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-kduI* 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 σ^A -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-kduI* 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.

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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 AAT(G/A)AAA(C/T)NN(T/C)(G/A)T-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).

Bacillus subtilis is a soil bacterium that has easy access to galacturonate as well as its metabolic precursors and derivatives present in the soil. A genetic locus that is essential for growth of *B. subtilis* on galacturonate as a carbon source has been identified (Mekjian *et al.*, 1999). This locus contains the *uxaA*, *uxaB* and *uxaC* genes, which

Abbreviations: DKI, 5-ketodeoxyuronate; DKII, 2,5-diketo-3-deoxygluconate; KDG, 2-keto-3-deoxygluconate.

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 (TCAAAATGTTAACGTTAACATTTTGA) located between the *exuP1* 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

Fig. 1. Possible biochemical pathways for degradation of pectin and galacturonate in *Er. chrysanthemi* or *B. subtilis*. The designations of genes that encode enzymes involved in catalysis of some steps of the degradative pathways are specified near the corresponding arrows. *kduL*, 5-keto-4-deoxyuronate isomerase; *kduD*, 2-keto-3-deoxygluconate oxidoreductase; *kdgK*, 2-keto-3-deoxygluconate kinase; *kdgA*, 2-keto-3-deoxygluconate-6-phosphate aldolase; *uxaC*, uronate isomerase; *uxaB*, altronate oxidoreductase; *uxaA*, altronate dehydratase. ExuT, KdgT, TogMNAB and ToqT are transporters.

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 ($\mu\text{g ml}^{-1}$): 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 *Hind*III and *Bam*HI sites was amplified by PCR and cloned between the *Hind*III and *Bam*HI 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 *Hind*III and *Bam*HI sites was amplified by PCR and cloned between the *Hind*III and *Bam*HI 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 *Bam*HI and *Hind*III sites was cloned between the *Bam*HI and *Hind*III 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 *Eco*RI and *Hind*III sites was amplified by PCR and cloned between the *Eco*RI and *Hind*III 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 *Bam*HI and *Hind*III sites was amplified by PCR and cloned between the *Bam*HI and *Hind*III sites of pLC4 (Ray *et al.*, 1985). Various DNA fragments flanked by *Sal*I and *Hind*III sites in plasmids pGS1574, pGS1575, pGS1560 and pGS1561 as shown in Fig. 7 were amplified by PCR and cloned individually between the *Sal*I and *Hind*III sites of the promoter probe vector pUB_{GAT} (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).

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'[traD36 proAB⁺ lacI^q lacZΔM15]</i>	Takara
<i>B. subtilis</i>		
168	<i>trpC2</i>	Laboratory stock
BM1048	<i>trpC2 kdgR::pGS1624 [Em^R]</i>	This work
BM1034	<i>trpC2 ccpA::pGS1601 [Em^R]</i>	This work
Plasmids		
pUB _{CAT}	Promoter probe vector, Ap ^R Km ^R	Wen <i>et al.</i> (1989)
pLC4	Promoter probe vector, Ap ^R Cm ^R	Ray <i>et al.</i> (1985)
pMAL-c2	Expression vector for producing fusions of maltose-binding protein, Ap ^R	New England Biolabs
pRN5101	Vector used for gene disruption, Ap ^R Em ^R	Fedhila <i>et al.</i> (2002)
pHY300PLK	Shuttle vector, Ap ^R Tc ^R	Takara
pGS1624	pRN5101 carrying the <i>kdgR</i> internal region	This work
pGS1601	pRN5101 carrying the <i>ccpA</i> internal region	This work
pGS1436	pMAL-c2 carrying the <i>kdgR</i> gene	This work
pGS1513	pHY300PLK with an insert containing the Shine–Dalgarno sequence plus the <i>kdgR</i> gene	This work
pGS1454	pLC4 carrying the promoter region and the N-terminal region of <i>kduI</i>	This work
pGS1560	pUB _{CAT} carrying the <i>kduI</i> promoter region with operator(<i>kdu</i>)	This work
pGS1561	pUB _{CAT} carrying the <i>kduI</i> promoter region without operator(<i>kdu</i>)	This work
pGS1574	pUB _{CAT} carrying the <i>kdgR</i> promoter region with operator(<i>kdg</i>)	This work
pGS1575	pUB _{CAT} carrying the <i>kdgR</i> promoter region without operator(<i>kdg</i>)	This work

The *kduI* transcriptional start site was determined by the previously described method of primer extension (Inoue & Cech, 1985) using synthetic oligonucleotide 5'-TTGTTTCAGGATGTACAGAA-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 resuspended 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 (*K_d*), ³²P-labelled DNA fragments at a concentration of 100 pM were titrated with various concentrations of KdgR. The KdgR protein was assumed to be a dimer in solution. Binding solutions were subjected to nondenaturing 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, 1975). 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.

Fig. 2. (a) Genetic organization of the *kdgRKAT* and *kduID* operons of *B. subtilis* and the nucleotide sequence of the *kdgR-kduI* intergenic region. A putative ρ -independent transcription terminator is shown at the 3' end of each operon. The -35 and -10 regions of the σ^A -like promoter of *kduI* and *kdgR* are shown. Two 18 bp imperfect inverted repeats are indicated by inverted pairs of arrows below the sequences. S/D, putative Shine-Dalgarno sequences. (b) Primer extension analysis of the transcriptional initiation site of the *kduID* operon. Total RNA was isolated from *B. subtilis* cells carrying pGS1454 and grown in LB medium to an OD₆₀₀ of 0.6. Lane 1 shows the primer extension product. Dideoxy sequencing ladders obtained with the same primer as used for primer extension analysis are shown in lanes G, A, T, C. The sequence shown is complementary to that read from the ladder. The arrow indicates the transcriptional initiation site.

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 *kduID* operon are involved.

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

Northern analysis was also carried out to test whether expression of the *kduID* operon is subject to catabolite repression by glucose. As shown in Fig. 4, 0.5 % galacturonate could induce expression of the *kduID* 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 *kduID* operon was reduced to a very low level, suggesting catabolite repression of the *kduID* 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 *kduID*, 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 *kduID* operon, indicating the involvement of the *ccpA* gene. Moreover, the expression level of *kduID* 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 *kdgRKAT* operon 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 *kduI*

We next used primer extension analysis to determine the transcriptional initiation site of the *kduID* 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 *kduI* 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 *kduI*. This transcriptional initiation site is at an appropriate distance from a σ^A -like promoter sequence (Hermann, 1995) (TTGACT for the -35 box and TAAAAT for the -10 box) (Fig. 2a), suggesting that expression of the *kduID* operon is likely to be driven by a σ^A -dependent promoter.

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

We then explored whether purified KdgR could bind to the *kduI* 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 *kduI* 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 *kduI* promoter region. The affinity of KdgR for the *kduI* 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 (K_d) was estimated to be 0.62 nM.

Fig. 4. Northern analyses of the role of *ccpA* in catabolite repression of *kduID*. 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 OD₆₀₀ of 0.6. A *kduI*-specific (a), *kduD*-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 ^{32}P , 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 -3 to +15) (Figs 2a and 6a). When the non-template strand of *kduI* DNA was end-labelled with ^{32}P , 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*

Fig. 5. Gel mobility shift assays of interaction of KdgR with various DNA probes. (a) A 0.2 kb control DNA fragment containing the *B. subtilis bscR* promoter region (Lee *et al.*, 2001) (lanes 1–5) and a 0.19 kb DNA fragment containing the *B. subtilis kduI* promoter region (positions -88 to +96) (lanes 6–10) were used as probes. About 100 pM ^{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 KdgR (0.05, 0.1, 0.2 and 0.4 ng, respectively). Samples were run on native 8% polyacrylamide gels. (b) The same control DNA fragment at the same concentration as described in Fig. 4(a) (lanes 1–5) and a 0.19 kb DNA fragment containing the *B. subtilis kdgR* promoter region (positions -72 to +119) (lanes 6–10) were used as probes. 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). (c) A double-stranded oligonucleotide containing the 18 bp 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 (shown at the bottom of the panel) was used as the probe in lanes 1–5 [Oligo(*kduI*), mutant]. About 3 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 KdgR (2, 4, 8 and 16 ng, respectively). Samples were run on native 10% polyacrylamide gels. (d) A double-stranded oligonucleotide containing the 18 bp 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 (2, 4, 8 and 16 ng, respectively).

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 [Oligo(*kduI*), wild-type] and a double-stranded oligonucleotide containing a 4 bp mutation in the inverted repeat [Oligo(*kduI*), mutant] (Fig. 5c) were used as probes in gel mobility shift assays. The result showed that KdgR was capable of binding to the wild-type Oligo(*kduI*) but not to the mutant Oligo(*kduI*), suggesting that the wild-type inverted repeat is a binding site for KdgR.

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

Fig. 6. DNase I footprinting analysis of KdgR binding to the *kduI* and *kdgR* promoter regions. (a) A 0.19 kb *Bam*HI–*Hind*III DNA fragment containing the *kduI* promoter region (positions –88 to +96) and labelled with 32 P at the *Hind*III 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 *Bam*HI–*Hind*III DNA fragment was labelled with 32 P at the *Bam*HI 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 *Hind*III–*Bam*HI DNA fragment containing the *kdgR* promoter region (positions –72 to +119) and labelled with 32 P at the *Bam*HI 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. (d) The same 0.19 kb *Hind*III–*Bam*HI DNA fragment was labelled with 32 P at the *Hind*III 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.

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 [Oligo(*kdgR*), mutant] (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*.

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

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.

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.

Effect of deletion of the KdgR-binding site in the *kdgR* promoter region on expression of the *kdgR* promoter-*cat* fusion *in vivo*

To examine the effect of deletion of the KdgR-binding site in the *kdgR* promoter region on expression of the *kdgR* promoter-*cat* transcriptional fusion *in vivo*, plasmids pGS1574 and pGS1575, which contain the *kdgR* promoter region with or without the 18 bp inverted repeat, respectively, were constructed (Fig. 7). As shown in Table 2, addition of galacturonate to LB medium could enhance expression of the *kdgR* promoter-*cat* fusion in pGS1574. Deletion of the 18 bp inverted repeat led to derepression of the *kdgR* promoter-*cat* fusion and galacturonate could not further enhance *kdgR* promoter-*cat* expression. These results are also in agreement with the notion that the 18 bp inverted repeat in the *kdgR* promoter region constitutes another KdgR-binding site and contributes to the control of *kdgRKAT* expression *in vivo*.

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*

Each value is the mean $\pm$ SD of at least four determinations.

Plasmid in <i>B. subtilis</i> *	Addition†	Specific activity of CAT‡ [mU (mg protein) ⁻¹]
pGS1560	None	224 $\pm$ 18
	GalU	362 $\pm$ 33
	GalU + Glu	201 $\pm$ 14
pGS1561	None	538 $\pm$ 49
	GalU	511 $\pm$ 56
	GalU + Glu	481 $\pm$ 57
pGS1574	None	202 $\pm$ 16
	GalU	294 $\pm$ 24
	GalU + Glu	180 $\pm$ 16
pGS1575	None	290 $\pm$ 21
	GalU	259 $\pm$ 33
	GalU + Glu	173 $\pm$ 17
pUB _{CAT}	None	9 $\pm$ 1
	GalU	9 $\pm$ 2
	GalU + Glu	8 $\pm$ 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.

operon. We have also identified two KdgR-binding sites: one functions as an operator for controlling *kduID* expression and the other serves as an operator for controlling *kdgRKAT* expression. The centres of these two KdgR-binding sites are separated by 131 bp (about 12.5 turns of the DNA helix) (Fig. 2a). Since these two KdgR-binding sites are on the opposite sides of the DNA helix, it is unlikely that binding of KdgR to them is cooperative. It is interesting to note that these two KdgR-binding sites also contain a 14 bp catabolite-responsive element (CRE)-like sequence (Fig. 2a). The consensus sequence 5'-(T/A)GNAA(C/G)CGN(T/A)(T/A)NCA-3' for CRE has been proposed previously (Hueck *et al.*, 1994). Deletion of the CRE-like sequence in the *kduI* promoter region almost abolished glucose repression of the *kduI* promoter-*cat* fusion (Table 2). This suggests that the CRE-like sequence in the *kduI* promoter region is involved in catabolite repression. However, deletion of the CRE-like sequence in the *kdgR* promoter region did not have a large effect on glucose repression of the *kdgR* promoter-*cat* fusion (Table 2). This is not unprecedented since the binding site for the aforementioned ExuR repressor of *B. subtilis* in the *uxaC* promoter region also contains a CRE-like sequence. Mutations in the CRE-like sequence also did not have a large effect on catabolite repression of *uxaC* by glucose (Mekjian *et al.*, 1999).

It is not difficult to imagine why KdgR controls expression of the *kdgRKAT* 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 KduI 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 KduI 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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