INTRODUCTION

Pectin is a constituent of plant cell walls. The main chain of pectin is composed of galacturonate residues, some of which are in the form of methyl or acetyl esters. Genetic pathways involved in pectin degradation are well studied in the plant pathogen Erwinia chrysanthemi, which causes soft-rot diseases by degradation of pectin in plant cell walls (Hugouvieux-Cotte-Pattat et al., 1996). This bacterium secretes several enzymes that degrade pectin into oligogalacturides. These oligogalacturides are transported into the bacterium via two transporters, TogT and TogMNAB (Hugouvieux-Cotte-Pattat et al., 2001; Hugouvieux-Cotte-Pattat & Reverchon, 2001), and are further metabolized into two kinds of monomers: galacturonate and DKI (5-ketodeoxyuronate). They are subsequently catabolized by two independent pathways to form a common intermediate, KDG (2-keto-3-deoxygluconate). DKI is isomerized into DKII (2,5-diketo-3-deoxygluconate) by the isomerase encoded by kduI (Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1989). DKII is then reduced to KDG by the reductase encoded by kduD (Chatterjee et al., 1985). On the other hand, uptake of galacturonate, DKI, DKII and KDG into Er. chrysanthemi cells is known to be mediated by two transport systems: ExuT for galacturonate uptake (Hugouvieux-Cotte-Pattat & Robert-Baudouy, 1987; San Francisco & Keenan, 1993) and KdgT for uptake of DKI, DKII and KDG (Condemine & Robert-Baudouy, 1987; Hugouvieux-Cotte-Pattat et al., 1996). These biochemical pathways are shown in Fig. 1.

In Er. chrysanthemi, the KdgR repressor controls transcription of most genes involved in pectin degradation (Rodionov et al., 2000, 2004). The consensus sequence of the KdgR box obtained from computer analysis of upstream regions of known KdgR-regulated genes from Er. chrysanthemi is AAATGAAACANTGTTTCATTT (Rodionov et al., 2000). It is wider than the previously proposed KdgR-binding consensus AATG(A/G)(A/G)NN(T/C)(G/A)TT(C/T)A (Nasser et al., 1994). KDG is the true inducer that binds to the KdgR repressor to cause dissociation of KdgR from its operator (Nasser et al., 1992).

Regulation of the kduID operon of Bacillus subtilis by the KdgR repressor and the ccpA gene: identification of two KdgR-binding sites within the kdgR-kduID intergenic region

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Transcription of the Bacillus subtilis kdgRKAT operon, which comprises genes involved in the late stage of galacturonate utilization, is known to be negatively regulated by the KdgR repressor. In this study, Northern analysis was carried out to demonstrate that the kdgR gene also negatively regulates the kduID operon, encoding ketodeoxyuronate isomerase and ketodeoxygluconate reductase. It has also been demonstrated that expression of the kduID operon can be induced by galacturonate and is subject to catabolite repression by glucose. The ccpA gene was found to be involved in this catabolite repression. Primer extension analysis identified a ρ^7-like promoter sequence preceding kduI. Gel mobility shift assays and DNase I footprinting analyses indicated that KdgR is capable of binding specifically to two sites within the kdgR-kduID intergenic region in vitro. Reporter gene analysis revealed that these two KdgR-binding sites function in vivo. One site is centred 33.5 bp upstream of the translational start site of kdgR and can serve as an operator for controlling expression of the kdgRKAT operon. The other is centred 57.5 bp upstream of the translational start site of kduI and can serve as an operator for controlling expression of the kduID operon. Possible physiological significance of this regulation is discussed.
encode enzymes responsible for degradation of galacturonate into KDG (Fig. 1). Expression of these genes, including exuT, is negatively regulated by the ExuR repressor encoded by the same locus (Mekjian et al., 1999). The operator for the ExuR repressor is a 26 bp perfect inverted repeat (TCAAAATGTTAAGTTACATTTTGAGA) located between the exuIP promoter and the uxaC gene. Another locus that contains two divergently transcribed operons, kdgRKAT and kduID, was also identified by homology with *Er. chrysanthemi* genes. These operons encode enzymes for conversion of DKI into KDG and KDG into pyruvate plus glyceraldehyde 3-phosphate (Pujic et al., 1998) (Fig. 1). It was demonstrated that the *kdgRKAT* operon of *B. subtilis* is negatively regulated by the KdgR repressor encoded by the first gene of the *kdgRKAT* operon. Expression of the *kdgRKAT* operon can be induced by galacturonate and repressed by glucose (Pujic et al., 1998). Amino acid sequence comparison revealed no significant similarity between the KdgR repressors of *B. subtilis* and *Er. chrysanthemi*. The operator for the *B. subtilis* KdgR repressor has not been identified yet. In this report, we present evidence that the KdgR repressor can also negatively regulate expression of the *kduID* operon. We show that expression of the *kduID* operon can be induced by galacturonate and is subject to catabolite repression by glucose. We have identified two *KdgR*-binding sites within the *kdgR*-kduI intergenic region, which serve as operators for controlling expression of the *kdgRKAT* and *kduID* operons, respectively.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *B. subtilis* cells were grown in Luria–Bertani (LB) medium (Sambrook et al., 1989). Antibiotics were used at the following concentrations (μg ml⁻¹): ampicillin, 100; chloramphenicol, 5; erythromycin, 2; kanamycin, 10; tetracycline, 5.

**Construction of plasmids.** To construct the *kdgR*-disruptive plasmid pGS1624, a 0.29 kb DNA fragment containing the internal region of *kdgR* and flanked by HindIII and BamHI sites was amplified by PCR and cloned between the HindIII and BamHI sites of the thermosensitive replicative plasmid pRN5101 (Fedhila et al., 2002). To construct the *ccpA*-disruptive plasmid pGS1601, a 0.32 kb DNA fragment containing the internal region of *ccpA* and flanked by HindIII and BamHI sites was amplified by PCR and cloned between the HindIII and BamHI sites of pRN5101.

To construct plasmid pGS1436, which can overproduce a maltose-binding protein (MalE)-KdgR fusion protein, a 1.06 kb DNA fragment carrying the coding sequence of *kdgR* and flanked by BamHI and HindIII sites was cloned between the BamHI and HindIII sites of pMAL-c2 (New England Biolabs).

To construct a plasmid that overproduces KdgR in *B. subtilis*, a 1.08 kb DNA fragment carrying the Shine–Dalgarno sequence plus the *kdgR* gene and flanked by EcoRI and HindIII sites was amplified by PCR and cloned between the EcoRI and HindIII sites of pHY300PLK (Takara Shuzo Co.) to generate plasmid pGS1513. The promoter of the tetracycline resistance gene present in pHY300PLK can thus drive the expression of *kdgR* in *B. subtilis*.

To construct plasmid pGS1454, a 0.49 kb DNA fragment that contains the promoter region plus the N-terminal coding region of *kduI* and is flanked by BamHI and HindIII sites was amplified by PCR and cloned between the BamHI and HindIII sites of pLC4 (Ray et al., 1985). Various DNA fragments flanked by SalI and HindIII sites in plasmids pGS1574, pGS1575, pGS1560 and pGS1561 as shown in Fig. 7 were amplified by PCR and cloned individually between the SalI and HindIII sites of the promoter probe vector pUBCAT (Wen et al., 1989).

**Disruption of the chromosomal *kdgR* and *ccpA* genes.** Disruption of the chromosomal *kdgR* gene or *ccpA* gene by integration of pRN5101-derived pGS1624 or pGS1601 through a Campbell-like single-crossover recombination was performed as described by Fedhila et al. (2002). Plasmid pGS1624 or pGS1601 was introduced into *B. subtilis* cells by the protoplast method (Chang & Cohen, 1979). Transformants were first grown at permissive temperature (30°C) and then transferred to nonpermissive temperature (39°C). Finally, integrants were selected on LB agar plates at 39°C for resistance to erythromycin. The correctness of integrants was verified by PCR.

**Primer extension and Northern analyses.** Total RNA was prepared by the previously described method (Zuber & Losick, 1983).
The kduI transcriptional start site was determined by the previously described method of primer extension (Inoue & Cech, 1985) using synthetic oligonucleotide 5'-TTGTTCAGGATGTACAGAA-3'. Northern analysis was carried out as described by Ausubel et al. (1994).

**Overproduction and purification of the MalE-KdgR fusion protein.** E. coli JM109 cells bearing plasmid pGS1436 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 suspended cells by sonication on ice, the disrupted cells were subjected to centrifugation at 15 000 g for 10 min. Purification of the MalE-KdgR fusion protein on an amylose column was carried out according to the instructions of the matrix manufacturer (New England Biolabs). In order to cleave the MalE from KdgR, 100 μg MalE-KdgR was incubated with 1 μg factor Xa protease (New England Biolabs) at 4°C for 2 days. The progress of cleavage was checked by SDS-13% PAGE. The released KdgR protein was used without further purification.

**Gel mobility shift assays.** Gel mobility shift assays to determine binding of KdgR to DNA were carried out as described by Fried & Crothers (1981) with slight modification (Lee et al., 2001). For determination of the apparent dissociation constant (Kd), 32P-labelled DNA fragments at a concentration of 100 μM were titrated with various concentrations of KdgR. The KdgR protein was assumed to be a dimer in solution. Binding solutions were subjected to non-denaturing 8% PAGE, and bands were visualized by using a Molecular Dynamics PhosphorImager. The phosphorimage was analysed with ImageQuant software.

**Other methods.** Transformation of B. subtilis cells by the protoplast method was carried out as described by Chang & Cohen (1979). DNase I footprinting analysis was performed exactly as previously described (Chiou et al., 2002). An established method was used for spectrophotometric measurement of CAT activity (Shaw, 1973). Protein concentrations were determined by the BCA protein assay method according to the instructions of the manufacturer (Pierce Biotechnology) with bovine serum albumin as the standard.

**RESULTS AND DISCUSSION**

**Induction of the kduID operon by galacturonate**

To determine whether galacturonate could induce expression of the kduI and kduD genes (Fig. 2a), Northern analysis with probes specific to the kduID region was performed to detect the corresponding transcript. RNA was prepared from B. subtilis cells growing exponentially in LB medium in the absence or presence of 0.5% galacturonate. As shown in Fig. 3(a), a transcript of about 1.7 kb was detected with a probe specific to the kduI gene. The band intensity in the presence of galacturonate was about 4.5-fold higher than that in its absence. Likewise, a transcript of similar size was detected and a similar fold-induction by galacturonate was observed when a kduD-specific probe was used instead (Fig. 3b). Since the kduI and kduD genes are 825 bp and 762 bp long, respectively, and the spacing between them is only 1 bp, the 1.7 kb transcript detected on Northern blots with either kduI-specific or kduD-specific probe is likely to be produced from co-transcription of kduID. Taken together, these observations suggest that kduI and kduD are organized as an operon whose expression can be induced by galacturonate.
Negative regulation of the kduID operon by the kdgR gene

To investigate whether the kdgR gene is involved in regulation of the kduID operon, a kdgR disruption mutant (BM1048) was constructed as described in Methods, and subjected to Northern analysis. As shown in Fig. 3(a, b), the band intensity of the kduID transcript from the kdgR mutant was substantially higher than that from the wild-type. Complementation of the kdgR mutant with a plasmid carrying the wild-type kdgR gene markedly repressed expression of the kduID operon no matter whether kduI- or kduD-specific probe was used (Fig. 3d, e). These results suggest that the kdgR gene is involved in negative regulation of kduID expression. Moreover, the level of kduID expression in the kdgR mutant grown in the presence of galacturonate was about 1.3-fold higher than that in its absence (Fig. 3a, b). As a control, a DNA fragment

Fig. 3. Northern analyses of induction of the kduID operon by galacturonate and the regulatory role of the kdgR gene. (a–c) Total RNA was isolated from wild-type B. subtilis cells (WT) or the kdgR mutant BM1048 grown in LB medium with (+) or without (−) the addition of 0.5% galacturonate (GalU) to an OD600 of 0.6. A kduI-specific (a), kduD-specific (b) or pgk-specific (c) probe was used for hybridization. (d, e) Total RNA was isolated from the kdgR mutant carrying kdgR-expressing plasmid pGS1513 (lane 2) or the control vector pHY300PLK (lane 1) grown in LB medium to an OD600 of 0.6. A kduI-specific (d) or kduD-specific (e) probe was used for hybridization.

Negative regulation of the kduID operon by the kdgR gene

To investigate whether the kdgR gene is involved in regulation of the kduID operon, a kdgR disruption mutant (BM1048) was constructed as described in Methods, and subjected to Northern analysis. As shown in Fig. 3(a, b), the band intensity of the kduID transcript from the kdgR mutant was substantially higher than that from the wild-type. Complementation of the kdgR mutant with a plasmid carrying the wild-type kdgR gene markedly repressed expression of the kduID operon no matter whether kduI-specific or kduD-specific probe was used (Fig. 3d, e). These results suggest that the kdgR gene is involved in negative regulation of kduID expression. Moreover, the level of kduID expression in the kdgR mutant grown in the presence of galacturonate was about 1.3-fold higher than that in its absence (Fig. 3a, b). As a control, a DNA fragment
corresponding to an internal region of the *B. subtilis* pgk gene, which encodes phosphoglycerate kinase, was also used as a probe in Northern analysis. Multiple RNA bands could be detected with the pgk-specific probe (Fig. 3c). This is in agreement with a previous report (Ludwig et al., 2001). No significant difference in band intensity was observed between the wild-type and *kdgR* mutant grown either in the presence of galacturonate or in its absence (Fig. 3c). This implies that both KdgR-dependent and KdgR-independent galacturonate induction of the *kdulD* operon are involved.

**Involvement of the ccpA gene in catabolite repression of the *kdulD* operon**

Northern analysis was also carried out to test whether expression of the *kdulD* operon is subject to catabolite repression by glucose. As shown in Fig. 4, 0.5 % galacturonate could induce expression of the *kdulD* operon in the wild-type *B. subtilis*, but when cells were grown in the presence of 0.5 % galacturonate plus 2 % glucose, expression of the *kdulD* operon was reduced to a very low level, suggesting catabolite repression of the *kdulD* operon. Previous studies have shown that the CcpA protein is a major transcription factor mediating catabolite repression in *B. subtilis* (Stulke & Hillen, 2000). To further test whether the *ccpA* gene is involved in glucose repression of *kdulD*, a *ccpA* disruption mutant (BM1034) was constructed as described in Methods. Fig. 4 shows that inactivation of the *ccpA* gene abolished catabolite repression of the *kdulD* operon. Previous studies have shown that the *ccpA* gene is involved in glucose repression of the *kdulD* operon, indicating the involvement of the *ccpA* gene. Moreover, the expression level of *kdulD* in the *ccpA* mutant grown in the presence of galacturonate was considerably higher than that observed in the wild-type. In a control experiment, using the pgk-specific probe did not detect a significant difference in the level of control RNA between the wild-type and *kdgR* mutant grown in the presence of galacturonate (Fig. 4c). A similar result was also observed with the *kdulD*-specific probe in the *ccpA* mutant grown in the presence of galacturonate (data not shown). The underlying mechanism remains to be explored.

**Identification of a σ^A^-like promoter sequence preceding *kdul***

We next used primer extension analysis to determine the transcriptional initiation site of the *kdulD* operon. RNA was isolated from *B. subtilis* cells carrying plasmid pGS1454 (Table 1) and growing exponentially in LB medium. A 19-mer synthetic oligonucleotide complementary to the 5′ end of *kdulD* was used as the probe. As shown in Fig. 2(b), one major extension product was detected. The size of the extension product indicates that the 5′ end of the transcript is located 63 bp upstream from the translational start site of *kdulD*. This transcriptional initiation site is at an appropriate distance from a σ^A^-like promoter sequence (Helmann, 1995) (TTGACT for the −35 box and TAAAT for the −10 box) (Fig. 2a), suggesting that expression of the *kdulD* operon is likely to be driven by a σ^A^-dependent promoter.

**Binding of KdgR to the *kdul* promoter region in vitro**

We then explored whether purified KdgR could bind to the *kdul* promoter region in vitro. To facilitate purification, we constructed plasmid pGS1436, which could overproduce a maltose-binding protein (MalE)-KdgR fusion protein. This MalE-KdgR fusion protein was purified from the crude extract of *E. coli* cells carrying pGS1436 by affinity chromatography on an amylose column. The KdgR protein released from cleavage of purified MalE-KdgR fusion protein with factor Xa protease was used without further purification. Gel mobility shift assays showed that the KdgR protein could bind to a 0.19 kb DNA fragment containing the *kdul* promoter region, but not to a 0.2 kb control DNA fragment containing the bscR promoter region (Lee et al., 2001) (Fig. 5a). These results indicated that the KdgR repressor could bind directly and specifically to the *kdul* promoter region. The affinity of KdgR for the *kdul* promoter region was also assessed in a gel mobility shift assay. For determination of the half-maximal binding, decreases in band intensity of the free DNA probe as a function of KdgR concentrations were measured. The apparent dissociation constant (Kd) was estimated to be 0.62 nM.

![Fig. 4](http://mic.sgmjournals.org) Northern analyses of the role of *ccpA* in catabolite repression of *kdulD*. Total RNA was isolated from wild-type *B. subtilis* cells (WT) or the *ccpA* mutant BM1034 grown in LB medium with (+) or without (−) the addition of 0.5 % galacturonate (GalU) or 2 % glucose (Glu) to an OD_600 of 0.6. A *kdul*-specific (a), *kdulD*-specific (b) or pgk-specific (c) probe was used for hybridization.
The location of the binding site for KdgR in the *kduI* promoter region was then determined by DNase I footprinting analysis. When the template strand of *kduI* DNA was end-labelled with 32P, the addition of KdgR protein protected the sequences from +3 to +16 (relative to the transcriptional initiation site of *kduI*) from digestion by DNase I. This region is a part of an 18 bp imperfect inverted repeat (from +2 to +15) (Figs 2a and 6a). When the non-template strand of *kduI* DNA was end-labelled with 32P, no protected region was detected under the assay conditions (Fig. 6b). It is not unprecedented that binding of a DNA-binding protein to DNA occurs only on one DNA strand. *In vitro* DNase I footprinting analysis showed that binding of the *Mycobacterium fortuitum* RepB protein to the origin of replication occurs on one DNA strand only (Stolt & Stoker, 1996). *In vivo* footprinting analysis revealed that protein–DNA interactions at a human DNA replication origin also occur on one DNA strand only (Dimitrova et al., 1996).

To further investigate whether KdgR could interact specifically with the 18 bp inverted repeat, a double-stranded oligonucleotide containing the inverted repeat (positions −3 to +15) in the *kduI* promoter region was used as the probe in lanes 6–10 [Oligo(*kduI*), WT]. The sequence of the non-template strand is shown at the bottom of the panel. A double-stranded oligonucleotide containing a 4 bp mutation in the inverted repeat (positions −9 to +26) in the *kdgR* promoter region was used as the probe in lanes 6–10 [Oligo(*kdgR*), WT]. A double-stranded oligonucleotide containing a 4 bp mutation in the inverted repeat was used as the probe in lanes 1–5 [Oligo(*kdgR*), mutant]. Lanes 1 and 6, DNA probe alone; lanes 2–5 and 7–10, DNA probe plus increasing amounts of KdgR (0.05, 0.1, 0.2 and 0.4 ng, respectively). About 3 nM 32P-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 KdgR (0.05, 0.1, 0.2 and 0.4 ng, respectively). Samples were run on native 10% polyacrylamide gels.

**Binding of KdgR to the *kdgR* promoter region in vitro**

It is known that KdgR can negatively regulate expression of the *kdgRKAT* operon in *B. subtilis*, but the operator for this
control has not been identified (Pujic et al., 1998).
Therefore, we attempted to use gel mobility shift assays to examine whether purified KdgR could also interact with the kdgR promoter region in vitro. As shown in Fig. 5(b), KdgR was capable of binding to the kdgR promoter region but not to the control DNA, suggesting that the binding is specific. The affinity of KdgR for the kdgR promoter region was determined in a similar way to that described for the kduI promoter. The apparent $K_d$ was estimated to be 0.49 nM.

DNase I footprinting analysis was also used to determine the location of the binding site for KdgR in the kdgR promoter region. When the template strand of kdgR DNA was end-labelled with $^{32}$P, the addition of KdgR protein protected the sequences from +12 to +31 [relative to the transcriptional initiation site of kdgR (Pujic et al., 1998)] from digestion by DNase I. This region is also a part of another 18 bp imperfect inverted repeat (from +9 to +26) (Figs 2a and 6c), whose nucleotide sequence is quite similar to that of the 18 bp inverted repeat present in the kduI promoter region (15 out of 18 nucleotides are identical). When the non-template strand of kdgR DNA was end-labelled with $^{32}$P, the KdgR protein was found to protect the segment from +8 to +28 (Figs 2a and 6d).

To further examine whether KdgR could interact specifically with the 18 bp inverted repeat, a double-stranded oligonucleotide containing the inverted repeat [Oligo(kdgR), wild-type] and a double-stranded oligonucleotide containing a 4 bp mutation in the 18 bp inverted repeat present in the kdgR promoter region (Fig. 5d) were used as probes in gel mobility shift assays. The result showed that KdgR was capable of binding to the wild-type Oligo(kdgR) but not to the mutant Oligo(kdgR), suggesting that the wild-type inverted repeat is also a binding site for KdgR.

We also used gel mobility shift assays to test whether galacturonate could exert its inductive effects by dissociating the KdgR–DNA complex directly. It was found that galacturonate at a concentration of up to 0.5 % (w/v) did not interfere with the formation of KdgR–DNA complex in vitro (data not shown). This result suggests that galacturonate per se is not the actual inducer; its metabolic derivative(s) may be the authentic inducer. It has been demonstrated that in Er. chrysanthemi, KDG is the true inducer that causes dissociation of KdgR from its operator (Nasser et al., 1992). It remains to be explored whether this is the case with B. subtilis.

Fig. 6. DNase I footprinting analysis of KdgR binding to the kduI and kdgR promoter regions. (a) A 0.19 kb BamHI–HindIII DNA fragment containing the kduI promoter region (positions −88 to +96) and labelled with $^{32}$P at the HindIII site of the template strand was incubated with or without the KdgR protein. Lanes 1 and 6, no KdgR protein; lanes 2–5 contained 100, 200, 400 and 800 ng KdgR, respectively. The numbers on the left indicate the positions of bases relative to the transcriptional initiation site of kduI. The protected regions are indicated by brackets. (b) The same 0.19 kb BamHI–HindIII DNA fragment was labelled with $^{32}$P at the BamHI site of the non-template strand and incubated with or without KdgR. Lanes 1 and 6, no KdgR protein; lanes 2–5 contained 100, 200, 400 and 800 ng KdgR, respectively. (c) A 0.19 kb HindIII–BamHI DNA fragment containing the kdgR promoter region (positions −72 to +119) and labelled with $^{32}$P at the BamHI site of the template strand was incubated with or without KdgR. Lanes 1 and 6, no KdgR protein; lanes 2–5 contained 100, 200, 400 and 800 ng KdgR, respectively. (d) The same 0.19 kb HindIII–BamHI DNA fragment was labelled with $^{32}$P at the HindIII site of the non-template strand and incubated with or without KdgR. Lanes 1 and 6, no KdgR protein; lanes 2–5 contained 100, 200, 400 and 800 ng KdgR, respectively.
Effect of deletion of the KdgR-binding site in the kduI promoter region on expression of the kduI promoter-cat fusion in vivo

To examine effect of deletion of the KdgR binding site in the kduI promoter region on expression of the kduI promoter-cat transcriptional fusion in vivo, DNA fragments containing the kduI promoter region with or without the 18 bp inverted repeat were amplified by PCR and transcriptionally fused to a promoterless cat gene on the promoter probe vector pUB_CAT (Wen et al., 1989). The resulting plasmids, pGS1560 and pGS1561 (Fig. 7), were individually introduced into B. subtilis cells and crude cell extracts were assayed for CAT activity. As shown in Table 2, when the 18 bp inverted repeat was present on plasmid pGS1560, galacturonate could enhance expression of the kduI promoter-cat fusion, probably by removing the KdgR repressor from this site. Deletion of the 18 bp inverted repeat led to derepression of the kduI promoter-cat fusion, and galacturonate could not further enhance kduI promoter-cat expression. These results are consistent with the idea that the 18 bp inverted repeat in the kduI promoter region acts as a KdgR-binding site and contributes to the control of kduID expression in vivo. The aforementioned Northern analysis revealed that the level of kduID expression in the kdgR mutant BM1048 was about 1.3-fold higher in the presence of galacturonate than that in its absence. However, results from CAT activity assays showed that galacturonate did not further enhance expression of the kduI promoter-cat fusion after deletion of the KdgR-binding site. These two observations imply that other galacturonate-responsive element(s) or repressor-binding site(s) may exist outside the kduI promoter region we tested.

Concluding remarks

In this study we have provided evidence that the B. subtilis KdgR repressor can directly regulate expression of the kduID

Table 2. Effect of deletion of KdgR-binding site on expression of kduI promoter-cat fusion or kdgR promoter-cat fusion in vivo

| Plasmid in B. subtilis* | Addition† | Specific activity of CAT‡ [mU (mg protein)] | D
|------------------------|-----------|---------------------------------------------|---
| pGS1560                | None      | 224 ± 18                                    | 1
|                        | GalU      | 362 ± 33                                    | 1
|                        | GalU + Glu| 201 ± 14                                    | 1
| pGS1561                | None      | 538 ± 49                                    | 1
|                        | GalU      | 511 ± 56                                    | 1
|                        | GalU + Glu| 481 ± 57                                    | 1
| pGS1574                | None      | 202 ± 16                                    | 2
|                        | GalU      | 294 ± 24                                    | 2
|                        | GalU + Glu| 180 ± 16                                    | 2
| pGS1575                | None      | 290 ± 21                                    | 2
|                        | GalU      | 259 ± 33                                    | 2
|                        | GalU + Glu| 173 ± 17                                    | 2
| pUB_CAT                | None      | 9 ± 1                                       | 2
|                        | GalU      | 9 ± 2                                       | 2
|                        | GalU + Glu| 8 ± 2                                       | 2

*The listed plasmids are shown in Fig. 7.
†Abbreviations: GalU, galacturonate; Glu, glucose.
‡B. subtilis cells carrying the listed plasmids were grown to an OD₆₀₀ of 0.8 in LB medium with or without 0.5% galacturonate, plus 2% glucose or not. After sonication and centrifugation, crude extracts were subjected to CAT assays.

Fig. 7. Schematic representation of plasmid constructs. The pUB_CAT-based plasmids contain a promoterless cat reporter gene preceded by various DNA fragments. The number above each DNA fragment denotes base position relative to the transcriptional initiation site of kduI or kdgR. The black bars below the kdgR–kduI intergenic region represent two 18 bp inverted repeats.
It is not difficult to imagine why KdgR controls expression of the kduID operon. Both KdgK and KdgA proteins are required to metabolize the metabolic derivatives of galacturonate (Pujic et al., 1998). When galacturonate is present, kdgK and kdgA are derepressed and more KdgK and KdgA proteins are synthesized to carry out their functions. On the other hand, the observation that KdgR also controls kduID expression raises an interesting question, since Kdul and KduD proteins are known to be involved in metabolism of DKI and DKII, which are not metabolic derivatives of galacturonate (Pujic et al., 1998). One possible explanation is as follows. When the kdgT gene is derepressed due to the presence of galacturonate, more DKI and DKII present in the environment can be transported into cells via the KdgT transporter (Condemine & Robert-Baudouy, 1987; Hugouvieux-Cotte-Pattat et al., 1996). Therefore, the kduID operon is derepressed in the presence of galacturonate and more Kdul and KduD proteins are synthesized to metabolize DKI and DKII.

It is thus conceivable that when both kdgRKAT and kduID operons are subject to negative control by the KdgR repressor, galacturonate can induce expression of both operons to carry out their functions.

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REFERENCES


Bacillus subtilis: evidence for the presence of multiple levels of control of the gapA operon. Mol Microbiol 41, 409–422.


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