Molecular Genetics of *S. thermonitrificans* ISP5579

by

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Summary

The majority of commercially important antibiotic producing Streptomyces are mesophiles, grown at 25-30°C. Fermentation at higher temperatures (ca. 45°C) leads to several process advantages eg. a significant reduction in cooling costs. Previously, entire antibiotic production pathways have been cloned from, and were heterologously expressed in, mesophilic streptomycetes. This work has tested the feasibility of expressing these cloned pathways in a thermophilic streptomycete.

S. thermonitrificans ISP55779, a thermophilic streptomycete, was identified as a suitable host. Methods for the transformation of S. thermonitrificans by plasmid DNA were developed and it can be transformed at frequencies of about $1x10^3$ transformants per ug. of pIJ702 DNA. A recombinant strain, S. thermonitrificans C5/pBROC139, which expressed genes associated with secondary metabolism in S. clavuligerus was constructed. S. clavuligerus was normally grown at 26°C but the cloned genes were expressed in S. thermonitrificans at temperatures higher than the maximum growth temperature of the donor. Thus the feasibility of expressing genes from a mesophilic species at a higher temperature, at which fermentation may be more efficient, has been proven.

A thermotolerant actinophage, TA, and a host range mutant, TAmt1, have been isolated and characterised. TAmt1 has been used to provide evidence for restriction endonuclease activity in S. thermonitrificans.

A plasmidogenic conjugative transposable element of S. thermonitrificans (STP1) has been isolated and aspects of its molecular biology have been investigated in both S.

thermonitrificans and S. lividans.

This work has provided a Streptomyces host-vector system for the heterologous expression of cloned genes using low and high copy-number vectors in both complex and minimal medium at higher, more efficient fermentation temperatures.

The work presented in this thesis is my own unless otherwise acknowledged. No part of this thesis has been previously submitted for examination leading to the award of a degree.

L. G. Czaplewski

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Abbreviations

bp.	Base pairs
CIAP	Calf Intestinal Alkaline Phosphatase
DNB	Difco Nutrient Broth

IPTG	Isopropyl thiogalactoside
Kbp.	Kilo-base pairs
L agar	Luria Bertani Agar
LB	Luria Bertani Broth
NA	Difco Nutrient Agar
OD	Optical density
PEG	Polyethylene Glycol
rpm	Revolutions per minute
RT	Room Temperature
SNA	Soft Nutrient Agar
TEMED	N,N,N',N'-Tetramethylene diamine
Tris	Tris (hydroxymethy) amino methane
TSB	Tripticase Soy Broth
v/v	Volume per volume
w/v	Weight per volume
Xgal	5-bromo-4-chloro-3-indolyl b, D-galactoside
579	S. thermonitrificans ISP5579

Chapter 1

1.1 Introduction

Streptomyces, are gram-positive aerobic soil bacteria which grow well in both submerged culture and on solid media. Streptomycetes form highly differentiated colonies on solid media with interconnected systems of two types of hyphae. Firstly, vegetative mycelia form followed, after the appropriate environmental signals, by aerial mycelia, which subsequently develop spores (Chater and Hopwood, 1973). It is this complex morphological differentiation which suggests that Streptomyces are phylogenetically distant from the Eubacteria, which include the bacteria with the simplest morphology eg. E. coli. The Eubacteria have been the subject of most physiological, biochemical and genetic analyses. Common features between these two distantly-related bacterial groups are likely to be shared by the majority of prokaryotes (Hopwood and Sermonti, 1962; Hopwood, 1967; Chater and Hopwood, 1984).

Associated with the onset of sporulation many streptomycetes produce antibiotics. Commonly 50% of soil isolates produce one or more active compounds (Omura, 1986). The first actinomycete antimicrobial compound, actinomycin, was isolated by Waksman and Woodruff (1940). Since then, many antibiotic-producing species making a number of structurally-different types of antibiotics have been isolated. Of the 6,000 antibiotics now characterised, 70 have been found to have practical applications. 90% of these antibiotics are produced by Streptomyces (Omura, 1986).

In an investigation of the range of bacteria able to undergo sexual recombination, Hopwood and Sermonti (1962) began a study of the genetics of Streptomyces. Since then, an impressive array

of manipulative techniques have been developed whereby it has been possible to characterise the genus Streptomyces more completely and to compare Streptomyces with other bacterial genera. These techniques have increased the exploitation of the healthcare opportunities presented by the prodigious capacity of Streptomyces to produce antibiotics (Chater and Hopwood, 1984).

Industry has provided some of the funding for academic groups provided that the work involves a species of industrial interest ie., producing an antibiotic. Progress in our understanding of streptomycete biology has been impeded by the diversity of Streptomyces species studied as a result. To date, streptomycete molecular biology has largely consisted of developing manipulative methodologies applicable to a number of different species. Literature and patent claims contain descriptions of plasmids isolated (reviewed Hopwood et al., 1986a), their development into cloning vectors (Hopwood et al., 1986a) and methods for protoplast formation, regeneration and transformation of many Streptomyces species (Sagara et al., 1971; Okanishi et al., 1974; Baltz, 1978; Bibb et al., 1978; Hopwood and Wright, 1978; Ochi et al., 1979; Bibb et al., 1980; Shirahawa et al., 1981; Baltz and Matsushima, 1981; Okanishi et al., 1982; Pigac et al., 1982; Thompson et al., 1982a; Baltz and Matsushima, 1983; Ikeda et al., 1983; Keller et al., 1983; Hunter and Friend, 1984; Larson and Hershberger, 1984; Ogata et al., 1984; Matsushima and Baltz, 1985; Acebal et al., 1986; Lampel and Strohl, 1986; Dominguez, 1987). Had the considerable expenditure of resources been focused upon one species of Streptomyces, a more detailed description of the genus would be possible. Conversely, if only one species had been studied intensely,

several streptomycete phenomena such as general transducing bacteriophages (Stuttard, 1979; Chung and Molner, 1983), classes of antibiotics (Crandall and Hamill, 1986) and many bioactive compounds (reviewed Demain, 1983) would have been missed. In addition, comparative streptomycete biology has been aided by the number of strains studied so that "unusual" phenomena such as plasmid pocking (Hopwood et al., 1986a), plasmidogenic sequences (Bibb, et al., 1981b; Hopwood et al., 1984), chromosomal instabilities (Hutter et al., 1981), antibiotic and bioactive compound production can be described as "normal" streptomycete behaviour.

The multispecies approach to streptomycete molecular biology, outlined above, has many advantages. However, it would seem prudent to work with one strain of Streptomyces providing all other considerations are equal (von Dohren, 1985). Hopwood has suggested that S. coelicolor A(3)2 derivative M145 should become a Streptomyces "E. coli K12" (Hunter, I. S. pers comm.). This suggestion has been met with enthusiasm as S. coelicolor A(3)2 is the most well-characterised streptomycete having been used to study chromosomal, plasmid, bacteriophage and developmental genetics as well as physiological and genetical studies of carbon catabolite repression, exoenzyme secretion and the production of antibiotics (Chater and Hopwood, 1984). S. coelicolor A(3)2 is capable of producing four antibiotic compounds, a polyketide - actinorhodin (Wright and Hopwood, 1976b), an epoxy cyclopentane - methylenomycin (Wright and Hopwood, 1976a) , undecylprodigiosin (Rudd and Hopwood, 1980) and a calcium dependent ionophore antibiotic (Rudd, 1978; Lakey, et al., 1983). The polyketide actinorhodin has been studied as a

model for other industrially-important polyketides. However, S. coelicolor A(3)2 does not produce many classes of industrially-important anti- microbial compounds eg. aminogylcosides, beta-lactams, macrolides, nucleosides, polyenes, polyethers etc. To study these one must choose other streptomycetes which produce the appropriate compound. Outwith this limitation S. coelicolor A(3)2 is expected to become the species of choice in future studies. It follows therefore that for the work described in this thesis choosing a new genetically-virgin species of Streptomyces as the focus of a long-term study requires justification.

1.2 Project Justification and Aims.

Streptomyces produce three classes of economically important products.

1) Enzymes Commercially-important enzymes include proteases for the tanning industry and glucose isomerase for the production of high fructose syrups from glucose.

2) Bioactive Microbial Compounds Compounds which may not necessarily have antimicrobial activity but possess biological activity eg., enzyme inhibitors (Umezawa, 1981), immunosuppressants, growth stimulants, and compounds affecting vasodilation, uterocontraction and diuresis (Demain, 1983).

3) Antibiotics Compounds used to inhibit the growth of undesirable organisms, "antibiotics", are, economically, the most-important class of compounds produced by Streptomyces. They are used extensively in both human and animal healthcare products.

These three different classes of compounds share not only

their origin but also the basic methodology of their manufacture. The biotechnological processes involved in their production at industrial scale are sufficiently similar that they may be analysed as one (Hacking, 1986). Cost analyses of all the stages, including product formation, extraction, packaging etc. in an industrial process can identify areas where research may lead to a reduction in costs. Such analyses reveal that reductions in capital outlay and operating costs are potentially obtainable by alterations in existing manufacturing practices (Aiba, 1983; Imanaka, 1983; Sonnleitner and Fiechter, 1983; Hacking, 1986).

Hacking (1986) suggested that the use of continuous culture, better instrumentation, more efficient recovery methods, improved reactor design, recombinant DNA technology and operation at higher temperatures would all be economically advantageous. Fermentation at higher temperatures reduces the coolant requirements and the other improvements apply equally well to a mesophilic or a thermophilic fermentation.

Existing industrial antibiotic production by fermentation of Streptomyces occurs at around 30°C in bioreactors of about 100 M^3 volume. As a result of the large culture volume the metabolic heat generated during fermentation is considerable and expensive cooling arrangements must be available to cool and maintain the correct fermentation temperature (Bader, 1986). Efficient cooling is achieved by maximising the temperature difference between the bioreactor (30°C) and the cooling fluid which is usually water. The water temperature is dependent on a number of factors such as the time of year ie. summer or winter, and the source of the water, whether from a reservoir or a cold deep well. In either

case, especially during summer, it may be necessary to further chill the cooling water using refrigeration units to increase the temperature gradient between reactor and coolant.

An alternative to expensive water chilling would be to increase the fermentation temperature. The temperature gradient between water at ambient temperature and the bioreactor at an elevated temperature of about 45°C would allow efficient cooling of such a fermentation even in summer. Aiba et al. (1983) have calculated that the requirement for cooling a thermophilic fermentation is about two thirds of a mesophilic fermentation. It has been estimated that a reduction in energy consumption would accrue, saving approximately 15 000 pounds $^{\circ}C^{-1}$ rise in fermentation temperature per large industrial fermenter per year (Stowell, 1983). Such a change in technology may increase the profitability of current fermentatons. It may improve the economics of previously unprofitable fermentations making them worthwhile, and might allow the production of antibiotics in hot climates where cooling is too costly or water is scarce.

The aim of the thermophilic streptomycete project at Glasgow is to test the technical feasibility of producing antibiotics at elevated temperature and to see if the expected process advantages materialise.

There are three approaches to elevating the temperature of an antibiotic fermentation.

1) A thermophilic organism producing the antibiotic may be identified during screening of wild isolates and used in thermophilic fermentation.

2) A mesophilic species which produces the antibiotic may be mutated and progeny screened for the ability to grow and produce antibiotic at elevated temperature.

3) The genes encoding the antibiotic production pathway from the mesophile may be transferred to a thermophilic host and antibiotic produced in a recombinant high temperature fermentation.

The commercially-important antibiotics have been discovered as a result of the screening of millions of soil isolates. Discovering a new and useful compound is a rare event. Historically, most screening programmes have been performed at mesophilic temperatures of around 30°C. Thermophiles would be excluded from such screens because of their slow growth at this temperature. With few exceptions, there is no compelling argument against the screening of thermophilic Streptomyces spp. in drug discovery programmes. There are limitations to this approach such as in screens to isolate new beta-lactam producers, where the compounds may be heat labile. At present there are fewer thermophilic than mesophilic Streptomyces spp. in culture collections. This probably reflects the historical preference for mesophilic cultivation temperatures. It is possible that thermophilic Streptomyces may provide a hitherto untapped resource of novel activities. The process advantages of thermophiles discussed below would apply to all these species.

Drug discovery programmes may adopt "thermophilic" screening methods but in order to improve existing fermentations the target of the screening programme would have to be a thermophilic streptomycete, capable of producing a chemically-identical structure to that produced by the mesophilic streptomycete which

it would replace. It would seem unlikely that all industrial mesophilic fermentations could be replaced by thermophilic species which make structurally identical compounds naturally. A thermophilic lincomycin producer has been isolated during a screening programme by Upjohn Chemical Co. (J. Coats, pers. comm.). The thermophilic producer compared favourably with the mesophilic S. espinosus strain which makes this commercially-important anticancer drug. The major difference between the species was the optimum production temperature of about 30°C for S. espinosus and 45°C for the thermophilic isolate. Upjohn chose not to pursue the option of the high temperature lincomycin fermentation available to them. Inertia was high amongst the reasons for this decision.

Using the second approach Lederle laboratories have attempted the isolation of thermotolerant S. aureofaciens strains. The normally mesophilic tetracycline producer could be mutated to grow at 40°C but tetracycline production ceased at 34°C in these strains (J. R. D. McCormick pers. comm.). Given the number of biochemical steps involved in antibiotic biosynthesis, in both the primary pathways leading to them and in the secondary metabolite-specific pathways, it is unremarkable that all could not be mutated to thermostability.

The third approach overcomes this problem by utilising the thermostable environment within the thermophilic streptomycete. All of the enzymes, structural proteins and macromolecular assemblies etc. are already adapted to growth at higher temperatures. The transfer of genes encoding antibiotic biosynthetic pathways into the thermophile reduces the problems of tailoring gene expression. If the pathway is not expressed at

elevated temperatures a good first candidate for mutagenesis would be the cloned pathway genes for the host organism, expression of its genes and their products are already thermostable.

Despite the number of Streptomyces spp. which have been the subject of genetic manipulation experiments, none had the required characteristics for this project thermophily - the ability to grow at elevated temperature. In this project a thermophilic streptomycete S. thermonitrificans ISP5579 (Desai and Dhala, 1967) has been selected and methodology has been developed for its genetic manipulation. The novel strain requirements of this project justified the development of genetic analysis and genetic manipulation for another species of Streptomyces.

No prior study of the physiology and molecular genetics of thermophilic Streptomyces had been published at the time of conception of this project, but there had been some interest in thermophilic Bacillus (Welker, 1978). Of note was the observation that at growth temperatures of less than 46°C some enzyme activities were labile, but stable to heat when grown at 50°C or greater (Campbell and Pace, 1968); Lauwers and Heinen, 1983). Amylases exhibiting this behaviour had different primary amino acid sequences (Isono, 1970). A thermotolerant streptomycete has been shown to exhibit similar growth temperature dependent cold-labile heat-stable enzymes (Heinen and Lauwers, 1983). It would appear that expression of heat-stable enzymes may be regulated by temperature in both Bacillus and Streptomyces.

Host/vector systems in thermophilic bacteria may be used to elucidate the mechanisms which control these temperature-regulated genes. In addition to examining thermophily, the capacity to genetically-manipulate thermophiles increases the potential number of microbial products available to industry and offers process advantages. These will be considered in greater detail.

1.3 Microbial Products from Thermophilic Bacteria

Novel enzymes are the most promising endogenous products from thermophilic bacteria. Enzymes isolated from thermophiles, particularly extreme thermophiles, tend to exhibit increased thermostability, enhanced resistance to denaturing agents and are more tolerant of high concentrations of reactants (Amelunxen and Murdock, 1978). The most quoted example of such a thermostable protein is the protease thermolysin from Bacillus thermoproteolyticus. The culture is grown at 55°C. but the protease is active at higher temperatures retaining 86% of its original activity after incubation at 70°C for 30 hours (Sonnlietner and Fiechter, 1983). Amylases, glucose isomerases, alcohol dehydrogenases and glucose-6-P-dehydrogenases are examples of thermostable enzymes which have been isolated from thermophiles and are finding practical applications (Sonnlietner and Fiechter, 1983). Despite the potential application of thermostable enzymes from thermophilic Streptomyces, the Glasgow thermophilic Streptomyces project is not concerned primarily with the endogenous products of a chosen strain, but rather with the development of a new Streptomyces cloning host, suitable for the expression of cloned genes at a higher temperature.

1.4 The Advantages and Disadvantages of Fermentation at Elevated Temperature

Above all other considerations industrial manufacturing processes must be profitable and the implementation of thermophilic fermentations will depend upon their economics. Most thermophilic studies have utilised tubes or shake flasks. Rarely have laboratory or large scale fermenters been used (Sonnleitner and Fiechter, 1983). Thermophilic organisms have been studied for a relatively short time so detailed knowledge of their metabolic control or of engineering problems associated with their cultivation is not available. Perhaps more important is the lack of strain development associated with thermophilic bacteria. Economic analysis of an industrial scale thermophilic fermentation is required before the cost effectiveness of this type of fermentation may be proven. Meanwhile it is possible to argue that a thermophilic fermentation ought to be more profitable by comparing the advantages and disadvantages of the proposed system.

1.4.1 Advantages of Fermentation at Elevated Temperature

1) Temperature Control

Heating may be required initially to maintain the fermentation broth at the chosen temperature, especially for fermentations at elevated temperature. The energy required during this stage of the fermentation has been calculated to be between 1.3 and 4.2% of that required for cooling later in the fermentation (Aiba et al., 1983). The cost of cooling is more important than that of heating as it represents a greater proportion of the total energy expended in temperature control. Thermophilic bacteria tend to have higher growth rates than

mesophiles (see below). Cooney et al. (1968) have shown that for mesophiles there is a linear correlation between specific rate of heat production and specific growth rate. If this linearity extends to thermophilic bacteria then a similar or higher production of heat must be expected in a thermophilic fermentation. Cooling systems will be required to maintain the optimum fermentation temperature. As previously described, for elevated fermentation temperatures the temperature gradient between the culture and coolant increases, thus enhancing cooling efficiency. Cooling systems may be of two designs. An open system is one where coolant, usually water, is pumped from the environment (perhaps chilled) and used as a sink into which heat from the reactor may be dumped. The warmer water is then returned to the environment. A closed system, as it suggests, works with a fixed volume of coolant, not necessarily water, which undergoes cycles of heating and cooling in heat exchangers. The energy expenditure in a closed system is proportionally greater as refrigeration is necessary. Closed systems are used because of the cost of large volumes of water via local authority tarriffs. A thermophilic fermentation reduces the volume of coolant required in an open system and the capacity of heat exchangers and their running costs in the closed system (Aiba et al., 1983). In both cases the energy used on cooling the fermentation is reduced in thermophilic bioprocesses.

2) Growth Rate

Arrhenius' law predicts that chemical reactions occur faster at elevated temperatures. This prediction holds true for microorganisms provided that the optimal temperature of growth for the organism is not exceeded. The rates of microbial growth

and product formation in a thermophilic fermentation are expected to be greater than for an equivalent mesophilic process (Sonnleitner and Fiechter, 1983). A direct consequence of the increased growth rates of thermophiles is that their fermentation batch times are reduced allowing more batches to be run per year.

3) Productivity

Sonnleitner and Fiechter (1983) found that thermophiles generally do not show the higher product formation rates expected of them. In the manufacture of high volume-low value products the formation rate is of primary importance in calculating overall productivity. The productivity of a process is dependent on many factors and only in special circumstances such as the production of volatile compounds, which may be easily recovered or when inhibitory concentrations of product are removed by evaporation, are advantages certain. The premise that thermophilic fermentations increase productivity when compared to mesophilic processes has not yet been proven. The effect of temperature change on productivity is probably process dependent and merits further study.

4) Broth Rheology

As fermentation temperature increases the viscosity of the broth decreases. The viscosity of water decreases by about 25% when heated from 30 to 45°C (Weast et al., 1972). A similar dramatic decrease in viscosity may be expected in fermentation broths. Less viscous broths will require less energy expenditure on aeration and agitation for the same degree of mixing.

5) Reactant Solubility

Non-gaseous reactants in microbial processes become more soluble as reaction temperature increases, allowing the use of higher substrate concentrations. The volumetric yield may be increased as more substrate per unit volume is available for conversion to product. The impact of this advantage will depend on the process. If the reactants are readily soluble at 30°C then the effect will be small, but if the reactants are relatively insoluble their increased solubility at elevated temperature may be valuable.

1.4.2 Disadvantages of fermentations at elevated temperatures

1) Reactant Solubility

The solubility of gaseous reactants falls dramatically with increasing temperature. The solubility of oxygen in water decreases by approximately 20% when it is heated from 30 to 45°C (Weast et al., 1972). A similar reduction of oxygen solubility in fermentation broths could lead to problems in aerobic fermentations. Provided that the mass transfer of oxygen to the broth is sufficient then the bacteria need not experience a lack of dissolved oxygen. Improvements in bioreactor design may be expected to overcome any problems caused by reduced oxygen solubility at higher temperature.

2) Thermostability of Product

The yield of a product is a function of its rates of formation and degradation. Whilst a moderately thermostable product could be manufactured in a thermophilic process its yield would be reduced because its rate of degradation would be expected to be significant. This may be offset by the faster batch time of a thermophilic fermentation. However, a

thermostable product with a reduced degradation rate will give better yields. This yield advantage will be enhanced further by the faster batch time. The thermostability of a product, whether enzyme or antibiotic, must be considered when choosing the temperature range of the expression system.

1.4.3 The Effect on Capital Investment and Running Costs

The major economic advantage to thermophilic fermentation is the reduction in the quantity of cooling energy required for temperature control (Aiba, 1983). Stowell (1983) has estimated the saving to be about 15 000 pounds per °C per fermenter per year. In addition, the size of the cooling plant may be reduced as the volume of required coolant is lower. This affects not only the running costs but also the capital investment in new fermentation and cooling plant.

For existing fermentations where the capital expenses have already been paid, the higher growth rate of thermophilic bacteria causes a reduction in fermentation batch time. It may be possible to produce more product by increasing the number of batches per year, or the same mass of product could be produced from fewer fermenters. This could release fermenters for the manufacture of new products. Capital investment would not be required for the new process as the fermenters have already been built.

Economically the cultivation of a thermophile is potentially more rewarding than that of a mesophile.

1.5 The Glasgow Thermophilic *Streptomyces* Project Strategy

The desired end product of the project is a fermentation system based upon a thermophilic streptomycete which will grow well over a wide range of temperatures. The streptomycete will also be amenable to genetic manipulation with both low- and high-copy number plasmid cloning vectors. This will allow the expression of cloned single gene products and the more complex pathways of antibiotic biosynthesis at elevated temperature to be tested. The expected process advantages of such a system may then be realised.

1.6 Selection of a thermophilic streptomycete suitable for the expression of heterologous gene products in a recombinant high temperature (c.45°C) fermentation.

Thermophilic *Streptomyces spp.* may be isolated from their natural habitats but are more readily available from culture collections (Williams *et al.*, 1983; American Type Culture Collection; Kaken Chemical Company etc.). About 20 thermophilic streptomycetes were obtained from these collections. The fermentation performances of these strains were evaluated in matrix experiments at shake flask level.

An ideal thermophilic streptomycete would have the following characteristics,

It should not:-

1) *Form Pellets*: The centre of a pellet is susceptible to nutrient limitation and reduced oxygen transfer. Pellet formation reduces the growth rate and should be avoided. The viscosity of the broth decreases if the culture pellets, but this advantage would not outweigh the mass transfer disadvantages of a

pelleted fermentation.

2) Produce Extracellular Polysaccharides: Production of extracellular polysaccharides increases the viscosity of the broth, and shunts both carbon and energy away from the biosynthesis of the required product.

3) Produce Proteases: The presence of extracellular proteases may reduce the yield of extracellular enzymes. This is especially important if heterologous proteins are to be overproduced and secreted.

4) Produce Antimicrobial or Bioactive Compounds: Production of compounds other than that required from the fermentation not only wastes carbon and energy in their biosynthesis, but also presents a problem in the purification of the desired compound. For healthcare, especially for human use, the final product must be free from the other activities.

It should possess:-

5) Resistance to Shear: The species must be sufficiently robust to survive the shearing forces present in fermentation broths.

6) A High Maximum Specific Growth Rate: As discussed earlier, the faster fermentation time reduces batch time. Either more product may be manufactured or the same amount may be produced from a smaller plant. Alternatively the extra capacity could be used for the manufacture of other products.

7) A High Growth Yield (g cells/g substrate): The species must be efficient in its conversion of nutrients to both biomass and product. This is especially true for fermentations involving expensive raw materials.

The strains were subjected to a variety of media at a range

of temperatures and scored for the above parameters. The strains were ranked according to their shake flask performance and the top three studied further in a fermenter trial. The same matrix experiment was performed on these three strains in both two and ten litre laboratory fermenters. One of the strains S. thermonitrificans ISP5579, also designated ATCC 233385, NCIB 10070, NCIM 2007, KCC S-0841 (Desai and Dhala, 1967) exhibited particularly good performance and was chosen for further study (L. Harvey pers. comm.)

1.7 The Organisation of this Thesis

The purpose of this section is to prepare the reader for the following chapters.

Chapter One has described the genus Streptomyces, why they are important academically and industrially and also why the thermophilic streptomycete project was started.

Chapter Two describes the materials and methods used to perform the work presented. It depends heavily on standard laboratory manuals such as Maniatis et al., (1982) and Hopwood et al., (1985). Modifications to protocols presented in these sources have been explained in detail, particularly where the modifications were necessary as a result of the peculiarities of the biology of S. thermonitificans ISP5579.

Chapter Three is concerned with the development of the methodology for the formation and regeneration of protoplasts of S. thermonitrificans. This methodology was central to the problem of plasmid transformation of S. thermonitrificans. The sensitivity of S. thermonitrificans to both lysozyme (3.3) and

sucrose (3.5) made protoplast formation and regeneration more problematic than expected.

Chapter Four describes the development of plasmid transformation systems for Streptomyces and how historical perspective allowed rapid advances to be made in the development of a plasmid transformation protocol applicable to S. thermonitrificans. Attempts were made to isolate plasmids from S. thermonitrificans (4.8) and other thermophilic Streptomyces spp. (4.9). These could have been used to construct thermostable cloning vectors if the cloning vectors used in mesophilic species did not replicate or were not stable at elevated temperatures. An interesting alternative to the use of mesophilic plasmids as transformation substrates was also considered (4.10). A thermotolerant actinophage TA (see chapter 5), able to infect S. thermonitrificans at high temperature, was used as the source of DNA in a cloning experiment designed to link the actinophage origin of DNA replication and a drug resistance gene. This attempt to construct a plasmid which contained an origin which was known to work in S. thermonitrificans failed due to the biology of the actinophage and the cloning host used.

Chapter Five is concerned with the isolation and characterisation of a thermotolerant actinophage, TA, and a host range mutant , TAmt1. TA was indirectly responsible for the choice of S. albusG 153 as a host strain from which to prepare pIJ702 for transformation into S. thermonitrificans (4.3.1). Without this, transformation of S. thermonitrificans may never have been possible. The host range mutant TAmt1 was used to estimate the level of restriction between S. lividans and S.

thermonitrificans. A similar level of restriction was observed for plasmid DNA on transformation into S. thermonitrificans.

Chapter Six describes the discovery, characterisation and biology of STP1, a plasmidogeneic transposable conjugative element of S. thermonitrificans. STP1 is a plasmid which normally resides in the chromosome, although it loops out to form cccDNA molecules at low frequency (6.12). When S. thermonitrificans was transformed with pIJ702, the copy number of the cccDNA form of STP1 increased which lead to it's discovery (6.2). STP1 transposed into pIJ702 when the two DNAs were cotransformed into S. lividans TK54 (6.5) and integrated into the chromosome whether pIJ702 was present or not (6.4). The biology of STP1 is discussed with respect to other plasmidogenic elements found in Streptomyces at the end of the chapter.

Chapter Seven is the last results chapter and is the culmination of the project. It describes the successful expression of antibiotic production genes at elevated temperature. The pathway for a yellow metabolite (thought to be holomycin) from the strict mesophile S. clavuligerus was kindly provided by Dr. M. Burnham of Beechams PLC. on plasmid pBROC139. pBROC139 prepared from S. albusG 153 was used to transform S. thermonitrificans C5. The recombinant strain produced a yellow compound similar to holomycin. However the chemical properties of the compound were somewhat different to those expected from holomycin. The structure of the yellow compound is therefore being determined.

Chapter Eight, the concluding remarks, describes the current state of the project and suggests what directions the project may take in the future.

Chapter 2. Materials and Methods

This chapter has been written with the assumption that the reader will have available the laboratory manuals by Maniatis et al.,(1982) and Hopwood et al., (1985). All of the materials used were good quality, AnalaR grade if possible. The sources of many of the chemicals used changed during the course of the three years of experimentation presented in this thesis. BDH Chemicals Ltd. Poole, Dorset, Difco Laboratories, Detroit, Michigan USA and Sigma, London Chemical Co. Ltd, Poole, Dorset were most commonly used as suppliers.

2.1 Making a Streptomyces Spore Suspension

A concentrated spore suspension was required for inoculating liquid cultures of Streptomyces spp.. For species used during this work the protocol described by Hopwood et al., (1985) was followed with minor modifications.

The solid growth medium which resulted in good sporulation was species-dependent. For S. thermonitrificans, a boiling tube containing a slant of Emerson's agar (Difco) (produced by pouring ca. 15 ml. of molten agar into the tube and allowing it to solidify with the tube placed nearly horizontal) was inoculated with 150 ul. of a spore or mycelial fragment suspension and incubated at 42°C. overnight, then moved to 37°C. After two further days incubation, the culture was covered in a dark grey mass of spores. The slant was frozen at -20°C; it could then be harvested immediately or stored indefinitely.

To harvest the spores, the frozen surface of the slant was rubbed with a 5 ml. pipette containing 5 ml. of sterile water. When the surface of the slant was scraped clean of spores the 5

ml. of water was used to wash the slant and to resuspend the spores. The spore suspension thus produced was dark grey in colour and contained little agar or mycelial fragments. Any of the latter were removed by passage through a cotton wool filter as described in Hopwood et al., (1985). The filtered spore suspension was aliquoted and frozen at -20°C. The aliquots were thawed and vortexed prior to use. The titre of the spore suspension was determined after storage at -20°C overnight and was found to be constant over the short term (3 months).

Other Streptomyces were treated similarly except that the slants contained either TSB agar eg. S. albus or R2YE agar eg. S. lividans. The mesophilic species were grown at a constant temperature of 30°C.

2.2 Growth of Streptomyces Mycelium

The methods described for the growth of mycelium by Hopwood et al., (1985) were used with minor modification. Spring flasks were not required for the dispersed growth of S. thermonitrificans, nor were glycine or $MgCl_2$ added to the culture broths for the preparation of S. thermonitrificans mycelium to be used for the formation of protoplasts. S. thermonitrificans and S. albus were grown in TSB and S. lividans in YEME. For the formation of protoplasts S. thermonitrificans was grown at 37°C. and the mesophilic species at 30°C. Routinely 100 ul. of a dense spore suspension was used to inoculate 100 ml. of broth. The volume of the culture grown depended on what the mycelium was to be used for. The formation of protoplasts required 50 ml. or less while for plasmid isolation 200 ml. or multiples of this were generally used. The flask volume was at least five times the volume of the broth to allow good aeration when shaken at

about 250 rpm.

2.2.1 Quantification of Growth

Due to the mycelial growth of Streptomyces optical density was not a good estimate of biomass. Dry weights were therefore determined according to L. Harvey (pers. comm.). Duplicate Whatmann No. 1 (GFC) filters were numbered with pencil and dried for 15 min. in a microwave oven (600 W) set on reheat and were allowed to cool in a dessicator. The weights of the filters were recorded and the appropriate filter was placed onto a standard membrane filtration unit. 5 ml. of culture was passed through the filter, followed by 15 ml. of water. The filter was dried in the microwave oven for 20 min. on reheat and cooled in the dessicator. The dry weight was recorded to three decimal places. The weight of the original filter deducted from the weight of the second reading, and the result multiplied by 200 provided the dry weight of the culture in g/l. An average dry weight was determined by taking several 5 ml. samples for each time point.

2.3 Growth of E. coli for Plasmid Isolation

The E. coli strain containing the desired plasmid was inoculated into L broth containing the appropriate antibiotic (usually ampicillin at 100 $ug.ml.^{-1}$ or carbenicillin at 50 $ug.ml.^{-1}$). The volume of the broth inoculated depended on the quantity of plasmid required, from 5 ml. for small to 1 litre for large scale preparations. The cultures were shaken overnight at 37°C. at 250 rpm in an orbital shaker.

2.4 Preservation of Streptomyces and E. coli Strains and Actinophage

For Streptomyces, agar slants covered in spores were frozen

at -20°C at which they could be stored indefinitely. This method used a lot of storage space but it did allow the harvesting of sufficient spores to begin work immediately. Smaller slopes made in 5 ml. bijous were also used for long-term storage of strains. These mini-slants were also frozen, but upon harvesting few spores could be collected and larger slants had to be inoculated, grown and harvested before work on the strain could begin. Spore suspensions could be stored frozen and these were found to be viable for at least one year when the spores were suspended in water alone. Mycelium of strains was also frozen in 20% glycerol (w/v). To revive this material the mycelium was thawed and washed with broth prior to plating.

E. coli strains were stored by growing an overnight culture (2.3) and adding an equal quantity (v/v) of sterile glycerol to an aliquot mixing and freezing at -70°C. The strains were revived by scraping the surface of the frozen suspension with a sterile toothpick and transferring the frozen cells to either liquid or steaking them onto solid medium containing drug if appropriate.

Actinophage TA and TAmt1 were stored by filter sterilising (0.22 uM filter) a cleared lysate (see 5.4.2.1). An air tight container was used to stop evaporation. The phage suspensions were kept at 4°C.

2.5 Isolation of Streptomyces Total DNA

The procedure 3 described by Hopwood et al., (1985) was used with minor modification for the production of total DNA from S. thermonitrificans and without modification for S. lividans and other mesophilic species. Cultural conditions applicable to S. thermonitrificans were used for the growth of mycelium (2.2). The

volumes were unchanged but the quantity of lysozyme used for the enzymatic degredation of S. thermonitrificans cell walls was reduced to 0.5 $mg.ml^{-1}$. Steps 6 and 7 (the removal of proteins from the cell extract by phenol/chloroform extraction) were repeated until the interface was clean.

2.6 The Isolation of Plasmid DNA

The alkaline lysis method of plasmid purification was used routinely as described by Maniatis et al., (1982). The solution volumes were kept constant but only 200 ml. of broth was used to grow cells rather than the 500 ml. suggested. The same method was used for both Streptomyces and E. coli with the exceptions that for Streptomyces the lysozyme incubation took place at 30°C. and used 0.5 $mg.ml^{-1}$ for S. thermonitrificans and 1 $mg.ml^{-1}$ lysozyme for S. lividans and S. albus.

The STET method of plasmid purification (Holmes and Quigley, 1981) as described by Maniatis et al., (1982) was also used for the analysis of E. coli clones without modification.

2.7 Preparation of Actinophage TA and TAmt1

see section 5.4

2.8 Large Scale DNA Purification from Actinophage TA and TAmt1

Actinophage TA and TAmt1 were prepared at large scale as above (2.7). After CsCl purification of the actinophage they were treated as if they were OC31 from step 11 onwards according to the purification procedure for actinophage DNA described by Hopwood et al., (1985).

2.9 Detection of Conjugative Plasmids by Pock Formation After Conjugal Transfer into S. lividans

The procedure for the detection of pock-forming conjugative

plasmids described by Hopwood et al., (1985) was used to assay for the presence of such plasmids in thermophilic Streptomyces and also to identify the pock-formation function in STP1.

2.10 Preparation of Competent E.coli Cells

The method described by Hopwood et al., (1985) was used for the preparation of competent cells without modification.

2.11 The Preparation of Protoplasts

2.11.1 Preparation of protoplasts from S. lividans

The procedure described by Hopwood et al., (1985) for the preparation of protoplasts from S. lividans was closely followed with minor modifications. Plastic Sterilin tubes (10 ml.) were used instead of 20 ml. screw cap tubes. 5 ml. of culture was used in each tube, with several tubes being used if many transformations were to be performed. The culture was centrifuged (room temperature, about 2000xg.) and the broth decanted. The mycelium was washed with 10.3% sucrose and centrifuged again. The pellet was resuspended in 5 ml. of filter-sterilised lysozyme (L) solution containing 1 $mg.ml^{-1}$ lysozyme. The suspension was incubated at 30°C and the appearence of protoplasts and disappearence of mycelium was followed by light microscopy. Step 6 was omitted and step 10 was followed by a repeat of step 9. The protoplasts were resuspended in 1 ml. of medium P. They were either used fresh for transformation or were aliquoted (150 ul) into small eppendorf tubes and frozen slowly according to Hopwood et al., (1985) for future use.

2.11.2 Preparation of Protoplasts from S. thermonitrificans Mycelium

The modifications described above (2.11.1) to the procedure described by Hopwood et al., (1985) were also used in the preparation of protoplasts from S. thermonitrificans except that the cultivation of mycelium was in TSB without additives at 37°C for 20 hours, and for lysozyme treatment 0.25 $mg.ml^{-1}$ of lysozyme was used in medium P. The concentration of protoplasts was determined by haemocytometry.

A Hawksley Cristalite "Improved Neubauer" (B.S. 748) haemocytometer was used. The haemocytometer grid consists of 25 large squares, each divided into 16 smaller squares. The area of the smallest square is 1/400 mm^2, whilst that of a larger square is 1/25 mm^2. The depth of the well is 0.1 mm. A tenfold dilution series of the protoplasts to be counted was prepared in medium P (Okanishi et al., 1974) or a derivative thereof. An excess of the suspension of protoplasts was placed onto the well and a coverslip pressed down firmly over the top. The protoplasts were left to settle before counting. This ensured that the protoplasts rested on the base of the well which facilitated focusing of the microscope. The number of protoplasts totally within each of five of the large squares was counted. Protoplasts lying on a line were ignored. From these data the arithmetic mean and standard error of the mean were calculated. Thus an estimate of the average number of protoplasts within $4x10^{-3}$ mm^3 of the protoplast suspension (or a dilution of it) was made. It was most convenient to consider the number of protoplasts per millilitre of the original protoplast suspension. Between 200 to 500 protoplasts in each large square

on the haemocytometer grid were counted. The dilution factor was adjusted to arrange this.

2.12 Plasmid Transformation of *E. coli* and *Streptomyces*

2.12.1 Transformation of Competent *E. coli* Cells with Plasmid DNA

The procedure described by Hopwood *et al.*, (1985) was followed with minor modifications. 200 ul. of competent cells were dispensed into 50 ml. Falcon tubes which contained the DNA and which had been cooled on ice. The mixture was incubated on ice for 20 min. The tubes were heat-shocked at 42°C. for 90 sec. and then placed on ice. 1 ml. of L-broth was added and the tubes incubated at 37°C. for one hour. The tubes were centrifuged at 3 000xg for 10 min. at room temperature and the supernatant poured off. The cells were resuspended in 200 ul L-broth and a portion (100 ul) and dilutions were plated onto antibiotic selection plates (usually L-agar + 100 $ug.ml^{-1}$ ampicillin). The plates were incubated overnight at 37°C.

2.12.2 Transformation of *Streptomyces* Protoplasts

2.12.2.1 *S. lividans*

The original procedure described by Hopwood *et al.*, (1985) for the plasmid transformation of *Streptomyces* protoplasts was used with minor modification. 100 ul. of fresh or thawed frozen protoplasts from 2.11.1 were used at step 5. The incubation period in step 7 was routinely 45 sec. Instead of adding medium P and centrifuging, 2 ml. of medium P was added to the transformed protoplasts and either this or dilutions of it were plated onto R2YE regeneration medium.

2.12.2.2 S. thermonitrificans

Protoplasts of S. thermonitrificans prepared as described in 2.11.2 were diluted in ice-cold medium P to a concentration of between $5x10^7$ and $1x10^8$ protoplasts $ml.^{-1}$. The plasmid DNA (10 ul.) to be transformed into the protoplasts was placed into ice-cold Eppendorf tubes and 100 ul of the protoplasts was added. After 15 sec. 500 ul. of 25% (w/v) PEG1500 in medium P (room temperature) was added. About 45 sec. later the mixture was sucked into a plastic pipette (Gilson blue tip) and dispensed into a plastic tube which contained 2 ml. of medium P at room temperature. The suspension was gently swirled and the transformed protoplasts or dilutions thereof were plated without delay.

2.12.3 Preparation of Protoplast Regeneration Plates

The method of preparation of regeneration medium for protoplasts was standardised. The media (refer to 2.28) were stored in two parts (A and B); the former solid and the latter liquid. Both were steamed until portion A melted. They were incubated at 50°C for about 30 min. to cool them and the B portion was poured into A and mixed by swirling. 1 ml. of a 1% KH_2PO_4 was then added followed by the addition of antibiotic if required. Regeneration medium was sometimes used for cultivation of plasmid containing strains. The mixture was swirled and poured into 9 cm. diameter petri dishes. 200 ml. of medium was sufficient for 8 plates on average. The plates were left half open to the air in a laminar flow hood for 45 min. when they were turned 180° and their position in the hood altered so that those at the front were positioned toward the back of hood. After a further 45 min. incubation the plates were removed to the 37°C.

incubator where they were left overnight. The next day any contaminated plates upon which colonies had developed were removed and the rest were used for the regeneration of protoplasts.

2.13 Selection of Recombinants of *Streptomyces* and *E. coli*

2.13.1 *Streptomyces*

The only drug resistance used for plasmid selection in *Streptomyces* was thiostrepton (provided by E. R. Squibb, New Jersey, U.S.A.). It was dissolved in DMSO to make a 1% (w/v) stock solution. Transformed protoplasts of mesophilic species were selected after about 22 hours non-selective growth at 30°C by overlaying the protoplast regeneration plates with 1 ml. of a 220 $ug.ml.^{-1}$ thiostrepton solution in a 10.3% (w/v) sucrose solution. Transformed protoplasts of *S. thermonitrificans* were selected after about 18 hours non-selective growth at 37°C. by either the above technique or by using 3 ml per plate of SNA or NB (Hopwood *et al.*, 1985) solidified with low melting point agarose (0.5% w/v) containing 75 $ug.ml^{-1}$ thiostrepton.

Recombinant strains were grown in liquid cultures with the addition of thiostrepton to a final concentration of 25 $ug.ml.^{-1}$.

Pock-forming plasmids were detected according to 2.9.

2.13.2 *E. coli*

The *E. coli* plasmids used in this thesis encoded resistance to both ampicillin and carbenicillin which were added to the media at a concentration of 100 or 50 $ug.ml^{-1}$ respectively.

2.14 Ethanol Precipitation of DNA

DNA was precipitated from solution by the addition of 0.1 volumes of 3 M sodium acetate and two volumes of ethanol. The solution was incubated on ice for 30 min. and centrifuged in a microfuge or larger scale depending on the final volume for at

least 30 min. at > 12 000xg. The supernatant was removed and the pellet washed with 70% (v/v) ethanol in water briefly, recentrifuged for 10 min. and the supernatant removed. The pellet was dried in a vacuum dessicator and resuspended in TE.

2.15 Phenol Chloroform Extraction of Protein-DNA Solutions

0.25 volumes of redistilled or AnalaR grade phenol containing a trace of 8 hydroxyquinoline was added and the mixture vortexed briefly. 0.25 volumes of chloroform was added and the mixture again vortexed. The phases were separated by centrifugation in equipment suitable for the scale of the purification and the upper aqueous phase removed into a sterile tube. The interface and any white denatured protein was left behind. 0.5 volumes of chloroform was added to the aqueous phase and the mixture again vortexed. The upper aqueous phase was removed to a new tube and was precipitated with ethanol (see 2.14).

2.16 Caesium Chloride Purification of Plasmid DNA

The method described by Maniatis et al., (1982) was followed with minor modifications. The plasmid DNA after ethanol precipitation was resuspended in a known volume of TE (usually 8 ml.). 9 g. of CsCl and 400 ul. of ethidium bromide (10 $mg.ml^{-1}$ in water) were added and mixed. When the CsCl dissolved the mixture was centrifuged at 20 000xg for 15 min. and the supernatant decanted into a new tube. This step removed protein precipitated by the addition of the salt and RNA precipitated by the combination of salt and EtBr. The density of exactly 1 ml of the solution was determined, and the solution was adjusted by the addition of TE or CsCl until the density was 1.56 $g.ml.^{-1}$ for E. coli and 1.58 $g.ml^{-1}$ for Streptomyces plasmids. The mixture was

placed into an ultracentrifuge tube (usually Beckmann Ti70 rotor tubes) and the tube filled with a CsCl-EtBr solution of the appropriate density. The tubes with caps were balanced so that the differences between balanced tubes were less than 0.05 g. The tubes were then sealed. The Ti70 rotor was centrifuged at 49 000 rpm. for at least 18 hours before the plasmid band was removed as described by Maniatis et al., (1982).

2.17 Quantification of DNA

The spectrophotometric and mini-gel methods of DNA quantitation described by Hopwood et al., (1985) were employed. For the latter, plasmid DNA was first linearised with restriction enzymes which digested the plasmid at one site. The markers were lambda bacteriophage cut with HindIII. 0.5 ug. of HindIII digested lambda DNA was loaded onto the minigel and the quantity of DNA in each band calculated by its proportion of the length of the genome. Comparison of the unknown DNA sample and dilutions of it with the standards allowed accurate determination of the DNA concentration in a way which was unaffected by any contaminants such as RNA.

2.18 Restriction Endonuclease Digestion of DNA

A description of this technique is well presented in Hopwood et al., (1985), but essentially the following rules were found to be important.

The buffers and temperatures recommended by the manufacturer were followed. The DNA concentration was not greater than 0.2 $ug.ul^{-1}$ and no more than 10% of the final volume was added as enzyme.

A standard reaction was

10X Reaction buffer	2 ul.
Enzyme (5-20 units)	1-2 ul.
DNA and H_2O	to 20 ul.

2.19 Agarose and Polyacrylamide Gel Electrophoresis of DNA

The methods described by Maniatis et al., (1982) and Hopwood et al., (1985) were used with minor modifications to allow for the equipment available. TBE buffer was used throughout and the concentration of the gel and the running conditions of time and voltage were altered to suit the size of the DNA fragments being studied.

2.20 Photography of Gels

The methods described by Hopwood et al., (1985) for the photography of DNA gels were used without alteration. The exposure time for Polariod Type 667 film was of the order of 1 sec. at f=5.6 and 60 sec. for Type 665 film at f=5.6.

2.21 Isolation of DNA from Agarose Gels

The method described in Hopwood et al., (1985) for the electroelution of DNA into dialysis tubing was followed without modification.

2.22 Ligation of DNA

The ligase buffer used was as described in Hopwood et al., (1985). The reaction mixture was incubated on ice in a 37°C incubator. During the course of the day the ice melted and the temperature of the water (and reaction) increased towards 37°C. At 37°C the ligation reaction was harvested, diluted 5 fold with TE, heated to 70°C for 5 mins. and cooled on ice. A portion of

the reaction was used to transform competent cells.

2.23 Use of Alkaline Phosphatase

Alkaline phosphatase was used according to Hopwood et al., (1985).

2.24 Radioactive Labelling of Probe DNA by Random Priming

The commercially-available kit for labelling DNA using random oligonucleotides as primers supplied by Boehringer Mannheim was used according to their instructions. alpha ^{32}p-dCTP was used as label.

2.25 Southern Transfer

Chromosomal or plasmid DNA was prepared and digested if appropriate as described above. About 1 ug. of chromosomal and 10 ng. of plasmid DNA was used per track for Southern analysis. Amersham Hybond-N nylon membrane was used as an alternative to nitrocellulose. The gel preparation and transfer techniques described in the Amersham handbook "Membrane Transfer and Detection Methods" were used throughout. The DNA was transferred using capillary blotting overnight. The DNA was crosslinked onto the membrane using a standard UV. transilluminator for 2 min. The prehybridisation was performed as described in the handbook (65°C) and the hybridisation of the probe was at 70°C. as described by Hopwood et al., (1985). The blots were hybridised overnight and washed the next day according to Hopwood et al., (1985). The stringency of the wash was such that duplexes with less than 84% homomlogy would be washed off. The washed filters were sealed into polythene bags and were used to expose X-ray film with or without an intensifying screen for the appropriate length of time (10 min. to 48 hours depending on the activity of the filter). Autoradiography over long periods of time was

carried out at -70°C. The film was developed in an Xomat machine.

2.26 Plasmid DNA Sequencing

Plasmid sequencing was performed on clones derived from the pTZ- series of plasmid vectors (Pharmacia). The sequencing protocol was as described in the Boehringer Mannheim handbook "Guidelines for quick and simple plasmid sequencing".

2.27 Solutions

Standard solutions, their ingredients and descriptions of their uses, used during this thesis may be found in either the appropriate handbook or the laboratory manuals by Maniatis et al., (1982) and Hopwood et al., (1985).

2.27.1 Medium P

Due to the central role this solution played in the development of methods for the formation of protoplasts, it is described below.

Per litre:

5.73 g TES pH to 7.4 with NaOH

103 g Sucrose (300 mM)

2.03 g $MgCl_2.6H_2O$ (10 mM)

3.68 g $CaCl_2.2H_2O$ (25 mM)

2 ml Trace element solution

(see Hopwood et al., (1985)

Autoclave 15 min. at 121°C.

Just before use add 1 ml of 1% (w/v) KH_2PO_4/100 ml.

2.28 Media

All media used in this thesis and not described below are described in either Maniatis et al., (1982) or Hopwood et

al., (1985).

2.28.1 R2YE

Made up in two parts.

R2/A per litre

0.5 g	K_2SO_4
20.2 g	$MgCl_2.6H_2O$
5.9 g	$CaCl_2.2H_2O$
20.0 g	Glucose
6.0 g	Proline
0.2 g	Casamino acids (Difco)
4.0 ml.	Trace elements
44.0 g	Agar (Difco)

R2/B per litre.

11.5 g	TES buffer. Adjust to pH 7.4 with NaOH.
10.0 g	Yeast extract (Difco)
203.0 g	Sucrose

Autoclave both A and B for 15 mins. at 121°C.

2.28.2 R9

R9/A per litre.

20.0 g	Glucose
0.5 g	K_2SO_4
8.2 g	$MgCl_2.6H_2O$
4.7 g	$CaCl_2.2H_2O$
4.0 g	$NaNO_3$
1.0 g	KCl
0.4 g	$MgSO_4$
0.8 g	Casamino acids
4.0 ml	Trace elements solution

2.0 ml of a 1% (w/v) $FeSO_4$ solution

44.0 g Agar

R9/B per litre

11.5 g MOPS pH 7.4

10.0 g Yeast extract

410.0 g Sucrose

2.28.3 ATCC172

per litre

10.0 g Glucose

20.0 g Soluble starch

5.0 g Tryptone

5.0 g Yeast extract

1.0 g $CaCO_3$

20.0 g Agar

pH 7.1

2.28.4 Emersons agar

Emersons agar was purchased as a powder from Difco and was used as directed by the manufacturers.

Emersons agar could be made up from its component parts as follows.

Per litre

4.0 g Beef extract (Difco)

1.0 g Yeast extract (Difco)

4.0 g Peptone (Difco)

10.0 g Glucose

2.5 g NaCl

20.0 g Difco-bacto agar

2.28.5 R2S

300 mM disodium succinate (81.0 g/l for the hexahydrate) was substituted for the sucrose in R2/B. This gave a final concentration of 150 mM disodium succinate in the regeneration medium.

2.28.6 EmR2S

R2/A was used without modification. An alternative B solution was used.

EmR2S/B

Per litre

11.5 g TES buffer. Adjust to pH 7.4 with NaOH

83.0 g Emersons agar (Difco)

81.0 g Disodium succinate. hexahydrate

2.28.7 EmR2SC

EmR2SC/A

Per litre.

0.5 g K_2SO_4

20.2 g $MgCl_2$

5.9 g $CaCl_2$

6.0 g Proline

0.2 g Casamino acids (Difco)

4.0 ml Trace elements

20.0 g Glucose

5.0 g NaCl

40.0 g Difco-bacto agar

EmR2SC/B

Per litre

11.5 g TES pH 7.4 with NaOH

81.0 g Disodium succinate.$6H_2O$

2.0 g Yeast extract (Difco)

8.0 g Beef extract (Difco)

8.0 g Peptone (Difco)

2.28.8 EmRM150

The disodium succinate in EmR2SC/B was replaced with Mannitol (54.65 g/l), giving a final concentration of 150 mM Mannitol. EmR2SC/A was used unaltered.

2.28.9 EmRM125

As above but Mannitol was used at 45.54 g/l to provide a final concentration of 125 mM Mannitol in the regeneration plates.

2.28.10 R579

R579/A

0.5 g K_2SO_4

6.0 g Proline

0.2 g Casamino acids (Difco)

4.0 ml Trace elements

20.0 g Glucose

5.0 g NaCl

40.0 g Difco-bacto agar

Made up to 900 ml and dispensed into 90 ml aliquots

R579/B

11.5 g TES pH 7.2 with NaOH

45.54 g Mannitol

2.0 g Yeast extract (Difco)

8.0 g Beef extract (Difco)

8.0 g Peptone (Difco)

Made up to 900 ml and dispensed into 90 ml aliquots

Parts A and B were autoclaved for 15 mins. at 121°C, after which they could be stored until required. At that time part A was melted and part B heated in a steamer. Both were cooled to 55°C before mixing. Then 1 ml of 1% (w/v) KH_2PO_4, 3.75 ml of 0.8 M $MgCl_2$, 6.25 ml. of 3.2 M $CaCl_2$ and 10 ml of water were added and mixed well. The agar was poured and dried according to 2.12.3.

2.29 Strains

Name	Comments
S. thermonitrificans ISP5579	Wild Type
S. thermonitrificans C5	see 4.6
S. thermonitrificans C51	see 4.6.1
S. thermonitrificans ISP5579/pIJ702	see 4.3.1
S. thermonitrificans C5/pIJ702	see 4.6
S. thermonitrificans C5/pIJ916	see 4.6
S. thermonitrificans C5/pIJ702	see 4.7
S. thermonitrificans C5/pBROC139	see 7.4

S. Thermohygroscopicus KCC-S-9017

Lab No. 017.

S. Thermohygroscopicus subsp. rubiginosus KCC-S-0918

Lab No. 018. Plasmids detected both physically and genetically (4.9.1, 4.9.2). Contains many plasmids.

S. thermogriseoviolaceus KCC-S-0919

Lab No. 019. Plasmids detected both physically and genetically (4.9.1, 4.9.2).

S. thermoolivaceus KCC-S-020

Lab No. 020. No plasmids identified.

S. thermoolovaceus KCC-S-021

Lab No. 021. No plasmids identified.

S. threomyceticus KCC-S-0935

Lab No. 035. Plasmids detected both physically and genetically. Produces threomycin.

S. thermovulgaris ISP5544

Lab No. 544. Plasmid detected genetically but not physically.

S. thermoflavus ISP5574

Lab No. 544. No plasmids detected.

S. albus Pfizer ISP5313

Lab No. 313. No plasmids detected.

S. lividans 1326 (Hopwood et al., 1983)

$SLP2^{+}$, $SLP3^{+}$

S. lividans TK24 (Hopwood et al. 1983)

Plasmid Free, str-6.

S. lividans TK54

Plasmid Free, his-2, leu-2, spc-1.

S. lividans TK64

Plasmid Free, pro-2, str-6.

S. lividans TC64 (Kieser pers. comm)

TK24 containing pIJ922 (Lydiate et al., 1985).

S. lividans TC73 (Kieser pers. comm)

TK24 containing pIJ61 (Thompson et al., 1982a).

The following strains were derived from S. lividans TK54

See section 6.5

Strain	Phenotype
GLW1	tsr⁻, White spores STP2⁺
GLW4	tsr⁻, White spores STP2⁺
GLW13	tsr⁺, pocking pIJ702 STP13
GLW16	tsr⁺, pocking pIJ702 STP16
GLW17	tsr⁺, pocking pIJ702 STP17
GLW18	tsr⁺, pocking pIJ702 STP18
GLW21	tsr⁺, pocking pIJ702 STP21
GLW16.4	see 6.10
GLW1654	see 6.10.1
GLW61	see 6.4.1 $STP1^{int}$
GLW62	see 6.4.1 $STP1^{int}$

S. lividans TK2418

TK24 containing the conjugative plasmid from 018

S. lividans TK2419

TK24 containing the conjugative plasmid from 019

2.30 Plasmids

pUC18	Viera and Messing, 1982
pGLW13	4.10.2
pGLW37	4.10.6
pGLW38	4.10.6
pGLW39	4.10.6
pIJ702	Katz et al., 1983
pBROC139	7.2.4
pIJ916	Lydiate et al., 1985
STP1	6.2
STP2	6.9

STP13	6.5
STP16	6.5
STP17	6.5
STP18	6.5
STP21	6.5
STP16.4	6.7
STP16.5	6.7
STP1654	6.10.1

Chapter 3.

The Formation and Regeneration of Protoplasts of S. thermonitrificans ISP5579

3.1 Introduction

The wealth of manipulative techniques applicable to E. coli have been used to study the structure, regulation and evolution of genes from a wide range of organisms. Their use to study streptomycete molecular biology would be both informative and useful in that important streptomycete products, such as enzymes, might be produced in E. coli. However, there are experiments which cannot be performed in this manner. The expression of whole streptomycete antibiotic production pathways in E. coli may be difficult (Hopwood et al., 1986). Fine structural analysis of promoters would be impossible without studying mutant promoters in their original host, and comparing their activities with that of the wild-type promoter. Although information can be gained using E. coli as a host, eventually the ability to transform Streptomyces and perform recombinant DNA experiments within Streptomyces becomes essential. Similarly, the molecular biology of thermophilic streptomycetes may be studied using E. coli and the mesophilic S. lividans as cloning hosts. However, the development of an endogenous cloning system for a thermophilic streptomycete is required for the optimal exploitation of their biotechnological potential and for a detailed examination of thermophily.

Exogenous DNA was first introduced into Streptomyces by Okanishi et al., (1966). They found that intact mycelium of S. kanamyceticus At-463 was not competent to absorb and express DNA from the actinophage PK-66. Protoplasts of S. kanamyceticus At-

462 were then produced and mixed with the actinophage DNA. The DNA entered the protoplasts and was able to direct the synthesis of new actinophage (Okanishi, et al., 1966). The assay was sensitive because each successful transfection event gave rise to a plaque on a plate. Unfortunately the formation of plaques is a destructive event producing new virion but not colonies containing the introduced DNA. Other DNA molecules were required which could be introduced into Streptomyces but which would not destroy the transformed cells. Preferably that DNA would also encode a selectable marker so that transformants could be recognised easily. The methodology of Okanishi et al., (1966) required improvement because the protoplasts made were incapable of regaining mycelial growth under the conditions employed.

Before 1975, DNA molecules with the required characteristics had not been isolated from Streptomyces. During the period between 1966 and 1975 the second problem of protoplast regeneration was studied. Okanishi and coworkers (1974) reported the first critical study of the factors affecting regeneration of protoplasts to a mycelial form. Studying S. griseus and S. venezuaelae, they found that when the bacterial cell wall was digested by treatment with lysozyme (Sigma Chemical Co.) and lytic enzyme No.2 (Kyowa Hakko Kogyo Co. Tokyo. Japan.) the protoplasts formed were osmotically-sensitive. They used solutions containing sucrose to stabilise them. They also studied the effects of cations Na^{+}, Mg^{2+} and Ca^{2+} in conjunction with sucrose to optimise the stability of protoplasts in suspension. When stable suspensions of protoplasts were available, they went on to study the regeneration of these protoplasts to a mycelial form. Using solid agar as a base, the

effects of different carbon and nitrogen sources, and amounts of sucrose, $MgCl_2$, $CaCl_2$, phosphate and casamino acids on regeneration were all investigated. On the appropriate media approximately 50% of the protoplasts of these two species were able to regenerate to a mycelial mode of growth. Their work has been used as a basis by others, upon which to develop regeneration media applicable to other Streptomyces.

The first problem, that of identifying a suitable DNA molecule for transformation studies was partially solved by Schrempf et al., (1975). They isolated a covalently closed circular deoxyribonucleic acid molecule, or plasmid, from S. coelicolor. The problem was fully resolved when Bibb et al., (1977) physically and genetically characterised the molecule, called SCP2, and showed that it was a fertility determinant. Furthermore, the plasmid SCP2 expressed a visual phenotype. Strains containing the plasmid could form 'pocks' on those that did not. SCP2 represented an ideal DNA molecule for transformation studies. It could be purified easily, it would not destroy transformants as did actinophage DNA and it had pocking as a visually recognisable marker. A year later Bibb et al., (1978) described the transformation of S. coelicolor and S. parvulus with SCP2 DNA using polyethylene glycol -mediated plasmid transformation of protoplasts.

Although plasmids are important in the development of transformation procedures for Streptomyces, plasmid transformations are not possible until the protoplasts can be regenerated to a mycelial mode of growth. The conditions for protoplast regeneration of each species to be transformed must be

determined before transformation studies proceed. Seldom do two species regenerate efficiently on the same medium. It is possible to achieve low levels of transformation of some streptomycetes under non-optimal regeneration conditions. However, if many transformants are required then a regeneration media must be developed to suit the species of interest.

As regeneration conditions are important for the development of a transformation system, the important variables and the parameters used to study their effect on regeneration frequency will be considered in detail.

3.1.1 Cultivation

Intuitively the physiological state of the mycelium will be dependent on the conditions employed during its cultivation. Sagara et al., (1971) found that the addition of glycine to the growth medium rendered streptomycete cell walls more susceptible to the action of lysozyme. The optimal concentration of glycine was species-dependent (Sagara et al., 1971;Thompson et al., 1982a; Matsushima and Baltz, 1987). Glycine reduces the growth rate of some strains. Although the same biomass yield may be achieved by prolonging the cultivation time the growth rate of the strain is probably also an important factor in determining the protoplast regeneration frequency.

In batch culture eg. shake flasks, the mycelium exists in a changing environment as nutrient resources are depleted and waste products increase during the fermentation. Baltz et al. (1978) found that protoplast regeneration frequencies of S. fradiae and S. griseofuscus were dependent on the stage of the growth phase at which mycelium was harvested. In both species protoplast

regeneration frequencies were higher when mycelium was harvested in the transition period between the exponential and stationary phases of growth.

The temperature of cultivation is also important. S. fradiae protoplasts regenerated with greater efficiency if the mycelium was cultivated at 34°C rather than at 29 or 37°C before protoplasting (Baltz, 1981). It is not clear whether the physiological differences causing enhanced regeneration after growth at 34°C are a result of temperature per se, or because of variations in the growth rate of S. fradiae as a result of these temperature alterations. Continuous culture at different dilution rates (giving defined growth rates) could be used as a source of mycelium to investigate this.

Work on the effect of composition of growth medium on protoplast regeneration frequency has not been published. In general, media supporting good growth of the streptomycete of interest have been used for cultivation, with the addition of glycine to enhance sensitivity to lysozyme. Thompson et al., (1982) also added 5mM $MgCl_2$ and 34% (w/v) sucrose to a complex medium containing yeast and malt extracts for the growth of S. lividans prior to protoplasting. The $MgCl_2$ and sucrose were used to encourage dispersed growth, but they may have also conditioned the mycelium to growth on media containing high concentrations of solutes, as would be found on regeneration media. Such conditioning would reduce the stress on the protoplasts when plated onto regeneration media.

3.1.2 Formation of Protoplasts

Okanishi et al., (1974) used lysozyme and lytic enzyme No.2 to digest the cell walls of S. griseus and S. venezuaelae. Lytic

enzyme No.2 was not easily obtainable outside Japan. Baltz, (1978) showed that this enzyme increased the rate of the formation of protoplasts of S. fradiae and S. griseofuscus, but was not an absolute requirement for their production. Since then, the use of lysozyme has continued but lytic enzyme No.2 has not been used frequently. Ogawa et al., (1983) used the enzyme achromopeptidase (Wako Pure Chemical Co.) for the production of protoplasts from species of Streptomyces refractory to protoplasting with lysozyme alone. Lysozyme and achromopeptidase together were found to increase the yield of protoplasts from S. hygroscopicus; up to 400 times more protoplasts could be formed compared to lysozyme alone or to a combination of lysozyme and lytic enzyme No.2. Achromopeptidase and lysozyme were absolute requirements for the formation of protoplasts from Micromonospora, a closely related genus (Ogawa et al., 1983). The amount of enzyme required for the formation of protoplasts was dependent upon the species, buffer and temperature. The optimal level therefore has to be determined empirically for each species.

The osmotically-sensitive protoplasts formed by enzymatic digestion of the cell wall must be stabilised in a suitable buffer. Okanishi et al., (1974) developed a solution called medium P (2.27.1) which was used for both production and stabilisation of protoplasts of S. griseus and S. venezuaelae. Medium P contains sucrose, Mg^{2+} and Ca^{2+} to stabilise the protoplasts osomotically. In addition, it also contains trace elements, phosphate, sulphate and a pH buffer. Whilst the constituents of medium P have been altered slightly by other authors, these alterations have been minor: the amounts of

divalent cations and sucrose have been changed and sodium chloride has been used in addition to the divalent cations. These refinements have allowed the protoplasting of many species of Streptomyces.

The first major changes to protoplasting methodology were made by Shirahawa et al., (1981) and Thompson et al., (1982a). They introduced the use of two solutions with different osmotic properties. The first solution, containing lysozyme, was hypotonic with respect to the protoplasts which tended to lyse in this solution. However it allowed rapid and efficient formation of protoplasts. When protoplasting was completed, a second solution containing increased concentrations of divalent cations was used to stabilise the protoplasts. The protoplasts could be centrifuged out of the first solution and resuspended in the osmotically-favourable second solution. This alteration in methodology was one of the steps which allowed efficient protoplast formation and regeneration of S. kasugaensis (Shirahawa et al., 1981) and efficient transformation of S. lividans (Thompson et al., 1982a).

3.1.3 Regeneration of Protoplasts

Okanishi et al., (1966) used protoplasts and actinophage DNA to show that transfection was possible in Streptomyces. If recombinant DNA experiments are performed using only vectors based on bacteriophage then this methodology is sufficient. If plasmid vectors need to be used, then the transformed protoplasts must be encouraged to regenerate their cell walls and form mycelia again so that colonies may form. In addition to transformation studies, the ability to regenerate protoplasts to

a mycelial form has allowed protoplast fusion to be used for genetic mapping (Rhodes, 1986) and strain improvement (Normansell, 1986). As previously described, Okanishi et al., (1974) developed regeneration media for S. griseus and S. venezuaelae by adding a sucrose and divalent cations (Mg^{2+} and Ca^{2+}) as osmotic buffers to a minimal medium. They found that addition of other nutrients in the form of casamino acids enhanced the regeneration frequencies of these two species. The optimal media for the two species differed slightly in the ratio of divalent cations and in the preferred nitrogen source. Minor changes to the R1 and R2 media developed by Okanishi et al. (1974) have allowed the efficient regeneration of many species. For example Baltz et al. (1978) found that the substitution of proline with asparagine in R2 improved the regeneration of protoplasts of S. fradiae and S. griseofuscus. An alternative strategy to the development of a regeneration medium from a minimal medium is to use a complex growth medium as the base, adding carbohydrate and divalent cations to buffer the media osmotically. Pigac et al., (1982) have used this approach to develop a regeneration medium for the oxytetracycline producer S. rimosus. They used sucrose, Mg^{2+} and Ca^{2+} to buffer the media osmotically and found that the addition of gelatin to 1 $g.l^{-1}$ improved regeneration three to five fold. However, despite a different approach towards the development of a regeneration media, the two types are similar.

The first major change to regeneration media was the replacement of sucrose by succinate for the efficient regeneration of protoplasts of S. kasugaensis by Shirahawa et al., (1981). They found that R1 and R2 were not suitable for the

regeneration of many streptomycetes or for Micromonospora. They developed a new regeneration medium, R3, similar to R2 but based upon sodium succinate instead of sucrose. They also altered the divalent cation concentrations and included sodium chloride. This was the first example which used an alternative to sucrose as the osmotic buffer in a regeneration medium.

Protoplast regeneration may be inhibited by mycelial fragments or, in some species, protoplasts which are regenerating quickly (Baltz and Matsushima, 1981). Plating the protoplasts in soft agar overlays prevents this inhibition and has been used to increase protoplast regeneration frequencies in a number of systems (Baltz and Matsushima, 1981; Shirahawa et al., 1983; Ikeda et al., 1983).

Although much attention has focused on the concentrations of sucrose and divalent cations in the regeneration media, it has been found that partial dehydration of the media also increases regeneration frequencies. Dehydrating plates to a mass loss of 15-22% increased the protoplast regeneration of S. fradiae (Baltz and Matsushima, 1981) and S. lividans (Thompson et al., 1982a) about ten and sixty-fold respectively. Regeneration frequencies were not enhanced when the concentrations of the constituents in the media were increased to concentrations similar to those found in the dehydrated media (Baltz and Matsushima, 1981). The dehydrated medium provides an environment which is more suitable for protoplast regeneration, perhaps by retarding the diffusion of autoinhibitory substances such as autolytic proteins.

The temperature at which protoplasts are regenerated influences the regeneration frequency. Generally, the temperature

used for the liquid culture of mycelia is also used for protoplast regeneration. However, Baltz (1981) showed that regeneration of S. fradiae protoplasts increased ten-fold when the regeneration temperature was reduced from 34 to 29°C.

3.1.4 Quantification

The variables which influence the regeneration frequency of Streptomyces protoplasts have been listed above. When each variable is studied, its effect on the regeneration of the protoplasts must be quantified in order that the optimal conditions may be identified.

3.1.4.1 Formation of Protoplasts

Quantity

Haemocytometry has been used to measure the number of protoplasts formed by enzymatic digestion of the streptomycete cell wall (Hopwood et al., 1986).

Quality

The total number of spherical vesicles may be counted but this does not provide a measure of the quality of the protoplasts. In addition to quantifying the number of protoplasts in a suspension, Okanishi et al., (1974) also estimated the number of protoplasts which had lysed, by measuring the amount of nucleic acid that leaked into the supernatant when protoplasts burst. The increase in the absorption of a solution at A_{260}nM was used to quantitate this leakage. The standard used was the absorption at A_{260}nM of a suspension of protoplasts which had been completely lysed in a medium P derivative without sucrose or divalent cations. The leakage measurement indicated which solutions allowed the efficient formation of protoplasts,

but did not provide an estimate of how robust the protoplasts were in those solutions. Okanishi et al., (1974) added a further refinement of a temperature incubation step of 1 hour at 28°C to stress the protoplasts prior to enumeration with a haemocytometer or leakage studies. This stress procedure differentiated protoplast suspensions which were robust from those only just stable enough to remain intact. The quality of the protoplasts could thus be evaluated.

3.1.4.2 Regeneration Efficiency

Quantity

In order to calculate the frequency of protoplast regeneration, the number of viable protoplasts plated, the number of non-protoplasts plated and the number of colonies formed on the regeneration media after incubation must be known.

In practice, the number of viable protoplasts cannot be determined. Instead the total number of protoplasts in suspension, as determined by haemocytometry, is used as an estimate of the number of viable protoplasts in the suspension. This approach underestimates the frequency of regeneration as non-viable protoplasts are counted by haemocytometry and are included in the calculation.

The number of non-protoplasts plated may be measured by preparing a tenfold dilution series of the protoplasts in water and plating them on media which do not contain an osmotic buffer. The environments of such media are not suitable for regeneration and only mycelial fragments will grow to form colonies. Usually few mycelial fragments survive the lysozyme treatment and filtration step in the preparation of protoplasts.

Quality

In some circumstances, a quantitative assessment of the regeneration frequency may not be the most suitable means of measuring the effect of a variable on the regeneration frequency. When different treatments encourage the protoplasts to regenerate at the same frequency, other parameters such as the length of time to form colonies, the amount of sporulation and the colony morphology may be used to differentiate between the treatments. Conditions in which the protoplasts regenerate into colonies similar to the parental colonies formed by outgrowth from spores should be preferred.

3.2 A Strategy for the Development of a Transformation System: I

At each stage of a typical streptomycete transformation procedure there are many factors which influence the eventual transformation frequency. Some of the factors affecting protoplast formation and regeneration have been discussed. If the established methods do not allow the efficient transformation of a new streptomycete species, then the protocols must be analysed and improved. The complexity of the problem demands that the analysis of variables in the transformation system be simplified by considering each step separately. This allows attention to be focused on the step most likely to improve the transformation efficiency. The order of the manipulations in a streptomycete plasmid transformation procedure does not reflect the relative importance of each step in the procedure.

1) Growth of Mycelia
2) Production and Stabilisation of Protoplasts
3) Transformation of Protoplasts by Plasmid DNA
4) Regeneration of Protoplasts and Selection of Transformed Regenerants

The suitability of a plasmid for use in transformation

studies cannot be assessed until the protoplasts can be formed and regenerated efficiently. These two steps (2 and 4 above) should be analysed first when a transformation system is not working. If after altering the solutions and media, the protoplasts can be produced and regenerated efficiently but are not transformed by the plasmid, then the choice of plasmid or method of selection of transformants may be questioned.

When each step is analysed and improvement sought, the best targets will be those which respond most to alteration. Due to the complexity of the buffers and media used in transformation studies it may be difficult to predict what those targets will be, but some suggestions may be possible.

Both the solutions and the regeneration media are used to protect osmotically-sensitive protoplasts. The constituents of the solutions and media responsible for their osmotic properties should be the first targets for study. Alterations in the types and amounts of carbohydrate, mono- and divalent cations should be made and the effect of these alterations on protoplast formation and regeneration quantified (see above).

The importance of a variable may be assessed quickly by altering its concentration, to both higher and lower levels, in two fold steps. For example, consider the investigation of the effect of the concentration of Mg^{2+} in the media used for protoplast regeneration. If 10mM Mg^{2+} is typical for other species it would be worth assessing regeneration frequencies in media containing 2.5, 5, 10, 20, and 40 mM Mg^{2+}. Such experiments quickly place the optimum value within an interval, or indicate the direction in which the optimum lies. The optimal levels may be further defined in subsequent experiments. The

value of such an experimental design is that it reduces the complexity of the experiment, making it both manageable and cheaper.

If regeneration frequency is independent of a variable, it is unlikely to be a good target for improvement. If the regeneration frequency decreases when the variable is altered, this is important and should be noted. When other variables have been altered and conditions are generally more favourable, examining that variable again may be worthwhile.

3.3 Development of a Method for the Efficient Formation of S. thermonitrificans Protoplasts

The formation of S. thermonitrificans protoplasts was attempted initially using protocols developed for mesophilic streptomycetes (Okanishi et al., 1974; Thompson et al., 1982a). Protoplasts from mycelium harvested during the transition period from exponential to stationary phases of growth have been found to regenerate efficiently in some mesophilic species (Baltz, 1978; Thompson et al., 1982a). In addition, suboptimal mycelial growth temperatures have been reported to improve the quality of the protoplasts produced (Baltz and Matsushima, 1981). The optimum growth temperature of S. thermonitrificans has been reported to be between 45-50°C (Desai and Dhala, 1967). The conditions of appropriate growth phase and sub-optimal growth rate may be arranged for S thermonitrificans by the cultivation of a spore inoculum (10^6 spores per 50 ml of broth) in TSB with vigorous shaking at 30°C for about 48 hours or 37°C for about 24 hours. The shorter cultivation time at 37°C was generally preferred. S. thermonitrificans grown in TSB produces dispersed three-dimensional mycelial mats. This morphology was ideal for studies on protoplasting as the matted structure allowed easy identification of the protoplasts. Had the growth been fragmented this may have been more difficult. Mesophilic streptomycete protoplasts are spherical bodies which on microscopic examination may be seen to move freely across the field of view. Protoplasts from the thermophilic S. thermonitrificans were expected to be similar to those of a mesophile.

S. thermonitrificans mycelium was harvested and washed in a 10.3% (w/v) sucrose solution. A range of lysozyme concentrations in both medium P (Okanish et al., 1974) and medium L (Thompson et al., 1982a) was added to the washed mycelium. Initially 1mg.ml^{-1} of lysozyme was used in the reactions at 30°C and 37°C. Under these conditions protoplasts were not visible by microscopic examination even after prolonged incubation. Increasing concentrations of lysozyme up to 10 mg.ml^{-1} failed to encourage protoplast formation. The mycelium of S. thermonitrificans was apparently resistant to the action of lysozyme.

The addition of glycine to the growth medium has been used to increase the sensitivity of the streptomycete cell wall to lysozyme (Sagara et al., 1971). The addition of glycine to 0.5% (w/v) in TSB reduced the growth rate of S. thermonitrificans and lowered the biomass yield between 4 and 5 fold at 30°C. This reduction was considered unacceptable.

Microscopic examination of the mycelium before and after lysozyme treatment (1mg.ml^{-1}) revealed a morphological change in the dispersed mycelial mats. On addition of the lysozyme to a suspension of S. thermonitrificans mycelium, in either medium P or medium L, the periphery of the mycelial mats collapsed rapidly, giving the appearance of a cuticle. The resulting structure which was enveloped in the cuticle seemed to be resistant to lysozyme. The macroscopic observation was that the suspension of S. thermonitrificans mycelia which was homogeneous initially clumped on the addition of lysozyme and that the clumps sedimented to the bottom of the tube. Identical observations

were made when the concentration of lysozyme was increased to 10mg.ml^{-1}.

The rapid formation of the cuticle could be stopped by the use of lower concentrations of lysozyme. When 0.5mg.ml^{-1} of lysozyme in medium P at 37°C was used, protoplasts of S. thermonitrificans formed rapidly and the lysozyme-resistant clumps were avoided. Microscopic examination of the protoplast suspension formed by this treatment showed that mycelial clumps were no longer visible after seven minutes of incubation, and by plating experiments the number of osmotically-insensitive colony forming units was constant by eight minutes. A reduction in the lysozyme concentration to 0.25mg.ml^{-1} and lowering the temperature to 30°C, slowed the protoplast formation rate so that an incubation time of between forty minutes and one hour was required for maximal conversion of the mycelium to protoplasts. This time of incubation is similar to those used in protocols for mesophiles.

3.4 Development of Solutions for the Manipulation of S. thermonitrificans Protoplasts

When the lysozyme concentration required for the efficient formation of S. thermonitrificans protoplasts had been determined, experiments similar to those of Okanishi et al. (1974) and Pigac et al., (1982) were performed. This work was undertaken in two phases. The first experiments were performed at the start of the project, prior to the optimisation of conditions for regeneration. The purpose of these experiments was to determine whether medium P (Okanishi, et al., 1974) was suitable for the formation and stability of S. thermonitrificans protoplasts. The second set of experiments were performed after

a low level of protoplast regeneration had been achieved by replacement of sucrose in the regeneration media by succinate or mannitol. This second set of experiments investigated the feasibility of replacing the sucrose in the protoplasting buffers with disodium succinate, mannitol or sorbitol.

Protoplasts of S. thermonitrificans were produced in a variety of medium P-like solutions, which contained different amounts of sucrose, $MgCl_2$ and $CaCl_2$. The mycelium was cultivated from approximately 10^6 spores in 50 ml of TSB at 37°C for 24 hours with vigorous shaking. The mycelium was harvested and washed with 10.3% (w/v) sucrose. The washed mycelia was incubated with 0.25 $mg.ml^{-1}$ of lysozyme at 30°C in a variety of medium P-like solutions until the protoplasting was complete. The efficiency of protoplast formation was determined by haemocytometry and the amount of leakage was measured by the increase in A_{260}nM in the protoplast supernatant (Okanishi, et al., 1974; Table 3.1).

. The addition of 3 mM $MgCl_2$ and 3mM $CaCl_2$ to basal medium P (solution 2, table 3.1) was sufficient to increase the stability of S. thermonitrificans protoplasts in solution. Protoplasts were produced efficiently but they were not as stable as protoplasts in other medium P-like solutions, which had lower leakage values. The concentrations of divalent cations in this solution were similar to those in the lysozyme solution medium L developed by Thompson et al., (1982a). The protoplasts produced in medium P (Okanishi, et al, 1974) had the lowest leakage value of all these derivative solutions (Table 3.1; solution 9).

Table 3.1

The Effect of Sucrose, Mg^{2+} and Ca^{2+} Concentrations on the Formation and Stability of S. thermonitrificans Protoplasts.

Basal Medium P	TES	5.73	$g.l^{-1}$
	K_2SO_4	0.5	$g.l^{-1}$
	Trace Elements	2	$ml.l^{-1}$
	after autoclaving		
	KH_2PO_4 (1% w/v)	10	$ml.l^{-1}$

Additives to basal medium P (mM)

Number	Sucrose	$MgCl_2$	$CaCl_2$	Protoplasts ml^{-1}	Leakage %
1	0	0	0	$2.5x10^7$	100
2	0	3	3	$5.4x10^8$	62
3	100	3	3	$6.1x10^8$	61
4	100	25	10	$1.2x10^8$	55
5	100	10	25	$9.3x10^7$	53
6	100	50	50	0	@
7	300	3	3	$4.0x10^8$	63
8	300	25	10	$3.3x10^8$	48
9	300	10	25	$4.2x10^8$	43 |
10	300	50	50	0	@

\| Medium P

@ No protoplasts were seen in these samples, lysozyme addition to 0.5 $mg.ml^{-1}$ and incubation at 37°C did not induce protoplast formation.

In order to differentiate further between the solution 3 of table 3.1 and medium P a temperature incubation step between protoplast formation and haemocytometry was used. Protoplasts were produced in these two solutions and they were centrifuged so that the supernatant could be removed. The protoplasts were then resuspended in the medium P-like solutions and incubated at 30°C for 1 hour before counting. When solution 3 was used for the production of S. thermonitrificans protoplasts (Table 3.2) the following observations were made.

The addition of 3 mM of each of the divalent cations to basal medium P was not sufficient to stabilise the protoplasts during the temperature incubation step. The presence of sucrose and higher divalent cation concentrations was required for optimal protoplast stability. The maximal number of intact protoplasts was obtained when solution 4 of table 3.1 was used for protoplast stabilisation. However, the performance of this solution was not better at the 5% level of significance when compared to medium P by the Mann-Whitney U test.

When medium P was used for protoplast formation similar results were obtained (Table 3.3). Higher sucrose and divalent cation concentrations enhanced protoplast stability during the temperature incubation step.

The constituents of the medium P-like solutions described above were chosen after considering the results of Okanishi et al., (1974) and Pigac et al., (1982).

Although useful, the studies of the effect of buffer constituents on protoplast formation and stability were not comprehensive. Further investigation of the parameters affecting

Table 3.2

The Number of S thermonitrificans Protoplasts in Suspension after Temperature Incubation: An Estimate of Stability.

The initial protoplasting solution was as in solution number 3 in Table 3.1.
Additives to basal medium P (mM)
Solution for Stability Measurement

Sucrose	$MgCl_2$	$CaCl_2$	Protoplasts ml^{-1}
0	0	0	$1.5x10^5$
0	3	3	$4.5x10^5$
100	3	3	N.A
100	25	10	$9.0x10^7$
100	10	25	$4.5x10^7$
300	3	3	$6.6x10^7$
300	25	10	$5.2x10^7$
300	10	25	$6.3x10^7$ |

| Medium P
NA Not available

protoplast formation and stability was possible by using matrix experiments, varying the concentrations of sucrose, $MgCl_2$ and $CaCl_2$. Previous results (i.e. Tables 3.1, 3.2, 3.3) indicated that divalent cations contributed more to the stabilising effect than sucrose. It therefore seemed reasonable to first vary the divalent cation concentrations and ratios at a constant lower sucrose concentration and to measure the effect of these alterations on the number of protoplasts formed by lysozyme treatment in these medium P-like solutions. Basal medium P containing 219mM sucrose with the addition of various amounts of $MgCl_2$ and $CaCl_2$ was used for the formation of S. thermonitrificans protoplasts. After incubation with lysozyme the protoplasts were washed in the corresponding medium P-like solutions and counted using a haemocytometer without a temperature incubation step. The matrix experiment compared the number of protoplasts formed in solutions containing 0, 1, 5, 10, 20 and 40mM $MgCl_2$ or $CaCl_2$. (Table 3.4) Medium P-like solutions containing 10 mM $MgCl_2$ or 20mM $CaCl_2$ tended (four out of six) to allow the formation of more S. thermonitrificans protoplasts than were formed in medium P.

To further define the optimal conditions for S. thermonitrificans protoplast formation, the effect of $CaCl_2$ concentration on protoplast formation in basal medium P, containing 219mM sucrose and 10mM $MgCl_2$ was investigated (Table 3.5). There would appear to be a maximum efficiency of S. thermonitrificans protoplast formation in $CaCl_2$ concentrations between 5 and 20mM, with an optimum in this experiment of 10mM. This was a lower concentration of $CaCl_2$ than was optimal in an earlier experiment (Table 3.4) and may reflect variability in the

Table 3.3

The Number of S thermonitrificans Protoplasts in Suspension after Temperature Incubation: An Estimate of Stability.

The initial protoplasting solution contained 300 mM sucrose, 10mM $MgCl_2$ and 25mM $CaCl_2$. (Medium P).

i.e. solution number 9 in table 3.1

Additives to basal medium P (mM)
Solution for Stability Measurements

Sucrose	$MgCl_2$	$CaCl_2$	Protoplasts ml^{-1}
0	0	0	$7.7x10^6$
0	3	3	$4.0x10^7$
100	3	3	$5.2x10^7$
100	25	10	$2.5x10^8$
100	10	25	$2.6x10^8$
300	3	3	$1.7x10^8$
300	25	10	$2.1x10^8$
300	10	25	$4.0x10^7$ |

\| Medium P

Table 3.4
The Effect of Divalent Cation Ratio and Concentration in Medium P-like Solutions on the Formation of S. thermonitrificans Protoplasts.

Basal Medium P (Table 3.1) containing 219mM (7.5% w/v) sucrose with the addition of the following divalent cations was used for the formation of S. thermonitrificans protoplasts. Lysozyme at 0.25 $mgml^{-1}$ was used at 30°C.

		$CaCl_2$ (mM)					
		0	1	5	10	20	40
$MgCl_2$ (mM)	0	20	56	140	75	179	113
	1	30	85	110	114	143	132
	5	44	108	123	129	212	156
	10	60	98	151	174	184	242
	20	53	120	149	159	284	59
	40	116	153	152	122	67	42

Protoplasts ml^{-1} $x10^7$

Medium P = $149x10^7$ protoplasts ml^{-1}

Table 3.5

The Effect of $CaCl_2$ Concentration on the Formation of Protoplasts of S. thermonitrificans in a Medium P-like Solution Containing Basal Medium P, 219 mM (7.5% w/v) Sucrose and 10mM $MgCl_2$.

$CaCl_2$ (mM)	Protoplasts ml^{-1}
1	$8.3x10^9$
5	$1.5x10^{10}$
10	$4.2x10^{10}$
20	$2.1x10^9$
40	@
Medium P	$3.2x10^{10}$

@ No protoplasts formed in this solution.

mycelium used for the formation of the protoplasts.

The effect of $MgCl_2$ concentration on protoplast formation in basal medium P, containing 219mM sucrose and 10mM $CaCl_2$ was investigated in the same manner (Table 3.6). The formation of S. thermonitrificans protoplasts appears to be less affected by the concentration of $MgCl_2$ in the presence of 10mM $CaCl_2$ than it was by $CaCl_2$ concentration in the presence of 10mM $MgCl_2$ (Table 3.5). A broad optimal level of $MgCl_2$ concentration was seen, and a concentration of 10 mM was chosen for further work.

The concentration intervals for divalent cations used in the matrix experiment were divided further, considering concentrations of 5, 10, 12.5, 15 and 20mM divalent cations. The experiments showed that further improvement in the protoplast yield might be possible, but that the differences between treatments were less significant, as one might expect when approaching optimal conditions. Microscopic examination of the protoplasts formed in the various medium P-like solutions showed that S. thermonitrificans protoplasts formed in a medium P-like solution containing 12.5mM $MgCl_2$ and 15mM $CaCl_2$ in addition to basal medium P and 219mM sucrose were of a more uniform size and appeared to clump less than protoplasts formed in other solutions.

The effect of changes in the sucrose concentration of the medium P-like solution containing basal medium P, 12.5mM $MgCl_2$ and 15mM $CaCl_2$ was investigated next. Concentrations of sucrose from 4% to 14% in 2% (w/v) steps were examined for efficient formation of S. thermonitrificans protoplasts (Table 3.7).

Table 3.6

The Effect of $MgCl_2$ Concentration on the Formation of Protoplasts of S. thermonitrificans in a medium P-like Solution Containing Basal Medium P, 219mM (7.5% w/v) Sucrose and 10mM $CaCl_2$.

$MgCl_2$ (mM)	Protoplasts ml^{-1}
1	$4.3x10^9$
5	$4.5x10^9$
10	$4.9x10^9$
20	$2.9x10^9$
40	$1.0x10^9$
Medium P	$4.1x10^9$

Table 3.7

The Effect of Sucrose Concentration on the Formation of Protoplasts of S. thermonitrificans in a Medium P-like Solution Containing Basal Medium P, 12.5mM $MgCl_2$ and 15 mM $CaCl_2$

Sucrose Concentration %	(mM)	Protoplasts ml^{-1}
4	(117)	$5.0x10^9$
6	(175)	$5.6x10^9$
8	(234)	$5.1x10^9$
10	(292)	$4.3x10^9$
12	(350)	$4.0x10^9$
14	(409)	$4.0x10^9$

A lower level of sucrose (6% w/v; 175 mM) than that used in medium P seemed optimal for the formation of S. thermonitrificans protoplasts.

The experiments described above measured the efficiency of S. thermonitrificans protoplast formation and the stability of the protoplasts formed, under a variety of conditions. Ideally the effect of these different conditions should be assayed by measuring the regeneration frequency of the protoplasts made in the different solutions. However, such an experiment would be time consuming and costly if all 45 medium P-like solutions were to be compared.

Hence, medium P was adopted for use with S. thermonitrificans protoplasts until the alternative medium P-like solutions could be analysed by regeneration frequency at a later date.

S. thermonitrificans grows poorly in the presence of sucrose on solid media or in liquid culture. Substitution of sucrose in the regeneration media with disodium succinate (3.5) or mannitol (3.8) enhanced the regeneration frequencies of S. thermonitrificans protoplasts. It was feasible that the presence of sucrose in medium P could inhibit protoplast regeneration and a derivative solution containing no sucrose might further enhance protoplast regeneration frequencies. Disodium succinate, mannitol and sorbitol were identified as alternative osmotic stabilisers for use in medium P-like solutions. Matrix experiments incorporating variations in the concentrations of carbohydrates and divalent cations were performed. S. thermonitrificans protoplasts were prepared in medium P using lysozyme (0.25 $mg.ml^{-1}$) at 30°C. Once the protoplasts were formed

they were washed with medium P to remove the lysozyme. Equal aliquots of protoplasts in medium P were dispensed into tubes and the protoplasts were pelleted by centrifugation (ca. 3000g). The supernatant was removed and the protoplasts resuspended in different medium P-like solutions in which sucrose was substituted by other osmotic supports. The protoplasts were stressed by a 90 minute incubation at 30°C. Then they were centrifuged out of suspension (ca. 10,000g) and the supernatants were subjected to spectrophotometry. The increased absorbance of the supernatants at A_{260}nm was used to estimate the leakage of nucleic acid from lysed protoplasts. Solutions which minimised the leakage of nucleic acid indicated the medium P-like solution in which the protoplasts were most robust.

Despite the standardised cultivation procedure for S. thermonitrificans mycelium, protoplasts made from the mycelium varied systematically between experiments. A medium P-like solution containing no sucrose, 50mM $MgCl_2$ and 50mM $CaCl_2$ in addition to basal medium P had a leakage value of 42% in one experiment (Table 3.8) and 7% in another (Table 3.9). However, the trend of enhanced protoplast stability in solutions containing higher levels of divalent cations was maintained between experiments. The medium P-like solutions which had the best performances were considered for further study. The study of leakage of nucleic acid from S. thermonitrificans protoplasts in suspension in solutions containing disodium succinate (Table 3.8), mannitol (Table 3.9) and sorbitol (Table 3.10) showed that alternative osmotic buffers could replace sucrose.

The interpretation of data and the choice of medium P-like solutions for further study were not easy. Due to the inclusion

of a temperature incubation step prior to leakage analyses of the supernatants, the leakage values were estimates of how robust the protoplasts were in suspension. Essentially, solutions hypotonic with respect to the S. thermonitrificans protoplasts were identified as those with high leakage values. Isotonic and hypertonic solutions were probably indistinguishable by this method of analysis. Hypertonic solutions were avoided because of the detrimental effect that the loss of water from S. thermonitrificans protoplasts could have on the physiology of those protoplasts. In fungi, both protein synthesis in, and secretion by protoplasts were sensitive to the osmolarity of the stabilising solution (Peberdy, 1979). In solutions of high osmolarity enzyme synthesis was inhibited and intracellular constituents leaked out of the protoplasts. A medium P-like solution isotonic with repect to the protoplasts, in theory at least, ought to provide the optimal environment for protoplast stability. Medium P-like solutions with the lowest osmolarity which provided satisfactory levels of protoplast stability, were therefore good candidates for further study. Consider table 3.8, where the results of the substitution of sucrose with disodium succinate in a medium P-like solution are presented. The leakage value of medium P in this experiment was 4%, i.e. lower than all the disodium succinate derivatives presented. However, the experimental aim was to identify likely solutions for further trial and it was possible that while S. thermonitrificans protoplasts in a disodium succinate version of medium P were less stable, they could regenerate more efficiently due to the absence of sucrose. A detailed analysis of the effect of medium P-like solutions on protoplast regeneration was not worthwhile at this

Table 3.8

Disodium Succinate as an Alternative to Sucrose in Medium P.

Additives to Basal Medium P (see Table 3.1) (mM)				
Disodium Succinate	$MgCl_2$	$CaCl_2$	Leakage %	
0	0	0	100	
0	5	5	87	
0	10	10	84	
0	25	25	82	
0	50	50	42	
75	0	0	109	
75	5	5	99	
75	10	10	98	
75	25	25	65	
75	50	50	12	*
150	0	0	78	
150	5	5	35	
150	10	10	26	
150	25	25	18	
150	50	50	6	*
225	0	0	58	
225	5	5	31	
225	10	10	23	
225	25	25	17	
225	50	50	8	*
300	0	0	40	
300	5	5	26	
300	10	10	20	
300	25	25	17	
300	50	50	15	
Medium P (Sucrose) 300	10	25	4	

* Worthy of further study

Table 3.9
Mannitol as an Alternative to Sucrose in Medium P

Additives to Basal Medium P (see Table 3.1) (mM)				
Mannitol	$MgCl_2$	$CaCl_2$	Leakage %	
0	0	0	100	
0	5	5	67	
0	10	10	59	
0	25	25	45	
0	50	50	7	*
0	100	100	1	
100	0	0	115	
100	5	5	67	
100	10	10	58	
100	25	25	40	
100	50	50	16	*
100	100	100	1	
200	0	0	100	
200	5	5	66	
200	10	10	54	
200	25	25	30	
200	50	50	11	*
200	100	100	1	
300	0	0	92	
300	5	5	60	
300	10	10	47	
300	25	25	20	*
300	50	50	15	*
300	100	100	12	
400	0	0	92	
400	5	5	28	
400	10	10	28	
400	25	25	18	*
400	50	50	17	
400	100	100	19	
500	0	0	112	
500	5	5	30	
500	10	10	18	*
500	25	25	17	
500	50	50	16	
500	100	100	17	
Medium P (Sucrose)				
300	10	25	NA	
Disodium Succinate				
150	50	50	13	

Table 3.10

Sorbitol as an Alternative to Sucrose in Medium P

Additives to Basal Medium P
(see Table 3.1). (mM)

Sorbitol	$MgCl_2$	$CaCl_2$	Leakage %	
75	5	5	13	
75	10	10	14	
75	25	25	10	
75	50	50	2	*
150	5	5	13	
150	10	10	13	
150	25	25	8	
150	50	50	2	*
225	5	5	15	
225	10	10	13	
225	25	25	7	
225	50	50	3	*
300	5	5	13	
300	10	10	5	
300	25	25	6	
300	50	50	3	

Medium P corrected leakage equivalent = 1.0
* Worthy of further study

time because it is possible that any improvement in protoplast regeneration through the use of alternative carbohydrates or improved divalent cation ratios or concentrations would be masked by the inefficient protoplast regeneration on the current regeneration medium.

In order to test the feasibility of using an alternative carbohydrate in the medium P-like solution the disodium succinate based medium P-like solution containing 150mM disodium succinate, 50mM $MgCl_2$ and 50mM $CaCl_2$ (P15) was used for the formation of S. thermonitrificans protoplasts and these protoplasts were compared to protoplasts made in medium P by regeneration studies.

S. thermonitrificans mycelium cultivated in TSB at 37°C for 20 hours formed protoplasts when incubated at 30°C in medium P with lysozyme (0.25 $mg.ml^{-1}$). Using the disodium succinate derivative P15, 1.75 $mg.ml^{-1}$ of lysozyme was required for the efficient formation of protoplasts from S. thermonitrificans mycelium. Essentially the same number of protoplasts could be formed in both solutions (between $5x10^8$ and 10^9 ml^{-1}). The protoplast regeneration medium used was EmR2S (3.7) in which sucrose was replaced with disodium succinate. S. thermonitrificans protoplast regeneration on this medium was poor (about 0.25%) and the colonies that formed were small. There was no significant difference in the regeneration frequencies of S. thermonitrificans protoplasts when the two solutions were used. Further examination of other alternative medium P-like solutions was postponed until an optimal regeneration medium for S. thermonitrificans protoplast regeneration had been developed.

The development of the regeneration medium is still an

ongoing process and improvements may still be made. In the future, when improvements to the regeneration medium are no longer possible, the optimised medium may be used to ask whether the divalent cation concentrations and ratios in medium P may be improved, or whether the presence of sucrose in medium P is inhibitory to S. thermonitrificans protoplast regeneration.

3.5 Development of a Regeneration Media Suitable for the Efficient Regeneration of S. thermonitrificans Protoplasts to a Mycelial Form.

"Probably regeneration is the biggest impediment to genetic work with protoplasts in any new system because, although some useful generalisation can be discerned, different nutritional and other conditions are needed for optimal regeneration even in quite closely related species."
D. A. Hopwood (1981).

This eloquent quote effectively summarises this section on the development of a medium for the efficient regeneration of S. thermonitrificans protoplast cell walls. The methodology for the efficient production of robust S. thermonitrificans protoplasts has been described (3.4). Regeneration media commonly used within the Glasgow laboratory, R2YE (Thompson et al., 1982a) and R9 (Butler et al., 1988), were tested for their abilities to promote good growth and sporulation from an inoculum of S. thermonitrificans spores. As controls for good growth, TSB agar, ATCC172, and Emerson's agar were also tested. These agars differ from the regeneration media because they do not contain high levels of carbohydrates and divalent cations as osmotic buffers. YEME which contains 34% w/v sucrose was also tested. The plates were incubated at 37°C for seven days and the growth scored. TSB agar, ATCC172 and Emerson's agar promoted good growth of S. thermonitrificans from a spore inoculum. Abundant spores could be harvested after growth on TSB and Emerson agars, with somewhat fewer from ATCC172 agar. The other media (R2YE, R9 and YEME agar) were poor media for the cultivation and sporulation of S.

thermonitrificans from a spore inoculum. They contain large amounts of sucrose (300mM, 600mM and 993mM respectively). When sucrose was added to TSB agar, an inhibition of S. thermonitrificans growth from a spore inoculum was obvious at 300mM sucrose but less apparent at 100mM sucrose. The inhibition of S. thermonitrificans growth by sucrose implied that if sucrose was to be used as the carbohydrate to provide osmotic buffering it would have to be used at lower concentrations than were currently used for the regeneration of protoplasts from mesophilic streptomycetes (i.e.<300mM sucrose). Protoplasts of S. thermonitrificans failed to regenerate efficiently on R2, or on derivatives in which the sucrose concentration in R2 was reduced to 100mM or 200mM. The addition of sucrose, $MgCl_2$ and $CaCl_2$ to TSB agar at a variety of concentrations also failed to promote the efficient regeneration of S. thermonitrificans protoplasts. However using these media an estimate of a low level of S. thermonitrificans protoplast regeneration of about 0.01% was possible. The regenerants were of poor quality and about 25% of them had unusual colony morphology. The apparent regeneration frequency was dependent on the dilution of the protoplasts plated. Very few isolated regenerants were found, implying that regeneration of S. thermonitrificans protoplasts on these inefficient media was a cooperative event. Furthermore, regeneration was slow, taking about three weeks of incubation before the colony counts on the regeneration media were reproducible. Clearly, improvements in the regeneration medium were required and as sucrose inhibited the growth of S. thermonitrificans, its replacement with an alternative carbohydrate was given high priority. Shirahawa et al., (1981) used disodium succinate as an alternative to sucrose for the

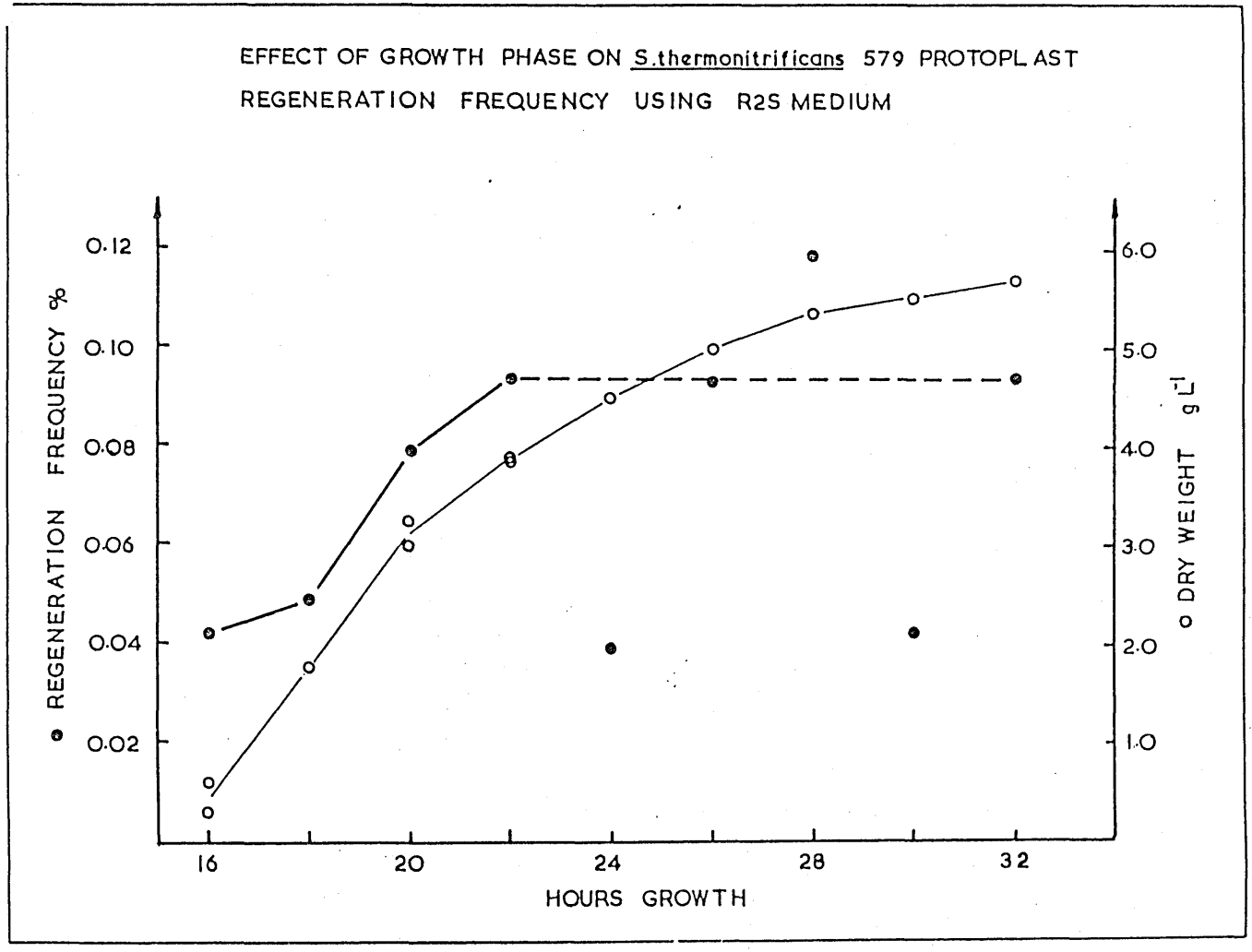
regeneration of S. kasugaensis protoplasts. The regeneration efficiency of S. thermonitrificans protoplasts was compared on regeneration media in which the sucrose in R2YE was replaced with 200mM, 400mM and 600mM disodium succinate. The highest concentration of disodium succinate (600mM) did not support efficient regeneration of S. thermonitrificans protoplasts, but the other two concentrations of disodium succinate (200mM and 400mM) performed better (estimated at about 0.05%). These media supported a higher level of S. thermonitrificans protoplast regeneration than previous regeneration media. As there was little difference between the 200mM and 400mM disodium succinate treatments an intermediate level of disodium succinate (300mM) was chosen for further study. This medium was called R2S.

3.6 The Effect of the S. thermonitrificans Growth Phase on the Efficiency of Protoplast Regeneration using R2S Regeneration Medium.

The effect of the growth phase of the culture when the mycelium was harvested for the formation of protoplasts, on the protoplast regeneration frequency was investigated. Previous experiments used S. thermonitrificans mycelium harvested after 24 hours growth in TSB at 37°C, when the culture was in the transition period between the exponential and stationary phases of growth (Fig. 3.1). Harvesting from this phase of growth was suitable for many mesophilic species of Streptomyces.

Two 250ml flasks containing 50ml TSB and 1.7×10^6 spores of S. thermonitrificans, shaken vigorously at 37°C, were used for each time point, with two samples taken for protoplast formation

Fig 3.1


EFFECT OF GROWTH PHASE ON S.thermonitrificans 579 PROTOPLAST
REGENERATION FREQUENCY USING R2S MEDIUM
● REGENERATION FREQUENCY %
0.12
0.10
0.08
0.06
0.04
0.02
o DRY WEIGHT g L⁻¹
6.0
5.0
4.0
3.0
2.0
1.0
16
20
24
28
32
HOURS GROWTH

and two for dry weight analysis (Fig. 3.1). From 16 to 22 hours post inoculation, (drawn as a solid dark line on the graph) There was an increase in regeneration frequency, which subsequently became variable. At 22 hours the average regeneration frequency on R2S regeneration medium was about 0.09%. This was an appreciable increase over previous regeneration media (0.01%) which used sucrose as an osmotic stabiliser. The results presented in figure 3.1 were obtained one week after inoculation of the regeneration medium, rather than the three weeks incubation period required in earlier experiments. The regeneration efficiency of S. thermonitrificans protoplasts appeared to increase when they were made from mycelium which had been grown for 20 to 22 hours. Mycelium from that stage of growth was used in subsequent experiments.

3.7 The Effect of Nutrients in Regeneration Media on the Efficiency of S. thermonitrificans Protoplast Regeneration.

Employing the logic of Pigac et al. (1982) who used a complex growth medium as a basis for the development of a protoplast regeneration medium for S. rimosus, the preferred agar for growth of S. thermonitrificans (Emerson's agar) was used to attempt to improve the regeneration medium R2S. R2YE is prepared as a binary medium in the Glasgow laboratory. The two parts, A and B, contain different constituents because if all are autoclaved together a precipitate forms. R2/A contains the agar, trace elements, casamino acids, glucose, proline, K_2SO_4, $MgCl_2$ and $CaCl_2$. R2/B contains a pH buffer (TES), yeast extract and sucrose. In the improved version of R2S, called EmR2S, the casamino acids and agar were removed from R2/A, the sucrose in

R2/B was replaced with 300mM disodium succinate and Emerson's agar (83.0 $g.l^{-1}$) was added to R2/B. After autoclaving the two parts were mixed and 1ml of a 1% (w/v) KH_2PO_4 solution was added per 200ml of mixture of A and B before the regeneration plates were poured. EmR2S was a much better regeneration medium than R2S. It increased the regeneration frequency of S. thermonitrificans protoplasts between 24- and 46- fold when compared to R2S in three experiments. The highest regeneration frequency achieved on EmR2S was 0.23%; R2S did not perform as well in these experiments as it had done previously.

To identify the important factor which enhanced the protoplast regeneration frequency the constituents of Emersons agar were further studied with respect to S. thermonitrificans protoplast regeneration efficiency.

Amount and Types of Nutrients

Emersons agar contains 4$g.l^{-1}$ beef extract, 1$g.l^{-1}$ yeast extract, 4$g.l^{-1}$ peptone, 10$g.l^{-1}$ glucose, 2.5$g.l^{-1}$ NaCl and 20$g.l^{-1}$ Difco Bacto agar. These were all added simultaneously to R2S in the development of EmR2S. Addition of the component parts of Emerson's agar to R2S showed that all of the components were required to enhance the protoplast regeneration frequency. The composition of parts A and B of the medium were reorganised forming a new medium EmR2SC. The effect of this reorganisation was shown to be insignificant in terms of protoplast regeneration efficiency.

Two avenues of further media improvement studies were apparent. The disodium succinate concentration and the divalent cation concentration and ratios could be optimised for the

regeneration of S. thermonitrificans protoplasts. However, there had been informal reports of a mannitol based medium suitable for the regeneration of both Streptomyces and Nocardia protoplasts (I. S. Hunter pers. comm). The protoplast regeneration frequencies on the mannitol based regeneration medium were species dependent and between 0.7 and 68% (I. S. Hunter, pers. comm.). Due to the immediate increase in S. thermonitrificans protoplast regeneration frequency obtained when disodium succinate was used instead of sucrose, a trial of regeneration media in which mannitol was used as an alternative to disodium succinate was considered worthwhile.

3.8 Can Mannitol be Used as an Alternative Osmotically Stabilising Carbohydrate for the Regeneration of S. thermonitrificans Protoplasts?

The disodium succinate in EmR2SC was replaced by 100, 200, 300, 400 and 500mM mannitol. S. thermonitrificans protoplasts prepared from mycelia cultivated under the standard conditions for 20 hours were plated onto the mannitol-based regeneration media and incubated at 37°C for one week. These initial experiments suggested that the lower levels of mannitol (100, 200 and 300mM) enhanced the regeneration frequency of S. thermonitrificans protoplasts. There appeared to be an optimum of 200mM mannitol. An examination of the effect of the growth phase of S. thermonitrificans at which mycelium was harvested for protoplasting, on the protoplast regeneration frequency on a mannitol based regeneration medium containing 150mM mannitol (EmRM150) was performed.

This experiment (Fig 3.2) was essentially the same as that

presented in Fig. 3.1 except that the regeneration medium was improved. These results are compared with those of the earlier work using R2S regeneration medium (Fig. 3.3). Approximately the same number of protoplasts were formed per gram dry weight of mycelium throughout the growth curve of S. thermonitrificans. EmRM150 supported a much higher regeneration efficiency of S. thermonitrificans protoplasts (about 0.9% on average) in the 20 hours growth treatment. The error bars are large because of the nature of protoplast regeneration. If plates were not uniformly dried, or if the protoplasts were not spread evenly and a count from a 10^{-3} dilution plate was used instead of a 10^{-4} dilution plate, then the apparent regeneration frequency, from the same population of protoplasts was changed. Protoplasts made from mycelium harvested after 20 hours growth had the optimum regeneration efficiency. This was in the early stages of the growth curve at a time before the mycelium had been harvested in experiments performed previously. In subsequent experiments the mycelium was harvested at this point in the growth curve of S. thermonitrificans.

The enhanced regeneration frequency obtained with mannitol instead of disodium succinate was a tremendous improvement. However, there was a further advantage. Because of the enhanced regeneration it was equally possible to identify conditions which were detrimental to protoplast regeneration efficiency. The regeneration media used previously had supported a low level of cooperative protoplast regeneration, i.e. the apparent protoplast regeneration frequency was higher on lower dilution plates. However, the regeneration of S. thermonitrificans protoplasts on EmRM150 was not cooperative because the protoplast regeneration

Fig 3.2

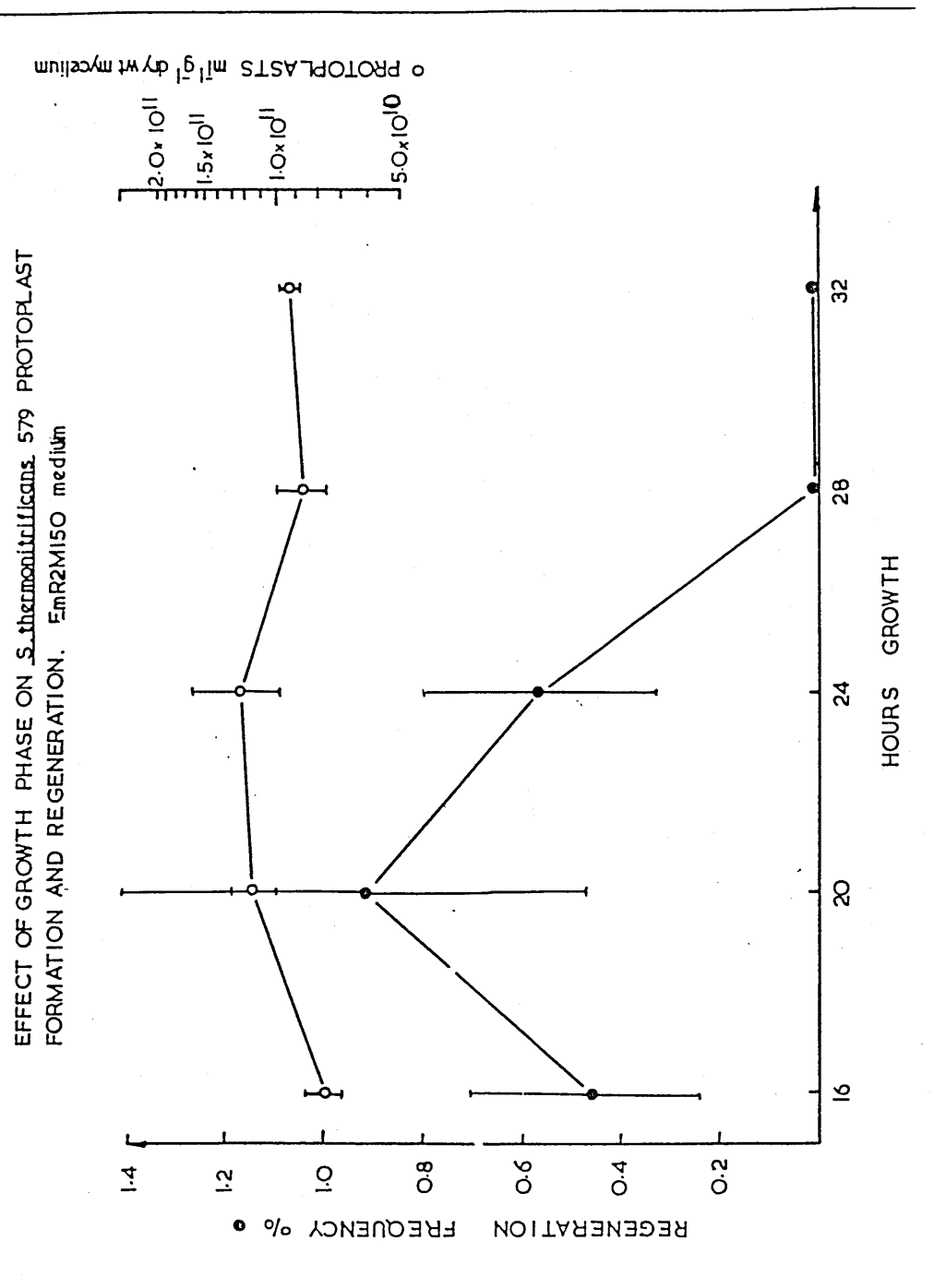
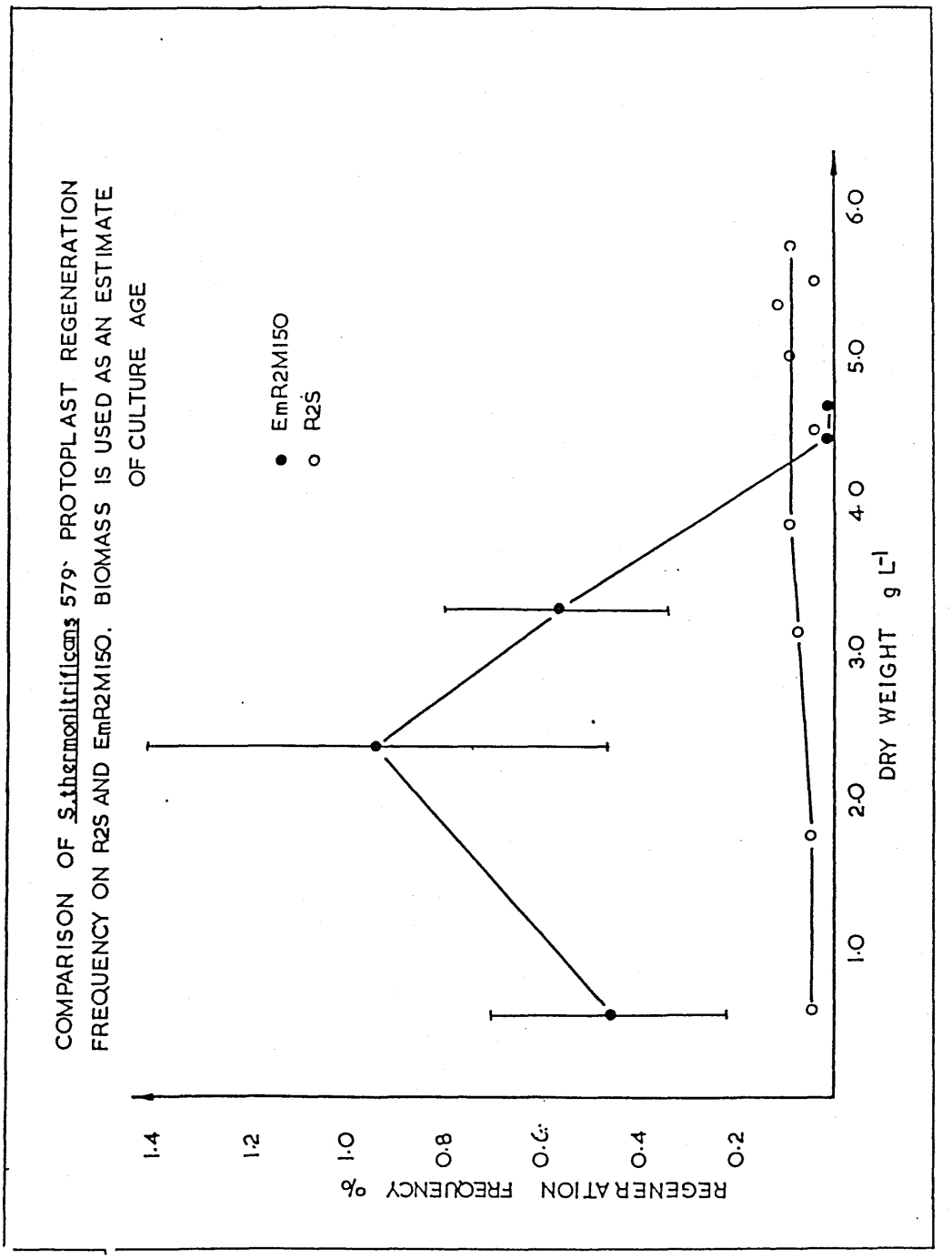

EFFECT OF GROWTH PHASE ON S. thermonitrificans 579 PROTOPLAST FORMATION AND REGENERATION. EmR2MI5O medium
REGENERATION FREQUENCY % ●
1·4
1·2
1·0
0·8
0·6
0·4
0·2
16
20
24
28
32
HOURS GROWTH
o PROTOPLASTS ml⁻¹ g⁻¹ dry wt mycelium
2·0 x 10¹¹
1·5 x 10¹¹
1·0 x 10¹¹
5·0 x 10¹⁰

Fig 3.3


COMPARISON OF S.thermonitrificans 579' PROTOPLAST REGENERATION FREQUENCY ON R2S AND EmR2M150. BIOMASS IS USED AS AN ESTIMATE OF CULTURE AGE
EmR2M150
R2S
REGENERATION FREQUENCY %
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·0
2·0
3·0
4·0
5·0
6·0
DRY WEIGHT g L⁻¹

frequency was higher when the protoplasts were plated at higher dilutions. This change in the character of S. thermonitrificans protoplast regeneration was significant, bringing the behaviour of S. thermonitrificans closer to that of the mesophilic systems already optimised.

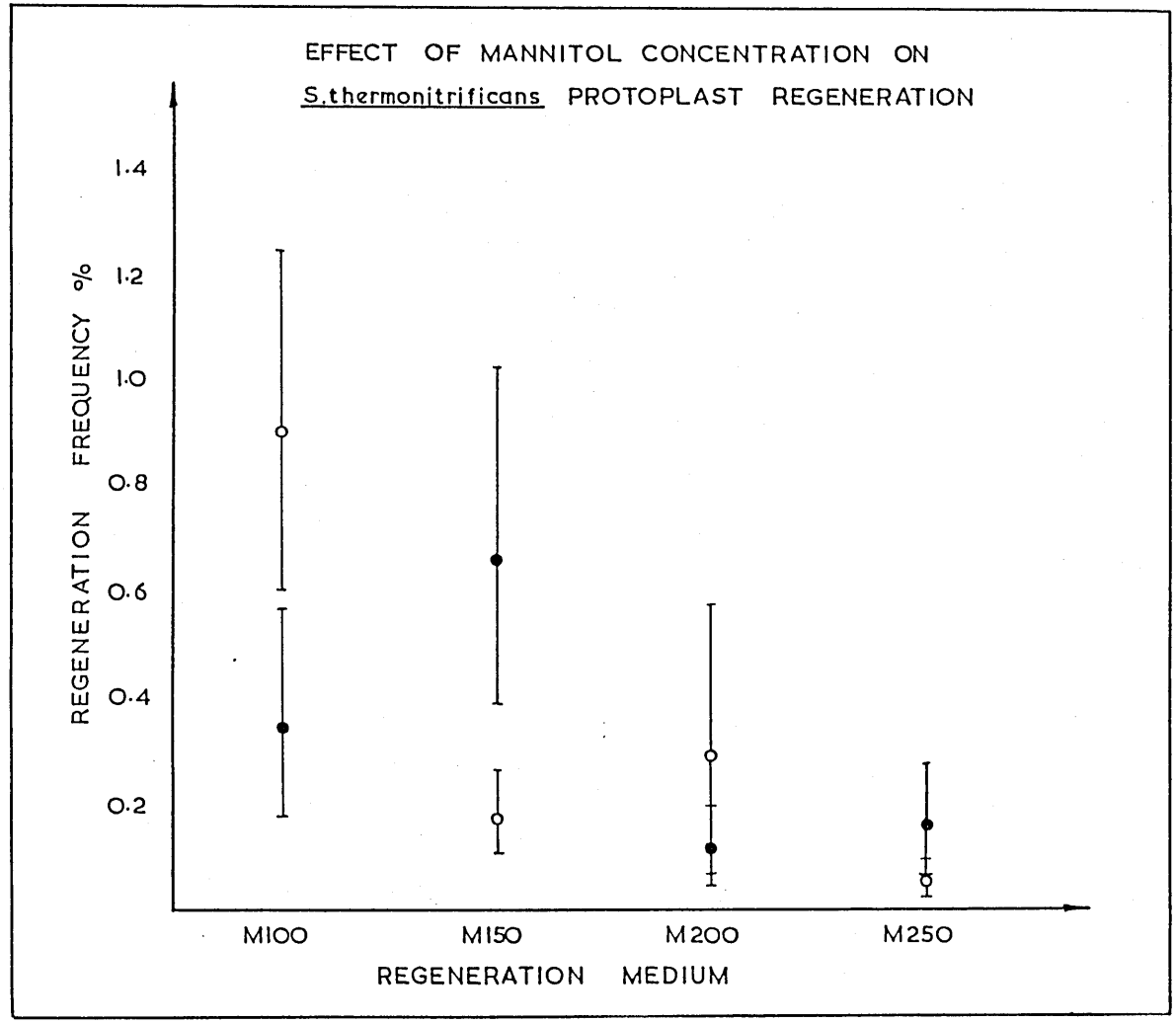
3.9 The Effect of Mannitol Concentration on the Regeneration Efficiency of S. thermonitrificans Protoplasts.

Further improvement in the regeneration efficiency of S. thermonitrificans protoplasts was achieved by the optimisation of the mannitol concentration in the regeneration medium. Media which contained 100, 150, 200 and 250mM mannitol were tested for their effect on S. thermonitrificans protoplast regeneration efficiency (Fig. 3.4). S. thermonitrificans protoplast regeneration was inhibited at higher concentrations of mannitol. By inspection it was likely that the lower levels of mannitol concentration improved the regeneration efficiency of S. thermonitrificans protoplasts. A slight alteration, replacing 150mM mannitol with 125mM mannitol was adopted for further analyses (EmRM125). In future, the effect of mannitol concentration should be further analysed after other media constituents have been optimised.

3.10 The effect of the Divalent Cation Concentration and Ratio in Regeneration Medium EmRM125 on the Regeneration Efficiency of S. thermonitrificans Protoplasts.

Employing the same strategy that worked well for the optimisation of the divalent cation concentration in medium P-like solutions, matrix experiments to investigate the effect of

Fig 3.4


EFFECT OF MANNITOL CONCENTRATION ON
S.thermonitrificans PROTOPLAST REGENERATION
REGENERATION FREQUENCY %
1.4
1.2
1.0
0.8
0.6
0.4
0.2
M100
M150
M200
M250
REGENERATION MEDIUM
O Experiment 1
● Experiment 2

increasing the concentration of divalent cations (in two fold steps) in EmRM125, on the efficiency of S. thermonitrificans protoplast regeneration were performed. Divalent cation concentrations of 10, 20, 40 and 80mM $MgCl_2$ and $CaCl_2$ in pairwise combinations were analysed. S. thermonitrificans protoplast regeneration frequency was enhanced in high $CaCl_2$ and low $MgCl_2$ concentrations in EmRM125 (Table 3.11).

The optimal concentrations of divalent cations were further defined by subdivision of the intervals between concentrations used in the regeneration media. Initially the influence of $MgCl_2$, from 0 to 30mM in 5mM steps in the presence of 80mM $CaCl_2$ was investigated. While the addition of some $MgCl_2$ was beneficial, there was relatively little increase in S. thermonitrificans protoplast regeneration efficiency over the range of $MgCl_2$ tested (Table 3.12). A concentration of 15mM $MgCl_2$ was chosen to study the effect of varying the concentration of $CaCl_2$ on the efficiency of S. thermonitrificans protoplast regeneration. Concentrations of $CaCl_2$ from 40mM to 160mM in 20mM steps were analysed in EmRM125 containing 15mM $MgCl_2$. A concentration of 100mM $CaCl_2$ in EmRM125 containing 15mM $MgCl_2$ was optimal for the regeneration of S. thermonitrificans protoplasts (Table 3.13). Although the optimal number of protoplast regenerants were obtained at 100mM $CaCl_2$, lower concentrations (60mM) supported slightly less efficient regeneration, but that regeneration took place at a faster rate; four instead of seven days. It is not known whether the total number of regenerants or the rate of regeneration is most important for the efficient transformation of Streptomyces protoplasts. In future, when the transformation procedure for S. thermonitrificans is being optimised,

Table 3.11

The Effect of the Concentration and Ratio of $MgCl_2$ and $CaCl_2$ in the Regeneration Media on S. thermonitrificans Protoplast Regeneration

$MgCl_2$	$CaCl_2$ (mM) 10	20	40	80
10	0.00	0.00	0.30	4.60
20	0.00	0.00	0.14	5.30
40	0.00	0.00	0.30	0.60
80	0.11	0.40	0.35	0.04

% S. thermonitrificans Protoplast Regeneration

The values are the averages of two determinations

Table 3.12

Optimisation of the $MgCl_2$ Concentration for the Efficient Regeneration of S. thermonitrificans Protoplasts in the Pres ence of 80 mM $CaCl_2$

$MgCl_2$ (mM)	% Regeneration of S. thermonitrificans Protoplasts
0	2.8
5	9.3
10	8.3
15	10.7
20	9.8
25	11.2
30	7.5
50 mM $MgCl_2$, 20 mM $CaCl_2$	4.8

The values are the average of two determinations.

Table 3.13

Optimisation of the $CaCl_2$ Concentration for Efficient Regeneration of S. thermonitrificans Protoplasts in the Presence of 15 mM $MgCl_2$

$CaCl_2$ (mM)	% Regeneration of S. thermonitrificans Protoplasts
40	7.0
60	7.3
80	8.1
100	14.2
120	14.4
140	5.9
160	5.4

Values are the average of two determinations

consideration of this observation may be worthwhile.

The protoplast regenerants on EmRM125, 15mM $MgCl_2$ and 100mM $CaCl_2$ had a much better colony morphology than regenerants on previous regeneration media. The colonies tended to be flatter, a common morphology for mesophilic species protoplast regenerants, and they often sporulated. This medium was the most efficient S. thermonitrificans protoplast regeneration medium developed, supporting regeneration frequencies of the order of 10%. This medium was named R579.

3.11 Discussion

S. thermonitrificans did not require growth in the presence of glycine or $MgCl_2$ for the efficient formation of protoplasts provided that the lysozyme concentration was lower than that normally used. 0.25 $mg.ml^{-1}$ of lysozyme in medium P at 30°C was routinely used for the formation of S. thermonitrificans protoplasts. Analysis of the effect of the stage of growth of the mycelium at harvesting on regeneration efficiency of S. thermonitrificans protoplasts, showed that mycelium harvested after 20 hours growth gave optimal protoplast regeneration. This stage of growth is earlier than the optimum harvesting time for many mesophilic species.

The choice of carbohydrate and the concentrations and ratio of divalent cations in medium P-like solutions were optimised to improve the yield and stability of S. thermonitrificans protoplasts. Solutions similar to both medium L and medium P were identified. The optimised solutions had comparable performances to medium P and it was unlikely that the increase in

protoplast regeneration frequency sought (100 to 1000-fold) would be achieved through the use of alternative medium P-like solutions. However, when other variables such as the regeneration medium are optimal, further analyses of the effects that medium P-like solutions may have on S. thermonitrificans protoplast regeneration efficiency may be rewarding.

Sucrose inhibited the growth of S. thermonitrificans spores and regeneration media containing appreciable amounts of it failed to support the efficient regeneration of S. thermonitrificans protoplasts. Disodium succinate and mannitol were identified as alternative osmotically buffering carbohydrates which increased the regeneration frequency of S. thermonitrificans protoplasts. The nutrients present in the regeneration medium were found to influence the protoplast regeneration frequency, which increased when the amount and diversity of nutrient sources in the media increased. The S. thermonitrificans protoplast regeneration frequency was dependent upon the concentrations and ratio of $MgCl_2$ and $CaCl_2$ in the regeneration medium. $CaCl_2$ concentrations higher than those reported for other Streptomyces regeneration media were optimal. The concentration of $MgCl_2$ was less critical. The initial S. thermonitrificans regeneration frequencies measured were of the order of 0.01%. The most efficient S. thermonitrificans regeneration medium developed, R579 was able to support regeneration of approximately 10% of the S. thermonitrificans protoplasts. The maximum recorded S. thermonitrificans protoplast regeneration frequency was 14.3%. This level of protoplast regeneration is comparable with many mesophilic species of Streptomyces on their optimal regeneration media.

3.12 Guidelines for the Development of Streptomyces Protoplast Regeneration Media.

If the development of a protoplast regeneration medium for a new species of Streptomyces was required, in hindsight the priorities would be:

1) To test currently used regeneration media for growth of the species from spores and for protoplast regeneration.
2) To identify alternative osmotically buffering carbohydrates if sucrose is unsuitable. R2YE or the preferred growth media should be used as the starting point for optimisation.
3) To investigate the effect of the growth phase of the mycelium at harvesting on protoplast regeneration frequency.
4) To optimise the concentrations and ratios of $MgCl_2$ and $CaCl_2$.
5) To optimise the concentration of osmotically buffering carbohydrate (sucrose, disodium succinate or mannitol).
6) To optimise the types of nutrients and their concentrations.

When the above have been attempted, a further round of optimisation of steps 3 to 6 may be worthwhile. After such a study, the influence of variables in the cultivation of mycelia and in the protoplasting solutions on protoplast regeneration efficiency could be analysed. Other factors such as the temperature of cultivation and regeneration, or the extent to which the regeneration plates are dried may be analysed throughout the schedule.

Chapter 4. The Plasmid Transformation of S. thermonitrificans ISP5579

4.1 The Introduction of DNA into Streptomyces.

The first report of the introduction of exogenous DNA into Streptomyces was by Okanishi et al., (1966). Previously it had been reported that spheroplasts of E. coli were sensitive to infection by purified bacteriophage DNA eg. Meyer et al., (1961). Okanishi et al., (1966) established similar methods for the penetration of biologically active DNA into Streptomyces. They studied the infectivity of DNA of the S. kanamyceticus At-463 actinophage PK-66 (Okanishi and Okami, 1966) to protoplasts of S. kanamyceticus. PK-66 DNA transfected protoplasts of S. kanamyceticus whereas virion could not. A prolonged incubation period, (3 to 4 hours) of DNA and protoplasts was required for transfection. Intact mycelium of S. kanamyceticus was resistant to transfection by PK-66 DNA. Polyethylene glycol (PEG) was not used to induce transfection. Konvalinokova et al., (1977) reported transfection of S. virginiae mycelium, showing that for S. virginiae at least, protoplasts were not an absolute requirement for transfection. Other Streptomyces spp. failed to exhibit similar competence (Bibb et al., 1980).

Polyethylene glycol (PEG) had been used to enhance the transformation frequencies obtained with E. coli spheroplasts and bacteriophage OX174 DNA. The use of PEG to induce transformation of Streptomyces protoplasts was investigated by Bibb et al., (1978). They attempted to transform PEG-treated protoplasts of derivatives of S. coelicolor A3(2) and S. parvulus with SCP2* DNA (see 3.1). Transformants were to be selected by their ability to

show SCP2*-determined lethal zygosis on a confluent SCP2*- lawn. Bibb et al., (1978) found that they could transform both species with SCP2* plasmid DNA using their methodology. They concluded that PEG was a requirement for reproducible transformation of Streptomyces protoplasts by plasmid DNA and that a concentration of about 20% (w/v) PEG 1000 (Koch-Light) was optimal. Between 4-20% of S. coelicolor A3(2) and 0.1% of S. parvulus ATCC12434 viable protoplasts could be transformed by SCP2* DNA. In optimal conditions about $1x10^6$ transformants per ug of SCP2* DNA were obtained (Bibb et al., 1980). The contribution of an efficient regeneration medium for S. coelicolor A3(2, to the development of transformation cannot be over emphasized.

Suarez and Chater (1980a) used the results of Bibb et al., (1978) as a basis for developing improved methodology for the transfection of Streptomyces with actinophage DNA. The second addition of 10% (w/v) PEG 1000 (Koch Light) was omitted. The transfected protoplasts were then plated in a soft top agar overlay of R2YE medium onto a base of hard R2YE medium and the plates were incubated at 30°C for 3 days. The transfection frequency was assayed by counting the number of plaques on the plates. The optimal concentration of PEG 1000 (Koch Light) was 20% (w/v) - identical to that for plasmid transformation (Bibb et al., 1978). Suarez and Chater (1980) used actinophage 0C31, VP5, R4, 0448 and S14 in their assay procedures. Irreversible binding of phage DNA to S. coelicolor protoplasts occurred quickly, between 45 and 60 seconds after DNA addition. The use of heterologous DNA reduced the efficiency of transfection. Other species of Streptomyces and their actinophages have been used in PEG assisted transfection experiments, eg. Krugel et al., (1980);

Isogai et al., (1980); Toyami et al., (1983) and Lampel and Strohl, (1986). The addition of small, positively-charged, DNA-free liposomes to the transfection mixture resulted in transfection frequencies as high as $5x10^7$ transfectants per ug of actinophage DNA (Rodicio and Chater, 1982).

Transformation frequency was dependent on the form of DNA used. Plasmid DNA in the cccDNA form was the most efficient form of DNA to use for transformations of Streptomyces by plasmids, open circle or linear forms with complementary sticky ends transformed at a 10- to 100-fold lower efficiency (Bibb et al., 1980). Plasmid DNA linearised with non-complementary sticky ends, or after treatment with alkaline phosphatase did not transform efficiently (Hopwood et al., 1986).

Thompson et al., (1982a) investigated parameters which increased the plasmid transformation frequency of S. lividans 66. They refined the protoplast transformation methodology reported by Bibb et al., (1978) so that 10^7 transformants per ug of SLP1.2 DNA could be routinely attained. The growth phase at which the culture was harvested had a dramatic effect on the competence of the protoplasts to be transformed. Cultures which had been growing for between 24 and 60 hours formed protoplasts which could be transformed most efficiently. The optimum harvesting time was during the transition period from logarithmic to stationary phases of growth (at 30 hours) when protoplasts could be transformed at a frequency of $4.6x10^6$ per ug with 0.7% of protoplasts regenerating.

Thompson et al., found that modifications to the growth medium and the solutions used in the manipulation of the

protoplasts enhanced the transformation frequency independently of the regeneration frequency of the protoplasts. The modified conditions were to grow S. lividans 66 in YEME (Bibb et al., 1977) containing 34% sucrose, 5 mM $MgCl_2$ and 0.5 mM glycine for 30 to 36 hours at 32°C in a 250 ml flask containing a coiled spring. Bibb et al., (1978) used 'S medium' (Okanishi et al., 1974) containing 5 mM $MgCl_2$ and 0.5% glycine for the cultivation of the mycelium. Previously 'medium P' (Okanishi et al., 1974) was used for handling the protoplasts; Two new solutions were developed by Thompson et al., 'Medium L' was used during the incubation of the mycelium with lysozyme, and 'medium T' was used instead of 'medium P' during the PEG-mediated plasmid transformation step. Medium P was still used for washing or stabilising the S. lividans 66 protoplasts osmotically. Medium L differed from medium P in that it contained a lower concentration of divalent cations. 2.5 mM $MgCl_2$ and $CaCl_2$ were used instead of 10 mM and 25 mM respectively. The reduction in divalent cation concentration allowed a more efficient degradation of the streptomycete cell wall by lysozyme. The protoplasts were not as stable in this solution as they were in medium P. When the lysozyme treatment had finished, the protoplasts were washed immediately with medium P which supported them osmotically. Medium T contained 2.5% (w/v) sucrose. It used 50 mM Tris-maleic acid (pH 8) instead of 25 mM TES (pH 7.2) for pH buffering and 100 mM $CaCl_2$. These alternative solutions resulted in an average of 24 times more transformants of S. lividans 66 per ug of SLP1.2 DNA.

Thompson et al., (1982) also showed that the density of the protoplasts on a plate during the regeneration of transformants and the moisture content of the plate influenced the

transformation efficiency of S. lividans 66 protoplasts. When more than $4x10^8$ protoplasts were inoculated per plate, the transformation frequency fell. Routinely, $4x10^9$ protoplasts were used per transformation. The transformed protoplasts were spread on ten plates to maximise the transformation frequency. Drying the regeneration plates in a laminar flow cabinet also enhanced the transformation frequency (about 60-fold) with an optimum at about four hours drying, when the plates had lost about 22.8% of their weight by evaporation. These two advances probably contributed most to the enhanced protoplast transformation frequencies, while the changes in the solutions played a minor role.

Matsushima and Baltz (1985) found that the methods for protoplast transformation of S. coelicolor A3(2) (Bibb et al., 1978) and S. lividans 66 (Thompson et al., 1982a) were not suitable for the efficient transformation of S. ambofaciens or S. fradiae. They further altered the transformation procedure of Bibb et al., (1978), but in different ways to the alterations of Thompson et al., (1982a). They did not use hypotonic solutions to encourage protoplast transformation (as did Thompson et al., (1982a)) but instead used heterologous DNA and protamine sulphate to increase the plasmid transformation frequency of these strains. They also investigated the effect of alterations in the methods of cell growth, protoplast formation and protoplast regeneration on the transformation frequencies of these species. The factors which affected the protoplast regeneration efficiencies of S. ambofaciens and S. fradiae also affected their transformation efficiencies. The S. lividans 66 plasmid transformation procedure concentrated the protoplasts prior to

the addition of DNA and PEG (Bibb et al., 1978; Thompson et al., 1982a). A similar 20-fold concentration of the protoplasts of S. ambofaciens or S. fradiae prior to the addition of DNA and PEG reduced the transformation frequency ten-fold, wheras a 2 to 3-fold dilution of the protoplasts increased the transformation frequency 2 to 3-fold.

Like Thompson et al. (1982a), Matsushima and Baltz (1985) found that inoculating a regeneration plate with too many protoplasts reduced the transformation frequency. They found that about 10^4 protoplasts per plate was optimal, not the $4x10^8$ that Thompson et al., (1982a) had found to be optimal for S. lividans 66.

The hypotonic solutions for protoplast formation and manipulation adopted by Thompson et al., (1982a) were detrimental to protoplast transformation when used on S. ambofaciens of S. fradiae, for which the use of medium P throughout resulted in a 2 to 3-fold improvement.

The source and batch of PEG also influenced the protoplast transformation efficiency of these species. The optimal concentration of PEG 1000 from Koch-Light was 20% (w/v) as reported by Thompson et al., (1982a) but PEG 1000 from Fluka Chemical Corp. gave the highest transformation frequency when 55% (w/v) was used in medium P.

The concentrations of protamine sulphate and heterologous DNA were optimised empirically. The addition of sodium acetate to 0.3 M in the DNA solution also enhanced the protoplast transformation efficiency of these species (Matsushima and Baltz,

1985).

The protoplast transformation procedure developed by Matsushima and Baltz (1985) worked equally well for S. lividans 66, and produced comparable results to the method of Thompson et al., (1982a). Since the method of Matsushima and Baltz (1985) used about 50-fold fewer protoplasts it must have yielded a higher frequency of transformants per viable protoplast. The efficiency of plasmid transformation of S. ambofaciens and S. fradiae was not correlated directly with the efficiency of protoplast regeneration, but may have been influenced more by the absolute number of viable protoplasts in the transformation reaction. The indirect correlation between protoplast regeneration efficiency and transformation frequency may be explained if the stage of the growth phase for optimal protoplast regeneration and that for optimal competence of the protoplasts to be transformed, did not coincide. However, it does not undermine the belief that the regeneration media must be optimal for optimal transformation frequencies, a point which could do with some clarification (Hopwood et al., 1986). Maximal transformation frequencies can only be obtained when the regeneration medium is optimal, ie. the medium allows the transformed protoplasts to regenerate at maximal efficiency. This statement holds true unless the regeneration conditions for transformed protoplasts differ from those for untransformed protoplasts. There is no evidence that this is the case.

4.1.1 Plasmid and Bacteriophage Vectors for Cloning in Streptomyces.

4.1.1.1 Plasmids

A large number of plasmids have been isolated from Streptomyces and their use in the construction of cloning vectors has been reported in both scientific and patent literature. Hopwood et al., (1986) describe over 80 plasmids in their review of Streptomyces plasmid biology, 14 of which have been used for the development of cloning vectors.

Several characteristics of plasmid biology should be considered before choosing a plasmid for use as a cloning vector.

1) Host Range of the Plasmid.

Preferably the plasmid would replicate in both the cloning host and the donor species so that expression of the cloned sequences could be examined in the donor.

2) Selectable Markers.

Antibiotic resistance determinants on the plasmid should allow selection of the plasmid in both hosts. They must be sensitive to the antibiotic used.

3) Unique Restriction Sites.

The plasmid should contain one or more unique restriction sites compatible with frequently cutting enzymes eg. Sau IIIa.

4) Selection of Recombinant Clones.

Insertional inactivation of an antibiotic resistance or a pigment producing gene has been used to allow an estimate of the recombinant frequency without resorting to DNA preparation and analysis. A positive selection system has also been used in

Streptomyces (Kieser and Melton, 1988) which uses the presence of a perfect inverted repeat at each end of the linear cloning vector pIJ699 in the ligation reaction to positively select for clones containing inserts on transformation. If the plasmid recircularises without an insert the repeats come together forming a structure which is lethal on transformation. Thus non-recombinant products of the ligation reaction are not recovered upon transformation. This positive selection cloning system should be a great advantage in Streptomyces cloning technology.

5) Copy Number.

High copy-number vectors are useful because they provide large quantities of vector and cloned DNA sequences for easy manipulation and analysis. However, some sequences may not be clonable on high copy-number vectors, but may be cloned on low copy-number vectors (Malpartida and Hopwood, 1984). More representative libraries may be made in low copy-number vectors. Fine analysis of the control of cloned genes may also be preferable in low copy-number vectors.

Most of the cloning vectors used in the academic community have been developed by workers at the John Innes Institute, Norwich, U.K. under the direction of D. A. Hopwood. Although others have been used successfully elsewhere (Hopwood et al., 1986), the collection of Norwich vectors provides examples of all the kinds of Streptomyces vectors in use. Their structures and uses have been described in a practical handbook on Streptomyces genetic manipulation written by the Norwich group (Hopwood et al., 1985).

4.1.1.1.1 High Copy Number Plasmids

pIJ101

A family of vectors based on derivatives of the small, self-transmissible, pock-forming, wide host-range, high copy-number plasmid pIJ101 (Kieser et al., 1982) have been developed. The DNA sequence of pIJ101 has been determined (Kendall and Cohen, 1988). The vectors usually contain the tsr (thiostrepton resistance) gene for the selection of the plasmid, and other features. For example, the mel gene (pIJ702 Katz et al., 1983) and the perfect indirect repeat (pIJ699, Kieser and Melton, 1988). They may also include promoterless genes which allow various types of gene fusion eg. pIJ424, pIJ425, pIJ486, pIJ487 (see Hopwood et al., 1985) to act as reporter genes to investigate the control of expression from promoters.

pJV1

pJV1 is a high copy-number (150 per chromosome), broad host-range, self-transmissible pocking plasmid of 10.8 Kbp in size, isolated from S. phaeochromogenes (Doull et al., 1983; Bailey et al., 1986). In vitro derivatives of pJV1 were constructed which were both non-transmissible and marked with the tsr gene. These plasmids (pWOR109 and pWOR120) were shown to be compatible with other known high-copy plasmids eg. pIJ101 and derivatives (Bailey et al., 1986). pJV1 has been included here because the ability to stably maintain combinations of cloned genes on different vectors within the same cell could be very important in the elucidation of mechanisms of gene control in Streptomyces.

4.1.1.1.2 Low Copy Number Plasmids

SLP1.2

This low-copy plasmid (4 to 5 copies per chromosome in S.

lividans) plasmid was isolated as a pocking variant of S. lividans after an interspecific mating with S. coelicolor and was subsequently shown to be a segment of the S. coelicolor chromosome which could exist in an autonomous or integrated state in S. lividans (Bibb et al., 1981a). The formation of this plasmid in S. lividans was complex, with a family of related plasmids being formed which were deletions of a larger plasmid SLP1 (Bibb et al., 1981a). SLP1 excised from the chromosome of S. coelicolor by a site specific process and formed the derivative plasmids by subsequent deletion events (Omer and Cohen, 1984). There are several other similar plasmids in Streptomyces, the most characterised of these being pIJ110 and pIJ408 (Hopwood et al., 1984). This type of Streptomyces plasmid is further described in chapter 6.

SLP1.2 was used for the early cloning experiments in Streptomyces (Thompson et al., 1982b). Since then a number of derivatives have been produced which contain antibiotic-resistance markers. pIJ61 is probably the most versatile containing the genes for thiostrepton (tsr) and neomycin (aph) resistance (Hopwood et al., 1986). The major problem with SLP1.2 vectors is their limited host range (Thompson et al., 1982b), which could be due to incompatibility with resident plasmids in the host, restriction endonuclease activity of the host or a requirement for host encoded functions absent in some species.

SCP2

SCP2 was the first Streptomyces plasmid to be isolated (Schrempf et al., 1975) and characterised (Bibb et al., 1977). It is 30 Kbp in size, has a copy-number of between 1 and 3 per

chromosome and is a resident sex factor in S. coelicolor. However, SLP1.2 was used in preference as a cloning vector for some time. Lydiate et al., (1985) in a detailed investigation of the molecular biology of SCP2 plasmids, developed several useful cloning vectors from SCP2. One of these vectors pIJ922, (24.5 Kbp, marked with the tsr gene) had several unique restriction sites which were available for cloning. pIJ922 was used for the cloning of the genes for the entire actinorhodin biosynthetic pathway from S. coelicolor into S. lividans (Malpartida and Hopwood, 1984).

4.1.1.2 Phages

Most actinophage-derived Streptomyces cloning vectors are derivatives of ΦC31, a broad host-range actinophage which infects about half of the streptomycetes on which it has been tested. The biology of ΦC31 has been reviewed (Chater, 1986). Other actinophages have been used to develop phage cloning vectors for Streptomyces spp. outwith the host range of ΦC31 (Floor and Morin, 1982; Morino et al., 1985; Klaus et al., 1981; Chung and Molnar, 1983).

The techniques for isolating and assaying actinophage from soil are similar to those used for eubacterial phage. Differences in detail usually result from the host's mycelial mode of growth (see chapter 5).

The development of ΦC31 derived cloning vectors has been described by Chater (1986a). Essentially, chimeric bifunctional vectors containing actinophage DNA and an E. coli plasmid were created. Inessential DNA was deleted by the use of chelating agents or by in vitro means and suitable restriction sites

introduced for the cloning of DNA. ØC31 derived cloning vectors are the most versatile of actinophage cloning vectors, with three basic types of vector available for use.

1) High capacity vectors

Suitable for cloning larger fragments of DNA eg. ØC31 KC402 (Harris et al., 1982). Removal of an internal dispensable 6.7 Kbp Bam HI fragment from ØC31 KC402 left 33.8 Kbp of DNA which is too small for phage DNA packaging. Insertion of donor DNA from 2 to 9 Kbp in size renders the phage packageable, thus providing a positive selection for recombinant phage. These types of vectors are either attP$^+$ or attP$^-$. The former allows lysogenisation of the host strain via the attB chromosomal sequence. The latter produces clear plaques in the absence of homology between the cloned DNA and the chromosome of the host. Lysogens are formed when homology is present (either by superinfection of a lysogen, or via insert homology).

2) Low capacity vectors

For example ØKC515 (Chater and Bruton, 1983) designed to clone relatively small fragments of donor DNA (<5 Kbp) for use in mutational cloning experiments (Chater 1986a).

3) Specialised Phage Vectors

Phages carrying chromosomal DNA segments other than attP which allow the selection of lysogens and the elegant in vivo manipulation of Streptomyces. ØC31 KC573 (Fisher, et al., 1987) contains a glucose kinase (glk) gene in addition to genes for thiostrepton and viomycin resistance. It may be used to select for deletions in the genome and to clone the deletion end points in vivo. Promoter-probe phage vectors ØC31 KC310 and ØC31 KC850

(Chater, 1986b) may be used to measure the strength of cloned promoters at a single copy number in lysogens. This alleviates the often aberrant effects of higher copy numbers on such experiments. In vivo recombination may be used to transfer cloned sequences from the high copy number cloning vectors to the phage vectors. The reader is referred to Chater (1986a and 1986b) for further information about actinophage-based vectors.

Although elegant, actinophage vectors have not been used for the interspecific transfer of antibiotic biosynthetic pathways because of insert size constraints in this type of vector. Whilst in future analysis of the biology of thermophilic Streptomyces these phage vectors may be useful, at present plasmid vectors are of more importance.

4.1.1.3 Cosmids

The cohesive (cos) ends of actinophage R4 have been used to construct a cosmid (Morino et al., 1985) based upon the multicopy plasmid pIJ365 (Kieser et al., 1982). Cosmid multimers (7-mers) were packaged into phage particles by infecting with R4. They could be transduced into species of Streptomyces within the host range of R4, but for which no protoplasting and regeneration methodology had been developed. Such a system has many advantages over the present plasmid transformation procedure for the introduction of recombinant plasmid DNA into Streptomyces. To date the use of the R4 cosmid for the cloning of genes involved in antibiotic biosynthesis, or the subcloning of cloned antibiotic biosynthetic pathways onto the cosmid has not been reported.

4.2 A Strategy for the Development of a Transformation System II

The importance of protoplast regeneration for plasmid transformation of Streptomyces protoplasts has been discussed previously (section 3.5). When R579, an efficient regeneration medium for S. thermonitrificans protoplasts had been developed (3.10), the investigation of other factors in the transformation procedure increased in priority. It was important to identify a suitable plasmid to develop the transformation system.

S. thermonitrificans is sensitive to thiostrepton and does not naturally secrete melanin. pIJ702 is a pIJ101 derivative which contains the thiostrepton-resistance and tyrosinase genes. It was therefore potentially suitable for the identification of S. thermonitrificans transformants. The high copy-number of pIJ702 would be useful in preparing large quantities of the plasmid DNA for transformation experiments and would presumably be helpful in isolating the plasmid from putative S. thermonitrificans transformants. Other plasmids (such as pIJ922 and SLP1.2) were also used as a contingency against S. thermonitrificans lying outwith the host range of pIJ702. The lower copy-number and reduced host-range of these vectors was a potential disadvantage. To improve the probability of identifying a replicon suitable for S. thermonitrificans transformation studies two other alternative approaches were taken. 1) S. thermonitrificans and other thermophilic Streptomyces were screened for plasmids. These could be marked with an antibiotic resistance gene and used in transformation assays. 2) A novel approach was attempted, using the origin of replication from a lambdoid thermotolerant actinophage, TA, to construct a plasmid for use in S. thermonitrificans

transformation assays.

The optimisation of the transformation frequency of S. thermonitrificans protoplasts was achieved by a similar approach to that used for the optimisation of regeneration.

4.3 Results

4.3.1 Obtaining the First S. thermonitrificans Transformant using pIJ702.

The transformation of S. thermonitrificans protoplasts with pIJ702 (purified from S. lividans) was attempted using the modified plasmid transformation protocols of Bibb et al., (1978) and Thompson et al., (1982a), with each of the early regeneration media (R2S, EmR2S, EmR2SC). At the time the medium for most efficient regeneration of S. thermonitrificans protoplasts was based on disodium succinate as an alternative osmotic buffer to sucrose (EmR2SC 2.28.7; 3.7). Although it supported protoplast regeneration frequencies of about 0.25%, no transformants of S. thermonitrificans were isolated when 5 ug. of pIJ702 DNA was used.

Putative transformants had been selected using a sucrose solution (10.3% w/v) containing 220 $ug.ml^{-1}$ of thiostrepton as an overlay. 1 ml of this solution was used to flood the surface of a plate after 20 to 24 hours incubation at 37°C. Previous evidence suggested that sucrose inhibited regeneration of S. thermonitrificans protoplasts. Disodium succinate solution (0.15 M) was used instead of the 10.3% (w/v) sucrose solution, without success.

The length of incubation between inoculation and drug

selection could have been critical to the transformation frequency. If too little time had elapsed the ribosomes would not be methylated by tsr, and cells would be killed by drugs. However, experience with mesophiles indicated that when the regenerating protoplasts formed a visible "mist" across the regeneration plates (at low dilution) they were ready to be overlayed.

pIJ702 had always been prepared from S. lividans TK24, an historically non-restricting host. It was possible that S. thermonitrificans had endonucleases which would restrict the entry of heterologous DNA. Alternative hosts to S. lividans TK24 could be used for the preparation of pIJ702 in the hope that a species with a suitably modified DNA could be identified. Experiments using an actinophage mutant, TAmt1, (see 5.7.2) suggested that there was a restriction barrier between S. lividans and S. thermonitrificans. They also identified S. albus as a suitable alternative species for preparation of pIJ702.

The next improved regeneration medium, EmRM150 (2.28.8; 3.8) supported a regeneration frequency of 0.95% for S. thermonitrificans protoplasts. Protoplasts were prepared and transformed with 2 ug of pIJ702 (prepared from S. albusG 153). They were incubated on regeneration plates at 37°C for 14 days. The mycelium and the few spores which had developed were scraped off 25 plates and used to inoculate 5 plates of Emerson's agar containing 25 $ug.ml^{-1}$ of thiostrepton. After 5 days at 42°C, 3 colonies had grown, which upon restreaking on Emerson's agar plus thiostrepton plates proved to be thiostrepton-resistant.

4.3.2 Analysis of Thiostrepton-Resistant Colonies of S. thermonitrificans.

4.3.2.1 Physical

The putative thiostrepton-resistant recombinants of S. thermonitrificans might have been the result of spontaneous mutations to thiostrepton-resistance. This was thought unlikely because the control transformation which included no DNA in the transformation procedure did not produce thiostrepton-resistant colonies. The three colonies were the result of at least two independent events (the products of two transformations were examined). Plasmid cccDNA was refractory to isolation from the putative recombinants by a number of protocols (alkaline lysis, Maniatis et al., 1982; alkaline lysis, Kieser, 1984; PEG precipitation, Hunter, 1985). However, adjusting the concentration of the lysozyme used in the enzymatic digestion of the streptomycete cell wall to 0.25-0.5 $mg.ml^{-1}$ improved the lysis of the S. thermonitrificans mycelium and pIJ702 was detected as cccDNA. Additional plasmids were also detected in S. thermonitrificans pIJ702 transformants. This will be discussed in chapter 6.

4.3.2.2 Temperature Range

The pIJ702 transformants of S. thermonitrificans grew on Emerson's agar plus 25 $ug.ml^{-1}$ thiostrepton at temperatures of up to 53°C (the highest tested). Untransformed S. thermonitrificans was sensitive to thiostrepton at this temperature. Thus the antibiotic thiostrepton was active at 53°C, the tsr gene on pIJ702 was expressed and the 23S rRNA of S. thermonitrifcans was methylated correctly at this temperature. The mel gene of pIJ702 was not expressed phenotypically in S. thermonitrificans.

4.3.2.3 Plasmid Stability

The plasmid stability of pIJ702 in S. thermonitrificans was analysed, but due to the nature of plasmids in mycelial organisms the results were difficult to interpret. At a gross level, S. thermonitrificans was transformed stably by pIJ702, which was not lost rapidly from the culture when grown non-selectively on solid or in liquid media. The temperature of incubation did not affect the stability of pIJ702 dramatically.

A pIJ702 containing spore suspension was plated at 37°C on Emerson's agar with and without thiostrepton. Liquid cultures of TSB with and without thiostrepton were also inoculated and grown at 37°C and 46°C. In each case the spores or mycelium produced were plated onto slopes of Emerson's agar with or without thiostrepton and grown at 37°C. The spores harvested from these slopes were used to inoculate plates of Emerson's agar with or without thiostrepton and the number of colonies on the selective medium compared to the number on the non-selective medium (Table 4.1).

One life cycle of non-selective growth of a pIJ702 containing strain of S. thermonitrificans resulted in the reduction of the number of plasmid containing spores to about 74% of the population (Table 4.1). If an overnight growth in non-selective liquid medium was included prior to the production of spores (regime 3) a further reduction to 37% of plasmid containing spores was found. The difference in plasmid stability may be due to the formation of plasmid free cells in the liquid culture. If the growth in liquid culture was selective (regime 5), then the % of plasmid free spores in the population fell, as might be expected.

Table 4.1
The Stability of pIJ702 in S. thermonitrificans

Growth Regime	Selective/Non-selective Spore Production	Plasmid Stability %
1) Emersons agar 37°C	-	74
2) Emersons agar 37°C	+	146
3) TSB 37°C	-	37
4) TSB 37°C	+	134
5) TSB+Thiostrepton 37°C	-	62
6) TSB+Thiostrepton 37°C	+	156
7) TSB 46°C	-	46
8) TSB 46°C	+	195
9) TSB+Thiostrepton 46°C	-	103
10) TSB+Thiostrepton 46°C	+	251

Plasmid Stability

$$\frac{\text{Number of Colonies on Selective Media}}{\text{Number of Colonies on Non-selective Media}} \times \frac{100}{1}$$

An elevated temperature of 46°C (regimes 7 and 9) did not alter the results qualitatively. If anything, the stability of pIJ702 in S. thermonitrificans was enhanced at the higher temperature. Selection with thiostrepton was required to maintain pIJ702 in a large proportion of S. thermonitrificans mycelium.

When spores were produced under thiostrepton selection "unusual" plasmid stability frequencies were estimated in all cases. The apparent frequency of plasmid containing spores was greater than the number of spores in suspension as estimated by the colony counts on the non-selective Emerson's agar. It is difficult to imagine that plasmid free spores were at a disadvantage in the absence of the drug, and plasmid-free spores were unlikely to be at an advantage in the presence of the drug. It is more plausible that plasmid$^+$ spores were at a disadvantage in the absence of the drug; although it is also possible that plasmid$^+$ spores were at an advantage in the presence of the drug. The tsr gene is expressed constitutively (Bibb and Cohen, 1982). By methylation of the 23S rRNA the tsr gene product inhibits the binding of thiostrepton to the ribosome (Thompson et al., 1982b). It is conceivable that a methylated ribosome in the absence of thiostrepton is at a disadvantage and that the disadvantage may be reflected in the survival of outgrowing spores. Such an explanation would account for the unusual plasmid stability frequencies presented (Table 4.1). Alternative plasmid selection markers may be more suitable for use in S. thermonitrificans.

4.4 A Comparison of the Transformation Efficiency of pIJ702 DNA When Prepared from S. thermonitrificans, S. lividans and S. albusG 153.

1 ug aliquots of pIJ702 DNA prepared from S. thermonitrificans, S. lividans or S. albusG 153 in 10 ul of medium P were used for the transformations. EmRM125 regeneration plates were inoculated with 100 ul of the appropriate transformation mix, or a dilution thereof. After 20 hours incubation at 37°C the regeneration plates were overlayed with 1 ml of a 10.3% (w/v) sucrose solution containing 220 $ug.ml^{-1}$ of thiostrepton to select for the transformed protoplasts. The plates were scored after a total of 6 days incubation (Table 4.2).

There was no apparent restriction barrier to the transformation of S. thermonitrificans with pIJ702 DNA prepared from S. albusG 153 when compared with DNA from S. thermonitrificans. However, pIJ702 DNA prepared from S. lividans was unable to transform S. thermonitrificans. This could have been due to restriction enzymes(s) of S. thermonitrificans digesting the DNA prepared from S. lividans, these enzymes not being active upon DNA prepared from S. albusG 153. This result was significant. S. albusG 153 was identified as a host which was able to alter the biological activity of the plasmid in such a way as to make it suitable for the transformation of S. thermonitrificans.

The efficiency of plasmid transformation was low, requiring approximately $1.6x10^6$ S. thermonitrificans protoplasts per pIJ702 transformant when 1 ug. of pIJ702 DNA prepared from S. thermonitrificans was used in the transformation.

Table 4.2

The Effect of the Source of the Plasmid DNA on the Transformation Frequency of S. thermonitrificans Protoplasts

Source of DNA	Transformation Frequency ($t.ug^{-1}$)
S. thermonitrificans	1235
S. albus	939
S. lividans	0

The average transformation frequencies of S. thermonitrificans protoplasts from three determinations are presented.

4.5 Preliminary Analysis of the Transformation Reaction: The Survival of Protoplasts after PEG Treatment and the Effect of Protoplast Concentration in the Reaction.

The efficiency of PEG-induced plasmid transformation of S. thermonitrificans protoplasts required improvement. To identify the steps within the transformation procedure which could lead to an increase in the transformation efficiency, the fate of the protoplasts throughout the transformation protocol was analysed. The number of protoplasts surviving the plasmid transformation procedure was determined by haemocytometry of the protoplast suspension before and after transformation. In two experiments an average of 10.5% (9 and 12%) of S. thermonitrificans protoplasts survived the addition of 25% PEG (w/v) required to induce plasmid transformation. The non-protoplasts were unaltered by the PEG treatment. The proportion of non-protoplasts in the population increased by a factor of ten.

When fewer (about 0.1x) protoplasts were used in the transformation reaction the S. thermonitrficans protoplast transformation efficiency increased. An average of $1.6x10^4$ $t.ug^{-1}$ of pIJ702 (prepared from S. thermonitrificans) was achieved in three experiments when $2.3x10^8$ protoplasts per transformation ($P.t^{-1}$) were used and an estimated $1.1x10^7$ protoplasts were inoculated per R579 regeneration plate. The maximum efficiency of protoplast transformation in these experiments was $1.8x10^4.ug^{-1}$ pIJ702, when $4x10^3$ total protoplasts were present per transformant. About 21% of the protoplasts which survived the PEG treatment during the transformation procedure regenerated. The calculated transformation efficiency per viable protoplast was therefore $3.2x10^2$ viable protoplasts per transformant.

These results indicated that the addition of PEG stressed the S. thermonitrificans protoplasts during the transformation procedure in such a way that fragile protoplasts, which were unlikely to regenerate, were selected against and the abundance of robust protoplasts and non-protoplasts was enhanced. Furthermore, these results indicated that enhancing the frequency of survival of the protoplasts through the PEG step in the transformation might lead to an improvement in the transformation frequency of S. thermonitrificans protoplasts.

4.5.1 Optimisation of the PEG-Induced Plasmid Transformation Protocol for S. thermonitrificans Protoplasts.

The preliminary transformation experiments described above suggested that the number of protoplasts in the transformation reaction and/or, the number of protoplasts plated on the regeneration plates were important factors in determining the transformation efficiency of S. thermonitrificans protoplasts. The survival of protoplasts through the transformation reaction was low (ca. 10.5%) (4.5). Improvements in protoplast survival were thought to be important if the transformation efficiency was to be increased. Thompson et al., (1982a) reported that alterations in the manipulation of buffers for protoplasts enhanced the transformation efficiency of S. lividans. In future, the alternative solutions developed in chapter 3 for protoplasts of S. thermonitrificans should be tested for enhancement of transformation frequencies after other variables in the transformation protocol have been optimised.

4.5.2 Order of Addition of Plasmid DNA, Protoplasts and Polyethylene Glycol in the Transformation Procedure.

Seven alternative transformation procedures were followed (Table 4.3). It was possible that the osmotic shock on protoplasts could differ between treatments and this could influence the transformation efficiency.

Procedure 4 was adopted for the transformation of S. thermonitrificans protoplasts by plasmid DNA (This method was used previously from the modified transformation procedure of Bibb et al., 1980). Order of addition had no dramatic influence on the transformation frequency.

4.5.3 The Effect of the Concentration of PEG (w/v) on the Transformation Frequency of S. thermonitrificans Protoplasts.

Using standard procedures (see methods), 150 ng of pIJ702 DNA prepared from S. thermonitrificans was used to transform protoplasts of S. thermonitrificans.

The quantity of PEG used in the transformation protocol for S. thermonitrificans was not critical (Table 4.4). Addition of PEG in the range of 20-50% (w/v) to the transformation resulted in about the same transformation frequency. On the basis of these observations there was no reason to alter the concentration of PEG from that used previously (25% w/v) for the transformation of S. thermonitrificans protoplasts.

Table 4.3

The Effect of the Order of Addition of Plasmid DNA, Protoplasts and Polyetylene Glycol to the Transformation Efficiency

Procedure	Transformation Frequency $t.ug^{-1}$
1) DNA added to protoplasts PEG onto protoplasts Medium P onto mixture	$1.6x10^3$
2) Protoplasts added to DNA PEG onto protoplasts Medium P onto mixture	$2.7x10^3$
3) DNA added to protoplasts Protoplasts onto PEG Mixture into medium P	$3.6x10^3$
4) Protoplasts added to DNA PEG onto protoplasts Mixture into medium P	$5.7x10^3$
5) DNA added to protoplasts Protoplasts into PEG Mixture into medium P	$4.5x10^3$
6) Protoplasts added to DNA Protoplasts into PEG Mixture into medium P	$3.9x10^3$
7) DNA added to protoplasts Protoplasts into medium P Medium P into mixture	$4.8x10^3$
8) As for procedure 4 but without DNA	0.0

Table 4.4

The Effect of the PEG Concentration in the Transformation Procedure on the Transformation of S. thermonitrificans Protoplasts

% of PEG added to the transformation	$t.ug^{-1}$	Total Protoplasts.t^{-1}.
10	$4.3x10^{2}$	$1.8x10^{6}$
20	$3.3x10^{3}$	$2.5x10^{5}$
30	$4.1x10^{3}$	$2.0x10^{5}$
40	$3.2x10^{3}$	$2.5x10^{5}$
50	$3.7x10^{3}$	$2.2x10^{5}$
60	$6.0x10^{2}$	$1.4x10^{6}$

Values are the average of two determinations.

Approximately $8.8x10^{8}$ S. thermonitrificans protoplasts were used per transformation, plating about $3x10^{7}$ protoplasts per plate.

4.5.4 The Effect of Density of Plating of Protoplasts on the Plasmid Transformation Frequency of S. thermonitrificans Protoplasts.

Approximately $8x10^8$ S. thermonitrificans protoplasts were transformed with 150 ng pIJ702 DNA purified from S. thermonitrificans. The mixture of transformed protoplasts and dilutions thereof were plated onto regeneration media and subjected to selection by a thiostrepton-sucrose overlay after 18 hours incubation at 37°C (Table 4.5).

When the number of protoplasts on the regeneration plates was reduced the transformation frequency of S. thermonitrificans protoplasts was enhanced. This was probably due to a reduction in the competition between protoplasts and with non-protoplasts on the regeneration medium. An optimal transformation frequency was obtained by plating a $3x10^{-1}$ dilution of the transformation mixture. It is not obvious why a further dilution of the transformation mixture (to $1x10^{-1}$) resulted in a reduced transformation frequency.

4.5.5 The Effect of the Number of S. thermonitrificans Protoplasts in the Plasmid Transformation Procedure on the Transformation Efficiency of S. thermonitrificans Protoplasts.

The concentration of protoplasts used in the transformation procedure was varied. 150 ng of pIJ702 DNA prepared from S. thermonitrificans was used with 25% PEG in each transformation procedure.

Table 4.5

The Effect of Protoplast Density on the Regeneration Plates on the Plasmid Transformation Frequency of S. thermonitrificans Protoplasts

Number of protoplasts per plate	$t.ug^{-1}$	Total Protoplasts. t^{-1}
$3.1x10^{7}$	$4.6x10^{3}$	$1.8x10^{5}$
$1.0x10^{7}$	$9.2x10^{3}$	$8.9x10^{4}$
$3.1x10^{6}$	$2.9x10^{3}$	$2.9x10^{5}$

The values are the averages of two determinations.

A reduction in the total number of protoplasts in the transformation procedure enhanced the plasmid transformation efficiency of S. thermonitrificans protoplasts (Table 4.6). The reduction in the concentration of protoplasts in the transformation also changed the number of protoplasts plated. Previously (4.5.3) when the number of protoplasts plated was altered the peak transformation efficiency was $9.2x10^3$ $t.ug^{-1}$ pIJ702, at a plating density of $1x10^7$ protoplasts per plate. At the corresponding protoplast plating density in the above experiment a maximum transformation frequency of $6.5x10^4$ $t.ug^{-1}$ pIJ702 was recorded. This maximum was not entirely due to the protoplast plating density because the same protoplasts were used for both experiments. The enhanced transformation frequency recorded in table 4.6 was likely to be the result of the reduced protoplast concentration in the transformation protocol. At the maximum transformation frequency of $6.5x10^4$ $t.ug^{-1}$ DNA, one in every $4.11x10^3$ protoplasts was transformed. When the protoplast concentration in the transformation was reduced further to $3.1x10^6$ protoplasts per transformation, one in $2.1x10^3$ protoplasts were transformed. This was at the expense of the absolute transformation frequency ($6.5x10^4$ to $3.8x10^4$ $t.ug^{-1}$). After consideration of the protoplast viability through the transformation procedure (ca. 10.5%) (4.5) and the ability of the survivors to regenerate (20-50%) (4.5), the number of viable protoplasts per transformant was estimated to be between 45 and 120 i.e. 1-2% of viable protoplasts were transformed. This efficiency is still lower than the transformation efficiency for S. coelicolor; when between 4-20% of viable protoplasts may be transformed (Bibb et al., 1978).

Table 4.6

The Effect of the Number of S. thermonitrificans Protoplasts in the Plasmid Transformation Procedure on the Transformation Efficiency of S. thermonitrificans Protoplasts

No. of Protoplasts Per Transformation	No. of Protoplasts Per Plate	$t.ug^{-1}$	$P.t^{-1}$
$8.2x10^8$	$3.1x10^7$	$5.0x10^3$	$1.6x10^5$
$2.7x10^8$	$1.0x10^7$	$6.5x10^4$	$4.1x10^3$
$8.2x10^7$	$3.1x10^6$	$3.8x10^4$	$2.1x10^3$
$2.7x10^7$	$1.0x10^6$	$3.8x10^3$	$7.1x10^3$

4.5.6 Selection of Transformants of S. thermonitrificans.

The time of selection of transformants on regeneration plates could have been critical in determining the overall transformation frequency of S. thermonitrificans. The incubation is required to allow the protoplast regeneration process to begin and the antibiotic resistance gene (usually thiostrepton) to express before stressing the cells with antibiotic. Thiostrepton was added in an overlay of 6 alternative media to a final concentration in each plate of 10 ug.ml^{-1} (table 4.7).

Overlays which do not contain osmotic buffers such as carbohydrate or divalent cations should be avoided. The most efficient overlay in these experiments was R medium agarose. Subsequently soft nutrient agar (0.5% w/v) was found to work equally well.

4.6 Transformation of S. thermonitrificans C5 by pIJ702 and pIJ916.

During the studies of stability of pIJ702, strains of S. thermonitrificans which had lost pIJ702 were isolated. These plasmid "cured" strains were used as recipients in further transformation assays to determine whether the competence of protoplasts derived from these strains was enhanced. One such strain S. thermonitrificans C5 (C5) was chosen for further use.

Protoplasts were prepared from S. thermonitrificans and C5 by the now established methods for S. thermonitrificans. They were transformed with pIJ702 prepared from S. thermonitrificans pIJ702 (100 ng) and with pIJ916 prepared from S. albus G153 (350

Table 4.7
The Effect of Different Transformant Selection Procedures on the Recovery of S. thermonitrificans Transformants.

pIJ702 prepared from S. thermonitrificans (100 ng) was used to transform $3.7x10^8$ S. thermonitrificans protoplasts. The transformed protoplasts were plated onto R579 regneration medium and incubated at 37°C for 20 hours. They were then overlayed with the different media containing thiostrepton, in each case thiostrepton was added to a final concentration in the plate of 10 $ug.ml^{-1}$.

Overlay	$t.ug^{-1}$
Sucrose (10.3%) 1 ml	$1.0x10^3$
Water	$6.0x10^2$
R medium Agar	$1.2x10^3$
R medium Agarose	$4.0x10^3$
Nutrient Broth Agarose	$1.6x10^3$
Water Agarose	$1.0x10^2$

Agar and Agarose was added to 0.5% (w/v)
R medium, the constituents of R579 but 0.5% (w/v) agar/agarose

ng). The regeneration and transformation frequencies of these protoplasts were determined (table 4.8).

During the course of other transformation experiments the above result was reproducible. S. thermonitrificans C5 protoplasts were more competent for transformation than S. thermonitrificans protoplasts. In each case the regeneration frequency of C5 protoplasts was reduced. It must be noted that the regeneration frequency and transformation efficiency of S. thermonitrificans protoplasts was lower than previously recorded (0.22% regeneration, compared to ca. 10% (table 3.13)). The reasons for the reduction are not known. If the previous regeneration efficiencies for S. thermonitrificans (section 3.13) were again attained, the relative transformation frequencies of S. thermonitrificans and C5 could be redetermined. This was not achieved therefore, S. thermonitrificans C5 was adopted for further work. The inability to transform S. thermonitrificans with plasmid DNA prepared from itself (Table 4.8) was not immediately explicable. Possibly the reduced protoplast regeneration frequency was the cause, a notion supported by the inability to transform S. thermonitrificans on early regeneration media which supported low regeneration frequencies (4.3.1). However, it was clear that C5 protoplasts were more competent than those from ISP5579.

4.6.1 Analysis of S. thermonitrificans C5/pIJ916

Plasmid DNA from a putative pIJ916 transformant of S. thermonitrificans C5 was purified by alkaline lysis and caesium chloride/ethidium bromide density gradient centrifugation. Restriction endonuclease digestion of the purified plasmid DNA and examination of the products of digestion by agarose gel

Table 4.8

A Comparison of the Transformation Frequencies of Protoplasts of S. thermonitrificans ISP5579 and C5 with pIJ702 and pIJ916 Prepared from S. thermonitrificans ISP5579 pIJ702 and S. albusG 153/pIJ916 Respectively

Strain	R%	$t.ug^{-1}$ pIJ702	pIJ916
ISP5579	0.22	0	0
C5	0.03	$3.9x10^3$ *	82

* $1.7x10^3$ Protoplasts per transformant
R% = Protoplast regeneration frequency

electrophoresis was consistent with the transformant containing pIJ916. The temperature range of resistance to thiostrepton conferred by pIJ916 on the S. thermonitrificans C5 transformant was measured. The recombinant was resistant to thiostrepton at 49°C but not at 53°C. As the thiostrepton-resistance marker had been shown previously to be active at the higher temperature (4.3.2.2), pIJ916 was not as temperature stable as pIJ702. The maximum temperature tested at which pIJ916 was stably maintained (49°C) was high enough to allow the feasibility of antibiotic biosynthetic pathways cloned on this or similar vectors (eg. pIJ922) to be tested.

The stability of pIJ916 in S. thermonitrificans C5 was determined. About 90% of spores maintained the thiostrepton-resisitance phenotype after a cycle of non-selective growth on solid media. An analogous experiment to the stability study on pIJ702 (4.3.2.3) was not performed on C5/pIJ916.

During the plasmid stability study, a plasmid-cured strain of C5/pIJ916 was isolated, it was named S. thermonitrificans C51.

4.7 Transformation of S. thermonitrificans C51 by pIJ702 Prepared From S. lividans

Protoplasts of S. thermonitrificans C51 were prepared by the standard procedure and were transformed with 1 ug of pIJ702 prepared from S. lividans. The transformation frequency of S. thermonitrificans C51 with this DNA was compared with the transformation frequencies of S. thermonitrificans and C5 using the same DNA. Previously (4.4), pIJ702 DNA prepared from S. lividans was unable to transform S. thermonitrificans. In two

transformation assays S. thermonitrificans C51 was transformed by pIJ702 whereas S. thermonitrificans and C5 were not. The transformation frequency of C51 by pIJ702 from S. lividans was of the order of $10^3.ug^{-1}$. This preliminary evidence suggests that C51 may have a reduced restriction barrier to DNA from S. lividans. If this is the case, then C51 should be adopted for future work. Analysis of the restriction status of S. thermonitrificans, S. thermonitrificans C5 and S. thermonitrificans C51 by actinophage TA studies could be used to clarify why strain C51 is competent to transformation by plasmid DNA directly from S. lividans.

4.8 Screening of S. thermonitrificans for Plasmids ?

A plasmid isolated from S. thermonitrificans could form the basis for the development of thermophilic streptomycete cloning vectors. It would be temperature-stable, and would obviously have S. thermonitrificans within its host range. It would have been essential to the thermophilic Streptomyces project if mesophilic plasmids were not suitable for use in S. thermonitrificans. In addition to the practical advantages which endogenous plasmids might have, the isolation and characterisation of a plasmid or plasmids from S. thermonitrificans would allow a comparison of thermophilic plasmid biology with mesophilic streptomycete plasmids.

S. thermonitrificans was subjected to several methods of analyses for the physical detection of plasmids, including alkaline lysis (Maniatis et al., 1982), the alkaline lysis method developed for S. lividans by Kieser (1984) and the polyethylene glycol (PEG) precipitation method of Hunter (1985) were used.

Total DNA of S. thermonitrificans was also prepared using methods of Smith (procedure 3, Hopwood et al., 1985) and Hunter (method 1, 1985). The methods were optimised for S. thermonitrificans by adjusting the concentration of lysozyme used for the lysis of the mycelium. In general 0.25 to 0.5 $mgml^{-1}$ of lysozyme was used. In all cases the DNA was analysed by both agarose gel electrophoresis and by ethidium bromide/caesium chloride density-gradient centrifugation. Only on one occasion (which was not reproduced) was physical evidence for a DNA molecule or molecules other than S. thermonitrificans chromosomal fragments obtained. When this DNA was analysed by agarose gel electrophoresis, two bands were seen of higher apparent molecular weight than the chromosomal smear. The plasmid was refractory to routine isolation and was apparently of low copy number. Such a plasmid was not ideal for the development of a cloning vector for use in S. thermonitrificans and there were no further attempts at the physical isolation of plasmid from S. thermonitrificans.

4.9 A Survey of Available Thermophilic Streptomyces spp. for Plasmids

Plasmids isolated from thermophilic Streptomyces spp. other than S. thermonitrificans would possess the same characteristics that made a plasmid from S. thermonitrificans so desirable, with the exception that the host range of the plasmids would need to be tested to ensure that they included S. thermonitrificans.

4.9.1 Physical Detection

The following thermophilic streptomycetes were analysed for the presence of plasmid DNA by the procedures outlined in 4.8.

		Lab' No.
S. thermohygroscopicus	KCC-S-0917	(017)
S. thermohygroscopicus	KCC-S-0918	(018)
S. thermogriseoviolaceus	KCC-S-0919	(019)
S. thermoolivaceus	KCC-S-0920	(020)
S. thermoolivaceus	KCC-S-0921	(021)
S. threomyceticus	KCC-S-0935	(035)
S. thermovulgaris	ISP5544	(544)
S. thermoflavus	ISP5574	(574)
S. albus (Pfizer)	ISP5313	(313)

Plasmids were not isolated when many of the plasmid isolation procedures were performed. This may have been a result of non optimal lysis conditions as the lysozyme concentration was not changed from the recommended levels. However, there was evidence for plasmids in species S. thermohygroscopicus subsp. rubiginosus KCC-S-0918, S. thermogriseoviolaceus KCC-S-0919 and S. threomyceticus KCC-S-0935, a producer of threomycin. When the DNA extracted from these species was analysed by ethidium bromide/caesium chloride density gradient centrifugation, bands distinct from the chromosomal DNA were identified and purified in each case.

S. thermohygroscopicus subsp. rubiginosus contained 5 or 6 cccDNA species. The two species of highest copy number had apparent sizes of about 7 and 17 Kbp with copy numbers of approximately 140 and 35 copies per chromosome respectively (plasmid copy number determination was as by Kieser et al., 1982). The largest plasmid was about 40 Kbp in size and had a low copy-number estimated at 2 to 3 per chromosome. The relationship between these plasmids was unknown and it is possible that the smaller plasmids were in vivo deletions of larger plasmids. In future, the relationships of these plasmid species could be resolved by using gel-purified plasmids as hybridisation probes against the other species. This species was

similar to the mesophilic S. lividans ISP5434 (Kieser et al., 1982) in that it contained several high copy number plasmids. It would be an ideal species to study for a comparison of the biology of thermophilic Streptomyces spp. plasmids with those of mesophilic species.

The other two strains S. thermogriseoviolaceus and S. threomyceticus contained plasmids of a similar size and apparent copy-number to the 40 Kbp plasmid of S. thermohygroscopicus subsp. rubiginosus. These strains did not contain smaller high copy number-plasmids.

4.9.2 Detection of Plasmids in Thermophilic Streptomyces spp. by Genetical Analysis

Some streptomycete plasmids were characterised genetically before physical methods for their detection were developed eg. SCP1 (Schrempf et al., 1975). In some cases plasmids have been difficult to isolate because of their large size (as in SCP1), because of their structure (linear as opposed to cccDNA (Hirochika et al., 1984)) or because of non-optimal conditions in the plasmid purification procedure. The last problem may be resolved by the introduction of a plasmid, such as a cloning vector, into the strain of interest to provide an internal control for the purification of plasmids from that strain (Hopwood et al., 1986). In addition to the isolation of a plasmid DNA molecule, phenotypes associated with streptomycete plasmids may be used to determine whether a species contains a plasmid or not. Streptomycete plasmids are often associated with the fertility of the host strain and with the ability of the strain to form pocks on other strains which do not contain the

same or related plasmids (Hopwood et al., 1986). Plasmid-free segregants of the parental strain may be identified by the ability of the parental strain to form pocks on the plasmid-free (ie. 'cured') segregants. The frequency of curing may be increased by using protoplast regenerants rather than single spores as the source of putative plasmid free segregants (Hopwood et al., 1983). Fertility, the formation of cured strains and the ability to form pocks are all indicative of plasmid presence in the parental strain.

4.9.2.1 Can S. thermonitrificans be Cured of a Plasmid ?

At the beginning of the thermophilic streptomycete project, protoplasts of S. thermonitrificans could not be regenerated because the efficient regeneration media had not yet been developed. For mesophilic species, plasmid curing was more efficient when protoplast regenerants were screened. However, it was considered worthwhile to screen strains derived from single spores in an attempt to identify plasmid-free segregants. A spore suspension of S. thermonitrificans was plated on Emerson's agar at a dilution to give about 200 colonies per plate. The plates were incubated at 37°C until the colonies had sporulated. 1,000 isolated colonies were patched into small squares on new Emersons agar plates and 5 ul of a spore suspension of parental S. thermonitrificans spores was placed in the centre of each patch. The plates were incubated until the patches had sporulated and were scored for the presence or absence of pocking. Putative cured strains were streaked out, repatched and checked again for pocking using the parental strain. No putative cured strain gave a positive result in the second screening ie.

plasmid deficient tstrains of S. thermonitrificans could not be isolated using this procedure. This would be the case if the partitioning of the plasmid through spores was highly efficient, if the plasmid did not possess a pocking phenotype or if S. thermonitrificans did not contain a plasmid. This approach was time-consuming and was not used to investigate the other thermophilic Streptomyces spp..

4.9.2.2 Pock Formation as an Indication of Plasmid Presence in Thermophilic Streptomyces spp. and Physical Analysis of Pock Forming Plasmids

A matrix experiment was performed to investigate pock formation by the thermophilic Streptomyces spp.. Strains were tested on each other or on S. lividans. S. lividans strains containing plasmids which form pocks on mesophilic species were tested for pock formation with the thermophilic Streptomyces spp.. Both R2YE and Emerson's agar were used throughout the experiment as the medium can affect the pocking phenotype (Hopwood et al., 1986).

Four thermophilic streptomycetes (018, 019, 035 and 544) formed pocks on S. lividans TK24 (Table 4.9). Three of these strains (018, 019 and 035) had been shown previously to contain plasmids by the physical isolation of cccDNA molecules (4.9.1) and it was proposed that these plasmids were responsible for the pocking phenotypes. When TK24 putative exconjugants were selected using streptomycin, inoculated into liquid cultures and the mycelium formed analysed physically, plasmids were isolated from the three strains of S. lividans TK24 which had been crossed with 018, 019 and 035. This indicated that a physical transfer

Table 4.9

Matrix Experiment to Investigate the Ability of Thermophilic <u>Streptomyces spp.</u> to Form Pocks on Each Other and of <u>S. lividans</u> Strains Containing Transmissible Plasmids to Form Pocks on Thermophilic <u>Streptomyces spp.</u>.

Recipient Strain	Donor Strain 017	018	019	020	035	544	574	579	313	1326	64	73
TK24	-	+	+/£	-	+/£	+	-	-	I/£	+	+	+
017	-	I	I	I	I	-	-	-	-	-	+	+
020	-	I	I	-	I	-	-	-	-	-	-	-
544	-	-	-	I	-	+/-	-/£	-	-	I	I	I
579	-	+/-	+/-	I	I	-	-	-	-	-	-	-
1326	-	-	-	-	-	-	-	-	-	-	+	-
64	+/-	+	£	-	£	-	-	-	-	+	-	+
73	-	+	+	-	+/£	-	-	-	-	+	+	-

+ Pock Formation
- Absence of Pock formation
I Inhibition of recipient growth around donor
£ Inhibition of recipient sporulation around donor, the aerial mycelium forms, but does not turn grey.
See 2.29 for details of the strains

of DNA from the plasmid-containing thermophilic Streptomyces spp. to the plasmid-free S. lividans TK24 had occurred. Plasmid DNA was not isolated from TK24:544 progeny.

The TK24 derivative which had received a plasmid from 018, TK2418, was analysed further because this strain had been identified as having interesting plasmid biology. The pock forming conjugative plasmid from 018 in TK2418 was purified by ethidium bromide caesium chloride density gradient centrifugation and studied by restriction enzyme analysis. The plasmid was approximately 40 Kbp in size and contained the following restriction endonuclease recognition sequences :- Bam HI (5), Bcl I (6), Bgl II (2), Hind III (1), Pst I (11), Sph I (4), Sal I (0), Xba I (1) and Xho I (8). The smaller higher copy number plasmids present in species 018 were not present in TK2418. The pocking phenotypes of TK2418 and TK2419 (produced by the transfer of the pock forming plasmid from species 019 into TK24) were different, suggesting that the plasmids were not identical. They showed different phenotypes when mated with other plasmid-containing strains. Neither TK2418 nor TK2419 formed pocks on 1326 (the S. lividans strain containing SLP2 and SLP3). TK2418 formed pocks on both strains 64 and 73 indicating that the plasmid was compatible with plasmids derived from SCP2 and SLP1.2, whilst TK2419 was not compatible with SCP2 derived plasmids (ie. it did not form pocks on 64), but was compatible with SLP1.2 plasmids (ie. it formed pocks on 73).

None of the S. lividans plasmid-containing strains formed pocks on S. thermonitrificans, indicating that introduction of plasmids from S. lividans to S. thermonitrificans would have to be via transformation rather than conjugal

transfer. Strains 018 and 019 did not form pocks on S. thermonitrificans in the same way that they did on S. lividans TK24, but there did appear to be some interaction between the strains resulting in the phenotype +/- (Table 4.9). It was possible that S. thermonitrificans was within the host range of the plasmids in strains 018 and 019. If mesophilic cloning vectors had not been suitable for the transformation studies of S. thermonitrificans, then the plasmids from other thermophilic Streptomyces identified here could have been used to develop thermophilic Streptomyces cloning vectors.

While the matrix experiment was being scored, other interesting zones of inhibition of growth were observed. Thermophilic species 018, 019, 020 and 035 produced diffusible substances which inhibited the growth of the recipient species. S. threomyceticus (035) was known to produce threomycin and it could have been this activity which caused this effect. The other streptomycetes might be producing other antimicrobial compounds. S. lividans strains 1326, 64 and 73 were able to inhibit the growth of 544, suggesting that S. lividans may have produced an antimicrobial compound to which 544 was sensitive but the other streptomycetes were not.

In several crosses, zones of inhibition of differentiation of the recipient were seen around the donor species. Species 019 and 035 produced diffusible compounds which affected the development of, or colouration of, TK24, TC64, TC73 (035 only) but not 1326 spores. A 3 to 5 mm halo of white mycelium around the donor growth was seen in lawns of grey recipient spores.

4.10 An Attempt to Clone the Origin of DNA Replication from S. thermonitrificans Actinophage TA.

The isolation of actinophage TA from S. thermonitrificans is described in chapter 5. TA was able to grow at all temperatures tested in the range of growth of the host S. thermonitrificans (30-53°C). All aspects of the life cycle of this actinophage must be temperature-stable. Of particular interest was the portion of the genome responsible for DNA replication. At this point in the project it was not known whether S. thermonitrificans was within the host range of mesophilic cloning vectors or whether these cloning vectors would be temperature-stable. If the origin of DNA replication from TA could be cloned in a mesophilic streptomycete, and marked with an antibiotic resistance determinant, the construct would be a suitable plasmid for initial transformation studies, as the DNA replication origin had already been proven to operate in S. thermonitrificans at elevated temperatures.

A precedent for the the consideration of this strategy was the construction of lambda dv plasmids (Boyd and Sherratt, 1985). These plasmids contain the cro, O, cI and P genes of lambda. The cI gene is superfluous as its expression is switched off by cro (Matsubara, 1981), but the other genes are required for the replication of the plasmid. In addition to these genes an antibiotic resistance determinant, in this case chloramphenicol resistance from pACYC184 (Chang and Cohen, 1978), and the pUC9 lacZ' coding region and multiple cloning site (Vieira and Messing, 1982) are included on the plasmid.

Plasmids of this type have several useful properties. They

are compatible with many other cloning vectors, they do not often contain homologous or analogous sequences to other vectors, their copy-number can be controlled (they may exist as multicopy episomes or integrated at single copy) and they can be used for the integration of plasmid and cloned sequences into the E. coli chromosome. When lambda dv plasmids are transformed into lambda lysogens, the expression of the cro gene is switched off by the action of the repressor protein gpcI. The plasmid is no longer able to replicate autonomously in the absence of cro. Selection for the drug resistance marker is by integration into the chromosome via recA dependent homologous recombination. This reduces copy-number from an intermediate level (30) to 1. Depending on the nature of the cloned sequences on the lambda dv plasmid and the site of integration into the chromosome, the integration event may be mutagenic. Lambda dv plasmids may therefore be used for "mutational cloning" in E. coli.

The construction of TA dv vectors was expected to provide Streptomyces genetics and in particular the Glasgow Thermophilic Streptomyces Project with some or all of these advantages, in addition to providing a plasmid for transformation assays.

4.10.1 Experimental Design

An `origin probe' vector containing an origin of replication and a selectable marker for E. coli in addition to a selectable marker for Streptomyces was required. When this vector had been produced it was used to clone partial Hind III digested fragments of TA DNA which were tested for DNA replication activity in Streptomyces. The efficiency of ligation was tested by transforming E. coli C1400 with 1/6 of the ligation reaction and the other 5/6 was transformed into protoplasts of S. lividans

TK24.

4.10.2 Construction of pGLW13; An *E. coli* Plasmid for the Detection of *Streptomyces* Origins of DNA Replication.

The thiostrepton (*tsr*) resistance gene from pIJ702 (Katz *et al.*, 1983) on a 1055 bp Bcl I fragment was cloned into the Bam HI site of pUC18. Clones containing a unique Eco RV site were sought as the *tsr* gene from pIJ702 contains the only Eco RV site in the recombinant plasmid. 5 of 15 clones which were analysed contained a unique Eco RV site. One such clone, pGLW13, was picked for further analysis.

The sequence data for the 1055 bp. Bcl I fragment containing the *tsr* gene in pIJ702 (Bibb *et al.*, 1985) suggested that a Sal GI restriction endonuclease fragment length polymorphism could be used to determine the orientation of the insert in pGLW13. If the *tsr* gene promoter directed RNA synthesis in the same direction as the *lacZ'* promoter a 433 bp. Sal GI fragment was expected. If the *tsr* gene promoter transcribed towards the *lac* promoter then a 650 bp. Sal GI fragment was expected after digesting pGLW13 with Sal GI. The 564 bp. lambda Hind III fragment was used as a convenient marker. Sal GI digestion of pGLW13 produced a fragment larger than the 564 bp. lambda Hind III fragment. This indicated that a 650 bp. fragment had been cleaved from pGLW13 on digestion of pGLW13 with Sal GI and that the direction of transcription of the *tsr* gene in pGLW13 was towards the *lacZ'* promoter. pGLW13 contained the following unique restriction sites; Sst I, Kpn I, Xma I/Sma I, Xba I, Pst I, Hinc II, Sph I, Hind III, Eco RV*, Sst II*, Cla I* and Xma II*; * denotes sites within the *tsr* gene. There were no unique

sites suitable for the cloning of Sau IIIa or Mbo I fragments of DNA.

4.10.3 Partial Digestion of TA DNA

The lack of restriction endonuclease recognition sites in pGLW13 suitable for the cloning of DNA fragments with 5'-GATC-3' sticky ends, made by digestion of DNA with Sau IIIa or Mbo I forced the decision to use Hind III to generate suitable fragments of TA DNA for cloning. Hind III was chosen because of the intermediate number of sites (17) in the TA genome and because the fragments were separable on a gel (with the exception of two doublets) which would aid in the analysis of clones. Pst I cut too frequently and an Eco RI digest produced too many fragments of similar sizes. The optimum concentration of Hind III per ug of TA DNA for partial digestion was determined empirically by the method of Maniatis et al., (1982), . A concentration of 6 units of Hind III per ug of TA DNA was chosen.

4.10.4 Cloning Partially Digested Hind III Fragments of TA DNA into pGLW13

pGLW13 (500 ng) was cut to completion with Hind III. The ends of the DNA were dephosphorylated using CIAP and ligated to partially digested Hind III DNA (4.10.3). One sixth of the ligation mix (2 ul.) was used to transform E. coli 1400, selecting for resistance to carbenicillin. The remaining 10 ul of the ligation mix was used to transform protoplasts of S. lividans TK24. A portion of the protoplasts were regenerated non-selectively; the remainder were selected by overlaying thiostrepton (2.13).

4.10.5 Results of the E. coli C1400 and S. lividans TK24, pGLW13 and Partially Hind III Cut TA DNA Ligation Transformations

1,600 E. coli C1400 transformants were obtained, giving a transformation frequency of about $2x10^4$ per ug of vector DNA in the ligation mix. The competent cells of C1400 were transformed to give $1x10^6$ transformants per ug of pUC18 cccDNA. 91 E. coli C1400 transformants from the ligation mixture were analysed by restriction endonuclease digestion and gel electrophoresis of plasmid DNA prepared by the STET procedure (Holmes and Quigley, 1981). 52% of the carbenicillin resistant colonies contained plasmids with inserts. 38% of these had one or more internal Hind III sites. Of the 17 DNA fragments formed by the complete Hind III digestion of the TA genome only four fragments were not identified in recombinants of pGLW13. It is possible that these fragments contain sequences which are unstable or detrimental to the growth of E. coli. Whilst they were not recovered in the E. coli recombinants, there was no reason to believe that these TA Hind III fragments should not be clonable in S. lividans.

Twenty-six protoplast regeneration plates of R2YE were inoculated with S. lividans TK24 protoplasts which had been transformed with 5/6 of the ligation mixture. In a control experiment with these protoplasts, $5x10^6$ transformants/ug. DNA ex. TK24 were obtained.

No thiostrepton-resistant transformants of S. lividans TK24 were isolated, regardless of whether the protoplasts were regenerated non-selectively and replica plated to thiostrepton or transformed protoplasts were selected directly with thiostrepton sucrose/overlays.

The ligation mix contained E. coli plasmid sequences prepared in E. coli (pGLW13). It was possible that recombinant molecules containing the thiostrepton resistance gene would be restricted on transformation into S. lividans TK24. However, bifunctional vectors containing pUC18 and pIJ486 sequences have been shown to transform into S. lividans TK24 efficiently when the DNA was prepared in E. coli, with a 5-fold reduction in transformation efficiency compared to the same vector prepared from S. lividans TK24 (Alves, T. pers. comm.). The transformation frequency of the S. lividans TK24 protoplasts was about 5 times that of the competent E. coli C1400 cells, so that approximately 4160 insert-containing recombinant molecules should have been transformed into the S. lividans TK24 protoplasts.

Intuitively, the experiment might not have been successful for a number of reasons.

1) The origin of DNA replication in the actinophage TA might not be active in S. lividans TK24.

2) The origin activity might require two or more discontinuous areas of the TA genome which were not present in the Hind III clones.

3) The origin might contain sites for Hind III and was thus not represented; although partial Hind III digests would be expected to have allowed such clones to be recovered.

4) The origin might have been cloned but the construct was unstable and could not be maintained. Perhaps partitioning and stability functions would need to be incorporated into the origin probe vector for successful isolation of DNA replication origin activities.

5) The TA DNA prepared from S. thermonitrificans may have been

restricted on entering S. lividans TK24. Historically S. lividans TK24 is a relatively non restricting host.

6) The pGLW13 origin probe vector might not express thiostrepton resistance in S. lividans TK24.

4.10.6 Cloning Sph I Fragments of TA DNA into pGLW13

In order to test the hypothesis that the origin of TA DNA replication contained a Hind III site or sites, another enzyme was used. Sph I was chosen because it cleaved the genome of TA into 5 fragments (4 sites) and they were easily separable on a gel. pGLW13 (500 ng) was cut to completion with Sph I and the DNA was dephosphorylated with CIAP. TA DNA (10 ug) was incubated with 0.5 units of T_4 DNA ligase at a final DNA concentration of 200 $ug.ml^{-1}$ to promote concatamerisation. The Sph I-digested and phosphatased pGLW13 and the ligated Sph I cut TA DNA were mixed and coprecipitated with ethanol. The ligation conditions were as described previously (2.22). The ligated mixture was diluted five-fold with TE and 10 ul of the diluted mixture was used to transform E. coli C1400. Minipreparations of DNA of twenty transformants were made using the STET method. The DNA was analysed by Sph I restriction endonuclease digestion and gel electrophoresis. All twenty colonies analysed contained recombinant plasmids. Three of the four Sph I TA DNA fragments were recovered in this experiment. The largest Sph I fragment was not found, nor was it found in another twenty transformants analysed. It is possible that the size of the fragment (about 28 Kbp) inhibited efficient transformation, or that sequences within the 28 Kbp Sph I fragment were detrimental to the cell or the plasmid. It is not known whether the Sph I 28 Kbp fragment includes sequences also present in the second largest Hind III

fragment of TA which was also not cloned in E. coli C1400. The recombinant containing the 5.6 Kbp. Sph I fragment was named pGLW37, that containing the 9 Kbp. Sph I fragment (corresponding to the Sph I-cos-Sph I structure) was named pGLW38 and that containing the 10.8 Kbp. Sph I fragment was named pGLW39.

4.10.7 Has the Origin of DNA Replication of Actinophage TA Been Cloned on pGLW13 in E. coli?

DNA prepared from E. coli recombinants containing TA DNA cloned into the origin probe vector pGLW13 was analysed for its ability to promote plasmid replication in S. lividans TK24. The three Sph I recombinants (pGLW37, pGLW38 and pGLW39) were tested individually for their ability to transform S. lividans TK24 protoplasts. The 1,600 transformed E. coli C1400 colonies produced during the cloning of partial Hind III TA DNA fragments into pGLW13 were collected by washing the colonies off the transformation plates with L broth. The cells were allowed to grow in L broth containing 50 $ug.ml^{-1}$ carbenicillin with vigorous shaking at 37°C for six hours prior to harvesting. Plasmid DNA from the pooled transformants was prepared by alkaline lysis (Maniatis, et al., 1982).

The three Sph I clones pGLW37, pGLW38 and pGLW39 did not yield thiostrepton resistant transformants of S. lividans TK24. However, several small morphologically abnormal colonies which were thiostrepton resistant were recovered from three of the five S. lividans TK24 transformations with the pooled, partially-digested Hind III TA DNA library. The colonies were not rigid, they did not form aerial mycelium or spores, nor did they produce pigments. They grew poorly in liquid culture whether

supplemented with thiostrepton or not. Furthermore, when the mycelium from liqiud culture grown in the presence of 25 $ug.ml^{-1}$ thiostrepton was examined for plasmids, no plasmid DNA molecules could be purified. Total DNA was prepared from these colonies by treatment with 1 $mg.ml^{-1}$ lysozyme in medium P, addition of S.D.S. to 1% (w/v) and phenol chloroform extraction followed by ethanol precipitation. Plasmid DNA could not be seen on agarose gels, but the DNA could be used to transform E. coli C1400 and carbenicillin resistant transformants were selected. The control experiments showed no carbenicillin resistant colonies in the absence of DNA prepared from thiostrepton-resistant S. lividans TK24. The presence of a thiostrepton resistant strain of S. lividans TK24 from which DNA could be prepared which was then capable of conferring resistance to carbenicillin on E. coli C1400 strongly suggests that a bifunctional vector had been formed. Despite numerous attempts to isolate plasmid DNA from carbenicillin resistant recombinants of E. coli or from S. lividans TK24 recombinants, no plasmid DNA was ever isolated. The E. coli C1400 strains resistant to carbenicillin also grew poorly and were difficult to work with.

Although S. lividans has generally been regarded as being restrictionless, the poor origin activity alluded to by the presence of a putative bifunctional replicon might be the result of deleted or otherwise rearranged cloned sequences after restriction by a putative S. lividans TK24 endonuclease. A restriction barrier between TA DNA from S. thermonitrificans and S. lividans TK24 has been discussed in chapter 5 and it is this which gives the most plausible explanation for the failure of the origin cloning experiments.

Even if the origin of DNA replication of TA had been cloned in this experiment, the clones were difficult to work with in both E. coli and S. lividans TK24, and so this line of approach for the formation of plasmids suitable for transformation experiments with S. thermonitrificans was discontinued.

4.11 Discussion

PEG-induced plasmid transformation of Streptomyces protoplasts has been used to transform a number of Streptomyces spp. efficiently (eg. Bibb et al., 1978, 1980; Thompson et al., 1982a; Matsushima and Baltz, 1985). Transformation provides a means by which the biology of Streptomyces might be better examined and exploited. New combinations of genes may be produced rapidly and the recombinant organisms tested for their ability to produce novel compounds with desirable characteristics.

The most important factors governing the overall transformation frequency of Streptomyces protoplasts are the regeneration frequency of the protoplasts (an efficient regeneration medium is required), the suitability of the vector and the correct transformation protocol.

The study of transformation of S. thermonitrificans by plasmid DNA began after relatively efficient regeneration media (R2S onwards) had been developed. During the development of the regeneration media, pIJ702 a high copy-number cloning vector (Katz et al., 1983) was chosen as a suitable plasmid on which to base the transformation studies. Other plasmids such as pIJ916 or pIJ922 and SLP1.2 were also considered as a contingency against S. thermonitrificans being outwith the host range of pIJ702.

However, they also might not include S. thermonitrificans in their host ranges. To increase the chances of success and to identify suitable plasmids for transformation assays, two alternative approaches were adopted. Plasmids endogenous to S. thermonitrificans and other thermophilic Streptomyces were identified and their potential for development into cloning vectors was assessed. If pIJ702 turned out to be unsuitable for S. thermonitrificans transformation assays it was hoped one of these plasmids might be used. Also a novel approach, using the origin of replication from a thermotolerant actinophage, TA to construct a plasmid for transformation assays was attempted. The plasmid could not be constructed, perhaps because of biological reasons which will be discussd in Chapter 5.

Transformation of S. thermonitrificans protoplasts by plasmid DNA is very similar to the transformation of mesophilic streptomycetes. S. thermonitrificans is within the host range of pIJ702, which is able to survive temperatures of at least 53°C in this species. During the course of this study pIJ702 was shown to be stable at 53°C in Thermomonospora fusca (Pidcock et al., 1985). This implies that the tsr gene is correctly expressed at this high temperature and that thiostrepton is stable to heat. The mel gene contained on pIJ702 was not expressed correctly in S. thermonitrificans pIJ702 recombinants. The reasons for this were not pursued.

S. thermonitrificans is also within the host range of pIJ916, a derivative of SCP2* (Lydiate et al., 1985). This low copy-number plasmid was stable in S. thermonitrificans at temperatures up to 49°C but not 53°C. The significance of obtaining pIJ916 transformants of S. thermonitrificans (Strain

C5) is that SCP2*-derived vectors have been used most effectively in cloning antibiotic pathways (Malpartida and Hopwood, 1984; Chen et al., 1986; Stanzak et al., 1896; Seno and Baltz, in press). As pIJ916 may be transformed into C5, these other pathways are potentially transferable into S. thermonitrificans.

S. thermonitrificans could be transformed with pIJ702 when the DNA was prepared from itself or from S. albusG 153, but not from S. lividans. A $>10^{-3}$ reduction in the transformation frequency was implied when the DNA was prepared from S. lividans. It is possible that S. thermonitrificans contains one or more restriction endonucleases and that pIJ702 contains sites for one or more of these enzymes. S. albusG 153 may possess a protecting (methylating?) activity which stops the restriction of pIJ702 from S. albusG 153 on entry into S. thermonitrificans protoplasts. This would explain the results presented in table 4.2 (4.4), where DNA from S. albusG 153 or S. thermonitrificans was shown to transform S. thermonitrificans equally well. Subsequent strains of S. thermonitrificans, C5 and C51, formed by curing strains of plasmids which they had been transformed with, had different transformation abilities. Strain C5 was transformed by plasmid pBROC139 (7.4) and pIJ916 (4.6) whereas S. thermonitrificans was not. Strain C51 was transformed with pIJ702 prepared from S. lividans, whereas S. thermonitrificans and C5 were not (4.7). If C5 had a reduced level of restriction which allowed transformation by pBROC139 and pIJ916 from S. albusG 153, then the presence of at least two restriction endonucleases in S. thermonitrificans was implied. One activity was negated by protection of the DNA by S. albusG 153, and the second activity was possibly inactivated by a

spontaneous mutation in the pIJ702 transformant which gave rise to C5 (Hunter and Friend, 1984), although such a spontaneous mutation would had have to occured at a relatively high frequency (>1 in 10^6 protoplasts). The single C5/pIJ916 transformant may have been the result of such an unlikely event in another activity, reducing the barrier to the larger plasmid. This event might have allowed the transformation of C51 by pIJ702 DNA prepared from S. lividans, which none of the other strains were competent to do. This is evidence for a spontaneous mutation in the first restriction endonuclease activity which was inactive on DNA prepared from S. albusG 153. Analysis of the restriction system present in S. thermonitrificans may be possible by using the thermotolerant actinophage TA isolated in this study.

The transformation protocol was analysed in order to identify alterations which increased the transformation frequency of S. thermonitrificans. The most important factors identified were the protoplast regeneration frequency and the protoplast density in the transformation reaction and on the regeneration plates. Maximal transformation was achieved when between $5x10^6$ and $1x10^7$ protoplasts were used per transformation. The maximum transformation frequency of S. thermonitrificans protoplasts measured was $6.5x10^4$ $t.ug^{-1}$ pIJ702 prepared from S. thermonitrificans and the maximum transformation efficiency estimated at between 45 and 120 viable protoplasts per transformant. These figures are high enough to allow reproducible plasmid transfer from hosts which do not present restriction problems and also the screening of secondary libraries in S. thermonitrificans. The production of a primary library in S. thermonitrificans at this level of transformation

efficiency would be possible but expensive in time and effort.

Chapter 5 Isolation and Characterisation of a Thermotolerant Actinophage, TA, and a Host Range Mutant, TAmt1.

5.1 Introduction

Actinophage have played a major role in the study of Streptomyces. They have been used as gene cloning vectors, as a means to study restriction-modification systems and as model systems to study the control of gene expression in this genus (reviewed by Lomovskaya et al., 1980; Chater, 1986a). It was thought prudent to assess the application of actinophage methodology to the thermophilic Streptomyces project. Thus, the range of techniques with which to study these organisms (and S. thermonitrificans in particular) might be increased.

5.1.1 Transfection

Okanishi et al. (1966) studied the infectivity of naked DNA of the actinophage PK-66 on the protoplasts of S. kanamyceticus. After prolonged incubation, infection occurred in the absence of PEG. This was the first report of the introduction of exogenous DNA into Streptomyces and formed the basis for much of the transfection and transformation development which occurred later (reviewed 3.1, 4.1). Suarez and Chater (1980a) developed improved methodology for the transfection of protoplasts of a number of Streptomyces with actinophage DNA. Many species of Streptomyces and their actinophage have now been used in transfection experiments eg. Krugel et al., (1980); Isogai et al., (1980); Toyami et al., (1983); Lampel and Strohl, (1986).

As actinophage have been used to determine conditions optimal for the entry of exogenous DNA into Streptomyces protoplasts (Suarez and Chater, 1980) the availability of

actinophage which infect S. thermonitrificans might be advantagous during the development of a transformation system for this species. Development of transformation methodology using a plasmid vector requires that it is introduced from a heterologous and usually that a drug-resistance marker be placed on the vector to recognise and select for transformants. Until the vector is established in the host under study, it is not known if the vector is capable of replication in that host or if the resistance marker will be active. Using an actinophage circumvents these problems: actinophage virions can be shown to infect the strain, thus proving that it is capable of replication in the host; and transformants can be recognised by lysis of the mycelium, removing the requirement for a resistance marker. Since the DNA will have been prepared from the host strain, there is no possibility of it being restricted upon entry. Also, there is no absolute requirement for protoplast regeneration. Transfection requires only that stable protoplasts are formed and that the protoplasts are competent to absorb naked DNA and correctly express it to produce new virions. Transfection might therefore be used to identify the time during mycelial growth when protoplasts could be formed which are optimally competent, and to refine the transfection procedure where actinophage DNA and protoplasts are mixed with PEG to encourage DNA entry. It was anticipated that these experiments might be successful even in the absence of a high frequency of S. thermonitrificans protoplast regeneration. It was reasonable to expect that procedures developed for transfection would also be optimal or near optimal for transformation.

5.1.2 Actinophages and Their Interactions with Restriction-Modification Systems.

A potentially serious limitation to interspecific gene cloning is the presence of restriction endonucleases in the recipient. It has previously been shown that restriction endonucleases can inhibit the development of actinophage in Streptomyces (Chater and Carter, 1978; Chater and Carter, 1979; Chater and Wilde, 1976; Chater and Wilde, 1980; Klaus et al., 1981; Cox and Baltz, 1984). The thermophilic Streptomyces project is dependent upon the interspecific transfer of antibiotic production pathways into the thermophilic streptomycete. Thus restriction endonuclease activity in S. thermonitrificans could potentially limit the success of the project.

A good example of the analysis of restriction-modification systems in Streptomyces is provided by the work on S. ambofaciens, S. griseofuscus, S. parvulus and S. fradiae. The first three formed protoplasts which were able to regenerate and could be transformed efficiently with plasmid vectors (Baltz and Matsushima, 1983; Hopwood et al., 1977; Bibb et al., 1978; Hershberger et al., 1983; Richardson et al., 1982). They propagated all ten of the actinophage studied by Cox and Baltz (1984). S. fradiae propagated six of the ten actinophage, restricting four. While S. fradiae could be protoplasted and regenerated, it was transformed at a low efficiency with plasmids prepared from other strains. Plasmid prepared from rare S. fradiae transformants could be reintroduced into S. fradiae by transfection at enhanced (1000-fold) efficiency compared to heterologous DNA. This was indicative of S. fradiae possessing

both restriction and modification systems (Cox and Baltz, 1984).

It was anticipated that similar experiments on S. thermonitrificans would indicate whether this strain posessed restriction-modification systems. Using actinophage it might also be possible to identify an alternative host which modified the DNA in such a way as to prevent the restriction activity of S. thermonitrificans. Plasmids to be transformed into S. thermonitrificans could be passaged through such a strain prior to introduction into S. thermonitrificans.

5.1.3 Actinophage-Based Gene Cloning Vectors for Streptomyces

Actinophage-based gene cloning vectors have been used to clone a number of genes either by complementation using recombinant lysogens containing the gene or by a process known as mutational cloning (reviewed by Chater 1985, 1986b; section 4.1.1.2). It was important to establish whether these vectors were useful for thermophilic Streptomyces. However, packaging constraints have prevented actinophage being used to transfer large sequences (>10 Kbp.) between streptomycetes. Therefore, they are not so useful for the transfer of complete antibiotic production pathways, which was of prime importance to this project. Two alternative uses of actinophage DNA in the construction of vectors was the production of a Streptomyces cosmid vector pR4C1 (Morino et al., 1985) and a high frequency transduction (hft) plasmid pRBH101 (McHenney and Baltz, 1988). pR4C1 was derived from a multicopy plasmid pIJ365 (Kieser et al., 1982) and the ligated cohesive ends of actinophage R4 (Chater and Carter, 1979). pRHB101 was derived from a segment (hft) of actinophage FP43, a 68 Kbp. wide host-range actinophage (McHenney

and Baltz, 1988) which was cloned into pIJ702 (Katz et al., 1983). Actinophage FP43 packages DNA via a "headful" mechanism and transducing particles contains linear concatamers of pRHB101 DNA. With pR4C1 and pRHB101, transduction may be considered as an alternative means of genetic transfer in Streptomyces in addition to conjugation, protoplast fusion, transfection and transformation.

The process of transduction does not depend upon the ability to prepare and regenerate protoplasts. However, the bacteriophage used in the cosmid construction must have the recipient strain within its host range. Both R4 and FP43 have a wide host range (Chater and Carter, 1979; McHenney and Baltz, 1988) and are thus ideal for the formation of this type of vector. If S. thermonitrificans was within the host range of R4 or FP43, transduction of plasmids might be a productive alternative to the development of a transformation system. If R4 or FP43 were not suitable, then alternative actinophage might be isolated and a cosmid developed to allow transduction in thermophilic Streptomyces.

5.2 Actinophage OC31, R4 and R4g2 Do Not Infect S. thermonitrificans.

The wide host range actinophage OC31 (Lomovskaya et al., 1980) and R4 (Chater and Carter 1979) together with an R4 host range mutation R4g2 (Chater, pers. comm) were plated on a lawn of S. thermonitrificans spores employing a variety of conditions. The spores of S. thermonitrificans were mixed into a soft nutrient agar overlay containing either 4 mM or 25 mM calcium nitrate and 10 mM magnesium sulphate and poured over nutrient

agar plates containing the same additives. Sufficient spores to provide a confluent lawn of growth were used. The experiment was repeated with S. lividans spores as a positive control. Aliquots (10 ul) of actinophage suspensions prepared from S. lividans plate lysates were spotted onto the plates once they had set. The plates were incubated at 30, 37 and 42°C. with S. thermonitrificans spores and at 30 and 37°C. when S. lividans spores were used. OC31 infected only S. lividans at both temperatures and ionic strengths. R4 and R4g2 infected only S. lividans at 30°C. at both ionic strengths. None of the actinophage infected S. thermonitrificans at any temperature or ionic strength. Actinophage FP43 was not available for host range analysis. Thus the application of the actinophage techniques described in 5.1.1, 5.1.2 and 5.1.3 would not be available immediately for the study of S. thermonitrificans. From these results it was deduced that either S. thermonitrificans was outwith the host range of these actinophage or that S. thermonitrificans possessed one or more restriction endonuclease activities. In retrospect the fact that the actinophage OC31, R4 and R4g2 were prepared from S. lividans only, rather than from a number of streptomycetes, limits the scope of this observation. It is possible that a peculiar aspect of S. lividans and/or S. thermonitrificans biology was responsible for this result rather than the hypothetical restriction activity. To provide an actinophage for the analysis of restriction-modification systems in S. thermonitrificans, an actinophage able to infect S. thermonitrificans over a wide range of temperatures was isolated.

5.3 Isolation of a Thermotolerant Actinophage TA

Actinophage TA was isolated by an enrichment procedure from top soil taken from a flower pot situated outside the Institute of Genetics, University of Glasgow. 5g of soil was added to 100 ml of Trypticase Soy Broth (TSB) in a 250 ml flask. About 10^7 spores of S. thermonitrificans were added and the culture shaken overnight at 37°C. The supernatant from a low-speed spin (1000xg) was sterilised by passage through a 0.22 um filter. Aliquots were plated on lawns of S. thermonitrificans spores in soft top nutrient agar containing 4 mM calcium nitrate and 10 mM magnesium sulphate. Duplicate plates were incubated at 37, 42 and 53°C. Plaques were formed at all three temperatures and those at 53°C were isolated for further analysis. All plaques at 53°C had identical morphologies. One of these plaques was repeatedly streaked to form single plaques and was designated TA.

Due to the enrichment procedure an accurate estimate of the number of actinophage TA particles in the soil was not possible. However, the number of TA actinophage must have been greater than 0.2 virion per gram of soil.

5.4 Methods for Propagation and Purification of Virus Particles of Actinophage TA.

5.4.1 Propagation

The host strain S. thermonitrificans was maintained on Difco Emersons Agar slopes at 37°C for several days until sporulation was complete. The slopes were then frozen at -20°C until the spores were required.

Actinophage TA was plated on Difco Nutrient Agar plates (NA) in a layer of Soft Top Nutrient Agar (SNA) which both contained 4

mM calcium nitrate and 10 mM magnesium sulphate. NA and SNA were made by the addition of Difco Bacto agar to 1.5% and 0.6% (w/v) respectively to Difco Nutrient Broth (DNB). SNA also contained sufficient S. thermonitrificans spores to provide a confluent lawn of growth. Usually the spores harvested from one slope were used in 50 ml of SNA. Standard procedures for the titration of phage stocks were employed (Maniatis et al., 1982) except that chloroform was omitted. Plates were incubated at 42°C overnight. Typically, 3 ml of SNA was poured onto each 9 cm diameter petri dish containing a layer of NA.

5.4.2 Purification of Virus Particles of Actinophage TA

Using the actinophage purification methods described for OC31 (Hopwood et al., 1985) as a starting point, the following protocols were developed for the purification of actinophage TA.

5.4.2.1 Small Scale Lysate Preparation

Actinophage TA stocks were prepared as 'soak outs' from single plaques in 1 ml of DNB or as filtered liquid lysates on 9 cm. petri dishes from soft top overlays which had been lysed almost confluently. The latter were cleared by centrifugation (18 000g, 10 mins.) to remove agar prior to filter sterilisation (0.22 um).

5.4.2.2 Large Scale Lysate Preparation

The large scale purification of TA was via the production of a large volume of soft top overlays on which there was near-confluent lysis. This was preferred to liquid lysis because the quantification was easier eg. If the overlays did not contain lysed mycelium after overnight incubation at 42°C then the preparation could be aborted (for propagation in liquid, the

lysate had to be harvested and titred before continuation). 140 ml of SNA (4 mM calcium nitrate and 10 mM magnesium sulphate) containing the spores from three slopes of S. thermonitrificans was seeded with approximately $4x10^5$ plaque forming units (pfu.) of actinophage TA. Aliquots (7 ml) of this mixture were pipetted onto 15x15 cm square petri dishes which contained NA plus the salt additives as above. The plates were incubated at 42°C overnight. The next day the plaques were well-formed and nearly or just touching each other, ie. " near-confluent lysis". The soft top overlays from 20 large plates were scraped into 200 ml of NB and left to soak for 4 hours. The agar was removed by centrifugation at 18 000 g for 10 mins. The supernatant was collected and the virus particles were concentrated by a high speed spin (34 000g, >12 hours). The resulting pellet was resuspended in phage buffer (Maniatis et al., 1982). Caesium chloride was added to the resuspended TA to a final density of 1.5 $gm.ml^{-1}$. The suspension was centrifuged in a Beckman VTi 65 rotor at 267 000g for 4 hours. The phage band was removed with a hypodermic needle and syringe. The phage particles were dialysed extensively against 10 mM Tris pH 7.5, 1mM $MgCl_2$ and stored at 4°C. Approximately 10^{13} TA particles were isolated in a typical purification.

5.4.2.3. TA DNA Purification

This was essentially as described in Hopwood et al., (1985); see 2.8.

5.5 Physical Characterisation of Actinophage TA.

5.5.1 Morphology

To identify TA and to determine if it had been isolated

previously, the morphological characteristics of this actinophage were studied. Electron microscopy of purified TA revealed that it was a member of Type B of the Bradley classification (Bradley, 1967) (Fig. 5.1). The icosahedral phage head was 59.4+/-2.6 nm. across (face to face) and 74.4+/-2.3 nm. from apex to apex. The phage tail was 263+/-5.7 nm. long and was flexible. There was no evidence of a large base plate structure.

5.5.2 Restriction Endonuclease Analysis of Actinophage TA DNA

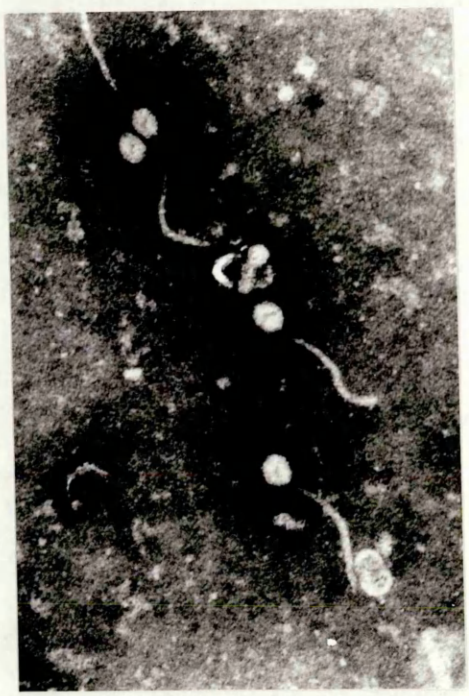
TA DNA was cleaved by the following restriction endonucleases: ClaI (9 sites), BamHI (11), BclI (16), BglII (10), EcoRI (10), EcoRV (5), HindIII (15), PstI (7), PvuII (18), SphI (4), SstII (2), XbaI (2), XhoI (17). Single and double digests with enzymes which cut infrequently, eg. EcoRV, SphI, SstII and XbaI were used to establish a restriction map of TA (5.5.2.1). The data were consistent with a linear double-stranded DNA molecule of about 51.5 Kbp. in length. Comparison of gel patterns using unheated DNA and DNA heated to 70°C. for 15 mins. prior to gel electrophoresis demonstrated that actinophage TA had cohesive ends.

5.5.2.1 Restriction Map Analysis of Actinophage TA from First Principles.

All combinations of single and double digestions with enzymes EcoRV, SphI, SstII and XbaI were performed on TA DNA. 1.2 ug. of TA DNA was digested in each case. 0.4 ug. was analysed by agarose gel electrophoresis (0.5% w/v agarose) and the remaining 0.8 ug. run on a 5% (w/v) polyacrylamide gel (Fig 5.2, 5.3). The fragment sizes were determined by comparison with lambda HindIII or ΦX174 HaeIII fragments. Actinophage TA contains cohesive ends which are partially separated by heating

Fig. 5.1

Electron Micrograph of Actinophage TA



to 70°C. for 15 mins. prior to electrophoresis. Therefore when considering the digest patterns, fragments corresponding to annealed products would also be present.

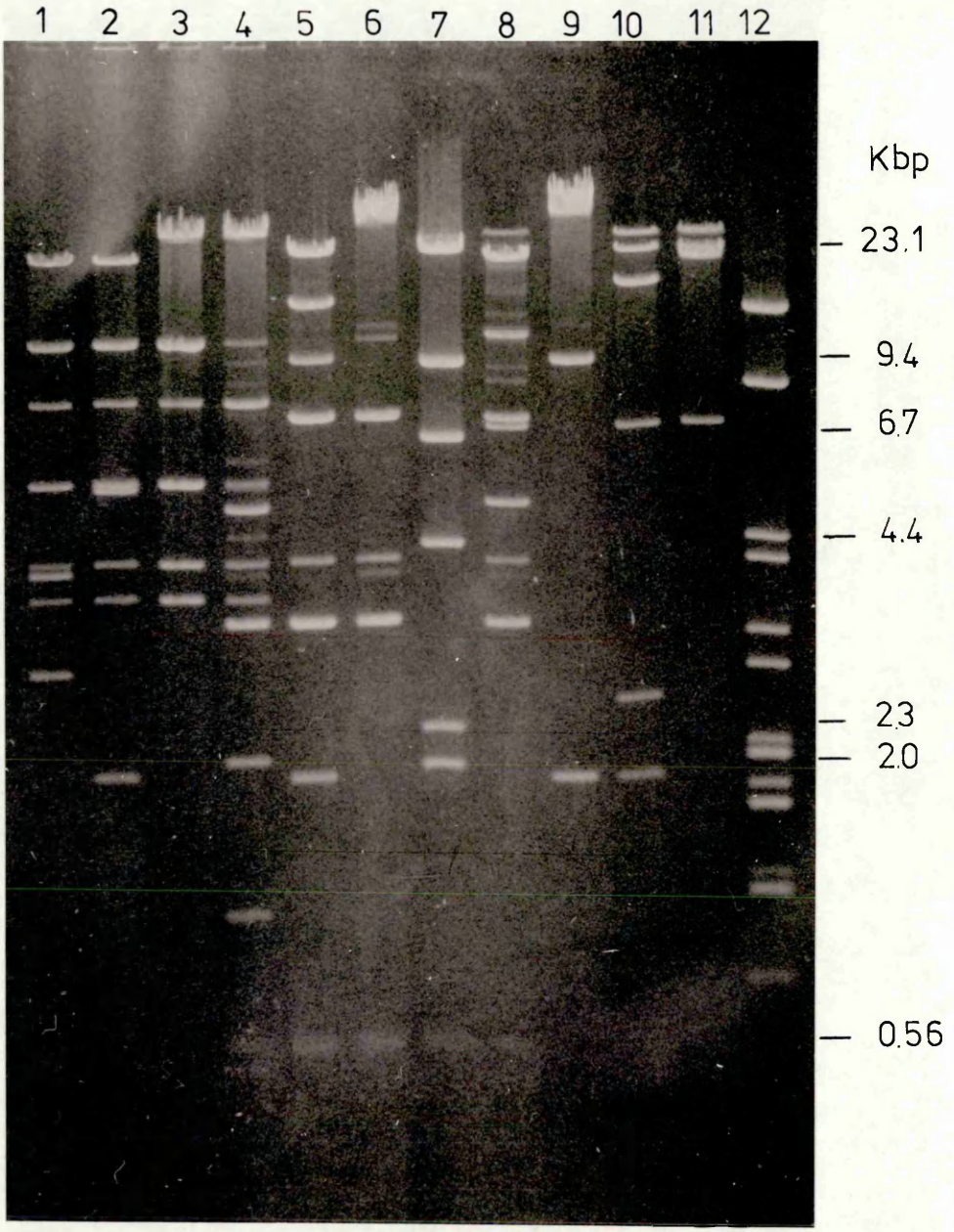
First consider EcoRV digestion.

There were 5 major bands indicating 4 EcoRV sites in the linear molecule (Fig 5.2). However, the 3.33 Kbp. band was at a higher intensity suggesting that a doublet was present and that there were actually 5 EcoRV sites. The EcoRV digestion gave fragments of about 35, 7.17, 4.16, 3.33x2 and 0.53 Kbp. A comparison of heated and unheated DNA digest patterns (not shown) indicated that the 4.16 Kbp. fragment had an EcoRV site at one end and a cohesive (cos) end at the other. As no other band changed intensity on heating, the other cos end was probably associated with the largest EcoRV fragment.

There were three bands of reduced intensity apparently formed by the partial digestion of the DNA. Their sizes were 11.95, 10.93 and 3.96 Kbp. By consideration of the completely digested fragments it could be deduced that the fragments of reduced intensity were most likely the result of partial digestion by the enzyme. eg. 4.16 + 7.17 + 0.53 = 11.86 (11.95); 7.17 + 3.33 + 0.53 = 11.03 (10.93); 3.33 + 0.53 = 3.86 (3.96). As it was deduced that the 0.53 Kbp. fragment was present in all of the partial digestion products, it followed that it must be central to these fragments. The 4.16 Kbp. fragment contained a cos end and was therefore at one extreme. Drawing this fragment at the left hand end of the actinophage TA DNA molecule, the next fragment could be either the 0.53 Kbp. or the 7.17 Kbp. fragment (deduced from the 11.95 Kbp. partial digestion product).

Fig. 5.2

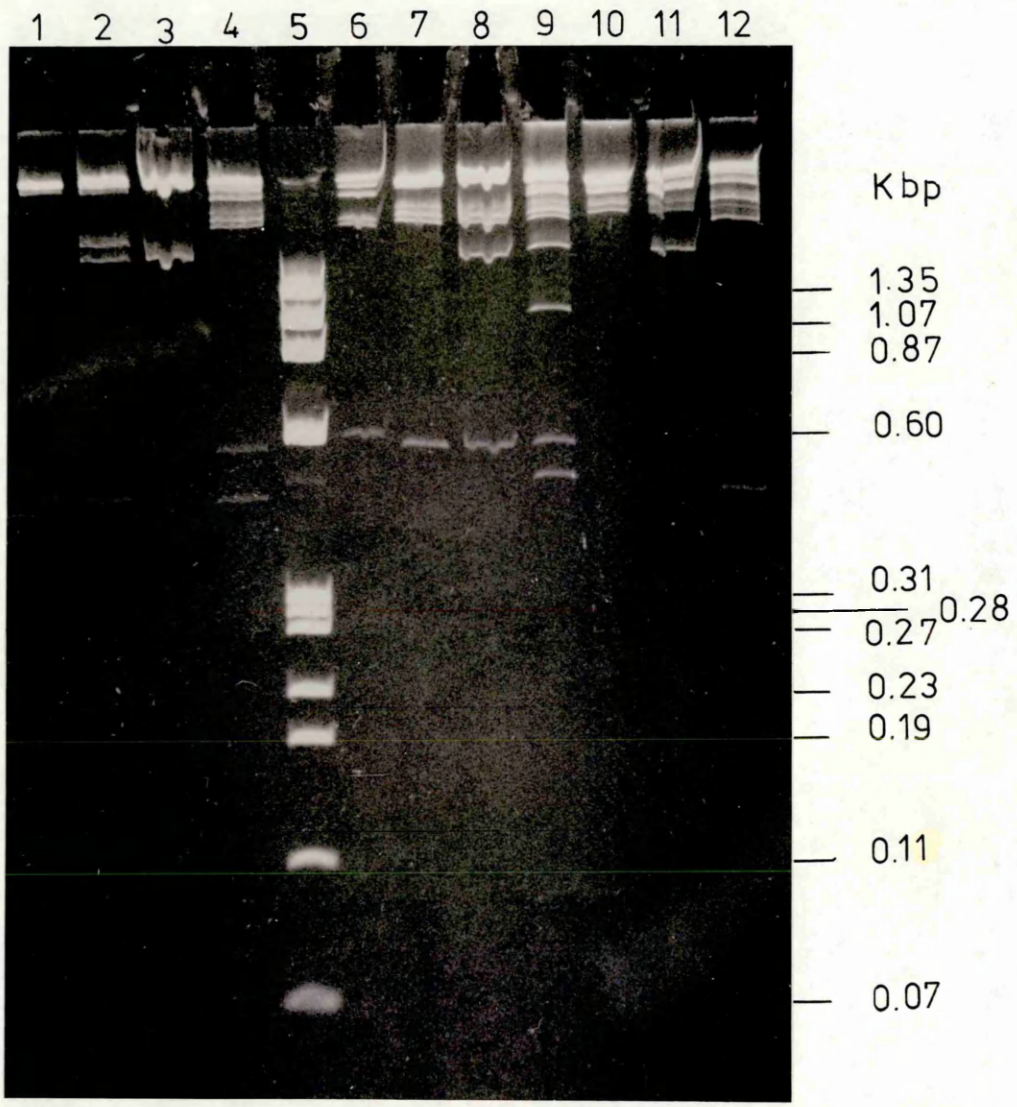
Agarose Gel of Actinophage TA DNA Restriction Fragments



1. Xba I/Sph I
2. Sph I/Sst II
3. Sph I
4. Sph I/Eco RV
5. Eco RV/Sst II
6. Eco RV
7. Lambda Hind III
8. Xba I/Eco RV
9. Sst II
10. Xba I/Sst II
11. Xba I
12. Hind III

Fig. 5.3

Polyacrylamide Gel of Actinophage TA DNA Restriction Fragments



1. Xba I
2. Xba I/Sst II
3. Sst II
4. Xba I/Eco RV
5. Ox174 Hae III
6. Lambda Hind III
7. Eco RV
8. Eco RV/Sst II
9. Sph I/Eco RV
10. Sph I
11. Sph I/Sst II
12. Xba I/Sph I

However, the 0.53 Kbp. band was associated with both the 7.17 and the 3.33 Kbp. bands in addition to the 4.16 Kbp. band; therefore, it most likely lay distal to the 4.16 Kbp. fragment. The deduced order of the EcoRV fragments thus far, from the left hand end of the actinophage DNA was therefore, cos, 4.16, 7.17, 0.56, 3.33. If the large EcoRV fragment contained the second cos end and there was indeed a 3.33 Kbp. doublet, then the order of the EcoRV restriction of TA was:

cos, 4.16, 7.17, 0.56, 3.33, 3.33, 35, cos

Now consider XbaI digestion of TA DNA.

XbaI digestion of TA DNA produces 5 fragments of 29.2, 23.5, 20.7, 7.0 and 0.43 Kbp. in length. The largest of these bands was probably the product of the 23.5 and 7.0 Kbp. XbaI-cos fragments reannealing. There were therefore 4 XbaI fragments produced by 3 XbaI sites. On double digestion with XbaI and EcoRV the 23.5 Kbp. XbaI fragment was cleaved by EcoRV thus placing the XbaI 23.5 Kbp fragment in the left hand side of TA as it is shown above. The 20.7, 7.0 and 0.42 Kbp. XbaI fragments contained no EcoRV sites and were therefore placed towards the right-hand side of the TA restriction map. The 7.0 Kbp. XbaI-cos fragment was placed at the extreme right of TA, leaving the order of the remaining two XbaI fragments undetermined.

Consider SstII digestion of TA DNA.

SstII digestion of TA DNA gave three fragments of 40, 9.52 and 1.92 Kbp. in length. In double digests with SstII and XbaI, the 9.52 Kbp. SstII band was cleaved into a 7.0 Kbp. XbaI-cos and a 2.57 Kbp. XbaI-SstII fragment. This placed an SstII site 9.52 Kbp. from the right hand end of the TA restriction map. The next SstII site would then be either 1.92 or 40 Kbp. away. If the 1.92

Kbp. SstII fragment contained a cos end at one of its termini, there would be a 1.92 Kbp. SstII fragment at the left hand end of TA. Double digestion of TA DNA with EcoRV and SstII showed that the 4.16 Kbp. cos-EcoRV fragment was not cleaved by SstII, implying that the 1.92 Kbp. SstII fragment did not have a cos end and was therfore internal. The order of the SstII sites was therefore cos, 40, 1.96, 9.52, cos.

Once the positions of the EcoRV, XbaI and SstII sites were determined, they were checked by predicting the digest patterns of all single and double digests using these three enzymes. With the exception of the small XbaI fragment there were no ambiguities and all bands on the gels could be explained by either complete and partial digestion or reannealing of the cos fragments.

Consider SphI digestion of TA DNA.

A comparison of heated and unheated TA DNA digested by SphI showed that the 4.1 and 3.6 Kbp. fragments were SphI-cos fragments and that their annealed product was the 7.8 Kbp. fragment. There were therefore four SphI sites which upon digestion produced fragments of 28, 10.75, 5.43, 4.14 and 3.60 Kbp. in length. The largest SphI band was cleaved by XbaI to produce fragments of 20.15, 3.96, 2.77 and the 0.43 Kbp. XbaI fragment. The 7.0 Kbp. XbaI-cos fragment from the right of the phage map was cleaved by SphI to yield fragments of 4.1 Kbp. (the 4.1 Kbp. SphI-cos) and 2.77 Kbp. (an XbaI-SphI fragment), placing one SphI site 4.1 Kbp. from the right hand end of the TA restriction map. By deduction, there must have been another SphI site 3.6 Kbp. from the left hand end. The 10.75 and 5.43 Kbp. SphI fragments were cleaved by EcoRV, but not XbaI or SstII,

placing these fragments to the left hand end of the TA restriction map. The order of the 10.75 and 5.43 Kbp. fragments was determined by considering the products of double digestion with EcoRV and SphI. If the 10.75 Kbp fragment were to the left of the 5.43 Kbp. fragment, the right hand end of the 10.75 Kbp. fragment would lie within one of the 3.33 Kbp. EcoRV fragments and this was clearly not the case. When the map was drawn such that the order of the SphI fragments was cos, 3.6, 5.43, 10.75, 28, 4.1, cos, then the products of all single and double digestions of SphI and the enzymes already mapped could be predicted accurately. The deduced restriction map of actinophage TA DNA (Fig 5.4) contains 2 uncertainties; The position of the small XbaI fragment has been assigned to the middle XbaI site because of size constraints at the right hand end of the map. However, no formal proof of this is available. In addition there was a small SphI fragment of approximately 110 bp. on the polyacrylamide gel (Fig 5.3). No attempt was made to map the position of this fragment.

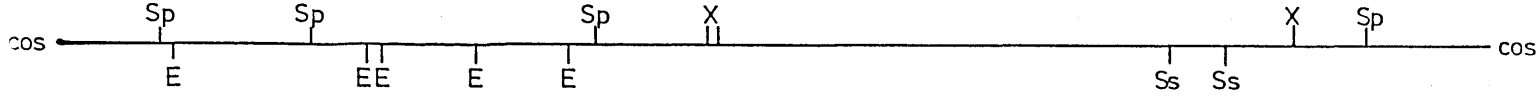
5.6 Biological Characterisation of Actinophage TA.

5.6.1 Optimisation of the Ionic Conditions for Plaque Formation by TA on S. thermonitrificans.

Actinophage are sensitive to the divalent cation concentration of the growth medium. There is often an absolute requirement for one or both of the commonly-used cations, Mg^{2+} and Ca^{2+}. Some actinophage require threshold levels of cations before infection proceeds eg. 0C31 infected S. lividans at both 4 and 25 mM $Ca(NO_3)_2$, but infected S. rimosus only at the higher concentration (LGC, unpublished observation).

FIG 5.4

Restriction Endonuclease Cleavage Site Map of Actinophage TA



2 Kbp.

	Enzyme	Approx. coordinates in BP				
E	Eco RV	4 130	11 160	11 700	15 000	18 300
Sp	Sph 1	3 660	9 090	19 350	47 330	
Ss	Sst 1	40 080	42 000			
X	Xba1	23 330	23 760	44 560		

Based on a calculated total length of 51 510 bp.

To optimise the cultural conditions such that the efficiency of plating (E.O.P) of TA on S. thermonitrificans was maximised, the effect of alterations in the ionic content of the plating media on the E.O.P. of TA on S. thermonitrificans was determined.

Initial studies indicated that actinophage TA infected S. thermonitrificans grown on NA plates in SNA overlays in the absence of additional Mg^{2+} or Ca^{2+}. Furthermore, the addition of glucose (0.5% w/v) reduced the size of the plaques. Subsequently a more detailed examination was made of the effect of divalent cations on E.O.P. of TA actinophage (Tables 5.1, 5.2).

The addition of a small amount of $Ca(NO_3)_2$ (ca. 4 mM) enhanced the E.O.P. while high levels (> 20 mM) inhibited infection. Similarly additional $MgSO_4$ (ca. 4 mM) increased the E.O.P. and high concentrations of this salt (> 50 mM) were inhibitory. The addition of both salts at 4 mM to NA and SNA was adopted for the titration of actinophage TA on S. thermonitrificans. The inhibition of the E.O.P. caused by high concentrations of divalent cations in the media would probably preclude the use of TA in S. thermonitrificans transfection studies; S. thermonitrificans protoplast regeneration medium R579 contains a high level of Ca^{2+}.

5.6.2. Host Range of Actinophage TA

The host range of actinophage TA was determined using a limited number of Streptomyces spp. readily available in the Institute of Genetics, University of Glasgow (Table 5.3). The purpose of the host range determination was two-fold: firstly, to further characterise the biological infectivity of actinophage TA and secondly to identify any well-characterised mesophilic

Table 5.1

The Effect of the Calcium Nitrate Concentration on the Efficiency of Plating of TA on S. thermonitrificans

Additional $Ca(NO_3)_2$ to Nutrient Agar (mM)	E.O.P
0	1.00
2	2.9+/-0.8
4	2.9+/-0.8
10	2.9+/-0.6
20	1.8+/-0.5
50	0.4+/-0.1

E.O.P given +/- SEM, where n=4

Table 5.2

The Effect of the Magnesium Sulphate Concentration on the Efficiency of Plating of TA on S. thermonitrificans

Additional $MgSO_4$ to Nutrient Agar + 4 mM $Ca(NO_3)_2$	E.O.P.
0	1.0
2	2.9+/-0.8
4	5.4+/-1.5
10	2.9+/-0.8
20	1.0+/-0.8
50	0.4+/-0.8

Results are given as means +/- SEM (with 4 determinations)

Table 5.3

A Comparison of the Host Ranges of Actinophage TA and TAmt1

Strain	Growth of TA	Growth of TAmt1
S.coelicolor G4	-	+
S. thermohygroscopicus 018	+	+
S. thermogriseoviolaceus 019	+	-
S. threomyceticus 035	+	+
S. thermonitrificans 579	+	+
S. albusG 148 SalI R^- M^-	-	+
S. vinaceus 155	-	+
S. lividans TK24	-	+
S. lividans 1326	-	+

streptomycetes which TA infected. Such species would serve as controls with which the E.O.P. of TA between S. thermonitrificans and other species might be compared. Due to the absolute and threshold requirements for divalent cations which some actinophage have (5.6.1), the host range studies were performed using the medium containing 4 mM $Ca(NO_3)_2$ and 10 mM $MgSO_4$ and a derivative containing 25 mM $Ca(NO_3)_2$, 10 mM $MgSO_4$. TA was observed to have a fairly-limited host range which was confined to thermophilic streptomycetes.

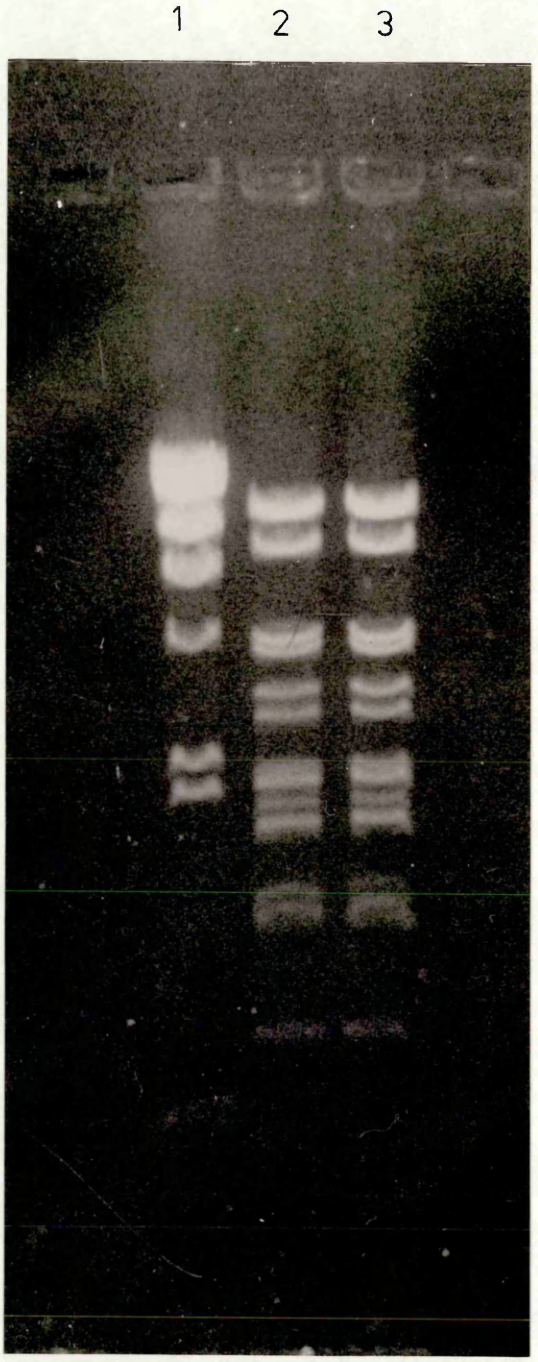
The test for mesophilic host range had been done at 30°C, whereas the thermophilic species had been screened at 42°C. It was possible that some function of TA was cold-sensitive (although unlikely since TA had been isolated from Scottish soil!). Infection of ISP5579 by TA was demonstrated at 30°C, although prolonged incubation was necessary. This was probably a result of the reduced growth rate of S. thermonitrificans at 30°C. TA was also unable to infect the mesophiles at 37°C on either medium.

5.7 Isolation of TAmt1: A Host Range Mutant of Actinophage TA.

During the course of the host range study of TA (5.6.2) a single plaque was observed on S. lividans TK24. The actinophage from this plaque was purified by repeated plating on S. lividans TK24. Its host range was compared to that of TA (Table 5.3). It had a different host range pattern, which included several mesophilic hosts. The host ranges were so dissimilar that the actinophage was originally thought to be a contaminant. However, DNA isolated from the actinophage grown on S. thermonitrificans was identical to that of TA when their restriction endonuclease cleavage patterns were compared (Fig 5.5). It was assumed that

Fig. 5.5

A Comparison of Restriction Endonuclease Digests of DNA of TA and TAmt1 using Hind III



1. Lambda Hind III
2. TA DNA Hind III
3. TAmt1 DNA Hind III

this new actinophage was a host range mutant of actinophage TA. It was named actinophage TAmt1.

TAmt1 was passaged through S. lividans TK24 and S. thermonitrificans ISP5579 and purified from single plaques. Both TA and TAmt1 were virulent in S. thermonitrificans. When TAmt1 infected S. lividans TK24 it formed turbid plaques which became overgrown after prolonged incubation, suggesting lysogeny. Mycelium from the centre of such plaques was streaked out to form single colonies and, after two days growth, the plate was flooded with SNA containing spores of S. lividans TK24. After overnight incubation at 37°C. most colonies had a halo of clearing surrounding the colony. This was also indicative of lysogeny in S. lividans but was not investigated further by Southern analysis of DNA of putative lysogens. Because actinophage TA had been isolated for its ability to form plaques on S. thermonitrificans at high temperature, the effect of incubation temperature on the E.O.P. of TAmt1 and TA on S. thermonitrificans was investigated. E.O.P.'s were similar for both actinophage (Table 5.4).

5.7.1 Optimisation of the Ionic Conditions for Plaque Formation by TAmt1 on S. lividans

To measure the E.O.P. of TAmt1 on S. thermonitrificans and S. lividans, and thus gain some insight into the level of restriction endonuclease activity between these two species, the plating conditions had to be optimal for both species. Therefore, experiments similar to those described in 5.6.1 were performed on TAmt1.

Table 5.4

The Effect of Incubation Temperature on the Plating Efficiency of TA and TAmt1 on Spores of S. thermonitrificans

Incubation Temperature (°C.)	Efficiency of Plating	
	TA	TAmt1
37	0.89	0.77
42	1.00	1.00
46	1.15	0.79
53	0.49	0.83

Preliminary experiments suggested that TAmt1 was unable to form plaques on S. lividans spores when plated in SNA on NA without additional divalent cations. The addition of 2 mM $MgSO_4$ to the media allowed infection and plaquing by TAmt1 on S. lividans. Increased $MgSO_4$ concentrations enhanced the E.O.P. such that a concentration of between 10 and 20 mM (15 mM) appeared optimal (Table 5.5). Using 20 mM $MgSO_4$ in the media, the concentration of $Ca(NO_3)_2$ was optimised at between 20 and 50 mM (Table 5.6). SNA and NA containing 15 mM $MgSO_4$ and 50 mM $Ca(NO_3)_2$ were adopted for the titration of TAmt1 on S. lividans and the conditions previously determined for TA were used for S. thermonitrificans (5.6.1). TAmt1 had similar plating requirements on S. thermonitrificans in that Mg^{2+} was not an absolute requirement for TAmt1 infection of this species.

5.7.2 Interaction of TAmt1 with Restriction-Modification Systems.

Initial passaging of TAmt1 between S. lividans and S. thermonitrificans suggested that there was a reduction in the E.O.P. between species. The observation was that actinophage TAmt1 soaked out from single plaques (5.4.1) produced either few or no plaques when plated on the alternative host. This indicated that restriction had occurred. The E.O.P. was measured accurately using the optimised plating conditions (5.6.1; 5.7.2) developed for each species. For both hosts, there was a reduction in plating efficiency when the phage had been propagated in the other such that only 1 in 100,000 actinophage successfully developed (Fig 5.6). This putative restriction barrier, particularly when phage propagated in S. lividans were introduced into S. thermonitrificans might represent a major problem in the

Table 5.5

The Effect of the $MgSO_4$ Concentration on the Efficiency of TAmt1 Plating on S. thermonitrificans

Additional $MgSO_4$ to Nutrient Agar (mM)	E.O.P.
0	0.0
2	1.0
4	1.3
10	1.3
20	1.5
50	0.6

Table 5.6

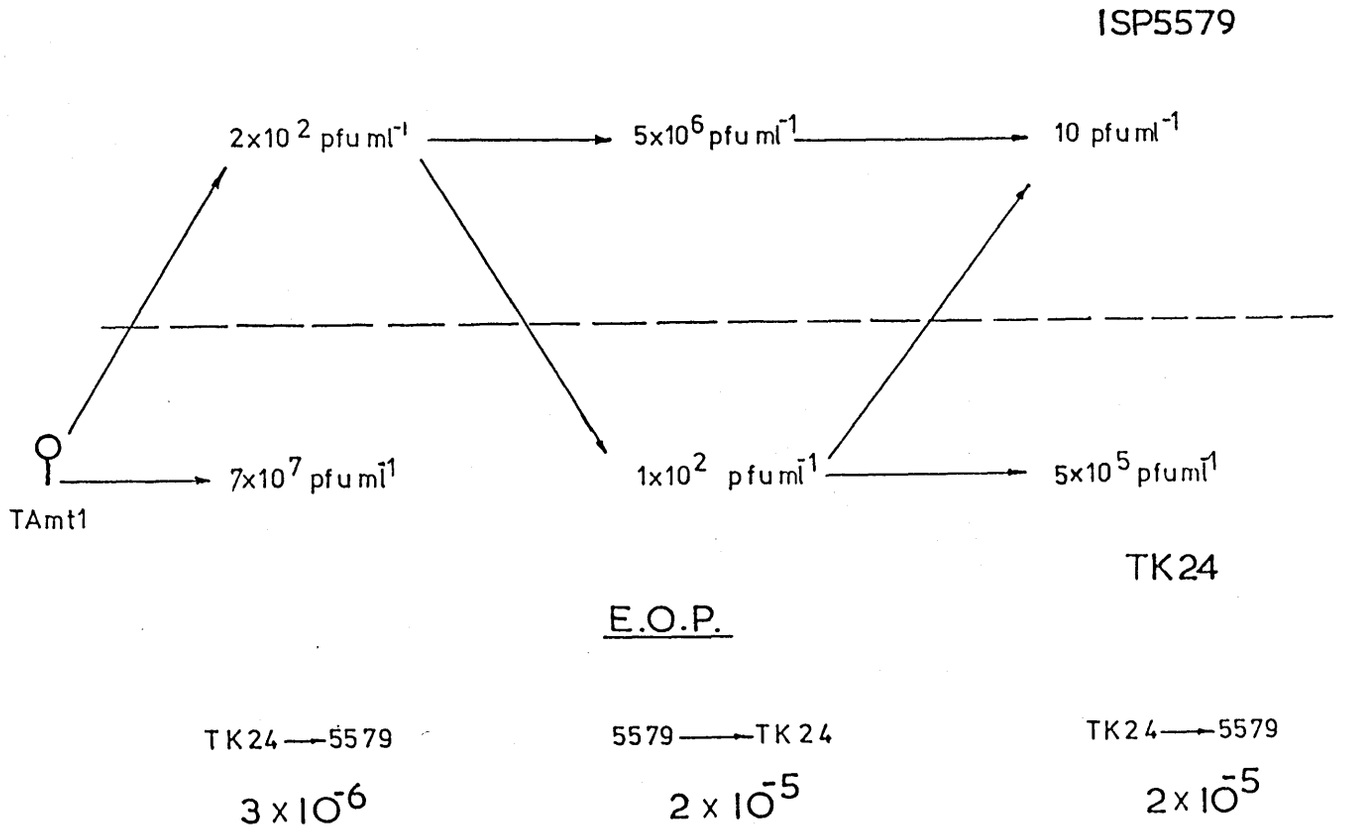
The Effect of the $Ca(NO_3)_2$ Concentration on the Efficiency of TAmt1 Plating on S. lividans in the Presence of 20 mM $MgSO_4$

Additional $Ca(NO_3)_2$ to Nutrient Agar + 20 mM $MgSO_4$	E.O.P.
0	1.0
2	1.4
4	1.5
10	1.8
20	2.0
50	2.2

The values are the average of two determinations.

Fig 5.6

Restriction of Actinophage Development Between S. lividans and S. thermonitrificans: Evidence for the Presence of in vivo Restriction and Hence Restriction Enzymes in These Species.



introduction of cloned antibiotic production pathways into S. thermonitrificans.

Initial restriction endonuclease cleavage analysis of TA DNA indicated that there were no recognition sequences for Sal G1. Although possible, it seemed unlikely intuitively that in 51.5 Kbp. of DNA from the actinophage there should be an absence of sites for Sal G1, unless there had been selective pressure against actinophage containing Sal G1 sites during the original isolation. Sal G1 is the product of a streptomycete, S. albus G. It seemed reasonable to assume that S. thermonitrificans might produce Sal G1 or an isoschizomer of this enzyme, and that TA was able to infect S. thermonitrificans because it had no Sal G1 sites within its genome. It was this observation which led to the hypothesis that plasmid DNA prepared from S. albus G, rather than from S. lividans, be used in attempts to transform protoplasts of S.thermonitrificans (Section 4.3.1). The plasmid DNA from S. albus G would be methylated at the Sal G1 sites and would thus be protected from a Sal G1, or isoschizomer activity in S. thermonitrificans. Subsequently, preparations of TA DNA cleaved when cut with Sal G1 indicating that the original preparation was probably impure, although it did cleave with a number of other enzymes. Thus the above argument, suggesting that S. thermonitrificans produced Sal G1 or an isoschizomer of this enzyme was invalid. However, in the interim period between the conception and experimental testing of this idea, the use of plasmid DNA prepared from S. albus G had been central to the production of the first transformants of S. thermonitrificans (4.3.1). Indeed, this provided further evidence for the presence of a restriction endonuclease active upon plasmid DNA derived

from S. lividans after entry into S. thermonitrificans whilst pIJ702 plasmid DNA from S. albus G could be used to transform S. thermonitrificans (Table 4.2). An activity (methylase?) of S. albus G was able to alter the biological properties of the plasmid DNA in such a way (methylation?) that it was now able to transform S. thermonitrificans (ie. it was no longer restricted). However, this activity was not the methylase associated with the Sal GI restriction/modification system (Chater and Wilde, 1976). Perhaps a second (Sal G2) system is present in S. albus G.

5.8 Discussion

A thermotolerant actinophage, TA, was isolated. This actinophage was able to infect S. thermonitrificans virulently over a wide range of temperatures (30-53°C). The host range of TA was limited to thermophilic and thermotolerant species of Streptomyces (5.6.2). The morphological characteristics (5.5.1), host range (5.6.2) and restriction endonuclease cleavage map (5.5.2) of actinophage TA have been compared to those for actinophage isolated previously and described in the literature. No satisfactory correlations have been found, suggesting that TA has not been described before.

The primary reason for the isolation of TA was to investigate the restriction barrier present between S. thermonitrificans and mesophilic species such as S. lividans which possess more advanced genetic methodology. TA was an unsuitable actinophage for such analysis because it had a limited host range. However, an actinophage with altered host range, TAmt1, was identified fortuitously (5.7). TAmt1 was thought to be a host-range mutant of TA because it had an identical

restriction map and was also thermostable (5.7). The increased host range of TAmt1 included many mesophilic species of Streptomyces such as S. coelicolor G4 and S. lividans.

Analysis of restriction between S. lividans and S. thermonitrificans was interesting because attempts at plasmid transformation of S. thermonitrificans had been unsuccessful when the plasmid was prepared from S. lividans. It was possible that S. thermonitrificans contained restriction endonuclease activity. To test this hypothesis experimentally, cell extracts of S. thermonitrificans were analysed for in vitro restriction endonuclease activity (data not shown). The results of these experiments were negative, suggesting that S. thermonitrificans either did not possess such activity or the assays were unable to detect it. Perhaps the biochemical conditions of the assays were incorrect. In vitro evidence, although inconclusive, suggested that S. thermonitrificans did not possess an overwhelming restriction activity. To test the hypothesis experimentally in vivo, actinophage TAmt1 was used to assess whether there was a reduction in E.O.P. between S. lividans and S. thermonitrificans. A restriction of actinophage development was observed, such that approximately 1 in 100,000 of actinophage plated was able to infect the alternative host (5.7.2). Such a level of restriction, and the observation that phage could reinfect the second host effectively when propagated on it (presumably by modification of the DNA) was strongly suggestive of the presence of restriction endonuclease activities in both S. lividans and S. thermonitrificans. TAmt1 DNA prepared from the two hosts might provide ideal substrates to use in future in vitro restriction endonuclease activity assays with cell-free extracts from both

species. In this way the presence of the putative restriction enzymes Sli 1 and Sth 1 from S. lividans and S. thermonitrificans respectively, may be confirmed.

The in vivo restriction data explained several results obtained previously. The inability of pIJ702 prepared from S. lividans to be used successfully in transformation of protoplasts of S. thermonitrificans (4.10) was likely to be due to restriction of the plasmid DNA by S. thermonitrificans. The presence of restriction activity in S. thermonitrificans might also explain why the wide host range actinophage 0C31, R4 and R4g2 failed to infect S. thermonitrificans (5.2). The restriction barrier observed in the opposite direction, when DNA was prepared from S. thermonitrificans might explain why the attempted production of a TAdv vector in S. lividans was unsucsessful (4.10). Thus, a TAmt1dv vector may be somewhat easier to produce as the DNA could be actinophage propagated in S. lividans. Unfortunately most of these experiments had been performed before the isolation of TAmt1, and the discovery of the restriction barrier between S. lividans and S. thermonitrificans.

However, the isolation of actinophage TA was responsible for the identification of S. albus G as an alternative to S. lividans for the preparation of plasmid DNA for transformation studies. Without this, the development of plasmid transformation for S. thermonitrificans would have taken longer. This was fortunate because the plating conditions required for TA infection of S. thermonitrificans and those for the regeneration of protoplasts were not compatible (5.6.1). The actinophage could not then be used to develop transfection methodologies.

Actinophage TA and TAmt1 were useful therefore in measuring

in vivo restriction activity and the identification of *S. albus* G as a host in which to prepare DNA but have not been useful for transfection studies. The restriction map of TA (and hence TAmt1) showed few sites for restriction enzymes which cut infrequently (5.5.2). Thus it seemed unlikely that a useful *Streptomyces* cloning vector could be developed easily from these actinophage. The *cos* ends of TA might be used to construct a cosmid analogous a pR4C1 (Morino *et al.*, 1985) to allow plasmids to be transduced into and out of *S. thermonitrificans* rather than using transformation for construction of recombinants. By analogy with bacteriophage lambda these actinophage might also be a source of promoters which are controllable and have strong activity. They could be incorporated into expression vectors for use at elevated temperatures.

The most immediate use of TAmt1 would be in the isolation of mutant strains of *S. lividans* and *S. thermonitrificans* which no longer possessed endogenous restriction activities. Such restrictionless strains might be expected to be improved hosts for cloning experiments. In the case of *S. thermonitrificans* restrictionless strains may be required for the introduction of large clones such as pIJ2303 (Malpartida and Hopwood, 1984). It is possible that *S. thermonitrificans* strain C51 is already such a mutant as it accepts pIJ702 DNA from *S. lividans* (4.7) and can be transformed by pIJ916 whereas the wild type cannot (4.6). The efficiency of plating by TAmt1 prepared from *S. lividans* on strain C51 was not tested, but one might expect it to be increased. This test should clearly be carried out if this aspect of the project were to be continued. Restrictionless mutants of both *S. thermonitrificans* and *S. lividans* may be made

by two routes. Chater and Wilde (1980) and Matsushima et al., (1987) used actinophage, plated in agar media, at concentrations such that wild type strains survived and restrictionless mutants were killed. Thus, by replica plating colonies onto such actinophage plates, the absence of colonies could be assumed to indicate restriction-deficient mutants. The mutants could be recovered from the master plates. The second route used by Hunter and Friend, (1984) and Matsushima and Baltz, (1987), relied on the use of transformation conditions which usually result in few transformants. Rounds of transformation and curing, using mutagenesis at each round to increase the percentage of restrictionless mutants, lead to the derivation of restrictionless mutants of Streptomyces which once contained as many as five restriction activities (Matsushima and Baltz, 1987). When these restrictionless mutants were tested for their ability to restrict actinophage, they were now found to be non-restricting for several actinophage which they had previously restricted efficiently. Similar experiments with S. thermonitrificans might result in the formation of strains into which it will prove easier to transfer heterologous antibiotic production pathways for their expression at elevated, more profitable, temperatures.

6.0 The Discovery, Characterisation and Biology of STP1: A Plasmidogenic Conjugative Transposable Element of *S. thermonitrificans* ISP5579

6.1 Introduction

Although plasmid DNA could not be isolated from parental *S. thermonitrificans* ISP5579 (4.8), recombinants containing pIJ702 also contained a 13 kbp. plasmid (STP1; 4.3.2). The discovery of STP1, its characterisation and a preliminary examination of its biological activity are the subject of this chapter.

Many *Streptomyces spp.* contain extrachromosomal elements and/or sequences capable of forming them; the latter are often referred to as plasmidogenic (Bibb *et al.*, 1981b; Hopwood *et al.*, 1984; Omer and Cohen, 1984,1986; Pernodet *et al.*, 1985; Cohen *et al.*, 1985; Madon *et al.*, 1987; Simonet *et al.*, 1987; Brown *et al.*, 1988). Indeed, it may be that the majority of species of *Streptomyces* contain such elements because, to date, no natural isolate has been shown to be devoid of them. *S. coelicolor* A3(2) contains type-examples of extrachromosomal and plasmidogenic elements found in *Streptomyces* (reviewed by Chater *et al.*, 1988).

Like many other bacteria, streptomycetes often contain covalently-closed circular (ccc) DNA species capable of autonomous replication. *S. coelicolor* A3(2) contains only one such plasmid, SCP2. It is a low copy number plasmid, 30 Kbp. in size, which acts as a sex factor (Schrempf *et al.*, 1975; Bibb *et al.*, 1977).

The other elements found within this organism are not so straightforward. *S. coelicolor* A3(2) also contains a giant (340

Kbp.) linear plasmid called SCP1. The presence of this plasmid was first determined genetically (Kirby et al., 1975) and was later isolated physically using orthogonal field agarose gel electrophoresis (Kinashi et al., 1987). SCP1 can recombine with the S. coelicolor A3(2) chromosome either by integration or by forming SCP1-primes (Chater and Hopwood, 1984). SCP1 can therefore exist in integrated or linear autonomous forms. SCP1 also recombines with SCP2* forming a family of larger linear plasmids with one or multiple copies of the SCP2 sequence (Kinashi et al., 1988).

SLP1 and SLP4 form another class of plasmid-like elements found in the S. coelicolor A3(2) chromosome and were identified by their phenotypes upon transfer to S. lividans 66 (Hopwood et al., 1983). Little is known about SLP4, but SLP1 has been studied extensively (Bibb et al., 1981b; Omer and Cohen, 1984,1986). SLP1, which exhibits some prophage-like properties, is a 17 Kbp. sequence present in the chromosome of S. coelicolor A3(2). SLP1 is integrated into the chromosome (containing an attB site), via an attP site (by analogy with bacteriophage lambda). The two att sites are nearly perfectly homologous; 111 out of 112 bp. are identical. SLP1 has been studied mostly in S. lividans which is free from SLP1-related sequences but retains the attB homology required for chromosomal integration. Conjugal transfer of SLP1 from S. coelicolor A3(2) into S. lividans resulted in the formation of a variety of progeny (Bibb et al., 1981b). Some exconjugants contained SLP1 integrated into the S. lividans chromosome at the attB site and others contained deleted forms of SLP1 as autonomous plasmids which were no longer able to integrate. These deletion products of SLP1 clearly showed that

SLP1 is able to replicate as a plasmid in S. lividans.

Another S. coelicolor A(3)2 element, the 2.6 Kbp. minicircle (Lydiate et al., 1986, 1987), has many features in common with SLP1. Both exist as integrated copies in the parental strain and are capable of excision from the chromosome. A free supercoiled form of the minicircle has been observed (Lydiate et al., 1986). No such form of SLP1 has been observed in S. coelicolor A3(2) but its existance, perhaps at a very low copy number, has been postulated (Omer and Cohen, 1986). However the two elements are sufficiently different in their methods of integration to warrant their separate classification. The minicircle does not integrate via a attP-B mechanism but instead employs a mechanism similar to those of some transposable elements (Chater, 1988).

These four genetic elements present in S. coelicolor A3(2) provide a basis with which to classify elements in other streptomycetes. Several elements have been found which are similar to SLP1; these will be discussed in more detail and compared to STP1 later.

The sudden appearance of a plasmid in a strain of Streptomyces which was previously plasmid-free need not be extraordinary, but may be explained by the "induction" (a term used loosely here) of an integrated plasmidogenic element. The appearance of STP1 was therefore studied against this background of information.

6.2 The Discovery of STP1

No endogenous plasmids could be isolated from S. thermonitrificans ISP5579 using a variety of techniques (4.8,

4.9.2). However, S. thermonitrificans recombinants containing pIJ702 contained an additional plasmid STP1 (4.3.2, Fig. 6.1). The appearance of the additional plasmid STP1 after transformation of S. thermonitrificans ISP5579 was investigated.

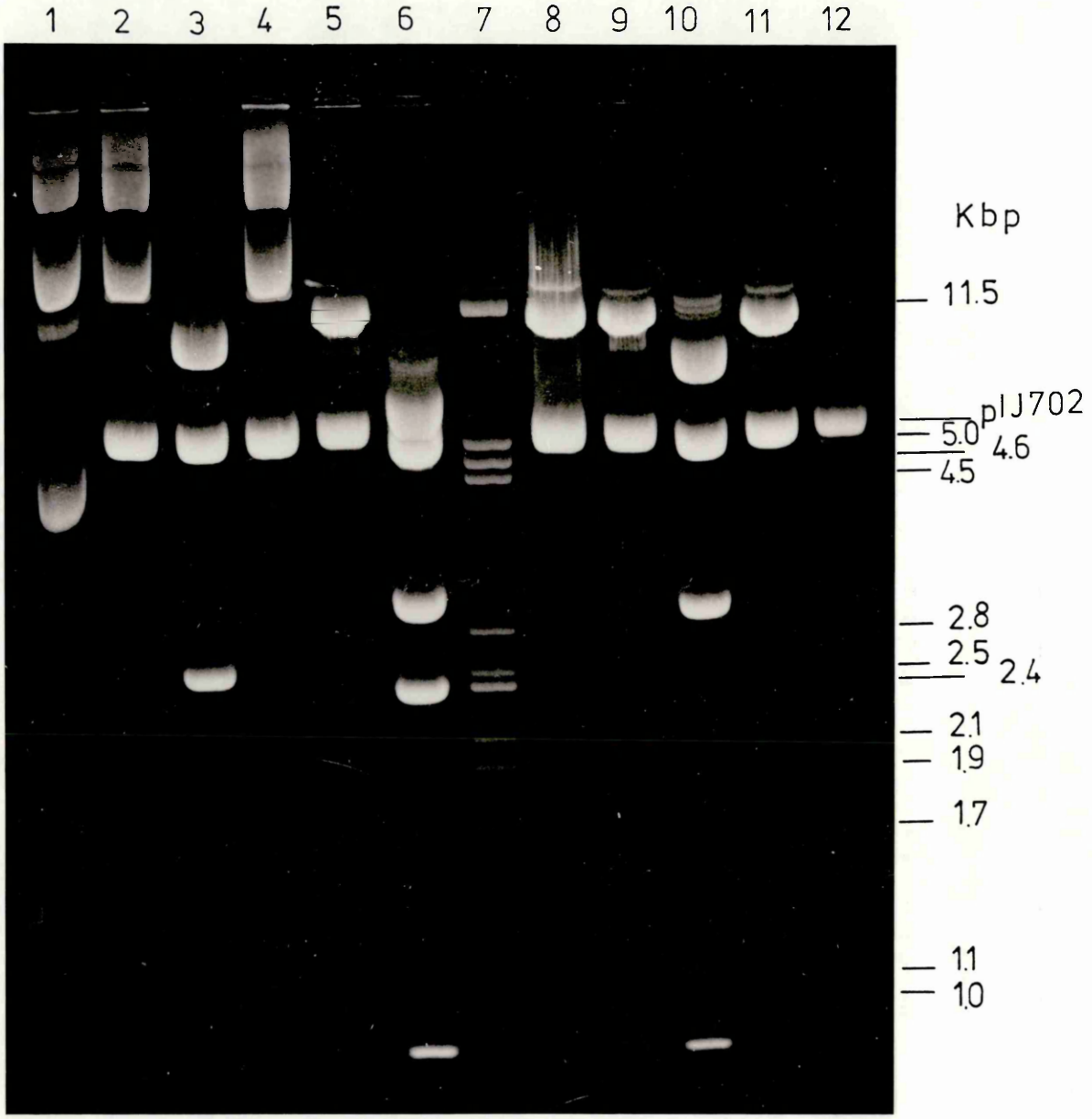
6.3 Restriction Endonuclease Cleavage Analysis of STP1

STP1 migrated more slowly than pIJ702 during agarose gel electrophoresis, implying that STP1 was larger than pIJ702. However, it was possible that STP1 was a stable dimer, a rearrangement or other related form of pIJ702 and not a novel S. thermonitrificans ISP5579 genetic element. The restriction endonuclease digestion pattern of STP1 was therefore analysed and compared to pIJ702 (Fig. 6.1). Linear pIJ702 was present in each lane (eg. see lane 12). STP1 had sites for BamHI (1), BglII (2), EcoRI (1), HindIII (1), KpnI (1), PvuII (3), SphI (4), SstI (1), XhoI (1), there were no sites for EcoRV or ClaI (data obtained from several gels). In addition there were several discrete fragments present at low copy-number.

Restriction enzymes which cut pIJ702 once did not reduce STP1 to a single band of pIJ702 linear size, indicating that STP1 was not a stable dimer of pIJ702. STP1 did not contain sites for EcoRV or ClaI, both of which cut in the thiostrepton resistance (tsr) gene of pIJ702, further indicating that STP1 and pIJ702 were not related physically. The two plasmids did contain several restriction sites in common, but this was not unlikely considering the size and G+C content of the plasmids and organism. The detailed mapping of restriction sites in STP1 was postponed until a strain containing STP1 alone was constructed. The presence of pIJ702 in the recombinant would confuse the

Fig. 6.1

Restriction Endonuclease Cleavage Analysis of STP1



1. STP1/pIJ702 Uncut
2. Cla I
3. Bgl II
4. Eco RV
5. Xho I
6. Sph I
7. Lambda Pst I
8. Bam HI
9. Sst I
10. Pvu II
11. Kpn I
12. pIJ702 Pst I

assignment of fragments to STP1.

6.4 Transfer of Gel Purified STP1 to S. lividans, and Analysis of the Recombinants

To isolate STP1, cccDNA from an S. thermonitrificans ISP5579/pIJ702 recombinant was separated by agarose gel electrophoresis and the STP1 monomer band was extracted by electroelution. The purified DNA was used to transform protoplasts of S. lividans TK54. The transformed protoplasts were plated onto R2YE regeneration medium and allowed to regenerate and grow at 30°C until sporulation had occurred. Some of the transformation plates were replica-plated to R2YE plates containing thiostrepton to determine the purity of the DNA used in the transformation. No thiostrepton-resistant transformants were recovered, indicating that pIJ702 and STP1 had been separated efficiently during electrophoresis and subsequent purification. Examination of the sporulated lawns of putative transformants allowed the identification of small patches exhibiting a pocking phenotype often associated with natural streptomycete plasmids (Hopwood et al., 1985). Restreaking of these patches onto lawns of freshly plated S. lividans TK54 spores showed that the pocking phenotype was stable. Several of these strains (eg., GLW61) were kept for further analysis.

6.4.1 Characterisation of S. lividans GLW61

In the S. thermonitrificans ISP5579/pIJ702 recombinant, STP1 coexisted with pIJ702. Both plasmids were present at high copy-numbers; STP1 was at about half the copy number of pIJ702. STP1 was therefore expected to be present at a similar copy-number in the pocking strains of S. lividans formed during this experiment. However, cccDNA could not be isolated physically

from these strains (typified by GLW61) using a variety of techniques (see 4.8). The "pocking" phenotype was stable and could be transferred to S. lividans TK64 by conjugal mating. Almost 100% of the streptomycin-resistant exconjugants of TK64 displayed the "pocking" phenotype, indicating that the frequency of transfer was high. Thus STP1 is a conjugative plasmid, able to mobilise the pocking phenotype. The effect of STP1 on the transfer of chromosomal markers was not determined.

It was possible that STP1 required the presence of pIJ702 to exist as a high copy-number autonomous plasmid in S. thermonitrificans ISP5579 and, because of this requirement, STP1 was not present at high copy in S. lividans GLW61. To test this hypothesis pIJ702 was transformed into S. lividans GLW61 and plasmid DNA prepared from six thiostrepton-resistant pocking transformants. None of the six transformants contained high copy-number STP1; only pIJ702 was present as an autonomous plasmid. This indicated that a requirement of a pIJ702 function in trans was not the reason for the absence of cccDNA of STP1 in S. lividans GLW61. The event which lead to a "pocking" phenotype in some strains of S. lividans TK54 after transformation with gel-purified STP1 DNA was obviously not the establishment of autonomous high copy-number STP1 in these strains. Control plates in which no STP1 DNA had been introduced did not have pocking zones indicating that the pocking phenotype was DNA-dependent. All or part of STP1 could probably be found at low copy-number in the genome of S. lividans GLW61, possibly integrated in high molecular weight DNA rather than as an autonomous plasmid. Of note was the observation that the introduction of pIJ702 into S. lividans GLW61 did not "induce"

the presence of high copy number STP1, whereas the same experiment did in S. thermonitrificans ISP5579. This implied that either a species difference or a physical difference in the (putatively) integrated STP1 element had altered the STP1 response to pIJ702. Southern analysis of chromosomal DNA from S. lividans GLW61 showed that the STP1 sequences integrated in this strain differed from the integrated sequences in another strain, S. lividans GLW62 (6.12.1). The affect of the introduction of pIJ702 into S. lividans GLW62 was not determined.

To investigate if STP1 and pIJ702 could coexist in S. lividans, in the same way as observed in S. thermonitrificans ISP5579/pIJ702 recombinants, a cotransformation experiment was performed.

6.5 The Cotransformation Experiment

Attempts to transform gel-purified STP1 into S. lividans TK54 recombinants containing pIJ702 failed to recreate the coexistance of STP1 and pIJ702 in S. lividans.

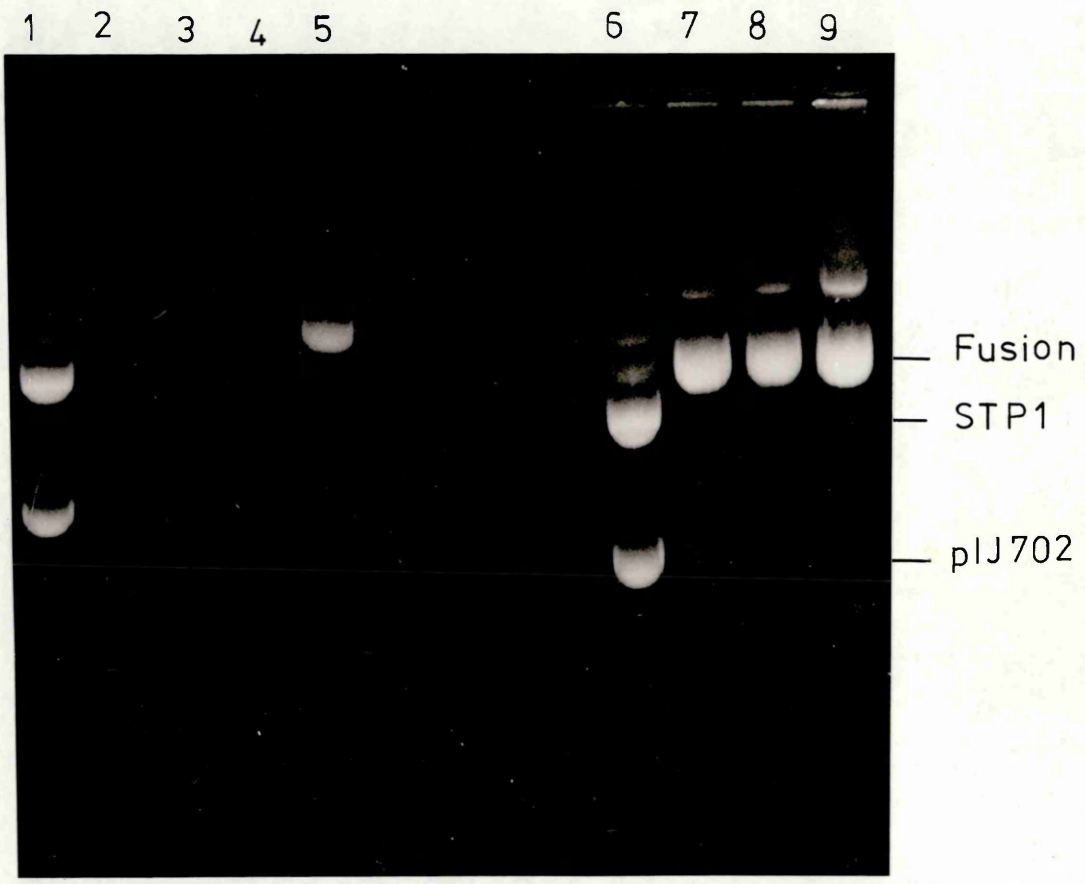
Plasmid DNA prepared from a S. thermonitrificans ISP5579/pIJ702 recombinant, was used to transform protoplasts of S. lividans TK54. At a high DNA concentration (> 5 ug plasmid per transformation) a high proportion of transformants were likely to have received both plasmid DNA's (see Suarez and Chater, 1980). The putative transformants were regenerated non-selectively. Within the lawns of protoplast regenerants, three types of patches were observed. One type (represented by strains GLW1 and GLW4) had white patches where the spores apparently did not have the grey colouration associated with their maturity, the second type of patch (represented by GLW16 and GLW17) was

identical to the "pocking" patches formed by S. lividans GLW61 and the third type was a composite of the first two, exhibiting both pocking and the white spore phenotypes. Spores from within these patches were restreaked onto lawns of S. lividans TK54 spores and their phenotypes were stable. Several examples of each type were retained and plasmid DNA was prepared from them.

The purified plasmid DNA was subjected to agarose gel electrophoresis (Fig. 6.2). The plasmid profiles of these strains were different. The strains which possessed the white spore phenotype either contained no plasmid or plasmid(s) were present at very low copy-number. When cccDNA was prepared from these strains and samples were analysed by gel electrophoresis prior to caesium chloride gradient purification, a faint doublet above chromosomal DNA could be observed. The doublet co-migrated with the upper bands present in cccDNA from S. thermonitrificans pIJ702 recombinants which also contained STP1. The white phenotype may therefore have been associated with this/these plasmids. The restriction endonuclease cleavage sites present in the high molecular weight cccDNA's putatively associated with the white spore phenotype were identical to those found in STP1 (Fig. 6.1). Restriction endonucleases which cut STP1 to form a single electrophoretic band also cleaved the high molecular weight plasmid, but no other bands of appropriate intensity were present indicating that the more slowly-migrating high copy-number plasmids were multimers or some other form of STP1. Thus there was a problem in assigning the phenotypes of the plasmids and their relatedness. A resolution to this problem will be presented later (6.8, 6.9).

Fig.6.2

Uncut Plasmid DNA from Strains Made During the Cotransformation Experiment



Uncut cccDNA from cotransformant numbers

1. STP1/pIJ702
2. GLW1
3. GLW4
4. GLW13
5. GLW16
6. STP1/pIJ702
7. GLW17
8. GLW18
9. GLW21

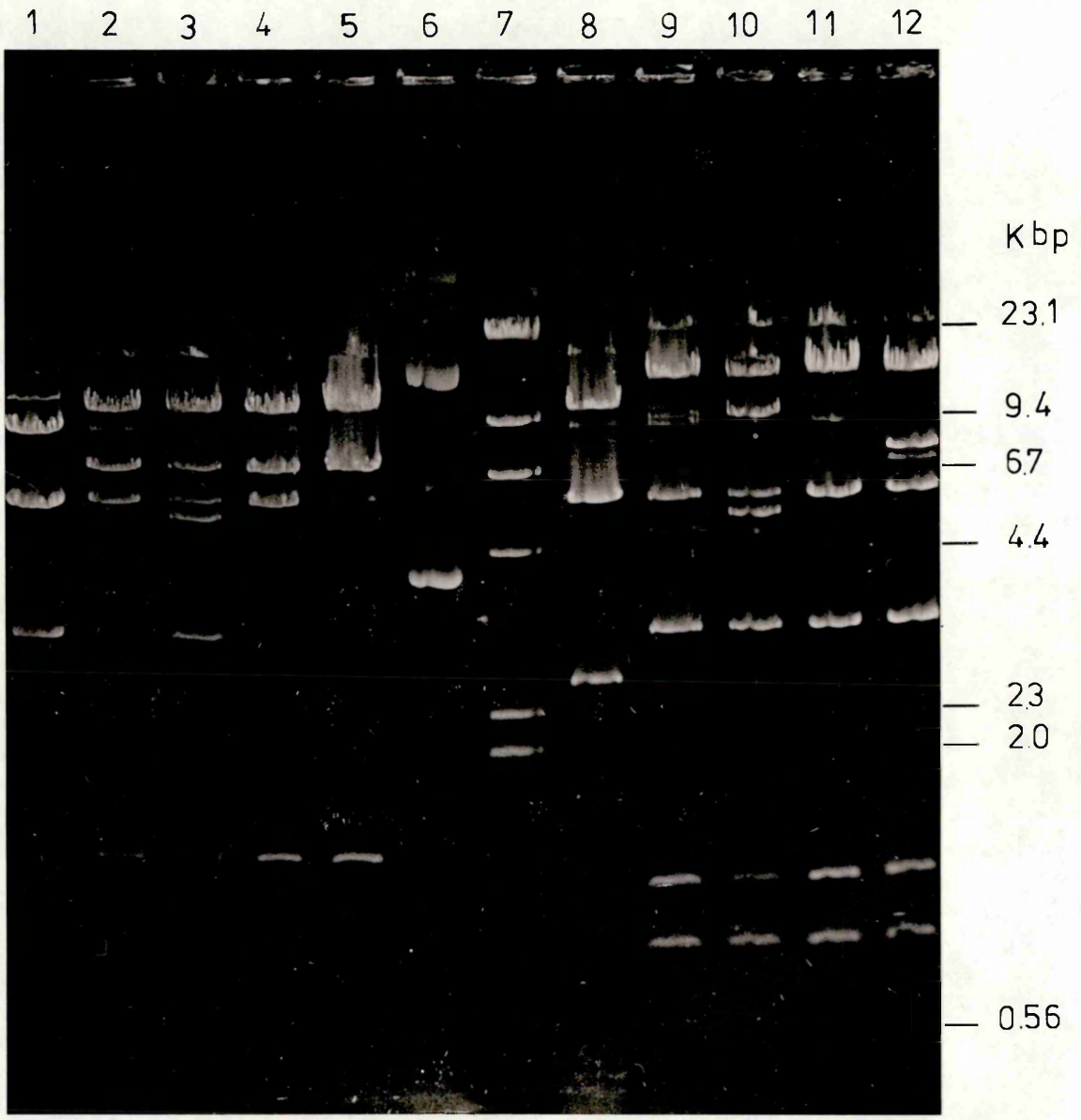
The pocking strains had a different plasmid profile and contained a high copy-number plasmid larger than STP1 and variable quantities of cccDNA monomer of pIJ702. None of the five pocking strains analysed contained ccc STP1 or had pIJ702 and STP1 in the coexistance observed in S. thermonitrificans ISP5579. The nature of this larger plasmid was of interest and was investigated further.

6.5.1 Restriction Endonuclease Analysis of the Large High Copy Number Plasmids Formed During the Cotransformation Experiment.

Several strains of S. lividans containing both the large novel plasmid and pIJ702 (eg., S. lividans GLW17) were analysed further by restriction endonuclease digestion (Fig. 6.3). The cccDNA was digested with BglII and PvuII restriction enzymes, both of which cut pIJ702 once. Agarose gel electrophoresis of the digestion products showed that many fragments were formed in each case, one of which was linear pIJ702. The relative abundance of pIJ702 DNA when compared to the other DNA fragments was consistent and it was of approximately equivalent copy number to the novel larger plasmids. Previously, in the gel of the unrestricted DNA (Fig. 6.2) the quantity of cccDNA pIJ702 monomer was variable and reduced. Since the same source of DNA was used in both cases, it was implied that pIJ702 in these strains was migrating anomalously either as multimers or topoisomers which spread the pIJ702 plasmid up the gel, effectively reducing its apparent concentration. Such a situation was not normally observed with pIJ702. The apparent sizes of the undigested novel cccDNA's in pocking strains were constant (Fig. 6.2), but when digested it was apparent that their restriction maps were not identical (Fig. 6.3). While some bands were present in all of

Fig.6.3

Restriction Analysis of Plasmids Formed During the Cotransformation Experiment



1. STP1/pIJ702 Pvu II
2. GLW13 Bgl II
3. GLW16 Bgl II
4. GLW17 Bgl II
5. GLW21 Bgl II
6. STP1/pIJ702
7. Lambda Hind III
8. GLW1/pIJ702 Bgl II
9. GLW13 Pvu II
10. GLW16 Pvu II
11. GLW17 Pvu II
12. GLW21 Pvu II

the appropriate tracks, several novel bands were apparent in each isolated pocking strain. Furthermore, when the fragment patterns of the large novel plasmids were compared with the pattern obtained when pIJ702 and STP1 mixed DNA's were cleaved with the same enzyme, a relationship between STP1 and the large plasmids was observed. The small STP1 BglII and the large PvuII fragments were absent in the digestion patterns of the larger plasmids whilst the large BglII and the other PvuII fragments were present. This was indicative of insertions into the small BglII (and large PvuII) fragments of STP1 which disrupted them. New fragments were also present in the digest pattern as would be expected if this hypothesis were correct. Thus it appeared as if the larger high copy number plasmids identified in these pocking strains were related to STP1 and contained additional DNA sequences which made them larger. The variability of the fragment pattern exhibited in Fig. 6.3 eg., GLW16, GLW17 and GLW21, indicated that the event which lead to the formation of the larger high copy number plasmid was variable and because of the ease of isolation of these strains was also frequent. The simplest and most complex fragment patterns generated by PvuII digestion of the cccDNA from these strains were found in GLW17 and GLW16 respectively. These strains were characterised further to investigate the events which lead to their formation.

6.6 Restriction Endonuclease Site Mapping of STP17: An *in vivo* Fusion Between STP1 and pIJ702.

Purified plasmid DNA prepared from *S. lividans* GLW17 was cleaved with a number of restriction enzymes. STP17 had sites for BamHI (2), BglII (3), ClaI (1), EcoRI (1), EcoRV (1), HindIII (1), KpnI (1), PstI (1), PvuII (4), SphI (5) SstI (2) and XhoI

(2); STP17 possessed many sites for SalI, SmaI and SstII and no sites for DraI or XbaI (data collected from a number of experiments).

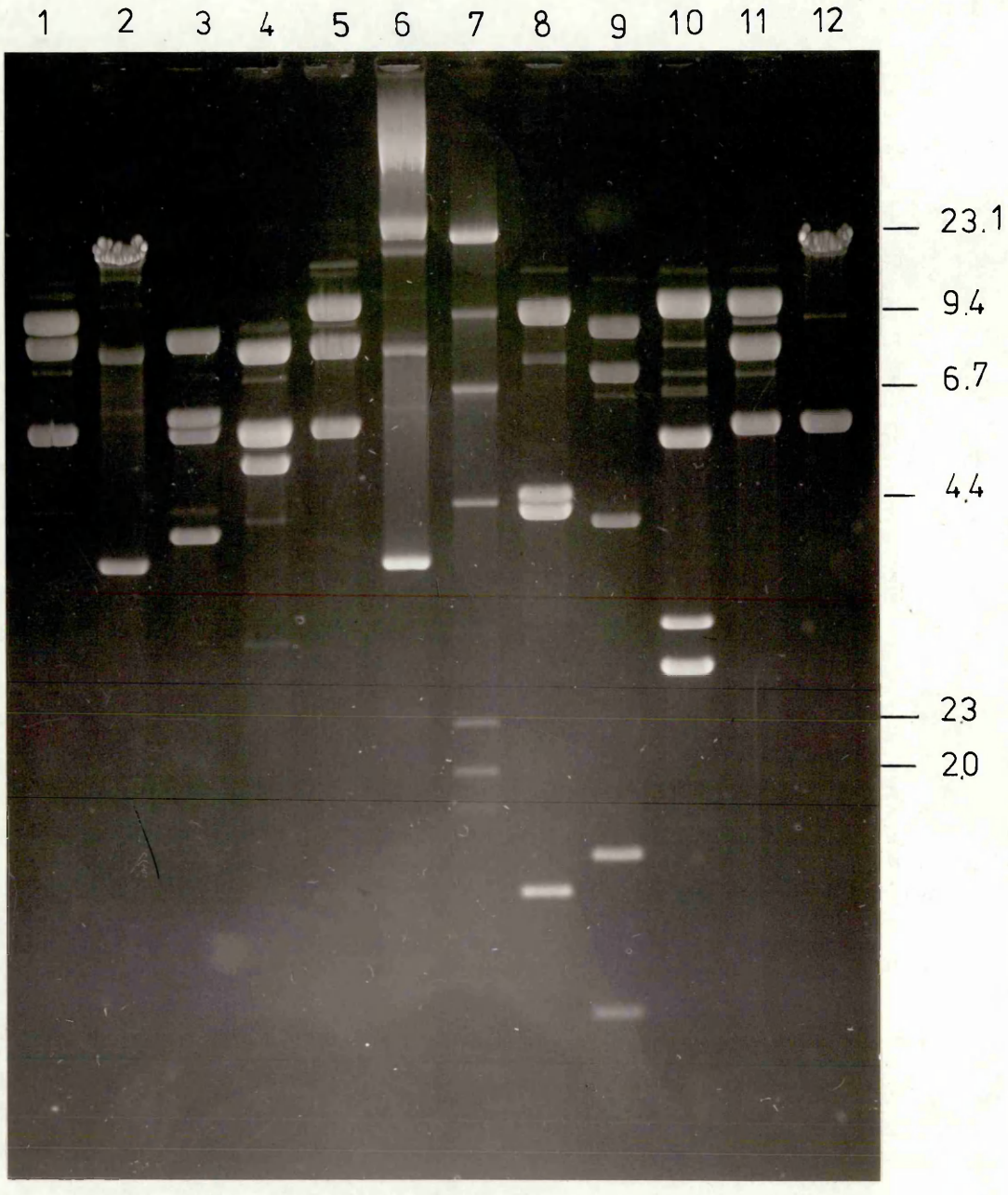
The restriction sites present in STP17 appeared to be a composite of those in pIJ702 and those in STP1. This observation resulted in the hypothesis that STP17 was a fusion plasmid between pIJ702 and STP1. Using enzymes which cut it infrequently, a restriction map of STP17 was developed as follows.

Restriction enzymes EcoRV, HindIII, SstI and XhoI in single digests and all possible pairwise combinations were used to digest cccDNA from S. lividans GLW17 (Fig. 6.4). The cccDNA from this strain contained both pIJ702 and STP17 cccDNA. The presence of pIJ702 in this DNA complicated interpretation of the results and many STP17 derived DNA fragments seemed to comigrate with pIJ702 derived DNA fragments.

EcoRV linearised both pIJ702 and STP17 DNA. HindIII cut the EcoRV linearised STP17 DNA into two fragments 10.5 and 8.3 Kbp. in length, but did not cut the pIJ702 DNA. XhoI cut STP17 and pIJ702 producing 10.0 and 7.8 Kbp. STP17-derived fragments and a 5.6 Kbp. linear pIJ702 molecule. HindIII digestion of the XhoI-cut DNA formed two novel bands of 5.6 and 5.0 Kbp. in length. The 5.6 Kbp. XhoI-HindIII STP17-derived DNA fragment comigrated with the XhoI linearised pIJ702 DNA. Thus, it was deduced that the XhoI sites were positioned 5.0 and 5.6 Kbp. away from the unique HindIII site of STP17. The orientation of the XhoI sites with respect to the unique EcoRV site was deduced from the XhoI-EcoRV double digest of STP17 DNA. EcoRV and XhoI digestion of

Fig. 6.4

Restriction Analysis of GLW17 with Eco RV, Hind III, Sst I and XhoI



1. Sst I
2. Hind III
3. Sst I/Hind III
4. Xho I/Hind III
5. Hind III/Eco RV
6. STP17 uncut
7. Lambda Hind III
8. Sst I/Eco RV
9. Xho I/Sst I
10. Xho I/Eco RV
11. Xho I
12. Eco RV

STP17 DNA produced DNA fragments of 10.6, 5.4, 3.1 and 2.7 Kbp. in length. The 2.7 Kbp. fragment was a doublet containing DNA from pIJ702 and STP17. The 3.1 Kbp. fragment was derived from pIJ702. The EcoRV/XhoI DNA fragments derived from STP1 (10.6, 5.4 and 2.7 Kbp.) could only be produced, and be consistent with the mapping data above, if the order of the restriction sites in STP17 was:-

EcoRV -5.5- XhoI -5.0- HindIII -5.6- XhoI -2.7- EcoRV.

SstI cut pIJ702 once and cut STP17 twice, to produce two STP17-derived DNA fragments of 9.6 and 8.3 Kbp. in length. HindIII digestion of the SstI-cut DNA produced two novel fragments of 6.0 and 4.0 Kbp. in length indicating that the 9.6 Kbp. SstI fragment contained the unique HindIII Site. The orientation of the two SstI sites was resolved by the analysis of the products of EcoRV digestion of SstI-cut STP17 DNA. When the pIJ702-derived SstI-EcoRV digestion products were accounted for, the SstI sites were positioned thus:-

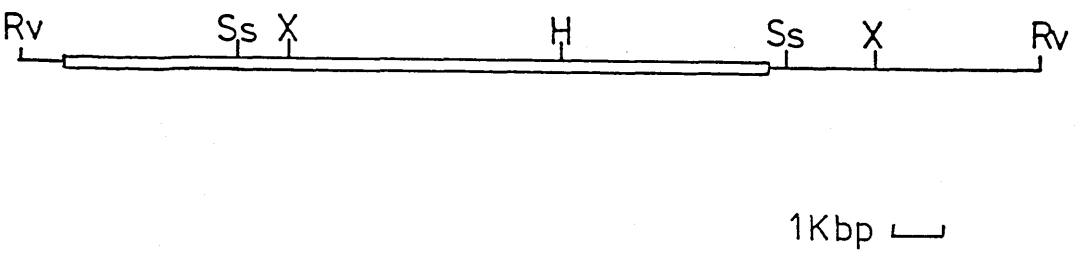
EcoRV -4.4- SstI -6.0- HindIII -4.0- SstI -4.3- EcoRV

The expected XhoI-SstI digestion propducts were consistent with those observed. Thus the restriction map of STP17 for EcoRV, HindIII, SstI and XhoI was obtained (Fig 6.5). The predicted map of STP17 for these restriction enzymes was analysed using ZBLATTNER, a computer programme designed to minimise error in restriction site alignments. The analysis showed no anomalies in the STP17 restriction map.

STP17 appeared to be an 18.7 Kbp. plasmid fusion between pIJ702 and STP1 (Fig. 6.1), with DNA inserted into pIJ702 between its unique SstI and EcoRV sites. If this model was correct then

Fig 6.5

The Restriction Map of STP17 for EcoRV, HindIII, SstI and XhoI



Box indicates STP1 sequences
Thin line indicates pIJ702 sequences

H	HindIII
RV	EcoRV
SsI	SstI
X	XhoI

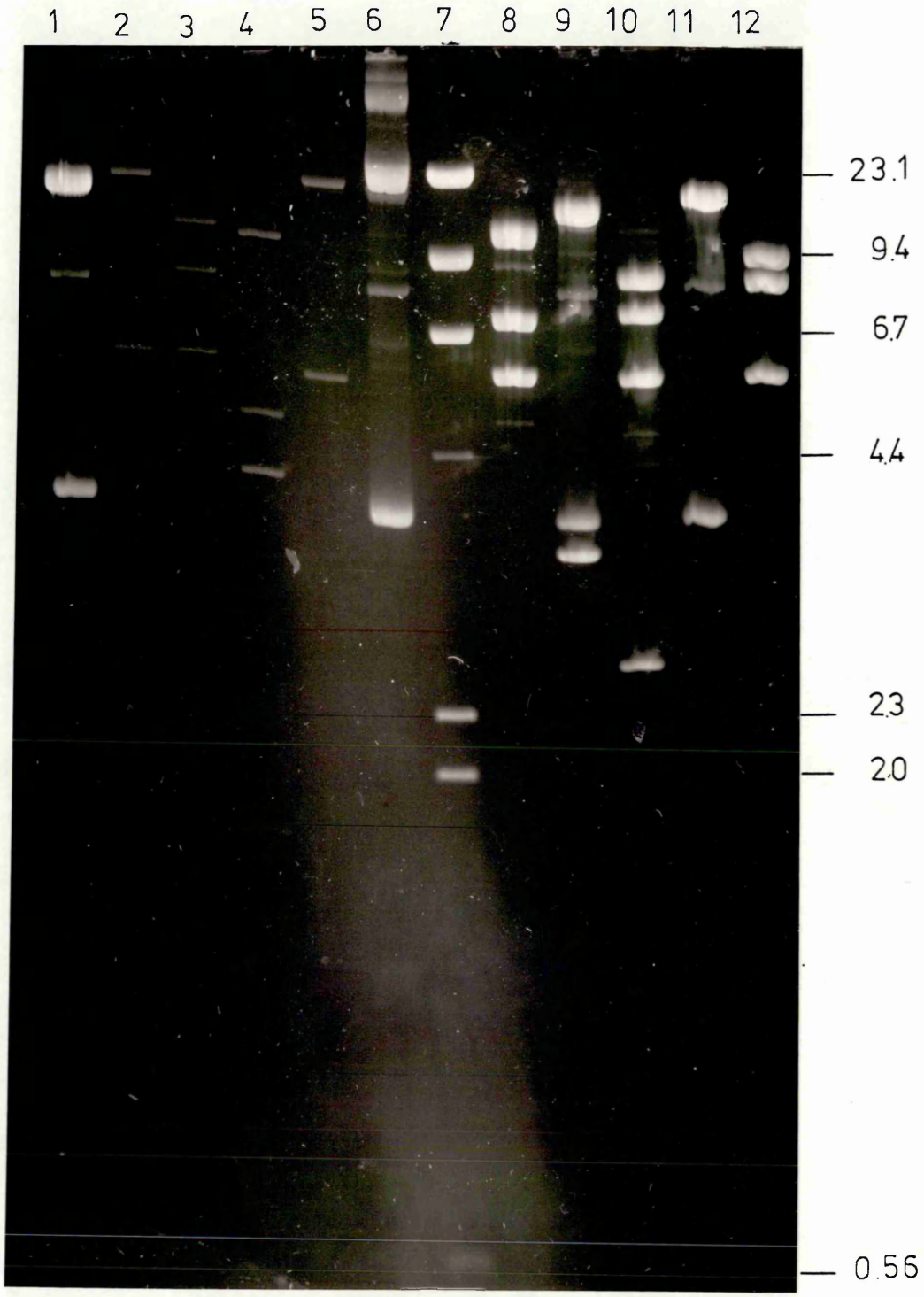
STP17 should also contain unique ClaI and EcoRI sites (from pIJ702 and STP1 respectively), and the ClaI to EcoRV map distance should be identical with that found in the tsr gene of pIJ702.

The sites for restriction enzymes ClaI and EcoRI in STP17 were mapped with respect to the HindIII, SstI and EcoRV sites (Fig. 6.6). Again, pIJ702 derived fragments complicated the interpretation of the results. HindIII digestion of ClaI-cut STP17 DNA produced two fragments 12.5 and 9.2 Kbp. in length. The position of the ClaI site was determined by digesting SstI-cut STP17 with ClaI, which resulted in the formation of new bands. They were consistent with the fragments predicted from an SstI-ClaI digest if the ClaI site were located at the right hand side of the STP17 map (Fig. 6.5) close to the EcoRV site, as is found in pIJ702. All of the digestion products of ClaI-cut STP17 DNA cut with second enzymes were consistent with this map position for the ClaI site. Similarly, the EcoRI site was mapped. HindIII and EcoRI digestion of STP17 DNA produced a 3.4 Kbp. fragment. The position of the EcoRI site was determined by examining the EcoRI/EcoRV and the EcoRI/ SstI double digestion products. The large SstI fragment was cleaved by EcoRI producing two fragments 7.3 and 3.5 Kbp. in length, thus assigning the EcoRI site 3.4 Kbp. to the left of the HindIII site (Fig. 6.8).

Using similar logic, the two BamHI and two KpnI sites in STP17 were mapped (Fig. 6.7). BamHI digestion of STP17 DNA produced two fragments of 12.0 and 6.2 Kbp. in length. HindIII digestion of BamHI-cut STP17 DNA resulted in the digestion of the 6.2 Kbp. fragment and the formation of two new fragments of 5.6 Kbp. and ca. 380 bp. (visualised on a 5% (w/v) polacrylamide gel, data not shown). Thus one BamHI site lay close to the unique

Fig.6.6

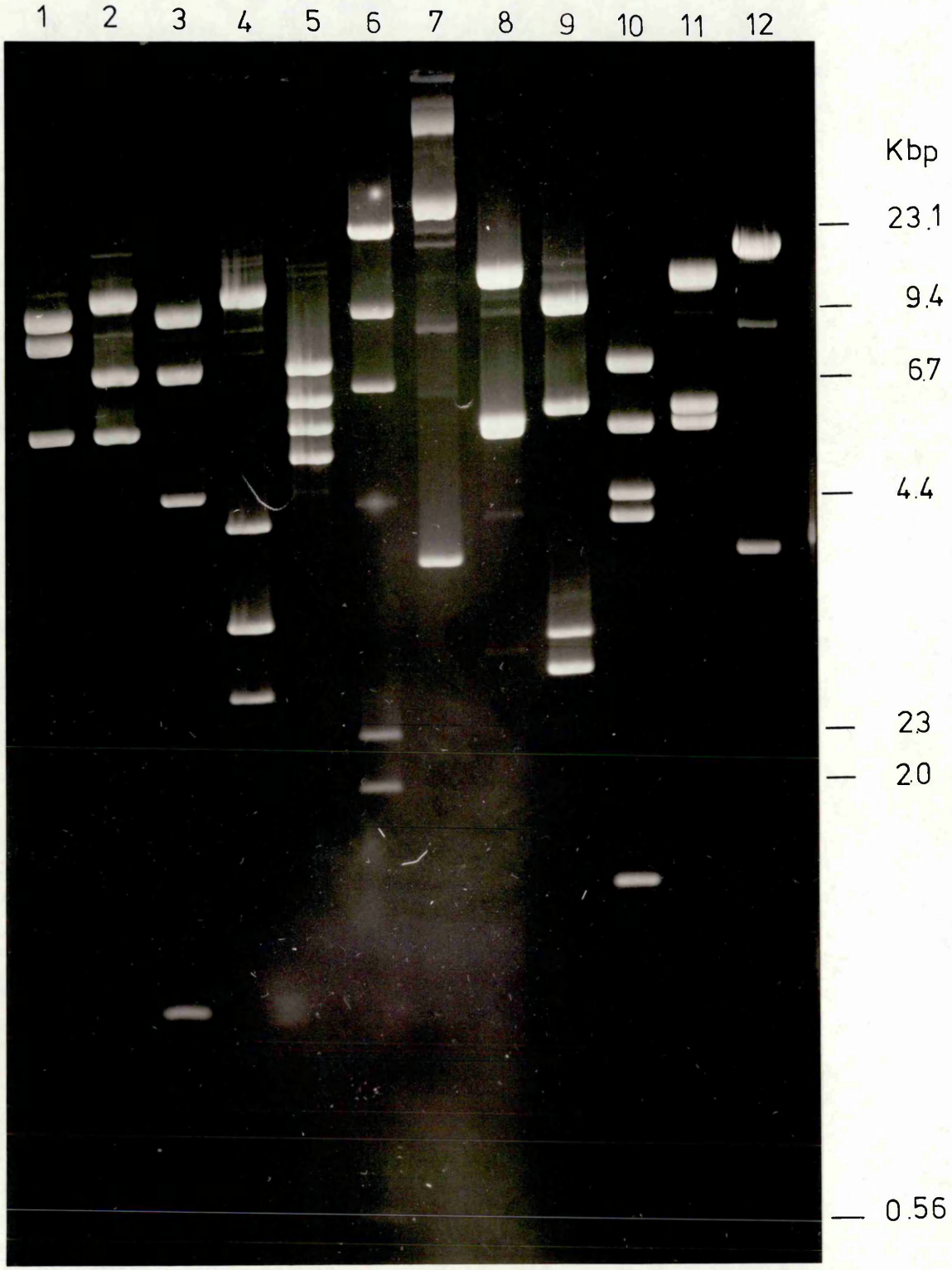
Mapping the Cla I and Eco RI Sites in STP17



1. Hind III
2. Cla I
3. Cla I/Hind III
4. Cla I/Sst I
5. Cla I/Eco RV
6. STP17 uncut
7. Lambda Hind III
8. Eco RI/Eco RV
9. Eco RV/Sst I
10. Eco RI/Sst I
11. Eco RI
12. Sst I

Fig.6.7

Mapping the Bam HI and Kpn I Sites in GLW17



1. Sst I
2. Kpn I
3. Kpn I/Sst I
4. Kpn I/Eco RV
5. Kpn I/Hind III
6. Lambda Hind III
7. GLW17 uncut
8. Bam HI/Hind III
9. Bam HI/Eco RV
10. Bam HI/Sst I
11. Bam HI
12. Hind III

HindIII site.

The second BamHI site therefore was 5.6 Kbp. to the right of the HindIII site (as drawn in Fig. 6.8). It was positioned close to the "right-hand" side XhoI site. The position of this right-hand BamHI site was consistent with the "right-hand" SstI, BamHI, XhoI, ClaI and EcoRV sites being derived from pIJ702 DNA. The KpnI sites were mapped by assuming that the right-hand KpnI site of STP17 was positioned according to the pIJ702 map, between the SstI and BamHI sites, which implied that the left hand KpnI site would be 7.1 Kbp. away. When the KpnI sites were thus positioned (Fig. 6.8) the double-digestion products of KpnI with SstI, EcoRV and HindIII were all consistent with those predicted. The restriction map of STP17 for BamHI, ClaI, EcoRI, EcoRV, HindIII, KpnI, SstI and XhoI was thus constructed (Fig. 6.8).

The restriction map of STP17 was entirely consistent with the hypothesis that STP17 was a fusion comprising both pIJ702 and STP1. In STP17 the fusion was formed in such a way that the interval between the pIJ702 SstI and EcoRV sites was disrupted by the addition of STP1 DNA. The sum of the sizes of STP1 and pIJ702 matched that of the fusion plasmid STP17, indicating that all of STP1 was involved in the fusion and that extensive duplication of sequences had not occurred.

6.7 Restriction Endonuclease Site Mapping of STP16: A Complex *in vivo* Fusion Between STP1 and pIJ702.

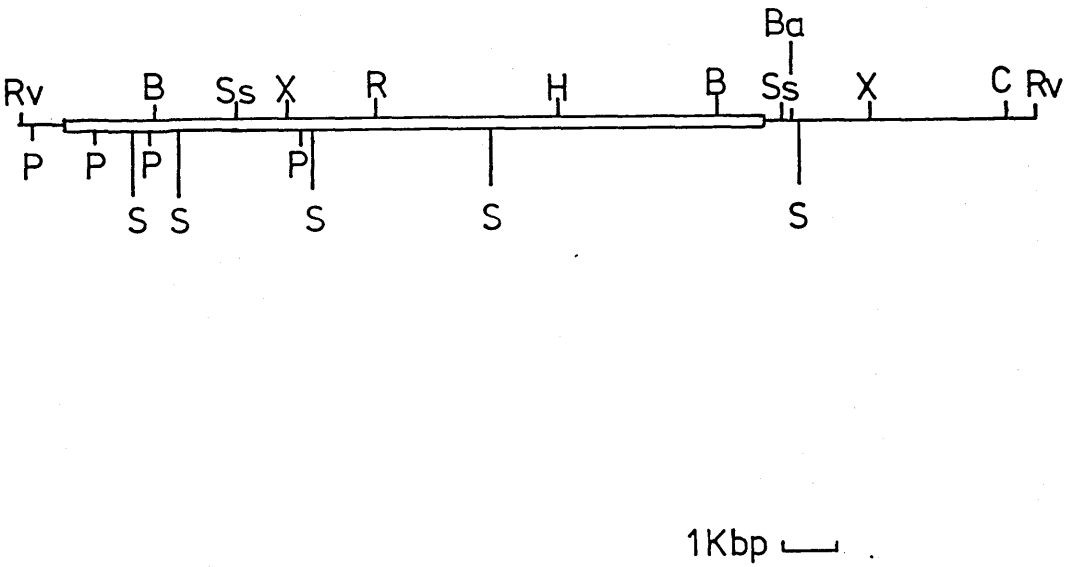
The pocking strains of *S. lividans* formed during the cotransformation experiment (6.5.1) contained plasmids which had different restriction maps (6.5.2, Fig. 6.3). *S. lividans* GLW17 contained cccDNA which had a relatively simple restriction

pattern, whilst cccDNA prepared from S. lividans GLW16 was more complex (Fig. 6.3). The BglII and PvuII digestion patterns of STP16 and STP17 had some fragments in common (Fig. 6.3) leading to the hypothesis that S. lividans GLW16 contained two plasmids. One of the plasmids was identical or very similar to STP17 and the other different. Restriction digests of cccDNA from GLW16 and GLW17 were compared for EcoRI, EcoRV and HindIII sites (Fig. 6.9). The restriction map of STP17 for these enzymes was confirmed in this experiment. Three observations were made on STP16. Firstly, enzymes which cut STP17 once cleaved cccDNA of STP16 to produce a linear molecule of about 19 Kbp. in length which included the appropriate pIJ702 restriction pattern. Secondly, some double digests using restriction enzymes which cut STP17 DNA once produced four digestion products with STP16. In each case, two of the products appeared similar to those in STP17. Thirdly, when enzymes which cut only within STP1 sequences were used in the comparison of STP16 and STP17, the restriction patterns appeared identical.

Digestion of STP16 DNA with EcoRI and HindIII resulted in the formation of two fragments of 3.4 and 16.5 Kbp. in length. When STP16 DNA was digested with EcoRV and HindIII, four digestion products of 15.5, 10.7, 8.4 and 4.4 Kbp. were formed. An EcoRV/HindIII digest of STP17 resulted in two STP17-derived fragments of 10.2 and 8.3 Kbp. If these fragments were similar or identical to the 10.7 and 8.4 Kbp. fragments in the STP16 digestion, then the novel 15.5 and 4.4 Kbp. fragments had to be explained. As the internal organisation of STP1 in the fusion seemed intact (the EcoRI-HindIII fragment was still 3.4 Kbp.) it was possible that STP1 had integrated into another region of

Fig. 6.8

The Restriction Map of STP17

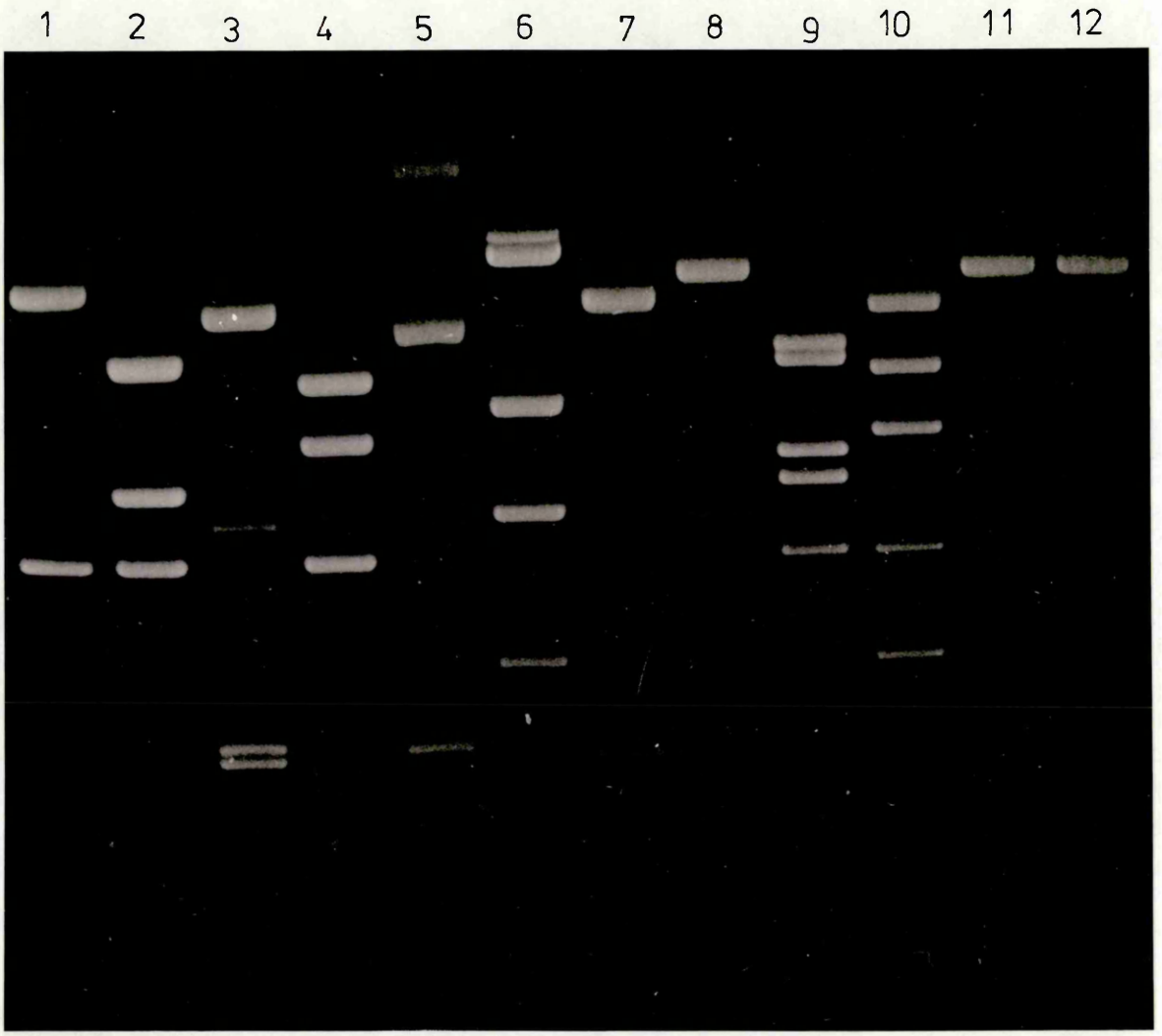


Box indicates STP1 sequences, thin lines pIJ702 sequences.

B	BglII	P	PvuII	Ss	SstI
Ba	BamHI	R	EcoRI	S	SphI
C	ClaI	Rv	EcoRV		
H	HindIII	X	XhoI		

Fig.6.9

A Comparison of cccDNA from GLW16 and GLW17.



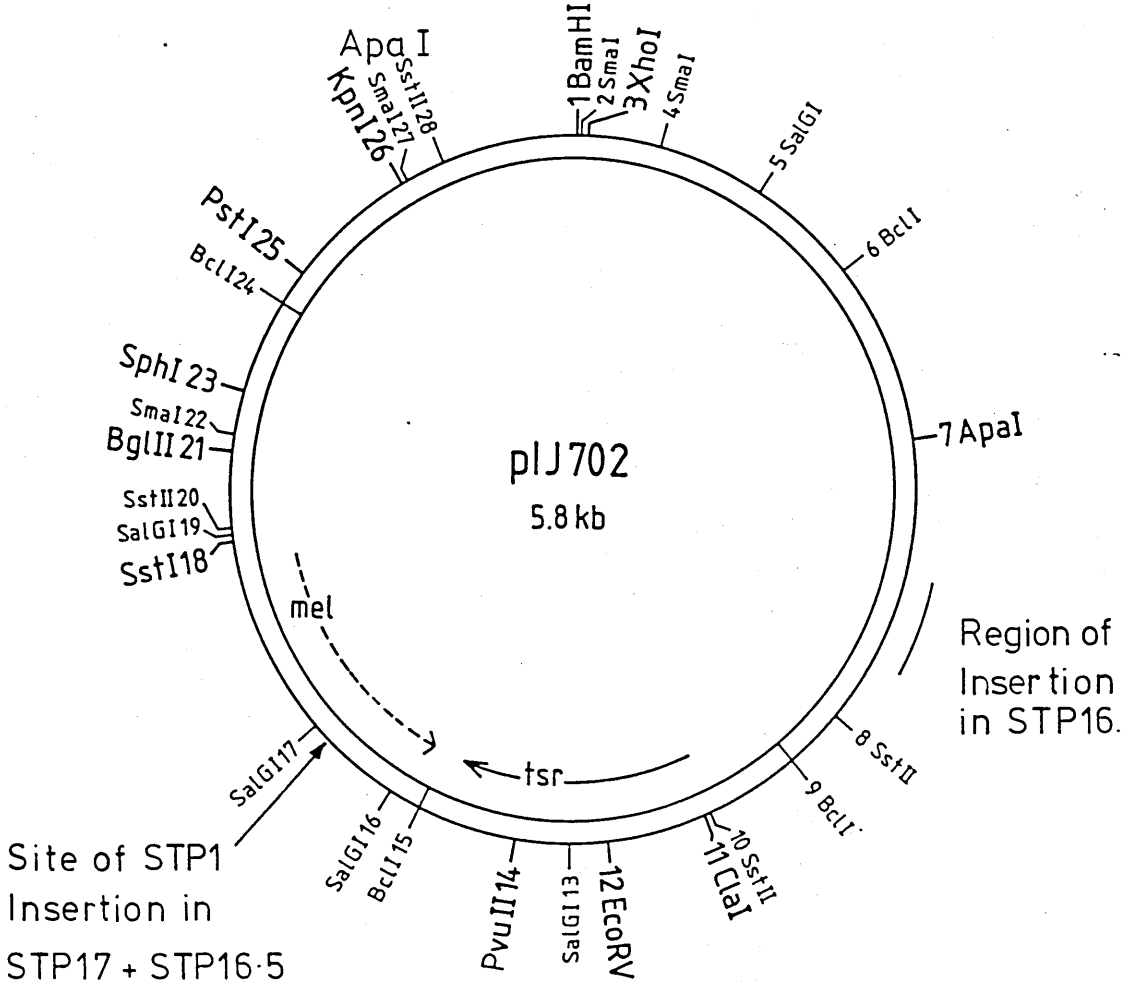
1. GLW17 Eco RV
2. GLW17 Eco RI/Eco RV
3. GLW17 EcoRI/Hind III
4. GLW17 Hind III/Eco RV
5. GLW17 uncut
6. Lambda Hind III
7. GLW16 Eco RI/Hind III
8. GLW16 Eco RI
9. GLW16 Eco RI/Eco RV
10. GLW16 Eco RV/Hind III
11. GLW16 Hind III
12. GLW16 Eco RI

pIJ702, altering the EcoRV/HindIII restriction digest pattern. When the expected fragments from STP17 digestions were removed from the analysis of STP16 the problem was simplified and an alternative fusion could be drawn (Fig. 6.10). This alternative fusion plasmid (STP16.4) was constructed such that STP1 had inserted into pIJ702 between the ApaI and ClaI sites. Thus the formation of STP1::pIJ702 fusion plasmids was not a site-specific event, although the observation that S. lividans GLW16 and S. lividans GLW17 both contained fusion plasmids of a similar structure indicates that the events might not have been entirely random. The gross structure of STP16.4, as presented above (Fig. 6.10), was refined by repeating the restriction digests used to map STP17 and comparing the observed digest products with the predicted fragment sizes. The calculated and the observed maps of STP16.4 agreed closely (data not shown), allowing the detailed comparison of the fusion plasmids STP17, STP16.5 (the STP17 like fusion in S. lividans GLW16) and STP16.4.

6.8 Further Analysis of the Cotransformation Experiment

The restriction map of STP17 showed that it was a fusion consisting of both pIJ702 and STP1 DNAs. The unique ClaI and EcoRV restriction sites of the thiostrepton resistance gene (tsr) of pIJ702 were absent from the high molecular weight cccDNA found in S. thermonitrificans ISP5579/pIJ702 recombinants (Fig. 6.1), indicating that these plasmids were not fusion plasmids like STP17, but were probably multimers or some other form of STP1(see 6.5). Thus the white spore phenotype associated with some of the strains formed during the cotransformation experiment was most likely due to another plasmid. To determine whether there was another plasmid present in the cccDNA of S. thermonitrificans

Fig 6.10
Two Alternative STP1 Integration Sites Into pIJ702



adapted from Hopwood et al., 1985

ISP5579/pIJ702 recombinants, as was implied by the cotransformation experiment, a second gel purification experiment was performed.

6.9 The Physical Identification of STP2

In an analogous experiment to that described in 6.4.1, purified cccDNA from a S. thermonitrificans ISP5579/pIJ702 recombinant was prepared by CsCl gradient ultracentrifugation and was separated by agarose gel electrophoresis. In this case, the two major bands which migrated more slowly than STP1 cccDNA were purified by electroelution (see Fig. 6.1). These fractions were used for the transformation of S. lividans TK54. The protoplasts were allowed to regenerate non-selectively at 30°C. until the lawn of regenerants had sporulated. Against the grey of the lawn of spores, several white patches were observed which appeared to be identical to the white patches observed during the cotransformation experiment (6.5). No patches exhibiting the pocking phenotype of S. lividans strains GLW61 or GLW17 were apparent in this second gel purification experiment although STP1 DNA was present in the fractions used for the transformation.

The above results indicated that a low copy-number plasmid was present in S. thermonitrificans ISP5579/pIJ702 recombinants, and that this plasmid encoded the "white spore" phenotype. This putative plasmid was named STP2. Furthermore, this observation confirmed that the plasmids of a higher molecular weight than STP1 were not fusion plasmids like STP17 because their transformation into S. lividans TK54 did not result in the pocking phenotype associated with STP17. Nor did they contain the EcoRV or ClaI restriction endonuclease recognition sequences associated with the tsr gene of pIJ702. Finally this experiment

also provided evidence that only monomeric STP1 was able to integrate into the S. lividans chromosome, as transformation with multimeric or other form of STP1 failed to result in the pocking phenotype.

6.10 Stability of the STP1::pIJ702 Fusion Plasmids

The heritable stability of the tsr$^+$ phenotype of S. lividans strains GLW16 and GLW17 was analysed. Spores of both strains were prepared using non-selective conditions and the spore suspensions titred on selective and non-selective media. In both strains the tsr$^+$ phenotype could result from the stable maintenance of either a fusion plasmid or pIJ702 (or both). S. lividans GLW17 stably maintained the tsr$^+$ phenotype whilst S. lividans GLW16 frequently lost the tsr$^+$ phenotype (about 50% of the colonies were tsr$^+$). As the S. lividans GLW16 strain was more unstable than S. lividans GLW17 it was more suitable for the isolation of a thiostrepton-sensitive strain. S. lividans GLW16 spores were plated on non-selective media and after sporulation the colonies were replica-plated onto both selective and non-selective media. Colonies which were sensitive to thiostrepton were analysed. Most of the colonies had lost the fusion plasmids and pIJ702, but one such colony was thiostrepton-sensitive and retained the ability to form pocks on S. lividans TK54. The strain was examined for the presence of cccDNA using both small-scale and large-scale plasmid purification methods, the latter followed by CsCl gradient centrifugation. No cccDNA was detected from this strain indicating that the STP1 pocking phenotype was no longer linked physically to the thiostrepton-resistance gene of pIJ702 and that STP1 was presumably in the chromosome of S. lividans as in S. lividans GLW61 and GLW62 (6.4.1).

The structural stabilities of the fusion plasmids present in S. lividans GLW16 and GLW17 were analysed by a gel purification experiment similar to those used earlier (6.4). The gel-purified fusion plasmids of ca. 19 Kbp. were used to transform protoplasts of S. lividans TK54. The protoplasts were allowed to regenerate non-selectively and pocking transformants were restreaked onto media containing thiostrepton. All of the pocking transformants were also thiostrepton-resistant. Despite gel purification of the fusion plasmids, all of the transformants obtained from DNA purified from S. lividans GLW17 contained both the fusion plasmid and pIJ702. The transformants from the purified GLW16 DNA were of two types. One type exhibited the same plasmid profile as was obtained from purified STP17 DNA (STP16.5 and pIJ702), whilst the second type exhibited only the alternative fusion present in S. lividans GLW16 (no cccDNA pIJ702 was observed). This strain containing only the alternative fusion plasmid was named GLW16.4. In this way the two types of fusion plasmid present in S. lividans GLW16 were separated.

The fusion plasmid STP16.4 (with one exception, 6.10.1) was structurally-stable, surviving repeated transformation and was maintained in spores in the absence of selection. No pIJ702 cccDNA was detected and no plasmid cured strains were obtained from 600 colonies of S. lividans GLW16.4 tested. Thus when STP1 was integrated in this position in pIJ702, the fusion did not break down to form STP1 and pIJ702. The fusion plasmid in S. lividans GLW17 was not structurally stable. The gel-purified 19 Kbp. fusion cccDNA should not have contained any pIJ702 monomer, yet pIJ702 monomer was observed in all pocking strains derived from transformation with gel-purified fusion DNA of STP17. This

implied that the fusion plasmid underwent breakdown into pIJ702 and STP1. STP1 cccDNA was never observed in S. lividans and presumably if it formed, it integrated into the chromosome as was observed in S. lividans GLW61 and GLW62 (6.4.1) and implied in the thiostrepton-sensitive pocking strain of S. lividans (above).

The major difference between the two fusion plasmids was the site of integration of STP1 into pIJ702. It seemed reasonable to expect the difference in structural stability to be a result of the differences in integration sites.

6.10.1 Isolation of STP1654: An *in vivo* Deletion of STP16.5

During the analysis of the structural stability of STP16.5 (6.10) one transformant strain, GLW1654, in which the thiostrepton-resistance plasmid was smaller than STP16.5 was identified. About 10 Kbp. of DNA was deleted from the STP1 portion of the fusion plasmid. This smaller plasmid, STP1654, was useful in determining the site of integration of STP1 into pIJ702 in this strain (6.11).

6.11 Detailed Restriction Analysis and Comparison of the Structures of STP1 and the Fusion Plasmids STP17, STP16.4, STP16.5 and STP1654

The plasmids already described in this chapter had been mapped for a number of restriction enzymes (6.6, 6.7). Using similar logic, the fusion plasmid STP16.4 was subjected to more detailed restriction mapping using a greater number of enzymes. STP16.4 was chosen for the analysis because cccDNA prepared from S. lividans STP16.4 did not contain any pIJ702 cccDNA (6.10), thus reducing both the complexity of the gels and the number of doublet bands. The restriction map of STP16.4 determined from

these experiments was used to predict the restriction map of the other plasmids. The predicted fragment sizes from single and double digests of these enzymes were in good agreement. The restriction map of STP1654 was determined from first principles. The restriction maps of these plasmids are compared in Fig. 6.11.

Two conclusions can be drawn from this information. Firstly, the same region of STP1 was involved at the STP1/pIJ702 junctions in two independent integration events. This suggested that the integration was a site-specific event with respect to STP1. Secondly, STP1 integrated into two different regions of pIJ702 indicating that the integration was not site specific with respect to pIJ702.

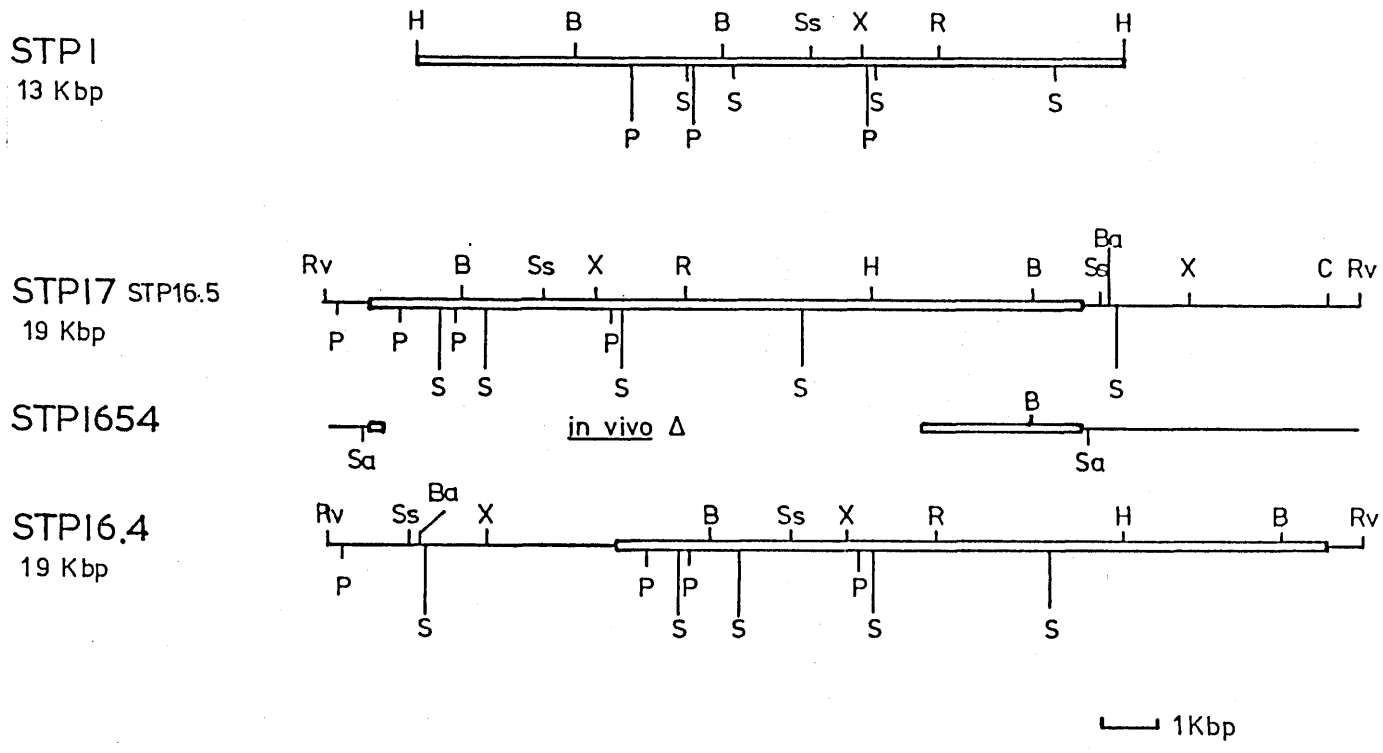
6.12 Southern Blotting Experiments

During the analyses of the pock-forming strains of S. lividans described above, it was assumed that STP1 resided within the chromosome of S. lividans as well as being part of the fusion plasmids (6.4, 6.6, 6.10). Likewise, the plasmidogenic nature of STP1 in S. thermonitrificans ISP5579 implied, that in the parental strain, STP1 would also be found associated with high molecular weight DNA (ie. integrated), rather than as autonomous DNA. This hypothesis was tested using Southern hybridisation. The fusion plasmid STP16.4 was radiolabelled using random oligonucleotide sequences to prime the synthesis of the probe DNA (2.24). The probe contained both pIJ702 and STP1 sequences; thus in the hybridisation experiments both DNA's were detected.

Fig. 6.11

A Comparison of the Structures of STP1 and the Fusion Plasmids

STP17, STP16.4, STP16.5 and STP1654



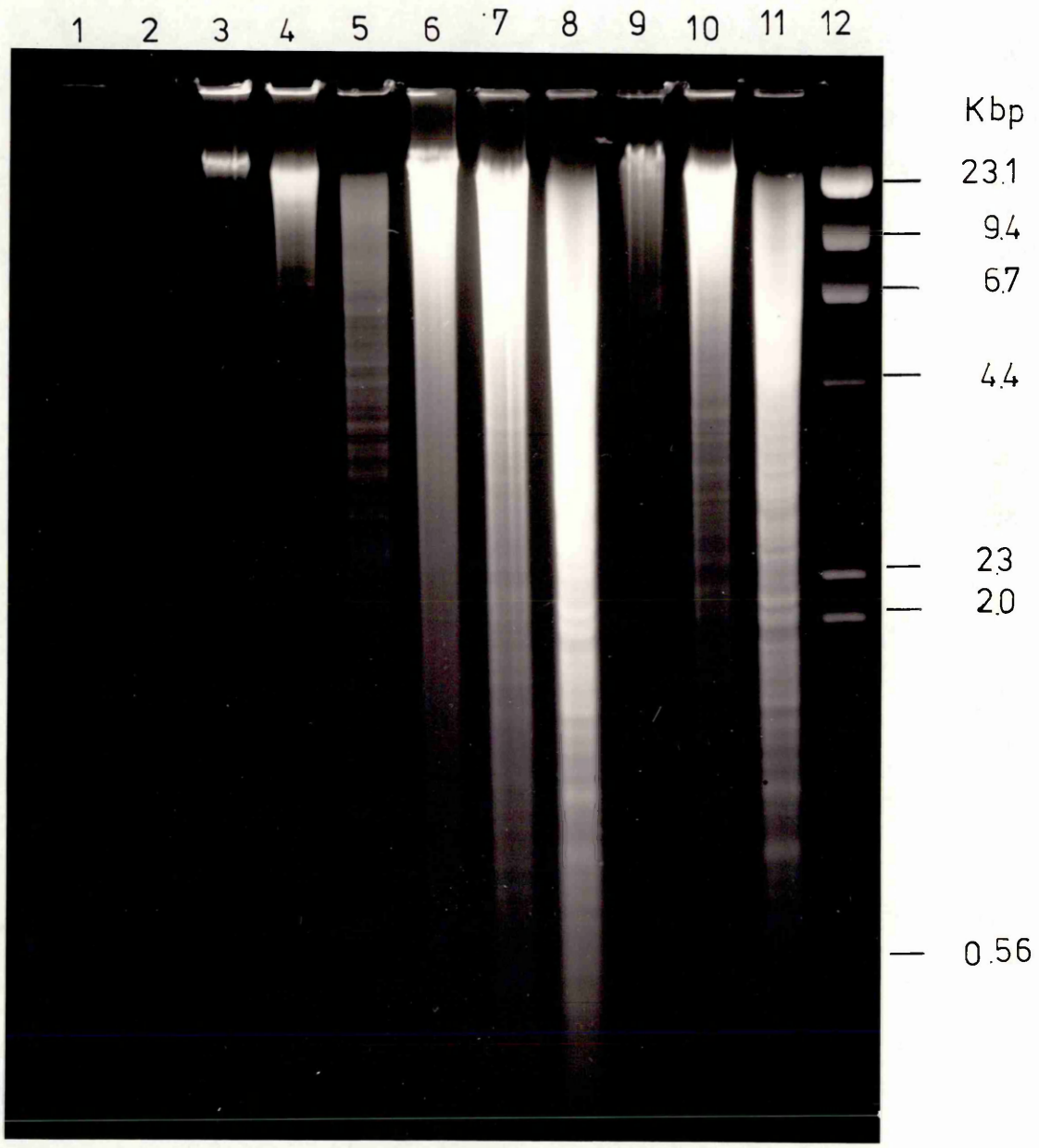
B	BglII	P	PvuII	Sa	SalI
Ba	BamHI	R	EcoRI	Ss	SstI
C	ClaI	Rv	EcoRV	S	SphI
H	HinDIII	X	XhoI		

6.12.1 STP1 is Integrated into High Molecular Weight DNA in S. thermonitrificans ISP5579 and S. lividans strains GLW61 and GLW62

The formal proof of the plasmidogenic nature of STP1 was obtained by Southern hybridisation (Fig. 6.12). Genomic DNA of S. thermonitrificans ISP5579, S. lividans GLW61 and S. lividans GLW62 was prepared and subjected to one of three treatments prior to gel electrophoresis. The samples were either left undigested, they were digested with restriction enzymes ClaI and EcoRV (which did not digest within STP1 DNA) or they were digested with SphI which cleaved autonomous STP1 into four fragments. STP1 was associated with high molecular weight DNA when the DNA was untreated (Fig. 6.12). It was possible that the STP1 DNA was in the cccDNA form but was running anomalously slowly in the gel. To test this, the DNA was digested with ClaI and EcoRV. The size of the hybridising band was reduced, suggesting that STP1 was integrated in a larger molecule, presumably within the chromosome. If the STP1 cccDNA had been migrating anomalously, digestion with ClaI and EcoRV would not have been expected to alter its mobility. Finally, digestion with a restriction enzyme which cut within STP1 (SphI) showed that the STP1 DNA could be cut and that the plasmid was therefore integrated. Autonomous STP1 DNA was cut by SphI into 4 fragments (Fig. 6.12, lane 2, linear pIJ702 also present). The S. thermonitrificans genomic DNA contained 5 SphI fragments which hybridised to the probe DNA (plus two fainter fragments which may be partial digestion products or homology elsewhere in the genome). Three of the 5 genomic fragments correspond to SphI fragments present in the autonomous form of STP1 and the two novel fragments appeared to be formed by the interruption of the large SphI fragment (lane 2).

Fig.6.12A

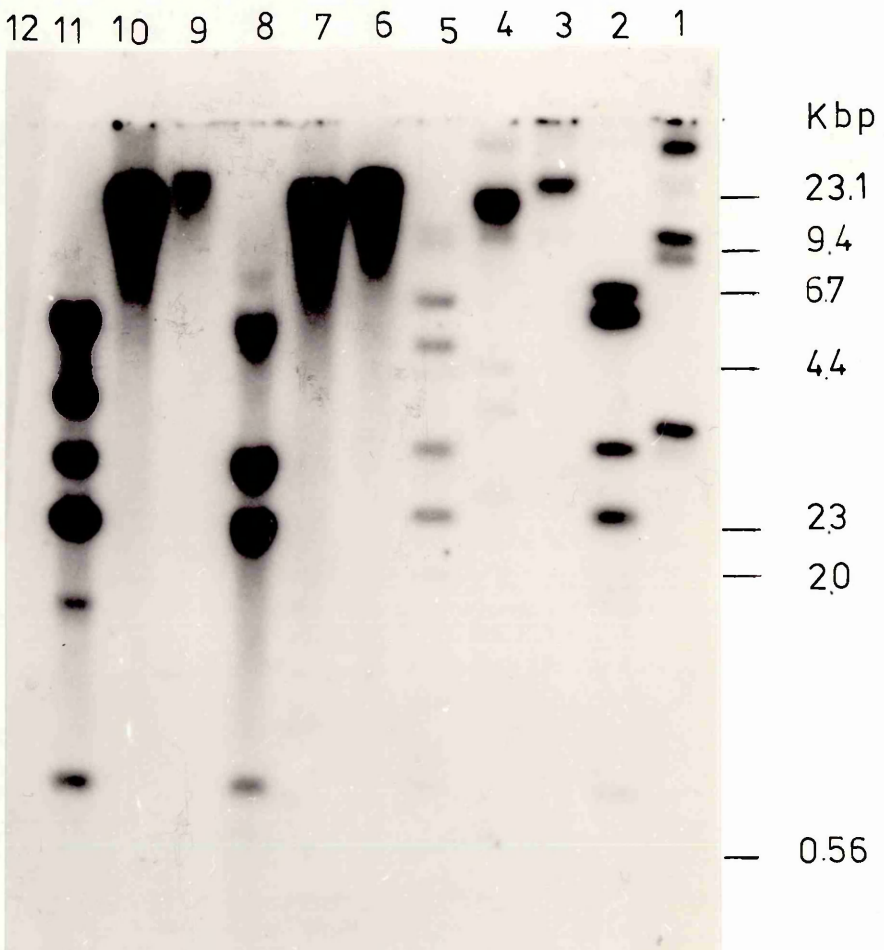
Agarose Gel and Southern Analysis of *S. thermonitrificans*, *S. lividans* GLW61 and *S. lividans* GLW62 Chromosomal DNA



1. STP1/pIJ702 uncut
2. STP1/pIJ702 Sph I
3. 579 uncut
4. 579 Eco RV/Cla I
5. 579 Sph I
6. GLW61 uncut
7. GLW61 Cla I/Eco RV
8. GLW61 Sph I
9. GLW62 uncut
10. GLW62 Cla I/Eco RV
11. GLW62 Sph I
12. Lambda Hind III

Fig.6.12B

Agarose Gel and Southern Analysis of *S. thermonitrificans*, *S. lividans* GLW61 and *S. lividans* GLW62 Chromosomal DNA



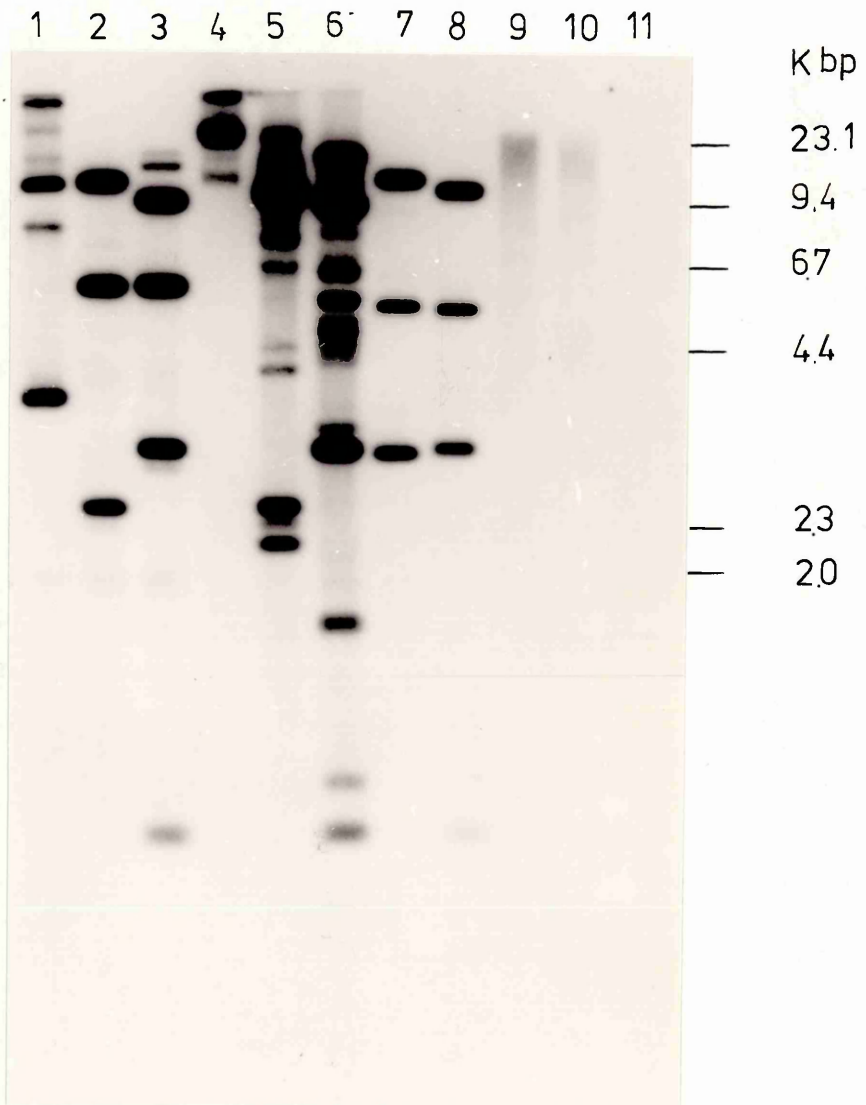
1. STP1/pIJ702 uncut
2. STP1/pIJ702 Sph I
3. 579 uncut
4. 579 Eco RV/Cla I
5. 579 Sph I
6. GLW61 uncut
7. GLW61 Cla I/Eco RV
8. GLW61 Sph I
9. GLW62 uncut
10. GLW62 Cla I/Eco RV
11. GLW62 Sph I
12. Lambda Hind III

These results confirmed that STP1 was integrated in the chromosome of parental S. thermonitrificans ISP5579. It excised upon transformation by pIJ702 to form cccDNA and the cccDNA could be transformed into S. lividans TK54 where it integrated to form S. lividans GLW61. Although there were only 4 hybridising bands in SphI digested GLW61 DNA (lane 8) the STP1 sequences were probably integrated. The large cccSTP1 SphI fragment (lane 2) was not present in lane 8 and one novel band was formed. The second novel fragment expected as a result of integration was not observed. The two junction fragments in S. thermonitrificans were of similar intensity, indicating that there was a similar length of STP1 sequence on both junction fragments. Therefore, if the same integration site for STP1 was used in the formation of GLW61, the second novel fragment must have been absent for a reason other than a lack of homology eg, a deletion after integration. The pattern of hybridisation obtained with S. lividans GLW62 DNA (lane 11) was consistent with the integration model. The large cccSTP1 SphI fragment (lane 2) was absent and two novel fragments were observed (lane 11). An additional new fragment, the fifth largest, was also present at reduced intensity. The origin of this fragment was not obvious. The uncut chromosomal S. thermonitrificans DNA contained a small amount of hybridising DNA which comigrated with purified STP1 cccDNA, indicating that at a low frequency STP1 was able to excise from the chromosome in the absence of pIJ702.

Further analysis of STP1 in the S. thermonitrificans ISP5579 chromosome (Fig. 6.13) showed that the large STP1 PvuII and SphI fragments may not be present in the chromosome implying a role in the integration event. These two fragments of STP1 DNA

Fig.6.13

Southern Analysis of the Integrated STP1 Element in *S. thermonitrificans* Chromosomal DNA



1. STP1/pIJ702 uncut
2. STP1/pIJ702 Bgl II
3. STP1/pIJ702 Pvu II
4. 579 uncut
5. 579 Bgl II
6. 579 Pvu II
7. STP16.4 Bgl II
8. STP16.4 Pvu II
9. *S. lividans* TK54
10. *S. coelicolor* G94
11. *S. rimosus* 4018

were disrupted in the chromosomal integration suggesting that the integration site was contained within them. The segment of DNA which was overlapped by the large SphI and PvuII fragments of STP1 was therefore considered to contain the integration site. This segment was contained within the small STP1 BglII fragment. Fig. 6.13 shows that it was not disrupted by integration. An explanation would be that the small BglII fragment was disrupted by integration, but a chromosomal BglII site was situated such that a hybridising fragment of the same size was produced. Unambiguous interpretation of these results was not possible, mainly because of the presence of partial digestion products in Fig. 6.13 lanes 5 and 6. The interpretation given is consistent with the previous results and with data not shown. It has not been possible to show point to point correspondence between cccSTP1 and its integrated form in S. thermonitrificans. The integrations of STP1 into S. lividans to form S. lividans GLW61 and GLW62 (6.4.1) were not identical. The biological properties of GLW61 were studied (6.4.1), with the assumption that GLW61 and GLW62 would have similar characteristics. However, the above results, particularly Fig. 6.12b show that GLW61 and GLW62 were physically different. It would appear that a comparison of GLW61 and GLW62 may be worthwhile to determine whether the physical difference between the strains is accompanied by a biological difference.

6.12.2 The Restriction Map of STP1 Integrated in the S. thermonitrificans ISP5579 Chromosome.

The same region of STP1 (The small BglII fragment) contained the site of integration in the formation of the fusion plasmids with pIJ702. More detailed restriction mapping of the resident

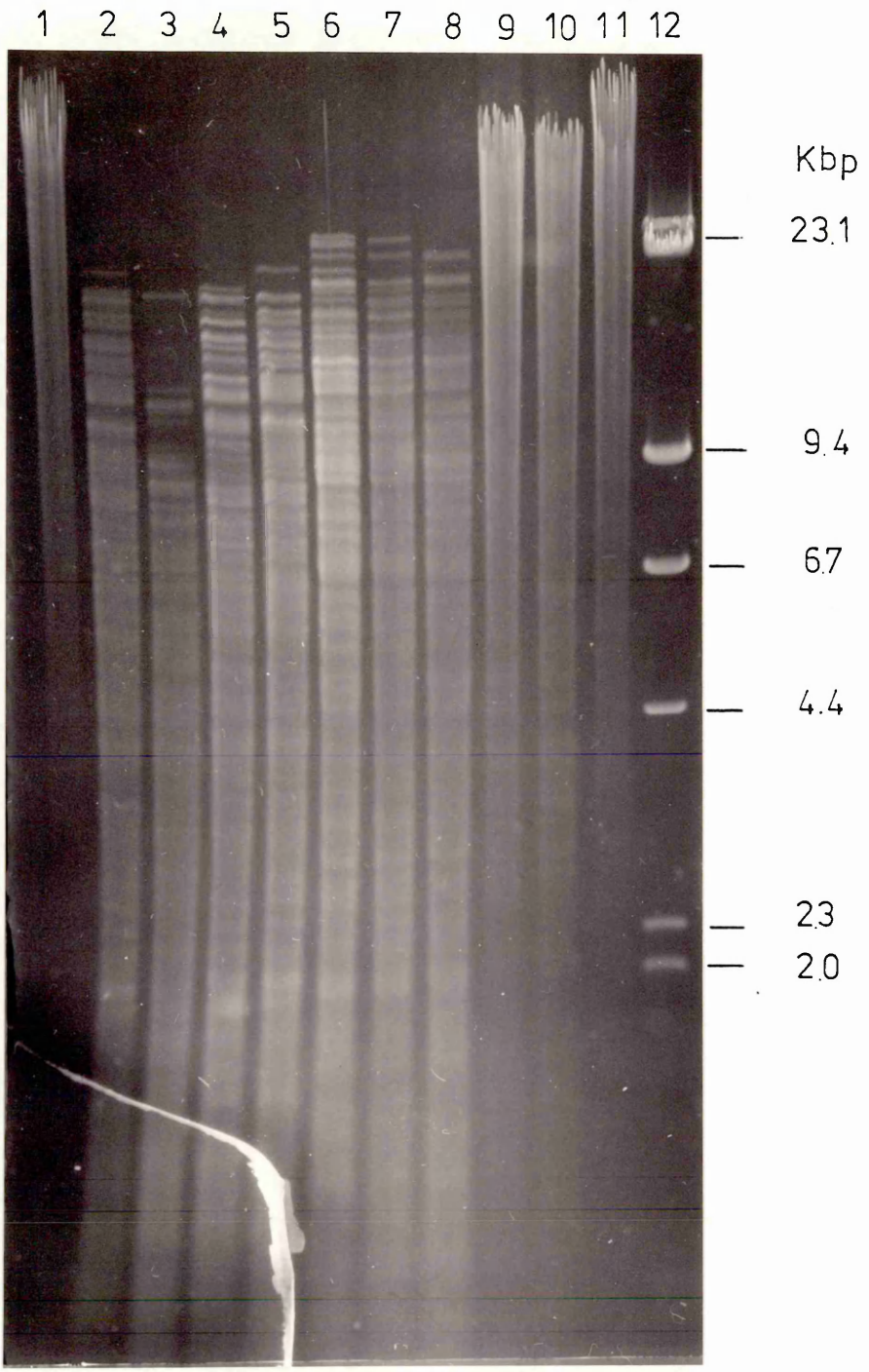
(integrated) STP1 sequences in S. thermonitrificans ISP5579 was performed using Southern hybridisation to investigate whether the same region of STP1 was used for integration into the chromosome (Fig. 6.14).

The first observation was that there were many fainter bands implying that STP1 or part of it existed as a repeated sequence or a family of STP1-like elements with some sequence similarity in S. thermonitrificans ISP5579. Other plasmidogenic elements have tended not to be repeated in this manner (Hopwood et al., 1984) although pSAM2 may have some homology elsewhere in the S. ambofaciens chromosome (Rao, pers. comm). The pattern of the faint bands made it unlikely that they were the product of partial digestion of the chromosomal DNA. A limited restriction map of the chromosomal region containing the integrated STP1 DNA was produced (Fig. 6.15) but the map was far from satisfactory. The problem was traced to the non-randomness of the random priming technique. In one case a 500 bp. region of the probe had three to five times the specific activity of the adjacent 500 bp. This meant that the intensity of the hybridising bands was no longer proportional to the length of the complimentary sequences. This complicated the assignment of fragments as junctions between STP1 and chromosomal DNA as these fragments would normally exhibit a reduced intensity in proportion to their length.

STP1 was certainly integrated into the high molecular weight DNA in S. thermonitrificans ISP5579 and S. lividans strains GLW61 and GLW62. The restriction map of integrated STP1 and the chromosomal DNA surrounding the integration site was not unambiguously determined. It was possible that more than one copy (including partial copies) of STP1 were integrated. The

Fig.6.14A

Restriction Mapping the Integrated STP1 Element by Southern Analysis

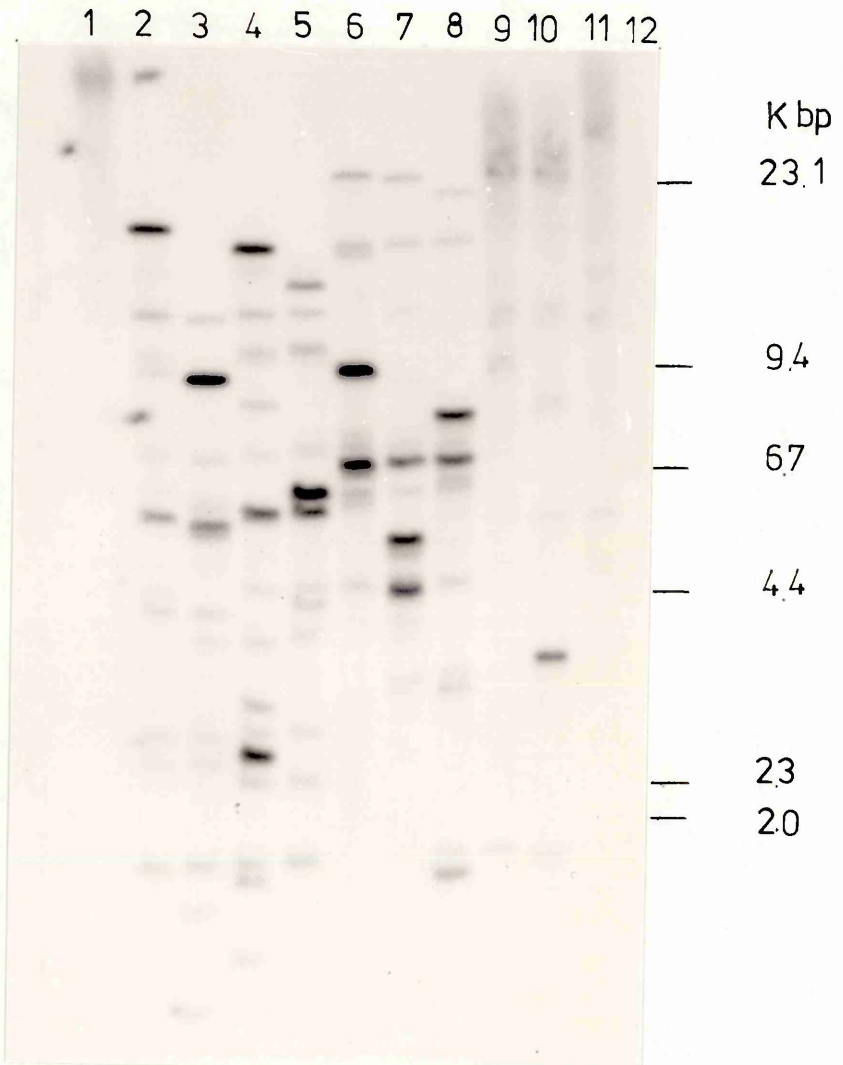


579 Chromosomal DNA

1. Uncut
2. Sst I/Xho I
3. Sst I/Eco RI
4. Sst I/Eco RI
5. Hind III/Sst I
6. Xho I
7. Xho I/Hind III
8. Eco RI/Xho I
9. Eco RI
10. Eco RI/Xho I
11. Hind III
12. Lambda Hind III

Fig.6.14B

Restriction Mapping the Integrated STP1 Element by Southern Analysis



579 Chromosomal DNA

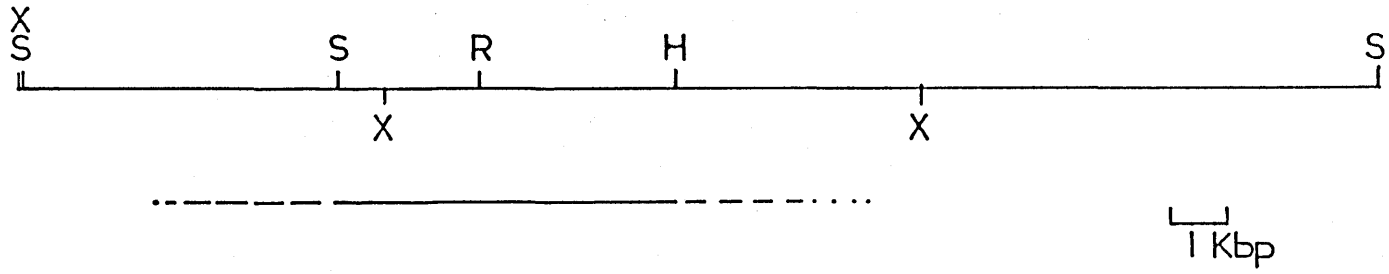
1. Uncut
2. Sst I/Xho I
3. Sst I/Eco RI
4. Sst I/Eco RI
5. Hind III/Sst I
6. Xho I
7. Xho I/Hind III
8. Eco RI/Xho I
9. Eco RI
10. Eco RI/Xho I
11. Hind III
12. Lambda Hind III

Fig. 6.15

Interpretation of the Southern Analysis Results:

A Partial Restriction Map of the Integrated STP1 Element

in the S. thermonitrificans Chromosome



Major Homology to STP16.4 in the ISP5579 Chromosome

H-HindIII S-SstI R-EcoRI X-XhoI

preliminary data for the integrated STP1 in S. lividans suggested that these strains (GLW61 and GLW62) might be more useful for mapping studies. There were fewer background bands on the autoradiographs suggesting that STP1 was not a repeated sequence in these strains.

6.13 The Site of Integration of STP1 into pIJ702 in the Fusion Plasmid STP16.5

The fusion plasmids STP17 and STP16.5 could not be separated physically from cccDNA of pIJ702 because the fusion plasmid broke down to yield pIJ702 as a product when STP1 was inserted into this position in pIJ702 (6.10). The constant presence of pIJ702 in the cccDNA preparations made the identification of the integration site difficult, because the DNA fragment into which STP1 had inserted was present intact in the contaminating pIJ702. The isolation of STP1654 (the in vivo deletion of STP16.5) (6.10.1) effectively removed the contaminating pIJ702 DNA. The restriction map of STP1654 (6.10.1, 6.11, Fig. 6.11) showed that the deletion was internal to STP1, leaving the two junctions between STP1 and pIJ702 DNA intact.

Comparison of STP1654 and pIJ702 using restriction enzyme SalI, showed that STP1 had integrated into the 267 bp. SalI fragment within the mel gene of pIJ702. This was consistent with the observation that in STP1654, the novel SalI fragment produced by the deletion contained a single BglII STP1-derived restriction site. The two ends of the junction between STP1 and pIJ702 were sub-cloned on SalI-BglII fragments into SalI-BamHI cut pTZ18 and pTZ19 vector DNA (Pharmacia). These vectors were chosen because synthetic oligonucleotide primers were available for double

stranded DNA plasmid sequencing. The sequence across both the junctions was determined for one strand only and must therefore be treated as very preliminary data. The site of STP1 insertion was already determined to be within the open reading frame of the mel gene of pIJ702 from restriction mapping. The sequence data confirmed this and using the published mel sequence (Bernan et al., 1985) it was possible to read the sequencing gel until new DNA unrelated to mel was observed. These changes in the sequence pattern were regarded as the junctions between STP1 and pIJ702 DNA. This preliminary data is presented in Fig. 6.16a and summarised in Fig. 16.6b. It will be discussed with respect to other Streptomyces plasmidogenic elements and the sequences of their insertion sites (6.14).

Fig 6.16

The Site of Integration of STP1 into pIJ702 in the Fusion Plasmid STP16.5

a) The Sequence of the Junctions of the STP1 Insertion in STP1654

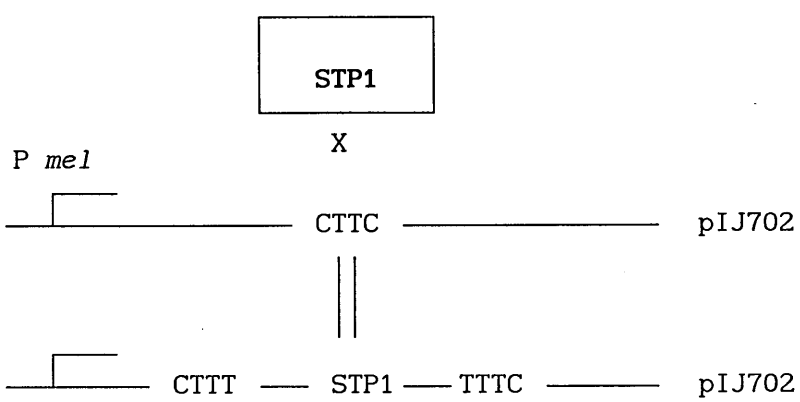
Tyrosinase gene (*mel*)* TCC GAC GGC TTC CGC AAC CAT

Left Junction TCC GAC GGC TTT CAA GAC CAA

Right Junction CAT AAN GGT TTC CGC AAC CAT

* from Bernan et al., (1985)

b) Integration of STP1 into the *mel* of pIJ702



6.14 Discussion

Many plasmids have been isolated from Streptomyces (Hopwood et al., 1986a). Several of these plasmids, and others isolated more recently, have been shown to interact physically with the chromosome eg. SCP1, SLP1, pIJ110, pIJ408, pSE101, pMEA100, pSAM2 and pSG1 (Bibb et al., 1981; Hopwood et al., 1984; Cohen et al., 1985; Madon et al., 1986; Omer and Cohen, 1986; Brown et al., 1988 and Boccard et al., 1988). All of these plasmids may be found integrated into the chromosome of their natural host. The integrated copy/ies may coexist with autonomous cccDNA of the plasmid (Cohen et al., 1985; Pernodet et al., 1984), or as in other cases, the cccDNA form of the plasmid may only be found after conjugal mating into a plasmid free recipient (Bibb et al., 1981b; Hopwood et al., 1984). Lee et al. (1989) have shown that the SLP1 functions responsible for the excision of the element from the chromosome are induced during conjugal transfer.

In every case when an integrated plasmidogenic element has been studied carefully, a complete linear sequence of the plasmid has been identified in either the natural or recombinant hosts. The plasmid has undergone site specific recombination into the chromosome (Omer and Cohen et al., 1986; Madon et al., 1987; Simonet et al., 1987; and Brown et al., 1988). In some cases a small quantity of cccDNA has been observed in the parental strain implying that the integrated plasmid has "looped out" or excised from the chromosome at low frequency (Hopwood et al., 1984; Brown et al., 1988). These cccDNA forms of the plasmids were not replicating autonomously indicating that the replication functions were repressed.

STP1 has been found integrated into high molecular weight DNA of S. thermonitrificans ISP5579 (6.12.2). A small amount of STP1 cccDNA was observed in S. thermonitrificans during Southern hybridisation experiments, implying that STP1 could excise or loop out from the chromosome (6.12.2) like other plasmidogenic elements. The cccDNA form of STP1 was present at very low copy-number indicating that it did not replicate autonomously in the parental strain. When S. thermonitrificans was transformed with pIJ702 the copy number of STP1 cccDNA increased to approximately half that of pIJ702 (4.3.2, 6.2). Either pIJ702 provided replication functions required by STP1 in trans or the introduction of pIJ702 released STP1 from the repression of replication by the integrated element. The gel purification experiment (6.4) showed that the cccDNA form of STP1 present in S. thermonitrificans ISP5579 pIJ702 was able to integrate into the chromosome of S. lividans after transformation (6.4.1, 6.12.1). STP1 was not transferred from S. thermonitrificans into S. lividans during interspecific matings (4.9.3, table 4.9). Transfer of STP1 might have been expected but it was possible that the cultural conditions did not favour mating between the two species. However, when STP1 was integrated into the S. lividans chromosome it could be transferred at high efficiency into another S. lividans strain (6.4.1) providing evidence that STP1 was conjugative. STP1 exhibits characteristics which are similar to those of the other Streptomyces plasmidogenic elements. It is therefore reasonable to conclude that STP1 is another example.

The most unusual activity associated with STP1 was the formation of fusion plasmids when STP1 became integrated into

pIJ702. The fusion event only took place when both plasmids were cotransformed into S. lividans (6.5). Two sites of STP1 insertion into pIJ702 have been studied in detail (6.6, 6.7). The same region of STP1 was involved in the integration event in both cases implying that a site-specific integration function is present on STP1. Whilst no other Streptomyces plasmidogenic element has yet been shown to possess this ability, it is possible that this was a result of the experimental cotransformation procedure required for its expression. Why this should be so remains a matter for speculation. In similar experiments Lee et al. (1989) showed that SLP1 would not integrate into pIJ702 when pIJ702 was resident in the recipient strain. They did not report the results of cotransformation of pIJ702 and SLP1. pSE101 has been shown to integrate into many sites in the S. lividans chromosome (Brown et al., 1988). They speculated that the integration sites may be analogous to the secondary site attachment of bacteriophage lambda in E. coli cells which have lost the attB site. Alternatively, the integration may be due to the presence of an insertion sequence on pSE101 (and possibly on STP1) but as yet this is unproven. Regardless of the mechanism of integration, STP1 has been shown to integrate into at least two different positions of pIJ702 (see 6.11), the chromosomal integration site of S. lividans GLW61 and another site in S. lividans GLW62, although these may be the same; GLW61 having undergone a deletion after the integration event (6.12). These observations justify the description of STP1 as a transposable element, which was supported further by the isolation of a strain of S. lividans in which STP1 was integrated when the STP1 element was introduced as a fusion plasmid (6.10). The formation of this strain implied that STP1 had transposed

from the fusion plasmid into the chromosome. This strain did not contain pIJ702 (it was tsr$^-$) perhaps indicating a non-replicative mode of STP1 transposition where the donor DNA was destroyed.

The above evidence suggests that STP1 is a plasmidogenic conjugative transposable element of S. thermonitrificans ISP5579.

The structural stability of the fusion plasmids depended on the site of integration of STP1 into pIJ702. Integrations into the mel gene of pIJ702 were unstable (STP17 and STP16.5) (6.10), whilst the alternative integration was stable (STP16.4). It is possible that transcription from the mel promoter across the junction of the fusion might increase instability. Fusion plasmid STP16.4 was very stable in S. lividans suggesting that the ability to create fusion plasmids may have a general application. An example would be the use of the fusion methodology to integrate STP1 into pIJ702 derived clones, enhancing their stability in the absence of thiostrepton and also making the fusion plasmid conjugative.

The differential stability of the fusion plasmids may be a result of their different sites of integration. Preliminary DNA sequence information on the site of integration of STP1 in STP16.5 (STP1654) was summarised in Fig. 6.16b.

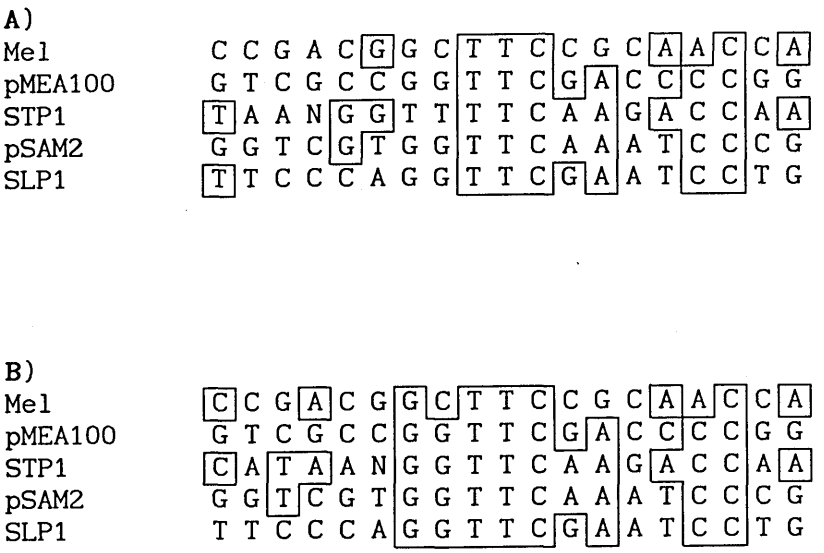
The integration event may result in the duplication of target sequences, or it may not, depending on the mechanism of STP1 integration. The sequence of the STP1 attP site was not available but two alternative STP1 attP sites were reconstructed from the available sequence data. If the duplication of target sequences was not assumed, the STP1 attP site was determined to

be 5'-GGTTTTCAA-3" and when a single base pair target DNA duplication at each end of the STP1 insertion was assumed, the STP1 attP site would be 5'-GGTTCAA-3'. Larger target sequence duplications were not feasible. These alternative STP1 attP sites have been compared to other plasmidogenic element attP sites and to the mel integration site (Fig. 6.17a, 6.17b). With either of the assumptions (none or a 1 bp. duplication at each end of the insertion) there was significant sequence similarity between the derived STP1 attP site and the attP sites of other plasmidogenic elements and the mel integration site. The similarity was greater in the right hand part of the attP site shown (Fig. 6.17 a and b) and was improved when the sequence duplication was assumed (Fig. 6.17b). The seqence similarity implies that STP1 is related to the other plasmidogenic elements and that the integration of STP1 into the mel gene of pIJ702 may be the result of an aberrant form of the site specific recombination reaction which integrates this type of element into the attB sites. Should analysis of STP1 continue, the determination of the sequence of the STP1 attP site sequence and the sequence of the attB integration site are high priorities.

STP1 contains six unique restriction enzyme sites (6.3), five of which are also present in the pUC polylinker (New England Biolabs catalogue 1986/87). It would be relatively simple to subclone STP1 into pUC vectors using these sites to form a series of bifunctional plasmids which would allow the effect of disrupting STP1 sequences on the biology of the plasmid to be determined. If pGLW13 (4.10.2) were used the bifunctional plasmids would be marked with thiostrepton resistance. Such an analysis may locate the segments of the plasmid required for

Fig. 6.17

A Comparison of the STP1 *att*P Site with the *att*P Sites of Other Actinomycete Plasmidogenic Elements.



A) Assumes no duplication of target sequences.

B) Assuming a 1 bp. duplication at each end of the insertion.

site-specific integration into the S. lividans chromosome, pock formation and conjugation. In addition, STP1 derivatives marked with antibiotic resistance genes such as neo should be constructed to speed the selection of fusion plasmids. At present, the tsr gene of pIJ702 and the pocking phenotype of STP1 have to be analysed and found to be linked physically before the presence of a fusion plasmid could be verified. If STP1 were marked with a resistance gene such as neo, then the inheritance of both neomycin (STP1neo) and thiostrepton (pIJ702) resistances after transformation or conjugal mating would be indicative of fusion, rather than having to repatch the tsr colonies onto plasmid-free lawns of S. lividans in order to assay for pocking.

A great deal of work could be performed on STP1 to bring our knowledge about STP1 up to that available about other plasmidogenic elements. However, this may be a poor use of resources which may be better directed towards understanding the formation and breakdown of the fusion plasmids presented here, and how STP1 can be used as a tool.

Some evidence describing the presence of STP2 in S. thermonitrificans ISP5579 has been presented in this chapter (6.3, 6.9) and in retrospect also in chapter four (4.8). STP2 was present at low copy number and appeared to be relatively large. It was associated with the white spore phenotype found in some strains of S. lividans produced by the cotransformation and gel purification experiments (6.5, 6.8, 6.9). STP2 was not transferred to S. lividans by interspecific mating.

The plasmid biology of S. thermonitrificans is unremarkable. As might be expected, the plasmids STP1 and STP2

isolated from S. thermonitrificans possess similar characteristics to their mesophilic counterparts. The formation of the fusion plasmids by STP1 may merit further analysis, but it is possible that other plasmidogenic elements are also capable of this activity if the cotransformation experiment were repeated on them. The use of STP1 as another tool for either the insertional mutagenesis of cloned sequences, or as a method to stabilise clones in the absence of drug selection may be worthwhile.

Chapter 7. The Heterologous Expression of the Holomycin Biosynthetic Pathway at Elevated Temperature

7.1 Introduction

The primary aim of this work was to test the feasibility of expressing antibiotic production pathways, cloned from mesophilic streptomycetes, in a thermophilic species. In this way the process advantages expected from fermentation at elevated temperatures (1.4) could be used to improve antibiotic production.

7.2 A Summary of Streptomycete Antibiotic Biosynthetic Pathways Which Have Been Cloned and Expressed in Heterologous Hosts; Candidates for use in the Thermophilic Streptomyces Project.

Streptomyces genomes have been estimated to be between 1.5 and 2.0 times the size of the E. coli genome (Gladek and Zakryewska, 1984). The E. coli genome has been mapped physically and measures approximately 4,700 Kbp in length (Kohara et al., 1987) from which the size of a streptomycete genome may be infered to be between 6,550 and 9,400 Kbp in length. This may be compared with the estimated sizes of the haploid genomes of Saccharomyces (Botstein and Davis, 1982) and Aspergillus (Timberlake, 1978) of about 15,000 and 27,000 Kbp respectively. 340 E. coli genes on a total of 450 Kbp of DNA have now been sequenced and entered into the GenBank data base (ca. 1986) (Foley et al., 1986). Assuming that the percentage of coding DNA in E. coli and Streptomyces is similar, Streptomyces genomes probably contain approximately 5300 to 7100 genes. A portion of the streptomycete genome must be devoted to encoding the control

mechanisms and structural proteins etc. for the complex morphological differentiation that Streptomyces undergo during their life cycle. The rest of the relatively large genome could accomodate many other complex biosynthetic and degradative capabilities, such as the ability to produce antimicrobial and bioactive microbial compounds, in addition to the necessary primary metabolic pathways required for their existance.

The structural genes for antibiotic biosynthetic pathways, and often other linked DNA, deletes from the chromosome of the producing species to give deleton variants which do not produce the antibiotic and are often sensitive to the compounds (Cox et al., 1986; Hintermann et al., 1984; Butler et al., 1989). That such deletions were viable implied that the genes were not essential to the organism, nor was it reported that these deletion strains were less vigorous than the wild types. The ability to produce antibiotics, in the laboratory at least, was not advantageous to the producing organisms. Whether the ability to produce antibiotics would be advantageous in a more natural environment, or under what conditions the production of antibiotics (or bioactive microbial products) confers selective advantages over non-producers, are interesting questions, worthy of attention.

Clustering of genes involved in the biosynthesis of compounds which confer a selective advantage on the producer would increase the chance of the stable inheritance of that characteristic. Clustering would mininise the loss or independent transfer of part of the biosynthetic pathway. Close linkage of the resistance gene or genes to the biosynthetic pathway would likewise minimise the chance of loosing the

resistance gene whilst maintaining the biosynthetic capability, a lethal situation. In all antibiotic biosynthetic pathways studied to date where good mapping data is available, evidence for the clustering of the structural genes involved in the biosynthesis and in some cases the resistance genes have been found (Rudd and Hopwood, 1979; Rhodes et al., 1981; Hintermann et al., 1984; Malpartida and Hopwood, 1984; Chater and Bruton, 1985; Feitelson et al., 1985; Stanzak et al., 1986; Chen et al., 1986; Murakami et al., 1986; Stonesifer et al., 1986; Motamedi and Hutchinson, 1987; Vats et al., 1987). By analogy with characters with selective advantage in the Gram-negative bacteria, it would be reasonable to expect antibiotic biosynthetic pathways to be present on mobilisable genetic elements such as plasmids, transposons and temperate bacteriophages. In only one case has an antibiotic biosynthetic pathway been mapped to a plasmid which is well defined genetically; methylenomycin biosynthesis on SCP1 in S. coelicolor A3(2) (see below) (Kirby and Hopwood, 1977). Whilst there have been arguments that antibiotic biosynthetic pathway genes are clustered in order to control their expression (Motamedi and Hutchinson, 1987) there are examples of other streptomycete genes which also must be controlled, such as those required for differentiation, which are not clustered. Clearly clustering is not a prerequisite for gene control in Streptomyces and the above arguments of selection driving the close association of antibiotic production and resistance genes are favoured by the author.

Outwith the intellectual importance associated with the clustering of genes involved with antibiotic biosynthetic pathways there is a tremendous practical advantage associated

with clustering. If one gene could be cloned, then the adjacent DNA could be readily identified and analysed for the presence of genes involved in antibiotic biosynthesis. This approach has been used for nearly all the antibiotic biosynthetic pathways cloned (see below).

The Glasgow thermophilic Streptomyces project required the availability of cloned antibiotic biosynthetic pathways to test the feasibility of their heterologous expression at elevated temperatures. Such pathways are described below. During the course of the study new cloned pathways were published and now, at the end of the project, there are more cloned pathways available.

7.2.1 Actinorhodin

S. coelicolor A3(2) produces two pigmented antibiotics, one of which is actinorhodin. It belongs to the isochromanequinone group of antibiotics which are formed via a polyketide pathway (Rudd and Hopwood, 1979).

The genes encoding the entire biosynthetic pathway for the production of actinorhodin were cloned on a low-copy number vector, pIJ922 (Lydiate et al., 1985) by Malpartida and Hopwood (1984). They originally obtained two clones by complementation of S. coelicolor A3(2) act mutants. The clones contained overlapping inserts which between them complemented all seven classes of act mutants. A single insert able to complement all act mutants and which also encoded sufficient information to direct the biosynthesis of actinorhodin in S. parvulus, a non-producing host, was constructed in vitro (pIJ2303). Subcloning

experiments defined 25 Kbp of the 32 Kbp insert as being involved in actinorhodin biosynthesis (Malpartida and Hopwood, 1984). pIJ922 is a relatively large vector of 24 Kbp which when recombined with the insert (32 Kbp) in pIJ2303 results in a large plasmid (56 Kbp). The yield of plasmid DNA was fairly low when low-copy number vector DNA was prepared and such a large construct was more likely to encounter restriction problems when introduced into a heterologous host. Despite these disadvantages and because actinorhodin biosynthesis has been the model polyketide pathway to study and Malpartida and Hopwood have made the clone pIJ2303 available for use in this project, it was chosen as a target for introduction into S. thermonitrificans to investigate the feasibility of actinorhodin expression at elevated temperature.

7.2.2 Cephamycin C

Chen et al. (1986) have cloned the entire biosynthetic pathway for the production of cephamycin C, a beta-lactam antibiotic from S. cattleya. Instead of identifying the clone by complementation of mutants blocked in production of cephamycin C or analysing DNA contiguous to a cephamycin C resistance gene, they screened for direct production of cephamycin C in a heterologous host, S. lividans. They used a low-copy number vector, pIJ943 to clone large random segments of the S. cattleya genome. One of the 30,000 recombinant S. lividans transformants produced an antibiotic compound indistinguishable from cephamycin C. The insert DNA (29.3 Kbp) was sufficiently large to encode the nine steps thought to be involved in the biosynthetic pathway of precursors to cephamycin C.

The cephamycin C biosynthetic pathway offered no obvious advantages over the use of the actinorhodin biosynthetic pathway for use in the thermophilic Streptomyces project. Due to the lack of data about the clone eg. the architecture of the cephamycin C clone, the actinorhodin clone was chosen in preference. The availability of the cephamycin C clone was not determined.

7.2.3 Erythromycin

Erythromycin is a macrolide antibiotic produced by Saccharopolyspora erythraea (Labeda, 1987), formerly Streptomyces erythreus (Seno and Hutchinson, 1986). The erythromycin biosynthetic pathway is complex requiring as many as 30 steps. Genetic analysis of erythromycin non-producing mutants suggests that at least some of the mutants are clustered (Weber et al., 1985). An erythromycin resistance gene (ermE) from Saccharopolyspora erythraea was cloned and sequenced (Thompson et al., 1982a; Uchiyama and Weisblum, 1985; Bibb et al., 1986). Stanzak et al., (1986) used the cloned ermE as a probe to identify homologous sequences in an E. coli bifunctional cosmid library of Saccharopolyspora erythraea DNA. Cosmids which contained the erythromycin resistance gene also contained flanking DNA. One of the cosmids, pKC488, produced a macrolide antibiotic indistinguihable from erythromycin A when transformed into S. lividans. Thus all or most of the genes required for the formation of erythromycin A were clustered on the 35 Kbp of insert DNA present on pKC488. The single resistance gene was present near the middle of the insert. However, although the compound from the S. lividans/pKC488 recombinant appeared indistinguishable from erythromycin A its chemical structure was

not determined. The recombinant plasmid was unstable and could not be reisolated in an intact form from S. lividans/pKC488. The pKC488 plasmid was made available to the thermophilic Streptomyces project with the warning that it was probably unsuitable for our purposes (Seno pers. comm.), so the offer was declined.

7.2.4 Holomycin

Holomycin, which was first isolated from S. griseus (Ettlinger et al., 1959), is a member of the pyrrothine group of antibiotics. Holomycin and related componds which contain the pyrrolinonodithiole nucleus have been isolated from several Streptomyces spp. (Ettlinger et al., 1959; Okamura et al., 1977; Kenig and Reading, 1979) and have been loosely associated with streptomycetes which have the ability to produce beta-lactam compounds (Kenig and Reading, 1979). The mutation of holomycin-producing strains (Hol^+) to non-producing strains (Hol^-) has been empiracly implicated as a factor involved with the development of beta lactam overproducing strains of Streptomyces (Burnham pers. comm.), suggesting a link in their biosynthetic pathways.

Holomycin was studied briefly (Ettlinger et al., 1959) because of interest in its wide spectrum of activity. They recorded activity against Micrococcus pyrogenes var aureus (100), Streptococcus pyrogenes (1), Streptococcus faecalis (100), Escherichia coli (10), Salmonella schottmulleri (10), Pseudomonas aeruginosa (100), Klebsiella Type A (10), Vibrio cholerae el Tor (10), Mycobacterium tuberculosis (10), Candida vulgaris (100), Endomyces albicans (100), Entomoeba histolytica (10), Trichomonas

foetus (1); Numbers in brackets refer to ug.ml^{-1} of holomycin. However, holomycin has not found widespread use in medical or veterinary practice.

S. clavuligerus is one of the beta-lactam producing streptomycetes which also produce holomycin (Kirby, 1978). The genetics of holomycin production in S. clavuligerus was the subject of a doubtful paper by Kirby (1978). UV-induced mutants of S. clavuligerus which did not produce holomycin were constructed. Of ten Hol$^-$ mutants analysed, only one retained the Hol$^-$ phenotype on both solid and in liquid culture. The optimal growth conditions for holomycin production by S. clavuligerus were not used by Kirby. The effect of Hol$^-$ mutations on the production of other metabolites by S. clavuligerus was "investigated" and found to be minimal, implying that the biosynthesis of holomycin was distinct to that of other metabolites in this species (Kirby, 1978). However, neither the data nor the growth and assay procedures for this investigation were described. Kenig and Reading (1979) found that the growth conditions for the production of holomycin excluded the formation of other compounds. The single good Hol$^-$ mutant obtained by Kirby was mapped to the S. clavuligerus chromosome btween phe-1 and ade-1, at about 2°Clock on the genetic map.

Kenig and Reading (1979) purified holomycin from cuture broths of S. clavuligerus when grown in a chemically-defined medium. The beta lactams produced by S. clavuligerus (penicillin N, deacetoxycephalosporin C and the carbamoyloxymethyl analogue of cephalosporin C) were not detected in the S. clavuligerus culture broths under these conditions. Holomycin-producing cultures of S. clavuligerus may be extracted with butanol,

yielding a yellow butanol phase (Burnham, pers. comm.), from which the holomycin may be purified according to the methods of Kenig and Reading (1979).

Thus holomycin biosynthesis may be separated from beta-lactam production physiologically (Kenig and Reading, 1979) and perhaps genetically (Kirby, 1978), but holomycin non-producing strains may possess higher beta lactam titres than holomycin producers (Burnham pers. comm.).

The biosynthetic pathway for the formation of pyrrothine antibiotics has not been determined. The above arguments indicate that holomycin may be produced in the absence of beta lactam biosynthesis and that holomycin production in some way reduces the accumulation of the beta lactam because holomycin non-producers tend to have higher beta lactam titres. By inspection of the holomycin structure, a biosynthetic route via cystine may be suggested (Fig. 7.1a). Cystine would be formed by condensation of two cysteines via the formation of a disulphide bond. Cyclisation of the cystine molecule via a terminal carbonyl group and the distal amino group leads to a large ring structure. The remaining carbonyl group would have to be removed, involving the breakage of a single carbon-carbon bond and the formation of an internal carbon-carbon bond, creating the two rings present in holomycin. Finally acetylation of the amino group would lead to the formation of holomycin.

Cysteine is required for the formation of alpha-aminoadipyl cysteinyl valine (ACV) which is an intermediate in the biosynthesis of beta-lactam antibiotics (Martin et al., 1985). In Streptomyces spp. which produce both holomycin and a beta-

lactam there may be competition for the available cysteine. During holomycin production the rate of beta-lactam formation may be reduced because of this. This model would explain the empirical link between holomycin and beta-lactam biosynthesis (see above).

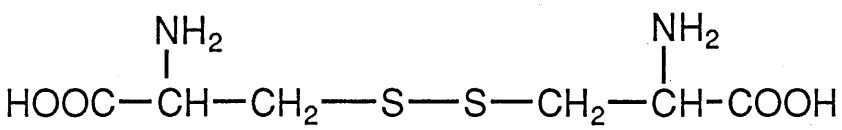
An alternative pathway which begins with a breakdown product of penicillin biosynthesis, N-acetyl- penicillamine, would link the formation of holomycin to the beta lactam biosynthetic pathway. Such a linkage could explain why holomycin production is associated with beta lactam producing streptomycetes. N-acetyl-penicillamine , which is present in penicillin producing organisms (Crooks, 1949), may combine with cysteine via the formation of a disulphide bond to form N-acetyl-penicillamine-cysteine disulphide (Fig. 7.1b). This molecule contains the acetylated amino group present in holomycin, whereas in the alternative biosynthetic (Fig. 7.1a) acetylation is a later step. Cyclisation of N-acetyl-penicillamine-cysteine disulphide by the same process as the alternative pathway produces an equally large ring structure. The formation of the two ring structure of holomycin is more problematic in this biosynthetic pathway. The central carbonyl group and two methyl groups must be removed and a carbon-carbon bond formed to produce holomycin. Thus three carbon-carbon bonds must be broken to form holomycin from N-acetyl-penicillamine. Energetically the former biosynthetic route (Fig. 7.1a) via cystine is preferable and implies that holomycin could be formed in Streptomyces which do not produce beta lactams.

It is of course possible that beta lactam and holomycin

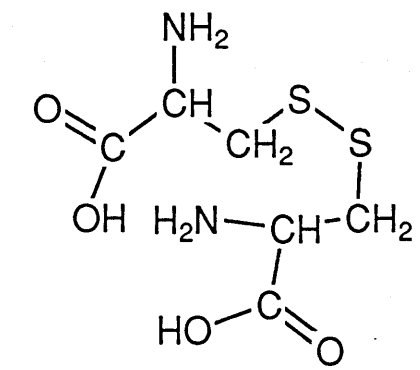
Fig 7.1a

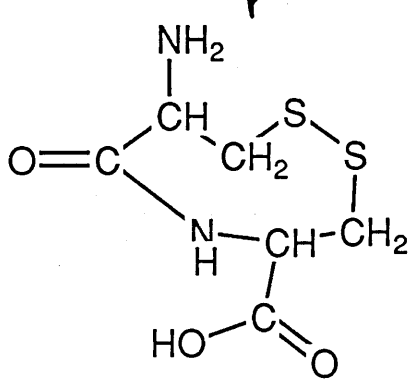
Proposed Alternative Pathways for Holomycin Production -via cysteine.

condensation of two cysteines to form cystine



cyclysation





break C-C bond to remove carbonyl
from internal C-C bond create 2 rings
acetylate amino group

Holomycin

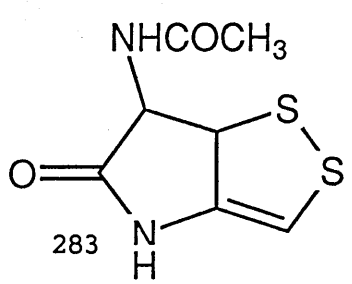
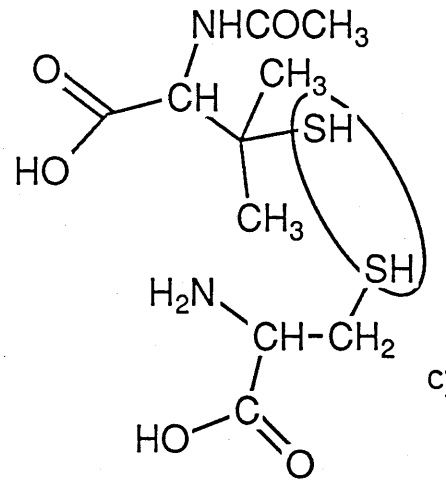


Fig 7.1b

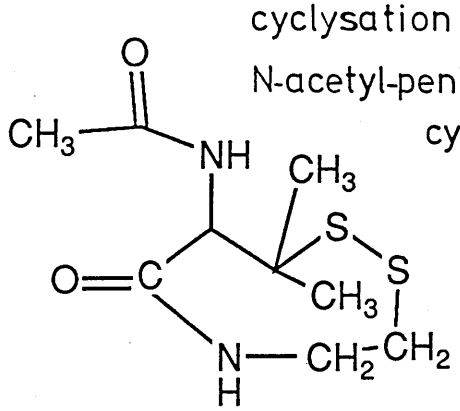
via β-lactam pathway

N-acetyl-penicillinamine



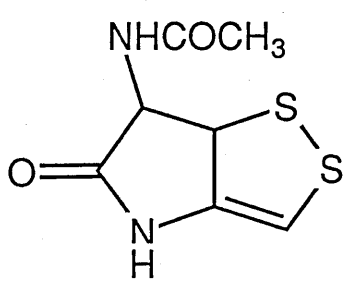
cysteine

cyclysation of
N-acetyl-penicillinamine
cysteine disulphide



central carbonyl and 2
methyl groups removed.
internal C-C bond formed.

Holomycin



production abilities arose in the same progenitor species and the relationship between the two compounds is an evolutionary rather than functional one.

Recently, a segment of DNA from S. clavuligerus was cloned which confers the ability to produce a yellow compound on S. lividans. The DNA was cloned using pIJ702-a high copy-number cloning vector suitable for S. thermonitrificans. The yellow compound formed by the S. lividans recombinant was typical of pyrrothine antibiotic compounds, suggesting that at least part or perhaps all of the holomycin biosynthetic pathway from S. clavuligerus had been cloned. The recombinant plasmid (pBROC139) was kindly made available to the thermophilic Streptomyces project by Dr. M. Burnham of Beechams PLC.

7.2.5 Methylenomycin

Methylenomycin is an epoxy-cyclopentane antibiotic produced by SCP1$^+$ strains of Streptomyces eg. S. coelicolor A3(2) and S. violaceus-ruber SANK95570 (Kirby and Hopwood, 1977; Aguilar and Hopwood, 1982). Methylenomycin is a diffusible growth inhibitor to which SCP1$^-$ strains of Streptomyces (Vivian, 1971) and a number of gram-positive bacteria (Kirby et al., 1975) are sensitive. Sixteen mutations, in 5 phenotypic classes, which were blocked in the biosynthesis of methylenomycin (mmy) were linked to SCP1 by the transfer of mmy to SCP1$^-$ recipient strains (Kirby and Hopwood, 1977). An actinophage vector 0C31 KC400 was used to clone genes involved in methylenomycin biosynthesis (Chater and Bruton, 1983). "Mutational cloning" was used to disrupt transcripts involved with methylenomycin biosynthesis by the integration of att site deleted phage. The phage contained

insert DNA entirely within the transriptional units to act as substrates for homologous recombination. Integration events were identified by selection for tsr expression (a tsr gene was present on the vector). Nine mutations were found in the cloning procedure, identifying 3 non-overlapping segments of DNA (7 Kbp in total) involved with methylenomycin biosynthesis. A fourth fragment encoding resistance to methylenomycin (mmr) was also cloned and was indistinguishable from a previous mmr clone (Bibb et al., 1980). Southern hybridisation experiments showed that the 3 mutagenic fragments were present on a contiguous 14 Kbp segment of SCP1 DNA.

Recently a fragment of SCP1 DNA encoding the entire methylenomycin biosynthetic pathway has been cloned on pIJ922 (Seno and Baltz, in press) and has been used to produce methylenomycin in SCP1$^-$ hosts. The clone was obtained too recently to be included in the thermophilic Streptomyces project, but as the insert is large (about 31 Kbp) in a large low-copy number vector, the clone offered no obvious advantages over the use of the actinorhodin clone pIJ2303.

7.2.6 Tetracenomycin

Tetracenomycin C (Tcm C) is a polyketide antibiotic of the anthracycline class produced by S. glaucescens (Motamedi et al., 1986). 34 mutants blocked in the biosynthesis of TcmC have been isolated and classified into ten complementation groups by consideration of their cosynthesis, their biotransformation properties and their accumulated products (Motamedi et al., 1986). Shotgun cloning of S. glaucescens DNA into the high-copy number pIJ702 and tranformation of tcmC mutants, identified two

plasmids containing inserts of 7 and 12 Kbp which both complemented class II mutants. The inserts contained a 6.3 Kbp common sequence which also complemented class II mutants. The library was used to transform S. lividans which is normaly sensitive to TcmC. Three recombinants were obtained which were resistant to TcmC. The six plasmids defined 24 Kbp of contiguous S. glaucescens DNA involved with the biosynthesis of TcmC. All 10 tcmC mutants were complemented by 2 or more of the 6 plasmids. Cofermentation of S. lividans recombinants containing plasmids pHM57 and pHM152, which between them span the whole 24 Kbp region of DNA analysed, resulted in the production of TcmC. Subcloning from pHM57 and pHM152 into pIJ702 and complementation of the tcmC mutants defined the structural organisation of the TcmC biosynthetic gene cluster. All the tcmC mutations were contained within a 14 Kbp section of DNA, with an apparent subclustering of genes involved with early, middle and late biosynthetic steps (Motamedi and Hutchinson, 1987; Rhodes et al., 1981).

This was the first report of the cloning of an antibiotic biosynthetic pathway on a high copy-number cloning vector in Streptomyces (albeit that the pathway was not available on one plasmid; two clones used in cosynthesis were required for heterologous expression of the TcmC pathway in S. lividans.). TcmC was a good candidate with which to test the feasibility of antibiotic production at elevated temperature. However, one of the clones pHM152 was unstable and as the transformation protocol demanded that clones be passaged through S. albus prior to their transformation into S. thermonitrificans the instability was thought to be a problem. Dr. R. Hutchinson kindly provided pHM57 and pHM152 for this study.

7.2.7 Undecylprodigiosin

Undecyprodigiosin is a red, non-diffusable pigment of S.. coelicolor A3(2), first observed in act mutants (Rudd and Hopwood, 1980). Mutants blocked in the biosynthesis of undecyprodigiosin (red) were found in five complementation groups (redA - redE), later redF was isolated (Rudd and Hopwood, 1980; Feitelson et al., 1985). The red mutants mapped to the chromosome of S. coelicolor A3(2) in a cluster. Cosynthesis studies between red mutants and also intergeneric cosynthesis studies between S. coelicolor A3(2) red mutants and Serratia maracescens mutants blocked in the biosynthesis of prodigiosin (a related compound) allowed the probable identification of the chemical blocks in some of the red mutants.

Shotgun cloning in high and low copy-number vectors identified 18 Kbp of DNA which restored undecylprodigiosin production to all the classes of red mutants and which resulted in the formation of undecylprodigiosin in S. parvulus, a non-producer (Seno and Baltz, in press).

These clones were obtained too recently to have been included in the thermophilic Streptomyces project and they offer no obvious advantage over the use of the actinorhodin clone as a model pathway. The availability of these clones was not determined.

7.3 Heterologous Expression of Antibiotic Biosynthetic Pathways in Streptomyces.

The rate-limiting steps in the heterologous expression of an antibiotic biosynthetic pathway are probably the initial cloning

of the entire pathway and the introduction of those genes into the required heterologous host. The Glasgow thermophilic Streptomyces project had avoided the first "bottleneck" by considering only those pathways which have been cloned and made available for use in this project. The second "bottleneck", that of the introduction of the cloned DNA, has been the main subject of this thesis.

There are two main reasons why cloned DNA may not be introduced into a heterologous host.

1) Host Range of the Vector: If the vector used in the cloning of the antibiotic biosynthetic pathway does not function in the host for heterologous expression ie. the host is not within the host range of the vector, then the cloned genes may not be stably introduced.

2) Restriction Endonucleases: If the host contains one or more efficient restriction endonucleases, recombinant DNA would probably be restricted on entry and transformation frequencies would be low (perhaps non-existant). If this were the case then the larger the recombinant plasmid the more likely that it would contain restriction endonuclease recognition sequences and thus be restricted. The choice of vector and cloned pathway for use in the feasibility studies ought to therefore include a consideration of the size of the recombinant plasmid. For this reason a number of different pathways ought to be used.

There are other possible limitations to the heterologous expression of antibiotic biosynthetic pathways which may become important as the taxonomic distance between the producer and the heterologous host increases.

Although many of the cloned pathways have been expressed in heterologous hosts such as S. lividans and S. parvulus (Malpartida and Hopwood, 1984; Chen et al., 1986; Stanzak et al., 1986; Motamedi and Hutchinson, 1987; Burnham pers. comm.; Seno and Baltz, in press), not all of the genes involved in the biosynthesis of the antibiotics were encoded on the cloned DNA. Host- encoded gene products such as RNA polymerase, sigma factors, ribosomal RNAs, ribosomal proteins etc. were also required for the expression of the cloned genes. If the antibiotic producing streptomycete and the heterologous host were taxonomically distant - it is conceivable that some host-encoded gene products might not interact correctly with the cloned sequences eg. the correct sigma factor may not be present, or although a promoter was recognised, it was not utilised at the correct level. If either were true then the antibiotic biosynthetic pathway would not be expressed usefully in the heterologous host. Furthermore, the temperature stability of the gene products involved in the biosynthesis of antibiotics from mesophilic streptomycetes must be questioned. If the proteins were thermolabile, then although the genes in the pathway were correctly expressed in a thermophilic heterologous host, the amount of active protein in the cells may be so low that the heterologous production of the antibiotic may not be detected. For this reason well characterised pathways, with known transcriptional maps were preferred.

Apart from the questions associated with the control of gene expression in a heterologous environment, the formation of antibiotics requires the condensation of precursor molecules into a larger structure with biological activity. The heterologous

host must contain these precursors in sufficient quantity for the expression of the cloned pathway to be efficient. In the absence of precursors, the expression of the genes from the cloned pathway would not be detectable by screening for antibiotic activity and northerns or S1 analysis would be required.

7.4 Construction of S. thermonitrificans C5/pBROC139

S. thermonitrificans C5 (4.6) was transformed with pBROC139, the pIJ702 derived plasmid containing the holomycin biosynthetic pathway from S. clavuligerus (7.2.4). pBROC139 was prepared from S. albusG to improve the transformation quality of the DNA (4.4). Thiostrepton resistant transformants were analysed for their ability to secrete holomycin (a yellow compound) into the media.

7.5 Expression of the Holomycin Production Pathway at Elevated Temperature from S. thermonitrificans C5 pBROC139.

S. clavuligerus is normally grown at 26°C. and grows poorly at 30°C. Expression of the S. clavuligerus holomycin production pathway cloned on pBROC139 at temperatures in excess of 30°C. would therefore be considered to be elevated.

S. thermonitrificans C5 pBROC139 was grown at 37°C. in Luria-Bertani (LB) broth containing thiostrepton to select for pBROC139. The mel phenotype of pIJ702 was not expressed in S. thermonitrificans ISP5579 (4.3.2), allowing pIJ702 to be used as a control. If the mel gene had been expressed the broth would have been coloured due to the formation of melanin. The mel inactivity left the broth light in colour allowing the detection of holomycin production.

After overnight incubation supernatant from cultures of S.

thermonitrificans C5 pBROC139 and C5 pIJ702 were compared by spectrophotometry using the C5 pIJ702 supernatant as a blank (Fig. 7.2). S. thermonitrificans C5 pBROC139 clearly alters the absorption spectra of the culture filtrate. The higher level of absorption at about 370 nM indicated that the insert in pBROC139 directed the synthesis of a compound at 37°C. As the insert was known to contain the holomycin biosynthetic pathway (7.2.4) the yellow pBROC139 dependent compound produced was assumed to be holomycin.

7.6 Preliminary Characterisation of the Yellow Compound

Holomycin can be purified from culture filtrates according to the method of Kenig and Reading (1979). The initial small scale isolation of holomycin was by the phase partitioning of holomycin into butanol (M. Burnham pers. comm.). Attempts to partition a yellow compound from LB filtrates of S. thermonitrificans C5 pBROC139 into butanol were unsuccessful. A possible explanation for this observation would be if the yellow compound was not holomycin and was instead either an unrelated compound or a related but not completely formed molecule. This observation implied that either pBROC139 did not contain the holomcycin production pathway, that the pathway was incomplete or that the pathway was complete, but incompletely expressed. The structure of the pBROC139 dependent compound should be identified in future work.

7.7 Discussion

The primary aim of the Glasgow Thermophilic Streptomyces project was to test the feasibility of the heterologous expression of antibiotic production pathways, cloned from

Fig. 7.2

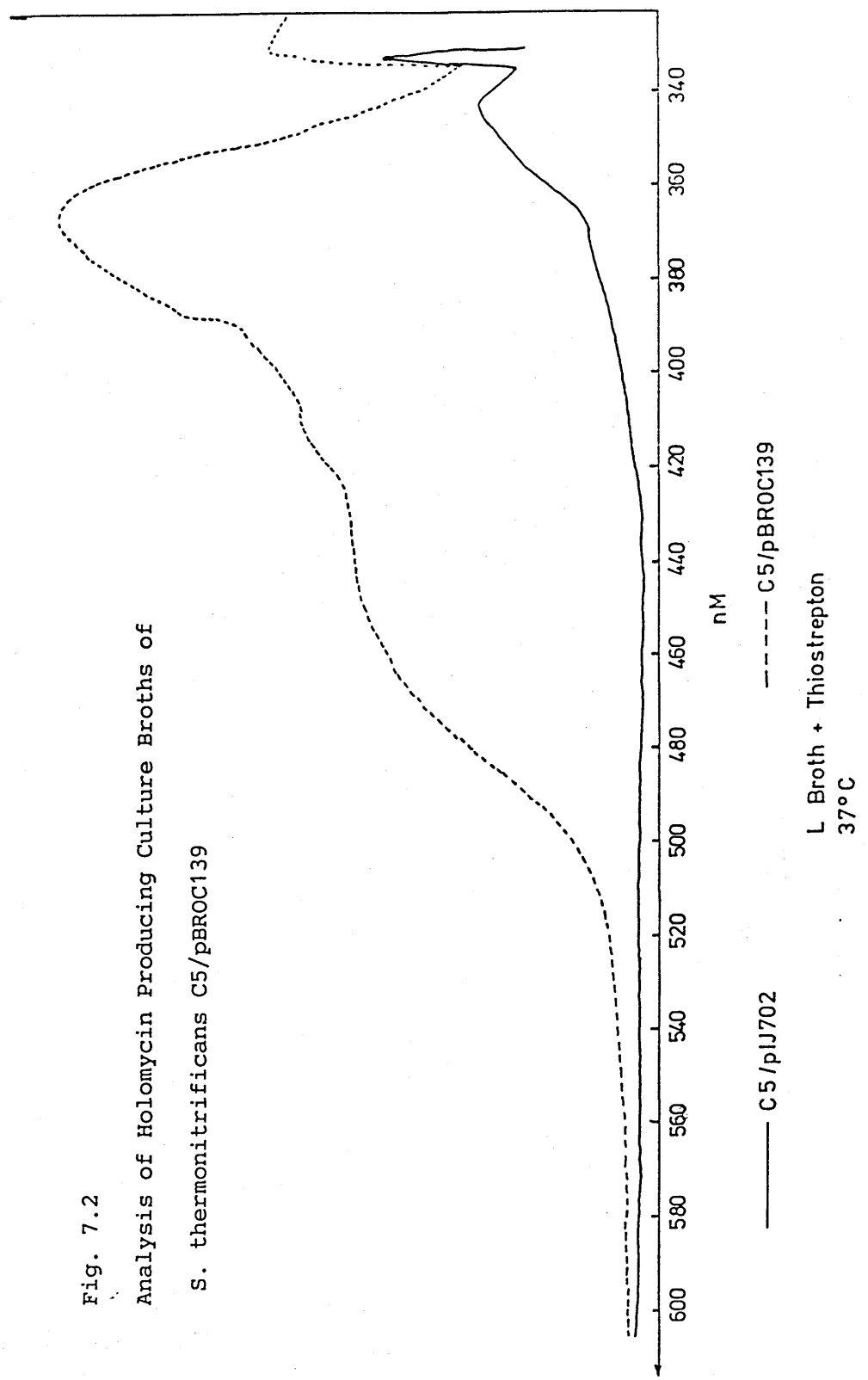


Fig. 7.2
Analysis of Holomycin Producing Culture Broths of
S. thermonitrificans C5/pBROC139

mesophilic species, in a thermophilic species. The bulk of this thesis describes work which was necessary to develop the methodology required to fulfill the project aims. During this work the number of antibiotic production pathways which have been cloned from mesophilic streptomycetes has steadily increased and a summary of these has been provided. Two in particular, actinorhodin and holomycin, were viewed as suitable candidates for transfer into S. thermonitrificans. Preliminary attempts to transfer the actinorhodin biosynthetic pathway into S. thermonitrificans were unsuccessful. This was due to instability of the pIJ2303 clone provided by John Innes (data not shown). The holomycin production pathway was transferred into S. thermonitrificans C5 (7.4). The transformants produced a yellow compound at 37°C. which was similar in colour to holomycin produced by S. clavuligerus (Burnham pers. comm.). However the yellow compound did not have the same chemical properties as holomycin (7.6). The pBROC139 clone produced the same yellow compound in S. lividans (data not shown) implying that the differences between the yellow compound and holomycin were clone- and not species- or temperature specific.

The importance of the expression of genes involved in secondary metabolism from S. clavuligerus in S. thermonitrificans at 37°C. is that the genes and proteins are active in a heterologous host at a temperature higher than the parental species grows. S. clavuligerus is normally grown at 26°C. More recently the yellow compound has been expressed at 45°C. in S. thermonitrificans grown in minimal medium, proving the feasibility of expressing antibiotic production genes at elevated temperatures (Burke pers. comm).

Now that the heterologous expression of mesophilic antibiotic production genes at elevated temperature has been proven, the system needs to be tested with an antibiotic production pathway which produces either a commercially important compound (such as oxytetracycline, Binnie et al., 1989), or a structurally similar compound (eg. actinorhodin). The problems encountered with the actinorhodin biosynthetic clone (pIJ2303) may have been peculiar to the isolate received from the John Innes Institute. Alternatively pIJ2303 may not have been transformed into S. thermonitrificans because of the presence of restriction endonucleases. S. thermonitrificans C51 which was the least restricting isolate constructed in this work could not be transformed either. Methods have been suggested for the isolation of improved restrictionless mutants of S. thermonitrificans (5.9).

When the actinorhodin or another academically or industrially-important biosynthetic pathway has been transferred into S. thermonitrifcans the high temperature expression system would need to be tested at industrial scale so that the cooling characteristics and product yield of the fermentation could be analysed. Only then would we know whether this fermentation system was economically advantageous. This would require the collaboration of industry which, fortunately and with my thanks, has not been shortcoming over the past years.

Chapter 8

8.0 Concluding Remarks

The purpose of the Glasgow thermophilic Streptomyces project was to test the feasibility of expressing antibiotic biosynthetic pathways cloned from mesophiles in a thermophilic species. It may then be possible to investigate whether the process advantages expected from thermophilic bioprocesses may be applicable to recombinant thermophilic antibiotic fermentations at industrial scale.

The immediate aims of this thesis were to identify a suitable thermophilic Streptomyces species and to develop methods for its transformation by plasmid DNA. Chapters Three and Four describe how this goal was achieved. S. thermonitrificans can now be reproducibly transformed at frequencies of about $1x10^3$ transformants per ug. of pIJ702 DNA.

The transformation methodology allowed the construction of S. thermonitrificans pBROC139; A strain of S. thermonitrificans containing a plasmid which encodes the ability to form a yellow compound thought to be closely related to holomycin. The holomycin production pathway was cloned from S. clavuligerus, a mesophilic streptomycete, which is normally grown at 26°C. (M. Burnham, pers. comm.). S. clavuligerus does not grow above 30°C. S. thermonitrificans pBROC139 produced a yellow compound at 37°C and more recently at 45°C (F. Burke, pers. comm.) proving that genes involved in antibiotic biosynthesis from mesophilic species can be expressed at temperatures higher than the parental species can survive. Thus the long term aim of the Glasgow thermophilic Streptomyces project, testing the feasibility of the

concept, has been achieved. The results presented in this thesis prove that it is therefore worthy of further funding to see whether the methodology developed here may be applied to economically important compounds and fermentations.

Outwith the commercial importance of this work lies an ethical responsibility to ensure that the enhanced cooling characteristics of the recombinant thermophilic fermentation system developed here are made available to the lesser well developed countries of the world, which are frequently warmer than Great Britain. In such countries the reduced coolant requirement of this fermentation system may allow the production of antibiotics where they were previously impossible to make. This seemingly charitable act may seem counter to the economic interests of the antibiotic manufacturers but I think one can argue strongly for such a transfer of technology.

In addition to these results the molecular biology of S. thermonitrificans was studied at a wider level so that a description of its plasmid (chapter 6) and actinophage (chapter 5) biology might be made. S. thermonitrificans contains a plasmidogenic conjugative transposable element STP1, which has properties similar to other streptomycete elements. However, a property unique to this type of Streptomyces element is STP1's ability to transpose into plasmids as shown in chapter 6. The isolation of a thermophilic actinophage TA provides further evidence that many Streptomyces genes may be expressed at temperatures higher than they are normally expressed. Scottish soil, the inoculum used for actinophage isolation, is rarely expected to reach 45-50°C. but TA plaques at these temperatures.

All the evidence to date suggests that it is the thermotolerance of the species in which the genes are being expressed rather than the thermotolerancy of the parental species which tends to determine whether the genes will be correctly expressed at elevated temperature.

Within the discussion sections at the end of each chapter, suggestions for future experiments may be found. However, these suggestions relate to the particular subjects of the chapters and not to the future direction of the thermophilic Streptomyces project. The development of media and solutions for the efficient transformation of S. thermonitrificans should not cease because improvement is an ongoing process. The alternative medium P-like solutions ought to be tested to investigate whether their use improves transformation efficiency. The regeneration medium itself could probably still be improved, perhaps by removing the carbohydrate (mannitol) and optimising the divalent cations before the re-introduction of an osmotically buffering carbohydrate at a lower concentration.

In retrospect, had an intermediate stage to the project been included more publishable results might have been generated. In particular if the high temperature recombinant fermentation system had been tested using the heterologous expression of a protein, such as a thermostable protease to prove the usefulness of the system, more serious industrial interest may have been forthcoming. Unfortunately, time constraints did not allow such thoroughness in proving the system if the final goals of the project were to be achieved. Nevertheless the development of a minimal medium for S. thermonitrificans (F. Burke, pers. comm.) allows many physiological experiments studying the mechanisms of

thermophily or the production of single or multiple gene products to be investigated over a wide range of temperature in both complex and minimal medium using low and high copy number cloning vectors.

Cloned antibiotic production pathway genes for commercially important compounds such as oxytetracycline should be used to investigate whether S. thermonitrificans can be used produce this class of compounds (polyketide) at elevated temperature. The future of the thermotolerant Streptomyces project really depends on the success of this or similar experiments with pathways of commercial importance.

It would be reasonable to describe the project as successful, in terms of both results and in the production of a host-vector system for the heterologous expression of cloned genes at a higher and more efficient fermentation temperature.

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