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Identification and Characterization of a New β-Glucoside Utilization System in *Bacillus subtilis*

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A new catabolic system in *Bacillus subtilis* involved in utilization of β-glucosidic compounds has been investigated. It consists of five genes encoding phosphotransferase system (PTS) enzyme II (*licB* and *licC*) and enzyme IIA (*licA*), a presumed 6-phospho-β-glucosidase (*licH*), as well as a putative regulator protein (*licR*). The genes map around 334° of the *B. subtilis* chromosome, and their products are involved in the uptake and utilization of lichenan degradation products. These five genes are organized in two transcriptional units. A weak promoter precedes gene *licR*, and transcription is obviously terminated at a secondary structure immediately downstream of the reading frame, as shown by Northern RNA blot analysis. Genes *licB*, *licC*, *licA*, and *licH* constitute an operon. Initiation of transcription at the promoter in front of this operon presumably requires activation by the gene product of *licR*. The LicR protein shows an unusual domain structure, i.e., similarities to (i) the conserved transcriptional antiterminator BglG family signature and (ii) PTS enzyme II. Using RNA techniques and transcriptional *lacZ* fusions, we have shown that the expression of the *licBCAH* operon is inducible by products of lichenan hydrolysis, lichenan and cellobiose. The presence of excess glucose prevents the induction of this operon, indicating the control by carbon catabolite repression. Moreover, the expression of the operon requires the general PTS components and seems to be negatively controlled by the specific *lic* PTS enzymes.

Soil bacteria such as many members of the family *Bacillaceae* are able to use a wide variety of carbohydrates, among them several glucans such as cellulose, the most abundant carbohydrate in nature; β-1,3-glucan (laminarin); and β-1,3-1,4-glucan (lichenan). The polymers are degraded by extracellular enzymes with distinct linkage specificities (44). However, only limited information on the steps which initiate metabolism of the degradation products of those polymers in bacteria has become available.

In *Bacillus subtilis*, several genes and operons which are involved in β-glucoside utilization are known. A monocistronic gene (*bglA*) encodes a phospho-β-glucosidase whose expression is induced by the aryl-β-glucosides salicin and arbutin (59) (SwissProt accession no. P42973). In the course of the sequencing project of the *B. subtilis* genome, a gene product with high similarity to the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) enzyme IIBC proteins was deduced from its coding sequence (*ywbA*, formerly *ipa-16d* [15]) (SwissProt accession no. P39584). Salicin seems to be an inducer of the expression of this gene. The *bglPH* operon (31) is another system for the β-glucoside utilization in *B. subtilis*, consisting of a PTS enzyme IIBCA protein (SytA/BglP; SwissProt accession no. P40739) and a phospho-β-glucosidase (BglH; SwissProt accession no. P40740). Arbutin and salicin are substrates of the gene products of the *bglPH* operon. The β-1,3-1,4-glucan lichenan is degraded by the gene product of the *licS* gene of *B. subtilis* (previously *bglS*) (41) (SwissProt accession no. P04957) to tetrasaccharides which are probably taken up via the PTS and become hydrolyzed intracellularly (50). The *licS* gene forms an operon with *licT* (50) (SwissProt accession no. P39805) encoding an antiterminator protein. The LicT protein is involved in the regulation of gene expression of both the *licTS* operon and the *bglPH* operon (27, 31, 50).

β-Glucosides such as lichenan and its hydrolysis products can be considered alternative carbon sources which are utilized under conditions of glucose limitation. The expression of the respective catabolic genes is controlled by different mechanisms in *B. subtilis* (51). Transcription of genes encoding proteins for utilization of sucrose and aryl-β-glucosides is regulated by transcriptional antitermination involving specific antitermination proteins which control transcription elongation in response to substrate availability (4, 9, 27). Degradation of the fructose polymer levan requires induction of the levanase operon by the activator LevR in the presence of small amounts of fructose (10, 36). The utilization of these carbon sources includes sugar-specific PTS components with both uptake and regulatory functions, i.e., in the absence of substrates the activity of the respective regulator (antiterminators or LevR) is controlled negatively by the PTS enzyme II (8, 31, 37). Moreover, the activity of such regulators is altered by phosphorylation by HPr (3, 54).

Another important mechanism for control of catabolic gene expression is known as carbon catabolite repression (14). The availability of rapidly metabolized carbon sources represses the expression of operons such as *sacPA*, *licTS*, *bglPH*, and *lev* (26, 27, 33, 36). There is an operator sequence (catabolite responsive element [CRE]) presumably serving as target for the DNA binding protein CcpA (11, 19, 23). Moreover, additional CcpA-independent mechanisms for catabolite repression have been reported (28, 38).

The *B. subtilis* genome sequencing project has proved to be a great resource for new, interesting genetic information (29). The identification of a system with high similarities to the *Escherichia coli* and *Bacillus stearothermophilus cel* operons led us to include these genes in our investigations about the β-glu-

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coside utilization network of *B. subtilis*. In *E. coli*, there are two cryptic operons for the utilization of cellobiose (*asc* and *cel*) which have been described in some detail (17, 42). Both systems contain genes for uptake of cellobiose via the PTS and intracellular cleavage by phospho-β-glucosidases. Transcription of the *celABCDF* operon can be activated by integration of insertion sequences or by mutations in *celD* encoding a repressor of the system (43). Once activated, the gene products of *celABC* direct uptake and phosphorylation of cellobiose, and the phospho-β-glucosidase CelF mediates degradation into glucose-6-phosphate and glucose (42). In the case of *B. stearothermophilus*, a DNA fragment containing four open reading frames sharing similarities to the *E. coli cel* operon has been identified (30). Genes *celA* and *celB* were identified as PTS enzyme II sharing similarity to *celA* and *celB* of *E. coli*, respectively. The *celD* gene shows similarity with PTS enzymes IIA and with *celC* of *E. coli*. Furthermore, a gene for a putative hydrolase, *celC*, was identified. Upstream from these genes presumably forming an operon, an incomplete regulatory gene was sequenced (30).

In this paper, we report the characterization of a new catabolic system in *B. subtilis*. The deduced gene products (SwissProt accession numbers are given in parentheses) of the five coding sequences of this system were previously named CelR (P46321), CelA (P46318), CelB (P46317), CelC (P46319), and CelD (P46320; in the SubtiList database, this protein is named CelF) (16). The experimental data represented here led us to redesignate this system. It consists of an oligosaccharide-specific PTS involved in uptake of degradation products of the β-glucan lichenan (*licB*, *licC*, and *licA*), a possible phospho-β-glucosidase (*licH*), and a putative regulator with an unusual domain structure (*licR*). The expression of these genes was investigated by means of RNA techniques and transcriptional *lacZ* fusions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed in Table 1. *E. coli* RR1 [*F*$^-$ (*gpt-proA*)*62 mcrB mrr ara-14 lacY1 leuB6 galK2 rpsL20 xyl-5 mtl-1 supE44*] (6) was used as the host for plasmid construction. This strain was grown in nutrient broth medium (26). For selection, ampicillin was added to a final concentration of 100 mg/liter. *B. subtilis* cells were grown in amino acid limitation medium (ASM) (53) under vigorous agitation at 37°C. For growth rate determination, the Belitzky minimal medium was modified as described by Krüger et al. (26), and the following carbon sources were used: 0.3% glucose, 0.05% cellobiose, 0.1% lichenan hydrolysate, and 0.2% lichenan. The hydrolysate of lichenan was obtained as described previously (50). Antibiotics were supplemented at the following concentrations: chloramphenicol, 5 mg/liter; erythromycin, 1 mg/liter; lincomycin, 25 mg/liter; kanamycin, 10 mg/liter.

Plasmid constructions. Plasmid pST1 was obtained by cloning a 1.8-kb PCR product containing an internal part of *licR* into the *Bam*HI site of pBluescriptSK$^-$ (Stratagene) treated with Klenow enzyme. The PCR was carried out with chromosomal DNA of *B. subtilis* IS58 as the template and primers Pr-cel5 (5′-GGAACTTCAAGGTGTGCTAAG-3′) and Pr-cel6 (5′-CATCCGAAAGC TCTTGATATG-3′) (Fig. 1B [b]).

A 3.3-kb DNA fragment containing the *licBCAH* genes was produced from chromosomal DNA of *B. subtilis* IS58 with primers Pr-cel2 (5′-GTGCAGCAG GCATGTCTACTAG-3′) and Pr-cel4 (5′-TGTTTGGCCACAGTATCGGAC-3′) (Fig. 1B [c]) and cloned into pBluescriptSK$^-$ treated with *Bam*HI and Klenow enzyme. The resulting plasmid was pST2.

The upstream region of *licR* was cloned by means of inverse PCR with primers Pr-cel7 (5′-GCAAAAAACGAACTTGTCACCG-3′) and Pr-cel5 (Fig. 1B) and religated chromosomal DNA of IS58 which was digested with *Pvu*II. The resulting 1.5-kb PCR fragment was recut with *Pvu*II, and the 0.7-kb fragment containing the complete promoter region was cloned into pBluescriptSK$^-$ modified as described above, yielding pST8.

Plasmid pST9 was obtained by isolation of a 0.8-kb *Eco*RV-*Pvu*II fragment from pST3 (Fig. 1B [a]) and subsequent ligation with pKL2 (1a) digested with *Sma*I.

The pDH32M (25) derivative pST13 was constructed as follows. A 2-kb PCR fragment was obtained with primers Pr-cel11 (5′-GATCGATTGGGAGAGTC AGCG-3′) and Pr-cel14 (5′-GTTCCATTTCCTCATTCACG-3′) (Fig. 1B [d]) and cloned into the *Sna*BI site of pDH32M.

To construct pST11 and pST21, the 1.3-kb *Xba*I fragment containing the neomycin resistance gene was isolated from pBEST501 (24). After treatment with Klenow enzyme, this fragment was cloned into the *Hpa*I site of pST1 and into the *Sty*I site of pST2 (Fig. 1B [b and c]), giving pST11 and pST21, respectively.

Genetic techniques. Standard techniques were used for plasmid extraction from *E. coli* (21), isolation of chromosomal DNA from *B. subtilis* (35), transformation of *E. coli* RR1 by the $CaCl_2$ method (47), and transformation of *B. subtilis* strains with naturally competent cells (20).

Treatment of DNA with restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase, and T4 polynucleotide kinase was performed as recommended by the supplier. DNA fragments and PCR products were purified with the Wizard Prep kits (Promega) or recovered from agarose gels with the GeneClean II kit (Bio 101, Inc.). DNA sequences were determined by the chain termination method with dideoxyribonucleotides (48), using modified T7 DNA polymerase (U.S. Biochemicals Corp.) and plasmid DNA as the template.

PCR products were obtained under the following conditions: denaturation, 1.1 min, 94°C; annealing, 1.2 min, 50°C; extension, 5 min, 72°C; 30 cycles. DNA fragments from plasmid DNA or from chromosomal DNA (200 ng) were amplified with 2.5 U of *Taq* polymerase in the appropriate buffer by adding 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, and 100 pmol of each primer in a final volume of 100 μl. The samples were overlaid with 70 μl of light mineral oil. Prior to ligation of the PCR products with vector plasmids, the DNA was modified according to the Double GeneClean procedure (Bio 101, Inc.). The resulting plasmids were subsequently sequenced to exclude potential PCR artifacts in the course of the *Taq* polymerase reaction.

Primer extension and Northern blot analysis. Total RNA was isolated according to the method of Völker et al. (55) by acid-phenol extraction from exponentially growing *B. subtilis* cells (optical density at 550 nm of about 0.4). Cultures were grown in ASM with 0.1% ribose, 0.3% glucose, 0.2% salicin, 0.1% lichenan hydrolysate, or 0.05% cellobiose. Samples of 25 ml were taken and mixed with ice containing 20 mM sodium azide. The resulting RNA pellets were dissolved in H_2O and stored at −80°C.

Primer Pr-cel7 (5′-GCAAAAAACGAACTTGTCACCG-3′) hybridizing from +153 to +132 with respect to the transcriptional start site of *licR* (Fig. 1B) and primer Pr-cel10 (5′-CATCTTGCTCACCAGTAAGC-3′) hybridizing from +107 to +88 with respect to the transcriptional start site of *licBCAH* (Fig. 1B) were 5′ end labeled with [γ-^{32}P]ATP and used for the primer extension experiments with

TABLE 1. *B. subtilis* strains

Strain	Genotype[a]	Source or reference[b]
IS58	*trpC2 lys-3*	Laboratory stock
BGT2/4	*trpC2 lys-3 licC*::*neo*	pST21 tf→ IS58
BGT5/6	*trpC2 lys-3 licR*::*neo*	pST11 tf→ IS58
BGT63	*trpC2 lys-3 licH′-lacZ cat*	pST9 tf→ IS58
BGT633	*trpC2 lys-3 licH′-lacZ cat* Δ*ptsGHI*::*erm*	BGW60 tf→ BGW63
BGT65	*trpC2 lys-3 licH′-lacZ cat licR*::*neo*	BGT5/6 tf→ BGW63
BGT67	*trpC2 lys-3 amyE*::(*licB′-lacZ cat*)	pST13 tf→ IS58
BGT671	*trpC2 lys-3 amyE*::(*licB′-lacZ cat*) *licC*::*neo*	BGT2/4 tf→ BGT67
BGT672	*trpC2 lys-3 amyE*::(*licB′-lacZ cat*) Δ*ptsGHI*::*erm*	BGW60 tf→ BGT67
BGT673	*trpC2 lys-3 amyE*::(*licB′-lacZ cat*) *licR*::*neo*	BGT5/6 tf→ BGT67
BGW60	*trpC2 lys-3* Δ*ptsGHI*::*erm*	52

[a] *cat*, chloramphenicol acetyltransferase gene; *erm*, erythromycin resistance; *neo*, neomycin resistance.
[b] tf→ indicates transformation.

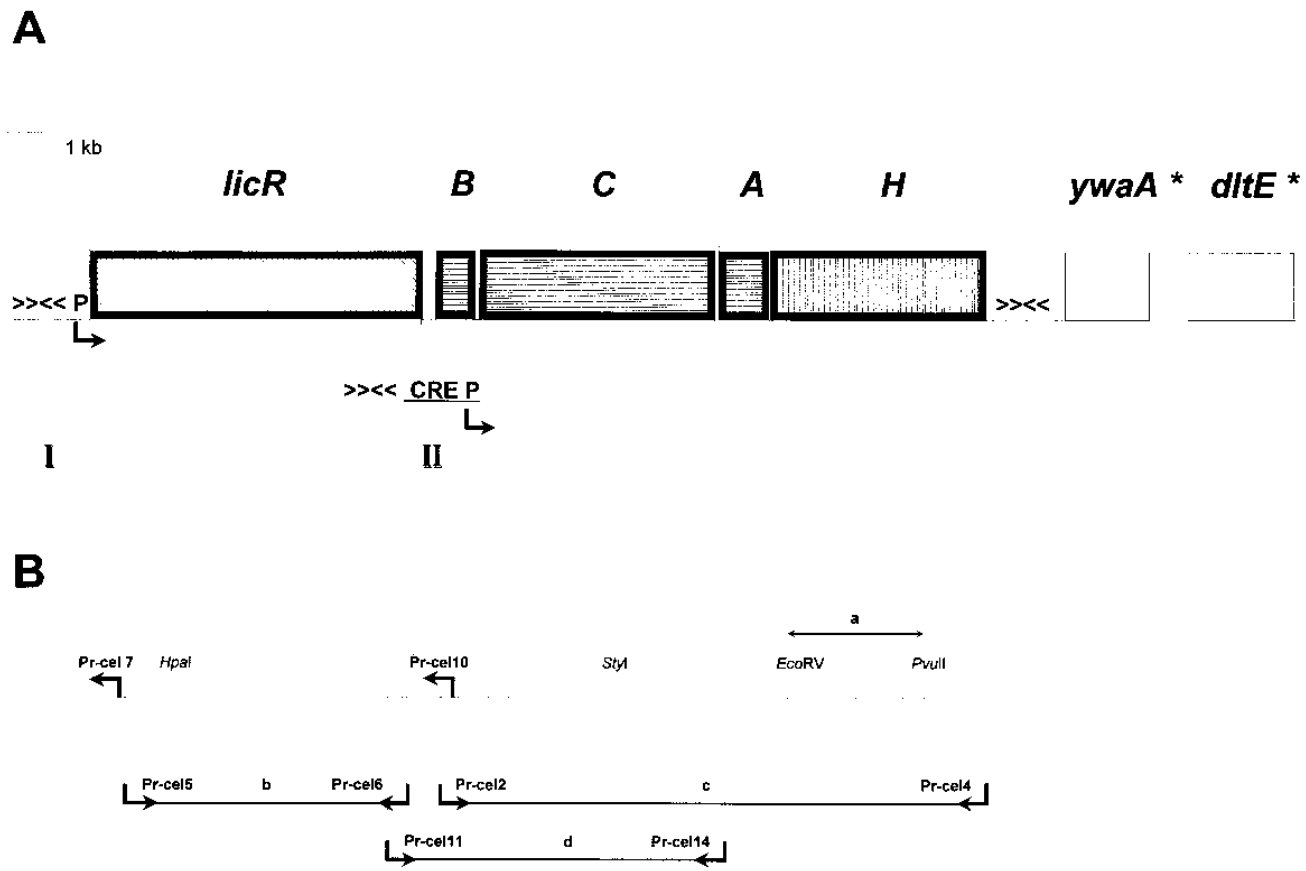


FIG. 1. Organization of the *lic* operon from *B. subtilis*. (A) The open reading frames named *licR*, *licB*, *licC*, *licA* and *licH* are indicated by striped bars. Putative factor-dependent terminators or sequences with dyad symmetry are indicated (>><<). The broken arrows upstream from *licR* and *licB* indicate the transcriptional start sites of these reading frames. The predicted target of the CcpA-dependent catabolite repression (CRE) upstream from *licB* is shown. *, see reference 15; I and II, see the legend to panel C below. (B) Restriction map of the *lic* operon with restriction enzymes, primers, and DNA fragments used (same scale as in panel A). Oligonucleotides are indicated by Pr-cel (see also Materials and Methods), and a through d show DNA fragments used in plasmid constructions. (C) I and II, DNA sequences of the upstream region of *licR* and *licB*, respectively. The −35 and −10 boxes of the promoters, transcriptional start sites (+1), and translational start codons are indicated. The potential RBS are underlined. The proposed terminator structures and the sequence with dyad symmetry are indicated (>><<). The putative CRE is double underlined.

10 μg of RNA. The same primers were used for DNA sequencing reactions which served as size standards. The products of reverse transcriptase extension were separated on a denaturing 6% polyacrylamide sequencing gel and visualized by autoradiography.

Digoxigenin-labeled RNA probes were produced by runoff transcription with T7 or T3 RNA polymerase according to the recommendation of the supplier (Boehringer, Mannheim, Germany). Probe C1 was obtained from plasmid pST1 linearized with *Xba*I prior to in vitro transcription, using T7 RNA polymerase. The plasmid pST2 was linearized with *Sca*I prior to in vitro transcription, using T3 RNA polymerase for probe C2. Northern blot analysis was performed according to the method of Wetzstein et al. (57), with modifications as described previously (28).

Assay of β-galactosidase activity. For enzyme assays, *B. subtilis* cultures were grown in ASM supplemented with the carbon source of interest (see Results). Cells were harvested (2-ml samples) 1.5 h after the culture entered stationary phase. The samples were stored at −20°C until the assay was carried out according to the method of Miller (39). The cells were permeabilized with toluene.

Computer analysis of sequence data. The sequence data analyses were performed with the Genetics Computer Group sequence analysis software package (Genetics Computer Group, Inc.). The deduced protein sequences of the open reading frames were compared with databases with the BLAST program (2).

Nucleotide sequence accession number. The nucleotide sequence including *licR* (formerly called *celR*) and *licBCAH* (formerly called *celABCD*) has been entered into the EMBL/GenBank/DDBJ databases under accession number Z49992 (16).

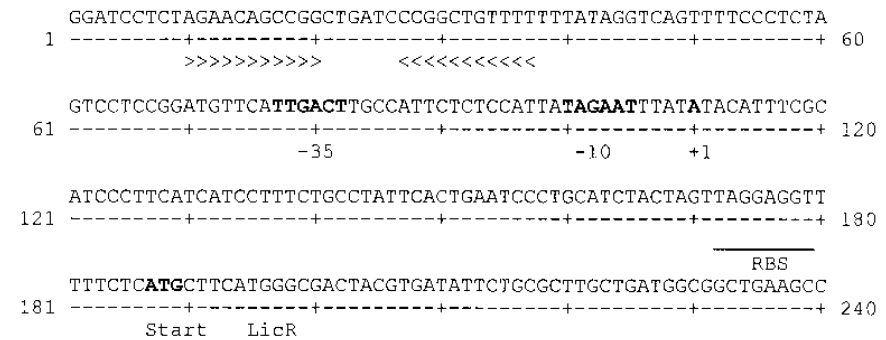
RESULTS

Identification of open reading frames by homology search and DNA sequence analysis. In the course of the project aimed at the sequencing of the *B. subtilis* genome, a 7-kb DNA fragment located immediately upstream of the 97-kb region described previously (15) was sequenced. Actually, this DNA region is located in the *B. subtilis* genome analyzed during the sequencing project by Fujita et al. (14a, 29). We have investigated a 6.8-kb fragment of this DNA region, which is situated directly upstream of a putative coding sequence called *ywaA* (*ipa-0r* [15]). The DNA sequence of the 6.8-kb fragment (GenBank accession no. Z49992) was translated in the six frames, and possible coding sequences (CDS) were identified. This chromosomal region contained five putative CDS all transcribed in the same direction (Fig. 1A) and in opposition to *ywaA*. The choice of the translational start codons for these putative proteins was based on the position of potential ribosomal binding sites (RBS) and similarities to known proteins. The predicted gene products of these reading frames are similar to known proteins from *B. subtilis* and other bacteria. The similarities were detected with the BLAST algorithm (2) and are summarized in Table 2.

The first CDS encodes a 641-amino-acid protein. Interestingly, the predicted protein seems to be composed of several domains, because it showed similarities to different protein families classified by function (Fig. 2). Two putative helix-turn-helix motifs (HTH) can be predicted in the N terminus of the

C

I



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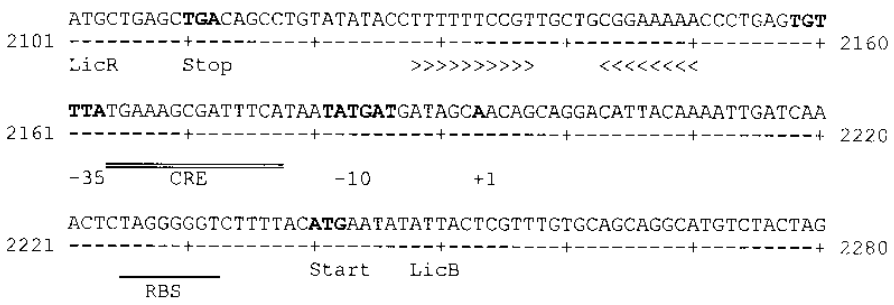

FIG. 1—*Continued.*

protein. The HTH at position 20 showed similarity to the consensus sequence of the HTH of the DeoR family (56) (Fig. 2 [A]). The second motif started with amino acid 108 and was similar to the consensus sequence of the LysR family HTH (18) (Fig. 2 [B]). Two other regions of the predicted protein (180 to 280 in Fig. 2 [C] and 315 to 400 in Fig. 2 [D]) shared similarities to the conserved regions of several antitermination proteins and the transcriptional activator LevR (for an overview, see reference 50) (Table 2). Furthermore, similarities to general mannitol-specific PTS enzymes IIA could be found in the C terminus of this polypeptide (Table 2) (Fig. 2 [E]). These similarities were particularly significant to the phosphorylation site signature of PTS enzyme IIA domains (46). Based on the unusual composition, we suggest that this CDS encodes a regulator protein, which was confirmed by our experimental data (see below). Therefore, it was designated *licR* (*celR*). Just upstream of the deduced *licR* promoter region, a potential factor-dependent transcription terminator structure was identified (Fig. 1A and 1C [I]).

A protein of 102 amino acids could be translated from the second CDS (designated *licB* [*celA*]) (Fig. 1A). It was highly similar to cellobiose-specific enzyme IIB and, to a lower degree, to the IIB domain of several enzymes $IICB^{Lac}$ (Table 2). The third CDS (designated *licC* [*celB*]) (Fig. 1A) encodes a 452-amino-acid polypeptide. The protein sequence revealed similarity to the *ywbA* gene product (*ipa-16d*) (15) localized 22 kb downstream in the same orientation which is presumably a PTS enzyme II protein (Table 2). The deduced amino acid sequence of *licC* from *B. subtilis* also shared significant similarities to the membrane-spanning enzyme IIC^{Cel} and to the IIC domain of enzyme $IICB^{Lac}$ (Table 2). The fourth CDS of 110 codons (designated *licA* [*celC*]) (Fig. 1A) showed similarities to several PTS enzyme IIA proteins (Table 2).

The last CDS with 442 codons (designated *licH* [*celD/F*]) (Fig. 1A) was not in the same translational reading frame as *licA*. The stop codon of *licA* overlapped the potential RBS of *licH*, indicating a translational coupling. The predicted amino acid sequence of *licH* was similar to different hydrolytic en-

TABLE 2. Comparison of predicted amino acid sequences for *licR*, *licB*, *licC*, *licA*, and *licH* from *B. subtilis* with homologous polypeptides from *B. subtilis* and other organisms

Protein[a]	Length (aa); MM (kDa); pI[b]	Proposed function	% Identity (aa)	Reference
LicR[c](CelR)	641; 73.3; 6.15	Regulator	32.7 (101) SacY *B. subtilis*[d]	60
			22.7 (308) BglG *E. coli*[d]	49
			21.3 (254) SacT *B. subtilis*[d]	9
			30.0 (100) MtlA *B. subtilis*[e]	1
			22.1 (163) MtlA *E. coli*[e]	32
			21.2 (156) MtlF *Enterococcus faecalis*[e]	13
LicB(CelA)	102; 10.9, 6.25	PTS enzyme IIB	59.2 (98) CelA *B. stearothermophilus*	30
			45.7 (94) CelA *E. coli*	42
			25.6 (82) LacE[f] *Streptococcus mutans*	45
			25.3 (83) LacE[f] *Staphylococcus aureus*	7
LicC(CelB)	452; 48.5; 8.44	PTS enzyme IIC	75.1 (437) YwbA *B. subtilis*	15
			65.9 (449) CelB *B. stearothermophilus*	30
			32.2 (428) LacE[g] *S. mutans*	45
			29.5 (448) CelB *E. coli*	42
LicA(CelC)	110; 12.2; 4.8	PTS enzyme IIA	61.5 (104) CelD *B. stearothermophilus*	30
			40.0 (100) CelC *E. coli*	42
			37.4 (99) LacF *Lactococcus lactis*	12
			35.4 (99) LacF *S. mutans*	45
LicH(CelD,CelF)	442; 48.7; 5.35	Hydrolase	54.7 (397) CelF *E. coli*	42
			24.2 (462) MelA *E. coli*	34

[a] In addition to the new nomenclature, the previous name of the respective gene product is given in parentheses.
[b] aa, amino acids; MM, molecular mass; pI, predicted pI.
[c] See Fig. 2.
[d] See Fig. 2 [C] and [D].
[e] See Fig. 2 [E].
[f] Similarities were found with the C-terminal domain.
[g] Similarities were found with the N-terminal domain.

zymes (Table 2). Thus, the *licH* gene is presumed to encode a hydrolase (6-phospho-β-glucosidase).

The intergenic region between *licR* and *licB* extended over 50 nucleotides. A sequence with a dyad symmetry and a putative target site for carbon catabolite repression (CRE) (23) were found in this region (Fig. 1A and 1C [II]). A potential factor-dependent transcription termination structure was located immediately downstream of the translational stop signal of *licH*.

Utilization of different carbon sources in *lic* mutants. To investigate the phenotype of a strain carrying a mutation in the *licC* gene encoding a PTS component, plasmid pST21 was constructed (see Materials and Methods) and integrated into the chromosome of the *B. subtilis* wild-type strain IS58, yielding the strain BGT2/4 (*licC*::*neo*). Growth rates were determined in a modified synthetic medium for the wild-type strain IS58 and BGT2/4 with different carbon sources as described in Materials and Methods. The wild-type and mutant strains grew with a growth rate (μ, measured per hour) of about 1 and 0.8, respectively, when glucose was used as the carbon source (Fig. 3). Interestingly, strain BGT2/4 (*licC*::*neo*) could utilize cellobiose as the sole carbon source like the wild type (Fig. 3). Therefore, the operon is not essential for cellobiose uptake. However, the *licC* mutant strain did not grow if lichenan hydrolysate (Fig. 3B) or lichenan (data not shown) was supplemented.

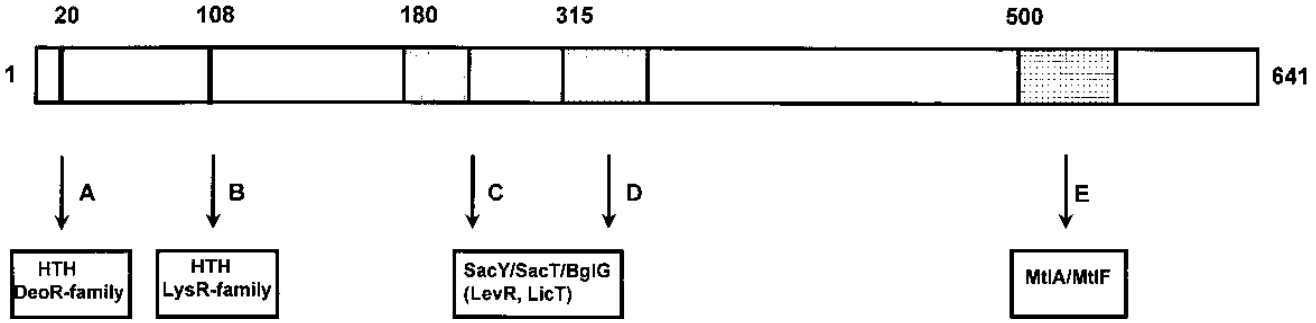

FIG. 2. Domain structure of LicR and the position of the domains in the polypeptide. The two putative HTH sharing similarities to the consensus sequences of the HTH of the DeoR family (A) and the LysR family (B) are indicated by bars. Domains C and D are similar to positive regulator proteins (antiterminators) and are represented as striped boxes. The cross-striped box (E) indicates a domain in the C terminus of LicR which shows similarities to PTS enzyme II proteins. The numbers show the first amino acid of a certain domain within the polypeptide.

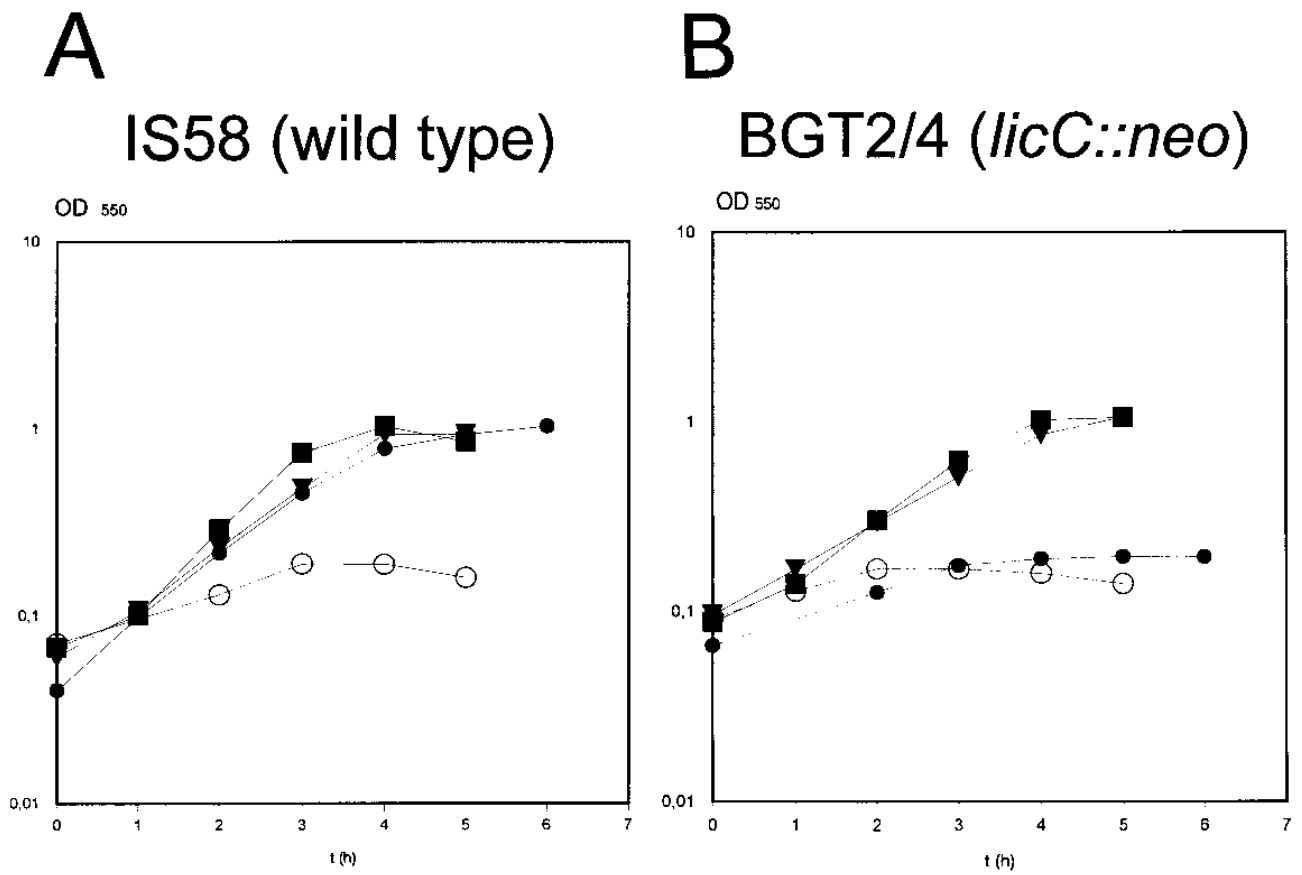

FIG. 3. Growth of the wild-type strain IS58 (A) and the *licC*::*neo* mutant strain BGT2/4 (B). Cells were grown in a modified synthetic medium as described in Materials and Methods. The following carbon sources were supplemented: 0.3% glucose (squares), 0.05% cellobiose (triangles), and 0.1% lichenan hydrolysate (circles). The residual growth of the control (medium without carbon sources, open circles) was due to traces of glucose present in the inoculum. These experiments were carried out in triplicate; representative data are shown. OD_{550}, optical density at 550 nm; t (h), time in hours.

A *licH* mutant grew on all substrates tested (data not shown). Thus, it is possible that other β-glucosidases with similar substrate specificities take over the function of the *licH* gene product (58).

Strain BGT5/6 derived from integration of plasmid pST11 containing an insertion of the *neo* gene in the unique *Hpa*I site of *licR* (Fig. 1B) was also unable to grow in this synthetic medium supplemented with lichenan hydrolysate or lichenan as the sole carbon source. The growth rates of BGT5/6 obtained with glucose or cellobiose were comparable to those of strain IS58 (data not shown).

From these experiments we conclude that the gene products of *licB*, *licC*, and *licA* (specific PTS components) and the LicR protein (regulator) are essential for the uptake of the extracellular hydrolysis products of lichenan.

Determination of specific transcripts by Northern blot analysis. The DNA sequence analysis suggested that genes *licBCAH* might be cotranscribed and that *licR* is transcribed alone. To investigate this assumption, Northern (RNA) blot analyses were performed. First, probe C1 covering an internal part of *licR* was used. A signal corresponding to a transcript of about 2,000 nucleotides was present under all conditions tested, but the intensity is extremely low in the presence of ribose or glucose (Fig. 4A, all lanes). This result suggests that the gene *licR* is expressed as a single transcription unit at a low level.

Probe C2 was used to detect transcription of *licBCAH*. A unique transcript of about 3,400 nucleotides could be detected if either lichenan hydrolysate or cellobiose was supplemented (Fig. 4B, lanes 4 and 5), but no signal was visible when ribose, glucose, or salicin was used as the carbon source (Fig. 4B, lanes 1, 2, and 3). These results confirmed that genes *licBCAH* constitute an operon as deduced from the nucleotide sequence. The intensity of the signal was very strong if cellobiose or lichenan hydrolysate was present (Fig. 4B, lanes 5 and 4, respectively). Thus, the operon appeared to be induced by lichenan hydrolysate and cellobiose and was not expressed in the presence of ribose, glucose, or salicin (see also below).

To verify these conclusions, we used probe C3, containing an internal part of *licH*. RNA from the same sources was used as described above. The transcriptional signals detected with probe C3 were consistent with the results obtained with the Northern blot analysis with probe C2 (data not shown).

Reverse transcriptase mapping of mRNA start points. The location of the promoters and the start sites of *licR* and *licBCAH* were determined by primer extension experiments. *B. subtilis* cells from strain IS58 were grown in ASM, and total RNA was isolated as described in Materials and Methods. The mRNA start point of *licR* was mapped with the primer Pr-cel7 (Fig. 1B). The transcriptional start point of *licR* was detected at a single A preceded by −10 (TAGAAT) and −35 (TTGACT) boxes resembling a vegetative promoter structure (σ^A) (40) (Fig. 1C [I]; Fig. 5A).

The start site of the *licBCAH* mRNA and the regulation at

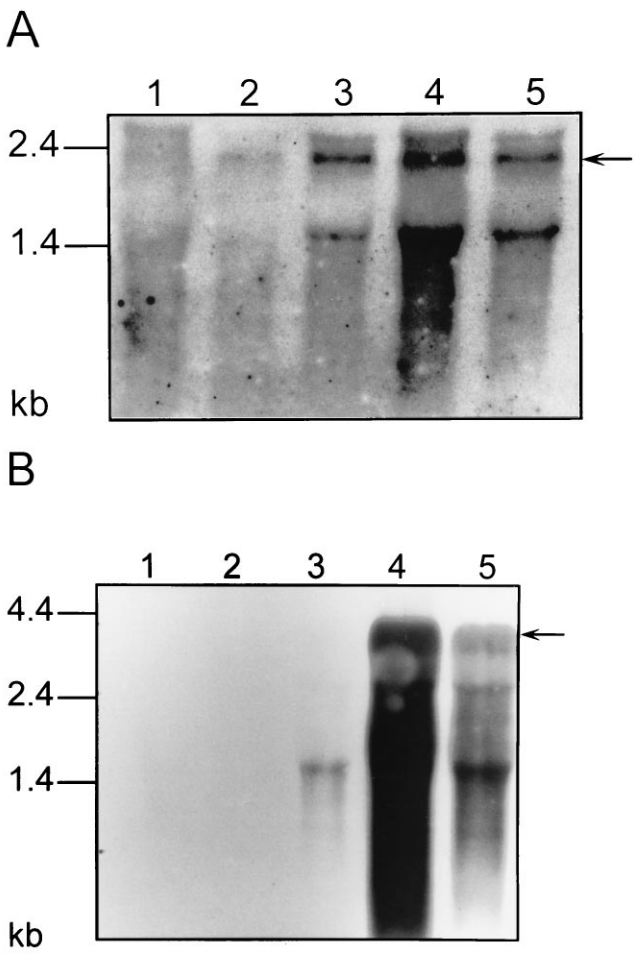

FIG. 4. Northern (RNA) blot analysis with digoxigenin-labeled RNA probes C1 (A) and C2 (B). Total RNA from *B. subtilis* wild-type strain IS58 grown in ASM with 0.1% ribose (lane 1), 0.3% glucose (lane 2), 0.2% salicin (lane 3), 0.1% lichenan hydrolysate (lane 4), or 0.05% cellobiose (lane 5) was used. The arrows indicate transcripts of about 2,000 (A) and 3,400 (B) nucleotides. rRNA migrated as follows: 23S rRNA, 2,900 nucleotides; 16S rRNA, 1,500 nucleotides.

the promoter were studied with primer Pr-cel10 (Fig. 1B). A single mRNA start point was identified (Fig. 1C [II]; Fig. 5B). A potential −10 region (TATGAT) could be identified, but the corresponding −35 box (TGTTTA) revealed only weak identity to promoters recognized by the vegetative RNA polymerase (Fig. 1C [II]) (see also Discussion). Transcription was not initiated if ribose, glucose, or salicin was added as the carbon source (Fig. 5B, lanes 1, 2, and 3). The mRNA from cells grown with cellobiose gave a strong signal (Fig. 5B, lane 5). The amount of mRNA increased when lichenan hydrolysate was used as the carbon source (Fig. 5B, lane 4). These experiments support the conclusions of the Northern blot experiments as mentioned above.

Analysis of transcriptional *lacZ* fusions and the involvement of putative *trans*-acting factors on expression of *licBCAH*. To investigate the regulation of the *licBCAH* promoter in more detail, plasmid pST9 carrying a *licH′-lacZ* fusion was introduced into the *licH* gene of *B. subtilis* IS58. The resulting strain BGT63 (reference strain) was grown in ASM supplemented with either 0.1% ribose, 0.1% glucitol, 0.3% glucose, 0.2% salicin, 0.05% cellobiose, 0.1% lichenan hydrolysate, or 0.2% lichenan (Table 3). The presence of cellobiose, lichenan hydrolysate, or lichenan resulted in a strong increase of β-galactosidase activity (cellobiose, 40-fold; lichenan hydrolysate and lichenan, 60-fold) compared to the control (ribose). Salicin had no effect on expression of the *licH′-lacZ* fusion. The activity of β-galactosidase decreased with respect to the control, if the cells were grown with 0.3% glucose as the sole carbon source. Thus, these results were consistent with the observations from Northern blotting and primer extension experiments, i.e., the transcription from the promoter in front of *licB* is inducible by cellobiose, lichenan, and lichenan hydrolysate.

The effect of rapidly metabolized carbon sources (e.g., glucose) on the induction of the operon was investigated by supplementary addition of 0.3% glucose to the inducing carbon sources. β-Galactosidase was not expressed (Table 3), suggesting that the *licBCAH* promoter is strongly regulated by carbon catabolite repression.

Furthermore, we examined the activity of the *licH′-lacZ* fusion in a Δ*ptsGHI* genetic background. Strain BGT63 was transformed with chromosomal DNA from *B. subtilis* BGW60 (Δ*ptsGHI*), yielding strain BGT633. The same growth conditions were used as described for BGT63, but glucitol was used instead of glucose. As shown in Table 3, the expression of the *licH′-lacZ* fusion is completely abolished. This result indicates that the PTS components are required for the expression of the *licBCAH* operon. On the other hand, this phenotype could be caused by prevention of the uptake of a possible internal inducer (see below).

Investigating *licH′-lacZ* expression in a *licR* mutant (BGT65), we observed no β-galactosidase activity with either the inducing carbon sources or glucose (Table 3). Thus, the LicR protein might be a positive regulator of the operon. This is in agreement with the deficiency of the *licR* mutant in growth on lichenan hydrolysate.

To analyze the influence of *trans*-acting factors on the regulation of *licBCAH* expression in more detail, a *licB′-lacZ* fusion (plasmid pST13) including the intergenic region of *licR* and *licB* was introduced in the *amyE* gene of *B. subtilis* IS58, yielding strain BGT67 (reference strain). The strain was grown in ASM with 0.1% ribose (control), 0.05% cellobiose, 0.1% lichenan hydrolysate, or 0.2% lichenan. The β-galactosidase activity of the *licB′-lacZ* fusion increased sevenfold if cellobiose was supplemented (Table 4). The strongest induction of β-galactosidase activity was obtained with lichenan hydrolysate (35-fold) and lichenan (27-fold) (Table 4). This indicates that the target for this kind of regulation seems to be located in the regulatory region of *licB*. The different amounts of β-galactosidase activity in strains BGT63 (Table 3) and BGT67 might be due to the insertion of the *lacZ* transcriptional fusions at different sites of the chromosome of *B. subtilis* IS58 (*licH* and *amyE*, respectively).

Investigating the influence of the proposed *lic* PTS components on the induction of their own promoter, chromosomal DNA from strain BGT2/4 (*licC*::*neo*) was transformed in BGT67 which contained a *licB′-lacZ* fusion integrated in *amyE*. The resulting strain (BGT671) was grown under the same conditions as the reference strain BGT67. Interestingly, the β-galactosidase activity in BGT671 obtained with the noninducing sugar ribose was similar to the activity of the *licB′-lacZ* fusion if an inducing carbon source (cellobiose, lichenan hydrolysate, or lichenan) was supplemented (Table 4). The expression of the *licBCAH* operon seems to be constitutive at a high level in this strain. Thus, the specific PTS components of the operon (LicC or LicA) are obviously involved in the negative regulation of its own expression, i.e., the induction of the *licBCAH* operon is altered via the *lic* PTS dependent upon the availability of an inducer. Moreover, these data show that even

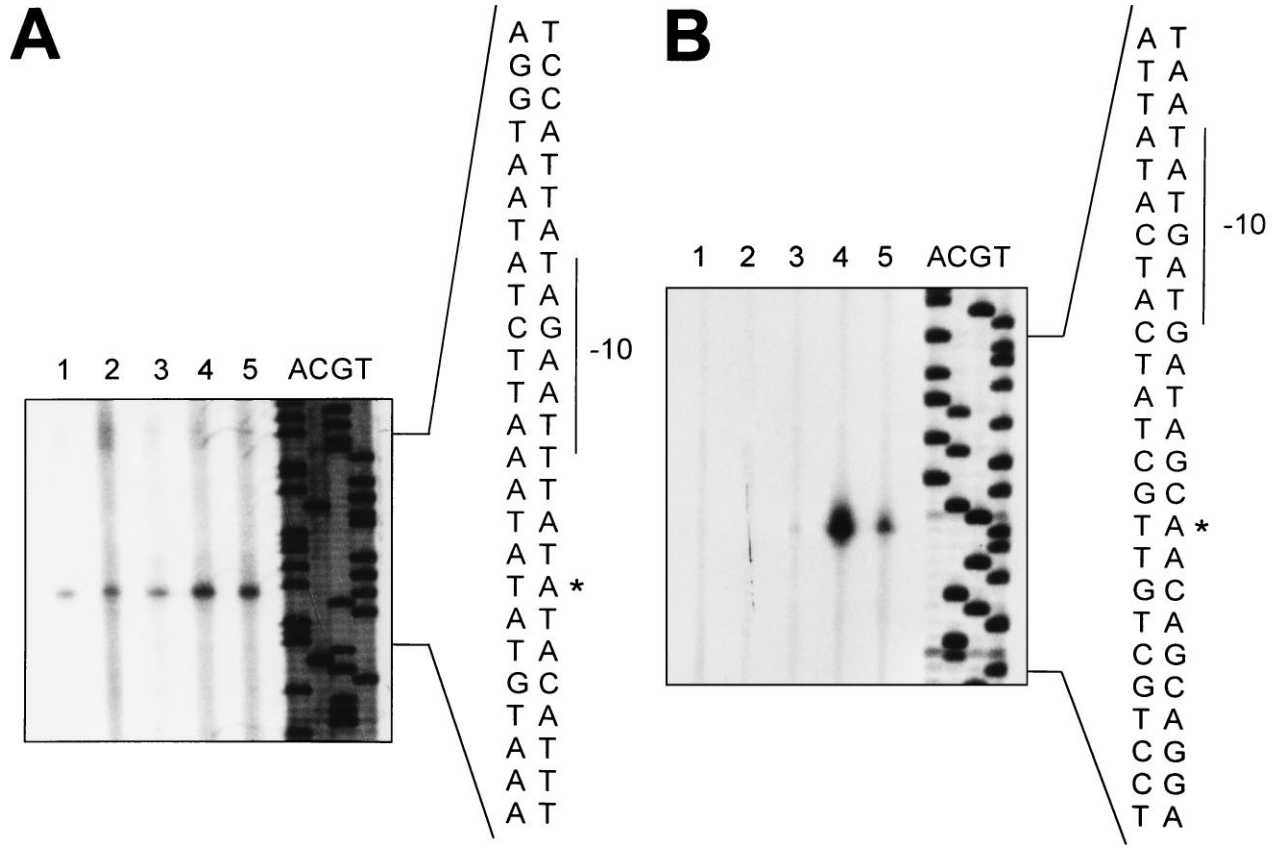

FIG. 5. Reverse transcriptase mapping of the transcriptional start points using the primer Pr-cel7 for *licR* (A) and the primer Pr-cel10 for *licBCAH* (B). RNA was isolated from strain IS58 grown with 0.1% ribose (lane 1), 0.3% glucose (lane 2), 0.2% salicin (lane 3), 0.1% lichenan hydrolysate (lane 4), or 0.05% cellobiose (lane 5). Sequencing reactions (ACGT) were done by using the respective oligonucleotides as primers. The sequences surrounding the initiation sites (indicated by asterisks) are shown on the right. The deduced −10 boxes are indicated.

in the absence of an internal inducer, a high level of gene expression occurred.

The activity of the *licB′-lacZ* fusion is completely abolished in a Δ*ptsGHI* or a Δ*licR* genetic background (BGT672 and BGT673, respectively) (Table 4) under the same growth conditions as described for BGT67. The results underline the conclusions that a functional PTS and the LicR protein are necessary for the expression of the *licBCAH* operon (compare BGT633 and BGT65 [Table 3]).

DISCUSSION

In this study, the characterization of an oligosaccharide utilization system in *B. subtilis* has been reported. It consists of five open reading frames sequenced in the course of the sequencing project of the *B. subtilis* genome and shares high similarities to the *cel* operons from *E. coli* (42) and *B. stearothermophilus* (30). Despite these apparent similarities, the genes were named *licR*, *licB*, *licC*, *licA*, and *licH*, since *licBCA*

TABLE 3. Influence of different carbon sources and effect of mutations in *ptsGHI* and *licR* on *licH* expression[a]

Strain	Relevant genotype	β-Galactosidase activity (Miller units)						
		0.1% Ribose	0.1% Glucitol	0.3% Glucose	0.2% Salicin	0.05% Cellobiose	0.1% LH[b]	0.2% Lichenan
BGT63	*licH′-lacZ*	3.9	0.9	0.7	4.6	172 (1.2)[d]	257 (1.6)[d]	264 (0.8)[d]
BGT633	*licH′-lacZ* Δ*ptsGHI*::*erm*	0.9	1.1	ND[c]	1.5 (0.5)[e]	1.4 (0.8)[e]	1.3 (0.8)[e]	1.6 (0.9)[e]
BGT65	*licH′-lacZ licR*::*neo*	0.5	0.2	0.1	0.1	0.4	0.5	0.6 (0.2)[d]

[a] Cells were grown in ASM supplemented with the carbon sources as indicated. For β-galactosidase assay, 2-ml samples were taken after cultures entered the stationary phase (optical density at 550 nm of about 1). Growth rate did not influence the expression of the *lacZ* fusions, because the activities of β-galactosidase in stationary phase did not differ significantly from those in exponential phase (data not shown). Three independent experiments were performed, and representative results are shown.

[b] LH, lichenan hydrolysate; 0.1% LH is equivalent to 0.1% glucose. The amount of glucose equivalents was determined by the dinitrosalicylic acid method (53).

[c] ND, not determined.

[d] 0.3% glucose was supplemented additionally.

[e] 0.1% glucitol was supplemented additionally.

TABLE 4. Expression of a *licB′-lacZ* fusion and influence of *trans*-active regulators[a]

Strain	Relevant genotype	β-Galactosidase activity (Miller units)			
		0.1% Ribose	0.05% Cellobiose	0.1% LH	0.2% Lichenan
BGT67	*licB′-lacZ*	13	84	459	363
BGT671	*licB′-lacZ licC*::*neo*	362	200	511	675
BGT672	*licB′-lacZ* Δ*ptsGHI*::*erm*	1.0	0.8	0.8	1.4
BGT673	*licB′-lacZ licR*::*neo*	0.5	0.5	0.6	0.8

[a] Growth conditions, sample collection, and determination of lichenan hydrolysate (LH) are equivalent to those described in Table 3. Representative results from three independent experiments are shown.

encode a specific PTS responsible for the uptake of lichenan hydrolysis products. Here, we have shown by Northern blot analysis that the genes *licB*, *licC*, *licA*, and *licH* constitute an operon. This operon is inducible by cellobiose, products of lichenan hydrolysis, and lichenan, as shown by different techniques, including measurement of mRNA levels and transcriptional *lacZ* fusions. The target required for induction is probably located upstream of the *licB* gene.

The polymeric carbohydrate lichenan isolated from *Cetraria islandica* is degraded by the extracellular β-1,3-1,4-endoglucanase LicS (*bglS* [41]; *licS* [50]). This reaction is the initial step of β-glucan utilization by *B. subtilis*. The synthesis of β-glucanase is two- to threefold stimulated by lichenan and its degradation products, and the induction mediated via transcriptional antitermination requires the positive regulator protein LicT, which is a member of the BglG family of antiterminators (50). We found that the products of lichenan degradation are a mixture of oligosaccharides as penta-, tetra-, and trisaccharides (data not shown). For the understanding of β-glucan utilization, it is crucial to find the genes whose products are necessary for uptake and hydrolysis of β-glucan degradation products. So far, neither a transport system nor a cleavage enzyme has been identified for those oligosaccharides. The results presented in this paper suggest that the *licR-licBCAH* system might encode these unknown proteins necessary for transport and hydrolysis as well as regulation, because *licR* and *licC* mutants are strongly impaired in the utilization of lichenan hydrolysis products. Schnetz et al. (50) proposed that the LicT protein not only is a positive regulator of *licS* gene expression but also is involved in the regulation of genes encoding proteins for lichenan or lichenan hydrolysate utilization. However, there is no sequence motif (RAT) in this system which is typical for the BglG antiterminator family (5). Preliminary experiments performed to investigate the role of the antiterminator LicT indicate an involvement in the regulation of the *licBCAH* operon (our unpublished results) which will be analyzed in further studies.

The transcriptional start point of the *licBCAH* mRNA was identified by primer extension. The deduced −35 region (TG TTTA) shares rather weak similarity to promoters recognized by the vegetative RNA polymerase (40). This observation and the strong induction of *licBCAH* gene expression are in agreement with the requirement of a transcriptional activator (presumably LicR). Such an activator could recognize its target upstream from the promoter, as has been described for LevR (10). Moreover, no sequence similarities to cognate promoter consensus sequences of alternative σ factors (40) could be identified.

The availability of glucose represses the expression of the operon, i.e., the operon is subject to carbon catabolite repression. In *B. subtilis*, the CcpA protein, the HPr protein, and a CRE are involved in the regulation of gene expression by carbon catabolites (see references 22 and 26). A putative operator sequence (TGAAAGCGATTTCA) following the CRE consensus sequence was identified in the spacer region of the *licBCAH* promoter. This arrangement is quite similar to the situation in the *bglPH* system for aryl-β-glucoside utilization, which is controlled by carbon catabolite repression (27), indicating a similar regulatory mechanism, i.e., inhibition of transcription initiation, for *licBCAH* expression.

The first CDS (*licR*) encodes a protein with an interesting domain structure. In the N terminus of the predicted amino acid sequence, two putative HTH could be identified. These motifs share similarities to the consensus sequences of the HTH of two transcriptional regulator subfamilies: the DeoR family (56) and the LysR family (18). It is possible that one or both motifs are necessary for LicR-DNA interaction. However, there are no sequence motifs resembling target sites for both types of regulator families within the *lic* region. Two regions of the LicR protein are highly similar to the regulatory domains (phosphorylation sites) of antiterminator proteins. Based on the strength of these similarities and the results obtained with the *licR* mutant strain, we suggest that the *licR* gene product is a regulator protein. Furthermore, a domain in the C terminus of LicR shows a phosphorylation site signature of PTS enzymes IIA (46). The structure of the *licR* gene product is therefore quite unusual, with two regulatory motifs (conserved phosphorylation sites) of different protein families located within the same protein. We propose that LicR activity might be regulated by (i) phosphorylation of the antiterminator-similar domain by the general PTS enzymes (possibly HPr), as has been described previously (3, 54); (ii) phosphorylation of the PTS enzyme II phosphorylation site domain by specific PTS enzymes (presumably $EIIA^{Lic}$) or other regulator proteins; or (iii) protein-protein interaction with proteins which are involved in the regulation of β-glucoside utilization (e.g., LicT [27]). These assumptions are supported by the facts that the *licBCAH* operon is not expressed in Δ*ptsGHI* strains but it is constitutive in a *licC* mutant. Further studies will be carried out to define the regulatory mechanisms mediated via LicR.

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