Characterization of Two New Glycosyl Hydrolases from the Lactic Acid Bacterium *Carnobacterium piscicola* Strain BA

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Three genes with homology to glycosyl hydrolases were detected on a DNA fragment cloned from a psychrophilic lactic acid bacterium isolate, *Carnobacterium piscicola* strain BA. A 2.2-kb region corresponding to an α-galactosidase gene, *agaA*, was followed by two genes in the same orientation, *bgaB*, encoding a 2-kb β-galactosidase, and *bgaC*, encoding a structurally distinct 1.76-kb β-galactosidase. This gene arrangement had not been observed in other lactic acid bacteria, including *Lactococcus lactis*, for which the genome sequence is known. To determine if these sequences encoded enzymes with α- and β-galactosidase activities, we subcloned the genes and examined the enzyme properties. The α-galactosidase, *AgaA*, hydrolyzes *p*aret-nitrophenyl-α-D-galactopyranoside and has optimal activity at 32 to 37°C. The β-galactosidase, *BgaC*, has an optimal activity at 40°C and a half-life of 15 min at 45°C. The regulation of these enzymes was tested in *C. piscicola* strain BA and activity on both α- and β-galactoside substrates decreased for cells grown with added glucose or lactose. Instead, an increase in activity on a phosphorylated β-galactoside substrate was found for the cells supplemented with lactose, suggesting that a phospho-galactosidase functions during lactose utilization. Thus, the two β-galactosidases may act synergistically with the α-galactosidase to degrade other polysaccharides available in the environment.

Glycosyl hydrolases (EC 3.2.1 to 3.2.3) cleave the glycosidic bond(s) between two or more carbohydrates or the bond between a carbohydrate moiety and a noncarbohydrate moiety. Traditionally, glycosyl hydrolases were grouped together based on substrate specificity. For example, all β-galactosidases were combined into one group (EC 3.2.1.23) because of their shared ability to hydrolyze lactose. However, classification based on substrate specificity is complicated by the fact that some enzymes hydrolyze more than one substrate. Some glycosyl hydrolases have activity on both phosphorylated and nonphosphorylated substrates (3, 21) or on β-glucosides and β-galactosides (2) and some β-galactosidases have activity on β-fucosides and β-galacturonides (11, 15, 25).

The increase in the number of sequenced glycosyl hydrolases and the availability of new analytical methods has permitted the reorganization of these enzymes into families based on amino acid sequence similarities and hydrophobic cluster analysis (12, 13, 14). There are presently four families containing enzymes with β-galactosidase activity, families 1, 2, 35, and 42, and three families which contain enzymes with α-galactosidase activity, families 4, 27, and 36. New glycosyl hydrolases which have been sequenced can be grouped into a specific family on the basis of DNA or deduced amino acid similarity. In many cases, however, there is no information to verify the substrate specificity of the enzymes within these groups or their possible role(s) in cellular metabolism.

The glycosyl hydrolases found in lactic acid bacteria have been of special interest because of their importance to the dairy and food processing industries. In contrast to most other bacteria, nearly all lactic acid bacteria transport and utilize lactose via the phosphoenolpyruvate-dependent phosphotransferase system, which requires the concomitant activity of a phospho-β-galactosidase. β-Galactosidases belonging to a different family, and sharing sequence similarity with the well-characterized *Escherichia coli lacZ*-encoded enzyme, have also been detected in lactic acid bacteria such as *Streptococcus thermophilus* or *Lactococcus lactis* (7).

The genus *Carnobacterium* is a recent taxonomic addition to the lactic acid bacteria group (4, 5). Most *Carnobacterium* species were isolated from meat or fish (1, 23) and are similar to those in the *Lactobacillus* genus but do not grow on acetate and have a higher tolerance to oxygen and high pH (24). Research on *Carnobacterium* species has centered on their ability to produce bacteriocins (8, 19). Recently, during our investigation of psychrophilic organisms, we isolated from soil a new *Carnobacterium piscicola* strain, BA, which hydrolyzed the β-galactosidase chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 4°C. Initial work discovered a gene, *bgaB*, encoding a family 42 glycosyl hydrolase that had a temperature optimum of 30°C (6). This was the first report of a gene from this family in any lactic acid bacterium.

Additional sequencing of this cloned fragment suggested that the *bgaB* gene is centered between two regions with homology to other glycosyl hydrolases. The gene *agaA* is located in the region adjacent to the N-terminal end of *bgaB*, and shared sequence homology with a group of α-galactosidases characterized from other bacteria and some eukaryotes, including a sequence from the lactic acid bacterium *Lactobacillus plantarum*. Adjacent to the C-terminal end of the *bgaB* β-galactosidase gene was a second, unrelated β-galactosidase.
gene, bgaC. Genes similar to bgaC have not been reported in the lactic acid bacteria. This includes L. lactis, for which the sequence of the entire genome is known.

In order to explore the functions encoded by these two new putative genes, they were subcloned and their ability to produce enzymes with α- and β-galactosidase activities was tested. The arrangement of these genes on a single fragment suggested that they might function together to degrade saccharides containing both alpha and beta linkages rather than being involved in lactose hydrolysis. We examined the regulation of these enzymes in the native C. piscicola strain BA and found that their activities decreased when the medium was supplemented with either glucose or lactose. In contrast, a phosphogalactosidase activity increased during growth with lactose. These results suggest that a phosphogalactosidase is responsible for lactose utilization and that the unusual cluster of glycosyl hydrolyse genes reported here might be involved in the degradation of other polysaccharides.

MATERIALS AND METHODS

Construction of plasmids with individual genes. Subclones were created for both the α-galactosidase, designated agaA, and the β-galactosidase, or bgaC, genes. Construct BA-α1 carrying the α-galactosidase gene was created by digesting plasmid DNA from the original transformant at native NcoI and PstI (Promega, Madison, Wis.) restriction sites, followed by gel purification of the DNA fragment (Qiagen, Valencia, Calif.), ligation into the pΔα vector (26) (Epicentre Fast Link ligase; Epicentre Technologies, Madison, Wis.), and transformation into E. coli DH5α cells. Insert DNA prepared from the resulting DH5α subclones (Wizard kit; Promega) was verified through restriction analysis (Promega) and activity of the expressed enzyme was assayed using 4-nitrophenyl-β-D-galactopyranoside as a substrate. The enzyme demonstrated peak activity at 37°C as a substrate. The enzyme demonstrated peak activity at 37°C.

Analysis of enzyme activity. The AgaA enzyme was assayed in crude cell lysate for thermal dependence of activity on the substrate pNP α-galactoside (Sigma). One milliliter of the reaction buffer (20 mM β-mercaptoethanol) was added to 200 µl of pNP α-galactoside (4 mg/ml) and preincubated at the assay temperature. The reaction was started by adding 10 µl of a 1:10 dilution of cell lysate. The assays were stopped with 500 µl of Na2CO3 and the intensity of the color change was measured at 420 nm. Substrate specificity of two of the three encoded enzymes had previous work analyzed the family 42 enzyme (6); however, the substrate specificity of two of the three encoded enzymes had not yet been verified. These two genes were subcloned into E. coli DH5α cells, and their expressed enzyme activities were examined independently.

Thermal dependence and specificity of BgaC. E. coli DH5α transformants carrying the DNA fragment BA-α4-4 were able to hydrolyze the β-1,4-linked chromogenic β-galactosidase substrate X-Gal, as well as ONPG. Thermal dependence of activity assays of expressed BgaC were performed using ONPG as a substrate. The enzyme demonstrated peak activity at 37°C (Fig. 1A). The BgaC enzyme was stable at 40°C for at least 60 min, but rapidly became inactivated when incubated at 45°C (Fig. 1B).

The BgaC enzyme was stable in cell lysates at 4°C for several weeks and did not require the stabilizing presence of glycerol as did the previously reported BgaB enzyme (6). Another notable difference is that its activity is unaffected by 0.5 M NaCl and 0.04 M imidazole. BgaC was dialyzed against different metals in preparation for iminodiacetic acid (IDA) affinity purification. Unfortunately, the enzyme was inactivated by dialysis against 50 mM concentrations of CuCl2 and lost 17% of its activity when dialyzed against ZnCl2 and 38% when dialyzed against NiCl2 (data not shown). CuCl2 and NiCl2 are known to have detrimental effects on some proteins. The effect of ZnCl2 is intriguing, however, since Zn2+ is known to interact positively with some groups of β-galactosidases.

Thermal dependence and specificity of AgaA. Transformants carrying the subcloned α-galactosidase gene agaA were unable to hydrolyze 5-bromo-3-chloro-2-indolyl-β-D-galactopyranoside (X-β-Gal) on Luria-Bertani-ampicillin plates. However, permeabilized whole cells and cell extracts did contain an activity which hydrolyzed the chromogen ONPG-α-Gal. The enzyme was also active on pNP α-galactoside and this was used for comparison with other pNP substrates. The specific activity with pNP α-galactoside was 2.3 U/mg. All substrates containing β-linkages (pNP β-fucoside, pNP β-galactoside, pNP β-mannoside, and pNP β-cellobioside) had less than 0.001% of the pNP α-galactosidase activity. When cell lysates were tested for the ability to hydrolyze pNP α-glucoside, no activity above the background found in the DH5α control cells was observed. Thermal dependence of activity of the AgaA enzyme (Fig. 2) indicated that it was most active within a range of 32 to 37°C.

Effect of growth conditions on enzyme activity in the native organism. Like all other members of the Carnobacterium genus, strain BA requires a rich medium for growth and does not
grow on a minimal medium, even when supplied with vitamins, minerals, and amino acids. Of all the common types of complex media tested (Luria-Bertani broth, nutrient broth, TSB, R2, yeast extract-malt [YM], Terrific broth), the organism had the highest cell yield on TSB. An ingredient of TSB, phytone, is a hydrolysate of soy containing a high carbohydrate content (35% dry weight). Other soy hydrolysates were tested by adding them to M9 medium in 1% (wt/vol) final concentrations to examine organism growth and gene expression. The yield and activity with ONPG of cells grown with the four best soy additives are shown in Fig. 3. While many of the complex additives permitted growth of the organism, the total cell yield remained highest with TSB. With all four of the media tested, addition of either 2% glucose or 2% lactose caused the cell yield to double, while additional raffinose did not cause a change in cell yield from that of controls with no additional carbohydrate.

TSB was supplemented with a variety of carbon sources (2% [wt/vol]) in order to examine their effects on enzyme levels in the native organism. The addition of the α-galactoside raffinose to the medium had no significant effect on the observed α- and β-galactosidase activities (Fig. 4A), whereas supplementing the growth medium with the α-galactoside stachyose caused a reduction in both α- and β-galactosidase activities (data not shown). Glucose, and more interestingly, lactose, both decreased α- and β-galactosidase activities (Fig. 4A).

Though no other β-galactosidase activities were observed during the creation of the C. piscicola strain BA chromosomal libraries, it is possible that the chromogens used in screening (X-Gal and ONPG) would not have detected them. Because other lactic acid bacteria use phospho-β-galactosidases during lactose utilization, we assayed C. piscicola strain BA cells for pNP 6-phospho-β-galactosidase activity. Growth of cells in TSB supplemented with glucose still caused reduction of activity towards the phosphorylated substrate; however, unlike assays using pNP β-galactosidase, activities on pNP phospho-β-galactoside were not reduced when cells were grown in the presence of galactose or lactose (Fig. 4B). This indicates that a third β-galactosidase, a phospho-β-galactosidase, was produced by the cells and that this enzyme would most likely be responsible for lactose utilization and the increased cell yield.

**DISCUSSION**

The discovery of two genes belonging to different families of β-galactosidases and their arrangement with an α-galactosidase gene on a single DNA fragment cloned from C. piscicola strain BA is presently unique among the lactic acid bacteria. This arrangement may be related to the interdependent function of the three encoded enzymes. If so, the study of these enzymes may help us understand the normal function of glycosyl hydrolases that have so far been identified only by their sequence homology with other enzymes. Characterization of the BgaB β-galactosidase was presented in a previous work (6).
Here we have concentrated on the second β-galactosidase, BgaC, and the α-galactosidase, AgaA. The sequence of the β-galactosidase BgaC is homologous to family 35 glycosyl hydrolases. Interestingly, the enzyme appears to be absent from most of the well-characterized lactic acid bacteria, including *L. lactis*, where a search of the entire genome detected no sequences homologous to BgaC. Assays at different temperatures with ONPG demonstrated that the enzyme has a thermal optimum of about 40°C. This optimum is lower than values for some other mesophilic enzymes, such as LacZ from *E. coli* (50 to 55°C) (17), BglI and II from *Bacillus circulans* (45°C and 60°C, respectively) (27), or the β-galactosidase from *Bacteroides polygagnatus* (45°C) (22). In addition, this thermal optimum is nearly 10°C higher than that of the cold-active BgaB β-galactosidase encoded by the gene found directly upstream of *bgaC*. Despite the fact that the thermal optimum of BgaC is higher than that of the other β-galactosidase, it is still quite thermolabile, showing rapid inactivation at temperatures of 45 and 50°C.

The other gene that is part of this cluster encodes a family 36 α-galactosidase, AgaA. Similar enzymes have also been detected in two different strains of the thermophile *Bacillus steaothermophilus*, the AgaN enzyme from strain NUB3621 (9) and the GalA enzyme from strain MCA2184 (accession number AF038547). The optimal temperature for activity of the *C. piscicola* AgaA enzyme is 32 to 37°C, which is much lower than that of the *B. stearothermophilus* GalA enzyme with a peak activity at 75°C. The GalA enzyme was not characterized with respect to thermal dependence; however, it retained full catalytic activity after incubation at 60°C for 24 h and therefore is likely to be much more stable than the *C. piscicola* BA enzyme. No data on thermal dependency of activity are available in the literature for the homologous α-galactosidases from the mesophiles *L. plantarum* (accession number AF189765) or *Pediococcus pentosaceus* (accession number L32093), and therefore the biochemical characteristics of these...
related enzymes from these mesophilic organisms cannot be compared.

The inability of *C. piscicola* strain BA to grow on minimal media made testing carbohydrate utilization difficult. In order to determine whether a carbon source was used by the cells, cultures were grown in rich medium (TSB) with and without added carbohydrate and then examined for increases in cell yield. When cells were grown in the presence of excess glucose, the cell yield increased. Furthermore, a simultaneous reduction in ONPG activity in these cultures suggested that the measured enzymes were subject to catabolite repression during growth with glucose. Cultures grown in TSB plus lactose also demonstrated an increased cell yield and decreased activity on ONPG. The similar decrease in the measured enzyme activities for cells grown with lactose also suggested that these enzymes were catabolite repressed. Because these enzyme activities were reduced rather than increased when lactose was added, it is unlikely that they are involved in lactose utilization.

None of the X-Gal-hydrolyzing transformants from chromosomal libraries of *C. piscicola* strain BA contained genes with homology to a family 1 phosphoenolpyruvate-dependent phospho-β-galactosidase. However, it is possible that some phospho-β-galactosidases might not hydrolyze X-Gal and would be undetectable in these screens. Because chromogenic substrates for the phosphorylated glycosidases are not commercially available, we obtained small quantities from J. Thompson that allowed us to test the possibility that a phospho-β-galactosidase might exist in our *C. piscicola* strain. Assays performed on lactose-grown cells using pNP phospho-β-galactopyranosidase showed that there was indeed a phospho-β-galactosidase activity present in *C. piscicola* strain BA cells, one which was undetectable without the use of a phosphorylated chromogen. Thus, it seems likely that the glycosyl hydrolases discovered on our cloned gene fragment have a function beyond that of lactose hydrolysis.

It is often assumed that enzymes with the ability to hydrolyze X-Gal or ONPG function in the utilization of lactose by the cell. However, the disaccharide lactose, found almost exclusively in mammal milk, is relatively rare in soils, streams, etc. In contrast, oligosaccharides are common components of microbrial cell walls and capsules as well as several eukaryotic structures. Thus, many of these enzymes may instead have important functions for providing alternative carbon sources. For example, recent work with β-galactosidases has indicated that these enzymes fill an essential role in the breakdown of side chains from large oligosaccharides such as hemicellulose and soy (10, 16). These enzymes are intracellular and are induced after breakdown of the target substrate’s polymer backbone by extracellular enzymes such as β-mannanase (18). Both α- and β-linkages are common in the side chains of sugar polymers produced by plants and in the complex saccharides adsorbed to humic acid substances in the soil. Therefore the by-products from the breakdown of these larger sugar molecules may be targets of the *C. piscicola* strain BA enzymes.

The clustering of the genes encoding novel β- and α-galactosidases suggests that the enzymes may function in concert to degrade oligosaccharides containing both alpha and beta galactosidase linkages. Preliminary results suggest that these genes are cotranscribed and may be regulated as an operon. The unique arrangement of the alpha and two different beta galactosidases together may provide a useful tool for helping us understand the prevalence and function of these enzymes in a variety of other microorganisms.

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**REFERENCES**


