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Cloning, Expression, and Characterization of a Peculiar Choline-Binding β-Galactosidase from *Streptococcus mitis*▽

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A *Streptococcus mitis* genomic DNA fragment carrying the SMT1224 gene encoding a putative β-galactosidase was identified, cloned, and expressed in *Escherichia coli*. This gene encodes a protein 2,411 amino acids long with a predicted molecular mass of 268 kDa. The deduced protein contains an N-terminal signal peptide and a C-terminal choline-binding domain consisting of five consensus repeats, which facilitates the anchoring of the secreted enzyme to the cell wall. The choline-binding capacity of the protein facilitates its purification using DEAE-cellulose affinity chromatography, although its complete purification was achieved by constructing a His-tagged fusion protein. The recombinant protein was characterized as a monomeric β-galactosidase showing a specific activity of around 2,500 U/mg of protein, with optimum temperature and pH ranges of 30 to 40°C and 6.0 to 6.5, respectively. Enzyme activity is not inhibited by glucose, even at 200 mM, and remains highly stable in solution or immobilized at room temperature in the absence of protein stabilizers. In *S. mitis*, the enzyme was located attached to the cell surface, but a significant activity was also detected in the culture medium. This novel enzyme represents the first β-galactosidase having a modular structure with a choline-binding domain, a peculiar property that can also be useful for some biotechnological applications.

Streptococcus mitis belongs to the viridans group of streptococci and is a relevant microorganism because it is both an opportunistic pathogen and phylogenetically close to *Streptococcus pneumoniae*, a major respiratory human pathogen. Although *S. mitis* isolates usually produce only mild infections, some *S. mitis* strains have acquired increased virulence and are one of the main causes of infectious endocarditis (15, 36). Remarkably, *S. mitis*, like only a few other streptococci, displays phosphorylcholine residues in its cellular envelope (3). This aminoalcohol is used for the anchorage of proteins belonging to the so-called "choline-binding proteins" (CBPs), which fulfill important physiological functions in these bacteria. CBPs bind to phosphorylcholine residues present in the teichoic and lipoteichoic acids located at the surface of *S. pneumoniae* and some streptococci of the mitis group. CBPs share a modular organization consisting of a biologically active domain and a conserved choline-binding domain (CBD), which contains 6 to 18 imperfect 20-amino-acid tandem repeats each located either at the carboxy- or amino-terminal ends of the proteins (26). This CBD is able to specifically bind to choline or its structural analogues like DEAE (diethylaminoethanol), which permits purification by affinity chromatography in a single step using DEAE-cellulose supports (38). Crystallographic studies of CBPs have shown that a typical CBD consists of several β-hairpins organized as a left-handed superhelix and that the linkage of CBPs to the choline-containing cell wall substrate is carried out through the binding of choline residues to the interface of two consecutive choline-binding repeats, named choline-binding sites (9, 13, 14).

β-D-Galactosidases (β-D-galactoside galactohydrolase; EC 3.2.1.23) constitute a large family of proteins that cleave the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety, e.g., lactose and related chromogens, like *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG), or 6-bromo-2-naphthyl-galactopyranoside. β-D-galactosidases belong to the glycosyl hydrolase (GH) superfamily, which contains 114 families (see http://www.CAZY.org) classified on the basis of amino acid sequence similarity (12). The enzymes exhibiting β-galactosidase activity are currently classified within four different families: GH-1, GH-2, GH-35, and GH-42. β-Galactosidases are widely distributed in nature and are present in numerous microorganisms (yeasts, fungi, bacteria, and archaea), plants, and animals (34, 44). These enzymes are of great interest for several industrial or biotechnological processes; the hydrolytic activity has been applied in the food industry for decades to reduce the lactose content of milk products in order to circumvent lactose intolerance, which is prevalent in more than half of the world's population (27). More recently, interest in β-galactosidases has increased due to their ability to synthesize β-galactosyl derivatives that have received a great deal of attention owing to their important roles in many biological processes (27).

In this study, we report the purification and biochemical characterization of a peculiar β-galactosidase encoded by the SMT1224 gene of *S. mitis* that represents a new type of β-galactosidase within this paradigmatic enzyme family.

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s)[a]	Sequence (5′→3′)[b]	Reference or source
Strains			
S. mitis NCTC 12261^T	Type strain		NCTC
S. pneumoniae R6	D39 derivative		2
E. coli			
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150* (Str^r) *relA1 flbB5301 deoC1 ptsF25 rbsR*		41
DH10B	F$^-$ *endA1 recA1 galE15 galK16* Δ*lacX74* ϕ80*lacZ*ΔM15 *araD139* Δ*(ara leu)7697 mcrA* Δ*(mrr-hsdRMS-mcrBC)* λ$^-$		Invitrogen
JM109	*endA1 recA1 gyrA96 thi-1 hsdR17*(r_k^- m_k^-) *relA1 supE44* Δ(*lac-proAB*), [F′ *traD36 proAB laqI^{q}Z*ΔM15]		Promega
Plasmids			
pUC19	Cloning vector; Ap^r		Fermentas
pGEM-T Easy	Cloning vector with T/A tails for PCR amplicons; Ap^r		Promega
pBSF01	pUC19 derivative; Ap^r; contains the gene encoding the entire *S. mitis* β-galactosidase		This study
pBSF02	pUC19 derivative; Ap^r; contains the gene encoding the mature *S. mitis* β-galactosidase		This study
pHGM01	pGEM-T Easy derivative; Ap^r; contains the gene fragment encoding the His-tag		This study
pHGM02	pUC19 derivative; Ap^r; contains the gene encoding the mature *S. mitis* His- β-galactosidase		This study
Primers			
DB5		*CGTCTAGATAAGGAGGATTAGATG*CAAAAAGGTAATTGGAAC	This study
bgsm-PS3		*GCGATATCGAGAAA*TTACATTCCTCAAATCAATCTTATCCG	This study
DB5SP		*CGTCTAGATAAGGAGGATTAGATG*GAAAATGCTGAAGAAATTGTG	This study
HISGAL 3′		*GGACTAGT*TGGCCATTGACGAATACTTG	This study
HISGAL 5′		*CGTCTAGATAAGGAGGATTAGATGCACC ACCACCACCACCAC*GAAAATGCTGAAGAAATTGTGTTAAC	This study

[a] Ap^r, β-lactamase gene.
[b] Underlining indicates the restriction enzyme sequences introduced for cloning purposes. Changed nucleotides are in italics.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. The *S. mitis* NCTC 12261^T type strain was grown in a brain heart infusion broth or in C medium (23) supplemented with yeast extract (0.8 mg/ml; Difco Laboratories) (C+Y medium) and incubated at 30°C or 37°C without shaking. *S. pneumoniae* R6 strain was grown in C+Y medium at 37°C. *Escherichia coli* strains were grown in Luria-Bertani broth containing ampicillin (100 μg/ml) at 37°C with aeration. To produce the *S. mitis* β-galactosidase, the *E. coli* MC4100 strain was chosen as the host because it lacks endogenous β-galactosidase activity. Plasmid pGEM-T Easy (Promega) was used for the construction of pHGM01 and pHGM02 to clone the PCR-amplified DNA fragments.

Recombinant DNA techniques. Recombinant DNA techniques were carried out following conventional protocols (37). DNA amplifications were done in a 2720 Thermal Cycler (Applied Biosystems), and DNA fragments were purified with a PCR purification kit (Roche, Basel, Switzerland). The PCR amplification reaction of the DNA fragment containing the β-galactosidase gene was performed using an Expand Long Template PCR System (Roche, Basel, Switzerland) using 500 ng of *S. mitis* NCTC 12261^T DNA as a template and a 300 nM concentration of oligonucleotide primers DB5 (or DB5SP for the fragment encoding the protein without the signal peptide) and bgsm-PS3. Both XbaI-EcoRV-cut amplified fragments were cloned into pUC19 digested with XbaI and SmaI to give rise to pBSF01 and pBSF02 (Fig. 1). Both plasmids were sequenced to confirm the correct gene composition.

To create a gene encoding a recombinant His-tagged β-galactosidase containing six His residues in the N-terminal region, the plasmid pBSF02 was used as a template for PCR amplification of a fragment of the β-galactosidase-encoding gene with HISGAL 5′ and HISGAL 3′ primers. The amplified 758-bp fragment was ligated to pGEM-T Easy and transformed into *E. coli* DH10B. The resultant recombinant plasmid (pHGM01) was sequenced and digested with XbaI and SpeI. The digested 758-bp DNA fragment was purified and ligated to pBSF02 vector, which had been previously digested with the same restriction enzymes. The final recombinant plasmid, pHGM02, was retransformed into *E. coli* MC4100 (Fig. 1). DNA sequencing was performed by SecuGen using an automatic sequencer (ABI Prism model 377; Applied Biosystems). The BLAST program (1) was used for database searches and sequence comparisons.

Enzymatic assays and biochemical properties. β-Galactosidase assays were performed with ONPG as a substrate, as previously described (32), and measurements were subtracted by the absorbance of blanks containing all reactants except enzyme to correct for the thermal hydrolysis of ONPG. Assays using PNPG were performed under the same conditions. The enzyme activity on lactose or lactulose was also assayed under the same conditions but using 1 mM concentrations of substrates. The amount of liberated glucose (from lactose) or fructose (from lactulose) was measured using amperometric biosensors for glucose (7) and fructose (5), respectively. One unit of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 nmol of substrate per min under the standard reaction conditions. Specific activity is defined in units per mg of protein. The kinetic parameters (K_m and V_{max}) were calculated from Lineweaver-Burk plots using different concentrations of substrates. The substrate concentration ranges used were 0.5 to 15.0 mM for ONPG and 5.0 to 160.0 mM for lactose. Enzymatic assays were conducted in triplicate. The pH dependence of *S. mitis* β-galactosidase activity was determined at 30°C in Z buffer (0.1 M sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 5 mM mercaptoethanol) using ONPG as a substrate over a pH range of 5.0 to 8.0. For ionic strength dependence, enzymatic activity was assayed in different buffers containing various salt concentrations. Specificity studies of the β-galactosidases from *Aspergillus oryzae*, *E. coli*, and *S. mitis* on different substrates were performed in Z buffer at the optimum pH reported for each enzyme (*A. oryzae*, pH 4.5; *E. coli*, pH 7.4)

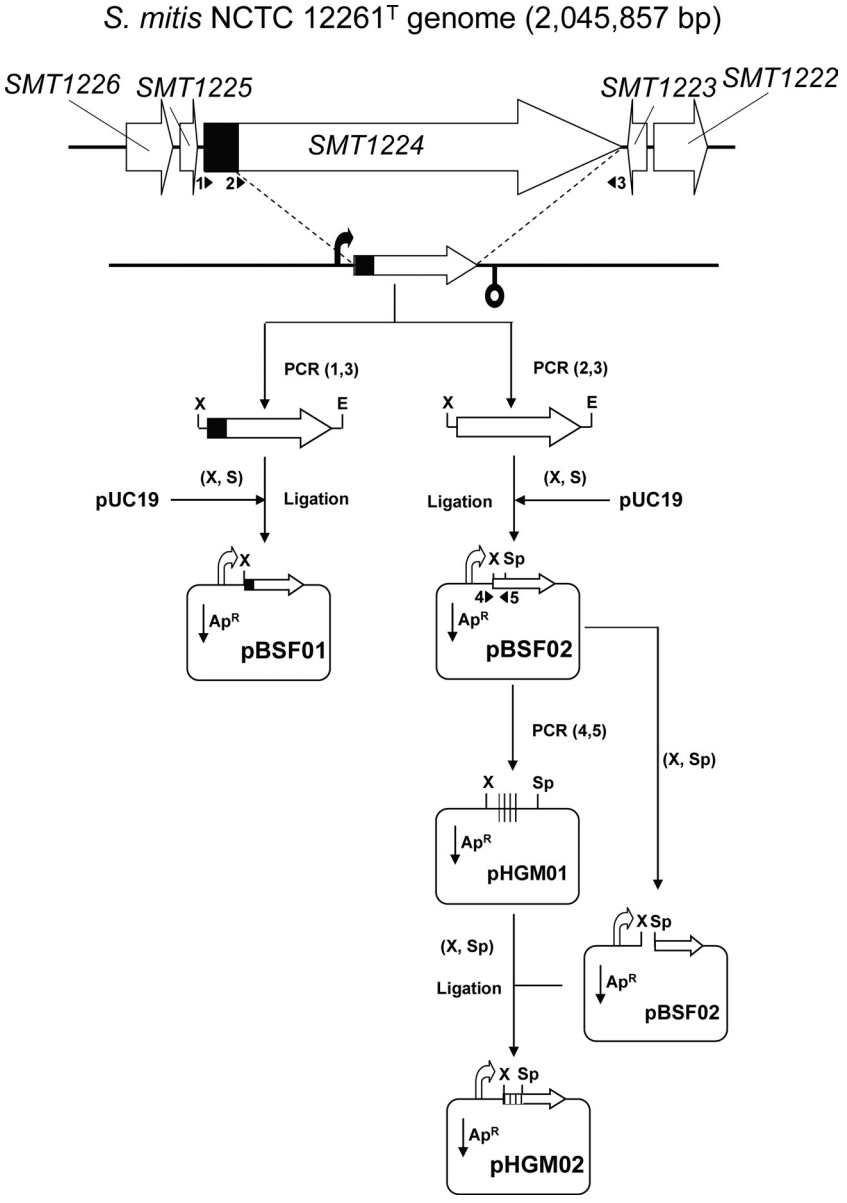

FIG. 1. Genetic organization around the SMT1224 gene encoding the β-galactosidase and construction of pBSF01, pBSF02, and pHGM02. Genes are represented as arrows showing their length and the direction of transcription. Primers are depicted as black triangles: 1, DB5; 2, DB5SP; 3, bgsm-PS3; 4, HISGAL5′; 5, HISGAL3′. Abbreviations and symbols: X, XbaI; E, EcoRV; S, SmaI; Sp, SpeI; Ap^r represents the β-lactamase gene; ⚲, transcription terminator; ■, signal peptide; ||||, N-terminal His tag; ↱, SMT1224 promoter; ↱, pUC19 promoter.

and at pH 6.5 for the mature *S. mitis* enzyme. The optimum temperature for the enzymatic activity was determined by the standard assay method described above at temperatures ranging from 20 to 60°C. To estimate thermal stability, the enzyme was preincubated for 5 min in 20 mM phosphate buffer (pH 6.5) at temperatures ranging from 20 to 60°C. Then, aliquots were withdrawn to measure the residual activity at 30°C under standard conditions.

Enzyme purification. *E. coli* MC4100 (pBSF02) cells were cultured at 37°C for 16 h. Cells were harvested and broken in a French pressure cell, and the crude extract was clarified by centrifugation (20 min at 15,000 × *g* at 4°C). The soluble protein was loaded onto a DEAE-cellulose column, which was extensively washed with 20 mM phosphate buffer, pH 6.9, containing 1.5 M NaCl until no protein was detected in the eluate. β-Galactosidase was eluted with 20 mM phosphate buffer, pH 6.9, containing 1.5 M NaCl plus 2% choline chloride. Alternatively, the protein was also purified using a batch procedure by centrifugation.

To further purify the enzyme, we used a preparative chromatographic column for size exclusion, taking advantage of the high molecular mass of the protein. Active fractions obtained after DEAE-affinity chromatography were pooled, dialyzed against 20 mM phosphate buffer, pH 6.9, and concentrated 10 times in a dialysis bag with polyethylene glycol 6000. The concentrated enzyme solution (1 ml) containing 4.5 mg of protein was applied onto a Sephacryl S-300 column (90 by 3 cm) previously equilibrated with 20 mM sodium phosphate buffer, pH 6.9, and eluted with the same buffer. Fractions showing the highest β-galactosidase activity were pooled. The recombinant His–β-galactosidase was produced in *E. coli* MC4100 (pHGM02) cells under the same conditions specified above. The fusion protein was purified in a single step using Bio-Scale Mini Profinity immobilized metal affinity chromatography (IMAC) cartridges (Bio-Rad) following the recommendations of the suppliers. Elution of the His–β-galactosidase protein occurred at a final imidazole concentration of 200 mM.

Analytical ultracentrifugation. Sedimentation velocity experiments were performed at 40,000 rpm and 20°C in an Optima XL-A analytical ultracentrifuge (Beckman-Coulter) equipped with UV-visible light absorbance optics using a double-sector Epon-charcoal centerpiece, as previously described (43). In these

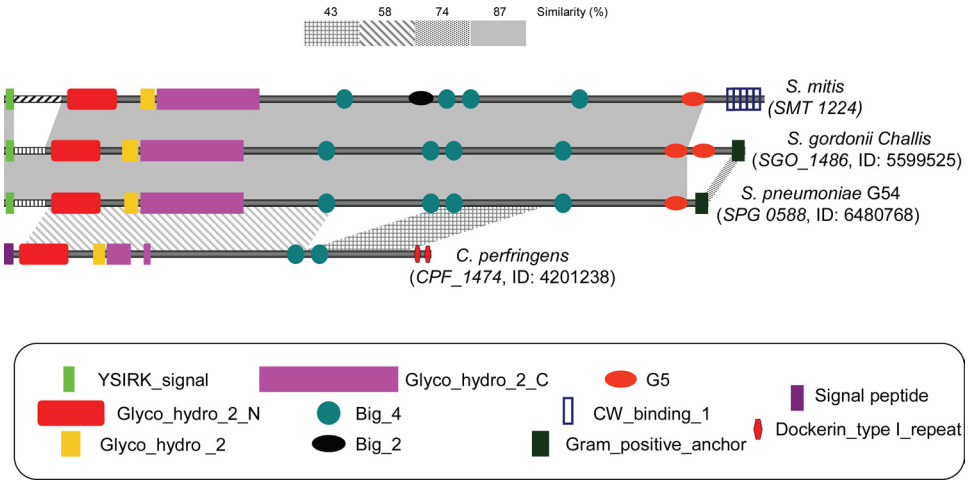

FIG. 2. Schematic representation of different β-galactosidase structures. Gray shadowing corresponds to different levels of amino acid similarity among the proteins. Symbols represent different domains or signatures according to the Pfam protein families database (10). Locus tags and GeneID numbers are given in parentheses.

experiments, protein solutions of 170 μg/ml in 20 mM phosphate buffer, pH 6.9, containing 50 mM NaCl, in the absence or in the presence of 2% choline chloride were used. Differential sedimentation coefficient distributions were calculated by least-squares boundary modeling of sedimentation velocity data using the SEDFIT program (40). The sedimentation coefficients were corrected for buffer composition using the SEDNTERP program (24) to obtain the corresponding standard values (H_2O and 20°C). Frictional coefficient ratios of the protein species were also calculated with the programs SEDFIT and SEDNTERP.

Enzyme localization. *S. mitis* was grown in C+Y medium at 37°C, and 10-ml aliquots were centrifuged. The culture supernatants were filtered, treated with trichloroacetic acid to pellet the proteins, and resuspended in 0.25 ml of Z buffer. The cells were washed once with distilled water, and pellets were resuspended in 0.4 ml of Z buffer, sonicated, and centrifuged at 10,000 × *g* for 10 min. Supernatants corresponded to the cytoplasmic soluble fraction, and pellets corresponded to the insoluble fraction. These latter samples were treated overnight with Z buffer containing 2% choline to release the choline-associated proteins from the envelope cell fraction. The β-galactosidase contained in the cell fractions was determined both by enzymatic assays and by Western blotting.

Miscellaneous methods. Western blot analyses were performed with anti-C-LytA, a polyclonal antiserum that recognizes the CBD, used at a 1:1,000 dilution, or anti-His–β-galactosidase, the fusion protein described in this work, used at a 1:500 dilution, following a previously described protocol (38). N-terminal amino acid sequence analyses were performed after proteins were transferred to a membrane (Immobilon-P; Millipore), according to a published procedure (42). Proteins were localized by brief staining with amido black, and paper strips were excised for sequencing. DEAE-cellulose filters used for immobilization of *S. mitis* β-galactosidase were Whatman chromatography papers (DE 81; Sigma-Aldrich).

RESULTS

Identification of a gene encoding a β-galactosidase in the *S. mitis* genome. Although the complete *S. mitis* NCTC 12261^T genomic sequence has not been published yet, a preliminary sequence is accessible at http://www.oralgen.lanl.gov. Analysis of this genome revealed the presence of at least 14 open reading frames (ORFs) coding for putative CBPs, some of them sharing similarities with those from pneumococcus. One of these *S. mitis* genes, SMT1224, putatively encodes a 2,411-amino-acid protein with noticeable similarity to the β-galactosidase from a pneumococcus of the GH-2 family. Remarkably, the SMT1224 protein presents at its C-terminal region five choline-binding repeats which may constitute a putative CBD. This indicates that the SMT1224 protein should be bound to the cell wall through a choline-binding mechanism. In addition, the *S. mitis* enzyme contains a putative signal peptide, suggesting that it is secreted through a signal peptide-mediated mechanism. All these data strongly suggest that the SMT1224 protein is designed to perform its function at the cellular envelope, in contrast with the typical intracellular β-galactosidases.

Comparison of the *S. mitis* β-galactosidase with other related proteins. Once SMT1224 from *S. mitis* was identified as a plausible gene encoding a β-galactosidase, we analyzed its relationship to other similar genes and their corresponding gene products. As expected, the deduced proteins that present a higher percentage of identity are those belonging to phylogenetically related bacteria like *Streptococcus gordonii* (73%) or strains of *S. pneumoniae* (72%) (Fig. 2). All of these proteins share a similar size, a putative signal peptide, and a modular organization with the same functional motifs, like the sugar binding domain (Pfam Glyco_hydro_2_N), the TIM barrel domain (Glyco_hydro_2_C), and the immunoglobulin-like fold domain (Big_4). Nevertheless, although they also might share the same cell surface localizations, they present important differences concerning the molecular traits responsible of their specific cell wall anchoring systems. Thus, β-galactosidases from *S. pneumoniae* and *S. gordonii* harbor the typical LPXTG motif that directs the covalent linkage of the protein to the muramic acid residues of the cell wall, a process mediated by a sortase (30). In contrast, the β-galactosidase from *S. mitis* has changed this covalent binding system by a noncovalent hydrophobic binding to choline residues of the (lipo)teichoic acids mediated by the five choline-binding repeats located at the C-terminal region of the protein. Interestingly, no other β-galactosidases in the database appear to harbor this type of molecular anchor to the cell wall, representing a striking peculiarity within the whole β-galactosidase family. It is noteworthy that all orthologous β-galactosidases belonging to streptococci and the mitis group share a large molecular size, about 250 kDa, and the similarity decreases with the smaller β-galactosidases, like that of *Clostridium perfringens*, and is negligible with those of gram-negative bacteria, including the well-studied *E. coli* β-galactosidase (114 kDa in size and 27%

identity). Concerning the signal peptide, a strong correlation has been observed between proteins carrying the motif YSIR KXXXGXXS at the N terminus and those carrying the gram-positive anchor domain with the LPXTG sortase processing site at the C terminus of the protein. In this sense, *S. mitis* β-galactosidase is an exception because it has replaced the sortase motif by the choline-binding repeats as an anchor element to the cell wall.

Transcriptional architecture of the β-galactosidase gene. It is also noteworthy that the *S. mitis* β-galactosidase gene appears to be transcribed as a monocistronic element having its own putative promoter, a consensus ribosome binding site, and a transcriptional terminator. A nearly perfect sigma 70 box sequence (TTGACA-16 bp-TAAAAT) located 73 bp upstream of the ATG start codon can be found. In this upstream region, five short repeated sequences (TTTTTA) between −86 and −194 bp from the ATG start codon are also present, suggesting the involvement of other proteins on its transcription regulation. Moreover, a palindromic sequence with a ΔG of −29.7 kcal is located downstream of the TAA stop codon that might act as a transcription terminator. Although the existence of a monocistronic transcription unit for the SMT1224 gene will require experimental support, this arrangement is in sharp contrast to that of the typical *lac* operon found in *E. coli*, where the *lacZ* gene forms an operon together with *lacY* and *lacA* (17). In *S. mitis*, the analysis of the ORFs located at the 5′ and 3′ ends of the β-galactosidase coding gene did not suggest the proximity of genes encoding enzymes, regulators, transporters, or other elements that could be related to its glycolytic function.

Production and purification of the mature *S. mitis* β-galactosidase. Preliminary results had suggested that *E. coli* did not tolerate the use of strong promoters for the overproduction of the *S. mitis* β-galactosidase protein. For this reason, we cloned the entire SMT1224 gene, as well as the gene devoid of the fragment encoding the putative signal peptide, into pUC19 and transformed the corresponding recombinant plasmids in the *E. coli* MC4100 strain (Fig. 1). The enzymatic activity assays performed in different fractions (culture medium, cytoplasmic/periplasmic, and pellet) showed that a significant activity was found only in the cytoplasmic/periplasmic soluble fraction of these clones (data not shown). These results strongly suggested that the signal peptide cannot be efficiently used to translocate the protein to the culture medium in *E. coli*. Thus, further studies were carried out with only the recombinant clone without signal peptide (pBSF02). Its nucleotide sequence showed, compared to the annotated ORF in the corresponding contig, three mutations that provoked two amino acid changes: Ala539Val and Glu1963Gly. The third nucleotide mutation, a transition of A to G at position 1480, was silent.

The β-galactosidase contained in the crude lysate supernatant from an *E. coli* MC4100 (pBSF02) culture was purified following the procedures described in Materials and Methods (Fig. 3, lanes 3 and 7, for example). A Western blot assay using anti-C-LytA revealed the existence of several accompanying proteins in the choline-eluted fraction. Analysis of the N-terminal sequence of the two major protein bands (Fig. 3, lane 3) confirmed that the largest one corresponded to the entire mature β-galactosidase (A-E-E-I-V-L residues), whereas the second major protein was indeed the product of an internal restart translation, beginning at the Met-1170 residue, which yielded the N-terminal sequence T-S-Q-L-G-D-R. Both proteins had a gel mobility that agreed perfectly with their theoretical molecular masses, i.e., 264 kDa and 137 kDa for the full-length and truncated β-galactosidases, respectively. Nevertheless, the appearance of additional faint bands in the choline-eluted fraction strongly suggested the presence of internal restart proteins or degradation products formed during fermentation or during the first steps of the downstream process. Similar protein bands have also been observed in the case of the chimeric β-galactosidases from *E. coli* (38) and from *Thermus* sp. strain T2 (44). Since the full-length β-galactosidase and the rest of accompanying proteins showed a great difference in sizes, we purified the full-length β-galactosidase using size exclusion chromatography in a Sephacryl-S300 column (Fig. 3, lane 4). To completely eliminate the contaminating N-terminally truncated proteins, we constructed an N-terminal His tag fusion protein of the mature β-galactosidase (Fig. 1). When the crude extract from an *E. coli* MC4100 (pHGM02) culture was subjected to IMAC chromatography, the His–β-galactosidase-purified protein could be recovered in a single step with a yield of 50% (Table 2).

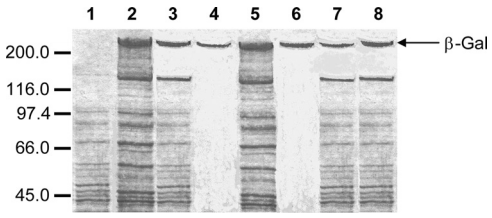

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%) analysis of *S. mitis* β-galactosidase production, immobilization, and purification processes. Lanes: 1, crude extract from *E. coli* MC4100 (pUC19); 2, crude extract from *E. coli* MC4100 (pBSF02); 3, mature β-galactosidase after DEAE-cellulose chromatography eluted with choline buffer; 4, mature β-galactosidase after DEAE-cellulose and Sephacryl S-300 column purification; 5, crude extract from *E. coli* MC4100 (pHGM02); 6, His–β-galactosidase after IMAC chromatography; 7, His–β-galactosidase after DEAE-cellulose in batch eluted with choline buffer; 8, His–β-galactosidase after DEAE-cellulose filter paper eluted with choline buffer. The molecular masses of the standard marker proteins (in kDa) are indicated at the left.

To take advantage of the C-terminal CBD and to explore alternative methods to immobilize the β-galactosidase for biotechnological applications, we tested its binding capacity to DEAE-cellulose paper filters. Total crude extracts from *E. coli* MC4100 (pBSF02) and MC4100 (pHGM02) cultures, producing the mature and His–β-galactosidases, respectively, were loaded into a DEAE-cellulose paper filter. The recombinant β-galactosidases were strongly retained in this support and became very stable since, after an extensive washing with 1.5 M NaCl, the proteins remained attached to the filters (Fig. 3, lane 8) and retained around 60% of the initial enzymatic activity after 1 month at room temperature (data not shown).

Biochemical properties of the recombinant *S. mitis* β-galactosidases. The purified mature β-galactosidase and the His–β-galactosidase presented similar specific activities and biochemical properties, demonstrating that the His tag did not affect activity (Table 2). The β-galactosidase activity was maximal at pH 6.0 to 6.5 in Z phosphate buffer (i.e., the standard buffer used to test β-galactosidases). Nevertheless, it is interesting

TABLE 2. Purification of mature and His-β-galactosidases from *S. mitis*

Enzyme type	Fraction	Enzyme activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Mature form	Crude extract (from 2 liters)	44,000	49	898	1	100
	Active fraction after DEAE-cellulose purification	29,920	20	1,496	1.7	68
	Active fraction after Sephacryl S-300 purification	1,760	0.72	2,444	2.7	4
N-terminally His-tagged fusion	Crude extract (from 2 liters)	45,900	42	1,093	1	100
	Active fraction after IMAC purification	24,350	8.2	2,969	2.7	53

that *S. mitis* β-galactosidase displays similar activities when assayed in 20 mM phosphate buffer, pH 6.0, within a broad range of ionic strength conditions. The enzyme activity on ONPG was essentially identical when the enzyme was assayed in buffers containing 0 to 100 mM KCl. In agreement with the behavior of the β-galactosidase from *S. pneumoniae* (16), no stimulating ions were found, but Cu^{2+}, Zn^{2+}, and Fe^{2+} at 5 mM were inhibitors (residual activities were 46%, 60%, and 68%, respectively). The optimum temperature was found to be in the range of 30 to 40°C, and above 50°C the activity rapidly decreased. Nevertheless, the enzyme was stable at room temperature in 20 mM phosphate buffer, pH 6.9, and its enzymatic activity remained stable for at least 55 days without the addition of protein stabilizers and for at least 2 years at −20°C. In addition, the ONPGase activity of the recombinant enzyme was enhanced by reducing agents, e.g., 32% by 10 mM dithiothreitol. This result is consistent with the presence of a sulfydryl (SH) group in the active site as described for the β-galactosidase from *Thermus* strain 41A (6) and suggests that −SH groups may be involved in the catalytic process or at least that they are important for maintaining the active conformation of the protein (34). When *S. mitis* β-galactosidase was preincubated with EDTA for 60 min, its activity remained essentially intact (87% of the ONPGase control activity), in clear contrast with the behavior of the *S. pneumoniae* β-galactosidase, which was almost completely inhibited by this chelating compound (16). Among the detergents tested, 1% sodium dodecyl sulfate strongly inhibited the ONPGase activity.

The effect of carbohydrates on the ONPGase activity was evaluated by preincubating the purified protein for 60 min at 30°C with the corresponding mono- or disaccharide (10 mM) and then measuring its activity on ONPG. Table 3 shows that the activity was weakly inhibited by galactose and strongly inhibited by lactose, but, surprisingly and in clear contrast with other β-galactosidases, it was not affected by glucose, even at 200 mM. We also compared the rates of hydrolysis among β-galactosidases from *E. coli*, *A. oryzae*, and recombinant *S. mitis* using PNPG, ONPG, lactose, and lactulose as substrates (Table 4). Interestingly, the substrate specificity of *S. mitis* β-galactosidase agrees fairly well with that of *S. pneumoniae* β-galactosidase (46).

Analytical ultracentrifugation studies of the mature purified *S. mitis* β-galactosidase showed a main peak that accounted for 62% of total signal at 230 nm, compatible with a 252-kDa protein monomer with a frictional ratio of 1.86 (data not shown). Interestingly, the addition of 2% choline chloride did not affect this result, in clear contrast to other CBPs which form dimers in the presence of choline (43). It is worth mentioning that other well-studied β-galactosidases behave as oligomers; e.g., the β-galactosidase from *E. coli* is a 464-kDa tetramer (31).

Subcellular localization of the *S. mitis* β-galactosidase. To localize the β-galactosidase in *S. mitis*, we carried out an enzymatic survey of cell fractions of *S. mitis* NCTC 12261^T strain taken at different growth phases (e.g., early exponential, mid-exponential, and stationary). As shown in Fig. 4A, β-galactosidase activity is detected in both the supernatant of the culture and the insoluble fraction in all the phases tested. As expected, the protein embedded in the insoluble fraction can be released after incubation with 2% choline. In contrast, the *S. pneumoniae* β-galactosidase activity was localized mainly in the insoluble fraction, and it was not detected in the supernatant (data not shown). These results were confirmed by Western blot analysis (Fig. 4B). Therefore, since the β-galactosidase of *S. mitis* is noncovalently attached to the cell wall, it can be partly released into the culture medium, at least under the tested conditions.

DISCUSSION

β-Galactosidases are paradigmatic and widespread GH enzymes which have been extensively studied, leading to a deep knowledge of their catalytic mechanisms, three-dimensional structures, and physiological functions (29, 31; see also at http://www.expasy.ch/cgi-bin/lists?glycosid.txt). This is particularly true for *E. coli* β-galactosidase, the best-known member of this family of proteins (31). Purified β-galactosidases have been

TABLE 3. Effect of carbohydrates on mature *S. mitis* β-galactosidase activity

Addition (concn)	Specific activity (U/mg of protein)
Control (no addition)	2,420 ± 120
D-(+)-Glucose (10 mM)	2,470 ± 125
D-(+)-Glucose (200 mM)	3,125 ± 155
D-(+)-Lactose (10 mM)	870 ± 45
D-(+)-Galactose (10 mM)	1,380 ± 70

TABLE 4. Initial rates of hydrolysis of substrates by β-galactosidases

Substrate	Concn (mM)	Hydrolysis rate with β-galactosidase from[a]:		
		E. coli	*A. oryzae*	Mature *S. mitis*
PNPG	5	1 (119,134 U)	1 (21,787 U)	1 (2,651 U)
ONPG	5	2.100	0.240	0.830
Lactose	1	0.070	0.013	0.360
Lactulose	1	0.027	0.028	0.007

[a] Results are normalized to the reaction rate measured with PNPG per mg of protein per min (in parentheses).

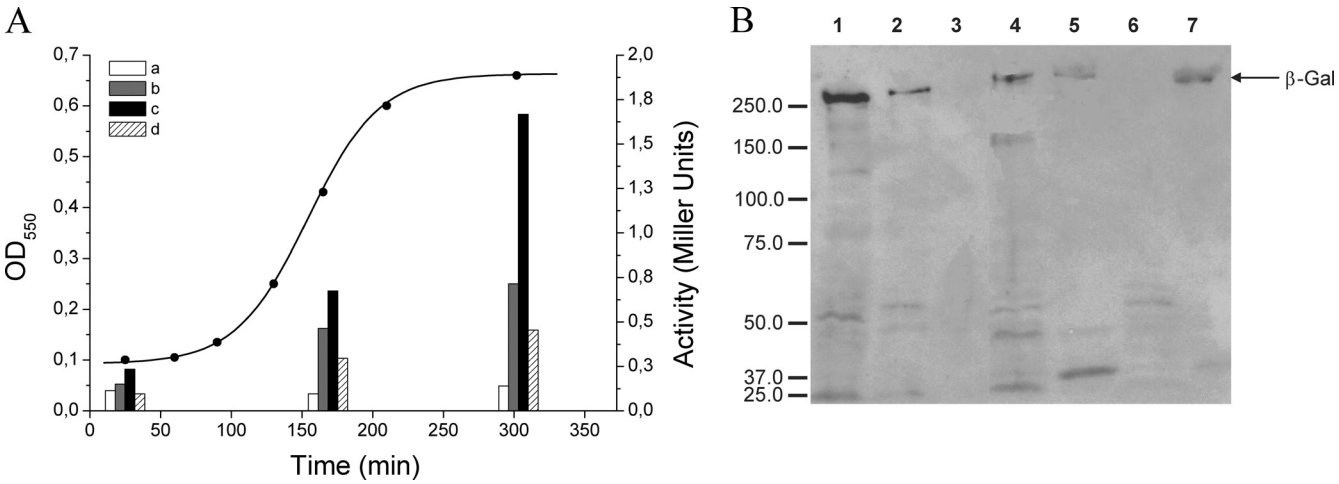

FIG. 4. Localization of β-galactosidases. (A) Growth curve of *S. mitis* NCTC 12261^T strain in C+Y medium at 37°C and β-galactosidase (β-Gal) activity of different fractions, according to the protocol described in Materials and Methods. Samples: a, cytoplasmic soluble fractions; b, insoluble fractions; c, culture supernatants; d, choline-associated protein fractions. (B) Western blot developed with anti-β-galactosidase, corresponding to cultures at an optical density at 550 nm (OD_{550}) of 0.65. Lanes 1 to 3 use samples from the *S. pneumoniae* R6 strain, and lanes 4 to 7 use samples from the *S. mitis* NCTC 12261^T strain. Lanes 1 and 4, insoluble fractions; lanes 2 and 6, cytoplasmic soluble fractions; lanes 3 and 7, culture supernatant fractions; lane 5, choline-associated protein fraction. The molecular masses of the standard marker proteins (in kDa) are indicated at the left.

obtained from different origins and used for a variety of applications. Typical β-galactosidases, like the β-galactosidase of *E. coli*, comprise approximately 1,000 amino acids and are cytoplasmic proteins. In contrast, we have described here a new member of a group of streptococcal β-galactosidases that share a larger size (about 2,200 amino acids long) than typical β-galactosidases, a similar domain organization, and a surface protein localization. To date, *S. mitis* β-galactosidase is the only protein of this family that harbors the consensus choline-binding repeats of CBPs responsible for its noncovalent attachment to the cell wall. The CBD facilitates the development of affinity-like purification and immobilization methods for this enzyme using a variety of supports, including the use of DEAE-cellulose paper filters. In fact, we had anticipated the usefulness of this domain by creating chimeric CBD proteins with the β-galactosidases from *E. coli* and *Thermus* sp. (28, 38, 44), but this is so far the first example of a naturally occurring β-galactosidase having this domain.

The results of this study demonstrate that the β-galactosidase from *S. mitis* can be efficiently synthesized in a biologically active form in *E. coli* and retain its catalytic properties. The recombinant proteins synthesized here possess additional characteristics, facilitating the development of a rapid and inexpensive procedure for their purification on an industrial scale. In addition, an important property that has received little attention in the literature is the level of purity of commercial preparations of β-galactosidases, especially with regard to the presence of other enzymes, such as proteases. These contaminants could have a severe impact on the stability of the enzyme, leading to undesirable changes in dairy products during storage. The high affinity of the CBD for choline or its structural analogs, such as DEAE (39), allowed the initial purification of *S. mitis* β-galactosidase in a single step by affinity chromatography on DEAE-cellulose (38). On the other hand, the negligible product inhibition by higher concentrations of glucose, the weak inhibition by galactose in comparison with other β-galactosidases (25, 33, 35), and this enzyme's high substrate specificity toward lactose are likely to make the *S. mitis* β-galactosidase a good candidate for biotechnological use in the treatment of milk and related products requiring lactose hydrolysis (34). Additionally, as β-galactosidases catalyze both hydrolysis and transgalactosylation reactions, the novel β-galactosidase described in this work might also be applied in synthesizing galactose-containing chemicals, e.g., diverse oligosaccharides, glycoconjugates, alkyl-glycosides, and other biologically active compounds that play important roles in the industry of food additives, cosmetics, and medicines (4).

With respect to physiological functions, most β-galactosidases play a major role in lactose metabolism and can be induced by lactose. Nevertheless, the first reported pneumococcal β-galactosidase (BgaA) is not involved in lactose metabolism but, rather, with a virulence factor in the interaction with host cells (21, 45). Very recently, a second β-galactosidase protein has been described, BgaC, which shows noticeable similarity to eukaryotic β-galactosidases as well as to pathogenic microbial enzymes (19). The BgaA has been previously shown to cleave human glycoproteins and to be important to adherence by this pathogen (22). In this sense, the size similarity, the common functional motifs, and the surface localizations of the *S. mitis* and *S. pneumoniae* β-galactosidases might point to an analogous physiological function for *S. mitis* β-galactosidase. Nevertheless, an important difference between these β-galactosidases is that the pneumococcal enzyme is covalently bound to the cell envelope, whereas the *S. mitis* β-galactosidase is noncovalently linked to the cell wall. In this sense, it is worth mentioning that the release of *S. mitis* β-galactosidase to the culture medium is similar to that described for the pneumococcal LytC lysozyme, another CBP that acts as an autolysin and is involved in the "fratricide" process (8). Moreover, *S. pneumoniae* and *S. mitis* colonize the upper hu-

man respiratory tract, where they are able to carry out an intense interchange of genes, which may explain the high degree of relatedness. Previously, it has been demonstrated that recombinational events occurred in vitro more frequently from *S. mitis* or *Streptococcus oralis* to *S. pneumoniae* (18). This transfer of genetic material has been well documented for several genes, for example, those encoding penicillin-binding proteins, where horizontal transfer of internal fragments of these genes leads to the formation of mosaic proteins, and, thus, to penicillin resistance (11). More recently, it has been reported that bacteria that are competent for natural transformation, like pneumococci and their commensal relatives *S. mitis* and *S. oralis*, possess a mechanism for active acquisition of homologous DNA that dramatically increases the efficiency of gene exchange between and within such streptococcal species (20).

Little is known about how *S. pneumoniae* and other related streptococci acquire energy during the colonization of the human upper airway, where the concentration of free sugar is generally low; however, the glycosidase-dependent release of monosaccharides from glycoconjugates in close proximity to the bacterial surface may provide these streptococci with a source of carbon in vivo (21). Therefore, while exoglycosidase activity may contribute to pneumococcal pathogenesis, it may also provide pneumococci with a source of carbon in vivo. Thus, exoglycosidase activity against glycoconjugates may contribute directly to the ability of these bacteria to acquire energy to establish colonization and therefore persist in the airway.

It is worth mentioning that the β-orthologous galactosidase enzyme from *S. pneumoniae* has been shown to be almost specific for β-1,4 linkages, and it is known to be useful for the structural studies of glycoprotein-linked oligosaccharides. This property together with the already mentioned high specific activity on lactose, the absence of glucose inhibition, and the easy purification opens new scenarios for different biotechnological applications of the novel β-galactosidase enzyme described in this work. In this context, we have under development for application in the dairy industry a promising integrated amperometric lactose biosensor which contains the recombinant *S. mitis* β-galactosidase, glucose oxidase, peroxidase, and tetrathiafulvalene coimmobilized by cross-linking with glutaraldehyde in a gold disk electrode modified with a 3-mercaptopropionic acid self-assembled monolayer.

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