The regulator protein PyrR of *Bacillus subtilis* specifically interacts *in vivo* with three untranslated regions within *pyr* mRNA of pyrimidine biosynthesis

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In vitro experiments have shown that the genes of the *de novo* pyrimidine biosynthetic pathway of *Bacillus subtilis*, the *pyr* genes, are regulated by a transcriptional attenuation mechanism. Specific regulatory sequences (binding loops, BLs) are located within three untranslated leader sequences at the beginning of *pyr* mRNA. These binding loops, BL1, BL2 and BL3, act as anti-antiterminators of transcription when stabilized by the regulator protein PyrR. In this work, the interaction of PyrR with BL1, BL2 and BL3 was qualitatively and quantitatively analysed *in vivo* using the yeast three-hybrid system. The results indicate that PyrR specifically binds to BL1, BL2 and BL3. Furthermore, the data suggest that the strength of interaction between PyrR and the three different BLs *in vivo* is within the same dimension. The yeast three-hybrid system also proved to be useful for the rapid analysis of structural requirements for PyrR–BL binding. Point mutations within the predicted critical regions of BL1, BL2 and BL3 led to drastically reduced binding of PyrR. In summary, it is shown that the yeast three-hybrid system is well suited to qualitatively and quantitatively analyse bacterial regulatory systems that are based on factor-independent transcriptional attenuation.

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INTRODUCTION

Regulation by transcription termination/antitermination, known as transcription attenuation, is a commonly used regulatory strategy in bacteria. It is most often based on the selective formation of either of two alternative base-paired structures in a nascent mRNA transcript, one of which causes termination of transcription (Switzer *et al.*, 1999; Yanofsky, 2000). In prokaryotes, there are two mechanisms of transcription termination: intrinsic termination and factor-dependent termination (Henkin, 1996; Yarnell & Roberts, 1999). The pyrimidine nucleotide biosynthesis (*pyr*) operon in *Bacillus subtilis* is regulated by an intrinsic termination mechanism involving the RNA-binding protein PyrR (Switzer *et al.*, 1999), and initiation of transcription at the *pyr* promoter is believed to be constitutive (Lu *et al.*, 1995). The *pyr* operon of *B. subtilis* contains 10 genes. The first gene in the operon encodes the bifunctional protein PyrR, which has been shown to be (i) the regulator for the operon, and (ii) a uracil phosphoribosyltransferase (EC 2.4.2.9) (Turner *et al.*, 1994). The second gene in the operon encodes PyrP, which is a uracil permease. The remaining eight genes encode the six enzymes responsible for the *de novo* synthesis of UMP (Kahler & Switzer, 1996; Quinn *et al.*, 1991; Turner *et al.*, 1994). Regulatory attenuation sites are

located in the 5' untranslated leader of the operon upstream of *pyrR*, between the first (*pyrR*) and second (*pyrP*) genes of the operon, and between the second and third (*pyrB*) genes of the operon. Following transcription, the three attenuation sites within the nascent *pyr* mRNA are each predicted to fold into either an anti-antiterminator/ ρ -independent transcription terminator, or a so-called antiterminator structure. In the absence of PyrR, the attenuation sites fold predominantly into the antiterminator structure, which is favoured over the other structures kinetically (because it is formed first) and thermodynamically (because it is predicted to be more stable). The antiterminator structures allow the RNA polymerase to elongate the *pyr* transcript. PyrR is stimulated by UMP, UDP and UTP to bind to the anti-antiterminators of the attenuation regions, which are also called binding loops or BLs. Binding of activated PyrR stabilizes the secondary structures of BL1, BL2 and BL3, and prevents the formation of the antiterminators. Concurrently with the anti-antiterminators, ρ -independent intrinsic transcription terminators are able to form, and these reduce the rate of transcription of the structural genes of the operon. The three different attenuation regions operate independently, and, consequently, there are three sequential regulatory decisions to allow expression of the full-length mRNA (Lu *et al.*, 1995, 1996; Lu & Switzer, 1996a, b).

Many aspects of the mechanism of regulation of the *pyr* operon in *B. subtilis* have been studied by analysing mutants

Abbreviations: ADVP16, activation domain from herpes simplex VP16; BL, binding loop.

that have lost normal regulation, or by using *in vitro* biochemical assays (Bonner *et al.*, 2001; Turner *et al.*, 1998). In this work, we describe the application of the yeast three-hybrid system (Putz *et al.*, 1996; SenGupta *et al.*, 1996) for the characterization of the PyrR–*pyr*-mRNA interaction *in vivo*.

METHODS

Cloning procedures, and other DNA manipulations. Plasmid construction, transformation and growth of *Escherichia coli*, and DNA sequencing, were carried out according to standard procedures (Sambrook *et al.*, 1989). Site-directed mutagenesis was done using the QuikChange II XL kit (Stratagene). Plasmids were amplified in *E. coli* Top 10F' (Invitrogen).

Yeast three-hybrid constructs. All constructs used were based on plasmids of the RNA-Protein Hybrid Hunter System from Invitrogen (catalogue no. K5100). The resulting plasmids (pBH) are listed in Table 1. The plasmids pRH3'-IRE and pYesTrp3-IRP were obtained from Invitrogen, and they served as positive controls. The plasmid pYESTrp3 was used to produce a fusion of *B. subtilis* PyrR and an N-terminal activation domain from virus herpes simplex VP16 (ADVP16) in a transformant yeast cell. The corresponding construct was obtained by introducing the *pyrR* gene of *B. subtilis* as an *EcoRI*–*XhoI* fragment into the multiple cloning site of pYESTrp3. The gene *pyrR* was amplified by PCR using genomic DNA from *B. subtilis* Marburg 168 (wild-type strain) as a template, and the oligonucleotides *pyrR*-forward 5'-CTATGAATTCTGTTGAATCAAAAA-GCTGTCATTCTCG-3' and *pyrR*-reverse 5'-ATATCTCGAGTTATTCGTTTTTCATAAATGGCGACG-3' (the sites for restriction endonucleases are underlined); for the *in vivo* production of hybrid RNAs, the plasmids pRH5' and pRH3', respectively, were used. The attenuation regions BL1 and BL2 to be tested were produced by PCR using the oligonucleotide pairs (forward and reverse primers)

listed in Table 2, and genomic DNA of *B. subtilis* Marburg 168. The PCR products were treated with *PmeI*/*XmaI*, and ligated into *PmeI*/*XmaI*-digested pRH5', and into *PmeI*/*XmaI*-treated pRH3'. BL3 was produced by hybridizing two oligonucleotides, BL3linker forward and BL3linker reverse (Table 2), to each other, and ligating the resulting linker into *PmeI*/*XmaI*-digested pRH5', and also into *PmeI*/*XmaI*-treated pRH3'. The plasmids pBH12, pBH13, pBH14 and pBH15 (Table 1), which produce variant forms of BL2, were generated by site-directed mutagenesis using the primer pairs listed in Table 2 (BL2mut forward/BL2mut reverse, and BL2del forward/BL2del reverse). A variant form of BL1 (10A>G) arose through a spontaneous mutation during cloning (pBH20). The same was true for a variant form of BL3 (Δ U2203/ Δ U2214) (pBH17) and a variant form of BL2 (Δ U719/716C>A) (pBH16). The plasmids pBH23, pBH24, pBH25, pBH26, pBH27 and pBH28 (Table 1) producing variant forms of BL1 were generated by site-directed mutagenesis using the primer pairs listed in Table 2 (BL1mut30 forward/BL1mut30 reverse, BL1mut31 forward/BL1mut31 reverse, and BL1del forward/BL1del reverse).

Yeast three-hybrid analysis. Yeast three-hybrid analysis was performed in *Saccharomyces cerevisiae* strain L40-*ura3* [*MATa ura3-52 leu2-3112 his3 Δ 200 trp1 Δ 1 ade2 LYS2::(*LexA op*)₄-HIS3 ura3::(*LexA-op*)₈-LacZ*] (Invitrogen) carrying pHybLexA/Zeo-MS2 (zeocin^R). The hybrid RNA expression constructs, and the construct expressing the activation domain fusion ADVP16/PyrR, or the activation domain only, were cointroduced into *S. cerevisiae* L40-*ura3* cells carrying pHybLexA/Zeo-MS2. Transformants were grown on synthetic complete medium (YC) lacking uracil, tryptophan and histidine, and containing zeocin (100 μ g ml⁻¹), for selection of the *URA3*, *TRP1* and *HIS3* marker genes. The YC medium used contained 0.12 % (w/v) yeast nitrogen base, 0.5 % ammonium sulfate, 1 % succinic acid, 2 % glucose, 0.01 % each of adenine, arginine, cysteine, leucine, lysine and threonine, 0.005 % each of aspartic acid, isoleucine, methionine, phenylalanine, proline, serine, tyrosine and valine.

Testing of reporter gene activation in the yeast three-hybrid system. Growing colonies were qualitatively analysed for the reporter gene product β -galactosidase (LacZ) using an overlay assay with X-Gal staining (Duttweiler, 1996; Jaeger *et al.*, 2004). To quantify the level of *lacZ* expression, β -galactosidase activity was measured in *S. cerevisiae* L40-*ura3* cells carrying pHybLexA/Zeo-MS2 producing both the ADVP16/PyrR fusion and the hybrid RNAs to be tested. Measurements were done with cells prepared from two independent transformants. For each transformant, three independent assays were carried out. Mean LacZ activities are given in Tables 3 and 4, including standard deviations. Cell material was taken from a colony of a freshly transformed yeast strain, and used as an inoculum for 5 ml YC lacking uracil and tryptophan, and containing zeocin (100 μ g ml⁻¹). Cells were grown overnight at 30 °C. The next day, liquid cultures (15 ml) were inoculated to an OD₆₀₀ of 0.15–0.2 using the overnight cultures, and grown at 30 °C to an OD₆₀₀ of 0.5–0.8. The β -galactosidase assay was then carried out according to Schneider *et al.* (1996).

RESULTS

Yeast three-hybrid analysis

In this work, the yeast three-hybrid system (Putz *et al.*, 1996; SenGupta *et al.*, 1996) was employed for the *in vivo* analysis of the interaction of the regulator protein PyrR from *B. subtilis* with the three anti-antiterminator structures BL1, BL2 and BL3 of *pyr* mRNA (Fig. 1). In the yeast three-hybrid system, the binding of a bifunctional hybrid RNA to each of

Table 1. Yeast three-hybrid constructs

Plasmid (based on)	Size (bp)	Description
pBH2 (pYESTrp3)	6330	ADVP16–PyrR
pBH8 (pRH5')	7957	MS2–BL1
pBH6 (pRH3')	7957	BL1–MS2
pBH9 (pRH5')	7983	MS2–BL2
pBH7 (pRH3')	7983	BL2–MS2
pBH11 (pRH5')	7954	MS2–BL3
pBH10 (pRH3')	7954	BL3–MS2
pBH14 (pRH5')	7983	MS2–BL2 726A>C
pBH12 (pRH3')	7983	BL2 726A>C–MS2
pBH15 (pRH5')	7982	MS2–BL2 Δ U719
pBH13 (pRH3')	7982	BL2 Δ U719–MS2
pBH16 (pRH5')	7982	MS2–BL2 Δ U719/716C>A
pBH17 (pRH5')	7952	MS2–BL3 Δ U2203/ Δ U2214
pBH20 (pRH5')	7957	MS2–BL1 10A>G
pBH23 (pRH3')	7957	BL1 30G>A–MS2
pBH25 (pRH5')	7957	MS2–BL1 30G>A
pBH24 (pRH3')	7956	BL1 Δ U24–MS2
pBH26 (pRH5')	7956	MS2–BL1 Δ U24
pBH27 (pRH3')	7957	BL1 31A>C–MS2
pBH28 (pRH5')	7957	MS2–BL1 31A>C

Table 2. Oligonucleotides used for the construction of hybrid-RNA-expressing plasmids

Oligonucleotide	Sequence (5'–3')
BL1 forward	AGC TTT GTT TAA ACC GAA GCT GAA TAG ATT CTT TAA AAC
BL1 reverse	TAT ACC CGG GCG CAT CCC GTC TAT CCG TTA TC
BL2 forward	AGC TTT GTT TAA ACC CAT TTA TGA AAA CGA ATA ATA GAT CAC C
BL2 reverse	TAT ACC CGG GGC CGA ATA CTT TTT GGG
BL3linker forward	AAA CAC ATC ATA TTT AAA ACC TTT TAA TGA AAG TCC AGA GAG GCT TGG AAG GGT TAT GAA GAG AAG GAC
BL3linker reverse	CCG GGT CCT TCT CTT CAT AAC CCT TCC AAG CCT CTC TGG ACT TTC ATT AAA AGG TTT TAA ATA TGA TGT GTT T
BL2mut forward	CCT TTT TAA GGG CAA TCC AGA GCG GTT GCA AAG AGG TGC ACA AC
BL2mut reverse	GTT GTG CAC CTC TTT GCA ACC GCT CTG GAT TGC CCT TAA AAA GG
BL2del forward	CAC CTT TTT AAG GGC AAC CAG AGA GGT TGC AAA GAG GTG CAC
BL2del reverse	GTG CAC CTC TTT GCA ACC TCT CTG GTT GCC CTT AAA AAG GTG
BL1mut30 forward	CTT TAA AAC AGT CCA GAA AGG CTG AGA AGG ATA ACG G
BL1mut30 reverse	CCG TTA TCC TTC TCA GCC TTT CTG GAC TGT TTT AAA G
BL1del forward	GAA TAG ATT CTT TAA AAC AGC CAG AGA GGC TGA GAA GG
BL1del reverse	CCT TCT CAG CCT CTC TGG CTG TTT TAA AGA ATC TAT TC
BL1mut31 forward	CTT TAA AAC AGT CCA GAG CGG CTG AGA AGG ATA ACG G
BL1mut31 reverse	CCG TTA TCC TTC TCA GCC GCT CTG GAC TGT TTT AAA G

two hybrid proteins activates transcription of reporter genes. The yeast strain used, L40-*ura3*, contained as reporters a *HIS3* gene and a *lacZ* gene under the control of *lexA* operator sites. The plasmid pHybLexA/Zeo-MS2 was used for the production of the fusion LexA-DBD-MS2 coat protein (Fig. 1). This hybrid protein, in which the bacterial LexA DNA-binding domain (LexA DBD) was fused to the MS2 coat protein, is known to recognize RNA stem-loop structures in MS2 RNA (Fig. 1). For the production of the second hybrid protein a construct was generated (pBH2; Table 1) that gave rise to the PyrR protein fused to ADVP16

(Fig. 1). As hybrid RNAs supposed to bridge the above-described two fusion proteins, BL1, BL2 or BL3, and variant forms of BL1, BL2 or BL3, respectively, fused to two tandemly repeated bacteriophage MS2 RNA recognition sites were used (Fig. 1) (see Table 1 for plasmids). Since the relative order of the MS2 sites and the RNAs to be tested can affect signal strength (Zhang *et al.*, 1999), the hybrid RNA molecules were produced in both possible orientations. For generating hybrid RNAs of BL1, BL2 and BL3 fused downstream to tandemly repeated bacteriophage MS2 RNAs, pRH5' was used (Table 1). For generating hybrid

Table 3. β -Galactosidase (LacZ) activity in yeast transformants: wild-type BLs

Units of β -galactosidase activity are defined according to Schneider *et al.* (1996). The two components present in each hybrid factor are depicted in the proper polarity, either N→C terminal, or 5'→3'. Fusion proteins are indicated in bold. LexA, transcriptional repressor from *E. coli*; MS2 (bold), coat protein from bacteriophage MS2; MS2 (not bold), MS2 RNA; AD, ADVP16; PyrR, PyrR from *B. subtilis* RNA-binding protein and regulator of pyrimidine biosynthesis; IRE, iron-responsive element (rat); IRP, iron-regulatory protein (rabbit); BL, RNA sequences within the attenuation regions of *B. subtilis pyr* mRNA.

Hybrid protein 1	Hybrid RNA	Hybrid protein 2	β -Galactosidase	Plasmid (hybrid RNA)
LexA-MS2	BL1-MS2	AD-PyrR	18.2 ($\pm$ 3.4)	pBH6
LexA-MS2	MS2-BL1	AD-PyrR	9.3 ($\pm$ 1.8)	pBH8
LexA-MS2	BL2-MS2	AD-PyrR	5.6 ($\pm$ 1.2)	pBH7
LexA-MS2	MS2-BL2	AD-PyrR	7.6 ($\pm$ 2.1)	pBH9
LexA-MS2	BL3-MS2	AD-PyrR	9.5 ($\pm$ 1.1)	pBH10
LexA-MS2	MS2-BL3	AD-PyrR	7.2 ($\pm$ 3.8)	pBH11
LexA-MS2	IRE-MS2	AD-IRP	23.2 ($\pm$ 6.0)	Invitrogen
LexA-MS2	-MS2	AD-PyrR	0.10 ($\pm$ 0.02)	pRH3'
LexA-MS2	MS2-	AD-PyrR	0.24 ($\pm$ 0.05)	pRH5'
LexA-MS2	-	AD-PyrR	0.17 ($\pm$ 0.03)	-
LexA-MS2	-	AD	0.11 ($\pm$ 0.02)	-

Table 4. β -Galactosidase (LacZ) activity in yeast transformants: mutated BLs

See Table 3 legend.

Hybrid protein 1	Hybrid RNA	Hybrid protein 2	β -Galactosidase	Plasmid (hybrid RNA)
LexA-MS2	BL2 726A > CMS2	AD-PyrR	0.45 ($\pm$ 0.2)	pBH12
LexA-MS2	MS2-BL2 726A > C	AD-PyrR	0.55 ($\pm$ 0.3)	pBH14
LexA-MS2	BL2 Δ U719-MS2	AD-PyrR	0.11 ($\pm$ 0.03)	pBH13
LexA-MS2	MS2-BL2 Δ U719	AD-PyrR	0.09 ($\pm$ 0.03)	pBH15
LexA-MS2	BL1 30G > A-MS2	AD-PyrR	0.40 ($\pm$ 0.05)	pBH23
LexA-MS2	MS2-BL1 30G > A	AD-PyrR	0.26 ($\pm$ 0.04)	pBH25
LexA-MS2	BL1 Δ U24-MS2	AD-PyrR	0.10 ($\pm$ 0.04)	pBH24
LexA-MS2	MS2-BL1 Δ U24	AD-PyrR	0.18 ($\pm$ 0.02)	pBH26
LexA-MS2	BL1 31A > C-MS2	AD-PyrR	0.85 ($\pm$ 0.16)	pBH27
LexA-MS2	MS2-BL1 31A > C	AD-PyrR	1.05 ($\pm$ 0.14)	pBH28
LexA-MS2	MS2-BL2 Δ U719/716C > A	AD-PyrR	0.08 ($\pm$ 0.01)	pBH16
LexA-MS2	MS2-BL3 Δ U2203/ Δ U2214	AD-PyrR	0.75 ($\pm$ 0.1)	pBH17
LexA-MS2	MS2-BL1 10A > G	AD-PyrR	10.7 ($\pm$ 4.5)	pBH20

RNAs of BL1, BL2 and BL3 fused upstream to tandemly repeated bacteriophage MS2 RNAs, pRH3' was used (Table 1).

To illustrate the *in vivo* production of the hybrid RNAs, the nucleotide sequence of one construct, BL1, fused downstream to MS2 RNA, is shown as an example (Fig. 2). In addition, the predicted secondary structures of the three BLs are depicted in Fig. 3A, B and C.

One of the MS2 hybrid-RNA-encoding plasmids to be tested (see Table 1), and pBH2 (ADVP16-PyrR fusion), were cointroduced into *S. cerevisiae* L40-*ura3* cells carrying pHybLexA/Zeo-MS2. The resulting transformants were analysed for reporter gene activity. Yeast strains that were able to grow on plates lacking histidine, and that produced LacZ, as shown by X-Gal staining, indicated an *in vivo* interaction between the BLs and PyrR (data not shown). The strength of the interaction between PyrR and the BLs was subsequently quantified using a LacZ liquid assay. The results of these experiments are summarized in Table 3: transformants carrying the LexA-MS2 coat protein and activation domain ADVP16-PyrR hybrids, along with the hybrid-RNA BL1-MS2, showed strong β -galactosidase activity (Table 3, row 1). The β -galactosidase activity was 18.2 units ($\pm$ 3.4), indicating that BL1 was capable of binding to PyrR *in vivo*. Strong activity was also detected for the hybrid-RNA MS2-BL1, where BL1 was produced downstream of the MS2 RNAs (Table 3, row 2); the β -galactosidase activity in this case, however, was only about 50 % (9.3 ± 1.8 units) of that of the BL1-MS2 construct. As shown in rows 3 and 4 (Table 3), the BL2-PyrR interaction was readily detectable *in vivo*; however, it did not appear to be as strong as the BL1-PyrR interaction: similar β -galactosidase activities were detected with the BL2 and MS2 RNA sites in either orientation with respect to one another (BL2-MS2, 5.6 ± 1.2 units; MS2-BL2, 7.6 ± 2.1 units). The BL3 hybrid RNAs (BL3-MS2 and

MS2-BL3) produced β -galactosidase activities within the same range as the β -galactosidase activities of the BL1 and BL2 constructs (Table 3, rows 5 and 6) (BL3-MS2, 9.5 ± 1.1 units; MS2-BL3, 7.2 ± 3.8 units).

MS2 RNAs without BL sequences did not produce β -galactosidase activity in transformant yeast strains. Thus, activation of the yeast reporter genes was dependent on the presence of the BL sequences in the hybrid RNAs, and this showed that the BL interactions with PyrR were specific (Table 3, rows 8 and 9). Furthermore, the activation domain and PyrR, and the activation domain alone, were not able to stimulate lacZ expression in the absence of hybrid RNAs (Table 3, rows 10 and 11). In contrast, the interaction between the iron responsive element and the iron regulatory protein produced strong β -galactosidase activity (23.2 ± 6.0 units), and served as a positive control (Table 3, row 7).

In order to provide further 'proof of principle' for the application of the yeast three-hybrid system to the analysis of transcription attenuation regulatory systems, mutagenesis experiments were carried out to determine the structural requirements for RNA binding by PyrR *in vivo*. The data are summarized in Table 4. Two structural variants of BL2 were tested in this work; these variants have been reported to show reduced binding activity *in vitro* (Bonner *et al.*, 2001) (Table 4, rows 1-4). As expected, the single nucleotide exchange 726A > C within BL2 (see Fig. 3B) resulted in drastically reduced affinity of BL2 towards PyrR (by a factor of > 12). The β -galactosidase activity of the 5' construct BL2 726A > C-MS2 was 0.45 units ($\pm$ 0.2) compared with 5.6 units ($\pm$ 1.2) for BL2-MS2. The β -galactosidase activity of the 3' construct MS2-BL2 726A > C was down to 0.55 units ($\pm$ 0.3), compared with 7.6 units ($\pm$ 1.2) for MS2-BL2. The deletion of U719 in the stem-loop of BL2 almost completely abolished PyrR binding; this was deduced from the virtually absent β -galactosidase activity of

Fig. 1. Experimental approach for the *in vivo* analysis of the interaction of PyrR from *B. subtilis* with the BL1, BL2 and BL3 of *pyr* mRNA using the yeast three-hybrid system and *S. cerevisiae* L40-*ura3*. A hybrid protein containing a DNA-binding domain (LexA DBD) and an RNA-binding domain (MS2 coat protein) localizes to the promoter of the reporter genes *HIS3/lacZ* through the LexA operator. This hybrid protein is produced by pHybLexA/Zeo-MS2. A second hybrid protein encoded by pBH2 (Table 1), and containing the transcriptional activation domain ADVP16 and PyrR from *B. subtilis*, activates transcription of the reporter genes when in close proximity to the genes' upstream regulatory sequences. Plasmid pRH5' or pRH3' is used for the *in vivo* production of a variety of hybrid RNA molecules to be tested for bridging the fusion proteins. The hybrid RNAs are composed of tandem repeats of MS2 RNA, and one of the BLs (BL1, BL2 or BL3). The hybrid RNAs are generated in both possible orientations, and they contain sites that are recognized by the two RNA-binding proteins. The resulting tripartite complex results in expression of the reporter genes (*HIS3/lacZ*). Only if an interaction between PyrR and one of the BLs occurs are the corresponding yeast transformants able to grow on medium lacking histidine. The strength of interaction between PyrR and the BLs can be quantified using the second reporter *lacZ*.

0.11 units (± 0.03) and 0.09 units (± 0.03) for the transformants of the 5' and 3' constructs.

Mutations in BL1, equivalent to those in BL2, were analysed, and the data are summarized in Table 4 (rows 5–10). The nucleotide exchange 30G>A within BL1 (see Fig. 3A) resulted in drastically reduced affinity of BL1 towards PyrR (by a factor of >36). The β -galactosidase activity of the 5' construct BL1 30G>A-MS2 was 0.40 units (± 0.05), compared with 18.2 units (± 3.4) for the wild-type BL1-MS2. The β -galactosidase activity of the corresponding 3' construct was down to 0.26 units (± 0.04), compared with 9.3 units (± 1.8) for MS2-BL1. The deletion of U24 in the stem-loop of BL1 strongly reduced PyrR binding; this was deduced from the low β -galactosidase activity of 0.10 units (± 0.04) and 0.18 units (± 0.02), respectively, within the transformants of the 5' and 3' constructs. The nucleotide

exchange 31A>C within BL1 also resulted in reduced β -galactosidase activity. The 5' construct produced 0.85 units (± 0.16), and the 3' construct produced 1.05 units (± 0.14) β -galactosidase activity.

During cloning of the RNA hybrid constructs of BL1, BL2 and BL3, spontaneous mutations occurred. These structural variants were analysed, and the respective data are shown in Table 4 (rows 11–13). In BL2 (see Fig. 3B), the combination of $\Delta U719$ and the nucleotide exchange 716A>C led to a completely inactive BL (0.08 ± 0.01 units). For BL3, the variant hybrid RNA BL3 $\Delta U2203/\Delta U2214$ (see Fig. 3C) also produced a drastically less active binding loop (0.75 ± 0.1 units). In contrast, the mutation (10A>G) (see Fig. 3A) in BL1 had no significant effect on PyrR binding; this was deduced from the strong residual β -galactosidase activity (10.7 ± 4.5 units) within the corresponding transformant.

DISCUSSION

The interaction of the *B. subtilis* regulatory protein PyrR with BL1, BL2 and BL3, which are located within the three different attenuation sites of *pyr* mRNA, has been analysed in great detail *in vitro* using gel mobility shift assays (Bonner *et al.*, 2001). It was found in that work that the apparent strength of the interaction between PyrR and the three different BLs was very different. In those experiments, PyrR bound significantly more tightly to the BLs of the second (BL2) and third (BL3) attenuation site than to the BL of the first (BL1) attenuation site. In the absence of uridine nucleotide cofactors, the apparent dissociation constants (K_d) for PyrR-BL interactions were 10 000 nM (BL1), 3 nM (BL2) and 200 nM (BL3)s. According to Bonner *et al.* (2001), the use of gel mobility shift assays imposed limitations for the determination of PyrR-BL interactions. They discussed that, since dissociation of nucleic-acid-protein complexes during electrophoresis was known to occur, the values listed as 'apparent' dissociation constants might well not be thermodynamically valid. In particular, it was surprising that BL1 apparently had a very low affinity towards PyrR, as compared with the other BLs (by factor of >3000), although the predicted secondary structures of BL1, BL2 and BL3 have a very similar size and shape (Bonner *et al.*, 2001) (see Fig. 3 of this work).

Our *in vivo* data, generated by using the yeast three-hybrid system, draw a somewhat different conclusion. The affinity of the wild-type forms of BL1, BL2 and BL3 towards PyrR was within the same range, with BL1 showing the strongest interaction. Thus, it seems that BL1, being the first anti-terminator of the *pyr* mRNA, is the most important regulatory element of the system. The termination through BL1 reflects the need for an early termination of transcription if pyrimidine nucleotides are present in sufficient amounts. The gene encoding the regulator PyrR is the first gene of the *pyr* operon, and is directly downstream of the untranslated attenuator region, which includes BL1. A

RNAse P leader sequence
 1 GUUUUACGUU UGAGGCCUCG UGGCGCACAU GGUACGCUGU GGUCUCGCG GCUGGGAACG
 61 AAACUCUGGG AGCUGCGAUU GGCAGAAUUC CAGCUUGCAU GCCUGCAGGU CGACUCUAGA MS2 1
 121 AAACAUGAGG AUCACCCAUG UCUGCAGGUC GACUCUAGAA AACAUAGAGGA UCACCCAUGU MS2 2
 181 CUGCAGGUCG ACUCUAGAGG AUCGCCCGUU UAAACcgaaG CUGAAUAGAU UCUUUAAAAC Up BL1
 241 AGUCCAGAGA GGCUGAGAAG GAUAACGGAU AGACGGGAUG cgCCCCGGGG AUCCACUAGU Down
 301 UCUAGCCGGA AUUCCCCCAU AUCCAACUUC CAAUUUAAUC UUUCUUUUUU

Fig. 2. The yeast three-hybrid system was used for the *in vivo* production of hybrid RNAs of MS2 and the BL1, BL2 and BL3 of *B. subtilis* *pyr* mRNA. As an example, the fusion between tandem repeats of MS2 (underlined, see arrows MS2 1 and MS2 2) and BL1 (underlined) is shown. For the generation of this hybrid RNA, pRH5' was used. As shown, this resulted in BL1 fused downstream to tandem repeats of MS2. In addition to MS2 and BL1, the protective RNase P leader sequence (underlined) is shown at the 5' end. The boxed nucleotides ('up' and 'down') indicate where additional nucleotides, as compared with the BL sequences BL1, BL2 and BL3 depicted in Fig. 3, may be located in the various hybrid-RNA constructs. As shown, the BL1 hybrid RNA contained four additional nucleotides upstream, and three additional nucleotides downstream (see up and down). The BL2 hybrid RNAs contained five nucleotides upstream, and eight nucleotides downstream. BL3 did not contain additional nucleotides.

marginal readthrough of the first attenuation site should provide the cell with sufficient amounts of PyrR to secure regulation.

The yeast three-hybrid system also proved to be useful for the rapid analysis of structural requirements for PyrR–BL binding. The mutation BL2 726A>C within the terminal hexaloop of BL2 (Fig. 3B) caused a reduction in PyrR binding (by factors of 12 and 14, depending on the orientation of the hybrid RNAs). Using gel mobility shift assays (Bonner *et al.*, 2001), this mutation has been described to produce a drastically higher K_d for the PyrR/BL2 726A>C interaction (20 000 nM), as compared with the wild-type BL2 (0.7 nM). Another mutation (Δ U719) located in the upper stem-loop of BL2 also diminished PyrR binding in our experiments (by factors of 51 and 84, depending on the orientation of the hybrid RNAs). In comparison, the K_d for the PyrR–BL2 Δ U719 interaction has been determined to be 100 000 nM using gel mobility shift assays.

Considering that BL1 seems to be more important than BL2 as an anti-antiterminator, mutant equivalents to those of BL2 were made for BL1. The mutation BL1 31A>C (Fig. 3A), which corresponds to BL2 726A>C, apparently weakened PyrR binding (by factors of 21 and 9, depending on the orientation of the hybrid RNAs). The deletion BL1 Δ U24, a mutation corresponding to BL2 Δ U719, also reduced the affinity of PyrR towards BL1 (by factors of 182 and 52, depending on the orientation of the hybrid RNAs). The mutation BL1 30G>A also resulted in less LacZ activity within the transformant yeast strains. The apparent binding of BL1 towards PyrR in this case was reduced by factors of 46 and 36, depending on the orientation of the hybrid RNAs.

Using gel mobility shift assays, the equivalent mutation in the binding loop BL2 (BL2 725G>A) had a smaller effect on PyrR binding (K_d 600 nM for BL2 725G>A, compared with 0.7 nM for the wild-type BL2).

As examples, predictions are shown for how the three mutations may affect the secondary structures of the corresponding RNA molecules for BL1 (Fig. 4). The deletion of uracil (BL1 Δ U24/BL2 Δ U719) converts the terminal hexaloop into a stable pentaloop, which is a dramatic structural change. The binding of PyrR seems to be strongly affected by this altered structure. The mutation A>C reduces PyrR binding to BL1 and also to BL2; however, the reduction is not as much as that for the uracil deletion. Interestingly, the A>C mutation may also produce a pentaloop (Fig. 4). This pentaloop, however, is more able to easily switch into the hexaloop state than the pentaloops of BL1 Δ U24 and BL2 Δ U719 (Bonner *et al.*, 2001). Consequently, PyrR binding is enhanced. The G>A mutation within BL1 significantly affects PyrR binding, although the purine–purine exchange is conservative. Obviously, PyrR interacts very specifically with single nucleotides within the BL terminal loop.

We show that the yeast three-hybrid system is very well suited to qualitatively and quantitatively analyse bacterial regulatory systems that are based on factor-independent transcriptional attenuation. To the best of our knowledge, this is the first report using the yeast three-hybrid system to address this type of investigation (Jaeger *et al.*, 2004). The limitations of electrophoretic gel mobility shift assays to quantify RNA–protein interactions are mentioned above. Our experiments also reveal that the yeast three-hybrid system is sensitive to a number of variables. Since folding of

Fig. 3. Nucleotide sequences and predicted secondary structures of anti-antiterminators BL1 (A), BL2 (B) and BL3 (C) of *B. subtilis* *pyr* mRNA, according to Bonner *et al.* (2001). BL1, BL2 and BL3 are located within the regulatory attenuation sites of the 5' untranslated leader of the operon upstream of *pyrR* (BL1), between the first (*pyrR*) and second (*pyrP*) genes (BL2), and between the second and third (*pyrB*) genes of the operon (BL3). Numbers denote the *pyr* nucleotide number, where +1 is the transcriptional start site (Quinn *et al.*, 1991). The mutations (arrows) introduced during this work are in bold.

the 5' end of an RNA molecule in the time course of transcription is kinetically favoured, the hybrid RNAs to be tested were produced upstream as well as downstream of the MS2 RNA. For the wild-type BLs BL2 and BL3, no significant differences were detected. For these BLs, the thermodynamically favoured formation of the strong tandemly arranged MS2 RNAs had no influence on BL formation. For BL1, however, the data obtained from the

liquid assay showed that if the BL is located at the 5' end of the transcript, the interaction with PyrR is almost twice as high as that for BL2 and BL3. This effect has been observed for other three-hybrid experiments (SenGupta *et al.*, 1996).

Regulation of gene expression by attenuation involving RNA-binding proteins seems to be a common theme in prokaryotic systems (Yanofsky, 2000). For *B. subtilis* alone,

Fig. 4. Predicted secondary structures of variants of anti-antiterminator BL1. The structures were generated using the DINAMelt Server (Markham & Zuker, 2005).

203 attenuation regions are known or predicted (<http://www.bork.embl-heidelberg.de/Docu/attenuation/table1.html>), and, in some cases, it is possible that a protein is involved in stabilizing important secondary structures. These attenuation regions, and the corresponding regions of other organisms, could be used as baits to identify as yet unknown RNA-binding proteins by screening genomic libraries using the yeast three-hybrid system.

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