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ETAMYCIN RESISTANCE IN *STREPTOMYCES*

BY

KEQIAN YANG

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

at

Dalhousie University

Halifax, Nova Scotia

July, 1993

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ABSTRACT

Streptomyces griseoviridus showed a low level of etamycin resistance before the onset of etamycin production, and a higher level of resistance after its onset.

Streptomyces griseofuscus was chosen as the host for expression of cloned etamycin resistance determinants. It was inherently sensitive to etamycin, but exhibited low-level resistance after induction by the antibiotic. A search for *ermE*-hybridizing DNA in *S. griseoviridus* identified *Sal*I fragments of 0.6 and 4.3 kb. The 4.3-kb fragment was cloned within a 17.4-kb segment of genomic DNA and the *ermE*-hybridizing region was sequenced. The 0.6-kb fragment was not present on the 17.4-kb segment and was not investigated. A region within the cloned 4.3-kb fragment showed a high degree of nucleotide sequence similarity to *ermE*, but did not encode an ErmE-like product. When introduced into *S. griseofuscus* in the *Streptomyces-E. coli* shuttle vector pHJL400, the 17.4-kb fragment conferred significantly higher etamycin resistance than was observed in transformants containing pHJL400 alone. The resistance phenotype associated with the 17.4-kb fragment was conferred in part by a 2.1-kb sub-fragment. By sequencing this sub-fragment, two intact ORFs were identified. Database searches failed to associate the ORF products with any known resistance genes. Overall, the results suggested that the elevated etamycin resistance conferred by the 17.4-kb fragment and by several other cloned DNA fragments was due to activation of an indigenous etamycin resistance gene in the host.

Streptomyces lividans was shown to possess an etamycin-inactivating enzyme. *S. griseofuscus* showed "temporal" resistance to erythromycin and lincomycin.

LIST OF ABBREVIATIONS

aa	amino acid
bp	base pair
BSA	bovine serum albumin
ccc	covalently closed circular
cfu	colony forming unit
cpm	counts per minute
dATP	deoxyadenine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanidine 5'-triphosphate
dITP	deoxyinosine 5'-triphosphate
dNTP	deoxynucleotide 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
ddATP	dideoxyadenine 5'-triphosphate
ddCTP	dideoxycytidine 5'-triphosphate
ddGTP	dideoxyguanidine 5'-triphosphate
ddTTP	dideoxythymidine 5'-triphosphate
DMSO	dimethylsulfoxide
DTT	dithiothreitol
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediamine tetracetic acid
$_f$M	formylmethionine
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-thiogalactopyranoside
kb	kilobase
MIC	minimum inhibitory concentration
MLS	macrolide, lincosamide and

	streptogramin B
MTC	maximum tolerated concentration
M_r	relative molecular mass
nt	nucleotide
ORF	open reading frame
PEG	polyethylene glycol
pfu	plaque forming unit
PVP	polyvinylpyrolidone
PMSF	phenylmethylsulfonyl fluoride
RBS	ribosomal binding site
R_f	relative mobility by TLC
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SSC	sodium chloride-sodium citrate
SSPE	sodium chloride-sodium phosphate -ethylene diamine tetraacetate
TEMED	N,N,N',N'-tetramethylenediamine
TES	N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid
TLC	thin-layer chromatography
tris	tris-(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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INTRODUCTION

Streptomycetes are Gram-positive soil bacteria, notable for their ability to produce a large variety of secondary metabolites, particularly antibiotics. Interest in antibiotics is due in a large part to their medical potential. In the past two decades, much effort has been devoted to developing techniques for genetic manipulation of streptomycetes; this has resulted in the molecular characterization of numerous antibiotic biosynthesis and resistance genes (Seno and Baltz, 1989). Extension of this research will lead to a better understanding of the genetic control of antibiotic production, and will further our ability to produce these compounds at low cost. It will also lead to greater understanding of antibiotic resistance mechanisms.

With the increase in drug-resistant microorganisms and the decrease in the number of new antibiotics being discovered, it will become increasingly difficult to maintain an armamentarium of effective antibiotics. A challenge facing us today is, therefore, to design bioactive compounds that can evade resistance mechanisms and yet maintain effectiveness. Molecular characterization of drug-resistance mechanisms should provide useful guidance for the development of such compounds.

Etamycin is a type-B member of the streptogramin family of peptidolactone antibiotics. Streptogramins are of two

chemically distinct types, A and B, synthesized via separate pathways (Cocito, 1979). Interestingly, both types are usually produced together and act synergistically against target bacteria (Cocito, 1979). They represent a noteworthy example of nature's ability to prescribe an effective combination of antibiotics. Streptogramins, like other peptide antibiotics, are synthesized by a thiotemplate pathway (Okumura, 1983). However, their biosynthesis and resistance have not been characterized genetically.

The immediate goal of the present research was to clone and characterize an etamycin resistance gene of a producing strain, *S. griseoviridus*. There is considerable evidence that most, if not all, antibiotic biosynthesis genes and resistance genes in streptomycetes are clustered on the genome. For example, in *Streptomyces rimosus*, the oxytetracycline biosynthesis gene cluster is flanked at each end by a resistance gene (Butler *et al.*, 1989). A similar arrangement has been described for resistance genes in the tylosin producer *Streptomyces fradiae* (Seno and Baltz, 1989). Among numerous other examples of the clustering of resistance and biosynthesis genes are those for erythromycin in *Saccharopolyspora erythraea* (Stanzak *et al.*, 1986); spiramycin in *Streptomyces ambofaciens* (Richardson *et al.*, 1990); bialaphos in *Streptomyces hygrocopicus* (Murakami *et al.*, 1986) and streptomycin in *Streptomyces griseus* (Ohnuki *et al.*, 1985a; Distler *et al.*, 1987). Given the high probability that

clustering of the two types of genes is a general phenomenon, and given also the relative ease with which resistance genes can be isolated by shotgun cloning and positive selection of transformants for the resistance phenotype, cloning of a resistance gene should provide a convenient path to cloning the etamycin biosynthesis genes of *S. griseoviridus*.

The approach initially used was to shotgun clone fragments of genomic DNA from *S. griseoviridus* in the streptomycete-derived vector pIJ702 (Katz *et al.*, 1983). These experiments failed because of the high frequency with which etamycin-resistant variants arose in *Streptomyces lividans*, the host into which the cloned genomic DNA was introduced for expression of the resistance phenotype. It subsequently became clear that *S. lividans* contained, at a relatively high frequency, super-resistant strains that were selected during the shotgun cloning procedure, and must have substantially outnumbered any transformants carrying a cloned etamycin resistance gene.

Many bacteria are resistant to antibiotics they do not themselves produce, and some possess resistance mechanisms not found in producing strains. For example, *Streptomyces coelicolor* Muller, although it does not produce any known antibiotic, carries out 3-O-phosphorylation of clindamycin (Coats and Argoudelis, 1971), O-acylation of chloramphenicol (Argoudelis and Coats, 1971), O-adenylation of clindamycin (Argoudelis *et al.*, 1977) and O-phosphorylation of macrolides

(Wiley *et al.*, 1987). Of particular relevance in view of its wide use as a cloning host, *S. lividans* has been shown to possess an MLS resistance gene (Jenkins and Cundliffe, 1991), a macrolide inactivating gene (Jenkins and Cundliffe, 1991), a tetracycline resistance gene (Kessler *et al.*, 1989) and a chloramphenicol resistance gene (Dittrich *et al.*, 1991). To this list can now be added an etamycin resistance gene. A preliminary investigation of the etamycin resistance phenotype in *S. lividans* indicated that it was due to inactivation of the antibiotic.

Because *S. lividans* was unsuitable as a host for selecting a cloned etamycin resistance gene, the characteristics of *Streptomyces griseofuscus* were investigated. The intrinsic resistance of this species to etamycin was substantially lower than that of *S. lividans*, and highly resistant variants were not detected. Therefore, *S. griseofuscus* was chosen as a cloning host for subsequent experiments. Among these experiments was a second attempt, made near the end of the research project, to isolate an etamycin resistance gene from *S. griseoviridus* by shotgun cloning. This experiment also benefitted from the use of a positive selective vector, pIJ699 (Kieser and Melton, 1988), which is maintained in the host only when two repeated sequences are separated by a DNA insert. Construction in this vector of a library of *S. griseoviridus* DNA fragments, and transformation of *S. griseofuscus* as the host, yielded several clones resistant to

greater than 50 $\mu g.ml^{-1}$ of etamycin. Although this approach appeared promising it was not pursued to completion due to lack of time.

As the producer of a streptogramin-type antibiotic, *S. griseoviridus* is potentially able to withstand the effects of endogenous etamycin through the possession of an rRNA methylase of the macrolide-lincosamide-streptogramin B (MLS) resistance type. Therefore, the next approach taken was to use *ermE*, a typical MLS methylase gene, to probe the genomic DNA of *S. griseoviridus*, and look for hybridization at high stringency. Although hybridizing fragments were identified, cloning and sequencing of one such *ermE*-hybridizing DNA region did not identify an ORF-encoded product related to ErmE. The high degree of nucleotide sequence similarity proved to be accidental. Nevertheless, *S. griseofuscus* transformants containing a recombinant vector in which the *ermE*-hybridizing region had been subcloned showed significantly higher etamycin resistance than did the host alone.

The reason for enhanced etamycin resistance in the transformant was investigated by further subcloning of segments from the original fragment in an attempt to locate the region responsible. This approach was supplemented by sequencing the DNA segment that appeared to confer resistance, and by introducing mutations *in vitro* into this segment. Since resistance was not associated exclusively with either of the two intact open reading frames (ORFs) present in the DNA

segment, enhanced etamycin resistance in the transformants may have resulted from activation of a normally silent host resistance gene, the product of which may inactivate etamycin.

LITERATURE REVIEW

I. The Streptogramin Family of Antibiotics

Because streptogramins A and B act synergistically, the biosynthesis of streptogramins may be coregulated to maximize the pair's effectiveness. The synergism is of two kinds: lowering of the minimum effective concentration of each component by its partner, and making irreversible the action of both components in the mixture (Cocito, 1979). Separately, each component causes a reversible inhibition. Streptogramins inhibit protein synthesis by binding to the peptidyltransferase region of the 50S ribosomal subunit (Cocito, 1979). Those of type A inactivate the donor and acceptor sites of peptidyltransferase, thus interfering with the corresponding functions of the enzyme (Di Giambattista *et al.*, 1989). Those of the type B inhibit peptide bond formation by binding at a domain different from the type A (Di Giambattista *et al.*, 1989). The presence of a type-A streptogramin increases the ribosome's affinity for type B.

The generalized structure of type-B streptogramins is shown in Fig 1A. Many variations are due to differences in the side groups R1 and R2. The structure of etamycin is shown in Fig 1B.

II. Etamycin

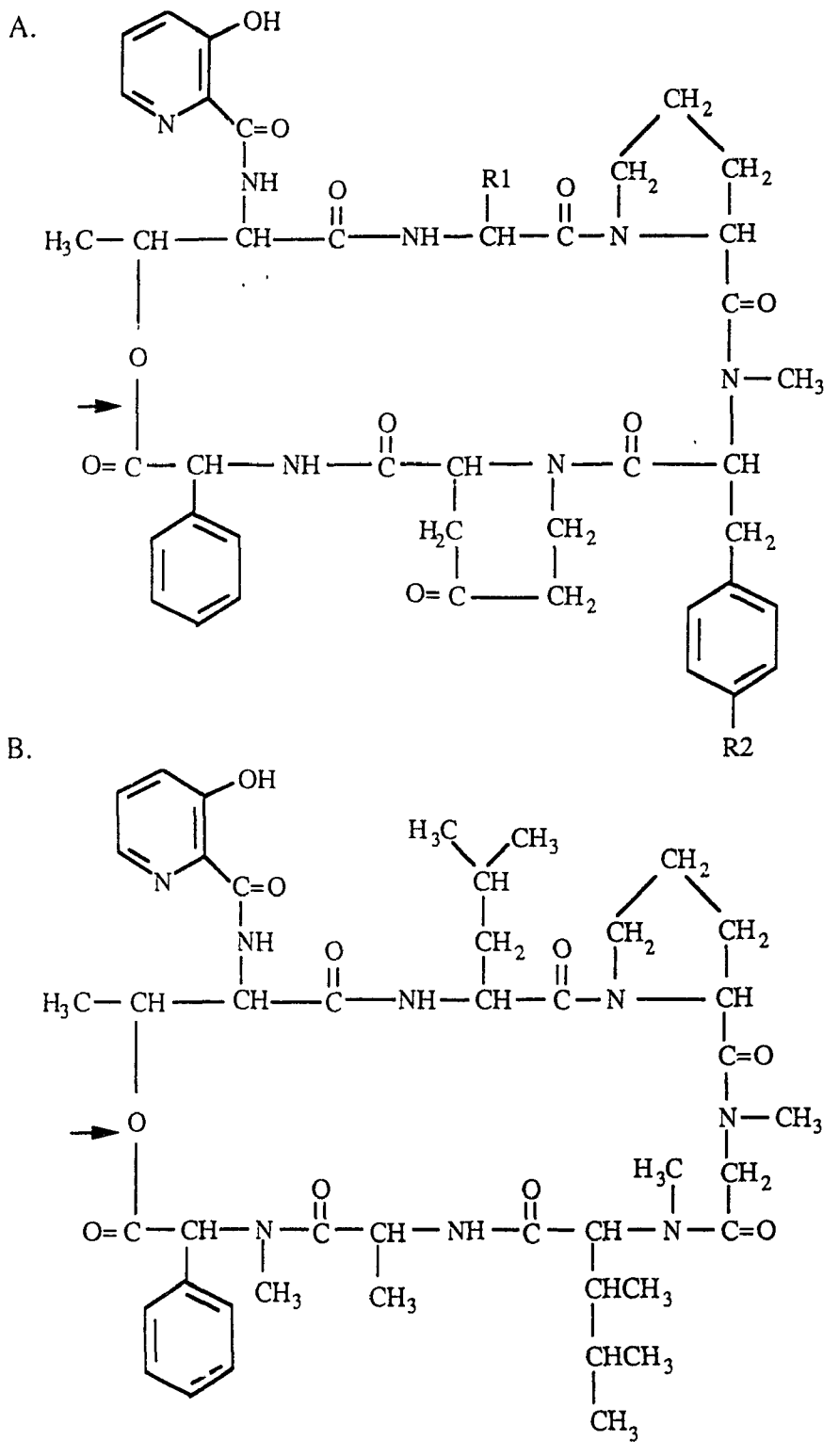

Fig. 1. A. General structure of group B streptogramin; B. Structure of etamycin. The ester bond is indicated by an arrow.

A. Structure

The antibiotic was discovered independently by three research groups and given three different names. Heinemann and co-workers (1955) isolated an antibiotic from cultures of a soil isolate, *Streptomyces lavendulae,* and named it etamycin. At about the same time, Bartz *et al.* (1955) isolated two antibiotics from culture filtrates of both an unidentified *Streptomyces* (P-D 04955) and a strain of *Streptomyces griseus* (P-D 04797; Ehrlich *et al.*, 1955). The former was identified by Anderson *et al.* (1956) as *Streptomyces griseoviridus*. Of the two antibiotics, one proved to be a type-A streptogramin and was named griseoviridin (Vazquez, 1967). The other was a type-B streptogramin, and was named viridogrisein. Sheehan and coworkers (1957, 1958) showed etamycin and viridogrisein to be identical. From *Actinomyces daghestanicus*, Toropova *et al.* (1959) later isolated antibiotic #6613, also identified as etamycin (Krugylark *et al.*, 1961).

Etamycin is hydrolysed by alkali at the ester bond between threonine and phenylsarcosine (see Fig. 1B) to generate a linear polypeptide which, by acid hydrolysis, gives in equimolecular yield eight amino acids: 3-hydroxypicolinic acid, L-threonine, D-leucine, allo-hydroxy-D-proline, sarcosine, N,β-dimethyl-L-leucine, L-alanine, and L-α-phenylsarcosine. L-α-phenylsarcosine and N,β-dimethyl-L-leucine were not known in nature before their discovery in etamycin (Heinemann *et al.*, 1955, Haskell *et al.*, 1955;

Sheehan *et al.*, 1957, 1958). The sequence of amino acids shown in Fig. 2 was established by Edman degradation (Sheehan *et al.*, 1958) and the structure was confirmed by Arnold and coworkers (1958).

Etamycin is an amorphous white solid, sparingly soluble in water (less than 1 $mg.ml^{-1}$) and soluble in most common organic solvents, but not in petroleum ether. The UV absorption spectrum shows a maximum at 305 nm in chloroform (Haskell *et al.*, 1955) and 304 nm in ethanol (Bartz *et al.*, 1955) or methanol (Heinemann *et al.*, 1955).

B. Biological activity

Etamycin is a broad-spectrum antibiotic and is the most active type-B streptogramin (Vazquez, 1967). *In vitro*, it is most active against Gram-positive bacteria but does show activity against some Gram-negative species. The target bacteria include pathogens such as *Bacillus anthracis* and species of *Clostridium*. Dickinson and coworkers (1955) reported low toxicity for experimental animals; the LD_{50} in mice was 273 $mg.kg^{-1}$ when administered intraperitoneally, and 1000 $mg.kg^{-1}$ orally. It was 4000 $mg.kg^{-1}$ in rabbits. Low toxicities were also reported by Heinemann *et al.* (1955). Local administration protected against clinical bovine mastitis caused by both staphylococci and streptococci. Etamycin was effective orally against experimental pneumococcal, streptococcal and staphylococcal infections in

mice; and against intestinal amoebiasis in rats and dogs (Ehrlich *et al.*, 1955). Etamycin does have the drawback of causing leucopenia in dogs, but this symptom is reversible. Orally administered, etamycin is excreted unchanged in the urine (Heinemann et al., 1955; Dickinson, 1955).

C. Mode of action

Type-A streptogramins have a clearly defined action on protein biosynthesis. They block two of the peptide chain elongation steps: binding of aminoacyl-tRNA (AA-tRNA) to the A site of ribosomes, and peptide bond formation between AA-tRNA and peptidyl-tRNA at the ribosomal P site. They function by preventing stable interaction of the aminoacyl component with peptidyl-tRNA, and by causing abnormal release of AA-tRNA from the A site and of peptidyl-tRNA from the P site upon translocation. A detailed molecular mechanism has been postulated for this action (Di Giambattista et al., 1989).

Type-B streptogramins have a more complex action, similar to that of the 14-membered macrolides (e.g., erythromycin). These two families and the lincosamides, which together form the so-called MLS group, compete in binding to ribosomes, and so are believed to have overlapping binding sites. The binding region has been mapped within the peptidyltransferase domain; it consists of segments (loop V and domain II) of 23S rRNA and various L ribosomal proteins (Di Giambattista *et al.*, 1987). Different groups of antibiotics have different binding sites,

which overlap to various degrees with one another. The binding site of type-B streptogramins overlaps that of the macrolides; this was reflected in the ability of both groups to inhibit peptide bond formation and to cause premature release of peptide chains in a template-dependent fashion (specific prevention of the polymerization of basic amino acids and proline).

D. Biosynthesis

Etamycin is a cyclic peptide containing both ester and amide bonds. Like most other peptide antibiotics it possesses unique constituents, such as D-amino acids. The D-leucine in etamycin is derived from the L-isomer, presumably by α-epimerisation (Bycroft, 1969). Walker and co-workers (1970) and Walker and Perlman (1971) established that the unusual N,β dimethyl-L-leucine is synthesized by methylation of L-leucine at the β-carbon and amino positions, using L-methionine as the methyl donor. Hook and Vining (1973) showed that L-threonine and L-alanine are incorporated into the peptide without alteration; glycine or sarcosine are precursors of the sarcosine moiety; phenylalanine is a precursor of L-α-phenylsarcosine. The methyl groups required in sarcosine, phenylsarcosine and N,β-dimethyl-L-leucine are all obtained from L-methionine. L-Proline and 4-hydroxy-L-proline are precursors of *allo*-4-hydroxy-D-proline. L-Lysine and 3-hydroxypicolinic acid are precursors of the 3-hydroxypicolinic

acid moiety. By feeding L-[^{14}C]leucine and L-[$^{14}CH_3$]methionine, Kamal and Katz (1976) also found that L-leucine is a direct precursor of the D-enantiomer. D-Leucine, on the other hand, inhibits etamycin formation.

Peptide antibiotics are synthesized through an enzymatic mechanism different from ribosomal peptide synthesis. Etamycin is no exception. Inhibitors of protein synthesis, such as chloramphenicol, do not affect peptide antibiotic synthesis (Katz and Weissbach, 1963; Mach *et al.*, 1963; Katz *et al.*, 1965; Froyshov *et al.*, 1970). DNase and RNase also have no effect on the synthesis of peptide antibiotics in cell-free systems (Gevers *et al.*, 1968, 1969; Kleinkauf and Gevers, 1969).

Tyrocidine is biosynthesized in *Bacillus brevis* by two peptide synthetases differing in their response to amino acid analogues (Mach *et al.*, 1963). In *Streptomyces chrysomallus*, which produces the peptidolactone antibiotic actinomycin, two multifunctional peptide synthetases have been identified (Keller, 1987). Actinomycin synthetase II (M_r 225,000) activates the first two amino acids of the peptide chain (threonine and valine or isoleucine) as thioesters via their corresponding adenylates. Actinomycin synthetase III (M_r 280,000) activates proline, glycine and valine (the remaining three amino acids in the antibiotic) as thioesters. It also carries out the methyltransferase functions for N-methylation of thioesterified glycine and valine. In addition, it

catalyzes the formation of *cyclo* (sarcosyl-N-methyl-L-valine) from glycine, L-valine, and S-adenosyl-L-methionine at the expense of ATP. A third enzyme, 4-methyl-3-hydroxyanthranilic acid-activating enzyme, is responsible for activating the nonamino acid component of actinomycin. This enzyme could use several structural analogues of 4-methyl-3-hydroxy anthranilic acid, and hence could synthesize novel compounds.

A 3-hydroxypicolinic acid-activating enzyme from an etamycin-producing strain of *S. griseoviridus* has been identified (Schlumbohm and Keller, 1990). The enzyme catalyzes both 3-hydroxypicolinic acid-dependent ATP-pyrophosphate exchange and the formation of 3-hydroxypicolinic acid adenylate from 3-hydroxypicolinic acid and ATP. Like the 4-methyl-3-hydroxyanthranilic acid-activating enzyme of actinomycin synthesis, it can use several 3-hydroxypicolinic acid analogues as substrates to synthesize etamycin analogues.

Investigations of numerous peptide antibiotics have shown similar enzymatic thiotemplate pathways to be responsible for their biosynthesis. The amino acid precursors are activated by ATP in the presence of Mg^{2+} to form amino-acyl adenylates with the concomitant release of inorganic phosphate. The amino acid is then transferred to an enzyme-bound thiol group as an activated thioester, followed by transfer of the acyl group to an acceptor and release of AMP. Sequential addition of the correct amino acids to the growing thioester-bound nascent peptide chain, and cyclization where appropriate, complete the

process. As in the ribosomal system, a large enzyme complex, normally aggregated from a few components, can ensure the correct sequential addition of amino acids. The enzymes are often multifunctional, and are characterized by their ability to modify naturally occurring amino acids.

Another noticeable feature of peptide antibiotic synthesis is that certain enzymes in the enzyme complex exhibit group specificity for certain amino acids instead of absolute specificity. This allows flexibility in a small number of positions in the peptide chain and results in the formation of congeners. Congener synthesis can be controlled by the nutrient supply. The production of different actinomycins and etamycins on various media is a good example of the effect of the nitrogen source on congener synthesis (Katz *et al.*, 1958; Katz and Goss, 1958; Waksman *et al.*, 1958; Chopra *et al.*, 1979; Okumura et al., 1979). The congeners form a family of structurally related components and provide variation in biological activity.

III. Antibiotic Resistance in *Streptomyces*

A common feature of antibiotic-producing organisms is their resistance to the antibiotic they produce. Each producing organism has a range of defensive options available. As described by Cundliffe (1989), these include inactivation of the antibiotic, modification of the target site, and exclusion

of the antibiotic by efficient efflux. Among other mechanisms are altered cell permeability (Fierro *et al.*, 1988), antibiotic-binding proteins (Gatignol *et al.*, 1988), and over-production of the enzyme that forms the target for antibiotic interference (Behrmann *et al.*, 1990).

A. Enzymatic inactivation of streptogramins

In *Streptomyces mitakaensis*, the organism producing mikamycins A and B, a lactonase specifically inactivates mikamycin B (Kim *et al.*, 1974). Another streptogramin producer, *Actinoplanes missouriensis*, possesses a similar enzyme (Hou *et al.*, 1970), and an enzyme of the same type has been detected in a clinical isolate of *Staphylococcus aureus* (Le Goffic *et al.*, 1977a). The gene responsible was located on a plasmid with several antibiotic resistance genes, and was cloned and sequenced (Allignet, *et al.*, 1988). The deduced amino acid sequence did not show significant similarity to any known protein. Recently, streptogramin-inactivating activity was also detected in three other producing streptomycetes: *Streptomyces diastaticus*, *Streptomyces loidensis* and *Streptomyces olivaceus* (Fierro, *et al.*, 1989). Although the exact mechanism has not yet been determined, evidence that inactivation did not depend on externally added cofactors suggests it is probably catalyzed by a hydrolase.

Inactivation of type-A streptogramins in two *S. aureus* strains was the result of an acetyltransferase (Le Goffic *et*

al., 1977b; De Meester and Rondelet, 1976).

B. Target site modification

Antibiotics act by binding to specific target(s) within the cell or on the cell surface (Cundliffe, 1989). Their interaction with the target sites interferes with a normal biological function. Various antibiotics (e.g., etamycin, erythromycin and thiostrepton) bind to ribosomes and interfere with protein synthesis (Cocito, 1979); some antibiotics bind to DNA gyrase (e.g., novobiocin, Thiara and Cundliff, 1988), or RNA polymerase (e.g., rifamycin, Watanabe and Tanaka, 1976); many antibiotics inhibit the various enzymes involved in synthesis of nucleic acids, amino acids, and fatty acids. The target site, if defined precisely, refers to a very small region that directly interacts with the antibiotic. Antibiotics that bind to the same target do not necessarily bind to the same target site. For example, chloramphenicol and streptomycin both bind to ribosomes, but the former binds to the 50S ribosomal subunit while the latter binds to the 30S ribosomal subunit (Cocito, 1979). The MLS group of antibiotics bind to similar but not identical regions of the 50S ribosomal subunit and have overlapping target sites. When the targets or target sites are modified to weaken or prevent their interaction with an antibiotic, the cell loses its sensitivity to that antibiotic. This mode of defense is employed by various organisms, whether or not they produce the antibiotic.

The MLS antibiotics are considered together because organisms resistant to one are often resistant to all three. It is now clear that, despite structural differences, MLS antibotics bind to similar target sites in the peptidyltransferase region of the 50S ribosomal subunit. True MLS resistance, first characterized in antibiotic-nonproducing staphylococci (Lai *et al.*, 1973), is associated with the action of an rRNA methylase capable of dimethylating 23S rRNA. The resulting ribosomes are resistant to members of all three groups of antibiotics (Lai *et al.*, 1973).

Genes conferring MLS resistance have been identified in two macrolide producers, *Saccharopolyspora erythraea* (erythromycin producer) (Bibb *et al.*, 1986) and *Streptomyces fradiae* (tylosin producer) (Birmingham *et al.*, 1986; Kamimiya and Weisblum, 1988). These genes, are designated *ermE* and *ermSF*, respectively. The gene products resemble the *ermC* product of staphylococci in dimethylating residue A2058 of 23S rRNA (Skinner *et al.*, 1983; Zalacain and Cundliffe, 1989); *ermA* from an erythromycin-producing strain of *Arthrobacter* hybridizes with *ermE* at high stringency, and so may also encode an RNA methylase that dimethylates 23S rRNA (Roberts *et al.*, 1985). N6,N6-Dimethylation at residue A2058 was indeed detected in that strain (Cundliffe, 1989). In *Sac. erythraea* *ermE* appears to be the sole resistance determinant and is expressed constitutively. In contrast, *ermSF* is only one of four resistance determinants identified in *S. fradiae*

(Birmingham *et al.*, 1986) and *ermSF* is expressed only when induced by tylosin; the control mechanism is similar to attenuation (Kamimiya and Weisblum, 1988). All MLS resistance genes analyzed display appreciable deduced amino acid sequence similarity (see references in Table 1). At the nucleotide sequence level, the similarity in the most highly conserved (N-terminal) region varies from 71% between *ermE* and *ermA* to 61% between *ermE* and *carB*.

A mechanism related to but different from the classical MLS resistance involves monomethylation of the N^6-amino group of residue A2058 in 23S rRNA. Genes responsible for this type of resistance are listed in Table 1. Their phenotypes differ from those of classical MLS resistance genes in not conferring strong resistance to all three groups of antibiotics; for example, the *clr* gene of *Streptomyces caelestis* confers resistance to a high concentration of lincosamides but to only low concentrations of macrolides.

C. Antibiotic efflux

Each antibiotic-producing strain must possess a mechanism for secreting the newly synthesized product. This mechanism could also function to expel any previously exported drug that re-entered the cell. Such a mechanism is particularly important in those producing strains in which the targets remain sensitive and in which antibiotic-inactivating activity is absent.

Table 1. *Streptomyces* genes that confer "partial MLS" resistance.

Resistance gene	Source (phenotype)	References
lrm	*S. lividans* TK21 (inducible lincomycin resistance)	Jenkins & Cundliffe, 1991
lrm	*S. lividans* 66 (lincomycin resistance)	Hara & Hutchinson, 1990
"MLS"	*S. coelicolor* A3(2) (lincomycin resistance)	Hara & Hutchinson, 1990
mdmA	*S. mycarofaciens* ATCC 21544 (midecamycin resistance)	Hara & Hutchinson, 1990
carB	*S. thermotolerans* (carbomycin resistance)	Epp *et al.*, 1987
tlrD	*S. fradiae* (tylosin resistance)	Zalacain & Cundliffe, 1991
clr	*S. caelestis* (celesticetin resistance)	Calcutt & Cundliffe, 1990

In general, two types of efflux mechanisms have been characterized: one involves a transmembrane export pump powered by transmembrane electrochemical gradients - e.g., the tetracycline resistance proteins of *E. coli* (McMurry, *et al.*, 1980). The other involves a family of ATP-binding transport proteins such as the human *mdr* (multidrug resistance) proteins. A *Staphylococcus aureus* plasmid gene (*vga*) conferring resistance to type-A streptogramins encodes a product with significant sequence similarity to ATP-binding domains of numerous proteins (Allignet *et al.*, 1992); the *vga* protein may therefore have an ATP-dependent transport function. Surprisingly, Vga does not contain long stretches of hydrophobic amino acids that might represent potential membrane-spanning domains.

IV. Regulation of Resistance

Many antibiotic producers have more than one gene coding for resistance to the endogenous antibiotic. Normally, at least one resistance gene is clustered with the antibiotic biosynthesis genes. Expression of resistance genes is either constitutive or regulated. It is reasonable to expect that genes specifying resistance to an endogenous antibiotic could be activated coordinately with, or prior to, activation of the antibiotic production genes. However, there are circumstances when resistance needs to be turned on regardless of antibiotic

production. Therefore, alternative regulatory strategies would be expected. In general, an antibiotic induces expression of its cognate resistance gene, though there are many different inducing mechanisms.

A. Induction mechanisms

Expression of a resistance gene may be regulated by a divergently transcribed repressor gene; this has been well-studied for *E. coli* tetracycline resistance (Klock *et al.*, 1985). Initially, constitutive, low-level synthesis of the repressor mRNA generates sufficient repressor protein to repress transcription of both the repressor and resistance genes by binding to the operator sites upstream of those genes. As the repressor concentration in the cell reaches a point where repressor binding sites are completely occupied, less transcription of repressor mRNA is allowed. Therefore, repressor protein autoregulates its own expression. An inducer antibiotic will bind to the repressor and thereby prevent it from binding to the operator sites. This allows expression of the resistance gene as well as the repressor gene. A high concentration of the repressor protein in the cell would ensure that further transcription of both the repressor and resistance genes would quickly cease once the concentration of antibiotic in the cell dropped. Similar systems were found in the actinorhodin repressor-resistance gene pair of *S. coelicolor* (Fernandez-Moreno *et al.*, 1991; Caballero *et al.*,

1991), and in the tetracenomycin C repressor-resistance pair of *Streptomyces glaucescens* (Guilfoile and Hutchinson, 1992a, 1992b).

The novobiocin resistance gene *gyrB* of *S. sphaeroides* encodes a novobiocin-resistant DNA gyrase B subunit (Thiara and Cundliffe, 1988). Expression of *gyrB* in *S. sphaeroides* is induced by novobiocin as well as by antibiotics such as ciprofloxacins that act on the DNA gyrase A subunit. Similar results were observed when *gyrB* was expressed in *S. lividans* where possible trans-acting factors were absent (Thiara and Cundliffe, 1989). It was postulated that *gyrB* is controlled by a promoter that responds to changes in DNA topology; the promoter is activated when the superhelical density of DNA falls due to inhibition of DNA gyrase. Therefore, induction is indirect, and markedly different from the classical induction model involving an inducer and a repressor.

The MLS resistance gene *ermC* of *S. aureus* is regulated post-transcriptionally via a so-called translational attenuation mechanism. The gene is transcribed as an mRNA molecule with a leader sequence that can adopt alternative conformations. In the absence of inducing antibiotics, translation stalls at a position in the leader sequence; this favors a downstream conformation that prevents translation of the *ermC* coding region. In the presence of inducing antibiotics, translation stalls at an alternative position favoring a downstream conformation that allows translation of

ermC. Sequencing *ermSF* (*tlrA*) revealed a leader region with the potential to adopt alternative conformations. Subsequent studies involving mutants in which the previously inducible *ermSF* (*tlrA*) gene had become constitutive confirmed that the leader region is crucial for induction (Kamimiya and Weisblum, 1988).

V. Cloning of Antibiotic Resistance Genes from Producing Organisms

Streptomyces have a genome size of 6.5-8.0 X 10^3 kb (Leblond *et al.*, 1990b; Leblond *et al.*, 1993), supporting a complex life cycle (spore to vegetative mycelium to aerial mycelium to spore) and incredible biochemical versatility. Over two-thirds of the naturally occurring antibiotics are produced by species of this genus (Berdy, 1980). Considerable attention has thus been drawn to the biochemical and genetic mechanisms of metabolic differentiation that make such versatility possible. The development of cloning vectors and efficient transformation systems in streptomycetes (Hopwood *et al.*, 1985) has allowed the molecular cloning of genes involved in these processes, among them antibiotic resistance genes.

The earliest genes isolated from streptomycetes were those for thiostrepton, neomycin and viomycin resistance; they were obtained by shotgun cloning procedures (Thompson *et al.*, 1980, 1982a), and proved useful as selective markers in the

construction of a second generation of cloning vectors. Most high copy-number streptomycete plasmid vectors are derived from the 8.9-kb plasmid pIJ101 of *S. lividans* ISP5434; pIJ101 is self-transmissible and is stably maintained in a wide range of *Streptomyces* (Kieser *et al.*, 1982). Functional characterization of different regions of pIJ101 allowed deletion of nonessential DNA segments and insertion of markers with easily identifiable phenotypes. The most widely used of all pIJ101-derived vectors is pIJ702 (Katz *et al.*, 1983; Fig. 2). It was constructed from a segment of pIJ101 by introducing *tsr* (a thiostrepton resistance gene from *Streptomyces azureus*) and *mel* (a melanin-producing tyrosinase gene from *Streptomyces antibioticus*). Cloning sites were created in front of the *mel* gene. Insertion of foreign DNA in these sites usually abolishes *mel* function; failure to produce the black melanin pigment allows visual recognition of transformants containing recombinant plasmids.

Also widely used are *Streptomyces-E. coli* shuttle vectors, which can be readily manipulated and propagated in *E. coli* and be maintained in *Streptomyces*. The shuttle vector pHJL400 (Larson and Hershberger, 1986; Fig. 3) contains the minimum essential replicon of plasmid SCP2* from *S. coelicolor* A 3(2), and the *tsr* marker selectable in streptomycetes. It also contains an *E. coli* replicon, an ampicillin resistance (*amp*) marker functional in *E. coli*, and a multiple cloning site internal to *lacZ*. Since it lacks the *par* region of SCP2* and

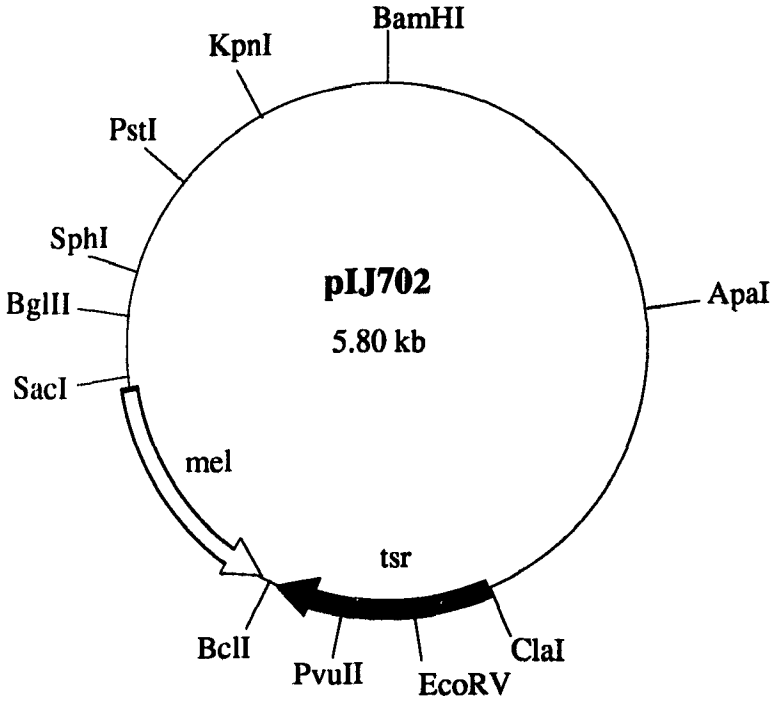

Fig. 2. Restriction map of pIJ702. The arrows represent direction of transcription of genes for melanin formation (*mel*, Katz *et al.*, 1983) and thiostrepton resistance (*tsr*, Thompson *et al.*, 1982a).

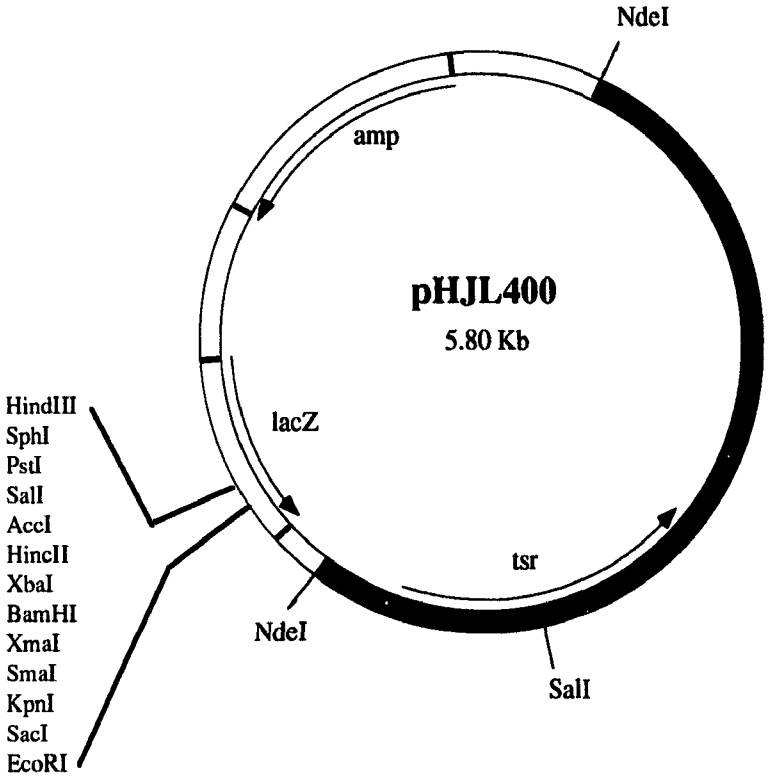

Fig. 3. Restriction map of pHJL400. The filled segment represents DNA from *Streptomyces*; the unfilled segment represents DNA from the *E. coli* vector pUC18 (Larson and Hershberger, 1986). Arrows represent direction of transcription.

is therefore segregationally unstable in streptomycetes, it can be maintained in the host only on media containing thiostrepton. This makes it a useful tool for gene disruption experiments.

Selection of a suitable cloning host is of paramount importance to the success of a cloning experiment. If the host is deficient in the function of a gene, the gene can be cloned by complementation of the function. In practice, hosts defective for a function should have a reversion rate lower than 10^{-8} cfu (Hunter and Friend, 1984). To obtain a usable host it may be necessary to mutagenize a wild-type organism. Other factors to be considered in choosing a cloning host are its transformability and the stability of cloned DNA in it. Homologous cloning using a mutant host defective in the desired trait is often complicated by integration and rearrangement of cloned DNA sequences in the host. This could be overcome by using a recombination-deficient host, but streptomycete strains of this type are not available. Therefore, most streptomycete genes have been cloned in heterologous hosts where the different genomic DNA sequence normally prevents homologous recombination.

Many streptomycetes have effective restriction systems (Matsushima and Baltz, 1989, and references therein); hence heterologous cloning is hindered by low transformation frequency due to restriction of foreign DNA. To overcome this, restrictionless hosts have been chosen or developed (Hunter

and Friend, 1984; Hopwood, 1986; Matsushima *et al.*, 1987; Matsushima and Baltz, 1989); the most widely used are *S. lividans* (Hopwood, 1986) and *S. griseofuscus* (Cox and Baltz, 1984). They have been used routinely as hosts for cloning streptomycete genes, including most resistance genes isolated so far.

In recent years, the development of molecular cloning techniques in *Streptomyces* has allowed the cloning of genes governing several antibiotic biosynthetic pathways, and numerous antibiotic resistance genes. However, genes involved in the biosynthesis of the streptogramin family of antibiotics have not been cloned and characterized. Antibiotic biosynthesis genes are normally clustered with the corresponding resistance gene(s). However, *S. griseoviridus* P-D 04955, the etamycin-producing strain used in this research, has never been characterized genetically, and its etamycin resistance mechanism has not been studied. Since resistant clones should be easily recognized by the presence of a resistance phenotype not shown by an appropriate host, the cloning of an etamycin resistance gene offers a promising approach to characterizing the biosynthesis gene system.

MATERIALS AND METHODS

I. Bacterial Strains and Plasmids

Bacteria and plasmids used are described in Table 2.

II. Chemicals and Biochemicals

Etamycin was a gift from Parke, Davis and Co., Ann Arbor, MI; thiostrepton was a gift from S. J. Lucania of E.R. Squibb and Sons, New Brunswick, NJ; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was from Diagnostic Chemicals Limited, Charlottetown, P.E.I.; polyethelene glycol (PEG) 1,000 used for transformation was from Koch-Light, Haverhill, UK; sodium dodecyl sulfate (SDS) was a specially pure grade from B.D.H. Inc, Toronto, Ont.

Reagent grade solvents and chemicals were used unless otherwise stated. Lysozyme, Pronase, Proteinase K, DNase I, RNase A, deoxyribonucleoside triphosphates (dNTPs), ethidium bromide and the random primer kit were purchased from Boehringer-Mannheim, Montreal, PQ. Ampicillin, lincomycin, kanamycin, erythromycin, chloramphenicol, tris-(hydroxymethyl) aminomethane (Tris), N-tris-(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES), phenylmethylsulfonyl fluoride (PMSF), herring sperm DNA, bovine serum albumin (BSA), Ficoll of molecular weight 400,000,

Table 2. Bacterial strains and plasmids

Organism	Genotype/phenotype	Source/reference
Streptomyces griseoviridus		
P-D04955	wild-type	PDC[a]
300E	selected on 300 μg.ml^{-1} etamycin	this study
400E	selected on 400 μg.ml^{-1} etamycin	this study
Streptomyces lividans		
TK23	SLP1$^-$ SLP2$^-$ *spc*	JII[b]
TK24	SLP1$^-$ SLP2$^-$ *str*	JII[b]
3131	contains pIJ702	Katz *et al.*, 1983
JG10	SLP$^-$ SLP2$^-$ *str pab*	Gil & Hopwood, 1983
ESR	Etamycin superresistant strain derived from TK24	this study
Streptomyces griseofuscus		
C581	wild-type	Cox & Baltz, 1984
FK400	C581 containing pHJL400	this study
FK1	C581 containing pDQ310	this study
FK2	C581 containing pDQ311	this study
FK3	C581 containing pDQ312	this study
FK4	C581 containing pDQ316	this study
FK5	C581 containing pDQ322	this study
FK6	C581 containing pDQ323	this study
FK7	C581 containing pDQ317	this study
FK8	C581 containing pDQ330	this study
FK9	C581 containing pDQ331	this study
FK10	C581 containing pDQ332	this study
FK11	C581 containing pDQ333	this study
FK12	C581 containing pDQ334	this study
FK13	C581 containing pDQ400	this study
Escherichia coli		
TG1	del (*lac,pro*) *supE thi hsdD*5 F'*traD*36	Carter *et al.*, 1985
LE392	del (*lacIZY*)6, *galK*2, *galT*22, *metB*1, *trpR*55 lambda$^-$	Maniatis, *et al.*, 1982

Plasmids

pDQ310-314	*tsr*, *amp*, from pHJL400	this study
pDQ315	*amp*, from pTZ18R	this study
pDQ316	*amp*, *tsr*, from pDQ315A and pIJ702	this study
pDQ317-323	*amp*, from pDQ311	this study
pDQ324	*amp*, from pBluescript SK+	this study
pDQ325A	*amp*, from pBluescript SK-	this study
pDQ326	*amp*, from pBluescript KS+	this study
pDQ327	*amp*, from pDQ314A	this study
pDQ328	*amp*, from pBluescript KS+	this study
pDQ329	*amp*, from pBluescript SK+	this study
pDQ330-334	*amp*, *tsr*, from pDQ322 or pDQ323	this study
pDQ400	*amp*, *tsr*, from pHJL400	LH[c]
pIJ702	*tsr*, *mel*$^+$	Katz *et al.*, 1983
pHJL400	*tsr*, *amp*, *lacZ*	Larson & Hershberger, 1986
pTZ18R	*amp*, *lacZ*	Mead and Kemper, 1988
pBluescript	*amp*, *lacZ*	Stratagene

Phage

VCSM13	*km*, derivative of M13K07	Stratagene
KY1	from lambda GEM-11 containing a 17.4-kb insert from *S. griseoviridus*	this study
KY2	from lambda GEM-11 containing a 12.7-kb insert from *S. griseoviridus*	this study
KY3	from lambda GEM-11 containing 14.6-kb insert from *S. griseoviridus*	this study
KY4	from lambda GEM-11 containing an 18.0-kb insert from *S. griseoviridus*	this study
KY5	from lambda GEM-11 containing a 17.1-kb insert from *S. griseoviridus*	this study
KY6	from lambda GEM-11 containing an 18.3-kb insert from *S. griseoviridus*	this study

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a. PDC - Parke, Davis and Co., Detroit, MI.
b. JII - John Innes Institute, Norwich, UK.
c. LH - Lei Han, Department of Biology, Dalhousie University.

polyvinylpyrrolidone (PVP) of molecular weight 360,000, PEG of molecular weight 6,000 and 8,000, and isopropyl-β-D-thiogalactopyranoside (IPTG) were all purchased from Sigma Chemical Company, St. Louis, Mo. Bacto-Agar, Bacto-Peptone, Bacto-Tryptone, nutrient broth, nutrient agar, yeast extract, malt extract, casamino acids and Tryptic Soy Broth were purchased from Difco Laboratories, Detroit, MI. Ultrapure DNA-grade agarose and electrophoresis-grade acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylenediamine (TEMED), urea and ammonium persulphate were purchased from Bio-Rad Laboratories, Mississauga, Ont.

Restriction enzymes, DNA ligase, Klenow fragment, mungbean nuclease and DNA exonuclease III were from Bethesda Research Laboratories, Burlington, Ont. The lambda GEM-11 cloning system was from Promega Co., Madison, WI. Hybond N nylon membrane was from Amersham Canada, Ltd; Oakville, Ont. The Geneclean kit was from Bio 101 Inc., LaJolla, CA. The random primers DNA labeling system was purchased from BRL, Gaithersburg, MD.

III. Media

All media were heat-sterilized in an autoclave at 121°C and 15 psi for 15 min.

Minimal medium (MM, Hopwood *et al.*, 1985), used in the controlled inoculum assay, contained:

Maltose	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
$FeSO_4.7H_2O$	0.01 g
Asparagine	0.5 g
Agar	15.0 g
Distilled water to	1000 ml

MYM medium (Stuttard, 1982), used for strain maintenance, contained:

Maltose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
Agar	20.0 g
Distilled water to	1000 ml

The pH was adjusted to 7.3 with NaOH before addition of agar.

To select thiostrepton-resistant *Streptomyces* colonies, MYMT was used; it was prepared by adding thiostrepton to MYM at a final concentration of 50 $\mu g.ml^{-1}$. For testing etamycin resistance, MYME was used; it was buffered with 0.573% (w/v) TES, pH 7.0, and supplemented with etamycin at various concentrations. For testing erythromycin and lincomycin resistance, MYM was supplemented with erythromycin at 50 $\mu g.ml^{-1}$, or lincomycin at 25 $\mu g.ml^{-1}$.

GYM medium, also used for maintaining *S. griseoviridus*, contained:

D-Glucose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
Agar	20.0 g
Distilled water to	1000 ml

The pH was adjusted to 7.3 with NaOH before addition of

agar.

YEME medium (Hopwood *et al.*, 1985), used to grow *Streptomyces* cultures for preparing protoplasts, for isolating genomic DNA, for producing etamycin or for the controlled inoculum assay, contained:

Yeast extract	3.0 g
Bacto-Peptone	5.0 g
Malt extract	3.0 g
Glucose	10.0 g
Sucrose	103.0 g
Distilled water to	1000 ml

The medium was adjusted to pH 7.5 with NaOH before autoclaving.

For plasmid isolation, the medium was supplemented with 25 $\mu g.ml^{-1}$ of thiostrepton. For growing cultures to prepare protoplasts, sucrose was increased to 340 g.

R5 medium (Hopwood *et al.*, 1985), used for protoplast regeneration, consisted of:

Sucrose	103.0 g
K_2SO_4	0.25 g
Glucose	10.0 g
Casamino acids	0.1 g
Yeast extract	5.0 g
TES buffer	5.73 g
Distilled water to	1000 ml
Agar	22.0 g

The pH of the medium was adjusted to 7.0 with NaOH before addition of agar. 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

The 10X trace element solution contained:

$ZnCl_2$	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	1000 ml

Soft nutrient agar (SNA, Hopwood *et al.*, 1985), used to overlay protoplasts during transformation experiments and to prepare bioassay plates, contained:

Nutrient broth powder	8.0 g
Agar	3.0 g
Distilled water to	1000 ml

The mixture was microwaved to melt the agar, and divided into small aliquots before autoclaving.

GNY medium, used to grow *Micrococcus luteus*, consisted of:

Glycerol	20 ml
Nutrient broth	8.0 g
Yeast extract	3.0 g
K_2HPO_4	5.0 g
Distilled water to	1000 ml

TB medium (Sambrook *et al.*, 1989), used for *E. coli* cultures, contained:

Bacto-Tryptone	10.0 g
NaCl	5.0 g
Distilled water to	1000 ml

LB medium (Sambrook *et al.*, 1989) contained:

Bacto-Tryptone	10.0 g
Bacto-Yeast extract	5.0 g
NaCl	5.0 g
Distilled water to	1000 ml
Agar	15.0 g

The pH was adjusted to 7.5 with NaOH.

TB top agar (Sambrook *et al.*, 1989), used for phage titration, contained:

Bacto-Tryptone	1.0 g
NaCl	0.5 g
Agar	0.8 g
Distilled water to	100 ml

Immediately before use, the agar was melted in a microwave oven; when the solution had cooled to 60°C, 1 ml of 1 M $MgSO_4$ was added.

TB top agarose, used when phage DNA was isolated, was prepared in the same way as TB top agar except that agar was replaced with agarose.

TBG medium (Sambrook *et al.*, 1989) was used to grow *E. coli* for isolating single-stranded templates for sequencing. It contained:

Bacto-Tryptone	12.0 g
Yeast extract	2.0 g
Glycerol	4.0 ml
KH_2PO_4	1.15 g
K_2HPO_4	6.25 g
Glucose	3.6 g
Distilled water to	1000 ml

IV. Buffers and Solutions

Etamycin stock solution used for enzyme assays was prepared in distilled water at 1 $mg.ml^{-1}$ (w/v). Etamycin stock solution used for preparing media was prepared in dimethylsulfoxide (DMSO) at 100 $mg.ml^{-1}$ (w/v). Ampicillin stock solution was made in distilled water at 100 $mg.ml^{-1}$ and filter sterilized through a Millipore membrane (pore size 0.22 μm). Thiostrepton stock solution was made in DMSO at 50 $mg.ml^{-1}$.

Hepes buffer, used for preparing streptomycete cell

extracts, consisted of 50 mM Hepes-KOH (pH 7.6), 10 mM $MgCl_2$, 60 mM NH_4Cl, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol and 0.5 mM phenylmethylsulfonyl fluoride (PMSF).

STET buffer (Sambrook *et al.*, 1989), used in plasmid DNA isolation, consisted of 0.1 M sodium chloride, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 5% (v/v) Triton X-100.

Lysis (L) buffer, protoplasting (P) buffer and basal transformation (T) buffer were prepared as described by (Hopwood *et al.*, 1985) without modification.

TE (pH 8.0) buffer was used to dissolve DNA; it contained 1 mM ethylene diamine tetraacetic acid (EDTA) and 10 mM Tris-HCl.

TES buffer was prepared by dissolving 5.73 g of N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) in 80 ml of water and adjusting the pH to 7.2 with NaOH; the volume was then made to 100 ml with water.

Tris-maleic buffer was prepared by adjusting 1 M Tris to pH 8.0 with maleic acid.

Lysozyme buffer (pH 8.0), used to lyse cells for DNA isolation, consisted of 0.3 M sucrose, 25 mM Tris-HCl, and 25 mM EDTA. Immediately before use lysozyme was added at 2 $mg.ml^{-1}$.

Buffers for restriction endonucleases, T4 DNA ligase, mungbean nuclease and exonuclease III were supplied by the manufacturer.

Acid phenol-chloroform used for plasmid isolation as well

as neutral phenol-chloroform and chloroform-isoamyl alcohol for DNA purification were prepared according to Hopwood *et al.* (1985). Buffered phenol for purification of single-strand DNA was prepared according to Sambrook *et al.* (1989).

PEG 1000 used for transformation was melted in a microwave oven, divided into 0.5 ml aliquots and autoclaved.

TAE (Tris-acetate) buffer (Sambrook *et al.*, 1989) for agarose gel electrophoresis was prepared as 50X stock containing 2 M Tris-acetate and 0.05 M EDTA.

TBE buffer (Sambrook *et al.*, 1989) for agarose gel electrophoresis and polyacrylamide gel electrophoresis was prepared as 5X stock containing 0.45 M Tris-acetate and 0.01 M EDTA. For polyacrylamide electrophoresis it was diluted to 1X before use; for agarose electrophoresis it was mixed with 10% SDS to give a final concentration of 1X TBE buffer and 0.1% sodium dodecyl sulphate (SDS).

Acrylamide stock solution (30% w/v) was made by dissolving 28.5 g acrylamide and 1.5 g bis-acrylamide in 100 ml water. The mixture was filtered through a 0.45 µ pore-size membrane and degassed under vacuum. The solution was stored in a dark brown bottle at 4°C.

Standard sequencing gel solution was made by combining 63 g urea, 30 ml of 5X TBE buffer, 25 ml of 30% acrylamide stock and 40 ml water. The urea was dissolved by stirring and the final volume was adjusted to 150 ml with water. The solution was filtered through a 0.45 µ pore-size membrane and degassed

under vacuum.

Ammonium persulphate (25% w/v) for polymerization of polyacrylamide was dissolved in water immediately before use.

Solutions used for hybridization: 20X SSC contained 3 M NaCl and 0.3 M trisodium citrate; 20X SSPE contained 3.6 M NaCl, 0.2 M sodium monobasic phosphate and 0.02 M EDTA; denaturing solution contained 1.5 M NaCl and 0.5 M NaOH; neutralizing solution contained 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2) and 1 mM EDTA; 100X Denhardt's reagent contained 2% (w/v) BSA, 2% (w/v) Ficoll and 2% (w/v) polyvinylpyrolidone; denaturing solution contained 1.5 M NaCl and 0.5 M NaOH; neutralizing solution contained 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2) and 1 mM EDTA. Pre-hybridization solution contained 5X Denhardt's reagent, 0.5% (w/v) SDS, 5X SSPE and 100 $\mu g.ml^{-1}$ of denatured herring sperm DNA. Hybridization solution was the same as pre-hybridization solution but supplemented with ^{32}P-labelled probe DNA.

V. Maintenance of Stocks

A. Bacterial stocks.

a. *E. coli*

E. coli stocks were prepared by mixing an overnight culture with sterile 100% glycerol in a 4:1 ratio. The mixture was kept at -20°C for short-term storage or -70°C for long-term storage.

b. *M. luteus*

M. luteus was grown in GNY medium overnight; the culture (4 parts) was mixed with sterile 100% glycerol (1 part), and stored at -20°C or -70°C.

c. *Streptomyces*

Spores were dislodged from *Streptomyces* strains grown in a Petri plate by adding 10 ml of sterile distilled water or 10% sucrose, and scraping the culture surface with an inoculation loop. The suspended spore mixture was agitated briefly by vortexing, then filtered through nonabsorbent cotton. Spores were pelleted by centrifugation, resuspended in 20% (v/v) aqueous glycerol and stored at -20°C or -70°C.

B. Phage Stocks

a. Lambda phage

For normal maintenance, lambda phage were eluted into 1 ml sterile SM buffer with a drop of chloroform added, and stored at 4°C. For long term storage, lambda phages were eluted into SM buffer containing 7% DMSO, and stored at -70°C.

b. Genomic DNA library

Recombinant phage particles (3×10^5) were used to infect *E. coli* LE392. Phages were allowed to develop in TB top agar by incubating at 37°C for 6-10 h. They were eluted into sterile SM buffer and the mixture was centrifuged at 2,000 g for 5 min. Supernatant containing phage particles was

transferred to a new glass tube and DMSO was added to 7% (v/v). The library was stored at -70°C.

VI. Culture Conditions

A. Cultures for genomic DNA isolation

For genomic DNA isolation, *Streptomyces* were grown in YEME supplemented with 10.3% (w/v) sucrose. Before inoculation, $MgCl_2.6H_20$ was added to a final concentration of 0.5% (w/v). Large amounts of spore suspension were used for inoculation to obtain dispersed growth. Cultures were harvested after 48 h incubation at 30°C on a rotary shaker at 220 rpm.

B. Cultures for plasmid isolation

a. *Streptomyces*

For routine plasmid screening, *Streptomyces* strains containing plasmids were either patched on MYMT agar or grown in 10 ml YEME medium supplemented with 15 $\mu g.ml^{-1}$ thiostrepton. Mycelium was harvested at the early logarithmic phase of growth (18-24 h).

For large-scale plasmid isolation, *Streptomyces* were grown in 500 ml YEME medium supplemented with 25 $\mu g.ml^{-1}$ thiostrepton. Before inoculation, $MgCl_2.6H_20$ was added to a final concentration of 0.5% (w/v). Cultures were harvested by centrifugation after 48 h incubation at 30°C with constant shaking at 220 rpm.

b. *E. coli*

For routine plasmid screening, *E. coli* colonies were either patched on LB plates containing 50 $\mu g.ml^{-1}$ of ampicillin or inoculated in 2 ml LB medium supplemented with 50 $\mu g.ml^{-1}$ of ampicillin. Cells were harvested by centrifugation after overnight incubation at 37°C.

For large-scale plasmid isolation, *E. coli* strains were grown in 50 ml LB medium supplemented with 50 $\mu g.ml^{-1}$ of ampicillin. Cells were harvested by centrifugation after overnight incubation at 37°C.

C. Cultures for protoplasting

Streptomyces were grown in YEME with 34% sucrose. Before inoculation, glycine was added to 0.5% (w/v) for *S. lividans*. For *S. griseofuscus*, glycine was added at 1% (w/v). Cultures were shaken at 30°C and 220 rpm for 36 h.

D. Controlled inoculum assay for antibiotic resistance.

To test etamycin resistance, spores of *Streptomyces* strains were added to 10 ml YEME medium containing 15 $\mu g.ml^{-1}$ of thiostrepton (omitted for strains that did not have a thiostrepton resistance gene). Samples (10-20 μl) of the culture were withdrawn at intervals and pipetted as single drops on MYM agar, buffered with TES at pH 7.0 and containing etamycin at 40 or 50 $\mu g.ml^{-1}$. These test inocula were incubated at 30°C to allow mycelium to grow. Alternatively, the samples

were pipetted on TSB agar containing 20 or 40 $\mu g.ml^{-1}$ of etamycin, or on MYM agar containing 50 $\mu g.ml^{-1}$ of erythromycin or 25 $\mu g.ml^{-1}$ of lincomycin.

E. Assaying antibiotics for minimum inhibitory concentrations (MIC).

Antibiotics were added to MYM agar to give final concentrations of 200, 100, 50, 40, 30, 20, 10 or 5 $\mu g.ml^{-1}$, and the agar was poured into Petri plates. Spores of *Streptomyces* strains were spread on the agar surface and the presence of mycelium was recorded after 3 days incubation at 30°C. The lowest antibiotic concentration that prevented growth was recorded as the MIC. Due to variations in etamycin resistance within spore populations, MICs depended on the size of spore inocula (with large spore inocula the small fraction of highly resistant spores grew as a lawn and gave misleading MIC values). The amount of spores used was predetermined by plating to give single colonies on MYM agar and adjusting the inoculum to 10^2 spore cfus.μl^{-1}. Ideally the etamycin concentration at which the majority of spores failed to grow was recorded as the MIC, but where this endpoint was difficult to judge the MIC was recorded as a concentration range.

F. Assaying *S. griseofuscus* for the maximum tolerated concentration (MTC) of etamycin.

Samples (15 μl) from seed cultures of *S. griseofuscus*

strains grown in 10 ml YEME medium were pipetted as single drops on MYM agar containing 5, 10, 20, 30, 40, 50 or 60 $\mu g.ml^{-1}$ of etamycin. After incubation at 30°C for 3 days, the highest etamycin concentration at which growth had occurred was recorded as the MTC. To increase reproducibility, the cfu/μl and OD_{640} of spore suspensions used to initiate the seed cultures were predetermined, and each seed culture was started with approximately 10^6 cfu of spores. Samples were withdrawn from each seed culture at three different times (e.g. 18, 21 and 24 h) when the OD_{640} was in the range 3-5, and each sample was used as inoculum for an MTC assay. In this way each transformant was tested three times, and the MTC of the transformants was recorded as the range obtained with the inoculum samples from different growth stages. The overall assay was repeated at least three times for each transformant, and the MTC values so obtained were found to be reproducible. Although statistical analysis was not appropriate, because the etamycin concentrations tested were discontinuous, without dose gradient variations, the differences in resistance levels obtained for transformants were highly consistant.

G. Induction of etamycin resistance in *S. griseofuscus*

Cultures of *S. griseofuscus* grown for 21 h in 10 ml YEME medium were supplemented with 0.5 $\mu g.ml^{-1}$ of etamycin and incubated again for 3 h to induce etamycin resistance before use in assays.

H. Cultures for etamycin production

YEME liquid medium was used routinely to grow *S. griseoviridus*. To determine whether etamycin was degraded after cultures had ceased to grow, four additional media were tested.

I. Cultures for single-stranded DNA.

The procedure described by Karger and Jessee (1990) was modified. An *E. coli* colony containing a phagemid was used to inoculate 2 ml of TBG medium supplemented with 100 $\mu g.ml^{-1}$ ampicillin. Helper phage VCSM13 was added at 5 X 10^8 to 1 X 10^9 pfu ml^{-1}. After the phages had infected the cells during a 1.5-h incubation at 37°C with constant shaking at 220 rpm, kanamycin was added to a final concentration of 75 $\mu g.ml^{-1}$ and incubation was continued for 16-20 h.

VII. Assays

A. Bioassay of etamycin

A sample (1 ml) of an *M. luteus* culture, grown overnight in GNY medium, was combined with 25 ml of melted soft nutrient agar, and the mixture was poured on MYM agar. The plates were stored at 4°C. Before use they were exposed without lids in a sterile air flow for 30 min; sterile paper discs (13 mm diameter) were placed on the agar surface and etamycin samples were pipetted on the discs. The plates were incubated at 30°C

until *M. luteus* had grown.

B. Thin-layer chromatography of etamycin and related compounds

Etamycin samples were dissolved in ethyl acetate or methanol. The solutions were applied to silica gel (Sil 60 F254, E. Merck, Darmstadt, Germany), and chromatographed in benzene: acetic acid: water:: 42:24:1 (Chopra *et al.*, 1979). Etamycin was detected by viewing the chromatogram under long wavelength ultraviolet light (Chromato-Vue cabinet, Ultraviolet Products Inc., San Gabriel, CA).

C. Assay of etamycin-inactivating activity

Samples of crude enzyme dissolved in Hepes buffer were incubated with 100 $\mu g.ml^{-1}$ etamycin at 37°C for 3 h. After incubation, 20 µl of each incubation mixture was added to a filter paper disk on MYM agar seeded with *M. luteus* as before. The residual antibiotic activity was determined by measuring the diameters of the inhibition zones. Alternatively, the assay mixture was extracted at both neutral and acidic pH with ethyl acetate, and the absorbance of the extracts at 305 nm was measured.

D. HPLC detection of etamycin

Etamycin was chromatographed on a 4.6 X 25 mm reverse phase C-18 column with a methanol-water solvent system. The presence of etamycin in the eluate was detected by measuring absorbance at 305 nm. The solvent consisted of a methanol gradient from

5% to 30% in water (1 min), from 30% to 100% (7 min), 100% methanol (2 min), and then a decreasing concentration of methanol, dropping from 100% to 5% (1 min) and remaining at 5% (1 min). The gradient was programmed by System Gold solvent module 126; absorbance was monitored with System Gold detector module 166 (Beckman, Palo Alto, CA). Compounds suspected to have pH dependent retention times were tested in solvents where water was replaced with sodium acetate buffer (0.1 M, pH 6.0).

VIII. DNA Manipulations

A. Isolation of plasmid DNA

a. *Streptomyces*

The alkaline lysis procedure of Kieser (1984) was used to isolate plasmid DNA. For rapid screening of *Streptomyces* colonies, mycelium was resuspended in 500 µl of a freshly prepared lysozyme solution and incubated for 30 min at 37°C; then 250 µl of 2% SDS in 0.3 M NaOH was added. The mixture was vigorously agitated to achieve rapid lysis. The lysate was incubated at 65°C for 15 min and allowed to cool at room temp for 30 min. Acid phenol/chloroform (100 µl) was then added and the phases were mixed by vortexing for 1 min. After centrifugation, the upper aqueous layer was transferred to a new 1.5-ml Eppendorf tube. To it, 1/10 volume of 3 M sodium acetate and 1 volume of isopropanol were added. The mixture

was inverted several times and then kept at -20°C for 15 min. The DNA was pelleted by centrifugation and resuspended in TE buffer.

For large-scale isolation, mycelium grown in 500-ml cultures was harvested. The extraction procedure was scaled up according to the wet weight of mycelium. The plasmid DNA was further purified by a cycle of caesium chloride centrifugation (Bibb *et al.*, 1977).

b. *E. coli*

E. coli plasmids were routinely isolated by the rapid boiling method (Sambrook *et al.*, 1989). The bacterial pellet from a 2-ml culture was resuspended in STET buffer supplemented with lysozyme at 1 $mg.ml^{-1}$. The mixture was agitated briefly to disperse cells, kept at room temperature for 10 min, and heated at 100°C for 50 s; it was then centrifuged at 14,000 rpm for 15 min. The bacterial debris was removed with a sterile toothpick and the DNA was precipitated with 1/10 volume of 3M sodium acetate and 1 volume of isopropanol.

For large-scale isolation, the alkaline lysis procedure of Kieser (1984) was used. The lysozyme treatment was at 0°C instead of at 37°C.

B. Isolation of *Streptomyces* genomic DNA

Streptomyces genomic DNA was isolated as described by Hopwood *et al.* (1985). Mycelium from a 100-ml culture was

collected by centrifugation and washed twice with 10 ml of 10.3% sucrose. It was resuspended in 10 ml of lysozyme buffer. After the mixture had been incubated at 37°C for 1 h, 2.4 ml of 0.5 M EDTA and 0.26 ml of pronase (2 $mg.ml^{-1}$) were added and the contents were mixed gently. Then 1.4 ml of a 10% SDS solution was added and the contents were mixed gently by inversion until the two phases became uniform. Phase separation was achieved by centrifugation at 10,000 g for 10 min.

The clear upper layer was transferred with a wide-mouth pipette to a new tube and re-extracted once with neutral phenol/chloroform and once with chloroform/isoamyl alcohol (24:1) until the interphase was clear. The upper aqueous phase was transferred with a wide-mouth pipette to 0.1 volume of 3 M sodium acetate, mixed gently and layered with 2.5 volumes of absolute ethanol. DNA was spooled on a glass rod by gently stirring the suspension with the rod. The DNA was air-dried for 15 min in a laminar air flow hood and redissolved in 5 ml TE.

C. Isolation of lambda DNA

a. Large scale

A modification of the procedure described by Sambrook *et al.* (1989) was used. Pre-titred phage stock was mixed with *E. coli* LE392 grown overnight at 37°C in 50 ml TB medium supplemented with 0.5 ml of 1 M $MgSO_4$ and 0.5 ml of 20% (w/v)

maltose. The mixture was used to inoculate 100 ml LB medium supplemented with 10 mM $MgSO_4$, and the culture was incubated at 37°C until complete lysis had occurred.

To the phage lysate, solid NaCl (final concentration 1 M) was added and dissolved by swirling. After 1 h on ice the mixture was centrifuged at 11,000 g for 10 min to remove cell debris. To the supernatant, solid PEG 8000 was added to a final concentration of 10% (w/v), and dissolved by slowly stirring at room temperature. The mixture was allowed to cool on ice for 2 h. Phages were pelleted by centrifugation and resuspended in 5 ml SM buffer to which DNase and RNase were added, each to a final concentration of 10 $\mu g.ml^{-1}$. The mixture was incubated at room temperature for 30 min and then extracted once with 5 ml chloroform. To the aqueous portion EDTA was added to 20 mM, SDS to 0.5% (w/v) and proteinase K to 50 $\mu g.ml^{-1}$; the mixture was incubated at 56°C for 1 h, then extracted once with 5 ml neutral phenol/chloroform and once with 5 ml chloroform/isoamyl alcohol (24:1). From the clear aqueous phase remaining, DNA was precipitated with 1/10 volume of 3M sodium acetate and 2.5 volume of ethanol.

b. Small scale

The method described by Meese *et al.* (1990) was used. Pretitred phage was mixed with *E. coli* LE392 and incubated for 15 min at 37°C. The mixture was added to 7 ml molten TB top agarose supplemented with 10 mM $MgSO_4$ and used to overlay LB agar in a 15-cm plate. After the top agarose had hardened, it

was inverted and incubated at 37°C overnight. Phage lysates were eluted into 10 ml SM buffer by gently shaking for 2 h at room temperature. The phage eluates (approximately 7 ml) were applied to a DEAE-cellulose (Whatman DE52, Mandel Scientific Company, Guelph, Ont.) column and the column was washed with 2 ml LB medium. To the combined effluent NaCl was added to 0.07 M and the phage was precipitated with 2 volumes of ethanol. Phage particles were recovered by centrifugation and washed with 70% ethanol. The pellet was resuspended in 2 ml TE buffer containing 0.2% SDS and lysed by adding 2 ml buffered phenol. The phases were mixed by shaking for 2 min and separated by centrifugation at 10,000 g for 5 min. Extraction was repeated and the DNA was eventually precipitated with 2 volumes of ethanol.

D. Caesium chloride-gradient centrifugation

To plasmid DNA dissolved in 8 ml TE buffer, 8.4 g of caesium chloride were added and gently mixed in until dissolved (Hopwood *et al.*, 1985). After 0.4 ml of aqueous ethidium bromide (10 $mg.ml^{-1}$) had been added, the mixture was poured into a 1.5 X 7.5-cm polyallomer tube and overlayed with paraffin oil. The tube was tightly capped and centrifuged (Beckman model L-55B ultracentrifuge) in a type 70.1 rotor at 45,000 rpm for 24 h at 18°C. The polyallomer tube was viewed under UV light and the plasmid band was recovered by piercing the side with a sterile syringe. Ethidium bromide was

extracted from the plasmid DNA solution with an equal amount of water-saturated *n*-butanol by inverting the tube several times. The butanol was removed and the extraction was repeated until the ethidium bromide was eliminated (disappearance of pink color). Two volumes of water were added to the aqueous phase, followed by 6 volumes of ethanol. After 30 min at -20°C the DNA precipitate was recovered by centrifugation and redissolved in TE buffer.

E. Sucrose-gradient centrifugation

As described by Hopwood *et al.* (1985), predigested DNA (up to 150 µg) dissolved in 100 µl TE buffer was loaded on a 10-40% sucrose gradient (12 ml) prepared in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 M NaCl in a Beckman polyallomer tube (14 X 89 mm). After centrifugation (Beckman model L8-70M ultracentrifuge and model SW41 rotor) at 35,000 rpm for 16 h at 18°C, fractions of approximately 400 µl were collected by puncturing the bottom of the centrifuge tube with a sterile needle. The size of the DNA in each fraction was estimated by agarose gel electrophoresis using lambda DNA digested with *Hind*III as size standards.

F. Isolation of single-stranded DNA template for sequencing

The procedure of Vieira and Messing (1987) was modified: to 1.2 ml of culture broth (containing phage) in a centrifuge tube, 200 µl of 20%(w/v) PEG 8000 in 2.5 M NaCl was added. The

contents were mixed by inverting several times and briefly vortexed. The tube was kept at 4°C for 30 min, and the phage particles were recovered by centrifuging at 12,000 g for 5 min at 4°C. The supernatant was decanted, and the tube centrifuged again for 30 s to allow all of the residual supernatant to be withdrawn with a pipette. The pellet was resuspended in 100 ml TE buffer by tapping and vortexing. The phage suspension was extracted twice with buffered phenol/chloroform (see earlier) and once with chloroform/isoamyl alcohol (24:1). The aqueous phase was transferred to a new tube and the DNA was precipitated by adding sodium acetate and ethanol.

G. Electrophoresis of DNA

Agarose gels (0.6-1.4%) in 1X TAE buffer were used routinely for electrophoresis of DNA. Agarose was dissolved in TAE buffer, poured into a gel tray and submerged in TAE buffer in an electrophoresis tank. Electrophoresis was normally carried out at room temperature at 70 volts.

For restriction mapping, a 0.4% agarose gel was prepared in Tris-phosphate-EDTA buffer (80 mM tris-phosphate, pH 8.0, 8 mM EDTA) at 4°C. Electrophoresis was carried out at 35 volts for 72 h at 4°C.

H. Restriction enzyme digestion

The DNA solution in TE buffer was mixed with 10X restriction buffer and sterile water to give the appropriate buffer

concentration. The quantity of enzyme added depended on the amount and purity of DNA. Normally, one unit of restriction enzyme was used for complete digestion of 1 µg DNA. The reaction was usually incubated for 2 h at the temperature recommended by the manufacturer.

I. Elution of DNA from agarose gels

Ultrapure agarose was used. The appropriate DNA bands were located under UV light and excised with a sterile razor blade. The agarose slice was suspended in 2-3 volumes of saturated sodium iodide solution in an Eppendorf tube and dissolved by incubation at 55°C for 2-3 min. The solution was then mixed with 5 µl of Geneclean glass milk and cooled on ice for 5 min. The glass milk with DNA adsorbed was pelleted by brief centrifugation and the supernatant was discarded. The pellet was washed three times with 0.5 ml of chilled Geneclean wash solution. The DNA was eluted by incubating the pellet in 10 µl TE buffer for 5 min at 55°C. The mixture was again centrifuged and the supernatant was collected.

J. Nested deletions

DNA fragments were cloned in the phagemid vectors pBluescript SK(+,-) or KS(+,-). The recombinant phagemids were restricted with two enzymes within the polylinker region at the priming end of the cloned fragments to obtain a 5′-overhang close to the DNA fragment to be sequenced, and a 3′-

overhang closer to the priming end. Approximately 2 µg doubly-restricted DNA in exonuclease III buffer was digested with 40 units of exonuclease III at 37°C. Samples were removed at 15 s intervals and pooled in a tube containing 5X mungbean nuclease buffer. The final mixture in 1X mungbean nuclease buffer was digested with 20 units of mungbean nuclease for 10 min at 37°C, neutralized with Tris-HCl (1.0 M, pH 8.0) and heated at 70°C for 5 min to inactivate the nuclease. A portion of the mixture was examined by agarose gel electrophoresis along with the linear phagemid and linear phagemid with insert. The single-stranded ends of the DNA were filled in by incubation at room temperature for 30 min with the Klenow fragment in 5 mM $MgCl_2$ containing 1 mM dNTPs. The "polished" DNA fragments were circularized by incubating in 1X ligase buffer with T4 DNA ligase at 12°C overnight. A portion of the ligation mixture was used to transform *E. coli* TG1, and the transformants were screened for phagemids that differed progressively in size by approximately 200-bp through the entire DNA fragment to be sequenced.

K. Rapid screening of *E. coli* plasmids

The method developed by Sekar (1987) was used. Small portions of well-separated colonies were collected with a tooth pick and each was suspended in 10 µl of protoplasting buffer (30 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM sodium chloride, 20% w/v sucrose, 1 $mg.ml^{-1}$ of lysozyme and 50 $\mu g.ml^{-1}$

of RNase) in a microtitre plate. After incubation at room temperature for 15-30 min, the cell lysates were loaded into the wells of an agarose gel prepared in 1X TBE buffer containing 0.05% SDS. The wells were preloaded with 3 µl of TBE/SDS loading buffer (1X TBE, 20% w/v SDS, 5% w/v sucrose and 0.04% w/v bromophenol blue). Electrophoresis was carried out for 15 min at 30 volts followed by 45 min at 100 volts. The gel was stained in 0.1% ethidium bromide and the plasmids were visualized under UV light.

L. DNA sequencing reactions

A sequencing kit from United States Biochemical Corporation, Cleveland, OH, was used. In the first step, 1-2 µg of single-stranded template dissolved in 7 µl TE was mixed with 1 µl of the synthetic primer (2.5 pmol) and 2 µl of reaction buffer in a final volume of 10 µl. The mixture was heated at 65°C for 2 min, allowed to cool to 30°C at room temperature, then placed on ice and used within 4 h. Just before the labelling reaction was started, four microfuge tubes received 2.5 µl of a termination mix containing either dideoxy G, A, T or C. The tubes were pre-warmed at 37°C for 1 min.

The labelling reaction required the following:

Annealed template-primer	10.0 µl
DTT (0.1 M)	1.0 µl
Diluted labelling mix	2.0 µl
[α-^{35}S]dATP (1000-1500 Ci. mmol^{-1}, 10 µCi. µl^{-1})	0.5 µl
Diluted Sequenase	2.0 µl

The labelling mix was normally diluted with distilled water and stored at -20°C. Sequenase (recombinant T7 DNA polymerase) was diluted by mixing 0.5 µl of the enzyme solution supplied with 4.0 µl of ice-cold Enzyme Dilution Buffer (supplied in the kit). The labelling reaction reagents were mixed thoroughly, and incubated at room temperature for 3 min; the reaction was terminated by transferring 3.5 µl of the mixture into each of four microfuge tubes containing a termination mix. The components were mixed and incubated at 45°C for 5 min before 4 µl of stop solution was added. The samples could then be stored at -20°C for up to 1 week; they were heated at 95°C for 2 min and cooled on ice for 2 min before being loaded on a sequencing gel. The acrylamide gel (6%) was prepared in 1X TBE. Electrophoresis was carried out at a constant temperature of 55°C for 1-3 h. The voltage was adjusted during the electrophoresis to keep the temperature constant. Regions of uncertainty were resolved by repeating the same sequencing reaction with 7-deaza dGTP or dITP.

The gel was fixed in an aqueous solution of methanol (10% v/v) and acetic acid (10% v/v) for 30 min and vacuum dried (Bio-Rad Dryer, Model 583) for 1 h. The completely dried gel was autoradiographed by exposure to an X-ray film (Kodak, X-omat AR) at room temperature for 18 h. The film was developed in an automated X-ray film developer.

M. Sequence analysis

Sequence data were analyzed with various programs of the Genetics Computer Group (GCG) package (University of Wisconsin Biotechnology Center, Madison, Wisconsin; version 7.1; Devereux *et al.*, 1984): CODONPREFERENCE, FASTA, LINEUP, PILEUP, BESTFIT, PROFILE SCAN, MOTIFS, COMPARE and DOTPLOT. Unless otherwise stated, default settings were used. Amino acid sequences of genes were compared using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) programs with sequences in NR (the non-redundant protein database, selected automatically by the BLASTP program, and including SWISS-PROT, PIR, GENPEPT and GENPEPT updates), and also with sequences in Genbank and EMBL. Sequence data were also analyzed with the DNA Strider program (Marck, 1988).

IX. Transformation

A. Formation and regeneration of *Streptomyces* protoplasts

The procedure was described by Hopwood *et al.* (1985). Mycelium from 25 ml cultures was washed twice with 10.3% sucrose, resuspended in 10 ml P buffer containing 2 $mg.ml^{-1}$ lysozyme, and incubated at 30°C for 1-2 h until observations by microscopy indicated that protoplast formation was complete. The protoplasts were washed twice in P buffer, resuspended in 2 ml P buffer, dispensed as 100 µl aliquots and stored at -70°C.

B. Transformation of *Streptomyces* protoplasts

The procedure of Hopwood *et al.* (1985) was used. A 100 µl protoplast suspension and up to 10 µl of DNA solution were thoroughly mixed with 100 µl of 25% PEG 1000 in T buffer. After 2 min at room temperature, 1 ml of P buffer was added; the protoplasts were pelleted by centrifugation, resuspended in 200 µl P buffer and plated on regeneration medium. The plates were incubated at 30°C for 18 h before being overlayed with 2.5 ml of soft nutrient agar containing thiostrepton (final plate concentration, 25 $\mu g.ml^{-1}$). Incubation was resumed for 4-5 days until thiostrepton-resistant transformants appeared.

C. Preparation of competent *E. coli* cells

Competent cells were prepared as described by Hopwood *et al.* (1985). An overnight *E. coli* culture (0.5 ml) was used to inoculate 50 ml of L broth supplemented with 0.4 ml of 2.5 M $MgCl_2$. The culture was shaken at 37°C for 1.5 h, then cooled on ice for 10 min, transferred to a cold centrifuge tube and centrifuged for 10 min at 4°C. The cells were resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and kept on ice for 30 min. The suspension was again centrifuged for 10 min at 4°C and resuspended in 2 ml of 0.1 M $CaCl_2$. For long-term storage, 100% glycerol was added to constitute 20% of the cell suspension; the competent cells were dispensed in 100 µl

aliquots and stored at -70°C.

D. Transformation of *E. coli*.

Plasmid DNA in 5-20 µl TE buffer was mixed with 100 µl of competent cells and the mixture was kept on ice for 30 min. Cells were heat-shocked at 45°C for 90 s and then chilled on ice for 2-3 min. The cells were diluted with 0.9 ml LB medium and incubated at 37°C for 60 min to allow the cells to recover. Up to 200-µl portions of cell suspension were plated on LB containing 100 $\mu g.ml^{-1}$ ampicillin and incubated at 37°C to detect ampicillin-resistant transformants.

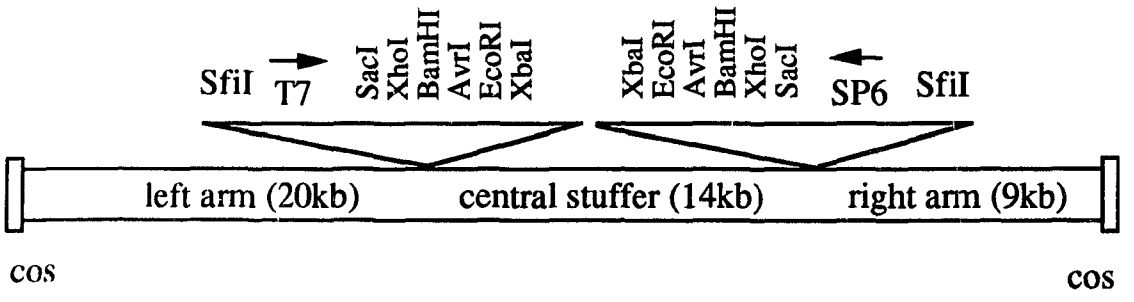
X. Construction of *S. griseoviridus* Genomic Library

The method used reagents supplied by Promega and is outlined in Fig. 4. Genomic DNA (100-200 µg) from *S. griseoviridus* was partially digested with *Sau*3A1 and fractionated by sucrose density-gradient centrifugation. DNA fragments in the 9-23 kb range were pooled, purified by extraction with neutral phenol/chloroform and redissolved in 50 µl of TE buffer. The DNA single-strand ends were partially filled in by incubating the following mixture at 37°C for 30 min:

Partially digested genomic DNA	10.0 µl
10X Fill-in buffer containing dGTP and dATP	2.5 µl
Klenow fragment (5 unit/µl)	1.0 µl
Distilled water	11.5 µl

The mixture was then extracted once with neutral

A.



B.

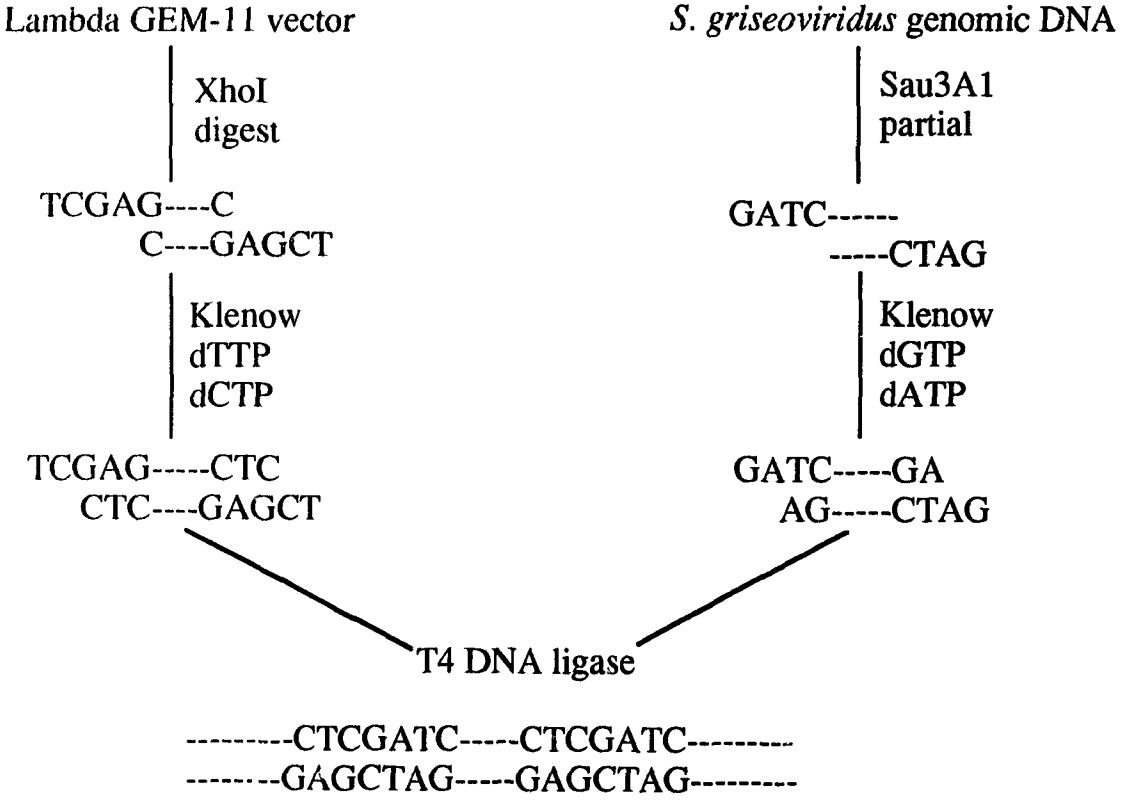


Fig. 4. Cloning with Lambda GEM-11 *Xho*I half-site arms (see text for details): A, the Lambda GEM-11 vector; B, the cloning strategy. Arrows in A represent direction of transcription. Dashed lines represent DNA.

phenol/chloroform and once with chloroform/isoamyl alcohol (24:1); DNA was precipitated with 7.5 M NH_4OAc and ethanol. The DNA pellet was redissolved in 10 µl TE buffer, and ligated with *Xho*I half-site lambda GEM-11 arms as follows:

Half-filled-in genomic DNA	2.0 µl
Lambda GEM-11 *Xho*I half-site arms	0.5 µl
10X Ligation buffer	0.5 µl
T4 DNA ligase	1.0 µl
Distilled water	1.0 µl

The ligation mixture was incubated for 3 h at 22°C. A sample (50 µl) of the Packagene *in vitro* packaging system was mixed in, and incubation was continued for 2 h. Phage buffer was added to a final volume of 0.5 ml, followed by 25 µl of chloroform. After thorough mixing and allowing the chloroform to settle out, 1 µl of the packaged phage was titred on LB agar. The number of recombinant phages in the library was calculated. The library was amplified according to the procedure described earlier and stored at -70°C.

XI. Southern Hybridization

A. Transfer of DNA from agarose gels to nylon membranes

After electrophoresis the agarose gel was stained with ethidium bromide and photographed. It was then soaked in 0.25 M HCl for 15-20 min to depurinate the DNA and rinsed with distilled water. The gel was immersed twice for 15 min in denaturing solution at room temperature with gentle agitation, then rinsed with distilled water and submerged in neutralizing

solution. The DNA was transferred to a nylon membrane (Hybond-N) by vacuum blotting (American Bionics) at 30 mm of Hg in 10X SSC for 1 h. The nylon membrane was air-dried for 10 min, sandwiched between two sheets of Whatman 3 MM paper and baked in a vacuum oven at 80°C for 2 h. Alternatively, the DNA was fixed to the nylon membrane by cross-linking (exposing the membrane to 300 nm UV irradiation for 2-5 min).

B. Radioactive labelling of the DNA probe

The Bethesda Research Laboratories random priming kit was used. DNA (up to 50 ng) was denatured in boiling water for 5 min and immediately cooled on ice for 2-3 min. The labelling reaction contained the following:

Denatured template DNA (50 ng)	5 µl
dATP solution	2 µl
dGTP solution	2 µl
dTTP solution	2 µl
Random primers buffer	15 µl
[α-^{32}P] dCTP (3000 Ci.mmol^{-1}, 10 µCi.µl^{-1})	5 µl
Distilled water	19 µl

These were mixed with a pipette and then 1 µl of Klenow fragment was mixed in. The reaction was carried out at room temperature for 1-2 h, and then 5 µl of stop solution was added. Before hybridization, the probe was denatured in boiling water for 5 min and chilled on ice.

C. Hybridization

The nylon membrane on which the DNA was immobilized was rolled in nylon mesh soaked in 2X SSC, and placed in a

hybridization bottle containing 15 ml of prewarmed (65°C) hybridization solution containing:

20X SSPE	6.25 ml
100X Denhardt's solution	1.25 ml
10% (w/v) SDS	1.25 ml
Denatured salmon sperm DNA (10 $mg.ml^{-1}$)	0.25 ml
Distilled water to	25 ml

The membrane was "pre-hybridized" at 65°C for 1-2 h; then the denatured DNA probe was introduced into the bottle and hybridization was allowed to proceed for 14-16 h at 65°C. The membrane was then washed once with a 2X SSPE, 0.1% SDS mixture for 30 min at room temperature, once with a 1X SSPE, 0.1% SDS mixture at 65°C for 1 h and finally once with a 0.1X SSPE, 0.1% SDS mixture at 60°C for 1 h. After the washing was complete, the membrane was autoradiographed by exposing it to an X-ray film (Kodak, X-omat AR) at -70°C. The exposure time depended on the intensity of the signals. The film was developed with photographic solutions from Kodak Canada Inc., Toronto.

XII. Plaque Hybridization

Pre-titred phage library and *E. coli* LE392 cells were mixed in 7 ml TB top agarose and plated on LB agar in a 15-cm Petri dish. Infection was allowed to develop overnight at 37°C until clear plaques were seen. A membrane was carefully placed on the agarose surface, and its alignment was marked by piercing the membrane and agar with a sterile needle. The membrane was

removed after 1 min and placed for 7 min, plaque side up, on sterile filter paper saturated with denaturing solution. It was then transferred, plaque side up, to a filter paper saturated with neutralizing solution for 3 min. This step was repeated, and the membrane was finally transferred to a fresh filter paper and air-dried for 15 min. The DNA was fixed to the membrane either by UV crosslinking or by baking (see earlier).

XIII. Construction of Restriction Maps

The procedure described by Kohara *et al.* (1987) was followed. Recombinant lambda clones were partially digested with *Sal*I, for which there was no site in the two lambda arms. Lambda DNA partially digested with *Ava*I was used to give size markers. The 2.7 kb *Eco*RI fragment situated at the right end of lambda DNA was used as the hybridization probe. The partial digests were resolved on a 0.4% agarose gel in Tris-phosphate-EDTA buffer as described earlier. The electrophoresis was carried out in a cold room at 15 volts for 72 h.

XIV. Purification of the Etamycin-inactivating Enzyme from *S. lividans* ESR.

A. Preparation of cell extract

Spores of *S. lividans* ESR was inoculated in 500 ml of YEME

medium and incubated for 48 h at 27°C. The mycelium was harvested by centrifugation for 10 min at 5,000 g, and washed twice with Hepes buffer. The cells, resuspended in Hepes buffer, were disrupted by sonication (Branson Sonifier, six pulses of 6 s at 50 MHz with intervening cooling) and centrifuged at 11,000 g for 30 min. The supernatant represented the mycelium extract.

B. Purification of the enzyme

The mycelium extract was fractionated by ammonium sulphate precipitation; the active fraction precipitated between 25% and 85% saturation was collected by centrifugation at 11,000 g for 30 min and redissolved in Hepes buffer (pH 7.6). The solution was heated at 95°C for 10 min and centrifuged at 11,000 g for 30 min. The supernatant was passed through an XM50 ultrafiltration membrane (Amicon Corporation, New Bedford, MA). The eluate contained the partially purified etamycin-inactivating enzyme.

RESULTS

I. Resistance Characteristics of an Etamycin-producing Strain

Streptomyces griseoviridus P-D 04955 produces both griseoviridin, a type-A streptogramin, and etamycin, a type-B streptogramin (Bartz et al., 1955; Anderson *et al.*, 1956; Vazquez, 1967). Recently, it was shown to produce additional but unidentified antibiotics (Zhuge *et al.*, 1992). The strain has not been genetically characterized and its resistance to antibiotics has not previously been investigated.

A. Resistance of *S. griseoviridus* P-D 04955 to various antibiotics

To assess the overall antibiotic resistance phenotype of *S. griseoviridus*, spores were grown on MYM agar containing increasing amounts of various antibiotics, and MICs for the antibiotics were determined. The strain was strongly resistant to etamycin and griseoviridin, but relatively sensitive to all nonautogenous antibiotics, particularly viomycin, streptomycin and erythromycin (Table 3). Since *S. griseoviridus* showed high-level resistance to etamycin, low-level resistance to lincomycin and sensitivity to erythromycin, it does not exhibit a classical MLS resistance phenotype; its phenotype does correspond to the partial MLS resistance conferred by 23S

Table 3. Resistance of *S. griseoviridus* to various antibiotics*.

Antibiotic	MIC ($\mu g.ml^{-1}$)
Griseoviridin	>200
Etamycin	>100
Lincomycin	30-40
Chloramphenicol	10-20
Erythromycin	<10
Viomycin	<10
Streptomycin	<10

* Measured as the minimum concentration of antibiotic at which growth is suppressed (MIC).

rRNA monomethylases such as those encoded by *carB*, which is associated with resistance to lincomycin, vernamycin B and certain macrolides (e.g., carbomycin), but not erythromycin.

B. Frequency of resistant variants

Antibiotic resistance genes are unstable in many *Streptomyces* species and are commonly lost at high frequency when selection pressure is removed (Schrempf *et al.*, 1988). To obtain a strain containing a stably expressed resistance gene, colonies highly resistant to etamycin were selected from the wild type by plating equal numbers of *S. griseoviridus* spores on GYM agar supplemented with increasing concentrations of etamycin. The number of colonies present after 5 days (Table 4) showed that the *S. griseoviridus* spore population is a mixture of types with different degrees of resistance to etamycin. Only a small proportion of the cfu had a high level of resistance (300 $\mu g.ml^{-1}$), but about 50% were resistant to a moderate (50 $\mu g.ml^{-1}$) concentration.

A colony of *S. griseoviridus* that grew on GYM agar supplemented with 300 $\mu g.ml^{-1}$ of etamycin was propagated on the same medium and used to prepare a spore suspension. The spores of this strain (300E) were plated on GYM agar and GYM agar containing 300 $\mu g.ml^{-1}$ etamycin. About 47% of the spores gave colonies on the latter medium. Spores from 300E were then plated on GYM agar containing 400 $\mu g.ml^{-1}$ etamycin and grown until they sporulated. A single colony (designated *S.*

Table 4. Frequency in a wildtype *S. griseoviridus* spore population of strains resistant to various concentrations of etamycin.

Etamycin ($\mu g.ml^{-1}$)	Resistant colonies	
	Number	Frequency
0	2.02×10^3	100%
50	1.06×10^3	52.4%
200	1.55×10^2	7.68%
300	50	2.48%

griseoviridus 400E) was selected and propagated on the same medium to obtain a spore suspension. When portions of the suspension were plated on GYM agar, and on GYM agar containing 300 $\mu g.ml^{-1}$ etamycin, almost equal numbers of colonies were obtained on the two media. The molecular or genetic basis for the increased etamycin resistance in a small fraction of the natural population was not investigated. It may well result from several genetic changes, as observed in variants of *S. fradiae* with increased resistance to tylosin (Baltz and Stonesifer, 1985; Baltz and Seno, 1988). The *S. griseoviridus* genomic DNA used later for cloning experiments was isolated exclusively from strain 400E.

The wild-type *S. griseoviridus* and strain 400E were grown in liquid YEME medium and the culture supernatants were bioassayed against *M. luteus* to determine etamycin production. The wild-type culture showed little activity after 96 h of incubation. In contrast, the supernatant of the 400E culture at 72 h showed strong antibiotic activity. When supernatants of the cultures were extracted with ethyl acetate and the extracts were examined by TLC, samples from 400E cultures showed a strong fluorescent spot at the same R_f value (R_f 0.52) in the benzene:acetic acid:water 42:24:1 solvent system as an etamycin standard; samples from the wild-type cultures showed no detectable fluorescence at this R_f value. These results suggest that selection for etamycin resistance also selected for etamycin biosynthesis.

C. Expression of etamycin resistance during etamycin production

After 48 h incubation at 30°C in liquid YEME medium, *S. griseoviridus* 400E started to produce etamycin. The concentration in the culture increased to approximately 50 $\mu g.ml^{-1}$ at 96 h, and remained unchanged during further incubation. To investigate whether the resistance of *S. griseoviridus* 400E to etamycin differed during different antibiotic production phases, YEME medium alone and supplemented with 100 $\mu g.ml^{-1}$ of etamycin were inoculated with either spores, 24-h (nonproducing) mycelium or 72-h (antibiotic-producing) mycelium. Growth was compared by measuring the OD_{640} of the cultures at 24-h intervals. Those inoculated with spores or with 24-h mycelium grew much more slowly in YEME medium containing 100 $\mu g.ml^{-1}$ etamycin than in YEME medium alone (Fig. 5A and 5B). However, cultures inoculted with 72-h mycelium grew equally well in YEME medium and YEME containing 100 $\mu g.ml^{-1}$ of etamycin (Fig. 5B), suggesting the 72-h mycelium was more resistant than spores and 24-h mycelium. Since spore inocula of *S. griseoviridus* 400E did grow, albeit slowly, whereas spore inocula of *S. griseofuscus* showed no growth after incubation for a week in YEME medium containing 100 $\mu g.ml^{-1}$ of etamycin, *S. griseoviridus* 400E possesses a basal level of resistance at the spore germination and early logarithmic stage. The results indicate that *S. griseoviridus* 400E becomes more resistant to

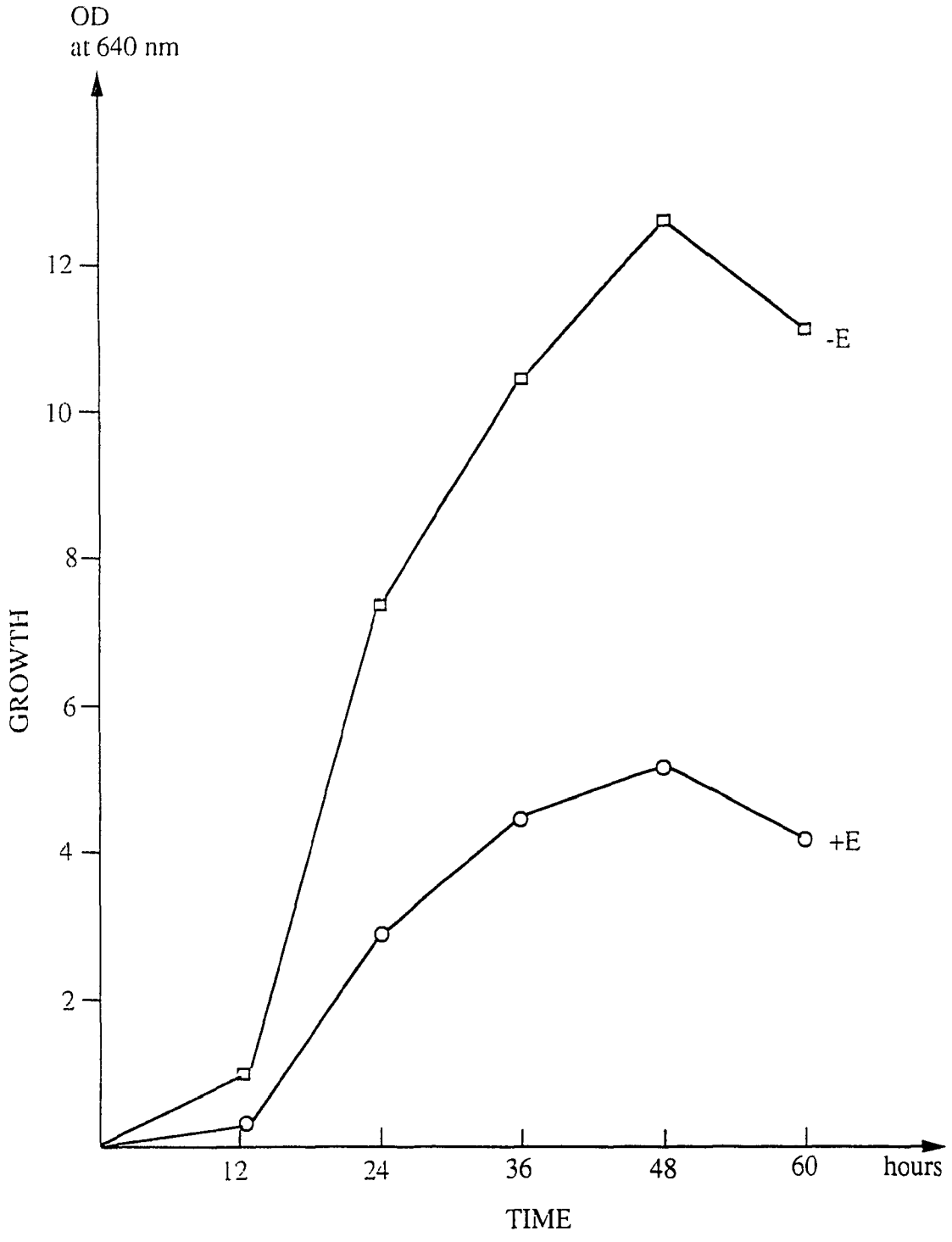


Fig. 5A. Effect of etamycin on the growth (OD at 640 nm) of *S. griseoviridus* 400E. The same number of spores was added to each flask; each OD value is the average from duplicate cultures grown in YEME medium (□), or in YEME medium supplemented with 100 ug.ml^{-1} etamycin (○).

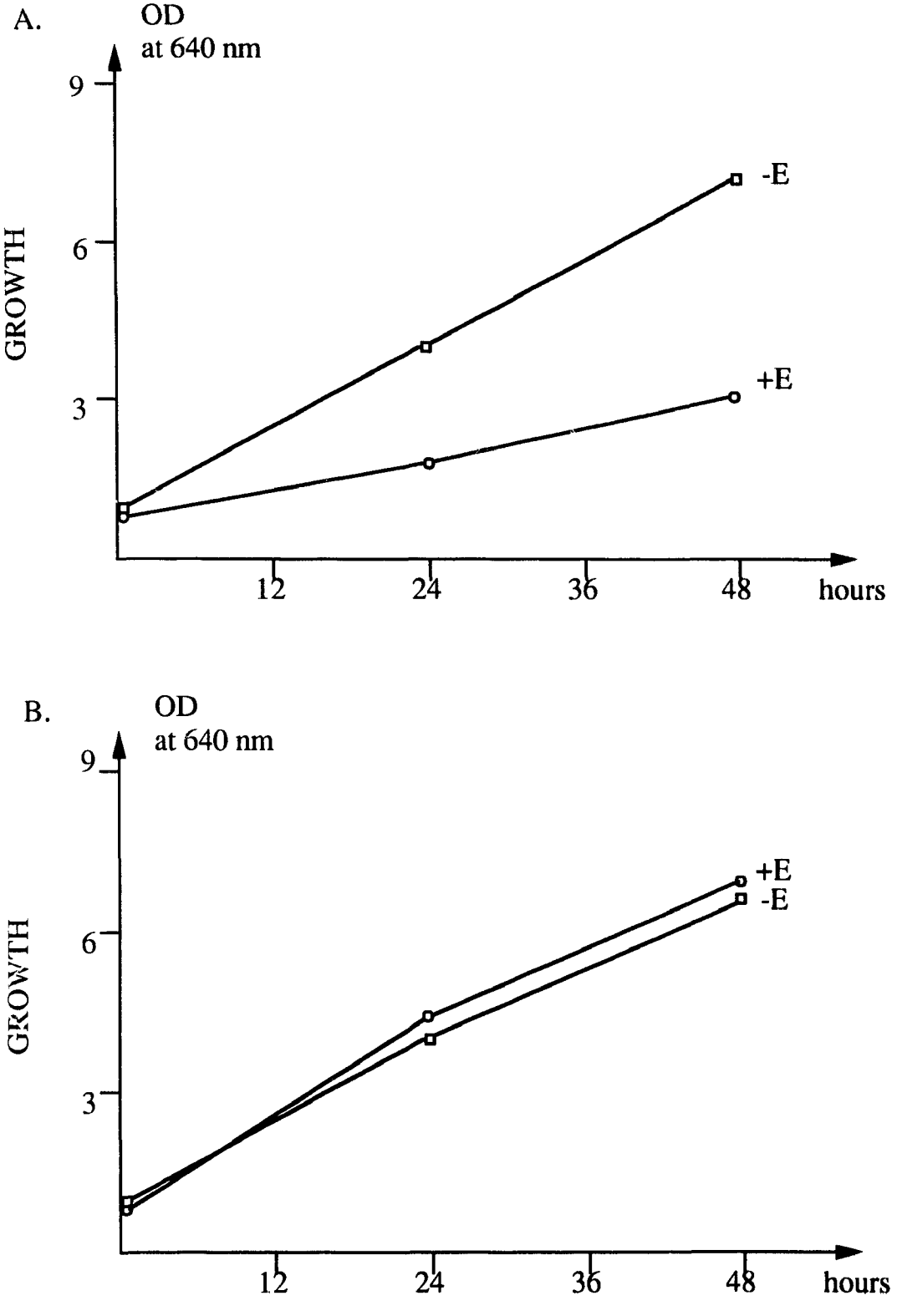


Fig. 5B. Effect of etamycin on the growth *S. griseoviridus* 400E cultures inoculated with A: 24-h old mycelium; B: 72-h old mycelium. Squares represent growth in YEME medium; circles in YEME supplemented with 100 ug.ml^{-1} of etamycin.

etamycin after the onset of etamycin production, and also imply that etamycin can enter the cells during the early growth stage.

II. Resistance Phenotypes of Other Streptomycetes

To assess the value of *S. lividans* and *S. griseofuscus* as heterologous hosts for cloning an etamycin resistance gene, it was necessary to know whether they were resistant to the antibiotic; if they proved to be sensitive, it was important to know how often resistant variants arose, particularly during the conditions used for transformation. When tested on MYM agar, the wild-type *S. lividans* TK24 spore population yielded colonies resistant to 50 $\mu g.ml^{-1}$ of etamycin at a frequency of 1.7×10^{-2}. After protoplasting and regeneration, *S. lividans TK24* gave rise at a frequency $>10^{-6}$ to variants resistant to 200 $\mu g.ml^{-1}$ of etamycin. To determine whether enhanced resistance was due to amplification of a resistance gene, genomic DNA was isolated from the parental *S. lividans* TK24 and from one of the highly resistant variants (referred to as *S. lividans* ESR). After digestion with *Bam*HI and fractionation by agarose gel electrophoresis, the two genomic DNA samples were indistinguishable (data not shown); the resistant variant showed no intense bands indicative of highly amplified DNA sequences.

The wild type *S. griseofuscus* spore population yielded

colonies resistant to 50 $\mu g.ml^{-1}$ of etamycin at a frequency of 4.4 X 10^{-7} cfu. On protoplasting and regeneration, *S. griseofuscus* did not give superresistant variants similar to those obtained from *S. lividans*. This suggested that it would be a better host for cloning etamycin resistance gene(s) than *S. lividans*.

A. MLS resistance phenotypes of *S. lividans* and *S. griseofuscus*

Kamimiya and Weisblum (1988) reported that *S. griseofuscus* is resistant to erythromycin and clindamycin at certain stages of vegetative growth. Conceivably, therefore, *S. griseofuscus* has a gene similar to *lrm*, which in *S. lividans* confers partial MLS resistance. When the resistance of *S. lividans* and *S. griseofuscus* spores to etamycin, lincomycin and erythromycin was compared (Table 5), the phenotype of *S. lividans* proved to be similar to that reported by Jenkins and Cundliffe (1991) in that it showed moderate resistance to all three antibiotics. In contrast, the spores of *S. griseofuscus* were relatively sensitive to all three antibiotics, suggesting that the previously reported resistance to erythromycin and lincomycin was expressed only during growth.

B. Resistance assays using *S. griseofuscus*

a. Inoculum effect

To verify that the resistance of *S. griseofuscus* is

Table 5. Resistance of *S. lividans* and *S. griseofuscus* to MLS antibiotics during outgrowth of spores.

Antibiotic	MIC ($\mu g.ml^{-1}$)[a]	
	S. lividans[b]	*S. griseofuscus*
Etamycin	30-50	<5
Lincomycin	30-50	10-20
Erythromycin	30-50	<10

[a] Spores of *S. lividans* and *S. griseofuscus* were spread on MYM agar containing increasing amounts of an antibiotic; growth was observed after 72 h at 30°C.

[b] Strains of *S. lividans* tested included TK24, TK23, JG10 and 3131; they showed identical phenotypes.

expressed in a growth-dependent fashion, samples from a culture in YEME medium were tested at 12-h intervals on MYM agar containing etamycin (50 $\mu g.ml^{-1}$), lincomycin (25 $\mu g.ml^{-1}$) or erythromycin (50 $\mu g.ml^{-1}$). Under the conditions of the controlled inoculum assay, *S. griseofuscus* failed to grow on the etamycin-containing medium; however, it showed an inoculum age-dependent resistance on both the erythromycin and the lincomycin-containing media (Table 6). Resistance occurred only with logarithmic-phase inocula; mycelium from the stationary phase was considerably more sensitive to both antibiotics. The age of inoculum cultures when resistance was at a maximum was typically from 24 to 48 h. The OD_{640} values of such cultures were between 3 and 8.

To determine the maximum tolerated concentration (MTC) of the antibiotics, mycelium from 24-h *S. griseofuscus* cultures growing logarithmically (OD_{640} in the 3-5 range) was used as the test inoculum. For *S. griseofuscus* the MTC of etamycin was 5-10 $\mu g.ml^{-1}$, and of lincomycin or erythromycin was greater than 50 $\mu g.ml^{-1}$. The increase in resistance of 24 h-old mycelium to etamycin (growth at 5-10 $\mu g.ml^{-1}$) compared with that of spores (growth only at < 5 $\mu g.ml^{-1}$) might be explained by physiological differences between spores and mycelium rather than by the presence of a resistance gene. However, the increase in lincomycin-erythromycin resistance seemed too large to be accounted for by physiological differences. Resistance to both antibiotics was expressed in a similar

Table 6. Resistance of *S. griseofuscus* to MLS-type antibiotics in the controlled inoculum assay.

Inoculum sample (h)	Antibiotic*		
	Ery	Lin	Eta
12	-	-	-
24	+	+	-
36	+	+	-
48	+	+	-
60	-	-	-
72	-	-	-

* Ery = erythromycin; Lin = lincomycin and Eta = etamycin. + = growth; - = no growth of inoculum.

temporal fashion, suggesting that a single genetic determinant might be responsible for both resistances. Since the putative determinant did not confer resistance to etamycin as well, it is unlikely to be a true MLS-type resistance gene.

b. Induction by etamycin

Antibiotic resistance is often induced by the corresponding antibiotic; the presence of an antibiotic resistance gene is then indicated by the resistance phenotype of the organism under inducing conditions. To compare the resistance of induced and uninduced *S. griseofuscus*, seed cultures were grown in YEME medium for 21 h; to a portion of the culture, 0.5 $\mu g.ml^{-1}$ etamycin was added and incubation of both the induced and uninduced portions was resumed for 3 h. Visual examination and OD_{640} measurements indicated that etamycin at 0.5 $\mu g.ml^{-1}$ slightly inhibited the growth of *S. griseofuscus*. The MTC of the induced culture (10-20 $\mu g.ml^{-1}$) was significantly higher than that of the uninduced culture (5-10 $\mu g.ml^{-1}$). Preliminary observations indicated that resistance could be further increased by increasing the amount of etamycin used for induction and the length of the induction period, but the increases were not quantified. The results suggested that *S. griseofuscus* possesses a normally silent etamycin resistance gene.

C. Inactivation of etamycin

a. Etamycin inactivation by *S. griseoviridus* and *S. lividans*

To investigate whether etamycin was inactivated by a lactonase of the type reported in several streptogramin-producing streptomycetes (Hou *et al.*, 1970; Kim *et al.*, 1974), the ability of *S. griseoviridus* 400E and *S. lividans* ESR1 to inactivate etamycin was examined. Because enzymatic inactivation of streptogramin has not been reported in *S. lividans* TK24, this species was included as a potentially negative control. Etamycin-inactivating activity was assayed in both culture supernatants and mycelial extracts at intervals during growth in YEME medium (pH 7.3). No activity was detected in either culture supernatants or cell extracts of *S. griseoviridus* (Table 7). In contrast, strong activity was detected in cell extracts of *S. lividans* ESR1. The activity was present throughout growth. Cell extracts of *S. lividans* TK24 showed weak etamycin-inactivating activity.

To further investigate etamycin inactivation in *S. griseoviridus* 400E, the antibiotic activity in supernatants of cultures grown for 192 h in four different media buffered with 0.1 M MOPS at pH 7.3 was bioassayed against *M. luteus* at intervals (Table 8). Media 2 and 4, which contained soybean meal, supported the highest etamycin production. The antibiotic activity in three of the media (1, 2 and 4) dropped slightly during the incubation period; since *S. griseoviridus* produces several antibiotics in addition to etamycin, the drop in antibiotic activity was not necessarily due to a decrease in etamycin concentration. In cultures grown in media 1, 2 and

Table 7. Antibiotic activity[a] in etamycin inactivation assay mixtures.

Sampling time (h)	*S. lividans* TK24		*S. lividans* ESR1		*S. griseoviridus* 400E	
	CS	ME	CS	ME	CS	ME
0	26	-	26	-	26	-
24	26	25	26	ni	26	26
48	26	21	26	ni	26	26
72	26	21	26	ni	26	26
96	26	21	26	ni	27[b]	26

Culture supernatant (CS) was obtained by centrifuging cultures at 11,000 g; mycelium extract (ME) was prepared as described in Materials and Methods.

[a] Antibiotic activity was measured as the inhibition zone diameter in mm; the diameter of the paper disk used for bioassay was 13 mm. Ni, no inhibition zone. As a control, etamycin (100 $\mu g.ml^{-1}$) was incubated in HEPES buffer (pH 7.6) at 37°C for 3 h. The inhibition zone diameter given by the control was 26 mm.

[b] The small increase in antibiotic activity in 96-h CS of *S. griseoviridus* was due to etamycin produced by the strain.

Table 8. Antibiotic activity[a] in culture supernatants of *S. griseoviridus* 400E grown in four media.

Medium[b]	Sampling time (h)							
	24	48	72	96	120	144	168	192
1	ni	ni	15	16	17	19	18	18
2	ni	19	20	20	23	23	22	20
3	ni	ni	ni	ni	16	16	16	16
4	ni	18	21	21	21	22	22	21

[a] Antibiotic activity was measured as the diameter of the zone of inhibition against *M. luteus*. The diameter of the paper disk used for bioassay was 13 mm; ni, no inhibition zone. Details of the media are given in Materials and Methods.

[b] The compositions of the media were: 1 - glucose 1%, sucrose 4%, yeast extract 0.3 %, malt extract 0.3 % and peptone 0.5%; 2 - sucrose 5%, yeast extract 0.1 %, malt extract 0.1%, peptone 0.1% and soybean meal 0.5%; 3 - glucose 1%, lactose 4%, yeast extract 0.3%, malt extract 0.3% and peptone 0.5%; 4 - glucose 1%, lactose 4%, yeast extract 0.1%, malt extract 0.1%, peptone 0.1% and soybean meal 0.5%.

4, etamycin was specifically assayed for 144 h by HPLC; no appreciable changes in the amounts of this antibiotic were observed after it reached its maximum concentration (Fig. 6). The results suggested that *S. griseoviridus* does not possess an etamycin-inactivating enzyme.

b. Etamycin inactivation in *S. griseofuscus*

Since spores of *S. griseofuscus* are extremely sensitive to etamycin, the putative etamycin resistance gene is most likely expressed in cultures at the mycelium stage. The ability of *S. griseofuscus* to inactivate etamycin was tested by measuring etamycin concentrations in cultures grown in YEME medium. Etamycin was added at a final concentration of 50 $\mu g.ml^{-1}$ to the culture after it had grown for 24 h; incubation was then resumed for 144 h. At intervals, the etamycin concentration in the culture supernatant was measured by HPLC. The etamycin peak area slowly decreased with time and a new peak was observed at a shorter retention time than etamycin. The retention time matched that of an etamycin metabolite formed by *S. lividans* ESR, suggesting the two compounds might be identical.

c. Etamycin inactivation in *S. lividans* ESR

To determine whether the etamycin-inactivating activity in cell extracts of *S. lividans* ESR was due to an enzyme, the extract was heated at 50°C, 65°C, 85°C, 95°C or 100°C for 30 min. Another sample was incubated with proteinase K. Enzyme activity was monitored by two methods: (i) by bioassaying the

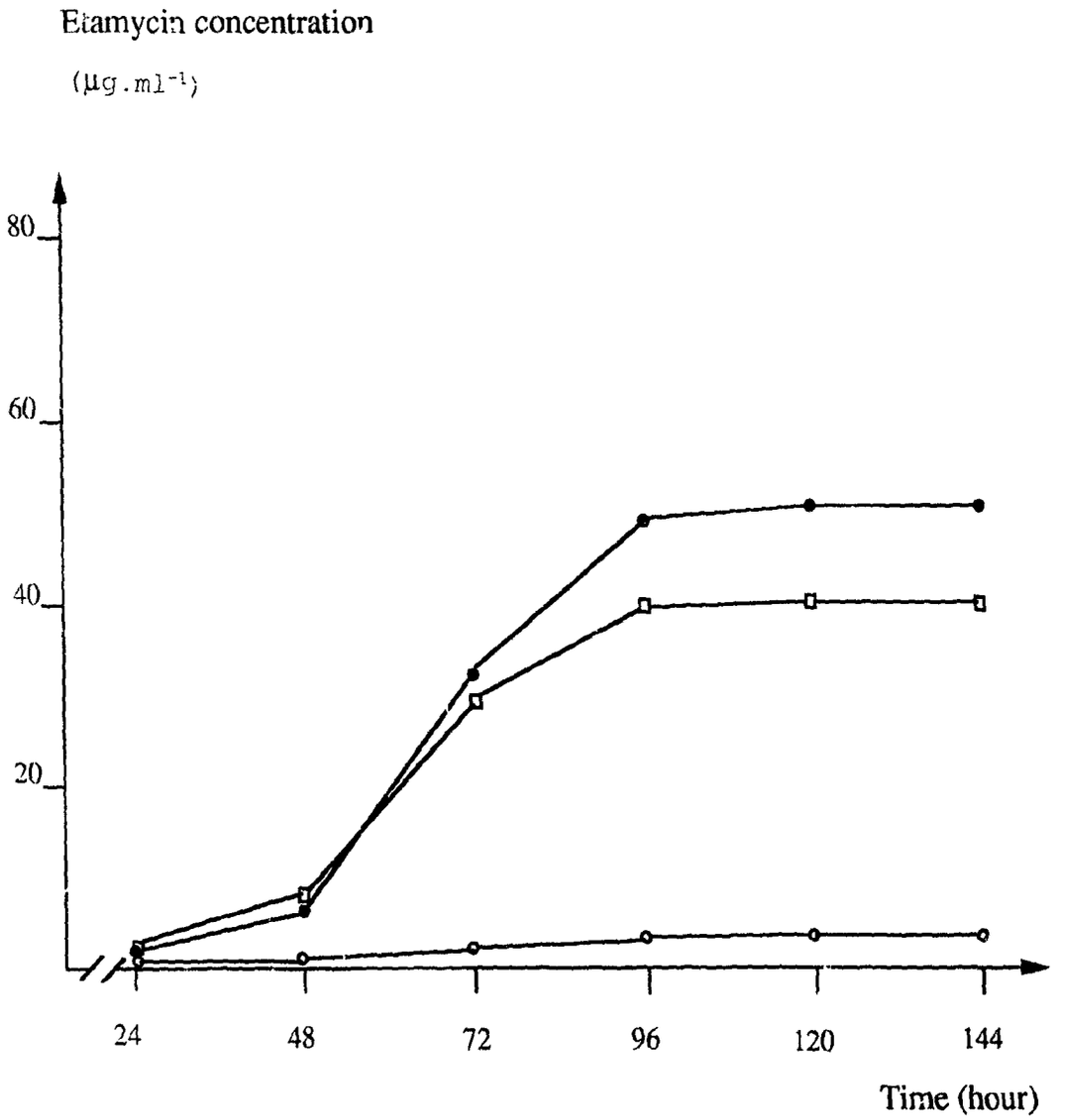


Fig. 6. Etamycin concentration in culture supernatants of *S. griseoviridus* 400E grown in media 1 (○), 2 (●) and 4 (□) of Table 8.

antibiotic activity in a reaction mixture containing etamycin as the substrate and buffered cell extract or treated samples thereof; (ii) by extracting the reaction mixture at neutral and then acidic pH with ethyl acetate and measuring the absorbance at 305 nm of both extracts; an increased absorbance in the acidic extract indicated the presence of enzyme activity. Each sample was assayed by both methods and the values were compared. The bioassay method was reliable but time consuming; the absorbance method was quicker but was subject to interference from other substances that absorb at 305 nm in the enzyme preparations. Both methods showed that heating at 100°C for 30 min or treatment with proteinase K completely abolished the ability of the cell extracts to inactivate etamycin. However, the enzyme activity was relatively heat-resistant; heating at 65-95°C for 30 min had little effect.

The enzyme was partially purified by the procedure outlined in Fig. 7. The heating step and subsequent centrifugation eliminated 95% of the soluble protein and PAGE showed only a few protein species in the remaining active fraction. After further fractionation the sample contained predominantly one protein with a size of approximately 40 kDa. Samples examined by both denaturing and native PAGE showed a single band, indicating that the protein was a monomer (data not shown).

Attempts were also made to characterize the inactivated product of etamycin. To examine whether the compound was the

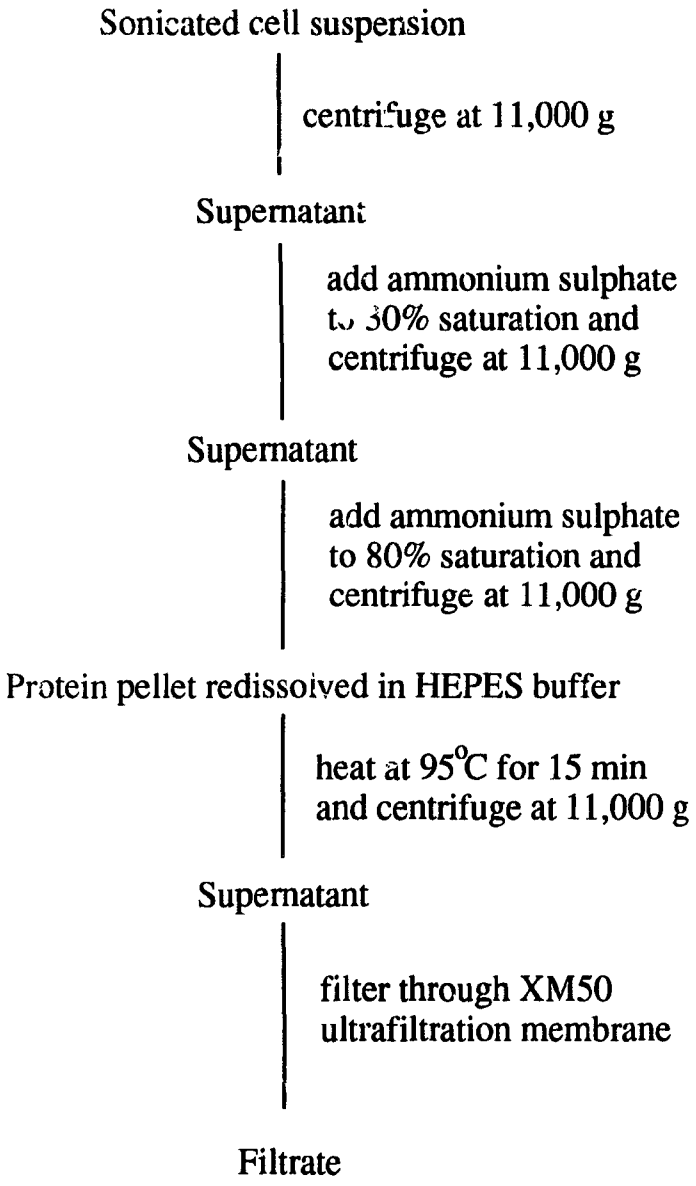


Fig. 7. Procedure used to partially purify the etamycin-inactivating enzyme from *S. lividans* ESR.

same as etamycinic acid generated by alkaline hydrolysis of etamycin, the latter was compared by HPLC and TLC with a sample prepared by incubation of the antibiotic with a cell extract from *S. lividans* ESR. The results suggested that the two compounds were different. The unknown compound was considerably more polar than etamycin and it was extractable into ethyl acetate only at acidic pH. Its retention time by HPLC was affected by pH but not to the same extent as the etamycinic acid.

III. Hybridization of *S. griseoviridus* Genomic DNA with an *ermE* Fragment

An earlier experiment (Fig. 5) showed that *S. griseoviridus* could grow in a medium containing etamycin, but growth was slowed significantly. This suggested that the ribosomes were still accessible to the antibiotic, but were not severely inhibited by the intracellular concentrations present. One possible explanation for this result was that the ribosomes of *S. griseoviridus* were modified by a methylase of the type encoded by *carB*. The presence of a methylase gene in *S. griseoviridus* might be detected by hybridization of genomic DNA with a probe derived from a known methylase gene. Since only the *ermE* and *ermSF* genes were available for use at the time this project started, *ermE* was chosen.

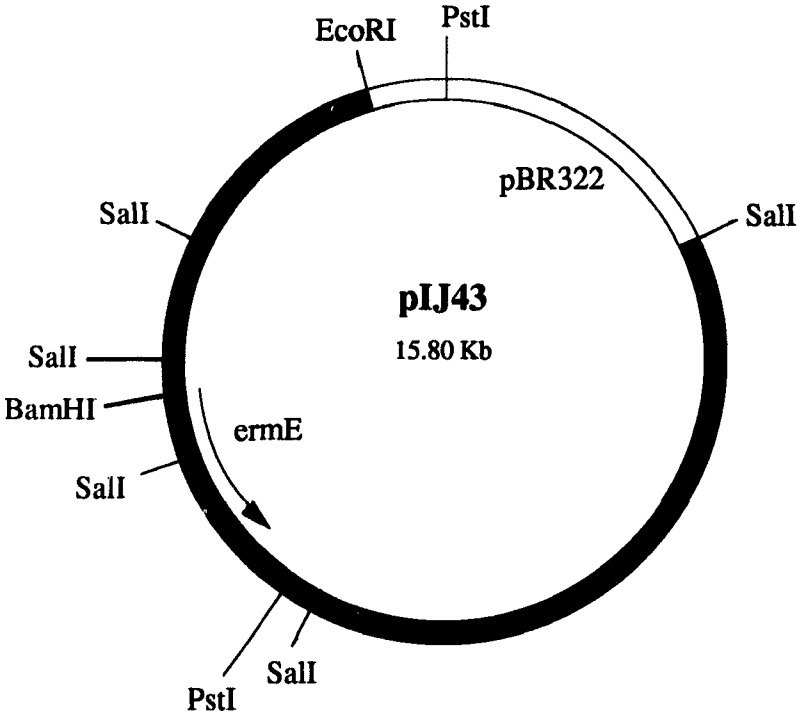
A. Preparation of the probe

The *ermE* gene was available on plasmid pIJ43 (Fig. 8A) (Thompson *et al.*, 1982a). The coding region of *ermE* (Fig. 8B) has been localized to a 2.2-kb *Bam*HI:*Pst*I fragment, within which is 580-bp *Bam*HI:*Sal*I fragment encoding most of the conserved amino acids of the known *erm* proteins; these are near the N-terminus (Uchiyama and Weisblum, 1985). The 2.2-kb *Bam*HI:*Pst*I was isolated and digested with *Sal*I. The 580-bp *Bam*HI:*Sal*I fragment was isolated and purified for use as a hybridization probe.

B. Southern hybridization with *S. griseoviridus* genomic DNA

To assess whether *S. griseoviridus* contains DNA with sequence similarity to *ermE*, samples of genomic DNA from two streptomycetes (*S. griseoviridus* and *S. lividans*) and from *Sac. erythraea* (the source of *ermE*) were hybridized with the *ermE* probe in a slot blot and washed at various stringencies. The genomic DNA of *S. griseoviridus* showed stronger hybridization than did that of *S. lividans*, but weaker hybridization than did the DNA of *Sac. erythraea* (Fig. 9). The hybridization signals with *S. griseoviridus* survived washing in 0.1X SSPE at 60°C, a stringency that allows hybridization of DNA sharing 60-70% sequence similarity based on the calculation proposed by Hopwood *et al.* (1985). The result suggested that an *ermE*-related gene was present in *S. griseoviridus*.

A.



B.

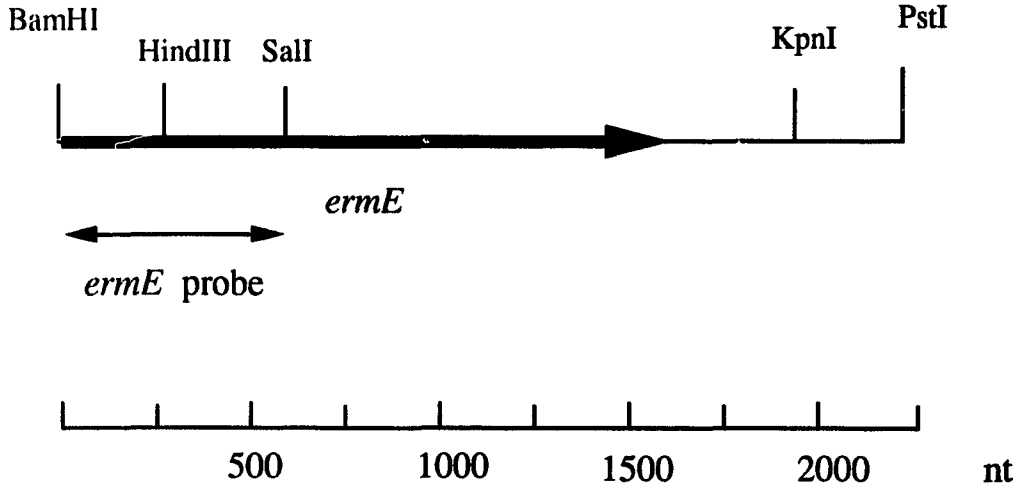


Fig. 8. A: Restriction map of pIJ43. The bifunctional plasmid carries SLP1.2 and pBR322 replicons and *ermE* of *Sac. erythraea*. The unfilled segment represents DNA from pBR322; the filled segment represents DNA from SLP1.2 and *ermE*. B: Linear map of the *ermE* region showing the fragment used as a probe.

Fig. 9. Hybridization of pIJ43, and genomic DNA from *Sac. erythraea*, *S. griseoviridus* 400E and *S. lividans* DNA with the ^{32}P-labelled *ermE* probe. DNA samples were collected on the membrane by vacuum filtration. The amount of genomic DNA applied (in µg) is indicated at the right hand margin. The amount of pIJ43 DNA applied was 0.01 µg. The membrane was washed with a mixture of 0.1X SSPE and 0.1% SDS at 60°C. The DNA samples were: a, pIJ43; b, *Sac. erythraea*; c, *S. griseoviridus*; d, *S. lividans* TK24.

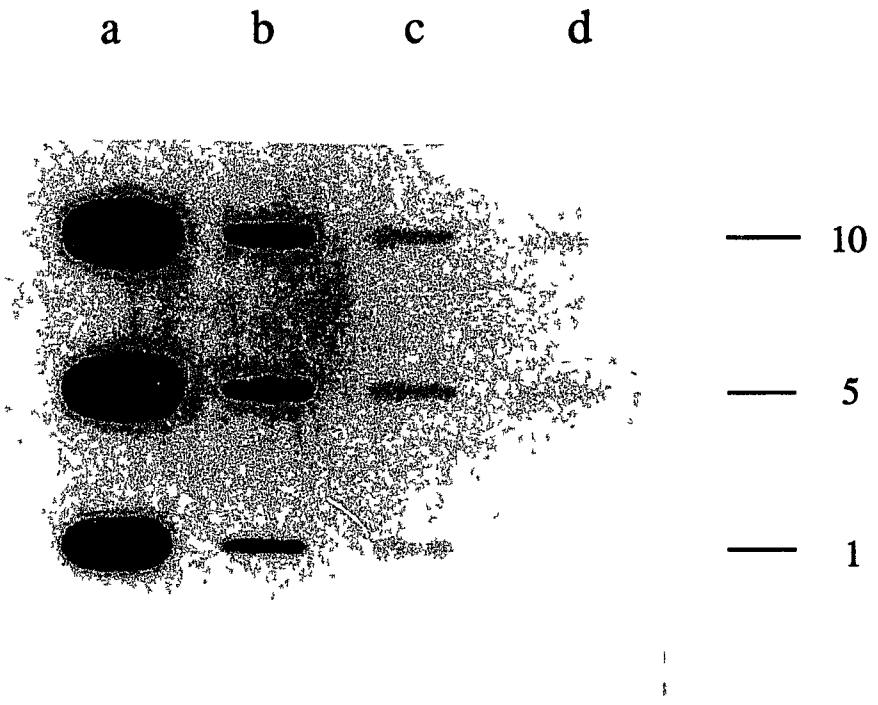


Fig. 9.

*Sau*3A1 and *Sal*I digests of *S. griseoviridus* 400E genomic DNA were resolved by electrophoresis on an agarose gel (Fig. 10A), transferred to a nylon membrane and hybridized to the *ermE* probe. After the membrane was washed in 0.1X SSPE at 60°C, the *Sau*3A1 digest of *S. griseoviridus* 400E genomic DNA did not show distinct hybridization signals, probably because the DNA fragments in the digest were too small. The *Sal*I digest gave two distinct bands, one of 4.3 kb and the other of 0.6 kb (Fig. 10B). To clone the potentially *ermE*-like resistance gene(s) in these bands, two alternative strategies were considered: one required excising each hybridizing fragment from an agarose gel, ligating it into a plasmid vector and identifying the desired plasmid by colony hybridization; the other was based upon construction of an *S. griseoviridus* genomic library in a lambda replacement vector and identification of the correct clones by plaque hybridization. The latter was chosen because it is usually a less time-consuming procedure, and might also give adjacent biosynthesis genes if these were clustered with the resistance gene. Moreover, it was anticipated that the 0.6 and 4.3-kb fragments might represent segments of a single hybridizing sequence, or might be closely linked on the *S. griseoviridus* genome.

C. Construction of an *S. griseoviridus* genomic library in lambda GEM11

Fig. 10. Southern hybridization of *S. griseoviridus* 400E genomic DNA.

Left panel: Agarose gel electrophoresis of DNA samples: lanes a and f, pIJ43 digested with both *Bam*HI and *Pst*I; lane b, *S. griseoviridus* genomic DNA (10 µg) digested with *Sal*I; lane c, *S. griseoviridus* genomic DNA (20 µg) digested with *Sal*I; lane d, *S. griseoviridus* genomic DNA (10 µg) digested with *Sau*3A1; lane e, lambda DNA digested with *Hin*dIII.

Right panel: Autoradiogram after transfer of the electrophoresed DNA samples to a nylon membrane and hybridization with the ^{32}P-labelled *ermE* probe. In lanes a and f, the probe hybridized predominantly with the 2.2-kb *Bam*HI:*Pst*I fragment of pIJ43, which contains the probe sequence. Other signals in lane a and f were due either to partial digestion of pIJ43 or to contamination of the probe with other pIJ43 fragments.

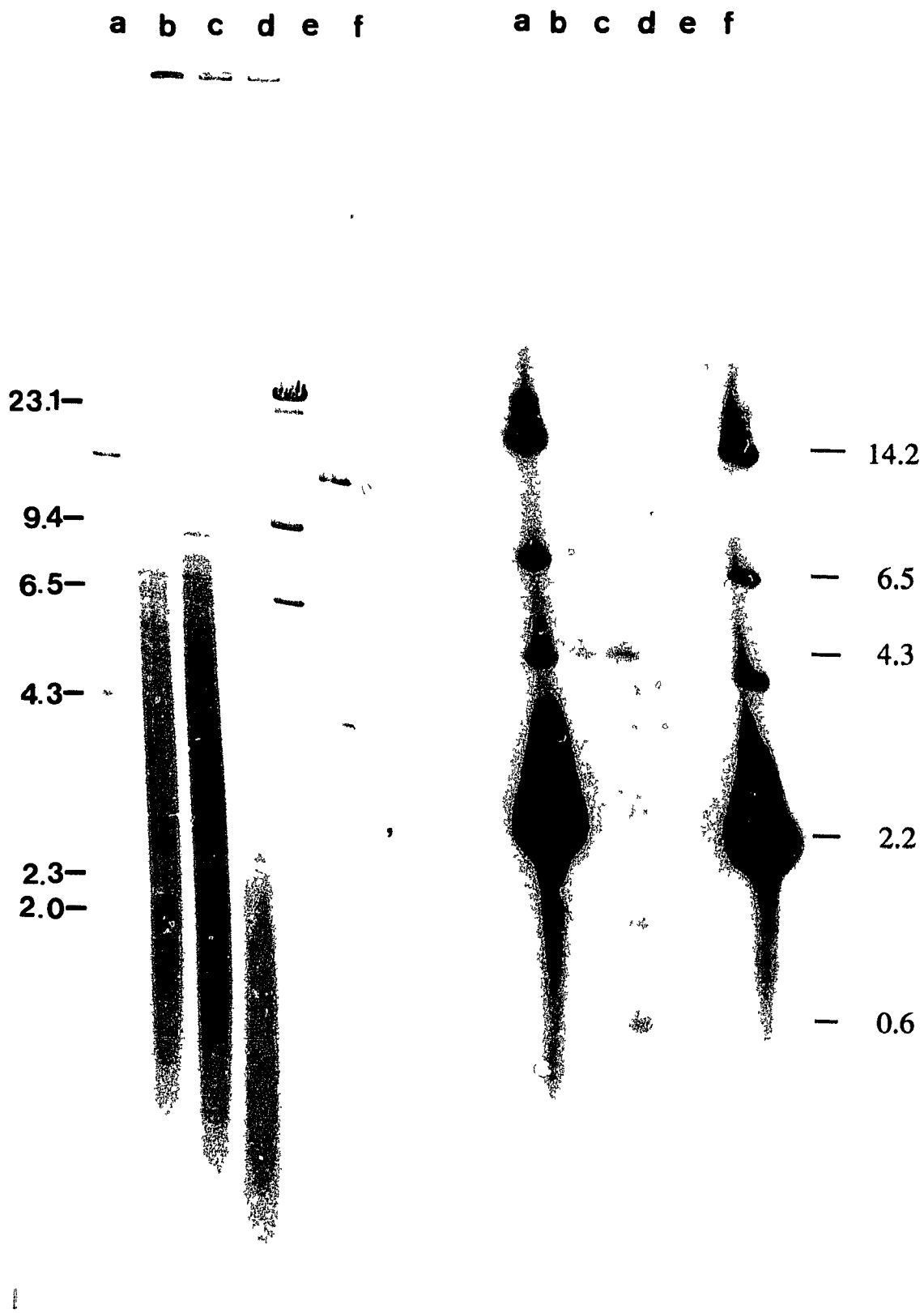


Fig. 10.

The procedure used to construct the library is outlined in Materials and Methods (see Fig. 4). Genomic DNA from *S. griseoviridus* 400E was partially digested with the restriction enzyme *Sau*3A1 and size-fractionated by sucrose-density-gradient centrifugation. DNA fragments in the size range 9 to 23 kb were ligated to the arms of the lambda GEM11 vector using the *Xho*I half-site cloning strategy, and were packaged into phage particles. The library contained 50,000 recombinant phage particles (10^6 pfu.ml^{-1}), as determined by plating a small portion of it. A library of 30,000 phage particles was amplified and stored. The rest was used to screen for lambda clones that hybridized with *ermE*.

D. Probing the lambda library with the *ermE* probe.

To isolate recombinant phages that hybridized with the *ermE* probe, up to 20,000 phages were plated on agar in two 15-cm Petri plates and incubated until the phage plaques were fully developed. Phage DNA was blotted on a nylon membrane and hybridized with the *ermE* probe. The membrane was washed at high stringency (0.1X SSPE/0.1% SDS, 60°C). Many potentially positive signals were detected on the two blots, one of which is shown in Fig. 11. All positive signals were excised from the plates and six were chosen for further purification. Phages collected from the area corresponding to each of the six positive isolates were plated at low density to allow well separated plaques to develop. The phage DNA was blotted again

Fig. 11. Autoradiogram of the nylon membrane blotted on the *S. griseoviridus* genomic DNA library in lambda GEM 11 and hybridized with the ^{32}P-labeled *ermE* fragment.

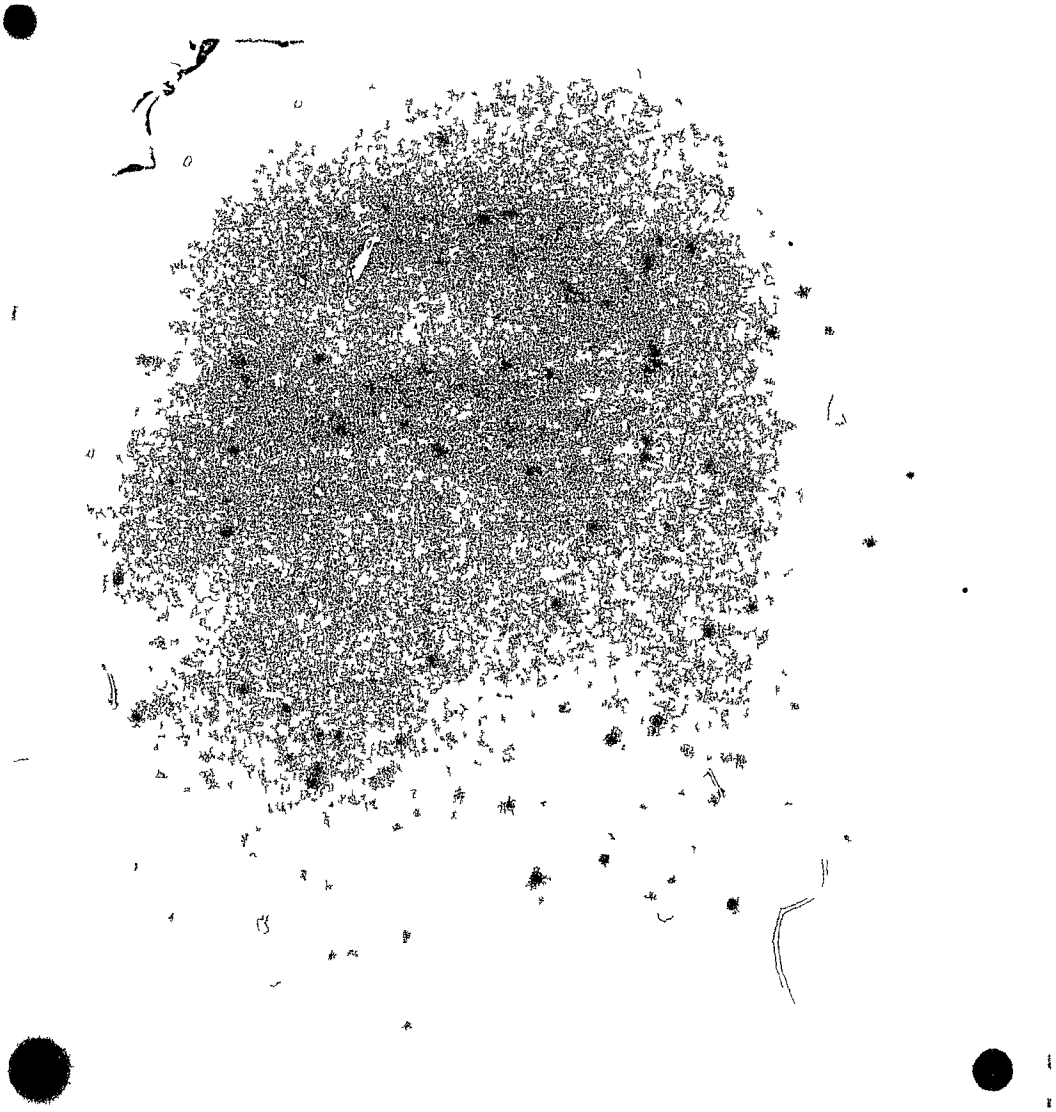


Fig. 11.

on a nylon membrane and hybridized with the *ermE* probe; hundreds of hybridizing plaques were obtained. Single plaques that hybridized with *ermE* were identified and several were saved. The homogeneity of phage in the single plaques was tested by plating each isolate at low density and hybridizing with *ermE;* a recombinant phage was considered pure when a hybridization signal was obtained from every plaque.

E. Restriction analysis of lambda clones and localization of the *ermE*-hybridizing fragment

The six purified lambda clones, designated lambda KY1 to KY6, were amplified and stored. DNA was isolated from each and was digested with various restriction enzymes. Since *SalI*, *SacI* and *SfiI* could cut the lambda vector DNA only in the polylinker regions, digestion of the recombinant lambda DNA with these enzymes provided information about the sizes of the cloned inserts. The presence of more than one fragment additional to the vector DNA indicated that the insert contained sites for the enzmye (Table 9). Since *SacI* cut rarely in the *S. griseoviridus* genome, the insert size could usually be estimated from the combined sizes of *SacI* digest fragments. Although *SfiI* recognizes an 8-base site and is designed for retrieving the cloned insert, the site is rich in G+C; *SfiI* cuts the *S. griseoviridus* insert more frequently than *SacI*.

SalI restriction maps were constructed for the DNA inserts from lambda clones KY1 and KY2 (Fig. 12). The two clones

Table 9. Size (in kb) of restriction fragments from recombinant lambda clones KY1-KY6.

Source of DNA	Restriction enzyme			Insert size (kb)
	*Sac*I	*Sfi*I	*Sal*I	
KY1	9.6; 7.8	6.7; 6.1; 4.3	4.3; 3.6; 2.5; 1.8; 1.5; 1.4; 1.1; 0.8; 0.7; 0.5; 0.45; 0.2	17.4
KY2	12.7	N/T	4.3; 1.0; 0.9; 0.8; 0.7; 0.6; 0.5; 0.3	12.7
KY3	9.0; 5.6	N/T	4.3; 3.6; 2.5	14.6
KY4	18.0	N/T	4.1; 3.6	18.0
KY5	11.0; 6.1	N/T	4.3; 3.6; 2.5; 1.8; 1.5; 1.4; 1.1; 0.5	17.1
KY6	11.5; 6.8	N/T	4.3; 3.6; 2.5; 1.8; 1.5; 1.4; 1.1	18.3

N/T, not tested. Digests of the DNA from all clones gave the 9-kb and 20-kb lambda arms of the recombinant phages. The combined *Sal*I fragments from KY2-KY6 did not match the insert size based on *Sac*I digestion because many small *Sal*I fragments were not included.

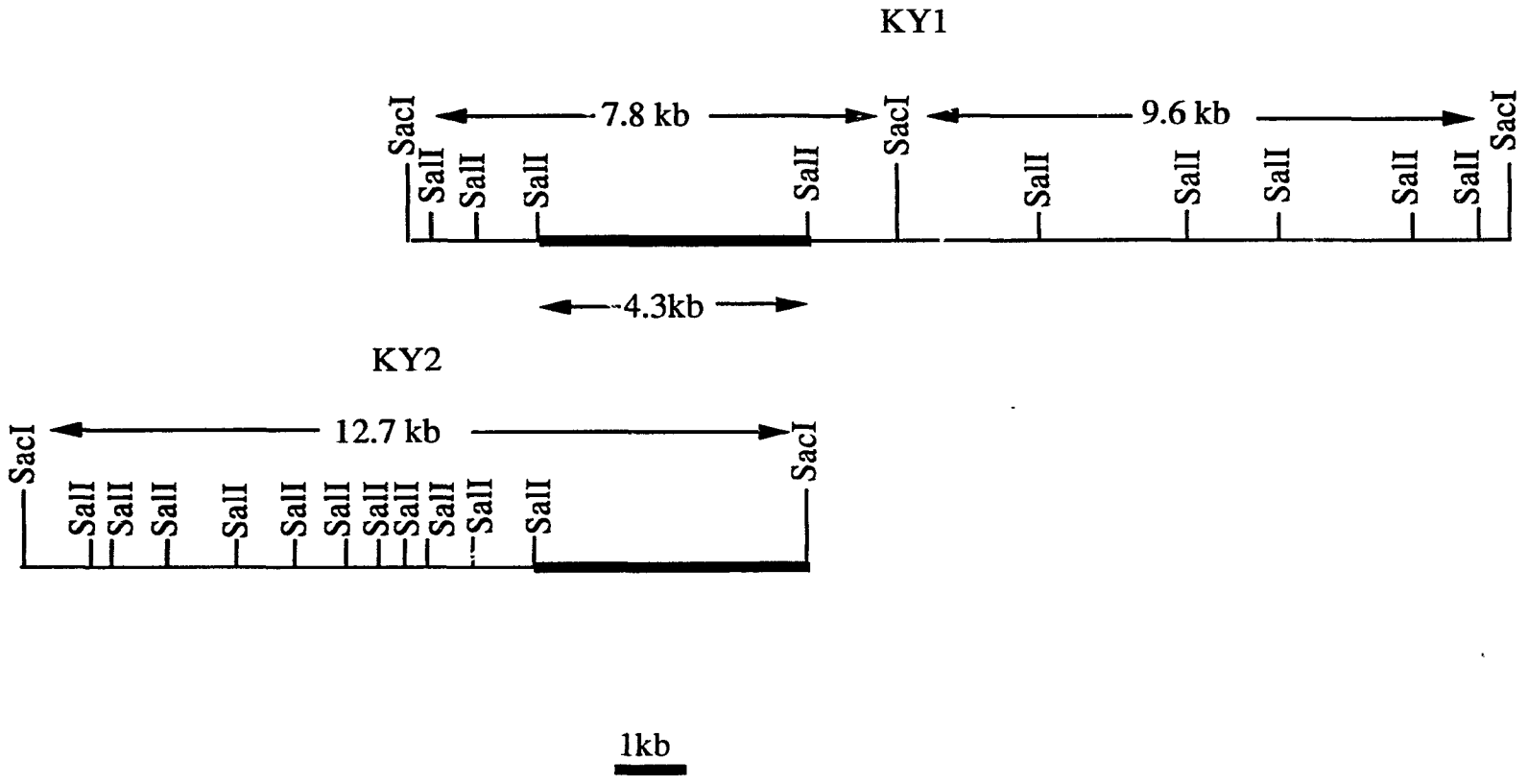


Fig. 12. *Sal*I restriction maps of the DNA from lambda clones KY1 and KY2. The *Sac*I sites at the left and right ends of each insert are derived from the polylinker region of the lambda vector. The *ermE*-hybridizing fragment is shown as a black bar.

shared an overlapping region containing a 4.3-kb *Sal*I fragment. The size of the latter corresponded to that of a *Sal*I fragment detected during Southern hybridization of *S. griseoviridus* genomic DNA with the *ermE* probe (see Fig. 10).

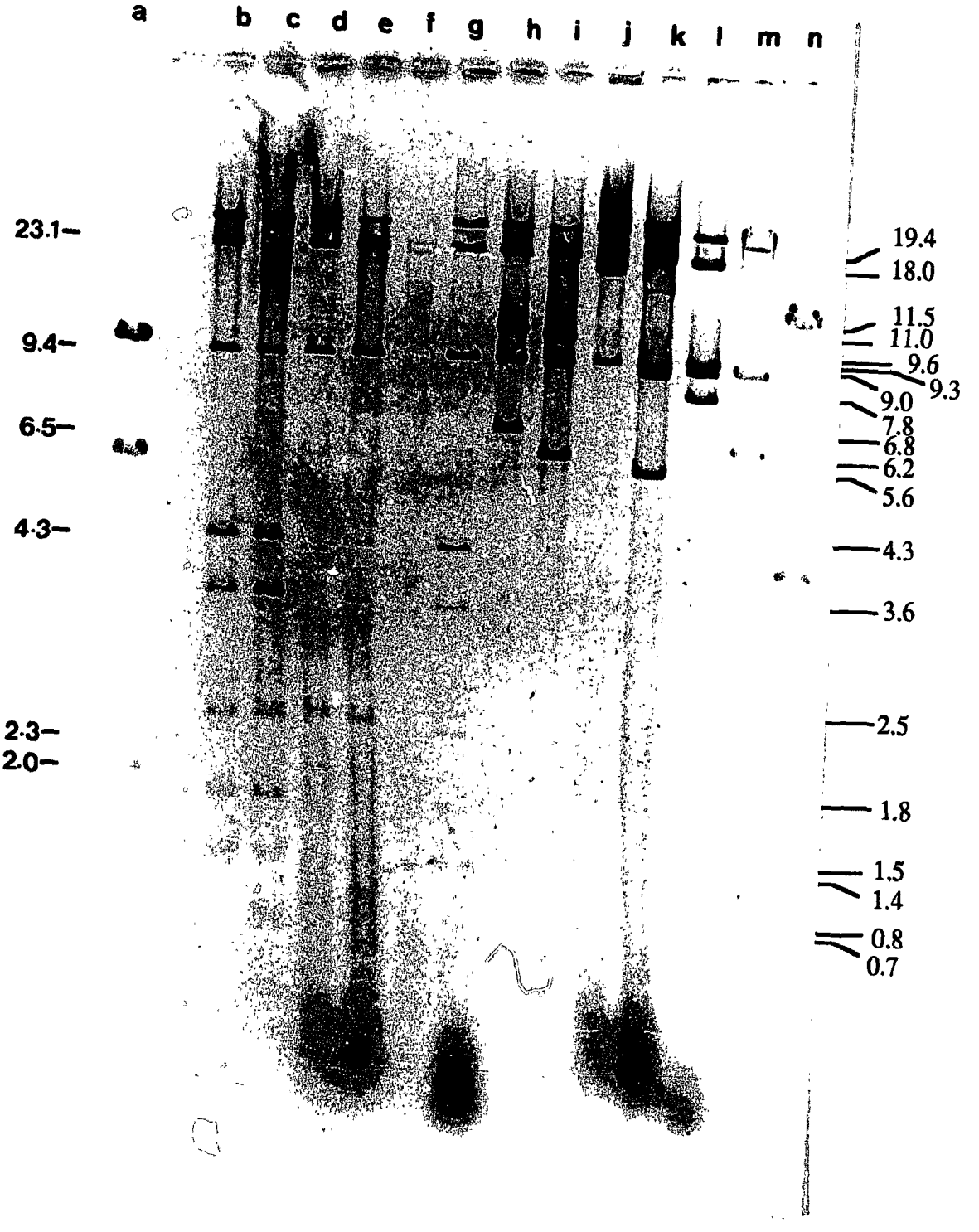
Within all six lambda clones the region(s) of recombinant DNA that hybridized with the *ermE* probe were located by Southern hybridization (Fig. 13A). In restriction enzyme digests of the DNA from KY1, KY3, KY5 and KY6, hybridization was detected with a 4.3-kb *Sal*I fragment (Fig. 13B). In KY4 the *Sal*I-fragment that hybridized to *ermE* was slightly smaller than 4.3 kb. The overall distribution of *Sal*I restriction fragments was similar to that of KY2 (data not shown), indicating that KY2 and KY4 shared overlapping DNA fragments. To confirm that the 4.3-kb *Sal*I fragment hybridized specifically with the *ermE* probe, the 7.7-kb *Sac*I fragment of KY1 (containing the 4.3-kb *Sal*I fragment) was used to probe a *Bam*HI and *Sal*I double digest of pIJ43 by Southern hybridization. A signal was detected only at 580 bp, corresponding to the *ermE* probe fragment. When the same probe was used to hybridize with the *Sal*I digest of *S. griseoviridus* DNA, hybridization signals were observed at the expected positions (0.5 kb, 0.7 kb, 0.8 kb, 3.7 kb and 4.3 kb) indicating that the 7.7-kb fragment originated from *S. griseoviridus*.

Although an earlier Southern hybridization experiment had identified two *ermE*-hybridizing fragments (one of 4.3 kb and

Fig. 13. Southern hybridization of DNA from pIJ43, lambda and lambda clones KY1-KY6 using the *ermE* probe. Fragment sizes (kb) are indicated in the left-hand (lambda) and right-hand (lambda clones) margins.

(A). Gel electrophoresis of the DNA samples: lane a, from pIJ43 digested with *Sal*I and *Bam*HI; lane b, from KY6 digested with *Sal*I; lane c, from KY5 digested with *Sal*I; lane d, from KY4 digested with *Sal*I; lane e, from KY3 digested with *Sal*I; lane f, from KY2 digested with *Sal*I; lane g, from KY1 digested with *Sal*I; lane h, from KY6 digested with *Sac*I; lane i, from KY5 digested with *Sac*I; lane j, from KY4 digested with *Sac*I; lane k, from KY3 digested with *Sac*I; lane l, from KY1 digested with *Sac*I; lane m, from lambda digested with *Hin*dIII; lane n, from pIJ43 digested with *Pst*I and *Bam*HI. Due to difficulty in isolating sufficient DNA from KY2, the SacI digest of this sample was omitted.

(B). Autoradiogram after transfer of the electrophoresed DNA samples to a nylon membrane and hybridization with the *ermE* probe. The signals in lane a and n were due to contamination of the *ermE* probe with other pIJ43 fragments.


a
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6.5
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11.0
9.6
9.3
9.0
7.8
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4.3
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1.8
1.5
1.4
0.8
0.7

Fig. 13A.

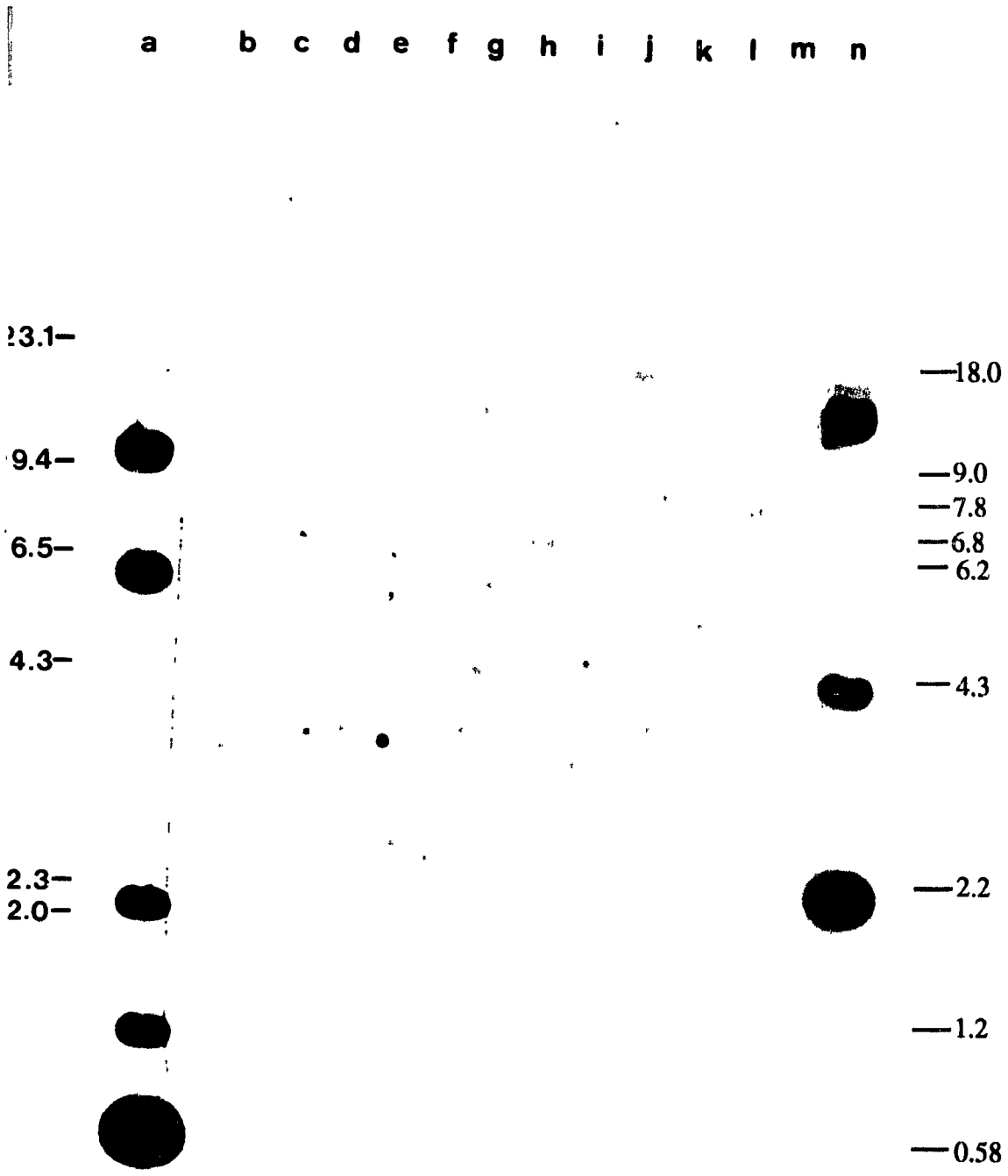


Fig. 13B.

the other of 0.6 kb) in a *Sal*I digest of *S. griseoviridus* genomic DNA, the six lambda clones examined contained only the 4.3-kb *Sal*I fragment. Since the 0.6-kb fragment is small and might be lost in DNA transfer, the hybridization experiment was repeated taking special care to effectively transfer small fragments from agarose gels to nylon membranes; however, no signal corresponding to a 0.6- kb fragment was detected. The combined evidence from restriction analysis, *Sal*I restriction maps of KY1 and KY2, and the location of the 4.3-kb *Sal*I fragment on the *Sac*I insert fragments indicated that the physical relationships of the DNA inserts in the six lambda clones were as shown in Fig. 14. The absence of the 0.6-kb hybridizing fragment from the approximately 30-kb genomic DNA sequence cloned in the lambda vector indicated that it was not linked to the 4.3-kb fragment. At this point in the study, attention was focused on the cloned region containing the 4.3-kb fragment, and the 0.6-kb fragment was not investigated further.

F. Sub-cloning of DNA fragments in a *Streptomyces-Escherichia coli* shuttle vector.

To investigate whether the cloned *ermE*-hybridizing fragments from *S. griseoviridus* did indeed contain an *ermE*-like gene, efforts were made to subclone the recombinant lambda clone insert in plasmid vectors that usually allow expression of resistance genes in a *Streptomyces* host. The

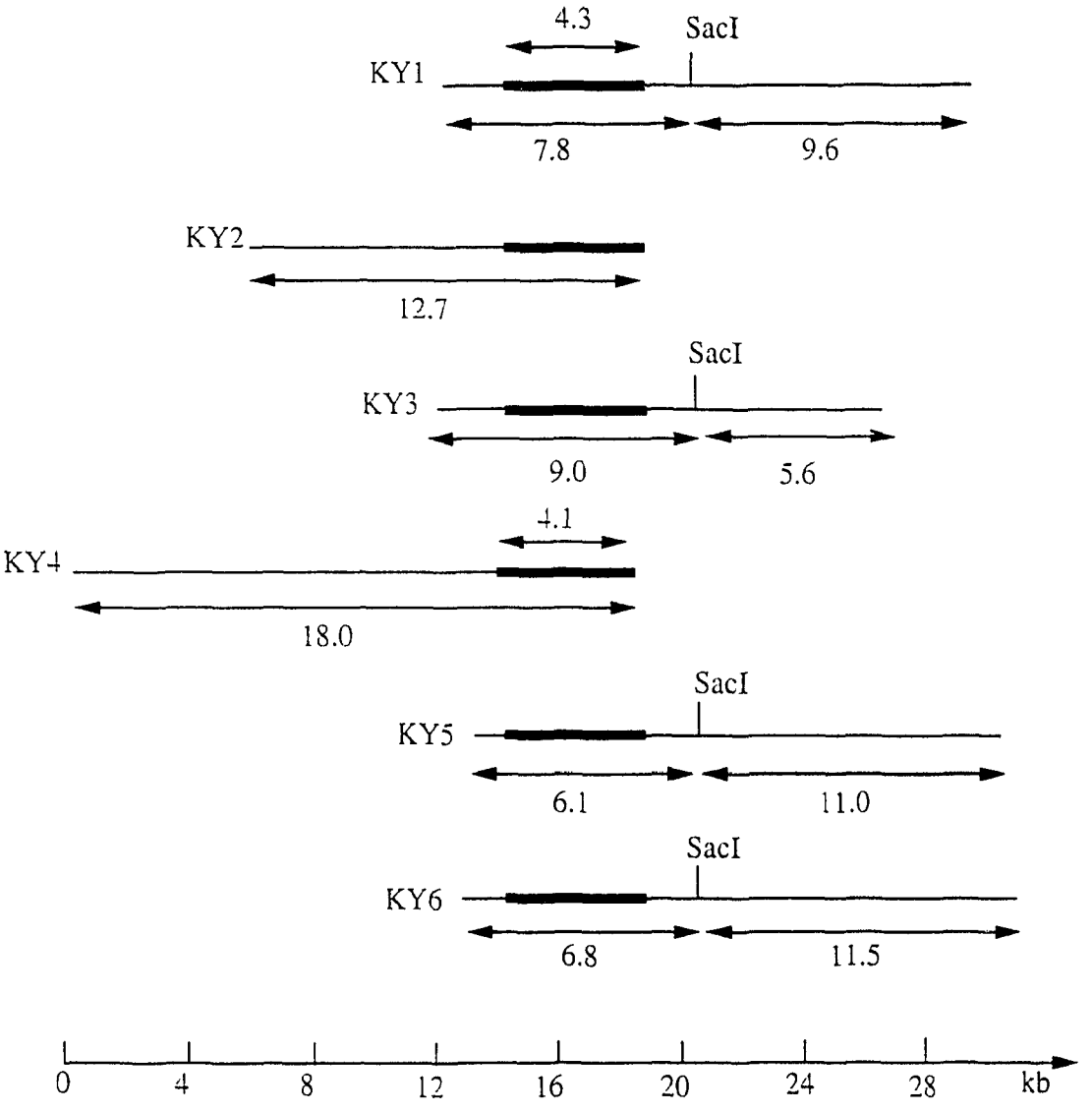


Fig. 14. Physical relationships of the *S. griseoviridus* DNA inserts in six *ermE* -hybridizing lambda clones. The 4.3-kb *ermE*-hybridizing *Sal*I fragment common to each is shown as a black bar.

17.4-kb insert KY1, as well as the component 7.8-kb and 9.6-kb *Sac*I fragments of the insert, were ligated into the *Sac*I site within the polylinker region of shuttle vector pHJL400. In the procedure outlined in Fig. 15, the KY1 DNA was partially digested with *Sac*I and ligated with the *Sac*I-digested vector; the ligation mixture was used to transform *E. coli* TG1. Electrophoresis of *Sac*I digests of recombinant plasmid DNA from 20 white colonies confirmed that they contained either 17.4, 7.8 or 9.6-kb inserts. The recombinant plasmids were designated pDQ310, pDQ311 and pDQ312, respectively (see Fig. 15).

The 12.7-kb insert of KY2 and the 18-kb insert of KY4 were similarly subcloned in the *Sac*I site of pHJL400 to give pDQ313 and pDQ314, respectively. The presence of the inserts in the subclones was confirmed by digesting the recombinant plasmids with *Sal*I and *Sac*I. All of the inserts could be cloned in two orientations. The orientations of the inserts in pDQ310, pDQ312, pDQ313 and pDQ314 were not determined. The orientation of the 7.8-kb *Sac*I fragment in pDQ311 was determined by agarose gel electrophoresis of a *Sal*I digest. This gave fragments of 4.9, 4.3, 1.4, 1.35, 0.8 and 0.7 kb, consistent with the orientation shown in Fig. 16, where the 4.9-kb fragment was derived from the vector, the 4.3-kb, 1.4-kb, 0.8-kb and 0.7-kb fragments were from the insert and the 1.35-kb fragment was a vector-insert hybrid. Double digestion with *Sal*I and *Sac*I gave similar fragments except that, as

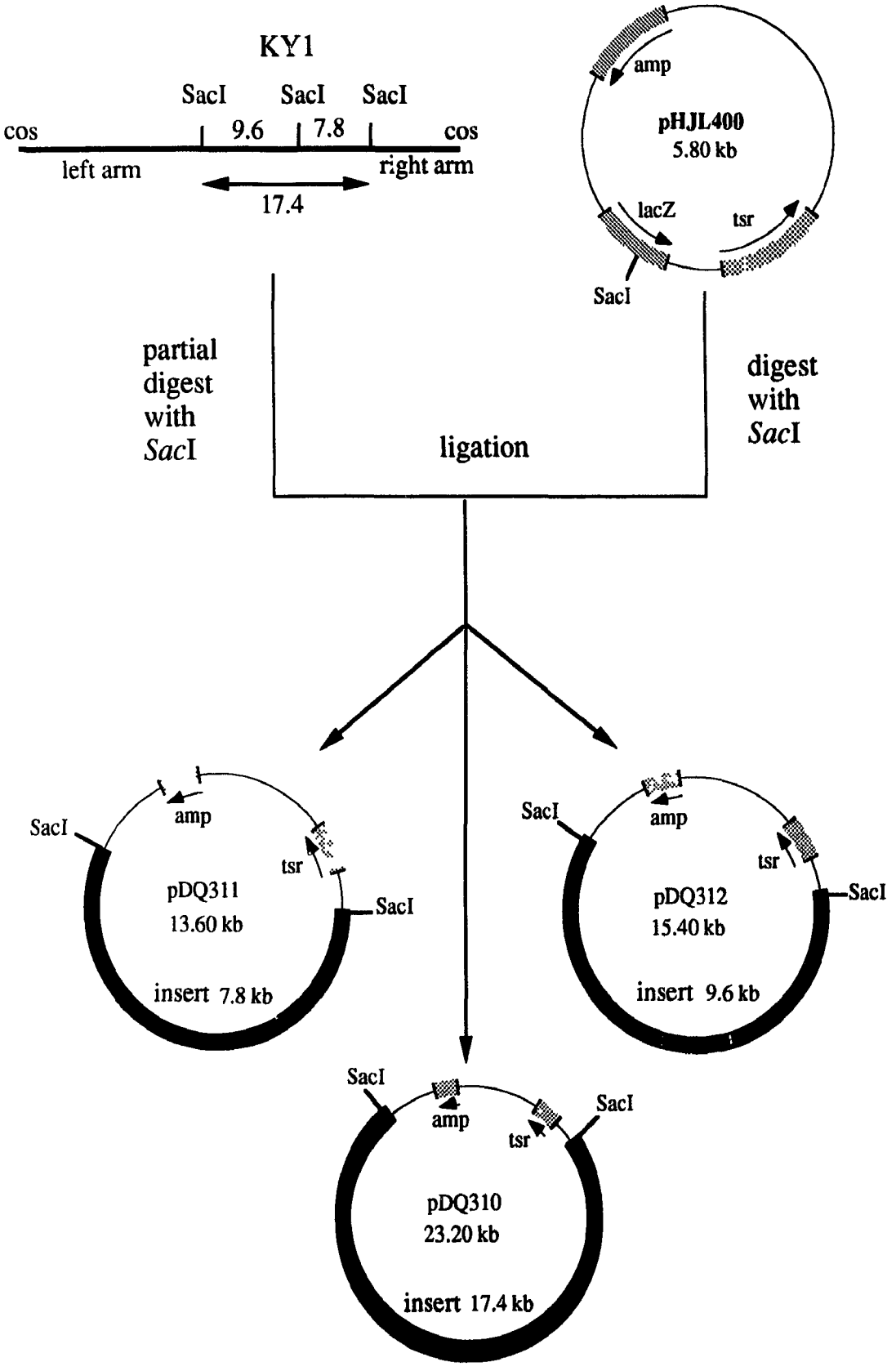


Fig. 15. Construction of pDQ310, pDQ311 and pDQ312.

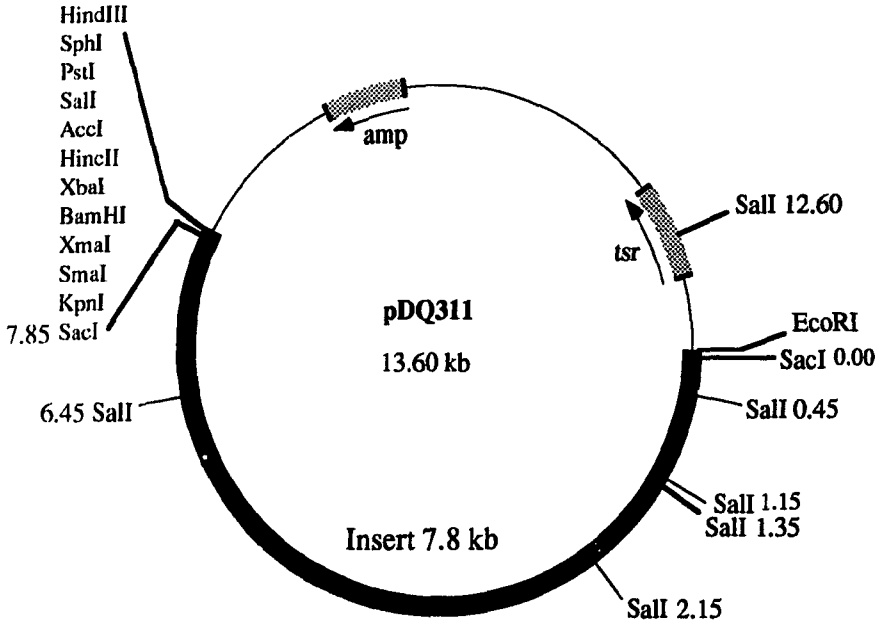


Fig. 16. Circular restriction map of pDQ311.

predicted, the 1.35-kb vector-insert fragment was replaced by 0.9 and 0.45-kb fragments. The alternative orientation of the insert would have given 4.9, 4.3, 2.3, 0.8, 0.7 and 0.45-kb fragments, the distinguishing feature being the size (2.3 kb) of the vector-insert hybrid.

Since an *ermE*-related gene seemed most likely to be located on the 4.3-kb *Sal*I fragment, this piece of DNA was subcloned in a shuttle vector using a procedure (Fig. 17) in which the 4.3-kb fragment was first subcloned in the *E. coli* vector pTZ18R, and the recombinant plasmid was then combined with the *Streptomyces* vector pIJ702 in a way that retained functional *E. coli* and *Streptomyces* replicons. The 4.3-kb DNA fragment was obtained by excising the appropriate band from an agarose gel, and was ligated with *Sal*I-digested pTZ18R. The ligation mixture was used to transform *E. coli* TG1, and plasmids were isolated from six white colonies. *Sal*I digests of these plasmids each regenerated the 4.3-kb *Sal*I fragment and the 2.8-kb vector fragment.

To analyze restriction sites on the 4.3-kb *Sal*I fragment, one of the recombinant plasmids (pDQ315A, see Fig. 17) was digested with a variety of restriction enzymes. *Bgl*II and *Xho*I had no effect; therefore, the plasmid lacked recognition sites for these enzymes. *Bam*HI and *Eco*RI digests gave a 7.1-kb fragment (the size of insert plus plasmid), indicating a single site for each enzyme. Since both enzymes have unique sites within the polylinker of pTZ18R, the 4.3-kb *Sal*I insert

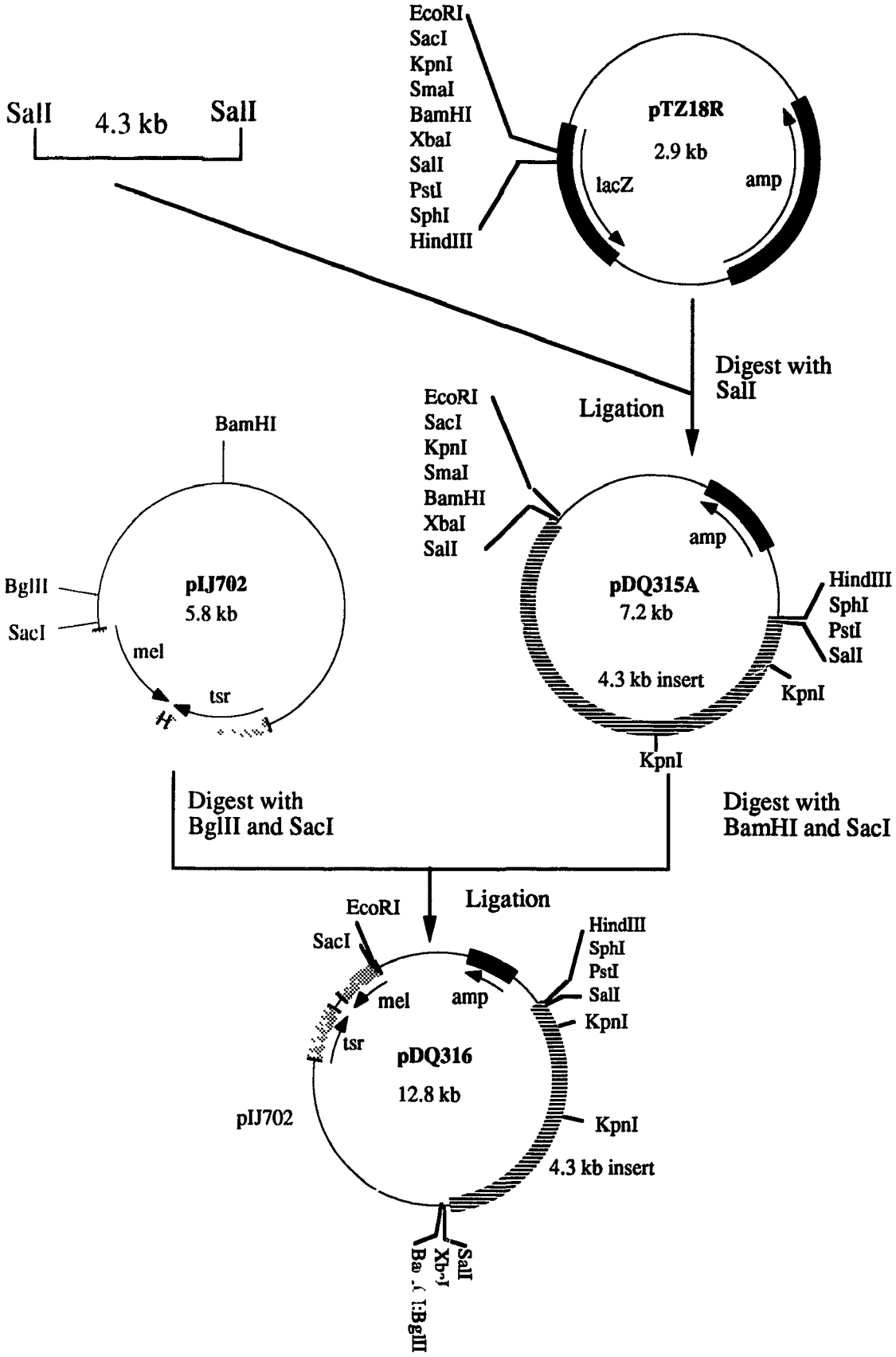


Fig. 17. Construction of pDQ315A and pDQ316. The solid-filled (black) boxes represent *E. coli* genes, dot-filled boxes represent *Streptomyces* genes and line-filled boxes represent *S. griseoviridus* 400E DNA.

lacks sites for these enzymes as well. A *Kpn*I digest gave three fragments; since there was a unique *Kpn*I site in the polylinker region of pTZ18R, the 4.3-kb *Sal*I fragment must contain two *Kpn*I sites.

To determine the position of these *Kpn*I sites, the six recombinant plasmids containing the 4.3-kb *Sal*I fragment cloned in pTZ18R were digested with *Kpn*I; two sets of *Kpn*I fragments (three of each set) representing two orientations of the insert in pTZ18R were obtained. The three plasmids that gave 1.4, 2.5 and 3.3-kb fragments were designated pDQ315A; the remaining three plasmids gave 0.4, 1.4 and 5.3-kb and were designated pDQ315B. When the *Kpn*I digest of pDQ315A was probed with the 0.58-kb *ermE* fragment, hybridization was detected only with the 1.4-kb *Kpn*I fragment. The 1.4-kb fragment was present in *Kpn*I digests of plasmids containing the insert in both orientations; therefore, it was internal to the 4.3-kb *Sal*I fragment, and the *Kpn*I restriction map of the latter must be as shown in Fig. 18.

The *Bam*HI and *Sac*I-digested pDQ315A was ligated to *Bgl*II and *Sac*I-digested pIJ702 to obtain pDQ316 (see Fig. 17). The restriction map of pDQ316 was confirmed by digesting with *Sal*I, and by double digestion with *Sac*I and *Hin*dIII.

G. Transformation of *S. griseofuscus* with recombinant vectors

Previous tests indicated that *S. griseofuscus* was more sensitive than *S. lividans* to etamycin; therefore, *S.*

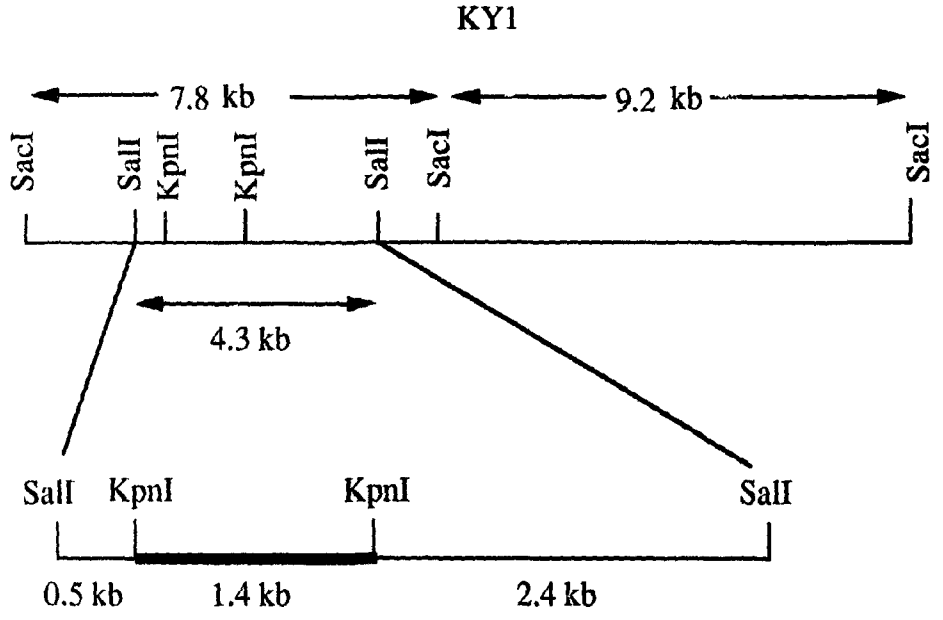


Fig. 18. Partial restriction map of the DNA insert in recombinant lambda clone KY1 showing the location of *Kpn*I sites within the 4.3-kb *Sal*I fragment of *S. griseoviridus* genomic DNA. The *ermE*-hybridizing region is indicated by a black bar.

griseofuscus was chosen as the cloning host for expressing the anticipated etamycin resistance gene. When the recombinant pHJL400 plasmids (pDQ310-314) and the additional shuttle plasmid pDQ316 were used under the same conditions to transform *S. griseofuscus*, transformants were obtained with pDQ310, pDQ311, pDQ312 and pDQ316 but not with pDQ313 or pDQ314. Further attempts to transform *S. griseofuscus* with pDQ313 and pDQ314 also failed. Since both plasmids contained fragments cloned from the same region of *S. griseoviridus* genomic DNA (inserts in lambda clones KY2 and KY4, see Fig. 14), this region may encode a function lethal to the host.

To confirm that all of the colonies obtained were genuine transformants, they were grown in YEME medium supplemented with 15 $\mu g.ml^{-1}$ of thiostrepton, and plasmid DNA was extracted from each of them. As a negative control, *S. griseofuscus* was transformed with the vector pHJL400, yielding transformant FK400. Transformants designated FK1, FK3 and FK4 gave plasmids identifiable by agarose gel electrophoresis with reference plasmids pDQ310, pDQ312 and pDQ316, respectively. Samples matching pDQ316 contained more DNA, probably because the plasmid was derived from the multicopy vector pIJ702. Restriction digests of the extracted plasmids established that they were not altered in their new host. In the extract of transformant FK2, a plasmid was not readily detected. However, the plasmid extract was able to transform *S. griseofuscus* to thiostrepton resistance, and *E. coli* TG1 to ampicillin

resistance; in addition, the plasmid DNA isolated from the *E. coli* transformants was electrophoretically identical to the parent plasmid pDQ311, confirming that this plasmid was stably maintained in strain FK2. The relationships between these plasmids and transformants are summarized in Table 10.

H. Assays for resistance in transformants

In the initial test for resistance, spores of the transformants and of wild type *S. griseofuscus* were streaked on MYM agar containing 5 $\mu g.ml^{-1}$ of etamycin. Under these assay conditions, all strains showed the same etamycin-sensitive phenotype (i.e., MIC <5 $\mu g.ml^{-1}$). The transformants were then tested using the controlled inoculum assay, in which spore inocula were compared with vegetative inocula obtained from cultures at different stages of growth. The results showed that etamycin resistance was exhibited by FK1, FK2 and FK3 when the assay inoculum was from cultures in a specific growth stage (Table 11).

The etamycin resistance levels in the transformants were also compared by determining the maximum tolerated concentration of etamycin (MTC) for each transformant using an exponential phase vegetative inoculum (Table 12). Parenthetically it may be noted that the term MIC, and the procedure associated with it, is widely used to indicate the efficacy of an antibiotic and to compare antibiotics with one another as inhibitors of selected test organisms. The emphasis

Table 10. Plasmid vectors, transformants and DNA fragments derived from recombinant lambda clones

Lambda clone	Fragment (kb)	Plasmid[a]	Transformant
-	-	pHJL400	FK400
KY1	17.4	pDQ310	FK1
	7.8	pDQ311	FK2
	9.6	pDQ312	FK3
	4.3	pDQ315	-
	4.3	pDQ316	FK4
KY2	12.7	pDQ313	-
KY3	14.6	-	-
KY4	18.0	pDQ314	-
KY5	17.1	-	-
KY6	18.3	-	-

a, except for pDQ315 and pDQ316, which were derived from pTZ18R and pIJ702, respectively, all of the plasmids were derived from pHJL400.

-, not obtained.

Table 11. Etamycin resistance of *S. griseofuscus* and various transformants of *S. griseofuscus* tested with the controlled inoculum assay[a].

Inoculum sample (h)	Strains					
	FK1	FK2	FK3	FK4	FK400	*S. griseofuscus*
0	-	-	-	-	-	-
6	-	-	-	-	-	-
12	-	-	-	-	-	-
18	+	+	+	-	-	-
24	+	+	+	-	-	-
30	+	+	+	-	-	-
36	+	+	+	-	-	-
42	-	-	-	-	-	-
48	-	-	-	-	-	-
60	-	-	-	-	-	-

[a] Growth (+) or absence of growth (-) on MYM agar supplemented with 40 $\mu g.ml^{-1}$ etamycin was assessed after incubation for 3 days at 30°C.

Table 12. MTC of etamycin for *S. griseofuscus* and transformants of *S. griseofuscus*.

Strain	Plasmid	MTC ($\mu g.ml^{-1}$)*	
		Induced	Uninduced
FK1	pDQ310	>60	50-60
FK2	pDQ311	40-50	30-40
FK3	pDQ312	40-50	30-40
FK4	pDQ316	20-30	10-20
FK400	pHJL400	20-30	10-20
S. griseofuscus		10-20	5-10

* Vegetative inocula from transformant cultures in early exponential growth (OD_{640} 3-5) were transferred to MYM agar containing increasing amounts of etamycin; growth was recorded after 3 days at 30°C. Induced inoculum cultures were supplemented with etamycin as described in Materials and Methods.

is on the properties of the antibiotic, not on the target organism. In contrast, the term MTC is focused on the properties of the organism. In the present study, the importance of the distinction between MTC and MIC values is that the former measure the ability of an organism to grow in the presence of etamycin under conditions that optimize expression of indigenous resistance genes. As noted above, in a medium containing 5 $\mu g.ml^{-1}$ etamycin the spore inocula used in the standard MIC assay procedure failed to grow (implying no resistance) whereas the vegetative inocula from a specific growth stage, as used in the MTC assay procedure, grew abundantly. Varying the concentration of etamycin in the test media used in the MTC assay therefore provided a more accurate assessment of the resistance of the organism.

Since pDQ316 differed from the pDQ310-pDQ314 series of plasmids by having the replication function of pIJ101 instead of SCP2*, it was expected to be maintained at higher copy number in *S. griseofuscus*. The amount of plasmid DNA extracted from transformant FK4 was indeed higher than the yields from FK1-FK3. Therefore, a resistance gene in pDQ316 should be expressed at a higher level than one in plasmids pDQ310, pDQ311 and pDQ312. Since plasmids pDQ310 and pDQ311 contain the same 4.3-kb *SalI* fragment as pDQ316, and the number of copies in pDQ316 should be much higher than in the first two, the relatively low resistance of FK4 (similar to that of FK400) compared with FK1-FK3 suggests that either a resistance

determinant, if present on the 4.3-kb fragment, was not expressed in pDQ316, or alternatively that any resistance determinant present in the cloned DNA was located elsewhere on the 17-kb insert in lambda clone KY1. The resistance of FK1 was significantly higher than that of both FK2 and FK3, which showed approximately equal resistance. Importantly, FK400 showed significantly higher etamycin resistance than untransformed *S. griseofuscus*. Since it is unlikely that pHJL400 contains an etamycin resistance gene, the enhanced resistance level might be a physiological effect related, for example, to a decrease in plasmid copy number allowing more rapid growth, or might be caused by the activation of a silent etamycin resistance gene in *S. griseofuscus*.

To learn the effect of the recombinant plasmids on erythromycin and lincomycin resistance in *S. griseofuscus*, each transformant was tested by the controlled inoculum assay on MYM agar containing 25 $\mu g.ml^{-1}$ of lincomycin or 50 $\mu g.ml^{-1}$ of erythromycin. Inocula withdrawn from seed cultures over a period of 60 h, showed the same resistance patterns as *S. griseofuscus* (Tables 13A and 13B), indicating that the presence of the plasmids did not affect the temporal expression of resistance in the inoculum culture (i.e., resistance to erythromycin was exhibited only with inocula from 24-44 h cultures with OD_{640} values in the 3-7 range, and resistance to lincomycin was shown only with 28-40 h inocula). However, expression of resistance to these antibiotics seemed

Table 13A. Controlled inoculum assay of growth on MYM agar containing erythromycin (50 $\mu g.ml^{-1}$).

Sampling time (h)	Strains				
	FK1	FK2	FK3	FK400	*S. griseofuscus*
16	-	-	-	-	-
20	-	-	-	-	-
24	+	+	+	+	+
28	+	+	+	+	+
32	+	+	+	+	+
36	+	+	+	+	+
40	+	+	+	+	+
44	+	+	+	+	+
48	-	-	-	-	-
60	-	-	-	-	-

Table 13B. Controlled inoculum assay of growth on MYM agar containing lincomycin (25 $\mu g.ml^{-1}$).

Sampling time (h)	Strains				
	FK1	FK2	FK3	FK400	*S. griseofuscus*
16	-	-	-	-	-
20	-	-	-	-	-
24	-	-	-	-	-
28	+	+	+	+	+
32	+	+	+	+	+
36	+	+	+	+	+
40	+	+	+	+	+
44	-	-	-	-	-
48	-	-	-	-	-
60	-	-	-	-	-

to occur at a different time during growth from expression of etamycin resistance. To learn whether the resistance to erythromycin and lincomycin reflected a physiologically based temporal resistance to all antibiotics, and to facilitate comparison, inoculum samples from the same seed culture were assayed on MYM agar containing 40 $\mu g.ml^{-1}$ of etamycin, 50 $\mu g.ml^{-1}$ of chloramphenicol and 30 $\mu g.ml^{-1}$ of viomycin. No growth occurred with chloramphenicol or viomycin. The results for etamycin are shown in Table 13C. The period of growth during which etamycin resistance developed was 20-32 h. Resistance to erythromycin and lincomycin was manifest in 24-44 and 28-40-h mycelium, respectively. Therefore, etamycin resistance, whether from the host or from the cloned insert, is independent of resistance to erythromycin and lincomycin. Resistance to all three antibiotics cannot be due to a single MLS resistance gene. Since all the samples were sensitive to 50 $\mu g.ml^{-1}$ of chloramphenicol and to 30 $\mu g.ml^{-1}$ of viomycin, the presence of plasmid and the age of the inoculum culture had no effect on resistance to these antibiotics.

I. Subcloning of fragments adjacent to the 4.3-kb *SalI* fragment

The possibility that the 4.3-kb *SalI* fragment might not be specifically associated with etamycin resistance, and the observation that pDQ311 conferred higher etamycin resistance on *S. griseofuscus* than did pDQ316, suggested that an etamycin

Table 13C. Controlled inoculum assay of growth on MYM agar containing etamycin (40 $\mu g.ml^{-1}$).

Sampling time (h)	Strains				
	FK1	FK2	FK3	FK400	*S. griseofuscus*
16	-	-	-	-	-
20	+	+	+	-	-
24	+	+	+	-	-
28	+	+	+	-	-
32	+	+	+	-	-
36	-	-	-	-	-
40	-	-	-	-	-
44	-	-	-	-	-
48	-	-	-	-	-
60	-	-	-	-	-

resistance determinant was located within the 7.7-kb insert of pDQ311, but partially or completely outside its internal 4.3-kb region. For this to be true, the resistance determinant should be in the 2.1-kb or 1.4-kb *Sac*I:*Sal*I fragments on either side of the 4.3-kb fragment or span the junctions (see Fig. 19A). Attempts were made to clone these fragments.

Digestion of pDQ311 with a variety of restriction enzymes showed that the 7.7-kb insert had no sites for *Bam*HI, *Eco*RI and *Hin*dIII, but too many *Pst*I and *Kpn*I sites for convenient subcloning. Therefore, pDQ311 was partially digested with *Sal*I and recircularized by ligation. The ligation mixture was used to transform *E. coli* TG1 and plasmids of various sizes were screened and isolated for further characterization. In the series of plasmids so obtained (pDQ317, pDQ318, pDQ319, pDQ320, pDQ321, pDQ322 and pDQ323), the original plasmid containing the 7.7-kb *Sac*I fragment had undergone a variety of deletions. Analysis by agarose gel electrophoresis of *Sal*I digests of the resulting plasmids is shown in Fig. 19B. All plasmid digests contained the expected 4.9-kb vector fragment (see restriction map of pDQ311 in Fig. 16). Digests of pDQ317 (lane b), pDQ318 (lane g), pDQ319 (lane d), pDQ320 (lane a) and pDQ321 (lane c) contained a 1.4-kb fragment corresponding in size to the 1.4-kb *Sal*I:*Sac*I segment in the 7.7-kb insert. The fragment was absent from pDQ322 and pDQ323 digests. All digests except those of pDQ320 (lane a) and pDQ321 (lane c) contained a 1.3-kb fragment corresponding in size to the

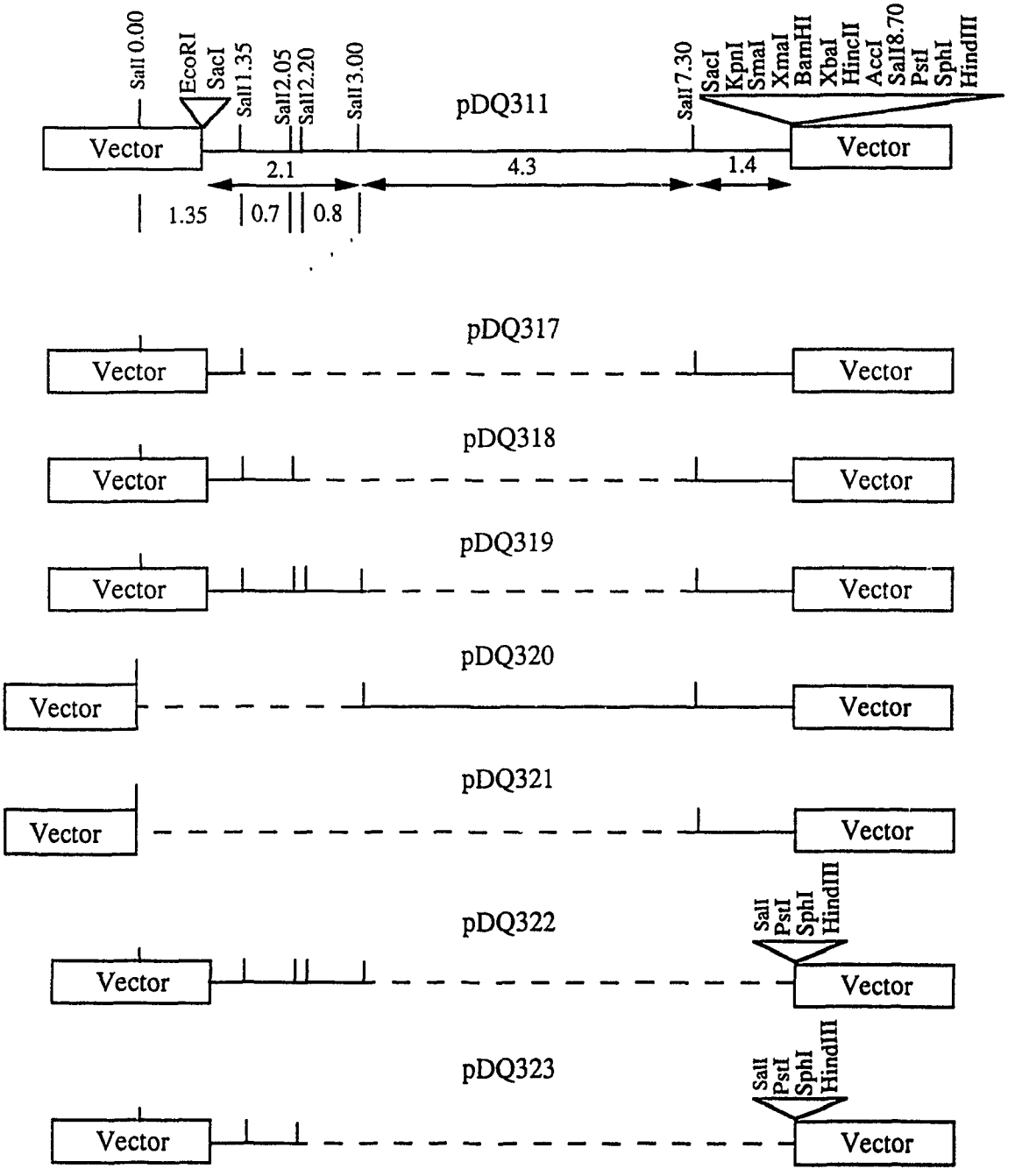


Fig. 19A. Deletion plasmids obtained from pDQ311 by religation of a partial *SalI* digest. Dashed lines represent the region deleted. The size of regions in the 7.7-kb insert in pDQ311 is indicated in kb.

Fig. 19B. Gel electrophoresis of *Sal*I-digested recombinant plasmids derived from pDQ311. The DNA samples were: lane a, pDQ320; lane b, pDQ317; lane c, pDQ321; lane d, pDQ319; lane e, pDQ322; lane f, pDQ323; lane g, pDQ318. The size of DNA is indicated at the right. The top band in all plasmid digests is the 4.9-kb vector fragment. In plasmids pDQ320 and pDQ321 a 0.9-kb vector fragment had been deleted. These plasmids lacked a 1.35-kb *Sal*I fragment (0.9-kb vector and 0.45-kb insert).

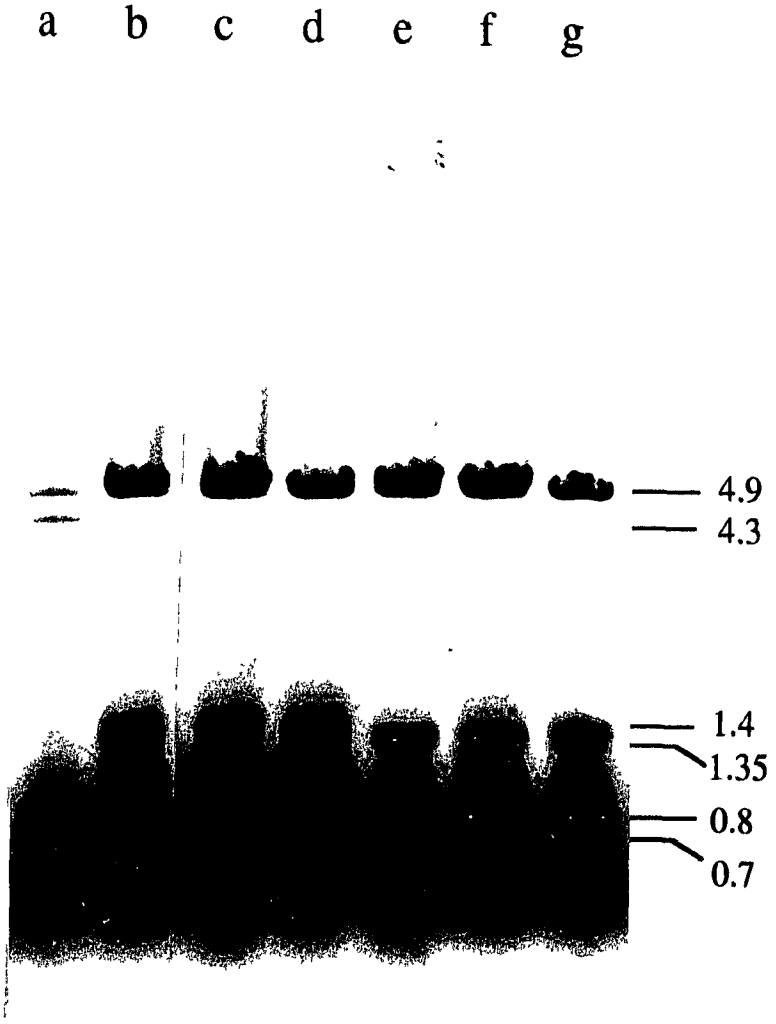


Fig. 19B.

hybrid insert-vector *SalI* fragment of pDQ311. Digests of pDQ319 (lane d) and pDQ322 (lane e) contained a 0.8-kb insert fragment corresponding in size to the component of the 2.1-kb *SalI*:*SacI* insert fragment. Digests of pDQ318 (lane g), pDQ319 (lane d), pDQ322 (lane e) and pDQ323 (lane f) contained a 0.7-kb fragment corresponding in size to a second component of the 2.1-kb *SalI*:*SacI* insert fragment. These 0.8 and 0.7-kb fragments were obscured in the digests shown in Fig. 19B by the presence of RNA, but were unambiguously identified in other samples in which the RNA was eliminated by digestion with RNase. In pDQ320 and pDQ321 digests, deletion of a 0.9-kb vector segment as well as an adjacent 0.45 kb of the insert accounted for the absence of the 1.35-kb *SalI* fragment.

J. Resistance of *S. griseofuscus* transformed with deletion plasmids

Plasmid pDQ322 in which the 1.4 and 4.3-kb *SalI* segments of the insert in pDQ311 had been deleted, plasmid pDQ323 lacking in addition, the adjacent 0.8-kb *SalI* segment, and plasmid pDQ317 in which the 4.3-kb fragment and an adjacent 1.5-kb region had been lost from the 2.1-kb SalI:SacI segment (see Fig. 19A) were used to transform *S. griseofuscus*. The transformants obtained (FK5, FK6 and FK7, respectively) were tested by the controlled inoculum assay for growth on MYM agar containing 40 $\mu g.ml^{-1}$ of etamycin. They exhibited resistance in an inoculum age-dependent manner; the temporal pattern was

similar to that observed previously with transformants FK1, FK2 and FK3 (see Table 11).

When the MTC values for etamycin were determined, FK5 and FK6 showed stronger etamycin resistance than FK7, against which the MTC of etamycin was similar to that against FK4 and FK400 (Table 14). The resistance of FK5 and FK6 to etamycin was similar to that shown by FK2, suggesting that the resistance conferred on the latter by pDQ311 might be caused solely by the 2.1 and 1.1-kb subfragments retained in pDQ322 and pDQ323. Since these two plasmids conferred equal resistance, the resistance determinant could be localized in the 1.1-kb region.

Plasmids pDQ320 and pDQ321 had lost a 0.9-kb vector fragment that contained part of the thiostrepton resistance gene; because *Streptomyces* transformed with these plasmids would no longer be selectable by growth in the presence of thiostrepton, they were not examined further. Plasmids pDQ318 and pDQ319 were similar to pDQ323 and pDQ322, respectively, except that they each retained as well the 1.4-kb segment of pDQ311. Since the presence of this 1.4-kb segment in pDQ317 did not confer resistance on FK7, they also were not used to transform *S. griseofuscus*.

IV. Sequencing and Analysis of Cloned DNA

Analysis of the nucleotide sequence of cloned DNA offered

Table 14. MTC of etamycin for the *S. griseofuscus* transformants.

Transformant	Plasmid present	MTC ($\mu g.ml^{-1}$)*	
		Induced	Uninduced
FK400	pHJL400	20-30	10-20
FK4	pDQ316	20-30	10-20
FK5	pDQ322	40-50	30-40
FK6	pDQ323	40-50	30-40
FK7	pDQ317	20-30	10-20

* Vegetative inoculum from transformant cultures in early exponential growth (OD_{640} 3-5) and MYM agar containing increasing amounts of etamycin were used. The presence or absence of growth was recorded after incubation at 30°C for 3 days. Inoculum cultures were induced with etamycin as described in Materials and Methods.

a direct way of finding out whether pDQ316, which contained the *ermE*-hybridizing 4.3-kb *SalI* fragment but did not confer etamycin resistance on *S. griseofuscus*, did indeed contain an *ermE*-like gene. The nucleotide sequence of the 2.1-kb *Sac*I:*Sal*I fragment of pDQ311 was also expected to reveal the cause of the resistance conferred by pDQ322 and pDQ323.

A. Sequencing strategy

a. Subcloning of fragments for sequencing.

The 4.3-kb *SalI* fragment was recovered from a digest of KY1 by agarose gel electrophoresis and ligated with *SalI*-digested pBluescript SK+ (Fig. 20). The ligation mixture was used to transform *E. coli* TG1. Of ten white colonies examined, all contained recombinant plasmids of the same size (7.2 kb). *SalI* digests of the plasmids regenerated the 4.3-kb insert and 2.96-kb vector fragments. *KpnI* digests of the plasmids showed the 4.3-kb *SalI* fragment to be cloned in two orientiations (Fig. 21). These plasmids (pDQ324A and pDQ324B) were not immediately useful because digestion with *KpnI* to protect the priming end would also cut within the 4.3-kb fragment.

The 4.3-kb *SalI* fragment was also cloned into the *SalI* site in the polylinker region of pBluescript SK-. More than sixty white colonies were examined. Most contained recombinant plasmid pDQ325A with the 4.3-kb fragment inserted in the orientation shown in Fig. 22. Those with the 4.3-kb fragment in the opposite orientation were altered; their *KpnI* digests

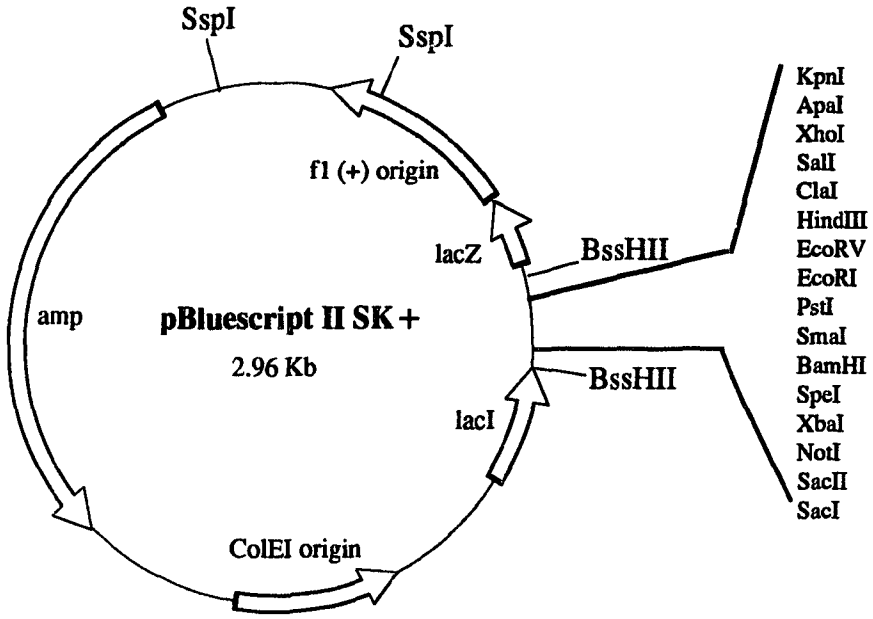


Fig. 20. Restriction map of pBluescript II SK+ (courtesy of Stratagene).

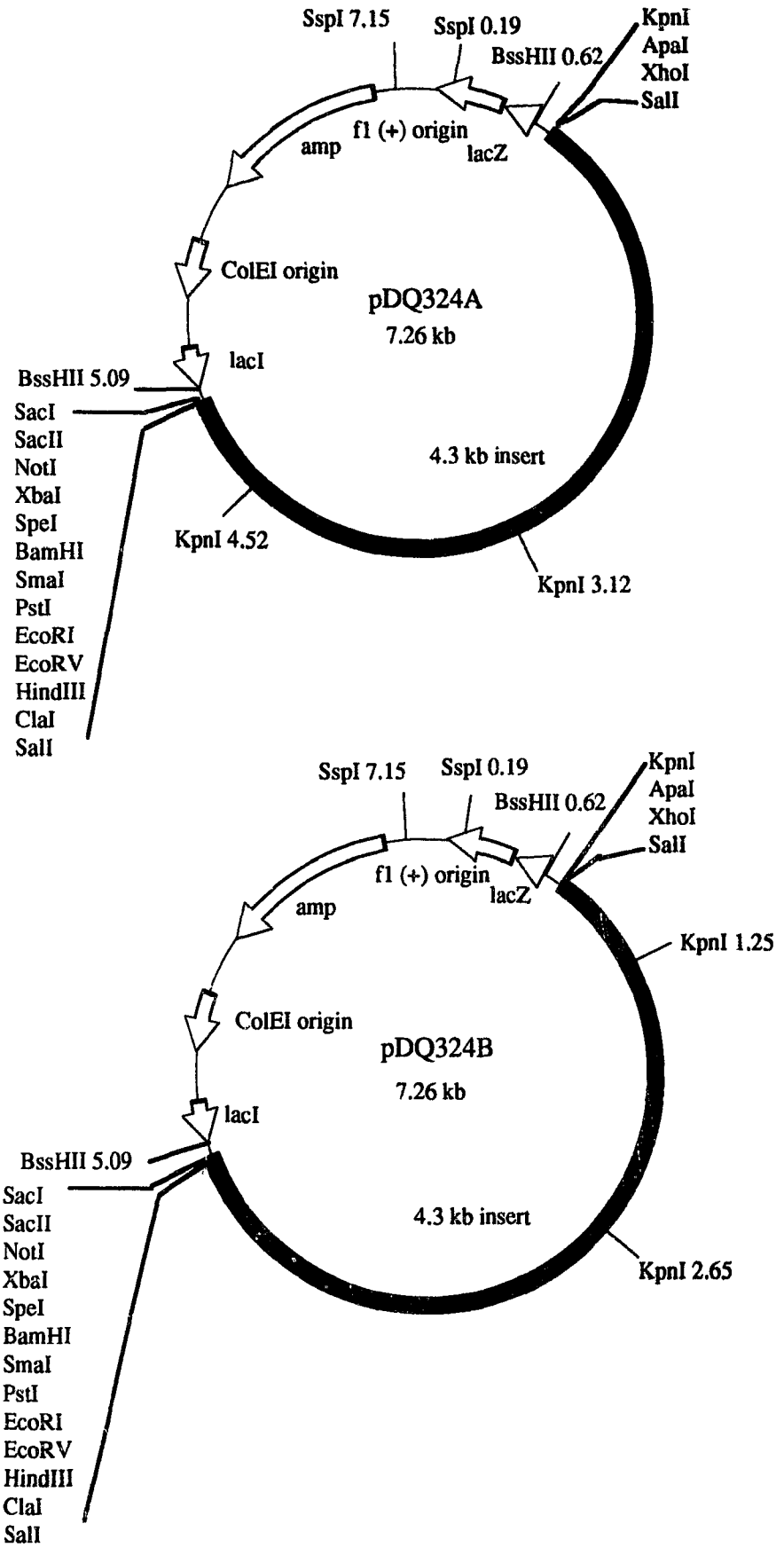


Fig. 21. Restriction maps of pDQ324A and pDQ324B.

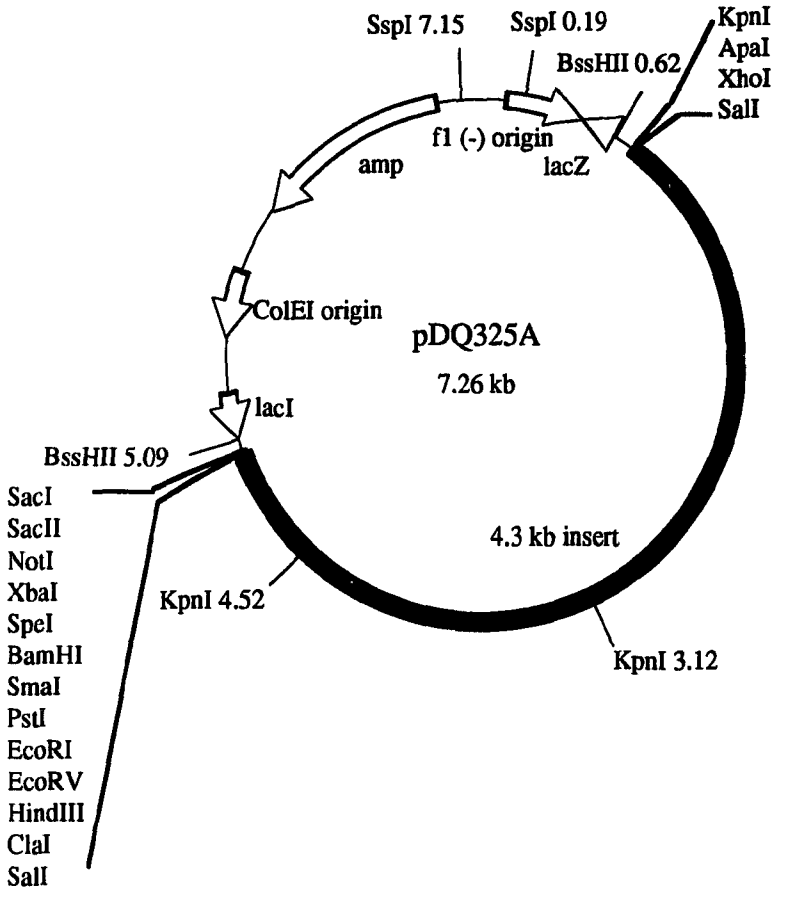


Fig. 22. Restriction map of pDQ325A.

gave 0.4, 1.4 and 7-kb fragments instead of the predicted 0.4, 1.4 and 5.5-kb fragments. Only pDQ325A was used to generate deletion clones for sequencing. In an effort to sequence the opposite strand, pBluescript KS+ was used to clone the 4.3-kb *Sal*I fragment; however, insertion was again found only in one orientation and this would have given sequence for the same strand as pDQ325A. To sequence part of the opposite strand, the 1.4-kb *Kpn*I fragment was cloned in the *Kpn*I site of pBluescript KS+. Three of the recombinant plasmids obtained were examined by gel electrophoresis and sequencing (sequence of one strand was already available); one plasmid (designated pDQ326A) contained the insert in the desired orientation. To sequence the second strand of the 0.5-kb *Sal*I:*Kpn*I region, pDQ324A was digested with *Kpn*I and religated to give a recombinant plasmid (pDQ327) containing only the 0.5-kb *Sal*I:*Kpn*I fragment.

To subclone the 2.1-kb insert of pDQ322 for sequencing, the DNA was liberated from the plasmid by *Sac*I and *Hin*dIII double digestion. The fragment was excised from an agarose gel and purified. It was then filled in by treatment with the Klenow fragment of DNA polymerase I and ligated into the EcoRV site of pBluescript KS+. Recombinant plasmids obtained from *E. coli* transformants were digested with *Sal*I and examined by agarose gel electrophoresis; one (pDQ328, Fig. 23) contained the correct insert. Other plasmids isolated contained the insert in the opposite orientation, but were altered and were not

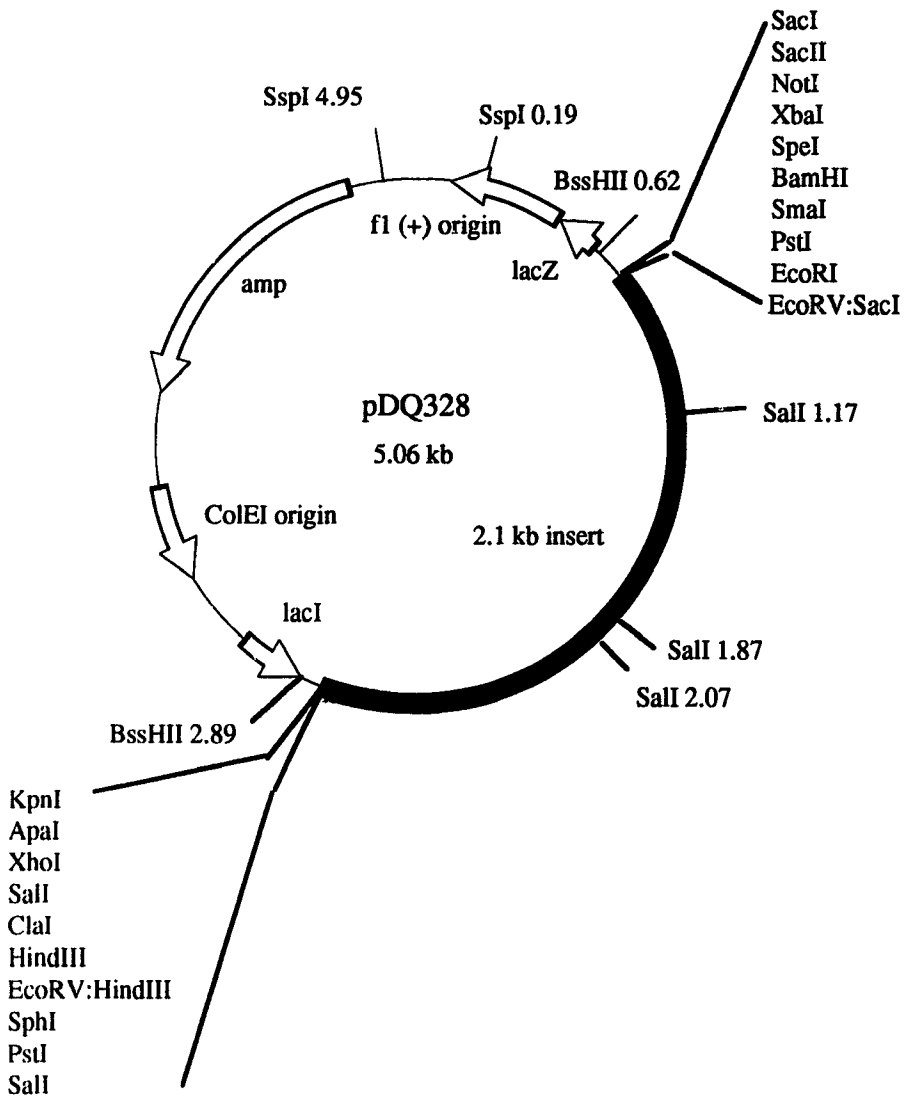


Fig. 23. Restriction map of pDQ328.

useful. To sequence the opposite strand, the 2.1-kb *Sac*I:*Hin*dIII fragment was ligated into *Sac*I and *Hind*III-digested pBluescript SK+. Only one type of recombinant plasmid (pDQ329, Fig. 24) was expected; the identity of the insert was confirmed by examining restriction digests.

b. Creation of deletion clones

pDQ325A, pDQ326A and pDQ328 were digested with *Bam*HI and *Sac*I; deletions were introduced from the *Bam*HI end by digestion with exonuclease III. The mixtures of linearized plasmid DNA, deleted to various extents from one end, were religated and used to transform *E. coli* TG1. For each plasmid 300 single colonies were screened to obtain a complete set of deletion clones. pDQ329 was digested with *Kpn*I and *Hind*III, and deletions were introduced from the *Hind*III end. Again, 300 single colonies were screened for desirable deletion clones. The deletion clones and the primers used to sequence gaps where deletion clones could not be found are listed in Fig. 25. Both strands of the 2.1-kb *Sac*I:*Sal*I fragment of pDQ322 were sequenced completely; one strand of the 0.4-kb *Sal*I:*Kpn*I and 1.4-kb *Kpn*I fragments internal to the 4.3-kb *Sal*I fragment was completely sequenced but each had an unsequenced gap on the opposite strand. The sequences at *Sal*I junctions (see Fig. 25) within the 2.1-kb fragment were compared with that obtained by site-specific double-strand sequencing of pDQ311 using synthetic oligonucleotide primers. The sequence data obtained by double-strand sequencing were identical to those

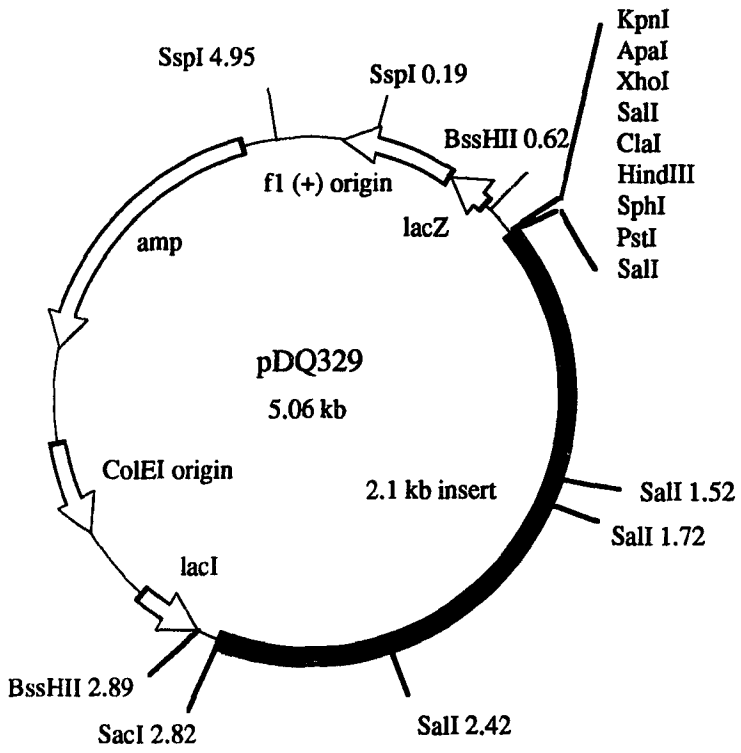


Fig. 24. Restriction map of pDQ329.

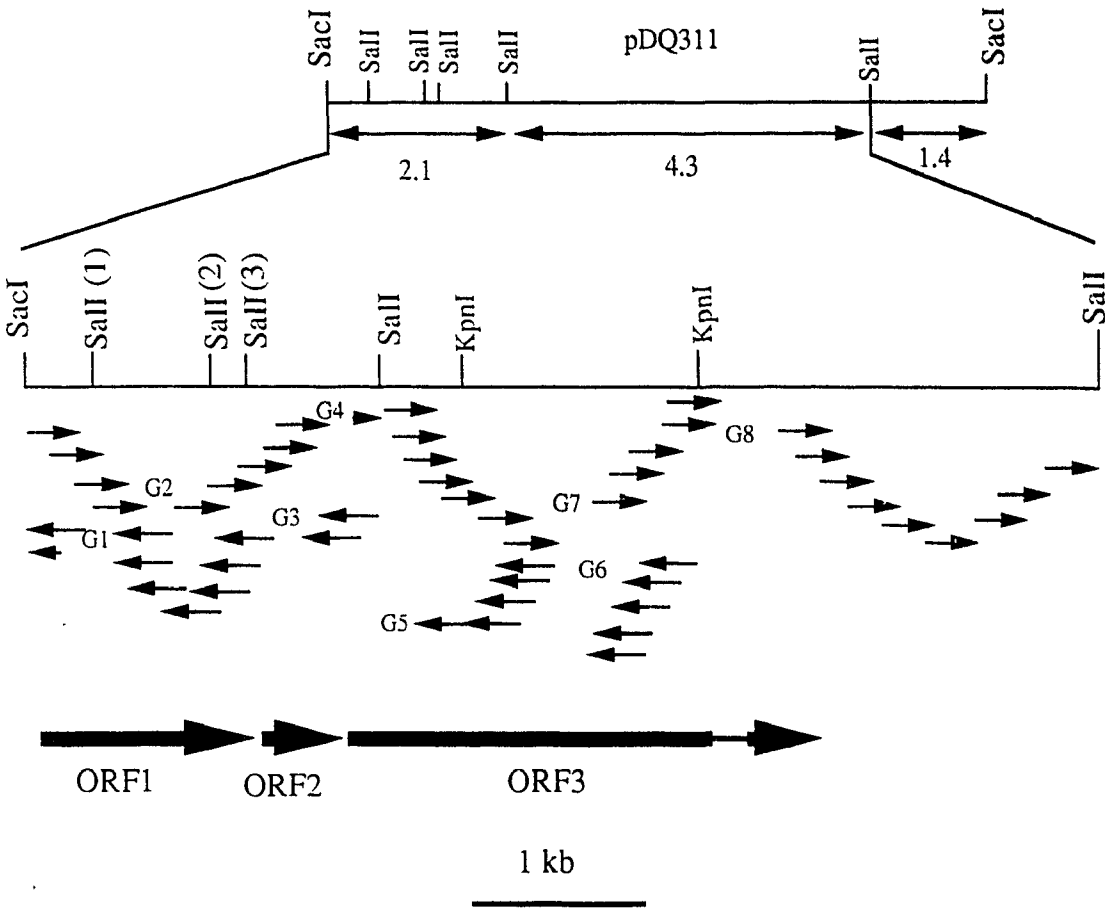


Fig. 25. Strategy used for sequencing the 6.4-kb fragment from *S. griseoviridus*. Small arrows represent deletion clones. G1-G8 are gaps where deletion clones could not be found. The heavy arrows are putative ORFs deduced from nucleotide sequence data; the direction of the arrows is the direction for transcription. Primers used to sequence the gaps and to confirm the sequences at thejunctions of three *SalI* sites (designated 1-3) of the pDQ322 insert are: G1, 5'-TGCCACGAGCCGAACATC-3'; G2, 5'-ATGT TCGGCTC GTGG CAG-3'; G3, 5'-ATGAACTCGCGGAAGTGC-3'; G4, 5'- AG TTCATCGAGGGCGTGG-3'; G7, 5'-CGTCACCCAGCACAAGC A-3'; junction (1), 5'-TGCCACGAGCCGAACATC-3'; junction (2), 5'-AG GTCTGCTCGTACTGG-3'; junction (3), 5'-ACTGGTGATGCAGAAC-3'. Gaps, G5, G6 and G8 were not sequenced.

obtained by sequencing the 2.1-kb insert of pDQ322; they confirmed that the insert in pDQ322 had been cloned in pHJL400 without rearrangement.

B. Nucleotide sequence

The 4117 nucleotide (nt) sequence obtained for a large part of the 7.8-kb insert in pDQ301 is shown in Fig. 26. In a segment of approximately 4 kb at one end of the insert, both strands were almost completely sequenced, and regions containing uncertainties were re-examined using dITP or 7-deaza dGTP in place of dGTP in the sequencing reactions. Complementary sequences were obtained for the two strands, confirming the accuracy of the data.

C. Sequence analysis

a. Identification of open reading frames

Using the CODONPREFERENCE program, potential open reading frames (ORFs) were identified from GC bias at the second and third codon positions, codon usage and the positions of start and stop codons in all six reading frames (Fig. 27). On one strand, the coding regions identified from GC bias in the second and third codon position agreed very well with the coding regions defined by start and stop codons; also codon usage in these regions conformed to the general trends for *Streptomyces* genes. The ORFs were designated ORF1 to ORF3 (see Fig. 27). On the reverse strand, coding regions identified

Fig. 26. Sequence of the region of *S. griseoviridus* DNA suspected to contain an etamycin resistance determinant. The sense strand is shown 5′ to 3′ from nt 1-4117. Selected restriction enzyme sites are listed above their underlined restriction sequences. Ribosomal binding sites (RBS) and translational start and stop codons are indicated by asterisks. The perfect inverted repeat from nt 65 to 84 is indicated with arrow heads. Each ORF is preceded by an arrow head indicating the translational direction. Three reiterated AEPA sequences within the ORF1 product are underlined.

```
 SacI
GAGCTCGATCCTGGGCGAACGCCACGGCACCCGCACCACGAGGCCGACGACGAAGACGTT 60

    >>>>>>>>    <<<<<<<<
CTTCTTTTCCTCAACAGAGGAAAATCTCTCCCTCCCGAGGGCGGCGGAGTACCCTGCCTC 120

                              RBS         >ORF1
                           **********     ***
GTCACGGCCAACCTCGCCGCACCGGAGAGGAGCTATGCGATGGGCACCCCACCCAGGCAC 180
                                        fM  G  T  P  P  R  H

GCCAGGCCGAGGTCGCGCGCCCTGCGGGCCGGTGTCCTCCTCGTCTCGGGGGCCGTCGTG 240
A  R  P  R  S  R  A  L  R  A  G  V  L  L  V  S  G  A  V  V

ACGACGGCTCTCTCGCTCGCCGGCACCGCGAACGGCGACCCGCCCGGCCCCCGTGTCACC 300
T  T  A  L  S  L  A  G  T  A  N  G  D  P  P  G  P  R  V  T

CAGCTCGGCCTCGCGCCGGACCACCTCACCGCGGGACACCGCGCTCCGGCCCGGCTGGAA 360
Q  L  G  L  A  P  D  H  L  T  A  G  H  R  A  P  A  R  L  E

                        .SmaI
CTGACGGCCGACCGGTGCCTGACCGCGGCCCGGGTCACCGTCGCCGTACGCGACCGCGAC 420
L  T  A  D  R  C  L  T  A  A  R  V  T  V  A  V  R  D  R  D

          . SalI   SmaI
GGCCGCAACGTCGACTTCCCGGGCGCCGCCTCCGACGTACGGGTGTGCCCCTCGGGTCAC 480
G  R  N  V  D  F  P  G  A  A  S  D  V  R  V  C  P  S  G  H

ACCTTCACCTCCGGGGCGCGCGCGTTCGCCGCCGGGTCGTACACGATGTTCGGCTCGTGG 540
T  F  T  S  G  A  R  A  F  A  A  G  S  Y  T  M  F  G  S  W

CAGGACGGGGAGGGGCGGTGGCACCCGCTGACGCCGATCACGCTGAGCGTGGCGGAGCCG 600
Q  D  G  E  G  R  W  H  P  L  T  P  I  T  L  S  V  A  E  P

GCCGGGCAGGCCGAGCCGGCCGATGCCGGGGCTCCGTCCGGTCCCGGTGAGCCCTCCGGG 660
A  G  Q  A  E  P  A  D  A  G  A  P  S  G  P  G  E  P  S  G

                                          MluI
TCGGCGGAGCCGGCCGAGGCGGACGGCGGCGGTCCGTCGTGGGACGCGTCCGGCGGGTGG 720
S  A  E  P  A  E  A  D  G  G  G  P  S  W  D  A  S  G  G  W

CGGCGGCGGTTCACCGACGAGTTCGACGGGACCGCGCTGGACACCTCGAAGTGGAACACC 780
R  R  R  F  T  D  E  F  D  G  T  A  L  D  T  S  K  W  N  T

GGCTGGTTCGGCACCGGCGTCACCGGGCCGGTGAACACGGCCGAGGACGCCTGCTACGAC 840
G  W  F  G  T  G  V  T  G  P  V  N  T  A  E  D  A  C  Y  D

ACGCGCAACGCCGTCGTGTCCGAGGGCGCGCTGCATCTGCGGCTGACCGGCGCGAGCGCC 900
T  R  N  A  V  V  S  E  G  A  L  H  L  R  L  T  G  A  S  A

                                    NcoI
ATCTGCAAGGGGGAGACCCGCCCGTACAGCGGGGCGCACGTGAACACCATGGGCCTGTTC 960
I  C  K  G  E  T  R  P  Y  S  G  A  H  V  N  T  M  G  L  F

                        .ApaI
TCCTACAGCTACGGAGCGGTGGAGTACCGGGCCCGCGTCCCGTCGGTGGACGGGGCGGTG 1020
S  Y  S  Y  G  A  V  E  Y  R  A  R  V  P  S  V  D  G  A  V

GCGAACTGGCCGGCGCTCTCCCACACCGGCTCCCTCTGGCCCCGCGACGGCGAGATCGAC 1080
A  N  W  P  A  L  S  H  T  G  S  L  W  P  R  D  G  E  I  D

                                       SalI
ACCCTGGAGGGCCTGTCCGGCGAGGTCTGCTCGTACTGGCACAGCACGCGCGTCGACCGG 1140
T  L  E  G  L  S  G  E  V  C  S  Y  W  H  S  T  R  V  D  R
```

Fig. 26.

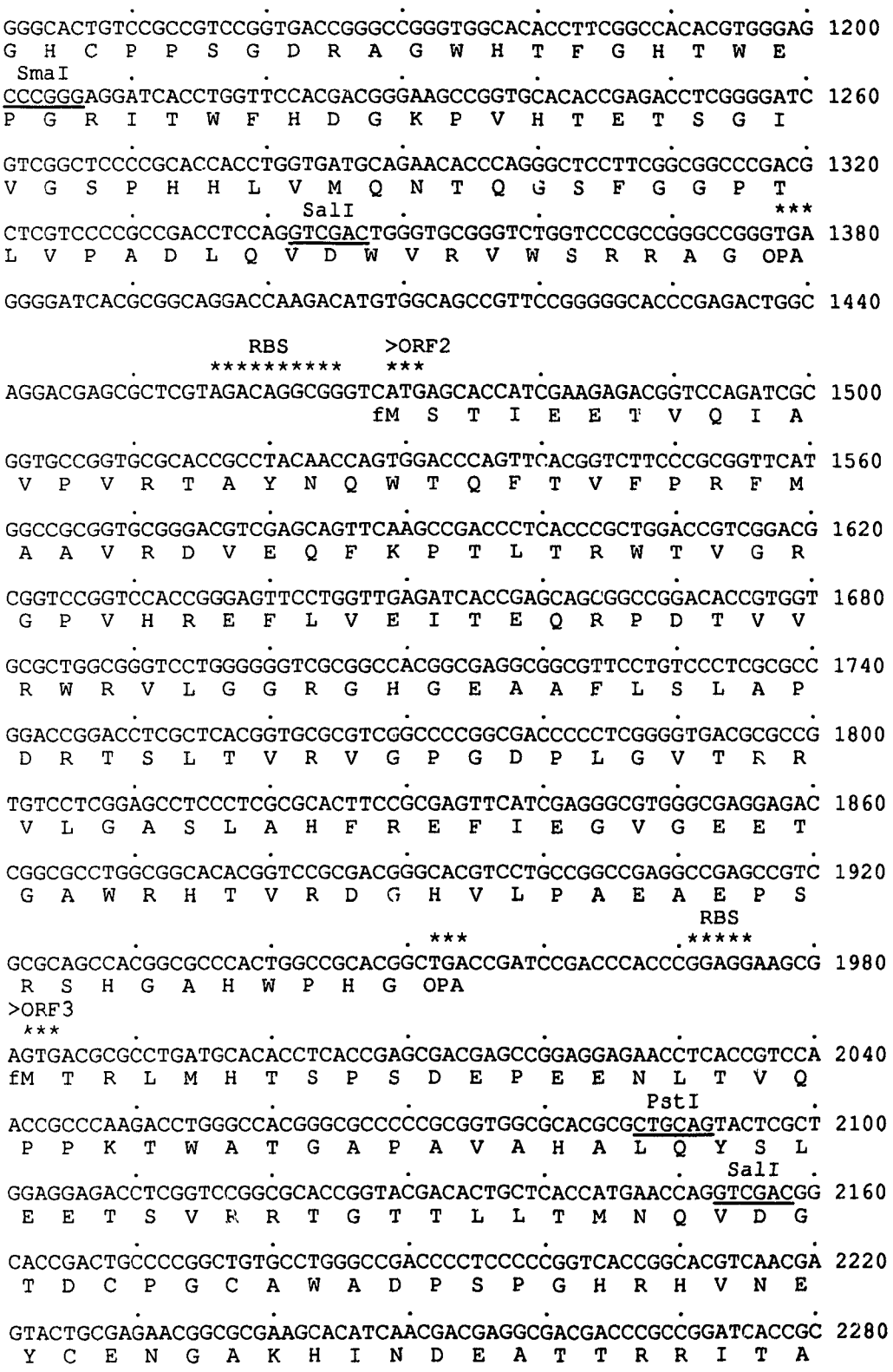

GGGCACTGTCCGCCGTCCGGTGACCGGGCCGGGTGGCACACCTTCGGCCACACGTGGGAG 1200
G H C P P S G D R A G W H T F G H T W E
SmaI
CCCGGGAGGATCACCTGGTTCCACGACGGGAAGCCGGTGCACACCGAGACCTCGGGGATC 1260
P G R I T W F H D G K P V H T E T S G I
GTCGGCTCCCCGCACCACCTGGTGATGCAGAACACCCAGGGCTCCTTCGGCGGCCCGACG 1320
V G S P H H L V M Q N T Q G S F G G P T
SalI
CTCGTCCCCGCCGACCTCCAGGTCGACTGGGTGCGGGTCTGGTCCCGCCGGGCCGGGTGA 1380
L V P A D L Q V D W V R V W S R R A G OPA
GGGGATCACGCGGCAGGACCAAGACATGTGGCAGCCGTTCCGGGGGCACCCGAGACTGGC 1440
RBS
>ORF2
AGGACGAGCGCTCGTAGACAGGCGGGTCATGAGCACCATCGAAGAGACGGTCCAGATCGC 1500
fM S T I E E T V Q I A
GGTGCCGGTGCGCACCGCCTACAACCAGTGGACCCAGTTCACGGTCTTCCCGCGGTTCAT 1560
V P V R T A Y N Q W T Q F T V F P R F M
GGCCGCGGTGCGGGACGTCGAGCAGTTCAAGCCGACCCTCACCCGCTGGACCGTCGGACG 1620
A A V R D V E Q F K P T L T R W T V G R
CGGTCCGGTCCACCGGGAGTTCCTGGTTGAGATCACCGAGCAGCGGCCGGACACCGTGGT 1680
G P V H R E F L V E I T E Q R P D T V V
GCGCTGGCGGGTCCTGGGGGGTCGCGGCCACGGCGAGGCGGCGTTCCTGTCCCTCGCGCC 1740
R W R V L G G R G H G E A A F L S L A P
GGACCGGACCTCGCTCACGGTGCGCGTCGGCCCCGGCGACCCCCTCGGGGTGACGCGCCG 1800
D R T S L T V R V G P G D P L G V T R R
TGTCCTCGGAGCCTCCCTCGCGCACTTCCGCGAGTTCATCGAGGGCGTGGGCGAGGAGAC 1860
V L G A S L A H F R E F I E G V G E E T
CGGCGCCTGGCGGCACACGGTCCGCGACGGGCACGTCCTGCCGGCCGAGGCCGAGCCGTC 1920
G A W R H T V R D G H V L P A E A E P S
RBS
GCGCAGCCACGGCGCCCACTGGCCGCACGGCTGACCGATCCGACCCACCCGGAGGAAGCG 1980
R S H G A H W P H G OPA
>ORF3
AGTGACGCGCCTGATGCACACCTCACCGAGCGACGAGCCGGAGGAGAACCTCACCGTCCA 2040
fM T R L M H T S P S D E P E E N L T V Q
PstI
ACCGCCCAAGACCTGGGCCACGGGCGCCCCCGCGGTGGCGCACGCGCTGCAGTACTCGCT 2100
P P K T W A T G A P A V A H A L Q Y S L
SalI
GGAGGAGACCTCGGTCCGGCGCACCGGTACGACACTGCTCACCATGAACCAGGTCGACGG 2160
E E T S V R R T G T T L L T M N Q V D G
CACCGACTGCCCCGGCTGTGCCTGGGCCGACCCCTCCCCCGGTCACCGGCACGTCAACGA 2220
T D C P G C A W A D P S P G H R H V N E
GTACTGCGAGAACGGCGCGAAGCACATCAACGACGAGGCGACGACCCGCCGGATCACCGC 2280
Y C E N G A K H I N D E A T T R R I T A

Fig. 26.

```
CGACTTCTTCCGCAGGTACTCCGTCTCCGACCTCGCCTCCCGCTCCGACCAGTGGCTGAA 2340
 D  F  F  R  R  Y  S  V  S  D  L  A  S  R  S  D  Q  W  L  N

CCAGCAGGGCCGGCTCACCGAACCCGTGGTGAAGCGTCCGGGCTCCGACCACTACGAGCC 2400
 Q  Q  G  R  L  T  E  P  V  V  K  R  P  G  S  D  H  Y  E  P

CATCGGCTGGCAGGAGGCGCTCGGGCTGCTCGCCGGGGAACTGAACTCCCTCGACTCGCC 2460
 I  G  W  Q  E  A  L  G  L  L  A  G  E  L  N  S  L  D  S  P

CGACGAGGCGGTCTTCTACACCTCCGGCCGGGTCAGCAACGAGGCGGCATTCGTCCTCCA 2520
 D  E  A  V  F  Y  T  S  G  R  V  S  N  E  A  A  F  V  L  Q

GCTCTTCGCGCGGGCGTACGGCACCAACAACCTGCCCGACTGCAGCAACATGTGCCACGA 2580
 L  F  A  R  A  Y  G  T  N  N  L  P  D  C  S  N  M  C  H  E

                          KpnI
GTCCAGCGGCTTCGCCCTGCACGAGACGCTCGGTACCGGCAAGGGCACGGTGACCCTGGA 2640
 S  S  G  F  A  L  H  E  T  L  G  T  G  K  G  T  V  T  L  E

GGACCTCCACCACGCCGACCTGATCTTCGTCGTCGGGCAGAACCCCGGGTCCAACCATCC 2700
 D  L  H  H  A  D  L  I  F  V  V  G  Q  N  P  G  S  N  H  P

GCGCCAGCTCAGCGCCCTGGAGGCGGCCAAGCGCAACGGCGCCCGCGTGATCGCGGTCAA 2760
 R  Q  L  S  A  L  E  A  A  K  R  N  G  A  R  V  I  A  V  N

CCCGCTGCCGGAGGCGGGACTGCTGCGGTTCAAGAACCCGCAGAAGCCGCGCGGGGTGAT 2820
 P  L  P  E  A  G  L  L  R  F  K  N  P  Q  K  P  R  G  V  I

CGGCCGCGGCGTGGAGATCGCCGACCGGTTCCTGCACATCCGCGTCGGCGGGGACCTCGC 2880
 G  R  G  V  E  I  A  D  R  F  L  H  I  R  V  G  G  D  L  A

CCTGTTCCAGGGGCTGAACCGGCTGCTGCTGGAGGCGGAGGACGCCCGTCCGGGGACCGT 2940
 L  F  Q  G  L  N  R  L  L  L  E  A  E  D  A  R  P  G  T  V

CCTCGACCATGACTTCATCCGGGCGAGCACCTCCGGTTTCGAGGAGTTCTCCCGGCACGC 3000
 L  D  H  D  F  I  R  A  S  T  S  G  F  E  E  F  S  R  H  A

CCGGACCGTCGCCTGGGAGGACGTCCTCCGCGCGACCGGCCTGACCCGGGCGGAGATCGA 3060
 R  T  V  A  W  E  D  V  L  R  A  T  G  L  T  R  A  E  I  E

GAAGGTGCGCGACGACGTGCTGGCCAGCGAGCGGGTCATCGTCTGCTGGGCCATGGGCGT 3120
 K  V  R  D  D  V  L  A  S  E  R  V  I  V  C  W  A  M  G  V

CACCCAGCACAAGCACGGCGTGCCCACCATCCGGGAGATCGTCAACTTCCTGCTGCTGCG 3180
 T  Q  H  K  H  G  V  P  T  I  R  E  I  V  N  F  L  L  L  R

CGGCAACCTCGGCCGGGCCGGGGCCGGGGCCTGCCCGGTGCGCGGGCACAGCAATGTGCA 3240
 G  N  L  G  R  A  G  A  G  A  C  P  V  R  G  H  S  N  V  Q

GGGCGACCGCACGGTGGGCATCTGGGAGCAGATGCCGGACTCCTTCCTGGACGCGCTGCG 3300
 G  D  R  T  V  G  I  W  E  Q  M  P  D  S  F  L  D  A  L  R

CGACGAGTTCGGGTTCGAGCCGCCCCGGCACCACGGGCTGGACGCGGTGAACTCGATCAA 3360
 D  E  F  G  F  E  P  P  R  H  H  G  L  D  A  V  N  S  I  K

GGCCATGCGGGAGGGGCGGGTCAGGTTCTTCCTCGGCGTGGCCGGCAACTTCGTGCGGGC 3420
 A  M  R  E  G  R  V  R  F  F  L  G  V  A  G  N  F  V  R  A

CGCCCCGGACAGCGACGTCACCGAGGAGGCGATGCGGCGCTGCCGTCTCACCGCGCACAT 3480
 A  P  D  S  D  V  T  E  E  A  M  R  R  C  R  L  T  A  H  I
```

Fig. 26.

```
CTCCACCAAGCTCAACCGGTCGCACACCGTGTGCGGCGACACCGCGCTGATCCTGCCGAC 3540
 S  T  K  L  N  R  S  H  T  V  C  G  D  T  A  L  I  L  P  T

GCTGGGCCGTACCGAGCGGGACATCCAGGCCACCGGCGACCAGTTCGTCACGGTGGAGGA 3600
 L  G  R  T  E  R  D  I  Q  A  T  G  E  Q  F  V  T  V  E  D

CTCGATGAGCGAGGTGCACACCTCGCGCGGACGGCTGGAGCCGGCCTCCCGGCTGCTGCT 3660
 S  M  S  E  V  H  T  S  R  G  R  L  E  P  A  S  R  L  L  L

CAGCGAGGTCGCGATCCTGTGCCGGCTGGCCCGCCGGACCCTGGAGGGCCGGACGGAGAT 3720
 S  E  V  A  I  L  C  R  L  A  R  R  T  L  E  G  R  T  E  I

CCCCTGGGACCGGTTCGAGGCCGACTACGGCGTGATCCGCGACAGGATCGCCCGGATCGT 3780
 P  W  D  R  F  E  A  D  Y  G  V  I  R  D  R  I  A  R  I  V

GCCGGGGCTGCACGACTACAACCGGCGGGTCGTCCGCCCCGGCGGCATCAGGCTGCCGAA 3840
 P  G  L  H  D  Y  N  R  R  V  V  R  P  G  G  I  R  L  P  N

CCCGGTCAACGAGGGCGTCTTCGGCACCGAGAGCGGCAAGGCCCTGTTCACCCGCAACGA 3900
 P  V  N  E  G  V  F  G  T  E  S  G  K  A  L  F  T  R  N  D

CTGGCAGATGCCGCGCGTGCCCGAGGGGCACCTGCTGCTCCAGACGCTGCGCTCGCACGA 3960
 W  Q  M  P  R  V  P  E  G  H  L  L  L  Q  T  L  R  S  H  D
                          KpnI
CCAGTGGAACACGGTGCCGTACACCACCGACGACCGGTACCGGGGCATCCACGGCAGCCG 4020
 Q  W  N  T  V  P  Y  T  T  D  D  R  Y  R  G  I  H  G  S  R

CCGGGTGGTCATGGTCAACCCCGACGACCTCACGGAGCTGGGGCTGGCCGACGGGCAGCG 4080
 R  V  V  M  V  N  P  D  D  L  T  E  L  G  L  A  D  G  Q  R

GGTGGACCTGGTGAGCGTGTGGTCCGACGACGTCGAG 4117
 V  D  L  V  S  V  W  S  D  D  V  E
```

Fig. 26.

Fig. 27. CODONPREFERENCE analysis of a 4117 nt sequence shown in Fig. 26. The X axis is the number of nucleotides. The Y axis is the score of third position GC bias of each codon in a frame. ORFs are shown as boxes beneath the plot for their repective translation frames. Potential start codons are shown as short lines that extend above the height of the box, and stop codons extend below the bottom of the box. Only the start and stop codons at the end of ORFS are shown in the frame display. The codon table used was derived by Bibb and Cohen (1982). A is an analysis of the DNA sequence shown in Fig. 26; B is the analysis of the DNA sequence read in the reverse direction from that shown in Figure 26.

Fig. 27.

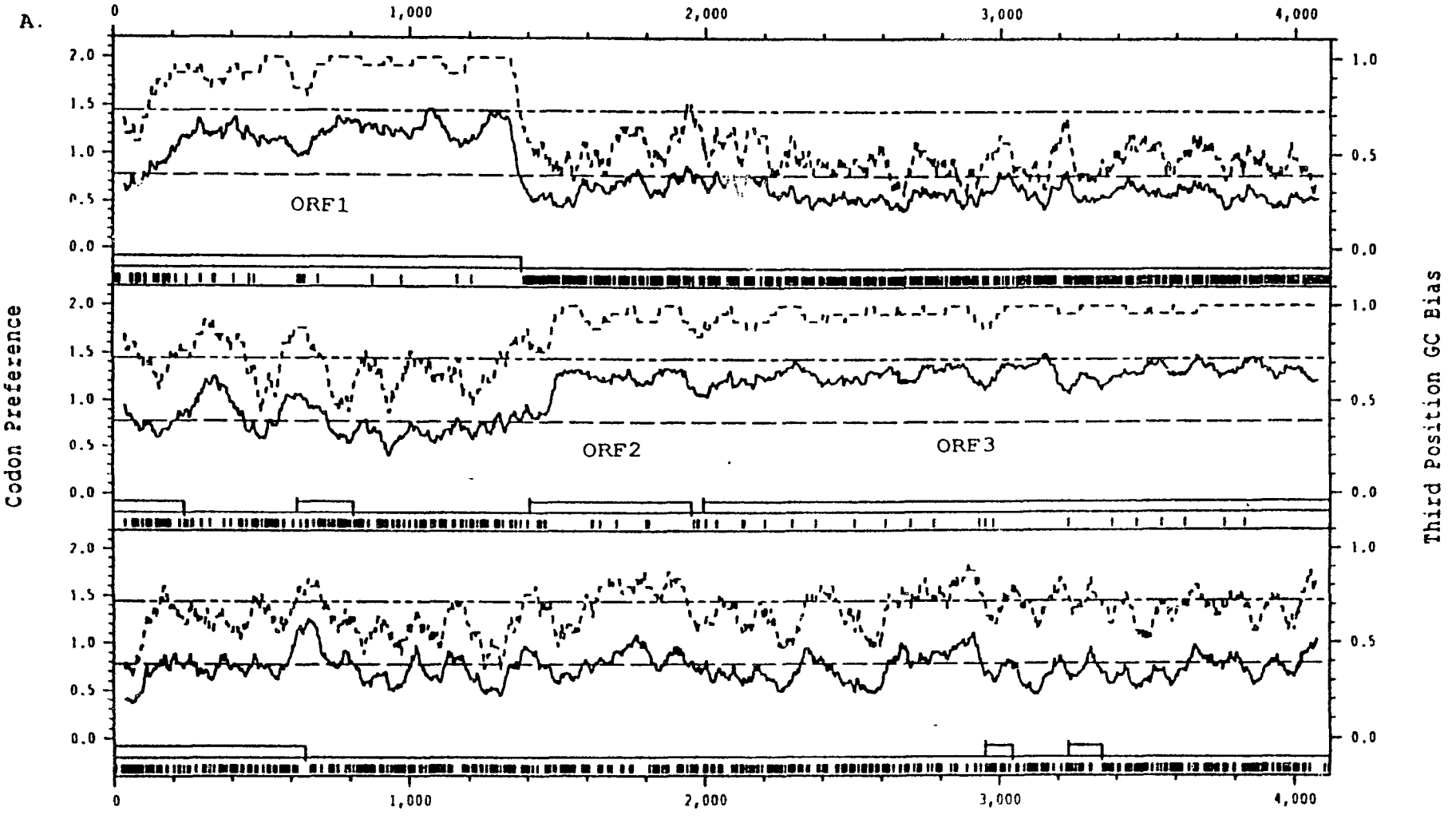
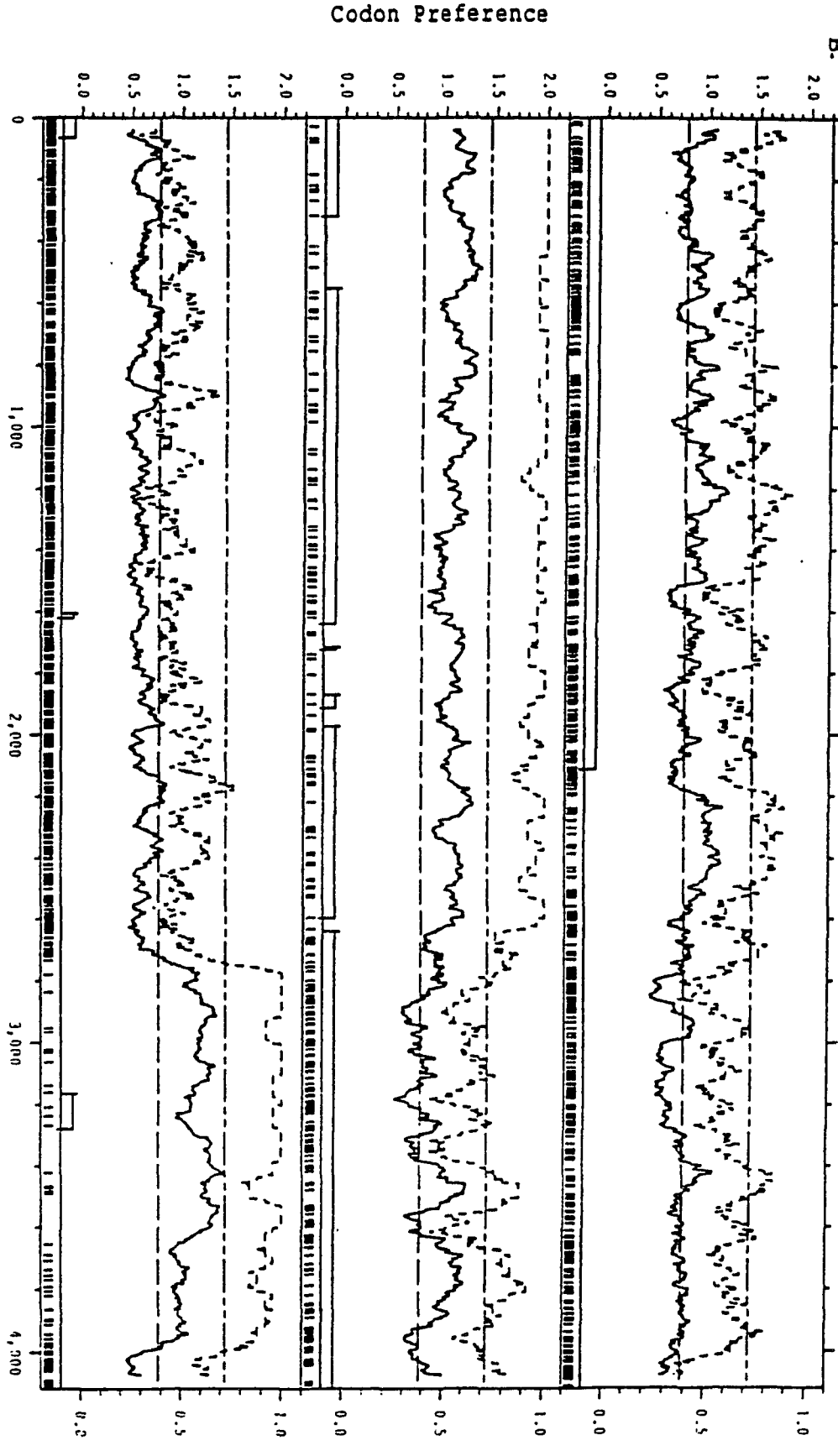

A.
0
1,000
2,000
3,000
4,000
2.0
1.5
1.0
0.5
0.0
ORF1
ORF2
ORF3
Codon Preference
Third Position GC Bias

Fig. 27.


Codon Preference
B.
Third Position GC Bias
0
1,000
2,000
3,000
4,000
0.0
0.5
1.0
1.5
2.0

from GC bias in the second and third codon positions either did not coincide with those defined by start and stop codons, or the occurrence of rare codons (Wright and Bibb, 1992) was too high (6-9%) for the sequence to be an ORF (2-3%).

Translational start codons for the ORFs were assigned by selecting the most upstream ATG or GTG (Seno and Baltz, 1988) codon preceded by a putative Shine-Dalgarno sequence. The latter is defined by its complementarity to the 3′-end of 16S rRNA from *S. lividans* (Bibb and Cohen, 1982). All ORFs have potential ribosomal binding sites (RBS) positioned 3-8 nt upstream of their translational start codons (see Fig. 26). Comparison with the sequence complementary to the 3′ end of 16S rRNA of *S. lividans* is shown in Table 15. ORF1 starts from the ATG codon at nt 160 and ends with a TGA codon at nt 1378. ORF2 has a probable ATG translational start codon at nt 1469, and its first in-frame stop codon is the TGA at nt 1952. ORF3 starts from the GTG at nt 1982 but continues after nt 4117. Sequence (data not shown) 300-400 nt downstream of (i.e., 3′ to) the sequence presented is AT-rich, suggesting that a noncoding region follows ORF3. An eight-base, perfect inverted repeat (shown as oppositely facing arrow heads in Fig. 26) was detected approximately 80 nt upstream of the putative translational start of ORF1. No typical inverted repeats that might function as transcription terminators were found downstream of either ORF1 or ORF2.

b. Similarity to the *ermE* probe sequence

Table 15. Comparison of putative ribosome-binding sites for the three ORFs in Fig. 26. with the sequence complementary to the 3′-end of *S. lividans* 16S rRNA.

ORF1	5′- GAGAGGAGCT
ORF2	5′-AGACAGGCGG
ORF3	5′- GGAGG
S. lividans 16S rRNA	5′-AGAAAGGAGGTGATC

Comparison of the DNA sequence of the 1.4-kb *Kpn*I fragment with sequences recorded in databases identified a region (nt 3340-3840) with strong sequence similarity to the *ermE* probe. Over this stretch of 500 nt (nt 3340-3840), the FASTA program after introduction of gaps to optimize alignment scored about 60% nucleotide identity between *ermE* and the 1.4-kb *Kpn*I fragment. This included a 22 nt (nt 3541-3562) perfect match between the two sequences (Fig. 28). However, a tentative translation using the 22-nt perfect match as frame guide (same reading frame as ORF3) did not give a protein resembling the *ermE* product. Only the seven amino acids encoded by the 22-nt sequence showed strong identity, and since none of these is conserved among MLS resistance methylases (Jenkins and Cundliffe, 1991), the region is evidently not functionally significant. It was concluded that the 4.3-kb *ermE*-hybridizing fragment did not encode an *ermE*-related gene. The 60% identity between the *ermE* probe and the 1.4-kb *Kpn*I fragment determined by FASTA was lower than the 65-75% identity calculated for duplex formation from the maximum hybridization stringency (Hopwood *et al.* 1985); the discrepancy may be accounted for by the presence of the 22-bp perfect match between the two sequences and the strong similarity in the region surrounding these 22 bases.

c. Codon usage

Codon usage in ORF1 and ORF2 is summarized in Table 16A and 16B. The frequency (Table 17) with which each base was used in

```
ermE  180 gcgagaggtgcgggg-aggatctgaccgacgcg-gtcca-ca----cgtg-gcaccgcgat
          ::: :::: :::::  ::: :::  ::   ::: : ::  ::    :::: :  ::::
     3362 gcgggaggggcgggtcaggttctt-cctcggcgtggccggcaacttcgtgcgggccgcccc

ermE  233 gctgttgtgggctg-gacaatcgtgccggttggtaggatcca--gcggtga-gcagt-tcg
                  :: : : :::  ::   :::      ::::  :   :::: :  :: :: ::
     3422 --------ggacagcgacg-tca--ccg------aggaggcgatgcggcgctgccgtctca

ermE  289 ga-cga----cgagcagcc--gcgcccgcgtcgccgca-accaggatcggcagcaccccaa
             ::          :  ::  :: :   ::  : :::: :::  :  ::::   :: ::
     3466 ccgcgcac--atctccaccaagctcaaccg--gtcgcacaccgtgtgcggcg--acaccgc

ermE  342 ccagaaccggccggtgctgggccgtaccgagcgggac-----cgcaaccggcgc-cagttc
           : :: :: ::::  ::::::::::::::::::::::      :: :::::::  ::::::
     3516 gctgatcctgccgacgctgggccgtaccgagcgggacatccaggccaccggcgagcagttc

ermE  397 gggcagaacttcctccgcgacc-gcaagac-----catcgcgcgcatcgccgagacagccg
          :  ::          : :::   :: ::       : ::::::: : :: :  ::  ::::
     3576 gt-cacggtggaggactcgatgagcgaggtgcacacctcgcgcgga-cggctgga--gccg

ermE  452 agctgcggcccgatctgc-cggtgc----tggaa-----gccggc---cccgtcgaa----
            :: : : : :  :::: : : :       ::      ::::::   :::: :: :
     3633 gcctcccggctg--ctgctcagcgaggtcgcgatcctgtgccggctggcccgccggaccct

ermE  496 ---gggctgctcaccagggaactcgcc---gaccgcg-cgcgtcaggtgacgtcgtacgag
             :::: :      : :::  :: ::   :::::   :: : : :   ::: :::    :
     3692 ggagggccgg-----acggagatcccctgggaccggttcgaggccgactacggcgt----g

ermE  550 atcgacccccggctggcgaag-tcgttgcggg----------agaagctt----tccggc
          :::  :  : :: : ::   : ::::  ::::          : :: :      :: : :
     3744 atccgcgacaggatcgcccggatcgtgccggggctgcacgactacaaccggcgggtc-gtc
```

Fig. 28. Comparison by FASTA of the *ermE*-hybridizing region of *S. griseoviridus* with *ermE* probe. Nucleotides are numbered in the left hand margin. Identical nucleotides are indicated by dots. The two sequences shared about 60% nucleotide identity.

Table 16A. Codon usage of ORF1

Amino acid	Codon	Total	Amino acid	Codon	Total
Phe	TTC	11	Tyr	TAC	7
	TTT	0		TAT	0
Leu	CTC	11	His	CAT	1
	CTG	14		CAC	15
	CTA	0			
	CTT	0	Gln	CAG	6
	TTA	0		CAA	0
	TTG	0			
			Asn	AAC	8
Ile	ATC	5		AAT	0
	ATT	0			
	ATA	0	Lys	AAG	3
				AAA	0
Met	ATG	5			
			Asp	GAT	1
Val	GTC	16		GAC	23
	GTG	12			
	GTA	2	Glu	GAA	1
	GTT	0		GAG	16
Ser	TCC	14	Cys	TGT	1
	TCG	12		TGC	5
	AGC	4			
	TCT	0	Trp	TGG	14
	TCA	0			
	AGT	0	Arg	CGT	1
				CGC	12
Pro	CCC	9		CGG	15
	CCA	1		AGG	4
	CCG	21		AGA	0
	CCT	0		CGA	0
Thr	ACC	24	Gly	GGT	6
	ACG	11		GGG	20
	ACA	0		GGC	26
	ACT	0		GGA	2
Ala	GCT	3	Term	TGA	1
	GCC	26		TAG	0
	GCG	18		TAA	0
	GCA	0			

Table 16B. Codon usage of ORF2

Amino acid	Codon	Total	Amino acid	Codon	Total
Phe	TTC	8	Tyr	TAC	1
	TTT	0		TAT	0
Leu	CTC	6	His	CAC	8
	CTG	4		CAT	0
	CTT	0			
	CTA	0	Gln	CAG	5
	TTA	0		CAA	0
	TTG	0			
			Asn	AAC	1
Ile	ATC	4		AAT	0
	ATA	0			
	ATT	0	Lys	AAG	1
				AAA	0
Met	ATG	2			
			Asp	GAC	5
Val	GTT	1		GAT	0
	GTC	10			
	GTG	8	Glu	GAA	1
	GTA	0		GAG	12
Ser	TCC	2	Cys	TGT	0
	TCG	2		TGC	0
	AGC	2			
	AGT	0	Trp	TGG	5
	TCT	0			
	TCA	0	Arg	CGC	10
				CGG	7
Pro	CCG	9		CGT	1
	CCC	2		CGA	0
	CCT	0		AGG	0
	CCA	0		AGA	0
Thr	ACC	10	Gly	GGG	3
	ACG	5		GGC	9
	ACA	0		GGA	2
	ACT	0		GGT	2
Ala	GCC	7	Term	TGA	1
	GCG	6		TAG	0
	GCA	0		TAA	0
	GCT	0			

Table 17. Percent base compositions of ORF1 and ORF2

	Codon position[a]					
	ORF1			ORF2		
Base	1	2	3	1	2	3
T	15.8	18.8	3.2	11.2	26.7	2.5
C	25.7	34.4	53.2	32.4	26.7	52.8
A	15.8	20.0	1.5	15.5	21.1	1.9
G	42.6	27.2	42.1	41.0	25.4	42.8
G+C	68.3	61.3	95.3	73.4	52.1	95.6

[a]Percent base compositions were calculated from the information in Tables 16A and 16B.

the three codon positions was calculated from these codon usage Tables. The overall G+C contents of ORF1 and ORF2 were 74.9% and 73.7%, respectively.

D. Comparison of ORF1 with known gene sequences

Database searches in which the amino acid (aa) sequences deduced for the three ORFs were compared with known sequences showed that the C-terminal half of the ORF1 product (aa 170-406) was similar to several bacterial β-1,3-1,4 glucanases and a β-1,3 glucanase (Fig. 29). The closest sequence similarity was to a β-1,3 glucanase (GlcA) of *Bacillus circulans* (Watanabe *et al.*, 1992). When compared by the BESTFIT software programme, the ORF1 and *glcA* products showed 58.5% sequence similarity and 34.8% identity. The score for the quality of the comparison was 175.9, while the average score for quality based on 10 comparisons between the ORF1 sequence and *glcA* product after it had been randomized was 134.1 +/- 3.5. This indicated a significant match between ORF1 and *glcA* product in the BESTFIT comparison. The two products were also compared using the PILEUP (Fig. 30A), and COMPARE and DOTPLOT (Fig. 30B) software programmes; significant sequence similarity was detected in the C-terminal regions. The N-terminus of the ORF1 product showed typical characteristics of a signal peptide for excreted proteins.

```
          +   + + +   +                              ** *
fMGTPPRHARPRSRALRAGVLLVSGAVVTTALSLAGTANGDPPGPRVTQL
```

```
       * + * **   +                              * * *  * +
GluB  DGKLKLSLTS PA..... .NNKFDCGEY RSTNN..YGY GLYEVSMKPA
GluA  lgemrlalts ps..... .ynkfdcgen rsvqt..ygy glyevrmkpa
ORF1  EGALHLRLT. GASAICKGET R..PYSGAHV NTMGLFSYSY GAVEYRARVP 281

         * *                          ** *                  *
GluB  KNTGIVSSFF TYTGPSHGTQ WD......EI DIEFLG.... ......KDTT
GluA  kntgivssff tytgptdgtp wd......ei dieflg.... ......kdtt
ORF1  SVDGAVANWP ALWDTG.... .SLWPRDGEI DTLEG...LS GEVCSYWHST 323

       *                              **     * *+ *  *  **   *
GluB  KVQFNYYTNG VGGHEKIINL GFDASTSFHT YAFDWQPGYI KWYVDGVLKH
GluA  kvqfnyytng agnhekivdl gfdaanayht yafdwqpnsi kwyvdgqlkh
ORF1  RVDRGHCPPS GDR..... .....AGWHT FGHTWEPGRI TWFHDGKPVH 361

      * *  *   *     *                       *      ***+  +
GluB  TATTNIPSTP GKIMMNLWNG TGVDSWLGSY NGANPLYAEY DWVKYTSN..
GluA  tatnqipttp gkimmnlwng tgvdewlgsy ngvnplyahy dwvrytkk..
ORF1  TETSGIVGSP HHLVMQNT.Q GSFGG..... PTLVPADLQV DWVRVWSRRA 406


GluB  ..
GluA  ..
ORF1  GZ
```

Fig. 29. Comparison by PILEUP of the ORF1 protein with GluB of *Bacillus polymyxa* (Gosalbes *et al.*, 1991) and GluA of *Bacillus subtilis* (Murphy *et al.*, 1984). Similar amino acids are defined according to Gribskov and Burgess (1986). Amino acids identical in all four proteins are indicated with asterisks; amino acids of the ORF1 product identical to an amino acid in any other protein are indicated by plus signs. Both GluA and GluB are β-1,3-1,4-glucanases.

```
          1                                                A1->          50
GlcA      mkpshftekr fmkkvlqlfl vvvmlasvgv lptskvqaag ttvtsmeyfs
ORF1      .......... .......... .......... .......... ..........

          51                                                           100
GlcA      padgpvisks gvgkasygfv mpkfnggsat wndvysdvgv nvkvgnnwvd
ORF1      .......... .......... .......... .......... ..........

          101                                                A2->      150
GlcA      idqaggyiyn qnwghwsdgg fngywftlsa tteiqlyska ngvkleyqlv
ORF1      .......... .......... .......... .......... ..........

          151       *                 **   *        *       *      200
GlcA      fqninkttit amnptqgpqi tasftggagf typtfnndsa vtyeavaddl
ORF1      ...MGTPPRH ARPRSRALRA GVLLVSGAVV T.......TA LSLAGTANG.

          201                                    * *          *      250
GlcA      kvyvkpvnss swididnnaa sgwiydhnfg ..qftdgg.g gywfnvtesi
ORF1      .......... .......DPP GPRVTQLGLA PDHLTAGHRA PARLELTADR

          251    *A3->           *   * *      * ***   *         300
GlcA      nvklesktss anlvytitfn eptrnsyvit pyegttftad angsigiplp
ORF1      CLTAARVTVA VRDRDGRNVD FPGAASDVRV CPSGHTFTSG AR........

          301                                      *  *   *       350
GlcA      kidggapiak elgnfvyqin ingqwvdlsn ssqskfaysa ngynnmsdan
ORF1      .......... .......... .......... ......AFAA GSY......T

            * * *         *         *        **          A4->* *400
GlcA      qwgywadyiy glwfqpiqen mqirigypln qqaggnignn fvnytfignp
ORF1      MFGSWQD..G EGRWHPL... ..TPITLSVA EPAGQAEPAD ......AGAP

            *               * *    *  *       *** **  *******  *450
GlcA      nap..rpdvs dqedisigtp tdpaiagmnl iwqdefngtt ldtskwnyet
ORF1      SGPGEPSGSA EPAEADGGGP SWDASGGWRR RFTDEFDGTA LDTSKWN..T

          *           *  *          * *  *   * *                  500
GlcA      gyylnndpat wgwgnaelqh ytnstqnvyv qdgklnikam ndsksfpqdp
ORF1      GWF...GTGV TGPVNTAEDA CYD.TRNAVV SEGALHLR.L TGASAICKGE

           *   **     **    *   ** *  **   *  **    * ****      550
GlcA      nryaqyssgk intkdklslk ygrvcerakl ptgdgv...w palwmlpkds
ORF1      TR..PYSGAH VNTMGLFSYS YGAVEYRARV PSVDGAVANW PALWDTG...

          551 *   ** **  *    *  * *    *              **        600
GlcA      vygtwaasge idvmeargrl pgsvsgtihf g.gqwpvnqs sggdyhfpeg
ORF1      ..SLWPRDGE IDTLEG...L SGEVCSYWHS TRVDRGHCPP SGDR......

          601    *      **   *  *  ***                           650
GlcA      qtfandyhvy svvweednik wyvdgkffyk vtnqqwysta apnnpnapfd
ORF1      ....AGWHTF GHTWEPGRIT WFHDGK.... .........P VHTETSGIVG

           *  * *      * * *          **   *** ***              694
GlcA      epfylimnla vggnfdggrt pnasdipatm qvdyvrvyke q...
ORF1      SPHHLVMQ.N TQGSFGG... ..PTLVPADL QVDWVRVWSR RAGZ
```

Fig. 30A. Comparison by PILEUP of the ORF1 protein with the *glcA* product of *Bacillus circulans*. Similar amino acids are defined according to Gribskov and Burgess (1986). Amino acids identical in both proteins are indicated with asterisks. The signal peptide of GlcA is underlined; the starts of the four glucanase species (A1 to A4) derived from GlcA are indicated with arrows.

Fig. 30B. Comparison of the predicted ORF1 protein sequence with the *glcA* product of *Bacillus circulans* by COMPARE and DOTPLOT (Devereux, *et al.*, 1984). A window of 30 amino acids and a stringency of 16 were used. The ORF1 protein sequence is plotted along the X axis; the *glcA* sequence is plotted along the Y axis. Numbers along axes refer to the number of amino acids from the N-terminus of each protein. *glcA* encodes a β-1,3-endoglucanase.

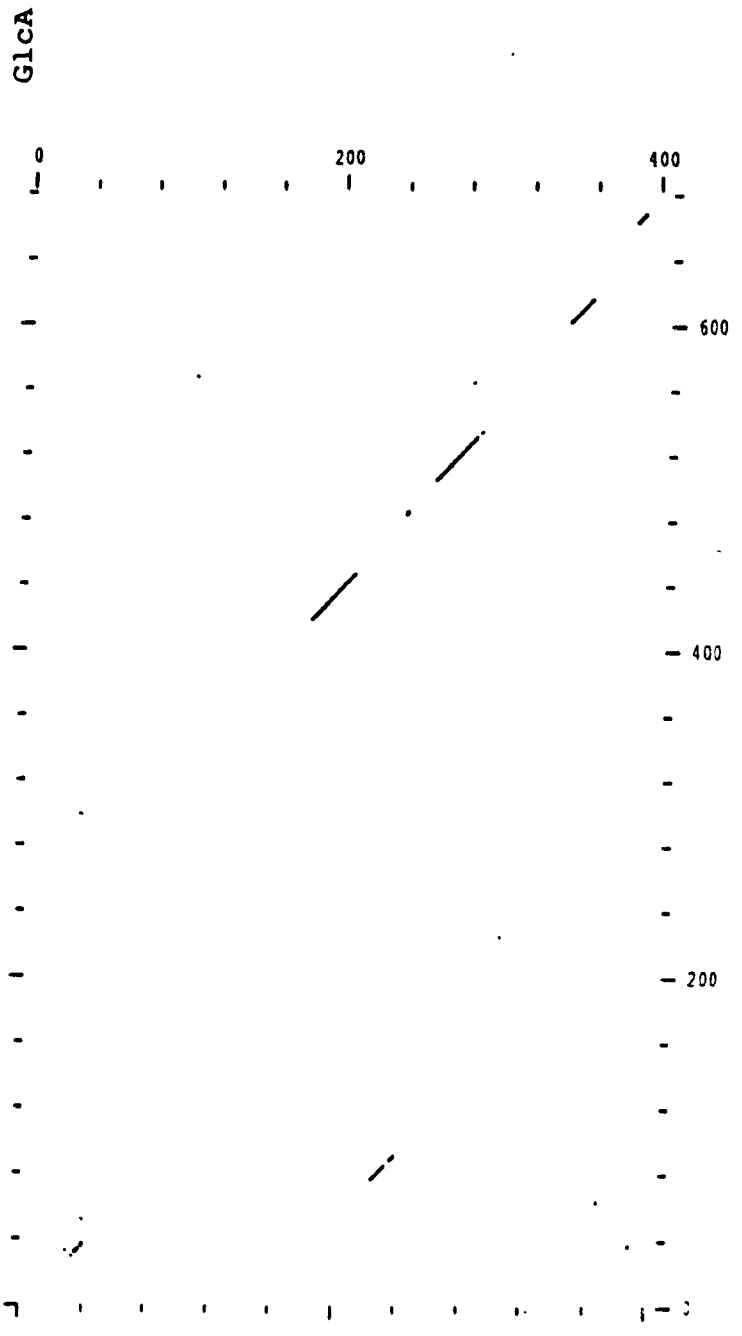


Fig. 30B.

Amino acids 1-16 are strongly hydrophilic and the region is rich in arginine; the arginines are highlighted above with pluses. Following these is a stretch of 21 amino acids (17-37) that are hydrophobic (underlined) except for two serines, which interrupt the sequence at separate places. Three prolines are clustered in the next region, and are highlighted with asterisks.

E. Comparison of the ORF3 product with known protein sequences

Database searches found significant aa sequence similarity between the N-terminal half of the ORF3 product and various formate dehydrogenases. A BESTFIT comparison of this region (aa 50-440 of ORF3) with the *fdhF* product (benzylviologen formate dehydrogenase; Zinoni *et al.*, 1986) of *E. coli* scored 55.3% similarity and 32.3% identity; the BESTFIT quality score was 208.7 (the average quality score from 10 comparisons of randomized ORF3 with FdhF was 180 +/- 3.5). The same region of the ORF3 product also showed significant similarity to FdhA (formate dehydrogenase α-subunit; Shuber *et al.*, 1986) of *Methanobacterium formicicum* and FdhA (large subunit of the membrane-bound formate dehydrogenase; Bokranz *et al.*, 1991) of *Wolinella succinogenes*. When the ORF3 product was compared with FdhF of *E. coli* and FdhA of *M. formicicum* using PILEUP (Fig. 31A), and with FdhF using COMPARE and DOTPLOT (Fig. 31B), sequence similarities were apparent in the N-terminal regions of these proteins. FdhF of *E. coli* and FdhA of M.

```
      1                                                                50
FdhA   msealsgrg ndrrkflkms alagvagvsg avgsdqskvl rpatkqelie
FdhA   .......... .......... .......... .......... ..........
FdhF   .......... .......... .......... .......... ..........
ORF3   MTRLMHTSPS DEPEENLTVQ PPKTWATGAP AVAHALQYSL EETSVRRTGT

      51     *   * *+ *+                      +     *+  +  + 100
FdhA   kypvskkv.k tictycsvg. ......cgii aevvdgvwvr qevaqdhpis
FdhA   ..mdikyv.p ticpycgvg. ......cgmn lvvkdekvvg vepwkrhpvn
FdhF   ....mkkv.v tvcpycasg. ......ckin vvvdngkivr aea.aqgktn
ORF3   TLLTMNQVDG TDCPGCAWAD PSPGHRHVNE YCENGAKHIN DEATTRRITA

      101     +    + + +++        **+  *  +        ++  * +*+150
FdhA   qgghcckgad midka.rset ......rlry piek..vggk wrktswdsam
FdhA   egklcpkgnf cyeiihred. ......rltt plik..enge freatwdeay
FdhF   qgtlclkgyy gwdfindtqi l...tprlkt pmirrqrggk lepvswdeal
ORF3   D...FFRRYS VSDLASRSDQ WLNQQGRLTE PVVKRPGSDH YEPIGWQEAL

       + *  *  +      *++   +  + +  ++ *   + + ** ++  **+*+ 200
FdhA   dkiakqlqdl tqkygpdsvm figgskc.si eqsyyfrkfa a.ffgtnnld
FdhA   dliasklga. ...ydpneig ffccars.pn eniyvnqkfa rivvgthnid
FdhF   nyvaerlsai kekygpdaiq ttgssrgtgn etnyvmqkfa ravigtnnvd
ORF3   GLLAGELNSL D...SPDEAV FYTSGR.VSN EAAFVLQLFA RA.YGTNNLP

       +   **  +    ++ ++*  *  +  + +   +++++*+ *  *+  +**  250
FdhA   tiarichapt vagvsntlgy ggmtnhladm mhskaifiig gnpavnhpvg
FdhA   hcarlchgpt vaglaasfgs gamtnsyasf edadlifsig ansleahplv
FdhF   ccarvchgps vaglhqsvgn gamsnainei dntdlvfvfg ynpadshpiv
ORF3   DCSNMCHESS GFALHETLGT GKGTVTLEDL HHADLIFVVG QNPGSNHPRQ

      251+  *++ **  * + *        ++     +           +**    300
FdhA   mvhilrakea gakiivvdp. ......hfsr tat....... ...kadhyvr
FdhA   grklmrakmn gayfivadp. ......rytp tak....... ...qadqyip
FdhF   anhvinakrn gakiivcdp. ......rkie tar....... ...iadmhia
ORF3   LSALEAAKRN GARVIAVNPL PEAGLLRFKN PQKPRGVIGR GVEIADRFLH

       + * + *+   +  +   +           *   *++  + + +**       350
FdhA   lrngtdvafm ygmirhivk  .....ngled kefirqrlfg yeeilkeceq
FdhA   fktgtdvalm nammnviis. .....egled kefiekrtkn yeelkevvsk
FdhF   lkngsniall namghviie. .....enlyd kafvasrteg feeyrkiveg
ORF3   IRVGGDLALF QGLNRLLLEA EDARPGTVLD HDFIRASTSG FEEFSRHART

      351* +   * +    ++                  + +*+*+ + +*+ + + 400
FdhA   ytpevveevt gvpaqqliei teifakakpa sliwgmgltq httgtsntrl
FdhA   ytpemaeeit qvpadvirdi aikyakadka aivyslgite hshgvdnvmq
FdhF   ytpesvedit gvsaseirqa armyaqaksa ailwgmgvtq fyqgvetvrs
ORF3   VAWEDVLRAT GLTRAEIEKV RDDVLASERV IVCWAMGVTQ HKHGVPTIRE

         +   + ** +*+ ++*  + +**+ ****     *                 450
FdhA   apilqmilgn igkrgggtnv lrghdnvqga tdmgnladsl pgyygldkna
FdhA   tanlamltgn igrlgtgvnp lrgqnnvqga cdmgalptdy pgyrkvadqe
FdhF   ltslamltgn lgkphagvnp vrgqnnvqga cdmgalpdty pgyqyvkdpa
ORF3   IVNFLLLRGN LGRAGAGACP VRGHSNVQGD RTVGIWEQMP DSF.......
```

Fig. 31A. Comparison by PILEUP of the ORF3 product with the *fdhA* product of *W. succinogenes* (top line), the *fdhA* product of *M. formicicum* (second line) and the *fdhF* product of *E. coli*. Amino acids identical in all four proteins are indicated with asterisks; amino acids of ORF3 identical to either of the FdhAs or FdhF are indicated with plus signs. *fdhA* of *W. succinogenes* encodes the largest subunit of its formate dehydrogenase. *fdhA* of *M. formicicum* encodes the α-subunit of its formate dehydrogenase; *fdhF* encodes the benzylviologen-linked formate dehydrogenase of *E. coli*.

```
        451      +  +  + + ++       + +    + ++                  500
FdhA    wnhfcgiwkv dfeamqkrf. ktpdmmhkkg fsvstwrygv teeeniphna
FdhA    vmedvtctwg csdlgcepgl kipemidaaa kgdlkvlyit gedpvisdpd
FdhF    nrekfakawg veslpahtgy riselphraa hgevraayim gedplqtdae
ORF3    .LDALRDEFG FEP.PRHHGL DAVNSIKAMR EGRVRFFLGV AGNFVRAAPD

        501 +++      +   +         +        +*+      +     + 550
FdhA    gtklrsliv. .vgsgistia rvdttk.dal dkmdlvvffd pyfndaaalt
FdhA    thhveealn. .nldffvvqd ifmtdt.aef advvlpaacw aeqegtftng
FdhF    lsavrkafe. .dlelvivqd ifmtkt.asa advilpstsw gehegvftaa
ORF3    SDVTEEAMRR CRLTAHISTK LNRSHTVCGD TALILPTLGR TERDIQATGE

        551   +     +   +                        + +  ++        600
FdhA    nrkdnlyilp aatqmetsgr va..... .....atnr syqwrsmv..
FdhA    errvqlirka vdapgeskyd we..... .....ifcd lakkmgadpe
FdhF    drgfqrffka vepkwdlktd wq..... .....iise iatrmgyp..
ORF3    Q.....FVTV EDSMSEVHTS RGRLEPASRL LLSEVAILCR LARRTLEGRT

        601           +                   +  +++ +   +         650
FdhA    mkplfecrpd eeilfdlakr lgfyeey... trslgdgkgn fv..wpddat
FdhA    mftyesaqdi feevrtvtpq yagmnre... rldrpealhw pc..psedhp
FdhF    .mhynntqei wdelrhlcpd fygatye... kmgelgfiqw pcrdtsdadq
ORF3    EIPWDRFEAD YGVIRDRIAR IVPGLHDYNR RVVRPGGIRL PNP.......

        651      +  +  + + +    +++ +       +  + +  + +       700
FdhA    revakairtv gfqgrtperl kahaenwhmf dkftlrgkgg pvkgeyyglp
FdhA    gtammhiekf ahpdglgifm pleeqgpmet pddeyplilt ttrllfhyha
FdhF    gtsylfkekf dtpnglaqff tcdwvapidk ltdeypmvls tvrevghysc
ORF3    ....VNEGVF GTESGKALFT RNDWQMPR.. .VPEGHLLLQ TLRSHDQWNT

        701+                    +  +  ++ + ++   +    +        750
FdhA    wpcwsekhpg tpnlwddsip vmdgglgfrv rwgdvsptge sllasqdssl
FdhA    .amtrraatl dr.evptgyv eintedaael giankekvkv ksrrgeieia
FdhF    rsmtgncaal aaladepgya qintedakrl giedealvwv hsrkgkiitr
ORF3    VPYTTD.DRY RGIHGSRRVV MVNPDDLTEL GLADGQRVDL VSVWSDDVE.

        751                                                  800
FdhA    pgskfkgghs mitdknveai tgialteeek akvagktwat dttnilveka
FdhA    arvtddivkg ivnipmhfre csaniltnaa aidpksgmpe ykacavaisk
FdhF    aqvsdrpnkg aiymtyqwwi gacnelv.te nlspitktpe ykycavrvep
ORF3    ......... ......... ......... ......... .........

        801                          828
FdhA    laaglspmgn graraivwew tdqipkhrep iytirhdlis qyptfkdkpn
FdhA    megsk..... ......... ........
FdhF    iadqraaeqy videynklkt rlreaala
ORF3    ......... ......... ........

FdhA    hfraniryes rqkekdwtke fplnmlsgrl vaqfgtgtet rsahylaevq
        pemfveihpe tatdlgvkhg dmvwvhgtng akilvkarhs ykvnktsvfl
        pqnfggmyqg eslvpyhiag tepyvigesc ntitsdaydi ntstpetkcg
        lcrieka 939
```

Fig. 31A. Comparison by PILEUP of the ORF3 product with the *fdhA* product of *W. succinogenes* (top line), the *fdhA* product of *M. formicicum* (second line) and the *fdhF* product of *E. coli*. Amino acids identical in all four proteins are indicated with asterisks; amino acids of ORF3 identical to either of the FdhAs or FdhF are indicated with plus signs. *fdhA* of *W. succinogenes* encodes the largest subunit of its formate dehydrogenase. *fdhA* of *M. formicicum* encodes the α-subunit of its formate dehydrogenase; *fdhF* encodes the benzylviologen-linked formate dehydrogenase of *E. coli*.

Fig. 31B. Comparison of the predicted ORF3 protein sequence with the *fdhF* product of *E. coli* by using COMPARE and DOTPLOT (Devereux, *et al.*, 1984). A window of 30 amino acids and a stringency of 16 were used. The ORF3 protein sequence is plotted along the X axis; the *fdhF* protein sequence is plotted along the Y axis. Numbers along axes refer to the number of amino acids from the N-terminus of each protein. *fdhF* encodes the benzylviologen-linked formate dehydrogenase.

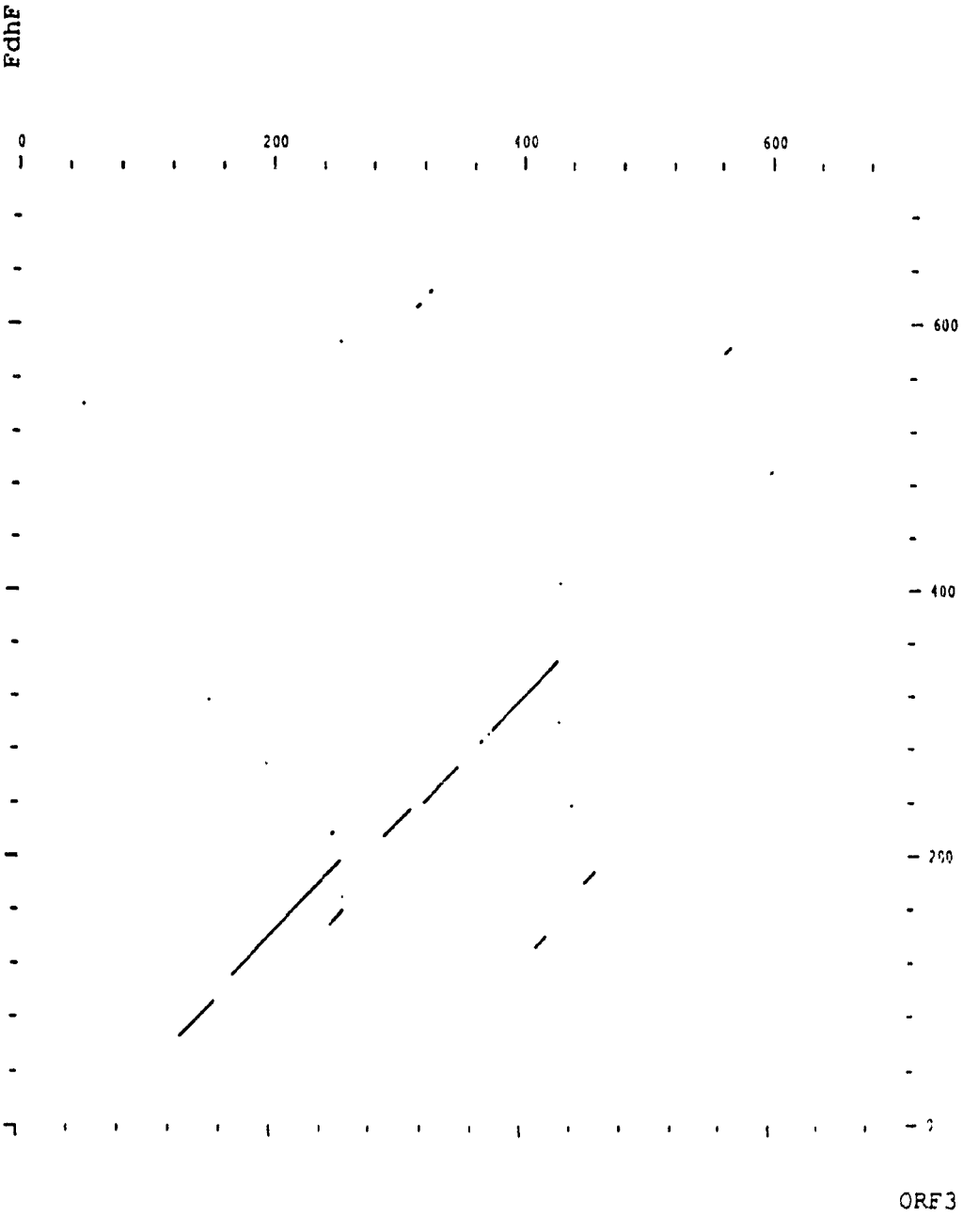


Fig. 31B.

formicicum showed end-to-end similarity with each other, but the ORF3 product, like FdhA of *W. succinogenes*, shared similarity only with the N-terminal half of the other two formate dehydrogenases.

The N-terminal region of the ORF3 product contained a cysteine-rich sequence as well as a cysteine corresponding to the selenium-cysteine and its surrounding conserved amino acids found in other formate dehydrogenases.

	*
FdhA	CarlCH
FdhF	CarvCH
ORF3	CsnmCH

The selenium-cysteine position is indicated by an asterisk. The amino acids conserved in all three proteins are capitalized.

F. Search for other possible ORFs

To minimize the possibility of failing to identify potential coding regions on each strand, the 4117 bp sequence was analysed using DNA STRIDER to identify all possible start (ATG and GTG for streptomycetes) and stop codons. Putative ORFs of reasonable length were identified and translated into amino acid sequences. Besides ORF1-3, which had already been identified by the CODONPREFERENCE program, seven other ORFs were assigned; they were sent to databases to search for homologous sequences. One ORF product showed low-level similarity to the *ermE* product; the ORF was in the reverse

direction from ORF3 and spanned the *ermE*-hybridizing region. Since it was strongly biased towards A and T at the third codon position, the ORF is probably not a genuine streptomycete gene. Because the ORF3 product showed significant homology to several formate dehydrogenases and the ORF met all the coding criteria for *Streptomyces*, it is most likely a valid ORF. To find another ORF on the reverse strand of ORF3 would be unexpected.

V. Deletion and Disruption of ORFs in Cloned DNA.

Sequencing data revealed two putative ORFs in the 2.1-kb *Sac*I:*Sal*I fragment present as the cloned DNA insert of pDQ322. Previous subcloning results indicated that pDQ323, now seen to contain the upstream region and a truncated 5'-terminal segment of ORF1, was able to confer low-level etamycin resistance similar to that conferred by pDQ322. If the truncated ORF1 is indeed responsible for etamycin resistance, further disruption of ORF1 might be expected to abolish this resistance. Furthermore, if ORF1 is the only ORF responsible for etamycin resistance, plasmids containing only ORF2 should not confer etamycin resistance. To test these assumptions, subclones defective in ORF1 were constructed (Fig. 32).

A. Deletion/disruption strategies

To disrupt ORF1, pDQ322 was digested with *Mlu*I and the

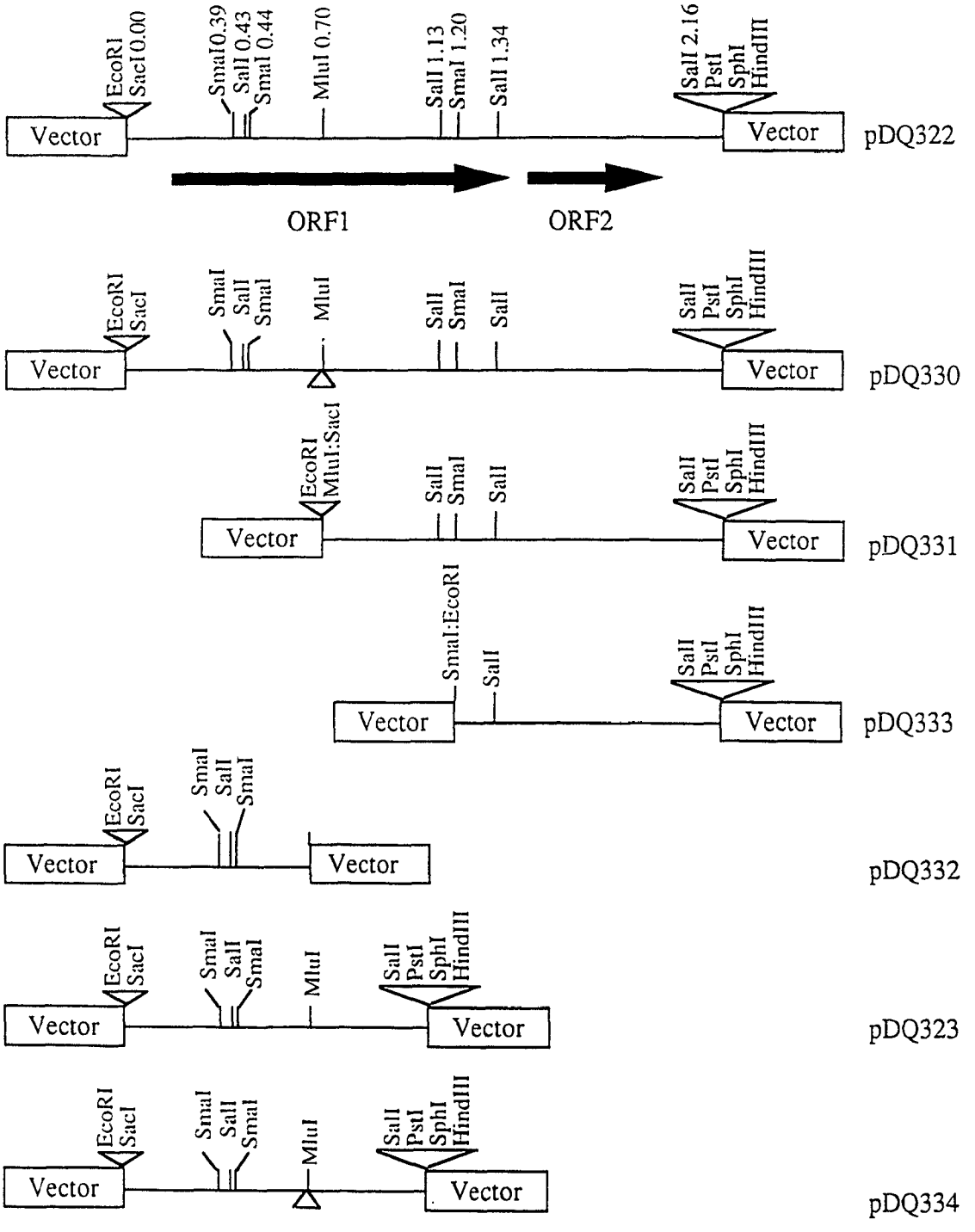


Fig. 32. Construction of plasmids with mutations in ORF1 and/or ORF2. Distances (kb) from the left hand *Sac*I (0.00) to each restriction site are shown for pDQ322.

single-stranded ends were filled in by treatment with the Klenow fragment of DNA polymerase I. Religation of the filled pDQ322 fragments generated pDQ330, which was no longer susceptible to digestion with *Mlu*I. This treatment should have created a frameshift mutation in ORF1. A similar frameshift mutation was introduced into pDQ323 by digesting the plasmid with *Mlu*I and filling in the reaction site by Klenow treatment. Religation of the blunt ends gave pDQ334, which was no longer susceptible to *Mlu*I digestion and which was predicted from the sequence data to contain ORF1 truncated close to the 3′ terminus.

In a second modification of pDQ322, the plasmid was digested with *Mlu*I and *Sac*I. The ends of the linear fragments so formed were filled in by Klenow treatment and religated. Transformation of *E. coli* TG1 with the ligation mixture gave pDQ331. This plasmid had a large segment of DNA deleted from the 5′ end of its insert and thus lacked the N-terminal half of ORF1 and its upstream sequences, but contained an intact ORF2.

By a prodedure similar to that used to construct pDQ331, the *Mlu*I:*Hin*dIII fragment was deleted from pDQ322 by double digestion with *Mlu*I and *Hin*dIII; the linear fragments remaining were filled in by Klenow treatment and religated to give pDQ332. This plasmid contained a truncated ORF1 and completely lacked ORF2. The deletion in ORF1 included most of the C-terminal region.

The procedure was followed again to obtain pDQ333 from pDQ331. The *Sma*I:*Eco*RI fragment was deleted from pDQ331 by double digestion with *Sma*I and *Eco*RI. The sticky ends were then filled by Klenow treatment and religated. Plasmid pDQ333 retained an intact ORF2 but most of ORF1 and its upstream region had been deleted.

All of these plasmids were shown by appropriate restriction enzyme digestions to yield the expected DNA fragments.

B. Transformation of *S. griseofuscus* with the modified vectors and resistance of the transformants

Transformation of *S. griseofuscus* with the modified plasmids gave strains FK8 (containing pDQ330), FK9 (containing pDQ331), FK10 (containing pDQ332), FK11 (containing pDQ333) and FK12 (containing pDQ334). The many thousands of transformants from each strain were allowed to sporulate *en masse*, and the spores were collected. Examination of etamycin resistance in these transformants with the controlled inoculum assay showed age-dependent phenotypes that resembled those of other *S. griseofuscus* transformants obtained earlier (see Table 11). In a comparison of MTCs for etamycin, transformants FK8 and FK9 exhibited low-level resistance (MTC of 30-40 $\mu g.ml^{-1}$ of etamycin), thus resembling FK2-3 and FK5-6. However, in FK10-12 resistance was lower and resembled that of FK400 (MTC of 10-20 μg etamycin ml^{-1}).

Since both pDQ330 and pDQ331 contained a disrupted ORF1

but had an intact ORF2, it might be suggested that ORF2 was responsible for enhanced etamycin resistance. However, pDQ333, which still contained the complete coding region of ORF2, did not confer the same level of etamycin resistance as pDQ330 and pDQ331. Although this could be attributed to the absence of an upstream regulatory sequence necessary for transcription of ORF2; a sequence presumably overlapping ORF1 and thus still functional in pDQ330 and pDQ331, the requirement for ORF2 is called into question by the ability of pDQ323, which contains only a truncated ORF1 missing 80 amino acids from the C-terminal region (see Fig. 32), to confer etamycin resistance (see Table 14).

In support of alternative conclusion, that a region in ORF1 was responsible for resistance, both further deletion in pDQ332 of the C-terminal region of ORF1, and in pDQ334 a frame-shift mutation at the *Mlu*I site in ORF1 weakened the degree of etamycin resistance conferred by pDQ323. In addition, the large deletion in pDQ333 that removed most of ORF1 also eliminated most of the resistance to etamycin. However, neither the reading frame mutation in pDQ330 that should have inactivated the C-terminal region of ORF1, nor the deletion in pDQ331 that deleted the N-terminal region completely eliminated the etamycin resistance phenotype. Thus the evidence for either conclusion tends to be mutually contradictory. An alternative explanation of the results might be that a DNA sequence, rather than an ORF product, was

responsible for the enhanced etamycin resistance. The presence of an intact sequence corresponding to the region between the *Mlu*I and *Sal*I sites at 0.7 and 1.13 kb in the insert of pDQ312 (Fig. 32) was shared by all plasmids that conferred etamycin resistance at or above 30-40 $\mu g.ml^{-1}$. The sequence of this piece of DNA did not contain direct repeats, inverted repeats or other special features.

The MTC of etamycin for each transformant was tested under induced and uninduced conditions (Table 18). Etamycin resistance was induced by exposing the inoculum cultures to 0.5 $\mu g.ml^{-1}$ of etamycin for 3 h prior to assay. To address the possibility that the resistance level was correlated with the size of the insert in a plasmid rather than with the presence in it of a resistance determinant, a recombinant pHJL400 plasmid vector (pDQ400) containing a 17-kb insert from *S. venezuelae* (L. Han, personal communication) was also used to transform *S. griseofuscus*. The transformant, designated, FK13, showed etamycin resistance higher than FK400 but lower than FK1 (containing pDQ310). Since FK1 and FK13 have similarly sized plasmids, the resistance level was not strictly correlated with insert size. The evidence that even a foreign DNA insert could confer some resistance was consistant with the hypothesis that resistance was due to activation of a host resistance gene.

C. Effect of assay media on etamycin resistance

Table 18. MTC of etamycin for the *S. griseofuscus* transformants.

Transformants	Plasmid present	MTC ($\mu g.ml^{-1}$)*	
		Induced	Uninduced
FK400	pHJL400	20-30	10-20
FK1	pDQ310	>60	50-60
FK6	pDQ323	40-50	30-40
FK8	pDQ330	40-50	30-40
FK9	pDQ331	40-50	30-40
FK10	pDQ332	20-30	10-20
FK11	pDQ333	20-30	10-20
FK12	pDQ334	20-30	10-20
FK13	pDQ400	40-50	30-40

* MYM agar containing increasing amounts of etamycin was inoculated with vegetative mycelium from transformant cultures in early exponential growth (OD_{640} 3-5). The presence or absence of growth was recorded after incubation at 30°C for 3 days. Inoculum cultures induced with etamycin were treated as described in Materials and Methods.

Mycelium at different ages was assayed on TSB agar, MYM agar and MM agar media containing etamycin. Surprisingly, samples that showed high-level etamycin resistance on both MYM and MM agar were completely sensitive to etamycin on TSB agar. However, when the inoculum was induced with etamycin, strains showing stronger etamycin resistance on MYM agar also showed stronger resistance on TSB agar (Table 19).

The inducible etamycin resistance phenotype of all the *S. griseofuscus* transformants (Table 20) supported the existence of a normally silent host resistance gene. The relatively small differences in resistance levels between the transformants suggested that the resistance was not determined by a cloned gene, but instead that inserts differed in their ability to enhance the activity of a host resistance gene.

D. Etamycin inactivation by *S. griseofuscus* transformants

The ability of *S. griseofuscus* transformants FK1, FK3, FK5, FK6 and FK400 to inactivate etamycin was compared in cultures grown in YEME medium. Spores of the transformants were used to inoculate medium supplemented with 15 $\mu g.ml^{-1}$ of thiostrepton, and the cultures were incubated at 30°C for 24 h. Etamycin (50 $\mu g.ml^{-1}$) was then added, incubation was resumed and etamycin concentrations in the cultures were measured at intervals by HPLC. The results indicated that etamycin was slowly degraded by each of the transformants. The decreased etamycin concentration in culture supernatants was associated with the

Table 19. Comparison on MM, MYM and TSB agar of the resistance (MTC) of *S. griseofuscus* transformants to etamycin.

Transformant	Plasmid present	MTC ($\mu g.ml^{-1}$)[a]		
		MM	MYM	TSB
FK400	pHJL400	5-10	10-20	10-20
FK1	pDQ310	40-50	40-50	40-50
FK5	pDQ322	20-30	20-30	20-30

[a] Vegetative inocula from transformant cultures in early exponential growth (OD_{640} 3-5) were assayed on MM, MYM and TSB agar containing various amounts of etamycin. The presence or absence of growth was recorded after incubation at 30°C for 3 days. MTCs on MM and MYM agar were obtained with uninduced inoculum cultures; MTCs on TSB agar were obtained using inoculum cultures induced with etamycin as described in Materials and Methods. With uninduced vegetative inocula, no growth was observed on TSB agar containing 5 $\mu g.ml^{-1}$ of etamycin after incubation at 30°C for a week.

Table 20. Resistance to etamycin (measured as MTC) of the *S. griseofuscus* transformants on TSB agar.

Transformant	Plasmid present	MTC ($\mu g.ml^{-1}$)[a]
FK400	pHJL400	10-20
FK1	pDQ310	40-50
FK2	pDQ311	20-40
FK3	pDQ312	20-40
FK4	pDQ316	10-20
FK5	pDQ322	20-40
FK6	pDQ323	20-40
FK7	pDQ317	10-20
FK8	pDQ330	20-40
FK9	pDQ331	20-40
FK10	pDQ332	10-20
FK11	pDQ333	10-20
FK12	pDQ334	10-20
FK13	pDQ400	20-40

[a] Vegetative inocula from transformant cultures in early exponential growth (OD_{640} 3-5) were assayed on TSB agar containing various amounts of etamycin. The presence or absence of growth was recorded after incubation at 30°C for 3 days. Inoculum cultures were induced with etamycin as described in Materials and Methods. With uninduced vegetative inocula, all strains failed to grow on TSB agar containing 5 $\mu g.ml^{-1}$ of etamycin after incubation at 30°C for a week.

appearance of a new peak with a retention time (4.85 min) identical to that of the etamycin metabolite formed in *S. lividans* ESR. That the new peak was derived from etamycin was supported by the following evidence: (1) the new peak (retention time 4.85 min) absorbed light at the same wavelength (305 nm) as etamycin; (2) the increase in area of the new peak was proportional to the decrease in area of the etamycin peak (retention time 8.41 min); (3) no comparable peaks were detected in all transformants cultures to which etamycin was not added.

DISCUSSION

Streptomyces lividans and *S. griseofuscus* are widely used as hosts for cloning antibiotic resistance genes from antibiotic-producing actinomycetes because they lack severe restriction barriers and are easily transformed. However, cloning can be complicated by the presence in these strains of genes that confer resistance to antibiotics they do not normally produce. Unlike resistance genes directed against the endogenous antibiotic in a producing strain, they may not be used very often, and though no doubt occasionally advantageous, they are expressed under normal circumstances at a low level or not at all. When these strains are used as cloning hosts, it is important to know whether they possess intrinsic resistance to the antibiotic of interest.

I. Resistance Phenotype of *S. lividans*

A. MLS resistance

Although etamycin resistance has not previously been investigated in *S. lividans*, much has been learned in recent years about the resistance of this species to macrolide and lincosamide antibiotics. *S. lividans* strains are resistant to high levels of lincosamide and low levels of various macrolides (Jenkins *et al.*, 1989). Resistance to lincomycin is due to the presence of a resistance gene, *lrm*, which encodes an rRNA monomethylase related to the *erm* family of rRNA

dimethylases (Jenkins and Cundliffe, 1991). The *lrm* gene may also confer resistance to certain macrolides, but is not the sole determinant of macrolide resistance. Jenkins and Cundliffe (1991) also cloned from *S. lividans* an *mgt* gene that encodes a glycosyltransferase capable of inactivating macrolides by glycosylation of a 2'-hydroxyl group. The two genes (*lrm* and *mgt*) are physically linked on about 2.4 kb of DNA. Whether *lrm* confers etamycin resistance has not been tested, but it might be expected to because a similar gene, *carB*, was shown to confer resistance to vernamycin B, a type-B streptogramin (Epp *et al.*, 1987). Assuming this is so, *lrm* would not be the only etamycin resistance determinant. As the present work shows, *S. lividans* produces an enzyme capable of inactivating etamycin. The presence of multiple resistance mechanisms for this antibiotic thus conforms to the expected pattern (Freeman and Hopwood, 1978).

B. Etamycin-inactivating enzyme

The normal low level of etamycin resistance (MIC 30-50 $\mu g.ml^{-1}$) may be due to low-level expression of *lrm* (high level expression requires induction by certain macrolides or celesticetin), or it may result from low-level expression of the etamycin-inactivating enzyme discovered in the present investigation. Super-resistant variants are also present at low frequency. The apparent increase in the number of resistant variants after protoplasting and regeneration

(notably the super-resistant ESR strains that were not detected by plating out spores), is consistent with other observations of enhanced variability associated with the protoplasting procedure (Ikeda *et al.*, 1983; Ishikawa *et al.*, 1988). The formation of host resistance variants at frequencies higher than the frequencies of transformation achieved causes difficulty in identifying transformants carrying plasmid-encoded cloned resistance genes.

The strong etamycin-inactivating activity in cell extracts of ESR strains indicates that these variants produce the enzyme so abundantly that they can tolerate as high as 200 $\mu g.ml^{-1}$ of etamycin. The elevated expression of etamycin-inactivating activity does not appear to be due to amplification of the corresponding resistance gene, a mechanism reported in some other systems (Altenbuchner and Cullum, 1984; Ishikawa *et al.*, 1988). This conclusion is based only on observation of electrophoresis gels after fractionation of restriction digests of genomic DNA; a low degree of amplification might have been overlooked. Nevertheless, the most probable cause of the enhanced level of etamycin-inactivating is a regulatory mutation similar to that described by Ishikawa *et al.* (1988).

The unusually high thermal tolerance of the etamycin-inactivating enzyme allowed a convenient partial purification, but the enzyme was not studied in detail. It would now be worthwhile to characterize the enzyme activity and the

corresponding gene. One possible strategy would be to initially purify the enzyme and sequence the N-terminal region. This could be followed up by making an oligonucleotide probe based on the amino acid sequence, and eventually by isolating the gene from *S. lividans* ESR genomic DNA after hybridization with the probe. Further characterization of the type of enzyme activity would be best achieved by isolation, purification and structural elucidation of the etamycin breakdown product.

II. Resistance Phenotype of *S. griseofuscus*

A. Stability of the etamycin resistance phenotype

No variants similar to the *S. lividans* ESR strains, which exhibit strong resistance to etamycin, were detected in *S. griseofuscus* after protoplasting and regeneration. Therefore, it appears that *S. griseofuscus* is not only inherently more sensitive than *S. lividans* to etamycin, but it is also more stable towards protoplasting. For these reasons, it was considered to be a better host for cloning an etamycin resistance gene than *S. lividans*.

B. Temporal expression of antibiotic resistance

S. griseofuscus is reported to be resistant to certain macrolides, such as erythromycin, chalcomycin and oleandomycin, but relatively sensitive to lincomycin and

clindamycin (Kamimiya and Weisblum, 1988; Epp *et al.*, 1987). Results obtained in this study generally confirmed these results. Even though spores of *S. griseofuscus* were quite sensitive to all MLS-type antibiotics, mycelium of *S. griseofuscus* in the late log phase became strongly resistant to both erythromycin and lincomycin. This type of resistance may reflect temporal expression of the resistance gene; the similar growth stage response of the inoculum suggests that the two antibiotics might have the same resistance determinant. In contrast, inocula that showed resistance to erythromycin and lincomycin were not resistant to etamycin, chloramphenicol or viomycin. Therefore, lincomycin-erythromycin resistance was specific, and not merely a side effect of rapid growth.

Temporal gene expression may indicate the presence of a regulatory mechanism. Employing a *Vibrio harveyi* luciferase reporter cassette, Schauer (1988) and Sohaskey *et al.*, (1992a,b) constructed transposon-based promoter-probe vectors with which they could isolate transposon insertion mutants of *S. coelicolor*. Some of these mutants expressed the luciferase gene (emitted light) in a spatial and temporal fashion. Temporal gene expression is ultimately due to temporal activation of transcription, which can depend on *trans*-acting sigma factors or positive regulators and *cis*-acting promoter sequences. Tan and Chater (1993) described two developmentally regulated promoters of *S. coelicolor* A3(2) that resembled the

major class of motility-related promoters in other bacteria. The two promoters depended on the *whiG* sporulation gene, which encodes a putative sigma factor important in sporulation of aerial hyphae (Chater *et al.*, 1989). Farkasovsky *et al.* (1991) reported the isolation of a temporally regulated promoter from the *Streptomyces aureofaciens* 2201 plasmid pSA2201; expression from this promoter depended on the growth phase.

In *E. coli*, alterations in nucleotide pools, especially ATP, GTP and ppGpp, occur as a result of amino acid or carbon source starvation. This results in global changes in gene expression affecting 50% of all gene products (VanBogelen *et al.*, 1990), an effect known as the stringent response (Cashel and Rudd, 1987). From an investigation of the stringent responses in *Streptomyces*, Strauch *et al.* (1991) and Holt *et al.* (1992) proposed that metabolic equilibria determine the rates of synthesis of hundreds of cellular proteins during balanced growth. Nutrient limitation or toxic waste accumulation upset the balance, and hence induce the stringent response, which is usually revealed by a transient rise in intracellular ppGpp concentration. The magnititude and complexity of the changes during the stringent response were demonstrated by two-dimensional gel electrophoresis of cellular proteins. Cluster analysis of protein expression patterns identified groups of proteins having the same temporal pattern of expression (Holt *et al.*, 1992). It is apparent from the evidence cited above that temporal gene

expression is a well established phenomenon in streptomycetes.

C. Evidence for a silent etamycin resistance gene

Mycelium of *S. griseofuscus* could grow at low concentrations of etamycin (5-10 $\mu g.ml^{-1}$), but resistance to higher concentrations was induced by previous exposure to the antibiotic. The extent of induction was positively correlated with the inducing etamycin concentration (data not shown). Similar observations have been made for expression of the tetracycline resistance genes, *tetA* and *tetB*, from *S. rimosus* (Ohnuki *et al.*, 1985b). The ultimate level of tetracycline resistance expressed by each gene depended upon the concentration of tetracycline used for induction. However, increasing the inducing concentration from 5 to 50 $\mu g.ml^{-1}$ increased resistance only 1.5-2 fold. Ohnuki *et al.* (1985b) suggested that this type of regulation would provide a mechanism for increasing the resistance of the producing organism as its biosynthetic activity accelerated. Although *S. griseofuscus* is not known to produce etamycin, it appears to regulate etamycin resistance in a way similar to that used by *S. rimosus* to regulate tetracycline resistance. A positive correlation of this type between the resistance level expressed and the antibiotic concentration encountered should be advantagous to the organism.

The inducibility of etamycin resistance in *S. griseofuscus* is similar to the inducibility of lincomycin resistance in *S.*

lividans. Both species are induced by antibiotics they do not produce, but the degree of induction in *S. griseofuscus* is much weaker than in *S. lividans* where lincomycin induces a 100-fold increase in resistance (Jenkins *et al.*, 1989). The evidence for inducible resistance to etamycin is consistent with other results indicating that a normally silent etamycin resistance gene is present in *S. griseofuscus*. Among these is the observation that all transformants, regardless of the vector used or the size of DNA insert, showed significantly higher etamycin resistance than did the untransformed host. Since the resistance of all *S. griseofuscus* transformants was increased further by induction with etamycin, the gene is evidently not fully activated by the plasmid present.

Exogenous etamycin added to *S. griseofuscus* and transformant FK400 cultures gradually disappeared and a new peak was detected by HPLC analysis; this peak increased with time, suggesting that etamycin was being slowly broken down. The observation may account for the low-level etamycin resistance observed in *S. griseofuscus*. Also noteworthy were the similar retention times during HPLC analysis of the suspected etamycin break-down product from *S. griseofuscus* cultures and the etamycin break-down product of *S. lividans* ESR. Whether *S. griseofuscus* possesses a similar etamycin-inactivating activity to that of *S. lividans* could be investigated by the same approach as used for *S. lividans*. This was not pursued due to time constraints.

Inocula of *S. griseofuscus* transformants that exhibited resistance on MYM agar were completely sensitive to etamycin on TSB agar. Presumably TSB media contains ingredient(s) that inhibit expression of etamycin resistance. Since inocula of various transformants pre-exposed to etamycin were able to grow on TSB agar containing etamycin, the inhibitory effect of TSB could be relieved through induction. Possibly etamycin and an unknown ingredient of TSB medium might control expression of the silent etamycin resistance gene through a classical inducer-repressor mechanism, but this needs to be tested experimentally.

The enhanced resistance to etamycin in all transformants of *S. griseofuscus* shows a similarity to the increased resistance to doxorubicin and daunorubicin reported in *S. lividans* after transformation with pIJ702 (Guilfoile and Hutchinson, 1991; Colombo *et al.*, 1992). The authors concluded that this was caused by selection for thiostrepton resistance. That the etamycin resistance of *S. griseofuscus* transformants depended on the presence of thiostrepton was indicated by two observations (data not shown): (1) Seed cultures of the transformants grown in YEME liquid medium without thiostrepton showed the same degree of etamycin resistance (MTC 5-10 $\mu g.ml^{-1}$) as the host. Although this might be attributed to loss of pHJL400-based plasmids in the absence of selection, a complete loss would not be expected from the mycelium used in the controlled inoculum assay. (2) Transformants replica-plated on

MYM agar containing 10 $\mu g.ml^{-1}$ thiostrepton and 30 $\mu g.ml^{-1}$ etamycin grew better than those replica-plated on MYM agar containing only 30 $\mu g.ml^{-1}$ etamycin. Thiostrepton-induced gene expression has previously been reported in *S. lividans* (Murakami *et al.*, 1989). From four thiostrepton-induced proteins identified, one was purified and its N-terminal sequence was used to isolate the corresponding gene (*tipA*). Sequence analysis of *tipA* revealed a unique promoter from which transcription was induced at least 200-fold by thiostrepton. It is possible that induction was due to an autoregulator (e.g., A factor) impurity in the thiostrepton preparation, but no evidence for or against this has been published.

Little is yet known about the function of the thiostrepton-induced proteins. The genes encoding them are examples of silent genes not normally expressed. It is possible that one of them might confer resistance to doxorubicin and daunorubicin in *S. lividans*. It is equally possible that one might confer resistance to etamycin in *S. griseofuscus*.

III. Resistance Phenotype of *S. griseoviridus*

A. Expression of etamycin resistance

Antibiotic producers must not only defend against chance exposure to antibiotics in their enviroments, they must also defend against their own antibiotics. It is logical that they

should have more than one genetic mechanism specifying resistance to a particular antibiotic, or that they should have more than one way of regulating a single resistance mechanism. Most antibiotic producers characterized thus far do indeed possess more than one resistance gene or more than one form of regulation. For instance, *ermE* in the erythromycin biosynthesis gene cluster, although the sole resistance determinant, has multiple promoters upstream of the open reading frame (Bibb *et al.*, 1985). The different levels of etamycin resistance observed in *S. griseoviridus* are consistent with activation of additional resistance at the onset of etamycin production. This is a widespread phenomenon in antibiotic producing cultures (Seno and Baltz, 1989). It may be a result of coordinated positive regulation of resistance and biosynthesis genes by an autoregulator (e.g., A-factor), by a newly synthesized sigma factor, or by an enviromental stimulus transmitted through a two-component signal transducing system. In many cases, induction by the antibiotic appears to be a key element in the control mechanism (Seno and Baltz, 1989).

In *S. griseoviridus* the low level of etamycin resistance was constitutively expressed, and might be conferred by ribosomal protection. Because cultures grew more slowly in a medium containing 100 $\mu g.ml^{-1}$ etamycin than in unsupplemented medium, etamycin can evidently reach target sites in *S. griseoviridus*. This contrasted with *S. griseofuscus*, which

showed no signs of growth in YEME containing 100 $\mu g.ml^{-1}$ etamycin. The ribosomes of *S. griseoviridus* must, therefore, be less accessible or more resistant to etamycin than the ribosomes from a fully sensitive species such as *S. griseofuscus*. The high-level resistance exhibited in *S. griseoviridus* during etamycin production could be due either to the turning on of an additional resistance gene (or genes), or to increased expresssion of the same gene. Resistance might involve an efflux pump that actively expels etamycin, or a membrane barrier that prevents it from reentering the cells. However, no mechanism of either kind has so far been reported in a streptogramin producer.

Spores from a wild-type *S. griseoviridus* population varied markedly in their degree of resistance to etamycin. No evidence is available to indicate whether this variability is in gene composition or in gene expression. The resistance trait does appear to be associated with etamycin biosynthesis, and it may be significant that the resistance and biosynthesis genes in streptomycetes are clustered (Seno and Baltz, 1989). Several phenotypic traits have been repeatedly described as unstable in *Streptomyces* species (Hutter and Eckhardt, 1988). These traits include aerial mycelium formation, spore production, antibiotic resistance and/or production and formation of extracellular enzymes. The instability is associated with genomic rearrangements such as deletions and amplification of specific DNA sequences (Baltz and Seno, 1988;

Birch *et al.*, 1991; Dyson and Schrempf, 1987., Leblond *et al.*, 1989, 1990a,b, 1991; Simonet *et al.*, 1992).

B. Inactivation of etamycin

In streptogramin-producing strains, streptogramin resistance has not been systematically investigated. However, two such strains are reported to produce a lactonase that specifically cleaves the ester bond in streptogramin B (Hou *et al.*, 1970; Kim *et al.*, 1974). Although inactivation of type-B streptogramin has been reported in several other streptogramin producers (Fierro *et al.*, 1989), the enzyme responsible has not been identified. In the present research, efforts to detect etamycin-inactivating activity in culture supernants and cell extracts of *S. griseoviridus* gave negative results. The absence of systems causing inactivation of indigenous antibiotics has been noted in many antibiotic-producing streptomycetes (Cundliffe, 1989).

C. MLS resistance phenotype

Both spores and 72-h etamycin-producing mycelium of *S. griseoviridus* are resistant to much higher concentrations of etamycin than are *S. lividans* and *S. griseofuscus*. *S. griseoviridus* is inhibited at relatively low concentrations of lincomycin, and is extremely sensitive to erythromycin. Since the classical (*erm*-like) MLS resistance gene confers resistance to all three antibiotics it seems unlikely that *S.*

griseoviridus possesses such a gene. The conclusion is supported by evidence (Cundliffe, personal communication) that the rRNA of *S. griseoviridus* lacks dimethylation at A2058. Epp *et al.* (1987) reported that the carbomycin producer, *S. thermotolerans*, is resistant to carbomycin, lincomycin and vernamycin B, but sensitive to several macrolides, including erythromycin. They described this phenotype as "partial MLS resistance". The gene responsible, *carB*, has been cloned and sequenced (Epp *et al.*, 1987). It was shown to monomethylate 23S rRNA at A2058, as does the *lrm* gene. Since *S. griseoviridus* showed resistance to lincomycin and etamycin but sensitivity to erythromycin, its resistance phenotype can also be described as "partial MLS resistance", and so it may possess a resistance gene similar to *carB*.

Recently, a lincomycin resistance gene (*lmrB*), cloned from a lincomycin-producing strain of *S. lincolnensis*, was shown to encode a product that strongly resembled previously characterized Erm and Lrm proteins (Zhang *et al.*, 1992). However, the resistance phenotype of *lmrB* was completely different from that of *erm* or *lrm;* resistance was conferred only to lincomycin, and not to other lincosamides (clindamycin and celesticetin) or to a macrolide (erythromycin). The lmrB product is an example of a protein related to Erm and Lrm that actually specifies a function different from both.

IV. Attempts to Clone an Etamycin Resistance Gene from *S.*

griseoviridus

A. Shotgun cloning

The most commonly used strategy to clone antibiotic resistance genes is shotgun cloning. Initially, this approach was taken using the high copy-number plasmid pIJ702 as the cloning vector and *S. lividans* as the cloning host. It was unsuccessful mainly because etamycin super-resistant clones were recovered at a frequency greater than 10^{-6} cfu from *S. lividans* after protoplast regenerations. In a second attempt at shotgun cloning, an alternative cloning host, *S. griseofuscus* was used. Although this species did not generate super-resistant variants, all transformants did acquire increased etamycin resistance. In retrospect, it seems likely that this increased etamycin resistance might have been induced by thiostrepton, which was used to select for introduction of the vector. To circumvent this problem, either a vector selectable with a different antibiotic, or a different cloning host could be used. However, the observation that putative thiostrepton-induced etamycin resistance was not expressed on TSB medium suggests that further shotgun cloning could be simply achieved by replica-plating transformants on TSB agar containing etamycin.

B. Hybridization

The indication that *S. griseoviridus* ribosomes might be

modified to confer resistance to etamycin led us to use *ermE* to probe *S. griseoviridus* DNA for a gene with a similar nucleotide sequence. Unexpectedly, *S. griseoviridus* DNA was poorly digested by and apparently lacked recognition sites for *Sac*I, a restriction enzyme that usually cuts frequently in *Streptomyces* DNA. *Sac*I was, therefore, useful in retrieving the DNA inserts from lambda clones. Hybridization signals were found at relatively high stringency against a background of other genomic DNA fragments in a *Sal*I digest of *S. griseoviridus* genomic DNA. After plaque hybridization with a genomic DNA library in lambda, and subsequent plaque purification, six *ermE*-hybridizing clones were successfully isolated from 20,000 plaques. Each contained a 4.3-kb *Sal*I fragment corresponding to that detected in the Southern hybridization experiment. This fragment hybridized to the *ermE* probe with sufficient specificity to be picked out against a background of 10,000 recombinant phage plaques.

Although random alignment of two nucleotide sequences with the same overall nucleotide contents would statistically be expected to show only 25% similarity (Boswell and Lesk, 1988), unbalanced nucleotide sequences (e.g., biased for G+C) might show a higher degree of similarity. However, this would not account for the hybridization observed between the *ermE* probe and *S. griseoviridus* genomic DNA fragments. The high-stringency washes (0.1X SSPE/0.1% SDS at 60°C) used to select hybridizing recombinant lambda clones would remove sequences

sharing less than 70% similarity. That it is possible to detect DNA sequences with the anticipated degree of sequence similarity is indicated by the results of Kamimiya and Weisblum (1988), who used the same *ermE* probe to isolate *ermSF* from *S. fradiae*. The probe hybridized predominantly to one fragment in a *Bam*HI digest of *S. fradiae* genomic DNA. Although the hybridization conditions were not described, subsequent comparison of the *ermE* probe and cloned *ermSF* showed only 63% nucleotide sequence identity. Zalacain and Cundliffe (1991) have also reported the cloning from *S. fradiae* of *tlrD*, another MLS resistance gene that shared even less nucleotide sequence identity than *ermSF* with the *ermE* probe.

Madu and Jones (1989) used the phenoxazinone synthase gene (*phs*) of *Streptomyces antibioticus* (Jones and Hopwood, 1984a) to probe a genomic DNA library of *S. lividans* at moderate hybridization stringency (washed with 1X SSC/0.1% SDS, temperature not specified), and isolated two *phs*-hybridizing fragments. *In vitro* transcription and translation of the cloned fragments suggested that only one contained a *phs* gene; the other fragment showed no functional relationship with the probe.

Stutzman-Engwall and Hutchinson (1989) used polyketide synthase genes (e.g., *actI*) to probe a genomic library of *S. peucetius* and identified four hybridizing regions. Their highest stringency wash was with 2X SSC/0.1% SDS at 65°C. Only one of the four regions identified proved to be responsible

for daunorubicin biosynthesis (Otten *et al.*, 1990). Whether the remaining three regions contained *actI*-related genes has not been reported. The point of interest is that the stringencies used in these experiments and in the work by Madu and Jones (1989) were lower than the stringencies used in the present investigation to probe the *S. griseoviridus* genomic library. It is also noteworthy that the sequence determined for the *ermE*-hybridizing region showed that the similarity between the probe and its target DNA sequence was considerably less than predicted from the stringency formula (Hopwood *et al.*, 1985) widely used as a basis for such calculations.

The resistance phenotypes specified by most of the DNA fragments from the recombinant lambda phage were ascertained by subcloning the fragments in the expression shuttle vector pHJL400. The 4.3-kb *Sal*I fragment was individually subcloned as part of a shuttle vector construct (pDQ316). When transformants harbouring pDQ316 unexpectedly did not show strong etamycin resistance, the most straightforward route to an explanation of the hybridization results was to compare the DNA sequence of the *ermE*-hybridizing region with DNA sequences in the GenBank database. This revealed strong similarity between *ermE* and a sequence within a 1.4-kb *Kpn*I segment of the 4.3-kb *Sal*I fragment. Presumably this is why this region of *S. griseoviridus* DNA hybridized with the *ermE* probe.

For comparison, the nucleotide sequence of the 580 nt *Bam*HI:*Sal*I fragment from *ermE* used as the probe was screened

against all sequences in databases (NR) by the BLASTN program. The strongest similarity was to *ermA* of *Arthrobacter* sp. (366/514, 71% identity); there was less similarity to *ermSF* (*tlrA*) of *S. fradiae* (303/477, 63.5% identity), *carB* of *S. thermotolerans* (302/493, 61% identity), *lrm* of *S. lividans* (260/407, 63%) and *lmrB* (Zhang *et al.*, 1992) of *Streptomyces lincolnensis* (47/66, 71%). The comparison was colinear (no gaps allowed for alignment of the two sequences) and was therefore very stringent. The nucleotide identity between *ermE* and *Streptomyces erm*-related genes obtained in this way was around 60% over 500 nt, which is not very high in terms of hybridization strength, but indicates a significant evolutionary relationship. In contrast, the 500-bp region that could hybridize to *ermE* with a strength close to that observed for the *erm* genes noted above, and scored 60% identity to the *ermE* probe in the FASTA programme when gaps were introduced, did not code for an ErmE-related polypeptide and thus was not truly homologous to *ermE*.

Although the sequence identity was apparently fortuitous, the hybridization with *ermE* would have particular strength in the region of the 22-bp perfect match. It may be noted that a colinear comparison of the two sequences would score less than 40% sequence identity. The reason that the *ermE* probe hybridized more strongly to undigested genomic DNA of *S. griseoviridus* than to that of *S. lividans*, which is known to contain the *lrm* gene, might be explained by the presence in *S.*

griseoviridus of two fragments sharing high sequence similarity with *ermE*. Although the similarity shown by the 4.3-kb *SalI* fragment was likely accidental, the similarity shown by the 0.6-kb *SalI* fragment could well have reflected true homology; a hybrid of the 0.6-kb fragment and the 0.58-kb *ermE* probe is less likely to have contained loops that would have aligned the two sequences. Further examination of the genomic DNA region in which the 0.6-kb fragment is located might, therefore, identify an *erm*E-related resistance gene in *S. griseoviridus*.

V. Analysis of the Nucleotide Sequence

A. Transcriptional regulation

The nucleotide sequences that control initiation and termination of transcription and translation in streptomycetes are still imperfectly understood. However, the features discovered so far include: (1) the presence in streptomycetes of several classes of promoters, some of which resemble *E. coli* consensus promoters (Strohl, 1992); (2) a similarity between the stem-loop mRNA structure signalling rho-independent termination of transcription and that of *E. coli*, even though DNA sequences 3′ to the transcription terminators lack the poly T sequence found in *E. coli* (Rosenberg and Court, 1979); (3) in most (but not all) streptomycetes genes, translation starts are preceded by an RBS (Strohl, 1992; Jones *et al.*, 1992).

Jaurin and Cohen (1985) described a class of *E. coli*-like *Streptomyces* promoters that contained sequences resembling typical *E. coli* promoters in the -10 and -35 regions upstream of the transcriptional start; most of these sequences were functional in *E. coli*. Regions surrounding them had novel structural features such as short direct or inverted repeat sequences; they also had an A+T content much higher than that of total streptomycete DNA. As a generalization, although noncoding regions are not always A+T rich, A+T-rich regions are always noncoding.

ORF1 was preceded by an A+T rich region that probably contained regulatory sequences. Examination of this region did not reveal sequences resembling *E. coli*-like promoters, nor any of the presently known *Streptomyces* promoters; however, a short A+T rich stem-loop structure was present about 80 nt upstream of the putative translational start codon; this may well be involved in transcriptional regulation of ORF1. Other potential functions for such a stem-loop structure include transcriptional termination and enhancement of mRNA stability.

B. Initiation and termination of translation

Shine and Dalgarno (1974) showed that an mRNA sequence complementary to a sequence near the 3′-end of 16S rRNA is required for the ribosome to bind mRNA and initiate translation in *E. coli*. Similar conclusions were reached about *Streptomyces* translation by Bibb and Cohen (1982). The

sequence at the 3′-end of *E. coli* 16S rRNA is GAUCACCUCCUUA-3′ (Kozak, 1983); the GAUCACCUCCUUUCU-3′ sequence at the 3′-terminus of *S. lividans* 16S rRNA (Bibb and Cohen, 1982), is identical to that of *B. subtilis* (Hopwood *et al.*, 1986), but differs from that of *E. coli* in the last three bases. In *S. griseoviridus* DNA, sequences resembling RBS were found three to six bases before putative translational starts of all three ORFs (see Table 12). The RBS upstream of ORF2, although not immediately evident, could be recognized by examination of a 10-base sequence that showed good complementarity to the 3′-end of *S. lividans* 16S rRNA. Another potential start codon (nt 1406) in ORF2 is preceded by a potential RBS (AGGA, 7 bases upstream of the ATG start codon); it was not chosen because translation from it to the second ATG would use many rare codons (Wright and Bibb, 1992). The putative RBSs of ORF1, ORF2 and ORF3 are 10, 10 and 5 bases, respectively, in length; this is well within the range of 4 to 12 bases suggested by Seno and Baltz (1989).

In *Streptomyces* sp., translation starts most commonly with AUG (82%), but GUG is used more frequently (18%) than in *E. coli* (3%) (Hopwood *et al.*, 1986). Therefore, the use of GUG as a start codon in ORF3 is not unusual. Both ORF1 and ORF2 terminated with a UGA codon; this is consistent with stop codon usage in *Streptomyces*: UGA 68%, UAG 29% and UAA 4% (Seno and Baltz, 1989). The higher frequency of UGA and UAG reflects the high (>70%) G+C content of streptomycete DNA.

C. Codon usage

Because of the high G+C content of streptomycete DNA, codons biased for G+C should be preferred, and this preference will be most evident in the third (degenerate) codon position. The bias has indeed been found (Bibb *et al.*, 1984; Wright and Bibb, 1992). Analysis of many streptomycete genes has given an average G+C content of 69.9% for the first codon position, 49.9% for the second codon position and 90.6% for the third codon position (Seno and Baltz, 1989).

ORF1 and ORF2 have G+C contents of 68.3% and 73.3%, respectively, at the first codon position; 61.3% and 52.1%, respectively, at the second codon position (the unusally high value for ORF1 suggests a possible sequencing error) and 95.3% and 95.6%, respectively, at the third codon position. Overall G+C contents of ORF1 and ORF2 are 74.9% and 73.7%, respectively, slightly higher than the average G+C content of *Streptomyces*. ORF1 contains a region (nt 623-650 of Fig. 31) where four rare codons are used in a row; the sequence in this region was therefore resequenced with alternative deletion clones and using alternative reaction conditions to confirm its accuracy.

The average use of G or C at the first codon position in streptomycete genes is 42.3% and 27.4%, respectively (Seno and Baltz, 1989). The bias of G over C was also observed in ORF1 (42.6% G and 25.7% C) and ORF2 (41% G and 32.4% C). At the second codon position, the four bases occur at roughly equal

frequencies (Seno and Baltz, 1989). This trend was followed in ORF1 and ORF2, but both ORFs showed a slight bias for G over C. At the third codon position, C (54.8%) is usually preferred to G (35.8%) (Seno and Baltz, 1989). Both ORF1 (53.2% C and 42.1% G) and ORF2 (52.8% C and 42.8% G) conformed to this trend. Since the sequence of ORF3 is not complete yet, similar analysis of codon usage was not done. However, analysis of ORF3 sequence using CODONPREFERENCE showed it to follow the general trend.

VI. Comparison of the Amino Acid Sequence of Putative ORF Products with Related Proteins

ORF1 starts at ATG (nt 160) and stops at TGA (nt 1378). It is 1221 nt in length and encodes a polypeptide of 407 aa. The N-terminal region of the ORF1 product contains a signal peptide similar to that described for other extracellular proteins of Gram-positive bacteria (Eckhardt *et al.*, 1987). The signal peptide encoded by ORF1 has an amino acid composition and features typical of such sequences (Perlman and Halvorson, 1983): a positively-charged hydrophilic N-terminus, followed by a long hydrophobic domain and proline residues that provide a turn to free the signal peptide from the core protein and so expose the cleavage site to the signal peptidase. Signal peptides are commonly associated with bacterial polysaccharide hydrolases that are excreted to

digest their polymeric substrates into small easily assimilated sugar molecules. Since the deduced ORF1 product showed significant similarity to two such enzyme classes (β-1,3 and β-1,3-1,4 glucanases, it would be expected to contain a signal peptide.

β-1,3 glucanases are widely distributed in plants and bacteria; however, those of bacterial origin share little sequence similarity with their counterparts in plants. For example, GlcA (a β-1,3 glucanase of *B. circulans;* Watanabe *et al.*, 1992) showed significant similarity to bacterial lichenases (endo β-1,3-1,4 glucanase) (Hofmeister *et al.*, 1986; Murphy *et al.*, 1984; Gosalbes *et al.*, 1991; Borriss *et al.*, 1990) and β-1,3 glucanase of *Arthrobacter* (Vrsanska *et al.*, 1977). Similarly, the bacterial lichenases showed strong sequence similarity with each other (Hofmeister *et al.*, 1986) but little with barley endo-β-1,3-1,4 glucanase (Fincher *et al.*, 1986).

Two distinct β-1,3 glucanase genes (*glcA* and *glcB*) from *B. circulans* WL-12 were cloned in *E. coli* (Watanabe *et al.*, 1992). Four active species (A1, A2, A3 and A4, see Fig. 30A) of β-1,3 glucanase were detected in the periplasmic space of *E. coli* cells carrying the *glcA* gene. The sizes of the four species corresponded to those of four glucanases detected in culture supernatants of *B. circulans*. Watanabe *et al.* (1992) demonstrated that intact GlcA (A1, see Fig. 30A) contains four structural domains (N-terminus-1,2,3,4-C-terminus) linked by

regions prone to protease attack. The sizes of the four glucanase species generated by *in vitro* protease treatment were similar to the sizes of corresponding species observed in *E. coli* and *B. circulans*. N-terminal aa sequence analysis of the four glucanase species indicated that proteins corresponding to A2, A3 and A4 were derived by sequential loss of three domains from A1, begining at the N-terminus. All three of these domains contributed to the binding of substrates (which could be either β-1,3 glucan, laminarin or pachyman), and the highest binding activity occurred when all three were present, as in A1. The C-terminal domain (A4, the smallest protein that retained enzyme activity) was the catalytic center.

Domains 1 (aa 39-143) and 2 (aa 156-261) shared extensive sequence identity and apparently arose by a gene duplication. Domain 3 (aa 275-339) did not show sequence similarity to domains 1 and 2 but retained the ability to bind to pachyman; it was also required for maximum catalytic activity, because A4 (lacking domain 3) showed considerably lower activity than A3 (containing domain 3), A2 and A3, which were about equal. Amino acid sequence identity between GlcA and bacterial lichenases was strictly limited to a C-terminal region (part of domain 4; aa 542-682); whereas the sequence similarity between GlcA and the ORF1 product cloned from *S. griseoviridus* extended over the whole of domain 4.

The region of sequence similarity between GlcA and the ORF1

product suggests that the latter is a β-1,3 glucanase. If this is confirmed, it will be the first time a gene for such an enzyme has been identified in a *Streptomyces* species. Since the N-terminal region of the putative ORF1 product showed little similarity to any of the N-terminal domains of GlcA, the ORF1 product may differ in function from these domains. Near the *Mlu*I site in ORF1, there are three direct imperfect 12 nt repeat sequences (nt 592 to 603, nt 610 to 621 and nt 664 to 675). Consequently, the region contains three reiterated 4-aa sequences (aa 145 to 148, 151 to 154 and 169 to 172) that may be functionally important. To prove that the cloned ORF1 is indeed a β-1,3 glucanase, the initial effort should be directed to expressing ORF1 in a β-1,3 glucanase-negative host. If the ORF conferred β-1,3 glucanase activity, the specificity of the enzyme could be determined. It would be desirable to purify the enzyme to confirm that its aa sequence corresponded to the DNA sequence.

ORF2 encodes a small peptide of 161 aa. Its deduced aa sequence at the N-terminus did not resemble a typical signal peptide, and since it showed no similarity to any protein in sequence databases, it is difficult to discern its function.

The N-terminal half of the ORF3 product strongly resembles formate dehydrogenases. Since ORF3 has not been completely sequenced, the exact size of its deduced peptide product is not known; however, based on the presumed presence of a transcription termination site downstream of ORF3, the size is

in the 715 to 850 aa range of other formate dehydrogenases.

Two types of formate dehydrogenase are thought to exist (Ferry, 1990): those in aerobic eubacteria and eukaryotes are not known to contain metals and cofactors; those in anaerobic archaebacteria and eubacteria, on the other hand, contain a complex inventory of redox centers and metals. For example, the formate dehydrogenase of *E. coli* contains cytochrome b, molybdenum, selenium and iron (Ferry, 1990); that of *M. formicicum* contains FAD, pterin, molybdenum, zinc and iron (Schauer and Ferry, 1986). The selenium is present in a selenocysteine residue encoded by a UGA (opal) codon, normally read as stop a codon (Zinoni *et al.*, 1986). The selenocysteine was incorporated cotranslationally by a selenocysteine-tRNA (Zinoni *et al.*, 1987). Incorporation of selenocysteine into proteins requires not only the opal codon but alsc the existence of a stem-loop structure in the mRNA region flanking the UGA at its 3′ side (Zinoni *et al.*, 1990; Berg *et al.*, 1991; Heider and Bock, 1992). Four genes that direct the incorporation of selenium were identified: *selA* and *selB* with unknown function, *selC* encoding the functional tRNA that inserts selenocysteine into protein, and *selD* promoting the incorporation of selenium into protein or tRNA (Leinfelder *et al.*, 1988; Sawers *et al.*, 1991). Recognition of UGA as a selenocysteine codon has not been a particularly rare event (Berry *et al.*, 1991); therefore, selenocysteine is sometimes called the 21st amino acid (Bock *et al.*, 1991).

Relatively little is known of the genetics of formate dehydrogenase in aerobic organisms but the genes for two such enzymes from anaerobic bacteria (FdhA, Zinoni *et al.*, 1986; and FdhF, Shuber *et al.*, 1986) have been cloned and sequenced. The two proteins showed end-to-end sequence similarity. A third formate dehydrogense (FdhA) from *W. succinogenes* showed similarity only in its N-terminal half to FdhA of *M. formicicum* and to FdhF of *E. coli*.

Selenium is a common but not an essential component of formate dehydrogenases. FdhA of *M. formicicum* contained a cysteine at the position corresponding to the selenocysteine of FdhF. Also, a mutant with FdhF in which the selenocysteine was replaced with a cysteine retained enzyme activity (Axley *et al.*, 1991). ORF3 in the DNA cloned from *S. griseoviridus* has a UGC (cysteine) codon at the position corresponding to the FdhF selenocysteine and FdhA cysteine codons; conserved amino acids around the cysteine residue were also present. A cysteine-rich region near the N-terminus of ORF3 was similar in location to a cysteine-rich region of other formate dehydrogenases. Conservation of these cysteine residues presumably reflects their functional importance.

Like FdhA of *W. succinogenes* (Bokranz *et al.*, 1991), the ORF3 product showed similarity to FdhF of *E. coli* and FdhA of *M. formicicum* only in the N-terminal half; the C-terminal half of the *W. succinogenes* FdhA also lacked sequence similarity to the C-terminal half of the ORF3 product. The divergence in the

C-terminal regions of these enzymes suggests that the catalytic domain is towards the N terminus. Detection of formate dehydrogenase has not been reported in *Streptomyces*.

It would be of interest to find out whether formate dehydrogenase is common in streptomycetes or whether its putative presence in *S. griseoviridus* is a rare event. This might be approached initially by hybridization probing of streptomycete genomic DNAs. If formate dehydrogenase is widely distributed and streptomycetes are able to metabolize formate, ORF3 could be expressed in a formate-dehydrogenase negative host and the enzyme characterized. Formate dehydrogenase may provide a mechanism whereby streptomycetes can survive anaerobic conditions.

VII. Examination of ORF1 and ORF2 as Etamycin Resistance Determinants.

A. ORF1

In an effort to characterize a putative resistance determinant, the 2.1-kb insert of pDQ312 was sequenced and two complete ORFs were identified. Testing of plasmids containing various ORF components suggested that, if specific DNA sequences did, in fact, contribute to etamycin resistance, these sequences were present within both ORF1 and ORF2. For instance pDQ323, which contained a truncated ORF1 lacking 80 amino acids at the C-terminus, conferred low level etamycin

resistance. If an ORF1 product was responsible for the resistance, the truncated product would have to be functional. Production of β-1,3 glucanase is believed to be a strategy used by some plants against harmful fungal infection (Edington *et al.*, 1991; Jutidamrongphan *et al.*, 1991; Ward *et al.*, 1991). Glucanases induced by fungal or viral infection are presumed to digest both plant and fungal cell walls, and help to combat the invader. However, it is more difficult to perceive how a glucanase could confer resistance to etamycin. Possibly it could alter cell walls of bacteria, including streptomycetes, but any digestion of the cell wall would have to be limited to avoid self-destruction. Participation of an ORF1 product thus seems unlikely.

B. ORF2

The different etamycin resistance levels conferred by pDQ331 and pDQ333 are difficult to explain in terms of ORF functions. Both plasmids contained an intact ORF2 and differed only in the upstream region. If the ORF2 product were indeed responsible for etamycin resistance, the result would imply that ORF2 was expressed in pDQ331 but not pDQ333. Since the intergenic sequence between ORF1 and ORF2 is intact in both pDQ331 and pDQ333, any promoter in this region should be intact in both plasmids; thus unless pDQ331 contained a regulatory sequence internal to ORF1 that was not present in pDQ333, ORF2 should be transcribed (or not transcribed) in

both plasmids.

The conclusion from these results was that the resistance was not conferred by a specific ORF product or a specific DNA sequence.

C. Is there an etamycin resistance gene on the 17.4-kb insert of pDQ311?

To explain the differences in etamycin resistance (MTC) of various plasmids two hypotheses could be proposed: in one the cloned inserts contain an etamycin resistance gene or genes; in the other, the cloned inserts differ in their ability to induce the host etamycin resistance gene. While cloning the phenoxazinone synthase gene from *S. antibioticus* into *S. lividans*, Jones and Hopwood (1984a) discovered two fragments of the *S. antibioticus* genome, in addition to a 2.4-kb structural gene, that transformed *S. lividans* to production of phenoxazinone synthase. Neither of these two DNA fragments synthesized proteins similar to phenoxazinone synthase in coupled in vitro transcription-translation systems, and yet in the transformants phenoxazinone synthase activity and a protein similar to the synthase in immunologic and electrophoretic properties could be detected. Jones and Hopwood (1984b) concluded that the enzyme was synthesized due to activation of a silent phenoxazinone synthase gene in the *S. lividans* genome. Although the case has not been fully proven, and the mechanism by which these fragments activate

the silent gene is yet to be determined, the conclusion is supported by other evidence (Murakami *et al.*, 1989) that *S. lividans* possess silent genes.

When the strong etamycin resistance (MTC 50-60 $\mu g.ml^{-1}$) associated with the 17.4-kb insert of pDQ310 was thought to be due to a resistance gene, attempts were made to localize the gene by testing various segments of it for their ability to confer etamycin resistance. Although differences in resistance level were observed in transformants containing the segments, a detailed analysis of one region (the 2.1-kb insert of pDQ322) did not provide strong support for a resistance gene. Moreover, pHJL400 containing a 17-kb insert from *S. venezuelae* (pDQ400), conferred etamycin resistance as high as many plasmids containing fragments of the 17.4-kb insert of pDQ310. Therefore, it appears that a random piece of DNA introduced in a plasmid can confer higher resistance than the plasmid alone. Since pDQ310 conferred still higher etamycin resistance than pDQ400, and higher than all other plasmids tested, the question of whether it contains a resistance gene remains open. Phenotypically, the resistance conferred by pDQ310 was similar to that of other plasmids in that it was temporally expressed and medium-dependent. This suggests that the high level etamycin resistance of FK1 (containing pDQ310) is due to higher ability of the 17.4-kb insert of pDQ310 to activate the silent gene.

CONCLUSIONS

All three streptomycetes investigated in this study showed etamycin resistance. The etamycin producing strain, *S. griseoviridus,* showed the highest resistance, but here resistance was regulated to maximize the activity in coordination with etamycin production. The resistance and biosynthesis phenotype were unstable, but selection for resistance also selected for etamycin production. This result was consistant with a genetic linkage between resistance and production genes. Both *S. lividans* and *S. griseofuscus* possessed inducible etamycin resistance genes. *S. lividans* was inherently more resistant to etamycin than *S. griseofuscus,* perhaps because it alone possessed the *lrm* gene in addition to an etamycin-inactivating enzyme. Etamycin resistance in *S. griseofuscus* was normally repressed, but was inducible by both thiostrepton and etamycin. The inducible etamycin resistance was temporally expressed. In addition, *S. griseofuscus* showed temporal resistance to erythromycin and lincomycin.

Whereas both *S. lividans* and *S. griseofuscus* appeared to inactivate etamycin, *S. griseoviridus* lacked etamycin-inactivating activity. The activity in *S. lividans* was confirmed to be caused by an enzyme.

An *ermE*-hybridizing fragment isolated from *S. griseoviridus* was functionally unrelated to *ermE*, the sequence identity being apparently accidental. Sequencing of

approximately 4 kb of *S. griseoviridus* DNA in the region containing the hybridizing sequence identified three ORFs; one of these encoded a product showing sequence similarity to β-1,3 glucanase, and the second encoded a putative formate dehydrogenase. The identity of the first two products and their functions in *S. griseoviridus* need to be confirmed and further defined.

Overall, the research described in this thesis clarified the etamycin resistance phenotypes of three streptomycetes, and provided the foundations for designing a successful strategy to clone an etamycin resistance gene from *S. griseoviridus*. The cloned and sequenced DNA identified ORFs with predicted functions not previously reported in streptomycetes. Their characterization should increase our understanding of streptomycete metabolism.

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