Regulation of the *Bacillus subtilis* Pyrimidine Biosynthetic (*pyr*) Gene Cluster by an Autogenous Transcriptional Attenuation Mechanism

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A complete transcript of the *Bacillus subtilis* *pyr* operon contains the following elements in 5' to 3' order: a 151-nucleotide (nt) untranslated leader; *pyrR*, encoding a 20-kDa protein; a 173-nt intercistronic region; *pyrP*, encoding a 46-kDa protein; a 145-nt intercistronic region; and eight overlapping cistrons encoding all of the six enzymes for de novo pyrimidine biosynthesis. Transcription is controlled by the availability of pyrimidines via an attenuation mechanism. There are three transcription terminators within the operon, each of which is preceded by another stem-loop structure, the antiterminator, whose formation would prevent formation of the terminator stem-loop. These are located in the leader, the *pyrR-pyrP* intercistronic region, and the *pyrP-pyrB* intercistronic region. Northern (RNA) blot analysis has identified transcripts of lengths which coincide with termination at these proposed attenuation sites and whose relative abundances vary in the expected pyrimidine-dependent manner. Each antiterminator contains a 50-base conserved sequence in its promoter-proximal half. Various transcriptional fusions of the *pyr* promoter and surrounding sequences to promoterless reporter genes support an attenuation mechanism whereby when pyrimidines are abundant, the PyrR protein binds to the conserved sequence in the *pyr* mRNA and disrupts the antiterminator, permitting terminator hairpin formation and promoting transcription termination. Deletion of *pyrR* from the chromosome resulted in the constitutive, elevated expression of aspartate transcarbamylase, which is encoded by *pyrB*, the third gene in the operon. Complementation of an *E. coli upp* mutant, as well as direct enzymatic assay, has demonstrated that *pyrR* also confers uracil phosphoribosyltransferase activity. Analysis of *pyrR* and *upp* deletion mutants demonstrated that *upp*, not *pyrR*, encodes the quantitatively important uracil phosphoribosyltransferase activity. The *pyrP* gene probably encodes an integral membrane uracil permease.

The genes encoding the enzymes of pyrimidine nucleotide biosynthesis de novo in *Escherichia coli* and *Salmonella typhimurium* are scattered around the chromosome and are regulated by multiple, different mechanisms (29, 47). In *Bacillus subtilis*, they are located in a single cluster on the chromosome (23, 35, 37). The nucleotide sequence of the entire *B. subtilis* *pyr* gene cluster has been determined previously (24, 37). All of the genes in the cluster appear to be expressed as a single transcriptional unit; *pyr* gene expression is coordinate and is strongly regulated by pyrimidines (33). Two putative transcription terminators, similar in structure to *E. coli* rho-independent terminators, were identified in the 5' end of the *pyr* cluster, one immediately downstream from the point of transcription initiation (37) and the other immediately preceding the *pyrB* gene (24). The presence of these terminators prompted the suggestion that the regulation of *B. subtilis* *pyr* gene expression might involve a transcriptional attenuation mechanism, but this proposal was not established experimentally (24, 37).

In the present study, it is shown that the 5' end of the *B. subtilis* *pyr* gene cluster encodes mRNA that has the potential to form three sets of mutually exclusive antiterminator/terminator structures. These structures are located, respectively, in the 5' untranslated leader region following the start of transcription, in a 173-nucleotide (nt) intercistronic region between the first (*pyrR*) and second (*pyrP*) open reading frames, and in a 145-nt intercistronic region between the second and

third (*pyrB*) open reading frames. It is proposed that transcriptional attenuation in each region is regulated by a protein-mediated equilibrium between termination and antitermination, as has been shown for the *B. subtilis* *trp* operon (4, 20, 32, 43). Evidence that the first open reading frame, *pyrR*, encodes a regulatory protein that strongly affects *pyr* expression is presented. We suggest that this protein may modulate transcriptional attenuation at the first of the antiterminator/terminator pairs, and probably at the other two as well. Remarkably, the product of this open reading frame also possesses uracil phosphoribosyltransferase activity. The product of the second open reading frame, *pyrP*, has been tentatively identified as a membrane-bound uracil permease, functionally equivalent to the *uraA*-encoded protein from *E. coli* (1).

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. When pQS183 (36) and pQS28 (36) were propagated in *E. coli*, the low-copy, *pcnB* host KE94 was used because of toxicity of *pyrP* (37).

Luria-Bertani medium (27) was used as a rich liquid medium for both *E. coli* and *B. subtilis* and as a solid medium (1.5% agar) for *E. coli*. Tryptose blood agar base (Difco) was used as a rich solid medium for *B. subtilis*. For chemically defined media, Vogel-Bonner E medium (49) containing 0.3% glucose was used for *E. coli* and buffered minimal medium (6) containing 0.3% glucose was used for *B. subtilis*. Supplements to minimal medium to meet auxotrophic requirements included amino acids (50 µg/ml), pyrimidines (uracil or uridine, 50 µg/ml), adenine (50 µg/ml), and thiamine (10 µg/ml). To grow

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TABLE 1. Strains used in this study

Strain	Genotype or phenotype	Source (reference)
<i>B. subtilis</i>		
DB104	<i>his nprR2 nprE18 ΔaprA3</i>	Laboratory stock (18)
DB104Δ <i>pyrR</i>	HC11 <i>pyrB</i> ⁺ Spec ^s Δ <i>pyrR</i>	This study
DB104 <i>pyrR</i> ⁺	HC11 <i>pyrB</i> ⁺ Spec ^s <i>pyrR</i> ⁺	This study
ED290	<i>purH1 trpC2 hisA1 furA2 spoIIG279</i>	P. Nygaard (31)
HC11	DB104 <i>pyrB</i> ::Spec ^r	P. Hu (16)
HH204	<i>trpC2 upp-11 (upp::cat)</i>	H. H. Saxild (41)
JH862	<i>trpC2</i>	Laboratory stock
MO199	<i>trpC2 pheA amyE::erm</i>	J. Grandoni (12)
MO1991	MO199 <i>amyE</i> ::pTS290	This study ^a
MO1993	MO199 <i>amyE</i> ::pTS290B	This study
MO1994	MO199 <i>amyE</i> ::pTS290C	This study
MO200	MO199 <i>amyE</i> ::pDH32	This study
MO204	MO199 <i>amyE</i> ::pLS44	This study
MO207	MO199 <i>amyE</i> ::pLS47	This study
<i>E. coli</i>		
JM83	<i>ara Δ(lac-proAB) rpsL thi φ80 lacZΔM15</i>	Laboratory stock (52)
KE94	<i>hsdS20(r_B⁻m_B⁻) recA13 ara-14 proA2 lacYI galK2 rpsL20 xyl-5 mtl-1 supE44 pcnB80 zad::Tn10</i>	Laboratory stock
SØ408	<i>relA1 rpsL254 metB1 upp-11</i>	CGSC ^b (15)

^a Details on the construction of plasmids are given in Materials and Methods.

^b CGSC, B. J. Bachmann, *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn.

B. subtilis in the presence of excess pyrimidines, 200 μg of uracil was used per ml, unless otherwise indicated. To starve a *B. subtilis pyr* auxotroph for pyrimidines, 5 μg of uridine plus 100 μg of orotate were used per ml. For a transition from a rich solid medium to a chemically defined liquid medium for *B. subtilis*, a starter culture of Stansly broth (45) was used. *E. coli* pUC- and pBR-based plasmids were selected for with 50 μg of ampicillin per ml.

Bacterial transformations. Transformation of *E. coli* was carried out as described by Sambrook et al. (39). *B. subtilis* transformations were carried out according to the procedure of Contente and Dubnau (7).

DNA isolation, manipulations, and analysis. Plasmid DNA was isolated as described by Sambrook et al. (39). *B. subtilis* chromosomal DNA was isolated by the protocol of Wilson (51), except that lysozyme (1.3 mg/ml) and additional EDTA (35 mM) were added, the incubation was performed for 5 min at 65°C, and three phenol-chloroform-isoamyl alcohol (25:24:1) extractions were done. All enzymes used in the manipulation of DNA were used according to the manufacturer's specifications. DNA was sequenced by the dideoxynucleotide method of Sanger et al. (40), with Sequenase v.2.0 (United States Biochemical Corp.). PCR was performed with *Taq* DNA polymerase in a Perkin-Elmer Cetus DNA thermal cycler.

Plasmids. Plasmids used in this study are listed in Table 2. Further details on the construction of novel plasmids are given below.

Promoter-indicator plasmid constructions. The promoter-indicator plasmids were made by subcloning various fragments, depicted in Table 3, into the *B. subtilis* vector pWP18 (50). This plasmid, which replicates only in gram-positive bacteria and confers resistance to 12 μg of kanamycin per ml, contains a promoterless subtilisin gene (*aprE*) preceded by a multiple cloning site. All of the constructions listed in Table 3 contained the *B. subtilis pyr* promoter and various flanking sequences. These plasmids were used to transform *B. subtilis* DB104, a strain deficient in the two predominant extracellular proteases (18), and were tested for pyrimidine-regulated subtilisin expression.

The *EcoRI* site in pTS182 was generated by PCR and is the

source of this restriction site in all of the indicated plasmids. The nucleotide designation -51 represents the number of the first nucleotide, relative to +1 designating the start of transcription, upstream of the promoter that is homologous to *pyr* DNA; it does not represent the point of cleavage of the *EcoRI* site, which is 2 additional nucleotides upstream. pQS183 (36) was used as the template; oligonucleotide primers, a mutagenic 32-mer starting at -59 (ATCGAATTCCTCAGAAAAACG GTTGACAGAG) and another 32-mer starting complementary to +230 (CGTTCGATCATTTCTGTGAGCAATCCTG GTCAG), were designed to amplify the leader region. The resultant 0.29-kb fragment was treated with the Klenow fragment of *E. coli* DNA polymerase and then digested with *EcoRI* and cloned into pUC19 (30), which had been digested with *EcoRI* and *SmaI*, generating pUC19/290. The sequence of the PCR-amplified region in pUC19/290 was verified by DNA sequencing. The *EcoRI*-*BamHI* fragment was then subcloned into similarly digested pWP18, generating pTS182.

pTS182 was used to produce both pTS183 and pTS184. pTS183 was constructed by replacing the 0.17-kb *BssHII*-*PstI* fragment of pTS182 with the corresponding 1.22-kb fragment from pQS183. pTS184 was constructed by replacing the 0.12-kb *EcoRI*-*BssHII* fragment of pTS182 with the corresponding 0.61-kb fragment from pQS183.

pTS185 was constructed by removing the 0.56-kb *ApaLI*-*PstI* fragment from pTS183, blunt ending the vector with mung bean nuclease, and then recircularizing it with T4 DNA ligase.

***pyr-lacZ* transcriptional fusion integrant constructions.** The plasmids used to produce the *pyr-lacZ* transcriptional fusion integrants were constructed by subcloning various fragments, depicted in Table 4, into the *E. coli* vector pDH32 (13). This vector was designed to permit cloning of *B. subtilis* promoters in transcriptional fusion to a *lacZ* gene. The plasmids are constructed in an *E. coli* host, with 50 μg of ampicillin per ml for selection, because they do not replicate in *B. subtilis*. The plasmid contains the *B. subtilis amyE* 5' half and 3' half flanking a promoterless *lacZ* gene, which is preceded by the restriction sites *EcoRI* and *BamHI* and a *cat* gene. Transcriptional fusions to *lacZ* are constructed by cloning various regions surrounding the promoter into the *EcoRI* and *BamHI*

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pBR322	Ap ^r Tet ^r	39
pLS2	3.1-kb <i>Pst</i> I- <i>Cla</i> I fragment of <i>pyr</i> DNA containing the 3' portion of <i>pyrP</i> through the 5' portion of <i>pyrC</i> in pBR322	23
pQS28	2.8-kb <i>Cla</i> I- <i>Hind</i> III fragment of <i>pyr</i> DNA containing the <i>pyr</i> promoter and upstream region, <i>pyrR</i> , and <i>pyrP</i> in pBR322	36
pDH32	Ap ^r Cm ^r <i>amyE</i> front <i>amyE</i> back <i>lacZ</i>	13
pLS43	0.61-kb <i>Eco</i> RI- <i>Bss</i> HII (Klenow) fragment of pQS18CP, containing the <i>pyr</i> promoter and upstream region, in the <i>Eco</i> RI- <i>Bam</i> HI (Klenow) sites of pDH32	This study
pLS44	0.24-kb <i>Eco</i> RI- <i>Bam</i> HI fragment of pLS34 in pDH32	This study
pLS47	0.13-kb <i>Eco</i> RI- <i>Bam</i> HI fragment of pLS372 in pDH32	This study
pTS290	0.28-kb <i>Eco</i> RI- <i>Bam</i> HI fragment of pUC19/290 in pDH32	This study
pTS290B	0.25-kb <i>Eco</i> RI- <i>Bam</i> HI fragment of pUC19/290B in pDH32	This study
pTS290C	0.22-kb <i>Eco</i> RI- <i>Bam</i> HI fragment of pUC19/290C in pDH32	This study
pEB112	Ap ^r Kn ^r Phl ^r P _{lac} <i>E. coli</i> - <i>B. subtilis</i> shuttle vector	22
pTS1121A	1.29-kb <i>Xmn</i> I- <i>Pst</i> I fragment of pTS183 containing the leader region through the 5' portion of <i>pyrP</i> , in the <i>Sma</i> I- <i>Pst</i> I sites of pEB112	This study
pTS112Δ1A	1.29-kb <i>Xmn</i> I- <i>Pst</i> I fragment of pQS183Δ1A in the <i>Sma</i> I- <i>Pst</i> I sites of pEB112	This study
pTS1121A2	0.85-kb <i>Xmn</i> I- <i>Bam</i> HI fragment of pQS800, containing the leader region and <i>pyrR</i> , in the <i>Sma</i> I- <i>Bam</i> HI sites of pEB112	This study
pTS1121A2stp	0.85-kb <i>Xmn</i> I- <i>Bam</i> HI fragment of pQS8001Astp in the <i>Sma</i> I- <i>Bam</i> HI sites of pEB112	This study
pTS1121A1B	0.93-kb <i>Pst</i> I- <i>Hind</i> III fragment of pTS112PH in pTS112Δ1A, adding in the 3' portion of <i>pyrP</i>	This study
pTS1121AYB	1.09-kb <i>Hind</i> III- <i>Hind</i> III fragment of pLS2 containing <i>pyrB</i> and the 5' portion of <i>pyrC</i> in the appropriate orientation in pTS1121A1B (see Fig. 7)	This study
pTS112PH	0.93-kb <i>Pst</i> I- <i>Hind</i> III fragment of <i>pyr</i> DNA containing a 3' portion of <i>pyrP</i> in pEB112	This study
pTS1BOX	0.45-kb <i>Hinc</i> II- <i>Pst</i> I fragment of <i>pyr</i> DNA, containing a 5' portion of <i>pyrP</i> , in the <i>Sma</i> I- <i>Pst</i> I sites of pTS112PH	This study
pTZ18U and pTZ19U	Ap ^r P _{T7}	26
pLS33	0.61-kb <i>Eco</i> RI- <i>Bam</i> HI fragment from pLS43 in pTZ18U	This study
pLS34	pLS33 with a 0.41-kb <i>Eco</i> RI (Klenow)- <i>Msc</i> I deletion, removing much of the region upstream of the <i>pyr</i> promoter	This study
pLS372	pYSP01-1 with a 0.16-kb <i>Bss</i> HII (Klenow)- <i>Bam</i> HI (Klenow) deletion, removing the leader terminator and the 5' portion of <i>pyrR</i>	This study
pYSP01	pYSP01-1 with a 0.19-kb <i>Bss</i> HII (Klenow)- <i>Hind</i> III deletion, removing the leader terminator and the 5' portion of <i>pyrR</i>	This study
pYSP01-1	0.32-kb <i>Eco</i> RI- <i>Hind</i> III fragment from pUC19/290 in pTZ19U	This study
pYSP04	0.45-kb <i>Sma</i> I- <i>Sst</i> I fragment containing part of <i>pyrB</i> in pTZ19U	This study
pYSP05	0.45-kb <i>Pst</i> I- <i>Hinc</i> II fragment containing part of <i>pyrP</i> in pTZ19U	This study
pUC18 and pUC19	Ap ^r	30
pQS183	1.8-kb <i>Cla</i> I- <i>Pst</i> I fragment of <i>pyr</i> DNA containing the <i>pyr</i> promoter and upstream region, <i>pyrR</i> , and a 5' portion of <i>pyrP</i> in the <i>Acc</i> I- <i>Pst</i> I sites of pUC18	36
pQS183Δ1A	pQS183 with a 0.15-kb <i>Acc</i> I (mung bean nuclease) deletion in <i>pyrR</i>	This study
pQS800	0.88-kb <i>Hinc</i> II fragment in the <i>Hinc</i> II site of pUC18 containing the <i>pyr</i> promoter and <i>pyrR</i> in the opposite orientation of transcription from P _{lac}	36
pQS8001Astp	pQS800 with a stop codon at codon 4 in <i>pyrR</i> (see Materials and Methods)	This study
pUC19/290	0.28-kb fragment of <i>pyr</i> DNA containing the <i>pyr</i> promoter and leader region and a 5' portion of <i>pyrR</i> (see Materials and Methods)	This study
pUC19/290B	pUC19/290 with a 0.030-kb deletion within the leader region (see Materials and Methods)	This study
pUC19/290C	pUC19/290 with a 0.064-kb <i>Xmn</i> I- <i>Bss</i> HII (Klenow) deletion within the leader region	This study
pWP18	Kn ^r <i>aprE</i>	50
pQS18CP	1.8-kb <i>Cla</i> I- <i>Pst</i> I fragment of <i>pyr</i> DNA containing the <i>pyr</i> promoter and upstream region, <i>pyrR</i> , and a 5' portion of <i>pyrP</i> in the <i>Acc</i> I- <i>Pst</i> I sites of pWP18	37
pTS182	0.29-kb <i>Eco</i> RI- <i>Bam</i> HI fragment of pUC19/290 in pWP18	This study
pTS183	pT182 with the 0.17-kb <i>Bss</i> HII- <i>Pst</i> I fragment replaced by the corresponding 1.22-kb fragment from pQS183	This study
pTS184	pT182 with the 0.12-kb <i>Eco</i> RI- <i>Bss</i> HII fragment replaced by the corresponding 0.61-kb fragment from pQS183	This study
pTS185	pTS183 with a 0.56-kb <i>Apa</i> LI (Klenow)- <i>Pst</i> I (Klenow) deletion to remove the first intercistronic region and the 5' portion of <i>pyrP</i>	This study
pTS186	pTS183 with a 0.030-kb deletion in the leader region (see Materials and Methods)	This study
pTS1BOX3	pTS183 with the 0.50-kb <i>Pst</i> I- <i>Hind</i> III fragment replaced by the corresponding 0.93-kb fragment from pTS112PH	This study

sites. Upon transformation of linearized plasmid (with *ScaI* and *PstI*) into a *B. subtilis* host, the *amyE* front and back flanking regions permit homologous recombination of linearized plasmid into the amylase locus on the *B. subtilis* chromosome, with 10 µg of chloramphenicol per ml for selection. *B. subtilis* MO199 contains an *amyE* gene that carries a macro-lide-lincosamide-streptogramin resistance (MLS^r) gene insertion, so that integration at *amyE* deletes this gene and could be checked by testing for an MLS^r phenotype with 1 µg of erythromycin and 20 µg of lincomycin per ml (12).

pTS290 was constructed by placing the 0.28-kb *EcoRI*-*Bam*HI fragment of pUC19/290 directly into pDH32.

pTS290B was produced by PCR to construct a new *Bss*HII site at +42, with pTS183 as the template. A 0.11-kb fragment from -59, including the *EcoRI* site, to +53 was amplified by PCR with the 5' primer ATCGAATTCCTCAGAAAAACGGTTGACAGAG and the 3' mutagenic primer CTATCCGCGCGCCTTCTCAG. The product was then digested with *EcoRI* and *Bss*HII and ligated to the vector of similarly digested pTS183 to generate pTS186. The fragment was verified by DNA sequencing, and then the 0.095-kb *EcoRI*-*Bss*HII fragment was transferred to similarly digested pUC19/290 to generate pUC19/290B. The 0.25-kb *EcoRI*-*Bam*HI fragment was then removed from pUC19/290B and placed into similarly digested pDH32, yielding pTS290B.

pTS290C was constructed by partial digestion of pUC19/290 with *XmnI* to digest only the *XmnI* site at +8 and not the alternate site within the *bla* gene within the vector. The plasmid was then digested with *Bss*HII. The *Bss*HII site was blunt ended with Klenow fragment of DNA polymerase, and the vector was recircularized with T4 DNA ligase, creating pUC19/290C. The DNA sequence at the splicing junction of this plasmid was verified by DNA sequencing. The 0.22-kb fragment of *EcoRI*-*Bam*HI-digested pUC19/290C was then placed into pDH32, generating pTS290C.

pLS44 was constructed through several intermediates. First, pQS18CP (37) was digested with *Bss*HII and blunt ended with Klenow fragment of DNA polymerase. The 0.61-kb fragment was removed by digestion with *EcoRI*, ligated into pDH32 that had been digested with *Bam*HI, blunt ended with Klenow fragment of DNA polymerase, and then digested with *EcoRI*. This generated pLS43, which was then used to create pLS33 by removing the 0.61-kb *EcoRI*-*Bam*HI fragment and placing it into similarly digested pTZ18U (26). This was then used to construct pLS34 by removing the 0.41-kb *EcoRI*-*MscI* fragment, blunt ending the vector with Klenow fragment of DNA polymerase, and religating the vector. The final product, pLS44, was then constructed by removing the 0.24-kb *EcoRI*-*Bam*HI fragment from pLS34 and ligating it into similarly digested pDH32.

pLS47 was also constructed through several intermediates. The 0.32-kb *EcoRI*-*Hind*III fragment of pUC19/290 was ligated into similarly digested pTZ19U (26) to create pYSP01-1. This was then digested with *Bss*HII and *Bam*HI, and the vector was then treated with Klenow fragment of DNA polymerase. The blunt-ended vector was then recircularized with T4 DNA ligase to yield pLS372. The 0.13-kb *EcoRI*-*Bam*HI fragment was then placed into similarly digested pDH32, yielding pLS47.

In-frame deletion of *pyrR*. The in-frame deletion of *pyrR* was accomplished as described in Fig. 7. Plasmid pTS1121AYB was constructed in multiple steps. pQS183 was digested with *AccI* to remove the 0.15-kb insert. The vector was then blunt ended with mung bean nuclease and recircularized by use of T4 DNA ligase, generating the plasmid pQS183Δ1A. The DNA sequence at the splicing junction of this plasmid was confirmed by DNA sequencing. The 1.29-kb *XmnI*-*PstI* fragment was then

subcloned into *SmaI*-*PstI*-digested pEB112 (22), producing pTS112Δ1A. The 0.93-kb *PstI*-*Hind*III fragment of pTS112PH, which contained the same *PstI*-*Hind*III fragment of pQS28 in the vector pEB112, was then placed into similarly digested pTS112Δ1A to yield pTS1121A1B. The 1.09-kb *Hind*III-*Hind*III fragment of pLS2 (23) was then placed into *Hind*III-digested, calf intestine alkaline phosphatase-treated pTS1121A1B to yield pTS1121AYB.

Double recombination was selected for by conversion to *pyr* prototrophy. Candidates were then checked for sensitivity to 100 µg of spectinomycin per ml. Chromosomal DNA of chosen candidates was then isolated and subjected to PCR analysis, with the oligonucleotide primers (-306)GCTTGAGCGTTTTCAGG(-290) and (783)GCATACAAAGACCTGCC(767) to amplify the chromosomal DNA to confirm the presence of the deletion.

Complementation of fluorouracil-resistant mutants. Complementation of fluorouracil-resistant mutants was performed as described in Table 5. pTS185 and pTS112Δ1A have already been described. pTS1BOX3 was constructed by partial *Hind*III digestion of pTS183, followed by digestion with *PstI* to remove the 0.50-kb fragment inclusive of the 5' end of *aprE*, leaving the 3' 0.80-kb *Hind*III-*Hind*III DNA. The 0.93-kb *PstI*-*Hind*III fragment of pTS112PH was then ligated into the digested vector, yielding pTS1BOX3. pTS1121A2 was constructed by ligating the 0.85-kb *XmnI*-*Bam*HI fragment of pQS800 into *SmaI*-*Bam*HI-digested pEB112. pTS1BOX was constructed by ligating the 0.45-kb *HincII*-*PstI* fragment of pTS183 into *SmaI*-*PstI*-digested pTS112PH. pTS1121A2stp contains a stop codon, introduced by PCR, into codon 4 of *PyrR* and was constructed by multiple steps. PCR was first used to introduce the stop codon (changing codon 4 from AAA to UAA) into pQS800, creating pQS8001Astp. A 0.26-kb fragment, from base 369 in the pUC18 vector to +166 in the *pyrR* reading frame, was amplified with the vector primer GTTTTCCCA GTCACGAC and the mutagenic primer CAGCTTATTGATT CAATGTG. The product of this PCR reaction was then used as a primer in the subsequent PCR reaction, along with the primer CAGGAAACAGCTATGAC, whose 5' end is complementary to base 467 in the pUC18 vector, yielding a 1.00-kb product. The product was then digested with *EcoRI* and *Hind*III and ligated into the vector of similarly digested pQS800. The mutation was confirmed by DNA sequencing, and the lack of overexpressed *PyrR* was verified on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 0.85-kb *XmnI*-*Bam*HI fragment of pQS8001Astp was then placed into *SmaI*-*Bam*HI-digested pEB112, generating pTS1121A2stp. pTS1121A was constructed by placing the 1.29-kb *XmnI*-*PstI* fragment of pTS183 into *SmaI*-*PstI*-digested pEB112.

Computer analyses. Nucleotide and amino acid sequence analyses were performed with the University of Wisconsin Genetics Computer Group software packages (9) on the VAX computer (Digital Equipment Co.). RNA secondary structure predictions were made with the FOLD program of Zuker and Stiegler (53). Alignment of deduced amino acid sequences was carried out with the BESTFIT program, which employs the local homology algorithm of Smith and Waterman.

Northern (RNA) blot analysis of *pyr* transcripts. *B. subtilis* RNA was harvested from strain JH862 grown in buffered minimum medium, with or without 0.2 mg of uridine per ml as the exogenous pyrimidine source, to a density of approximately 70 Klett units. Twenty milliliters of total culture was mixed, on ice, with 10 ml of frozen buffered minimal medium containing 15 mM sodium azide. The cells were harvested by centrifugation (15 min, 20,000 × g, 4°C), the supernatant was decanted,

and the cell pellet was frozen in liquid nitrogen for 5 min. The pellet was thawed by addition of 2.5 ml of protoplasting buffer (15 mM Tris-HCl [pH 8.0], 6 mM EDTA, 0.45 M sucrose, 2 mg of lysozyme per ml) (34) at room temperature. The RNA was then isolated by hot phenol extraction (43). The resulting RNA pellet was dissolved in H₂O and stored at -80°C.

[α -³²P]ATP-labeled RNA probes were produced by runoff transcription (as described by Post and Switzer [34]) of the appropriate segments of DNA placed in the polylinker of pTZ19U, behind the T7 promoter. The probe 1 plasmid (pYSP01), which contains *pyr* DNA nucleotides +75 to -51, was constructed from pYSP01-1 (described above) by removing the 0.19-kb *Bss*HII-*Hind*III fragment, blunt ending the vector with Klenow fragment of *E. coli* DNA polymerase, and ligation with T4 DNA ligase. The probe 2 plasmid (pYSP05), which contains *pyr* DNA nucleotides +1296 to +845, was constructed by placement of the 0.45-kb *Pst*I-*Hinc*II fragment into similarly digested pTZ19U. The probe 3 plasmid (pYSP04), which contains *pyr* DNA nucleotides +2842 to +2393, was constructed by placing the 0.45-kb *Sma*I-*Sst*I fragment into similarly digested pTZ19U. Plasmids were linearized by digestion with the appropriate restriction enzyme and transcribed with T7 RNA polymerase. The restriction enzymes used to linearize pYSP01, pYSP05, and pYSP04 were *Xmn*I, *Sau*3AI, and *Eco*RI, and their sites of cleavage were nt +8 and +1108 and in the vector, respectively.

Fifteen micrograms of total cellular RNA was electrophoresed, vacuum blotted, and hybridized with the radiolabeled probes as described by Post et al. (34), except that hybridization was carried out in a Mini Oven MK II (National Labnet Co.). After hybridization with the appropriate probe, the membrane was exposed to X-ray film for periods ranging from 15 min to overnight. *E. coli* 23S, 16S, and 5S ribosomal RNA (3.7, 1.7, and 0.12 knt, respectively) served as size standards.

Enzyme assays. Unless otherwise noted, 40 ml of cell culture was harvested at approximately 100 Klett units by centrifugation (14,000 $\times$ g, 4°C), and the cell pellet was resuspended in 5.5 ml of the appropriate assay buffer. The cells were then sonicated, on ice, with 3 20-s pulsed blasts with a Heat Systems (Ultrasonics Inc.) cell disruptor, model W-375, set at 50% duty and control output level 5, with the microtip. Cell debris was removed by centrifugation (6,000 $\times$ g, 4°C).

Subtilisin activity was determined by assay with the substrate carbobenzoxy-L-alanyl-L-alanyl-L-leucine *p*-nitroanilide (Peptides International, Louisville, Ky.) according to Sheehan's (42) modification of the Stepanov protocol (46). Cells were grown in buffered minimal medium, with or without 0.2 mg of uracil per ml as the exogenous pyrimidine source, to a density of approximately 100 Klett units, and the extracellular culture fluid was harvested and assayed. The assay mix, 1 ml total volume, contained 300 μ l of 100 mM Tris-HCl-4 mM CaCl₂ (pH 8.5), substrate (125 μ l, 0.5 mg/ml in dimethylformamide), and 325 μ l of sample. The reactions were run for a fixed time of 1.5 h and stopped by the addition of 250 μ l of 1 M sodium citrate, pH 2.0. The A_{410} was read against zero time controls. Specific activity was determined by normalizing the activities to culture density (Klett units).

β -Galactosidase activity was determined by a protocol modified from that of Miller (27). Typically, 5 ml of cell culture was harvested by centrifugation (14,000 $\times$ g, 4°C), and the cell pellet was resuspended in 5 ml of 0.1 M potassium phosphate buffer, pH 7.0. The cell density was measured at A_{600} , and then cells were treated with 1/100 volume of toluene for 10 min at room temperature, with shaking. The resultant permeabilized cells were then assayed for β -galactosidase activity. Samples were added to 0.1 M potassium phosphate buffer, pH 7.0, to total

500 μ l, and each mixture was then added to 500 μ l of the assay mix (0.1 M potassium phosphate buffer [pH 7.0], 0.1 M NaCl, 20 mM MgCl₂, 50 mM β -mercaptoethanol, and 6 mM *o*-nitrophenyl β -D-galactopyranoside) and incubated at 37°C. The reaction was terminated by the addition of 500 μ l of 1 M sodium carbonate. The A_{420} was read against zero time controls. The specific activities were determined in Miller units (27).

Aspartate transcarbamylase (ATCase) activity was determined by the protocol of Bond et al. (6), with 0.4 M Tris-acetate (pH 8.2) and the colorimetric determination of carbamylaspartate described by Shindler and Prescott (44).

Uracil phosphoribosyltransferase activity was determined by the method of Rasmussen et al. (38), except that the assay mix did not contain sodium phosphate, the [2-¹⁴C]uracil was 4 μ Ci/ μ mol, and the reaction was stopped by placement in a boiling water bath for 1 min. A 5- μ l sample of the 50- μ l reaction mixture was spotted onto a polyethylenimine-cellulose plate and chromatographed for 2 cm in methanol and then to the top of the 10-cm plate in water. The UMP spot, which does not migrate, was cut out, placed in 5 ml of scintillation fluid consisting of 75% mixed xylene isomers, 25% Triton X-114, 0.3% 2,5-diphenyloxazole, and 0.02% 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene, and counted in a Beckman LS7000 scintillation counter. A 5- μ l sample of the reaction mixture was also spotted out onto a separate plate, cut out, and counted, to determine the total counts in the reaction for calculation of the percentage of the original uracil converted to UMP. The reactions were blanked with controls that lacked 5-phosphoribosyl-1-pyrophosphate (PRPP) in the reaction mixture.

Protein assays were done with the Coomassie protein assay reagent (Pierce, Rockford, Ill.).

Chemicals and enzymes. [2-¹⁴C]uracil and [α -³²P]ATP were obtained from ICN Biomedicals. Uracil, uridine, PRPP, GTP, 5-fluorouracil, *o*-nitrophenyl β -D-galactopyranoside, kanamycin, erythromycin, lincomycin, and chloramphenicol were acquired from Sigma Chemical Company. Isopropyl- β -D-thiogalactopyranoside (IPTG) and Sequenase v2.0 were purchased from United States Biochemical Corporation. [α -³⁵S]dATP α S was procured from Amersham. Restriction enzymes were purchased from either Gibco BRL or New England Biolabs. *Taq* polymerase was obtained from Perkin-Elmer Cetus and used in accordance with the manufacturer's specifications. Mung bean nuclease, T7 RNA polymerase, and Klenow fragment of DNA polymerase were acquired from Gibco BRL. T4 DNA ligase and lysozyme were purchased from Boehringer Mannheim. Low-molecular-weight protein standards were procured from Bio-Rad. Oligonucleotides were synthesized by the University of Illinois Biotechnology Center with an Applied Biotechnology Systems DNA synthesizer.

Nucleotide sequence accession number. Corrections to the nucleotide sequence of the *B. subtilis pyr* operon have been deposited in the GenBank data base under the accession number M59757.

RESULTS

Identification of the two open reading frames at the 5' end of the *Bacillus subtilis pyr* gene cluster. The previously published sequence of the *Bacillus subtilis pyr* gene cluster predicted the presence of a single long open reading frame, called *orf1*, that would encode a protein with an M_r of 72,396 at the 5' end of the cluster (37). However, the consistent observation of an overproduced protein with an apparent molecular mass of about 26 kDa in *E. coli* cells transformed with plasmids

634
 GTT ATG GTA CAG CTT GAT GAG GTA GAC CAA AAC GAT CTC GTC GCC ATT TAT GAA AAC
 V M V Q L D E V D Q N D L V A I Y E N

GAA TAA TAGATCACCTTTTAAAGGGCAATCCAGAGAGGTTGCAAAGAGGTGCACAACAAGGC
 E ← end of *pyrR*

CCAAAAAGTATTTCGGCAGGCTCTTGTATGCCTCTTTCGCTAAAAAAGCAAAGAGGTTTTTTATA

CAGTCATTGAGTCATCCTAAAATGAAAGTCAACAATCAGGGGGAAATCATC ATG AGT AAG AAA
 start of *pyrP* → M S K K

AAA GTA AAT TTA GGG GTC AGG GAT GTC CCG ACA CCT TTC TCT TGG GTT TCA TTC AGC
 K V N L G V R D V P T P F S W V S F S

CTT CAG CAT TTG TTT GCC ATG TTT GGC TCA ACC ATT TTG GTT CCG AAG CTC GTC GGA
 L Q H L F A M F G S T I L V P K L V G 995

FIG. 1. Corrections to the *B. subtilis* *pyr* gene sequence and their consequences for deduced protein products. The sequence from nt +634 to +995, numbering from the start of transcription (37), are shown. Three nucleotides that were added to the previously published sequence (37) are indicated by an asterisk above each; the location of a G residue deleted from the previous sequence is shown by the G above the sequence. The termination codon for *pyrR* and the initiation codon for *pyrP* are underlined, and the proposed ribosome binding site for *pyrP* is overlined.

bearing the corresponding segment of *B. subtilis* chromosomal DNA led us to reexamine the nucleotide sequence of this region. Four corrections to the published (37) sequence were found (Fig. 1). The consequence of these corrections is that the region previously described as encoding *orf1* was shown to encode two open reading frames, which we call *pyrR* and *pyrP*, that are separated by a 173-nt intercistronic region (Fig. 2).

The amino terminus of the PyrR protein was shown to be the same as that deduced for the product of *orf1* by Quinn et al. (37). That is, *pyrR* translation is initiated at a UUG codon at nt

151, which is appropriately placed downstream from a good ribosome binding sequence (14, 37). This was documented by analysis of PyrR overproduced in *E. coli* JM83 cells transformed with plasmid pQS800, which contains a *HincII* fragment of *B. subtilis* DNA (nt -38 to +845) encoding all of *pyrR* in a pUC18 vector (36). These cells produced large amounts of a protein that migrated on SDS-PAGE at a position corresponding to a mass of about 26 kDa (Fig. 3, lane 8). Extracts from these cells were subjected to SDS-PAGE, and the entire gel was blotted onto a polyvinylidene difluoride membrane as described by Matsudaira (25). The membrane was then stained with Coomassie brilliant blue R-250 (Sigma), and the band was cut out and subjected to automated gas-phase microsequence analysis by the University of Illinois Biotechnology center in an Applied Biosystems 470A gas-phase sequencer. The sequence determined, MNQKAVILDE, matched exactly the sequence predicted by the above initiation codon. PyrR is predicted from the nucleotide sequence to be terminated at tandem translation stop codons (UAA and UAG in Fig. 1), leading to a 20.2-kDa polypeptide. This value is significantly smaller than that observed for PyrR on SDS-PAGE when overexpressed in either *E. coli* or *B. subtilis* (Fig. 3, lanes 4, 5, and 8). We do not have a satisfactory explanation for this discrepancy, but it is noteworthy that a similar difference between predicted and observed *M_s* for the corresponding *pyrR*-encoded protein from *Bacillus caldolyticus* was observed by Ghim and Neuhaud (10).

The sequence encoding *pyrP* is predicted to begin at an AUG codon (nt +870), which is also appropriately spaced (11 bases) downstream from a potential ribosome binding sequence ($\Delta G = -14.2$ kcal/mol [48]) (Fig. 1). All of the amino acid sequence predicted for PyrP is the same as that originally deduced for the corresponding region of Orf1 by Quinn et al. (37), except for codons 20 through 35 (Fig. 1). The molecular mass predicted for PyrP is 45.6 kDa. Attempts to overexpress PyrP in *E. coli* or *B. subtilis* with plasmids bearing the corresponding *B. subtilis* DNA segments were unsuccessful. In fact, it was evident that overexpression of PyrP was toxic to both *E. coli* and *B. subtilis* cells. In *E. coli*, this was shown with plasmid

FIG. 2. Schematic representation of the *B. subtilis* *pyr* gene cluster. The entire cluster is shown at the top of the figure; the 3-kb region at the 5' end is expanded at the bottom to reveal details. Dark bars represent open reading frames. Numbers indicate the length of the DNA in kilobases. The arrow at 0 kb represents the start of transcription. Locations of proposed antiterminator/terminator structures are indicated by A/T. The hatched lines labeled P1, P2, and P3 indicate the regions corresponding to the single-stranded RNA probes designed to hybridize to *pyr* transcripts in the Northern blot analysis shown in Fig. 6.

FIG. 3. Expression of the *pyrR*-encoded and *pyrP*-encoded proteins in *B. subtilis* and *E. coli*. SDS-PAGE was performed by the method of Laemmli (21). Lane 6 contains molecular weight standards of the sizes shown, in thousands, on the left-hand side of the figure. All other lanes contain equivalent amounts of extract from cells that were harvested at 100 Klett units or after the cessation of growth, as indicated, if this culture density was not reached. Overexpression of PyrR and PyrP in *B. subtilis* was attempted by starving the cells for pyrimidines. Plasmid pTS1BOX3, which contains the *pyrR* and *pyrP* genes under the control of the *pyr* promoter and their own regulatory elements, was placed in the *pyr* auxotroph HC11 and starved for pyrimidines by growth in minimal medium and addition of only 5 μ g of uridine per ml and 0.1 mg of orotate, which is very slowly utilized, per ml. Lanes 1 and 2, *B. subtilis* HC11 bearing the plasmid pWP18 (vector only) grown with excess uracil (0.2 mg/ml) and starvation conditions, respectively; lanes 3 to 5, *B. subtilis* HC11 bearing the plasmid pTS1BOX3, grown in minimal medium with excess uracil (lane 3) and starvation conditions (lanes 4 and 5); lane 4, cell extract from cells that were harvested immediately after the cessation of growth; lane 5, cell extract from cells which were harvested 1 h later. The arrow at the right-hand side of the figure points to overexpressed PyrR, which migrates at 26 kDa. PyrP could not be detected. PyrR was overexpressed in *E. coli* by growing JM83 cells bearing pQS800, which encodes *pyrR*, in Vogel-Bonner E medium (lane 8). Lane 7, the vector (pUC18) control.

pTS1BOX, which contained a *HincII*-*HindIII* fragment of *B. subtilis* DNA (nt +845 to +2225) encoding all of PyrP together with its putative ribosome binding sequence under the control of the inducible *tac* promoter in the pEB112 shuttle vector (22). Induction of PyrP expression in *E. coli* cells bearing pTS1BOX by addition of even low levels of IPTG (0.02 mM) led to the immediate cessation of growth without obvious accumulation of a protein with a molecular mass of about 46 kDa on SDS-PAGE (data not shown). In *B. subtilis* cells bearing the plasmid pTS1BOX3, which contained both *pyrR* and *pyrP* under the control of the *B. subtilis pyr* promoter (nt -51 to +2225), overexpression of PyrR was readily observed on SDS-PAGE in *B. subtilis* DB104 grown in minimal medium in the absence of exogenous pyrimidines, but PyrP was not (data not shown). When the plasmid was placed in the *pyr* auxotroph HC11 and additional overexpression was attempted by starving the cells for pyrimidines, PyrR was once again easily observed, but PyrP was not (Fig. 3, lanes 4 and 5). Starvation of cells bearing plasmid pTS1BOX3 also resulted in a much earlier cessation of growth relative to those bearing the vector alone, indicating PyrP toxicity in *B. subtilis* as well.

The 5' end of the *B. subtilis pyr* mRNA is predicted to be capable of forming three pairs of mutually exclusive stem-loop structures in untranslated regions. Nucleotide sequence features characteristic of rho-independent transcription termina-

tors in the *B. subtilis pyr* mRNA, which were located in the 5' untranslated leader region and in the *pyrP-pyrB* intercistronic region, were previously pointed out by Lerner and Switzer (24) and Quinn et al. (37). A more thorough analysis of possible secondary structures in *pyr* mRNA in these regions and in the *pyrR-pyrP* intercistronic region was carried out with a computer program for RNA folding predictions (53). Three strikingly similar pairs of mutually exclusive stem-loop structures were identified, one in each segment (Fig. 4). In each case, a promoter-proximal stem-loop structure (antiterminator) that is predicted to be more stable than a promoter-distal, transcription terminator stem-loop can form. Formation of the antiterminator stem-loop would prevent formation of the terminator structure because the downstream segment of the antiterminator stem would also be required to form the upstream segment of the terminator stem (Fig. 4). Also striking is the presence of conserved A+G-rich sequences at the 5' end of the upstream stem of each of the antiterminators (Fig. 4 and 5). The location of these conserved segments is such that, in each case, the binding of a regulatory factor to these segments would tend to destabilize the antiterminator structure and displace its downstream stem, thereby favoring formation of the terminator structure. The presence of these elements in the *B. subtilis pyr* mRNA clearly provides the potential for regulation of *pyr* gene expression by a transcriptional attenuation mechanism analogous to that described for the *B. subtilis trp* operon (4, 20, 32, 43) (see Discussion). Experimental evidence supporting this hypothesis and identifying *pyrR* as the gene encoding the regulatory protein is presented in the following sections.

Analysis of *B. subtilis pyr* mRNA by Northern hybridization. If the three transcription terminators identified from the preceding analysis of *pyr* mRNA sequences actually function in vivo, it could be expected that *pyr* transcripts of the predicted sizes (approximately 0.12, 0.81, and 2.28 kilonucleotides [knt] plus the full-length 11.88-knt *pyr* transcript) would be detectable on Northern hybridization analysis. RNA was extracted from *B. subtilis* cells that had been grown on minimal medium with or without 0.2 mg of uridine per ml, separated by agarose gel electrophoresis, and subjected to Northern blot hybridization with three different single-stranded radioactive RNA probes, which were designed to hybridize specifically to the predicted transcripts (Fig. 2). The hybridization clearly detected four distinct RNA species, whose lengths estimated from a standard curve (0.12, 0.65, 2.3, and 12 knt) matched very well to the sizes of the predicted *pyr* transcripts, although the size of the longest transcript was difficult to determine with the rRNA standards (3.7, 1.7, and 0.12 knt) employed (Fig. 6). The three longer transcripts were reduced in abundance or were undetectable when RNA from cells grown in the presence of excess uridine was probed. This observation indicates that transcription termination is significantly affected by uridine in the medium and supports the identification of the bands as *pyr* RNA. Termination of *pyr* transcripts at the terminator in the *pyrP-pyrB* intercistronic region was also previously shown to occur in vivo by Quinn et al. (37). Further attempts to obtain quantitative estimates of the abundance of the various *pyr* transcripts by Northern hybridization gave variable results, which suggested that the RNA species being analyzed were unstable. For this reason, we chose to characterize the regulation of *pyr* transcription further by use of plasmids and chromosomal integrants in which various portions of the 5' end of the *pyr* cluster were transcriptionally fused to the genes of indicator enzymes, and we analyzed expression in vivo.

Expression of *pyr-aprE* fusion plasmids in *B. subtilis* cells. Initial characterization of the role of the 5' end of the *pyr*

FIG. 4. Proposed attenuation structures for all three untranslated regions of the *B. subtilis* *pyr* operon. The antiterminator and terminator structures are labeled with their calculated free energies of formation. The nucleotides outlined by the dashed lines identify the 50-base consensus sequence present in all three antiterminators. The nucleotides outlined by the solid lines highlight the regions of overlap between the corresponding antiterminator and terminator structures.

cluster in regulation of *pyr* expression by exogenous pyrimidines was conducted with the pWP18-pWP19 promoter-indicator plasmid system previously described by Wang (50) and used by Quinn et al. (37) to identify the *pyr* promoter. Segments of *B. subtilis* DNA surrounding the *pyr* promoter were inserted into pWP18, which contains a multiple cloning site upstream from a promoterless *aprE* (subtilisin) gene. *B. subtilis* DB104 ($\Delta aprA3$ *nprE18* *nprR2*) cells transformed with such plasmids excrete subtilisin into the culture medium, which can then be analyzed with a simple colorimetric assay (46). The structures of the plasmids tested and the levels of subtilisin produced by cells grown with and without 0.2 mg of uracil in the medium are shown in Table 3. As shown previously by Quinn et al. (37), the sequences around the *pyr* promoter from -538 to +1296 conferred pyrimidine-regulatable expression of subtilisin (pQS18CP). Sequences 5' to nt -51 from the start of transcription appear to have little effect on regulation by pyrimidines (pTS183 versus pQS18CP and pTS182 versus

Region	Sequence
Leader	8 AGAUUCUUUAAAACAGUCCAGAGAGGCUGAGAAGGAUAACGGAUAGACGG
<i>pyrR</i> - <i>pyrP</i> intercistronic	703 ACCUUUUUUAAAGGCCAAUCCAGAGAGGUUGCAAAGAGGUGCACAACAAGG
<i>pyrP</i> - <i>pyrB</i> intercistronic	2175 AACCUUUUUAUAGAAAGUCCAGAGAGGCUUGGAAGGGUUUAUGAAGAGAAGG
Consensus	A - - YUYUU - A - RR - ARUCCAGAGAGGYU - - RAAGR - - RYR - A - - RA - GG

FIG. 5. Consensus sequence for the proposed PyrR-binding site. The sequences represented by the dashed lines in Fig. 4 are shown here, giving the resulting consensus sequence shown at the bottom. R represents conserved purine nucleotides, and Y represents conserved pyrimidine nucleotides.

FIG. 6. Northern blot analysis of *B. subtilis* *pyr* mRNA. The probe designations (as given in Fig. 2) are given above the relevant lanes. -U and +U lane designations represent lanes where RNA was harvested from cells grown in the absence and presence of 0.2 mg of uridine per ml, respectively. The positions of the proposed *pyr* transcripts, as well as their nucleotide lengths, as determined by a standard curve of *E. coli* 23S, 16S, and 5S rRNA, are indicated by arrows at the right. Arrows at the left side point out the positions of the 16S and 23S rRNA, to which probes P1 and P2 hybridize nonspecifically. No nonspecific hybridization to 5S rRNA was detected with probes 2 and 3, but nonspecific hybridization to probe 1 may be masked by specific hybridization to the smallest transcript.

TABLE 3. Expression of multicopy *pyr-aprE* fusion plasmids in *B. subtilis*

		Subtilisin Activity ($\Delta A_{410} \cdot \text{ml}^{-1} \cdot 100 \text{KU}^{-1}$) ^a			ATCase Activity ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)		
		-Uracil	+Uracil	-/+ ^b	-Uracil	+Uracil	-/+
Host Strain DB104							
Plasmid							
pWP18		(1.5) ^c	(1.4)	-	90	15	5.8
pQS18CP		1.1	<0.1	>11	nd ^d	nd	-
pTS182		6.2	5.3	1.2	419	450	0.9
pTS183		2.2	0.3	7.2	nd	nd	-
pTS184		5.4	5.0	1.1	nd	nd	-
pTS185		9.2	1.9	4.9	131	14	9.3
Host Strain DB104 Δ <i>pyrR</i>							
Plasmid							
pWP18		nd	nd	-	2120	2000	1.1
pTS185		nd	nd	-	157	11	14

^a KU, Klett units.^b -/+ , -uracil activity/+uracil activity, i.e., fold repression.^c The background level of protease activity assayed from DB104 carrying the vector (pWP18) alone was subtracted from the level of activity assayed in the strains bearing the other fusion plasmids, yielding the values shown in the table.^d nd, not determined.

pTS184). Thus, if a *pyr* operator exists, it must occur within the promoter or 3' to it. Plasmids that contained the *pyr* promoter and 5' leader but did not contain the entire coding sequence of *pyrR* exhibited high levels of subtilisin expression that was not repressible by exogenous uracil (or uridine; data not shown) (pTS182 and pTS184). On the other hand, plasmid pTS185, which was engineered to contain the complete *pyrR* coding region but not the *pyrR-pyrP* antiterminator/terminator sequences or any of the *pyrP* coding region, gave subtilisin expression that was very well regulated by exogenous uracil. These results indicated that the complete *pyrR* coding region was essential for normal *pyr* regulation by exogenous pyrimidines, at least in this multicopy plasmid promoter indicator system. To determine whether the *pyrR*-encoded product could act in *trans* to affect expression of the normal chromosomal *pyr* genes in DB104, the effects of pTS182 and pTS185 on the expression of ATCase activity were assayed (Table 3). It was evident that multiple copies of pTS182 led to substantially elevated ATCase activity and that this activity could not be repressed by excess uracil. Since pTS182 does not encode a *pyrR*-related protein, this observation indicates that multiple copies of the 5' *pyr* leader DNA or, more likely, RNA transcribed from it, lead to derepression of *pyr* genes, presumably by binding and sequestering a regulatory protein. That this regulatory protein is encoded by *pyrR* is suggested by the observation that cells bearing multiple copies of pTS185, which does encode *pyrR*, displayed normal repression of the plasmid-borne *pyr-aprE* fusion (subtilisin) and the chromosomal *pyr* genes (ATCase) (Table 3).

Expression of *pyr-lacZ* fusions integrated into the *B. subtilis* chromosome. Since the results described in the preceding section relied on the use of multicopy plasmids, it was neces-

sary to determine which of the conclusions, if any, might have been affected by variations in plasmid copy number. Furthermore, we wanted to learn whether the requirement for the *pyrR* coding region would be seen in cells containing only one additional copy of the *pyr* promoter-leader sequence. For these reasons, experiments were conducted with *pyr-lacZ* transcriptional fusions which had been integrated in a single copy into the *B. subtilis* chromosome. Segments of *B. subtilis pyr* DNA were fused to a promoterless *lacZ* gene in the pDH32 plasmid (Cm^r) (13). Chromosomal integrants of the *pyr-lacZ* fusions into the *amyE* locus could be selected by transforming *B. subtilis* MO199 (*amyE::erm*) and screening for $\text{MLS}^s \text{Cm}^r$ transformants (12).

Several important conclusions could be drawn from the results obtained with a series of *pyr-lacZ* fusion integrant strains (Table 4). First, normal regulation of *pyr-lacZ* expression by exogenous uracil was obtained even when the fusion lacked the *pyrR* coding region (MO1991). This is in contrast to the failure of the analogous *pyr-aprE* fusion to be regulated when it was present in multiple copies (Table 3, pTS182). We conclude that the product of the single copy of *pyrR* is adequate to regulate a single copy of *pyr-lacZ* in *trans*, but not when many copies are present. This supports the conclusion that *pyrR* encodes a regulatory protein. *pyr-lacZ* fusion integrants in which the portion of the 5' leader predicted to form a transcription terminator in *pyr* mRNA is deleted (MO204 and MO207 versus MO1991) displayed elevated, constitutive *lacZ* expression. This observation indicates that this terminator limits *pyr* expression and that it is required for normal regulation by exogenous uracil. Comparison of these strains also indicates the lack of an operator sequence in and around the promoter region. A *pyr-lacZ* fusion integrant in which the

TABLE 4. Expression of *pyr-lacZ* fusion integrants in *B. subtilis*

Strain		β-Galactosidase Activity (Miller Units)		
		-Uracil	+Uracil	-Uracil/+Uracil
MO200		(7) ^a	(5)	-
MO1991		198	38	5.3
MO204		619	581	1.1
MO207		600	563	1.1
MO1993		584	649	0.9
MO1994		<1	<1	-

^a The background level of β-galactosidase assayed in strain MO200, which contained the pDH32 vector integrated into the chromosome, was subtracted from the levels of activity assayed in the other integrant strains, yielding the values shown in the table.

entire leading stem and loop of the putative antiterminator was deleted (MO1994) expressed no detectable β-galactosidase, whether or not uracil was present in the medium. We interpret this result as indicating that the antiterminator stem-loop does exist *in vivo* and that its formation is essential to allow transcription readthrough. Deletion of a portion of the ascending stem of the putative antiterminator in the *pyr-lacZ* fusion MO1993 led to constitutive elevated expression. In this construction, a stable antiterminator ($\Delta G = -18.2$ kcal/mol) is predicted to form and to be able to prevent terminator formation, but since the deletion removes 15 of the 50 nt of the consensus sequence, we suggest that PyrR is unable to interact with the antiterminator to promote termination. Clearly, a more detailed deletion analysis will be needed to analyze fully the structural elements needed in the leader region for regulation by pyrimidines.

Biochemical functions of *pyrR* and *pyrP*. While the studies described above were in progress, we were informed by Ghim and Neuhaud of results of their studies on the *pyr* gene cluster of the thermophile *B. caldolyticus* (10, 11), which established that the *pyr* genes in that organism were organized in a cluster that was very similar to that found in *B. subtilis* and indicated that *pyrR* and *pyrP* in *B. caldolyticus* encoded functions analogous to the *upp* (uracil phosphoribosyltransferase) (2) and *uraA* (uracil permease) (1) genes of *E. coli*, respectively. Given the high degree of sequence similarity between the *pyrR* and *pyrP* genes of *B. subtilis* and *B. caldolyticus* (72 and 63% amino acid identity, respectively), we tested whether the *B. subtilis* genes also encoded uracil phosphoribosyltransferase and uracil permease activities.

We first tested whether plasmids encoding *pyrR* or *pyrP* could complement *E. coli upp* mutants. *upp* mutants are resistant to 5-fluorouracil, whereas *upp*⁺ strains do not grow in the presence of the same concentration of 5-fluorouracil. As seen in Table 5, plasmids bearing *pyrR*, but not the vector plasmid or disrupted forms of *pyrR*, complemented an *E. coli upp* mutant. Furthermore, significant levels of uracil phosphoribosyltransferase activity could be detected in a crude extract

of *E. coli upp* strain SØ408 (15) bearing the *pyrR*-encoding plasmid pTS1121A2 when expression of *pyrR* was induced with IPTG ($7.2 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$), but not in uninduced cells or in cells that carried the vector plasmid pEB112 alone ($<0.1 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$). The uracil permease of *B. subtilis* was recently shown to be encoded by the *furA* gene (31), which had been mapped near or within the *pyr* gene cluster (47). Strain ED290 (*furA2*) is resistant to $1 \mu\text{g}$ of 5-fluorouracil per ml; a plasmid bearing *pyrP* and another bearing both *pyrR* and *pyrP*, but not plasmids bearing *pyrR* alone or the vector, converted this strain to fluorouracil sensitivity (Table 5). Thus, we conclude that, like *B. caldolyticus*, *B. subtilis pyrR* and *pyrP* genes encode uracil phosphoribosyltransferase (*upp*) and uracil permease (*uraA* and *furA*) activities, respectively. This raised an interesting question: does *pyrR* encode both a uracil

TABLE 5. Complementation of *upp* and *furA* (*uraA*) mutants by *B. subtilis pyrR* and *pyrP* genes

Host strain (relevant genotype)	Plasmid	Plasmid-borne gene(s)	Complementation ^a
<i>B. subtilis</i> ED290 (<i>furA2</i>)	pTS1121A2	<i>pyrR</i>	No
	pTS1BOX	<i>pyrP</i>	Yes
	pEB112		No
	pTS185	<i>pyrR</i>	No
	pTS1BOX3	<i>pyrR</i> and <i>pyrP</i>	Yes
	pWP18		No
<i>E. coli</i> SØ408 (<i>upp</i>)	pTS1121A	<i>pyrR</i>	Yes
	pTS112Δ1A	<i>pyrR</i> (156-bp deletion)	No
	pTS1121A2	<i>pyrR</i>	Yes
	pTS1121A2stp	<i>pyrR</i> (ochre in codon 4)	No
	pEB112		No

^a Complementation was scored by the ability of the plasmid to confer sensitivity of the host to $1 \mu\text{g}$ of 5-fluorouracil per ml when grown on minimal medium with the appropriate antibiotic selection.

FIG. 7. Construction of the in-frame chromosomal deletion of *pyrR*. (A) The chromosomal structure of the 5' end of the *pyr* operon in *B. subtilis* HC11 (*pyrB::spec^c*). B' and 'B' represent the 5' and 3' segments of *pyrB* from which part of the coding sequence was deleted. (B) The structure of the overlapping region in plasmid pTS1121AYB. *B. subtilis* HC11 was transformed with pTS1121AYB, which had been thoroughly linearized with *EcoRI* (in which there were three sites within the vector) and *XmnI* (in which there were two sites within the vector in addition to the site shown at the end of the insert). To obtain *pyr* prototrophy and spectinomycin sensitivity, homologous double recombination must occur within the *SphI*-to-*HindIII* region at the 3' end and the *XmnI*-to-*DraII* region at the 5' end of the plasmid-borne insert. (C) The desired deletion mutant is obtained only if recombination occurs within the *XmnI*-to-*AccI* region at the 5' end. A wild-type strain is obtained if recombination occurs within the *AccI*-to-*DraII* region at the 5' end. Solid bars indicate *pyr* genes; the hatched bar indicates the spectinomycin resistance (*spec^c*) gene. The thick arrow represents the *pyr* promoter, and the thin arrow represents the *Spec^c* promoter.

phosphoribosyltransferase activity and a protein involved in regulation of *pyr* gene expression?

To examine the biochemical functions of *pyrR* further, a *B. subtilis* strain carrying an in-frame deletion of the chromosomal *pyrR* gene was constructed as described in the legend to Fig. 7. In brief, *B. subtilis* strain HC11 (*pyrB::Spec^c*) was transformed with linearized DNA from plasmid pTS1121AYB, which carried the complete 5' end of the *pyr* cluster from nt +8 through the 5' end of *pyrC* but with a 156-bp in-frame deletion within *pyrR*. *pyr⁺ Spec^c* transformants were selected. Two types of double crossover recombinants with this phenotype are expected: those which incorporate the deleted form of *pyrR* and those which do not. The two classes could be distinguished by PCR amplification of a segment of the *pyrR* region, with primers which should give rise to a 1,088-bp amplified product if the *pyrR* gene were intact and a 932-bp product if the deletion in *pyrR* were incorporated. Of 10 transformants screened by PCR,

two carried the 156-bp deletion. Properties of one of these two Δ *pyrR* strains (DB104 Δ *pyrR*) and of a representative recombinant with a normal *pyrR* gene (DB104*pyrR⁺*) are shown in Table 6, along with those of the wild-type parent of HC11, DB104. The Δ *pyrR* strain produced very high levels of ATCase and was refractory to repression by very high levels of exogenous pyrimidines. In contrast, cells with a normal *pyrR* gene produced a 12-fold-lower level of ATCase during growth on minimal medium, presumably because of partial repression by endogenous pyrimidines, and were fully repressed by exogenous pyrimidines. Furthermore, the regulation of ATCase in the Δ *pyrR* strain was restored to normal by introduction of a plasmid carrying the *pyrR* gene (pTS185, Table 3), demonstrating that the defects in the Δ *pyrR* strain are not the result of polar or other *cis*-acting effects. Finally, the Δ *pyrR* strain produced uracil phosphoribosyltransferase at a specific activity equal to those of strains with a normal *pyrR* gene. Altogether, these observations establish conclusively that the *pyrR*-encoded protein plays a crucial role in regulation of *pyr* gene expression by pyrimidines and that this regulatory function is not simply an indirect consequence of its uracil phosphoribosyltransferase activity. The uracil phosphoribosyltransferase activity of the Δ *pyrR* strain must be encoded by a second, predominant *upp* gene. In fact, a *B. subtilis* *upp* gene encoding the major uracil phosphoribosyltransferase has very recently been cloned, sequenced, and mapped near 325° on the *B. subtilis* chromosome (41). This gene is specifically disrupted in strain HH204 (Table 6), in which we were able to detect only trace amounts of uracil phosphoribosyltransferase activity in extracts under our assay conditions. This strain contains normal levels of ATCase activity when grown in minimal medium and is fully repressed by exogenous uridine, which can be converted to UMP by direct phosphorylation, i.e., independently of uracil phosphoribosyltransferase. Presumably, the very low level of uracil phosphoribosyltransferase activity encoded by *pyrR* or some other pathway permits some conversion of uracil to UMP since partial repression by exogenous uracil was observed.

It was expected that HH204 cells would be resistant to 5-fluorouracil since they are defective in conversion of the toxic analog to the nucleotide. Attempts to analyze the ability of *pyrR*-containing plasmids to complement this *B. subtilis* *upp* strain, as demonstrated with an *E. coli* *upp* strain (Table 5), were hampered by the ability of vector controls (pEB112 and pWP18) to confer 5-fluorouracil sensitivity on this strain. Interestingly, the Δ *pyrR* mutant was also resistant to 5-fluorouracil, even though it possessed normal levels of uracil phosphoribosyltransferase activity. We suggest that resistance arises in this case from overproduction of normal uracil nucleotides, which reduce inhibition of biosynthetic enzymes by fluorouracil nucleotides via competition.

DISCUSSION

The results presented here lead to a clarification and more complete picture of the structure and function of the *B. subtilis* *pyr* gene cluster. The 5' end of the cluster, which was previously thought to encode a single large open reading frame (37), is now known to encode two open reading frames, *pyrR* (regulatory) and *pyrP* (permease), with a 173-nt intercistronic region. A very similar structure was independently determined by Ghim and Neuhaud (10) for the corresponding segment of the *B. caldolyticus* *pyr* gene cluster.

The findings of Ghim and Neuhaud contributed substantially to our identification of the biochemical function of *pyrP*, namely, that it encodes a uracil permease. The experimental evidence for this conclusion for the *B. subtilis* *pyrP* rests on the

TABLE 6. A *B. subtilis* Δ *pyrR* strain has lost *pyr* regulation, but not uracil phosphoribosyltransferase activity

Strain	<i>pyrR</i>	5-FU ^a	Pyr ^b	ATCase		Uracil phosphoribosyltransferase	
				Sp act (nmol · min ⁻¹ · mg ⁻¹)	-Pyr/+Pyr ^c	Sp act (nmol · min ⁻¹ · mg ⁻¹)	-Pyr/+Pyr
DB104 Δ <i>pyrR</i>	-	+	None	2,270		10	
			Uracil	2,420	0.9	12	0.8
			Uridine	2,660	0.9	nd ^d	
DB104 <i>pyrR</i> ⁺	+	-	None	185		11	
			Uracil	17	11	15	0.7
			Uridine	8.2	22	nd	
DB104	+	-	None	147		11	
			Uracil	16	9.2	14	0.8
			Uridine	7.3	20	nd	
HH204	+	+	None	192		<1	
			Uracil	112	1.7	<1	
			Uridine	13	15	nd	

^a 5-FU^r, resistance to 1 μ g of 5-fluorouracil per ml. + indicates the ability to grow in the presence of 5-fluorouracil on buffered minimal medium.

^b Cultures were grown in the presence or absence of 0.2 mg of uracil or uridine per ml, as indicated.

^c Specific activity without Pyr/specific activity with Pyr (uracil or uridine).

^d nd, not determined.

observation that *pyrP* is able to complement a *B. subtilis* *furA2* mutant, which is defective in uracil permease (31), but it is also supported by the similarity of *B. subtilis* *pyrP* to *E. coli* *uraA* (44% amino acid identity). Like *B. caldolyticus* *pyrP* (10), *B. subtilis* *pyrP* is predicted to contain 12 hydrophobic segments (data not shown), which could serve as transmembrane helical elements, and is probably an integral membrane protein. The toxicity of overexpressed *pyrP* in *E. coli* hosts accounts for the difficulty encountered by Quinn et al. (37) in cloning this segment of the *B. subtilis* *pyr* cluster.

Establishing the biochemical function of *pyrR* was initially more confusing. The protein clearly possesses uracil phosphoribosyltransferase activity despite its low sequence identity to other known uracil phosphoribosyltransferases (27 and 22% to *E. coli* [2] and *Saccharomyces cerevisiae* [19], respectively, inclusive of many gaps), which are more identical to each other (35%, with fewer gaps). Furthermore, the results of our experiments with *pyr-aprE* fusion plasmids and *pyr-lacZ* fusion integrants (Tables 3 and 4) indicated a role for PyrR in regulation of *pyr* gene expression. Disruption of the *B. subtilis* *pyrR* gene established its regulatory role and demonstrated that PyrR contributes only a very minor fraction of the cell's total uracil phosphoribosyltransferase activity, showing clearly that its function in regulation was not simply an indirect consequence of its uracil phosphoribosyltransferase activity. The major uracil phosphoribosyltransferase of both *B. subtilis* (41) and *B. caldolyticus* (28) is now known to be contributed by another *upp* gene. The amino acid sequence of the *B. subtilis* *upp* gene product shows a much higher degree of identity to the uracil phosphoribosyltransferases of other species (41). Thus, PyrR is a bifunctional protein, both an enzyme and a regulatory protein. This is an unusual finding, but not without precedent. The PutA protein from *E. coli* possesses proline oxidase and pyrroline-5-carboxylate dehydrogenase activities and serves as a DNA-binding protein for the *put* operon (8). Also, the mammalian iron-responsive-element-binding protein, which regulates expression of ferritin and transferrin receptor genes by binding to their mRNAs to control their stabilities, has been shown to be identical to cytoplasmic aconitase (17). It is not yet clear whether the uracil phosphoribosyltransferase activity of PyrR is important to either the

uracil metabolism of *B. subtilis* cells grown under some conditions or to the ability of the protein to regulate *pyr* gene expression. It is possible that PyrR is a regulatory protein that has evolved from an ancestral uracil phosphoribosyltransferase which retains vestigial enzymatic activity.

On the basis of the observations in this paper, we propose the following model for the regulation of *B. subtilis* *pyr* gene expression by transcriptional attenuation (Fig. 8). *pyr* mRNA is capable of forming one of two mutually exclusive secondary structures at three points: the 5' leader, the *pyrR-pyrP* intercistronic region, and the *pyrP-pyrB* intercistronic region. When intracellular pyrimidine nucleotide pools are low, no regulatory factor binds to the *pyr* mRNA. This allows the kinetically and thermodynamically favored antiterminators to form, resulting in transcription readthrough and expression of the downstream genes. When intracellular pyrimidine pools are high, a pyrimidine nucleotide binds to a regulatory factor, which we propose to be the PyrR protein, which is then capable of binding to the conserved sequence in the upstream stem of each antiterminator (Fig. 5). This binding is predicted to prevent the promoter-proximal stem of the antiterminator from base pairing with its promoter-distal stem, therefore freeing the upstream stem of the terminator to base pair with its downstream stem, resulting in termination of transcription and reduced expression of the downstream genes.

We have presented evidence for the critical roles of the antiterminator and terminator structures in the regulation of *pyr* gene expression only in the case of the 5' leader region (Table 4), but we propose that similar mechanisms act at all three attenuation regions identified above. As is evident from the Northern blot analysis (Fig. 6), termination at each individual site is not complete but is cumulative. The effectiveness of each attenuation site is not likely to be equal but instead is likely to be optimized to allow the desired amount of readthrough of the corresponding downstream genes. The level of repressibility at each site is predicted to be dependent upon two main factors which appear to differ in each of the three sites. First, attenuation depends on the relative stabilities of the antiterminator versus terminator structures. As is evident in Fig. 4, the stabilities of the attenuation structures within the leader region and the second intercistronic region

FIG. 8. Proposed mechanism for regulation of transcriptional attenuation by PyrR in the *B. subtilis* *pyr* 5' leader region. Numbers indicate segments of the stems of proposed RNA secondary structures found by base pairing between sequences designated by opposing arrows. Further details are given in the text.

are very similar to each other but substantially different from those within the first intercistronic region. Also, modulation of the attenuation structures depends on the binding affinity of PyrR to the specific binding sequence within each region. The consensus sequence depicted in Fig. 5 shows that these proposed binding sequences are very similar, but the sequences are not identical. We believe that the stepwise regulation of transcription termination at these three points can be rationalized from a consideration of the physiological functions of the proteins encoded by *pyrR*, *pyrP*, and the downstream *pyr* genes. A significant level of the PyrR regulatory protein is needed even when pyrimidines are abundant to continue termination of newly initiated transcription from the *pyr* promoter. Likewise, some expression of uracil permease is required even when uracil is abundant in the medium to allow its continued entry into the cell. On the other hand, when pyrimidine nucleotides are abundant, little if any expression of the downstream biosynthetic genes is necessary. This laboratory is currently studying the relative contributions of the three attenuation regions by use of a family of *pyr-lacZ* fusion integrants containing them in various combinations.

It is evident that the full range of potential regulation of *pyr* gene expression is not revealed by comparing the levels of ATCase in cells grown without pyrimidines to the levels in cells grown with excess pyrimidines. Many terminated transcripts were found in cells grown without uridine (Fig. 6), and much higher levels of *pyrB* expression were observed in the *pyrR* deletion strain than in wild-type cells grown without pyrimidines (Table 6). We are currently examining the expression of *pyr-lacZ* transcriptional fusion integrants in pyrimidine-starved cells. The results obtained so far suggest that during severe pyrimidine starvation, little transcription termination occurs at any of the three attenuator sites (24a).

The binding of PyrR to *pyr* mRNA is postulated to be dependent on a pyrimidine nucleotide, which is probably a uridine nucleotide (UMP, UDP, or UTP), because *B. subtilis* *pyr* genes cannot be repressed by cytidine in a *cdd* (cytidine deaminase) mutant but are still repressed by uracil (3). We suggest that the regulatory nucleotide is likely to be UMP; PyrR presumably contains a UMP-binding site, since UMP is

the product of the uracil phosphoribosyltransferase reaction. It is not likely that uracil plays a significant role in regulating PyrR binding to *pyr* mRNA, even though it presumably possesses the ability to bind to PyrR as well. First, a *B. subtilis* *upp* mutant (HH204, Table 6) is defective in repression by exogenous uracil but not by uridine. Also, it is not likely that uracil levels accumulate significantly within the cell because uracil must be rapidly converted to UMP by uracil phosphoribosyltransferase for efficient transport into the cell (2). There is the interesting possibility, however, that attenuation may be modulated not only by UMP, but by PRPP as well. PRPP may antagonize UMP binding, which would suggest that PRPP may stimulate *pyr* gene expression. PRPP may be a physiologically important modulator of *pyr* gene expression because it is a necessary substrate for UMP biosynthesis. Furthermore, the PRPP pool in *B. subtilis* cells is known to drop sharply when glucose in the medium is exhausted (5) and thus may serve as a good indicator of the capacity of the bacteria for rapid growth.

Our proposed model makes a number of explicit predictions which have not yet been tested experimentally. PyrR should bind to *pyr* mRNA at specific sequences that interfere with antiterminator formation, i.e., at the conserved sequence or some portion of it. Binding of PyrR to RNA should be dependent on the presence of a pyrimidine nucleotide. Regulation of termination of *pyr* gene transcription should be demonstrable in vitro with a simple transcription system involving RNA polymerase and PyrR, if no additional protein factors are involved. This prediction is best tested with purified PyrR protein. Purification and characterization of *B. subtilis* PyrR is currently under way in our laboratory. Mutations in the proposed PyrR-binding sites should also have predictable effects on repression of *pyr* genes in vivo. Characterization of *pyrR* and non-*pyrR* *B. subtilis* *pyr* regulatory mutants is currently under way in our laboratory, as well.

It is evident that the proposed model is very similar to the mechanism for regulation of the *B. subtilis* *trp* operon by the *trpB*-encoded protein TRAP proposed by Yanofsky and his colleagues, Kuroda et al. (20) and Shimotsu et al. (43). This model has been strongly supported by recent biochemical

experiments of Babitzke and Yanofsky (4) and Otridge and Gollnick (32). The most important differences between our proposed mechanism and that deduced for the *B. subtilis* *trp* system are our belief that the PyrR protein interacts at three attenuation sites instead of one and the finding that PyrR is a bifunctional protein.

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