Identification of *aecA* mutations in *Bacillus subtilis* as nucleotide substitutions in the untranslated leader region of the aspartokinase II operon

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Recent genetic mapping of the aspartokinase II (*lysC*) operon of *Bacillus subtilis* [M. Petricek, L. Rutberg & L. Hederstedt (1989) *FEMS Microbiology Letters* 61, 85–88; N. Y. Chen, J. J. Zhang & H. Paulus (1989) *Journal of General Microbiology* 135, 2931–2940] has shown its chromosomal location to be close to the *aecA* locus, the mutation of which leads to highly increased levels of aspartokinase II. In order to examine the relationship between *lysC* and *aecA*, we have cloned the control regions of the *lysC* operon from several independent *aecA* mutants and determined their nucleotide sequences. The nucleotide sequences of the *aecA* mutants differed from the wild-type sequence by the substitution of one or two nucleotides at two widely separated sites in the transcribed leader region of the *lysC* operon. To confirm that the observed nucleotide changes are indeed responsible for the AecA phenotype and not simply the reflection of sequence polymorphisms in different *B. subtilis* strains, we introduced the same nucleotide substitutions as those observed in the *aecA* strains into the leader region of the wild-type *lysC* operon by oligonucleotide-directed mutagenesis. The expression of the mutagenized genes was analysed after transcriptional or translational fusion to *lacZ* in a single-copy integration vector. The levels of β -galactosidase were greatly elevated by the nucleotide substitutions, with similar increases observed in transcriptional and translational fusions. The high level of expression of β -galactosidase in the *lysC*–*lacZ* strains with nucleotide substitutions corresponding to the *aecA* mutations was resistant to repression by L-lysine but was completely abolished by the inactivation of the *lysC* promoter. These results suggest that the sites identified by nucleotide sequencing of the *aecA* mutants are important regulatory sites in the *lysC* operon, which exert their effect at the level of transcription involving the normal *lysC* promoter.

Introduction

The enzyme aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) catalyses the first step in the aspartate pathway, a complex biosynthetic pathway that leads to the biosynthesis of lysine, methionine and

threonine. *Bacillus subtilis* has three aspartokinase isoenzymes, one of which, aspartokinase II, is controlled by lysine through repression and feedback inhibition (Moir & Paulus, 1977; Zhang *et al.*, 1990). The growth of *B. subtilis* is inhibited by the lysine analogue S-(2-aminoethyl)cysteine (AEC), and mutants resistant to AEC can readily be isolated (Vold *et al.*, 1975). One class of AEC-resistant mutants, designated *aecA*, has about 50-fold elevated levels of aspartokinase II (Vold *et al.*, 1975; Yeh & Steinberg, 1978). Other *aecA* mutants have been isolated and classified by genetic mapping, but their aspartokinase II levels have not been examined (Mattioli *et al.*, 1979; Magnusson *et al.*, 1983). Originally, *aecA* was thought to be a *trans*-acting regulatory gene (e.g. Piggot & Hoch, 1985), but genetic mapping of the aspartokinase II operon (*lysC*, formerly *ask*) suggests

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Abbreviation: AEC, S-(2-aminoethyl)cysteine.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number J03294.

Table 1. *B. subtilis* strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
GSY225	<i>pheA1 trpC2</i>	Yeh & Steinberg (1978)
GSY225-40	<i>pheA1 trpC2 amyE::lysC'-lac'Z</i> (translational fusion)	This study
GSY225-41	Same as GSY225-40, except G to A change at 320* of <i>lysC</i> †	This study
GSY225-42	Same as GSY225-40, except C to T change at 476* of <i>lysC</i> †	This study
GSY225-47	Same as GSY225-41, except ΔA270* in <i>lysC</i> †	This study
GSY225-60	<i>pheA1 trpC2 amyE::lysC'-lac'Z</i> (transcriptional fusion)	This study
GSY225-61	Same as GSY225-60, except G to A change at 320* of <i>lysC</i> †	This study
GSY225-62	Same as GSY225-60, except C to T change at 476* of <i>lysC</i> †	This study
BGSC 1A8	<i>aroD120 lys-1 trpC2</i>	Dedonder <i>et al.</i> (1977)
1A8-40	<i>aroD120 lys-1 trpC2 amy::lysC'-lac'Z</i> (translational fusion)	This study
1A8-41	Same as 1A8-40, except G to A change at 320* of <i>lysC</i> †	This study
1A8-60	<i>aroD120 lys-1 trpC2 amy::lysC'-lac'Z</i> (transcriptional fusion)	This study
1A8-61	Same as 1A8-60, except G to A change at 320* of <i>lysC</i> †	This study
VB217	<i>pheA1 trpC2 sul^R aecA3</i>	Yeh & Steinberg (1978)
FB59 (BGSC 1A432)	<i>aecA4</i>	Mattioli <i>et al.</i> (1979)
KA120	<i>pheA1 trpC2 aecA5</i>	Magnusson <i>et al.</i> (1983)
pJH101-AskNB	pJH101 with <i>lysC</i> † (1350-1645)* insert	Zhang <i>et al.</i> (1990)
M13-40	M13mp19 carrying <i>lysC</i> † (212-654)* in <i>Sma</i> I site	Chen & Paulus (1988)
M13-41	Same as M13-40, except G to A change at 320*	This study
M13-42	Same as M13-40, except C to T change at 476*	This study
M13-47	Same as M13-41, except ΔA270*	This study
pIS112	<i>lacZ amp cat</i>	Lewandoski & Smith (1988)
pIS112-40	pIS112 with <i>lysC</i> † (212-654)* from M13-40 inserted between the <i>Eco</i> RI and <i>Bam</i> HI sites	This study
pIS112-41	Same as pIS112-40, except G to A change at 320*	This study
pIS112-42	Same as pIS112-40, except C to T change at 476*	This study
pIS112-47	Same as pIS112-41, except ΔA270*	This study
pDH32	<i>spoVG-lacZ amp cat amyE(front) amyE(back)</i>	D. J. Henner
pDH32-40	pDH32 with a <i>Eco</i> RI- <i>Cla</i> I fragment containing the translational <i>lysC'-lac'Z</i> fusion from pIS112-40 in place of the original <i>Eco</i> RI- <i>Cla</i> I segment	This study
pDH32-41	Same as pDH32-40, except G to A change at 320*	This study
pDH32-42	Same as pDH32-40, except C to T change at 476*	This study
pDH32-47	Same as pDH32-41, except ΔA270*	This study
pDH32-60	pDH32 with <i>lysC</i> † (212-654)* from M13-40 inserted between the <i>Eco</i> RI and <i>Bam</i> HI sites	This study
pDH32-61	Same as pDH32-60, except G to A change at 320*	This study
pDH32-62	Same as pDH32-60, except C to T change at 476*	This study

* Nucleotide residues are numbered according to the system of Chen *et al.* (1987) (see Fig. 3).

† Formerly referred to as the *ask* gene (Zhang *et al.*, 1989) but now renamed *lysC* to conform with the designation of the gene for the lysine-sensitive aspartokinase of *E. coli*.

that *aecA* is located in the 5'-untranslated region of the aspartokinase II transcription unit (Petricek *et al.*, 1989; Chen *et al.*, 1989).

The recent cloning and sequencing of the entire aspartokinase II operon and adjacent regions of the *B. subtilis* chromosome (Chen *et al.*, 1987, 1989) allowed us to address the question whether *aecA* mutations are due to changes in the nucleotide sequence of *lysC* or its control region. In this paper, we describe the cloning and nucleotide sequence analysis of the upstream portion of the *lysC* operon from three independent *aecA* mutants, originally isolated by Yeh & Steinberg (1978), Mattioli *et al.* (1979) and Magnusson *et al.* (1983) respectively. The nucleotide sequence of the transcribed *lysC* leader region

of the *aecA* mutants was found to differ from that of the *aecA*⁺ parent strain by the replacement of one or two nucleotides at two widely separated sites. The same nucleotide substitutions were then introduced into the leader region of the wild-type *lysC* operon by oligo-nucleotide-directed mutagenesis to determine whether these nucleotide changes are indeed responsible for the AecA phenotype and define potential *cis*-acting regulatory sites in the *lysC* operon.

Methods

Bacterial strains and growth conditions. The strains of *Bacillus subtilis* and the plasmids used in this work are listed in Table 1. LB was used as

a rich medium; the minimal medium was that of Anagnostopoulos & Spizizen (1961), supplemented with FeSO_4 (10 mg l^{-1}) CaCl_2 (20 mg l^{-1}), and glucose (5 g l^{-1}), and with amino acids and antibiotics when appropriate. The effect of lysine limitation or lysine excess was studied using strains derived from the lysine auxotroph *B. subtilis* 1A8 in minimal medium supplemented with 0.016 mM - or 5 mM -L-lysine, respectively; other supplements were 0.3 mM each of L-phenylalanine, L-tyrosine and L-tryptophan, and $5 \mu\text{g}$ chloramphenicol ml^{-1} . The minimal medium was inoculated with 0.025 vol. of starter culture in the rich medium of Stansly *et al.* (1947) and the cells were harvested 1 h after growth had stopped due to lysine limitation in the lysine-deficient medium or at the corresponding density ($70\text{--}80$ Klett units with filter no. 54) in the lysine-rich medium. Plasmids were propagated and maintained in *Escherichia coli* DH5 [*deoR recA1 endA1 hsdR17(r_m⁺) supE44 λ^- gyrA96*] or *E. coli* HB101 [*Δ(gpt-proA)62 leuB6 thi-1 lacY1 hsdS₁₈20 recA rpsL20(Str^r) ara-14 galK2 xyl-5 mtl-1 mcrB_B supE44*].

Isolation and sequence analysis of the *lysC* operon from *aecA* strains. The upstream portions of the *lysC* operon of the *aecA* strains VB217, FB59 and KA120 were isolated from the *lysC::cat* derivatives of these strains, obtained by the method of Ferrari *et al.* (1983) through integration of plasmid pJH101-AskNB as described by Zhang *et al.* (1990). Briefly, DNA was isolated from the *lysC::cat* strains, digested with either *Pst*I or *Eco*RI and self-ligated at low DNA concentrations. The ligation mixtures were used to transform *Escherichia coli* HB101 or DH5, and chloramphenicol-resistant colonies were selected for plasmid isolation. The plasmids thus obtained were subjected to restriction analysis to ascertain the presence of DNA surrounding the *lysC* locus. A positive plasmid isolate derived from each of the *aecA* strains was selected for nucleotide sequence determination by the dideoxynucleotide method (Sanger *et al.*, 1977) using modified T7 DNA polymerase (Tabor & Richardson, 1987). DNA sequencing was done either after transfer of a 1.6 kb *Pst*I–*Bam*HI fragment of the cloned genes to M13mp18 or M13mp19 (for *B. subtilis* VB217), or directly on the double-stranded plasmids (for *B. subtilis* FB59 and KA120), using appropriate synthetic sequencing primers. The nucleotide sequence was determined for both strands of the *lysC* promoter and leader regions. Additional sequences, which ranged from residues 1 to 1360 for strain VB217, and from residues 235 to 714 for strains FB59 and KA120, were obtained by the analysis of only one strand.

Nucleotide residues in *lysC* and adjacent regions are referred to by the numbering system of Chen *et al.* (1987).

Oligonucleotide-directed mutagenesis. Site-directed mutagenesis was carried out by the phosphorothioate method of Nakamaye & Eckstein (1986), employing a commercial reagent kit (Amersham), or by the method of Kunkel (1985). Mutagenesis employed the following deoxyribo-oligonucleotides, which were synthesized by the phosphoramidite method using the BioSearch Cyclone DNA synthesizer (the residues that differ from the wild-type sequence are underlined): oligonucleotide 1, TATGGTGAAGATAGAGATGC (residues 304–323); oligonucleotide 2, GAGGGITATCTCGTTGTTCA (residues 471–490); oligonucleotide 3, GATAGAGATGCGAACTTC (residues 313–330). Mutagenesis was carried out on a derivative of bacteriophage M13mp19 (phage M13-40), constructed, as described by Chen & Paulus (1988), by ligating a blunt-ended *Dde*I fragment, corresponding to residues 212–654, into the *Sma*I site of the M13mp19 polylinker (Fig. 1). The nucleotide sequence of the mutagenized M13mp19 derivatives was confirmed by direct sequence determination as described by Zhang *et al.* (1990). The following mutant bacteriophages were obtained, with the mutagenic oligonucleotide used and the sequence differences from the parental phage M13-40 indicated in parentheses: M13-41 (oligonucleotide 1; $\text{G}^{320} \rightarrow \text{A}^{320}$), M13-42 (oligonucleotide 2; $\text{C}^{476} \rightarrow \text{T}^{476}$), and M13-47 (oligonucleotide 3; $\text{G}^{320} \rightarrow \text{A}^{320}$ and ΔA^{270}).

The double mutant M13-47 was obtained serendipitously, perhaps on account of partial complementarity of oligonucleotide 3 with residues 266–280.

Construction of single-copy *lacZ* fusion vectors. The *lysC* leader region from M13-40 or from its mutagenized derivatives was excised from the polycloning sites by cleavage with *Eco*RI and *Bam*HI and used in the construction of either transcriptional or translational *lacZ* fusions (Fig. 1). For transcriptional fusions, the *Eco*RI–*Bam*HI fragment carrying the *lysC* leader together with the first 15 codons of aspartokinase II was ligated to the corresponding sites of the single-copy integration vector pDH32, which is similar to ptpBGI (Shimotsu & Henner, 1986) except that it carries a *spoVG-lacZ* fusion instead of *trpE-lacZ* (D. Henner, personal communication). The nucleotide sequence at the fusion junction is shown in Fig. 2. Plasmid pDH32 and its derivatives can replicate in *E. coli* but not in *B. subtilis*, where it integrates into the chromosomal *amyE* locus through the 3' and 5' segments of *amyE* which flank the *lacZ* fusion protein and the *cat* gene of pDH32 (Shimotsu & Henner, 1986). Accordingly, the desired *lysC-lacZ* transcriptional fusion plasmid pDH32-60 and its mutagenized derivatives were isolated by transforming *E. coli* DH5, selecting for chloramphenicol-resistant transformants, and screening of the plasmids obtained from selected transformants by restriction analysis to confirm that they were the desired recombinants. After linearization by cleavage at the unique *Pst*I site, pDH32-60 and its derivatives were used to transform *B. subtilis* GSY225 or 1A8. *amyE::lysC-lacZ* derivatives of *B. subtilis* GSY225 or 1A8 were isolated as chloramphenicol-resistant colonies and were screened for the absence of amylase activity to ascertain that integration had indeed occurred at the amylase locus.

Translational *lysC-lacZ* fusion plasmids were constructed along similar lines, except that the *Eco*RI–*Bam*HI fragment from M13-40 or its derivatives was first inserted into the corresponding sites of pSI112 (Lewandoski & Smith, 1988) in order to achieve the in-phase fusion of codon 15 of aspartokinase II with the codons for the amino-terminal sequence of β -galactosidase (Fig. 1). The sequence of the fusion junction is shown below, with the residues derived from *lysC* in italics:

<i>lysC</i>		<i>lacZ</i>
Gly Ser Gly Asp Pro Val Leu		
GGC TCA GGG GAT CCC GTC GTT TTA		

The resulting *lysC-lacZ* fusion plasmid (pSI112-40 and derivatives) was used to transform *E. coli* DH5, with selection for ampicillin resistance. The DNA segment encoding the *lysC* leader region and the amino-terminal portion of the aspartokinase II– β -galactosidase fusion protein was excised from pSI112-40 with *Eco*RI and *Cla*I and used to replace the *Eco*RI–*Cla*I segment of the single-copy integration vector pDH32. The desired translational *lysC-lacZ* fusion plasmid pDH32-40 and its mutagenized derivatives were isolated and integrated into the *amyE* locus of *B. subtilis* GSY225 or 1A8 to yield *amyE::lysC-lacZ* derivatives in a manner similar to that described above for the corresponding transcriptional fusions.

Enzyme assays. For measurements of aspartokinase, cells were disrupted by passage through a French pressure cell. The lysate was centrifuged at $100\,000 g$ for 1 h and the supernatant solution was assayed for aspartokinase II by measuring the lysine-sensitive and aspartate-dependent formation of acyl phosphate from (γ - ^{32}P)ATP as described previously (Paulus & Gray, 1967). For the measurement of β -galactosidase, cultures of *B. subtilis* were harvested in mid-exponential phase (70 Klett units with no. 54 filter), washed and suspended in 0.1 M -potassium phosphate, pH 7.0, and permeabilized by treatment with 1% (v/v) toluene at 25°C for 10 min. β -Galactosidase was then assayed essentially as described by Miller (1972), except that incubations were carried out at 37°C . Protein concentration was determined by the method of Bradford (1976).

Fig. 1. Construction of single-copy integration vectors for *lysC'*-*lac'*Z fusions. See text for explanation. RBS, ribosome-binding site.

lysC

Gly Ser Gly Asp Pro Gln Leu Glu Leu Ile His .
 GGC TCA GGG GAT CCC CAG CTT GAA TTG ATA CAC TAA

spoVG RBS

.....

TGCTTTTATATAGGGAAAAGGTGGTGAAGTACT

spoVG'-lac'Z

Fmet Glu Val Thr Asp Val Arg Leu Arg Val Asp Arg Glu
 GTG GAA GTT ACT GAC GTA AGA TTA CGG GTC GAC CGG GAA

Fig. 2. Nucleotide sequence of the fusion junction of the *spoVG'-lac'Z* fusion. The residues derived from *lysC* are shown in italics. RBS, ribosome-binding site.

Results and Discussion

Effect of aecA mutations on aspartokinase levels

Two classes of mutants of *B. subtilis* resistant to the lysine analogue AEC have been characterized, which differ in the levels of aspartokinase II (Vold *et al.*, 1975) and in the overproduction of lysine (Mattioli *et al.*, 1979). Mutants of the *aecB* class have normal levels of aspartokinase II, do not overproduce lysine, and map near the threonine operon at 280°. In contrast, the *aecA* mutants, which are the subject of this paper, have elevated levels of aspartokinase II, overproduce lysine, and map near the aspartokinase II operon (*lysC*) at 250°. However, of three *aecA* mutants that have been described in the literature, only one has actually been shown to produce derepressed levels of aspartokinase II (Yeh & Steinberg, 1978), whereas the others (Mattioli *et al.*, 1979; Magnusson *et al.*, 1983) were classified as *aecA* primarily on the basis of genetic mapping. It was therefore of interest to compare the aspartokinase II levels of these *aecA* strains to determine whether they indeed represent a biochemically homogeneous group.

The levels of aspartokinase II in extracts of three independently isolated *aecA* mutants, *B. subtilis* strains VB217 (Yeh & Steinberg, 1978), FB59 (Mattioli *et al.*, 1979) and KA120 (Magnusson *et al.*, 1983), were examined and found to be between 33 and 66 times higher than in extracts of the wild-type (*aecA*⁺) strain during exponential growth in rich medium, where aspartokinase II is ordinarily repressed (Table 2). On the other hand, the sensitivity of aspartokinase II to feedback inhibition by L-lysine is not significantly altered in *aecA* strains, the values reported in Table 2 being lysine-sensitive aspartokinase activities. Indeed, the derepressed *aecA* strain VB217 served as the original source for the purification of lysine-sensitive aspartokinase II (Moir & Paulus, 1977). It is of interest that, in spite

Table 2. Aspartokinase II activity in *aecA* mutants of *B. subtilis*

Strain	<i>aecA</i> allele	Aspartokinase II activity* (nmol min ⁻¹ mg ⁻¹)
GSY225	<i>aecA</i> ⁺	<9
VB217	<i>aecA3</i>	630
FB59	<i>aecA4</i>	290
KA120	<i>aecA5</i>	470

* Aspartokinase II activity was measured as lysine-sensitive aspartokinase activity in extracts of cells harvested in the mid-exponential phase of growth in LB medium as described in Methods.

of the diverse origins of the three *aecA* strains, their aspartokinase II levels differ not more than twofold. We can therefore conclude that derepressed levels of aspartokinase II are a common feature of *aecA* mutants and that the extent of derepression in the mutants studied varies over a relatively narrow range.

Nucleotide sequence of the lysC leader region of aecA mutants

The promoter-proximal portion of the *lysC* operon of the three *aecA* strains was cloned by the method of Ferrari *et al.* (1983) after inactivation of the aspartokinase II gene by recombination with a pJH101 derivative carrying a segment of the aspartokinase II coding region. The upstream portion of the *lysC* gene and adjoining regions was then recovered from chromosomal *EcoRI* or *PstI* libraries of the aspartokinase II-negative strains (*lysC::cat*) after self-ligation and screening for plasmids that conferred chloramphenicol-resistance upon transformation of *E. coli*. The same procedure had been applied earlier to the *lysC* operon of *B. subtilis* ATCC 6051 and GSY225, and subsequent sequence determination showed no differences between the *lysC* operons of these strains and of *B. subtilis* 168, from which the *lysC* operon was originally isolated (Zhang *et al.*, 1990). [A sequence difference was found in the coding region for the UvrB protein just upstream of the *lysC* operon, with strains ATCC 6051 and GSY225 differing from strain 168 by the insertion of C at residue 201 and a deletion of G at residue 210, with a resulting short frame-shift in the *uvrB* coding sequence and substitution of Ala-Ala-Ala for Gln-Arg-Arg at residues 578-580 of the UvrB protein (N. Y. Chen & H. Paulus, unpublished results). This substitution occurs in a relatively unconserved region of the UvrB protein (Chen *et al.*, 1989) and may be an instance of sequence polymorphism between different strains of *B. subtilis* that has no functional significance.] On the other hand, the nucleotide sequences of the *lysC* leader region from the *aecA* mutants differed from that of

Fig. 3. Nucleotide sequence of the *lysC* leader region from *B. subtilis* 168 and from the *aecA* strains VB217, FB59 and KA120. The *lysC* sequences of strain 168 is shown in its entirety, but the *lysC* sequences of the *aecA* mutants are shown only where they differ from strain 168. The sequence is annotated to show reading frames and elements of potential regulatory significance (Chen *et al.*, 1987). The arrows indicate regions with dyad symmetry. RBS, ribosome-binding site.

the *aecA*⁺ strains by one or two nucleotide substitutions: a G to A change at position 320 in strain VB217 (*aecA3*), a C to T change at position 476 in strain FB59 (*aecA4*), and a T to A change at position 315 as well as a G to A change at position 320 in strain KA120 (*aecA5*). These nucleotide substitutions do not affect any of the known or potential control elements identified earlier in *lysC* (Chen *et al.*, 1987), which include the sigma-43 promoter region, the transcription start site, the putative transcription attenuator, and the translation start site (Fig. 3). Their function in the control of *lysC* expression is thus not immediately obvious and it was important to determine whether the observed nucleotide changes are indeed responsible for the AecA phenotype or are merely the reflection of DNA sequence polymorphism in different strains of *B. subtilis*.

Effect of nucleotide substitutions in the *lysC* leader region on the expression of *lysC'*-*lacZ* fusions

The question whether the nucleotide replacements observed in the *aecA* mutants are responsible for enhanced gene expression can be answered most directly by introducing the corresponding nucleotide changes into the wild-type *lysC* leader region and examining the resulting phenotype. Accordingly, we used oligonucleo-

tide-directed mutagenesis to effect nucleotide changes corresponding to those observed in *aecA* strains VB217 (G to A change at residue 320) and FB59 (C to T change at residue 476). In addition, we combined the nucleotide change characteristic of strain VB217 (G to A at residue 320) with a deletion in the -10 consensus region of the *lysC* promoter ($\Delta\Delta$ at residue 270), which leads to inactivation of the promoter. The effect of these mutations on gene expression was studied in a *lysC'*-*lacZ* fusion to allow the measurement of β -galactosidase as a convenient indicator enzyme. In order to avoid possible complications due to gene dosage effects and variation in plasmid copy number, we made use of a single-copy integration vector which allowed insertion of the *lysC'*-*lacZ* fusion into the *amyE* locus of *B. subtilis* (Shimotsu & Henner, 1986).

Our results show that nucleotide substitutions at residues 320 or 476 in the *lysC* leader, corresponding to the ones found in the *aecA* strains VB217 and FB59, respectively, led to greatly increased levels of β -galactosidase expression, both in translational and transcriptional *lysC'*-*lacZ* fusions (Table 3). The G to A substitution at residue 320 caused a 100- to 200-fold increase in gene expression, and the replacement of C by T at residue 476 produced a somewhat smaller (40- to 100-fold) effect. Small but significant differences were

Table 3. Effect of *lysC* leader mutations on gene expression in *lysC'*-*lacZ* fusions

Strain	Mutation	Type of fusion	β -Galactosidase activity* ($A_{420} \text{ min}^{-1} \text{ mg}^{-1}$)
GSY225-40	None (wild-type)	Translational	0.8
GSY225-41	G ³²⁰ →A ³²⁰	Translational	118
GSY225-42	C ⁴⁷⁶ →T ⁴⁷⁶	Translational	31
GSY225-47	G ³²⁰ →A ³²⁰ ; ΔA^{270}	Translational	0.0
GSY225-60	None (wild-type)	Transcriptional	0.6
GSY225-61	G ³²⁰ →A ³²⁰	Transcriptional	154
GSY225-62	G ⁴⁷⁶ →A ⁴⁷⁶	Transcriptional	60

* β -Galactosidase was measured in toluene-treated cells harvested in the mid-exponential phase of growth in LB medium as described in Methods.

Table 4. Effect of L-lysine on gene expression in *lysC'*-*lacZ* fusions

Strain	Mutation	Type of fusion	β -Galactosidase activity ($\Delta A_{420} \text{ min}^{-1} \text{ mg}^{-1}$) in cells grown with:	
			Excess L-lysine*	Limiting L-lysine*†
1A8-40	None (wild-type)	Translational	0.12	14 (117)
1A8-41	G ³²⁰ →A ³²⁰	Translational	53	44 (0.83)
1A8-60	None (wild-type)	Transcriptional	0.15	23 (153)
1A8-61	G ³²⁰ →A ³²⁰	Transcriptional	142	127 (0.89)

* β -Galactosidase was measured in toluene-treated cells harvested after growth in minimal medium supplemented with either limiting levels (0.016 mM) or an excess (5 mM) of L-lysine as described in Methods.

† Numbers in parentheses: degree of derepression due to lysine limitation.

also seen in the β -galactosidase levels of translational and transcriptional fusions, but these may in part be a reflection of the relative efficiencies of the *lysC* and the *spoVG* ribosome binding sites, which direct β -galactosidase synthesis in the translational and transcriptional fusions, respectively. These observations provide strong evidence that the nucleotide replacements observed on sequencing the *lysC* leader region of *aecA* strains are indeed responsible for the derepression of aspartokinase II observed in *aecA* mutants.

The effect of L-lysine on the expression of *lysC'*-*lacZ* fusions was studied in lysine auxotrophs cultured in minimal medium with L-lysine present either at growth-limiting concentrations or in excess. Whereas the presence of an excess of L-lysine repressed β -galactosidase synthesis more than 100-fold in both translational and transcriptional fusion strains carrying the wild-type *lysC* leader region, no repression by lysine was seen in strains with a nucleotide substitution at residue 320 corresponding to *aecA3* (Table 4). Similarly, no repression by L-lysine was seen in strains with a nucleotide substitution at residue 476 corresponding to *aecA4* (data not shown). It is interesting, however, that the levels of β -galactosidase in *lysC'*-*lacZ* fusions expressed in the strains with an altered *lysC* leader region are three to five

times higher than those observed in fusion strains with a wild-type *lysC* leader under conditions of lysine starvation (Table 4). This observation implies either that the degree of lysine starvation achieved in our experiments was not sufficient for complete derepression or that other signals, besides the availability of lysine, may be involved in the control of the *lysC* operon.

The data presented in Tables 3 and 4 also allow certain conclusions concerning the general mechanism by which the *aecA* mutation brings about derepression of *lysC*. The observation that the degree of repression by L-lysine is the same in translational and transcriptional *lysC'*-*lacZ* fusions (Table 4) shows that it does not involve control of *lysC* translation but rather the modulation of levels of functional transcripts. Does the increased transcription in the *aecA* mutants involve the same promoter as normal *lysC* transcription or is it due to the activation of a new promoter? Earlier studies on transcript mapping and promoter analysis of the *lysC* operon showed that normal transcription initiation occurs near residue 280 and that the promoter straddles the *Bgl*II site at residue 262, which is flanked by the -35 and -10 consensus sequences associated with promoters recognized by the major *B. subtilis* RNA polymerase (Chen *et al.*, 1987). Our observation that strain GSY225-47, which differs

from the derepressed mutant GSY225-41 by the deletion of a single residue (270) in the -10 consensus element, completely lacked the ability to express the *lysC'*-*lacZ* fusion protein (Table 3) demonstrates that both wild-type and mutant *lysC* expression involve the same promoter.

Conclusions

The results presented here show that *aecA* mutations involve the replacement of one or two nucleotide residues in the *lysC* leader region. The mutations studied define two distinct sites in the *lysC* leader, the alteration of which causes approximately 100-fold increases in the levels of functional *lysC* mRNA and which must, therefore, play an important role in the control of aspartokinase II levels. Whether these control sites, located near residues 320 and 476, act independently or together, and how they relate to other control elements, such as the putative transcription attenuator (Chen *et al.*, 1987), are important questions that remain to be addressed.

Although this work has identified the *aecA* loci as important control sites in the *lysC* operon, very little can be said at this time about the molecular mechanisms by which these loci participate in the control of transcription. Examination of the nucleotide sequence of the *lysC* leader region (Fig. 3) fails to reveal clear-cut relationships between the nucleotides surrounding residues 320 and 476 and other potential control elements, such as the promoter and the putative transcription attenuator. Except for a region of imperfect dyad symmetry centred on residue 320, no indications of potential secondary structure or possible base-pairing interactions with other sites in the *lysC* leader region, at the level of either DNA or RNA, can be discerned. Indeed, it is hard to see how a substitution of A for G, or of T for C, the basis of the two *aecA* mutations studied, could produce major alterations in nucleic acid secondary structure, and the possibility that the sites defined by the *aecA* mutations may be the targets of as yet unidentified *trans*-acting control elements merits serious consideration. The 330 nucleotide length of the *lysC* leader region is exceptionally large for prokaryotic operons, suggesting that it may be the site of unusual or novel control mechanisms. Interestingly, the *lysC* leader region of *E. coli*, at 308 nucleotide residues, is almost as extensive as that of *B. subtilis* but differs from the latter by the absence of elements resembling a transcription attenuator (Cassan *et al.*, 1983). Further analysis of the control sites in the *lysC* operon of *B. subtilis* defined by the *aecA* mutations should provide interesting insights into the regulation of this complex operon.

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