The genes controlling sucrose utilization in *Clostridium beijerinckii* NCIMB 8052 constitute an operon

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The sucrose operon of *Clostridium beijerinckii* NCIMB 8052 comprises four genes, which encode a sucrose-specific enzyme IIBC^{scr} protein of the phosphotransferase system (ScrA), a transcriptional repressor (ScrR), a sucrose hydrolase (ScrB) and an ATP-dependent fructokinase (ScrK). The *scrARBK* operon was cloned in *Escherichia coli* in three stages. Initial isolation was achieved by screening a *C. beijerinckii* genomic library in *E. coli* for clones able to utilize sucrose, while the remainder of the operon was isolated by inverse PCR and by plasmid rescue of flanking regions from a *scrB* mutant constructed by targeted gene disruption. Substrate specificity assays confirmed that the sucrose hydrolase was a β -fructofuranosidase, able to hydrolyse sucrose and raffinose but not inulin or levans, and that the *scrK* gene encoded an ATP/Mg²⁺-dependent fructokinase. Both enzyme activities were induced by sucrose in *C. beijerinckii*. Disruption of the *scr* operon of *C. beijerinckii* by targeted plasmid integration into either the *scrR* or the *scrB* gene resulted in strains unable to utilize sucrose, indicating that this was the only inducible sucrose catabolic pathway in this organism. RNA analysis confirmed that the genes of the *scr* operon were co-transcribed on a 5 kb mRNA transcript and that transcription was induced by sucrose, but not by glucose, fructose, maltose or xylose. Primer extension experiments identified the transcriptional start site as lying 44 bp upstream of the *scrA* ATG start codon, immediately adjacent to the imperfect palindrome sequence proposed to be a repressor binding site. Disruption of the *scrR* gene resulted in constitutive transcription of the upstream *scrA* gene, suggesting that *scrR* encodes a transcriptional repressor which acts at the *scrA* operator sequence. The *scrR* gene is therefore itself negatively autoregulated as part of the polycistronic *scrARBK* mRNA.

Keywords: *Clostridium beijerinckii*, sucrose PTS, sucrose hydrolase, fructokinase, coordinated induction

INTRODUCTION

Solventogenic strains of the *Clostridium acetobutylicum*/*C. beijerinckii* group have been used for production of acetone and butanol in the classical acetone-butanol-ethanol (ABE) fermentation (Woods, 1993). Industrial ABE fermentations were initially based

on starch, but subsequently molasses became the primary substrate, and saccharolytic clostridial strains from three distinct taxonomic groups were isolated (Johnson & Chen, 1995; Jones & Keis, 1995; Woods, 1993). *C. beijerinckii* NCIMB 8052 (formerly *C. acetobutylicum*; Keis *et al.*, 1995) was one of these mesophilic, obligately anaerobic spore-forming bacteria isolated for its ability to ferment molasses. Although the disaccharide sucrose is a major constituent of molasses and optimal utilization of the carbohydrate substrate is essential for high yields in the ABE fermentation, the genes involved in sucrose utilization in the saccharolytic clostridia have not been identified and characterized.

Abbreviation: PTS, phosphoenolpyruvate-dependent phosphotransferase system.

The GenBank accession number for the *C. beijerinckii* *scrARBK* operon is AF059741.

Sucrose metabolism has been well characterized in several Gram-positive bacterial species (Steinmetz, 1993) and sucrose has been shown to be metabolized by extracellular as well as intracellular enzyme systems. Extracellular enzymes capable of sucrose hydrolysis include β -fructosyltransferases and α -glucosyltransferases, which catalyse the hydrolysis of sucrose and the synthesis of fructose and glucose polymers respectively (Steinmetz, 1993). Where sucrose is catabolized intracellularly, the predominant mechanism facilitating sucrose uptake is the phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Saier & Reizer, 1992), which results in the accumulation of intracellular sucrose 6-phosphate. Transport and phosphorylation of the carbohydrate is mediated by the sucrose-specific Enzyme II (EII^{Ser}). The sucrose 6-phosphate is further metabolized by sucrose-6-phosphate hydrolases, yielding glucose 6-phosphate and fructose. In some species, fructokinases phosphorylate the fructose generated by sucrose hydrolysis, so that both products can be incorporated into the glycolytic pathway.

The components of the glucose PTS have been identified in *C. beijerinckii* 8052 (Mitchell *et al.*, 1991), and the presence of Enzyme I, HPr and the glucose-specific membrane-associated Enzyme II proteins was confirmed by complementation of cell extracts with extracts prepared from PTS mutants of *Escherichia coli* or *Bacillus subtilis*. Studies on the metabolism of several other sugars by *C. beijerinckii* have established that PTS-mediated sugar uptake appears to be the predominant carbohydrate transport mechanism in this bacterium and PTS-dependent transport mechanisms have been specifically proposed for sucrose, fructose, glucitol, mannitol and lactose. Non-PTS uptake systems, however, are thought to play a role in galactose and maltose uptake (Mitchell *et al.*, 1995; Tangney *et al.*, 1998). Recent evidence confirmed the presence of all three components of a sucrose-specific PTS in *C. beijerinckii* (Tangney *et al.*, 1998).

PTS permeases contain two peripheral membrane domains (IIA and IIB) as well as an integral membrane domain (IIC), all of which may be contained within a single polypeptide chain (Saier & Reizer, 1992). Analysis of several sucrose transport systems indicates that in *Streptococcus mutans* (Sato *et al.*, 1989), *Streptococcus sobrinus* (Chen *et al.*, 1993) and *Pediococcus pentosaceus* (GenBank accession L32093) the EII^{Ser} proteins consist of EIIB, EIIC and EIIA domains, fused by linker regions (EIIBCA^{Ser} proteins). In other cases, such as *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Vibrio alginolyticus*, the EII^{Ser} proteins simply consist of fused EIIBC^{Ser} domains (Blatch *et al.*, 1990; Lengeler *et al.*, 1994).

Sucrose-catabolic genes are often clustered on the genome, as is the case with *V. alginolyticus* (Blatch *et al.*, 1990), *B. subtilis* (Fouet *et al.*, 1986, 1987) and *Strep. mutans* (Sato *et al.*, 1989), or on transposons or plasmids, as in enteric bacteria (Bockmann *et al.*, 1992; Tittgemeyer *et al.*, 1996). The arrangement and transcrip-

tional organization of these clusters differ in each species, suggesting a modular evolution of these systems. These sucrose-utilization regions invariably contain genes encoding a sucrose hydrolase and a sucrose transport protein, but also may be clustered with other *scr*-specific genes, such as fructokinase and regulator genes. The genes encoding regulatory proteins associated with sucrose-catabolic genes are functionally diverse. In *B. subtilis*, the expression of sucrose-catabolic genes is regulated by transcriptional antiterminators (Arnaud *et al.*, 1992), while in *Staphylococcus xylosus*, *V. alginolyticus*, *Sal. typhimurium* and *K. pneumoniae* regulation is achieved by transcriptional repressors of the GalR-LacI family (Blatch *et al.*, 1990; Bockmann *et al.*, 1992; Jahreis & Lengeler, 1993). In most other sucrose-utilization regulons, with the exception of *Lactococcus lactis* (Rauch & de Vos, 1992), the corresponding *scrR* genes are independently transcribed, and no examples where all the genes are co-ordinately controlled as an operon have hitherto been described.

In this paper we report the isolation and characterization of a sucrose-utilization operon from *C. beijerinckii* NCIMB 8052, and demonstrate the genetic organization and transcriptional regulation of the genes. The *scr* operon of *C. beijerinckii*, unlike the other sucrose-utilization regulons reported to date, contains all the genes required for sucrose uptake, hydrolysis and the subsequent phosphorylation of the products, under co-ordinated control of the repressor gene, *scrR*.

METHODS

Bacterial strains, plasmids and culture conditions. *C. beijerinckii* NCIMB 8052 was routinely cultured at 37 °C using Clostridial Basal Medium (CBM) (Jones *et al.*, 1982) or Reinforced Clostridial Medium (RCM) (Difco) in an anaerobic cabinet with a gas phase of 5% H₂, 10% CO₂ and 85% N₂ (Forma Scientifica). Where appropriate, erythromycin was used at a final concentration of 10 µg ml⁻¹. Carbohydrate studies were performed on cells grown in GSMM medium (Jones *et al.*, 1982) supplemented with 0.2% Casamino acids (Difco) and with the appropriate carbohydrate (0.25%, w/v).

E. coli strains and plasmids are listed in Table 1. JM109 (Sambrook *et al.*, 1989) was used for all cloning purposes and was routinely cultured at 37 °C on Yeast Tryptone (YT) or Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). MacConkey agar medium (Difco) supplemented with either sucrose (1%, w/v) or lactose (1%, w/v) was used to assess carbohydrate fermentation phenotypes. *E. coli* CA474, a λ cl857 lysogen of *E. coli* HB101 (Wilkinson & Young, 1994) was used as a donor in transconjugation experiments and was cultured on Brain Heart Infusion (BHI) medium (Difco). Ampicillin (100 µg ml⁻¹) and kanamycin (100 µg ml⁻¹) were included in media where appropriate.

Nucleic acid isolation and manipulation. DNA and RNA manipulations were carried out by standard methods (Sambrook *et al.*, 1989). Plasmid DNA was extracted and purified from *E. coli* using the Nucleobond AX KIT (Macherey-Nagel). T4 DNA ligase and DNA restriction endonucleases were obtained from Boehringer and were used according to

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Reference or source
<i>C. beijerinckii</i>		
NCIMB 8052	Prototrophic	NCIMB Culture Collection
CBSCRB	<i>scrB::erm</i>	This study
CBSCRR	<i>scrR::erm</i>	This study
<i>E. coli</i>		
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ⁻ Δ(lac-proAB) (F', traD36 proAB lacI^qΔM15)</i>	Sambrook <i>et al.</i> (1989)
CA474	F ⁻ <i>λcl857 hsdS20(r_B⁻ m_B⁻) supE44 aro-14 galK2 lacY1 proA2 rpsL20(Sm^R) xyl-5 mtl-1 recA13</i>	Wilkinson & Young (1994)
Plasmids		
pBluescript SK (pSK)	Amp ^R <i>lacZ'</i>	Stratagene
pEcoR251	Amp ^R (<i>EcoRI</i>)	Zabeau & Stanley (1982)
pACYC177	Amp ^R Km ^R	Chang & Cohen (1978)
R702	Km ^R ; Tra ⁺ Mob ⁺	Wilkinson & Young (1994)
pMTL30	Amp ^R Em ^R ; Tra ⁻ Mob ⁺	Williams <i>et al.</i> (1990)
pCTC1	Amp ^R Em ^R ; Tra ⁻ Mob ⁺	Williams <i>et al.</i> (1990)
pCBS1	Amp ^R ; 6.8 kb <i>Sau3A</i> <i>C. beijerinckii</i> fragment in pEcoR251	This study
pCBS2	Amp ^R ; 3 kb <i>ScaI</i> fragment from pCBS1 in pSK <i>EcoRV</i> site	This study
pCBS3	Amp ^R Em ^R ; 3.2 kb <i>HindIII</i> - <i>NdeI</i> fragment recovered from CBSCRB	This study
pCBS4	Amp ^R Km ^S ; 1.2 kb isolated from <i>C. beijerinckii</i> by PCR	This study
pCBMU1	Amp ^R Em ^R ; pMTL30 with internal 435 bp <i>scrB</i> fragment	This study
pCBMU3	Amp ^R Em ^R ; pMTL30 with internal 456 bp <i>scrR</i> fragment	This study

*Sm^R, Amp^R, Km^R, Em^R, resistance to streptomycin, ampicillin, kanamycin and erythromycin respectively; Tra, Mob, ability of plasmids to transfer or to be mobilized by conjugation respectively.

the manufacturer's recommendations. Chromosomal DNA was isolated from *C. beijerinckii* as described by Collett *et al.* (1997). The *C. beijerinckii* genomic library was constructed by partially digesting chromosomal DNA with *Sau3A*, and ligating DNA fragments of between 5 and 11 kb into the *Bgl*III site of the positive selection vector, pEcoR251 (Table 1). Total RNA was isolated from *C. beijerinckii* as described previously (Youngleson *et al.*, 1995). DIG-labelled RNA probes, specific for the transcripts of the *scrA*, *scrR*, *scrB* and *scrK* genes, were prepared and hybridized according to standard techniques specified by Boehringer. Primer extension experiments were carried out according to Ausubel *et al.* (1989), with the addition of actinomycin D (0.225 mg ml⁻¹). The primers used were: primer c (*scrA* gene: 5'-TTCTTTTGCTACTAT-TTGTTC-3') and primer d (*scrK* gene: 5'-TTATCTAC-ACAAAGCATATC-3').

Targeted integration of plasmids into the *C. beijerinckii* genome. The targeted integration system developed by Wilkinson & Young (1994) involves the mobilization of the suicide vector, pMTL30, carrying short regions of *C. beijerinckii* 'target' DNA, into *C. beijerinckii*. pCBMU1 was constructed by cloning the 435 bp *NdeI* fragment internal to

scrB from pCBS1 into the *StuI* site of pMTL30, and pCBMU3 by cloning the 456 bp *HindIII*-*ScaI* fragment internal to *scrR* from pCBS1 into pMTL30 (Table 1, Fig. 1). *E. coli* CA474, containing the mobilizing plasmid R702 and the relevant pMTL30 derivative, was conjugated with *C. beijerinckii* cultures as described by Wilkinson & Young (1994), with incubation anaerobically at 30 °C for 18 h to prevent induction of the *λ* prophage of CA474 (Table 1). Selection of *C. beijerinckii* transconjugants was facilitated by plating onto CBM agar plates containing 10 µg erythromycin ml⁻¹ and incubating anaerobically at 42 °C for 18 h to enhance counter-selection of the donor strain by induction of the *λ* prophage. Southern blot analysis was used to verify that the *scrR* and *scrB* genes had been disrupted in *C. beijerinckii* CBSCRR and CBSCRB respectively. The hybridization pattern indicated that in both cases, multiple copies of the plasmids had integrated into the genes.

Recovery of integrated plasmids from the *C. beijerinckii* genome. Genomic DNA was isolated from CBSCRB, the *C. beijerinckii* strain carrying chromosomally integrated copies of pCBMU1 in the *scrB* gene (Table 1). The isolated DNA was digested with *HindIII*, and self-ligated (10 ng DNA per 30 µl

ligation reaction). *E. coli* JM109 transformants were screened for ampicillin resistance on YT agar medium containing ampicillin ($100 \mu\text{g ml}^{-1}$).

PCR amplification of DNA from the *C. beijerinckii* genome.

An inverse-PCR strategy as described by Sambrook *et al.* (1989) was used to amplify the 5' region of the *C. beijerinckii* sucrose-utilization system. Chromosomal DNA was digested with *Xmn*I and self-ligated (200 ng DNA per 50 μl ligation). Two divergent primers, primer a (5'-TATTATAGGTAC-AAATACATCTCC-3') and primer b (5'-ATATGTGAGC-GAAGAAAATGC-3') were designed from the known sequence of the *scrR* gene, and were used for inverse PCR with the self-ligated chromosomal DNA as a template (Fig. 1). PCR reactions were conducted using an OmniGene thermocycler (Hybaid) and *Taq* DNA polymerase (Advanced Biotechnologies). The amplified fragment was cloned into the *Sma*I site of the low-copy-number vector pACYC177 (Chang & Cohen, 1978).

Nucleotide sequencing. DNA sequencing was carried out as described previously (Collett *et al.*, 1997) or with the Sequi-therm kit (Epicentre Technologies) using CY-5 labelled M13 primers and an OmniGene thermocycler (Hybaid). Where CY-5 label was used, nucleotide sequence was resolved using an Alflexpress automated DNA sequencer (Pharmacia). The current databases were screened for similarities to the DNA and deduced protein sequences using the BLAST program of the National Centre for Biotechnology Information (NCBI), and further analysed with the programs in the Genetics Computer Group (GCG) sequence analysis package (Devereux *et al.*, 1984). Phylogenetic analysis was conducted using the CLUSTAL w algorithm of Thompson *et al.* (1994), and the results were rendered using the TreeView software package (R. D. M. Page, 1996, Institute of Biomedical and Life Sciences, University of Glasgow).

Enzyme and protein assays. Sucrose hydrolase activity was assayed using an adaptation of the dinitrosalicylic acid method as described by Blatch & Woods (1993). Sucrose hydrolase activities were expressed as μmol reducing sugar produced min^{-1} (mg protein^{-1}). The substrate specificity assays were carried out as in the standard saccharolytic assay, except that all substrates (sucrose, raffinose, melezitose, levan and inulin) were supplied at a concentration of 1% (w/v). Fructokinase activity was assayed using the coupled assay described by Sprenger & Lengeler (1988). Fructokinase activities were expressed as nmol or μmol fructose 6-phosphate produced min^{-1} (mg protein^{-1}). Protein concentrations were determined using the method of Bradford (1976), with bovine serum albumin as the standard.

DNA-directed cell-free protein synthesis. Plasmid-encoded proteins were investigated using an *E. coli* DNA-directed cell-free transcription/translation system (Promega) according to the manufacturer's protocol. The proteins were labelled with L-[^{35}S]methionine and resolved on 12% SDS-PAGE gels as described by Laemmli (1970). Molecular mass standards were obtained from Pharmacia.

RESULTS

Isolation of the truncated *scr* gene cluster

The *C. beijerinckii* genomic library constructed in pEcoR251 was screened in *E. coli* JM109 on sucrose-MacConkey agar plates for sucrose-fermenting clones. After a 36 h incubation period, a single red colony was

Fig. 1. Organization of the *scr* genes of the *C. beijerinckii* sucrose-utilization system. Transcriptional polarities are indicated by solid arrows. pCBS1 was isolated from a *C. beijerinckii* genomic library by its ability to confer a sucrose-positive phenotype on *E. coli* and has an insert of 6.8 kb. pCBS2 was constructed by cloning the 3 kb *Sca*I fragment into pSK. pCBS3 was recovered from the genome of *C. beijerinckii* strain CBSCRb, carrying multiple copies of a pMTL30 derivative integrated into the *scrB* gene. pCBS4 was constructed by amplifying a 1.2 kb fragment containing the 5' region of *scrA* by inverse PCR, using primers a and b, and cloning into the unique *Sma*I site in pACYC177.

identified out of approximately 3000 colonies screened. The plasmid isolated from this colony (pCBS1) was found to contain a 6.8 kb insert (Fig. 1). The sequence that conferred a sucrose-positive phenotype was located on a 3 kb *Sca*I fragment (pCBS2) and DNA sequencing revealed a truncated ORF followed by two complete ORFs in the same orientation (Fig. 1). The truncated ORF (*scrR*) specified a protein with homology to members of the GalR-LacI family of transcriptional regulators, and the two ORFs downstream of *scrR* specified proteins with homology to sucrose hydrolases (*scrB*), and to fructokinases (*scrK*), respectively. No sucrose-transport genes were present on the insert in pCBS2 despite the fact that pCBS2 was able to confer a sucrose-positive phenotype on *E. coli*. This may be due to limited cell lysis releasing the cloned sucrose hydrolase.

Isolation of the complete *scrR* gene by plasmid rescue

The complete *scrR* gene was isolated by plasmid recovery from *C. beijerinckii* strain CBSCRb, which had been shown to carry multiple copies of a pMTL30 derivative integrated into the *scrB* gene (Table 1). The recovered plasmid, containing the adjacent chromosomal DNA, was designated pCBS3 (Fig. 1). Sequence analysis of the *C. beijerinckii* DNA inserted in pCBS3 indicated that although the complete *scrR* gene had been isolated, a truncated ORF was present upstream of *scrR*,

Table 2. Substrate specificity of sucrose hydrolase activity conferred on *E. coli* JM109 by pCBS2

Specific activity is expressed as μmol reducing sugar min^{-1} (mg protein) $^{-1}$. Values are means from three experiments, with standard deviations in parentheses. ND, No reducing sugars were detected after exposure to the indicated substrate for 60 min.

Strain	Specific enzyme activity				
	Sucrose	Raffinose	Melezitose	Levan	Inulin
<i>E. coli</i> JM109(pCBS2)	15.60 (0.31)	8.84 (0.51)	ND	ND	0.24 (0.08)
<i>E. coli</i> JM109(pSK)	ND	0.22 (0.04)	ND	ND	0.18 (0.07)

specifying a protein with homology to EIIBC^{Scr} PTS proteins (*scrA*).

Isolation of the 5' region of the *scrA* gene by inverse PCR

Attempts were made to clone the *scrA* gene using the plasmid recovery strategy outlined above, but while plasmids equivalent to pCBS3 were readily recovered, regions further upstream could not be isolated. This suggested that the region upstream of the *scr* genes contained a strong promoter or DNA motif which was lethal to *E. coli*. Such an observation has been made for the sucrose-utilization system of *L. lactis*, where the promoter region of the *scrB* gene was found to be unstable when cloned on high-copy-number plasmids in *E. coli* (Rauch & de Vos, 1992). It has also been noted that overexpression of membrane-bound PTS proteins may be lethal to host cells (Lengeler *et al.*, 1994). An inverse-PCR strategy was therefore developed to amplify the 5' region of the *scrA* gene and clone it into a low-copy-number plasmid, pACYC177, yielding pCBS4 (Fig. 1).

Sucrose hydrolase and fructokinase activities conferred on *E. coli* by pCBS2

Analysis of enzyme activities in cell-free extracts prepared from *E. coli* cells carrying pCBS2 confirmed the presence of both sucrose hydrolase and fructokinase activities. Investigation of the substrate specificity of the sucrose hydrolase indicated that it catalysed the hydrolysis of sucrose and raffinose but not melezitose, inulin and levan (Table 2). These data are consistent with the presence of a β -fructofuranosidase, able to hydrolyse the fructose moieties from low-molecular-mass sugars (sucrose and raffinose), but not from the (β 2 $\rightarrow$ 1)-linked fructose polymer inulin or the (β 2 $\rightarrow$ 6)-linked fructose polymer levan. The sucrose hydrolase activity had a pH optimum slightly below pH 6.0, which corresponds almost exactly with that reported for the intracellular invertase activity of *C. acetobutylicum* IFP 912 (Looten *et al.*, 1987). Maximum sucrose hydrolase activity was recorded between 35 and 40 °C, and the sucrose hydrolase was relatively stable at temperatures up to 30 °C for 2 h. Exposure to temperatures higher

than 30 °C resulted in a substantial loss of activity (data not shown).

The *scrK* gene product was proposed, on the basis of homology, to be a fructokinase of the ribokinase/*pfkB* sugar kinase family. Fructokinase activity could be demonstrated in cell-free extracts from *E. coli* JM109(pCBS2), with no detectable activity in the controls with plasmid vector only (results not shown). No fructokinase activity could be detected when ATP or MgCl₂ was omitted from the assay mixture, in agreement with the reported characteristics of other fructokinases of this family (Fennington & Hughes, 1996; Sprenger & Lengeler, 1988). Maximum fructokinase activities were recorded between 20 and 35 °C, and the enzyme was unstable with respect to temperature, particularly when exposed to temperatures higher than 20 °C (results not shown). Subsequent fructokinase assays were conducted at 20 °C, which gave relatively high levels of activity while minimizing enzyme denaturation.

Gene organization of the complete *scrARBK* gene cluster

The *scrA*, *scrR*, *scrB* and *scrK* genes were clustered and present in the same orientation on the *C. beijerinckii* genome. As indicated above, *scrA* was proposed to encode an EIIBC^{Scr} protein, *scrR* to encode a regulatory protein of the GalR-LacI family, *scrB* to encode a sucrose hydrolase, and *scrK* to encode a fructokinase. The largest intergenic region (190 bp) was present between *scrA* and *scrR*, while *scrR* and *scrB* were separated by only 26 bp, and *scrB* and *scrK* overlapped (Fig. 2). Primer extension experiments using a *scrA*-specific primer, primer c, showed that *scrA* transcripts were initiated at an adenine residue 44 bp upstream of the *scrA* ATG start codon (Fig. 3). The deduced -35 and -10 promoter sequence (5'-TTGACA-N₁₈-TATCAT) lies between 51 and 80 bp upstream of the *scrA* initiation codon (Fig. 2a), and shows considerable similarity to *E. coli* σ^{70} and clostridial consensus promoter sequences (Young *et al.*, 1989). A potential stem-loop structure which could act as a transcriptional terminator was identified 21–59 bp downstream of the proposed *scrK* termination codon (-15 kcal mol⁻¹;

Fig. 2. Nucleotide sequence of the promoter regions preceding the *scrA* gene (a) and the *scrK* gene (b) of the *C. beijerinckii* *scrARBK* gene cluster, indicating the overlap between the *scrB* and *scrK* genes (b). Deduced amino acid sequences are shown in single-letter code below the coding sequences. Putative initiation and termination codons are indicated in bold type. Putative promoters (–35 and –10 regions) and ribosome-binding sites (SD) are in bold type and underlined. The transcriptional start site of the *scrARBK* operon as determined by primer extension analyses is marked by a vertical arrow. The facing arrows, 31 to 42 bp upstream of the proposed *scrA* initiation codon, indicate an imperfect palindrome which may act as an operator site for a GalR-LacI family regulatory protein. Primers c and d were used for primer extension experiments of the *scrA* and *scrK* genes respectively.

–63 kJ mol^{–1}) (Zuker & Stiegler, 1981). An imperfect palindrome (G₆A₅A₄A₃A₂C₁/G₁G₂T₃T₄T₅C₆) was identified between the putative *scrA* promoter and initiation codon (Fig. 2a), which may function as an operator at which a transcriptional regulator of the GalR-LacI family could bind (Weickert & Adhya, 1992).

Analysis of the deduced *C. beijerinckii* Scr proteins

The deduced amino acid sequences encoded by the *C. beijerinckii* *scrA*, *scrR*, *scrB* and *scrK* genes were used to search the nucleic acid and protein databases using BLAST (NCBI; Devereux *et al.*, 1984).

The *scrA* gene specifies a deduced protein of 451 aa with a calculated molecular mass of 47 641 Da and homology to previously studied EIIScr proteins, with the highest amino acid sequence identity to the *Strep. sobrinus* ScrA (55.1% identity over the corresponding EIIBC domains). Several particularly well-conserved regions have been identified for EIIBCScr proteins (Titgemeyer *et al.*, 1996) and these regions are present in the deduced *C. beijerinckii* ScrA. The most highly conserved regions include the motif containing the proposed EIIB phosphorylation site and the GITE motif which has been proposed to be involved in phosphate and/or substrate binding (Lengeler *et al.*, 1994). Hydrophobicity analysis revealed that the deduced ScrA protein has an N-

terminal hydrophilic domain of approximately 112 aa, corresponding to the intracellular EIIB^{Scr} domain and linker region, while the remainder of the protein is essentially hydrophobic and corresponds to the EIIC^{Scr} domain which is likely to be an integral membrane component (data not shown).

The *C. beijerinckii* *scrR* gene specifies a deduced protein of 330 aa with a calculated molecular mass of 36 602 Da and showing significant homology to members of the GalR-LacI family of transcriptional regulators (31.8% and 26% identity to *Staph. xylosus* and *P. pentosaceus* ScrR proteins respectively). Multiple sequence alignment of the ScrR proteins demonstrated that the region which was most highly conserved was the N-terminal helix–turn–helix DNA-binding motif. In addition, the *C. beijerinckii* and *Staph. xylosus* ScrR proteins share several other conserved domains, corresponding to the regions of the LacI protein identified as being involved in inducer binding (Weickert & Adhya, 1992). This suggests that the *C. beijerinckii* and *Staph. xylosus* ScrR proteins respond to the same ligand, probably sucrose 6-phosphate (Gering & Brückner, 1996).

In order to establish the relationship between the deduced *C. beijerinckii* ScrR and other GalR-LacI family members, a phylogenetic analysis was conducted using the CLUSTAL W algorithm of Thompson *et al.* (1994). The recently isolated ScrR proteins were analysed, and the GalR-LacI regulatory proteins selected by Nguyen &

Fig. 3. Identification of the *scr* operon promoter and transcription initiation site: primer extension analysis of *scr* transcription in *C. beijerinckii* 8052 grown in GSMM containing sucrose (0.25 %, w/v). A DNA sequence ladder of the promoter region is shown on the left. PE, primer extension reaction product. Oligonucleotide primer c was used as the primer for both the DNA sequence and the primer extension reactions. The transcription initiation site at an A residue is indicated.

Saier (1995) were included for comparison. The resulting phylogenetic tree suggests that within the GalR-LacI family, proteins involved in the regulation of sucrose-catabolic genes have evolved at least four times (A to D in Fig. 4). The first evolutionary line involves the *Staph. xylosus* and *C. beijerinckii* ScrR proteins (A), while the second line involves the *P. pentosaceus* and *Strep. mutans* ScrR proteins (B), which appear to have diverged from one another more recently than the ScrR proteins of *Staph. xylosus* and *C. beijerinckii*. The third and fourth lines lead to the CscR of *E. coli* (C) and the closely related ScrR proteins of *K. pneumoniae* and *Sal. typhimurium* respectively (D). The proteins of the four evolutionary lines appear to be no more related to each other than to other GalR-LacI family members, despite their common role as regulators of sucrose metabolism.

The *scrB* gene specifies a deduced protein of 485 aa with a molecular mass of 57 128 Da, which agrees with the value of 53.2 kDa determined after cell-free transcription/translation and PAGE analysis (data not shown). The putative *C. beijerinckii* ScrB protein showed homology to β -fructofuranosidases of the glucosyl hydrolase

family 32 (Henrissat & Bairoch, 1993), with highest identity to a group of sucrose-6-phosphate hydrolases including the ScrB proteins from *B. subtilis* (35.6 %), *V. alginolyticus* (35.5 %) and *Staph. xylosus* (35.2 %) (Blatch *et al.*, 1990; Brückner *et al.*, 1993; Fouet *et al.*, 1986). This would suggest that the product of the *scrB* gene normally hydrolyses sucrose 6-phosphate rather than sucrose. Seven regions of distinct homology have been reported for sucrose hydrolases from various organisms (Gunasekaran *et al.*, 1990), and all of these are present in the putative *C. beijerinckii* ScrB protein.

The *scrK* gene specifies a deduced protein of 312 aa with a calculated molecular mass of 34 297 Da. Analysis of the translation products of pCBS2 confirmed the presence of a protein band with an apparent molecular mass of 32 kDa, corresponding to the ScrK protein. The deduced ScrK protein showed significant homology to several prokaryotic as well as three eukaryotic ribokinase/*pfkB* family fructokinases, the highest being to those of *K. pneumoniae* (30.3 % identity) and *V. alginolyticus* (29.9 % identity). Multiple sequence alignment of the *C. beijerinckii* ScrK protein with several other members of the ribokinase/*pfkB* fructokinases revealed several relatively well-conserved regions; three of these are possibly involved in ATP binding (Bork *et al.*, 1993) and two other domains may play a role in fructose binding (Fennington & Hughes, 1996).

Disruption of *scrR* and *scrB* genes in *C. beijerinckii*

In order to investigate the role of the *scrR* gene product in transcriptional regulation, and to establish the importance of the *scr* operon to sucrose metabolism, the *scrR* and *scrB* genes were disrupted in *C. beijerinckii* by targeted integration, giving strains designated CBSCR_R and CBSCR_B respectively. Both mutant strains grew on media containing glucose (10 mM) as the sole carbon source, but neither could grow when sucrose (10–100 mM) was supplied as the sole carbon source. The result clearly indicated that an intact *scr* operon was essential for growth on sucrose.

Regulation of expression of the *scrB* and *scrK* genes in *C. beijerinckii*

Sucrose hydrolase and fructokinase activities were assayed in *C. beijerinckii* cultures grown to OD₆₀₀ 0.4 in the presence of sucrose, glucose, fructose, maltose or xylose. Both activities were induced in the presence of sucrose, but were low or absent when cells were grown on the other carbohydrates (data not shown). This regulation of enzyme activities associated with sucrose metabolism confirms previous findings by Tangney *et al.* (1998), where PEP-dependent sucrose phosphorylation activity was reported to be induced in sucrose-grown *C. beijerinckii* cells, but not in xylose- or fructose-grown cells.

Northern blot analysis was undertaken to investigate the transcription of the *scr* gene cluster and to confirm the role of the *scrR* gene, encoding a GalR-LacI-like

Fig. 4. Phylogenetic tree for the proteins of the GalR-LacI family. Branch lengths are approximately proportional to the amount of sequence difference. The scale of 0.1 indicates 10% amino acid sequence divergence. The four evolutionary lines involving regulators of sucrose-catabolic genes are shown (A to D), and bootstrap values, as a percentage of 1000 trials, are indicated. Sequence abbreviations and accession numbers are: [1] RbtRKae, *Klebsiella aerogenes* ribitol repressor, sp: P07760; [2] CraSty, *Salmonella typhimurium* catabolite repressor/activator protein, sp: P21930; [3] ScrRCbe, *Clostridium beijerinckii* ScrR; [4] ScrRSxyl, *Staphylococcus xylosus* ScrR, EMBL: X67744; [5] XylRMmo, *Morganella morganii* xylitol repressor, gb: L34345; [6] TreRSty, *Sal. typhimurium* trehalose repressor, sp: P36674; [7] MalIEco, *E. coli* maltose repressor, sp: P18811; [8] MalRSxy, *Staph. xylosus* maltose repressor, PIR: S44187; [9] CcpABme, *Bacillus megaterium* catabolite control protein A (CcpA), gb: L26052; [10] CcpABsu, *Bacillus subtilis* CcpA, sp: P25144; [11] RegACac, *Clostridium acetobutylicum* RegA, gb: L14685; [12] DegABsu, *B. subtilis* degradation enzyme activator, sp: P37947; [13] PurREco, *E. coli* purine repressor, sp: P15039; [14] RbsREco, *E. coli* ribose repressor, gb: L10328; [15] CytREco, *E. coli* cytidine repressor, sp: P06964; [16] EndRBpo, *Bacillus polymyxa* endoglucanase repressor, sp: P27871; [17] EbgREco, *E. coli* evolved β -galactosidase repressor, sp: P06846; [18] ScrRmmu, *Streptococcus mutans* ScrR, gb: U46902; [19] ScrRPpe, *Pediococcus pentosaceus* ScrR, sp: P43472; [20] RbsRBSu, *B. subtilis* ribose repressor, sp: P36944; [21] GalREco, *E. coli* GalR, sp: P03024; [22] GalRHin, *Haemophilus influenzae* GalR, sp: P31766; [23] CcpBBSu, *B. subtilis* Catabolite control protein B (CcpB), sp: P37517; [24] CscREco, *E. coli* CscR, sp: P40715; [25] LacIEco, *E. coli* lactose repressor, sp: P03023; [26] RafREco, *E. coli* raffinose repressor, sp: P21867; [27] ScrRKpn, *K. pneumoniae* ScrR, sp: P37076; [28] ScrRSty, *Sal. typhimurium* ScrR, sp: P37077.

regulatory protein. Probes specific for the *scrA*, *scrR*, *scrB* and *scrK* transcripts were hybridized against RNA extracted from *C. beijerinckii* cells grown on sucrose, glucose, fructose, xylose or maltose. A predominant 5.0 kb transcript was identified by each of the *scr* probes in RNA isolated from sucrose-grown cells, but was not present in RNA extracted from cells grown on glucose, fructose, xylose or maltose (Fig. 5). The size of the transcript (5.0 kb) correlates with the size of the four

genes involved (4946 bp from sequence data). These data confirm that the *scr* genes are expressed as an operon and that transcription is induced by sucrose.

RNA analysis of the *scrB* and *scrR* mutant strains

The effect of *scrR* and *scrB* disruption on the expression of the *scr* operon was determined by Northern blot analysis using a probe specific for the *scrA* transcript.

Fig. 5. Northern blot hybridization of RNA isolated from *C. beijerinckii* NCIMB 8052 grown to exponential phase in GSMM medium containing 0.25% (w/v) of the respective carbohydrate. Lanes 1–5, sucrose, glucose, fructose, xylose and maltose respectively. Panels (a), (b), (c) and (d) were hybridized with probes specific for the *scrA*, *scrR*, *scrB* and *scrK* transcripts respectively.

The *scrA* gene lies upstream of *scrR* and *scrB* and would therefore not be subject to the polar effects of disruptions in either of these genes. Total RNA was extracted from wild-type *C. beijerinckii* cells grown with 0.25% (w/v) sucrose, glucose or xylose. Since the *C. beijerinckii* *scrR* and *scrB* mutants were unable to grow on sucrose, RNA could only be extracted from cells grown on 0.25% (w/v) glucose and xylose. As shown in Fig. 6, the *scrA* transcript was detected in the wild-type *C. beijerinckii* strain only in RNA from sucrose-grown cells, and not in that from glucose- or xylose-grown cells (Fig. 6a, lanes 1, 2 and 3). In the *scrR* mutant, CBSCRR, *scrA* transcripts were detected in RNA extracted from both glucose- and xylose-grown cells. The 5.0 kb *scr* operon transcript was not present and transcripts with a wide range of sizes were detected, probably representing transcription initiated at the *scrA* promoter which had extended into the multiple tandem repeats of plasmids integrated into the *scrR* gene. Clearly, a disruption of *scrR* had resulted in expression of the *scr* operon under non-inducing conditions, confirming that *scrR* encoded a negative regulator of *scr* operon transcription. A *scrA* transcript was not detected in the *scrB* mutant grown on either glucose or xylose, because the intact *scrR* gene product would have repressed *scr* operon transcription.

While Northern blot analysis of wild-type *C. beijerinckii* indicated that all four *scr* genes were co-transcribed as a 5.0 kb operon, an additional 1.1 kb transcript was detected with a *scrK*-specific probe (Fig. 5d). This transcript was constitutively expressed in the presence of the different carbohydrate substrates; however, the intensity of the band was significantly greater in RNA from sucrose-grown cells. Analysis of RNA isolated from the CBSCRR and CBSCRB mutants using the *scrK* probe indicated that the 1.1 kb transcript was expressed despite disruption of the *scr* operon under different growth conditions (Fig. 6b). A possible explanation is that this transcript represents an additional *scrK* tran-

Fig. 6. Northern blot analysis of *C. beijerinckii* wild-type (WT) and mutant strains CBSCRR and CBSCRB. RNA was extracted from cells grown in GSMM with carbohydrates supplied at 0.25% (w/v), and hybridized with (a) a *scrA*-specific probe or (b) a *scrK*-specific probe. (a) RNA extracted from wild-type *C. beijerinckii* cells grown on sucrose, glucose or xylose (lanes 1, 2 and 3 respectively); or from *C. beijerinckii* CBSCRR or CBSCRB cells grown on glucose (lanes 4 and 6) or xylose (lanes 5 and 7) respectively. (b) RNA extracted from wild-type *C. beijerinckii* cells grown on sucrose or glucose (lanes 1 and 2) or from *C. beijerinckii* CBSCRR or CBSCRB cells grown on glucose (lanes 3 and 5) or xylose (lanes 4 and 6).

script expressed from a promoter within the *scr* operon. A putative promoter has been identified upstream of *scrK*, within the coding region of *scrB* (Fig. 2b), but primer extension analysis using primer d (Fig. 2) failed to confirm this as a functional promoter. The 1.1 kb transcript is therefore more likely to represent the mRNA of an independent gene with homology to *scrK*.

DISCUSSION

Nucleotide sequence analysis indicated that the *C. beijerinckii* sucrose-utilization cluster consisted of four genes, *scrA*, *scrR*, *scrB* and *scrK*, encoding proteins capable of catalysing all the steps required for sucrose catabolism. In addition, this was the only sucrose-catabolic pathway in *C. beijerinckii* because mutants in either *scrR* or *scrB* were unable to grow on sucrose as a sole carbohydrate source. These findings confirm that sucrose utilization involves PTS-dependent sucrose transport, resulting in the accumulation of intracellular sucrose 6-phosphate. This would in turn be hydrolysed by the sucrose-6-phosphate hydrolase, releasing glucose 6-phosphate and fructose, which would be incorporated into the glycolytic pathway once the fructose had been phosphorylated by the product of the *scrK* gene, the fructokinase. Unlike the other sucrose-utilization regulators reported thus far, the *scr* genes constitute an operon.

RNA analysis confirmed that the genes were co-transcribed on a 5 kb mRNA transcript with the transcriptional start site situated 44 bp upstream of the *scrA* gene. To our knowledge, this is the first example of co-ordinated expression of all four genes involved in sucrose catabolism in bacteria.

The deduced *C. beijerinckii* ScrA protein showed the highest degree of homology at the amino acid level to *Strep. sobrinus* ScrA (55.1% identity over the corresponding EIIBC domains). However, the *C. beijerinckii* ScrA represents an EIIBC^{Scr} protein while the *Strep. sobrinus* ScrA represents an example of a fused EIIBCA^{Scr} protein. *Strep. sobrinus* ScrA is structurally similar to the *Strep. mutans* and *P. pentosaceus* ScrA proteins, which are also fused EIIBCA^{Scr} proteins (Lengeler *et al.*, 1994), and undoubtedly share a common origin, but the *C. beijerinckii* EIIBC^{Scr} protein shares only 34.5–34.8% identity with the equivalent EIIBC^{Scr} domains of these two proteins. Since the *scrA* of *C. beijerinckii* appears to encode an EIIBC^{Scr} protein, it must interact with an EIIA domain encoded elsewhere on the chromosome. It is possible that a separate EIIA^{Scr} domain is present in *C. beijerinckii*; however, in *B. subtilis* the EIIA^{Glc} domain of the EIICBA^{Glc} protein has been shown to phosphorylate EIIB^{Scr} (Sutrina *et al.*, 1990). In studies with the *K. pneumoniae*, *Sal. typhimurium* and *V. alginolyticus* sucrose-utilization systems cloned into *E. coli*, it was demonstrated that EIIBC^{Scr}-dependent sucrose uptake required the presence of a functional *E. coli* EIIA^{Glc} (Blatch *et al.*, 1990; Lengeler *et al.*, 1982; Sprenger & Lengeler, 1988). It is therefore conceivable that the *C. beijerinckii* EIIB^{Scr} domain interacts with a shared EIIA domain, probably that of the glucose PTS.

The *scrK* gene product indicated homology to several prokaryotic as well as three eukaryotic fructokinases of the ribokinase/*pfkB* family. ATP-dependent fructokinases occur in two non-homologous families, the ribokinase/*pfkB* family and the ROK/hexokinase family (Bork *et al.*, 1993; Titgemeyer *et al.*, 1994). Genes encoding proteins from both families have been found to be associated with sucrose-utilization gene clusters. However, it is worth noting that the *C. beijerinckii* ScrK represents the first complete sequence of a ribokinase/*pfkB* family fructokinase associated with a sucrose-utilization gene cluster in a Gram-positive organism. In other Gram-positive bacteria, fructokinase-encoding genes are either not clustered with sucrose-catabolic genes or encode ROK/hexokinase family fructokinases (Thompson *et al.*, 1991; Sato *et al.*, 1993; GenBank accession L32093).

The *C. beijerinckii* *scr* gene cluster has a number of similarities to the sucrose PTS regulon of *Staph. xylosus*. The *Staph. xylosus* system has been reported to consist of an independent *scrA* and a *scrRB* gene cluster (Brückner *et al.*, 1993; Gering & Brückner, 1996; Wagner *et al.*, 1993). However, it was noted during the sequence analysis that a gene encoding a ribokinase/*pfkB* family fructokinase appears to lie immedi-

ately downstream of the *Staph. xylosus* *scrB*, making the final gene arrangement *scrRBK*. This is precisely the same order as the equivalent genes of the *C. beijerinckii* system except that the *scrA* of *Staph. xylosus* is not associated with the remaining *scr* genes. The similarity in the molecular organization of the two gene clusters suggests a common evolutionary origin.

The presence of *scrR* in the *scr* operon, encoding a GalR-LacI-like regulatory protein, suggested that the transcription of the *C. beijerinckii* *scr* system was subject to negative regulation by the *scrR* gene product. Northern blot analysis demonstrated that the *scrARBK* genes were transcribed when *C. beijerinckii* had been grown in the presence of sucrose but were repressed in the presence of other carbohydrate substrates. In the *C. beijerinckii* *scrR* mutant, the *scr* operon was constitutively transcribed under non-inducing conditions, confirming that the *scrR* gene product played a negative regulatory role. While the precise details of this mechanism remain unclear, analogy with other GalR-LacI-like regulatory proteins suggests that in the absence of sucrose, the *C. beijerinckii* ScrR may repress transcription by binding to imperfectly palindromic operator sites in the vicinity of the *scr* operon promoter. A potential operator site was identified between the putative *scrA* promoter and initiation codon. It is of interest to note that this operator sequence differs from the consensus sequence of catabolite-responsive element (CRE) sites by only 1 bp (Hueck *et al.*, 1994; Weickert & Chambliss, 1990). Genes encoding CcpA homologues have been identified from both *C. acetobutylicum* P262 (Davison *et al.*, 1995) and *C. beijerinckii* (RegB). It is possible that the operator site may also be the target of a regulatory protein such as CcpA, which mediates catabolite repression in several Gram-positive bacteria (Henkin *et al.*, 1991; Hueck *et al.*, 1994; Saier *et al.*, 1996). Gering & Brückner (1996) demonstrated that the deletion of 4 bp of the right arm of a similar operator site in the *scrB* gene of *Staph. xylosus* eliminated not only the sucrose-specific regulation of *scrB* expression but also the glucose-mediated repression observed in the wild-type strain.

The location of the gene for the ScrR regulatory protein itself, within the inducible *scrARBK* operon, is somewhat unusual. In other sucrose-utilization regulons, with the exception of that of *L. lactis* (Rauch & de Vos, 1992), the corresponding *scrR* genes are independently transcribed. The *C. beijerinckii* regulator protein has highest sequence homology to the ScrR protein of *Staph. xylosus*; however, the regulation of the gene clusters differs. In *Staph. xylosus*, the *scrR* and *scrB* genes are independently transcribed, and inactivation of the *scrR* gene leads to constitutive expression of the downstream *scrB* gene (Gering & Brückner, 1996). In contrast, the *C. beijerinckii* *scrR* is transcribed as part of the *scr* operon and therefore is negatively autoregulated. It would be unnecessary for large amounts of ScrR to be synthesized, as *scr* operon transcripts accumulate under inducing conditions. Indeed, if large amounts of ScrR were produced, this could conceivably lead to the repression

of *scr* operon transcription, even in the presence of inducer. Gram-positive bacteria are stringent in their requirements for high-level initiation of protein synthesis. In *B. subtilis*, translational yield can be substantially reduced if ribosome-binding sites and initiation codons are separated by less than 6 bp (Vellanoweth & Rabinowitz, 1992). While the proposed ribosome-binding site and initiation codon of the *scrA*, *scrB* and *scrK* genes in *C. beijerinckii* are separated by the conventional 7–9 bp, only 5 bp separate the ribosome-binding site and initiation codon in *scrR*. It is possible that this suboptimal spacing may limit translation of *scrR*, in order to allow for optimal expression of the *scr* operon and the build-up of gene products other than ScrR while the inducer is present.

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