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Characterization of a Psychrotrophic *Arthrobacter* Gene and Its Cold-Active β-Galactosidase

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Enzymes with high specific activities at low temperatures have potential uses for chemical conversions when low temperatures are required, as in the food industry. Psychrotrophic microorganisms which grow at low temperatures may be a valuable source of cold-active enzymes that have higher activities at low temperatures than enzymes found for mesophilic microorganisms. To find cold-active β-galactosidases, we isolated and characterized several psychrotrophic microorganisms. One isolate, B7, is an *Arthrobacter* strain which produces β-galactosidase when grown in lactose minimal media. Extracts have a specific activity at 30°C of 2 U/mg with *o*-nitrophenyl-β-D-galactopyranoside as a substrate. Two isozymes were detected when extracts were subjected to electrophoresis in a nondenaturing polyacrylamide gel and stained for activity with 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal). When chromosomal DNA was prepared and transformed into *Escherichia coli*, three different genes encoding β-galactosidase activity were obtained. We have subcloned and sequenced one of these β-galactosidase genes from the *Arthrobacter* isolate B7. On the basis of amino acid sequence alignment, the gene was found to have probable catalytic sites homologous to those from the *E. coli lacZ* gene. The gene encoded a protein of 1,016 amino acids with a predicted molecular mass of 111 kDa. The enzyme was purified and characterized. The β-galactosidase from isolate B7 has kinetic properties similar to those of the *E. coli lacZ* β-galactosidase but has a temperature optimum 20°C lower than that of the *E. coli* enzyme.

Cold-active enzymes that have high catalytic rates at low temperature have many potential uses in processes which must occur in the cold or in environments impractical to heat. One such application would be the removal of lactose from refrigerated milk or whey so that it can be used by people who are lactose intolerant because they lack a β-galactosidase in the small intestine and cannot digest lactose (21). A β-galactosidase which could hydrolyze lactose in milk or whey at refrigerated temperatures could be used to produce low-lactose products during shipping and storage. An ideal enzyme for treating milk would work well at 4 to 8°C; be active at pH 6.7 to 6.8; not be inhibited by sodium, calcium, or galactose; and be specific for lactose (20). The current commercial enzyme is from the yeast *Kluyveromyces lactis*, has a temperature optimum of 35°C, requires manganese or magnesium and potassium or sodium, and is inhibited by calcium at concentrations greater than 0.1 mM (5) and by galactose with a K_i of 42 mM (19). The *K. lactis* enzyme was originally chosen because it was already commercially available, and a process which removed about 70% of the lactose during incubation for a few hours at 35 to 40°C was developed (20).

In addition to searching for enzymes that might be useful for treating refrigerated milk products, our work had other long-range objectives. One is to test the rationale that psychrotrophic organisms could be a source of cold-active enzymes. Another is to compare new enzymes with those from organisms growing at higher temperatures with the long-term goal of understanding which changes influence an enzyme's response to temperature.

To meet these objectives, we isolated and screened several psychrotrophic organisms for ones producing cold-active β-galactosidases. Three isolates (B7, D2, and D5) obtained from fields which were spread with whey were further characterized (14). These strains are nonmotile aerobes, do not produce acid with glucose as a carbon source, grow at 0°C, do not form isolated colonies above 30°C, have a rod-coccus morphological cycle during growth, and contain lysine as the diamino acid in their peptidoglycan (14). On the basis of these traits, these isolates have been classified as psychrotrophic *Arthrobacter* strains. There are no other reports of *Arthrobacter* strains making β-galactosidase, and our work with the type strains, *Arthrobacter globiformis* ATCC 8010 and NRRL B-2979 and *Arthrobacter citreus* NRRL B-14091, demonstrated that they are unable to use lactose as a carbon source (14). Because our isolates produced cold-active enzymes and the presence of β-galactosidase may be an unusual trait for *Arthrobacter* strains, we chose one, B7, for further characterization.

Initial β-galactosidase assays using permeabilized cells and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as a chromogenic substrate suggested that the isolate had a β-galactosidase activity with a broad temperature range and an optimum of 30°C. Further study demonstrated that when the isolate is grown with lactose as a carbon source, two enzymes with β-galactosidase activity are produced. Extracts subjected to electrophoresis on nondenaturing polyacrylamide gels and subsequent in situ staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) showed two bands of activity (14).

In order to further examine these β-galactosidase isozymes, we prepared chromosomal DNA from the *Arthrobacter* isolate B7 and used it to transform an *Escherichia coli* recipient.

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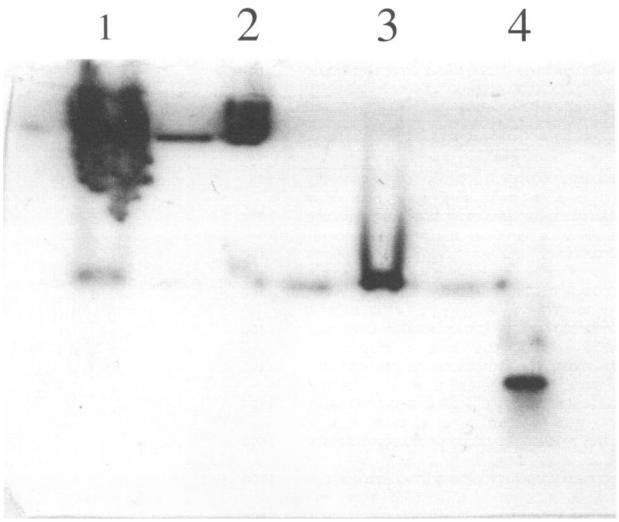

FIG. 1. Expression of β-galactosidases from genes cloned from *Arthrobacter* isolate B7. Extracts from isolate B7 and *E. coli* transformants carrying different genes cloned from isolate B7 were subjected to electrophoresis in a 7.5% nondenaturing polyacrylamide gel and stained for in situ activity with X-Gal. The predominant and more slowly migrating β-galactosidase activity in isolate B7 extracts was added in excess in order to detect the minor lower band. Lanes: 1, isolate B7; 2, *E. coli* with clone B7-15; 3, *E. coli* with clone B7-12; 4, *E. coli* with clone B7-14. The scanned image was generated as described in Materials and Methods.

Interestingly, we obtained three different genes, numbered 12, 14, and 15, each encoding a β-galactosidase activity capable of hydrolyzing ONPG and X-Gal. As part of our characterization of these genes and their physiological roles, we have sequenced the gene encoding the major β-galactosidase isozyme found when the isolate is grown with lactose. In addition, we expressed the gene in *E. coli*, purified the recombinant protein, and determined its biochemical and thermal properties. Comparison of the amino acid sequence deduced from the DNA sequence showed that its probable catalytic site is homologous to that found for the β-galactosidase *lacZ* gene from *E. coli*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Arthrobacter* isolate B7 was isolated in our laboratory as described elsewhere (14). Cultures were grown on tryptic soy broth (Difco) at 25°C. *E. coli* JM109 (30) and MC1061 (15) have been described previously and were maintained on M9 minimal medium (29). Chromosomal DNA was cloned into plasmid pΔα18, which we had constructed by deleting the *Nde*I-*Hin*dIII fragment containing the coding sequence of the *lacZ* α fragment from pUC18 (30). Subclones were inserted into pUC18 for sequencing. Plasmid-containing strains were grown in LB broth (16) containing 100 μg of ampicillin per ml.

DNA preparation. Chromosomal DNA was isolated from the *Arthrobacter* isolate B7 by a modification of the method of Brahamsha and Greenberg (2). The lysozyme concentration was increased to 5 mg/ml, and the cells were treated for 30 min and then incubated in 1% sodium dodecyl sulfate (SDS) at 60°C for 1 h. From 100 ml of cells grown in tryptic soy broth, 0.5 to 1.0 mg of DNA was recovered as determined by A_{260} measurements. The quality of DNA was determined after electrophoresis in 0.4% agarose gels.

Cloning protocol. Chromosomal DNA from isolate B7 was partially digested with *Sau*3AI to give an average fragment size of 4 to 6 kb. The digested B7 DNA was separated on a 0.4% agarose gel. DNA fragments between 4 and 6 kb were cut out of the gel and purified by using the USBioclean MP kit (United States Biochemical, Cleveland, Ohio). Plasmid pΔα18 was digested with *Bam*HI and then treated with calf intestine alkaline phosphatase. The digested vector was purified from an agarose gel with the USBioclean MP kit. Chromosomal and vector DNA were mixed at a 1:1 ratio and then ligated with T4 DNA ligase at 14°C. Competent cells of strain JM109 were prepared by the method of Hanahan et al. (8), transformed by the ligation reaction, and then plated onto LB agar containing 0.01% X-Gal, 0.1 mM IPTG (isopropyl-thio-β-D-galactopyranoside), and 100 μg of ampicillin per ml. Plates were incubated at 37°C overnight to allow the colonies to form and then transferred to either 25°C or 4°C to allow expression or activation of any heat-labile proteins. Blue colonies were picked and transferred to fresh LB ampicillin plates.

DNA sequencing. Plasmid DNA was isolated by the method of Sambrook et al. (22). DNA sequencing was performed by the procedure of Tabor and Richardson (27) using the Sequenase version 2.0 sequencing kit (United States Biochemical) with the following modifications: the reaction temperature was increased to 42°C, and 7-deaza-dGTP replaced dGTP.

DNA hybridization. *Bam*HI restriction endonuclease digests of DNA samples were separated on 0.5% agarose gels and then transferred to Immobilon-S membranes (Millipore Corporation, Bedford, Mass.) by the method of Southern (26). DNA was labeled with biotin by using the PolarPlex chemiluminescent blotting kit (Millipore Corporation). Hybridizations were carried out at 68°C for 16 h as described elsewhere (26).

Enzyme purification. Strain MC1061/pΔαB7-15.12 was grown for 8 h in 2 ml of LB broth with ampicillin at 25°C. This culture was inoculated into 500 ml of TYP medium (16 g of tryptone, 16 g of yeast extract, 5 g of NaCl, and 2.5 g of K_2HPO_4 per liter) containing 100 μg of ampicillin per ml. The cultures were

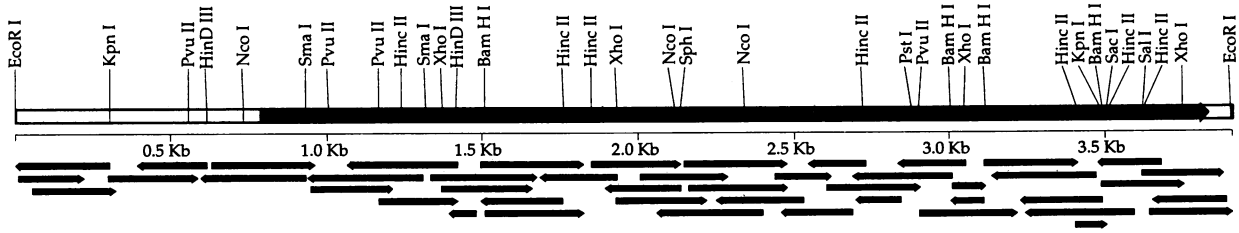

FIG. 2. Summary of the sequencing strategy for the β-galactosidase gene. Restriction map of the 3.9-kb *Eco*RI fragment in pΔαB7-15.9, with the coding region for the β-galactosidase gene indicated (thick arrow). The lengths and directions of the sequencing fragments are indicated by arrows below the map.

FIG. 3. Nucleotide sequence of the *Eco*RI fragment of pΔαB7-15.9 containing the β-galactosidase gene from isolate B7. The deduced amino acid sequence of the coding region is given below the nucleotide sequence. A putative ribosome binding site (RBS) is indicated.

incubated for 16 h, and the cells were harvested by centrifugation at 22,000 × *g* for 15 min at 4°C and resuspended in 12 ml of Z buffer (16). The cells were broken by passage through a French pressure cell (16,000 lb/in^2), and debris was removed by centrifugation (22,000 × *g* for 15 min at 4°C).

Ammonium sulfate was added to the crude extract to 25% saturation, and the mixture was incubated for 30 min and centrifuged at 31,000 × *g* for 20 min at 4°C. The pellet was discarded, and additional ammonium sulfate was added to 50% saturation. The solution was centrifuged again, and the supernatant was discarded. The pellet was resuspended and dialyzed overnight in Z buffer. Following dialysis, the sample was applied to High-Q anion exchange resin (Bio-Rad Laboratories, Melville, N.Y.) equilibrated with a 200 mM NaCl–20 mM sodium phosphate buffer (pH 7.0). The enzyme was eluted with a 200 to 500 mM NaCl linear gradient.

The active fractions were diluted twofold with 20 mM sodium phosphate buffer (pH 7.0) and then added to an affinity matrix of agarose derivatized with *p*-aminobenzyl-1-thio-β-D-galactopyranoside (Sigma, St. Louis, Mo.). The matrix contain-

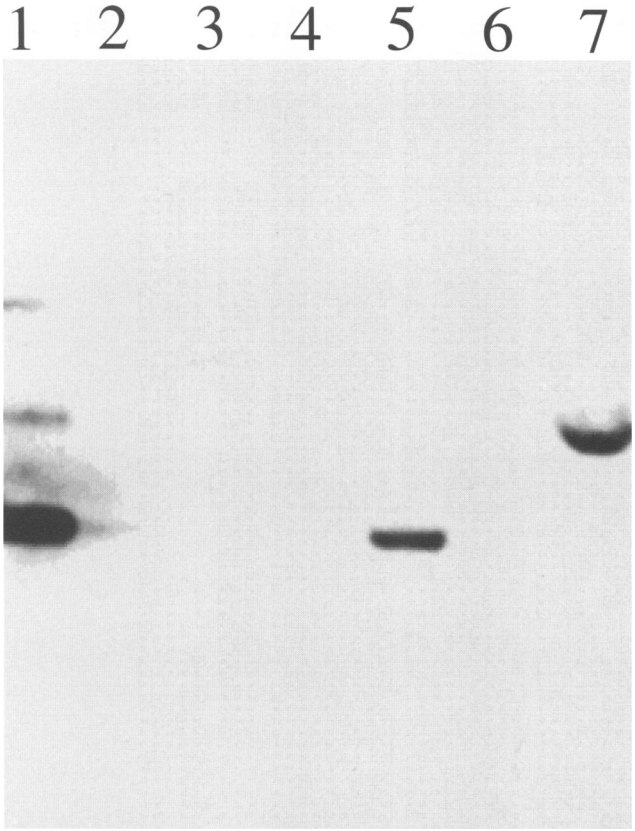

FIG. 4. Southern blot analysis with the *Arthrobacter* isolate B7 *lacZ* DNA. *Bam*HI digests of chromosomal DNAs from *E. coli* JM109 and isolates B7, D2, and D5 were probed with an internal 1.5-kb *Bam*HI fragment from the *Arthrobacter* isolate B7 clone 15 *lacZ* gene. Lanes 1 to 3, plasmid preparations carrying DNA cloned from isolate B7; lanes 4 to 7, total DNAs extracted from the specified strains. Lanes: 1, pΔαB7-15; 2, pΔαB7-14; 3, pΔαB7-12; 4, *E. coli*; 5, isolate B7; 6, isolate D2; 7, isolate D5. The scanned image was generated as described in Materials and Methods.

ing the sample was washed with 300 mM NaCl and eluted with 600 mM NaCl in 20 mM sodium phosphate buffer (pH 7.0). The active fractions were pooled and concentrated by using a Macroprep concentrator 30,000 MW (Micron Separations Inc., Westboro, Mass.). The N-terminal amino acid sequence was determined at the Hershey Medical Center of The Pennsylvania State University.

Enzyme assays. β-Galactosidase activity was determined by measuring the release of *o*-nitrophenol from ONPG at 420 nm. Enzyme reactions were performed by diluting the enzyme in Z buffer at 30°C and starting the reaction with the addition of ONPG at a final concentration of 2.2 mM. After 1 to 5 min the reaction was stopped by the addition of 1 M Na_2CO_3. One unit is defined as 1 μmol of *o*-nitrophenol released per min. Specific activity is given in units per milligram of protein. Activity with lactose as the substrate was determined in Z buffer at 30°C with the addition of lactose at 2 to 20 mM. After 10 to 20 min the reaction was stopped by heating the sample to 60°C. Galactose concentrations were determined with the galactose dehydrogenase assay (23). Specific activity is defined as 1 μmol of galactose released per min per mg of protein. Protein concentrations were determined by the method of Bradford (1), with reagents from Bio-Rad Laboratories and bovine serum albumin as a standard.

Gel electrophoresis and Western blots (immunoblots). Protein samples were analyzed on 7.5% polyacrylamide gels by the method of Laemmli (12), and the gels were stained with Coomassie blue to detect proteins. Staining for β-galactosidase activity was done by incubating nondenaturing gels in an assay buffer containing X-Gal instead of ONPG to detect in situ activity. The hydrolysis of X-Gal produced blue bands within the polyacrylamide gels.

Polyclonal antibodies against the recombinant β-galactosidase were produced by injecting purified protein emulsified with Freund's complete adjuvant into New Zealand White rabbits. After 6 weeks the rabbits were bled and the serum was absorbed with whole-cell extracts from *E. coli* MC1061. Western blot analyses were performed by the method of Towbin et al. (28) using goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (13).

Presentation of scanned images. Figures 1, 4, and 6 were prepared by scanning the results obtained on the gels or membranes. The scanned image was then generated by using an Apple Color One scanner and the application Ofoto, version 2.0. The file was saved in the tagged-image file format.

Chemicals and reagents. Growth medium components were obtained from Difco. Restriction enzymes, polymerases, and nucleotides were from Gibco-BRL (Gaithersburg, Md.) and Boehringer Mannheim (Indianapolis, Ind.). Radionucleotides were from New England Nuclear Corporation; X-Gal and ONPG were obtained from Sigma.

Nucleotide sequence accession number. The GenBank accession number for the sequence reported in this article is U12334.

RESULTS

Cloning of the *Arthrobacter* isolate B7 β-galactosidase genes into *E. coli*. Plasmid pΔα18 was constructed from pUC18 by deleting the *lacZ* α fragment to prevent α complementation of the β-galactosidase in the recipient. Transformants of JM109 containing the resulting vector, pΔα18, produced white colonies on X-Gal plates. A genomic library of *Arthrobacter* isolate B7 DNA was constructed by partial restriction digestion of DNA with *Sau*3AI and ligation into the *Bam*HI site of the vector pΔα18. Over 1,000 colonies were screened, and five β-galactosidase-positive colonies were obtained. The DNA inserts in three of the β-galactosidase-positive transformants were unique on the basis of analysis of the restriction digests. These inserts, numbered 12, 14, and 15, encoded genes for different β-galactosidases, as demonstrated by their separate patterns of migration in nondenaturing polyacrylamide gel electrophoresis (PAGE) (Fig. 1). Clone B7-15 (7.2-kb fragment) produced a β-galactosidase which corresponded to the major activity in isolate B7 and was chosen for further study. Transformant colonies containing B7-15 remained white when incubated on media containing X-Gal at 37°C but turned blue within 10 min when transferred to 20°C. This indicated that this clone encoded a cold-active enzyme with a temperature optimum lower than that of the *E. coli* β-galactosidase.

A restriction map of clone B7-15 was generated, and fragments were subcloned to determine the smallest fragment which could encode the β-galactosidase gene. A clone, B7-15.9, containing a 3.9-kb *Eco*RI fragment which produced β-galactosidase activity was found. The 3.9-kb *Eco*RI fragment cloned into pΔα18 in the opposite orientation produced less β-galactosidase activity, indicating that the gene in the original orientation had been transcribed in that direction from the vector's promoter.

FIG. 5. Alignment of β-galactosidase protein sequences of *Arthrobacter* strain B7 LacZ, *E. coli* EbgA (7), *E. coli* LacZ (11), *Klebsiella pneumoniae* LacZ (3), *Kluyveromyces lactis* (18), *Clostridium acetobutylicum* (9), *Streptococcus thermophilus* (25), *Lactobacillus bulgaricus* (24), and *Leuconostoc lactis* LacL and LacM (4). Sequences were aligned by using the Clustal method with the Ktuple setting at 1 and the gap penalty at 10. Consensus residues are boxed. The proposed active-site residues, Glu-461 and Glu-537, are indicated (arrows).

Nucleotide sequence of the isolate B7 β-galactosidase gene *lacZ*. A restriction map of the 3.9-kb *Eco*RI fragment from B7-15.9 was determined by using a number of common enzymes (Fig. 2). A series of overlapping subclones using appropriate restriction sites was constructed in pUC18 and then sequenced. The nucleotide sequence for the 3.9-kb *Eco*RI fragment is shown in Fig. 3. A single large open reading frame was found starting with ATG at nucleotide 787 and ending with TAG at nucleotide 3835. The open reading frame coded for a 1,016-amino-acid protein with a calculated M_r of 111,000, which was slightly smaller than the estimated molecular mass of 139 kDa determined by SDS-PAGE (see later results in Fig. 6). The gene was designated *lacZ* on the basis of its sequence homology with the *lacZ* from *E. coli*.

The start site was confirmed by determining the N-terminal sequence both for the β-galactosidase purified from the *Arthrobacter* isolate and for that purified from the *E. coli* transformant containing the cloned gene 15. Both proteins had the amino acid sequence of MSSSY that had been predicted at the N-terminal region of the protein. These results not only confirmed the start codon for the nucleotide sequence but verified that the protein purified from the *E. coli* transformant began at the same site as the enzyme produced in the original isolate.

In order to demonstrate that gene 15 had originated from the *Arthrobacter* isolate, an internal 1.5-kb *Bam*HI fragment was used to probe B7 chromosomal DNA (Fig. 4). The Southern blot showed that the fragment did hybridize to the B7 DNA but did not hybridize to either of the other two genes, 12 and 14, encoding β-galactosidase activity that were cloned from this isolate. In addition, the fragment from gene 15 showed no hybridization with chromosomal DNA prepared from the host *E. coli* strain or another *Arthrobacter* strain, D2, which we had characterized (14). The fragment did hybridize with DNA from another isolate, D5 (14).

Analysis of the β-galactosidase protein sequence. The predicted amino acid sequence of the *lacZ* gene was compared with other β-galactosidase sequences in the Swiss-Prot protein database. The *Arthrobacter* β-galactosidase was found to be a member of the *E. coli lacZ* family of β-galactosidases. The protein sequences were aligned by the Clustal method (Fig. 5), and similarities are shown in Table 1. The *Arthrobacter* β-galactosidase was most similar to the *E. coli* EbgA, with 22% sequence similarity. The alignment shows that the proposed active-site residues in *E. coli* LacZ, Glu-461 (10) and Glu-537 (6), are conserved in the *Arthrobacter* sequence. In addition, a tyrosine residue that may also be important in the reaction is conserved at site 503 (17).

Characterization of the β-galactosidase activity. The nucleotide sequence results showed that the open reading frame started at nucleotide 787 of the 15.9 subclone. In order to eliminate a portion of this upstream sequence, a new subclone, 15.12, was constructed by deleting the 0.6-kb region between the *Eco*RI site and the first *Hin*dIII site (Fig. 2). Since strains carrying subclone 15.12 had increased enzyme expression, the subclone was used for enzyme purification. In addition, an analysis of the DNA sequence revealed that the *Arthrobacter* gene contained an amber stop codon. The *E. coli* host strain, JM109, contains an amber suppressor mutation which might allow some read-through and complicate the results of the protein characterization. Thus, the plasmid pΔα carrying the subclone 15.12 was transformed into *E. coli* MC1061 (22), which lacks a suppressor mutation. The β-galactosidase was purified from the *E. coli* MC1061 transformant by ammonium sulfate precipitation, anion exchange chromatography, and affinity chromatography (Table 2). Although the affinity column decreased the yield of active enzyme, it increased the purity by removing other proteins. Following this procedure, the enzyme was 80% pure as determined by SDS-PAGE and had a subunit with an estimated molecular mass of 139 kDa (Fig. 6).

Antibodies were prepared against this enzyme preparation and used to show that the enzyme purified from the cloned gene in *E. coli* migrated at the same position during electrophoresis as the enzyme of the original *Arthrobacter* isolate. A reaction of the antibodies with an *Arthrobacter* crude extract which had been subjected to PAGE produced a single band. This band migrated in the same position as the recombinant enzyme prepared from the *E. coli* transformant (Fig. 6, lanes 5 and 6).

The activity of the purified enzyme was determined in phosphate, Tris, and glycine buffers at different pH values. The enzyme had a pH optimum of 7.2 in phosphate buffer (data not shown). The enzyme preparation was treated with EDTA to remove metal ions and desalted by chromatography on a Sephadex G-25 column. The enzyme showed less than 0.01%

TABLE 1. Sequence similarity of β-galactosidase

Enzyme source	% Similarity with enzyme from:								
	Arthrobacter isolate B7	*E. coli ebgA*	*E. coli lacZ*	*K. pneumoniae*	*C. acetobutylicum*	*S. thermophilus*	*L. bulgaricus*	*L. lactis*	*K. lactis*
Arthrobacter isolate B7	100.0	22.0	19.1	19.7	17.3	17.9	15.5	15.4	20.8
E. coli ebgA		100.0	21.5	20.1	20.0	16.2	16.9	16.1	21.5
E. coli lacZ			100.0	54.1	24.2	20.4	19.7	18.5	19.4
K. pneumoniae				100.0	19.6	20.4	18.2	17.0	17.4
C. acetobutylicum					100.0	43.7	33.2	33.0	17.9
S. thermophilus						100.0	39.6	28.4	14.7
L. bulgaricus							100.0	25.9	15.5
L. lactis								100.0	13.6
K. lactis									100.0

TABLE 2. Purification of the *Arthrobacter* isolate B7 β-galactosidase produced by clone 15

Step	Vol (ml)	Total protein (mg)	Total activity (μmol/min)	Sp act (μmol/min/mg)	Recovery (%)	Purification (fold)
Crude extract	11.6	1,241.2	4,454.4	3.6	100.0	1.0
Ammonium sulfate (25–50%)	10.6	291.1	2,618.2	9.0	58.8	2.5
Anion exchange	4.0	4.6	559.6	121.7	12.6	33.9
Affinity	0.8	0.4	46.0	108.7	1.0	30.3

of its normal activity without ion addition when assayed in a 0.1 M sodium phosphate buffer. Potassium, but not sodium, at a concentration of 100 mM restored 47% of the activity found with the Z buffer, which contains 10 mM KCl and 1 mM $MgSO_4$. The enzyme required either magnesium or manganese at 1 mM for maximum activity (Table 3). The effect of other ions on activity was tested by adding 1 mM solutions to a reaction mixture. The activity was completely inhibited by Cu^{2+}, but 61% of the activity remained with 1 mM $CaCl_2$ (data not shown). Measurements comparing the activities in the basic Z buffer with and without β-mercaptoethanol showed that the addition of β-mercaptoethanol stimulated activity over sevenfold.

The β-galactosidase had an apparent K_m of 0.4 mM and a V_{max} of 1,182 U/mg of protein with ONPG as the substrate. The K_m for lactose was 16 mM, and the V_{max} was 117 U/mg of protein. The ability of the enzyme to cleave a number of *p*-nitrophenyl (PNP)-β-glycoside substrates (PNP-β-D-galactoside, PNP-α-D-galactoside, PNP-β-D-glucoside, PNP-β-D-mannoside, PNP-β-L-fucoside, PNP-β-xyloside, PNP-β-L-arabinoside, PNP-β-D-galuronide, PNP-β-glucuronide, PNP-β-D-lactoside, and PNP-β-D-cellobioside) was tested at concentrations of 0.5 mM. The enzyme was specific for the β-galactosidase. The activity with β-galuronide as a substrate was only 1.4% of that found with PNP-β-D-galactoside, and all other substrates had less than 0.1% of the β-galactosidase activity.

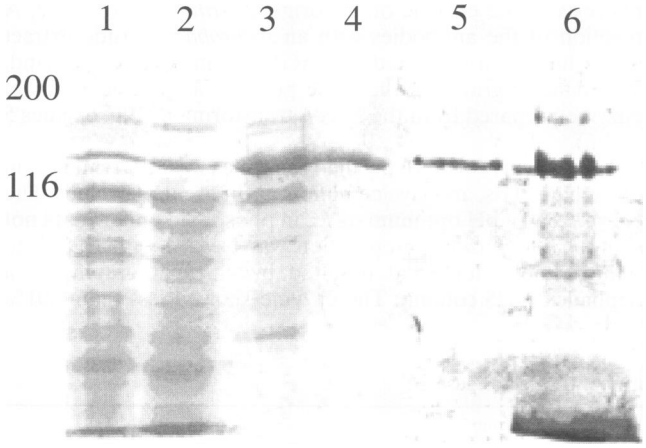

FIG. 6. Purification and Western blot analysis of the β-galactosidase from the *Arthrobacter* isolate B7 expressed in *E. coli*. The levels of purity are monitored at different stages by SDS–7.5% PAGE and Coomassie blue staining. The sizes (in kilodaltons) of molecular weight standards are indicated on the left. Lanes: 1, crude extract (54 μg of protein loaded); 2, ammonium sulfate precipitation (55 μg); 3, anion exchange chromatography (5.7 μg); 4, affinity chromatography (1.2 μg); 5, Western blot analysis of antibody binding with crude extract from isolate B7; 6, Western blot analysis of antibody binding with extract from *E. coli* MC1061 containing a plasmid with gene 15 from isolate B7. The scanned image was generated as described in Materials and Methods.

The effect of temperature on the enzyme activity was determined by assaying the enzyme at increments between 4 and 60°C. Maximum activity was found at about 40°C, and the enzyme retained approximately 25% of its activity at 10°C, a temperature at which the *E. coli* enzyme was inactive (data not shown). The enzyme's thermal stability was determined by incubating it at different temperatures and periodically withdrawing samples for assay at 30°C. The activity was stable at 30°C (or less) for over 2 h, but at 40°C it had a half-life of 90 min (Fig. 7). Incubation at 50°C caused inactivation within 10 min.

DISCUSSION

Microorganisms that grow at low temperatures have not been extensively studied, even though knowledge of their metabolism, enzymes, and chemical products could be extremely useful. Psychrotrophic microorganisms may be of particular importance, since the preliminary data suggest that they will be an important source of enzymes with lower temperature optima. We are especially interested in investigating the properties of cold-active enzymes in order to gain basic insight into the function of enzymes at a low temperature and in discovering enzymes that may be commercially useful. To initiate this work, we chose β-galactosidase as a model enzyme that could be easily monitored and had the potential for being used to remove lactose from milk at refrigeration temperatures.

TABLE 3. Effects of ions on the β-galactosidase activity encoded by the isolate B7 gene 15

Ion (mM)	Sp act	% of control[a]
None	<0.001	<0.01
$MgSO_4$		
0.1	6	1
1.0	604	111
$MgCl_2$		
0.1	10	2
1.0	628	116
$MnCl_2$		
0.1	113	21
1.0	865	159
KCl		
1.0	<0.001	<0.01
10	106	20
100	257	47
NaCl		
1.0	<0.001	<0.01
100	<0.001	<0.01

[a] The control activity in Z buffer with 10 mM KCl and 1 mM $MgSO_4$ was 542 U/mg of protein. The values are averages of three assays.

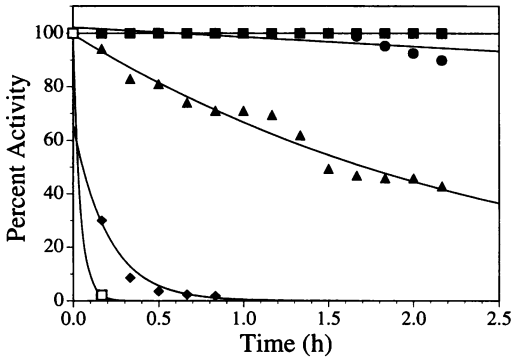

FIG. 7. Thermostability of β-galactosidase from isolate B7. The enzyme was incubated in Z buffer at 50 (□), 45 (◆), 40 (▲), 35 (●), or 30°C (■). Samples were removed at different times and assayed for ONPG activity at 30°C.

In searching for cold-active enzymes, we reasoned that psychrotrophic organisms which grow at low temperatures would produce enzymes with higher catalytic activities at temperatures lower than those for enzymes from mesophilic microbes such as *E. coli*. To examine this possibility, we isolated and characterized psychrotrophic *Arthrobacter* strains which grow at 0°C, produce β-galactosidase activities, and grow on lactose. No other *Arthrobacter* species have been reported to grow on lactose as a sole carbon source. The temperature optimum of the total β-galactosidase activity in whole-cell assays for one of these strains, B7, was 30°C (14), which is about 20°C below that for the *E. coli lacZ* enzyme. In addition to the lower-temperature profile, this isolate appeared interesting because two bands stained for X-Gal activity following electrophoresis of crude extracts on native gels, suggesting the presence of at least two β-galactosidase isozymes.

The initial characterization of the enzymes produced by isolate B7 was hampered by their low levels and the production of multiple β-galactosidases in the original isolate. We therefore cloned the genes encoding the β-galactosidases into *E. coli* to increase expression, to separate the activities, and to allow sequencing. The cloning of the recombinant genes not only confirmed that the organism produced two β-galactosidases but revealed a gene for a third enzyme. Two genes, 12 and 15, which encoded enzymes corresponding to those expressed during growth on lactose minimal media and separated by electrophoresis were found. In addition, a third gene, 14, for which no enzyme had been observed in isolate B7 extracts, was cloned. The functions of these three enzymes are not yet understood, but studies of their regulation and expression during growth at different temperatures and with various carbon sources are in progress.

Because clone 15 encoded the major enzyme expressed in cells growing with lactose, we chose it for characterization. The open reading frame of this gene encoded a 1,016-amino-acid protein with a calculated M_r of 111,000. This is slightly smaller than the *E. coli lacZ* enzyme, with 1,023 amino acids, and slightly larger than the *ebgA* protein, with 964. Of special interest is the conservation of sequences for amino acids involved in catalysis. Gebler et al. (6) identified Glu-537 as the nucleophilic amino acid and suggested that Glu-461 serves as the general acid/base catalyst which protonates the galactosyl transition state intermediate and deprotonates the attacking water in the *E. coli lacZ* protein. On the basis of this conservation at the catalytic site with other genes in the *lacZ* family, we also designated gene 15 from isolate B7 as a *lacZ*. Although the B7 gene has some similarity to those from organisms with quite diverse physiologies, it is not identical and is thus not the same as any that has been studied. It will be interesting to trace the evolution and transfer of the *lacZ* genes in these different microorganisms.

The enzyme encoded by the isolate gene 15 has a temperature optimum at about 20°C below that of the *E. coli* enzyme and requires either magnesium or manganese for activity. Its K_m for ONPG is slightly higher than that found for the *E. coli* enzyme (0.4 versus 0.1) and is one-sixth the K_m (2.5) for the *K. lactis* enzyme. The isolate B7 β-galactosidase has the highest V_{max} of the three enzymes with ONPG as a substrate (1,182 versus 390 for *E. coli* and 425 for *K. lactis*). With lactose as a substrate, the enzyme from B7 has the same K_m value, 16, as the *K. lactis* enzyme, which is significantly higher than the value of 1.4 reported for *E. coli*. In preliminary tests in milk, the B7 β-galactosidase activity was comparable to that of a commercial preparation of the *K. lactis* enzyme used to hydrolyze lactose in milk (unpublished data). Although further work is needed to determine if there are situations in which the *Arthrobacter* enzyme outperforms the current commercial enzyme, these results demonstrate that it is possible to find new enzymes that might be useful for removing lactose from milk.

The key features dictating enzyme stability and activity in response to temperature are unknown. Comparisons of cold-active enzymes with homologous sequences from mesophilic and thermophilic enzymes should identify properties affecting protein thermostability. Several thermophilic proteins and genes have been studied, but few genes from psychrotrophic organisms have been sequenced. The addition of information on psychrophilic proteins provides new examples which increase the temperature range that can be studied. Furthermore, cold-active enzymes have the advantage of permitting the selection and study of mutations which increase thermostability. Since psychrophilic enzymes are denatured at the normal growth temperatures of *E. coli*, one can directly select for thermostable mutations. For example, *E. coli* transformants containing clone 15 remained white at 37°C on media containing X-Gal. Thermostable mutants could be isolated either by looking for blue colonies or by selecting for colonies that can use lactose as a carbon source at 37°C. The results of these studies may lead to our ability to engineer proteins with desired temperature optima for activity.

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