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The Occurrence of Duplicate Lysyl-tRNA Synthetase Gene Homologs in *Escherichia coli* and Other Procaryotes

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The lysyl-tRNA synthetase (LysRS) system of *Escherichia coli* K-12 consists of two genes, *lysS*, which is constitutive, and *lysU*, which is inducible. It is of importance to know how extensively the two-gene LysRS system is distributed in procaryotes, in particular, among members of the family *Enterobacteriaceae*. To this end, the enterics *E. coli* K-12 and B; *E. coli* reference collection (ECOR) isolates EC2, EC49, EC65, and EC68; *Shigella flexneri*; *Salmonella typhimurium*; *Klebsiella pneumoniae*; *Enterobacter aerogenes*; *Serratia marcescens*; and *Proteus vulgaris* and the nonenterics *Pseudomonas aeruginosa* and *Bacillus megaterium* were grown in AC broth to a pH of 5.5 or less or cultured in SABO medium at pH 5.0. These growth conditions are known to induce LysRS activity (LysU synthesis) in *E. coli* K-12. Significant induction of LysRS activity (twofold or better) was observed in the *E. coli* strains, the ECOR isolates, *S. flexneri*, *K. pneumoniae*, and *E. aerogenes*. To demonstrate an association between LysRS induction and two distinct LysRS genes, Southern blotting was performed with a probe representing an 871-bp fragment amplified from an internal portion of the coding region of the *lysU* gene. In initial experiments, chromosomal DNA from *E. coli* K-12 strain MC4100 (*lysS*$^+$ *lysU*$^+$) was double digested with either *Bam*HI and *Hin*dIII or *Bam*HI and *Sal*I, producing hybridizable fragments of 12.4 and 4.2 kb and 6.6 and 5.2 kb, respectively. Subjecting the chromosomal DNA of *E. coli* K-12 strain GNB10181 (*lysS*$^+$ Δ*lysU*) to the same regimen established that the larger fragment from each digestion contained the *lysU* gene. The results of Southern blot analysis of the other bacterial strains revealed that two hybridizable fragments were obtained from all of the *E. coli* and ECOR collection strains examined and *S. flexneri*, *K. pneumoniae*, and *E. aerogenes*. Only one *lysU* homolog was found with *S. typhimurium* and *S. marcescens*, and none was obtained with *P. vulgaris*. A single hybridizable band was found with both *P. aeruginosa* and *B. megaterium*. These results show that the dual-gene LysRS system is not confined to *E. coli* K-12 and indicate that it may have first appeared in the genus *Enterobacter*.

It is well established that two genes for lysyl-tRNA synthetase (LysRS) exist in *Escherichia coli* K-12 (13, 21, 22, 25, 50). One gene, designated *lysS*, is expressed constitutively and is located at 62 min on the *E. coli* chromosome (13). The second gene, designated *lysU*, has been shown to map at approximately 93.5 min on the *E. coli* chromosome (50). The level of expression of this gene is low when *E. coli* is grown in a minimal or enriched minimal medium at neutral pH, but its expression is induced by several factors, which include heat shock (50); addition of small, hydrophobic leucine peptides, such as Gly-L-Leu, to minimal medium (36); addition of L-alanine or L-leucine to minimal medium (23, 24); growth to a low pH in broth (18, 20); anaerobiosis (27); and a mutation in the leucine-responsive regulatory gene (*lrp*) (29).

It is not apparent why two LysRS genes are extant in *E. coli* K-12. Studies on strains lacking the *lysU* gene indicate that it is not essential for growth of *E. coli* at temperatures in the range of 42 to 45°C (19, 27). Examination of the duplicate LysRS gene system has been confined to *E. coli* K-12. From an evolutionary perspective, if a duplicate gene such as *lysU* has no advantageous function, it will probably be lost from the genome through a recombination event or at least functionally through random genetic drift. Therefore, studying the LysRS system in other procaryotes may provide insight into the following more general evolutionary questions. (i) Is LysRS activity inducible in procaryotes other than *E. coli* K-12? (ii) Do two genes for lysyl-tRNA synthetase exist in procaryotes other than *E. coli* K-12? (iii) Can evidence of the apparent initial appearance of the duplicate LysRS system in procaryotes be found?

Since it is relatively rare to find duplicate genes in *E. coli*, there appears to be significance to the genomic maintenance of *lysU*, which, as noted above, is responsive to certain environmental signals. Dissemination of the duplicate LysRS genes among various procaryotes would suggest that the *lysU* gene product plays a physiologically significant role.

In this study, we demonstrated that lysyl-tRNA synthetase activity is inducible not only in *E. coli* K-12 but also in *E. coli* B, eight *E. coli* reference collection (ECOR) isolates, *Shigella flexneri*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*. In those organisms in which LysRS activity was clearly inducible, two *lysU* homologs were discernible as the result of Southern blot analysis of restriction-digested chromosomal DNA with a probe containing only internal *lysU* coding sequences. In this study, the organism most ancestral to *E. coli* in which two *lysU* homologs could be detected was in the genus *Enterobacter*.

MATERIALS AND METHODS

Bacterial strains. All of the bacterial strains used in this study are listed in Table 1. Strain GNB10181, an *E. coli* strain derived from MC4100 by Mud *lac* fusion, has been shown to be deficient in inducible lysine decarboxylase (the *cadA* gene product) (1), inducible LysRS activity (LysU), melibiose utilization (*mel* operon), and inducible arginine decarboxylase (*adi*) (19). Strain JA200/pLC4-5 is a Clarke-Carbon colony bank strain (9) which has been shown to express LysU by maxicell analysis (50).

Media and culture conditions. All bacterial strains, except transformants, were maintained on Luria-Bertani plates (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar). *E. coli* transformants were maintained on Luria-Bertani plates

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Bacteria		
Enterobacteriaceae		
Escherichia coli K-12 strain MC4100	*lysU*$^+$	G. N. Bennett[a]
Escherichia coli K-12 strain GNB10181	Δ*lysU*	G. N. Bennett
Escherichia coli K-12 strain AT2092	*lysU*$^+$	This laboratory
Escherichia coli K-12 strain JM105		Pharmacia Biotech, Inc.
Escherichia coli NC3		This laboratory
Escherichia coli EC2		ECOR[b]
Escherichia coli EC10		ECOR
Escherichia coli EC16		ECOR
Escherichia coli EC49		ECOR
Escherichia coli EC50		ECOR
Escherichia coli EC65		ECOR
Escherichia coli EC68		ECOR
Shigella flexneri		ATCC 35660
Salmonella typhimurium		ATCC 14028
Klebsiella pneumoniae		This laboratory
Enterobacter aerogenes		This laboratory
Serratia marcescens		ATCC 29632
Proteus vulgaris		This laboratory
Nonenterics		
Pseudomonas aeruginosa		This laboratory
Bacillus megaterium		This laboratory
Plasmids		
pLC4-5	Clarke-Carbon; *lysU*$^+$	F. C. Neidhardt[c]
pLURS28-n	*lysU*$^+$ subclones	This laboratory
pLUP87-1	Source of *lysU* probe	This laboratory
pBR322		Pharmacia Biotech, Inc.
pT7T318U		Pharmacia Biotech, Inc.

[a] Rice University, Houston, Tex.
[b] D. Dykhuisen, State University of New York at Stony Brook.
[c] University of Michigan, Ann Arbor.

supplemented with 100 μg of ampicillin per ml. Bacterial cultures that were used as a source of either plasmid or chromosomal DNA were grown in Terrific broth (49). In experiments done to measure lysyl-tRNA synthetase activity, cultures were grown in the minimal medium of Davis and Mingioli (10) supplemented with amino acids (minus lysine), vitamins, and bases (SMM) with 0.36% glucose as the sole carbon source (36). To determine the amount of inducible LysRS activity, cultures were grown in either AC broth (Difco), SABO medium (39), or SMM supplemented with 3 mM glycyl-L-leucine (Gly-L-Leu; Sigma Chemical Co.).

Enzyme assays. LysRS activity in whole-cell extracts was determined by the standard assay conditions described previously (18).

Recombinant DNA techniques. General cloning techniques were as previously described (2, 17, 42). Restriction and modifying enzymes were used in accordance with the manufacturer's (Pharmacia Biotech, Inc.) recommendations. Chromosomal DNA was prepared by the procedure of Best and Bender (3). Plasmid miniprep DNA was prepared either by purification by the Qiagen Tip-20 column method or by a modification of the procedure of Birnboim and Doly (4). Oligonucleotides used for PCR amplifications and sequencing were synthesized on a Gene Assembler Plus DNA Synthesizer (Pharmacia Biotech, Inc.) with phenoxyacetyl base-protected β-cyanoethyl phosphoramidites (43, 44). After synthesis, the oligonucleotides were deprotected at 70°C for 1 h in 1 ml of ammonium hydroxide and then purified by gel filtration over a NAP 10 (Sephadex G-25; Pharmacia Biotech, Inc.) column. An aliquot of the column-purified oligonucleotide solution was diluted in sterile distilled water, and the A_{260} was measured for quantitation on an UltroSpec III spectrophotometer (Pharmacia Biotech, Inc.). The primers synthesized for amplification were 5′-GAGCGGAT AACAATTTCACACAGG-3′-OH (sense strand) and 5′-GTTGGGTAACGC CAGGGTTTTCCC-3′-OH (antisense strand).

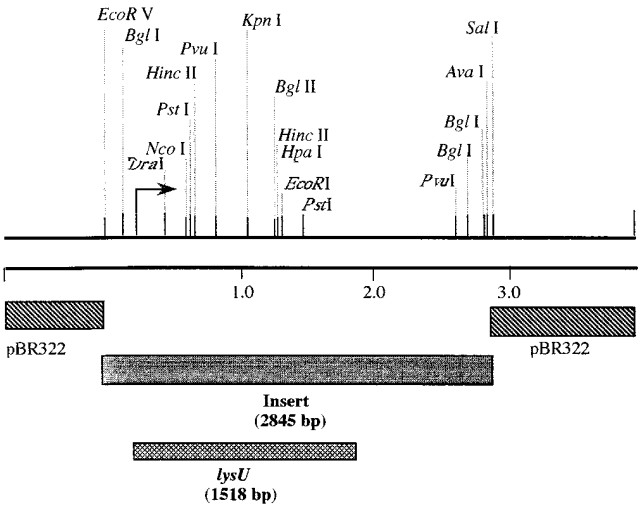

FIG. 1. Restriction map of *lysU*-expressing subclone pLURS28-9. The arrow indicates the direction of transcription of the *lysU* gene. The divisions are marked in kilobase pairs.

Cycle sequencing was carried out as described previously (12, 34, 35), with an automated DNA sequencer for detection (A.L.F. DNA Sequencer; Pharmacia Biotech, Inc.) and fluorescein-labelled primers (FluorePrime; Pharmacia Biotech, Inc.). The primers used to sequence the *lysU* probe were as follows: 5′-fluor-TTTCACACAGGAAACAGCTATGAC-3′-OH, 5′-fluor-CTGGTTGT AGGCGGTTTTGAACGG-3′-OH, and 5′-fluor-CTGAATCTATCGGTATTA CGGTAG-3′-OH (sense strands) and 5′-fluor-CGACGTTGTAAAACGACGG CCAGT-3′-OH, 5′-fluor-CTCTGTGACAATACGTCCCAACCC-3′-OH, and 5′-fluor-CGTGGTAATCCGCATACGCCATGT-3′-OH (antisense strands). Sequence analysis was performed with the DNASIS package (Hitachi), and the results were compared to published sequences for the *lysU* gene (8, 28).

Isolation of a LysU-expressing subclone. Plasmid pLC4-5, isolated from strain JA200/pLC4-5, served as a source of DNA for cloning of the *lysU* gene. A 2.8-kb *Eco*RV-*Sal*I fragment previously shown to contain the *lysU* gene (8, 27, 28) was initially cloned into the *Eco*RV-*Sal*I site of plasmid pBR322 (Pharmacia Biotech, Inc.). Plasmid DNA from a LysU-expressing subclone (pLURS28-9) was isolated, and a restriction enzyme map was constructed (Fig. 1). Several isolates of this subclone were found to have a five-to eightfold increase in LysRS activity compared with control strain JM105 under LysU-noninducing and -inducing conditions (41). An 871-bp *Pst*I fragment, representing an internal portion of the *lysU* gene, was isolated from pLURS28-9 and cloned into the *Pst*I site of phagemid pT7T318U (Pharmacia Biotech, Inc.) to generate *lysU* subclone pLUP87-1.

PCR amplification. Amplification of purified pLUP87-1 miniprep DNA was carried out on a Perkin-Elmer 480 thermocycler with the GeneAmp PCR Reagent Kit and Amplitaq DNA polymerase (40). After amplification, the product was purified by filtration (with sterile distilled water as the eluant) through a 30,000 NMWL Ultrafree-MC Filter Unit (Millipore Corp.) in accordance with the manufacturer's instructions. The 1,081-bp amplified product contained the entire cloned DNA segment (871 bp) and 210 bp of flanking vector DNA. Several microliters of the purified PCR product was examined for purity, homogeneity, and size on a 1.2% agarose gel containing 0.5 μg of ethidium bromide per ml. The purified product was quantitated with a DNA minifluorometer by following the manufacturer's (Hoefer) recommendations.

Southern blot hybridization analysis. Southern blot analysis was performed in accordance with standard protocols (2, 48), with a *lysU* DNA probe amplified from plasmid pLUP87-1 as described above and 2.5 to 5 μg of chromosomal DNA (digested to completion with various restriction enzymes). For use as a DNA probe in hybridization experiments, the purified 1,081-bp PCR product was nonradioactively labelled with a T7 QuickPrime Kit (Pharmacia Biotech, Inc.) by substituting biotinylated dCTP (Enzo, Inc.) for radiolabelled [^{32}P]dCTP in the reaction mixture. Southern hybridization and detection of biotinylated hybrids were carried out by using modifications of existing protocols for biotin-labelled probes (26, 33). The hybridized membranes were analyzed by using a streptavidin–alkaline phosphatase (Bethesda Research Laboratories)–nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co.) detection method (26, 33).

Two-dimensional gel electrophoresis of polypeptides. Cell extracts were prepared as described previously (20). Total cellular protein was determined by the method of Lowry et al. (30). Approximately 15 μg of total cellular protein was loaded on each gel and resolved by two-dimensional gel electrophoresis as described by O'Farrell (38). Silver staining of the second-dimension gels was done by the method of Wray et al. (52).

TABLE 2. Lysyl-tRNA synthetase activities of bacterial strains grown under LysU-noninducing and -inducing conditions

Bacterium	LysRS activity (U/mg of protein)[a]	
	Noninducing[b]	Inducing[c]
E. coli K-12 strains		
MC4100	63	187
GNB10181	57	58
AT2092	78	179
E. coli B strain NC3	95	225
ECOR strains		
EC 2	57	373
EC 49	87	261
EC 65	44	209
EC 68	61	274
Other enterics		
S. flexneri	90	246
K. pneumoniae	42	103
E. aerogenes	23	179
S. typhimurium	117	159
S. marcescens	82	16, 77[d]
P. vulgaris	12	18
Nonenterics		
P. aeruginosa	65	94
B. megaterium	75	62

[a] All LysRS assays were done at least in duplicate, and values for all duplicates are within 15% of each other.
[b] LysRS activity in SMM.
[c] Unless otherwise noted, induction of LysRS activity by growth in AC broth to pH 5.3.
[d] LysRS activity in SABO medium at pH 5.0.

RESULTS

Assay of lysyl-tRNA synthetase activity in various *E. coli* strains and other procaryotes. It is well established that the LysU, but not the LysS, protein is induced in *E. coli* K-12 upon its growth in AC broth to a culture pH of 5.5 or lower (18, 20). To assess the induction of LysRS (hence, presumably, LysU) activity in microorganisms other than *E. coli* K-12, especially in other members of the family *Enterobacteriaceae*, representatives of several genera were examined (Table 2). In these experiments, each organism was grown in SMM (noninducing condition) and in AC broth to an external pH of 5.5 (inducing condition; 19). In the *E. coli* K-12 control strain, AT2092, there was a typical two- to fourfold enhancement of LysRS activity upon its growth in the AC broth compared with SMM. All of the other *E. coli* strains tested had inducible LysRS activity; stimulation of LysRS activity among the ECOR strains was 3.0- to 6.5-fold. Two-dimensional gel electrophoretic analysis of ECOR strains EC2 and EC68 has corroborated that the LysU protein is induced and is in the same position relative to the LysS protein observed in two-dimensional gels of *E. coli* K-12 (41).

Of the organisms tested from other genera, *E. aerogenes* had the strongest induction of LysRS activity. Induction of LysRS activity in *S. flexneri* and *K. pneumoniae* was comparable to that of *E. coli* K-12. In contrast, there was no or only marginal induction of LysRS activity in *Salmonella typhimurium*, *Serratia marcescens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Bacillus megaterium* by the criterion used above or by growth in SABO medium at pH 5.0. The low level of LysRS activity in extracts from *P. vulgaris* is presumed to be due to the heterologous LysRS assay system, i.e., the acceptor $tRNA^{Lys}$ is from *E. coli* B.

Sequencing of the *lysU* probe. The sequence generated in this study for the 871-bp *Pst*I site from the *lysU* gene was compared by DNASIS with two separate sequences of the gene that were previously published (8, 28). The results of this comparison indicate discrepancies between our sequence data and those generated by the other two groups. With the results obtained in this investigation as the basis for comparison, most of the 871-bp sequence generated, with the exception of a single base inversion (CG instead of GC) at positions 859 and 860 (data not shown), is in accord with the corresponding 871-bp region sequenced by Lévêque et al. (28). Most of the differences seen in the sequences are single base changes that differ from the *lysU* sequence of Clark and Neidhardt (8). However, the most dramatic difference when the three sequences are compared is the absence of nine bases in the region corresponding to bases 295 to 330 of the *lysU* sequence of Clark and Neidhardt (8). These bases are present in both the sequence of Lévêque et al. (28) and the sequence of the same region obtained in this laboratory. More detailed information on the *lysU* region of the *E. coli* chromosome can be found by examining the sequence given GenBank-EMBL accession number ECOUW93.

Assignment of the *lysS* and *lysU* genes of *E. coli* K-12 to specific DNA restriction fragments. A critical tool for analyzing which restriction fragment from *E. coli* K-12 contained the *lysU* gene was strain GNB10181. There is no induction of the LysU protein in this strain, and it is suspected of harboring a deletion that includes the *lysU* gene (1, 19). The chromosomal DNAs of GNB10181 and its isogenic parent, MC4100, were double digested with either *Bam*HI-*Hin*dIII or *Bam*HI-*Sal*I, enzymes which do not cut into either the *lysS* or the *lysU* gene of *E. coli* K-12. Hybridization of the MC4100 chromosomal DNA restriction digests to the *lysU* probe, under both low- and high-stringency conditions, resulted in the detection of two bands (Fig. 2). They were approximately 12.4 and 4.2 kb for the *Bam*HI-*Hin*dIII digest and 6.6 and 5.2 kb for the *Bam*HI-*Sal*I digest. The stronger signal in each digest was found with the higher-molecular-weight fragment, suggesting that this fragment contains the *lysU* gene. This suspicion was reinforced by hybridization analysis of the chromosomal DNA of GNB10181 double digested with the same two sets of restriction enzymes. For both digests, the higher-molecular-weight fragment was missing. These results establish that the *lysU* gene resides on the larger of the two fragments that bind to the probe and that a stronger signal is generated in the hybridization of the *lysU* probe to the *lysU*-containing fragment. These results directly confirm that the lack of expression of *lysU* in strain GNB10181 is due to a deletion that includes this gene. The sizes of DNA fragments detected by Southern blot hybridization with a *lysU* probe by these restriction digests for all strains are given in Table 3.

Southern blot analyses of *E. coli* strains. Chromosomal DNA isolated from *E. coli* K-12 strain AT2092, *E. coli* B, or ECOR collection strain EC2, EC49, EC65, or EC68 was hybridized under low- and high-stringency conditions with the *lysU* probe. In all of the hybridization experiments, the *Bam*HI-*Hin*dIII digests generated two hybridizing fragments of 12.4 and 4.2 kb, with the larger fragment giving the stronger signal (Fig. 3). The *Bam*HI-*Sal*I digest resulted in two bands of 6.6 and 5.2 kb, with the larger fragment again generating the stronger hybridization signal. The only exceptions to the results of the *Bam*HI-*Sal*I double digest were found in EC65 and EC68. In both of these strains, the 6.6-kb band is conserved whereas the 5.2-kb band is absent. In EC65, the latter is replaced by a 5.5-kb band, whereas a 3.5-kb band is present in EC68. These results indicate that with the *E. coli* strains tested,

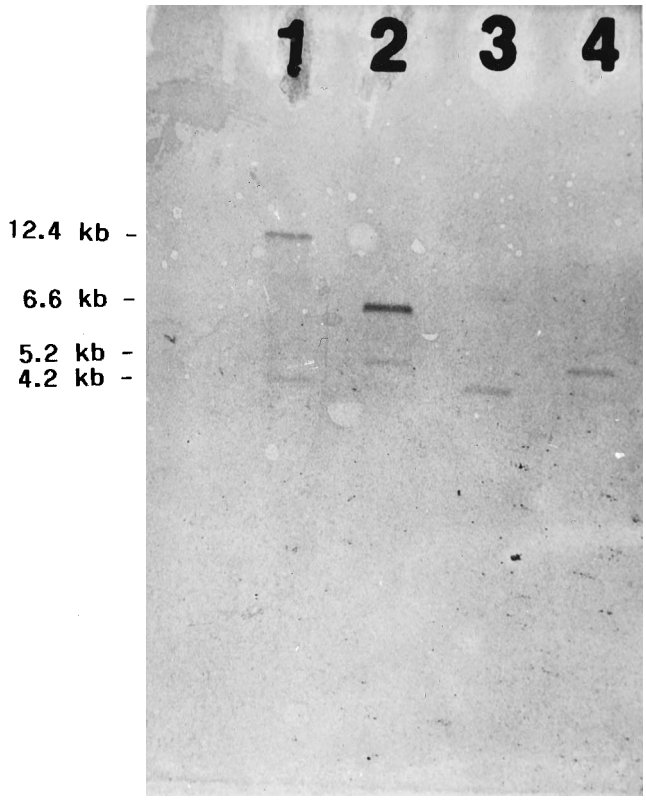

FIG. 2. Southern blot of MC4100 and GNB10181 with the *lysU* probe at low stringency. Lanes: 1, MC4100, *Bam*HI-*Hin*dIII; 2, MC4100, *Bam*HI-*Sal*I; 3, GNB10181, *Bam*HI-*Hin*dIII; 4, GNB10181, *Bam*HI-*Sal*I.

the regions surrounding *lysU* are somewhat better conserved than those surrounding *lysS*.

Southern blot analyses of members of the family *Enterobacteriaceae* other than *E. coli*. The remaining representative genera of the family *Enterobacteriaceae* examined for LysRS induction, *Shigella*, *Salmonella*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Proteus*, were analyzed by Southern blot analysis with the *lysU* probe under low- and high-stringency conditions (Fig. 3 to 6). When chromosomal DNA isolated from *S. flexneri* ATCC 35660 was double digested with *Bam*HI-*Hin*dIII, two bands, one greater than 23 kb and the second 5.2 kb long, were detected (Fig. 3). For reasons unknown, digestion of the DNA with the *Bam*HI-*Sal*I combination was not successful; only DNA from this organism presented this problem.

Hybridizing restriction fragments generated from *K. pneumoniae* genomic DNA had the same signal intensity under both low- and high-stringency conditions with all of the double digests performed (Fig. 4). The *Bam*HI-*Hin*dIII digest resulted in detection of 12.4- and 2.3-kb bands, and the *Bam*HI-*Sal*I digest yielded bands of 5.9 and 4.1 kb. Additional double digests were performed with the restriction enzyme combinations *Ban*II-*Sac*I and *Ban*II-*Xba*I, which do not cleave either *lysS* or *lysU* from *E. coli* K-12. These digests each produced two hybridizable fragments of 4.6 and 4.2 kb.

The Southern blot analysis of the genomic DNA of *E. aerogenes* initially presented unexpected results. The original studies with either *Bam*HI-*Hin*dIII or *Bam*HI-*Sal*I digests resulted in the production of three fragments hybridizable to the *lysU* probe under both low- and high-stringency conditions. With

TABLE 3. Sizes of fragments detected with the 1,081-bp *lysU* probe upon hybridization of genomic DNA restriction digests

Bacterium	Fragment size(s) (kb)			
	*Bam*HI-*Hin*dIII	*Bam*HI-*Sal*I	*Ban*II-*Sac*I	*Ban*II-*Xba*I
E. coli MC4100	12.4, 4.2	6.6, 5.2		
E. coli GNB10181	4.2	5.2		
E. coli AT2092	12.4, 4.2	6.6, 5.2		
E. coli B strain NC3	12.4, 4.2	6.6, 5.2		
E. coli EC2	12.4, 4.2	6.6, 5.2		
E. coli EC49	12.4, 4.2	6.6, 5.2		
E. coli EC65	12.4, 4.2	6.6, 5.5		
E. coli EC68	12.4, 4.2	6.6, 3.5		
S. flexneri	>23.0, 5.2	ND[a]		
K. pneumoniae	12.4, 2.3	5.9, 4.1	4.6, 4.2	4.6, 4.2
E. aerogenes	11.7, 8.9, 1.9	12.4, 5.2, 4.2	13.0, 5.2	13.0, 5.2
S. typhimurium	6.6	10.0	10.0	10.0
S. marcescens	5.9	10.0		
P. vulgaris	ND	ND		
P. aeruginosa	15.0, 8.5[b]	6.9, 4.4[b]		
B. megaterium	9.2	15		

[a] ND, no bands detected.
[b] Low-stringency conditions.

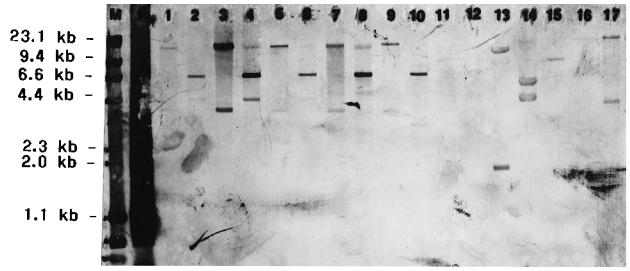

FIG. 3. Southern blot of members of the family *Enterobacteriaceae* with the *lysU* probe at high stringency. Lanes: M, λ*Hin*dIII-ϕX174 *Hin*cII; P, the *lysU* amplified product; 1, AT2092, *Bam*HI-*Hin*dIII; 2, AT2092, *Bam*HI-*Sal*I; 3, EC2, *Bam*HI-*Hin*dIII; 4, EC2, *Bam*HI-*Sal*I; 5, EC49, *Bam*HI-*Hin*dIII; 6, EC49, *Bam*HI-*Sal*I; 7, EC65, *Bam*HI-*Hin*dIII; 8, EC65 *Bam*HI-*Sal*I; 9, EC68, *Bam*HI-*Hin*dIII; 10, EC68, *Bam*HI-*Sal*I; 11, *E. coli* B, *Bam*HI-*Hin*dIII; 12, *E. coli* B, *Bam*HI-*Sal*I; 13, *E. aerogenes*, *Bam*HI-*Hin*dIII; 14, *E. aerogenes*, *Bam*HI-*Sal*I; 15, *S. typhimurium*, *Bam*HI-*Hin*dIII; 16, *S. typhimurium*, *Bam*HI-*Sal*I; 17, *S. flexneri*, *Bam*HI-*Hin*dIII.

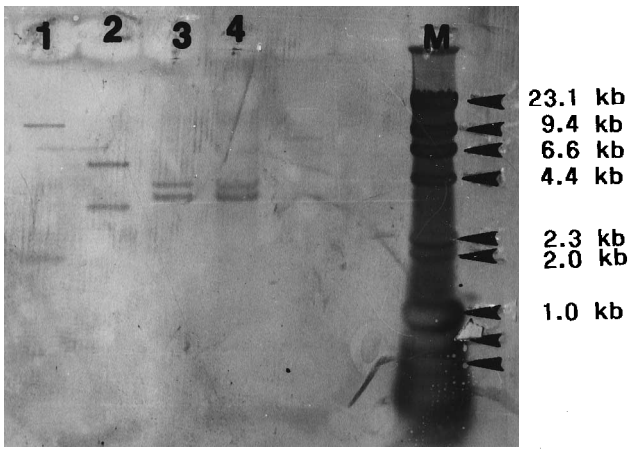

FIG. 4. Southern blot of *K. pneumoniae* at low stringency. Lanes: M, λ*Hin*dIII-ϕX174 *Hin*cII; 1, *Bam*HI-*Hin*dIII; 2, *Bam*HI-*Sal*I; 3, *Ban*II-*Sac*I; 4, *Ban*II-*Xba*I.

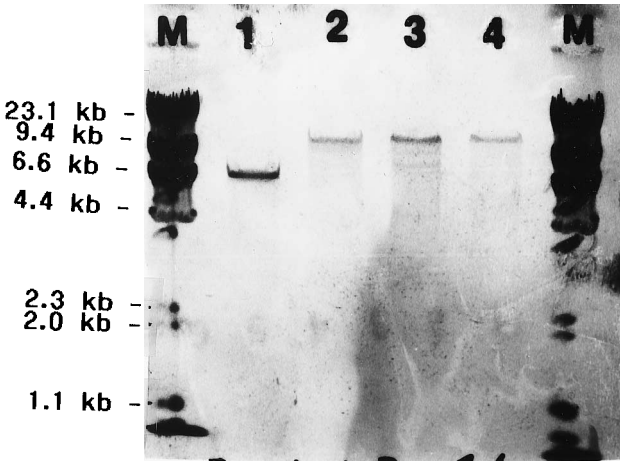

FIG. 5. Southern blot of *S. typhimurium* 14028 with the *lysU* probe at high stringency. Lanes: M, λ*Hin*dIII-ϕX174 *Hin*cII; 1, *Bam*HI-*Hin*dIII; 2, *Bam*HI-*Sal*I; 3, *Ban*II-*Sac*I; 4, *Ban*II-*Xba*I.

the first combination of digests, 11.7-, 8.9-, and 1.9-kb fragments were observed, whereas with the second combination of digests, 12.4-, 5.2-, and 4.2-kb bands were found. These results suggested the following possibilities: (i) that one of the restriction enzymes cleaves a site in either the *lysS* or *lysU* homolog, (ii) that incomplete digestion of the largest fragment resulted in detection of three bands, or (iii) that three *lysU* homologs actually exist in this strain. To gain further information, the chromosomal DNA was cleaved with two additional sets of restriction enzymes: *Ban*II-*Sac*I and *Ban*II-*Xba*I. These digests resulted in identical 13.0- and 5.2-kb bands. This outcome indicates that there are not three *lysU* homologs in the genus *Enterobacter* and that there is likely an additional cleavage site for one of the original restriction enzymes, probably *Bam*HI, in one of the two genes homologous to the *lysU* probe.

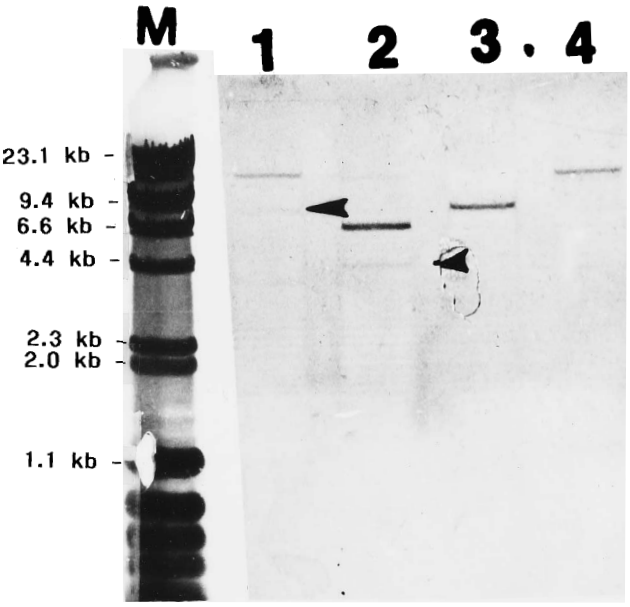

FIG. 6. Southern blot of *P. aeruginosa* and *B. megaterium* with the *lysU* probe at low stringency. Lanes: M, λ*Hin*dIII-ϕX174 *Hin*cII; 1, *P. aeruginosa*, *Bam*HI-*Hin*dIII; 2, *P. aeruginosa*, *Bam*HI-*Sal*I; 3, *B. megaterium*, *Bam*HI-*Hin*dIII; 4, *B. megaterium*, *Bam*HI-*Sal*I. The arrowheads indicate the positions of faint bands.

Since *E. coli* and the *Shigella*, *Klebsiella*, and *Enterobacter* spp. tested all appear to have two LysRS gene homologs, it was anticipated that the *Salmonella* sp. tested would give a similar profile. However, Southern blot analysis of *S. typhimurium* ATCC 14028 genomic DNA digested with *Bam*HI-*Hin*dIII or *Bam*HI-*Sal*I consistently yielded only one LysRS gene homolog either 6.6 or 10 kb long, respectively (Fig. 5). This result suggested either that there is only one LysRS gene homolog in the genus *Salmonella* or that the *lysS* and *lysU* genes are very close together on the *Salmonella* chromosome and thus present on the same restriction fragment. To reduce the probability of the latter, the *Salmonella* genomic DNA was double digested with either *Ban*II-*Sac*I or *Ban*II-*Xba*I; these digestions also resulted in only one observable hybridizable band.

S. marcescens ATCC 29632 genomic digests, upon hybridization, yielded only a single band of 5.9 (*Bam*HI-*Hin*dIII) or 10 (*Bam*HI-*Sal*I) kb at low or high stringency. *Proteus vulgaris* genomic DNA digested with either the *Bam*HI-*Hin*dIII or the *Bam*HI-*Sal*I combination at either low or high stringency yielded no hybridizable bands. These results suggest that there are considerable differences between the *E. coli* and *Proteus* genomes such that no homolog is detected with the *lysU* probe.

Southern blot analysis of nonenterics. Digestion of *P. aeruginosa* genomic DNA with *Bam*HI-*Hin*dIII or *Bam*HI-*Sal*I and hybridization to the *lysU* probe resulted in the detection of 15- and 8.5-kb bands with the former and 6.9- and 4.4-kb bands with the latter digest under low-stringency conditions (Fig. 6). The higher-molecular-weight fragment had a moderately strong signal, and the lower-molecular-weight fragment gave a weak signal at low stringency. Repetition of these digests at high stringency led to the detection of only the higher-molecular-weight fragment (result not shown).

Digestion of *B. megaterium* genomic DNA with the same sets of restriction enzymes resulted in the detection of only a single band of 9.2 or 15 kb, respectively, at either high or low stringency. Thus, only one LysRS homolog appears to exist in this *Bacillus* strain.

DISCUSSION

As a generalization, induction of LysRS activity by growth of microorganisms in AC broth to an external pH of 5.5 (early stationary phase) was a good prognosticator of whether two LysRS gene homologs would be observed by Southern blot analysis. Only in organisms in which a twofold or greater induction of LysRS activity was seen was there evidence by hybridization analysis of two LysRS gene homologs. Perhaps the only exception is *P. aeruginosa*, in which two potential LysRS gene homologs were seen under low-stringency conditions.

In all of the *E. coli* strains tested, there was ample induction of LysRS activity and evidence of two *lysU* homologs. This includes analysis of the four ECOR strains, EC2, EC49, EC65, and EC68, which represent three of the six ECOR phylogenetic groups as designated by Selander et al. (45). Additionally, LysRS was normally induced in ECOR strains EC10, EC16, EC40, and EC50, suggesting the presence of two LysRS genes in these strains as well. The two distinct fragment sizes for the two *lysU* homologs is consistent with the widely separated genomic locations of the *lysS* and *lysU* genes in *E. coli* K-12 (13, 19, 50). By the above two criteria, it is clear that two LysRS homologs are present in representative strains of the five of the six ECOR phylogenetic groups that were examined.

LysRS enzyme assays and Southern blot analyses indicate

that two LysRS gene homologs also exist in *S. flexneri*, *K. pneumoniae*, and *E. aerogenes*. *Enterobacter* was the genus most ancestral to *E. coli* in which we obtained firm evidence of two LysRS gene homologs.

E. coli K-12 has been our paradigm for the study of the dual-gene LysRS system, and it is known that regulation of the *lysU* gene is complex and environmentally sensitive. Expression of *lysU*, but not that of *lysS*, is stimulated by nutritional or environmental factors such as L-leucine (23), L-leucine dipeptides (23, 24), L-alanine (23, 24), heat shock (21, 50), anaerobiosis (28), or growth to low pH in rich medium (18, 20). We used only the last criterion in this effort since it appears to be the strongest inducer of *lysU* expression (18, 20). The observations that in the genus *Enterobacter* there appear to be two LysRS gene homologs and LysRS activity is induced upon the strain's growth to pH 5.5 in AC broth are noteworthy because they suggest the possibility that induction of LysRS by low external pH has been an important selective factor in maintaining an inducible lysyl-tRNA synthetase.

Shigella has been afforded the status of a genus separate from *Escherichia*, but this appears to be prejudiced by its clinical importance. Studies indicate that shigellae are not sufficiently distinctive genetically from escherichias to warrant status as a separate genus (37, 45). Nevertheless, we have found some differences between *S. flexneri* and *E. coli* with respect to the LysRS system. *Bam*HI-*Hin*dIII digestion of all of the *E. coli* strains tested produced 12.4- and 4.2-kb fragments, whereas in *S. flexneri*, this double digestion produced a higher-molecular-weight fragment greater than 23 kb long. Two-dimensional gel electrophoresis of whole-cell extracts prepared from *S. flexneri* suggested that the LysS polypeptide is in a position on the gel equivalent to that of its *E. coli* K-12 counterpart. However, it is clear that the positions of the LysU polypeptide on the two gels are different (41).

It was surprising that on the basis of both LysRS assays and Southern hybridization, no evidence of two LysRS gene homologs in *S. typhimurium* ATCC 14028 was obtained. This was not anticipated because of the results which indicate that two LysRS gene homologs exist in genera which are ancestral to or have more recently diverged from *Salmonella*. It is, of course, possible that the strain examined in this study is anomalous, and future work will examine this question. Alternatively, on the basis of available information (47), it is conceivable that our results reflect ecological habitat probability. The factors involved in regulation of the *lysU* gene have led to the suggestion that this gene's importance is tied to the existence of *E. coli* in the mammalian colon (19, 20, 27). Bacterial examination of the large intestine has shown that *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. are present at a high frequency; *Shigella* groups A to D are present at a low frequency, but *S. typhimurium* is only rarely present (31, 47). *S. typhimurium* would only be expected in the colon under pathogenic conditions.

There was essentially no detectable hybridization with the *lysU* probe on blots prepared with chromosomal digests from *P. vulgaris*, even at low stringency. We can rule out difficulty in the preparation of *P. vulgaris* genomic DNA or its cleavage by the restriction enzymes as a source of this problem, as they were normal.

Because of the lack of hybridization of the *lysU* probe to *P. vulgaris* genomic DNA, it was assumed that similar results would be found with other microorganisms, such as *P. aeruginosa* and *B. megaterium*, that are even more distantly related to *E. coli* than is *P. vulgaris*. With *B. megaterium*, a hybridizing DNA fragment was found, but only at low stringency, whereas with *P. aeruginosa* DNA, a hybridizing fragment was found at high stringency, and possibly two hybridizing fragments were observed at low stringency (Fig. 6). It is not clear why there was better hybridization of the *E. coli* K-12 *lysU* probe to the genomic DNA digests of the *Bacillus* and *Pseudomonas* species than to those of *P. vulgaris*. One possibility with respect to *P. aeruginosa* may be its habitat. *P. aeruginosa* is grouped together with other members of the family *Enterobacteriaceae* in the gamma subdivision of purple sulfur bacteria (51) and has been reported to occur in mammalian colons at a surprisingly high frequency (47). This is presumably because this bacterium is ubiquitous in nature and is found on foods such as fresh vegetables. Although *P. aeruginosa* is probably normally only a transient species in the colon, it is conceivable that a rare gene transfer and recombination event occurred between *P. aeruginosa* and either *E. coli* or other enterics that contain *lysU*. Recently, there have been indications that interspecies gene transfer and recombination have occurred in nature (5–7, 11, 16, 32).

At least two other *E. coli* duplicate gene systems have been evolutionarily analyzed. The first, and the most extensively examined, is the *tufA-tufB* (elongation factor Tu) system. These duplicate genes were found to be widespread among gram-negative bacteria but not to be present in gram-positive bacteria (14, 15). More recently, Smith et al. (46) have analyzed the dual glutamate decarboxylase genes *gadA* and *gadB*. These two genes were found in all of the *E. coli* strains examined, including ECOR strains EC20, EC26, EC37, and EC46. However, no species cross-hybridizing to the *gad* probe was detected in *Salmonella* sp.

Through our hybridization studies on the dual-gene LysRS system, the earliest indication of two LysRS genes is in the genus *Enterobacter*. Thus, there is a possibility that a duplication of lysyl-tRNA synthetase that has been maintained evolutionarily occurred in the genus *Enterobacter*. In *E. coli* K-12, the *lysU* gene is inducible and regulated by a variety of factors that do not influence the expression of *lysS* (19, 20, 21, 23, 50). It remains to be determined if all of the factors that induce *lysU* expression in *E. coli* K-12 can induce LysRS activity (presumably, increased *lysU* expression) in the other genera or even strains of *E. coli* other than *E. coli* K-12. All of the microorganisms that appear to have two LysRS gene homologs have inducible LysRS activity when grown in AC broth to low external pH. Hence, it appears that this physiological function has also been evolutionarily conserved. The actual physiological need for the duplicate, inducible LysRS remains an enigma, but its apparent evolutionary conservation suggests that it plays a distinct metabolic role.

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