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Structural and functional studies of the tryptophan operon of *Bacillus pumilus*

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Structural and Functional Studies of the Tryptophan Operon of *Bacillus pumilus*

by

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Abstract

Structural and Functional Studies of the Tryptophan Operon of *Bacillus pumilus*

by

Miriam V. Rivas

Advisor: Dr. Rivka Rudner

The nucleotide sequence of the *trpE*, *trpD*, and 5'*trpC* genes of the *Bacillus pumilus* tryptophan operon was determined for a comparative evaluation. DNA comparisons to the tryptophan operon of a related species, *Bacillus subtilis*, showed 66% base homology. The overlapping of translational start and stop signals indicate that, similar to *Escherichia coli*, the *trpE*, *trpD*, and *trpC* genes of the *B.pumilus trp* operon are translationally coupled. The values for amino acid identity between the two Bacilli showed 68, 62, and 65% for *trpE*, *trpD* and 5'*trpC* genes, respectively. Charge related residues increased the % similarity to 79, 76, and 72%. Amino acid comparisons to the *E.coli trpE*, *trpD*, and 5'*trpC* gene products showed an average of 30 and 50% for %

amino acid identity and % amino acid similarity, respectively. Genetic analyzes with a 3.6-kb B. pumilus trp DNA fragment cloned in opposite orientations in plasmids pRR106 and pRR103 revealed the presence of an internal promoter upstream to trpC. A search for consensus-like promoter sequences upstream to trpC did not reveal an obvious promoter sequence. In the trpE gene three potential promoter sequences were identified by homology to the consensus. Two of these tandem promoter-like elements were able to facilitate the expression of β-galacto-saidase in E.coli 71-18 when cloned upstream to a plasmid promoterless lacZ gene.

The amidotransferase activity of anthranilate synthetase was assayed from B.subtilis trp mutants transformed with pRR106. The anthranilate synthetase complex showed sensitivity to Trp and to regulation by the mtr locus. Since mtr regulation is believed to act on the trp leader mRNA and the trp leader is not present on pRR106, this result indicates that additional areas or levels of regulation may be involved with mtr.

The 2.733-kb sequenced portion of the trpE, trpD, and 5'trpC genes of B.pumilus was cloned into the integrable plasmid pJH101 as a molecular probe for

preliminary experiments into the nature of heterologous integration in *Bacillus subtilis*.

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Introduction

Amino acids are the building blocks of proteins as well as precursors of other important biomolecules such as purines, pyrimidines, vitamins, and hormones. When ingested in excess they can serve as a source of energy. Tryptophan, a nonpolar aromatic amino acid, is one of the ten essential amino acids required by man and the albino rat. The other nine are threonine, methionine, lysine, valine, isoleucine, leucine, arginine, histidine, and phenylalanine. The primary biological source of tryptophan comes from biosynthetic pathways of bacteria, fungi, and plants (Lehninger, 1979).

The pathway of tryptophan biosynthesis is similar in all of the procaryotic and eucaryotic organisms studied. The last common intermediate in the aromatic synthetic pathway of tyrosine, phenyalanine and tryptophan is chorismate. From this branching point, there is a series of seven enzymatic functions culminating in the synthesis of tryptophan. The first enzyme unique to tryptophan biosynthesis, anthranilate synthetase (AS), catalyzes the conversion of chorismate to anthranilate by

an enolpyruvyl elimination reaction and amide addition (Figure 1; Lehninger, 1979; Crawford, 1975). The enzyme complex (AS) is composed of two nonidentical subunits. The amidotransferase subunit coded by the trpE gene catalyzes the enolpyruvyl elimination reaction and amide addition. The glutamine amidotransferase subunit, coded by the trpG gene, catalyzes the removal of the amide group from glutamine. The products are glutamate, pyruvate and anthranilate (Figure 1). At high pH (pH 8.0) and in the presense of NH_4Cl the aminotransferase subunit (trpE) alone can use free ammonia (NH_3) to produce pyruvate and anthranilate from chorismate (Patel et al., 1974). The next step towards tryptophan synthesis is the addition of the phosphoribosyl moiety of 5-phosphoribosyl-1-pyrophosphate to the 3-position of anthranilate forming phosphoribosyl-anthranilate. This reaction is catalyzed by the trpD gene product, phosphoribosyl transferase (PRT). Phosphoribosyl-anthranilate undergoes an Amadori rearrangement catalyzed by the trpF gene product, phosphoribosylanthranilate isomerase (PRAI), to form 1- (o-carboxyphenylamino)-1-deoxyribulose phosphate. This rearrangement is followed by a decarboxylation and ring closure to yield indoleglycerol phosphate, catalyzed by the trpC gene product, indoleglycerol phosphate synthetase

(InGPS). In the final reaction the *trpB* and *trpA* gene products forming the tryptophan synthetase enzyme complex (TS), catalyze the removal of the glycerolphosphate side chain from indoleglycerol phosphate and replace it with the alanyl moiety of L-serine to produce L-tryptophan (Crawford, 1975).

Although the pathway of trpytophan synthesis is similar, the arrangements, gene fusions, and regulatory mechanisms of the tryptophan (*trp*) genes are variable among the organisms studied (Yanofsky, 1984; Yanofsky and Crawford, 1987). To illustrate just a few, discussion will be limited to those organisms in which comparisons have uncovered relevant similarities or differences to the tryptophan operon of *Bacillus pumilus*.

The arrangements of the *trp* genes in several different organisms are shown in Figure 2. Of the organisms studied only the Enterobacteria (for example, *Escherichia coli* and *Salmonella typhimurium*) contain the seven functional units for tryptophan biosynthesis in a single operon (*trpE,(G)D,C(F),B,A*). Fusion coding for four enzymatic functions have occurred between *trpG* and *trpD* (*trp(G)D*), and *trpC* and *trpF* (*trpC(F)*). In *Bacillus subtilis* the *trpG* is located nearly 180 degrees away from the other six genes (*trpE,D,C,F,B,A*) and fusion has not

occurred between any of the them (Kane et al. 1972). Nor has fusion been observed in the trp genes of Acinetobacter calcoaceticus, yet they are found in three different locations (trpE/ trpG,D,C/ trpF,B,A) (Sawula and Crawford, 1972). In the fungus, Saccaromyces cerevisiae, the trp genes are located in five loci (trpE/ trpG(C)/ trpD/ trpF/ trpB(A)) (Doy and Cooper, 1966) and gene fusions between trpG and trpC, (trpG(C)) and trpB and trpA, (trpB(A)) have occurred. In Psuedomonas aeroginosa there are 4 trp gene loci, no gene fusions, and an additional trpI gene. The trpI gene codes for an inducer of the trpB,A locus (Calhoun et al., 1973). These few samples serve to illustrate the various arrangements and fusion events that have occurred during the evolution of the trp genes. As expected, the mechanisms which regulate trp gene expression also shows considerable variation (Yanofsky, 1984).

The trp operon of E.coli is the most studied and the one where the most information about regulation is known. There are two mechanisms involved in the control of trp gene expression in response to cellular tryptophan availability in E.coli, repression and attenuation (Yanofsky and Crawford, 1987).

In repression, the amino acid tryptophan is a corepressor and allosterically binds to the Trp apo-repressor protein to form the active Trp repressor. The Trp repressor whether in its active or inactive form exists as a dimer. The active Trp repressor inhibits transcription from the E.coli trp operon. This inhibition is achieved by the physical exclusion of RNA polymerase from the trp promoter as the Trp repressor binds on to the overlapping trp operator. The structural and chemical interactions between the trp operator and Trp repressor have been deduced down to a 2.4Å resolution by X-ray crystallography. It is interesting to note that the interaction is not between the sequence specific nitrogenous bases of the operator and the amino acid residues of the Trp repressor, except in one case. But rather the interaction involves spatially specific hydrogen bonding from amino acid side chain constitutants of the repressor onto unesterified oxygens of the sugar phosphate backbone of the operator. Even the pyrole ring nitrogen of the corepressor tryptophan is involved in hydrogen bonding to an unesterified oxygen (Otwinowski et al., 1988). The specific DNA sequence is required to produce the correct alignment between repressor and operator. The Trp repressor also recognizes and binds to the aroH and the trpR operators

(Gunsalus and Yanosky, 1980). The activity of the Trp repressor depends on the concentration of free tryptophan in the cell. Under tryptophan deprivation the corepressor tryptophan, dissociates from the Trp repressor complex leaving an inactive Trp aporepressor. The aporepressor unable to recognize and bind to the *trp* operator exposes the adjacent *trp* promoter allowing RNA polymerase to bind and initiate transcription (Yanofsky and Crawford, 1987).

Transcription of the *trp* operon is also regulated by a second mechanism called attenuation. Attenuation allows for transcription termination of the *trp* operon. Between the transcript start site and the first structural gene (*trpE*) is a leader region of 162 bp. The following special features of this region are responsible for attenuation (Lee *et al.*, 1978). As the *trp* leader (*trpL*) region is transcribed, the mRNA forms secondary structures that serve as signals to RNA polymerase. The formation of these secondary structures depend on the location of the translating ribosome that immediately attaches to the mRNA while RNA polymerase synthesizes the message. For instance when mRNA regions 1 and 2 form hydrogen bond contacts this serves as a pause signal for the RNA polymerase to temporarily halt synthesis until the ribosome moves along to melt out regions 1 and 2 (Oxender *et al.*, 1979). The movement of the

ribosome depends on the availability of charged tRNA for amino aids coded on the trpL sequence. The leader sequence contains a pair of tandem trp codons located in a strategic location that can change the formation of alternate secondary structures. If the level of tryptophan is so low that tRNA for trp remains uncharged, translation of the trpL will be stalled at the trp codons. The stalled ribosome aids in the formation of the secondary structure between trpL regions 2 and 3. For RNA polymerase this is a signal to continue transcription into the trp structural genes. On the other hand, if there is sufficient charged tRNA for trp, the ribosome will follow behind the RNA polymerase allowing for secondary structure formation between trpL regions 3 and 4. The structure is a G C rich area of dyad symmetry followed by a series of U's. The formation of this secondary structure signals rho independent transcription termination. At this point RNA polymerase stops transcription before entering into the trp structural genes. Only a short peptide of 16 amino acids is synthesized by the ribosome. In effect the transcription and translation of the trp operon has been attenuated (Landick and Yanofsky, 1987).

The two mechanisms for trp operon expression in E.coli are sensitive to different cellular levels of tryptophan. Repression regulates trp gene expression at

high to moderate tryptophan levels. While attenuation controls the trp operon over mild to severe tryptophan starvation (Yanofsky and Crawford, 1987). Repression and attenuation were separately measured to determine the relative contributions of each to the regulation of the trp operon (Yanofsky, 1985; Yanofsky et al., 1984). Repression was relieved by a 50-70 fold increase in trp gene expression in tryptophan starved cultures relative to tryptophan containing cultures without attenuation relief (Jackson and Yanofsky, 1972). Under severe tryptophan starvation, attenuation was also relieved and trp operon expression was increased 600 fold above cultures growing in tryptophan excess (Jackson and Yanofsky, 1973; Bertrand and Yanofsky, 1976). In terms of relative contributions repression can reduce transcription up to 60 fold while attenuation can reduce transcription by at most 10 fold (Yanofsky, 1981).

Other features of the E.coli trp operon are the presence of an internal promoter upstream to the trpC gene (Horowitz and Platt, 1982; Morse and Yanofsky, 1968), the overlapping of translational start and stop codons (Oppenheim and Yanofsky, 1980) and the presence of rho independent and rho dependent terminators at the end of the trp operon (Wu et al., 1981).

In E.coli the internal promoter is a low level constitutive promoter (p2) (Horowitz and Platt, 1982) that is insensitive to the tryptophan availability of the cell. The low level of expression may be attributed to its lack of consensus sequence generally seen in other E.coli promoters. The downstream trpC(F), trpB and trpA gene products are always available in low amounts to act immediately towards the synthesis of tryptophan, once the trpE and trp(G)D gene products are made. This added feature may be critical in times of severe tryptophan starvation to allow for the quick recovery of the cell.

The translational coupling of the trp genes of E.coli by the overlapping start and stop codons of adjacent genes is believed to insure a 1:1 stoichiometric amount of the gene products of the trp operon. This insures the coordinate production of the genes involved in tryptophan biosynthesis (Oppenheim and Yanofsky, 1980).

The rho-independent and rho-dependent terminators located 36 and 1400 bases from the end of the trpA gene are believed to aid in the stability of the trp mRNA. Degradation of the trp mRNA from the rho-dependent terminator by a 3' to 5' exonuclease is temporarily halted at the secondary structure formed by the rho-independent terminator (Wu et al., 1981).

The trp operon of B.subtilis, a gram positive organism, is regulated by a trp repressor protein, the gene product of the 5-methyltryptophan resistant locus (mtr) (Hoch et al., 1971; Hoch, 1974). When the trp promoter of the B.subtilis trp operon was removed and replaced with foreign promoters, regulation by the tryptophan availability of the cell continued. This observation led Shimotsu et al., (1986) to postulate a novel form of tryptophan regulation called transcript attenuation. The promoter-operator structure seen in the E.coli trp operon does not exist in the trp operon of B.subtilis. Analogous to the trp operon of E.coli, a leader sequence located 5' to the trp structural genes is transcribed by RNA polymerase. The presence of a leader sequence appears to be the only similarity between trp gene regulation in E.coli and the Bacilli.

Based on DNA sequence analysis the leader transcripts of both B.pumilus and B.subtilis trp operons do not contain tandem trp codons in strategic locations (Shimotsu et al., 1986; Kuroda et al., 1986). Tandem trp codons in the leader of E.coli are required for the coupled transcription translation attenuation of the trp operon involving RNA polymerase, the ribosome, and charged $tRNA^{trp}$. This leads one to believe that the ribosome may not be

involved in attenuation in the B.subtilis trp operon. However, the transcript has the potential to form two alternate secondary structures, one which resembles a rho independent transcript terminator, the other an anti-terminator. In the presence of excess tryptophan, the trp repressor is believed to bind to direct repeats on the trpL mRNA sequence. Deletion analyses of these direct repeats abolished regulation by tryptophan creating a constitutive mutation. The binding of an activated trp repressor to these direct repeats, prevents the formation of the antiterminator, facilitating the formation of the terminator. At this signal the RNA polymerase dissociates attenuating the transcript at the leader. During tryptophan starvation, tryptophan is unavailable to facilitate Trp repressor action on the leader transcript. This allows the formation of the antiterminator which excludes the formation of the potential downstream terminator (Shimotsu et al., 1986; Kuroda et al., 1986). The transcript is synthesized through the trp structural genes and possibly into the adjacent hisH, tyrA, and aroE genes. The trp operon of B.subtilis is located near other genes responsible for the synthesis of aromatic compounds. Some evidence exists for transcript initiation upstream to the trp promoter. This leads to the possibility that the trp genes

are part of a super aromatic operon (Roth and Nester, 1971; Henner et al., 1986). It is not clear what relationship if any trp operon expression has to the super operon theory.

A primary difference between the Bacilli and E.coli is the ability of the former to differentiate into a spore. Sporulation is the last resort of the Bacilli in response to carbon or nitrogen or phosphate source depletion in the environment (Sonenshein, 1989). The growth and developmental stage of these gram positive organisms dictate which genes are expressed. The selective expression of genes during the growth and development of the Bacilli involve at present nine sigma factors for RNA polymerase (Moran, 1989). These sigma factors allow the RNA polymerase holoenzyme to recognize different promoters. The mechanism to regulate trp operon expression in B.pumilus and B.subtilis operates during the exponential vegetative stage. During stationary phase amino acids can be generated by extracellular proteases that are exclusively produced at this growth phase (for example, subtilisin, Valle and Ferrari, 1989). In E.coli the mechanisms of adaptation during stationary phase are just beginning to be studied and it is not known whether they have similar kinds of proteases (Shultz et al., 1988). The differences in the regulatory mechanism of the

tryptophan operons of these two organisms may in part be due to the available or unavailable option to develop into a spore (Zalkin and Ebbole, 1988).

In A.calcoaceticus the trpE and trpG,D,C loci seem to be coordinately regulated by tryptophan levels in the cell. Conversely the trpF,B,A locus show different levels of derepression for trpF than for the trpB and trpA gene products. This indicates that two regulatory regions within this locus repond differently to tryptophan availability. It also indicates these regions differ in some way to the regulatory regions of the other two trp loci (Cohn and Crawford, 1975).

Regulation of the five trp loci in the fungus S.cerevisiae is under a general control that include the genes for many amino acid biosynthetic operons (Schurch et al., 1974). Deprivation for any of these amino acids stimulates all the amino acid biosynthetic operons, not just the one signaling depletion. This regulation has been shown to occur at the transcriptional level. Upstream to the promoters of hisH, trpE trpC(G) and trpB(A) loci a nine base consensus recognition sequence (5'A(A or T)GTGACTC3') in one to three copies is found (Donahue et al., 1983; Hinnebusch and Fink, 1983). A positive acting regulatory protein called GCN4 binds to this

sequence and aids in the transcription of amino acid biosynthetic operons (Struhl *et al.*, 1985).

In the fluorescent bacteria *Psuedomonas aeroginosa* the *trpE* and *trpG,C,D* loci are coordinately regulated by tryptophan. The *trpF* locus is constitutive. The *trpI,B,A* locus is induced by the gene product of *trpI*. The *trpI* gene product is activated by indolyglyerol phospate, the substrate of tryptophan synthetase (Calhoun *et al.*, 1973; Essar *et al.*, 1990, 1,2,3; Chang and Crawford 1990).

A general mechanism operative in many biosynthetic pathways is feedback inhibition of the first enzyme in the pathway by the final product. The anthranilate synthetases of many organisms including the ones mentioned above are feedback inhibited by tryptophan (Pabst *et al.*, 1973; Zalkin, 1980; Hoch *et al.*, 1969). Feedback inhibition is a regulatory control mechanism operating at the posttranslational level which further adds to the fine-turning of tryptophan regulation in the cell.

The elucidation of the different *trp* gene arrangements and regulatory mechanisms came about by studying structural and functional features of the operons. As the number of different organisms studied increased, the opportunity for comparative evaluation

between them became greater. Structural and functional features overlooked either as oddities or as unique to a particular organism, reveal themselves over and over again. When reviewed from a comparative perspective more attention is brought to them. Ultimately the bits and pieces of information add to the greater picture and the better understanding of the basic principles underlying gene regulation. In this spirit a 3.6-kb portion containing the *trpE*, *trpD*, *trpC*, and *trpF* genes of the tryptophan operon of the gram positive bacteria *Bacillus pumilus* has been analyzed and is presented in this thesis.

Purposes of this Study

1. To determine the DNA sequence of a section of the tryptophan operon of *Bacillus pumilus* for a comparative evaluation between gram positive and gram negative *trp* structural genes.

2. To determine whether unique structural and functional features exist in the tryptophan operon of *Bacillus pumilus*.

3. To study the extent of genomic heterologous integration and whether there are sequence barriers which limit this event.

Materials and Methods

Bacterial Strains and Plasmids

B.pumilus, B.subtilis, and E.coli strains and plasmids used in this study are described in Table I. Heterologous B.subtilis integrants strains were created by plasmid transformation with pMR111 and pMR131 (Figure 11). Construction of plasmids are described below.

Chromosomal DNA Isolation

DNA was isolated by the procedure of Marmur, (1961) as modified by Rudner et al. (1967). Isolation of B.pumilus and B.subtilis chromosomal DNA commenced with a starter day culture grown in 2 ml of veal infusion broth and 0.5% yeast extract (VY) (Difco). Heterologous integrant strains were grown in VY supplemented with 10 ug/ml of chloramphenicol (Cm) (Sigma). The cells were spun down in a clinical centrifuge, washed 2 times with sterile dilution saline, (0.15M NaCl, 0.02M K_2HPO_4 pH 7.0) and spot-tested for the required amino acids or Cm^R

when appropriate. 0.2 ml of the starter culture was inoculated into a 2 liter flask containing 500 ml of VY or VY + 10 ug/ml Cm and aerated overnight on a gyratory floor shaker at 37°C. Cultures were pelleted in 250 ml centrifuge bottles at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 4-5 ml of 0.15M NaCl, 0.1M EDTA pH 8.0 per gram of wet weight of cells. The cells were transferred into a 250 ml flask and incubated in a 37°C water bath shaker with 7-10 mg of solid lysozyme for 30 to 60 minutes. Sodium dodecyl sulfate (SDS) was added to the lysate at a final concentration of 2.5% and heated for 10 minutes at 60°C. The suspension was cooled on ice and brought to 1M sodium perchlorate with a 5M stock solution. An equal volume of chloroform-isoamyl (24:1) was added and allowed to shake by wrist action or by a table-top shaker for 30 minutes. The emulsion was centrifuged at 10,000g for 5 minutes and the upper aqueous layer was carefully pipeted into a chilled 250 ml beaker on ice. Two volumes of cold 95% ethanol was gently layered onto the aqueous layer and mixed with a glass rod. The thread-like nucleic acids spooled on the rod are drained of excess ethanol and transferred into a clean 50 ml glass stopper flask. Five to 10 ml of 0.1xSSC was

added and the flask was placed on a rotary shaker until it dissolved. Several hours later or the next day RNAase A and RNAase T1 were added to a final concentrations of 50 ug/ml and 500 total units respectively and incubated for 30 minutes at 37°C. Pronase was added to a final concentration of 0.5 mg/ml and incubated for at least 2 hours at 37°C. An equal volume of chloroform-isoamyl was added, shaken for 10 minutes, and centrifuged at 10,000g for 10 minutes. The upper aqueous phase was removed to a small glass-stopper flask and an equal volume of 80% buffered phenol was added and shaken for 10 minutes then centrifuged at 10,000g for 10 minutes. An ether extraction was performed with equal volumes and the bottom aqueous layer was transferred to a 50 ml beaker on ice after centrifugation at 10,000g for 10 minutes. The aqueous layer was adjusted to 1xSSC from a stock 10xSSC solution, 2 volumes of cold ethanol were added, and the DNA was spooled as was stated before. Two to 5 ml of 0.1xSSC was added to the spool and it was allowed to dissolve. Extensive dialysis was carried out in dialysis buffer (DB) (10mM Tris-HCl, 4mM NaCl, 1mM EDTA pH 7.0). The concentration of DNA was determined at 260nm assuming 1 OD unit = 50 ug/ml. A stock of 2.0 OD at 260nm in DB was made for DNA

restrictions and a stock of 0.4 OD in 1xSSC, kept over a drop of choloroform, was made for transformation assays.

Plasmid DNA Isolation from B.subtilis

Large-scale plasmid DNA isolation from B.subtilis was by the method of Guerry et al. (1973), as modified by Gryczan et al. (1978) and Tackney (1981). A 0.2 ml aliquot of day starter culture was inoculated into a 2 liter flask containing 500 ml of VY + 5 ug/ml kanamycin (Km) and grown overnight on a gyratory floor shaker. The following morning, the culture was chilled on ice and pelleted by centrifugation at 5000 rpm in 250 ml centrifuge bottles for 10 minutes. The pellet was resuspended in 20 ml of buffer A (25% sucrose, 0.1M NaCl, 0.05M Tris-HCl pH 7.5), containing 0.5 mg/ml lysozyme, and incubated at 37°C for 15 minutes without agitation. In the order presented, the following solutions were added: 4.8 ml of 5M NaCl, 1.2 ml of 0.5M EDTA pH 8.5, and 26 ml of 2% SDS, 0.7M NaCl. The suspension was gently inverted once and stored at 4°C for 18 hours. The following day the lysate was centrifuged at 18,000 rpm for 2 hours in 50

ml plastic polycarbonate tubes. Immediately after the centrifuge stopped, the supernatant was collected into a graduated cylinder. Extra care was taken to avoid the viscous blob of cellular DNA associated with the pellet. The volume was recorded and Na Acetate was added to a final concentration of 0.3M. Two volumes of cold 95% ethanol was added and the mixture was placed in a -20°C freezer for 2 hours or overnight. The precipitate was collected by centrifugation at 5000 rpm for 30 minutes in 150 ml corex centrifuge tubes. All traces of ethanol were poured off and the tube was placed in a dry vacuum for 1 hour. The dry crusty precipitate was dissolved in 5-10 ml of TES buffer (30mM Tris-HCl pH 7.5, 50mM NaCl, 5mM EDTA). RNAse-A and RNAse T1 were added to the suspension at final concentrations of 50 ug/ml and 1 unit/ml respectively, and incubated at 37°C for 30 minutes. Predigested pronase was added at a 0.5 mg/ml final concentration and incubated for 3 to 7 hours until the lysate was nearly clear. The clear lysate was suitable and highly active for plasmid transformation experiments. A 3 to 5 ml portion was used for further plasmid purification by CsCl-EtBr buoyant density gradient centrifugation.

Rapid Plasmid DNA Isolation from B.subtilis

Rapid plasmid isolation from B.subtilis followed the procedure of Rodriguez and Tait, (1983). B.subtilis cells containing plasmids were grown overnight in 5 ml of VY + 5 ug/ml Km, pelleted in a clinical centrifuge for 10 minutes and resuspended in 1.5 ml of SET buffer (20% Sucrose, 50mM Tris-HCl pH 7.6, 50mM EDTA). Each culture was transferred to a 1.5 ml microfuge tube and spun for 1 minute. The pellet was resuspended in 150 ul of SET buffer to which 20 ul of RNAse-A (10 mg/ml in 0.5M Na Acetate, 0.3M EDTA pH 4.8 heated to 80°C for 10 minutes) and 50 ul of lysozyme (5 mg/ml in 10mM Tris-HCl pH 7.6, 1mM EDTA, 10mM NaCl) were added and vortexed. Three hundred fifty ul of lytic mix (1% SDS, 0.2N NaOH made fresh every week) was added at room temperature, vortexed, and placed on ice for 10 minutes. To precipitate the chromosomal DNA, 250 ul of Na Acetate (3.0M Na Acetate pH 4.8) was added, tubes were inverted 15 times and placed on ice for 1 hour. The solution was microfuged for 6 minutes at 4°C and the supernatant was saved in a clean microfuge tube. An equal volume of isopropanol was added, mixed by inversion, and microfuged for 10 minutes at room temperature. Isopropanol was removed and the plasmid pellet was washed 2 times

with 1 ml 70% ethanol. The pellet was vacuum dried in a speed vacuum desicator for 10 minutes, resuspended in 50-100 ul of dH_2O, and stored at -20°C.

Plasmid Isolation from E.coli

Large-scale isolation was performed by the procedure of Tanaka and Weisblum, (1975). A 20 ml starter culture of E.coli containing parental or recombinant plasmid DNA was inoculated into 500 ml Luria Broth (LB: 1% bactotryptone, 1% NaCl, 0.5% yeast extract, 0.1% glucose, with the appropriate antibiotics added, ampicillin 100 ug/ml (Ap) or tetracycline 10 ug/ml (Tc)). The starting colorimeter readings (red filter) was 30 to 35 klett units. The culture was incubated at 37°C in a 2 liter flask and aerated on a floor shaker. It was grown for 4 hours or until Klett readings reached 180 units. At this time 90 mg of solid Cm was added and the flask was returned to the floor shaker for overnight amplification of plasmid DNA (Clewell and Helsinki, 1972). Cultures were chilled in an ice slurry, pelleted at 5000 rpm for 10 minutes, and resuspended in 8 ml of cold Tris-Sucrose buffer (50 mM Tris-HCl pH 8.0, 25% sucrose). The suspension was transferred to 50 ml

polycarbonate tubes and placed on ice. Solid lysozyme (7mg) and 20 ug/ml RNAse-A (Cooper Biochemicals) were added and kept on ice for 15 minutes. Gently 0.8 ml of 250 mM EDTA pH 8.0 and 8 ml of Triton solution (0.3% Triton X-100 (Sigma), 188mM EDTA, 150mM Tris-HCl pH 8.0) were added and kept on ice for 15-30 minutes. The lysate was pelleted by centrifugation at 20,000 rpm for 90 to 120 minutes. The supernatant was immediately saved and mixed with an equal volume each of water and 80% phenol. The 80% phenol was buffered with 2M Tris-HCl to pH 8.0 with added 0.1% 8-hydroxyquinoline to prevent oxidation (Gallard Schlesinger). The mixture was centrifuged at 10,000 rpm for 10 minutes in 30 ml corex tubes. The aqueous phase was transferred to a glass stopper flask and Na Acetate was added to a final concentration of 0.3M. Three volumes of cold ethanol were added and the plasmid was precipitated overnight at -20°C. The precipitate was pelleted at 8000 rpm in 250 ml plastic centrifuge bottles for 10 minutes and washed with 70% ethanol before it was vacuum dried. The pellet was resuspended in 2-3 ml of TE buffer (10mM Tris-HCl pH 8.0, 50 mM EDTA). Typically 2-3 mg of plasmid were isolated from a 500 ml culture.

Mini Rapid Isolation of Plasmid from E.coli

Plasmid and replicative forms of M13 (RF) DNA were isolated by the rapid alkaline lysis method of Birnboim and Doly (1979). Plasmid bearing or phage infected E.coli cells were cultured overnight in 5 ml LB plus antibiotic or 2xYT respectively (1xYT: 1% bactotryptone, 1% NaCl, 0.5% yeast extract), pelleted in a table-top centrifuge for 10 minutes, resuspended in 1.5 ml dilution saline, and transferred to 1.5 ml microfuge tubes. Cells were microfuged for 1 minute, resuspended in 200 ul of solution I (50mM glucose, 10 mM EDTA, 25mM Tris-HCl pH 8.0, with freshly added 2 mg/ml lysozyme, 250 ug/ml RNAse-A) and kept on ice for 30 minutes. Afterwards 400 ul of freshly made solution II (0.2N NaOH, 1% SDS) was added, mixed by inversion, and incubated on ice for 5 minutes. To precipitate chromosomal DNA 300 ul of 3.0M Na Acetate pH 4.8 was added and placed on ice for 60 minutes. The chromosomal DNA and proteins were pelleted in a cold microfuge for 15 minutes, with the subsequent transfer of the supernatant into a clean microfuge tube. Plasmid DNA was precipitated with 2 volumes of cold 95% ethanol and placed at -70°C for 30 minutes. The ethanol was removed and the pellet was resuspended in 0.3M Na Acetate, 10mM EDTA and reprecipitated with two volumes of

ethanol. After an additional 30 minutes at -70°C the precipitate was pelleted, washed 2 times with 70% ethanol and dried in a vacuum desicator for 10-15 minutes. The pellet was resuspended in 100 ul dH_2O, heat inactivated at 68°C for 10 minutes, and stored at -20°C until needed.

Large-scale Isolation of Replicative Form and Single-Stranded M13 phage DNA

A soft agar plate was seeded with a fresh lawn of E.coli strain 71-18 or JM101 in early log phase of growth (50-80 Klett units). A loopful of desired lysogen was placed on the lawn. In 4-6 hours or overnight, a bit of the plaque area from the lawn of cells was scrapped off with a loop and added to 2 ml of 2xYT to which 0.2 ml of fresh host cells were added. This step was performed to insure infection of E.coli by phage. The phage cell mixture was added to 500 ml of 2xYT in a 2 liter flask and grown with vigorous shaking at 37°C for no more than 16-18 hours. The cells and supernatant were harvested and saved. The cell pellet was processed exactly as the E.coli plasmid isolation procedure mentioned above.

To the supernatant aliquots of 10 ml were poured into 15 ml corex tubes and centrifuged at 5000 rpm for 10

minutes. This was repeated twice to get rid of any residual E.coli cells. The supernatant was transferred into a fresh 15 ml corex tube and 2 ml of a (1:1) mixture of 20% PEG (polyethylene glycol 6000) and 2.5M NaCl were added, vortexed, and allowed to precipitate at room temperature for 10 minutes. The tubes were centrifuged at room temperature for 10 minutes at 5000 rpm. The supernatant was poured off and any remaining drops of PEG were removed by careful aspiration. To insure PEG removal a pasture pipet was covered with a kimwipe and used to clean the sides without disturbing the phage pellet. The precipitated phage was dissolved in 300 ul of M13 buffer (50mM Tris-HCl pH 8.0, 10mM NaCl, 1mM EDTA) and transferred to 1.5 ml microfuge tubes. An equal volume of buffered 80% phenol pH 8.0 was added, vortexed, and placed in a 42-45°C water bath for 10 minutes. The mixture was vortexed again followed by the addition of 200 ul of phenol and 200 ul of chloroform isoamyl (24:1) revortexed, and microfuged for 10 minutes. The aqueous phase was transferred into a clean microfuge tube and 1/10 the volume of 3.0M Na Acetate was added. Two to three chloroform isoamyl extractions were performed. To the final aqueous extract about 800 ul of 200 proof ethanol was added and placed at -70°C for 2 hours or

overnight at -20°C. The precipitate was microfuged for 5 minutes and washed 2 times with cold 80% ethanol before drying in a vacuum desicator for 10 minutes. The final dried pellet was resuspended in 50 ul of TE buffer. On a mini gel 2 ul were checked for the characteristic single-strand puff.

CsCl-EtBr Buoyant Density Gradient Centrifugation

Plasmid purification by CsCl-EtBr buoyant density gradient centrifugation was as described by Ullrigh et al. (1977). Two to five ml of plasmid lysate isolated from B.subtilis or E.coli strains were brought up to 6 ml with buffer used to dissolve the original plasmid pellet. In a 50 ml beaker 7.3 gms of CsCl (Gallard), was dissolved in 6 ml of the plasmid lysate. In subdued light, 2 ml of 1 mg/ml EtBr was added and the entire mixture was transferred into polyallomer tubes, topped with heavy mineral oil, and sealed with rim-gripping mechanical caps. Tritium labeled cell and plasmid copy number was determined by the method described by Tackney (1981). Ultra centri-fugation was in a Ti 50 rotor, at 38,000 rpm, 15°C, for 48 hours, without brakes. The plasmid DNA was visualized

with a UV lamp as the lower of two bands. It was removed with a pasteur pipet and transferred into a conical centrifuge tube. Et-Br was removed by 3 consecutive extractions with NaCl saturated isopropanol. The plasmid was precipitated with two volumes of ethanol for 30 minutes at -70°C, washed twice with 70% ethanol, vacuum dried, and resuspended in 1 to 2 ml of TE buffer.

Preparation of B.subtilis Competent Cells For Transformation Assays

Competent cells of B.subtilis were prepared by the method of Anagnostopoulos and Spizizen (1961), as modified by Rudner et al. (1967). A culture from a single colony was grown during the day in 2.5 ml VY, washed twice in dilution salts and spot-tested for the auxotrophic markers. A 0.2 ml aliquot was transferred to 5 ml of VY for overnight growth at 37°C on a rollodrum. The culture was pelleted and resuspended in 1 ml of Spizizen I synthetic media (1x Spizizen salts: 0.044M KH_2PO_4, 0.08M K_2HPO_4, 0.0034M Na Citrate, 0.0001M $MgSO_4$ (Spizizen 1958) supplemented with 0.5% glucose, 0.02% vitamin-free casamino acid (VFCA), 0.1% yeast extract, 0.8% L-arginine, and 100 ug/ml of each required amino acid). In a 250 ml side-arm flask 20 ml of Spizizen I

medium was brought up to a Klett reading of 20-25 units (red filter) with the concentrated cell suspension. After 4 to 4.5 hours of growth at 37°C with aeration, the culture was diluted 1:10 into Spizizen II synthetic media (1x Spizizen salts, 0.5% glucose, 0.01% VFCA, 0.05% yeast extract, 2.5mM $MgCl_2$, 1mM $CaCl_2$, 0.05mM spermine tetrahydrochloride, 5 ug/ml of each required amino acid) and returned to the 37°C water bath for 90 minutes. At this time the cells were immediately used in a transformation assay or frozen for storage (see below).

In a transformation assay 0.9 ml of fresh or thawed competent cells were mixed with 0.1 ml of plasmid (5-10 ug) or chromosomal DNA (0.2 to 2.0 ug), and incubated at 37°C for 30 minutes in a rollodrum. DNAse I (Cooper Biomedical) (100 ug/ml in 0.25M $MgSO_4$) was added to a final concentration of 10 ug/ml followed by a 10 minute incubation period. Samples were diluted in dilution saline and plated at 0.05 to 0.2 ml aliquots on appropriate selective minimal plates (Spizizen 1x salts supplemented with 0.5% glucose, 1% glutamate, and 1.5% agar, Difco) or LB plates containing 10 ug/ml Km or Cm. Plates were incubated at 37°C for 24 to 48 hours.

Frozen competent B.subtilis cells were prepared by centrifugation in sterile centrifuge tubes, resuspended in 1/10 volume of Spizizen II with 10% glycerol, and quick-frozen in 2 ml aliquots in a dry-ice ethanol bath. Frozen cells were quickly thawed at 65°C with subsequent addition of 7-10 ml of Spizizen II media.

Preparation of E.coli Competent Cells

Competent E.coli cells were prepared by the method of Cohen et al. (1972). Cells started at 10 Klett units (red filter) were grown until 50-90 units, chilled on ice for 10 minutes and pelleted at 5000 rpm for 5 minutes. The pellet was resuspended in 1/2 the volume of sterile cold 0.01M $CaCl_2$ then repelleted with the same time and speed. The pellet was gently resuspended in an equal volume of cold sterile 0.03M $CaCl_2$ and placed on ice for 20 minutes. Cells were centrifuged and resuspended in 1/10 volume of cold sterile 0.03M $CaCl_2$. At this point cells were either aliquoted at 0.2 ml volumes to tubes containing plasmid DNA for

transformation assays or quick-frozen in 1/10 the volume 0.03M $CaCl_2$ in 40% glycerol.

Fresh or thawed competent E.coli cells mixed with plasmid DNA were kept on ice for 30 minutes, then heat-shocked at 42°C for 2 minutes. One ml aliquots of LB was added to the cells and placed in a rollodrum at 37°C for a minimum of 90 minutes. After appropriate dilution of cells were made, they were plated at 0.05 to 0.2 ml aliquots on antibiotic selective LB agar plates.

Construction of Plasmids pMR111 and pMR131

Plasmids pMR111 and pMR131 were constructed by ligating via shotgun cloning the 2.733-kb and 0.9-kb EcoRI-HindIII B.pumilus restriction fragments of pRR106 (Figure 3; Rudner et al., 1982; Figure 11) into the same sites in plasmid pJH101 (Ferrari et al., 1983; Figure 11). The procedures followed for enzyme restrictions and ligation reactions were as instructed by the suppliers (Biolabs and Boehringer). These recombinants were selected in E.coli strain HB101 as conferring Ap^R, Cm^R, and Tc^S. The desired recombinant was grown to mass culture,

isolated from the host cell in a crude lysate and used to transform competent B.subtilis trp mutants.

It was shown by Canosi et al. (1978) that plasmid transformation efficiencies in B.subtilis were increased as the multimeric form of the plasmid serving as donor increased. For this reason concatenated forms of plasmids pMR111 and pMR131 were created as follows. After CsCl purification the plasmids were restricted with HindIII and ligated with DNA T4 ligase under concatenating conditions. The recommendation of the manufacturer (IBI) were followed. The plasmid DNA and ligase concentration were 0.2 ug/ul and 0.05 units/ul, respectively, with incubation at 15°C for 2 hours.

Construction of Plasmid pMR500

Plasmid pMR500 was constructed by ligating the 504 bp EcoRI-BclI purified fragment of pRR106 (Figure 3) into the EcoRI-BamHI site of promoter search plasmid pSK10Δ6 (Zuber and Losick, 1983; Figure 8). The promoterless beta-galactosaidase gene of pSK10Δ6 requires that the fragment contains its own ribosomal binding site (RBS), as well as an initiation methionine in the same reading frame as the beta-galactosaidase gene for expression.

Southern Analysis

Southern analysis was performed by the method of Southern, (1975) as modified by Ostapchuk et al., (1980). On a 0.8% agarose gel (w/v Sigma), prepared with 1xTris Borate EDTA buffer (TBE: 89mM Tris-HCl 89mM Boric acid, 1mM EDTA pH 8.0), 2.6 ug of restricted chromosomal DNA and 0.5 ug of plasmid DNA were loaded per well. Submarine gels were electrophoresed in 1xTBE containing 0.5 ug/ml ethidium bromide for 4 hours at 50 mAmp. Bacteriophage lambda (λ) DNA restricted with HindIII served as molecular weight standard markers for all gels. Subsequent to electrophoreses, gels were immersed in denaturing solution (0.5M NaOH, 1.5M NaCl) for 1 hour with gentle shaking, followed by 1 hour in neutralizing solution (1.5M NaCl, 1M Tris-HCl pH 7.5) with gentle shaking. The separated DNA was transferred to nitrocellulose membranes (type BA-85, 0.45 um pore size; Schlesinger and Schuell) by capillary action from 10xSSC troughs. The DNA was bound to the filter by baking in vacuo at 80°C for 2 hours. Filters were prehybridized inside heat-sealable freezer bags with prehybridized solution: 40% formamide (Sigma),

4xSSC, 1% glycine, 50mM phosphate buffer pH 6.5, 250 ug/ml sheared herring DNA, (Sigma), 5x Denhardt's solution (Ostapchuk et al., 1980) (1x Denhardt's is 0.02% ficoll, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin fraction V) at 2 ml/cm for 2 hours to overnight, at 42°C. Prehybridization solution was removed and replaced by hybridization solution: 40% formamide, 4xSSC, 20mM phosphate buffer pH 7.0, 100 ug/ml herring testes DNA, and 5x Denhardt's solution, at 50 ul/cm, to which $0.5x10^6$ to $10x10^6$ cpm of ^{32}P labeled DNA probe was added. Hybridized filters were incubated overnight at 42°C. High stringency washing of filters was performed at 68°C for 2 hours in 2xSSC with 0.1% SDS. Filters were dried, covered in plastic wrap, placed in film cassettes with Kodak XAR-5 film, and exposed at room temperature or at -70°C with a Dupont Cronex lightning plus intensifying screen. Sizes of DNA fragments were determined from autoradiograms by measuring the migration distances and applying the relationship of Bearden (1979).

Radioactive Probes

DNA fragments for ^{32}P labeling were isolated on mini 0.8% agarose gels in Tris Acetate buffer (TAE: 40mM Tris-acetate, 2mM EDTA pH 8.0). The restriction fragment was bound to DEAE membranes (type NA-4S, S&S) by cutting a slit and placing membranes in the gel just before and after the fragment of interest. The gel was further electrophoresed for an additional 15-30 minutes at 2X the running voltage. The DNA of interest is bound to the membrane. The DNA was released from the DEAE membrane according to the procedure described by the supplier (S&S). Restricted lambda DNA, singly restricted plasmid DNA and isolated fragments were labeled with ^{32}P-dCTP (3000 Ci/mmole, New England Nuclear (NEN), Boston, Mass), using the random primed synthesis kit (Feinberg and Volgelstein, 1983) as directed by supplier (Boehringer).

Enzymatic Assays

a. Beta-galactosaidase

E.coli strain 71-18 containing plasmid pMR500, pUC19 (Yanish-Peron *et al.*, 1985) or pSK10Δ6 were

assayed for beta-galactosidase activity by the method of Miller (1972). Samples of 1 ml from cultures grown in minimal media 1A (60mM K_2HPO_4, 30mM KH_2PO_4, 7.0mM $(NH_4)_2SO_4$, 1.7mM sodium citrate) (Miller, 1972) with or without tryptophan (50 ug/ml) and in the presence or absence of isopropyl-thio-beta-galactoside (10 ug/ml) (IPTG, Sigma), were taken at various stages of log phase growth as determined by absorption at 600nm (Spectronic 21 spectrophotometer). Cells were resuspended in 1 ml Z buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 50mM β-mercaptoethanol pH 7.0) and permeabilized with 2 drops of tolulene for 15-30 minutes. The reaction was started by the addition of 0.2 ml o-nitrophenyl-beta-D-galactoside (ONPG: 4 mg/ml in 100mM phosphate buffer pH 7.0) and stopped with 0.5 ml 1M Na_2CO_3 when sufficient yellow color developed. The cell debris was pelleted in a clinical centrifuge before reading absorption at 420nm and 550nm. Miller units were calculated by the following formula.

$$1 \text{ unit} = 1000 \times \left(\frac{(A_{420} - A_{550})}{(v \times t \times A_{600})} \right)$$

where v = volume of lysate used (1 ml) and t = incubation time of reaction in minutes.

b. The Amidotransferase Activity of Anthranilate Synthetase

1. Growth conditions: B.subtilis strains E78, E78R and BR151 as well as their transformed derivatives containing plasmid pRR106 (Table I; Table VI; Table VII and Table VIII) were cultured from single colonies picked from minimal glucose plates or from tryptone blood agar base (TBAB) (Difco) containing an additional 0.5% agar supplemented with 5.0mM $MgCl_2$ and 0.02mM $MnCl_2$. In cases where strains contained plasmid, 5 ug/ml and 10 ug/ml Km were added to minimal and TBAB plates, respectively. The colonies were inoculated for day growth in 2.5 ml penassay broth (Difco). Strains containing plasmid pRR106 were inoculated in penassay + 10 ug/ml Km. After five to six hours of growth at 37°C in a rollodrum, cells were pelleted in a clinical centrifuge, washed 2X with sterile dilution saline, and diluted 1:100. A 0.2 ml aliquot was transferred into 5 ml of Spizizen minimal media supplemented with 0.5% glucose, 0.05% vitamin-free casamino acid and the required amino acids at 100 ug/ml. The cultures were incubated overnight in a 37°C rollodrum. The following

morning three side-arm flasks were prepared containing 20 ml of supplemented Spizizen minimal media. Each flask was inoculated with 0.1 ml or more of the overnight grown culture to a starting Klett of 25-30 units. Into one of these flasks 50 ug/ml tryptophan (Trp) was added. Cultures were incubated at 37°C in an aerating water bath and followed hourly with a Klett Summerson colorimeter containing a 660nm red filter. When the three cultures reached a Klett reading ranging from 60-75 units, one of the Trp starved cultures was prepared for indole acrylic acid (IAA) addition. The contents of the flask were spun down in a sterile tube, resuspended in an equal volume of fresh minimal media of the same composition, and transferred to another side-arm flask. a 40 ul aliquot of a freshly prepared solution of IAA (10 mg/ml in ethanol) was added to this flask. An additional 20 ml of minimal media was added to the IAA containing and to the Trp starved flasks. The third flask containing Trp was also brought up to 40 ml with minimal media containing 50 ug/ml Trp. The flasks were returned to the 37°C water bath shaker. After another 2.5 to 3 hours of growth, or when Klett readings reached 140-160 units, cultures were chilled on ice for 10-15

minutes. Cultures were then pelleted at 10,000g for 5 minutes (supernatant discarded), washed in a solution of 0.1M K_2HPO_4 and 10% glycerol, repelleted at 10,000g for 5 minutes (supernatant discarded), quick-frozen in a dry ice ethanol bath, and placed in a -70°C freezer for storage until ready for the anthranilate synthetase assay.

2. Amidotransferase (trpG) Activity: Frozen pellets were thawed on ice and resuspended in 2-3 ml of buffer D (0.04M K_2HPO_4 pH 7.5, 0.1mM EDTA, 30% glycerol, 6mM beta-mercaptoethanol, and 20mM L-glutamine). Cells were disrupted by freshly prepared lysozyme (1 mg/ml in buffer D). DNA viscosity was removed by DNAase in 0.2M $MgCl_2$ at a final concentration of 100 ug/ml. The lysozyme and DNAase treatment was incubated at 37°C for 30 minutes. The extract was spun down at 29,000g for 20 minutes in the ultra centrifuge and the supernatant was kept for the assay. In a 13x100 mm test tube the following reagents were added in the following order: 0.1ml of 0.05M glutamine, 0.1 ml of 0.1M $MgSO_4$, 0.05 ml of extract, 0.7 ml of 0.1M Tris-HCl pH 7.6, and 0.05 ml of 1 mg/ml chorismate. The reaction was incubated at

37°C for 20 minutes and stopped by the addition of 0.1 ml of 1M HCl forming a white precipitate. The reaction mixture was extracted with 4 ml of ethyl acetate in a conical tube, vortexed and spun in a clinical centrifuge for 2 minutes. The upper ethyl acetate phase was saved for measurements of anthranilate activity. A standard curve was performed by preparing anthranilate (Sigma) in 1 ml at concentrations of 50 to 1000 picomoles/ml in dH_2O, followed by ethyl acetate extraction in the same manner as the reaction mixtures. The formation of anthranilate from chorismate and glutamine was monitored on an Amico-Bowmman spectrophotofluorometer at an excitation wavelength of 313nm and an emission wavelength of 393nm, at machine settings range = 0.1 (the range can be set from 0.1 to 10, 0.1 is the most sensitive range) and mode = ratio (the other mode is standard, ratio returns to the blank to rezero the machine before reading). Protein concentration was determined colorimetrically by the Lowry method (Lowry, 1951) on a 1:10 and 1:2 dilution of extract. The protein standard was bovine serum albumin at concentrations of 20-200 ug/ml. One unit of anthranilate synthetase activity was defined as the formation of 1 umole of anthranilate per minute. Specific activity was defined as units/mg of protein.

DNA Sequencing

The 2.733-kb EcoRI-HindIII fragment of pRR106 was cloned into the EcoRI-HindIII sites of Rf M13mp18 and M13mp19 (Yanish-Perron et al., 1985) by T4 DNA ligase (Boehringer) in a 3:1 target to vector ratio. Transformation of E.coli JM101 with the ligated products was as described above for plasmid transformation in E.coli, except after the 2-minute heat shock treatment, 2 ml of molten 0.6% LB top agar (45°C), 20 ul 100mM isopropanol-thio-beta-D-galactoside (IPTG, Sigma) and 100 ul of a 2% 5-bromo 4-chloro 3-indolyl-beta-D-galactopyranoside (X-gal, Boehringer) dissolved in N,N,dimethylformamide were added. The mixture was poured onto prewarmed thick YT plates, and incubated at 37°C overnight. White plaques from recombinant phages were picked, and transferred into a tube with 5 ml of 2xYT containing 0.2 ml of log phase JM101 cells. The plaque cell mixture was incubated at 37°C for 5-6 hours on a rollodrum and used to isolate double and single-strand phage DNA as described above.

DNA sequencing was by the Sanger dideoxy chain-termination method (Sanger et al., 1977). Materials for

DNA sequencing were either the premixed kits, (Pharmacia ^{35}S or T7 sequencing kits) or from the individual deoxy and dideoxy nucleotide triphosphates (Pharmacia). The solid nucleotide triphosphates were dissolved to give 10mM stock solutions as estimated by the UV absorption (Maniatus et al., 1982). Stock solutions were mixed according to an Amersham protocol for DNA sequencing, using ^{35}S dATP (400 Ci/mmole, NEN). Nucleotide mixtures were adjusted accordingly: 25 ul G + 25 ul 0.3mM ddGTP; 25 ul A + 18 ul 0.1mM ddATP; 30 ul T + 20 ul 0.5mM ddTTP; 35 ul C + 20 ul 0.1mM ddCTP. The annealing reaction entailed mixing 5 ul of single-stranded (ss) template DNA, 1 ul of M13 17-mer primer DNA (Pharmacia), 1.5 ul 10x Klenow buffer (100mM Tris-HCl pH 8.5, 100mM $MgCl_2$) and enough ddH_20 to bring the total volume up to 10 ul. This mixture was heated in a boiling water bath for 10 minutes, then slow cooled to room temperature for 15-30 minutes. From 1 to 2 ul of ^{35}S dATP and 1 to 2 units of Klenow enzyme (Boehringer) was added, gently mixed, microfuged, and distributed at 2.5 ul aliquots into 1.5 ml microfuge tubes. Two ul of the appropriate N/ddNTP mixes were added to the rim of the tube and pulse microfuged to synchronize and initite the reactions.

The reaction mixtures were incubated at 37°C for 15-25 minutes, chased with 2 ul of a 0.5mM mixture of all four dNTPs, then reincubated, at 37°C for 15 minutes, stopped by adding 4 ul of formamide dye mix (0.03% xylene cyanol, 0.03% bromophenol blue, 20mM EDTA in deionized formamide) and stored at -20°C or loaded on to a sequencing gel.

Two glass plates one 33 X 40 cm and the other 33 X 37.5 cm were cleaned with the following reagents in the following order: alconox detergent, deionized water, 95% ethanol and acetone. To the smaller plate 2 ml of 2% dichlorodimethylsilane in $CHCl_4$ was spread on to the plate with a kimwipe and allowed to dry in a fume hood for 10 minutes. During this time the polyacrylamide gel solution was prepared. For a 6% polyacrylamide gel 42 gms of ultra pure urea (Bethesda Research Laboratories, BRL), 14.5 ml of a 40% acrylamide stock solution (38:2, acrylamide: bis-acrylamide), 10 ml of 10x TBE and ddH_20 for a final volume of 100 ml was stirred with low heating until the urea dissolved. The glass plates were assembled by placing a 0.8mm thick bottom spacer and 0.4-0.8mm wedge spacers between them (International Biochemical Industries,

IBI). Edges were sealed with 3 inches of plastic packing tape and secured with binder clips. Just prior to casting the acrylamide solution between the glass plates, 0.8 ml of 10% ammonium persulfate solution (made fresh) and 50-70 ul of tetramethyl-ethylenediamine (TEMED, Kodak) were added. Fifty ml of acrylamide solution was drawn up in a 50 ml plastic syringe without a needle and squeezed in between the plates which were tilted at a 45 to 60 degree angles. This was repeated until the cavity was filled. Trapped air bubbles were eliminated as they appeared by halting the pouring process and gently rocking glass plates from side to side. When the acrylamide solution overflowed slightly, the flat side of 0.4mm sharkstooth comb (BRL) was inserted and clamped. Gels were allowed to polymerize for 45 to 60 minutes. The comb was removed and the flat surface was moistened with 1x TBE. The polymerized gel was stored in a wet autoclaved bag overnight at room temperature or in the refrigerator for up to 2 weeks. When the gel was ready to be run, the bottom spacer was removed and the sharkstooth comb was reinserted so that the pointed side touched the flat surface of the gel forming wells (without puncturing

it). The gel was mounted on to a lucite gel stand and 1x TBE buffer was added to the top and bottom reservoirs. Air bubbles at the bottom of the gel were removed by squirting 1x TBE buffer with a 25 ml syringe fitted with a bent needle (45^0 angle). A gel surface thermometer was attached (IBI) and the gel was prerun at 1200 volts, 50 mAmp (Savant high voltage power supply) or until it reached a running temperature of 48-50^{0}C (30 to 60 minutes). The sample wells were rinsed out three times with 1x TBE to remove urea diffusing from the gel. The sequencing reactions were heated to 95^{0}C for 3 minutes and 2 ul (microtiter syringe Hamilton model 710 with narrow needle) of the samples was loaded per well. Electrophoresis was for 2.5 hours or when the second dye is 1/4 the distance from the bottom of the gel. At this point it was either stopped or a second loading of the sequencing mixtures was applied. The run was stopped when the first dye of the second loading almost reached the bottom. The gel was disassembled by breaking the suction with a flat spatula. The gel usually sticks to the larger glass plate. The gel was fixed for 20 minutes in 10% acetic acid, 10% methanol. A Whatman

3MM paper cut to gel size was rolled and pressed on to the gel in order to transfer the gel from plate to paper. The gel was peeled off the glass plate via Whatman 3MM paper. The other side of the gel was covered with plastic wrap, and dried under vacuum for 1.5 to 2 hours on a slab gel dryer (Bio Rad) at 80°C.

Synthetic Oligonucleotide Primers

In order to sequence the 2.733-kb fragment without subcloning it into smaller fragments, six oligonucleotide primers were synthesized on an applied Biosystems model 380A in the sequencing and synthesis facility at Hunter College. For the M13mp19-2.7-kb template three 15mer oligonucleotides were synthesized with the following sequences: 19a; 5'GTCTGCCAACGTGA3', 19b; 5'ATCTCCTTATATGTA3', and 19c; 5'AGTTGATGCGCTCAT3'. The M13mp19-2.733-kb template was sequenced in the direction from the EcoRI to HindIII site. The M13mp18-2.733-kb template is the reverse compliment strand. It was sequenced from the HindIII to EcoRI direction. The three 15mer oligonucleotides synthesized for the M13mp18-2.733-kb template are as follows: 18a;

5'TAATCGTTTCAATTGC3', 18b; 5'AATTCATCCAGTCCA3', and 18c; 5'TTTCATAGATGAATG3'. The crude synthetic primers were supplied at 0.2 umole amounts. For purification, primers were incubated overnight in 1 ml of ammonium hydroxide at 55°C, in a sealed vial to deprotect the bases. The liquid was transferred into a 1.5 ml microtubes and evaporated in a Savant speed-vac centrifuge. Dried samples were resuspended in 100 ul of 0.05 M triethlammonium acetate pH 7.0 (TEA-Ac, diluted from a 1 M stock TEA, 1 M Acetic Acid titrated to pH 7.0 with TEA). Gel filtration with sephadex G-50 fine beads (Pharmacia) was used to desalt the primers. The beads were swelled overnight in 0.05 M TEA-Ac (20 ml/gm of beads). In a 10 ml plastic disposable pipet plugged with glass wool, a 10 ml column was poured and prerunned with 0.05 M TEA-Ac for 30 minutes before the primer was loaded. Twenty 0.5ml fractions were collected while keeping the buffer above the gel bed at all times. The absorbance at 260 nm and 280 nm was determined for all fractions by diluting 20 ul into 1 ml 0.05 M TEA-Ac. Generally one to three tubes within fractions 9-14 contained the peak of absorbance. These fractions were pooled, dried in a

speed vacuum desicator and resuspended in 200 ul of sterile ddH_2O and stored at -20°C. A 1 to 3 ul aliquot of the peak fraction was used for the annealing reaction in DNA sequencing.

Computer Analysis

The Genetic Computer Group program for nucleic acids from the University of Wisconsin was used to analyze, align, and translate the B.pumilus DNA trytophan operon sequences (Denereux et al., 1984) (see appendix for the programming strategy used to generate Figure 10).

Results and Discussion

Orientation of Plasmids pRR106 and pRR103

In order to study genes of interest it is useful to clone them away from the rest of the chromosome. Isolated genes can be more clearly scrutinized on a molecular level and their structural and functional relationships can be determined. A 3.6-kb EcoRI fragment containing the trpE, trpD, trpC, and trpF genes of B.pumilus had been cloned previously in this laboratory (Tackney and Rudner, 1981; Rudner et al., 1982; Figure 3). An identical plasmid pSL103 has also been described (Keggins et al., 1980). The restriction endonuclease cleavage site map of pRR106 with selected restriction sites relevant to this study is shown in Figure 4. The upper line illustrates the 4.5-kb parental plasmid pUB110 which contains the Km^R gene (Ehrlich, 1978) the lower bold line represents the 3.6-kb EcoRI fragment of B.pumilus DNA (Figure 3 and Figure 4).

An indirecet method for ascertaining the presence of a promoter element in a cloned fragment of DNA is to reclone the fragment in the reverse orientation. Plasmid pRR103 was isolated by restriction of plasmid pRR106 with EcoRI, religation with T4 DNA ligase, and selection for TrpC+ transformants in B.subtilis strain SB25 (trpC2, hisH2). Rapid plasmid isolation was performed on 8 clones and screened by restriction analysis for the presence of the 3.6-kb B.pumilus trp fragment in the reverse orientation. The BamHI site of the parent vector and the HindIII site of the B.pumilus insert were used to determine fragment orientation. In the case of pRR106 a double endonuclease restriction with BamHI and HindIII generated 4.9-kb and 3.2-kb fragments (Figure 4 and Figure 5). In the reverse orientation the HindIII site should be closer to the BamHI site generating 6.3-kb and 1.8-kb fragments. Of the 8 clones 2 had the fragment cloned in the reverse orientation. One of these was isolated and designated pRR103 (Figure 4 and Figure 5). In either orientation an EcoRI digest generates identical patterns of 4.5-kb and 3.6-kb fragments as expected for both plasmids (Figure 5). The isolation of pRR103 indicated the

presence of a promoter element within the cloned fragment.

Biological Activities of Plasmids pRR106 and pRR103

The biological activities of plasmid pRR106 and plasmid pRR103 were compared in order to localize additional promoter elements. The transforming activity of plasmids pRR106 and pRR103 were tested using B.subtilis trp mutants as recipients. The parent vector (pUB110) transformed all the trp recipients with an efficiency of 2-5x10^4 Km^R transformants/ug DNA (Tackney, 1981). Plasmid pRR106 contained the B.pumilus trpE, D, C and F genes as determined by complementation analysis to B.subtilis trpE, D, C, F, B, and A mutants (Tackney, 1981). The results obtained with both plasmids are shown in Table II. Transformants were initially selected for either Trp$^+$ or Km^R, followed by replica plating to determine the percent cotransformation of the unselected marker. Transforming pRR106 into all four B.subtilis recipients containing mutations in the trp genes (trpE, trpD, trpC, or trpF) and selecting initially for Km^R, resulted in 99-100% cotransformation of the Trp$^+$

phenotype (Table II). Primary selection for Trp$^+$ transformants containing pRR106 were also Km^R irrespective of the *trp* recipient strain used (Table II). However the Trp$^+$ primary transformants compared to the transformants from homologous chromosomal DNA resulted in smaller size colonies.

All strains could be transformed with pRR103 for Km^R. However, only *trpC* recipients gave rise to Trp$^+$ colonies irrespective of the primary selection (Table II). Even after a 90-minute delay, no other strains produced Trp$^+$ transformants. The biological behavior of pRR103 in *trpC* recipients suggested that an internal promoter located upstream of *trpC* may be involved.

The Structure of the B.pumilus trpE, trpD and 5'trpC Genes

For the purpose of a comparative evaluation between the *trp* operons of gram positive bacteria analogous to the ones made for gram negative bacteria, (Crawford *et al.*, 1980; Nichols and Yanofsky, 1979; Yanofsky and van Cleemput, 1982) the sequence of the *B.pumilus trp* operon was determined. If an internal

promoter does exist upstream to the B.pumilus trpC gene, it was anticipated that by homology to other Bacilli promoters it can be located. The HindIII site of the analogous plasmid pSL103 is located in the trpC gene as determined by an insertional inactivation experiment by Keggins et al., (1979). This HindIII site is also present in pRR106 (Figure 3). A 2.733-kb EcoRI-HindIII fragment of the B.pumilus trp operon suspected of containing the trpC gene and 5' sequences to it was sequenced. The sequencing strategy of the 2.733-kb fragment is shown in Figure 6. The location and extent of sequence overlap is indicated in parenthesis. The DNA sequence is shown in Figure 7. Comparisons of the published B.pumilus trp promoter leader sequence (Kuroda et al., 1986) to the one shown in Figure 7 reveals an identical overlap from +272 to +355 beginning at the EcoRI site in trpE. The numbering system started by Kuroda et al. (1986) for the B.pumilus trp leader was continued into the trpE, trpD, and 5'trpC genes (Figure 7). According to the sequences published by Kuroda et al. (1986), the cloned B.pumilus trp fragment in pRR106 as well as the identical plasmid pSL103 (Keggins et al., 1980) do not contain the promoter of the trp operon. Therefore,

the complementation analysis obtained for pRR106 (Table II) can only be mediated by one of the pUB110 promoters. The vector is known to contain at least four open reading frames (ORF), all with the same transcriptional orientation as the B.pumilus trp insert in pRR106 (McKenzie et al., 1986 and 1987). The trp insert of pRR106 was cloned within the ORF- of pUB110, but the insert was not in the same frame as the ORF's codon sequence (McKenzie et al., 1986; 1987). For expression it most likely utilizes an internal methionine located 15 bases upstream from the EcoRI cloning site (see legend of Figure 7). Upstream of the cloned trp fragment and proximal to the methionine is a weak putative RBS (AAAGcGuGauGUGA; G= -8.4 kcal) which is not stringent enough for efficient ribosome binding (Band and Henner, 1984) but sufficient to generate the observed small colony size (Table II). The capital letters of the RBS written above indicates the bases involved in binding the 3' end of the B.subtilis 16S rRNA (Murray and Rabinowitz, 1982).

The trpF gene is only partially represented (approximately 57% of the gene) in the B.pumilus insert (Tackney and Rudner, 1981; Keggins et al., 1979). Both

orientations should pose a problem with respect to the expression of the trpF gene. In the pRR106 orientation the vector could provide a transcriptional terminator which would not be possible in the pRR103 orientation. Therefore, complimenting activity of the plasmid specific trpF protein is possible only in the pRR106 orientation (Table II). The T12 mutation has been mapped to the approximate middle of the trpF gene (Carlton and Whitt, 1969). From the above results with trpF the most logical conclusion is that the enzymatic activity of the trpF gene product resides in the amino terminal half and can function as a truncated protein.

It seems unlikely that trpC from pRR103 could be initiated from a vector promoter since no ORF's were found in the opposite orientation of pUB110 (McKenzie et al., 1986, 1987). More likely as the functional evidence suggest, similar to E.coli and S.typhimurium (Margolin, 1971), a low efficiency internal promoter of novel sequence may exist upstream of the trpC gene. Supporting evidence for the existence of an internal promoter in the trp operon of B.pumilus comes from Kane and Hill (1980) who used the analogous plasmid pSL103. They showed that only the trpE gene product was regulated by tryptophan and by the product of the mtr

locus. The gene products of the trpD, trpC, and trpF were not. They concluded that there must be two promoters in the cloned B.pumilus trp insert: one regulated by tryptophan and one not. In view of the results presented below on the measurements of anthranilate synthetase activity, the DNA sequence of the trpE, trpD, and 5'trpC genes of B.pumilus, and the sequence data of Kuroda et al., (1986) it appears that the tryptophan sensitivity of anthranilate synthetase (the trpE gene product) from pRR106 is probably due to feedback inhibition (Patel et al., 1974) and not to a Trp regulated promoter. The cloned B.pumilus trpE gene product was regulated in an mtr background (see Measurements of Anthranilate Synthetase, pg 64 and 71 for definition of mtr, and discussion of Table VI, VII, and VIII). The mtr effect measured by us and Kane and Hill (1980) indicates that the mtr gene product may act at other locations besides the trp leader transcript (Shimotsu et al., 1986). The conclusion made by Kane and Hill (1980) of an additional promoter, not regulated by Trp or the mtr gene product is supported by the biological activities of pRR106 and pRR103 (Table II).

Sequence analysis was undertaken to search for consensus promoter-like elements within the structural genes. Internal promoters are known to serve a dual function: at times the sequence is part of a protein and is transcribed, while at other times it serves as a regulatory element (Horowitz and Platt, 1982). Originally an internal promoter was identified by Bauerle and Margolin (1967) by genetic and enzymatic analyzes in trp mutants of S.typhimurium. The location was upstream to the trpC gene in the structural sequences of the trpD gene. An analogous site was found a year later in the trp operon of E.coli (Morse and Yanofsky, 1968). The sequence of the E.coli internal promoter did not resemble the typical consensus promoters. From S1 nuclease analysis it was identified as GTGACA at the -35 region and TACAAG at the -10 region (Horowitz and Platt, 1982). To determine whether the B.subtilis trp operon contained additional promoters Shimotsu and Henner (1986) removed the promoter upstream to trpE and fused a lacZ gene to the end of the trpF gene and integrated the construct into the amyE locus. Their results did not show significant expression of beta-galactosaidase above background levels, which led them to conclude that either there is no internal

promoter in the B.subtilis trp operon or that the expression of a possible promoter may not be high enough to detect above background levels. Upstream to the initiation Met codon of the B.pumilus trpC gene (ATG at +2870-2872; Figure 7), there is also no recognizable consensus promoter element, nor sequences resembling the E.coli internal promoter. Although consensus-like promoter elements were not found immediately 5' to trpC, three consensus promoter-like sequences were found in the trpE gene of B.pumilus at the following locations: 1) +337-342 (TTGAAA) -35, +361-366 (TCTCAC) -10; 2) +379-384 (TTGAAA) -35, +424-429 (TGTATT) -10; and 3) +1728-1733 (CTGAAA) -35, +1752-1757 (CATATT) -10 (Table III). The first two were tested in a promoter search plasmid (see next section).

Measurements of beta-galactosaidase Activity from pMR500

To determine whether the first two (1 and 2) promoter-like elements within the B.pumilus trpE gene had any promoter activity, an EcoRI-BclI 504 bp fragment was cloned upstream to a promoterless lacZ gene of plasmid pSK10Δ6 resulting in the recombinant plasmid

pMR500 (Figure 8). This fragment served as a promoter and expressed beta-galactosaidase when transformed into E.coli host 71-18. In Table V the results of the measurements of beta-galactosaidase activity in Miller units (Miller, 1972) of plasmid pMR500 compared to parental plasmid pSK10 6, and positive control plasmid pUC19 are shown. Plasmid pUC19 (Yanish-Perron et al., 1985) contains the endogenous beta-galactosaidase promoter (β-gal). Compared to the endogenous β-gal promoter, the promoter activity of pMR500 is moderate to low. In E.coli there is no tryptophan sensitivity. Parental plasmid pSK10Δ6 gives the expected negative results, since it does not contain a promoter. The primary structure of consensus promoter sequences recognized by vegetative RNA polymerase is identical in E.coli (McClure et al., 1985) and B.subtilis (Moran et al., 1982). Although the capability of this B.pumilus trp fragment to show promoter activity in E.coli is suggestive, it does not unequivocally prove that it functions as an internal promoter in B.pumilus. If the conclusion by Shimotsu and Henner (1986) that an internal promoter may not exist in the trp operon of B.subtilis is true, our observations may represent a difference in the regulation of the trp operons between

the two Bacilli species. A better system currently available for testing promoters in B.subtilis is the plasmid pDH32, a recent unpublished derivative of ptrpIGI (Shimotsu and Henner, 1986).

Organization of the B.pumilus trp Genes

The organization of the B.pumilus trp genes were analyzed with respect to their initiation signals, termination signals, ribosomal binding sites (RBS) and homology to the B.subtilis trp genes. Since the B.pumilus trp operon is organized much like that of B.subtilis and is regulated by the same novel form of transcription attenuation (Kuroda et al., 1986; Shimotsu et al., 1986), the published B.subtilis trp sequence (Henner et al., 1985) was used as a reference to locate the beginnings and ends of the trpE, trpD, and 5'trpC genes of B.pumilus. When the two sequences were maximally aligned, a value of 66% homology emerged between the two species (Figure 9). Similarly, the trp promoter-leader regions were compared between B.pumilus and B.subtilis and the sequences were found to be 62% homologous (Kuroda et al., 1986).

The RBS and the start codon for trpE sequenced by Kuroda et al., (1986) were not present on the insert of pRR106. As was found in the B.subtilis and E.coli trp operons, the termination codon for the B.pumilus trpE gene overlaps the coding sequences of trpD. There are 29 nucleotides (nt) between the beginning of trpD and the end of trpE (Figure 7 and 9). The termination codon of trpD also overlaps the initiation codon of trpC by 8 nt. Both of these overlaps are similar to the situation found in the B.subtilis trp operon (Figure 9; Henner et al., 1985). In the E.coli trp operon there is one nucleotide separating the end of trpE and the beginning of trpD, and 3 nucleotides between the end of trpD and the beginning of trpC (Yanofsky et al., 1981). In the E.coli trp operon, the overlapping of intercistronic regions is associated with translational coupling (Oppenheim and Yanofsky, 1980). The sequence data suggest that in B.pumilus the trpE, trpD, and trpC genes are also translationally coupled. Although it is impossible to be certain about the overlapping sequences in B.pumilus without protein data, it is felt that this finding is common to many bacterial trp operons (Yanofsky and Crawford, 1987). A potential trpD RBS, which has the

proper sequence AGaAAAGGAGaGUG, was located in trpE. The calculated free energy (G) for that RBS and the 3' end of the 16S rRNA of B.subtilis was -18.0 kcal (Tinoco et al., 1973). The potential RBS of the trpC gene AGaGAGGaaGA has a calculated G of -13.8 kcal. Its location is within the end of the trpD gene (Figure 7).

Comparisons of the trp Coding Sequence of B.pumilus B.subtilis and E.coli

The deduced amino acid sequences for the first three Bacilli trp genes were easily aligned with their E.coli counterparts (Figure 10). Among the Bacilli species, the values for amino acid identity are 68, 62, and 65% for the trpE, trpD and 5'trpC genes, respectively. When the charged related residues were included the % similarities were 79, 76, and 72%, respectively. Approximately a third of the amino acids match exactly between the two Bacilli and E.coli. The charge related residues of the two Bacilli and E.coli trp proteins averages to 55%.

A critical serine residue, part of a conserved sequence LLES (Leu-Leu-Glu-Ser) (Matsui et al., 1987)

located upstream in the trpE gene, was found in the Bacilli species alignment at residues 47-50 [i.e. (89-92)-42] and in E.coli at 37-40 (Figure 10 A). Apparently this region is essential for the allosteric regulation of the trpE gene product of the anthranilate synthetase complex. In Brevibacterium lactofermentum (B.lactofermentum), a substitution of R (Arg) for the S (Ser) caused the desensitization to feed back inhibition by tryptophan and also conferred resistance to 5-fluorotryptophan (an mtr mutation) (Matsui et al., 1987). Since regulation of the Bacillus trp operon is governed by the product of the mtr locus (Shimotsu et al., 1986), the conservation of this sequence may have functional importance. Since the mtr mutation was caused by this amino acid substitution in the B.lactofermentum trpE gene at an area not believed to be a leader or an operator sequence, it indicates that mtr regulation may act on the structural gene. Resistance to the tryptophan analogs 5-methytryptophan and 5-fluorotryptophan have been localized to the trpR and trp operator locus in E.coli (Cohen and Jacob, 1959; Hiraga, 1969). The trpR codes for the regulatory protein (Trp aporepressor) in E.coli (Zubay et al., 1972). In the Bacilli, 5-methyltryptophan resistance

is believed to be the result of a mutated Trp regulatory protein localized to the mtr locus (Hoch, 1974). The site of action of the Trp regulatory proteins is on the DNA sequence (Operator) in E.coli, on the trp mRNA transcript in B.subtilis and B.pumilus and possibly on the trpE gene in B.lactofermentum. Whether the effect of a proposed Trp regulatory protein in B.lactofermentum is on the mRNA level or on the protein level of the trpE gene remains to be determined. As presented below in the section on the Measurements of Anthranilate Synthetase Activity of B.pumilus, the mtr gene product is apparently also having an effect on the trpE gene. The location, isolation and characterization of the mtr gene from the Bacilli and B.lactofermentum should resolve whether there is a single site of action or multiple sites of action for this gene product.

In E.coli, the arrangement of the trpD and trpG gene differs from the Bacilli (Figure 2; Yanofsky et al., 1971; Jackson and Yanofsky, 1974). In E.coli the two genes are fused while in the Bacilli species the trpG function is unlinked to the trp operon (Hoch et al., 1969; Kane et al., 1972). Therefore, the alignment shown in Figure 10 begins after amino acid

number 200 in E.coli, where the trpD genes of the Bacilli species were maximally aligned. A remarkable conserved block of 50 amino acids was found in the alignment from 276-325 of the trpD gene (Figure 10 B). A computer search from GenBank for that sequence showed that other microorganisms like A.calcoaceticus, B.lactofermentum, S.typhimurium and S.cerevisiae contain the highly conserved block (Figure 10 C). The 5' portion of the conserved region from 279-284, coding for the amino acids GTGGDG, was invariant and found in all microorganisms compared including S.cerevisiae. It was noted that this sequence GTGGDG (Gly-Tyr-Gly-Gly-Asp-Gly) is characteristic of an ATP-binding site (GXGXXG-16-23 amino acids-K) found in almost all known protein kinases and in some nucleotide binding proteins (Hunter and Cooper, 1985). A K (Lys) was found in the trpD gene product (anthranilate phosphoribosyl transferase) after amino acid number 20-22 from the last G (Gly) of the ATP-binding site in all organisms available, except A.calcoaceticus (T, Tyr; Figure 10 C). In some protein kinases, it has been demonstrated that this K (Lys) binds the gamma PO_4 of ATP while preliminary evidence indicate that the

other two PO_4 sit in the GXGXXG loop (Taylor et al., 1990). Although protein kinases transfer PO_4 groups from ATP onto Ser, Thr, or Tyr residues of substrate proteins (Taylor et al., 1990) this sequence may be playing a similar but not identical role in the phosphoribosyl transferase activity of the trpD gene product (Figure 1). By analogy it is likely that the pyrophosphates, of PRPP (Figure 1) may be held by the K (Lys) and G (Gly) loop while the phosphoribosyl moiety is transferred to anthranilate.

At the 3' portion of the conserved block at amino acids 312-320 a unique sequence with the following amino acids: RSVSSKSGS (Arg-Ser-Val-Ser-Ser-Lys-Ser-Gly-Ser; Figure 10 C) is observed. The richness of serine residues may have functional significance in the phosphoribosyl transferase activity of the trpD gene product since phosphorylated serines in proteins have been associated with regulation (Taylor et al., 1990). Its proximity to the invariant sequence of PO_4 acceptor sites make this hypothesis plausible.

Codon Usage

Codon usage in the B.pumilus trp sequences were compared with published tables reported for B.subtilis and E.coli by Henner et al., (1985) and Yanofsky et al., (1981), respectively (Table V). Overall, codon utilization in both Bacilli was similar especially for the trpE genes. However, differences were found in B.pumilus with certain favored codons involving A in the third position. For example, the B.pumilus sequence of the trpD gene product only contained two of the four codons for proline, (CCG 40% and CCA 60%); while B.subtilis used all four, with CCA being the least utilized (8%). For glutamine, with only two choices, B.pumilus favored CAA (80%) and B.subtilis CAG (78%). There is a complete absence of codons with GG in the second and third positions in the trpE and trpD gene products in E.coli. This may have functional or structural implications considering that G is the base which is most mutable and subject to transitional and frameshift mutations. In addition TGG is the only codon for Trp and Trp codons are not found in the E.coli trpE, trpD, and trpA nor in the B.subtilis

trpC and trpA gene products (Yanofsky et al., 1981; Henner et al., 1985).

The presence of tryptophan residues were evaluated for the sequenced portion of B.pumilus. There were 3 and 0 Trp codons in trpE and trpD, respectively, compared to 2 and 1 for B.subtilis trpE and trpD genes. The physiological or evolutionary implications of this observation and the variation between the three species remains unknown.

According to Grantham et al., (1981), the non-random pattern of codon usage is characteristic of highly expressed genes in bacteria. The use of U over C in the third position defines the level of gene expression as high and non-random (Grantham et al., 1981). The U(T)/C ratios of nucleotide choices in the third position of 16 pairs of codons for the trpE and trpD genes were calculated. They were found to be 1.44 and 1.44 for B.pumilus and 1.94 and 1.44 for B.subtilis trpE and trpD genes, respectively. For E.coli the ratios were 0.85 and 0.75 for the trpE and trpG-D genes, respectively. These ratios exceeded expectation based on the composition of Bacillus genomes (T/C=1.27) and were below the expected value of 0.92 for E.coli

(Yanofsky et al., 1981; Henner et al., 1985). Therefore we do not agree with Henner et al., (1985) who concluded that the B.subtilis trp operon is not expressed at high levels because the codon usage seems more random than that seen for E.coli.

Measurements of Anthranilate Synthetase Activity

To resolve whether the cloned B.pumilus trpE gene product was regulated by the tryptophan availability in the host cell, measurements of anthranilate synthetase activity were performed. The crude extracts prepared from B.subtilis contain both the aminotransferase subunit (trpE) and the amidotransferase subunit (trpG) of the anthranilate synthetase complex. The trpG subunit (amidotransferase) cannot synthesize anthranilate alone although the trpE subunit (at pH 8.0 and in the presence of NH_4Cl) can. Patel et al. (1974) showed that the B.pumilus trpE subunit forms an active anthranilate synthetase complex with the B.subtilis trpG subunit. Thus measuring the amidotransferase activity is equivalent to measuring the activity of the hybrid anthranilate synthetase complex. The B.subtilis

trp recipients chosen, BR151 (trpC2, metB10, lys-3), E78 (trpE24) and E78R (trpE24, mtr), were transformed either with B.subtilis chromosomal DNA from strains NCTC3610 or W23, or plasmid pRR106, or with both chromosomal DNA and plasmid (pRR106). It has been shown that the endogenous aminotransferase subunit trpE of B.subtilis E78R (trpE24, mtr) does not complex with the endogenous trpG subunit (Kane and Hill, 1980). Strain BR151 was chosen because it contains an intact chromosomal trpE gene. It serves as a positive control for the measurements of ASase activity in response to the presence or absence of tryptophan. Strains E78 and E78R are isogenic trpE deletion mutants (Kane, 1977). These strains serve as trpE mutants for the measurements of ASase specific activity from the B.pumilus trpE/ B.subtilis trpG complex.

An additional feature of strain E78R, is its mtr mutation. This strain serves the purpose of a regulatory mutant. The mtr gene is believed to code for the Trp aporepressor. Bacilli strains carrying the mtr mutation constitutively produce the enzymes of the tryptophan operon regardless of the tryptophan availability in the cell. ASase specific activity

measurements of transformed BR151, E78, and E78R in response to the presence or absence of tryptophan, and in the presence of the tryptophan analogue 3-indolyacrylic acid (IAA) are shown in Table VI, VII, and VIII. The analogue 3-indolyacrylic acid (IAA) is capable of prolonging the 1/2 life of the tryptophan message in E. coli (Morse et al., 1969). Hershfield et al., (1974) used IAA to show complete depression of all plasmid derived copies of tryptophan synthetic enzymes in E.coli. Whereas studies by Nagahari et al., (1977) showed that the repression state of the host cell had a greater effect in controlling trp gene expression over the effects of gene dosage and derepression by IAA. In this study IAA was used to determine whether the repression state of the host cell would have the greater effect in controlling trpE gene expression or whether complete derepression of all plasmid derived B.pumilus trpE gene product is obtainable.

The results in Table VI, VII, and VIII clearly show that strains transformed with chromosomal DNA have the expected response to the presence or absence of tryptophan and to IAA. In one case (see Table VIII #1) E78R was also transformed to the wild type with respect to its sensitivity to 5-CH_3-Trp. This is entirely

possible since transformation crosses between trp and mtr mutants showed the two genes to be approximately 60 map units from each other (Hoch, 1974). In other words, both markers can be cotransformed 40% of the time. The two other E78R TrpE+ chromosomal transformants shown in Table VIII (#2 and #3) retained the mtr mutation and showed lack of ASase regulation in response to tryptophan availability. There is a 2-3 fold increase in ASase specific activity induced by IAA when the trpE gene is present in one copy (Table VI).

In E78/pRR106 as compared to E78R/pRR106 there appears to be regulation of ASase activity in response to tryptophan (Table VII and Table VIII). Yet in E78/pRR106 ASase is not totally repressed in the presence of tryptophan, nor totally derepressed in the presence of IAA. It is not expected for ASase in E78R/pRR106 to respond to tryptophan and yet some repression seems to be occurring. But its lack of total derepression in the presence of IAA is unexpected since Hershfield et al., (1974) suggested that in E.coli, IAA totally derepressed all plasmid derived ASase. Plasmid copy number was determined to estimate the number of plasmids in B.subtilis. By CsCl-EtBr and gel electro-

phoresis 20 to 30 copies of pRR106 were found in B.subtilis (Table IX; unpublished results provided by Miriam Reches). Therefore, if all plasmids are transcribing ASase message one would expect at least a 20 to 30 fold increase in specific activity. No such significant increase in specific activity was observed (Table VI, VII, and VIII). A maximum 3 fold increase in ASase activity relative to the derepressed level was obtained in strains transformed with chromosomal DNA and plasmid pRR106 (Table VII and VIII). This result is not higher than that observed with BR151 containing plasmid pRR106 in the presence of IAA (Table VI). These results seem to indicate that the repression system of the host cell is highly efficient even at high gene dosage.

It is possible that part of the repression system may indirectly involve trpG, since the trpG gene is present in only one copy and it is required for the amidotransferase reaction of ASase. Although trpG is not regulated by tryptophan nor by the mtr gene product, it is regulated by the gene products of the trpE and pabA loci. An increase in trpE gene product increases trpG gene product in the cell (Kane, 1977). It is possible that the amount of trpG gene product made is

insufficient to contribute to the expected high levels of the trpE gene product from the plasmid, although 20 to 30 copies of pRR106 are present in the cell and theoretically can produce 20 to 30 copies of the trpE gene product. The amount that can be assayed with the amidotransferase reaction may be limited to the amount of the trpG gene product that is produced. Kane and Hill (1980) measured the aminotransferase activity (trpE) alone (without trpG) using the strain E78 carrying identical plasmid pSL103. The level they observed of 0.2 nanomoles of anthranilate formed per mg protein in the absence of tryptophan is within the level observed by us (Table VII).

Another component of the repression system may involve feedback inhibition by tryptophan on ASase. Feedback inhibition may account for some of the observed tryptophan regulation of ASase specific activity in the absence of the trp promoter and the trpL region in pRR106 (Table VII). Feedback inhibition cannot be the sole reason for tryptophan regulation of ASase in pRR106 containing cells. Otherwise one would not observe an mtr effect on the specific activity of ASase as shown

in Table VIII. Where ASase specific activity continues to be measured in the presence of tryptophan.

Another aspect of the host repression system may involve the stability of the B.pumilus trp mRNA. The fragment of the B.pumilus trp operon on pRR106 does not contain any trp operon 5' or 3' sequences. These sequences have been shown to contribute to the stability of the E.coli trp mRNA during tryptophan deprivation (Schlessinger et al., 1977; Mott et al., 1985). To date, in the Bacilli the role of 5' and 3' sequence and their contribution to the stability of the trp mRNA have not been investigated. If by analogy to E.coli these sequences are important to the stability of the Bacilli trp mRNA then it is likely that a majority of the mRNA produced from pRR106 may be degraded before it is translated.

The mtr gene product has an effect on the amidotransferase reaction of the hybrid anthranilate synthetase by its sensitivity to the presence or absence of Trp (Table VII) and by its continued synthesis in an mtr mutant (Table VIII). Since the B.pumilus trp fragment on pRR106 does not contain any of the trp leader sequence, the mtr gene product may

be having an effect on the ASase protein or on another area of the trp mRNA. A number of direct repeats are believed to be involved in the mtr regulation on the B.subtilis trpL sequence (Shimotsu et al., 1986). These repeats were searched for in the B.pumilus trp sequences. Only one such sequence (AGAGAA) was found twice in the B.pumilus trp sequence at nucleotide locations +1233-1238 in trpE and at +2205-2210 in trpD (Figure 7). Whether they are involved in the process of mtr regulation remains to be determined by deleting these sequences and by following the subsequent mtr effect.

Heterologous Integration

Transformation efficiency of heterologous DNA has been used to measure the taxonomic relationship between organisms (Wilson and Young, 1972). The factors that influence the mechanism of foreign DNA integration in B.subtilis has been of interest to several investigators (Young, 1983; Harris-Warrick and Letterberg, 1978). For example, % homology, length, concentration, and conformation of foreign DNA as well

as other factors have been proposed to effect integration. With the available DNA sequence of the B.subtilis trp operon (Henner et al., 1985) and 2.733kb of the B.pumilus trp operon (Rivas et al., 1990 and this thesis), it may be now possible to describe what is occurring at the molecular level. Presented in this section are preliminary experiments aimed toward this goal.

Transformation Efficiencies

Two integrative plasmid derivatives of pJH101 were constructed (Figure 11 and 12). Plasmid pJH101 is a bifunctional plasmid made of a fusion of pBR322 and pCR194 (Ferrari et al., 1985). It contains single restriction sites in the Ap^R and Tc^R genes that are selectable via inactivation by a cloned fragment. Plasmid pJH101 can integrate into the B.subtilis chromosome only if a segment of homologous B.subtilis DNA is available. The B.subtilis cells are selected as Cm^R transformants. The only way to express Cm^R is if the plasmid integrates into the chromosome via a Campbell-like mechanism, in this case via heterologous

recombination provided by the B.pumilus trp sequences. Plasmid pMR111 contains the sequenced 2.733-kb EcoRI-HindIII fragment cloned into the Tc^R gene. Plasmid pMR131 contains the 0.9-kb fragment cloned in the opposite orientation relative to pMR111 also in the Tc^R gene (Figure 11). To increase the efficiencies of the plasmids multimeric forms of both plasmids were produced as stated in materials and methods. It was reported by Canosi et al., (1978) that multimeric forms of plasmid are required in order to efficiently transform B.subtilis recipients. Plasmid pRR106 gave the expected results namely, it transformed B.subtilis trpE, D, C, and F mutants to Km^R and Trp^+ at nearly equal efficiencies (see also Table II; Table X). Integration of plasmids pMR111 and pMR131 have barely occurred as demonstrated by the low efficiency of Cm^R transformants (1-15 Cm^R transformants/ml; Table X). These transformants were rarely Trp^+. To illustrate this phenomenon several Cm^R transformants were picked and grown in minimal media. The cultures did not grow overnight to full turbidity. Instead there was light growth in all tubes. These were subsequently washed 2X in dilution salts and spot-tested on LB plates containing 10ug/ml Cm and on minimal plates

containing histidine. The phenotypes of 17 E88/pMR111 transformants are shown in Table XI. There appeared to be several trp (+/-) colonies. A similar result was obtained for 17 BD170/pMR111 transformants. Only 1 out of 17 Cm^R transformants was Trp^+ in each recipient strain. These results indicate that the rare pMR111 B.subtilis integrant is very unstable and made up of a mixed population. Plasmid pMR111 transformed strains E88 (trpE46, hisB2) and DB83 (trpD2), to Trp^+ at very low efficiencies: 30 and 115 transformants/ml, respectively, (Table X). In DB83 the Trp^+ transformants were of small colony size indicating slow growth. In BD170 (trpC2, thr-5) and T12 (trpF12) no Trp^+ transformants were obtained. The low primary Trp^+ transformants of E88 and DB83 may indicate that they are the result of a spontaneous reversion. Since both of these strains are known to be revertible (Carlton and Whitt, 1969).

Chromosomal DNA was prepared from 2 transformants of strain E88 one Cm^R Trp^+ and the other Cm^R trp- designated MR188 and MR189, respectively. The DNAs were analyzed biologically in transformation assays and physically by Southern blotting.

To test whether the integrated B.pumilus trp fragment is intact and linked to Cm^R, integrant $TrpE^+$ DNA (MR188; E88/pMR111) was used to transform the original parental B.subtilis recipient (E88). For comparison DNA from a trpE- integrant (MR189; E88/pMR111) was also tested. The Cm^R character of both DNAs was transferred at 1/10 the efficiency of prototrophic homologous DNA (NCTC3610; Table XII). There was no difference in the efficiency of Trp^+ transformants between NCTC3610 and MR188 DNA. Upon replica plating primary Cm^R transformants, only a few showed the Trp^+ character. For example, 22 of 68 Cm^R transformants were also Trp^+ (Table XII). These results may indicate that in some of these transformants only a small segment of the B.pumilus trp sequences facilitated the integration. Another possibility is that part of the integrated trp sequences were deleted or rearranged so that it can no longer be expressed. Southern analysis of MR188 DNA show that B.pumilus trp sequences integrated into the B.subtilis chromosome (Figure 13; see below). Whether all or a portion of the 2.733-kb was integrated remains to be determined.

Harris-Warrick and Letterberg (1978), calculated a minimum length of 3.0×10^6 daltons (40 kb) of heterologous DNA from B.globigii was required to facilitate integration. In our studies, 0.9-kb and 2.733-kb with 66% homology on a plasmid were hardly able to facilitate integration (Tables X, XI, and XII). This conclusion is based on the low transformation efficiencies of sequences from B.pumilus compared to homologous B.subtilis sequences in pJH101 (La Fauci et al., 1986; Jarvis et al., 1988). In the integrated state the B.pumilus trp sequences are either not totally present or are unable to express. In pJH101 derivatives with homologous leucine fragments this was not the case, both markers were nearly 100% cotransformed. Therefore, transformation efficiency via heterologous integration may be limited by the size of the donor DNA, not because a larger piece is required to facilitate the integration event (pMR111 and pMR131), but because a larger piece is required to carry a complete foreign gene for efficient expression.

Southern Analysis of an Integration Event

To gain insight into the nature of an integration event, DNAs MR188 and MR189 were restricted with HindIII and subjected to Southern Analysis. If the integration event occurred via a Campbell-like mechanism, then restriction analysis should generate predictable band sizes on Southern blots. For instance it is known that in the trp operon of B.subtilis there are 3 HindIII sites (Henner et al., 1985). A 4.2-kb HindIII fragment can be generated containing the trpE,D,C,F and 5'trpB genes. To verify the 4.2-kb band, the parental and integrant DNA were probed with B.subtilis sequences (Figure 13). The parental DNA (prior to plasmid integration) are shown in Figure 13 A. Parental strains like BD170 or the prototroph NCTC3610 exhibited the 4.2-kb band while strain E88 showed a 6.0-kb band. Assuming the HindIII site in trpE is absent in E88 and the plasmid pMR111 finds partial homology and integrates into the trpE gene, then the predicted bands in a HindIII digest should be 5.7 kb and 8.4 kb (Figure 14). In the case of MR188 DNA, a 6.0-kb and 8.2-kb hybridized to probe pTrp-H3B2 and is

in good agreement with the predicted sizes (Figure 13 B). However with M13mp 19-2.7-kb as probe 8.2-kb and 11.1-kb bands are observed (Figure 13 C). With pJH101 as probe only the 8.4-kb should be observed (Figure 14). Again the apparent same size bands observed with M13mp19-2.7-kb (8.2-kb and 11.1-kb) hybridized to pJH101 (Figure 13 D). A simple model based on a Campbell-like integration with a monomermic form of the plasmic cannot account for the observed results from Southern analysis. Nor can the observed bands be explained by the integration of a linear plasmid by a double crossover event. Additional restriction analysis with another enzyme like BglII (B2) may give information as to what occurred on the molecular level. Additionally, plasmids rescued from integrant chromosomal DNA by restriction with EcoRI for 5' sequences and by HindIII for 3' sequences, and a sequence determination of the rescued sequences would lead to an understanding of the molecular events that occurred during the heterologous integration event.

Concluding Remarks and Summary

The comparative evaluation of the DNA sequence of the tryptophan operon of *B.pumilus* to its relative *B.subtilis* has revealed that a difference of 33% (66% homology) is enough to present a barrier to heterologous integration as measured by low transformation efficiencies. These low transformation efficiencies may be due to the absence of a complete foreign gene that may be needed for efficient expression. Southern analysis of a single integration event (MR188; Figure 13) indicates that it cannot be explained by a Campbell-like or by a double crossover mechanism of a monomermic plasmid. Additional analysis of this and other integration events may uncover the structure of the plasmid and/or whether a different mechanism of heterologous integration had occurred.

There are a number of similar characteristics between the two species that have been retained through their divergence as two separate species. Both regulate *trp* operon expression by transcript attenuation (Shimotsu *et al.*, 1986; Kuroda *et al.*, 1986). Over-

lapping of translational start and stop signals for the trpE, trpD, and trpC genes were found for both and may indicate a role for translational coupling in the trp operon of both species. The gene cluster for purine biosynthesis in B.subtilis also appears to be translationally coupled (Zalkin and Ebbole, 1988). Translational coupling appears to be a general mechanism whose role may be to insure a 1:1 stoichiometry for proteins produced from a monocistronic message and not just as a single observation made in the E.coli trp operon (Oppenheim and Yanofsky, 1980).

The protein sequences show that certain amino acids residues which are important either for function or structure have been conserved. This conservation is not just between related species but between unrelated organisms as well. For example the sequence LLES in trpE is believed to be the site for feedback inhibition and for the mtr mutation in B.lactofermentum (Matsui et al., 1987). The same may be true in B.pumilus and B.subtilis and can be easily tested by site directed mutagenesis and the subsequent tryptophan insensitivity of the assayed ASase. The conserved amino acid sequence shown in Figure 10 C for trpD is the same sequence

reported to be important for protein kinase function (Taylor et al., 1990). Although the trpD gene product is not believed to be a kinase, conservation of this amino acid sequence may be important for proteins involved in phosphate transfer in general and have been conserved from procaryotic to eucaryotic organisms.

The detection of an internal promoter in the tryptophan operon of B.pumilus by genetic means awaits further verification through S1 nuclease analysis. If one of the two consensus promoter sequences in the trpE gene does function in vivo, it would have to take into account the lack of expression of the gene immediately following it (the trpD gene). Translational coupling may be responsible for its lack of expression. As stated by Zalkin and Ebbole (1988) there appears to be two primary requirements for translational coupling. One is that the upstream gene should terminate within or slightly beyond the initiation region of the down-stream gene. Two, the ribosome binding site is required for high level coupled translation. If the trp mRNA is not translated it will fold up to achieve its lowest energy conformation. The folding of the mRNA obscures the ribosome binding site for the trpD gene (data not

represented). The movement of the translating ribosome may unmask the RBS for *trpD* allowing for its expression whereas lack of translation makes it unavailable for *trpD* expression. This is a possible explanation that can be tested by specific mutational manipulation on the now known sequence of the *trpE*, *trpD*, and 5'*trpC* genes of *B.pumilus*. For instance sequences suspected as contributors to the masking (via hydrogen bonding) of the *trpD* RBS can be changed so that the RBS is no longer masked. After in vitro mutatgenesis, one should be able to assay for the expression of *trpD* in a pRR103 orientation. With the now available translational fusion cloning vector pDH32 derived from p*trp*IGI (Shimotsu and Henner, 1986) the 504-bp *Eco*RI-*Bcl*I fragment tested in *E.coli* can now be tested in *B.subtilis*.

In Summary the Studies Showed:

1) The trpEDC and part of trpF of the B.pumilus trp operon was cloned into pUB110 in both orientations.

2) In B.subtilis the two plasmids, pRR106 and pRR103, behave differently with respect to their expression in trpE, D, and F recipients.

3) The two plasmids, pRR106 and pRR103 behave identically in trpC recipients indicating the presence of an internal promoter.

4) The sequence of trpE, D, and 5'trpC genes of B.pumilus was determined.

5) A 66% homology exist between the trpE, D, and 5'trpC genes of B.pumilus and B.subtilis.

6) Two RBS sites were localized upstream to the B.pumilus trpD and trpC genes.

7) By homology to other B.subtilis promoters, three possible promoters were identified in the structural sequence of trpE.

8) Promoter activity is present in a 504 bp EcoRI-BclI of trpE by its ability to turn on a promotorless lacZ gene in E.coli and was insensitive to the presence of tryptophan and IPTG.

9) Between the B.pumilus and B.subtilis trpE, trpD and 5'trpC genes there are 68, 62, and 65% amino acids identity, respectively.

10) The amino acid similarity between the B.pumilus and B.subtilis trpE, trpD and 5'trpC genes there are 79, 76, and 72%, respectively.

11) Between the two Bacilli and E.coli there is an average of 55% amino acid similarity for the trpE, trpD and 5'trpC genes and only a third of the amino acids are exactly alike.

12) The sequence of LLES believed to be necessary for feedback inhibition in B.lactofermentum was also identified in the B.pumilus trpE gene product.

13) In the amino acid sequence of trpD a consensus ATP-binding site was identified by its identity to other ATP-binding sites in protein kinases.

14) The relatively high copy numer (20-32) of the cloned B.pumilus trp fragment did not result in the over-production of the enzyme anthranilate synthetase.

15) The tryptophan analogue, 3-indolyacrylic acid (IAA) caused a 2-3 fold increase in enzyme activity.

16) The effect of the mtr gene on the anthranilate synthetase activity indicated that it may act on other regions besides the B.pumilus trpL region.

17) The 2.733-kb and 0.9-kb B.pumilus trp fragments cloned into the integrable plasmid pJH101 barely facilitated integration into the B.subtilis

chromosome as shown by low transformation efficiencies.

18) The generation of odd patterns of DNA on Southern blots could not be explained by a traditional Campbell-like integration.

Table I

A. Bacterial Strains

	Genotype	Source
B.pumilus		
RUB502	*bio*, Rif^R, Ery^R, Str^R	F.E. Young
Bp503	*bio*, *trpC*	P.S. Lovett
Bp8A1	*bio*	P.S. Lovett
B.subtilis[b]		
E78	*trpE*24	J. Kane
E78R	*trpE*24, *mtr*	J. Kane
E88	*trpE*46, *hisH*2	P.S. Lovett
DB83	*trpD*2	D.H. Dean
BD170	*trpC*2, *thrA*5	D. Dubnau
SB25	*trpC*2, *hisH*2	D. Dubnau
BR151	*trpC*2, *metB*10, *lys*-3	R. Yasbin
T12	*trpF*12	P.S. Lovett
W23	Str^R	BGSC[a]
NCTC3610	prototroph	A. Sonenshein
Heterologous integrants		
MR188	E88::pMR111, *hisH*2, Cm^R	This study
MR189	E88::pMR111, *trpE*46 *hisH*2, Cm^R	This study
E.coli		
HB101	*hsdS*20, *recA*13, *ara*-14, *proA*2, *lacY*1, *galK*2, *rpL*20, *xyl*-5, *mtl*-1, *supE*44	K. Bott
JM101	(*lac*-*proAB*), *thi*1, *supE*, (F'*traD*36, ProAB+, $lacI^q$, Z M15)	E. Dubnau
71-18	(*lac*-*proAB*), *thi*1, *supE*, (F' ProAB+, $lacI^q$, Z M15)	C. Squires

Continuation of Table I

B. Recombinant plasmids

pUB110	Km^R	D. Dubnau
pRR106	*B.pumilus* TrpEDCF, Km^R	R. Rudner
pRR103	*B.pumilus* TrpFCDE, Km^R	This study
pJH101	Tc^R, Ap^R, Cm^R	K. Bott
pSK10Δ6	Ap^R, promotorless *lacZ*	E. Dubnau
pMR111	*B.pumilus* TrpEDC, Ap^R, Tc^R	This study
pMR131	*B.pumilus* TrpCF, Ap^R, Tc^R	This study
pMR500	*B.pumilus* TrpE, Ap^R, LacZ+	This study
pTrpH3B2	*B.subtilis* TrpED, Ap^R, Cm^R	BGSC[a]

[a] Bacillus Genetics Stock Center, Columbus, Ohio
[b] Strains E78 to T12 were used for plasmids pRR106 and pRR103 transformations, see text for details.

Table II: [a] The B.subtilus recipients were made competent by the method of Anagnostopoulos and Spizizen (1961) as modified by Rudner et al., (1967). Selection for Km^R transformants was on LB agar plates supplemented with 10 ug Km/ml. Selection for trp prototrophy was on minimal plates supplemented with the appropriate amino acid at 100 ug/ml.

[b] Cotransformation frequency (%) was determined by replica plating. Plates were scored after 24 hours of incubation at 37°C.

N.A. Not applicable.

Table II

Transforming activities of a cloned *trp* insert from *B. pumilus* in both orientations

Plasmid:		pRR106		pRR103	
Orientation:		(Km^R)*trpE,D,C,F*		(Km^R)*F,C,D,E trp*	
Recipients [a]	Phenotype	Transformants No./ugDNA	Cotransformation [b]	Transformants No./ugDNA	Cotransformation [b]
E88 *trpE46 hisH2*	Trp^+	6.8×10^5	100	0	N.A.
	Km^R	1.8×10^4	100	2.2×10^4	0
DB83 *trpD2*	Trp^+	3.9×10^5	100	0	N.A.
	Km^R	2.3×10^4	99	2.5×10^4	0
SB25 *trpC2 hisH2*	Trp^+	6.6×10^5	100	7.3×10^4	100
	Km^R	9.2×10^3	100	5.5×10^3	100
T12 *trpF12*	Trp+	5.5×10^5	99	0	N.A.
	Km^R	1.8×10^4	100	1.6×10^4	0

Table III

REGULATORY SEQUENCES FOUND IN trp OPERONS

I. REGULAR PROMOTERS

SPECIES	-35	BP	-10
B.pumilus	TTGACA	18	TACGAT
B.subtilus	TTGACA	18	TACGAT
E.coli	TTGACA	18	TAACTA

II. INTERNAL PROMOTERS

B.pumilus [a]	TTGAAA	18	TCTCAC
	TTGAAA	18	TGTATT
	CTGAAA	18	CATATT
B.subtilis		?	
E.coli	GTGACA	15	TACAAG

CONSENSUS PROMOTER

TTGACA	15-19	TATAAT

[a] For comparison, possible candidates for internal promoters are presented.

The locations of these sequences in the B.pumilus trpE gene are the following in Figure 7:

1. +337-342 (-35) +361-366 (-10)
2. +379-384 (-35) +403-408 (-10)
3. +1728-1733 (-35) 1752-1757 (-10)

Promoter references: B.pumilus (Kuroda et al., 1986) B. subtilis (Shimotsu et al., 1986) E.coli (Yanofsky, et al., 1981).

Table IV

Codon Frequency Table

Amino Acid	Codon	S.aureus freq.		S.subtilis freq.		E.coli freq.	
		Number	Fraction	Number	Fraction	Number	Fraction
Gly/G	GGG	5.00	0.20	7.00	0.25	2.00	0.07
Gly/G	GGA	6.00	0.24	4.00	0.14	3.00	0.11
Gly/G	GGT	2.00	0.08	7.00	0.25	11.00	0.39
Gly/G	GGC	12.00	0.48	10.00	0.36	12.00	0.43
Glu/E	GAG	14.00	0.31	21.00	0.48	5.00	0.14
Glu/E	GAA	31.00	0.69	25.00	0.56	30.00	0.86
Asp/D	GAT	21.00	0.72	10.00	0.48	21.00	0.60
Asp/D	GAC	8.00	0.28	12.00	0.48	14.00	0.40
Val/V	GTG	12.00	0.48	7.00	0.21	14.00	0.42
Val/V	GTA	6.00	0.20	5.00	0.15	8.00	0.24
Val/V	GTT	6.00	0.20	13.00	0.39	5.00	0.15
Val/V	GTC	6.00	0.20	8.00	0.24	6.00	0.18
Ala/A	GCG	6.00	0.19	4.00	0.12	14.00	0.38
Ala/A	GCA	11.00	0.35	11.00	0.33	4.00	0.11
Ala/A	GCT	7.00	0.23	11.00	0.33	12.00	0.32
Ala/A	GCC	7.00	0.23	7.00	0.21	10.00	0.26
Arg/R	AGG	2.00	0.10	3.00	0.16	0.00	0.00
Arg/R	AGA	6.00	0.29	6.00	0.32	1.00	0.03
Ser/S	AGT	3.00	0.08	1.00	0.05	4.00	0.11
Ser/S	AGC	8.00	0.28	9.00	0.24	13.00	0.35
Lys/K	AAG	10.00	0.28	8.00	0.21	3.00	0.20
Lys/K	AAA	26.00	0.72	30.00	0.79	12.00	0.80
Asn/N	AAT	12.00	0.75	10.00	0.59	10.00	0.59
Asn/N	AAC	4.00	0.25	7.00	0.41	7.00	0.41
Met/M	ATG	15.00	1.00	19.00	1.00	12.00	1.00
Ile/I	ATA	4.00	0.14	8.00	0.28	0.00	0.00
Ile/I	ATT	25.00	0.61	21.00	0.51	12.00	0.71
Ile/I	ATC	12.00	0.29	12.00	0.29	5.00	0.29
Thr/T	ACG	10.00	0.38	6.00	0.19	3.00	0.12
Thr/T	ACA	13.00	0.39	19.00	0.59	4.00	0.16
Thr/T	ACT	2.00	0.06	3.00	0.09	4.00	0.16
Thr/T	ACC	8.00	0.24	4.00	0.13	14.00	0.56
Trp/W	TGG	3.00	1.00	2.00	1.0	0.00	0.00
End/*	TGA	0.00	0.00	1.00	1.00	1.00	1.00
Cys/C	TGT	5.00	1.00	4.00	0.00	5.00	0.42
Cys/C	TGC	0.00	0.00	1.00	0.20	7.00	0.58
End/*	TAG	0.00	0.00	0.00	0.00	0.00	0.00
End/*	TAA	1.00	1.00	0.00	0.00	0.00	0.00
Tyr/T	TAT	0.00	0.44	11.00	0.55	0.00	0.06
Tyr/T	TAC	10.00	0.56	9.00	0.45	5.00	0.36
Leu/L	TTG	2.00	0.05	3.00	0.07	5.00	0.07
Leu/L	TTA	16.00	0.37	0.00	0.00	7.00	0.10
Phe/F	TTT	12.00	0.71	15.00	0.71	0.00	0.00
Phe/F	TTC	5.00	0.29	6.00	0.29	12.00	0.00
Ser/S	TCG	3.00	0.08	7.00	0.16	5.00	0.14
Ser/S	TCA	10.00	0.25	6.00	0.11	4.00	0.11
Ser/S	TCT	8.00	0.20	12.00	0.32	6.00	0.16
Ser/S	TCC	8.00	0.20	5.00	0.13	5.00	0.14
Arg/R	CGG	2.00	0.10	2.00	0.11	0.00	0.00
Arg/R	CGA	4.00	0.19	3.00	0.16	1.00	0.03
Arg/R	CGT	4.00	0.19	3.00	0.16	17.00	0.43
Arg/R	CGC	3.00	0.14	2.00	0.11	21.00	0.52
Gln/Q	CAG	8.00	0.36	6.00	0.40	13.00	0.62
Gln/Q	CAA	14.00	0.64	9.00	0.60	8.00	0.38
His/H	CAT	15.00	0.75	10.00	0.63	7.00	0.64
His/H	CAC	5.00	0.25	6.00	0.38	4.00	0.36
Leu/L	CTG	8.00	0.19	12.00	0.29	35.00	0.52
Leu/L	CTA	5.00	0.12	2.00	0.05	4.00	0.06
Leu/L	CTT	6.00	0.14	10.00	0.24	6.00	0.09
Leu/L	CTC	6.00	0.14	6.00	0.15	10.00	0.15
Pro/P	CCG	5.00	0.22	10.00	0.48	15.00	0.54
Pro/P	CCA	13.00	0.57	2.00	0.10	5.00	0.18
Pro/P	CCT	5.00	0.22	6.00	0.29	3.00	0.11
Pro/P	CCC	0.00	0.00	3.00	0.14	5.00	0.18

Amino Acid	Codon	S.aureus freq.		S.subtilis freq.		E.coli freq.	
		Number	Fraction	Number	Fraction	Number	Fraction
Gly/G	GGG	6.00	0.23	7.00	0.25	8.00	0.17
Gly/G	GGA	10.00	0.38	10.00	0.33	5.00	0.11
Gly/G	GGT	2.00	0.08	9.00	0.30	15.00	0.33
Gly/G	GGC	6.00	0.31	4.00	0.13	18.00	0.39
Glu/E	GAG	6.00	0.20	16.00	0.53	7.00	0.24
Glu/E	GAA	24.00	0.80	14.00	0.47	22.00	0.76
Asp/D	GAT	8.00	0.80	5.00	0.62	12.00	0.57
Asp/D	GAC	2.00	0.20	3.00	0.38	9.00	0.43
Val/V	GTG	3.00	0.17	3.00	0.18	15.00	0.43
Val/V	GTA	3.00	0.28	3.00	0.18	3.00	0.09
Val/V	GTT	3.00	0.17	3.00	0.47	7.00	0.21
Val/V	GTC	7.00	0.39	3.00	0.18	8.00	0.24
Ala/A	GCG	4.00	0.14	6.00	0.22	21.00	0.47
Ala/A	GCA	12.00	0.29	11.00	0.31	10.00	0.15
Ala/A	GCT	14.00	0.34	13.00	0.36	6.00	0.09
Ala/A	GCC	11.00	0.27	4.00	0.11	19.00	0.29
Arg/R	AGG	0.00	0.00	0.00	0.00	0.00	0.00
Arg/R	AGA	4.00	0.33	4.00	0.36	1.00	0.04
Ser/S	AGT	3.00	0.13	1.00	0.08	7.00	0.23
Ser/S	AGC	3.00	0.13	11.00	0.33	10.00	0.32
Lys/K	AAG	3.00	0.22	5.00	0.44	3.00	0.21
Lys/K	AAA	10.00	0.78	10.00	0.56	11.00	0.79
Asn/N	AAT	13.00	0.87	9.00	0.75	8.00	0.35
Asn/N	AAC	2.00	0.13	3.00	0.25	15.00	0.65
Met/M	ATG	12.00	1.00	12.00	1.00	13.00	1.00
Ile/I	ATA	3.00	0.17	2.00	0.11	1.00	0.04
Ile/I	ATT	11.00	0.61	12.00	0.67	20.00	0.74
Ile/I	ATC	4.00	0.22	4.00	0.22	6.00	0.22
Thr/T	ACG	5.00	0.26	6.00	0.31	4.00	0.16
Thr/T	ACA	10.00	0.53	6.00	0.31	5.00	0.20
Thr/T	ACT	2.00	0.11	5.00	0.19	9.00	0.04
Thr/T	ACC	2.00	0.11	5.00	0.19	15.00	0.60
Trp/W	TGG	0.00	0.00	1.00	1.00	1.00	1.00
End/*	TGA	0.00	0.00	1.00	1.00	0.00	0.00
Cys/C	TGT	1.00	0.25	0.00	0.00	4.00	0.00
Cys/C	TGC	3.00	0.75	2.00	1.00	1.00	0.20
End/*	TAG	0.00	0.00	0.00	0.00	0.00	0.00
End/*	TAA	1.00	1.00	0.00	0.00	1.00	1.00
Tyr/T	TAT	6.00	0.86	4.00	0.67	0.00	0.62
Tyr/T	TAC	1.00	0.14	2.00	0.33	5.00	0.38
Leu/L	TTG	4.00	0.11	2.00	0.06	6.00	0.09
Leu/L	TTA	11.00	0.31	7.00	0.22	9.00	0.14
Phe/F	TTT	9.00	0.90	10.00	0.77	8.00	0.53
Phe/F	TTC	1.00	0.10	3.00	0.23	7.00	0.47
Ser/S	TCG	1.00	0.06	3.00	0.09	3.00	0.10
Ser/S	TCA	10.00	0.63	7.00	0.21	2.00	0.06
Ser/S	TCT	3.00	0.13	9.00	0.27	4.00	0.13
Ser/S	TCC	3.00	0.13	2.00	0.06	5.00	0.16
Arg/R	CGG	2.00	0.17	3.00	0.27	0.00	0.00
Arg/R	CGA	3.00	0.25	1.00	0.09	1.00	0.04
Arg/R	CGT	2.00	0.17	1.00	0.09	7.00	0.28
Arg/R	CGC	1.00	0.08	2.00	0.18	16.00	0.64
Gln/Q	CAG	3.00	0.20	7.00	0.78	14.00	0.54
Gln/Q	CAA	12.00	0.80	2.00	0.22	12.00	0.46
His/H	CAT	7.00	0.70	4.00	0.67	12.00	0.52
His/H	CAC	3.00	0.30	2.00	0.33	11.00	0.48
Leu/L	CTG	4.00	0.11	5.00	0.16	37.00	0.56
Leu/L	CTA	3.00	0.08	5.00	0.16	2.00	0.03
Leu/L	CTT	6.00	0.17	11.00	0.34	4.00	0.06
Leu/L	CTC	8.00	0.22	2.00	0.06	8.00	0.12
Pro/P	CCG	4.00	0.40	6.00	0.50	25.00	0.79
Pro/P	CCA	6.00	0.60	1.00	0.08	2.00	0.07
Pro/P	CCT	0.00	0.00	3.00	0.25	1.00	0.03
Pro/P	CCC	0.00	0.00	2.00	0.17	3.00	0.10

Table V

The Levels of B- galactosidase Activity in E.coli 71-18 Containing Plasmids With or Without Promoter Fragments

	Enzyme Units (b)		
	Plasmid		
Growth Conditions (a)	pUC19	pSK10Δ6	pMR500
IPTG, tryptophan	131	0	61.3
IPTG	71.5	1.6	43.9
tryptophan	35	0	70.2
no addition	34	2.5	78.7

(a) Cells grown in A media, 50ug of ampicillin per ml

(b) Assayed by the method of Miller[1]

$$\text{Units} = 1000X \frac{OD420 - 1.750D550}{\text{tmin} \times \text{vol ml} \times OD600}$$

[1] Miller, J.H. (1972) Experiments in Molecular Genetics Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 352-355.

Table VI

Anthranilate Synthetase (AS) Levels in a *trpC* Mutant Strain Containing Plasmid pRR106-*trp*

strain and relevant genotype[a]	growth supplement[b]	specific activity ASase[c]	activity relative to depressed levels (%)
I. *BR151*			
	0	0.25	100
trpC$^{+(W23)}$, *metB10*, *lys-3*	Trp	0	--
	IAA	0.64	256
II. *BR151/pRR106*			
	0; Km	0.45	100
	Trp	0.04	9
trpC2, *metB10*, *lys-3*	Trp; Km	0.04	9
	IAA	1.48	328
	IAA;Km	1.20	266

(a) *trpC*$^{+(W23)}$ was isolated by transformation with DNA from strain W23.

(b) Tryptophan (Trp) was added to a final concentration of 100 mg/ml; Kanomycin (Km) to 5 mg/ml; and 3-indolylacrylic acid (IAA) to 10 mg/ml.

(c) Specific activity is expressed as nanomoles of anthranilate formed per minute per milligram of protein.

Table VII

Anthranilate Synthetase (AS) Levels in
a *trpE* Mutant Strain Containing Plasmid pRR106-*trp*

strain and relevant genotype[a]	growth supplement	specific activity ASase[b]	activity relative to depressed levels (%)
I. *E78*			
	0	0.51	100
+(W23)			
trpE	Trp	0	--
	IAA	0.77	151
II. *E78/pRR106*			
	0; Km	0.48	100
	Trp	0.06	12
trpE24	Trp; Km	0.04	8
	IAA	0.68	141
	IAA; Km	0.83	173
III. E78/*pRR106*			
	0	0.46	100
+(3610)			
trpE	Trp	0.11	24
	IAA	0.97	211

(a) *trpE*$^{+(W23)}$ or *trpE*$^{+(3610)}$ were isolated by transformation with DNAs from the donor strains W23 or 3610.

(b) Specific activity is expressed as nanomoles of anthranilate formed per minute per milligram protein.

Table VIII

Anthranilate Synthetase (AS) Levels in a Consitutive Derepressed Strain (E78R) Containing Plasmid pRR106-*trp*

strain and relevant genotype[a]	growth supplement	specific activity ASase[b]	activity relative to depressed levels (%)
I. *E78R*			
	0	0.56	100
+(W23)[1]			
trpE , *mtr*	Trp	0	--
	IAA	1.03	184
	0	0.40	100
+(W23)[2]			
trpE , *mtr*	Trp	0.31	77
	IAA	0.70	176
	0	0.34	100
+(3610)			
trpE , *mtr*	Trp	0.39	113
	IAA	1.04	306
II. *E78R/pRR106*			
	0; Km	0.28	100
	Trp	0.12	43
trpE24, *mtr*	Trp; Km	0.13	46
	IAA	0.36	129
	IAA; Km	0.34	121
III. *E78R/pRR106*			
	0	0.34	100
+(W23)[2]			
trpE , *mtr*	Trp	0.36	104
	IAA	1.18	347

(a) *TrpE*$^{+(W23)}$ or *trpE*$^{+(3610)}$ were isolated by transformation with DNAs from the donor strains W23 or 3610.

(b) Specific activity is expressed as nanomoles of anthranilate formed per minute per milligram protein.

(1) and (2) Separate clones which were purified as individual strains.

Table IX

Estimation of pRR106-*trp* Copy Number in Plasmid-Containing Strains of *B. subtilis*

	strain and genotype	technique	Km (5 ug)	Chromosomal DNA (cpm)	CCC DNA (cpm)	Copy No. [a]
I.	BR151 *trpC2, metB10, lys-3*	CsCl-EtBr centrifugation	-	38,580	2,940	28
			+	6,930	350	22
		gel electrophoresis	-	1,194	80	31
			+	1,294	91	32
II.	E78 *trpE24*	CsCl-EtBr	-	8,500	620	24
			+	13,360	1,020	26
III.	E78R *trpE24, mtr*	CsCl-EtBr	-	11,840	670	20
			+	12,980	1,030	25

(a) To calculate copy number, the molecular weights of pRR106-*trp* and the *B. subtilis* chromosome were taken as 5.4×10^6 and 2.5×10^9, respectively.

Table X

Transformation of *B. subtilis trp* Strains with pRR106, pMR111 and pMR131 Plasmid DNAs

Strain Used	Relevant Genotype	Plasmids	No. Transformants/ml Trp+	Cm^R	Km^R
E88	*trpE46, hisB2*	pRR106	3.5×10^4	0	1.1×10^4
		pMR111	30	12	0
		pMR131		N.D.	
DB83	*trpD2*	pRR106	6.4×10^4	0	5.9×10^4
		pMR111	115^S	5	0
		pMR131		N.D.	
BD170	*trpC2, thr-5*	pRR106	1.5×10^5		1.3×10^5
		pMR111	0	15	0
		pMR131		N.D.	
T12	*trpF12*	pRR106	4.2×10^4	0	2.9×10^4
		pMR111		N.D.	
		pMR131	0	1.3	0

S = Small Colonies

N.D. = Not Determined

Table XI

Analysis of Chloramphenicol Transformants

	E88:pMR111		BD170:pMR111	
	Cm^R	Cm^S	Cm^R	Cm^S
Trp +	1	3	1	4
trp + -	3	6	3	3
trp -	1	3	5	1

Strains E88 and BD170 were transformed with plasmid pMR111. 17 originally chloramphenicol resistant colonies from each cell line were grown in minimal media with the appropriate amino acids. They were spot-tested for the retention of the chloramphenicol resistant marker and the cotransformation of the Trp+ phenotype. Shown are the results indicating the relative instability of the integrated plasmid in these transformants.

Table XII

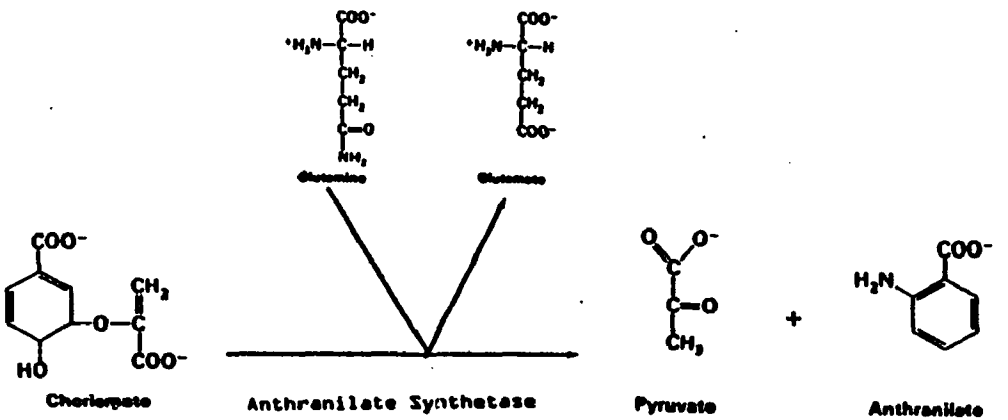
Transforming Activity of DNAs Containing Integrated pMR111 with *trp* sequences from *B. pumilus*

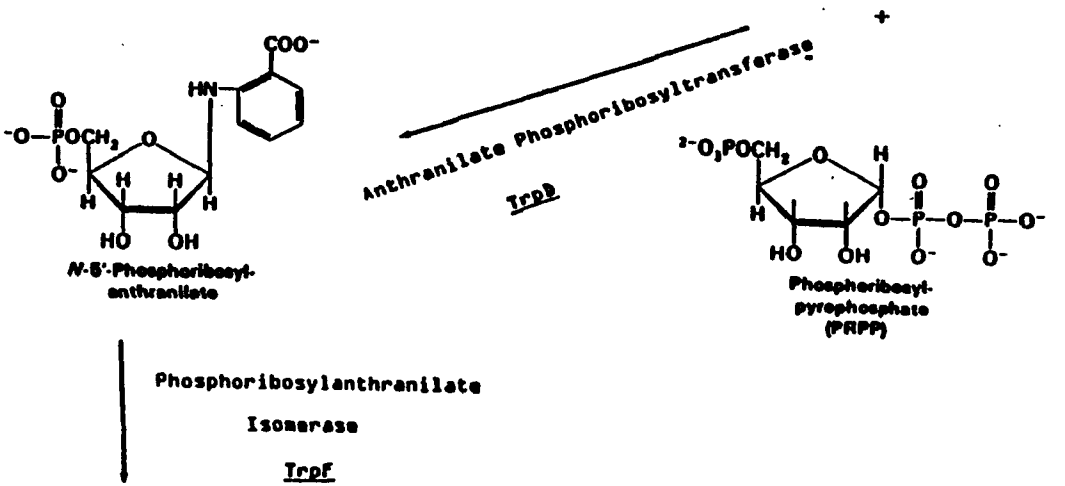
Donor DNA and Relevant Genotype	No. Transformants/ml			Recombinant Class No.		
	Trp$^+$	His$^+$	Cm^R	Trp$^+$ Cm^S	Trp$^+$ Cm^R	trp$^-$ Cm^R
NCTC3610	4.9×10^5	3.3×10^5	0			
MR188 TrpE$^+$, *hisH2*::Cm^R	2.5×10^5	0	9.5×10^3	307	6 22	68
MR189 *trpE46*, *hisH2*::Cm^R	0	0	1.8×10^4		0	158

Recipient Strain E88 *trpE46*, *hisH2*

Figure 1: **The biosynthetic pathway of tryptophan synthesis. Shown are the reactions unique to tryptophan biosynthesis from the branching point to the aromatic intermediate, chorismate.**

Figure 1


Chorismate
Anthranilate Synthetase
Pyruvate
Anthranilate
TrpE & TrpG


N-5'-Phosphoribosyl-anthranilate
Anthranilate Phosphoribosyltransferase
TrpD
Phosphoribosyl-pyrophosphate (PRPP)
Phosphoribosylanthranilate Isomerase
TrpF

Continuation of Figure 1

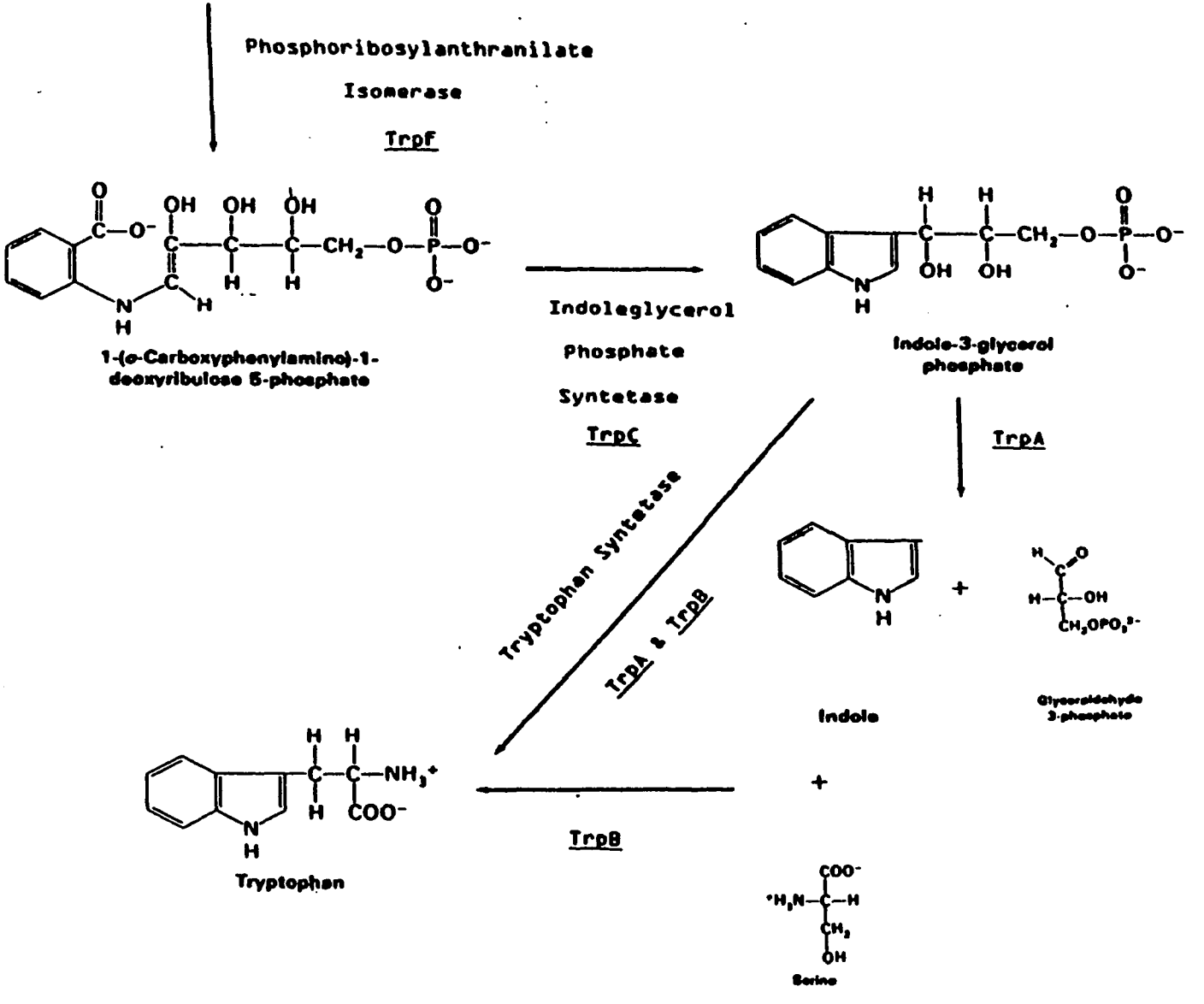


Figure 2: A schematic representation of the arrangements of the genes involved in the biosynthetic pathway of tryptophan in Bacillus pumilus, Bacillus subtilis, (Henner et al., 1985) Escherichia coli, (Yanofsky, et al., 1981) Acinetobacter calcoaceticus (Sawula and Crawford, 1972) Saccharomyces cerevisiae, (Doy and Cooper, 1966) and Pseudomonas aeruginosa (Calhoun et al., 1973). The following capitol letters denote either the regulatory region or the gene and its gene product:

P = Promoter of the tryptophan operon

L = Leader of the tryptophan operon

O = Operator of the tryptophan operon

p2 = Internal promoter of the tryptophan operon

E = The trpE gene coding for subunit E of Anthranilate Synthetase

G = The trpG gene coding for subunit G of Anthranilate Synthetase

D = The trpD gene coding for Anthranilate Phosphoribosyl-transferase

C = The trpC gene coding for Indoleglycerol Phosphate Synthetase

F = The trpF gene coding for Phosphoribosylanthranilate Isomerase

Figure 2: (continued)

B - The trpB gene coding for the B subunit of Tryptophan Synthetase

A = The trpA gene coding for the A subunit of Tryptophan Synthetase

Figure 2

Arrangement of Tryotophan Genes

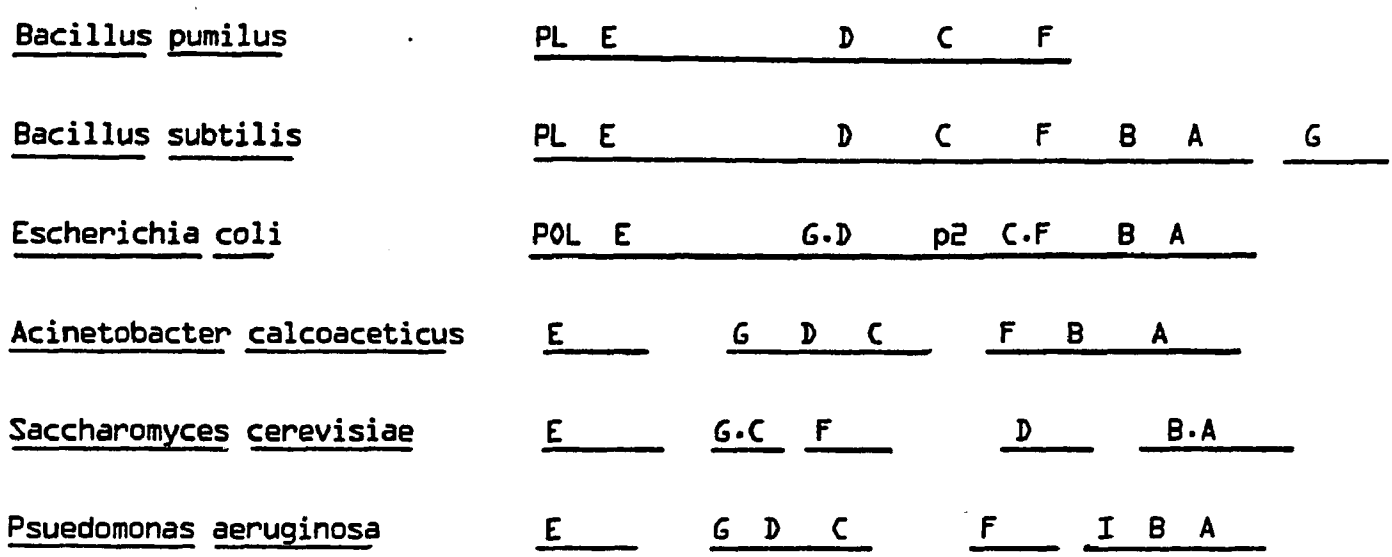


Figure 3: The restriction site map of plasmid pRR106. The bold lower line indicates the 3.6-kb *B.pumilus trp* fragment cloned into the *EcoRI* site of parental plasmid pUB110.

Kindly provided by Luisa DiGiovani.

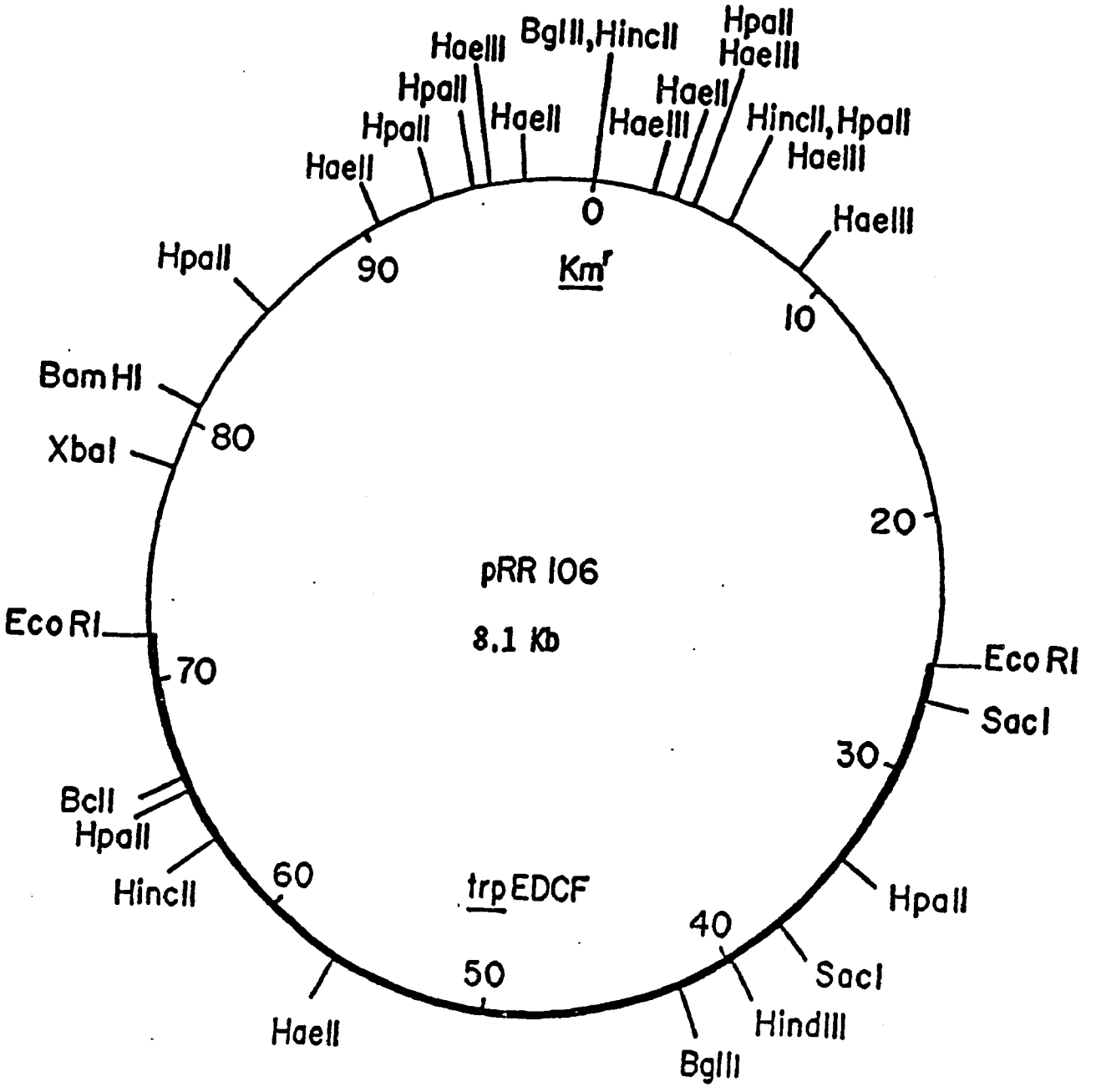


Fig. 3 Restriction endonuclease cleavage site map of pRR106. The bold line indicates the 3.6 Kb insert of Bacillus pumilus DNA.

Figure 4: The restriction maps of plasmids pRR106 and pRR103. Shown are restriction sites present once or twice on the plasmids. The bold lower line indicates the 3.6-kb B.pumilus trp fragment cloned into the EcoRI site of plasmid pUB110 (4.5-kb). The inner arrow represents the 2.733-kb (EcoRI-HindIII fragment which was sequenced. The outer circle denotes restriction fragment sizes generated by a double digestion with HindIII and BamHI to determine the orientation of the cloned trp genes.

Figure 4

The Restriction Maps of Plasmids pRR106 and pRR103

Containing the trp Genes of Bacillus pumilus

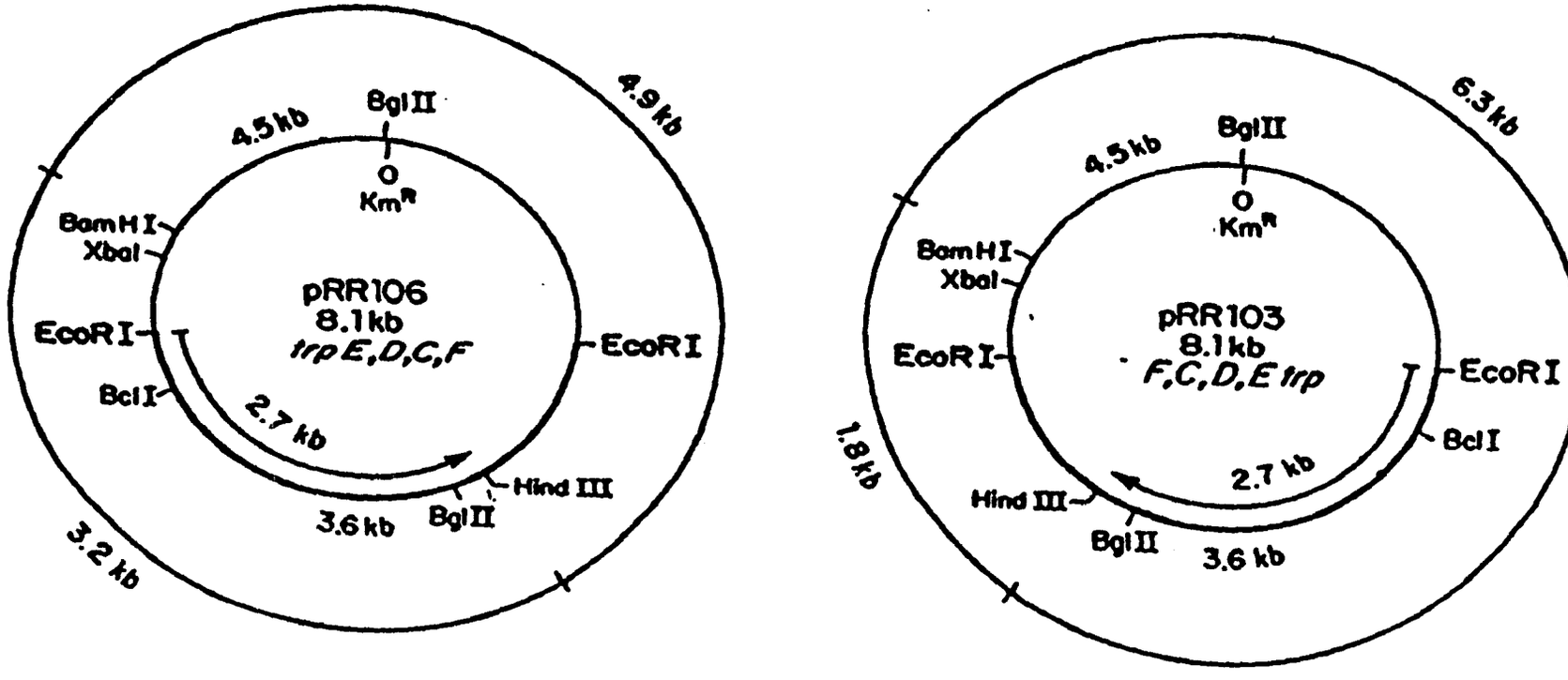
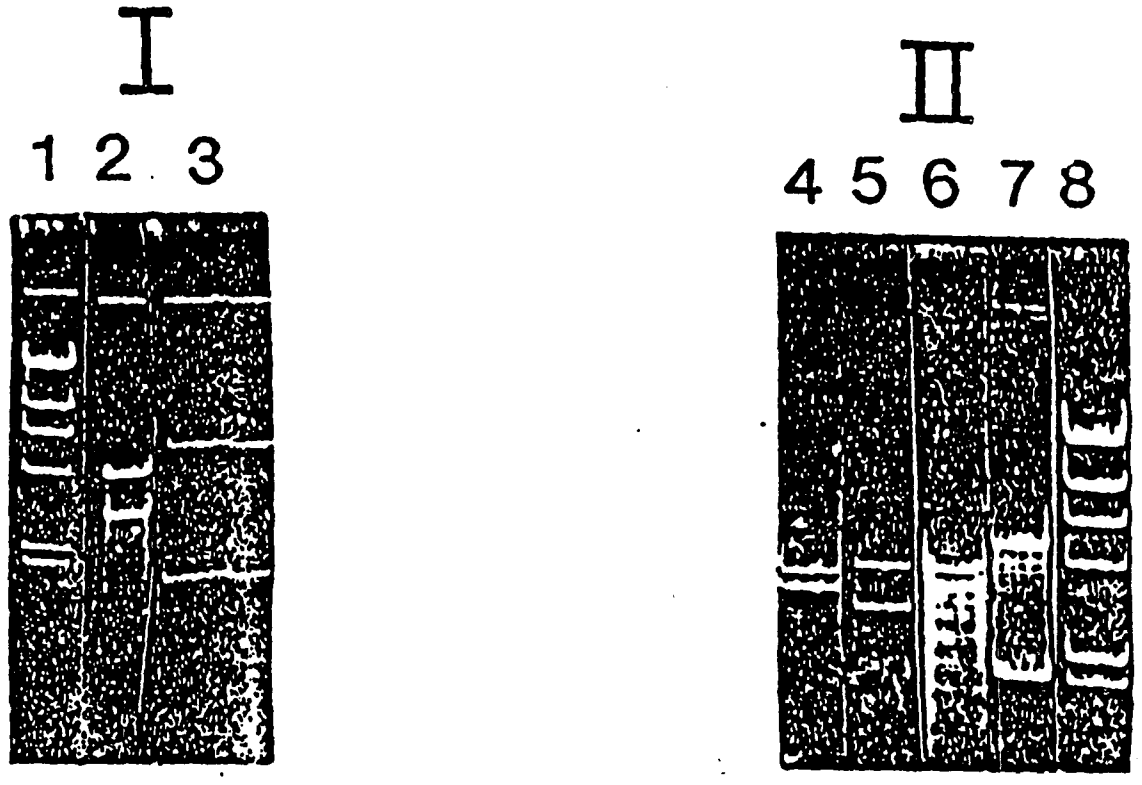


Figure 5: The electrophorectic patterns of pRR106 and pRR103 after digestion with EcoRI and HindIII-BamHI. Panels I and II show two separate preparations of plasmids. Lanes 1 and 8 are bands used as molecular weight markers generated from a HindIII digestion of λDNA. Lanes 2 and 5; 3 and 7 are pRR106 and pRR103, respectively, digested with HindIII and BamHI. Lanes 4 and 6 are pRR106 and pRR103, respectively, digested with EcoRI.

Figure 5

ELECTROPHORETIC PATTERNS OF pRR106 AND pRR103 AFTER DIGESTIONS WITH EcoRI AND HindIII-BamHI



I AND II : TWO SEPARATE PREPARATIONS OF THE PLASMIDS.
1;8 : LAMBDA DIGESTED WITH HindIII.
2 AND 5; 3 AND 7: pRR106 AND pRR103 DIGESTED WITH HindIII-BamHI RESPECTIVELY.
4;6 : pRR106 AND pRR103 DIGESTED WITH EcoRI RESPECTIVELY.

Figure 6: The sequencing strategy of the trpE, trpD and 5'trpC genes of Bacillus pumilus. The 2.733-kb EcoRI-HindIII fragment was cloned into the EcoRI-HindIII sites of the replicative forms of M13mp18 and M13mp19 and designated M13mp18-2.7-kb and M13mp19-2.7-kb, respectively. The single stranded forms of the recombinants were sequenced by the dideoxy - chain termination method with the Klenow fragment of DNA polymerase, (Sanger, 1977) or the modified T7 polymerase (Pharmacia). The fragment was sequenced without sub-cloning with M13 and six synthetic primers. The sequence of the synthetic primers were determined during the course of the project. For M13mp18-2.7-kb recombinant, 18a; 5'AATCGTTTCAATTC3', 18b; 5'AATTCATCCAGTCCA3' 18c; 5'TTTCATAGATAATG3' and for M13mp19-2.7-kb recombinant, 19a; 5'GTCTCCAACGATGA, 19b; 5'ATCTCCTTATATTA3', 19c; 5'AGTTATCGCTCAT3', were made. The arrows indicate the direction, relative length, and extent of sequence overlap obtained from each primer. The numbers in parenthesis are the numbers assigned to the nucleotide in the tryptophan operon of B.pumilus sequenced with the indicated primer.

Figure 6

The Sequencing Strategy of the *trpE*, *trpD* and 5' *trpC* Genes of *Bacillus pumilus*

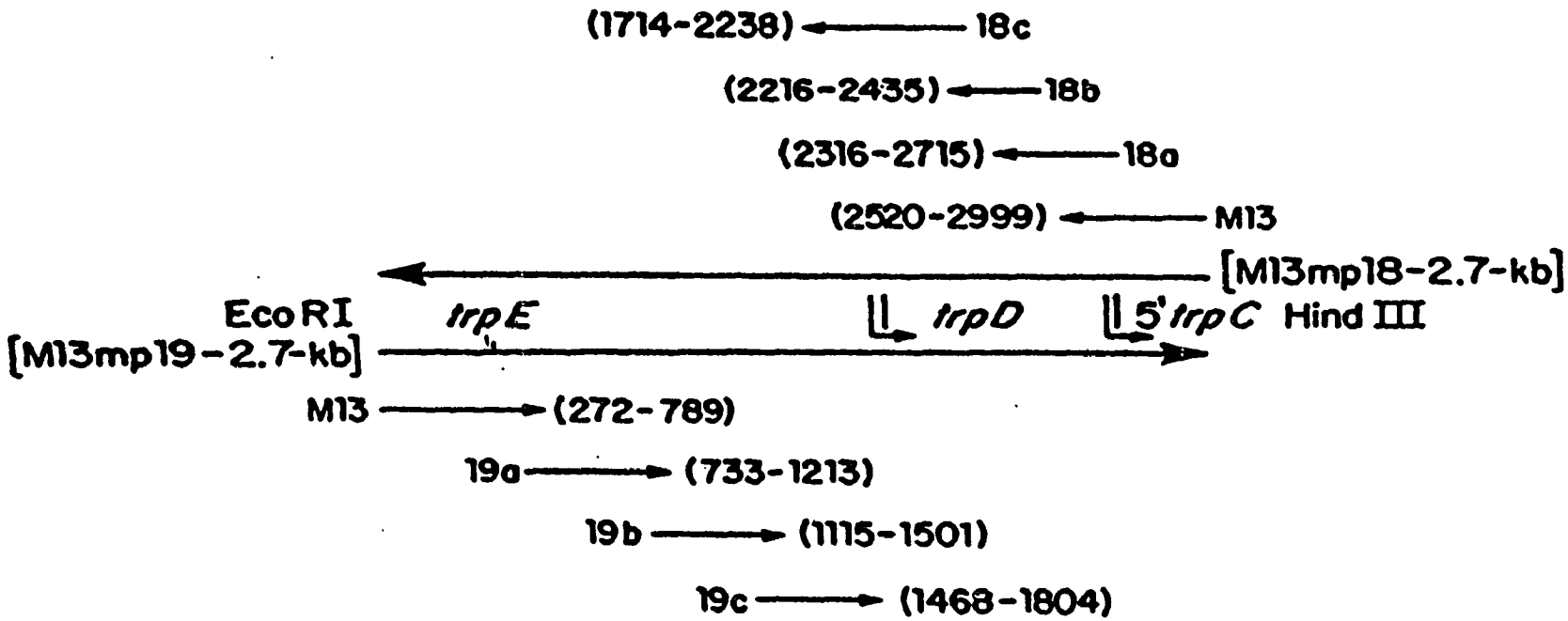
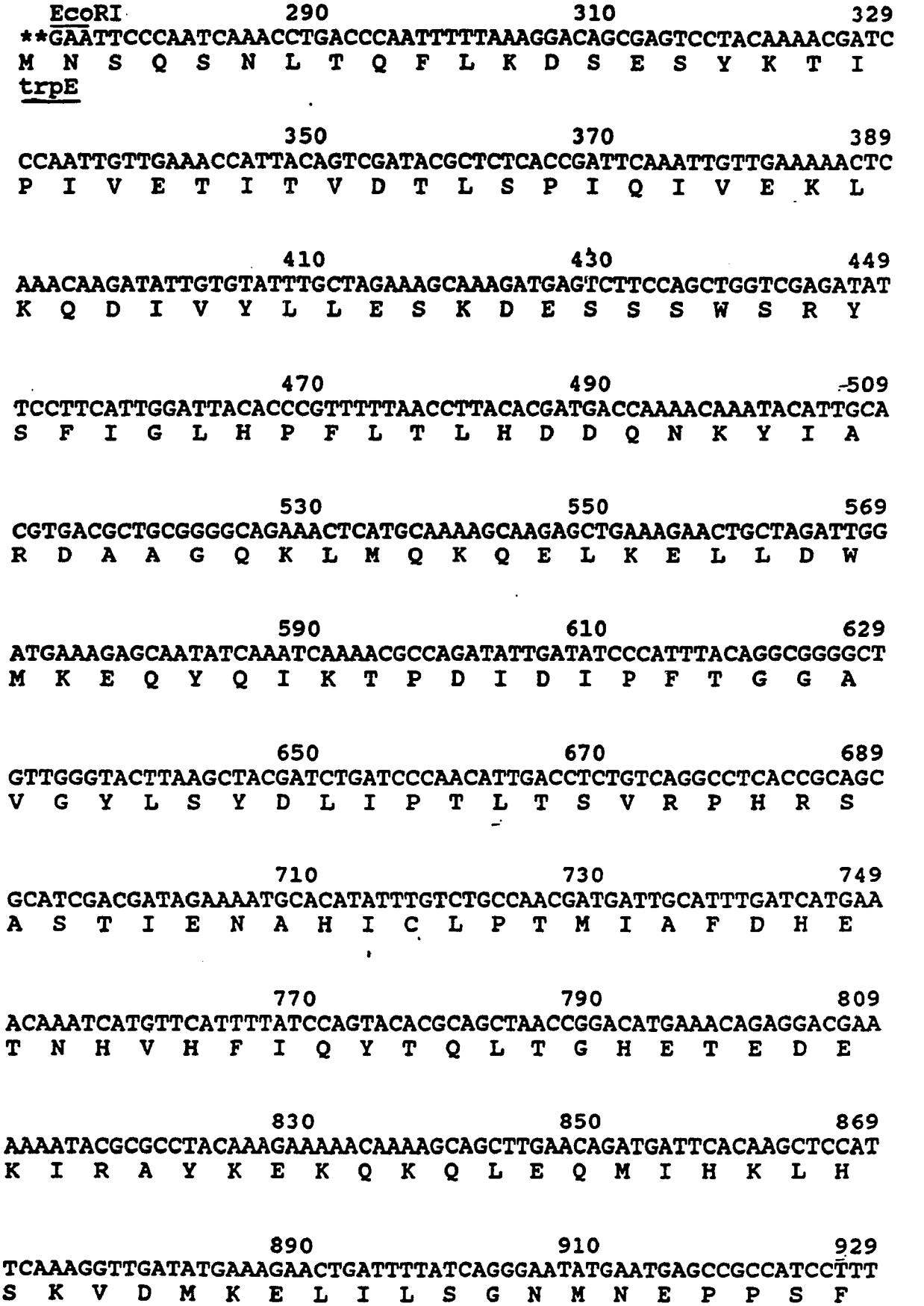


Figure 7: The nucleotide sequence and deduced amino acid sequence of the B.pumilus trpE, trpD, and 5'trpC genes. The start and stop codons are underlined. The ribosome binding sites (RBS) are indicated with lines above and below the sequence. The flanking EcoRI and HindIII sites are indicated. **The following amino acid sequence upstream of the cloning site within ORF- , M-K-A-L-R, is present next to the second amino acid (N) or trpE (McKenzie et al., 1986 and 1987).

Figure 7

The DNA sequence of the trpE, trpD and 5'trpC genes of B.pumilus



```
                    950                 970                 989
GAACATGTGACGTCAACCTATGAAAAAGCGCAATTTTTAAAAGACGTAGAAAAAATCAAA
E  H  V  T  S  T  Y  E  K  A  Q  F  L  K  D  V  E  K  I  K

                   1010                1030                1049
GAATACATTCGAGCGGGTGACATTTTTCAAGGCGTACTCTCGCAGCGATTTGATATCCCT
E  Y  I  R  A  G  D  I  F  Q  G  V  L  S  Q  R  F  D  I  P

                   1070                1090                1109
GTATCAGTGAGTTCATTTGAGTTATACCGCGTGCTTCGGATTGTGAATCCATCTCCTTAT
V  S  V  S  S  F  E  L  Y  R  V  L  R  I  V  N  P  S  P  Y

                   1130                1150                1169
ATGTATTTTATGAAATTAAAAGATCGTGATTTAGTCGGCAGCTCACCAGAACGATTAATC
M  Y  F  M  K  L  K  D  R  D  L  V  G  S  S  P  E  R  L  I

                   1190                1210                1229
CATGCCAAAAATGGGCATTTAGAAATTCATCCTATTGCTGGCACAAGAAAACGAGGAACA
H  A  K  N  G  H  L  E  I  H  P  I  A  G  T  R  K  R  G  T

                   1250                1270                1289
ACAAGAGAAGAGGATGCTGAACTAGCAAGAGAGCTGCTTGAAGATGAAAAGGAAAAAGCC
T  R  E  E  D  A  E  L  A  R  E  L  L  E  D  E  K  E  K  A

                   1310                1330                1349
GAGCATTACATGTTAGTGGATCTTGCCAGAAATGATGTAGGCCGTGTGGCAGAATACGGC
E  H  Y  M  L  V  D  L  A  R  N  D  V  G  R  V  A  E  Y  G

                   1370                1390                1409
AGTGTGTCCGTACCAACCTTTACAAAAGTAGTGAACTTCTCTCATGTCATGCACATCATC
S  V  S  V  P  T  F  T  K  V  V  N  F  S  H  V  M  H  I  I

                   1430                1450                1469
TCCATTGTGACAGGAAAGCTAAAGCGGGATACACATCCAGTTGATGCGCTCATGTCCGCA
S  I  V  T  G  K  L  K  R  D  T  H  P  V  D  A  L  M  S  A

                   1490                1510                1529
TTCCCAGCAGGCACATTAACAGGTGCCCCGAAAATAAGAGCGATGCAATTATTAAATGAA
F  P  A  G  T  L  T  G  A  P  K  I  R  A  M  Q  L  L  N  E

                   1550                1570                1589
ATGGAGCCTGAGCCAAGGGAAACGTATGGCGGCTGTATTGCTTATATTGGATTCGACGGC
M  E  P  E  P  R  E  T  Y  G  G  C  I  A  Y  I  G  F  D  G

                   1610                1630                1649
AATATCGACTCTTGTATTACGATTCGTACGATGAGCGTCAAAAATCATACCGCTTCTATA
N  I  D  S  C  I  T  I  R  T  M  S  V  K  N  H  T  A  S  I
```

```
                    1670                1690                1709
CAAGCAGGCGCCGGCATTGTGGCTGATTCTGTCCCAGAAAATGAATGGGAAGAGACTTGT
Q  A  G  A  G  I  V  A  D  S  V  P  E  N  E  W  E  E  T  C

                    1730                1750                1769
AACAAGGCAGGAGCACTTCTGAAAGCCATTCAGCTTGCGGAACATATTTTCTCAGAAAAG
N  K  A  G  A  L  L  K  A  I  Q  L  A  E  H  I  F  S  E  K

RBS                 1790                1810                1829
GAGAGTGTGCAGGATGAATCACCGACTATCAGCTCTTGTTAATGGAGGCTTTCTATCAGA
E  S  V  Q  D  E  S  P  T  I  S  S  C  *
            M  N  H  R  L  S  A  L  V  N  G  G  F  L  S  E
            trpD                    end trpE

                    1850                1870                1889
AAATGAAGCAAATAAACTCATGCATGATATGATGAGCGGCTTTTTAACAGATGCTGAAGT
 N  E  A  N  K  L  M  H  D  M  M  S  G  F  L  T  D  A  E  V

                    1910                1930                1949
CGCTGCTAGTCTCTCGATTTTAGCGCATAGAGGAGAAACCGCTGAAGAAATGACGGGTTT
 A  A  S  L  S  I  L  A  H  R  G  E  T  A  E  E  M  T  G  F

                    1970                1990                2009
TGTGAAAGCTATGCGGCAAAATGCAGCACCAATGGAACGAGCGCTTGATGTAGTAGATAC
 V  K  A  M  R  Q  N  A  A  P  M  E  R  A  L  D  V  V  D  T

                    2030                2050                2069
TTGCGGCACAGGAGGCGATGGACTCTCCACCTTTAATATATCAACTGCCGCTGCCATTGT
 C  G  T  G  G  D  G  L  S  T  F  N  I  S  T  A  A  A  I  V

                    2090                2110                2129
CCGTTCCGCTGCTGGAGCCAAAATCGCCAAACACGGAAATCGATCAGTTTCTTCTAAAAG
 R  S  A  A  G  A  K  I  A  K  H  G  N  R  S  V  S  S  K  S

                    2150                2170                2189
CGGGAGTGCCGACGTACTAGAGTGTCTTGGGATTCATATTCAATCCACACCAGAAGAAAC
 G  S  A  D  V  L  E  C  L  G  I  H  I  Q  S  T  P  E  E  T

                    2210                2230                2249
AAGAAGACAAATACAAGAGAAAAACATGGGCTTTTTATTTGCACCATTGTATCATTCATC
 R  R  Q  I  Q  E  K  N  M  G  F  L  F  A  P  L  Y  H  S  S

                    2270                2290                2309
TATGAAACAAGTAGCCGCAGTCCGCAAGCAGCTCGGCTTCCGGACGGTATTTAATCTCTT
 M  K  Q  V  A  A  V  R  K  Q  L  G  F  R  T  V  F  N  L  L
```

Continuation of Figure 7

```
                    2330                2350                2369
AGGCCCGCTTTCCCACCCAATGCAAGCCAAAAAGCAAATCATCGGTGTCTACTCAAAGGA
 G  P  L  C  H  P  M  Q  A  K  K  Q  I  I  G  V  Y  S  K  E

                    2390                2410                2429
AAAGGCAAAATTAATGGCAGAAGCCCTTGCACCGTTAGAGCCAGAACATGTGCTGTTTGT
 K  A  K  L  M  A  E  A  L  A  P  L  E  P  E  H  V  L  F  V

                    2450                2470                2489
TTGCGGAGAAGATGGACTGGATGAATTAACGATTACAGCAAATTCATATGTGATTGAACT
 C  G  E  D  G  L  D  E  L  T  I  T  A  N  S  Y  V  I  E  L

                    2510                2530                2549
CAAAAAAGATGTCATGACAGAATATACACTCAATCCAGAAGACTTTGGGCTGCAAAAAGG
 K  K  D  V  M  T  E  Y  T  L  N  P  E  D  F  G  L  Q  K  G

                    2570                2590                2609
ATATTTATCAGAAATTCAGGTCCAATCACCGGAAGAGAGTGCTAAATTGATTCAGAATAT
 Y  L  S  E  I  Q  V  Q  S  P  E  E  S  A  K  L  I  Q  N  I

                    2630                2650                2669
ATTGAATCATCAAACAGAAGGGGCGCCGCTTCATATTACAGCCCTGAATGCTGGAGCGGC
 L  N  H  Q  T  E  G  A  P  L  H  I  T  A  L  N  A  G  A  A

                    2690                2710                2729
TTTATATGTCGCAGGGAAGTCAGAAAGCCTCATGGCCGGGACATTAAAAGCAATTGAAAC
 L  Y  V  A  G  K  S  E  S  L  M  A  G  T  L  K  A  I  E  T

                    2750                2770                2789
GATTAAAAAACGGCGCAGCCAAAGAACAATTGGCTCGTTTAAAACAAAAAACGAGAGAGGA
 I  K  N  G  A  A  K  E  Q  L  A  R  L  K  Q  K  T  R  E  E

RBS                 2810                2830                2849
AGAGATCTATGCTTAATCAAATCATTGCCCGCAAAAAAGAACATATTCAAACATTGCAGT
 E  I  Y  A  *
          M  L  N  Q  I  I  A  R  K  K  E  H  I  Q  T  L  Q  L
    trpC      end trpD

                    2870                2890                2909
TACCAGTAGATGGACACTTTGAAAGACGATCATTTAAAGAAGCACTCATGAATCCTCATC
 P  V  D  G  H  F  E  R  R  S  F  K  E  A  L  M  N  P  H  R

                    2930                2950                2969
GCTCAATTGGTCTCATTGCGGAGGTGAAAAAAGCGTCTCCTTCCAAAGGAATCATTCAAC
 S  I  G  L  I  A  E  V  K  K  A  S  P  S  K  G  I  I  Q  P

                    2990      HindIII
CGAATTTTGATCCTTTACAAACAGCAAAAGCTT
 N  F  D  P  L  Q  T  A  K  A
```

Figure 8: Construction of lacZ fusion plasmid pMR500. Promoter search plasmid pSK10Δ6 (Zuber and Losick, 1983) was restricted with EcoRI and BamHI and plasmid pRR106 was restricted with EcoRI and BclI, mixed and ligated with T4 DNA polymerase. The mixture was used to transform E.coli 71-18 to Ap^R blue colored colonies on minimal media M9 X-gal plates.

Figure 8

Construction of a *lacZ* Fusion Plasmid pMR500

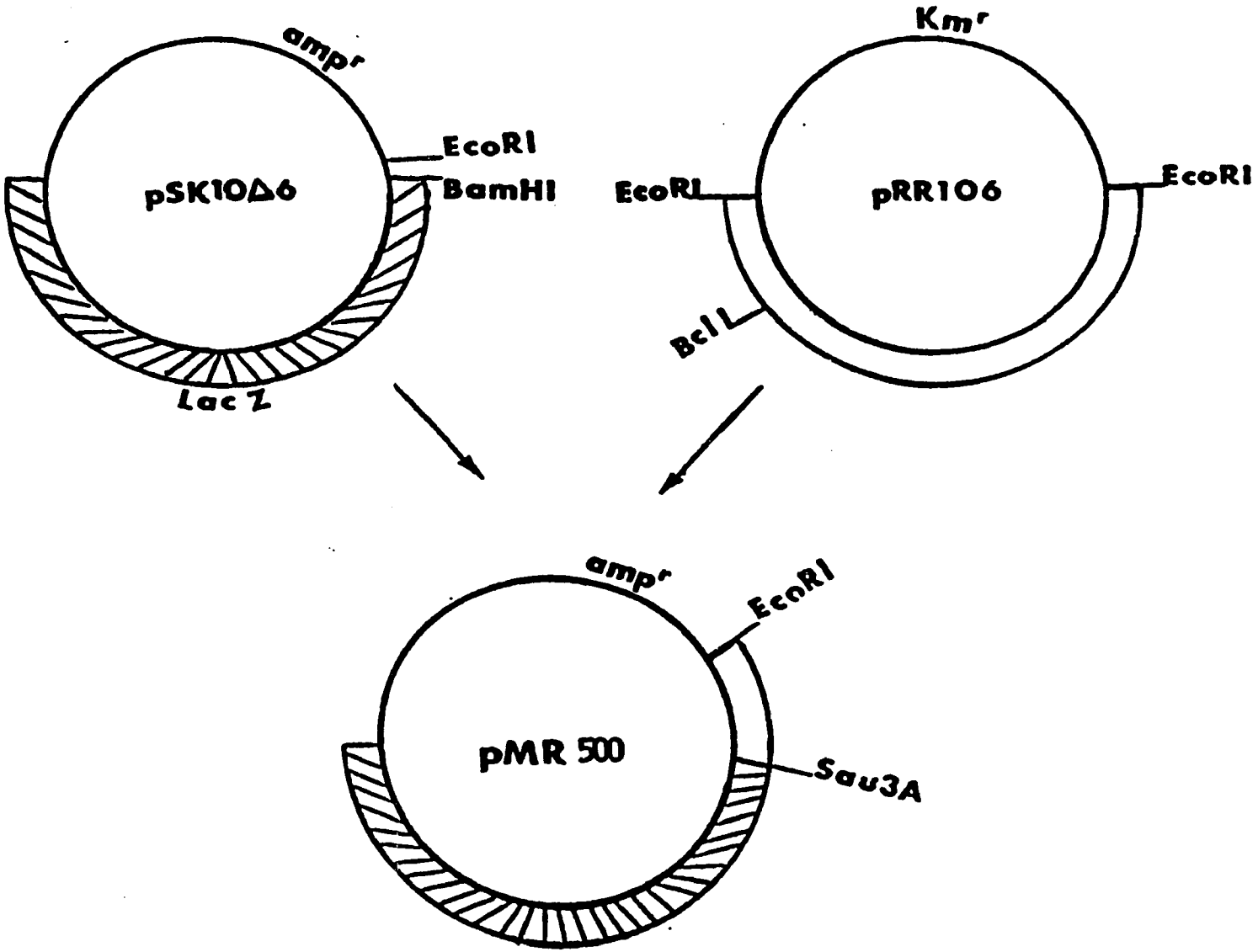


Figure 9: Comparison of the nucleotide sequences of B.pumilus and B.subtilis trp operons. The Genetics Computer Group of the University of Wisconsin programs for nucleotide and amino acid sequences were used to analyze, align, and translate the B.pumilus sequence (Devereux et al., 1984). To maximally align the sequences the gaps introduced are denoted by dots. The RBS, start and stop codons of the two sequences are indicated. The sequence data of B.subtilis trp genes was taken from Henner et al., (1985). To increase the reported G by -.2 kcal the B.subtilis trpD RBS was extended by 4 nucleotides.

Figure 9 The Comparison of the Tryptophan Genes of B. pumilus and B. subtilis

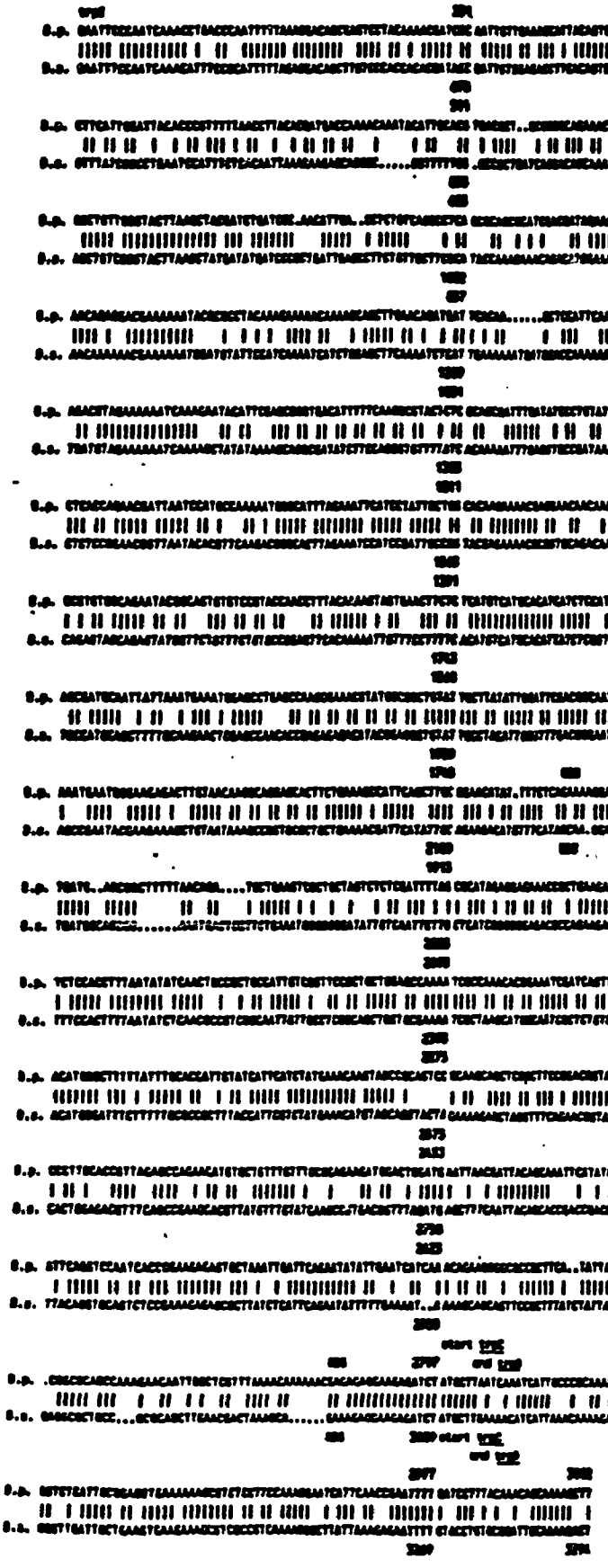


Figure 10: Comparison of the amino acid sequences of the trpE (Panel A) and trpD and 5'trpC (Panel B) genes of B.pumilus (B.p.), B.subtilis (B.s.), and E.coli (E.c.). According to Jackson and Yanofsky, (1974) in E.coli the trpG is fused to the trpD gene whereas in the Bacilli this is not the case. Therefore maximal alignment between the Bacillis and E.coli occur at the latter 2/3 of the E.coli trp(G)D gene. Gaps introduced to maximally align the sequences are denoted by dots. Identical and charge-related residues are in capital letters. "Con" denoted conserved amino acids, lack of Con is indicated by dashes. Conserved sequences are either underlined (Panel A) or presented as a comparison between seven organisms obtained from GenBank (Panel C). The amino acid sequences of B.subtilis and E.coli were obtained from Henner et al., (1985) and Yanofsky et al., (1981), respectively.

Figure 10: The Amino Acids Comparison of the *trpE* and *trpD* genes

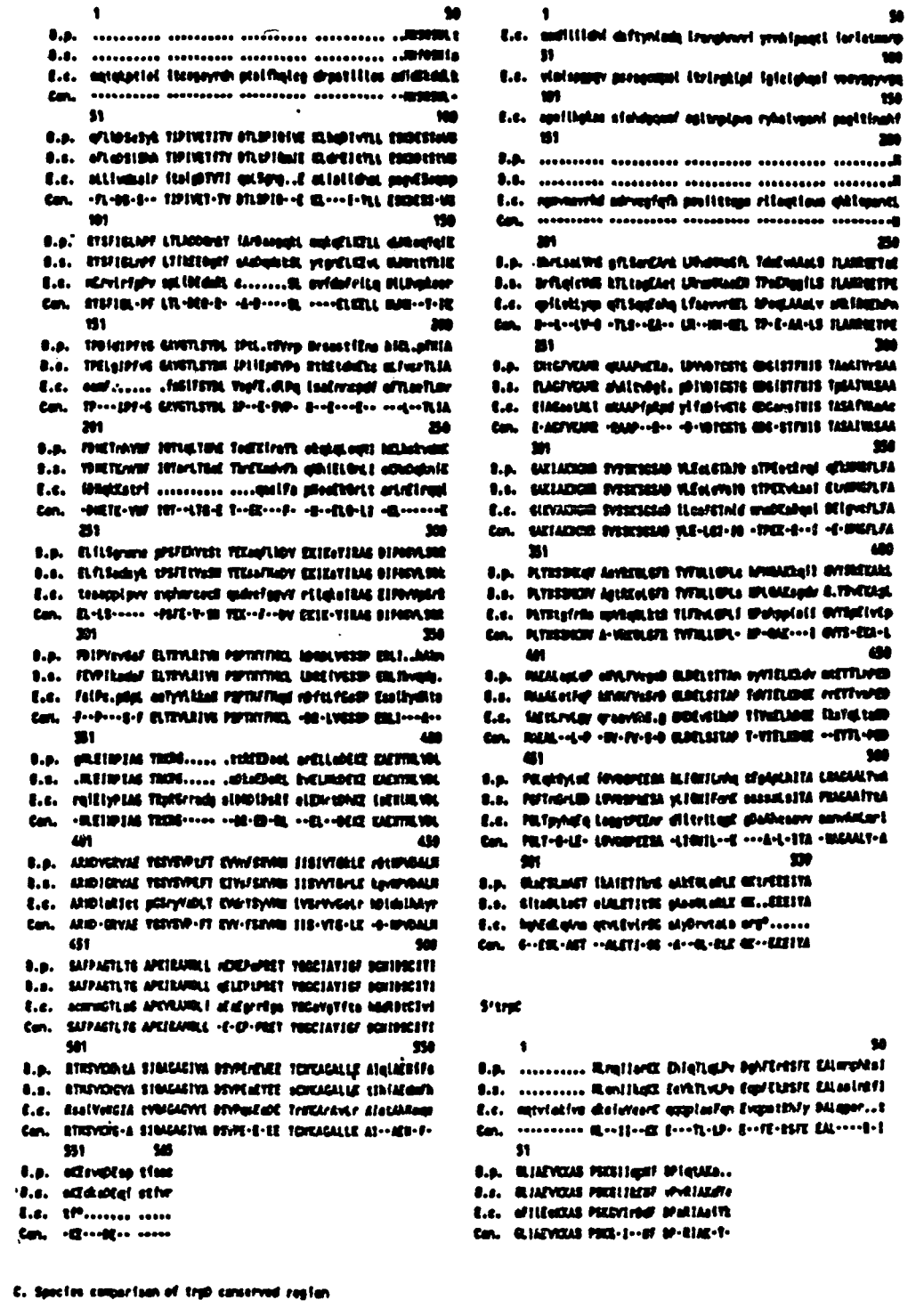
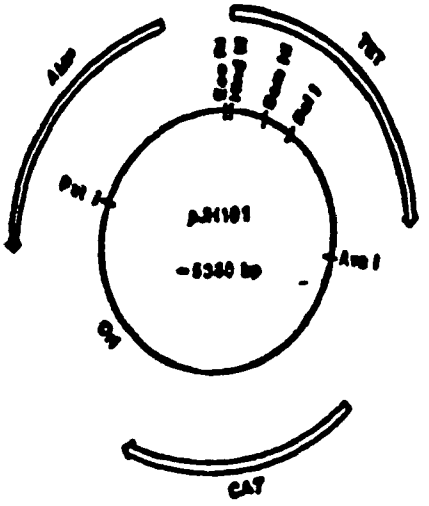
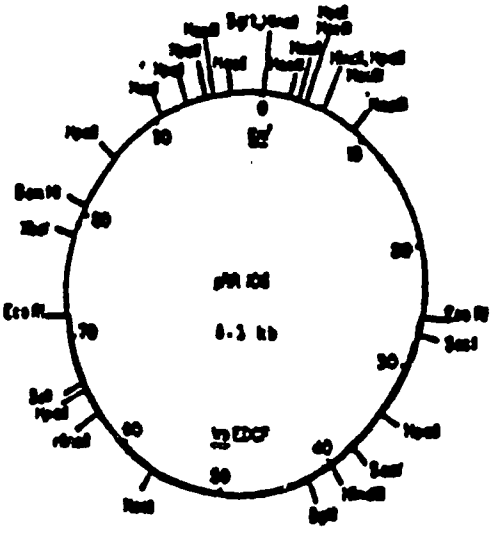


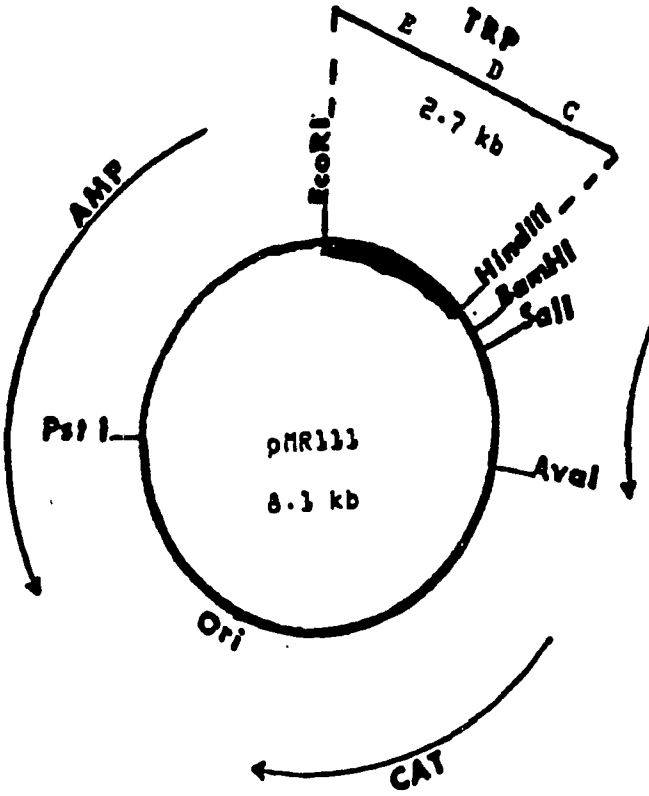
Figure 11: The construction of integrable plasmids pMR111 and pMR131 containing the trp operon of B. pumilus. Plasmids pJH101 (Ferrari et al., 1983) and pRR106 (Rudner et al., 1982) were restricted with HindIII and EcoRI, mixed and ligated with T4 DNA ligase. Two plasmids were isolated containing the 2.733-kb fragment (pMR111) and the 0.9-kb fragment (pMR131) of B.pumilus trp DNA.

Figure 11

Construction of Integrable Plasmids Containing the trp Operon of Bacillus pumilus







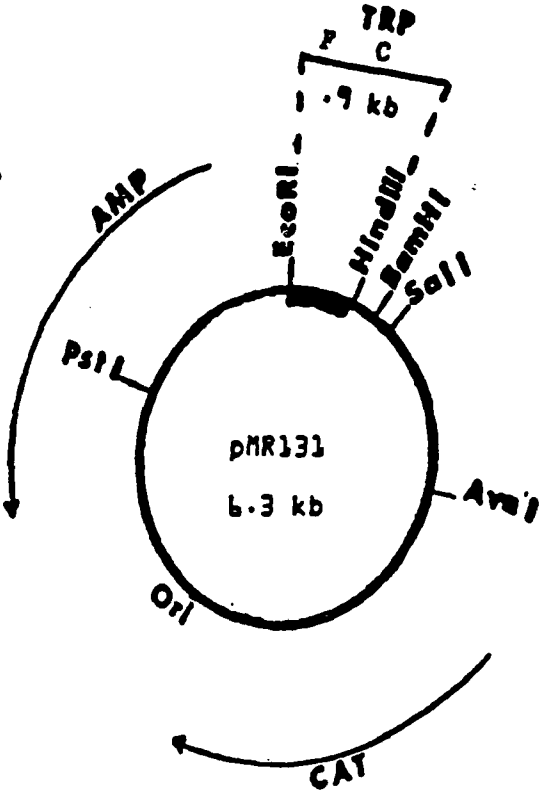
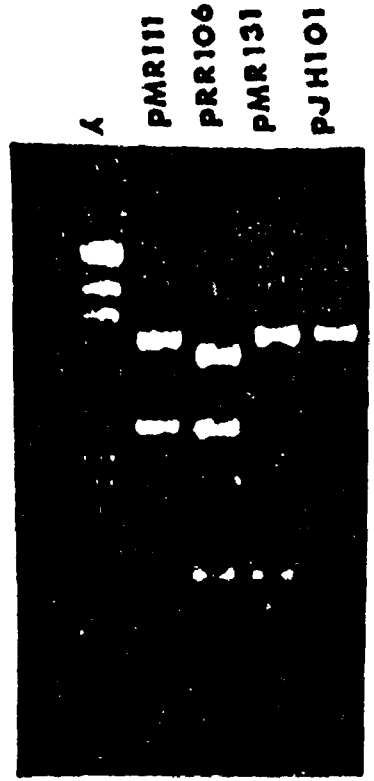


Figure 12: The electrophorectic pattern of pMR111, pMR131, pRR106 and pJH101 after restriction with EcoRI and HindIII. The molecular weights marker is DNA restricted with HindIII.

Figure 12

The electrophorectic pattern of pMR111, pRR106, pMR131 and pJH101



Lane 1 = Molecular weigth markers of λDNA restricted with HindIII

Lane 2 = pMR111 restricted with HindIII and EcoRI

Lane 3 = pRR106 restricted with HindIII and EcoRI

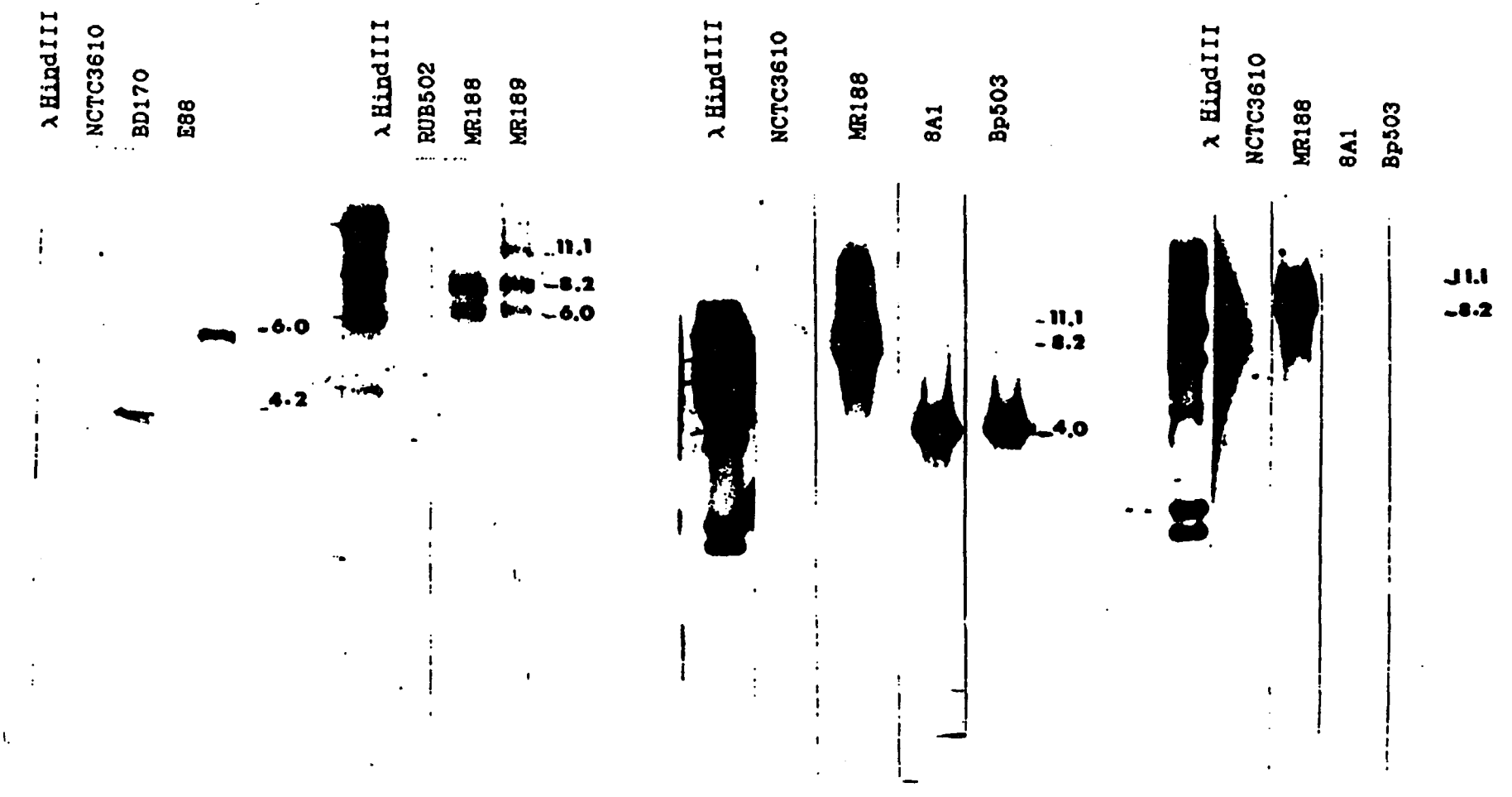
Lane 4 = pMR131 restricted with HindIII and EcoRI

Lane 5 = pJH101 restricted with HindIII and EcoRI

Figure 13: The Southern blots of HindIII restricted DNA. The DNA of the strains used are denoted at the top of the lanes. Parental B.subtilis strains are shown in Panel A (Table I). B.pumilus is presented in Panels B, C, and D. Panels B, C, and D contain DNA from heterologous integrants of B.subtilis E88 (Table I). Panel A and B were hybridized with the probe pTrpH3B2 (contains parts of the B.subtilis trpE and trpD genes). Panel C was hybridized with M13mp19-2.7-kb (contains the sequenced 2.733-kb of B.pumilus trp DNA (Figure 6 and 7). Panel D was hybridized with pJH101.

Figure 13

The Southern Blots of HindIII Restricted DNA Hybridized with pTrpH3B2, M13mp19-2.7-kb and pJH101



Panel A

ptrpH3B2

Panel B

ptrpH3B2

Panel C

M13mp19-2.7-kb

Panel D

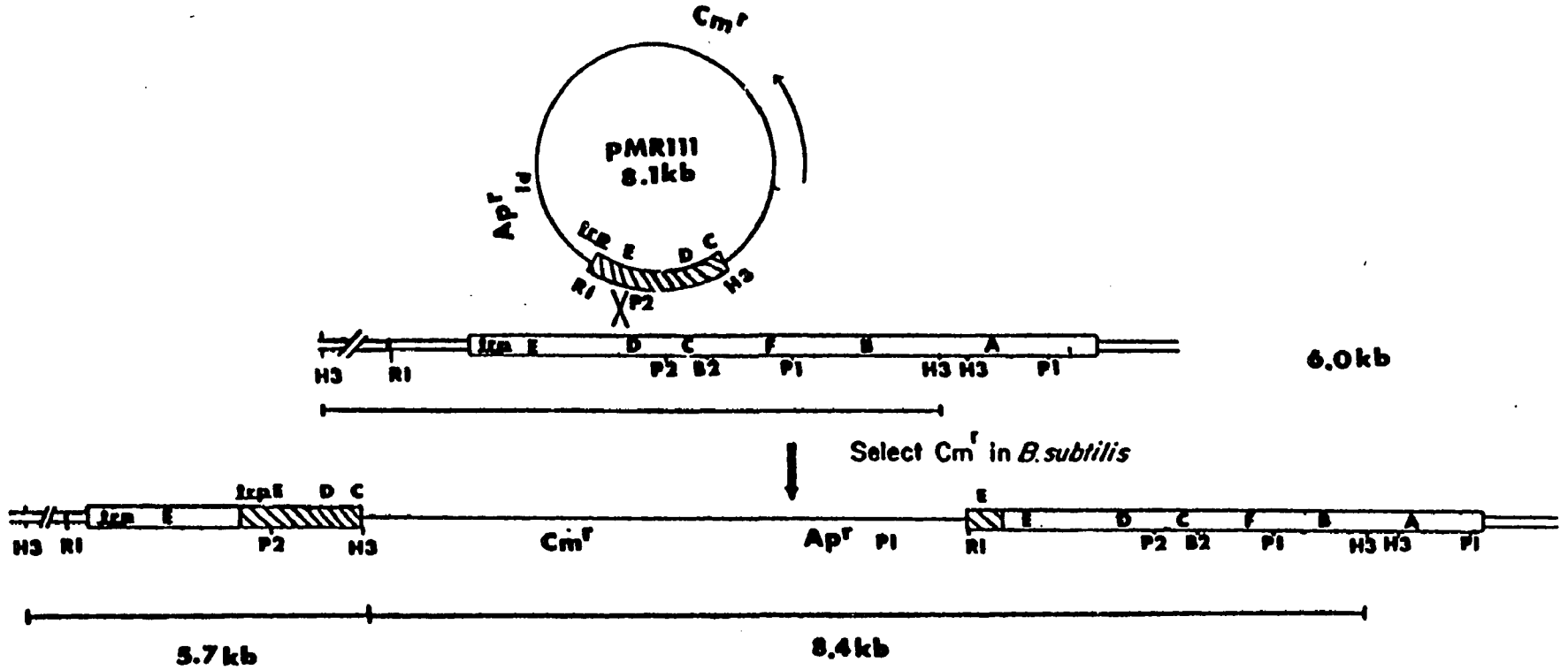
pJH101

Figure 14: A model of a Campbell-like integration event of pMR111 into the trp operon of the B. subtilis chromosome of strain E88. Figure 14 A shows the 6.0-kb fragment assuming the HindIII present in trpE of strain 168W (Henner et al., 1985) is missing in strain E88. In Figure 14 B the result of a single plasmid integration in trpE. Below the integrated configuration are the predicted band sizes of a HindIII digestion of the DNA. The following abbreviations represent selected restriction sites: H3 = HindIII, RI = EcoRI, P2 = Pvu2, P1 = Pst1, and B2 = BgIII. In Figure 14C are the predicted bands sizes that should hybridige to probes pTrpH3B2, M13mp19-2.7-kb, and pJH101, followed by the actual size bands observed in Figure 13. The results indicate that this hypothetical model is inadequate to explain integration of pMR111 into the trp operon of E88.

Figure 14

A Model of a Campbell-like Integration of pMR111

Into the trp operon of the B.subtilis Chromosome of Strain E88



A

B

C

Probes	Predicted size of band(s)	Actual size of band(s)
pTrpH3B2	5.7 kb and 8.4 kb	6.0 kb and 8.2 kb
M13mp19-2.7-kb	5.7 kb and 8.4 kb	8.2 kb and 11.1 kb
pJH101	8.4 kb	8.2 kb and 11.1 kb

Appendix

The computer program of Denereux *et al.*, (1984) called GAP cannot align more than two sequences at a time. In order to print the alignment of the amino acid sequence of the *trpE*, *trpD*, and 5'*trpC* gene products (Figure 10) of *B.pumilus*, *B.subtilis*, and *E.coli* the following programming strategy was performed. The DNA sequence was translated from an open reading frame. To find other proteins with similar amino acid sequence the WORDSEARCH program was executed. This program will assess all protein sequences entered in GenBank. The search can take a long time if it is not limited. For this thesis it was limited to bacterial, fungal and plant sequences. As expected the *B.subtilis trp* operon gave the best match. Several other organisms including the *E.coli trp* operon showed 50% or greater amino acid sequence similarity. At this point it was decided to limit the presentation to the closest relative of *B.pumilus* (*B.subtilis*) and to the best studied *trp* operon (*E.coli*). The *B.subtilis trpE* gene product was "GAPPED" against the *B.pumilus trpE* gene

product. The output is saved into two separate files. For example in Figure 15 (Trpe.Gap1) is the file of the sequence of the B.subtilis trpE protein gapped to the B.pumilus trpE. In Figure 16 is the second file showing the sequence of the B.pumilus trpE protein gapped against the B.subtilis trpE protein (Trpe.Gap2). The gaps are denoted by dots. The Trpe.Gap1 file is then gapped against the E.coli trpE protein. The output is saved into two more files Trpe.Gap3 (sequence of Trpe.Gap1 after alignment with the E.coli trpE protein, Figure 17) and Trpe.Gap4 (sequence of the E.coli trpE protein after alignment with Trpe.Gap1, Figure 18). The final gap algorhithm is between Trpe.Gap2 and Trpe.Gap4 with output files Trpe.Gap5 (sequence of Trpe.Gap2 after alignment with Trpe.Gap4, Figure 20) and Trpe.Gap6 (sequence of Trpe.Gap4 after alignment with Trpe.Gap2, Figure 21). A "PRETTY" file is created with Trpe.Gap5, Trpe.Gap3, and Trpe.Gap6 with the command CREATE FILE @pretty.fil. To obtain the consensus sequence the program called "PRETTY" is prompted. The output is saved into a pretty.txt file which can be edited by the "EDIT" program and printed as it is presented in Figure 10

after cutting and splicing. The same steps were taken for the *trp*D and 5'*trp*C gene products.

Figure 15

GAP of: Pumtrpe.Pep check: 5269 from: 1 to: 513
after alignment with: Bsutrp.Pep check: 4137 from: 1 to: 516

Symbol comparison table: GenDataBase:NWSGapPep.Cmp CompCheck: 1254

Gap Weight:	5.000	Average Match:	0.540
Length Weight:	0.300	Average Mismatch:	-0.396
Quality:	574.2	Length:	516
Ratio:	1.119	Gaps:	2
Percent Similarity:	78.752		

trpe.gap2 Length: 516 June 13, 1989 20:58 Check: 3128

```
   1  MNSQSNLTQF LKDSESYKTI PIVETITVDT LSPIQIVEKL KQDIVYLLES
  51  KDESSSWSRY SFIGLHPFLT LHDDQNKYIA RDAAGQKLMQ KQELKELLDW
 101  MKEQYQIKTP DIDIPFTGGA VGYLSYDLIP TL.TSVRPHR SASTIENAHI
 151  CL.PTMIAFD HETNHVHFIQ YTQLTGHETE DEKIRAYKEK QKQLEQMIHK
 201  LHSKVDMKEL ILSGNMNEPP SFEHVTSTYE KAQFLKDVEK IKEYIRAGDI
 251  FQGVLSQRFD IPVSVSSFEL YRVLRIVNPS PYMYFMKLKD RDLVGSSPER
 301  LIHAKNGHLE IHPIAGTRKR GTTREEDAEL ARELLEDEKE KAEHYMLVDL
 351  ARNDVGRVAE YGSVSVPTFT KVVNFSHVMH IISIVTGKLK RDTHPVDALM
 401  SAFPAGTLTG APKIRAMQLL NEMEPEPRET YGGCIAYIGF DGNIDSCITI
 451  RTMSVKNHTA SIQAGAGIVA DSVPENEWEE TCNKAGALLK AIQLAEHIFS
 501  EKESVQDESP TISSC.
```

Figure 16

GAP of: Bsutrp.Pep check: 4137 from: 1 to: 516

after alignment with: Pumtrpe.Pep check: 5269 from: 1 to: 513

Symbol comparison table: GenDataBase:NWSGapPep.Cmp CompCheck: 1254

Gap Weight:	5.000	Average Match:	0.540
Length Weight:	0.300	Average Mismatch:	-0.396
Quality:	574.2	Length:	516
Ratio:	1.119	Gaps:	2
Percent Similarity:	78.752		

trpe.gap1 Length: 516 June 13, 1989 20:58 Check: 4137

```
   1  MNFQSNISAF LEDSLSHHTI PIVETFTVDT LTPIQMIEKL DREITYLLES
  51  KDDTSTWSRY SFIGLNPFLT IKEEQGRFSA ADQDSKSLYT GNELKEVLNW
 101  MNTTYKIKTP ELGIPFVGGA VGYLSYDMIP LIEPSVPSHT KETDMEKCML
 151  FVCRTLIAYD HETKNVHFIQ YARLTGEETK NEKMDVFHQN HLELQNLIEK
 201  MMDQKNIKEL FLSADSYKTP SFETVSSNYE KSAFMADVEK IKSYIKAGDI
 251  FQGVLSQKFE VPIKADAFEL YRVLRIVNPS PYMYYMKLLD REIVGSSPER
 301  LIHVQDGHLE IHPIAGTRKR GADKAEDERL KVELMKDEKE KAEHYMLVDL
 351  ARNDIGRVAE YGSVSVPEFT KIVSFSHVMH IISVVTGRLK KGVHPVDALM
 401  SAFPAGTLTG APKIRAMQLL QELEPTPRET YGGCIAYIGF DGNIDSCITI
 451  RTMSVKNGVA SIQAGAGIVA DSVPEAEYEE SCNKAGALLK TIHIAEDMFH
 501  SKEDKADEQI STIVR*
```

Figure 17

```
GAP of: Ecotrp.Pep  check: 2889  from: 1  to: 521
after alignment with: Trpe.Gap1  check: 4137  from: 1  to: 516

Symbol comparison table: GenDataBase:NWSGapPep.Cmp  CompCheck: 1254

        Gap Weight:  5.000      Average Match:  0.540

     Length Weight:  0.300   Average Mismatch: -0.396

           Quality:  270.9             Length:    566
             Ratio:  0.525               Gaps:      8
Percent Similarity: 53.928

    trpe.gap4  Length: 566  June 13, 1989  21:01  Check: 5738

      1  MQTQKPTLEL LTCEGAYRDN PTALFHQLCG DRPATLLLES ADIDSKDDLK

     51  SLLLVDSALR ITALGDTVTI QALSGNG..E ALLALLDNAL PAGVESEQSP

    101  NCRVLRFPPV SPLLDEDARL C,.....SL SVFDAFRLLQ NLLNVPKEER

    151  EAMF..... .FSGLFSYDL VAGFE.DLPQ LSAENNCPDF CFYLAETLMV

    201  IDHQKKSTRI ..........  ....QASLFA PNEEEKQRLT ARLNELRQQL

    251  TEAAPPLPVV SVPHMRCECN QSDEEFGGVV RLLQKAIRAG EIFQVVPSRR

    301  FSLPC.PSPL AAYYVLKKSN PSPYMFFMQD NDFTLFGASP ESSLKYDATS

    351  RQIEIYPIAG TRPRGRRADG SLDRDLDSRI ELEMRTDHKE LSEHLMLVDL

    401  ARNDLARICT PGSRYVADLT KVDRYSYVMH LVSRVVGELR HDLDALHAYR

    451  ACMNMGTLSG APKVRAMQLI AEAEGRRRGS YGGAVGYFTA HGDLDTCIVI

    501  RSALVENGIA TVQAGAGVVL DSVPQSEADE TRNKARAVLR AIATAHHAQE

    551  TF*.....  ......
```

Figure 18

GAP of: Trpe.Gap1 check: 4137 from: 1 to: 516

after alignment with: Ecotrp.Pep check: 2889 from: 1 to: 521

Symbol comparison table: GenDataBase:NWSGapPep.Cmp CompCheck: 1254

Gap Weight:	5.000	Average Match:	0.540
Length Weight:	0.300	Average Mismatch:	-0.396
Quality:	270.9	Length:	566
Ratio:	0.525	Gaps:	8
Percent Similarity:	53.928		

trpe.gap3 Length: 566 June 13, 1989 21:01 Check: 7901

```
   1  .......... .......... .......... .......... ..MNFQSNIS
  51  AFLEDSLSHH TIPIVETFTV DTLTPIQMIE KLDREITYLL ESKDDTSTWS
 101  RYSFIGLNPF LTIKEEQGRF SAADQDSKSL YTGNELKEVL NWMNTTYKIK
 151  TPELGIPFVG GAVGYLSYDM IPLIEPSVPS HTKETDMEKC MLFVCRTLIA
 201  YDHETKNVHF IQYARLTGEE TKNEKMDVFH QNHLELQNLI EKMMDQKNIK
 251  ELFLSADSYK TPSFETVSSN YEKSAFMADV EKIKSYIKAG DIFQGVLSQK
 301  FEVPIKADAF ELYRVLRIVN PSPYMYYMKL LDREIVGSSP ERLIHVQDG.
 351  .HLEIHPIAG TRKRG..... .ADKAEDERL KVELMKDEKE KAEHYMLVDL
 401  ARNDIGRVAE YGSVSVPEFT KIVSFSHVMH IISVVTGRLK KGVHPVDALM
 451  SAFPAGTLTG APKIRAMQLL QELEPTPRET YGGCIAYIGF DGNIDSCITI
 501  RTMSVKNGVA SIQAGAGIVA DSVPEAEYEE SCNKAGALLK TIHIAEDMFH
 551  SKEDKADEQI STIVR*
```

Figure 19

GAP of: Trpe.Gap2 check: 3128 from: 1 to: 516

after alignment with: Trpe.Gap4 check: 5738 from: 1 to: 566

Symbol comparison table: GenDataBase:NWSGapPep.Cmp CompCheck: 1254

Gap Weight:	5.000	Average Match:	0.540
Length Weight:	0.300	Average Mismatch:	-0.396
Quality:	306.8	Length:	566
Ratio:	0.598	Gaps:	2
Percent Similarity:	52.452		

trpe.gap5 Length: 566 June 13, 1989 21:03 Check: 6822

```
   1  .......... .......... .......... .......... ..MNSQSNLT
  51  QFLKDSESYK TIPIVETITV DTLSPIQIVE KLKQDIVYLL ESKDESSSWS
 101  RYSFIGLHPF LTLHDDQNKY IARDAAGQKL MQKQELKELL DWMKEQYQIK
 151  TPDIDIPFTG GAVGYLSYDL IPTL.TSVRP HRSASTIENA HICL.PTMIA
 201  FDHETNHVHF IQYTQLTGHE TEDEKIRAYK EKQKQLEQMI HKLHSKVDMK
 251  ELILSGNMNE PPSFEHVTST YEKAQFLKDV EKIKEYIRAG DIFQGVLSQR
 301  FDIPVSVSSF ELYRVLRIVN PSPYMYFMKL KDRDLVGSSP ERLI..HAKN
 351  GHLEIHPIAG TRKRG..... .TTREEDAEL ARELLEDEKE KAEHYMLVDL
 401  ARNDVGRVAE YGSVSVPTFT KVVNFSHVMH IISIVTGKLK RDTHPVDALM
 451  SAFPAGTLTG APKIRAMQLL NEMEPEPRET YGGCIAYIGF DGNIDSCITI
 501  RTMSVKNHTA SIQAGAGIVA DSVPENEWEE TCNKAGALLK AIQLAEHIFS
 551  EKESVQDESP TISSC.
```

Figure 20

```
GAP of: Trpe.Gap4  check: 5738  from: 1  to: 566
after alignment with: Trpe.Gap2  check: 3128  from: 1  to: 516

Symbol comparison table: GenDataBase:NWSGapPep.Cmp  CompCheck: 1254

        Gap Weight:  5.000      Average Match:  0.540
     Length Weight:  0.300   Average Mismatch: -0.396

           Quality:  306.8             Length:    566
             Ratio:  0.598               Gaps:      2
Percent Similarity: 52.452

   trpe.gap6  Length: 566  June 13, 1989  21:03  Check: 5738

     1  MQTQKPTLEL LTCEGAYRDN PTALFHQLCG DRPATLLLES ADIDSKDDLK

    51  SLLLVDSALR ITALGDTVTI QALSGNG..E ALLALLDNAL PAGVESEQSP

   101  NCRVLRFPPV SPLLDEDARL C.......SL SVFDAFRLLQ NLLNVPKEER

   151  EAMF...... .FSGLFSYDL VAGFE.DLPQ LSAENNCPDF CFYLAETLMV

   201  IDHQKKSTRI .......... ....QASLFA PNEEEKQRLT ARLNELRQQL

   251  TEAAPPLPVV SVPHMRCECN QSDEEFGGVV RLLQKAIRAG EIFQVVPSRR

   301  FSLPC.PSPL AAYYVLKKSN PSPYMFFMQD NDFTLFGASP ESSLKYDATS

   351  RQIEIYPIAG TRPRGRRADG SLDRDLDSRI ELEMRTDHKE LSEHLMLVDL

   401  ARNDLARICT PGSRYVADLT KVDRYSYVMH LVSRVVGELR HDLDALHAYR

   451  ACMNMGTLSG APKVRAMQLI AEAEGRRRGS YGGAVGYFTA HGDLDTCIVI

   501  RSALVENGIA TVQAGAGVVL DSVPQSEADE TRNKARAVLR AIATAHHAQE

   551  TF*....... ......
```

TRANSFORMATION OF INTEGRABLE PLASMIDS CONTAINING *B. PUMILUS TRP* SEQUENCES

STRAIN	DNA	Number/ml		Test of Clones				
		Cm^R	Trp^+	Cm^RTrp^+	Cm^RTrp^+	Cm^RTrp^-	Trp^+Cm^R	Trp^+Cm^S
E88	pMR111	215	530	0/ 43	0/ 43	43/ 43	0/106	106/106
tryE46,hisH2	pMR131	450(S)	280(S)	0/ 90	0/ 90	90/ 90	0/ 84	84/ 84
	E88/pM111	3.2×10^3	700	2/244	0/244	242/244	0/ 70	70/ 70
	B. pumilus		230(S)					
DB83	pMR111	103	460	2/ 31	0/ 31	29/ 31	0/ 92	92/ 92
trpD2	pMR131	260(S)	260(S)	0/ 52	0/ 52	52/ 52	0/ 58	58/ 58
	E88/pM111	3.3×10^3	1.0×10^5	0/104	10/104	94/104	0/500	500/500
	B. pumilus		230(S)					
SB25	pMR111	90	305	0/27	6/27	21/27	0/61	61/61
trpC2,hisH2	pMR131	290(S)	105(S)	0/87	2/87	85/87	0/21	21/21
	E88/pM111	2.8×10^2	1.5×10^5	2/57	3/57	52/57	n.d.	n.d.
	B. pumilus		20					
T12	pMR111	10; 80(S)	530(S)	1/ 24	7/ 24	16/ 24	0/106	106/106
trpF12	pMR131	1033(T)	230(S)	0/413	12/413	399/413	0/ 46	46/ 46
	E88/pMR111	1.3×10^3	2.8×10^4	1/ 71	12/ 71	58/ 71	0/ 56	56/ 56
	B. pumilus		0					

S = Small
T = Tiny
n.d. = not determined

The table on page 149 (Transformation of Integrable Plasmids Containing B.pumilus trp Sequences) (unpublished data provided by Rivka Rudner) represents another experiment similar to the one described in Table X. In this experiment unlike the one in Table X the plasmids pMR111 and pMR131 were grown in the E.coli strain JC9604 (Cohen and Clark, 1986). The attractive feature of strain JC9604 is that it generates linear plasmid multimers. The isolation of the plasmids from this strain eliminates the in vitro generation and the ambiguity of the presence of the multimeric form. The results show that the transformation efficiencies for Cm^R or Trp^+ of these plasmids have increased by a factor of 10 compared to Table X. However, neither the double recombinant class nor the transformation efficiencies observed for integrable plasmids containing homologous B.subtilis sequences (Jarvis et al., 1988; La Fauci et al., 1986) increased at all. Therefore the low transformation efficiencies observed with plasmids pMR111 and pMR131 were most likely due to the heterologous sequence of the B.pumilus trp genes and not to the integrity and integration ability of the plasmids.

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