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Characterization of *cis*-Acting Mutations in the First Attenuator Region of the *Bacillus subtilis pyr* Operon That Are Defective in Pyrimidine-Mediated Regulation of Expression

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A transcriptional attenuation mechanism for the regulation of *pyr* operon expression in *Bacillus subtilis* in which the PyrR regulatory protein binds *pyr* mRNA at three sites with similar sequences to cause transcription termination in response to elevated pyrimidine nucleotide pools has been proposed (R. J. Turner, Y. Lu, and R. L. Switzer, J. Bacteriol. 176:3708–3722, 1994). Twenty-seven mutants with *cis*-acting defects in the repression by pyrimidines of β-galactosidase expression of a *pyr-lacZ* fusion-integrant were isolated as blue colonies on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) agar plates containing uracil and uridine after UV irradiation or treatment with mutagens or following *mutD* mutagenesis. These mutants showed normal repression of the chromosomal *pyr* operon by exogenous pyrimidines. Sequence analysis revealed 12 unique sites of mutation, which occurred in the conserved putative PyrR binding sequence (10 of the 12) or in the stem of the transcriptional terminator structure. These mutants strongly support the proposed model for regulation of the *pyr* operon.

Expression of the pyrimidine nucleotide biosynthetic (*pyr*) operon of *Bacillus subtilis* is regulated by a transcriptional attenuation mechanism (15, 21). Largely on the basis of molecular genetic evidence, Turner et al. (21) and Lu et al. (15) concluded that exogenous pyrimidines act to promote termination at three attenuation sites located in the promoter-proximal end of the operon. These sites are located in the 5′ leader, between the first (*pyrR*) and second (*pyrP*) genes of the operon, and between the second and third (*pyrB*) genes of the operon. For each attenuator the mRNA is predicted to fold into one of two mutually exclusive secondary structures, one of which can function as a factor-independent transcription terminator and the other of which is an antiterminator structure whose formation prevents formation of the terminator. The ratio between terminator and antiterminator structures is proposed to be regulated by the protein product encoded by the first gene of the operon, *pyrR*. The PyrR protein is believed to act by binding in a pyrimidine nucleotide-dependent manner to the 5′ stem of the antiterminator, thereby interfering with formation of the antiterminator stem-loop and promoting formation of the alternative terminator structure, which leads to transcriptional termination. A clue to the identity of the putative PyrR binding site was obtained from identification of a highly conserved nucleotide sequence located in the 5′ stem of each of the three proposed antiterminators of the *pyr* operon (21).

Many aspects of the mechanism of regulation of the *pyr* operon can be studied by analysis of mutants that have lost normal regulation. We describe in this work the isolation and characterization of a collection of mutants which were defective in pyrimidine repression of expression of *pyr-lacZ* fusions. These mutants were obtained by screening for defects in repression of the expression of β-galactosidase by a *pyr-lacZ* fusion in which the promoter and 5′ leader attenuator sequences from the *pyr* operon were transcriptionally fused to the *lacZ* gene and integrated into the *B. subtilis* chromosome. The mutants resulted from single base replacements that were heavily concentrated in the sequences previously identified as a putative PyrR binding site or in the stem of the postulated terminator structure. The results provide strong experimental support for the transcriptional attenuation model for regulation of the *pyr* operon proposed by Turner et al. (21).

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are described in Table 1.

Luria-Bertani (LB) medium (3) was used as a rich medium for growth of both *Escherichia coli* and *B. subtilis*. For synthetic minimal media, CH medium (7) and Spizizen medium (20) were used for *B. subtilis*. Solid medium contained 1.5% agar. When necessary, the following nutrients were added: uracil (50 μg/ml), uridine (50 μg/ml), starch (1%), and individual amino acids (50 μg/ml). Liquid media and agar plates were supplemented with antibiotics for selection as follows: ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml). The β-galactosidase chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 80 μg/ml.

Construction of *pyr-lacZ* fusion-integrant strains. The vector used to construct a transcriptional fusion of the *B. subtilis pyr* promoter and 5′ leader region to the *E. coli lacZ* gene was the *E. coli* vector pDH32 (10). The pDH32 vector cannot replicate in *B. subtilis*, but it has flanking *amyE* front and back gene segments, which allow integrative recombination of the linearized plasmid at the *amyE* locus of the *B. subtilis* chromosome. We constructed two *pyr-lacZ* transcriptional fusion integrants, BGH39 and BGH64. BGH39 was constructed from pTS790 (22) and carries the *B. subtilis pyr* promoter, 5′ leader, and *pyrR* gene fused to *lacZ* (Fig. 1). BGH64 was constructed from pTS290 (21) and carries only the *pyr* promoter and 5′ leader fused to *lacZ*. In both cases the plasmids were linearized by digestion with *Pst*I and *Sca*I and used to transform strain DB104 to chloramphenicol resistance as described below.

Mutagenesis. For mutagenesis of strain BGH39, treatment with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) or 2-aminopurine and irradiation with UV were performed according to the procedures described by Miller (17).

For *mutD* mutagenesis, the pTS290 plasmid was transformed in the *E. coli mutD* strain KD1067. The transformants were grown in multiple, separate tubes containing 5 ml of LB medium at 37°C overnight. The mutagenized plasmids were extracted, linearized, and transformed into *B. subtilis* DB104.

Selection and characterization of mutants. For chemical mutagenesis, mutagenized cells were plated on CH agar plates containing X-Gal and chloramphenicol and supplemented with uracil and uridine. Blue colonies that also lacked amylase activity, as determined by iodine staining of colonies restreaked on LB agar plates containing 1% starch, were isolated as candidates for mutants with defects in regulation of *pyr* expression. To ensure that only mutations in the *pyr-lacZ* fusion region were being studied, chromosomal DNA from the candi-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
B. subtilis		
DB104	*his nprR2 nprE18 ΔaprA3*	14
BGH39	DB104 *amyE*::pTS790	This work
BGH64	DB104 *amyE*::pTS290	This work
E. coli		
KD1067	F$^-$ *arg his mutD5 sup*	J. Cronan
Plasmids		
pDH32	Ap^r Cm^r *amyE* front *amyE*back *lacZ*	10
pTS290	0.28-kb *Eco*RI-*Bam*HI fragment of pUC19/290 (21) containing the *pyr* promoter, leader, and 5′ portion of the *pyrR* region in the *Eco*RI-*Bam*HI sites of pDH32	21
pTS790	0.8-kb *Eco*RI-*Apa*LI (Klenow) fragment of *pyr* DNA containing the *pyr* promoter, leader, and *pyrR* region in the *Eco*RI-*Bam*HI (Klenow) sites of pDH32	22

date strains was prepared and used to transform to chloramphenicol resistance another culture of *B. subtilis* DB104, which had not been exposed to mutagens. The transformants were rescreened for blue color on X-Gal plates containing excess pyrimidines as before.

For *mutD* mutagenesis, *B. subtilis* DB104 transformants obtained from mutagenized and linearized plasmids were plated on LB agar plates containing chloramphenicol to select for integration of the plasmid into the chromosome at the *amyE* locus and then replica plated onto CH agar plates containing X-Gal with or without uracil and uridine and LB agar plates containing 1% starch and chloramphenicol. Blue colonies growing on plates supplemented with repressive levels of pyrimidines and showing no amylase activity on starch-containing plates were isolated as the attenuator mutant candidates. All purified mutants were further characterized by β-galactosidase and aspartate transcarbamylase (ATCase) assays.

PCR amplification and sequencing of *pyr* attenuator DNA. PCR amplification was performed by using AmpliTaq DNA polymerase (Perkin-Elmer) for DNA amplification. Each 100-μl reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 50 μM each deoxynucleoside triphosphate, 0.5 pmol of each primer, 1 μg of template DNA per ml, and 2 U of polymerase. The reaction mixture was heated to 94°C for 3 min and passed through 30 reaction cycles as follows: denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, and extension at 72°C for 45 s. After the completion of the cycles, the reaction was extended at 72°C for 15 min. Sequencing-grade *Taq* DNA polymerase (Promega, Madison, Wis.) was used for direct sequencing of PCR products as described by the manufacturer. DNA sequencing was carried out with a [γ-^{32}P]ATP-labeled primer, purified DNA template from PCR amplification, and *Taq* DNA polymerase in a DNA Thermal Cycler (Perkin-Elmer Cetus) (13). The Cycler conditions used for DNA sequencing were 95°C for 2 min followed by 30 reaction cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 1 min. DNA fragments were separated on 6% polyacrylamide–35% formamide slab gels.

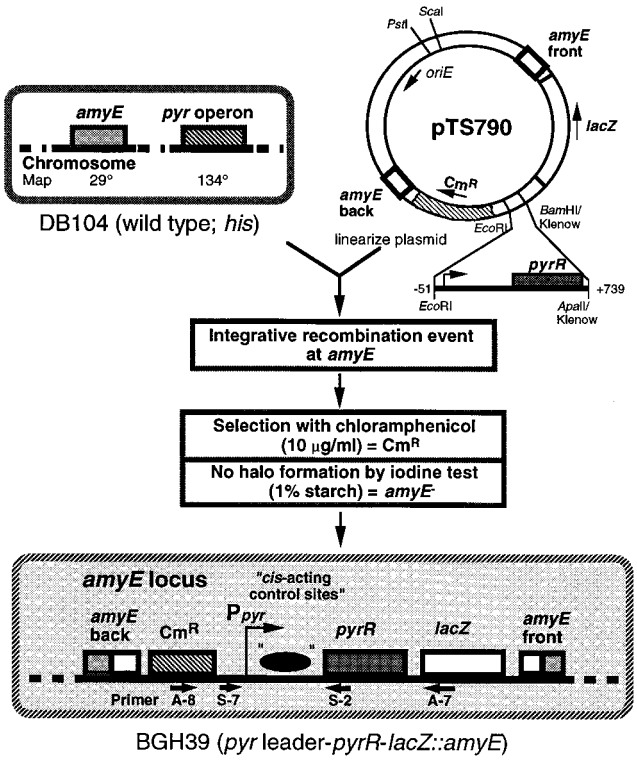

FIG. 1. Construction of strain BGH39.

Two synthetic primers, 5′-GGAGGCTTACTTGTCTGCTTTCTTC-3′ (primer A-8) and 5′-CCCGGTCGACCCGTAATCTTACG-3′ (primer A-7), were used for amplification of the target DNA region, and two [γ-^{32}P]ATP 5′-end-labeled oligonucleotides, 5′-GCCGATGATAAGCTGTC-3′ (primer S-7) and 5′-GTTCGTCGAGAATGACAG-3′ (primer S-2), were used for the sequencing of both DNA strands. The locations of these primers on the DNA integrated into the *amyE* locus are shown in Fig. 1.

Recombinant DNA techniques. For *B. subtilis* preparation of competent cells and transformation were performed as described by Boylan et al. (5), and for *E. coli* these procedures were performed as described by Cohen et al. (6). For purification of amplified DNA fragments, PCR products were electrophoresed on 0.8% agarose gels and gel pieces of the proper band were cut out with a scalpel. The gel pieces were extracted with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Plasmid preparations used Qiagen columns according to the manufacturer's instructions. *B. subtilis* chromosomal DNA was isolated as described by Wilson (23). Other DNA techniques were performed as described previously (19).

Enzyme assays. For ATCase assays, 55-ml portions of cell cultures grown in Spizizen minimal medium with and without pyrimidines were harvested by centrifugation at a cell density corresponding to a turbidity of about 100 Klett units (no. 66 filter; late exponential growth phase) and the precipitated cells were washed with 0.9% NaCl and resuspended in 5.5 ml of 10 mM Tris-HCl–1 mM EDTA buffer (pH 8.0). The cells were disrupted by sonication and centrifuged at 16,000 × *g* for 10 min, and the supernatant liquid was collected. ATCase activity was determined by the procedure of Bond et al. (4) and the colorimetric method of Prescott and Jones (18).

β-Galactosidase activity was determined as described previously (15, 16). Cultures were grown in Spizizen minimal medium to a culture turbidity of approximately 100 Klett units, the cell density was measured (A_{600}), and 1 ml of cell culture was mixed with 10 μl of toluene with vigorous vortexing. A 0.1-ml sample of the toluenized cells was used for assay. Specific activities were determined in Miller units (16).

Protein content was determined by using the Coomassie protein assay reagent (Pierce, Rockford, Ill.) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

The first group of mutants with mutations in the *pyr* 5′ leader attenuator region was isolated after chemical mutagenesis of strain BGH39, which contained a transcriptional fusion of the *B. subtilis pyr* promoter, 5′ leader, and *pyrR* gene to the *E. coli lacZ* gene integrated into the chromosome at the *amyE* locus (Fig. 1). A fusion that contained the *pyrR* gene was used so that

TABLE 2. *pyr-lacZ* and chromosomal *pyrB* expression in *pyr* attenuator mutants derived from chemical mutagenesis

Mutation[a]	Strain	Mutagen	β-Galactosidase activity (Miller units)		β-Galactosidase repression ratio	ATCase repression ratio[b]
			No pyrimidines	+ Uracil and uridine (50 μg/ml each)		
Parent strain	BGH39	None	390	40	9.8	8
G-23→A	BGH45	MNNG	1,200	760	1.6	9
	BGH54	MNNG	1,160	740	1.6	9
G-28→A	BGH51	MNNG	840	610	1.4	8
G-30→A	BGH44	MNNG	1,140	990	1.2	8
	BGH47	MNNG	1,090	990	1.1	6
	BGH49	MNNG	1,010	1,050	1.0	7
G-32→A	BGH48	MNNG	1,230	1,020	1.2	8
	BGH50	MNNG	1,260	1,010	1.2	10
	BGH56	MNNG	1,310	1,020	1.3	13
	BGH59	MNNG	1,160	940	1.2	8
	BGH60	2-AP[c]	1,180	960	1.2	8
G-61→A	BGH40	UV	430	120	3.6	10
G-113→A	BGH43	MNNG	580	320	1.8	11
	BGH55	MNNG	550	290	1.9	7
G-114→A	BGH52	MNNG	600	340	1.8	9
	BGH53	MNNG	580	330	1.8	7
	BGH57	MNNG	590	330	1.8	8
	BGH58	MNNG	550	310	1.8	12

[a] Bases are numbered from the start of transcription of *pyr* mRNA as +1.
[b] The specific activity for ATCase under repressing conditions in all strains listed averaged 27 nmol/min/mg of protein and ranged from 20 to 30 nmol/min/mg of protein.
[c] 2-AP, 2-aminopurine.

the presence of two copies of the gene (one is also present in the normal chromosomal *pyr* operon) would reduce the chance of isolating mutations in the *pyrR* gene. Colonies of strain BGH39 were blue when they were grown on minimal medium plus X-Gal and white when the plates also contained uracil and uridine, demonstrating normal regulation of the *pyr-lacZ* fusion by exogenous pyrimidines. Mutant colonies were selected as those which remained blue even when grown on the indicator plates containing pyrimidines. The colonies were purified by restreaking, and DNA from the mutants was extracted and used to transform a culture of strain DB104 to chloramphenicol resistance, which is tightly linked to the *pyr-lacZ* fusion (Fig. 1), so as to reduce the likelihood of isolating strains bearing multiple mutations. Nearly all of the transformants also carried resistance to repression of the *pyr-lacZ* fusion, as indicated by their blue color on X-Gal plates containing pyrimidines. Eighteen such mutant strains were purified and characterized (Table 2). All of the mutant strains displayed normal regulation of the chromosomal *pyr* operon, as evidenced by ATCase assays of the cells grown on minimal medium with and without uracil plus uridine, but all were defective in the normal repression of β-galactosidase expression from the *pyr-lacZ* fusion. This demonstrated that the mutations were *cis* acting and were not located in the *pyrR* gene.

DNA specifying the *pyr* promoter and 5′ leader from the *pyr-lacZ* fusions of the mutant strains was amplified by PCR using primers that were complementary to fusion vector sequences that flank this region, so that the corresponding region from the normal *pyr* operon was not amplified. The amplified DNA from all 18 mutant strains was sequenced. In each case the sequence corresponding to nucleotides −35 through 150 of the *pyr* operon, numbering from the start of transcription as +1, was determined. Mutations in seven loci were identified (Table 2). That is, most of the mutations that we identified were isolated independently several times; only two of the seven mutations were isolated only once. All of the mutations were G-to-A transitions, as might be expected from the nature of the mutagens used.

In an attempt to isolate other types of *cis*-acting *pyr* regulatory mutants, we devised a protocol for *mutD* mutagenesis. Plasmid pTS290, which carries a transcriptional fusion of the *B. subtilis pyr* promoter and 5′ leader to *lacZ*, was grown in the *E. coli mutD* strain KD1067, and the mutagenized plasmids were then linearized and transformed into *B. subtilis* DB104, with selection for chloramphenicol-resistant integrants at the *amyE* locus. Colonies displaying defects in the repression of *pyr-lacZ* expression by pyrimidines were selected on X-Gal plates. Nine mutants were isolated and shown to contain *cis*-acting mutations affecting repression of the *pyr-lacZ* fusion (Table 3). The DNA specifying the *pyr* promoter and attenuator from these mutants was amplified by PCR and sequenced as described above; eight different mutational sites were identified (Table 3). Three of these mutations were identical to mutations which had been generated by the chemical mutagenesis procedure. Thus, 12 unique mutations in the *pyr* 5′ leader region that lead to *cis*-acting defects in repression by pyrimidines were characterized.

Determination of the sites of mutations leading to regulatory defects within the *pyr* 5′ leader-attenuator region gives valuable clues to the location of functionally important regions within this RNA. The mutations were not randomly distributed throughout the 5′ leader region but were clustered within two relatively short segments (Fig. 2). Most of the mutants were isolated multiple times from independent mutagenesis experiments. The properties of the mutants are quite readily interpreted in terms of the transcriptional attenuation model previously proposed for the *B. subtilis pyr* operon (21).

Two of the 12 mutations (seven isolates) were located within the stem of the predicted stem-loop structure of a transcription terminator near the 3′ side of the attenuator. Previous deletion mutagenesis had demonstrated that this region behaves as a transcription terminator in vivo and that it plays an important role in the repression by uracil of a *pyr-lacZ* fusion (21). Both mutations in the terminator would disrupt G · C base pairs

TABLE 3. *pyr-lacZ* and chromosomal *pyrB* expression in *mutD*-derived *pyr* attenuator mutants

Mutation[a]	Strain	β-Galactosidase activity (Miller units)		β-Galactosidase repression ratio	ATCase repression ratio[b]
		No pyrimidines	+ Uracil and uridine (50 μg/ml each)		
Parent strain	BGH64	220	40	5.5	11
A-5→G	BGH85	360	190	1.9	11
G-23→A	BGH84	1,070	1,080	1.0	11
C-25→U	BGH80	1,080	460	2.3	11
C-26→U	BGH82	990	890	1.1	12
G-32→A	BGH79	960	930	1.0	11
A-39→G	BGH72	820	750	1.1	11
A-43→G	BGH76	380	180	2.1	11
	BGH83	390	190	2.1	11
G-114→A	BGH81	420	270	1.6	12

[a] Bases are numbered from the start of transcription of *pyr* mRNA as +1.
[b] The specific activity for ATCase under repressing conditions in all strains listed averaged 24 nmol/min/mg of protein and ranged from 20 to 30 nmol/min/mg of protein.

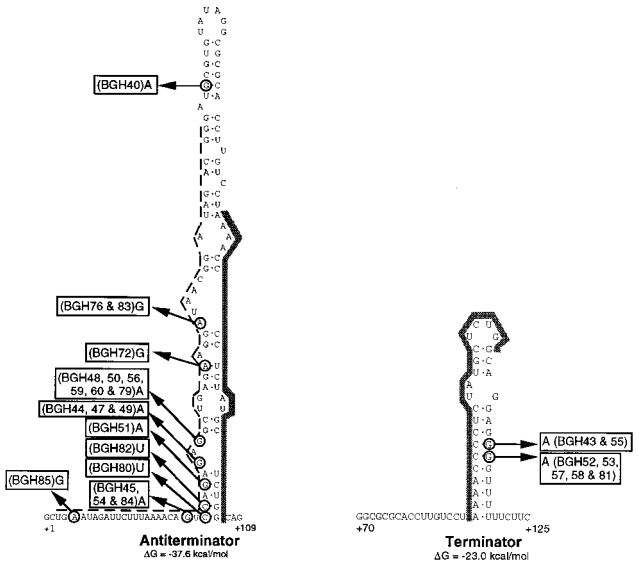

FIG. 2. Mapping of *cis*-acting regulatory mutations in the *pyr* leader region. Mutated sites are marked on proposed antiterminator and terminator structures (21). Conserved sequences for the putative PyrR-binding sites (21) are marked with the dashed line on the antiterminator structure. The nucleotides indicated by the stippled lines indicate the regions of overlapping between the antiterminator and terminator structures, which are mutually exclusive.

located in the middle of the stem and are predicted by a computer RNA folding program (24) to reduce the stability of the stem-loop structure by about 40%. The expected consequence of disrupting the terminator stem would be decreased transcriptional termination, leading to increased expression, whether or not PyrR is bound to the antiterminator stem. This prediction is consistent with the properties of the mutants in the present study. The fact that the terminator mutants could still be repressed about 1.8-fold by exogenous uracil indicates that some residual termination of transcription can take place with these mutants and that it can still be regulated by PyrR and the antiterminator.

Ten of the 12 mutations (20 isolates) were located within or immediately adjacent to a sequence that is highly conserved in all three *pyr* attenuators (21). The central portion of this conserved region forms part of the 5′ half of the putative antiterminator stem-loop structure in each of the three regulatory regions, which is located so that base pairing of that segment with the 3′ segment of the antiterminator hairpin prevents formation of the terminator stem-loop (21) (Fig. 2). Turner et al. (21) have proposed that this conserved region forms the recognition site for the binding of the PyrR protein and that binding of PyrR prevents base pairing of this RNA segment with the 3′ stem, thus permitting the terminator to form. The identification of so many mutations in this limited sequence that have defects in repression by pyrimidines confirms the region's importance in attenuation. The properties of these mutants can be easily explained by proposing that the mutated RNA strand has greatly reduced affinity for the PyrR protein. An important part of the PyrR binding sequence, as defined by these mutants, lies between G-23 and A-39. The mutations lying 5′ to this region (A-5 to G) and 3′ to it (A-43 to G and G-61 to A) were less derepressed than the others and generally showed somewhat greater residual repressibility (1.9- to 3.7-fold) than the others (1.0- to 1.6-fold, except for C-25 to U). This central region is precisely the region in which the regulatory sequences are most strongly conserved (Fig. 3). The fact that mutations causing derepression ranging all of the way from A-5 to G-61 were isolated, however, indicates that the PyrR binding site or the attenuation mechanism may involve elements of sequence recognition or RNA secondary structure throughout this region. It should also be noted that 4 of the 10 mutations in this region are not in nucleotides whose sequence is conserved in all three of the *pyr* attenuators (Fig. 3). All of these mutations lie outside of the 10-nucleotide core conserved sequence. If these mutations exert their effects on *pyr* expression by reducing PyrR binding, we suggest that they might do so by adversely affecting the folding of the RNA into a structure that is optimal for PyrR recognition. Further definition of the sequence and secondary structure elements required for PyrR binding will require in vitro mutagenesis and direct studies of RNA binding by the purified protein.

If the attenuator mutations in the antiterminator do indeed define the PyrR binding site, the nature of that site is quite different from that of the RNA sequence elements recognized by the *B. subtilis trp* RNA-binding attenuation protein, TRAP, which regulates transcriptional attenuation of the *trpEDCFBA* operon by a mechanism that is formally very similar to that proposed for the *pyr* operon (1, 2, 9). TRAP has been shown to bind in a highly cooperative manner to a region of single-stranded RNA that contains tandem repeats of up to 11 GAG or UAG segments, optimally separated by 2-nucleotide spacer segments (1, 2). No such pattern of repeated sequences is evident in the corresponding sequences of the three *pyr* attenuators (Fig. 3). If several repeated sequences were critical for PyrR binding, mutations leading to loss of regulation would not be expected to be so highly concentrated in a short segment of the RNA. If cooperative binding of PyrR to several repeated sequences were a feature of *pyr* attenuation, as has been shown for TRAP binding to *trp* RNA, it is unlikely that single-base-substitution mutations would lead to major defects in PyrR binding and regulation of expression in vivo. It has been shown that 3 of the 11 G/UAG repeats in *trp* RNA can be deleted with

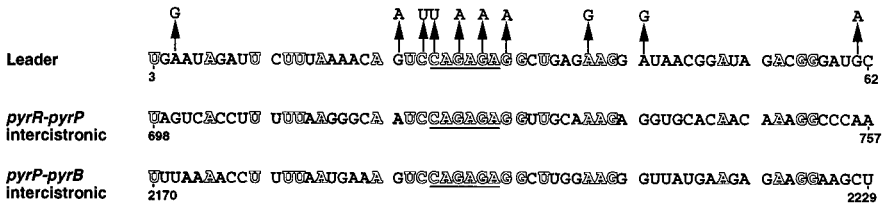

FIG. 3. Mutations in the putative PyrR-binding site of the 5′ leader attenuator sequence. Shown below are the corresponding sequences from the other two *pyr* attenuators (21). Outlined letters indicate nucleotides that are identical in all three attenuators. Underlining indicates the sequence that matches the AG box described by Henkin (11, 12; also see the text).

only a minor reduction in TRAP binding and that RNA segments containing only 6 or 7 repeats still bind reasonably well (1). Our results suggest that the PyrR binding site is confined to a shorter sequence and that PyrR binding is highly sensitive to single base changes in the RNA to which it binds.

We considered the possibility that the mutations in the antiterminator region might exert their effects through alterations in the secondary structure of the RNA in this region. Several of the mutations in the antiterminator would be predicted to disrupt base pairs in the antiterminator stem (Fig. 2), but we believe that it is unlikely that the effect of these mutations on *pyr* expression is solely a consequence of the breaking of these base pairs. Such effects would be predicted to weaken the antiterminator structure and favor terminator formation, which would have led to reduced expression, especially when the cells were grown without pyrimidines, but all of the mutants showed increased expression. Furthermore, the effects of those mutations predicted to disrupt base pairing (C-25 to U, C-26 to U, G-28 to A, A-39 to G, and G-61 to A) were no greater than those of most of the mutations that were not predicted to disrupt base pairing. Finally, the calculated free energies of formation (24) of the antiterminator stem-loop structures in the mutants differ from that of the wild-type structure by 10% or less in all but three cases. The G-61–to–A mutation was predicted to destabilize the antiterminator structure somewhat more than the others, but it caused the smallest defects in regulation of any of the mutants. Two mutations, A-39 to G and A-43 to G, were predicted to form antiterminator structures of roughly 20% greater stability than the wild type, but the effects of these mutations were not obviously different from those of others for which the stability of the antiterminator was predicted to be decreased. The failure of the regulatory defects in these mutants to correlate with the calculated free energies of formation of their antiterminator structures suggests that the mutations do not exert their effects simply as a result of alterations in the secondary structure of this segment of the *pyr* mRNA.

We have noticed that the conserved CAGAGA sequence (underlined in Fig. 3.) in the *pyr* attenuator sequence is identical to a segment of the *B. subtilis tyrS* gene leader, known as the AG box, which has been identified as a conserved element in the 5′ leaders of several *B. subtilis* aminoacyl-tRNA synthetase genes that are controlled by transcriptional attenuation (11). Henkin (12) has suggested that the AG box might be important in the control of expression of these genes, because mutations of two or three positions of that sequence in *tyrS* resulted in a significant drop in the level of expression, but a specific biochemical role for this sequence in regulation of *tyrS* expression has not been identified. The presence of the AG box in the *pyr* attenuators might suggest a more general role for this sequence element in attenuation in *B. subtilis*. If the AG box serves as a binding site for a regulatory protein in both cases, the consequences of binding must be different in the two systems, because mutations in the AG box caused elevated expression in *pyr-lacZ* fusions but led to reduced expression of *tyrS*.

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