PrcR, a PucR-type transcriptional activator, is essential for proline utilization and mediates proline-responsive expression of the proline utilization operon *putBCP* in *Bacillus subtilis*

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The soil bacterium *Bacillus subtilis* can utilize exogenous proline as a sole nitrogen or carbon source. The proline-inducible *putBCP* (formerly *ycgMNO*) operon encodes proteins responsible for proline uptake and two-step oxidation of proline to glutamate. We now report that a gene (formerly *ycgP*, now designated *prcR*) located downstream of the *putBCP* operon is essential for *B. subtilis* cells to utilize proline as a sole nitrogen or carbon source. Disruption of the *prcR* gene also abolished proline induction of *putB* transcription. *prcR* expression is not subject to autoregulation and proline induction. The PrcR protein shows no significant amino acid sequence similarity to the known transcriptional activators for proline utilization genes of other bacteria, but it does show partial amino acid sequence similarity to the transcriptional regulator PucR for the purine degradation genes of *B. subtilis*. PrcR orthologues of unknown function are present in some other *Bacillus* species. Primer-extension analysis suggests that both *putB* and *prcR* are transcribed by a σ^A -dependent promoter. Deletion and mutation analysis revealed that an inverted repeat (5'-TTGTGG-N5-CCACAA-3') centred at position -76 relative to the transcriptional initiation site of *putB* is essential for *putB* expression. Electrophoretic mobility shift assays showed that the purified His-tagged PrcR was capable of binding specifically to this inverted repeat. Altogether, these results suggest that PrcR is a PucR-type transcriptional activator that mediates expression of the *B. subtilis putBCP* operon in response to proline availability.

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INTRODUCTION

The soil bacterium *Bacillus subtilis* can use exogenous proline as a sole nitrogen or carbon source. The gene products of the *putBCP* (formerly *ycgMNO*) operon are responsible for proline uptake and two-step oxidation of proline to glutamate (Bremer, 2002). The *putB*, *putC* and *putP* genes encode proline dehydrogenase, Δ^1 -pyrroline-5-carboxylate dehydrogenase, and a proline uptake protein, respectively. Disruption of the *putBCP* operon abolishes the ability of *B. subtilis* cells to use exogenous proline as a sole nitrogen or carbon source (Bremer, 2002). Proline can also serve as a source of glutamate for *B. subtilis* mutants deficient in glutamate synthase, since proline can be metabolized to glutamate by PutB and PutC (Atkinson *et al.*, 1990). Exogenous proline can induce the expression of the *putBCP* operon and proline dehydrogenase activity in *B. subtilis* (Atkinson *et al.*, 1990; Bremer, 2002).

Abbreviations: EMSA, electrophoretic mobility shift assay; RT-qPCR, real-time quantitative PCR.

A supplementary table, showing the oligonucleotide primers used in this study, is available with the online version of this paper.

However, the regulatory mechanism for proline induction of *putBCP* expression in *B. subtilis* remains unknown.

In *Escherichia coli*, *Salmonella typhimurium* and some other bacteria, the *putA* gene encodes a bifunctional proline utilization protein with both the proline dehydrogenase and Δ^1 -pyrroline-5-carboxylate dehydrogenase activities for the two-step oxidation of proline to glutamate (Menzel & Roth, 1981; Lee *et al.*, 2003; Tanner, 2008). In some of these bacteria, the PutA protein can also act as a transcriptional repressor for controlling expression of the *putA* and *putP* genes. PutA switches between its roles as a transcriptional repressor and as a catabolic enzyme in response to proline availability (Ostrovsky de Spicer & Maloy, 1993; Muro-Pastor *et al.*, 1997; Soto *et al.*, 2000; Gu *et al.*, 2004; Commichau & Stülke, 2008). In *Agrobacterium tumefaciens*, *Rhodobacter capsulatus* and *Vibrio vulnificus*, expression of the *putA* gene is activated by the Lrp/AsnC-type transcriptional activator PutR in response to exogenous proline (Keuntje *et al.*, 1995; Cho & Winans, 1996; Lee & Choi, 2006). In *Pseudomonas aeruginosa* PAO1, the *putAP* operon is positively regulated by the AraC/XylS-type transcriptional activator PruR (Nakada *et al.*, 2002). In this study, we have

found that the *prcR* gene is essential for *B. subtilis* cells to utilize proline as a sole nitrogen or carbon source. PrcR shows no significant amino acid sequence similarity to the above-mentioned transcriptional regulators of proline utilization genes from various bacteria. We present evidence that PrcR is a transcriptional activator that mediates proline-responsive expression of the *B. subtilis putBCP* operon.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. The oligonucleotide primers are listed in Supplementary Table S1. *E. coli* and *B. subtilis* cells were grown in Luria–Bertani (LB) medium (Sambrook & Russell, 2001) or in modified M9 minimal medium, which consisted of 0.05 % NaCl, 0.05 % MgSO₄, 0.1 % NH₄Cl, 0.0001 % FeSO₄, 0.0002 % MnSO₄, 0.002 % CaCl₂, 0.3 % KH₂PO₄, 1.7 % Na₂HPO₄ · 12H₂O and 0.4 % glucose, unless specified otherwise. Tryptophan was supplemented at a final concentration of 50 µg ml⁻¹. Antibiotics were used at the following concentrations (µg ml⁻¹): ampicillin, 100; chloramphenicol, 5; erythromycin, 1.

Plasmid construction. To construct plasmid pGS2047, a DNA fragment carrying an internal region close to the N terminus of *prcR* and flanked by *Hind*III and *Bam*HI sites was amplified by PCR and cloned between *Hind*III and *Bam*HI sites of the thermosensitive plasmid pRN5101 (Fedhila *et al.*, 2002). To construct plasmids

pGS2090, pGS2097 and pGS2098, DNA fragments carrying various lengths of the regulatory region plus the N-terminal coding region of *putB* and flanked by *Eco*RI and *Bam*HI sites were amplified by PCR and cloned individually between *Eco*RI and *Bam*HI sites of the integrative promoter probe vector pDL (Yuan & Wong, 1995). To construct plasmid pGS2140, a DNA fragment carrying the regulatory region plus the N-terminal coding region of *prcR* and flanked by *Eco*RI and *Bam*HI sites was amplified by PCR and cloned into pDL. To construct plasmid pGS2135, which carries a 4 bp mutation in the right arm of the inverted repeat (with sequence alteration from CCAC to GGTG), a two-step PCR method (Higuchi *et al.*, 1988) was used for site-directed mutagenesis. The PCR-amplified DNA fragment was restricted with *Eco*RI plus *Bam*HI and then cloned into pDL. To construct plasmid pGS2111, which can overproduce His-tagged PrcR, a DNA fragment carrying the coding sequence of *prcR* and flanked by *Bam*HI and *Hind*III sites was amplified by PCR and cloned between *Bam*HI and *Hind*III sites of pQE80 (Qiagen). The correctness of sequences of PCR-amplified DNA fragments was verified by DNA sequencing.

Disruption of the chromosomal *prcR* gene. Disruption of the chromosomal *prcR* gene by integration of plasmid pRN5101-derived pGS2047 through a Campbell-like single-crossover recombination was performed as described previously (Fedhila *et al.*, 2002). The integration plasmid was introduced into *B. subtilis* cells by the protoplast method (Chang & Cohen, 1979). The correctness of integrants was verified by PCR.

Construction of strains with a *bgaB* fusion integrated at the *amyE* locus. Competent cells of *B. subtilis* were prepared and

Table 1. Strains and plasmids used in this study

Abbreviations: Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance.

Strain or plasmid	Genotype or description	Source or reference
<i>E. coli</i> strain		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	Takara
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	Laboratory stock
BM1657	<i>trpC2 amyE::pGS2090</i> [Cm ^R]	This work
BM1660	<i>trpC2 amyE::pGS2090 prcR::pGS2047</i> [Cm ^R Em ^R]	This work
BM1663	<i>trpC2 prcR::pGS2047</i> [Em ^R]	This work
BM1667	<i>trpC2 amyE::pGS2097</i> [Cm ^R]	This work
BM1668	<i>trpC2 amyE::pGS2098</i> [Cm ^R]	This work
BM1704	<i>trpC2 amyE::pGS2135</i> [Cm ^R]	This work
BM1715	<i>trpC2 amyE::pGS2140</i> [Cm ^R]	This work
BM1736	<i>trpC2 amyE::pGS2140 prcR::pGS2047</i> [Cm ^R Em ^R]	This work
Plasmids		
pDL	<i>amyE</i> integration vector for generation of transcriptional fusions to <i>bgaB</i> , Ap ^R Cm ^R	Yuan & Wong (1995)
pQE80	Expression vector for producing His-tagged proteins, Ap ^R	Qiagen
pRN5101	Vector used for gene disruption, Ap ^R Em ^R	Fedhila <i>et al.</i> (2002)
pGS2047	pRN5101 carrying an internal region close to the N terminus of <i>prcR</i> ; <i>prcR</i> disruption plasmid	This work
pGS2090	pDL carrying the regulatory region of <i>putB</i>	This work
pGS2097	pDL containing the inverted repeat in the regulatory region of <i>putB</i>	This work
pGS2098	pDL containing half of the inverted repeat in the regulatory region of <i>putB</i>	This work
pGS2111	pQE80 expressing the <i>prcR</i> gene	This work
pGS2135	pDL carrying a mutation in the inverted repeat of the regulatory region of <i>putB</i>	This work
pGS2140	pDL carrying the regulatory region of <i>prcR</i>	This work

transformed by pDL-derived integrative plasmids, as described elsewhere (Contente & Dubnau, 1979). Transformants were selected by growth on chloramphenicol-containing LB agar plates. Correct integrants were screened by a method described elsewhere (O'Kane *et al.*, 1986).

RNA isolation, cDNA synthesis and primer-extension analysis.

Total RNA was isolated according to a procedure described elsewhere (Zuber & Losick, 1983). Isolated RNA was treated with RQ1 RNase-free DNase (Promega) at 37 °C for 30 min. The reaction solution was then incubated with RQ1 stop buffer at 65 °C for 10 min to inactivate DNase. AMV reverse transcriptase (Promega) and random hexamer were used to synthesize cDNA for RT-PCR and real-time quantitative PCR (RT-qPCR) according to the instructions of the manufacturer. Determination of the transcriptional initiation sites of *putB* and *prcR* by primer-extension was carried out as described elsewhere (Inoue & Cech, 1985), using primers A251 and A364, respectively.

RT-PCR and RT-qPCR. RT-PCR and RT-qPCR were performed with the above-mentioned synthesized cDNA as the template and a *putB*-specific PCR primer pair (A365 and A368) plus a 16S rRNA-specific PCR primer pair (A366 and A367). RT-qPCR was carried out with the StepOnePlus Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR Master Mix kit (Applied Biosystems) according to the instructions of the manufacturer. The 16S rRNA level was used as an internal control to normalize the *putB* mRNA level. A non-template negative control in which cDNA was replaced with water was also included in each PCR run.

Overproduction and purification of the His-tagged PrcR protein. *E. coli* JM109 cells bearing plasmid pGS2111 were grown in LB medium. After the OD₆₀₀ had reached 0.5, IPTG was added at a final concentration of 0.3 mM and incubation was continued for 2 h. After harvesting cells by centrifugation and disrupting resuspended cells by sonication on ice, the disrupted cells were subjected to centrifugation at 15 000 g for 10 min. The resulting supernatant was used for the purification of His-tagged PrcR protein by affinity chromatography on a nickel-nitrilotriacetic acid (Ni-NTA) agarose column according to the instructions of the manufacturer (Qiagen).

Electrophoretic mobility shift assays (EMSAs). EMSAs used to determine the binding of PrcR to DNA were carried out as described elsewhere (Fried & Crothers, 1981). Binding solutions were run on 5% native polyacrylamide gels. Bands were visualized by using a Molecular Dynamics PhosphorImager. The phosphorimage was analysed with ImageQuant software.

RESULTS AND DISCUSSION

Genetic organization and sequence analysis of the flanking genes of the *putBCP* operon

The *ycgL* gene, located adjacent to and upstream from the *putBCP* operon of *B. subtilis* (Fig. 1a), encodes a potential protein of 260 aa predicted to be a putative member of the nucleotidyltransferase superfamily. The spacing between *ycgL* and *putB* is 190 bp. The *ycgP* gene, located downstream of the *putBCP* operon and separated from *putP* by a 152 bp intergenic region (Fig. 1a), is now designated *prcR* (for proline catabolism regulator), because *prcR* is absolutely required for *putB* expression as shown below. A potential ρ -independent transcription terminator ($-20.1 \text{ kcal mol}^{-1}$; $-84.1 \text{ kJ mol}^{-1}$) is present within the *putP*–*prcR* intergenic region. Further downstream of *prcR* is the convergently

orientated *ycgQ* gene (Fig. 1a), which encodes a putative protein of 285 aa of unknown function. These features suggest that the *prcR* gene constitutes a monocistronic operon. PrcR (411 aa) shows no significant amino acid sequence similarity with the known proline catabolism-related transcriptional activator PruR (250 aa) of *P. aeruginosa* PAO1 or PutR activators (154–164 aa) of various bacteria, whereas it shows partial amino acid sequence similarity with the transcriptional regulator PucR (531 aa) of *B. subtilis* purine degradation genes (Schultz *et al.*, 2001; Beier *et al.*, 2002) (22.2% identity and 43.8% similarity between amino acids 112–409 of PrcR and amino acids 227–528 of PucR). A potential helix–turn–helix DNA-binding motif (Dodd & Egan, 1990) is present at the C terminus of PrcR (amino acids 361–382). A similar helix–turn–helix DNA-binding motif is present at the corresponding positions of PucR (amino acids 480–501).

Role of *prcR* in utilization of proline as a sole nitrogen or carbon source for growth

The *putBCP* operon is known to be responsible for proline utilization in *B. subtilis* (Bremer, 2002). To examine whether its downstream gene *prcR* plays a role in utilization of proline as a sole nitrogen or carbon source for cell growth, a *prcR* disruption mutant (BM1663) was constructed as described in Methods. It was found that the wild-type *B. subtilis* cells and the *prcR* mutant grew equally well (doubling time about 60 min) in modified M9 minimal medium containing 0.4% glucose and 20 mM NH₄Cl as the sole carbon and nitrogen sources, respectively. However, when 20 mM proline was used to substitute for 20 mM NH₄Cl as the sole nitrogen source, the *prcR* mutant could not grow within 24 h, whereas the wild-type grew with a doubling time of about 95 min. When 0.4% proline was used to substitute for 0.4% glucose as the sole carbon source, the wild-type grew with a doubling time of about 120 min, whereas the growth of the *prcR* mutant was undetectable under the same growth condition. These observations indicate that the *prcR* gene is essential for *B. subtilis* cells to utilize proline as a sole nitrogen or carbon source.

prcR is necessary for expression of the *putBCP* operon

To determine whether the requirement of *prcR* for proline utilization is due to the fact that *prcR* is required for expression of the *putBCP* operon, a transcriptional fusion of the regulatory region of *putB* to *bgaB* was constructed (pGS2090) and integrated at the *amyE* locus of the wild-type cells and the *prcR* mutant to generate strains BM1657 and BM1660, respectively. Cells were grown in LB medium for various periods of time. It was found that expression of the *putB* promoter region–*bgaB* fusion was abolished in the *prcR* mutant when compared with that in the wild-type (Fig. 2a). We also analysed the effect of disruption of *prcR* on *putB* expression by RT-PCR in a qualitative manner and by RT-qPCR. RNA was isolated from wild-type *B. subtilis*

Fig. 1. Construction of gene cassettes. For clarity in visualizing smaller features, the diagram is not to scale. The three dots on each side of the type D, E and G arrays indicate that they are much larger than depicted here. Note that the ELAN reactions also yield linear concatemers, which for simplicity are not shown in the scheme.

cells and the *prcR* mutant grown in LB medium for 5 h. As shown in Fig. 2(b, c), disruption of *prcR* abolished *putB* expression at the RNA level. Taken together, these results indicate that *prcR* is necessary for expression of the *putBCP* operon.

Proline induction of *putB* expression is PrcR-dependent

It is known that proline can induce the expression of the *putBCP* operon and proline dehydrogenase activity in *B. subtilis* (Atkinson *et al.*, 1990; Bremer, 2002). We next attempted to investigate whether proline induction of *putB* expression is mediated through PrcR. The above-mentioned strains BM1657 and BM1660 were grown in modified M9 minimal medium containing 0.4% glucose to OD₆₀₀ 0.3, and then treated or not treated with 1 mM proline. It was found that proline could induce expression of the transcriptional fusion of the *putB* promoter region to *bgaB* in the wild-type cells (BM1657) (Fig. 3a), but not in the *prcR* mutant (BM1660) (Fig. 3b). These results indicate that proline induction of *putB* expression is PrcR-dependent.

Identification of the transcriptional initiation sites of *putB* and *prcR*

We next performed primer-extension analysis to determine the transcriptional initiation sites of *putB* and *prcR*. A synthetic oligonucleotide complementary to the 5' end of *putB* or *prcR* was used as the probe. RNA was isolated from *B. subtilis* cells grown in LB medium to OD₆₀₀ 0.5. As shown in Fig. 4(a), one major extension product was detected for *putB*. The size of the extension product indicates that the 5' end of the transcript is located 40 bp upstream from the translational start site of *putB*. This transcriptional initiation site is at an appropriate distance from a σ^A -dependent promoter with a spacing of 18 bp between the -35 box (TTGTGA) and the -10 box (TACAAT) (Fig. 1b). One major extension product was also detected for *prcR* (Fig. 4b). The 5' end of the *prcR* transcript is deduced to be located 24 bp upstream from the translational start site of *prcR*. This transcriptional initiation site is at an appropriate distance from a σ^A -dependent promoter with a spacing of 17 bp between the -35 box (TATACA) and the -10 box (TATGAT) (Fig. 1c).

Fig. 2. Effect of disruption of *prcR* on *putB* expression. (a) Expression of a transcriptional fusion of the regulatory region of *putB* to *bgaB* (pGS2090) integrated at the *amyE* locus of the wild-type cells (BM1657) (●) or the *prcR* mutant (BM1660) (■). Cells were grown in LB medium for various periods of time as indicated. Solid lines, BgaB activities; dotted lines, growth curves. Each value is the mean $\pm$ SD of three determinations. (b) Transcriptional analysis of *putB* expression by RT-PCR with *putB*-specific primers (A365 and A368) and 16S rRNA-specific primers (A366 and A367). RNAs isolated from wild-type *B. subtilis* cells and the *prcR* mutant grown in LB medium for 5 h were used for cDNA synthesis. PCR products were run on a 2% agarose gel and stained with ethidium bromide. (c) Relative expression of *putB* in wild-type *B. subtilis* cells (1) and the *prcR* mutant (2) analysed by RT-qPCR. Each value is the mean and SD of three determinations.

Effect of deletion or mutation of the inverted repeat in the regulatory region of *putB* on *putB* expression *in vivo*

Inspection of the sequence of the regulatory region of *putB* revealed a 17 bp inverted repeat (TTGTGG-N₅-CCACAA) that is centred at position -76 relative to the transcriptional initiation site of *putB* (Fig. 1b). To examine whether

Fig. 3. Effect of disruption of *prcR* on proline induction of *putB* expression. Expression of a transcriptional fusion of the regulatory region of *putB* to *bgaB* (pGS2090) integrated at the *amyE* locus of wild-type cells (BM1657) (a) or the *prcR* mutant (BM1660) (b). Cells were grown in modified M9 minimal medium containing 0.4% glucose to OD₆₀₀ 0.3 and then divided into two parts. One part was left untreated (●), and the other was treated with 1 mM proline at time 0 (■). The cells were then grown for various periods of time as indicated. Solid lines, BgaB activities; dotted lines, growth curves. Each value is the mean $\pm$ SD of three determinations.

this inverted repeat plays a regulatory role in *putB* expression, we constructed three plasmid pDL-based derivatives as shown in Fig. 5(a), and integrated them individually at the *amyE* locus of the wild-type cells. Fig. 5(b) shows that either deletion of the left arm or mutation in the right arm of the inverted repeat abolished the expression of the *putB* promoter region-*bgaB* fusion. These results indicate that the inverted repeat centred at position -76 is important for *putB* expression. It is interesting to note that the transcriptional activator PurR of *Lactococcus lactis* binds to a PurBox that is also centred at position -76 relative to the transcriptional initiation site of *purC* and *purD* (Kilstrup *et al.*, 1998). This PurBox has been found to be required for high-level promoter activity.

Interaction of PrcR with the inverted repeat in the regulatory region of *putB* *in vitro*

To explore whether PrcR could bind to the regulatory region of *putB* *in vitro*, we constructed plasmid pGS21111,

Fig. 4. Primer-extension analyses of the transcriptional initiation sites of *putB* (a) and *prcR* (b). Total RNA was isolated from *B. subtilis* cells grown in LB medium to OD₆₀₀ 0.5. Lanes 1 show the primer-extension products. Dideoxy sequencing ladders obtained with the same primer as used for primer-extension analysis are shown in lanes A, T, C and G. The sequences shown are complementary to those read from the ladders. The arrows indicate the transcriptional initiation sites.

which was able to overproduce His-tagged PrcR in *E. coli*, and then purified His-tagged PrcR by affinity chromatography on an Ni-NTA agarose column. A ³²P-labelled DNA fragment containing the *putB* promoter region was used as the probe. A ³²P-labelled DNA fragment containing the *proH* promoter region (Belitsky *et al.*, 2001) was used as the control probe. An EMSA showed that purified His-tagged PrcR could retard the DNA fragment containing the *putB* promoter region, but could not retard the control probe at the assay concentrations (Fig. 6a). We also used the unlabelled DNA fragment containing the

Fig. 5. (a) Schematic representation of the pDL-based plasmid constructs integrated at the *amyE* locus in *B. subtilis*. A four-base substitution (CCAC to GGTG, as shown in parentheses) within the inverted repeat (shown by an inverted pair of arrows) in the *putB* promoter region is represented by a cross. (b) BgaB activities of *B. subtilis* cells carrying pGS2097 (●), pGS2098 (■) or pGS2135 (▲) and grown in LB medium for various periods of time, as indicated. Solid lines, BgaB activities; dotted lines, growth curves. Each value is the mean $\pm$ SD of three determinations.

putB promoter region as the specific competitor DNA and the unlabelled DNA fragment containing the *proH* promoter region as the non-specific competitor DNA. The ³²P-labelled DNA fragment containing the *putB* promoter region was used as the probe in an EMSA. It was found that the specific competitor DNA could reduce the formation of PrcR–DNA complex, whereas the non-specific competitor DNA at the same range of concentrations could not (Fig. 6b). Taken together, these results indicate that PrcR can bind to the regulatory region of *putB* in a specific manner.

To further investigate whether PrcR is able to interact specifically with the inverted repeat in the regulatory region of *putB*, a double-stranded oligonucleotide containing the inverted repeat [oligo(*putB*), wild-type] and a double-stranded oligonucleotide containing a mutation in the inverted repeat [oligo(*putB*), mutant] (Fig. 6c) were used as the probes in an EMSA. It was found that PrcR could form a complex with the wild-type oligo(*putB*) but could not form a complex with the mutant oligo(*putB*) under similar assay conditions (Fig. 6c). These results indicate that PrcR can bind to the inverted repeat in a specific manner.

Fig. 6. EMSAs of the interaction of PrcR with various DNA probes. (a) A DNA fragment containing the *putB* promoter region (spanning from -163 to $+17$ relative to its transcriptional initiation site) was used as the probe in lanes 1–4. A DNA fragment containing the *proH* promoter region (Belitsky *et al.*, 2001) (spanning from -42 to $+123$) was used as the control probe in lanes 5–8. About 0.5 nM ^{32}P -labelled DNA probe was used in each reaction mixture (final volume, 30 μl). Lanes 1 and 5, DNA probe alone; lanes 2–4 and 6–8, DNA probe plus increasing amounts of the purified His-tagged PrcR (100, 200 and 400 ng, respectively). (b) A ^{32}P -labelled DNA fragment (about 0.5 nM) containing the *putB* promoter region was used as the probe in lanes 1–8. The same but unlabelled DNA fragment was used as the specific competitor DNA in lanes 2–4. An unlabelled DNA fragment containing the *proH* promoter region was used as the non-specific competitor DNA in lanes 6–8. Lanes 1 and 5, DNA probe plus PrcR; lanes 2–4 and 6–8, DNA probe plus PrcR and increasing amounts of the competitor DNA (10-, 50- and 150-fold molar excess, respectively). Four-hundred nanograms of PrcR was used in each reaction mixture in lanes 1–8. (c) A ^{32}P -labelled double-stranded oligonucleotide containing the inverted repeat (shown by a pair of inverted arrows) in the *putB* promoter region was used as the probe in lanes 1–5 [Oligo(*putB*), WT]. The sequence of the non-template strand is shown at the bottom of the panel. A ^{32}P -labelled double-stranded oligonucleotide containing mutations (shown by dotted lines) in the inverted repeat was used as the probe in lanes 6–10 [Oligo(*putB*), Mutant]. About 1 nM ^{32}P -labelled DNA probe was used in each reaction mixture (final volume, 30 μl). Lanes 1 and 6, DNA probe alone; lanes 2–5 and 7–10, DNA probe plus increasing amounts of PrcR (100, 200, 400 and 800 ng, respectively).

B. R. Belitsky had also independently reported the characterization of YcgP, which he designated PutR (Belitsky, 2011).

prcR expression is not autoregulated and is not subject to proline induction

To investigate whether *prcR* expression is subject to autoregulation, a transcriptional fusion of the regulatory region of *prcR* to *bgaB* (pGS2140) was constructed and integrated at the *amyE* locus of the wild-type cells and the *prcR* mutant. Cells were grown in LB medium for various periods of time. It was found that there was no difference in the expression level of the *prcR* promoter region–*bgaB* fusion between the wild-type cells and the *prcR* mutant (data not shown). We also tested whether *prcR* expression is subject to proline induction. Cells were grown in modified M9 minimal medium as described above. No proline induction of *prcR* expression was observed (data not shown).

Concluding remarks

In conclusion, proline utilization in *B. subtilis* is PrcR-dependent. PrcR mediates proline-responsive expression of the proline utilization operon *putBCP* through interaction with the inverted repeat in the regulatory region of *putB*. During the submission of this manuscript, we were aware that

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