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CLONING AND SEQUENCING OF P-AMINOBENZOIC ACID SYNTHETASE GENES FROM LACTOCOCCUS LACTIS SUBSP. LACTIS NCDO496 AND STREPTOMYCES LIVIDANS 1326

by

Francis Felix Arhin

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Canada

To my daugther, Araba, that she will grow to appreciate the value of education.

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ABSTRACT

Genes involved in the biosynthesis of p-aminobenzoic acid (PABA) were cloned from Lactococcus lactis subsp. lactis NCDO496 and Streptomyces lividans 1326. The L. lactis subsp. lactis gene was cloned by complementation of a pab mutant of S. lividans using pIJ41 as a vector. A deletion of about 1.2-kb from the vector sequence was required to allow transcriptional readthrough from a vector promoter. Attempts to complement pab mutants of Escherichia coli with the cloned L. lactis subsp. lactis fragment resulted in complementation of the pabB but not the pabA mutation. The nucleotide sequence of the cloned fragment contained a single open reading frame (ORF) similar to pabB genes from other organisms. A putative ribosome binding site (RBS) was also located. There was no consensus promoter sequence within the sequenced region. The S. lividans PABA synthetase genes were cloned as a single fragment that complemented the pabA and pabB mutations of E. coli. The cloned genes were expressed in E. coli when present in opposite orientations in the vector, indicating that an S. lividans promoter on the cloned fragment was being used. Attempts to express the cloned gene in pab mutants of S. lividans led to integration into the host chromosome. Use of a 2.7-kb pab-complementing fragment of the cloned gene as a probe at high stringency showed a hybridizing sequence in S. lividans strains 1326 and M252. At lower stringency, these S. lividans strains, as well as other Streptomyces, showed multiple hybridizing sequences. The nucleotide sequence of the 2.7-kb fragment revealed two ORFs similar to pabA and pabB genes from other organisms. Putative RBSs were located immediately upstream of each ORF. A putative promoter sequence was also located upstream of the pabB ORF. Comparison of codon usage in the PABA synthetase genes of L. lactis subsp. lactis, S. lividans and related genes from other organisms showed a bias in the direction of the G+C content of the organism.

LIST OF ABBREVIATIONS

bp	base pair
BSA	bovine serum albumin
CAT	chloramphericol acetyl transferase
ccc	covalently closed circular
cml	chhloramphenicol
cpm	conts per minute
dATP	deoxyadenine 5′-triphosphate
dCTP	deoxycytidine 5′-triphosphate
dGTP	deoxyguanidine 5′-triphosphate
dITP	deoxyinosine 5′-triphosphate
ddATP	dideoxyadenine 5′-triphosphate
ddCTP	dideoxycytidine 5′-triphosphate
ddGTP	dideoxyguanidine 5′-triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine 5′-triphosphate
EDTA	ethylenediamine tetracetic acid
IPTG	isopropyl-β-thiogalactopyranoside
kb	kilobase
nt	nucleotide
ORF	open reading frame
PABA	p- aminobenzoic acid
PEG	polyethylene glycol
p.s.i	pounds per square inch
RBS	ribosome binding site
RNA	ribonucleic acid

rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TES	N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid
TLC	Thin-layer chromatography
tris	tris-(hydroxymethyl)aminoethane
tsr	thiostrepton
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Almighty, whose special relationship with me leads me on in all I do: Ase da nka Ewuradze.

INTRODUCTION

p-Aminobenzoic acid (PABA) is a microbial growth factor required for the biosynthesis of folic acid. Its absence halts biochemical processes such as the biosynthesis of purines, formation of the pyrimidine thymidine and of N-formylmethionyl tRNA, and the interconversions of serine and glycine, or serine and methionine. These are all activities in which tetrahydrofolate participates to transfer one-carbon units and are vital for the life of the cell (Lehninger, 1975). PABA is also involved in secondary metabolism in the biosynthesis of the polyene macrolides candicidin in Streptomyces griseus, and fungimycin in Streptomyces coelicolor var. aminophilus (Gil et al., 1985a). In these streptomycetes, it has been suggested that there are two sets of genes for PABA biosynthesis, one for primary and the other for secondary metabolism (Gil et al., 1990).

The biosynthesis of PABA diverges from the primary aromatic metabolites at chorismic acid and involves three genes (Nichols et al., 1989; Slock et al., 1990). Genes involved in the biosynthesis have been cloned and sequenced from Escherichia coli (Kaplan and Nichols, 1983; Goncharoff and Nichols, 1984), Salmonella typhimurium (Kaplan et al., 1985; Goncharoff and Nichols, 1988), Klebsiella aerogenes (Kaplan et al., 1985; Goncharoff and Nichols, 1988), Serratia marcescens (Kaplan et al., 1985) and Bacillus subtilis (Slock

et al., 1990). Those from Streptomyces griseus (Gil and Hopwood, 1983) and Streptomyces venezuelae (Aidoo, 1989) have been cloned but not sequenced. Differences in organization of the genes have been observed in different organisms. For example, in E. coli the genes have been mapped at different locations on the chromosome indicating that they are not linked (Huang and Gibson, 1970). A similar arrangement appears to be the case in other enteric bacteria (Kaplan et al., 1985; Goncharoff and Nichols, 1988). On the other hand, nucleotide sequencing of the B. subtilis pab- complementing fragment suggests linkage of all three genes involved in PABA biosynthesis (Slock et al., 1990). There is some evidence to suggest that in S. griseus as well, the genes are linked (Gil et al., 1985a).

In enteric bacteria, the genes involved in the biosynthesis of PABA share a marked similarity with genes for the biosynthesis of anthranilic acid of the tryptophan pathway at both the nucleotide and amino acid sequence levels (Kaplan et al., 1985; Goncharoff and Nichols, 1988). This has led to the suggestion that the two sets of genes evolved from a common ancestor.

In the present study, the organization and characterization of PABA synthetase genes in Lactococcus lactis subsp. lactis and Streptomyces lividans was investigated by cloning and sequencing.

In considering the potential routes available for cloning the gene(s) of L. lactis subsp. lactis, account was taken of the following factors: (i) no efficient transformation system for L. lactis subsp. lactis had been developed and (ii) no pab mutants of L. lactis subsp. lactis were available. To circumvent these problems, cloning of the PABA synthetase gene(s) was approached by complementation of pab mutants of S. lividans and E. coli. The attempt to clone and express the L. lactis subsp. lactis gene in S. lividans tested the validity of a suggestion that the high G+C content of Streptomyces DNA (73%) would not allow expression of genes low in G+C content in Streptomyces (Bibb et al., 1985). Since the G+C content of L. lactis subsp. lactis is about one-half that of Streptomyces (37% versus 73%), this represented a crucial test of the suggestion by Bibb et al. (1985).

Since pab mutants of S. lividans and E. coli were available and also transformation systems for these organisms have been well characterized (Hopwood et al., 1985; Maniatis et al., 1982), the approach to cloning the S. lividans PABA synthetase genes involved complementation of pab mutants of S. lividans and E. coli.

From the nucleotide sequences of the cloned genes, regulatory signals as well as codon usage in the open reading frames (ORFs) of the genes can be obtained. By comparing the regulatory signals and codon usage in the ORFs of the L.

lactis subsp. lactis and S. lividans PABA synthetase genes to each other and also to related genes from other organisms, we can learn how much evolutionary divergenece has been introduced into the cellular processes that convert genetic information into phenotypic activities.

Since S. lividans does not use PABA to make secondary metabolites (Gil et al., 1990) its PABA synthetase genes should be involved solely in primary metabolism. Obtaining the PABA synthetase genes of S. lividans and comparing them with PABA synthetase genes cloned from organisms that use PABA to form a secondary metabolite will shed some light on the evolution of secondary metabolite genes.

LITERATURE REVIEW

I. p-AMINOBENZOIC ACID (PABA)

A. Biosynthesis.

Davis (1950) suggested that this bacterial growth factor might be formed by reactions involved in the biosynthesis of aromatic amino acids. Later, phosphoenolpyruvate, erythrose-4-phosphate and chorismic acid were found to be required for both PABA and aromatic amino acid biosynthesis (Gibson and Jackman, 1963; Gibson and Gibson, 1964). Using enzyme extracts from Enterobacter aerogenes, Streptomyces aminophilus and S. coelicolor, Johanni et al. (1989) observed that extracts that lacked isochorismate synthetase activity were able to make PABA whereas extracts lacking chorismate synthetase activity could not. Thus although both chorismic acid and isochorismic acid were converted to PABA, isochorismic acid was not an obligatory intermediate. Earlier, Weiss and Srinivasan (1959) had reported that PABA could be formed from shikimate-5-phosphate and L-glutamine by cell-free extracts of baker's yeast. Participation of L-glutamine was inferred from evidence that structural analogues such as 6-diazo-5-oxo-L-norleucine inhibited the biosynthesis of PABA (Srinivasan and Weiss, 1961). Furthermore, isotopic labelling indicated that the amino group of PABA was derived from the amide nitrogen of L-glutamine (Srinivasan and Weiss,

1961).

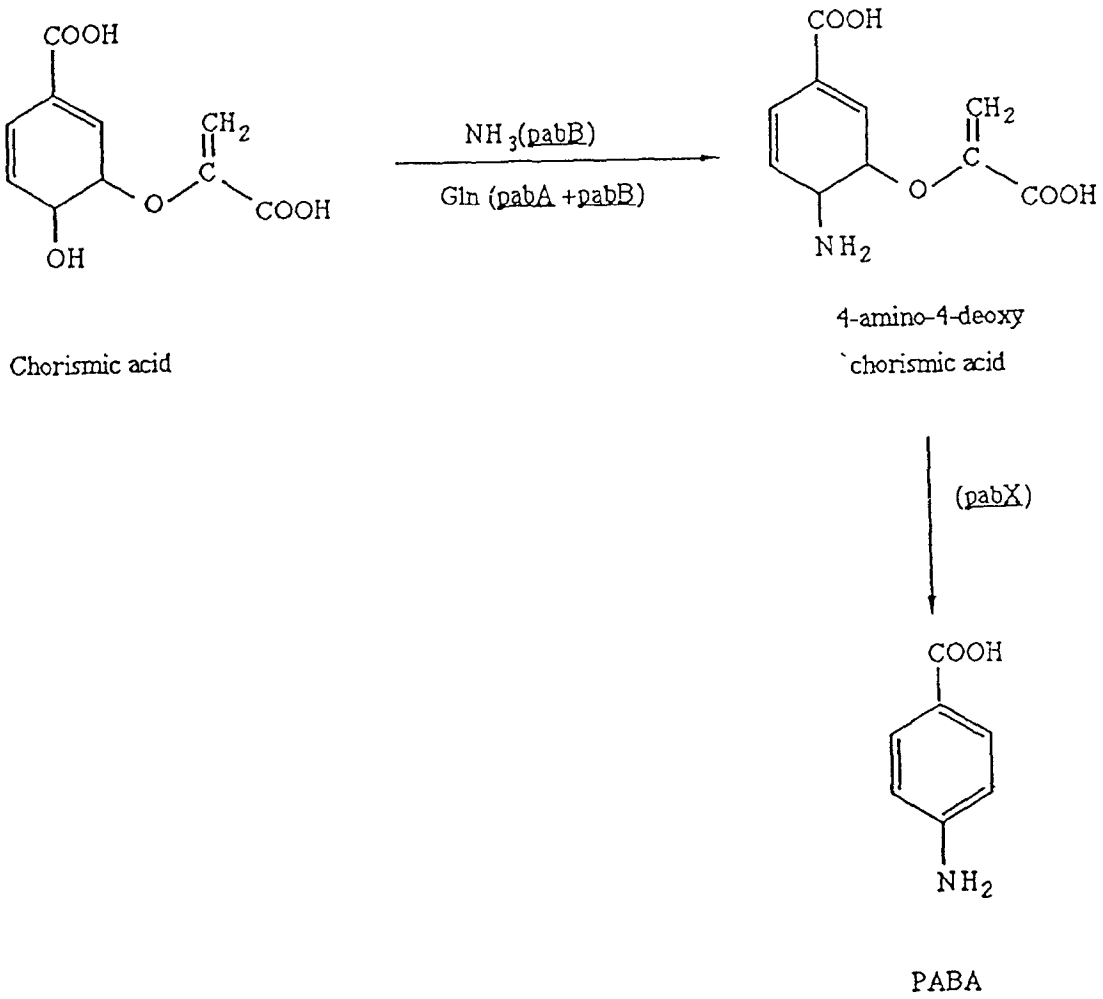
Genetic analysis of E. coli mutants that required PABA for growth indicated that at least two genes were involved in PABA biosynthesis (Huang and Pittard, 1967). Two distinct loci, designated pabA and pabB were mapped. Also, the PABA-synthesizing enzymes were separated into two components with apparent molecular weights of 9,000 and 48,000, corresponding to the pabA and pabB gene products, respectively (Huang and Gibson, 1970). In Neurospora crassa also, pab mutants mapped at two distinct chromosomal loci (Drake, 1956).

Classification of mutants into two distinct groups led Huang and Gibson (1970) to suggest that there could be a biosynthetic intermediate between chorismic acid and PABA. No cross-feeding was observed between the two classes of E. coli pab mutants (Huang and Pittard, 1967) but Hendler and Srinivasan (1967) did report cross-feeding between the two classes of N. crassa pab mutants. Also, the formation of an intermediate between chorismate and PABA has been reported in E. aerogenes (Altendorf et al., 1969) and S. venezuelae (Teng et al., 1975). The existence of an intermediate in E. coli was confirmed by Nichols et al. (1989) who observed two steps in the biosynthesis of PABA and obtained evidence for a diffusible intermediate. Partially purified pabA and pabB gene products alone were unable to make PABA and a third factor (enzyme X) was required. In dialysis experiments pabA and pabB gene products were shown to physically interact in

converting chorismic acid and L-glutamine to the dialyzable product that was converted to PABA by enzyme X. Failure to identify enzyme X in previous experiments was explained by the fact that enzyme X with a molecular weight of 49,000 copurified with the pabB gene product (molecular weight 48,000) and so was overlooked in fractionation studies. Based on these observations, Nichols et al. (1989) proposed that the pabB gene product in the presence of ammonia converts chorismic acid to an intermediate, possibly the 4-amino-4-deoxychorismic acid described by Teng et al. (1985), which is then converted to PABA by enzyme X. When L-glutamine is the nitrogen source, the pabA gene product (a glutamine amidotransferase) is required to transfer the amide nitrogen from glutamine to the pabB gene product to allow formation of the intermediate. The intermediate is then converted to PABA by enzyme X. Figure 1 summarizes the postulated pathway.

B. Involvement of PABA in the biosynthesis of folic acid

The biological role of PABA was first recognized by Woods (1940) who observed that PABA antagonized the bacteriostatic effects of sulphonamides. It was suggested that PABA was an intermediate in the biosynthesis of an essential metabolite. Purification of folic acid and structural determination revealed that PABA was a component of the molecule (Angier et al., 1946; Stokstad et al., 1948). Also, a number of microorganisms used PABA or folic acid as



Chorismic acid

4-amino-4-deoxy chorismic acid

PABA

Figure 1: Pathway for the biosynthesis of p-aminobenzoic acid.

alternative growth factors, supporting the conclusion that PABA was an intermediate in folic acid biosynthesis. Folate-like compounds are formed when bacterial cell extracts are incubated with 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine and either PABA or p-aminobenzoyl glutamate (PABAG) in the presence of Mg^{2+} and ATP (Brown et al., 1961; Shiota and Disraley, 1961; Wolf and Hotchkiss, 1963). Similar results were obtained using yeast extracts (Jaenicke and Chan, 1960). Shiota and Disraley synthesized the pyrophosphate ester of 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine and showed that ATP was not required when it was used with cell extracts for the synthesis of folate-like compounds. Based on this and other observations, Ortiz and Hotchkiss (1966) suggested that the condensation of PABA or PABAG with 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine to form folate-like compounds was preceded by pyrophosphokinase-catalyzed pyrophosphorylation of the pteridine derivative. Ortiz and Hotchkiss (1966) showed that the monophospate derivative could not be used, and also that the pyrophosphate derivative was not formed from the monophosphate in the presence of ATP. Shiota and Disraley (1961) established that a reduced pteridine was involved in the condensation, and showed that the dihydro- rather than the tetrahydropteridine was the substrate. They also showed that dihydrofolic acid was first formed and then reduced to tetrahydrofolic acid.

There is some confusion in the literature as to whether

PABA or PABAG is the substrate that condenses with the pteridine derivative to form folate compounds. Enzyme extracts from mutant and wild-type strains showed major differences in the ability to use PABAG but not PABA for the condensation (Ortiz and Hotchkiss, 1966), suggesting that PABAG might be the normal substrate. On the other hand, Brown et al. (1961) reported that conjugation of glutamate to PABA was not an obligatory step before condensation. Crude extracts of E. coli converted di- and tetrahydropteroic acids into tetrahydrofolic acid, suggesting that the conjugation with glutamate occurred after condensation. Also, enzyme extracts of E. coli used PABA ten times more effectively than PABAG as a substrate and the enzyme catalyzing formation of dihydrofolic acid from glutamic acid and dihydropteroic acid was found in extracts of E. coli whereas no enzyme for the conversion of PABA and glutamic acid to PABAG could be detected. A similar observation was made by Richey and Brown (1970) who also showed that dihydropteroate synthetase from E. coli was a single enzyme that used PABA more effectively than PABAG.

Dialysis experiments showed that the formation of tetrahydrofolic acid from pteridines depended on glutamate (Griffin and Brown, 1964). The results also suggested that glutamate condensed with dihydropteroic acid rather than with tetrahydropteroic acid. As further evidence for this the purified enzyme that catalyzed this reaction used

dihydropteroic acid as a substrate but not pteroic acid or tetrahydropteroic acid (Griffin and Brown, 1964). The overall conclusions from these results are summarized in figure 2 which shows the reactions involved in the biosynthesis of folate compounds and the involvement of PABA in the pathway.

C. Organization and expression of genes for PABA biosynthesis.

1. Enteric bacteria

Three E. coli strains auxotrophic for PABA were isolated by Huang and Pittard (1967) from a prototrophic strain treated with the mutagen N-methyl-N'-nitrosoguanidine. Mapping of the mutations by conjugation and transduction suggested that at least two genes were involved in PABA biosynthesis and that these genes were not linked (Huang and Pittard, 1967). Conjugational analysis placed the pab mutation in two of the three mutants in the same general region as xyl, aroB, str and malA (Huang and Pittard, 1967). Transduction using phage P1 mapped the mutation between aroB and str at about 74 min on the E. coli map (Huang and Pittard, 1967; Huang and Gibson, 1970). This mutation was designated pabA (Huang and Pittard, 1967). The other mutation, designated pabB, was mapped by conjugational analysis between his and gal, at about 40 min (Huang and Pittard, 1967).

Involvement of two separate genes in the biosynthesis of PABA implicated at least two enzymatic reactions in the

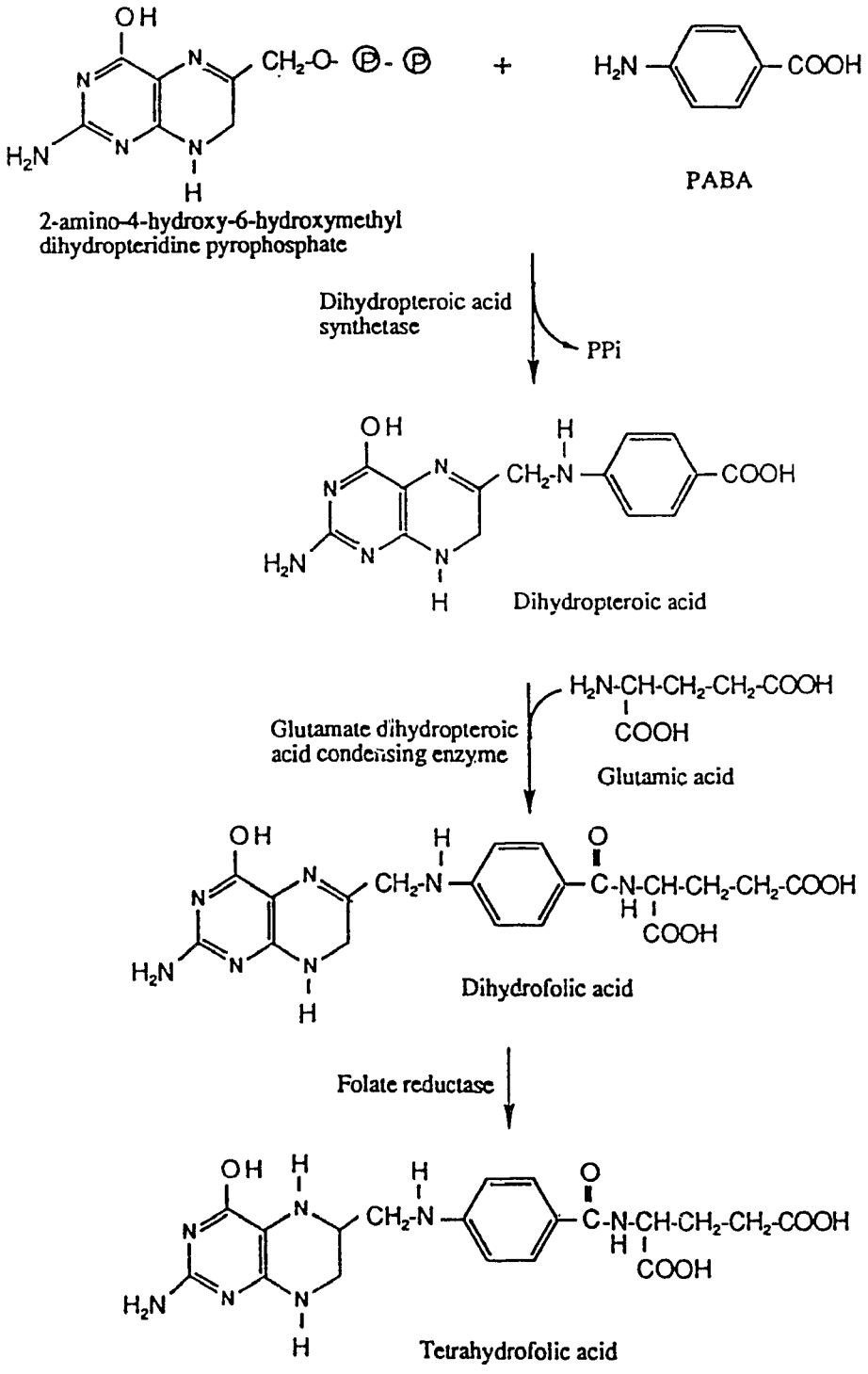


Figure 2: Pathway for the biosynthesis of tetrahydrofolic acid.

conversion of chorismate to PABA in E. coli (Huang and Pittard, 1967). Since none of the pab mutants secreted a diffusible intermediate that could stimulate growth of any other mutant, Huang and Gibson (1970) proposed that the polypeptide products of the two genes were involved in only one step in converting chorismic acid to PABA. This was confirmed when Nichols et al. (1989) showed that the pabA and pabB gene products act together to convert chorismic acid and glutamine to a diffusible intermediate which is then converted by another enzyme (X) to PABA.

The pabA gene of E. coli has been cloned and sequenced (Kaplan and Nichols, 1983). It features an open reading frame (ORF) encoding 187 amino acids representing a polypeptide of molecular weight 20,752. The sequence preceding the pabA ORF includes two additional ORFs, orf1 and fic (Tran et al., 1990). Moreover, pabA incorporates two transcriptional units; the one initiated from promoter P2 includes orf1 and fic, whereas that initiated from promoter P1 contains only pabA. When compared with the E. coli consensus sequence, the two promoters have a good -10 but a poor -35 sequence (Tran et al., 1990). Directed mutagenesis and gene fusion experiments showed pabA to be expressed primarily from P1; P2 was dispensible for PABA metabolism (Tran et al., 1990). Although the steady state levels of the two pabA transcripts were approximately the same, those initiated from P1 were translated more efficiently than those from P2. Possibly, the

P2-initiated transcript could form a secondary structure that sequestered the ribosome-binding site of the pabA transcript and decreased the efficiency of translation (Tran et al., 1990).

The pabB gene of E. coli has also been cloned and sequenced (Goncharoff and Nichols, 1984). A 1.62-kb fragment complemented the pabB mutation and only one of all the possible translational frames, exceeded a total length of 300 bp. This continous frame of 1,359 bp could encode a protein containing 453 amino acids with a molecular weight of 50,958, close to the figure of 48,000 determined for the pabB gene product of E. coli by Huang and Gibson (1970) using gel permeation chromatography. The region upstream of the pabB gene contained a promoter-like sequence with -10 and -35 regions similar to the E. coli consensus sequences (Goncharoff and Nichols, 1984). The 5'-leader sequence also contained a Shine-Dalgarno sequence for ribosome binding (Goncharoff and Nichols, 1984). Additionally, the 5'-flanking region included an ORF oriented in the opposite direction to that for the pabB gene while the 3'-flanking region contained an ORF 3-bp from the termination codon of the pabB gene (Goncharoff and Nichols, 1988). Transcriptional fusion experiments showed that the two reading frames flanking pabB were transcribed (Goncharoff and Nichols, 1988). The functions of these two genes are not known.

The gene for enzyme X has not been cloned but the

product has a molecular weight of 49,000 (Nichols et al., 1989) and consists of two identical subunits of 25,000 daltons (Slock et al., 1990).

As expected for enteric bacteria, the pab genes of S. typhimurium and K. aeroqenes are organized in a similar way to those of E. coli. The pabA gene of S. typhimurium was cloned from a lambda hybrid pool containing randomly generated fragments of S. typhimurium DNA. That of K. aeroqenes was cloned from a library of K. aeroqenes genomic DNA constructed in pBR322. Both cloned fragments complemented the pabA mutation in E. coli (Kaplan et al., 1985). The nucleotide sequences as well as the deduced amino acid sequences of these pabA genes are highly conserved (Kaplan et al., 1985). Of the differences 61 to 74% occur in the third position of codons where they do not grossly change the amino acid sequences of proteins (Kaplan et al., 1985). Similar observations have been made for other genes from this group of organisms (Crawford et al., 1980). The G+C content of pabA codons in the enteric bacteria is higher than that of the genome as a whole (Kaplan et al., 1985).

The 5'-flanking region of the S. typhimurium pabA gene contains ORFs similar in size to orf1 and fic and are, as in E. coli, transcribed in the same direction as pabA (Tran et al., 1990). Although an ORF that terminates 31 bp upstream of the pabA gene and is transcribed in the same direction as pabA has been found in K. aeroqenes (Kaplan et al., 1985) it is not

known if genes similar to orf1 and fic are present. It is important to note, however, that in all three organisms, the upstream reading frame(s) as well as the 31-bp region between the last reading frame and the pabA gene are highly conserved (Kaplan et al., 1985). This conserved region has dyad symmetry which might be functionally important; it may be involved in regulating expression of pabA (Kaplan et al., 1985). The 3'-sequence flanking the pabA genes of the three enteric bacteria also contains a conserved ORF that displays the same high proportion of third position codon differences that are without effect on the amino acid sequence (Kaplan et al., 1985). The evidence suggests that this region encodes a functional protein of unknown function. The region between pabA and the next downstream gene contains sequences with similarity to conserved promoter sequences in these organisms (Kaplan et al., 1985).

The S. typhimurium pabB gene was isolated from a hybrid lambda gt7 library of S. typhimurium genomic DNA (Goncharoff and Nichols, 1988). The K. aerogenes pabB gene was isolated from a pBR322 library of K. aerogenes genomic DNA (Goncharoff and Nichols, 1988). Both cloned fragments complemented the pabB mutation in E. coli. The nucleotide sequences as well as the deduced amino acid sequences of pabB from these two organisms and from E. coli showed strong sequence similarity, the major differences being at the amino terminus. Of the nucleotide substitutions, 60% were in the third position of

the codons and did not change the amino acid sequence. A 43-bp stretch identical in the three organisms was capable of forming a stable secondary structure at the mRNA level. The pattern of codon usage in the pabB genes of the three enteric bacteria showed a comparatively high proportion of codons that are rarely used (Goncharoff and Nichols, 1988). Consistent with this, the pabB gene in E. coli is not expressed at high levels (Nichols et al., 1989).

The oppositely oriented ORF in the 5′ flanking region of the pabB gene of E. coli is also found with the pabB genes of S. typhimurium and K. aerogenes (Goncharoff and Nichols, 1988). Also, the downstream ORF with an intercistronic spacing of 3 bp that appears to be transcribed as a polycistronic message with pabB is conserved in all three organisms (Goncharoff and Nichols, 1988).

Whether there is a gene in S. typhimurium and K. aerogenes equivalent to the E. coli gene coding for enzyme X is not yet known.

2. Bacillus subtilis.

Kane and O'Brein (1975) reported that PABA synthetase from B. subtilis was composed of two nonidentical subunits, designated A and X. Subunit A with an estimated molecular weight of 31,000 had aminase but no amidotransferase activity and is thus the equivalent of pabB in E. coli. Subunit X, a glutamine amidotransferase of molecular weight 19,000, is the

equivalent of pabA in E. coli. In a previous report, Kane et al. (1972) had observed that subunit X was a component of both PABA synthetase and anthranilate synthetase and named the gene encoding it trpX. Mutations in trpX abolished the ability to make both PABA and anthranilic acid when glutamine was used as the nitrogen source (Kane et al., 1972). Mutations in trpX did not map in the tryptophan operon but within genes involved in the biosynthesis of folic acid (Kane et al., 1972; Slock et al., 1990). Kane (1977) reported that trpX mapped between sul (the gene encoding dihydropteroate synthetase) and the gene for subunit A. Expression of trpX was regulated by the gene products of trpE (which encodes the aminase subunit of anthranilate synthetase) and pabB (which encodes the aminase subunit of PABA synthetase).

McDonald and Burke (1982) cloned a 4.9-kb fragment of B. subtilis chromosomal DNA that conferred resistance to sulphonamides. A portion of the cloned fragment also complemented the trpX mutation. Slock et al. (1990) have suggested that the trpX gene should be called trpG to conform with the nomenclature used in other bacteria. The entire 4.9-kb fragment with genes conferring resistance to sulphonamides and complementing the trpG mutation has been sequenced (Slock et al., 1990). Five complete ORFs and one incomplete one (referred to as orf2) were found. Genes identified in the complete ORFs were pabB (encoding the larger subunit of PABA synthetase), trpG (the amphibolic gene encoding the glutamine

amidotransferase subunit of both PABA synthetase and anthranilate synthetase), pabC (the equivalent of the gene encoding enzyme X in E. coli) and sul (encoding dihydropteroate synthetase). The fifth complete ORF (orf1) was thought to be also involved in the biosynthesis of folic acid.

The ORF encoding the larger subunit of PABA synthetase was identified by the similarity of the deduced amino acid sequence to that of pabB gene of E. coli (Slock et al., 1990). Furthermore, in vitro insertional inactivation of the gene and integration of the mutant form into the chromosome of a prototrophic strain partially abolished the ability to synthesize PABA. The trpG gene was also identified by the similarity of its deduced amino acid sequence to those of the trpG genes of E. coli and Acinetobacter calcoaceticus. Also, insertional inactivation of the gene and its integration into the chromosome of a prototrophic host led to a complete requirement for tryptophan and a partial requirement for PABA (Slock et al., 1990). A complete requirement for PABA ensued when pabC was insertionally inactivated and introduced into the chromosome. The deduced amino acid sequence of pabC was similar to that of ilvE of E. coli (Kuramitsu et al., 1985); ilvE encodes a branched-chain amino acid transaminase that participates in the biosynthesis of isoleucine and valine. That pabC is analogous to enzyme X of PABA biosynthesis in E. coli is supported by their similar size; the subunit molecular

weight of enzyme X indicates a polypeptide of about 227 amino acids while there are 293 amino acids encoded by pabC (Slock et al., 1990).

The sul gene of B. subtilis showed amino acid similarity with the dihydropteroate synthetase of Streptococcus pneumoniae (Slock et al., 1990). Both orf1 and orf2 showed amino acid sequence homology to the amino acid sequence of the gene encoding dihydropteridine hydroxymethyl pyrophosphokinase of S. pneumoniae (Lopez et al., 1990; Slock et al., 1990). It is not known if either or both of these genes perform(s) the same function in B. subtilis.

Since no B. subtilis consensus promoter sequences were detected in the 4.9-kb fragment, Slock et al. (1990) suggested that all the genes on this fragment are transcribed from a single promoter and thus belong to an operon. Four of the six genes are involved in the biosynthesis of folic acid and the other two (orf1 and orf2) show similarity to a gene that is involved in the folate pathway; therefore it was proposed that the complete assemblage be referred to as a folic acid operon. The operon may contain other genes not present on the cloned fragment.

3. Streptomyces griseus

The PABA synthetase gene of S. griseus was cloned from a candicidin-producing strain as a 4.5-kb fragment that complemented a pab mutation in S. lividans as well as the pabA

and pabB mutations in E. coli (Gil and Hopwood, 1983). In E. coli, the gene was not expressed from its own promoter; rather, a 1-kb portion of the cloned fragment was lost spontaneously to allow expression by readthrough from a vector promoter (Gil and Hopwood, 1983). Complementation of the unlinked pabA and pabB mutations of E. coli, suggested that the cloned fragment carried both genes from S. griseus. On partial purification, the enzyme did not separate into two active fractions (Gil et al., 1985a) as described for E. coli PABA synthetase (Huang and Gibson, 1970). The single active fraction was of molecular weight 50,000, comparable to the combined molecular weights of the two subunits of the enzymes from E. coli (Huang and Gibson, 1970) and B. subtilis (Kane and O'Brein, 1975). The single active fraction exhibited both aminase and amidotransferase activities, although the amidotransferase activity was 5- to 10-fold higher than the aminase activity (Gil et al., 1985a).

It has been suggested that the PABA synthetase gene cloned from the S. griseus candicidin producer is a secondary metabolism gene, and that a separate PABA synthetase exists for the biosynthesis of folic acid (Gil et al., 1985a). This will be discussed below.

4. Other microorganisms.

The report by Altendorf et al.(1969) that in E. aerogenes an intermediate was formed during the biosynthesis

of PABA from chorismic acid and L-glutamine implicates at least two genes in that bacterium. A similar observation was made in Neurospora crassa (Drake, 1959; Hendler and Srinivasan, 1967).

As in B. subtilis (Kane et al., 1972), A. calcoaceticus (Sawula and Crawford, 1973) and Pseudomonas acidovorans (Buvinger et al., 1981) express a single glutamine amidotransferase that functions in the biosynthesis of both PABA and anthranilic acid. In these two organisms, the amphibolic glutamine amidotransferase gene (trpG) is linked to the trpC and trpD genes for indoleglycerol phosphate synthetase and phosphoribosyl transferase, respectively, but not to other genes involved in the biosynthesis of tryptophan (Sawula and Crawford, 1973; Buvinger et al., 1981). It is not known whether the other genes involved in the biosynthesis of PABA are linked with trpG as in B. subtilis (Slock et al., 1990).

D. PABA synthetase as an antibiotic biosynthesis gene.

By definition, antibiotics are produced by microorganisms and interfere with the growth of other microorganisms. They are produced by secondary metabolism, a process distinguished from primary metabolism not only in showing major differences between organisms but also in not being essential for growth (Martin and Demain, 1980). Generally, expression of antibiotic biosynhesis genes is repressed during active growth

and derepressed at low specific growth rates. Thus, the biosynthesis of antibiotics often follows a biphasic pattern with an initial growth phase where little or no antibiotic is made, followed by a production phase (Walker, 1974).

In organisms that produce polyene macrolide antibiotics with PABA as a component of the structure, the enzyme PABA synthetase has been postulated to be associated with secondary metabolism (Gil and Hopwood, 1983). Consequently, the PABA synthetase gene cloned from S. griseus, which produces candicidin, a polyene macrolide derived biosynthetically from PABA (Gil and Hopwood, 1983) may well be a secondary metabolite pathway gene. In support of this, detectable levels of PABA synthetase were found in cell extracts from candicidin-producing strains but not in those from non producing mutants (Gil et al.. 1985a). The enzyme was also detected in cell extracts of S. coelicolor var. aminophilus which produces fungimycin, another PABA-derived polyene macrolide antibiotic (Gil et al., 1985a). The enzyme was not detected in cell extracts from several other streptomycetes that do not produce polyene macrolides (Gil et al., 1985a).

Expression of the PABA synthetase gene of the candicidin-producing S. griseus was strongly repressed but not inhibited by phosphate (Gil et al., 1985a). Similar repression of antibiotic biosynthesis by phosphate has been widely reported (Martin, 1977). Because derepression of PABA synthetase occurred at low phosphate concentrations (Gil et al., 1985a)

and the onset of candicidin biosynthesis was triggered by phosphate depletion, regulation of PABA synthetase is postulated to be a key step in the biosynthesis of candicidin (Liras et al., 1977; Martin, 1977; Gil et al., 1985a).

The region of the S. griseus chromosome involved in the response to phosphate has been localized within a 114-bp fragment of the PABA synthetase gene that has promoter activity (Rebollo et al., 1989). Expression of the S. griseus PABA synthetase gene in S. lividans from the S. griseus promoter was strongly repressed by phosphate; however, no phosphate regulation was found when a 1-kb upstream sequence that contained the promoter was deleted and the gene was expressed from a vector promoter (Rebollo et al., 1989). The 114-bp fragment from this 1-kb upstream sequence was cloned into the promoter-probe plasmid pIJ424 where it controlled expression of the kanamycin phosphotransferase gene conferring kanamycin resistance (Rebollo et al., 1989); in this construct, kanamycin resistance was regulated by phosphate (Rebollo et al., 1989). Sequence analysis of the 114-bp promoter-active fragment revealed regions similar to the "pho boxes" that regulate expression of pho genes in E. coli (Makino et al., 1986; Liras et al., 1990). Twelve out of eighteen nucleotides were identical to those of the consensus E. coli pho box. Also, two similar pho boxes were found in tandem in the S. griseus sequence; this arrangement of pho boxes is associated with phoS in E. coli (Torriani and

Ludtke, 1985).

Regulation of the S. griseus PABA synthetase gene by phosphate has been shown to occur at the transcriptional level (Asturias et al., 1990). Quantitation of the specific mRNA for the PABA synthetase gene of S. griseus using an internal fragment of pab as a probe showed that synthesis of the mRNA was repressed at high phosphate and derepressed at low phosphate concentrations. Also, synthesis of PABA synthetase mRNA peaked after 12 h incubation in production medium, just before the onset of candicidin production (Asturias et al., 1990). Similar transcriptional regulation of other antibiotic biosynthesis genes has been reported (see Jones, 1985 and references therein).

Following the finding that a gene involved in the biosynthesis of a polyketide antibiotic could be used as a hybridization probe to detect genes directing the biosynthesis of other polyketides (Malpartida et al., 1987), the PABA synthetase gene of the candicidin-producing S. griseus was tested as a probe for polyene macrolide production by other Streptomyces strains. Of the sixteen tested, six hybridized to the probe (Gil et al., 1990). All of these showed high levels of PABA synthetase activity whereas those that did not had no detectable activity (Gil et al., 1990). When the positive strains were grown in a candicidin-production medium, candicidin was formed (Gil et al., 1990). The fact that the pab gene was found only in Streptomyces that produced

candicidin suggests that this gene is directly involved in the biosynthesis of the antibiotic. It is not clear if it is also involved in the biosynthesis of folic acid. It is noteworthy, however, that under conditions where pab mutants of S. coelicolor and S. lividans required an exogenous supply of PABA for growth candicidin-nonproducing mutants with no detectable PABA synthetase activity grew on minimal medium without the addition of PABA (Gil et al., 1985a). Presumably, these latter mutants received PABA from an undetectable residual level of PABA synthetase activity. Although this may have been due to leakiness in the mutant gene, it may also have represented the activity of the primary metabolic gene. The existence of two genes for the biosynthesis of PABA in candicidin-producing strains of S. griseus - one involved in primary metabolism and the other in secondary metabolism, is consistent with a report that Alegre et al. (cited in Gil et al, 1990) have isolated a pab gene from S. griseus that appears to be involved only in the biosynthesis of folic acid.

E. Comparison of PABA and anthranilate synthetases.

Because of the similarity between PABA and anthranilate biosynthesis, the enzymes involved in these pathways are expected to be functionally and structurally related. Evidence for this has come from the cross reaction between antibodies raised against anthranilate synthetase and fractionated extracts of PABA synthetase (Reiners et al.,

1978). The reactions catalyzed by the two enzymes use chorismic acid and L-glutamine as substrates and produce glutamate, pyruvate and either p-aminobenzoic acid (PABA synthetase) or o-aminobenzoic acid (anthranilate synthetase). The major end-products of these reactions are intermediates in the biosyntheses of essential compounds in the organisms that produce them; PABA for the biosynthesis of folic acid and anthranilic acid for the biosynthesis of tryptophan.

It must be noted that the term "PABA synthetase" has been used to refer to the aminase and amidotransferase activities of the PABA synthesizing system (Huang and Gibson, 1970) without reference to enzyme X (Nichols et al., 1989). Although an intermediate similar to the one reported for the biosynthesis of PABA from chorismic acid and glutamine has been postulated for the biosynthesis of anthranilic acid, there has been no report of the equivalent of enzyme X in the biosynthesis of anthranilic acid (Nichols et al., 1989). Thus, it is not clear if the two subunits of anthranilate synthetase generate an intermediate or are capable of forming the aromatic end-product directly.

Excluding the issue of enzyme X, there is remarkable similarity between the PABA and anthranilate synthetases of E. coli. Both enzymes are composed of two nonidentical subunits; the larger subunit, component I (CoI), has a molecular weight of approximately 50,000 and catalyzes the formation of PABA or anthranilate from chorismic acid and ammonia. The smaller

subunit, component II (CoII), has a molecular weight of approximately 20,000 and is a glutamine amidotransferase that confers on the enzyme complex the ability to use the amide group of L-glutamine in place of ammonia. The similarity between PABA synthetase and anthranilate synthetase is underscored by the ability of some microorganisms to use a single amphibolic glutamine amidotransferase in the biosynthesis of both PABA and anthranilate (Sawula and Crawford, 1973; Kane, 1977; Buvinger et al., 1981).

PABA synthetase and anthranilate synthetase are not only related structurally, functionally and immunologically but also in the nucleotide sequence of their genes. A comparison of E. coli pabA (CoII) and trpG (CoII) nucleotide and deduced amino acid sequences shows 44% homology at the amino acid and 53% homology at the nucleotide sequence level (Kaplan and Nichols, 1983). There are regions of high nucleotide sequence homology in which most differences are in the third position of the codons and do not affect the amino acid sequence (Kaplan and Nichols, 1983). Regions of the two genes lacking DNA sequence homology appear to have arisen by single base substitutions as well as by rearrangement of small regions through deletion, inversion and duplication (Kaplan and Nichols, 1983). The marked sequence similarity between pabA and trpG of E. coli has led Kaplan and Nichols (1983) to suggest that the two genes arose from a common ancestral sequence through gene duplication. Mutation and selection in

the sister nucleotide sequences led eventually to the evolution of genes for two new pathway-specific enzymes. Comparison of the glutamine amidotransferase nucleotide sequences of a variety of prokaryotic and eukaryotic organisms, including glutamine amidotransferases that are PABA synthetase-specific, anthranilate synthetase-specific and amphibolic, suggests that these genes have indeed evolved from a common ancestor (Kaplan et al., 1985).

Despite the high degree of homology between pabA and trpG of E. coli, and the amphibolic nature of the gene in some organisms, the two genes are not functionally interchangeable (Zalkin and Murphy, 1975). In an attempt to account for the amphibolic glutamine amidotransferase subunit, Crawford (1975) suggested that loss of one of the pathway-specific glutamine amidotransferase genes was followed by mutations that allowed the remaining enzyme to function in both enzyme complexes. Crawford's model also includes mutations in the genes for the larger subunits of the two enzyme complexes to facilitate interactions. It accomodates the evidence that in B. subtilis, the amphibolic glutamine amidotransferase gene is linked to genes involved in PABA biosynthesis, while in A. calcoaceticus and P. acidovorans where the glutamine amidotransferase is amphibolic, it is linked to other trp genes.

Yanofsky (1974) offered an entirely different proposal for the evolution of the amphibolic glutamine amidotransferase

gene; according to this, a primitive prokaryotic ancestor contained a single amphibolic glutamine amidotransferase gene that duplicated and diverged, giving rise to the glutamine amidotransferases that are pathway-specific. Organisms such as B. subtilis, A. calcoaceticus and P. acidovorans retained the ancestral amphibolic gene. Comparison of the nucleotide and amino acid sequences of several glutamine amidotransferases supports Yanofsky's model (Kaplan et al., 1985).

Nucleotide and deduced amino acid sequence comparison between E. coli pabB (CoI) and trpE (CoI) genes suggest that these two genes also arose from a common ancestor (Goncharoff and Nichols, 1984). They are 26% homologous at the amino acid level and 40% at the nucleotide level. Homology is not present throughout the sequence but is primarily at the carboxy-terminal portion of the genes. Goncharoff and Nichols (1984) pointed out that a higher degree of similarity between the CoII subunits (pabA and trpG) than between the CoI subunits (pabB and trpE) is expected because the CoII subunits have identical roles in transferring the amino group from glutamine to the CoI subunit. When pabB genes of three enteric bacteria were compared with the trpE genes of several organisms, several regions of high homology were found (Goncharoff and Nichols, 1988). Also, secondary structure predictions of the pabB and trpE genes based on deduced amino acid sequences showed several regions of identical structure

(Goncharoff and Nichols, 1988).

Nichols et al. (1989) have noted that despite the high degree of similarity in the biosyntheses of PABA and anthranilate, there are several differences in the enzymology of the two reactions. First, the interaction between CoI and CoII in anthranilate synthetase is reported to be strong (Ito and Yanofsky, 1969) whereas the PABA synthetase CoI and CoII subunits interact only weakly (Nichols et al., 1989). Secondly, the CoII component of anthranilate synthetase has been found to enhance the ammonia-dependent reaction catalyzed by CoI (Zalkin, 1973) but this does not occur in the PABA synthetase complex (Nichols et al., 1989). Thirdly, it remains to be established if the product of the anthranilate synthetase-catalyzed reaction is non aromatic, whereas the PABA synthetase-catalyzed reaction has been shown to give a non aromatic product (Nichols et al., 1989). Thus, although two subunits of PABA and anthranilate synthetases each evolved from a common ancestor, they have acquired different subunit interactions and catalytic properties (Nichols et al., 1989).

II. Sulphonamides

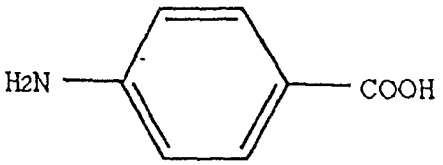
A. Mechanism of action of sulphonamides.

The chemotherapeutic action of Prontosil was recognized not to be due to its properties as a dye but to the formation

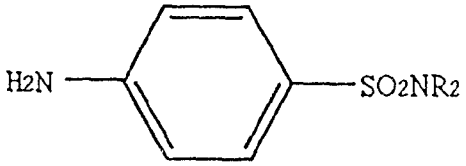
from it *in vivo* of an active metabolite, sulphanilamide (Daniel and Norris, 1947). Experiments with several bacterial species showed that sulphonamides inhibit the synthesis of folic acid compounds (Lascelles *et al.*, 1954). It had earlier been observed that PABA could nullify the inhibitory effects of sulphanilamide (Woods, 1940) and evidence that inhibition by sulphonamides could be reversed by supplying folic acid suggested that folic acid was the product of the inhibited reaction (Lampen and Jones, 1946). This reversal was not observed in *E. coli* and *Neurospora crassa* (Hutchings and Burchall, 1965). However, in these organisms exogenous folic acid could not be transported and assimilated (Hutchings and Burchall, 1965).

Reversal by PABA of the inhibitory effects of sulphonamides, coupled with the finding that PABA formed part of the folic acid molecule, suggested that sulphonamides might act on the folic acid biosynthesis pathway. The target proposed was the condensation of PABA with 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphate to form dihydropteroic acid. The structural similarity between PABA and sulphonamides (Fig. 3) could allow competition for the active site of the enzyme catalyzing the condensation.

Brown (1962) confirmed that sulphonamides inhibit folate synthesis by inhibiting the condensation reaction. He also found that various sulphonamides inhibit folate synthesis to different degrees. Although inhibition appears to result from



PABA



Sulphonamide

Figure 3: Structural similarity between p-aminobenzoic acid and sulphonamide.

direct competition between PABA and the sulphonamide for the active site of the enzyme, once bound the sulphonamide can react with the pteridine to form inactive folate derivatives (Brown, 1962; Buck et al., 1973; Swerdberg et al., 1979). The activity of sulphonamides could thus be explained in terms not only of competitive inhibition of dihydropteroic acid formation but also of the drain on the cellular supply of 2-amino-4-hydroxy-6-hydroxymethyl didydropteridine pyrophosphate. Other analogues of PABA have been reported to condense with pteridine compounds to form inactive folate compounds. Wacker et al. (1958) observed that p-aminosalicylate was converted by certain enterococci into inactive folate compounds. Hotchkiss and Evans (1960) observed a similar phenomenon in pneumococci.

It is interesting to note that sulphonamides are more potent inhibitors of the condensing enzyme than of growth (Brown, 1962). Sulphanilic acid was totally ineffective as a growth inhibitor but quite potent as an inhibitor of enzymatic synthesis of folic acid compounds. The difference may be due to difficulty in transporting these highly ionized molecules across cell membranes (Brown, 1962).

B. Resistance to sulphonamides

Under the pressure of an inhibitor, mutants that escape inhibition accumulate spontaneously in any microbial population (Cavalli-Sforza, 1962). Resistance to

sulphonamides is of clinical importance, especially in urinary tract infections, where 25-80% of the causative bacteria are 100-1000 times more resistant than normal strains (Otaya et al., 1972). Strains of several microorganisms that show complete resistance to sulphonamides have been isolated.

Earlier attempts to characterize the mechanism of resistance focused on identifying a by-pass mechanism whereby the organism as a result of its inability to produce folic acid gains the ability to use preformed dietary folic acid (Hitchings and Burchall, 1965). Attempts to demonstrate this mechanism in bacteria have failed. However, it appears to operate in plasmodia since sulphonamide-resistant strains were cross-resistant to pyrimethamine and other inhibitors of dihydrofolate reductase. Bishop (1959) concluded that the plasmodia had developed an ability to use the reduced forms of folic acid available in the host's erythrocytes.

Several organisms are intrinsically resistant to sulphonamides by virtue of the fact that they do not synthesize folic acid and acquire it in their diet. Absence of the folic acid pathway has been used as a basis for chemotherapy capitalizing on a major difference between host and parasite (Hitchings and Burchall, 1965). The parasite (which biosynthesizes folic acid) can be inhibited by sulphonamides with no consequence for the appropriate host.

Another mode of resistance to sulphonamides involves modification of the target enzyme, dihydropteroic acid

synthetase, whereby the enzyme has diminished affinity for sulphonamides but unchanged or enhanced affinity for the natural substrate PABA. This mode of resistance was proposed when E. coli strains were found to be resistant to a broad spectrum of sulphonamides (Davis and Mass, 1952). Wacker et al. (1957) observed that resistant strains had an enzyme that bound PABA more tightly and sulphonamides less tightly than the corresponding enzyme of the sensitive line. In sulphonamide-resistant strains of pneumococci, dihydropteroic acid synthetase had twice the affinity for PABA and a seven-fold lower affinity for sulphanilamide compared to the wild-type (Ortiz and Hotchkiss, 1966; Ortiz, 1970). Swerdberg et al. (1979) compared the chromosomally-mediated dihydropteroic acid synthetases of sulphonamide-resistant and wild-type strains. The wild-type enzyme had a lower affinity for the natural substrate than for sulphonamides, implying that the enzyme favours the drug over PABA. In resistant strains, the affinity for the drug was reduced 150-fold whereas the affinity for PABA was reduced only 10-fold. That this type of resistance is caused solely by a mutation altering the relative substrate affinities of dihydropteroic acid synthetase has been challenged by Lopez et al. (1987). In mutants of Streptococcus pneumoniae resistant to sulphonamides and altered in dihydropteroic acid synthetase, deletions in the cloned DNA containing the gene allowed expression of the mutant dihydropteroic acid synthetase but

eliminated sulphonamide resistance *in vivo*. They suggested that dihydropteroic acid synthetase may not be the only enzyme involved in the resistance, and that another step of the folate biosynthetic pathway may be inhibited by free or conjugated sulphonamides.

Plasmid-mediated resistance to sulphonamides is often associated with resistance to various antibiotics, suggesting that R-plasmids are involved (Benveniste and Davies, 1973). Wise and Abou-Donia (1975) showed that sulphonamide resistance in clinically isolated strains harbouring R-plasmids could be explained by the expression of a plasmid-encoded drug-resistant dihydropteroic acid synthetase that was distinct from the chromosomally expressed enzyme. Similar observations were made by Skold (1976) and Nagate *et al*. (1978). Swerdberg and Skold (1980) demonstrated two types of plasmid-mediated resistance to sulphonamides. In the first, a plasmid-encoded dihydropteroic acid synthetase was involved. This enzyme was different from its chromosomal counterpart in heat stability and molecular size. It conferred resistance through its higher affinity for PABA than for the drug. In the second class, sulphonamide resistance was due to a drug permeability barrier. Nagate *et al*. (1978) have also reported sulphonamide resistance mediated by a permeability barrier.

Sulphonamide resistance could also be achieved by deregulation of the normal low expression of PABA synthesizing enzymes so that large amounts of PABA are produced to

outcompete sulphonamides for the condensing enzyme's active site. Gil and Hopwood (1983) isolated a sulphonamide-resistant mutant of S. griseus that apparently owed its phenotype to overproduction of PABA. The resistant strain was able to stimulate the growth of PABA-requiring Streptomyces mutants, suggesting that it released PABA into the medium (Gil and Hopwood, 1983). White and Woods (1965) have also reported that a sulphonamide-resistant isolate of Staphylococcus aureus greatly increased its PABA production. Similar observations have been made for sulphonamide-resistant isolates of Neisseria gonorrhoeae (Landy and Gerstrong, 1944) and Pneumococcus sp. (Tillet et al., 1943). The ability of large amounts of PABA to overcome the antagonistic effects of sulphonamides is underscored by the use of sulphonamides in the isolation of pab mutants (Gil and Hopwood, 1983; Atkinson, 1987; Aidoo, 1989).

III. Gene cloning in Streptomyces.

Streptomyces are Gram-positive soil bacteria with a genome size of about 10^4 kb. The best characterzed example is S. coelicolor A3(2); the organization of its genome has been studied by a variety of methods and a detailed genetic map of functions has been developed. A wide variety of genes involved in differentiation, auxotrophy, secondary metabolism and drug resistance has been allocated map positions (Hopwood

et al., 1973). Streptomyces have received considerable attention over the last two decades for two main reasons. First, Streptomyces provide a model for the study of prokaryote differentiation since they not only grow as vegetative mycelia but also differentiate to form aerial mycelia from which spores are generated. The available information on differentiation in Streptomyces suggests mechanisms quite different from those by which endospores are formed in bacilli (Hardisson and Manzanal, 1976). Secondly, many Streptomyces elaborate antibiotics as secondary metabolites; over two-thirds of the naturally occurring antibiotics are produced by species of this genus (Berdy, 1980). Attention has thus been drawn to the physiological and genetic mechanisms by which these antibiotics are biosynthesized.

Progress in the application of molecular genetic techniques to streptomycetes has in a large measure been due to the efforts of Hopwood and his associates (Hopwood et al., 1985). These advances have made available procedures for molecular cloning in the genus. In this section, some factors considered important for gene cloning in Streptomyces, as well as the strategies employed and examples of foreign gene expression in Streptomyces will be reviewed.

A. Choice of host strain.

The choice of a suitable host is of paramount importance

in any cloning experiment. It is sometimes advantageous to select a host deficient in expressing the phenotype of the gene to be cloned. Complementation of the absent function in the host by the introduction of foreign DNA is used to detect a successful cloning event. The reversion rate of the host has to be lower than 10^{-8} (Hunter, 1984). In some cases, it is necessary to mutagenize a wild-type organism to obtain a suitable host; for example, to acquire an auxotrophic host. For cloning of genes using plasmid or phage vectors, a desirable host will usually be sensitive to an inhibitory drug, resistance to which has been engineered into the cloning vector (Hunter, 1984). Because a large number of transformant colonies will have to be screened to find one with the desired phenotype, the host should be amenable to transformation and regeneration at high efficiencies.

The recombinogenic nature of some streptomycetes may lead to integration and rearrangement of cloned DNA sequences. This could be overcome by using a recombination-deficient host. However, no useful recombination-deficient Streptomyces strains are yet available. To circumvent the problem, Streptomyces genes may be cloned in an organism, such as a heterologous Streptomyces, where the difference in genomic DNA sequences will prevent homologous recombination.

The use of heterologous hosts has the disadvantage of exposing an unmodified vector and/or its foreign DNA insert to restriction activity. Many Streptomyces have effective

restriction systems (see Matsushima and Baltz, 1989 and references therein). Hunter (1984) has suggested that the high restriction activity of streptomycetes protects them from phage infections. There are two reported ways of overcoming restriction systems. In one way, protoplasts of the strain are 'heat-shocked' to reduce restriction activity. Matsushima et al. (1989) have reported that the frequency with which Streptomyces phaeochromogenes and Streptomyces fradiae are transformed is higher if protoplasts are allowed to regenerate at 39°C instead of 27°C, and suggest that the higher temperature reduces restriction. Engel (1987) has reported that Streptomyces tendae could not be transformed with the vector pIJ702 using normal procedures, but when the protoplasts were incubated at 50°C for 30 min before transformation, a frequency of 10^2 ug^{-1} DNA was obtained. With plasmid DNA from these primary transformants, S. tendae was transformed at a frequency of 10^6 to 10^7 ug^{-1} DNA. Engel (1987) suggests that incubation at 50°C inactivated the restriction system and that the primary transformants underwent modification and could then escape the restriction barrier.

A second way of overcoming restriction systems is to use a restrictionless mutant as the host. After identifying a restriction system in Streptomyces fradiae (Matsushima and Baltz, 1985), Matsushima et al. (1987) selected a mutant lacking the restriction barrier. Spores mutagenized with N-

methyl-N'-nitroso-N-nitrosoguanidine (MNNG) were replica-plated on a medium seeded with phage FP43, which does not infect the wild-type due to a restriction barrier; strains sensitive to the phage were thereby identified. Assay of the sensitive strains for infection by three other phages showed four different patterns of plating efficiency, suggesting that there were at least four different restriction systems in S. fradiae.

The β-lactam producer Streptomyces lipmani has at least two restriction systems (Matsushima and Baltz, 1987). Mutagenesis with MNNG led to the isolation of a strain lacking one of these and able to be transformed with several plasmids (Matsushima and Baltz, 1987). Restrictionless strains of the oxytetracycline producer Streptomyces rimosus have also been obtained by MNNG mutagenesis (Hunter and Friend, 1984). Transformation with the plasmid pP224 gave a few thiostrepton-resistant transformants; these were cultured nonselectively on a medium without thiostrepton to obtain thiostrepton-sensitive colonies. These had lost the plasmid spontaneously but retained their restrictionless phenotype.

Streptomyces lividans (Hopwood, 1986) and Streptomyces griseofuscus (Cox and Baltz, 1985) have been reported to lack restriction-modification systems. Not suprisingly, these species have been used routinely as hosts for cloning streptomycete genes. A potential disadvantage associated with S. lividans, is the presence of an active gene amplification

system (Altenbuchner and Cullum, 1984) which could amplify a cloned segment of DNA should it contain an amplifiable unit recognized by the amplification system (Altenbuchner and Cullum, 1987).

B. Choice of vectors.

Several plasmid and phage vectors have been developed and used to clone streptomycete genes. Depending on the vector, they can be introduced into the host by transformation or transfection, and are derived from plasmids and phages that are found naturally in streptomycetes.

1. High copy number plasmid vectors.

Many high copy number streptomycete plasmid vectors are derived from the 8.9-kb pIJ101, a plasmid naturally occurring in S. lividans ISP5434 (Kieser et al., 1982). These vectors have the advantage of being well characterized and usually transform a suitable host with high efficiency (Hunter, 1984). Because of their high copy number and small size, they are easy to detect and isolate from small scale DNA preparations. However, over-expression of some genes cloned into high copy number vectors of this type may be harmful to the host (Malpartida and Hopwood, 1984).

pIJ101 has a wide host range; it was stably maintained in thirteen of the eighteen strains tested originally (Kieser et al., 1982) and has subsequently been shown to be maintained in

several other strains. Since pIJ101 is self-transmissible and exhibits 'lethal zygosis' (Bibb et al., 1977)- a phenomenon whereby plasmid-containing colonies are surrounded by a clear zone (pock) - non-essential segments of DNA encoding such characteristics have been removed and other markers have been introduced to enhance its value as a cloning vector. One such marker is thiostrepton resistance; most streptomycetes are sensitive to this antibiotic. Thus useful vectors such as the 4.1-kb plasmid, pIJ350, have been constructed by deleting portions of pIJ101 and inserting the thiostrepton resistance gene from Streptomyces azureus (Kieser et al., 1982). The most widely used of all the pIJ101-derived vectors is pIJ702 (Katz et al., 1983; Fig.4). It was constructed by introducing the mel gene for tyrosinase from Streptomyces antibioticus into pIJ350 (Katz et al., 1983). The mel gene allows colonies carrying the plasmid to be identified by the brownish-black pigmentation they produce on media containing tryptone and copper ions. There are three unique restriction sites within the mel gene; insertion of foreign DNA into these sites inactivates the gene, so colonies carrying plasmids with inserts are white. Thus the pigmentation of transformant colonies provides a good visual indication of the presence of inserts in the vector.

2. Low copy number plasmid vectors.

Many versatile low copy number vectors commonly used for

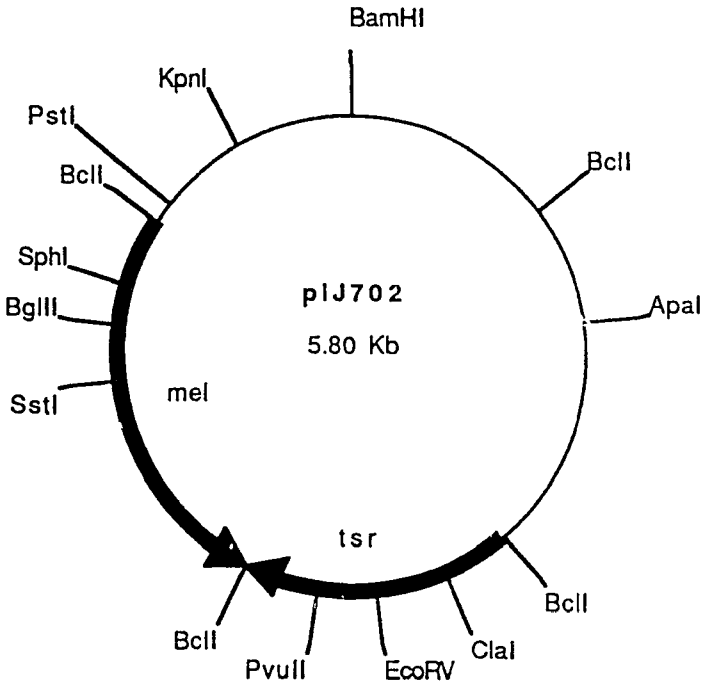


Figure 4: Circular map of pIJ702.

cloning in Streptomyces are derived from the naturally occurring plasmids SLP1 and SCP2.

SPL1 was detected in matings between S. coelicolor A3(2) and S. lividans 66 (Bibb et al., 1981). It was present in chromosomal sequences of S. coelicolor A3(2) and became autonomous when transferred into S. lividans 66 (Bibb et al., 1981). There are several variants of SLP1 differing in size and restriction pattern. They are present in S. lividans at copy numbers of four to five per chromosome. One disadvantage of SLP1-derived vectors is that they have a narrow host range; thus they are almost exclusively used for cloning in S. lividans. The most widely used low copy number SLP1-derived vector is the 14.8-kb pIJ41, derived from the variant SLP1.2 and containing the thiostrepton-resistance gene from S. azureus as well as the neomycin-resistance gene from S. fradiae (Thompson et al., 1982; Fig. 5). There are unique sites for the restriction endonucleases BamHI and PstI within the neomycin-resistance gene; insertion of foreign DNA into these sites inactivates the gene and thereby allows transformants carrying plasmids with inserts to be identified.

Another low copy number plasmid vector, pIJ941, was derived from SCP2*. SCP2* is a sex plasmid from S. coelicolor A3(2) where it occurs autonomously as a 31-kb molecule (Bibb et al., 1977; Schrempf and Goebel, 1977). pIJ941 was constructed by Lydiate et al. (1985) as a 25-kb plasmid containing the thiostrepton resistance gene from S. azureus

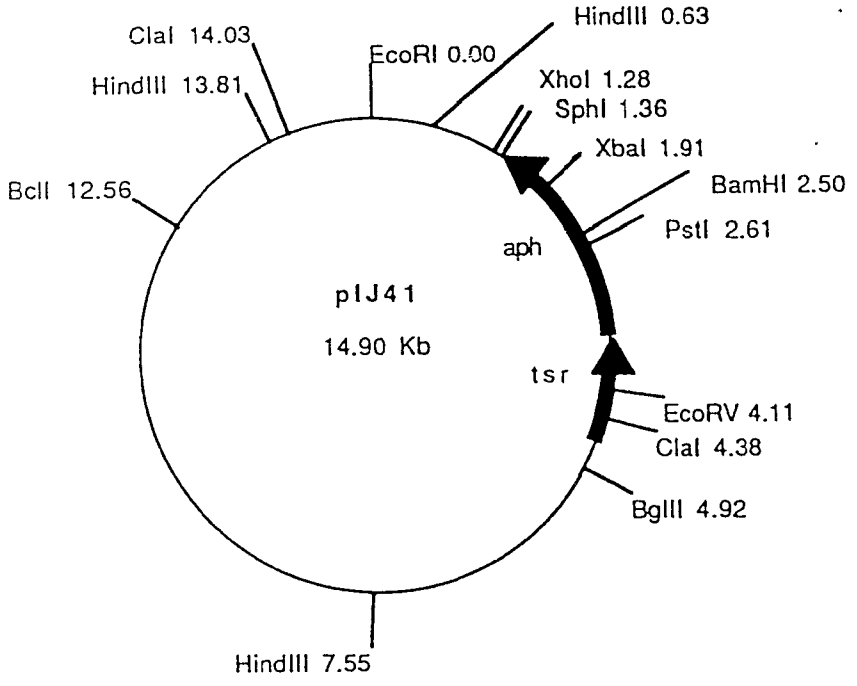


Figure 5: Circular map of pIJ41.

and the hygromycin resistance gene from S. antibioticus. There are unique sites in both resistance genes to allow insertional inactivation.

3. Phage vectors.

The broad host range S. coelicolor A3(2) temperate phage ϕC31 has a genome size of 41 kb and has cohesive ends. It is lysogenized by recombination between a phage attachment site (attP) and a chromosomal attachment site (attC). Harris et al. (1983) have developed cloning vectors from ϕC31 in which the attP site is deleted so that the phage DNA is incapable of integrating into the chromosome. Construction of ϕC31 chimeras with the E. coli plasmid pBR322 allow expression in both Streptomyces and E. coli hosts (Harris et al., 1983). The ampicillin resistance and tetracycline resistance genes on pBR322 serve as useful markers in E. coli. The viomycin resistance gene of Streptomyces vinaceus was introduced into an attP-deleted ϕC31 to facilitate selection in Streptomyces (Harris et al., 1983).

C. Approaches to cloning of Streptomyces genes.

Most streptomycete genes that have been cloned are involved in the production of or resistance to antibiotics. The incentive for such work comes from the economic importance of antibiotics; however, research aimed at overproducing them, or at creating novel hybrid antibiotics by combining

antibiotic biosynthetic genes from different sources, have been supplemented with much basic research on the organization and regulation of antibiotic biosynthesis genes. Some of the strategies used to clone antibiotic biosynthesis genes are described.

1. Complementation of blocked mutants.

The use of blocked mutants of a producing strain as the cloning host allows genes involved in production of the antibiotic to be cloned by direct complementation of the mutations. Feitelson and Hopwood (1983) were the first to describe the cloning of a streptomycete antibiotic biosynthetic gene by this method. Mutants (red) of S. coelicolor A3(2) blocked in the biosynthesis of undecylprodigiosin had been obtained by uv mutagenesis (Rudd and Hopwood, 1980). Cosynthesis experiments had established five complementation groups, A - E. Characterization of the mutants suggested that the redE mutation involved a block in O-methyltransferase activity (Rudd and Hopwood, 1980). This was supported by assaying the transfer of [^{3}H] methyl from ^{3}H-[methyl]-S-adenosylmethionine to prodigiosin-like products in wild-type S. coelicolor A3(2) and in red mutants. No transfer was detected in redD and redE mutants. A 1.73-kb cloned segment of the wild-type genomic DNA restored undecylprodigiosin biosynthesis in redE mutants (Fietelson and

Hopwood, 1983). Using this 1.73-kb fragment as a probe, a 20-kb sequence was obtained from a cosmid library prepared in a phage lambda derivative (Feitelson et al., 1985). When cloned in pIJ702 and pIJ922 the 20-kb sequence complemented the redA, redB and redE mutations in S. coelicolor A3(2). As well, it complemented another class of mutants, redF, not previously described (Feitelson et al., 1985). Another S. coelicolor wild-type sequence adjoining the 20-kb sequence was subsequently cloned and shown to complement other known red mutations (Feitelson et al., 1985).

Genes directing the biosynthesis of actinorhodin in S. coelicolor A3(2) have also been cloned by complementation. Rudd and Hopwood (1979) classified act mutants into seven complementation groups of which one, actII, was thought to have a regulatory function. Shotgun cloning of wild-type S. coelicolor A3(2) DNA into pIJ922 followed by transformation of act mutants led to the isolation of two plasmids that together complemented all known act mutations (Malpartida and Hopwood, 1984). By combining the cloned DNA in the two plasmids to give a continous stretch of 32.5 kb of S. coelicolor A3(2) DNA in a third plasmid, pIJ2303, Malpartida and Hopwood (1984) complemented all act mutations. That all the genes required for actinorhodin biosynthesis were present in pIJ2303 was established by introducing the plasmid into Streptomyces parvulus, an actinorhodin nonproducing strain. Transformants containing pIJ2303 produced actinorhodin. The gene cluster

for actinorhodin biosynthesis has subsequently been localized within a 22-kb segment (Malpartida and Hopwood, 1986).

Other antibiotic biosynthetic genes that have been cloned by complementing blocked mutants include a gene cluster from S. hygroscopicus that complements all of the known mutations in bialaphos biosynthesis (Murakami et al., 1986), a gene that complements the tcmII mutation in the tetracenomycin C producer Streptomyces glaucescens (Motamedi and Hutchinson, 1987), a gene restoring the production of clavulinic acid in a blocked mutant of Streptomyces clavuligerus (Bailey et al, 1984), genes for the biosynthesis of streptomycin in S. griseus (Ohnuki et al., 1985) and a gene involved in the biosynthesis of streptomycin in Streptomyces bikiniensis (Kumada et al., 1986).

2. Using a cloned antibiotic resistance gene to identify linked biosynthetic genes.

Expression of antibiotic biosynthesis genes is closely regulated to avoid suicide of the producing organism. It is now apparent that this regulation is achieved by linking genes involved in antibiotic biosynthesis to those that specify resistance. Because resistance can be detected by positive selection, cloning of such genes is facilitated. A cloned antibiotic resistance gene can then be used to probe for adjacent biosynthetic genes.

Stanzak et al. (1986) have used the erythromycin

resistance gene to clone genes involved in the biosynthesis of erythromycin. After constructing a genomic library of the erythromycin producer Saccharopolyspora erythrea in the E. coli-Streptomyces bifunctional cosmid vector pKC426a, transductants that hybridized to the erythromycin resistance gene previously cloned by Thompson et al. (1982) were isolated. DNA from one of the clones was used to construct a plasmid, pKC488, containing 35 kb of S. erythrea DNA and the plasmid was transformed into S. lividans. Erythromycin-resistant transformants obtained from this experiment produced an antibiotic substance identified as erythromycin. The cloned DNA not only complemented S. erythrea blocked mutants but also hybridized strongly to DNA digests from five erythromycin producers (Stanzak et al., 1986). The cloned sequence did not hybridize to a genomic digest of the erythromycin nonproducer S. lividans so Stanzak et al. (1986) concluded that the cloned 35-kb segment of S. erythrea DNA contained the genes for the biosynthesis of erythromycin.

Motamedi and Hutchinson (1987) have used cloned DNA conferring resistance to tetracenomycin C in S. lividans to clone the entire gene cluster for tetracenomycin C biosynthesis from S. glaucescens. Restriction analysis on the cloned DNA followed by cloning of all or parts of it into blocked mutants of S. glaucescens showed that a 24-kb segment that included the resistance determinant could complement all of the known blocked mutants. When the entire 24-kb fragment

was cloned into S. lividans, transformants produced tetracenomycin C, indicating that the 24-kb fragment contained all the genes required to produce tetracenomycin C.

Genes for oxytetracycline biosynthesis in S. rimosus have also been cloned using the resistance determinant (Rhodes et al., 1984).

3. Mutational cloning.

Chater and Bruton (1983) have used mutational cloning to isolate S. coelicolor A3(2) genes involved in the biosynthesis of methylenomycin A. These genes were located on the SCP1 plasmid (Kirby and Hopwood, 1977). The ϕC31-derived phage KC400, which lacks the attP site and carries the phosphotransferase gene from S. vinaceus conferring resistance to viomycin, was used as a vector. The absence of the attP site allowed lysogeny only when the phage recombined at a chromosomal site sharing homology with a sequence on the phage DNA. To introduce this homology, Chater and Bruton (1983) cloned genomic DNA fragments of an SCPI$^+$ strain of S. parvulus into phage KC400 and used the phage to transfect S. lividans. Since S. parvulus and S. lividans share little homology (Aguillar and Hopwood, 1982), only the shared sequences of SCP1 would allow lysogeny of the phage by recombination. Plaques of interest were identified by their ability to transduce viomycin resistance into indicator strains. Viomycin-resistant transductants were examined for any changes

in methylenomycin A biosynthesis. Since the lysogens were obtained by homologous recombination, methylenomycin A mutants would be obtained if SCP1 fragments had integrated into the recipient, provided integration occurred within the trancriptional unit. Chater and Bruton (1983) observed that of 278 lysogens, nine had a greatly reduced capacity to produce methylenomycin A. DNA from two of these plaques hybridized to DNA fragments of $SCP1^+$ but not $SCP1^-$ strains. Some pairwise combinations of the nine mutants produced the antibiotic in cosynthesis cultures indicating that they carried some of the genes involved in biosynthesis of the antibiotic.

4. Use of oligonucleotide hybridization probes.

For antibiotic biosynthesis enzymes of which at least part of the amino acid sequences are known, the amino acid sequence can be used to construct an oligonucleotide probe with which to search a gene bank for hybridizing sequences.

Anzai et al. (1987) used polyacrylamide gel electrophoresis to examine the protein profiles of a bialaphos-producing and a blocked mutant of S. hygroscopicus. A 32-kDa protein absent from the mutant profile, was isolated and purified from the producing strain and the amino-terminus sequence was determined. Based on this sequence, a mixed oligonucleotide was synthesized and used to probe a genomic digest of a bialaphos producer. A hybridizing fragment thus

identified was cloned, and from it, an internal fragment that specified the 32-kDa protein was obtained (Anzai et al., 1987).

Jensen et al. (1986) purified isopenicillin N synthetase from the β-lactam producing S. clavuligerus and Leskiw et al. (1988) determined its amino-terminal sequence. A mixed oligodeoxyribonucleotide probe based on this sequence with G and C as uncertain third letters of the codons (reflecting the 95% G + C content of the third position codons of streptomycete genes) was used to isolate a 9.6-kb DNA fragment from a genomic library of S. clavuligerus DNA (Leskiw et al., 1988). That this fragment encoded isopenicillin N synthetase was demonstrated by introducing it into an isopenicillin N synthetase-deficient mutant of S. clavuligerus. Transformants containing the 9.6-kb fragment were able to produce cephamycin. The cloned isopenicillin N synthetase gene of S. clavuligerus has been used as a hybridization probe to identify isopenicillin N synthetase genes in the genomic DNA of the β-lactam producers S. lipmani and Streptomyces jumonjinensis (Shiffma et al., 1988).

Raymer et al. (1990) cloned an extracellular esterase gene from the plant pathogen Streptomyces scabies using a synthetic oligonucleotide hybridization probe. A mixed synthetic oligonucleotide which corresponded to amino acid residues 18 through 28 of the excreted esterase protein was labelled and used to probe a genomic library of S. scabies DNA

in a phage lambda derivative. A 10-kb S. scabies DNA fragment from a positively hybridizing plaque was purified; restriction analysis as well as further hybridization experiments localized the esterase gene on a 2.5-kb SmaI fragment.

Other antibiotic biosynthetic genes that have been cloned using oligonucleotide probes corresponding to amino acid sequences of biosynthetic enzymes include the macrocin-O-methyltransferase gene of the tylosin producer S. fradiae (Fishman et al., 1987) and the ATC oxygenase gene of the oxytetracycline producer S. rimosus (Butler et al., 1989).

5. Using cloned DNA as a probe to locate and clone a related antibiotic.

Malpartida et al. (1987) used cloned fragments of the actI and actIII genes of S. coelicolor A3(2) as probes to clone genes from other streptomycetes producing polyketides. In addition, the DNA from some organisms not hitherto known to be polyketide producers hybridized to the probes.

In a similar approach, Gil et al. (1990) used the pabS gene of the candicidin producing S. griseus to identify other organisms that produce PABA-containing polyene macrolides.

D. Expression of nonstreptomycete genes in Streptomyces.

The commercial importance of Streptomyces has led to a rapid application of molecular biological techniques in this genus (Hopwood et al., 1985). Streptomyces have some

advantages as hosts for foreign DNA production in that active protein synthesis occurs well into stationary phase, and some of the strains are able to reach very high cell densities. These features should allow efficient expression of cloned heterologous genes.

A possible barrier to the expression of nonstreptomycete genes in Streptomyces might be the high guanine and cytosine (G+C) content of streptomycete DNA; for example, the G+C content of S. coelicolor and S. lividans is 73%. DNA sequence analysis of cloned streptomycete genes has shown that the high G+C content leads to a biased codon usage whereby the third position of each codon is about 95% G or C (Bibb et al., 1985). This codon usage may reflect the availability of tRNAs that are complementary to codons with Gs or Cs in the third position.

Bibb and Cohen (1982) observed that one group of streptomycete DNA fragments with promoter activity in streptomycetes did not function as promoters in E. coli. The nucleotide sequences of these streptomycete promoters did not resemble the promoter sequences identified widely in E. coli and B. subtilis. However, another group of streptomycete promoters was functional in E. coli and responded to mutations in a way similar to E. coli promoters (Jaurin and Cohen, 1984). Using E. coli promoter-probe plasmids, Jaurin and Cohen (1985) isolated several streptomycete promoters that functioned in E. coli. These were referred to as

Streptomyces-E. coli-type promoters (SEPs). The RNA polymerase of S. lividans recognized and utilized promoter sequences of E. coli, Serratia marcescens and Bacillus lichenformis (Bibb and Cohen, 1982). Additional evidence that a streptomycete RNA polymerase recognizes E. coli-type promoters was provided by Westpheling et al. (1985). From these results it is evident that Streptomyces are capable of recognizing and utilizing transcription initiation signals from other organisms. However, foreign gene expression in Streptomyces often depends on Streptomyces vector promoters, as illustrated in some of the examples described below.

1. Expression of the chloramphenicol and kanamycin resistance genes from E. coli in S. lividans.

Schottel et al. (1981) constructed hybrid plasmids by restricting the S. lividans plasmid pSLP111 and ligating it separately to the E. coli plasmids pACYC184 and pACYC177. The latter contain as antibiotic-resistance markers the genes for chloramphenicol acetyltransferase [CAT]) and the phosphotransferase conferring resistance to kanamycin, respectively; both genes are derived from the E. coli plasmid R6-5 (Timmis et al., 1978). The hybrid plasmids could be maintained in both E. coli and S. lividans. When pSLP120 (pACYC184 +pSLP111) and pSLP125 (pACYC177 + pSLP111) were introduced into S. lividans, chloramphenicol resistance and kanamycin resistance were phenotypically expressed. A 1-kb

fragment containing the entire structural gene for chloramphenicol resistance but lacking the sequences that serve as the gene's promoter in E. coli was cloned into pSLP111; the resultant plasmid also specified chloramphenicol resistance in S. lividans. Schottel et al. (1981) concluded from this observation that a Streptomyces promoter was used to initiate transcription of the chloramphenicol resistance gene. The results also provided evidence that the ribosome-binding site and the start codon used in translating the gene in E. coli were functional in S. lividans.

2. Expression of E. coli tetracycline resistance genes in S. albus G.

Suarez and Chater (1980) constructed a chimaeric prophage comprising ϕC31 and the E. coli plasmid pBR322 which contains genes for ampicillin resistance (bla) and tetracycline resistance (tet). This chimaeric prophage was shown to exist as a plasmid in E. coli. Chater et al. (1982) showed that the chimaeric phage transduced tetracycline-sensitive S. albus G to tetracycline resistance whereas phage ϕC31 lacking pBR322 could not do so. That the tetracycline resistance of the S. albus G lysogen resulted from expression of the tetracycline resistance gene on pBR322 was established by introducing the viomycin-resistance gene from S. vinaceus into the tetracycline resistance gene in the chimaera. Chimaeric phages with this modification transduced to viomycin

resistance but not to tetracycline resistance. When the viomycin resistance gene was removed, transduction to tetracycline resistance was restored. Chater et al. (1982) have suggested that since expression of the tetracycline resistance gene was observed in lysogens, where phage promoters were most likely repressed, the Streptomyces RNA polymerase recognized the E. coli tet promoter. This observation contrasts with that of Schottel et al. (1981) where expression of an E. coli gene appeared to involve a Streptomyces promoter.

3. Expression in Streptomyces ambofaciens of an E. coli gene that confers resistance to hygromycin B.

The hygromycin B resistance gene on the E. coli plasmid pT1104 was characterized by Rao et al. (1983) as a phosphotransferase. Kuhstos and Rao (1983) constructed shuttle vector pKC203 comprising sequences from the E. coli plasmid pBR322, Streptomyces plasmid pFJ103, the viomycin-resistance gene from S. vinaceus, the hygromycin-resistance gene from pT1104 and the Tn5 neomycin resistance gene that had previously been expressed in S. lividans (Bibb et al., 1983). The origins of replication of pBR322 and pFJ103 allowed pKC203 to replicate in both E. coli and Streptomyces, respectively. When pKC203 was introduced into S. ambofaciens, transformants selected on viomycin-supplemented plates were resistant to hygromycin B. Cell extracts prepared from pKC203-containing

S. ambofaciens colonies had a high phosphotransferase activity capable of phosphorylating both hygromycin and viomycin. A region of the hygromycin resistance gene known to contain the promoter sequence was inverted to yield another plasmid, pKC305. Cell extracts of S. ambofaciens transformants carrying pKC305 were only 10% as active in phosphorylating hygromycin B as extracts from transformants carrying pKC293. Since the promoter of the hygromycin resistance gene recognized in E. coli appeared to be also functional in S. ambofaciens, Kuhstoss and Rao (1983) suggested that residual hygromycin B phosphotransferase activity in colonies carrying pKC305 could be due to readthrough transcription from an upstream sequence.

4. Expression of an E. coli sulphonamide resistance gene in S. lividans.

Shareck et al. (1984) constructed E. coli-Streptomyces shuttle vectors based on pIJ101 and the E. coli plasmid pSAS1206 which conferred linked resistance to sulphonamides and streptomycin (Shareck et al., 1983). The 8.9-kb pIJ101 contains a unique restriction site for the endonuclease KpnI in a non-essential region whereas the unique KpnI site of the 5.9-kb pSAS1206 is within the streptomycin resistance gene. Shuttle vector pFSH101 was constructed by ligating pIJ101 and pSAS1206, both linearized with KpnI. After transforming S. lividans with the ligation mixture, colonies habouring the

shuttle vector were selected on a medium containing sulphonamide. Restriction analysis of plasmid DNA isolated from the transformants indicated that whereas the entire 8.9-kb pIJ101 was present, 5.1 kb of the 5.9-kb pSAS1206 had been deleted. Shareck et al. (1984) suggested that the remaining 800 bp of pSAS1206 contained the structural gene conferring sulphonamide resistance. The size of the sulphonamide resistance gene was estimated from the molecular weight of its protein (31,000) to be about 840 bp. Because the promoter for the sulphonamide resistance gene was located about 100-bp upstream of the structural gene, Shareck et al. (1984) suggested that the 800 bp of pSAS1206 retained in pFSH101 lacked the promoter and was transcribed from a promoter within pIJ101. Deletion of some of the pSAS1206 sequence may have been necessary to put the sulphonamide resistance gene in frame with the pIJ101 promoter.

The above examples indicate that although heterologous genes can be expressed in Streptomyces, in some cases there may be barriers in the use of heterologous transcription initiation signals for expression. To circumvent this, deletions that align cloned genes with transcription initiation signals of Streptomyces vectors may be required for expression.

MATERIALS AND METHODS

I. Organisms.

The characteristics and sources of organisms used in this study are listed in Table 1.

II. Chemicals and Biochemicals.

Reagent grade solvents and chemicals were used unless otherwise stated. Bacto-Agar, Bacto-Peptone, Bacto-Tryptone, nutrient broth, nutrient agar, yeast extract, malt extract and casamino acids were purchased from Difco Laboratories, Detroit, MI. Lysozyme and ethidium bromide were from Boehringer-Mannheim, Montreal, P.Q. p-Aminobenzoic acid, p-dimethylaminobenzaldehyde, neomycin, ampicillin, tetracycline, sulphanilamide, tris-(hydroxymethyl)-aminoethane (Tris), N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonate (TES), herring sperm DNA, ribonuclease A, Triton X-100, bovine serum albumin (BSA), Ficoll of molecular weight 400,000, polyvinylpyrrolidone (PVP) of molecular weight 360,000, polyethylene glycol (PEG) of molecular weights 1,000 and 8,000 and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma Chemical company, St. Louis, Mo.

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was from Diagnostic Chemicals Limited, Charlottetown, P.E.I. Thiostrepton was a gift from S. J. Lucania of E. R. Squibb and Sons, New Brunswick, N.J. Agarose (Seakem ME) was from Mandel

Table 1. Bacterial strains used in this study.

Organism	Genotype/phenotype	Source/reference
Lactococcus lactis subsp. *lactis*		
NCDO496	wild-type	NCDO[a]
AV117	plasmid-free	Aidoo, 1985
Streptomyces lividans		
1326	wild-type	JII[b]
TK23	SLP1$^-$ SLP2$^-$ *spc*	JII[b]
TK24	SLP1$^-$ SLP2$^-$ *str*	JII[b]
JG10	SLP1$^-$ SLP2$^-$ *str pab*	Gil and Hopwood, 1983
AP3	*pab*	A. Paradkar, this lab.
M252	*cml*^{s}	JII[b]
FA1	JG10 containing pDQ250	This study
FA2	AP3 containing pDQ250	This study
FA3	JG10 containing pIJ41	This study
FA4	AP3 containing pIJ41	This study
FA5	JG10 containing pDQ254	This study
FA6	AP3 containing pDQ254	This study
FA7	JG10 containing pDQ255	This study
FA8	AP3 containing pDQ255	This study
FA9	JG10 containing pDQ256	This study
FA10	AP3 containing pDQ256	This study
FA11	JG10 containing pDQ293	This study

Table 1 (cont'd)

FA12	AP3 containing pDQ293	This study
Streptomyces venezuelae		
ISP5230	wild-type	Stuttard, 1982
13S	wild-type	Ahmed and Vining, 1983
Streptomyces griseofuscus		
C581	wild-type	Cox and Baltz, 1985
FA1	C581 containing pDQ293	This study
Streptomyces griseus		
IMRU3572	wild-type	IMRU[c]
Streptomyces griseoviridus		
P-D 04955	wild-type	PDC[d]
Escherichia coli		
AB3292	*proA2 his-4 pabA1 ilvC7 argE3 thi-1*	EcGSC[e]
AB3295	*his-4 pabB3 ilvC7 argE3 thi-1*	EcGSC[e]
TG1	del (*lac*,*pro*) *supE thi hsdD5 F'traD36*	Carter et al., 1988
ADA1	AB3295 containing pDQ251	This study
ADA2	AB3292 containing pDQ251	This study
ADA3	AB3295 containing pDQ252	This study
ADA4	AB3292 containing pDQ252	This study
ADA5	AB3295 containing pDQ253	This study

Table 1 (cont'd)

ADA6	TG1 containing pDQ257	This study
ADA7	TG1 containing pDQ258	This study
ADA9	TG1 containing pDQ259	This study
ADA10	TG1 containing pDQ260	This study
ADA11-13	TG1 containing progressive deletions of about 200-nt from the ClaI site of pDQ257 into the pab gene	This study
ADA14-21	TG1 containing progressive deletions of about 200-nt from the ClaI site of pDQ258 into the pab gene	This study
ADA22-24	TG1 containing progressive deletions of about 200-nt from the XbaI site of pDQ259 into the pab gene	This study
ADA25-32	TG1 containing progressive deletions of about 200-nt from the XbaI site of pDQ260 into the pab gene	This study
AKA1	AB3295 containing pDQ290	This study

Table 1 (cont'd)

AKA2	AB3292 containing pDQ290	This study
AKA3	AB3295 containing pDQ291	This study
AKA4	AB3292 containing pDQ291	This study
AKA5	TG1 containing pDQ292	This study
AKA6	AB3295 containing pDQ292	This study
AKA7	AB3295 containing pDQ292	This study
AKA8	TG1 containing pDQ293	This study
AKA9	AB3295 containing pDQ293	This study
AKA10	AB3292 containing pDQ293	This study
AKA11	TG1 containing pDQ294	This study
AKA12	TG1 containing pDQ295	This study
AKA13-27	TG1 containing progressive deletions of about 200-nt from the ClaI site of pDQ294 into the pab gene	This study
AKA28-42	TG1 containing progressive deletions of about 200-nt from the XbaI site of pDQ295 into the pab gene	This study

a. NCDO - National Collection of Dairy Organisms, Reading, U.K.

b. JII - John Innes Institute, Norwich, U.K.

c. IMRU - Institute of Microbiology, Rutgers University, Piscataway, N.J. USA.

d. PDC - Parke, Davis and Co., USA.

e. EcGC - E. coli Genetics Stock Centre, Yale University School of Medicine, New Haven, CT.

Scientific Company, Rockwood, Ontario. Ultrapure DNA-grade agarose, acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylenediamine (TEMED), urea and ammonium persulphate were all of electrophoresis grade and were purchased from Bio-Rad Laboratories, Richmond, CA.

Restriction enzymes, T4 DNA ligase and ultrapure BSA were from Bethesda Research Laboratories, Gaithersburg, MD.

III. Media.

Sterilization of media and some solutions that were required sterile was carried out in an autoclave at 121°C and 15 p.s.i for 20 min. Amino acid and vitamin growth factors were sterilized by filtration through a 0.22 um cellulose acetate membrane.

Lactococcus lactis subsp. lactis strains were grown in SM17 broth (Terzaghi and Sandine, 1975) which contained :

Phytone peptone	5.0 g
Plypeptone peptone	5.0 g
Yeast extract	2.5 g
Beef extract	5.0 g
Sucrose	5.0 g
K_2HPO_4	2.0 g
$MgSO_4.7H_2O$ (1 M)	1.0 mL
Distilled water to	1000 mL.

The pH of the medium was adjusted to 7.0 with 1 M HCl before

sterilization.

To prepare SM17 agar medium, 15 g. L^{-1} of agar was added to SM17 before autoclaving.

Streptomyces lividans strains were maintained on PDA medium which contained :

Potato dextrose agar	39.0 g
Yeast extract	15.0 g
Distilled water to	1000 mL

The pH of the medium was adjusted to 7.0 with 1 M NaOH before sterilization.

Streptomyces lividans strains were also maintained on K1 agar (Aidoo, 1989) which contained :

Maltose	10.0 g
Yeast extract	5.0 g
Casamino acids	0.2 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
$FeSO_4.7H_2O$	0.1 g
Agar	15.0 g
Distilled water to	1000 mL

To maintain thiostrepton-resistant colonies of S. lividans, PDAT and K1T were used. They were prepared by adding thiostrepton to a final concentration of 25 ug mL^{-1} to PDA and K1 media, respectively.

Other Streptomyces strains were maintained on MYM (Stuttard, 1982) containing :

Maltose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
Agar	15.0 g
Distilled water to	1000 mL

To maintain thiostrepton-resistant colonies of Streptomyces strains, MYMT was used; it was prepared by adding thiostrepton to MYM at a final concentation of 25 ug mL^{-1}.

Streptomyces minimal (SM) medium was used to test sulphanilamide resistance levels and PABA synthetase activity of Streptomyces strains; it was prepared as described by Hopwood (1967) except that maltose replaced glucose. The following partial medium (S) was first prepared :

Asparagine	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
$FeSO_4.7H_2O$ (0.2% w/v)	9.0 mL
Agar	15.0 g
Distilled water to	900 mL

Asparagine was sometimes replaced with 0.5 g ammonium sulphate. Sterile 10% maltose solution (100 mL) was added to the sterile S solution before it was used.

YEME medium (Hopwood et al., 1985) was used to grow S. lividans and S. griseofuscus for preparing protoplasts and for DNA isolation. It contained :

Yeast extract	3.0 g

Bacto-Peptone	5.0 g
Malt extract	3.0 g
Glucose	10.0 g
Sucrose	340 g
Distilled water to	1000 mL

For some experiments, sucrose was reduced from 340 g to 103 g. Aliquots (25 mL) of the medium were dispensed into 250-mL Ehrlenmeyer flasks and, just before use, 0.05 mL of 2.5 M $MgCl_2.6H_2O$ and 1.95 mL of 20% glycine were added to each flask.

For regenerating S. lividans and S. griseofuscus protoplasts, R2YE (R5) was used. It contained :

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.6H_2O$	10.1 g
Glucose	10.0 g
Casamino acids	0.1 g
Yeast extract	5.0 g
TES	5.73 g
Agar	15.0 g
Distilled water to	1000 mL

The pH of the medium was adjusted to 7.6 with 1 M NaOH and, just before pouring, the following sterile solutions were added :

Trace element solution (10X)	0.2 mL
KH_2PO_4 (5%)	10.0 mL

$CaCl_2.2H_2O$ (5 M)	4.0 mL
L-Proline (20%)	15.0 mL

The plates were dried for 5-6 h in a laminar airflow hood before use.

Soft nutient agar (SNA) (Hopwood et al., 1985) was used to overlay transformats to select thiostrepton-resistant colonies. It contained :

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

After the agar had been dissolved by heating, the solution was dispensed in 100-mL aliquots and autoclaved.

Glucose-isoleucine medium (Chatterjee et al., 1983) was used to test for PABA production by Streptomyces cultures. It contained :

Glucose	30.0 g
$MgSO_4.7H_2O$	0.2 g
KH_2PO_4	4.5 g
K_2HPO_4	10.5 g
Isoleucine	7.5 g
NaCl (1% solution)	9.0 mL
$CaCl_2.2H_2O$ (1% solution)	9.0 mL
$FeSO_4.7H_2O$ (0.2% solution)	9.0 mL
$ZnSO_4.7H_2O$	4.0 mg
$CuSO_4.5H_2O$	180 ug
H_3BO_3	26.0 ug

$(NH_4)_6Mo_7O_{24}.4H_2O$	17.0 ug
$MnSO_4.4H_2O$	17.0 ug
Distilled water to	1000 mL

L-broth was used for growing E. coli. It contained :

Bacto-Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Glucose	1.0 g
Distilled water to	1000 mL

When required, agar was added at a concentration of 1.5%. Ampicillin and tetracycline were added to L-broth or L-broth agar at concentrations of 100 ug mL^{-1} and 10 ug mL^{-1}, respectively, when required.

To test for PABA dependence, and also to maintain the F' plasmid in E. coli TG1, M9 medium was used. It contained:

Na_2HPO_4	6.0 g
KH_2PO_4	3.0 g
NaCl	0.5 g
NH_4Cl	1.0 g
Agar	15.0 g.

The pH was adjusted to 7.4 before autoclaving. Just before use, the following sterile solutions were added :

$MgSO_4$ (1 M)	2 mL
Glucose (20%)	10 mL
$CaCl_2.2H_2O$ (1 M)	0.1 mL

In some experiments, NH_4Cl was replaced with 1 g of

asparagine.

Medium B (Zalkin and Murphy, 1975) was used to grow E. coli pab mutants for enzyme assays. It contained :

Na_2SO_4	1.06 g
$MgSO_4.7H_2O$	0.1 g
Glucose	5.0 g
$(NH_4)_2SO_4$	0.1 g
Distilled water to	1000 mL

$(NH_4)_2SO_4$ was added as a filter-sterilized solution. Before the solution was made to 1000 mL with distilled water, the pH was adjusted to 6.0, 7.0 or 8.0 using a 0.1 M NaH_2PO_4-Na_2HPO_4 buffer.

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

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

IV. Solutions and Buffers.

Lysis (L) buffer (Thompson et al., 1982) was used to prepare protoplasts of S. lividans and S. griseofuscus. It consisted of:

Sucrose (10.3% w/v)	100 mL
TES, pH 7.2 (5.73% w/v)	10 mL
K_2SO_4 (2.5% w/v)	1.0 mL
KH_2PO_4 (0.5% w/v)	1.0 mL
$MgCl_2.6H_2O$ (2.5 M)	1.0 mL
$CaCl_2.2H_2O$ (3.68% w/v)	1.0 mL
Trace element solution (10X)	0.02 mL

Trace element solution (10X) contained :

$ZnCl_2$	0.4 g
$FeCl_3.6H_2O$	2.0 g
$CuCl_2.2H_2O$	0.1 g
$MnCl_2.4H_2O$	0.1 g
$Na_2B_4O_7.10H_2O$	0.1 g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.1 g
Distilled water to	1000 mL

Osmotic stability of protoplasts during protoplast formation and transformation was maintained using protoplast (P) buffer (Hopwood et al., 1985). The following solution was prepared:

Sucrose	103 g
K_2SO_4	0.25 g
$MgCl_2.2H_2O$	2.02 g
Trace element solution (10X)	0.2 mL

Distilled water to 800 mL

This was divided into 40-mL portions and sterilized. At the time of use, the following sterile solutions were added to each portion in the order shown :

KH_2PO_4 (0.5% w/v) 0.5 mL

$CaCl_2.2H_2O$ (3.68% w/v) 5.0 mL

TES, pH 7.2 (5.73% w/v) 5.0 mL

TES pH 7.2 was prepared by dissolving 5.73 g of TES in 80 mL of water. The pH of the solution was adjusted to 7.2 using 1 M NaOH and the volume made to 100 mL using distilled water. The solution was autoclaved and stored at room temperature.

Basal transformation (T) buffer (Thompson et al., 1982) was used in the transformation of S. lividans and S. griseofuscus protoplasts; it contained :

Sucrose (10.3% w/v) 25 mL

K_2SO_4 (2.5% w/v) 1.0 mL

Trace element solution (10X) 0.02 mL

Distilled water to 100 mL

At the time of use, the following sterile solutions were added to a 4.65 mL portion of basal T-buffer :

$CaCl_2.2H_2O$ (5 M) 0.1 mL

Tris-maleic acid buffer, pH 8.0 (1 M) 0.25 mL

Tris-maleic buffer was prepared by adjusting a 1 M Tris solution to pH 8.0 with maleic acid. In transformations, three parts of supplemented basal T-buffer (by volume) were mixed with one part (by weight) of sterile PEG 1,000.

Buffers for restriction endonucleases and T4 DNA ligase were supplied by the manufacturer. Buffers for mung bean nuclease and exonuclease III were prepared according to the recipes given by the manufacturers. The buffers were stored at -20°C until required.

To dissolve and store DNA, TE buffer was used; it contained 1.0 mM EDTA pH 8.0, and 10 mM Tris-HCl, pH 8.0.

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

To denature and extract protein during DNA isolation, acid phenol-chloroform (Hopwood et al., 1985) was used. This was prepared by mixing 90% aqueous phenol and chloroform in a 1:1, v/v ratio and adding 1.0 mg mL^{-1} of 8-hydroxyquinoline to the mixture to act as an antioxidant. This solution was stored in a brown bottle at room temperature.

Neutral phenol-chloroform was used to extract protein and renature DNA. It was prepared by first equilibrating acid phenol-chloroform with 0.5 volume of 1 M Tris-HCL, pH 8.8. The two phases were allowed to separate overnight and the aqueous layer was removed and replaced with an equal volume of 0.1 M Tris-HCl, pH 8.0. After separation of the two phases, the organic layer was removed and stored in a brown bottle at room temperature and used as neutral phenol-chloroform.

Phenol equilibrated with Tris-HCl was used to isolate

single strand DNA and was prepared by shaking liquid phenol with a half volume of 1 M Tris-HCl, pH 8.0. The mixture was allowed to separate into the two phases overnight and the phenol layer was removed and stored in a brown bottle as phenol equilibrated with Tris-HCl pH, 8.0.

Chloroform-isoamyl alcohol was used to remove traces of phenol in DNA preparations and also to extract protein. It was made by mixing chloroform and isoamyl alcohol in a ratio of 24:1 (v/v).

For assaying PABA synthetase activity in E. coli, cell extracts were resuspended in 0.05 M Tris-HCl buffer, pH 7.8.

TAE buffer (Sambrook et al., 1989) was used for agarose gel electrophoresis. It was prepared as a 50X stock solution containing 2 M Tris-HCl and 0.1 M EDTA; the pH of both solutions was adjusted to 8.0 with glacial acetic acid. At the time of use, the stock solution was diluted to 1X. Stop buffer [50% (w/v) sucrose, 0.1% (w/v) bromophenol blue and 0.1 M EDTA] was used to introduce samples into gel slots as well as stop restriction enzyme activity. The dye assisted loading and allowed the progress of electrophoretsis to be monitored.

TBE buffer (Sambrook et al., 1989) was used for polyacrylamide gel electrophoresis. It was prepared as a 10X stock solution containing 108 g Tris-HCl, 55 g H_3BO_3, 40 mL of 0.5 M EDTA, pH 8.0, and distilled water to 1 L. At the time of use, it was diluted to 1X.

Stock acrylamide solution (30% w/v) was used to prepare

standard gel stock solution for polyacrylamide gel electrophoresis. It was prepared by combining 28.5 g acrylamide, 1.5 g bis-acrylamide and 60 mL of distilled water. The mixture was warmed to 37°C with constant stirring to dissolve the components and then made to 100 mL with distilled water. The solution was filtered through a 0.45 u pore-size membrane and degassed under vacuum. It was stored in a dark brown glass bottle at 4°C.

Standard gel stock solution was prepared by combining 63 g of urea, 15 mL of 10X TBE, 25 mL of 30% acrylamide stock solution and 40 mL of distilled water. The components were stirred until dissolved and then made to 150 mL with distilled water. The solution was filtered through a 0.45 u pore size membrane and degassed under vacuum. It was stored in a dark brown glass bottle at 4°C.

Ammonium persulphate (25% w/v), used for polyacrylamide gels, and was always prepared fresh by dissolving 0.025 g in 100 uL of distilled water.

To transfer DNA from agarose gels to nylon membranes, 20X SSC was used. It contained 3.0 M NaCl and 0.3 M sodium citrate adjusted to pH 7.0 with 1.0 M HCl. Before transfer, DNA was first depurinated by soaking the gel twice for 15-min periods in 0.25 M HCl. The gel was then washed in water to remove excess acid and the DNA was denatured by gently agitating the gel for two 15-min periods in a solution of 0.5 M NaOH and 1.5 M NaCl. The gel was then agitated gently for

two 15-min periods in neutralizing solution containing 0.5 M Tris-HCl pH 7.2, 1.5 M NaCl and 0.001 M EDTA.

For prehybridization and hybridization 20X SSPE was used. It contained 3.6 M NaCl, 0.2 M Na_2HPO_4 and 0.02 M EDTA, pH 7.0. Denhardt's reagent was used in hybridization experiments and was prepared at 100-fold concentration containing 2% (w/v) Ficoll, 2% (w/v) BSA and 2% (w/v) PVP. The reagent was prepared in a sterile container by mixing 6% (w/v) solutions of the above components in equal proportions. It was stored as 10 mL aliquots at -20°C. Prehybridization solution contained 5X Denhardt's reagent (a 1:20 dilution of the 100X stock solution), 0.5% (w/v) SDS, 5X SSPE and 100 ug mL^{-1} of denatured salmon sperm DNA. Hybridization solution was the same as prehybridization solution but supplemented with ^{32}P-labelled DNA.

The Lowry method, used to estimate the concentration of protein in cell extracts, required Biuret's reagent. This was always freshly prepared and contained 1 mL $CuSO_4.5H_2O$ (2%, w/v), 1 mL sodium tartrate (4%, w/v) and 100 mL Na_2CO_3 (4%, w/v).

V. Culture conditions.

A. Glycerol stock suspensions.

1. Lactococcus lactis subsp lactis.

Stock suspensions of L. lactis subsp. lactis were prepared by mixing 0.4 mL of an overnight culture in SM17

medium with 0.1 mL of sterile 100% glycerol. They were stored at -20°C or -70°C.

2. Escherichia coli.

Stock suspensions of E. coli were prepared by mixing 0.4 mL of an overnight culture in L-broth with 0.1 mL of sterile 100% glycerol. They were stored at -20°C or -70°C.

3. Streptomyces spore suspensions.

Spores of a confluently sporulating Streptomyces culture grown in a Petri plate were dislodged by adding 10 mL of sterile distilled water and gently scraping the surface with a sterile toothpick. The resulting suspensions were agitated briefly and then filtered through sterile nonabsorbent cotton wool to remove mycelial fragments and agar. After centrifuging at 5,000 rpm for 10 min, the pelleted spores were resuspended in 20% (v/v) aqueous glycerol and stored at -20°C or -70°C.

B. Vegetative inoculum

Vegetative cultures of L. lactis subsp. lactis were prepared by inoculating SM17 broth with a single colony from an SM17 plate and incubating without shaking at 27°C.

Vegetative cultures of E. coli were prepared by inoculating L-broth with a single colony from L-agar and incubating at 37°C with constant shaking at 220 rpm.

Vegetative cultures of Streptomyces were obtained by inoculating 25 mL of YEME or MYM in a 250-mL Erlenmeyer flask with 100 uL of a stock spore suspension and incubating at 30°C with constant shaking at 220 rpm.

C. Cultures for PABA production.

To test for PABA production in Streptomyces, 100-mL portions of glucose-isoleucine medium in 500-mL Erlenmeyer flasks were inoculated with a 2% (v/v) vegetative culture that had been washed twice with a 10.3% sucrose solution. These were incubated with constant shaking at 220 rpm for 3-5 days at 30°C.

D. Cultures for characterizing pab mutants.

1. Escherichia coli.

Single colonies of the pab mutants were used to inoculate 10 mL of L-broth and incubated at 37°C overnight with constant shaking. The cells were harvested by centrifugation and washed two times with 10 mL of 0.9% saline. Enough inoculum from the wahed cells was added to phosphate buffered medium adjusted to pH 6.0 or 8.0 and supplemented with 1mM filter-sterilized ammonium sulphate to give an initial OD reading of 0.05 at 600 nm.

2. Streptomyces lividans.

Spores of the pab mutants were streaked on SM medium containing ultrapure agarose. Some plates were supplemented

with a nitrogen source (ammonium sulphate and/or asparagine), PABA and/or sulphanilamide.

E. Cultures for genomic DNA isolation.

To isolate genomic DNA from L. lactis subsp. lactis, cultures were prepared by inoculating 25 mL of SM17 broth with a single colony. The culture was incubated at 27°C for 16-18 h.

Cultures of Streptomyces for genomic DNA isolation were prepared by inoculating 25 mL of YEME medium in a 250-mL Erlenmeyer flask with a spore suspension from a confluently sporulating Petri plate. They were incubated at 30°C for 36 h with constant shaking at 220 rpm.

F. Cultures for plasmid DNA isolation.

1. Small scale

Strains of E. coli were patched on L-agar containing 100 ug ml^{-1} ampicillin or 10 ug ml^{-1} tetracycline and incubated at 37°C. In some experiments, 2 mL of L-broth containing 100 ug mL^{-1} of ampicillin or 10 ug ml^{-1} of tetracycline was inoculated with a single colony of the desired strain and grown at 37°C overnight with constant shaking at 220 rpm.

Streptomyces strains were patched on PDAT, K1T or MYMT and incubated at 30°C until substrate mycelium appeared.

2. Large scale

For E. coli strains, 50 mL of L-broth supplemented with 100 ug mL^{-1} ampicillin was inoculated with a single colony and incubated at 37°C for 16-18 h with constant shaking at 220 rpm.

Cultures of Streptomyces were prepared by inoculating 500-mL YEME medium supplemented with 25 ug mL^{-1} thiostrepton in a 2-L Erlenmeyer flask with a 25 mL vegetative inoculum grown in the same medium.

G. Cultures for single strand DNA isolation.

A modification of the procedure described by Karger and Jessee (1990) was used. An isolated single colony was used to inoculate 2 mL of TBG medium supplemented with 100 ug mL^{-1} ampicillin and containing 5 X 10^8 to 1 X 10^9 pfu mL^{-1} of helper phage VCMS13. The culture was incubated at 37°C with constant shaking at 220 rpm for 1.5-2 h. Kanamycin (75 ug mL^{-1}) was added and incubation was continued for 14-20 h. The bacterial cells were pelleted tightly by centrifugation and 1.2 mL of the supernatant was used for single strand DNA isolation.

VI. Assays.

A. Chemical assay for aromatic amines.

Aromatic amines were assayed by a modification of the colorimetric procedure of Levine and Fischbach (1951). To 1 mL of culture filtrate, 0.5 mL of $NaNO_2$ (5%, w/v) and 0.1 mL of concentrated HCl were added. After 5 min, 0.1 mL of urea

solution (50%, w/v) was added. This was allowed to react for 10 min at room temperature and then 2.5 mL of sulfamic acid solution (2%, w/v in 2.2 M disodium hydrogen phosphate) was added. After 5 min, 1 mL of N-1-napthylethylenediamine hydrochloride (0.2%, w/v) was added to form the chromophore. After 15 min at room temperature, the absorbance of the coloured product at 550 nm was measured. Known concentrations of PABA were used as a standard.

B. Extraction of aromatic amines with ethyl acetate.

To the clarified broth from cultures grown in glucose-isoleucine medium, a half volume of ethyl acetate was added and the mixture was shaken. After the two layers had separated, the ethyl acetate was removed and the aqueous solution was extracted twice more with ethyl acetate; the ethyl acetate extracts were pooled and evaporated in vacuo. The residue was dissolved in methanol at 100X the concentration of the combined ethyl acetate extract; it was stored tightly sealed at 4°C.

C. Thin-layer chromatography.

The concentrated extracts and PABA as a reference were applied to 5 X 20-cm thin layers of silica gel F254 on glass plates (E. Merck, Darmstadt). The chromatograms were developed with either benzene-acetic acid-water (42:24:1, v/v/v) or n-butanol-acetic acid water (12:3:5, v/v/v), then

air-dried and viewed under UV-light (254 nm) to identify fluorescence-quenching zones. To confirm the location of aromatic amines (yellow zones), the chromatograms were sprayed with 1% (w/v) acidic p-dimethylaminobenzaldehyde in ethanol-concentrated HCl (9:1).

D. Enzyme assay.

1. Preparation of cell extracts.

Cells were washed twice with 0.9% saline and resuspended in 2 mL of 0.05 M Tris-HCl for each gram (wet weight) of cells. The cells were disintegrated by four 15-sec exposures to ultrasound using a sonicator at maximun intensity. The cell sonicate was centrifuged for 30 min at 30,000 X g to remove debris and the cell extract was stored at -20°C.

2. PABA synthetase assay.

The reaction mixture for PABA synthetase (amidotransferase) activity contained chorismic acid (1 umol), Tris-HCl, pH 8.2 (50 umol), L-glutamine (100 umol) and crude cell extract containing 5 mg of protein, in a final volume of 1 mL. PABA synthetase (aminase) activity was detected by replacing L-glutamine with 100 umol of NH_4Cl.

Reactions were started by adding the crude cell extract. Assay mixtures were incubated at 37°C for 30 min and reactions were stopped by adding 0.4 mL of 10% trichloroacetic acid. The precipitated protein was removed by centrifugation and 1 mL of the supernatant was used in the assay for aromatic

amines to determine the amount of PABA formed. A standard curve was prepared with 1-100 nmol of authentic PABA.

To test for complementation, a crude extract containing 4 mg protein from each of two mutants was mixed and incubated with the substrates as described above.

E. Protein assay.

The Lowry method was used. A sample of the cell extract was made to 500 uL with distilled water; 5 mL of Biuret's reagent was added and mixed in immediately by vortexing. After 5 min at room temperature, 500 uL of Folin's reagent was added with mixing. The chromophore was allowed to develop for 10-20 min and the absorbance at 700 nm was read. The concentration of protein in the cell extract was determined from a standard curve using known concentrations of BSA.

F. Growth stimulation experiment.

To test for growth stimulation of one Streptomyces strain by another, washed spores of the strains were streaked close to and at right angles to each other (but without touching) on SM agar containing 0.25 ug mL-1 of sulphanilamide.

VII. Isolation and processing of DNA.

A. Isolation of plasmid DNA.

The procedure of Kieser et al. (1984), which is based on

alkaline lysis, was used. To screen plasmids from E. coli or Streptomyces, colonies patched on L-agar containing ampicillin (for E. coli) or PDAT, K1T or MYMT (for Streptomyces) were scraped off with a sterile toothpick and resuspended in 500 uL of lysozyme solution in a 1.5-mL microcentrifuge tube. This was incubated on ice (for E. coli) or at 37°C (for Streptomyces) for 30 min and then 250 uL of 2% SDS in 0.3 M NaOH was added. After rapid mixing to complete lysis, the lysate was incubated at 70°C for 15 min (for plasmids smaller than 20 kb) or 55°C for 35 min (for plasmids larger than 20 kb) and then allowed to cool to room temperature. Acid phenol-chloroform (100 uL) was mixed in on a vortex mixer for 1 min. The aqueous and organic phases were then separated by centrifugation for 2 min and the aqueous phase was transferred to a new centrifuge tube. After adding 70 uL of 3 M unbuffered sodium acetate, 700 uL of isopropanol was added to precipitate the DNA. The mixture kept at room temperature for 5 min; then the DNA was pelleted by centrifuging for 5 min. The DNA was resuspended in 100 uL TE buffer, and then 10 uL of unbuffered 3 M sodium acetate and 50 uL of neutral phenol-chloroform were added. The mixture was vortexed for 1 min and the two phases were separated by centrifugation for 2 min. The aqueous phase was removed to a new tube and 100 uL of isopropanol was added to precipitate the DNA. The DNA was pelleted by centrifugation and washed with 1 mL of 70% ethanol. The pellet was allowed to dry and then resuspended

in 20 uL TE buffer.

For restriction analysis, DNA samples were further purified by one neutral phenol-chloroform extraction followed by an extraction with chloroform-isoamyl alcohol. Two volumes of absolute ethanol was used to precipitate the DNA. After pelleting, the DNA was washed in 70% ethanol, dried and resuspended in 20 uL TE buffer.

For isolation of plasmid DNA on a large scale, the procedure for rapid screening of plasmids was scaled up by a factor of 10. The DNA was further purified by two cycles of ultracentrifugation in caesium chloride density gradients (Sambrook et al., 1989).

B. Isolation of genomic DNA.

1. Lactococcus lactis subsp. lactis.

The method of Anderson and McKay (1981) for extracting large plasmid molecules was modified to isolate chromosomal DNA. A single colony was used to inoculate 25 mL of SM17 medium and the culture was grown overnight at 27°C to provide inoculum for 500 mL M17 broth. The culture was grown for 4-6 h and then harvested. The cells were washed twice in a 6.7% sucrose solution containing 50 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0, and then resuspended in 10 mL of the same solution. The cell suspension was warmed to 37°C and 2.5 mL of a 10 mg mL^{-1} solution of freshly prepared lysozyme in 25 mM Tris-HCl, pH 8.0, was added. After incubation at 37°C for 5

min, 1.25 mL of 0.25 M EDTA-50 mM Tris-HCl, pH 8.0, was added. Then 1 mL of a 20% (w/v) SDS solution in 50 mM Tris-HCl, pH 8.0, containing 20 mM EDTA, pH 8.0, was mixed in immediately. Incubation was continued for 5 to 10 min to complete lysis, after which the suspension was thoroughly but gently mixed.

An equal volume of neutral phenol-chloroform (approximately 15 mL) was added and the mixture was shaken until the two phases were mixed thoroughly. After centrifuging at 10,000 rpm for 15 min at room temperature, a clear aqueous layer formed and was transferred with a wide-mouthed pipette to a new tube and then re-extracted with an equal volume of neutral phenol-chloroform. To the aqueous layer from the second extraction, 1.5 mL of 3 M sodium acetate and 30 mL of ethanol were added and mixed in gently but thoroughly. The mixture was kept at -20°C for 15 min to complete the precipitation of DNA. The DNA was spooled on a sterile glass rod, transferred to a new tube and washed twice with 70% ethanol. It was dissolved in 5 mL TE buffer and treated with 100 ug mL^{-1} RNase for 1 h. After this treatment, the DNA was further purified by extracting twice with neutral phenol-chloroform and then once with chloroform-isoamyl alcohol. The DNA was then precipitated with ethanol, spooled on a sterile glass rod and washed with 70% ethanol. When dry, the DNA was dissolved in 2 mL of TE buffer.

2. *Streptomyces species*.

The rapid small scale procedure for genomic DNA isolation (Hopwood et al., 1985) was used to isolate genomic DNA from Streptomyces. Mycelium (50 mg wet weight) was resuspended in 500 uL of lysozyme solution containing 2 mg mL^{-1} lysozyme and 50 ug mL^{-1} RNase and incubated at 37°C until the cells became translucent. At this time, 250 uL of 2% SDS was added and the mixture was vortexed for 1 min; then 250 uL of neutral phenol-chloroform was added and the mixture was vortexed for 30 sec. After centrifugation for 2 min, the aqueous layer was removed and re-extracted with neutral phenol-chloroform; the reextraction was repeated until no interface was seen when the two phases were separated by centrifugation. The aqueous layer was transferred to a new tube and then mixed with 70 uL of 3 M sodium acetate, pH 4.8, and 700 uL of isopropanol to precipitate the DNA. The mixture was left at room temperature for 5 min and the DNA was pelleted by centrifuging for 3 min. The pelleted DNA was washed in 70% ethanol, dried and dissolved in 100 uL of TE buffer.

For large scale isolation of Streptomyces genomic DNA, the process was scaled up ten times but the extractions with neutral phenol-chloroform were done gently; also, after the final neutral phenol-chloroform extraction, the aqueous phase was extracted once with chloroform-isoamyl alcohol before the DNA was precipitated.

D. Caesium chloride gradient ultracentrifugation.

Plasmid DNA extracted from large scale cultures was further purified by caesium chloride gradient ultracentrifugation following the procedure of Maniatis et al. (1982). The plasmid to be purified was dissolved in 8 mL of TE buffer and 8.4 g of caesium chloride was weighed in. After mixing had dissolved the caesium chloride, 0.4 mL of ethidium bromide (10 mg mL^{-1}) was added. The mixture was poured into a 1.5 X 7.5 cm polyallomer tube which was then filled to the top with paraffin oil. The tube was capped and the sample was centrifuged at 100,000 X g in a Beckman model L55B ultracentrifuge using a type 70.1 rotor for 24 h at 20°C.

After centrifugation, the tubes were viewed under uv light to locate the band containing nicked forms of the plasmid as well as chromosomal DNA and the lower band containing covalently-closed circular (ccc) plasmid DNA. The upper band was discarded and the lower band was carefully removed to a new tube. It was resuspended in 7 mL of caesium chloride solution (1.05 g mL-1 of TE buffer) and after 0.4 mL of ethidium bromide had been added, it was recentrifuged to further separate the ccc DNA from chromosomal DNA and nicked forms of the plasmid. The ccc DNA was carefully collected and the ethidium bromide was extracted into an equal volume of water-saturated n-butanol. The aqueous phase was collected and repeatedly extracted with water-saturated n-butanol until all the ethidium bromide had been removed. It was then mixed with two volumes of water, followed by six volumes of ethanol.

After at least 1 h at 20°C to allow precipitatation, the DNA was pelleted by centrifuging at 12,000 rpm for 30 min and then washed once with 70% ethanol and twice with absolute ethanol. The DNA was dried and dissolved in 1 mL TE buffer.

E. Isolation of single-strand DNA templates for sequencing.

The procedure described by Vieira and Messing (1987) was followed. To 1.2 mL of supernatant obtained by centrifugation of phage-infected E. coli culture, 300 uL of 20% PEG 8,000 in 2.5 M NaCl was added and mixed by inversion and gentle vortexing. The mixture was kept at room temperature for 15 min, then centrifuged at 12,000 X g for 4 min at 4°C to pellet the single strand DNA. After the supernatant was removed, the single strand DNA was resuspended in 100 uL TE buffer by vigorous vortexing. An equal volume of phenol equilibrated with 1 M Tris-HCl, pH 8.0, was mixed in by vortexing for 30 s. After 1 min at room temperature vortexing was resumed, for another 30 sec. The mixture was centrifuged and the aqueous phase was removed and extracted with chloroform-isoamyl alcohol. The aqueous phase from this extraction was mixed with two volumes of ethanol to precipitate the single stranded DNA. After 15 min at room temperature, the DNA was pelleted by centrifuging and washed in 70% ethanol. It was pelleted again, dried and resuspended in 20 uL TE buffer.

F. Measurement of DNA concentration.

For rough estimation of double stranded DNA concentration, a sample of the DNA was electrophoresed along with known concentrations of phage lambda DNA as a standard. For accurate estimations, the absorbance of the DNA solution in TE buffer was determined at 260 nm and 280 nm. An absorbance of 1.0 at 260 nm was considered to represent approximately 50 ug mL^{-1} of double stranded DNA (Maniatis et al., 1982). The ratio between the readings at 260 nm and 280 nm provided an estimate of protein or other contaminants in the DNA solution. Pure preparations of DNA give a ratio of 1.8 (Maniatis et al., 1982). A ratio of 1.70 - 1.90 was considered adequate. Otherwise, the DNA was further purified by extracting with neutral phenol-chloroform and chloroform-isoamyl alcohol.

The concentration of single stranded DNA was estimated by electrophoresing with known concentratios of single stranded M13mp18 DNA.

G. Electrophoresis of DNA.

1. Agarose gel electrophoresis.

Agarose gels (0.7%) in 1X TAE buffer were used routinely for electrophoresis. Agarose was dissolved in the buffer by boiling in a microwave oven for 2 to 5 min. The solution was allowed to cool to 50°C and poured into a tray taped at both ends and with a comb fixed at one end. The gel was allowed to set at room temperature and then submerged in TAE buffer in an

electrophoresis tank. Loading buffer (5 uL) was added to DNA samples before they were loaded into the wells created in the gel by the comb. Electrophoresis was carried out at 70 volts for 2 h. To separate fragments that were similar in size, a lower voltage was used for a longer time. For Southern blots, larger gels were used and electrophoresis was carried out at 35 volts for 12 to 16 h.

After electrophoresis the gels were stained in ethidium bromide (1 mg mL^{-1}) for 20 min and then soaked in water for 10 min to remove excess stain. They were then viewed under transillumination at 300 nm.

2. Polyacrylamide gel electrophoresis.

The SEQUI-GEN sequencing apparatus from Bio-Rad was used with polyacrylamide gels for electrophoresis of DNA sequencing reactions.

A gel plug solution was formed by mixing 10 mL of standard gel stock with 50 uL of 25% (w/v) ammonium persulphate and 50 uL of TEMED. The mixture was poured along the entire length of the "sealing strip" on a cushion in the "casting tray" of the apparatus. The "Integral Plate/Chamber" (IPC) assembly was placed on top of the "sealing strip" and the screws of the "casting tray" were tightened. Gentle but firm pressure was applied to the top of the IPC assembly to allow the gel solution to move by capillary action across the full width of the glass plates of the IPC assembly. The gel

between the plates was allowed to set for 5 min to form a plug.

To 40 mL of standard gel stock solution, 40 uL of 25% (w/v) ammonium persulphate and 40 uL of TEMED were added and swirled quickly but gently. Using a 50-mL syringe with a wide bore, this mixture was slowly added to the top of the IPC assembly near one corner, tilting the IPC assembly occasionaly to allow even distribution of the solution. When the gel solution filled the IPC assembly, a comb was quickly inserted between the plates at the top and the gel was allowed to polymerize for at least 3 h before it was used. After polymerization, the "casting tray" was detached and the comb was gently pulled out. The wells created by the comb were immediately washed with 1X TBE to prevent any unpolymerized acrylamide from solidifying in the wells. The IPC assembly containing polymerized gel between the plates was then placed in the "universal base" and the screws of the "universal base" were tightened to fit the IPC assembly. The lower buffer chamber was filled with 400 mL of 1X TBE and the upper chamber (in the IPC) was filled with 1X TBE to about 1/4 inch from the top portion.

The gel was pre-run at 1,900 V until the temperature was near 55°C before samples were loaded into the wells. After loading, the gel was run for about 1.5 h, always ensuring that throughout the period, the temperature of the gel was between 50-60°C. The IPC assembly was then disassembled and the gel,

attached to the outer plate of the IPC assembly, was fixed in a solution of 10% methanol and 10% acetic acid in water for 20 min. The fixing solution was carefully siphoned out and the outer plate with the attached gel was placed glass down on a piece of Whatman 3 MM filter paper. Folds and distortions on the gel were smoothed out with a gloved finger and any excess fixing solution was wiped off with Kimwipes. A piece of Whatman 3 MM filter paper slightly larger than the gel in both dimensions was placed on top of the gel and gentle pressure was applied over the surface of the gel to allow the gel to adhere to the paper. The Whatman 3MM filter paper with the adhering gel was peeled away. A piece of Saran Wrap was laid firmly on top of the gel and any creases and bubbles were smoothed out. The Whatman 3 MM filter paper and Saran Wrap were trimmed to the size of the gel and the gel was dried under vacuum at 80°C for 2 h. After drying, the Saran Wrap was removed and the gel was exposed to Kodak X-Omat film for 24 h at room temperature.

H. Restriction enzyme digestions.

The instructions given by the supplier of the restriction enzyme were followed. Routine restriction enzyme reactions contained 1-2 ug of DNA, 2 uL of the restriction enzyme's reaction buffer (10X), 1-2 units of the restriction enzyme and distilled water to a total volume of 20 uL. For complete digestions, the mixture was incubated overnight at the

required temperature. For partial digestions, the reaction mixture was incubated for 5-60 min at the required temperature. Samples of the digests were analyzed by agarose gel electrophoresis.

When the digested DNA was required for other analyses, it was purified either by using the GENECLEAN procedure (see below) or by extracting with neutral phenol-chloroform and chloroform-isoamyl alcohol. Otherwise, the restriction enzyme digestion was stopped by adding 5 uL of loading buffer and then examined by electrophoresis on an agarose gel.

I. Elution of DNA from agarose gels.

The GENECLEAN procedure for which the reagents are supplied in a kit from BIO/CAN Scientific was used to elute and purify DNA from agarose gels. The DNA fragment of interest was located by illuminating the stained gel with light at 300 nm and the gel segment was carefully excised using a sharp razor blade. The approximate volume of the gel slice was determined and 2.5X volume of SODIUM IODIDE solution was added. The mixture was incubated at 55°C until the gel slice was completely dissolved. After cooling, 5 uL of GLASS MILK (silica matrix) was added and mixed by tapping; the mixture was kept on ice for 5 min with intermittent mixing. The silica matrix with the bound DNA was pelleted by centrifuging for 5 s. After the supernatant had been decanted, the pellet was washed three times with 500 uL of

ice-cold WASHING SOLUTION by mixing and centrifugation. The bound DNA was eluted by suspending the pellet in 10 uL TE buffer at 55°C for 3 min. After centrifuging to pellet the silica matrix, the supernatant containing the DNA was transferred to a new tube. Any remaining bound DNA was recovered by a second elution and the two DNA solutions were pooled. A portion of the pooled sample was electrophoresed on an agarose gel to assess the adequacy of the DNA elution.

J. Ligation of DNA.

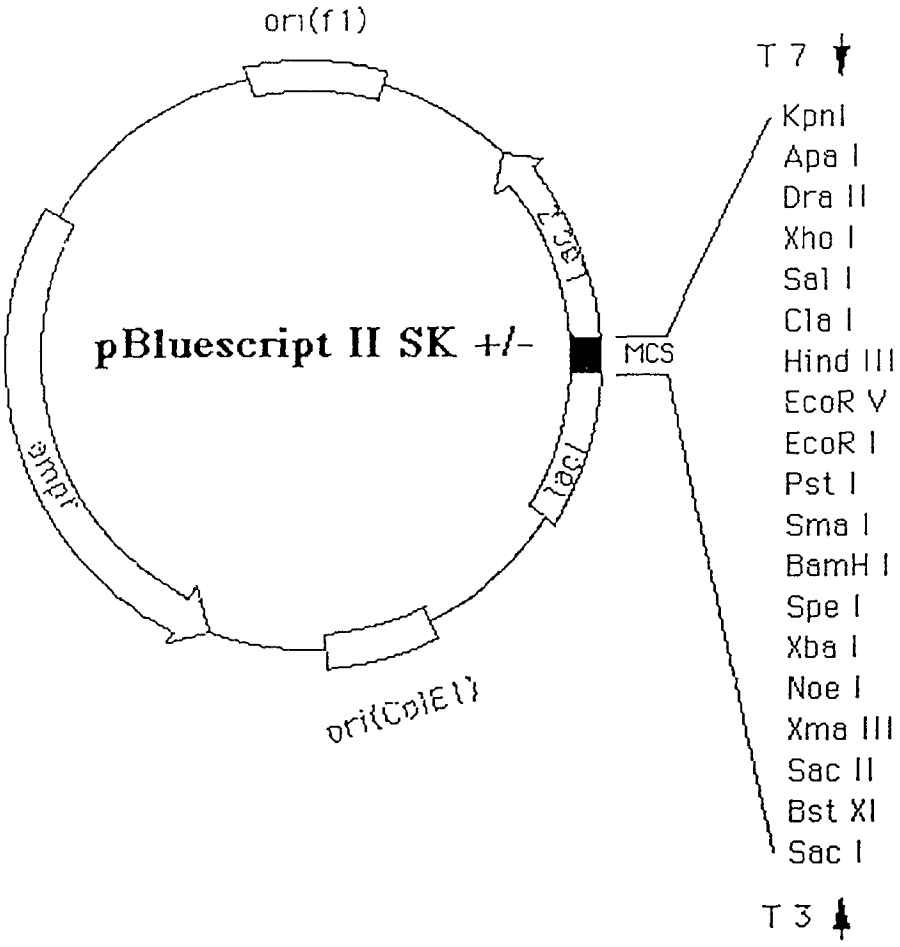
Ligation conditions described by Thompson et al. (1982) were used. For "shotgun" cloning experiments, suitably digested genomic and plasmid DNA samples in TE buffer were mixed in a ratio of 5:1. For subcloning experiments, vector and target DNA

samples were mixed in a ratio of 1:1. The mixtures were incubated at 65°C for 15 min and then placed on ice for 5 min. Ligation buffer and distilled water were added to give a total DNA concentration of 40 ug mL^{-1}. The mixture was incubated with 1 unit of T4-DNA ligase at 16°C for 12-16 h. The DNA was precipitated with ethanol and washed twice with 70% ethanol. It was then dried and resuspended in 10-20 uL of TE buffer. A sample was electrophoresed on an agarose gel to ascertain the results of the ligation.

K. Directed nested deletions of DNA in recombinant plasmids.

DNA fragments to be sequenced were cloned into the phagemid vectors pBLUESCRIPT SK(+) and SK(-) (Fig. 6). To create nested deletions of the DNA fragments, recombinant pBLUESCRIPT plasmids were restricted with two enzymes within the polylinker region to yield a 5'-overhang closer to the DNA fragment to be sequenced and a 3'-overhang closer to the primer regions of the vector. To 2 ug of the doubly restricted recombinant phagemid in 5 uL exonuclease III buffer, 13 uL of distilled water and 2 uL of exonuclease III (20 U uL^{-1}) were added and the mixture was incubated at 37°C. Under these conditions, exonuclease III removes 800 nucleotides per min. At 15-s intervals, 2 uL samples were taken and pooled in a tube containing 6 uL of 5X mung bean nuclease buffer. To the pooled exonuclease III products, 2 uL of distilled water and 2 uL of mung bean nuclease (10 u uL^{-1}) were added and the mixture was incubated for 10 min at 37°C. Tris-HCl (1.0 M, pH 8.0, 1.5 uL) was added and the mung bean nuclease was inactivated by heating at 70°C for 5 min. A portion of the mixture (5 uL) was assessed by agarose gel electrophoresis for coverage of the target DNA fragment range; the linear phagemid plus the DNA fragment and the linear phagemid were used as markers. To 26.5 uL of the remaining mung bean nuclease products, 2.5 uL of 100 mM $MgCl_2$, 5 uL of a mixture of dNTPs (each of concentration 5 mM in Tris-HCl, pH 7.0), 15 uL of distilled water and 2 uL of Klenow fragment (1

Figure 6: Diagram showing the features of the E. coli sequencing vectors pBluescript SK(+) and SK(-). The vector contains a polylinker and the lacZ' gene. Cloning into the polylinker inactivates the lacZ' gene and allows selection of transformants carrying recombinant plasmids by plating on media containing ampicillin, IPTG and X-gal. The recombinant transformants appear as white colonies while colonies with the unaltered vector are blue. The f1 origin of replication allows generation of single-strand templates when cells carrying the vector are infected with a helper phage. The presence of the f1 origin in two orientations (+ or -) allows the generation of single-strand templates that are complementary. In this way both strands of a cloned DNA fragment can be sequenced.


ori(f1)
pBluescript II SK +/-
lacZ
ampr
ori(ColE1)
MCS
T 7
KpnI
Apa I
Dra II
Xho I
Sal I
Cla I
Hind III
EcoR V
EcoR I
Pst I
Sma I
BamH I
Spe I
Xba I
Noe I
Xma III
Sac II
Bst XI
Sac I
T 3

u uL^{-1}) were added. The mixture was incubated at room temperature for 20 min. To 20 uL of this mixture, 5 uL of 10X ligase buffer, 23 uL of distilled water and 2 uL of T4 DNA ligase (1 U uL^{-1}) were added and the mixture was incubated at room temperature overnight. The ligated preparation (20 uL) was used to transform competent cells of E. coli TG1 and transformants were screened for plasmids that differed progressively in size by approximately 200-bp through the entire DNA fragment to be sequenced.

L. DNA sequencing reactions.

Sequenase version 2.0, purchased as a kit from United States Biochemicals, Cleveland, OH, was used for sequencing reactions. The procedure involved annealing a single strand DNA template to a synthetic primer, a labelling reaction and a chain termination reaction.

In the annealing reaction, 1 uL of the synthetic primer (2.5 pmol) was added to 1 ug of the template and 2 uL of annealing reaction buffer in a final volume of 10 uL. The mixture was heated at 65°C for 2 min and allowed to cool on a heating block to 30°C. The annealed template was placed on ice and used within 4 h.

The labelling reaction was started by combining the following on ice :

Annealed template-primer	10.0 uL
DTT (0.1 M)	1.0 uL

Diluted labelling mix	2.0 uL
[α-^{35}S]dATP (1000-1500 uCi mmol^{-1}, 10 uCi uL^{-1})	0.5 uL
Diluted Sequenase	2.0 uL

The labelling mix was diluted 5-fold with distilled water and stored at -20°C. Sequenase was diluted by adding 0.5 uL of the enzyme to 4.0 uL of ice-cold Enzyme Dilution Buffer. The diluted enzyme was stored on ice for no more than 60 min.

The components of the labelling reaction were mixed thoroughly and incubated at room temperature for 3 min. Just before starting the labelling reaction, four microfuge tubes were marked G, A, T and C and received 2.5 uL of the appropriate termination mix. The tubes were prewarmed at 37°C for 1 min.

Chain termination reactions were started by placing the microfuge tubes marked G, A, T and C in a microcentrifuge and adding 3.5 uL of the labelling reaction mixture to the inner wall of each tube. After centrifugation for 5 sec to mix the solutions, the reactions were incubated at 37°C for 5 min. The tubes were placed in the microcentifuge again and 4 uL of stop solution was mixed into each sample as before. The samples were then stored at -20°C for up to 1 week. They were heated at 85°C for 2 min and placed on ice before they were loaded on a sequencing gel.

All sequencing reactions were done in pairs with one set containing dGTP and the other dITP.

M. DNA sequence analysis.

Sequence data were analyzed with the DNA Strider program (Marck, 1988) and the Genetics Computer Group program (University of Wisconsin Biotechnology Center, Madison, Wis.) (Devereux et al., 1984). Amino acid sequences of genes were compared with sequences of related proteins after appropriate alignment using the FASTA program (Pearson and Lipman, 1988).

The nucleotide sequences reported in this study have been submitted to Genbank under accession numbers M64859 (for the S. lividans fragment) and M64860 (for the L. lactis subsp. lactis fragment).

VII. Transformations.

A. Formation and regeneration of Streptomyces protoplasts.

The procedure described by Hopwood et al. (1985) was used to form and regenerate protoplasts of S. lividans and S. griseofuscus. YEME medium (25 mL) supplemented with 0.05 mL of 2.5 M $MgCl_2$ was inoculated with 100 uL of a concentrated spore preparation of S. lividans or S. griseofuscus and incubated at 30°C for 36 h with constant shaking. The mycelium was harvested by centrifugation, washed twice with 15 mL of 10.3% sucrose solution and incubated with 4 mL of filter-sterilized L-buffer containing 1 mg mL^{-1} lysozyme. Incubation was carried out for 30-45 min at 30°C with

trituration every 15 min. To monitor protoplast formation, samples were examined with a phase-contrast microscope. When enough protoplasts had been formed, 5 mL of P-buffer was added and the mixture was triturated and filtered through sterile cotton wool. The filtrate was collected into a sterile tube and centrifuged to pellet the protoplasts. The pellet was then resuspended in 10 mL of P-buffer, and the suspension was centrifuged to pellet the protoplasts, which were finally resuspended by tapping the tube several times to disperse them in the drop of buffer left after decantation. To assess the number of protoplasts formed, a sample was removed, diluted and then counted in a haemocytometer with a phase-contrast microscope. A specified number of protoplasts were used immediately for transformation; alternatively, the sample was diluted with P-buffer and aliquoted into portions giving 4 X 10^9 protoplasts and stored at -70°C.

Protoplasts were regenerated by diluting with P-buffer and plating on R5 medium which had been partially dried in a laminar airflow cabinet for 5-6 h. The plates were incubated at 30°C.

B. Transformation of Streptomyces protoplasts.

Streptomyces protoplasts were transformed by the procedure of Thompson et al. (1982) as described by Hopwood et al. (1985). DNA in up to 20 uL of TE buffer was added to a specified number of protoplasts resuspended in a drop of P-

buffer, and immediately diluted by mixing with 0.5 mL of T-buffer. P-buffer (5 mL) was added and mixed in by pipetting. The protoplasts were pelleted by centrifuging and the pellet was resuspended in 1 mL of P-buffer. Portions (0.1 mL) of this suspension were plated on partially dried R5 plates and incubated at 30°C for 12-18 h until regeneration of protoplasts was just visible. When required, regenerated protoplasts were overlaid with 2.5 mL of SNA containing enough thiostrepton to give a final plate concentration of 25 ug mL^{-1}. The transformants were incubated until the regenerated protoplasts appeared as distinct colonies or until the colonies sporulated.

C. Preparation of competent E. coli cells.

The procedure described by Hopwood et al. (1985) was used. A single E. coli colony was used to inoculate 10 mL of L-broth and incubated at 37°C overnight with constant shaking. A portion (0.1 mL) of the culture was used to inoculate 10 mL of L-broth supplemented with 20 mM $MgCl_2$. This was incubated at 37°C for 2.5-3 h with constant shaking at 220 rpm. The cells were cooled on ice for 10 min and kept at 0-4°C from then on. They were pelleted (3,000 rpm for 10 min.) and the pellet was resuspended in the drop of liquid left after decanting; then 10 mL of ice-cold 0.1 M $CaCl_2$ was added. After a further 10 min on ice, the cells were pelleted again (3,000 rpm for 10 min) and resuspended in 1 mL of ice-cold 0.1

M $CaCl_2$. For "shotgun" cloning experiments, the entire 1 mL of competent cells was used. Otherwise, 100 uL portions of the competent cells were used immediately and the remainder were prepared for long-term storage at -70°C by adding 20 uL of sterile 100% glycerol to 80 uL aliquots.

D. *Transformation of competent E. coli cells*.

DNA in up to 20 uL of TE buffer was added to competent cells and the mixture was kept on ice for 20 min. The cells were then "heat-shocked" for 2 min at 42°C and immediately placed on ice. For "shotgun" cloning experiments, the heat-shocked cells were diluted with 4 mL of L-broth. Otherwise, 0.9 mL of L-broth was added. The cells were then incubated at 37°C for 90 min to allow expression of antibiotic resistance genes and then spread on L-agar plates containing appropriate supplements. The plates were incubated overnight at 37°C.

VIII. Genomic libraries.

A. Construction of an L. lactis subsp. lactis NCDO496 genomic library in pIJ41.

Genomic DNA from L. lactis subsp. lactis and plasmid pIJ41 were separately digested with the endonuclease BclI. The genomic digest (10 ug DNA) was mixed with 2 ug of the digested plasmid DNA and the mixed DNA was precipitated with ethanol. After centrifugation, the pelleted DNA was washed

with 70% ethanol and resuspended in 358 uL of distilled water. Ligation was brought about by adding 40 uL of 10X ligation buffer and 2 units of T4-DNA ligase. The ligation reaction was allowed to proceed at 16°C overnight and the DNA, precipitated with ethanol, was pelleted by centrifugation and washed with 70% ethanol. It was redissolved in 20 uL of TE buffer and used to transform protoplasts of S. lividans JG10. Thiostrepton-resistant colonies obtained in this transformation were screened for resistance to sulphanilamide.

B. Construction of an S. lividans genomic library in pBR322.

Genomic DNA from S. lividans was digested with the endonuclease BamHI for 1 h. Plasmid pBR322 was digested completely with the same enzyme. The partial genomic digest (10 ug) and the complete digest of the plasmid DNA (2 ug) were mixed, prepared for ligation and ligated as described in the preceeding section (VIII.A).

The ligation preparation, in 20 uL TE buffer, was used to transform competent cells of E. coli AB3295. The transformants were spread on L-agar containing 100 ug mL^{-1} ampicillin and incubated at 37°C overnight. Ampicillin-resistant colonies were replicated on L-agar containing 10 ug mL^{-1} tetracycline and, to identify Pab^{+} clones, on M9 agar containing appropriate supplements but lacking PABA.

IX. Southern hybridization.

A. Transfer of DNA from agarose gels to nylon membranes.

After the gel had been stained and observed under uv illumination, it was trimmed and photographed. It was then soaked in 0.25 M HCl for two 15-min periods to depurinate the DNA and thereby facilitate transfer of high molecular weight molecules. The gel was rinsed with water to remove excess acid and treated for two 15 min periods with denaturing solution under gentle but constant shaking. After this, the gel was treated with neutralizing solution for two 15-min periods. A nylon membrane (Hybond-N, Amersham Canada Ltd., Oakville, Ont.) and 3MM Whatman filter papers were cut with dimensions 1 cm longer than the gel on each side. These were pre-wet in distilled water.

A Vacublot apparatus (American Bionetics, Hayward, CA.) was used to transfer DNA from gels to nylon membranes. The neutralized gel was placed on top of the nylon filter resting on a sheet of 3MM Whatman filter paper on the Vacublot setup. The chamber of the setup containing the gel and the membrane was filled with 10X SSC and a vacuum was applied for 45 min to transfer the DNA from the gel to the membrane. Transfer of the DNA was assessed by restaining the gel and viewing under uv illumination. After transfer, the nylon membrane was allowed to dry, placed between two sheets of Whatman 3MM filter paper, and baked in a vacuum oven at 80°C for 2 h to

immobilize the DNA.

B. Labelling of DNA probes.

A Random Primers DNA labelling kit (GIBCO BRL, Burlington, Ontario) was used. The reaction was started by combining the following on ice : 2 uL each of dATP, dGTP and dTTP (0.5 mM in 3 mM Tris-HCl, pH 7.0, 0.2 mM EDTA), 15 uL of Random Primers Buffer mixture, 5 uL (approximately 50 uCi) of [α-^{32}P]dCTP (3,000 Ci mmol-1), 1 uL of Klenow fragment (3 U uL^{-1}) and 30 ng of DNA in 5-20 uL of TE buffer. The reaction mixture was made up to 50 uL with distilled water and incubated at room temperature for 2 h. Stop buffer (5 uL of 0.2 M EDTA, pH 7.5) was added to stop the reaction.

The extent of incorporation was determined by diluting a 2-uL sample of the mixture with 489 uL of distilled water and placing 5 uL of the diluted solution on a glass fibre filter disk (Whatman GF/C). The filter was washed three times with ice-cold trichloroacetic acid (TCA, 10% w/v containing 1% (w/v) sodium pyrophosphate) and then once with 95% ethanol (50 mL). Radioactivity on the filter was measured with a liquid scintillation spectrometer and multiplied by 2750 to assess the total incoporation of ^{32}P into the probe.

When required, labelled DNA was separated from unincorporated nucleotides by chromatographing the reaction mixture on a column of Sephadex G-50 equilibrated with 10 mM Tris-HCl, pH 8.0, 50 mM NaCl and 0.1 mM EDTA, pH 8.0.

C. Probing.

The membrane with the immobilized DNA was prehybridized for 1-2 h at 65°C in a Hybaid hybridization oven (BIO/CAN Scientific, Mississauga, Ont.). The prehybridization solution contained 5X SSPE, 5X Denhardt's solution, 0.5% (w/v) SDS and sheared herring sperm DNA (100 ug ml^{-1}) which was denatured by boiling for 5 min and kept on ice. Following prehybridization, the labelled DNA probe (10^8 cpm ug^{-1} DNA) was added and incubation at 65°C was continued overnight.

After hybridization, the membranes were washed in solutions containing SSPE and SDS. For moderate stringency washings, the membranes were washed for two 15-min periods with 5X SSPE containing 0.1% SDS and then two times with 2X SSPE containing 0.1% SDS at 65°C with constant shaking. For high stringency washings, 0.1X SSPE containing 0.1% SDS was used for two additional treatments. The membranes were wrapped in Saran Wrap after washing.

D. Autoradiography.

The washed membranes were secured in place with Scotch tape in Radelin T-2 lead blocker screens and exposed to X-ray film (Kodak X-Omat) for 1-3 days. After exposure, the X-ray films were treated in the dark with Kodak liquid X-ray developer for 5 min with shaking, agitated in Kodak stop bath for 1 min and then immersed in Kodak rapid fixer for 5 min. Finally, the films were washed in running water for 15 min.

RESULTS

Streptomyces pab mutants on minimal medium lacking PABA grow after 72 h (Atkinson, 1987; Aidoo, 1989). To facilitate selection for pab mutations, sulphanilamide can be included in the medium to antagonize PABA utilization. Under such conditions, Streptomyces pab mutants are unable to grow for up to one week (Aidoo, 1989).

I. Selection of a streptomycete host for cloning a PABA synthetase gene from L. lactis subsp. lactis.

To select a suitable cloning host, several streptomycetes were tested for their ability to grow on minimal medium containing sulphanilamide. Whereas most were resistant to concentrations up to 15 ug mL^{-1} (Table 2), the PABA-requiring auxotrophic mutant S. lividans JG10 was extremely sensitive and was unable to grow on minimal medium containing 0.25 ug ml^{-1} of sulphanilamide. Therefore, it was chosen as the cloning host.

II. Resistance of L. lactis subsp. lactis strains to sulphanilamide

Lactococcus lactis subsp. lactis strains NCDO496 and AV117 grown overnight in SM17 broth were tested for their ability to grow when streaked on SM17 agar containing various concentrations of sulphanilamide. Both strains were able to

Table 2. Minimun inhibitory concentrations of sulphanilamide for various streptomycetes.*

Strain	Sulphanilamide concentration (ug mL^{-1})							
	0	0.25	0.5	1	2	5	15	25
S. lividans TK24	+	+	+	+	+	-	-	-
S. lividans TK23	+	+	+	+	+	-	-	-
S. lividans JG10	+	-	-	-	-	-	-	-
S. venezuelae 10712	+	+	+	+	+	+	+	-
S. venezuelae 13S	+	+	+	+	+	+	+	-
S. griseofuscus C581	+	+	+	+	+	+	+	-

* Washed spores of the various *Streptomyces* strains were streaked on minimal medium and minimal medium containing appropriate amounts of sulphanilamide. The cultures were incubated at 30°C for 48h and scored for growth (+) or no growth (-).

grow at up to 150 ug mL^{-1} of sulphanilamide but were inhibited at 200 ug mL^{-1}.

III. Molecular cloning and characterization of the PABA synthetase gene from L. lactis subsp. lactis

1. Shotgun cloning with S. lividans JG10.

The strategy used was based on the evidence that the pab mutant S. lividans JG10 was particularly sensitive to sulphanilamide and on the report (Gil and Hopwood, 1983) that overproduction of PABA expressed from a cloned PABA synthetase gene could overcome the sensitivity.

Of several restriction enzymes used to digest genomic DNA of L. lactis subsp. lactis NCDO496, BclI gave a high proportion of fragments in the 5-25 kb range and so was chosen for the cloning experiment. Plasmid pIJ41, which contains the tsr gene from S. azureus and the aph gene from S. fradiae as well as a unique BclI site was used as a vector.

Lactococcus lactis subsp. lactis NCDO496 genomic DNA was completely digested with BclI and the fragments were ligated into the BclI site of pIJ41. The ligation mixture was used to transform approximately 2 x 10^9 protoplasts of S. lividans JG10; approximately 4 x 10^7 protoplasts from the transformation mixture were spread on each of 50 partially-dried R5 agar plates. After 18 h incubation at 30°C to allow expression of the tsr gene, the plates were overlayed with SNA containing enough thiostrepton to give a final plate

concentration of 25 ug mL^{-1}. About 15,000 thiostrepton-resistant colonies were obtained. The colonies were incubated until they had sporulated and were then replicated on minimal medium and on minimal medium containing 0.25 ug mL^{-1} of sulphanilamide.

Two transformants, FA1 and KB10, were able to grow on the medium containing sulphanilamide. Plasmid DNA was extracted from them by the rapid alkaline lysis procedure. Comparison of the mobilities during agarose gel electrophoresis of these plasmids and pIJ41 (Fig. 7) showed that the plasmid in strain FA1 was bigger than pIJ41 whereas the plasmid in strain KB10 was similar in size to pIJ41. Strain KB10 was suspected to be a spontaneous resistant variant (possibly a revertant) of the host generated during protoplasting and subsequently transformed with pIJ41. It was not examined further. The plasmid from strain FA1 proved to be a recombinant form with an insert in the BclI site of pIJ41 (see below) and was designated pDQ250.

2. Transformation of S. lividans strains JG10 and AP3 with pDQ250.

Plasmid pDQ250 was used to transform protoplasts of S. lividans strains JG10 and AP3 (a pab mutant derived from S. lividans TK24 by NTG mutagenesis). A similar number of protoplasts of both strains was also transformed with pIJ41 to serve as a control. Each transformation yielded about 1,500

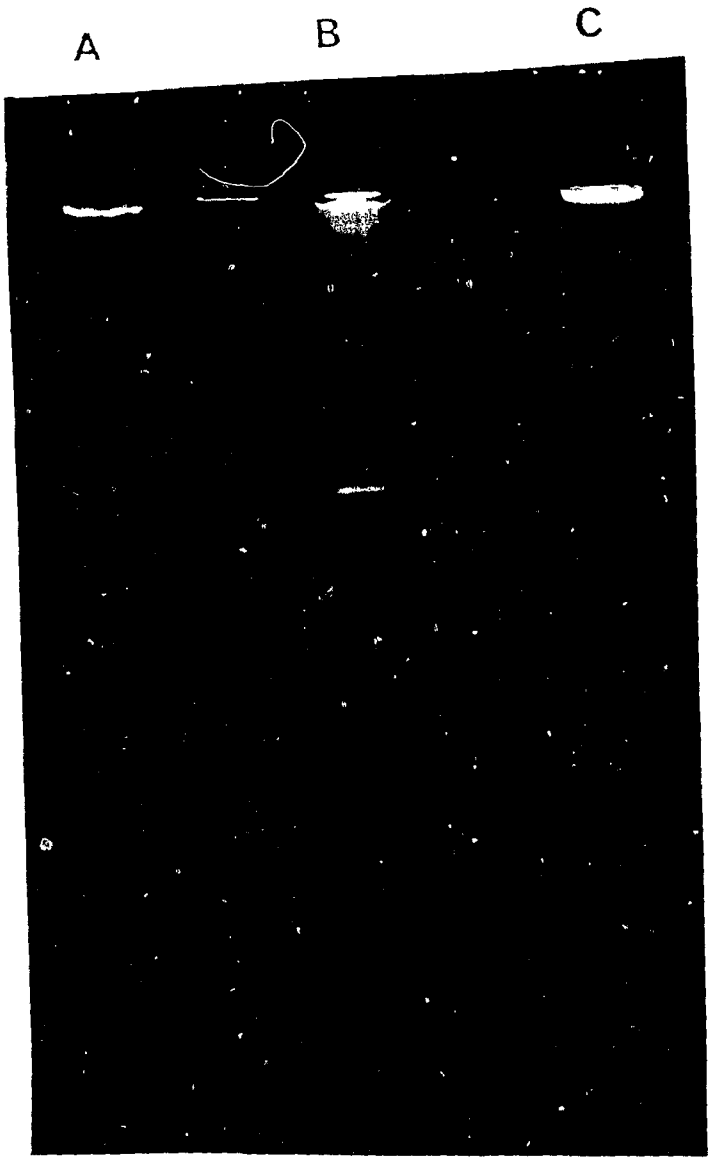


Figure 7: Agarose gel electrophoresis of (A) pIJ41, and of ccc DNA extracted from (B) S lividans FA1; and (C) S. lividans KB10

thiostrepton-resistant colonies. The transformants were replicated on minimal agar medium and on minimal agar medium supplemented with 0.25 ug mL^{-1} of sulphanilamide. With both strains JG10 and AP3, transformants containing pDQ250, but not those containing pIJ41, grew on minimal medium supplemented with sulphanilamide. From this it was possible that the insert in pDQ250 contained information complementing the pab mutations in S. lividans strains JG10 and AP3, by causing overproduction of PABA thereby relieving the sensitivity to sulphanilamide.

3. Characterization of pDQ250.

Both pIJ41 and pDQ250 were digested with BclI and the digests were examined by agarose gel electrophoresis. It was expected that the recombinant plasmid pDQ250 would be restricted by BclI to generate at least two fragments, one of which would have the same mobility as pIJ41 restricted with BclI. Although two fragments were obtained, neither had the same mobility as BclI-restricted pIJ41 (Fig. 8). The sizes of the two fragments generated from pDQ250 were estimated to be 18.3 and 13.6-kb by comparing them with size markers of lambda DNA restricted with HindIII and PstI. The size of pIJ41 was, as expected, 14.8-kb. Elution and purification of the 13.6-kb pDQ250 fragment from agarose gels followed by restriction analysis showed that although some pIJ41 sequences were present, a region of about 1.2-kb encompassing part of the aph

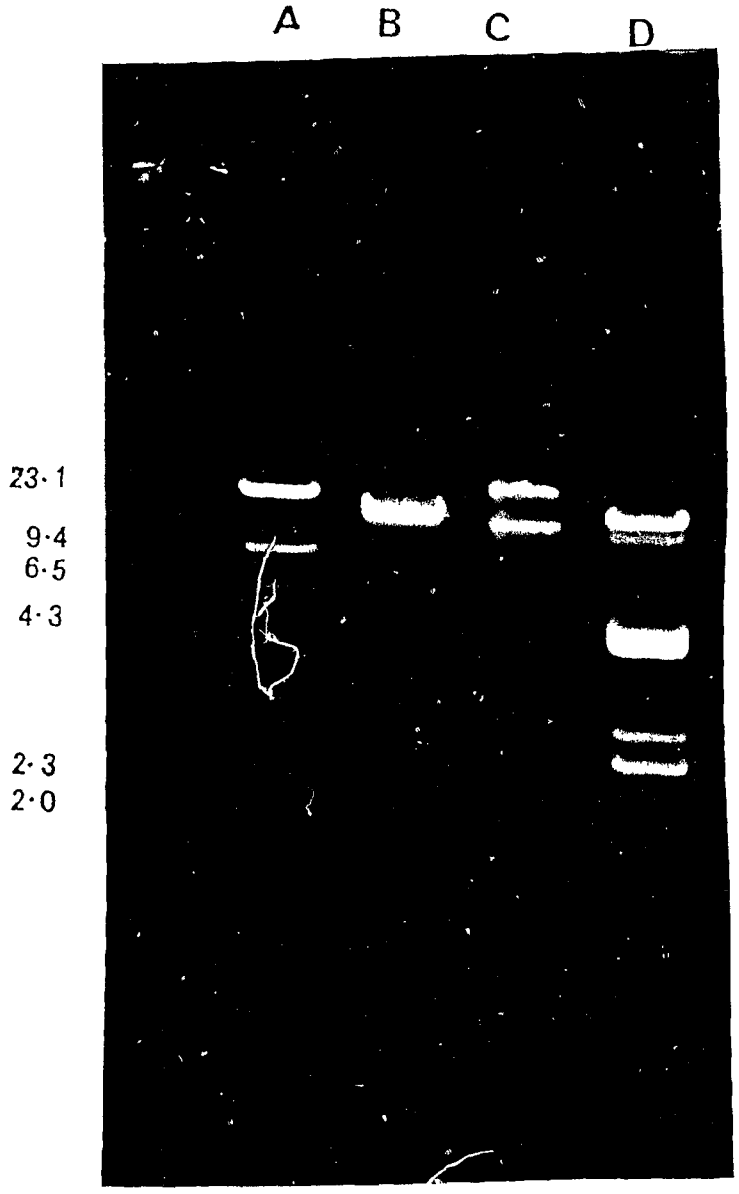


Figure 8: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; of BclI-digests of (B) pIJ41 and (C) pDQ250; and of (D) lambda DNA digested with PstI.

gene had been deleted from the vector (see Appendix). The 13.6-kb fragment lacked the XbaI site within the aph gene and also the SphI site which is about 100-bp from the carboxy-terminus of the aph gene. Consistent with deletion from pIJ41 of a 1.2-kb segment that included part of the aph gene, S. lividans FA1 was unable to grow on minimal medium supplemented with 20 ug mL^{-1} of neomycin.

4. Subcloning of the 18.3-kb fragment in pIJ41 and pIJ702.

The entire 18.3-kb fragment, or subfragments excised with BamHI, BglII or double digests involving BamHI, BclI and BglII were ligated into suitable sites on pIJ41 and pIJ702. Each derivative plasmid was introduced into protoplasts of S. lividans strains JG10 and AP3. A similar number of protoplasts of the two strains were also transformed with pIJ41 and pIJ702 to serve as controls. Thiostrepton-resistant colonies obtained from each transformation were allowed to sporulate and then were replicated on minimal medium and on minimal medium supplemented with 0.25 ug mL^{-1} of sulphanilamide. No growth of either test or control replicas was observed with sulphanilamide present. Apparently, the insert DNA alone was unable to confer the Pab^+ phenotype in S. lividans, and required vector sequences for expression.

IV. Expression of the L. lactis subsp. lactis NCDO496 PABA synthetase gene in E. coli.

1. Subcloning in pBR322.

To ascertain whether the DNA fragment from L. lactis subsp. lactis that complemented the pab mutation(s) in S. lividans could be expressed in E. coli, a 6.0-kb BglII-BglII restriction fragment internal to the 18.3-kb insert of pDQ250 was eluted from an agarose gel and ligated into the BamHI site of pBR322. Plasmid pBR322 has resistance markers for ampicillin and tetracycline. Insertion of DNA fragments into the BamHI site inactivates the tetracycline resistance gene. The ligation mixture was introduced into competent cells of E. coli strains AB3292 and AB3295 which have lesions in the pabA and pabB genes respectively.

Of approximately 500 ampicillin-resistant colonies obtained from the transformation of strain AB3295, 25 were sensitive to tetracycline indicating that these possessed recombinant plasmids. Of the 25, eight were able to grow on appropriately supplemented minimal medium lacking PABA. Plasmid DNA extracted from these colonies was digested with several restriction enzymes and the digests were examined by agarose gel electrophoresis. Two internal BamHI sites 0.7-kb apart within the 6.0-kb BglII-BglII fragment were used to orient the insert in the vector after double digestions involving BamHI. The results indicated that in all eight clones, the 6-kb fragment had been inserted into the BamHI

site of pBR322 in the same orientation with respect to the vector (Figs. 9 and 10; Table 3). The recombinant plasmid was designated pDQ251 and AB3295 strains carrying this plasmid were designated E. coli ADA1.

Restriction endonuclease analysis of plasmid DNA extracted from the seventeen ampicillin-resistant, tetracycline-sensitive colonies that were unable to grow on appropriately supplemented minimal medium lacking PABA showed that the 6.0-kb fragment was present in the BamHI site in an orientation opposite to that of pDQ251 (Figs. 9 and 11; Table 3). Plasmid DNA contained in these strains was designated pDQ252.

Although plasmids identical to pDQ251 and pDQ252 were isolated from ampicillin-resistant, tetracycline-sensitive colonies obtained in the transformation of E. coli AB3292, there was no complementation of the pabA mutation in this host.

The above observations indicated that the 6.0-kb fragment contained genetic information that complemented the pabB but not the pabA mutation in E. coli. Because the pabB mutation was complemented only when the 6.0-kb fragment was in one orientation within the vector, the gene was probably expressed not from its own promoter but from a vector promoter.

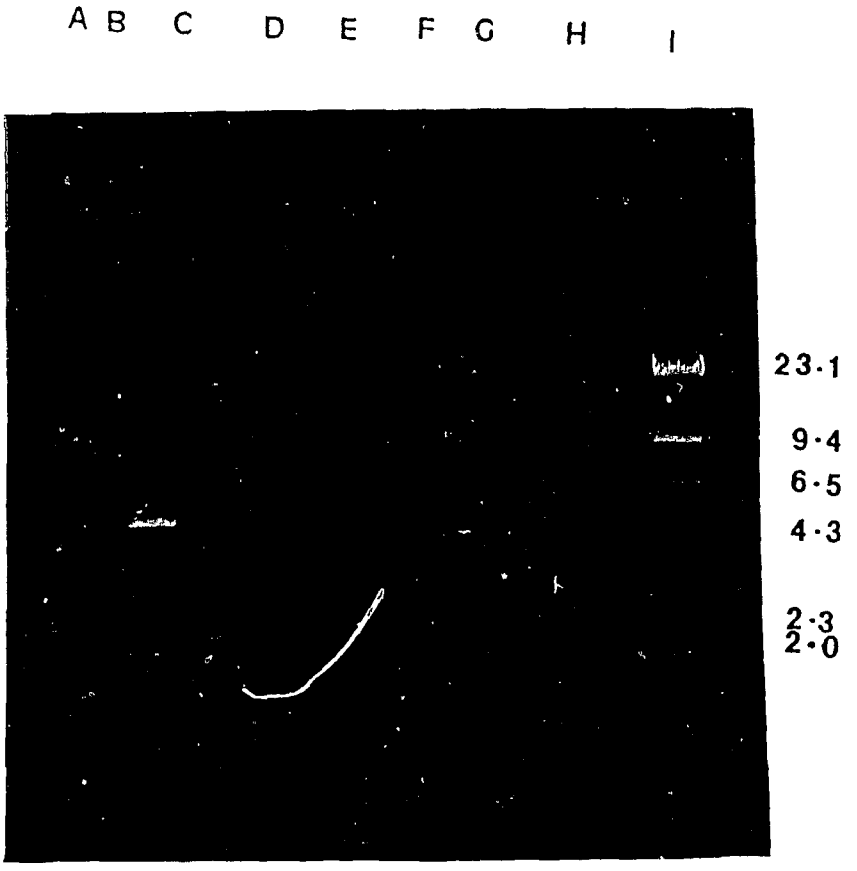


Figure 9: Agarose gel electrophoresis of pDQ252 digested with (A) BamHI and PstI, (B) BamHI and EcoRI, (C) BamHI, (D) EcoRI; of pDQ251 digested with (E) BamHI and PstI, (F) BamHI and EcoRI, (G) BamHI, (H) EcoRI; and of (I) lambda DNA digested with HindIII.

Table 3. Fragments generated by digesting pDQ251 and pDQ252 with restriction enzymes.

Restriction enzyme(s)	Size of fragments (kb) pDQ251	pDQ252
EcoRI	10.3	10.3
BamHI	9.6	9.6
	0.7	0.7
BamHI + EcoRI	7.3	5.2
	2.3	4.4
	0.7	0.7
BamHI + PstI	8.0	5.1
	1.6	4.5
	0.7	0.7

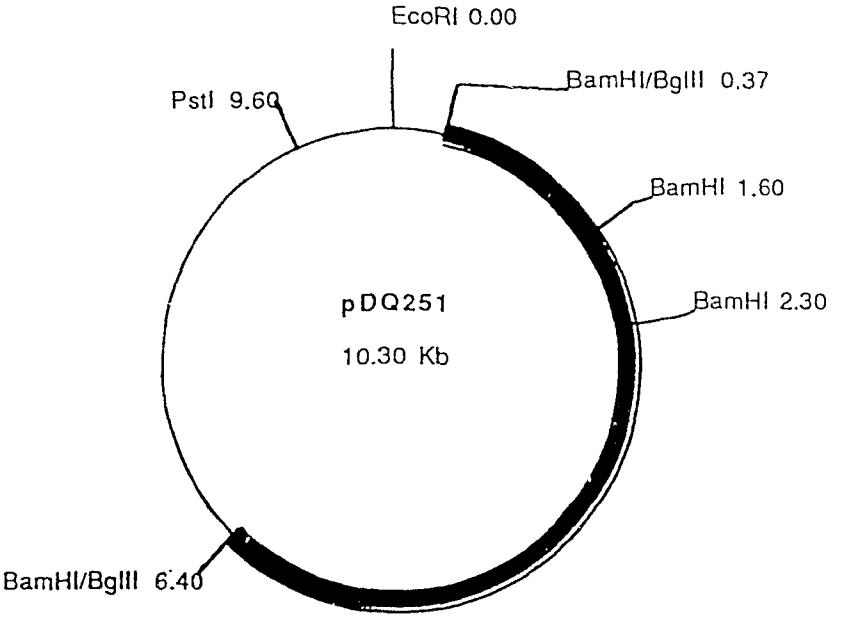


Figure 10: Circular restriction map of pDQ251. The thin line represents sequences from pBR322. The thick line represents sequences cloned from L. lactis subsp. lactis. The numbers indicate the map positions (in kb) of restriction enzyme sites relative to the EcoRI site (0.0).

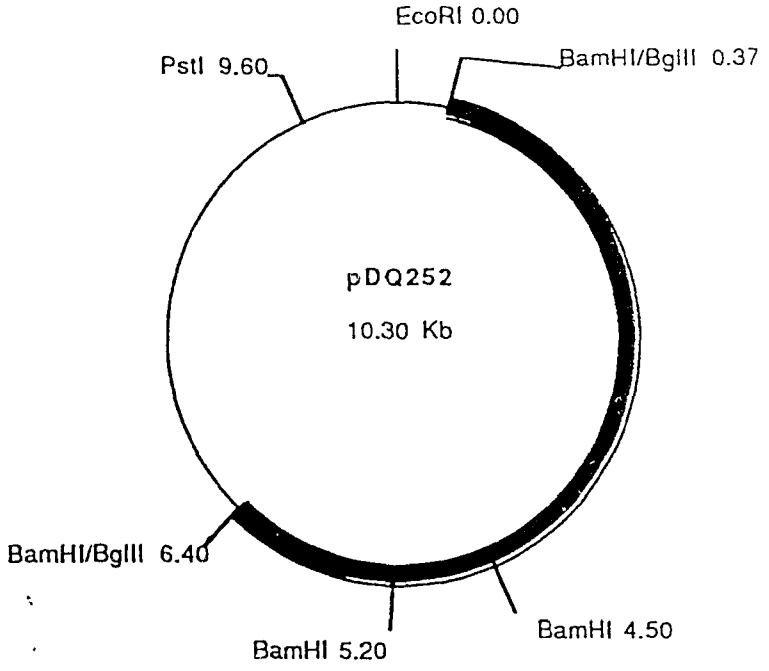


Figure 11: Circular restriction map of pDQ252. The thin line represents sequences from pBR322. The thick line represents sequences cloned from L. lactis subsp. lactis. The numbers indicate the map positions (in kb) of restriction enzyme sites relative to the EcoRI site (0.0).

2. Localization of the L.lactis subsp. lactis PABA synthetase gene within the cloned fragment.

To determine the extent of the DNA region coding for pabB, the 6.0-kb BglII-BglII fragment was partially digested with BamHI and ligated with BamHI-linearized pBR322. The ligation mixture was used to transform competent cells of E. coli AB3295.

Of approximately 400 ampicillin-resistant colonies obtained, 20 were sensitive to tetracycline indicating that foreign DNA was present in the BamHI site of the vector. Only one colony (strain ADA5) was able to grow on appropriately supplemented minimal medium lacking PABA. Restriction endonuclease analysis of the plasmid (designated pDQ253) extracted from strain ADA5 showed that a 1.9-kb fragment had been inserted into the BamHI site of pBR322 and that there was an internal BamHI site within this 1.9-kb fragment (Fig. 12; Table 4). It was concluded that the pabB gene of L. lactis subsp. lactis was present on the 1.9-kb BglII-BamHI fragment (Fig. 13).

V. Expression of the L.lactis subsp. lactis PABA synthetase gene using pDQ254.

To establish that the 13.6-kb fragment obtained from pDQ250 contained sequences from pIJ41 that allowed it to act as an expression vector, this BclI-derived fragment as well as the 6.0-kb BglII-BglII fragment derived from pDQ250 were

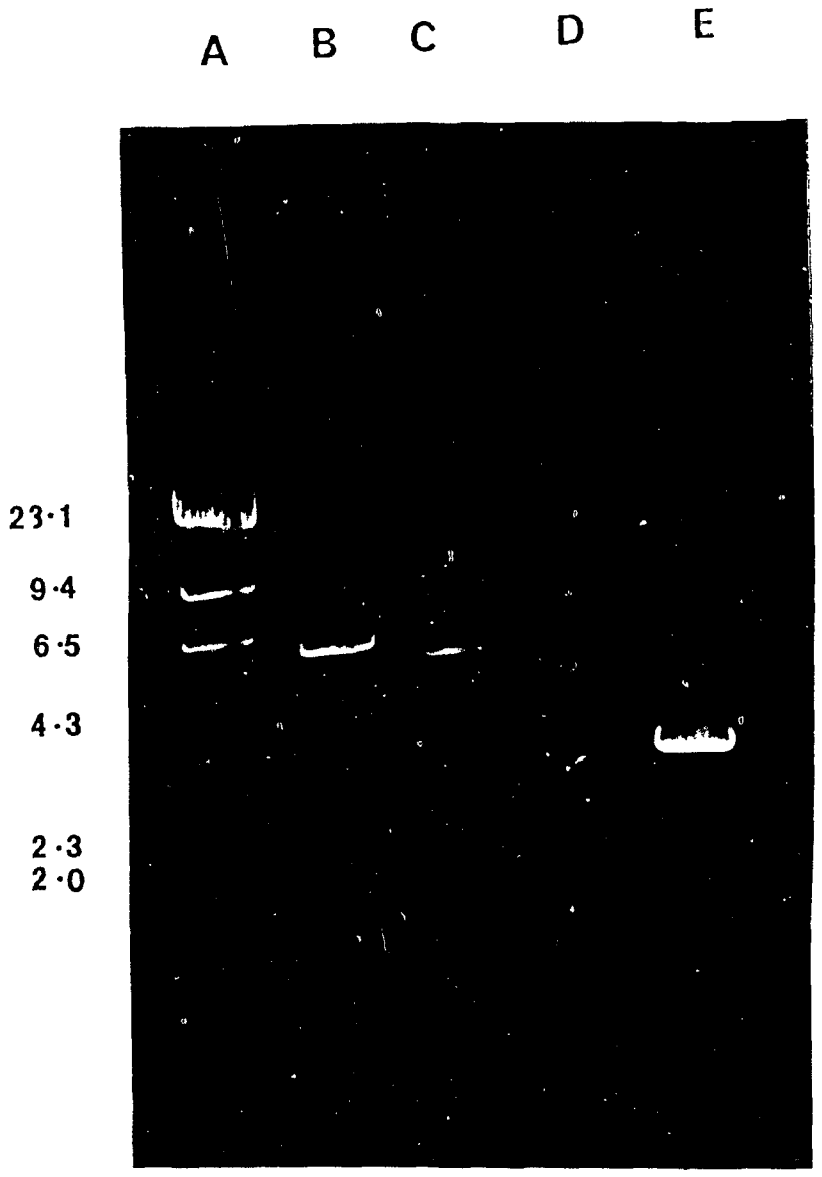


Figure 12: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; and of pDQ253 digested with (B) EcoRI, (C) BamHI, (D) BamHI and EcoRI and (E) BamHI and PstI.

Table 4. Fragments generated by digesting pDQ253 with restriction enzymes.

Restriction enzyme(s)	Size of fragments (kb)
EcoRI	6.2
BamHI	5.5
	0.7
BamHI + EcoRI	3.9
	1.6
	0.7
BamHI + PstI	3.2
	2.3
	0.7

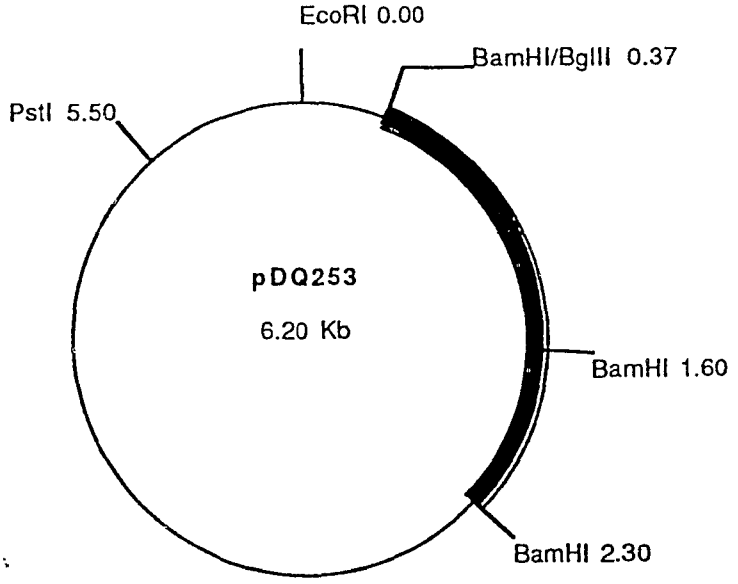


Figure 13: Circular restriction map of pDQ253. The thin line represents sequences from pBR322. the thick line represents sequences cloned from L. lactis subsp. lactis. The numbers indicate the map positions (in kb) of restriction enzyme sites relative to the EcoRI site.

eluted from agarose gels and ligated together (BclI and BglII generate the same protuding ends and so can be ligated together) to obtain a plasmid in which the PABA synthetase gene was aligned in the correct reading frame with a vector promoter. The ligation mixture was used to transform protoplasts of S. lividans strains JG10 and AP3. Thiostrepton-resistant colonies obtained from each transformation were allowed to sporulate and then replicated on minimal medium and on minimal medium supplemented with 0.25 ug mL^{-1} of sulphanilamide.

Fifteen colonies from the transformation of strain JG10 and twelve colonies from the transformation of strain AP3 were able to grow on minimal medium supplemented with sulphanilamide. Restriction endonuclease analysis of the plasmid DNA isolated from these colonies showed that in each of them the 6.0-kb fragment was linked to the BclI site of the 13.6-kb fragment in the same relative orientation (Figs. 14 and 15; Table 5). Plasmid DNA isolated from these colonies was designated pDQ255.

Restriction endonuclease analysis of plasmid DNA from thiostrepton-resistant, sulphanilamide-sensitive strains indicated that some of the plasmids (designated pDQ254) consisted of the 13.6-kb fragment circularized by ligation of the BclI ends and in others (designated pDQ256) the 6.0-kb fragment had ligated to the BclI site of the 13.6 kb fragment in an orientation opposite to that of pDQ255 (Figs. 14 and 16;

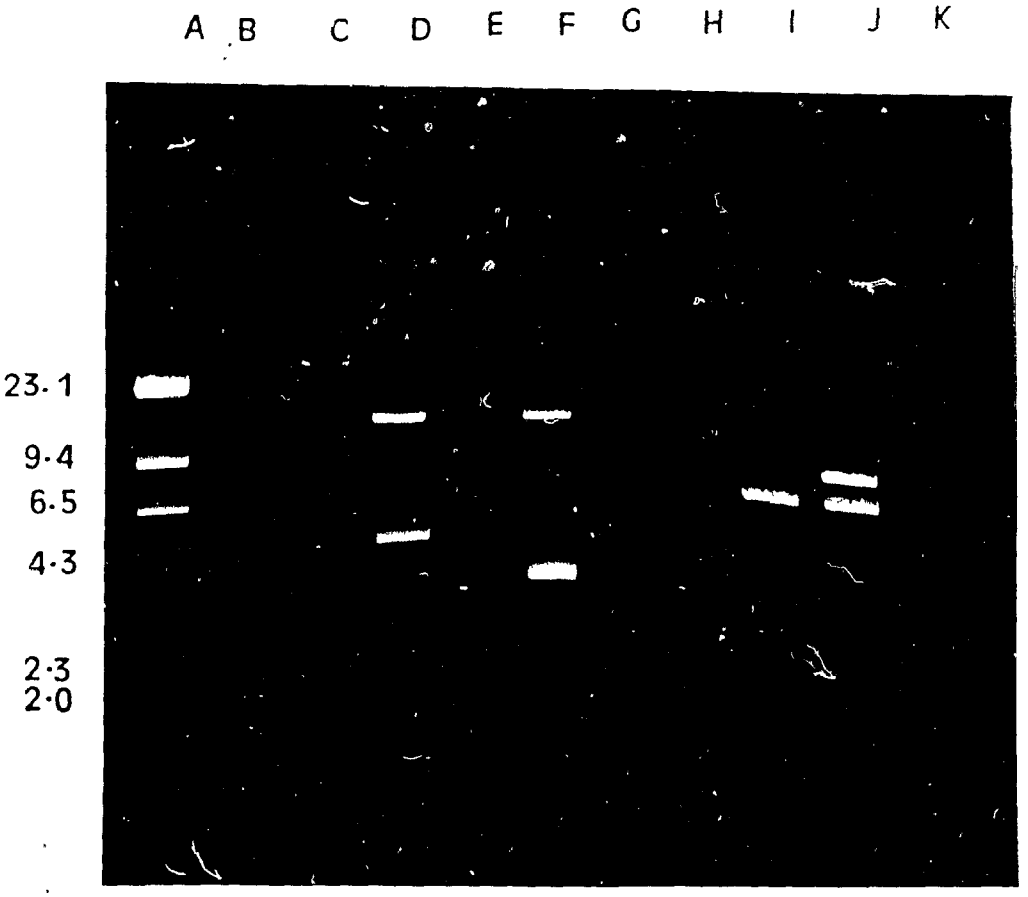


Figure 14: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; and of pDQ255 digested with (B) EcoRI, (C) BglII, (D) BamHI, (E) BamHI and BglII and (F) BamHI and EcoRI; and of pDQ256 digested with (G) EcoRI, (H) BglII, (I) BamHI, (J) BamHI and BglII, and (K) BamHI and EcoRI.

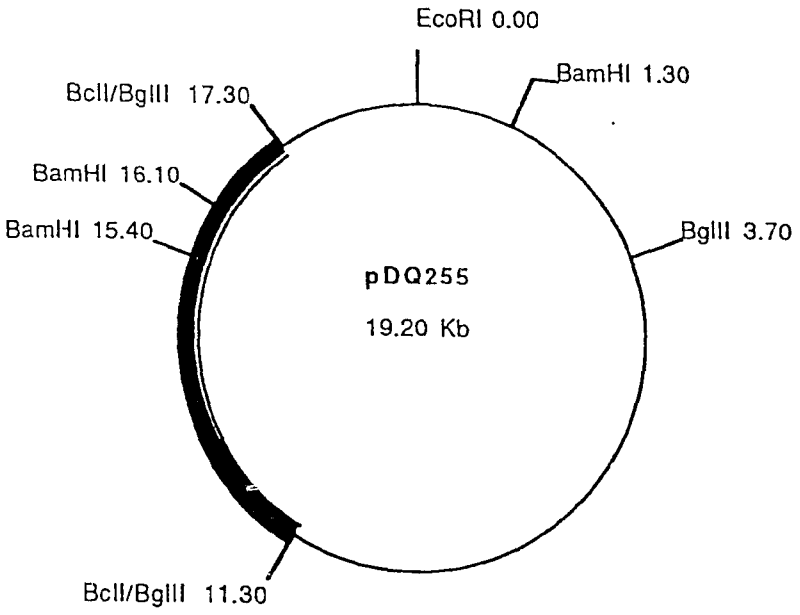


Figure 15: Circular restriction map of pDQ255. The thick line represents sequences cloned from L. lactis subsp. lactis. The thin line represents sequences from pDQ254. The numbers indicate the map positions (in kb) of restriction enzyme sites relative to the EcoRI site (0.0).

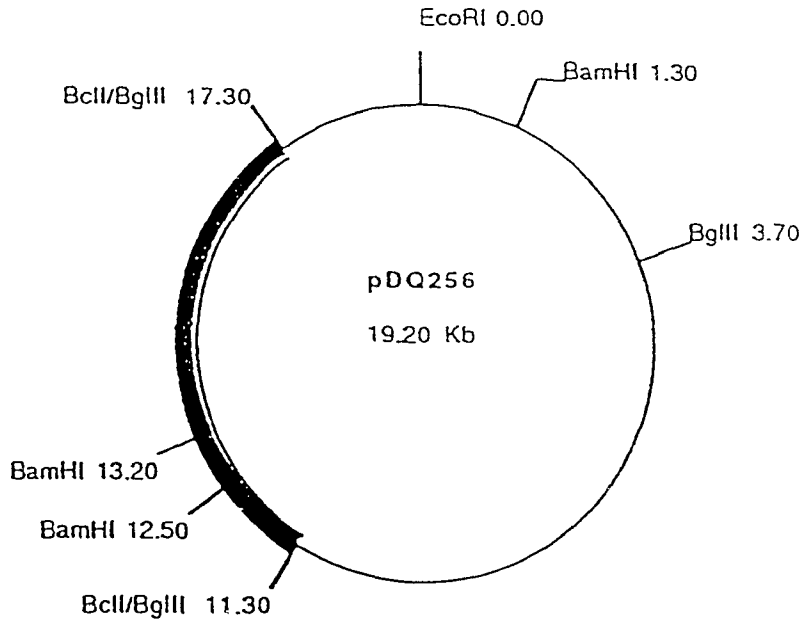


Figure 16: Circular restriction map of pDQ256. The thick line represents sequences cloned from L. lactis subsp. lactis. The thin line represents sequences from pDQ254. The numbers indicate the map positions (in kb) of restriction enzyme sites relative to the EcoRI site (0.0).

Table 5).

Protoplasts of S. lividans strains JG10 and AP3 were retransformed with pDQ255. As a control, protoplasts of both strains were also transformed with pDQ254. After thiostrepton-resistant colonies from each transformation had sporulated, they were replicated on minimal medium and on minimal medium supplemented with 0.25 ug mL^{-1} of sulphanilamide. Only transformants containing pDQ255 were able to grow on medium containing sulphanilamide. From these observations it was concluded pDQ254 was being used as an expression vector and that the 6.0-kb DNA fragment containing the L. lactis subsp. lactis PABA synthetase gene was expressed in S. lividans only when it was present in one orientation. If a L. lactis subsp. lactis promoter is present on this fragment, it is not recognized for transcription initiation by the Streptomyces lividans RNA polymerase.

VI. Growth stimulation of S. lividans pab mutants by S. lividans strains FA1 and FA2.

To test for PABA production by S. lividans strains FA1 (JG10 containing pDQ250) and FA2 (AP3 containing pDQ250), washed spores of the two strains were streaked separately on one side of a minimal medium plate containing 0.25 ug mL-1 of sulphanilamide. Washed spores of strains JG10 and AP3 were streaked at right angles to but without touching the streaks of strains FA1 and FA2. As a control washed spores of S.

Table 5. Fragments generated by digesting pDQ255 and pDQ256 with restriction enzymes.

Restriction enzyme(s)	Size of fragments (kb) pDQ255	pDQ256
EcoRI	19.6	19.6
BamHI	14.1	11.2
	4.8	7.7
	0.7	0.7
BglII	19.6	19.6
BamHI + BglII	11.7	8.8
	4.8	7.7
	2.4	2.4
	0.7	0.7
BamHI + EcoRI	14.1	11.2
	3.5	6.4
	1.3	1.3
	0.7	0.7

lividans TK24 (parent of strains JG10 and AP3) were also streaked on a minimal medium plate containing 0.25 ug mL-1 of sulphanilamide and washed spores of strains JG10 and AP3 were streaked at right angles to them. After 48 h incubation at 30°C, there was heavy growth of strains JG10 and AP3 at sites closest to strains FA1 and FA2 but no growth at distant sites (Fig. 17). There was no growth of strains JG10 and AP3 in the neighbourhood of strain TK24. The results indicated that strains FA1 and FA2, which contain the pab-complementing fragment from L. lactis subsp. lactis secreted a product that satisfied the growth requirements of mutant strains JG10 and AP3.

VII. Production of PABA by S. lividans strains.

To confirm that S. lividans strains expressing the PABA synthetase gene of L. lactis subsp. lactis did indeed produce PABA, S. lividans strains FA1 (JG10 containing pDQ250), FA7 (JG10 containing pDQ255), FA2 (AP3 containing pDQ250), FA8 (AP3 containing pDQ255), FA3 (JG10 cotaining pIJ41), FA5 (JG10 containing pDQ254), FA4 (AP3 containing pIJ41) and FA6 (AP3 containing pDQ254) were grown in glucose-isoleucine medium for five days. When the clarified broth of each strain was examined for aromatic amines, strains FA1, FA2, FA7 and FA8 gave positive assays (Table 6). No aromatic amines were detected in the broths of strains FA3, FA4, FA5 and FA6.

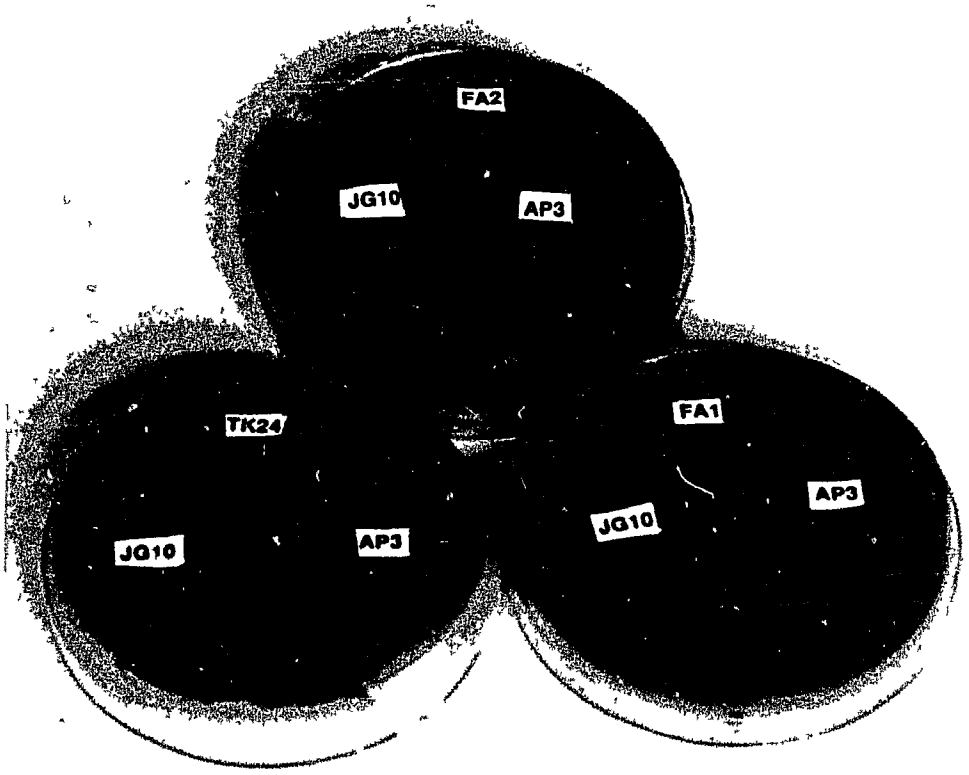


Figure 17: Cross-feeding experiments on S. lividans pab mutants by S. lividans strains FA1, FA2 and TK24.

Table 6. Production of aromatic amines by cultures of S. lividans strains.[a]

Strain	Aromatic amines (ug mL^{-1})[b]
FA1	15
FA2	14
FA3	Nil
FA4	Nil
FA5	Nil
FA6	Nil
FA7	13
FA8	14

a. Aromatic amines were detected by diazotization and coupling to naphthylethylenediamine.

b. Yields were measured as ug equivalents of PABA.

The clarified broths of the strains were extracted with ethyl acetate and the concentrated extracts were examined along with authentic PABA as a reference compound by TLC using two solvent systems. The chromatograms were exposed to UV light to detect quenching zones and then sprayed with acidic p-dimethylaminobenzaldehyde to detect aromatic amines. A single quenching zone that reacted with p-dimethylaminobenzaldehyde was obtained for the extracts of strains FA1, FA2, FA7 and FA8 all of which contain and express the cloned PABA synthetase gene. The R_f value of this quenching zone corresponded to that of authentic PABA.

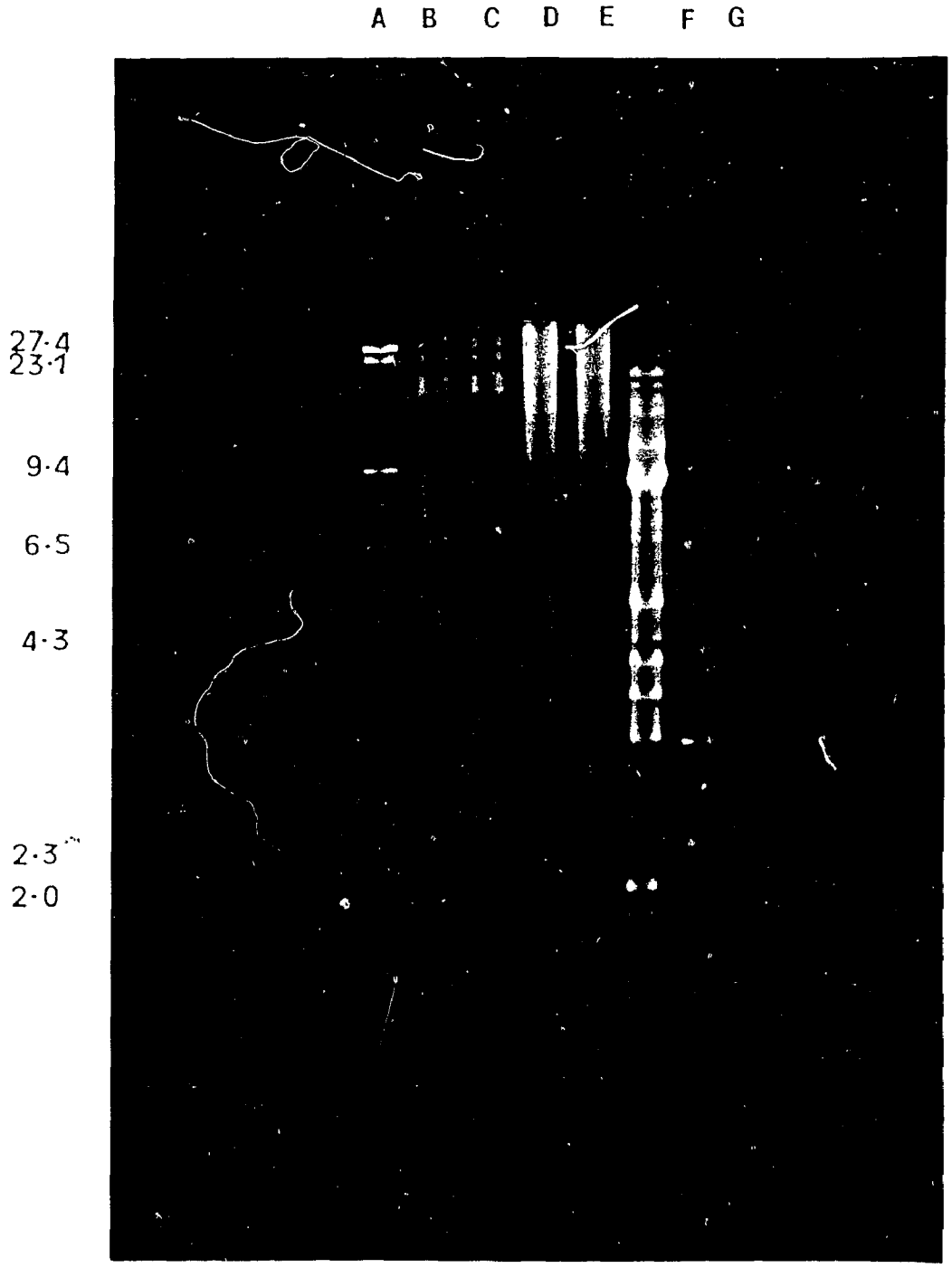
No quenching zones or colour reaction typical of aromatic amines was observed with extracts of S. lividans strains FA3, FA4, FA5 and FA6, which do not contain the cloned PABA synthetase gene. It was concluded that expression of the cloned DNA sequence from L. lactis subsp. lactis resulted in the production of PABA.

VIII. Hybridization of L. lactis subsp. lactis PABA synthetase gene to genomic digests.

The source of the cloned PABA synthetase gene was investigated by Southern hybridization. Genomic DNA samples from L. lactis subsp. lactis strains NCDO496 and AV117 were each digested individually with BclI and with BglII; a sample from S. griseofuscus C581 was digested with BamHI (Fig. 18).

Figure 18: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; of BclI-digested genomic DNA of (B) L. lactis subsp. lactis NCDO496 and (C) L. lactis subsp. lactis AV117; of BglII-digested genomic DNA of (D) L. lactis subsp. lactis NCDO496 and (E) L. lactis subsp. lactis AV117; of (F) BamHI-digested genomic DNA of S. griseofuscus C581; and of (G) ccc DNA of pDQ253. The DNA fragments, blotted on a nylon membrane, were those used (Fig. 19) for hybridization analysis.

Figure 18



The digests were immobilized on a nylon membrane and probed with a ^{32}P-labelled sample of the 6.0-kb BglII-BglII fragment from pDQ250 containing the PABA synthetase gene. Hybridization was carried out at high stringency.

The probe hybridized to an 18.3-kb fragment in the BclI-digests of strains NCDO496 and AV117 and to a 6.0-kb fragment in the BglII-digests of strains NCDO496 and AV117 (Fig. 19). It also hybridized to pDQ253, but not to the BamHI digest of S. griseofuscus C581.

These results indicated that the PABA synthetase gene was from L. lactis subsp. lactis and that it had little or no sequence similarity to PABA synthetase genes presumed to be present in S. griseofuscus.

IX. Characterization of E. coli pab mutants.

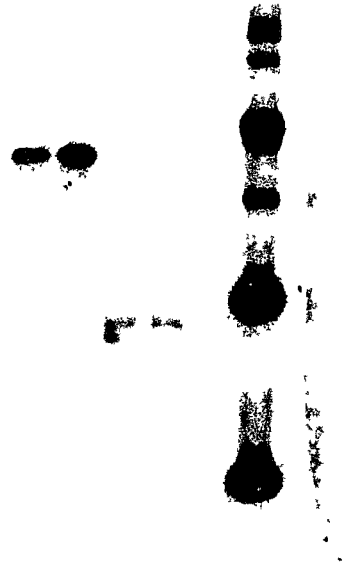
The method used was based on the biochemical functions of the pabA and pabB gene products. The pabA gene product is a glutamine amidotransferase which is required only when L-glutamine is the sole nitrogen source for the enzyme reaction. In its absence, biosynthesis of PABA can be achieved by the pabB gene product provided enough free ammonia is present.

To characterize the mutants, strains AB3292 (pabA) and AB3295 (pabB) were grown from washed cells in a medium adjusted to pH 6.0 or 8.0 and supplemented with 1 mM filter-sterilized ammonium sulphate. Control cultures were identical except that they received PABA at a concentration of 50 ug mL^{-}

Figure 19: Autoradiogram of the DNA blot from the gel in Fig. 18 after hybridization with the ^{32}P-labelled 6-kb BglII fragment from pDQ250.. The samples were: (A) HindIII-digested lambda DNA; BclI-digested genomic DNA of (B) L. lactis subsp. lactis NCDO496, (C) L. lactis subsp. lactis AV117; BglII-digested genomic DNA of (D) L. lactis subsp. lactis NCDO496, (E) L. lactis subsp. lactis AV117; (F) BamHI-digested genomic DNA of S. griseofuscus C581 and (G) cccDNA of pDQ253.

Figure 19

A B C D E F G



[1]. Table 7 shows that both strains were unable to grow at pH 6.0 where ammonia is largely protonated. However, at pH 8.0, enough free ammonia was apparently available to allow strain AB3292, which has a functional aminase activity (the pabB gene product) to make PABA and thus to grow. Strain AB3295 (pabB), which lacks the aminase activity was unable to make PABA even when free ammonia was available, and did not grow at either pH value.

X. PABA synthetase assay for E. coli pab mutants.

The reaction mixture for measuring PABA synthetase activity with L-glutamine as the nitrogen source contained chorismic acid, Tris-HCl, pH 8.2, L-glutamine and crude cell extract. Aminase activity was detected by replacing L-glutamine with 100 umol of ammonium chloride. After incubation at 37°C for 30 min, the PABA formed was measured colorimetrically. For complementation studies, crude extract containing 4 mg of protein from each of the mutants was included in the reaction mixture.

The results (Table 8) indicate that crude extract from strain AB3295 (pabB) could not make PABA with either L-glutamine or ammonium chloride as nitrogen source. However, when a mixture of the crude extracts of the two mutants was used, either L-glutamine or ammonium chloride served as the nitrogen source to make PABA. Crude extract from strain AB3292 (pabA) used ammonium chloride but not L-glutamine to

Table 7. Optical density at 600nm (OD_{600}) of E. coli strains AB3292 and AB3295 grown overnight in a defined medium with 1mM ammonium sulphate at pH 6.0 and 8.0.

Medium condition[a]	Optical density[b] AB3292	AB3295
pH 6.0	0.09	0.08
pH 6.0 + PABA	0.12	0.13
pH 8.0	0.62	0.17
pH 8.0 + PABA	0.74	0.70

a. At pH 6.0, the ammonium salt is >99.9% NH_4^+. At pH 8.0, 5.2% of the ammonium salt is unprotonated (Zalkin and Murphy, 1975).

b. Optical density values are averages of three readings. The standard deviation was +/- 0.03. Measurements were made after growth for 16 h at 37°C. All cultures were initially at OD_{600} = 0.05

Table 8. PABA synthetase activity in E. coli strains AB3292 and AB3295.

Crude extract	Amount of PABA formed (nmol/mg protein/h)[a] amidotransferase[b]	aminase[c]
AB3292 (pabA)	-	6
AB3295 (pabB)	-	-
AB3292 + AB3295	12	10

a. Values were averages of three readings. The standard deviation was +/- 1.0.

b. Assayed with glutamine in the incubation mixture.

c. Assayed with ammonium chloride in the incubation mixture.

make PABA. The results agree with those obtained from the growth studies; strain AB3292 can use its aminase function to make PABA when free ammonia (from ammonium chloride at pH 8.2) is available. When L-glutamine is the nitrogen source, the glutamine amidotransferase function from strain AB3295 was required together with the aminase function from strain AB3292 to make PABA.

XI. Characterization of S. lividans pab mutants.

Enzyme assays for PABA synthetase in Streptomyces cell extracts gave values too low for accurate measurement. Growth assays in liquid cultures were also abandoned because S. lividans strains JG10 and AP3 grew as clumps in the defined medium used and measurements of optical density were inaccurate and unreliable.. Growth assays were thus carried out on agar plates.

Table 9 shows that in the absence of PABA, strain JG10 could use ammonium sulphate but not asparagine for growth, whereas strain AP3 could not grow with either nitrogen source. When sulphanilamide was added to the medium, both strains did not grow, regardless of the nitrogen source. The ability of strain JG10 to use ammonium sulphate but not asparagine for growth suggests that it has functional aminase and PabX activities, and that the mutation could be in the glutamine amidotransferase (pabA) gene. Since strain AP3 was unable to grow when ammonium sulphate was the nitrogen source,

Table 9. Growth studies on S. lividans strains JG10 and AP3

Supplement*	Strains[a] JG10	AP3
No nitrogen source	-	-
No nitrogen source + PABA	-	-
Ammonium sulphate	+++	-
Ammonium sulphate + PABA	++++	++++
Ammonium sulphate + sulphanilamide	-	-
Asparagine	+	-
Asparagine + PABA	++++	++++
Asparagine + sulphanilamide	-	-
Asparagine + ammonium sulphate	+++	-
Asparagine + ammonium sulphate + PABA	++++	++++

* For each culture, Streptomyces minimal medium (SM) was supplemented as shown. Final concentrations of supplements were: ammonium sulphate, 5 mM; asparagine, 3 mM; sulphanilamide, 0.25 ug mL^{-1} and PABA, 50 ug mL^{-1}.

a. - indicates no growth; + indicates poor growth; +++ indicates moderate growth; ++++ indicates good growth.

its mutation could be in the pabB or pabX gene(s).

XII. Molecular cloning and characterization of the S. lividans 1326 PABA synthetase gene.

1. Shotgun cloning in E. coli AB3295.

The strategy was to obtain genomic DNA fragments of S. lividans that complemented the pab mutations in E. coli. The E. coli plasmid pBR322, which possesses genes conferring resistance to ampicillin (bla) and tetracycline (tet), was used as a vector. Plasmid pBR322 contains a unique BamHI site within the tet gene; insertion of foreign DNA into this site inactivates the gene.

A BamHI partial digest of S. lividans 1326 genomic DNA was ligated into the BamHI site of pBR322. The ligation mixture was used to transform competent cells of E. coli AB3295 and the transformation mixture was spread on L-agar containing 100 ug mL^{-1} of ampicillin. After incubation overnight at 37°C, approximately 12,000 ampicillin-resistant colonies were obtained. These were replicated on L-agar containing 15 ug mL^{-1} of tetracycline and on appropriately supplemented M9 medium lacking PABA. Approximately 3,000 of the 12,000 ampicillin resistant colonies were unable to grow on medium containing tetracycline indicating insertion of foreign DNA into the BamHI site of pBR322. One of these, strain AKA1, was able to grow on M9 medium lacking PABA indicating that it contained information that complemented the

pabB mutation in strain AB3295. Plasmid DNA isolated from strain AKA1 (pDQ290) was bigger than pBR322 (Fig. 20) indicating that foreign DNA had been introduced.

2. Transformation of E. coli strains AB3292 and AB3295 with pDQ290.

Plasmid pDQ290 was used to transform competent cells of E. coli strains AB3292 and AB3295. As a control, competent cells of strains AB3292 and AB3295 were also transformed with pBR322. Each transformation yielded approximately 600 ampicillin-resistant colonies. Transformants containing pBR322 did not grow when replicated on appropriately supplemented M9 medium lacking PABA. However, transformants of both strains AB3292 and AB3295 containing pDQ290 were able to grow on supplemented M9 medium lacking PABA. This indicated that pDQ290 contained information that complemented the pab mutations in both strains AB3292 and AB3295. Strain AB3292 containing pDQ290 was designated E. coli AKA2.

3. Characterization of the cloned DNA fragment in pDQ290.

Both pBR322 and pDQ290 were digested with BamHI and the digests were examined by agarose gel electrophoresis. Figure 21 shows that BamHI-restricted pBR322 had the expected size of 4.3-kb. Plasmid pDQ290 restricted with BamHI gave an additional fragment estimated to be 4.8-kb in size. This

Figure 20: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; of (B) pBR322 and (C) ccc DNA of pDQ290 extracted from E. coli transformant AKA1.

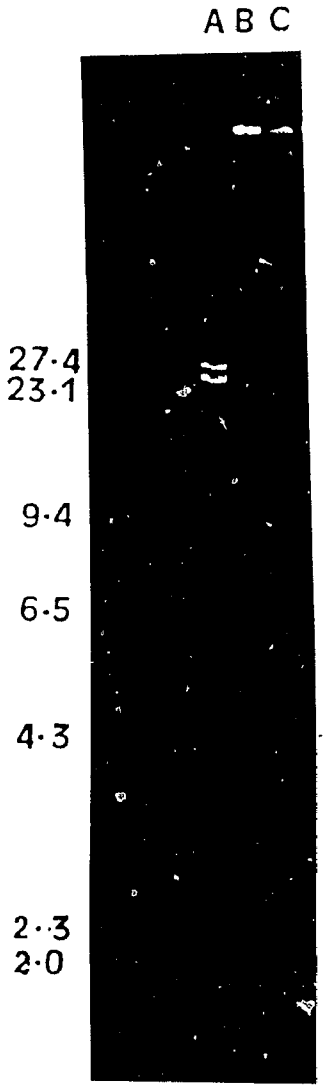


Figure 21: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; and of BamHI-digests of (B) pBR322 and (C) pDQ290.

fragment was presumed to contain the information complementing the pab mutations in E. coli strains AB3292 and AB3295.

To determine the orientation of the 4.8-kb BamHI fragment, pDQ290 was digested with the restriction endonucleases EcoRI and SstI. The restriction endonuclease EcoRI has no recognition site within the 4.8-kb insert but recognizes a single site on pBR322. On the other hand, SstI has no recognition site in pBR322 but recognizes a single site in the 4.8-kb insert. Figure 22 shows that when pDQ290 was restricted with both SstI and EcoRI, two bands of approximate sizes 6.0 and 3.1-kb were obtained. From the circular map of pBR322, a circular map of pDQ290 was constructed (Fig. 23) indicating the SstI site within the insert relative to the EcoRI site on pBR322.

4. Cloning of the 4.8-kb BamHI fragment in an orientation opposite to that of pDQ290.

To determine whether the pab gene(s) were expressed from a promoter on the 4.8-kb fragment, the fragment was excised from pDQ290 and reinserted in the opposite orientation. Excision was achieved by digestion with BamHI and the resultant digest was religated. The ligation mixture was used to transform competent cells of E. coli AB3295. Ampicillin-resistant, tetracycline-sensitve colonies obtained from this transformation were selected and the plasmids present were screened by electrophoresis of EcoRI and SstI digests for

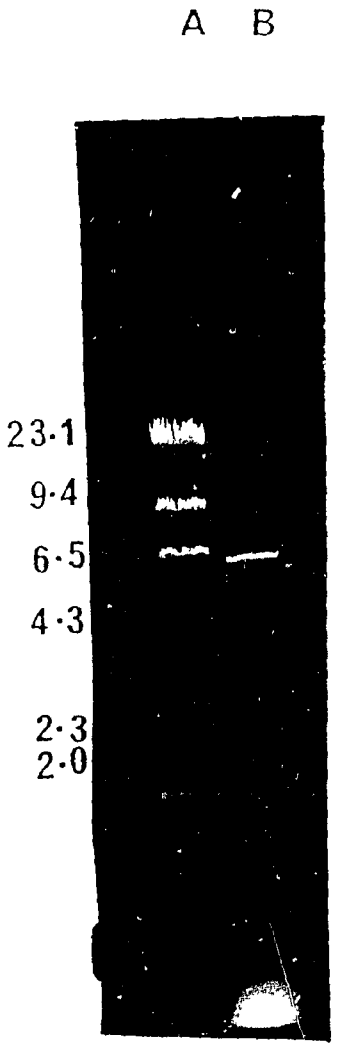


Figure 22: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; and of (B) pDQ290 digested with EcoRI and SstI.

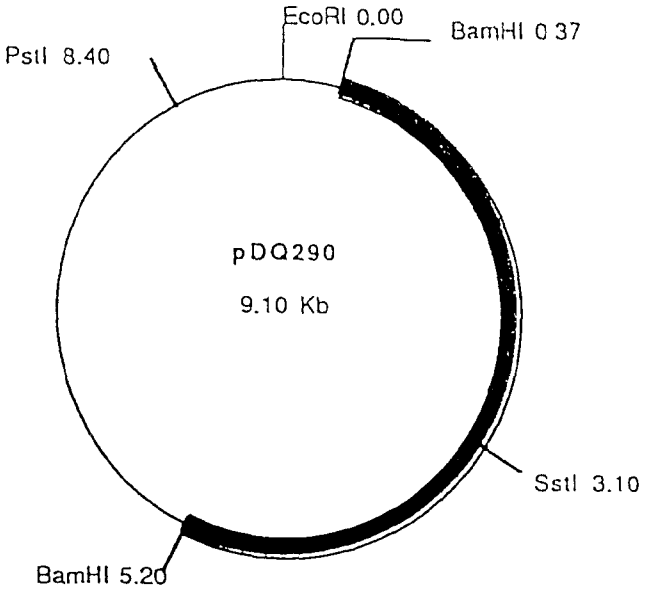


Figure 23: Circular restriction map of pDQ290. The thick line represents sequences cloned from S. lividans. The thin line represents sequences from pBR322. The numbers indicate the map positions (in kb) of restriction enzyme sites relative to the EcoRI site (0.0).

those in which the 4.8-kb insert was in an orientation opposite to that of pDQ290. One such colony (strain AKA3) contained a plasmid (pDQ291) which when digested with EcoRI and SstI gave two bands of estimated sizes 6.6 and 2.5 kb (Fig. 24), as predicted for an orientation opposite to that of pDQ290 (Fig. 25).

When pDQ291 was introduced into competent cells of E. coli AB3292 and AB3295, the transformants obtained were able to grow on supplemented M9 medium lacking PABA. Expression of the S. lividans PABA synthetase gene(s) in E. coli when the 4.8-kb fragment was cloned in two opposite orientations suggested that a promoter sequence on the 4.8-kb fragment was used for expression and therefore that the E. coli RNA polymerase recognized and used a Streptomyces promoter.

5. Subcloning of the PABA synthetase genes into pTZ18R.

To localize the portion of the 4.8-kb BamHI fragment that complemented the pab mutations in E. coli, the 2.7-kb BamHI-SstI segment of the 4.8-kb BamHI fragment was eluted and ligated into pTZ18R restricted with BamHI and SstI. The ligation mixture was used to transform competent cells of E. coli TG1 and the transformants were spread on L-agar containing ampicillin. Transformants that contained plasmid in which foreign DNA had been inserted into pTZ18R were detected as Lac^- (white) by including X-gal and IPTG in the medium.

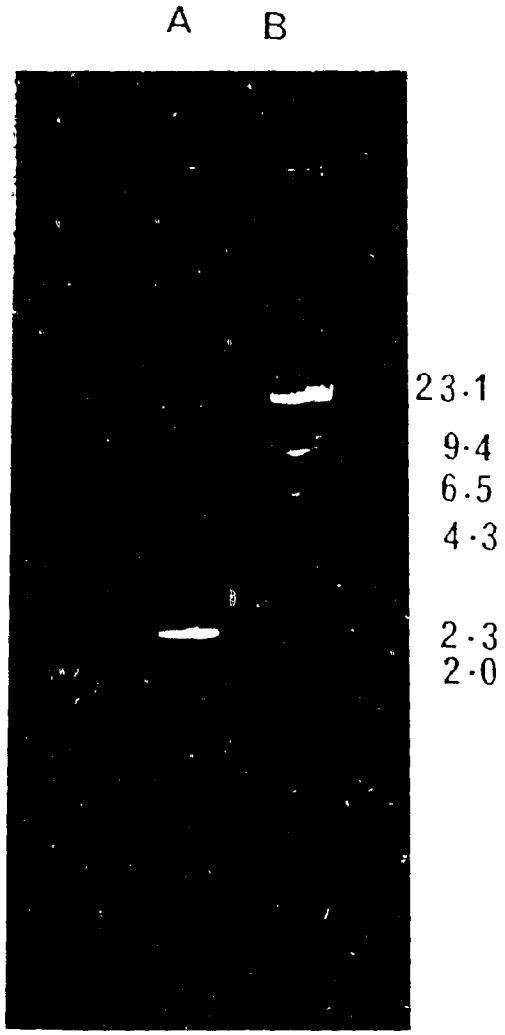


Figure 24: Agarose gel electrophoresis of (A) pDQ291 digested with EcoRI and SstI; and of (B) lambda DNA digested with HindIII.

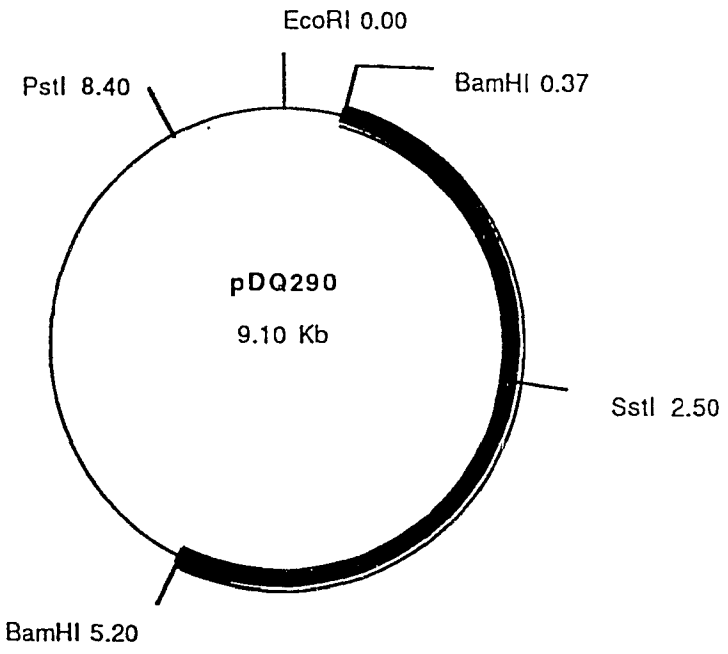


Figure 25: Circular restiction map of PDQ291. The thick line represents sequences cloned from S. lividans. The thin line represents sequences from pBR322. The numbers indicate the map positions (in kb) of restriction enzyme sites relative to the EcoRI site (0.0).

Plasmid DNA isolated from white (Lac$^-$) ampicillin-resistant transformants (pDQ292) contained the 2.7-kb BamHI-SstI fragment from pDQ290 inserted into pTZ18R (Fig. 26). When pDQ292 was used to transform E. coli strains AB3292 and AB3295, the ampicillin-resistant transformants obtained from each transformation were able to grow on supplemented M9 medium lacking PABA. This indicated that the 2.7-kb BamHI-SstI fragment contained information that complements both pab mutations in E. coli.

6. Hybridization of the cloned S. lividans PABA synthetase gene(s) to genomic digests.

The source of the cloned gene was investigated by hybridizing the 2.7-kb BamHI-SstI fragment from pDQ292 to genomic digests. Genomic DNA samples from several Streptomyces spp. were digested with BamHI and a sample from L. lactis subsp. lactis was digested with BglII (Fig. 27). The digests were immobilized on a nylon membrane and probed with the radiolabelled 2.7-kb fragment containing the PABA synthetase gene(s). Hybridization was carried out at low and high stringencies.

The results showed that at high stringency, the probe hybridized to 4.8-kb fragments in the digests of S. lividans strains 1326 and M252 (Fig. 28). It also hybridized weakly to fragments from S. venezuelae 10712, S. griseus IMRU3572 and S. griseofuscus C581. Although no hybridizing signals were

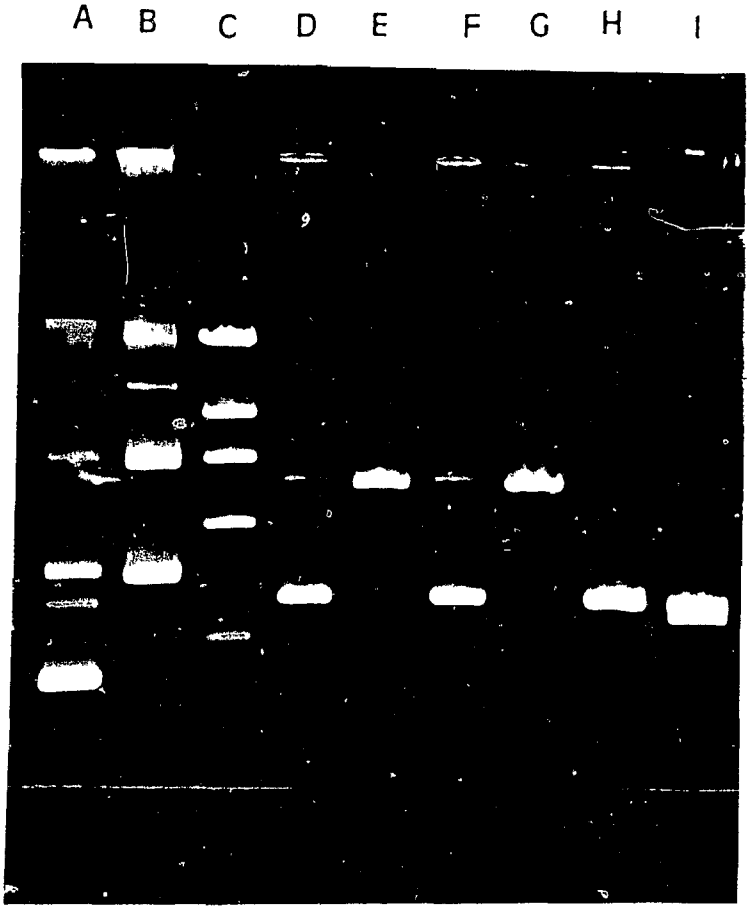


Figure 26: Agarose gel electrophoresis of cccDNA of (A) pTZ18R and (B) pDQ292; of (C) lambda DNA digested with HindIII; of BamHI-digests of (D) pTZ18R and (E) pDQ292; of SstI-digests of (F) pTZ18R and (G) pDQ292; and of BamHI- and SstI-double digests of (H) pTZ18R and (I) pDQ292.

Figure 27: Agarose gel electrophoresis of restriction enzyme digests of lambda DNA, genomic DNA and ccc DNA samples. The DNA samples are (A) HindIII digest of lambda; BamHI digests of (B) S. lividans 1326, (C) S. lividans M252, (D) S. venezuelae 10712, (E) S. griseus IMRU3572, (F) S. clavuligerus LCV21, (G) S. griseofuscus C581, (H) S. griseoviridus; BglII digest of (I) L. lactis subsp. lactis NCDO496; and of (J) ccc DNA of pDQ292. The DNA fragments in the gel were blotted on a nylon membrane and used in hybridization analysis (see Fig. 28).

Figure 27

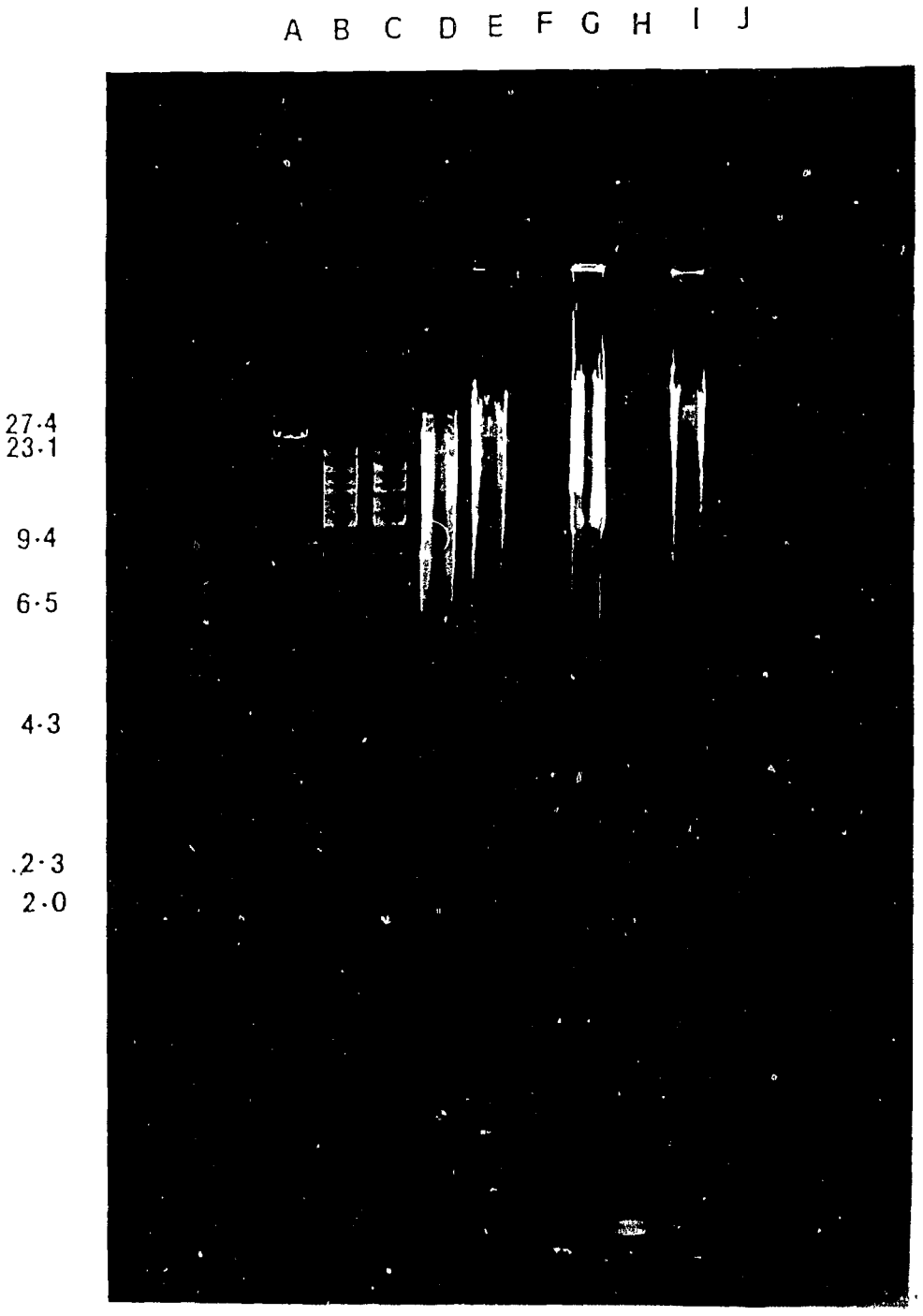


Figure 28: Autoradiogram of the DNA blot from the gel in Fig. 27 after hybridization at high stringency with the ^{32}P-labelled 2.7-kb fragment from pDQ290. The samples are (A) HindIII-digested lambda DNA; BamHI-digested genomic DNA of (B) S. lividans 1326, (C) S. lividans M252, (D) S. venezuelae 10712, (E) S. griseus IMRU3572, (F) S. clavuligerus LCV21, (G) S. griseofuscus C581, (H) S. giseoviridus; BglII digest of (I) L. lactis subsp. lactis NCDO496; and of (J) ccc DNA of pDQ292.

Figure 28

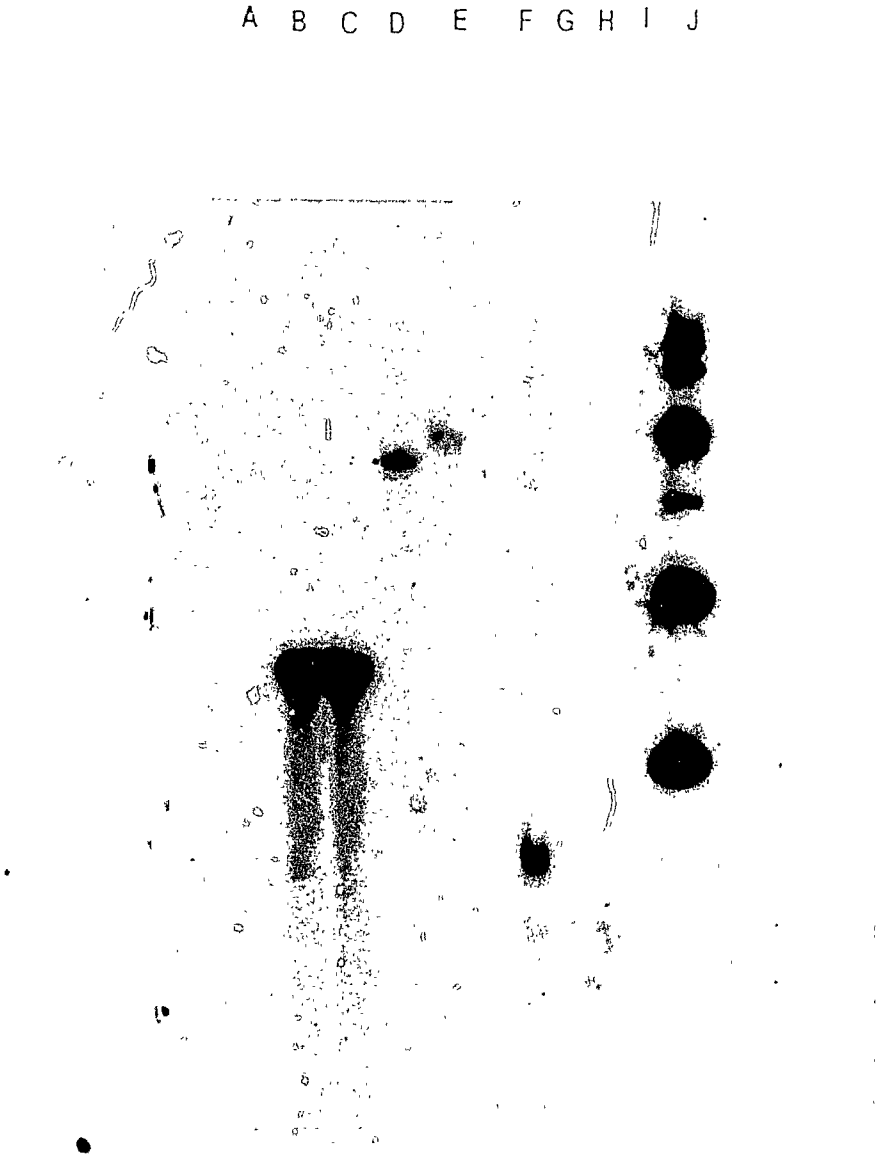
observed for the digests of S. clavuligerus LCV21 and S. griseoviridus, this may well have been due to there being insufficient DNA from each of these species on the gel. There was no hybridization to the digest of L. lactis subsp. lactis. At low stringency, the genomic DNA from each of the Streptomyces strains tested except S. griseoviridus and S. clavuligerus hybridized to the 2.7-kb probe (Fig. 29). Additional signals not observed at high stringency were detected. The results confirmed that the source of the 2.7-kb fragment was from S. lividans and indicated that the fragment shared some sequence similarity with DNA fragments from other Streptomyces spp. The fact that additional signals were obtained with some Streptomyces strains at low stringency suggests that multiple pab genes may be present or that there are be other genes in these strains with sequence homology to pab genes.

7. Expression of the S. lividans PABA synthetase gene in S. lividans.

The 2.7-kb BamHI-SstI fragment that complemented the pabA and pabB mutations in E. coli was subcloned into the BglII and SstI sites of pIJ702. When the resultant recombinant plasmid was introduced into protoplasts of S. lividans strains JG10 and AP3, thiostrepton-resistant, Pab$^+$ colonies were obtained. However, no plasmid DNA could be extracted from these colonies

Figure 29: Autoradiogram of the DNA blot from the gel in Fig. 27 after hybridization at low stringency with the ^{32}P-labelled 2.7-kb fragment from pDQ290. The samples are (A) HindIII-digested lambda DNA; BamHI-digested genomic DNA of (B) S. lividans 1326, (C) S. lividans M252, (D) S. venezuelae 10712, (E) S. griseus IMRU3572, (F) S. clavuligerus LCV21, (G) S. griseofuscus C581, (H) S. giseoviridus; BglII digest of (I) L. lactis subsp. lactis NCDO496; and of (J) ccc DNA of pDQ292.

Figure 29



by the alkaline lysis procedure.

To investigate the possibility that the recombinant plasmid had integrated into the host chromosome by homologous recombination, a Streptomyces-E. coli shuttle vector (pDQ293) was constructed by ligating SstI-restricted pDQ292 and SstI-restricted pIJ702 (Fig. 30). The shuttle vector was introduced into E. coli TG1 and plasmid DNA isolated from ampicillin-resistant transformants was shown to be a recombinant form of pDQ292 and pIJ702 (Fig. 31). The orientation of pDQ292 in the shuttle vector was deduced by digesting pDQ293 with BamHI. Two fragments with sizes of 6.9 and 4.5-kb were obtained (Fig. 32) indicating the orientation shown in figure 30.

Plasmid pDQ293 was used to transform protoplasts of S. lividans strains JG10 and AP3. As a control, protoplasts of S. griseofuscus C581 were also transformed with pDQ293. Transformants of the S. lividans strain JG10 (designated FA11) and transformants of AP3 (designated FA12) were thiostrepton-resistant and Pab$^+$. However, plasmid DNA could not be extracted from them. On the other hand, thiostrepton-resistant transformants of S. griseofuscus C581 (strain FA1) contained free pDQ293.

Total DNA from S. lividans strains JG10, AP3, FA11 and FA12 and S. griseofuscus FA1 was digested with BamHI (Fig. 33) and immobilized on a nylon membrane. The digests were hybridized with radiolabelled pDQ293 at high stringency.

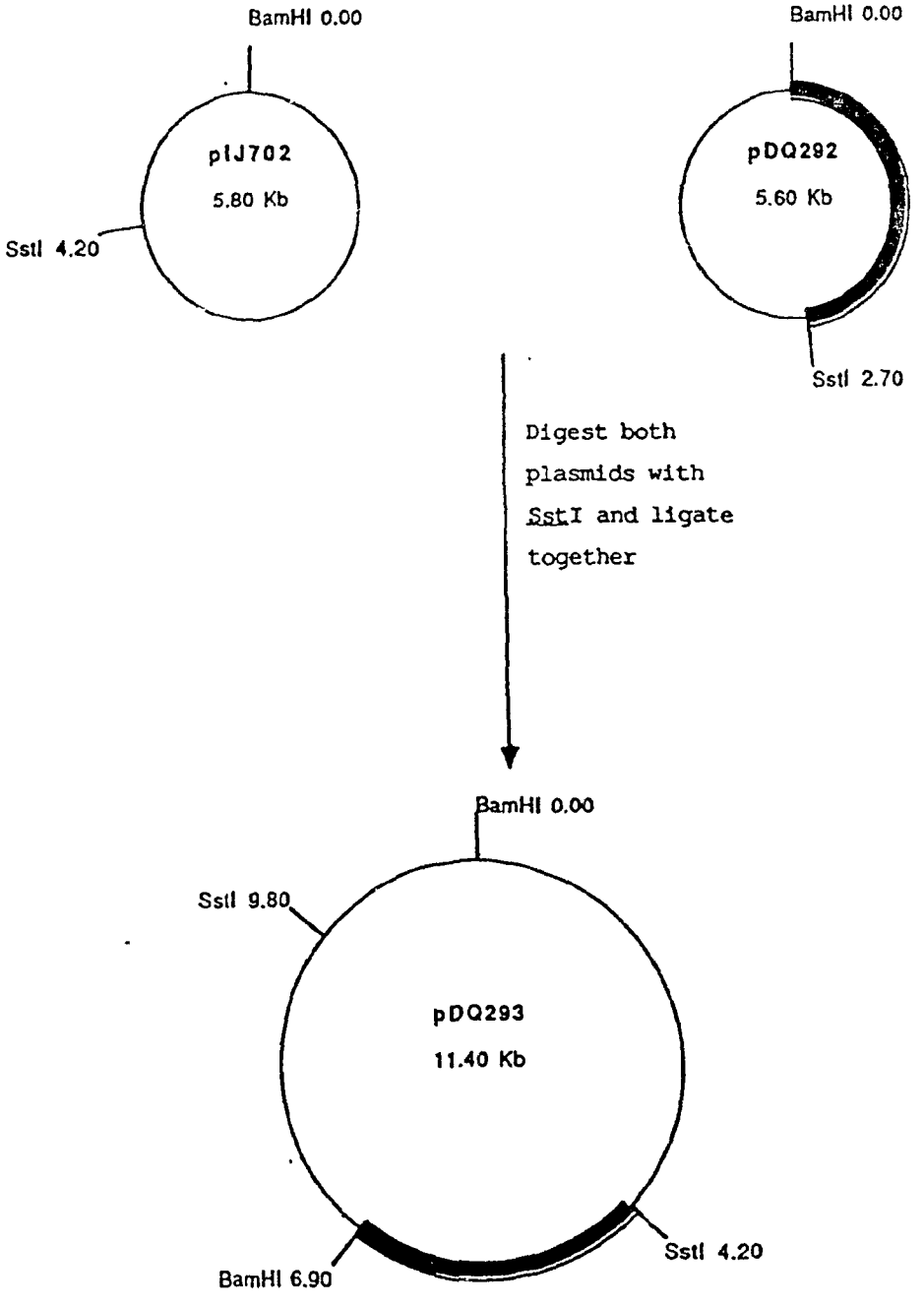


Figure 30: Construction of shuttle vector pDQ293. The thick line represents sequences from S. lividans that complemented the pab mutations in E. coli.

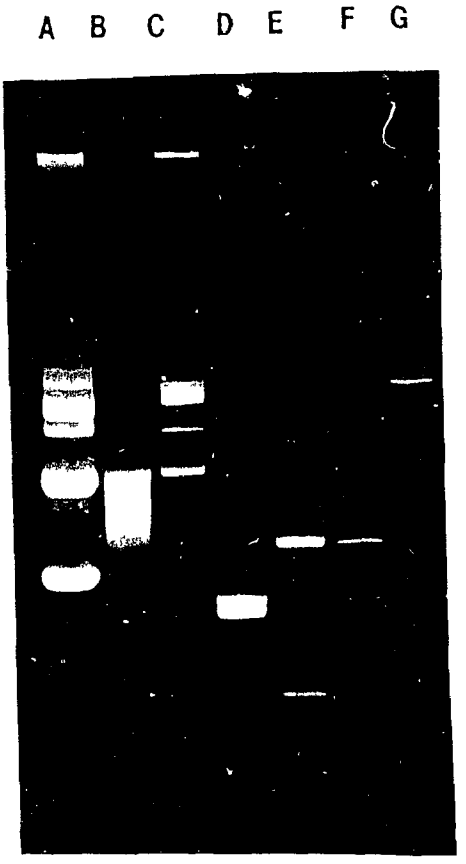


Figure 31: Agarose gel electrophoresis of cccDNA of (A) pDQ292, (B) pIJ702, (C) pDQ293; of BamHI- and SstI-double digests of (D) pDQ292, (E) pIJ702, (F) pDQ293; and of lambda DNA digested with (G) HindIII.

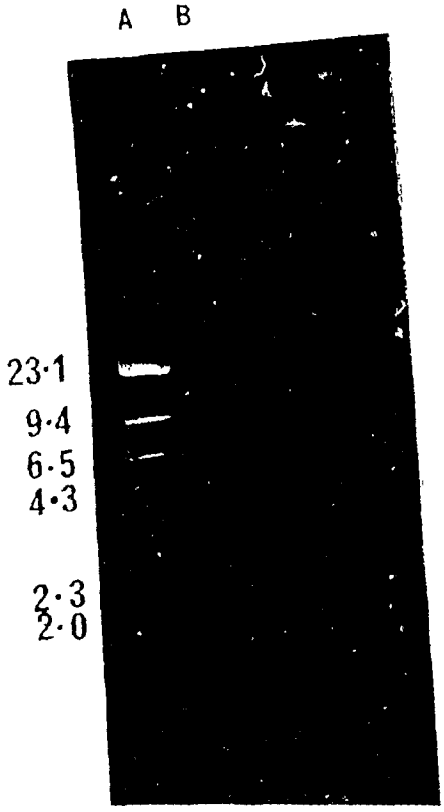


Figure 32: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; and of (B) pDQ293 digested with BamHI.

Figure 33: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; and of BamHI-digested genomic DNA of (B) S. lividans JG10, (C) S. lividans AP3, (D) S. lividans FA11, (E) S. lividans FA12 and S. griseofuscus FA1. The DNA fragments were blotted on a nylon membrane and used in hybridization analysis (see Fig. 34).

Figure 33

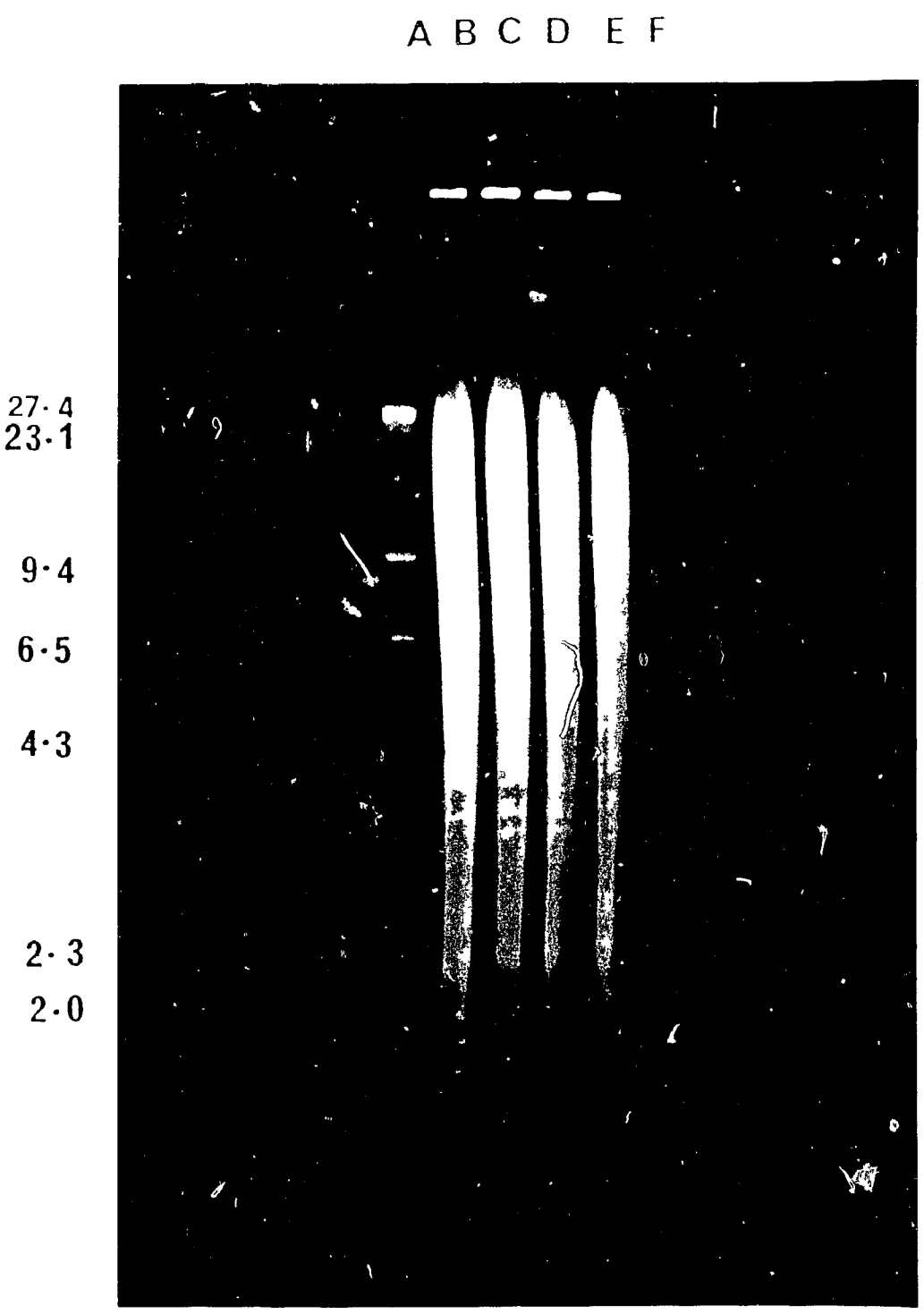


Figure 34 shows that two hybridizing signals of estimated sizes 6.9 and 4.5-kb were found in the digest of S. griseofuscus FA1. This is consistent with the generation of 6.9 and 4.5-kb fragments when pDQ293 is digested with BamHI. A single hybridizing signal of estimated size 4.8 kb was observed in the digests of S. lividans strains JG10 and AP3.

There appear to be three hybridizing signals of estimated sizes 6.9, 4.8 and 4.5 kb in the digests of S. lividans strains FA11 and FA12. This can be explained if pDQ293 has integrated into the host chromosme by a single cross-over event at the homologous region provided by the 2.7-kb fragment containing the pab gene(s). Integration of pDQ293 into the chromosome as depicted in Fig. 35 would generate three BamHI fragments of 6.9, 4.8 and 4.5 kb in which pDQ293 sequences would be present. This would account for the hybridizing pattern observed.

XIII. Sequencing and analysis of the L. lactis subsp. lactis PABA synthetase gene.

1. Sequencing of the gene.

To obtain the nucleotide sequence of both strands of the 1.9-kb BglII-BamHI fragment of L. lactis subsp. lactis genomic DNA that complemented the pabB mutation in E. coli, the 0.7-kb BamHI portion of the fragment was subcloned in the BamHI sites of the phagemid vectors pBLUESCRIPT SK(+) and SK(-) to generate pDQ257 and pDQ259, respectively. Also, a 1.4-kb

Figure 34: Autoradiogram of the DNA blot from the gel in Fig. 33 after hybridization with ^{32}P-labelled pDQ293. The samples are: (A) HindIII-digested lambda DNA; BamHI-digested genomic DNA of (B) S. lividans JG10, (C) S. lividans AP3, (D) S. lividans FA11, (E) S. lividans FA12 and (F) S. griseofuscus FA1.

Figure 34

A B C D E F

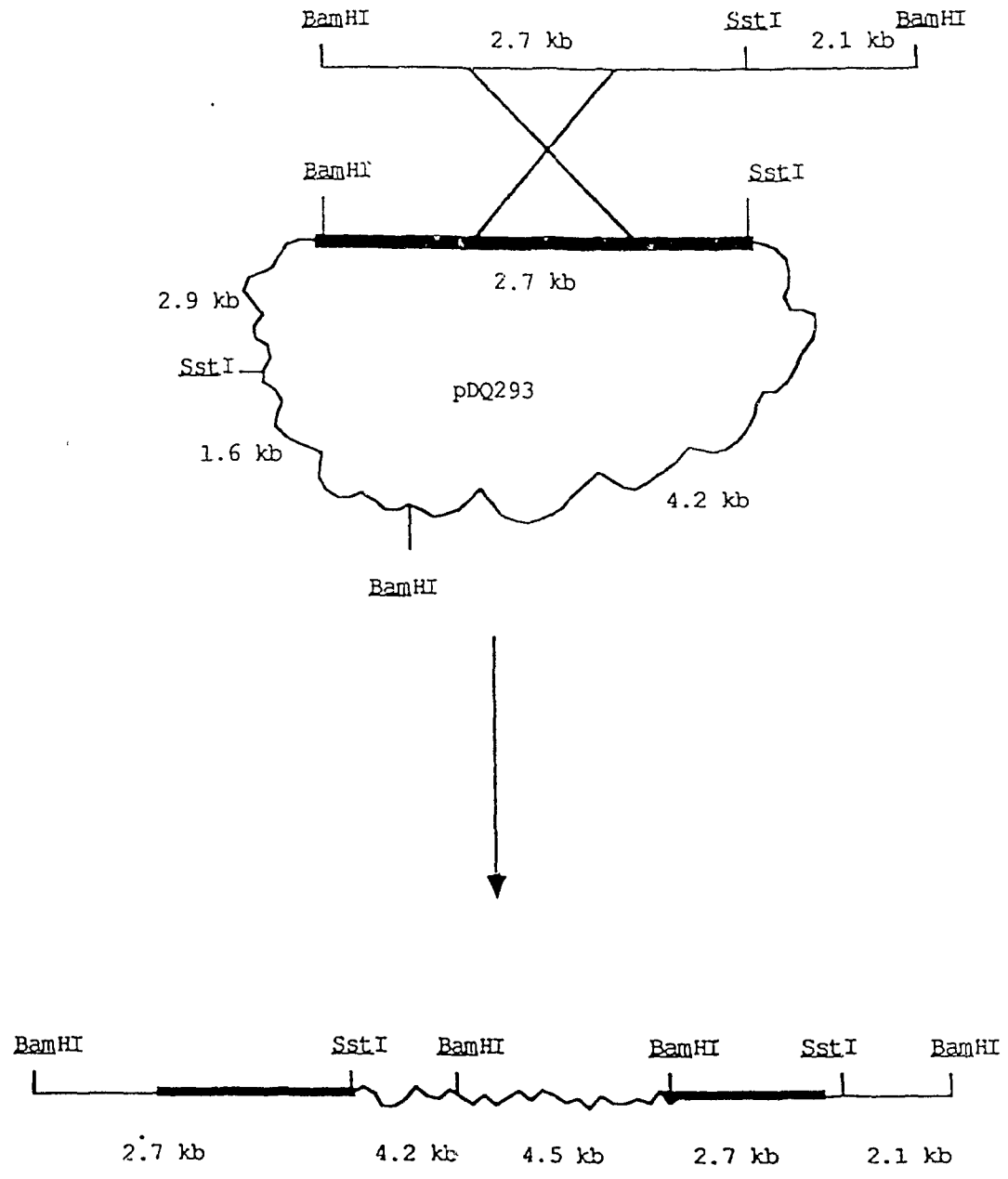


Figure 35: Model accounting for integration of pDQ293 into the chromosome by homologous recombination. The thin line (4.8 kb BamHI fragment) is a portion of the chromosome containing the pab genes. The thick line in pDQ293 is the cloned 2.7-kb pab-complementing fragment. (Diagram is not drawn to scale).

fragment containing the remaining 1.2-kb BglII-BamHI portion of the L. lactis subsp. lactis fragment together with the 200-bp EcoRV-BamHI fragment of pBR322 was subcloned into the EcoRV-BamHI sites of pBLUESCRIPT SK(+) and SK(-) to generate pDQ258 and PDQ260, respectively.

Nested deletions of the fragments cloned into pBLUESCRIPT SK(+) were obtained by digesting the resultant recombinant plasmids with ApaI to generate a 3'-overhang that protected the T7 primer from exonuclease III digestion, and with ClaI to generate a 5'-overhang that provided the substrate for exonuclease III to initiate digestion of the cloned fragment. Nested deletions of the fragments cloned into pBLUESCRIPT SK(-) were obtained by digesting with SstI to generate a 5'-overhang to protect the T3 primer from exonuclease III digestion and with XbaI to generate a 5'-overhang for initiation of exonuclease III digestion of the cloned fragment.

Colonies carrying deleted plasmids of desired sizes were used to generate single strand DNA templates by infecting them with the helper phage VCMS13. The templates were used for sequencing reactions using the T7 primer for those cloned into pBLUESCRIPT SK(+), or the T3 primer for those cloned into pBLUESCRIPT SK(-). The sequencing reaction mixtures were electrophoresed on polyacrylamide gels; after the nucleotide sequences were read from the gels, they were aligned using the overlaps in successive sequences obtained from clones with

plasmids differing in size by approximately 200-bp.

2. Open reading frame.

Figure 36 shows the nucleotide sequence of the 1.9-kb BglII-BamHI L. lactis subsp. lactis fragment that complemented the pabB mutation in E. coli. Within the sequence is an ORF that starts with the ATG at nt 145 and terminates with the TAA site at nt 1557. It encodes a 471 amino acid polypeptide with a size of 50,937 Daltons. The molecular weight of the polypeptide compares favorably with pabB genes from E. coli (48,000, [Nichols et al., 1989]) and B. subtilis (49,000 [Slock et al., 1990). The presence of a single ORF encoding a polypeptide of molecular weight comparable with pabB genes from other organisms explains the complementation of pabB but not pabA mutations in E. coli strains by the cloned fragment. The amino acid composition of the polypeptide (Table 10) shows that the enzyme contains 45.3% hydrophobic, 36.5% hydrophilic, 8.9% basic and 8.8% acidic amino acid residues. These values are close to the 42.2% hydropbobic, 33.2% hydrophilic, 11.45% basic and 12.9% acidic amino acid residues reported for the E. coli pabB gene (Goncharoff and Nichols, 1984).

Upstream of the ATG start site (nt 134-140), a sequence resembling the RBS for prokaryotes (Rosenberg and Court, 1979) was located. Sequences upstream of the RBS contain high A/T nucleotides typical of prokaryotic promoters. However, no consensus prokaryotic promoters could be discovered in this

Figure 36: Nucleotide and deduced amino acid sequence of the 1.9-kb BglII-BamHI L. lactis subsp. lactis fragment that complemented the pabB mutation in E. coli. The numbers on top of the nucleotide sequence represent the nucleotide/amino acid positions in the sequences. The putative RBS (nucleotides 134-141) is underlined. The start codon (ATG) is in bold face.

```
          1         11         21         31         41         51         61
          AGATCTCTTATAGAATAGCAATGAAATCTTGGGCGTATACGTAATATACTGAATATATCAATCGTAT

   71         81         91        101        111        121        131        141
TGAGCATGGTATATCACTGTGCAAAGACATACGGACTGACTGAACAGTAGCGTGGCATAAGTCGATCGAAAGGAATTAG
145/1                                       175/11
ATG TTC ACA ATC AGT GGA GTA GTA TTG ATA ACA AGA CCA GTA TAC GAT GAA GGA AGT TTG
Met phe thr ile ser gly val val leu ile thr arg pro val tyr asp glu gly ser leu
205/21                                      235/31
AAT TAC TGT CAG AGT GGA GCA ATG AAC AAC GGA ATC TTG TTG GAA AGT GTA GAA GGA AAC
asn tyr cys gln ser gly ala met asn asn gly ile leu leu glu ser val glu gly asn
265/41                                      295/51
AAG CCT CGA TAC AGT ATC GGA GGA GCA GAA CCT ATC GGA ACT ATC AAC GCA AAC GCA GTA
lys pro arg tyr ser ile gly gly ala glu pro ile gly thr ile asn ala asn ala val
325/61                                      355/71
TTG ACT GCA GCA ACT TAC GCA GAA GAT GTA AAG TTC ACT GAT GCA GAT CCT TTG AAC GGA
leu thr ala ala thr tyr ala glu asp val lys phe thr asp ala asp pro leu asn gly
385/81                                      415/91
ACT AGA GTA GCA ATC TGT AAC GGA GAA GAT ACT CAA CAA GAA GAA ATG GGA TTC CAA GGA
thr arg val ala ile cys asn gly glu asp thr gln gln glu glu met gly phe gln gly
445/101                                     475/111
GGA GCA TTG GGA TAC TTC GCA TAC GAT GTA GGA AGA AGA TTG GAA GGA TAC AAC GAT TTG
gly ala leu gly tyr phe ala tyr asp val gly arg arg leu glu gly tyr asn asp leu
505/121                                     535/131
GGA ATC GAA GAT TTG GCA ATC CCA GAT TTG GCA GGA AGT AGT TAC GAA ATC GGA GTA AGT
gly ile glu asp leu ala ile pro asp leu ala gly ser ser tyr glu ile gly val ser
565/141                                     595/151
GCA GAT CAT CAA AAC GAT GTA ATC GTA TTG ATC GCA CAT GCA AGT GCA GAT GGA AAC GAT
ala asp his gln asn asp val ile val leu ile ala his ala ser ala asp gly asn asp
625/161                                     655/171
GTA TTC ATC ACA AGT AGT AGA CAA TTG AGT ATG GTA GCA GGA CCA ACA TGT TGT GCA AGT
val phe ile thr ser ser arg gln leu ser met val ala gly pro thr cys cys ala ser
685/181                                     715/191
GGA GAT GTA GAA ATC TTG AGA AAC AAG TGG CAT TAC TAC GGA GTA ATC CCA TTC AGT CAA
gly asp val glu ile leu arg asn lys trp his tyr tyr gly val ile pro phe ser gln
745/201                                     775/211
GAT GAT TGT GGA TTC AAC AGA TTG AAG GAT TAC TTG GGA AGT GGA GAT ATG TAC CAA GTA
asp asp cys gly phe asn arg leu lys asp tyr leu gly ser gly asp met tyr gln val
805/221                                     835/231
AAC TTG GGA AAC AGA AAC GTA GGA GCA ATC GTA ATG ACA TTG TTC CAA GGA TAC AAC CAA
asn leu gly asn arg asn val gly ala ile val met thr leu phe gln gly tyr asn gln
865/241                                     895/251
TTG AGA TTG ATG AAC CCG GGA CCA TAC ATG GTA TTC TTG GAT GAA GCA AAC ATC ATC ATG
leu arg leu met asn pro gly pro tyr met val phe leu asp glu ala asn ile ile met
925/261                                     955/271
GCA AGT CCA GAA ATC GTA TTG GCA GAT GAA GCA AAC GAT TTG AAC ACA AGA CCA ATC GCA
ala ser pro glu ile val leu ala asp glu ala asn asp leu asn thr arg pro ile ala
985/281                                     1015/291
GGA ACA TTG ATG AGA TTG AAC GAG CAA GAT GAA GAT GGA GTA AAC GCA GCA TGT CTG GGA
gly thr leu met arg leu asn glu gln asp glu asp gly val asn ala ala cys leu gly
1045/301                                    1075/311
CAA CAT CAT AAG GAT AGA GCA GAA CAT ATG ATG ATC GTA GAT TTG GTA AGA AAC GAT TTG
gln his his lys asp arg ala glu his met met ile val asp leu val arg asn asp leu
1105/321                                    1135/331
GGA AGA GTG GGA AGA TTC GGA AGT GTA AAC GTA CAA GAA ATC GTA GGA GCT GAA AAC TAC
gly arg val gly arg phe gly ser val asn val gln glu ile val gly ala glu asn tyr
1165/341                                    1195/351
AGT GTA GTA ATG CAT ATC GTA AGT AGA GTA ACA GGA TCC TTG AAC GAA GCA TTC GAA GCA
```

Figure 36

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ser val val met his ile val ser arg val thr gly ser leu asn glu ala phe glu ala
1225/361                                  1255/371
ATG GAA ATC ATC AGA GCA GGA TTC CCA GGA GGA AGT ATC ACA GGA GCA CCA AAG GTA AGA
met glu ile ile arg ala gly phe pro gly gly ser ile thr gly ala pro lys val arg
1285/381                                  1315/391
GCA ATG GAA ATC ATC GAA GAA TTG GAA CCA CAA AGA CGA GAT GGA TGG GGA GGA AGT ATC
ala met glu ile ile glu glu leu glu pro gln arg arg asp gly trp gly gly ser ile
1345/401                                  1375/411
GGA TAC ATC GCA TAC AGA GGA AAC ATC GGT TAC AGA ATC GCA ATC AGA ACA TTG TTC GCT
gly tyr ile ala tyr arg gly asn ile gly tyr arg ile ala ile arg thr leu phe ala
1405/421                                  1435/431
TGT AAC GGA CAA TTG TTC GCA AGT AGT GGA GCA GGA TTG GTA GGA GAT AGT ATG GAA GAC
cys asn gly gln leu phe ala ser ser gly ala gly leu val gly asp ser met glu asp
1465/441                                  1495/451
GGA GAA TAC AAC GAA ACA TTC GAA AAG ATG AGA GCA TTG AGA AGT TTC TTC TGT GCA GCG
gly glu tyr asn glu thr phe glu lys met arg ala leu arg ser phe phe cys ala ala
1525/461                                  1555/471          1571      1581
GTA CAT ATG GGA AAG ACA CCA TAC TTG AGT TAA TTACGATCCGTAATATGTACTTATAAGCGTTGCAAT
val his met gly lys thr pro tyr leu ser OCH
       1601      1611      1621      1631      1641      1651      1661
ATGCGTAATTAGTACTTAATTACGTAATGTTTATTTCAATAGTAATAGATCATGACTAGAATATCGAATGTAATAGATCT
       1681      1691      1701      1711      1721      1731      1741
GATGTAATTAAATTCAGTATAATCGTATCATGACAAAATGCATGAATCGTTATCGGTAACTATAATGCTCAGTGATATGT
       1761      1771      1781      1791      1801      1811      1821
GAATCGTATCCGTGCCTAGCGATTTAACTAAAGCTAGCATGATAAATAGAATAGATAACTGATACAAATACTAGTCATAC
       1831      1841      1851      1861
ATTGACTATAGTACGCCGGTACGTATAGATTCGTAATCCGTACGTAGGATCC
```

Figure 36

Table 10. Amino acid composition of the L. lactis subsp. lactis pabB gene.

			n	n(%)	Amino acid type
A	ala	alanine	42	8.9	hydrophobic
C	cys	cysteine	8	1.7	hydrophilic
D	asp	aspartic acid	29	6.2	acidic
E	glu	glutamic acid	31	6.6	acidic
F	phe	phenylalanine	17	3.6	hydrophobic
G	gly	glycine	54	11.5	hydrophilic
H	his	histidine	8	1.7	basic
I	ile	isoleucine	33	7.0	hydrophobic
K	lys	lysine	8	1.7	basic
L	leu	leucine	35	7.4	hydrophobic
M	met	methionine	18	3.8	hydrophobic
N	asn	asparagine	30	6.4	hydrophilic
P	pro	proline	15	3.2	hydrophobic
Q	gln	glutamine	15	3.2	hydrophilic
R	arg	arginine	26	5.5	basic
S	ser	serine	27	5.7	hydrophilic
T	thr	threonine	18	3.8	hydrophilic
V	val	valine	34	7.2	hydrophobic
W	trp	tryptophan	2	0.4	hydrophobic
X	---	unknown	-	-	
Y	tyr	tyrosine	20	4.2	hydrophilic
Z	---	STOP	1	0.2	

n represents the number of residues of the amino acid present in the protein.

n(%) represents the percentage of the amino acid present in the protein.

region. The lack of a promoter explains why the gene would be expressed in E. coli only when it was cloned in one orientation in pBR322; in this orientation, the gene was transcribed from a vector promoter.

3. Codon usage in the ORF.

Table 11 shows the codon usage in the ORF of the L. lactis subsp. lactis pabB gene. Codons rich in A/T nucleotides were preferred to those of high G/C. The coding region contains 58.6% A/T and 41.4% G/C. The entire 1.9-kb sequence contains 60.8% A/T and 39.2% G/C. This value compares well with the 37% G+C content of L. lactis subsp. lactis genomic DNA.

Table 12 summarizes the G+C content of the three positions of each codon in the ORF. The 34% G+C content of the third (degenerate) position reflects the low G+C content of L. lactis subsp. lactis genomic DNA.

4. Comparison of the deduced amino acid sequence of the L. lactis subsp. lactis pabB gene with those of related genes.

The deduced amino acid sequence of the ORF was compared with amino acid sequences of other proteins using the Genbank FASTA program. Figure 37 shows the comparison between the deduced amino acid sequence of L. lactis subsp. lactis pabB

Table 11. Codon usage in the L. lactis subsp. lactis pabB gene.

TTT	phe	F	-	TCT	ser	S	-	TAT	tyr	Y	-	TGT	cys	C	8
TTC	phe	F	17	TCC	ser	S	1	TAC	tyr	Y	20	TGC	cys	C	-
TTA	leu	L	-	TCA	ser	S	-	TAA	OCH	Z	1	TGA	OPA	Z	-
TTG	leu	L	34	TCG	ser	S	-	TAG	AMB	Z	-	TGG	trp	W	2
CTT	leu	L	-	CCT	pro	P	3	CAT	his	H	8	CGT	arg	R	-
CTC	leu	L	-	CCC	pro	P	-	CAC	his	H	-	CGC	arg	R	-
CTA	leu	L	-	CCA	pro	P	11	CAA	gln	Q	14	CGA	arg	R	2
CTG	leu	L	1	CCG	pro	P	1	CAG	gln	Q	1	CGG	arg	R	-
ATT	ile	I	-	ACT	thr	T	6	AAT	asn	N	1	AGT	ser	S	26
ATC	ile	I	32	ACC	thr	T	-	AAC	asn	N	29	AGC	ser	S	-
ATA	ile	I	1	ACA	thr	T	12	AAA	lys	K	-	AGA	arg	R	24
ATG	met	M	18	ACG	thr	T	-	AAG	lys	K	8	AGG	arg	R	-
GTT	val	V	-	GCT	ala	A	2	GAT	asp	D	28	GGT	gly	G	1
GTC	val	V	-	GCC	ala	A	-	GAC	asp	D	1	GGC	gly	G	-
GTA	val	V	33	GCA	ala	A	39	GAA	glu	E	30	GGA	gly	G	53
GTG	val	V	1	GCG	ala	A	1	GAG	glu	E	1	GGG	gly	G	-

Table 12. Percent G+C composition of the coding region of the L. lactis subsp. lactis pabB gene.

Codon position	% G+C
1	49.3
2	39.7
3	34.9
Entire coding region	41.4

Figure 37: Comparison of the deduced amino acid sequence of the L. lactis subsp. lactis pabB gene with that of the E. coli pabB gene. Hyphens indicate gaps introduced to increase similarity. Two dots indicate the same amino acid in the two sequences at that position. One dot indicates similar amino acids in the two sequences at that position.

Figure 37


L. lactis MFTISGVVLITRPVYDEGSLNYCQSGAMNNGILLESVEGNKP--RYSI
E. coli MKTLSPAVITLLWRQDAAEFYFSRLSHLPWAMLLHSGYADHPYSFRDI
L. lactis GGAEPIGTINANAVLTAATYAEDVKFTDADPLNGTRVAICNGE--DTQ
E. coli VVAEPICTLTTFGKETVVSESEKRTTTTDDPLQVLQQVLDRADIRPTH
L. lactis QEENGFQGGALGYFAYDVGRRLEGYNDLGIEDLAIPDLAGSSYEIGVS
E. coli NEDLPFQGGALGLFGYDLGRRFESLPEIAEQDIVLPDMAVGIYDWALI
L. lactis ADHQNDVIVLIAHASADGNDVFITSSRQLSMVAGPTCCASGDVEILRN
E. coli VDHQRHTVSLLSHNDVNARRAWL-ESQQFS---------PQEDFTLTS
L. lactis KWHYYGVIPFSQDDCGFNRLKDYLGSGDMYQVNLGNR-VGAIVMTLFQ
E. coli DWQ--SNMTREQYGEKFRQVQEYLHSGDCYQVNLAQRFHATYSGDEWQ
L. lactis GWNQLRLMNPGPYMVF--LDEANIIMASPEIVLADEANDLNTRPIAGT
E. coli AFLQLNQANRAPFSAFLRLEQGAILSLSPERFILCDNSEIQTRPIKGT
L. lactis LMRLNEQDEDGVNAACLGQHHKDRAEHNMIVDLVRNDLGRVGRFGSVN
E. coli LPRLPDPQEDSKNAVKLANSAKDRAENLMIVDLMRNDIGRVAVAGSVK
L. lactis VQEIVGAENYSVVHMIVSRVTGSLNEAFEAMEIIRAGFPGGSITGAPK
E. coli VPELFVVEPFPAVHHLVSTITAQLPEQLHASDLLRAAFPGGSITGAPK
L. lactis VRAMEIIEELEPQRRDGWGGSIGYIAYRGNIGYRIAIRTLFACNGQLF
E. coli VRAMEIIDELEPQRRNAWCGSIGYLSFCGNMDTSITIRTLAAINGQIF
L. lactis ASSGAGLVGDSMEDGEYNETFEKM-RALRSFFCAAVHMGKTPYLS
E. coli CSAGGGIVADSQEEAEYQETFDKVNRILKQLEK

and that of E. coli pabB. Using this analysis, the deduced amino acid sequence of the L. lactis subsp. lactis pabB was found to share high identity with pabB and trpE genes from other organisms (Table 13). Identity with trpE genes is expected, since the reactions catalyzed by pabB and trpE are similar. Complementation of the pabB mutation in E. coli and similarity of the ORF with other pabB genes indicated that the cloned DNA encoded a protein with pabB function.

XIV. Sequencing and analysis of the S. lividans PABA synthetase gene(s).

1. Sequencing of the gene(s).

The single strand tails of the 2.7-kb BamHI-SstI fragment of S. lividans genomic DNA that complemented pab mutations in E. coli and S. lividans were removed by digesting with the single strand specific mung bean nuclease to generate blunt ends. The endonuclease SmaI generates blunt ends and fragments containing blunt ends can be cloned into SmaI sites. The intent in creating blunt ends on the 2.7-kb fragment was to remove the SstI site, thereby allowing the unique SstI site on pBLUESCRIPT SK(-) to be used for protecting the T3 primer (see below). The blunt-ended 2.7-kb fragment was cloned into the SmaI site of pBLUESCRIPT SK(+) to give pDQ294. It was thev recovered for cloning into pBLUESCRIPT SK(-) by digesting pDQ294 with ClaI and XbaI. The 2.7-kb fragment was ligated it into the ClaI and XbaI sites of pBLUESCRIPT SK(-) to obtain

Table 13. Comparison of the amino acid sequence of the L. lactis subsp. lactis pabB gene with those of other genes.

Gene	% Identity
E. coli pabB	39.3
B. subtilis trpE	39.2
B. subtilis pabB	39.0
S. typhimurium pabB	38.6
K. aerogenes pabB	37.8
Pseudomonas aeroginosa trpE	32.5
Clostridium sp. trpE	31.1

pDQ295. This allowed the generation of single strand templates that were complementary to those obtained from pDQ294 and its deleted versions.

Nested deletions of the fragment cloned into pBLUESCRIPT SK(+) were obtained by digesting pDQ294 with ApaI to generate a 3'-overhang that protected the T7 primer from exonuclease III digestion, and with ClaI to generate a 5'-overhang that served as a substrate for exonuclease III digestion into the cloned fragment. Nested deletions of the fragment cloned into pBLUESCRIPT SK(-) were obtained by digesting pDQ295 with SstI to generate a 3'-overhang that protected the T3 primer from exonuclease III digestion and with XbaI to generate a 5'-overhang that served as a substrate for exonuclease digestion into the cloned fragment.

Colonies carrying deleted plasmids of desired sizes were used to generate single strand templates by infecting with the helper phage VCMS13 and the templates were used for DNA sequencing reactions using the T7 primer (for nested deletions obtained from pDQ294) or the T3 primer (for nested deletions obtained from pDQ295). The sequencing reactions were run on polyacrylamide gels and after the gels were read, the sequence was aligned using overlaps from each two successive sequences. These were obtained from clones that contained plasmids differing in size by approximately 200-bp.

2. Open reading frames.

Figure 38 shows the nucleotide sequence of the 2.7-kb SstI-BamHI S. lividans fragment that complemented pab mutations in E. coli and S. lividans. Two ORFs were identified in this sequence. The first starts with the ATG start site at nt 119 and terminates with the TGA site at nt 1547. It encodes a 476 amino acid polypeptide of molecular weight 52,000. The second ORF starts with the ATG start site at nt 1552 and terminates with the TGA site at nt 2130. It encodes a 193 amino acid polypeptide of molecular weight 20,700. The stop site of the first ORF overlaps the RBS of the second (see below). The molecular weights of these polypeptides compare favourably with pabB and pabA polypeptides, respectively, from other organisms.

The presence of two ORFs encoding polypeptides similar in size to PabB and PabA enzymes of other organisms is consistent with the complementation of both pabB and pabA mutations in E. coli by the cloned S. lividans fragment.

3. Control sequences.

A putative promoter sequence comprising a -35 region, a 16-bp spacer and a -10 region was located upstream of the first ORF. This sequence is notably similar to the SEP sequences described for Streptomyces (Table 14). No promoter sequences were located upstream of the second ORF. The small intergenic region between the two ORFs suggests that they are

Figure 38: Nucleotide sequence of the 2.7-kb BamHI-SstI S. lividans 1326 fragment that complemented pab mutations in E. coli and S. lividans. The putative promoter sequence, and the RBSs of the the two ORFs are underlined. The two ORFs are indicated from their ATG (bold face) start codons. The termination codons of the two ORFs are also in bold face.

```
      |   10      |   20      |   30      |   40      |   50      |   60
    1 GAGCTCCGCA ATCGGTGCCT TGACCCGCTA  GGCTCCGCTA CTAGCTCCGC GCTTGCACTT 60
   61 GACGGCAATC GTGGACTGCG TAGAGTGGCA  TCGTGGACCG TGGCGAGTAC GGGAGGAAAT 120
  121 GGCGAGCTGC CGGATGGGCG CGCGGTCGGC  GCTGGAGCCG TGCCAGGTGG ACTGCCTAGA 180
  181 CGAGGCGGCG GACGAGCGGT GCGCTGAGAC  GCCGAGGTGC CACGCAGAGC TGCTGGAGAG 240
  241 CGTGACGGGC GCGTCGCGGA TGTCGCGGTA  CTCGATCATC GTGCTGGACC CGATCGGCAC 300
  301 GATCCGGGCG GCAGAGGCGC TGACGGCGCT  GGTGGACGCG GACGACGTGA TCTTCAAGGA 360
  361 CGAGGACCCG CTGAAGGGCA TCCGGTCGGT  GTTCGAGCTG GGCGACCTGG ACCCGACGAA 420
  421 CCACGAGGAA ATCGAGTTCC AGGGCGGCGC  GCTGGGCCGG TTCGCGTACG ACATCGCGCG 480
  481 GCGACTGGAG GCGATCCGGG ACCTGGGCGA  CCGGGAGCTG GCAGGCCCGG ACGCGGGCAC 540
  541 GGCGCTGTAC GACCTGATCC TGTACGACCA  CCAGGACGAC GTGATCTGGA TTCTGGTACC 600
  601 CAACGAGGCG GGCGAGCAGG ACCCAAGCGA  GGACTTCCGG GACCTGGTGA ACGCGTGGTC 660
  661 GTACGACGAC GAGTTCGACA TCGGCGCGGA  GTTCGGCGCG AACTACACGG ACGATGCGTA 720
  721 CGCGGACGGC GTGGACCGGC TGAAGGACTA  CCTGGGCTCG GGCGACATGT ACCAGGTGAA 780
  781 CCTGGCGCAG CGGCGGGTGG GCATGATCAG  CGCGGAGGAC TACCAGCTGT ACATCCGACT 840
  841 GCGGGACGCG AACCCGGCGC CGTACATGGC  GTACCTGGAC ATCGACGAGG GACTGCTGGT 900
  901 GGCGAGCCCG GAGCGGATCA TCCTGGACGA  GGCGTCGGAC CTGGACACGC GGCCGATCGC 960
  961 GGGCACGCTG CGAGGCCGGC CGCGGGCGGG  CGGAGACGAC GAGGACGACG GACGGGCGAT 1020
 1021 CGACCTGCTG CGGGTGGACA AGGACCGGGC  GGAGCGGATC ATGATCGTGG ACCTGGACCG 1080
 1081 GAACGACATC GCGCGGGTGG GCGTGGGCGG  AAGCGTGAAG GTGCGGGAGA TCATGGGCCT 1140
 1141 GGAGCGGTAC AGCGGCGTGA TGCACCTGGT  GAGCCAGGTG ACGGGCG TC TGCAGGAAGC 1200
 1201 GATCGAGGCG GTGGACCTGA TCCGGGCGGG  CTTCCCGGGA GGCACGCTGA CGGGCGCGCC 1260
 1261 GAAGGTGCGG ACGATGGAGA TCATAGACGA  GCTGGAGCCG CAGCGGCGAG CGGCCTACTG 1320
 1321 CGGCAGCATC GGCTACATCG CGTACAAGGG  CAACATCGAC TTCAAGATCG CGATCCCGAC 1380
 1381 GCTGTACGCA CTGGCGGGCC AGCTGTTCTG  CCAGGCGGGC GGAGGCGTGG TGGGAGACAG 1440
 1441 TGTGCCGGAC GGCGAGTACC GGGAGAGCTT  CGAGAAGGGA AACGCGCTGA TCCGGGGCCT 1500
 1501 GGAGATCCGG CATGGCGCGG TGGTGGCGCA  GTCGGAGGAC AAGTGAGGTA AATGACGAGC 1560
 1561 GTCCTGATGA TCGACAACTG CGACAGCTTT  ACGTACAACC TGGTGGACCA GTTCAGCCCG 1620
 1621 CACGGCACGA TCGTCATCGT GAAGCGGAAC  CACCCGTTCT ACGACGGCGA GATCGAAGCA 1680
 1681 ATCATGGCGC TGACGAGCAT CTGCATCACG  CCGGGACCGT GCTACCCGGC AGAGGCGGCA 1740
 1741 CTGAACTCGT GCAGCATCAT CGGACACCTC  GCGGGCCGCA TCCCGATCCT GGGCATCTGC 1800
 1801 CTTGGACAGC AGGCGTTGGG ACAGGCGCGC  GGCGGCTTGG TCATCTTCGC GCACGGAAAG 1860
 1861 TTGTCCAACA TCGAGCACAA CGGAATCTTC  GCGCCACTGT TCAACCCGCC GCGGGCGCTG 1920
 1921 CCGGCGGGCC GATACCACTC GCTGGTCGTC  GAGCCGGCGC GCATCGAGGT CACGGGCCAG 1980
 1981 TGCAACCAGC TGGAGGTCGT CCCGCAGGAG  ATCATGGCGA TCCGGCACCG GGACCTGCCG 2040
 2041 GTGGAGGGCG TGCAGTTCCA CCCGGAGTCG  ATCCTGTCGT CGAACGGCGC GGCGATCCTG 2100
 2101 GCGAACTTGA TCCACCGGCC GTGCCACTGA  CCGTGCAAGC TGACTTAGCC GTAGCCTAGC 2160
 2161 GATGCCATAT GGCACGGACG TAGCAGGGCA  TGCCAGGACT GCACCTGAAC GCGTCGATCC 2220
 2221 ATGGCCAGCA TCAGCCGTGC GGACGCCTGC  ATCAGCGGCG GATGGCGGTA GCGCTCGGCA 2280
 2281 CCTCGAAGCA TTCCGCGGTT GGCACAGCCA  AGGGCGGAAT GCTTCCGGTA CGCACCGCTG 2340
 2341 CGGAGCTAGC TCGTAGCGCT CCGAGGTCGC  CTGGACGTCG AGTTGCTCAG GCGGACCTGT 2400
 2401 CGTGCCGATG CAGGCGTGCG GCACCGTGCC  GTGCGTACCA GACCGTGACG GGCAGCGCAG 2460
 2461 CCGATGCACG GCGTGCGATG CATGCAGCGC  TGCCGGTGCC ATGCGGTCTG CGATGCTGAC 2520
 2521 GGCGGTGGCG GTGACGGTGG CGTCGTGGTC  GGTCCGACAT GCGGTGCAGT CGTATGCGTG 2580
 2581 ACGTGCACGT GCACCGGTGC GGCGACCCGT  GACTGCATGC GTGCTACGTG ACGGGCTGTG 2640
 2641 CGTGGACGTC CCGTGACGTG ACGTGGCGAG  CGGTGCGACG CGACCCGCGT GCGACGACCG 2700
 2701 CACTGCGGAT CC                                                     2712
      |   10      |   20      |   30      |   40      |   50      |   60
```

Figure 38

Table 14. Comparison of putative S. lividans pab promoter with other promoters.

Promoter	-35	spacer	-10
E. coli[1]	TTGACA	17-bp	TATAAT
Streptomyces[2]	TTGACA	18-bp	TAGGAT
SEP2[3]	TTGACG	18-bp	TAAAAT
SEP3[3]	TTGACA	16-bp	CATCAT
SEP6[3]	TGGACA	17-bp	TTATAT
S. lividans (pab)	TTGACA	16-bp	TAGAGT

1. Data obtained from Rosenberg and Court (1979).
2. Data obtained from Hopwood et al. (1985).
3. Data obtained from Jaurin and Cohen (1985).

transcribed as a polycistronic message. The presence of an E. coli-type promoter on the sequence would explain expression of the genes in E. coli when the fragment was cloned in the vector in opposite orientations. The genes are probably trarscribed in E. coli as well as in S. lividans from this promoter.

Immediately upstream of each ORF, a sequence that shares complementarity with the 3'-end of the 16s rRNA of S. lividans was identified, and these could serve as the RBS (Table 15).

4. Deduced amino acid sequences of the ORFs and comparison with those of other proteins.

The deduced amino acid sequence of the first ORF (ORF1) is shown in Fig. 39. The amino acid composition (Table 16) shows that the enzyme contains 42.2% hydrophobic, 26.9% hydrophilic, 19.4% acidic and 11.4% basic amino acids. These values compare well with those of the pabB polypeptides from E. coli and L. lactis subsp. lactis. The deduced amino acid sequence of ORF1 was compared with amino acid sequences of other polypeptides using the Genbank FASTA programme. Figure 40 shows the comparison with the amino acid sequence of the L. lactis subsp. lactis pabB polypeptide. The deduced amino acid sequence of ORF1 showed high similarity with pabB and trpE polypeptides from other organisms (Table 17). This indicated that ORF1 encoded a polypeptide with pabB function.

The deduced amino acid sequence of the second ORF (ORF2)

Table 15. Comparison of putative ribosome-binding sites of S. lividans pab genes with the S. lividans consensus sequence.

pabB	GAGGAA
pabA	GAGGTA
Region complementary to S. lividans 16S rRNA*	GAAAGA

* Data obtained from Seno and Baltz (1989).

Figure 39: Deduced amino acid sequence of the first ORF (pabB) in the pab-complementing 2.7-kb BamHI-SstI S. lividans fragment. The numbers on top of the nucleotide sequence represent the nucleotide/amino acid positions in the sequences.

```
                                                    1         11        21        31
                                                    GAGCTCCGCAATCGGTGCCTTGACCCGCTAGGCTCCGC
  41        51        61        71                  81        91        101
TACTAGCTCCGCGCTTGCACTTGACGGCAATCGTGGACTGCGTAGAGTGGCATCGTGGACCGTGGCGAGTACGGGAGGAA

119/1                                           149/11
ATG GCG AGC TGC CGG ATG GGC GCG CGG TCG GCG CTG GAG CCG TGC CAG GTG GAC TGC CTA
Met ala ser cys arg met gly ala arg ser ala leu glu pro cys gln val asp cys leu
179/21                                          209/31
GAC GAG GCG GCG GAC GAG CGG TGC GCT GAG ACG CCG AGG TGC CAC GCA GAG CTG CTG GAG
asp glu ala ala asp glu arg cys ala glu thr pro arg cys his ala glu leu leu glu
239/41                                          269/51
AGC GTG ACG GGC GCG TCG CGG ATG TCG CGG TAC TCG ATC ATC GTG CTG GAC CCG ATC GGC
ser val thr gly ala ser arg met ser arg tyr ser ile ile val leu asp pro ile gly
299/61                                          329/71
ACG ATC CGG GCG GCA GAG GCG CTG ACG GCG CTG GTG GAC GCG GAC GAC GTG ATC TTC AAG
thr ile arg ala ala glu ala leu thr ala leu val asp ala asp asp val ile phe lys
359/81                                          389/91
GAC GAG GAC CCG CTG AAG GGC ATC CGG TCG GTG TTC GAG CTG GGC GAC CTG GAC CCG ACG
asp glu asp pro leu lys gly ile arg ser val phe glu leu gly asp leu asp pro thr
419/101                                         449/111
AAC CAC GAG GAA ATC GAG TTC CAG GGC GGC GCG CTG GGC CGG TTC GCG TAC GAC ATC GCG
asn his glu glu ile glu phe gln gly gly ala leu gly arg phe ala tyr asp ile ala
479/121                                         509/131
CGG CGA CTG GAG GCG ATC CGG GAC CTG GGC GAC CGG GAG CTG GCA GGC CCG GAC GCG GGC
arg arg leu glu ala ile arg asp leu gly asp arg glu leu ala gly pro asp ala gly
539/141                                         569/151
ACG GCG CTG TAC GAC CTG ATC CTG TAC GAC CAC CAG GAC GAC GTG ATC TGG ATT CTG GTA
thr ala leu tyr asp leu ile leu tyr asp his gln asp asp val ile trp ile leu val
599/161                                         629/171
CCC AAC GAG GCG GGC GAG CAG GAC CCA AGC GAG GAC TTC CGG GAC CTG GTG AAC GCG TGG
pro asn glu ala gly glu gln asp pro ser glu asp phe arg asp leu val asn ala trp
659/181                                         689/191
TCG TAC GAC GAC GAG TTC GAC ATC GGC GCG GAG TTC GGC GCG AAC TAC ACG GAC GAT GCG
ser tyr asp asp glu phe asp ile gly ala glu phe gly ala asn tyr thr asp asp ala
719/201                                         749/211
TAC GCG GAC GGC GTG GAC CGG CTG AAG GAC TAC CTG GGC TCG GGC GAC ATG TAC CAG GTG
tyr ala asp gly val asp arg leu lys asp tyr leu gly ser gly asp met tyr gln val
779/221                                         809/231
AAC CTG GCG CAG CGG CGG GTG GGC ATG ATC AGC GCG GAG GAC TAC CAG CTG TAC ATC CGA
asn leu ala gln arg arg val gly met ile ser ala glu asp tyr gln leu tyr ile arg
839/241                                         869/251
CTG CGG GAC GCG AAC CCG GCG CCG TAC ATG GCG TAC CTG GAC ATC GAC GAG GGA CTG CTG
leu arg asp ala asn pro ala pro tyr met ala tyr leu asp ile asp glu gly leu leu
899/261                                         929/271
GTG GCG AGC CCG GAG CGG ATC ATC CTG GAC GAG GCG TCG GAC CTG GAC ACG CGG CCG ATC
val ala ser pro glu arg ile ile leu asp glu ala ser asp leu asp thr arg pro ile
959/281                                         989/291
GCG GGC ACG CTG CGA GGC CGG CCG CGG GCG GGC GGA GAC GAC GAG GAC GAC GGA CGG GCG
ala gly thr leu arg gly arg pro arg ala gly gly asp asp glu asp asp gly arg ala
1019/301                                        1049/311
ATC GAC CTG CTG CGG GTG GAC AAG GAC CGG GCG GAG CGG ATC ATG ATC GTG GAC CTG GAC
ile asp leu leu arg val asp lys asp arg ala glu arg ile met ile val asp leu asp
1079/321                                        1109/331
CGG AAC GAC ATC GCG CGG GTG GGC GTG GGC GGA AGC GTG AAG GTG CGG GAG ATC ATG GGC
arg asn asp ile ala arg val gly val gly gly ser val lys val arg glu ile met gly
1139/341                                        1169/351
CTG GAG CGG TAC AGC GGC GTG ATG CAC CTG GTG AGC CAG GTG ACG GGC GAC CTG CAG GAA
```

Figure 39

```
leu glu arg tyr ser gly val met his leu val ser gln val thr gly asp leu gln glu
1199/361                                1229/371
GCG ATC GAG GCG GTG GAC CTG ATC CGG GCG GGC TTC CCG GGA GGC ACG CTG ACG GGC GCG
ala ile glu ala val asp leu ile arg ala gly phe pro gly gly thr leu thr gly ala
1259/381                                1289/391
CCG AAG GTG CGG ACG ATG GAG ATC ATA GAC GAG CTG GAG CCG CAG CGG CGA GCG GCC TAC
pro lys val arg thr met glu ile ile asp glu leu glu pro gln arg arg ala ala tyr
1319/401                                1349/411
TGC GGC AGC ATC GGC TAC ATC GCG TAC AAG GGC AAC ATC GAC TTC AAG ATC GCG ATC CCG
cys gly ser ile gly tyr ile ala tyr lys gly asn ile asp phe lys ile ala ile pro
1379/421                                1409/431
ACG CTG TAC GCA CTG GCG GGC CAG CTG TTC TGC CAG GCG GGC GGA GGC GTG GTG GGA GAC
thr leu tyr ala leu ala gly gln leu phe cys gln ala gly gly gly val val gly asp
1439/441                                1469/451
AGT GTG CCG GAC GGC GAG TAC CGG GAG AGC TTC GAG AAG GGA AAC GCG CTG ATC CGG GGC
ser val pro asp gly glu tyr arg glu ser phe glu lys gly asn ala leu ile arg gly
1499/461                                1529/471
CTG GAG ATC CGG CAT GGC GCG GTG GTG GCG CAG TCG GAG GAC AAG TGA
leu glu ile arg his gly ala val val ala gln ser glu asp lys OPA
```

Figure 39

Table 16. Amino acid composition of the S. lividans pabB gene.

			n	n(%)	Amino acid type
A	ala	alanine	52	10.9	hydrophobic
C	cys	cysteine	7	1.5	hydrophobic
D	asp	aspartic acid	55	11.6	acidic
E	glu	glutamic acid	37	7.8	acidic
F	phe	phenylalanine	11	2.3	hydrophobic
G	gly	glycine	46	9.7	hydrophobic
H	his	histidine	5	1.1	basic
I	ile	isoleucine	35	7.4	hydrophobic
K	lys	lysine	10	2.1	basic
L	leu	leucine	44	9.2	hydrophobic
M	met	methionine	10	2.1	hydrophobic
N	asn	asparagine	9	1.9	hydrophilic
P	pro	proline	18	3.8	hydrophobic
Q	gln	glutamine	13	2.7	hydrophilic
R	arg	arginine	39	8.2	basic
S	ser	serine	20	4.2	hydrophilic
T	thr	threonine	14	2.9	hydrophilic
V	val	valine	29	6.1	hydrophobic
W	trp	tryptophan	2	0.4	hydrophobic
X	---	unknown	-	-	
Y	tyr	tyrosine	19	4.0	hydrophilic
Z	---	STOP	1	0.2	

n represents the number of residues of the amino acid present in the protein.

n(%) represents the percentage of the amino acid present in the protein.

Figure 40: Comparison of the deduced amino acid sequence of the S. lividans pabB gene with that of the L. lactis subsp. lactis pabB gene. Hyphens indicate gaps introduced to increase similarity. Two dots indicate same amino acid in the two sequences at that position. One dot indicates similar amino acids in the two sequences at that position.

Figure 40


S. lividans MASCRMGARSALEPCQDVCLDEAADERCAETPRCHAELLESVTGASR
L. lactis MFTISGVVLITRPVYDEGSLNYCQSGEMNNCILLESVEG--N
S. lividans MSRYSIIVLDPIGTIRAAEALTALVDADDVIFKDEDPLKGIRSVFEL
L. lactis KPRYSIGGAEPIGTINANAVLTAATYAEDVKFTDADPLNGTRVAICN
S. lividans GDLDPTNHEEIEFQGGALGRFAYDIARRLEAIRDLGDRELAGPDAGT
L. lactis G--EDTQQEEMGFQGGALGFYAYDVGRRLEGYNDLGIEDLAIPDLAG
S. lividans ALYDL-ILYDHQDDVIWILVPNEAGEKDP--SEDFRDL--VNAWSY-
L. lactis SSYEIGVSADHQNDVI-VLIAHASADGNDVFITSSRQLSMVAGPTCC
S. lividans -DDEFDIGAEFGANYTDDAYAD---GVDRLKDYLGSGDMYQVNLAQR
L. lactis ASGDVEILRNKWHYYGVIPFSQDDCGFNRLKDYLGSGDMYQVNLGNR
S. lividans RVGMISAEDYQLYIRLRDANPAPYMAYLDIDEGLLVASPERIILDEA
L. lactis NVGAIVMTLFQGYNQLRLLMNPGPYMVFLDEANIIMASPEIVLADEA
S. lividans SDLDTRPIAGTLRGRPRAGGDDEDDGRAIDLLRVDK-DRAERIMIVD
L. lactis NDLNTRPIAGTLM---RLNEQDEDGVNAACLGQHHKDDRAEHMMIVD
S. lividans LDRNDIARVGVGGSVKVREIMGLERYSGVHMLVSQVGTDLQEAIEAV
L. lactis LVRNDLGRVGRFGSVNVQEIVGAENYSVVHMIVSRVTGSLNEAFEAM
S. lividans DLIRAGFPGGTLTGAPKVRTMEIIDELEPQRRAAYCGSIGYIAYKGN
L. lactis EIIRAGFPGGSITGAPKVRAMEIIEELEPQRRDGVYGGSIGYIAYRGN
S. lividans IDFKIAIPTLYALAGQLFCQAGGGVVGDSVPDGEYRESFEKGNALIR
L. lactis IGYRIAIRTLFACNGQLFASSGAGLVGDSNEDGEYNETFEKMRALRS

Table 17. Comparison of the amino acid sequence of the S. lividans pabB gene with those of other genes.

Gene	% Identity
L. lactis subsp. lactis pabB	48.5
B. subtilis pabB	43.5
E. coli pabB	43.1
Clostridium sp. trpE	41.9
S. typhimurium pabB	41.8
E. coli trpE	41.1
K. aerogenes pabB	41.0
B. subtilis trpE	40.7

is shown in Fig. 41. The amino acid composition (Table 18) shows that it contained 48.7% hydrophobic, 32.1% hydrophilic, 10.9% basic and 7.8% acidic amino acid residues. These values are similar to the 45.9% hydrophobic, 29.9% hydrophilic, 13.3% basic and 10.6% acidic amino acid residues reported for the E. coli pabA polypeptide (Kaplan et al., 1985). Comparison of the deduced amino acid sequence of ORF2 with the amino acid sequence of other polypeptides (Fig. 42 is an example of this comparison with the E. coli pabA polypeptide) indicated high similarity with pabA and trpG polypeptides from other organisms (Table 19). Since both pabA and trpG encode a glutamine amidotransferase function, similarity with trpG polypeptides was expected. Complementation of the pabA mutation in E. coli and similarity with pabA polypeptides from other organisms indicated that ORF2 encoded a polypeptide with pabA function.

5. Codon usage in ORFs 1 and 2.

Tables 20 and 21 show the codon usage in ORFs 1 and 2. Codons rich in G or C were preferred to those containing high A or T in both ORFs. The G+C content was 69.1% and 64.4% for ORFs 1 and 2 respectively. The entire 2.7-kb sequence contains 68% G+C. This value is similar to the 73% G+C content of S. lividans genomic DNA.

Table 22 compares codon usage in the S. lividans pabA and pabB genes with average values for Streptomyces genes. Codons

```
1552/1                                  1582/11
ATG ACG AGC GTC CTG ATG ATC GAC AAC TGC GAC AGC TTT ACG TAC AAC CTG GTG GAC CAG
Met thr ser val leu met ile asp asn cys asp ser phe thr tyr asn leu val asp gln
1612/21                                 1642/31
TTC AGC CCG CAC GGC ACG ATC GTC ATC GTG AAG CGG AAC CAC CCG TTC TAC GAC GGC GAG
phe ser pro his gly thr ile val ile val lys arg asn his pro phe tyr asp gly glu
1672/41                                 1702/51
ATC GAA GCA ATC ATG GCG CTG ACG AGC ATC TGC ATC ACG CCG GGA CCG TGC TAC CCG GCA
ile glu ala ile met ala leu thr ser ile cys ile thr pro gly pro cys tyr pro ala
1732/61                                 1762/71
GAG GCG GCA CTG AAC TCG TGC AGC ATC ATC GGA CAC CTC GCG GGC CGC ATC CCG ATC CTG
glu ala ala leu asn ser cys ser ile ile gly his leu ala gly arg ile pro ile leu
1792/81                                 1822/91
GGC ATC TGC CTT GGA CAG CAG GCG TTG GGA CAG GCG CGC GGC GGC TTG GTC ATC TTC GCG
gly ile cys leu gly gln gln ala leu gly gln ala arg gly gly leu val ile phe ala
1852/101                                1882/111
CAC GGA AAG TTG TCC AAC ATC GAG CAC AAC GGA ATC TTC GCG CCA CTG TTC AAC CCG CCG
his gly lys leu ser asn ile glu his asn gly ile phe ala pro leu phe asn pro pro
1912/121                                1942/131
CGG GCG CTG CCG GCG GGC CGA TAC CAC TCG CTG GTC GTC GAG CCG GCG CGC ATC GAG GTC
arg ala leu pro ala gly arg tyr his ser leu val val glu pro ala arg ile glu val
1972/141                                2002/151
ACG GGC CAG TGC AAC CAG CTG GAG GTC GTC CCG CAG GAG ATC ATG GCG ATC CGG CAC CGG
thr gly gln cys asn gln leu glu val val pro gln glu ile met ala ile arg his arg
2032/161                                2062/171
GAC CTG CCG GTG GAG GGC GTG CAG TTC CAC CCG GAG TCG ATC CTG TCG TCG AAC GGC GCG
asp leu pro val glu gly val gln phe his pro glu ser ile leu ser ser asn gly ala
2092/181                                2122/191
GCG ATC CTG GCG AAC TTG ATC CAC CGG CCG TGC CAC TGA
ala ile leu ala asn leu ile his arg pro cys his OPA
```

Figure 41: Deduced amino acid sequence of the second ORF in the pab-complementing 2.7-kb BamHI-SstI S. lividans fragment. The numbers on top of the nucleotide sequence represent the nucleotide/amino acid positions in the sequence.

Table 18. Amino acid composition of the S. lividans pabA gene.

		n	n(%)	Amino acid type
A ala	alanine	17	8.8	hydrophobic
C cys	cysteine	7	3.6	hydrophobic
D asp	aspartic acid	5	2.6	acidic
E glu	glutamic acid	10	5.2	acidic
F phe	phenylalanine	7	3.6	hydrophobic
G gly	glycine	16	8.3	hydrophobic
H his	histidine	10	5.2	basic
I ile	isoleucine	21	10.9	hydrophobic
K lys	lysine	2	1.0	basic
L leu	leucine	18	9.3	hydrophobic
M met	methionine	4	2.1	hydrophobic
N asn	asparagine	10	5.2	hydrophilic
P pro	proline	15	7.8	hydrophobic
Q gln	glutamine	8	4.1	hydrophilic
R arg	arginine	9	4.7	basic
S ser	serine	11	5.7	hydrophilic
T thr	threonine	6	3.1	hydrophilic
V val	valine	12	6.2	hydrophobic
W trp	tryptophan	-	-	hydrophobic
X ---	unknown	-	-	
Y tyr	tyrosine	4	2.1	hydrophilic
Z ---	STOP	1	0.5	

n represents the number of residues of the amino acid present in the protein.

n(%) represents the percentage of the amino acid present in the protein.

```
S. lividans  MTSVLMIDNCDSFTYNLVDQFSPHGTIVIVKRNHPFYDGEIEAIMAL
                . ... .... ..   .   .  . ....       . .
             ..............  . ..  .. .........  ........
E. coli        MILLIDNYDSFTWNLYQYFCELGADVLVKRNDALTLADIDALKP-

S. lividans  TSICITPGPCYPAEAALNSCSIIGHLAGRIPILGICLGQQAIGQAAG
               . . .... . ..       . . ... .... ... ..  .. .
             .. ..... .......  ... . ..................... .
E. coli      QKIVISPGPCTPDEAGISL-DVIRHYAGRLPILGVCLGHQAMAQAFG

S. lividans  GLVI----FAHGKLSNIEHN--GIFAPLFNPPRALPAGRYHSLVVEP
             . .       ... . . ..  . .  . ..   .  .........
             . ..      ... . ....  ...  . ..   ............
E. coli      GKVVRAAKVHMGKTSPITHNGEGVFRGLANP---LTVTRYHSLVVEP

S. lividans  ARIEVTGQLEV--VPQEIMAI-HRDLPVEGVQFHPESILSSNGAAIL
                          ... . ..    ............  .   .
             ... .  ....  ....... ...  ..................  ...
E. coli      DSLPACFDVTAWSETREIMGIRHRQWDLEGVQFHPESILSEQGHQLL

S. lividans  ANLIHRPCH
             ..  ..
             ......
E. coli      ANFLHR
```

Figure 42: Comparison of the deduced amino acid sequence of the S. lividans pabA gene with that of the E. coli pabA gene. Hyphens indicate gaps introduced to increase similarity. Two dots indicate the same amino acid in the two sequences at that position. One dot indicates similar amino acids in the two sequences at that position.

Table 19. Comparison of the amino acid sequence of the S. lividans pabA gene with those of other genes.

Gene	% Identity
E. coli pabA	48.4
B. subtilis trpG	47.6
A. calcoaceticus pabA	46.7
Serratia marcescens pabA	45.9
S. typhimurium pabA	44.8
K. aerogenes pabA	40.1
E. coli trpG	40.0
Shigella dysenteriae trpD	39.4

Table 20. Codon usage in the S. lividans pabB gene.

TTT	phe	F	-	TCT	ser	S	-	TAT	tyr	Y	-	TGT	cys	C	-
TTC	phe	F	11	TCC	ser	S	-	TAC	tyr	Y	19	TGC	cys	C	7
TTA	leu	L	-	TCA	ser	S	-	TAA	OCH	Z	-	TGA	OPA	Z	1
TTG	leu	L	-	TCG	ser	S	9	TAG	AMB	Z	-	TGG	trp	W	2
CTT	leu	L	-	CCT	pro	P	-	CAT	his	H	1	CGT	arg	R	-
CTC	leu	L	-	CCC	pro	P	1	CAC	his	H	4	CGC	arg	R	-
CTA	leu	L	1	CCA	pro	P	1	CAA	gln	Q	-	CGA	arg	R	4
CTG	leu	L	43	CCG	pro	P	16	CAG	gln	Q	13	CGG	arg	R	34
ATT	ile	I	1	ACT	thr	T	-	AAT	asn	N	-	AGT	ser	S	1
ATC	ile	I	33	ACC	thr	T	-	AAC	asn	N	9	AGC	ser	S	10
ATA	ile	I	1	ACA	thr	T	-	AAA	lys	K	-	AGA	arg	R	-
ATG	met	M	10	ACG	thr	T	14	AAG	lys	K	10	AGG	arg	R	1
GTT	val	V	-	GCT	ala	A	1	GAT	asp	D	1	GGT	gly	G	-
GTC	val	V	-	GCC	ala	A	1	GAC	asp	D	54	GGC	gly	G	38
GTA	val	V	1	GCA	ala	A	4	GAA	glu	E	2	GGA	gly	G	8
GTG	val	V	28	GCG	ala	A	46	GAG	glu	E	35	GGG	gly	G	-

Table 21. Codon usage in the S. lividans pabA gene.

TTT	phe	F	1	TCT	ser	S	-	TAT	tyr	Y	-	TGT	cys	C	-
TTC	phe	F	6	TCC	ser	S	1	TAC	tyr	Y	4	TGC	cys	C	7
TTA	leu	L	-	TCA	ser	S	-	TAA	OCH	Z	-	TGA	OPA	Z	1
TTG	leu	L	4	TCG	ser	S	5	TAG	AMB	Z	-	TGG	trp	W	-
CTT	leu	L	1	CCT	pro	P	-	CAT	his	H	-	CGT	arg	R	-
CTC	leu	L	1	CCC	pro	P	-	CAC	his	H	10	CGC	arg	R	3
CTA	leu	L	-	CCA	pro	P	1	CAA	gln	Q	-	CGA	arg	R	1
CTG	leu	L	12	CCG	pro	P	14	CAG	gln	Q	8	CGG	arg	R	5
ATT	ile	I	-	ACT	thr	T	-	AAT	asn	N	-	AGT	ser	S	-
ATC	ile	I	21	ACC	thr	T	-	AAC	asn	N	10	AGC	ser	S	5
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	-	AGA	arg	R	-
ATG	met	M	4	ACG	thr	T	6	AAG	lys	K	2	AGG	arg	R	-
GTT	val	V	-	GCT	ala	A	-	GAT	asp	D	-	GGT	gly	G	-
GTC	val	V	8	GCC	ala	A	-	GAC	asp	D	5	GGC	gly	G	10
GTA	val	V	-	GCA	ala	A	3	GAA	glu	E	1	GGA	gly	G	6
GTG	val	V	4	GCG	ala	A	14	GAG	glu	E	9	GGG	gly	G	-

Table 22. Comparison of codon usage in S. lividans pabA and pabB genes with average values for Streptomyces.

Amino acid	Codon	% usage of codon in gene: S. lividans pabA	S. lividans pabB	Streptomyces (average)*
Phe	TTT	14.3	0	3.3
	TTC	85.7	100	96.7
Leu	TTA	0	0	0.6
	TTG	22.2	0	3.3
	CTT	5.6	0	2.8
	CTC	5.6	0	37.2
	CTA	0	2.3	0.4
	CTG	66.6	97.7	55.8
Ile	ATT	0	2.9	3.8
	ATC	100	94.2	91.5
	ATA	0	2.9	4.6
Met	ATG	100	100	100
Val	GTT	0	0	2.9
	GTC	66.7	0	56.2

Table 22 (cont'd).

	GTA	0	3.5	2.9
	GTG	33.3	96.5	37.9
Ser	TCT	0	0	1.6
	TCC	9	0	37.4
	TCA	0	0	4.2
	TCG	45.5	45	25.3
	AGT	0	5	4
	AGC	45.5	50	27.5
Pro	CCT	0	0	4.2
	CCC	0	5.5	48.6
	CCA	6.7	5.5	1.1
	CCG	93.3	91	46.1
Thr	ACT	0	0	2.4
	ACC	0	0	72.8
	ACA	0	0	3
	ACG	100	100	21.7
Ala	GCT	0	1.9	3.2
	GCC	0	1.9	60
	GCA	17.6	7.7	4.7
	GCG	83.4	88.5	32.1

Table 22 (cont'd).

Tyr	TAT	0	0	6.9
	TAC	100	100	93.1
His	CAT	0	20	11.1
	CAC	100	80	88.9
Gln	CAA	0	0	8.6
	CAG	100	100	91.4
Asn	AAT	0	0	8.1
	AAC	100	100	91.9
Lys	AAA	0	0	8.8
	AAG	100	100	91.2
Asp	GAT	0	1.8	7.7
	GAC	100	98.2	92.3
Glu	GAA	10	5.4	22
	GAG	90	94.6	78
Cys	TGT	0	0	10.3
	TGC	100	100	89.7
Trp	TGG	0	100	100

Table 22 (cont'd).

Arg	CGT	0	0	9.3
	CGC	33.3	0	39.9
	CGA	11.1	10.2	5.5
	CGG	55.6	87.2	35.8
	AGA	0	0	2.3
	AGG	0	2.6	7.2
Gly	GGT	0	0	7.9
	GGC	62.5	82.7	62.8
	GGA	37.5	17.3	10.1
	GGG	0	0	19.2
Term	TAA	0	0	3.7
	TAG	0	0	29.6
	TGA	100	100	66.7

* Data obtained from Seno and Baltz (1989).

that are used frequently in Streptomyces are also used often in the S. lividans pabA gene. Although a similar trend is found in the pabB gene, some codons rarely used on average by Streptomyces are used with high frequency in the pabB gene.

Table 23 summarizes the G+C content of the three positions of the codons for ORFs 1 and 2. The G+C content at each codon position is similar in both ORFs to the average value reported for Streptomyces. The pattern in which the third position of the codons in Streptomyces is >90% G+C, has been used to identify genes from Streptomyces (Seno and Baltz, 1989). This pattern was observed in both ORFs.

6. Comparison of codon usage in ORFs 1 and 2 with those of related polypeptides from other organisms.

Table 24 compares the codon usage in the S. lividans pabB gene (ORF1) with related genes from other organisms. Codon usage in each organism reflects the G+C content of the organism; the third position of each codon indicates this codon bias. Table 25 summarizes the G+C content of the three positions of each codon for these genes. Whereas the G+C content of the first two positions of the codons are identical, there is much variability in the G+C content of the third position for each organism.

Comparison of codon usage in the S. lividans pabA gene (ORF2) with related genes is shown on Table 26. As in the ORF1 comparison, codon usage reflects the G+C content of the

Table 23. Percent G+C composition of the coding regions of the S. lividans pab genes compared with average values for Streptomyces.

Codon Position	% G+C pabA	pabB	Streptomyces average*
1	60.6	71.8	69.7
2	40.0	41.6	49.9
3	92.7	94.3	90.6
Coding region	64.4	69.1	70.1

* Data obtained from Seno and Baltz (1989)

Table 24. Comparison of codon usage in S. lividans pabB gene with those of related genes from other organisms.

Amino acid	Codon	Codons in gene[a] S. lividans pabB	E. coli pabB[b]	E. coli trpE[b]	L. lactis pabB
Phe	TTT	0(0)	13(62)	8(40)	0(0)
	TTC	11(100)	8(38)	12(60)	17(100)
Leu	TTA	0(0)	9(19)	7(11)	0(0)
	TTG	0(0)	4(9)	5(8)	34(97)
	CTT	0(0)	6(13)	6(9)	0(0)
	CTC	0(0)	5(11)	10(15)	0(0)
	CTA	1(2)	3(6)	4(6)	0(0)
	CTG	43(98)	20(43)	35(52)	1(3)
Ile	ATT	1(3)	16(64)	12(71)	0(0)
	ATC	33(93)	7(28)	5(29)	32(97)
	ATA	1(3)	2(8)	0(0)	1(3)
Met	ATG	10(100)	8(100)	12(100)	18(100)
Val	GTT	0(0)	6(22)	5(15)	0(0)

Table 24 (cont'd)

	GTC	0(0)	6(22)	6(18)	0(0)
	GTA	1(4)	6(22)	8(24)	33(97)
	GTG	28(96)	9(33)	14(42)	1(3)
Ser	TCT	0(0)	4(13)	6(16)	0(0)
	TCC	0(0)	4(13)	5(14)	1(4)
	TCA	0(0)	3(10)	4(11)	0(0)
	TCG	9(45)	3(10)	5(14)	0(0)
	AGT	1(5)	4(13)	4(11)	26(96)
	AGC	10(50)	13(42)	13(35)	0(0)
Pro	CCT	0(0)	3(13)	3(11)	3(20)
	CCC	1(6)	3(13)	5(18)	0(0)
	CCA	1(6)	8(35)	5(18)	11(73)
	CCG	16(91)	9(39)	15(54)	1(7)
Thr	ACT	0(0)	8(31)	4(16)	6(33)
	ACC	0(0)	9(35)	14(56)	0(0)
	ACA	0(0)	2(8)	4(16)	12(67)
	ACG	14(100)	7(27)	3(12)	0(0)
Ala	GCT	1(2)	6(16)	12(23)	2(5)
	GCC	1(2)	14(37)	19(36)	0(0)
	GCA	4(8)	8(21)	6(11)	39(93)
	GCG	46(88)	10(26)	16(30)	1(2)

Table 24 (cont'd)

Tyr	TAT	0(0)	8(73)	9(64)	0(0)
	TAC	19(100)	3(27)	5(36)	20(100)
His	CAT	1(20)	7(58)	7(64)	8(100)
	CAC	4(80)	5(42)	4(36)	0(0)
Gln	CAA	0(0)	8(27)	8(36)	14(93)
	CAC	13(100)	22(73)	13(62)	1(7)
Asn	AAT	0(0)	10(67)	10(59)	1(3)
	AAC	9(100)	5(33)	7(41)	29(97)
Lys	AAA	0(0)	10(77)	12(80)	0(0)
	AAG	10(100)	3(23)	3(20)	8(100)
Asp	GAT	1(2)	10(67)	10(59)	28(97)
	GAC	54(98)	6(21)	14(40)	1(3)
Glu	GAA	2(5)	25(81)	30(86)	30(97)
	GAG	35(95)	6(19)	5(14)	1(3)
Cys	TGT	0(0)	1(17)	5(42)	8(100)
	TGC	7(100)	5(83)	7(58)	0(0)

Table 24 (cont'd).

Trp	TGG	2(100)	7(100)	0(0)	2(100)
Arg	CGT	0(0)	10(36)	17(43)	0(0)
	CGC	0(0)	15(50)	21(53)	0(0)
	CGA	4(10)	1(4)	1(3)	2(8)
	CGG	34(87)	3(11)	0(0)	0(0)
	AGA	0(0)	0(0)	1(3)	24(92)
	AGG	1(3)	0(0)	0(0)	0(0)
Gly	GGT	0(0)	8(32)	11(39)	1(2)
	GGC	38(83)	13(52)	12(43)	0(0)
	GGA	8(17)	2(8)	3(11)	53(98)
	GGG	0(0)	2(8)	2(7)	0(0)
Term	TAA	0(0)	1(100)	0(0)	1(100)
	TAG	0(0)	0(0)	0(0)	0(0)
	TGA	1(100)	0(0)	0(0)	0(0)

a. Numbers in parentheses show the percentage of residues of the indicated amino acid coded by the indicated codon.

b. Data obtained from Goncharoff and Nichols (1984).

Table 25. Comparison of percent G+C content of the codon positions and the coding region of the S. lividans pabB gene with those of related genes from other organisms.

	% G+C			
	L. lactis pabB	E. coli pabB[1]	E. coli trpE[2]	S. lividans pabB
Genome	37	51	51	73
Coding region	41	51	55	69
Position 1	49	62	67	72
Position 2	39	40	46	42
Position 3	35	52	54	94

1. Data obtained from Goncharoff and Nichols (1984).
2. Data obtained from Nichols et al. (1985).

Table 26. Comparison of codon usage in the S. lividans pabA gene with those of related genes from other organisms.

Amino acid	Codon	Number of codons in gene[a]			
		E. coli pabA[b]	E. coli trpG[b]	S. marcescens pabA[c]	S. lividans pabA
Phe	TTT	5(71)	3(60)	4(50)	1(14)
	TTC	2(29)	2(40)	4(50)	6(86)
Leu	TTA	1(5)	3(13)	0(0)	0(0)
	TTG	2(10)	3(13)	3(12)	4(22)
	CTT	6(28)	1(4)	2(8)	1(6)
	CTC	2(10)	6(26)	3(12)	1(6)
	CTA	0(0)	0(0)	0(0)	0(0)
	CTG	10(47)	10(44)	18(69)	12(67)
Ile	ATT	7(59)	11(79)	3(33)	0(0)
	ATC	4(33)	2(14)	6(67)	21(100)
	ATA	1(8)	1(7)	0(0)	0(0)
Met	ATG	4(100)	7(100)	3(100)	4(100)
Val	GTT	3(21)	2(18)	0(0)	0(0)

Table 26 (cont'd).

	GTC	3(21)	2(18)	1(6)	8(33)
	GTA	1(7)	1(9)	0(0)	0(0)
	GTG	7(50)	6(55)	16(94)	4(67)
Ser	TCT	1(11)	2(20)	0(0)	0(0)
	TCC	1(11)	2(20)	3(33)	1(9)
	TCA	2(22)	0(0)	0(0)	0(0)
	TCG	2(22)	1(10)	2(22)	5(45)
	AGT	1(11)	2(20)	0(0)	0(0)
	AGC	2(22)	3(30)	4(45)	5(45)
Pro	CCT	2(20)	1(9)	0(0)	0(0)
	CCC	1(10)	2(18)	2(25)	0(0)
	CCA	5(50)	0(0)	0(0)	1(7)
	CCG	2(20)	8(73)	6(75)	14(93)
Thr	ACT	0(0)	0(0)	1(13)	0(0)
	ACC	4(44)	6(67)	6(75)	0(0)
	ACA	2(22)	1(11)	0(0)	0(0)
	ACG	3(33)	2(22)	1(13)	6(100)
Ala	GCT	1(7)	3(16)	1(6)	0(0)
	GCC	5(33)	6(32)	9(53)	0(0)
	GCA	4(27)	2(10)	0(0)	3(18)
	GCG	5(33)	8(44)	7(41)	14(82)

Table 26 (cont'd)

Tyr	TAT	1(20)	2(40)	2(40)	0(0)
	TAC	4(80)	3(80)	3(80)	4(100)
His	CAT	7(78)	7(70)	5(56)	0(0)
	CAC	2(22)	3(30)	4(44)	10(100)
Gln	CAA	3(38)	2(22)	0(0)	0(0)
	CAG	5(62)	7(78)	8(100)	8(100)
Asn	AAT	2(33)	4(40)	1(17)	0(0)
	AAC	4(67)	6(60)	5(83)	10(100)
Lys	AAA	4(67)	2(67)	3(75)	0(0)
	AAG	2(33)	1(33)	1(25)	2(100)
Asp	GAT	7(64)	4(57)	6(60)	0(0)
	GAC	4(36)	3(43)	4(40)	5(100)
Glu	GAA	7(78)	8(100)	6(50)	1(10)
	GAG	2(22)	0(0)	6(50)	9(90)
Cys	TGT	2(50)	2(67)	2(50)	0(0)
	TGC	2(50)	1(33)	2(50)	7(100)
Trp	TGG	3(100)	1(100)	1(100)	0(0)

Table 26 (cont'd)

Arg	CGT	0(0)	3(33)	3(23)	0(0)
	CGC	8(80)	6(67)	9(69)	3(33)
	CGA	1(10)	0(0)	0(0)	1(11)
	CGG	1(10)	0(0)	1(8)	5(56)
	AGA	0(0)	0(0)	0(0)	0(0)
	AGG	0(0)	0(0)	0(0)	0(0)
Gly	GGT	4(27)	6(32)	1(7)	0(0)
	GGC	5(33)	8(42)	12(80)	10(63)
	GGA	1(7)	3(16)	0(0)	6(37)
	GGG	5(33)	2(10)	2(14)	0(0)
Term	TAA	1(100)	1(100)	1(100)	0(0)
	TAG	0(0)	0(0)	0(0)	0(0)
	TGA	0(0)	0(0)	0(0)	1(100)

a. Numbers in parentheses show the percentage of residues of the indicated amino acid coded by the indicated codon.

b. Data obtained from Kaplan and Nichols (1983).

c. Data obtained from Kaplan et al. (1985).

organism. The G+C content of the three codon positions also shows similarity in the first two positions but variability in the third (Table 27).

Table 27. Comparison of percent G+C content of the codon positions and the coding region of the S. lividans pabA gene with those of related genes from other organisms.

	% G+C			
	E. coli pabA[1]	E. coli trpG[1]	S. marcescens pabA[2]	S. lividans pabA
Genome	51	51	59	73
Coding region	54	55	63	64
Position 1	64	61	69	61
Position 2	40	42	39	40
Position 3	58	61	81	93

1. Data obtained from Kaplan and Nichols (1983).
2. Data obtained from Kaplan et al. (1985).

DISCUSSION

This chapter first considers the results obtained when a PABA synthetase gene from L. lactis subsp. lactis was cloned in plasmid vectors and expressed in S. lividans and E. coli. This is followed by an evaluation of the growth and enzyme assays carried out with pab mutants of E. coli and S. lividans. The next section discusses cloning and expression of the S. lividans PABA synthetase genes in E. coli and S. lividans. Finally, data obtained from the nucleotide sequences of the L. lactis subsp. lactis and S. lividans PABA synthetase genes are discussed.

I. Cloning of the L. lactis subsp. lactis PABA synthetase gene.

1. Expression in S. lividans.

Genetic transformation procedures for L. lactis have only recently been developed; the best appears to involve the use of high-voltage electroporation (McIntyre and Harlander, 1989). Hitherto, most L. lactis genes have been cloned by expression in a heterologous host (Dao and Ferretti, 1985; David et al., 1990), the approach used here to clone the L. lactis subsp. lactis PABA synthetase gene.

The PABA synthetase gene cloned from L. lactis subsp. lactis complemented the pab mutations in S. lividans strains JG10 and AP3; however, a segment of the vector DNA was deleted

to allow expression from a vector promoter, presumably that for the aph gene. A similar genetic rearrangement accompanied expression in E. coli of the S. griseus PABA synthetase gene (Gil and Hopwood, 1983), although here it was a portion of the cloned insert rather than vector DNA that was deleted. In both cases expression appears to involve transcriptional readthrough from vector promoters, as also reported for expression of the E. coli chloramphenicol acetyltransferase (CAT) gene in S. lividans (Schottel et al., 1981). In CAT gene expression the RBS and transcriptional start site normally used in E. coli were also functional in S. lividans. Although a sequence resembling a RBS was found upstream of the L. lactis subsp. lactis PABA synthetase translational start point, further experimentation will be required to confirm its function in S. lividans.

Genetic rearrangement allowing expression of genes via transcriptional readthrough from vector promoters may be a consequence of the inability of the host RNA polymerase to recognize and utilize promoters from other organisms. Characterization of promoters from several organisms indicate that not only do promoter sequences differ from one organism to another but also they differ within the same organism. Several classes of promoter sequences have been identified in B. subtilis (Helmann et al., 1988), E. coli (Grossman et al., 1984; Hirschman et al., 1985) and S. coelicolor A3(2) (Westpheling et al., 1985; Buttner et al., 1988; Buttner et

al., 1989). Recognition and utilization of a class of promoter sequences depends on the availability of an RNA polymerase holoenzyme containing a specific sigma factor. Some classes of promoter sequence from one organism can be recognized and utilized in other organisms; for example, the veg promoter from B. subtilis, which is recognized by the predominant form of RNA polymerase containing σ^A in B. subtilis is recognized and utilized in S. coelicolor (using RNA polymerase holoenzyme containing σ^{35}) (Westpheling et al., 1985) and in E. coli (using RNA polymerase holoenzyme containing σ^{70}) (Helmann and Chamberlain, 1988). On the other hand, there are certain classes of promoter sequences that are not recognized in other organisms because the appropriate sigma factor is lacking. Where this occurs and there is selection pressure for the phenotype encoded by a gene without a functional promoter, genetic rearrangements may occur and allow transcriptional readthrough from the host's vector promoter signals.

Although the high G+C content of Streptomyces DNA leads to a biased codon usage (Bibb et al., 1985), several genes from other organisms have been expressed in Streptomyces (Schottel et al., 1981; Chater et al., 1982; Kushstos and Rao, 1983; Shareck et al., 1984). It is also worthy of note that the genes for bovine growth hormone (Gray et al., 1984) and for human α2 interferon (Pulido et al., 1986) have been expressed in S. lividans from aphP, the promoter for an

aminoglycoside phosphotransferase gene in S. fradiae. Since S. lividans and L. lactis subsp. lactis have a markedly different G+C content (73% for S. lividans and 37% for L. lactis subsp. lactis), expression in S. lividans of the L. lactis subsp. lactis PABA synthetase gene indicates that the high G+C content of Streptomyces DNA is not an absolute barrier for the expression of foreign genes with a low in G+C content.

PABA was detected in culture broths of S. lividans clones containing the cloned PABA synthetase gene from L. lactis subsp. lactis. The clones were resistant to sulphanilamide at up to 5 ug mL^{-1}, and their ability to cross-feed pab mutants of S. lividans indicated that they overproduced PABA and excreted it into the medium. Under the same conditions the S. lividans TK24 parent of the pab mutants did not excrete PABA. This indicates that the cloned L. lactis subsp. lactis fragment is involved in PABA overproduction and suggests that the high resistance of L. lactis subsp. lactis strains NCDO496 and AV117 to sulphanilamide (up to 150 ug mL^{-1}) may involve overproduction of PABA. A similar mechanism of resistance has been observed in sulphonamide-resistant strains of Neisseria gonorrhoea (Landy and Gersrtong, 1944), Pneumococcus sp. (Tillet et al., 1943), Staphylococcus aureus (White and Woods, 1965) and S. griseus (Gil and Hopwood, 1983). In the case of S. griseus, the resistant strain also cross-fed pab mutants of Streptomyces. It is unlikely that the mechanism of

sulphanilamide resistance conferred by the cloned L. lactis subsp. lactis fragment involves the synthesis of a modified dihydropteroate synthetase that has a reduced affinity for sulphanilamide and an enhanced affinity for PABA since this would not result in overproduction of PABA.

2. Expression in E. coli.

Heterologous gene expression in microorganisms has centred on E. coli. This is due partly to the detail in which its genome organization has now been described and partly to the development of molecular biology techniques for introduction of heterologous genes. Genes from other prokaryotes such as B. subtilis and S. aureus, as well as lower eukaryotes such as Saccharomyces cerevisiae (Struhl and Davis, 1977) and Neurospora crassa (Vapnek et al., 1977) and higher eukaryotes (Mercereau-Puijalon et al., 1978; Villa-Komaroff et al., 1978) have been successfully expressed in E. coli.

Several L. lactis subsp. lactis genes have been cloned and expressed in E. coli (Dao and Ferretti, 1985). Expression of the L. lactis subsp. lactis PABA synthetase gene appears to involve an E. coli vector promoter, presumably the tet promoter of pBR322. Expression of the S. griseus PABA synthetase gene in E. coli also involved transcriptional readthrough from the tet promoter of pBR322. Insertion of Tn5 within the tet promoter of a pBR322 derivative containing the

S. griseus PABA synthetase gene abolished the Pab$^+$ phenotype. When the transposon was excised, the Pab$^+$ phenotype was restored (Gil and Hopwood 1983). Similar use of the pBR322 tet promoter for expression of the heterologous streptomycin-6-phosphotransferase gene from S. griseus in E. coli was reported by Ohnuki et al. (1985).

As in the expression of the L. lactis subsp. lactis PABA synthetase gene in S. lividans, transcriptional readthrough from a vector promoter in E. coli may be the result of failure to recognize a heterologous promoter signal. It must be noted that the nucleotide sequence of the entire 1.9-kb L. lactis subsp. lactis fragment that complemented the pabB mutation in E. coli did not reveal a canonical prokaryotic promoter sequence upstream of the translational start point. This may indicate that the cloned fragment lacks a promoter sequence. Alternatively, since the region upstream of the translational start point contains high A+T sequences typical of prokaryotic promoters (Rosenberg and Court, 1979), it is possible that a promoter sequence is present. Failure to locate a typical prokaryotic consensus promoter sequence within this region may be a result of the promoter being atypical and requiring ancillary proteins for expression. One of the promoters of the gal operon in E. coli (P1) has a typical -10 but a very poor -35 promoter consensus sequence. To compensate for the poor -35 region, the cAMP-CAP complex binds to the -35 region to allow the RNA polymerase to recognize this region as a

promoter sequence (Irani et al., 1983). Absence of the ancillary protein(s) required to transcribe the L. lactis subsp. lactis PABA synthetase gene from its own promoter sequence in S. lividans and E. coli could explain the need to use the host's vector promoter by transcriptional readthrough.

Complementation by the cloned L. lactis subsp. lactis fragment of only the pabB but not the pabA mutation in E. coli implies that the fragment encodes the aminase but not the amidotransferase function. This is confirmed by the presence of only a single ORF similar to pabB from other organisms within the 1.9-kb L. lactis subsp. lactis fragment, and the absence of an ORF corresponding to pabA from other organisms. The unlinked arrangement of pabA and pabB in L. lactis subsp. lactis contrasts with the arrangement in B. subtilis where pabA and pabB are not only linked but also form part of a folic acid biosynthetic operon (Slock et al., 1990). The pabA and pabB genes in S. lividans are also linked (see below). The arrangement of the pab genes in L. lactis subsp. lactis is similar to that in E. coli where the pabA and pabB mutations have been mapped at different sites of the chromosome (Huang and Gibson, 1970).

II. Characterization of E. coli and S. lividans pab mutants.

1. Escherichia coli.

Ito and Yanofsky (1969) used purified subunits of the

anthranilate synthetase complex to study substrate specificities of the subunits. They established that the trpE gene encoding one anthranilate synthetase subunit had aminase activity and that it used ammonium ions at pH between 7.5 and 8.0 for anthranilate synthesis. This subunit alone was unable to use L-glutamine as a nitrogen source for anthranilate synthesis. The second subunit encoded by the trpG(D) gene was required together with the first subunit when L-glutamine was used as the nitrogen source for anthranilate synthesis. From these observations, Ito and Yanofsky (1969) concluded that the trpG(D) product either activates a glutamine binding site on the trpE protein or provides this binding site itself. It was subsequently shown that trpG(D) is a glutamine amidotransferase, indicating that it provides the binding site itself (Kaplan and Nichols, 1983).

The results of the enzyme assays on the anthranilate synthetase subunits by Ito and Yanofsky (1969) were confirmed by Zalkin and Murphy (1975) using growth assays. An E. coli strain in which the gene encoding the glutamine amidotransferase subunit was deleted synthesized tryptophan using the ammonia-dependent anthranilate synthetase subunit. When ammonia was limiting, this strain was a tryptophan auxotroph. They also established that protonated ammonia (NH_4^+) could not be used for trptophan biosynthesis; rather, the ammonia must be in free form. Thus, at pH 6.0 where the ammonium salt used in the medium was >99.9% protonated, there

was no growth of the E. coli strain due to starvation for tryptophan. At pH values between 7.0 and 8.0 where a portion of the ammonium salt was unprotonated, some growth was observed. Zalkin and Murphy established that the free ammonia requirement for non-growth-limiting tryptophan biosynthesis was between 0.16 mM and 1.6 mM. As further proof that the aminase subunit alone used free ammonia for anthranilate synthesis, Zalkin and Murphy (1975) isolated mutants with increased aminase activity that grew in ammonia-limited media.

Because of the similarity between anthranilate synthetase and PABA synthetase, the approaches used by Ito and Yanofsky (1969) and by Zalkin and Murphy (1975) to characterize the functions of the two subunits by enzyme and growth assays seemed likely to be useful as means of characterizing pab mutants. To examine this possibility, they were used to confirm the identity of the mutations in the pab mutants of E. coli. When ammonium sulphate was used as the nitrogen source in growth assays using a defined medium, both strains AB3292 and AB3295 were unable to grow at pH 6.0. The inabilty to grow is presunably due to unavailability of free ammonia since at this pH >99.9% of the salt is protonated (Zalkin and Murphy, 1975). At pH 8.0 where 5.2% of the ammonium salt is unprotonated (Zalkin and Murphy, 1975), strain AB3292 but not strain AB3295 was able to grow. This is consistent with strain AB3292 having a functional aminase (pabB gene product) that allows it to use the free ammonia for PABA biosynthesis.

The mutation in strain AB3292 must therefore be in the pabA and/or pabX gene. Complementation of the mutation in strain AB3292 by the 2.7-kb S. lividans fragment, the nucleotide sequence of which revealed a gene identical to pabA genes from other organisms but not a pabX gene, places the mutation in the pabA gene. Enzyme assays in which cell extracts of strain AB3292 used ammonia for PABA synthesis supported the presence of a functional aminase (pabB) gene in strain AB3292.

Strain AB3295 did not grow when ammonium sulphate was used as the nitrogen source even though at pH 8.0 free ammonia was present. The absence of a functional aminase which this result implies places the mutation in pabB, or pabX, or in a combination of the genes involved in PABA biosynthesis. Complementation of the mutation in strain AB3295 by the L. lactis subsp. lactis fragment, the nucleotide sequence of which revealed a single gene identical to the pabB gene from other organisms, indicates that the mutation is in pabB. Also, cell extracts of strain AB3295 alone did not make PABA, but did so when mixed with an extract from strain AB3292 (which has a functional aminase). These results confirmed that the pabB gene product is lacking in strain AB3295.

2. Streptomyces lividans.

The pab mutants of S. lividans were both obtained by NTG mutagenesis of S. lividans TK24; strain JG10 was obtained by

Gil and Hopwood (1983) and strain AP3 was obtained by A. Paradkar in this laboratory. It was not possible to characterize the mutants by enzyme assays on cell extracts because of the low activity of the enzymes involved in the biosynthesis of PABA. Gil et al. (1985a) reported that PABA synthetase activity was detectable only in S. griseus strains that produced candicidin. That candicidin nonproducing strains of S. griseus that did not show PABA synthetase activity were not pab mutants was indicated by their growth on minimal medium lacking PABA. From these observations, Gil et al. (1985a) concluded that PABA synthetase was expressed in low, undetectable (by the assay procedure) amounts in the nonproducing strains and that this (or a second) enzyme was deregulated in producing strains allowed formation of enough PABA (detectable by the assay procedure) for candicidin biosynthesis. This conclusion was strengthened by the report of Gil et al. (1990) who showed that PABA synthetase activity was detectable in other species of Streptomyces that produce candicidin whereas nonproducing species showed undetectable amounts even though they were not pab mutants. PABA synthetase activity was also detectable by enzyme assay in cell extracts of S. coelicolor var. aminophilus producing fungimycin, a polyene macrolide that requires large amounts of PABA for biosynthesis (Gil et al., 1985a).

Since PABA synthetase activity could not be detected in S. lividans pab mutants by enzyme assays on cell extracts,

growth assays were carried out using agar cultures. Strain JG10 grew on SM medium containing ammonium sulphate as the sole nitrogen source but did not grow with asparagine. The ability to use ammonium sulphate to make PABA indicates the presence of functional aminase (pabB gene product) and pabX gene products. The mutation in strain JG10 is thus not in pabB or pabX. The inability to use asparagine as the sole nitrogen source suggests that the mutation in strain JG10 is in the glutamine amidotransferase (pabA) gene. However, nucleotide sequence analysis of the L. lactis subsp. lactis fragment that complemented the pab mutation in strain JG10 revealed only a single ORF that is identical to the pabB gene of other organisms. Moreover, the fragment complemented the pabB but not the pabA mutation in E. coli. A possible explanation for complementation of the pabA mutation in S. lividans JG10 by a pabB gene product from L. lactis subsp. lactis could be recruitment of a glutamine amidotransferase (maybe the mutant gene product in strain JG10 or some other glutamine amidotransferase) that interacts favourably with the L. lactis subsp. lactis pabB gene product. This would mean that no functional interaction occurs between the normal pabB gene product and the mutant pabA gene product or any other glutamine amidotransferase in strain JG10. The common evolution and high relatedness of glutamine amidotransferases (Kaplan et al., 1985) and the use of a single amphibolic glutamine amidotransferase for the biosynthesis of both PABA

and anthranilic acid in some microorganisms (Sawula and Crawford, 1973; Kane, 1977; Buvinger et al., 1981) argue in favour of recruitment. It is thus possible that in L. lactis subsp. lactis, the pabB gene product interacts with an amphibolic glutamine amidotransferase, the equivalent of which is present and functional in strain JG10.

Strain AP3 was unable to grow when ammonium sulphate was the sole nitrogen source. This indicates that the mutation could be in the pabB and/or the pabX gene(s). However, complementation of the mutation in strain AP3 by fragments from L. lactis subsp. lactis and S. lividans, the nucleotide sequences of which revealed genes identical to pabB genes of other organisms but not pabX, suggests that the mutation is in the pabB gene. To date, no pabX mutations have been described in any organism (Nichols et al., 1989; Slock et al., 1990).

III. Cloning of the S. lividans PABA synthetase genes.

1. Expression in E. coli.

The PABA synthetase genes of S. lividans were cloned as a single 4.8-kb fragment that complemented the pabA and pabB mutations in E. coli strains AB3292 and AB3295 respectively. A similar complementation of the pabA and pabB mutations in E. coli was obtained using a single fragment from S. griseus (Gil and Hopwood, 1983). Complementation of both mutations by a single fragment suggests linkage of the two genes in S.

lividans (see below). The S. lividans PABA synthetase genes were expressed when the 4.8-kb fragment was cloned in both orientations in the BamHI site of pBR322, suggesting that a Streptomyces promoter present on the fragment was used for expression in E. coli. This differs from the expression of the S. griseus PABA synthetase genes in E. coli where an E. coli vector promoter was implicated in expression.

There have been mixed results on the expression of Streptomyces genes, and the recognition and utilization of Streptomyces promoters in E. coli. Initial observations by Bibb et al. (1985) suggested that the high G+C content of Streptomyces genes will present problems in attempts to express these genes in organisms of lower G+C content, presumably due to lack of tRNAs that recognize high G+C codons. However, several Streptomyces genes have been successfully expressed in E. coli. Vara et al. (1985) reported expression in E. coli of the Streptomyces albonigер gene encoding puromycin N-acetyltransferase (PAC). Expression of the PAC gene in E. coli was from the lac promoter on the E. coli plasmid pUC19; evidence for this was obtained by expression in only one of the two possible orientations when the cloned fragment was inserted into pUC19 and also by the stimulation of expression when IPTG was added to the medium. A similar expression from the lac promoter was reported by Katz et al. (1987) in the expression of the MLS B resistance gene (ermE) from Saccharopolyspora erythrea in E. coli.

Expression of the phosphinothricin N-acetyltransferase gene from Streptomyces viridochromogenes in E. coli was also from the lac promoter (Strauch et al., 1988). The cloned fragment was expressed in only one of the two possible orientations when cloned downstream of the E. coli lac promoter. Paradkar et al. (1991) have also reported expression in E. coli of the S. venezuelae anthranilate synthetase genes by transcriptional readthrough from the lac promoter on the E. coli plasmid pTZ18R. When the cloned fragment was inserted in pTZ19R, in which the orientation of the lac promoter is reversed with respect to the insert, the genes were not expressed.

Gil et al. (1985b) have reported expression of the Streptomyces acrimycini CAT gene in E. coli. In this case, however, a spontaneous deletion of 0.7 kb of the cloned fragment was required to correctly align the gene for expression from the tet promoter of pBR322. A similar deletion allowing expression from the tet promoter of pBR322 was reported by Gil and Hopwood (1983) in the expression of the S. griseus PABA synthetase gene in E. coli.

The above examples show expression of Streptomyces genes in E. coli using E. coli vector promoters. On the other hand, Deng et al. (1986) reported that a promoter sequence from the Streptomyces multicopy plasmid pIJ101 was not only recognized in E. coli but also that initiation of RNA synthesis was at the same nucleotide in E. coli and S. lividans. Jaurin and Cohen (1985) also isolated several S. lividans promoter

signals that were active in E. coli.

Results obtained from the present study indicate that the S. lividans PABA synthetase genes were not only expressed in E. coli but they were expressed from a Streptomyces promoter.

2. Expression in S. lividans.

Attempts to express in S. lividans the cloned PABA synthetase genes of S. lividans that had already been expressed in E. coli led to the isolation of Pab$^+$ colonies; however, the recombinant plasmid had disappeared in these Pab$^+$ transformants. The recombinant plasmid was present in free form in transformants of E. coli and S. griseofuscus. Absence of free plasmid in transformants of S. lividans was probably due to its integration by homologous recombination into the S. lividans genome using the common sequences provided by the fragment containing the PABA synthetase genes. Although Streptomyces DNA fragments cloned in recombinant plasmids can be recovered when the recombinant plasmid is introduced into a homologous host (Malpartida et al., 1984; Fietelson and Hopwood, 1983, 1985; Ohnuki et al., 1985), there are several reports of recombination occurring during such circumstances. Murakami et al. (1986) reported that when DNA fragments involved in the production of bialaphos in S. hygroscopicus were cloned in plasmid vectors and introduced into a homologous host, recombination occurred between the cloned fragment and chromosomal sequences. A similar observation was

made by Schupp et al. (1988) during attempts to clone genes involved in the biosynthesis of desferrioxamine B in Streptomyces pilosus. Seno et al. (1984) have suggested that a recombination event between cloned DNA and the homologous chromosomal sequence resulted in a replicon fusion that placed the cloned genes of the glycerol utilization operon of S. coelicolor A3(2) under the control of the chromosomal promoter. Attempts to express cloned genes for pyridoxal biosynthesis (Aidoo, 1989) and anthranilate biosynthesis (A. Paradkar, personal communication) in S. venezuelae also led to integration of the cloned fragments into the chromosome by homologous recombination. The results obtained from probing genomic DNA from the thiostrepton-resistant, Pab^+ colonies of S. lividans with the recombinant plasmid were consistent with integration of the plasmid into the host chromosome.

The 2.7-kb S. lividans 1326 insert cloned in E. coli strains AB3292 and AB3295 complemented the pab mutations in both S. lividans strains JG10 and AP3. This supported the evidence that the cloned fragment contained information complementing both the pabA and pabB mutations, suggesting that the two genes are linked in S. lividans. Moreover, the nucleotide sequence of the fragment revealed linkage between two genes that are identical to pabA and pabB genes fom other organisms. Slock et al. (1990) have also reported linkage of the pabA and pabB genes in B. subtilis. This arrangement contrasts with that in enteric bacteria and in L. lactis

subsp. lactis where the pabA and pabB genes are not linked.

IV. Analysis of the nucleotide sequences of the L. lactis subsp. lactis and S. lividans PABA synthetase genes.

1. Lactococcus lactis subsp. lactis.

A. Regulatory regions.

No regions similar to E. coli (Rosenberg and Court, 1979) or L. lactis subsp. lactis (De Vos, 1987) consensus promoter sequences were found upstream of the L. lactis subsp. lactis pabB gene. However, a region upstream of the pabB gene is rich in A/T nucleotides, a feature typical of prokaryotic promoters (Rosenberg and Court, 1979), so it is possible that a noncanonical promoter is present. Expression of the gene in E. coli and S. lividans only when host vector promoters are used may be due to absence of a promoter sequence on the cloned fragment or to the presence of such a noncanonical promoter not recognized in E. coli or S. lividans. Noncanonical promoters have been located in other systems; they require transcriptional activators to aid in their recognition as promoter sites (Irani et al., 1983). These activators may be present in L. lactis subsp. lactis to allow recognition of the pabB gene but may be absent in E. coli and S. lividans.

The nucleotide sequence of the L. lactis subsp. lactis citrate permease gene, which was cloned and expressed in E.

coli, did not contain any regions resembling prokaryotic promoters (David et al., 1990). However, it was not clear from this report whether the gene was expressed from a vector promoter.

In the cloned DNA fragment from L. lactis subsp. lactis, a putative RBS complementary to the 16S rRNA of E. coli precedes the ATG initiation codon for the pabB ORF by four nucleotides. The termination codon in this ORF is TAA; the preference of TAA to TGA and TAG may reflect the low G+C content of the L. lactis subsp. lactis genome.

B. Codon usage.

The codons used in the L. lactis subsp. lactis pabB gene reflect the G+C content of the L. lactis subsp. lactis genome. Codons that are low in G and C are preferred (see Table 11). Thus, the overall G+C content is 41% (c/f 37% in the genome). The third (degenerate) position of the codon is especially low in G+C content (35%) (see Table 12). Since this position is degenerate, the choice of A or T nucleotides at this position will not grossly alter the amino acids specified by the codons. Consistent with this, the amino acid sequence of the L. lactis subsp. lactis pabB gene is very similar to that of the pabB gene of S. lividans, which has a G+C content twice that of L. lactis subsp. lactis, and also to pabB genes of other organisms that have different G+C contents.

2. Streptomyces lividans

A. Regulatory signals.

(i). Promoter sequence.

A sequence resembling prokaryotic promoters (Rosenberg and Court, 1979) was located upstream of the S. lividans pabB gene. The sequence not only resembles the E. coli canonical sequence with good -35 and -10 regions but also appears to be functional in E. coli since it is expressed when the fragment is cloned in both orientations. The promoter sequence resembles the SEPs described by Jaurin and Cohen (1985). From the frequency of isolation of the SEPs, Jaurin and Cohen (1985) suggested that they represented a minor fraction of S. lividans promoters. Of the fourteen Streptomyces SEPs, seven are involved in primary metabolism. This suggests that although a minor form of RNA polymerase is used for transcription from these promoters, the sigma factor attached to this polymerase is constitutively expressed. The fourteen Streptomyces SEPs have an average spacing of 18-bp between the -35 and -10 regions (Seno and Baltz, 1989) which is similar to the 17-bp spacing reported for E. coli promoters (Rosenberg and Court, 1979). The spacing between the -35 and -10 regions of the S. lividans PABA synthetase promoter is 16-bp.

Jaurin and Cohen (1985) noted that the SEPs were not as efficiently expressed in S. lividans as they were in E. coli. The use of a weak promoter to express the S. lividans PABA synthetase genes may have a regulatory function. Gil et al.

(1990) reported that PABA synthetase activity was not detectable in candicidin nonproducing Streptomyces suggesting a very low expression of these genes. A similar observation was made in E. coli (Nichols et al., 1989). Thus, the use of a weak promoter to express the S. lividans PABA synthetase genes may reflect the low levels at which these enzymes are required in the cell.

It is worthy of note that although the ermE P1 and ermE P2 promoters have good homology to the E. coli consensus promoter, they are not expressed in E. coli. This suggests that sequence homology alone is inadequate to explain promoter activity and that other factors may be involved (Seno and Baltz, 1989).

(ii). Ribosome binding site, translational initiation and translational termination.

Shine and Dalgarno (1974) reported that an mRNA sequence complementary to a sequence near the 3'-end of 16S rRNA is required for the ribosome to bind mRNA and initiate translation. In E. coli, the conserved sequence GGAGGA on mRNA was implicated (Rosenberg and Court, 1979). Bibb and Cohen (1982) found the same sequence to be complementary to the 3'-end of S. lividans 16S rRNA and suggested that it was required for ribosome binding in this organism. In all genes analyzed in Streptomyces, a sequence on the mRNA at least partailly overlaps the conserved sequence on the 16S rRNA.

The putative RBSs of the S. lividans pabA and pabB genes each have a match with 3 out of 6 bases in the conserved sequence.

Seno and Baltz (1989) reported that the length of the RBS varies from 4 to 12 bases with an average of 6.3+/-2.0. The putative RBSs of the S. lividans pabA and pabB genes have 6 bases.

That the start codon in both S. lividans PABA synthetase genes is ATG is not surprising since 82% of Streptomyces genes contain an ATG start codon; the remaining 18% use GTG (Seno and Baltz, 1989). The occurence of the GTG start codon in Streptomyces is higher than in E. coli (3%) (Hopwood et al., 1986).

Of the three available translational stop codons, TGA is used in both S. lividans PABA synthetase genes. This is consistent with frequencies of use for the stop codons in Streptomyces of 68% TGA, 29% TAG and 4% TAA (Seno and Baltz, 1989). The higher frequency for TGA, followed by TAG, reflects the high G+C content of streptomycete DNA.

B. Codon usage.

The high G+C content of streptomycete DNA suggests that amino acid codons with G or C in the third positions will be preferred. That this was the case was reported by Bibb et al. (1985) who showed that codon usage in streptomycetes was biased, by the high G+C content of the DNA. Compilation and analysis of data for several streptomycete genes showed that

the first position of the codons contained 70% G or C and the third positions contained >90% (Seno and Baltz, 1989). The S. lividans pabA and pabB genes have a G+C content of 61% and 72%, respectively, in the first codon positions and 93 and 95%, respectively, in the third codon positions. The average G+C content in the second position is 50% (Seno and Baltz, 1989). The S. lividans pabA and pabB genes contain 40 and 42% G or C, respectively, in the second position of the codon. The average G+C content of the coding regions of streptomycete genes is 70%. The S. lividans pabA and pabB genes have a G+C composition of 65 and 70%, repectively. These values show that codon usage in the S. lividans pabA and pabB genes conform to the general trend in Streptomyces.

The average use of C and G residues in the first position of the codons in Streptomyces genes is 27% and 42% respectively (Seno and Baltz, 1989). The S. lividans pabA and pabB genes follow this trend; the pabA gene has 22% C and 40% G residues in the first position of the codons while the pabB gene has 25% C and 47% G residues in this position. In the second position of the codons, the four bases occur in equal frequency in Streptomyces genes and this was also observed in both pabA and pabB genes of S. lividans. In the third positions of the codons, C (55%) is slightly preferred to G (36%) in Streptomyces genes (Seno and Baltz, 1989). The S. lividans pabA gene follows this trend with 53% C and 39% G residues in the third position of the codons. The pabB gene

however, deviates from this trend; it contains 39% C and 56% G residues in the third position. This use of rare codons in the pabB gene may have a regulatory function. Gouy and Gautier (1982) noted that in bacteria, genes that are highly expressed contain codons that are used frequently in that organism. Andersson and Kurland (1990) have also reported that rarely used codons may be exploited to regulate gene expression or to modulate the performance of the translation system. This may contribute to the low activity of PABA synthetase in S. lividans.

V. Comparison of codon usage in pab and trp genes in different organisms.

1. pabA and trpG genes.

There is substantial identity between the amino acid sequences of the pabA genes from S. lividans, E. coli and S. marcescens and the trpG gene of E. coli (see Table 19). However, the codon distribution in these genes is not similar. Codon usage in these genes reflect the G+C content of the organism containing the gene. This is evident in the third (degenerate) position of the codons (see Tables 26 and 27). Muto and Osawa (1987) have reported that in bacteria, codon usage follows the base composition of the genome. They observed that although there is variation in the G+C content of the third codon position of ribosomal protein genes from different bacteria, this variation is biased in the same

direction as the G+C content of the whole genome. A similar observation has been made in vertebrates; codon choice depends mainly on the G+C bias of the region of DNA harbouring the gene (Aota and Ikemura, 1986). Most of the nucleotide differences between the pabA and trpG genes occur in the third position of the codons where they have no gross consequences on the amino acid sequences of the polypeptides.

Table 27 shows that the G+C content of the pabA genes of E. coli and S. marcescens as well as the trpG gene of E. coli are slightly higher than the G+C content of the genome as a whole. This is especially evident in the third position of the codons. The S. lividans pabA gene has a slightly lower G+C content than the genome as a whole.

The second position of the codons in the pabA genes of S. lividans, E. coli and S. marcescens and the trpG gene of E. coli are low in G+C content (see Table 27). Kaplan et al. (1985) have noted that about 75% of the amino acids in the pabA polypeptides from enteric bacteria are either nonpolar or charged residues and most of these amino acids have codons with A or T in the second position.

Kaplan et al. (1985) also noted a strong bias for G or C in the first position of the codons in the S. marcescens pabA gene. The explanation offered for this was that S. marcescens shows a preference for amino acids such as leucine, arginine and valine, the codons of which begin with G or C. Despite the higher G+C content of the S. lividans genome, the first

position of the codons in the S. lividans pabA gene show a lower G+C content (61%) than that of S. marcescens (69%). This may reflect the preference in S. lividans for G or C residues in the third position of the codon rather than in the first.

The differences in codon usage observed in the pabA and trpG genes from different organisms do not grossly affect the amino acid composition of these polypeptides (see Table 19). This gives support to the view that glutamine amidotransferase genes evolved from a common ancestor (Kaplan et al., 1985); the genes have evolved to reflect the G+C content of the organisms containing them, with little effect on the amino acid composition.

2. pabB and trpE genes.

As with the pabA and trpG genes, there is substantial identity between pabB and trpE genes from different organisms, even though codon distributions in these genes are not similar (see Tables 17 and 20). Again, the codon usage reflects the G+C content of the organism containing the gene. For example, the L. lactis subsp. lactis pabB gene contains 41% G+C in the coding region, reflecting the 37% G+C content in the entire genome. On the other hand, the S. lividans pabB gene contains 69% G+C in the coding region, reflecting the 73% G+C content in the entire genome. The major differences in the codons of the different pabB genes and the trpE gene of E. coli occur in

the third position of the codons, and to a lesser extent in the first position. However these changes do not cause gross changes in the amino acid composition of the polypeptides. The second position of the codons in these genes are low in G+C content, reflecting a high number of non-polar and charged amino acid residues in the polypeptides, as in the pabA and trpG genes.

Goncharoff and Nichols (1988) observed a marked identity between pabB genes and trpE genes from enteric bacteria. From this they concluded that the genes coding for the aminase subunits of the PABA and anthranilate synthetase in enteric bacteria evolved from a common ancestor. The marked similarity of the L. lactis subsp. lactis and S. lividans pabB genes to each other and also to the pabB genes of enteric bacteria and B. subtilis suggests that in fact the genes encoding the aminase subunits in all these organisms evolved from a common ancestor. However, codon usage has reflected the G+C content of the organisms containing them.

SUMMARY AND CONCLUSIONS

Shotgun cloning of L. lactis subsp. lactis NCDO496 genomic DNA into S. lividans JG10 (pab) using the vector pIJ41 led to the isolation of a Pab$^+$ transformant. Plasmid DNA isolated from this transformant also complemented the pab mutation in S. lividans AP3. Restriction endonuclease analysis of this recombinant plasmid indicated that a 1.2-kb segment of the vector DNA was deleted in the recombinant plasmid and that the deletion encompassed part of the aph gene conferring resistance to neomycin. Thus, Pab$^+$ transformants of S. lividans strains JG10 and AP3 were sensitive to neomycin. The deletion was required to allow the PABA synthetase gene to be aligned to a vector promoter (presumably the aph promoter) so that the gene could be expressed by transcriptional readthrough.

Transformants of S. lividans strains JG10 and AP3 carrying recombinant plasmids containing the cloned gene from L. lactis subsp. lactis in the correct orientation cross-fed pab mutants of S. lividans. PABA was also detected in culture broths of these transformants. Streptomyces lividans TK24 (Pab$^+$ and parent of both strains JG10 and AP3) did not cross-feed pab mutants of S. lividans. Also PABA could not be detected in the culture broth of S. lividans TK24. From these observations, it was concluded that the high resistance of L. lactis subsp. lactis was due to overproduction of PABA.

The cloned L. lactis subsp. lactis fragment complemented the pabB but not the pabA mutation in E. coli. In this case too, expression of the gene appeared to involve a vector promoter since expression was achieved only when the fragment was cloned in one particular orientation. The inability to complement the pabA mutation suggested that in L. lactis subsp. lactis, the pabA and pabB genes are not linked. This was confirmed by analysis of the nucleotide sequence of the cloned fragment. Only one ORF, the deduced amino acid sequence of which showed identity to pabB genes from other organisms, was located within the sequence. The nucleotide sequence also revealed a putative RBS but not a typical prokaryotic promoter. A region upstream of the RBS contained A/T-rich sequences typical of prokaryotic promoters but no promoter consensus sequence could be distinguished in this region. It was concluded that either the cloned fragment lacked a promoter sequence or it contained an atypical promoter for which transcriptional activators were required.

To evaluate the use of biochemical characteristics to identify pab mutations, the pab mutants of E. coli were examined by growth and enzyme assays. Strain AB3292 utilized ammonia at pH >7.0 to make PABA indicating that it contained a functional aminase gene (pabB). The mutation should thus be in the pabA gene as expected. Strain AB3295 did not use ammonia at pH >7.0 to make PABA indicating that it lacked either the pabB and/or the pabX gene. Complementation of

this mutation by the L. lactis subsp. lactis fragment that contained only a pabB gene indicated that the mutation was, in fact in pabB.

Using growth assays, the S. lividans pab mutants (strains JG10 and AP3) were then characterized. The ability of strain JG10 to use ammonia to make PABA indicated that it had functional pabB and pabX genes. This suggested that the mutation in strain JG10 was in pabA. Complementation of this mutation by an S. lividans fragment that contained the pabA and pabB but not the pabX gene indicated the mutation in strain JG10 could indeed be in pabA. Complementation of the putative pabA mutation in strain JG10 by a pabB gene contained in the L. lactis subsp. lactis fragment was attributed to recruitment of a glutamine amidotransferase in strain JG10 that functioned along with the pabB gene from L. lactis subsp. lactis. Strain AP3 was unable to use ammonia to make PABA, suggesting that the mutation was in pabB and/or pabX. Since this mutation was complemented by the cloned fragments from L. lactis subsp. lactis and S. lividans both of which contained pabB but not pabX, it was concluded that the mutation in strain AP3 was in pabB.

The PABA synthetase genes of S. lividans were cloned by complementation of the pab mutations in E. coli using a genomic library of S. lividans 1326 genomic DNA constructed in pBR322. A single DNA fragment complemented both the pabA and pabB mutations in E. coli suggesting that in S. lividans the

two genes are linked. The same fragment also complemented the pab mutations in strains JG10 and AP3. In the latter case, however, the recombinant plasmid containing the PABA synthetase genes of S. lividans recombined with homologous sequences on the chromosome. Thus, free plasmid could not be isolated from such Pab$^+$ transformants.

Analysis of the nucleotide sequence of the cloned S. lividans fragment revealed two ORFs closely resembling pabA and pabB genes from other organisms. A putative promoter sequence resembling the SEPs (Jaurin and Cohen, 1985) was located upstream of the pabB gene. Also, putative RBSs were located immediately upstream of the translational start codons of both pabA and pabB genes.

Comparison of codon usage in the PABA synthetase genes of L. lactis subsp. lactis with those of S. lividans as well as related genes from other organisms indicated a codon bias favouring the G+C content of the organism.

APPENDIX

Restriction mapping of pDQ254.

The 13.6 kb BclI fragment from pDQ250 was recircularized by ligation and introduced into protoplasts of S. lividans JG10 by transformation. Plasmid DNA was isolated from a thiostrepton-resistant colony obtained in this transformation and was subjected to restriction enzyme digestions. Digestions were also carried on pIJ41 for comparison. The digests were examined by agarose gel electrophoresis (Figs. 43 and 44; Table 28). The sizes of the restriction fragments were estimated by comparing their mobilities during electophoresis with those of fragments generated by digesting phage lambda with HindIII.

Taking into account the known restriction sites in the pIJ41 vector (see Fig. 5), the extent of deletion of pIJ41 sequences in pDQ254 was determined.

When pDQ254 was digested with BamHI, BclI, EcoRI, BglII and XhoI, a single fragment of approximate size 13.6 kb was obtained. These enzymes have single restriction sites on pIJ41 and generate a 14.8-kb fragment. No sites on pDQ254 were restricted by SphI and XbaI. These enzymes have single restriction sites on pIJ41 near the carboxy terminus of the aph gene (see Fig. 5). This indicated that the deletion of pIJ41 sequences in pDQ254 encompassed the carboxy terminus of the aph gene.

Figure 43: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; and of pIJ41 digested with (B) BclI, (C) BamHI, (D) EcoRI, (E) BglII, (F) SphI, (G) XhoI, (H) HindIII, (I) BamHI and BglII, (J) BamHI and EcoRI, (K) BglII and EcoRI, (L) BclI and HindIII, (M) BclI and XhoI and (N) HindIII and XhoI.

Figure 43

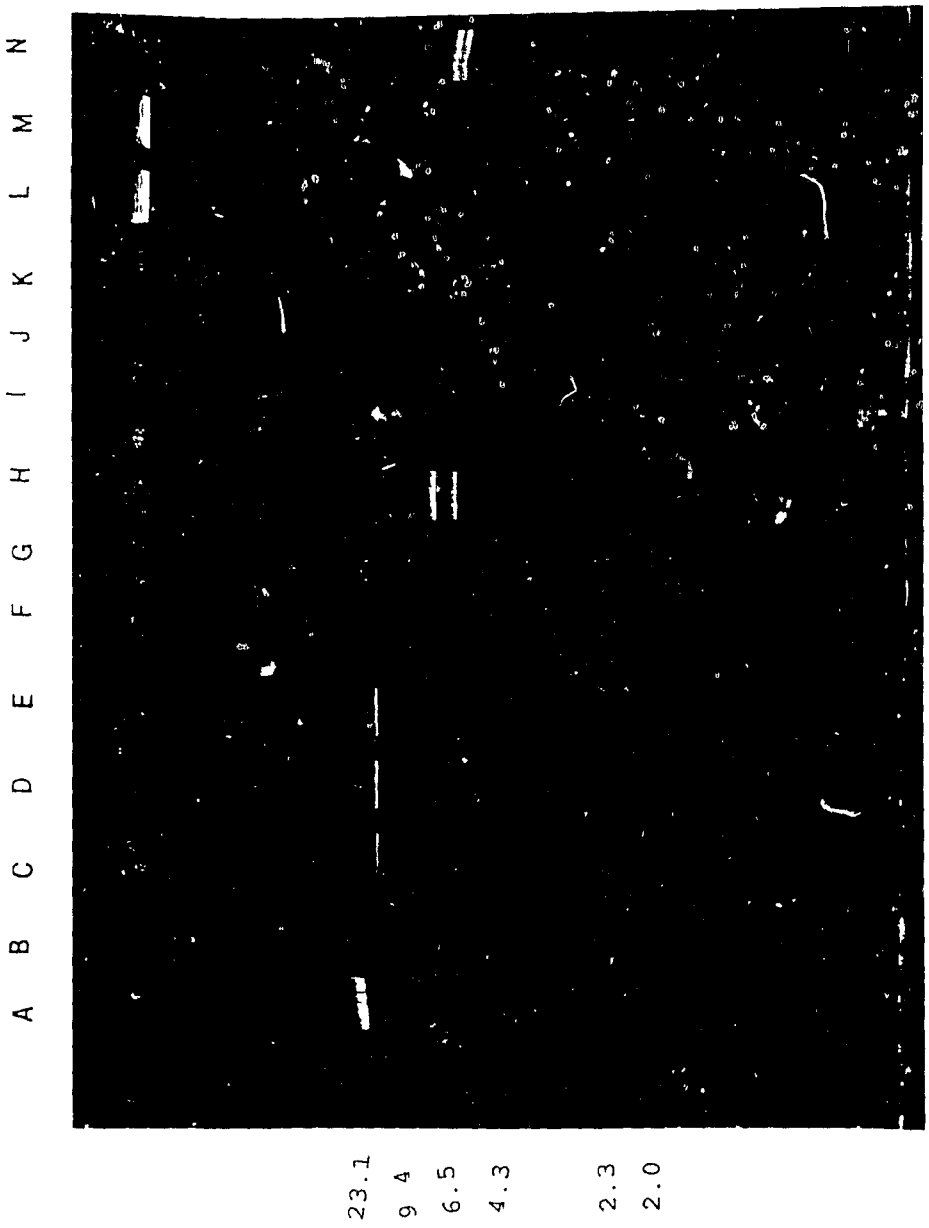

Figure 44: Agarose gel electrophoresis of (A) lambda DNA digested with HindIII; of (B) ccc DNA of pDQ254; and of pDQ254 digested with (C) BclI, (D) BamHI, (E) EcoRI, (F) BglII, (G) SphI, (H) XbaI. (I) HindIII, (J) BamHI and BglII (K) BamHI and EcoRI, (L) BglII and EcoRI, (M) BclI and HindIII, (N) BclI and XhoI and (O) HindIII and XhoI.

Figure 44

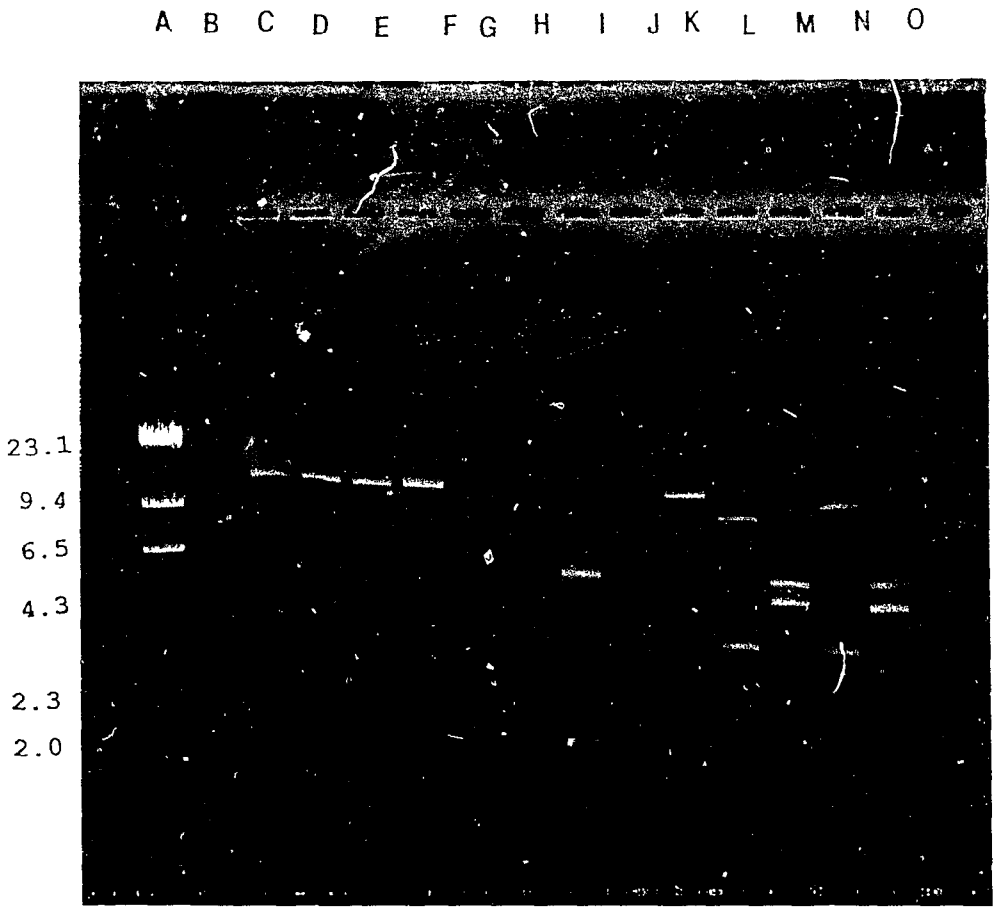

Table 28. Fragments generated by digesting pIJ41 and pDQ254 with restriction enzymes.

Restriction enzyme(s)	Sizes of fragments (kb) pIJ41	pDQ254
BamHI	14.8	13.6
BclI	14.8	13.6
BglII	14.8	13.6
EcoRI	14.8	13.6
SphI	14.8	No sites
XbaI	ND	No sites
XhoI	14.8	13.6
HindIII	6.9	6.3
	6.3	5.0
	1.6	1.6
BamHI + BglII	12.4	11.2
	2.4	2.4
BamHI + EcoRI	12.3	12.3
	2.5	1.3

Table 28 (cont'd).

BclI + HindIII	6.9	5.7
	5.0	5.0
	1.6	1.6
	1.3	1.3
BclI + XhoI	11.2	10.0
	3.6	3.6
BglII + EcoRI	9.9	9.9
	4.9	3.7
HindIII + XhoI	6.3	6.3
	6.2	5.0
	1.6	1.6
	0.7	0.7

ND - Not determined.

Digestion of pDQ254 with HindIII also indicated that the deletion was within the arc containing the aph gene. When pIJ41 was digested with HindIII, three fragments of sizes 6.9, 6.3 and 1.6 kb were obtained. With pDQ254, three fragments of size 6.3, 5.7 and 1.6 kb were obtained. Since the 6.3 and 1.6 kb fragments were common in both digestions, the deletion occured in the 6.9-kb HindIII fragment which is contained in the arc containing the aph gene. Digestion of pIJ41 with BamHI and BglII yielded two fragments of sizes 12.4 and 2.4kb, of which the 12.4-kb fragment contains the aph gene. Digestion of pDQ254 with BamHI and BglII generated the 2.4-kb fragment and an 11.2-kb fragment indicating that the deletion was in the 12.4-kb fragment of pIJ41. Digestion of pIJ41 with BamHI and EcoRI generated two fragments of sizes 12.3 and 2.5-kb, of which the 2.5-kb fragment contains the aph gene. When pDQ254 was digested with BamHI and EcoRI, the 12.3-kb fragment was obtained in addition to a 1.3-kb fragment. This is consistent with deletion of a 1.2-kb fragment in the arc containing the aph gene. Double digestions with BglII and EcoRI, BclI and XhoI, BclI and HindIII and HindIII and XhoI all indicate that the 1.2-kb deletion of pIJ41 sequences was in the arc containing the aph gene.

From the above observations, it was concluded that pDQ254 was a deleted version of pIJ41 and that the deletion encompassed the carboxy terminus of the aph gene.

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