Catabolite repression of β -glucanase synthesis in *Bacillus subtilis*

STEFFEN KRÜGER, JÖRG STÜLKE and MICHAEL HECKER*

Fachrichtung Biologie, Institut für Mikrobiologie und Molekularbiologie, Ernst-Moritz-Arndt-Universität Greifswald, D-17487 Greifswald, Jahnstraße 15, Germany

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β -Glucanase synthesis in *Bacillus subtilis* was repressed by glucose and other substrates of glycolysis. Experiments with different *pts* mutants showed that the phosphoenolpyruvate:sugar phosphotransferase system is not involved in carbon catabolite repression of β -glucanase synthesis. Carbon catabolite repression of β -glucanase synthesis was completely abolished in a *ccpA* mutant. An operator structure similar to those upstream of *amyE* and the *xyl* operon was found and was shown by site-directed mutagenesis to be the target for carbon catabolite repression of β -glucanase synthesis. The presence of this operator on a multi-copy plasmid resulted in a reduced repression of both β -glucanase and α -amylase synthesis. It seems likely that the gene encoding these enzymes are part of one regulon with respect to catabolite repression.

Introduction

In *Bacillus subtilis* many catabolic enzymes are subject to carbon catabolite repression by glucose and other readily metabolized carbon sources (Fisher & Sonenshein, 1991). While catabolite repression is a well-understood phenomenon in *Escherichia coli* (Magasanik & Neidhardt, 1987), only limited information is available on the mechanism(s) of carbon catabolite repression in *B. subtilis*. The phosphoenolpyruvate:sugar phosphotransferase system (PTS) (Reizer *et al.*, 1988) and glycolytic intermediates (Freese *et al.*, 1974) have been suggested to be involved. Furthermore, Rygus & Hillen (1992) demonstrated that carbon catabolite repression of the *Bacillus megaterium xyl* operon correlates with the growth rate that the bacterium can reach on a given sugar.

trans-active mutations have been isolated that led to glucose-resistant synthesis of one or more enzymes. The corresponding genes were defined as *ccpA* (Henkin *et al.*, 1991), *pai* (Honjo *et al.*, 1990) and *ptsH* (Eisermann *et al.*, 1988).

Nicholson & Chambliss (1985) identified a *cis*-active mutation, *gra-10*, in the *amy* locus of *B. subtilis* that

renders α -amylase synthesis glucose-resistant. The *gra-10* mutation is a base substitution in a palindromic operator-like structure. Site-directed mutagenesis revealed that this structure is the target of carbon catabolite repression of *amyE* expression (Weickert & Chambliss, 1990). A similar structure has been implicated in catabolite repression of the *B. subtilis xyl* operon (Jacob *et al.*, 1991).

We are interested in the regulation of the *bglS* gene of *B. subtilis* encoding endo- β -1,3-1,4-glucanase (EC 3.2.1.73). In an accompanying study it has been shown that the *bglS* gene is temporally activated in response to glucose or amino acid limitations in a GTP-pool-dependent manner (Stülke *et al.*, 1993). In this report we demonstrate that carbon catabolite repression of β -glucanase synthesis is not mediated by the PTS but probably by glycolytic intermediates. An operator structure similar to those of *amyE* and the *xyl* operon was found to be the target site for *bglS* carbon catabolite repression.

Methods

Bacterial strains and plasmids. These are listed in Table 1. The genetic markers of the strains were periodically confirmed.

Plasmid pEC021 was constructed as follows. After deletion of the 2.5 kb *PvuI* fragment from plasmid pEG1 (Fig. 1a), yielding pEG026, the 2.9 kb *HindIII* fragment of pHV33 (corresponding to pC194) was cloned into the unique *HindIII* restriction site of pEG026.

Plasmid pEC040 was obtained by inversion of the 3.8 kb *EcoRI* fragment of plasmid pHV33-012 (Fig. 1b) and subsequent deletion of

* Author for correspondence. Tel. 03834 77271 ext. 522; fax 0383 463345.

Abbreviation: PTS, phosphoenolpyruvate:sugar phosphotransferase system.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source (reference)
<i>B. subtilis</i>		
BG314	<i>trpC2 aroD120 bgl-35</i>	Borriess <i>et al.</i> (1986)
BGW2	<i>trpC2 lys-3 ccpA::Tn917</i>	This study
GM273	<i>trpC2 sacR::lacZ</i> <i>ΔptsXHI::ermC</i>	Crutz <i>et al.</i> (1990)
GM808	<i>trpC2 sacR::lacZ iol</i>	J. Deutscher
GR100	<i>trpC2 gra-100::Tn917</i>	G. Chambliss
IS58	<i>trpC2 lys-3</i>	Smith <i>et al.</i> (1980)
SA003	<i>trpC2 sacR::lacZ iol</i> <i>ptsHSA46A</i>	J. Deutscher
WLN29	<i>trpC2 aroG932 ccpA::Tn917</i>	Henkin <i>et al.</i> (1991)
WLN63	<i>rpsE rpsL1 sacA321</i> <i>gra32::Tn917</i>	G. Chambliss
WLN106	<i>metB5 sacA321 gra-48::Tn917</i>	G. Chambliss
<i>E. coli</i>		
RR1	F ⁻ <i>mcrB mrr hsdS20 ara-14</i> <i>proA2 lacY1 leu galK2</i> <i>rpsL20 xyl-5 mtl-1 supE44</i>	Bolivar <i>et al.</i> (1977)
Plasmids		
pHV33	Tc ^r Ap ^r Cm ^r	Primrose & Ehrlich (1981)
pUC19	Ap ^r	Yanisch-Perron <i>et al.</i> (1985)
pEG1*	Tc ^r Ap ^r <i>bglA</i> from <i>B. amyloliquefaciens</i>	Borriess <i>et al.</i> (1985)
pHV33-012†	Ap ^r Cm ^r <i>bglS</i> from <i>B. subtilis</i>	Borriess <i>et al.</i> (1986)
pEC021	Tc ^r Cm ^r <i>bglA</i>	This study
pEC040‡	Tc ^r Cm ^r <i>bglS</i>	This study
pEC041	Tc ^r Cm ^r <i>bglS</i>	This study
pEC042	Tc ^r Cm ^r <i>bglS</i>	This study
pUC19/2	Ap ^r <i>bglS</i>	This study

* Fig. 1(a). † Fig. 1(b). ‡ Fig. 2(a).

the 2.5 kb *PvuI* fragment. After religation of the purified 8.2 kb fragment the resulting plasmid was pEC040 (Fig. 2a).

For the purpose of conducting PCR reactions an *EcoRI*-*PstI* restriction fragment containing the *bglS* gene from pEC040 was cloned between the *EcoRI* and *PstI* sites of plasmid pUC19. The resulting plasmid was pUC19/2.

Plasmids pEC041 and pEC042, carrying modified operator sequences, were constructed as follows. Fragments of 1.8 kb produced in PCR reactions were treated with restriction enzymes *EcoRI* and *PstI*, resulting in fragments of 1.7 kb. The purified fragments were ligated with the 6.5 kb *EcoRI*-*PstI* fragment from plasmid pEC040. The resulting plasmid constructions pEC041 and pEC042 were verified by DNA sequencing with an oligonucleotide primer located near the translation initiation codon of the *bglS* gene.

Culture and growth conditions. *B. subtilis* cells were grown in amino acid limitation medium (ASM) (Stülke *et al.*, 1993) at 37 °C under vigorous agitation.

For the determination of growth rates, sodium citrate and potassium glutamate were omitted from the medium, and KCl was replaced by NaCl. If necessary, antibiotics were added to 1 µg ml⁻¹ (erythromycin), 5 µg ml⁻¹ (chloramphenicol), 12.5 µg ml⁻¹ (tetracycline) and 100 µg ml⁻¹ (ampicillin).

Media for the production of competent cells of *B. subtilis* were prepared according to Hoch (1991).

Fig. 1. Physical maps of plasmids pEG1 (a) and pHV33-012 (b). These plasmids, containing the *bglA* and *bglS* genes encoding β -glucanase of *B. amyloliquefaciens* and *B. subtilis*, respectively, were constructed by Borriess *et al.* (1985) (pEG1) and Borriess *et al.* (1986) (pHV33-012). They served for the construction of related plasmids used in this study. Regions of homology to the consensus operator sequence in front of the genes encoding β -glucanase are indicated (*Op.*).

E. coli was grown in nutrient broth containing 8.5 g peptone, 6.5 g yeast extract and 10 g NaCl per litre, pH 7.4. Agar (1.5%, w/v) was added for solid media.

Repression experiments. For these experiments the cultures were grown in ASM, divided at an OD₅₅₀ of 0.8 and the sugars to be tested were added at a final concentration of 0.2% unless stated otherwise. The residual activity is the percentage of β -glucanase synthesis in a

Fig. 2. (a) Physical map of plasmid pEC040. The position of the wild-type operator (Op.) upstream of the *bglIS* gene encoding β -glucanase is shown. (b) Scheme of primer arrangement in plasmid pUC19/2. The positions of the *bglIS* gene and the operator (Op.) are shown. The bold lines represent the 1.7 kb fragment obtained from plasmid pEC040. A convenient procedure for PCR reactions was achieved via this strategy.

culture containing a sugar compared to the control culture after 2.5 h of stationary phase.

Oligonucleotides. The sequences of the mutagenic oligonucleotides spanning the postulated operator structure are given below, with the mismatch bases underlined:

U1, 5'-d(GAATCATGTAAGCGCAACATAG)-3'
 U2, 5'-d(GCGTTTCTATGTTGCGCTTACATG)-3'
 D1, 5'-d(GAATCATGTAAGATCAATATAG)-3'
 D2, 5'-d(GCGTTTCTATATTGATCTTACATG)-3'

The flanking primers, F1 and F2, that hybridize upstream and downstream, respectively, of the polylinker region of plasmid pUC19, have the following sequences:

F1, 5'-d(AAAGGGGGATGTGCTGCAAGGCG)-3'
 F2, 5'-d(GCTTCCGGCTCGTATGTTGTGTG)-3'

The arrangement of the primers used in this study is shown schematically in Fig. 2(b).

General recombinant DNA techniques. Standard procedures were used for extracting plasmids from *E. coli* and *B. subtilis* (Holmes & Quigley, 1981; Sambrook *et al.*, 1989). Restriction enzymes and T4 DNA ligase were used according to the recommendations of the manufacturer. DNA fragments were purified from agarose gels by using the GeneClean II kit (Bio 101).

Chromosomal DNA was isolated as described by Meade *et al.* (1982).

The CaCl_2 method (Sambrook *et al.*, 1989) was used to transform *E. coli* RR1. *B. subtilis* strains were transformed with plasmid or chromosomal DNA using naturally competent cells (Hoch, 1991).

DNA sequences were determined by the dideoxy-chain termination method (Sanger *et al.*, 1977) with plasmid DNA as template and modified T7 DNA polymerase (US Biochemicals Corp.).

Generation of PCR products. PCRs were carried out using *Taq* polymerase (Promega). Amplification of DNA fragments from plasmid template was achieved by adding 200 ng of template DNA, 50 mM-KCl, 10 mM-Tris/HCl pH 9.0, 1.5 mM-MgCl₂, 0.01 % (w/v) gelatin, 0.1 % (v/v) Triton X-100, 200 μM of each dNTP, 25 pmol of each primer and 2.5 units of *Taq* polymerase in a final volume of 100 μl . The samples were overlaid with 60 μl light mineral oil (Sigma).

In each mutagenic strategy two overlapping gene fragments were synthesized in separate primary amplifications with plasmid pUC19/2 as template. PCR conditions were chosen as follows: denaturation 1 min, 94 $^{\circ}\text{C}$; annealing 1 min, 50 $^{\circ}\text{C}$; extension 3 min, 72 $^{\circ}\text{C}$; 30 cycles. For amplification of 5' fragments of the *bglIS* gene, mutagenic primers U2 or D2 and the flanking primer F1 were used. The 3' part of the *bglIS* gene was amplified using mutagenic primers U1 or D1 and the flanking primer F2.

In the second round of PCR, equivalent amounts of purified overlapping gene fragments from the two primary amplifications were mixed in samples as described above. Twenty PCR cycles of 94 $^{\circ}\text{C}$ for 1 min, 45 $^{\circ}\text{C}$ for 2 min and 72 $^{\circ}\text{C}$ for 3 min were carried out to produce full-length DNA fragments using primers F1 and F2.

The products of the reactions were analysed on an agarose gel containing 1 % (w/v) agarose and 0.5 μg ethidium bromide ml^{-1} in Tris/borate buffer (90 mM-Tris/borate, 1 mM-EDTA, pH 8.0).

Enzyme assays. β -Glucanase activities were assayed by the dinitro-salicylic acid method as described previously (Stülke *et al.*, 1993). One enzyme unit was defined as 1 mol glucose equivalent produced ($\text{OD}_{550} \text{ unit}^{-1}$ and $\text{ml}^{-1} \text{ s}^{-1}$ (= 1 kat).

The measurement of α -amylase activity was based on a modification of the procedure of Tsukagoshi *et al.* (1984). A 150 μl portion of culture supernatant was mixed on ice with 100 μl 0.2 % (w/v) soluble starch in 100 mM-sodium acetate pH 6.0 and then incubated at 37 $^{\circ}\text{C}$ for 60 min. The reaction was stopped by adding 50 μl of 0.2 % (w/v) I₂, 1 % (w/v) KI in 1.75 M-sodium acetate pH 4.0. After the addition of 1 ml distilled water the absorbance at 570 nm was compared with that of a blank (same composition as above but no incubation). One unit corresponds to the amount of enzyme necessary for degradation of 6 mg starch h^{-1} .

Results

Carbon catabolite repression of β -glucanase synthesis by various sugars

The repression of β -glucanase synthesis exerted by the carbon sources glucose, fructose, sucrose, glucitol, glycerol, ribose, xylose, gluconate and methyl α -glucoside (final concentration 0.2 %) in *B. subtilis* IS58 was determined (Table 2). The strongest repression was

Table 2. Carbon catabolite repression of β -glucanase synthesis in IS58

The β -glucanase activity in *B. subtilis* IS58 was 0.62 nkat under unrepressed conditions. Experiments were performed three times. Representative results are shown. NA, Not applicable.

Sugar	Residual activity (%)	Specific growth rate, μ (h ⁻¹)
Glucose	7	1.25
Fructose	15	1.25
Sucrose	13	1.25
Glucitol	54	1.25
Glycerol	26	1.25
Ribose	180	0.71
Gluconate	130	1.11
Methyl α -glucoside	100	NA

found with glucose (7% residual activity); no repression was obtained with ribose and gluconate. The addition of glucose to a final concentration of 0.4% resulted in complete repression of β -glucanase synthesis. No repression was found with the slowly metabolizable PTS substrate methyl α -glucoside (Table 2). The addition of this sugar led to a cessation of growth and to an immediate induction of β -glucanase synthesis (data not shown).

Catabolite repression of β -glucanase synthesis in *pts* mutants

For a more detailed analysis of PTS involvement in carbon catabolite repression, β -glucanase synthesis was determined in PTS mutants in the presence of either the PTS sugar glucose or the non-PTS carbohydrate glucitol. In strain GM273 ($\Delta ptsGHI::ermC$), glucitol repressed β -glucanase synthesis fourfold. With the *ptsHS46A* mutant SA003 no repression was observed with glucose, but the

residual activity with glucitol was 57%, similar to that in the wild-type strain GM808 (Table 3).

Determinations of growth rates of strains GM808, GM273 and SA003 with either glucose or glucitol as single carbon sources revealed that GM273 did not grow on glucose, and strain SA003 grew more slowly on glucose than on glucitol (Table 3).

Expression of *bglS* in *gra* mutants

Studies to find the *trans*-acting element of catabolite repression of α -amylase synthesis led to the isolation of four glucose-resistant mutants, among them a *ccpA* mutant (Henkin *et al.*, 1991).

Experiments were performed to determine glucose repression of β -glucanase synthesis and growth rates of these mutants (Table 4). β -Glucanase synthesis was repressed twofold in the *ccpA* mutant WLN29 in the presence of glucose. In the *gra* mutant strains glucose repression of β -glucanase synthesis was comparable to that of *B. subtilis* IS58.

The insufficient utilization of glucose or glycerol led us to ask whether *ccpA* is the only mutation of *B. subtilis* WLN29 affecting growth and catabolite repression. To transfer the *ccpA* allele into a defined genetic background, chromosomal DNA of WLN29 was purified and used to transform strain IS58. Transformants carrying a *ccpA::Tn917* insertion were isolated by selection for erythromycin resistance. The resulting strain was BGW2. Neither glucose nor glycerol repressed the synthesis of β -glucanase (Table 4) or α -amylase (data not shown) in this strain.

Identification of a regulon

Using the multi-copy vector pEC040 transformed into strain IS58 a more than 100-fold increase in β -glucanase synthesis in comparison with IS58 and IS58(pHV33) was

Table 3. Repression of synthesis of β -glucanase in *pts* mutants

Representative results of three separate experiments are shown.

Strain	β -Glucanase activity* (nkat)	Glucose		Glucitol	
		Residual activity (%)	Specific growth rate, μ (h ⁻¹)	Residual activity (%)	Specific growth rate, μ (h ⁻¹)
GM273 ($\Delta ptsGHI::ermC$)	1.14	—	—	24	0.53
SA003 (<i>ptsHS46A</i>)	1.51	98	1.0	57	1.1
GM808 (<i>pts</i> ⁺)	1.5	20	1.25	53	1.25

* Under unrepressed conditions.

Table 4. Glucose repression of synthesis of β -glucanase in *ccpA* and *gra* mutants and IS58

Experiments were repeated at least twice; representative data are shown. ND, Not determined.

Strain	β -Glucanase activity* (nkat)	Residual activity (%)	Specific growth rate, μ (h ⁻¹)		
			Citrate	Glucose	Glycerol
WLN29 (<i>ccpA</i> ::Tn917)	1.31	50	0.62	0.45	0.42
WLN63 (<i>gra-32</i> ::Tn917)	0.4	12	0.62	1.1	ND
WLN106 (<i>gra-48</i> ::Tn917)	0.64	9	0.5	0.71	ND
GR100 (<i>gra-100</i> ::Tn917)	1.71	15	0.25	0.62	ND
BGW2† (<i>ccpA</i> ::Tn917)	2.8	127	ND	0.75	0.68
IS58 (<i>ccpA</i> ⁺ <i>gra</i> ⁺)	0.62	7	1.0	1.25	1.25

* Under unrepressed conditions.

† In repression experiments using 0.2% (w/v) glycerol a residual activity of 148% was measured.

Table 5. Effect of a multi-copy operator on carbon catabolite repression of β -glucanase and α -amylase synthesis in IS58

Three separate experiments were performed and representative results are shown.

Plasmid	β -Glucanase		α -Amylase	
	Enzyme activity* (nkat)	Residual activity† (%)	Enzyme activity* (units per OD ₅₅₀ unit)	Residual activity‡ (%)
—	0.62	7	0.15	26
pHV33	1.14	5.7	0.15	27
pEC040	147.0	23	0.18	40

* Under unrepressed conditions.

† Media supplemented with 0.2% (w/v) glucose.

‡ Media supplemented with 0.4% (w/v) glucose.

observed. As shown in Table 5 repression of *bglS* expression was lowered when the operator was present in multiple copies. It was assumed that an active *trans*-acting element occurs in limiting amounts under experimental conditions.

Moreover, the presence of plasmid pEC040 also influenced the stringency of carbon catabolite repression of α -amylase synthesis (Table 5). α -Amylase synthesis was not efficiently repressed in the presence of 0.2% glucose. Bacteria were grown in ASM under repressing (0.4% glucose) and non-repressing conditions and α -amylase activity measured. *amyE* gene expression was repressed fourfold in IS58 and IS58(pHV33), whereas in IS58(pEC040) a repression factor of only 2.5 was obtained.

Site-directed mutagenesis and analysis of mutants

An operator-like sequence was found via sequence alignment of the *amyO* operator with the upstream region of the *bglS* gene (Weickert & Chambliss, 1990). The postulated operator is located between -47 and -35 (with reference to the translation initiation codon) and shows three mismatch bases and one gap in comparison with *amyO* (Fig. 3).

To analyse the contribution of the operator-like sequence to catabolite repression of β -glucanase synthesis, site-directed mutagenesis using the recombinant PCR technique was performed. Two sets of primers were synthesized (U1/U2 and D1/D2) containing nucleotides representing the base substitutions to mutagenize the region of homology. The former primer set should generate an operator allowing stronger catabolite re-

Consensus sequence	T G W N A N C G N T N W C A
Optimal operator	T G T A A G C G C T T A C A
<i>B. subtilis</i> genes	
<i>amyE</i> (Weickert & Chambliss, 1990)	T G T A A G C G T T A A C A
<i>xyIA</i> (Jacob <i>et al.</i> , 1991)	T G A A A G C G T A A A C A
<i>bglS</i> (Murphy <i>et al.</i> , 1984)	T G T A A G A T C # A A C A
<i>gnt</i> (Miwa & Fujita, 1990)	T G A A A G C G G T A C C A
<i>glpD</i> (Holmberg & Rutberg, 1991)	T G T A A G C A T G C A C T
<i>xynCB</i> (Hastrup, 1988)	T G T T T G A T C # A A C A
<i>B. amyloliquefaciens</i> genes	
<i>bglA</i> (Hofemeister <i>et al.</i> , 1986)	T G T A T G A T C # A A T A
<i>apr</i> (Vasanthi <i>et al.</i> , 1984)	C G T A A G C G A A A T C A
<i>B. megaterium</i> xylose isomerase (<i>xyIA</i>)	
(Rygus & Hillen, 1992)	T G A A A G C G C A A A C A
<i>B. licheniformis</i> α -amylase (<i>amyL</i>)	
(Laoide <i>et al.</i> , 1989)	T A A C A G C G T C A G C A
<i>B. stearothermophilus</i> α -amylase	
(Nakajima <i>et al.</i> , 1985)	T G A A A A C G A T C A C A

Fig. 3. Comparison of sequences with homology to the consensus operator. (W = A or T; N = any base; # = gap).

Table 6. Effect of base substitutions on β -glucanase expression

Experiments were done at least twice; representative results are shown.

Plasmid strain	Operator sequence	β -Glucanase activity* (nkat)	Residual activity† (%)
IS58‡	TGTAAGATCAACA	0.62	0
BG314(pEC040)	TGTAAGATCAACA	84.7	61
BG314(pEC041)	TGTAAGATCAATA	484.7	94
BG314(pEC042)	TGTAAGCGCAAACA	27.3	48
BG314(pEC021)	TGTATGATCAATA	81.0	117

* Under unrepresed conditions.

† The glucose concentration used in these experiments was 0.4%.

‡ Control strain, in which the *bglS* system only occurs on the chromosome.

pression than the wild-type sequence ('up' mutation). The latter was used to form a structure abolishing the effect of catabolite repression of β -glucanase ('down' mutation). The primary amplifications and the fusion reactions were done as described above. Mutants were screened by sequence analysis and a frequency of mutation of 75% was obtained. In addition to base substitutions according to the primer design other single-base changes occurred (frequency 0.25%).

The recombinant plasmids pEC041 and pEC042, representing the 'down' and 'up' mutations, respectively, were transformed into *B. subtilis* BG314 and the effect of glucose on β -glucanase expression was measured. In the case of BG314(pEC041) glucose repression was nearly completely abolished, whereas in BG314(pEC042) the repression of β -glucanase synthesis was stronger than in the wild-type BG314(pEC040) (Table 6).

The β -glucanase gene of *Bacillus amyloliquefaciens* shows extensive homology with that of *B. subtilis* (Hofemeister *et al.*, 1986). The operator sequence for catabolite-mediated repression of *bglS* can also be found in the *bglA* system but involves two further base changes (Fig. 3). Plasmid pEC021, carrying the entire *bglA* gene and the upstream region, was transformed into *B. subtilis* BG314. Catabolite repression of β -glucanase synthesis was fully abolished in this strain as in the 'down' mutant BG314(pEC041) (Table 6).

Discussion

Carbon catabolite repression is a well-documented phenomenon in bacteria, but its mechanism in *B. subtilis* is poorly understood.

While the EIIA^{Glc} of the PTS is the sensor of glucose availability in *E. coli*, this is probably not the case in *B.*

subtilis. The repression data from experiments with PTS and non-PTS carbohydrates, and methyl α -glucoside, and with PTS mutants indicated that the PTS is not involved in carbon catabolite repression of β -glucanase synthesis in *B. subtilis*.

The HPr proteins of Gram-positive bacteria are unique in possessing a second phosphorylation site (Ser-46, Reizer *et al.*, 1989) besides the ubiquitous phosphorylation site His-15. A substitution of this Ser-46 by an alanine renders the expression of the *B. subtilis* gluconate operon glucose-resistant. The same applied to β -glucanase synthesis, but repression by the non-PTS carbohydrate glucitol was not influenced by the *ptsH* mutation. Furthermore, the reduced growth rate of the *ptsH* mutant SA003 indicates that this mutant might somehow be defective in glucose uptake and/or utilization.

Only those sugars that are metabolized via the glycolytic pathway (Table 2) led to catabolite repression of β -glucanase synthesis. Therefore it is possible that the intracellular signal for carbon catabolite repression is derived from glycolytic intermediates. This hypothesis is in accordance with the data concerning the *gra* mutants. The only pleiotropic mutation, *ccpA*, resulted in a very slow growth on both glucose and glycerol (Table 4). The glycerol-resistant expression of *amyE* is in contrast to previously published observations (Henkin *et al.*, 1991). This might be due to the genetic backgrounds of the strains studied. Indications of the involvement of glycolysis in carbon catabolite repression were also obtained by Freese *et al.* (1974).

The target of carbon catabolite repression of β -glucanase expression in *B. subtilis* was identified by site-directed mutagenesis. Data obtained by Weickert & Chambliss (1990) for the *cis*-active site of α -amylase repression were used to construct defined *bglS* operator mutants. The results (Table 6) clearly indicate that the postulated sequence is indeed the target for glucose repression of β -glucanase synthesis. Preliminary experiments proved the same to apply to repression by glucitol. In the case of pEC042, representing the operator 'up' mutation, an extra A at position 11 occurred which might be caused by the *Taq* polymerase during recombinant PCR (Table 6). On the other hand this operator consists of 14 base pairs, providing an additional adjustment to the consensus operator sequence. A region of weak homology to the consensus operator sequence is present in front of the *bglA* gene of *B. amyloliquefaciens* (Fig. 3). That region is very similar to the operator generated in the 'down' mutation approach but includes a further base substitution at position 5. It was not surprising that no catabolite repression of β -glucanase synthesis could be detected with strain BG314(pEC021) (Table 6).

The presence of the multi-copy plasmid pEC040

resulted in an increase of enzyme activity (about 140-fold). The lower activity obtained with strains harbouring pEC042 may be due to an amino acid exchange (Lys $\rightarrow$ Glu) in the leader peptide of the β -glucanase leading to an inefficient transport through the cytoplasmic membrane. The reason for the high enzyme level in the case of pEC041 will have to be determined in further investigations.

Strains BG314 and IS58 differed in catabolite repression efficiency. Enzyme synthesis was less repressed in strain BG314 than in IS58. An additional mutation in BG314 is thought to be the cause of that behaviour.

The dependence of catabolite repression on the concentration of the carbon source is another interesting observation. Both β -glucanase and α -amylase syntheses were more strongly repressed by 0.4% glucose in comparison to 0.2% (data not shown).

The phenomenon of the titration of a *trans*-factor due to the presence of the multi-copy plasmid pEC040 observed for β -glucanase synthesis (Tables 5 and 6) made it possible to investigate whether *amyE* expression is repressed by the same *trans*-factor. Plasmid pEC040 carries the entire *bglS* gene and the operator for catabolite repression of *bglS* expression. The binding of the postulated repressor to its operator sequence on the multi-copy plasmid pEC040 should also influence α -amylase repression. The results (Table 5) indicate that one type of negative regulator is responsible for glucose repression of both *amyE* and *bglS* expression. In addition, catabolite repression of β -xylosidase (encoded by *xynB*; Hastrup, 1988) is reduced by the presence of multicopy operator (C. Lindner, personal communication). Therefore all three genes may be part of one carbon catabolite repression regulon.

With the data presented in this paper the molecular mechanism of carbon catabolite repression for another degradative enzyme has been established. A *cis*-active sequence similar to those previously identified as targets of carbon catabolite repression of *amyE* and the *xyl* operon (Weickert & Chambliss, 1990; Jacob *et al.*, 1991) was found to be the regulatory element for *bglS* catabolite repression. Sequences resembling the consensus operator are present in front of many catabolic genes of *Bacillus* spp. (Fig. 3). All these genes are possibly part of a global carbon catabolite repression regulon. The identification of the *trans*-factor will lead to a more precise understanding of that regulatory phenomenon.

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