Lysine-induced premature transcription termination in the *lysC* operon of *Bacillus subtilis*

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The expression of the *Bacillus subtilis lysC* operon, which encodes the first specific enzyme of lysine biosynthesis, is controlled by the availability of the end product, lysine. The question of whether lysine exerts its control by inducing premature termination of transcription was addressed using Northern blot analysis. Whereas *lysC*-specific RNA from lysine-starved *B. subtilis* consisted primarily of the expected full-length mRNA (1.6 kb), that from bacteria grown with an excess of lysine consisted of a truncated 0.27 kb RNA in place of the full-length 1.6 kb transcript. On the other hand, a *B. subtilis aecA* mutant, in which the *lysC* operon was derepressed owing to a single nucleotide substitution in the region corresponding to the *lysC* leader transcript, produced full-length *lysC* mRNA, but no 0.27 kb RNA, even during growth with excess lysine. Mapping of the truncated 0.27 kb *lysC* RNA by hybridization with oligonucleotide probes showed that it corresponded to the upstream portion of the *lysC* leader transcript, extending from the transcription initiation site to a putative rho-independent terminator element. Quantitative transcript analysis by hybridization with specific oligonucleotides showed that lysine did not affect the number of *lysC*-specific RNA molecules but promoted the stoichiometric replacement of full-length mRNA with truncated 0.27 kb molecules. These results indicate that lysine regulates the expression of the *lysC* operon by effecting the premature termination of transcription at a rho-independent terminator site in the *lysC* leader region and that the site of the *aecA* mutation, far upstream of the putative terminator element, must play an essential role in premature transcription termination by a mechanism which is not yet understood.

Keywords: aspartokinase, attenuation, lysine biosynthesis, *aecA* locus

INTRODUCTION

The *Bacillus subtilis lysC* operon, which encodes the in-phase overlapping genes for the subunits of the lysine-sensitive aspartokinase, has an extensive untranslated leader region which appears to play a crucial role in the negative control of the operon by lysine (Paulus, 1993). When lysine is present in excess, the *lysC* operon is repressed more than 100-fold, but mutations at specific sites in the *lysC* leader can completely overcome this repressive effect (Lu *et al.*, 1991, 1992). Examination of the nucleotide sequence of the *lysC* leader transcript (Chen *et*

al., 1987) reveals the potential for considerable secondary structure; this includes a stem-loop structure followed by a run of uridylates, characteristic of rho-independent transcription terminators (Fig. 1). The putative rho-independent terminator is preceded by additional stem-loops which can assume an alternative conformation in which the terminator is no longer functional, reminiscent of the transcription attenuators found in many biosynthetic operons of *Escherichia coli* (Kolter & Yanofsky, 1982). Indeed, like the transcription attenuators controlling amino acid biosynthesis in *E. coli*, the *lysC* leader contains an open reading frame encoding a lysine-rich oligopeptide that overlaps the first of the stem-loops. However, in spite of the striking similarity to the enterobacterial transcription attenuators, there has been no evidence to support the involvement of premature

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transcription termination in the control of the *B. subtilis* *lysC* operon. On the contrary, the majority of *cis*-acting mutations that lead to derepression, including those known as *aecA* (Magnusson *et al.*, 1983; Mattioli *et al.*, 1979; Vold *et al.*, 1975; Yeh & Steinberg, 1978), are at a site which has no obvious structural or functional connections with any of the putative transcription attenuator elements (Lu *et al.*, 1991, 1992).

In this paper, we describe the results of transcript analyses which show that lysine promotes the accumulation of prematurely terminated RNA molecules, suggesting that lysine-dependent transcription attenuation plays a major role in the control of the *lysC* operon of *B. subtilis*.

METHODS

Materials. Restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs; T3 RNA polymerase and Bluescript II plasmid pBC KS⁺ from Stratagene; and [γ -³²P]ATP and [α -³²P]CTP from Du Pont. Oligonucleotides were synthesized on a Milligan Expedite DNA synthesizer using phosphoramidite chemistry.

Bacterial strains and growth conditions. The following strains of *B. subtilis* were used in this work: strain 1A8 (*aroD120 lys-1 trpC2*) (Dedonder *et al.*, 1977) and *B. subtilis* strain VB217 (*pheA1 trpC2 Sul^R aecA3*) (Yeh & Steinberg, 1978). Strains were grown in the minimal medium of Anagnostopoulous & Spizizen (1961) as described earlier (Lu *et al.*, 1991). Cells growing in limiting lysine (0.012 mM) were harvested 1 h after the culture reached a plateau density of 70 Klett units (filter no. 54); cells growing with an excess of lysine (1 mM) were harvested during exponential phase at the same density.

RNA isolation and Northern blotting. Bacterial cultures (25 ml) were harvested at a density of 70 Klett units (filter no. 54), which corresponds to mid-exponential phase when lysine is present in excess, by pouring onto 8 ml frozen medium plus 0.25 ml 1 M sodium azide, followed by centrifugation at 12000 *g* for 10 min at 4 °C. The cells were frozen at -70 °C and RNA was extracted by a modification of the methods of Wu *et al.* (1989) and Grandoni *et al.* (1992). This involved suspending the cells in 2 ml of LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris/HCl, pH 7.4 and 1%, w/v, SDS), adding 2 ml phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and 1 g acid-

washed glass beads (425–600 μ m), and vortexing the mixture at 25 °C for 4 min, followed by heating at 75 °C for 10 min with intermittent shaking. The aqueous layer was then collected and subjected to two additional phenol extractions. The final aqueous phase was supplemented with 0.1 vols 2 M LiCl and precipitated with 2 vols of ethanol. The resulting RNA precipitate was washed with 70% (v/v) ethanol and redissolved in 0.1 ml diethylpyrocarbonate-treated water. RNA electrophoresis was done on 1.25% (w/v) agarose gels in 20 mM MOPS, pH 6.5, 5 mM sodium acetate, 1 mM EDTA and 2% (v/v) formaldehyde, together with 0.16–1.77 kb RNA size standards (Gibco/BRL). After electrophoresis at 90 V for 3.5 h the agarose gels were washed three times for 30 min each with diethylpyrocarbonate-treated water and the RNA was electrophoretically transferred to Hybond N membranes (Amersham) using a Genie electroblotting apparatus (Idea Scientific) in 20 mM MOPS, pH 6.5, 5 mM sodium acetate and 0.5 mM EDTA for 2 h at 6 V. The RNA blots were air-dried and crosslinked for 3 min in a Spectronics XL-1000 UV crosslinker.

Probes for Northern blots and hybridization conditions. An antisense RNA probe for the *lysC* leader region was prepared by cloning the 0.42 kb *Bgl*II–*Mun*I fragment of *lysC* DNA [nucleotides 261–689; nucleotides are numbered as in Chen *et al.* (1987)] into the *Bam*HI and *Eco*RI sites of the pBluescript II pBC KS⁺ polylinker region, followed by linearization by *Xba*I cleavage and transcription with bacteriophage T3 RNA polymerase, using [α -³²P]CTP as the labelled substrate. Antisense oligonucleotide probes, complementary to the regions indicated in Fig. 1, were prepared by labelling the 5' ends of the following oligonucleotides using bacteriophage T4 polynucleotide kinase and [γ -³²P]ATP: probe 305–324, 5'-CGCACCTCTATCTTC-ACCAT-3'; probe 360–379, 5'-CCTTTCAGCCTTTTTCACAG-3'; probe 471–490, 5'-TGAACAACGAGATAGCCCTC-3'; probe 550–569, 5'-AAGAGCGCTTTGTCCACAAT-3'; probe 597–616, 5'-CCCATGTATTACCACCTTT-3'. The blots were prehybridized for 3 h at 43 °C with 2 $\times$ SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1 $\times$ Denhardt's reagent (0.02% each of Ficoll, polyvinylpyrrolidone and BSA), and 1.5 mg tRNA ml⁻¹, followed by hybridization in the same mixture supplemented with radioactive probe (about 500 000 c.p.m.) for at least 15 h at 43 °C. The blots were washed repeatedly with 1 $\times$ SSC containing 0.1% SDS (three 15 min cycles at 25 °C and two 15 min cycles at 33 °C) and then subjected to autoradiography using a storage phosphor screen and analysed on a Molecular Dynamics PhosphorImager SF with ImageQuant software (Molecular Dynamics).

Fig. 1. Nucleotide sequence of the *lysC* leader region from *B. subtilis*, showing the postulated secondary structure of a putative transcription terminator element, the site of the *aecA* mutation in *B. subtilis* VB217, and the oligonucleotide probes used in this work. The transcription terminator structure is as proposed earlier (Chen *et al.*, 1987) and can exist in equilibrium with an antiterminator structure in which stem B pairs with C, and D with E. The coding region for the aspartokinase II α subunit starts at nucleotide 612. Nucleotides are numbered as in Chen *et al.* (1987).

RESULTS

Effect of lysine and *aecA* mutations on *lysC* transcription

The *lysC* operon can be derepressed either by starvation for lysine or by a class of *cis*-acting mutations known as *aecA*. Earlier studies using translational and transcriptional *lysC'::lac'Z* fusion strains suggested that both modes of derepression occurred at the level of transcription (Lu *et al.*, 1991). To ascertain whether lysine indeed controlled *lysC* transcription, we directly examined the levels of *lysC* mRNA under various conditions by the

use of Northern blots. Cells of *B. subtilis* strain 1A8 (*lys-1*) were grown either with limiting (0.012 mM) or saturating (1 mM) lysine, and RNA was extracted, subjected to Northern blotting and visualized by hybridization with a synthetic RNA probe which was complementary to 400 residues at the 5' end of the *lysC* transcript. Under conditions of lysine limitation, the major transcript was full-length *lysC* RNA (1.6 kb), whereas in saturating lysine, the full-length transcript almost completely disappeared and was replaced by a short (0.27 kb) *lysC*-specific RNA (Fig. 2). Interestingly, in *B. subtilis* strain VB217, which is genetically derepressed owing to an *aecA* mutation, the full-length *lysC* transcript was the major RNA species even under conditions of lysine excess and little 0.27 kb transcript was produced (Fig. 2).

Fig. 2. Northern blot analysis of RNA extracted from *B. subtilis* strains 1A8 and VB217, grown with limiting (0.012 mM) or saturating (1 mM) lysine. The blots were hybridized with a 32 P-labelled antisense RNA probe complementary to nucleotides 261–689 of the *lysC* operon (Chen *et al.*, 1987) as described in Methods.

Mapping of the site of premature transcription termination with oligonucleotide probes

To map the 0.27 kb *lysC*-specific transcripts within the *lysC* operon, we hybridized Northern blots of RNA obtained from cells of *B. subtilis* 1A8, harvested during either lysine starvation or growth with saturating lysine, with 32 P-labelled oligonucleotides complementary to specific segments of the *lysC* leader region as shown in Fig. 1. All oligonucleotide probes hybridized with the 1.6 kb full-length *lysC* transcript that was produced under conditions of lysine starvation, but only the probes upstream of nucleotide 490 and not those downstream of nucleotide 550 hybridized with the short transcript (0.27 kb) that was produced during growth with saturating lysine (Fig. 3). This is consistent with the notion that the 0.27 kb RNA molecules extend from the site of transcription initiation (nucleotide 281) through the rho-independent terminator (nucleotide 550). Attempts to map the 3' end of the 0.27 kb RNA more precisely by using oligonucleotide probes between nucleotides 490 and 550 were unsuccessful because these probes failed to hybridize even with full-length *lysC* mRNA, perhaps because extensive secondary structure in the nucleotide

Fig. 3. Northern blot analysis of RNA extracted from *B. subtilis* strain 1A8 grown with limiting (0.012 mM) (lanes L) or saturating (1 mM) (lanes S) lysine, using as probes 32 P-labelled oligonucleotides complementary to the segments of the *lysC* leader region indicated in Fig. 1, as described in Methods.

Table 1. Relative molar amounts of full-length and short *lysC* transcripts synthesized in the presence of limiting and saturating lysine

The relative amounts of radioactivity of the high and low molecular mass RNA components in the corresponding panels of Fig. 2 were estimated by integration using ImageQuant software. Image intensities are expressed as pixel values and no correction was made for background variation (± 1000 pixels).

Transcripts	Probe 305–324		Probe 360–379	
	hybridization (pixels)		hybridization (pixels)	
	0.012 mM lysine	1 mM lysine	0.012 mM lysine	1 mM lysine
Full-length	36200	1200	25700	0
Short	16500	50900	8100	37800
Total	52700	52100	33800	37800

490–550 region of the mRNA molecules (see Fig. 1) prevented stable hybrid formation (data not shown).

The fact that the upstream oligonucleotide probes could hybridize in their entirety with both the full-length and the short *lysC* transcripts (and therefore produced equivalent signals irrespective of the length of the RNA target) allowed comparison of the relative molar amounts of the various *lysC* RNA species synthesized by cells grown with excess or limiting lysine. As shown in Table 1, lysine had no effect on the molar amounts of total *lysC* RNA synthesized but rather caused a quantitative shift of full-length 1.6 kb *lysC* mRNA to the truncated 0.27 kb RNA species.

DISCUSSION

Like most other operons that function in amino acid biosynthesis in bacteria, the *B. subtilis lysC* operon, which encodes the first enzyme in the biosynthesis of lysine, is controlled by the intracellular concentration of its end product. When *B. subtilis* was starved for lysine, the predominant transcript of the *lysC* operon was a 1.6 kb RNA species, which is the expected size for full-length *lysC* mRNA (Chen *et al.*, 1987). When lysine was present in excess, full-length *lysC* mRNA was almost completely replaced by a truncated 0.27 kb *lysC* RNA species. Mapping of the 0.27 kb RNA by hybridization to oligonucleotides complementary to various regions of the *lysC* leader region suggested that it corresponds to the 5'-terminal portion of *lysC* mRNA, extending from the transcription start site (nucleotide 281) to the putative rho-independent transcription terminator (nucleotide 549).

Lysine had no effect on the total number of *lysC*-specific RNA molecules but caused a stoichiometric replacement of full-length mRNA by truncated RNA molecules. This suggested that lysine controls *lysC* expression by promoting the premature termination of transcription rather

than by inhibiting the initiation of transcription. (Another possible explanation, though not very likely, is that lysine promotes the decay of *lysC* mRNA, but that the high degree of RNA secondary structure arrests the progression of 3'-exonucleolytic degradation at the putative transcription terminator.) The observation that the lysine-induced accumulation of truncated transcripts was completely suppressed by an *aecA* mutation involving a single G-to-A substitution in the leader transcript [nucleotide 320; Lu *et al.* (1991)] also supports the notion of a post-transcriptional control mechanism. Indeed, the fact that the *lysC* operon transcript has an unusually long untranslated leader region with the potential for alternative secondary structures similar to the terminator/antiterminator structures that play a role in the control of amino acid biosynthetic operons in *Enterobacteriaceae* (Kolter & Yanofsky, 1982), had led to the earlier prediction that the *lysC* operon of *B. subtilis* is controlled by a similar transcription attenuation mechanism (Chen *et al.*, 1987). An important aspect of this prediction, that premature termination of transcription should occur at a rho-independent terminator that ends at nucleotide 549, is confirmed by the results presented in this paper.

On the other hand, the mechanism by which lysine promotes transcription attenuation is not yet clear. Originally, it was thought that control of premature transcription termination might be effected through the translation of a short open reading frame encoding a 24-residue lysine-rich peptide, which overlaps the first stem-loop of the putative terminator structure (Chen *et al.*, 1987). Such a mechanism is prevalent in the *Enterobacteriaceae*, where amino acid control of transcription is effected by the stalling of ribosomes at the cognate amino acid codons during translation of the leader peptide, thereby destabilizing the RNA secondary structure required for transcription termination (Kolter & Yanofsky, 1982). However, experiments involving in-frame translational fusions of the putative leader peptide with β -galactosidase provided no evidence for translation of this open reading frame under a variety of growth conditions (Yang Lu & H. Paulus, unpublished results). Another observation that speaks against this type of terminator/antiterminator control mechanism is the complete suppression of premature transcription termination by a G-to-A substitution at nucleotide 320, a position remote from both the open reading frame for the putative leader peptide (nucleotides 362–436) and the terminator stem-loop structures (nucleotides 393–544) (Fig. 1). Rather, our results suggest that the RNA segment around nucleotide 320, the site of many *aecA* mutations (Lu *et al.*, 1992), is an essential component of the transcription terminator.

The results on the *lysC* operon presented here bring the number of amino acid biosynthetic operons in *B. subtilis* that are known to be controlled by amino-acid-induced premature transcription termination to three, the others being the *trp* operon (Shimotsu *et al.*, 1986) and the *ilv-leu* operon (Grandoni *et al.*, 1992). [A fourth example may be *cysE*, which encodes the first enzyme for cysteine biosynthesis and is part of a complex operon also encoding

the aminoacyl-tRNA synthetases for glutamate and cysteine (Gagnon *et al.*, 1994).] It is interesting that transcription attenuation in each of these three *B. subtilis* operons occurs by entirely different mechanisms, in contrast to the situation in *E. coli*, where attenuation control of many amino acid biosynthetic operons involves a single basic mechanism (Kolter & Yanofsky, 1982). In the *trp* operon of *B. subtilis*, the transcription terminator in the leader transcript is stabilized by an RNA-binding protein that is active only when complexed with tryptophan (Babitzke & Yanofsky, 1993; Gollnick, 1994); in the *ilv-leu* operon, an antiterminator structure seems to be specifically stabilized by uncharged tRNA^{Leu} but not by leucyl-tRNA^{Leu} (Grandoni *et al.*, 1993; Grundy & Henkin, 1993); in the *lysC* operon, transcription attenuation seems to involve the lysine-dependent interaction between the site of the *aecA* mutation and a remote transcription terminator element by a mechanism yet to be defined. Clearly, the simple RNA secondary structure model for transcription attenuation in the *lysC* operon presented earlier (Chen *et al.*, 1987), which was based on the *E. coli* paradigm, must be modified or extended to account for the role of nucleotide 320 in the structure of the terminator and the mechanism by which lysine modulates its function. Among the possibilities that need to be considered are more complex RNA structures than those shown in Fig. 1, especially ones involving tertiary structure elements such as pseudoknots as well as RNA-binding proteins whose activity may be modulated by lysine.

ACKNOWLEDGEMENTS

This work was supported by Grant R01 GM45879 from the National Institutes of Health.

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Received 20 November 1995; revised 12 February 1996; accepted 22 February 1996.