Catabolite repression of *dra-nupC-pdp* operon expression in *Bacillus subtilis*

Xianmin Zeng,¹ Anne Galinier² and Hans H. Saxild¹

Author for correspondence: Hans H. Saxild. Tel: +45 25 24 95. Fax: +45 88 26 60.
e-mail: imhhs@pop.dtu.dk

¹ Department of Microbiology, Technical University of Denmark, Building 301, DK-2800 Lyngby, Denmark

² Institut de Biologie et Chimie des Protéines, CNRS, 7 Passage du Vercors, F-69376 Lyon, Cedex 07, France

Expression of the *Bacillus subtilis dra-nupC-pdp* operon is subject to catabolite repression by glucose. It was shown that a *cis*-acting catabolite-responsive element (CRE) sequence located 64 bp downstream of the transcription-start site mediated catabolite repression of the *dra-nupC-pdp* operon as it does for many other *B. subtilis* genes. Point mutations in the CRE sequence caused the loss of catabolite repression of the operon. Catabolite repression of *dra-nupC-pdp* expression was relieved in a *ccpA* mutant and was found to be dependent on both HPr and the HPr-like protein Crh. Furthermore, a transcription-repair coupling factor, Mfd, was also found to be involved in the glucose repression of *dra-nupC-pdp* expression. By the use of *in vitro* gel mobility shift analysis, a specific HPr-P dependent binding of CcpA to the *dra* CRE site was demonstrated.

Keywords: Glucose catabolite repression, nucleoside catabolism, *dra-nupC-pdp* operon, *Bacillus subtilis*, CRE

INTRODUCTION

Catabolite repression is a general regulatory mechanism of gene expression in response to the availability of carbohydrates in the growth medium. In the presence of a rapidly metabolized carbon source, the synthesis of enzymes involved in the utilization of other carbohydrates is inhibited.

Recent publications present evidence that catabolite repression in *Bacillus subtilis* involves transcriptional repression mediated by a negative-control system (Hueck & Hillen, 1995; Saier *et al.*, 1996). Three components are found to be involved in this process: a *cis*-acting sequence called the catabolite-responsive element (CRE), a *trans*-acting factor called catabolite control protein A (CcpA), which is a member of the LacI-GalR family of transcription regulatory proteins, and a second *trans*-acting factor HPr, which is a component of the phosphoenolpyruvate-carbohydrate phosphotransferase system. The HPr protein of *B. subtilis* can be phosphorylated at two sites: one at His-15 by enzyme I of the phosphotransferase system and the other at Ser-46 by an ATP-dependent protein kinase. Resistance to glucose repression in strains carrying the

ptsH1 mutation, which changes the Ser-46 residue of HPr to alanine and thereby prevents phosphorylation of the protein by the ATP-dependent protein kinase, has been reported for several catabolite-repressed genes (Deutscher *et al.*, 1994; Kruger *et al.*, 1996; Martin-Verstraete *et al.*, 1995). A homologue of HPr encoded by the *crh* gene has been reported also to be involved in catabolite repression of inositol dehydrogenase, levanase and β -xylosidase expression (Galinier *et al.*, 1997). The seryl-phosphorylated Crh protein has a similar function to P-Ser-HPr; it induces the binding of CcpA to CRE sequences (Martin-Verstraete *et al.*, 1999; Galinier *et al.*, 1999).

It is suggested that catabolite repression of target genes in Gram-positive bacteria is achieved by the following mechanism: the metabolite-activated HPr(ser) kinase phosphorylates Ser-46 in HPr, converting it to a form that can bind to the transcription factor, CcpA. These two proteins, probably together with a metabolite such as fructose 1,6-bisphosphate, form a complex, which binds to CREs of catabolite-sensitive target genes to promote catabolite repression (Deutscher *et al.*, 1995; Fujita *et al.*, 1995). But the finding of P-Ser-HPr independent binding of CcpA complexed with glucose 6-phosphate leads to the proposal that P-Ser-HPr triggers non-cooperative binding of CcpA to CRE sequences, whereas glucose 6-phosphate activates co-operative binding of CcpA to CRE sequences (Fujita *et*

Abbreviations: CRE, catabolite-responsive element; O_{deor}, DeoR operator.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Characteristics*	Reference/source
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	C. Anagnostopoulos†
QB5407	<i>trpC2 ccpA::Tn917 Δ(lacZ-erm) spc</i>	J. Stülke‡
QB5223	<i>trpC2 ptsH1</i>	Martin-Verstraete <i>et al.</i> (1995)
QB7096	<i>trpC2 crh::aphA3</i>	I. Martin-Verstraete§
SF22CDH	<i>trpC2 mfd22::Tn10</i>	Zalieskas <i>et al.</i> (1998b)
XM15	<i>trpC2 amyE::dra-lacZ</i> (pJOY5)	Zeng & Saxild (1999)
XM45	<i>trpC2 amyE::dra-lacZ</i> (pJOY5) <i>ccpA::Tn917, Spc^R</i>	XM15 transformed with QB5407
XM268	<i>trpC2 amyE::pDG268 (neo)</i>	168 transformed with pDG268 <i>neo</i>
XM512	<i>trpC2 amyE::dra-lacZ</i> (pJOY512), Neo ^R	168 transformed with pJOY512
XM2681	<i>trpC2 amyE::pDG268 (neo)::pMutin7756 (cre⁺) (erm)</i>	XM330 transformed with pMutin7756
XM2683	<i>trpC2 amyE::pDG268 (neo)::pMutin7757 (cre57) (erm)</i>	XM330 transformed with pMutin7757
XM5156 <i>erm</i>	<i>trpC2 amyE::dra-lacZ</i> (pJOY5156 <i>erm</i>)	168 transformed with pJOY5156 <i>erm</i>
XM5156 <i>neo</i>	<i>trpC2 amyE::dra-lacZ</i> (pJOY5156 <i>neo</i>)	168 transformed with pJOY5156 <i>neo</i>
XM5156A	<i>trpC2 ccpA::Tn917 Δ(lacZ-erm) spc amyE::dra-lacZ</i> (pJOY5156 <i>neo</i>)	XM5156 <i>neo</i> transformed with QB5407
XM5156C	<i>trpC2 crh::aphA3 amyE::dra-lacZ</i> (pJOY5156 <i>erm</i>), Neo ^R	XM5156 <i>erm</i> transformed with QB7096
XM5156M	<i>trpC2 mfd22::Tn10 amyE::dra-lacZ</i> (pJOY5156 <i>erm</i>), Cm ^R	XM5156 <i>erm</i> transformed with SF22CDH
XM5156P	<i>trpC2 ptsH1 amyE::dra-lacZ</i> (pJOY5156 <i>erm</i>)	QB5223 transformed with XM5156 <i>erm</i>
XM5156PC	<i>trpC2 ptsH1 crh::aphA3 amyE::dra-lacZ</i> (pJOY5156 <i>erm</i>), Neo ^R	XM5156P transformed with QB7096
XM5157	<i>trpC2 amyE::dra-lacZ</i> (pJOY5157, <i>cre57</i>), Neo ^R	168 transformed with pJOY5157
XM5158	<i>trpC2 amyE::dra-lacZ</i> (pJOY5158, <i>cre58</i>), Neo ^R	168 transformed with pJOY5158
XM5158A	<i>trpC2 ccpA::Tn917 Δ(lacZ-erm) spc amyE::dra-lacZ</i> (pJOY5158, <i>cre58</i>)	XM5158 transformed with QB5407
XM5161	<i>trpC2 amyE::dra-lacZ</i> (pJOY5161, <i>Δcre</i>), Neo ^R	168 transformed with pJOY5161
XM5161A	<i>trpC2 ccpA::Tn917 Δ(lacZ-erm) spc amyE::dra-lacZ</i> (pJOY5161, <i>Δcre</i>)	XM5161 transformed with QB5407
XM7756	<i>trpC2 amyE::pDG268 (neo)::pMutin7756 (erm)</i>	XM330 transformed with pMutin7756
XM7757	<i>trpC2 amyE::pDG268 (neo)::pMutin7757 (erm)</i>	XM330 transformed with pMutin7757
<i>E. coli</i> strains		
MC1061	F ⁻ <i>araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsI</i> (Str ^R) <i>hsdR2</i> (r ⁻ m ⁻) <i>mcrA mcrB</i>	Laboratory stock
Plasmids		
pDG268 <i>neo</i>	Ap ^R (<i>E. coli</i>), Neo ^R (<i>B. subtilis</i>); integration vector for construction of transcriptional <i>lacZ</i> fusion integrated into the <i>amyE</i> gene of <i>B. subtilis</i>	Zeng & Saxild (1999)
pDG268 <i>erm</i>	Ap ^R (<i>E. coli</i>), Er ^R (<i>B. subtilis</i>); integration vector for construction of transcriptional <i>lacZ</i> fusion integrated into the <i>amyE</i> gene of <i>B. subtilis</i>	Zeng & Saxild (1999)
pMutin4	Ap ^R (<i>E. coli</i>), Er ^R (<i>B. subtilis</i>); integrational vector for knockout mutations and formation of transcriptional <i>lacZ</i> fusions; the IPTG-inducible P _{spac} promoter is introduced to ensure expression of downstream genes	Vagner <i>et al.</i> (1998)
pJOY512	<i>EcoRI</i> – <i>Bam</i> HI PCR fragment generated by using primers 1 and 2, ligated to pDG268 <i>neo</i> digested with <i>EcoRI</i> / <i>Bam</i> HI	This work
pJOY5156 <i>erm</i>	<i>EcoRI</i> – <i>Bam</i> HI PCR fragment generated by using primers 2 and 3, ligated to pDG268 <i>erm</i> digested with <i>EcoRI</i> / <i>Bam</i> HI	This work
pJOY5156 <i>neo</i>	<i>EcoRI</i> – <i>Bam</i> HI PCR fragment generated by using primers 2 and 3, ligated to pDG268 <i>neo</i> digested with <i>EcoRI</i> / <i>Bam</i> HI	This work
pJOY5157	<i>EcoRI</i> – <i>Bam</i> HI PCR fragment generated by using primers 2 and 6, ligated to pDG268 <i>neo</i> digested with <i>EcoRI</i> / <i>Bam</i> HI	This work
pJOY5158	<i>EcoRI</i> – <i>Bam</i> HI PCR fragment generated by using primers 2 and 7, ligated to pDG268 <i>neo</i> digested with <i>EcoRI</i> / <i>Bam</i> HI	This work
pJOY5161	<i>EcoRI</i> – <i>Bam</i> HI PCR fragment generated by using primers 2 and 4, ligated to pDG268 <i>neo</i> digested with <i>EcoRI</i> / <i>Bam</i> HI	This work
pMutin7756	<i>EcoRI</i> – <i>Bam</i> HI PCR fragment generated by using primers 5 and 3, ligated to pMutin4 digested with <i>EcoRI</i> / <i>Bam</i> HI	This work
pMutin7757	<i>EcoRI</i> – <i>Bam</i> HI PCR fragment generated by using primers 5 and 6, ligated to pMutin4 digested with <i>EcoRI</i> / <i>Bam</i> HI	This work
pHH1011	Plasmid containing a 760 bp <i>Pvu</i> II– <i>Hinc</i> II fragment containing the 3' end of <i>deoR</i> and the 5' end of <i>dra</i>	Saxild <i>et al.</i> (1996)

* Ap, ampicillin; Cm, chloramphenicol; Neo, neomycin; Er, erythromycin; Spc, spectinomycin.

† CNRS, Jouy-en-Josas, France.

‡ Universität Erlangen, Erlangen, Germany.

§ Institut Pasteur, Paris, France.

al., 1995; Gösseger *et al.*, 1997; Miwa *et al.*, 1997; Zalieckas *et al.*, 1998b). However, cofactor-independent sequence-specific DNA binding by CcpA has also been reported (Kim *et al.*, 1995).

Recently, in *B. subtilis*, several other factors such as CcpB (Chauvaux *et al.*, 1998), CcpC (Jourlin-Castelli *et al.*, 2000), Crh (Galinier *et al.*, 1997), and a transcription-repair coupling factor, Mfd (Zalieckas *et al.*, 1998b), have been reported to be involved in catabolite repression. CcpB, which exhibits 30% sequence identity to CcpA, is found to mediate catabolite repression of *gnt* and *xyl* in cells grown on solid medium or in liquid medium with little agitation (Chauvaux *et al.*, 1998). CcpC, which belongs to the LysR family of transcriptional regulators was found to participate in carbon repression of *citB* (Jourlin-Castelli *et al.*, 2000). Crh, which exhibits 45% sequence identity to HPr, is not phosphorylated by phosphoenolpyruvate and enzyme I, but is phosphorylated by ATP and the HPr kinase at Ser-46 and it is required for catabolite repression of certain catabolite operons (Galinier *et al.*, 1997). The Mfd protein, encoded by *mfd*, is known to promote strand-specific DNA repair by displacing RNA polymerase stalled at a nucleotide lesion and directing the (A)BC excinuclease to the DNA damage site. Resistance towards glucose repression in an *mfd* mutant has been reported for catabolite-repressed genes containing the CRE sequence located downstream of the transcription-start site (Zalieckas *et al.*, 1998b). A model for how catabolite repression is relieved in an *mfd* mutant was proposed by Zalieckas *et al.* (1998b). The Mfd protein is proposed to displace RNA polymerase stalled at downstream CRE sites that function as transcription road blocks. The *mfd* mutation relieves catabolite repression of *hut* and *gnt* expression at the CRE sequences located downstream of the transcriptional start sites, but does not affect catabolite repression at sites located at the promoters (Zalieckas *et al.*, 1998b).

In *B. subtilis*, three enzymes required for the utilization of deoxyribonucleosides as carbon sources are encoded within an operon, *dra-nupC-pdp* (Saxild *et al.*, 1996). The expression of the operon is induced by deoxyribonucleosides and deoxyribose (Saxild *et al.*, 1996; Zeng & Saxild, 1999). Transcription of this operon is negatively regulated by the DeoR repressor protein encoded by the gene *deoR* (Saxild *et al.*, 1996). The operator for DeoR contains a palindromic sequence and a direct repeat sequence to the 3' half of the palindrome located from -58 to -20 bp relative to the transcription-start point (Zeng & Saxild, 1999). The metabolite deoxyribose 5-phosphate is suggested to be the internal inducer for the expression of the operon (Zeng & Saxild, 1999; Zeng *et al.*, 2000).

Expression of the *dra-nupC-pdp* operon is repressed by glucose. A CRE sequence located in the start of *dra* was found to be involved in catabolite repression of the operon (Zeng & Saxild, 1999). In this study, we show that CcpA-mediated catabolite repression of the operon

works via this downstream CRE sequence and that HPr, Crh and Mfd participate in catabolite control of *dra-nupC-pdp* operon expression.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this work are listed in Table 1. *B. subtilis* was grown in Spizizen minimal salts medium (Saxild *et al.*, 1996) supplemented with 50 µg L-tryptophan ml⁻¹ and with either 0.4% glucose or 0.4% succinate as the carbon source. L broth (Difco) was used as a rich medium for both *Escherichia coli* and *B. subtilis*. Culturing of cells was performed at 37 °C. For selection of antibiotic resistance, antibiotics were used at the following final concentrations: ampicillin, 100 µg ml⁻¹; chloramphenicol, 6 µg ml⁻¹; neomycin, 5 µg ml⁻¹; erythromycin, 1 µg ml⁻¹; lincomycin, 25 µg ml⁻¹; spectinomycin, 100 µg ml⁻¹.

DNA manipulations and genetic techniques. *B. subtilis* chromosomal DNA and plasmid DNA was isolated as described previously (Saxild *et al.*, 1996). Transformation of *E. coli* and *B. subtilis* was performed as previously described (Saxild *et al.*, 1996). Treatment of DNA with restriction enzymes and T4 DNA ligase was performed as recommended by the supplier. DNA sequences were obtained using the Amersham Pharmacia Biotech Thermo Sequenase radio-labelled termination cycle sequencing kit. All sequencing analysis was done on double-stranded plasmid or PCR product DNA templates and performed as described by the supplier. Standard PCR reactions were performed as described previously (Zeng & Saxild, 1999).

Construction of *dra-lacZ* fusions. Different deletion derivatives of the *dra* control region were amplified by PCR using plasmid pHH1011, which contains the 3' end of *deoR* and the 5' end of *dra*, as template DNA (Zeng & Saxild, 1999). The forward and reverse oligonucleotide primers were synthesized with an *EcoRI* and *BamHI* 5'-linked restriction site, respectively (Table 2). The various *dra* DNA fragments used for the construction of *dra-lacZ* fusions were amplified with primer 2 together with one of the primers 1, 3, 6, 7 or 4. The PCR products were digested with *EcoRI/BamHI*, ligated to *EcoRI/BamHI*-digested plasmid pDG268*erm* or pDG268*neo* and transformed into *E. coli* MC1061 selecting for ampicillin resistance. Plasmids extracted from *E. coli* (pJOY512, pJOY5156*erm*, pJOY5156*neo*, pJOY5157, pJOY5158 and pJOY5161; Table 1) were linearized by *KpnI* and recombined into the *amyE* locus of *B. subtilis* 168 by transformation and selection for neomycin resistance. The correct integration into the *amyE* locus was confirmed by the amylase-negative phenotype of the transformants. The resulting *B. subtilis* strains were XM512, XM5156*erm*, XM5156*neo*, XM5157, XM5158 and XM5161 (Table 1).

Construction of P_{spac}-*lacZ* fusions. Two 71 bp PCR products amplified by primers 5+3 and 5+6, containing the *dra* ribosome-binding site and either wild-type or mutant CRE sequence of *dra*, were cloned in the *EcoRI* and *BamHI* sites of pMutin4. The resulting plasmids, pMutin7756 and pMutin7757, were transformed into *E. coli* MC1061 selecting for ampicillin resistance. Plasmids extracted from *E. coli* were transformed into XM268 (which contains a promoterless *lacZ* gene integrated in the *amyE* locus) by a single crossover between the two copies of *lacZ* following selection for erythromycin resistance. The correct integration into the *lacZ*

Table 2. Oligonucleotides used for PCR amplifications

Primer name	Linked restriction site	Sequence (5'–3')*	Coordinates†
Deletion analysis			
1	5' <i>Bam</i> HI	GCGGGATCCCCATGTCGGATTGACAC	+ 154
2	5' <i>Eco</i> RI	GCCGGAATTCCCAATTTACATATGTTCAAAAG	– 40
3	5' <i>Bam</i> HI	GCGGGATCCTTGTGTATGCGGTTTCAAAGC	+ 81
4	5' <i>Bam</i> HI	GCGGGATCCAAGCTGTATGATCAATTA	+ 64
5	5' <i>Eco</i> RI	GCCGGAATTCCAAAAAAGGAAGTGTGCG	+ 11
Site-directed mutagenesis			
6	5' <i>Bam</i> HI	GCGGGATCCTTGTGTATGAGGTTTCAAAGC	+ 81
7	5' <i>Bam</i> HI	GCGGGATCCTTGTGTATGCAGTTTCAAAGC	+ 81
Mobility-shift assay			
8	5' <i>Eco</i> RI	GCCGGAATTCCCTTTCATTGAACAAAATTTTC	– 66
9	5' <i>Eco</i> RI	GCCGGAATTCCCTTTCATCGAACAAAATTTTC	– 66

*Underlined letters indicate the position of the restriction site sequence. Bold letters indicate nucleotides that differ from the wild-type sequence.

†The number indicates the position of the 5' proximal nucleotide of the primer relative to the *dra* transcription-start site (Zeng & Saxild, 1999). The transcription-start point is genome nucleotide number 4051089 (Kunst *et al.*, 1997).

gene located at the *amyE* locus of XM268 was confirmed by direct DNA sequencing of specific PCR products from the transformants covering the flanking regions of the *lacZ* gene. The resulting *B. subtilis* strains were XM2681 and XM2683 (Table 1).

Site-directed mutagenesis. This was performed by using oligonucleotides with a single mismatch as PCR primers. The primer sequences are shown in Table 2. A standard PCR reaction was performed with one of these primers together with primer 2. The resulting 122 bp PCR products were cloned into pDG268*neo* and transformed into *B. subtilis* as described above. The resulting strains are listed in Table 1.

β -Galactosidase assay. β -Galactosidase activity was measured according to the method of Miller (1972). Specific enzyme activities are expressed as U (mg protein)^{–1}. One unit is defined as 1 nmol substrate converted min^{–1}. The presented values are means of at least three different experiments. The variation was less than 15%. Total protein concentration was determined by the Lowry method.

Mobility-shift assay. The standard PCR mixture containing 25 μ mol [α -³²P]dATP (25 μ Ci) was used to produce the radiolabelled operator-containing DNA probes. The 148 bp labelled fragments were generated by combining primers 8 + 3 and 9 + 3. Each binding reaction mixture contained 10 mM Tris/HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT (pH 7.5), 1 U ml^{–1} double-stranded poly(dI–dC), 250 μ g BSA ml^{–1} and 5% (v/v) glycerol in a final volume of 10 μ l. Approximately 10–100 pM labelled DNA probe and various concentrations of proteins were used in each binding reaction. After incubation for 20 min at room temperature, samples were loaded onto a 5% polyacrylamide gel and electrophoresed at 7 V cm^{–1} for 1 h. Dried gels were visualized with a Packard Instant Imager. CcpA and Hpr proteins were both purified on Ni-nitrilotriacetate-agarose columns (Galinier *et al.*, 1998). Hpr was phosphorylated by HprK in the presence of ATP as described by Galinier *et al.* (1998).

RESULTS

Localization of the *dra*–*nupC*–*pdp* CRE site using deletion analysis and site-directed mutagenesis

A set of *dra*–*lacZ* transcriptional fusions was constructed for deletion analysis of the catabolite control region of the *dra*–*nupC*–*pdp* operon. These fusions were introduced into the *amyE* locus on the chromosome in a single copy as described in Methods. The upstream end point of all the fusions was at position –40 relative to the transcription-start site and therefore the fusions do not include the DeoR operator (O_{deoR}) site. The positions of the downstream end points were different and are given in Table 3. Catabolite repression of these fusions was monitored during growth in media containing either glucose or succinate as the carbon source (Table 3). The β -galactosidase activity in strains containing a *dra*–*lacZ* fusion including the potential CRE sequence (XM512 and XM5156*neo*) was repressed approximately fivefold in glucose-grown cells compared to succinate-grown cells, whereas no glucose repression was observed in strains containing a *dra*–*lacZ* fusion not including the CRE sequence (XM5161). Point mutations were introduced into the CRE sequence by site-directed mutagenesis. Two mutant strains, XM5157 and XM5158, each having a single-base-pair mutation in the central conserved C or G residue of the CRE sequence, were constructed and β -galactosidase activity was assayed after growth in minimal media with either glucose or succinate as the carbon source. The introduction of a point mutation in strains XM5157 and XM5158 reduced the level of regulation to only 1.2- and 1.4-fold, respectively. This should be compared to the 5.4-fold repression in the comparable wild-type strain

Table 3. β -galactosidase activity of *B. subtilis* strains carrying various *dra-lacZ* fusions integrated into the *amyE* locus in the wild-type and different genetic backgrounds

Cells were grown in minimal medium supplemented with either glucose or succinate as the carbon source. Cells were collected and assayed for β -galactosidase activity as described in Methods. Deoxyribose (dRib; where used) was added to a final concentration of 1 mg ml⁻¹.

Strain	Relevant genotype	Region fused to <i>lacZ</i>	O _{deoR} status	CRE status*	Inducer added	β -Galactosidase activity† with carbon source:	
						Succinate	Glucose‡
XM512	Wild-type	-40 to +154	—	+	None	146	34 (4.3)
XM5156neo	Wild-type	-40 to +81	—	+	None	151	28 (5.4)
XM5161	Δcre	-40 to +64	—	—	None	189	186 (1.0)
XM5157	<i>cre57</i>	-40 to +81	—	Mut	None	120	101 (1.2)
XM5158	<i>cre58</i>	-40 to +81	—	Mut	None	147	102 (1.4)
XM15	Wild-type	-80 to +154	+	+	None	22	30 (0.7)
					dRib	248	36 (6.8)
XM45	<i>ccpA</i>	-80 to +154	+	+	None	27	24 (1.1)
					dRib	190	253 (0.7)
XM5156A	<i>ccpA</i>	-40 to +81	—	+	None	149	116 (1.3)
XM5161A	<i>ccpA</i> Δcre	-40 to +64	—	—	None	164	159 (1.0)
XM5158A	<i>ccpA cre58</i>	-40 to +81	—	Mut	None	158	162 (1.0)
XM5156P	<i>ptsH1</i>	-40 to +81	—	+	None	163	73 (1.9)
XM5156C	<i>crh</i>	-40 to +81	—	+	None	170	34 (5.1)
XM5156PC	<i>ptsH1 crh</i>	-40 to +81	—	+	None	183	152 (1.2)
XM5156M	<i>mfd</i>	-40 to +81	—	+	None	203	79 (2.5)

* Mut indicates that the CRE element contains either the *cre57* or *cre58* point mutation described in Methods.

† nmol min⁻¹ (mg protein)⁻¹.

‡ Numbers in parentheses indicate level (fold) of repression and were calculated by dividing the enzyme activity in succinate-grown cells by the enzyme activity in glucose-grown cells.

XM5156neo. These results indicated that catabolite repression of *dra-nupC-pdp* expression is mediated by the CRE sequence located at position +64. Inspection of the nucleotide sequence and additional deletion analysis revealed no other potential CRE elements in the region from position -80 to +154 (data not shown). We therefore conclude that the *B. subtilis dra-nupC-pdp* operon most likely carries a single *cis*-acting CRE sequence at position +64 relative to the transcription-start site.

To investigate whether DeoR affected catabolite repression control at the *dra* CRE site a *lacZ* fusion including O_{deoR} was constructed (XM15). It was observed that *dra* expression was not totally repressed during growth on glucose in the absence of deoxyribose. The level of 20–30 U (mg protein)⁻¹ of β -galactosidase activity seen in non-induced strains growing on glucose is significantly higher than in a wild-type strain containing no *dra-lacZ* fusion [approximately 1 U (mg protein)⁻¹ (data not shown)]. In the absence of inducer, that is when the fusion is repressed by DeoR, it appears that there is no catabolite control of *dra* expression (XM15, Table 3). One explanation for this phenomenon could be that some minimum number of transcription complexes arriving at the CRE site is required for

catabolite repression to operate. This hypothesis was tested using two transcriptional fusions between the IPTG-inducible promoter, P_{spac}, and *lacZ*. These fusions, which contain neither the *dra* promoter nor O_{deoR}, are composed of a 71 bp fragment containing the wild-type or mutant CRE sequence of *dra* inserted between the P_{spac} promoter and *lacZ*, respectively (see Methods). Expression of β -galactosidase from these fusions as regulated by catabolite repression was monitored during growth in media containing either glucose or succinate as the carbon source. We found that catabolite repression was observed at all levels of IPTG tested up to 1 mM in the fusion strain containing the wild-type CRE, whereas no repression was observed in the fusion strain carrying the mutant CRE (data not shown). This indicated that catabolite control was turned on even at very low transcription-initiation frequencies and did not require some minimum amount of transcription dosage for normal function.

Role of CcpA and the effect of the *ptsH1*, *crh* and *mfd* mutations on *dra-nupC-pdp* expression

A *ccpA* gene-disruption mutation was introduced into strains XM5156neo and XM5161. XM5156neo and XM5161 carry a transcriptional *dra-lacZ* fusion with or

without CRE, respectively. β -Galactosidase activity was measured for the *ccpA* derivatives XM5156A and XM5161A grown either in glucose or succinate (Table 3). It was shown that glucose repression was almost completely relieved in a *ccpA* genetic background containing a *dra-lacZ* fusion either with or without the CRE sequence. The level in succinate-grown cells was unchanged while the level in glucose was increased compared to the wild-type derivatives grown under the same conditions. Essentially the same effect was observed when the *cre58* allele was substituted for Δcre in the *ccpA* genetic background (XM5158A). To test whether the glucose catabolite repressor protein CcpA in some way affected the observed constitutively low level of *dra* expression in the absence of the inducer deoxyribose (see previous paragraph), a *ccpA* derivative of XM15 (containing the *dra-lacZ* fusion including the O_{DeoR} sequence) was constructed (XM45) and analysed for *dra* expression during growth with glucose or succinate. The *dra* expression level in strain XM45 during growth on glucose, in the absence of deoxyribose, was similar to that of the wild-type, which means that the low level of *dra* expression is not a result of the action of CcpA. On the other hand, in the presence of inducer, high constitutive levels were found in both glucose- and succinate-grown cells, indicating that CcpA is involved in catabolite control of *dra* expression, at least under induced conditions. All the results presented above indicate that CcpA is responsible for the glucose control of the *dra-nupC-pdp* operon expression.

To study whether HPr and Crh play a role in catabolite repression of the *dra-nupC-pdp* operon, a *ptsH1* mutation was introduced into strain XM5156erm carrying a transcriptional *dra-lacZ* fusion, resulting in strain XM5156P. Similarly, a *crh* gene disruption mutation (Galinier *et al.*, 1997) was introduced into XM5156erm resulting in XM5156C. The β -galactosidase activity of both glucose- and succinate-grown cells was measured for these two strains (Table 3). Glucose repression was partially relieved resulting in a reduction to 1.9-fold of regulation in the *ptsH1* mutant, while disruption of the *crh* gene in the wild-type background did not alter the pattern of catabolite repression of the operon expression. However, disruption of the *crh* gene in the *ptsH1* background (XM5156PC) almost totally abolished catabolite repression. These results suggested that, in addition to HPr, which plays the major role in signalling the presence of glucose to CcpA, Crh also participated in catabolite repression of the *dra-nupC-pdp* operon as it does for other *B. subtilis* catabolite-repressed genes such as *lev* and *xyn* (Martin-Verstraete *et al.*, 1999; Galinier *et al.*, 1999). To investigate the role of Mfd in catabolite repression of *dra-nupC-pdp* operon expression, an *mfd*: Tn10 mutation was introduced into XM5156erm resulting in strain XM5156M, and β -galactosidase activity was measured in both glucose- and succinate-grown cells (Table 3). The results showed that the glucose repression was only 2.5-fold in the *mfd* mutant compared to 5.4-fold in the wild-type cells. This result

Fig. 1. Binding of CcpA to the CRE of the *dra-nupC-pdp* operon. A profile of a mobility-shift assay is shown. DNA fragments containing either the wild-type *cre* (lanes 1–3) or the mutant *cre57* element (lanes 4–6) were incubated with various combinations of CcpA and P-Ser-HPr.

was similar to what has been observed for the expression of the *hut* and *gnt* operons in an *mfd* genetic background (Zalieskas *et al.*, 1998b). So CcpA most likely mediates catabolite repression of *dra* expression via the binding to a single downstream CRE, thus functioning by a transcriptional road-block mechanism.

Specific interaction between CcpA and CRE

The presumed interaction between CcpA and the CRE of the *dra-nupC-pdp* operon was demonstrated by an *in vitro* gel mobility-shift experiment. The DNA probes were two [32 P]-labelled 148 bp fragments containing either the wild-type CRE sequence or the mutant CRE sequence with an A substituted for the central C (*cre57*). As shown in Fig. 1, the mobility of the labelled DNA containing the wild-type CRE was shifted in the presence of CcpA and P-Ser-HPr, but no shift was observed for the DNA with the mutant CRE. When CcpA was present alone in the assay, no interaction was observed (Fig. 1, lane 2); only in the presence of P-Ser-HPr did CcpA specifically recognize the CRE (Fig. 1, lane 3). The results presented in Fig. 1 clearly demonstrate the specific P-Ser-HPr dependent binding of CcpA to the *dra* CRE site. The results complement the previous genetic data obtained using *lacZ* fusions and various regulatory mutations (Table 3).

DISCUSSION

By the use of both genetic and biochemical means we have demonstrated that the *B. subtilis dra-nupC-pdp* operon carries a downstream CRE sequence that is involved in CcpA-mediated catabolite repression. Moreover, HPr, Crh and Mfd were proved to participate in catabolite repression of the operon expression. The pattern of catabolite repression of the *dra-nupC-pdp* operon provides us with a fine example in which all the factors found to be involved in the negative-control system of catabolite repression in *B. subtilis* play a role in catabolite control of this operon.

An interesting aspect of our study is that catabolite repression of the *dra-nupC-pdp* operon is absent when the expression is repressed by the DeoR repressor. Only in a *deoR* strain or in a wild-type strain induced with deoxyribose (Zeng & Saxild, 1999) does catabolite

repression take place. We suggested a mechanism to explain this phenomenon and designed an experiment to test this suggestion. However, the experiment did not confirm that catabolite repression of the *dra-nupC-pdp* operon requires a minimum amount of transcription arriving at the CRE site.

A special feature of the catabolite repression of the *dra-nupC-pdp* operon exerted by DeoR and CcpA is that it is leaky [approximately 20–30 nmol min⁻¹ (mg protein)⁻¹ β -galactosidase activity during the repressed condition (Table 3)]. It means that neither CcpA or DeoR binds sufficiently strongly to their operators to totally prevent transcription initiation (DeoR) or transcription elongation (CcpA). One reason for maintaining a low level of deoxyriboaldolase could be that accumulation of the substrate, deoxyribose 5-phosphate, is toxic for the cell (Saxild *et al.*, 1996). Deoxyribose 5-phosphate is produced constitutively at a low level by deoxyribomutase, encoded by *drm* (Schuch *et al.*, 1999). Maybe therefore the level of catabolite repression of *dra-nupC-pdp* operon expression is quite low (fivefold) compared to 17-fold for *acsA* (Zalieckas *et al.*, 1998a), 16-fold for *xyl* (Gösseringer *et al.*, 1997), 32-fold for *lev* (Martin-Verstraete *et al.*, 1995) and 25-fold for *hut* (Wray *et al.*, 1994).

The structure with one and only one CRE site located more than 50 nt downstream of the transcription start has also been reported for the *xyn* gene (Galinier *et al.*, 1999). Even though the *xyn* gene is repressed approximately 110-fold by glucose (Galinier *et al.*, 1999), the unity and the location of the *dra* CRE site may account for some of the observed leakiness of the glucose repression of *dra-nupC-pdp* operon expression.

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