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Nucleotide Sequence of the *Pseudomonas fluorescens* Signal Peptidase II Gene (*lsp*) and Flanking Genes

LESLIE ISAKI,† RICHARD BEERS, AND HENRY C. WU*

Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20889-4799

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The *lsp* gene encoding prolipoprotein signal peptidase (signal peptidase II) is organized into an operon consisting of *ileS* and three open reading frames, designated genes *x*, *orf149*, and *orf316* in both *Escherichia coli* and *Enterobacter aerogenes*. A plasmid, pBROC128, containing a 5.8-kb fragment of *Pseudomonas fluorescens* DNA was found to confer pseudomonic acid resistance on *E. coli* host cells and to contain the structural gene of *ileS* from *P. fluorescens*. In addition, *E. coli* strains carrying pBROC128 exhibited increased globomycin resistance. This indicated that the *P. fluorescens lsp* gene was present on the plasmid. The nucleotide sequences of the *P. fluorescens lsp* gene and of its flanking regions were determined. Comparison of the nucleotide sequences of the *lsp* genes in *E. coli* and *P. fluorescens* revealed two highly conserved domains in this enzyme. Furthermore, the five genes which constitute an operon in *E. coli* and *Enterobacter aerogenes* were found in *P. fluorescens* in the same order as in the first two species.

The *ileS-lsp* operon in *Escherichia coli* is located at 0.5 min on the *E. coli* genetic map (17). The operon consists of *ileS*, the structural gene for isoleucyl-tRNA synthetase; *lsp*, the gene that encodes prolipoprotein signal peptidase (4, 26); and three open reading frames designated *x* (8), *orf149*, and *orf316* (11). Gene *x* encodes a soluble protein with an apparent M_r of 35,000. The gene products of *orf149* and *orf316* have not been identified. Signal peptidase II (SPase II), an inner membrane enzyme, cleaves lipid-modified prolipoprotein to form apolipoprotein, which is further modified by N-acylation to yield mature Braun's lipoprotein (22). Although there is no apparent physiological connection between the activation of isoleucine by isoleucyl-tRNA synthetase and the proteolytic cleavage of lipid-modified prolipoprotein by SPase II, these two genes are cotranscribed with the three open reading frames in the operon. In a previous paper, we showed that all five of these genes present in the *E. coli ileS-lsp* operon were also found in *Enterobacter aerogenes* in the same order as in *E. coli* (6).

Braun's lipoprotein and homologous lipoproteins appear throughout the gram-negative bacteria (reviewed in reference 24). In a study by Nakamura et al. (16), antisera raised against *E. coli* Braun's lipoprotein cross-reacted with homologous lipoproteins in all members of the family *Enterobacteriaceae* tested, but no cross-reactivity was observed with *Pseudomonas aeruginosa* and with three other nonenteric bacteria. These results indicated that the homolog of Braun's lipoprotein in *P. aeruginosa*, lipoprotein I (14), is antigenically unrelated to those in enteric bacteria. Although glyceride-modified cysteine has not been unequivocally demonstrated in *P. aeruginosa* lipoprotein, glycerol and fatty acid incorporations into lipoprotein I (14) and lipoprotein H, a peptidoglycan-associated lipoprotein (13), have been detected. A recent study by Duchêne et al. (2) has shown that in lipoprotein I, the amino acid sequence at the SPase II cleavage site is Leu-Ala-Thr-Gly-Cys-Ser-Ser, which is very similar to the Leu-Ala-Gly-Cys-Ser-Ser sequence in *E. coli* Braun's lipoprotein. Therefore, although the lipoprotein substrates of SPase II may be antigenically unrelated among the gram-negative bacteria, the recognition site for prolipoprotein modification enzymes and SPase II appears to be similar and may be a conserved feature. Consequently, SPase II may also be a conserved enzyme among these bacteria.

Pseudomonic acid, a competitive inhibitor of isoleucyl-tRNA synthetase (3), is lethal for *E. coli*; in contrast, it does not inhibit isoleucyl-tRNA synthetase from *Pseudomonas fluorescens*, which produces the antibiotic. We obtained from Beecham Pharmaceuticals an *E. coli* strain containing a cloned *ileS* gene from *P. fluorescens* on pBROC128 that conferred increased pseudomonic acid resistance on the *E. coli* strain (M. Burnham and D. Winstanley, personal communication).

In this study, we identified the presence of the *P. fluorescens lsp* gene on plasmid pBROC128 and determined the nucleotide sequence of the *lsp* gene and its flanking genes to ascertain whether those genes composing the *E. coli ileS-lsp* operon are also present in pBROC128. We compared the DNA sequences of the *lsp* gene in *E. coli*, *Enterobacter aerogenes*, which is closely related to *E. coli*, and *P. fluorescens*, which is not an enteric bacterium and is only distantly related to *E. coli*. This comparison enabled us to determine the highly conserved regions of the *lsp* gene which may correspond to the amino acid sequences involved in the catalytic and/or recognition site(s) of SPase II.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1. All bacteria were routinely grown on proteose-peptone-beef extract (PPBE) medium. Ampicillin was used at a concentration of 50 μg/ml.

DNA manipulations. Genomic and plasmid DNA were isolated by methods described by Silhavy et al. (18). Ligations were performed in low-melting-point agarose (19). Competent cells for transformation were prepared by $CaCl_2$ treatment, and competent *E. coli* DH5α cells were pur-

* Corresponding author.
† Present address: Division of Basic Research, National Institute on Alcohol Abuse and Alcoholism, Rockville, MD 20857.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
P. fluorescens NCIB 10586	Wild type	3
E. coli		
DH5α	*recA1* Δ(*argF-lacZYA*) φ80d*lacZ* ΔM15	BRL[a]
JM103	Δ(*lac pro*) (F′ *lacI*^{q}ZΔM15 *proA*$^+$*B*$^+$)	10
331c$^-$	*ileS*(Ts)	7
Plasmids		
pBROC128	Ap^r *ileS*$^+$	Beecham
pLSP222	*lsp*$^+$ Ap^r	12
pMT521	*ileS*$^+$ *lsp*$^+$ Ap^r	20
pUC18	Ap^r	25
pUC19	Ap^r	25
pBK1	Ap^r	6
pSF2518	Ap^r	This study
pSF2519	Ap^r	This study
pSF15	Ap^r	This study
pSF10	Ap^r	This study

[a] Bethesda Research Laboratories.

TABLE 2. Expression of *lsp* and *ileS* in *E. coli*

Strain (plasmid)	Globomycin concn (μg/ml)[a]	Complementation[b] at:		Pseudomonic acid concn (μg/ml)[c]
		30°C	42°C	
DH5α	25			25
DH5α(pUC18)	25	+	−	25
DH5α(pMT521)	>100	+	+	25
NCIB 10586[d]				>200
DH5α(pBROC128)	>100	+	+[e]	>200[e]
DH5α(pSF2518)	25			
DH5α(pSF2519)	>100	+	−	25
331c$^-$		+	−	

[a] Globomycin resistance in DH5α clones was assayed. The concentration of globomycin allowing saturated growth was measured after overnight incubation at 37°C.

[b] Complementation of the IleS(Ts) phenotype was performed with strain 331c$^-$.

[c] Pseudomonic acid resistance in DH5α clones was assayed. Overnight cultures were diluted 1:800 in 100 μl of PPBE broth containing various concentrations of pseudomonic acid. The concentration of pseudomonic acid allowing saturated growth was measured after overnight incubation at 37°C.

[d] Strain NCIB 10586 is a pseudomonic acid-producing strain of *P. fluorescens* and is resistant to this antimicrobial agent.

[e] Confirms observations of Burnham and Winstanley (personal communication).

chased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Southern hybridization analysis was performed as previously described (6).

For sequencing, a series of deletion plasmids was constructed with *Bal* 31. Digestion mixtures were prepared according to the specifications of the manufacturer and incubated at 37°C. Samples of digested DNA were removed at 30-s intervals for a total elapsed time of 10 min and then pooled. After Klenow enzyme treatment to produce blunt ends, the *Bal* 31-digested DNA was ligated to pUC18 and transformed into DH5α cells. Plasmid DNA was prepared from the resulting transformants. After digestion with appropriate restriction enzymes, the linearized plasmids were electrophoresed and screened for size. Twenty-three clones carrying inserts of approximately 300 to 2,300 nucleotides (nt) were selected for sequencing.

Enzymes, chemicals, and oligonucleotides. Restriction enzymes were purchased from American Allied Biochemicals (Aurora, Colo.) and Bethesda Research Laboratories. T4 DNA ligase and *E. coli* polymerase I were purchased from Bethesda Research Laboratories. Calf intestine phosphatase was purchased from NEN Research Products, Du Pont Co. (Boston, Mass.). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was purchased from Sigma Chemical Co. (St. Louis, Mo.). [α-^{32}P]dCTP and [α-^{35}S]dATP were purchased from Amersham (Arlington Heights, Ill.). Oligonucleotide primers for sequencing were purchased from the Oligonucleotide Synthesis Facility at the Department of Microbiology, Uniformed Services University of the Health Sciences.

DNA sequencing and computer analyses. DNA sequencing of double-stranded templates was performed as previously described (6). Compressions in the sequencing gels were resolved with dITP or 7-deaza-dGTP, which were substituted for dGTP. The DNA data were analyzed on a VAX computer with the University of Wisconsin Genetics Computer Group program package, version 5 (1), and the Protylyze software program of D. Ward.

Nucleotide sequence accession numbers. The sequence data presented in Fig. 2 and 3 will appear in the EMBL/GenBank nucleotide sequence data base under the accession numbers M35366 and M35367, respectively.

RESULTS AND DISCUSSION

Subcloning of the *P. fluorescens lsp* gene. To determine whether the *lsp* gene was located on pBROC128, the clone was assayed for increased globomycin resistance (21). SPase II is specifically inhibited by globomycin, a cyclic peptide antibiotic (5). *E. coli*, cells containing pBROC128 exhibited increased resistance to globomycin at concentrations exceeding 100 μg/ml (Table 2), inidicating that the *lsp* gene was located on the 5.8-kb insert, and functional SPase II was synthesized.

The 5.8-kb insert was excised from pBROC128 with *Cla*I and restriction mapped. The DNA fragments generated by the restriction enzymes were analyzed by a low-stringency modification of the Southern hybridization method as described previously (6). The *P. fluorescens lsp* gene was located on a 2.5-kb *Pst*I-*Cla*I fragment (Fig. 1). To facilitate subcloning, a 2.5-kb *Pst*I-*Eco*RI fragment, which carried 23 bp of the original vector, was excised, ligated to pUC18 and pUC19, and transformed into DH5α. These subclones of pUC18 and PUC19 were designated pSF2518 and pSF2519, respectively. To determine whether gene *x* and a promoter were present on pBROC128, two additional clones (pSF15 and pSF10) were obtained. pSF15 was constructed by excising a 1.2-kb *Cla*I-*Eco*RI fragment from pBROC128 and ligating the isolated DNA segment to pUC18 digested with *Acc*I and *Eco*RI. pSF10 was constructed by excising a 1.1-kb *Hin*dIII-*Pst*I fragment from pBROC128 and ligating the fragment to pUC18 at the *Hin*dIII-*Pst*I site.

To sequence the *P. fluorescens lsp* and downstream genes, plasmids carrying shorter inserts were constructed by *Bal* 31 nuclease digestion of pSF2518. In this way, the same primer, which consisted of vector sequences, was used to sequence all of these plasmids in one direction. Plasmid pSF2518 was cleaved with *Pst*I and digested with *Bal* 31, and the duplex termini were repaired with Klenow enzyme to generate blunt ends. After addition of *Hin*dIII linkers, the fragments were digested with *Hin*dIII and *Eco*RI, ligated to pUC18, and

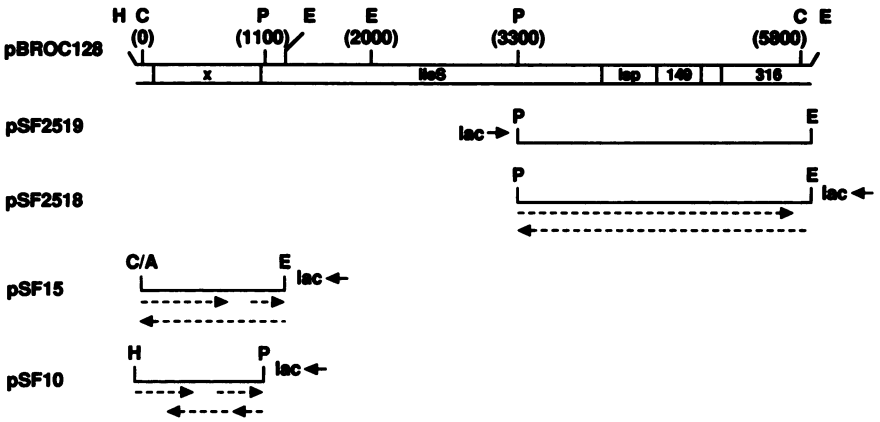

FIG. 1. Strategy used to subclone and sequence the *P. fluorescens* genes from pBROC128. The numbers in parentheses indicate the coordinates in base pairs. Restriction enzymes are abbreviated as follows: H, *Hin*dIII; C, *Cla*I; P, *Pst*I; E, *Eco*RI; and A, *Acc*I. Only sites relevant to the subcloning procedures are shown. The orientation of transcription from the *lac* promoter on the vectors is indicated by solid arrows. Dashed arrows indicate the direction of sequencing from oligonucleotide primers.

transformed into DH5α. These plasmids were used with sequencing primers in the orientation opposite to that of *lac* transcription. To create plasmids for sequencing in the reverse direction, graduated deletions at the *Eco*RI end (distal to the *Pst*I site) were generated by treating pSF2518 as previously described, except that the plasmid was first cleaved with *Eco*RI. After addition of *Eco*RI linkers, the fragments were cleaved with *Eco*RI and *Pst*I.

Expression of the *lsp* and *ileS* genes of *P. fluorescens*. To evaluate expression of the *P. fluorescens lsp* gene, SPase II activity was assayed by using the antibiotic globomycin (Table 2). pBROC128 and pSF2519 clones displayed resistance to globomycin at concentrations exceeding 100 μg/ml, similar to that of the *E. coli* clone pMT521, which encodes *E. coli* SPase II. pSF2518 subclones, however, failed to exhibit increased globomycin resistance. Since the same insert was

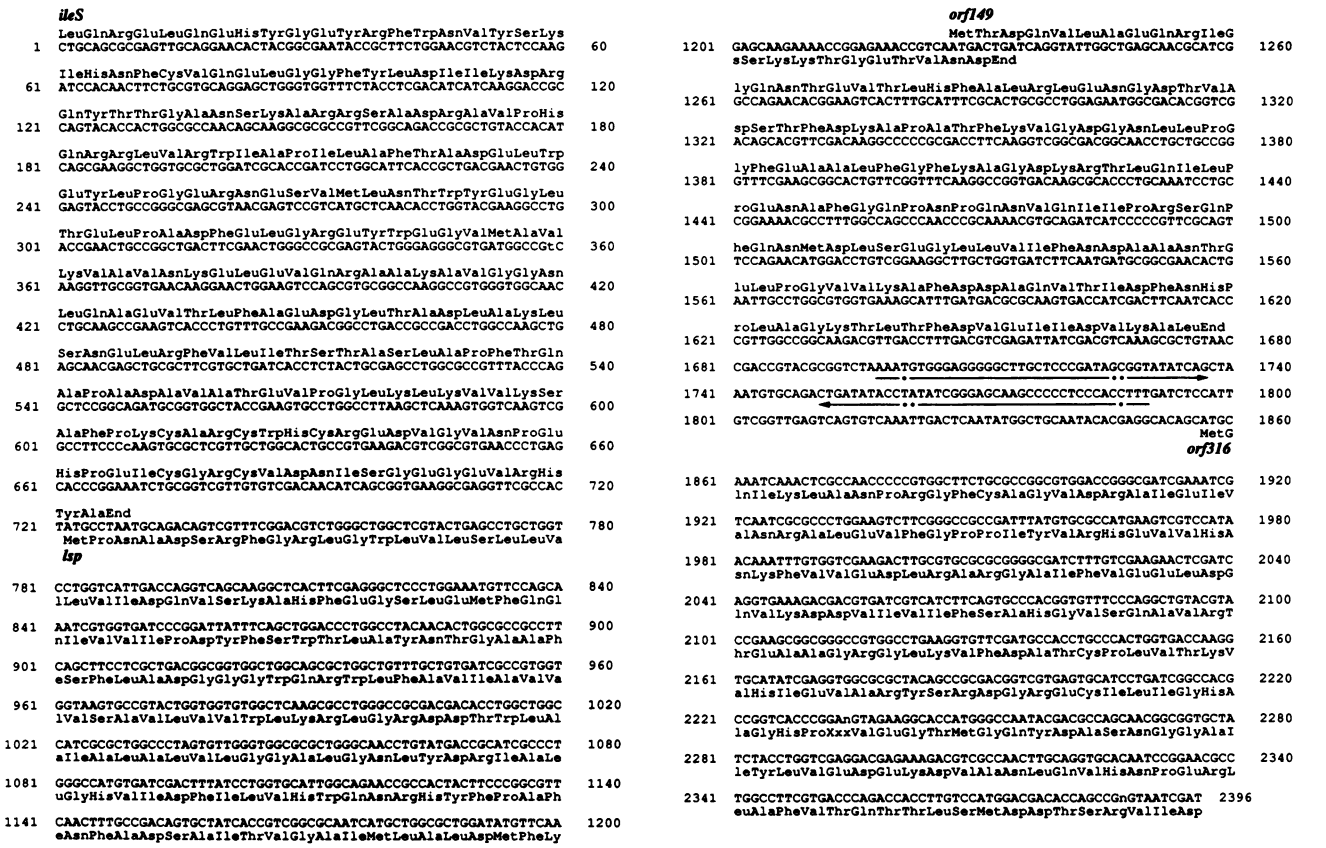

FIG. 2. Nucleotide and predicted amino acid sequences of the *P. fluorescens lsp* and *orf149* and flanking sequences. The sequence numbering starts with the *Pst*I site at the 3′ terminus of *ileS*. The structural gene for *lsp* begins at nt 722 and ends at nt 1231. The *orf149* gene starts at nt 1227 and ends at nt 1676. The *orf316* open reading frame begins at nt 1857, and the cloned sequences end at the *Cla*I site (nt 2396). Arrows indicate inverted repeats; dots indicate mismatched bases.

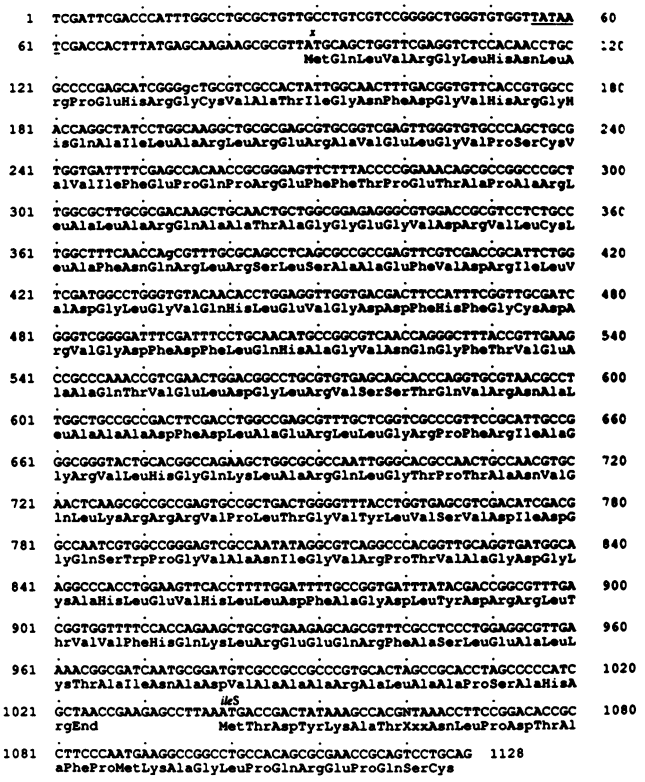

FIG. 3. Nucleotide and predicted amino acid sequences of the *P. fluorescens x* gene. The putative −10 promoter region is underlined (nt 56 to 61). Translation of the *x* gene begins at nt 90 and ends at nt 1025. The 5′-terminal sequence of *ileS* is shown.

cloned into both pUC18 (yielding pSF2518) and pUC19 (yielding pSF2519), this failure to display increased resistance by pSF2518 clones was apparently due to the insert being cloned in the orientation opposite to that of the *lac* promoter on the vector. These results with pSF2518 and pSF2519 indicate that a *P. fluorescens* promoter for *lsp* expression either is absent from the insert or is not functional in *E. coli*.

Expression of the *P. fluorescens ileS* gene was analyzed by two procedures. First, to determine whether functional isoleucyl-tRNA synthetase was made, the plasmids were transformed into *E. coli* 331c$^-$ to complement the *ileS*(Ts) mutation in this strain. A second assay, using pseudomonic acid, was performed to detect activity of *P. fluorescens* IleS. Pseudomonic acid is a competitive inhibitor of isoleucyl-tRNA synthetase with respect to isoleucine. Of the clones tested, only pBROC128 complemented the temperature-sensitive mutation in strain 331c$^-$ and showed increased resistance to pseudomonic acid. These results indicated that only the original clone, pBROC128, carried sufficient genetic information to encode functional isoleucyl-tRNA synthetase and that this gene is expressed in *E. coli* (Table 2).

Nucleotide sequence analyses of pBROC128 subclones. The sequencing strategy of *lsp* and of its flanking genes is shown in Fig. 1. The insert on pSF2518 was found to consist of 2,396 bp and was sequenced in both directions except for about 200 bp at the 3′ terminus of the insert. Plasmid pSF2518 was found to contain all of the *lsp* and *orf149* genes and 540 nt of *orf316* (Fig. 2). pSF10 and pSF15 (Fig. 1) were sequenced to determine the presence of gene *x* and of a promoter and, when gene *x* was detected, to determine the junction between *x* and *ileS*. All five genes homologous to the *E. coli* operon, *x-ileS-lsp-orf149-orf316*, were present on pBROC128 and the same order as in *E. coli* (Fig. 2 and 3). At the DNA level, the homologies ranged from 49% for *orf149* to 67% for the 5′ terminus of *ileS*, while the amino acid homologies ranged from 63% for *x* and *lsp* to 81% for the N-terminal region of *orf316* (Table 3). The translation products of *P. fluorescens x*, *lsp*, and *orf149* are predicted to be proteins composed of 312, 170, and 150 amino acids, respectively. These proteins are similar in size to the *E. coli x*, *lsp*, and *orf149* gene products, which are composed of 311, 164, and 149 amino acids, respectively.

The nucleotide sequences at the intercistronic junctions reveal many similarities and important differences between the *E. coli* and *P. fluorescens x-ileS-lsp-orf149-orf316* gene regions (Table 4). The *x-ileS* junctions in both organisms are similar. At the second junction, the *E. coli lsp*, ATG is nested within TGATGA, which functions as stop signals for *ileS* translation. In *P. fluorescens*, the initiation ATG of *lsp* is upstream of the stop TAA sequence of *ileS*. This arrangement occurs again at the *lsp-orf149* junction in *P. fluorescens*, where the translation initiation and stop sequences are separated by two nucleotides. The junctures of the *E. coli lsp-orf149* and the *P. fluorescens orf149-orf316* appear similar in that the stop and start sequences are separated by 121 and 177 nucleotides, respectively. This spacing is sufficient to impose stem-loop structures between these genes (Fig. 2). It is not known, however, whether these secondary structures function as termination signals.

The hydropathic index for the deduced amino acid sequence (Fig. 4) of the *lsp* gene from *P. fluorescens* was examined by the Kyte and Doolittle (9) algorithm. The plots of SPase II from all three organisms were strikingly similar (data not shown), and four hydrophobic domains were discerned for *P. fluorescens* (Fig. 4). These regions, which are composed mainly of hydrophobic residues, presumably represent the transmembrane regions similar to those postulated for *E. coli* (4).

Depicted in Fig. 5 is a schematic model of the SPase II molecule embedded in the cytoplasmic membrane. When the SPase II amino acid sequences of *P. fluorescens*, *E. coli*, and *Enterobacter aerogenes* are compared, two highly homologous regions are identified (domains I and II in Fig. 4). In the model, these two regions are located on opposing domains within the periplasm. The postulated topology of SPase II is supported by recent evidence of Muñoa and Wu (F. Muñoa and H. C. Wu, FASEB J. 4:A2245, 1990), who employed PhoA-SPase II and β-galactosidase–SPase II fusions. Although it is tempting to speculate that these two domains fold to form one active site, the possibility that these two domains represent two separate active sites for binding and catalysis cannot be excluded. Aside from these two regions of high homology, there are four regions of significant

TABLE 3. Similarity between *P. fluorescens x-ileS-lsp-orf149-orf316* genes and the corresponding *E. coli* genes

P. fluorescens gene	Homology (%) with *E. coli*	
	DNA	Amino acid
x	57	63
ileS (5′ 125 bases)	67	77
ileS (3′ 730 bases)	60	68
lsp	53	63
orf149	49	65
orf316 (5′ 552 bases)	66	81

TABLE 4. Comparison of the intercistronic junctions of the *x-ileS-lsp-orf149-orf316* operon in *E. coli* and *P. fluorescens*

Junction	Sequence[a] in:	
	E. coli	*P. fluorescens*
x-ileS	TAA–42 nt–TGATGA	TAA–11 nt–TAAATG
ileS-lsp	TGATGA	ATG CC TAA TG
lsp-orf149	TAATAA–121 nt–ATG	ATG AC TGA
orf149-orf316	TAA C ATG	TAA–177 nt–ATG

[a] The stop codon(s) (TAA or TGA) of the first gene indicated in the first column is overlined, while the initiation codon (ATG) of the second gene is underlined.

hydrophobicity, a requirement imposed by the membrane in which this enzyme is located.

The *ileS-lsp* operon in *P. fluorescens*. Nucleotide sequence analyses and DNA comparisons with the *E. coli ileS-lsp* operon indicate that the genes that compose the *E. coli* operon are also present in *P. fluorescens* in the same order. Since pBROC128 was constructed by cloning a 5.8-kb DNA fragment into the *Cla*I site of vector pAT153 (23), the tetracycline promoter on the vector was inactivated and thus unavailable for transcription of the *P. fluorescens* genes. That expression of the *ileS* and *lsp* genes on pBROC128 occurred indicates that these genes were probably transcribed by an endogenous promoter. Examination of sequences upstream of the translation start site of the *P. fluorescens x* gene revealed a TATAAT (Fig. 3), which corresponds with the consensus sequence for the −10 RNA polymerase-binding domain of constitutive *E. coli* promoters (15). However, a −35 region in *P. fluorescens* was not as easily detected. Three putative −35 elements which differed significantly from the *E. coli* consensus, TTGACA, were located 15, 18, and 22 nt upstream of the −10 region. The predicted strengths of these potential promoter sites were calculated by using the program described by Mulligan et al., which compares a given sequence to that of the "ideal" promoter (15). The values obtained for the −35 elements located 15, 18, and 22 nt upstream in combination with the −10 region were 40, 44, and 50%, respectively (15). Two

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          0                A                                  49
Lspeco      MSQSICST GLRWLWLVVV VLIIDLGSKY LILQNFALGD TVPLFPSLNL
Lspent      MSKSICST GLRWLWVVVA VLIIDLGSKF LILQNFALGE TVSLFPSLNL
Lsppsf    MPNADSRFGR LGWLVLSLLV LVIDQVSKAH FEGSLEMFQQ IVVIPDYFSW
Consensus  ..ms.Sicst glrwlw.vvv vlIidlgsk. lilqnfalg. tV.lfpslnl

          50                          B                      99
Lspeco    HYARNYGAAF SFLADSGGWQ RWFFAGIAIG ISVILAVMMY RSKATQKLNN
Lspent    HYARNYGAAF SFLADSGGWQ RWFFAGIAVG ICVVLAVLMY RSKATQKLNN
Lsppsf    TLAYNTGAAF SFLADGGGWQ RWLFAVIAVV VSAVLVVWLK RLGRDDTWLA
Consensus hyArNyGAAF SFLADsGGWQ RWfFAqIAvg isvvLaV.my Rskatqklnn
                    I
          100  C                                        D 149
Lspeco    IAYALIIGGA LGNLFDRLWH GFVVDMIDFY VGDWHFATFN LADTAICVGA
Lspent    IAYALIIGGA LGNLFDRLWH GFVVDMIDFY VGDWHFATFN LADSAICIGA
Lsppsf    IALALVLGGA LGNLYDRIAL GHVIDFILVH WQNRHYFPAF NFADSAITVG
Consensus IAyALiiGGA LGNLfDRlwh GfVvDmIdfy vgdwHfatfn lad.aic.ga
                  II
          150                 172
Lspeco    ALIVLEGFLP SRAKKQ*
Lspent    ALIVLEGFLP SSDKKTS**
Lsppsf    AIMLALDMFK S..KKTGETV ND*
Consensus Alivlegflp S..KKt.... ND*
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FIG. 4. Comparisons of the deduced amino sequences of SPase II from *E. coli* (Lspeco), *Enterobacter aerogenes* (Lspent), and *P. fluorescens* (Lsppsf). Asterisks in the sequences indicate translation stop signals. In the consensus rows, periods indicate no consensus, lowercase letters indicate identity in two of three residues, and uppercase letters indicate complete consensus. The numbers above the sequence refer to the amino acid residues relative to the *P. fluorescens* sequence. The postulated transmembrane domains for *E. coli* are overlined (A to D). The two domains of high homology among the three organisms are underlined (I and II).

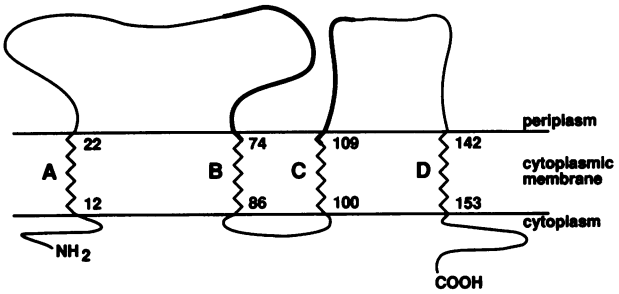

FIG. 5. Model of transmembrane structure of SPase II. The numbers refer to the *E. coli* amino acid residues identified in reference 4. The four transmembrane domains (A to D) are identified and presented as β-sheet structures embedded in the cytoplasmic membrane. The two regions of high homology (structures I and II in Fig. 4) are presented as boldface lines.

other regions corresponding to the *E. coli* site 2 promoter located within the *x* gene and the *lsp* internal promoter, were examined for near consensus promoter sequences; however, none were detected by inspection of the DNA sequences. Unlike those of *E. coli* and *Enterobacter aerogenes*, the *P. fluorescens lsp* promoter is either absent or not functional in *E. coli*.

The intervening sequences between *lsp* and *orf149* in *E. coli* and between *orf149* and *orf316* in *P. fluorescens* (Fig. 2) suggest formation of stem-loop structures which may function as transcription termination signals. Miller et al. (11) detected an *ileS-lsp* cotranscript which apparently terminated in this region in *E. coli*. Since the intervening sequences in *P. fluorescens* occur after *orf149*, we predict that a longer transcript which terminates after *orf149* will be detected.

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