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

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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

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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 isoleucyltRNA 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. fluo*rescens 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 μ g/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* DH5 α cells were pur-

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Strain or plasmid	Relevant genotype or phenotype	Source or reference	
P. fluorescens NCIB 10586	Wild type		
E. coli			
DH5a	$recA1 \Delta(argF-lacZYA) \phi 80 dlacZ \Delta M15$	BRL ^a	
JM103	$\Delta(lac \ pro) (F' \ lacI^{q}Z\Delta M15)$ $proA^{+}B^{+})$	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	Apr	This study	
pSF10	Apr	This study	

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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 DH5 α 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.). $[\alpha^{-3^2}P]dCTP$ and $[\alpha^{-3^5}S]dATP$ 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

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

Strain (plasmid)	Globomycin concn	Complemen- tation ^b at:		Pseudomonic acid concn	
(plasmid)	(µg/ml) ^a	30°C	42°C	(µg/ml) ^c	
DH5a	25			25	
DH5a(pUC18)	25	+	-	25	
DH5α(pMT521)	>100	+	+	25	
NCIB 10586 ^d				>200	
DH5a(pBROC128)	>100	+	$+^{e}$	>200°	
DH5α(pSF2518)	25				
DH5α(pSF2519)	>100	+	_	25	
331c ⁻		+	-		

^a Globomycin resistance in DH5a clones was assayed. The concentration of globomycin allowing saturated growth was measured after overnight incubation at 37°C

Complementation of the IleS(Ts) phenotype was performed with strain

 $331c^{-}$. ^c Pseudomonic acid resistance in DH5 α 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. Strain NCIB 10586 is a pseudomonic acid-producing strain of P. fluo-

rescens and is resistant to this antimicrobial agent. Confirms observations of Burnham and Winstanley (personal communi-

cation).

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 μ g/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 ClaI 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 PstI-ClaI fragment (Fig. 1). To facilitate subcloning, a 2.5-kb PstI-EcoRI fragment, which carried 23 bp of the original vector, was excised, ligated to pUC18 and pUC19, and transformed into DH5 α . 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 ClaI-EcoRI fragment from pBROC128 and ligating the isolated DNA segment to pUC18 digested with AccI and EcoRI. pSF10 was constructed by excising a 1.1-kb HindIII-PstI fragment from pBROC128 and ligating the fragment to pUC18 at the HindIII-PstI 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 PstI and digested with Bal 31, and the duplex termini were repaired with Klenow enzyme to generate blunt ends. After addition of HindIII linkers, the fragments were digested with *HindIII* and *EcoRI*, ligated to pUC18, and

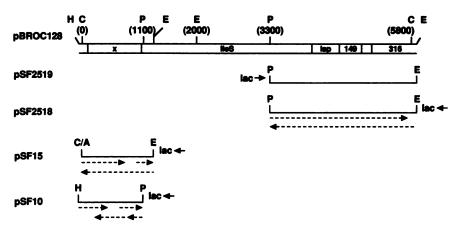


FIG. 1. Strategy used to subclone and sequence the P. fluorescens genes from pBROC128. The numbers in parentheses indicate the coordinates in base pairs. Restriction enzymes are abbreviated as follows: H, HindIII; C, ClaI; P, PstI; E, EcoRI; and A, AccI. Only sites relevant to the subcloning procedures 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 DH5 α . 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 EcoRI end (distal to the PstI site) were generated by treating pSF2518 as previously described, except that the plasmid was first cleaved with EcoRI. After addition of EcoRI linkers, the fragments were cleaved with EcoRI and PstI.

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 μ g/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

	iteS			orf1 4 9	
1	LeuGlnArgGluLeuGlnGluHisTyrGlyGluTyrArgPheTrpAsnValTyrSerLys CTGCAGCGCGAGTTGCAGGAACACTACGGCGAATACCGCTTCTGGAACGTCTACTCCAAG	60	1201	GAGCAAGAAAACCGGAGAAACCGTACTGATCAGGTATTGGCTGAGCAACGCATCG GAGCAAGAAAACCGGAGAAACCGTCATGACTGATCAGGTATTGGCTGAGCAACGCATCG SSerLysLysThrGlyGluThrValkanAspEnd	1260
61	IleHisAsnPheCysValGlnGluLeuGlyGlyPheTyrLeuAspIleIleLysAspArg ATCCACAACTTCTGCGTGCAGGAGCTGGGTGGTTCTACCTCGACATCATCAAGGACCGC	120	1261	lyGlnAsnThrGluValThrLeuHisPheAlaLeuArgLeuGluAsnGlyAspThrValA GCCAGAACACGGAAGTCACTTTGCATTCGCACTGCGCCTGGAGAATGGCGACACGGTCG	1320
121	GlnTyrThrThrGlyAlaAsnSerLysAlaArgArgSerAlaAspArgAlaValProHis CAGTACACCACTGGCGCCAACAGCAAGGCGCGCCGTTCGGCAGACCGCGGTTGACCACAT	180	1321	spSerThrPheAspLysAlaProAlaThrPheLysValGlyAspGlyAsnLeuLeuProG	1380
181	GlnArgArgLeuValArgTrpIleAlaProIleLeuAlaPheThrAlaAspGluLeuTrp CAGCGAAGGCTGGTGCGCTGGATCGCACCGATCCTGGCATTCACCGCTGACGAACTGTGG	240	1381	lyPheGluAlaAlaLeuPheGlyPheLysAlaGlyAspLysArgThrLeuGlnIleLeuP GTTTCGAAGCGCACTGTTCGGTTTCAAGGCCGGTGACAAGCGCACCCTGCAAATCCTGC	1440
241	GluTyrLeuProGlyGluArgAsnGluSerValMetLeuAsnThrTrpTyrGluGlyLeu GAGTACCTGCCGGGCGAGCGTAACGAGTCCGTCATGCTCAACACCTGGTACGAAGGCCTG	300	1441	roGluAsnAlaPheGlyGlnProAsnProGlnAsnValGlnIleIleProArgSerGlnP CGGAAAACGCCTTTGGCCAGCCCAACCCGCAAAACGTGCAGATCATCCCCGTTCGCAGT	1500
301	ThrGluLeuProAlaAspPheGluLeuGlyArgGluTyrTrpGluGlyValMetAlaVal ACCGAACTGCCGGCTGACTTCGAACTGGGCCGCGAGTACTGGGAGGGCGTGATGGCCGtC	360	1501	heGInAsnMetAspLeuSerGluGlyLeuLeuValIlePheAsnAspAlaAlaAsnThrG TCCAGAACATGGACCTGTCGGAAGGCTTGCTGGTGATCTTCAATGATGGGGGGAACACTG	1560
361	LysValAlaValAsnLysGluLeuGluValGlnArgAlaAlaLysAlaValGlyGlyAsn AAGGTTGCGGTGAACAAGGAACTGGAAGTCCAGCGTGCGGCCAAGGCCGTGGGTGG	420	1561	luLeuProGlyValValLysAlaPheAspAspAlaGlnValThrIleAspPheAsnHisP AATTGCCTGGCGTGGAAAGCATTTGATGACGCGCAAGTGACCATCGACTTCAATCACC	1620
421	LeuGInAlaGluValThrLeuPheAlaGluAspGlyLeuThrAlaAspLeuAlaLysLeu CTGCAAGCCGAAGTCACCCTGTTTGCCGAAGACGGCCTGACCGCCGACCTGGCCAAGCTG	480	1621	roLeuAlaGlyLysThrLeuThrPheAspValGluIleIleAspValLysAlaLeuEnd CGTTGGCCGGCAAGACGTTGACCTTTGACGTCGAGATTATCGACGTCAAAGCGCTGTAAC	1680
481	SerAsnGluLeuArgPheValLeuIleThrSerThrAlaSerLeuAlaProPheThrGln AGCAACGAGCTGCGCTTCGTGCTGATCACCTCTACTGCGAGCCTGGCGCCGTTTACCCAG	540	1681	CGACCGTACGCGGTCTAAAATGTGGGAGGGGGGCTTGCTCCCGATAGCGGTATATCAGCTA	1740
541	AlaProAlaAspAlaValAlaThrGluValProGlyLeuLysLeuLysValValLysSer GCTCCGGCAGATGCGGTGGCTACCGAAGTGCCTGGCCTTAAGCTCAAAGTGGTCAAGTCG	600	1741 1801	ANTGTGCAGACTGATATACCCTATATCGGGAGCAAGCCCCCCCC	1800 1860
601	AlaPheProLysCysAlaArgCysTrpHisCysArgGluAspValGlyValAsnProGlu GCCTTCCCcAAGTGCGCTCGTTGCTGGCACTGCCGTGAAGACGTCGGCGTGAACCCTGAG	660		MetG orf316	2000
661	HisProGluIleCysGlyArgCysValAspAsnIleSerGlyGluGlyGluValArgHis CACCCGGAAATCTGCGGTCGTTGTGTGTGGACAACATCAGCGGTGAAGGCGAGGTTCGCCAC	720	1861	AAATCAAACTCGCCAACCCCCGTGGCTTCTGCGCCGGCGTGGACCGGGCGATCGAAATCG lnllelysleuklakanProkrgGlyPheCysAlaGlyValkspkrgAlaIleGlulleV	1920
721	TyrAlaEnd TATGCCTAATGCAGACAGTCGTTTCGGACGTCTGGGCTGGCT	780	1921	TCAATCGCGCCCTGGAAGTCTTCGGGCCGCCGATTATGTGCGCCATGAAGTCGTCCATA alAsnArgAlaLeuGluValPheGlyProProIleTyrValArgHisGluValValHisA	1980
	lsp		1981	ACAAATTTGTGGTCGAAGACTTGCGTGCGCGGGGGGGATCTTTGTCGAAGAACTCGATC snLysPheValValGluAspLeuArgAlaArgGlyAla1lePheValGluGluLeuAspG	2040
781	CCTGGTCATTGACCAGGTCAGCAAGGCTCACTTCGAGGGCTCCCTGGAAATGTTCCAGCA 1LeuVal11eAspGlnValSerLysAlaHisPheGluGlySerLeuGluMetPheGlnGl	840	2041	AGGTGAAAGACGACGTGATCGTCATCTTCAGTGCCCACGGTGTTTCCCAGGCTGTACGTA lnVallysAspAspVallleValllePheSerAlaHisGlyValSerGlnAlaValArgT	2100
841	AATCGTGGTGATCCCGGATTATTTCAGCTGGACCCTGGCCGCCTACAACACTGGGCCGCCCT nIleValValleProAspTyrPheSerTrpThrLeuAlaTyrAsnThrGlyAlaAlaPh	900	2101	CCGAAGCGGCCGGGCCCTGGCCTGAAGGTGTTCGATGCCACCTGCCCACTGGTGACCAAGG hrGluAlaAlaGlyArgGlyLeuLysValPheAspAlaThrCysProLeuValThrLysV	2160
901	CAGCTTCCTCGCTGACGGCGGTGGCTGGCTAGCGCCGGTGTTGCTGTGATCGCCGTGGT eSerPheLeuAlaAspGlyGlyGlyTrpGlnArgTrpLeuPheAlaValIleAlaValVa	960	2161	TGCATATCGAGGTGGCGCGCTACAGCCGCGGCGGTGGTGGAGTGCATCCTGATCGGCCACG alHisIleGluValAlaArgTyrSerArgAspGlyArgGluCysIleLeuIleGlyHisA	2220
961	GGTAAGTGCCGTACTGGTGGTGTGCGCTCAAGCGCCTGGGCCGCGACGACACCTGGCCGGC IValSerAlaValLeuValValTrpLeuLysArgLeuGlyArgAspAspThrTrpLeuAl	1020	2221	CCGGTCACCCGGAnGTAGAAGGCACCATGGGCCAATACGACGCCAGCAACGGCGGTGCTA laGlyHisProXxxValGluGlyThrMetGlyGlnTyrAspAlaSerAsnGlyGlyAlaI	2280
1021	CATCGCGCTGGCCCCTAGTGTGGGTGGCGCGCGGGGCAACCTGTATGACCGCATGGCCCT allahaLuuxlaLeuValLeuGlyGlyAlaLeuGlyAsnLeuTyrAspArgIleAlaLe	1080	2281	TCTACCTGGTCGAGGACGAGAAAGACGTCGCCAACTTGCAGGTGCACAATCCGGAACGCC leTyrLeuValGluAspGluLysAspValAlaAsnLeuGlnValHisAsnProGluArgL	2340
1081	GGGCCATGTGATCTTTATCCTGGTGCATTGGCAGAACCGCCACTACTTCCCGCGTT uGlyHisVallleAspPhelleLeuValHisTrpGlnAsnArgHisTyrPheProAlaPh	1140	2341	TGGCCTTCGTGACCCAGACCACCTTGTCCATGGACGACACCAGCCGnGTAATCGAT 239 euAlaPheValThrGlnThrThrLeuSerMeCAspAspThrSerArgValIleAsp	6
1141	CAACTTTGCCGACAGTGCTATCACCGTCGGCGCAATCATGCTGGCGCTGGATATGTTCAA eAsnPheAlaAspSerAlaIleThrValGlyAlaIleMetLeuAlaLeuAspMetPheLy	1200			

FIG. 2. Nucleotide and predicted amino acid sequences of the P. fluorescens lsp and orf149 and flanking sequences. The sequence numbering starts with the PsII 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 ClaI site (nt 2396). Arrows indicate inverted repeats; dots indicate mismatched bases.

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1	TCGATTCGACCCATTTGGCCTGCGCGCTGTGCCTGCCGGGCCGGGGCGGGGTGGGGT	60
61	TCGACCACTTTATGAGCAAGAAGCGCGCTTATGCAGCTGGTTCGAGGTCTCCACAACCTGC	120
	MetGlnLeuValArgGlyLeuHisAsnLeuA	
121	GCCCCGAGCATCGGGGCTGCGTCGCCACTATTGGCAACTTTGACGGTGTTCACCGTGGCC	180
	rgProGluHisArgGlyCysValAlaThrIleGlyAsnPheAspGlyValHisArgGlyH	
181	ACCAGGCTATCCTGGCAAGGCTGCGCGAGCGTGCGGTCGAGTTGGGTGTGCCCAGCTGCG	240
101	isGlnAlaIleLeuAlaArgLeuArgGluArgAlaValGluLeuGlyValProSerCysV	
• • •	TGGTGATTTTCGAGCCACAACCGCGGGAGTTCTTTACCCCGGAAACAGCGCCGGCCCGCT	
241	alValllePheGluProGlnProArgGluPhePheThrProGluThrAlaProAlaArgL	300
301	TGGCGCTTGCGCGACAAGCTGCAACTGCTGGCGGAGAGGGCGTGGACCGCGTCCTCTGCC	360
	euklaLeuklaArgGlnAlaAlaThrAlaGlyGlyGluGlyValAspArgValLeuCysL	
361	TGGCTTTCAACCAgCGTTTGCGCAGCCTCAGCGCCGCCGAGTTCGTCGACCGCATTCTGG	420
	euAlaPheAsnGlnArgLeuArgSerLeuSerAlaAlaGluPheValAspArgIleLeuV	
421	TCGATGGCCTGGGTGTACAACACCTGGAGGTTGGTGACGACTTCCATTTCGGTTGCGATC	480
	alAspGlyLeuGlyValGlnHisLeuGluValGlyAspAspPheHisPheGlyCysAspA	
	GGGTCGGGGGATTTCGATTTCCTGCAACATGCCGGCGTCAACCAGGGCTTTACCGTTGAAG	
481	rgValGlyAspPheAspPheLeuGlnHisAlaGlyValAsnGlnGlyPheThrValGluA	540
541	CCGCCCAAACCGTCGAACTGGACGGCCTGCGTGTGAGCAGCACCCAGGTGCGTAACGCCT	600
	${\tt laklaGlnThrValGluLeuAspGlyLeuArgValSerSerThrGlnValArgAsnAlaL}$	
601	TGGCTGCCGCCGACTTCGACCTGGCCGAGCGTTTGCTCGGTCGCCCGTTCCGCATTGCCG	660
	$eu \\ A \\ la \\ A \\ la \\ A \\ sp \\ Phe \\ A \\ sp \\ Leu \\ A \\ la \\ G \\ lu \\ A \\ rg \\ Leu \\ Leu \\ G \\ ly \\ A \\ rg \\ Pro \\ Phe \\ A \\ rg \\ Ie \\ A \\ la \\ G \\ a \\ $	
661	GCCGGGTACTGCACGGCCAGAAGCTGGCGCGCCAATTGGGCACGCCAACTGCCAACGTGC	720
	lyArgValLeuHisGlyGlnLysLeuAlaArgGlnLeuGlyThrProThrAlaAsnValG	
	AACTCAAGCGCCGCCGAGTGCCGCTGACTGGGGTTTACCTGGTGAGCGTCGACATCGACG	780
721	AACTCAAGCGCCGCCGAGTGCCGCTGACTGGGGGTTTACCTGGTGAGCGTCGACATCGACG InLeuLysArgArgArgArgValProLeuThrGlyValTyrLeuValSerValAspIleAspG	/80
781	GCCAATCGTGGCCGGGAGTCGCCAATATAGGCGTCAGGCCCACGGTTGCAGGTGATGGCA lyGlnSerTrpProGlyValAlaAsnIleGlyValArgProThrValAlaGlyAspGlyL	840
	lyginsertrpproglyvalklaksnilegiyvalkrgprotnrvalklagiykspolyL	
841	AGGCCCACCTGGAAGTTCACCTTTTGGATTTTGCCGGTGATTTATACGACCGGCGTTTGA	900
	ysAlaHisLeuGluValHisLeuLeuAspPheAlaGlyAspLeuTyrAspArgArgLeuT	
901	CGGTGGTTTTCCACCAGAAGCTGCGTGAAGAGCAGCGTTTCGCCTCCCTGGAGGCGTTGA	960
	$hr Val Val Phe {\tt His Gln Lys Leu Arg Glu Glu Gln Arg Phe {\tt Ala Ser Leu Glu Ala Leu L}$	
961	ANACGGCGATCAATGCGGATGTCGCCGCCGCCGTGCACTAGCCGCACCTAGCCCCCATC	1020
301	ysThrAlaIleAsnAlaAspValAlaAlaAlaArgAlaLeuAlaAlaProSerAlaHisA	
	· · · · · · · · · · · ·	
1021	GCTAACCGAAGAGCCTTAAÄTGACCGACTATAAAGCCACGNTAAACCTTCCGGACACCGC rgEnd MetThrAspTyrLysAlaThrXxxAsnLeuProAspThrAl	1080

1081 CTTCCCAATGAAGGCCGGCCTGCCACAGCGCGAACCGCAGTCCTGCAG aPheProMetLysAlaGlyLeuProGlnArgGluProGlnSerCys

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 isoleucyltRNA synthetase with respect to isoleucine. Of the clones tested, only pBROC128 complemented the temperaturesensitive 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-orf149orf316 genes and the corresponding E. coli genes

P. fluorescens	Homology (%) with E. coli			
gene	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 ileS-lsp lsp-orf149 orf149-orf316	TAA-42 nt-TG <u>ATG</u> A TG <u>ATGA</u> TAATAA-121 nt- <u>ATG</u> TAA C <u>ATG</u>	TAA-11 nt-TAAATG ATG CC TAA TG AC TGA ATG AC TGA 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 ClaI 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

0 A 49							
Lspeco	MSQSICST	GLRWLWLVVV	VLIIDLGSKY	LILONFALGD	TVPLFPSLNL		
Lspent	MSKSICST	GLRWLWVVVA	VLIIDLGSKF	LILONFALGE	TVSLFPSLNL		
Lsppsf	MPNADSRFGR	LGWLVLSLLV	LVIDOVSKAH	FEGSLEMFOO	IVVIPDYFSW		
Consensus		glrwlw.vvv		lilqnfalg.	tV.lfpslnl		
	50			В	99		
Lspeco	HYARNYGAAF	SFLADSGGWQ	RWFFAGIAIG	ISVILAVMMY			
Lspent	HYARNYGAAF		RWFFAGIAVG				
Lsppsf	TLAYNTGAAF	SFLADGGGWO					
Consensus	hyArNyGAAF	SFLADsGGWO	RWfFAqIAvq	isvvLaV.my	Rskatgklnn		
		I			•		
	100 C	-			D 149		
Lspeco	IAYALIIGGA	LGNLFDRLWH	GFVVDMIDFY	VGDWHFATFN	LADTAICVGA		
Lspent	IAYALIIGGA	LGNLFDRLWH	GFVVDMIDFY	VGDWHFATFN	LADSAICIGA		
Lsppsf	IALALVLGGA	LGNLYDRIAL	GHVIDFILVH	WONRHYFPAF	NFADSAITVG		
Consensus	IAYALIIGGA	LGNLfDRlwh	GfVvDmIdfy	vqdwHfatfn	lad.aic.ga		
	150		172				
Lspeco	ALIVLEGFLP	SRAKKQ*					
Lspent	ALIVLEGFLP	SSDKKTS**					
Lsppsf	AIMLALDMFK	SKKTGETV	ND*				
Consensus	Alivlegflp	SKKt	ND*				

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).

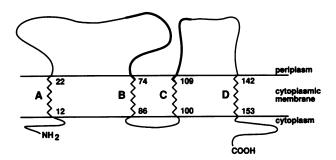


FIG. 5. Model of transmembrane structure of SPase II. The numbers refer to the *E. coli* amino acid residues identified in reference 4. The four transmembrane domains (A to D) are identified and presented as β -sheet structures embedded in the cytoplasmic membrane. The two regions of high homology (structures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