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Genetic Location of Two Mutations Affecting the Lysyl-Transfer Ribonucleic Acid Synthetase of *Bacillus subtilis*

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Two mutations (*lysS1* and *lysS2*), each independently resulting in a thermosensitive, lysyl-transfer RNA synthetase (L-lysine: tRNA ligase [adenosine 5'-monophosphate] EC 6.1.1.6), have been mapped on the *Bacillus subtilis* chromosome between *purA16* (adenine requirement) and *sul* (sulfanilamide resistance). They are linked by transformation with *sul* (70 to 74% cotransfer) in the order *purA16-lysS1-lysS2-sul*. The mutant loci are either in the same gene or in two closely linked genes. They are not linked to the tryptophanyl-tRNA synthetase structural gene or to the *lys-1* locus.

As part of a study on the roles of aminoacyl-transfer ribonucleic acid (tRNA) synthetases in sporulation, we recently isolated and characterized two mutants of *Bacillus subtilis* with altered, thermosensitive, lysyl-tRNA synthetase (LRS) activities (11). The LRS enzymes produced by strains harboring either the *lysS1* or *lysS2* alleles differ from each other with respect to their molecular weights, the capacity to carry out the amino acid-dependent adenosine triphosphate-pyrophosphate (ATP-PP_i) exchange reaction, and the ATP-dependent attachment of amino acid to tRNA (11).

To be able to make meaningful comparisons between the spore and vegetative enzymes and also to provide a functional basis for the isolation of additional LRS mutants, it was necessary to know the genetic sites of the defects and whether the two mutant loci were located in the same structural gene. Since both mutations in the LRS produce a thermosensitive phenotype, we used this property to map the two mutation sites. (This work was part of a thesis submitted by F.M.R. in partial fulfillment of the requirements for a Ph. D. at the University of Virginia, Charlottesville, Va.)

MATERIALS AND METHODS

Strains. The strains used in this study are listed in Table 1. All strains are derivatives of *B. subtilis* 168M. The isolation of the *lysS* mutants has been described (11).

Media. Strains were maintained on AK-2 agar slants (BBL, Baltimore, Md.), supplemented with thymine (10 μg/ml). Difco antibiotic medium #3 (PAB) was used to grow recipients for transduction. For transformation experiments, the minimal glucose (MG) medium of Spizizen (13) was employed. In preparation for transformation experiments, recipient strains were grown on Difco tryptose blood agar base at one-half the recommended strength, with 1.5% agar. Solid media employed were MG medium with 1.7% agar, or NAT medium which contained (per liter) 8 g of Difco nutrient broth, 1 g of glucose, 10 mg of thymine, and 15 g of agar.

PBS-1 phage transduction and transformation. Procedures for PBS-1 phage transduction and for transformation have been described (16). The first-stage transformation medium was supplemented with 0.01% Difco yeast extract (J. Hoch, personal communication). All steps in transformation and transduction were carried out at 30 C.

Selection of recombinants. The techniques used to select recombinants from transduction and transformation experiments were the same. In selection for thermoresistance, appropriately diluted samples of the transduced or transformed cells were spread on NAT medium and incubated at 43 C overnight. For prototrophic recombinants, MG agar medium containing the appropriate nutritional requirements was used. Sulfanilamide-resistant recombinants were selected on MG medium containing 500 μg of sulfanilamide per ml. All recombinants were cloned once under the same conditions used in the initial selection. They were then tested for secondary markers by replica plating (9).

Materials. L-Amino acids (A grade) were obtained from Calbiochem (Los Angeles, Calif.). Sulfanilamide (*p*-aminobenzene sulfonamide) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Pancreatic deoxyribonuclease I (92,400 U/mg) was purchased from Worthington Biochemicals (Freehold, N.J.).

RESULTS

Localization of lysS1 and lysS2 mutant sites by PBS-1 transduction. PBS-1 phage lysates were prepared from the *lysS* mutants,

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and these were used to transduce a series of strains with auxotrophic mutations distributed over the *B. subtilis* chromosome. Prototrophic recombinants were selected and tested for thermosensitivity. The results (Table 2) showed that the *lysS* locus was linked to *purA16* and *cysA14* (Fig. 1). There was 19% contransfer of *purA16* with *lysS1* and 21% with *lysS2*. The linkage to *cysA14* was 83% cotransfer with *lysS1* and 76% with *lysS2* (Table 2). These results suggested two possible orders for the three markers, either *purA-lysS-cysA* or *purA-cysA-lysS*. The order *lysS-purA-cysA* was eliminated because *lysS* was closer to *cysA14* than to *purA16*. To distinguish between the two configurations, three-point transduction crosses were carried out (Table 3). If one assumes that the class of recombinants that occurs with the least frequency is the one that results from a double crossover, then the class *pur*$^+$, *cysA*$^+$, *lysS*$^+$ must be the result of such a crossover. From this it was deduced that the correct order of these markers was *purA16-lysS-cysA14*. A recombination map constructed from the data of Table 3 is shown in Fig. 1. All distances are expressed as percent recombination and were calculated after normalizing the number of transductants in each primary selection class so that the

TABLE 1. *Bacterial strains used*[a]

Strain	Genotype	Origin or Reference
TD235	*lysS2, thy, trpC2*	(11)
TD246	*lysS1, thy, trpC2*	(11)
VB101	*trp C2, lys-1*	GSY225 by transformation with GSY251 as donor
VB103	*lys-1, metB3*	VB101 by transformation with GSY266 as donor
VB104	*lysS2, lys-1*	VB103 by transformation with TD235 as donor
VB106	*lysS2, metB3*	VB103 by transformation with TD235 as donor
VB133	*cysA14, ery-1*	Dubnau
VB134	*cysA14*	Takahashi
VB138	*purA16, cysA14, metB5*	Mu8u5u16 by transformation with VB133 as donor
VB151	*lysS1, metB5, leuA8*	Mu8u5u16 by transformation with VB122 as donor
VB157	*sul, trpC2*	Goldthwaite
VB161	*lysS2, sul, metB3*	VB106 by transformation with VB157 as donor
VB162	*lysS1, sul, metB3, leuA8*	VB151 by transformation with VB157 as donor
VB176	*trpC2, lys-1*, PR13	Spontaneous partial revertant of VB101[b]
VB122	*lysS1, lys-1*, PR13	VB167 by transformation with TD246 + GSY266 as donors[c]
VB124	*lysS2, lys-1*, PR13	VB167 by transformation with TD235 + GSY266 as donors[c]
GSY225	*pheA1, trpC2*	Anagnostopoulos
GSY251	*lys-1*	Anagnostopoulos
GYS266	*metB3*	Anagnostopoulos
168M	*trpC2*	Anagnostopoulos
Mu8u5u16	*purA16, leuA8, metB5*	Sueoka

[a] Nomenclature in accordance with Demerec et al. (5). Abbreviations: *cys*, cysteine; *ery*, erythromycin; *lys*, lysine; *leu*, leucine; *met*, methionine; *phe*, phenylalanine; *pur*, purine (*purA* denotes a strict adenine requirement); *sul*, sulfanilamide; *thy*, thymine; *trp*, trytophan.

[b] The designation PR13 denotes a partial revertant of *lys-1*, selected for the ability to form small colonies on MG medium without lysine.

[c] Prepared by congression. High deoxyribonucleic acid concentrations (2 μg/ml from strain TD246 and 1 μg/ml from strain GSY266) were used to obtain double transformants. Primary selection was for *trp*$^+$.

TABLE 2. *Linkage of lysS1 and lysS2 to purA and cysA by PBS-1 transduction*

Expt[a]	Donor genotype	Recipient genotype	Thermosensitive recombinants among prototrophic transductants	
			purA$^+$	*cysA*$^+$
1	*lysS1, lys-1PR13*	*purA16, leuA8, metB5*	76/411 (19)[b]	
2	*lysS2, lys-1PR13*	*purA16, leuA8, metB5*	43/203 (21)	
3	*lysS1, lys-1PR13*	*cysA14*		200/242 (83)
4	*lysS2, lys-1PR13*	*cysA14*		286/376 (76)

[a] Expt 1: donor, VB122; recipient, Mu8u5u16. Expt 2: donor, VB124; recipient, Mu8u5u16. Expt 3: donor, VB122; recipient, VB134. Expt 4: donor, VB124; recipient, VB134.

[b] Numbers in parenthesis are percent thermosensitive recombinants among prototrophic transductants.

contribution from each class would be equivalent.

Transformation linkage of the lysS mutation. Table 4 shows the linkage of the *lysS1* and *lysS2* mutations to *sul* and *cysA14* obtained by transformation. The *lysS* mutations are closely linked to *sul* (70 to 74% cotransformation). These results suggested two possible configurations, either *lysS-sul-cysA* or *sul-lysS-cysA*. The order *sul-cysA14-lysS* was eliminated since it was shown by transduction (Table 3, Fig. 1) that the *lysS* mutations were between *purA* and *cysA14*.

Table 5 shows the results of reciprocal three-point transformation crosses to distinguish between the two configurations. Only with the configuration *lysS-sul-cysA* does the least frequent class correspond to a double crossover. After normalizing the data, a map was constructed (Fig. 2).

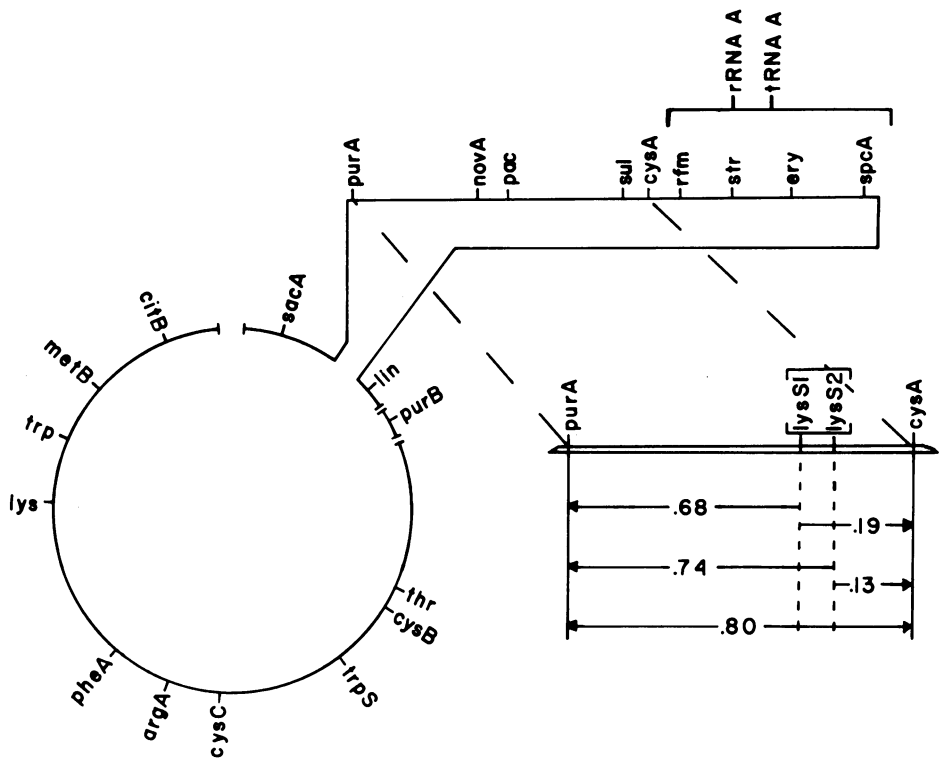

FIG. 1. *A genetic map of the B. subtilis chromosome. The positions of the lysS1 and lysS2 markers with respect to the purA16 and cysA14 loci (as determined by PBS-1 transduction) are shown. The values are percent recombination obtained after normalizing the data of Table 3. The head of the arrow indicates the recipient. The order of lysS1 relative to lysS2 is indefinite since both markers were not present simultaneously in the crosses. This map is derived from Young and Wilson (19).*

TABLE 3. *Analysis of three-factor PBS-1 transduction crosses involving purA16, lysS, and cysA14*

Expt[a]	Primary selection	*purA*$^+$ *cysA*$^+$, *lysS*$^+$	*purA*$^+$ *cysA*$^+$, *lysS*	*purA*$^+$ *cysA*, *lysS*$^+$	*purA*$^+$ *cysA*, *lysS*	*purA* *cysA*$^+$, *lysS*$^+$	*purA* *cysA*$^+$, *lysS*
1	*purA*$^+$	15/674[b]	64/674	505/674	90/674		
	cysA$^+$	9/469	92/469			38/469	330/469
2	*purA*$^+$	16/410	87/410	269/410	38/410		
	cysA$^+$	27/490	75/490			90/490	298/490

[a] Expt 1: donor, VB122 (*lysS1, lys-1PR13*); recipient, VB138 (*purA16, cysA14, metB5*). Expt 2: donor, VB124 (*lysS2, lys-1PR13*); recipient, VB138 (*purA16, cysA14, metB5*).

[b] Ratios represent the number of transductants of the indicated phenotype (numerator) and the total number of transductants (denominator).

TABLE 4. *Transformation linkage of lysS1 and lysS2 to cysA14 and sul*

Expt[a]	Donor genotype	Recipient genotype	*cysA* or *sul* recombinants among thermoresistant transformants	
			cysA	*sul*
1	*purA16, cysA14, metB5*	*lysS1, metB, leuA8*	30/459 (6.5)[b]	
2	*purA16, cysA14, metB5*	*lysS2, metB3*	4/410 (1)	
3	*sul, trpC2*	*lysS1, metB5, leuA8*		200/269 (74)
4	*sul, trpC2*	*lysS2, metB3*		166/236 (70)

[a] Expt 1: donor, VB138; recipient, VB151. Expt 2: donor, VB138; recipient, VB106. Expt 3: donor, VB157; recipient, VB151. Expt 4: donor, VB157; recipient, VB106.
[b] Numbers in parenthesis are percent *cysA* or *sul* recombinants among thermoresistant transformants.

TABLE 5. *Analysis of three-factor transformation crosses involving lysS, cysA14, and sul*

Expt[a]	Primary selection	*lysS*$^+$, *sul*, *cysA*$^+$	*lysS*$^+$, *sul*, *cysA*	*lysS*$^+$, *sul*$^+$, *cysA*$^+$	*lysS*$^+$, *sul*$^+$, *cysA*	Total
1	*lysS*$^+$	116	0	300	14	430
2	*lysS*$^+$	131	3	274	13	421
Expt[a]	Primary selection	*cysA*$^+$ *lysS*, *sul*	*cysA*$^+$ *lysS*, *sul*$^+$	*cysA*$^+$ *lysS*$^+$, *sul*	*cysA*$^+$ *lysS*$^+$, *sul*$^+$	Total
3	*cysA*$^+$	25	4	85	427	541
4	*cysA*$^+$	15	4	122	408	549

[a] Expt 1: donor, VB134 (*cysA14*); recipient, VB162 (lysS1, sul, metB3, leuA8). Expt 2: donor, VB134 (*cysA14*); recipient, VB161 (*lysS2, sul, metB3*). Expt 3: donor, VB162 (*lysS1, sul, metB3, leuA8*); recipient, VB134 (*cysA14*). Expt 4: donor, VB161 (*lysS2, sul, metB3*); recipient, VB134 (*cysA14*).

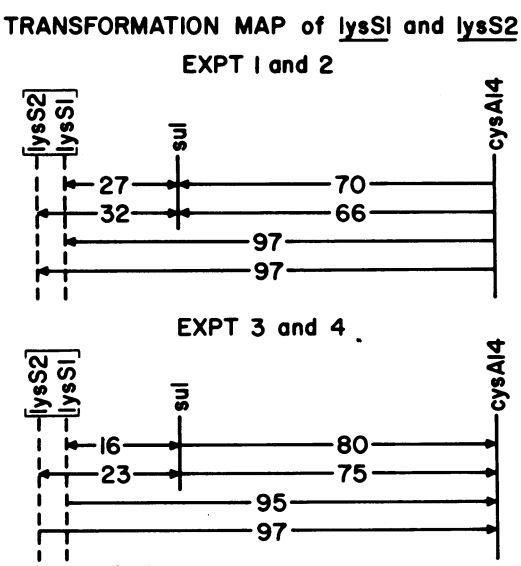

FIG. 2. *A transformation map of lysS1 and lysS2. The values are percent recombination, obtained after normalizing the data of Table 5. The head of the arrow indicates the recipient. The order of lysS1 relative to lysS2 is indefinite since both markers were not present simultaneously in the crosses.*

Order of lysS1 and lysS2 relative to each other. From the data presented thus far it was not possible to determine an order for *lysS1* relative to *lysS2* since the two markers were not present simultaneously in any of the previous crosses. Figure 3 shows the two possible configurations for *lysS1* and *lysS2* relative to *sul*. The four experiments shown in Table 6 were carried out to distinguish between these two configurations. Experiments 1 and 2 are reciprocal transformation crosses in which sulfanilamide resistance was selected. Experiments 3 and 4 are the same two crosses, with selection for temperature resistance.

In experiment 1, if configuration I were correct, the proportion of thermoresistant recombinants (*lysS*$^+$) among sulfanilamide-resistant recombinants (*sul*) would be high in relation to the proportion expected if order II were correct, since with configuration II thermoresistant, sulfanilamide-resistant recombinants must be the result of a double crossover (Fig. 3). In the reciprocal experiment (experiment 2), the opposite would be true. In this case, if configuration I were correct, the *lysS*$^+$ *sul* recombinants would be present at a low frequency, the result of a double crossover. The data in Table 6 support configuration I.

The same type of rationale applied to experiments 3 and 4 also supports configuration I, *purA16-lysS1-lysS2-sul*.

DISCUSSION

The *lysS1* and *lysS2* strains each possess a thermosensitive LRS (11). As judged by the relatively high reversion frequency ($\sim 10^{-7}$), and the fact that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) is not known to produce deletions or frameshifts, it would appear that the defects in the LRS activities are most

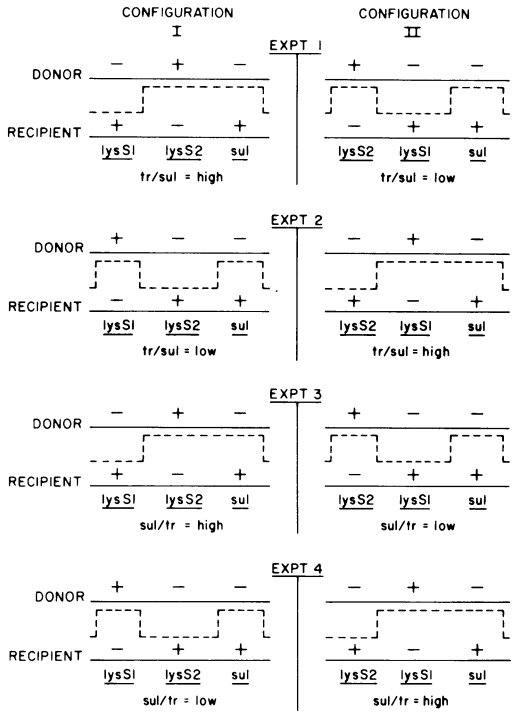

FIG. 3. *Possible configurations of the lysS1, lysS2, and sul loci. Dashed lines represent recombination events yielding thermoresistant, sulfanilamide-resistant clones. Experiments 1 through 4 refer to experiments in Table 6.*

TABLE 6. *Test for order of lysS1 and lysS2 with respect to sul by three-factor transformation crosses*

Expt[a]	Primary selection	*lysS*$^+$/*sul*[b]	*sul*/*lysS*$^+$ [c]
1	*sul*	32/296	
2	*sul*	1/288	
3	*lysS*$^+$		139/188
4	*lysS*$^+$		43/184

[a] Expt 1 and 3: donor, VB162 (*lysS1, sul, metB3, leuA8*); recipient, VB106 (*lysS2, metB3*). Expt 2 and 4; donor, VB161 (*lysS2, sul, metB3*); recipient, VB151 (*lysS1, metB5, leuA8*).

[b] Number of temperature-resistant recombinants (numerator) among sulfanilamide-resistant recombinants (denominator).

[c] Number of sulfanilamide-resistant recombinants (numerator) among temperature-resistant recombinants (denominator).

likely the result of single point mutations. Although NTG mutagenesis can produce closely linked multiple mutations (6), the additivity of the recombination frequencies in the crosses reported here would argue against multiple site mutations in the region examined.

The mutations are almost certainly in structural and not regulatory genes (11). It has not been possible to determine whether the *lysS1* and *lysS2* mutations are located in the same cistron. The small differences in recombination frequencies between *lysS1* and *lysS2* suggest that they are very close. Based on recombination values obtained for mutations in the tryptophan gene cluster of *B. subtilis* (1, 2), it is not unreasonable to suggest that the *lysS1*, *lysS2* mutational sites are in the same or adjacent cistrons. However, despite this proximity of the two sites, the phenotypes of *lysS1* and *lysS2* strains are dramatically different. The *lysS1* mutation results in an LRS which is approximately one-half the molecular weight of the wild-type enzyme and completely defective in the attachment of lysine to tRNA (11). It is suggested that the *lysS1* mutation is not a nonsense mutation since it is doubtful that a severely truncated enzyme could function at all in vivo. In contrast, the LRS of *lysS2* strains is approximately the same size as the wild-type enzyme. It can carry out both the ATP-PP_i exchange and the attachment reactions, but at reduced levels. Although both mutations result in a failure to grow at 43 C (11), there is a different response to the restrictive temperature when cells are incubating in a rich medium. Cells bearing the *lysS2* marker remain viable at 43 C whereas cells of the *lysS1* phenotype undergo irreversible thermal injury (W. Steinberg, unpublished data).

The lack of any attachment activity in *lysS1* strains suggests that either there is only one gene for LRS in *B. subtilis*, or that the *lysS1* mutation affects a component common to more than one LRS species. The latter alternative could be supported by the evidence indicating that this enzyme is composed of subunits (11). Although only single-enzyme species have been observed for most of the aminoacyl-tRNA synthetases of prokaryotic organisms, there is evidence for the existence of two active forms of the lysyl- and arginyl-tRNA synthetases of *Escherichia coli* (8, 18). Heterogeneity has been found in preparations of LRS from *E. coli* (8) and *Saccharomyces cerevisiae* (3). However, it has not been demonstrated that any of these multiple LRS fractions are actually due to more than one unique enzyme. Heterogeneity could also be inferred from the biphasic kinetics of *E. coli* LRS (7, 10). Using the techniques of phosphocellulose and hydroxylapatite chromatography, and gel filtration, Steinberg (15) found only a single LRS component in *B. subtilis*. Furthermore, Racine and Steinberg (11), study-

ing the kinetic parameters of the *B. subtilis* enzyme, found no indication of biphasic kinetics.

The results presented here indicate that the *lysS* gene is not located near the only known region coding for a lysine biosynthetic enzyme (*lys-1*). This is not surprising since, with the exception of the seryl-tRNA synthetase of *E. coli* (4), the genes coding for aminoacyl-tRNA systhetases are not associated with the genes for their corresponding biosynthetic enzymes (13, 17). The *lysS* markers are not linked to the *B. subtilis* tryptophanyl-tRNA synthetase structural gene (16); however, they are located on a PBS-1 phage transducing fragment which contains several tRNA cistrons (19). Unlike the *trpS1* mutation which results in a defective regulatory system for enzymes of the tryptophan pathway (14), both *lysS1* and *lysS2* strains produce normal amounts of aspartokinase, the first enzyme of the lysine pathway (W. Steinberg, unpublished data).

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