Evidence that the *Bacillus subtilis* Pyrimidine Regulatory Protein PyrR Acts by Binding to *pyr* mRNA at Three Sites In Vivo

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The *Bacillus subtilis* *pyr* operon is regulated by a transcriptional attenuation mechanism that requires the PyrR regulatory protein. Multicopy plasmids that could be transcribed to yield segments of RNA from the attenuation regions of the *pyr* operon induced derepression of chromosomal *pyr* genes, whereas plasmids that could not yield *pyr* RNA did not. We conclude that *pyr* RNA acts by titrating the PyrR protein and preventing it from regulating *pyr* attenuation.

The genes of pyrimidine nucleotide biosynthesis in *Bacillus subtilis* are organized in an operon whose expression is governed by a single promoter and a regulatory protein encoded by *pyrR*, which is the first gene of the operon (22). Repressive control of expression of the *pyr* operon by exogenous pyrimidines operates by an attenuation mechanism in which termination of transcription can occur at one of three sites located near the 5' end of the operon (18, 22). These sites are located in the 5' untranslated leader of the operon (attenuator 1), between the first (*pyrR*) and second (*pyrP*) genes (attenuator 2), and between the second and third (*pyrB*) genes (attenuator 3) of the operon. We have proposed that the PyrR regulatory

protein acts to promote termination of transcription by binding in a pyrimidine nucleotide-dependent manner to specific regions in the *pyr* mRNA and suppressing antitermination (22). Evidence supporting this hypothesis includes the observation that introduction of multicopy plasmids bearing portions of *pyr* DNAs encoding the attenuator regions resulted in constitutive expression of *pyr* genes unless the *pyrR* gene was also included on the plasmid (22). This result was interpreted as indicating that the many copies of *pyr* attenuator RNA produced by these plasmids acted to titrate the PyrR protein and prevent it from acting to regulate the single chromosomal copy of the *pyr* operon. However, it is also possible that the PyrR protein

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Strains		
DB104	<i>his nprR2 nprE18 ΔaprA3</i>	14
MO199	<i>trpC2 pheA amyE::erm</i>	8
MO220	MO199 <i>amyE::pLS60</i>	18
Plasmids		
pYSP01-1		22
pLS372	pYSP01-1 with a 0.16-kb <i>Bss</i> HII (Klenow)- <i>Bam</i> HI (Klenow) deletion	This study
pSP72 and pSP73	<i>Ap^r, P_{TR}, P_{SP6}</i>	Promega
pLS693	0.14-kb <i>Eco</i> RI- <i>Hind</i> III fragment from pLS691 (16) in pSP72	This study
pLS703	0.16-kb <i>Eco</i> RI- <i>Hind</i> III fragment from pLS701 (16) in pSP72	This study
pHPS9	<i>Cm^r Em^r, pUC ori, pTA1060 rep</i>	10
pLS11	<i>Xma</i> I (+8)- <i>Bam</i> HI (+230) fragment from pUC19/290 (22) in pHPS9	This study
pLS12	<i>Eco</i> RI (–51)- <i>Bam</i> HI (+230) fragment from pUC19/290 (22) in pHPS9	This study
pLS13	<i>Eco</i> RI (+670)- <i>Bam</i> HI (+850) fragment from pLS621 (18) in pHPS9	This study
pLS14	<i>Eco</i> RI- <i>Bam</i> HI fragment from pLS622 (18) in pHPS9	This study
pLS15	<i>Eco</i> RI (+2132)- <i>Bam</i> HI (+2381) fragment from pLS382E (18) in pHPS9	This study
pLS16	<i>Eco</i> RI- <i>Bam</i> HI fragment from pLS601 (18) in pHPS9	This study
pLS17	68 bp of <i>Eco</i> RI- <i>Bam</i> HI from pLS612 (16) in pHPS9	This study
pEB112	<i>Ap^r Kan^r or Neo^r, pBR322 ori, pUB110 ori, lacI^q</i>	9 and 15
pLS21	<i>Pvu</i> II- <i>Bam</i> HI fragment from pLS11 in <i>Sma</i> I- <i>Bam</i> HI-digested pEB112	This study
pLS22	<i>Pvu</i> II- <i>Bam</i> HI fragment from pLS12 in <i>Sma</i> I- <i>Bam</i> HI-digested pEB112	This study
pLS23	<i>Pvu</i> II- <i>Bam</i> HI fragment from pLS13 in <i>Sma</i> I- <i>Bam</i> HI-digested pEB112	This study
pLS24	<i>Pvu</i> II- <i>Bam</i> HI fragment from pLS14 in <i>Sma</i> I- <i>Bam</i> HI-digested pEB112	This study
pLS25	<i>Pvu</i> II- <i>Bam</i> HI fragment from pLS15 in <i>Sma</i> I- <i>Bam</i> HI-digested pEB112	This study
pLS26	<i>Pvu</i> II- <i>Bam</i> HI fragment from pLS16 in <i>Sma</i> I- <i>Bam</i> HI-digested pEB112	This study
pLS27	<i>Pvu</i> II- <i>Bam</i> HI fragment from pLS17 in <i>Sma</i> I- <i>Bam</i> HI-digested pEB112	This study

^a Parenthetical numbers preceded by a + or – indicate nucleotide number in the *pyr* operon, with +1 being the site of the start of transcription.

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TABLE 2. Elevated levels of expression of *B. subtilis* *pyr* genes and a chromosomal *pyr-lacZ* fusion in strains bearing high-copy-number plasmids that transcribe *pyr* attenuators^a

Plasmid		β -Galactosidase Activity (Miller Units)			Aspartate Transcarbamylase Activity (nmole $\cdot$ mg ⁻¹ $\cdot$ min ⁻¹)		
		-Uracil	+Uracil	-U/+U	-Uracil	+Uracil	-U/+U
pEB112		137 $\pm$ 22	38 $\pm$ 5	3.6	348 $\pm$ 2	44 $\pm$ 16	7.9
pLS27		216 $\pm$ 15	34 $\pm$ 3	6.4	456 $\pm$ 8	47 $\pm$ 2	9.7
pLS21		148 $\pm$ 9	45 $\pm$ 13	3.3	373 $\pm$ 110	88 $\pm$ 20	4.3
pLS22		236 $\pm$ 31	189 $\pm$ 51	1.2	1,420 $\pm$ 75	1,392 $\pm$ 109	1.0
pLS23		249 $\pm$ 23	38 $\pm$ 6	6.5	415 $\pm$ 120	54 $\pm$ 22	7.6
pLS24		450 $\pm$ 8	468 $\pm$ 10	1.0	2,411 $\pm$ 462	2,351 $\pm$ 362	1.0
pLS25		203 $\pm$ 18	34 $\pm$ 4	6.0	305 $\pm$ 31	68 $\pm$ 17	4.5
pLS26		379 $\pm$ 2	373 $\pm$ 24	1.0	1,700 $\pm$ 62	1,685 $\pm$ 9	1.0

^a The plasmids are described in Table 1. The host strain was *B. subtilis* MO220 in all cases. The arrows indicate the location of the plasmid-borne *pyr* promoter, when present, and lines indicate the location of *pyr* DNA inserted into a plasmid. Numbers beneath the indicated restriction enzymes are nucleotide numbers in the *pyr* operon, with +1 being the site of the start of transcription. Values are means $\pm$ average deviation from the mean. -U/+U, average of the value without uracil over the value with uracil.

bound to the *pyr* DNAs in the plasmids. Further understanding of the mechanism of regulation by PyrR requires knowing whether it binds to DNA or RNA.

Multicopy plasmids must form *pyr* transcripts to induce derepression of *pyr* genes. In this work the issue of whether PyrR binds to DNA or RNA was resolved by examining the effects on *pyr* expression of introducing into *B. subtilis* cells multicopy plasmids carrying each of the *pyr* attenuator regions with or without the *pyr* promoter fused immediately upstream. Thus, only those plasmids bearing the upstream promoter were able to express large amounts of the corresponding attenuation region RNA, but the amounts of *pyr* DNA produced by both sets of plasmids were approximately equal. A series of plasmids was constructed in which *pyr* DNA encoding attenuation region 1, attenuation region 2, or attenuation region 3 either without (pLS21, pLS23, or pLS25, respectively [Table 1]) or with (pLS22, pLS24, or pLS26, respectively [Table 1]) the *B. subtilis* *pyr* promoter fused upstream was inserted into plasmid pEB112 (15). These plasmids were transformed into the *B. subtilis* host strain MO220, which has a normal chromosomal *pyr* operon as well as a *pyr-lacZ* fusion with a copy of attenuation region 3 inserted between the *pyr* promoter and the *lacZ* gene integrated into the chromosome at the *amyE* locus (18). Thus, in strain MO220, regulation of the chromosomal *pyr* genes can be measured by assays (4, 20) of aspartate transcar-

bamylase activity (encoded by *pyrB*) and regulation of the *pyr-lacZ* fusion can be monitored by β -galactosidase assays (19). Both genes have been shown to be regulated in *trans* by *pyrR* (18). As seen in Table 2, plasmids in which the three attenuation regions could be transcribed led to elevated and constitutive levels of expression of both *pyrB* and the *pyr-lacZ* fusion, but multiple copies of the same DNA that could not be transcribed did not. In the latter case, normal regulation of the *pyr* genes was observed, as it was when the vector plasmid alone was transformed into strain MO220. To test the possibility that deregulation of *pyr* gene expression by the plasmids in strains bearing pLS22, pLS24, and pLS26 results from PyrR binding to the promoter DNA, a derivative of pEB112 with only the *pyr* promoter inserted, pLS27 (Table 1), was transformed into strain MO220. β -Galactosidase and aspartate transcarbamylase activities were regulated normally in this strain (Table 2).

The experiments whose results are shown in Table 2 were conducted with pEB112, which has a relatively high copy number (9). The experiments were repeated with analogous segments of *pyr* DNA inserted into pHPS9, a plasmid with a copy number estimated to be about five copies per cell (10, 11). These plasmids were transformed into strain MO220, and *pyr* gene expression in the presence and absence of exogenous uracil was measured as described above (Table 3). As seen above, only those plasmids that contained the *pyr* promoter

TABLE 3. Expression of *B. subtilis* *pyr* genes and a chromosomal *pyr-lacZ* fusion as differentially affected by low-copy-number plasmids that transcribe mRNAs from the different *pyr* attenuator regions^a

Plasmid		β -Galactosidase Activity (Miller Units)			Aspartate Transcarbamylase Activity (nmole $\cdot$ mg ⁻¹ $\cdot$ min ⁻¹)		
		-Uracil	+Uracil	-U/+U	-Uracil	+Uracil	-U/+U
pHPS9		200 $\pm$ 3	62 $\pm$ 7	3.2	400 $\pm$ 5	64 $\pm$ 4	6.3
pLS17		212 $\pm$ 14	62 $\pm$ 11	3.4	390 $\pm$ 7	71 $\pm$ 7	5.5
pLS11		173 $\pm$ 5	71 $\pm$ 19	2.4	542 $\pm$ 82	942 $\pm$ 25	5.8
pLS12		187 $\pm$ 5	78 $\pm$ 16	2.4	518 $\pm$ 21	104 $\pm$ 20	5.0
pLS13		168 $\pm$ 2	74 $\pm$ 20	2.3	441 $\pm$ 10	81 $\pm$ 10	5.4
pLS14		272 $\pm$ 18	238 $\pm$ 1	1.1	1,445 $\pm$ 48	1,608 $\pm$ 87	0.9
pLS15		284 $\pm$ 90	74 $\pm$ 15	3.8	497 $\pm$ 51	72 $\pm$ 8	6.9
pLS16		180 $\pm$ 2	98 $\pm$ 10	1.8	5314 $\pm$ 3	154 $\pm$ 8	3.4

^a The plasmids are described in Table 1. The host strain was *B. subtilis* MO220 in all cases. The arrows indicate the location of the plasmid-borne *pyr* promoter, when present, and lines indicate the location of *pyr* DNA inserted into a plasmid. Numbers beneath the indicated restriction enzymes are nucleotide numbers in the *pyr* operon, with +1 being the site of the start of transcription. Values are average deviation from the mean. -U/+U, average of the value without uracil over the value with uracil.

upstream of attenuation region sequences were able to cause deregulation of *pyr* expression, but in a low-copy-number vector the three attenuation regions differed markedly in their abilities to cause deregulation. The plasmid that was able to transcribe attenuation region 2 caused high-level, constitutive expression (Table 3, pLS14), whereas the plasmid that could transcribe attenuation region 3 had only a small effect on *pyrB* expression (pLS16) and the plasmid that transcribed attenuation region 1 did not alter normal regulation of the *pyr* gene (pLS12 versus pLS11). A plasmid in which only the *pyr* promoter was inserted into the pHPS9 vector (pLS17) did not affect *pyr* gene expression.

Levels of *pyr* mRNA in cells bearing multicopy plasmids correlate with the ability of the plasmids to induce derepression of *pyr* genes. The results presented in Table 3 may indicate differences in the abilities of the three different attenuation region RNA sequences to bind to the limited amount of PyrR protein from the chromosomal *pyrR* gene. Alternatively, the amounts of RNA produced by the various plasmids or the levels of stability of the three RNA species in vivo may differ, so that their steady-state levels may differ substantially under the conditions studied. We tested this latter possibility by slot blot hybridization assays for each of the three RNA species, using radioactive single-stranded RNA probes that hybridized specifically to each of the *pyr* RNAs produced by the plasmids described in Tables 2 and 3. Figure 1A shows the relationship between amounts of total RNA analyzed and the corrected levels of radioactivity specifically hybridizing to the probes in the strains that contained high-copy-number titration plasmids. Figure 1B shows similar experiments for the strains that con-

tained low-copy-number titration plasmids. Both the high- and low-copy-number plasmids containing the *pyrR-pyrP* intercistronic region generated the highest steady-state levels of *pyr* mRNA. The plasmids containing the *pyrP-pyrB* intercistronic region generated lower levels of *pyr* mRNA. The plasmids containing the 5' leader region generated the lowest levels of *pyr* mRNA. These results parallel the extent of derepression by the low-copy-number plasmids (Table 3). Above a certain level of *pyr* mRNA, which is roughly the amount found in strain MO220 bearing pLS22, maximal derepression of both aspartate transcarbamylase and *pyr-lacZ* expression was observed.

Conclusions. Plasmids capable of expressing RNA from all three *pyr* attenuation regions were able to induce derepression of chromosomal *pyr* genes. Plasmids that could not express these RNAs did not induce derepression. With the low-copy-number plasmids there was a correlation between the amount of *pyr* RNA in the cells and the extent of derepression. Given that *pyr* repression is known to be totally dependent on the presence of the PyrR protein (22), we conclude that PyrR acts by binding to *pyr* RNA to cause repression and that overexpression of plasmid-encoded *pyr* RNA causes derepression by titrating the PyrR protein so that insufficient free PyrR protein is available to regulate the chromosomal *pyr* genes. Since this work was completed, additional biochemical evidence in support of this conclusion has been obtained. Purified PyrR when complexed to UMP has been shown to cause termination of transcription from *pyr* templates in vitro (17). Furthermore, UMP-dependent binding of PyrR to *pyr* RNA has been demonstrated by electrophoretic gel mobility shift analysis (21).

The extent of derepression caused by low-copy-number plas-

FIG. 1. Steady-state levels of plasmid-encoded *pyr* mRNA detected by slot blot hybridization analysis of total RNA extracted from *B. subtilis* MO220 cells bearing the high-copy-number plasmid pLS22, pLS24, or pLS26 (A) or *B. subtilis* DB104 cells bearing the low-copy-number plasmid pLS12, pLS14, or pLS16 (B). Total cellular RNAs were prepared with a Qiagen Total RNA Midi kit (catalog number 14142). Strains used for RNA preparation were MO220 bearing pLS22, pLS24, or pLS26 for the high-copy-number titration plasmids and DBL412, DBL414, and DBL416 for the low-copy-number titration plasmids. DBL412, DBL414, and DBL416 are the counterparts of MO220 bearing pLS12, pLS14, and pLS16, respectively, but the host strain was changed from MO220 to DB104 to avoid possible interference of transcripts from the chromosomal *pyr-lacZ* fusion. The levels of regulation of the *pyr* genes by the low-copy-number titration plasmids were similar in both MO220 and DB104. The 32 P-labeled RNA probes were synthesized with a commercial Ambion Maxiscript kit. Templates for the probes for the 5' leader region, the *pyrR-pyrP* intercistronic region, and the *pyrP-pyrB* intercistronic region were pLS372, pLS693, and pLS703, respectively (Table 1). RNA samples were transferred onto a nitrocellulose membrane according to published procedures (5, 6, 13) and hybridized to the appropriate radioactive probes, and the membrane was exposed to a PhosphorImager cassette (Molecular Dynamics). The counts were corrected for the number of AMP residues in each of the radioactive RNA probes and were proportional to the molar concentration of RNA in each sample. In panel B, the amounts of *pyr* RNA transcribed from plasmids pLS12, pLS14, and pLS16 have been corrected for the background of chromosomally specified *pyr* RNA by subtracting the amounts of specifically hybridizing RNA detected in DB104 cells bearing the promoterless plasmids pLS11, pLS13, and pLS15, respectively, which ranged from 11 to 14% of the total detected. For the high-copy-number plasmids used in the experiments whose results are shown in panel A, this background was on the order of 1% and was not subtracted.

mids was dependent on which attenuation region was transcribed, which was a consequence of surprisingly great differences in the steady-state levels of *pyr* RNA in cells bearing these plasmids. We did not investigate whether differences in the rates of synthesis or rates of degradation accounted for this large variation in RNA levels. The observation serves as a

warning, however, that an attempt to map the specificities of RNA-binding proteins for RNA with a collection of plasmids expressing a series of structurally related RNAs could give highly misleading results unless steady-state levels of RNA are also carefully determined.

Other proteins are known to regulate attenuation by binding to mRNA. Two well-documented examples are the *trp* RNA-binding attenuation protein (TRAP) from *B. subtilis*, which promotes termination of the *trp* operon (2, 3, 7), and the *Escherichia coli* BglG protein, which acts by preventing attenuation of the *bglGFB* operon (1, 12). The potential for regulation of gene expression by attenuation mechanisms involving mRNA-binding regulatory proteins is very general.

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