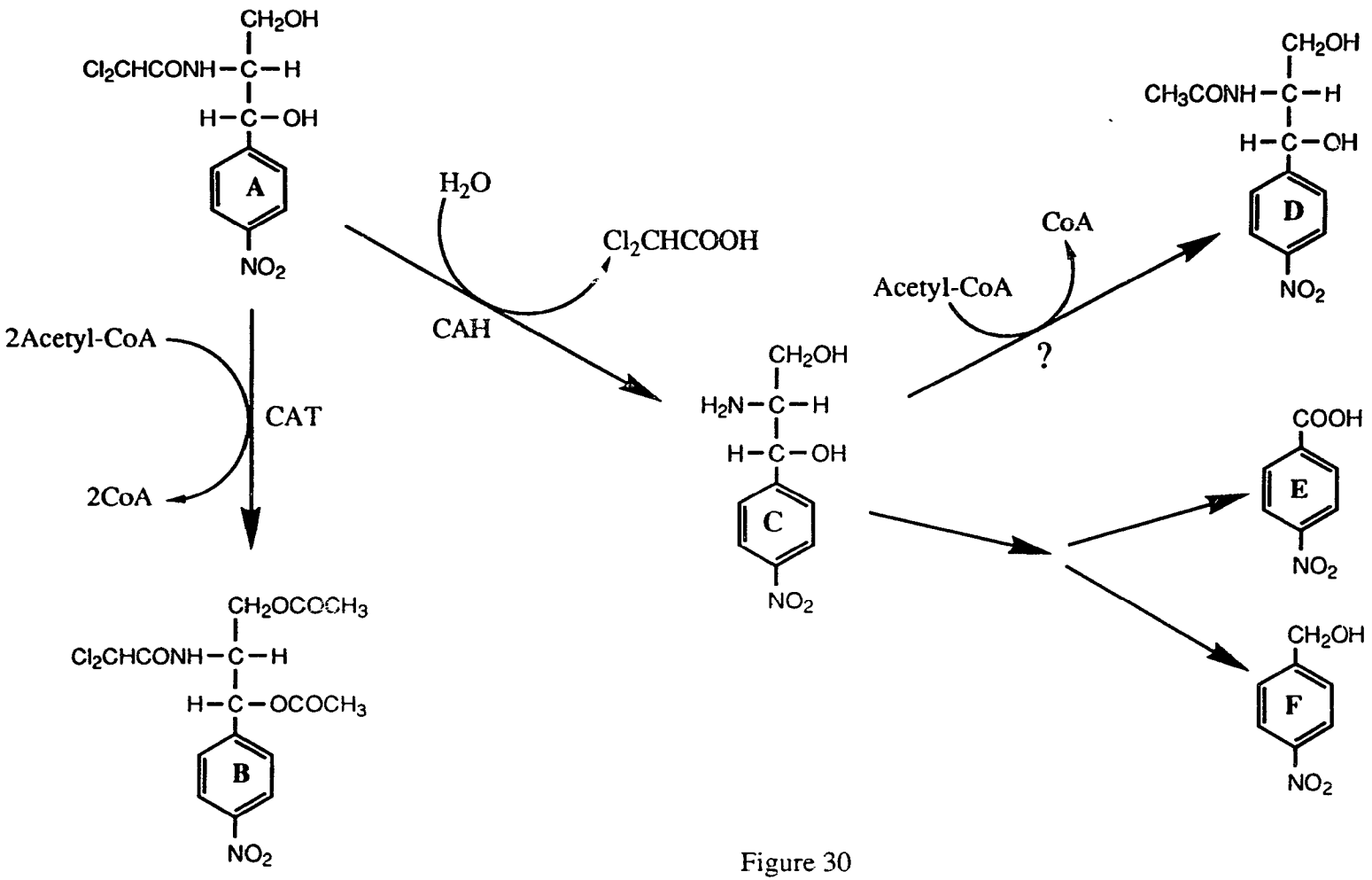

CH2OH
Cl2CHCONH–C–H
H–C–OH
A
NO2
H2O
Cl2CHCOOH
CAH
2Acetyl-CoA
CAT
2CoA
CH2OCOCH3
Cl2CHCONH–C–H
H–C–OCOCH3
B
NO2
CH2OH
H2N–C–H
H–C–OH
C
NO2
Acetyl-CoA
CoA
?
CH2OH
CH3CONH–C–H
H–C–OH
D
NO2
COOH
E
NO2
CH2OH
F
NO2

Figure 30

pensions in water or buffer of the *S. lividans* transformants RM3 and RM4 rapidly metabolized chloramphenicol to one major product, cmpd.X, and it seemed likely that the conversion of chloramphenicol to cmpd.X was a one step process. None of the products of chloramphenicol metabolism observed by Malik (1970) or Mosher *et al.* (1990) were detected in culture supernatants of either organism. The inability to detect *p*-nitrobenzyl alcohol (PNBA) or N-acetyl-*p*-nitrophenylserinol (N-acetyl-PNPS) in the culture supernatants of RM3 and RM4 by HPLC might have been due to the relatively low levels at which these metabolites were formed. Mosher *et al.* (1990) reported that PNBA and N-acetyl-PNPS represented only 3.9 and 5.6% of the total products formed, respectively, after chloramphenicol metabolism. Since the initial concentration of antibiotic in cultures and mycelial suspensions used in the present investigation was 50 $\mu g.mL^{-1}$, the expected final concentration of PNBA and N-acetyl-PNPS would have been 1.8 and 2.8 $\mu g.mL^{-1}$ respectively. For these metabolites to have been recognized as significant peaks distinguishable from baseline noise in the HPLC analysis reaction mixtures, the initial concentration of chloramphenicol should have been increased by at least 5-fold.

Mosher *et al.* (1990) observed that cultures of *S. lividans* M252 were quite sensitive to PNPS, while RM3 was relatively resistant; this was confirmed in the present study. The results obtained earlier indicated that both cultures converted PNPS to N-acetyl-PNPS and PNBA, and suggest that M252 possessed enzymes capable of metabolizing PNPS. The reason for the enhanced resistance of RM3 to PNPS was not explained, but might reasonably be attributed to the presence of genes cloned from *S. venezuelae*. Gottlieb *et al.* (1956) reported that *S. venezuelae* cultures rapidly metabolized exogenously supplied PNPS to N-acetyl-PNPS. In the present study, *S. lividans* M252 was more sensitive to PNPS than either *S. lividans* RM3 or RM4, and rapidly metabolized PNPS to a product that by HPLC appeared to be N-acetyl-PNPS; however, no PNBA was observed. Surprisingly, both RM3 and RM4 metabolized PNPS at a much slower rate than M252, and none of the putative products could be identified as known metabolites. The reason for the

differences from the findings of Mosher *et al.* (1990) has not been established but it is noteworthy that the growth of both RM3 and RM4 was unaffected by the concentration of PNPS used. Possibly RM3 and RM4 possess a permeability barrier to PNPS. It is also possible that the unidentified products were phosphorylated.

The differences between the earlier results and those of the present study may be due to the different concentrations of PNPS used in the two experiments. Mosher *et al.* (1990) supplemented M252 cultures with 15 $\mu g\ mL^{-1}$ PNPS, whereas in the present study the concentration was 50 $\mu g.mL^{-1}$. At the lower concentration, M252 may grow more rapidly and metabolize PNPS to a greater extent.

Mosher *et al.* (1990) supplemented RM3 cultures with 190 $\mu g.mL^{-1}$ PNPS, whereas in the present study cultures of RM3 the supplement was 50 $\mu g\ mL^{-1}$ If both RM3 and RM4 possess a permeability barrier to PNPS the higher concentration used in the earlier work may have partially overcome the barrier; PNPS penetrating to the cytoplasm might then have been metabolized by intracellular enzymes. However, the report (Mosher *et al.*, 1990) that all of the PNPS used to supplement RM3 cultures had been metabolized after 24 h growth does not agree well with uptake due to a leaky permeability barrier. One possibility could be that the relatively low antibiotic activity of PNPS might not correlate with its effect on protein synthesis. Perhaps PNPS, like streptomycin, causes the synthesis of aberrant membrane proteins, especially those that control cellular uptake (Davis, 1987), and the consequent effect on PNPS permeability eventually leads to complete conversion of PNPS to N-acetyl-PNPS in RM3

Failure to detect expression of the *cml*^{r} gene(s) when the 6.5-kb *Sst*I-*Sst*I insert of pJV3 was subcloned in the *E. coli* vector pTZ18R, in both orientations with respect to the *lac* promoter, and the lack of expression when the 2.4-kb *Kpn*I-*Sst*I insert of pJV4 was subcloned directly downstream of the *lac* promoter of pTZ18R or pTZ19R, suggested that a transcriptional terminator might be located between the *lac* promoter and the *cml*^{r} gene(s). The results indicated that, if a promoter was present on the 2.4-kb DNA

fragment, it was not recognized by the *E. coli* transcriptional apparatus. These conclusions were supported by the inability of *E. coli* TG1, transformed by pJV7 or pJV8, to metabolize sublethal concentrations of chloramphenicol (Mosher *et al.*, 1990).

The lack of expression of streptomycete genes from their native promoters in *E. coli* is not uncommon. Although some streptomycete genes possess $E\sigma^{70}$-like promoters upstream of their structural sequences, most lack the proper -10 and -35 consensus sequences recognized by the $E\sigma^{70}$ holoenzyme (Strohl, 1992). Nevertheless, selective pressure has in some cases allowed the *in vivo* deletion of noncoding regions upstream of streptomycete genes and the alignment of these genes with strong *E. coli* promoters. An example of this type of rearrangement was observed when the chloramphenicol acetyltransferase (*cat*) gene (see Fig. 30) of *Streptomyces acrimycini* was subcloned downstream of the *tet* promoter in pBR322. High level expression of the gene was observed only after a 0.7-kb fragment between the *tet* promoter and the *cat* gene was spontaneously deleted under chloramphenicol selection. Sequencing of the 0.7-kb DNA fragment revealed the presence of a short repeat sequence in the vector DNA and in the DNA sequence upstream of the *cat* gene; it was suggested that deletion of the intervening sequence occurred via homologous recombination between the two repeats (Murray *et al.*, 1988). Similar attempts to select for adventitious expression of the *S. venezuelae* cml^r gene(s) were unsuccessful.

II. Sequence analysis of the cloned DNA fragment

Nucleotide sequencing of the 2.4-kb *Kpn*I-*Sst*I fragment showed that it consisted of 2355 bp and possessed 73.6% G+C nucleotides. The high G+C content of the cloned DNA was consistent with an average 74% G+C content of *Streptomyces* genomes overall (Wright and Bibb, 1992).

The discovery of three complete open reading frames on the 2.4-kb *Kpn*I-*Sst*I fragment raised the possibility that chloramphenicol resistance in *S. lividans* transformants

RM3 and RM4 might be determined by more than one gene. Earlier studies of chloramphenicol resistance in these transformants had indicated that antibiotic resistance was mediated by chloramphenicol hydrolase activity (Mosher, 1986; Mosher *et al.*, 1990). However, the strong amino acid sequence similarity between Orf4 and the chloramphenicol resistance proteins CmlG and CmlR of *S. lividans* and *R. fascians*, suggested that resistance in RM3 and RM4 might also involve antibiotic efflux. Both CmlR and CmlG are putative integral membrane proteins, and both are thought to confer chloramphenicol resistance by promoting active efflux of the antibiotic from the cell (Desomer *et al.*, 1992; Dittrich *et al.*, 1991). Experiments, using everted membrane vesicles have confirmed this prediction for CmlG (H. Schrempf, personal communication) and it is likely, therefore, that Orf4, CmlR, and CmlG are functionally analogous.

Antibiotic efflux was first proposed as a mechanism for chloramphenicol resistance in the chloramphenicol-producing *S. venezuelae* 13s (Malik, 1970). The active efflux of chloramphenicol from the cell that would occur during biosynthesis of the antibiotic might function as one component of a permeability barrier. Recent work has shown that many putative antibiotic efflux protein genes are closely clustered with antibiotic production genes. Some of these genes are thought to function as export proteins, involved mainly in excretion of the completed antibiotic from the cell. Thus, Orf4 could be a component of a specific inducible chloramphenicol resistance mechanism of *S. venezuelae* ISP5230 (Doull, 1984) or merely a biosynthetic gene involved in export of the antibiotic from the cell.

The presence of an ATP/GTP binding site motif close to the amino terminus of Orf3 strongly suggested a biological process requiring a high energy co-factor such as a phosphotransferase reaction. Initial searches of the SWISSPROT and Genpept protein databases yielded no significant matches to the deduced amino acid sequence of *orf-3*. However, a search of these databases using the BLITZ alignment program showed that the amino acid sequence of Orf3 possessed a limited and highly localized similarity to the amino-termini of several ATP-requiring proteins. The amino acid sequences of ATP/GTP

binding proteins typically show only limited similarity to each other and this similarity is usually restricted to those amino acids involved in the nucleotide binding site (Saraste *et al.*, 1990).

The tandem arrangement of *orf-3* and *orf-4* on the 2.4-kb fragment suggested that these genes might be cotranscribed. Inspection of the DNA sequence upstream of the translational start site for *orf-4* did not detect any likely promoter sequences. However, because there are no consensus sequences for the majority of streptomycete promoters, the presence of a non $E\sigma^{70}$-like promoter cannot be discounted (Strohl, 1992). Examination of the DNA sequence immediately downstream of the translational stop codon for *orf-4* located an imperfect inverted repeat followed by a smaller but perfect inverted repeat. The free energies of formation for these inverted repeats indicated that they could form stable stem-loop structures, perhaps capable of causing rho-independent transcriptional termination. Therefore, *orf-3* and *orf-4* could be transcribed on separate transcripts.

Supporting this suggestion was the discovery of two $E\sigma^{70}$-like -10 hexamers upstream of each of the possible translational start sites for *orf-3*. One of these hexamers was located 10 bp upstream of the GTG start codon; if this hexamer was to act as a promoter for RNA polymerase binding, transcription and translation would most likely start at the same nucleotide, producing a leaderless transcript. Many streptomycete genes, especially antibiotic resistance and developmental genes, produce leaderless transcripts that are efficiently translated in streptomycete hosts despite lacking an obvious ribosome-binding site (Strohl, 1992). Work by Janssen and co-workers has suggested that such transcripts can also be translated in *E. coli* (Wu and Janssen, 1992). Interestingly, the ATG that constitutes a potential second translational start codon of *orf-3* is preceded by a possible Shine-Dalgarno sequence that is in turn preceded by a -10 hexamer. These observations could mean that *orf-3* possesses more than one promoter and perhaps more than one translational start site. A number of streptomycete genes, including resistance genes and genes involved in primary and secondary metabolic functions, have been

reported to have multiple promoters; the presence of such promoters for a gene may represent a requirement of the host cell to carefully regulate the level or timing of gene expression (Seno and Baltz, 1989; Strohl, 1992)

These observations might explain why chloramphenicol resistance was not detected when the 2.4-kb fragment was subcloned in *E. coli*. If *orf-3* is the resistance determinant, transcriptional read-through from the upstream *lac*-promoter would have been possible only with pJV8. However, transcription from the *lac*-promoter might have been blocked by the potential rho-independent transcriptional terminator located downstream of *orf-4*. Furthermore, although there are -10 consensus hexamers upstream of *orf-3*, they lack the corresponding -35 consensus hexamers that make up the binding site for the $E\sigma^{70}$ RNA polymerase.

III. Identification of compound X

HPLC analysis suggested that cmpd.X was the major product of chloramphenicol metabolism in *S. lividans* RM3 and RM4 cultures. It also suggested that cmpd.X was a very acidic metabolite. The acidic properties of cmpd.X were exploited in the two methods used to purify it. Compound X was successfully bound to QAE-Sephadex and could be completely eluted from the anion exchanger with 1 *M* ammonium formate (pH 2.5). However, the preferred method of purification was C18 reversed phase chromatography, primarily because cmpd.X could be recovered in a salt-free aqueous solution.

Both ^{1}H-NMR and ^{13}C-NMR spectroscopy indicated that cmpd.X was structurally very similar to chloramphenicol. However, the complexity of the signals for the carbon-1' protons and the downfield shift of the carbon-1' signal, suggested the presence of an electron-withdrawing group attached to carbon-1'. Although the most common mechanism of chloramphenicol resistance is by acetylation of C-1', the carbon-spectrum of cmpd.X failed to show chemical shifts consistent with this type of modification.

Concurrent with these experiments a collaborative investigation by D. Camp at the University of Leicester showed that chloramphenicol added to unsupplemented cell lysates of *S. lividans* RM4 was converted at a low rate to cmpd.X. However, addition to the lysates of Mg^{2+} and ATP, alone among a number of co-factors, increased the conversion rate by a factor of four. When considered with the discovery of an ATP/GTP binding site motif in the deduced amino acid sequence of *orf-3* this result suggested that cmpd.X might be the product of kinase activity. A combination of ^{31}P-NMR and mass spectroscopy established that the electron-withdrawing moiety on carbon-1' of cmpd.X was indeed a phosphate group.

To test the hypothesis that the insert of pJV3 and pJV4 encodes a chloramphenicol kinase that specifically inactivates chloramphenicol by catalyzing O-phosphorylation at carbon-1', cell extracts of RM4 have been supplemented with [γ-^{32}P]ATP and incubated with chloramphenicol, 1-deoxychloramphenicol, or 3-deoxychloramphenicol. By TLC and autoradiography, the radioactive product from chloramphenicol was identified as compd.X. The 3'-deoxychloramphenicol was almost completely converted to a radio-active product, whereas only 10% of the 1'-deoxychloramphenicol was converted (D. Camp, personal communication). Thus, the chloramphenicol kinase activity in RM4 cell extracts preferentially catalyzes O-phosphorylation of chloramphenicol at carbon-1' and also to a lesser extent at carbon-3' (Fig. 31).

IV. Chloramphenicol kinase

Further evidence that the ATP/GTP binding site motif in the deduced amino acid sequence of *orf-3* is functional and that *orf-3* encodes the chloramphenicol kinase activity detected in cell extracts of *S. lividans* RM4 has been obtained by digesting pJV7 with *Sma*I (D. Camp, personal communication). The enzyme cuts twice within the 2.4-kb insert of pJV7, producing a 1.67-kb *Sma*I-*Sma*I fragment carrying the intact *orf-3* sequence and portions of *orf-4* and *orf-2* (see Fig. 8). The *Sma*I-*Sma*I fragment has been subcloned in

Figure 31: The proposed reaction catalyzed by chloramphenicol kinase.

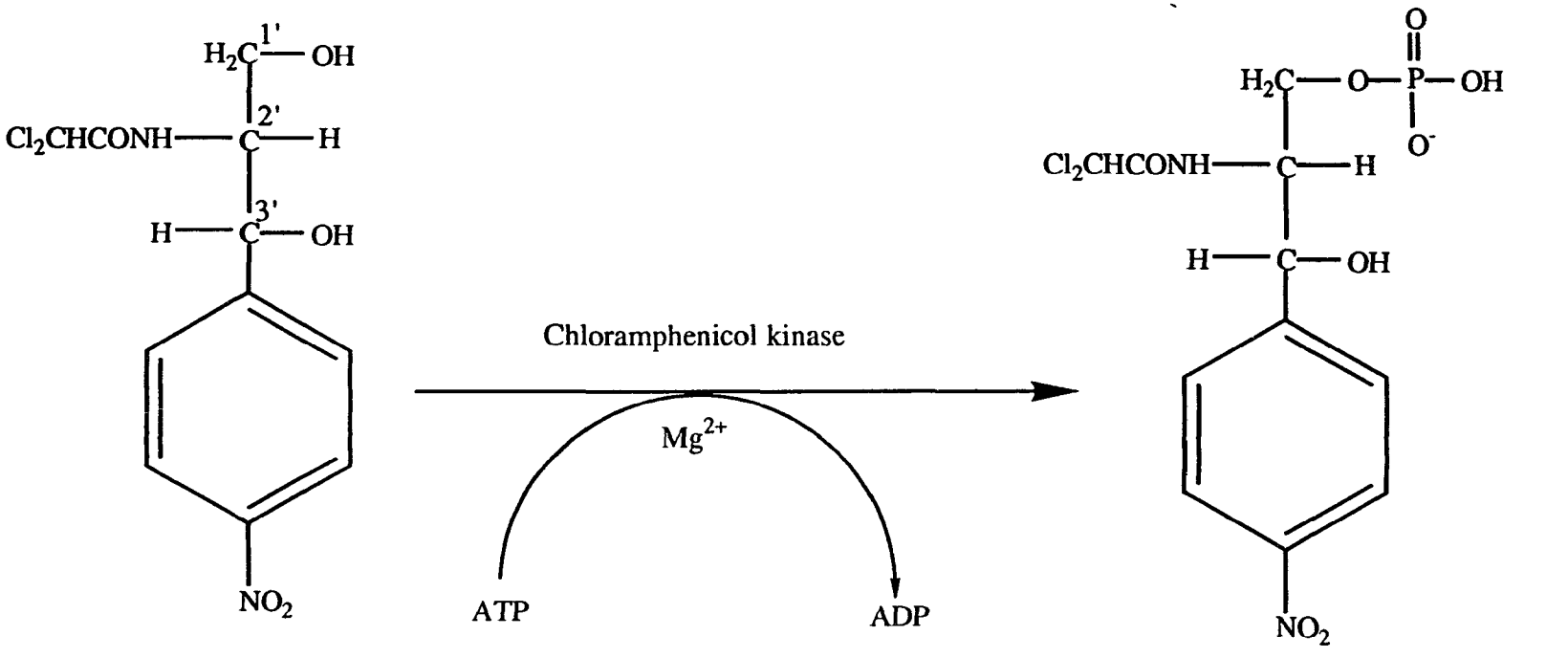

H2C 1' OH
Cl2CHCONH C 2' H
H C 3' OH
NO2
Chloramphenicol
Chloramphenicol kinase
Mg2+
ATP
ADP
O
H2C O P OH
O-
Cl2CHCONH C H
H C OH
NO2
Chloramphenicol-1'-phosphate

Figure 31

the *E. coli* vector pUC19 to create the recombinant plasmid pPD16 A shuttle vector capable of replicating in *E. coli* and *S. lividans*, and carrying *orf-3* downstream of a strong streptomycete promoter was formed by ligating pPD16 to a streptomycete vector carrying a thiostrepton inducible promoter (*tip*). Transformants of *S. lividans* M252 containing this plasmid (pPD16/tip) were all chloramphenicol resistant. The mycelium from cultures of pPD16/tip transformants showed comparable chloramphenicol kinase activity to that of RM4. These results strengthen the evidence that *orf-3* encodes a chloramphenicol kinase activity.

The 2.4-kb insert of pJV7 was digested with *Bst*EII and *Sst*I (D. Camp, personal communication), *Bst*EII cuts between nucleotides 1410 and 1411, immediately upstream of, and adjacent to the GTG translational start codon of *orf-3* (see Fig. 13) The resulting 0.95-kb *Bst*EII-*Sst*I fragment was treated with Klenow fragment to fill in the cohesive ends and the blunt-ended fragment was ligated to a pUC18 derivative carrying the *tac* promoter (a hybrid of the *E. coli lac* and *trp* promoters) and a good rbs The vector had been linearized with *Nco*I at a site directly downstream of the rbs, and then treated with Klenow fragment to create blunt ends; this treatment produced an in frame ATG codon at the 3' terminus of the filled in restriction site The mixture obtained by ligating the vector to the blunt-ended *Bst*EII-*Sst*I fragment was used to transform *E. coli* JM101. Transformants containing a plasmid, pDC13, in which *orf-3* was aligned immediately downstream of the *tac* promoter were resistant to 20 $\mu g.mL^{-1}$ chloramphenicol and yielded cell extracts exhibiting chloramphenicol kinase activity From this evidence it seems likely that *orf-3* alone encodes chloramphenicol kinase activity and confers chloramphenicol resistance. However, amino-terminal sequencing of the purified chloramphenicol kinase protein should unambiguously identify which open reading frame encodes the chloramphenicol kinase protein.

V. Chloramphenicol kinase and chloramphenicol resistance

The results obtained in this investigation indicate that chloramphenicol resistance in the *S. lividans* transformants RM3 and RM4 is mediated by a chloramphenicol kinase. Sequence analysis of the cloned 2.4-kb *Kpn*I-*Sst*I fragment of *S. venezuelae* DNA in pJV4 has revealed the presence of two open reading frames, *orf-3* and *orf-4*, encoding putative chloramphenicol kinase and efflux proteins, respectively. The role of *orf-4* in the resistance mechanism is uncertain since some results have suggested that *orf-3* alone is sufficient to confer chloramphenicol resistance. In addition, expression of *orf-4* may not be essential.

D Camp (personal communication) has introduced a frame-shift mutation in *orf-4* by digesting pJV4 at its sole *Eco*RI site (between nucleotides 153 and 154; see Fig. 8) and filling in the cohesive ends using Klenow fragment; because the *Eco*RI site is located very close to the 5' end of *orf-4*, a frame-shift mutation at this point should result in a totally aberrant protein product. When the blunt-ended plasmid was ligated and used to transform *S. lividans* M252, all of the transformants were chloramphenicol resistant. Since the plasmid DNA extracted from these transformants was not digested by *Eco*RI, it presumably contained the mutation; therefore, *orf-4* either has an alternative translational start site downstream of the *Eco*RI site, or its expression is not essential for chloramphenicol resistance.

The 1.67-kb *Sma*I-*Sma*I fragment subcloned in pPD16 still conferred chloramphenicol resistance on M252, although it was missing the first 375 bp of *orf-4*. Furthermore, the *Bst*EII-*Sst*I fragment, subcloned in pDC13, lacked *orf-4* altogether and yet still conferred chloramphenicol resistance on *E. coli*. However, chloramphenicol kinase appears to be an intracellular enzyme whereas nearly all of the choramphenicol-1'-phosphate produced by cultures of RM3 and RM4, was found in the culture supernatants. It may be that Orf4 transports chloramphenicol-1'-phosphate out of the cell. This hypothesis could

be tested by measuring the amount of chloramphenicol-1'-phosphate that accumulates in a culture supernatant of *S. lividans* M252 containing pPD16.

The role of chloramphenicol kinase in *S. venezuelae* ISP5230 is worthy of further investigation. One function might be to phosphorylate potentially toxic intermediates of chloramphenicol biosynthesis. Chloramphenicol-1'-phosphate may be the final product of the biosynthetic process, a hypothesis that implies the presence of an extracellular chloramphenicol-activating enzyme, perhaps analogous to the streptomycin-phosphate phosphatase produced by *S. griseus* (Mansouri and Piepersberg, 1991). An investigation of the fate of [U-^{14}C]chloramphenicol added to cultures of *S. venezuelae* 13s has shown that a large proportion of the radioactive metabolites formed are poorly extracted by ethyl acetate, and then only under acidic conditions. A preliminary TLC analysis of these metabolites has shown one dominant zone of radioactivity; this exhibits chromatographic properties similar to those of cmpd.X (L.C. Vining, personal communication). If the identity of this product is confirmed, it will implicate phosphorylation as an important resistance mechanism in the producing species.

The discovery of a putative chloramphenicol efflux protein gene directly upstream of a chloramphenicol kinase gene suggests that these genes might be part of the chloramphenicol biosynthetic gene cluster. This possibility could be explored by subcloning the 2.4-kb *Kpn*I-*Sst*I fragment of pJV4 into the segregationally unstable shuttle vector pHJL400 (Larson and Hershberger, 1986). Use of the recombinant plasmid to transform auxotrophically marked strains of *S. venezuelae* ISP5230 should be followed by integration of the plasmid into the chromosome by homologous recombination. This should permit the mapping of the vector-encoded thiostrepton resistance (*tsr*) gene by either conjugation or phage SV1-mediated transduction (Paradkar *et al.*, 1993). Since the chloramphenicol biosynthesis genes have previously been mapped between the *cys* and *pdx* genes on the *S. venezuelae* chromosome (Vats *et al.*, 1987), it should be possible to locate the position of the *tsr* gene in relation to these genes. The initial steps towards this goal

have been achieved through construction of the recombinant plasmid pJV11 by insertion of the 2.4-kb *Kpn*I-*Sst*I fragment from pJV7 into pHJL400.

SUMMARY AND CONCLUSIONS

The chloramphenicol resistance determinant of pJV3 was localized in pJV4 as a 2.4-kb *Kpn*I-*Sst*I fragment of *S. venezuelae* DNA by digesting pJV3 DNA with *Kpn*I and *Sst*I. The restriction fragments produced were ligated and used to transform the chloramphenicol-hypersensitive strain *S. lividans* M252. Selection and examination of chloramphenicol-resistant transformants established that all possessed the 7.1-kb plasmid, pJV4. Restriction analysis showed that a 5.2-kb *Kpn*I-*Sst*I fragment of pJV3, consisting of 4.1 kb of *S. venezuelae* DNA and 1.1 kb of vector DNA, had been deleted *in vitro* during the digestion and ligation reactions.

The nucleotide sequence of both strands of the 2.4-kb *Kpn*I-*Sst*I fragment was determined. The DNA fragment contained of 2355 bp, and had an overall G+C content of 73.6%. CODONPREFERENCE analysis of the sequenced DNA predicted the presence of three complete open reading frames and one incomplete open reading frame. The largest of the open reading frames, *orf-4*, consisted of a 1238-bp sequence encoding a polypeptide (Orf4) of 412 amino acids with a deduced M_r of 41,479. The deduced amino acid sequence of Orf4 showed significant similarity to the deduced amino acid sequences of the choramphenicol resistance proteins CmlG and CmlR from *S. lividans* and *R. fascians*, respectively; both CmlG and CmlR are putative chloramphenicol efflux proteins. Significant similarity was also seen with various other antibiotic resistance proteins believed to function as antibiotic efflux pumps. The amino acid sequence of Orf4 was extremely hydrophobic, and a Kyte-Doolittle hydropathy plot suggested that Orf4 could form 12 α-helical transmembrane segments.

Located immediately downstream of *orf-4* was *orf-3*. It consisted of 536 bp and encoded a polypeptide (Orf3) of 178 amino acids with a deduced M_r of 18,804. The amino acid sequence of Orf3 showed no overall similarity to any other protein in current databases; however, it did show localized similarity to a number of proteins that require nucleotide co-factors. A sequence of amino acids fitting the conserved ATP/GTP binding

site "motif A" was identified near the amino terminus of Orf3, and suggested a biological process such as a phosphotransferase activity requiring a high energy co-factor. The last complete open reading frame, *orf-2*, was located downstream of *orf-3* but on the opposite DNA strand. It consisted of 269 bp and encoded a polypeptide (Orf2) of 89 amino acids with a deduced M_r of 9,762. The amino acid sequence of Orf2 showed no significant similarity to that of any database protein. The incomplete open reading frame, *orf-1*, consisted of the first 75 bp adjacent to the *Sst*I terminus of the cloned 2.4-kb fragment. It encoded the final 24 amino acids of an open reading frame that was only partially cloned on the original 6.5-kb fragment of *S. venezuelae* DNA.

Streptomyces lividans transformants RM3 and RM4 both rapidly metabolized chloramphenicol to one major product, compound X. HPLC analysis suggested that compound X was an acidic metabolite containing an aromatic nitro group. Failure of the metabolite to bind to the cation exchanger SP-Sephadex suggested that it lacked a free amino group. Compound X was purified to near homogeneity by reversed phase chromatography ^{1}H-NMR and ^{13}C-NMR spectroscopy showed that it was structurally very similar to chloramphenicol, but differed in possessing an electron-withdrawing group attached to carbon-1'. The discovery of an ATP/GTP binding site motif near the amino terminus of Orf3, suggested that the electron-withdrawing group attached to carbon-1' might be a phosphate group A ^{31}P-NMR spectrum of compound X supported this deduction, and a low resolution mass spectrum confirmed the identity of compound X as chloramphenicol-1'-phosphate. It was concluded that *S. lividans* RM3 and RM4 possess an intracellular chloramphenicol kinase activity that inactivates chloramphenicol by O-phosphorylation. The presence of an ATP/GTP binding site motif in the derived amino acid sequence of *orf-3*, indicated that it encodes the chloramphenicol kinase protein.

REFERENCES

Baldwin, S.A. (1992). Mechanisms of active and passive transport in a family of homologous sugar transporters found in both prokaryotes and eukaryotes. In *Molecular aspects of transport proteins*. Edited by J.J.H.H.M. De Pont. New York: Elsevier Science Publishers B.V.; p. 169-217.

Bibb, M.J., P.R. Findlay, and M.W. Johnson (1984). The relationship between base composition and codon usage in bacterial genes and its use for simple and reliable identification of protein-coding sequences. Gene **30**: 157-166.

Bissonnette, L., S. Champetier, J.-P. Buisson, and P.H. Roy (1991). Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In*4* integron of Tn*1696*: similarity of the product to transmembrane transport proteins. J. Bacteriol. **173**(14): 4493-4502.

Brenner, S. (1988). The molecular evolution of genes and proteins: a tale of two serines. Nature **334**: 528-530.

Brown, M.P. (1991). Subcloning and partial sequencing of the chloramphenicol hydrolase gene of *Streptomyces venezuelae*. Honours thesis; Dept. of Biology, Dalhousie University.

Butler, M.J., E.J. Friend, I.S. Hunter, F.S. Kaczmarek, D.A. Sugden, and M. Warren (1989). Molecular cloning of resistance genes and architecture of a linked gene cluster involved in biosynthesis of oxytetracycline by *Streptomyces rimosus*. Mol. Gen. Genet. **215**: 231-238.

Caballero, J.L., E. Martinez, F. Malpartida, and D.A. Hopwood (1991) Organisation and functions of the *actVA* region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor*. Mol. Gen. Genet. **230**: 401-412.

Calcutt, M.J. and F.J. Schmidt (1991). Bleomycin biosynthesis: characterization of the resistance region of the producer streptomycete. Abstract P1-43 International symposium on biology of actinomycetes. August 11-16, 1991; University of Wisconsin, Madison, WI.

Chater, K.F. (1992) Genetic regulation of secondary metabolic pathways in *Streptomyces* In *Secondary metabolites: their function and evolution* Edited by D J Chadwick and J Whelan Ciba Foundation Rexdale, Ontario John Wiley & Sons (Canada) Ltd , p 144-162

Coque, J.J.R., P. Liras, and J.F. Martín (1993) Genes for a β-lactamase, a penicillin-binding protein and a transmembrane protein are clustered with the cephamycin biosynthetic genes in *Nocardia lactamdurans* EMBO J **12**(2) 631-639

Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering Nucleic Acids Res **16**(22) 10881-10890

Cundliffe, E. (1989) How antibiotic-producing organisms avoid suicide Annu Rev Microbiol **43** 207-233

Cundliffe, E. (1990) Recognition sites for antibiotics within rRNA In *The ribosome: structure, function, and evolution* Edited by W E Hill, A Dahlberg, R A Garrett, P B Moore, D Schlessinger, and J R Warner Washington, D C American Society for Microbiology, p 479-490

Cundliffe, E. (1992) Self-protection mechanisms in antibiotic producers In *Secondary metabolites: their function and evolution* Edited by D J Chadwick and J Whelan Ciba Foundation Rexdale, Ontario John Wiley & Sons (Canada) Ltd , p 199-214

Davies, J. (1986) A new look at antibiotic resistance FEMS Microbiol Rev **39** 363-371

Davies, J. (1992) Another look at antibiotic resistance J Gen Microbiol **138** 1553-1559

Davies, J.E. and M. Yagisawa (1983) The aminocyclitol glycosides (aminoglycosides) In *Biochemistry and genetic regulation of commercially important antibiotics* Edited by L C Vining Don Mills, Ontario Addison-Wesley Publishing Company, p 329-354

Davis, B.D. (1987) Mechanism of bactericidal action of aminoglycosides Microbiol Rev **51**(3). 341-350

Desomer, J., D. Vereecke, M. Crespi, and M. Van Montagu (1992) The plasmid-encoded chloramphenicol-resistance protein of *Rhodococcus fascians* is homologous to the transmembrane tetracycline efflux proteins Mol Microbiol **6**(16) 2377-2385

Distler, J., A. Ebert, K. Mansouri, K. Pissowotzki, M. Stockmann, and W. Piepersberg (1987). Gene cluster for streptomycin biosynthesis in *Streptomyces griseus* nucleotide sequence of three genes and analysis of transcriptional activity Nucleic Acids Res **15**(19) 8041-8056

Distler, J., K. Mansouri, G. Mayer, M. Stockmann, and W. Piepersberg (1992) Streptomycin biosynthesis and its regulation in streptomycetes Gene **115** (1 and 2) 105-111

Dittrich, W., M. Betzler, and H. Schrempf (1991) An amplifiable and deletable chloramphenicol-resistance determinant of *Streptomyces lividans* 1326 encodes a putative transmembrane protein Mol Microbiol **5**(11) 2789-2797

Doran, J.L., B.K. Leskiw, S. Aippersbach, and S.E. Jensen (1990) Isolation and characterization of a β-lactamase-inhibitory protein from *Streptomyces clavuligerus* and cloning and analysis of the corresponding gene J Bacteriol **172**(9) 4909-4918

Doull, J., Z. Ahmed, C. Stuttard, and L.C. Vining (1985) Isolation and characterization of *Streptomyces venezuelae* mutants blocked in chloramphenicol biosynthesis J. Gen Microbiol **131** 97-104

Doull, J.L. (1984) Genetic studies of chloramphenicol biosynthesis in *Streptomyces* Ph D thesis, Dept of Microbiology, Dalhousie University

Endicott, J.A. and V. Ling (1989) The biochemistry of P-glycoprotein mediated multidrug resistance Annu Rev Biochem **58** 137-171

Forsman, M., B. Häggström, L. Lindgren, and B. Jaurin (1990) Molecular analysis of β-lactamases from four species of *Streptomyces*. comparison of amino acid sequences with those of other β-lactamases J Gen Microbiol **136** 589-598

Gale, E.F., E. Cundliffe, P.E. Reynolds, M.H. Richmond, and M.J. Waring (1981) *The molecular basis of antibiotic action* Second edition Toronto John Wiley & Sons Ltd

Ghuysen, J.-M. (1991) Serine β-lactamases and penicillin-binding proteins Annu Rev Microbiol **45** 37-67

Gil, J.A., H.M. Kieser, and D.A. Hopwood (1985) Cloning of a chloramphenicol acetyltransferase gene of *Streptomyces acrimycini* and its expression in *Streptomyces* and *Escherichia coli* Gene **38** 1-8

Gottlieb, D., P.W. Robbins, and H.E. Carter (1956) The biosynthesis of chloramphenicol II Acetylation of *p*-nitrophenylserinol J Bacteriol **72** 153-156

Gribskov, M., J. Devereux, and R.R. Burgess (1984) The codon preference plot graphic analysis of protein coding sequences and prediction of gene expression Nucleic Acids Res **12** 539-549

Guilfoile, P.G. and C.R. Hutchinson (1991) A bacterial analog of the *mdr* gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin Proc Natl Acad Sci USA **88** 8553-8557

Guilfoile, P.G. and C.R. Hutchinson (1992) Sequence and transcriptional analysis of the *Streptomyces glaucescens tcmAR* tetracenomycin C resistance and repressor gene loci J Bacteriol **174**(11) 3651-3658

Hancock, R.E.W. (1987) Role of porins in outer membrane permeability J Bacteriol **169**(3) 929-933

Heinzel, P., O. Werbitzky, J. Distler, and W. Piepersberg (1988) A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin-3"-phospho-

transferase relationships between antibiotic and protein kinases Arch Microbiol **150** 184-192

Hopwood, D.A., M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, H.M. Kieser, D.J. Lydiate, C.P. Smith, J.M. Ward, and H. Schrempf (1985) *Genetic manipulation of Streptomyces: a laboratory manual* Norwich The John Innes Foundation

Horinouchi, S. and T. Beppu (1992) Regulation of secondary metabolism and cell differentiation in *Streptomyces* A-factor as a microbial hormone and the AfsR protein as a component of a two-component regulatory system Gene **115**(1 and 2) 167-172

Jähnig, F. (1990) Structure predictions of membrane proteins are not that bad TIBS **15** 93-95

Jaurin, B., M. Forsman, B. Häggström, and L. Lindgren (1988) Molecular aspects of extracellular β-lactamases of *Streptomyces* In *Proceedings of seventh international symposium on biology of actinomycetes* Edited by Y Okami, T Beppu, and H Ogawara Tokyo Japan Scientific Societies Press, p 457-462

Jensen, S.E. (1986) Biosynthesis of cephalosporins CRC Crit Rev Biotechnol **3** 277-301

Karger, B.D. and J. Jessee (1990) Preparation of single strand DNA from phagemids Focus **12** 28-29

Kieser, T. (1984) Factors affecting the isolation of cccDNA from *Streptomyces lividans* and *Escherichia coli* Plasmid **12** 19-36

Kieser, T. and D.A. Hopwood (1991) Genetic manipulation of *Streptomyces* integrating vectors and gene replacement Methods Enzymol **204** 430-458

Kirby, R. (1990) Evolutionary origin of aminoglycoside phosphotransferase resistance genes. J Mol Evol **30** 489-492

Knoch, M., K.-H. Van Pée, L.C. Vining, and F. Lingens (1989) Purification, properties and immunological detection of a bromoperoxidase-catalase from *Streptomyces*

venezuelae and from a chloramphenicol-nonproducing mutant J Gen Microbiol **135** 2493-2502

Kumada, Y., H. Anzai, E. Takano, T. Murakami, O. Hara, R. Itoh, S. Imai, A. Satoh, and K. Nagaoka (1988) The bialaphos resistance gene (*bar*) plays a role in both self-defense and bialaphos biosynthesis in *Streptomyces hygroscopicus* J Antibiot **41**(12) 1838-1845

Kyte, J. and R.F. Doolittle (1982). A simple model for displaying the hydropathic character of a protein J Mol Biol **157** 105-132

Lacalle, R.A., J.A. Tercero, and A. Jiménez (1992) Cloning of the complete biosynthetic gene cluster for an aminonucleoside antibiotic, puromycin, and its regulated expression in heterologous hosts EMBO J **11**(2) 785-792

Lambert, H.P. and F.W. O'Grady (1992) *Antibiotic and Chemotherapy* Sixth Edition New York Churchill Livingstone, p 2-5 and p 136-139

Larson, J.L. and C.L. Hershberger (1986) The minimal replicon of a streptomycete plasmid produces an ultrahigh level of plasmid DNA Plasmid **15** 199-209

Levy, S B. (1992) Active efflux mechanisms for antimicrobial resistance Antimicrob Agents Chemother **36**(4) 695-703

Lipman, D.J. and W.R. Pearson (1985) Rapid and sensitive protein similarity searches Science **227** 1435-1441

Malik, V.S. (1970) Chloramphenicol resistance in the producing streptomycete Ph D thesis, Dept of Biology, Dalhousie University

Malik, V.S. (1983) Chloramphenicol In *Biochemistry and genetic regulation of commercially important antibiotics* Edited by L C Vining Don Mills, Ontario Addison-Wesley Publishing Company, p 293-309

Malik, V.S. and L.C. Vining (1970) Metabolism of chloramphenicol by the producing organism Can J Microbiol **16** 173-180

Malik, V.S. and L.C. Vining (1971). Metabolism of chloramphenicol by the producing organism. Some properties of chloramphenicol hydrolase. Can. J. Microbiol. **17**: 1287-1290.

Malik, V.S. and L.C. Vining (1972). Chloramphenicol resistance in a chloramphenicol-producing *Streptomyces*. Can. J. Microbiol. **18**: 583-590.

Marsouri, K. and W. Piepersberg (1991). Genetics of streptomycin production in *Streptomyces griseus*: nucleotide sequence of five genes, *strFGHIK*, including a phosphatase gene. Mol. Gen. Genet. **228**: 459-469.

Marck, C. (1988). 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple MacIntosh family of computers. Nucleic Acids Res. **16**(5): 1829-1836

Marconi, R.T., J.S. Lodmell, and W.E. Hill (1990). Identification of a rRNA/chloramphenicol interaction site within the peptidyltransferase centre of the 50S subunit of the *Escherichia coli* ribosome. J. Biol. Chem. **265**(14): 7894-7899.

Mazodier, P. and J. Davies (1991). Gene transfer between distantly related bacteria. Annu. Rev. Genet. **25**: 147-171.

Medeiros, A.A. (1989). Plasmid-determined beta-lactamases In *Handbook of experimental pharmacology*. Vol. 91: *Microbial resistance to drugs* Edited by L.E. Bryan. New York: Springer-Verlag; p. 101-127.

Mosher, R.H. (1986). Molecular cloning of a chloramphenicol resistance gene from *Streptomyces venezuelae*. M.Sc. thesis; Dept. of Biology, Dalhousie University.

Mosher, R.H., N.P. Ranade, H. Schrempf, and L.C. Vining (1990). Chloramphenicol resistance in *Streptomyces*: cloning and characterization of a chloramphenicol hydrolase gene from *Streptomyces venezuelae*. J. Gen. Microbiol. **136**: 293-301.

Murray, I.A., J.A. Gil, D.A. Hopwood, and W.V. Shaw (1989) Nucleotide sequence of the chloramphenicol acetyltransferase gene of *Streptomyces acrimycini* Gene **85** 283-291

Neal, R.J. and K.F. Chater (1987). Nucleotide sequence analysis reveals similarities between proteins determining methylenomycin A resistance in *Streptomyces* and tetracycline resistance in eubacteria Gene **58** 229-241

Neu, H.C. (1992) The crisis in antibiotic resistance Science **257** 1064-1072

Nikaido, H. (1989) Role of the outer membrane of Gram-negative bacteria in antimicrobial resistance In *Handbook of experimental pharmacology* Vol 91 *Microbial resistance to drugs* Edited by L E Bryan New York Springer-Verlag, p 1-34

Nikaido, H. and M.H. Saier Jr. (1992) Transport proteins in bacteria common themes in their design Science **258** 936-942

Ogawara, H. (1993) Sequence of a gene encoding β-lactamase from *Streptomyces cellulosae* Gene **124** 111-114

Ogawara, H. (1981) Antibiotic resistance in pathogenic and producing bacteria, with special reference to β-lactam antibiotics Microbiol Rev **45**(4) 591-619

Ogawara, H. and S. Horikawa (1980) Penicillin-binding proteins of *Streptomyces cacaoi*, *Streptomyces olivaceus*, and *Streptomyces clavuligerus* Antimicrob Agents Chemother **17**(1) 1-7

Paradkar, A.S., C. Stuttard, and L.C. Vining (1993) Location of the genes for anthranilate synthase in *Streptomyces venezuelae* ISP5230 genetic mapping after integration of the cloned genes. J Gen. Microbiol. **139** 687-694

Paulsen, I.T. and R.A. Skurray (1993). Topology, structure, and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes - an analysis. Gene **124** 1-11

Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA **85** 2444-2448

Pongs, O. (1979) Chloramphenicol In *Antibiotics, Vol. V / Part 1: Mechanism of action of antibacterial agents* Edited by F E Hahn New York Springer-Verlag; p. 26-42

Pulido, D. and A. Jiménez (1987) Optimization of gene expression in *Streptomyces lividans* by a transcriptional terminator Nucleic Acids Res **15**(10) 4227-4240

Raibaud, A., M. Zalacain, T.G. Holt, R. Tizard, C.J. Thompson (1991) Nucleotide sequence analysis reveals linked N-acetyl hydrolase, thioesterase, transport, and regulatory genes encoded by the bialaphos biosynthetic gene cluster of *Streptomyces hygroscopicus* J Bacteriol **173**(14) 4454-4463

Reithmeier, R.A.F. and C.M. Deber (1992) Intrinsic membrane protein structure principles and prediction In *The structure of biological membranes* Edited by P. Yeagle London CRC Press, Inc , p 337-393

Russell, A.D. and I. Chopra (1990) *Understanding antibacterial action and resistance*. Toronto Ellis Horwood Ltd

Saier, M.H. Jr. (1992) Mechanisms of carbohydrate transport in bacteria and comparisons with those in eukaryotes In *The structure of biological membranes* Edited by P Yeagle London CRC Press, Inc , p 833-891

Sambrook, J., E.F. Fritsch, T. Maniatis (1989) *Molecular cloning a laboratory manual* Second edition Cold Spring Harbor Cold Spring Harbor Laboratory Press.

Saraste, M., P.R. Sibbald, and A. Wittinghofer (1990) The P-loop - a common motif in ATP- and GTP-binding proteins TIBS **15** 430-434

Schoner, B., M. Geistlich, P. Rosteck Jr., R.N. Rao, E. Seno, P. Reynolds, K. Cox, S. Burgett, and C. Hershberger (1992) Sequence similarity between macrolide-resistance determinants and ATP-binding transport proteins Gene **115**(1 and 2) 93-96

Seno, E.T. and R.H. Baltz (1989) Structural organization and regulation of antibiotic biosynthesis and resistance genes in actinomycetes In *Regulation of secondary metabolism in actinomycetes* Edited by S Shapiro Boca Raton CRC Press Inc , p 1-48.

Shaw, K.J., P.N. Rather, R.S. Hare, and G.H. Miller (1993) Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes Microbiol Rev **57**(1) 138-163

Shaw, W.V. (1983) Chloramphenicol acetyltransferase enzymology and molecular biology CRC Crit Rev Biochem **14**(4) 1-46

Shaw, W.V. and D.A. Hopwood (1976) Chloramphenicol acetylation in *Streptomyces* J Gen Microbiol **94** 159-166

Shaw, W.V. and A.G.W. Leslie (1989) Chloramphenicol acetyltransferases In *Handbook of experimental pharmacology* Vol 91 *Microbial resistance to drugs* Edited by L E Bryan New York Springer-Verlag, p 313-324

Shaw, W.V. and A.G.W. Leslie (1991) Chloramphenicol acetyltransferase Annu Rev Biophys Biophys Chem **20** 363-386

Silver, L.L. and K.A. Bostian (1993) Discovery and development of new antibiotics the problem of antibiotic resistance Antimicrob Agents Chemother **37**(3) 377-383

Silver, S., G. Nucifora, L. Chu, and T.K. Misra (1989) Bacterial resistance ATPases primary pumps for exporting toxic cations and anions TIBS **14** 76-80

Silver, S. and M. Walderhaug (1992) Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria Microbiol Rev **56**(1) 195-228

Stanzak, R., P. Matsushima, R.H. Baltz, and R.N. Rao (1986) Cloning and expression in *Streptomyces lividans* of clustered erythromycin biosynthesis genes from *Streptomyces erythreus* Bio/technol **4** 229-232

Strohl, W.R. (1992) Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20**(5): 961-974

Tinoco Jr., I., P.N. Borer, B. Dengler, M.D. Levine, O.C. Uhlenbeck, D.M. Crothers, and J. Gralla (1973). Improved estimation of secondary structure in ribonucleic acids Nature (New Biology) **246**: 40-41.

Trieu-Cuot, P., M. Arthur, and P. Courvalin (1987). Origin, evolution and dissemination of antibiotic resistance genes. Microbiol. Sci. **4**(9): 263-266.

Turner, D.H., N. Sugimoto, J.A. Jaeger, C.E. Longfellow, S.M. Freier, and R. Kierzek (1987). Improved parameters for prediction of RNA structure. Cold Spring Harbor Symposia on Quantitative Biology. **52**: 123-133.

Vats, S., C. Stuttard, and L.C. Vining (1987). Transductional analysis of chloramphenicol biosynthesis genes in *Streptomyces venezuelae*. J. Bacteriol. **169**(8): 3809-3813

Vining, L.C. and D.W.S. Westlake (1984). Chloramphenicol: properties, biosynthesis and fermentation. In *Biotechnology of Industrial Antibiotics*. Edited by E.J. Vandamme. New York: Marcel Dekker; p. 387-409

Waley, S.G. (1988). β-Lactamases: a major cause of antibiotic resistance. Sci. Prog. Oxf. **72** 579-597.

Walker, J.E., M. Saraste, M.J. Runswick, and N.J. Gay (1982). Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1**(8). 945-951

Wright, F. and M.J. Bibb (1992). Codon usage in the G+C-rich *Streptomyces* genome. Gene **113**: 55-65.

Wu, C.-J. and G.E. Janssen (1992). *Streptomyces* viomycin phosphotransferase (*vph*) gene can be translated from a message lacking its 5' leader sequence in both *Streptomyces lividans* and *Escherichia coli*. Program and Abstracts. Fifth ASM conference

on genetics and molecular biology of industrial microorganisms. October 11-15, 1992. Bloomington, Indiana, USA.

Yunis, A.A. (1988). Chloramphenicol: relation of structure to activity and toxicity. Annu. Rev. Pharmacol. Toxicol. **28**: 83-100.

Zhang, H.-Z., H. Schmidt, and W. Piepersberg (1992). Molecular cloning and characterization of two lincomycin-resistance genes, *lmrA* and *lmrB*, from *Streptomyces lincolnensis* 78-11. Mol. Microbiol. **6**(15): 2147-2157.