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Identification and Characterization of Extrachromosomal Elements from *Streptomyces* spp.

by

© Donald John Netolitzky

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Microbiology

Edmonton, Alberta

Spring 1995

ISBN 0-612-01739-7

University of Alberta

Library Release Form

Name of Author: Donald John Netolitzky

Title of Thesis: Identification and Characterization of Extrachromosomal Elements from *Streptomyces* spp.

Degree: Doctor of Philosophy

Year this Degree Granted: 1995

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University of Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Identification and Characterization of Extrachromosomal Elements from *Streptomyces* spp. submitted by Donald John Netolitzky in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dedicated to all those who weren't here
to see the end of the matter.

You're missed.

Abstract

In this research project, a survey was conducted of extrachromosomal elements contained by a number of β-lactam antibiotic producing *Streptomyces* spp. *Streptomyces clavuligerus* NRRL 3585 contained two giant linear plasmids of 120 (pSCL2) and 430 kb (pSCL3), in addition to the well-characterized 11.7 kb linear plasmid pSCL1 (formerly pSCL). *Streptomyces griseus* NRRL 3851 contained a single giant linear plasmid of 120 kb (pSGL1), and *Streptomyces jumonjinensis* NRRL 5741 and ATCC 29864 contained two giant linear plasmids, pSJL3 and pSJL4 (220 and 280 kb), and one or two smaller linear plasmids pSJL1 and pSJL2 (11.7 and 17.5 kb). No plasmids were identified in *Streptomyces cattleya* NRRL 3841 or *Streptomyces lipmannii* NRRL 3584. Southern hybridization did not reveal any homology shared by these plasmids, and β-lactam antibiotic synthesis gene clusters were located on the chromosome, though pSCL3 was observed to hybridize to the *S. clavuligerus* chromosome. Restriction maps generated for pSJL1 and pSJL2 showed no common sites, nor were any sites shared with pSCL1.

Using a novel extraction procedure which isolates material from the interface of phenol/water extractions, an unusual pair of extrachromosomal elements which possess a hydrophobic component but retain a supercoiled structure as their native form was isolated from *Streptomyces lividans* TK19. These structures (pSL1A and pSL1B) which have apparent lengths of 11 and 9.2 kb, share homology as detected by Southern transfer and hybridization,

and react to nucleases in a similar fashion. However, pSL1A and pSL1B do not share homology to the other plasmids in this survey, SLP2, nor the *S. lividans* 1326 and TK19 chromosomes. Three topological forms of these plasmids were identified by electrophoretic migration, rate zonal sedimentation, and electron microscopy: a native supercoiled form, an open circle form, and a linear form. pSL1A and pSL1B appear to contain a short region of single stranded DNA, and do not possess termini accessible to exonuclease action. The observed features of these structures do not match those of any known extrachromosomal element, suggesting the identification of a new class of plasmid.

ACKNOWLEDGEMENTS

"The scientific concept of the dictatorship of the proletariat means nothing more nor less than authority untrammeled by any laws, absolutely unrestricted by any rules whatever, and based directly on violence."

-V. I. Lenin.

"KILL EVERYONE."

-J. Stalin. (attributed)

But personally, I like fudge. Lots of people influenced the dynamics of this project and bounced around my life for these few years. Those who contributed to my genome are at least in part to blame for this mess, and should be credited for their remarkable tolerance and support; in turn I'll forgive 'em. Similarly, Drs Kenneth L. Roy and Susan E. Jensen took a significant risk with me, their support and patience has been crucial, and a continued surprise. Lab staff including the Pats (Murrey and Kenyon), Heather Jenkins, and fellow zeks Bill Henry, Domenic Spadafora, and Frank Behrend all made work and life at least interesting and certainly helped keep my liver in practice!

Excellent and notable oddities include Robin Barnett/Bourdon/?, Christina Blanchard, Kevin Brown, Sandra Carson, Shell Giesbrecht, Michelle Hay, Heather AustA, Heather AustB, Shawn Kerr, Bill Kournikakis, Erin M., Angela Melano, David M. N., Chelsea P., Fay Schmaltz, and Kelly Torrance. The survivors of Radio Free Albemuth (whichever version) are sent a warm greeting, you people performed quiet miracles for me, with me, and upon me. The high thought and low amusement prizes go to J. S. Bach, J. G. Ballard, CapCom, David Cronenberg, David Drake, Jim Dunnigan, the Feral Electronics Corporation, Reid Fleming - worlds toughest milkman, Dr. Clayton Forrester and TV's Frank (and the rest of the Brains), Robert Frezza, Steve Gallacci, GDW, Philip Glass, Glenn Gould, Matt Howarth, K. W. Jeter, Konrad Lorenz, David Lynch, Louise McMaster-Bujold, Erich von Manstein, Lucius Shepard, Lisa Simpson, SPI, The Church of the Subgenius, Ed Wood Jr., and cichlids everywhere.

However, the lynchpin to my all too often tangled psyche must go to those always loving, often maniacal, most faithful companions of mine, Shockwave and Fallout. And someday, I'll see about getting that rosegarden, ladies, I promise! And there will be lotsa birds... lots!

The financial support which kept me solvent (at times) came from The Alberta Heritage Foundation for Medical Research. This project was brought to you by the genus *Chaetognatha*, the nucleotide Adenosine, the plasmid pSCL1, and is guaranteed to be chock full of Hypno Helio Static Stasis (with ZX-14!).

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List of Abbreviations

3' OH	3' hydroxyl end of a DNA molecule
5' OH	5' hydroxyl end of a DNA molecule
A	Adenosine
AUD	Amplifiable unit of DNA
bp	Base pairs
CCC	Covalently closed circular
CHEF	Contour-clamped Homogeneous Electric Field
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DS	Double stranded
EDTA	Ethylenediaminetetracetic acid
G+C	Guanosine + cytidine bases
GLP	Giant linear plasmid
kb	1,000 base(s) or base pairs
Leu	Leucine
LET	Low EDTA Tris buffer
Mb	1,000,000 base(s) or base pairs
mw	Molecular weight
PEG	Polyethelene glycol
RNAse	Ribonuclease
SDS	Sodium dodeclysulfate
T	Thymidine
TE	Tris-EDTA buffer
TEA	Tris-EDTA acetate buffer
TEB	Tris-EDTA borate buffer
TIR	Terminal inverted repeats
tRNA	Transfer RNA
TSB	Trypticase soy broth
UV	Ultraviolet

1 INTRODUCTION:

1.1 Extrachromosomal Elements

In addition to the chromosome, many prokaryotes and eukaryotes possess independently replicating, extrachromosomal genetic elements called plasmids. Structurally and genetically diverse, plasmids have proven a useful, if not crucial tool with a wide variety of research and industrial applications. Most gene cloning, transfer, and expression systems are plasmid based, making plasmids critical for even the basic processes of molecular biology. The use of engineered plasmids to alter and improve the characteristics of organisms of industrial interest has also made plasmid technologies of considerable industrial value.

The term plasmid has been applied to a wide variety of genetic elements, indeed the definition is more functional than structural. The distinction between a plasmid and a chromosome is at best vague; among most prokaryotes a single chromosome is present per cell, and any other genetic elements are considered plasmids. Given that the eukaryotic genome is segmented among a number of chromosomes, the plasmid/chromosome distinction becomes even less specific. In general, a plasmid is considered to be a genetic element whose presence is not required for cell viability, while the chromosome contains genetic elements most of which cannot be eliminated. In short, a plasmid can be removed (or cured) from a cell with retention of cell viability while the chromosome cannot. This definition is less than ideal, given that a number of plasmids have been identified which contain genes normally encoded by the chromosomal genome (Banfalvi *et al.*, 1981; Fornari *et al.*, 1984; Frantz and Chakrabarty, 1986; Sobral *et al.*, 1991; Willey *et al.*, 1991; Choudhary *et al.*, 1994; Pardo *et al.*, 1994). The recent discovery that *Brucella melitensis* has a genome which is divided into two independantly replicating circular chromosomes (2.1 and 1.15 Mb) (Michaux *et al.*, 1993), and

observations which indicate that the *Rhodobacter sphaeroides* genome is composed of two distinct circular molecules suggest that prokaryote genome organization may also be more complex than originally thought.

The distinction between plasmids and viruses is also blurred, especially considering that some viral prophages are functionally identical to many plasmids. Both circular (e.g. ϕSF1 phage; Chung, 1982) and linear (e.g. coliphage N15; Svarchevsky and Rybchin, 1984) viruses can maintain their prophage within a cell in a 'plasmid-like' state. Differentiating plasmids and viruses by the ability to transfer between host cells is also unsuccessful; while viruses produce infectious particles many plasmids encode genes for production of conjugative pili, facilitating plasmid transfer between cells. The similarities between many eukaryotic and prokaryotic plasmids and viruses indicate that these categories are not rigid; these entities have likely crossed phylogenic boundaries during their evolutionary history. The observation of conjugative transfer of plasmids between organisms as diverse as *Escherichia coli* (Gram negative rod, mid G+C DNA) and *Streptomyces lividans* (Gram positive filamentous, high G+C DNA) (Mazodier *et al.*, 1989; Gormley and Davies, 1991; Bierman *et al.*, 1992; Tabakov *et al.*, 1994) illustrates this point.

The discussion of plasmids which follows will emphasize plasmids found in prokaryotes, but given the apparent similarities between plasmids of both prokaryotic and eukaryotic origin, as well as viruses, information concerning other structures will be included when judged to be useful and applicable. Since this project involves studies of *Streptomyces* plasmids, a focus will be placed upon actinomycete examples, where possible.

Plasmids have a wide variety of observed sizes ranging from 0.5 kb to over a megabase. Larger plasmids (commonly those over 50 kb in length) are often referred to as 'giant' plasmids, though no evidence exists of any essential differences between these classes other than their size. Typically, plasmids are composed of DNA, like the chromosome, though a number of double stranded RNA

plasmids have been reported (Wickner, 1980; Zhang and Brown, 1993).

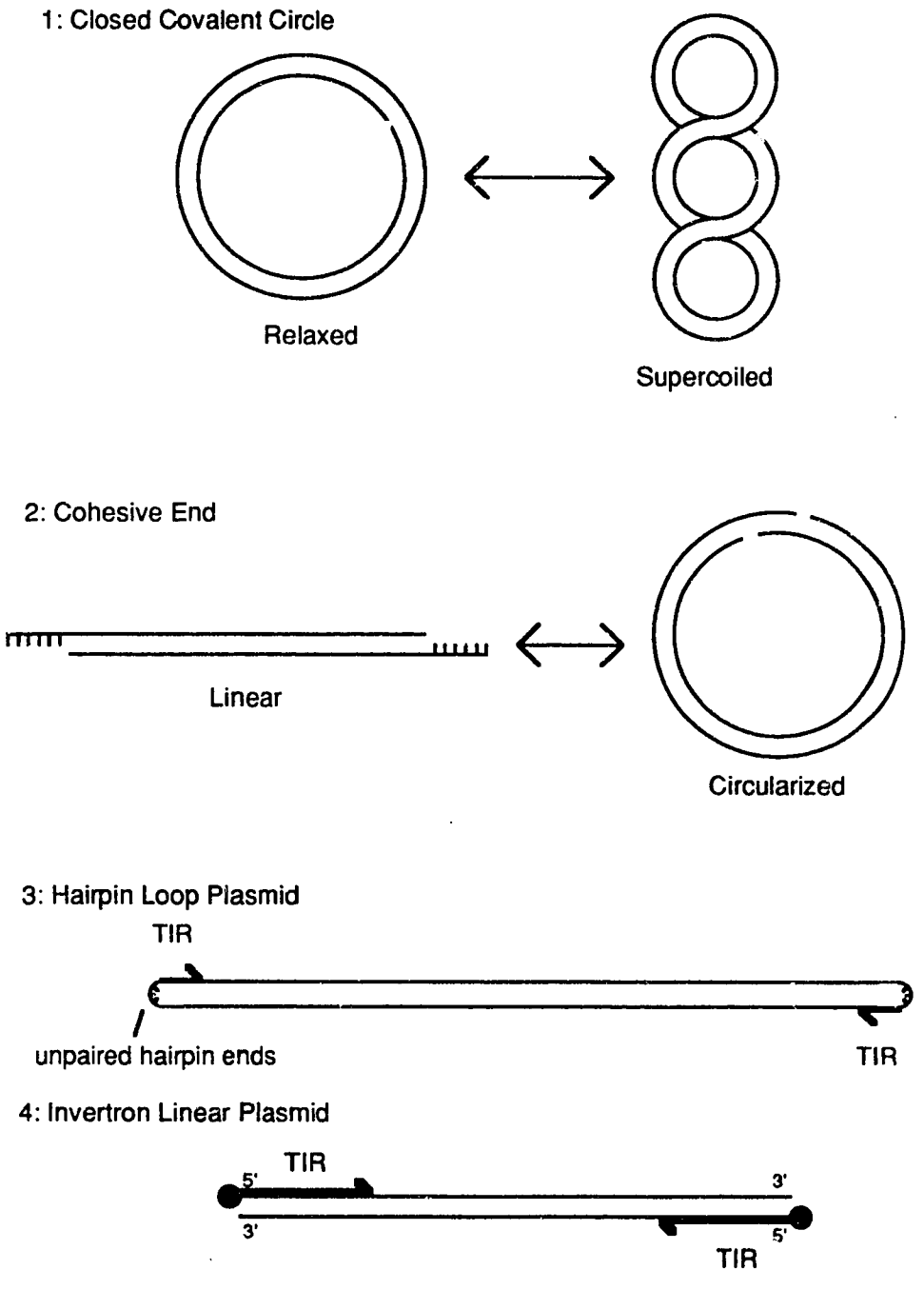
Like chromosomes, plasmids have been found to adopt a number of distinct topologies. Figure 1 illustrates the forms which have been identified. The most commonly observed and best characterized plasmid topology is a covalently closed circular (CCC), double-stranded (DS) DNA molecule, such as pBR322. These structures are typically supercoiled *in vivo*, and functionally closely resemble the classic bacterial chromosome.

A second class of extrachromosomal elements are linear double stranded molecules with short single-stranded cohesive ends. This type of DNA molecule is capable of forming a circular molecule, a state which is typically an intermediate in the replicative process. This structure has been commonly found among bacteriophage, including the well studied λ phage (Skalka, 1977). A number of actinophages, including MPϕWR-1 (*Micromonospora purpurea*) (Tilley *et al.*, 1990), VWB (*Streptomyces venezuelae*) (Anne *et al.*, 1984, 1985, 1990) and the temperate *Streptomyces rimosus* phage RP2, RP3 (Rausch *et al.*, 1993) also fall into this category. Experimentally, these extrachromosomal elements are easily recognized by the formation of a 'ladder' of DNA molecular multimers linked by their cohesive ends.

Another class of plasmid structure is a linear molecule which has 'hairpin' loop ends. These structures are essentially a single-stranded DNA circle containing two long complementary sequences which form a duplex, leaving a short unpaired DNA loop at each end of the DNA duplex. Only a limited number of examples of this structure have been identified from a variety of apparently unrelated and diverse sources: the N15 prophage (*Escherichia coli*) (Svarchevsky and Rybchin, 1984), the *Borrelia* bacterial chromosomes and plasmids (Barbour and Garon, 1987; Ferdows and Barbour, 1989), pox viruses (Baroudy *et al.*, 1982), african swine fever viruses (Gonzalez *et al.*, 1986), fungal mitochondrial linear plasmids (Miyashita *el al.*, 1990), and a few mitochondrial DNA

Fig. 1. Simplified representations of the molecular structures of various extrachromosomal elements. TIR; terminal inverted repeats, 5' and 3'; 5' and 3' OH ends of DNA polynucleotide.

Fig. 1 Simplified representation of the molecular structures of plasmids



molecules of yeast (Dinouel *et al.*, 1993). A variety of these structures have been sequenced, and they share remarkably few sequence similarities. In particular, the sequences associated with the single stranded terminal hairpins show little homology other than the presence of short AT rich terminal inverted repeats (TIR). Sequencing the *Borrelia* plasmids has identified 19 bp long AT rich TIR (Barbour and Garon, 1987; Kitten and Barbour, 1990), though the sequences outside this area share no homology, even between telomeres of the same plasmid (Hinnebusch *et al.*, 1990). Similarly, the N15 prophage contains short 28 bp terminal inverted repeats (Hinnebusch and Tilly, 1993) in addition to the palindromic hairpin loops which span the single-stranded ends.

Another phylogenetically widely dispersed linear plasmid structure consists of a linear double-stranded DNA molecule which has two attached prot ns. These proteins are linked by a covalent bond between the 5' phosphate of the DNA polymer and the OH group of a serine, threonine, or tyrosine residue of the terminal protein. These structures, named invertrons (Sakaguchi, 1990), also possess one other characteristic feature; the ends of the linear DNA molecule contain homologous inverted repeat sequences. While these TIR sequences are of varying length, and frequently contain numerous potential secondary structures of unknown significance, their presence has been demonstrated as crucial for DNA replication (Tamanoi, 1986; Salas, 1988). For the purposes of this discussion, the term 'telomere' will be used not only to describe the eukaryotic chromosome ends but also the TIR/terminal protein complex of invertron type structures. An interesting feature of these molecules is that conventional DNA extraction techniques fail to isolate invertrons, as phenol extractions remove both the DNA and attached proteins from the aqueous phase. Extensive proteinase K treatment removes enough of the terminal protein to allow recovery of the linear plasmid (Hirochika and Sakaguchi, 1982). *In situ* lysed pulsed-field gel electrophoresis DNA preparations also detect these structures. The first identified and best characterized invertrons are the adenoviruses and bacteriophage ϕ29, though

additional examples from a wide variety of phylogenic sources have been identified. These include a number of linear plasmids from the actinomycetes (Hirochika and Sakaguchi, 1982; Chater and Hopwood, 1983; Keen *et al.*, 1988; Chen *et al.*, 1993b), several *Streptomyces* spp. chromosomes (Leblond *et al.*, 1993; Lin *et al.*, 1993.) , and *Rhodococcus* sp. (Crespi *et al.*, 1992; Kalkus *et al.*, 1993; Dabrock *et al.*, 1994) and possibly *Thiobacillus versutus* plasmids (Wiodarczky and Nowicka, 1988; Jagusztyn-Krynicka *et al.*, 1990)

number of eukaryotic invertron linear plasmids have also been identified and are widely scattered among the eukaryotes, with examples from the invertebrates (Ruan and Emmons, 1984), higher plants (Pring *et al.*, 1977), algae (Turmel *et al.*, 1986), and fungi (Gunge *et al.*, 1982; Mohan *et al.*, 1984). These plasmids are often very short, as small as 1.1 kb, and are frequently associated with organelles such as the mitochondria (Pring *et al.*, 1977; Mohan *et al.*, 1984) or chloroplast (Turmel *et al.*, 1986). Interestingly, these plasmids are often associated with some deleterious effect such as male sterility (Pring *et al.*, 1977), senescence (Bertrand and Griffiths, 1989) and killer toxins (Gunge *et al.*, 1982).

The terminal inverted repeat sequences of these plasmids have been the subject of considerable study, and are very diverse. Terminal inverted repeat lengths vary widely, ranging from 44 bp in the SLP2 plasmid of *Streptomyces lividans* (Chen *et al.*, 1993b) to the extensive 80 kb TIR of the SCP1 plasmid of *Streptomyces coelicolor* (Kinashi and Shijami-Murayama, 1991; Kinashi *et al.*, 1991). More typically, the length of these TIR is around 0.5 to 1 kb; *Rhodococcus* sp. pHG207, 600 bp (Kalkus *et al.*, 1990), *Streptomyces clavuligerus* pSCL1, 900 bp (Wu and Roy, 1993), *Streptomyces rochei* pSLA2, 600 bp (Hirochika *et al.*, 1984), and *Morchella conica*, 700 bp (Rohe *et al.*, 1991). Analyses of the homology between these TIR, both eukaryotic and prokaryotic, have detected no apparent universal features. Though some limited homology has been detected between eukaryotic mitochondrial linear plasmids and the *Bacillus* phage ϕ29 (Levings and Sederoff, 1983), and a short terminal common conserved sequence has been noted among

the adenovirus (Tamanoi, 1986), these examples of limited similarities are exceptions rather than the rule. Similarly, sequenced actinomycete linear plasmid TIR show only limited homology (Hirochika *et al.*, 1984; Chen *et al.*, 1993b; Wu and Roy, 1993), with few discernible common sequence features. An extreme example of this lack of homology among TIR is seen in the *Kluyveromyces lactis* plasmids pGK11 and pGK12, which show no detectable sequence homology even though the two plasmids share replication enzymes within the same cell (Hishinuma *et al.*, 1984). Interestingly, the invertron linear plasmid group is frequently associated with integration of the plasmid into the host's chromosome and such integration and excision events are often linked to inverted repeat structures (Chater and Hopwood, 1989; Sakaguchi, 1990). Invertrons observed associated with these recombination events include the bacteriophage HB-3 (Romero *et al.*, 1990) fungal and plant mitochondrial plasmids (Meinhardt *et al.*, 1990), adenovirus (Horowitz, 1990), and the *S. coelicolor* linear plasmid SCP1 (Kinashi *et al.*, 1992). An unusual shift between a protein linked linear and circular plasmid structure has been reported in the pSA1 plasmid of *Streptomyces azureus* (Ogata *et al.*, 1983; Mioshi *et al.*, 1986) though the significance of this change is not understood.

Another distinct linear DNA structure is found in linear eukaryotic chromosomes. Eukaryotic chromosomes are terminated by sequences called telomeres; structures consisting of short tandem repeat sequences of 5 to 8 bp (Blackburn, 1991a) which form a stable DNA quadriplex structure *in vivo* (Blackburn 1991b). No prokaryotic examples of this telomeric system have been identified.

In addition to having a number of different possible structures, plasmids also vary in many other traits. The plasmid copy number (the ratio of plasmid to chromosomes within the cell) varies considerably, from high (50-100) to low copy number (1-2). An anomalous plasmid with a copy number of less than 1 has also

been observed in *S. coelicolor*, as will be discussed later in greater detail.

Frequently, more than one different plasmid is found within a single cell. In these cases, each plasmid's mechanisms for controlling the copy number, plasmid replication, and plasmid segregation into daughter cells may interfere if the DNA sequences and proteins involved in these processes recognize each other. The groups of plasmids which cannot co-exist, or incompatibility groups, usually reflect a high degree of relatedness between the genes and proteins responsible for stable maintenance of a plasmid. Interference between plasmids may result in either immediate elimination of one plasmid, a typical result in low copy number plasmids, or a gradual loss of one plasmid over time due to unequal segregation of hig copy number plasmids. In some cases, the presence of mul le plasmids may also indicate that a recombination, inser on, or deletion event has occurred, but over extended subculturing these strains rarely prove stable. What would otherwise be unstable plasmid groupings may, however, persist under conditions where plasmid-borne traits are actively selected and thus cured cells are not viable.

Many plasmid-like genetic elements and a few plasmids (e.g. F of *E. coli*; Kline, 1988, and SCP1 of *Streptomyces coelicolor*; Kinashi *et al.*, 1992) have been observed to integrate into the genome. These integration events may occur at random, but more commonly occur at a specific site on the bacterial chromosome (typically named attB) via homologous recombination with a site on the plasmid (attP). In some cases, both the integrated and free form may coexist (e.g. pSAM2 of *Streptomyces ambofaciens*; Boccard *et al.*, 1988), in others, only one of the two forms may exist per cell (e.g. SLP1 of *Streptomyces coelicolor*; Hopwood and Kieser, 1993). Integration offers obvious advantages for successful segregation and maintenance of plasmids, and is a state adopted by many viruses (e.g.. λ of *E. coli*; Horowitz and Hagen, 1980; Friedman *et al.*, 1984) for long-term persistence.

As plasmids divert cellular resources from cellular functions, the elimination of a plasmid is generally believed to be selectively advantageous. Plasmids have adopted a wide variety of strategies to avoid elimination from their host cells. These can be divided into two general classes; cases where a plasmid provides a selective advantage to its host cell, and those where the plasmid DNA is essentially 'selfish' and encodes functions which prevent the plasmid's loss. Typical examples of plasmid encoded selective functions are the antibiotic resistance characteristics encoded by the R factors and other antibiotic resistance plasmids (Guiney, 1984; Clewell, 1990), and the bacteriocin systems noted in certain plasmids (Govan, 1986). Other plasmid encoded functions have less clearly defined advantages, such as the conjugation systems encoded in plasmids such as the F plasmid of *E. coli* (reviewed by Frost *et al.*, 1994) and many *Streptomyces* plasmids (Hopwood and Kieser, 1993). Other plasmids appear to be strictly 'selfish' DNA, and encode genes which are designed to kill any daughter cells which do not contain the plasmid. The classic model for this gene system is the kil/kor gene system found in the RK2 plasmid of *E. coli*. (Kornacki *et al.*, 1984; Thomas *et al.*, 1985; Young *et al.*, 1987), in which a plasmid produced repressor prevents the action of a cytoplasmic cytotoxic protein. A final possible model for plasmid persistence is where an organism may have a segmented genome in which essential chromosomal DNA is spread between several DNA structures, much as eukaryotic genomes are spread among several chromosomes. This model has recently been observed in several prokaryotes (Suwanto and Kaplan, 1992; Michaux *et al.*, 1993); where it occurs, the smaller segment of the genome might be considered a plasmid, though in a classic sense this description is not accurate.

1.2 Plasmid Replication

Plasmid replication has been found to employ a variety of mechanisms, many of which are specific to a certain type of

plasmid structure. The following discussion of plasmid replication includes certain DNA virus replication mechanisms, but given the close relationship of many plasmids and viruses, common mechanisms are not only possible, but likely. For example, the invertron replication system was first observed and characterized in adenovirus, but later found in linear plasmids as well.

Circular plasmids have been observed to use three primary methods of replication. Linear DNA structures which recircularize prior to replication, such as the λ phage, can also use these methods. The first method is the 'theta' bi-directional replication mechanism (reviewed by Lewin, 1987) observed in bacterial chromosomes, and many bacterial plasmids, as well as eukaryotic chromosomes. This mechanism, as illustrated in Figure 2, involves strand dissociation, initiation and the formation of two replication forks, one moving in each direction. Each fork includes a site of leading and lagging strand DNA synthesis. The site of strand dissociation and DNA replication initiation, the oriC, appears to be present in one copy per bacterial genome or plasmid, though multiple origins of replication are present on eukaryotic chromosomes. Two complete double-stranded DNA molecules are produced once the replication forks meet. In eukaryotic chromosomes, this mechanism is unable to replicate all the telomeric sequence of the lagging strand. To maintain telomeric sequences, a specific enzyme, telomerase, engages in non-DNA template dependent synthesis of the telomeric repeat sequences (Blackburn, 1991b), a process which adds repeat sequences to the telomere and thus prevents a gradual reduction in the length of the terminal telomeric repeats.

Alternatively, circular DNA molecules may replicate each DNA strand separately. The rolling circle mechanism is a plasmid replication mechanism which involves individual strand replication (Fig. 3), and has been examined in detail during the study of DNA replication in the M13 and λ phage. Known examples of *Streptomyces* plasmids which utilize this replication mechanism include pIJ101, pSB24, pJV1 (Servin-Gonzalez, 1993), and pSN22 (Kataoka *et al.*, 1994). Here, one strand of the DS circular DNA is

Fig. 2. Simplified representation of the bidirectional DNA replication mechanism. oriC; origin of circular replication. Black indicates parental DNA, dark grey indicates newly synthesized DNA, and light grey indicates RNA primers.

Fig. 3. Simplified representation of the rolling circle DNA replication mechanism. Black indicates parental DNA, dark grey indicates newly synthesized DNA, and light grey indicates RNA primers. See figure key for protein identifications.

Fig. 2 Simplified representation of bi-directional DNA replication

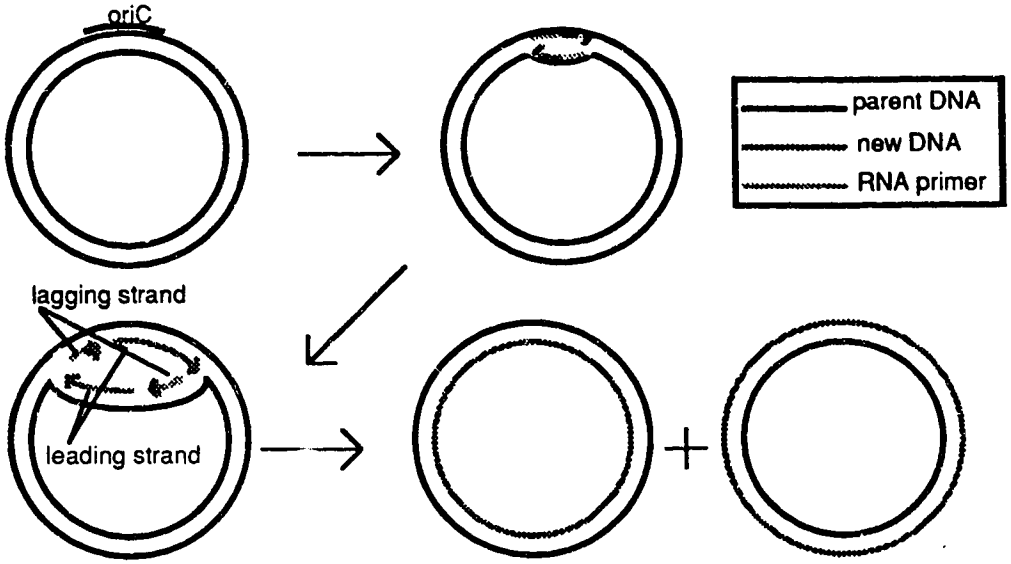


Fig. 3 Simplified representation of rolling circle DNA replication

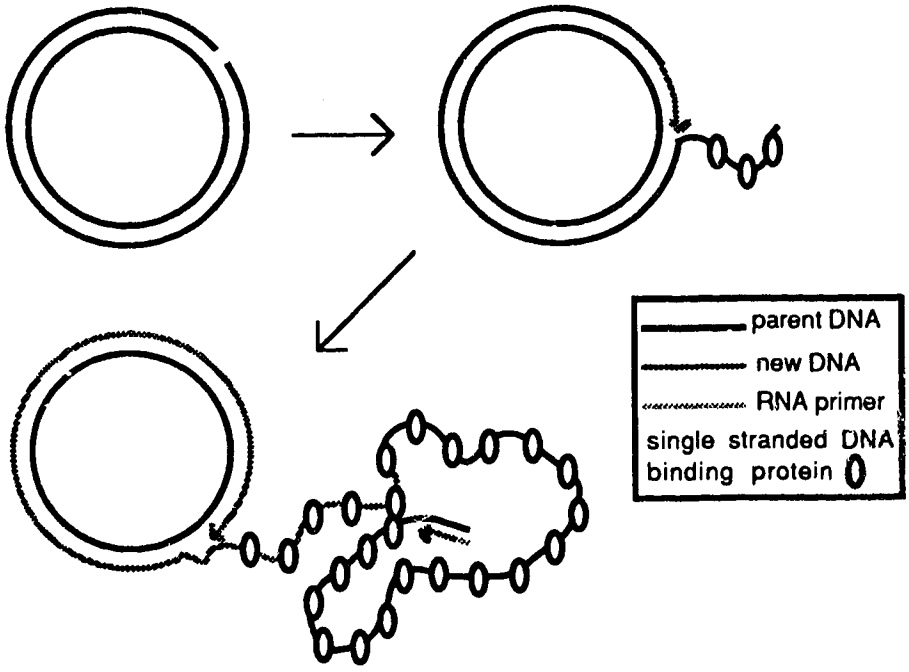
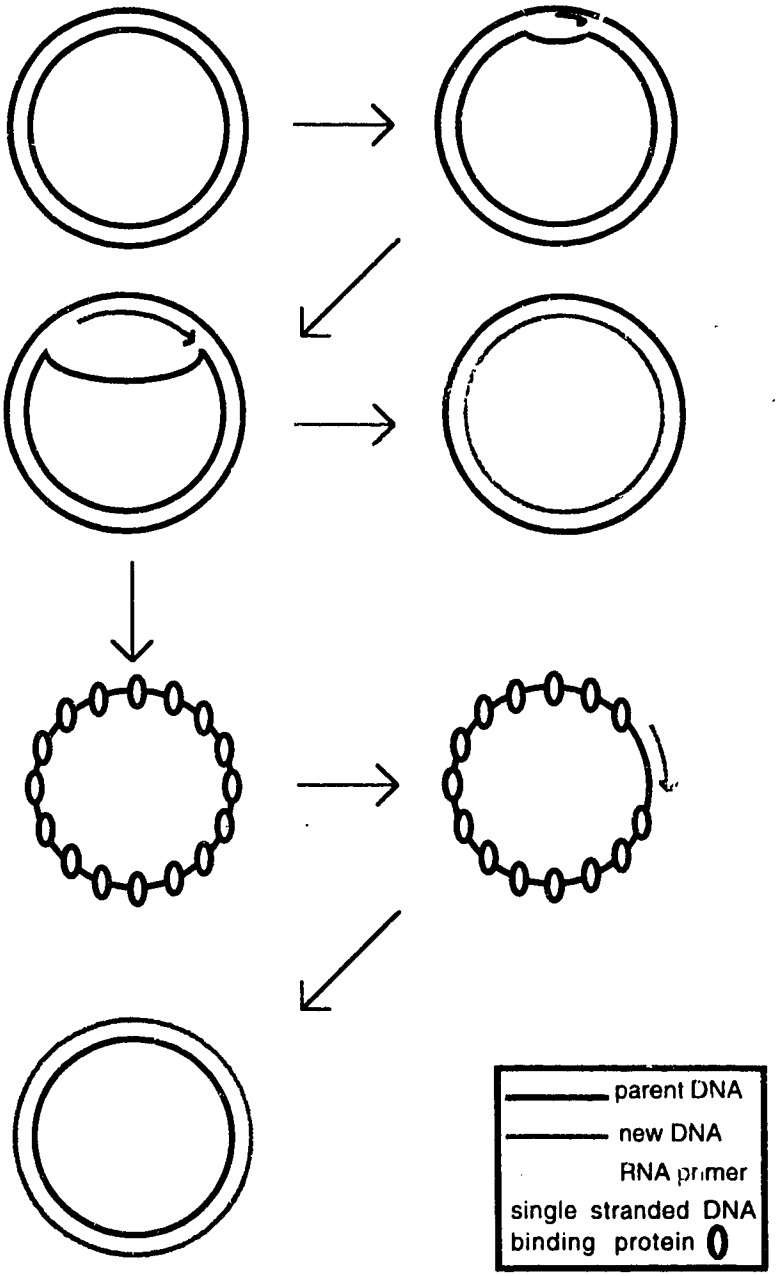


Fig. 4. Simplified representation of the displacement loop DNA replication mechanism. oriC; circular origin of replication. Black indicates parental DNA, dark grey indicates newly synthesized DNA, and light grey indicates RNA primers. See figure key for protein identifications.

Fig. 4 Simplified representation of displacement loop DNA replication



nicked, and the free 3' OH end produced is used as the site for initiation of DNA replication. As the new strand of DNA is extended, the original parental strand is displaced, ultimately producing amultimeric single-stranded copy of the plasmid genome. As with other replication systems which produce a single-stranded DNA intermediate, the unpaired DNA is typically bound by a single-stranded DNA binding protein. A complementary strand for this single-stranded structure may be synthesized by a variety of mechanisms including conventional RNA primed DNA replication, and the resulting DS multimer is cut into a monomeric form.

The displacement loop (or D-loop) system (fig 4) is a second single-strand replication form used by *Tetrahymena* plasmids (reviewed by Clayton, 1982). This mechanism involves strand dissociation, synthesis of a single new leading strand, and displacement of one of the single-stranded circular strands of the original plasmid, which is also replicated in a 5' to 3' direction.

Hairpin loop plasmids and similar structures use a replication system first suggested by Cavalier-Smith (1974) and Bateman (1975) as a mechanism for eukaryotic chromosome replication. While not utilized in eukaryotic chromosome replication, this mechanism was later confirmed as being used by the poxviruses (Baroudy *et al.*, 1982), and several *Borrelia* linear plasmids (Hinnebusch *et al.*, 1990). The mechanism (fig. 5) begins by nicking the DNA near the hairpin loops, causing the loops to unfold. DNA polymerase extends the complementary strand from the free 3' OH along the now exposed single stranded telomere sequence. The newly synthesized DNA disassociates from its parent strand to reform two stem loops, and the newly synthesized strand primes DNA replication extending into the template DNA duplex. DNA synthesis continues until the replication forks meet, ultimately producing two complete double stranded daughter genomes. The example illustrated in Fig. 5 depicts the intermediate states formed when replication occurs at both ends, simultaneously. Experimentally, replication has been observed from one end, resulting in large concatemers which are later resolved. This

Fig. 5. Simplified representation of the hairpin loop DNA replication mechanism. TIR; terminal inverted repeats, cTIR; sequence complementary to the terminal inverted repeat sequence, 5' and 3'; 5' and 3' OH ends of DNA polynucleotide. Black indicates parental DNA, and dark grey indicates newly synthesized DNA.

Fig. 5 Simplified representation of hairpin loop DNA replication

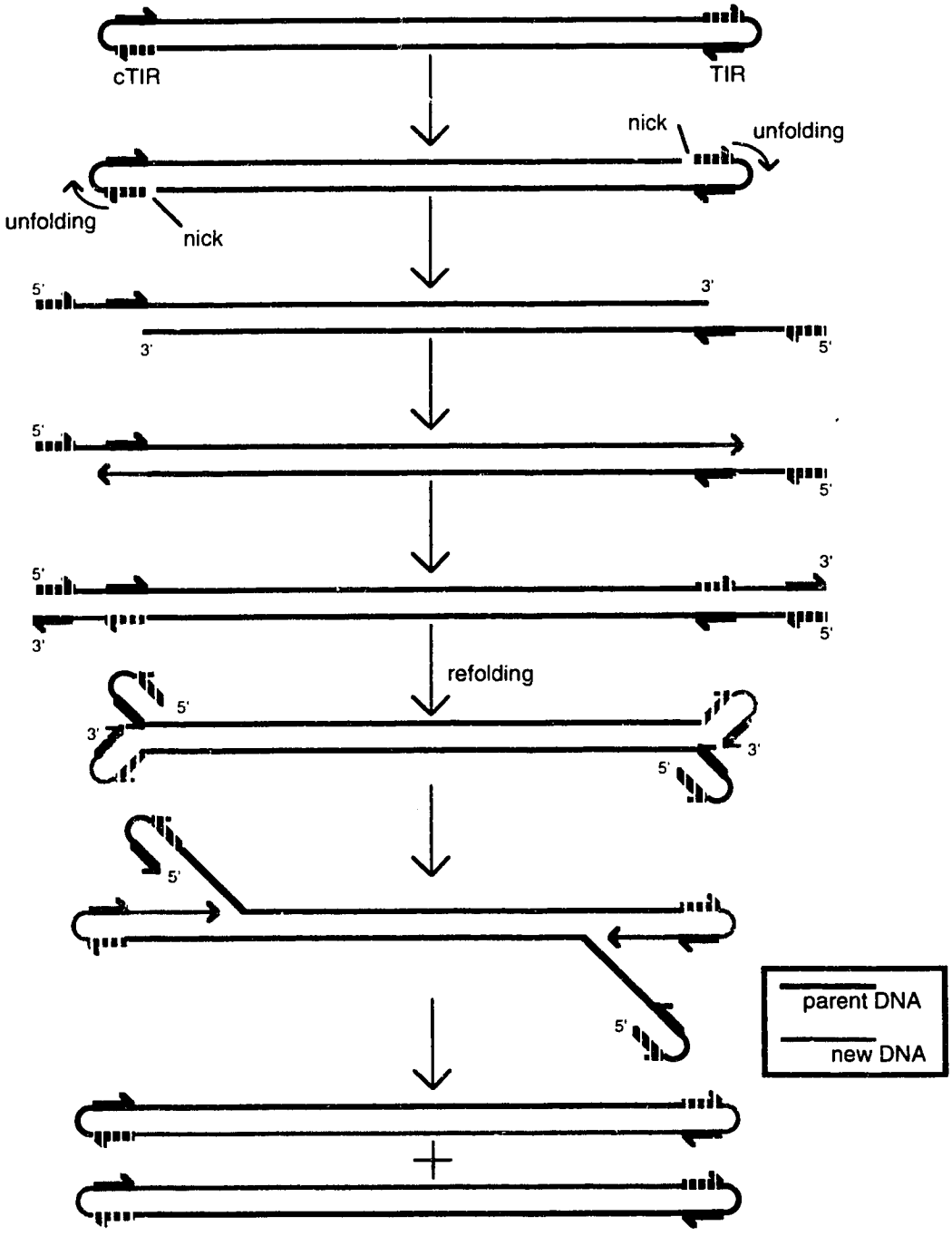
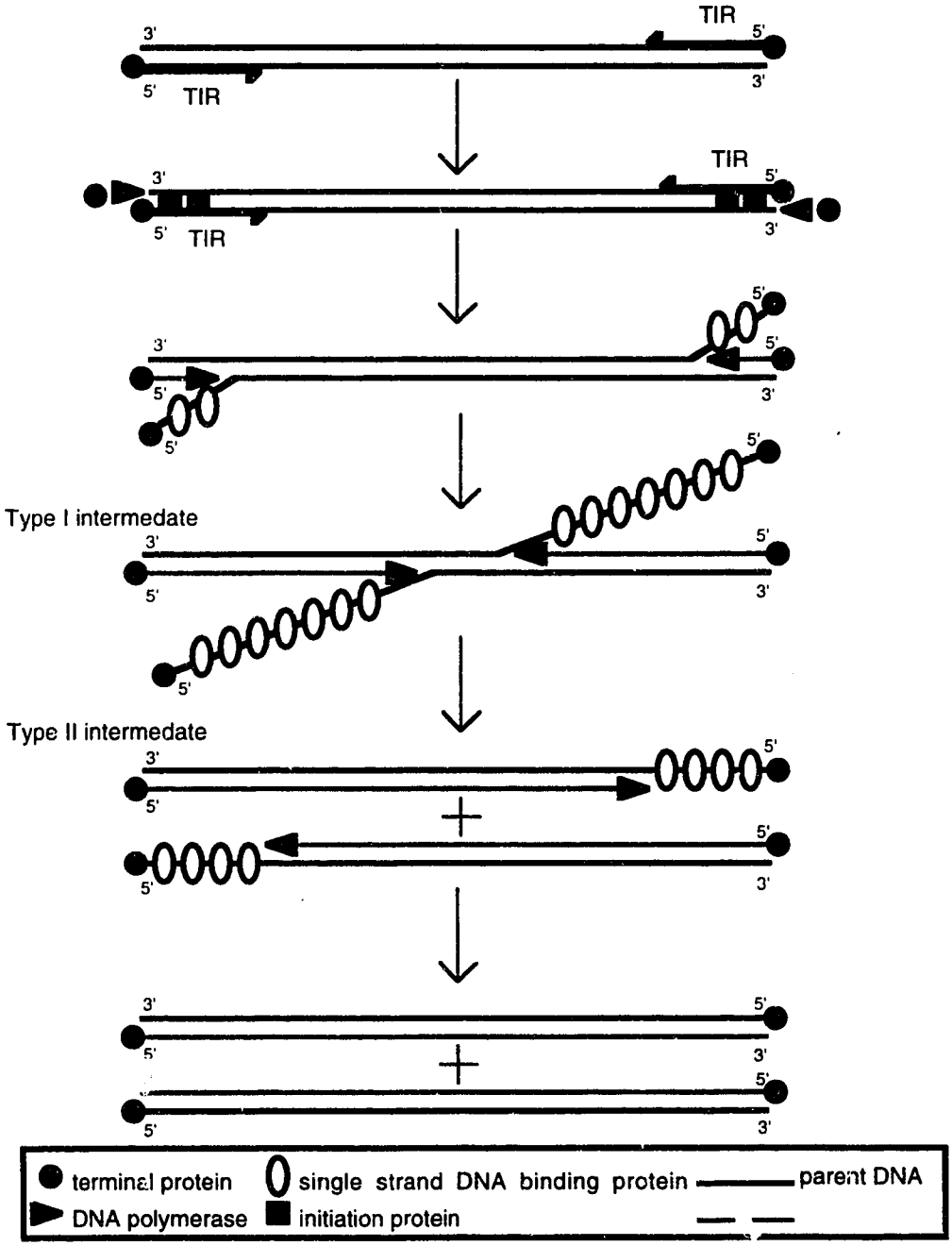


Fig. 6. Simplified representation of the invertron protein primed DNA replication mechanism. TIR; terminal inverted repeats, 5' and 3'; 5' and 3' OH ends of DNA polynucleotide. Black indicates parental DNA, and dark grey indicates newly synthesized DNA. See figure key for protein identifications.

Fig. 6 Simplified representation of inverton protein primed DNA replication

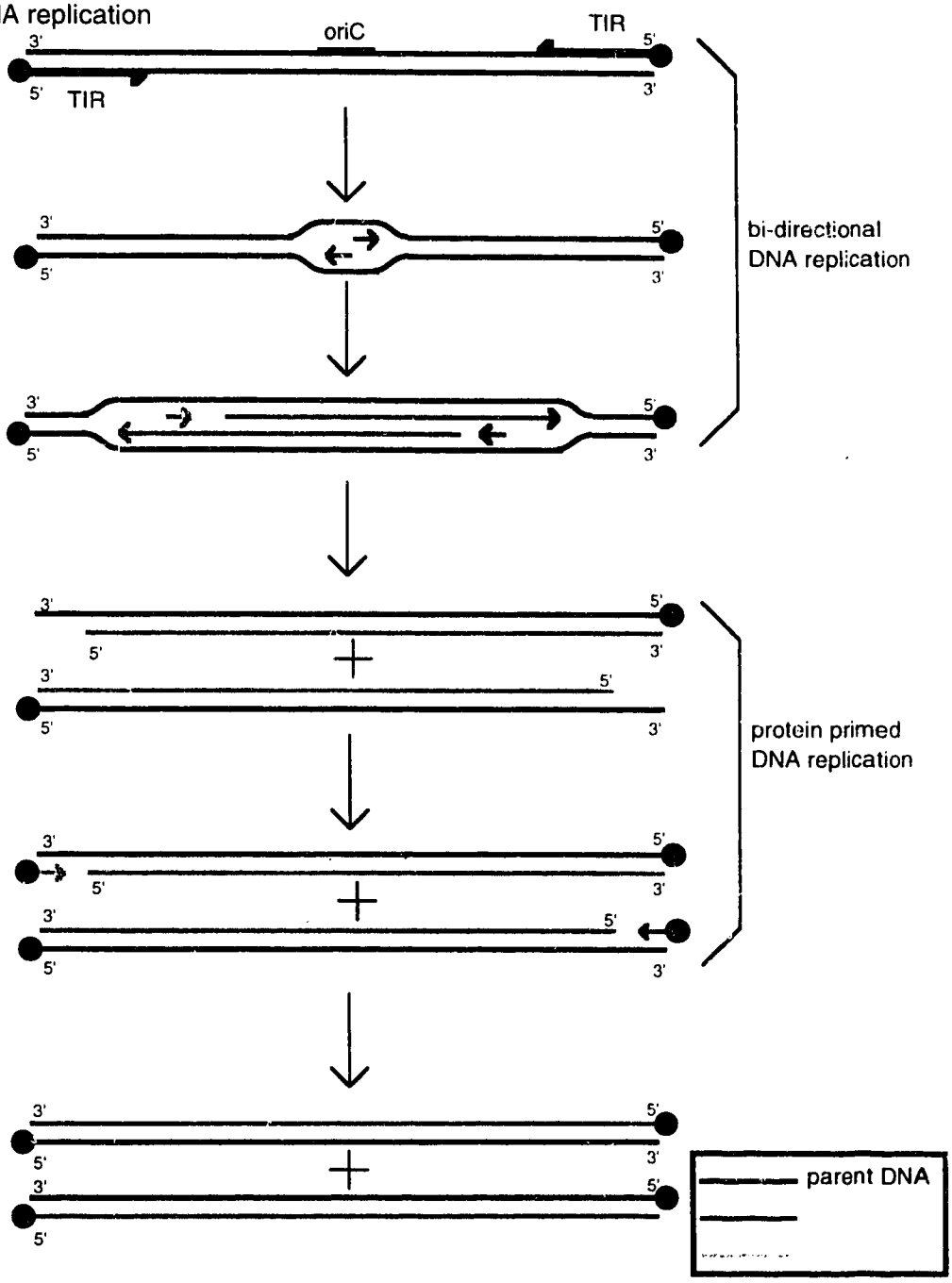


system of using terminal repeats to initiate DNA replication is also used by a number of viruses with terminal redundancies, such as the T7 phage (Richardson, 1983).

The invertron linear DNA type plasmids have been identified as using two different DNA replication mechanisms. The better understood mechanism is the terminal protein primed system, studied in detail in the adenovirus, and bacteriophage ϕ29 DNAs (reviewed by Salas, 1991). This mechanism, illustrated in Figure 6, involves the initiation of replication from both ends of the linear DNA molecule. An additional copy of the terminal protein acts as the primer by providing the 3' OH for 5' to 3' DNA polymerase activity. During this process, the other parent strand is displaced, and bound by a single-stranded DNA binding protein. When the two DNA polymerase complexes meet, the individual parent strands separate into two complexes, each containing a complete parent strand, and an incomplete daughter strand. DNA synthesis continues, displacing the single stranded DNA binding protein, until two mature DS linear molecules are produced. At no time is a lagging strand DNA synthesis process used; there are no Okazaki fragments. Electron microscopic examination of replicating invertrons has revealed the presence of two intermediate forms, named type 1 and type 2 (Salas, 1991), representing the replicative structure prior to and after the two replicative forks meet (Fig. 6). In the well studied adenovirus, bacteriophage ϕ29 and PRD1 cases, a limited number of proteins are required for viral replication. These include a virus encoded protein-primed replication specific DNA polymerase (Stillman *et al.*, 1982; Watabe *et al.*, 1984; Savilahti *et al.*, 1987), a terminal protein (Stillman *et al.*, 1982; Savilahti *et al.*, 1987), a single-stranded binding protein (Kruijer *et al.*, 1981; Gutierrez *et al.*, 1986), and the several host or virus encoded initiation proteins (Nagata *et al.*, 1983; de Vries *et al.*, 1985; Pastrana *et al.*, 1985; Zaballos, *et al.*, 1986). Typically, the initiation proteins bind to the linear DNA at the TIR and assist in separation of the parent strands. The DNA polymerase and free terminal protein of ϕ29 have been demonstrated to form dimers

Fig. 7. Simplified representation of the hybrid bi-directional protein primed DNA replication mechanism. oriC; origin of bi-directional replication, TIR; terminal inverted repeats, 5' and 3'; 5' and 3' OH ends of DNA polynucleotide. Black indicates parental DNA, dark grey indicates newly synthesized DNA, and light grey indicates RNA primers. See figure key for protein identifications.

Fig. 7 Simplified representation of hybrid bi-directional / protein primed DNA replication



(Watabe *et al.*, 1983), and form a covalent linkage to the priming nucleotide (dAMP for ϕ29), indicating that these two proteins likely form a complex which initiates DNA polymerization. The DNA polymerase genes of adenovirus and ϕ29 have been sequenced, and share greater homology with the eukaryotic DNA polymerase-α than the prokaryotic DNA polymerase I (Salas, 1991).

Recently, an alternative replicative system for linear DNA plasmids with covalently attached proteins has been described for the *S. rochei* pSLA2 plasmid (Chang and Cohen, 1994). This mechanism uses a combination of the bi-directional theta structure closed circular plasmid system and the protein-primed replication system (Fig. 7). The initial stages of replication involve the formation of a replication bubble at a conventional 'circular' origin followed by bi-directional replication towards either end of the linear plasmid. The mechanism appears to involve both leading and lagging strand synthesis (Okazaki fragment synthesis), likely using the chromosomally encoded bi-directional DNA replication enzymes. In both replication forks the leading strand replication complex synthesizes a complementary strand through to the 3' end of the parent chromosome. However, the lagging strand replication complex stops short of the 5' end of the parent chromosome, leaving about 280 bp of the parent strand unpaired. These remaining 280 bases are then synthesized by a protein-primed DNA polymerase activity which simply fills in the unpaired terminal DNA sequence without displacement of the previously synthesized DNA duplex. As this DNA replication mechanism has only been observed in pSLA2, the distribution and significance of the hybrid bi-directional/protein primed DNA replication mechanism is uncertain. Some observations suggest this mechanism may be common among *Streptomyces* plasmids and perhaps, chromosomes. The sequenced TIR of the *Streptomyces* plasmids pSCL1 (Wu and Roy, 1993), and SLP2 (Lin *et al.*, 1993) show significant sequence homology to the telomeres of pSLA2, suggesting a common ancestry and replicative system. Furthermore, the *S. lividans* chromosome has been demonstrated to

be linear (Leblond *et al.*, 1993) and to share sequences identical to the SLP2 TIR (Lin *et al.*, 1993), again suggesting a common replication system. A conventional circular origin of replication of the same type used by pSLA2 to initiate bi-directional DNA synthesis has also been predicted (Keen *et al.*, 1988) and demonstrated as being functional (Shiffman and Cohen, 1992) in pSCL1. The overall significance and distribution of this replication mechanism as well as the enzymes await further investigation.

Most plasmids and viruses do not encode all of the proteins necessary for their replication and maintenance, but instead rely on some host encoded factors. In certain cases, proteins encoded by other plasmids may also be required for replication. This scenario has a number of implications; the loss of the 'helper' plasmid providing necessary proteins will obviously lead to the elimination of the 'satellite' plasmid.

1.3 *Streptomyces* Genetics

The organisms which are the focus of study in this research project, the *Streptomyces*, are members of the soil-dwelling actinomycete group, a Gram-positive high G+C subdivision of the eubacteria (Woese, 1987). When grown on solid media *Streptomyces* spp. show a complex life cycle; during exponential growth these organisms grow as mycelia, followed by the development of aerial hyphae which in turn differentiate into spores (reviewed by Kutzner, 1981). During mycelial growth conventional 'cells' are largely absent though some cross walls do exist. Instead a syncytium containing several chromosomes per mycelial compartment has been observed, though interestingly, removal of the cell wall produces protoplasts containing single chromosomes. Spore formation is accompanied by the segregation of chromosomes into discrete compartments; ultrastructure studies have concluded that only one chromosome is found per spore (Hopwood and Glauert, 1960). These organisms have been the focus of considerable attention, largely due to the fact that they

produce over 70% of all known antibiotics, hence representing a focus of considerable industrial and medical interest (Berdy, 1980). These antibiotics are largely secondary metabolites, compounds the organisms produce during differentiation into aerial hyphae and spores following logarithmic growth (Chater, 1989). The secondary metabolites produced are numerous and diverse; their significance and function are largely obscure (Omura, 1992). The antibiotic activity of some of these secondary metabolites has been suggested to be coincidental by some researchers (Horinouchi and Beppu, 1990; Hunter, 1991), indeed some evidence linking antibiotics to a signal or differentiation control function has been found (Chater *et al.*, 1991). Little evidence exists that the antibiotic concentrations produced in the natural environment are sufficient for anti-bacterial action (Horinouchi and Beppu, 1990). The degree of similarity among the *Streptomyces* spp. is extensive, to the point that differentiating among species can be very difficult. This observation has led to the suggestion that the diversity within this group is due more to recombination and a general plasticity in gene expression than to actual phylogenic distance (Luehrsen et al., 1989).

The genetic properties of the *Streptomyces* spp. also show a wealth of unusual features (reviewed by Hopwood and Kieser, 1990). The G+C content of DNA within this group is among the highest observed in any organism, typically ranging between 70 and 74% (Gladek and Zakrzewska, 1984). This base bias appears to be quite uniform throughout the genome, for example there is little evidence of any G+C rich satellite DNA (Usdin *et al.*, 1984). While explanations for this high G+C bias include reducing genetic damage caused by ultraviolet light and minimizing the number of stop codons generated by frame shift mutations, thus favoring rapid evolution of new genes (Uchiyama and Weisblum, 1985), no definitive evolutionary advantage to this base bias has been identified. This extreme DNA base bias has a number of interesting implications. First, the high G+C content creates a strong codon usage bias, especially in the third base of any codon (Bibb and

Cohen, 1982). Statistical analysis of *Streptomyces* open reading frames has revealed that the first base of a codon is 70% likely to be G or C, the second base 50%, and the final base of the codon 90%. This strong statistical bias allows the rapid analysis of DNA sequence for the presence of open reading frames likely to represent actual protein-coding sequences (Bibb *et al.*, 1984). The extreme bias towards certain codons used to encode proteins has even led to the use of a $tRNA^{Leu}$ (UUA codon) as a regulatory factor in *Streptomyces coelicolor* (Leskiw *et al.*, 1991a, 1991b). The relative rarity of A and T residues also limits the number of restriction sites recognized by restriction endonucleases with target sequences rich in A and T residues. Thus, infrequently and frequently cutting enzymes are easily chosen when digesting *Streptomyces* DNA.

Another consequence of the extreme base bias in *Streptomyces* is the unusually high melting temperatures of the G+C rich DNA as the three hydrogen bonds joining the more common G-C base pairs are more numerous than the two hydrogen bonds binding the rarer A-T base pairs. The high melting temperatures of this DNA also lead to unusually stable secondary structures. These very stable secondary structures can interfere with common molecular biology techniques, especially sequencing, as the separation of single stranded DNA by polyacrylamide gel electrophoresis encounters anomalous gel migration caused by the strong secondary structures in these molecules (Mizusawa *et al.*, 1986).

Furthermore, recent research has indicated that high and low G+C DNA may, under certain conditions, migrate electrophoretically in a fashion different from 50% G+C DNA, leading to difficulties in accurately estimating the size of DNA molecules (Cantor *et al.*, 1988; Maniloff, 1989; Gravius *et al.*, 1994a) by pulsed field electrophoresis. This observation is particularly unfortunate as λ phage ladders (≅50% G+C content (Sanger *et al.*, 1982)) and yeast chromosomes (≅38% G+C content), the standard molecular weight markers used for pulsed field electrophoresis, have a much lower

G+C content than actinomycete DNA. Quantitative estimates by (Gravius *et al.*, 1994a) indicate that the size of G+C rich DNA is consistently underestimated when compared to 50% G+C DNA markers by pulsed field electrophoresis. This distortion is more pronounced for smaller pieces of DNA, up to a 30% underestimation of size for 60 kb pieces of DNA, though larger DNA molecules show less distortion; 7.5% for a 500 kb piece of high G+C DNA. A possible solution for this problem is the use of other high G+C DNA molecular markers, such as an RP2 or RP3 cohesive end actinophage ladder (Rausch *et al.*, 1993).

Another unusual feature present in *Streptomyces* DNA is a variety of DNA base modification systems. *Streptomyces* spp. have been found to contain extensive DNA restriction systems which frequently interfere with transformation and transfection of DNA between species, leading to the practice of passaging DNA through intermediate species or strains to avoid restriction endonuclease digestion by the addition of suitable base modifications (example, Qin *et al.*, 1994). As well as conventional DNA protection by the methylation modification of certain nucleotides (Matsushima *et al.*, 1987; MacNeil, 1988), an unusual DNA modification which produces non-enzymatic site specific DNA cleavage has been observed in *S. lividans* 66 (Zhou *et al.*, 1988). DNA cleavage was reported to occur when Tris buffers were contaminated by iron which likely originated as a trace contaminant in EDTA. Interestingly, the actual DNA damage seems to have taken place only during electrophoresis. A mutant strain of *S. lividans* was detected which did not show this DNA cleavage phenomenon, suggesting the inactivation or deletion of the gene or genes responsible for the DNA modification responsible for the cleavage effect. The nature of this modification to the DNA has not been reported. A survey of other *Streptomyces* detected only one other species which demonstrated the same characteristics, *Streptomyces avermitilis* NRRL 8165 (Kieser *et al.*, 1992; Evans *et al.*, 1994). Research by Ray *et al.* (1992) into the features of the *S. avermitilis* modification indicated a different active agent was responsible for the DNA cleavage reactions; the

Tris buffer. After changing the electrophoresis conditions to replace Tris buffer with HEPES, Ray *et al.* (1992) observed that the DNA cleavage stopped. Furthermore, Tris buffer was found to retain the ability to promote DNA cleavage even after electrophoresis stopped; the addition of this 'activated' buffer to sensitive DNA caused DNA degradation. The active component of the buffer has been noted to originate at the anode, and migrate towards the cathode during electrophoresis. The addition of thiourea, a radical scavenger and reducing agent, was also found to neutralize the reaction. As these observations suggested that the active agent is produced by the electric current at the anode, and then migrates in the electric field through the electrophoresis chamber, it probably carries a positive charge. Ray *et al.* (1992) believe an oxidising or free radical species is a likely candidate for this active agent, which possibly interacts in some fashion with Tris to produce the DNA breaking activity, and hence iron is not involved in the process.

The genome structure of the *Streptomyces* spp. has been the centre of considerable attention, and has demonstrated a number of unusual features. First, the bacterial genome has been estimated to be considerably larger than the 4.7 Mb genome of *E. coli*, with initial estimates of 5 to 7 Mb (Gladek and Zakrzewska, 1984). More detailed estimates obtained by physical mapping have indicated an even larger size; *S. coelicolor* 8 Mb (Kieser *et al.*, 1992), *S. lividans* 8 Mb (Kieser *et al.*, 1992), and *S. ambofaciens* strains ranging from 6.5 to 8.2 Mb (Leblond *et al.*, 1990).

The best characterized genetic linkage map for an actinomycete genome is that of *S. coelicolor* (Chater and Hopwood, 1983; Hopwood and Kieser, 1990) and it contains a number of unusual features which appear to be shared by *S. lividans* (Kieser *et al.*, 1992). First, two large areas of the genome, the "three and nine o'clock" regions, seem to be largely devoid of identifiable genes. Also, the pattern of organization and order of the genes between the three and nine o'clock and the nine and three o'clock regions is similar, leading to the suggestion that the genome may have

undergone a duplication event at some time in the past (Hopwood and Kieser, 1990).

Another unusual observation is that at least some *Streptomyces* spp. possess linear chromosomes. The topology of bacterial chromosomes (initially *E. coli*) was first examined by physical and genetic means and found to be circular. Both early and later more detailed genetic linkage maps of the *S. coelicolor* genome were also circular (Chater and Hopwood, 1983), as was the first published physical map (Kieser *et al.*, 1992). However, research by Leblond *et al.* (1993) indicated quite a different situation, detecting a physical discontinuity within the 3 o'clock region of the genome of *S. lividans* 66. Further investigations confirmed this result, and demonstrated that the linear genomes in *S. coelicolor* and *S. lividans* have proteins linked to their telomeric sequences (Chen *et al.*, 1993a; Lin *et al.*, 1993). According to Lin *et al.* (1993), all the other Streptomycetes surveyed (*Streptomyces antibioticus*, *S. coelicolor*, *Streptomyces lipmanii*, *Streptomyces moderatus*, *Streptomyces parvulus*, and *Streptomyces rochei*) share this linear topology, though these conclusions are not supported by data nearly as conclusive as the physical mapping data of *S. lividans* (Leblond *et al.*, 1993). The closely related organism *Rhodococcus fascians* also appears to possess a linear chromosome (Crespi *et al.*, 1992). While these actinomycetes are not the only examples of linear bacterial chromosomes (the spirochaete *Borrelia burgdorferi* (Baril *et al.*, 1989; Ferdows and Barbour, 1989) has been demonstrated to have a linear hairpin loop structure) this research reported the first case in which a linear chromosome with proteins covalently linked to its termini was identified. Interestingly, the *S. lividans* and *S. coelicolor* chromosomes are known to contain a functional oriC origin of circular replication (Kieser *et al.*, 1992; Zakrzewska-Czerwinska and Schrempf, 1992). Using a recombination-deletion system, the linear chromosome of *S. lividans* was recircularized without a loss of viability (Lin *et al.*, 1993), raising the question of what adaptive function a linear chromosome structure may provide.

Another characteristic of the *Streptomyces* genome is the unusually high degree of genetic instability, genome rearrangement, and phenotypic plasticity encountered within the genus (Hutter *et al.*, 1988). Observations of the high degree of gene transfer among *Streptomyces* spp. within a soil environment indicate that genetic exchange is a normal element of the lifestyle of these bacteria (Wellington *et al.*, 1992). Certain phenotypic traits have been noted as being particularly unstable (Hutter and Eckhardt, 1988). Interestingly, many of the recombination events occur in the three o'clock region, in the sequences near the linear chromosome telomeres (Kieser *et al.*, 1992). While a wide variety of amplification and deletion events have been reported, the mechanism producing these chromosomal alterations is poorly understood (Leblond *et al.*, 1991). Chromosomal deletions map to the 3 o'clock region of the bacterial chromosome and can be very large, ranging up to 800 kb in *Streptomyces glaucescens* (Birch *et al.*, 1989), and even 2 Mb in *S. ambofaciens* (Leblond *et al.*, 1991). Amplification events have been reviewed (Chater *et al.*, 1988; Hutter and Eckhardt, 1988; Birch *et al.*, 1990; Hornemann *et al.*, 1993), and typically involve small amplifiable units of DNA (AUD) amplifying to produce a tandem repeat array of several hundred copies. A typical example is a well studied *Streptomyces fradiae* amplification event in which a 10.5 kb AUD was amplified approximately 500 times (Fishman and Hershberger, 1983). Once again, these events are usually found in the 3 o'clock region (Betzler *et al.*, 1987; Kieser *et al.*, 1992; Redenback *et al.*, 1993). Some researchers have suggested these events are linked; deletions occur first, followed by amplification of short repeated sequences (Birch *et al.*, 1989; Hausler *et al.*, 1989). No satisfactory explanations have been offered as to how these bacteria can survive after such radical genomic modifications, let alone what selective advantage they may offer. Suggestions have been made that DNA encoding dispensable secondary metabolite genes are lost (Birch *et al.*, 1990) during these events. The observation that recombination, deletion, and amplification events cluster near the telomeres of the known linear

actinomycete genomes seems hardly coincidental, especially as a stable recircularized *S. lividans* chromosome was generated experimentally by a deletion mutation (Lin *et al.*, 1993). Recircularization and other recombination events in telomeric sequences may account for much of the genomic plasticity which has been observed, though mechanisms for these events remain obscure. No direct evidence exists of extrachromosomal elements having any role in these processes (Birch *et al.*, 1990). A number of similar recombination, deletion and amplification events have also been observed in various actinomycete plasmids. The circular *S. coelicolor* plasmid SCP2* had four 9 kb duplications (Bibb and Hopwood, 1981), pSLP101 of *S. lividans* has been observed to contain 1.1 kb amplifications (Jaurin and Cohen, 1984), and a number of spontaneous deletion events have been observed in pIJ101 (Kieser *et al.*, 1982) and pSN2 (Hussain *et al.*, 1990). The *Rhodococcus* sp. giant linear plasmids (Kalkus *et al.*, 1993) have been demonstrated to undergo recombination *in vivo* forming novel recombination products. Also, integration and amplification/integration events between SCP1 and SCP2 of *S. coelicolor* have been reported (Lydiate *et al.*, 1985). Curiously, many of the DNA rearrangements and recombination events observed in both plasmids and chromosomes are associated with apparently normal biological processes such as sporulation, temperature changes or shifts between nutrients, hardly conventional mutagenic conditions (Leblond *et al.*, 1990). Protoplast formation and regeneration is similarly often associated with these events.

1.4 *Streptomyces* Plasmids

The actinomycetes, and *Streptomyces* spp. in particular, have been recognized to contain an unusually large and diverse range of extrachromosomal elements (Hopwood *et al.*, 1986). During surveys, plasmids have been detected in as many as one in five of the strains tested (Toyama *et al.*, 1982; Daniel and Tiraby, 1983).

Investigations of the G+C content of these elements has consistently revealed % G+C contents similar to that of their host organisms (Hirochika *et al.*, 1984; Kinashi and Shimaji-Murayama, 1991; Wu and Roy, 1993), suggesting a long evolutionary association between host and plasmid. Many covalently closed circular plasmids (reviewed by Hopwood *et al.*, 1986) have been identified in these organisms; Table 1 lists some of the identified plasmids, their host strains, sizes, and phenotypes, with a focus upon those organisms and plasmids discussed. A wide variety of linear plasmids have also been identified; these plasmids and some of their features are summarized in Table 2.

While many circular and linear actinomycete plasmids have been detected, a phenotype has been assigned in very few cases. The most frequently observed traits are pock formation, and conjugation. As reviewed by Hopwood and Kieser (1993), plasmid loss is generally accompanied by a significant decrease in gene transfer during inter-strain matings, even in cases where transfer of the plasmid itself is not observed. *Streptomyces* conjugation appears quite distinct from the pilus-mediated systems typified by the *E. coli* F plasmid, but rather appears to involve direct contact between mycelial filaments. The suggestion has been made (Hopwood and Kieser, 1993) that transfer of Streptomycete plasmids and at least one virus (ϕSF1; Chung, 1982) occurs along filaments with transfer genes mediating movement between mycelial compartments. This conjugation capability is not intrinsic to all DNA molecules in *Streptomyces*, but rather has been demonstrated to be encoded by specific genetic loci (*tra*, *spd*) carried by the plasmids (Omer and Cohen, 1986; Kendall and Cohen, 1988; Hopwood and Kieser, 1993; Zotchev and Schrempf, 1994), and at least one *Streptomyces* virus [ϕSF1 (Chung, 1982)]. The formation of pocks, a colony morphology frequently observed in mixed cultures of plasmid-bearing and plasmid-less *Streptomyces* strains, is poorly understood. Pocks typically appear as circular areas of different colony morphology caused by a temporary inhibition in growth (Bibb *et al.*, 1977), and are suggested to be

Table 1. List of some *Streptomyces* circular plasmids. "Size" indicates the length of the plasmid. The phenotype catagories include: "Transfer" has ability to facilitate own transfer, "Pock" has ability to produce pocks, "Fertility" encodes ability to stimulate conjugation between strains, and "Integrates" can integrate into genome and excises upon conjugation. Copy number indicates the copy number of the plasmid per chromosome. Other characteristics include: not visualized - this plasmid has not been visually identified and is detected by phenotype alone, detected EM only - plasmid has been identified by electron microscopy only, prophage - plasmid is a stable virus prophage, antibiotic production - plasmid is implicated in antibiotic synthesis.

Table 1

List of Some *Streptomyces* Circular Plasmids and Traits

Plasmid	Host Organism/Strain	Size (kb)	Phenotype: Transfer	Fertility: Pock	Fertility: Integrates	Copy Number	Other	REF
pSA1	*S. ambofaciens* KA-1028	80	+	+		2		Omera *et al.*, 1981
pSAM2	*S. ambofaciens*	11.1	+	+	+	+		Pernodet *et al.*, 1985
pSAR1	*S. arenae* TU469	80				variable		Braxenthaler *et al.*, 1991
SCP2	*S. coelicolor* A3(2)	31	+	+	+	- 1-2		Schrempf *et al.*, 1975; Bibb *et al.*, 1977
SLP1	*S. coelicolor* A3(2)	14.5	+	+	+	+ 1		Bibb *et al.*, 1981
SLP4	*S. coelicolor* A3(2)		+	+			not visualized topology uncertain*	Hopwood *et al.*, 1983
minicircle	*S. coelicolor* A3(2)	2.6				<1		Lydiate *et al.*, 1986; Lydiate *et al.*, 1989
small	*S. fradiae* ATCC 10745	65					detected EM only	Okanishi *et al.*, 1980
large	*S. fradiae* ATCC 10745	94					detected EM only	Okanishi *et al.*, 1980
pUC1	*S. fradiae* ATCC 10745	77				1-2	ϕSF1 prophage	Chung *et al.*, 1982
	S. griseus 45						detected EM only, antibiotic production	Zhuang *et al.*, 1980
pSG1	*S. griseus* NRRL 3851	16.6			+			Cohen *et al.*, 1985
pGIF3	*S. incarnatus*	8.7			+	2		Malina&Roberi-Gero, 1992
SLP1	*S. lividans* 66	9.4-14.5	+	+	+	+ 4-5		Bibb *et al.*, 1981
SLP3	*S. lividans* 66		+	+	-		not visualized topology uncertain*	Hopwood *et al.*, 1983
pIJ110	*S. lividans* 66	13.6	+	+	+	+		Hopwood *et al.*, 1983
pIJ408	*S. lividans* 66	15	+	+	+	+		Hopwood *et al.*, 1983
pIJ101	*S. lividans* ISP5434	8.9	+	+	+	-		Kieser *et al.*, 1982
pSN22	*S. nigrifaciens*	11	+	+		-		Kataoka *et al.*, 1991
pSN2	*S. niveus* ATCC 19793	32	+	+		-		Hussain *et al.*, 1990

*may not be circular topology

Table 2. Some *Streptomyces* linear plasmids. "Size" indicates the length of the plasmid. "Repeats" indicates the length of the terminal inverted repeats, if present. "Protein" indicates whether the presence or absence of a terminal covalently linked protein has been demonstrated.

Table 2

List of Some *Streptomyces* Linear Plasmids and Traits

Plasmid	Host Organism/Strain	Size (kb)	Terminal Inverted Repeats	Terminal Proteins	Reference
pSAM1	*S. ambofaciens*	80	cohesive ends	no	Leblond *et al.*, 1990
pSA1	*S. avermitilis* ATCC 31267	100			Evans *et al.*, 1994
pSA2	*S. avermitilis* ATCC 31267	250			Evans *et al.*, 1994
pSA1	*S. azureus*	9, 14.5			Ogata *et al.*, 1983
pBL1	*S. bambergiensis*	43		yes	Zotchev *et al.*, 1992; Zotchev & Schrempf, 1994
	S. bambergiensis	640			Zotchev *et al.*, 1990
pSCL1	*S. clavuligerus* NRRL 3585	11.7	900 bp	yes	Keen *et al.*, 1988; Wu and Roy, 1993
SCP1	*S. coelicolor* A3(2)	350	80 kb	yes	Kinashi&Shimaji-Murayama, 1991
	S. fradiae ATCC 10745	420			Kinashi and Shimaji, 1987
pSGF2	*S. griseofuscus* C581	200			Hershberger *et al.*, 1989; Solenberg&Baltz, 1991
pSGF3	*S. griseofuscus* C581	65			Solenberg and Baltz, 1991
pKSL	*S. lasaliensis* NRRL3382R	520			Kinashi and Shimaji, 1987
SLP2	*S. lividans* 1326	50	44 bp	yes	Chen *et al.*, 1993b
pSPA1	*S. parvulus* M226	650		yes	Chen *et al.*, 1993
	S. parvulus ATCC 12434	520,560,580			Kinashi and Shimaji, 1987
pSRM	*S. rimosus*	43		yes	Chardon-Loriaux *et al.*, 1986
pPZG101	*S. rimosus* R6-500	387	95 kb		Gravius *et al.*, 1994b.
pSLA2	*S. rochei* 7434-AN4	17	614 bp	yes	Hayakawa *et al.*, 1979; Hirochika and Sakaguchi, 1982; Hirochika *et al.*, 1984
	S. rochei 7434-AN4	90,180			Kinashi and Shimaji, 1987
	S. venezuelae ATCC 10712	130			Kinashi and Shimaji, 1987
	S. violaceoruber JCM 4979	410,440,470,500, 530,560,590			Kinashi and Shimaji, 1987

analogous to the zones of lethal zygosis produced by viruses or bacteriocins (Kieser *et al.*, 1982). Pock formation has been demonstrated to depend upon plasmid transfer, and not a diffusable extracellular compound (Bibb *et al.*, 1977). Indeed, the close relationship between the pock formation trait and the conjugative plasmid transfer genes on many *Streptomyces* plasmids has been well illustrated (Hopwood *et al.*, 1986; Akagawa, 1987; Kendall and Cohen, 1987; Kendall and Cohen, 1988; Kataoka *et al.*, 1991; Zotchev and Schrempf, 1994). *Streptomyces* plasmids have been reportedly cured by a wide variety of techniques, including the conventional ethidium bromide or acridine orange methods, and also by less conventional techniques such as production of protoplasts followed by cell wall regeneration (Hopwood, 1981; Hopwood *et al.*, 1983).

Rather than discuss all the known *Streptomyces* linear plasmids, four plasmids will be discussed in detail; pSLA2, SLP2, SCP1 and pSCL1. These four plasmids are by far the best characterized of the *Streptomyces* linear plasmids, and feature many of the range of traits which have been observed in *Streptomyces* linear plasmids.

pSLA2:

This *S. rochei* linear plasmid was the first identified bacterial linear plasmid with covalently attached terminal proteins (Hirochika and Sakaguchi, 1982). The plasmid is 17 kb long (Hayakawa *et al.*, 1979), of high copy number, and has TIR which are 614 bp long (Hirochika *et al.*, 1984). Little is known of this plasmid; aside from the TIR sequence, this plasmid has not been sequenced and has no known phenotype. Most notably, pSLA2 is the only linear DNA structure demonstrated to use the hybrid bi-directional protein primed replication method described by Chang and Cohen (1994). This fact will likely result in a more detailed investigation of this plasmid in the near future.

SLP2:

SLP2 is a *S. lividans* 1326 plasmid which was identified phenotypically (Hopwood *et al.*, 1983) long before it was identified physically. This plasmid had been associated with two traits; it promoted conjugation between *Streptomyces* strains and species 300 fold, and caused pock formation (Hopwood *et al.*, 1983). Attempts to physically identify SLP2 and SLP3 (another *S. lividans* 1326 plasmid) failed initially, likely due to the degradation inducing DNA modification system described previously, and a failure to anticipate that the plasmid might have a linear invertron structure. Ultimately, SLP2 was detected after conjugation and transfer to *Streptomyces parvulus* M226 (Chen *et al.*, 1993b), where SLP2 is maintained stably. SLP2 was found to be a 50 kb linear plasmid with covalently attached proteins and contained a previously unidentified 5.4 kb transposon, Tn4811 (Chen *et al.*, 1992). Analysis identified copies of this transposon on both SLP2 and the *S. lividans* chromosome, an observation which led to the discovery of the *S. lividans* linear chromosome, and the finding that the chromosome and SLP2 share identical terminal sequences (Chen *et al.*, 1993a; Lin *et al.*, 1993). Restriction analysis indicated that the terminal 16 kb of the right end of SLP2 is also the telomeric sequence for both ends of the bacterial chromosome (Lin *el al.*, 1993), although surprisingly the TIR shared by the two ends of SLP2 are extremely short, only 44 bases long. The exact phylogenic relationship between the SLP2 and *S. lividans* chromosomal telomeres is not understood, though the common sequences suggest these structures use the same covalently attached terminal proteins and DNA replication systems.

SCP1:

SCP1 of *S. coelicolor* is the best characterized *Streptomyces* giant linear plasmid. This plasmid is 350 kb in size, and contains extensive 80 kb TIR. The mechanism and location of SCP1's integration and excision from the *S. coelicolor* genome has been well characterized (reviewed by Chater and Hopwood, 1983), and the

plasmid has been observed periodically to incorporate chromosomal DNA during these events. In the wild type organism this plasmid is found in a free form and has a copy number of about four (Kinashi and Shimaji-Murayama, 1991). Relatively little of this plasmid has been sequenced; some terminal sequences and a number of genes. Unlike most *Streptomyces* plasmids, a number of genetic traits have been localized on SCP1. In addition to a conjugative role discussed previously (Hopwood *et al.*, 1985b), the methylenomycin gene cluster (Kirby and Hopwood, 1977; Chater and Bruton, 1985; Kinashi *et al.*, 1987) and genes encoding three spore associated proteins (sapC, sapD, sapE) (Willey *et al.*, 1991) have been located on SCP1. The spore associated proteins are synthesized during the production of spores from aerial mycelia, though their precise role in this process is not understood. This plasmid is the only *Streptomyces* plasmid which has been conclusively demonstrated to encode the biosynthetic gene cluster for an antibiotic. The detection of the genes encoding the sap protein is particularly surprising for two reasons, these genes were found within the TIR, and the genes encoding the sap protein would be normally considered 'chromosomal' genes. Interestingly, curing the host of this plasmid does not affect normal bacterial differentiation.

pSCL1:

This *S. clavuligerus* plasmid is 11.7 kb long, linear, with terminal covalently attached proteins (Keen *et al.*, 1988) and with no known phenotype. pSCL1 is the only *Streptomyces* linear plasmid for which the entire DNA sequence is known, and it shows a number of unexpected features (Wu and Roy, 1993). Analysis of the 7-8 probable open reading frames encoded by pSCL1 revealed neither a DNA polymerase, nor any protein showing homology to known invertron terminal proteins. Two RNAs transcripts were identified from pSCL1, one encoding a protein sharing homology to the korA protein of the *S. lividans* plasmid pIJ101 (Kendall and Cohen, 1988). The other RNA did not correspond to an open reading frame, but rather appears to represent an antisense RNA,

which likely functions as a repressor (Wu and Roy, 1993). Other open reading frames encoded on the plasmid show homology to a replication origin protein of *Bacillus subtilis*, and a plasmid partition protein (Motalebi-Veshareh *et al.*, 1990). Unexpectedly, pSCL1 can be maintained in *S. lividans* following circularization with active selection (Shiffman and Cohen, 1992), indicating the presence of a functional origin of circular replication. This observation suggests pSCL1 may use the novel bi-directional/protein primed, replication system observed in pSLA2. pSCL1 appears to be an example of a parasitic DNA molecule, which uses a kil/kor system to prevent plasmid loss; attempts to cure *S. clavuligerus* of this plasmid have proven unsuccessful (Michaluk *et al.*, 1994).

In addition to plasmids, several unusual classes of genetic elements have been described in *Streptomyces*. The first is a group of molecules which can exist either in an autonomous plasmid state, or as an integrated form within the host genome, a behaviour more typically associated with viruses. Eight elements of this type have been identified in Streptomycetes (reviewed by Kieser and Hopwood, 1991), along with the similar non-conjugative e14 element of *E. coli* (Brody *et al.*, 1985). The best characterized are SLP1 of *S. coelicolor* and pSAM2 of *S. ambofaciens*. SLP1, a 17.2 kb sequence (Omer and Cohen, 1984) originating as an integrated form within *S. coelicolor*, was first identified in *S. lividans* as a free covalently closed circular autonomously replicating plasmid following conjugation with *S. coelicolor*. Excision from the chromosome was mediated by plasmid-encoded proteins homologous to the *int* and *xis* genes of integrative viruses such as λ (Chater *et al.*, 1988; Hutter and Eckhardt, 1988). Integration may also occur in new hosts (Omer and Cohen, 1984) at specific sites within plasmid (attP) and chromosome (attB) (Omer and Cohen, 1986). Sequence analysis of the integration sites has revealed that an apparently functional and essential $tRNA^{Tyr}$ gene is located at the attB site. This tRNA gene is reformed during integration by recombination with a copy of the tRNA gene contained in the attP site (Vogtli and Cohen, 1992). Interestingly, SLP1 exists in only

the integrated or the autonomous form, never both (Hopwood and Kieser, 1993), a system controlled by the *imp* locus (Shiffman and Cohen, 1993). The 11 kb pSAM2 seems to be very similar to SLP1, again integrating at a $tRNA^{Pro}$ gene (Boccard *et al.*, 1989; Kuhstoss *et al.*, 1989; Mazodier *et al.*, 1990), though pSAM2 may coexist within a cell in both integrated and free forms (Boccard *et al.*, 1988). These two elements, and the others of their group, have been demonstrated to be conjugative, promote chromosomal gene transfer and to cause pock formation, but to have no other known phenotypes (Smokvina *et al.*, 1991). The strong functional and sequence similarities between these plasmids and many integrative viruses has led to the suggestion that the integrative elements are simply defective phage which have acquired conjugation as a method of dispersal; certainly their close similarity to the behaviour of many phage is obvious.

Another unusual *Streptomyces* genetic element is the *S. coelicolor* mini-circle. This poorly understood element is a 2.6 kb DNA sequence which is integrated into the *S. coelicolor* A3(2) chromosome in two locations (Lydiate *et al.*, 1986), but also exists free within the cell as a very low copy number CCC form, with less than one free copy per chromosome (Henderson *et al.*, 1989). While this structure resembles a transposon in many ways, free cytoplasmic forms of transposons have not been observed. The free form has been suggested to act as a vector, a mechanism for minicircles to move between host chromosomes within a single filament (Henderson *et al.*, 1989); site specific integration to a plasmid borne target sequence has been observed (Henderson *et al.*, 1990). A mechanism for the production of the CCC form of the minicircle and its function has not been conclusively demonstrated.

1.5 Research Goals

Previous research in our laboratory led to the discovery of the invertron linear plasmid pSCL1 of *S. clavuligerus* (Keen *et al.*, 1988). Determination and analysis of the DNA sequence of this

plasmid, along with an investigation of RNA transcripts of pSCL1 were conducted by Wu and Roy (1993). These investigations indicated that the proteins encoded by this plasmid did not appear to include all of the factors necessary for its own replication. Particularly notable was a lack of any identifiable gene for the terminal protein and a gene for a DNA polymerase. These observations suggested that an additional protein-linked linear DNA plasmid or plasmids which could act as a 'helper' plasmid might be present in *S. clavuligerus*, much like the pGK11, pGK12 system of *Kluyveromyces lactis* (Hishinuma *et al.*, 1984; Wilson and Meacock, 1988). The more recent discovery that some, if not all, *Streptomyces* chromosomes are linear (Leblond *et al.*, 1993; Lin *et al.*, 1993) offered an alternative possible explanation; pSCL1 may use some or all of the enzymes of a linear chromosome replication system.

To obtain a better understanding of the plasmid system in *S. clavuligerus* and other β-lactam producing *Streptomyces* spp., a survey of extrachromosomal elements found within *Streptomyces cattleya* NRRL 3841, *Streptomyces clavuligerus* NRRL 3585, *Streptomyces griseus* NRRL 3851, *Streptomyces jumonjinensis* NRRL 5741, and *Streptomyces lipmannii* NRRL 3584 was conducted using a variety of DNA preparative techniques including *in-situ* cell lysis followed by pulsed-field gel electrophoresis. Given the economic importance of the antibiotics produced by these organisms, a comparison of the extrachromosomal elements identified in these organisms would be of considerable interest, especially should the gene clusters for antibiotic biosynthesis be located on plasmids carried by these organisms.

Since several of the organisms within the survey are considered to be very closely related (especially *S. clavuligerus* and *S. jumonjinensis*), a comparison of any extrachromosomal elements detected might also prove instructive. Given that among the actinomycetes, some *Streptomyces* plasmids have an apparent wide host range (e.g. pIJ101; Kendall and Cohen, 1987), the possibility that plasmids found among these actinomycetes could be related, if

not identical, seemed high. Therefore, hybridization studies and restriction mapping were planned to allow the assessment of similarities among any extrachromosomal elements found in this survey.

A survey of extrachromosomal elements within the *Streptomyces lividans* strains 1326, TK19, and TK24 was also planned in an attempt to detect the previously uncharacterized SLP2 and SLP3 plasmids (Hopwood *et al.*, 1983). *S. lividans* 1326 was known to contain both SLP2 and SLP3, strain TK19 contained only SLP3, and strain TK24 was cured of both plasmids (Hopwood *et al*, 1985). As the plasmid profiles of these three strains are different, the detection of plasmid species within these strains should allow an assessment and identification of any plasmids detected. As the project research progressed, the 50 kb linear plasmid SLP2 was detected in our lab (Netolitzky *et al.*, 1991a; 1991b) and reported at the same meeting in which its discovery was reported by Chen *et al.* (1993). Research continued in an attempt to identify and characterize SLP3, focusing on the TK19 strain.

2) METHODS AND MATERIALS

2.1) Materials

2.1.1) Enzymes, Fine Chemicals, and Supplies

Nylon membrane (Hybond-N) used for Southern transfers was obtained from Amersham. Kodak X-ray film (XAR5) was obtained from the Eastman Kodak Co. Nucleotides were purchased from Pharmacia, and [^{32}P] labeled nucleoside triphosphates were obtained from ICN Biochemicals.

Oligonucleotides used were obtained from two different sources. Random primer oligonucleotides were purchased from Boehringer Mannheim. The remaining oligonucleotides were synthesized within the lab using a 381A or a 391EP DNA synthesizer manufactured by Applied Biosystems.

Restriction endonucleases were obtained from Boehringer Mannheim, and New England Biolabs (NEB). The Klenow fragment of DNA polymerase I, DNAse free pancreatic RNAse, Exonuclease III, and terminal deoxynucleotidyl transferase were purchased from Boehringer Mannheim. DNAse I and Exonuclease VII were obtained from Sigma. BAL 31 nuclease was purchased from NEB. β-Agarase was obtained from either Cedar Lane Biologicals, or NEB.

2.1.2 Bacterial Strains, Culture Conditions

Streptomyces cattleya NRRL 3841, *Streptomyces clavuligerus* NRRL 3585, *Streptomyces griseus* NRRL 3851, *Streptomyces lipmannii* NRRL 3584 were obtained from NRRL, Peoria, Illinois. *Streptomyces jumonjinensis* NRRL 5741 was received both from NRRL, Peoria, Illinois and ATCC, Rockville, Maryland. The ATCC designation for *S. jumonjinensis* NRRL 5741 is ATCC 29864, and the isolate information indicated this isolate is the same as NRRL 5741. *Streptomyces lividans* 1326, TK19 and TK24 strains were provided by Dr. T. Kieser, John Innes Foundation, Norwich, UK.

These organisms were maintained on Tomato Oatmeal Agar plates at 27°C as per Keen *et al.* (1985), or in Trypticase Soy Broth (TSB) media following Hopwood *et al.* (1985a). Preparation of spores was conducted using the techniques described by Hopwood *et al.* (1985a). When preparing mycelia for *in situ* lysis and phenol-protein interface extracts, liquid cultures of all organisms were grown in TGS broth [30 g trypticase soy (BBL), 20 g glycerol, 15 g sucrose per l] and incubated at 28°C on a rotary shaker at 250 rpm. Fifty ml amounts of TGS broth in 500 ml Erlenmeyer flasks were inoculated with spore stocks, incubated for 48-72 hours, then added to 250 ml amounts of sterile TGS broth in 4 l Erlenmeyer flasks. To assist in dispersing mycelia masses, a 1 cm diameter coiled stainless steel spring was placed within the 500 ml and 4 l Erlenmeyer flasks. These cultures were incubated for 24-48 hours, then mycelia were harvested by vacuum filtration or centrifugation at 10,000 x *g*.

2.2 DNA Isolation and Manipulation Techniques

2.2.1 DNA Isolation Techniques

Several protocols were used to prepare whole genomic DNA, each of which tended to recover selectively certain types of DNA. To minimize confusion, each of these techniques has been given a specific name, which will be used in the discussion of the research which follows.

Conventional phenol-chloroform: Conventional phenol-chloroform DNA extraction (Sambrook *et al.*, 1989) was used to isolate relatively small fragments of the total cellular DNA. As has been previously demonstrated, DNA with covalently attached proteins is selectively lost during this isolation procedure (Hirochika and Sakaguchi, 1982).

Low molecular weight (mw) DNA preparation by proteinase K: To include DNA with covalently attached proteins, the method of Fishman and Hershberger (1983) was utilized. In brief, this technique involved digestion of washed mycelia with lysozyme,

followed by treatment with proteinase K and sodium dodecyl sulfate (SDS, Sigma). The resulting lysate was cleared by gh speed centrifugation after increasing the NaCl concentration to 0.5 M, followed by precipitation of the nucleic acids by isopropanol. This technique, previously used to isolate the small linear plasmid pSCL1 from *S. clavuligerus*, proved efficient in recovering small linear plasmid DNAs. This technique will be referred to as the proteinase high mw DNA preparation technique in the remainder of the thesis.

High molecular weight (mw) DNA preparation by proteinase K: To prepare high molecular weight DNA samples suitable for the isolation of giant linear plasmids, a gentle lysis protocol was used. Mycelia were harvested by centrifugation and filtration, then 1 gram (wet weight) of mycelia was resuspended in 4 ml of a solution of 15% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA followed by the addition of 1.5 ml of 0.5 M EDTA and 5 mg of lysozyme, mixing by gentle inversion, and a 15 minute incubation at 21°C. Proteinase K (2.5 mg) was added, mixed by gentle inversion, followed by the addition of 0.5 ml of 20% sodium lauryl sarcosinate, inversion, and incubation at 55°C for 2 to 3 hours. The resulting cell lysate was cleared by the addition of 1.4 ml of 5 M NaCl, gentle inversion, and centrifugation in a Ti 50 rotor (Beckman) at 40,000 rpm for 45 minutes. The pellet was discarded, and the supernatant could then be fractionated directly using a sucrose gradient without further preparative steps. To minimize shearing of large DNA molecules such as the GLPs, this preparative technique avoided vortexing or any other harsh mechanical stresses; all mixing was achieved by gentle inversion. This technique will be referred to as the proteinase low mw DNA preparation technique in the remainder of the thesis.

Phenol-protein interface: This technique is a novel protocol developed by Frank Behrend and Dr. Kenneth L. Roy which selectively recovers DNA molecules which have covalently attached proteins, such as many linear plasmids, and the telomeres of certain linear chromosomes (Behrend *et al.*, 1995). Liquid cultures were

harvested and resuspended in 10 ml per g (wet weight) mycelia of STE [25% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. One half volume of 0.5 M EDTA (pH 8.0), and one half volume of 10 mg/ml lysozyme in STE were added and incubated at 21°C for 15 minutes. 20% SDS solution was added to give a final concentration of 1% SDS, followed by a 2 hour incubation at 37°C. One half volume of phenol was added, followed by 10 minutes of shaking. The phenol and aqueous phases were separated by centrifugation at 1000 x G for 15 minutes, after which the phenol layer and the aqueous layer were removed separately, leaving a thick layer of white material at the phase interface. Equal volumes of TE buffer and phenol were added to the interface layer, followed by another cycle of 10 minutes of shaking, centrifugation, and removal of the the phenol and aqueous layers. This extraction procedure was repeated a total of 4 times, selectively removing material contained within each of the aqueous and phenol phases, leaving only the interface between these phases, which should contain material with both lipid and water soluble components, such as a covalently linked protein-DNA complex. The remaining interface material was centrifuged at 5000 x *g* for 15 minutes in capped 40 ml centrifuge tubes, and any aqueous or phenol layer produced was selectively removed. One volume of TE buffer, 1/10 volume of 3 M sodium acetate and two volumes of 2-propanol were added, mixed, and DNA-protein conjugates were precipitated at -20°C for 1 hour. Following centrifugation at 5000 x *g* for 30 minutes, the pellet was resuspended and allowed to dissolve for 12 or more hours in a minimal volume of TE buffer. The thick white suspension which was produced was placed in 1.5 ml microfuge tubes and centrifuged at 14000 rpm for 10 minutes, resolving the preparation into a white pellet and a clear supernatant. The clear supernatant contained the protein-DNA complexes and could either be used directly, or treated with proteinase K for 1 hour at 55°C to produce DNA without the covalently attached protein.

In situ pulse field ge. (PFG): *In situ* cell lysis and DNA isolation techniques were used to prepare samples with large intact

DNA molecules, such as GLPs and chromosomes, by embedding the lysing cells in agarose to avoid shearing DNA (Smith *et al.*, 1988). For this protocol harvested cells were resuspended in 0.5 M EDTA (Mallinckrodt AR), 0.01 M Tris-HCl (pH 8.0) (LET) buffer, in proportions of 1, 0.5, 0.25, 0.125 g wet weight of cells/ml of LET buffer. Cell suspensions were heated in a water bath to 37°C, then mixed with an equal volume of molten 2% (w/v) InCert agarose (FMC Bioproducts) in 0.1 x LET buffer at 65°C. The resulting mixture was poured a Bio-Rad CHEF sample plug mold and allowed to solidify. Blocks were transferred to a solution of 5 mg/ml lysozyme (Sigma), 10 mg/ml sodium lauryl sarcosinate (Sigma) in LET buffer solution, allowing 0.5 ml of solution per block, and incubated at 37°C for 24 hours. Blocks were washed twice for 15 minutes with 0.5 M EDTA (2 ml per block) at 21°C, then transferred to 1% (w/v) SDS [(Sigma) 0.5 ml per block] in LET buffer and incubated at 37°C for 24 hours. Blocks were again washed twice in 0.5 M EDTA for 15 minutes at 21°C, then incubated in 1% SDS in LET buffer containing 0.5 mg/ml proteinase K (Boehringer-Mannheim), 0.5 ml per block, for 24 hours at 50°C. After proteinase K treatment the blocks were washed twice in 0.5 M EDTA, then incubated in 0.5 M EDTA at 21°C for 24 hours before storage in 0.5 M EDTA, at 4°C.

The *in situ* agarose microbeads cell lysis protocol of Koob and Szybalski (1992) was used on a trial basis, for comparison with the conventional agarose embedded cell preparations.

2.2.2 Sucrose Gradient Separation and Purification of Plasmids

Purification of both small and giant linear plasmids proved possible using sucrose gradient centrifugation techniques. DNA prepared by the proteinase high mw DNA preparation procedure was the primary source material for these experiments, though other DNA preparations were examined with this protocol. Gradients of 13 ml (SW40) or 38 ml (SW28) 10%-35% sucrose in 1 M NaCl, 20 mM Tris-HCl (pH 8.0), 2 mM EDTA were produced using a two-chamber gradient maker. Samples of 0.1-0.5 ml (SW40

rotor) or 1-2 ml (SW28 rotor) were layered on top of the gradient, and then centrifuged under conditions which varied depending on the size of DNA molecules which were to be recovered. For small plasmids up to 50 kb, samples were centrifuged for 15-24 hours at 36000 rpm (SW40 rotor) or 28000 rpm (SW28 rotor). Larger sized plasmids and other DNA fragments were recovered after 48 hours at 18000 rpm (SW40 rotor) or 12000 rpm (SW28 rotor). Gradients were fractionated by repeatedly pipetting 0.7 ml fractions from the top of each gradient. The DNA in each fraction was then precipitated with 0.7 ml of 2-propanol at -20°C.

2.2.3 Agarose Gel Electrophoresis of DNA Samples

DNA fragments in the range of 0.5 to 40 KB were separated and their sizes determined using conventional agarose gel electrophoresis techniques (Sambrook *et al.*, 1989). Various appropriate concentrations (0.4 to 1.5%) of Ultrapure Electrophoresis grade agarose (Gibco/BRL) in a 1 x TEA buffer [1 x TEA contains 20 mM Tris-HCl (pH 7.2), 50 mM sodium acetate and 2 mM EDTA] were prepared in a vertical gel apparatus. Typical electrophoresis conditions ranged from 1 to 5 V/cm, for durations between 3 and 18 hours.

Two dimensional agarose gel electrophoresis (Hintermann *et al.*, 1981) was used to assess topology present in certain DNA bands. Samples were electrophoresed on a 0.4% agarose gel, stained with ethidium bromide and illuminated with UV light for 10 minutes. Following illumination, the lane of the agarose gel containing the sample was cut out as an agarose strip, re-orientationed at right angles to the direction of the first run, and electrophoresed into a 0.7% agarose gel in 1 x TEA.

SDS agarose gel electrophoresis, a modified agarose gel electrophoresis procedure in which a 0.1% SDS component was added to the buffer and gel, was used to assess the possible impact of proteins covalently attached to DNA. All gels included molecular weight marker lanes, typically those produced by *Bst*EII, *Bsp*HI,

*Cla*I, *Hin*dIII, *Apa*I, and *Kpn*I digests of λ DNA (Boehringer-Mannheim).

Higher molecular weight DNA fragments and GLPs were separated and their sizes determined using a pulsed field gel electrophoresis technique, the Contour-clamped Homogeneous Electric Field (CHEF) apparatus (Chu *et al.*, 1986; Chu, 1989; Maule and Green, 1990), capable of separating DNA fragments up to at least 2 MB in size. CHEF gels were prepared in 0.5 x TEB buffer [1 x TEB contains 100 mM Tris (pH 8.3), 2.5 mM EDTA (Mallinckrodt AR), 100 mM borate (BDH)] using 1.2 % low melting temperature agarose (Boehringer-Mannheim), and electrophoresed at 10-15°C in a Bio-Rad CHEF DR II apparatus. Typical pulse settings for 20-250 kb analysis were 5 second pulses for 24 hours, at 165 V. Typical conditions for 50-2000 KB fragment analysis were a ramp of 5 to 80 second pulses for 24 hours, at 165 V. Molecular weight markers used include *Saccharomyces cerevisiae* chromosomes (Bio-Rad), and λ concatameric DNA molecules (New England Biolabs).

During electrophoresis of DNA samples isolated from *S. lividans*, 0.1 mM thiourea was added to the gel buffers to minimize base modification induced DNA degradation (see Introduction.)

All DNA bands on CHEF and conventional gels were visualized by illumination with ultraviolet light after ethidium bromide staining.

2.2.4 Plasmid Copy Number Estimation

The copy number of the plasmids pSCL1, pSJL1 and 2 were estimated by a comparison of the intensity of the chromosomal DNA with the plasmid bands over several DNA dilutions (Kalkus *et al.*, 1993). As the relative lengths of the chromosome and the linear plasmids are known and the relative fluorescent intensity of ethidium stained DNA depends on the quantity of DNA, measurement of the relative fluorescence of the sheared chromosomal DNA and the plasmid bands allows approximation of the plasmid copy number. Proteinase low mw DNA samples were sheared by passage through a 1.5 mm gauge needle, then diluted in

ten fold dilutions from one to 10^7 fold, and electrophoresed on a conventional 0.7% agarose 1 x TEA gel. After ethidium bromide staining and photography the intensities of the plasmid and chromosome bands were determined densitometrically.

2.2.5 DNA Restriction Endonuclease Digestion Techniques

Restriction enzyme digestion of DNA in aqueous solution was conducted following conventional protocols (Sambrook *et al.*, 1989), using the restriction enzyme manufacturer's recommended conditions and supplied buffers. When digesting DNA with two or more enzymes which require incompatible digestion buffers, digestion with one enzyme was followed by precipitation, and centrifugation. Sedimented DNA fragments were then redissolved and digested with the second enzyme.

Several protocols were used in an attempt to digest agarose embedded DNA with restriction enzymes, including the protocols of Smith *et al.* (1988), Chen *et al.* (1990), Nuijten *et al.* (1990), Grothues and Tummler (1991), Solenberg and Baltz (1991), Heath *et al.* (1992), Kieser *et al.* (1992), and Kalkus *et al.* (1993),. Blocks containing whole cell DNA as well as isolated DNA fragments excised from agarose gels were used in these experiments. Agarose embedded DNA samples were also digested with β-agarase (NEB) according to the manufacturer's specifications, followed by restriction endonuclease digestion, and gel electrophoresis.

Agarose microbeads containing whole-cell *Streptomyces* spp. DNA were digested following the protocol of Koob and Szybalski (1992) as an alternative method of conducting restriction site mapping of plasmid and chromosome DNA.

Phenol-interface extract DNA samples from *S. lividans* TK19 were treated with a variety of enzymes including BAL 31 nuclease, Exonuclease III, Exonuclease VII, DNAse I, DNAse free pancreatic RNAse, Nuclease S1, and proteinase-K to investigate the nature of pSL1A and pSL1B. These enzymes were incubated in the enzyme manufactors' recommended conditions and buffers.

2.2.6 Recovery of DNA Embedded in Agarose Gels

A number of plasmids were identified in the *Streptomyces* spp. studied, and these were selectively recovered by several procedures. DNA used for preparation of pSCL2, pSCL3, pSGL1, pSJL3, pSJL4 and SLP2 probes was obtained by cutting out pieces of CHEF gels containing ethidium bromide stained giant linear plasmid bands. A similar technique employing conventional agarose gels was used to cut out and isolate pSJL1, pSJL2, pSL1A and 1B DNA. The pieces of agarose were washed twice with TE buffer [10 mM Tris-HCl pH 8.0, 1 mM EDTA (1 ml per band)] for 15 minutes at 4°C, then melted at 60°C for 15-30 minutes, and incubated at 40°C with 10 units of β-agarase (New England Biolabs) for 2-3 hours. This agarase digested material was used for random primer labeling without further precipitation or preparation.

2.2.7 Recovery of Extracellular Material

Extracellular material, including possible virus particles, was recovered by polyethylene glycol (PEG) precipitation. Streptomycete liquid cultures were grown under standard conditions, and mycelia were harvested by filtration and centrifugation. The supernatant was retained, and PEG was added to a concentration of 6-8%, and NaCl was added to produce a concentration of 1 M. The resulting solution was stored at 5°C for 12 hours, then centrifuged at 6000 x g for 30 minutes. The pellet was resuspended in a minimum volume of TE buffer. This material was used directly for electron microscopic examination. For analysis of the DNA content of the precipitate, proteinase-K (5 mg/ml) and SDS (final concentration 1%) were added, then incubated at 55°C for 3 hour, followed by conventional agarose gel electrophoresis.

2.3 Preparation and Hybridization of Radioactive DNA Probes

2.3.1 Preparation of DNA Probes

Radioactive DNA probes used in hybridization analysis of Southern transfers were obtained by a number of methods. Solution samples of pSCL1 used for the preparation of probes were isolated from proteinase low mw DNA preparations using sucrose gradient sedimentation (Keen *et al.*, 1988). DNA was labeled with $\alpha[^{32}P]$-dATP using the random primer techniques (Feinberg and Vogelstein, 1983, 1984). Agarase prepared DNA samples were also labeled by random primer techniques, but used a larger sample volume of DNA (30-35 µl) in a total volume of 50 µl. Oligonucleotide probes were end labeled with T4 polynucleotide kinase and γ-$[^{32}P]$-ATP as described by Maxam and Gilbert (1980). All probes were passed through a 3 ml Sephadex G-50 column after labeling to remove unincorporated nucleotides.

2.3.2 Southern Transfer Protocols

DNA from conventional agarose gels was transferred to nylon membranes (Amersham) following a standard weakly alkaline buffer Southern blotting procedure (Southern, 1979) as modified for nylon membranes (Reed and Mann, 1985; Rigaud *et al.*, 1987). The procedure included a 10 minute 0.25 M HCl digestion, a 10 minute wash in denaturing solution (1.5 M NaCl, 0.5 M NaOH), a 10 minute wash in transfer buffer (1 M ammonium acetate, 20 mM NaOH), followed by blotting transfer for 12-18 hours.

Efficient transfer of CHEF gels required a somewhat modified protocol; 15 minute HCl digestion, 30 minute alkaline denaturing solution wash, 30 minute transfer buffer wash, followed by a blotting transfer for 3-4 days. Electroblotting (Anderson and Young, 1985) of CHEF gels was attempted, but proved inefficient. Attempts to conduct hybridization of nucleic acids directly within CHEF gels (unblots) (Tsao *et al.*, 1983) were also made, but the method proved unreliable.

2.3.3 Hybridization Techniques

Nylon membranes containing transferred DNA were baked at 80°C for 2-4 hours, and the DNA was cross-linked to the membrane by exposure to ultraviolet light using a Bio-Rad GS Gene Linker UV chamber following the manufacturer's suggested conditions for a dry Southern blot (Anderson and Young, 1985). Filters were prehybridized at an appropriate temperature (typically 60°C) in a heat-sealable plastic bag (Dazey Corp.) with a prehybridization solution containing 6 x SSPE [1 x SSPE is 0.15 M NaCl, 20 mM sodium phosphate (pH 7.0), and 2 mM EDTA], 5 x Denhardt's solution (Denhardt, 1966), 100 µg/ml denatured, sheared salmon sperm DNA (Sigma) for at least 1 hour. Variable concentrations (0, 10, 20, 30% vol/vol) of deionized formamide (Sigma) were incorporated into the prehybridization solution for certain experiments. After prehybridization the $\alpha[^{32}P]$-dATP labeled DNA probe was denatured by heating to 95°C for 5 minutes and added to the prehybridization solution. Hybridization was conducted for 3 to 18 hours. After hybridization, the filter was removed and washed twice with 4 l of 2 x SSPE at 50°C for 15 minutes, followed by a wash in 4 l of a 0.2 x SSPE, 0.1 x SDS solution for 15 minutes at 50°C. The filter was wrapped in Saran Wrap and exposed to Kodak XAR5 X-ray film with a Dupont Cronex Lightning Plus intensifying screen at -80°C for a variable period of time.

For probes produced by random primer techniques, hybridization conditions of 60°C and 20% formamide were typically used. For terminally labeled oligonucleotide probes, conditions of 45°C and no formamide were typical.

To reuse blots previously hybridized to different probes, the blot were placed in 4 litre volumes of 2 x SSPE, 0.1% SDS at 95°C for 5-15 minutes to remove the bound probe.

2.4 Electron Microscopy

Extracellular material was examined for the possible presence of viruses by Richard Sherburne using transmission electron

microscopy. Samples were negative stain preparations of the extracellular material isolated by PEG precipitation. Transmission electron microscopic examination of cytochrome-C coated DNA samples for the *S. lividans* plasmids pSL1A and pSL1B were also conducted by Richard Sherburne.

3 RESULTS:

3.1 Survey of Extrachromosomal Elements

A variety of DNA preparation and DNA gel electrophoresis techniques were used to survey the extrachromosomal element profile of the β-lactam producing *Streptomyces* strains *S. cattleya*, *S. clavuligerus*, *S. griseus*, *S. jumonjinensis*, and *S. lipmannii*. Figure 8 illustrates the DNA species revealed by fractionation of *in situ* PFG DNA preparations using CHEF techniques. Plasmid sizes were estimated by comparison with the known sizes of λ virus concatamers and *S. cerevisiae* chromosomes. In addition to the previously identified 11.7 kb pSCL plasmid, *S. clavuligerus* was found to contain two larger, previously unidentified plasmids, 120 and 430 kb in size. These new plasmids were designated pSCL2 and pSCL3, and the previously identified pSCL plasmid was renamed pSCL1. Giant linear plasmids were also observed in *S. griseus* (pSGL1, 120 kb) and *S. jumonjinensis* (pSJL3, 220 kb and pSJL4, 280 kb). No plasmids of any size were detected in *S. cattleya* and *S. lipmannii*. The apparent sizes of the plasmids observed remained consistent relative to the linear DNA molecular weight markers as the CHEF pulse conditions were altered (data not shown).

Figure 9 illustrates the DNA observed by conventional gel electrophoresis of both proteinase low mw and conventional phenol chloroform extraction DNA preparations of the organisms surveyed. Small linear plasmids were detected from *S. jumonjinensis* within the proteinase-K digested DNA preparations, but other than the previously identified pSCL1 plasmid, the other strains did not contain any additional extrachromosomal elements in the range of 1-50 kb. The larger plasmids identified in Figure 8 are not visible. As DNA with covalently attached proteins is not recovered by conventional phenol chloroform DNA preparations, the absence of a DNA species within the aqueous phases from conventional phenol chloroform preparations indicates that the plasmids seen in *S.*

Fig. 8. CHEF gel electrophoresis of whole cell *in situ* pulse field gel DNA preparations of a variety of β-lactam prod[illegible] *Streptomyces spp.* CHEF gel was run for 24 hours at 165 V, 5 to 80 second pulse ramp. Lanes one to ten: 1) λ ladder molecular weight marker, 2) *S. cerevisiae* chromosomes, 3) *S. cattleya*, 4) *S. clavuligerus*, 5) *S. griseus*, 6) *S. jumonjinensis* NRRL 5741, 7) *S. jumonjinensis* ATCC 29864, 8) *S. lipmannii*, 9) *S. lividans* 1326, 10) λ ladder molecular weight marker. Numbers to the side of the figures indicate molecular weight marker positions (size in kb), C indicates zone of compression.

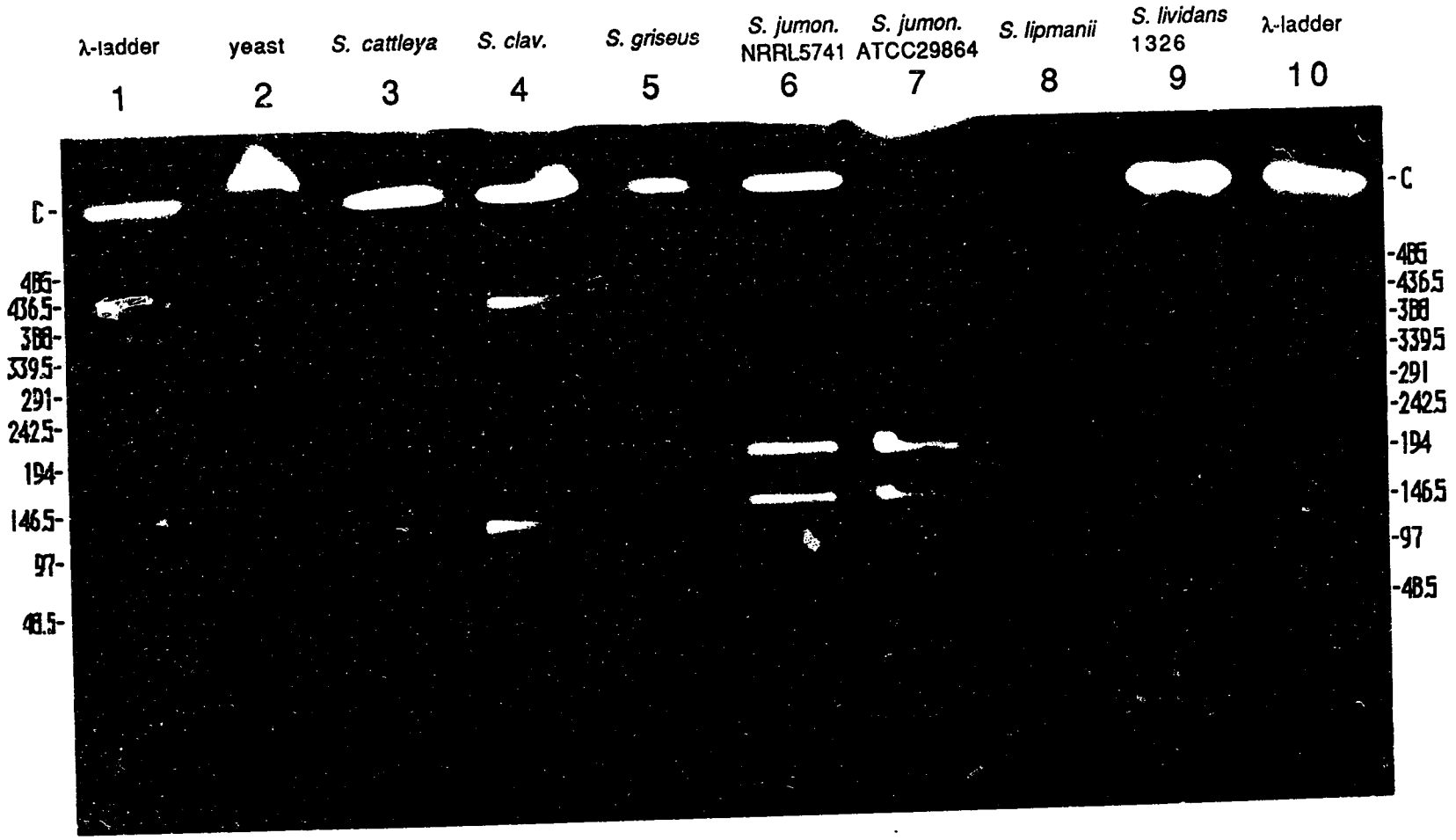
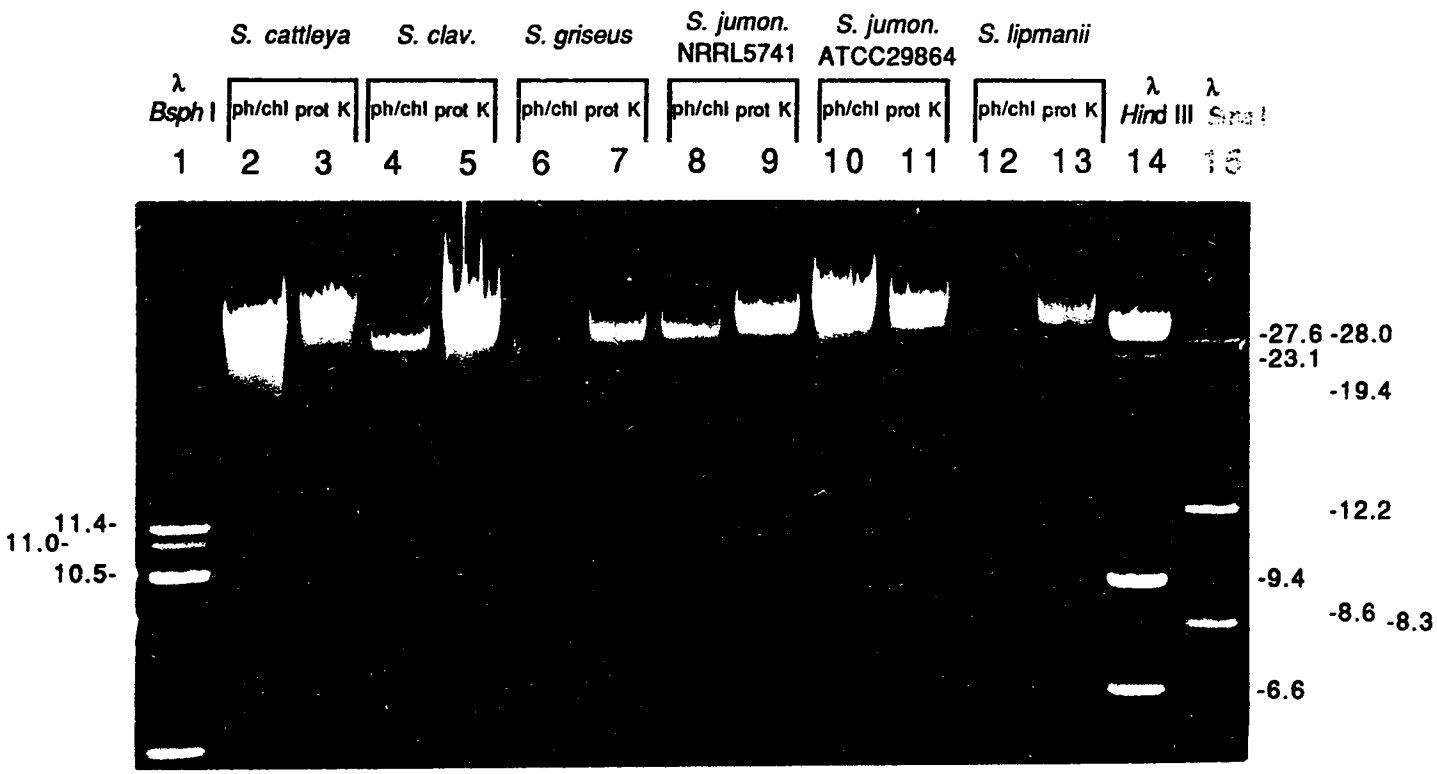

λ-ladder
yeast
S. cattleya
S. clav.
S. griseus
S. jumon. NRRL5741
S. jumon. ATCC29864
S. lipmanii
S. lividans 1326
λ-ladder
1
2
3
4
5
6
7
8
9
10
C-
486-
436.5-
388-
339.5-
291-
242.5-
194-
146.5-
97-
48.5-
-C
-486
-436.5
-388
-339.5
-291
-242.5
-194
-146.5
-97
-48.5

Fig 9. Conventional gel electrophoresis of whole cell DNA prepared from β-lactam producing *Streptomyces spp.* by conventional phenol chloroform and proteinase low mw methodologies. Marker lanes: 1) λ *BSph* I digest, 14) λ *Hind* III, and 15) λ *Sma* I digests. Sample lanes 2 to 13 are duplicates of conventional phenol chloroform preparations (even) and proteinase low mw preparation (odd) for: 2-3) *S. cattleya*, 4-5) *S. clavuligerus*, 6-7) *S. griseus*, 8-9) *S. jumonjinensis* NRRL 5741, 10-11) *S. jumonjinensis* ATCC 29864, and 12-13) *S. lipmannii.* Electrophoresis was within a 0.5% agarose gel in a 1 x TEA buffer at 1.2 V/cm for 16 hours followed by staining with ethidium bromide. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


S. cattleya
S. clav.
S. griseus
S. jumon. NRRL5741
S. jumon. ATCC29864
S. lipmanii
λ Bsph I
ph/chl prot K
λ Hind III
λ Sma I
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
11.4-
11.0-
10.5-
-27.6
-28.0
-23.1
-19.4
-12.2
-9.4
-8.6
-8.3
-6.6

clavuligerus and *S. jumonjinensis* are linear with covalently attached terminal proteins, and do not possess a conventional circular plasmid topology (Fig. 9). Table 3 summarizes the sizes and designations of the DNA bands identified either by pulsed field or conventional gel electrophoresis techniques. The unusual pSJX band, which is visible as a rapidly migrating band at the bottom of lanes 6 and 7 in Figure 8, and as a band with an apparent size 22 kb in Figure 9 is not considered to be a plasmid, and will be discussed in detail later.

Initial studies of *S. jumonjinensis* NRRL 5741 indicated the presence of four plasmids (12, 17.5, 225, 275 kb). A new culture isolate was ordered from ATCC (*S. jumonjinensis* ATCC 29864) following contamination of the original stock culture, and upon analysis it was found to contain a distinct plasmid profile different from the initial isolate (three plasmids; 12, 225, 275 kb) despite the fact that both the NRRL and ATCC cultures supposedly represented a single isolate. These two sets of plasmid appear to be maintained stably after repeated growth in liquid media and sporulation. These isolates are designated *S. jumonjinensis* NRRL 5741 and ATCC 29864 in the discussions that follow.

An unusual low intensity band of ethidium bromide stained material was also identified from *S. jumonjinensis* during these procedures. No size estimate is given for this unusual band due to the variable migration characteristics observed during electrophoresis within conventional agarose gels, but it appears to be approximately 15 kb in Figure 8, and 22 kb in Figure 9. The material was designated pSJX to indicate that in some respects it resembled a plasmid, though its exact nature was uncertain. Figure 10 illustrates this phenomenon, showing migration within 0.4, 0.5, 0.6, and 0.7% agarose gels. The pSJX bands are indicated by an arrow in all photographs, and are present in both the NRRL 5741 and ATCC 29864 strains, though the ATCC 29864 form consistently appears to be slightly larger. As a point of clarification in panel A of Figure 10, the pSJX band comigrates with pSJL2 of the *S. jumonjinensis* NRRL 5741 isolate. As the concentration of agarose

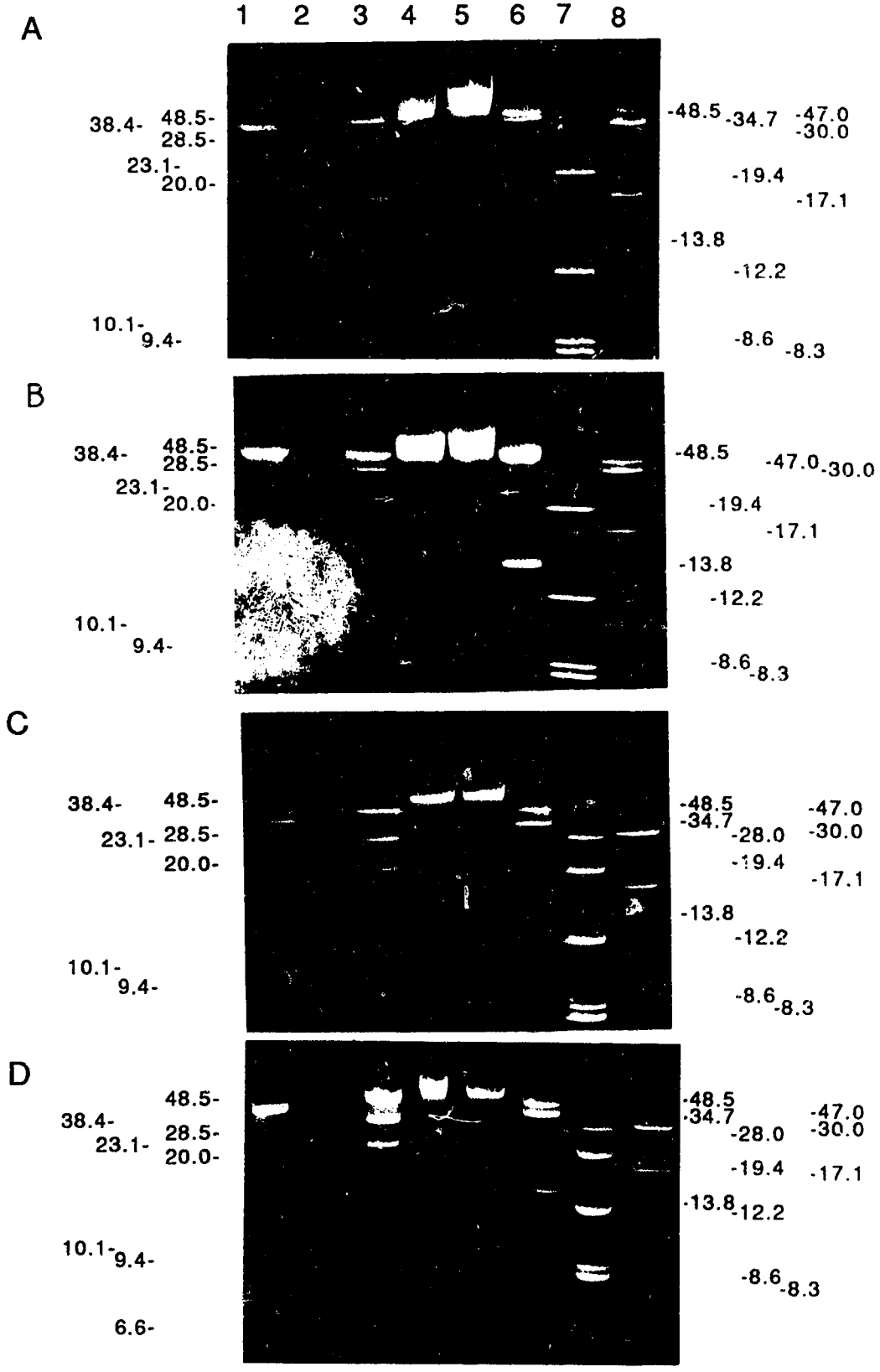
Table 3 Plasmids detected within the *Streptomyces* spp. surveyed

Organism	Plasmid name	Size *(kb)	Conformation
S. cattleya	nd		
S. clavuligerus NRRL 3585	pSCL1	11.7	linear
	pSCL2	120	linear
	pSCL3	430	linear
S. griseus NRRL 3851	pSGL1	120	linear
S. jumonjinensis NRRL 5741	pSJL1	11.7	linear
	pSJL2	17.5	linear
	pSJL3	220	linear
	pSJL4	280	linear
	pSJX	uncertain	uncertain
S. jumonjinensis ATCC 29864	pSJL1	11.7	linear
	pSJL3	220	linear
	pSJL4	280	linear
	pSJX	uncertain	uncertain
S. lipmannii NRRL 5741	nd		
S. lividans 1326	SLP2	50	linear
S. lividans TK19	pSL1A	6.3	non-linear
	pSL1B	5.0	non-linear
	pSL2	3	unknown
S. lividans TK24	nd		

nd = none detected

* Lengths based on comparison with linear standards

Fig. 10. Effect of varying concentrations of agarose upon the electrophoretic migration of the *S. jumonjinensis* pSJX band in conventional gels. The four photograph panels show the same samples electrophoresed within different concentrations of agarose (A=0.4% agarose, B=0.5% agarose, C=0.6% agarose, D=0.7% agarose). Lanes in all four panels represent identical samples: 1) λ *Apa* I digest, 2) λ *Hin*d III digest, 3) λ *Nae* I digest, 4) *S. jumonjinensis* NRRL 5741 proteinase K DNA preparation, 5) *S. jumonjinensis* ATCC 29864 proteinase K DNA preparation, 6) λ *Nhe* I digest, 7) λ *Sma* I digest, and 8) λ *Kpn* I digest, respectively. In all four panels wherever the pSJX band is visible in lanes 4 and 5 the pSJX band is indicated by a white arrowhead. Other bands visible in lanes 4 and 5 are the plasmids pSJL1 (11.7 kb) and pSJL2 (17.5 kb). Electrophoresis was on an agarose gel in a 1 x TEA buffer at 1.2 V/cm for 16 hours Gels were stained with ethidium bromide. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


A
1 2 3 4 5 6 7 8
38.4-
48.5-
28.5-
23.1-
20.0-
10.1-
9.4-
-48.5
-34.7
-47.0
-30.0
-19.4
-17.1
-13.8
-12.2
-8.6
-8.3
B
38.4-
48.5-
28.5-
23.1-
20.0-
10.1-
9.4-
-48.5
-47.0
-30.0
-19.4
-17.1
-13.8
-12.2
-8.6
-8.3
C
38.4-
48.5-
23.1-
28.5-
20.0-
10.1-
9.4-
-48.5
-34.7
-47.0
-28.0
-30.0
-19.4
-17.1
-13.8
-12.2
-8.6
-8.3
D
48.5-
38.4-
28.5-
23.1-
20.0-
10.1-
9.4-
6.6-
-48.5
-34.7
-47.0
-28.0
-30.0
-19.4
-17.1
-13.8
-12.2
-8.6
-8.3

increases, so does the apparent size of the pSJX band as estimated by comparison with molecular weight markers. In gels containing agarose concentrations of 0.7% or more, the pSJX band runs within the sheared chromosomal DNA. Attempts to introduce a radioactive label into β-agarase treated isolates of this band by random primer techniques proved unsuccessful, preventing any attempts to use this band as a radioactively labelled probe. None of the other radioactively-labeled DNA probes used during this project hybridized to this band.

A novel phenol-protein interface method for the isolation of covalently linked protein and DNA complexes has been developed within our lab by F. Behrend (Behrend *et al.*, 1995). The selective effect of this process is illustrated in Figure 11, in which the DNA content of the phenol interface extract and the corresponding aqueous phase of several cultures is compared. DNA isolated from both fractions was treated with proteinase K following isolation and contained plasmids previously identified using proteinase low mw DNA preparations (pSCL1, pSJL1, pSJL2). In addition to these previously identified plasmids, the TK19 strain of *S. lividans* was observed to contain two strong bands with apparent sizes of 6.3 and 5.0 kb, respectively designated pSL1A and pSL1B, and a very weakly staining band of 3 kb (pSL2) (Figure 14, lanes 5 and 10). None of these three *S. lividans* TK19 species were detected in conventional phenol chloroform and proteinase low mw digest DNA preparations of any *S. lividans* strains (data not shown).

The identical size of pSCL1 and pSJL1 (both *S. jumonjinensis* NRRL 5741 and ATCC 29864) suggested the possibility these plasmids might be identical. To minimize confusion and simplify further research, a number of restriction endonuclease digestions of sucrose gradient purified phenol-interface extract pSCL1 and pSJL1 samples were conducted to compare the profile of these three DNA samples. Figure 12 illustrates a representative set of digests, demonstrating that while the pSJL1 DNA isolated from the two *S. jumonjinensis* strains show identical profiles, their restriction endonuclease digestion products are distinct from those of pSCL1.

Fig. 11. Conventional gel electrophoresis comparison of whole cell DNA from *S. clavuligerus* and *S. jumonjinensis* extracted from the aqueous and phenol interface phases of a phenol extraction. Following extraction, but prior to electrophoresis, test samples were digested with proteinase K. Lanes: 1) λ *Hin*d III, 2) *S. jumonjinensis* NRRL 5741, aqueous phase, 3) *S. jumonjinensis* NRRL 5741, phenol-interface extract, 4) *S. clavuligerus*, aqueous phase, 5) *S. clavuligerus*, phenol-interface extract. Electrophoresis was within a 0.5% agarose gel in a 1 x TEA buffer at 1.5 V/cm for 8 hours followed by staining with ethidium bromide. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.

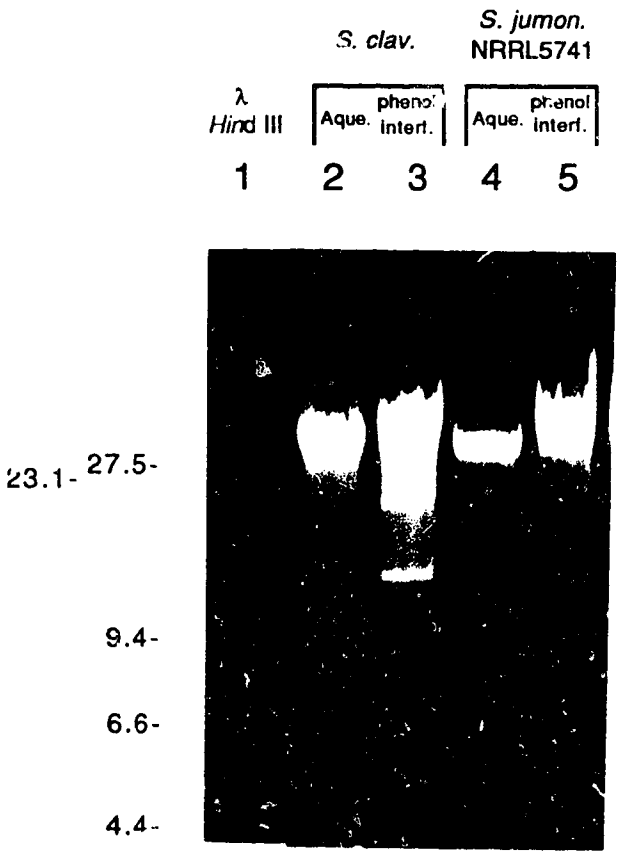
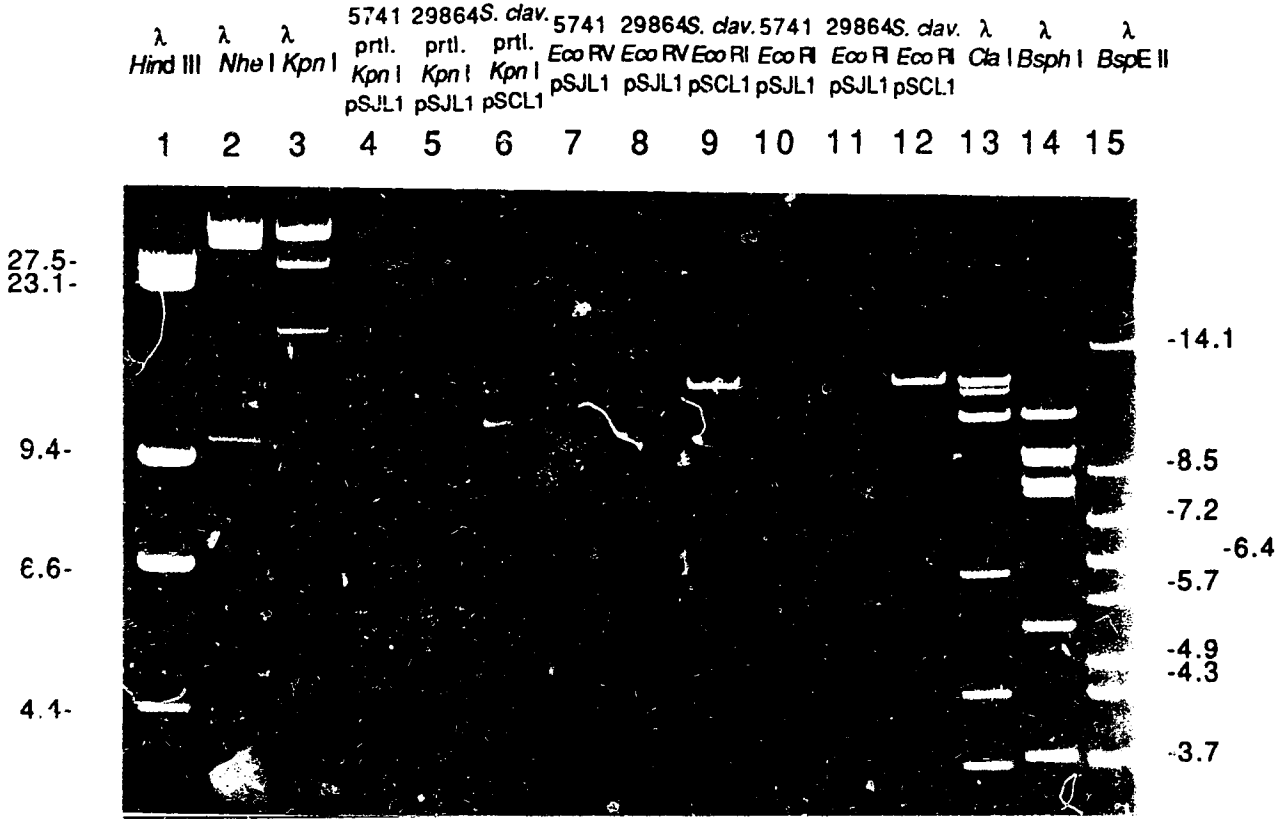

S. clav.
S. jumon. NRRL5741
λ Hind III
Aque.
phenol interf.
Aque.
phenol interf.
1 2 3 4 5
23.1-
27.5-
9.4-
6.6-
4.4-

Fig 12. Conventional gel electrophoresis of restriction endonuclease digests of purified phenol interface preparations of pSCL1 and pSJL1 (source strains NRRL 5741 and ATCC 29864) DNA. These samples were purified by sucrose gradient centrifugation and precipitation prior to restriction endonuclease digestion. Lanes: 1) λ *Hind* III, 2) λ *Nhe* I 3) λ *Kpn* I, 4) incomplete *Kpn* I digest of pSJL1 (*S. jumonjinensis* NRRL [illegible] incomplete *Kpn* I digest of pSJL1 (*S. jumonjinensis* ATCC 29864), 6) incomplete *Kpn* I digest of pSCL1 (*S. clavuligerus*), 7) *Eco* RV digest of pSJL1 (*S. jumonjinensis* NRRL 5741), 8) *Eco* RV digest of pSJL1 (*S. jumonjinensis* ATCC 29864), 9) *Eco* RV digest of pSCL1 (*S. clavuligerus*), 10) *Eco* RI digest of pSJL1 (*S. jumonjinensis* NRRL 5741), 11) *Eco* RI digest of pSJL1 (*S. jumonjinensis* ATCC 29864), 12) *Eco* RI digest of pSCL1 (*S. clavuligerus*), 13) λ *Cla* I, 14) λ *Bsp* HI, 15) λ *Bst* EIII. Electrophoresis was within a 0.5% agarose gel in a 1 x TEA buffer at 1.5 V/cm for 6 hours followed by staining with ethidium bromide. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


λ Hind III
λ Nhe I
λ Kpn I
5741 prtl. Kpn I pSJL1
29864 prtl. Kpn I pSJL1
S. clav. prtl. Kpn I pSCL1
5741 Eco RV pSJL1
29864 Eco RV pSJL1
S. clav. Eco RI pSCL1
5741 Eco RI pSJL1
29864 Eco RI pSJL1
S. clav. Eco RI pSCL1
λ Cla I
λ Bsph I
λ BspE II
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
27.5-
23.1-
9.4-
6.6-
4.4-
-14.1
-8.5
-7.2
-6.4
-5.7
-4.9
-4.3
-3.7

Further restriction endonuclease characterization and hybridization studies thus treated pSJL1 samples isolated from *S. jumonjinensis* NRRL 5741 and ATCC 29864 as identical but different from pSCL1.

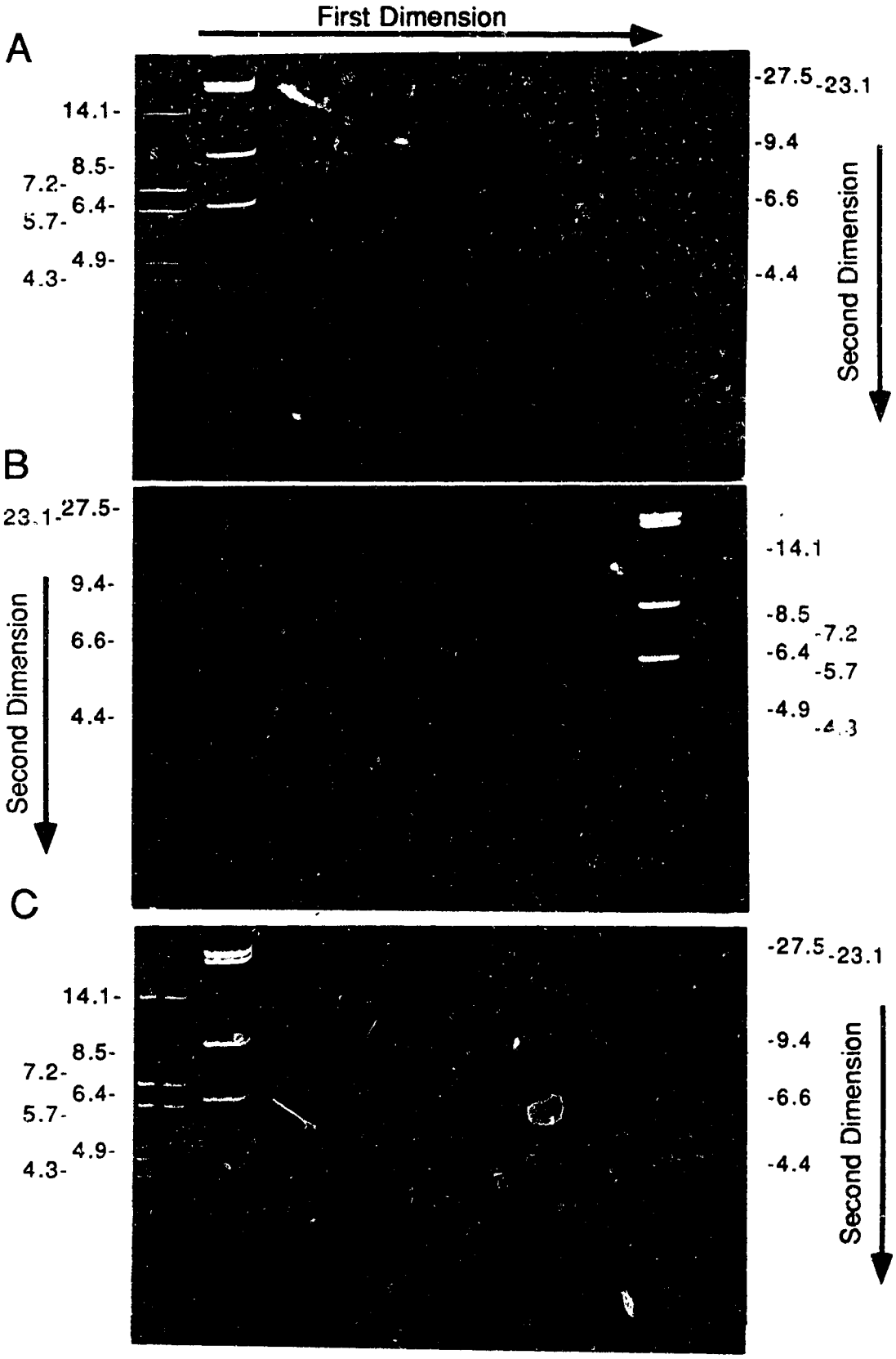
3.2 Possible Extracellular (Virus) Forms from *S. clavuligerus*, *S. griseus*, and *S. jumonjinensis*

The extrachromosomal DNA species detected during the preceeding experiments could represent either plasmids, or the prophage of previously undetected viruses. Several experiments were conducted to examine this possibility. Examination of negatively stained extracellular material in PEG precipitates by transmission electron microscopy failed to detect any viral particles. Agarose gel electrophoresis of proteinase-K treated extracellular material detected a smear of DNA, which did not contain any distinct ethidium bromide staining bands (data not shown). Agarose gel electrophoresis of restriction digestion fragments of proteinase-K treated extracellular extracts also failed to detect any distinct bands (data not shown).

3.3 Topology of pSJL1, pSJL2, pSL1A and pSL1B

The topological conformation of the smaller linear plasmids identified by the previous research was examined by two-dimensional agarose gel electrophoresis. Figure 13 depicts the profile observed after electrophoresis of proteinase low MW preparations of the two *S. jumonjinensis* strains NRRL 5741 and ATCC 29864 (pSJL1 and pSJL2) and the phenol-protein interface preparation from *S. lividans* TK19 (pSL1A and pSL1B). The plasmids pSJL1 and pSJL2 formed bright spots which migrated on the 'diagonal' of DNA, a pattern shared by pSCL1, demonstrating that their structure was not altered by exposure to UV irradiation, thus indicating that these plasmids are not topologically constrained but rather linear structures. However, each of the pSL1A and 1B bands was observed to form two spots per plasmid band, one located on the sheared linear DNA diagonal, and the second moving with significantly retarded migration. These slower moving spots

Fig. 13. Two-dimensional gel electrophoresis analyses of DNA species in *S. jumojinensis* NRRL 5741, *S. jumonjinensis* ATCC 29864, and *S. lividans* TK 19. Individual photograph panels depict: A) proteinase low mw *S. jumonjinensis* ATCC 29864 DNA, B) proteinase low mw *S. jumonjinensis* NRRL 5741 DNA, C) *S. lividans* phenol-protein interface DNA TK19. Samples were electrophoresed on a 0.4% agarose gel in 1 x TEA at 2.5 V/cm, stained with ethidium bromide and illuminated with UV light for 10 minutes. Following illumination, the gel and sample were re-oriented at right angles to the direction of the first run, and electrophoresed at 1.2 V/cm for 16 hours in a 0.7% agarose gel in 1 x TEA. Arrows indicate the direction of the first and second electrophoresis. The white arrowheads on panel C indicate the locations of the slower migrating spots created following UV irradiation. The numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


First Dimension
A
14.1-
8.5-
7.2-
6.4-
5.7-
4.9-
4.3-
-27.5
-23.1
-9.4
-6.6
-4.4
Second Dimension
B
23.1-
27.5-
9.4-
6.6-
4.4-
Second Dimension
-14.1
-8.5
-7.2
-6.4
-5.7
-4.9
-4.3
C
14.1-
8.5-
7.2-
6.4-
5.7-
4.9-
4.3-
-27.5
-23.1
-9.4
-6.6
-4.4
Second Dimension

migrated with sizes estimated at 15 kb for pSL1A, and 10 kb for pSL1B. The appearance of the slower migrating spots suggested that a new conformation had been produced by UV irradiation of the ethidium bromide stained bands, most likely the result of a conversion from a supercoiled structure to a relaxed circle or linear DNA structure which possessed a lower migration during conventional agarose gel electrophoresis. Attempts to use analogous techniques with CHEF gel samples proved unsuccessful, producing small DNA fragments, most likely breakdown products (data not shown).

3.4 Effects of Covalently Attached Terminal Proteins Upon Electrophoretic Mobility of Plasmids

The ability of the phenol interface protocol to isolate DNA with intact covalently attached proteins allowed an assessment of what effect these proteins have upon the electrophoretic migration of their attached DNA species. Figure 14 depicts electrophoresis of phenol interface extracts of *S. clavuligerus*, *S. jumonjinensis* (NRRL 5741 and ATCC 29864) and *S. lividans* TK19 after a two hour digestion with proteinase K for two hours or a two hour 1% SDS incubation. The effect of removing the terminal protein upon electrophoretic mobility is minimal, producing slightly sharper and more rapidly migrating bands.

3.5 Copy Number of pSCL1, pSJL1, and pSJL2

Given the approximate known size of the *Streptomyces* genome (8 Mb) and a plasmid's size, a comparison of the relative fluorescence of ethidium bromide stained chromosomal and plasmid DNAs should allow an estimation of the plasmid copy number. Figure 15 illustrates sets of serial dilutions of proteinase K low molecular weight whole cell DNA samples from *S. clavuligerus* and *S. jumonjinensis* NRRL 5741. Densitometric analysis of these lanes 3 to 5 in panel A indicated that pSCL1 DNA represents 3.5 to 4% of the cellular DNA as measured by DNA flourescence, giving copy number estimated to range between 24 and 27. Densitometric

Fig 14. Conventional gel electrophoresis of linear DNA molecules from various *Streptomyces* spp. as DNA or protein/DNA complexes. Lanes: 1) λ *Bst* E2, 2) λ *Hind* III 3) *S. clavuligerus* phenol interface extract, 4) proteinase-K digested *S. clavuligerus* phenol interface extract, 5) *S. jumonjinensis* ATCC 29864 phenol interface extract, 6) proteinase K digested *S. jumonjinensis* ATCC 29864 phenol interface extract, 7) *S. jumonjinensis* NRRL 5741 phenol interface extract, 8) proteinase-K *S. jumonjinensis* NRRL 5741 phenol interface extract, 9) *S. lividans* TK19 phenol interface extract, 10) proteinase-K digested *S. lividans* TK19 phenol interface extract. All samples were incubated for two hours in 1%SDS supplimented with proteinase K in even numbered samples. Electrophoresis was within a 0.4% agarose gel in a 1 x TEA buffer at 1.8 V/cm for 6 hours followed by staining with ethidium bromide. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.

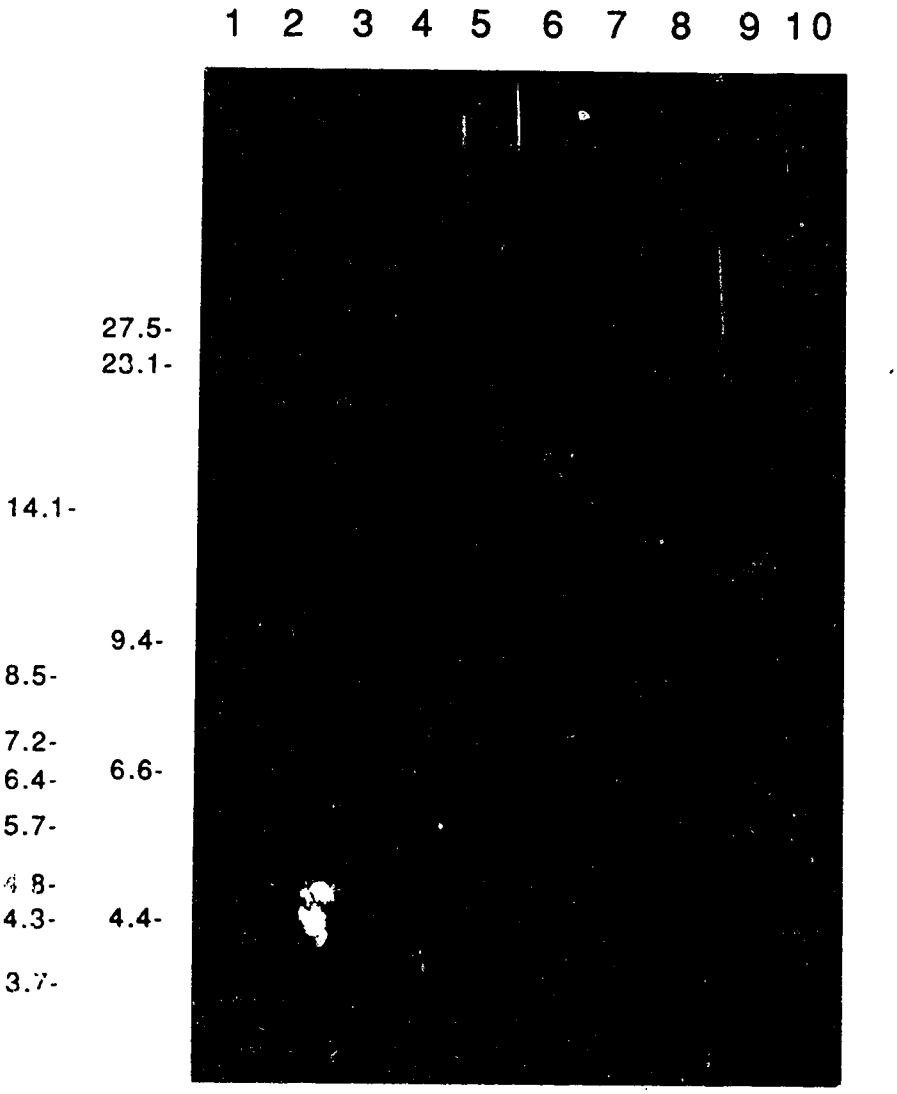
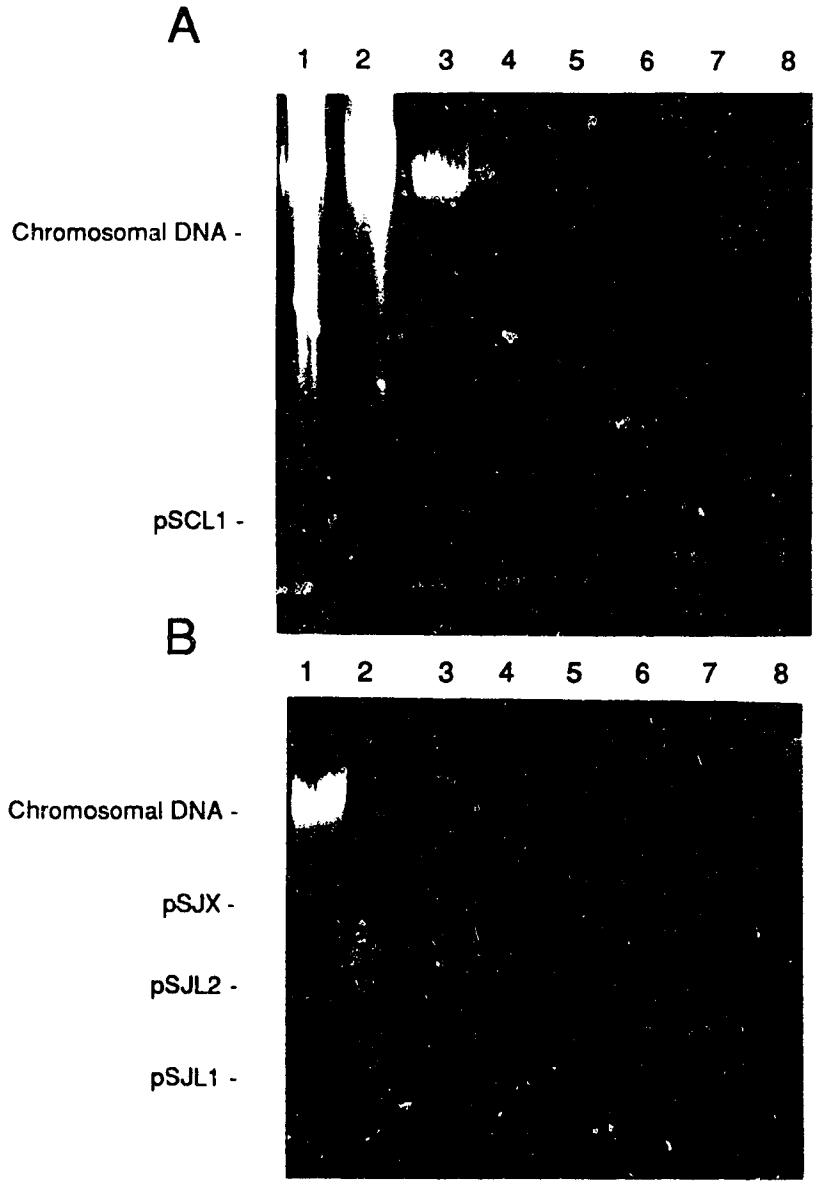

1 2 3 4 5 6 7 8 9 10
27.5-
23.1-
14.1-
9.4-
8.5-
7.2-
6.4-
6.6-
5.7-
4.8-
4.3-
4.4-
3.7-

Fig. 15. Copy number estimation of pSCL1 and pSJL1 by serial dilution of total cellular DNA samples. Panel A represents an agarose gel electrophoresogram of *S. clavuligerus* serial dilutions, panel B represents *S. jumonjinensis* ATCC 29864 dilutions. Lanes 1 to 8) 10 fold dilutions (1 to 10^7) sample DNA. Electrophoresis was within a 0.5% (panel A) or 0.7% (panel B) agarose gel in a 1 x TEA buffer at 1.8 V/cm for 6 hours followed by staining with ethidium bromide. Names beside the gels indicate the identity of the various bands.

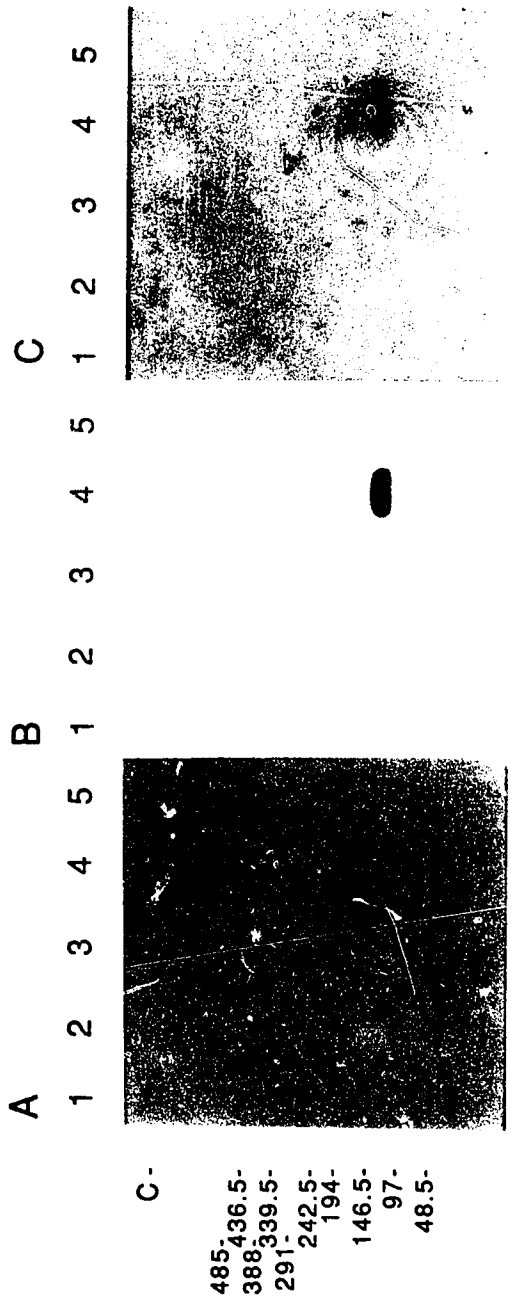

A
1 2 3 4 5 6 7 8
Chromosomal DNA -
pSCL1 -
B
1 2 3 4 5 6 7 8
Chromosomal DNA -
pSJX -
pSJL2 -
pSJL1 -

analysis of these lanes 1 to 3 in panel B indicated pSJL1 DNA represents 3.1 to 3.9% of the cellular DNA, giving a copy number estimated to range between 21 and 26, while pSJL2 DNA makes up 3.4 to 4.4% of the total cellular DNA, and has an estimated copy number of 15 to 20. The copy number of pSJL1 within *S. jumonjinensis* ATCC 29864 is similar to that observed within *S. jumonjinensis* NRRL 5741. The calculated ratio of pSJL1 to pSJL2 copy number ranged from 1.33 to 1.42. Given the reported syncytial nature of the *Streptomyces* spp., these copy numbers reflect the ratio of plasmids per chromosome.

3.6 Homology Between Linear Plasmids

The identification of previously undetected plasmids within the test species raised the question as to whether they shared any common sequences. Some homology was anticipated, as previous research (Hirochika *et al.*, 1984, Lin *et al.*, 1993, Wu and Roy, 1993) had indicated that some terminal sequences were shared among known *Streptomyces* plasmids. As well, the close taxonomic relationship between the species studied (*S. clavuligerus* and *S. jumonjinensis* example) and the similar sizes of many of the plasmids in this research suggested the possibility that some might have a common ancestor, or even provide evidence for to conducting homology studies among the , conditions for specific hybridization were examined. Figure 16 shows a 60°C hybridization of *in situ* PFG DNA preparations of *S. clavuligerus*, *S. griseus*, *S. jumonjinensis* NRRL 5741, and *S. lividans* 1326 with a pSJL3 ^{32}P random primer probe in hybridization solutions containing 10, 20, and 30% formamide. Specific hybridization was noted only to the positive control and demonstrated similar intensity under all three conditions. This result is representative of those observed using the same conditions with pSCL1, pSCL2, pSCL3, pSGL1, pSJL1, pSJL2, and pSJL4 probes (data not shown). Test conditions of 60°C and 20% formamide were used as the standard conditions when conducting random primer labeled radioactive probe hybridization

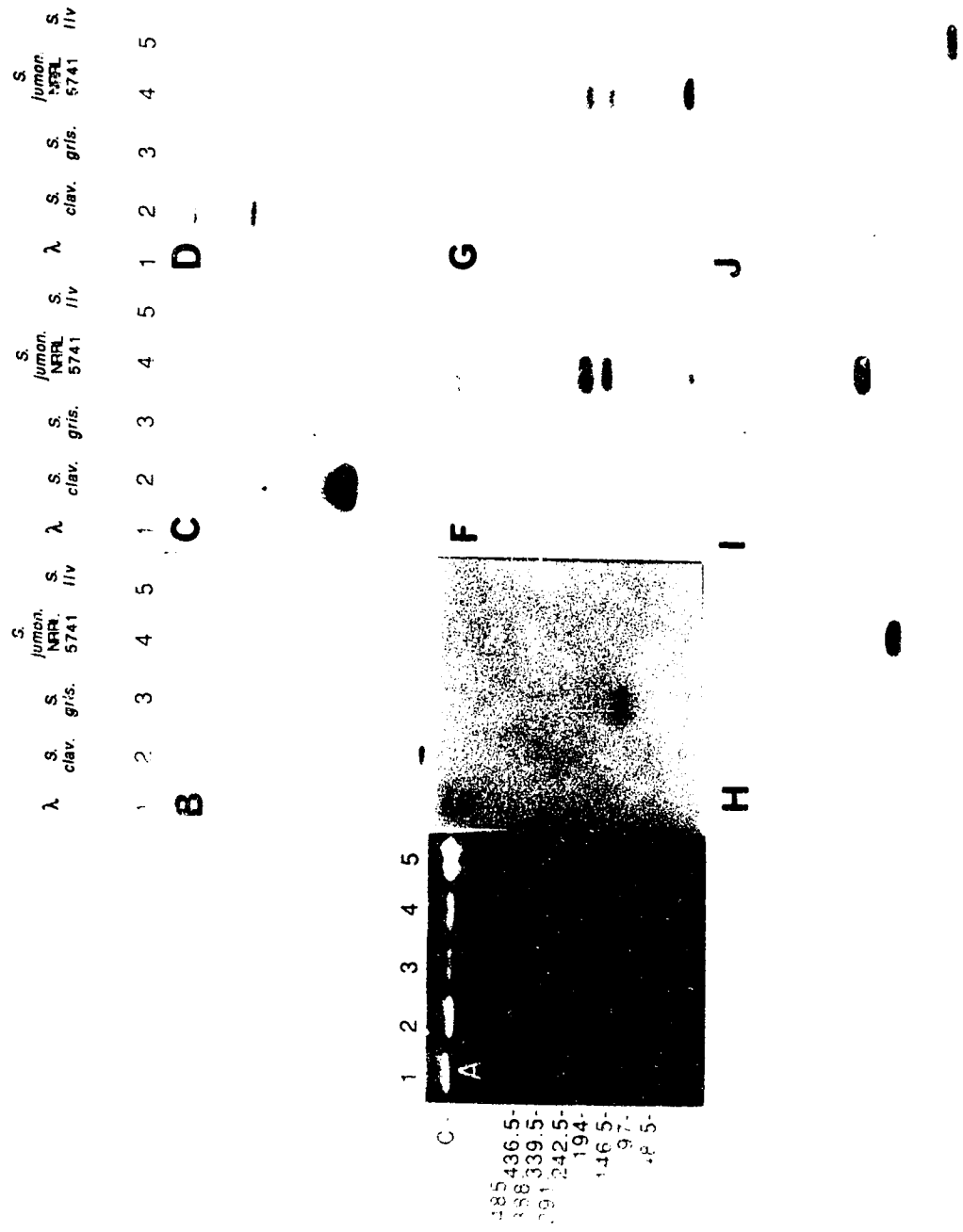
Fig. 16. Southern transfer and hybridization of pSJL3 under varying hybridization stringency conditions. Panels A, B, and C represent the gel in Figure 17, panel A (ethidium bromide stained CHEF gel run for 24 hours at 165 V, 5 to 80 second pulse, with the samples: 1) λ concatamer ladder molecular weight marker, 2) *S. clavuligerus*, 3) *S. griseus*, 4) *S. jumonjinensis* NRRL 5741, and 5) *S. lividans* 1326) transferred to nylon membrane, and probed by a pSJL3 probe under conditions of 60°C, and 10%, 20%, and 30% formamide, respectively. Scale and lane contents remain constant between the panels. Numbers to the side of the figures indicate molecular weight marker positions (size in kb). C indicates zone of compression.


A
B
C
1 2 3 4 5
C-
485-
436.5-
388-
339.5-
291-
242.5-
194-
146.5-
97-
48.5-

studies. When stringency was reduced by conducting hybridizations at 60°C or lower in the absence of formamide, non-specific hybridization was observed to all high G+C (*Streptomyces* spp.) DNA (data not shown).

Hybridizations were conducted using each of the linear plasmids detected in this survey as both target and probe DNA. Evaluation of homology between the giant linear plasmids of the *Streptomyces* spp. in this survey and all of the plasmids listed in Table 3 except pSL2 is shown in Fig. 17. The pSL1A and 1B plasmids from *S. lividans* TK19 are not present on the transferred gel as these plasmids were not detected within *in situ* lysis DNA preparations. As well, the results of hybridization studies using pSL1A, and pSL1B probes are not shown, as these probes did not hybridize to any part of the Southern blot. In almost all the remaining experiments, hybridization was observed only to the positive control; no cross hybridization was observed between plasmid probes and other plasmid species or chromosomal DNA with the following exceptions: pSCL3 hybridized to the sheared *S. clavuligerus* chromosomal DNA found in the zone of compression, and pSJL1 and pSJL2 probes hybridized to all of the *S. jumonjinensis* DNA bands but not to DNA from any other species. In both of these cases, the possibility existed that the probe DNA was contaminated with other cellular DNA species. The signal intensity from the pSCL3 and *S. clavuligerus* sheared chromosomal DNA bands are comparable to the intensity of their respective DNA bands, while the pSJL1 and pSJL2 ethidium bromide stained bands were weaker than their respective radioactive signals. In the case of pSCL3 the source of the β-agarase treated probe DNA appeared to be quite pure, as judged by agarose gel electrophoresis. The *S. clavuligerus* lane in Figure 8 was representative of the limited amount of sheared chromosomal DNA contamination typically seen within the samples. Thus, the hybridization seen between pSCL3 and the *S. clavuligerus* chromosomal DNA likely represents genuine homology. The pSJL1 and pSJL2 DNA samples used for probe production were purified from proteinase high mw DNA

Fig. 17. Southern transfer and hybridization analysis of total cellular DNA *in situ* preparations of *S. clavuligerus*, *S. griseus*, *S. jumonjinensis*, and *S. lividans* 1326 with linear plasmid probes. A. Ethidium bromide stained CHEF gel run for 24 hours at 165 V, 5 to 80 second pulse ramp, with the samples: 1) λ concatamer ladder molecular weight markers, 2) *S. clavuligerus*, 3) *S. griseus*, 4) *S. jumonjinensis* NRRL 5741, and 5) *S. lividans* 1326. Panels B to J represent autoradiographs of the gel shown in panel A after hybridizations with random primer labeled probes: B) pSCL1, C) pSCL2, D) pSCL3, E) pSGL1, F) pSJL1, G) pSJL2, H) pSJL3, I) pSJL4, J) SLP2. Scale and lane contents remain constant between panels. Numbers to the side of the figures indicate molecular weight marker positions (size in kb), C indicates zone of compression.

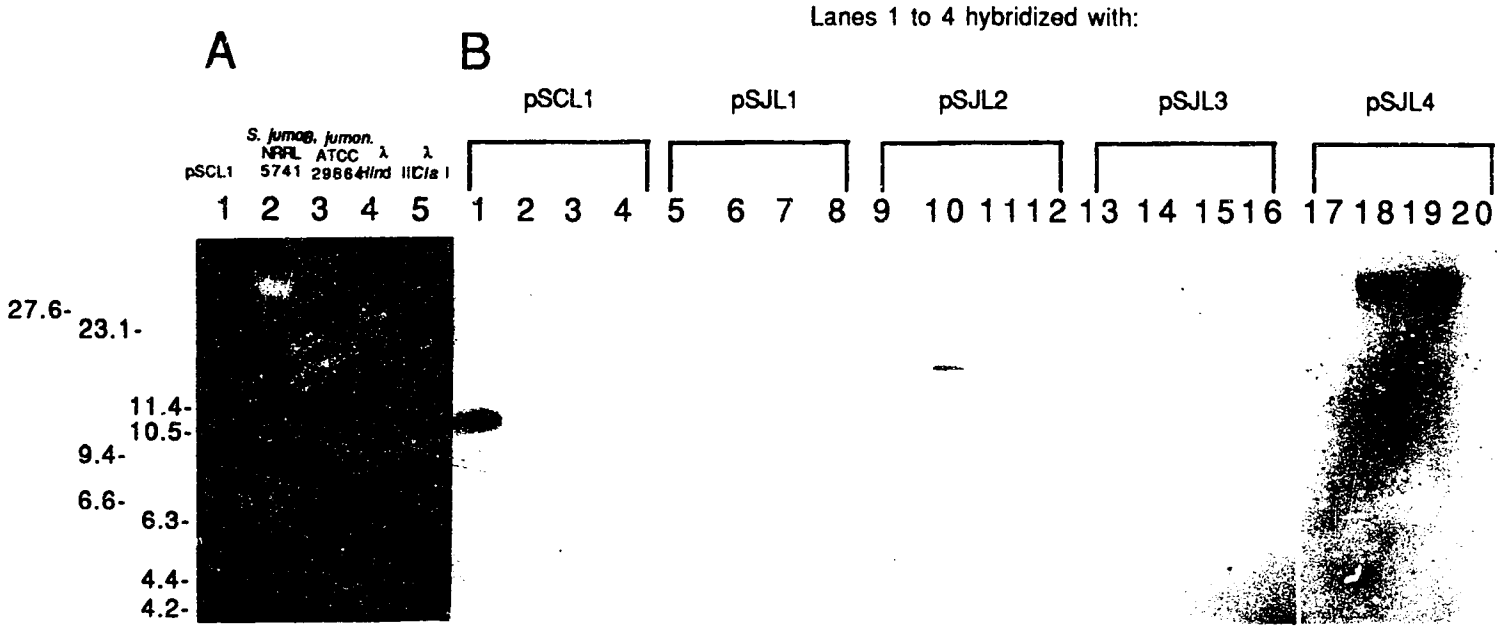

λ
S. clav.
S. gris.
S. lumon. NRRL 5741
S. liv
1
2
3
4
5
A
B
C
D
F
G
H
I
J
436.5-
339.5-
242.5-
194-
146.5-
97-

preparations by sucrose gradient centrifugation and agarose gel electrophoresis followed by agarase treatment of cut-out DNA bands from gels. These preparations were contaminated by noticeable quantities of sheared background DNA which could account for the generalized hybridization to all *S. jumonjinensis* DNA species, indicating these hybridizations do not represent genuine homology.

Although pSCL2 and pSGL1 are similar in size, the apparent lack of homologous sequences indicated that these plasmids were distinct and different. In contrast, the two giant linear plasmids pSJL3 and pSJL4 observed in both *S. jumonjinensis* strains NRRL 5741 and ATCC 29864 were found to cross hybridize to the identical sized plasmid in the other strain, confirming that these plasmids are likely to be identical (data not shown). In essence, the only difference between the two *S. jumonjinensis* strains appears to be the fact the ATCC 29864 strain does not contain the pSJL2 plasmid.

Comparisons of homology among the smaller linear plasmids detected in this study were also conducted by hybridization to Southern transfers of conventional agarose gels of *S. clavuligerus*, *S. jumonjinensis*, and *S. lividans* 1326 proteinase low mw DNA preparations. These studies of the smaller plasmids are shown in Figures 18 (pSCL1, pSJL1, pSJL2) and 19 (pSL1A, pSL1B). In Figure 18, the pSCL1, pSJL1, and pSJL2 probes were observed to hybridize only to their own plasmid bands. The pSJL3 and pSJL4 probes hybridized to the band of sheared chromosomal and giant linear plasmid DNA running near the top of the gel. The other probes (pSCL2, pSCL3, pSGL1, SLP2, pSL1A, pSL1B) were not observed to hybridize to any of the lanes on the blots, and hence are not included in Figure 18. Though pSCL1 and pSJL1 appear to have an identical size, these plasmids did not share homologous sequences, indicating that the common size is merely coincidental, and confirming the restriction endonuclease data of Figure 12. Furthermore, the 11.7 kb bands (pSJL1) in both *S. jumonjinensis* strains cross hybridized, once again indicating that these two bands

Fig. 18. Southern transfer and hybridization analysis of pSCL1 and proteinase low mw DNA preparations of *S. jumonjinensis* NRRL 5741 and ATCC 29864 with linear plasmid probes. A. Ethidium bromide stained conventional 1 x TEA 0.6% agarose gel run for 12 hours at 1.2 V/cm with the samples: 1) purified pSCL1 2) *S. jumonjinensis* NRRL 5741, and 3) *S. jumonjinensis* ATCC 29864, 4) λ *Hin*d III digest molecular weight marker, 5) λ *Cla* I digest molecular weight marker. Panel B represents autoradiographs of lanes 1 to 4 of the gel shown in Panel A after separate hybridizations with random primer labeled probes: 1 to 4) pSCL1, 5 to 8) pSJL1, 9 to 12) pSJL2, 13 to 16) pSJL3, 17 to 20) pSJL4. Scale and lane contents remain constant between panels. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


A
B
Lanes 1 to 4 hybridized with:
pSCL1
pSJL1
pSJL2
pSJL3
pSJL4
S. fumos. fumon.
pSCL1 NRRL 5741 ATCC 29864 λ Hind III λ Cla I
1 2 3 4 5 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
27.6-
23.1-
11.4-
10.5-
9.4-
6.6-
6.3-
4.4-
4.2-

were the same plasmid. The observation that the pSJL3 and 4 probes did not recognize the pSJL1 and pSJL2 bands strongly supports the hypothesis that the non-specific hybridization patterns observed with the pSJL1 and pSJL2 probes in Figure 17 were artifacts caused by contaminant DNA within the probe DNA.

In Figure 19, both pSL1A and pSL1B probes were observed to hybridize to each other as well as to self, while the SLP2 probe hybridized only to the sheared chromosomal DNA. Other probes (pSCL1, pSCL2, pSCL3, pSGL1, pSJL1, pSJL2, pSJL3, pSJL4) were not observed to hybridize to any *S. lividans* TK19 DNA (data not shown). Longer exposures of the pSL1A and pSL1B hybridization studies are depicted in Figure 19 and reveal a number of additional weaker bands, including one at the position of pSL2, and several of apparent sizes of approximately 8.5, 10, 12, 14, and 18 kb. The general background hybridization in panel D represents non-specific hybridization visible due to overexposure of the autoradiograph.. The pSL2 band is clearly detected via autoradiography, but was also visible as a very weak ethidium bromide stained band of 3 kb on gel photographs, while the larger weakly hybridizing bands were not successfully visualized by ethidium bromide staining. The hybridization of the SLP2 probe to the high molecular weight DNA from *S. lividans* TK19 may seem unexpected as the TK19 strain is believed to have been cured of the SLP2 plasmid. However, the fact that the *S. lividans* linear chromosome telomeres share sequences with the SLP2 plasmid (Lin *et al.*, 1993) likely accounts for this observation.

While the previous hybridization studies would appear to indicate that little, if any, homology exists between the linear plasmids detected in this study, a more detailed examination of telomeric homology was attempted by the construction of oligonucleotide probes containing telomeric sequences. An oligonucleotide was constructed to contain sequences conserved between pSCL1, pSLA2 (*S. rochei*, see pg. 38), and SLP2 terminal sequences (Figure 20), and used to probe a Southern transfer of an agarose gel of undigested and restriction endonuclease treated

Fig. 19. Southern transfer and hybridization analysis of phenol-protein interface DNA preparations of *S. lividans* TK19 with plasmid probes. A [illegible]dium bromide stained conventional 0.6% agarose gel [illegible] 12 hours at 1.2 V/cm with the samples: 1) λ *Bsp* HI digest molecular weight marker, 2) *S. lividans* TK19 phenol-protein interface DNA, 3) λ *Hin*d III digest molecular weight marker Panels B to D depict audoradiographs of duplicates of lanes 1 and 2 of the gel in panel A transferred to nylon membrane, and each probed individually with one of a number of plasmid probes. Lanes in Panels B to D: 1, 3, 5, 7) λ digest molecular weight markers, 2) *S. lividans* TK19 phenol-protein interface DNA probed with pSL1A probe, 4) *S. lividans* TK19 phenol-protein interface DNA probed with pSL1B probe, 6) *S. lividans* TK19 phenol-protein interface DNA probed with SLP2 probe. Lanes 2, 4, and 6 were hybridized separately, then combined for autoradiography for varying exposures: B) 15 minutes, C) 1 hour, D) 4 hours. Scale and lane contents remain constant between panels. Numbers to the side of panel A indicate molecular weight marker positions, sizes in kb.

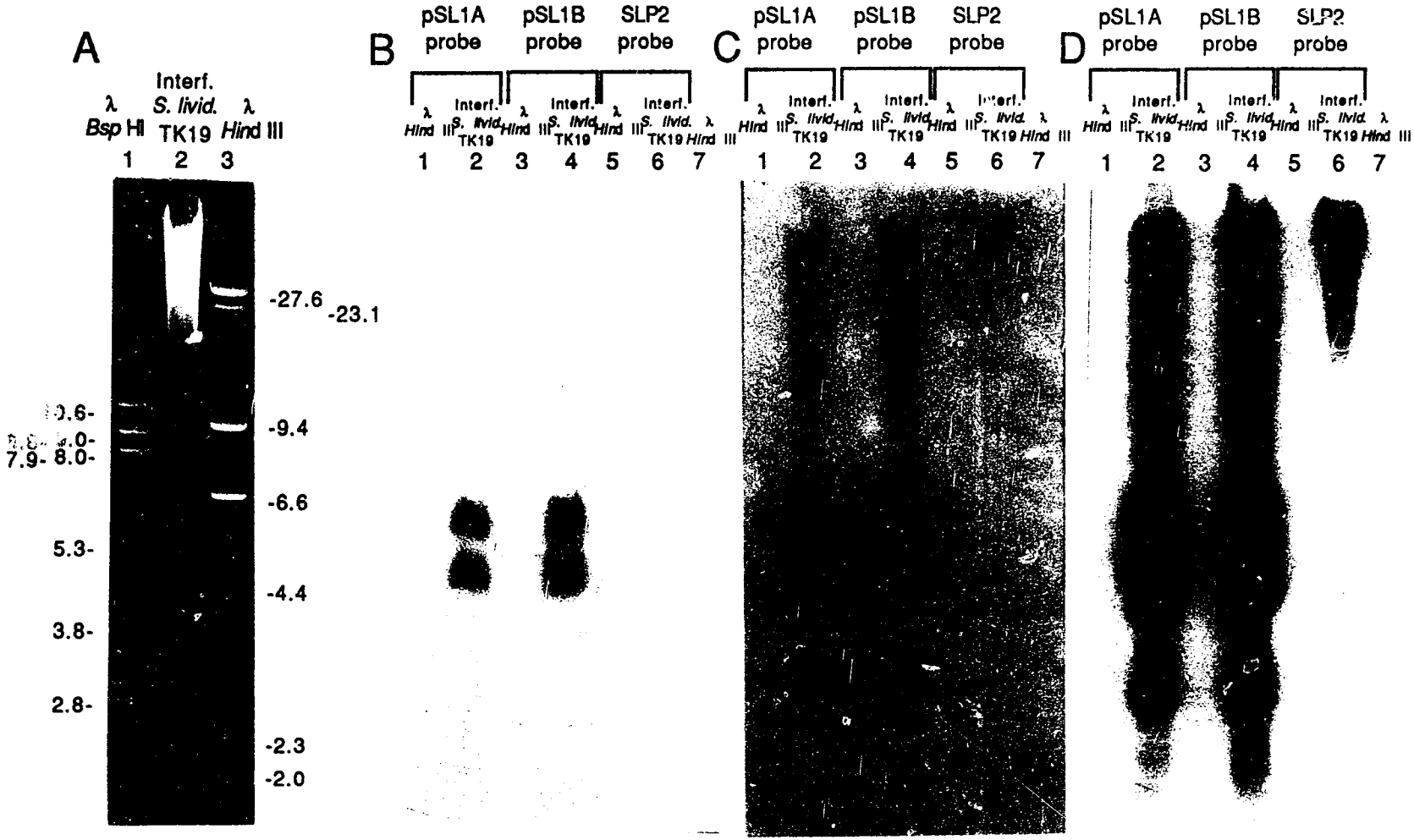

A
λ Bsp HI
Interf. S. livid. TK19
λ Hind III
1 2 3
-27.6
-23.1
-9.4
-6.6
-4.4
-2.3
-2.0
9.6-
8.0-
7.9-
5.3-
3.8-
2.8-
B
pSL1A probe
pSL1B probe
SLP2 probe
λ Hind III
Interf. S. livid. TK19
1 2 3 4 5 6 7
C
pSL1A probe
pSL1B probe
SLP2 probe
D
pSL1A probe
pSL1B probe
SLP2 probe

Fig. 20. Terminal inverted repeat oligonucleotide design. Numbers to the left and right of the pSCL1 sequences indicate the position of the terminal nucleotides within the gene sequence.

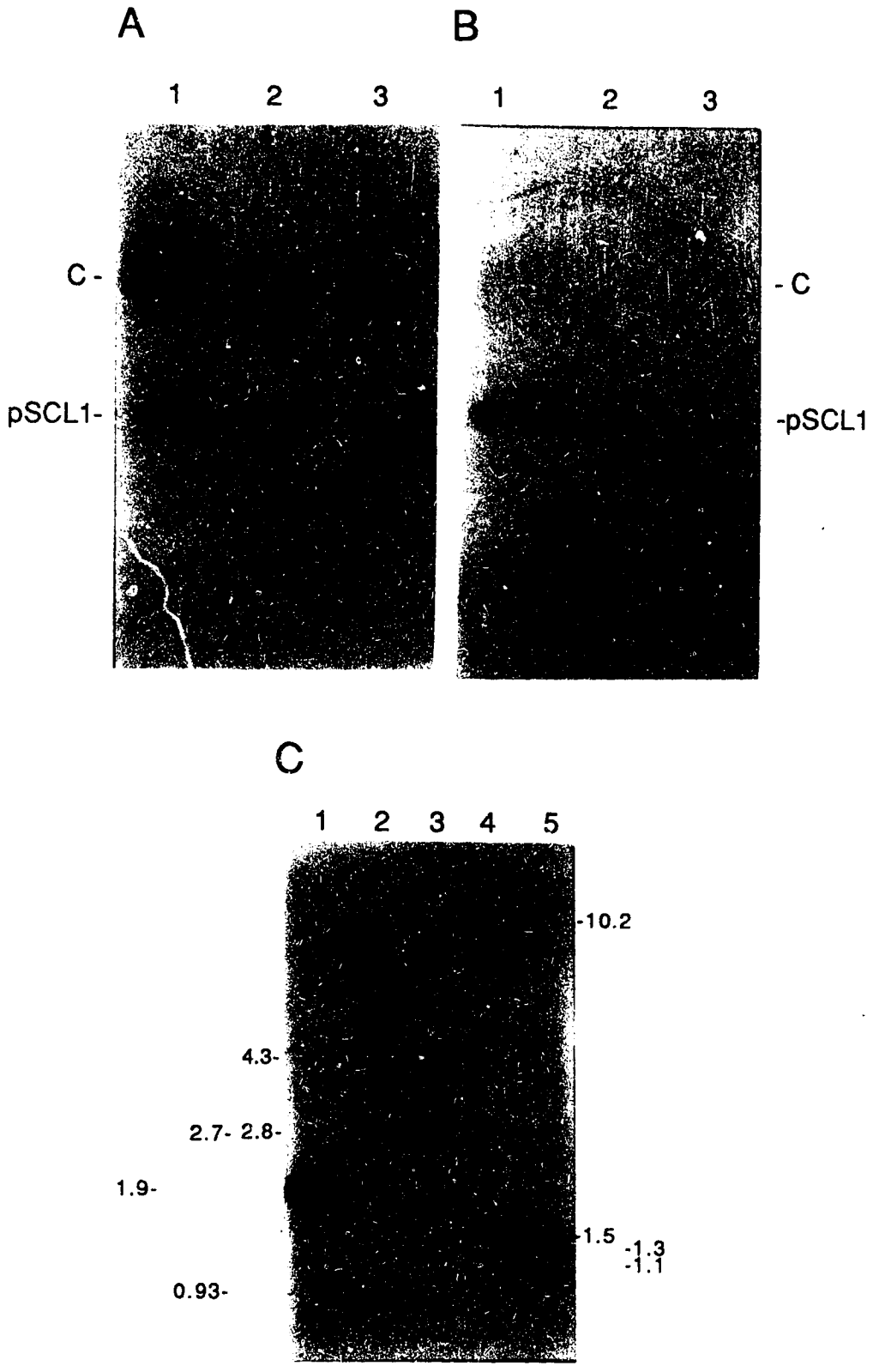
pSCL1 telomeric sequence

0 CCCGCGGAGCGGGTACCTAGGCGCTGCGCGCCTAGCGAGTGCATGCCCCCGCT. . . 54

GGAGCGGGTACCTAGGCGCTGCGCGCCTAGCGAGTGCATGCC

TIR oligonucleotide probe

Fig. 21. Southern transfer and hybridization analysis of proteinase low mw whole cell DNA preparations of *S. clavuligerus* with a linear plasmid terminal oligonucleotide probe. Panels A and B: Hybridization at 45°C of a Southern transfer of an 0.6% agarose gel (electrophoresed for 12 hours at 1.2 V/cm) of low mw DNA preparations of *S. clavuligerus* DNA with a telomeric sequence oligonucleotide (Fig. 20) probe: panel A, 20% formamide, panel B, 40% formamide. Lanes: 1) undiluted *S. clavuligerus* low mw whole cell DNA, 2) three fold dilution, and 3) nine fold dilution. C. Hybridization at 45°C of a Southern transfer of a 0.6% agarose gel (electrophoresed for 12 hours at 1.2 V/cm) of restriction endonuclease digested low mw DNA preparations of *S. clavuligerus* DNA with a telomeric sequence oligonucleotide (Fig. 20) probe: 1) *Sac* II, 2) *Sal* I, 3) *Pvu* I, 4) *Kpn* I, 5) *Bst* EII. The positions of pSCL1 and the sheared chromosomal and giant linear plasmid DNA is indicated by the pSCL1 and C labels to the sides of panels A and B. Numbers to the side of the panel C indicate molecular weight marker positions, sizes in kb.


A
B
1
2
3
1
2
3
C -
pSCL1-
- C
-pSCL1
C
1
2
3
4
5
-10.2
4.3-
2.7- 2.8-
1.9-
-1.5
-1.3
-1.1
0.93-

proteinase low mw *S. clavuligerus* DNA in an attempt to determine whether the termini of pSCL2, pSCL3 and a putative linear chromosome share these sequences (Figure 21). In Figure 21, panels A and B illustrate hybridizations of undigested *S. clavuligerus* DNA samples with the telomeric oligonucleotide probe at 45°C, 20% and 40% formamide, respectively. Hybridization to both pSCL1 and sheared chromosomal and giant linear plasmid DNA was observed in the 20% formamide hybridization, but, using a hybridization mix containing 40% formamide, hybridization to only pSCL1 was noted. A similar experiment using digests of whole *S. clavuligerus* DNA in a hybridization solution containing 20% formamide at 45°C is shown in panel C, and the only bands detected correspond to the digestion products of pSCL1, no additional bands are apparent. While hybridization did occur to the sheared chromosomal and GLP DNA at 45°C, 20% formamide, the failure of the same conditions to detect DNA bands other than those produced by digestion of pSCL1 indicates the hybridization observed in panel A was likely non-specific, and does not reflect hybridization to any homologous terminal sequences.

3.7 Location of the *pcbC* Gene

Except for *S. lividans*, all of the organisms in this survey have been previously identified as β-lactam antibiotic producing strains. Therefore it was of interest to examine the possibility that the antibiotic biosynthetic gene cluster might be located on a linear plasmid. While the penicillin and clavulanic acid antibiotic gene clusters of *S. clavuligerus*, and *S. jumonjinensis* have been determined to reside on a very large DNA fragment (Ward, and Hodgson, 1993), this cluster could be accommodated within either of the giant linear plasmids (pSCL2, pSCL3, pSJL3, pSJL4) identified by this survey. Less information was available concerning the *S. cattleya*, *S. griseus* and *S. lipmannii* antibiotic production clusters, again raising the possibility that their GLPs might encode these genes. Fig. 23 shows the result of a hybridization study in which the whole cellular DNA profile of these organisms, as separated by

Fig. 22. *pcbC* oligonucleotide probe design based on a consensus sequence among four sequenced *pcbC* genes. Numbers to the left and right of the aligned sequences indicate the position of the terminal nucleotides within the gene sequence.

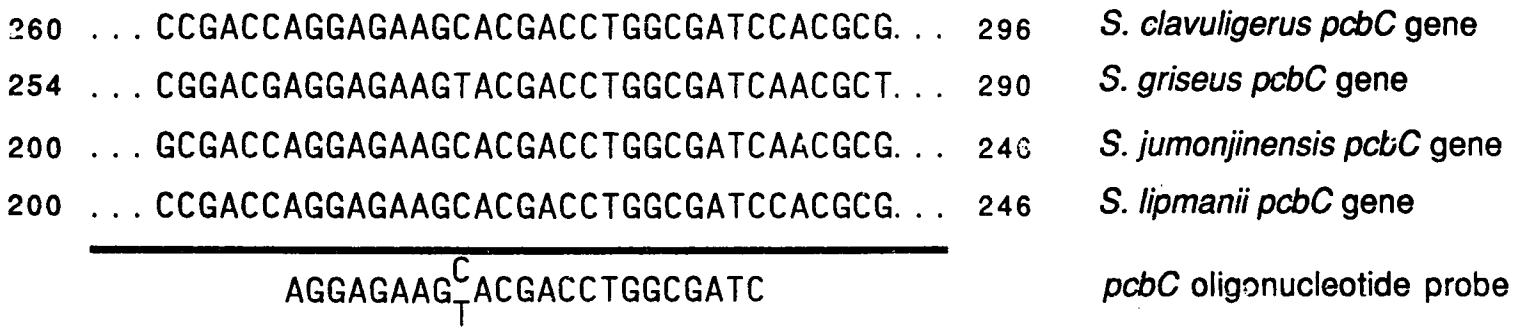
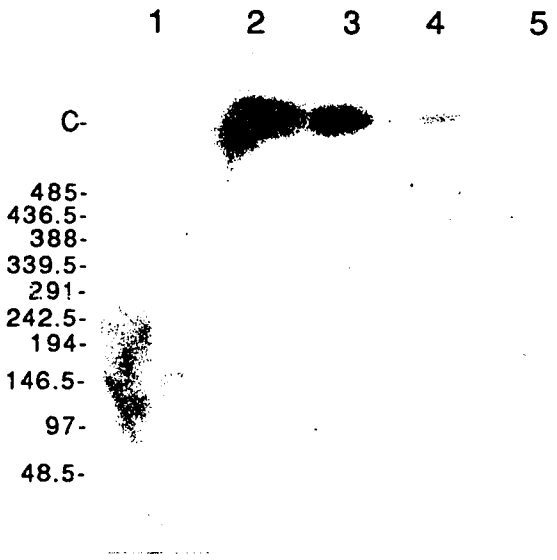

260 . . . CCGACCAGGAGAAGCACGACCTGGCGATCCACGCG. . . 296 S. clavuligerus pcbC gene
254 . . . CGGACGAGGAGAAGTACGACCTGGCGATCAACGCT. . . 290 S. griseus pcbC gene
200 . . . GCGACCAGGAGAAGCACGACCTGGCGATCAACGCG. . . 246 S. jumonjinensis pcbC gene
200 . . . CCGACCAGGAGAAGCACGACCTGGCGATCCACGCG. . . 246 S. lipmanii pcbC gene
AGGAGAAG C/T ACGACCTGGCGATC
pcbC oligonucleotide probe

Fig. 23. Southern transfer and hybridization analysis of whole cellular *in situ* DNA preparations of *S. clavuligerus*, *S. griseus*, and *S. jumonjinensis* with an oligonucleotide probe specific for the *pcbC* gene. The autoradiograph represents the CHEF gel in panel A, figure 17 [CHEF gel run for 24 hours at 165 V, 5 to 80 second pulse ramp, with the DNA samples: 1) λ concatamer ladder molecular weight markers, 2) *S. clavuligerus*, 3) *S. griseus*, 4) *S. jumonjinensis*, and 5) *S. lividans* 1326] transferred to nylon membrane, and probed by the *pcbC* oligonucleotide probe (Fig. 22) at 45°C, without formamide. Numbers to the side of the figures indicate molecular weight marker positions (size in kb), C indicates zone of compression.


1
2
3
4
5
C-
485-
436.5-
388-
339.5-
291-
242.5-
194-
146.5-
97-
48.5-

the CHEF technique, was probed with an oligonucleotide probe constructed to match a consensus sequence identified between the isopenicillin N synthase protein-encoding *pcbC* genes of *S. clavuligerus* (Leskiw *et al.*, 1988), *S. griseus* (Garcia-Dominguez *et al.*, 1991) *S. jumonjinensis* (Shiffman *et al.*, 1988) and *S. lipmannii* (Weigel *et al.*, 1988) (Fig. 22). In all cases, the sheared chromosomal DNA within the zone of compression hybridized with the probe; no signal was observed from any of the giant linear plasmids, indicating that the β-lactam biosynthetic gene clusters are located on the chromosome in these organisms.

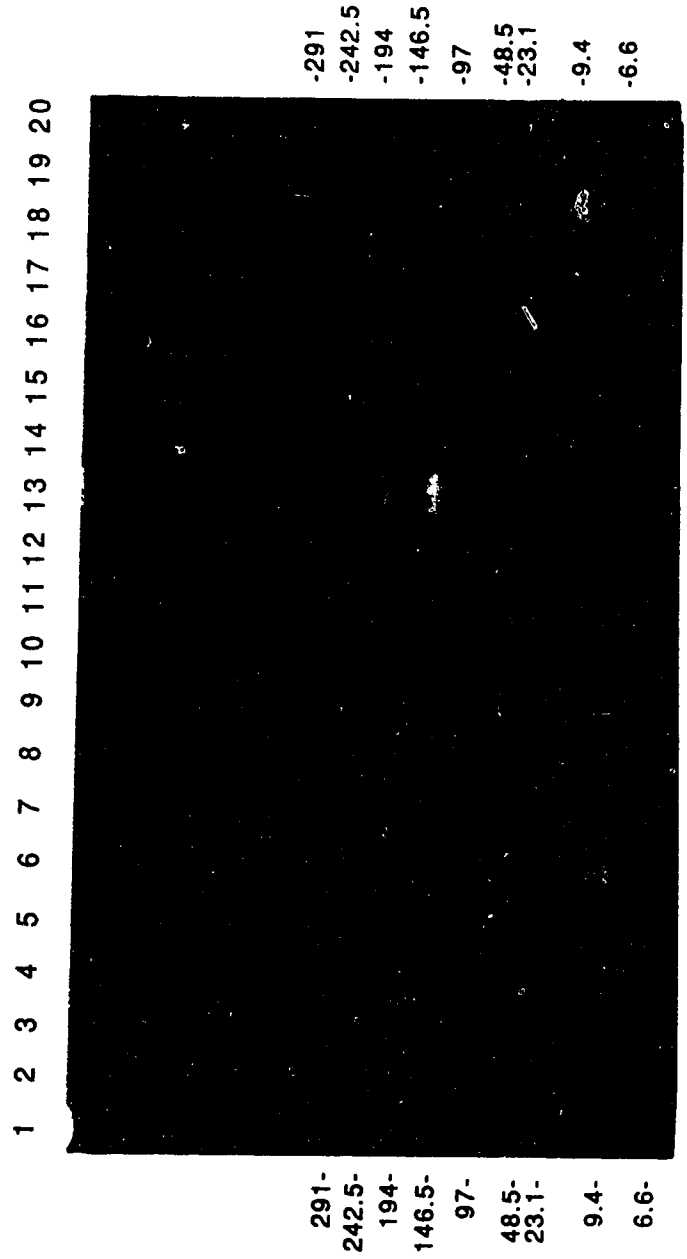
3.8 Purification of Linear Plasmid DNA by Sucrose Gradient Centrifugation

Restriction enzyme site mapping and other detailed analyses required some degree of separation and purification of the linear plasmids found in the various *Streptomyces* spp. surveyed. As ethidium bromide/CsCl gradients efficiently separate only topologically constrained molecules from sheared chromosomal DNA, another technique was required to isolate a more purified and concentrated sample of the linear plasmid DNA. Sucrose gradient separation of proteinase high mw DNA preparations was found to be an efficient method for the isolation of both the smaller linear plasmids, including pSCL1, pSJL1 and pSJL2, and the giant linear plasmids, such as pSCL2, pSJL3 and pSJL4. Figure 24 depicts a typical sucrose gradient separation of *S. clavuligerus* DNA. Purified pSCL1 and 2 are clearly visible as bands present in fractions 4 to 6 (lanes 5 to 7)and 11 to 13 (lanes 12 to 14), respectively. This technique proved inefficient for the recovery of pSCL3; it appears that this plasmid was too large to be recovered by this technique, likely due to mechanical shearing during the procedure.

3.9 Restriction Endonuclease Mapping of pSJL1 and pSJL2

Restriction analysis of the two small *S. jumonjinensis* plasmids pSJL1 and pSJL2 was conducted using single and double digests of material isolated by sucrose gradient fractionation of proteinase

Fig. 24. Sucrose gradient purification of linear plasmid DNA from a proteinase high mw DNA preparation of *S. clavuligerus*. Proteinase high mw *S. clavuligerus* DNA was fractionated on a 10 to 35% sucrose gradient by centrifugation at 18000 rpm for 48 hours in a SW40Ti rotor. Samples were precipitated for 12 hours, centrifuged, then the pelleted DNA was redissolved and electrophoresed in a CHEF gel at 165 V for 24 hours with a 5 to 80 second pulse ramp, using a 1.2% low melt agarose gel in 0.5 x TEB buffer and stained with ethidium bromide. Lanes 1 and 20 are a λ *Hin*d III + λ ladder molecular weight marker, lanes 2 to 18 correspond to sucrose gradient fractions, with lane 2 being the top of the gradient, lane 18 the bottom. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
-291
-242.5
-194
-146.5
-97
-48.5
-23.1
-9.4
-6.6
291-
242.5-
194-
146.5-
97-
48.5-
23.1-
9.4-
6.6-

high mw DNA preparations. Isolation of each plasmid independent of the other proved difficult, but fortunately the lack of homology between these two plasmids allowed the use of radioactive DNA probe hybridization to distinguish which DNA bands originated from each plasmid. The preliminary survey of restriction sites of pSJL1 plasmid determined that the restriction endonucleases *Bcl* I, *Hin*d III, *Hpa* I, *Kpn* I, *Nco* I, *Nde* I, *Nhe* I, *Pst* I, *Spe* I, *Xba* I, and *Xho* I did not produce any apparent digestion products. Figure 25 shows a number of single and double restriction endonuclease digestions of pSJL1. The sizes of the DNA fragments obtained by these digests are summarized in Table 4. Determination of the location of the restriction sites within the plasmid proved quite simple given the number of endonucleases which cut at only a single site. In particular, the terminal *Eco* RI site proved very useful, providing a reference point for locating other sites. Figure 26 is an illustration showing the size and location of restriction fragments produced by the digestions in Figure 25 in comparison to the map of all identified restriction sites on pSJL1. As a note, the pattern of restriction enzymes within the double digests is interrupted by the unintentional addition of *Eco* RI to the final digestion; within Figure 26 the site and fragments produced by this enzyme are shaded. In addition, the restriction enzymes *Bam* HI, *Cla* I, *Sac* I, *Sal* I and *Sca* I were tested and found to cut more frequently than proved useful for restriction mapping (data not shown) and were hence excluded from the double digest experiments.

Restriction analysis of pSJL2 followed a similar path. Figure 27 shows single digests using restriction enzymes identified as producing a number of easily analyzed fragments during the preliminary survey. Figure 28 shows a number of double digests using the same enzymes as in Figure 29. The data from these digests is summarized in Table 5. The enzymes *Bam* HI, *Bcl* I, *Bst* BI, *Hin*d III, *Hpa* I, *Kpn* I, *Nde* I, *Nhe* I, *Pst* I, *Pvu* I, *Pvu* II, *Sal* I, *Sca* I, *Sph* I, *Xba* I, and *Xho* I were not found to digest pSJL2. The single cuts produced by *Eco* RV and *Spe* I allowed the restriction sites to

Fig. 25. Southern transfer of restriction endonuclease digests of purified pSJL1 and pSJL2 probed with pSJL1. A. Sucrose gradient purified pSJL1 and pSJL2 DNA (enriched in pSJL1) digested by a variety of restriction enzymes and electrophoresed on an ethidium bromide stained conventional 0.7% agarose gel for 12 hours at 1.2 V/cm. Marker lanes: 1) λ *Bsp* HI, 18) λ *Cla* I. Sample lanes: 2) undigested, 3) *Bgl* II, 4) *Cla* I, 5) *Eco* RI, 6) *Eco* RV, 7) *Nru* I, 8) *Bgl* II + *Cla* I, 9) *Bgl* II + *Eco* RI, 10) *Bgl* II + *Eco* RV, 11) *Bgl* II + *Nru* I, 12) *Cla* I + *Eco* RI, 13) *Cla* I + *Eco* RV, 14) *Cla* I + *Nru* I, 15) *Eco* RI + *Eco* RV, 16) *Eco* RI + *Nru* I, 17) *Eco* RI + *Eco* RV + *Nru* I. Panels B, C and D represents 3 hour, 12 hour, and 36 hour exposures of the gel in panel A transferred to nylon membrane, and probed with a pSJL1 random primer probe under conditions of 60°C and 20% formamide. Scale and lane contents remain constant between the panels. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.

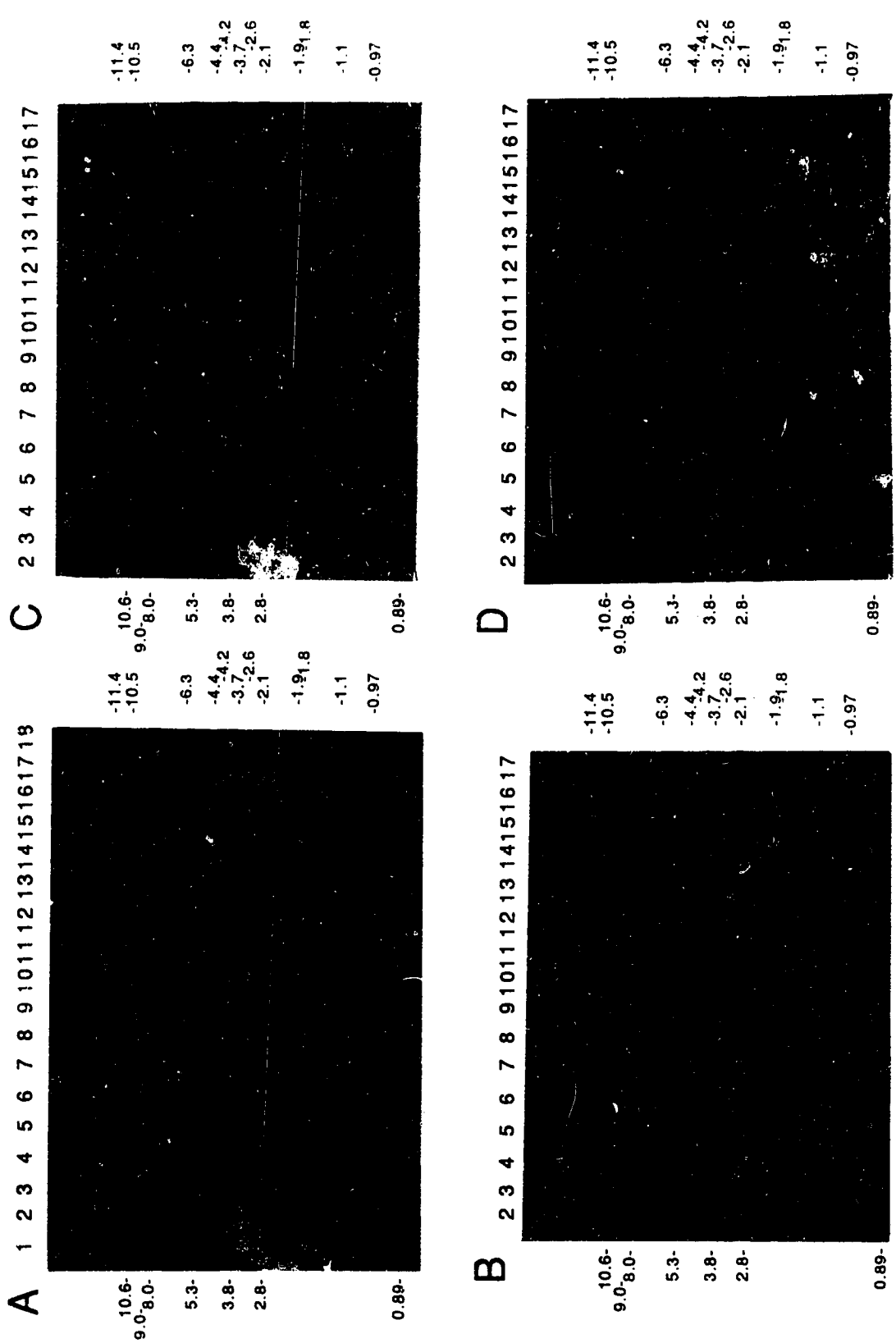

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
10.6-
9.0-
8.0-
5.3-
3.8-
2.8-
0.89-
-11.4
-10.5
-6.3
-4.4
-4.2
-3.7
-2.6
-2.1
-1.9
-1.8
-1.1
-0.97
C
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
10.6-
9.0-
8.0-
5.3-
3.8-
2.8-
0.89-
-11.4
-10.5
-6.3
-4.4
-4.2
-3.7
-2.6
-2.1
-1.9
-1.8
-1.1
-0.97
B
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
10.6-
9.0-
8.0-
5.3-
3.8-
2.8-
0.89-
-11.4
-10.5
-6.3
-4.4
-4.2
-3.7
-2.6
-2.1
-1.9
-1.8
-1.1
-0.97
D
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
10.6-
9.0-
8.0-
5.3-
3.8-
2.8-
0.89-
-11.4
-10.5
-6.3
-4.4
-4.2
-3.7
-2.6
-2.1
-1.9
-1.8
-1.1
-0.97

Table 4. Summary of restriction fragment sizes produced by digestion of pSJL1

Restriction Enzyme(s)	Number of Observed Fragments	Size of Fragments Observed (kb)
Bgl II	2	7.0, 4.8
Cla I	3	5.8, 4.8, 1.0
Eco RI	2	11.0, 0.8
Eco RV	2	6.6, 5.3
Nru I	3	7.1, 3.1, 1.7
Bgl II + *Cla* I	3	5.8, 4.8 1.0
Bgl II + *Eco* RI	3	7.0, 4.0, 0.8
Bgl II + *Eco* RV	3 (1 x doublet)	(5.1-4.9) x 2, 1.5
Bgl II + *Nru* I	3 (1 x doublet)	3.7, (3.2-3.1) x 2, 1.7
Cla I + *Eco* RI	4	5.8, 4.0, 1.0, 0.8
Cla I + *Eco* RV	3 (1 x doublet)	(5.0-4.8) x 2, 1.0
Cla I + *Nru* I	5 (1 x doublet)	(3.2-3.1) x 2, 2.6, 1.7, 1.0
Eco RI + *Eco* RV	3	5.9, 5.1, 0.8
Eco RI + *Nru* I	4	7.1, 3.1, 1.0, 0.8
Eco RI +*Eco* RV + *Nru* I	5	4.8, 3.1, 2.2,1.0, 0.8

Note: small fragments which were electrophoresed through the gel and thus were not directly observed are not included in this table.

Fig 26. Restriction map of pSJL1. Size and location of restriction fragments produced by the digestions in figure 25 as shown in comparison to the map of all known pSJL1 restriction endonuclease digestion sites. Numbers indicate fragment sizes in kb. The shaded information in the *Eco* RI, *Eco* RV and *Nru* I line designates the digestion products and sites of accidentally added *Eco* RI.

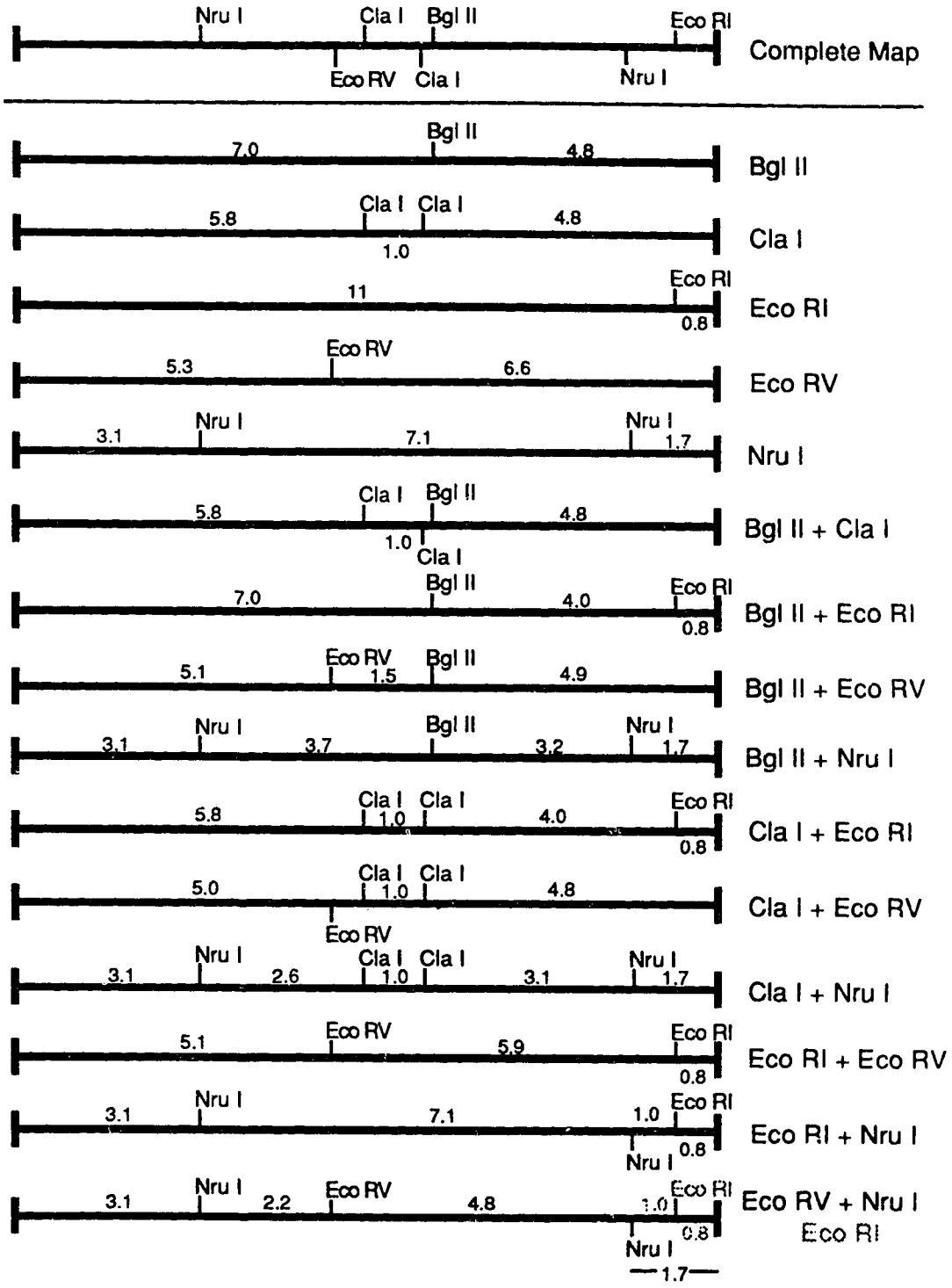

Nru I
Cla I
Bgl II
Eco RI
Eco RV
Cla I
Nru I
Complete Map
Bgl II
7.0
4.8
Bgl II
Cla I
Cla I
5.8
4.8
1.0
Cla I
Eco RI
11
0.8
Eco RI
Eco RV
5.3
6.6
Eco RV
Nru I
Nru I
3.1
7.1
1.7
Nru I
Cla I
Bgl II
5.8
4.8
1.0
Cla I
Bgl II + Cla I
Bgl II
Eco RI
7.0
4.0
0.8
Bgl II + Eco RI
Eco RV
Bgl II
5.1
1.5
4.9
Bgl II + Eco RV
Nru I
Bgl II
Nru I
3.1
3.7
3.2
1.7
Bgl II + Nru I
Cla I
Cla I
Eco RI
5.8
1.0
4.0
0.8
Cla I + Eco RI
Cla I
Cla I
5.0
1.0
4.8
Eco RV
Cla I + Eco RV
Nru I
Cla I
Cla I
Nru I
3.1
2.6
1.0
3.1
1.7
Cla I + Nru I
Eco RV
Eco RI
5.1
5.9
0.8
Eco RI + Eco RV
Nru I
Eco RI
3.1
7.1
1.0
0.8
Nru I
Eco RI + Nru I
Nru I
Eco RV
Eco RI
3.1
2.2
4.8
1.0
0.8
Nru I
Eco RV + Nru I
Eco RI
1.7

Fig. 27. Southern transfer of single restriction endonuclease digested preparations of purified pSJL1 and pSJL2 probed with pSJL2. A. Sucrose gradient purified pSJL1 and pSJL2 DNA digested by restriction enzymes, electrophoresed on an conventional 0.7% agarose gel for 12 hours at 1.2 V/cm, transferred to nylon membrane, and probed by a pSJL2 random primer labelled probe under conditions of 60°C and 20% formamide. Lanes: 1 and 4) undigested samples, 2) *Spe* I digest, 3) *Nru* I digest. B. Sucrose gradient purified pSJL1 and pSJL2 DNA digested by restriction enzymes, electrophoresed on an conventional 0.8% agarose gel for 10 hours at 1.5 V/cm transferred to nylon membrane, and probed by a pSJL2 random primer labelled probe under conditions of 60°C and 20% formamide. Lanes: 1) undigested samples, 2) *Eco* RV digest, 3) *Eco* RI digest. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.

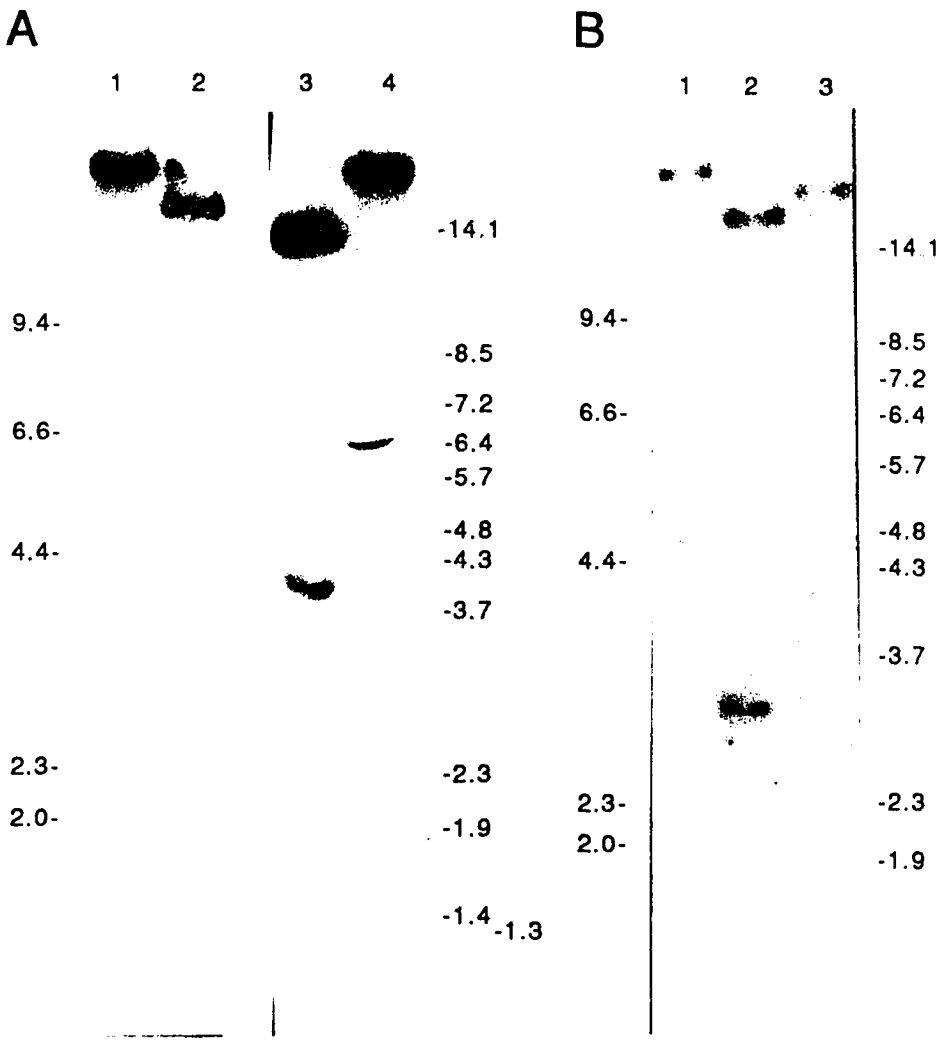

A
1
2
3
4
-14.1
9.4-
-8.5
-7.2
6.6-
-6.4
-5.7
-4.8
4.4-
-4.3
-3.7
2.3-
-2.3
2.0-
-1.9
-1.4
-1.3
B
1
2
3
-14.1
9.4-
-8.5
-7.2
6.6-
-6.4
-5.7
-4.8
4.4-
-4.3
-3.7
2.3-
-2.3
2.0-
-1.9

Fig. 28. Southern transfer of double restriction endonuclease digested preparations of purified pSJL1 and pSJL2 probed with pSJL2. A and D. Molecular weight marker lanes: A1) λ *Bst* EII A2), λ *Cla* I, D1) λ *Hin*d III, D2) λ *Apa* I, D3) λ *Nae* I, D4) λ *Nhe* I. B. Sucrose gradient purified pSJL1 and pSJL2 DNA digested by a variety of restriction enzymes and electrophoresed on an ethidium bromide stained conventional 0.7% agarose gel for 10 hours at 1.4 V/cm. Lanes: 1) undigested, 2) *Eco* RI + *Eco* RV, 3) *Eco* RI + *Nru* I, 4) *Eco* RI + *Spe* I, 5) *Eco* RV + *Nru* I, 6) *Eco* RV + *Spe* I. Panel C represents the gel in panel B transferred to nylon membrane, and hybridized with a pSJL2 random primer labelled probe under conditions of 60°C and 20% formamide. Scale and lane contents remain constant between panels B and C. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.

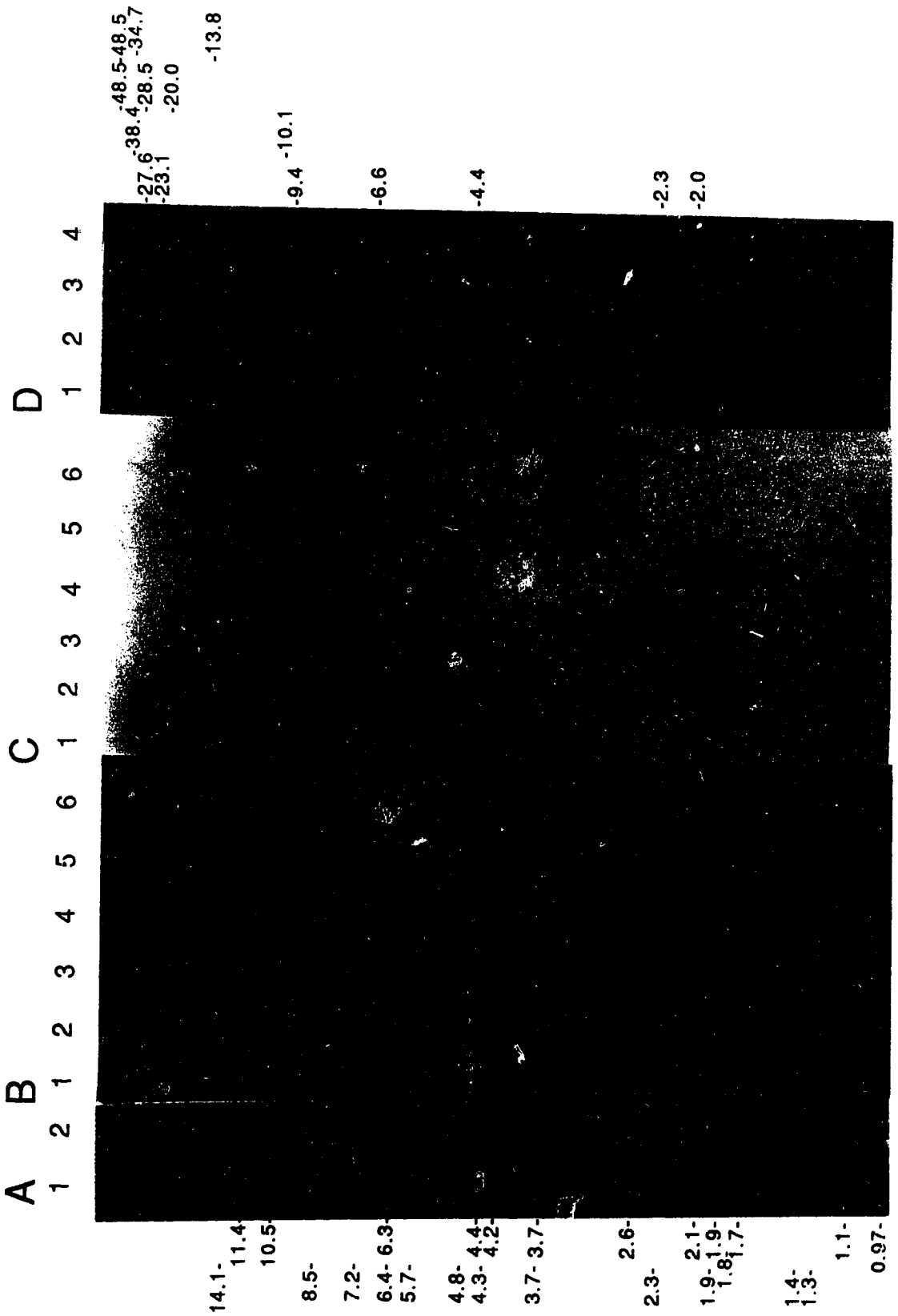
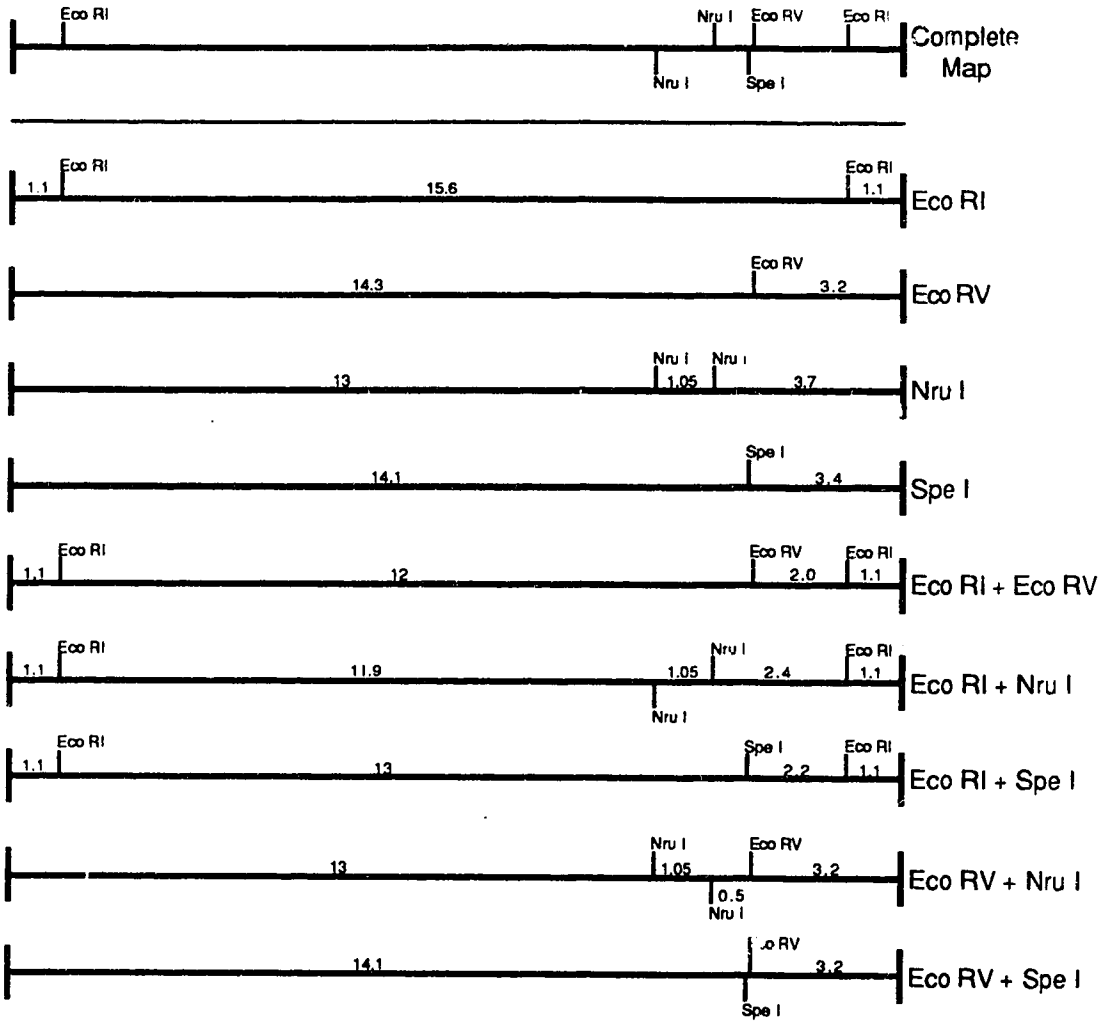

A B C D
1 2 1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4
14.1-
11.4-
10.5-
8.5-
7.2-
6.4-
6.3-
5.7-
4.8-
4.4-
4.3-
4.2-
3.7-
3.7-
2.6-
2.3-
2.1-
1.9-
1.9-
1.8-
1.7-
1.4-
1.3-
1.1-
0.97-
-48.5
-48.5
-34.7
-38.4
-28.5
-27.6
-20.0
-23.1
-13.8
-10.1
-9.4
-6.6
-4.4
-2.3
-2.0

Table 5. Summary of restriction fragment sizes produced by digestion of pSJL2

Restriction Enzyme(s)	Number of Observed Fragments	Size of Fragments Observed (kb)
Eco RI	1	15.6
Eco RV	2	14.3, 3.2
Nru I	3	13, 3.7, 1.05
Spe I	2	14.1 3.4
Eco RI + *Eco* RV	4 (1 x doublet)	12, 2, (1.1) x 2
Eco RI + *Nru* I	5 (1 x doublet)	11.9, 2.4, (1.1) x 2, 1.05
Eco RI + *Spe* I	4 (1 x doublet)	13, 2.2, (1.1) x 2
Eco RV + *Nru* I	3	13, 3.2, 1.05
Eco RV + *Spe* I	2	14.1, 3.2

Note: small fragments which were electrophoresed through the gel and thus were not directly observed are not included in this table.

Fig 29. Restriction map of pSJL2. Size and location of restriction fragments produced by the digestions in figures 27 and 28, as shown in comparison to the map of all known restriction endonuclease pSJL2 sites. Numbers indicate fragment sizes in kb.


Eco RI
Nru I
Eco RV
Eco RI
Nru I
Spe I
Complete Map
Eco RI
1.1
15.6
Eco RI
1.1
Eco RI
Eco RV
14.3
3.2
Eco RV
Nru I
Nru I
13
1.05
3.7
Nru I
Spe I
14.1
3.4
Spe I
Eco RI
1.1
12
Eco RV
2.0
Eco RI
1.1
Eco RI + Eco RV
Eco RI
Nru I
1.1
11.9
1.05
2.4
Eco RI
1.1
Nru I
Eco RI + Nru I
Eco RI
Spe I
1.1
13
2.2
Eco RI
1.1
Eco RI + Spe I
Nru I
Eco RV
13
1.05
3.2
0.5
Nru I
Eco RV + Nru I
14.1
3.2
Spe I
Eco RV + Spe I

dbe located on this plasmid. Figure 29 is an illustration showing the size and location of restriction fragments produced by the enzymes used for the digestions in Figures 27 and 28 in comparison to the map of all identified restriction sites on pSJL1. In addition, the restriction enzymes *Bsp* HI, *Cla* I, *Nco* I, and *Sac* I were tested and found to cut more frequently than proved useful for restriction mapping (data not shown) and were hence excluded from the ouble digest experiments. In both pSJL1 and pSJL2, the restriction sites observed are only consistent with a linear plasmid, and not a circular topology.

Figure 30 depicts the restriction maps of pSCL1, pSJL1 and pSJL2 using the restriction enzymes whose recognition sites were determined during the mapping experiments of the small *S. jumonjinensis* plasmids. Each plasmid has a very distinctive and unique restriction map; pSCL1 and pSJL1 are clearly different plasmids, even though their apparent sizes are very similar. Similarly, the apparent lack of homology between pSJL1 and pSJL2 indicated by hybridization studies is supported by the absence of common sites on the pSJL1 and pSJL2 restriction maps. While the only possible similarity involves the single terminal *Eco* RI site of pSJL1 and the two apparently identical terminal sites on pSJL2, the distance of these sites from the plasmid ends is not exactly the same.

3.10 Restriction Site Survey of pSCL2

The failure of repeated attempts to conduct *in situ* restriction digests of either cut out giant linear plasmid bands or whole cell DNA preparations led to an alternative approach to generating a restriction map for the giant linear plasmids. Sucrose gradient centriguation proved an efficient method of isolating pSCL2 intact and in solution (Fig. 24), allowing restriction digestion by conventional techniques. Figures 31 and 32 illustrates the digestion products detected in a restriction enzyme survey of pSCL2, with digestion products separated by both conventional agarose and CHEF techniques. Product visualization was conducted both by

Fig. 30. Restriction maps of pSCL1, pSJL1, and pSJL2. The figure depicts the restriction maps of the plasmids pSCL1, pSJL1, and pSJL2 using restriction sites for *Bcl* I, *Bgl* II, *Cla* I, *Eco* RI, *Eco* RV, *Kpn* I, *Nhe* I, *Nru* I, *Kpn* I and *Spe* I.

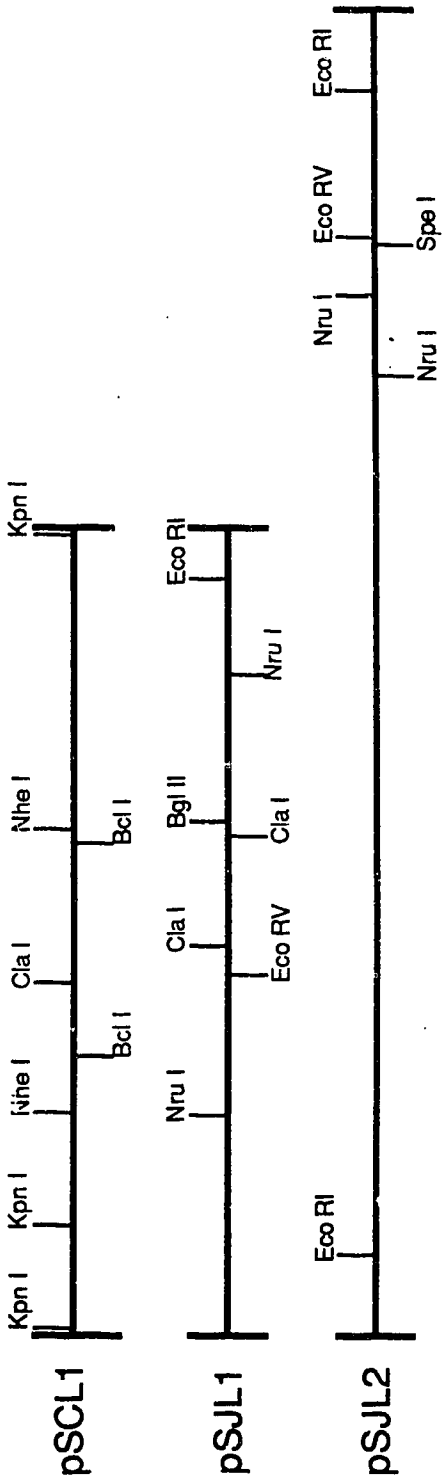

pSCL1
Kpn I
Kpn I
Kpn I
Xho I
Cla I
Nhe I
Bcl I
Bcl I
pSJL1
Nru I
Cla I
Eco RV
Bgl II
Cla I
Nru I
Eco RI
pSJL2
Eco RI
Nru I
Nru I
Eco RV
Spe I
Eco RI

Fig. 31. Southern transfer and hybridization analysis of CHEF gel electrophoresis of restriction endonuclease digests of sucrose gradient purified pSCL2 DNA with a pSCL2 random primer probe. A. Ethidium bromide stained CHEF gel run for 24 hours at 165 V, 5 to 80 second pulse ramp. Marker lanes: 1) λ ladder + λ *Hin*d III digest, 2) uncut λ, 3) λ *Cla* I, and 20) λ *Hin*d III. pSCL2 restriction digests lanes: 4) *Acc* I, 5) *Bcl* I, 6) *Bgl* II, 7), *Cla* I, 8) *Eco* RI, 9) *Eco* RV, 10) *Hin*d III, 11) *Hpa* I, 12) *Nde* I, 13) *Nsi* I, 14) *Sca* I, 15) *Spe* I, 16) *Xba* I, 17) *Ase* I, 18) *Dra* I, and 19) *Ssp* I. Panel B shows autoradiographs of the gel shown in Panel A after hybridization with a pSCL2 random primer labeled probes. Scale and lane contents remain constant between panels. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.

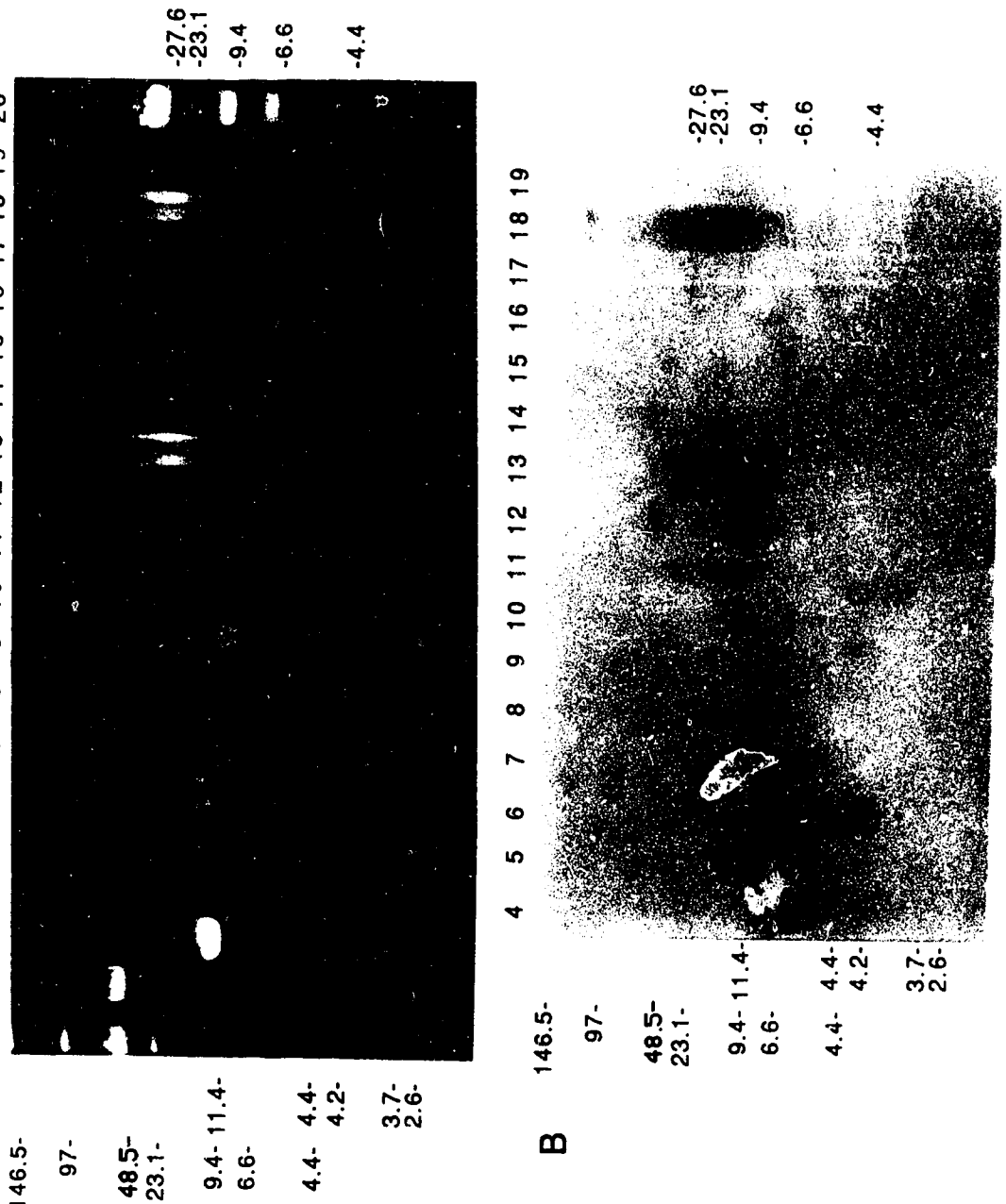
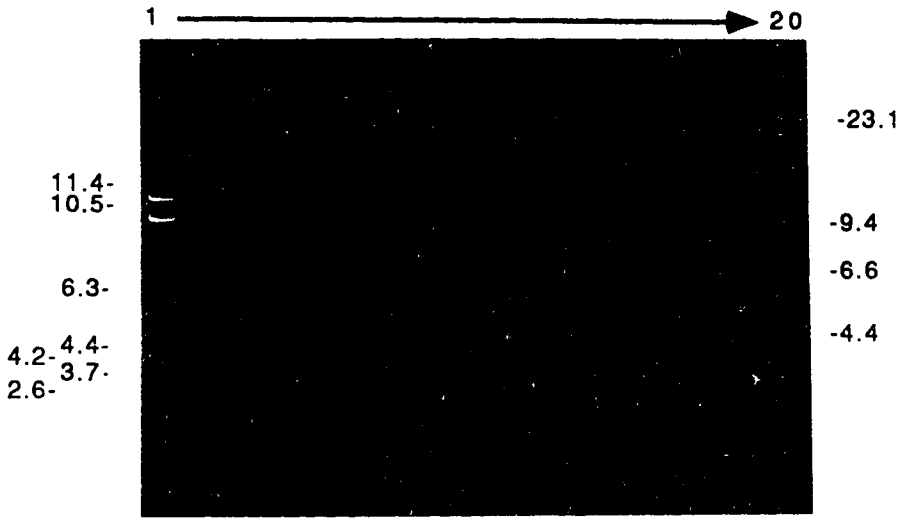
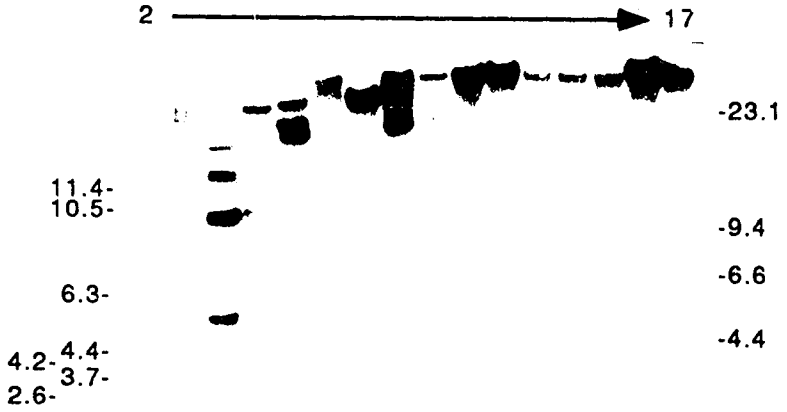

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
146.5-
97-
48.5-
23.1-
9.4-
6.6-
4.4-
11.4-
4.4-
4.2-
3.7-
2.6-
-27.6
-23.1
-9.4
-6.6
-4.4
B
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
146.5-
97-
48.5-
23.1-
9.4-
6.6-
4.4-
11.4-
4.4-
4.2-
3.7-
2.6-
-27.6
-23.1
-9.4
-6.6
-4.4

Fig. 32. Southern transfer and hybridization analysis of conventional gel electrophoresis of restriction endonuclease digests of sucrose gradient purified pSCL2 DNA with a pSCL2 random primer probe. A. Ethidium bromide stained conventional 1 x TEA 0.4% agarose gel run for 24 hours at 0.8 V/cm. Marker lanes: 1) λ *Cla* I, 18) uncut λ, 19) empty, and 20) λ *Hin*d III. pSCL2 restriction digests lanes: 2) *Acc* I, 3) *Bcl* I, 4) *Bgl* II, 5), *Cla* I, 6) *Eco* RI, 7) *Eco* RV, 8) *Hin*d III, 9) *Hpa* I, 10) *Nde* I, 11) *Nsi* I, 12) *Sca* I, 13) *Spe* I, 14) *Xba* I, 15) *Ase* I, 16) *Dra* I, and 17) *Ssp* I. Panel B shows autoradiographs of the gel shown in Panel A after hybridization with a pSCL2 random primer labeled probe. Scale and lane contents remain constant between panels. Numbers to the side of the figure indicate molecular weight marker positions, sizes in kb.

A


1
20
11.4-
10.5-
6.3-
4.2-
4.4-
3.7-
2.6-
-23.1
-9.4
-6.6
-4.4

B

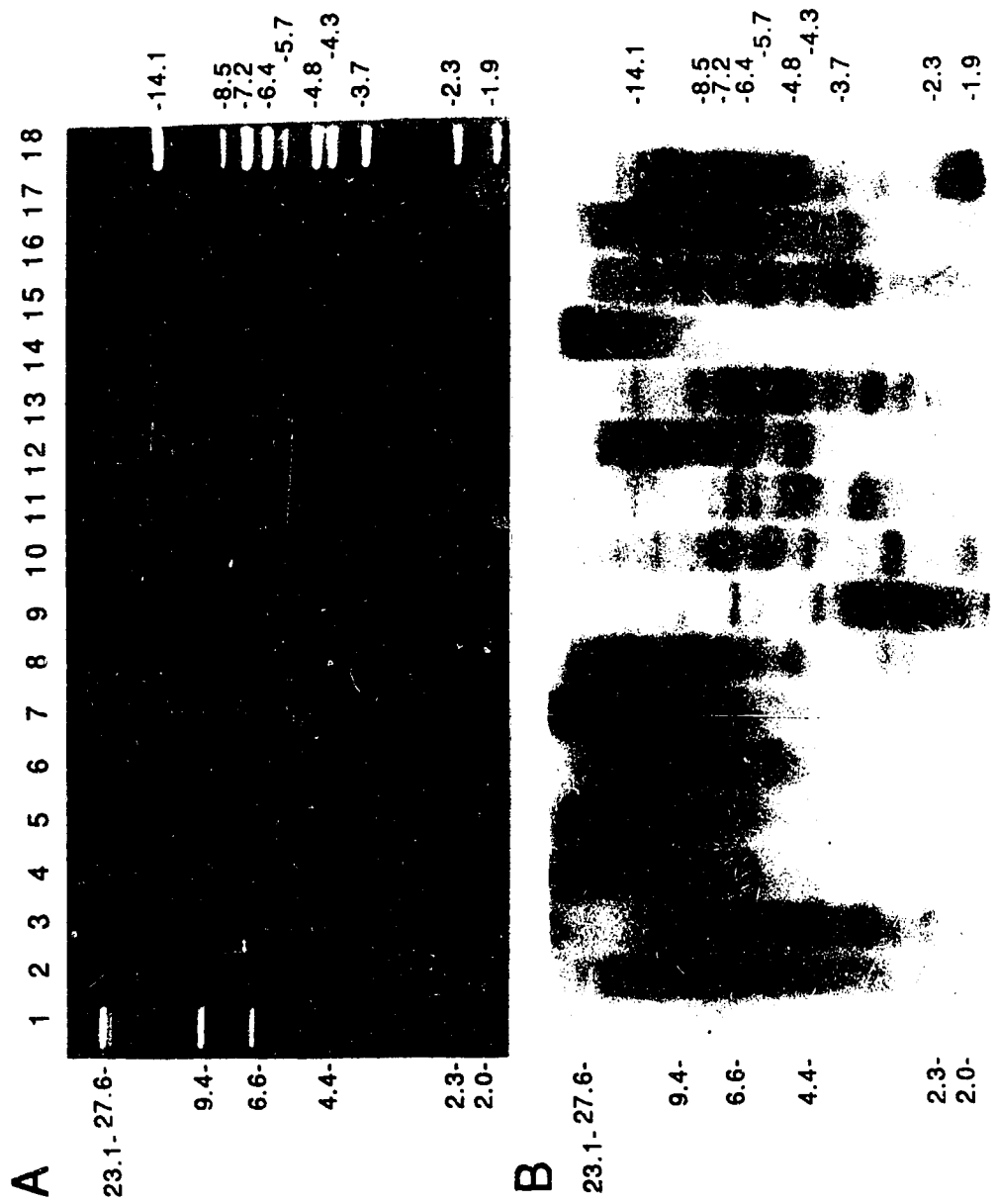

2
17
11.4-
10.5-
6.3-
4.2-
4.4-
3.7-
2.6-
-23.1
-9.4
-6.6
-4.4

ethidium bromide staining and by hybridization with random primer pSCL2 probes following Southern transfer. None of the enzymes within the survey appear to cut only once, and as a result, *Nde* I and *Spe* I, each of which produce three or four DNA fragments, were selected for further restriction analysis. Double restriction digests using these enzymes and radioactive probe hybridizations did not produce a definitive restriction map (data not shown).

3.11 Restriction Analysis of pSCL2 Telomere Sequences

The ability of the phenol-protein interface DNA preparation protocol to recover covalently linked DNA/protein complexes selectively could allow the analysis of the telomere sequences of linear plasmids and chromosomes. The terminal sequences of linear DNA structures too large to be recovered intact are selectively isolated in phenol-protein interface DNA preparation, and the remaining DNA segregates into the aqueous phase. The DNA fragments observed following restriction endonuclease digestion of the sheared DNA from the phenol-interface DNA preparation should be biased towards those from the ends of the plasmid. Figure 33 shows the DNA fragments which were observed following restriction endonuclease digestion of phenol-protein interface extract DNA from *S. clavuligerus*, Southern transfer, and hybridization with a pSCL2 probe. Given the lack of evident homology between most GLPs studied and the chromosome in *S. clavuligerus*, the bands detected in these lanes should originate from pSCL2. Further, the phenol-protein interface technique should only recover DNA fragments which have proteins covalently attached, creating a bias towards recovery of DNA fragments arising from the termini of the linear plasmids. Since pSCL2 was used as a probe, labeled bands should represent a pool of DNA restriction products enriched in the termini of the pSCL2 linear plasmid. Figure 33 demonstrates a large number of weak bands visible in each lane. Furthermore, these bands do not correspond to the prominent pSCL1 restriction endonuclease digestion product

Fig. 33. Restriction analysis of pSCL2 telomere sequences recovered from phenol-protein interface preparations. A. Restriction endonuclease digestions of *S. clavuligerus* phenol-protein interface DNA, separated on a conventional 0.6% agarose 1 x TEA gel, electrophoresed for 16 hours at 1.2 V/cm. Marker lanes: 1) λ *Hin*d III, 18) λ *Bst* EII. Restriction digested sample lanes: 2) undigested, 3) *Kpn* I, 4) *Hin*d III, 5) *Eco* RV, 6) *Eco* RI, 7) *Xho* I, 8) *Sph* I, 9) *Sal* I, 10) *Sac* I, 11) *Pvu* I, 12) *Pst* I, 13) *Nru* I, 14) *Nhe* I, 15) *Nco* I, 16) *Mlu* I, and 17) *Bam* HI. Panel B represents the gel in panel A following Southern transfer, and hybridization with a random primer pSCL2 probe. Since this includes the whole of pSCL2, it will reveal not only the terminal fragments but also internal fragments, especially those close to the termini. Scale and lane contents remain constant between the panels. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
23.1-
27.6-
9.4-
6.6-
4.4-
2.3-
2.0-
-14.1
-8.5
-7.2
-6.4
-5.7
-4.8
-4.3
-3.7
-2.3
-1.9
B
23.1-
27.6-
9.4-
6.6-
4.4-
2.3-
2.0-
-14.1
-8.5
-7.2
-6.4
-5.7
-4.8
-4.3
-3.7
-2.3
-1.9

clearly visible in the ethidium bromide stained gel, eliminating the possibility of cross-hybridization confusing the observations.

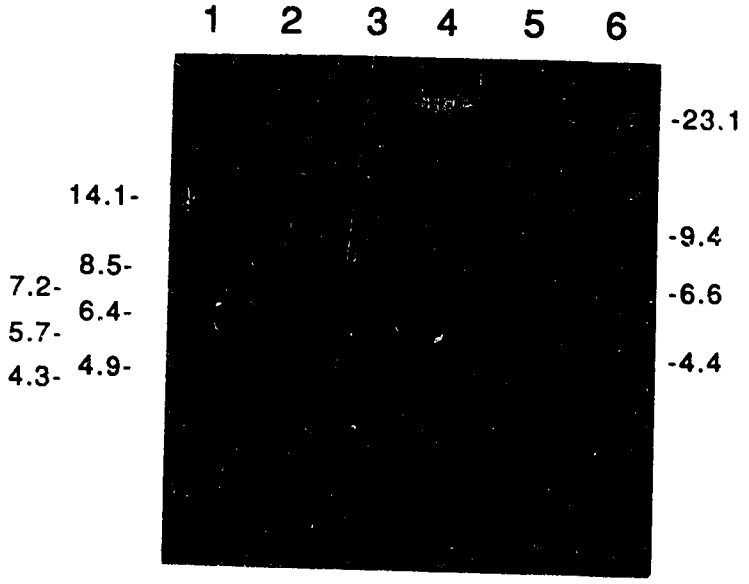
3.12 Analysis of pSL1A and pSL1B Topology and Structure

The results of two dimensional gel electrophoresis and hybridization studies of pSL1A and 1B indicated that these plasmids are quite distinct from the other plasmids detected in this survey. The two dimensional gel electrophoresis results suggested that the bands recovered from the phenol/water interface are in some way topologically constrained, and that ultraviolet irradiation of the ethidium bromide stained bands resulted in a change in structure, retarding electrophoretic migration. A conversion between a supercoiled DNA structure and a relaxed open circle would be consistent with the observed results. However, the fact that these bands were not observed in either phenol chloroform extraction or proteinase low mw DNA preparations indicates that these bands are not simple double stranded DNA circles. These bands were detected only in the interface extract samples, indicating that a phenol soluble component of the bands, likely a protein, was involved. Surprisingly, the proteinase K digest preparations also did not show any bands of this size. A number of exploratory studies were conducted to attempt to assess the nature of the pSL1A and B bands.

S. lividans TK19 interface extracts were either digested with proteinase K for two hours or left untreated, and then analyzed by agarose gel electrophoresis in the presence or absence of SDS (Fig. 34). Interestingly, no differences were observed between the results observed under these distinct conditions. Previous studies on linear plasmids have suggested (Kalkus *et al.*, 1990; Lin *et al.*, 1993) that the protein component of a covalently attached protein/DNA complex significantly retards migration, however this phenomenon was not observed. Similarly, since the presence or absence of SDS had no observable effect upon migration, protein-protein interaction does not seem to influence migration.

Fig. 34. Gel electrophoresis of *S. lividans* TK19 phenol interface extracts under various conditions. A. Ethidium bromide stained conventional agarose gel electrophoresis of *S. lividans* TK19 samples, B. ethidium bromide stained conventional agarose gel electrophoresis of *S. lividans* TK19 in the presence of 0.1% SDS. Lanes: 1) λ *Bst* EII, 2) λ *Bsp* HI, 3) *S. lividans* TK19 phenol interface extract DNA, 4) proteinase-K digested *S. lividans* TK19 phenol interface extract DNA, 5) λ *Cla* I, 6) λ *Hind* III. Electrophoresis was within a 0.7% agarose gel in a 1 x TEA buffer at 1.2 V/cm for 4 hours followed by staining with ethidium bromide. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.

A


1 2 3 4 5 6
-23.1
14.1-
-9.4
8.5-
7.2-
-6.6
6.4-
5.7-
4.3-
4.9-
-4.4

B

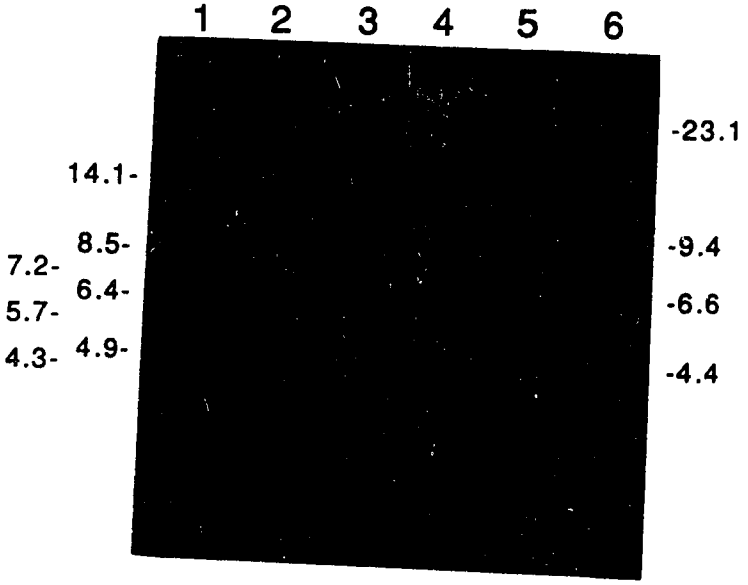
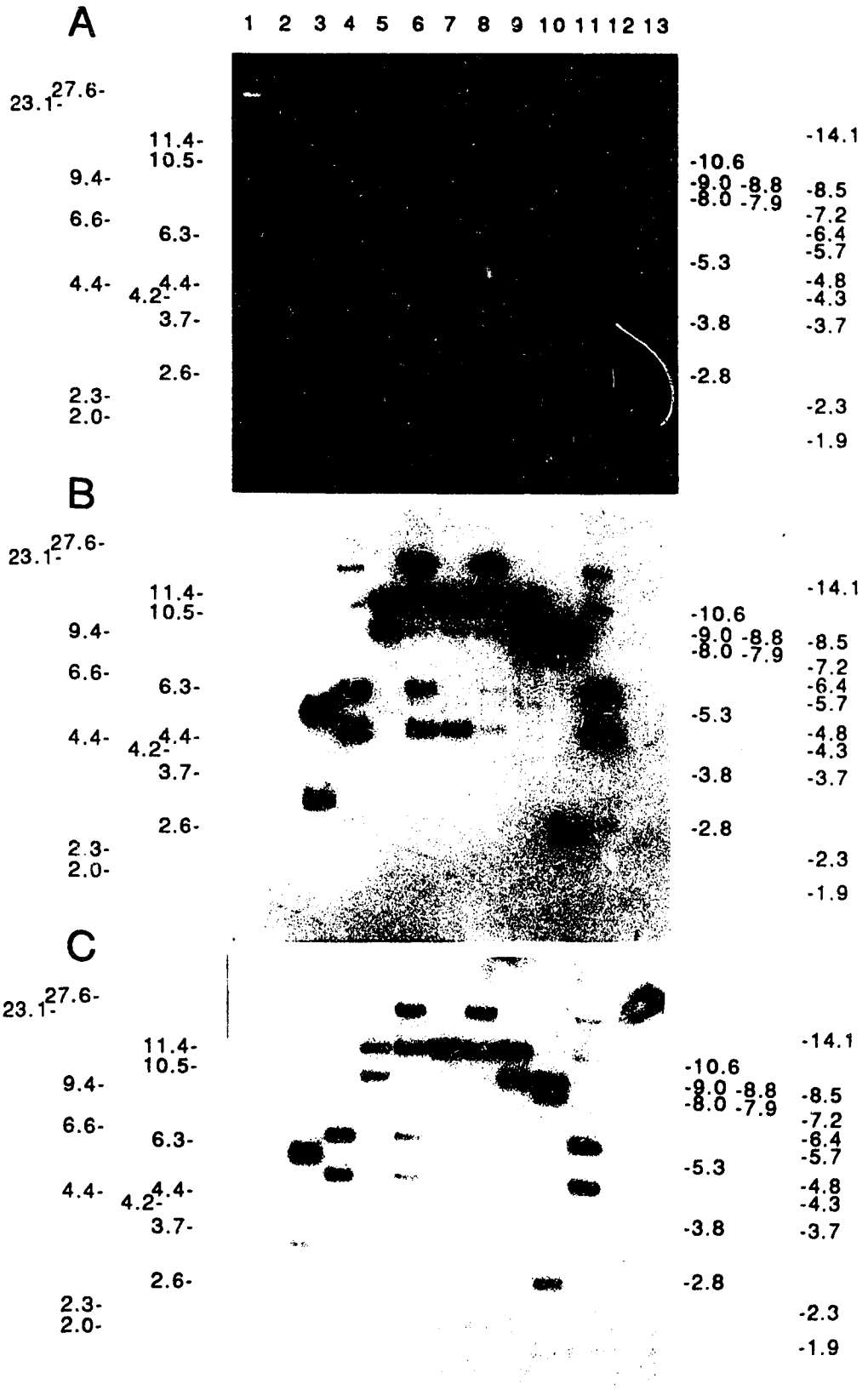

1 2 3 4 5 6
-23.1
14.1-
8.5-
-9.4
7.2-
6.4-
-6.6
5.7-
4.3-
4.9-
-4.4

Fig. 35. Southern transfer and hybridization analysis of restriction endonuclease digests of *S. lividans* TK19 phenol interface extract DNA preparations with pSL1A and pSL1B plasmid probes. A. Ethidium bromide stained conventional 1 x TEA 0.8% agarose gel run for 12 hours at 1.2 V/cm. Marker lanes: 1) λ *Hin*d III, 2) λ *Cla* I, 12) λ *Bsp* HI, and 13) λ *Bst* EII. *S. lividans* TK19 phenol interface DNA preparations digests lanes: 3) *Pst* I, 4) *Nsi* I, 5) *Kpn* I, 6) *Hin*d III, 7) *Eco* RV, 8) *Eco* RI, 9) *Bgl* II, 10) *Bam* HI, and 11) undigested.

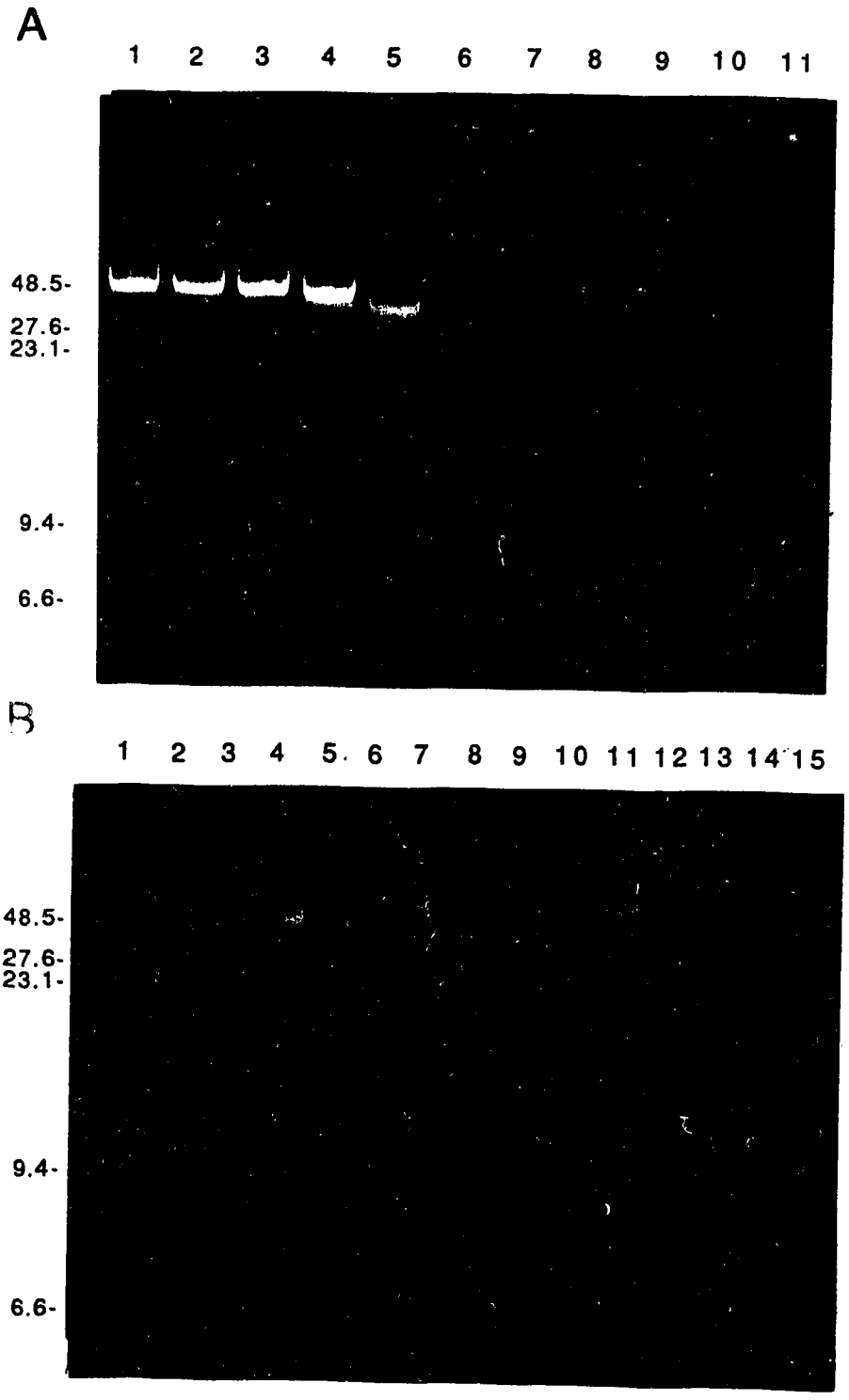
Panels B and C depict autoradiographs of the gel shown in Panel A after hybridizations with random primer labeled probes: B) pSL1A, and C) pSL1B. Scale and lane contents remain constant between panels. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


A
1 2 3 4 5 6 7 8 9 10 11 12 13
23.1-
27.6-
11.4-
10.5-
9.4-
6.6-
6.3-
4.4-
4.2-
4.4-
3.7-
2.6-
2.3-
2.0-
-14.1
-10.6
-9.0 -8.8
-8.0 -7.9
-8.5
-7.2
-6.4
-5.7
-5.3
-4.8
-4.3
-3.8
-3.7
-2.8
-2.3
-1.9
B
23.1-
27.6-
11.4-
10.5-
9.4-
6.6-
6.3-
4.4-
4.2-
4.4-
3.7-
2.6-
2.3-
2.0-
-14.1
-10.6
-9.0 -8.8
-8.0 -7.9
-8.5
-7.2
-6.4
-5.7
-5.3
-4.8
-4.3
-3.8
-3.7
-2.8
-2.3
-1.9
C
23.1-
27.6-
11.4-
10.5-
9.4-
6.6-
6.3-
4.4-
4.2-
4.4-
3.7-
2.6-
2.3-
2.0-
-14.1
-10.6
-9.0 -8.8
-8.0 -7.9
-8.5
-7.2
-6.4
-5.7
-5.3
-4.8
-4.3
-3.8
-3.7
-2.8
-2.3
-1.9

Upon digestion *S. lividans* TK19 interface extracts with restriction enzymes *Bam* HI, *Eco* RI, *Eco* RV, *Hin*d III, *Kpn* I, and *Nsi* I, several bands of identical size appeared regardless of which enzyme was used, and in each case the bands which appeared after digestion migrated more slowly than the native pSL1A and B bands (Fig. 35). These slower migrating bands should normally indicate a larger sized DNA fragment, a result not anticipated during restriction digestion. Since phenol-protein interface DNA extracts should also be enriched in *S. lividans* linear chromosome telomere DNA, these bands might represent digestion products from that source. However, these bands do not correspond to any of the restriction fragments described by Lin *et al.* (1993) during the analysis of SLP2 and the *S. lividans* linear chromosome telomeres. Hybridization of this digest with SLP2, pSL1A, and 1B probes demonstrated that the pSL1A and pSL1B probes produced identical patterns of hybridization to all the new, larger bands, but the SLP2 probe failed to hybridize to any digestion products (data not shown). This observation that several enzymes produced fragments of the same size suggests that each of the enzymes caused a single cut within a topologically constrained structure. This cutting may cause the structure to relax into a form of lower mobility (open circle or linear DNA). The alternative interpretation, that several different restriction enzymes all produced identically sized fragments seems very improbable. Furthermore, the only known examples of modified mobility of DNA on conventional gels following digestion with endonucleases are the result of topological changes, such as linearization of circular forms or relaxation of supercoiled forms.

Sucrose gradient separation of *S. lividans* TK19 interface extracts was conducted in an attempt to further characterize the nature of the pSL1A and pSL1B bands. Fig. 36 depicts the DNA species isolated from fractions of the gradient. The pSL1A and pSL1B bands are clearly visible in lanes 3 to 5 (6.3 kb) and 4 to 6 (5.0 kb) respectively, having migrated a much greater distance through the gradient than the sheared chromosomal DNA with

Fig. 36. Sucrose gradient analysis of *S. lividans* TK19 phenol interface DNA. Phenol interface extract DNA from *S. lividans* TK19 was fractionated as either an undigested or *Bgl* II digest on a 10 to 35% sucrose gradient by centrifugation at 36000 rpm for 20 hours in a SW40Ti rotor. Samples were precipitated for 12 hours, centrifuged, then the pelleted DNA was redissolved and electrophoresed in an ethidium bromide stained conventional 1 x TEA 0.4% agarose gel run for 12 hours at 1.2 V/cm. A. Undigested *S. lividans* TK19 DNA preparations, lanes 1 to 11 correspond to sucrose gradient fractions, with lane 11 being the top of the gradient, lane 1 the bottom. B. *Bgl* II digested *S. lividans* TK19 DNA preparations, lanes 1 to 15 correspond to sucrose gradient fractions, with lane 15 being the top of the gradient, lane 1 the bottom. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


A
1 2 3 4 5 6 7 8 9 10 11
48.5-
27.6-
23.1-
9.4-
6.6-
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
48.5-
27.6-
23.1-
9.4-
6.6-

similar electrophoretic mobility in agarose gel. A second pair of very weak bands whose size appeared to correspond to that of the slow migrating spots in the two dimensional gel (Fig. 13) was visible in lanes 5-7 (10.4 kb) and 6-7 (15 kb). These results clearly indicated that the intact pSL1A and pSL1B DNAs migrated in a non-linear fashion in the sucrose gradient, again indicating these structures were likely topologically constrained, possibly supercoiled. The fainter bands migrated in a fashion consistent with a more open structure than the native pSL1A and pSL1B bands, such as an open circle, but still migrated faster than the sheared chromosomal DNA. A second sucrose gradient centrifugation of *Bgl* II digests of *S. lividans* TK19 phenol interface extract material produced a different migration pattern, with bands of approximately 9.4 kb (lanes 8 to 9) and 11.6 kb (lanes 7 to 8) migrating through the sucrose gradient at the same speed as the sheared linear DNA, indicating these restriction digest products have a linear topology.

To test for the possibility that the pSL1A and 1B plasmids include a protein component that is either inaccessible to proteinase K or very proteinase resistant, preparations of *S. lividans* TK19 interface extract material were incubated at 55°C with and without proteinase K for extended periods, up to 4 days (Figure 37). Both the proteinase K treated and untreated samples show a gradual disappearance of the pSL1A and 1B bands, with a corresponding appearance of two larger bands which migrate at the same points as the slow migrating spots identified during two dimensional agarose gel electrophoresis. The change appears to occur slightly faster in the proteinase K treated samples.

The effects of proteinase K digestion led to an investigation of the hydrophobic component of the pSL1A and pSL1B plasmids. *S. lividans* TK19 phenol interface DNA preparations were incubated for 3 hours with 0.1% SDS in the presence and absence of proteinase K, and re-extracted with phenol, saving only the aqueous phase (Figure 38). Surprisingly, even the non-proteinase K digested samples of pSL1A and pSL1B did not return to the phenol phase or

Fig. 37. Gel electrophoresis of *S. lividans* TK19 phenol interface extracts after extended digestion by proteinase-K. Ethidium bromide stained conventional 0.6% agarose gel in a 1 x TEA buffer electrophoresed at 1.2 V/cm for 4 hours. Marker lanes: 1) λ *Bst* EII, 18) λ *Hin*d III. Proteinase K digests of *S. lividans* TK19 phenol interface extract DNA lanes: 2) 0 hours, 3) 3 hours, 4) 6 hours, 5) 12 hours, 6) 24 hours, 7) 48 hours, 8) 72 hours, and 9) 96 hours. 0.1% SDS incubation of *S. lividans* TK19 phenol interface extract DNA lanes: 10) 0 hours, 11) 3 hours, 12) 6 hours, 13) 12 hours, 14) 24 hours, 15) 48 hours, 16) 72 hours, and 17) 96 hours. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.

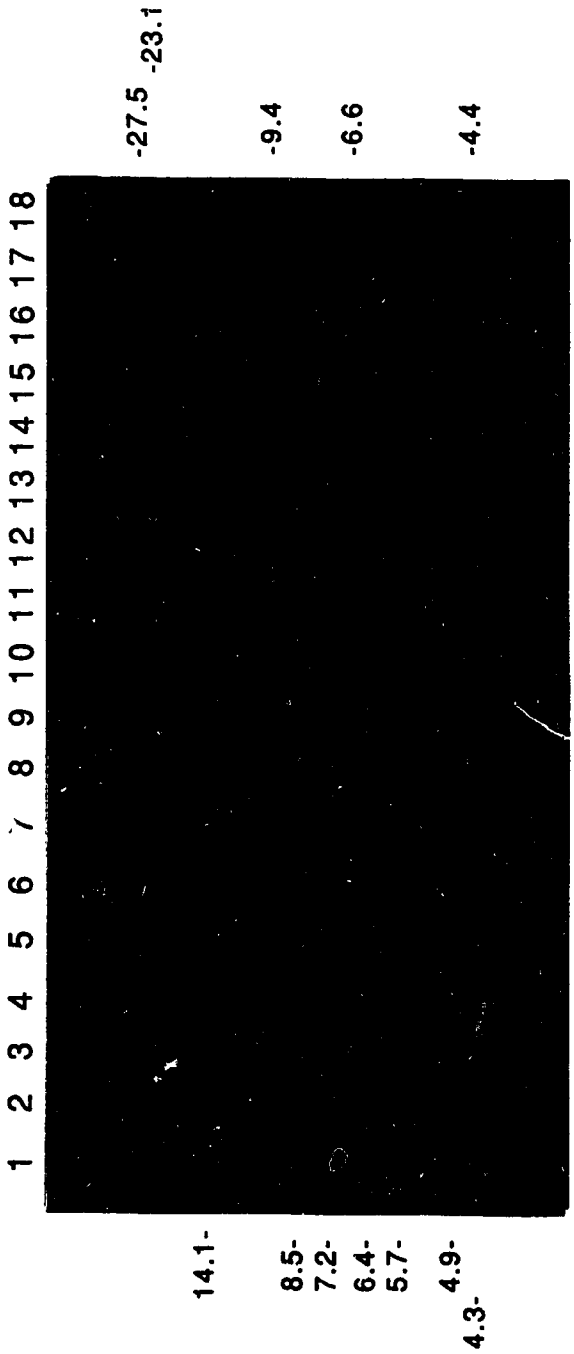
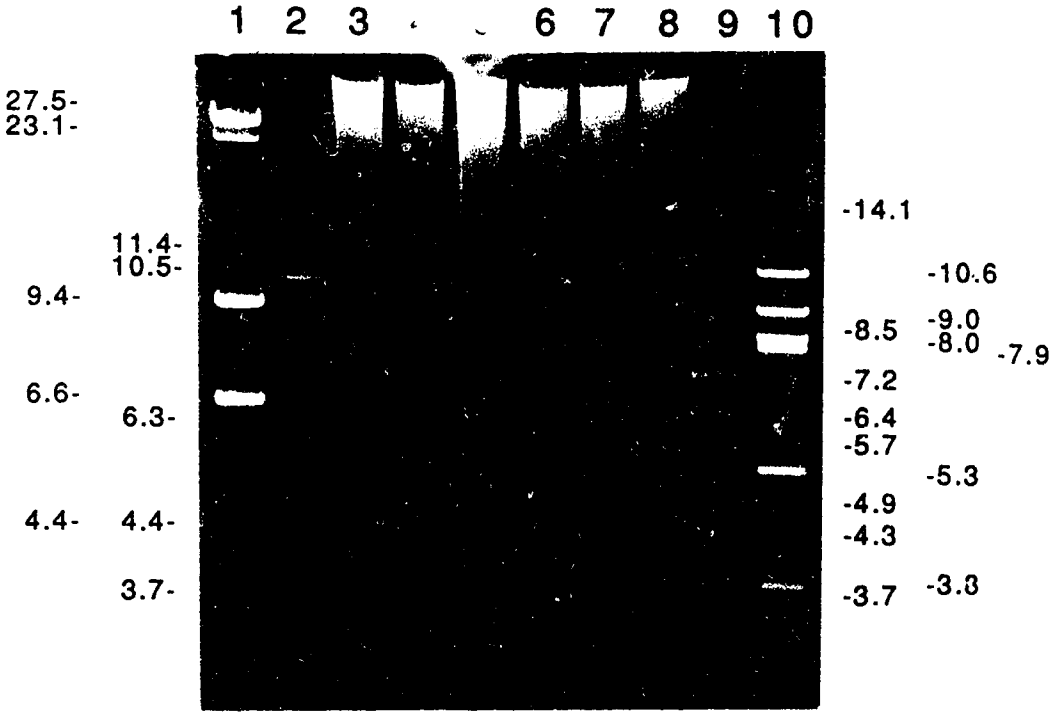

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
-27.5
-23.1
-9.4
-6.6
-4.4
14.1-
8.5-
7.2-
6.4-
5.7-
4.9-
4.3-

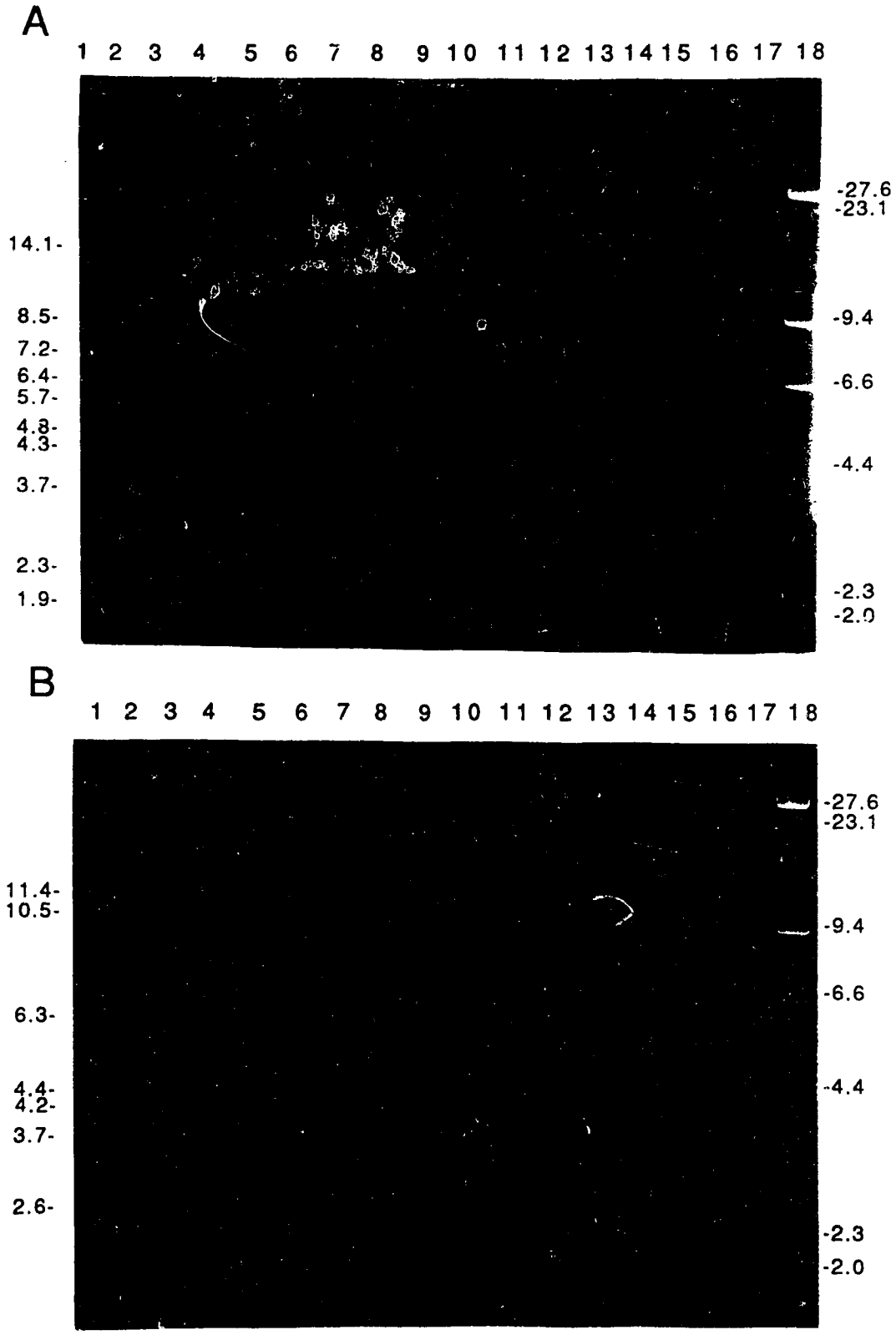
Fig. 38. Gel electrophoresis of *S. lividans* TK19 phenol interface extracts after re-extraction with phenol. Ethidium bromide stained conventional 0.4% agarose gel in a 1 x TEA buffer electrophoresed at 1.0 V/cm for 3 hours. Marker lanes: 1) λ *Hin*d III, 2) λ *Cla* I, 9) λ *Bst* EII, 10) λ *Bsp* HI. *S. lividans* TK19 phenol interface extract DNA lanes lanes: 3) undigested, no phenol extraction, 4) undigested, one phenol extraction, 5) undigested, two phenol extractions, 6) proteinase K digested, no phenol extraction, 7) proteinase K digested, one phenol extraction, 8) proteinase K digested, two phenol extractions. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


1 2 3 6 7 8 9 10
27.5-
23.1-
11.4-
10.5-
9.4-
6.6-
6.3-
4.4-
4.4-
3.7-
-14.1
-10.6
-9.0
-8.5
-8.0
-7.9
-7.2
-6.4
-5.7
-5.3
-4.9
-4.3
-3.7
-3.3

to the phenol-water interface after extraction, but rather remained in the aqueous phase without any apparent loss in plasmid concentration.

In an attempt to characterize further the pSL1A and pSL1B plasmids, phenol interface DNA extracts were digested by a variety of nucleases, including DNAse I, RNAse I, Exonuclease III, Exonuclease VII, BAL 31 nuclease, and Nuclease S1. The results of these digestions are shown in Figure 39. DNAse I rapidly digested all ethidium bromide staining material within the samples, while RNAse I did not have any apparent effect upon the plasmids, indicating that these structures are indeed DNA. Digestion by Exonuclease III and VII also failed to have any discernable effect upon pSL1A and pSL1B, indicating if these DNA molecules have free ends, they are protected from digestion in some manner. The results of BAL 31 nuclease digestion indicate the fast migrating species of pSL1A and pSL1B (apparent size, 6.3 and 5.0 kb respectively) are rapidly degraded, but trace amounts of the slower migrating species (apparent size, 15 kb, 10.4 kb) appear to remain at constant concentrations. There is no evidence of a conversion from one form to another. Digestion by Nuclease S1, a single-stranded polynucleotide specific enzyme, produces another interesting profile. The third (45 minute) sample in this set does appears not to have had Nuclease S1 added, due to experimental error, and should be disregarded. In the other lanes, the rapidly migrating 6.3 and 5.0 kb (apparent size) bands appear to be initially converted into the slow migrating 15 kb and 10.4 kb (apparent size) bands. As well, two bands with an apparent size of 11.4 and 9.2 kb appear in increasing concentrations over time. In effect, it appears that the rapidly migrating form is converted into the slow migrating form, and then into a third form with a slightly higher electrophoretic mobility. Once again, these shifts in electrophoretic mobility appear to be due to a shift in topological conformation, possibly from a supercoiled to relaxed circle to linear forms. The buffer control lane for BAL 31 nuclease indicates that incubation in the buffer alone caused a conversion from the fast

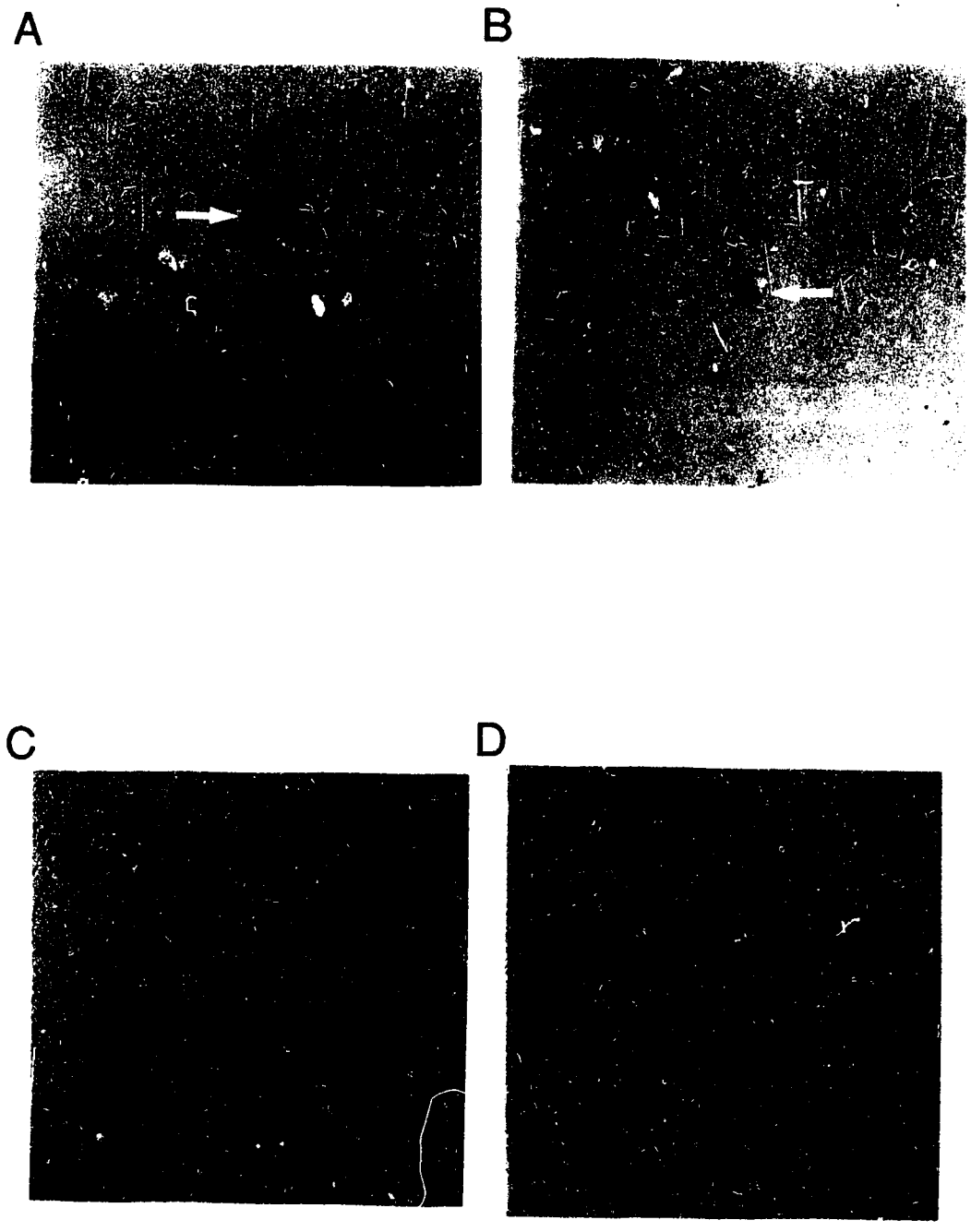
Fig. 39. Agarose gel electrophoresis of *S. lividans* TK19 phenol interface extracts digested by a variety of nucleases. Panels A and B. Ethidium bromide stained conventional 0.4% agarose gel in a 1 x TEA buffer electrophoresed at 1.2 V/cm for 6 hours. Marker lanes: A1) λ *Bst* EII, A2 and B1) λ *Cla* I, A18 and B18) λ *Hin*d III. *S. lividans* TK19 phenol interface extract DNA lanes: Panel A. 3) 15 minute DNAse I digestion, 4) 30 minute DNAse I digestion, 5) 45 minute DNAse I digestion, 6) 1 hour DNAse I digestion, 7) 15 minute RNAse I digestion, 8) 30 minute RNAse I digestion, 9) 45 minute RNAse I digestion, 10) 1 hour RNAse I digestion, 11) 15 minute Exonuclease III digestion, 12) 30 minute Exonuclease III digestion, 13) 45 minute Exonuclease III digestion, 14) 1 hour Exonuclease III digestion, 15) 1 hour incubation in DNAse I buffer, 16) 1 hour incubation in RNAse I buffer, 17) 1 hour incubation in Exonuclease III buffer. Panel B. 2) undigested, 3) 15 minute Exonuclease VII digestion, 4) 30 minute Exonuclease VII digestion, 5) 45 minute Exonuclease VII digestion, 6) 1 hour Exonuclease VII digestion, 7) 15 minute BAL 31 nuclease digestion, 8) 30 minute BAL 31 nuclease digestion, 9) 45 minute BAL 31 nuclease digestion, 10) 1 hour BAL 31 nuclease digestion, 11) 15 minute Nuclease S1 digestion, 12) 30 minute Nuclease S1 digestion, 13) 45 minute Nuclease S1 digestion, 14) 1 hour Nuclease S1 digestion, 15) 1 hour incubation in Exonuclease VII buffer, 16) 1 hour incubation in BAL 31 nuclease buffer, 17) 1 hour incubation in Nuclease S1 buffer. Numbers to the side of the figures indicate molecular weight marker positions, sizes in kb.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
14.1-
8.5-
7.2-
6.4-
5.7-
4.8-
4.3-
3.7-
2.3-
1.9-
-27.6
-23.1
-9.4
-6.6
-4.4
-2.3
-2.0
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
11.4-
10.5-
6.3-
4.4-
4.2-
3.7-
2.6-
-27.6
-23.1
-9.4
-6.6
-4.4
-2.3
-2.0

migrating to slow migrating forms of these plasmids. This may be due to the Ca^{2+} component of the BAL 31 buffer which is not found in any of the other buffers used in this experiment.

In an attempt to clarify the structure of pSL1A and pSL1B, sucrose gradient fractions enriched in the rapidly migrating (6.3 and 5.0 kb apparent size) forms were examined by transmission electron microscopy after coating with cytochrome c (Figure 40). Initial examination revealed highly supercoiled DNAs along with sheared chromosomal DNAs of random lengths. Upon brief DNAse treatment, the supercoiled structures relaxed to form a circular structure. The lengths of the relaxed structures were measured, and found to be distinctly different between pSL1A and pSL1B, in a ratio of 1.21 to 1.

Fig. 40. Transmission electron micrographs of pSL1A and pSL1B. Transmission electron micrographs of cytochrome-C coated samples of pSL1A and pSL1B isolated by sucrose gradients. Photographs: A) pSL1A, B) pSL1B, C) pSL1A DNAse treated, and D) pSL1B DNAse treated. Photographs one and two are magnified 5500 fold, three and four are magnified 11000 fold.


A
B
C
D

4. DISCUSSION:

In this study at least two very distinct types of extrachromosomal DNA elements were detected; conventional invertron type linear plasmids found within *S. clavuligerus*, *S. griseus*, and *S. jumonjinensis*, and two unusual plasmids (pSL1A and pSL1B) found within *S. lividans* TK19. Therefore, the discussion of the results from these two sets of plasmids will be largely conducted separately, with cross references where applicable and appropriate.

The plasmid survey of the β-lactam antibiotic producing bacteria *S. cattleya*, *S. clavuligerus*, *S. griseus*, *S. jumonjinensis*, and *S. lipmannii* detected extrachromosomal elements in some, though not all species. All of the plasmids detected appear to be linear plasmids with covalently attached terminal proteins, no conventional circular plasmids were identified. No apparent pattern in the presence or absence of plasmids, nor any particular size or number of plasmids was observed. Previous surveys, such as Kinashi and Shimaji (1987) also failed to detect any clear pattern in the linear plasmid content among the species studied. In general, the function and significence of plasmids in *Streptomyces*, especially the giant linear plasmids, is poorly understood, and this research can offer only minimal illumination of that topic.

The detection of multiple plasmids, both large and small, in several of the species examined during this survey was not unexpected Many *Streptomyces* spp. have been observed to carry a wide variety of extra-chromosomal genetic elements of various topologies, sizes, and copy numbers. In particular, the presence of additional plasmids within *S. clavuligerus* had been anticipated, given pSCL1's apparent failure to encode any of the enzymes required for the plasmid's replication (Wu and Roy, 1993). Analysis of the complete DNA sequence of pSCL1 plasmid had failed to identify any open reading frame with homology to either a

covalently attached terminal protein, nor a protein primed DNA polymerase, both proteins essential for pSCL1's maintanance. The multiple linear plasmids observed within *S. clavuligerus* and *S. jumonjin* ... are also not unprecedented, as other actinomycetes have been observed to contain more than one linear plasmid. For example, two *Rhodococcus* sp. have been described which contain several GLP's: M11 (pHG201 - 270 kb, pHG202 - 400 kb, pHG203 - 420 kb) and MR22 (pHG204 - 180 kb, pHG205 - 280 kb, pHG206 - 510 kb) (Kalkus *et al.*, 1990). As well, *S. rochei* 7434-AN4 contains three plasmids (17, ... 0 kb) (Hayakawa *et al.*, 1979; Kinashi and Shimaji, 1987) and ... *laceoruber* JCM 4979 contains seven giant linear plasmids (410, 440, 470, 500, 530, 560, 590 kb) (Kinashi and Shimaji, 1987). The apparently identical size of several of the plasmids discovered in this survey (pSCL1, pSJL1 ≅ 11.7 kb and pSCL2, pSGL2 ≅ 120 kb) suggested that these plasmids might be identical, or at least closely related. Given the fact that many *Streptomyces* linear plasmids appear to promote their own transfer between species (Hopwood *et al.*, 1986), and are stably maintained in a variety of host species, the possibility that these plasmids could be identical appeared significant.

The detection of two different *S. jumonjinensis* plasmid profiles (NRRL 5741, ATCC 29864) was unexpected, as the sources of these two isolates had indicated that these bacteria were in fact the same strain. No morphological or growth differences were noted between these isolates; thus pSJL2 does not appear to have any easily identified phenotype. The failure of the pSJL2 probe to hybridize with the *S. jumonjinensis* ATTC 29864 chromosomal DNA (Figure 18) indicates no copy of pSJL2 exists integrated within the chromosome. The absence of pSJL2 in ATCC 29864 offers the possibility of examining the transfer of this plasmid during mating, and whether any pock formation is induced by this plasmid. The difference betwe2 the plasmids observed in these two isolates was the only variation in plasmid profile detected in this study; at no other time were spontaneous alterations in plasmid number or size noted. Similarly, attempts to cure *S. clavuligerus* of pSCL1 and

pSCL2 by protoplasting (Michaluk *et al.*, 1994) failed despite repeated attempts. The plasmids detected in this research were maintained in a very stable fashion.

An assessment of plasmid topology was attempted using several approaches. First, the size of the plasmid bands observed indicated that cohesive end type molecules were not present. Cohesive end plasmids are easily recognized by the formation of a 'ladder' of bands, each differing by a given size equal to that of the plasmid monomer (*Streptomyces* examples; Rausch *et al.*, 1993; Gravius *et al.*, 1994a). While several plasmids were observed in *S. clavuligerus* and *S. jumonjinensis*, their sizes did not form any regular 'ladder' pattern. Second, a comparison of the presence or absence of plasmids within phenol extracted DNA preparations and proteinase K treated DNA preparations allows assessment of whether these DNA molecules have covalently attached proteins. As DNA partitions into the aqueous phase during phenol extractions, the phenol extraction technique is useful for removing contaminating proteins. Circular DNA molecules, and linear DNA molecules without attached proteins (such as sheared DNA, hairpin loop telomere plasmids, and cohesive end plasmids) all partition into the aqueous phase, and thus any DNA molecule detected following phenol extraction is assumed to fall into one of these topological classes. DNA molecules with covalently attached proteins are attracted to the phenol phase by their phenol soluble protein moiety, and to the aqueous phase by their DNA compoent and thus remain at the interface (Hirochika and Sakaguchi, 1982). Digestion with proteinase K prior to phenol extraction removes most (if not all) of the terminal protein, producing a DNA molecule which behaves functionally as a piece of linear DNA.

When the proteinase low mw and conventional phenol-chloroform DNA preparations from *S. cattleya*, *S. clavuligerus*, *S. griseus*, *S. jumonjinensis*, and *S. lipmannii* were examined using conventional agarose gel electrophoresis, pSCL1, pSJL1 and pSJL2 were only detected in proteinase K treated samples (Fig. 9). This observation indicates that these plasmids probably have covalently

attached proteins, and hence are likely to be linear. Confirmation of the linear topology was indicated by two-dimension agarose gel electrophoresis of these plasmids (Fig. 13), as the plasmid spots for pSJL1 and pSJL2 all migrated the same distance as the sheared (and hence linear) DNA without the formation of a second distinct spot. If these plasmids had a non-linear topology, exposure to UV light should have altered some of these DNA molecules, causing them to adopt a new topology and thus modify their migration during electrophoresis to a position outside of the sheared DNA diagonal (Hintermann *et al.*, 1981). Not only does the electrophoretic migration of these DNA species within agarose gels conform to a linear DNA topology, these plasmids also co-migrated with sheared linear DNA in sucrose gradients. The sample sucrose gradient in Figure 24 is representative of the many preparative sucrose gradients in which this observation was illustrated.

A topological analysis of the giant linear plasmids (pSCL2, pSCL3, pSGL1, pSJL3 and pSJL4) detected in this survey proved somewhat more difficult. While phenol extraction of DNA preparations proved an excellent technique for assessing the presence of terminal covalently attached proteins for smaller plasmids (those of approximately 50 kb or less), larger plasmids were sheared by the isolation process, and not recovered in detectable quantities. A more indirect approach proved necessary. Fortunately, one characteristic of circular DNA molecules is that the distance migrated during CHEF electrophoresis is dependent primarily on the length of the electrophoresis, and not on the electrophoresis pulse times (Hightower *et al.*, 1987; Matthew *et al.*, 1988). Thus, as the CHEF electrophoresis pulse times alter but total electrophoresis times remain, the electrophoretic migration of circular and supercoiled plasmids will change in respect to the linear molecular weight markers. Thus, the apparent size of circular and supercoiled DNA molecules change as CHEF pulse times change, a scenario not observed during this research. It should be noted that this fact does not demonstrate that these plasmids have

covalently attached proteins, only that these plasmid molecules are linear.

The phenol-protein interface DNA preparation technique offers a potentially powerful alternative to conventional methods for the isolation and identification of linear plasmids. This technique proved very efficient at isolating intact invertron type linear plasmids of 50 kb or less (Figure 11). Furthermore, since this technique should select only covalently attached protein-DNA complexes or DNA complexes with any other lipophilic moiety, the recovery of any plasmid using this process indicates that it probably has protein blocked telomeres, and does not maintain the structure and topology of a hairpin, cohesive end, or circular molecule. Furthermore, this technique offers a method of determining whether giant linear plasmids have covalently attached proteins, even though these plasmids are not likely to be recovered intact. Detection of homology between the DNA fragments isolated by the phenol-protein interface and isolated linear plasmid DNA can demonstrate the existence of a protein attached to the giant linear plasmid's telomeres. Fig. 33 demonstrates that pSCL2 DNA, for example, is recovered by the phenol-protein interface protocol, and hence this plasmid most likely has covalently attached proteins. The phenol-protein interface DNA preparation protocol also appears to recover what may represent a new class of genetic elements, as discussed in the text that follows (pSL1A, pSL1B).

The copy number estimation technique utilized obtained reproducible results for the smaller linear plasmids of *S. clavuligerus* and *S. jumonjinensis*. Unfortunately, calculation of the ratio of chromosomal to plasmid DNA content was not possible for analysis of giant linear plasmids, as the chromosomal DNA observed in CHEF separations does not include chromosomal DNA which fails to enter the CHEF gel and remains within the *in situ* DNA preparations agarose blocks The use of proteinase low mw digests might offer a successful alternative source material for copy number estimation after separation by CHEF techniques. The copy

numbers observed for the smaller linear plasmids pSCL1, pSJL1, and pSJL2 are all very similar, ranging approximately from 20-25, indicating these plasmids are moderately high copy number plasmids. Comparison of these values with other linear plasmids is difficult, as in only a few cases have actinomycete linear plasmid copy numbers been established: *Rhodococcus* sp. GLP pHG207 - 1-2 per chromosome (Kalkus *et al.*, 1993), *S. rochei* pSLA2 - 60 per chromosome (Hirochika and Sakaguchi, 1982), and *S. coelicolor* SCP1 - 4 per chromosome (Kinashi and Shimaji-Murayama, 1991). The high copy number of these plasmids suggests a relatively inefficient system for segregation of plasmids into both daughter cells, which matches well with the indications that pSCL1 ensures maintance with a *kil/kor* system (Wu and Roy, 1993). As the *Streptomyces* spp. studied are in part a multichromosomal syncytium, copy number in these cases represents a ratio of plasmids to chromosome. The conventional definition of number of plasmids per cell is not valid.

A faint ethidium bromide stained band which migrated in an anomalous and variable fashion was detected in both *S. jumonjinensis* isolates, though its size appeared to be slightly different in the two isolates. This band, named pSJX, has resisted all attempts at further analysis. At this time, any definite identification, or even suggestion of what pSJX may represent, or whether it is an experimental artifact is difficult. In summary, beyond the simple observation of the existance of the pSJX band, and it's variable migration, little suggestion to the identity or nature of this material can be offered, other than that it likely represents an unconventional structure.

Whether any of the plasmids detected in this survey share any common sequences with other plasmids or with the bacterial chromosomes is of considerable interest. In particular, given that pSCL1 is believed to use a plasmid replication system encoded by another plasmid (Wu and Roy, 1993), or possibly by the chromosome, similarities between the terminal sequences of these molecules were anticipated. As well, the similar size of plasmids

observed in different species raised the possibility that some of these plasmid might be related, if not identical. In other *Streptomyces* spp., investigations have demonstrated that where more than one plasmid is present, the plasmids may either be products of recombination, or perhaps an amplification, insertion, or deletion event (Kinashi and Shimaji, 1987; Kalkus *et al.*, 1993). Were this scenario true for the multiple plasmids seen in *S. clavuligerus* and *S. jumonjinensis*, cross-hybridization would be expected.

Cross-hybridization between plasmids in this study was minimal; only the positive control was detected in most cases. One possible exception is pSCL3, whose probe hybridized both to the pSCL3 band, and also to the *S. clavuligerus* chromosomal DNA. The possibility exists that this hybridization is an experimental artifact, caused by the contamination of pSCL3 probe DNA with sheared chromosomal DNA fragments. However, this explanation seems unlikely, as the background of sheared chromosomal DNA during CHEF electrophoresis of *S. clavuligerus in-situ* DNA samples was very limited; Figure 7 illustrates the results typically observed after electrophoresis. The apparent cross hybridization of the pSJL1 and pSJL2 probes to the larger *S. jumonjinensis* plasmids and chromosome separated by CHEF electrophoresis (Fig. 17) more likely represents a false result caused by contamination of the pSJL1 and pSJL2 probe DNA by sheared fragments of larger DNA molecules. When the reciprocal hybridizations were conducted (Fig. 18), the pSJL3 and pSJL4 probes failed to hybridize with either pSJL1 or pSJL2, not the expected result had genuine homology between these plasmids existed. Furthermore, the pSJL1 and pSJL2 DNA used for producing random primer probes was obtained from proteinase low mw preparations (eg. Fig. 9) which were observed to contain far more sheared DNA than the quite pure pSJL3 and pSJL4 probe DNA obtained from *in situ* PFG DNA preparations (eg. Fig. 9).

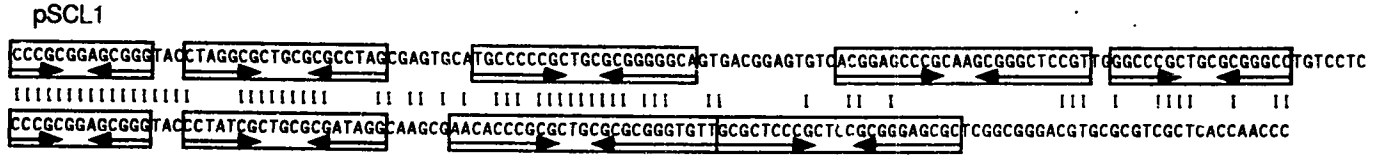
The apparent homology observed between pSCL3 and the *S. clavuligerus* chromosome has a number of possible implications. First, the possibility exists that pSCL3 may also be present in part

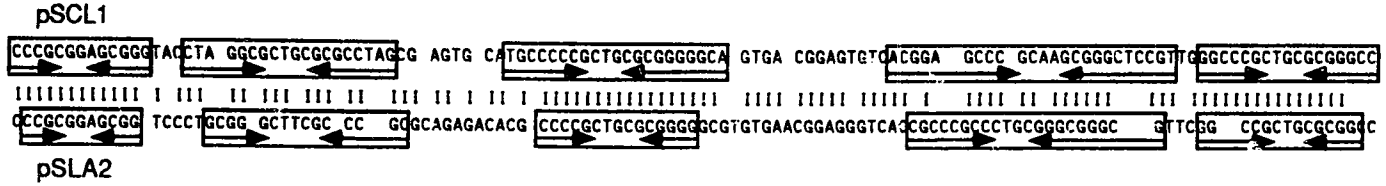
or whole as an intergrated copy, much like SCP1 (Chater and Hopwood, 1983) or SLP1 (Chater *et al.*, 1988; Hutter and Eckhardt, 1988) of *S. coelicolor*. Alternatively, should *S. clavuligerus* possess a linear chromosome, the termini of the plasmid and chromosome may share significant sequence homology, if not identical sequence, as do SLP2 and the *S. lividans* chromosome (Chen *et al.*, 1993; Lin *et al.*, 1993). Clarification of this situation may prove possible by comparison of DNA fragments detected by hybridization of a pSCL3 probe with restriction endonuclease digestion products of total *S. clavuligerus* DNA and isolated pSCL3 DNA.

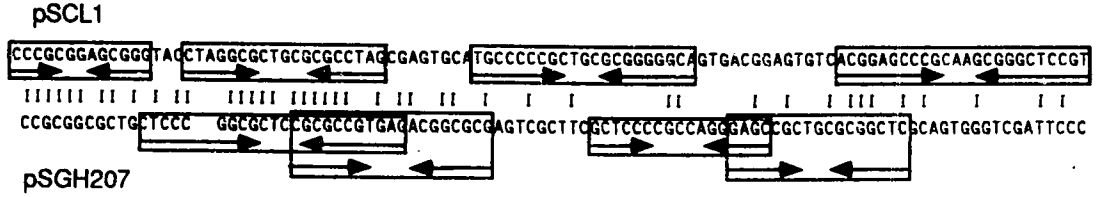
Another unexpected observation was the lack of hybridization between the sheared chromosomal DNA of *S. lividans* 1326 and the SLP2 probe in Figure 17, as Lin *et al.* (1993) report that the telomeric sequences of SLP2 and the linear chromosome are homologous. This lack of hybridization may be due to a dilution effect, as less than 2% of the probe DNA would hybridize to the sheared chromosomal target sequences. However, in other hybridization experiments (eg. Fig. 19, lane D6), hybridization between a random primer SLP2 probe and the linear *S. lividans* chromosome was observed.

The lack of homology observed between the various plasmids detected in this survey was quite unexpected, given the observed sequence similarities between the terminal sequences (Fig. 41) from pSCL1 (Wu and Roy, 1993) and the termini of other sequenced actinomycete linear DNA structures: pSLA2 (Hirochika *et al.*, 1984), SLP2 (Lin *et al.*, 1993) and *Rhodococcus sp.* pHG207 (Kalkus *et al.*, 1993) Nevertheless, the literature contains one similar report; a complete lack of cross hybridization was observed between the two giant linear plasmids of *S. avermitilis* (pSA1, and pSA2) (Evans *et al.*, 1994), and the *S. avermitilis* chromosome. Considering that the terminal inverted repeats of linear plasmids have been demonstrated to be crucial for replication via the protein primed invertron mechanism (Tamanoi, 1986; Salas, 1988), some conservation of TIR sequences would appear to be quite likely among the *S. clavuligerus* plasmids, especially in the case of pSCL1,

Fig. 41. Sequence alignments of pSCL1 and other sequenced actinomycete linear DNA structure termini. Sequences used for comparison are the S. lividans linear chromosome/SLP telomere (Lin *et al.*, 1993), the *S. rochei* plasmid pSLA2 (Hirochika *et al.*, 1984), and the *Rhodococcus* sp. plasmid pHG207 (Kalkus *et al*, 1993). Boxes around sequences indicate the limits of palindromes, arrows indicate the inverted repeat sequences. Vertical bars indicate identical bases.


pSCL1
S. lividans telomere/SLP2


pSCL1
pSLA2


pSCL1
pSGH207

which does not appear to encode its own replicative enzymes, nor terminal proteins (Wu and Roy, 1993). Furthermore, in some cases multiple linear plasmids have been demonstrated to be recombination products [eg. *Rhodococcus* sp. GLPs (Kalkus *et al.*, 1990; 1993), *S. violaceoruber* JCM 4979 GLPs (Kinashi and Shimaji, 1987)] suggesting the possibility that the multiple GLP's observed in *S. clavuligerus* and *S. jumonjinensis* may also be recombination, amplification, or deletion products of a single, original plasmid. The lack of observed homology within plasmids of the same species, let alone between species, seems to eliminate this possibility. The lack of homology suggests a variety of possible alternative explanations. Each will be discussed in some detail as follows:

1) Terminal sequences are not conserved.

This scenario is quite unexpected, as previously sequenced actinomycete linear plasmids do appear to share similarity in their terminal sequences (Hirochika *et al.*, 1984; Lin *et al.*, 1993; Wu and Roy, 1993). Furthermore, in the case of pSCL1, the terminal proteins and plasmid replication enzymes would appear to be encoded outside pSCL1, leading to the expectation that the telomeres of pSCL1 and its helper plasmid (or chromosome) should be very similar if enzyme recognition of telomeric features is to occur. A limited number of examples of linear DNA structures which share a common DNA replication apparatus yet possess very divergent telomeres have been reported. The *Kluyveromyces lactis* plasmids pGK11 and pGK12 contain inverted terminal repeats (202, 184 bp respectively) of similar length that are very rich in A and T residues yet share no common sequences or other common features (Hishinuma *et al.*, 1984; Wilson and Meacock, 1988). Even without any discernable telomeric homology, these two plasmids share the same DNA replication enzymes. This system may be similar to those of the *Streptomyces* spp. plasmids observed in this study.

2) Terminal secondary structure is conserved, but not sequence.

The terminal inverted repeat sequences of many linear DNA molecules with covalently attached proteins, such as adenovirus telomere sequences (Salas, 1991) and *Streptomyces* plasmid

telomere sequences (Hirochika *et al.*, 1984; Lin *et al.*, 1993; Wu and Roy, 1993), have been observed to contain extensive potential secondary structures. While the role of these secondary structures in plasmid replication has not been demonstrated experimentally, it is generally believed that they must have some significance in the process. Among the sequenced *Streptomyces* linear plasmid telomeres, these inverted repeats are areas of strong sequence homology (Fig. 41). The more distantly related *Rhodococcus* sp. plasmid HG207 (Kalkus *et al.*, 1993) shows less homology, but the sequence very close to the plasmid ends shows considerable conservation. Furthermore, in some cases the location and length of the inverted repeats remain a constant even though the actual sequences of these repeats share only limited homology. These observations suggest an alternative to conserved sequences being the crucial determining element in telomere function; perhaps the single stranded secondary structure of these regions is crucial to recognition by replication enzymes. This scenario would explain the apparent lack of homology among the linear plasmids observed, and allow the replicative mechanisms of one linear molecule to recognize and replicate another. Should secondary structure alone prove crucial to telomere function and stability, the high G+C content of *Streptomyces* DNA may provide an unusually favorable environment for the origin and evolution of linear plasmids. DNA with a high G-C bias not only has a higher probability of forming sequences capable of duplex formation, but alsosecondary structures with greater stability, given that the G-C base pairs have a higher melting point than the less common A-T base pairs.

3) Conserved telomeric sequences are very short

A third alternative possibility is that the actual number of bases conserved within the linear plasmid telomeres is extremely limited, and hence is simply too small to be detected by DNA hybridization techniques. The known sequence data for *Streptomyces* linear plasmids do not support this hypothesis (Fig. 41), though certain other linear DNA structures do conform to this pattern. The strongly conserved first 20 to 40 bases (Fig. 41) in the

actinomycete plasmids pSCL1, pSLA1, SLP2, and pHG207 may be representative of the extent of telomere homology found among these linear plasmids. Adenoviruses for example, has been noted as having one strongly conserved telomeric sequence of 14 bp, found throughout that virus group (Tamanoi, 1986). The degree to which a single DNA sequence allows recognization and replication by any particular adenovirus replication apparatus, however, is uncertain. The presence of one or more small but key DNA sequences within the linear plasmid telomeres could explain the observed lack of hybridization, especially considering how difficult a short DNA sequence might be to detect, given the G+C rich DNA sequence of the actinomycetes.

4) Terminal sequences are conserved but not detectable by conventional hybridization.

The extensive secondary structures found within the TIR of the streptomycete linear plasmids sequenced suggest a fourth possible explanation for the observed lack of hybridization. As hybridization involves base-pairing between probe and target DNA during annealing, both the target and the probe DNA must be single-stranded. Should the target and/or probe DNA contain large stable inverted repeats, these secondary structures will rapidly form stable duplexes (unimolecular reaction), far more rapidly than the annealing reaction between the probe and the target (bimolecular reaction). Typically, hybridization conditions are such that duplex structures have to be quite large to remain stable; for example, the hybridization conditions used for most of the experiments in this project (60°C, 20% formamide) had an effective temperature of 74°C. At that temperature, a duplex of 20-25 base pairs should be required for 70% G+C DNA to remain stable. The inverted repeats found in the telomeres of sequenced *Streptomyces* linear plasmids are usually much shorter and hence in principle should not interfere with the hybridization reaction. However, one of the potential stem loop structures (GCGAAAGC) found in the pSCL1 TIR sequence has been independently and coincidentally identified as a hyperstable secondary structure (Hirao *et al.*, 1992;

1994) with a melting temperature (76°C) far higher than would be predicted by conventional theory. Should a significant number of the other potential secondary structures in the TIR have comparable stability, or even stability significantly higher than would be traditionally expected, the ability of conventional hybridization techniques to detect homologous sequences within the TIR might be considerably reduced.

A novel linear DNA replication mechanism has been reported by Chang and Cohen (1994) for the pSLA2 plasmid of *S. rochei*. This plasmid replication mechanism involves an origin of replication within the linear plasmid acting as an initiation site for a conventional bi-direction replication bubble, instead of the protein-primed terminal initiation of the adenovirus model. According to this research, protein primed DNA replication is responsible for synthesis of only 280 terminal bases of the lagging strand. While this replication mechanism has thus far been observed in only one plasmid, it could explain many unusual observations about *Streptomyces* linear plasmids and chromosomes. First, the ability of some of these structures to interconvert between linear and circular forms is explained by the simple fact that in both topological states essentially the same replication mechanism is used. Second, many of the linear DNA structures analyzed have been found to contain what was thought to be a cryptic, yet fully functional circular origin of replication. Should the mechanism proposed by Chang and Cohen (1994) be used by these molecules, then the origin of bidirectional replication would be fully functional. Third, the invertron replication mechanism produces a free single strand of DNA which requires protection by a single-stranded DNA binding protein (Salas, 1991). While adequate for smaller linear plasmids and viruses, this replication mechanism could prove problematical for replicating larger linear plasmids, let alone entire bacterial genomes. The degree to which this mechanism is present among the streptomycetes is uncertain, but given the clear sequence homology between pSLA2, pSCL1, SLP2, and the telomeres of the *S. lividans* linear chromosome, it seems possible, if

not likely, that this mechanism is widespread. The question of how strictly telomere sequences would be conserved among linear DNA molecules using this mechanism awaits further research, but given the reduced role of terminal sequences in replication, sequence conservation may be less dramatic than within linear DNA molecules which replicate via the adenovirus model, with corresponding implications for the degree of telomeric homology observed between the plasmids in this survey. Elucidation of the distribution of this plasmid replication model, and its precise DNA sequence requirements awaits further research.

Another interesting observation which may indicate a reduced importance for TIR conservation is that following transformation, a linear DNA without any attached terminal proteins can have proteins attached to its telomeres, and replicate (Shiffman and Cohen, 1992). In their research, pSCL1 was transformed into *S. lividans* in a number of forms, including as a protein/DNA complex, and as an almost complete DNA molecule. Interestingly, the protein/DNA complex was never maintained, even under selective conditions, though the naked DNA molecule was transformed, replicated and maintained with a low copy number and frequency. While pSCL1 is never maintained at a high copy number, the fact that the presence of a foreign terminal protein prevented replication but the addition of the 'local' terminal protein allows a DNA molecule to replicate, suggests that the covalently linked protein is perhaps the key component, not the telomeric sequences. This suggestion has a number of interesting implications, especially when one considers the possible origin of linear plasmids in actinomycetes. Perhaps naked linear DNA molecules are simply recognized by the local linear DNA replication system, have proteins attached, and are then maintained by the host cell. In conjunction with the hybrid circular and protein primed replication system (Chang and Cohen, 1994) discussed previously, an origin of circular replication might be the other key requirement in the creation and maintenance of actinomycete linear DNA/protein complexes.

Generating restriction maps of the linear plasmids discovered during this survey proved relatively simple in the case of the smaller plasmids pSJL1 and pSJL2, and much more difficult for the larger GLPs pSCL2, pSCL3, pSJL3, and pSJL4. While it proved impossible to resolve pSJL1 and pSJL2 DNA using sucrose gradient protocols due to their similar size and migration, the lack of homology between these two plasmids allowed restriction site determination by hybridizing restriction endonuclease digests of preparations containing both pSJL1 and pSJL2 with either a pSJL1 or pSJL2 probe (Figs. 25, 27, and 28). The same digests could often be analyzed for both plasmids, simply by reprobing Southern transfers with a second radioactively labelled plasmid probe. Fortunately, a number of suitable sites were identified allowing analysis of the restriction map using single and double digests of the plasmid DNA.

Comparison of the restriction maps for pSJL1 and pSJL2 indicated no obviously common sites (Fig. 30). Similarly, neither of these plasmids shared any apparent similar sites with pSCL1, surprising considering the apparently identical size of pSCL1 and pSJL1. These results, however, do confirm the observations of the hybridization studies conducted on them; pSJL1 and pSJL2 are not products of a recombination or deletion process, but are apparently completely unrelated. The lack of any apparent similarity between these plasmids is once again unexpected; there are no apparent signs of shared telomeric sequences. While the presence of symmetrical *Eco* RI sites 1.1 kb from either end of the pSJL2 plasmid may be coincidential, it may also indicate the presence of long terminal inverted repeats. The actual extent of these putative repeats will require sequencing this plasmid, or at least the construction of a much more detailed restriction map.

Restriction mapping of the giant linear plasmids detected in this survey has been more problematic. The major problem encountered during this study involved difficulty in conducting restriction digestion of agarose embedded DNA, either samples prepared by *in situ* lysis of whole cells for PFG, or excised linear

plasmid bands. A wide variety of techniques was attempted (see Materials and Methods), all of which failed, producing either no discernable restriction digestion products, or a large smear of apparently randomly cut DNA. While the exact problem with these protocols was not determined, a number of possible explanations can be proposed. Several protocols for restriction digestion of *in situ* whole cell DNA preparations noted that SDS or similar detergents were removed by an unspecified method (example; Lin *et al.*, 1993); should these compounds remain with the agarose block, restriction enzyme activity may well be inhibited. Another observation was that in almost no published reports of studies of *Streptomyces* spp. are DNA bands cut out of a pulsed-field agarose gel, and then digested with restriction endonucleases. A recent paper (Gravius *et al.*, 1994b) however, seems to be an exception, as cut out bands of agarose embedded giant linear plasmid DNA were directly restriction mapped. The authors noted that the source bands were not ethidium bromide stained, but rather had their position estimated prior to excision by staining a duplicate lane to act as a guide. This technique appears very successful, and while Gravius *et al.* (1994b) did not indicate why they used material which had not been stained with ethidium bromide, their technique suggests that either ethidium bromide or exposure to UV light may have caused the problems observed during attempts to restriction digest excised giant linear plasmids. Should either of these digestion techniques have proven successful during this project, hybridization with random primer probes from isolated GLP DNA would have allowed the identification of which bands represent restriction digestion fragments of the target plasmid.

Fortunately, a protocol was developed to allow the isolation in aqueous solution of some of the GLPs detected in this project. Sucrose gradient centrifugation of high proteinase mw DNA preparations was an efficient way to separate giant linear plasmids from both chromosomal DNA and smaller linear plasmids (Fig. 24). The resulting isolated GLP's could then undergo restriction endonuclease digestion by conventional methods. While the

restriction analysis of these plasmids has not been completed, due in part to the lengthy preparation time required to obtain purified plasmids and the failure to detect any conveniently positioned restriction sites, this technique should ultimately allow for the construction of restriction maps for these plasmids. Again, purification of pSJL4 from pSJL3 by sucrose gradient centrifugation was not possible, though the lack of homology should allow restriction mapping following restriction endonuclease digestion, Southern transfer, and hybridization with individual pSJL3 and pSJL4 probes. The sucrose gradient technique appears to have a maximum plasmid size limitation; pSCL3 was not recovered in significant quantities, likely due to mechanical shearing during the high proteinase mw DNA preparation. Modifications to the procedure used will likely allow isolation pSCL3 and other larger plasmids intact

Attempts to create a restriction map for pSCL2 ultimately proved unsuccessful. A survey of the number and size of fragments produced by a selection of restriction endonucleases revealed very few enzymes which seemed suitable for further analysis. In particular, no enzymes cutting at a single site were identified. *Nde* I and *Spe* I were selected for further analysis, and although successful double digests were conducted, the fragments produced did not produce an unequivocal restriction map. Successful restriction mapping of this plasmid will likely require identification of additional suitable restriction endonuclease sites. While this sucrose gradient extraction procedure does appear feasible, hybridization to restriction digests of *in situ* DNA preparations would be preferable for simplicity and speed.

A second technique for analysis of giant linear plasmid restriction maps used the DNA isolated by the phenol-protein interface protocol (Fig. 33). While this experiment only represents an initial trial of the technique, the results appear promising. A large number of faint DNA bands were detected by Southern hybridization of restriction endonuclease digested phenol-protein interface DNA with a radioactively labelled pSCL2 probe. Since

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bands detected sh
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The general
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Rhodococcus sp.
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(Lydiate *et al.*,
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these bands did not correspond to any pSCL1 fragments (which were easily visualized on the ethidium bromide stained gel), the bands detected should represent pSCL2 DNA fragments. The large number of bands observed indicates that the phenol-protein interface protocol is not especially harsh (minimal shearing), and produced quite large DNA fragments. As the initial goal of this experiment was specifically to investigate the DNA sequences located close to the telomeres (and attached proteins), the lack of shearing was ultimately counterproductive. The original expectation was that the DNA fragments observed would be strongly biased toward the terminal few kb of DNA. These observations however suggest another possible refinement upon the original technique; restriction endonuclease digestion of the initial phenol-protein interface extracts, followed by a phenol extraction, retention of the phenol/water interface layer, then proteinase K digestion. This protocol should eliminate all DNA fragments except those with covalently attached proteins, thus identifying the terminal DNA fragment produced by digestion with any particular restriction endonuclease. Should this technique prove successful, rapid analysis of the restriction sites in the putatitive TIR should be possible, representing a source of data particularly useful in comparing telomeres of different linear plasmids. The fragments identified and isolated by this protocol might also be useful targets for cloning and sequencing efforts.

The general origin and function of the plasmids discovered in this project are uncertain. These plasmids do not appear to be rearrangements or variations of one or more GLP's, unlike some *Rhodococcus* sp. plasmids (Kalkus *et al.*, 1993), nor integrations of one plasmid into another forming a ladder (SCP1 and SCP2) (Lydiate *et al.*, 1985). The presence of the plasmids was not associated with any easily identifiable phenotypes. Had these plasmids been found to carry the *pcbC* gene or gene for other enzymes involved in antibiotic synthesis, the function and selection of these plasmids would be quite obvious. However, although these large antibiotic synthesis clusters could fit on the GLPs in *S.*

clavuligerus, *S. griseus* and *S. jumonjinensis*, hybridization indicated a chromosomal location for the *pcbC* gene in all cases, and by inference, the whole of the β-lactam biosynthetic gene cluster. The location of the methylenomycin gene cluster on SCP1 (Kirby and Hopwood, 1977; Kinashi *et al.*, 1987) appears to be an exception, rather than the rule.

Given that the giant linear plasmids in *Streptomyces* can account for a very significant fraction of the genome (up to 8% for the linear plasmids in *S. clavuligerus* and *S. jumonjinensis*), it would seem likely that some important functions are served by these plasmids. Attempts to cure pSCL1 and pSCL2 have failed repeatedly, and since no spontaneous plasmid loss events were observed during this study, some mechanism must exist which ensures the maintenance of these plasmids. The limited number of genes which have been identified as residing on *Streptomyces* linear plasmids are not typical of plasmid phenotypes. pSRM (43 kb) has been demonstrated to be necessary for the development of aerial hyphae in *Streptomyces rimosus*, a trait which would be expected to be encoded chromosomally (Chardon-Loriaux *et al.*, 1986). The sporulation associated proteins detected on SCP1 (Willey *et al.*, 1991) are other examples of proteins which would typically be encoded by chromosomal genes. These observations suggest that at least some of these plasmids may simply be mobilized segments of the bacterial chromosome which have been excised at some time in the past and are maintained because the genes they encode are indispensible. In effect, this hypothesis proposes that giant linear plasmids could be considered a part of a segmented genome. The ability of SCP1 to insert and excise from the bacterial chromosome is well recognized, as is the observation that this process may result in chromosomal sequences relocating into SCP1 (Chater and Hopwood, 1983). Another case of a linear plasmid carrying chromosomal genes is that of pPZG101, which has also been observed to both integrate into the host *S. rimosus* chromosome, and to exist in an enlarged (1 Mb vs 387 kb) form which contains the chromosomal sequences encoding the

oxytetracycline synthesis genes (Gravius *et al.*, 1994b). The integration and excision of SLP1 (Chater *et al.*, 1988; Hutter and Eckhardt, 1988) may illustrate another similar mechanism for the mobilization of chromosomal sequences. The statistical frequency of mobilization and transfer of chromosomal genes increases in strains which possess certain linear plasmids (eg. SCP1, SLP2, and SLP3) (Chater and Hopwood, 1983; Hopwood *et al.*, 1983), again indicating that these plasmids interact with the genome in some fashion, and facilitate movement of genetic material in and out the genome.

Alternatively, these plasmids may represent some form of 'selfish' DNA. The only fully sequenced *Streptomyces* plasmid, pSCL1, appears to follow this model, as it contains an ORF (ORF-L) encoding a protein similar to the *KorA* protein of pIJ101 (Wu and Roy, 1993) likely part of a classic *kil-kor* system which ensures plasmid maintenance. A *kil-kor* system is also a reasonable explanation for the persistant failures to cure *S. clavuligerus* of this plasmid. The method of integration of SLP1 [insertion into a crucial $tRNA^{tyr}$ gene (Vogtli and Cohen, 1992)] also suggests a selfish DNA 'element', implying that the inactivation of a crucial gene is necessary to prevent the plasmid from being lost. The common pock formation phenomenon associated with some of these linear plasmids [(SLP2 and SLP3 for example (Hopwood *et al.*, 1986)] resembles a bacteriocin like function, a trait once again associated with selfish DNA.

Another possible explanation for some of the plasmids identified involves a viral role; these plasmids might represent the prophage form of latent bacteriophages. While no extracellular forms of these plasmids were detected during an examination of material in liquid media by electrophoretic and electron microscopic means, this possibility cannot be eliminated. Should these phage be lysogenized, only the correct environmental stimuli might trigger a lytic growth cycle, explaining the absence of a free form in the supernatant. Some of the traits associated with these plasmids, such as pock formation and transfer of genetic material

could be explained by a prophage role. Another curious observation concerns the surprising frequency of similar sized linear DNA molecules identified in this survey; pSCL1 and pSJL1 are 11.7 kb, and pSCL2 and pSGL1 are both 120 kb. While these plasmids show no apparent homology, the similar size is reminiscent of the common sizes of DNA molecules packaged in certain 'head full' bacteriophage systems (Streisinger *et al.*, 1967). Even if these plasmids do not represent virus prophage, perhaps they were originally mobilized and packaged by a phage replication system at some time in the past.

One of the more interesting questions concerning *Streptomyces* linear DNA molecules is simply why the linear topology is utilized, and why does it appear to have such a widespread distribution? The discovery that not only plasmids, but also bacterial chromosomes may be linear indicates that this DNA topology is not only widespread, but perhaps the norm within this group of bacteria. While only one example has been studied extensively, the fact that the *S. lividans* linear chromosome and the SLP2 plasmid share identical terminal sequences strongly suggests a shared DNA replication machinery (Lin *et al.*, 1993), indicating that the linear DNA replication system is not strictly a plasmid or chromosome phenomenon, but rather a common, if not universal method for DNA replication in streptomycetes. The evolution of this DNA replication method by simple genetic drift also seems less likely than that the replication method affords some selective advantage to these bacteria. Nor is the linear structure apparently a developmental cul-de-sac from which the streptomycetes could not escape; the ability of *S. lividans* to remain viable after its chromosome was re-circularized by simple recombination (Lin *et al.*, 1993) indicates that these organisms could very rapidly return to a more conventional chromosome topology, should selective pressure favour such a development.

It is generally believed that linear DNA molecules form some type of circular structure *in vivo*, most likely by the non-covalent association of their terminal proteins, or proteins associated with

the chromosome or plasmid telomeres. Association of terminal proteins to form a circular or concatemeric structure has been well established in adenovirus and ϕ29 (Ortin *et al.*, 1971; Robinson *et al.*, 1973). A circular structure appears crucial, as the necessity of DNA molecules retaining a supercoiled state for gene transcription and DNA replication has been well understood (Orlica, 1984; Wang, 1985). Chromosomal supercoiling has also been implicated in response to changes in environmental conditions (Wang, 1985). One of the major differences between the conventional circular chromosome and a linear chromosome could be the topoisomerase systems used to introduce or remove supercoils from the DNA molecule. Instead of the well understood DNA strand breaking topoisomerases used by bacteria with circular chromosomes, bacteria which possess linear chromosomes might utilize an entirely different and distinct type of protein, one which does not break the DNA strands but rather introduces or removes supercoils at the already existing gap between the two telomeres. The enzymes involved in a telomere associated system would likely have little, if any, shared homology with the previously identified topoisomerases. This potential change in topoisomerase systems may represent a significant selective advantage to streptomycetes in their native environment, as the conventional topoisomerase enzymes are a frequent target of antimicrobial agents such as novobiocin, coumermycin and ciprofloxacin. While the degree to which antibiotics are a significant factor in the soil environment is of considerable controversy (Horinouchi and Beppu, 1990), this shift in topology and the associated topoisomerases may provide a selective advantage. The fact that these organisms can survive with a circularized chromosome, and the identification of a number of streptomycete DNA topoisomerases (Zimmer *et al.*, 1990) does suggest that conventional topoisomerase systems are still present, however, and studies of the presence and expression of these genes may prove of considerable interest. Studies of the topoisomerases of *Streptomyces noursei* have shown a far higher resistance to topoisomerase targetted antimicrobial agents than *E. coli*, suggesting

a selective advantage to developing resistant gyrase enzymes (Storl *et al.*, 1991; 1994). Similarly, the presence of a number of conventional circular plasmids in some *Streptomyces* spp. raises the question of how these structures regulate their own supercoiling, perhaps encoding their own topoisomerase systems. For example, the ability of pSCL1 to function as a CCC in *S. lividans* (Shiffman and Cohen, 1992), and to coexist with its host's linear chromosome and plasmids, offers an opportunity to investigate this phenomenon, as does the SCP2 circular plasmid, which exists in a host which contains a linear plasmid, and linear chromosome.

Another explanation for the advantages of maintaining a linear chromosome, and other linear DNA molecules, involves the possible association of a linear chromosomal topology with the high rate of DNA recombination, rearrangement, amplification and deletion events. Genomic mapping of various *Streptomyces* spp. has revealed that many of the rearrangements, amplifications and deletions are clustered in an area of the genome which early researchers have named the "arm" region (Kieser et al., 1992). Recently this region of the *S. lividans* and *S. coelicolor* chromosomes has been identified as the location of the chromosomal telomeres (Chen *et al.*, 1993; Lin *et al.*, 1993). While the evolutionary and adaptive role of these alterations in genome structure are poorly understood, they may serve to enable rapid response to changes in environmental conditions. The pPZG101 and SRP2 plasmids of *S. rimosus* have been observed to incorporate chromosomal sequences, thus produce altered gene expression levels, including that of the genes encoding enzymes for the biosynthesis of oxytetracycline antibiotic (Gravius *et al.*, 1994b). Given both linear plasmid and chromosomal molecules could exchange genetic information by a single crossing-over event, genetic exchange would be a far simpler process than the imprecise excision mechanism observed during integration of conventional circular chromosomes and plasmids (Hayes, 1986). Frequent recombination likely assists the creation and evolution of new genes, a function also suggested for the extreme base content of actinomycete DNA

(Uchiyama and Weisblum, 1985). Chromosomal telomeres have been implicated in switching the expression of variable surface antigen genes in eukaryotes (*Trypanosoma* spp.; Borst, 1986), and prokaryotes (*Borrelia hermsii*; Restrepo *et al.*, 1992) and perhaps an analogous function could also exist in these organisms as well. Nevertheless, the modifications to the streptomycete genomes during these events can be dramatic and extensive, examples amounting to as much as 25% of the total genome (Leblond *et al.*, 1990). This genomic flexibility likely provides some evolutionary advantage, and perhaps the linear DNA molecule structure facilitates this process.

In the previous discussion, while attempting to suggest an adaptive function for the unusual DNA structures observed in actinomycetes, recognition should be given to the unnatural conditions in which these organisms are grown and studied. The simple fact that the soil environment may be very different from any of the conditions the actinomycetes commonly encounter in the laboratory could lead to quite unexpected, if not unnatural responses. This observation may prove especially relevent to the high rate of recombination, deletion and amplification events encountered in the laboratory, which upon cursory examination appear quite maladaptive.

Another possible complication in the analysis of the role of linear DNA structures in *Streptomyces* is that the frequency of linear structures in the genus *Streptomyces* may be overestimated, as some of the evidence for linear chromosomes and plasmids may not be as definitive as some publications have indicated. In particular, the evidence for protein terminated linear chromosomes described in Lin *et al.* (1993) contradict the observed behaviour of other covalently linked protein/DNA complexes, and may have failed to examine all possible explanations for the observed phenomena of DNA failing to enter agarose gels. While the data indicating that the *S. lividans* chromosome is linear (Leblond *et al.*, 1993; Lin *et al.*, 1993) is quite definitive, Lin *et al.* (1993) state that during electrophoresis of *in situ* PFG DNA preparations, proteinase K

treated linear chromosomes and plasmids migrate into the gel, but without proteinase K digestion only circular chromosomes and plasmids migrate normally, while linear protein-terminated structures fail to enter into the gel. The rationale for this phenomenon is that the terminal proteins of linear plasmids prevent migration into the gel, while the circular structures migrate normally, as they have no attached proteins. The circular genome control used for this experiment was the *Escherichia coli* genome. This analysis fails to examine the possibility that proteins other than those covalently attached to the telomeres may be retarding migration. Evidence that terminal proteins dramatically reduce migration under conventional agarose gel electrophoresis appears absent; in fact migration shifts appear to be very limited. Kalkus *et al.* (1990) report electrophoresis of *Rhodococcus* pHG207 telomere DNA fragments with or without their attached proteins and observed that protein/DNA complexes migrated only slightly slower than the DNA alone. In our own research, a similar result was observed; linear plasmids with attached terminal proteins migrated at almost the same rate as the plasmid DNA alone, and were not excluded from the gel. Lin *et al.* (1993) have conducted a similar study and show a gel containing a 1.6 kb chromosomal telomeric DNA with or without covalently attached terminal protein. The migration of the protein/DNA complex is slightly retarded when compared to the DNA alone. The extent of the decrease in migration is impossible to assess, however, as the figure does not include any molecular weight markers. Furthermore, Lin *et al.* (1993) present results of an electrophoresis experiment in which non-proteinase K treated SLP2 plasmid failed to enter a CHEF gel, an observation attributed to its linear structure. The potential role of detergents in allowing electrophoresis of DNA molecules, either circular or linear, into gels by disassociation of attached proteins was also not addressed. The possibility that incomplete cell lysis may account for some of the observations of Lin *et al.*, (1993) is discussed only to note that lysis appeared incomplete in some of their preparations. While a quantitative analysis of the effect of

covalently attached terminal proteins upon DNA migration in CHEF gels has not been published, the results observed in conventional agarose gel electrophoresis do not directly support the conclusion that terminal proteins alone may be sufficient to eliminate entry of the linear DNA structures into the CHEF gel; other DNA binding proteins not found in *E. coli* may be responsible. While these proteins may interact with the telomeric sequences and or terminal proteins, thus retarding only linear DNA structures, the relationship between chromosome topology and a failure to enter the CHEF gel has not been conclusively demonstrated. The conclusions of Lin *et al.* (1993), and in particular their method of rapid determination of plasmid and chromosome topology, may provide a misleading assessment of the DNA topology in *Streptomyces* spp. In addition, the potential effects of the linear plasmid replication fork were not addressed. The use of a circular *Streptomyces* chromosome, such as the deletion mutant form of the *S. lividans* chromosome mentioned by Lin *et al.* (1993) could prove a useful control.

A third potential problem with the research presented here concerns the apparent sizes of DNA molecules as measured using CHEF electrophoresis. A significant difference between the migration of high G+C and low G+C DNA has been noted by Gravius *et al.* (1994a); the high G+C DNA is noted as migrating somewhat faster than anticipated, leading to underestimates of DNA fragment length by as much as 10%. Gravius *et al.* (1994a) noted that this situation is most pronounced in DNA fragments of under 100 kb, and the effect decreases as molecular length increases. In the research presented here, the CHEF molecular weight markers (*Saccharomyces cerevisiae* chromosomes, and λ concatameres) possess G+C contents of 38% and 50% (Sanger *et al.*, 1982) respectively. As a result, molecular weight determinations obtained via CHEF electrophoresis must be treated with a certain degree of caution. Since this variation in apparent molecular size is significant, though not overwhelming, any distortion does not necessarily invalidate our observations, but does reflect a need to recognize this variable. Use of high G+C molecular weight markers

would alleviate this problem; a number of cohesive end actinophages have been suggested to serve in just that role.

The discovery and analysis of the pSL1A and pSL1B has been accompanied by a number of unanticipated and anomalous observations, indicating that these structures are very distinct and different from the other extrachromosomal elements identified during this survey. pSL1A and pSL1B were recovered from the phenol-protein interface extract of *S. lividans* TK19, a strain believed to contain the uncharacterized genetic element SLP3 but to be cured of SLP2. Repeated examination of phenol-protein interface extracts from *S. lividans* 1326 detected the 50 kb SLP2 plasmid, but no trace of pSL1A and pSL1B was found by either direct visualization using ethidium bromide staining, or by Southern hybridization using *S. lividans* TK19 derived pSL1A and pSL1B plasmid probes. Preparations of DNA from *S. lividans* TK19 using the conventional phenol chloroform and proteinase low mw methods did not contain either of the pSL1A and pSL1B bands. The presence of pSL1A and pSL1B in the phenol-protein interface DNA preparations indicated the existence of a phenol soluble component, but the apparent failure of the proteinase low mw protocol to recover this DNA species was puzzling. Possible explanations for this phenomenon included a very proteinase resistant protein, a protein sequestered from proteinase K in some manner, or perhaps the presence of a non-proteinaceous phenol soluble element.

The research which followed was an attempt to clarify the nature of these DNA species, and to propose a model for these unconventional structures. The following discussion is an attempt to integrate these observations.

First, the pSL1A and pSL1B plasmids are clearly related, as is implied by their designation. Whether these plasmids are deletion or amplification products was impossible to determine at this point, but the fact these plasmids hybridize strongly to one another (Fig. 19), respond in the same fashion to DNA specific enzymes (Fig. 37, 39), and share some (*Bgl* II, *Kpn* I) but not all (*Bam* HI, *Eco* RV) restriction endonuclease sites (Fig. 35) indicates that pSL1A and

pSL1B are closely related but not identical. Characterization of pSL2 was limited, as insufficient quantities were isolated for detailed examination. Beyond the fact pSL2 may share sequence homology with pSL1A and pSL1B, little more can be concluded.

The native form of pSL1A and pSL1B appears to be a strongly supercoiled circular structure. These molecules consistantly migrate at apparent sizes of 6.3 kb (pSL1A) and 5.0 kb (pSL1B). Direct visualization of these plasmids using electron microscopy (Fig. 40) revealed a supercoiled DNA structure which relaxed into a circular molecule after a brief DNAse treatment. To assess if perhaps the pSL1A and pSL1B plasmids had terminal proteins which created a very strong association, forming in effect a circular structure, proteinase K and non-proteinase K digested *S. lividans* TK19 phenol-protein interface material were electrophoresed in the presence and absence of SDS. In all cases, the electrophoretic behaviour of the pSL1A and 1B bands remained unchanged; no slower migrating 'relaxed' structures appeared. This observation indicated that the apparent secondary structure was not the result of non-covalent protein-protein interactions typical of adenovirus and ϕ29 terminal proteins (Ortin *et al.*, 1971; Robinson *et al.*, 1973), as SDS should have disrupted those bonds, relaxing the topologically constrained structure. Sucrose gradient fractionation of phenol interface *S. lividans* TK19 DNA also demonstrated the compact nature of the native form of these plasmids, as both pSL1A and pSL1B (Fig. 36) clearly migrate faster than the sheared DNA. Thus, the natural form of this plasmid appears to be a strongly supercoiled circular molecule.

In addition to the native supercoiled form of these plasmids four additional versions of these plasmids were detected, with characteristic sizes when compared with linear molecular weight markers: pSL1A migrating at 15.0 and 11.4 kb, and pSL1B migrating at 10.4 and 9.2 kb. These new forms were created under a diverse variety of conditions and show traits which indicate they represent a relaxed circle and linear form of the native plasmid structure. A point which caused considerable confusion was the

very similar apparent sizes of the linear form of pSL1A (10.4 kb) and the relaxed circle form of pSL1B (11.4 kb), which under certain electrophoretic conditions migrate at very similar positions during agarose gel electrophoresis. An example of a clear resolution of these two bands is illustrated in Figure 40, panel B, lane 8.

Assessments of the topology of pSL1A and 1B by two dimensional agarose gel electrophoresis (Fig. 12) indicated that some of these plasmids have adopted a new topological state, as ultraviolet irradiation produced a new slower migrating species for both pSL1A (15 kb) and pSL1B (10 kb), likely an open circle. Sucrose gradient separation of *S. lividans* TK19 DNA (Fig. 36) also revealed two additional weak DNA species whose size correspond to the relaxed structure size seen in the two dimensional gels. While these bands did not sediment as far down the sucrose gradient, they did migrate further than the sheared linear DNA, indicating that this form of the pSL1A and 1B plasmids still has not adopted a linear topology, an observation again consistant with a relaxed circle structure. A similar sized species was seen after extended incubation at 55°C with or without proteinase K, an observation which is difficult to explain (Fig. 37). As well, these DNA species were also apparently produced by digestion with certain restriction enzymes (*Eco* RI, *Hind* III), an unexpected result as restriction enzymes can normally only cause linearization of DNA molecules, not relaxation of a supercoiled structure.

The linear DNA structure is visible in Figure 35 after restriction digestion with several enzymes (*Bgl* II, and *Kpn* I). Sucrose gradient fractionation of a *Bgl* II digest of *S. lividans* TK19 phenol interface extract DNA (Fig. 34, panel B) illustrates DNA species sedimenting through the gradient at the same rate as the sheared DNA, indicating their linear nature.

In an attempt to characterize the physical nature of these molecules, digestion with a variety of nucleases was used (Figure 39). Observations of rapid and complete digestion of pSL1A and pSL1B by DNAse, but of no apparent digestion with RNase, indicated these plasmids indeed do represent a DNA structure. Exonuclease

digestions failed to have any affect upon the plasmids, indicating if there are any DNA molecule ends, they are blocked in some fashion, and not accessable to the enzymes. BAL 31 nuclease produced an different result, rapidly degrading the supercoiled form of pSL1A and pSL1B, but not the relaxed circle, which persisted in trace amounts. As well, the native supercoiled form is not converted into the relaxed circle, but rather the quantity of relaxed circle form present in the original DNA sample persisted without digestion. BAL 31 acts as an exonuclease, an endonuclease in regions of single stranded polynucleotides, and also digests DNA which is under unusual topological stress. The last ability suggests an explanation for these observations; BAL 31 digested the strongly supercoiled native form of these plasmids, relaxing them and at the same time creating a nick for further exonuclease activity. The relaxed circle form does not offer a starting point for exonuclease activity, and thus persists. The effects of digestion with the single stranded polynucleotide specific enzyme nuclease S1 are illluminating, revealing two shifts in topology. Initially the supercoiled form of pSL1A and pSL1B is converted into the relaxed circle structure, and then gradually into the linear form. Clearly, nuclease S1 activity indicates some part of these plasmids is single stranded, but this domain is not accessible to Bal 31, or all forms these plasmids should have been degraded by Bal 31, not just the supercoiled form. Secondly a circular DNA structure containing any single stranded domain, or even a nick in either strand should not be able to maintain a supercoiled configuration, an apparent contradiction. The single stranded region of these plasmids is apparently quite small, as the sizes of the nuclease S1 products form a sharp band of consistant size throughout the digestion.

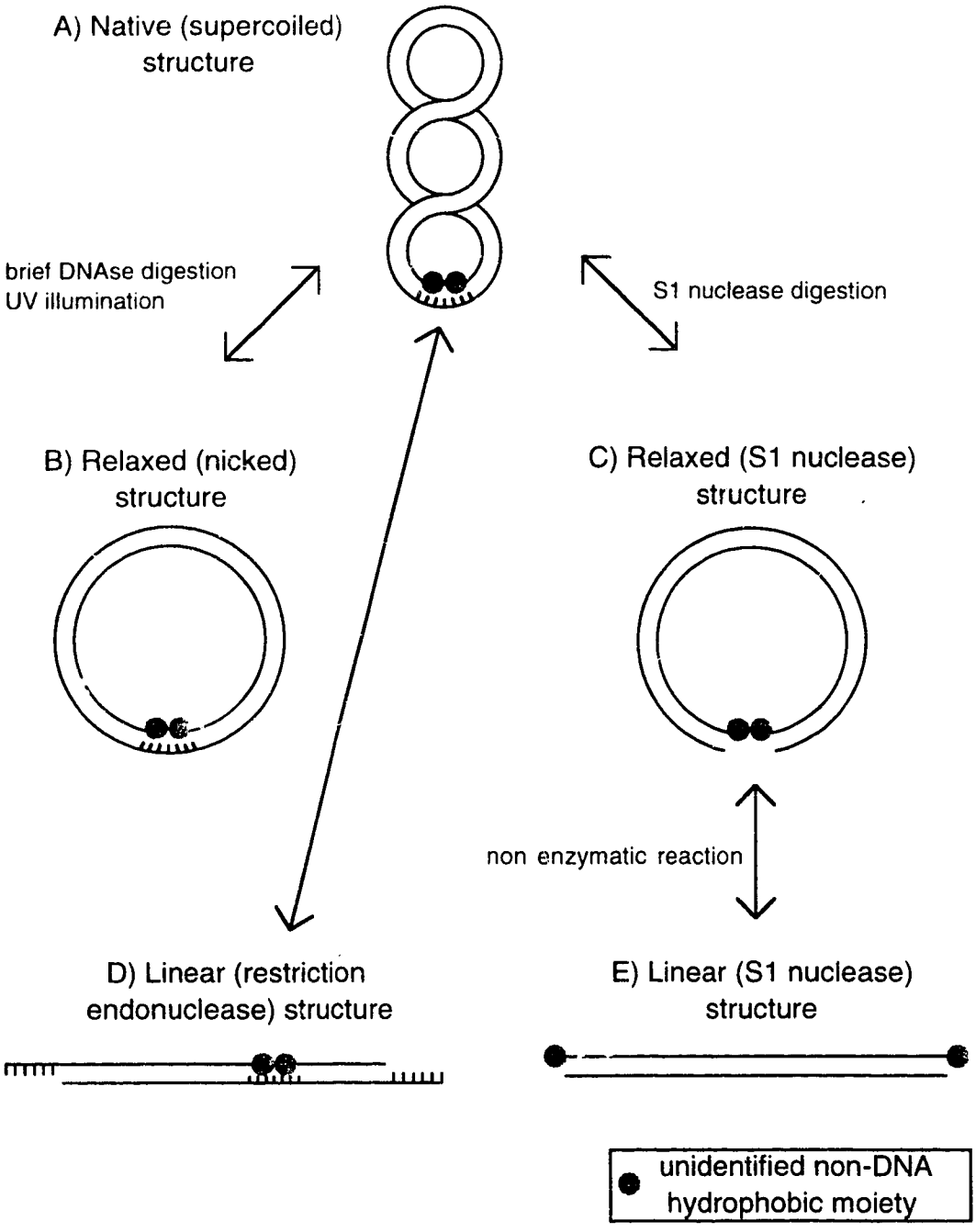
A model for a DNA plasmid structure including these features is depicted in Figure 42. This structure is a predominantly a double stranded DNA molecule with a short single stranded region. On each end of the incomplete DNA strand is a covalently attached non-DNA structure, which together bridge the single stranded region and form a non-covalent linkage. This model assumes the

non-DNA structure bridging the single stranded region has a number of properties; it blocks BAL 31 but not nuclease S1 activity upon the single stranded DNA region, and the interaction between these two elements is strong enough to retain supercoils in the DNA, even after phenol interface extraction.

While restriction mapping data for pSL1A and pSL1B is still very preliminary, a number of conclusions can be drawn. Both plasmids have single *Bgl* II and *Kpn* I sites, but a single *Eco* RV site exists only on pSL1A. The *Bam* HI digestion products indicate that one site exists on pSL1B, but two sites exist on pSL1A. The actual length of the DNA component of these molecules is approximately 11 kb and 9.2 kb (pSL1A and pSL1B respectively), the two *Bam* HI digestion products of pSL1A total 11 kb, again indicating that the DNA structures in question are linear. The ratio of the sizes of these linear structures as measured by electrophoresis (1.20 to 1) compares favorably with the relative lengths of pSL1A and pSL1B as measured by electron microscopy (1.21 to 1). The apparent relaxation of pSL1A and pSL1B into the open circle structure during some restriction e digestions is difficult to explain, perhaps an inter the buffer and the proposed non-DNA structure le stranded area of these plasmids is responsibl on.

The n ic moiety of pSL1A and pSL1B is difficult to a to sequester these plasmids into the interface, phenol did not appear to remove this plasmid f ous solutions (Fig. 38). This observation suggests that at least part of the hydrophobic component of these plasmids may be gradually lost or titrated away during the interface preparation process. Only further investigation of this phenomenon, especially by monitoring the localization of the pSL1A and pSL1B plasmids through the progress of the phenol interface extraction procedure, will clarify this anomaly. The failure of proteinase K digestion to have any dramatic effect upon these plasmids is also unexpected, especially in the failure to rapidly form a relaxed circular structure. Whether this indicates that the

Fig. 42. Proposed model of pSL1A and pSL1B structure. A) native supercoiled state, B) relaxed state, C) relaxed state (nuclease S1 digestion product), D) linear state (restriction digestion product), E) linear state (nuclease S1 digestion product).


A) Native (supercoiled) structure
brief DNAse digestion
UV illumination
S1 nuclease digestion
B) Relaxed (nicked) structure
C) Relaxed (S1 nuclease) structure
non enzymatic reaction
D) Linear (restriction endonuclease) structure
E) Linear (S1 nuclease) structure
unidentified non-DNA hydrophobic moiety

non-DNA hydrophobic component of these plasmids is not protein, or perhaps represents an extremely proteinase resistant protein awaits further investigation.

The origin and nature of the pSL1A and pSL1B plasmids is not obvious. These plasmids are obvious candidates for the previously undetected plasmid SLP3, a mobile, stable genetic element which had only been observed indirectly through pock formation (Hopwood *et al.*, 1983) and an increase in conjugative transfer of DNA (Kieser *et al.*, 1982). However, since pSL1A and 1B have not yet been observed in *S. lividans* 1326 (which does contain SLP3 according to genetic analysis), it is not likely that these plasmids are the same. The failure of pSL1A and pSL1B probes to hybridize to the *S. lividans* 1326 chromosome indicates that these plasmids are not present in an integrated form within the chromosome. One possible method of conclusively confirming whether pSL1A and/or pSL1B are equivalent to SLP3 would be to study plasmid transfer and pock formation by bacterial mating.

The observation that *S. lividans* 1326 does not appear to contain pSL1A and pSL1B, and that *S. lividans* TK19, the host strain for the pSL1A and pSL1B, is derived from *S. lividans* 1326 raises the question of the origin of these plasmids. According to Hopwood *et al.* (1983), the TK19 strain was derived by protoplast regeneration from *S. lividans* 1326, so these anomalous plasmids should be present, in some form, in *S. lividans* 1326. However, hybridization results indicate otherwise. The introduction of pSL1A and pSL1B into *S. lividans* TK19 by some form of contamination appears a possible explanation. If the lack of hybridization between the pSL1A and pSL1B probes and the *S. lividans* 1326 chromosomal DNA on CHEF gels is an artifact, the failure to detect pSL1A and pSL1B in *S. lividans* 1326 may be due to a failure to detect an integrated form of these plasmids. Integrated plasmids which may excise and exist in a free form have been reported previously, well studied examples being SLP1 from *S. coelicolor* (Chater *et al.*, 1988; Hutter and Eckhardt, 1988), the RP2 and RP3

phage (Rausch *et al.*, 1993) and pSAM2 from *S. ambofaciens* (Boccard *et al.*, 1989; Kuhstoss *et al.*, 1989; Mazodier *et al.*, 1990).

Regardless of the origin or the precise structure of pSL1A and pSL1B, these molecules represent a new class of genetic elements, ones which incorporate a single stranded domain, a hydrophobic element, and have a highly supercoiled circular native structure. Unlike the circular structure formed in vivo by invertron molecules such as adenovirus (Robinson *et al.*, 1973), these molecules appear to contain a complete DNA circle, which is neither proteinase nor exonuclease sensitive. No structures similar to pSL1A and pSL1B have been identified, and should the proposed model of these plasmids prove accurate, the Hepadnavirus structure may represent the closest analogous molecule. Regardless, an investigation of the precise structure of these molecules, their hydrophobic moiety, replication, and the relationship between pSL1A and pSL1B all promise to be productive areas for future research.

In conclusion, the research reported in this study offers a number of potential directions for further investigations. A more detailed examination of the conventional linear plasmids detected in this survey could clarify many of the questions raised in this study. Isolation and sequencing of the telomeric sequences of these plasmids should prove feasible using the phenol interface DNA preparation protocol, and allow direct comparison of these telomeric regions. Assessment of the exact phenotypes encoded by these plasmids will likely require sequencing of these plasmids, a technique which may not prove practical for some of the larger plasmids in question! A survey of genetic markers might also reveal the functions of these plasmids. The ability of the phenol interface extraction protocol to isolate intact DNA/protein complexes offers a method for the isolation of not only telomeric sequences, but also terminal proteins, possibly allowing comparison and study of the entire telomeric DNA/protein complex.

The plasmids identified within *S. lividans* TK19 offer a wealth of possible directions for investigation. Further elucidation of the actual structure of these molecules would be useful, and the hydrophobic component of these plasmids should be identified, and characterized. Generation of a detailed restriction map of these plasmids appears feasible, and could reveal the nature of the similarities and differences between pSL1A and pSL1B. Similarly, given the relatively small size of these plasmids, sequencing of the entire plasmids appears to be a feasible goal. A more detailed analysis of the origin of pSL1A and pSL1B is also desirable. Further investigations of the possible relationship between SLP3 and pSL1A and pSL1B, and whether the *S. lividans* TK19 plasmids are present in *S. lividans* 1326 are of interest.

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