Expression of the phospho- β -glycosidase ArbZ from *Lactobacillus delbrueckii* subsp. *lactis* in *Lactobacillus helveticus*: substrate induction and catabolite repression

Beate A. Weber, Jürgen R. Klein and Bernhard Henrich

Author for correspondence: Bernhard Henrich. Tel: +49 631 2052347. Fax: +49 631 2053799.
e-mail: henrich@rhrk.uni-kl.de

Universität Kaiserslautern,
Fachbereich Biologie,
Abteilung Mikrobiologie,
PO Box 3049, D-67653
Kaiserslautern, Germany

ArbZ from *Lactobacillus delbrueckii* subsp. *lactis* was previously shown to enable utilization of the β -glucoside arbutin by *Escherichia coli*. The *arbZ* gene was cloned and expressed in the industrially used β -glucoside-negative strain *Lactobacillus helveticus* 3036(62). The transformants were able to ferment not only arbutin, but also cellobiose, salicin and methyl- β -glucoside (M β Glc). Cleavage of β -glucosides by the transformants depended on the integrity of the cytoplasmic membrane, whereas in cell-free extracts only C₆-phosphorylated substrates were hydrolysed. This suggested that ArbZ is a phospho- β -glycosidase. ArbZ activity in transformants of *Lb. helveticus* was subject to substrate induction mediated by the β -glucosides arbutin, salicin and M β Glc, whereas cellobiose or the β -galactoside lactose had no inducing effect. Northern blot analysis proved that induction by M β Glc was due to enhanced transcription of *arbZ*. Catabolite repression of *arbZ* induction was observed with glucose, mannose, fructose and galactose. The induction kinetics observed in the presence of these sugars indicated that at least two different mechanisms are operative in catabolite repression of *arbZ* in *Lb. helveticus*.

Keywords: *Lactobacillus delbrueckii*, ArbZ phospho- β -glycosidase, substrate induction, catabolite repression

INTRODUCTION

Lactic acid bacteria are indispensable for the manufacture of fermented foods, especially of dairy products. Their metabolism and genetics have been extensively studied over the past years. Growth of lactic acid bacteria depends on the availability of appropriate carbohydrates and, since lactose is the predominant sugar in milk, two pathways for lactose utilization by lactic acid bacteria have been thoroughly investigated (deVos, 1996). One of them, which is operative in lactococci and in several lactobacilli, relies on lactose transport by the phosphotransferase system (PTS), and the resulting lactose 6-phosphate is hydrolysed by phospho(P)- β -galactosidase (deVos *et al.*, 1990). The second pathway has been observed in *Leuconostoc lactis*, *Streptococcus thermophilus* and a number of

thermophilic lactobacilli where lactose is transported via a specific permease and then cleaved by β -galactosidase (David *et al.*, 1992; Vaughan *et al.*, 1996).

Utilization of β -glucosides by bacteria occurs through pathways similar to those described for the β -galactoside lactose. In most cases, β -glucosides are hydrolysed by extracellular or cell-wall-associated β -glucosidases (Gräbnitz & Staudenbauer, 1988; González-Candelas *et al.*, 1989). If uncleaved β -glucosides are transported into the cells, this is achieved either by specific permeases (Helaszek & White, 1991) or via the PTS with subsequent hydrolysis of the resulting P- β -glucosides into glucose 6-phosphate and the respective aglycons (Schnetz *et al.*, 1987; Tobisch *et al.*, 1997).

Connections between the metabolism of β -glucosides and β -galactosides were suggested by Simons *et al.* (1993), who observed that, in revertants of *lacG* deletion mutants of *Lactococcus lactis*, a P- β -glucosidase seemed to be responsible for their slow growth on lactose. In these revertants, the P- β -glucosidase was not only

Abbreviations: M β Glc, methyl- β -glucoside; oNPGal, o-nitrophenyl β -D-galactopyranoside; oNPGalP, oNPGal 6-phosphate; pNPGlc, p-nitrophenyl β -D-glucopyranoside; PTS, phosphotransferase system.

induced by cellobiose but also by lactose. Relations between β -glucoside and β -galactoside utilization may also be deduced from sequence similarities of P- β -galactosidases and P- β -glucosidases which assign most of them to family I of glycosylhydrolases (Henrissat, 1991). Furthermore, several P- β -glucosidases and P- β -galactosidases cleave C₆-phosphorylated derivatives of both β -galactosides and β -glucosides (Witt *et al.*, 1993; Simons *et al.*, 1993).

Little is known about β -glucoside metabolism in lactobacilli. For *Lactobacillus plantarum*, a glucose-repressible P- β -glucosidase gene was recently identified (Marasco *et al.*, 1998). We previously achieved cloning and sequence analysis of *arbZ* from *Lactobacillus delbrueckii* subsp. *lactis*. ArbZ seems to be involved in β -glucoside metabolism since it conferred the ability to utilize the β -glucoside arbutin when expressed in *Escherichia coli* (Weber *et al.*, 1998). Here we report on the expression and control of *arbZ* in *Lactobacillus helveticus* strain 3036(62), which is used in industrial food production.

METHODS

Bacterial strains and media. *E. coli* ER1562 (Raleigh *et al.*, 1988) was grown in LB medium (Sambrook *et al.*, 1989) at 37 °C. When appropriate, chloramphenicol (10 µg ml⁻¹) was added. Fermentation of arbutin was screened on eosine methylene blue agar (EMB agar base; Difco) supplemented with 0.5% arbutin (EMB-arbutin agar). Positive colonies were identified by their dark-blue colour on this medium. *Lb. delbrueckii* subsp. *lactis* DSM 7290 (Süddeutsche Forschungsanstalt für Milchwirtschaft, Weihenstephan) and *Lb. helveticus* 3036(62) (Bundesanstalt für Milchforschung, Kiel) were grown in MRS broth (De Man *et al.*, 1960) at 37 °C. When appropriate, chloramphenicol (5 µg ml⁻¹) was added. Fermentation of carbohydrates (1% final concentration) was tested in sugar fermentation broth (SFB), which is MRS broth with 0.2% (instead of 0.8%) meat extract and without glucose. After 24–48 h cell growth in this medium, acidification was checked by addition of chlorphenol red (0.1 mg ml⁻¹ final concentration). Cleavage of aesculin was screened on aesculin-iron agar, which is aesculin broth (Merck), supplemented with 15 g agar l⁻¹ and 1 g Tween 80 l⁻¹. Positive colonies were identified on this agar by their brown colour due to the formation of an aesculetin–Fe(III) complex.

Recombinant DNA techniques. Restriction endonucleases and nucleic acid modifying enzymes (Roche) were used as recommended by the manufacturer. Cloning techniques were performed according to Sambrook *et al.* (1989). Plasmids were prepared from *Lb. helveticus* as described by Anderson & McKay (1983). Electroporation of *E. coli* was performed as described by Dower *et al.* (1988), and *Lb. helveticus* was electroporated according to Bhowmik & Steele (1993).

For PCR amplification of a 817 bp *arbZ* fragment (nt 1293–2109, accession number Z86115), the primers 5'-CCAC-AATATCGAGCTCACCGCACCCAAC-3' and 5'-GCCGG-CGATATCCCATGGAGTGAAGTCG-3' were used. The *cat* gene (encoded by pGK12 and pBW121) was amplified with the primers 5'-AAAGCACCCATTAGTTCAACAAACG-3' and 5'-AACCTTCTTCACTAACGGGGCAGG-3'. PCR reactions were carried out with *Taq* DNA polymerase as

recommended by the supplier (Appligene). The temperature profile (25 cycles) used for both amplifications was 1 min at 95 °C (denaturation), 1 min at 60 °C (annealing) and 1 min at 72 °C (elongation). As templates, 100 ng total bacterial DNA isolated with the Genomix scale-up kit (Talent, Triest) or 10 ng plasmid DNA (controls) was used.

Construction of pBW121. A 1474 bp *AccI*–*Xma*III fragment, containing *arbZ* and the 3'-end of the preceding *arbX* gene ('*arbX*') (Weber *et al.*, 1998), was made blunt at the *Xma*III site with Klenow fragment of *E. coli* DNA polymerase I and inserted between the *Hpa*II and *Sna*BI sites of the vector pGK12 (Kok *et al.*, 1984). The resulting plasmid, pBW121, was initially identified after transformation of *E. coli* ER1562 by screening on EMB-arbutin agar and then used to transform *Lb. helveticus*.

Northern and Southern hybridizations. For Northern blot analyses, total RNA (4 µg), isolated with the RNeasy total RNA kit (Qiagen), was electrophoretically separated and blotted as described previously (Henrich *et al.*, 1990). A 817 bp PCR-amplified *arbZ* probe was non-radioactively labelled, hybridized to the blotted RNA, and detected by using a digoxigenin DNA labelling and detection kit (Roche) as recommended by the supplier.

For Southern blot analyses, total DNA from *Lb. helveticus* 3036(62) was isolated using the Genomix scale-up kit. DNA fragments (2 µg), obtained by restriction with *Hind*III or *Cla*I, were separated on 0.7% agarose gels and blotted to nylon membranes (Sambrook *et al.*, 1989). The 817 bp *arbZ* probe was fluorescently labelled, hybridized to the blotted DNA fragments, and detected as recommended by the manufacturer (Amersham) of the ECL random prime labelling and detection system used.

In vivo assay of β -glycosidase activity. Strains of *Lb. helveticus* were grown in SFB supplemented with appropriate sugars. At OD₆₀₀ 0.6–0.8, cells from 2 ml aliquots of the cultures were collected by centrifugation, washed twice with 1 ml Z-buffer (Miller, 1972), and resuspended in 1.5 ml of the same buffer. When appropriate, cells were permeabilized by the addition of chloroform to a final concentration of 14% (v/v). Three hundred microlitres of these suspensions was mixed with 10 µl of the respective substrates (10 mg ml⁻¹ in Z-buffer) and incubated at 37 °C. When using the chromogenic substrates *p*-nitrophenyl β -D-glucopyranoside (pNPGlc) (Fluka) and *o*-nitrophenyl β -D-galactopyranoside (oNPGal) (Sigma), the reactions were stopped by the addition of 300 µl 2 M Na₂CO₃ as soon as a light-yellow colour had developed. Cells were subsequently removed by centrifugation, and the amounts of liberated *p*- or *o*-nitrophenol were determined photometrically at 410 nm or 420 nm, respectively. Cleavage of salicin or arbutin was assayed as described by Schaefer (1967). Each reaction was repeated at least four times. Units of ArbZ activity were expressed as nmol of the aglycons liberated from the respective substrates min⁻¹. Specific activities were calculated as units per OD₆₀₀ unit of the cell suspensions used in the assays.

Assay of (phospho) β -glycosidase activity in cell-free extracts. To prepare crude cell extracts, *Lb. helveticus* strains were cultivated in SFB supplemented with appropriate sugars. At OD₆₀₀ 0.4–0.7, cells were pelleted from 20 ml aliquots by centrifugation, washed twice with Z-buffer, and resuspended in 1.7 ml Z-buffer (mg cell wet wt)⁻¹. After addition of 2.5 ml glass beads (Ø 0.18 mm) (mg cell wet wt)⁻¹, the bacteria were disrupted by shaking in a Vibrogen cell mill (Bachhofer) for 20 min at 4 °C at a frequency of 70 Hz. The samples were

subsequently diluted by the addition of Z-buffer [1.5 ml (mg cell wet wt)⁻¹] and thoroughly mixed. Cell debris and glass beads were removed by centrifugation for 10 min at 14800g and 4 °C, and the protein concentration in the supernatants (cell-free extracts) was determined with Coomassie blue (Spector, 1978). To determine the activity of β -glycosidases and P- β -glycosidases in cell extracts (Weber *et al.*, 1998), the chromogenic substrates oNPGal, oNPGal 6-phosphate (oNPGalP) (Sigma) and pNPGlc (Fluka) were used. Each reaction was repeated at least three times. Enzyme units were defined as nmol nitrophenol liberated min⁻¹, and specific activities were expressed in units (mg protein)⁻¹.

RESULTS AND DISCUSSION

Cloning of *arbZ* in *Lb. helveticus*

The *arbZ* gene from *Lb. delbrueckii* subsp. *lactis* DSM 7290 was cloned in the industrially used *Lb. helveticus* strain 3036(62). We chose *Lb. helveticus* as a host for several reasons. (i) In contrast to *Lb. delbrueckii* subsp. *lactis* DSM 7290, strain 3036(62) is readily accessible to electrotransformation. (ii) Strain 3036(62) is definitively unable to ferment β -glucosides, and it does not spontaneously release β -glucoside-positive mutants during prolonged selection on β -glucosides as has been reported for *E. coli* (Parker & Hall, 1990; Hall & Xu, 1992). On the other hand, *arbZ* mutants of *Lb. delbrueckii* subsp. *lactis* DSM 7290 are difficult to construct due to its resistance to transformation, and it is not known whether additional systems for β -glucoside utilization are present in this strain. The occurrence of at least two β -glucoside utilization systems has been reported for *Lc. lactis* (Simons *et al.*, 1993). (iii) *Lb. helveticus* is more closely related to *Lb. delbrueckii* than are other transformable lactic acid bacteria, such as *Lactococcus* or *Lactobacillus casei*. In addition, all of the transformable *Lactococcus* and *Lactobacillus* strains for which characterized mutants in the catabolite control system exist are β -glucoside-positive.

Lb. helveticus 3036(62) was transformed with the *arbZ*-containing plasmid pBW121, or with the vector pGK12 as a control. Of about 100 chloramphenicol-resistant clones obtained from two independent transformations with pBW121, 24 were tested for utilization of the β -glucoside arbutin in SFB-arbutin. Of these transformants, only four (two from each transformation) were stably positive for arbutin fermentation. No arbutin-fermenting clones were detected among a similar number of pGK12 transformants, even after prolonged incubation.

Attempts to prepare plasmids from pBW121 transformants and pGK12 transformants (Anderson & McKay, 1983) did not yield electrophoretically detectable amounts of plasmid DNA. In a more sensitive assay, we used the material obtained from these preparations to retransform the restriction/modification-deficient *E. coli* strain ER1562. As estimated from control experiments with known concentrations of pBW121 and pGK12, fresh transformants of *Lb. helveticus*, after growth in chloramphenicol-con-

Fig. 1. Southern blot analysis of DNA from *Lb. helveticus* transformants. Plasmid pBW121 (lanes 5, 10) and total DNA from four arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones (lanes 1, 2, 6, 7) and from four *Lb. helveticus* 3036(62)(pGK12) transformants (lanes 3, 4, 8, 9) were restricted with *Clal* (a) or *HindIII* (b) and blotted to nylon membranes. Blots were probed with a fluorescently labelled 817 bp fragment of *arbZ*.

taining MRS broth for 12 h, contained less than one plasmid copy per cell. More than 50 % of these plasmids were clearly reduced in size as compared with pBW121 and pGK12, respectively. These effects pointed to structural instability of pBW121 and pGK12 in the *Lb. helveticus* host, since both plasmids were structurally stable during propagation in *E. coli*. After repeated subculturing of *Lb. helveticus* 3036(62)(pBW121) and *Lb. helveticus* 3036(62)(pGK12), no plasmid DNA at all was detectable in the *E. coli* retransformation assay.

When total DNA prepared from subcultures of each of the four arbutin-positive pBW121 transformants was used as template for PCR, *arbZ* as well as the plasmid-encoded *cat* gene could be amplified, whereas in pGK12 transformants only *cat* was detectable. The presence of *arbZ* in the arbutin-utilizing *Lb. helveticus* 3036(62)(pBW121) clones was also verified by Southern blot analysis (Fig. 1). An *arbZ* probe gave a single hybridization signal with total DNA from each of the four clones, digested with restriction enzymes (*HindIII* or *Clal*) which do not cut within *arbZ*. No signals were obtained with DNA from pGK12 transformants. When *Clal*- or *HindIII*-restricted plasmid pBW121 (one *Clal* site, no *HindIII* site) was probed, the positions of *arbZ*-specific signals were clearly different from those observed with total DNA from the arbutin-positive

Table 1. ArbZ activities of transformants of *Lb. helveticus* grown with different carbon sources

SD, Standard deviation calculated from at least three independent measurements; ND, not determined.

Carbohydrate in growth medium	<i>Lb. helveticus</i> transformed with:	In vivo with pNPGlc (U per OD ₆₀₀ unit $\pm$ SD)		In cell-free extracts [U (mg protein) ⁻¹ $\pm$ SD] with:		
		– Chloroform	+ Chloroform	pNPGlc	oNPGal	oNPGalP
Glucose	pBW121	0.2 $\pm$ 0.05	0.1 $\pm$ 0.04	5.3 $\pm$ 1.3	1.29 $\pm$ 0.3	4.0 $\pm$ 1.0
	pGK12	0.02 $\pm$ 0.006	0.025 $\pm$ 0.007	0.2 $\pm$ 0.06	1.44 $\pm$ 0.36	0.8 $\pm$ 0.23
Lactose	pBW121	0.42 $\pm$ 0.09	0.33 $\pm$ 0.09	6.1 $\pm$ 1.5	732 $\pm$ 95	537 $\pm$ 70
	pGK12	0.04 $\pm$ 0.008	0.055 $\pm$ 0.014	0.4 $\pm$ 0.1	728 $\pm$ 93	524 $\pm$ 64
Xylose	pBW121	1.0 $\pm$ 0.25	ND	5.1 $\pm$ 1.15	12.3 $\pm$ 3	14.5 $\pm$ 3.6
	pGK12	0.034 $\pm$ 0.01	ND	0.33 $\pm$ 0.08	12.0 $\pm$ 2.8	9.5 $\pm$ 2.4
Salicin*	pBW121	11.4 $\pm$ 2.8	0.24 $\pm$ 0.07	9.1 $\pm$ 2.3	14.2 $\pm$ 3.7	352 $\pm$ 50
Arbutin*	pBW121	12.2 $\pm$ 3.0	0.5 $\pm$ 0.11	ND	ND	ND

* Since transformants with pGK12 did not grow in the presence of salicin or arbutin, no activities could be determined in these cases.

pBW121 transformants (Fig. 1). This indicated that *arbZ* had been integrated into the chromosome of the transformants.

The vector pGK12, used to construct pBW121, has been reported to replicate in *Lb. helveticus* CNRZ32 (Bhowmik & Steele, 1993). Segregational instability of this replicon, however, may be suspected from its sigma replication mode (Kiewiet *et al.*, 1993), and structural instability of pBW121 was observed in transformants of *Lb. helveticus* 3036(62) (see above). We therefore suspect that chloramphenicol-resistance marker during the initial selection of transformants, whereas the non-selected *arbZ* gene was lost in most of the clones. Recombination between the *Lb. helveticus* genome and some homologous regions present in the insert or the vector part of the plasmid may be considered as a mechanism for chromosomal integration of pBW121. However, no sequences with notable homology to *arbZ* seem to be present in *Lb. helveticus* since no *arbZ*-specific signals were obtained in Southern hybridizations with DNA from *Lb. helveticus* 3036(62)(pGK12), even under non-stringent conditions (not shown). Furthermore, PCR and plasmid analyses of *Lb. helveticus* 3036(62)(pGK12) transformants indicated that the unmodified vector also integrated into the chromosome. This suggested that integration did not require the plasmid-encoded '*arbX*-*arbZ*' region, but probably occurred through sequences of the vector.

As *Lb. helveticus* 3036(62) is not able to ferment β -glucosides and has no native (P)- β -glucosidase (see Table 1), the presence of a functional *arbZ* homologue in this strain can be excluded. So, even if weak homology between the '*arbX*-*arbZ*' region in pBW121 and the *Lb. helveticus* chromosome was assumed, integration into a non-functional *arbZ*-like sequence of the host would most probably lead to inactivation and disruption of the plasmid-encoded *arbZ* gene. This was excluded by measurements of ArbZ activity in the transformants (see

Table 1) and by demonstrating the integrity of *arbZ* in PCR and Northern blot (Fig. 2b) analyses.

In addition to ordinary recombination, other pathways allowing for low-frequency DNA integration in the absence of obvious sequence homology seem to be operative in lactic acid bacteria (Rixon *et al.*, 1990; Leenhouts *et al.*, 1990).

Fermentation of β -glucosides by *Lb. helveticus* 3036(62)(pBW121)

Our previous studies showed that *arbZ*, when expressed in *E. coli*, allowed for utilization of arbutin, whereas fermentation of other β -glucosides could not be detected (Weber *et al.*, 1998). With the arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones a different substrate pattern was observed. In SFB, not only arbutin, but also the β -glucosides salicin and cellobiose, and, with a lower efficiency, methyl- β -glucoside (M β Glc) (Sigma), were utilized as carbon sources and converted to acidic fermentation products. The growth rates ranged between 0.003 and 0.004 min⁻¹ for cellobiose, salicin and arbutin and was 0.0012 min⁻¹ for M β Glc. Cleavage of arbutin and salicin was verified by assaying the hydrolytic activity of the transformants towards these substrates. The arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones, in addition, were able to cleave aesculin, as indicated by a positive reaction on aesculin-iron agar. The transformants with pGK12, in contrast, were not able to grow in the presence of arbutin, salicin, cellobiose or M β Glc or to hydrolyse arbutin, salicin or aesculin. When glucose or the β -galactoside lactose was used as carbon source, no differences in the growth rates (0.0056 min⁻¹ for glucose, 0.0053 min⁻¹ for lactose) were observed between transformants carrying pBW121 or pGK12, respectively. This indicated that the ability of *Lb. helveticus* to utilize β -glucosides depended on the presence of the cloned *arbZ* gene.

Fig. 2. Substrate induction of ArbZ in arbutin-positive *Lb. helveticus* 3036(62)(pBW121) transformants. (a) Cells were grown in SFB-xylose supplemented with chloramphenicol. At OD₆₀₀ 0.4–0.5 (time = 0 min), no β -glucoside (●), arbutin (◆), salicin (×), M β Glc (□) or cellobiose (▽) (1 mM final concentration) was added. ArbZ activity was determined in culture aliquots by the *in vivo* assay with pNPGlc as a substrate. Induction kinetics obtained with two independently isolated arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones were almost identical. (b) Total RNA, prepared from aliquots of a M β Glc-induced culture 0 min (1), 10 min (2), 30 min (3), 60 min (4), 120 min (5) and 240 min (6) after induction, was used for Northern hybridization with a digoxigenin-11-dUTP-labelled 817 bp fragment of *arbZ*. A 0.24–9.5 kb RNA ladder (Gibco-BRL) was used as a size standard (M).

arbZ confers P- β -glycosidase activity

Expression of *arbZ* was clearly detectable in the *in vivo* assay. After growth with glucose, lactose or xylose, each of the four arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones showed 10–30-fold higher activities towards the β -glucoside pNPGlc than the respective pGK12 transformants (Table 1). Permeabilization of the cytoplasmic membrane by chloroform prior to the assay resulted in a decrease of ArbZ activity, which was most pronounced (45-fold) after growing the cells in the presence of salicin. This suggested that ArbZ activity towards β -glycosides required the integrity of the cytoplasmic membrane. As possible explanations, membrane association of ArbZ or its requirement for phosphorylated substrates were considered. Since membrane location of ArbZ was

unlikely (Weber *et al.*, 1998), we chose the C₆-phosphorylated β -galactoside oNPGalP for activity tests with cell-free extracts. This substrate seemed appropriate, as chromogenic derivatives of phosphorylated β -glucosides were not available and cleavage of oNPGalP had previously been reported for P- β -glucosidases of *E. coli* (Witt *et al.*, 1993) and *Lc. lactis* (Simons *et al.*, 1993). Cell-free extracts prepared from the arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones, after growth with glucose or xylose, showed 5- or 1.5-fold higher activities towards oNPGalP than those from pGK12 transformants, which demonstrated that ArbZ contributed to cleavage of this substrate. Notably, extracts from salicin-grown cells were more than 20-fold less active towards pNPGlc or oNPGal than towards oNPGalP (Table 1). This clearly indicated that ArbZ was able to efficiently hydrolyse the phosphorylated substrate in the absence of a functional cytoplasmic membrane. We therefore classified ArbZ as a P- β -glycosidase.

After growth with lactose, extracts of transformants with both pBW121 or pGK12 contained more than 100-fold higher oNPGalP-hydrolysing activities, as compared with extracts of glucose-grown cells (Table 1). This suggested that, under these conditions, the activity of ArbZ towards oNPGalP was concealed by a native, lactose-inducible P- β -galactosidase of *Lb. helveticus* 3036(62). As deduced from *in vitro* assays (Table 1) and *in vivo* assays (not shown) with oNPGal after growth on lactose, this strain in addition has a native β -galactosidase. Coincidence of both activities in the same strain has previously been reported for other lactic acid bacteria (Premi *et al.*, 1972; Okamoto & Morichi, 1979).

Since *Lb. helveticus*, in the absence of cloned *arbZ*, did not grow at all with salicin as carbon source, we could not exclude that high oNPGalP-hydrolysing activities in extracts of salicin-grown pBW121 transformants were partly due to induction of the native P- β -galactosidase by salicin. This, however, seemed unlikely, since induction of oNPGalP cleavage by another β -glucoside, M β Glc, also required the expression of *arbZ*, and no induction of the native P- β -galactosidase by M β Glc was observed in this case (not shown).

Induction of ArbZ activity by β -glucosides

ArbZ activity of the arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones depended on the carbon source in the growth medium (Table 1). When salicin or arbutin was used, the *in vivo* activities were about 10-fold higher than in the presence of xylose and more than 25-fold higher than in the presence of lactose. This indicated that ArbZ is subject to substrate induction, which is a common feature in bacterial β -glucoside catabolism (Schnetz & Rak, 1988; Krüger *et al.*, 1996; Woodward & Wiseman, 1982).

Two independently isolated arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones were grown in SFB-

Fig. 3. Effects of hexoses on M β Glc-induced ArbZ activity. Arbutin-positive *Lb. helveticus* 3036(62)(pBW121) transformants were grown in SFB-xylose supplemented with chloramphenicol. At OD₆₀₀ 0.4–0.5 (time = 0 min), ArbZ was induced by the addition of M β Glc (1 mM final concentration), and simultaneously glucose (a), mannose (b), fructose (c) or galactose (d) were added. Concentrations (w/v) of the sugars were 0.3% (Δ), 0.2% (*), 0.1% (∇), 0.075% ($\diamond$) and 0.035% (+), respectively. To the control cultures only M β Glc ($\bullet$), or neither M β Glc nor an additional sugar ($\blacksquare$) was added. ArbZ activity was determined by the *in vivo* assay with pNPGlc as a

xylose, and the time courses of ArbZ induction were followed after addition of various β -glucosides. Since both clones showed very similar induction kinetics, only one set of curves is presented in Fig. 2(a). Cellobiose, although being a substrate for ArbZ, was only a poor inducer. A similar observation, previously reported for a β -glucosidase from *Botryodiplodia theobromae* (Woodward & Wiseman, 1982), has been ascribed to repression by glucose, which is liberated by cleavage of cellobiose in twofold higher amounts than from other β -glucosides. Arbutin, salicin and M β Glc led to strong increase of activity within 60 min after their addition (Fig. 2a). Arbutin was the most potent inducer, while induction by M β Glc occurred more slowly. The activities induced by arbutin and salicin slowly declined after reaching a maximum, probably due to cleavage of these β -glucosides. The inducing effect of M β Glc persisted longer, which is in accordance with low ArbZ activity towards M β Glc, as concluded from growth experiments.

Total RNA, prepared from arbutin-positive *Lb. helveticus* 3036(62)(pBW121) transformants after induction with M β Glc, was analysed in Northern hybridizations with an *arbZ*-specific probe. Increase of ArbZ activity after induction was found to be accompanied by simultaneous increase of a 960 nt *arbZ* transcript (Fig. 2b). A similar transcript was not found in pGK12 transformants. This demonstrated that β -glucoside-dependent control of ArbZ occurs at the transcriptional level. Antitermination may be considered as a possible mechanism to enhance *arbZ* transcription, since several other prokaryotic systems for β -glucoside metabolism are subject to this type of control (Schnetz & Rak, 1988; Krüger *et al.*, 1996). The *arbZ* upstream sequence, however, does not contain any stem-loop structures or RAT motifs (Aymerich & Steinmetz, 1992) typical of other transcriptional anti-terminators.

We previously demonstrated that the 1474 bp fragment of chromosomal DNA present in pBW121 contains a functional promoter between *arbX* and *arbZ* and a terminator downstream of *arbZ*. These signals determined the ends of a 960 nt *arbZ* transcript which we detected in *Lb. delbrueckii* subsp. *lactis* DSM 7290 after growth with arbutin or salicin (Weber *et al.*, 1998). It should be noted that the *arbZ* transcripts, detected after M β Glc induction of *Lb. helveticus* 3036(62)(pBW121) transformants (Fig. 2b), had exactly the same size. This clearly indicated (i) that *arbZ* had retained its physical integrity during integration into the *Lb. helveticus* genome and (ii) that substrate induction actually occurred at the native *arbZ* promoter and not at a promoter which might be located adjacent to the *arbZ* integration site.

substrate. The catabolite repression effects observed with two independently isolated arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones were almost identical.

Catabolite control of ArbZ

After growth with glucose, whole cells or extracts of the four arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones showed 5- or 3.6-fold lower activities towards pNPGlc or oNPGalP, respectively, than after growth with xylose (Table 1). ArbZ therefore seemed to be subject to catabolite repression in these transformants. To verify this, different hexoses were added to cultures of two independently isolated arbutin-positive *Lb. helveticus* 3036(62)(pBW121) clones grown in SFB-xylose, and simultaneously *arbZ* was induced with M β Glc. As shown in Fig. 3, each of the sugars tested (glucose, fructose, mannose, galactose) had an inhibitory effect on induction of ArbZ activity. There were, however, significant differences in the time courses of ArbZ induction, which allowed for classifying the sugars into two groups. (i) Inhibition by glucose and mannose was very effective. In the presence of 0.3% of these sugars no induction of ArbZ was observed within 7 h. The repressive effect was detectable immediately after addition of the sugars and resulted in a time delay of induction. The length of this delay was proportional to the concentration of the respective sugars whereas the final ArbZ activities were inversely related. This effect was more pronounced for glucose than for mannose (Fig. 3a, b). The immediate repression effect of glucose and mannose may be explained by a transport-dependent control mechanism such as inducer exclusion (Ye & Saier, 1996) or involvement of a transcriptional regulator with a PTS regulation domain (PRD) (Stülke *et al.*, 1998). It is also reminiscent of 'transient repression', previously described for the *lac* operon of *E. coli* (Tyler & Magasanik, 1969). (ii) Inhibition by fructose and galactose was more moderate; distinct ArbZ induction was still observed in the presence of 0.3% of the sugars (Fig. 3c, d). In contrast to glucose and mannose, the inhibitory effect of galactose and fructose only became visible about 60 min after addition of the sugars and resulted in flattening of the induction curves. Such effects would be expected for a repression mechanism that depends on formation of specific intermediates during catabolism of the respective sugars. The maximum ArbZ activities after addition of fructose or galactose showed a similar dependence on sugar concentrations as observed for glucose and mannose.

In conclusion, it appeared that, depending on the nature of the repressing sugars, at least two distinct mechanisms are involved in catabolite repression of ArbZ. Similar observations have been reported for the P- α -glucosidase TreA of *Bacillus subtilis* (Helfert *et al.*, 1995) and for a β -galactosidase from *Staphylococcus xylosus* (Egeter & Brückner, 1996). Although catabolite control in Gram-positive bacteria is often mediated by the transcription regulator CcpA (Saier *et al.*, 1996), several other mechanisms are known. These include inducer exclusion (Ye & Saier, 1996), inducer expulsion (Reizer, 1989) and involvement of a glucose kinase (Wagner *et al.*, 1995) or proteins with PRDs (Stülke *et al.*, 1998).

CcpA, through its interaction with intermediates of

carbohydrate catabolism, is able to respond not only to glucose but also to a number of other sugars (Saier *et al.*, 1996). Therefore, repression of ArbZ by each of the four sugars tested might be a reflection of a CcpA-related control mechanism. This option is further supported by the presence of a palindromic sequence (5'-TGAGAA-CGATGACT-3') upstream of *arbZ* (Weber *et al.*, 1998), which overlaps with the first nucleotide of the putative -35 region and matches the consensus sequence of catabolite responsive elements (*cre*) (Weickert & Chambliss, 1990) in 13 out of 14 positions. The *cre* sequence is known to interact with CcpA in a number of catabolite-controlled operons, and CcpA usually has a repressive effect if (as in the case of *arbZ*) *cre* overlaps the respective promoters or is located downstream of them (Weickert & Chambliss, 1990; Hueck *et al.*, 1994; Saier *et al.*, 1996). It is therefore tempting to speculate that *cre* and CcpA homologues are involved in catabolite repression of *arbZ*.

ACKNOWLEDGEMENTS

We thank Bernd Winkelmann for the synthesis of oligonucleotides. This work was in part supported by the Bundesministerium für Forschung und Technologie (grant 0319281B).

REFERENCES

- Anderson, D. G. & McKay, L. L. (1983). Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl Environ Microbiol* **46**, 549–552.
- Aymerich, S. & Steinmetz, M. (1992). Specificity determinants and structural features in the RNA target of the bacterial anti-terminator proteins of the BglG/SacY family. *Proc Natl Acad Sci U S A* **89**, 10410–10414.
- Bhowmik, T. & Steele, J. L. (1993). Development of an electroporation procedure for gene disruption in *Lactobacillus helveticus* CNRZ32. *J Gen Microbiol* **139**, 1433–1439.
- David, S., Stevens, H., van Riel, M., Simons, G. & deVos, W. M. (1992). *Leuconostoc lactis* β -galactosidase is encoded by two overlapping genes. *J Bacteriol* **174**, 4475–4481.
- De Man, J. C., Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.
- deVos, W. M. (1996). Metabolic engineering of sugar catabolism in lactic acid bacteria. *Antonie Leeuwenhoek* **70**, 223–242.
- deVos, W. M., Boerrigter, I., van Roojen, R. J., Reiche, B. & Hengstenberg, W. (1990). Characterization of the lactose specific enzymes of the phosphotransferase system in *Lactococcus lactis*. *J Biol Chem* **265**, 22554–22560.
- Dower, W. J., Miller, J. F. & Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16**, 6127–6145.
- Egeter, O. & Brückner, R. (1996). Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus*. *Mol Microbiol* **21**, 739–749.
- González-Candelas, L., Aristoy, M. C., Polaina, J. & Flors, A. (1989). Cloning and characterization of two genes from *Bacillus polymyxa* expressing β -glucosidase activity in *Escherichia coli*. *Appl Environ Microbiol* **55**, 3173–3177.

- Gräbnitz, F. & Staudenbauer, W. L. (1988). Characterization of two β -glucosidase genes from *Clostridium thermocellum*. *Bio-technol Lett* **10**, 73–77.
- Hall, B. G. & Xu, L. (1992). Nucleotide sequence, function, activation, and evolution of the cryptic *asc* operon of *Escherichia coli* K12. *Mol Biol Evol* **9**, 688–706.
- Helaszek, C. T. & White, B. A. (1991). Cellobiose uptake and metabolism by *Ruminococcus flavefaciens*. *Appl Environ Microbiol* **57**, 64–67.
- Helfert, C., Gotsche, S. & Dahl, M. K. (1995). Cleavage of trehalose-phosphate in *Bacillus subtilis* is catalysed by a phospho- α -(1-1)-glucosidase encoded by the *treA* gene. *Mol Microbiol* **16**, 111–120.
- Henrich, B., Monnerjahn, U. & Plapp, R. (1990). Peptidase D gene (*pepD*) of *Escherichia coli* K-12: nucleotide sequence, transcript mapping and comparison with other peptidase genes. *J Bacteriol* **172**, 4641–4651.
- Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **280**, 309–316.
- Hueck, C. J., Hillen, W. & Saier, M. H. (1994). Analysis of a cis-active sequence mediating catabolite repression in gram-positive bacteria. *Res Microbiol* **145**, 503–518.
- Kiewiet, R., Bron, S., de Jonge, K., Venema, G. & Seegers, J. F. (1993). Theta replication of the lactococcal plasmid pWVO2. *Mol Microbiol* **10**, 319–327.
- Kok, J., van der Vossen, M. B. M. & Venema, G. (1984). Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. *Appl Environ Microbiol* **48**, 726–731.
- Krüger, S., Lindner, C. & Hecker, M. (1996). Transcriptional analysis of *bglPH* expression in *Bacillus subtilis*: evidence for two distinct pathways mediating carbon catabolite repression. *J Bacteriol* **178**, 2637–2644.
- Leenhouts, K. J., Kok, J. & Venema, G. (1990). Stability of integrated plasmids in the chromosome of *Lactococcus lactis*. *Appl Environ Microbiol* **56**, 2726–2735.
- Marasco, R., Muscariello, L., Varcamonti, M., De Felice, M. & Sacco, M. (1998). Expression of the *bglH* gene of *Lactobacillus plantarum* is controlled by carbon catabolite repression. *J Bacteriol* **180**, 3400–3404.
- Miller, J. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Okamoto, T. & Morichi, T. (1979). Distribution of β -galactosidase and β -phosphogalactosidase activity among lactic streptococci. *Agric Biol Chem* **43**, 2389–2390.
- Parker, L. L. & Hall, B. (1990). Mechanisms of activation of the cryptic *cel* operon of *Escherichia coli*. *Genetics* **124**, 473–482.
- Premi, L., Sandine, W. E. & Elliker, P. R. (1972). Lactose-hydrolyzing enzymes of *Lactobacillus* species. *Appl Microbiol* **24**, 51–57.
- Raleigh, E. A., Murray, N. E., Revel, H., Blumenthal, R. M., Westaway, D., Reith, A. D., Rigby, P. W. J., Elhai, J. & Hanahan, D. (1988). McrA and McrB restriction phenotypes of some *E. coli* strains and implications for gene cloning. *Nucleic Acids Res* **16**, 1563–1575.
- Reizer, J. (1989). Regulation of sugar uptake and efflux in gram-positive bacteria. *FEMS Microbiol Rev* **63**, 149–156.
- Rixon, J. E., Hazlewood, G. P. & Gilbert, H. J. (1990). Integration of an unstable plasmid into the chromosome of *Lactobacillus plantarum*. *FEMS Microbiol Lett* **71**, 105–110.
- Saier, M. H., Jr, Chauvaux, S., Cook, G. M., Deutscher, J., Paulson, I. T., Reizer, J. & Ye, J. J. (1996). Catabolite repression and inducer control in gram-positive bacteria. *Microbiology* **142**, 217–230.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schaeffler, S. (1967). Inducible system for the utilization of β -glucosides in *Escherichia coli* I. Active transport and utilization of β -glucosides. *J Bacteriol* **93**, 254–263.
- Schnetz, K. & Rak, B. (1988). Regulation of the *bgl* operon of *Escherichia coli* by transcriptional antitermination. *EMBO J* **7**, 3271–3277.
- Schnetz, K., Toloczyki, C. & Rak, B. (1987). β -Glucoside (*bgl*) operon of *Escherichia coli* K12: nucleotide sequence, genetic organization and possible evolutionary relationship to regulatory components of two *Bacillus subtilis* genes. *J Bacteriol* **169**, 2579–2590.
- Simons, G., Nijhuis, M. & deVos, W. M. (1993). Integration and gene replacement in the *Lactococcus lactis* *lac*-operon: induction of a cryptic phospho- β -glucosidase in LacG-deficient strains. *J Bacteriol* **175**, 5168–5175.
- Spector, T. (1978). Refinement of the coomassie-blue method of protein quantitation. *Anal Biochem* **86**, 142–146.
- Stülke, J., Arnaud, M., Rapoport, G. & Martin-Verstraete, I. (1998). PRD – a protein domain involved in PTS-dependent induction and carbon catabolite repression of catabolic operons in bacteria. *Mol Microbiol* **28**, 865–874.
- Tobisch, S., Glaser, P., Krüger, S. & Hecker, M. (1997). Identification and characterization of a new β -glucoside utilization system in *Bacillus subtilis*. *J Bacteriol* **179**, 496–506.
- Tyler, B. & Magasanik, B. (1969). Molecular basis of transient repression of β -galactosidase in *Escherichia coli*. *J Bacteriol* **97**, 550–556.
- Vaughan, E. E., David, S. & deVos, W. M. (1996). The lactose transporter in *Leuconostoc lactis* is a new member of the LacS subfamily of galactoside-pentose-hexuronide translocators. *Appl Environ Microbiol* **62**, 1574–1582.
- Wagner, E., Marcandier, S., Egeter, O., Deutscher, J., Götz, F. & Brückner, R. (1995). Glucose kinase-dependent catabolite repression in *Staphylococcus xylosus*. *J Bacteriol* **177**, 6144–6152.
- Weber, B. A., Klein, J. R. & Henrich, B. (1998). The *arbZ* gene from *Lactobacillus delbrueckii* subsp. *lactis* confers to *Escherichia coli* the ability to utilize the β -glucoside arbutin. *Gene* **212**, 203–211.
- Weickert, M. J. & Chambliss, G. H. (1990). Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **87**, 6238–6242.
- Witt, E., Frank, R. & Hengstenberg, W. (1993). 6-Phospho- β -galactosidases of Gram-positive and 6-phospho- β -glucosidase B of Gram-negative bacteria: comparisons of structure and function by kinetic and immunological methods and mutagenesis of the *lacG* gene of *Staphylococcus aureus*. *Protein Eng* **6**, 912–920.
- Woodward, J. & Wiseman, A. (1982). Fungal and other β -D-glucosidases – their properties and applications. *Enzyme Microb Technol* **4**, 73–79.
- Ye, J. J. & Saier, M. H., Jr (1996). Regulation of sugar uptake via the phosphoenolpyruvate-dependent phosphotransferase system in *Bacillus subtilis* and *Lactococcus lactis* is mediated by ATP-dependent phosphorylation of seryl residue 46 in HPr. *J Bacteriol* **178**, 3557–3563.

Received 29 March 2000; accepted 28 April 2000.