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Molecular Genetics of Tryptophan Biosynthesis

in *Streptomyces venezuelae* ISP5230.

by

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Submitted in partial fulfillment of the requirements
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at

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To Aai and Baba

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ABSTRACT

From their growth requirements, the metabolic intermediates accumulated, and the enzymes present, ten auxotrophs of Streptomyces venezuelae and two of Streptomyces lividans were characterized and their trp mutations determined. All classes of trp mutations except trpF, trpE, and trpG were present. When considered with the relative location of trp mutations obtained in this and earlier studies, the results showed that in S. venezuelae trpC, trpB, and trpA are clustered near hisA and hisB while trpD is at another location near nicB. Fragments of S. venezuelae DNA encoding trpC and trpD were cloned by complementation of relevant S. lividans auxotrophs. A trpE-encoding DNA fragment was cloned by complementation of an E. coli trpE auxotroph. Growth studies and enzyme assays indicated that trpG was clustered with trpE on a 2.4-kb DNA fragment. By developing a technique for integration of a vector carrying a resistance marker and homologous DNA of the gene under study, and in conjunction with "classical" genetic analysis, trpEG was mapped close to but separate from the trpCBA cluster. The arrangement of trp genes found in S. venezuelae is unique among bacteria. An attempt was made to introduce a trpE or trpG mutation by in vitro modification of the cloned 2.4-kb DNA fragment containing trpEG and introduction of the modified fragment into the host chromosome by allele replacement. Although DNA replacement occurred, it did not result in tryptophan auxotrophy.

ABBREVIATIONS AND SYMBOLS

g	grams
mg	milligrams
µg	micrograms
l	litres
mL	millilitres
µL	microlitres
h	hours
min	minutes
s	seconds
M	molar
mM	millimolar
µM	micromolar
nmol	nanomoles
g/l	grams per litre
w/v	weight per volume
v/v	volume per volume
°C	degrees Celsius
u	units
cfu	colony forming units
pfu	plaque forming units
SDS	sodium dodecyl sulfate
TE	Tris-EDTA buffer
EDTA	ethylenediamine tetraacetic acid
Tris	tris-hydroxymethylaminomethane

TES	tris-(hydroxymethyl)methylaminoethanesulfonic acid
PEG	polyethylene glycol
DTT	dithiothreitol
ATP	adenosine 5′-phosphate
AS	anthranilate synthetase
GAT	glutamine amidotransferase
MM	minimal medium
AA	anthranilate
PABA	p-aminobenzoate
CDRP	1-(carboxyphenylamino)-1-deoxyribulose-5-phosphate
InGP	indoleglycerol phosphate
InG	indoleglycerol
PRA	N-phosphoribosylanthranilate
Trp	tryptophan

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INTRODUCTION

Tryptophan is an amino acid that is required for protein biosynthesis in all organisms. Animals obtain it from their diet but it is produced in microorganisms and plants by a highly conserved pathway. This pathway is well characterized and has been examined in a wide variety of microorganisms. Although every group of bacteria has adopted its own individual strategy to synthesize tryptophan, synthesis occurs via essentially the same reaction sequence. The different strategies vary over a wide spectrum and are reflected in the organization of tryptophan biosynthesis genes on the chromosome. They range from an operon-like arrangement on one hand to a more dispersed pattern with varying degrees of separation and various gene combinations on the other; every arrangement has a characteristic regulatory mechanism associated with it. Since all present-day gene arrangements have presumably evolved from a common ancestral one, a comparative study of these arrangements in diverse groups of bacteria should provide an indication of evolutionary relationships. Close analyses should reveal the kinds of chromosomal rearrangements that have taken place after divergence of the species.

Streptomycetes belong to the high G+C subdivision of the Gram-positive class of eubacteria (Woese, 1987). A study of trp gene organization in these organisms is attractive because

they are evolutionarily distant from the bacteria in which trp gene organization has been studied in detail so far. In only one other member of the high G+C Gram-positive sub-division, Brevibacterium lactofermentum, has trp gene arrangement been fully investigated; it was found to be operon-like (Matsui et al, 1986). Preliminary studies have indicated that the arrangement in streptomycetes is, in contrast, a dispersed one (Smithers and Engel, 1974; Stuttard, 1983a). A more detailed comparison of the structure and organization of trp genes in these two types of organisms is likely to provide insights into their relatedness and into the mechanisms by which their gene arangements evolved.

In streptomycetes the life cycle is developmentally programmed through vegetative growth, which consists of elongating and branching hyphae, to differentiation by formation of aerial branches that septate to form spore chains. This morphological development is usually coupled with production of a wide variety of secondary metabolites. These organisms are also attractive for the study of trp gene organization because they provide an excellent model system in which to compare the assembly and regulation of genes for primary metabolism, secondary metabolism and differentiation. Although much is known about the organization and regulation of genes involved in differentiation and antibiotic production, the genetics of primary metabolism has not yet been extensively investigated.

Among streptomycetes, S. venezuelae was chosen for two reasons: firstly, because it possesses a generalized transduction system that can be used to study gene organization at a fine structure level; secondly, because chorismic acid is the branch-point intermediate leading to the biosynthesis of tryptophan as well as to the antibiotic chloramphenicol. Knowledge gained about the gene organization and regulation of tryptophan biosynthesis should shed some light on the control mechanisms that regulate the flow of chorismic acid into these separate pathways.

Stuttard (1983a) initiated the study of trp gene organization in S. venezuelae by isolating trp auxotrophs and demonstrating, by cotransduction, at least two chromosomal locations for trp genes. The present study was designed to extend this initial information in addition by:

a) identifying the biochemical lesions present in the trp auxotrophs used in the earlier cotransduction experiments (Stuttard, 1983a; Stuttard et al., 1987; Stuttard, 1988) thereby establishing conclusively which trp genes belonged to the two linkage groups, and

b) isolating and characterizing other trp mutations and mapping them on the chromosome.

LITERATURE REVIEW

I. TRYPTOPHAN BIOSYNTHESIS : A COMMON PATHWAY

Tryptophan biosynthesis begins from chorismic acid, the branch-point intermediate in the shikimic acid pathway for aromatic amino acid biosynthesis, and thus the common precursor of all aromatic protein amino acids as well as of aromatic components in folic acid and ubiquinones. The biosynthesis of tryptophan requires five reactions and involves seven enzymatic activities (Figure 1). The pathway is highly conserved and all microorganisms examined so far use essentially the same reaction sequence. Even among widely divergent groups of bacteria there is a great deal of similarity in the structure and function of the enzymes involved (Crawford, 1975; 1989).

The first reaction is the conversion of chorismate and glutamine to anthranilate and pyruvate; it is catalysed by anthranilate synthetase, a multienzyme complex (Zalkin, 1980). In all bacteria, this complex consists of two nonidentical subunits referred to as ASI, the bigger subunit encoded by trpE, and ASII, the smaller one encoded by trpG. ASI has the binding site for chorismate while ASII is a glutamine amidotransferase (GAT). It binds glutamine and catalyzes transfer of the amido group to the active site of ASI. In the absence of ASII, ASI cannot use glutamine as the amido donor to convert chorismate to anthranilate. However, it can

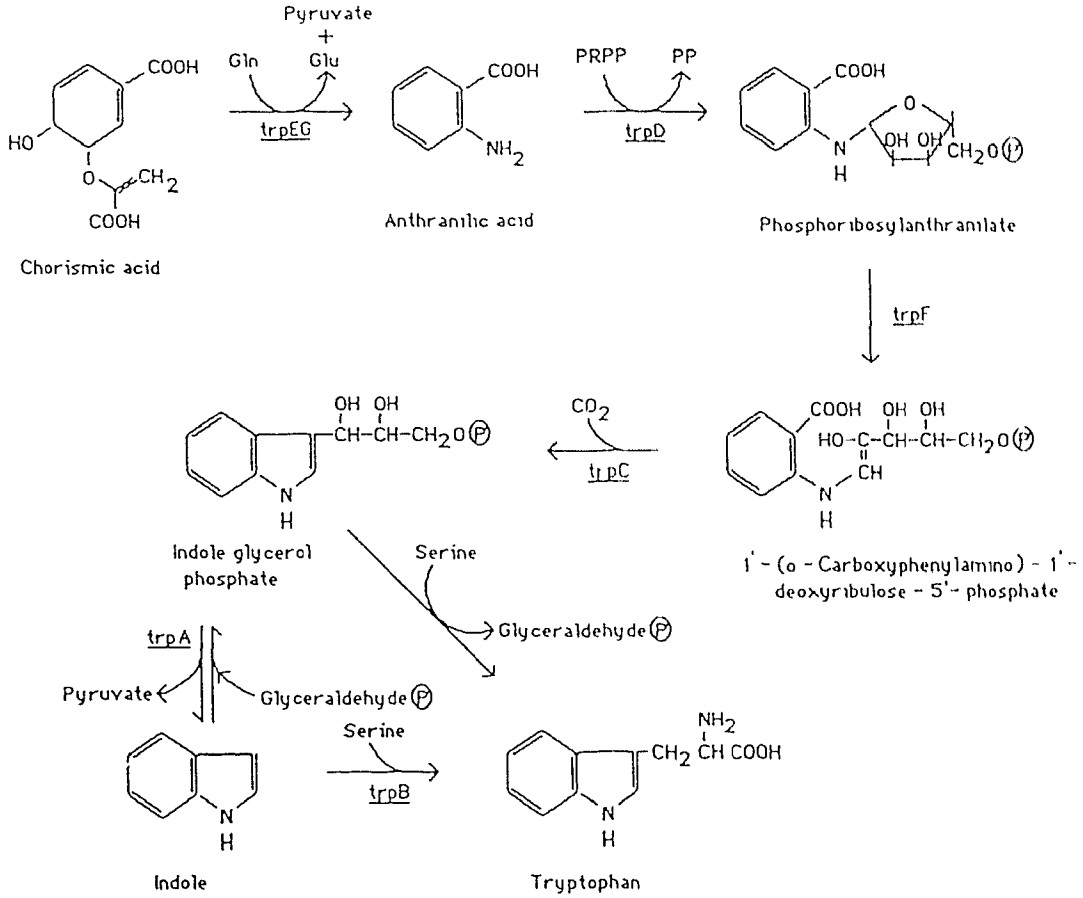


Figure 1. The tryptophan biosynthetic pathway.

catalyze this conversion in the presence of high concentrations of ammonia, although with less efficiency. Strainsthat lack either ASI or both ASI and ASII functions show a Trp^- phenotype on minimal medium (MM), while those that lack only ASII will grow on MM in the presence of high concentrations of ammonium. Zalkin (1975) devised a selective medium to distinguish between wild-type strains and those defective in ASII but possessing a functional ASI. It contained a low concentration of ammonium, which allowed strains that possessed only ASI to carry out tryptophan biosynthesis and grow at pH 8, where about 5% of ammonium salts are undissociated and could be assimilated. This medium did not allow such strains to grow at pH 6, where ammonium exists mostly as NH_4^+ and is not available for tryptophan biosynthesis. In all bacteria, feedback inhibition of anthranilate synthetase by tryptophan is used as a mechanism for regulating the pathway (Zalkin, 1980).

The second reaction in the tryptophan pathway is the conversion of anthranilate and 5-phosphoribosylpyrophosphate to N-phosphoribosyl anthranilate (PRA), catalyzed by phosphoribosyltransferase, a gene product of trpD. In the third step PRA is rearranged in the presence of phosphoribosyl-isomerase, encoded by trpF, to form 1-(o-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CDRP). The fourth step is the conversion of CDRP to indoleglycerol phosphate (InGP). It is catalyzed by trpC-encoded InGP synthase and involves

decarboxylation and ring closure.

The last step is the conversion of InGP to tryptophan. It is catalyzed by tryptophan synthetase, which in all bacteria is a tetramer consisting of two α (trpA-encoded) and two β (trpB-encoded) polypeptides; the α polypeptide catalyzes conversion of InGP to indole, while the β subunit has the ability to convert indole and serine to tryptophan. The reaction is peculiar in two respects : first, both conversions take place at a much higher rate when the subunits interact in a complex - the activity of each subunit decreases considerably in the absence of the other; and second, free indole is not an intermediate in the reaction. These observations are explained by the structure of the enzyme which has the active sites on the α and β subunits connected by an intramolecular tunnel through which indole is channelled (Hyde et al., 1988).

The pathway thus involves both phosphorylated (PRA, CDRP, InGP) and nonphosphorylated (anthranilate, indole) intermediates. Depending on the nature and the site of mutation, tryptophan-requiring auxotrophs accumulate intermediate/s prior to the block. The intermediate CDRP is generally accumulated only in the dephosphorylated form, while PRA is unstable and forms anthranilate. When exogenously supplied, anthranilate and indole, but not CDRP, InGP, nor their dephosphorylated forms, can be taken up and used for tryptophan biosynthesis (Crawford, 1975).

Despite the common features of tryptophan biosynthesis in various bacteria, there are differences in the structure of some pathway enzymes, and in the involvement of the first enzyme in the biosynthesis of other metabolites. Also, there is great diversity in the chromosomal organization and regulation of the genes encoding the pathway enzymes.

II. ENZYME DIVERSITY

In some bacteria, certain pathway enzymes are bifunctional proteins; whereas in a majority of species the catalytic functions are performed by two separate polypeptides. The first of these bifunctional enzymes was discovered simultaneously in E. coli and Salmonella typhimurium. The mutations in a class of auxotrophs unable to produce CDRP (trpF class) were mapped at the same locus as the mutations in a class that accumulated CDR (trpC class) (Smith and Yanofsky, 1960). Later, during enzyme purification, Creighton and Yanofsky (1966) found that InGP synthase-PRA isomerase, TrpC(F) activities were associated with a protein that consisted of a single polypeptide chain but exhibited two separate domains, the amino terminal one responsible for TrpC, and the carboxy terminal one responsible for TrpF activity (Smith, 196[illegible] Although covalently linked, the domains do not depend on each other for their individual functions. Missense mutations blocked in only one of the enzyme activities can be isolated (Smith, 1967; Pittard, 1987). Moreover, using a

plasmid merodiploid system Yanofsky et al. (1971) observed complementation between pairs of such missense mutants blocked in only one of the two activities. Finally, the purified protein can be separated by limited proteolysis into two separate polypeptides, one of which has TrpC and the other TrpF activity (Kirchner et al., 1980). An important feature of the fused TrpC(F) polypeptide and the corresponding trpC(F) gene is its presence without exception in all enteric bacteria that have been examined (Crawford, 1989). However, trpC(F) gene fusion is not limited to the enteric bacteria since it is present in Brevibacterium lactofermentum, a Gram-positive bacterium (Matsui et al., 1986); there are no reports describing the fused TrpC(F) polypeptide in B. lactofermentum.

A second well-characterized example of a bifunctional polypeptide is that carrying anthranilate synthetase-glutamine amidotransferase and phosphoribosyltransferase (Trp(G)D) activities. It is found in only one group of enteric bacteria, represented by E. coli and S. typhimurium. In other bacteria, represented by Serratia sp. and Aeromonas sp., the two activities are present on separate polypeptides. The bifunctional Trp(G)D polypeptide can be dissociated into two components by limited proteolysis, the amino-terminal third retaining the TrpG (ASII) activity and the carboxy-terminal two thirds exhibiting the TrpD activity (Hwang and Zalkin, 1971). As with the fused TrpC(F) polypeptide, there is no functional interaction between the two catalytic domains.

Missense mutations blocked in only one of the two activities can be isolated (Yanofsky et al., 1971).

A third example of a bifunctional enzyme is the TrpE(G) recently discovered in Rhizobium meliloti (Bae and Crawford, 1990). In this polypeptide both subunits of anthranilate synthetase are fused. The situation thus differs from that in TrpC(F) and Trp(G)D since cooperative interaction between the two catalytic domains on the fused polypeptide is expected. In all other organisms, the TrpE and TrpG subunits are present as separate polypeptides; however, they do interact in a multienzyme complex to catalyze the formation of anthranilate.

The catalytic machinery of the tryptophan pathway also shows variation in the nature and subunit composition of the anthranilate synthetase complex, as well as in its involvement with other biosynthetic pathways. Anthranilate synthetases can be classified as bifunctional in organisms where TrpG-TrpD activities are on a fused polypeptide, while in other organisms where TrpG and TrpD are separate they are monofunctional(Zalkin, 1980). The bifunctional enzymes such as those of E. coli, S. typhimurium and Citrobacter freundii, are tetrameric. The complex is $(ASI)_2$ $(ASII\text{-}TrpD)_2$; the subunits ASI and ASII-TrpD bind very tightly and dissociate in vitro only under stringent conditions (Zalkin,1980).

Monofunctional anthranilate synthetases may be present as dimeric enzymes, (ASI) (ASII), as in Acinetobacter calcoaceticus, Pseudomonas aeruginosa, Pseudomonas putida,

Bacillus subtilis and Bacillus pumilus, or they may be present as a tetrameric complex, $(ASI)_2$ $(ASII)_2$ as in Proteus sp. and Serratia sp. In dimeric enzymes, the subunit interaction is less avid than in the tetrameric ones, and they undergo facile association-dissociation in vitro. This reversibility in bacteria like B. subtilis, A. calcoaceticus and Pseudomonas acidovorans is correlated with the ability of ASII to also participate in the first reaction of p-aminobenzoate (PABA) biosynthesis. In bacteria, the first reaction in the biosynthesis of PABA is catalyzed by PABA synthetase, a multimeric enzyme which is very similar to anthranilate synthetase in its substrate requirement and enzyme composition. Like anthranilate synthetase, PABA synthetase requires chorismate and either ammonia or glutamine as the amino donor, and it consists of two nonidentical subunits. The smaller subunit (PabA) contains the glutamine amidotransferase activity; by mediating NH_2 transfer to the bigger subunit (PabB), it makes glutamine-dependent synthesis possible. PabB posseses the chorismate binding domain and alone can catalyze ammonia-dependent synthesis (Slock et al., 1990).

In B. subtilis, A. calcoaceticus and P. acidovorans, PABA synthetase and anthranilate synthetase share a common smaller subunit, referred to as amphibolic glutamine amidotransferase. TrpG in these organisms is capable of transferring the amido group from glutamine to either ASI-chorismate or PabB-chorismate. Thus auxotrophs in which it is

defective have a requirement for both tryptophan and PABA (Crawford, 1975). In all other bacteria examined so far, including enterics, there are separate glutamine amidotransferases for the biosynthesis of PABA and anthranilate. Also, neither ASI and PabA nor PabB and ASII interact productively to catalyze anthranilate and PABA formation, respectively. Nevertheless in E. coli (Goncharoff and Nichols, 1984), B. subtilis (Slock et al., 1990) and other bacteria, there is a considerable sequence similarity between the genes for anthranilate synthetase and the corresponding genes for PABA synthetase.

Diversity in the function of anthranilate synthetase extends to one member of the fluorescent pseudomonads, P. aeruginosa, which uniquely posseses two sets of this enzyme. Genes for both sets have been cloned and sequenced (Essar et al., 1990a). Isolation and characterization of specific mutations by insertional inactivation of these genes identified their functions. One set, trpE and trpG, is involved specifically in tryptophan biosynthesis while the other, phnA and phnB, directs production of a phenazine pigment, pyocyanin. Phenazines are secondary metabolites derived from anthranilate. PhnA is comparable to the bigger subunit TrpE, while PhnB, like TrpG, is a glutamine amidotransferase. The components of both sets of genes were capable of complementing the appropriate E. coli trpE and trpE(G) mutants, and under certain conditions the two sets

were functionally interchangeable. Thus insertionally inactivated phnA mutants still retained a low level of phenazine production whereas production completely ceased in phnA, trpE double mutants. This suggested that the tryptophan specific enzyme could provide some anthranilate for phenazine biosynthesis. Because trpE mutants did not grow on minimal medium, phnAB gene products evidently do not participate in tryptophan biosynthesis under normal conditions. However, Trp$^+$ revertants in which all the anthranilate synthetase activity was due to phnAB gene products arose spontaneously. Northern blotting indicated that under normal circumstances, phnAB mRNA increased through the growth phase but was maximal at the stationary phase, as might have been expected for a secondary metabolic enzyme; in contrast, trpG specific mRNA was maximal during early log phase.

III. DIVERSITY OF GENE ORGANIZATION

Examination of seven divergent subdivisions of eubacteria suggests that every major group of bacteria has its own unique arrangement of tryptophan biosynthetic pathway genes (Crawford, 1975; 1980; 1989). The disposition of genes on the chromosome ranges from a single cluster to a dispersed pattern (Figure 2). The information on which these conclusions are based has come from standard recombination analysis of trp

mutations using procedures such as transformation, conjugation and transduction. In many cases also, the genes have been cloned by complementation of trp auxotrophs and their nucleotide sequences have been analysed (Crawford, 1989).

In enteric bacteria, all trp genes are clustered at a single locus on the chromosome to form an operon which is under the control of a single regulatory region (Yanofsky and Crawford, 1987). The trp operons of E. coli and S. typhimurium trp were the first to be discovered and still remain the most completely studied. They were elucidated by transductional analysis of auxotrophic mutations using P1 and P22 phages (Yanofsky and Lennox, 1959; Demerec and Hartman, 1956). The entire operon in both organisms has been cloned, sequenced and thoroughly analysed (Yanofsky and Crawford, 1987). Interestingly, the trp operons in E. coli and S. typhimurium are transcribed in opposite directions with respect to chromosomal replication due to the location of the S. typhimurium trp operon within an inversion encompassing about 10-11% of the chromosome (Riley and Sanderson, 1990).
Other enteric bacteria in which the trp genes have been studied also show indications of an operon structure, e.g., the presence of polarity and coordinate regulation (Crawford, 1975). In many species where DNA fragments, including both structural and regulatory regions, have been cloned and sequenced, the trp genes show little difference from the corresponding regions in E. coli (Crawford, 1987). In the

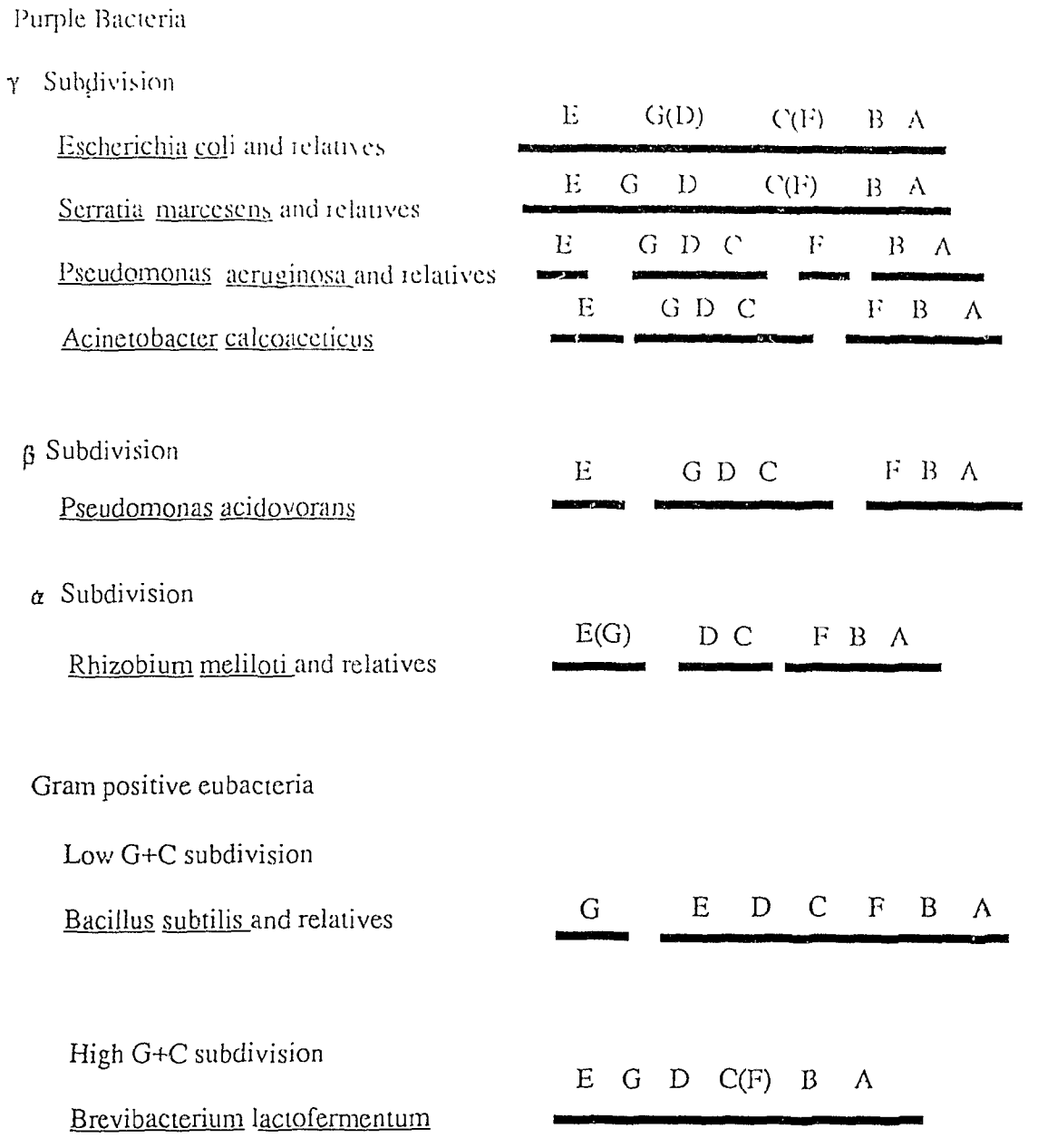


Figure 2. Chromosomal distribution of tryptophan biosynthesis genes in eubacteria. (modified from Crawford, 1989).

group of enteric bacteria represented by E. coli and S. typhimurium the seven enzyme activities of the pathway are encoded in five genes which include two gene fusions, trp(G)D and trpC(F). In the group represented by Serratia and Vibrio there are six genes with only trpC(F) representing a gene fusion. However, the trp gene order in all the enteric bacteria is essentially the same. In E. coli, a region spanning approximately 200 bp immediately upstream from the first structural gene, trpE, is the regulatory region; it consists of a trp promoter-operator and an attenuator. The trp operon is regulated by a dual system at the transcriptional level. This comprises a conventional repressor-operator component and a transcription attenuation mechanism that couples translation of the trp leader peptide to termination of transcription at the trp attenuator terminator sequence preceding the first structural gene (Yanofsky and Crawford, 1987).

Enteric bacteria are not the only ones to show clustering of all genes in an operon. This occurs also in Brevibacterium lactofermentum, a member of the high G+C subdivision of Gram-positive bacteria. The B. lactofermentum operon contains one gene fusion, trpC(F); since the gene order is the same as in enteric bacteria (Matsui et al., 1986), the arrangement matches that of the Serratia group. Moreover, the nucleotide sequence of the entire operon as well as the upstream flanking sequence is similar to that of enterics; the regulatory region

consists of an operator-like sequence and an attenuator which likewise show only minor differences from those of the enterics (Matsui and Sano, 1987).

In the Gram-positive bacterium B. subtilis, transformational mapping of trp mutations indicated that all of the genes except trpG were together in an operon, in the same order as found in the enteric bacteria (Carlton and Whitt, 1969). Interestingly, trpG, which in the species encodes an amphibolic glutamine amidotransferase was near the pab genes. It was part of an operon containing other genes involved in folic acid biosynthesis (Kane et al., 1972). An identical arrangement is found in B. pumilus (Hoch and Crawford, 1973). Both the tryptophan and folic acid biosynthesis operons of B. subtilis have been cloned and sequenced (Henner et al., 1984; Slock et al., 1990). Coordinate repression by tryptophan of both the trp operon and the differently located trpG gene is mediated by a regulatory RNA-binding molecule. Repression of the trp operon occurs by transcription termination while that of trpG is postulated to occur at the translational level without affecting expression of other genes in the folic acid operon (Kuroda et al , 1986; Slock et al., 1990).

Organisms in which trp genes are distributed in three clusters include A. calcoaceticus and R. meliloti. In A. calcoaceticus, transformation mapping suggested that trpGDC and trpFBA form two separate clusters, each distinct from trpE

(Sawula and Crawford, 1972). Both clusters have been cloned by complementation of the relevant E. coli trp auxotrophs. Expression of the trpGDC operon and trpE is regulated coordinately while trpFBA is under different regulation (Cohn and Crawford, 1976); however, the mechanisms are not fully understood. In the rhizobia, two clusters, trpFBA and trpDC, were mapped by examining the ability of various R-prime plasmids from R. meliloti to complement trp mutations in R. lequminosarum (Johnston et al., 1978). Subsequent cloning and sequence analysis of trpE revealed that it formed a fused trpE(G) pair (Bae et al., 1989). The deduced amino acid sequence of the proximal two thirds of the open reading frame showed similarity to the TrpE of other bacteria, while the distal one third resembled TrpG from the same bacteria. Among all bacteria examined so far, this is the only example of a fusion of trpE and trpG. In rhizobia only trpE(G) is regulated in response to tryptophan starvation or excess (Holmgren and Crawford, 1982). Regulation takes place solely by attenuation in a manner analogous to that in E. coli (Bae et al., 1991).

Among the Gram-negative bacteria, P. aeruginosa and P. putida are similar in having their trp genes dispersed in four locations (Gunsalus et al., 1968; Essar et al., 1990b; 1990c). At one location is trpGDC which is separated from trpE by only 2.2-kb in P. putida, but by at least 25 kb in P. aeruginosa (Shinomiya et al., 1983). At different locations and separated

from each other are trpBA and trpF. While trpE and trpGDC are regulated coordinately by tryptophan repression, trpF is not. The trpBA in Pseudomonas is uniquely regulated through induction by its substrate, InGP. The phnA and phnB set of anthranilate synthetase genes involved in phenazine biosynthesis are in a cluster and are cotranscribed but their chromosomal location is not known. (Essar et al., 1990a)

In other bacteria, only partial information is available about the chromosomal arrangement of trp genes. Of various difficulties reported, the absence of some classes of mutations from the collection of trp auxotrophs used for genetic analysis is commonly mentioned. In Caulobacter crescentus, genetic mapping indicated that the trp genes are dispersed in at least two locations (Winkler et al., 1984). One contained the trpFBA operon while at the other are trpC, trpD and trpE. Sequence analysis of the trpFBA operon indicates that its expression is dependent on usg, an unidentified open reading frame located just upstream from trpF (Ross and Winkler, 1988). The location of trpG is uncertain since mutants defective in this gene have not been isolated. Likewise in Staphylococcus aureus and Micrococcus luteus, the location of all trp genes, except trpG, is known. In S. aureus, mutations in trpE, D, C, F, B and A were mapped by transductional analysis to one location, and gave the same order as in enteric bacteria (Proctor and Kloos, 1970). In M. luteus, trpE,C,B and A were mapped in the same order by

transformation to one location, while trpD and trpF (see Figure 2) were at a separate location (Kloos and Rose, 1970).

In some bacteria there is a lack of genetic mapping systems. To circumvent this problem efforts have been made to clone trp-containing DNA fragments from such organisms by complementation of the relevant E. coli trp auxotrophs. Taking advantage of the observation that trp genes generally occur in clusters, nucleotide sequence analysis of the gene and its flanking regions can provide a means of identifying other linked trp genes. Among spirochetes and their relatives, the leptospira subdivision represented by Leptospira biflexa contains trpE and trpG in a cluster along with an unidentified open reading frame situated upstream from trpE (Yelton and Charon, 1984; Yelton and Cohen, 1986), while in the spirochete subdivision represented by Spirochete aurantia, trpE-containing DNA fragments do not contain trpG (Brahamsha and Greenberg, 1987). Presumably the two genes are separated from each other on the chromosome (Brahamsha and Greenberg, 1987). In the archaebacteria, trpE of Thermus thermophilus is adjacent to trpG while an attenuator-like sequence is present immediately upstream from trpE (Sato et al., 1988); in Methanococcus voltae, trpD, F, B, and A are present in this order in a cluster (Sibold et al., 1988).

IV. EVOLUTIONARY IMPLICATIONS

There are many features of the tryptophan biosynthetic pathway that suggest a common ancestry in all microorganisms. That the pathway is highly conserved is apparent from the evidence that in at least seven widely different groups of eubacteria and five groups of eukaryotes examined, essentially the same reaction sequence is present. Even in archaebacteria the trp genes that have been cloned and sequenced show the same pattern as in other organisms. The catalytic properties of the individual enzymes in the pathway and some of their pecularities have remained constant during evolution. These include the ability of anthranilate synthetase to use either ammonia or glutamine as a source of nitrogen and the cooperative interaction between tryptophan synthetase subunits to form tryptophan from InGP with indole as a captive intermediate.

At the molecular level also, many features of gene and gene product organization have been well conserved, again suggesting a common ancestry. When their amino acid sequences are compared, tryptophan pathway enzymes from different bacteria show the presence of conserved regions. Regulatory elements such as attenuators share common nucleotide sequences. The order in which trp genes are transcribed in operons or clusters is generally the same in different bacteria. In every species so far examined, trpB and trpA are adjacent and may overlap. The overlap is 1 bp in enteric

bacteria, 4 bp in P. aeruginosa, and 8 bp in B. subtilis. However, they are separated by 12 bp in C. crescentus and 37 bp in M. voltae (Sibold et al, 1988).

Superimposed on the high degree of uniformity, the chromosomal arrangement and regulation of trp genes shows remarkable diversity. During evolution, every group of bacteria has acquired a unique gene arrangement and regulatory scheme to satisfy its requirement for the amino acid. Each arrangement thus represents the natural history of the organism and its ancestors.

The trp genes show two types of differences. One is at the nucleotide sequence level where variable regions are interspersed with some essential conserved regions; this could be explained by spontaneous and undirected mutations (Lederberg and Lederberg, 1952). The second difference is in the chromosomal arrangement of the genes. Such differences can be accounted for only by more drastic rearrangements in the DNA sequences rearrangements big enough to mobilize one or more genes. They can be brought about by insertions, deletions, inversions and translocations, and are the result of illegitimate recombination, which can occur in bacteria at appreciable frequencies (Starlinger, 1977; Crawford, 1980). A different mechanism, namely lateral transfer en bloc from enteric bacteria via plasmids or phages is hypothesized to account for the Serratia-like arrangement and regulation of trp genes in B. lactofermentum, which belongs to the high G+C

subdivision of Gram positive bacteria (Crawford, 1989).

Once trp gene/s assume a new location, an appropriate control mechanism regulating expression to maintain the cellular economy could evolve (Crawford, 1980). Every unique arrangement shows distinct regulatory patterns in response to varying levels of tryptophan or pathway intermediates. These patterns range from coordinate regulation of all or some genes by transcriptional or translational mechanisms on the one hand, to absence of regulation in one or all of the genes on the other. In Chromobacterium violaceum, Wegman and Crawford (1968) found no apparent regulation of Trp enzymes in response to tryptophan starvation or excess. The chromosomal arrangement of the genes is not known.

There are two possible ways to explain why all trp genes occur as a single cluster in some bacteria while in others they are dispersed to varying degrees. Firstly, pathway intermediates or enzymes in some organisms may have had other functions during evolution thus generating a need for their genetic separation. This may have mandated a different location along with independent regulation. Alternatively, selective forces may have acted towards achieving coordinate regulation of all trp genes, thus resulting in the more compact trp gene arrangement observed in enteric bacteria (Yanofsky, 1984).

Another solution for achieving coordinate and equimolar synthesis of gene products is exemplified by the numerous gene

fusions observed. Many potential advantages of the resulting bifunctional enzymes have been proposed (Zalkin, 1980; Crawford, 1989). There is evidence to suggest that bifunctional enzymes are catalytically more efficient than their separate counterparts (Welch and Gartner, 1975). Secondly, fusion of two or more enzymes catalyzing reactions in sequence can channel the intermediates from one active site to another (Zalkin, 1980). This becomes important where intermediates are not compatible with their surroundings due to lability, hydrophobicity or toxicity. The channelling of indole between the active sites of the subunits of tryptophan synthetase without releasing a free intermediate is an example of this.

Examination of the intercistronic region of genes which are fused in other bacteria has suggested a mechanism by which fusion of two genes could have occurred (Crawford, 1989; Yanofsky, 1984). Alignment of nucleotide sequences for trpG and trpD of S. marcescens with those of E. coli trp(G)D fusion genes shows that the intercistronic space in S. marcescens contains a ribosome binding site, a start codon and a stop codon. A deletion and some base substitutions eliminating the trpG stop codon along with elimination of the ribosome binding site for trpD while maintaining the correct reading frame would explain the trp(G)D fusion.

Another interesting feature of tryptophan biosynthesis genes is their relationship to the evolution of genes for PABA

biosynthesis. The two pathways of biosynthesis are very similar. The pabA and pabB genes from various organism have been sequenced, and they show appreciable similarity to their trp counterparts. The two sets of genes are, therefore, thought to have had a common ancestry (Kaplan et al., 1985; Goncharoff and Nichols, 1988). Moreover, TrpG of P. aeruginosa and P. putida, which is involved only in tryptophan biosynthesis, resembles PabA of enteric bacteria and the amphibolic glutamine amidotransferase of B. subtilis and A. calcoaceticus more closely than the TrpG portion of the bifunctional Trp(G)D of enteric bacteria (Crawford, 1989). Two proposals have been put forward to explain this.

According to Crawford (1989), both sets of genes, trpE-trpG, and pabB-pabA, were present in the ancestral strain, probably as a result of duplication. In those organisms which now use an amphibolic glutamine amidotransferase, deletion of one of the two glutamine amidotransferase genes (either pabA or trpG) led to dependence on the remaining enzyme to perform both tryptophan-specific and PABA-specific reactions. Deletion of trpG would result in a B. subtilis arrangement where the amphibolic gene is located along with pab genes, while elimination of pabA would lead to the A. calcoaceticus arrangement in which the amphibolic gene is located with other trp genes.

The other suggestion (Yanofsky, 1984) is that the ancestral strain possessed only a single amphibolic glutamine

amidotransferase capable of performing both the tryptophan- and PABA-specific reactions. Whereas it was retained during evolution in organisms like B. subtilis and A. calcoaceticus, in E. coli or P. aeruginosa the single gene duplicated and diverged, giving rise to pathway specific glutamine amidotransferases.

The presence in P. aeruginosa of two independent but partially interchangeable sets of anthranilate synthetase genes suggests duplication followed by active site modification. Presumably this was a solution to the need to catalyze similar reactions in two functionally different pathways. However, the duplication is evidently not a recent one since the amino acid sequences of TrpE and TrpG in P. aeruginosa are more similar to those of TrpE and TrpG, respectively, in P. putida than to the PhnA and PhnB involved in phenazine biosynthesis in P. aeruginosa. Since P. putida does not contain the second set of anthranilate synthetase genes, a duplication resulting from the need for separately regulated enzymes for secondary metabolism is postulated to have taken place earlier than the speciation event that separated P. aeruginosa and P. putida (Crawford, 1989; Essar et al., 1990a).

V. TRYPTOPHAN BIOSYNTHESIS IN STREPTOMYCES

Streptomycetes belong to the high G+C subdivision of Gram positive bacteria (Woese, 1987). They have been

extensively investigated, but most studies have focussed on the economic importance of products such as antibiotics. There are fewer reports describing the primary metabolism of these bacteria. The availability of well characterized genetic systems has allowed a number of genes involved in biosynthetic or catabolic pathways and those responsible for differentiation and antibiotic synthesis to be mapped on the circular chromosome of several species (Hopwood and Kieser, 1990; Stuttard, 1988). However, the organization of *trp* genes in streptomycetes has not been studied extensively.

In *S. coelicolor* A3(2), conjugational mapping of *trp* auxotrophs suggested that *trp* genes were present in at least two positions on the chromosome; *trpA* and *trpB* were placed together at a location near *his* genes while *trpC* and *trpD* were at the other (Hopwood, 1973). Later, Engel mapped *trp* mutations in twenty eight auxotrophs and repositioned *trpC* in the same region as *trpA* and *trpB*; the newly isolated *trpF* mutation was placed near *trpD*. This revision has not been incorporated in recently published genetic maps of the *S. coelicolor* chromosome (Hopwood et al., 1985; Hopwood and Kieser, 1990). No *trpE* or *trpG* auxotrophs have yet been isolated. Evidence that *trpC*, *trpB* and *trpA* are physically close to each other in streptomycetes comes from the recent cloning of a 4.2-kb chromosomal DNA fragment of *S. griseus* which complemented *trpC*, *trpB* and *trpA* mutations in *E. coli* (Rivero-Lezcano et al., 1990). However, it is not known

whether these genes form an operon. In S. coelicolor A3(2) all Trp enzymes except TrpC (indoleglycerolphosphate synthase) are regulated in response to Trp starvation or excess, but the mechanism involved has not been elucidated (Smithers and Engel, 1974).

In S. venezuelae, conjugational mapping of genetic markers indicated that several trp mutations are located between his and str in the broad arc opposite the adeA marker (Doull et al., 1985). Because of the availability of generalized transduction, this species is particularly well suited for carrying out genetic analysis at a fine structure level, as exemplified by analysis of genes involved in chloramphenicol biosynthesis (Vats et al, 1987; Stuttard, 1988). Stuttard examined mutations in five trp auxotrophs for cotransduction and showed that the mutations were clustered in two locations on the S. venezuelae chromosome; trp-1, trp-3, trp-5 and trp-6 were each cotransducible with hisA and hisB, while trp-4 was cotransducible with nicB (Stuttard, 1983a). Relative to the location of other chromosomal markers, the two clusters corresponded well with the trp clusters in S. coelicolor A3(2). Markers used in this study were tentatively classified as defining trpA, B, C, or D from the auxotrophic requirements and cross-feeding properties of the mutants. The enzymatic lesions caused by the mutation in each of the trp auxotrophs was not established biochemically. Nonetheless, the auxotrophic requirements did suggest that none of the

auxotrophs was either trpE or trpG.

In another chloramphenicol-producing streptomycete, Streptomyces spp. 3022a subsequently identified as an S. venezuelae strain (L.C.Vining, personal communication), Francis et al. (1978) characterized anthranilate synthetase, the first enzyme in the pathway. This enzyme is composed of two nonidentical subunits. The bigger subunit, ASI (mol. wt. 72,000) could, by itself, catalyze the synthesis of anthranilate from chorismate in the presence of ammonia, while the smaller subunit, ASII (mol. wt. 28-29,000), formed a complex with ASI, which could then use glutamine as the source of nitrogen for anthranilate formation. Tryptophan regulated the activity of anthranilate synthetase by both inhibition and repression. The second enzyme in the pathway, phosphoribosyltransferase (TrpD), eluted separately from the anthranilate synthetase subunits during chromatography on DEAE-cellulose. Thus there is no evidence in Streptomyces for the presence of a bifunctional Trp(G)D protein as found in E. coli and S. typhimurium. This suggests that anthranilate synthetase in Streptomyces sp 3022a is of the monofunctional type (Francis et al., 1978).

VI. GENETIC ANALYSIS IN STREPTOMYCES

A. Conjugation

Conjugation is the principal means by which genetic recombination in streptomycetes can be brought about for the

purpose of establishing a gene linkage map. When two mutually fertile parental strains carrying different genetic markers are allowed to grow together in a mixed culture, progeny spores with new combinations of markers from both parents can be readily isolated. The process prabably involves fusion of mycelia from the two strains, which brings the genetic material of the two parents into the same cytoplasm (Stuttard, 1992). Recombination can then occur between the two sets of genes and subsequently the haploid genomes, consisting of parental types and a small number of recombinants, segregate into the spores.

Chromosomal recombination between the two parents is mediated by conjugative plasmids, and its frequency is often increased when the plasmid is present in only one parent (Hopwood et al., 1986; Doull et al., 1986). Streptomyces venezuelae is believed to possess three conjugative plasmids on the basis of "pocking" character istics, although none has yet been physically identified (the 130 kb giant linear plasmid in S. venezuelae may be one). Of these plasmids, SVP1 encodes the major fertility function while SVP2 and SVP3 promote chromosomal recombination to a lesser extent (Doull et al., 1986). Fertility plasmids are self-transmissible -i.e., in a cross between parents differing in the plasmid content, the plasmid is transferred to the plasmid-free parent, the transfer possibly being a consequence of hyphal fusion. However, hyphal fusion, though necessary, is not the only

factor responsible for chromosomal recombination in a cross because SLP3 plasmid of S. lividans is self-transmissible but does not promote chromosomal recombination (Hopwood et al., 1983). This suggests that other as yet unidentified function/s besides the transfer function are also responsible for mediating chromosomal recombination (Hopwood et al., 1986; Stuttard, 1992).

Although conjugation and its mechanisms in Streptomyces are not yet completely understood, conjugative recombination has provided an important technique for analyzing linkage relationships between chromosomal markers. A "four-on-four" procedure, which involves crossing parents differing in at least four markers and assessing the segregation of the recombinant progeny, is used to construct a chromosomal map of a previously unmapped species (Hopwood, 1967; Hopwood et al., 1985). Once a few markers have been mapped, others can be positioned in relation to them by a "single-selection" or "allele-gradient" procedure (Hopwood, 1967; Hopwood et al., 1985; Doull et al., 1986).

Of the chromosomal maps of various Streptomyces species constructed by these procedures, that of S. coelicolor A3(2) is the most extensively studied. So far at least 128 markers have been mapped on it; the next best known is that of S. venezuelae on which 38 markers have been mapped (Stuttard, 1988; 1991). When the circular maps of different species are aligned appropriately and compared, an overall conservation of

linkage relationships and genome structure is evident. More extensive knowledge of the similarities and differences in the genetic and physical maps of different species should shed light on the evolutionary relationships among them (Stuttard, 1992).

Genetic analysis using haploid recombinants is useful but it gives information about the arrangement of only well-separated markers. This is because in conjugational crosses relatively large DNA segments from the two parents are exchanged and therefore recombinants that show segregation of closely linked genes are rarely obtained. Nevertheless, it is possible by selecting and analyzing heteroclones from among the progeny spores to determine gene order among closely linked markers (Hopwood, 1967). Heteroclones are partial diploids in which a segment of genome of one parent added to the complete genome of the other (mechanism unknown). Analysis of parental types and recombinant spores originating from heteroclones provides recombination frequencies and thus linkage relationships between closely linked markers. However, heteroclone analysis is tedious and systems are not available for mapping genes in all species or strains. Therefore it has not been widely used (Hopwood, 1967; Stuttard, 1992).

B. <u>Transduction</u>

In streptomycetes, both, natural (Stuttard, 1992) and artificial (Chater, 1986; Hahn et al., 1991) means of phage-

mediated gene transfer are available. To study the "fine structure" arrangement of closely linked genes, the naturally occurring genetic exchange due to generalized transduction has been used. So far, generalized transducing phages have been reported only in S. fradiae (neomycin-producer) and S. venezuelae, and only phage SV1 of S. venezuelae has been extensively used for genetic mapping (Stuttard, 1983b; 1988; 1989; Vats et al., 1987; Stuttard et al., 1987). In S. venezuelae, cotransduction has been demonstrated with gene pairs located near each other in widely separated regions of the chromosome. Close linkage of markers in gene pairs based on cotransduction frequencies corresponds well with the linkage established for similar gene pairs in S. coelicolor A3(2) by heteroclone analysis (Stuttard, 1988).

Phage SV1 can package approximately 45 kb of DNA by a headful packaging mechanism (Stuttard, 1989). Thus during generalized transduction, the phage can carry about 0.67% of the S. venezuelae genome (assuming a size of 6.75 Mb) per particle (Stuttard et al., 1987). Any given marker can be transduced at frequency of about 10^{-6} per pfu using phage treated with UV irradiation to 0.1-0.5% survival. Although SV1 remains the most extensively used for genetic mapping, several other phages, SV3, SV9, SV11 and SV12 found with S. venezuelae as host are also capable of generalized transduction. However, all generalized transducing phages discovered in S. venezuelae have a narrow host range, limiting the use of this valuable

tool for genetic analysis to only this species and one type of S. phaechromogenes.

C. Analysis of cloned genes by homologous recombination

Streptomycetes can be transformed with a variety of plasmid vectors. These are stably maintained in an autonomous state provided they consist of heterologous DNA. Vectors that carry DNA homologous to the transformed host are usually not maintained autonomously (Murakami et al., 1986; Seno et al., 1984). They integrate into the host chromosome by a Campbell-type recombination that usually involves a single crossover, the result of which is a duplication of the homologous sequences and their separation by the heterologous vector sequence (Seno et al., 1984; Campbell, 1962) (Figure 3). However, the chromosomal region containing the integrated plasmid is unstable due to further recombination between the duplicate homologous sequences. This results in excision of the plasmid. In rare cases, integration may result from a double crossover recombination event. Allele exchange occurs between the plasmid-borne and the chromosomal homologous sequences, after which the nonhomologous vector sequences regain autonomy. The efficiency with which single or double crossover recombinations take place is proportional to the size of the homologous DNA sequence. Nevertheless, as little as 300 bp of homologous DNA present in pIJ702 was sufficient to achieve integration of the recombinant plasmid into the

Saccharopolyspora erythraea chromosome (Weber et al., 1990).

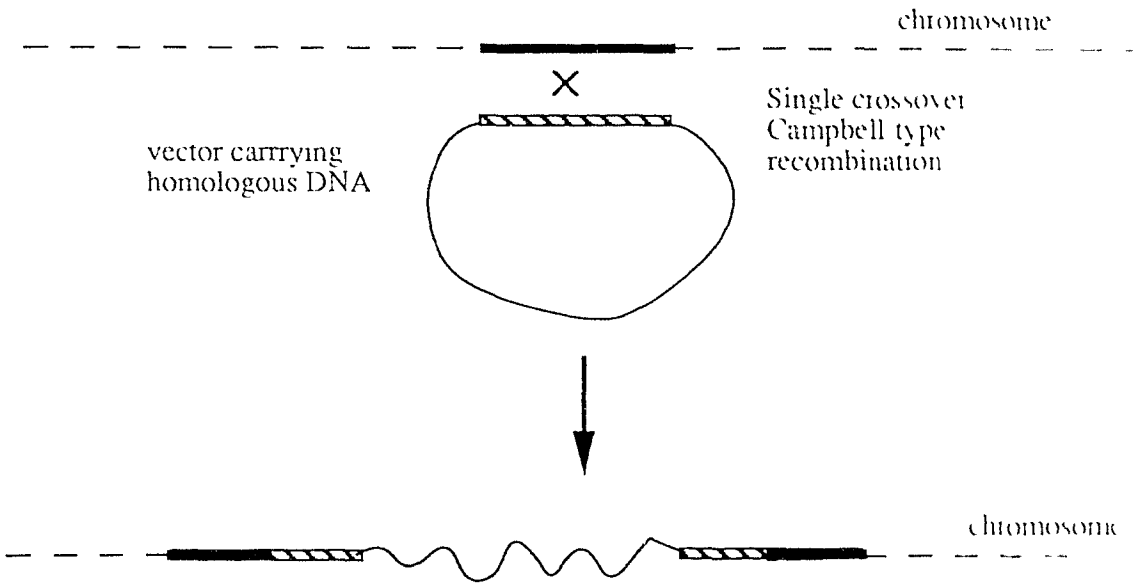
Integration and excision of plasmids by homologous recombination is the basis for many techniques used to analyze cloned DNA. Functional analysis can be carried out by gene disruption and/or gene replacement (Shortle et al., 1982; Chater and Bruton, 1983; Anzai et al., 1988). Moreover, specific mutants can be isolated by these techniques. If the homologous DNA is an internal fragment of the gene of interest, integration by a single cross over disrupts the chromosomal allele by separating the two ends of the transcriptional unit (Figure 3b). In gene replacement, a plasmid carrying the inactivated gene (obtained either in vitro or in vivo) is allowed to integrate into the chromosome. Due to homologous recombination between the directly repeated sequences (see Figure 3c), the integrated plasmid is excised and, depending upon the site of the crossover, either the wild type gene is restored or the inactivated gene is incorporated (Figure 3c). These techniques were employed to functionally characterize a 30-kb DNA fragment from the ermE region of Sac. erythraea involved in erythromycin biosynthesis; in the process five new mutant phenotypes were discovered (Weber et al., 1990). Since antibiotic biosynthesis genes are organized in clusters of transcriptional units, gene disruption analysis at various sites on the putative cloned DNA in conjunction with mutant characterization can identify the transcriptional organization of these genes.

Insert-directed integration of a plasmid vector carrying a selectable marker can be used in conjunction with standard genetic analysis to determine the chromosomal location of the insert. In Sac. erythraea, pIJ702 carrying tsr and an ermE DNA insert integrated into the ermE region of the chromosome. Conjugational mapping established the close linkage of tsr, and therefore ermE, to erythromycin biosynthesis genes (Weber and Losick, 1988).

By employing homologous recombination, relatively big chromosomal fragments can be cloned. The procedure has been used to clone a 38-kb fragment containing red genes for undecylprodigiosin biosynthesis (Malpartida et al., 1990). Two available DNA fragments from either end of the red cluster were placed adjacent to each other on pIJ941. When the recombinant plasmid was introduced into S. coelicolor A3(2), homologous recombination involving a double crossover occurred between the two red sequences on the plasmid and homologous red sequences located at the ends of the chromosomal red cluster. This gave a plasmid which now carried an insert encompassing the entire red cluster.

The above techniques depend upon efficient integration of plasmids carrying homologous DNA into the chromosome, and easy selection of host strains in which this event has occurred. For these purposes, a variety of host-vector systems have been developed for use in streptomycetes. All such plasmid vectors carry a selectable marker, usually an antibiotic resistance

(a) Plasmid integration by homologous recombination



(b) gene disruption

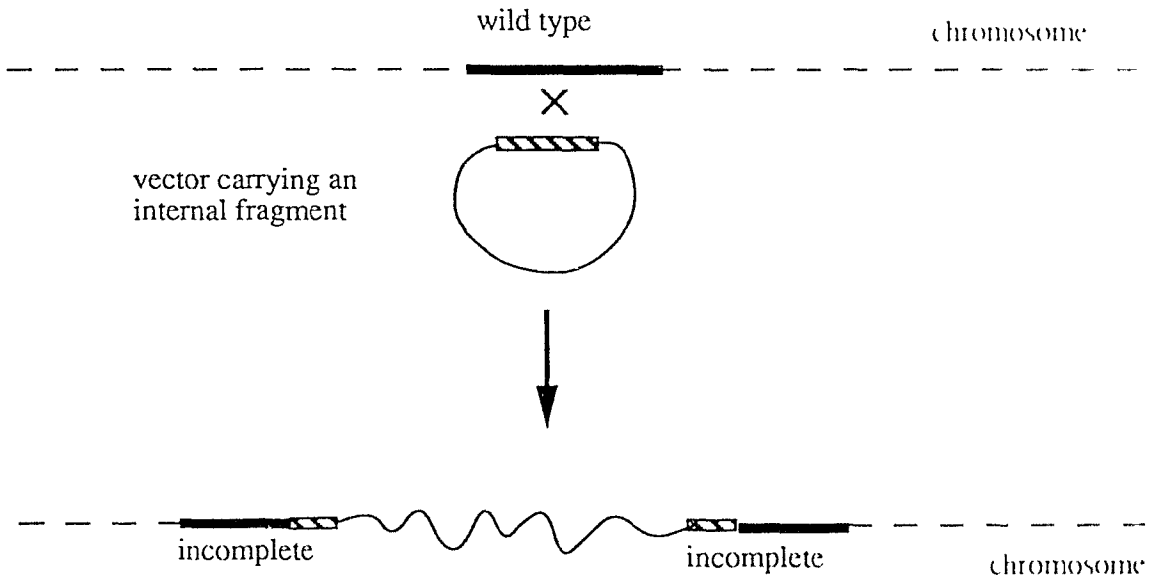
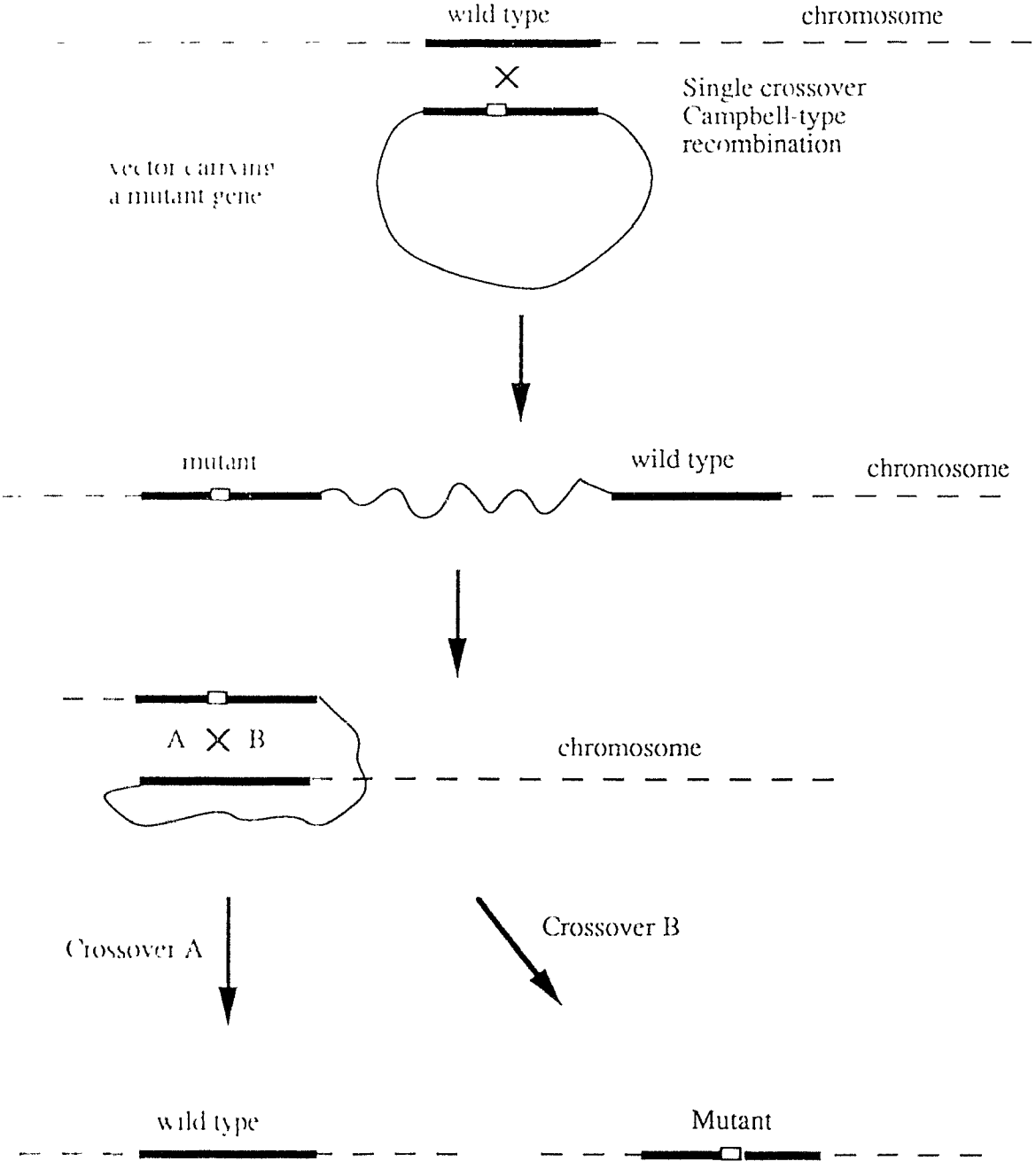


Figure 3. Model for integration of plasmids by homologous recombination and its applications : (a) plasmid integration (b) gene disruption and (c) gene replacement

(c) gene replacement



gene. The vector may be chosen for its inability, when it lacks an insert containing host homologous DNA, to give stable transformants. Such vectors may either lack a Streptomyces replicon (non-replicative vectors), or may replicate only under permissive conditions (conditional vectors). Others may have defective replication and/or segregation functions. Derivatives of these vectors carrying DNA inserts homologous to the host DNA give stable transformants in which the plasmid has been integrated into the chromosome. Such stable strains are isolated and selected under conditions in which strains carrying the vector alone are not stable.

Since nonreplicative vectors are usually E. coli plasmids lacking a Streptomyces replicon, the efficiency with which stable transformants containing integrated plasmid are obtained is a function not only of homologous recombination but also of the inherent transformation efficiency of the host strain. Nevertheless, such vectors have been used successfully to analyze by gene disruption and gene replacement the spiramycin and avermectin biosynthesis genes in S. ambofaciens and S. avermitilis, respectively (Streicher et al., 1989; Richardson et al., 1990). Recently, higher integration rates were obtained when a single stranded rather than a double stranded form of the nonreplicative vector was used to transform S. viridochromogenes (Hilleman et al., 1991). In these experiments, the nonreplicative vector had not only an E. coli replicon but also a phage F1 origin of replication for

production of single stranded plasmid DNA.

Among the conditional vectors are pGM plasmids which are based on the naturally temperature-sensitive replicon of a plasmid from S. ghanaensis (Muth et al., 1989). These vectors are maintained stably below 34°C, but at 39°C they are lost at a high frequency. They have been used for gene disruption of the S. viridochromogenes pat gene which is responsible for phosphinothricylalanylalanine resistance.

The vector pIJ702 gives stable transformants in most streptomycetes but, for unknown reasons, is lost from Sac. erythrea transformants efficiently; therefore, it has been widely used for gene disruption and gene replacement in this host (Vara et al., 1989; Weber et al., 1990). Another vector, pHJL400, contains the SCP2* replicon of S. coelicolor (Hopwood et al., 1985) but lacks par, the sequences involved in partition, and is therefore segregationally unstable (Larson and Hershberger, 1986). As a result, transformants segregate into plasmidless strains at a high frequency when grown without plasmid-based selection. This vector has been used for insert-directed integrations in S. griseofuscus (Larson and Hershberger, 1990).

Derivatives of the temperate phage, ϕC31, have been used extensively in techniques similar to those for plasmid vectors involving homologous recombination (Chater, 1986; Chater et al., 1985). During the lysogenic part of the phage life cycle, prophage is formed by site-specific recombination between attP

phage sequences and attB sequences of the host chromosome; the repressor product of the c gene shuts off the lytic functions. Derivatives of ϕC31 with attP-deletion fail to form prophage and enter lysogeny. However, such phages form prophages by homologous recombination if they carry fragments of host DNA in the available cloning sites. Lysogens can be easily identified by selecting for phage-encoded viomycin resistance. Using a procedure known as mutational cloning the attP-deleted phage was used to isolate mmy genes directing methylenomycin biosynthesis, in S. coelicolor A 3(2) (Chater and Bruton, 1983). The recombinant phage containing an internal mmy sequence was identified in a genomic library of S. coelicolor A3(2) constructed in the attP-deleted phage vector by its ability to lysogenize the wild type host and to confer an Mmy phenotype as a result of gene disruption. The phage carrying the mmy DNA was released spontaneously from the lysogen and was recovered. The attP-deleted phage vector has been used in disruption and replacement analysis of genes involved in the production of other antibiotics (Hopwood et al., 1985), in primary metabolism (Seno et al., 1984) and in differentiation (Piret and Chater, 1985; Buttner et al., 1990).

MATERIALS AND METHODS

I. Bacteria and Plasmids

The bacterial strains and plasmids used are listed in Table 1.

II. Chemicals and Biochemicals

Chorismic acid was isolated from culture filtrates of Aerobacter aerogenes 62-1 by the method of Gibson (1964). 1-(o-Carboxyphenylamino)-1'-deoxyribulose-5'-phosphate (CDRP) was synthesized by the procedure of Smith and Yanofsky (1963). Indoleglycerol phosphate (InGP) was prepared enzymatically (Wegman and Crawford, 1968).

Bacto-agar, Bacto-peptone, yeast extract, casamino acids, malt extract, and nutrient broth were purchased from Difco laboratories, Detroit, MI. Trichloroacetic acid, 5-phosphoribosyl-1-pyrophosphate (PRPP), pyridoxal-5-phosphate, ribose-5-phosphate, herring sperm DNA, N-methyl-N'-nitro-N-nitrosoguanidine (NTG), tris-(hydroxymethyl)aminomethane (Tris-HCl), N-tris-(hydroxymethyl)-2-aminoethane sulfonate (TES), Triton X-100, Ficoll of molecular weight 400,000, bovine serum albumin, polyvinylpyrrolidone of molecular weight 360,000, lysozyme, ampicillin, all amino acids and chemicals used in media preparation were purchased from Sigma Chemical Co., St. Louis, MO.; polyethelene glycol 1000 was from Koch-

Light, Haverhill, UK; thiostrepton was a gift from S.J.Lucania of E.R.Squibb and Sons, New Brunswick, NJ.; silica gel Sil-60 thin layer chromatography plates were from E.Merck AG, Darmstadt, FRG. All the restriction enzymes and DNA ligase were from Bethesda Research Laboratories, Burlington, Ont. Hybond-N nylon membrane was from Amersham Canada Ltd, Oakville, Ont. (α-^{32}P)dCTP was from ICN Chemicals Ltd. Random primer kit was purchsed from Boehringer-Mannheim, Montreal, PQ.

Table 1. Bacteria and Plasmids used.

Strain	Genotype /Phenotype	Source/Reference
S. venezuelae ISP5230		
10712	Wild-type	E.Wellington
VS19	nicB1	Stuttard (1983)
VS24	trpA1	Methoxypsoralen-near ultraviolet mutagenesis of 10712 (Stuttard, 1983a)
VS65	his-6	Stuttard (1983a)
VS153	trpC3,cm1-5	Methoxypsoralen-near ultraviolet mutagenesis of VS35 (cm15) (Stuttard,983a)
VS154	trpD4,cm1-5	same as VS153
VS160	ura-1,thr-1,trp-5	Stuttard (1983a)
VS161	tyr-2,thrC1,uraA1	Doull et al.(1986)
VS173	trp-6	Stuttard (1983a)
VS180	trpAB6	By transduction: his-6 x SV1(VS173)
VS181	trpB5	By transduction: his-6 x SV1(VS160)
VS194	hisA6,adeA10,	Doull et al.(1986) SVP1$^-$

		SVP2⁻
VS228	trpC7, cml-12	Ethylmethane sulfonate mutagenesis of VS532 (cml2) (S.Vats, 1987, Ph.D. thesis, Dalhousie University)
VS309	arg-6, thrC1, uraA1 adeA10, strA6	C. Stuttard
VS317	trpA9	Localized hydroxylamine mutagenesis of SV1 (10712) with VS65 as recipient (L.Atkinson, 1987.M.Sc. thesis, Dalhousie University)
VS420	trpA13, pdx-4 cml-12	Nitrosoguanidine mutagenesis of VS258 (Vats et al., 1987)
VS517	trpAB12	Nitrosoguanidine mutagenesis of 10712 L.Atkinson, 1987, M.Sc. thesis, Dalhousie University)
VS525	trpB14	Nitrosoguanidine mutagenesis of 10712: this study
AP11-14	VS154 (pDQ171)	This study

AP15	VS154(pIJ702)	This study
AP41-49	VS194(pDQ189)	This study
AP50	VS194(pHJL400)	This study
AP52-53, replacement; AP55-57	VS194 containing modified *trpEG*	Gene this study
S. venezuelae		
13s	Wild type	L. Vining
S. lividans		
TK24	$SLP1^-$, $SLP2^-$, *str*	Hopwood et al.(1985)
AP1	*trpD*	Nitrosoguanidine mutagenesis of TK24: this study
AP2	*trpC*	same as AP1
S. griseofuscus NRRL B5429	Wild type	R. H. Baltz
S. parvulus ISP 5048	Wild type	K.F.Chater
S. phaeochromogenes NRRL B2119	Wild type Wild type	A.R.S A.R.S
S. coelicolor A 3(2)	Wild type	Hopwood et al.(1985)
Escherichia coli		
TG1	*supE*, *hsdΔ5*, *thi* Δ(*lac*-	J.Wright

	proAB) F′(traD36,proAB$^+$, lacI^q,lacZΔM5)	
JA221	ΔtrpE5, leuB6,r$^-$m$^+$	C.Yanofsky
JA200	ΔtrpE5,	B.Bachmann
W3110 trpED23	W3110 his,cysB,ΔtrpED23	C.Yanofsky
W3110 trpD58	W3110 trpD58	C. Yanofsky
JA194 trpC9830	JMB9 r$^-$ m$^+$ thr$^+$ trpC9830 (TrpF$^-$)	C.Yanofsky
W3110 trpC55	W3110 trpC55	C.Yanofsky
W3110 trpB9579	W3110 tna2, trpB9579	C.Yanofsky
JA194 trpA33	JMB9 r$^-$ m$^+$ thr$^+$ trpA33	C.Yanofsky
Plasmids		
pIJ702 al.(1983)	tsr, mel$^+$	Katz et
pDQ101	tsr, hyg	Aidoo et al. (1990)
pHJL400	tsr, amp, lacZ′	Larson and Hershberger (1986)
pTZ18R	amp, lacZ′	J.Wright

A.R.S., U.S. Agricultural Research Station, Peoria, Il;

III. Media

1. MYM medium (Doull et al., 1985)

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

For liquid media, agar was omitted.

2. Minimal medium (Hopwood et al., 1985)

Maltose	10.0 g
Dipotassium hydrogen phosphate	0.5 g
Magnesium sulfate heptahydrate	0.2 g
Ferrous sulfate heptahydrate	0.01 mg
Asparagine	0.5 g
Agar	15.0 g
Distilled water	1000 mL

3. Minimal liquid medium (Hopwood et al., 1985)

Asparagine	0.5 g
Casamino acids	5.0 g
Magnesium sulfate heptahydrate	0.6 g
Minor elements solution*	1 mL
Distilled water	800 mL

This was dispensed in 80-mL aliquots and autoclaved. Before use, the following sterile solutions were added :

NaH_2PO_4/K_2HPO_4 buffer (0.1 M, pH 6.8)	15 mL
Maltose (20%)	2.5 mL

This is the same medium as described by Hopwood et al. (1985) except that PEG1000 was omitted and asparagine replaced ammonium sulfate.

*

Zinc sulfate heptahydrate	1 g
Ferrous sulfate heptahydrate	1 g
Manganese chloride tetrahydrate	1 g
Calcium chloride, anhydrous	1 g
Distilled water	1000 mL

4. YEME medium for protoplast formation (Hopwood et al., 1985)

Yeast extract	3.0 g
Malt extract	3.0 g
Bacto-peptone	5.0 g
Glucose	10.0 g

Sucrose	340.0 g
Distilled water	1000 mL

After the above solution had been autoclaved 0.05 mL of 2.5 M MgCl2.6H2O and 0.625 mL of 20% glycine were added. For protoplast preparation of S. venezuelae, the same medium was used except that 1.875 mL glycine was added and the sucrose concentration was reduced to 10% (Aidoo et al., 1990).

5. R2YE medium for protoplast regeneration (Hopwood et., 1985)

Sucrose	103.0 g
Potassium sulfate	0.25g
Magnesium sulfate heptahydrate	10.1 g
Glucose	10.0 g
Casamino acids	0.1 g
Yeast extract	5.0 g
TES buffer	5.73g
Agar	22 g
Distilled water	1000 mL

The medium was adjusted to pH 7 and autoclaved. Before use the following ingredients were added:

Dihydrogen potassium phosphate (0.5%)	5.0 mL
Calcium chloride dihydrate (5 M)	2.0 mL
Proline (20%)	7.5 mL
Trace elements solution*	2.0 mL

*	Zinc chloride	40 mg
	Ferric chloride hexahydrate	200 mg
	Cuprous chloride dihydrate	10 mg
	Manganese chloride tetrahydrate	10 mg
	Sodium borate	10 mg
	Distilled water	1000 mL

For regeneration of S. venezuelae protoplasts, the same medium was used except that 10.3% sucrose and glucose were replaced by 0.3 M sodium chloride and maltose respectively.

7. NCG agar for SV1 phage propagation (Stuttard, 1979)

Nutrient agar	2.3 g
Distilled water	100 mL

To the autoclaved solution the following sterile solutions were added :

Calcium nitrate (0.8 M)	0.5 mL
Glucose (50% w/v)	1 mL

8. Soft nutrient agar (Hopwood et al., 1985)

Nutrient broth powder	8.0 g
Agar	3.0 g
Distilled water	1000 mL

9. LB agar (Sambrook et al. 1989)

Bacto-tryptone	10.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Agar	15 g
Distilled water	1000 mL

For L broth, agar was omitted.

10. M9 minimal medium (Sambrook et al., 1989)

Sodium monohydrogen phosphate	6.0 g
Potassium dihydrogen phosphate	3.0 g
Sodium chloride	0.5 g
Ammonium chloride	1.0 g
Agar	15 g
Distilled water	1000 mL

After the solution had been autoclaved, the following sterile solutions were added :

Magnesium sulfate (1 M)	1 mL
Calcium chloride (0.01 M)	10 mL

11. Vogel-Bonner medium (Vogel and Bonner, 1956)

Magnesium sulfate heptahydrate	0.2 g
Citric acid monohydrate	2.0 g
Dipotassium hydrogen phosphate	10.0 g
Sodium ammonium hydrogen phosphate	3.5 g
Casamino acids	0.05 g
Distilled water	to 1000 mL

To the autoclaved solution, 20 mL of 25% (w/v) glucose was added.

12. Medium B (Zalkin and Murphy, 1974)

Sodium sulfate	0.1 g
Magnesium sulfate heptahydrate	0.1 g
Glucose (25% w/v)	20 mL
Casein hydrolysate	0.1 g
Ammonium sulfate (0.75 M)	1 mL

Potasssium phosphate buffer* (0.65 M) to 100 mL

* The pH of the phosphate buffer was adjusted to either 6.0 or 8.0 by mixing appropriate amounts of monobasic and dibasic potassium phosphates.

IV. Culture conditions

A. Streptomyces

1. Preparation of spore stocks

Cultures were maintained on solid media which supported sporulation. For S. venezuelae MYM medium was used while S. lividans cultures were maintained on R2YE (minus sucrose) medium. Spores were harvested, washed twice with sterile water, resuspended in 20% (v/v) glycerol and stored at -20 C. Thiostrepton-resistant transformed strains were maintained on similar media which contained 50 µg/mL of thiostrepton.

2. Vegetative inoculum

The vegetative inoculum was prepared by incubating S. venezuelae spores for 24-36 h at 30° C in 25 mL of MYM liquid medium in a 250-mL Erlenmeyer flask on a shaker rotating at 220 rpm. For S. lividans YEME medium was used under the same growth conditions.

3. Culture for accumulation of intermediates

The mycelium of the inoculum culture was centrifuged, washed twice and resuspended in sterile water to the original

volume. A portion (0.25 mL) of this suspension was used to inoculate 25 mL of minimal medium containing 10 µg/mL tryptophan and 1% (w/v) glucose. The culture was incubated at 30° C on a rotary shaker at 220 rpm for 12-16 h, and then centrifuged to pellet the mycelium. The supernatant was examined for accumulated intermediates.

4. Growth of cultures for preparation of cell extracts

Mycelium from a 2-mL portion of the vegetative inoculum culture was washed with 0.1 M potassium phosphate buffer, pH 6.8, and used to inoculate 400 mL of liquid minimal medium supplemented with containing 10 µg/mL tryptophan and 1% (w/v) glucose and contained in a 2-L flask. The culture was then incubated under the conditions described above for 16-20 h. Mycelium was harvested by centrifugation at 8000 rpm for 15 min, and then used for preparation of the cell extracts.

5. Growth of mycelia for protoplast preparation

Spores were incubated in 25 mL YEME medium at 30° C for 24-36 h on a shaker rotating at 220 rpm. Mycelium from this culture was washed twice with 10.3% sucrose and either used immediately or stored at -20° C.

6. Growth of mycelia for DNA Isolation

For isolating genomic DNA or plasmid DNA, 25 mL MYM liquid medium (for S. venezuelae) or YEME medium (S. lividans)

were inoculated with spores and then incubated at 30° for 24-36 h on a shaker at 220 rpm. For plasmid DNA isolation, 10 µg/mL thiostrepton was also added to the growth medium. To isolate larger amounts of DNA, cultures were scaled-up appropriately. Mycelium harvested from these cultures was either used immediately or stored at -20° C.

B. E. coli

1. Preparation of stock cultures

All E. coli strains were maintained on LB agar, and the cells were stored in 20 % (v/v) glycerol suspensions at -70 C.

2. Growth of cells for preparation of cell extracts

Cells obtained from an overnight culture grown in L broth at 37° C, were washed twice with 0.1 M potassium phosphate buffer, pH 7.8 and used at 1% (v/v) to inoculate 400 mL Vogel-Bonner medium. The inoculated culture was incubated at 37° C on a shaker at 220 rpm for 16-20 h. Cultures of auxotrophic strains were supplemented with appropriate amino acids or growth factors. Cells from these cultures were washed twice with the buffer described above, and used to prepare cell extracts.

3. Culture for preparing competent cells

A single colony was transferred to 10 mL L broth and shaken at 37° C overnight. A portion (0.1 mL) of this culture was used to inoculate 10 mL L broth, which was then shaken at 37° C for 2.5-3 h. This culture was used to prepare competent cells.

4. Growth of cells for DNA isolation

To isolate genomic DNA or plasmid DNA, cells were grown in appropriate volumes of L broth and shaken at 37° C overnight. Plasmid-bearing cultures were grown in the presence of 25 µg/mL ampicillin.

V. Preparation of cell extracts

Cultures of Streptomyces or E. coli were centrifuged (10,000 x g, 10 min, 4° C) and the pelleted cells were washed twice with the extraction buffer. The extraction buffer for Streptomyces contained 0.1 M potassium phosphate, pH 7.8, 0.8 M sucrose, 0.1 mM EDTA, 0.5 M potassium chloride and 6 mM 2-mercaptoethanol (Smithers and Engel, 1974) while that for E. coli contained 0.1 M potassium phosphate, pH 7.8, 1 mM EDTA, 1mM dithiothreitol (Smith and Yanofsky, 1962). The cells were resuspended in two volumes of the same buffer, chilled on ice and broken by sonication at maximum intensity: six 5-s bursts for Streptomyces and three 5-s bursts for E. coli. The sonicate was centrifuged (15,000 x g, 20 min, 4° C) and the

supernatant fluid was used as the cell extract.

VI. Assays

A. Enzyme Assays

Assays were performed with freshly prepared cell extracts. The assay mixtures were based on those described for S. coelicolor A3(2) and were incubated at 37° C for 30 min. A unit of specific activity is defined as 1 nmol of product formed or substrate used per min per mg of protein under these assay conditions.

1. Anthranilate synthetase (component I + component II) was assayed in a reaction mixture containing 100 nmol chorismic acid, 50 µmol Tris-HCl, pH 7.5, 10 µmol MgCl2, 20 µmol glutamine and cell extact in a final volume of 1 mL. Reaction was stopped by addition of 0.2 mL of 10 % (w/v) trichloroacetic acid. Precipitated proteins were removed by centrifugation and the supernatant solution was assayed for anthranilic acid content.

For assaying anthranilate synthetase component I, the reaction mixture and assay conditions were the same except that glutamine was replaced with 60 µmol of ammonium sulfate and the pH of the Tris-HCl buffer used was 8.2.

2. Phosphoribosyl(PR) transferase was assayed by measuring the amount of anthranilic acid consumed in a reaction mixture consisting of 100 nmol anthranilic acid, 300

nmol PRPP, 10 µmol MgSO4, 50 µmol Tris-HCl, pH 7.5, and cell extract in a final volume of 1 mL. The reaction was stopped by adding 0.2 mL of 10% (w/v) trichloroacetic acid. The mixture was centrifuged to remove the precipitated proteins and the supernatant was then assayed for anthranilic acid content.

3. Phosphoribisyl(PR) isomerase activity was measured by the formation of InGP from anthranilic acid and PRPP in the presence of an E. coli JA 194 trpC9830 ($TrpF^-$) cell extract which contained PR transferase and InGP synthetase but not PR isomerase. The reaction mixture contained 100 nmol anthranilic acid, 300 nmol PRPP, 2 µmol MgSO4, 120 µmol sucrose, the E. coli cell extract and the Streptomyces cell extract in a final volume of 1 mL. After the incubation, InGP was assayed in the mixture.

4. InGP synthetase was measured by the amount of InGP formed from CDRP. The reaction mixture contained 30 µmol potassium phosphate, pH 7.8, 120 µmol sucrose, 700 nmol CDRP and cell extract in a final volume of 0.5 mL.

5. Tryptophan synthetase A activity was measured as the amount of indole formed from InGP in the presence of hydroxylamine. The reaction mixture contained 200 nmol of InGP, 100 µmol potassium phosphate, pH 7.8, 120 µmol sucrose, 250 µmol hydroxylamine, pH 7, and cell extract in a final volume of 0.5 mL.

6. Tryptophan synthetase B activity was assayed by measuring the amount of indole consumed from a reaction

mixture which contained 200 nmol indole, 50 µmol potassium phosphate, pH 7.8, 120 ,25 µmol sucrose, 15 µmol serine, 20 nmol pyridoxal phosphate and cell extract in a final volume of 0.5 mL.

B. Chemical assays

1. Anthranilic acid. The assay was based on the procedure described by Jones and Westlake (1974). All incubations were at room temperature. To 1 mL of the sample 0.12 mL conc. HCl and 0.5 mL 5% sodium nitrite were added and then mixture was vortexed. After 5 min, 0.1 mL of 50 % (w/v) urea solution was added and the mixture was further incubated for 10 min. Then 2.5 mL sulfamic acid reagent (2% w/v sulfamic acid in 27.4% w/v sodium dihydrogen phosphate) was added and incubation was continued for 5 min. The absorbance of the coloured product formed by adding 1 mL of 0.2 % (w/v) N-napthylethylenediamine dihydrochloride was read at 550 nm and compared with appropriate controls.

2. InGP. InGP was oxidized to indole-3-aldehyde which was then measured colorimetrically, as described by Smith and Yanofsky (1963). A 0.4-mL portion of the sample was mixed with 0.1 mL of 1 M acetate buffer, pH 4, and 0.5 mL of 0.1 N sodium metaperiodate, and incubated for 20 min at room temperature. Then 0.25 mL of 1 N NaOH was added and the indole-3-aldehyde formed was extracted with 5 mL ethyl acetate. The ethyl acetate layer was clarified by brief centrifugation in a

clinical centrifuge and its absorbance was measured at 290 nm (indole-3-aldehyde extinction coefficient, 11,400).

3. CDRP

CDRP was assayed by measuring the amount of anthranilic acid produced from it by hydrolysis. A 10-uL sample of CDRP in 0.1 N NaOH was heated at 100° C for 5 min and the anthranilic acid produced was assayed. The difference in anthranilic acid content between the original and the hydrolyzed sample gave the amount of CDRP present in the original sample in terms of anthranilic acid equivalents.

4. Indole. Indole was converted into a coloured product by treatment with Ehrlich's reagent. To 1 mL of the sample, 0.1 mL of 1 N NaOH was added, followed by 4 mL of toluene. The tubes containing the mixture were shaken several times. When the two layers separated, 1 mL of the toluene layer was mixed with 4 mL of 95% ethanol and 2 mL of Ehrlich's reagent which had the following composition;

p-dimethylaminobenzaldehyde		36 g
Conc. HCl		180 mL
Ethanol	to	1000 mL

The mixture was incubated for 20-30 min at room temperature. The absorbance of the mixture containing the coloured product was read at 550 nm.

5. Protein. The protein content of the cell extracts was measured by the Lowry method using bovine serum albumin as the standard (Lowry et al., 1951).

VII. Isolation of Trp⁻ mutants

Spores of S. venezuelae or S. lividans were suspended in 5 mL of TM buffer and 10 mg of NTG was added. The spore suspension was then incubated at 30° C for 2 h to obtain 2-4% survival. After incubation, spores were washed with distilled water and resuspended in TM buffer. Appropriate dilutions were plated on complex medium (MYM for S. venezuelae and R2YE [minus sucrose] for S. lividans) to obtain 100-150 colonies per plate. The colonies were allowed to sporulate and then replica-plated on minimal medium and minimal medium containing tryptophan and p-aminobenzoic acid. Colonies that grew only in the presence of supplements were purified and characterized further.

VIII. Detection of intermediates in culture filtrates

The procedures were based on those described previously (Wegman and Crawford, 1968; Whitt and Carlton, 1968).

Anthranilic acid and CDR were detected by thin-layer chromatography. The culture filtrate was acidified to pH 5, and extracted with ethyl acetate. The extracted product was chromatographed on a thin layer of silica gel Sil-60 in methanol-butanol-benzene-water (2:1:1:1, v/v/v/v). Anthranilic

acid was detected under UV light (254 nm) as a blue-fluorescent zone at Rf 0.74, the same as that of an authentic sample. CDR gave a less intense blue-fluorescent zone, Rf 0.67, which when treated with triphenyltetrazolium formed a bright-red reduction product.

To detect indole the procedure described in the section on chemical assays was used.

InGP or InG were detected with $FeCl_3$ reagent as described by Smith and Yanofsky (1963). To 1 mL of the sample, 2 mL of $FeCl_3$ reagent was added. Formation of a pink colour within 5-10 min during incubation at room temperature indicated the presence of InGP or InG.

IX. Isolation of Genomic DNA from Streptomyces

A. Large scale

Genomic DNA was isolated by a procedure described by Hopwood et al. (1985). Approximately 1 g of mycelium was washed twice with 10.3% sucrose and resuspended in 5 mL freshly prepared lysozyme solution (2 mg/mL lysozyme and 50 µg/mL RNAase in 0.3 M sucrose, 25 mM Tris-HCl, pH 8.0, and 25 mM EDTA). The suspension was incubated at 37° C for 30-40 min and swirled every 15 min. Then 1.2 mL 0.5 M EDTA and 0.13 mL of pronase (2 mg/mL) were added and the contents were mixed gently by swirling the tube. To the suspension 0.7 mL of 10 %

(w/v) SDS was added. After 2 h at 37° C, 6 mL of neutral phenol/chloroform solution was added, the tube was shaken thoroughly several times for 5-10 min, and then centrifuged at 8000 rpm for 5 min. The upper (aqueous) phase was transferred with a wide-mouth pipette into a new tube and reextracted with 6 mL of neutral phenol/chloroform as before. The upper phase was transferred again to a sterile beaker and mixed with 0.1 volumes of 3 M sodium acetate. Two volumes of absolute ethanol were layered on top of the aqueous layer and the DNA was spooled on a glass rod by gently stirring the suspension with the rod. The DNA was air dried for 5-10 min and then redissolved in 5 mL TE buffer. The DNA was further purified by extractions, first with neutral phenol/ chloroform, and then with chloroform/isoamyl alcohol. It was precipated with ethanol as before, redissolved in TE buffer (10 mM Tris-HCl, 1mM EDTA) and stored at 4° C.

B. Small-scale

The procedure described by Hopwood et al.(1985) was used. Approximately 50 mg of mycelium was mixed with 0.5 mL lysozyme solution as used for large scale isolation) and incubated at 37° C for 30-40 min. Then 250 uL of 2% SDS was added to the mixture and vortexed for 1-2 min. The mixture was extracted twice with 250 uL neutral phenol/chloroform and then with same volume of chloroform/isoamyl alcohol. The DNA present in the aqueous phase was precipitated by adding 0.1 vol of 3 M sodium

acetate, pH 4.8, and 1 volume of isopropanol. After 5 min at room temperature the precipitate was collected by centrifugation for 2 min in a microfuge. The pellet was washed with 70% ethanol and then resuspended in 100 uL TE buffer.

X. Isolation of plasmid DNA from Streptomyces

A. Large scale

The alkaline lysis method described by Kieser (1984) was used. The mycelium from 500 mL of culture was resuspended in 50 mL lysozyme solution (as used for genomic DNA isolation) and incubated at 37° C. After 30-40 min, 25 mL SDS/NaOH solution (2% SDS, 0.3 M NaOH) was added and the suspension was thoroughly mixed. The resulting cell lysate was heated at 70° C for 15 min and cooled at room temperature. The suspension was extracted with 8 mL acid phenol/chloroform and centrifuged. The top (aqueous) phase was transferred to another bottle and mixed with 0.1 vol 3 M sodium acetate and 1 vol isopropanol to precipitate the DNA. The suspension was centrifuged and the pellet, washed with 70% ethanol, was redissolved in TE buffer. A sample was examined by agarose gel electrophoresis.

The plasmid DNA was purified furtherby dissolving 8.4 g caesium chloride in 8 mL TE buffer containing the sample and adding 4 mL of ethidium bromide (10 mg/mL). The solution was transferred to an ultracentrifuge tube and the top was layered with paraffin oil to exclude air bubbles before the tube was

sealed with a screw cap and centrifuged at 36,000 rpm at 4° C for 36 h. Chromosomal and plasmid DNA bands were visualized under UV light. Using a wide mouth pipette, chromosomal DNA was carefully removed and discarded; the plasmid DNA layer was then collected separately and extracted with water-saturated butanol until colorless to remove ethidium bromide. It was diluted with 2 volumes of water, followed by 6 volumes of ethanol; after 15 min at 4° C the precipitated DNA was collected by centrifugation and the pellet, washed with 70% ethanol, and redissolved in TE buffer was stored at 4° C.

B. Small scale

Mycelium scraped from the agar surface was resuspended in 0.5 mL of lysozyme solution. The rest of the procedure was the same as for the large scale isolation except that the volumes of buffers and reagents were scaled down.

XI. Isolation of plasmid DNA from E. coli

The cell pellet obtained from 500 mL of culture was resuspended in 4 mL lysozyme solution and kept on ice for 5 min before 10 mL of NaOH/SDS solution (0.2 M NaOH, 1% w/v SDS) was mixed in. After a further 5-10 min on ice, 7.5 mL ice-cold potassium acetate solution was added and the mixture was kept on ice again for 10 min. The tubes were centrifuged at 8000 rpm for 10 min and the supernatant solution was carefully decanted into another tube. The DNA was precipitated from it by adding 0.1 vol 3 M sodium acetate and two volumes of

ethanol. After 15 min at room temperature, the DNA was pelleted by centrifugation and was redissolved in TE buffer. A small portion was examined by agarose gel electrophoresis. Further purification of the plasmid DNA was carried out by caesium chloride gradient centrifugation, as described above.

For small scale isolations, the cells from 2 mL of an overnight culture were resuspended in 0.1 mL of TE buffer. The isolation procedure was the same as for large scale isolation except that the lysozyme treatment was omitted and the volumes of buffers and reagents were scaled down. To purify the plasmid DNA, the sample was extracted once with neutral phenol/chloroform and then again with chloroform/isoamyl alcohol. The DNA was precipitated, washed, and redissolved in TE buffer as before.

XII. Rapid screening of plasmid DNA in E. coli

For quick screening of E. coli colonies for the presence of recombinant plasmids, a method developed by Sekar (1987) was used. A small portion of the colony was picked up with a tooth pick and suspended in 5 uL of the protoplasting buffer (30 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM sodium chloride, 20% w/v sucrose and containing 50 ug/mL, of lysozyme and RNAase) in a microtitre plate. After incubation at room temperature for 15- 30 min, the cell lysate was loaded into wells of an 0.8% agarose gel (prepared in 1X TBE, 0.05% SDS; 5X TBE is 445mM Tris base, 445 mM boric acid and 12.5 mM sodium EDTA).

The wells were preloaded with 3 uL of TBE/SDS loading buffer (1X TBE, 2% w/v SDS, 5% w/v sucrose and 0.04% w/v bromophenol blue). Electrophoresis was carried out for 15 min at 30 v followed by 1 h at 100v. The gel was then stained and the DNA was visualized under UV light.

XIII. Transformation of Streptomyces

A. Preparation of protoplasts

Mycelium collected from a 36-h culture of S. lividans was washed twice with 10.3% sucrose and resuspended in 5 mL filter-sterlized lysis buffer containing 2 mg/mL of lysozyme. Lysis buffer had the following composition:

Sucrose (10.3%)	100 mL
TES buffer (5.73%, pH 7.2)	10 mL
Potassium sulfate (2.5%)	1 mL
Trace elements solution*	0.2 mL
Potassium dihydrogen phosphate (0.5%)	1 mL
Magnesium chloride hexahydrate (2.5 M)	0.1 mL
Calcium chloride (0.25 M)	1 mL

* same as used for preparing R2YE medium

The tube containing mycelium was incubated at 30° C for 30-45 min with gentle shaking every 15 min. The extent of protoplast formation was assessed by examining a small aliquot by microscopy. After the incubation, the protoplast suspension was mixed with 5 mL of P buffer which had the following composition:

Sucrose	103 g
Potassium sulfate	0.25 g
Magnesium sulfate heptahydrate	2.02 g
Trace element solution*	2 mL
Distilled water	800 mL

* same as used for preparing R2YE medium

The solution was dispensed in 40 mL aliquots and autoclaved. Before it was used, the following were added :

Dipotassium hydrogen phosphate (0.5%)	0.5 mL
Calcium chloride (3.68%)	5.0 ml
TES buffer (5.73%, pH 7.2)	5.0 mL

The protoplasts were mixed with P buffer by drawing the mixture into a pipette several times; they were then filtered through cotton to remove unprotoplasted mycelia. The protoplasts were pelleted by centrifugation in a clinical centrifuge for 15 min, washed twice with P buffer and then resuspended in the last drop that remained after decanting the supernatant.

To prepare S. venezuelae protoplasts, the modified procedure of Aidoo et al.(1990) was used. Sucrose was replaced in L buffer and P buffer with 0.3 M sodium chloride, and the mycelium was incubated in L buffer for 60-90 min at 37° C.

B. Transformation of protoplasts

To the freshly prepared S. lividans protoplasts, 1-2 µg DNA in TE buffer was added and mixed in by tapping the tube several times. Immediately, the contents of the tube were mixed with 0.5 mL of T buffer, which contained:

Sucrose (10.3%)	25 mL
Trace element solution*	0.2 mL
Potassium sulfate (2.5 %)	1 mL
Distilled water	75 mL

* same as used for preparing R2YE medium

A portion (4.65 mL) of this solution was mixed with 1 mL of sterilized PEG 1000 just before use to obtain T-buffer.

This was followed by addition of 5 mL of P buffer. Transformed protoplasts were then pelletted by centrifugation for 15 min, resuspended in 1 mL of P buffer and plated on regeneration medium. The plates were incubated at 30° C for 14-18 h or until the protoplats had begun to regenerate. The agar surface was overlayed with 2.5 mL of soft nutrient agar containing thiostrepton to give a final plate concentration of 25 µg/mL. Plates were further incubated for 4-5 days or until thiostrepton-resistant transformants appeared.

For transformation of S. venezuelae, an identical procedure was used except that the sucrose in T buffer was replaced with 0.3 M sodium chloride (Aidoo et al, 1990).

XIV. Transformation of E. coli

A. Preparation of competent cells

Competent cells of strain TG1 were prepared by the procedure described by Hopwood et al.(1985). The culture was chilled on ice for 15 min and then centrifuged in a clinical centrifuged for 15 min. The supernatant was discarded and the cells were resuspended in the last drop of the liquid. To this, 10 mL of chilled 100 mM calcium chloride was added and the cell suspension was mixed and kept on ice for 30 min. The suspension was centrifuged as before, and the cells were resuspended in 1 mL of 100 mM calcium chloride solution. The competent cells were dispensed in aliquots of 0.1 mL and

either used immediately or stored at -70° C.

Competent cells of all E. coli trp auxotrophic strains were prepared by a method described by Kushner (1978). The culture was chilled and then centrifuged as above. The cell pellet was washed with 4 mL of chilled MR buffer (10 mM morpholinopropane sulfonic acid (MOPS), pH 7.0, 10 mM rubidium chloride) and then resuspended in 8 mL of MCR buffer (100 mM MOPS, pH 6.5, 50 mM calcium chloride, 10 mM rubidium chloride) and kept on ice for 30 min. The suspension was then centrifuged and the cells, resuspended in 1 mL of MCR buffer, were effectively competent.

B. Transformation

Plasmid DNA in 5-10 uL TE buffer was mixed with 0.1 mL of competent cells and the mixture was kept on ice for 30 min. Cells were heat shocked at 42° C for 1 min, then transferred back on ice for 5 min and diluted with 0.9 mL of L-broth. The transformed cells were incubated at 37° C for 90 min and then plated on LB agar containing 100 µg/mL ampicillin. The plates were incubated overnight at 37° C to detect ampicillin-resistant transformants.

XV. Preparation of S. venezuelae genomic library

A. Genomic library in pTZ18R

Genomic DNA (50-100) µg of S. venezuelae genomic DNA was

mixed with 0.5 u of MboI in a 100 µL reaction mixture and incubated at 37° C. Portions (4 µL) were removed at 10, 20, 30 and 40 min and the extent of genomic DNA digestion was examined by agarose gel electrophoresis. The reaction time that gave the maximum yield of digestion products in the 8-12-kb range was determined. Additional digestions were carried out for the optimum time and the pooled digestion products were fractionated by sucrose density-gradient centrifugation.

A pollyallomer tube filled with a solution of the following composition:

Sucrose	25 g
Sodium chloride (5 M)	20 mL
Tris-HCl (2 M, pH 8.0)	1 mL
Disodium EDTA (0.5 M, pH 8.0)	1 mL
Distilled water	to 100 mL

was frozen at -70° C. It was then thawed, and the procedure was repeated several times. The MboI partial DNA digest in 0.5 mL TE buffer was layered on the top of the sucrose gradient which was then centrifuged in a SW41 rotor at 26,000 rpm for 24 h at 20° C. Samples (0.5 mL) were removed as sequential fractions after punching a hole at the bottom of the tube, and then diluted to 0.75 µL with TE buffer. The DNA present in the fractions was precipitated with isopropanol; the precipitate was dried and redissolved in 50 µL TE buffer. A sample (2-4 µL) from each fraction was analyzed by agarose gel electrophoresis along with HindIII-digested λDNA as size markers. The fractions containing 8-12-kb DNA fragments were pooled and further purified by extraction with neutral

phenol/chloroform. The DNA was precipitated with two volumes of ethanol, pelleted by centrifugation and redissolved in 50 µL TE.

The purified 8-12-kb genomic DNA fragments were mixed in a 5:1 ratio (excess genomic DNA) with pTZ18R, which had previously been linearized with BamHI and treated with calf intestine alkaline phosphatase. Ligation buffer and T4 DNA ligase were added in amounts recommended by the manufacturer's instructions, and the reaction was allowed to proceed at 16° C for 18-24 h. Subsequently, the ligated DNA was precipitated with ethanol, dried, and redissolved in TE buffer. A portion was used to transform E. coli TG1 and the transformation mixture was plated on LB-agar containing ampicillin, IPTG and X-gal which was then incubated overnight at 37° C. The ampicillin- resistant transformants included blue colonies, presumed to carry the vector alone, and white ones expected to carry a recombinant plasmid.

B. Genomic library in pIJ702

Genomic S. venezuelae DNA was partially digested with MboI for 20 min, extracted with phenol/chloroform and resuspended in TE buffer. To this, pIJ702 previously linearized with BglII was added to give a 5:1 insert:vector ratio. Appropriate amounts of ligase buffer and T4 DNA ligase were then added and ligation was allowed to proceed at 16° C for 18-24 h. The ligated DNA was precipitated with ethanol and a portion redissolved in TE buffer was used to transform S.

lividans protoplasts. Among the thiostrepton-resistant transformants were black colonies, presumed to carry the vector alone, and white ones thought to contain recombinant plasmids.

XVI. Restriction analysis of the inserts in recombinant plasmids

The recombinant plasmid was digested singly and in pairwise combinations with restriction enzymes. The DNA fragments obtained were analyzed by agarose gel electrophoresis and their sizes were estimated by comparison with HindIII and PstI digests of lambda DNA. Sites for the various restriction enzymes were mapped by rationalizing the unique arrangement of the various sized DNA fragments.

XVII. Elution of DNA fragments from agarose gels

The agarose gel slice containing the DNA fragment was suspended in 2-3 volumes of saturated sodium iodide solution in an Eppendorf tube and dissolved by incubation at 50° C for 2-3 min. Glass milk (5 µL) was mixed in and allowed to bind the released DNA at room temperature. After 5 min the glass milk was pelleted by brief centrifugation and the supernatant was discarded. The pellet was washed three times with 0.5 mL of chilled wash solution. The bound DNA was eluted by incubating the pellet in 10 µL TE buffer for 5 min at 50° C. The tubes were again centrifuged for 2 min and supernatant

containing the DNA was transferred to a fresh tube.

XVIII. Southern hybridization

A. Transfer of DNA from agarose gels to nylon membranes

After electrophoresis the agarose gel was stained with ethidium bromide and photographed. It was then soaked in 0.25 M HCl for 15-20 min to depurinate the DNA, and rinsed with distilled water. The gel was immersed twice for 15 min in denaturing solution (1.5 M NaCl, 0.5 M sodium hydroxide) at room temperature with gentle agitation. The gel was rinsed with distilled water and agitated gently (2 x 15 min) with neutralizing solution (1.5 M NaCl, 1 M Tris-HCl, pH 7.2, .05 M EDTA). The DNA was then transferred to a nylon membrane by suction (Vacublot apparatus, American Bionics) at 30 mm of Hg using 10X SSC (SSC is 0.15 M sodium chloride and 0.15 M sodium citrate, pH 7.0), for 30-40 min. The extent of transfer was determined by restaining the gel and examining it under UV light. The nylon membrane was air-dried for 15 min, sandwiched between two sheets of Whatman 3MM paper and baked in an oven at 80° C for 2 h.

B. Radioactive labelling of the DNA probe

The labelling reaction contained the following:

DNA (0.5 µg)	15 µL
dATP,dGTP and dTTP (2 µL each)	6 µL
Random primer buffer	15 µL
[^{32}p] CTP (3000 Ci/mmol)	5 µL
Klenow fragment	1 µL
Distilled water	to 50 µL

The mixture was incubated for 2 h at room temperature. The labelled DNA was then denatured at 100° C for 5 min, chilled on ice for 10 min and used for hybridization.

C. Hybridization

Hybridization was carried out in a Hybaid hybridization oven (Biocan Ltd.). The nylon membrane containing the immobilized DNA was rolled in a nylon wire mesh soaked in 2X SSC, and placed in a bottle containing 15 mL 2X SSC. The bottle was rotated gently to allow the mesh and the membrane to unroll against the walls. After 1-2 min the 2X SSC solution was replaced with 30 mL of prewarmed hybridization solution of the following composition:

20X SSPE (3.6 M sodium chloride, 0.2 M sodium phosphate, 0.02 M disodium EDTA)	6.25 mL
100X Denhardt's solution (2%(w/v) each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone)	1.25 mL
10% (w/v) SDS	1.25 mL
Denatured salmon sperm DNA (10 mg/mL)	0.5 mL
Distilled water	to 25 mL

The membrane was incubated at 65° C for 1-2 h for prehybridization. Then the denatured DNA probe was introduced into the bottle and hybridization was allowed to proceed by for 14-16 h. The nylon membrane was removed from the bottles for washing. For high stringency, the membrane was incubated twice with 2X SSC, 0.1% SDS for 10 min at room temperature. This was followed by a wash in 1X SSC, 0.1% SDS at 65° C for

15 min and a final wash in 0.1X SSC, 0.1% SDS at 65° C for 10 min. For a low stringency wash, the membrane was incubated twice in 5X SSC, 0.1% SDS solution at room temperature. After washing was complete, the membrane was covered with Saran wrap and autoradiographed.

D. Autoradiography

The membrane was exposed to an X-ray film (Kodak X-omat AR) in an X-ray cassette at -70° C for 6-16 h, the exposure time depending on the specific activity of the probe. The film was developed with appropriate photographic solutions.

XIX. Genetic Analysis

A. Conjugation

The single selection procedure for analyzing progeny from a cross (Hopwood et al., 1985; Doull et al., 1986; Stuttard, 1991) was used. Approximately 10^6-10^7 spores of a strain carrying fertility plasmids were mixed with 10-20 fold excess spores of the plasmidless (SVP1$^-$, SVP2$^-$) and allowed to proceed through a spore to spore cycle on MYM (non-selective) medium. The progeny spores were harvested, washed twice with distilled water and plated on a medium selecting one allele from each parent. Recombinants (approximately 200) were patched on the same selective medium, allowed to sporulate, and then replica-plated on various diagnostic media to determine the frequency of each member of each pair of nonselected alleles. These

"allele-ratioes" formed a clockwise and anticlockwise gradient on either side of the selected alleles. The "allele-ratio" of the new marker placed it on one of the gradients; the final position chosen was that requiring the minimum multiple crossovers.

B. Transduction

1. Preparation of high titre phage lysate

Approximately 10^6 pfu of phage SV1 grown on the donor strain was spread on NCG agar in two large (140-mm diameter) Petri plates. About 10^6-10^7 spores of the donor strain were mixed with 10 mL of molten soft nutrient agar which was then poured on each agar surface. The Petri plates were kept upright at 30° C for 24 h. Then 12 mL of phage buffer (10mM Tris-HCl, pH 7.5, 4 mM calcium nitrate and 0.5% gelatin) was added to each plate containing confluent plaques. The plates were kept at room temperature for 2 h before the buffer containing the phage particles was pooled from the two plates, filtered (0.45 u) to eliminate any spores or mycelium and centrifuged at 50,000 x g for 90 min at 4° C. Phage buffer (2 mL) was added to the tube containing the phage pellet and kept at 4° C overnight. The resulting phage suspension, which was then clarified by centrifugation for 15 min in a clinical centrifuge, represented the high titre phage lysate and contained approximately 10^{11} pfu/mL. The phage lysate was stored at 4° C.

2. Transduction procedure

The procedure for transduction has been described (Stuttard, 1979; Vats et al., 1987; Stuttard, 1992). High titre phage lysate (1 mL) containing 10^{10} pfu/mL was UV-irradiated at 254 nm to 0.1% survival. Of this phage, suspension, 0.05 mL was spread along with 0.05 mL of the recipient spores (10^{8} cfu/mL) on a medium that would support the growth of transductants, and allow germination of recipient spores but not growth to visible colony size. Control medium was spread separately with the recipient spores and UV-treated phage alone. The plates were incubated for 3-4 days at 30° C. Transductants were picked and patched on the transduction medium containing 10 mM sodium citrate (to prevent phage reinfection). After the transductants had sporulated they were phenotypically characterized by replica-plating on appropriate diagnostic media.

RESULTS

I. Isolation of Trp- auxotrophs of S. venezuelae and S. lividans

Spores of S. venezuelae were treated with NTG and the survivors were plated on MYM agar. After the colonies had sporulated, they were replica-plated on MM agar and MM agar supplemented with both tryptophan and p-aminobenzoic acid (PABA). p-Aminobenzoic acid was included because in Bacillus and Acinetobacter the trpG gene product has an amphibolic role and serves as a component of the enzymes catalyzing the first reactions in the biosynthesis of tryptophan and of PABA; if this were also true in streptomycetes, the absence of PABA would prevent growth. Examination of approximately 8000 colonies yielded only one that failed to grow on MM agar but could grow on supplemented agar. When this mutant, VS525, was examined further, it proved to be an auxotroph requiring tryptophan, but it would not grow on anthranilic acid with or without PABA. Attempts to isolate specific trpE or trpG mutants by screening with PABA-supplemented MM agar containing anthranilic acid proved unsuccessful.

In experiments similar to those carried out with S. venezuelae, spores of S. lividans TK24 were mutagenized with NTG and screened for auxotrophs able to grow on MM only when supplemented with tryptophan and PABA. Of approximately 6000 colonies examined, two, AP1 and AP2, were auxotrophic for

tryptophan alone, and one, AP3, was auxotrophic for PABA alone. Neither AP1 nor AP2 could grow in MM supplemented with anthranilic acid with or without PABA; therefore, they were not trpE or trpG mutants. The PABA-requiring mutant AP3 was not examined further.

II. Characterization of Trp- auxotrophs of S. venezuelae and S. lividans.

Mutants VS525, AP1 and AP2 along with the other previously isolated Trp- auxotrophs (Stuttard, 1983) were characterized more completely. Three properties were investigated: (a) their ability to grow on MM supplemented with tryptophan biosynthetic pathway intermediates, (b) the identity of the pathway intermediates and related metabolites that accumulated during growth and (c) the presence or absence of specific enzyme activities in cell extracts.

A. Growth studies

The growth requirements of the auxotrophs were determined by replica-plating sporulating cultures grown on MYM agar to MM agar supplemented with tryptophan, indole or anthranilic acid . Growth of cultures on replica plates was scored after 24-48 h. The results allowed the auxotrophs of S. venezuelae to be separated into two groups: in the first group were VS180, VS181, VS517 and VS525, which grew only if supplied with tryptophan. The second group contained VS24, VS153,

VS154, VS228, VS317 and VS420, which could grow in the presence of either tryptophan or indole but not anthranilic acid. The S. lividans auxotrophs AP1 and AP2 both grew in presence of either indole or tryptophan, and thus belonged to the second group.

B. Accumulation of intermediates

The auxotrophs were further classified into four classes based on the identity of pathway intermediates or related metabolites excreted during growth (Table 2). Class I consisted of VS181 and VS 525; these contained all of the intermediates and related metabolites in their culture filtrates. Since they grew on MM only in the presence of tryptophan, they were presumed to be blocked in the last step of tryptophan biosynthesis, the conversion of indole to tryptophan catalyzed by the TrpB subunit of tryptophan synthetase. Class II auxotrophs included VS24, VS180, VS317, VS517 and VS420: they accumulated AA, CDR and InG, indicating a block after InGP formation. A class IIa subgroup which consisted of VS180 and VS517 could be recognized by their failure to grow on indole, a response indicating a block in both the TrpA and TrpB subunits of tryptophan synthetase. Class IIb mutants (VS24, VS317 and VS420) formed a subgroup able to grow on indole and therefore possessing TrpB activity. They were presumed to have a defective TrpA subunit of tryptophan synthetase, and so to be unable to convert InGP to

indole. Class III mutants (VS228 and VS153) accumulated AA and CDR. Both grew on indole, and hence seemed to be blocked in InGP synthetase (TrpC)-mediated conversion of CDRP to InGP. The S. lividans mutant AP2 accumulated the same metabolites as the class III mutants of S. venezuelae and was, therefore, also a TrpC-deficient strain. Class IV contained only one strain, VS154, which could grow on indole and accumulated only AA. These characteristics could be due to a defect in either PR transferase (TrpD) or PR isomerase (TrpF). Mutants with a defective TrpF accumulate phosphoribosylanthranilate (PRA) but this is unstable and changes to anthranilic acid. Such mutants, therefore, show the same phenotype as trpD mutants. The S. lividans mutant AP1 also belonged to this category since it grew on indole and accumulated AA.

C. Enzyme assays

Of the enzyme activities for reactions in the tryptophan biosynthesis pathway of S. venezuelae all except one could be detected by assaying cell extracts (Table 3). The activity not detected was that of the TrpA subunit of tryptophan synthetase, which mediates the conversion of InGP to indole. The absence of detectable activity in S. venezuelae and S. lividans wild types may be because in general the level of enzyme activities in wild type extracts were several-fold lower than in mutant extracts. However, TrpA activity has been

Table 2. Growth supplements required and metabolites accumulated by trp auxotrophs of S. venezuelae ISP5230 and S. lividans TK24.

Strain*	Supplement required	Metabolites accumulated	Class
VS181, VS525	Trp	AA, CDR, InG, In	I
VS180, VS517	Trp	AA, CDR, InG	IIa
VS24, VS317, VS420,	In or Trp	AA, CDR, InG	IIb
VS153, VS228, AP2	In or Trp	AA, CDR	III
VS154, AP1	In or Trp	AA	IV

* The VS and AP designations identify derivatives of S. venezuelae and S. lividans TK24, respectively.

Table 3. Specific activity of tryptophan biosynthetic enzymes in cell extracts of S. venezuelae ISP5230, S. lividans TK24 and their trp auxotrophs.

Strain	Trp enzyme activity (units.mg protein^{-1}) E+G	F	D	C	B	A	Mutation
S. venezuelae							
ISP5230	0.06	1.6	0.12	0.80	0.08	ND	None
VS24 (trp-1)	1.8	2.0	0.60	1.9	0.38	ND	trpA
VS153 (trp-3)	8.4	0.55	0.90	ND	1.2	ND	trpC
VS154 (trp-4)	6.7	0.93	ND	1.4	1.7	ND	trpD
VS180 (trp-6)	8.1	1.1	1.4	1.6	ND	ND	trpA, trpB
VS181 (trp-5)	7.4	0.86	0.50	0.95	ND	ND	trpB
VS228 (trp-7)	6.5	1.5	0.96	ND	5.2	ND	trpC
VS317 (trp-9)	7.4	0.75	1.0	1.7	ND	ND	trpA
VS420 (trp-13)	5.8	1.3	1.0	2.0	0.30	ND	trpA
VS517 (trp-12)	6.7	1.9	1.2	2.5	ND	ND	trpA, trpB
VS525 (trp-14)	5.3	1.2	0.66	1.5	ND	ND	trpB
S. lividans							
TK24	0.16	0.5	0.15	1.3	0.75	ND	None
AP1	7.0	0.6	ND	0.75	3.2	0.15	trpD
AP2	7.6	0.7	0.50	ND	3.5	0.07	trpC

All specific activities are average values of duplicate assays that differed by less than 10% TrpA activity (the α subunit of tryptophan synthetase) was not measured. ND = no activity detected.

reported in trp mutants of S. coelicolor A3(2), and could be detected in cell extracts of S. lividans AP1 and AP2 mycelium (see Table 3) prepared by the same procedure as used for the wild-type strains. Trp A activity could also be detected in cell extracts of an E. coli trp auxotroph blocked in TrpF but with intact TrpC activity; this TrpA activity was not affected when S. venezuelae cell extracts were added to the E. coli assay mixture, indicating that the inability to detect TrpA activity in any S. venezuelae trp auxotrophs was not due to the presence of inhibitors in the cell extracts or to suboptimal assay conditions. Addition of 1 mM phenylmethyl-sulphonyl fluoride (a serine-ester type protease inhibitor) to the S. venezuelae cell extracts did not result in detectable TrpA activity.

Since TrpA activity could not be demonstrated in the wild- type S. venezuelae strain, or in VS181 (which excretes indole and so must possess intact TrpA activity in vivo), its absence from cell extracts could not be used as evidence for a trpA mutation. Despite the lack of this confirmatory test, mutants which grew on indole and excreted InGP were considered to have a lesion in that gene.

Enzyme assays gave results consistent with the metabolic blocks deduced from other evidence to be present in each of the S. venezuelae and S. lividans mutants (see Tables 2 and 3). Class I auxotrophs VS181 and VS525 lacked tryptophan synthetase TrpB subunit activity and therefore carried a

mutation in trpB. Among the presumptive trpA mutants, two subgroups could be distinguished based on whether or not they also lacked TrpB activity. The group represented by VS24 and VS420 showed TrpB activity in cell extracts so the requirement for tryptophan was assumed to be due to a defective trpA. Mutant VS317, though it failed to show TrpB activity in cell extracts, did grow slowly on indole; therefore, it was assumed to have weak TrpB activity and was included in this group as a trpA mutant. The other trpA group was represented by VS180 and VS517, which lacked TrpB activity in cell extracts. This was consistent with their inability to grow in the presence of either indole or anthranilic acid. The absence of both the TrpA and TrpB subunit activities of tryptophan synthetase suggests that they are either trpA trpB double mutants or polar trpA or trpB mutants. Because the direction of transcription is unknown, either of the latter is possible.

From the pattern of their accumulation of metabolic intermediates, the class III strains VS153 and VS228, as well as S. lividans AP2 were predicted to lack InGP synthetase activity. This was confirmed by enzyme assay, and hence these strains are trpC mutants. The class IV auxotrophs, VS154 and S. lividans AP1, gave cell extracts which showed PRisomerase but not PRtransferase activity; this confirmed their assignment as trpD mutants.

III. Transductional mapping of trpB14 in VS525

Generalized transduction allows mutations to be closely mapped relative to neighbouring mutations on the chromosome. In S. venezuelae mutations may be mapped to a distance shorter than the genome size of the transducing phage, SV1. The SV1 phage-mediated transduction system present in S. venezuelae is the only one used extensively in genetic analysis of streptomycetes. Mutations in all of the previously available tryptophan-requiring auxotrophs had already been mapped relative to other chromosomal mutations (Stuttard, 1983a). In the present study, the trpB14 mutation in the newly isolated strain VS525 was mapped relative to the trpC3 and hisB3 mutations (Table 4).

In a transductional cross, phage SV1 was grown on the donor strain VS34 (hisB3) and a high titre phage lysate was prepared; this was subsequently used to infect the recipient strain, VS525 (trpB14). Trp+ transductants were selected on MM containing histidine. A 11.8% cotransduction frequency was observed between hisB3 and trpB14. In a reciprocal cross using VS525 as the donor and VS34 as the recipient strain, 14% cotransduction of these markers was observed. A separate transductional cross where VS153 was the donor and VS525 was the recipient gave 50% cotransduction of the trpC3 and trpB14 markers. These frequencies for trpB14 cotransduction were similar to those obtained in crosses involving trpB5 (Stuttard, 1983a), which was as expected since in the present

study both were confirmed to be trpB auxotrophs. From the cotransduction frequencies obtained here and those reported earlier (Stuttard, 1983a), the cluster of his and trp genes mapping to the 12'clock region of the S. venezuelae chromosome can be ordered as shown in Figure 4.

Table 4. Cotransduction of *trpB14* with *hisB3* and *trpC3*

Cross*		Medium	Transductants	
Donor	Recipient		Number tested	Donor type (%)
VS34	VS525	MM + histidine	127	11.8
VS525	VS34	MM + tryptophan	131	14
VS153	VS525	MM + indole	128	50

* The markers present in the strains are : VS34, *hisB3*; VS525, *trpB14* and VS153, *trpC3*, *cml-5*.

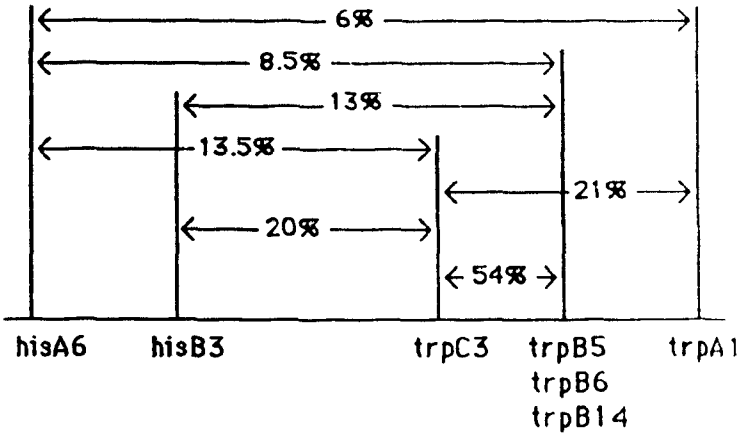


Figure 4. Relative locations of trp and his mutations in the ... venezuelae chromosome (modified from ... (1967). The values are the average ... at the allele pairs.

IV. Molecular cloning of trpE gene from S. venezuelae ISP 5230

Because auxotrophs defective in trpE or trpG had not been obtained by mutagenic treatment of S. venezuelae or other streptomycetes, alternative means of isolating such mutants were explored. As part of a strategy for locating trpE in S. venezuelae, an effort was made to clone wild type trpE DNA which could then be used in three ways. First, the DNA fragment containing trpE could be used as a probe in hybridization experiments to find out whether similar sequences were present in previously cloned regions of the S. venezuelae chromosome carrying trpC and trpD genes (Section X). Second, the chromosomal location of trpE or trpG genes could be determined by homologous recombination. In this procedure, a vector containing DNA of interest and a drug resistance marker is allowed to integrate into the chromosome of the homologous host. The chromosomal location of the integrated DNA is then determined by mapping the drug resistance marker using recombination analysis. Third, the results obtained by plasmid integration might be extended to isolate a trpE mutant by gene disruption or allele replacement.

The protocol used for cloning trpE is summarized in Figure 5. From the proportion of white colonies obtained when it was used to transform E. coli TG1 (ΔlacZ) the S. venezuelae genomic library constructed in pTZ18R contained 80%

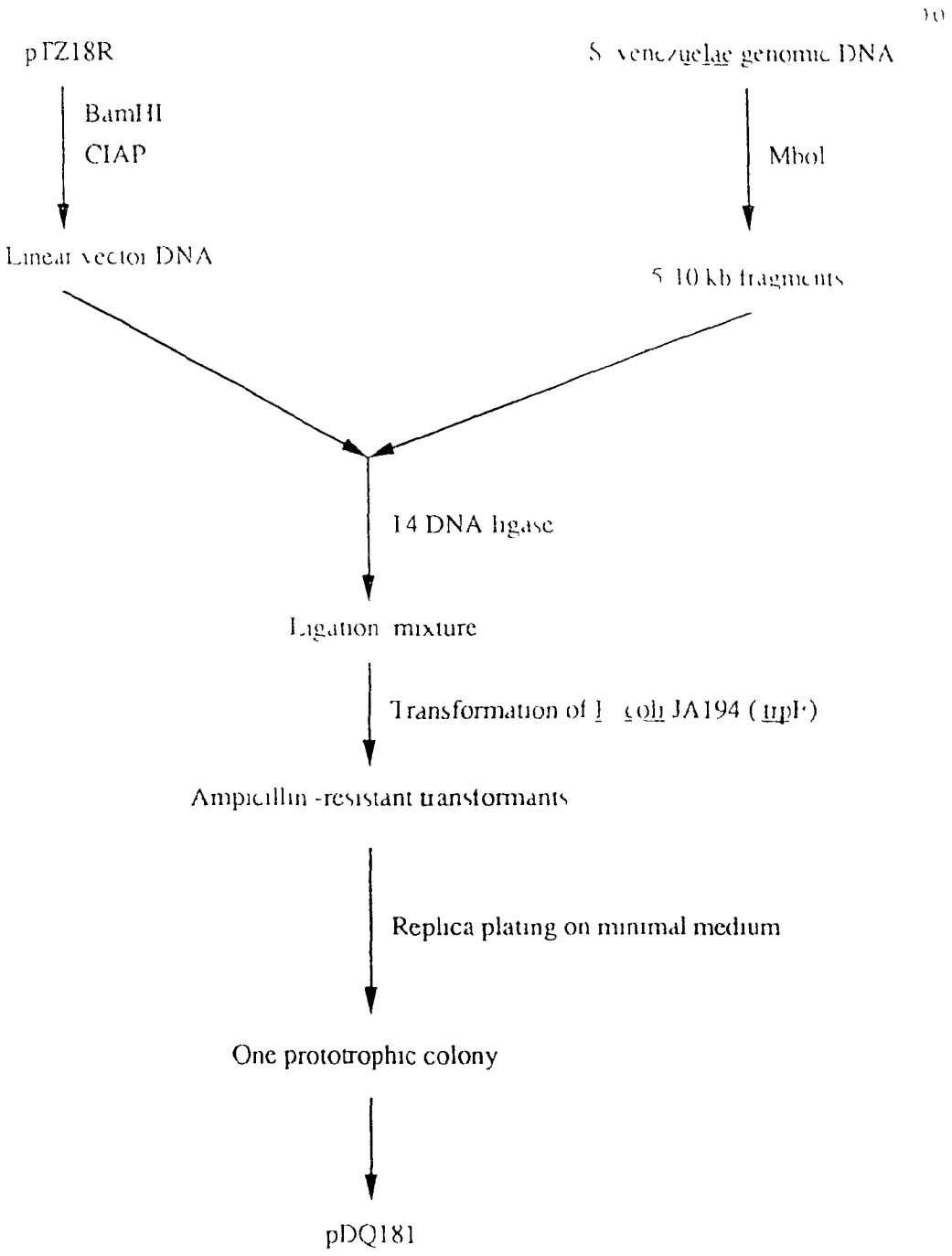


Figure 5. Protocol for the cloning of S. venezuelae trpE DNA by complementation of a trpE mutant of E. coli. CIAP, calf intestine alkaline phosphatase

recombinant plasmids. The library was introduced into E. coli JA194 (ΔtrpE) and approximately 6000 of the ampicillin-resistant transformants were tested for growth on minimal medium. A single prototrophic colony was isolated.

The colony-purified prototrophic transformant contained a recombinant plasmid. However, extraction of plasmid DNA from this strain and use of the plasmid preparation to transform JA194 gave a mixture of transformants, only 60% of which were prototrophic. Examination by gel electrophoresis of plasmid preparations from a number of the transformants showed that many of the plasmids had undergone deletions during the initial propagation in E. coli JA194. The original recombinant plasmid (pDQ181) was rescued by using the initial plasmid preparation to transform E. coli JA200 (trpE, recA) and selecting for prototrophy. In the new host, pDQ181 was stably maintained. When extracted from a JA200 transformant and used to transform JA200 again, it gave ampicillin-resistant transformants which were all prototrophic, confirming that the stable Trp^+ phenotype was plasmid linked.

A. pDQ181

Plasmid pDQ181 was digested with several restriction enzymes, singly or in pairwise combinations, and the sizes of the resulting DNA fragments were estimated by comparing their electrophoretic mobility with those of DNA fragments obtained by digesting lambda DNA separately with HindIII and PstI.

Digestion of pDQ181 with EcoRI, HindIII and KpnI gave a single band, estimated to be approximately 9.7 kb in size. Since the vector pTZ18R contained 2.9 kb of DNA, the size of the insert in pDQ181 was 6.8 kb. A restriction map of the plasmid is shown in Figure 6.

To determine whether the trpE gene present on the 6.8-kb insert was expressed from its own promoter, the insert was excised from pDQ181 as an EcoRI-HindIII fragment and recloned into pTZ19R cut with EcoRI and HindIII. This vector is identical to pTZ18R except that the multiple cloning cassette is oppositely oriented relative to the lac promoter. Insertion of the 6.8-kb insert gave pDQ180 which differed from pDQ181 only in the fragment orientation. When pDQ180 was used to transform E. coli JA200, the ampicillin-resistant transformants failed to grow on minimal medium. The inability of S. venezuelae trpE DNA in its reversed orientation to complement the JA200 trpE mutation suggests that the gene is expressed from the lac promoter in pDQ181.

B. Subcloning of trpE

To further locate the trpE gene present on the 6.8-kb insert, various segments of pDQ181 were deleted and the resulting plasmids tested for their ability to complement the JA200 trpE mutation. To construct these derivative plasmids, the desired restriction fragments were isolated from agarose

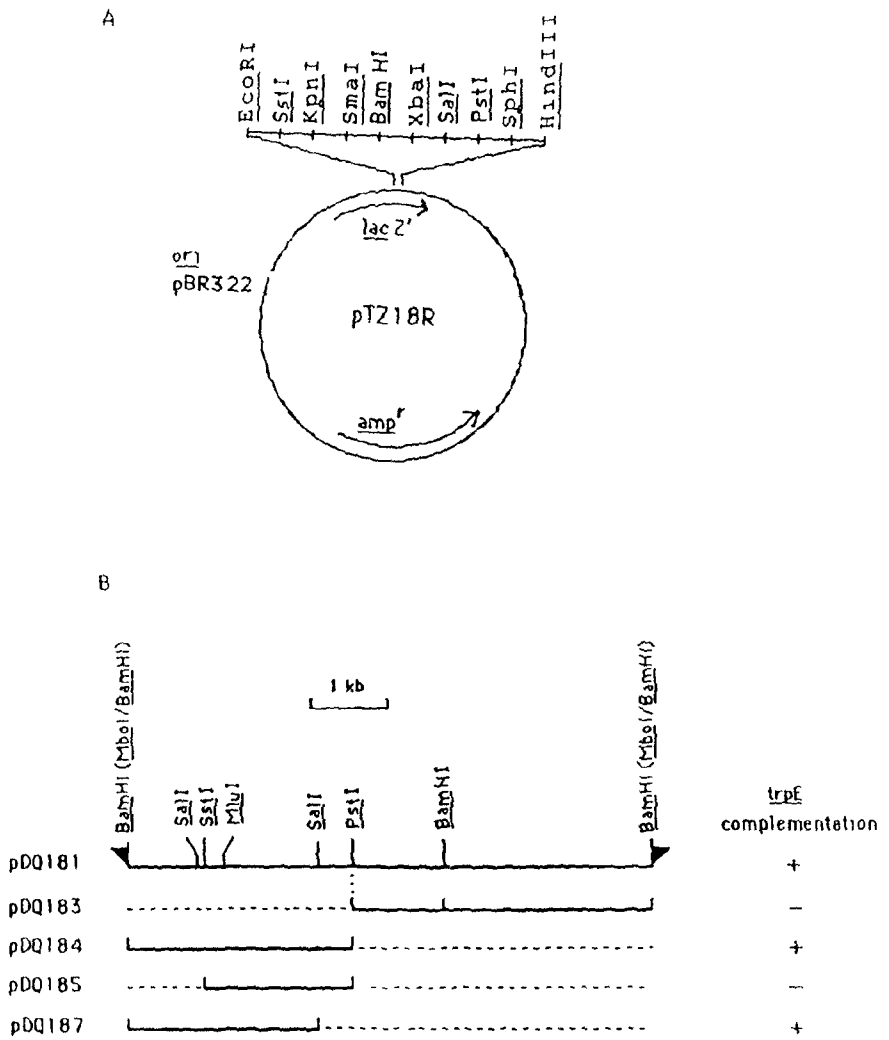


Figure 1. (A) Plasmid pTZ18R (B) Restriction map and deletion analysis of the S. venezuelae DNA insert in pDQ181

gels, purified by "geneclean" and ligated to pTZ18R cut with the same enzymes. Two BamHI-PstI fragments, one of 3 kb and the other of 3.8 kb, were cloned to form pDQ184 and pDQ183, respectively. When tested, only pDQ184 was able to complement the trpE mutation. To facilitate further deletion analysis, pDQ184 was mapped with the restriction enzymes SalI and SstI. When a 0.9-kb BamHI-SalI segment towards the left junction of the insert (Figure 3) was deleted from pDQ184, the resulting plasmid, pDQ185, failed to complement the trpE mutation. The trpE gene was confirmed to be within the 2.4-kb BamHI-SalI segment by constructing pDQ187 containing this segment and demonstrating its ability to complement the mutation in E. coli JA200.

C. Linkage of trpG and trpE

To determine whether the trpE-containing S. venezuelae DNA fragment also encoded the trpG function, pDQ181 was used to transform E. coli trpED23 which has a deletion in trpE extending into the first third of the trpD gene (thus inactivating the trpG component which comprises the 5'one-third of trpD in E. coli). All the transformants were prototrophic and grew on MM without tryptophan. In a control experiment, the ED23 host transformed with pTZ18R alone retained its requirement for tryptophan. However, since MM

contained ammonium as the nitrogen source, it was possible that the S. venezuelae trpE gene product alone catalyzed synthesis of anthranilate using ammonium as the amino donor. Therefore, further growth studies and in vitro enzyme assays were done to ascertain if a trpG function was indeed present on pDQ181. Using either ammonia (to detect ASI) or glutamine (to detect ASII) anthranilate synthetase activity was found in cell extracts of E. coli trpED23 transformed with pDQ181, pDQ184 or pDQ187, but not in extracts of the host transformed with pTZ18R (Table 5). This suggested that the DNA insert in these recombinant plasmids contained trpG as well as trpE coding regions.

The results from growth studies were also consistent with the presence of trpE and trpG on the cloned S. venezuelae DNA. In medium B, which contained a low concentration of ammonium, strains depending solely on the trpE function for tryptophan synthesis grow at pH 8 where about 5% of the ammonium salt is undissociated and can be assimilated. They do not grow in the same medium at pH 6 where most of the ammonium is present as NH4+ and is not available for tryptophan biosynthesis (Zalkin and Murphy, 1975). This selective medium has been used to detect the presence of trpG on the cloned DNA from Spirochaeta aurantia (Brahamsha and Greenberg, 1987). The ED23 transformants containing either pDQ181, pDQ184 or pDQ187 grew in medium B at both pH 6 and pH 8 (Table 6), indicating that in S. venezuelae, trpG was present along with trpE.

Table 5. Anthranilate synthetase activity in E. coli ED23 (trpED23) carrying pDQ181 or its deletion derivatives and pTZ18R.

Strain (plasmid)	Specific activity (units/mgprotein)	
	ASI	ASI + ASII
1. ED23 (pTZ18R)	Nil	Nil
2. ED23 (pDQ181)	0.19	1.34
3. ED23 (pDQ184)	0.24	1.23
4. ED23 (pDQ187)	0.20	1.46

Table 6. Growth of E. coli ED23 (trpED23) carrying pDQ181 or its deleted derivatives and pTZ18R, in medium B.

Strain (plasmid)	Growth* in medium B	
	pH 6.0	pH 8.0
ED23 (pTZ18R)	0.017	0.017
ED23 (pDQ181)	0.75	0.62
ED23 (pDQ184)	0.82	0.72
ED23 (pDQ187)	0.76	0.90

* Growth was measured as optical density at 660 nm after 24 h. The initial optical density was approximately 0.015.

D. Southern hybridization of trpEG to S. venezuelae and several other streptomycetes

To examine for sequence similarities between the cloned S. venezuelae trpEG DNA and regions in the genomic DNA of other streptomycetes, and to confirm that the trpEG-containing insert in pDQ187 had indeed originated from S. venezuelae ISP5230, genomic DNA of several streptomycetes was probed with the 2.4-kb DNA insert from pDQ187. Genomic DNA samples of S. venezuelae ISP5230, S. venezuelae 13s, S. phaeochromogenes, S. parvulus, S. griseofuscus, S. lividans and E. coli TG1 were digested with BamHI, fractionated on agarose gels by electrophoresis and then transferred to a nylon membrane. The membrane was probed with the radioactively labelled DNA which had been isolated from pDQ187 as 0.9-kb BamHI-SalI and 1.5-kb SalI fragments and mixed. When the membrane was washed at high stringency, hybridization was observed to genomic DNA fragments of S. venezuelae ISP5230 and 13s, S. phaeochromogenes, S. parvulus and S. griseofuscus, but not to those of S. lividans or E. coli (Figure 7). At lower stringency (data not included), genomic DNA of S. lividans showed weakly hybridizing bands. In a separate experiment, the same probe hybridized to S. coelicolor A3(2) genomic DNA even at high stringency (Figure 8). Although the insert DNA hybridized to the genomic DNA of all other streptomycetes tested, the degree of sequence similarity indicated by the intensity of the radioactive signals on autoradiographs showed

Figure 7. Southern hybridization of genomic DNA samples using as a probe the S. venezuelae 10712 BamHI:SalI DNA fragment containing trpEG.

(A) Separation of fragments in BamHI digests of genomic DNA samples by agarose gel electrophoresis; lane 1, S. venezuelae ISP5230; lane 2, S. venezuelae 13s; lane 3, S phaeochromogenes; lane 4, S. parvulus; lane 5, S. griseofuscus.

(B) Autoradiogram after transfer of the DNA fragments to a nylon membrane and hybridization with the probe.

Figure 7.

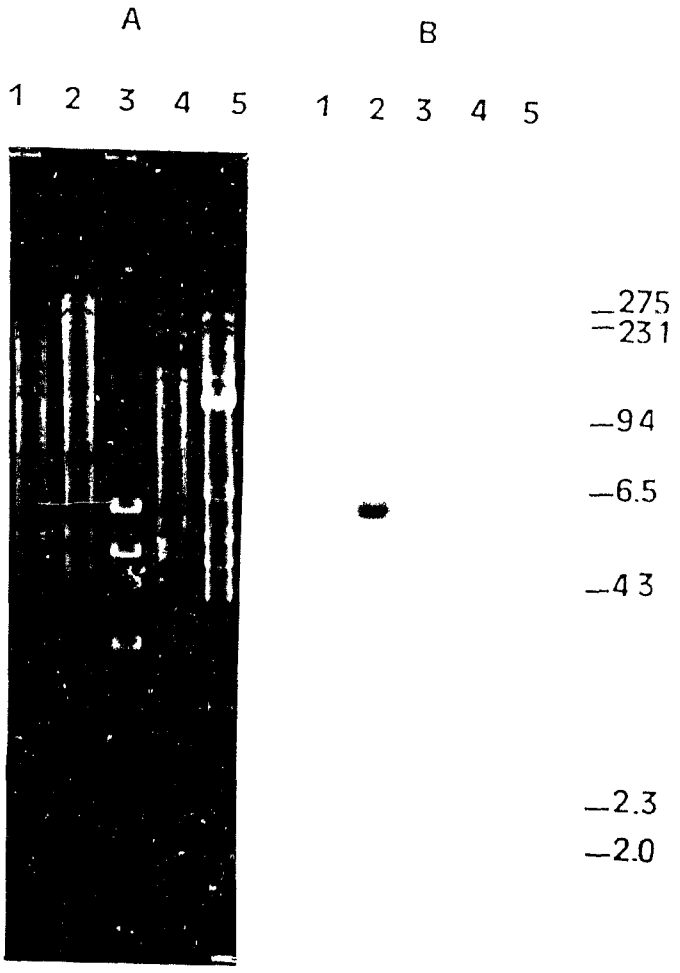
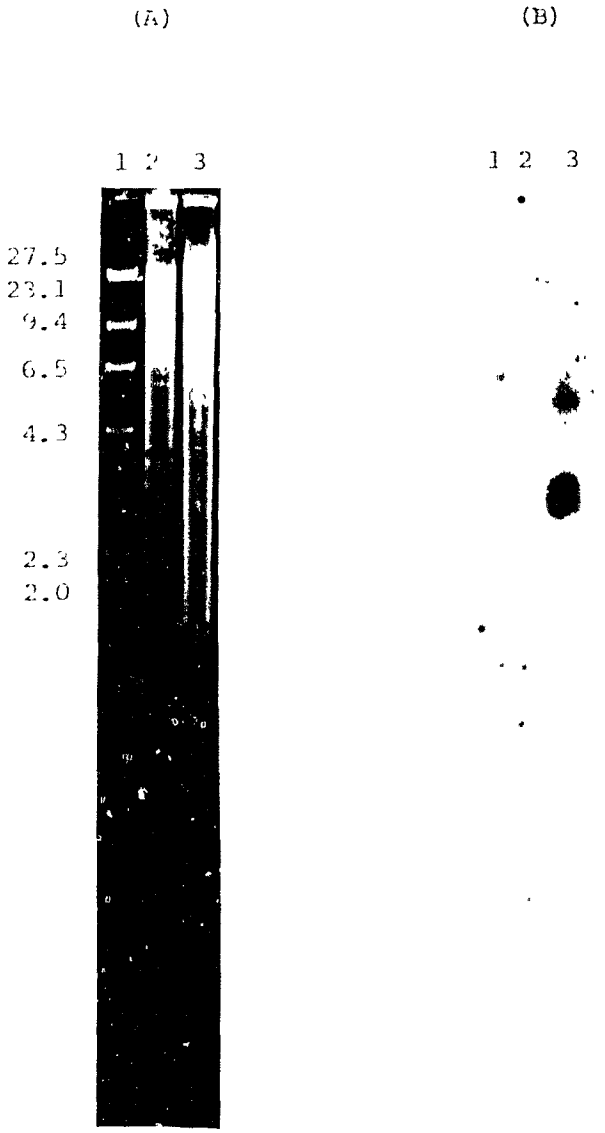


Figure 8. Southern hybridization of genomic samples using the S. venezuelae 10712 BamHI:SalI DNA fragment containing trpEG as a probe.

(A) Gel electrophoresis of genomic DNA samples: lane 1, Lambda DNA HindIII digest; lane 2, S. venezuelae ClaI+PstI digest; Lane 3, S. coelicolor BamHI digest.

(B) Autoradiogram after transfer of the DNA fragments to a nylon membrane and hybridization with the probe.

Figure 8.



marked differences. As expected there were also differences in the sizes of the BamHI restriction fragments.

V. **Attempts to locate trpEG on the S. venezuelae chromosome**

None of the trp auxotrophs of S. venezuelae described in the earlier section exhibited the characteristics expected of trpE or trpG mutants, and attempts to isolate such mutants by the usual mutagenic treatments were not successful. Since the lack of mutants hindered mapping of these genes, two alternative approaches to obtaining them were explored. The first was aimed at finding whether other trp genes (for which the chromosomal location was known) were present on the cloned 6.8-kb S. venezuelae DNA that complemented the trpE and trpG mutations in E. coli. Since there was no information suggesting that trpEG was clustered along with other trp genes, this was a "hit or miss" approach and was pursued by complementation and hybridization experiments. The second approach exploited homologous recombination. It was a more systematic attempt to determine the chromosomal location of trpEG. The results obtained using these approaches are discussed below.

A. Test for ability of pDQ181 to complement other trp mutations in E. coli

To determine if trpEG was clustered with other trp genes, the ability of pDQ181, which contains the original 6.8-kb S.

venezuelae insert, to complement other trp mutations was tested. The plasmid was introduced by transformation into trpD, trpF, trpC, trpB, and trpA auxotrophs of E. coli, and approximately 100 ampicillin-resistant transformants of each auxotroph were replica-plated on MM. None of the transformants was prototrophic. Although these negative results suggested that trpEG was not clustered with other trp genes, they could not be considered conclusive evidence. Thus other trp sequences might be adjacent to trpEG in the chromosome but not present on pDQ181, or not present in a complete form. The possibility that other trp genes or segments were present on pDQ181 but were not expressed due to experimental constraints was explored in the following hybridization experiment.

B. Test for hybridization between pDQ181 and other cloned trp sequences

Southern blots of plasmids pDQ171-176 which complemented the trpD mutation in S. lividans AP1, pDQ177 which complemented the trpC mutation in S.lividans AP2 (section X), and pDQ181 were probed at high stringency with radioactively-labelled plasmid pDQ181 linearized with EcoRI. The probe hybridized strongly to the sample of pDQ181 included as a positive control but no hybridization was detected to the plasmids carrying trpC or trpD DNA. This experiment showed that not only did the 6.8-kb S. venezuelae DNA fragment encoding trpEG not contain trpC or trpD sequences, but also

that trpE and trpG were not present on the cloned S. venezuelae DNA fragments containing trpC or trpD.

C. Approaches based on homologous recombination

Introduction of a vector carrying the cloned trpEG segment of DNA into S. venezuelae should allow the recombinant vector to integrate by homologous recombination into the chromosomal region containing trpEG. The use of a vector containing a selectable marker such as tsr would tag the chromosomal location of trpEG with the marker. The position of trpEG could then be determined by mapping tsr with respect to other chromosomal markers by classical genetic methods.

In the first attempt to integratively transform S. venezuelae in this way, a recombinant non-replicative vector was used; however, no transformants could be obtained. In the second attempt a segregationally unstable replicative vector was used successfully. The construction of the recombinant plasmid vectors, which was done using E. coli TG1 as the host strain, and results obtained with them, are described below.

1. Nonreplicative vector(pDQ188)

A fragment containing the tsr gene was cloned in pDQ181 to give the plasmid pDQ188, which had a 6.8-kb segment of S. venezuelae DNA containing trpEG, and a tsr marker selectable in streptomycetes. However, its origin of replication

functioned in E. coli but not in Streptomyces. With these features pDQ188 should not be able to replicate extrachromosomally in S. venezuelae but it was expected that stable thiostrepton-resistant transformants would be obtained by integration of the vector into the host chromosome via the 6.8-kb homologous DNA. Plasmid pDQ198 in which the tsr fragment alone was inserted in pTZ18R served as a non-homologous control.

Samples of pDQ188 and pDQ198 isolated from E. coli TG1 were used to transform S. venezuelae VS194 (hisA6, adeA10, SVP1$^-$, SVP2$^-$). The choice of the fertile strain VS194 as the host was intended to facilitate mapping of the integrated tsr by conjugation. However, in three different attempts using 1-5 µg of plasmid DNA, no thiostrepton-resistant transformants were obtained.

2. Segregationally unstable vector pDQ189

The shuttle vector pHJL400 contains the minimum essential replicon of plasmid SCP2* from S. coelicolor A 3(2), and the tsr marker selectable in streptomycetes. However, it lacks the par region and is therefore segregationally unstable (Larson and Hershberger, 1986). Growth of transformants in the absence of thiostrepton gives a high frequency of plasmidless thiostrepton-sensitive strains. Since pHJL400 also contains an E. coli replicon and an amp marker functional in E. coli, it can readily be manipulated and propagated in that host. To

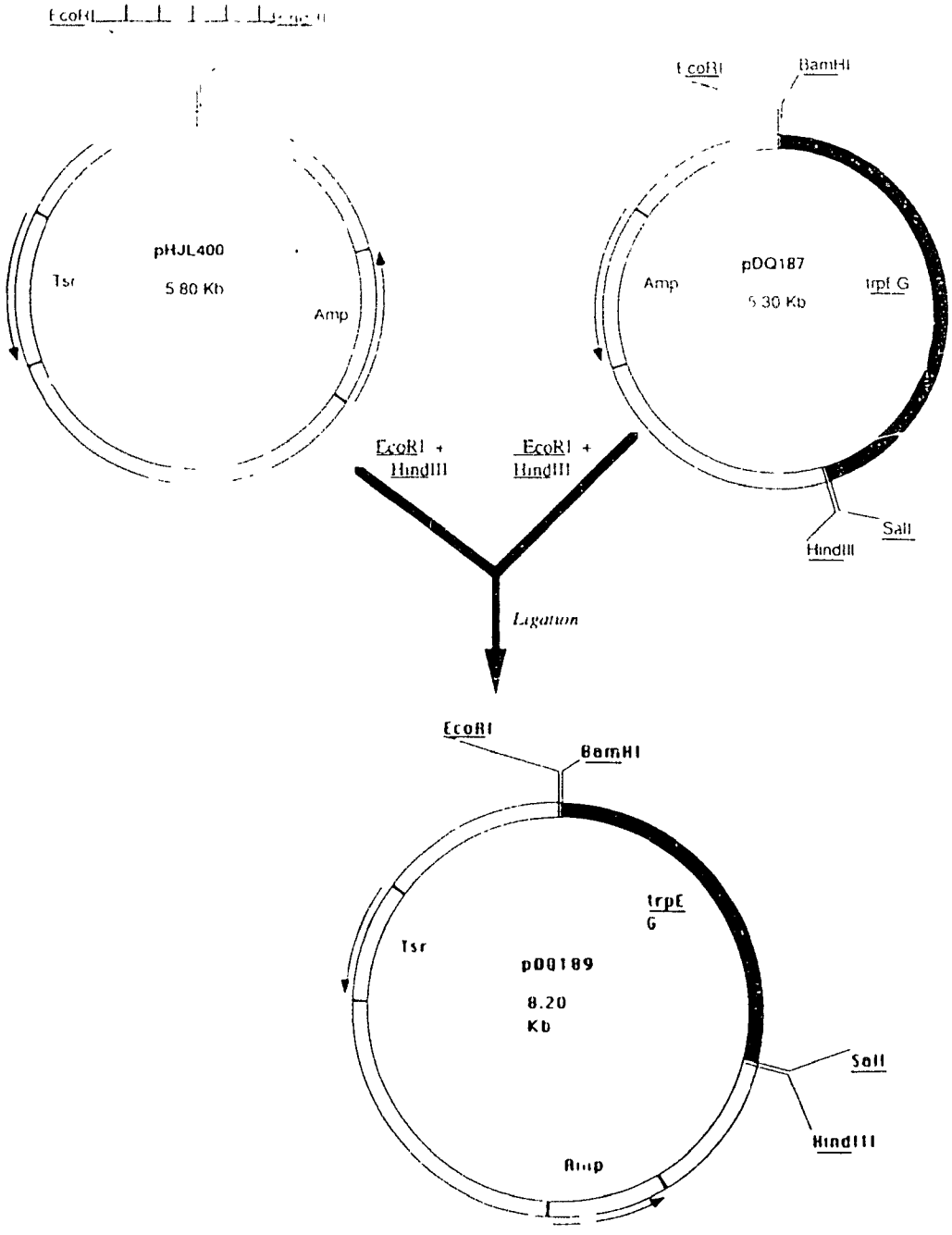
construct pDQ189 a 2.4-kb EcoRI-HindII fragment was excised from pDQ187 and ligated with pHJL400 linearized with the same enzymes (Figure 9).

Both pDQ189 and pHJL400 were isolated from E. coli TG1 and used to transform VS194 to thiostrepton resistance. The transformants were patched on MYM agar lacking thiostrepton and allowed to sporulate; the spores were again patched on MYM agar. The cultures were allowed to undergo another round of sporulation in absence cf selection before plating on MYM agar to give 100-150 colonies per plate. After the colonies had sporulated they were replica-plated in parallel to MYM agar and to MYM agar with thiostrepton (50 ug/mL). As predicted, growth of the transformants in the absence of thiostrepton selection yielded thiostrepton-sensitive colonies. From the number of colories that grew on MYM agar but not on MYM agar with thiostrept

on, the frequency of drug resistance loss in VS194 (pDQ189) under the conditions used was estimated to be 75%. In contrast, more than 98% of the colonies from VS194 transformed with pHJL400 had lost thiostrepton resistance under these conditions. These results indicated that most of the stable thiostrepton-resistant colonies of VS194 transformed with pDQ189 were likely to contain the plasmid integrated into the chromosome at the trpEG locus.

Figure 9. Construction of pDQ189. A 2.4-kb EcoRI-HindIII fragment was excised from pDQ187 and ligated with pHJL400 linearized with the same enzymes. When the ligation mixture was used to transform E. coli TG1, some of the transformants contained pDQ189.

Figure 9.


EcoRI
BamHI
pHJL400
5.80 Kb
Tsr
Amp
pDQ187
5.30 Kb
Amp
trpE G
EcoRI + HindIII
EcoRI + HindIII
SalI
HindIII
Ligation
EcoRI
BamHI
trpE G
Tsr
pDQ189
8.20 Kb
SalI
HindIII
Amp

VI. Integration of pDQ189 into the chromosome

Integration of pDQ189 into the S. venezuelae VS194 chromosome was confirmed by Southern hybridization. Single thiostrepton-resistant colonies were chosen from nine different stable transformants (integrants) that had been propagated in the absence of thiostrepton for three spore-to-spore cycles. The colonies were subcultured to sporulation on MYM agar and the spore preparations were used individually to inoculate 10 mL portions of MYM medium; after incubation for 24 h, the cultures were harvested and genomic DNA was isolated from the mycelium. Figure 10 shows the results of the Southern analysis, using pHJL400 as the probe, of uncut genomic DNA from the nine integrants (AP 41-49), from VS194 transformed with pHJL400 (AP50), from untransformed VS194 and from VS194 transformed with pHJL400 but no longer thiostrepton-resistant after nonselective growth (AP51). As expected, no hybridization was observed to DNA from untransformed VS194 or AP51. The radioactivity observed in the lanes containing DNA from these strains are probably due to contamination from adjacent lanes. This lack of hybridization is also evident in Figure 16B. As expected, strain AP50, containing pHJL400 gave two hybridizing bands corresponding to the CCC and OC forms of the freely replicating plasmid. No comparable hybridization was observed in the DNA samples from the integrants. Instead, the probe hybridized to the high molecular weight chromosomal DNA. This suggested that these

Figure 10. Southern hybridization at high stringency of uncut genomic DNA samples of S. venezuelae VS194, and its transformants using pHJL400 as the probe.

(A) Gel electrophoresis of DNA samples : lane 1, pDQ189 DNA; lane 2-10, AP41-49; lane 11, AP50; lane 12, VS194; lane 13, AP51; lane 14, VS194 DNA mixed with pHJL400 DNA; and lane 15, pHJL400 DNA.

(B) Autoradiogram after transfer of DNA to a nylon membrane and hybridization with ^{32}P-labelled pHJL400.

Figure 10.

(A)

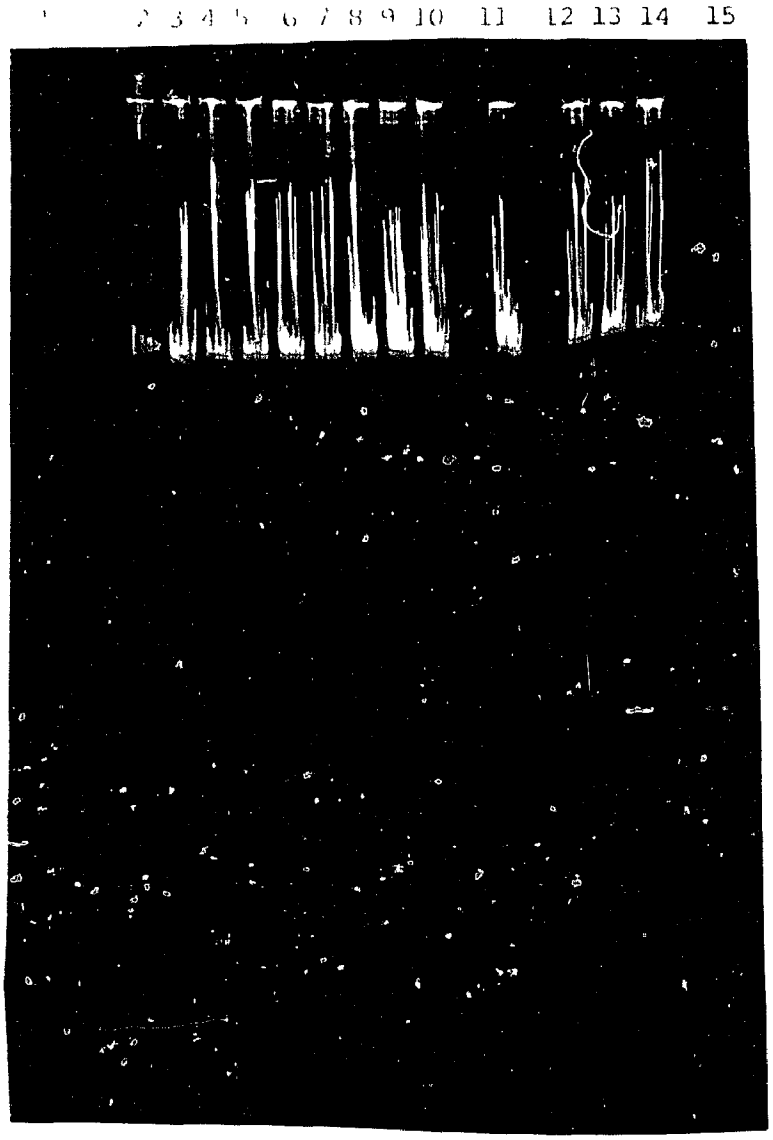
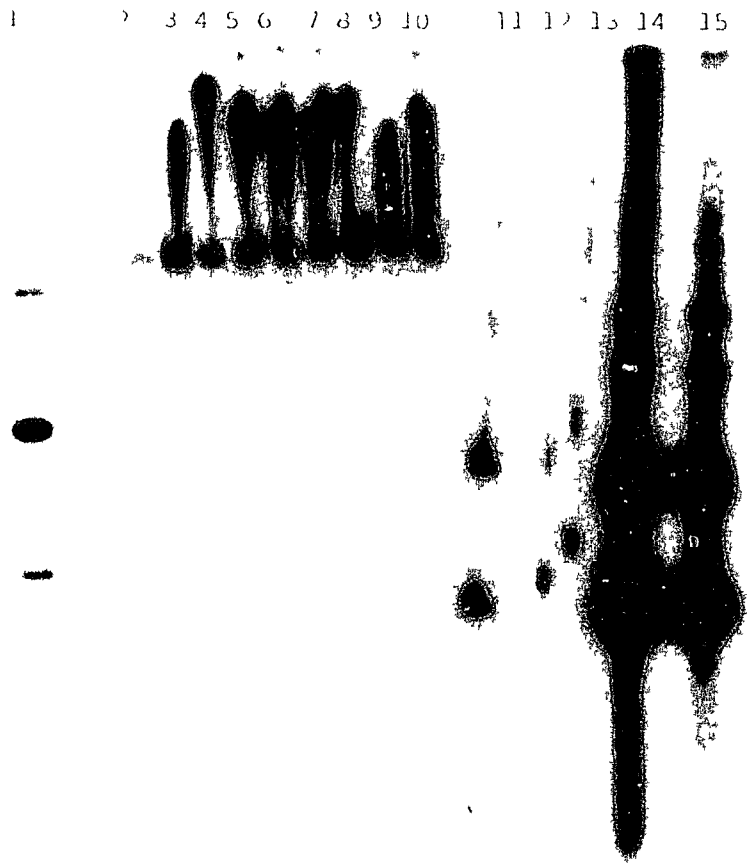


Figure 10.

(E)



strains lacked freely replicating pDQ189 and that the plasmid had either formed a high molecular weight multimer inseparable from chromosomal DNA under the electrophoretic conditions used, or had integrated into the chromosome.

To distinguish between these possibilities, Southern blots of genomic DNA of VS194 and an integrant, AP41, digested singly and in pairwise combinations with several restriction enzymes, was probed with pDQ189. Previous experiments had shown that pHJL400 does not hybridize to the genomic DNA of VS194 (see Figure 10). Since pDQ189 did hybridize, it was the 2.4 kb DNA insert from S. venzuelae present in pDQ189 that hybridized to VS194 DNA. The results (Figure 11) showed that pDQ189 hybridized to a single fragment from VS194 DNA digested with BglII, BglII+PstI, PstI+ClaI, PstI+KpnI or KpnI. This was consistent with previous evidence from restriction enzyme mapping of the cloned trpEG fragment in pDQ189 (see Figure 6) that there are no recognition sites for these restriction enzymes in the 2.4-kb fragment of S. venezuelae VS194 DNA in pDQ189. Based on the sizes of the fragments obtained with the above restriction enzymes (Table 7), a restriction map of the S. venezuelae chromosomal DNA in the trpEG region was constructed (Figure 12, top portion).

Integration of single or multiple copies of pDQ189 into the homologous chromosomal allele by single-crossover Campbell-type recombination should introduce the restriction sites present on pDQ189 into the chromosome, as shown in

Figure 12 a and b. Hybridization of radioactive pDQ189 to restriction digests of genomic DNA from AP41 and from the parental strain made it possible to test this model of integration, and also to determine if single or multiple copies of pDQ189 had integrated into the S. venezuelae chromosome.

Since there are no sites for the restriction enzyme BglII in the 2.4 kb-trpEG DNA or in the 5.8-kb vector portion of pDQ189, integration of a single copy of pDQ189 should result in an increase in the size of the chromosomal BglII fragment carrying the trpEG DNA by 8.2 kb. Alternatively, if two or more copies of pDQ189 have integrated in tandem, the chromosomal BglII fragment carrying trpEG should show an appropriate increase in size. Indeed, the 10.5-kb BglII fragment in DNA from the parental strain was replaced by a higher molecular weight fragment in DNA from AP41. In the region of the gel containing the AP41 fragment accurate size determination was not possible; however, comparison with lambda DNA fragments indicated the size to be in the 35-40 kb range. This is the size expected if 3-4 copies of pDQ189 had integrated in tandem into the chromosome.

Hybridization of radioactive pDQ189 with AP41 DNA digested with other restriction enzymes gave results consistent with integration of multiple copies of pDQ189 into the S. venezuelae chromosome. In BglII+PstI digests, a single hybridizing band corresponding to the 3.2-kb fragment in the

Figure 11. Southern hybridization at high stringency of genomic DNA samples from S. venezuelae VS194 and AP41 containing pDQ189 integrated into the chromosome; [^{32}P] - labelled pDQ189 was used as the probe.

(A) Gel electrophoresis of DNA samples: lanes 1 and 13, HindIII-digest of Lambda DNA; lanes 2 and 14, EcoRI-digest of lambda DNA; lane 15, pDQ189 DNA digested with ClaI. Different restriction enzyme digests of genomic DNA of AP41: lane 3, BglII; lane 5, BglII+PstI, lane 7, ClaI+PstI; lane 9, KpnI+PstI; and lane 11, KpnI; and strain VS194: lane 4, BglII; lane 6, BglII+ PstI; lane 8, ClaI+PstI; lane 10,KpnI+PstI; and lane 12, KpnI.

(B) Autoradiogram of the nylon membrane after transfer of the DNA samples and hybridization with the pDQ189 probe.

Figure 11 (A)

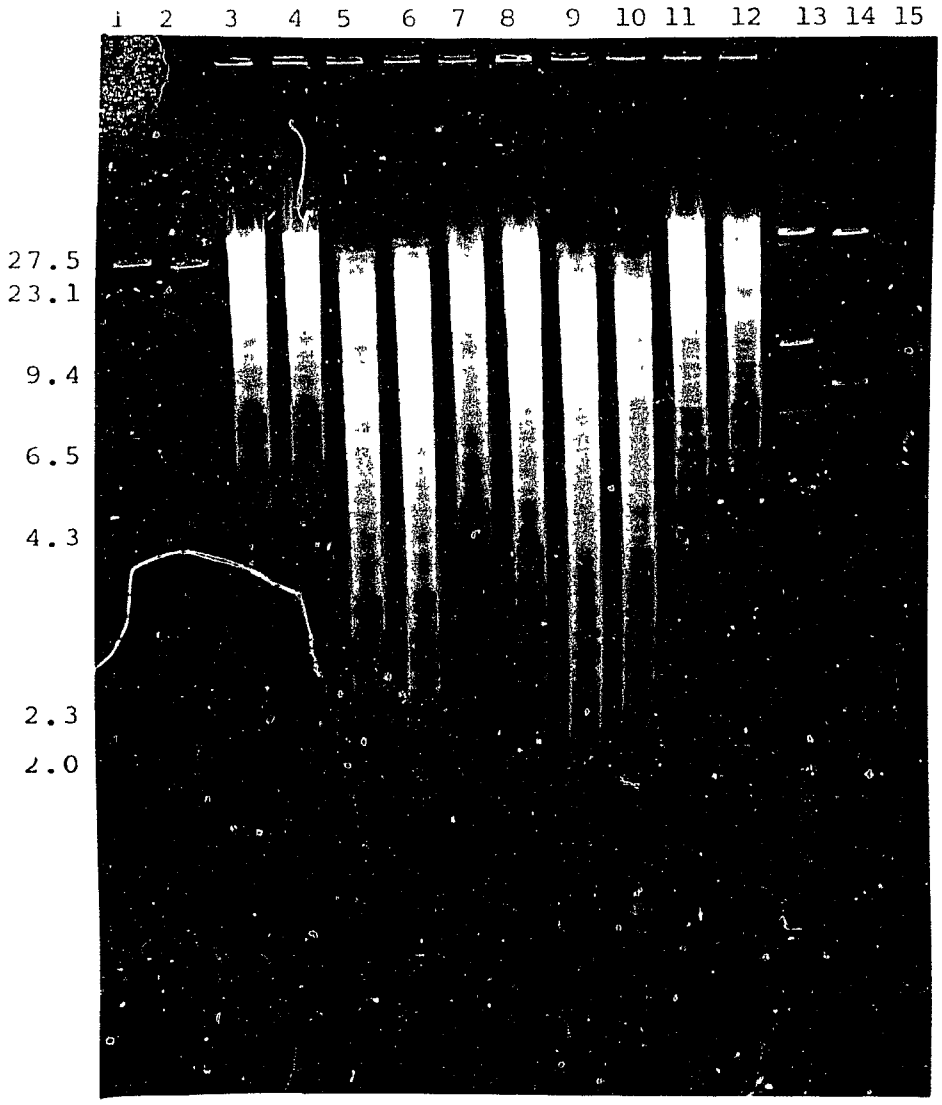


Figure 11(b)

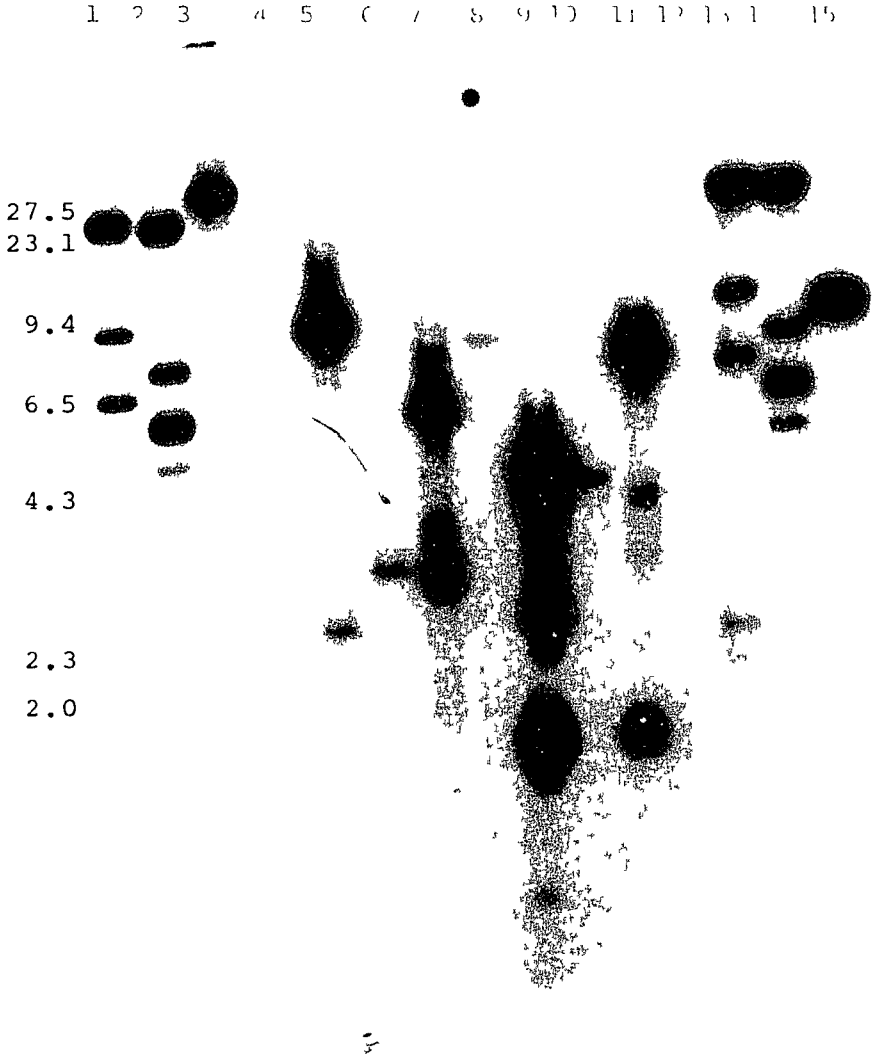


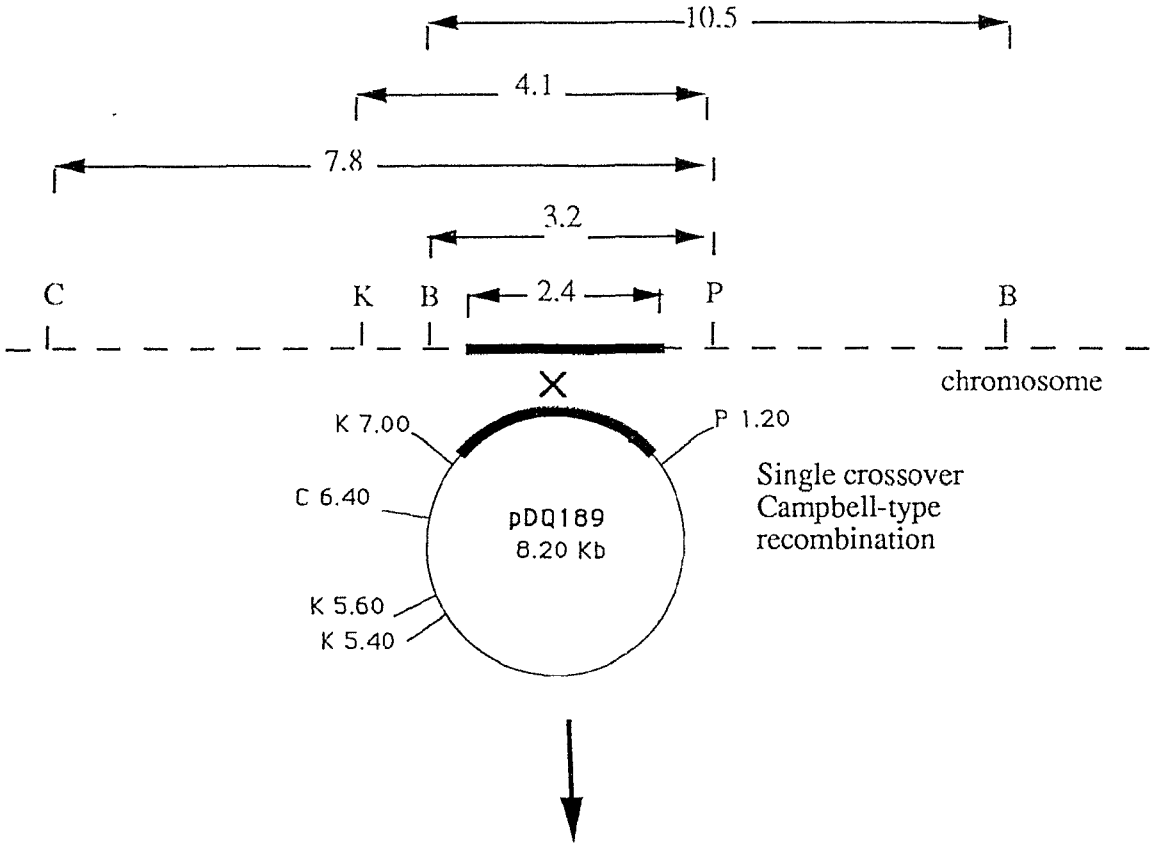
Table 7. Restriction fragments from the genomic DNA of S. venezuelae VS194 and from strain, AP41, a VS194 (pDQ189) integrant that hybridized to pDQ189 DNA.

Restriction enzyme used	Source of genomic DNA fragments	
	VS194	AP41
1. BglII	10.5	35-45
2. BglII+PstI	3.2	2.6 (8.2+8.7)
3. ClaI+PstI	7.8	3.0 3.5 5.2 7.3
4. KpnI+PstI	4.1	1.4 2.1* 2.4 2.9 3.6 4.2

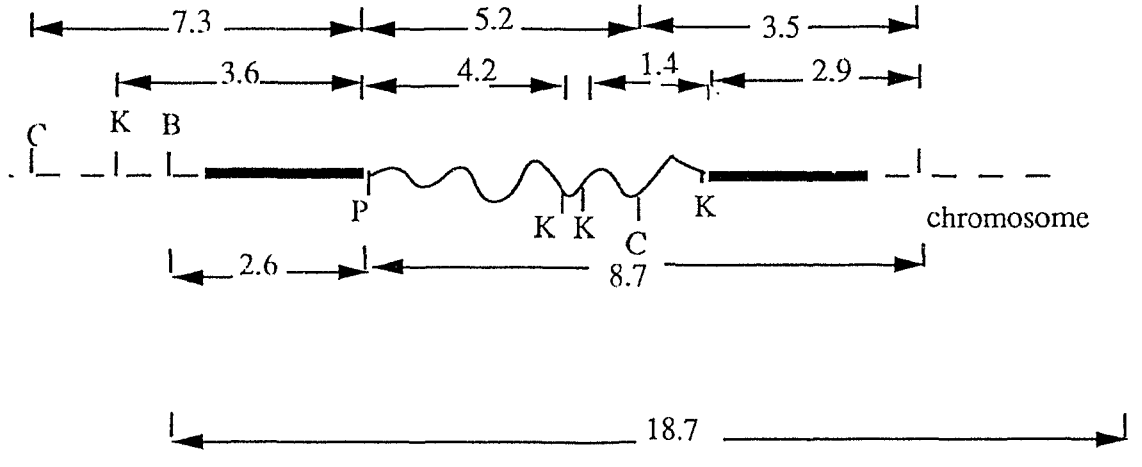
* may represent a partial digestion product
() may represent two , 8.2 and 8.7 kb fragments.

Figure 12. Model for the integration of (a) a single copy or (b) multiple copies of pQD189 carrying trpEG into the S. venzuelae chromosome by Campbell-type, single crossover recombination.

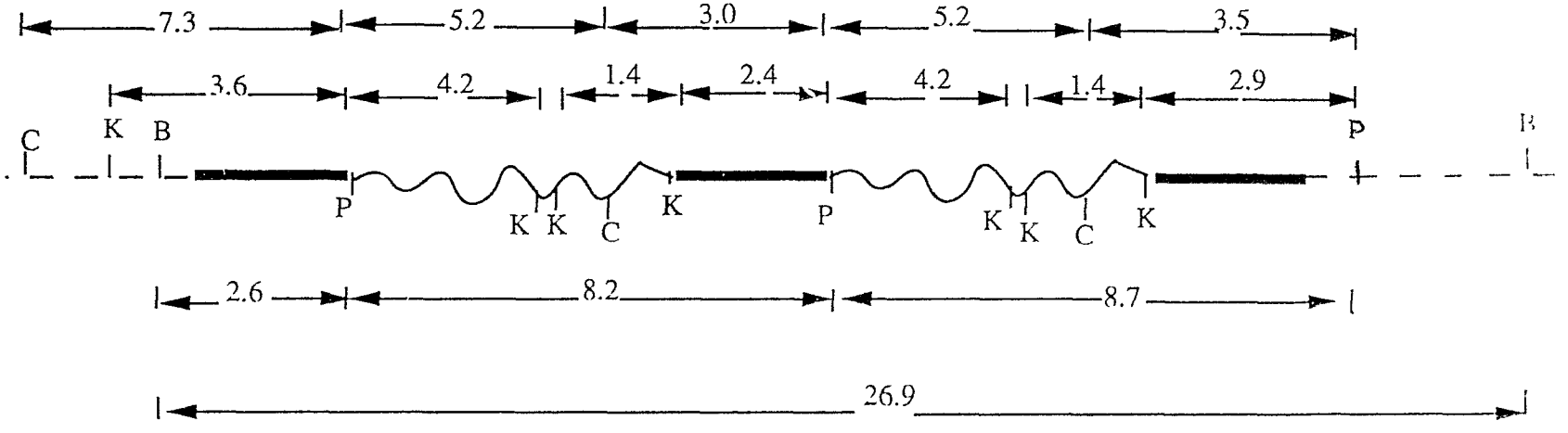
The dashed lines indicate the chromosomal region carrying trpEG; continuous lines, pHJL400 sequences; solid thick bar, trpEG sequences. C - ClaI; K - KpnI; B -BglII; P - PstI.



a) insertion of a single copy of pDQ189



b) Insertion of multiple (two) copies of pDQ189



parental strain was replaced by two hybridizing bands in theintegrant DNA. Since PstI cuts once in the vector portion of pDQ189, one band corresponds to the 2.6-kb BglII-PstI leftmost junction fragment (Figure 12b). The second band may be a mixture of two fragments, one being the 8.7-kb PstI rightmost junction fragment (Figure 12b), while the more intensly labelled component would be the complete pDQ189 sequence of 8.2 kb obtained by PstI digestion of a tandem multimer.

In ClaI+PstI digests, where ClaI, like PstI, has a single recognition site in pDQ189, integration of a single copy of pDQ189 into the chromosome as in Figure 12a, should replace the 7.8-kb fragment in the parental strain with three hybridizing fragments, two being the left and right junction fragments and one representing the portion of pDQ189 present in the middle. However, four hybridizing fragments were observed in the integrant DNA sample. The 7.3-kb and 3.5-kb fragments represent the left and the right junction fragments, respectively. The presence of 5.2-kb and 3.0-kb hybridizing fragments which together account for the complete pDQ189 sequence, and the higher intensity of the hybridization signals than observed with the junction fragments, is compatible with the integration of multiple copies of pDQ189 (Figure 12b).

Since KpnI has three sites on pDQ189, integration of a single copy of pDQ189 should give KpnI+PstI digests in which

the 4.1-kb fragment of the parental strain is replaced by five fragments: a left and a right junction fragment and three (at 4.2, 1.4, and 0.16 kb) that represent the pHJL400 vector portion of pDQ189. The integrant DNA samples gave five hybridizing fragments (Figure 11); of these the 3.6-kb and the 2.9-kb fragments represent those at the left and the right junctions. The remaining three (at 4.2, 2.4, and 1.4 kb) accounted for the pDQ189 sequence and were of higher intensity than the junction fragments. Another hybridizing fragment (0.16 kb), originating from the vector was expected; however, being a small fragment it may not have transferred from the gel to the nylon membrane in a sufficient amount to show a signal. This would be consistent with the faint signals obtained from the smaller fragments (less than 0.5 kb) in HindIII-digests of lambda DNA when probed with homologous sequences. The results obtained from other hybridizing fragments are again consistent with multiple copies of pDQ189 having integrated into the chromosome (Figure 12b). An additional faintly hybridizing 2.1-kb fragment may represent a partial digestion product.

In summary, Southern analysis of restriction enzyme digests of genomic DNA from the parental strain and AP41 suggested that multiple (three or four) copies of pDQ189 had integrated into the trpEG locus of the S. venezuelae chromosome by homologous recombination.

VII. Chromosomal location of trpEG

In S. venezuelae, it is usually possible to map a new chromosomal marker relative to known chromosomal markers by conjugation and by SV1 phage-mediated transduction. When Southern analysis established that pDQ189, carrying both tsr and trpEG had integrated into the trpEG locus of VS194, the chromosomal location of trpEG could be determined by mapping tsr relative to other chromosomal markers. In pursuing this goal, three different conjugational crosses were analysed by the single selection procedure (Doull et al., 1986). Details of the procedure are given only for the first cross.

A. Cross 1 : AP41 (hisA6, adeA10, tsr) X VS161 (tyr-2, thrC1,uraA1)

i. Selection for his+ and tsr

Spores of the integrant(hisA6, adeA10, tsr) and VS161 (tyr-2,thrC1, uraA1) were mixed and plated on a nonselective medium (MYM agar). After the culture had sporulated the spores were plated on MM medium supplemented with adenine, thiostrepton, threonine, uracil and tyrosine. This medium was counterselective for each parent but allowed the growth of His^+ $Thio^r$ recombinants. The choice of these two markers for selection was based on the low frequency of His^+ revertants and the ease of scoring for tsr. The frequency of recombinants was 2×10^{-4}.

Randomly picked recombinant colonies were patched on MYM agar, allowed to sporulate and replica-plated on the diagnostic media shown in Table 8 to determine their phenotypes. The allele ratios (wild type +/ mutant -) for each marker were calculated by scoring the growth of recombinants on the diagnostic and the control media. The markers were arranged on a gradient based on the calculated allele ratios. The order of the known markers in this gradient was consistent with that obtained in earlier studies (Doull et al., 1986). All of the 324 his+ tsr recombinants examined contained the tyr+ allele, indicating that the tyr and tsr loci were very close to each other, either as in model I or model II. Since the number of recombinants that required multiple crossovers according to either model I or model II was the same, i.e. 18, it was not possible to determine whether tsr was between his and tyr+ (model I) or between tyr+ and thrC1 (model II).

Table 8. Diagnostic media used to determine the frequencies of unselected markers among the His^+ Tsr^r Tyr^+ recombinants from the cross AP41 (hisA6,adeA10,tsr) x VS161 (tyr-2, thrC1 ,uraA1).

Media	Counterselected phenotypes[a]
1. MM + adenine, threonine, uracil, thiostrepton	Tyr^-
2. MM + threonine, tyrosine, uracil, thiostrepton	Ade^-
3. MM + adenine, threonine, tyrosine, thiostrepton	Ura^-
4. MM + adenine, tyrosine, uracil, thiostrepton	Thr^-
5. Control	
MM + adenine, tyrosine, threonine, uracil, thiostrepton	None

[a] in addition to His^- and $Thio^s$

Table 9. Mapping the tsr locus by the single selection procedure

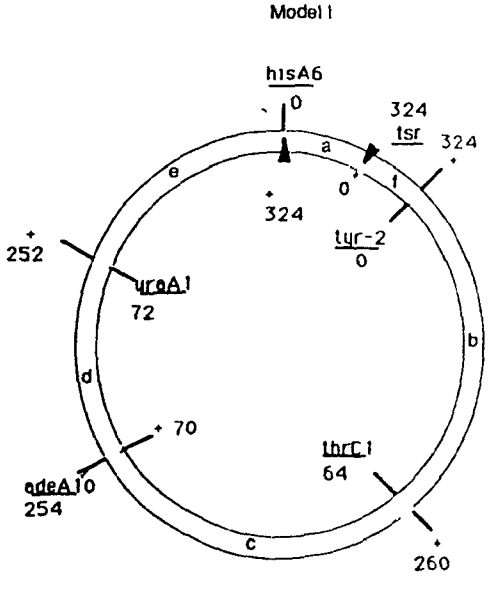
Cross 1. AP41 (hisA6, adeA10, tsr)
x
VS161 (tyr-2, thrC1, uraA1)

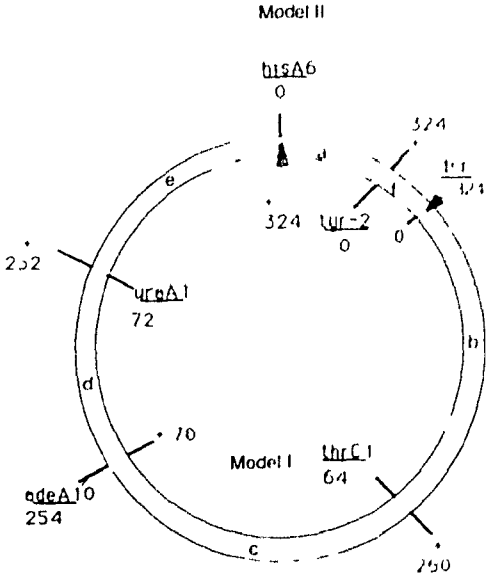
a. Selection for tsr and his+

Genotype of selectable recombinants [a]			Number of recombinant progeny	Regions of crossing-over	
				Model I	Model II
(1) +	+	+	6	a,c,d,e	a,c,d,e
(2) +	+	uraA1	7	a,c	a,c
(3) +	adeA10	uraA1	4	a,d	a,d
(4) +	adeA10	+	244	a,e	a,e
(5) thrC1	+	+	1	a,b,d,e	a,b,d,e
(6) thrC1	+	uraA1	58	a,b	a,b
(7) thrC1	adeA10	uraA1	3	a,b,c,d	a,b,c,d
(8) thrC1	adeA10	+	1	a,b,c,e	a,b,c,e

Total = 324

[a], Selection for the his+ and tsr markers is omitted; all recombinants were tyr-2+ although this was not specifically selected.





ii. Selection for ade+ and tsr

Because tsr mapped relatively close to his, and it is preferable in the single selection procedure to choose two markers on opposite sides of the circular chromosome, spores from the cross were plated on a medium selecting for the tsr and ade+ alleles. Although it was possible to identify the Tsr^{r} Ade^{+} recombinants, the adeA10 mutation in AP41 was leaky and did not allow plates to be incubated longer than 4 days. Recombinants were replica-plated on diagnostic media to score for the unselected alleles. As noted in the preceding selection, all the recombinants carried the tyr^{+} allele, and it was not possible by using minimum multiple crossover analysis to distinguish between the order his-tsr-tyr (model I) or his-tyr-tsr (model II) (Table 10).

The results from the cross suggested that tsr was tightly linked to tyr-2, but gave no information about the side of tyr-2 on which tsr was located. Since in previous studies, the tyr-2 locus was mapped between his and thr by single selection, this meant that in AP41 the tsr locus was between his and thr, in the arc opposite adeA10.

Table 10. Mapping the tsr locus by the single selection procedure

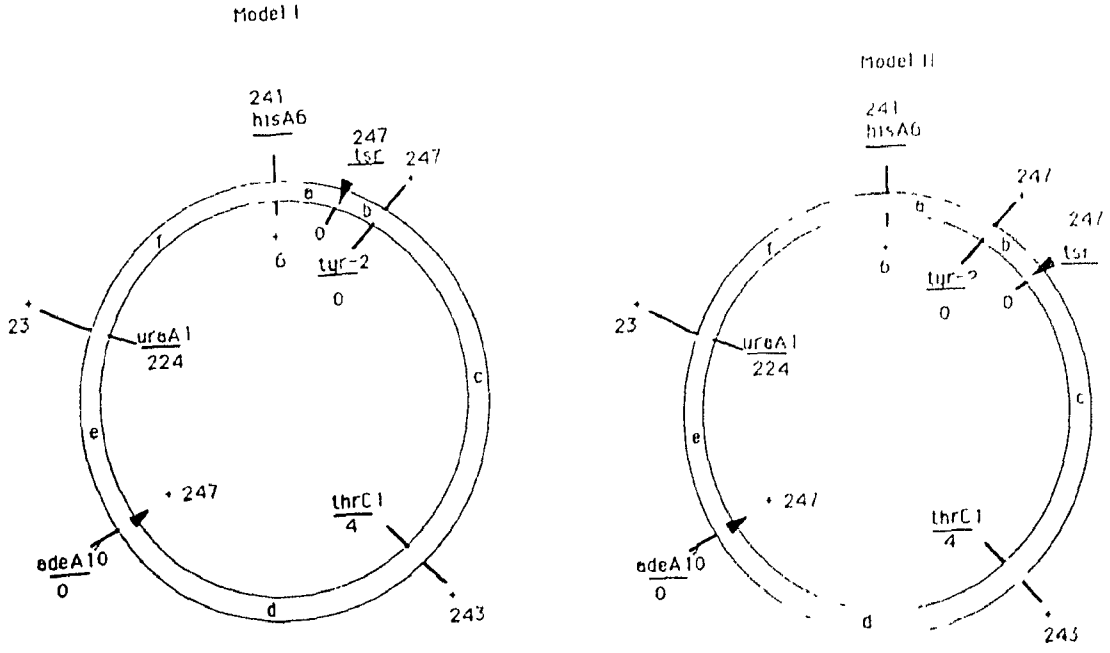
Cross 1 : AP41 (hisA6 adeA10 tsr)
x
VS161 (tyr-2 thrC1 uraA1)

b. Selection for tsr and ade+

Genotype of selectable recombinants[a]			Number of recombinant progeny	Region of Crossing over	
				Model I	Model II
(1) +	+	+	0	a,d,e,f	a,d,e,f
(2) +	+	uraA1	6	d,a	d,a
(3) +	thrC1	uraA1	0	a,c	a,c
(4) +	thrC1	+	0	a,c,e,f	a,c,e,f
(5) hisA6	+	+	23	d,e	d,e
(6) hisA6	+	uraA1	214	c,a	c,a
(7) hisA6	thrC1	uraA1	4	c,f	c,f
(8) hisA6	thrC1	+	0	c,e	c,e

Total = 247

[a], Selection for the his+ and tsr markers is omitted; all the recombinants were tyr-2+



B. Cross 2 : AP41 (hisA6,adeA10, tsr) X VS420 (pdx-4, trpA13)

The procedure selecting for his$^+$ and tsr was the same as used for cross 1. The adeA10 marker was leaky and therefore his$^-$ was counterselected. The highest allele ratio was 148 pdx$^+$: 74 pdx$^-$ (Table 11) suggesting that the pdx locus was closer to tsr than to any of the other marker loci; thus the tsr marker was either between trp$^+$ and pdx$^+$ (model I) or between pdx$^+$ and ade$^-$ (model II).

To distinguish between these possibilities, the number of recombinants in each recombinant class was scored. From these numbers and the pattern of crossing over required to obtain each recombinant, model I gave 5 recombinants requiring quadruple crossovers whereas in model II, 13 recombinants required quadruple crossovers. Assuming the number of recombinants requiring multiple crossovers to be always lower than those arising from double crossovers, model 1 is preferred. This gives the order trp-tsr-pdx.

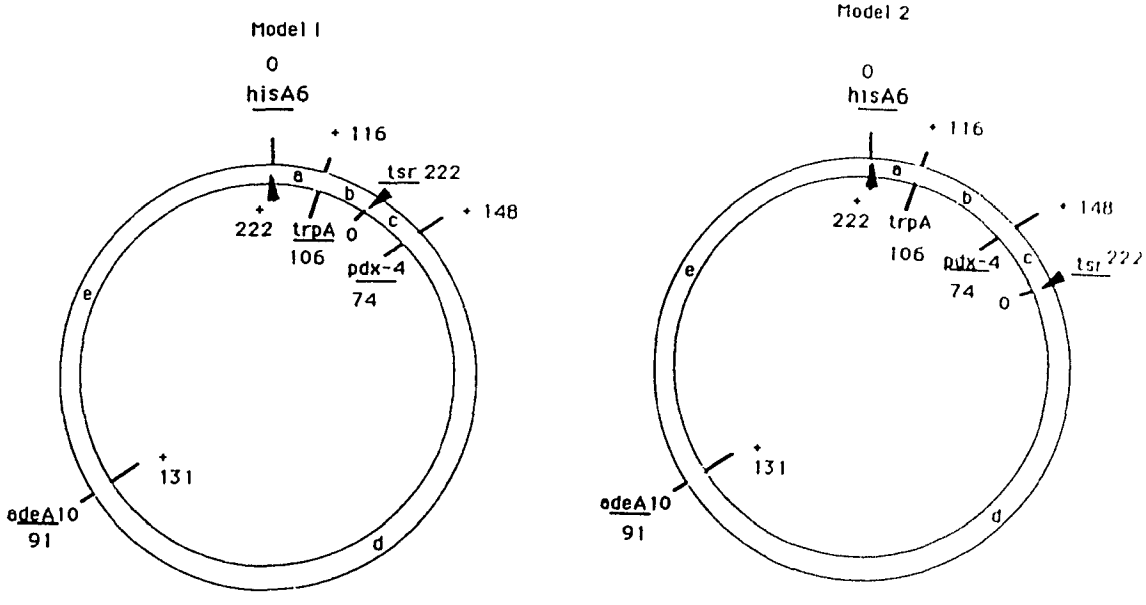
Table 11. Mapping the *tsr* locus by single selection for *his*$^+$ and *tsr* alleles.

Cross 2. AP41 (*hisA6 adeA10 tsr*) x VS420 (*pdx-4 trpA13*)

Genotype of selectable recombinants [a]			Number of recombinant progeny	Regions of cross-overs Model I	Model II
(1) +	+	+	16	a,d	a,d
(2) +	+	*adeA10*	83	a,e	a,e
(3) +	*pdx-4*	*adeA10*	0	a,c	a,b,c,d
(4) +	*pdx-4*	+	13	a,c	a,b,c,d
(5) *trpA13*	+	+	48	b,d	b,d
(6) *trpA13*	+	*adeA10*	3	b,e	b,e
(7) *trpA13*	*pdx-4*	*adeA10*	5	b,c,d,e	c,e
(8) *trpA13*	*pdx-4*	+	54	b,c	c,d

Total = 222

[a], Selective markers are omitted



C. Cross 3 : AP41 (hisA6 adeA10 tsr) x VS309 (arg-6 thrC1 adeA10 uraA1 str)

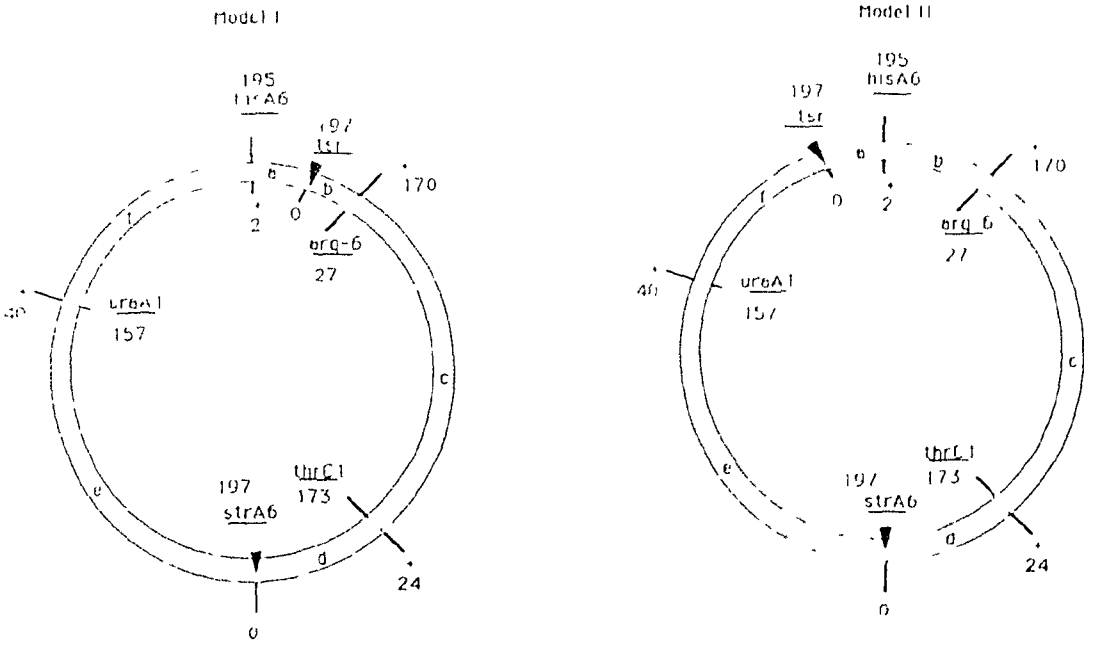
The procedure was again similar to that used for cross 1. Among the tsr, str recombinant progeny, the highest unselected allele ratio was 195 (his^-) : 2 (his^+); it suggested that tsr was either between his^- and arg^+ (model I) or between ura^+ and his^- (model II) (Table 12). Since in model I three recombinants would require multiple crossovers, whereas in model II five recombinants would require multiple crossovers, model I giving the order his--tsr-arg was chosen.

Table 12. Mapping tsr by single selection for strA6 and tsr alleles.

Cross : AP41 (hisA6, adeA10, tsr)
x
VS309 (arg-6, adeA10, thrC1, uraA1, strA6)

Genotypes of selectable recombinants[a]					Number of recombinant progeny	Regions of crossing-overs	
						Model 1	Model 2
(1)	hisA6	+	+		13	d,e	d,e
(2)	+	arg-6	+		0	b,c,d,e	a,c,d,e
(3)	+	+	thrC1	+	0	c,e,f,a	a,b,c,e
(4)	+	+	+	uraA1	0	d,a	a,b,d,f
(5)	hisA6	arg-6	+	+	2	b,c,d,e	b,c,d,e
(6)	hisA6	+	thrC1	+	16	c,e	c,e
(7)	hisA6	+	+	uraA1	8	d,f	d,f
(8)	+	arg-6	thrC1	+	0	b,e,f,a	a,e
(9)	+	arg-6	+	uraA1	0	b,c,d,a	a,c,d,f
(10)	+	+	thrC1	uraA1	3	c,a	a,b,c,f
(11)	hisA6	arg-6	thrC1	+	9	b,e	b,e
(12)	hisA6	arg-6	+	uraA1	1	b,c,d,f	b,c,d,f
(13)	hisA6	+	thrC1	uraA1	130	c,f	c,f
(14)	+	arg-6	thrC1	uraA1	0	b,a	a,f
(15)	hisA6	arg-6	thrC1	uraA1	15	b,f	b,f
(16)	+	+	+	+	0	d,e,f,a	a,b,d,e
				Total =	197		

[a], Selective markers are omitted



VIII. Cotransduction of trpB14(VS525) and tsr(integrant)

The cumulative results of conjugation suggested that pDQ189 carrying tsr and trpEG had integrated between the trpB14 and arg-6 loci on the S. venezuelae chromosome and indicated a close linkage between tsr and tyr-2. By extrapolation, the data suggested that tyr-2 might be between trpB14 and arg-6, a location consistent with that found in S. coelicolor A3(2) (Hopwood and Kieser, 1990). However, when VS525 (trpB14) was used as a donor strain for phage SV1 with VS161 (tyr-2) as the recipient, cotransduction between trpB14 and tyr-2 was not detected (Table 13).

To obtain information about the precise location of the integrated tsr marker on the chromosome, experiments were done to see if tsr was cotransducible with tyr-2 and/or trpB14. Using AP41 (tsr) as the donor strain and VS161 (tyr-2) as the recipient, no cotransduction between tsr and tyr-2 was detected. However, with the same donor and VS525 (trpB14) as the recipient strain, cotransduction between tsr and trpB14, although at a low frequency, was observed (Table 13). The possibility that the two thiostrepton-resistant colonies arose through spontaneous mutation was ruled out by two tests. In the first, approximately 10^7 spores of VS525 were plated on MYM agar containing thiostrepton; not a single colony was observed after incubation for 4 days. More significantly, in the second test, a Southern blot of a transductant (AP31) genomic DNA digested with KpnI was probed with pHJL400 at high

Table 13. Cotransduction of tsr (AP41) and tyr-2 (VS161) with other markers.

Cross*			Transductants	
Donor	Recipient	Medium	Number tested	Donor type(%)
VS41	VS161	MM + Thr + Ura + His + Ade	352	0
VS41	VS525	MM + His + Ade	531	2
VS525	VS161	MM + Thr + Ura + His + Ade	122	0

* The markers present in the strains are : VS41, hisA6, adeA10, tsr; VS161, tyr-2, thrC1, uraA1; and VS525, trpB14.

stringency. The probe did not hybridize to the genomic DNA of wild type S. venezuelae (see figures 13A and B) but it did hybridize to the transductant DNA (Figure 13, lane 2). The phage SV1 had indeed transduced integrated pDQ189 DNA sequences into VS525.

The observation that 13 out of 94 Trp^+ transductants of VS525 (approximately 14%) were His^- was expected and served as an internal control in the transduction experiment. However, the discovery that none of the His^- transductants was $Thio^r$, but both Trp^+ $Thio^r$ transductants were His+ provided useful new information and suggested that the relative gene order on the S. venezuelae chromosome was his-trp-tsr rather than tsr-his-trp or his-tsr-trp.

IX. Attempts to isolate trpEG auxotrophs by gene disruption or replacement

Gene disruption or allele exchange replacement procedures may be used to isolate specific mutants if the gene of interest is available on a cloned piece of DNA. The procedures take advantage of homologous recombination between the gene cloned on a suitable vector and its chromosomal allele. If the homologous DNA is an internal fragment of the gene of interest, the integration event results in disruption of the chromosomal allele (see Figure 3b). In the gene replacement procedure, the gene of interest is inactivated either in vitro or in vivo, and then allowed to integrate into the chromosome

Figure 13. Southern hybridization at high stringency of genomic DNA of transductants AP31 (from cross VS525 x SV1(AP41), section VIII) and AP32 (from cross VS19 x SV1(AP11), section XIIB) with pHJL400.

(A) Gel electrophoresis of genomic DNA: lane 2, AP31 DNA digested with KpnI; and lane 1, AP32 DNA digested with BclI.

(B) Autoradiogram of the nylon membrane after hybridization with the probe.

Figure 13.

(A) (B)

by homologous recombination along with the vector. This results in duplication of the cloned gene in the chromosome; the wild type chromosomal allele and the cloned inactivated allele recombine: the two recombinant alleles are separated by the vector sequence. Strains in which the wild type sequences are excised as a result of homologous recombination between the duplicate structures are recovered by screening for loss of the vector sequence. The inactivated allele remains in the chromosome (see Figure 3c).

Both the disruption and the replacement procedures were investigated as possible means of isolating trpE or trpG mutants in S. venezuelae. The vector pHJL400 was used since it had been established that pDQ189 (pHJL400 carrying the 2.4-kb trpEG DNA) integrated into the S. venezuelae chromosome presumably by homologous recombination.

A. Gene disruption

Plasmid pDQ187 was digested with EcoRI and HindIII; the 2.4-kb trpEG-containing DNA fragment was separated by gel electrophoresis, extracted from the gel and purified. The DNA was partially digested with Sau3A and the resulting digest was electrophoresed on an agarose gel. Fragments in the size range 0.7-0.9 kb were removed from the gel, purified and ligated to pHJL400 that had been cut with BamHI and treated with calf intestine alkaline phosphatase. The ligation mixture was used to transform E. coli TG1 and the transformation mixture was

plated on LB agar containing X-gal and ampicillin. Ampicillin-resistant transformants giving white colonies were picked and their plasmid content was examined by a rapid screening procedure. All 24 tested gave recombinant plasmids bigger than pHJL400. These plasmids were combined at random into pools of six.

Two pools were used separately to transform S. venezuelae VS194 to thiostrepton resistance. For each transformation, spores from 20 such transformants were mixed to form two pools of spore suspension. Spores from each pool were patched on MYM agar (without thiostrepton) and the culture was allowed to sporulate. Again, the culture was passed through a cycle of sporulation on MYM without thiostrepton selection. Finally, spores were harvested and plated on MYM agar containing thiostrepton to select for stable transformants in which, presumably, the plasmid carrying tsr and a portion of 2.4-kb trpEG had integrated into the chromosome. It was anticipated that integration into the chromosome by homologous recombination of any such plasmid carrying an internal fragment of trpE or trpG would disrupt the respective gene. As a result, the transformants with such an integration event were expected to show a Trp- phenotype. Four plates containing 500 colonies of stable thiostrepton-resistant transformants from each pool were replica-plated in parallel on MM and on MM supplemented with tryptophan. None of the colonies was Trp^-.

B. Gene replacement

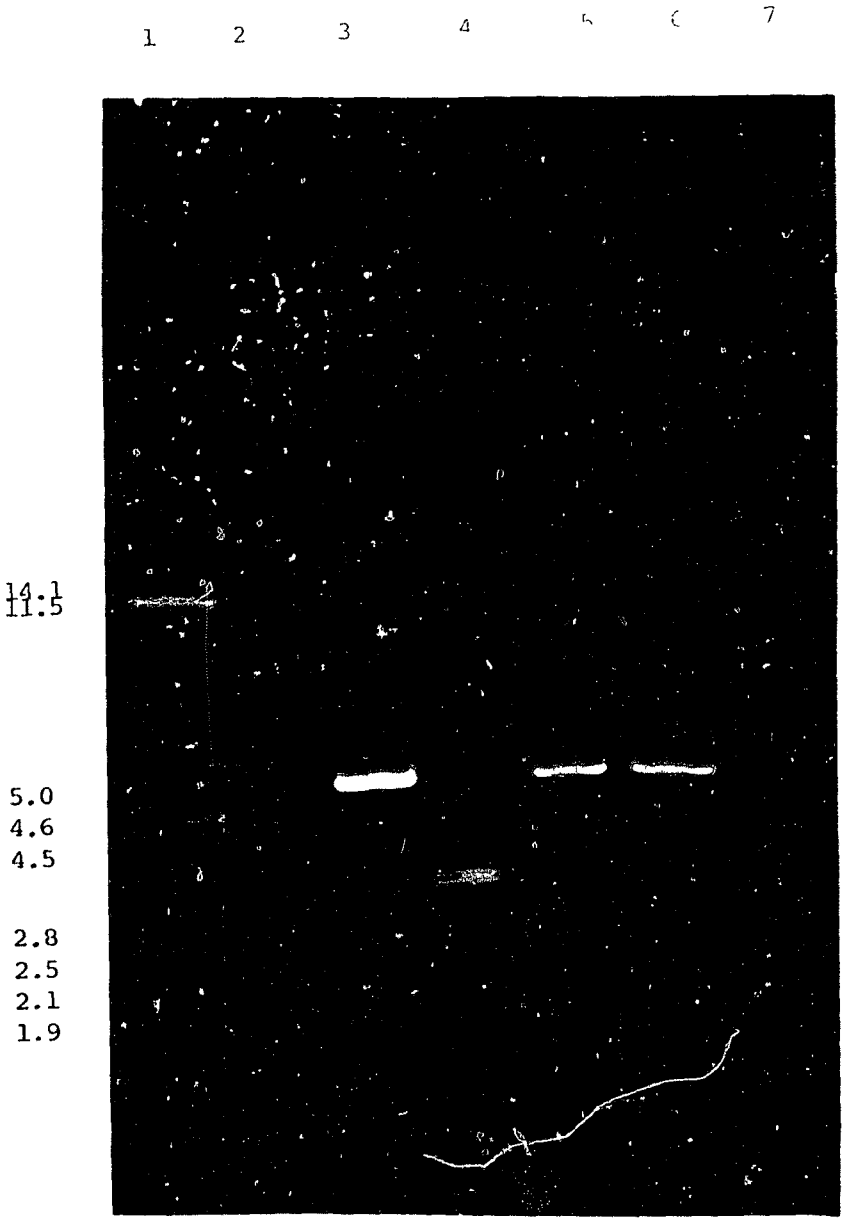
To create in vitro a mutation in trpEG, an approach that depended on modification of a restriction site, as used previously (Anzai et al., 1989), was chosen. Because there are no sites for the restriction enzyme MluI on pTZ18R, the unique site for this enzyme in the 2.4-kb DNA fragment containing trpEG was an appropriate target. The plasmid pDQ187, derived from pTZ18R by insertion of the 2.4-kb trpEG DNA, was digested with MluI and the single stranded portions of DNA formed were removed by treatment with mung bean nuclease. This was followed by treatment with the Klenow fragment of DNA polymerase in the presence of all four deoxyribonucleotides. The 5′- 3′ polymerase activity of the Klenow fragment was expected to repair any single stranded DNA formed by partial digestion with Mung bean nuclease. Thus it complemented the action of mung bean nuclease in forming blunt-ended DNA fragments. The DNA fragment obtained from these treatments, was incubated with excess T4 DNA ligase to promote blunt-ended ligation. When the ligation mixture was then used to transform E. coli JA200 (ΔtrpE), and the transformants were replica-plated on MM agar and MM agar containing tryptophan, about 50% showed a Trp^- phenotype. One Trp^- transformant was analysed for its plasmid content. The plasmid (pDQ190) it carried was identical in electrophoretic mobility to pDQ187. However, MluI failed to digest pDQ190 DNA, whereas pDQ187, extracted and digested under the same conditions, gave the expected 5.3-kb

fragment (Figure 14). Moreover, EcoRI, which used the same reaction buffer system, did digest pDQ190 to give the expected single fragment. Therefore, the inability of pDQ190 to complement the trpE mutation in JA200 was presumed to be due to modification of the MluI site.

The 2.4-kb DNA fragment containing the "inactivated" trpE from pDQ190 was isolated as an EcoRI-HindIII fragment and ligated into pHJL400 cut with the same enzymes. The ligation mixture was used to transform E. coli TG1 to ampicillin resistance. One of the transformants that formed a white colony on LB agar containing ampicillin and X-gal was examined for its plasmid content. It yielded the recombinant plasmid, pDQ191, consisting of pHJL400 containing the "inactivated" trpE gene; the plasmid was used to transform S. venezuelae VS194 to thiostrepton resistance. Thiostrepton-resistant transformants were pooled and grown through 3 spore-spore cycles on MYM agar (without thiostrepton selection) as described previously. Finally, spores were plated on MYM agar to give 100-150 colonies per plate and allowed to sporulate. When these cultures were replica-plated on MYM agar containing thiostrepton and MYM agar, only approximately 25% of the colonies were thiostrepton resistant. Five of these thiostrepton-resistant transformants were grown on MYM agar (without thiostrepton) to sporulation and the spores were harvested, pooled and resuspended in 20% glycerol.

Figure 14. Gel electrophoresis of uncut, and restriction enzyme-digested DNA samples of plasmids pDQ187 and pDQ190: Lambda DNA digested with PstI is in lane 1. Plasmid pDQ190 DNA: lane 2, MluI; lane 3, EcoRI; and lane 4, uncut sample. Plasmid pDQ187: lane 5, MluI; lane 6, EcoRI; and lane 7, uncut sample.

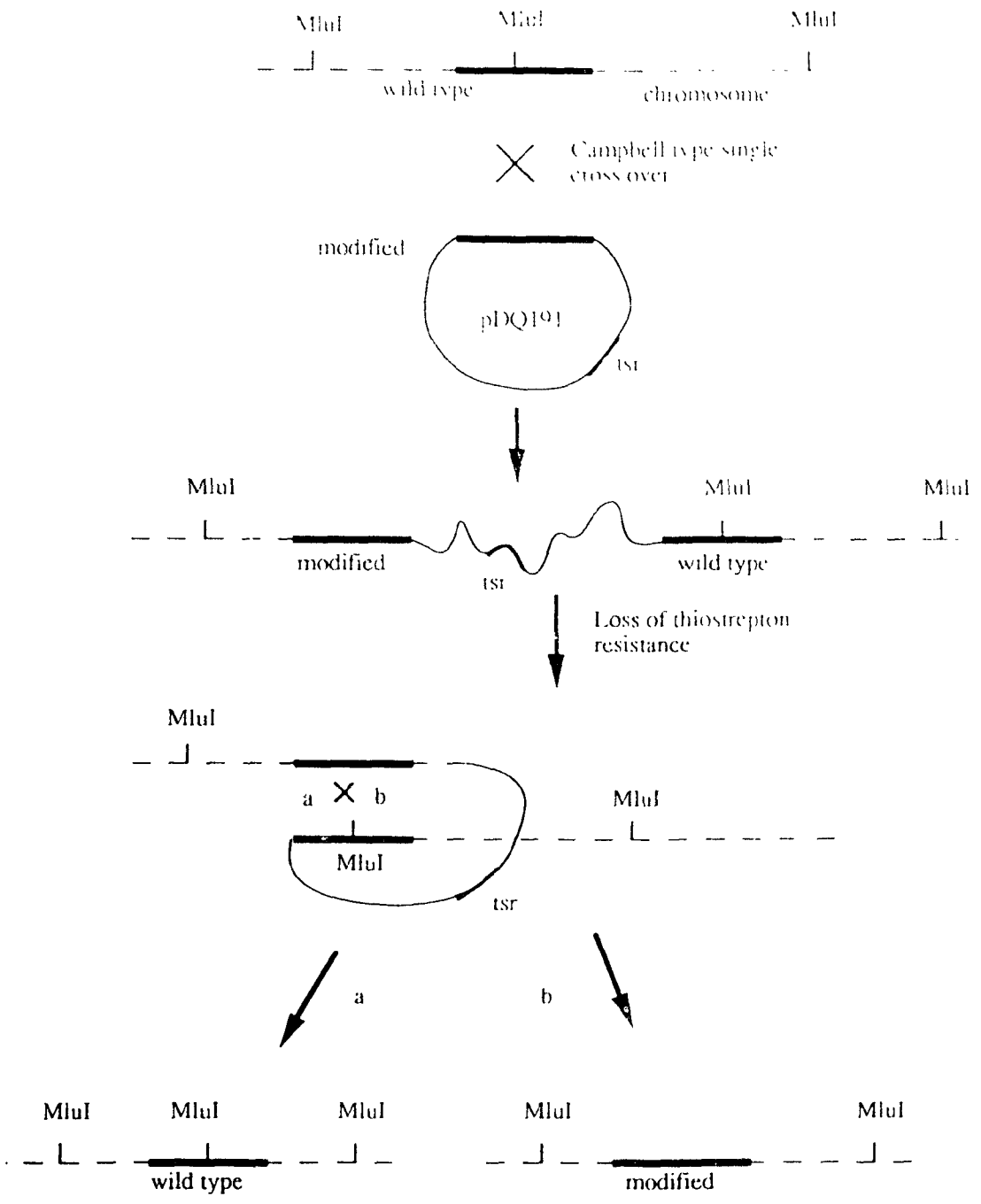
Figure 14.



The five strains that maintained thiostrepton resistance stably after nonselective growth were presumed by analogy with the results of similar experiments with pDQ189 to have integrated pDQ191 with its "inactivated" trpE into the trpEG region of the chromosome. As predicted, the transformants with integrated pDQ191 were protrophic; integration of one or more copies of pDQ191 into the chromosome maintained the wild-type chromosomal trpE allele while introducing copy/copies of the "inactivated" trpE and vector (pHJL400) sequences (Figure 15). This structure, with homologous DNA in tandem, should lose vector sequences due to excision after homologous recombination. As shown in Figure 15 crossover "a" during homologous recombination would restore wild type trpEG sequences; on the other hand, crossover "b" would retain "inactivated" trpEG in the chromosome. The net result of the genetic instability in the integrant, AP41, would then be gene replacement.

Since a strain modified within either the trpE or trpG structural gene was expected to require tryptophan, pooled spores from the stable thiostrepton-resistant transformants were plated on MYM agar to obtain 100-150 colonies per plate, allowed to sporulate and then replica-plated on MM, MM containing tryptophan and MYM agar containing thiostrepton. About 10% of the replicated colonies failed to grow on MYM agar containing thiostrepton, suggesting that the plasmid had been excised. However, none showed a requirement for

Figure 15. Model for the integration of pDQ189 into the chromosome and excision resulting in allele-exchange; dashed lines represent the chromosome; thick lines represent the trpEG; and thin lines denote the vector sequences.


MluI
MluI
MluI
wild type
chromosome
Campbell type single cross over
modified
pDQ191
tsr
MluI
MluI
MluI
modified
tsr
wild type
Loss of thiostrepton resistance
MluI
a
b
MluI
MluI
tsr
a
b
MluI
MluI
MluI
wild type
MluI
MluI
modified

tryptophan.

To see if allele exchange had indeed occurred, genomic DNA from seven such thiostrepton-sensitive strains (AP52-58) and VS194 was digested with MluI, separated on an agarose gel and transferred to a nylon membrane. The membrane was probed by Southern hybridization with pHJL400, and then with pDQ189. Although pHJL400 hybridized to pDQ189 (positive control), it did not hybridize to the genomic DNA of the parental strain or the eight thiostrepton-sensitive strains (Figure 16B). Since these eight strains were derived from stable thiostrepton-resistant transformants, this result meant that they had lost not only tsr but all of pHJL400. When probed with radioactive pDQ189, the genomic DNA of the parental strain showed hybridizing fragments of 16.5 kb and 12 kb as expected from the restriction map of the trpEG fragment (Figure 16C). The genomic DNA from the seven thiostrepton-sensitive strains showed patterns that divided them into two classes: two strains (Class I), AP54 and AP58, showed the same two hybridizing fragments (16.5 kb and 12 kb) as the parental strain, while five strains (ClassII), AP52-53, AP55-57, showed only a single hybridizing fragment of approximately 28.5 kb. Since the size of the hybridizing fragment in class II strains equalled the sum of the two fragments in the class I and parental strains, the class II strains were presumed to contain the "inactivated" trpE sequence that carried the mutation in the MluI site. This also suggested that in all of

Figure 16. Southern hybridization at high stringency of genomic DNA samples of S. venezuelae VS194 and strains AP52-58 digested with MluI, and probed with pHJL400, and pDQ189.

(A) Gel electrophoresis of DNA samples : lanes 1 and 12, Lambda DNA digested with HindIII; lanes 2 and 11, Lambda DNA digested with EcoRI; lane 3, VS194; lanes 4-10, strains AP52-AP58; lane 13, pDQ189 digested with MluI.

(B) Autoradiogram after transfer of DNA samples to nylon membrane and hybridization with pHJL400.

(C) Autoradiogram after reprobing the nylon membrane with pDQ189.

Figure 16. (A)

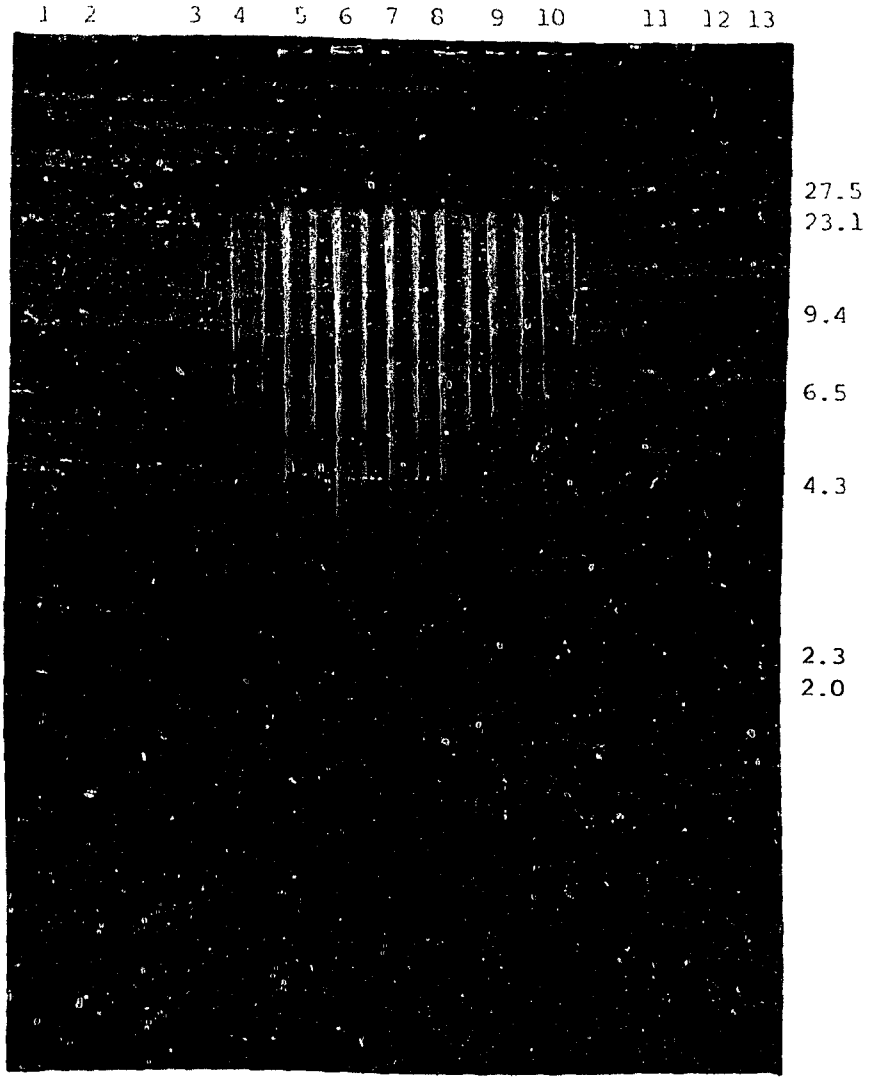
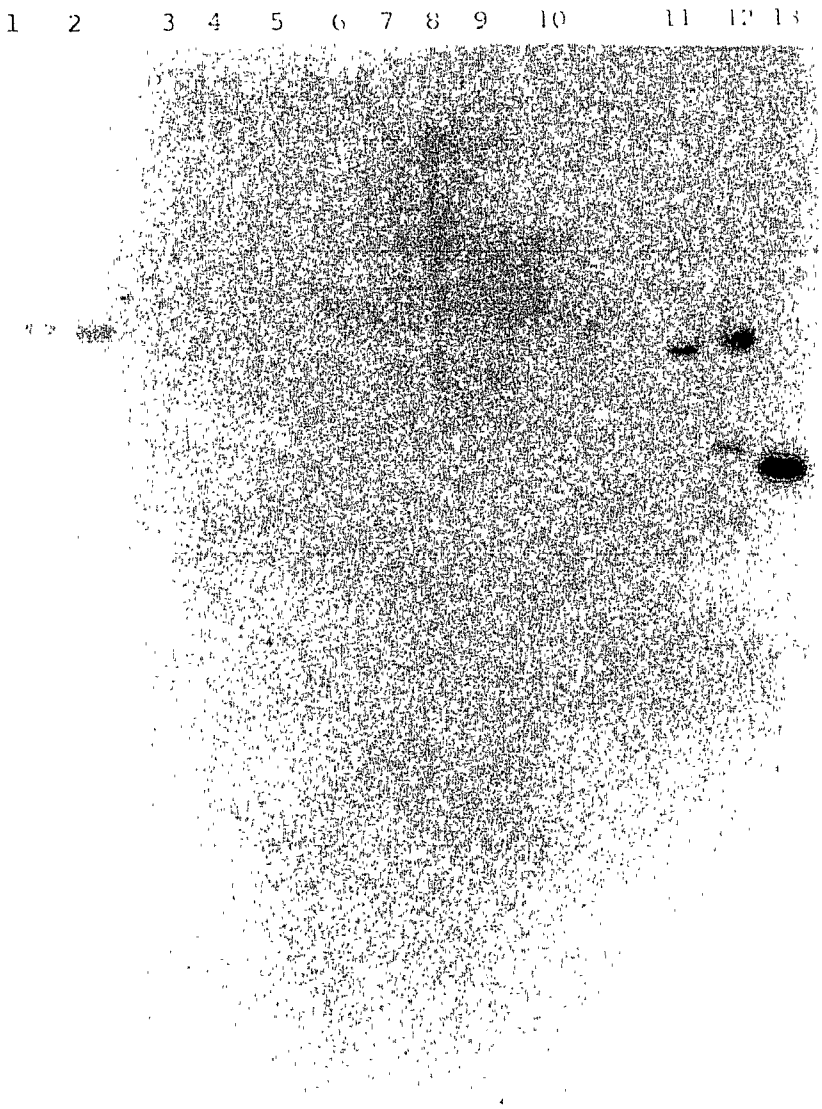


Figure 16. (B)



the thiostrepton sensitive strains, the tsr-carrying vector sequences were lost by either crossover A resulting in restoration of the wild type trpE class I strains, or crossover B resulting in introduction of the inactivated trpE class II strains. Possible reasons for not obtaining a Trp- phenotype despite successful replacement of the wild type trpE by the "inactivated" trpE gene are considered in the "Discussion" section.

X. Molecular cloning of S. venezuelae trpD and trpC genes

Aidoo et al (1990) observed that recombinant plasmids carrying homologous DNA fragments are highly unstable in S. venezuelae and appear to integrate into the chromosome. Therefore, despite the availability of trp auxotrophs of S. venezuelae, the cloning of trp genes by complementation with genomic DNA of this species presented difficulties. To circumvent the problem of homologous recombination, S. lividans trp auxotrophs, AP1 and AP2, were isolated (see section I) and used as heterologous hosts for shot-gun cloning of S. venezuelae genomic DNA fragments. Because the mutations in the host strains were in trpD and trpC, respectively, the DNA fragments cloned by complementation were expected to contain these genes. The protocol used is shown in Figure 17. Genomic DNA of S. venezuelae was partially digested with MboI and ligated into the BglII site of pIJ702, a vector that carries a thiostrepton resistance gene. When the ligation

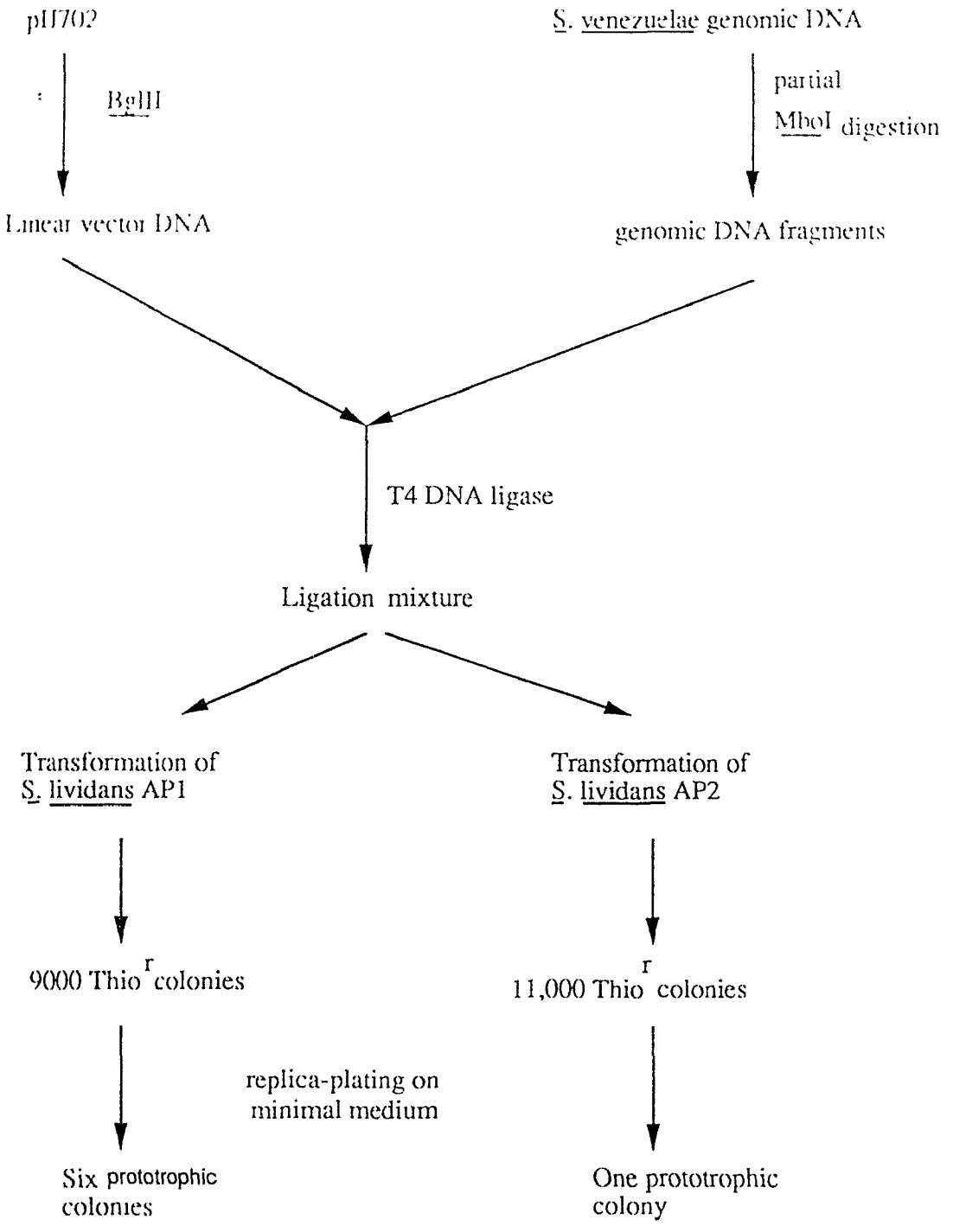


Figure 17. Protocol for cloning the trpD and trpC genes of S. venezuelae by complementation of S. lividans trp mutants

mixture was used to transform AP1, six of approximately 9000 transformants selected for thiostrepton resistance were prototrophic. Extraction of plasmid DNA from the transformants and its examination by gel electrophoresis showed that all six strains contained plasmids bigger than the vector. After they had been isolated, all of the plasmids transformed AP1 to prototrophy, indicating that the Trp^+ determinant was plasmid-linked. One of these plasmids, pDQ171, was studied further.

When the ligation mixture described above was used to transform AP2 (trpC), only a single prototrophic transformant was obtained out of approximately 11,000 thiostrepton-resistant colonies. Extraction of plasmid DNA from the prototrophic strain yielded a recombinant plasmid (pDQ177) bigger than pIJ702.

A. pDQ171

The plasmid, which contained a DNA insert of approximately 4 kb, was digested singly and in pairwise combinations with various restriction enzymes. The resulting DNA fragments were separated by agarose gel electrophoresis and their sizes were estimated by comparison with those of fragments from lambda phage DNA digested with HindIII. A restriction map of pDQ171 is shown in Figure 18.

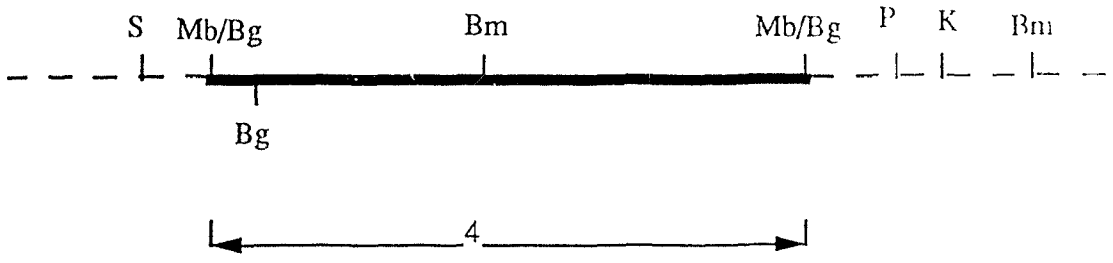


Figure 18. Restriction map of pDQ171 containing S. venezuelae trpD DNA. No sites were present for PstI, KpnI, Cla I, and EcoRI. The The dashed lines indicate pIJ702 sequences and solid lines denote the trpD sequences. The abbreviations used are : S=SstI, Mb=MboI, Bg= BglII, P= PstI, Bm= BamHI

B. Southern hybridization of S. venezuelae genomic DNA with pDQ171

To confirm the origin of the 4 kb DNA insert in pDQ171, S. venezuelae ISP5230 genomic DNA was digested with several restriction enzymes and then probed with radioactive pDQ171. The pIJ702 vector portion of pDQ171 does not hybridize to the genomic DNA of S. venezuelae (see Figure 23B, lane 3), but pDQ171 did show hybridization signals (Figure 19). The PstI, KpnI and EcoRI digests gave a single band; two bands were seen in BamHI digests. The hybridization signals were consistent with the restriction map of the insert in pDQ171 (see Figure 18), which showed that BamHI cuts within the insert once, while there are no sites for either PstI, KpnI or EcoRI. Therefore, the insert represents a contiguous segment of S. venezuelae genomic DNA and contains no false linkages.

C. pDQ177

The plasmid pDQ177, which carried an insert of 9.2 kb, was digested singly or in pairwise combinations with restriction enzymes. The digestion products were electrophoresed and their sizes calculated by comparison with lambda DNA digested with HindIII and PstI as size markers. Based on their sizes, a res- triction map of the insert in pDQ177 was constructed (Figure 20).

Figure 19. Southern hybridization at high stringency of genomic DNA samples of S. venezuelae digested with various restriction enzymes.

(A) Gel electrophoresis of DNA samples: Lane 1, plasmid pDQ171 digested with PstI; lane 2, lambda DNA digested with PstI; and S. venezuelae genomic DNA digested with: lane 3, KpnI; lane 4, PstI; lane 5, BamHI.

(B) Autoradiogram after transfer of DNA samples to the nylon membrane and hybridization with ^{32}P-labelled pDQ171.

Figure 19.

(A)

(B)

D. Southern hybridization of genomic DNA of S. venezuelae ISP 5230 with pDQ177

To confirm the origin of the 9.2-kb DNA insert in pDQ177, S. venezuelae ISP5230 genomic DNA, digested with different enzymes, was challenged with radioactive pDQ177 as the probe. Since the pIJ702 vector portion of pDQ177 does not hybridize to the genomic DNA of S. venezuelae (see Figure 23B, lane 3) whereas pDQ177 did hybridize (Figure 21) the cloned insert in pDQ177 originated from S. venezuelae. Moreover, the number and sizes of hybridizing fragments in each of the digests was consistent with the restriction map of the insert (see Figure 20). The BglII digest gave two hybridizing fragments, one of 6.8 and the other of 16 kb, which is consistent with there being only one BglII site within the insert. Similarly, KpnI digests contained 1.1, 1.2 and 10.5-kb hybridizing fragments; the number and sizes were consistent with there being two KpnI sites in the insert. There are no ClaI or PstI sites in the insert and accordingly only one hybridizing fragment (28 kb) was observed in the ClaI+PstI digest. The results indicated that the 9.2-kb insert was a contiguous piece of chromosomal DNA and did not have any false linkages.

Genomic DNA of S. venezuelae strains AP52 and AP54 was also included in this experiment to see if trpC could be detected in a region adjacent to trpEG in the chromosome. Since trpEG is present on two MluI fragments (of 12 and 16.5 kb) in strain AP54 but, as a result of gene replacement, on

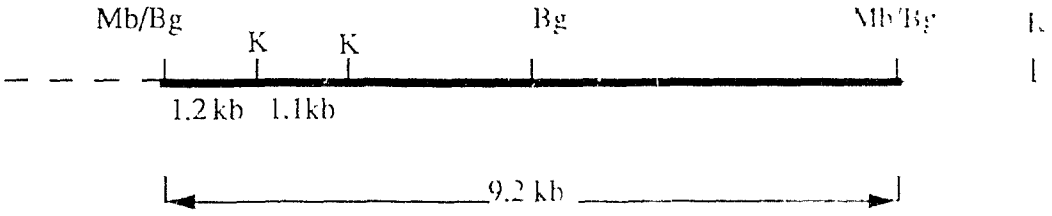


Figure 20. Restriction map of S. venezuelae trpC containing DNA insert in plasmid pDQ177.
The dashed lines indicates pIJ702 sequences and solid lines denote trpC DNA . Mb, MboI; K, KpnI; Bg, BglII;

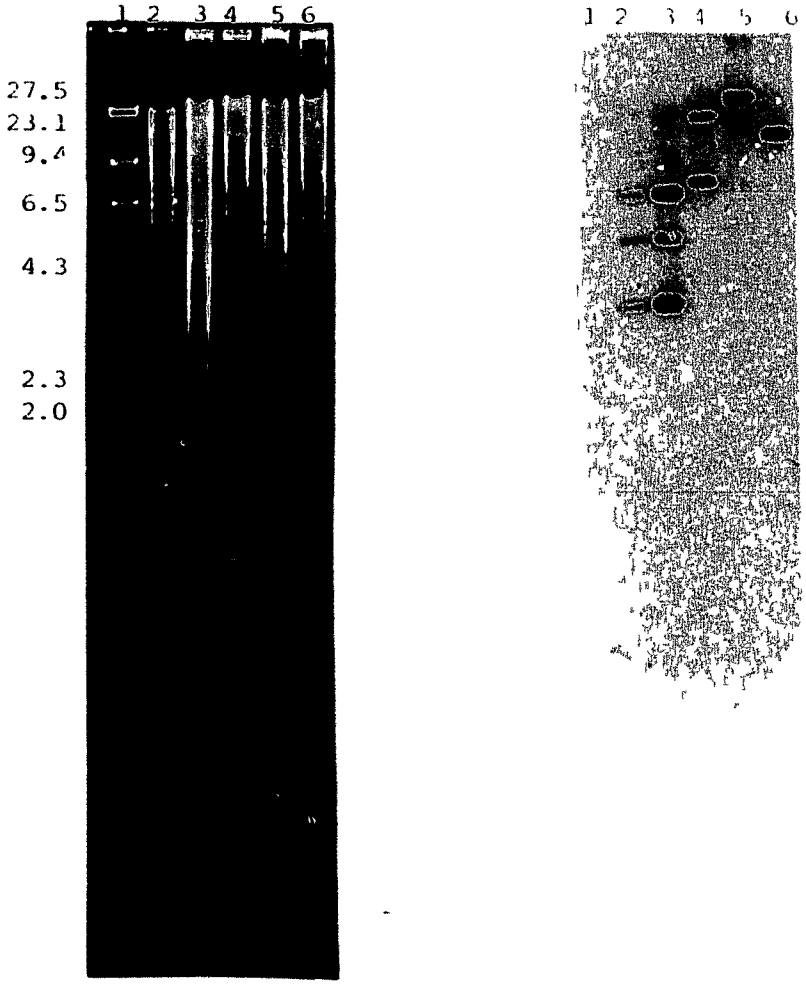
Figure 21. Southern hybridization at high stringency using pDQ177 as a probe of S. venezuelae genomic DNA.

(A) Gel electrophoresis of the DNA samples: lane 1, lambda DNA digested with HindIII; lane 2, AP54 DNA digested with MluI; lane 3, AP52 DNA digested with MluI; lane 4, VS195 digested with BglII; lane 5, VS195 DNA digested with ClaI+PstI; lane 6, VS194 DNA digested with KpnI.

(B) Autoradiogram after transfer of the DNA samples to a nylon membrane and hybridization with pDQ177.

Figure 21.

(A) (B)



only one (of 28.5 kb) in strain AP52 (see Section IXB), the presence of pDQ177-hybridizing signals corresponding to the two fragments in AP54 and one fragment in AP52 would place trpC within 12 kb on one side of trpEG and 16.5 kb on the other. However, hybridization patterns given by the MluI digests of strains AP52 and AP54 were identical, and showed hybridizing fragments at 3.2, 4.6 and 6.2 kb. The presence of three fragments is consistent with there being two MluI sites in the trpC insert, based on the number of fragments obtained by digesting pDQ177 and pIJ702 by MluI. These sites were not mapped precisely because information about the number and position of MluI sites on pIJ702 was not available and the information was unnecessary in view of the identical hybridization patterns given by strains AP52 and AP54. The results from the MluI digests and comparison of the restriction maps of the cloned trpC and trpEG sequences indicate that trpC does not lie within 12 kb on one side or 16.5 kb on the other side of trpEG.

XI. Complementation of S. venezuelae trpD4 by pDQ171

S. venezuelae VS154 (trpD4) was transformed with pDQ171 and pIJ702 to thiostrepton resistance. Transformants were patched on MYM agar containing thiostrepton, allowed to sporulate, and then replica-plated on MM agar. Because the trpD4 mutation in VS154 is leaky, growth of the transformants

was scored no later than 24-36 h, beyond which this mutant does not show a Trp- phenotype. Transformants containing pDQ171, but not those containing pIJ702, showed prototrophy. The ability of pDQ171 to complement the trpD4 mutation was confirmed by growing VS154 (pDQ171) and VS154 (pIJ702) in minimal liquid medium with or without tryptophan (Table 14). Only the transformants bearing pDQ171 showed growth comparable to that of the wild type after 24 h (Table 14). When the transformants were examined for the presence of plasmids, extrachromosomal CCC DNA was found only in VS154 transformed with pIJ702. Judged by the intensity of the plasmid DNA band obtained by gel electrophoresis, the copy number of pIJ702 in the VS154 transformants was much lower than in S. lividans TK24 transformed with pIJ702.

XII. Integration of pDQ171 into the S. venezuelae chromosome

A. Southern blot analysis

Ten independent transformants (AP11 to AP20) of VS154 transformed with pDQ171 and one (AP21) of VS154 transformed with pIJ702 were examined by Southern hybridization. Genomic DNA was isolated from the mycelia, fractionated by electrophoresis on a 0.5% agarose gel and transferred to a nylon membrane. The membrane was then probed with radioactive pIJ702, washed at high stringency and autoradiographed (Figure 22). A radioactive zone corresponding to supercoiled pIJ702 was present in AP21, but no comparable zone for supercoiled

Table 14. Growth characteristics of transformed cultures of *S. veenzuelae* VS154 (*trpD4*)

Strain	Initial O.D.	O.D. (24 h)	
		MM	MM+Trp
VS154 (pIJ702)	0.1	0.8	6.8
VS154 (pDQ171)	0.1	4.0	4.8
VS154 (pDQ177)	0.1	0.8	3.9

O.D., Optical density at 640 nm; MM, minimal medium; Trp, tryptophan.

circular DNA was present in any of the ten strains (AP11-20) transformed with pDQ171. In all ten transformants, hybridization signals were present in the region expected for high molecular weight chromosomal DNA. Two explanations were considered : either the plasmid was present in a high molecular weight (multimeric) form inseparable from chromosomal DNA, or the plasmid had integrated into the chromosome.

In Gram-positive bacteria plasmids that replicate via single stranded intermediates (described as single-stranded plasmids) often form high molecular weight multimers inseparable from chromosomal DNA during agarose gel electrophoresis. Insertion of foreign DNA into these plasmids has been reported to increase the frequency of multimer formation (Gruss and Ehrlich, 1988). The plasmid pIJ702 is a derivative of pIJ101, which generates single stranded DNA during replication (Gruss and Ehrlich, 1989). On the basis of deduced amino acid sequence similarity between the active sites of its Rep protein and the Rep proteins of other well characterized single-stranded plasmids, it is tentatively classifed in this group (Pigac et al, 1988; Gruss and Ehrlich, 1989). The formation of high molecular weight multimers of pIJ702 or pDQ171 was, therefore, to be expected. In S. venezuelae and in other streptomycetes, plasmids carrying homologous DNA inserts integrate into the chromosome by homologous recombination. Therefore, integration of pDQ171

Figure 22. Southern hybridization of uncut genomic DNA samples of S. venezuelae VS154 and VS154 transformed with pDQ171.

(A) Gel electrophoresis of DNA samples : lane 1, uncut plasmid pDQ171; lanes 2-11, strains AP11-AP20, all containing pDQ171; lane 12, AP21 containing pIJ702; lane 13, VS154; lane 14, VS154 DNA mixed with uncut pIJ702 DNA; and lane 15, uncut pIJ702.

(B) Autoradiogram after transfer to the nylon membrane and hybridization with ^{32}P-labelled pIJ702.

Figure 22.

(A)

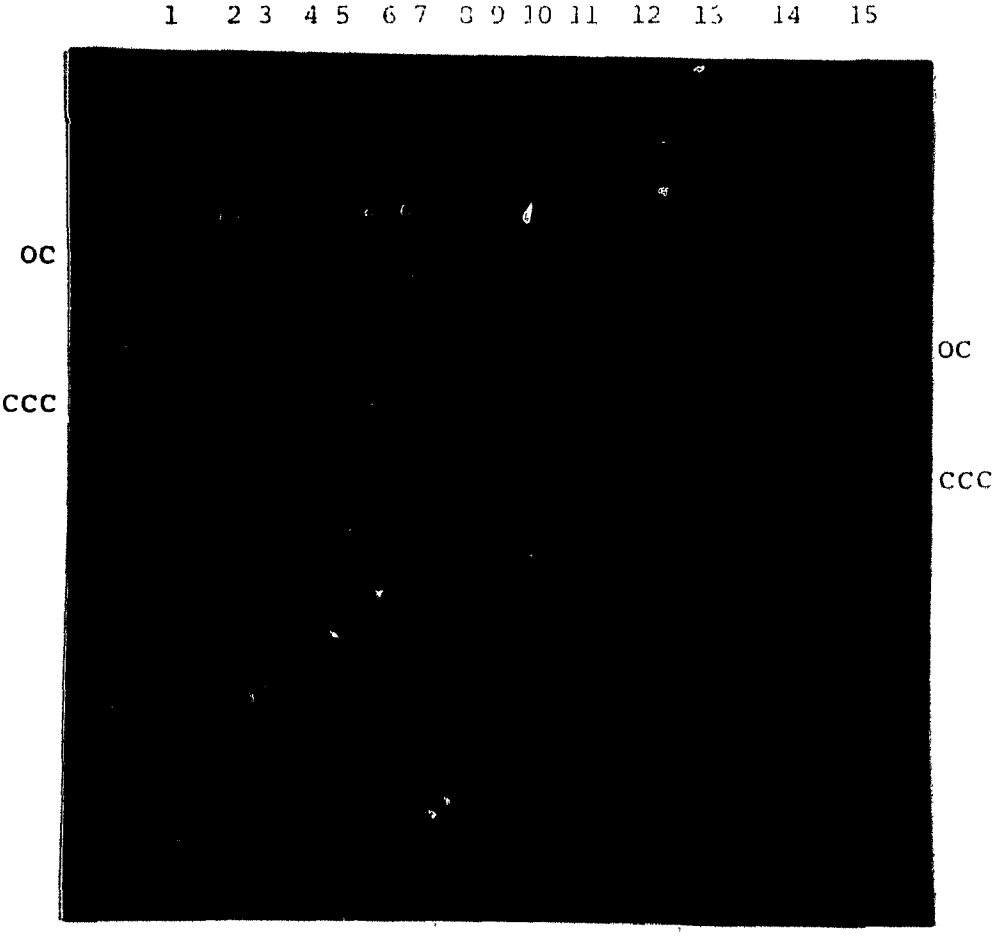
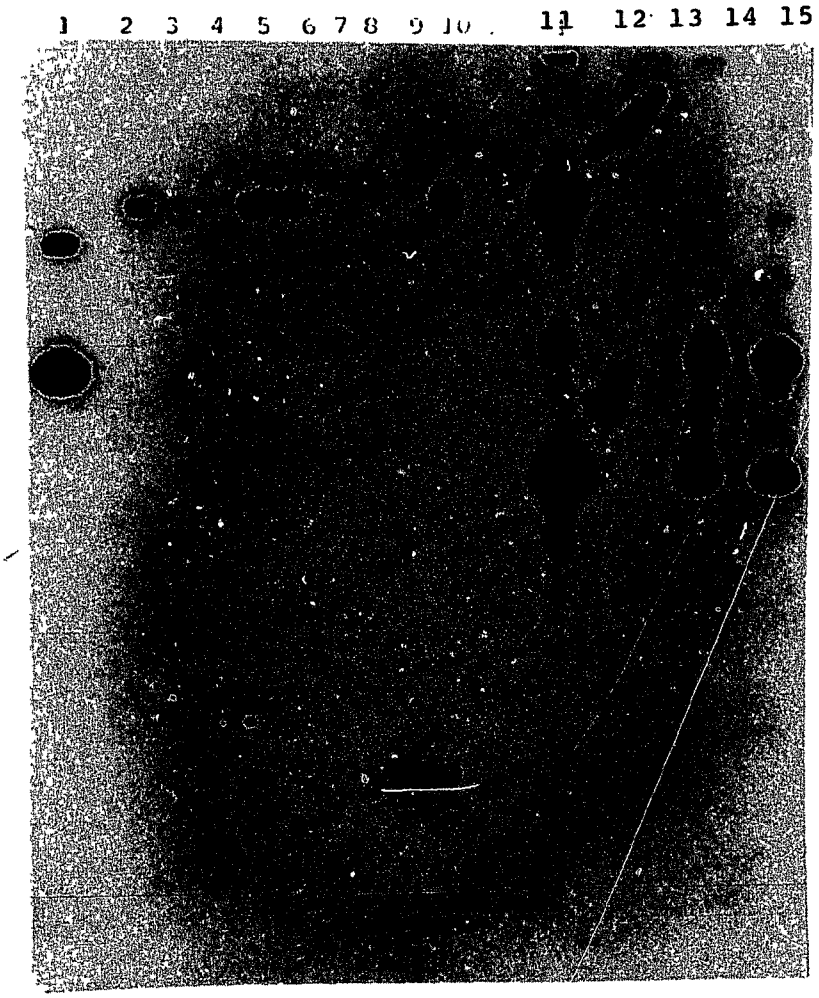


Figure 22.
(B)



into the chromosome was also a possibility. Since pIJ702 does not hybridize to S. venezuelae genomic DNA at high stringency, integration of this vector alone was much less likely to have occurred.

To distinguish between these possibilities, the genomic DNA of four pDQ171 transformants and VS154 (parent strain) was digested with PstI and analysed by Southern hybridization using either pIJ702 (Figure 23B) or pDQ171 (Figure 23C) as the probe. While pIJ702 did not hybridize to the genomic DNA of VS154 (Figure 23B, lane 3), pDQ171 did hybridize to an approximately 11.8-kb DNA fragment (Figure 23C, Lane 3). Among the pDQ171 transformants, two patterns of hybridization signals were observed, one given by AP11 and AP14, the other by AP12 and AP13; the two patterns could be distinguished with either probe. The pattern given by AP11 and AP14 (lanes 4 and 7) would be expected if pDQ171 integrated into the chromosome by a single crossover. Since there is only one PstI site in the vector portion of pDQ171, integration of the plasmid into the 11.8-kb PstI-PstI chromosomal DNA fragment should introduce an additional PstI site. Thus digestion with PstI should generate two fragments hybridizing to pDQ171 and pIJ702. The results were consistent with these predictions in that the 11.8-kb DNA fragment carrying the trpD gene in the parental DNA was replaced by fragments of approximately 11.4 and 10.4 kb. The combined size (21.8 kb) accomodates a single copy of pDQ171 (10 kb) integrated into the 11.8-kb chromosomal

Figure 23. Southern hybridization with pIJ702 and pDQ171 at high stringency of PstI-digested genomic DNA samples of S. venzuelae VS154 transformants containing pDQ171 (AP11-14).

(A) Gel electrophoresis of the DNA samples: lane 1, lambda DNA digested with HindIII; lane 2, pDQ171 digested with PstI; lane 3, VS154; and lanes 4-7, AP11-14.

(B) Autoradiogram after transfer of the DNA samples to a nylon membrane and hybridization with pIJ702.

(C) Autoradiogram after reprobing the nylon membrane with pDQ171

Figure 23.

(A)

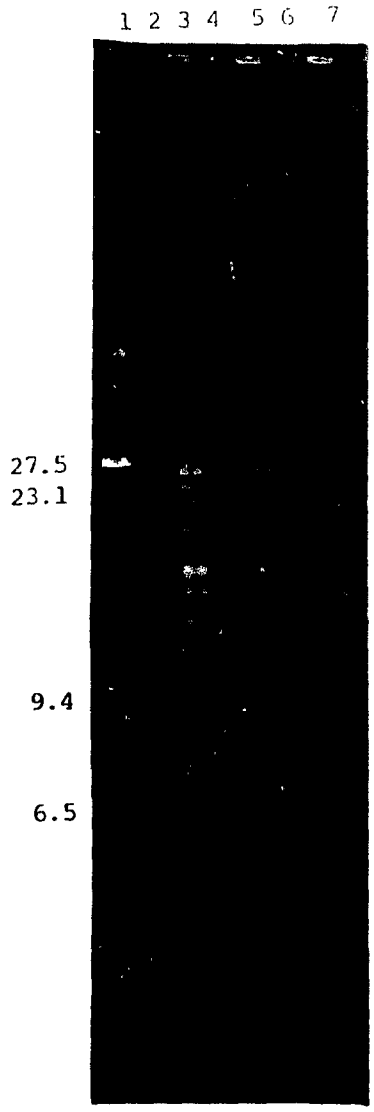
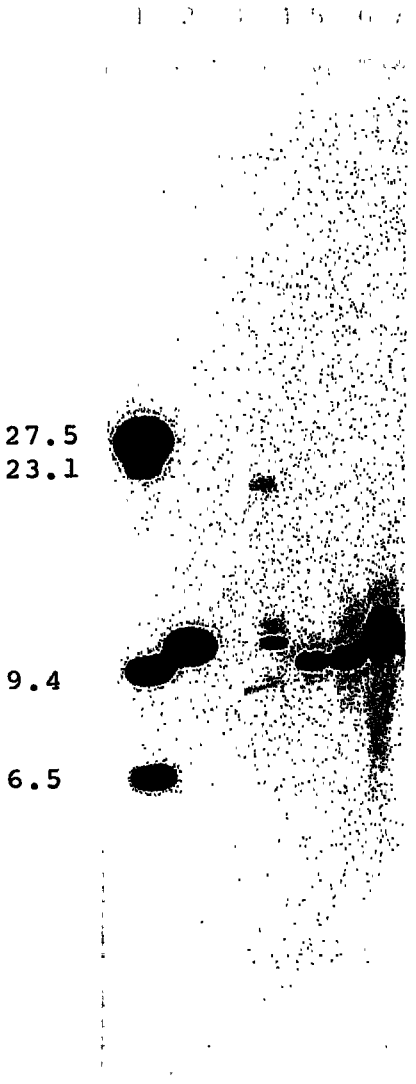
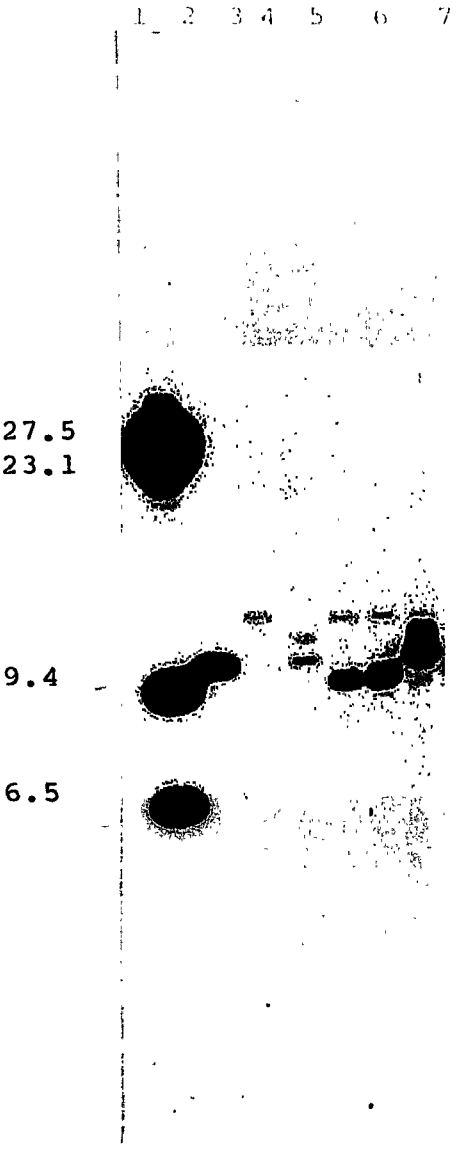


FIGURE (23).

(B)



(C)



DNA fragment. An additional band at approximately 22-kb can be interpreted as a product of partial digestion.

The hybridization patterns shown by the genomic DNA samples of AP12 and AP13 probed with pIJ702 and pDQ171 are more difficult to explain. One of the two hybridizing fragments (lanes 5 and 6) was identical to the 11.8-kb chromosomal DNA fragment from VS154 (lane 3); the other was of 9.5 kb and was more intense. A possible explanation is that pDQ171 is present as a multimeric plasmid, but has suffered a deletion of about 0.5 kb in a region outside trpD, tsr and sequences necessary for replication (AP12 and AP13 are thiostrepton-resistant and prototrophic). Deletions due to the instability of pIJ101-based plasmids have been reported (Butler et al., 1989; K. Madduri, Ph.D. thesis, Dalhousie University).

In a control experiment, genomic DNA of strain AP15, which carried pIJ702 alone, was digested with PstI and analyzed by Southern hybridization using radioactive pIJ702 as a probe. The autoradiogram showed a single band at 5.8 kb corresponding to linear pIJ702 (data not included). The results indicate that pIJ702 alone does not integrate into the S. venezuelae chromosome, but does so when it carries a segment of chromosomal DNA. In the light of this observation, the hybridization experiments in which intact genomic DNA samples of VS154 transformed with pDQ171 and pIJ702 were probed with pIJ702 (see Figure 22B) can be interpreted to mean

that pIJ702 can undergo multimer formation in S. venezuelae. The presence of high molecular weight pIJ702 multimers in S. venezuelae also explains the low amounts of supercoiled plasmids observed during gel electrophoresis, a phenomenon previously attributed to be due to a low copy number (Aidoo et al, 1990).

B. Cotransduction of tsr and nicB1

Marker linkage in transformants that showed the pattern expected for integration of pDQ171 into the chromosome by homologous recombination was investigated by genetic analysis. Stuttard (1983a) showed that trpD4 and nicB1 are cotransducible at a frequency of 10%. Integration of pDQ171 into the chromosome by homologous recombination should introduce the vector-based tsr marker into the trpD region. To see if tsr was cotransducible with nicB1, a transductional cross was performed. Phage SV1 was grown on the donor strain AP11 to produce a high titre phage lysate which was then used to infect strain VS19 (nicB1). Nic+ transductants were selected on minimal medium containing tryptophan. When replica-plated on MYM and MYM containing thiostrepton, four out of 403 transductants tested were thiostrepton resistant. Since not a single thiostrepton-resistant colony was observed when approximately 0.5×10^7 spores of the recipient strain, VS19, were plated on MYM agar containing thiostrepton and incubated for 3-4 days, the 1% thiostrepton resistance among

the transductants was presumed to be the result of transduction, and not a spontaneous mutation to drug resistance. To confirm this, the genomic DNA of one of the transductants digested with BclI was analysed by Southern hybridization. The DNA digest was probed with the plasmid pHJL400, which contains tsr on the same 1.1-kb BclI fragment present in pIJ702. As shown in Figure 13 (lane 2), the probe hybridized to a single 1.1-kb DNA fragment in the BclI-digested genomic DNA of the transductant. Since the transductant was obtained using SV1 phage grown on a donor strain that contained the pIJ702-based recombinant plasmid pDQ171 integrated into the chromosome, and since neither pHJL400 nor pIJ702 hybridize to S. venezuelae, it was concluded that the hybridizing fragment was derived from the genomic DNA of the donor strain, and that the nic^+ tsr strains recovered in the transduction experiment were true transductants.

DISCUSSION

I. Characterization of Trp⁻ auxotrophs of Streptomyces

As expected for the highly conserved tryptophan biosynthesis pathway, all tryptophan-requiring auxotrophs characterized from S. venezuelae and S. lividans belonged to phenotypic classes similar to those described for other bacteria. Characterization of mutants based on growth requirements, pathway intermediates accumulated, and enzyme assays yielded all known classes of trp mutations except those designated trpF, trpE, or trpG. All of the mutant phenotypes identified in this study, and in addition a trpF phenotype, have previously been described in S. coelicolor A 3(2) (Smithers and Engel, 1974). However, trpE and trpG mutants in streptomycetes still remain elusive.

The specific activities of pathway enzymes in S. venezuelae and S. lividans trp auxotrophs were several-fold higher than in the wild types. A similar increase in activity was observed in other bacteria (Smith and Yanofsky, 1962) and it was suggested that the enzyme activities were derepressed as a consequence of tryptophan starvation. In S. venezuelae and S. lividans, derepression of anthranilate synthetase, the first pathway enzyme, was higher than that of other enzymes; a similar observation was made in S. coelicolor A 3(2) (Smithers and Engel, 1974). The greater derepression of anthranilate synthetase activity suggests that in streptomycetes the flow of metabolites through this pathway

might be regulated mainly at the first step in tryptophan biosynthesis. In support of this, anthranilate synthetase is subject to both inhibition and repression by tryptophan in S. venezuelae sp. 3022a (Francis et al., 1978).

In S. venezuelae cell extracts all the Trp enzyme activities, except TrpA, were demonstrable. Although they failed to show activity in cell extracts, the auxotrophs carrying the trpB5 and trpB14 alleles evidently possessed TrpA subunit activity in vivo because their cells accumulated indole. The presence of TrpA activity in extracts of S. lividans and E. coli but not in those of S. venezuelae suggests that the TrpA subunit in S. venezuelae is inactivated during the preparation of cell extracts. A similar situation was reported for Bacillus subtilis and Staphylococcus aureus in which the TrpB subunit was inactivated during extraction (Proctor and Kloos, 1973).

Among presumptive trpA mutants, two sub-classes were observed. One showed weak TrpB activity in cell extracts compared to that in the trpC or trpD classes of mutants, while in the other TrpB activity was absent. Reduced TrpB activity in trpA mutants of S. venezuelae suggests a functional relationship between TrpA and TrpB gene products similar to that observed in other bacteria (Pittard, 1987). TrpA and TrpB are subunits of the multimeric enzyme, tryptophan synthetase, and catalyze the reactions InGP ---> indole and indole ----> tryptophan, respectively. However, the subunits interact with

each other during the overall conversion of InGP to tryptophan. The activity of one subunit decreases substantially in the absence of the other. Moreover, mutations affecting this interaction between the subunits result in considerable loss of both enzyme activities. The S. venezuelae mutants VS24 and VS420 accumulated InG/I[illegible] and possessed much weaker TrpB activity than strains with w[illegible] type TrpA and TrpB subunits (e.g., mutants carrying trpC3, trpC7, or trpD4 alleles). Presumably the trpA1 and trpA13 mutations in the TrpA subunit of VS24 and VS420, respectively, are not only defective in converting InGP to indole but also reduce the activity of the TrpB subunit. Probably also in this category is VS317 carrying the trpA9 mutation, which lacked detectable TrpB activity in vitro but grew slowly on indole and so must have possessed weak TrpB activity in vivo. Strains VS180 and VS517 which belong to the other category of trpA mutants and accumulated InG/InGP, grew on tryptophan but not indole, and showed no TrpB activity. Presumably these mutants have a defective TrpA as well as an inactive or nonexistent TrpB subunit, and therefore may be polar or double trpA trpB mutants.

II. Fine structure map of the his-trp region

Generalized transduction makes it possible to study gene organization at the fine structure level and has been used to study trp gene arrangements in E. coli (Yanofsky and Lennox,

1959), S. typhimurium (Demerec and Hartman, 1956), P. aeruginosa (Fargie and Holloway, 1965) and S. aureus (Proctor and Kloos, 1970). Using this technique in S. venezuelae, Stuttard (1983a) deduced that the available trp mutations could be placed in two chromosomal locations. The auxotrophs bearing these mutations were not then fully characterized; however, identification of the biochemical lesions in the present study confirms that in S. venezuelae trpA, trpB, and trpC are cotransducible with the hisA and hisB mutations while trpD is cotransducible with nicB. Moreover from relative cotransduction frequencies, the group of his and trp genes mapped to the 12′ clock region of the S. venezuelae chromosome can be arranged in the order: hisA-hisB-trpC-trpB-trpA. Confirmation of this order is needed but would require either a series of three-point crosses with strains carrying double mutations in trp and his loci, or molecular analysis. Progress in the latter approach was made during the present study with the cloning of an S. venezuelae DNA fragment complementing a trpC mutation in S. lividans.

Molecular analysis of trp genes in other streptomycetes has revealed that they are physically clustered on the chromsome. In S. griseus, trpA, trpB, and trpC genes were located on a 4.2-kb DNA chromosomal fragment (Rivero-Lezcano et al., 1990), while in S. coelicolor A 3(2), molecular cloning and nucleotide sequence analysis has located these genes in a cluster and arranged in the order trpC-trpB-trpA

(Hodgson, personal communication). This is the same gene order as deduced for S. venezuelae from cotransduction data and suggests that a common trp gene arrangement may be present within streptomycetes.

III. Molecular cloning of trpEG

In the absence of any trpE or trpG mutants in streptomycetes, the approach taken to clone the corresponding genes from S. venezuelae was to complement relevant E. coli trp auxotrophs. Because the tryptophan pathway is highly conserved it was anticipated that Streptomyces trp gene products would be functional in E. coli, as are the trp gene products from divergent groups of eubacteria and also archaebacteria (Crawford, 1989). Whether the S. venezuelae genes would be expressed was uncertain, however, since Streptomyces promoters, with only a few exceptions, are generally not recognized in E. coli. Most of the Streptomyces genes that have been expressed in E. coli depended upon transcription from adjacent promoters present on the cloning vectors. The exceptions to this are the promoters in S. lividans for argG (Meade, 1985) and pabAB (Arhin, personal comunication) and the S. coelicolor hisBd promoters (Limauro et al., 1990), which are presumably recognized in E. coli since the expression of these genes was observed when cloned in either orientation on plasmid vectors.

To maximize the chances of achieving expression in E. coli, random S. venezuelae genomic fragments were cloned downstream from the lac promoter of the E. coli plasmid pTZ18R, anticipating expression from the lac promoter in case the S. venezuelae promoter did not function. A recombinant plasmid, pDQ181, that complemented the trp mutation in E. coli, was isolated, and, as frequently observed for Streptomyces genes, S. venezuelae trpEG was expressed in only one orientation; presumably the plasmid-based lac promoter was used.

Plasmid pDQ181 was able to complement deletions in both the trpE and trp(G)D genes of E. coli, and the presence of both glutamine- and ammonia-reactive anthranilate synthetase activity in cell extracts of strains transformed with pDQ181 was consistent with the S. venezuelae DNA insert encoding both TrpE and TrpG functions. Such trpE- and trp(G)D-deleted E. coli strains have been used previously to clone trp genes from Leptospira biflexa (Yelton and Cohen, 1984), Spirochaeta aurantia (Brahamsha and Greenberg, 1987), and Thermus thermophilus (Sato et al., 1988). However, only in L. biflexa and T. thermophilus were trpE and trpG clustered. In S. venezuelae it is very likely that these two functions are encoded by two separate genes rather than one fused gene as observed in Rhizobium meliloti (Bae and Crawford, 1989) since TrpE and TrpG activities eluted separately during chromatography (Francis et al., 1978).

IV. A method for mapping genes in S. venezuelae

Aidoo (1989, Ph.D. thesis, Dalhousie University) observed that recombinant plasmids carrying S. venezuelae pab and pdx genes were unstable in the homologous. Subsequently he found cotransduction between the tsr and pdx markers in S. venezuelae transformants and demonstrated by Southern hybridization that DNA carrying the tsr gene was present. Aidoo et al., (1990) suggested that plasmids carrying homologous DNA inserts tended to integrate into the chromosome by recombination at high frequency. In the present study, the S. venezuelae trpD-carrying plasmid, pDQ171, was unstable as an autonomous entity and was shown by Southern analysis to be integrated into the chromosome. In one strain where integration had occurred, 1% cotransduction between the tsr and neighbouring nicB1 markers was detected.

This procedure promises to be very useful for locating the map position in streptomycetes of cloned genes for which a mutant phenotype does not exist. The gene of interest should be cloned on a suitable vector carrying a selectable marker and allowed to integrate into the chromosome. The location can then be determined by mapping the vector-based marker. The method has been used to map ermE in Sac. erythraea (Weber and Losick, 1988) and in this study was used successfully to determine the chromosomal location of trpEG in S. venezuelae.

V. Integration of pDQ189 into the S. venezuelae chromosome

Plasmid pHJL400 is an SCP2*-derived vector and is segregationally unstable owing to lack of the par function (Larson and Hershberger, 1986). The vector was used to achieve efficient plasmid integration into the chromosome by homologous recombination in S. griseofuscus (Larson and Hershberger, 1990). Since Aidoo et al. (1990) demonstrated that S. venezuelae could be transformed with the SCP2* derived vector, pDQ101, use of the more convenient pHJL400 to achieve plasmid integration in S. venezuelae was explored. The results established that pHJL400 was able not only to transform S. venezuelae to thiostrepton resistance but was lost from the transformants with more than 98% frequency when they were grown without selection. This high frequency of plasmid loss is comparable to that obtained in S. griseofuscus by Larson and Hershberger (1990) and indicated that the vector was suitable for use in gene disruption/replacement analysis in S. venezuelae.

When S. venezuelae transformants containing pDQ189 (pHJL400 with the trpEG insert) were grown without selection, a high proportion of stable transformants with pDQ189 integrated into the chromosome were recovered. Southern hybridization analysis established that pDQ189 integrated by single reciprocal homologous recombination. Integration by a single crossover has been observed in other streptomycetes (Vara et al., 1989; Weber and Losick, 1988; Weber et al.,

1990), and as in these cases, further growth of the strain without selection gave thiostrepton-sensitive colonies. This suggested that plasmid excision took place.

Southern hybridization analysis of a stable S. venezuelae transformant suggested that its chromosome contained 3-4 copies of pDQ189 integrated in tandem. Although rare, multiple tandem integrations have been reported in S. griseofuscus using a similar pHJL400-based plasmid (Larson and Hershberger, 1990). Likewise, in Sac. erythraea, a recombinant pIJ702 plasmid gave multiple tandem integration (Weber et al., 1990).

The phenomenon of multiple tandem integrations has not been well characterized. It is possible that a single copy of the multicopy replicative plasmid integrates first, followed by sequential integration of other copies into the first chromosomal copy. Thus integration involving a nonreplicative plasmid should give only single-copy integration. This could not be tested in the present study since S. venezuelae could not be transformed with the nonreplicative vector, pDQ188. Alternatively, multiple copies of the integrated plasmid might arise by amplification of the single integrated copy of the plasmid and be retained as a result of selective pressure. In S. typhimurium, multiple copies of a non-replicative vector carrying homologous DNA and a tetracycline resistance gene integrated in tandem into the chromosome. Moreover, the integrated plasmid copy number was proportional to the levels

of tetracycline in the growth medium (Gutterson and Koshland, 1983). During the present study, the S. venezuelae integrant was selected and maintained on medium containing 50 ug/mL of thiostrepton, and it would be of interest to determine if different copy numbers of the integrated pDQ189 can be selected at different concentrations of thiostrepton.

Failure of the nonreplicative vector to transform S. venezuelae may have been due to the low DNA uptake efficiency or to the size of the homologous DNA insert present on the vector, or both. Such vectors have been used for gene replacement and gene disruption in S. ambofaciens (Richardson et al., 1989). However, S. venezuelae, unlike S. ambofaciens, has a strong restriction system (Stuttard, 1982; Kuhstoss et al., 1989) and therefore the efficiency with which it could be transformed by a vector isolated from E. coli would be much lower.

VI. Chromosomal location of trpEG in S. venezuelae

The chromosomal location of pDQ189 carrying trpEG and tsr was mapped by conjugation in four different crosses. In all crosses the map position of tsr was constant and the cumulative results from these crosses indicated that tsr was located in the short arc between trpA13 and arg-6.

In crosses between the integrant AP41 (hisA6 adeA10 tsr) and VS161 (tyr-2 thrC1 uraA1), no segregation was observed between tyr^+ and tsr markers present in the integrant. Close

linkage of the two markers was also indicated by the Tyr' phenotype of all thiostrepton-resistant recombinants. However, because no cotransduction of the markers was detected, they appear not to be located within 45 kb (the approximate size of phage SV1 genomic DNA) of each other (Stuttard, 1989). Cotransduction was found, albeit at low frequency, between tsr and trpB14. This suggested that these two markers are within 45 kb of each other. The possibility that two independent phage particles, one carrying tsr and the other carrying trpB14 infected the same recipient is ruled out by the very low frequency with which such an event would occur. Under optimum conditions, any given donor marker can be transduced to the recipient at a frequency of 10^{-6} pfu^{-1}. Receipt of two transducing particles, at a frequency of 10^{-12} pfu^{-1}, would be unlikely under the experimental conditions since no more than 10^{10} pfu of phage were used for a transduction experiment. Moreover, if spurious cotransduction occurred it should be detected with other unlinked markers under similar conditions, but no cotransduction was detected between the tsr and tyr-2 markers.

Although trpEG is located within 45 kb of trpB it is not close enough to be clustered with trpCBA. That the two sets of trp genes form separate clusters is supported by the restriction maps of the chromosomal fragments carrying trpEG and trpC. Southern analysis indicated that trpEG sequences are present on two, 12- and 16.5-kb, MluI fragments. The failure

of these two fragments to hybridize to trpC sequences places trpC further than 12 kb on one side and 16.5 kb on the other side from trpEG. Based on the combined evidence, the trpEG cluster is separated from trpCBA by at least 12 kb but not more than 45 kb of DNA. Chromosomal walking using DNA from either cluster should establish the exact physical distance between them.

VII. Attempts to isolate a trpE/G auxotroph

Routine mutagenesis used in the first attempt to isolate trpE or trpG auxotrophs was not successful. When the cloned 2.4-kb trpEG DNA became available, it was possible to use a more specific procedure, namely gene replacement. A mutation was created in the 2.4-kb trpEG DNA sequence by modification of the unique MluI site. As evidence that this modification inactivated the TrpE function, the plasmid carrying the "modified" trpEG failed to complement a trpE mutation in E. coli. However, strains in which the wild type trpEG was replaced in the S. venezuelae chromosome by the "modified" sequence did not exhibit a Trp^- phenotype. Taking into consideration the present lack of information about the precise arrangement of trpE and trpG on the 2.4-kb DNA fragment, two explanations can be suggested for the failure of the modification at the MluI site to give a TrpE- phenotype in S. venezuelae whereas it does so in E. coli.

Firstly, the MluI site within the 2.4- kb trpEG DNA

fragment may not lie in the trpE structural gene and may be located upstream from it, between the trpE structural gene and the adjacent vector-encoded lac promoter. Modification of the MluI site (A↓CGCGT) by treatment with mung bean nuclease and the Klenow fragment could introduce either a point mutation if three base pairs are deleted, or a frame shift mutation if one, two or four base pairs are removed. A frame shift mutation at the MluI site would alter the entire trpE reading frame downstream from this site and would certainly result in the loss of TrpE function in E.coli where trpE is presumably transcribed from the lac promoter (since only one orientation of the insert complemented the host mutation). A similar frameshift effect could explain why deletion of the 0.9-kb BamHI-SstI lac-promoter-proximal fragment destroyed the ability of pDQ185 to complement the trpE mutation in E. coli. When present in the S. venezuelae chromosome, trpEG would be expressed from its native promoter; if the modified MluI site was outside the region encoding trpEG and its promoter(s), the mutation might not cause auxotrophy. Against this possibility is the relatively small size of the trpEG fragment downstream of the MluI site. The TrpE function would have to lie within 1.2 kb of DNA (since the MluI site lies approximately in the middle of the 2.4-kb fragment). In bacteria examined so far, the trpE gene product consists of a single polypeptide chain with molecular weight of approximately 60,000-80,000 (Zalkin, 1980). The molecular weight of the TrpE protein in S.

venezuelae sp 3022a (a strain closely related to 13s and similar in many characteristics to ISP5230) was estimated to be 72,000. If TrpE is a single polypeptide, at least 1.9 kb of DNA (taking the average molecular weight of an amino acid to be 110) is probably needed to encode it.

The second possibility is that the MluI site does lie within the trpE structural gene and that the gene replacement abolishes TrpE function at this site, both in E. coli and in S. venezuelae. However, lack of this TrpE activity in S. venezuelae strains does not cause a Trp^- phenotype because a second set of trpE genes is present elsewhere in the chromosome. There is a precedent for this in P. aeruginosa where there are two sets of anthranilate synthetases encoded by separate genes. One is involved in tryptophan biosynthesis while the other one takes part in the biosynthesis of phenazine secondary metabolites derived from anthranilic acid (Essar et al., 1990). Both sets of genes are able to complement the respective trpE and trpG mutations in E. coli, and under some conditions their functions are interchangeable. Mutations in the genes of one pathway could be suppressed by the normal function of genes of the other pathway.

If a similar situation existed in S. venezuelae the second set of anthranilate synthetase genes might be involved in the biosynthesis of an as yet unidentified secondary metabolite. Although S. venezuelae is not known to produce phenazines, other actinomycetes, including several species of

stretomycetes, do produce such compounds (Gerber, 1984). Besides phenazines, other streptomycete secondary metaboites likely to be derived from anthranilate include streptonigrin, an antibiotic produced by S. flocullus (Gould and Erickson, 1988; Erickson and Gould, 1987), 2-3-dihydro-3-hydroxyanthranilic, a metabolite from S. aureofaciens (Weiss and Edwards, 1980), and 3-hydroxy-anthranilate which is the precursor of antibiotic LL-C10037 from Streptomyces LL-C10037 (Whittle and Gould, 1987).

Southern hybridization at low stringency of S. venezuelae genomic DNA using the 2.4-kb trpEG DNA as probe did not show presence of a second hybridizing fragment (the first one containing the cloned trpEG) which could represent the second set of trpE gene. A more specific probe to detect the second set of trpE gene would be to use an internal DNA sequence from a conserved region of the cloned trpE gene. Nucleotide sequence analysis of the 2.4-kb trpEG DNA of S. venezuelae will assist in such an experiment and also allow a decision between the above two possibilites.

VIII. Organization of tryptophan biosynthesis genes in Streptomyces

The results obtained in this study have clarified the organization of all tryptophan biosynthesis genes in S. venezuelae except trpF. Although it is clearly desirable to characterize a trpF mutation and to map it on the chromosome,

information from work on other Streptomyces can be drawn upon for an overview of trp gene organization in this genus. Extrapolation of data from other species is reasonable because trp gene organization is generally the same within each particular group of bacteria (Crawford, 1980). Results obtained so far in a limited number of streptomycetes, namely, S. coelicolor (Smithers and Engel, 1975), S. griseus (Rivero-Lezcano et al., 1989) and S. venezuelae (Stuttard, 1983a; this study) support the generalization. Moreover, the chromosomal maps of different species of streptomycetes, despite some minor variations, show an overall conservation of linkage relationships (Stuttard, 1988).

In overview then, streptomycete trp genes are grouped on the chromosome at three locations. At one location are trpD and trpF but no data are yet available on whether these genes are physically adjacent and co-regulated. At a different location is the trpCBA cluster, while trpEG forms a third locus which maps to a location within 45 kb of trpCBA. This arrangement of trp genes is unique and represents another variation in the gene organization patterns discovered among bacteria. It is consistent with the view that, although their basic functional domains have remained constant, the trp genes have been subject during the evolution of species to a variety of chromosomal rearrangements such as translocation, inversion, fusion and gene splitting.

Unlike the arrangement in enteric bacteria, where trp

genes form an operon, the organization in streptomycetes is more dispersed, and shows similarity to that in A. calcoaceticus, P. acidovorans and R. meliloti which also have three clusters; however, it differs in the composition of these clusters. In having one distinct cluster for trpE and trpG, it most closely resembles the organization in R. meliloti. The other two clusters in this species differ in the substitution of trpF for trpC and vice versa.

The trp gene organization in streptomycetes is not representative of the high G+C subdivision of the Gram-positive bacteria, to which these organisms belong (Woese, 1987). In B. lactofermentum, which belongs to this subdivision, all trp genes are arranged in an operon, very similar to that in enteric bacteria. Such an arrangement could have resulted from a recent lateral transfer of genetic information. It was suggested that this possibility would be strengthened if the nucleotide sequences of trp genes in B. lactofermentum proved to be quite different from those of other members of the same subdivision. Nucleotide sequence analysis of the S. venezuelae trp genes isolated in this study will assist in the testing of this hypothesis. If S. venezuelae trp sequences are more similar to other Gram-positive bacteria - e.g., B. subtilis - than to B. lactofermentum, the notion of lateral transfer of trp genes from enterics to the latter would be supported. It will be necessary to study the trp gene arrangements in other members

of the high G+C subdivision to understand the diversity present among its members.

SUMMARY AND CONCLUSIONS

The growth requirements, accumulation of pathway intermediates, and enzyme activities, of tryptophan-requiring auxotrophs of S. venezuelae and S. lividans isolated previously and in this study were characterized. The results suggest that the tryptophan pathway in streptomycetes is biochemically identical to that found in other bacteria. All classes of tryptophan-requiring auxotrophs, except trpF, trpE, and trpG, were found. Identification of their biochemical lesions and additional information from the present studies confirmed previous evidence (Stuttard, 1983a) from transductional analysis of some of the strains that in S. venezuelae, trpC, trpB and trpA are each cotransducible with hisA and hisB. The data suggested that the order is hisA-hisB-trpC-trpB-trpA. In addition, trpD was confirmed to be cotransducible with nicB. This arrangement of trp genes at two location is similar to that present in S. coelicolor A 3(2) (Smithers and Engel, 1974).

Chromosomal DNA fragments carrying trp genes from both the regions were cloned by complementation of S. lividans trpC and trpD mutants. A trpE-carrying DNA fragment was cloned by complementation of an E. coli trpE auxotroph. Since the recombinant plasmid carrying trpE also complemented an E. coli auxotroph containing deletions in trpE and a portion of trpD

that encodes the TrpG function, trpE and trpG in S. venezuelae are physically clustered. The presence of both trpE and trpG on the cloned fragment was confirmed by enzyme assays and growth studies.

Introduction of the S. venezuelae trpD-carrying recombinant plasmid into the homologous host resulted in integration of the plasmid into the chromosome by homologous recombination. That integration occurred was demonstrated by Southern analysis and also by transduction. The results are similar to those of Aidoo (1990) who observed integration of recombinant plasmids carrying S. venezuelae pab and pdx genes. This indicates suggests that most, if not all, recombinant plasmids carrying homologous DNA inserts cannot be stably maintained in S. venzuelae.

The location of trpEG was determined by allowing a recombinant plasmid carrying trpEG and tsr to integrate into the S. venezuelae chromosome. The integrated tsr, and thus trpEG, was mapped by conjugation and transduction to a location near but distinct from the trpCBA cluster. The combined evidence from genetic mapping and restriction enzyme analysis of cloned DNA indicates that trpEG is within 45 kb of trpCBA but is separated from this cluster by at least 12 kb. These results demonstrate that the overall arrangement of trp genes in streptomycetes differs from that in all other bacteria examined so far.

Since routine mutagenesis failed to yield any trpE or

trpG mutants, more specific attempts to obtain these were made by gene disruption and replacement. A procedure was developed in S. venzuelae for creating a mutation on a cloned fragment, followed by replacement of the wild-type fragment with the mutated copy. However, strains carrying the mutation in the trpEG DNA fragment did not show a TrpE$^-$ phenotype.

REFERENCES

Aidoo, D.A., Barrett, K., and L.C. Vining (1990) Plasmid transformation of Streptomyces venezuelae : modified procedures used to introduce the gene(s) for p-aminobenzoate synthetase. J. Gen. Microbiol. 136: 657-662.

Anzai, H., Kumada, Y., Hara, O., Murakami, T., Itoh, R., Takano, S., Imai, S., Satoh, A., and K. Nagaoka (1988) Replacement of Streptomyces hygroscopicus genomic segments with in vitro altered DNA sequences. J. Antibiot. 41: 226-233.

Atkinson, L. (1987) A search for mutants of Streptomyces venezuelae blocked in synthesis of aromatic compounds due to mutations in glutamine amido-transferase. M.Sc. Thesis. Dalhousie University, Halifax, N.S., Canada.

Bae, Y. M., Holmgren, E., and I.P. Crawford (1989) Rhizobium meliloti anthranilate synthetase: cloning, sequence and expression in Escherichia coli. J. Bacteriol. 171: 3471-3478.

Bae, Y.M., and I.P. Crawford (1990) The Rhizobium meliloti trpE(G) gene is regulated by attenuation, and its product anthranilate synthetase, is regulated by feedback inhibition. J. Bacteriol. 172: 3318-3327.

Bae, Y.M., and G. Stauffer (1991) Genetic analysis of the attenuator of the Rhizobium meliloti trpE(G) gene. J.

Bacteriol. 173: 3382-3388.

Bentley, R. (1990) The shikimate pathway- A metabolic tree with many branches. Crit. Rev. Biochem. Mol. Biol. 25: 307-384.

Campbell, A.M. (1962) Episomes. Adv. Genet. 11: 101-145.

Carlton, B.C., and D.D. Whitt (1969) The isolation and genetic characterization of mutants of the tryptophan system of B. subtilis. Genetics 62: 445-460.

Brahamsha, B., and E.P. Greenberg (1987) Complementation of a trpE deletion in Escherichia coli by Spirochaeta aurantia DNA encoding anthranilate synthetase component I activity. J. Bacteriol. 169: 3764- 3769.

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

Buttner, M.J., Chater, K.F., and M.J. Bibb (1990) Cloning, disruption, and transcriptional analysis of three RNA polymerase sigma factor genes of Streptomyces coelicolor A3(2). J. Bacteriol. 172: 3367-3378.

Chater, K.F., and C.J. Bruton (1983) Mutational cloning in Streptomyces and isolation of antibiotic production genes. Gene 26: 67-78.

Chater, K.F., King, A.A., Rodicio, M.R., Bruton, C.J., Fisher, S.H., Piret, J.M , Smith, C.P. and S.G. Foster (1985)

Cloning and analysis of Streptomyces DNA in ϕC31-derived vectors. In Microbiology 1985. Edited by L.Leive. American Society for Microbiology. Washington, D.C. pp. 421-426.

Chater, K.F. (1986) Streptomyces phages and their applications to Streptomyces genetics. In The Bacteria. Vol. IX, Antibiotic-producing Streptomyces. Edited by S.W. Queener and L.E.Day. Academic Press, Orlando. pp. 119-158.

Cohn, W., and I.P.Crawford (1976) Regulation of enzyme synthesis in the tryptophan pathway of Acinetobacter calcoaceticus. J. Bacteriol. 127: 367-379.

Crawford, I.P. (1975) Gene rearrangements in the evolution of the tryptophan synthetic pathway. Bacteriol. Rev. 39: 87-120.

Crawford, I.P. (1980) Comparative studies on the regulation of tryptophan synthesis. Crit. Rev. Biochem. 8: 175-189.

Crawford, I.P. (1987) Synthesis of tryptophan from chorismate: Comparative aspects. In Methods in Enzymology 142. Edited by S. Kaufman, Academic Press. Inc. pp 293-299.

Crawford, I.P. (1989) Evolution of a biosynthetic pathway: the tryptophan paradigm. Ann. Rev. Microbiol. 43: 567-600.

Creighton, T.E., and C. Yanofsky (1966) Indole-3-glycerol phosphate synthetase of Escherichia coli, an enzyme of the tryptophan operon. J. Biol. Chem. 241: 4616-4624.

Demerec, M., and Z. Hartman (1956) Tryptophan mutants in Salmonella typhimurium. In Genetic studies with bacteria.

Carnegie Inst. Washington. Publ. 612: 5-13.

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

Doull, J.L., Vats, S., Chaliciopoulos, M., Stuttard, C., Wong, and L.C. Vining (1986) Conjugational fertility and location of chloramphenicol biosynthesis genes on the chromosomal linkage map of Streptomyces venezuelae. J. Gen. Microbiol. 132: 1327-1338.

Erickson, W.R., and S.J. Gould (1987) Streptonigrin biosynthesis. VIII. Evidence for the involvement of a new shikimate pathway product and a new route to quinolines. J. Am. Chem. Soc.109: 620.

Essar, D.W., Eberly, L., Hadero, A., and I.P. Crawford (1990a) Identification and characterization of genes for a second anthranilate synthetase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthases and evolutionary implications. J. Bacteriol. 172: 884-900.

Essar, D.W., Eberly, L., and I.P. Crawford (1990b) Evolutionary differences in chromosomal locations of four early genes of the tryptophan pathway in fluorescent pseudomonads: DNA sequences and characterization of Pseudomonas putida trpE and trpGDC. J. Bacteriol. 172: 867-883.

Essar, D. W., Eberly, L., Han, C.Y., and I.P. Crawford (1990c) DNA sequences and characterization of four early genes of the tryptophan pathway in Pseudomonas aeruginosa. J. Bacteriol. 172: 853-866.

Fargie, B., and B.W. Holloway (1965) Absence of clustering of functionally related genes in Pseudomonas aeruginosa. Genet. Res. 6: 284-299.

Francis, M. M., Vining, L.C., and D.W.S. Westlake (1978) Characterization and regulation of anthranilate synthetase from a chloramphenicol-producing streptomycete. J. Bacteriol. 134: 10-16.

Gerber, N (1984) Microbial Phenazines. In Handbook of Microbiology, edited by A.I. Laskin and H.E. Lechevalier, CRC Press, Boca Raton. pp 573-576.

Gibson, F (1964) Chorismic acid: purification and some chemical and physical studies. Biochem. J. 90: 256-261.

Goncharoff, P., and B.P. Nichols (1988) Evolution of aminobenzoate synthases: nucleotide sequences of Salmonella typhimurium and Klebsiella aerogenes pabB. Mol.Biol.Evol. 3: 191-204.

Gould, S.J., and W.R. Erickson (1988) Isolation of 4-aminoanthranilic acid: a new shikimate pathway product from Streptomyces flocculus. J. Antibiot. 41: 688.

Gruss, A., and S. Ehrlich (1988) Insertion of foreign DNA into plasmids from Gram-positive bacteria induces formation of high molecular-weight plasmid multimers. J. Bacteriol.

170: 1183-1190.

Gruss, A., and S. Ehrlich (1989) The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. Microbiol.Rev. 52: 231-241.

Gunsalus, I.C., C.F. Gunsalus, Chakrabarty, A.M., Sikes, S., and I.P. Crawford (1968) Fine structure mapping of the tryptophan genes in Pseudomonas putida. Genetics. 60: 419- 435.

Gutterson, N.I., and D.E. Koshland (1983) Replacement and amplification of bacterial genes with sequences altered in vitro Proc. Natl. Acad. Sci. 80: 4894-4898.

Hahn, D.R., Solenberg, P.J., McHenney, M.A., and R.H. Baltz (1991) Transposition and transduction of plasmid DNA in Streptomyces spp. J. Ind. Microbiol. 7: 229-234.

Henner, D.J., Band, L., and H. Shimotsu (1984) Nucleotide sequence of the Bacillus subtilis tryptophan operon. Gene. 34: 169-177.

Hilleman, D., Puhler, A., and W. Wohlleben (1991) Gene disruption and gene replacement in Streptomyces via single stranded DNA transformation of integration vectors. Nuc. Acids. Res. 19: 727-731.

Hoch, S.O., and I.P. Crawford (1973) Enzymes of the tryptophan pathway in three Bacillus species. J. Bacteriol. 116: 685- 693.

Holmgren, E., and I.P. Crawford (1982) Regulation of the tryptophan genes in Rhizobium leguminosarum. J.

Bacteriol. 149: 1135-1137.

Hopwood, D.A. (1967) Genetic analysis and genome structure in Streptomyces coelicolor. Bacteriol. Rev. 31: 373-403.

Hopwood, D.A., Kieser, T., Wright, H.M., and M.J. Bibb (1983) Plasmids, recombination and chromosome mapping in Streptomyces lividans 66. J. Gen. Microbiol. 129: 2257-2269.

Hopwood, D.A., Kieser, T., Lydiate, D.J., and M.J. Bibb (1986) Streptomyces plasmids: their biology and use as cloning vectors. In The Bacteria, Vol. IX, Antibiotic-producing Streptomyces, Edited by S.W.Queener and L.E.Day, Academic Press, Orlando, pp. 159-229.

Hopwood, D.A., Malpartida, F., Kieser, H.M., Ikeda, H., and S. Omura (1985) Cloning genes for antibiotic biosynthesis in Streptomyces spp : production of a hybrid antibiotic. In Microbiology 1985. Edited by L.Leive. American Society for Microbiology, Washington, D.C. pp. 409-413.

Hopwood, D.A., and T. Kieser (1990) The Streptomyces genome. In The bacterial chromosome. Edited by K. Drlica and M. Riley. American Society for Microbiology, Washington, D.C. pp. 147- 162.

Hopwood, D.A., Chater, K.F., Dowding, J.E., and A. Vivian (1973) Advances in Streptomyces coelicolor genetics. Bacteriol. Rev. 37: 371-405.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton,

C.J., Kieser, H.M., Lydiate, C.P., Smith, C.P., Ward, J.M., and H. Schrempf (1985) Genetic manipulation of Streptomyces : a laboratory manual. John Innes Foundation, Norwich.

Hwang, L.H., and H. Zalkin (1971) Multiple forms of anthranilate synthetase-anthranilate 5-phosphoribosyl-pyrophosphate phosphoribosyl transferase from Salmonella typhimurium. J. Biol. Chem. 246: 2338-2345.

Hyde, C.C., Ahmed, S.A., Padlan, E.A., Miles, E.W., and D.R. Davies (1988) Three-dimensional structure of the tryptophan synthase α_2-β_2 multienzyme complex from Salmonella typhimurium. J. Biol. Chem. 263: 17857-17871.

Johnston, A.W.B., Bibb, M.J., and [illegible]. Beringer (1978) Tryptophan genes in Rhizobium: their organization and their transfer to other bacterial genera. Mol. Gen. Genet. 165: 323-330.

Jones, A., and D.W.S. Westlake (1974) Regulation of chloramphenicol synthesis in Streptomyces spp. 3022a. Properties of arylamine synthetase, an enzyme involved in antibiotic synthesis. Can. J. Microbiol. 20: 1599-1611.

Kane, J.F., Holmes, W.M., and R.A. Jensen (1972) Metabolic interlock: the dual function of a folate pathway gene as an extra-operonic gene of tryptophan biosynthesis. J. Biol. Chem. 247: 1587-1596.

Kaplan, J.B., Merkel, W.K., and B.P. Nichols (1985) Evolution of glutamine aminotransferase genes: nucleotide sequences

of the pabA genes from Salmonella typhimurium, Klebsiella aerogenes and Serratia marscesens. J. Mol. Biol. 183: 327- 340.

Katz, E., Thompson, C.J., and D.A. Hopwood (1983) Cloning and expression of the tyrosinase gene from Streptomyces antibioticus in Streptomyces lividans. J. Gen. Microbiol. 229: 2703-2714.

Kirschner, K., Szadkowsky, H., Henshen, A., and F. Lottspeich (1980) Limited proteolysis of N-(5'phosphoribosyl) anthranilate isomerase:indole-glycerol synthase from Eschericha coli yields two different enzymatically active, functional domains. J. Mol. Biol. 143: 395-409.

Kloos, W.E., and N.E. Rose (1970) Transformation mapping of tryptophan loci in Micrococcus luteus. Genetics. 66: 595-605.

Kuhstoss, S., Richardson, M.A., and R.N. Rao (1989) Site-specific integration in Streptomyces ambofaciens: Localization of integration functions in S. ambofaciens plasmid pSAM2. J. Bacteriol. 171: 16-23.

Kushner, S.R. (1978) An improved method for transformation of Escherichia coli with ColE1 derived plasmids. In Genetic Engineering. Edited by H.W. Boyer and S. Nicosia, Elsevier/North-Holland Biomedical Press, Amsterdam, pp 17-23.

Larson, J.L., and C.L. Hershberger (1986) The minimal replicon

of a streptomycete plasmid produces an ultrahigh level of plasmid DNA. Plasmid 15: 199-209.

Larson, J.L., and C.L. Hershberger (1990) Insertion of plasmids into the chromosome of Streptomyces griseofuscus. Plasmid 23: 252-256.

Lederberg, J., and E.M. Lederberg (1952) Replica-plating and indirect selection of bacterial mutants. J. Bacteriol. 63: 399-406.

Limauro, D., Avitabile, A., Cappellano, C, Puglia, A.M., and C.B. Bruni (1990) Cloning and characterization of the histidine biosynthesis gene cluster of Streptomyces coelicolor A3(2). Gene 90: 31-41.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and R.J. Randall (1951) Protein measurement with the Folin phenol reagent. J.Biol.Chem. 193: 265-275.

Malpartida, F., Niemi, J., Navarrete, R., and D.A. Hopwood (1990) Cloning and expression in a heterologous host of the complete set of genes for biosynthesis of the Streptomyces coelicolor antibiotic undecylprodigiosin. Gene 93: 91-99.

Matsui, K., and K. Sano (1987) Structure and function of the trp operon control regions of Brevibacterum lactofermentum, a glutamic acid-producing bacterium. Gene. 53: 191-200.

Matsui, K., K. Sano, and E. Ohtsubo (1986) Complete nucleotide and deduced amino acid sequences of the Brevibacterium

lactofermentum tryptophan operon. Nuc. Acids. Res. 14: 10113-1014.

Meade, H.(1985) Cloning of argG from Streptomyces: loss of gene in Arg⁻ mutants of S. cattleya. Biotechnology 3: 917-918.

Murakami, T., Anzai, H., Imai, S., Satoh, A., Nagaoka, K., and C.J. Thompson (1986) The bialaphos biosynthetic genes of Streptomyces hygroscopicus: Molecular cloning and characterization of the gene cluster. Mol. Gen. Genet. 205: 42-50.

Muth, G., Nubbaumer, B., Wohlleben, W., and A. Puhler (1989) A vector system with tremperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. Mol. Gen. Genet. 219: 341-348.

Piret, J.M., and K.F. Chater (1985) Phage-mediated cloning of bldA, a region involved in Streptomyces coelicolor morphological development, and its analysis by genetic complementation. J. Bacteriol. 163: 965-972.

Pittard, A.J. (1987) Biosynthesis of the aromatic amino acids. In Escherichia coli and Salmonella typhimurium: cellular and molecular biology. Vol1. Edited by F.C.Neidhardt, J.L.Ingraham, K.B.Low, B.Magasanik, M.Schaechter and H.E.Umbarger. American Society of Microbiology, Washington, D.C. pp. 368-393.

Proctor, A.R., and W.E. Kloos (1970) The tryptophan gene cluster of Staphylococcus aureus. J. Gen. Microbiol. 64:

319-327.

Proctor, A.R., and W.E.Kloos (1973) Tryptophan biosynthetic enzymes of Staphylococcus aureus. J.Bacteriol. 114: 169-177.

Richardson, M.A., Kuhstoss, S., Huber, M., Ford, L., Godfrey, O., Turner, J.R., and R.N. Rao (1989) In Genetics and Molecular Biology of Industrial Microorganisms. Edited by C.L. Hershberger, S.W. Queener, and G. Hegemann, American Society for Microbiology, Washington, D.C., pp. 40-43.

Richardson, M.A., Kuhstoss, S., Huber, M.L.B., Ford, L., Godfrey, O., Turner, J.R.,, and R.N. Rao (1990) Cloning of spiramycin biosynthetic geens and their use in constructing Streptomyces ambofaciens mutants defective in spiramycin biosynthesis. J. Bacteriol. 172: 3790-3798.

Riley, M., and K.E. Sanderson (1990) Comparative genetics of Escherichia coli and Salmonella typhimurium. In The Bacterial Chromosome. Edited by K.Drlica and M.Riley. American Society for Microbiology, Washington, D.C. pp. 147- 162.

Rivero-Lezcano, O., Castillo, J.A., Nieto, J.L., and G.N. Carrasco (1990) Cloning and expression in Escherichia coli of tryptophan genes from Streptomyces griseus IMRU 3570. FEMS Microbiol. Lett. 68: 201-206.

Ross, C.M., and M.E. Winkler (1988) Structure of the Caulobacter crescentus trpFBA operon. J. Bacteriol. 170:

757-768.

Sambrook, J., E.F. Frisch, and T. Maniatis (1989) Molecular cloning : A laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New york.Sawula, R.V., and I.P. Crawford (1972) Mapping of the tryptophan genes of Acinetobacter calcoaceticus by transformation. J. Bacteriol. 112: 797-805.

Sato, S., Kakada, Y., Kanaya, S., and T. Tanaka (1988) Molecular cloning and nucleotide sequence of Thermus thermophilus HB8 trpE and trpG. Biochim. Biophys. Acta. 950: 303-312.

Seno, E.T., Bruton, C.J., and K.F. Chater (1984) The glycerol utilization operon of Streptomyces coelicolor: genetic mapping of gyl mutations and the analysis of cloned gyl DNA. Mol. Gen. Genet. 193: 119-128.

Shinomiya, T., Shiga, S., and M. Kageyama (1983) Genetic determinant of pyocin R2 in Pseudomona aeruginosa PAO. I.Localization of the pyocin R2 gene cluster between the trpCD and trpE genes. Mol. Gen. Genet. 189: 375-381.

Shortle, D., Haber, J.E., and D. Botstein (1982) Lethal disruption of the yeast actin gene by integrative DNA transformation. Science 217: 371-373.

Sibold, L., and L. Henriquet (1988) Cloning of trp genes from the archaebacterium Methanococcus voltae: nucleotide sequence of the trpBA genes. Mol. Gen. Genet. 214: 439-450.

Slock, J., Stahly, D.P., Han, C., Six, E.W., and I.P. Crawford (1990) An apparent Bacillus subtilis folic acid biosynthetic operon containing pab, an amphibolic trpG gene, a third gene required for sythesis of para-amino benzoic acid, and the Dihydropteroate synthetase gene. J. Bacteriol. 172: 721-7226.

Smith, O. H. (1967) Structure of the trpC cistron specifying indoleglycerol phosphate synthetase, and its localization in the tryptophan operon of Escherichia coli. Genetics. 57: 95-105.

Smith, O.H., and C. Yanofsky (1960) 1-(o-caraboxyphenylamino)-1-deoxyribulose 5-phosphate. A new intermediate in the biosynthesis of tryptophan. J. Biol. Chem. 235: 2051-2057.

Smith, O.H., and C. Yanofsky (1963) Intermediates in the biosynthesis of tryptophan. In Methods in Enzymology. Vol.6. Edited by S.P.Colowick and N.O.Kaplan. Academic Press Inc., New York. pp. 590-597.

Smithers, C.M. and P. Engel (1974) Gene-enzyme relationships of tryptophan mutants in Streptomyces coelicolor A3(2). Genetics. 78: 799-808.

Starlinger, P. (1977) DNA rearrangements in prokaryotes. Ann.. Rev. Genet. 11: 103.

Streicher, L.S., Ruby, P.S., Paress, P.S., Sweasy, J.B., Danis, S.J., MacNeil, D., Gewain, K., MacNeil, T., Foor, F., Morin, N., Cimis, G., Rubin, R., Goldberg, R.B.,

Nallin, M., Schulman, M.D., and P. Gibbons (1989) Cloning genes for avermectin biosynthesis in Streptomyces avermitilis In Genetics and Molecular Biology of Industrial microorganisms. Edited by C.L.Hershberger, S.W. Queener, and G. Hegemann, American Society for Microbiology, Washington, D.C., pp.44-48.

Stuttard, C.(1979) Transduction of auxotrophic markers in a chloramphenicol-producing strain of Streptomyces. J. Gen. Microbiol. 110: 479-482.

Stuttard, C.(1982) Temperate phages of Streptomyces venezuelae: lysogeny and host-specificity shown by phages SV1 and SV2. J. Gen. Microbiol. 128: 115-121.

Stuttard, C.(1983a) Cotransduction of his and trp loci by phage SV1 in Streptomyces venezuelae. FEMS Microbiol. Lett. 20: 467-470.

Stuttard, C.(1983b) Localized hydroxylamine mutagenesis and cotransduction of threonine and lysine genes in S. venezuelae. J. Bacteriol. 155: 1219-1223.

Stuttard, C. (1988) Transduction and genome structure in Streptomyces. Dev. Ind. Microbiol. 29: 69-76.

Stuttard, C. (1989) Generalized transduction in Streptomyces species. In Genetics and molecular biology of industrial microorganisms. Edited by C.Hershberger, S.W.Queener, and G.Hegeman. American Society for Microbiology, Washington, D.C. pp. 157-162.

Stuttard, C. (1992) Genetic analysis. In Streptomyces. Edited

by E.M.Wellington. Plenum Publishing Corp., New York. In press.

Stuttard, C., Atkinson, L., and S. Vats (1987) Genome structure in Streptomyces spp. adjacent genes on the S. coelicolor A3(2) linkage map have cotransducible analogs in S. venezuelae. J.Bacteriol. 169: 3814-3816.

Vara, J., Lewandowska-Skarbek, M., Wang, Y.G., Donadio, S., and C.R. Hutchinson (1989) Cloning of genes governing the deoxysugar portion of the erythromycin biosynthesis pathway in Saccharopolyspora erythraea (Streptomyces erythraea). J. Bacteriol. 171: 5872-5881.

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

Vogel, H.J., and D.M. Bonner (1956) Acetylorthinine of Escherichia. coli: partial purification and some properties. J. Biol. Chem. 218: 97-106.

Weber, J.M., and R. Losick (1988) The use of a chromosome integration vector to map erythromycin resistance and production genes in Saccharopolyspora erythraea (Streptomyces erythraea). Gene. 68: 173-180.

Weber, M.J., Leung, J.O., Maine, G.T., Pitenz, R.H.B., Paulus, T.J., and J.P. DeWitt (1990) Organization of a cluster of erythromycin genes in Saccharopolyspora erythraea. J. Bacteriol. 172: 2372-2383.

Wegman, J., and I.P. Crawford (1968) Tryptophan synthetic

pathway and its regulation in Chromobacteriium violaceum. J. Bacteriol. 95: 2325-2335.

Wegman, J., and J.A. DeMoss (1965) The enzyme conversion of anthranilate to indole glycerol phosphate in Neurospora crassa. J. Biol. Chem. 240: 3781-3788.

Weiss, U., and J.M. Edwards (1980) The biosynthesis of aromatic compounds. John Wiley and Sons, New York.

Whitt, D.D., and B.C. Carlton (1968) Characterization of mutants with single and multiple defects in the tryptophan biosynthetic pathway in Bacillus subtilis. J. Bacteriol. 96: 1273-1280.

Whittle, Y.G., and S.J. Gould (1987) The biosynthesis of LL-C110037α from the shikimate pathway. J. Amer. Chem. Soc. 109: 5043.

Winkler, M.E., Schoenlein, P.V., Ross, C.M., Barrett, J.T., and B. Ely (1984) Genetic and physical analysis of Caulobacter crescentus trp genes. J. Bacteriol. 160: 279-287.

Woese, C.R. (1987) Bacterial evolution. Microbiol. Rev. 51: 221- 271.

Yanofsky, C.(1956) The enzymatic conversion of anthranilic acid to indole. J. Biol. Chem. 223: 171-184.

Yanofsky, C. (1984) Comparison of regulatory and structural regions of genes of tryptophan metabolism. Mol. Biol. Evol. 1: 143-161.

Yanofsky, C., and E.S. Lennox (1959) Transduction and

recombination study of linkage relationships among the genes controlling tryptophan synthesis in Escherichia coli. Virology 8: 425-447.

Yanofsky, C., Horn, V., Bonner, M., and S. Stasiowski (1971) Polarity and enzyme functions in mutants of the first three genes of the tryptophan operon of Escherichia coli. Genetics 69: 409-433.

Yanofsky, C., and I.P. Crawford (1987) The tryptophan operon In Escherichia coli and Salmonella typhimurium: cellular and molecular biology. Vol. 2. Edited by F.C.Neidhardt, J.L.Ingraham, K.B.Low, B.Magasanik, M.Schaechter, and H.E.Umbarger, American Society for Microbiology, Washington, D.C. pp. 1453-1472.

Yelton, D.B., and N.C. Charon (1984) Cloning of a gene required for tryptophan biosynthesis from Leptospira biflexa serovar patoc into Escherichia coli. Gene 28: 147-152.

Yelton, D.B., and R.A. Cohen (1986) Analysis of cloned DNA from Leptospira biflexa serovar patoc which complements a deletion of the Escherichia coli trpE gene. J. Bacteriol.165: 41-46.

Yelton, D.B., and S.L. Peng (1989) Identification and nucleotide sequence of the Leptospira biflexa serovar patoc trpE and trpG genes. J. Bacteriol. 171: 2083-2089.

Zalkin, H., and T. Murphy (1975) Utilization of ammonia for tryptophan synthesis. Biochem. Biophys. Res. Commu. 67 :

1370-1377.

Zalkin, H. (1980) Anthranilate synthse: relationshiops between bifunctional and monofunctional proteins. In Multifunctional enzymes. Edited by H. Bisswanger and E. Schmincke-Ott, Wiley and sons, Inc. New York. pp 180-206.