Cloning and Nucleotide Sequence of the *Enterobacter aerogenes* Signal Peptidase II (*lsp*) Gene

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In *Escherichia coli*, prolipoprotein signal peptidase is encoded by the *lsp* gene, which is organized into an operon consisting of *ileS*, *lsp*, and three open reading frames, designated genes *x*, *orf-149*, and *orf-316*. The *Enterobacter aerogenes* *lsp* gene was cloned and expressed in *E. coli*. The nucleotide sequence of the *Enterobacter aerogenes* *lsp* gene and a part of its flanking sequences were determined. A high degree of homology was found between the *E. coli* *ileS-lsp* operon and the corresponding genes in *Enterobacter aerogenes*. Furthermore, the same five genes which constitute an operon in *E. coli* were found in *Enterobacter aerogenes* in the same order.

Of the genes encoding the enzymes of the prolipoprotein modification and processing pathway in *Escherichia coli* (17), only the prolipoprotein signal peptidase (or signal peptidase II [SPase II]) gene, *lsp*, has been mapped (11, 18), cloned, and sequenced (2). SPase II processes modified prolipoprotein to form apolipoprotein, which is further modified by N acylation to form a mature lipoprotein (14). On the *E. coli* chromosome, the *lsp* gene resides immediately downstream of the isoleucyl-tRNA synthetase gene, *ileS*. Although there is no apparent physiological connection between the activation of isoleucine by Ile-tRNA synthetase and the proteolytic cleavage of lipid-modified prolipoprotein by SPase II, these two genes are cotranscribed along with three open reading frames in an operon, *x-ileS-lsp-orf-149-orf-316*, located at 0.5 min on the *E. coli* genome (9).

Virtually all gram-negative bacteria possess Braun lipoprotein or other lipoprotein molecules in their inner and outer membranes, and these lipoproteins have been shown to possess N-acyl diglyceride-cysteine as the N-terminal residue (reviewed in reference 16). It is therefore highly likely that SPase II is a conserved enzyme among these bacteria. In this paper, we report the cloning, nucleotide sequence, and expression of the *lsp* gene from *Enterobacter aerogenes* in *E. coli*.

Cloning and subcloning of *Enterobacter aerogenes* *lsp* gene. *Enterobacter aerogenes* (ATCC 13048) genomic DNA was digested with *Hind*III and probed by Southern blot hybridization under low stringency with [α -³²P]dCTP-labeled 0.4-kilobase (kb) *E. coli* fragments carrying only *lsp* sequences. Fragments of the desired size (7.2 kb) were ligated to *Hind*III-digested and dephosphorylated pUC18 vector and transformed into *E. coli* DH5 α cells (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Southern hybridization analysis was performed on the colony blots, and a clone which carried the putative *lsp* on a 7.2-kb fragment was recovered (pBK1; Fig. 1). After restriction mapping and further Southern hybridization analysis, the *Enterobacter aerogenes* *lsp* gene was isolated on a 1.6-kb *Sal*I-*Eco*RV fragment, which was subcloned into pUC18 at the *Sal*I-*Sma*I site to generate pBK16. Attempts to subclone this fragment into pUC19 were unsuccessful. To evaluate whether the *lac*

promoter on the vector was necessary for transcription of the *lsp* gene, a 5-kb *Bam*HI-*Hind*III fragment was subcloned from pBK1 into both pUC18 and pUC19 plasmids to generate pBK185 and pBK195, respectively.

Expression of the *Enterobacter aerogenes* *lsp* gene. Globomycin, a cyclic peptide antibiotic, is a specific inhibitor of SPase II activity (3). To assess expression of the *Enterobacter aerogenes* *lsp* gene, strains of *E. coli* harboring the original clone and subclones were grown in the presence of various concentrations of globomycin. DH5 α clones containing pBK1, pBK16, pBK185, and pBK195 exhibited resistance to globomycin at levels exceeding 100 μ g/ml (Fig. 1B). *E. coli* JM103 (8) clones containing these plasmids were also resistant to >100 μ g of globomycin per ml, irrespective of the presence or absence of 2 mM IPTG (isopropyl- β -D-thiogalactopyranoside) (data not shown). These results indicate that the *Enterobacter aerogenes* *lsp* gene present in these clones is expressed in *E. coli* and confers increased resistance to globomycin. Furthermore, the expression of the *lsp* gene in pBK16, pBK185, and pBK195, with or without IPTG, suggests that the cloned fragment contains a promoter(s) for the expression of the *Enterobacter aerogenes* *lsp* gene.

Expression of *ileS* on pBK1. Western immunoblot assays were performed on soluble fractions which were prepared from 5-ml overnight L-broth cultures. After being pelleted, the cells were suspended in 1 ml of 10 mM sodium phosphate buffer (pH 7.0) and sonicated until a cleared lysate was obtained. The homogenate was centrifuged at 225,000 $\times$ g for 1 h, and the supernatant was lyophilized. Samples (100 μ g of protein) were electrophoresed in a sodium dodecyl sulfate-8% polyacrylamide gel (7). After electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% [vol/vol] methanol; pH 8.3) for 30 min. The proteins were electrophoretically transferred overnight onto nitrocellulose paper (0.45- μ m pore size; Schleicher & Schuell, Inc., Keene, N.H.) in transfer buffer at 10°C. The paper was then treated with Tween buffer (150 mM NaCl, 1 mM EDTA [ethylenediaminetetraacetic acid], 50 mM Tris, 0.05% [vol/vol] Tween 20) plus 3% bovine serum albumin for 1 h, incubated with anti-*E. coli* isoleucyl-tRNA synthetase serum for 1 h, rinsed three times in Tween buffer, and reacted with affinity-purified goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate for 1 h at room temperature. After the secondary antibody was removed by

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FIG. 1. Characterization of pBK1 and subclones. (A) Physical map of pBK1. Subclones are shown below the map. Orientation of transcription from the *lac* promoter on the vector is indicated by solid arrows. Dashed arrows indicate the sequencing strategy on pBK1 and pBK16. The predicted termination site on *orf-316* is indicated by a dashed vertical line. Restriction enzyme abbreviations: H, *Hind*III; B, *Bam*HI; E, *Eco*RV; S, *Sal*I. Only sites relevant to subcloning procedures are shown. (B) Expression of *lsp* and *ileS*. A pUC18 clone served as a negative control in which growth occurred only at a globomycin concentration of 25 μ g/ml. ^a+, Growth in concentrations up to at least 100 μ g of globomycin per ml. ^b+, Growth after overnight incubation at 42°C; -, no growth after such incubation.

three washes with Tween buffer plus 0.1% bovine serum albumin, the nitrocellulose paper was immunostained with 5-bromo-4-chloro-3-indolyl phosphate-Nitro Blue Tetrazolium reagent system (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). After visualization, the nitrocellulose paper was rinsed in distilled water and dried.

The Western blot with *E. coli* anti-isoleucyl-tRNA synthetase (Fig. 2) showed that the *Enterobacter aerogenes* *ileS* gene product (lane 5) migrated slightly faster than the *E. coli* Ile-tRNA synthetase (lanes 1, 2, and 4). Two species of isoleucyl-tRNA synthetase were detected in cells containing pBK1 (lane 3). The predominant protein comigrated with the *Enterobacter aerogenes* band and apparently corresponded to the amplified *Enterobacter aerogenes* *ileS* on pBK1. A second, fainter band comigrated with the *E. coli* Ile-tRNA synthetase and probably corresponded to the *ileS* encoded by the *E. coli* genomic *ileS*. This band was also observed with pBK16 (lane 4), which lacked coding sequences for the first two-thirds of the N-terminal region of the *Enterobacter aerogenes* *ileS*; therefore, only chromosomally encoded isoleucyl-tRNA synthetase was expected to be seen.

Plasmid pBK1 and subclones were introduced into *E. coli* 331c⁻ (4) by transformation to assess their abilities to complement the *ileS*(Ts) mutation in the strain (Fig. 1). After

overnight incubation at 42°C, growth was observed with pBK1 but not with pBK16, pBK185, or pBK195, indicating that complementation occurred only with pBK1. These results indicate that the *Enterobacter aerogenes* *ileS* is expressed in *E. coli* to form functional IleS-tRNA synthetase. Furthermore, the complementation data are in full agreement with the Western blot results regarding the presence or absence of an intact *Enterobacter aerogenes* *ileS* gene in these clones.

Nucleotide sequence analyses of pBK16 and pBK1. DNA was sequenced by the dideoxy-chain termination method of Sanger et al. (12) as modified by Kraft et al. (6) for double-stranded template sequencing with Sequenase (United States Biochemical Corp., Cleveland, Ohio). Compressions in the sequencing gels were resolved by substituting dITP for dGTP. The DNA data were analyzed on a VAX computer with the University of Wisconsin Genetics Computer Group program packages, version 5 (1). The sequencing strategy of *lsp* and its flanking genes in the operon is shown in Fig. 1. Initially, pBK16 was sequenced and oligonucleotide primers were prepared from these sequences to obtain the 3'-terminal sequences of *orf-149* and to identify *orf-316* in pBK1. The nucleotide sequence of a 1.6-kb segment of the *Enterobacter aerogenes* chromosome subcloned in pBK16 and shown to confer SPase II activity was determined on both strands of DNA. pBK16 was found to contain 719 bases of *ileS*, the entire *lsp* gene (495 base pairs), and 372 nucleotides, or 83% of the homolog of *E. coli* *orf-149*. A homolog of gene *x*, the first gene in the *E. coli* operon (5), was also located on pBK1. Analysis of the sequence data revealed, however, that the original clone (pBK1) lacked the first 240 nucleotides of gene *x* and the upstream promoter region. Additionally, a homolog of *orf-316* was detected on pBK1. Therefore, all five genes homologous to the *E. coli* operon *x-ileS-lsp-orf-149-orf-316* were present in *Enterobacter aerogenes* in the same order.

Considerable nucleotide and amino acid sequence homologies (88 and 96%, respectively) exist between *E. coli* and *Enterobacter aerogenes* *lsp* genes (Fig. 3). The remaining genes were not sequenced in their entirety; however, the sequences obtained displayed similar degrees of homology.

FIG. 2. Western immunoblot assay of isoleucyl-tRNA synthetase. Lanes: 1, DH5 α (no plasmid); 2, DH5 α (pUC18); 3, DH5 α (pBK1); 4, DH5 α (pBK16); 5, *Enterobacter aerogenes*; 6, DH5 α (pMT521) (13). Molecular mass markers are indicated in kilodaltons.

FIG. 3. Nucleotide and predicted amino acid sequences of *Enterobacter aerogenes* (Ea) *lsp* and flanking nucleotide sequences. The *lsp* sequence is compared with published *E. coli* (Ec) *lsp* sequences (2). *Enterobacter aerogenes* amino acids that are different from *E. coli* are indicated in bold type. The overlap of the stop and start codons at the cistronic junction of *ileS* and *lsp* is underlined, the proposed *lsp* promoter region is double underlined, and termination codons are indicated by asterisks. These sequence data will appear in the EMBL/GenBank/DBJ/NBRF-PIR/MIPS/JIPID nucleotide sequence data bases under accession number M26713.

In *E. coli*, the termination codon of the upstream *ileS* overlaps the initiation codon of *lsp*, and the latter gene terminates in the codons TAATAA (2). The same codon usages were detected in the *Enterobacter aerogenes* *lsp* gene. Although dissimilarities in amino acid sequences were found to be mainly conservative differences, the *Enterobacter aerogenes* *lsp* gene was found to contain one additional amino acid at the extreme carboxyl-terminal end (Fig. 3).

A putative promoter which apparently corresponds to the *lsp* internal promoter of *E. coli* (Fig. 3; 10) was detected upstream of *lsp* within the *ileS* gene. Increased SPase II activity in cells containing pBK1 subclones was probably derived from this promoter since all clones, regardless of the orientation of the *lac* promoter on the vector, conferred increased globomycin resistance on the transformants. In cells transformed with pBK1, expression of the *ileS* and *lsp* genes appears to be independent of the *lac* promoter on the vector on the basis of the direction of transcription of the cloned genes. In view of the high degree of homology between the two organisms, the *Enterobacter aerogenes* *ileS* and *lsp* genes may very well be organized into an operon, along with *x*, *orf-149*, and *orf-316*, similar to that of *E. coli*, and multiple promoters may exist, including a promoter located within gene *x* which may be responsible for the expression of *Enterobacter aerogenes* *ileS* and *lsp* in cells containing pBK1.

These results could have been predicted on the basis of the report of Woese et al. (15), who showed that *E. coli* and *Enterobacter aerogenes* have 98% homology with respect to their 16S rRNAs. However, since *ileS* and *lsp* appear to possess disparate physiological functions, it is surprising that these genes and three flanking genes of yet unknown functions are retained in tandem in another organism.

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