CHARACTERIZATION OF AN OPERON CONTAINING A RIBOSOMAL PROTEIN GENE AND LIPID BIOSYNTHETIC GENES IN *ESCHERICHIA COLI* K-12

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(ABSTRACT)

The *plsX50* mutation is required together with *plsX26* (encoding a K_m-defective glycerol 3-phosphate acyltransferase) for the conferral of a glycerol 3-phosphate auxotrophic phenotype. A 4.9 kb segment of DNA complementing the *plsX50* mutation have been cloned and sequenced. Six open reading frames (ORF's) were found with five reading in the same direction and one in the opposite direction relative to the *plsX* gene. Each ORF encoded a protein, as demonstrated by radiolabeling in maxicells. ORF1 (*orfY*) encodes a protein of unknown function. ORF2 and ORF3 (*rpmF*) were sequenced prior to this study and encode a protein called G30k of unknown function and L32, a protein of the large ribosomal subunit, respectively. ORF4 complemented the *plsX50* mutation. ORF5 was identified as *fabH* encoding 3-ketoacyl-ACP synthase III. ORF6 was identified as *fabD* encoding malonyl-CoA/ACP transacylase. The *fabG* gene encoding 3-ketoacyl-ACP reductase and the *acpP* gene encoding acyl carrier protein are located just downstream of the *fabD* gene. Northern and promoter activity analysis

demonstrated that the *rpmF-plsX-fabH-fabD-fabG-acpP* genes comprise an operon suggesting a coordinate control of the synthesis of a ribosomal protein (L32), PlsX protein, and fatty acid biosynthetic enzymes. However, several features were identified that are likely to be important for differential expression of the individual genes. These include the presence of multiple promoters, an internal terminator (attenuator), differential degradation of transcripts, and differential efficiency of translation initiation.

Portions of transcripts arising upstream of *rpmF* terminate at the attenuator located just downstream of the *plsX* initiation codon, and some of the transcripts continue into the *plsX-fab* genes. The *fabH-fabD-fabG-acpP* genes are also cotranscribed from a promoter located upstream of the *fabH* gene, within the *plsX* structural gene. There are additional cotranscripts responsible for the expression of the *fabD-fabG-acpP* genes. The *acpP* gene is encoded by several more transcripts. Transcription initiation sites upstream of *rpmF* were identified by primer extension analysis and the attenuator site was identified by S1 mapping analysis. N-terminal amino acid sequence analysis identified the translation initiation codons of *orfY, plsX, fabH, fabD* and *fabG*. Short intergenic distances (15 and 12 bp) found between *fabH* and *fabD* and between *fabD* and *fabG* implicate translational coupling as a mechanism for coordinate control of *fabHDG* expression. The *plsX50* mutation was identified as deletion of a single nucleotide from the 6th codon of *plsX* resulting in a frame shift nonsense mutation.

TO MY FATHER AND MY MOTHER

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List of Abbreviations

ACP	acyl carrier protein
Amp	ampicillin
bp	base pair
BSA	bovine serium albumin
dNTP	deoxynucleotide triphosphate
DDT	dithiothreitol
FAS	fatty acid synthase
G3P	*sn*-glycerol 3-phosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropylthio-β-D-galactopyranoside
kb	kilobase
KS	3-ketoacyl-ACP synthase
LB	Luria Broth
MTA	malonyl transacylase
MW	molecular weight
nt	nucleotide
ONPG	orthonitrophenyl-β-D-galactopyranoside
PAGE	polyacrylamide gel electrophoresis

ppGpp	guanosine tetraphosphate
p.s.i.	pounds per square inch
r-protein	ribosomal protein
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
TB	Terrific broth
TddR	2',3'-dideoxythymidine
U	units
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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INTRODUCTION

Escherichia coli is enclosed by inner cytoplasmic and outer membranes. Phospholipids are the major lipid components found in the membranes of *E. coli.* There should be global controls that coordinate the rate of production of phospholipid with the rate of cell growth such that membrane growth occurs with the cell growth. The rate of phospholipid synthesis in *E. coli* is likely to be regulated at an early stage of fatty acid biosynthesis. If so, fatty acyl-ACP synthesis is expected to be rate-limiting for phospholipid synthesis. The precise step of fatty acyl-ACP synthesis that is regulated has not been identified. It is known that the synthetic rates of macromolecules, especially those comprising the ribosomes (rRNA's, ribosomal proteins) in *E. coli* are subject to growth-rate-dependent regulation and to stringent control. It is also known that the rate of phospholipid synthesis decreases significantly during the stringent response.

The *plsB* gene encodes a G3P acyltransferase which catalyzes the initial step of phospholipid biosynthesis in *E. coli. plsB26* mutant strains contain a defective G3P acyltransferase with a 10-fold increased K_m value for G3P. A second mutation in *plsX (plsX50)* along with *plsB26* is required for the conferral of a G3P auxotrophic phenotype. Correction of either mutation results in the correction of the G3P auxotrophic phenotype. The exact biochemical role played by the *plsX* gene product has not been determined.

In order to identify the function of *plsX*, a segment of DNA containing the *plsX* gene was studied. It was found that the *plsX* gene is located just downstream of a gene encoding ribosomal protein L32 (*rpmF*) and immediately upstream of genes encoding fatty acid biosynthetic enzymes. Northern, S1 mapping, primer extension and promoter activity analysis were carried out to determine the transcriptional organization of these genes. N-terminal amino acid sequence analysis of the proteins was performed to figure out the translational organization of these genes. The organization of the *rpmF-plsX-fab* genes into an operon suggests a mechanism for the coordinate regulation of ribosome and lipid synthesis, therefore coordinate regulation of protein and membrane synthesis. Juxtaposition and cotranscription of *plsX* with *fabH* suggest a role for *plsX* in production of fatty acyl-ACP, which is one of the substrates for the glycerol 3-phosphate acyltransferase. The nature of the *plsX50* mutation was identified and its effect at the transcriptional level and at the translational level was determined.

LITERATURE REVIEW

E. coli is enclosed by an envelope consisting of inner cytoplasmic and outer membranes separated by the periplasm and peptidoglycan (1). All of the phospholipids, neutral lipids (diglyceride and free fatty acids), and lipoproteins of *E. coli* are membrane components; no cytoplasmic, extracellular or periplasmic lipid-containing structures are known (2). However, the early precursors of fatty acids are synthesized in the cytoplasm. The lipids of *E. coli* consist mainly of phospholipids and phospholipids contain about 90% of the total fatty acyl groups. A small amount of fatty acid is found in the lipid A component of lipopolysaccharide, in lipoproteins, and in traces of neutral lipids (3). The fatty acid synthetic enzymes, acyl carrier protein (ACP), and *sn*-glycerol-3-phosphate (G3P) synthase are cytoplasmic (2, 4, 5), whereas the enzymes of phospholipid synthesis are localized on the inner membrane (6, 7). Formation of phosphatidic acid, which is the key intermediate of phospholipid synthesis, can be considered to be the convergence of two pathways, a lengthy pathway of fatty acid synthesis and a single step pathway for synthesis of G3P. Phosphatidic acid is formed by the acylation of G3P by the acyl-ACP products of fatty acid synthesis and is then converted into the three major phospholipid classes: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) (2).

FATTY ACID BIOSYNTHESIS IN *ESCHERICHIA COLI*

The synthesis of fatty acids is essential for growth of the cellular membrane in *E. coli*. All known organisms can synthesize fatty acids from acetyl-CoA (8). Acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase. Acetyl-CoA carboxylase of *E. coli* is composed of three different components; biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC) and carboxyl transferase (CT). The genes for BCCP (*accB*) and BC (*accC*) are located at 72 min on the linkage map of *E. coli* and have been sequenced (9, 10, 11, 12). It was demonstrated that BCCP and BC are transcribed as a single mRNA species (12). The *accA* gene and *accD* gene encoding two subunits of CT were recently identified (13). The *accA* gene is located at min 4.3 and the *accD* gene is located at min 50 of the *E. coli* genetic map (13). Eucaryotic acetyl-CoA carboxylases contain all three components in a single multi-functional polypeptide (14).

The remaining fatty acid synthetic reactions are catalyzed by a group of enzymes known collectively as fatty acid synthase (FAS). FAS catalyzes a series of reactions, the combined effect of which is to elongate the primer acyl group by two carbon atoms, using malonyl-CoA as the carbon source. This sequence of reactions is repeated several times in a cyclical manner, to yield a 16- or 18-carbon fatty acid, which is removed from the cycle by a competing termination reaction (8). In most bacteria and in plant chloroplasts, each reaction is catalyzed

by a distinct individual enzyme (8). In addition, the acyl carrier which has been designated as the acyl carrier protein (ACP), is a separate, non-enzymic protein with a molecular weight of 8,847 (15). This type of organization has been called type II FAS. In animals, each reaction is catalyzed by a single multifunctional enzyme complex and acyl carrier is an integral part of this enzyme complex. This type of organization has been referred to as type I FAS. In animals, FAS is found to be a dimer consisting of identical polypeptides. In fungi, all functions are carried on two polypeptide chains, α and β, with the active complex having the stoichiometry $\alpha_6\beta_6$ (8).

The fatty acid biosynthetic pathway in *E. coli.* is as follows (Fig. 1) (2, 15, 16). Acetyl-CoA is the precursor for fatty acid biosynthesis. Once malonyl-CoA has been synthesized from acetyl-CoA by acetyl-CoA carboxylase, the malonyl group is transferred to the sulfhydryl group of 4-phosphopantetheine of acyl carrier protein by *fabD*-encoded malonyl CoA/ACP transacylase (MTA). Temperature-sensitive *fabD* mutant strains are totally deficient in fatty acid synthesis at the nonpermissive temperature (17), indicating that MTA catalyzes an essential step in fatty acid biosynthesis. Malonyl-ACP is a substrate for the condensing enzymes.

Three condensing enzymes have been found in *E. coli* (18, 19), called 3-ketoacyl-ACP synthase (KS) I, KS II, and KS III, which are encoded by the *fabB, fabF*, and *fabH* genes, respectively. These enzymes catalyze the condensation of malonyl-ACP with the growing acyl chain producing a 3-ketoacyl-ACP elongated

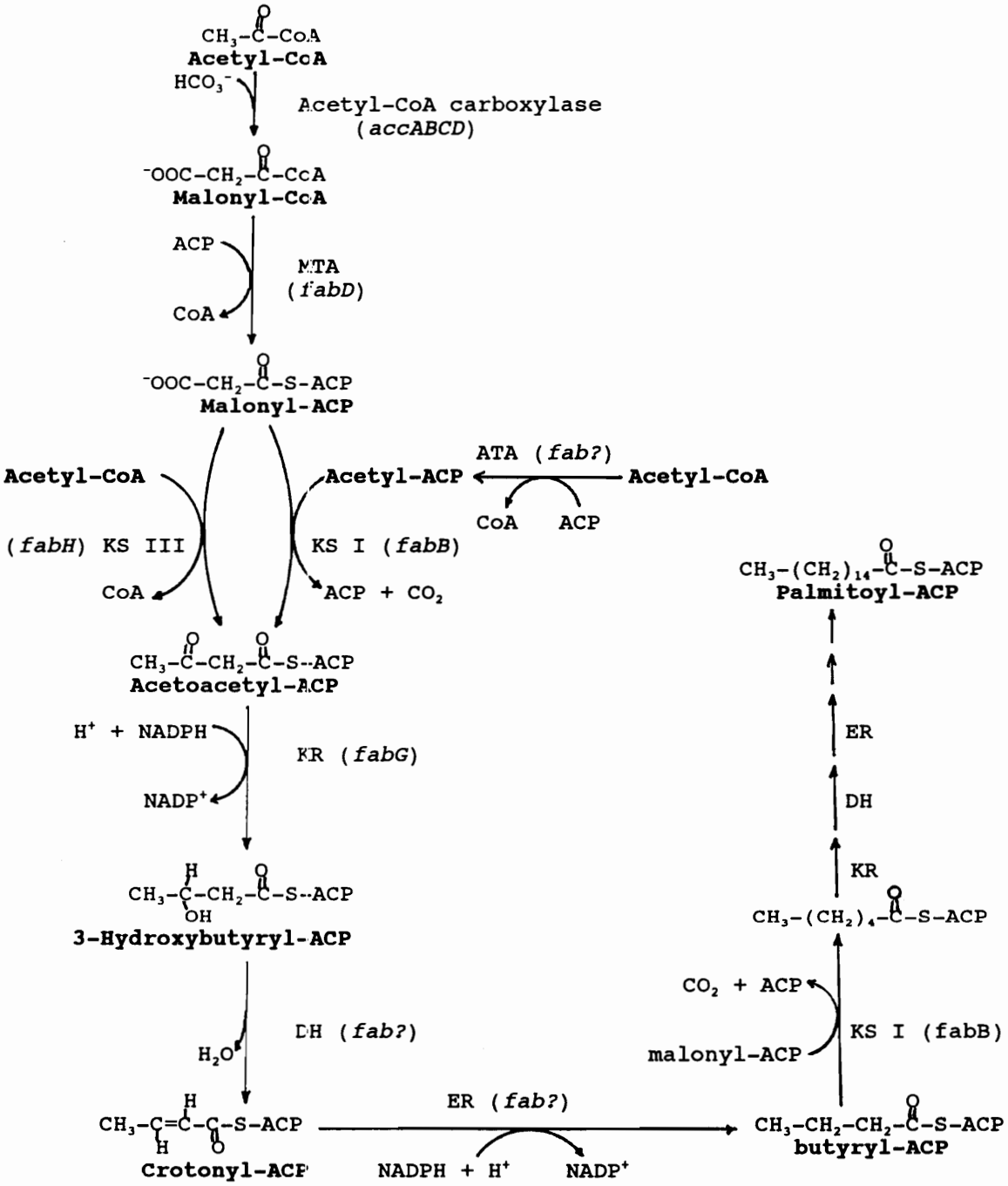

Fig. 1. Outline of the reactions for fatty acid synthesis. ATA, Acetyl transacylase; MTA, malonyl transacylase; KS, 3-ketoacyl-ACP synthase; KR, 3-ketoacyl-ACP reductase; DH, 3-ketoacyl-ACP dehydrase; ER, 2,3-trans-enoyl-ACP reductase.

by two carbon units. Both KS I and KS II can catalyze all of the condensations for synthesis of saturated fatty acids. But KS I is required for the elongation of *cis*-3-decenoyl-ACP. KS I *(fabB)* mutants require supplementation with unsaturated fatty acids for growth (20). KS II is responsible for the elongation of palmitoleate ($C_{16:1}$) to *cis*-vaccenate ($C_{18:1}$) (21). Mutants (*fabF*) lacking synthase II activity do not have a growth phenotype, but are unable to synthesize *cis*-vaccenate to regulate their fatty acid composition in response to temperature change (22).

Recently, evidence for the existence of a third condensing enzyme, acetoacetyl-ACP synthase (KS III) was reported (18, 23, 24). KS III catalyzes the first condensation reaction of fatty acid biosynthesis and is called acetoacetyl-ACP synthase because the major product of this enzyme is acetoacetyl-ACP in vitro (18). In contrast to KS I and KS II, KS III selectively catalyzes the condensation of malonyl-ACP with acetyl-CoA, rather than with acetyl-ACP (23). Both KS I and KS II are capable of transacylating all chain lengths of acyl-CoA/ACP except acetyl-CoA (25, 26). The purified KS III catalyzes both condensation (acetoacetyl-ACP synthase) and transacetylation (acetyl CoA/ACP transacylase) (24). KS III is proposed to be a potential regulator of fatty acid biosynthesis in bacteria because of its position at the beginning of the biosynthetic pathway (18). The fact that significant pools of malonyl-ACP and acetyl-CoA were present in vivo whereas the long-chain acyl-ACP pool was a minor fraction of the total ACP pool suggested that condensation of malonyl-ACP with acetyl-CoA is the slow step and rate-

controlling reaction in fatty acid biosynthesis (18).

It appears that there are two pathways for the initial condensation of fatty acid biosynthesis (16). KS III catalyzes the condensation of acetyl-CoA and malonyl-ACP producing acetoacetyl-ACP. The alternative pathway is the production of acetyl-ACP from acetyl-CoA by acetyl transacylase followed by the condensation of acetyl-ACP and malonyl-ACP by KS I.

The composition of fatty acids produced depends on the relative activities of the synthases I, II, and III (24). The absence of synthase I prevents the production of unsaturated fatty acids, whereas the overproduction of synthase I increases the fatty acid chain length. The absence of synthase II leads to defective temperature control and lack of the elongation of palmitoleate to *cis*-vaccenate. Decreased activity of synthase III leads to a small increase in average fatty acid chain length, whereas overproduction of synthase III causes a large increase in the amount of shorter chain fatty acids (myristate $C_{14:0}$) accompanied by a large decrease in the amount of cis-vaccenate ($C_{18:1}$).

In addition to the synthases, three other enzymes participate in each cycle of chain elongation. The 3-carbonyl group of 3-ketoacyl-ACP is reduced to a hydroxyl group by *fabG*-encoded 3-ketoacyl-ACP reductase (KR). Then a water molecule is removed by the 3-hydroxyacyl-ACP dehydrase (DH) producing a 2,3-*trans* double bond, which is further reduced by 2,3-*trans*-enoyl-ACP reductase (ER) to form a saturated acyl-ACP. This acyl-ACP in turn can serve as the substrate for

another condensation. There are no known mutations affecting these three steps (2). These condensation, reduction, dehydration, and reduction reactions cycle until the C_{10} intermediate 3-hydroxydecanoyl-ACP is reached. At this point, competition occurs between *fabA*-encoded 3-hydroxydecanoyl-ACP dehydrase, which forms a *cis*-3-decenoyl-ACP, and 3-hydroxyacyl-ACP dehydrase, which forms a *trans*-2-decenoyl-ACP. *trans*-2-decenoyl-ACP is the precursor of saturated fatty acids and is further elongated to yield a palmitic acid (16:0). *cis*-3-decenoyl-ACP is subsequently elongated to yield a palmitoleoyl-ACP ($16:1^{\Delta 9}$). KS I is thought to be absolutely required for one of the elongation reactions in this branch. The conversion of palmitoleoyl-ACP to *cis*-vaccenic acid ($18:1^{\Delta 11}$) involves KS II, which shows a preference for palmitoleoyl-ACP as a substrate. Palmitic, palmitoleic, and *cis*-vaccenic acids are the three major fatty acids produced by the *E. coli* fatty acid synthase system.

FATTY ACID INCORPORATION INTO MEMBRANE PHOSPHOLIPID

The glycerol backbone of *E. coli* phospholipids, *sn*-glycerol-3-phosphate (G3P), can be synthesized from glycerol by the reaction catalyzed by *glpK*-encoded glycerol kinase (27) or from dihydroxyacetone phosphate by the reaction catalyzed by *gpsA*-encoded G3P synthase (28). Acylation of G3P by the acyl-ACP products of fatty acid synthesis is catalyzed by acyltransferases (29) (Fig. 2). At least two

different enzymes are involved in the conversion of G3P to phosphatidic acid. The first acylation of G3P to form 1-acylglycerol-3-phosphate is catalyzed by G3P acyltransferase which is encoded by the *plsB* gene (30). The second fatty acid is added by another enzyme, 1-acyl-G3P acyltransferase which is encoded by the *plsC* gene (31), to form phosphatidic acid.

Mutant strains (*plsB26)* harboring a defective G3P acyltransferase were isolated as G3P auxotrophs (32). These mutants showed a 10 fold increased K_m value for G3P in in vitro acyltransferase assays (33). Therefore these mutants require a greater than normal intracellular G3P concentration for growth. The *plsB* gene is located near min 92 on the linkage map of *E. coli* (9, 34). The *plsB* gene has been cloned and sequenced (34, 35). G3P acyltransferase has been overproduced and purified to homogeneity (36). Subsequently it was demonstrated (37) that a second mutation in *plsX* (*plsX50*) is required for a *plsB26* strain to exhibit a requirement for G3P. Correction of either mutation corrected the G3P auxotrophic phenotype. The *plsX* gene was mapped near min 24 on the linkage map of *E. coli* (9, 37). The G3P acyltransferase enzyme activity measured in membranes from strains harboring the four possible configurations of *plsB* and *plsX* mutant and wild-type alleles is shown in Table 1. Neither the V_{max} nor the apparent K_m of the acyltransferase for G3P was influenced by *plsX50* or *plsX*$^+$ (37).

The gene for the second acyltransferase, 1-acyl-*sn*-glycerol-3-phosphate acyltransferase has recently been mapped near min 65 on the linkage map (38) and

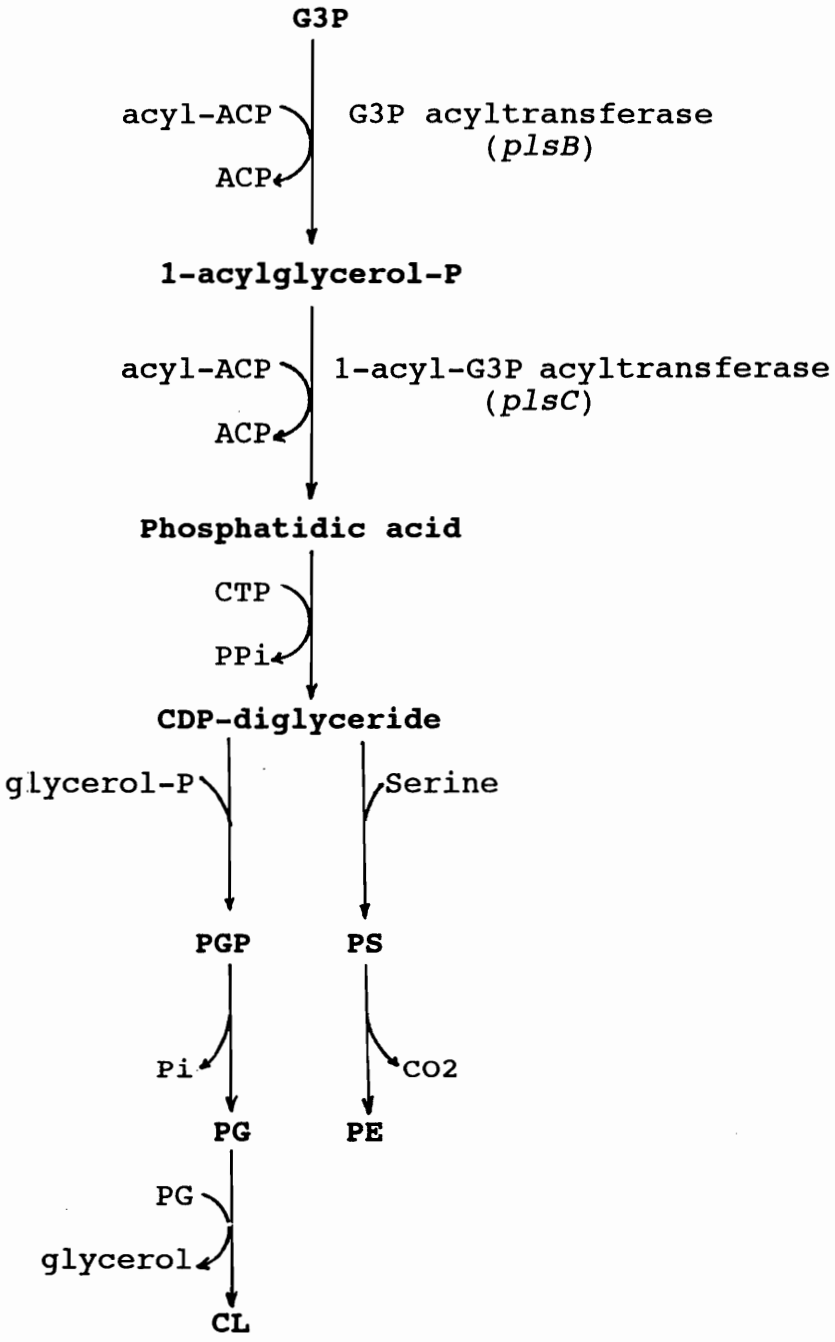

Fig. 2. Biosynthesis of phospholipids in *E. coli*: G3P; glycerol-3-phosphate, PGP;phosphatidylglycerolphosphate, PS; phosphatidylserine, PG; phosphatidylglycerol, PE; phosphatidylethanolamine, CL; cardiolipin

Table 1. Glycerol-P acyltransferase activity in membranes from wild-type and mutant strains[a]

Strain	Genotype	V_{max} (U/mg)	K_m (μM) for glycerol-P
TL48	*plsB⁺plsX⁺*	6.9	120
TL154	*plsB⁺plsX50*	6.9	120
TL145	*plsB26plsX⁺*	0.9	1,000
TL150	*plsB26plsX50*	0.7	1,000

a: Activities were determined with palmitoyl coenzyme A as the acyl donor.

*: This table is cited from reference 37

sequenced (31). Most naturally occurring phospholipids have a saturated fatty acid at position 1 and an unsaturated fatty acid at position 2 of the glycerol backbone. This acyl group asymmetry in phospholipid seems to be, although not absolute, due to the substrate specificity of both acyltransferases (2).

The branch point for phospholipid diversification occurs at CDP-diglyceride, the activated form of phosphatidic acid. The three major phospholipid species found in *E. coli,* PE (phosphatidylethanolamine) (75%), PG (phosphatidylglycerol) (15-20%), and CL (cardiolipin) (5-10%), are synthesized from CDP-diglyceride (Fig. 2) (2, 3, 39). CDP-diglyceride synthase utilizes either CTP or dCTP as a substrate, producing a mixture of dCDP-diglyceride and CDP-diglyceride. CDP-diglyceride has two metabolic fates. One fate for this compound is reaction with serine to form phosphatidylserine (PS) by PS synthase. PE is formed by decarboxylation of PS catalyzed by PS decarboxylase. In alternative pathway the CDP-diglyceride is converted to phosphatidylglycerol phosphate (PGP) by PGP synthase, then dephosphorylated by PGP phosphatase yielding PG. Cardiolipin can be made from two molecules of PG in a reaction catalyzed by CL synthase.

REGULATION OF RIBOSOMAL GENES

Ribosomes are essential machinery for protein biosynthesis in the cell. The *E. coli* ribosome is composed of 53 different ribosomal proteins (r-proteins)

(counting L7/L12 as two different proteins) and three species of rRNAs (23S, 16S, and 5S rRNAs). Each of the rRNA and r-protein is present in one copy per ribosome except for r-protein L7/L12, which is present in four copies per ribosome (40). The assembly of ribosomal components is cooperative and sequential. Binding of certain r-protein depends on prior binding or a simultaneous binding of other r-proteins (41).

Protein synthesis is essential for cellular growth. There should be some important regulatory mechanism to control the global rate of protein synthesis in relation with other regulatory mechanisms controlling cellular growth (42). The synthesis of ribosomes in *E.coli* is regulated coordinately as a function of growth rate. The amount of ribosomes produced per unit amount of cell mass increases with increasing growth rate to match the amount necessary for a given rate of translation.

Regulation of rRNA synthesis: The synthesis of the stable RNAs (rRNA and tRNA) are coordinately controlled with respect to the stringent response and growth rate (42). Guanosine tetraphosphate (ppGpp) accumulates when the stringent response (by amino acid starvation) is invoked in *rel*$^+$ but not *relA* cells. There is an inverse correlation between the level of ppGpp and the rate of rRNA synthesis during the stringent response. This stringent control of rRNA synthesis can not be observed in *relA* strains. ppGpp is proposed to influence the selectivity of RNA polymerase for stable RNA relative to other mRNA promoters, reducing

the stable RNA transcription at the level of promoter binding and transcription initiation by RNA polymerase (43).

For the growth rate-dependent control of stable RNA synthesis to adjust the concentration of ribosome to meet the cell's need for protein synthesis capacity, ribosome feedback regulation is proposed (44, 45, 46). Ribosomes which are in excess over the amount used for protein synthesis inhibit rRNA synthesis through their translational activities. That is, excess ribosomes cause a small excess in translation (or possibly just translation initiation), which in turn generates a signal leading to an eventual decrease in rRNA synthesis. Whether excess ribosomes directly inhibit rRNA synthesis or act indirectly through some intermediary regulatory molecule, such as ppGpp, is not clear.

Regulation of ribosomal protein synthesis: Each r-protein is encoded by a single gene. All of the genes for r-proteins in *E. coli* have been mapped. The majority of the r-protein (and rRNA) genes are in the one-half of the chromosome centered at the OriC. About half of the r-protein genes are clustered at the "*str-spc*" locus and the rest are scattered around the chromosome in clusters of one-to-six genes (47). In exponentially growing cells, the synthesis of r-proteins is regulated so that synthesis rates of all the r-proteins are identical and coordinately changed in response to environmental conditions. The synthesis of r-protein is coordinated very closely with the synthesis of rRNA to match the level of ribosome assembly such that there are essentially no free pools of ribosomal components

(48).

The synthesis rate of r-proteins is growth rate dependent but that of r-protein mRNA is not. Promoter activities of all the r-protein operons are not the same (42). To explain how all the r-proteins are synthesized in equimolar amount and balanced with the synthesis of the rRNAs, an autogenous translational feedback regulatory mechanism was proposed (42). The mechanisms are different for the various ribosomal protein operons, but in general, one of the r-proteins encoded by a given operon is a feedback translational repressor of most or all genes in the same operon. This regulatory r-protein can bind to its own mRNA or to rRNA at an early stage in ribosome assembly. Some significant structural homologies between r-protein mRNA and rRNA have been identified (47). When the production of this protein exceeds that needed for assembly into ribosomes, the protein binds to the leader part of its own mRNA and acts as a translational repressor. Many of the r-protein genes are organized into polycistronic operons. There is evidence indicating that the translation of downstream genes in the regulatory unit is dependent on the translation of the first gene in the unit via translation coupling between the neighboring genes (42). Interrupted translation of an upstream gene often leads to premature termination of mRNA synthesis (49) and also the stimulation of mRNA degradation (42). According to this scheme, blocking of the translation of the first gene in r-protein operon would result in the inhibition of translation of downstream genes. This translational feedback

mechanism ensures the balanced synthesis of all r-proteins by coupling the synthesis with the process of ribosome assembly. Translational feedback regulation also appears to be important in the growth rate-dependent control of r-protein synthesis by way of a secondary consequence of the control of rRNA synthesis (42).

Although an autogenous translational feedback mechanism functions in many r-protein operons (42, 50-53), there are examples of transcriptional regulation. The transcriptional organization and regulation of r-protein operons are quite complex (47). Some of the operons have multiple promoters. Also, there are internal promoters located within structural genes in several operons. Transcripts are processed in some operons by RNase III. Transcription attenuation sites are found in some operons. In the S10 operon, in addition to the L4-mediated translational feedback mechanism identified in vitro, excess L4 protein leads to transcription termination at the transcription terminator (attenuator) located upstream of the first structural gene in the operon. So, the primary effect of overproduced L4 protein in vivo is the reduction of mRNA synthesis (54, 55).

The role of a transcriptional control mechanism in the stringent control of r-protein synthesis has also been investigated (42). During the stringent response, both the amount and the synthesis rate of r-protein mRNA are reduced, and it has been suggested that such transcriptional control might be mediated by ppGpp. The stringent response of S10 operon is mediated at the promoter and does not require

the target for L4-mediated autogenous control (56). However, experiments with the L11 operon suggest that control by the stringent response in this operon is accomplished by the L1-mediated autogenous translational regulation (57). So, there is a possibility that stringent control of r-protein synthesis can be a secondary consequence of stringent control of r-RNA synthesis and can be accomplished through the translational feedback mechanism. The exact mechanism responsible for the stringent control of r-protein synthesis has not been resolved, but it is possible that different r-protein operons use different regulatory strategies for regulating the expression of their genes.

Many of the ribosomal protein operons contain other genes for nonribosomal proteins which are generally considered to be essential for growth (42, 47). These proteins are involved in such functions as replication, transcription, translation, tRNA maturation and protein secretion. For instance, the genes for DNA primase, α, β, β' and σ subunits of RNA polymerase, translation initiation factor IF3, translation elongation factor EF-G and EF-Ts, tRNA (m'G) methyltransferase, phenylalanyl-tRNA synthase, protein secretion factor SecY, and polynucleotide phosphorylase are included in some of the r-protein operons. While this association is not completely understood, the organization of the genes into polycistronic transcription units might be related to their coordinate regulation.

However, synthesis of nonribosomal proteins is not always coordinately regulated with those of cotranscribed r-proteins. Multiple promoters, attenuation

and processing of transcripts may play important roles in the differential expression of nonribosomal proteins in r-protein operons. Synthesis of β and β' RNA polymerase subunits of the L10 operon is uncoupled from that of r-proteins by transcription attenuation and mRNA processing (58). The gene for the α-subunit of the RNA polymerase is regulated independently of the r-protein genes in the α-operon, probably by regulation at the level of mRNA translation (59). The synthesis of tRNA methyltransferase protein (TrmD) responded differently from that of the cotranscribed two r-proteins during the stringent response, although accumulation of the mRNA molecule responded similarly to the rate of synthesis of two r-proteins. This differential and noncoordinate expression in the *trmD* operon is suggested to be achieved by regulation at the level of mRNA translation (60).

COORDINATION OF PHOSPHOLIPID AND MACROMOLECULAR SYNTHESIS

The rate of phospholipid synthesis in *E. coli* seems to be regulated at an early stage of fatty acid biosynthesis (16). Control of phospholipid synthesis at the level of fatty acid supply appears to be logical, because fatty acids in *E. coli* are primarily used for phospholipid synthesis. Furthermore, 94% of the ATP required for phospholipid synthesis is expended in the biosynthesis of fatty acids. However,

the precise step of fatty acyl-ACP synthesis that is regulated has not been identified. β-ketoacyl-ACP synthase III is proposed to be a regulatory enzyme of fatty acid biosynthesis in bacteria (18). Comparison of acyl-ACP pools during logarithmic growth demonstrated that comparable concentrations of acetyl-ACP, malonyl-ACP, and nonesterified ACP were found while long-chain acyl-ACP comprised a minor fraction, suggesting that supply of acyl-ACP limits the rate of phospholipid synthesis. Acetyl-CoA carboxylase which catalyzes the first committed step of fatty acid biosynthesis might be another target of potential regulators like in the mammalian case.

Cellular metabolism in exponentially growing cells is well coordinated and balanced (16). The regulatory mechanisms for the coordination have not been proved, but some progress has been made using amino acid starvation to perturb the metabolic system. The stringent response in *E. coli*, elicited by deprivation of a required amino acid, results in a strong inhibition of the synthesis of stable RNA, ribosomal proteins, and translation elongation factors (61). These regulatory effects are mediated by ppGpp that accumulates during stringent control of wild-type (*rel*$^+$) but not *relA* mutant strains.

It has been shown that the rate of phospholipid synthesis decreases after amino acid starvation in *rel*$^+$ but not *relA* strains (135, 136). Measurement of both the ppGpp concentration and the rate of phospholipid biosynthesis shows that there is a quantitative correlation between the decreased rate of phospholipid synthesis

and increased ppGpp concentration (62). ppGpp appeared to be an effector of stringent control of phospholipid synthesis in *E. coli*, and the effects of ppGpp on several enzymes of phospholipid synthesis were examined in vitro, but no significant inhibition was observed (29). G3P acyltransferase was inhibited in vitro, but acyltransfer from acyl-CoA is affected whereas acyl transfer from the in vivo acyl donor, acyl-ACP is unaffected (63, 64). Even though the identity of the enzyme(s) affected is still unproven, two sites are proposed for the stringent control of phospholipid synthesis in *E. coli* (2). Regulation at an early step in fatty acid biosynthesis controls phospholipid production when fatty acids are synthesized de novo. The other possibility is regulation at the G3P acyltransferase step when exogenous fatty acids are utilized for phospholipid synthesis.

MATERIALS AND METHODS

Materials: Restriction endonucleases were purchased from U.S. Biochemical, Promega, New England Biolabs, Pharmacia LKB Biotechnology Inc. or BRL. Calf intestinal alkaline phosphatase, Sequenase DNA sequencing kit, Prime a gene random labeling kit, X-gal and IPTG were obtained from U.S. Biochemical. T4 DNA ligase, AMV reverse transcriptase, S1 nuclease and the RNA molecular weight standards were from BRL. Magic Minipreps DNA purification kit was from Promega. Geneclean II kit for DNA extraction was obtained from BIO 101. GeneAmp PCR kit and GeneAmp PCR system 9600 were from Perkin Elmer Cetus. Immobilon-N and Immobilon-P transfer membranes were purchased from Millipore. Du Pont-New England Nuclear supplied [α-^{35}S]dATP (1000-1500 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol) and [^{35}S]methionine (1176 Ci/mmol). Amersham supplied [2-^{14}C]malonyl coenzyme A (50 mCi/mmol). Yeast extract, tryptone, casamino acids and bacto agar were from Difco Laboratories. Various salts, glucose, glycerol, chloroform, 2-propanol, isoamyl alcohol, phenol, agarose and acrylamide were from Fisher Scientific Company. Antibiotics, amino acids, protein molecular weight standards, ONPG and MOPS were obtained from Sigma Chemical Company. Oligonucleotides were synthesized using an Applied Biosystems model 381A DNA synthesizer and purified using oligonucleotide

purification cartridges (Cruachem) as recommended by the manufacturer. Oligonucleotides used in this study are listed in Table 2.

Bacterial strains and growth media: The bacterial strains used in this study were derivatives of *E. coli* K-12 and are listed in Table 3. Strain DH5αF' was used as host during construction of recombinant plasmids or propagation of recombinant M13 phages. LB (66) medium was routinely used to grow bacteria and to propagate phages. Terrific broth (67) was used for the isolation of plasmids. For the maxicell technique and complementation tests, AB minimal medium (68) was used. Supplements were: thiamine, 4 μg/ml; glucose, 0.2%; G3P, 0.05%; Amp, 50 μg/ml; casamino acids, 0.05% unless indicated otherwise. YT top agar (69) was used for the transfection of phage DNA. When supplementation of antibiotics was required, the concentrations were as follows: ampicillin, 100 μg/ml; tetracycline, 20 μg/ml; spectinomycin, 50 μg/ml. When phenotypic expression of β-galactosidase was tested, 40 μg/ml of X-gal was added to the medium.

Agarose gel electrophoresis: DNAs were digested with restriction endonucleases in appropriate buffers recommended by suppliers and incubated at 37 °C for 2 hr to overnight. Restriction fragments were separated on a horizontal flat bed gel containing 0.7 to 1.3% low melting or high melting temperature agarose. Electrophoresis was carried out at 8-12 V/cm in TBE (0.05 M Tris, 0.05 M Borate, 0.01 M EDTA) buffer containing 0.5 μg/ml of ethidium bromide. High melting

Table 2. Synthetic oligonucleotides used in this study

Serial number #	Nucleotide sequence (5′ → 3′)	Position in sequence(Fig.3)
238804	CCTTCATCCATCGGAGC	70 to 54[1]
225522	GCATCCCCTGCACTGCG	381 to 365[1]
253436	CATTTCCGTCACCTGAGC	431 to 414[1]
231121	AAACAGCGTAATGCCAA	324 to 340[1]
227252	CCTTCACTCTTAAAGCTA	343 to 360[1]
219668	CCGCGCAGTGCAGGG	362 to 376[1]
235857	CGCCAGTCCAGTATAGA	486 to 502[1]
110319	GCGTATTGATGATAGCC	827 to 843[1]
221812	GGCAACCACAGGCTGCC	943 to 959[1]
258961	AAAGTTAATATGCGCGC	985 to 1001[1]
276086	GCATAGGGCGCGCATAT	1008 to 992[1]
272697	AGATACCCTGGTAATCAAGGCG	1080 to 1059[1,2]
219623	CGAATTCGTTAACCTCAATCGG	1347 to 1326[1,2]
236290	CCGCTTCGGACACTTCACAGTGTT	1446 to 1423[2]
288603	TGGCTAATACGGCAAATGGG	1509 to 1490[2]
111277	AAGGTCCATGGCCGTACAAC	1571 to 1590[3]
207919	GGTCAGCGCGTCATGGGAACGACG	1646 to 1623[2]
091568	ATGAAGATCTGACCGCAGTCACCAGCC	1641 to 1659[3]
056025	CCTCACTAAGCTTGGC	1770 to 1782[4]
091971	GAAGGATCCGCCTGCAATGCTGCAGGC	1911 to 1894[3]
027240	GCATTGAACTCCCACGA	2093 to 2077[1]

310398	TGACACAGGCTTGCGCT	2141 to 2125[1]
016509	ATAGCCGATATAATTGA	2473 to 2457[1]
054362	GCAATTCGCTGAGGAAC	2829 to 2813[1]
316348	CCCAGCTGGTTTTGAGCT	2851 to 2868[1]
219821	GTGCGTAGCAGAAGTCGTTG	3224 to 3205[3]
043682	CATGCATATGAGCACTATCGAAGAACG	5825 to 5845[3]
056178	CTACAAGCTTCACTTACGCCTGGTGGC	6049 to 6067[3]
019049	CTATAGTGAGTCGGGATC	pTL61T primer[5]

1. Primers used for sequencing
2. Primers used for primer extension analysis
3. Primers used for PCR reactions
4. Primer used for S1 mapping
5. Primer used for sequencing of pTL61T clones

Table 3. Bacterial strains used in this study

Strain	Genotype	Source or Reference
DH5αF′	(F′ϕ80*dlacZΔM15) endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1 Δ(lacZYA-argF)U169*	70
HB101	*supE44 hsdS20*($r_B^-m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	71
ECL8	HfrC *phoA8 glpD3 glpR2 relA1 spoT1 pit-10 fhuA22 ompF627 fadL701* (λ)	72
MC4100	F⁻ *araD139 Δ(argF-lac)U169 rpsL150 deoC1 relA1 ptsF25 flbB5301 rbsR*	66
SH305	MC4100 Δ*glpD102 recA1 srl*::Tn*10*	73
HS3018	MC4100 *malT*^{c}-1 Δ*malE444*	74
LA-12	F⁺ *ptsG21 thi-1 relA1 spoT1*	75
TL73	MC4100 *glpR2 recA1*	76
TL85	ECL 8 *glpK*$^+$ *plsB26 plsX50 gyrA*	37
TL129	TL85 *zjb-729*::Tn*10 plsB*$^+$	37
XA100	F⁻ *ara Δ(lacpro) nalA metB argE-am rif thi*	77
TL400	XA100 *glpD3 glpR2 plsB26 plsX50 recA56*	XA100 deritive[1]
TL212	HS3018 *zce-726*::Tn*10*	37
TL226	HS3018 *zce-728*::Tn*10*	37
TL248	LA-12 *zce-727*::Tn*10*	37
L48	F⁻ *fabD89 gltA5 rpsL20 thi-1 lct-1 ara-14 lacY1 galK12* xyl-5 *mtl-1 tsx-57 tfr-5 supE44* (λ)	78
LW48	L48 *recA1 srl*::Tn*10*	P1.SH305→L48
BL21(DE3)	F⁻ *hsdS gal* (λ*cI857 ind1 Sam7 nin5 lacUV5*-T7 gene 1)	79
TD105	*metB leu hisA lacY malA xyl mtl rpsL dcd-1 cdd-50 tmk-1*	80

1; TL400 was constructed by using a series of P1 transductions essentially as described by Larson *et al.*(37).

temperature agarose gels were run at room temperature and low melting temperature agarose gels were run at 4 °C.

Extraction of DNA fragments and ligation: DNA bands were extracted from low melting temperature agarose gels using phenol/chloroform (81). Geneclean II was also used to extract DNA bands from high melting temperature agarose gels following the instruction supplied by the manufacturer. Ligation was performed by extraction of insert and vector DNA in a tube, adding ligase buffer and 1 unit of T4 DNA ligase in a 15 μl reaction, and incubation overnight at 18 °C. For blunt end or single restriction site ligation, vector DNA was usually dephosphorylated using calf intestinal alkaline phosphatase (82). After ligation, the reaction mixture was used directly for transformation or transfection of cells.

Construction of recombinant DNA: Plasmids and M13 clones were constructed by extraction of restriction fragments from agarose gels followed by ligation into appropriately digested vectors. pBluescript KS$^+$ (Stratagene), pGEM-4 (Promega), pGEM-3Z (Promega), pCB267 (83), pTL61T (84), pSH76 (85), pT7-7 (87), M13mp19 and M13mp18 (86) were used as vectors. A description of each clone constructed is shown in Table 4.

Transformation and Transfection: Cells were made competent by treatment with

Table 4. Plasmids and M13 clones constructed in this study

Plasmids or phages	Insert (Position in the sequence)	Source and Restriction sites	
		to cut insert	to cut vector
pBluescript KS$^+$			
pWO102	XhoI-NsiI(1215-3319)	λ_{235} (XhoI-NsiI)	pBS (XhoI-PstI)
pWO103	[1]HIII-NsiI(1770-3319)	pWO102(HIII-BamHI)	pBS (HIII-BamHI)
pWO104[2]	HIII (1770-4042)	λ_{235} (HIII)	pBS (HIII)
pWO105	XhoI-NruI(1215-2775)	λ_{235}(XhoI-NruI)	pBS (EcoRV-SalI)
pWO106[3]	HIII-NruI(1770-2775)	pWO104(BamHI-NruI)	pBS (BamHI-EcoRV)
pWO107	EcoRI-NruI(1915-2775)	pWO106(EcoRI-SalI)	pBS (EcoRI-SalI)
pWO108	HIII-SspI(1770-2381)	pWO104(BamHI-SspI)	pBS (BamHI-SmaI)
pWO109	XhoI-PstI(1215-1896)	pWO105(XhoI-PstI)	pBS (XhoI-PstI)
pWO110	XhoI-PstI(1215-2009)	pWO105(XhoI-PstI[5])	pBS (XhoI-PstI)
pWO112[6]	PstI (1-1896)	λ_{235} (PstI)	pBS (pstI)
pWO113	NruI-SalI(2775-4803)	λ_{235} (NruI-SalI)	pBS (EcoRV-SalI)
pWO114	NruI-HIII(2775-4042)	pWO113(BamHI-HIII)	pBS (BamHI-HIII)
pWO115[7]	PstI-NsiI(2009-3319)	pWO113(NsiI-PstI)	pBS (PstI)
pWO116[8]	PstI-NsiI(2009-3319)	pWO113(NsiI-PstI)	pBS (PstI)
pWO118	SalI-KpnI(4803-5300)	pWO610(SalI-EcoRI)	pBS (SalI-EcoRI)
pWO121	EcoRI-HIII(3569-4042)	pWO114(EcoRI-SalI)	pBS (EcoRI-SalI)
pWO122	EcoRI-SspI(1915-2381)	pWO603(EcoRI-SspI)	pBS (EcoRI-SmaI)
pWO123	HIII-HincII(1770-2491)	pWO103(HIII-HincII)	pBS (HIII-HincII)
pWO124	SalI-HIII(711-1770)	pWO602(HIII-KpnI)	pBS (HIII-KpnI)
pWO125	XhoI-HincII(1215-2491)	pWO404(BamHI-HIII)	pWO123(BamHI-HIII)
pWO126[4]	HIII-NruI(1770-2775)	pWO606(HIII-KpnI)	pWO124(HIII-KpnI)
pWO127	PstI-XmnI(4487-5079)	pWO618(PstI-XmnI)	pWO115 (PstI-SmaI)
pCB267			
pWO401	SalI-HIII(711-1770)	pWO112(SalI-HIII)	pCB (SalI-HIII)
pWO402[9]	SalI-XhoI(711-1215)	pWO112(SalI-XhoI)	pCB (SalI)
pWO403[10]	SalI-XhoI(711-1215)	pWO112(SalI-XhoI)	pCB (SalI)
pWO404	XhoI-HIII(1215-1770)	pWO105(XhoI-HIII)	pCB (SalI-HIII)
pWO407	XhoI-SphI(1215-1618)	pWO801(BamHI-HIII)	pCB (BamHI-HIII)
pWO408	XhoI-PstI(1215-1896)	pWO109(XhoI-BamHI)	pCB (SalI-BglII)
pWO409	XhoI-PstI(1215-2009)	pWO110(XhoI-BamHI)	pCB (SalI-BglII)
pWO412	S3A[13]-TaqI(1543-2121)	pWO805(SmaI-HIII[5])	pCB (SmaI-HIII)
pWO416	S3A-PstI(3777-4487)	pWO609(S3A-HIII[5])	pCB (BamHI-HIII)
pWO417	EcoRI-HIII(3569-4042)	pWO121(BamHI-HIII)	PCB (BamHI-HIII)

pWO418	NruI-NsiI(2775-3319)	pWO102(NruI-XbaI)	pCB (SmaI-XbaI)
pWO419	NsiI-PstI(3319-4487)	pWO116(SalI-BamHI)	pCB (SalI-BglII)
pWO420	EcoRI-SspI(1915-2381)	pWO122(SalI-BamHI)	pCB (SalI-BglII)
pWO421	SspI-NruI(2381-2775)	pWO107(SspI-HIII)	pCB (SmaI-HIII)
pWO422	EcoRI-NruI(1915-2775)	pWO107(HIII-BamHI)	pCB (HIII-BamHI)
pWO423	SspI-EcoRI(2381-3569)	pWO111(SspI-HIII)	pCB (SmaI-HIII)
pWO424	SalI-KpnI(4803-5300)	pWO118(SalI-BamHI)	pCB (SalI-BglII)
pWO425	PstI-XmnI(4487-5079)	pWO127(EcoRV-BamHI)	pWO402(SmaI-BglII)

pGEM-4

pWO601	SalI-NsiI(711-3319)	λ_{235} (SalI-NsiI)	pWO603(XhoI-PstI)
pWO602	SalI-HIII(771-1770)	pWO601(HIII)	pWO601(HIII)
pWO603	XhoI-NsiI(1215-3319)	pWO102(KpnI-XbaI)	pGEM-4(KpnI-XbaI)
pWO604	HIII-NsiI(1770-3319)	pWO103(KpnI-XbaI)	pGEM-4(KpnI-XbaI)
pWO605	EcoRI-NsiI(1915-3319)	pWO604(EcoRI-BamHI)	pGEM-4(EcoRI-BamHI)
pWO607[11]	NruI-SalI(2775-4803)	pWO113(KpnI-XbaI)	pGEM-4(KpnI-XbaI)
pWO608	EcoRI-SalI(3569-4803)	pWO113(EcoRI-SalI)	pGEM-4(EcoRI-SalI)
pWO610	SalI-KpnI(4803-5300)	λ_{235} (SalI-KpnI)	pGEM-4(SalI-KpnI)
pWO611	NruI-HIII(2275-4042)	pWO113(BamHI-HIII)	pGEM-4(BamHI-HIII)
pWO612	S3A-SalI(3777-4803)	pWO608(S3A-SphI)	pGEM-4(BamHI-SphI)
pWO613	NruI-NruI(2775-5790)	λ_{235} (NruI)	pGEM-4(SmaI)
pWO615	XhoI-PstI(1215-1896)	pWO102(XhoI-PstI)	pWO603(XhoI-PstI)
pWO616	XhoI-PstI(1215-2009)	pWO102(XhoI-PstI[5])	pWO603(XhoI-PstI)
pWO617	PstI-EcoRI(1-1341)	pWO112(PstI-EcoRI)	pGEM-4(PstI-EcoRI)
pWO618	EcoRI-KpnI(3569-5300)	pWO613(EcoRI-KpnI)	pGEM-4(EcoRI-KpnI)
pWO619	PstI-HIII(1-1770)	pWO112(PstI-HIII)	pGEM-4(PstI-HIII)
pWO620[12]	NruI-SalI(2775-4803)	λ_{235}(NruI-SalI)	pGEM-4(SmaI-SalI)
pWO621	PstI-SalI(1-711)	pWO617(PstI-SalI)	pWO615(PstI-XhoI)
pWO622	EcoRV-SalI(-xxx-711)	λ235(EcoRV-SalI)	pWO612(SmaI-SalI)
pWO623	HIII-NsiI(1771-3319)	pWO102(HIII-BamHI)	pWP612(HIII-BamHI)
pWO624	SphI-S3A(1618-3194)	PCR of TL85(*plsX50*) (SphI-S3A)	pGEM-4(SphI-BamHI)
pWO625	PstI-XhoI(1-1215)	pWO619(EcoRI-XhoI)	pWO608(EcoRI-SalI)

pGEM-3Z

pWO801	XhoI-SphI(1215-1618)	pWO105(XhoI-SphI)	pGEM3Z(SalI-SphI)
pWO802	XhoI-HinpI(1215-1476)	pWO801(BamHI-HinpI)	pGEM3Z(BamHI-AccI)
pWO804	SalI-SphI(711-1618)	pWO124(KpnI-SphI)	pGEM3Z(KpnI-SphI)
pWO805	S3A-TaqI(1543-2121)	pWO105(S3A-TaqI)	pGEM3Z(BamHI-AccI)
pWO806	SalI-HIII(711-1770)	pWO602(KpnI-HIII)	pGEM3Z(KpnI-HIII)

pTL61T

pWO902	SalI-HIII(711-1770)	pWO112(SalI-HIII)	pTL61T(XhoI-HIII)
pWO903	SalI-PstI(711-1896)	pWO102(SphI-PstI)	pWO902(SphI-PstI)
pWO904	SalI-PstI(711-1896)	pWO624(SphI-PstI[5])	pWO902(SphI-PstI)
pWO905	SalI-PstI(711-2009)	pWO102(SphI-PstI)	pWO902(SphI-PstI)
pWO906	SalI-PstI(711-2009)	pWO624(SphI-PstI[5])	PWO902(SphI-PstI)

pSH76

pWO951	*plsX*[+](1641-1911)	PCR of pWO105 (BglII-BamHI)	pSH76 (BamHI)
pWO952	*plsX50*(1641-1911)	PCR of pWO624 (BglII-BamHI)	pSH76 (BamHI)

M13 Phages

19W-1	HIII-SalI(-488-711)	λ_{235}(HIII-SalI)	mp19(HIII-XhoI)
19W-2	HaeIII-XhoI(170-1215)	pWO617(HaeIII-XhoI)	mp19(SmaI-SalI)
18W-3	XhoI-HinpI(1215-1476)	pWO802(BamHI-HIII)	mp18(BamHI-HIII)
18W-4	XhoI-SphI(1215-1618)	pWO105(XhoI-SphI)	mp18(SalI-SphI)
19W-5	XhoI-NruI(1215-2775)	pWO102(XhoI-NruI)	mp19(SalI-SmaI)
19W-6	SphI-S3A(1618-3194)	PCR of ECL8(SphI-S3A)	mp19(SphI-BamHI)
19W-7	SphI-S3A(1618-3194)	PCR of TL85(SphI-S3A)	mp19(SphI-BamHI)
18W-8	HIII-S3A(1770-3194)	PCR of ECL8(HIII-S3A)	mp18(HIII-BamHI)
18W-9	HIII-S3A(1770-3194)	PCR of TL85(HIII-S3A)	mp18(HIII-BamHI)
19W-10	PstI-NruI(2009-2775)	PWO103(PstI-NruI)	mp19(PstI-SmaI)
19W-11	NsiI-HaeIII(3319-3800)	pWO113(NsiI-HaeIII)	mp19(PstI-SmaI)
19W-12	HIII-XhoI(4042-4857)	λ_{235}(HIII-XhoI)	mp19(HIII-SalI)
19II-1	HIII-TaqI(1770-2121)	pWO104(HIII-TaqI)	mp19(HIII-AccI)
19PE	PstI-EcoRI(1-1341)	pWO112(PstI-EcoRI)	mp19(PstI-EcoRI)

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1. HIII represents *Hind*III.
2. Unknown source of *Nru*I site was created downstream of the *Hind*III(nt 4042).
3, 4. Expression of the *plsX* is the same (3) or the opposite (4) direction with the *lacZ*.
5. Fragment was obtained by partial digestion.
6. Unknown source of the *Pst*I fragment (1.7kb) was inserted into the *Pst*I (nt 1) site.
7, 8. Expression of the *fabD* is the opposite (7) or the same (8) direction with the *lacZ*.
9, 10. Transcription goes from *Sal*I to *Xho*I (9) or from *Xho*I to *Sal*I (10) direction.
11, 12. Expression of the *fabH* is the same (11) or the opposite (12) direction with the T7 promoter
13. S3A represents *Sau*3A

calcium chloride (66) and stored at -70 °C after adding glycerol to be a final concentration of 15%.

Transformation was done using a standard protocol (66). A 2 to 8 μl aliquot of a ligation mixture or 1-200 ng of plasmid DNA was mixed with 100 μl of competent cells and incubated on ice for 40 min. After 2 min of heat shock at 42 °C, 1 ml of LB medium was added, incubated at 37 °C for 1 hr, and plated on a selective medium.

Transfection was performed by mixing competent cells with recombinant RF M13 DNA. Heat shock was at 42 °C for 2 min, and 2 ml of 45 °C molten top agar was added. The mixture was spread on solid LB X-gal medium.

Isolation of DNA: Phage lambda DNA and chromosomal DNA from bacterial cells were isolated using the methods described by Silhavy *et al.* (66). Plasmid DNA was isolated using the miniprep technique (88) or using the Magic Minipreps kit. Plasmid DNA isolated from 1.5 ml of terrific broth (67) culture was dissolved in 50 μl TE. Aliquots of 1-3 μl or 10-20 μl were used for the restriction mapping or for the sequencing reactions respectively. M13 single stranded DNA was isolated by the procedure described in the Sequenase manual (U.S. Biochemical).

DNA sequencing: Double stranded plasmids or single stranded M13 clones were used as templates for the dideoxynucleotide chain termination method of Sanger

et al. (89). Universal primer or synthetic oligonucleotides (Table 2) were used as primers. Sequencing reactions were made following the protocol provided in the Sequenase kit and were analyzed on gels containing 6% polyacrylamide and 7 M urea. Sequencing gels were run in TBE buffer for 1.5 h to read up to 250 nucleotides and for 5 h to read up to 350 nucleotides.

SDS gel electrophoresis: Protein samples were dissolved in 2 x loading buffer to an approximate protein concentration of 1 μg/μl. Thirty μl samples were analyzed on 12-18 % SDS-polyacrylamide gels using the buffer system of Laemmli (90). Protein bands were visualized with Coomassie brilliant blue.

Analysis of protein synthesis in maxicells: The maxicell technique adapted by Silhavy *et al.* (66) was used to identify plasmid-encoded proteins. Plasmids harboring each ORF (Table 5) were introduced into strain HB101. Overnight cultures were diluted into AB minimal medium with 0.4% glucose, 1% casamino acid, 30 μg/ml thiamine and 100 μg/ml Amp. Cells were grown until the A_{600} reached 0.7-1.0. The cells were irradiated with germicidal UV light at 25 cm distance for 40 sec. After shaking for 30 min at 37 °C in the dark, freshly made cycloserine was added to a final concentration of 200 μg/ml. After overnight incubation in the dark, cells were washed with AB minimal medium and suspended in AB minimal medium supplemented with 0.4% glucose. Cells were incubated

at 37 °C for 1 h, and then [^{35}S]methionine was added (30 μCi/ml). Incubation was at 37 °C for 30 min. After washing with 10 mM Tris-HCl (pH 8), 1 mM EDTA, 100 mM NaCl, cells were resuspended in 2 x SDS gel loading buffer. Samples (30 μg) were boiled for 5 min before analysis on 12-18% SDS polyacrylamide gels.

Complementation and Recombination test: In order to localize the *plsX* gene, plasmids containing a series of restriction fragments were introduced into strain TL85 (*plsX50*) or TL400 (*plsX50, recA56*) by selection on LB Amp medium supplemented with glucose (0.4%). Ampicillin resistant colonies were picked and streaked on three test plates: glucose Amp minimal, glucose Amp plus G3P minimal, and LB Amp glucose (0.4%). In addition, minimal media for strain TL400 were supplemented with methionine (45 μg/ml), proline (230 μg/ml), arginine (126 μg/ml) and casamino acids. All colonies grew well on minimal glucose medium with G3P and on LB Amp glucose medium. Colonies growing confluently on glucose minimal medium indicated complementation of the *plsX50* mutation in TL400 (TL85). Colonies forming revertant papillae on glucose minimal medium indicated rescue of the *plsX50* mutation by recombination in TL85.

In order to localize the *fabD* gene, strain L48 (*fabD89*) or LW48 (*fabD89, recA1*) was transformed with a series of plasmids by selection on LB Amp medium after overnight incubation at 30 °C. Each single colony was streaked on duplicate

LB Amp plates. One plate was incubated at 30 °C and the other at 42 °C overnight to score the correction of temperature sensitive *fabD89* mutation. The possibility that *tmk*, which encodes thymidine monophosphate kinase, is equivalent to *orfX, orfY, g30k*, or *plsX* was tested by introducing appropriate plasmids into strain TD105 (*tmk-1*). Ampicillin resistant colonies were selected on LB Amp medium after overnight incubation at 30 °C and streaked on duplicate LB Amp plates. One plate was incubated at 30 °C and the other at 42 °C to score the correction of the temperature sensitive *tmk-1* mutation on rich media.

Genetic transduction using P1vir: Preparation of a P1 lysate of SH305 and genetic transduction were carried out as described by Silhavy *et al.* (66).

Strain LW48 (*fabD89 recA1*) was made using a P1 lysate of strain SH305 (*srl*::Tn*10 recA1*) as donor and strain L48 (*fabD89*) as recipient. To screen for cotransduction of the Tn*10* and *recA1*, tetracycline resistant colonies were streaked on duplicate plates, and one was irradiated with germicidal UV light at 25 cm distance for 20 sec. After overnight incubation at 30 °C in the dark, UV sensitive cells were presumed to contain the defective recA1 allele.

To measure the cotransduction frequency of the *tmk* gene with other known Tn*10* genetic markers, transduction was performed using strain TD105 (*tmk-1*) as recipient and P1 lysates of strains TL212, TL226, and TL248 as donors. After adding 2.5 ml of molten top agar (45 °C) to the mixture of recipient and P1 lysate,

the mixture was plated on LB medium containing 20 mM sodium citrate and 5 μg/ml tetracycline and incubated overnight at 30 °C. Tetracycline resistant colonies were selected and streaked on glucose minimal medium containing 3 μg/ml tetracycline with or without 20 μg/ml TddR. These plates were incubated overnight at 30 °C. Based on the resistance of the *tmk-1* strain to TddR, the TddR sensitive colonies were counted to determine the cotransduction frequency of *tmk* with the Tn *10* genetic markers.

Purification of acyl carrier protein: Strain MC4100 was grown in 24 l of terrific broth overnight in a 30 l fermenter and harvested by using a cell concentrator producing 175 g of cells (wet weight). Acyl carrier protein was purified following the method of Rock *et al.* (91) except that cells were broken using a French pressure cell instead of using lysozyme and DEAE-sephadex was used instead of DEAE-cellulose.

Preparation of cell extracts and Assay of malonyl coenzyme A-acyl carrier protein transacylase (MTA) : Strain L48 harboring pWO618 or pGEM-4 vector was grown overnight in LB Amp medium at 30 °C to stationary phase. Cells were harvested by centrifugation and washed with buffer containing 50 mM potassium phosphate (pH 6.8), 1 mM EDTA and 10 mM DTT. The washed pellet was resuspended in the same buffer and lysed in a French pressure cell at 16,000-

20,000 p.s.i. The lysate was centrifuged at 8,000 rpm for 10 min using a Beckman JA-20 rotor to remove unbroken cells.

MTA activity was determined by measuring the formation of acid insoluble [^{14}C]malonyl-ACP resulting from the malonyl transfer from [^{14}C]malonyl-CoA to ACP. The assay conditions described by Ruch *et al.* (92) were used with certain modifications. The incubation mixture contained 50 mM potassium phosphate (pH 6.8), 1 mM EDTA, 10 mM DTT, 100 μM reduced ACP, 100 μM [2-^{14}C]malonyl-CoA (specific activity 10 μCi/μmol), and crude extract containing 0 to 15 μg of protein in a final volume of 40 μl. A mixture of ACP, DTT, and buffer was preincubated at 37 °C for 30 min to insure complete reduction of the ACP, then aliquoted into the assay tubes containing [2-^{14}C]malonyl-CoA and water. The assays were started by the addition of crude extracts. Reaction mixtures were incubated at 25 °C for 5 min and then deposited on Whatman 3MM filter discs. The discs were washed with three changes (20 min each) of 10 to 5 to 1% of ice-cold trichloroacetic acid. In each washing, 20 ml of trichloroacetic acid was used for each filter.

Determination of protein concentration: Protein concentration was determined by the Bradford method (93) using BSA as the standard.

Overexpression of polypeptides: The T7 RNA polymerase expression system of

Studier and Moffat (79) was used for the overexpression of polypeptides. Appropriate restriction fragments were cloned into pBS^+ or pGEM-4 downstream of the T7 promoter. Strain BL21(DE3) was transformed with recombinant plasmid and grown in 3 ml of LB Amp medium until the A_{600} reached 0.7 to 1.0. The culture was divided into two tubes. IPTG (0.4-1 mM) was added to one of the tubes, and growth was continued for 2.5 h. Cells were harvested and suspended in 2 x SDS sample loading buffer. Overexpression of polypeptides was identified by analysis of the IPTG induced and non-induced cells on adjacent lanes of SDS-polyacrylamide gels.

Purification of* plsX-lacZ *fusion protein: Purification of the *plsX-lacZ* fusion protein was carried out using affinity chromatography (94). Strain TL73 (pWO951) was grown in 400 ml of LB Amp medium at 30 °C until the A_{600} reached 0.6. Four hundred ml of fresh LB Amp medium (65 °C) was added and growth was continued for 4 more hours at 42 °C. β-galactosidase activity was measured at each step to monitor the fusion protein. Cells were harvested by centrifugation at 5,000 rpm with a Beckman JA 14 rotor at 4 °C for 10 min, washed in 100 ml of buffer B (0.05 M Tris-HCl, pH 7.6, 0.1 M NaCl, 0.01 M $MgCl_2$), and stored at -20 °C. Frozen cells were thawed, suspended in 13 ml of buffer B, and broken by passage through a French pressure cell. The unbroken cells were removed by centrifugation at 8,000 rpm in a Beckman JA 20 rotor for

15 min (4 °C). The supernatant was centrifuged at 40,000 rpm in a rotor at 4 °C for 100 min. The supernatant was mixed with 5 ml bed volume of *p*-aminophenyl β-D-thiogalactopyranoside agarose which was equilibrated with buffer B at room temperature. The mixture was rotated in a roller drum at low speed for 1.5 h at 4 °C and then centrifuged at 1,000 rpm with a Beckman JA 20 rotor for 1 min at room temperature. The pellet was washed with 25 ml of buffer B, suspended in 10 ml of buffer B and poured into a column. The column was washed with 150 ml of buffer B until the A_{280} of the eluent declined to 0.078. Elution was achieved with 0.1 M sodium borate, pH 10. β-galactosidase activity was highest in the fractions where the protein concentration was highest. Fractions having high β-galactosidase activity were pooled (~4 ml) and concentrated 5 fold by using a Centricon 30 (Amicon).

N-terminal amino acid sequence analysis: Overexpressed polypeptides were used for N-terminal amino acid sequence analysis of the OrfY, FabD, and FabG proteins. Overexpression of the G30k and PlsX polypeptides was apparently not possible using the T7 RNA polymerase system. BL21 (DE3) containing whole or part of *g30k* ORF (pWO806, pWO617, or pWO804) grew on plates but did not grow or lost the plasmids during growth in liquid medium. But G30k protein was overproduced in larger amount in BL21 (DE3, pWO124) without IPTG induction than with IPTG induction. pWO124 carries the *g30k* ORF but is in the opposite

direction relative to the T7 promoter. The promoter for *g30k* seems to be strong enough to overproduce G30k protein from a multicopy plasmid. But when IPTG is added, the strong T7 promoter acts against the *g30k* promoter because they are convergent. So pWO124 was introduced into HB101 and grown 2.5 h more after cell density reached A_{280} ~0.8. A 34 kDa protein was overproduced without IPTG induction. This presumed G30k protein was subjected to N-terminal sequence analysis.

Several recombinant plasmids (pWO123, pWO125, pWO126, and pWO623) were used in an attempt to overexpress the intact or truncated PlsX protein by the T7 RNA polymerase system. But there was no difference in protein profiles between the induced and non-induced cells on SDS-gels. Therefore, the *plsX-lacZ* translational fusion was constructed. The resulting protein was partially purified as described above and subjected to N-terminal sequence analysis.

OrfY and G30k proteins were overexpressed in BL21 (DE3, pWO625) and strain HB101 (pWO124) respectively, and whole cells were fractionated on a SDS-gel. Strain TL73 (pWO951) was used to overexpress the *plsX-lacZ* fusion protein. Strain BL21 (DE3, pWO115) and BL21 (DE3, pWO610) produced truncated FabD and truncated FabG polypeptides respectively. The truncated polypeptides formed inclusion bodies and were partially purified as described by Sambrook *et al.* (95). Cells were harvested, washed (50 mM Tris-HCl, pH 7.6, 5 mM β-mercaptoethanol, 1 mM EDTA) and then broken by a single passage through a French pressure cell.

The truncated FabD and FabG polypeptides aggregated and were recovered by centrifugation at 5,000 g for 10 min. Pellets were suspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl, and 0.5% Triton X-100) and incubated at room temperature for 15 min. After centrifugation at 1,000 g for 15 min at 4 °C, pellets were resuspended in 2 x SDS sample loading buffer. Protein samples were loaded on 13% or 6.5% (*plsX-lacZ* fusion) polyacrylamide gels. Electrophoresis was carried out using the buffer system of Laemmli (90) as described by Moos *et al.* (96). Proteins were electrophoretically transferred to Immobilon-P membrane at 400 mA (for 2 hr for *plsX-lacZ* fusion protein and for 1 h for other proteins). Membranes were stained with Coomassie Brilliant blue R-250, and then washed with destainer followed by HPLC grade water as described by Matsudaira (97) and by Kounnas et al. (98). Membranes were dried at room temperature for 30 min and the regions of the membrane containing the expected polypeptides were cut out and subjected to sequence analysis by using the method of Tarr (99) in the Biology Department at Virginia Polytechnique Institute and State University or in the Protein Chemistry Laboratory, Department of Biochemistry and Molecular Biophysics at Washington University in St. Louis.

Promoter activity analysis: To locate the promoters responsible for the expression of each gene, inter- and intragenic DNA restriction fragments were cloned into promoter probe vector pCB267 (83) upstream of a promoterless *lacZ* gene. In

order to orientate the insert such that the direction of transcription was the same as *lacZ* gene, preliminary cloning into other vectors was sometimes required. Plasmids with transcriptional *lacZ* fusions were introduced into strain TL73 (Δ*lac*) and β-galactosidase activity was measured to assess promoter strength.

β-galactosidase assay: β-galactosidase activity was measured following the procedures of Miller (69) with some modifications. Cells (1.5 ml) were grown in LB medium until the A_{578} reached 0.2 - 1.0 and cooled on ice. Cells were harvested, resuspended in 1 ml of Z buffer (60 mM Na_2HPO_4, 40mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0), and the A_{578} was measured. Cells (10 to 500 μl) were diluted into Z buffer to a final volume of 800 μl. Cells were permeabilized by adding 1 drop of 0.1% SDS and 2 drops of chloroform, vortexed for 10 seconds, and preincubated for 10 min at 28 °C. Reactions were started by adding 160 μl of ONPG (4 mg/ml in 0.1 M phosphate buffer, pH 7.0) and stopped by adding 0.4 ml of 1 M Na_2CO_3 when appropriate yellow color (A_{420} = 0.4 - 0.8) had developed. The absorbance at 420 nm was measured using a blank containing no cells. The specific activity of β-galactosidase was expressed in Miller units:

$$\text{Specific activity} = \frac{A_{420} \times 800}{A_{578} \times \text{Cell vol.} \times (\Delta T)}$$

Cell vol. = cell volume used in assay in ml

ΔT = reaction time in min

Isolation of RNA: Total RNA was isolated from *E. coli* by the hot phenol extraction method (100). RNA was stored in 3 volumes of ethanol at -70 °C.

Primer extension analysis: In order to map the 5' end of transcripts upstream of *plsX* and *rpmF*, primer extension analysis was performed by the procedures of Alam *et al.* (101) with certain modifications. Total RNA was isolated from the strains HB101, HB101(pWO401), or HB101(pWO105). Five synthetic oligonucleotides (Table 2. # 272692, 219623, 236290, 288603, and 207919) were used. Twenty five μg of total RNA and 25 ng of primer were combined in 6.5 μl of water and boiled for 2 min, followed by immediate transfer to an ice water bath. After 30 min of incubation, 5 μl of 5 x reverse transcriptase buffer (0.25 M Tris-HCl, pH 8.3, 0.375 M KCl, 15 mM $MgCl_2$), 2.5 μl of 0.1 M DTT, 1.5 μl of [α-^{35}S]dATP and 2.5 μl of each three dCTP, dGTP, and dTTP (10 mM stock solution) were added to this mixture. The reaction was started by adding 2 μl of M-MLV reverse transcriptase (200 U/μl) and incubated at 42 °C. After 30 min, 3.5 μl of dATP (10 mM) and 2.5 μl of dCTP, dGTP, and dTTP mixture (10 mM each) were added and the mixture was incubated at 42 °C for an additional 30 min. The reaction was stopped by the addition of 20 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). Samples were

heated for 5 min at 80 °C before loading and 5 μl of each sample was analyzed on a sequencing gel.

S1 mapping: In order to map the 3' ends of transcripts at the attenuator site, S1 mapping was utilized following the method of Wang *at al.* (102) with some modifications. A probe uniformly radiolabeled on the strand complementary to the mRNA was made from an M13 clone (Fig. 13, panel A). The *Hin*dIII (nt 1770) - *Taq*I (nt 2121) restriction fragment was cloned into the *Hin*dIII-*Acc*I sites of M13mp19 (19II-1). Single stranded DNA of 19II-1 was isolated from 3 ml of culture supernatant and dissolved in 8 μl of water. Synthetic oligonucleotide (Table 2. #056025) complementary to the sequence across the *Hin*dIII site of 19II-1 was annealed in 0.5 x Sequenase buffer in a final volume of 10 μl by heating to 65 °C for 2 min and cooling slowly to 30 °C. The resulting M13-oligo duplex was cleaved with *Hind*III using an overnight incubation at 37 °C. Universal primer (2 pmole) and 1.8 μl of 5 x Sequenase buffer were added to this reaction mixture in a final volume of 13 μl. The annealing reaction was performed by heating at 65 °C for 5 min and cooling slowly to 30° C. To the above mixture, 1.4 μl of 100 mM DTT, 1 μl of dCTP, dGTP, and dTTP mixture (1 mM each), and 5.3 μl of [α-^{35}S]dATP were added. The reaction was started by adding 1 μl (3.3 units) of Sequenase to the above reaction mixture, followed by incubating at 37°C for 10 min. One μl of all four dNTPs (1 mM each) was added as chase and incubation

was continued for 5 min. The reaction was stopped by adding 1 μl of 0.5 M EDTA. An aliquot (5 μl out of 24 μl) of the above reaction mixture was mixed with 250 μg of total RNA from strain ECL8 (*plsX*$^+$) or strain TL129 (*plsX50*). Ammonium acetate was added to a final concentration of 2.5 M and 2.5 volume of 95% ethanol was added, incubated -70 °C for 20 min., and centrifuged for 15 min at 4 °C. The pellet was washed with 80% ethanol and dissolved in 50 μl of freshly prepared hybridization solution (80% formamide, 2 x SSC, 0.4 mM HEPES, pH 7.0, and 0.1% SDS). The solution was heated at 55 °C for 5 min, then transferred to a heat block and incubated at 39 °C overnight. After hybridization, the solution was diluted with 250 μl of S1 buffer (50 mM sodium acetate, pH 4.8, 0.5 mM $ZnCl_2$, 50 mM NaCl). S1 nuclease (55 units) was added and the reaction mixture was incubated at 25 °C for 30 min. After ethanol precipitation, the DNA/RNA hybrids were dissolved in 6 μl of TE and mixed with 6 μl of stop solution. An aliquot (1 μl) was analyzed on a 6% sequencing gel.

Northern analysis: Cells were grown in LB medium until the A_{600} reached ~0.8. Total RNA was isolated from strain ECL8 (*plsX*$^+$) or strain TL129 (*plsX50*) by the method described above. RNA (40 μg per lane) was fractionated by electrophoresis on a 1.2 % agarose/formaldehyde gel as described by Sambrook *et al.* (95). RNA molecular weight standards were run in parallel on each gel. Electrophoresis was performed at 3.5 V/cm for 10-11 hours with constant circulation of electrophoresis

buffer from cathode chamber to anode chamber. RNAs were transferred to Immobilon-N membrane by capillary flow in 20 x SSC buffer overnight. After transfer, the membrane was baked at 80 °C for 2 hours in a vacuum oven. The lane with RNA molecular weight standards was cut and stained as described by Sambrook *et al.* (95). Hybridization with radiolabeled DNA probes and washing of the membrane were performed following the instructions provided by the manufacturer (Millipore). Thirteen different probes (Table 8) were radiolabeled with [α-^{32}P]dCTP using a random-priming labeling kit (U.S. Bochemical). DNA restriction fragments were extracted from the gel and 25 ng was used as a template for random priming in a 50 μl reaction. The entire reaction mixture was used in 10 ml of hybridization solution in a plastic bag.

Polymerase chain reaction: Two μg of chromosomal DNA or 100 ng of plasmid DNA was used as template and 100 pmol of each synthetic oligonucleotide were used in 100 μl reactions. Primers were usually 20 mers in the case of a perfect match between template and primer or 27-mers in the case of a partial mismatch to create a restriction site. DNA amplification was carried out using a GeneAmp PCR kit and a GeneAmp PCR system 9600 (Perkin Elmer Cetus) according to the manufacturer's instruction. The PCR cycles were as follows: In the case of a perfect match between primers and template, the first cycle was a 3 min denaturation at 94.5 °C. Subsequent cycles (30 total) were 30 sec denaturation at 94 °C, 30 sec annealing at 50 °C, 30 sec polymerization at 72 °C. The last cycle

held the samples at 5 °C. In the case of a partial mismatch between primers and template, the first cycle was a 3 min denaturation at 94.5 °C. Subsequent cycles (30 total) were 30 sec denaturation at 94 °C, 30 sec annealing at 45 °C, 30 sec polymerization at 72 °C (5 cycles) and 30 sec denaturation at 94 °C, 30 sec annealing at 55 °C, 30 sec polymerization at 72 °C (25 cycles). The last cycle held the samples at 5 °C. Amplified DNA was purified by using the Geneclean II or by phenol/chloroform extraction for further cloning.

RESULTS

NUCLEOTIDE SEQUENCE OF THE *plsX* REGION AND IDENTIFICATION OF EACH ORF

In order to study the molecular basis for the requirement of the *plsX50* mutation for the conferral of a G3P auxotrophic phenotype in *plsB26* strains of *E. coli,* a molecular genetic approach has been taken. Knowledge of the precise map location of the *plsX* gene near min 24 of the *E. coli* linkage map (37) allowed prediction of a small subset of λ phages from the Kohara library (9) that would carry *plsX*. Phages 234 and 235 were found to complement the glycerol-P auxotrophy of a *plsB26 plsX50* mutant. Prior to my work, DNA fragments common to both phages had been cloned into plasmid and M13 vectors and sequenced in our lab. For my work, it was necessary to complete the DNA sequences, identify the open reading frames (ORF's), and study each ORF.

1. Cloning and Sequencing of DNA:

From λ phage 235, fragments from the 4808 bp region of DNA (*Pst*I-*Sal*I) containing *plsX* was cloned into pBluescript KS$^+$, pCB267, pGEM-4, pGEM-3Z, pTL61T, pSH76, pT7-7, or M13 vectors. Subclones generated for this study are listed in Table 4. The nucleotide sequence of 4.8 kb DNA and translation of each

ORF are shown in Fig. 3. In order to study the *plsX* gene, cloning of a DNA fragment big enough to contain all of *plsX* and its flanking genes was tried but it was not possible to clone a DNA fragment larger than the 2609 bp *Sal*I-*Nsi*I (nt 711-3319) fragment. This clone was also very toxic to the cells so it was hard to interpret the results using this clone. Cloning of the 4093 bp *Sal*I-*Sal*I (nt 711-4803) or 2273 bp *Hin*dIII-*Hin*dIII (nt 1779-4042) fragments was tried but these clones were too toxic for the cells to grow or insertion or deletion mutations occurred too frequently. The largest clone that contained the whole *plsX* gene and gave reliable results was the 2105 bp *Xho*I-*Nsi*I (nt 1215-3319) fragment. The altered mRNA stability (*ams*) gene and a gene of unknown function (*orfX*) are located upstream of the *Pst*I (nt 1) site (103, 104). The 3-ketoacyl-ACP reductase (*fabG*) and acyl carrier protein (*acpP*) genes are located downstream of the *Sal*I (nt 4803) site (105). The DNA (4808 bp) sequence shown in Fig. 3 was proved to be connected with these upstream and downstream sequences by generation of the appropriate clones and sequencing the DNA across the *Pst*I (nt 1) and *Sal*I (nt 4803) sites (19W-1 and 19W-12 clones respectively).

2. Identification of each ORF:

Sequence analysis of 4.8 kb of DNA (*Pst*I-*Sal*I) revealed 6 ORF's (ORF1-ORF6). Five ORF's are translated in the same direction with *plsX* and one ORF is translated in the opposite direction. The six ORF's in relation with *ams* (103),

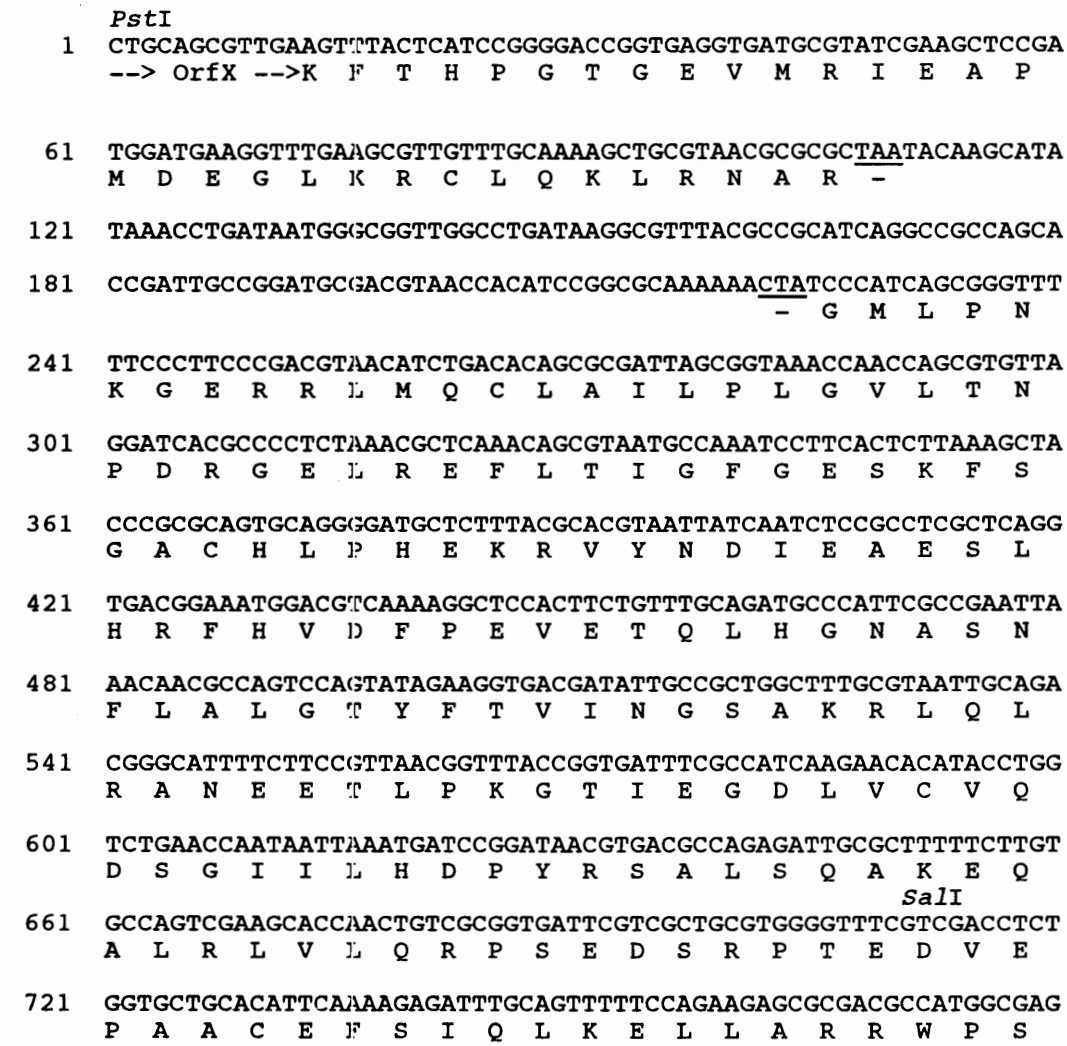
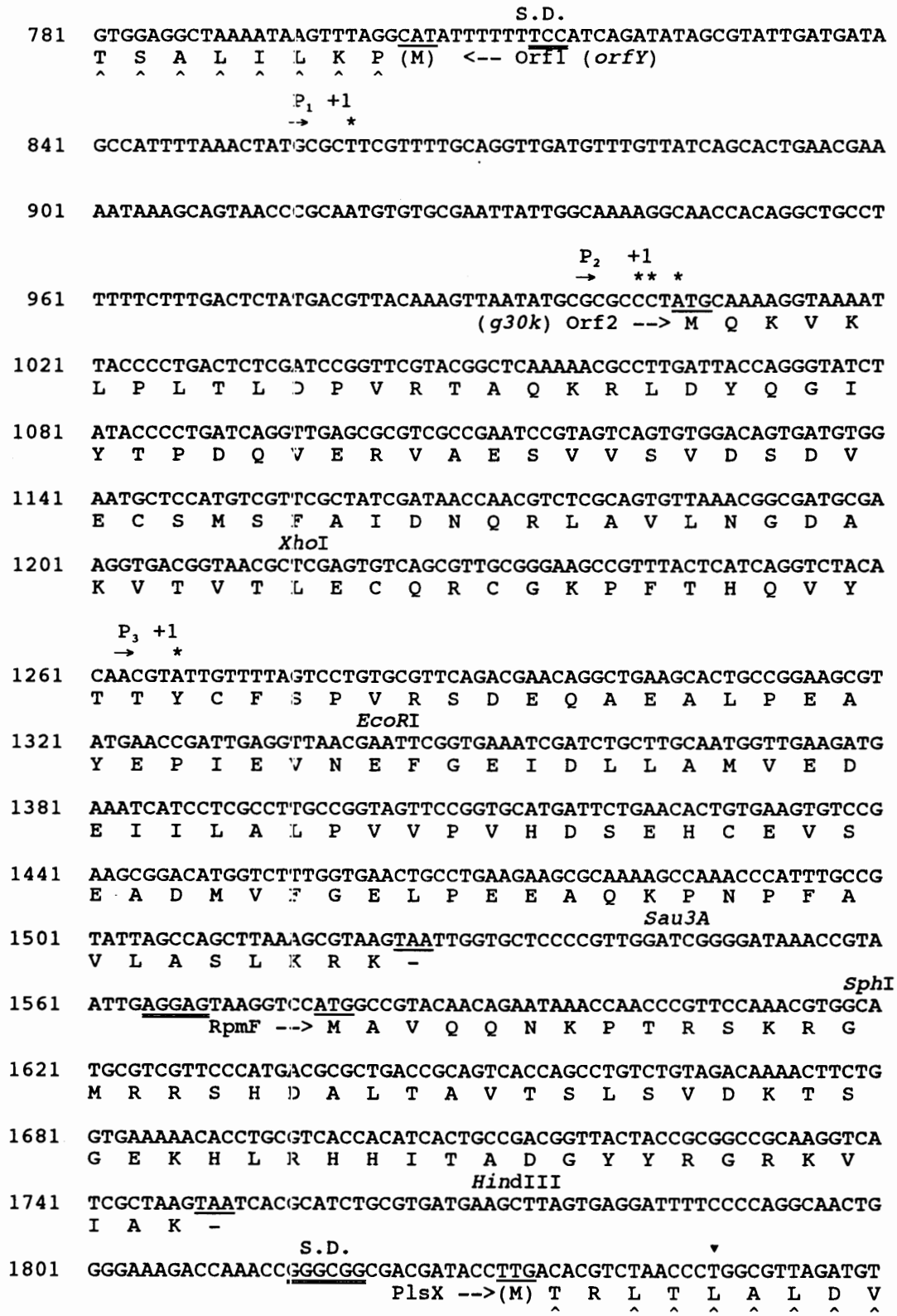
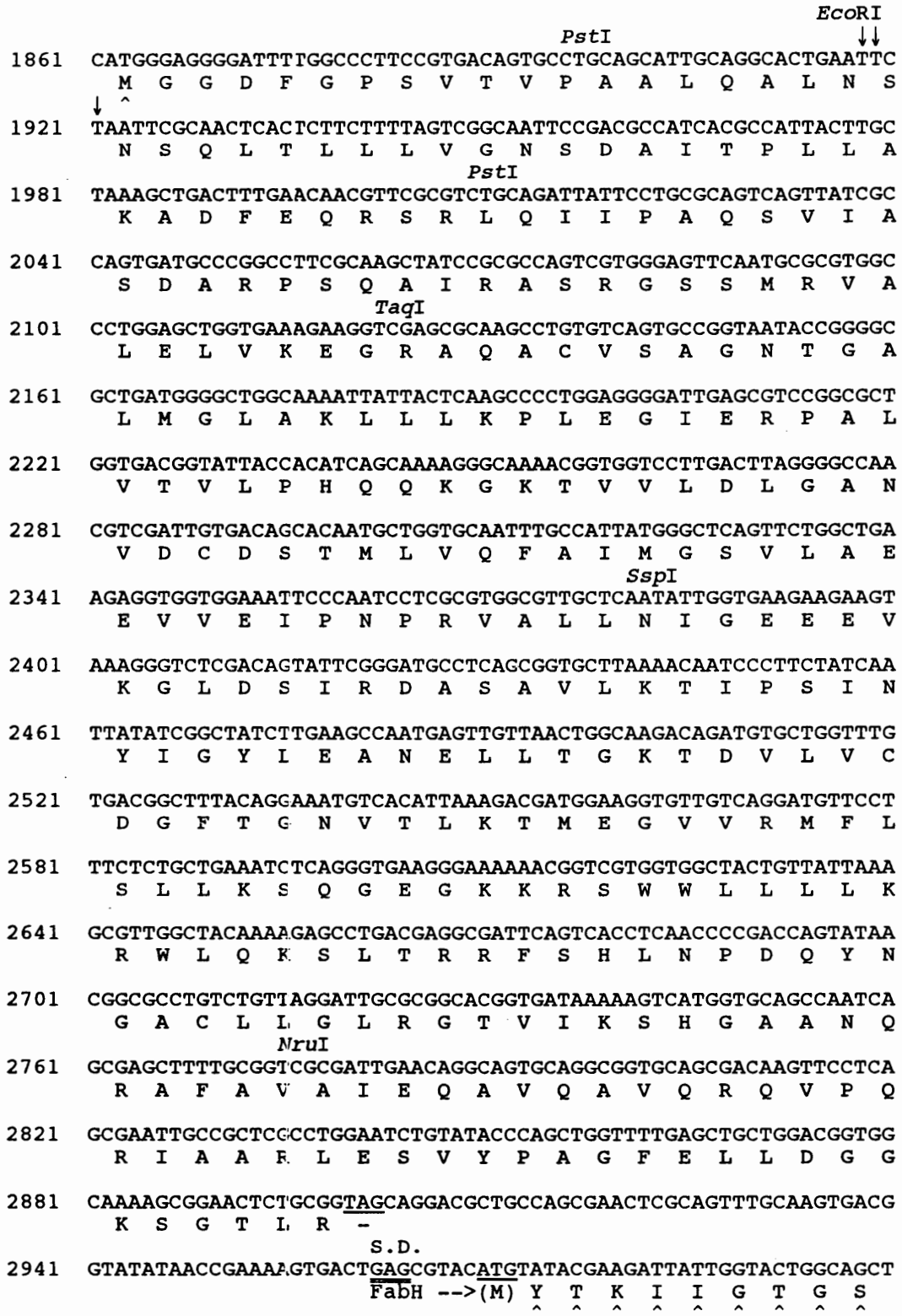
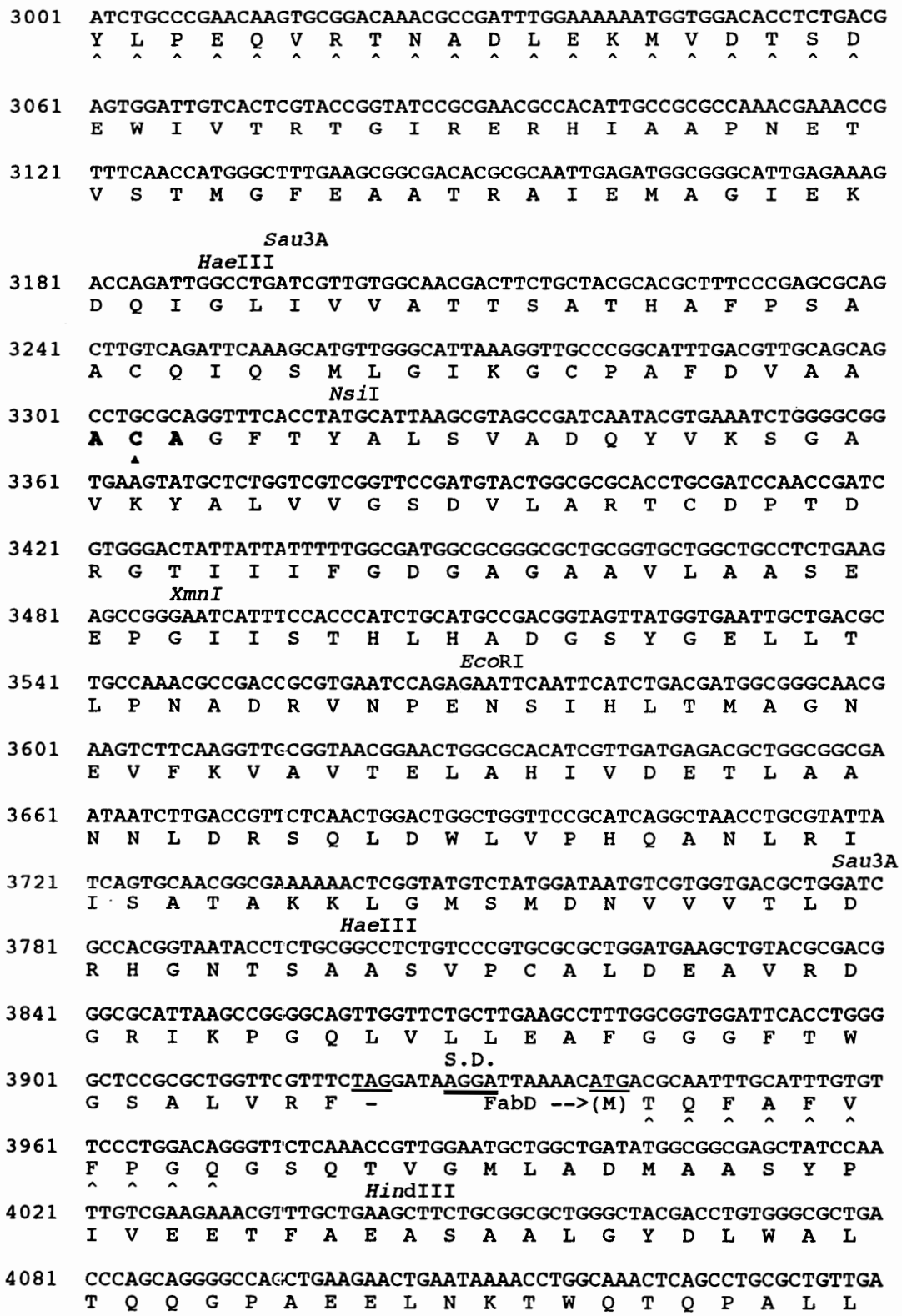
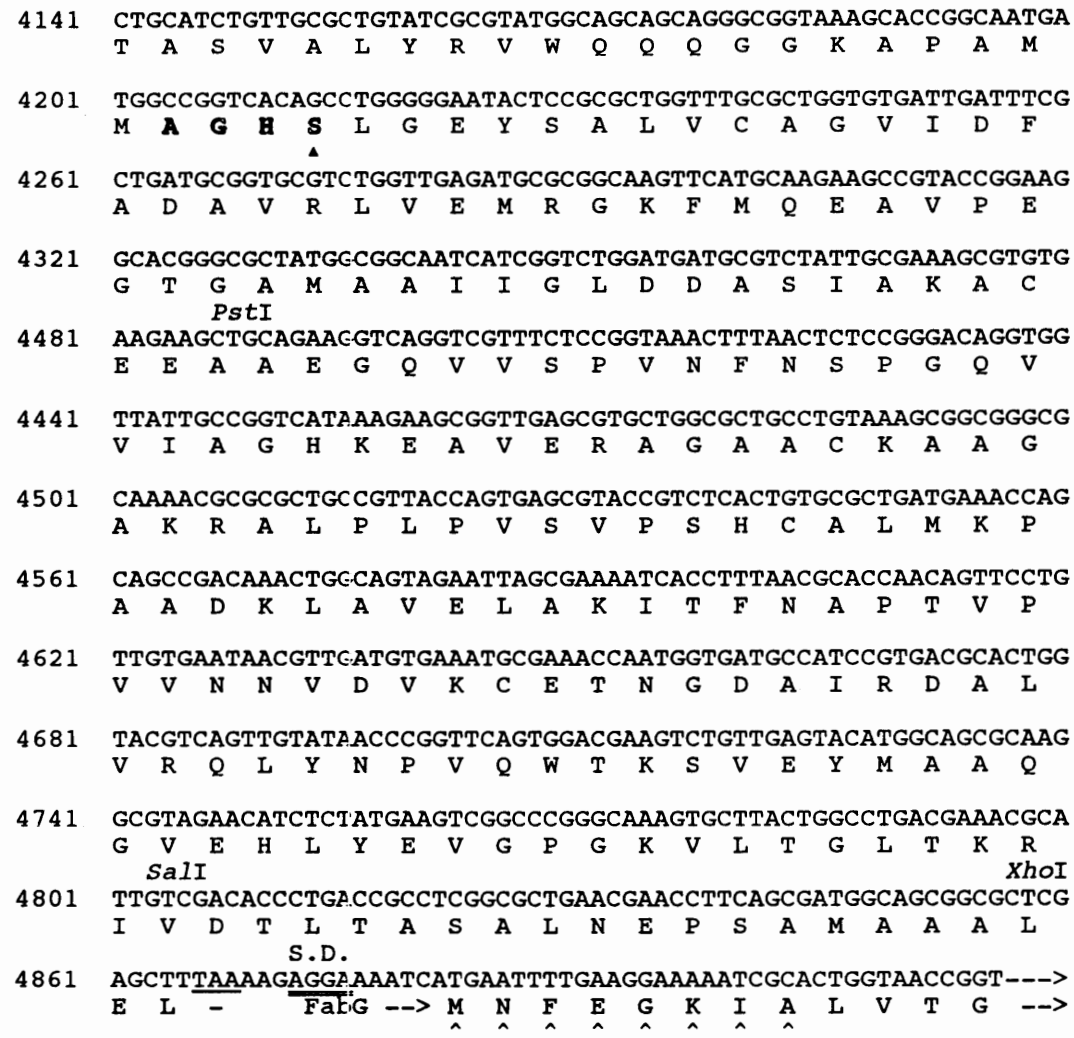


Fig. 3. Nucleotide sequence of 4.8 kb DNA (PstI-SalI) and deduced amino acid sequences of 6 ORF's. *Orf1, Orf2, rpmF, plsX, fabH,* and *fabD.* Numbering of nucleotides starts from the first base of the *Pst*I site. OrfX mentioned by Claverie-Martin *et al.* (103. 104) terminates at nt 108. ORF1 is translated from the opposite strand starting at nucleotide 806. ORF2, *rpmF, plsX, fabH,* and *fabD* are translated from the same strand and start at nucleotide positions 1005, 1578, 1832, 2970, and 3939 respectively. The sequence downstream of *Xho*I (nt 4856) is provided by Magnuson *et al.* (116). The restriction endonuclease sites mentioned in the text are indicated above the sequence. N-terminal amino acid sequences of each ORF identified by sequencing of the overproduced peptides are marked by ^. Putative ribosome binding sites (S.D.) are double underlined. The T residue deleted in the DNA in *plsX50* strains is marked by ▾. The potential transcription start sites upstream of *rpmF* identified by primer extension analysis are indicated by P_1, P_2, and P_3. Transcription attenuator sites identified by S1 mapping are indicated by vertical arrows ↓. The putative active site cysteine of FabH and serine of FabD are denoted by ▲.


S.D.
781 GTGGAGGCTAAAATAAGTTTAGGCATATTTTTTTCCATCAGATATAGCGTATTGATGATA
T S A L I L K P (M) <-- Orf1 (orfY)
P1 +1
841 GCCATTTTAAACTATGCGCTTCGTTTTGCAGGTTGATGTTTGTTATCAGCACTGAACGAA
901 AATAAAGCAGTAACCCGCAATGTGTGCGAATTATTGGCAAAAGGCAACCACAGGCTGCCT
P2 +1
961 TTTTCTTTGACTCTATGACGTTACAAAGTTAATATGCGCGCCCTATGCAAAAGGTAAAAT
(g30k) Orf2 --> M Q K V K
1021 TACCCCTGACTCTCGATCCGGTTCGTACGGCTCAAAAACGCCTTGATTACCAGGGTATCT
L P L T L D P V R T A Q K R L D Y Q G I
1081 ATACCCCTGATCAGGTTGAGCGCGTCGCCGAATCCGTAGTCAGTGTGGACAGTGATGTGG
Y T P D Q V E R V A E S V V S V D S D V
1141 AATGCTCCATGTCGTTCGCTATCGATAACCAACGTCTCGCAGTGTTAAACGGCGATGCGA
E C S M S F A I D N Q R L A V L N G D A
XhoI
1201 AGGTGACGGTAACGCTCGAGTGTCAGCGTTGCGGGAAGCCGTTTACTCATCAGGTCTACA
K V T V T L E C Q R C G K P F T H Q V Y
P3 +1
1261 CAACGTATTGTTTTAGTCCTGTGCGTTCAGACGAACAGGCTGAAGCACTGCCGGAAGCGT
T T Y C F S P V R S D E Q A E A L P E A
EcoRI
1321 ATGAACCGATTGAGGTTAACGAATTCGGTGAAATCGATCTGCTTGCAATGGTTGAAGATG
Y E P I E V N E F G E I D L L A M V E D
1381 AAATCATCCTCGCCTTGCCGGTAGTTCCGGTGCATGATTCTGAACACTGTGAAGTGTCCG
E I I L A L P V V P V H D S E H C E V S
1441 AAGCGGACATGGTCTTTGGTGAACTGCCTGAAGAAGCGCAAAAGCCAAACCCATTTGCCG
E A D M V F G E L P E E A Q K P N P F A
Sau3A
1501 TATTAGCCAGCTTAAAGCGTAAGTAATTGGTGCTCCCCGTTGGATCGGGGATAAACCGTA
V L A S L K R K -
SphI
1561 ATTGAGGAGTAAGGTCCATGGCCGTACAACAGAATAAACCAACCCGTTCCAAACGTGGCA
RpmF --> M A V Q Q N K P T R S K R G
1621 TGCGTCGTTCCCATGACGCGCTGACCGCAGTCACCAGCCTGTCTGTAGACAAAACTTCTG
M R R S H D A L T A V T S L S V D K T S
1681 GTGAAAAACACCTGCGTCACCACATCACTGCCGACGGTTACTACCGCGGCCGCAAGGTCA
G E K H L R H H I T A D G Y Y R G R K V
HindIII
1741 TCGCTAAGTAATCACGCATCTGCGTGATGAAGCTTAGTGAGGATTTTCCCCAGGCAACTG
I A K -
S.D.
1801 GGGAAAGACCAAACCGGGCGGCGACGATACCTTGACACGTCTAACCCTGGCGTTAGATGT
PlsX -->(M) T R L T L A L D V


EcoRI
PstI
1861 CATGGGAGGGGATTTTGGCCCTTCCGTGACAGTGCCTGCAGCATTGCAGGCACTGAATTC
M G G D F G P S V T V P A A L Q A L N S
1921 TAATTCGCAACTCACTCTTCTTTTAGTCGGCAATTCCGACGCCATCACGCCATTACTTGC
N S Q L T L L L V G N S D A I T P L L A
PstI
1981 TAAAGCTGACTTTGAACAACGTTCGCGTCTGCAGATTATTCCTGCGCAGTCAGTTATCGC
K A D F E Q R S R L Q I I P A Q S V I A
2041 CAGTGATGCCCGGCCTTCGCAAGCTATCCGCGCCAGTCGTGGGAGTTCAATGCGCGTGGC
S D A R P S Q A I R A S R G S S M R V A
TaqI
2101 CCTGGAGCTGGTGAAAGAAGGTCGAGCGCAAGCCTGTGTCAGTGCCGGTAATACCGGGGC
L E L V K E G R A Q A C V S A G N T G A
2161 GCTGATGGGGCTGGCAAAATTATTACTCAAGCCCCTGGAGGGGATTGAGCGTCCGGCGCT
L M G L A K L L L K P L E G I E R P A L
2221 GGTGACGGTATTACCACATCAGCAAAAGGGCAAAACGGTGGTCCTTGACTTAGGGGCCAA
V T V L P H Q Q K G K T V V L D L G A N
2281 CGTCGATTGTGACAGCACAATGCTGGTGCAATTTGCCATTATGGGCTCAGTTCTGGCTGA
V D C D S T M L V Q F A I M G S V L A E
SspI
2341 AGAGGTGGTGGAAATTCCCAATCCTCGCGTGGCGTTGCTCAATATTGGTGAAGAAGAAGT
E V V E I P N P R V A L L N I G E E E V
2401 AAAGGGTCTCGACAGTATTCGGGATGCCTCAGCGGTGCTTAAAACAATCCCTTCTATCAA
K G L D S I R D A S A V L K T I P S I N
2461 TTATATCGGCTATCTTGAAGCCAATGAGTTGTTAACTGGCAAGACAGATGTGCTGGTTTG
Y I G Y I E A N E L L T G K T D V L V C
2521 TGACGGCTTTACAGGAAATGTCACATTAAAGACGATGGAAGGTGTTGTCAGGATGTTCCT
D G F T G N V T L K T M E G V V R M F L
2581 TTCTCTGCTGAAATCTCAGGGTGAAGGGAAAAAACGGTCGTGGTGGCTACTGTTATTAAA
S L L K S Q G E G K K R S W W L L L L K
2641 GCGTTGGCTACAAAAGAGCCTGACGAGGCGATTCAGTCACCTCAACCCCGACCAGTATAA
R W L Q K S L T R R F S H L N P D Q Y N
2701 CGGCGCCTGTCTGTTAGGATTGCGCGGCACGGTGATAAAAAGTCATGGTGCAGCCAATCA
G A C L L G L R G T V I K S H G A A N Q
NruI
2761 GCGAGCTTTTGCGGTCGCGATTGAACAGGCAGTGCAGGCGGTGCAGCGACAAGTTCCTCA
R A F A V A I E Q A V Q A V Q R Q V P Q
2821 GCGAATTGCCGCTCCCCTGGAATCTGTATACCCAGCTGGTTTTGAGCTGCTGGACGGTGG
R I A A R L E S V Y P A G F E L L D G G
2881 CAAAAGCGGAACTCTGCGGTAGCAGGACGCTGCCAGCGAACTCGCAGTTTGCAAGTGACG
K S G T L R -
S.D.
2941 GTATATAACCGAAAAGTGACTGAGCGTACATGTATACGAAGATTATTGGTACTGGCAGCT
FabH -->(M) Y T K I I G T G S


3001 ATCTGCCCGAACAAGTGCGGACAAACGCCGATTTGGAAAAAATGGTGGACACCTCTGACG
Y L P E Q V R T N A D L E K M V D T S D
3061 AGTGGATTGTCACTCGTACCGGTATCCGCGAACGCCACATTGCCGCGCCAAACGAAACCG
E W I V T R T G I R E R H I A A P N E T
3121 TTTCAACCATGGGCTTTGAAGCGGCGACACGCGCAATTGAGATGGCGGGCATTGAGAAAG
V S T M G F E A A T R A I E M A G I E K
Sau3A
HaeIII
3181 ACCAGATTGGCCTGATCGTTGTGGCAACGACTTCTGCTACGCACGCTTTCCCGAGCGCAG
D Q I G L I V V A T T S A T H A F P S A
3241 CTTGTCAGATTCAAAGCATGTTGGGCATTAAAGGTTGCCCGGCATTTGACGTTGCAGCAG
A C Q I Q S M L G I K G C P A F D V A A
NsiI
3301 CCTGCGCAGGTTTCACCTATGCATTAAGCGTAGCCGATCAATACGTGAAATCTGGGGCGG
A C A G F T Y A L S V A D Q Y V K S G A
3361 TGAAGTATGCTCTGGTCGTCGGTTCCGATGTACTGGCGCGCACCTGCGATCCAACCGATC
V K Y A L V V G S D V L A R T C D P T D
3421 GTGGGACTATTATTATTTTTGGCGATGGCGCGGGCGCTGCGGTGCTGGCTGCCTCTGAAG
R G T I I I F G D G A G A A V L A A S E
XmnI
3481 AGCCGGGAATCATTTCCACCCATCTGCATGCCGACGGTAGTTATGGTGAATTGCTGACGC
E P G I I S T H L H A D G S Y G E L L T
EcoRI
3541 TGCCAAACGCCGACCGCGTGAATCCAGAGAATTCAATTCATCTGACGATGGCGGGCAACG
L P N A D R V N P E N S I H L T M A G N
3601 AAGTCTTCAAGGTTGCGGTAACGGAACTGGCGCACATCGTTGATGAGACGCTGGCGGCGA
E V F K V A V T E L A H I V D E T L A A
3661 ATAATCTTGACCGTTCTCAACTGGACTGGCTGGTTCCGCATCAGGCTAACCTGCGTATTA
N N L D R S Q L D W L V P H Q A N L R I
Sau3A
3721 TCAGTGCAACGGCGAAAAAACTCGGTATGTCTATGGATAATGTCGTGGTGACGCTGGATC
I S A T A K K L G M S M D N V V V T L D
HaeIII
3781 GCCACGGTAATACCTCTGCGGCCTCTGTCCCGTGCGCGCTGGATGAAGCTGTACGCGACG
R H G N T S A A S V P C A L D E A V R D
3841 GGCGCATTAAGCCGGGGCAGTTGGTTCTGCTTGAAGCCTTTGGCGGTGGATTCACCTGGG
G R I K P G Q L V L L E A F G G G F T W
S.D.
3901 GCTCCGCGCTGGTTCGTTTCTAGGATAAGGATTAAAACATGACGCAATTTGCATTTGTGT
G S A L V R F - FabD -->(M) T Q F A F V
3961 TCCCTGGACAGGGTTCTCAAACCGTTGGAATGCTGGCTGATATGGCGGCGAGCTATCCAA
F P G Q G S Q T V G M L A D M A A S Y P
HindIII
4021 TTGTCGAAGAAACGTTTGCTGAAGCTTCTGCGGCGCTGGGCTACGACCTGTGGGCGCTGA
I V E E T F A E A S A A L G Y D L W A L
4081 CCCAGCAGGGGCCAGCTGAAGAACTGAATAAAACCTGGCAAACTCAGCCTGCGCTGTTGA
T Q Q G P A E E L N K T W Q T Q P A L L


4141 CTGCATCTGTTGCGCTGTATCGCGTATGGCAGCAGCAGGGCGGTAAAGCACCGGCAATGA
T A S V A L Y R V W Q Q Q G G K A P A M
4201 TGGCCGGTCACAGCCTGGGGGAATACTCCGCGCTGGTTTGCGCTGGTGTGATTGATTTCG
M A G H S L G E Y S A L V C A G V I D F
4261 CTGATGCGGTGCGTCTGGTTGAGATGCGCGGCAAGTTCATGCAAGAAGCCGTACCGGAAG
A D A V R L V E M R G K F M Q E A V P E
4321 GCACGGGCGCTATGGCGGCAATCATCGGTCTGGATGATGCGTCTATTGCGAAAGCGTGTG
G T G A M A A I I G L D D A S I A K A C
PstI
4481 AAGAAGCTGCAGAAGGTCAGGTCGTTTCTCCGGTAAACTTTAACTCTCCGGGACAGGTGG
E E A A E G Q V V S P V N F N S P G Q V
4441 TTATTGCCGGTCATAAAGAAGCGGTTGAGCGTGCTGGCGCTGCCTGTAAAGCGGCGGGCG
V I A G H K E A V E R A G A A C K A A G
4501 CAAAACGCGCGCTGCCGTTACCAGTGAGCGTACCGTCTCACTGTGCGCTGATGAAACCAG
A K R A L P L P V S V P S H C A L M K P
4561 CAGCCGACAAACTGGCAGTAGAATTAGCGAAAATCACCTTTAACGCACCAACAGTTCCTG
A A D K L A V E L A K I T F N A P T V P
4621 TTGTGAATAACGTTCATGTGAAATGCGAAACCAATGGTGATGCCATCCGTGACGCACTGG
V V N N V D V K C E T N G D A I R D A L
4681 TACGTCAGTTGTATAACCCGGTTCAGTGGACGAAGTCTGTTGAGTACATGGCAGCGCAAG
V R Q L Y N P V Q W T K S V E Y M A A Q
4741 GCGTAGAACATCTCTATGAAGTCGGCCCGGGCAAAGTGCTTACTGGCCTGACGAAACGCA
G V E H L Y E V G P G K V L T G L T K R
SalI XhoI
4801 TTGTCGACACCCTGACCGCCTCGGCGCTGAACGAACCTTCAGCGATGGCAGCGGCGCTCG
I V D T L T A S A L N E P S A M A A A L
S.D.
4861 AGCTTTAAAAGAGGAAAATCATGAATTTTGAAGGAAAAATCGCACTGGTAACCGGT--->
E L - FabG --> M N F E G K I A L V T G -->
^ ^ ^ ^ ^ ^ ^ ^

fabG and *acpP* (105) are shown in Fig. 4, panel A.

Identification of proteins produced by each ORF: The proteins encoded by each ORF were identified by the maxicell technique. Subclones used in this experiment and proteins produced by each ORF are represented in Table 5 and in Fig. 5. The results indicated that each ORF in fact encoded a protein of expected size except for ORF2. ORF2 produced a 34 kDa protein on a 15% SDS-PAGE which is much larger than the 18 kDa protein predicted from the deduced amino acid sequence of ORF2. This result is consistent with that observed by Tanaka *et al.* (106), where the size of this protein was estimated to be 30 kDa.

Complementation and Recombination tests: To determine the function of each ORF, complementation tests were performed based on the previous results indicating that *plsX* is located within this 4.8 kb DNA and knowledge that *fabD* has been mapped at min 24 near *plsX* (120). A series of subclones containing each ORF were introduced into strains defective in *plsX* (TL85: *plsB26 plsX50* and TL400: *plsB26 plsX50 recA1*) or *fabD* (L48: *fabD89* and LW48: *fabD89 recA1*) function. Results are shown in Fig. 4, panel B. pWO601 and pWO603, which contain entire ORF4, complemented the *plsX50* mutation while other subclones did not. pWO604, even though it contains the entire ORF4, did not complement the *plsX50* mutation, nor produce the PlsX protein in maxicells (Fig 5, lane 6). These

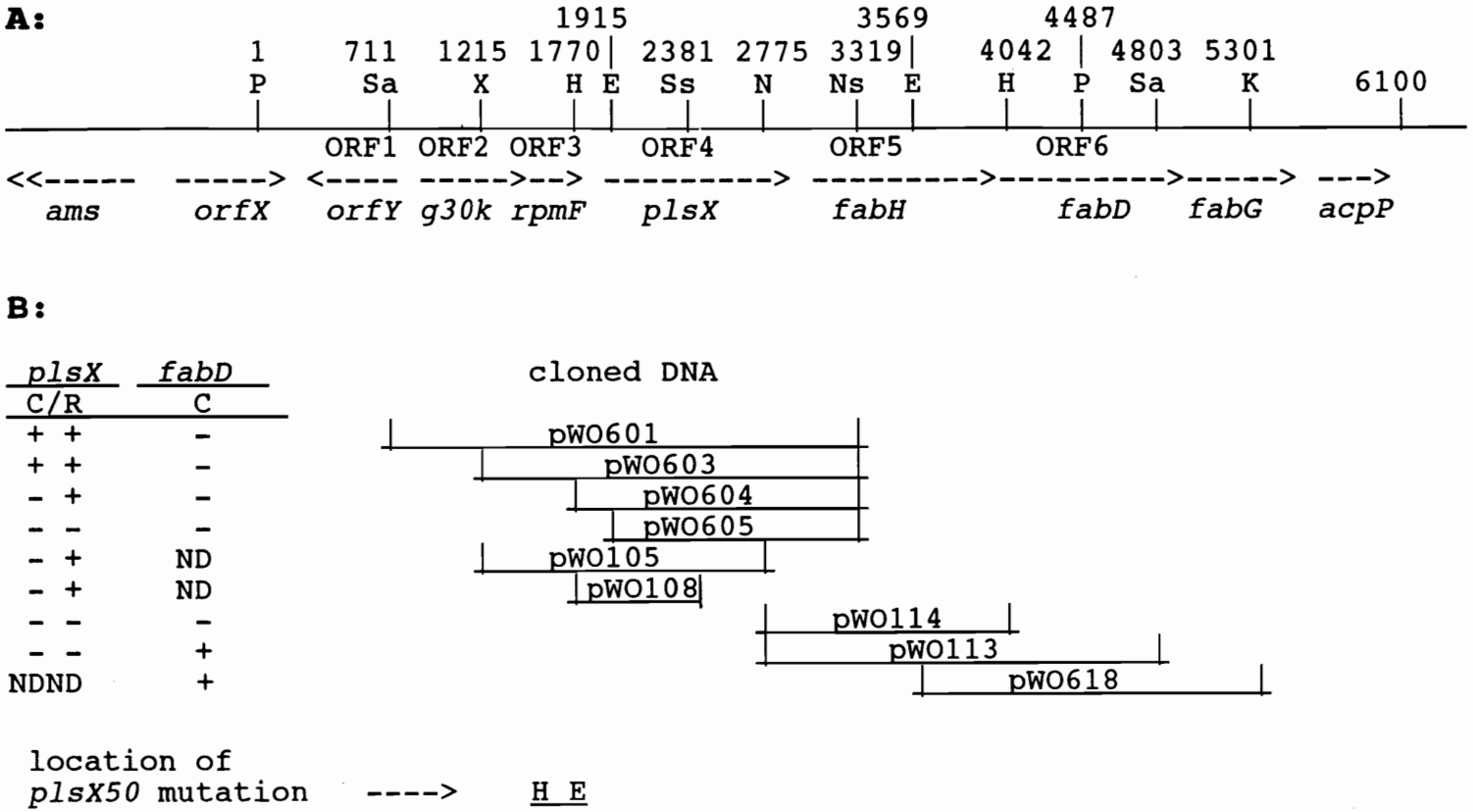


Fig. 4. A: Physical and genetic map of the *ams-fab* region. Restriction sites: P, *Pst*I; Sa, *Sal*I; X, *Xho*I; H, *Hin*dIII; E, *Eco*RI; Ss, *Ssp*I; N, *Nru*I; Ns, *Nsi*I; K, *Kpn*I. **B: Complementation and recombination tests;** Each subclone was introduced into mutant strains deficient in *plsX* or *fabD* function. C = Complementation, R = Recombination, ND = Not determined.

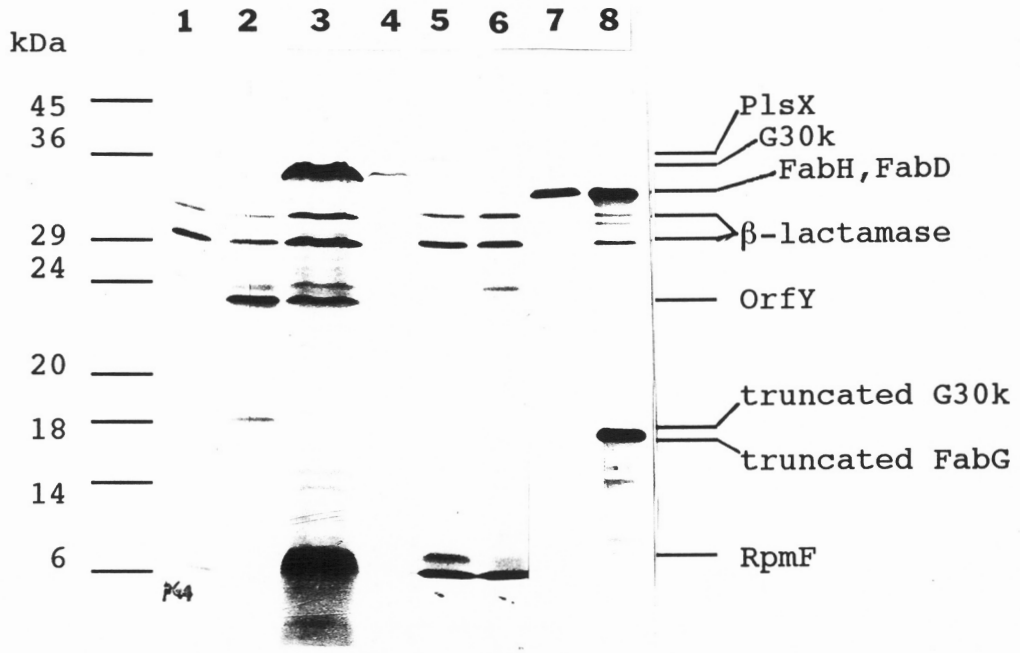


Fig.5. Analysis of proteins synthesized in maxicells derived from HB101. The proteins were labeled with [^{35}S]methionine and analyzed by SDS-PAGE. Lane 1; Proteins synthesized in cells harboring plasmid pGEM-4 were analyzed as control. β-lactamase and precursor of β-lactamase were produced. Lane 2; pWO617 carries OrfY and truncated G30k. Land 3; pWO619 carries G30k, OrfY and RpmF. Lane 4; pWO602 carries G30k and RpmF. Lane 5; pWO603 carries PlsX and RpmF. Lane 6; pWO604 contains *plsX* ORF but downstream of *Hin*dIII (nt 1770). pWO604 did not produce PlsX protein. Lane 7; pWO611 carries FabH. Lane 8; pWO618 carries FabD and truncated FabG

Table 5. Plasmid-directed protein synthesis in maxicells

ORF	gene	subclones	Size (kDa) Predicted	Size (kDa) Observed
1	*orfY*	pWO617 pWO619	21.7	23
2	*g30k*	pWO602 pWO619	19.3	34
3	*rpmF*	pWO602 pWO619	6.4	7
4	*plsX*	pWO603	38.2	35.5
5	*fabH*	pWO611	33.5	32.5
6	*fabD*	pWO618	32.4	32.5

results suggest that ORF4 encodes the PlsX protein and that a promoter element for *plsX* is located upstream of *Hin*dIII (nt 1770) or DNA upstream of *Hin*dIII (nt 1770) is required for the expression of *plsX*. The results of complementation tests in strain TL85 and TL400 were the same but recombination tests using strain TL400 were negative in all subclones as expected for this *recA1* host. The results of recombination tests suggest that the *plsX50* mutation might be located between the *Hin*dIII (nt 1770) and *Eco*RI (nt 1915) sites, because pWO604 rescued the mutation by recombination while pWO605 did not. This was proven at the molecular level by sequence analysis of PCR amplified chromosomal DNA from a *plsX50* mutant strain (Figs. 15-16).

pWO113 and pWO618 complemented the temperature sensitive phenotype of strain L48 deficient in malonyl CoA-ACP transacylase activity, indicating that ORF6 is *fabD*. This was proved so by demonstrating that the MTA activity is encoded by ORF6 and by comparing the ORF6 sequence with other transacylase sequences in GenBank. Strain LW48 (*fabD89 recA1*) grew very slowly and showed negative results in both complementation and recombination tests. This might be due to the fact that strain LW48 was so unhealthy that transformants overproducing MTA could not grow at 42 °C.

Comparison of amino acid sequences of each ORF with protein sequences in GenBank: To gain insight into the function of each ORF, comparison of the

amino acid sequences encoded by each ORF with those in GenBank was performed. ORF1 did not show any significant sequence similarity with the translated sequences in GenBank. ORF2 and ORF3 have been already sequenced (106). The published DNA sequences were exactly the same as our sequences between the *Sal*I (nt 711) and *Hin*dIII (nt 1770) sites. ORF2 encodes a so-called 30 kDa (G30k) protein whose function is not known. ORF3 is *rpmF* which encodes L32, a protein of the large ribosomal subunit (106). The amino acid sequence of ORF4 *(plsX)* showed greatest sequence similarity (34.2% identity) with a protein of undefined function (called ORF2) of *Rhodobacter capsulatus* (107). ORF2 in *R. capsulatus* was flanked by open reading frames of unknown functions whose protein sequences showed significant sequence similarity with our ORF3 (*rpmF*) and ORF5 (*fabH*) (46% and 48% identity, respectively). Although these observations do not solve the enigma of *plsX* function, they do indicate that the juxtaposition of *rpmF, plsX,* and *fabH* in an operon is evolutionarily conserved and may be important for their coordinate regulation. These genes in *R. capsulatus* are located just upstream of a gene encoding an integration host factor-like protein (107). No other protein sequences were found that exhibited significant sequence similarity to the PlsX protein.

The protein encoded by ORF5 in our sequence showed greatest sequence similarity to chalcone synthase (*CHS*), a condensing enzyme from plants (108), with 21% identity and 43% similarity if conservative substitutions are considered

over the entire sequence of ORF5. The second highest score was observed with the NodE protein of *Rhizobium meliloti* (109, 110) with 14% identity and 33% similarity. Previous work (111) had indicated that the sequence of NodE was similar to the *E. coli fabB* gene product (3-ketoacyl-ACP synthase I ; KS I). These observations, along with mapping data indicating that the gene encoding 3-ketoacyl-ACP synthase III is located in this vicinity (112) suggested that ORF5 might encode KS III. ORF5 was shown to encode KS III when extracts from a KS III mutant strain harboring a plasmid containing only ORF5 (pWO114) elevated the level of acetoacetyl-ACP synthase activity (24). Also, KS III protein was overproduced and purified from a strain carrying pWO114 using the T7 RNA polymerase dependent system (24). Purified KS III also catalyzed both acetoacetyl-ACP synthase and acetyl transacylase activities (24). The putative active site cysteine residue of KS III was identified by comparison of active site amino acid sequences of other known condensing enzymes (Fig. 6).

Plasmid clones containing ORF6 (pWO113 and pWO618) complemented the temperature sensitive phenotype conferred by the *fabD*89 (malonyl CoA/ACP transacylase) mutation (Fig. 4). When GenBank was searched for proteins having sequences similar to ORF6, significant sequence similarity was observed in the acyltransferase domains of the multifunctional polyketide synthase of *Saccharopolyspora erythraea* (30% identity) (113), and a segment of the multifunctional 6-methyl salicyclic acid synthase of *Penicillum patulum* (23%

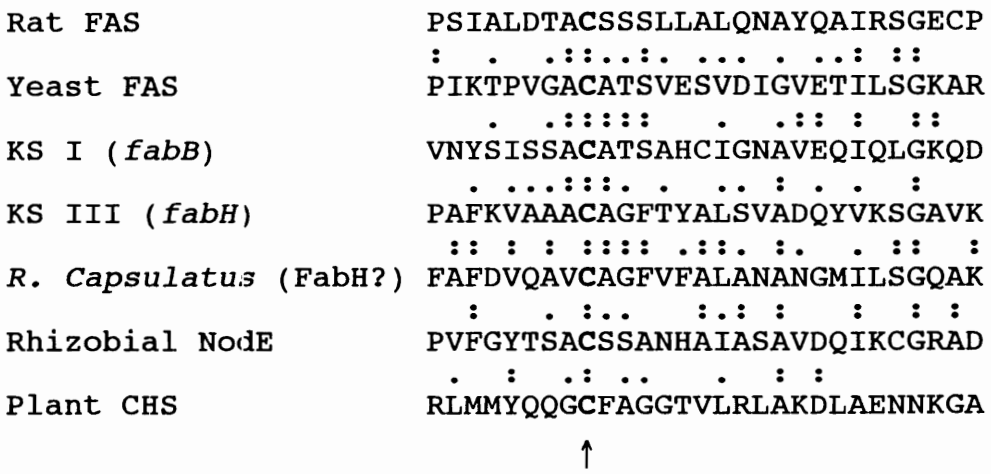


Fig. 6. Amino acid sequence similarity of the active site region of FabH to the condensing enzyme domains of other enzymes. The active site cysteine is indicated by the arrow. FAS: fatty acid synthase, CHS: chalcone synthase. For assignment of conservative substitutions, amino acids were placed in the following groups; GASTP, QNED, FYW, ILVM, HRK.

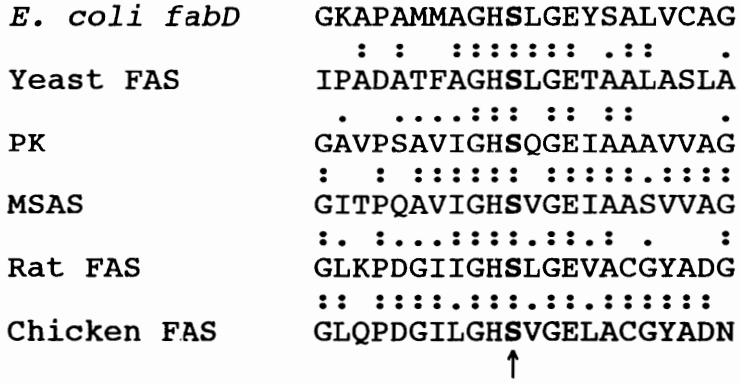


Fig. 7. Amino acid sequence similarity of the active site region of FabD to the acyltransferase domains of other enzymes. The active site serine is indicated by the arrow. FAS: fatty acid synthase, PK: polyketide synthase, MSA: methyl salicyclic acid synthase.

identity) (114). The acyltransferase active site of ORF6 was identified by comparison of active site sequences of other acyltransferases (Fig. 7). The predicted active site sequence contains the amino acids A, G, H, and S that were previously identified as the malonyl binding site of the enzyme by amino acid analysis of a proteolytic ^{14}C-peptide derived from a [^{14}C]malonyl-enzyme intermediate (115). The serine residue of malonyl transacylase was identified as the covalent binding site of malonyl group in the malonyl-enzyme intermediate (115). The molecular mass (32.4 kDa), pI (4.9) and amino acid composition from the calculated amino acid sequence of ORF6 matched those (35.5 kDa, pI=4.65) reported for purified malonyl transacylase (92). Malonyl transacylase activity was measured in extracts from strain L48 (*fabD89*) harboring plasmid pWO618 which contains only ORF6 and strain L48 harboring pGEM-4 vector only. MTA specific activity from pWO618 (0.029 μmol/min/mg) was 3 times higher than that (0.0098 μmol/min/mg) obtained from strain L48 harboring only the vector. Morever, cloning of the *fabD* gene downstream of the *tac* promoter resulted in 155-fold overproduction of MTA activity upon IPTG induction (116). These results indicate that ORF6 is in fact *fabD* (116).

Identification of translation initiation sites: The translation initiation sites of OrfY, PlsX, FabD, and FabG were identified by sequencing 8 to 10 amino acids of the respective N-termini. The N-terminus of FabH was sequenced by Tsay *et*

al. (24). ORFY and truncated FabD and FabG proteins were overproduced by using the T7 RNA polymerase system. Truncated FabD and FabG proteins formed inclusion bodies and were partially purified. Overproduction of the PlsX protein was not possible using the T7 RNA polymerase system. Therefore, a *plsX-lacZ* translational fusion was constructed (see below, Fig. 18) and the resulting protein was partially purified by affinity chromatography. These proteins were subjected to N-terminal sequence analysis. The results of sequencing and the expected amino acid sequences deduced from DNA sequence are shown in Table 6.

The first methionines of OrfY, PlsX, and FabD protein seem to be removed by methionine-specific aminopeptidase in the cell. This is consistent with the observation that Ala, Ser, Gly, Pro, Thr, or Val as second amino acids permit N-terminal Met to be removed (117). The first Met of FabG protein remains intact because the second amino acid is asparagine.

The concentration of each amino acid on the chromatogram was 29-131 pmol for OrfY, 11-37 pmol for PlsX, and 109-303 pmol for FabG. The N-terminal amino acid sequences from amino acid sequence analysis are identical with those predicted from the DNA sequences in the case of OrfY, FabD and FabG proteins. The concentrations of these proteins were high enough to produce strong bands on SDS gels compared to that of the PlsX protein. In the case of the PlsX protein, there was no obvious peak for the second amino acid on the chromatogram, and for the eighth amino acid, the serine peak was more prominant than any other peak

Table 6. N-terminal amino acid sequences of OrfY, PlsX, FabD and FabG

amino acid number	ORF (clones used in analysis)							
	OrfY(pWO625)		PlsX(pWO951)		FabD(pWO115)		FabG(pWO610)	
	AA[1]	DNA[2]	AA	DNA	AA	DNA	AA	DNA
1	(M)[3]	M	(M)	M	(M)	M	M	M
2	P	P	T/M	T	T	T	N	N
3	K	K	?[4]	R	Q	Q	F	F
4	L	L	L	L	F	F	E	E
5	I	I	T	T	A	A	G	G
6	L	L	L	L	F	F	K	K
7	A	A	A	A	V	V	I	I
8	S	S	L	L	F	F	A	A
9	T	T	S	D	P	P		
10			V	V	G	G		
11			M	M	Q	Q		

[1]AA; amino acid sequence determined by chemical analysis.
[2]DNA; amino acid sequence deduced from DNA sequence.
[3]Parentheses indicate M was not detected.
[4]No clear residue was detected.

and the expected aspartic acid peak was almost negligible. The reason for the discrepancy in the sequence obtained from amino acid sequence analysis and that predicted from the DNA sequence for the eighth amino acid of PlsX might be due to; i) Contamination from other proteins because the PlsX protein was not pure enough to produce a sharp band. ii) Effect of protein overexpression on mistranslation. *E. coli* strains engineered to overproduce proteins have been observed to exhibit increased mistranslation by incorporation of a wrong amino acid into the growing polypeptide chain (118). From the N-terminal amino acid sequence analysis it was demonstrated that the intergenic regions between *rpmF* and *plsX, plsX* and *fabH, fabH* and *fabD*, and *fabD* and *fabG* are 80 bp, 67 bp, 15 bp, and 12 bp respectively.

HB101 carrying *g30k* ORF in multicopy plasmid was used to overproduce G30k protein and was subjected to N-terminal analysis. N-terminal sequencing of an overproduced protein thought to be the G30k protein revealed the sequence AEIYNK?G which was not found in any possible reading frame of the *g30k* ORF. This sequence was compared to the translated sequences in GenBank and was found to be identical to the sequence (after removal of the signal sequence) of the *E. coli* major outer membrane protein OmpF (137). So the overproduced ~34 kDa protein was proved to be mature OmpF protein and not G30k protein. The overproduction of this protein might be due to the perturbation of normal cell physiology by introduction of pWO124.

***Possibility that an ORF of unknown function is* tmk:** The *tmk* gene, which encodes thymidine monophosphate kinase, has been mapped near min 24 on the *E. coli* linkage map (119). In order to figure out if any of the ORF's of undefined function (*orfX, orfY, g30k,* or *plsX*) encodes *tmk*, complementation tests were carried out by introducing plasmids carrying each ORF into strain TD105 (*tmk-1*). None of the plasmids (pWO601, pWO602, pWO603, pWO617, or pWO622) corrected the temperature sensitive phenotype of the *tmk-1* mutation on rich media. To map the *tmk* gene more precisely, P1 transduction using well defined Tn*10* genetic markers near *plsX* was performed. Strain TD105 was used as recipient and strain TL212 (*zce-726* ::Tn*10*), strain TL226 (*zce-728*::Tn*10*), and strain TL248 (*zce-727*::Tn*10*) were used as P1 donors. The cotransductional frequencies of the above genetic markers with the *plsX* gene are 83%, 96% and 98% respectively. Based on the resistance of the *tmk-1* mutant strain to TddR, the cotransductional frequency of *tmk* with each Tn*10* marker was measured. The results demonstrated that *tmk* is 99% (106/107) cotransducible with *zce-726*::Tn*10,* 76% (50/74) cotransducible with *zce-728*::Tn*10*, and 70% (78/111) cotransducible with *zce-727*::Tn*10*. These data, along with the complementation tests, suggest that none of the ORF's near *plsX* is *tmk* and that *tmk* is located downstream of *acpP*. It has been reported that *zce-726*::Tn*10* is 98% cotransducible with a *Kan* cassette inserted approximately 570 bp downstream of *acpP* (105).

TRANSCRIPTIONAL ORGANIZATION OF THE *g30k-rpmF-plsX-fab* REGION

The results from the previous section identified 6 ORF's; undefined ORF1 and ORF2 (so called g30k), *rpmF, plsX, fabH*, and *fabD*. The *fabG* and *acpP* genes are located just downstream of the *fabD* gene (105). The *fabH, fabD, fabG*, and *acpP* gene products encode proteins that are involved in fatty acid biosynthesis in *E. coli.* Functionally related genes are frequently cotranscribed as a polycistronic operon in procaryotes as one of the ways to control gene expression coordinately at the transcriptional level. Sequence analysis of the *rpmF-plsX-fab* region demonstrated that the intergenic regions between these genes are short, especially between *fabH* and *fabD* (15 bp) and between *fabD* and *fabG* (12 bp). Search for hairpin RNA secondary structures using the PC/GENE program (HAIRPIN) did not reveal any potential rho-independent transcription terminator sequences downstream of any of the genes. Such sequences contain a G-C rich region capable of forming a RNA stem-loop structure followed by four to eight uridine residues (65). A putative transcription termination site was identified downstream of the *acpP* gene (105). These observations suggest that these genes might be cotranscribed and be coregulated at the transcriptional level and by translational coupling at the translational level. In order to gain insight into how

these genes are organized and regulated, identification of the promoter sites and transcripts of this region was necessary. Therefore in this study, promoters carried by a number of DNA fragments were analyzed and Northern, primer extension, and S1 mapping analysis were performed to characterize the transcripts of this region.

1. Identification of promoters

Promoter activity analysis: A series of restriction fragments was cloned into promoter probe vector pCB267 and β-galactosidase activity was measured to determine the promoter strengths (Table 7, Fig. 8). pWO401, pWO402, pWO404 and pWO497 containing the DNA upstream of *rpmF* showed various levels of promoter activity while pWO412 containing the intergenic region between *rpmF* and *plsX* showed no promoter activity, suggesting that strong promoters are located upstream of *rpmF* and *rpmF* and *plsX* may be cotranscribed. Promoter activities of pWO408 and pWO409 decreased significantly compared to that of pWO404 indicating that transcripts terminate between *Hin*dIII (nt 1770) and *Pst*I (nt 2009) sites. Considering the fact that there is no promoter for *plsX* except those upstream of *rpmF*, a portion of the transcripts originating upstream of *rpmF* may read through the terminator and go into the *plsX* gene. This was confirmed by S1 mapping analysis (see below). Weak promoter activity was detected between *Ssp*I (nt 2381) - *Nru*I (nt 2775) in pWO421 and pWO422. This promoter is upstream of *fabH* and within *plsX* and may be partly responsible for expression of the *fab*

Table 7. Promoter activity analysis

Fusion plasmids	Restricion site (nucleotide position)	β-galactosidase (Miller units)
pWO401	SalI-HindIII (711-1770)	471
pWO402*	SalI-XhoI (711-1215)	106
pWO403**	SalI-XhoI (711-1215)	3.3
pWO404	XhoI-HindIII (1215-1770)	16
pWO407	XhoI-SphI (1215-1618)	4
pWO408	XhoI-PstI (1215-1896)	2.4
pWO409	XhoI-PstI (1215-2009)	0.1
pWO412	Sau3A-TaqI (1543-2121)	0
pWO416	Sau3A-PstI (3777-4487)	0.1
pWO417	EcoRI-HindIII (3569-4042)	3
pWO418	NruI-NsiI (2775-3319)	0.2
pWO419	NsiI-PstI (3319-4487)	0.2
pWO420	EcoRI-SspI (1915-2381)	0
pWO421	SspI-NruI (2381-2775)	22
pWO422	EcoRI-NruI (1915-2775)	22
pWO424	SalI-KpnI (4803-5300)	6
pWO425	PstI-XmnI (4487-5079)	0.6

*; SalI-XhoI fragment is cloned with *g30k* and *lacZ* transcribed in the same directions.

**; SalI-XhoI fragment is cloned with *g30k* and *lacZ* transcribed in the opposite directions.

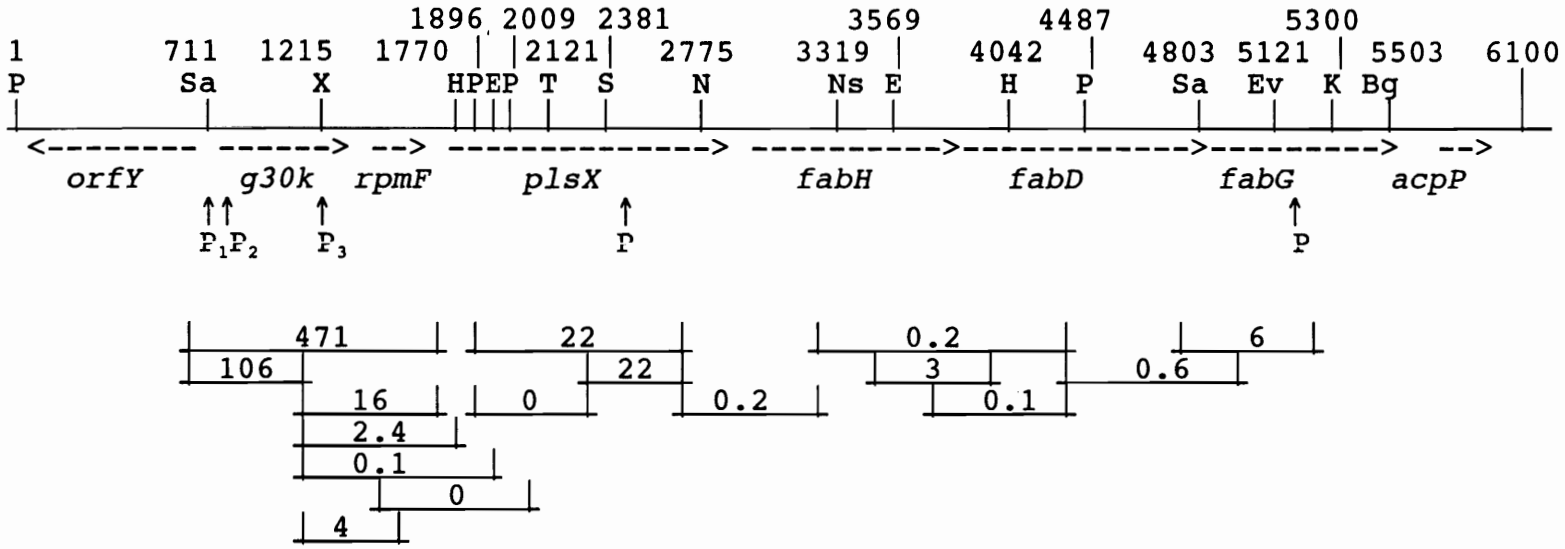


Fig. 8. Promoter locations. Promoter activity was sought by cloning of restriction fragments into promoter-probe vector pCB267 upstream of the promoterless *lacZ* gene. The specific activity of ß-galacatosidase (Miller units) expressed from each potential transcriptional fusion is indicated above each of the cloned fragments. Restriction sites are; P, *Pst*I; Sa, *Sal*I; X, *Xho*I; H, *Hin*dIII; E, *Eco*RI; T, *Taq*I; S, *Ssp*I; N, *Nru*I; Ns, *Nsi*I; Ev, *Eco*RV; K, *Kpn*I; Bg, *Bgl*I. P represents a potential promoter, and P_1, P_2 and P_3 potential promoter sites identified by primer extension analysis.

genes. Very low β-galactosidase activity encoded by pWO417 may be due to a weak promoter upstream of *fabD* or may be an artifact because that of pWO419 is very low. Weak promoter activity was detected in pWO424 but almost no promoter activity in pWO425 suggesting that a weak promoter might be located within *fabG* upstream of *acpP* and be responsible for the expression of *acpP* but not for *fabG*. This was confirmed by Northern analysis (see below). Weak promoter activity detected by pWO403 seems to be a real promoter because OrfY was expressed in maxicells and was overproduced by the T7 RNA polymerase system. Furthermore, the N-terminus of OrfY was identified. Transcription of *orfY* is in the opposite direction relative to that of the other ORF's. Three transcripts were identified by an *orfY*-specific probe in Northern analysis.

Primer extension analysis: Promoter activity analysis demonstrated that a promoter for *plsX* is located upstream of the *Sau*3A (nt 1543) site. Strong promoter activity was detected in the *Sal*I (nt 711)-*Hin*dIII (nt 1770) fragment. To identify the 5' ends of the transcripts upstream of *plsX,* primer extension analysis using reverse transcriptase was performed. Primer extension products were run on a sequencing gel next to ladders produced with the same region of DNA and the same primers so that the location of the 5' ends of mRNAs could be identified directly on the sequence (Fig 9). Major bands appeared at nucleotide position 860 (P_1), 1002, 1003, 1005 (P_2). A weak band at nucleotide position 1267 (P_3) was detected consistently in several experiments. Repeated primer extension

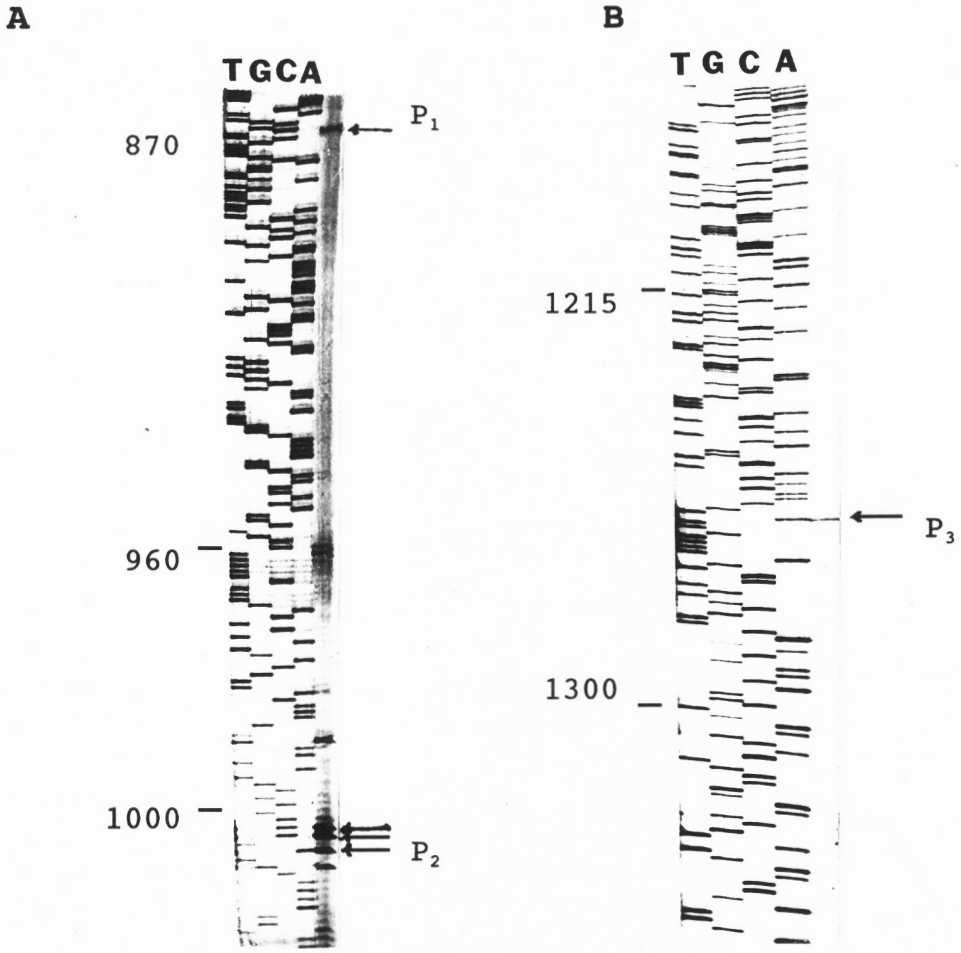


Fig. 9. Primer extension analysis. The 5′ ends of transcripts upstream of *rpmF* were mapped using reverse transcriptase. Sequencing ladders using the same region of DNA and primers were run next to the primer extension products. TGCA refers to the sequencing ladder. Numbers represent the nucleotide position on the sequence (Fig. 3). Transcription initiation sites are indicated by horizontal arrows for promoters P_1, P_2, and P_3. **A;** RNA was isolated from strain HB101(pWO401). Oligonucleotide # 272697 was used as a primer. **B;** RNA was isolated from strain HB101 (pWO105). Oligonucleotide # 236290 was used as a primer.

experiments failed to reveal any other consistent bands downstream of P_3 using these experimental conditions even though Northern analysis demonstrated 2 more transcripts. The P_1 and P_2 sites are identical to those mapped by Tanaka *et al.* (24). P_1 and P_2 initiate transcription upstream of *g30k*. P_3 initiates transcription upstream of *rpmF*. P_1 has good -10 but poor -35 consensus sequences and seems to be responsible for *g30k* because P_2 overlaps with the putative translational start site of *g30k*. P_2 has good -10 and -35 consensus sequences and showed a characteristic ribosomal protein promoter sequence; a G-C rich region immediately downstream of the -10 sequence. This feature was suggested to be important for stringent control (121). Even though the P_3 site does not match with the consensus promoter sequence found in *E. coli,* repeated primer extension analysis always identified this band.

2. Characterization of transcripts

Northern analysis: Thirteen probes used in Northern analysis and the autoradiogram of the transcripts are shown in Table 8 and Fig. 10. The weak bands don't photograph well but are visible on the autoradiogram. The interpretation of the results is given in Fig. 11. A long transcript (~5.2 knt), even though very weak, was detected by the probes downstream of *Sal*I (nt 711) indicating the presence of cotranscripts for the *rpmF-plsX-fab* genes. Probe 1, probe 2, and probe 3 detected a ~4.2 knt transcript. This transcript is very weak

Table 8. Probes used for Northern analysis and the bands detected in autoradiograms.

Probe #	Restriction fragment (position in sequence)	sizes of bands on autoradiogram (knt)
1	HaeIII-SalI (170-711)	4.2, 1.2, 0.8, 0.7
2	SalI-XhoI (711-1215)	5.2, 4.2, 3.7, 1.05, 0.95
3	XhoI-SphI (1215-1618)	5.2, 4.2, 3.7, 1.05, 0.95 0.7, 0.5, 0.4
4	SphI-SspI (1618-2381)	5.2, 4.2, 3.7, 1.05, 0.95 0.7, 0.5, 0.4
5	PstI-NruI (2009-2775)	5.2, 3.7
6	HaeIII-HaeIII(3189-3800)	5.2, 3.7
7	HindIII-SalI (4042-4803)	5.2, 3.7, 2.4, 2.2
8	EcoRV-BglI (5121-5503)	5.2, 3.7, 2.4, 2.2, 0.95, 0.85
9	*acpP* (PCR product of 5824-6066)	5.2, 3.7, 2.4, 2.2, 1.8, 0.95, 0.85, 0.7, 0.6, 0.35
10	XmnI-HindIII(3487-4803)	5.2, 3.7, 2.6, 2.4
11	HindIII-PstI (4803-4487)	5.2, 3.7, 2.6, 2.4
12	PstI-XmnI (4487-5079)	5.2, 3.7, 2.6, 2.4
13	PstI-SspI (2009-2381)	5.2, 3.7

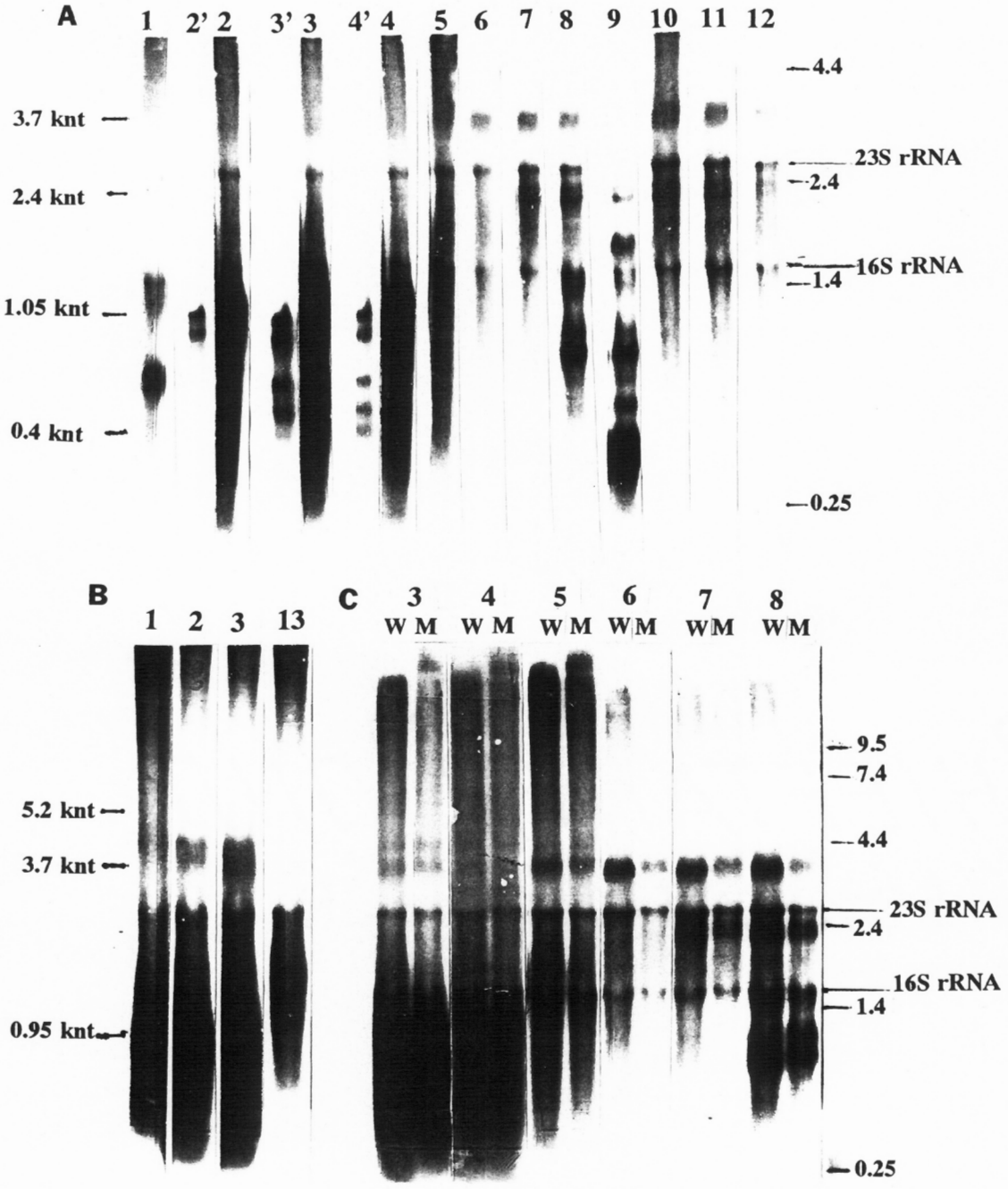


Fig. 10. Northern analysis of *orfY-acpP* transcripts. Total RNA was hybridized with the 13 random primed probes (Table 8). Numbers on the top of each lane indicate the probe numbers. A, B, and C are separate gels. RNA standards (in knt) are provided on the right. **A:** 15 x 20 cm gel. 2', 3', and 4' are short exposures of lane 2, 3, and 4 respectively. **B:** 15 x 15 cm gel. **C:** 15 x 15 cm gel. W, RNA was isolated from strain ECL8 (*plsX*$^+$). M, RNA was isolated from strain TL129 (*plsX50*).

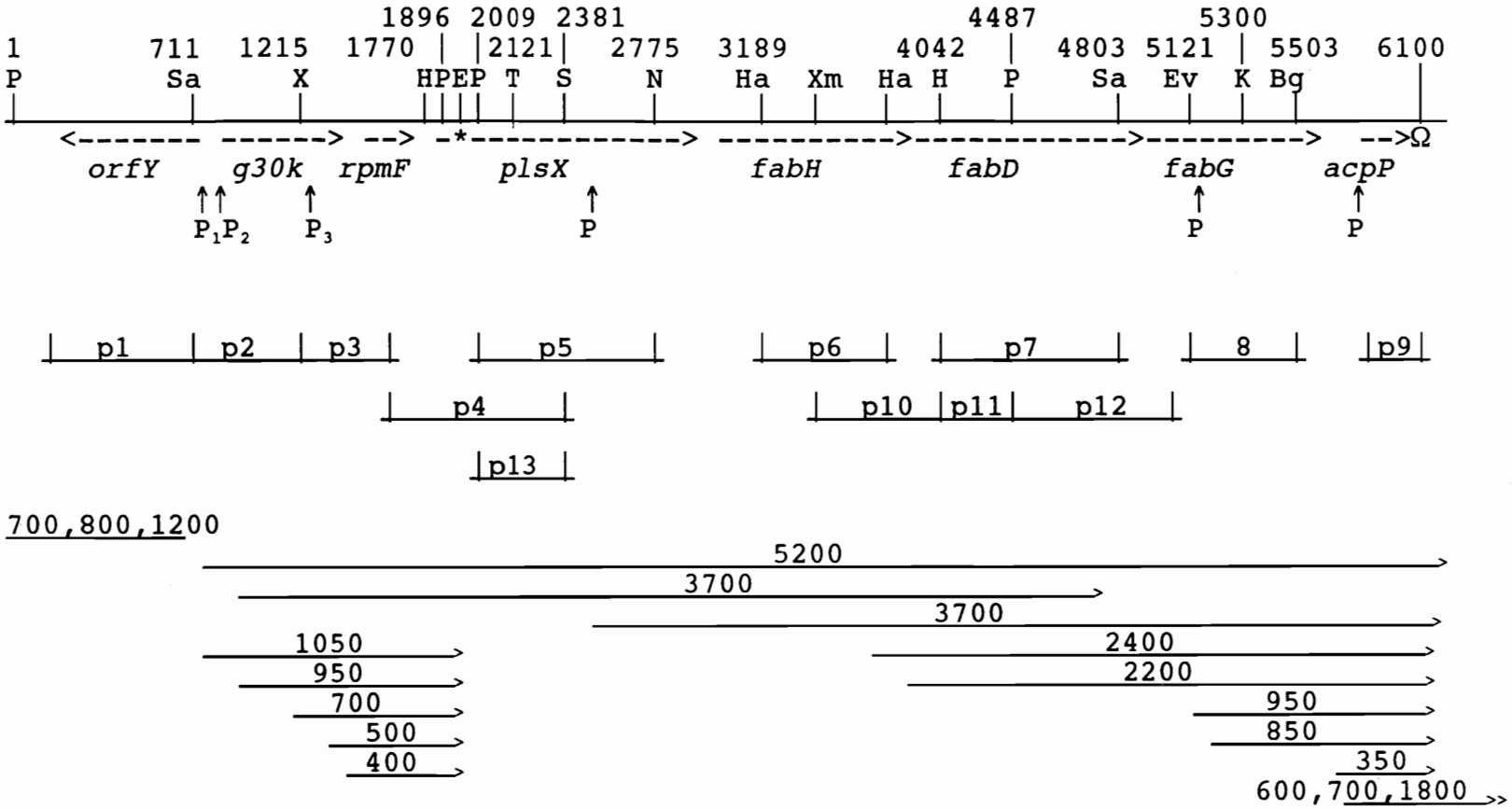


Fig. 11 Interpretation of Northern analysis. Restriction sites are as defined earlier (Fig. 8) and Ha, *HaeIII*; Xm, *XmnI*. p1-p13 are the templates used for random primed probes. Transcripts are drawn with lines. Numbers above the lines indicate approximate lengths. P represents a potential promoter, and P_1, P_2, and P_3 are promoters mapped by a primer extension. The * indicates the potential attenuator and Ω indicates the potential terminator.

for probe 1, a little stronger for probe 2 and probe 3. At this point, it is not clear where it originates and which direction it is transcribed. Transcripts of about 3.7 knt were detected also by the probes downstream of *Xho*I (nt 1215). Probe 1 did not detect this transcript. It is not clear whether probe 2 detected this band or not. So this transcript might originate downstream of *Xho*I (nt 1215) or possibly of *Sal*I (nt 711). This transcript hybridizes weakly to probes upstream of *fabH* and relatively strongly to probes from the *fabH-fabD-fabG-acpP* region. But the size (~3.7 knt) of this transcript is not big enough to cover *rpmF* to *acpP*. The promoter probing study suggested that there is a promoter upstream of *fabH* between *Ssp*I (nt 2381) and *Nru*I (nt 2775). From these data, two similarly sized transcripts (3.7 knt) seem to overlap in this region. One originates upstream of *rpmF* and terminates within or downstream of *fabD* and the other originates upstream of *fabH* and terminates at the potential terminator downstream of *acpP*. So, *rpmF-plsX-fabH* are cotranscribed from a relatively rare 3.7 knt transcript and *fabH-fabD-fabG-acpP* are cotranscribed from a relatively abundant 3.7 knt transcript. No other transcripts except 5.2 knt and 3.7 knt were detected by *plsX* or *fabH* specific probe (probe 5 or probe 6 respectively). The *fabD* (probe 7), *fabG* (probe 8), and *acpP* (probe 9) specific probes detected 2.4 knt and 2.2 knt transcripts. These transcripts were also detected by probe 10 indicating that they originate upstream of *Hin*dIII (nt 4042) and encode *fabD, fabG and acpP*. Promoter probing (see above) did not detect a promoter(s) that could be responsible

for these transcripts. There is a potential RNase E cleavage site around nucleotide position 3755 (Fig. 12). Considering these facts, the 2.2 and/or 2.4 knt transcripts might be the result of processing of long transcripts rather than of promoters. There was a relatively weak promoter activity in pWO424. This promoter activity seems to be responsible for *acpP* rather than for *fabG* because probe 8 detected two extra 0.95 and 0.85 knt transcripts but probe 12 did not. An *acpP* specific probe (probe 9) detected four more 350, 600, 700, and 1800 knt transcripts in addition to those mentioned above. Thus, at least 10 transcripts are responsible for expression of the *acpP* gene. The 350 nt transcript is most abundant and is specific for only *acpP*. Probe 8 did not detect the 600, 700, and 1800 nt transcripts. Hence, these transcripts seem to originate upstream of *acpP* and continue through the *acpP* terminator and into *fabF* which is thought to be located downstream of *acpP* (105).

3. S1 mapping of the attenuator between rpmF and plsX:

Promoter activity (Table 7) around the 5' end of *plsX* (pWO412; nt 1543-2121) is almost zero indicating that there in no apparent promoter which could express *plsX* independently of the promoters upstream of *rpmF*. The promoter activity of pWO409 (nt 1215-2009) decreased significantly compared to that of pWO404 (nt 1215-1770) suggesting that transcripts terminate between the *Hin*dIII (nt 1770) and *Pst*I (nt 2009) sites. Results of Northern analysis also demonstrated

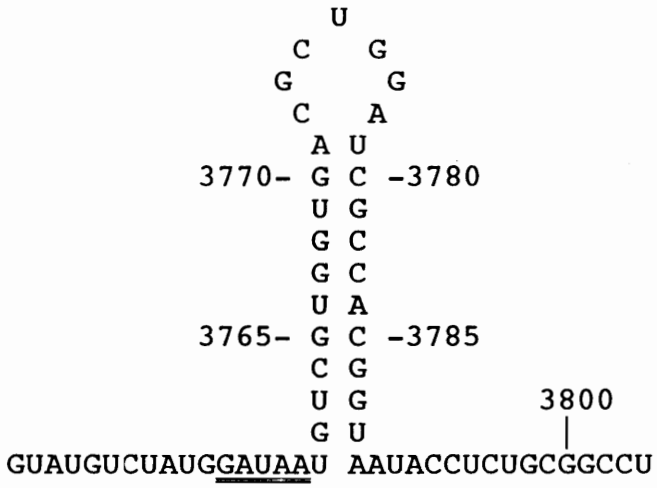


Fig. 12. Potential RNase E cleavage site found upstream of the *fabD* gene. ΔG = -9.1 Kcal/mol. Numbers indicate the nucleotide position in the sequence (Fig. 3). The potential cleavage site is double underlined. RAUUW (R=A or G; W=A or U) is proposed to be the RNase E site consensus sequence (140). All of the processed RNAs have the potential to form a stable secondary structure slightly downstream of this sequence (140).

that most of the transcripts terminate in this region but a small portion of transcripts read through this terminator site producing 5.2 and 3.7 knt transcripts. The results of Northern analysis and promoter probing study indicate that the sequence between *Hin*dIII (nt 1770) and *Pst*I (nt 2009) contains an attenuator, and no promoter for *plsX* in this vicinity. An attenuator in this context is defined as a transcription terminator which can be regulated in a way that some of the transcripts continue on into the downstream *plsX* region. Sequence analysis of this region revealed no good consensus promoter. Instead, mRNA encoded by this region is predicted to assume a stable secondary structure with a potential terminator near the *Eco*RI (nt 1915) site (see below). S1 mapping analysis was performed to locate the transcription termination site and to identify the transcripts that read through this terminator. A DNA probe radiolabeled in the strand complementary to the mRNA (Fig. 13 panel A) was hybridized with total RNA and S1 digested products were run next to a sequence ladder (which was independent of this region). As indicated in Fig. 13, panel B, three products (band A, B, and C) protected from S1 nuclease digestion were expected on a sequencing gel if there is an attenuator between the *Hin*dIII and *Taq*I sites with a portion of the transcripts reading through this attenuator. The longest band A would be the result of reassociation of the full-length probe (406 nt) with its template DNA. The expected size of band A is 406 nt (355 nt *Hin*dIII-*Taq*I fragment + 34 nt M13 fragment between *Acc*I and universal primer + 17 nt universal primer). Band B has

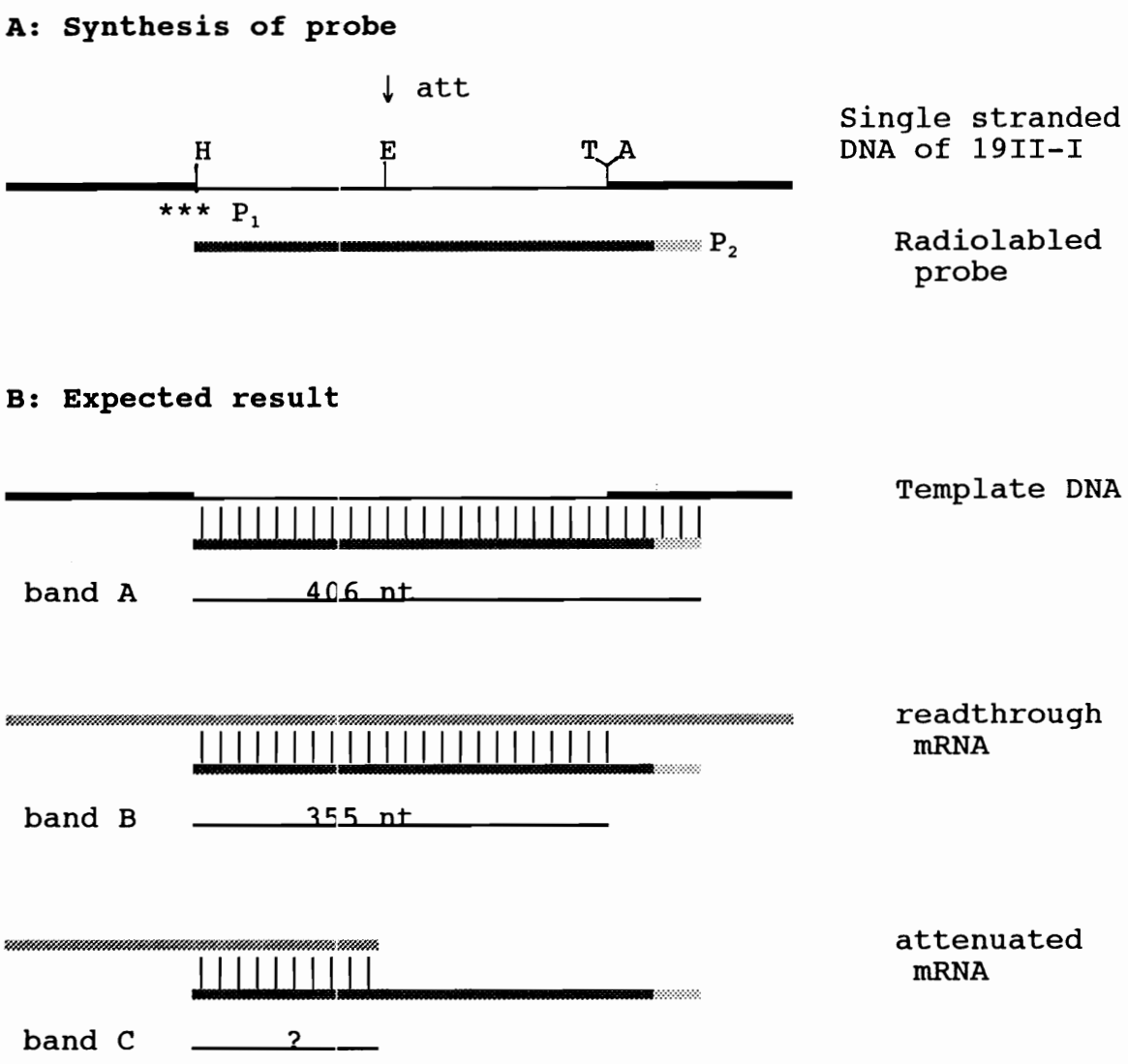


Fig. 13. S1 mapping strategy. Restriction sites; H, *Hin*dIII; E, *Eco*RI; T, *Taq*I; A, *Acc*I. **A:** Radiolabled probe (406 nt) complementary to the mRNA was made using M13mp19II-1 as template. Synthetic primer P_1 was annealed to single stranded DNA of 19II-1. After digestion with *Hin*dIII, uniformly radiolabled single strand DNA was synthesized by using universal primer P_2 and Seqenase. **B:** Bands A, B, and C are expected to be generated after S1 digestion if portions of transcripts read through the potential attenuator site (near *Eco*RI). Band A; reassociation with template. Band B; hybridization with readthrough mRNA. Band C; hybridization with attenuated mRNA at the attenuator.

an expected size of 355 nt (*Hin*dIII-*Taq*I fragment) and would be the result of hybridization of the probe with the mRNA that read through the attenuator site. The smallest band C would be the result of hybridization of the probe with the mRNA that terminated at the attenuator site. The bands protected by S1 digest are shown in Fig. 14, panel A. The size of each band deduced from the sequencing ladder is ~408 nt, ~354 nt, 148 nt, 149 nt, and 151 nt. The biggest band (~408 nt) is the expected size of band A and the middle band (~354 nt) is the expected size of band B. The smallest (triplet) band is the expected band C which locates the attenuator site just downstream of the *Eco*RI (nt 1915) site. The 3' ends of transcripts terminated at the attenuator are positioned at nt 1918, nt 1919, and nt 1921, as deduced from the sizes of the three bands C (Fig. 14, panel B). The fact that band B was detected by S1 mapping strongly indicates that a portion of the transcripts originating upstream of *Hin*dIII (nt 1770) continue into the *plsX* gene. This result is consistent with the results of Northern and promoter probing analysis and indicates that *rpmF* and *plsX* comprise an operon.

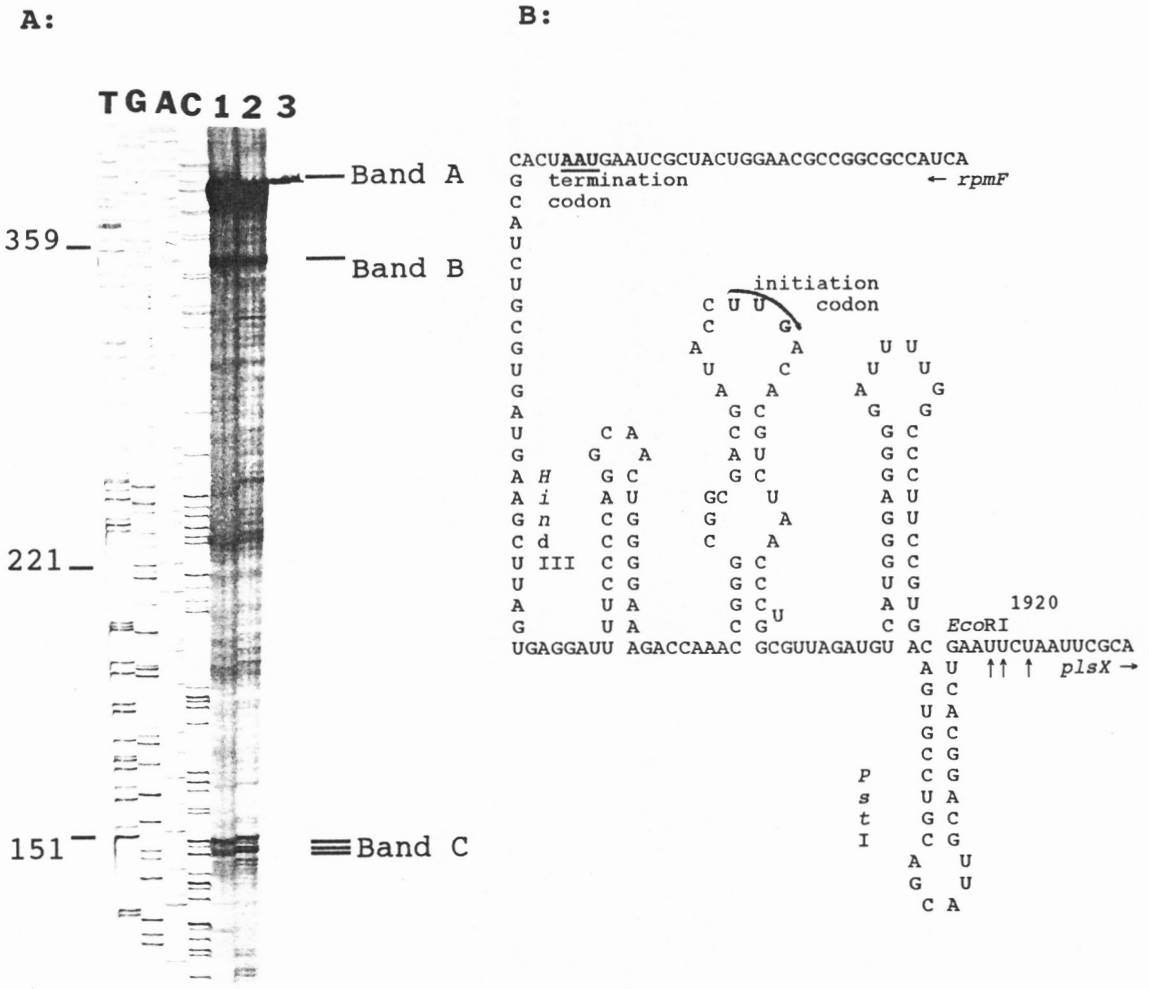


Fig. 14. S1 analysis of the 3′ end of the transcripts originating upstream of *plsX*. **A;** S1 protected bands were run next to the independent sequencing ladder (19W-6 with universal primer), which was used as size standard and is indicated as T, G, A, and C. Probe was hybridized with total RNA from strain ECL8 (lane 1) or from strain TL129 (lane 2). Uniformly radiolabeled single stranded probe was run on lane 3. Numbers indicate the sizes of bands. From the sizes of bands C, 3′ ends of the transcripts were located at nt position 1918, 1919, and 1921. **B;** Secondary structure found in a potential attenuator. Translation termination codon of *rpmF* and initiation of *plsX* are underlined. The vertical arrows indicate the 3′ ends of transcripts.

CHARACTERIZATION OF THE *plsX50* MUTATION

The *plsX50* mutation is required along with the *plsB26* mutation for the conferral of a glycerol-3-phosphate auxotrophic phenotype in *E. coli* (37). In order to gain insight into the function of the *plsX* gene product, the molecular basis of the *plsX50* mutation was sought.

1. Identification of the plsX50 *mutant allele*

Chromosomal DNAs from strain ECL8 (*plsX*$^+$) and strain TL85 (*plsX50*) were amplified by using PCR reactions and cloned into M13mp19 and M13mp18. The entire *plsX50* mutant allele was sequenced using 4 primers (Table 2; # 310398, 016509, 054362, and universal primer). The *plsX50* mutation was identified as the deletion of a single base pair (T at nt 1848, Fig. 15). This deletion was found in both strands of two independently amplified *plsX50* DNAs. The location of *plsX50* is consistent with the results of recombination tests which localized the *plsX50* mutation between the *Hin*dIII (nt 1770) and *Eco*RI (nt 1915) sites (Fig. 4).

The location of the *plsX50* mutation in the secondary structure found in the 5' end of *plsX* (Fig. 16) suggests that the mutation may play a role at the transcriptional or at the translational level. This deletion may stabilize the proposed secondary structure at the attenuator site, increasing the transcription termination at the attenuator, and may decrease the level of the *plsX* transcript in *plsX50* strains. Five potential translation initiation codons of *plsX* are located

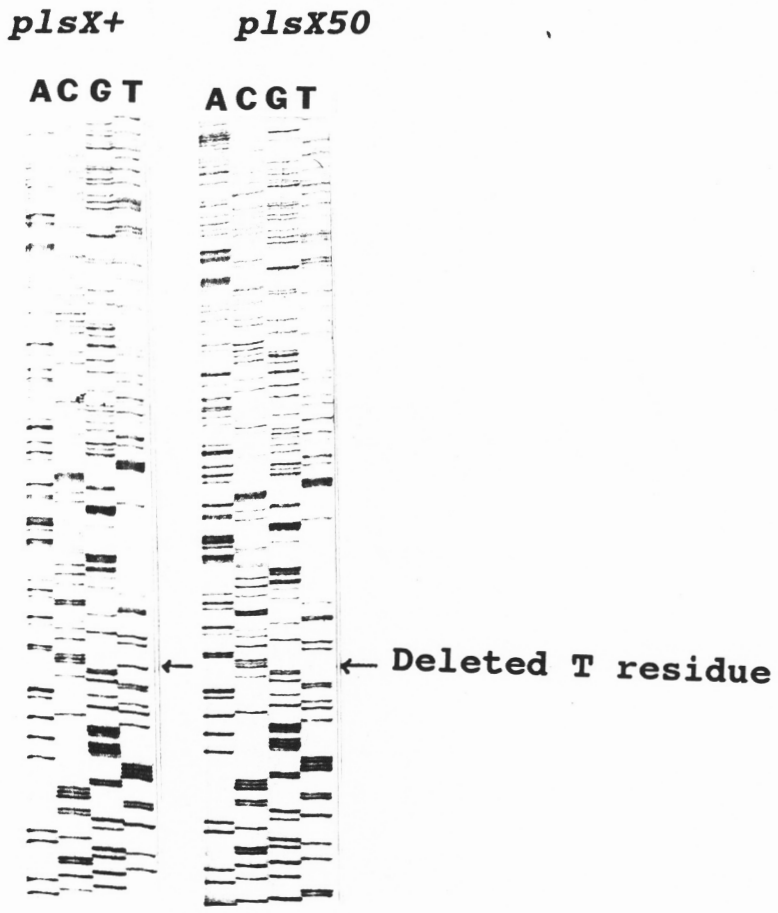


Fig.15. Identification of the *plsX50* mutation. The *plsX50* mutation was identified as the deletion of the T nucleotide at position 1848 by sequencing of PCR-amplified chromosomal DNA. The arrow indicates the T residue deleted in the DNA from the *plsX50* strain.

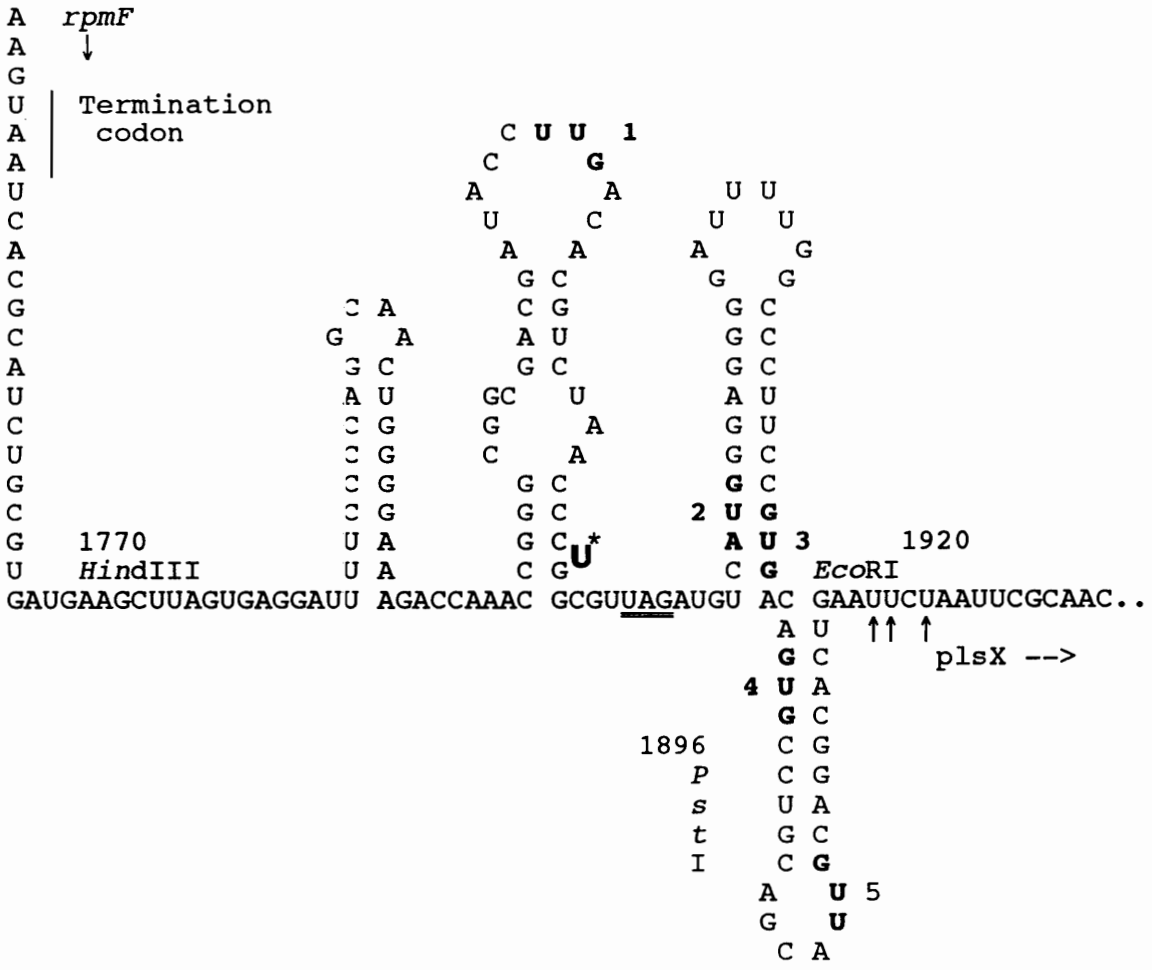


Fig. 16. Potential secondary structure found in the intergenic region between *rpmF* and *plsX*. Potential translation initiation codons in the correct reading frame for *plsX* are indicated in bold and numbered 1-5. The first initiation codon (UUG) was proved to be the correct one by N-terminal sequencing of the *plsX-lacZ* fusion protein. The * indicates the U residue which is deleted in the mRNA from *plsX50* strains. Deletion of U residue results in the termination of *plsX* translation at the UAG stop codon marked by double underline. The 3′ ends of transcripts mapped by S1 analysis are indicated by small vertical arrows (↑).

within the secondary structure (Fig. 16) where the *plsX50* mutation occurs. The second possibility is that if the first proposed translation initiation site (UUG at nt 1832) is the real one, then translation of the *plsX* protein in *plsX50* strain would terminate at the UAG termination codon (nt 1854) after translation of 7 codons. This would result in the absence of the PlsX protein in *plsX50* strains. The third possibility is that if the deletion influences the efficiency of translation by changing the secondary structure around the ribosome binding site of the second potential initiation codon, then translation of *plsX* would be affected in *plsX50* strains. To test these possibilities, transcriptional and translational fusions of *plsX*$^+$ and *plsX50* DNA with the *lacZ* gene were made and the expression of β-galactosidase was measured.

2. Effect of plsX50 *mutation at the transcriptional level*

Transcriptional fusions: The *Sal*I (nt 711) - *Pst*I (nt 1896) and *Sal*I (nt 711) - *Pst*I (nt 2009) fragments from both *plsX*$^+$ and *plsX50* DNAs were cloned into improved promoter probe vector pTL61T (84) upstream of the promoterless *lacZ* gene (pWO903, pWO904, pWO905, and pWO906). The pTL61T containing *Sal*I-*Hin*dIII fragment (nt 711-1770) is pWO902. A synthetic oligonucleotide (Table 2, # 091949) was used to sequence and verify that these clones were correct. pWO902-pWO906 and pTL61T were introduced into strain TL73 (Δ*lacZ recA1*)

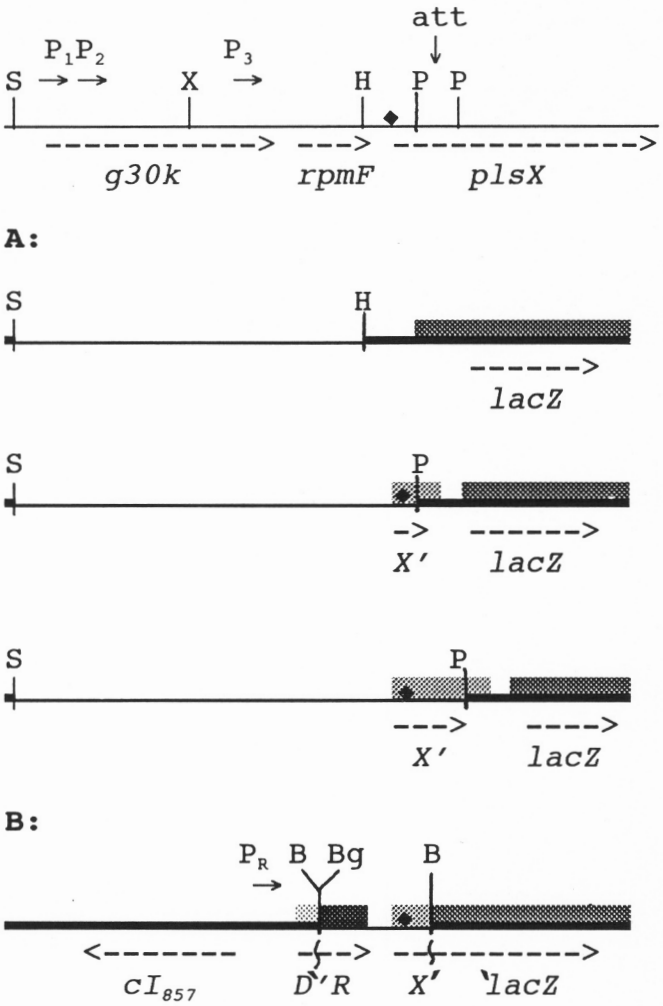
and β-galactosidase activities were measured (Fig. 17). The results demonstrated that the *plsX50* mutation decreases the level of transcription into *plsX* by a small amount. The β-galactosidase activities of pWO904 and pWO906 (*plsX50*) are 89% and 92% of pWO903 and pWO905 (*plsX*$^+$) respectively.

It was not anticipated that the β-galactosidase activity of pWO903 (nt 711-1896) is higher than that of pWO902 (nt 711-1770), which suggests there may be another promoter between *HindI*II and *Pst*I (nt 1770-1896). However, the results of Northern analysis and the promoter probe study exclude the possibility of a promoter specific for *plsX*.

The results obtained with the transcriptional fusions may be used to estimate the efficiency of the attenuator. The β-galactosidase activity of pWO905 is 72% of that of pWO902 and 49% of that of pWO903. This result suggests that approximately 50-70% of transcripts originated upstream of *rpmF* read through the attenuator and go into the *plsX* gene.

3. Effect of plsX50 *mutation at the translational level*

Translational fusions: Plasmid pSH76 (85) which has the temperature inducible strong λ P_R promoter upstream of the *lacZ* gene was used as a vector to make translational fusions (Fig. 18). Two synthetic oligonucleotides (Table 2. # 091568 and # 091971) were used as primers to amplify DNA fragments containing



strain	*plsX*	β-gal
pWO902		12,200
pWO903	+	17,800
pWO904	-	15,800
pWO905	+	8,800
pWO906	-	8,100
pWO951	+	71.3/0.05
pWO952	-	1.14/0.03

Fig. 17. Effect of *plsX50* on the expression of the *plsX* gene at the transcriptional (A) and at the translational (B) level. Restriction endonuclease sites are: S, *Sal*I; X, *Xho*I; H, *Hin*dIII; P, *Pst*I. The ◆ indicates the location of the *plsX50* mutation and "att", the position of the attenuator. Each gene is symbolized as followes: *D, glpD; R, rpmF; X, plsX*, where the prime indicates a missing portion of a gene. Thick lines represent vector DNA (A; pTL61T, B; pSH76) and thin lines represent insert DNA. Fusion points are indicated by vertical lines. P_1, P_2, P_3, and P_R represent promoters 1, 2, and 3 upstream of *rpmF* and promoter P_R of λ respectively. + indicates *plsX-lacZ* and - indicates *plsX50-lacZ* fusions. β-galactosidase activity corrected by the basal level (370 for pTL61T) is indicated in Miller units under β-gal. In the case of pWO951 and pWO952, the activity is represented as induced (42°C)/non-induced (30°C).

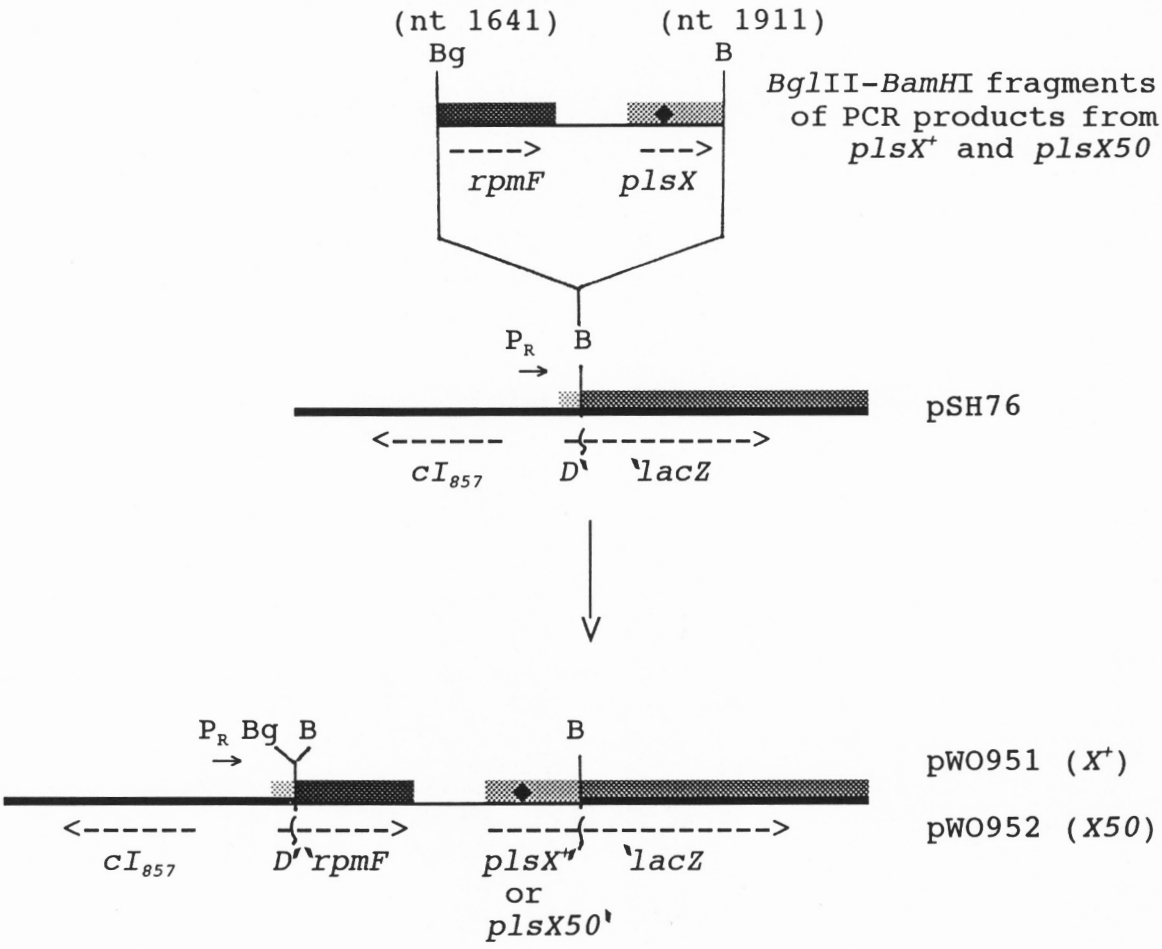


Fig. 18. Construction of translational *plsX*$^+$*-lacZ* (pWO951) and *plsX50-lacZ* (pWO952) fusion plasmids. *plsX*$^+$ DNA from pWO102 and *plsX50* DNA from pWO624 were PCR amplified. *Bgl*II-*Bam*HI fragments of PCR products were cloned into *Bam*HI site of pSH76. The ◆ indicates the location of the *plsX50* mutation. Restriction sites are: Bg, *Bgl*II; H, *Hind*III; B, *Bam*HI. *D'*, *X*$^+$*'*, and *X50'* represent portions of *glpD*, *plsX*$^+$, and *plsX50* respectively, where the prime indicates a portion of the gene.

the potential translation start codons of *plsX*. pWO102 (*plsX*$^+$) and pWO624 (*plsX50*) were used as templates for PCR reactions. PCR primers were designed so that *Bgl*II and *Bam*HI sites were created at the 5' and 3' ends respectively to clone amplified DNA fragment into the unique *Bam*HI site of pSH76. In order to keep the original space between the *rpmF* and *plsX* genes, the 5' PCR primer was designed such that translation started upstream of *plsX* would be terminated by the *rpmF* termination codon. To prevent the attenuation that occurs within the *plsX* gene, the 3' PCR primer was designed to be annealed with the 5' half of the secondary structure at the attenuator site and maintain translation of *plsX* in frame with the *lacZ* gene. By restriction mapping and sequence analysis using primer # 091568 (Table 2), the desired translational fusions with *plsX*$^+$ (pWO951) and *plsX50* (pWO952) were identified.

pWO951 (*plsX*$^+$) and pWO952 (*plsX50*) were introduced into strain TL73 and β-galactosidase activities were measured under inducing and non-inducing conditions to determine the effect of the *plsX50* mutation on the translation of *plsX*. Cells were grown at 30 °C until cell density reached A_{600} ~ 0.4. Half of the culture was transferred to 42 °C. After growing the cells at 30°C or 42 °C for 2.5 hours more, β-galactosidase activities were measured (Fig. 17, panel B). The β-galactosidase activity of pWO952 (*plsX50*) is ~63 times lower than that of pWO951 (*plsX*$^+$) at induced condition indicating that the *plsX50* mutation affects the expression of the *plsX* gene at the translational level. The β-galactosidase

activity of pWO951 increased 1400 fold when the λ P_R promoter of pWO951 was activated by increasing the temperature while that of pWO952 increased 38 fold. These results suggest that even though the λ P_R promoter strengths are the same in both pWO951 and pWO952, translation in the case of *plsX*$^+$ is much more efficient than that of *plsX50.*

The effect of the *plsX50* mutation at the translational level was confirmed by N-terminal sequence analysis of the PlsX protein. Five potential translation initiation sites (Fig 15) are located in the secondary structure found in the intergenic region between *rpmF* and *plsX*. Initiation from one of these codons would result in production of proteins ranging in the size from 38.2 kDa (start site 1) to 35.8 kDa (start site 5). A 35.5 kDa protein was identified as the *plsX* gene product in maxicells (Fig. 5). The 6th potential start codon is 190 bp downstream of the 5th and would produce a 29 kDa protein. The N-terminal amino acid sequence analysis (Table 6) demonstrated that the first potential translation initiation codon (UUG at nt 1832) is utilized. Therefore, the *plsX50* mutation would result in a frame shift nonsense mutation by deletion of a single nucleotide. Because translation is initiated upstream of the *plsX50* deletion, translation of the PlsX protein will stop at the UAG stop codon after 7 codons are translated (Fig. 16). Intact PlsX protein will not be produced in *plsX50* strains. Furthermore, the *plsX50* mutation is expected to prevent translation of the *plsX-lacZ* translational fusion protein, which was observed (Fig. 17).

DISCUSSION

SEQUENCING AND IDENTIFICATION OF EACH ORF

Nucleotide sequence of orfY-g30k-rpmF-plsX-fabH-fabD

The *plsX* gene maps near minute 24 on the linkage map of *E. coli* (9, 37). Approximately 4,900 base pairs of DNA surrounding the *plsX* gene was sequenced in our lab (Fig. 19, from coordinates 1,163.3 to 1,168.2 kb). The DNA sequence obtained overlapped partly with the neighboring sequences in GenBank and thus closed a gap in the sequence of the chromosome (139). A map of the region based on the merged nucleotide sequences is shown in Fig. 19, panel A. The physical map of Kohara *et al.* (9; Fig. 19, panel B) is in relatively good agreement with the restriction map predicted by the nucleotide sequence, except the *Hin*dIII and *Eco*RV sites were missing in the *rne* (*ams*) region.

The left-most DNA sequence contains the *Pst*I (nt 1) site within *orfX* (104). Knowledge of the complete nucleotide sequence allowed translation of *orfX* to a termination codon at nt 108 which would result in a protein of 320 amino acids with a predicted molecular weight of 36,008. This protein may be the 31 kDa protein identified by Claverie-Martin *et al.* (103, 104). The right-most DNA

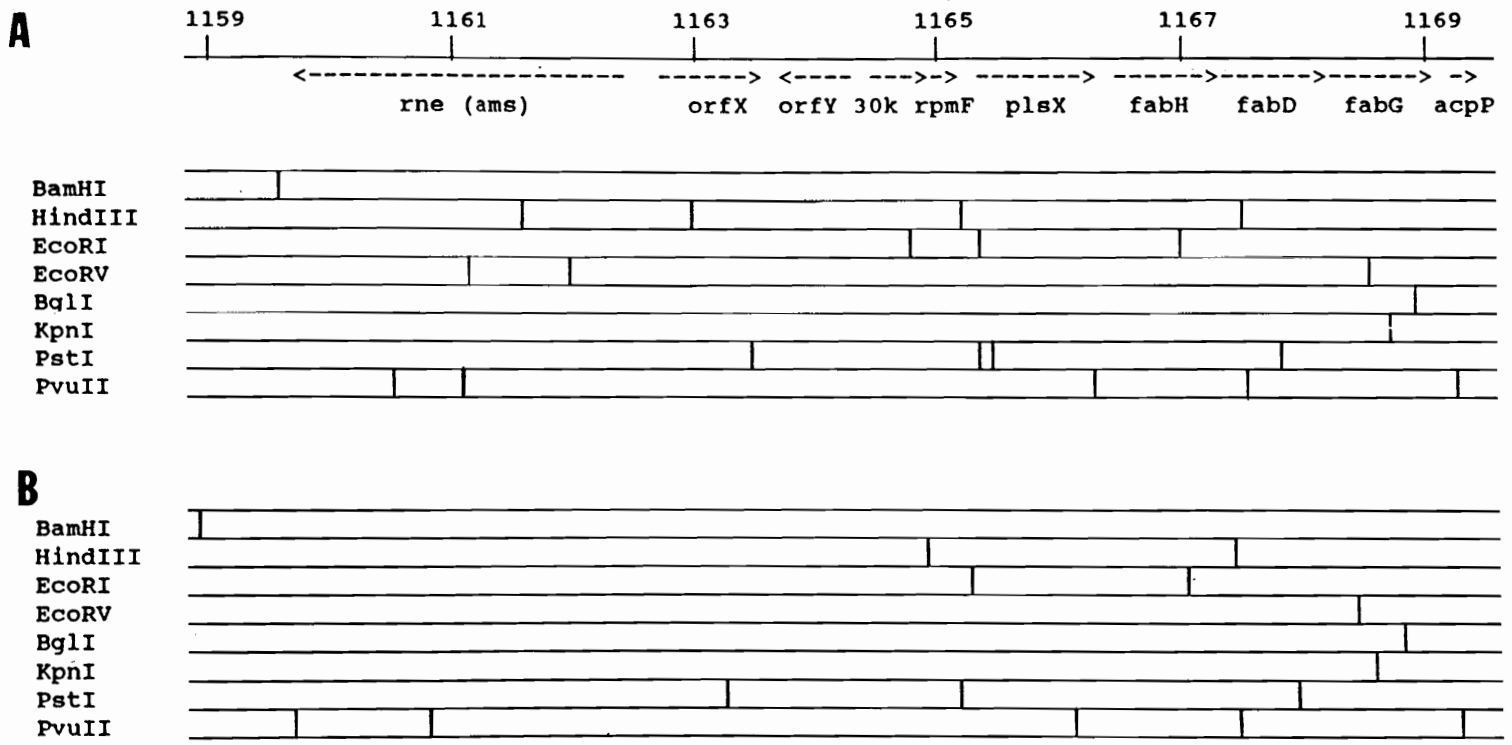


Fig. 19. Physical map of the *rne(ams)-rpmF-plsX-fab* region of the *E. coli* chromosome. **A:** The scale of Kohara *et al.*(9) is given at the top in kilobase pairs. The restriction map is based on the nucleotide sequence, with the positions of the genes indicated above the map. **B:** The physical map of Kohara *et al.*(9) is reproduced

sequence contains the *Sal*I (nt 4803) site within *fabD* (105, 116). Translation of *fabD* terminates 64 base pairs downstream of the *Sal*I (nt 4803) site.

With the closure of this gap in the nucleotide sequence, a continuous sequence of 10,113 base pairs of DNA in this region is now complete. The positions of all of the genes are known and all of the gene products have been identified.

Characterization of each ORF

orfY: *orfY* is composed of 194 amino acids with a predicted molecular weight of 21,691 and pI value of 5.8. A protein of 20 - 23 kDa was identified in the maxicell system and an overexpression system using T7 RNA polymerase. The concentration of the protein in both systems was not high compared to the G30k, RpmF, FabD or FabG proteins, suggesting that translation of *orfY* is relatively inefficient. *orfY* is transcribed divergently from the *g30k-plsX-rpmF-fab* genes.

g30k: *g30k* ORF encodes a protein of 173 amino acids with a predicted molecular weight of 19,315 and pI value of 4.2 if the first potential translation start codon (AUG at nt 1005) is used. The product of *g30k* in the maxicell system migrates aberrantly on SDS-polyacrylamide gel electrophoresis where a protein of 34 kDa (Fig. 5, lane 3 and lane 4) was produced. Truncation of the *g30k* ORF at *Eco*RI (nt 1341) resulted in loss of the 34 kDa protein and production of a new 22 kDa protein instead of a 13 kDa protein deduced from the DNA sequence (Fig. 5,

lane 2). The reason for the aberrant migration of G30k protein on SDS-gels is unknown.

The expression of G30k protein seems to be very efficient as seen from the fact that the protein encoded by *g30k* is expressed at high levels in the maxicell system. Also, the G30k protein is overproduced by multicopy plasmid in strain BL21(DE3, pWO124) and can be identified on SDS-gels without IPTG induction. The high level of G30k expression seems to be attributed to its strong promoter(s). The first (nt 1,005) and the second (nt 1,125) potential translation initiation codons do not have any obvious SD consensus sequence. If the first initiation codon is used, the transcription start site of promoter 2 overlaps with the translation start site of *g30k*.

Introduction of the *Sal*I-*Hin*dIII (nt 711-1770) fragment in multicopy plasmid into a cell appears to be very toxic, significantly decreasing the cellular growth rate. Overproduction of G30k protein seems to perturb the normal cell physiology because strain HB101 harboring pWO124 overproduced OmpF protein at the expense of OmpC protein. OmpF and OmpC are two of the major outer membrane porins. The ratio of OmpF/OmpC is inversely related to the osmolarity of the growth medium (138). Regulation of the respective genes is controlled at the transcriptional level by the envZ/ompR two component system (138).

From the result that *g30k* is cotranscribed with the ribosomal protein *rpmF*, the *g30k* gene product may be involved in an essential cellular process.

rpmF: *rpmF* encodes ribosomal protein subunit L32 which is assembled into the ribosome at a late stage (123). L32 is composed of 57 amino acids and has a predicted molecular weight of 6,446. The pI value of L32 is 11.6 which is typical for that of a ribosomal protein. Multiple promoters are responsible for the expression of *rpmF*, and sequences matching the promoter (P_2) and ribosome binding site consensus sequences are obvious, which would ensure the high level expression of *rpmF*.

plsX: The function(s) of *plsX* still remains to be defined, although some progress has been made. PlsX is composed of 356 amino acids and has a predicted molecular weight of 38,196 and pI value of 9.7.

PlsX protein (intact or truncated) could not be overproduced by the T7 RNA polymerase system and was not detectable on SDS-gels. Also, translation of the *plsX-lacZ* fusion protein expressed from a strong phage λ promoter was inefficient so that the *plsX-lacZ* fusion protein comprised less than 5% of the total protein after 1,000 fold purification. Large amounts of PlsX protein seem to be toxic to the cell, because pWO601 (encoding G30k, RpmF and PlsX) which has strong promoters for *plsX* could not be stably propagated as a multicopy plasmid due to spontaneous deletion or extremely low growth rate. Cloning of the *Hin*dIII-*Hin*dIII fragment (nt 1770-4042), which contains the *plsX* and *fabH* ORF's, into pBluescript could not be achieved although cloning of *plsX* alone (pWO102) or *fabH* alone (pWO114) was feasible.

The low level of PlsX protein appears to be maintained by inefficient translation as well as transcription attenuation. It appears that most of the transcripts which originate from strong and multiple promoters upstream of *plsX* are attenuated (nt 1918) just downstream of the translation initiation codon of *plsX* (nt 1832). If translation of *plsX* were efficient, then plenty of truncated PlsX protein would be produced. Inefficient translation of *plsX* is likely to be the result of three factors; use of an unusual translation initiation codon, a poor Shine-Dalgarno sequence in the ribosome binding site, and secondary structure around the ribosome binding site and initiation codon.

The translation initiation codon of *plsX* is UUG which is rarely found in *E. coli*. In prokaryotes, more than 90% of translation initiation codons contain the AUG triplet. The remainder contain GUG (~8%), UUG(~1%) and a very few examples of AUA and AUU (124). Peabody suggested two potential advantages in the ability to initiate translation at sites other than AUG (125); 1) Since initiation at a non-AUG triplet is generally less efficient than at AUG, it could provide a means for negatively modulating the synthesis of a protein that is required (or tolerated) only in small quantities. 2) Initiation at a non-AUG triplet situated upstream of an initiator AUG provides a mechanism for the production of more than one protein from a single mRNA. In case of *plsX* the former seems more likely because DNA harboring *plsX* (pWO603) produced only a 35.5 kDa protein in the maxicell system. Also, the *plsX50-lacZ* translational fusion plasmid

(pWO952) expressed a very low level of β-galactosidase under inducing conditions, which means that translation initiated at the first initiation codon stops at UAG (nt 1854) and the other four potential codons, all located downstream of nt 1854, are not used. Translation initiated at the 6th potential start codon would produce a 29 kDa protein which could be discriminated, if it were produced, from the 35.5 kDa protein identified in maxicells.

Usually ribosome binding sites provide for more than three (including G:U pairs) uninterrupted base pairs between the 3' end of the rRNA and the mRNA. The Shine-Dalgarno (SD) sequence (5'UAAGGAGGUGA3') is found within the region -15 to -2 relative to the initiation codon. The degree of base pairing probably affects the affinity of the ribosome for the mRNA and hence the efficiency of translation. The Shine-Dalgarno region responsible for *plsX* expression is not obvious, where GGGCGG appears to be the most probable candidate but C is present instead of A between the conserved G's.

There is a strict correlation between the stability of a local secondary structure in the ribosome binding site and the efficiency of translation (126). Shielding the SD signal, initiation codon, or both in a secondary structure strongly reduces translation (124). Part of the SD sequence of *plsX* seems to be sequestered by the potential secondary structure in the ribosome binding site. The initiation codon of *plsX* is not directly involved in forming a secondary structure. But the overall secondary (or tertiary) structure around ribosome binding site might

decrease the efficiency of translation of *plsX*.

fabH: The gene downstream of *plsX* was identified as *fabH* encoding β-ketoacyl-ACP synthase (KS III) in collaboration with Tsay *et al.* (24). *fabH* encodes a protein of 317 amino acids with a predicted molecular weight 32,417 and pI value of 4.7. KS III selectively catalyzes the formation of acetoacetyl-ACP from acetyl-CoA and malonyl-ACP (18). Its position at the beginning of the biosynthetic pathway suggested that it might play a role in governing the total rate of fatty acid production (24). But the fact that the *fabH1* mutant does not have any growth phenotype and the resulting decreased amount of synthase III does not change the fatty acid composition significantly suggests that KS III might not be an essential enzyme in fatty acid biosynthesis.

Comparison of the predicted FabH polypeptide sequence with those in Genbank revealed that FabH was most closely related to plant chalcone synthase (108). In several plants, chalcone synthase (CHS) catalyzes the condensation reactions in the formation of some pigmented product. Interestingly, neither ACP nor specific acyltransferases appeared to be involved: all of the substrates for chain building are the CoA esters of the building units rather than ACP-linked residues (127). The sequence of FabH was also similar to that of the NodE protein from *Rhizobium* species. The *nod* genes are involved in the formation of nodules during plant symbiosis in *Rhizobium* species. A subset of nod genes, *nodE, nodF,* and *nodG* determine the host-specificity of the process. It is proposed that *nodE*

encodes a condensing enzyme, *nodF* an acyl carrier protein and *nodG*, where present (*R. meliloti*), a keto-reductase. These genes are involved in the biosynthesis of an unusal fatty acid (128) which is added to a nodulation factor produced by the bacterial cell giving it the host-specificity.

During the condensation reaction, the acyl-group is attached to the -SH group of cysteine of the synthase, forming an acyl-enzyme intermediate (2). The FabH protein contains 5 cysteine amino acids. One of these, Cys (nt 3303) has an adjacent amino acid sequence motif with a high degree of homology with the active cysteines of other known condensing enzymes (Fig. 6). These observations point to this Cys as the active site cysteine of β-ketoacyl-ACP synthase III.

fabD: The gene downstream of *fabH* was identified as *fabD* encoding malonyl-CoA/ACP transacylase (MTA) in collaboration with Magnuson *et al.* (116). *fabD* encodes a protein of 309 amino acids with a predicted molecular weight of 32,417 and pI value of 4.2. MTA catalyzes an essential step in fatty acid biosynthesis providing malonyl-ACP which is the two carbon donor in the elongation steps. N-terminal sequence analysis shows that translation of *fabD* starts 16 bp downstream of the termination codon of *fabH*, implying that translational coupling might occur. But there are two more transcripts (2.4 knt and 2.2 knt) which are specific for *fabD-fabG-acpP* but not for *fabH*, which indicates that expression of *fabD* is not absolutely dependent on expression of *fabH*.

The specific activity of MTA measured from strains harboring pWO618

(*fabD*$^+$) increased only 3 times compared to that of control. The unexpected low level of MTA expressed by pWO618 might be due to lack of promoters strong enough to express FabD protein. But this result is consistent with others (116, 129). When *tac* promoter was placed upstream of *fabD,* MTA activity increased 155 fold upon IPTG induction (116). Along with the observation that this DNA encodes MTA, several other lines of evidence confirm that this ORF encodes MTA. These include homology to the acyl transferase domain of other enzymes, comparable predicted and determined amino acid composition, and other physical characteristics.

Genetic organization of fatty acid biosynthetic genes:

The genes *fabG* encoding 3-ketoacyl-ACP reductase, *acpP* encoding acyl carrier protein, and may be *fabF* encoding 3-ketoacyl-ACP synthase II are located immediately downstream of *fabD*, and are read in the same direction (105). So the genetic order of this fatty acid biosynthetic gene cluster in *E. coli* is *fabH-fabD-fabG-acpP-fabF* (clockwise on the physical map). It may be of evolutionary significance that the information in *E. coli* is encoded in the same order as the information encoding the domains of the multifunctional fatty acid synthase (131) and many of the modules of polyketide synthase (Fig. 20) (127, 130). It must be pointed out, however, that the genetic order of FAS in yeast is quite different. FAS of the yeast, *S. cervisiae,* is composed of α and β multifunctional

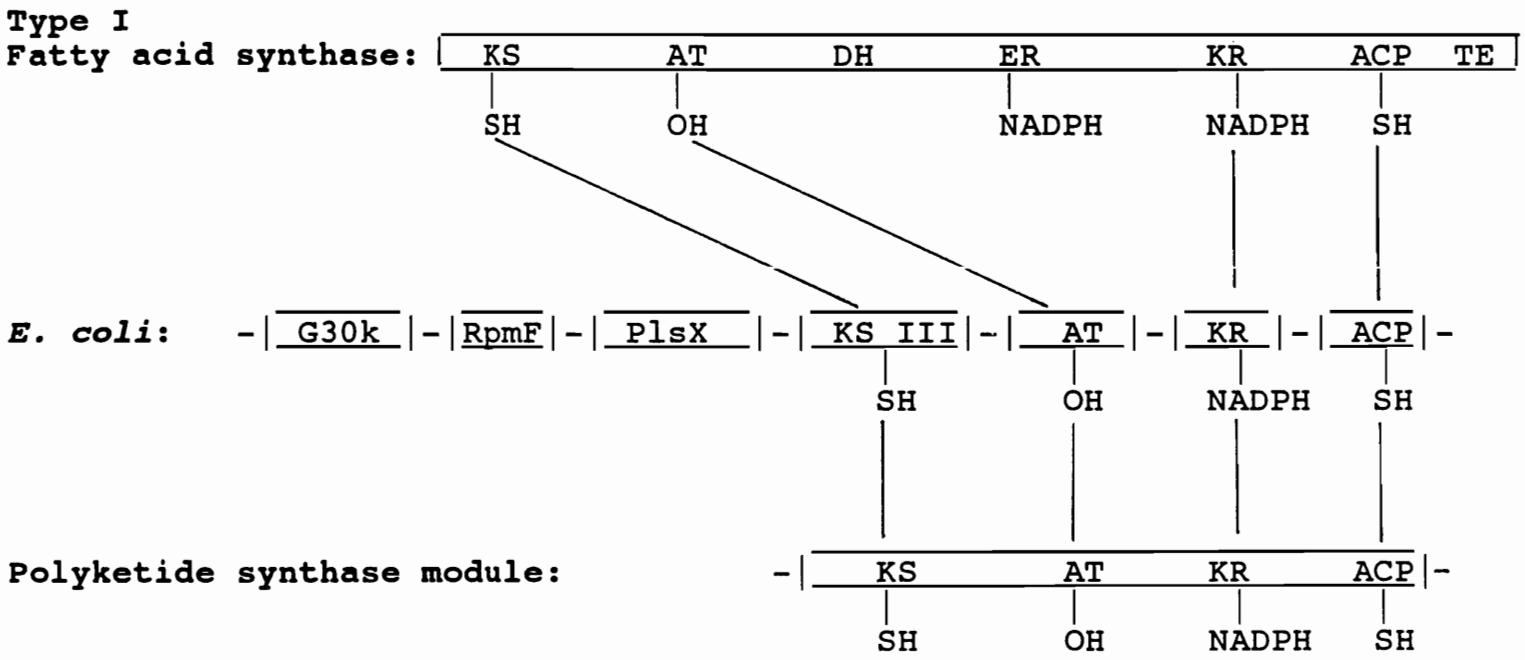


Fig. 20. Alignment of FAS functional domains.
Abbreviations: KS, 3-ketoacyl-ACP synthase; AT, acyltransferse; DH, dehydrase; ER, enoyl reductase; KR, 3-ketoacyl-ACP reductase; ACP, acyl carrier protein; TE, thioesterse. The -SH and -OH of the active sites and the NADPH binding sites are indicated.

polypeptides. The order of the catalytic domain of α-subunit is AT-ER-DH-MT/PT and that of β-subunit is ACP-KR-KS. The α- and β-subunit aggregate to form the functional $\alpha_6\beta_6$ FAS (127).

Other known *fab* genes, such as *fabA* (at min 22), *fabB* and *accD* (at min 50), and *accBC* (at min 72) are scattered around the chromosome (1). The gene for the 3-ketoacyl-ACP dehydrase and enoyl reductase have not yet been mapped in *E. coli*.

TRANSCRIPTIONAL ORGANIZATION OF THE *g30k-rpmF-plsX-fab* GENES

In *E. coli,* many of the functionally related genes are cotranscribed and cotranscription is one way to regulate gene expression in a coordinate manner. The *g30k, rpmF, plsX,* and *fab* genes comprise an operon. But the relative level of transcripts for each gene is not the same because of multiple promoters (sometimes within a structural gene), attenuation, and possibly processing and differential degradation of transcripts, which are typical for many of the ribosomal protein and other complex operons. In addition to these characteristics, extremely low levels of some transcripts, relatively large number of genes (8 genes) involved in cotranscription make the organization of transcripts quite complex. But some

conclusions may be derived from the results of Northern, S1 mapping, and primer extension analysis (Fig. 21).

There are multiple promoters upstream of *rpmF*. Ribosomal proteins are abundant within a cell and the rate of synthesis should increase promptly when cells grow fast to match the need for protein synthesis. But *plsX*, if it is a regulatory protein or an enzyme in a biosynthetic pathway, and fatty acid biosynthetic genes do not need to be expressed at high level. The exception is acyl carrier protein, probably the most abundant soluble protein (~6 x 10^4 molecules per cell) (15) in *E. coli*. Northern results support this idea. There are at least five abundant transcripts responsible for the expression of *rpmF* and at least ten transcripts for *acpP*. The *rpmF* gene (24 min) is located farthest from the bidirectional origin of replication (OriC, 84 min) while the majority of the r-protein (and rRNA) genes are in the one-half of the chromosome centered at the OriC. In order to ensure the equimolar synthesis of r-proteins, transcription or/and translation of *rpmF* should be very active to compensate for lower gene dosage imposed by its chromosomal location (24). It is possible that some of the *rpmF* promoters are constitutive and some are regulated by growth rate or stringent response. Promoter 2, which has a GC rich consensus sequence (47) [CN(g/c)C(g/c)cc] around the transcription start site that has been proposed to be important in stringent control, might be the one that responds to environmental signals.

Many of the transcripts originating upstream of *rpmF* terminate at the

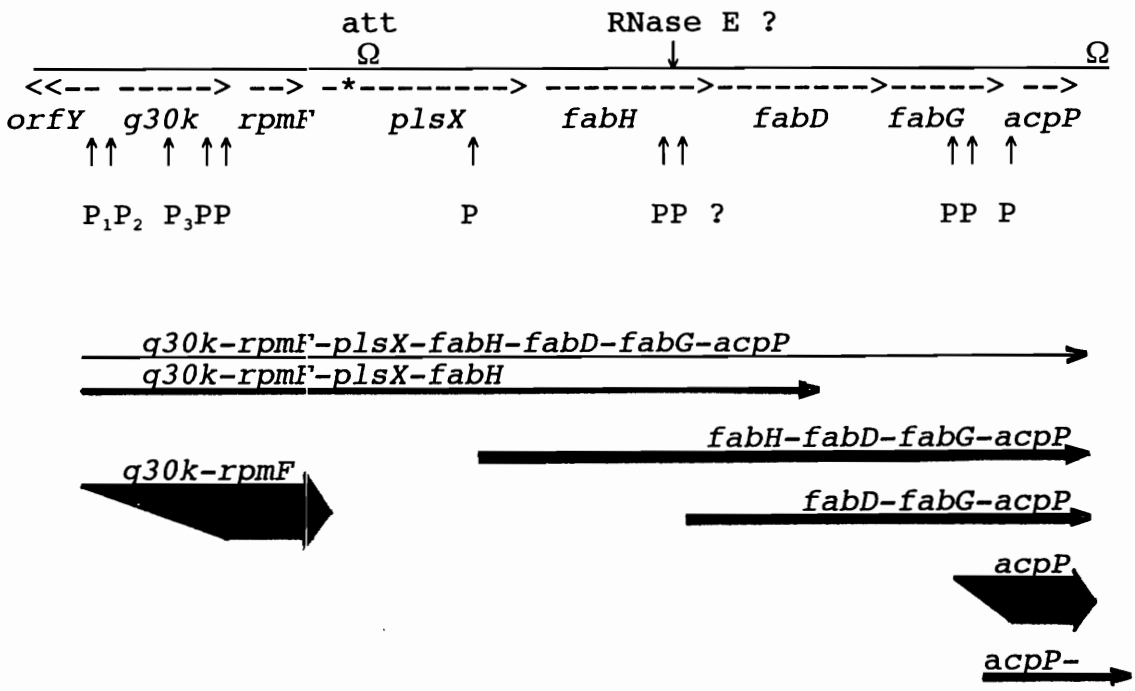


Fig. 21. Transcriptional organization of *g30k-rpmF-plsX-fabH-fabD-fabG-acpP* genes. The thickness of each line indicates the relative abundance of each transcript determined by visidual inspection of autoradiogram. The P's represent potential promoters. P_{1-3} indicate the transcription initiation sites identified by primer extension analysis. The * indicates the position of the *plsX50* mutation. The Ω's represent transcription terminators, with the attenuator labeled "att".

attenuator located downstream of the *plsX* translation initiation codon, and a portion of the transcripts go into the *plsX* and *fab* genes. The 3' part of the readthrough transcripts seems to be degraded rapidly by 3'→5' exonuclease, but the 5' parts of the transcripts specific for *g30k, rpmF, plsX, fabH,* and possibly *fabD* may be protected from degradation by stable secondary structure within *fabD* or *fabG*. The rare 5.2 knt transcript might be the intact full length transcript and the 3.7 knt transcript might be the most stable degradation product. There is no evidence for promoters which could express *plsX* independently of the promoters upstream of *rpmF*. Cotranscription of *g30k, rpmF, plsX, fabH*, and possibly *fabD* might be important for the coordinate control of ribosomal protein synthesis and fatty acid, and accordingly membrane synthesis.

Attenuation occurs within the *plsX* gene, which may be partly responsible for the low level of transcript specific for *plsX*. S1 mapping analysis revealed a relatively strong transcript (Fig. 14, band B) which read through the attenuator compared to those attenuated (Fig. 14, band C). Analysis of *plsX-lacZ* transcriptional fusions where *lacZ* is fused upstream or downstream of the attenuator indicated 50-70% read through at the attenuator (Fig. 17). However, attenuation may be regulated according to the growth rate or stringent control. It is possible that the transcripts originating from the promoter(s) which is subjected to the growth rate or stringent control may read through the attenuator while the transcripts originating from constitutive promoter(s) terminate at the attenuator.

There is a possibility that the secondary structure of the attenuator may serve as regulatory signal. This may act as site of interaction with accessory proteins. Regulatory protein can bind to the secondary structure of an attenuator, like in the S10 operon, where excess production of one of the products of the S10 operon, L4, binds to the attenuator and increases the termination of transcription (54, 55). The level of attenuation may be autoregulated by one of the products of the *g30k-rpmF-plsX-fab* operon. The location of the transcription attenuation site downstream of the translation initiation codon of *plsX* is not understood but suggests another model whereby translation of *plsX* may influence attenuation by disruption of the stable secondary structure by moving ribosomes. Two possibilities can be considered. Most of the transcripts arising upstream of *plsX* terminate at the attenuator. mRNA which is not translated degrades fast. In order to ensure the transcripts needed for the expression of *plsX*, which are low level, transcripts should be protected by the translating ribosomes. In this way, coordinate regulation of *rpmF* and *plsX* can be achieved more closely. Alternatively, transcription attenuation may be influenced by the translation of *plsX*. One scenario is that translation of *plsX* is coordinated with the production of ribosome by the translational coupling with *rpmF*, which may not be possible due to a relatively large separation (80 bp) between *rpmF* and *plsX* but secondary structure might work to reduce the actual space, or by the increased level of ribosome at fast growth, which may increase the global translation including *plsX*. Increased

translation of *plsX* may increase the readthrough at the attenuator by altering the secondary structure resulting in an increased amount of transcript responsible for the *plsX* and *fab* region.

Northern analysis demonstrates the level of transcripts for the expression of *g30k-rpmF-plsX-fab* genes (5.2 knt, and 3.7 knt) are very low. The primary cotranscripts for the *fab* genes are another 3.7 knt transcript for *fabH-fabD-fabG-acpP* genes, and 2.4 knt and 2.2 knt transcripts for the *fabD-fabG-acpP* genes. Coordinate expression of *fabH, fabD*, and *fabG* genes, which are all involved in the initial three steps of fatty acid biosynthesis, appears to be achieved by cotranscription and translational coupling between *fabH* and *fabD*, and between *fabD* and *fabG*. Transcripts for the fatty acid biosynthetic genes are more abundant than those for the *plsX* gene. However, transcripts for *fabH* are less abundant than the other *fab* genes, implicating FabH as an enzyme catalyzing the rate-limiting step in fatty acid biosynthesis.

A number of transcripts are responsible for the expression of *acpP*. Rawlings *et al.* (105) reported that despite the high level of expression of the *acpP* gene, no sequence matching the promoter and ribosome binding site consensus sequence were obvious. High level expression of *acpP* might be achieved by these multiple transcripts along with an abundant *acpP*-specific transcript (350 nt). Some of the transcripts (600, 700, and 1,800 nt) seem to read through the potential terminator downstream of *acpP* and continue into what may be the *fabF* gene

which encodes 3-ketoacyl-ACP synthase II. *fabF* produces a 43 kDa protein in a maxicell system (105), which would require about 1,200 bp of DNA. So the 1,800 nt transcript might be the cotranscript of *acpP* and *fabF*. Synthase II is required in the conversion of palmitoleate to *cis*-vaccenate and for the temperature-dependent regulation of fatty acid composition. There should be some mechanism to regulate the expression of *fabF*. Inefficient translation initiation was proposed to cause premature transcription termination (49). Differential expression of polycistronic genes in some cases is accomplished by the differential degradation of individual transcript segment (132, 133). It is possible that the *acpP* and *fabF* genes are cotranscribed to some extent but when the *fabF* gene is not translated, premature transcription termination might occur or the 3' portion of these transcripts might be degraded rapidly to give rise to stable mRNA remnants which are usually upstream from a hairpin loop structure. The 600 nt and 700 nt transcripts might be the premature transcripts or the most stable products of differential degradation. The *orfY* upstream of *rpmF* is transcribed divergently from the rest of genes in this region. Three transcripts, which were not detected by other probes, were detected by the *orfY* specific probe in Northern analysis. Considering the fact that the *orfX* (~1,100 bp) is located adjacent to and transcribed convergently toward *orfY*, the 1,200 nt transcript might be responsible for *orfX*, and the 600 nt and 700 nt transcripts are responsible for *orfY*.

In summary, the organization of the transcripts in this region is as follows:

There are very low amount of transcripts cotranscribing *g30k-rpmF-plsX-fabH-fabD-fabG-acpP* genes and *g30k-rpmF-plsX-fabH*-possibly *fabD* genes. There are additional transcripts cotranscribing the *fabH-fabD-fabG-acpP* genes, *fabD-fabG-acpP* genes, and *acpP*-putative *fabF* genes. The level of transcripts appears to be regulated according to the cellular need by promoters, attenuation, deferential degradation of the transcript, and possibly processing by endonuclease. Fatty acid biosynthetic genes may be coordinately regulated by cotranscription and translational coupling. Coordinated regulation of macromolecules and membrane lipid synthesis may be achieved by placement of the ribosomal protein gene and fatty acid biosynthetic genes in an operon or via the role of *plsX*.

POSSIBLE ROLE OF *plsX*

Characterization of the plsX50 *mutation*

Identification of the *plsX50* mutation as the frameshift nonsense mutation may be helpful in understanding the role of *plsX*. The recombination tests, complementation tests, sequence analysis of *plsX50* DNA, and the results from transcriptional and translational fusions are consistent in relation with the nature of the *plsX50* mutation. The finding that clones containing the *plsX* gene alone complemented the G3P auxotrophic phenotype and that the *plsX50* mutation is a

nonsense mutation indicate that the *plsX* gene product plays a role, and excludes the possibility that *plsX50* increases significantly termination at the attenuator by stabilizing the secondary structure. Increased termination would result in decreased levels of transcripts of the *plsX-fabH-fabD* portion of the *rpmF-plsX-fabH-fabD* operon, and therefore decreased fatty acid biosynthetic capacity. The insignificant effect of *plsX50* on the level of attenuation is also supported by the results of transcriptional fusions where the β-galactosidase activity of the *plsX50-lacZ* fusion was not significantly different from that of the *plsX-lacZ* fusion.

Intact PlsX protein will not be made in *plsX50* strains, indicating that the PlsX protein might not be essential for cellular growth. However, it is possible that a truncated PlsX protein could be made using one of the next translational initiation codons, and fulfill the role of *plsX* in the *plsX50* strains so that the cells can survive (as long as *plsB* is wild type). The *plsX50-lacZ* translational fusion plasmid (pWO952) contains five potential translational initiation codons for *plsX*. The low β-galactosidase activity produced by *plsX50-lacZ* even when induced indicates that none of the next four downstream translation initiation codons are used as an alternative. The 6th potential initiation codon is located at nt 2,090 which would produce a 29 kDa protein. A 33 kDa protein was identified in the clone containing DNA downstream of *Eco*RI (nt 2009) of *plsX* (pWO605) using the maxicell system (data not shown), which indicates the sixth potential translation initiation codon might be used.

possible role of **plsX**

The role of *plsX* for the conferral of G3P auxotrophic phenotype in *plsB26* strains still remains to be elucidated. But some progress has been made to gain insight into the function of *plsX*. G3P acyltransferase catalyzes the initial step of phospholipid biosynthesis in *E. coli* using two substrates; glycerol-3-phosphate and fatty acyl-ACP. The enzyme kinetic data of Larson *et al.* (37) (Table 1) demonstrated that neither the V_{max} nor the apparent K_m of the acyltransferase for G3P was influenced by *plsX50* or *plsX*$^+$. Also, the presence of *plsX*$^+$ did not influence thermolability of *plsB26* encoded G3P acyltransferase significantly (37). These data suggest that *plsX*$^+$ may exert its effect by elevating the intracellular substrate concentrations for the G3P acyltransferase, G3P or fatty acyl-ACP, thereby permitting the defective G3P acyltransferase to function, rather than modulating the G3P acyltransferase activity.

The PlsX protein seems more likely to influence the concentration of fatty acyl-ACP in light of the organization and cotranscription of *plsX* with the fatty acid biosynthetic gene cluster. The *plsX* gene product may play a role in the biosynthesis of fatty acids or may influence the incorporation of fatty acids into phospholipid by influencing one of the enzymes in the process, or participating as one of the enzymes in the process. Juxtaposition of the genes for a ribosomal protein and fatty acid biosynthetic enzymes into an operon may provide a mechanism for the coordinate regulation of protein and membrane synthesis during

balanced growth and during perturbations such as nutritional shifts and the stringent response. The *plsX* gene product might mediate this coordinate regulation of macromolecule and fatty acid, therefore membrane, synthesis.

One scenario of how *plsX* is involved in fatty acid biosynthesis may be that PlsX exerts its influence by affecting the expression or activity of KS III. KS III has been implicated in catalyzing the rate-limiting step in fatty acid biosynthesis. The high pI value (9.69) of PlsX suggests the possibility of interaction with FabH (pI=4.71), FabD (pI=4.17) or nucleic acids. The PlsX protein may affect the expression of KS III by acting as a transcriptional or translational activator protein. Alternatively, PlsX protein may affect the KS III activity by post-translational modification such as phosphorylation or dephosphorylation. A third possibility is that the *plsX* product may play a role as an antiterminator at the attenuator site located upstream of *plsX*, increasing the level of transcripts for the *plsX-fabH-fabD* portion of the operon. However, it is possible that KS III might not be absolutely required for cellular growth. There is an alternative pathway to produce acetoacetyl-ACP via KS I (Fig 1). It is possible that a decrease in KS III may reduce the intracellular fatty acyl-ACP concentration but not totally block fatty acid synthesis. Another possibility is that *plsX* controls the activity or expression of KS III partly, for instance, only the part related to coordinate regulation with ribosomal protein synthesis. Therefore, the *plsX50* mutation may decrease the fatty acid

would be the reason why both the *plsX50* mutation and the decreased affinity for G3P specified by *plsB26* are required for the G3P auxotrophic phenotype.

There was not a big difference in the level of transcripts in this region (Fig. 10, Fig. 14) between *plsX*$^{+}$ and *plsX50* strains in Northern and S1 mapping analysis. Maybe if the amount of transcripts for *fab* genes is lower in the *plsX50* strains, it is no less than 50% of wild type. A more objective method than visual inspection should be used to determine the level of transcripts, however.

It is possible that the *plsX* gene product may influence an enzyme other than KS III in the fatty acid biosynthetic pathway, or itself be one of the enzymes in phospholipid biosynthetic pathway. However, the absence of NADPH binding site in the PlsX sequence eliminates the possibility of PlsX being an enoyl reductase. The low level of PlsX protein within a cell suggests the role of *plsX* is more likely a regulator of some pathway. On the other hand, the cotranscription of *plsX* gene with the ribosomal protein gene *rpmF* suggests that the *plsX* gene product may be involved in almost any important cellular process.

LITERATURE CITED

1. Raetz, C. R. H. (1986) Molecular genetics of membrane phospholipid synthesis. *Ann. Rev. Genetics* **20,** 235-295.

2. Cronan, J. E., Jr., and Rock, C. A. (1987) Biosynthesis of membrane lipids. p. 474-497. *In* Neidhardt, F. C. *et al.* (eds.) "*Escherichia coli* and *Samonella typhimurium.* Cellular and Molecular Biology", American Society for Microbiology, Washington, D. C.

3. Cronan, J. E., Jr., and Vagelos, P. R. (1972) Metabolism and function of the membrane phospholipids of *Escherichia coli. Biochim. Biophys. Acta* **265,** 25-60.

4. Jackowski, S., Edwards, H. H., Davis, D., and Rock, C. O. (1985) Localization of acyl carrier protein in *Escherichia coli. J. Bacteriol.* **162,** 5-8

5. Kito, M., and Pizer, L. I. (1969) Purification and regulatory properties of biosynthetic L-glycerol-3-phosphate dehydrogenase from *Escherichia coli. J. Biol. Chem.* **244,** 3316-3323.

6. Bell, R. M., Mavis, R. D., Osborn, M. J., and Vagelos, P. R. (1971) Enzymes of phospholipid metabolism: localization in the cytoplasmic and outer membrane of the cell envelope of *Escherichia coli* and *Salmonella typhimurium. Biochim. Biophys. Acta* **249,** 628-635.

7. White, D. A., Albright, F. A., Lennarz, W. J., and Schnaitman, C. A. (1971) Distributation of phospholipid-synthesizing enzymes in the wall and membrane subfractions of the envelope of *Escherichia coli. Biochim. Biophys. Acta* **249,** 636-642.

8. Hardie, D. G., and McCarthy, A. D. (1986) Evolution of fatty acid synthase systems by gene fusion. p. 229-258. *In* Hardie, D. G. and McCarthy, A. D. (eds.) "Multidomain Proteins-Structure and Evolution", Elsevier Science Publishing company, Inc., NY.

9. Kohara, Y., Akiyama, K., and Isono, K. (1987) The physical map of the whole *E.coli* chromosome. Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50,** 495-508.

10. Muramatsu, S., and Mizuno, T. (1989) Nucleotide sequence of the *fabE* gene and flanking regions containing a bent DNA sequence of *Escherichia coli. Nucleic Acids Res.* **17,** 3982.

11. Kondo, H., Shiratsuchi, K., Yoshimoto, T., Masuda, T., Kitazono, A., Tsuru, D., Anai, M., Sekiguchi, M., and Tanabe, T. (1991) Acetyl-CoA carboxylase from *Escherichia coli:* Gene organization and nucleotide sequence of the biotin carboxylase subunit. *Proc. Natl. Acad. Sci. USA* **88,** 9730-9733.

12. Li, S.-J., and Cronan, J. E., Jr. (1992) The gene encoding the biotin carboxylase subunit of *Escherichia coli* acetyl-CoA carboxylase. *J. Biol. Chem.* **267,** 855-863.

13. Li, S.-J., Rock, C. O., and Cronan, J. E., Jr. (1992) The gene encoding the two carboxyltransferase subunits of *Escherichia coli* acetyl-CoA carboxylase. *J. Biol. Chem.* **267,** 16841-16847

14. Takai, T., Yokoyama, C., Wada, K., and Tanabe, T. (1988) Primary structure of chicken liver acetyl-CoA carboxylase deduced from cDNA sequence. *J. Biol. Chem.* **263,** 2651-2657.

15. Boom, J. V., and Cronan, J. E., Jr. (1989) Genetics and regulation of bacterial lipid metabolism. *Annu. Rev. Microbiol.* **43,** 317-343.

16. Jackowski, S., Cronan, J. E., Jr., and Rock, C. O. (1991) Lipid metabolism in procaryotes. p. 43-85. *In* D. E. Vance and J. Vance (eds.) "Biochemistry of Lipids, Lipoproteins and Membranes", Elsevier Science Publishing Co. Inc., NY.

17. Harder, M. E., Beacham, I. R., Cronan, J. E., Jr., Beacham, K., Honegger, K. L., and Silbert, D. F. (1972) Temperature-sensitive mutants of *Escherichia coli* requiring saturated and unsaturated fatty acids for growth: Isolation and properties. *Proc. Natl. Acad. Sci. USA* **69,** 3105-3109.

18. Jackowski, S., and Rock, C. O. (1987) Acetoacetyl-acyl carrier protein synthase as potential regulator of fatty acid biosynthesis in bacteria. *J. Biol. Chem.* 262, 7927-7931.

19. D'Agnolo, G. D., Rosenfeld, I. S. and Vagelos. P. R. (1975) Multiple forms of β-ketoacyl-acyl carrier protein synthetase in *Escherichia coli. J. Biol. Chem.* **250,** 5289-5294.

20. Clark, D. P., and Cronan, J. E., Jr. (1981) Bacterial mutants for the study of lipid metabolism. Methods Enzymol. **72,** 693-707.

21. Garwin, J. L., Klages, A. L. and Cronan, J. E., Jr. (1980) β-ketoacyl-acyl carrier protein synthase II of *Escherichi coli:* Evidence for function in the thermal regulation of fatty acid synthesis. *J. Biol. Chem.* **255,** 3263-3265.

22. de Mendoza, D., and Cronan. J. E., Jr. (1983) Thermal regulation of membrane lipid fluidity in bacteria. *Trends Biochem. Sci.* **8,** 49-52.

23. Jackowski, S., Murrphy, C. M., Cronan,, J. E., Jr., and Rock, C. O. (1989) Acetoacetyl-acyl carrier protein synthase: a target for the antibiotic thiolactomycin. *J. Biol. Chem.* **264,** 7624-7629.

24. Tsay, T.-J., Oh, W., Larson, T. J., Jackowski, S., and Rock, C. O. (1992) Isolation and characterization of the 3-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. *J. Biol. Chem.* **267,** 6807-6814.

25. Cooper, C. L., Jackowski, S., and Rock, C. O. (1987) Fatty acid metabolism in *sn*-glycerol-3-phosphate acyltransferase (*plsB*) mutants. *J. Bacteriol.* **169,** 605-611.

26. Prescott, D. J., and Vagelos, P. R. (1970) Acyl carrier protein. XIV. Further studies on β-ketoacyl acyl carrier protein synthase from *Escheria coli. J. Biol. Chem.* **245,** 5484-5490.

27 Lin, E. C. C. (1976) Glycerol dissimilation and its regulation in bacteria. *Annu. Rev. Microbiol.* **30,** 535-578.

28. Cronan, J. E., Jr., Bell, R. M. (1974) Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: Mapping of the structural gene for L-Glycerol-3-phosphate dehydrogenase. *J. Bacteriol.* **118,** 598-605.

29. Rock, C. O., and Cronan, J. E. Jr. (1982) Regulation of bacterial membrane lipid synthesis. *Curr. Top. Membr. Transp.* **17,** 207-233.

30. Larson, T. J., Lightner, V. A., Green, P. R., Modrich, P., Bell, R. M. (1980) Membrane phospholipid synthesis in *Escherichia coli*. Identification of the *sn*-glycerol-phosphate acyltransferase as the *plsB* gene product. *J. Biol. Chem.* **255,** 9421-9425.

31. Coleman, J. (1992) Characterization of the *Escherichia coli* gene for 1-acyl-*sn*-glycerol-3-phosphate acyltransferase (*plsC*). *Mol. Gen. Genet.* **232,** 295-303.

32. Bell, R. M. (1974) Mutants of *Escherichia coli* defective in membrane phospholipid synthesis. Macromolecular synthesis in an *sn*-glycerol-3phosphate acyltransferase K_m mutant. *J. Bacteriol.* **117,** 1065-1076.

33. Bell, R. M. (1975) Mutants of *Escherichia coli* defective in membrane phospholipid synthesis: Properties of wild type and K_m defective *sn*-glycerol-3-

phosphate acyltransferase activities. *J. Biol. Chem.* **250,** 7147-7152.

34. Lightner, V. A., Larson, T. J., Tailleur, P., Kantor, G. D., Raetz, C. R. H., Bell, R. M., and Modrich, P. (1980) Membrane phospholipid synthesis in *Escherichia coli.* Cloning of a structural gene (*plsB*) of the *sn*-glycerol-3-phosphate acyltransferase. *J. Biol. Chem.* **255,** 9413-9420.

35. Lightner, V. A., Bell, R. M., and Modrich, P. (1983). The DNA sequences encoding *plsB* and *dgk* loci of *Escherichia coli. J. Biol. Chem.* **258,** 10856-10861.

36. Green, P. R., Merrill, A. H., Jr., and Bell, R. M. (1981) Membrane phospholipid synthesis in *Escherichia coli.* Purification, reconstitution, and characterization of *sn*-glycerol-3-phosphate acyltransferase. *J. Biol. Chem.* **256,** 11151-11159.

37. Larson, T. J., Ludtke, D. N., and Bell, R. M. (1984) *Sn*-glycerol-3-phosphate auxotrophy of *plsB* strains of *Escherichis coli:* Evidence that a second mutation, *plsX*, is required. *J. Bacteriol.* **160,** 711-717.

38. Coleman, J. (1990) Characterization of *Escherichia coli* cells deficient in 1-acyl-*sn*-glycerol-3-phosphate acyltransferase activity. *J. Biol. Chem.* **265,** 17215-17221.

39. Raetz, C. R. H. (1978) Enzymology, genetics, and regulation of membrane phospholipid synthesis in *Escherichia coli. Microbiol. Rev.* **42,** 614-659.

40. Hardy, S. J. S. (1975) The stoichiometry of the ribosomal proteins of *Escherichia coli. Mol. Gen. Genet.* **140,** 253-274.

41. Noller, H. F., and Nomura, M. (1987) Ribosomes. p. 104-125 *In* Neidhardt, F. C. *et al.* (eds.) "*Escherichia coli* and *Samonella typhimurium.* Cellular and Molecular Biology", American Society for Microbiology, Washington, D. C.

42. Jinks-Robertson, S., and Nomura, M. (1987) Ribosome and tRNA, pp 1358-1385. *In* Neidhardt, F. C. *et al.* (eds.) "*Escherichia coli* and *Salmonella typhimurium.* Cellular and Molecular Biology", American Society for Microbiology, Washington, D.C.

43. Travers, A. A., Lamond, A. I., and Mace, H. A. F. (1982) ppGpp regulates the binding of two RNA polymerase molecules to the *tyrT* promoter. *Nucleic Acids Res.* **10,** 5043-5057.

44. Jinks-Robertson, S., Gourse, R. L. and Nomura, M. (1983) Expression of rRNA and tRNA genes in *Escherichia coli*: Evidence for feedback regulation by products of rRNA operons. *Cell* **33,** 865-876.

45. Nomura, M. (1990) History of ribosome research: a personal account, p. 3-55. In Hill, *et al.* (eds.) "The Ribosome. Structure, Function, and Evolution", American Society for Microbiology, Washington, D.C.

46. Cole, J. R., Olsson, C. L., Hershey, W. B., Grunberg-Mango, M., and Nomura, M. (1987) Feedback regulation of rRNA synthase in *E. coli:* Requirement for initiation factor IF2. *J. Mol. Biol.* **198,** 383-392.

47. Lindahl, L., and Zengel, J. M. (1986) Ribosomal genes in *Escherichia coli. Ann. Rev. Genet.* **20,** 297-326.

48. Gausing, K. (1974) Ribosomal protein in *E. coli*: Rate of synthesis and pool size at different growth rates. *Mol. Gen. Gent.* **129,** 61-75.

49. Stanssens, P., Remaut, E., and Fiers, W. (1986) Inefficient translation initiation causes premature transcription termination in the *lacZ* gene. *Cell* **44,** 711-718.

50. Baughman, G., and Nomura, M (1983) Localization of the target site for translational coupling in *Escherichia coli. Cell* **34,** 979-988.

51. Mattheakis, L., Vu, L., Sor, F., and Nomura, M, (1989) Retroregulation of the synthesis of ribosomal protein L14 and L24 by feedback repressor S8 in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **86,** 448-452.

52. Portier, C., Dondon, L., and Grunberg-Mango, M. (1990) Translational autoregulation of the *Escherichia coli* ribosomal protein S15. *J. Mol. Biol.* **211,** 407-414.

53. Skouv, J., Schnier, J., Rasmussen, M. D., Subramanian, A. R., and Pedersen, S. (1990) Ribosomal protein S1 of *Escherichia coli* is the effector for the regulation of its own synthesis. *J. Biol. Chem.* **265,** 17044-17049.

54. Lindahl, L., Archer, R., and Zengel, J. M. (1983) Transcription of the S10 ribosomal protein is regulated by an attenuator in the leader. *Cell* **33,** 241-248.

55. Janice, M., Lindahl, Z., and Lindahl, L. (1990) Ribosomal protein L4 stimulates in vitro termination at a NusA-dependent terminator in the S10

operon leader. *Proc. Natl. Acad. Sci. USA* **87,** 2675-2679.

56. Freedman, L. P., Zengel, J. M., and Lindahl, L. (1985) Genetic dissection of stringent control and nutritional shift-up response of *Escherichia coli* S10 ribosomal protein operon. *J. Mol. Biol.* **185,** 701-712.

57. Cole, J. R., and Nomura, M. (1986) Translational regulation is responsible for growth rate-dependent and stringent control of the synthesis of ribosomal L11 and L1 in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **83,** 4129-4133.

58. Barry, G., Squires, C., and Squires, C. L. (1980) Attenuation and processing of RNA from the rplJL-rpoBC transcription unit of *Escherichia coli. Proc. Natl. Acad. Sci. USA* **77,** 3331-3335.

59. Thomas, M. S., Bedwell, D. M., and Nomura, M. (1987) Regulation of α-operon gene expression in *Escherichia coli.* A novel form of translational coupling. *J. Mol. Biol.* **196,** 333-345.

60. Wikström, P. M., and Björk, G. R. (1988) Noncoordinate translation-level regulation of ribosomal and nonribosomal protein genes in the *Escherichia coli trmD* operon. *J. Bacteriol.* **170,** 3025-3031.

61. Schreiber, G., Metzger, S., Aizenman, E., Roza, S., Cashel, M., and Glaser, G. (1991) Overexpression on the *relA* gene of *Escherichia coli. J. Biol. Chem.* **266,** 3760-3767.

62 Nunn, W. D., and Cronan, J. E., Jr. (1976) Regulation of membrane phospholipid synthesis by the *relA* gene: Dependence on ppGpp levels. *Biochemistry* **15,** 2546-2550.

63. Ray, T. K., and Cronan, J. E., Jr. (1975) Acylation of *sn*-glycerol 3-phosphate in *Escherichia coli*: Study of reaction with native palmitoyl-acyl carrier protein. *J. Biol. Chem.* **250,** 8422-8427.

64. Lueking, D. R., and Goldfine, H. (1975) The involvement of guanosine 3'-diphosphate in the regulation of phospholipid biosynthesis in *Escherichia coli:* Lack of ppGpp inhibition of acyl transfer from acyl-ACP to *sn*-glycerol 3-phosphate. *J. Biol. Chem.* **250.** 4911-4917.

65. Albrechtsen, B., Ross, B. M., Squires, C., and Squires, C. L. (1991) Transcriptional termination sequence at the end of the *Escherichia coli* ribosomal RNA G operon: Complex terminators and antitermination. *Nucleic Acids Res.* **19,** 1845-1852.

66. Silhavy, T. J., Berman, M. L., and Enquist, L. W. (1984) *In* Experiments with Gene Fusions. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.

67. Tartof, K. D., and Hobbs, C. A. (1987) Improved media for growing plasmids and cosmid clones. *Focus* **9** (2), 12.

68. Clark, D. J., and Maaløe, O. (1976) DNA replication and the division cycle in *Escherichia coli*. *J. Mol. Biol.* **23,** 99-112.

69. Miller, J. H. (1972) Formulas and recipes. *In* Experiments in Molecular Genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY

70. Liss, L. R. (1987) New M13 host. DH5αF' competent cells. *Focus* **3,** 13.

71. Boyer, H. W., and Roulland-Dussoix, D. (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* **41,** 459-472.

72. Hayashi, S., Koch, J. P., and Lin, E. C. C. (1964) Active transport of L-α-glycerophosphate in *Escherichia coli*. *J. Biol. Chem.* **239,** 3098-3105.

73. Schweizer, H., Boos, W., and Larson, T. J. (1985) Repressor for the *sn*-glycerol-3-phosphate regulon of *Escherichia coli* K-12: Cloning of the *glpR* gene and identification of its product. *J. Bacteirol.* **161**, 563-566.

74. Semple, K. S., and Silbert, D. F. (1975) Mapping of the *fabD* locus for fatty acid biosynthesis in *Escherichia coli*. *J. Bacteriol.* **121,** 1036-1046.

75. Loomis, W. F., and Magasanik, B. (1965) Genetic control of catabolite repression of the *lac* operon in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **20**, 230-234.

76. Larson, T. J., Schumacher, G., and Boos, W. (1982) Identification of the *glpT*-encoded *sn*-glycerol-3-phosphate permease of *Escherichia coli*, an oligomeric integral membrane protein. *J. Bacteriol.* **152,** 1008-1031.

77. Coulondre, C., and Miller, J. H. (1977) Genetic studies of the *lac* repressor. III. Additional correlation of mutational sites with specific amino acid residues. *J. Mol. Biol.* **117,** 525-575.

78. Shuman, H. A. (1982) Active transport of maltose in *Escherichia coli* K-12. Role of the periplasmic maltose-binding protein and evidence for a substrate recognition site in the cytoplasmic membrane. *J. Biol. Chem.* **257,** 5455-5461.

79. Studier, F. W., and Moffatt, B. A. (1986) Use of bacterophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189,** 113-130.

80. Daws, T. D., and Fuchs, J. A. (1984) Isolation and characterization of an *Escherichia coli* mutant deficient in dTMP kinase activity. *J. Bacteriol.* **157,** 440-444.

81. Wieslander, L. (1979) A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature gels. *Anal. Biochem.* **98,** 305-309.

82. Maniatis, F., Fritsch, E. F., and Sambrook, J. (1982) *In* Molecular cloning : A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

83. Schneider, K., and Beck, C. F. (1987) New expression vectors for identifying and testing signal structures for initiation and termination of transcription. *Methods Enzymol.* **153,** 452-461.

84. Linn, T., and Pierre, R. S. (1990) Improved vector system for constructing transcriptional fusions that ensures independent translation of *lacZ*. *J. Bacteriol.* **172,** 1077-1084.

85. Schweizer, H., and Larson, T. J. (1987) Cloning and characterization of the aeorbic *sn*-glycerol-3-phosphate dehydrogenase structural gene *glpD* of *Escherichia coli* K-12.

86. Hackett, P. B., Fuch, J. A., and Messing, J. W. (1988) *In* An Introduction to Recombinant DNA Techniques. Benjamin/Cummings Publishing Co, Inc., Menlo Park, CA.

87. Ausubel, F. M. et al. (eds.) (1989) *In* Current Protocols in Molecular Biology. vol. 2. Molecular Biology-Laboratory Manuals. Greene Publishing Associates and Wiley-Interscience.

88. Morelle, G. (1989) A plasmid extraction procedure on a miniprep scale. *Focus.* **11,** 7-8.

89. Sanger, F., Nicklen, S., and Coulson, A. R. (1987) DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. U.S.A* **774,** 5463-5467.

90. Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

91. Rock, C. O., and Cronan, J. E., Jr. (1980) Improved purification of acyl carrier protein. *Anal. Biochem.* **102,** 362-364.

92. Ruch, F. E., and Vagelos, P. R. (1973) The isolation and general properties of *Escherichia coli* malonyl coenzyme A-acyl carrier protein transacylase. *J Biol. Chem.* **248,** 8086-8094.

93. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248-254.

94. Steers, E., Jr., Cuatrecasas, P., and Pollard, H. B. (1971) The purification of β-galactosidase from *Escherichia coli* by affinity chromatography. *J. Biol. Chem.* **246,** 196-200.

95. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *In* Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

96. Moos, M., Jr., Nguygen, N. Y., and Liu, T.-Y. (1988) Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. *J. Biol. Chem.* **263,** 6005-6008.

97. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262,**10035-10038.

98. Kounnas, M. Z., Wolz, R. L., Gorbea, C. M., and Bond, J. S. (1991) Meprin-A and -B. Cell surface endopeptidases of the mouse kidney. *J. Biol. Chem.* **266,** 17350-17357.

99. Tarr, G. E. (1977) Improved manual sequencing methods. *Methods Enzymol.* **47,** 335-357.

100. Bialkowska-Hobrzanska, H., Gilchrist, C. A., and Denhardt, D. T. (1985) *Escherichia coli rep* gene: Identification of the promoter and N-terminus of the Rep protein. *J. Biol. Chem.* **164,** 1004-1010.

101. Alam, J., Whitaker, R. A., Krogmann, D. W., and Curtis, S. E. (1986) Isolation and Sequence of the gene for ferredoxin I from the *Cyanobacterium anabaena* sp. strain PCC7120. *J. Bacteriol.* **168,** 1265-1271.

102. Wang, S.-Z., Chen, J.-S., and Johnson, J. L. (1988) The presence of five *nif*-like sequences in *Clostridium pasteurianum:* Sequence divergence and

transcription properties. *Nucleic Acids Res.* **16,** 439-452.

103. Claverie-Martin, F. C., Diaz-Torres, M. R., Yancey, S. D., and Kushner, S. R. (1989) Cloning of the altered mRNA stability (*ams*) gene of *Escherichia coli* K-12. *J. Bacteriol.* **171,** 5479-5486.

104. Claverie-Martin, F. C., Diaz-Torres, M. R., Yancey, S. D., and Kushner, S. R. (1991) Analysis of the altered mRNA stability (*ams*) gene from *Escherichia coli. J. Biol. Chem.* **266,** 2848-2851.

105. Rawlings, M, and Cronan, J. E., Jr. (1992) The gene encoding *Escherichia coli* acyl carrier protein lies within a cluster of fatty acid biosynthetic genes. *J. Biol. Chem.* **267,** 5751-5754.

106. Tanaka, Y., Tsujimura, A., Fujita, N., Isono, S., and Isono, K. (1987) Cloning and analysis of an *Escherichia coli* operon containing the *rpmF* gene for ribosomal protein L32 and the gene for a 30-kilodalton protein. *J. Bacteriol.*. **171,** 5707-5712.

107. Toussaint, B., Bosc, C., Richaud, P. Colbeau, A., and Vignais, P.M. (1991) A Mutation in a *Rhodobacter capsulatus* gene encoding an integration host factor-like protein impairs in vivo hydrogenase expression. *Proc. Natl. Acad. Sci. U.S.A* **88,** 10749-10753.

108. Koes, R. E., Spelt, C. E., Reif, H. J., van Den Elzen, P. J. M., Veltkamp, E., and Mol, J. N. M. (1986) Floral tissue of *Petunia hybrida* (V30) expresses only one member of the chalcone synthase multigene family. *Nucleic Acids Res.* **14,** 5229-5239.

109. Debelle, F., and Sharma, S. B. (1986) Nucleotide sequence of *Rhizibium meliloti* RCR 2011 genes involved in host specificity of nodulation. *Nucleic Acids Res.* **14,** 7453-7472.

110. Fisher, R. F., Swanson, J. A., Mulligan, J. T., and Long, S. R. (1987) Extended region of nodulation genes in *Rhizobium meliloti* 1021. II. Nucleotide sequence, transcription start sites and protein products. *Genetics* **117,** 191-201.

111. Bibb, M. J., Biro, S., Motamedi, M., Collins, J. F., and Hutchinson,C.R. (1989) Analysis of the nucleotide sequence of the *Streptomyces glaucescens tcmI* gene provides key information about the enzymology of polyketide antibiotic biosynthesis. *EMBO J.* **8,** 2727-2736.

112. Tsay, J.-T., Jackowski, S., and Rock, C. O. (1991) Role of 3-ketoacyl-ACP

synthases in thiolactomycin resistance. *FASEB J.* **5:**A1156, Abst. 4551.

113. Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J., and Katz, L. (1991) Modular organization of genes required for complex polyketide biosynthesis. *Science.* **252,** 675-679.

114. Beck, J., Ripka, S., Siegner, A., Schiltz, E., and Schweizer, E. (1990) The multifunctional 6-methylsaliccylic acid synthase gene of *Penicillium patulum*: Its gene structure relative to other polyketide synthase. *Eur. J. Biochem.* **192,** 487-498.

115. Ruch, F. E., and Vagelols, P. R. (1973) Characterization of a malonyl-enzyme intermediate and identification of the malonyl binding site in malonyl coenzyme A-acyl carrier protein transacylase of *Escherichia coli. J. Biol. Chem.* **248,** 8095-8106.

116. Magnuson, K., Oh, W. Larson, T. J., and Cronan, J. E. Jr. (1992) Cloning and nucleotide sequence of the *fabD* gene encoding malonyl coenzyme A-acyl carrier protein transacylase of *Escherichia coli. FEBS Lett.* **299,** 263-266.

117. Tsunasawa, S., Stewart, J. W., and Sherman, F. S. (1985) Amino-terminal processing of mutant forms of yeast iso-1-cytochrom c: the specificities of methionine aminopeptidase and acetyl-transferase. *J. Biol. Chem.* **260,** 5382-5391.

118. Bogosian, G., Violand, B. N., Jung, P. E., and Kane, J. F. (1990) Effect of protein overexpression on mistranslation in *Escherichia coli,* p. 546-558. *In* The Ribosome. Structure, Function, and Evolution. Hill, W. E. *et al.*(eds). American Society for Microbiology, Washington, D.C.

119. Binkley, J. P., and Kuempel, P. L. (1986) Genetic mapping in *Escherichia coli* of *tmk*, the locus for dTMP kinase. *J. Bacteriol.* **168,** 1457-1458.

120. Semple, K. S., and Silbert, D. F. (1975) Mapping of the *fabD* locus for fatty acid biosynthesis in *Escherichia coli. J. Bacteriol.* **121,** 1036-1046.

121. Travers, A. A. (1980) Promoter sequence for stringent control of bacterial ribonucleic acid synthesis. *J. Bacteriol.* **141,** 973-976.

122. Schweizer, H., and Larson, T. J. (1987) Cloning and characterization of the aerobic *sn*-glycerol-3-phosphate dehydrogenase structural gene *glpD* of *Escherichia coli* K-12. *J. Bacteriol.* **169,** 507-515.

123. Franceschi, F. J., and Nierhaus, K. H. (1990) Ribosomal proteins L15 and

L16 are mere late assembly proteins of the large ribosomal subunit. *J. Biol. Chem.* **265,** 16676-16682.

124. Knippenberg, P. H. (1990) Aspects of translation inition in *Escherichia coli,* p. 265-274. *In* Hill, W. E. et al. (eds) "The Ribosome. Structure, Function, and Evolution." American Society for Microbiology. Washington, D.C.

125. Peabody, D. S. (1989) Translation initiation at non-AUG triplets in mammalian cells. *J. Biol. Chem.* **264,** 5031-5035.

126. De Smit, M. H., and Duin, J. V. (1990) Control of prokaryotic translational initiation by mRNA secondary structure. *Prog. Nucleic Acid Res. Mol. Biol.* **38,** 1-35

127. Hopwood, D. A., and Sherman, D. H. (1990) Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annu. Rev. Genet.* **24,** 37-66.

128. Roche, P., Lerouge, P., Ponthus, C., and Prome, J.-C. (1991) Structural determination of bacterial nodulation factors involved in the *Rhizobium meliloti*-alfalfa symbiosis. *J. Biol. Chem.* **266,** 10933-10940.

129. Verwoert, I. I. G. S., Verbree, E. C., VAN DER Linden, K. H., Nijkamp, H. J. J and Stuitje, A. R. (1992) Cloning, nucleotide sequence, and expression of *Escherichia coli fabD* gene, encoding malonyl coenzyme A-acyl carrier protein transacylase. *J. Bacteriol.* **174,** 2851-2857.

130. Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J., and Katz, L. (1991) Modular organization of genes required for complex polyketide biosynthesis. *Science* **252,** 675-679.

131. Chang, S.-I., and Hammes, G. G. (1989) Homology analysis of the protein sequences of fatty acid synthases from chicken liver, rat mammary gland, and yeast. *Proc. Natl. Acad. Sci. USA* **86,** 8373-8376.

132. Klug, G., and Cohen, S. N. (1991) Effects of translation on degradation of mRNA segments transcribed from the polycistronic *puf* operon of *Rhodobacter capsulatus. J. Bacteriol.* **173,** 1478-1487.

133. Belasco, J. G., Beatty, J. T., Adams, C. W., von Gabain, A., and Cohen, S. N. (1985) Differential expression of photosynthesis genes in *R. capsulata* results from segmental differences in stability within the polycistronic *rxcA* transcript. *Cell* **40,** 171-181.

134. Rock, C. O., and Cronan, J. E., Jr. (1979) Re-evaluation of the solution structure of acyl acrrier protein. *J. Biol. Chem.* **254,** 9778-9785.

135. Nunn, W. D., and Cronan, J. E., Jr. (1974) *rel* gene control of lipid synthesis in *Escherichia coli.* Evidence for eliminating fatty acid synthesis as the sole regulatory site. *J. Biol. Chem.* **249,** 3994-3996.

136. Nunn, W. D., and Cronan, J. E., Jr. (1976) Evidence for a direct effect on fatty acid synthesis in *relA* gene control of membrane phospholipid synthesis. *J. Mol. Biol.* **102,** 167-172.

137. Inokuchi, K., Mutoh, N., Matsuyama, S., and Mizushima, S. (1982) Primary structure of the *ompF* gene that codes for a major outer membrane protein of *Escherichia coli* K-12. *Nucleic Acids Res.* **10,** 6957-6968.

138. Ingraham, J. (1987) Effect of temperature, pH, water, activity, and pressure on growth. p. 1543-1554. *In* Neidhardt, F. C. *et al.* (eds.) "*Escherichia coli* and *Samonella typhimurium.* Cellular and Molecular Biology", American Society for Microbiology, Washington, D. C.

139. Oh, W., and Larson, T. J. (1992) Physical location of genes in the *rne(ams)-rpmF-plsX-fab* region of the *Escherichia coli* K-12 chromosome. *J. Bacteriol.* paper submitted.

140. Rock, C. O., and Cronan, J. E., Jr. (1981) Acyl carrier protein from *Escherichia coli. Methods Enzymol.* **71,** 341-351.

APPENDIX

CONSTRUCTION OF ACP OVEREXPRESSION VECTOR

In order to overproduce acyl carrier protein, the ACP coding sequence was amplified from 2 ug of strain ECL8 chromosomal DNA and cloned into pT7-7 vector that contains the T7 promoter and translational initiation signals upstream of the multiple cloning site. Two 27-mer (100 pmole each) synthetic oligonucleotides were used as primers in 100 μl reactions. The *Nde*I site was created on the 5' end primer sequence (Table 2, #043682) and the *Hin*dIII site was created on the 3' end primer sequence (Table 2, #056178). The PCR cycles were as follows: The first cycle was a 5 min denaturation at 94 °C. Subsequent cycles were (30 total) 1 min denaturation at 94 °C, 1 min annealing at 45 °C , 2 min polymerization at 72 °C, 5 cycles and 1 min at 94 °C, 1 min at 55 °C , 2 min at 72°C, 20 cycles and 1 min at 94 °C, 1 min at 55 °C, 3 min at 72 °C with changing temperature and time, 5 cycles. The last cycle held the sample at 5 °C. Amplified DNA was purified using the Geneclean II kit, digested with *Nde*I and *Hin*dIII and ligated into the same sites of pT7-7 cloning vector. The resulting plasmid clone (pWO961) was introduced into strain BL21 (DE3, CB424) and ACP was overproduced by the procedure described in "Materials and Methods". Cells overexpressing ACP were dissolved in SDS sample loading buffer and analyzed on

an 18% SDS-polyacrylamide gel. The overproduced ACP represented about 20% of total cellular protein as estimated by visual inspection of the Coomassie blue stained SDS-gel. ACP migrated aberrantly on the SDS-gel as reported before (105, 134). ACP migrated as if it were a 20 kDa protein even though its molecular weight is 8,847. However, most of the overproduced ACP was an apo-ACP which is not posttranslationally modified by a 4'-phosphopantetheine coenzyme. This was determined by comparison of overproduced ACP with commercial holo-ACP (Sigma) in a native gel (140). The ACP produced in this way was therefore of limited usefulness for assay of KSIII or MTA activity.

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Publications and Abstracts:

1. Oh, W., and Larson, T. J. (1992) Physical location of genes in the *rne(ams)-rpmF-plsX-fab* region of the *Escherichia coli* K-12 chromosome. *J. Bacteriol.* paper submitted.

2. Tsay, J.-T., Oh, W., Larson, T. J., Jackowski, S., and Rock, C. O. (1992) Isolation and characterization of the β-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. *J. Biol. Chem.* **267,** 6807-6814.

3. Magnuson, K., Oh, W., Larson, T. J., and Cronan, J. E., Jr. (1992) Cloning and nucleotide sequence of the *fabD* gene encoding malonyl coenzyme A-acyl carrier protein transacylase of *Escherichia coli.* *FEBS Lett.* **299,** 262-266.

4. Oh, W., and Larson, T. J. (1992) Genes encoding ribosomal protein L32 and fatty acid biosynthetic enzymes are cotranscribed in *Escherichia coli* K-12. *FASEB J.* **6,** A216, Abst. 1244.

5. Oh, W., and Larson, T. J. (1991) Characterization of a fatty acid biosynthetic operon of *Escherichia coli* K-12. *FASEB J.* **5,** A813, Abst. 2572.

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