JOURNAL OF BACTERIOLOGY, May 1984, p. 615–620
0021-9193/84/050615-06$02.00/0

Vol. 158, No. 2

Escherichia coli K-12 Lysyl-tRNA Synthetase Mutant with a Novel Reversion Pattern

IRVIN N. HIRSHFIELD,[1]* RAMON TENREIRO,[1] RUTH A. VANBOGELEN,[2] AND FREDERICK C. NEIDHARDT[2]

Department of Biological Sciences, St. John's University, Jamaica, New York 11439,[1] and Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109[2]

Received 10 November 1983/Accepted 17 February 1984

Fast-growing revertants have been selected from a slow-growing lysyl-tRNA synthetase mutant. All of the revertants had increased lysyl-tRNA synthetase activity compared with the mutant (5- to 85-fold), and in some revertants this amounted to two to three times the wild-type synthetase activity. Two-dimensional gel electrophoresis of a whole-cell extract of revertant IH2018 (1.5- to 2-fold wild-type synthetase activity) showed that the increase in synthetase activity is due to the induction of cryptic lysyl-tRNA synthetase forms and not to a change in the constitutive lysyl-tRNA synthetase. Genetic studies have shown that a locus termed *rlu* (for regulation of *lysU*) which is cotransducible with *purF* at 49.5 min influences the amount of the cryptic lysyl-tRNA synthetase.

Prior studies on the lysyl-tRNA synthetase (LysRS) of *Escherichia coli* K-12 by two-dimensional (2D) gel electrophoresis have indicated that there may be two distinct genes for this enzyme (10). A constitutive gene, *lysS*, codes for LysRS forms I and III, and a second gene, *lysU*, can be induced which codes for LysRS forms II and IV (10, 33). Recently, *lysU* has been mapped at 92 to 93 min (33). Forms II and IV have equivalent isoelectric points but a higher apparent molecular weight than do forms I and III. Forms II and IV can be induced by different stimuli. These include small molecules such as L-alanine (11, 15) or L-leucine dipeptides (2, 13), high temperature (10, 26), or a mutation in *metK*, the structural gene for *S*-adenosylmethionine synthetase (12).

Revertant analysis of aminoacyl-tRNA synthetase mutants has proved to be a valuable tool for gaining insight into their regulation. A variety of synthetase revertants have been isolated, including operator mutants (4, 17), gene duplications (6), and mutations at sites distant from synthetase genes which are not tRNA suppressor mutations (3, 7, 17, 32, 34). Revertant analysis of LysRS mutant IH2017 (14) was chosen as a means of gaining further evidence that *E. coli* contains two independently regulated genes for this synthetase. Previous work has shown that, due to the low activity of LysRS in this strain, the in vivo level of charged $tRNA^{lys}$ is low and consequently rate limiting for growth on supplemented minimal medium (SMM) (1, 14). Any alteration which increases the amount or activity of LysRS in IH2017 results in a strain which would outgrow IH2017 on SMM and be selectable by its larger size.

Specifically, the question posed was whether a revertant could be obtained in which there would be loss of control of the inducible synthetase forms II and IV without influencing the expression of the constitutive forms I and III. That this can be accomplished is demonstrated in this report.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used in this study are listed in Table 1.

Media. *E. coli* was grown in the minimal medium of Davis and Mingioli (5) or in this minimal medium supplemented with amino acids, vitamins, purines, and pyrimidines (27). The supplemented medium is referred to as SMM, and glucose (0.4% [wt/vol]) was used as the carbon and energy source for these media. Luria broth was prepared according to Miller (22).

Bacterial growth. All cultures were grown aerobically on a rotary shaker at 37°C. Growth of cells in minimal medium and in SMM or Luria broth was monitored spectrophotometrically at 490 and 580 nm, respectively. Cells to be assayed for LysRS were grown to an absorbance of 0.1 to 0.13 in either minimal medium or SMM.

LysRS assay. The activity of the synthetase was determined in crude sonic extracts (12, 15). Protein was determined by the method of Lowry et al. (21) with crystalline bovine serum albumin as a standard. The assay was run at 37°C for 3 min at pH 7.8 (Tris, 0.1 M) with a solution of 10^{-3} M ATP, 10^{-2} M $MgCl_2$, 0.4 mg of crude *E. coli* B tRNA, 5×10^{-6} M lysine (0.04 μCi of L-[^{14}C]lysine), and 1.0 μg of crude extract protein in a total volume of 0.1 ml. For the in vitro heat stability test, each sample was measured at 36 and 42°C as described above with all substrates present.

Selection of revertants. Ten independent single-colony cultures of mutant strain IH2017 were grown overnight in SMM at 37°C, diluted, and grown to log phase. The cultures were then streaked on 2% agar SMM plates and incubated at 37°C. Revertant colonies, which were distinguished by their larger size, were picked and purified on SMM agar plates for further study. Only three colonies from each culture were examined.

Rapid mapping. Rapid mapping was done by the method of Low (20).

Conjugation. The protocol for the conjugation experiments was that of Miller (22). The plates were incubated at 37°C for 48 to 72 h to allow sufficient time for the recombinants to grow. All Hfr strains were counterselected with streptomycin (100 μg/ml).

Replica plating. Replica plating was performed as described by Lederberg and Lederberg (18).

Transduction. Titers of P1 *vir* and lysate preparations were determined according to Miller (22). The protocol for transduction was that of Miller (22) or Lennox (19).

2D gel electrophoresis. Strains were grown in morpholine-

* Corresponding author.

TABLE 1. *E. coli* K-12 strains

Strain	Genotype or Phenotype	Source
WT	*arG pheA purF his lysS*$^+$ *Str*^{r} F$^-$	I. N. Hirshfield
IH2017 (LysRS mutant)	*argG pheA purF his lysS Str*^{r} F$^-$	I. N. Hirshfield (14)
PK191	*argG*$^+$ *pheA*$^+$ *purF*$^+$ *his*$^+$ *lysS*$^+$ *Str*^{s} *Hfr* po42′ counterclockwise	B. Low[a]
KL983	*argG*$^+$ *pheA*$^+$ *purF*$^+$ *his*$^+$ *lysS*$^+$ *Str*^{s} *Hfr* po51′ clockwise	B. Low
PK18	*argG*$^+$ *pheA*$^+$ *purF*$^+$ *his*$^+$ *lysS*$^+$*Str*^{s} *Hfr* po66′ clockwise	B. Low

[a] Yale University, New Haven, Conn.

propanesulfonic acid minimal medium (24) to early log phase. 2D gel electrophoresis was run by the method of O'Farrell (28). Cultures were labeled with [^{35}S]methionine (1,026.9 Ci/mmol; 30 μCi/ml) for several generations.

RESULTS

LysRS activity in the revertant strains. Revertants of mutant IH2017 were isolated as described above. The LysRS activity of revertants grown in SMM was up to 85 times greater than the LysRS activity of mutant IH2017, and in some instances 2 to 3 times greater than that of parent strain AT2092 (wild type [wt]). The results were similar when the revertants were grown in minimal medium instead of SMM (Table 2).

In vitro heat stability of LysRS from the revertants. Studies have shown that the presence of LysRS forms II and IV confers increased heat stability to LysRS in crude extracts (12). Heat inactivation studies with crude extracts of the revertants demonstrated that the LysRS from these strains was clearly heat stable compared with the WT enzyme (which should be composed only of forms I and III). This result is indicative of the presence of LysRS forms II and IV in the revertants (Table 3). The apparent increase in LysRS forms II and IV was not the result of a *metK* mutation since all revertants were inhibited by methionine analogs (16) to which *metK* strains are resistant.

2D gel analysis. In all figures, LysRS form I is labeled A and form II is labeled B. LysRS forms III and IV are not labeled, but are located immediately to the right of forms I and II, respectively. Figure 1 shows autoradiograms of gels of parent strain AT2092 (WT), mutant IH2017, and revertant IH2018, respectively, from cells grown in minimal medium to log phase and labeled with [^{35}S]methionine. In both WT and mutant IH2017, there are very low levels of LysRS forms II and IV. Of further interest is the observation that in mutant IH2017, LysRS forms I and III have undergone an acidic isoelectric shift. It is clear (Fig. 1) that in revertant IH2018 there is a distinct increase in the amounts of forms II and IV but no change in the positions or amounts of I and III.

Temperature sensitivity of the revertants. In addition to having increased LysRS activity, all revertants were found to be temperature sensitive on SMM plates or liquid medium at 42°C. Temperature was found to be a critical factor in the selection of the revertants, as none were found when mutant IH2017 was grown at 42°C. Apparently, the growth of any potential revertant was prevented at 42°C by its temperature-sensitive phenotype.

Conjugation studies with Hfr strains PK191 and PK18. Since the revertants acquired both a temperature-sensitive phenotype as well as elevated LysRS activity, the possibility was considered that both characteristics resulted from one event. In the initial effort to define this locus, temperature-resistant recombinants were selected by rapid mapping (20), and it was found that the Hfr strains which donated the temperature resistance locus most effectively were PK191 (po42′) and PK18 (po66′) (Fig. 2).

To further define this locus and to determine whether one locus was responsible for both events, two-factor matings were done with these Hfr strains with revertant IH2018 as a recipient, and *phe*$^+$ (56 min), *purF*$^+$ (Ade$^+$; 49.5 min), and *his*$^+$ (44 min) recombinants were individually selected. In each recombinant group, LysRS activity and the temperature-resistant or temperature-sensitive phenotype were tested as unselected markers. This analysis showed that the locus responsible for the temperature-sensitive phenotype

TABLE 2. LysRS activity in revertants grown in SMM or minimal medium

Strain	LysRS sp act			
	SMM		Minimal medium	
	U/mg[a]	% of strain AT2092[b,c]	U/mg	% of strain AT2092
WT (AT2092)	35.5–51.2 (9)[b]	100	30.5	100
Original mutant (IH2017)	1.0–1.5 (2)	3	1.0	3
Revertant				
IH2018	66.1–111.5 (4)	194	82.1	269
IH2020	82.1–103.6 (2)	209	—[d]	—
IH2021	4.4–8.8 (4)	15	3.6	12
IH2022	14.1–27.8 (3)	48	—	—
IH2023	35.3–41.5 (2)	87	—	—
IH2024	111.8 (1)	253	88.0	288
IH2025	74.0–120.0 (2)	220	78.0	256
IH2026	80.2–113.9 (2)	219	69.6	228
IH2027	107.8 (1)	244	—	—

[a] Data represent a range of specific activity.
[b] Numbers in parentheses represent the number of trials for each strain in SMM.
[c] The data represent the average specific activity with WT at 100%.
[d] —, Not tested in minimal medium.

TABLE 3. Heat stability of LysRS in WT and revertant strains

Strain	LysRS sp act (U/mg)		% Activity at 42°C/36°C
	36°C	42°C	
WT (AT2092)	41.7	19.2	42[a]
Revertants			
IH2018	75.9	83.5	110
IH2020	77.6	60.5	78
IH2021	4.2	4.0	95
IH2022	20.0	20.2	101
IH2025	69.6	70.3	101
IH2026	75.9	69.1	91

[a] The WT value is the average of four experiments in which the LysRS activity retained at 42°C ranged from 32 to 51% of the activity at 36°C.

and the regulation of LysRS are distinct because recombinants had a level of LysRS activity as high as or higher than that of revertant IH2018 (Table 4).

Analysis of Ade$^+$ (*purF*$^+$) recombinants of revertant IH2018 from matings with PK18 or PK191 indicated that three recombinant classes were present in terms of their LysRS activity: class 1 had activity like that of revertant IH2018, class 2 had WT activity, and class 3 had activity that was double that of revertant IH2018 and up to four times greater than the WT activity (Table 4). The class 2 strain R4 and the class 3 strain R8 were selected for further study. Heat stability tests in vitro on the LysRS activity in these recombinants showed them to be different. The R4 srain LysRS retained only 32% of its LysRS activity at 42°C compared with that at 36°C, a result typical of WT activity, but the LysRS of strain R8 retained 91% of its activity, which was indicative of the presence of forms II and IV.

A 2D gel of strain R4 was indistinguishable from that of the WT with respect to LysRS (data not shown). With strain R8, a multitude of events was recorded by the gel (Fig. 3). Not only had LysRS forms I and III shifted to the WT position but also forms II and IV were present at an even higher level than that seen in revertant IH2018. In addition, lysine decarboxylase and two unknown proteins were present at elevated levels compared with WT, mutant IH2017, or revertant IH2018. Since the Hfr strains PK191 and PK18 transfer in opposite directions, it is apparent that the gene which restores LysRS forms I and III to their normal positions is in the region bounded by these Hfr strains. In addition, the gene which influences LysRS form II and IV would also be in this region.

Conjugation with Hfr strain KL983. To further define the location of the latter gene, KL983 (po51′) was mated with revertant IH2018 for 20 min, thereby selecting *his*$^+$ recombi-

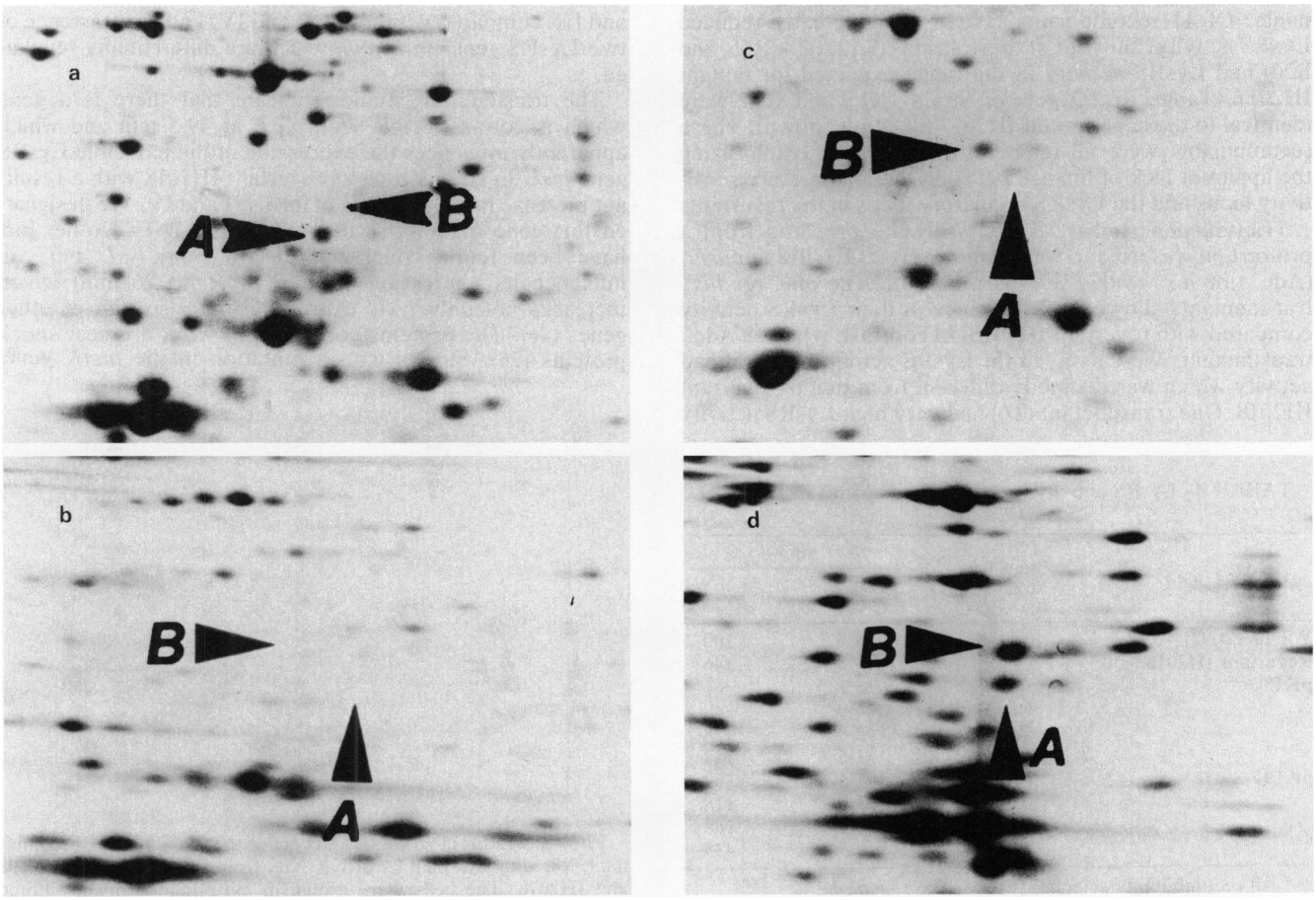

FIG. 1. Autoradiograms of 2D gels of WT and mutant LysRS strains. The cells were grown in morpholinepropanesulfonic acid minimal medium and labeled with [^{35}S]methionine. (a) WT strain AT2092; (b) mutant strain IH2017; (c) revertant strain IH2018; (d) WT strain AT2092 grown with 3 mM glycyl-L-leucine. (A) Form I of LysRS; (B) form II of the synthetase.

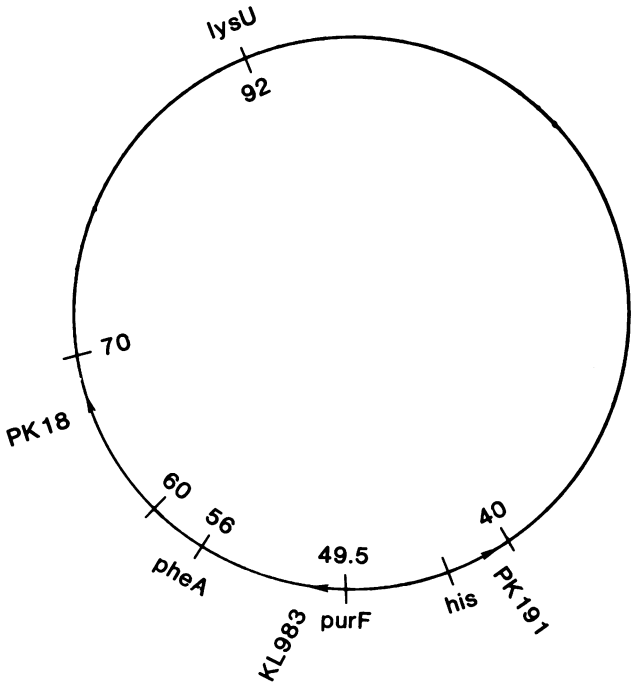

FIG. 2. Representation of the *E. coli* genome with relevant loci.

nants. Of 11 recombinants tested, 5 had greatly reduced LysRS activity, and four of these strains (R12, R14, R15, and R20) had LysRS activity in the range expected for mutant IH2017 (Table 5). 2D gels of strains R15 and R20 were identical to those of mutant IH2017 (data not shown). These recombinants were all temperature sensitive, emphasizing the apparent lack of linkage between the temperature sensitivity locus and the LysRS regulatory locus in the revertant.

Transduction studies. A P1 *vir* lysate prepared from a prototroph was used to transduce revertant IH2018 to *purF*$^+$ (Ade$^+$) or *his*$^+$ with *phe*+ as a control. The *phe*$^+$ or *his*$^+$ transductants showed no difference in their LysRS activity compared with revertant IH2018. In contrast, when 18 Ade$^+$ transductants were assayed for LysRS activity, several had activity which was distinctly different from that of revertant IH2018. One transductant (T6) had very high LysRS activity (three to four times that of WT), whereas others (e.g., T9 and T18) had LysRS activity identical to that of the LysRS mutant IH2017 (data not shown). A 2D gel of T6 (not shown) showed all of the changes seen in the ultrahigh recombinant R8, except there was no shift in LysRS forms I and III back to their WT positions. The gels of T9 and T18 were identical and were indistinguishable from a 2D gel of mutant IH2017 (data not shown).

TABLE 4. LysRS activity in Ade$^+$ recombinants of revertant IH2018[a]

Hfr strain used	Recombinant strain[b]	Sp act	
		LysRS[c] (U/mg)	% of WT (AT2092)
WT (AT2092)		29.7	100
Revertant IH2018		43.5	146
pK18	R1	18.1	61
	R2	44.0	148
	R3	42.8	178
	R4	33.5	113
pK191	R5	107.6	362
	R6	35.2	118
	R7	56.0	188
	R8	129.3	435

[a] All recombinants selected for Ade$^+$ are also *phe*$^+$.
[b] All of these recombinants are temperature resistant.
[c] For the synthetase assay, the strains were grown in glucose minimal medium at 37°C to an absorbance of 0.1 to 0.13 at 490 nm.

DISCUSSION

Revertant analysis of aminoacyl-tRNA synthetase mutants has been a valuable means of isolating synthetase-regulatory mutants. Some of these mutations have been shown to map closely to the synthetase structural gene (4, 17); others are clearly distant (3, 7, 17, 32, 34), but not tRNA suppressor mutations. Studies on LysRS in the revertant IH2018 demonstrate a unique pseudoreversion pattern in that the apparent regulatory mutation promotes an increase in LysRS forms II and IV, which are usually not expressed, rather than in the constitutive forms. Fortuitously, on 2D gels, forms I and III from mutant IH2017 show an acid isoelectric shift (to the right) compared with a WT strain (Fig. 1a and b). Thus, it is easy to visualize in revertant IH2018 that forms II and IV are in the same position seen in a WT strain stimulated to produce these forms (e.g., by glycyl-L-leucine, compare Fig. 1c and d) without a shift in position of forms I and III. These results as well as prior studies (10) are consistent with a separate origin for forms I and III, compared with forms II and IV, and the existence of two LysRS genes in *E. coli* which are differentially regulated.

The transduction studies indicate that there is a gene which is cotransducible with *purF* at 49.5 min and which apparently influences the expression of the inducible LysRS gene *lysU* in strains such as revertant IH2018 with a resultant increase in the amounts of forms II and IV. We designated this gene *rlu* for "regulation of *lysU*." Two other loci have been found which are distant from *lysU* but can influence its expression. One is *htpR* (at 76 min) which increases not only *lysU* expression but also that of other genes (26). The proteins controlled by *htpR* are heat shock proteins (26). Strains with a mutation in the *metK* gene,

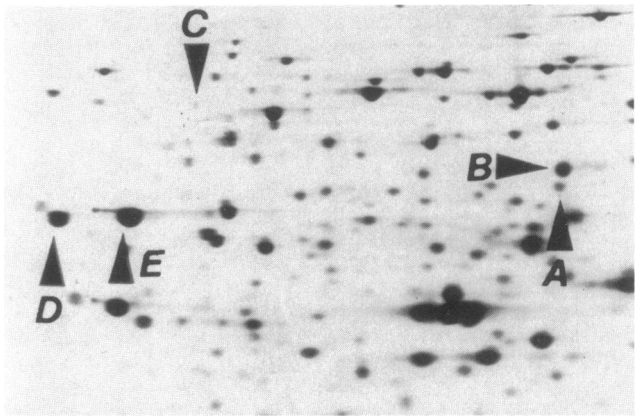

FIG. 3. Autoradiogram of a 2D gel of the class 3 Ade$^+$ recombinant, R8, with ultrahigh LysRS activity from a cross between PK191 and IH2018. The cells were grown in morpholinepropanesulfonic acid minimal medium and labeled with [^{35}S]methionine. (A) Form I of LysRS; (B) form II of LysRS; (C) lysine decarboxylase; (D, E) unidentified proteins.

TABLE 5. LysRS activity in *his*$^+$ recombinants of revertant IH2018 upon mating with Hfr strain KL983

Strain[a]	Sp act		
	LysRS[b] (U/mg)	% of WT	Type
WT (AT2092)	30.1	100.0	
Mutant IH2017	1.4	4.7	
Revertant IH2018	81.3	270.0	
Recombinant			
R12	2.1	7.0	
R13	11.2	37.2	
R14	1.1	3.7	Like IH2017
R15	1.7	5.7	
R20	1.8	6.0	
R16	75.5	251.0	
R17	72.9	242.0	
R18	75.8	252.0	Like IH2018
R19	70.3	233.5	
R21	76.6	254.5	
R22	66.0	219.0	

[a] All recombinants are temperture sensitive.
[b] For the synthetase assay, the cells were grown in glucose minimal medium to an absorbance of 0.1 to 0.13 at 490 nm.

which results in a decrease in cellular methylation (12), also show a marked increase in the expression of *lysU* (10).

Originally, it was thought that the locus responsible for the characteristic temperature-sensitive phenotype of the revertants would be the same locus responsible for the increase in LysRS activity. At least with revertant IH2018 this is not true (Tables 4 and 5), and whereas *rlu* is linked to *purF* at 49.5 min, the temperature-sensitive locus is more closely linked to *pheA* at 56 min (data not shown).

Perhaps the most unforeseen event was the observation that a locus which is cotransducible with *purF*$^+$ at 49.5 min can stimulate the expression of LysRS (forms II and IV) to an even greater degree than observed in revertant IH2018. Additionally, this event was pleiotropic in that lysine decarboxylase and two unknown proteins were also expressed at a higher level on 2D gels than was observed in revertant IH2018, mutant IH2017, or WT. Normally, lysine decarboxylase is expressed at a very low level in minimal medium (8, 31), although mutants have recently been reported in which the enzyme is constitutively produced in minimal medium (29, 31). However, in these strains, the mutant loci are not cotransducible with *purF* at 49.5 min (29, 31), and where it was measured, there was no increase in LysRS activity (29). Since both the pleiotropic locus and *rlu* are cotransducible with *purF*$^+$, it will require further effort to resolve whether the loci are allelic or just closely linked.

The results of the conjugation studies show that a locus responsible for the shift in the isoelectric positions of LysRS forms I and III lies between 42 and 66 min. Most likely, this locus represents *lysS*, and whereas determining its exact location will require further effort, it is clearly different from *lysU*, which maps between 92 and 93 min (33).

ACKNOWLEDGMENTS

I.N.H. thanks Ivan Lee for assistance in the initial phase of this work and St. John's University for support.

This work was supported in part by Public Health Service grant GM 17892 from the National Institute of General Medical Sciences to F.C.N. and by an equipment grant (PRM-8118134) from the National Science Foundation to I.N.H.

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