

A GH170 phosphoglycosidase links phosphotransferase system transport to N,N'-diacetylchitobiose catabolism in *Lacticaseibacillus paracasei*

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Research Article

Keywords: *Lacticaseibacillus paracasei*, GH170, phosphotransferase system, chitooligosaccharide metabolism, phosphoglycosidase

Posted Date: October 16th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11142743/v1>

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Additional Declarations: No competing interests reported.

Abstract

Lactocaseibacillus paracasei utilizes *N,N'*-diacetylchitobiose (GlcNAc- β 1,4-GlcNAc; ChbNAc) through the action of a phosphotransferase system (PTS) encoded by the *chbABC* genes and the glycosyl hydrolase ChbE, a member of the recently established GH170 family, whose substrate specificity remains largely unexplored. Although genetic evidence suggested a role for ChbE in ChbNAc metabolism, its biochemical function remained unknown. Here, purified ChbE showed no activity towards non-phosphorylated ChbNAc or *N,N',N''*-triacetylchitotriose (TriChbNAc), suggesting that substrate phosphorylation is required for catalysis. To identify the physiological substrate of ChbE, a $\Delta chbR/E$ double mutant constitutively expressing the *chb* operon was constructed. When grown in the presence of ChbNAc, this strain accumulated an intracellular sugar that disappeared after incubation with purified ChbE, concomitantly with the appearance of GlcNAc and GlcNAc-6-phosphate, suggesting that the accumulated metabolite was phosphorylated ChbNAc. This assignment was confirmed by MS/MS analyses. Similarly, cells grown with TriChbNAc accumulated phosphorylated TriChbNAc that was hydrolyzed by purified ChbE to generate GlcNAc-6-phosphate and ChbNAc. These results show that ChbE likely functions as a phospho-glycosyl hydrolase acting on phosphorylated chitooligosaccharides produced during PTS-mediated transport. Genome context analyses further revealed that GH170 homologues are predominantly associated with PTS genes, supporting the existence of specialized PTS-GH170 modules for carbohydrate utilization. Our findings identify ChbE as the first GH170 enzyme involved in chitooligosaccharide metabolism and the second biochemically characterized member of this family, expanding the known functional diversity of GH170 enzymes and providing new insights into amino-sugar utilization by lactobacilli.

Key points

- ChbE hydrolyzes phosphorylated chitooligosaccharides generated by a PTS.
- ChbNAc-P and TriChbNAc-P are substrates of the GH170 enzyme ChbE.
- GH170 enzymes show a strong genomic association with PTS systems.

Introduction

Lactocaseibacillus paracasei is a probiotic species commonly found in the gastrointestinal tract (Bengoa et al. 2021; Mejia-Caballero and Marco 2026). Its adaptation to this environment is supported by a repertoire of genes involved in the utilization of host- and diet-derived glycans, including human milk oligosaccharides and glycans released from mucosal glycosylated proteins (Becerra et al. 2020; Bidart et al. 2014; Bidart et al. 2018). *L. paracasei* BL23 has been reported to metabolize the disaccharide-amino acid fucosyl- α -1,6-*N*-acetylglucosamine-Asn (fucosyl- α -1,6-GlcNAc-Asn), that forms part of the core fucosylated *N*-glycans in mammals (Becerra et al. 2020). This strain is also able to metabolize *N,N'*-diacetylchitobiose (GlcNAc- β -1,4-GlcNAc; ChbNAc), the conserved core disaccharide of *N*-glycans and a major product of chitin hydrolysis, one of the most abundant carbohydrate resources in nature (Meibom

et al. 2004). BL23 also utilizes TriChbNAc (*N,N',N''*-triacetylchitobiose; GlcNAc- β -1,4-GlcNAc- β -1,4-GlcNAc) but not chitooligosaccharides of higher degree of polymerization and lacks detectable extracellular chitinase activity (Garcia-Telles et al. 2026). The genes responsible for ChbNAc and TriChbNAc utilization are located within the *chb* operon (Garcia-Telles et al. 2026), which encodes a dedicated phosphotransferase system (PTS) for carbohydrate uptake and a gene, *chbE*, predicted to encode a glycosyl hydrolase belonging to the recently described GH170 family within the CAZy classification system (www.cazy.org/GH170.html; (Drula et al. 2022)). *L. paracasei* is unlikely to function as degrader for complex glycoproteins or chitin. Instead, it probably depends on the activity of other members of the intestinal microbiota that release low-molecular-weight GlcNAc-containing oligosaccharides. In this regard, the *chb* pathway of *L. paracasei* may represent a scavenging system for exploiting carbohydrates made available by neighboring microorganisms, a common trait found in the intestinal niche (Culp and Goodman 2023; Flint et al. 2012; Tailford et al. 2015).

To date, the MupG protein from *Staphylococcus aureus* is the only GH170 enzyme whose biochemical activity has been experimentally characterized (Kluj et al. 2018). MupG participates in peptidoglycan recycling by acting on *N*-acetylmuramic acid- β -1,4-GlcNAc (MurNAc-GlcNAc) after uptake and phosphorylation by a specific PTS, generating MurNAc-6-phosphate-GlcNAc, for which it represents a novel 6-phospho-*N*-acetylmuramidase. In the present work, through purification of ChbE and the use of an *L. paracasei* strain that accumulates the internalized ChbNAc or TriChbNAc, we demonstrate that the GH170 ChbE is a 6-phospho-*N*-acetylglucosaminidase. These findings expand the functional characterization of the GH170 family and support the concept of coordinated PTS-GH170 systems involved in the utilization of amino sugar-containing carbohydrates.

Materials and Methods

Bacterial strains, culture conditions and plasmids

L. paracasei strains were cultured at 37 °C under static conditions in MRS (Difco) medium for routine assays. *E. coli* DH10b was used as a host in cloning experiments and *E. coli* BL21(DE3) was used for protein expression. Both strains were cultured in Luria-Bertani (Condalab) medium at 37 °C under constant agitation at 230 rpm. The corresponding solid media were prepared by adding 1.8% agar. *E. coli* transformants were selected with ampicillin (100 $\mu\text{g ml}^{-1}$) or erythromycin (100 $\mu\text{g ml}^{-1}$). *L. paracasei* transformants were selected with erythromycin (5 $\mu\text{g ml}^{-1}$).

A pRV300-derived vector (Leloup et al. 1997) was used for gene insertion and inactivation in *L. paracasei*. The pQE80 vector (Qiagen) was used for protein overproduction in *E. coli*. The *E. coli* and *L. paracasei* strains were transformed by electroporation using a Gene Pulser apparatus (Bio-Rad Laboratories), according to the manufacturer's recommendations for *E. coli* and the methods described earlier for *L. paracasei* (Posno et al. 1991).

Construction of an *L. paracasei* *chbR chbE* strain

The plasmid pRVchbE (Garcia-Telles et al. 2026), carrying a disrupted *L. paracasei* BL23 *chbE* gene, was introduced into cells of a previously constructed *L. paracasei* $\Delta chbR$ mutant (Garcia-Telles et al. 2026) by electroporation. Chromosomal integration and subsequent excision of the plasmid were achieved through a double-crossover recombination strategy as previously described (Bidart et al. 2014). Antibiotic-sensitive colonies resulting from a second recombination event were screened by PCR. A double mutant $\Delta chbR/E$ was subsequently confirmed by amplification of the targeted loci followed by DNA sequencing and was selected for further analyses.

Expression and purification of His-tagged ChbE

The coding region of *chbE* were amplified by PCR using chromosomal DNA of *L. paracasei* BL23 as a template and the primer pair 5'-TTTTGGATCCATGAAACGACTAGGAATATCGCTC/5'-TTTTAAGCTTACTAGCATTCGTTTTTTCAAGAACAAATG. The obtained PCR fragment was treated with BamHI/HindIII (introduced restriction sites in the oligonucleotides are underlined) and cloned into pQE80 digested with the same enzymes using *E. coli* DH10b. The resulting plasmid, pQEchbE, was confirmed by DNA sequencing and used to transform *E. coli* BL21(DE3). One selected clone was cultured in 250 mL of Luria-Bertani medium at 37°C with agitation. When the culture reached an OD₅₉₅ of 0.5, isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to 0.2 mM for induction of protein production and incubation was continued at 18 °C for 18 h. Cells were harvested by centrifugation and resuspended in lysis buffer (phosphate buffered saline (PBS); lysozyme, 1 mg ml⁻¹; dithiothreitol, 1 mM; phenylmethylsulphonyl fluoride, 1 mM) and incubated for 30 min at room temperature. Cell extracts were prepared, loaded onto a Ni Sepharose 6 Fast Flow column (GE Healthcare) and the proteins purified as previously described (Bidart et al. 2014). The fractions containing 6x(His)ChbE were analyzed using SDS-PAGE gels, then combined and dialyzed against PBS containing 20% glycerol. The purified proteins were kept frozen at -80°C. Protein concentrations were determined with the Protein Assay Dye Reagent Concentrate (BioRad).

Growth of *L. paracasei* with ChbNAc and TriChbNAc and analysis of extracts

L. paracasei strains were cultured overnight at 37°C under static conditions in sugar-free MRS basal medium. This medium contained bactopectone (Gibco), 10 g L⁻¹; yeast extract (Life Technologies), 4 g L⁻¹; sodium acetate, 5 g L⁻¹; triammonium citrate, 2 g L⁻¹; magnesium sulphate 7-hydrate, 0.2 g L⁻¹; manganese sulphate monohydrate, 0.05 g L⁻¹; and Tween 80, 1 ml L⁻¹. Overnight cultures were diluted to an OD₅₅₀ of 0.1 in 200 μ l of MRS basal medium supplemented with 30 mM ribose. This pentose supported sustained bacterial growth of both the wild type and the $\Delta chbR/E$ strains without eliciting strong carbon catabolite repression (Viana et al. 2000), thus minimizing potential repression of *chb* PTS genes expression. Bacterial growth was monitored for 25 h by spectrophotometric measurements every 30 min at 550 nm on 96-well plates at 37°C without shaking in a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany). At least three independent biological replicates were obtained for each growth curve.

After 11 h of growth, when cultures had reached the exponential growth phase, *N,N'*-diacetylchitobiose (ChbNAc) or *N,N',N''*-triacetylchitotriose (TriChbNAc) (obtained from Biosynth Ltd., Compton, Berkshire, UK) were added to a final concentration of 4 mM. Cultures were subsequently incubated until the stationary phase. Cells were harvested by centrifugation, washed once with PBS 1X, and resuspended in 200 μ l of PBS 1X with mutanolysin (10 U μ l⁻¹) and lysozyme (20 mg ml⁻¹). Samples were incubated for 1 h at 37°C. Cells were then disrupted using a FastPrep-24TM5^G with two cycles of 60 s. Cell debris was removed by centrifugation and the resulting supernatant was filtered. Each intracellular extract was subsequently divided into two aliquots. One aliquot was incubated with purified ChbE at a concentration of 10 μ g ml⁻¹, whereas the second aliquot with no ChbE served as a control. Both samples were incubated for 3 h at 37°C. Following incubation, samples were diluted 1:2 and analyzed by high-performance anion-exchange chromatography (HPAEC) as described below.

HPAEC analysis of intracellular chitooligosaccharides

Twofold diluted samples from *L. paracasei* (wild type and the Δ *chbR/E* mutant) intracellular extracts non-treated and treated with ChbE were analyzed by HPAEC with pulsed amperometric detection (HPAEC-PAD) with a Dionex ICS-6000 system (Thermo Fisher Scientific) fitted with a CarboPacTM PA100 analytical column and a CarboPacTM PA100 guard column (Dionex Corp., Sunnyvale, CA, USA), at a column temperature of 27°C. Separation was achieved using a gradient of 1 M NaOH (eluent B) and 1 M sodium acetate (eluent C) in water (eluent A) at a flow rate of 1 mL min⁻¹: 10% B (0–2 min), 30% B (2–5 min), and a linear increase to 30% C (5–20 min). Carbohydrates were identified by comparison of their retention times with those of ChbNAc 0.2 mM, TriChbNAc 0.2 mM, GlcNAc 0.2 mM and GlcNAc-6-phosphate 2 mM standards (Biosynth Ltd).

UPLC-HRMS analysis of intracellular metabolites

UPLC-HRMS analysis was performed using a Vanquish UPLC system coupled to an Orbitrap Exploris 120 mass spectrometer. Chromatographic separation employed an Acquity UPLC BEH Amide column (3.0 x 100 mm, 1.7 μ m). The mobile phases consisted of water (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid and 10 mM ammonium formate. The gradient initiated at 90% B for 2 min, decreased to 80% at 8 min, 70% at 12 min, and 50% at 14 min. A washing step at 20% B was applied from 17 to 20 min, followed by re-equilibration at 90% B from 20.5 to 25 min. The column was maintained at 35°C, with a flow rate of 0.35 ml min⁻¹ and an injection volume of 3 μ l.

Mass spectrometric detection was conducted in both positive and negative ionization modes, using separate acquisition methods for each polarity. In both methods, the sheath and auxiliary gas flows were set to 30 and 10 (arbitrary units), respectively, and the ion transfer tube and vaporizer temperatures were maintained at 300°C. Full MS scans were acquired at a resolution of 120,000 over an *m/z* range of 150–1000, with automatic maximum injection time. A data-dependent acquisition strategy was applied, excluding precursor ions below an intensity threshold of 1×10^4 and enabling dynamic exclusion to

enhance coverage. MS/MS spectra were collected at a resolution of 30,000 using stepped collision energies of 20, 30, and 40%. Data processing was performed using FreeStyle v1.8.91.0 (Thermo Fisher Scientific). Features were first assigned based on theoretical precursor masses (MS^1) within a 5 ppm tolerance, and structural confirmation was achieved through inspection of corresponding MS^2 spectra.

Genome context analysis

The Enzyme Function Initiative Enzyme Similarity Tool (EFI-EST) and Genome Neighborhood Tool (EFI-GNT) available through the EFI server (efi.igb.illinois.edu; (Oberg et al. 2023)) were used to identify homologs of *L. paracasei* ChbE and analyze their genomic context. The ChbE protein sequence (WP_012492176) was used as the query to retrieve homologous proteins and generate a sequence similarity network (SSN) comprising 996 nonredundant sequences (BLAST E-value thresholds ranging from 2×10^{-131} to 4×10^{-23}). Genome neighborhood analysis was performed with EFI-GNT based on the generated SSN. The presence of PTS-related domains was assessed within a genomic window spanning five genes upstream and five genes downstream of each *chbE* homolog. Functional assignments were inferred from the protein family and superfamily annotations provided by EFI-GNT.

Results

The glycosyl hydrolase ChbE from *L. paracasei* does not hydrolyze ChbNAc

In *L. paracasei* BL23, the disaccharide ChbNAc (*N,N'*-diacetylchitobiose) and the trisaccharide TriChbNAc (*N,N',N''*-triacetylchitobiose) require a functional *chbE* gene for their metabolism (Garcia-Telles et al. 2026). We purified ChbE after overexpression in *E. coli* (Fig. 1). The recombinant protein was unable to release *N*-acetylglucosamine when it was incubated with ChbNAc or TriChbNAc (data not shown). The lack of activity of ChbE with these substrates suggested that enzymatic hydrolysis of the GlcNAc- β 1,4-GlcNAc glycosidic bond probably required a phosphorylated substrate.

ChbE purification. Coomassie brilliant blue-stained 10% SDS-PAGE showing the purified His-tagged protein ChbE. Lane P, protein standards. The numbers on the left are molecular masses.

Construction and analysis of a Δ *chbR/E* mutant in *L. paracasei*

We postulated that ChbE was probably acting only on phosphorylated substrates. As phosphorylated derivatives of ChbNAc or TriChbNAc are not commercially available, we designed a mutant *L. paracasei* strain that would accumulate the internalized ChbNAc. A strain lacking the transcriptional repressor ChbR constitutively expresses the *chb* genes in the absence of ChbNAc in the growth medium (Garcia-Telles et al. 2026). We used a previously constructed mutant devoid of *chbR* to disrupt *chbE*. We hypothesized that, in the resulting Δ *chbR/E* double mutant, ChbNAc uptake would be enhanced due to the absence of the transcriptional regulator ChbR, whereas its catabolism would be abolished, since ChbE is essential for ChbNAc utilization. When ChbNAc was added to exponentially-growing cells of the

wild-type strain, an increased growth was observed, probably resulting from ChbNAc metabolism. Such growth enhancement was not evident in the $\Delta chbR/E$ mutant (Fig. 2), supporting the fact that the disaccharide was not catabolized by this strain.

Growth curves of *L. paracasei* BL23 wild type and $\Delta chbR/E$ mutant strains following supplementation with chitooligosaccharides. Wild type strain BL23 and $\Delta chbR/E$ mutant strain grown in MRS basal medium without added sugar (blue) or ribose only at 30 mM (orange). At 11 h of growth, cultures with ribose were supplemented with either 4 mM *N,N'*-diacetylchitobiose (ChbNAc) (green) or *N,N',N''*-triacetylchitotriose (TriChbNAc) (purple). The black arrows indicate the time of oligosaccharide addition. Growth with ribose excluded potential carbon catabolite repression effects that would eventually decrease *chb* genes expression even in the absence of ChbR. Data presented are mean values based on at least three replicates. Error bars indicate standard deviations.

Sugar analysis in cells grown in the presence of ChbNAc

The cellular extracts of the wild type and $\Delta chbR/E$ mutant strains from the experiments in Fig. 2 were analyzed for sugar contents by chromatography at the end of the growth phase (24h). A clear distinguishable peak was found in the double mutant that was absent in the wild type (Fig. 3a). When these extracts were treated with purified ChbE, the disappearance of this additional peak coincided with the appearance of peaks corresponding to GlcNAc and GlcNAc-6-phosphate, respectively (Fig. 3a). This suggested that the additional peak present in the $\Delta chbR/E$ strain was ChbNAc-P, that was produced through transport by the ChbNAc PTS (PTS^{chb}) and hydrolyzed by ChbE resulting in phosphorylated and non-phosphorylated GlcNAc. Of note, extracts from the mutant strain showed increased accumulation of ChbNAc compared with the wild-type strain.

Intracellular sugar analysis. High-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) of (a) cell extracts of cells grown with ribose plus *N,N'*-diacetylchitobiose (ChbNAc) or (b) ribose plus *N,N',N''*-triacetylchitotriose (TriChbNAc). Chromatograms were obtained using a Dionex ICS-6000 high-performance anion-exchange chromatography system equipped with a CarboPac PA100 column. Standards included GlcNAc-6-phosphate (GlcNAc-6P), GlcNAc, ChbNAc and TriChbNAc. Cytoplasmic extracts from wild type and $\Delta chbR/E$ mutant strains treated with or without ChbE were analyzed. Black arrows indicate peaks appearing after ChbE treatment, corresponding to hydrolysis products that were identified by comparison with the retention times of the standards. The red arrows indicate unidentified peaks appearing only in the $\Delta chbR/E$ strain that disappeared after ChbE treatment. WT, wild-type *L. paracasei* BL23; nC, nanoCoulomb.

In order to characterize the compound disappearing from the extracts after ChbE treatment, UPLC-MS/MS analysis was performed. A compound with the expected mass of ChbNAc-P (*m/z* 503.12) was detected in the extracts of the $\Delta chbR/E$ strain that was absent from the wild type (data not shown) and it was completely depleted following incubation with purified ChbE (Fig. 4a), MS/MS fragmentation of this

compound yielded phosphate-derived ions (m/z 96.96) and fragments consistent with GlcNAc-P-containing species (m/z 300.04), supporting its assignment as ChbNAc-P (Fig. 4b).

UPLC-MS/MS analysis of extracts. Extracted ion chromatograms (EIC) for the expected m/z corresponding to (a) ChbNAc-P and (c) TriChbNAc-P in extracts of $\Delta chbR/E$ strain grown in the presence of ChbNAc or TriChbNAc, respectively. Samples untreated or treated with ChbE are represented, showing that treatment with the enzyme resulted in the disappearance of the signals with m/z 503.1259 (ChbNAc-P) and m/z 706.20 (TriChbNAc-P), respectively. MS/MS fragmentation analyses of the putative ChbNAc-P (b) and TriChbNAc-P (d) ions. The MS/MS spectra show the sequential loss of GlcNAc masses (203.079 Da) and the presence of phosphate ions (m/z 96.96), supporting compound identification. The blue squares represent GlcNAc molecules.

ChbE also acts on the product of TriChbNAc uptake

Similar growth experiments were performed with TriChbNAc and wild-type and $\Delta chbR/E$ strains (Fig. 2). Comparable to the wild type strain supplemented with ChbNAc, addition of TriChbNAc to growing cells of the wild type resulted in a slight increase in growth. This increase was not evidenced in the $\Delta chbR/E$ mutant. The sugar analysis of cell extracts revealed peaks corresponding to GlcNAc-6-phosphate and ChbNAc after ChbE treatment of $\Delta chbR/E$ extracts, whereas these peaks were absent in the wild-type (Fig. 3b). The appearance of these two peaks coincided with the disappearance of an unidentified peak absent from wild-type extracts, which likely corresponded to TriChbNAc-P. In addition, no peak corresponding to GlcNAc was detected after the enzymatic treatment, supporting again the fact that ChbNAc is not hydrolyzed by ChbE. In the same way to the results obtained with ChbNAc, a marked accumulation of TriChbNAc relative to the wild-type strain was observed in the $\Delta chbR/E$ double mutant (Fig. 3b). UPLC-MS analysis identified a metabolite displaying the expected m/z of TriChbNAc-P, which accumulated in the mutant strain and disappeared after ChbE treatment (Fig. 4c). MS/MS analysis of this metabolite revealed sequential losses of approximately 203 Da, corresponding to GlcNAc residues, together with phosphate signals (Fig. 4d), supporting its assignment as TriChbNAc-P.

ChbNAc and TriChbNAc catabolism in *L. paracasei*

The experimental observations obtained in this study support a model in which ChbNAc and TriChbNAc are transported and phosphorylated by the PTS^{chb} encoded by the *chbABC* genes, and the resulting intracellular phosphorylated oligosaccharides are hydrolyzed by ChbE, which acts specifically on phosphorylated substrates. Hydrolysis of ChbNAc-P yields GlcNAc and GlcNAc-6-phosphate, which can subsequently enter the known GlcNAc catabolic pathway of *L. paracasei* (Garcia-Telles et al. 2026), whereas hydrolysis of TriChbNAc-P generates ChbNAc and GlcNAc-6-phosphate. The formation of ChbNAc as an intracellular product of TriChbNAc-P hydrolysis indicates that additional reaction(s) must occur to complete its utilization. A schematic representation of the proposed pathway is shown in Fig. 5.

Schematic representation of N,N'-diacetylchitobiose (ChbNAc) and N,N',N''-triacetylchitotriose (TriChbNAc) metabolism in *L. paracasei* BL23. ChbNAc and TriChbNAc are transported and phosphorylated by the PTS^{chb}. Phosphorylated di- and trisaccharides are hydrolyzed by ChbE, which generates GlcNAc-6-phosphate plus GlcNAc or GlcNAc-6-phosphate plus ChbNAc, respectively. The monosaccharides are channeled through glycolysis, while intracellular ChbNAc can follow different alternative pathways (labelled with question marks): phosphorylation by a sugar kinase, intracellular phosphorylation by the PTS^{chb} or leakage from the cells and translocation and phosphorylation by the PTS^{chb}. In these three cases, ChbNAc-P would be obtained that could then be hydrolyzed by ChbE. ChbC, ChbB and ChbA are enzyme II domains of the cellobiose-type phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS); HPr, Histidin-containing protein of the PTS; EI, enzyme I of the PTS; PEP, phosphoenolpyruvate; GlcNAc, *N*-acetylglucosamine; ChbE, phospho- β -*N*-acetylglucosaminidase.

The GH170 family and the PTS

More than 9000 entries are currently assigned to the GH170 family in the CAZy database (www.cazy.org/GH170.html), although MupG from *S. aureus* remains the only biochemically characterized member to date. GH170 proteins are predicted to adopt a $(\beta/\alpha)_8$ barrel fold. Although this structure had previously been elucidated by crystallography in *Bacillus cereus* (PDB 1X7F) and *Enterococcus faecalis* (PDB 2P00) homologs, they remained functionally uncharacterized and were classified as members of the domain of unknown function family DUF871 until the characterization of MupG. Sequences encoding GH170 proteins are predominantly distributed within the phylum Bacillota (formerly Firmicutes) and are associated with members of the class Bacilli, particularly the orders Caryophanales and Lactobacillales, although this distribution may reflect biases in the availability of sequenced genomes. In *S. aureus*, *mupG* is associated with a gene encoding a PTS transporter of the glucose-glucoside family [TCDB 4.A.1.2, Transporter Classification Database (<https://www.tcdb.org/>)], namely MurP (Borisova et al. 2016; Kluj et al. 2018). MurP contains the IIB and IIC domains required for substrate transport and phosphorylation, but requires an additional, as yet unidentified, IIA component for full activity in the uptake and phosphorylation of MurNAc-GlcNAc (Kluj et al. 2018). By contrast, the *L. paracasei chbABC* genes, encoding the IIA, IIB, and IIC components of the PTS involved in the uptake and phosphorylation of ChbNAc and TriChbNAc (PTS^{chb}) and located adjacent to *chbE*, belong to the cellobiose family (TCDB 4.A.3.2). Interestingly, inspection of the genomic context of other GH170-encoding genes revealed a strong association with genes encoding PTS components. Analysis of the genomic neighborhoods of 996 *chbE* homologues showed that 915 (92%) were associated with one or more PTS-related genes (e.g., IPR003352; IIC). Overall, 73% of the loci encoding PTS components carried glucose-glucoside family transporters containing an IIC domain, most commonly displaying a typical IIBC architecture. A subset of these loci also encoded an additional IIA domain, either as part of an IIBCA fusion protein or as a separate IIA component. Another 22% of the loci contained lactose-cellobiose family PTS transporters. Among these, 14% encoded the three PTS domains [IIA (IPR003188), IIB (IPR013012), and IIC (IPR004501)] as separate proteins, as occurred in PTS^{chb}. The remaining 5% of the loci were associated with PTS transporters from other functional classes, including mannose-, sorbose-,

and glucitol-type systems. Furthermore, many genomic regions also contained additional carbohydrate-active enzymes, including putative phospho- β -glucosidases belonging predominantly to the GH1 and GH4 families.

Discussion

The present work provides the first characterization of ChbE from *L. paracasei* BL23 and the second characterization of a member of the recently established GH170 glycosyl hydrolase family, following the description of the 6-phospho-*N*-acetylmuramidase MupG from *S. aureus* (Kluj et al. 2018). While MupG hydrolyzes MurNAc-6-phosphate-GlcNAc generated after uptake of peptidoglycan-derived MurNAc-GlcNAc by a PTS, our results show that ChbE likely acts on phosphorylated derivatives of ChbNAc and TriChbNAc generated during PTS^{chb}-mediated transport in *L. paracasei*, acting as a 6-phospho-*N*-acetylglucosaminidase. Thus, despite acting on structurally different substrates and participating in distinct metabolic pathways, MupG and ChbE share the common feature of hydrolyzing phosphorylated amino sugar-containing oligosaccharides and they are both linked to PTS-dependent carbohydrate utilization pathways.

The inability of purified ChbE to hydrolyze ChbNAc or TriChbNAc directly indicated that substrate phosphorylation is an absolute requirement for catalysis. In addition, the genetic context of GH170-encoding genes showed that association to PTSs represents a general feature of this family, and reinforced its proposed activity on phosphorylated substrates. ChbE-catalyzed ChbNAc-P and TriChbNAc-P hydrolysis rendered GlcNAc-6-phosphate, establishing that ChbE specifically cleaves the glycosidic bond adjacent to phosphorylated non-reducing-end residues. This catalytic behavior resembles that reported for MupG and suggests that phosphorylation of the non-reducing sugar residue may be a general recognition determinant for members of the GH170 family. The results confirmed the central role of the ChbABC PTS in ChbNAc and TriChbNAc utilization. Because ChbE acts exclusively on phosphorylated substrates, uptake through PTS^{chb} appears to be mechanistically linked to the subsequent intracellular hydrolysis step. This organization resembles the classical phospho- β -glycosidase systems described for lactose, cellobiose and other sugars in Bacillota, where transport and phosphorylation by a PTS are tightly coupled to intracellular phosphoglycosidase activity (Deutscher et al. 2006; Plaza-Vinuesa et al. 2023; Zeng and Burne 2009). The detection of non-phosphorylated ChbNAc and TriChbNAc in extracts of the $\Delta chbR/E$ mutant could reflect partial dephosphorylation of the corresponding phosphorylated intermediates during sample preparation, mediated by endogenous phosphatases acting on sugar-phosphates (Boulanger et al. 2021). ChbNAc and TriChbNAc were also present in extracts of the wild-type strain. However, their lower abundance compared to the $\Delta chbR/E$ mutant, along with the lack of other detectable phosphorylated intermediates, indicates that phosphorylated metabolites accumulated to measurable levels only in the $\Delta chbR/E$ mutant background.

An unresolved aspect of the pathway is how the ChbNAc generated from TriChbNAc-P hydrolysis is further metabolized. Since ChbE does not act on non-phosphorylated ChbNAc, utilization of this intermediate requires an additional conversion step preceding its hydrolysis (Fig. 5). One possibility is

the existence of an intracellular kinase capable of phosphorylating ChbNAc, although no obvious candidate gene was identified within the *chb* locus. Alternatively, ChbNAc may be intracellularly phosphorylated by PTS^{chb} independently of transport or may undergo release and subsequent reuptake by the transporter, generating ChbNAc-P. Similar mechanisms have been proposed in other PTS-dependent carbohydrate utilization pathways (Benthin et al. 1994; Lanz and Erni 1998; McCoy et al. 2016). Further studies will be required to clarify the fate of ChbNAc and to establish how *L. paracasei* completes TriChbNAc catabolism.

In conclusion, the present study demonstrates that ChbE is a phospho-glycosyl hydrolase of the GH170 family that hydrolyzes phosphorylated derivatives of ChbNAc and TriChbNAc generated during transport by the PTS^{chb}. These findings expand the known functional range of GH170 enzymes and reveal a mechanism for the metabolism of GlcNAc-containing oligosaccharides in lactobacilli. Such metabolic capabilities may contribute to the ability of lactobacilli to exploit carbohydrates released by other members of the gut microbiota through glycan degradation and cross-feeding processes (Flint et al. 2012; Tailford et al. 2015). Moreover, our findings support the existence of PTS-GH170 modules specialized in the utilization of amino sugar-containing carbohydrates derived from cell wall turnover (MurP-MupG) or host glycans and chitin degradation products (PTS^{chb}-ChbE). The frequent association of GH170 homologs with PTS genes in genomes, suggest that coupling to PTS-dependent carbohydrate uptake may represent a general functional trait of this glycosyl hydrolase family.

Declarations

Data availability

All data supporting the findings of this study are available within the paper.

Funding

This work was supported by Grant PID2023-1480 940B (C21 and C22) funded by the Spanish Ministry of Science and Innovation (MICIN)/Spanish State Research Agency (AEI)/<https://doi.org/10.13039/501100011033>.

Acknowledgements

VG-T was supported by a pre-doctoral Contract CIACIF/2022/353 by the Valencian Government. IATA-CSIC is a Center of Excellence Accreditation Severo Ochoa (CEX2021-001189-S MCIN/AEI/10.13039/501100011033). We are grateful to José M. Coll for technical support.

Contributions

VM, MJY and JR-D conceived and designed research. VG-T and JS conducted experiments and prepared figures. VM drafted the manuscript. VG-T, JS, JR-D and MJY helped improving the draft. All authors read and approved the manuscript.

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Figures

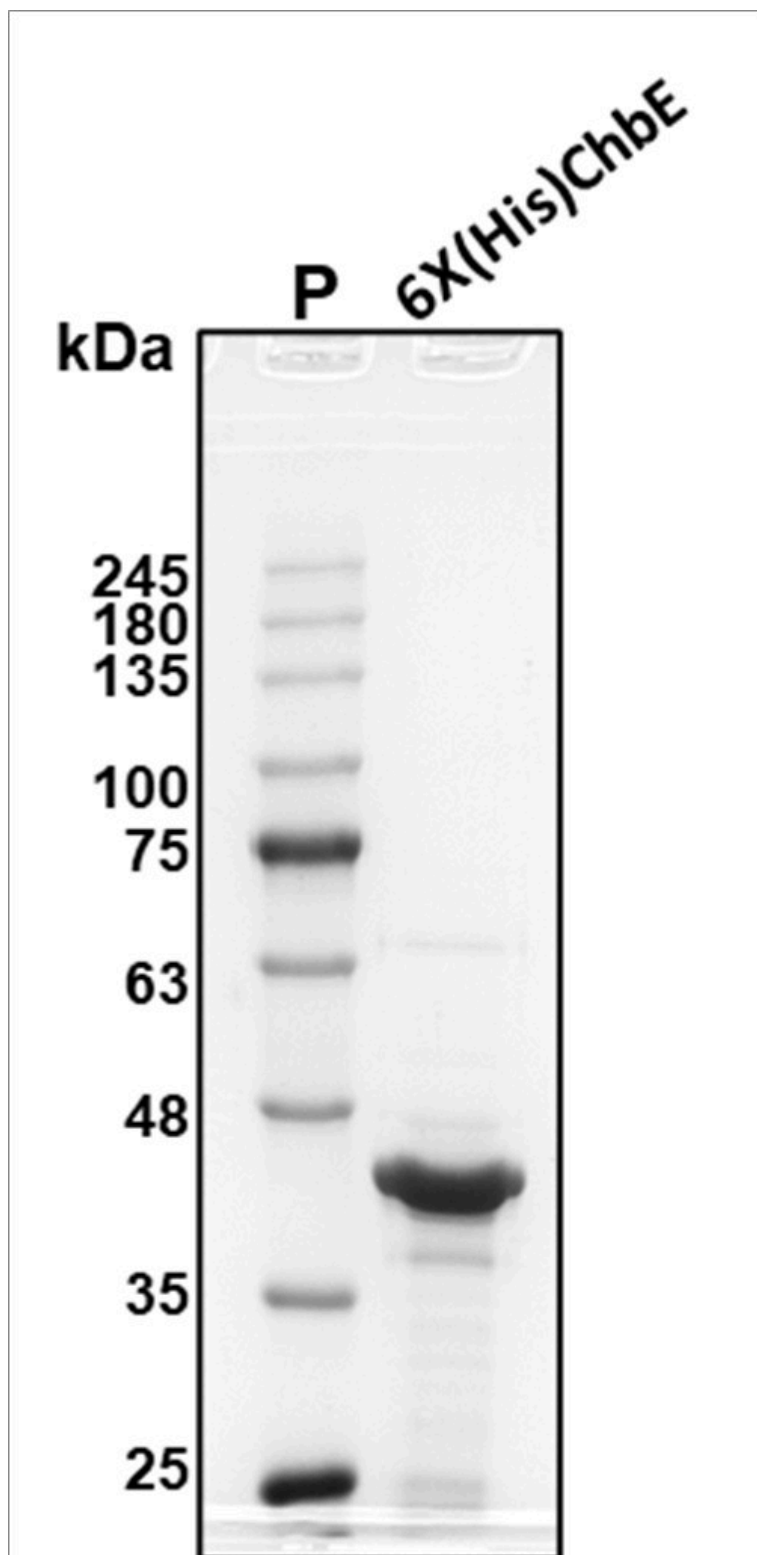


Figure 1

ChbE purification. Coomassie brilliant blue-stained 10% SDS-PAGE showing the purified His-tagged protein ChbE. Lane P, protein standards. The numbers on the left are molecular masses.

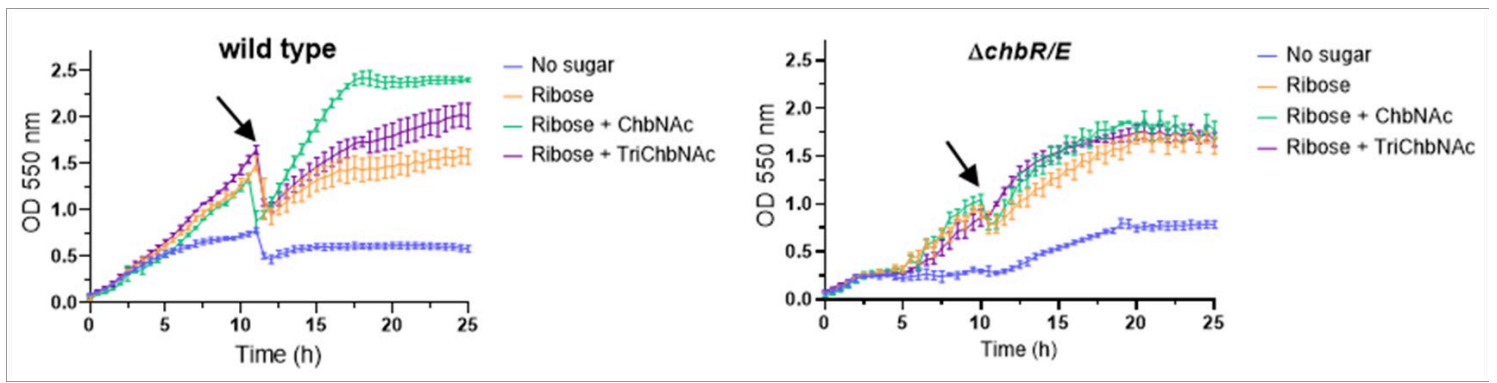


Figure 2

Growth curves of *L. paracasei* BL23 wild type and $\Delta chbR/E$ mutant strains following supplementation with chitooligosaccharides. Wild type strain BL23 and $\Delta chbR/E$ mutant strain grown in MRS basal medium without added sugar (blue) or ribose only at 30 mM (orange). At 11 h of growth, cultures with ribose were supplemented with either 4 mM *N,N'*-diacetylchitobiose (ChbNAc) (green) or *N,N',N''*-triacylchitotriose (TriChbNAc) (purple). The black arrows indicate the time of oligosaccharide addition. Growth with ribose excluded potential carbon catabolite repression effects that would eventually decrease *chb* genes expression even in the absence of ChbR. Data presented are mean values based on at least three replicates. Error bars indicate standard deviations.

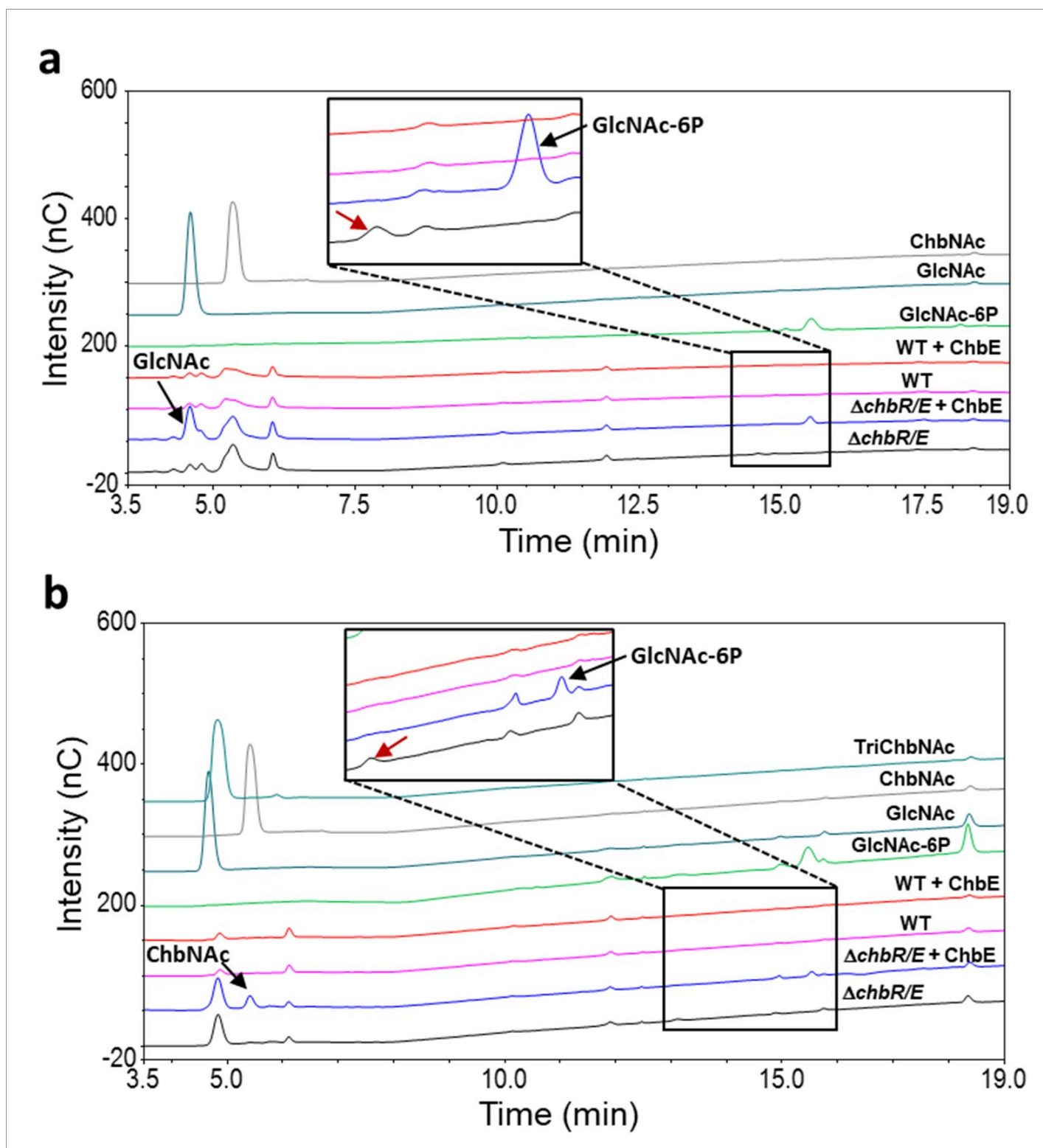


Figure 3

Intracellular sugar analysis. High-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) of (a) cell extracts of cells grown with ribose plus *N,N'*-diacetylchitobiose (ChbNAc) or (b) ribose plus *N,N',N''*-triacetylchitotriose (TriChbNAc). Chromatograms were obtained using a Dionex ICS-6000 high-performance anion-exchange chromatography system equipped with a CarboPac PA100 column. Standards included GlcNAc-6-phosphate (GlcNAc-6P),

GlcNAc, ChbNAc and TriChbNAc. Cytoplasmic extracts from wild type and $\Delta chbR/E$ mutant strains treated with or without ChbE were analyzed. Black arrows indicate peaks appearing after ChbE treatment, corresponding to hydrolysis products that were identified by comparison with the retention times of the standards. The red arrows indicate unidentified peaks appearing only in the $\Delta chbR/E$ strain that disappeared after ChbE treatment. nC, nanoCoulomb.

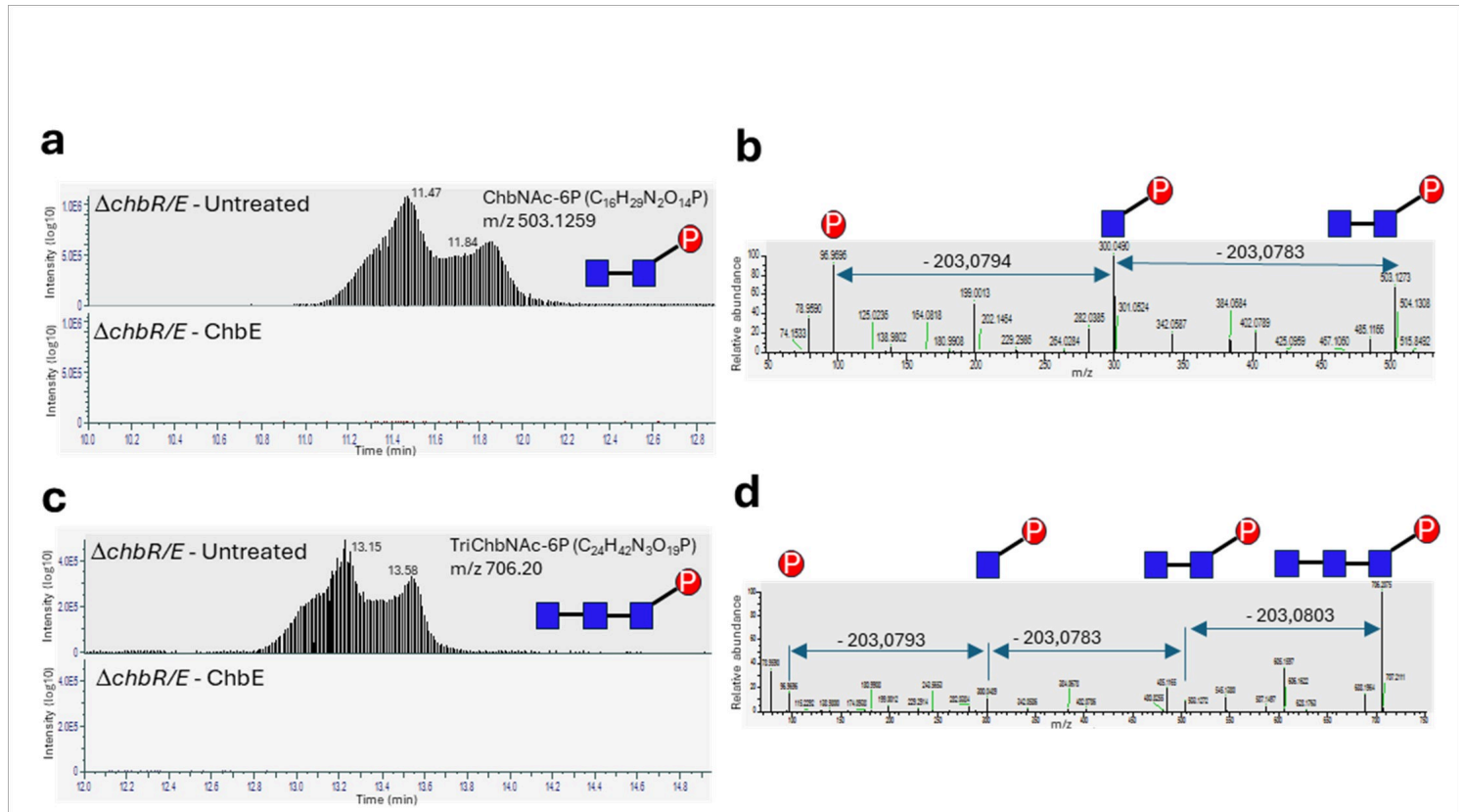


Figure 4

UPLC-MS/MS analysis of extracts. Extracted ion chromatograms (EIC) for the expected m/z corresponding to (a) ChbNAc-P and (c) TriChbNAc-P in extracts of $\Delta chbR/E$ strain grown in the presence of ChbNAc or TriChbNAc, respectively. Samples untreated or treated with ChbE are represented, showing that treatment with the enzyme resulted in the disappearance of the signals with m/z=503.1259 (ChbNAc-P) and m/z=706.20 (TriChbNAc-P), respectively. MS/MS fragmentation analyses of the putative ChbNAc-P (b) and TriChbNAc-P (d) ions. The MS/MS spectra show the sequential loss of GlcNAc masses (203.079 Da) and the presence of phosphate ions (m/z=96.96), supporting compound identification. The blue squares represent GlcNAc molecules.

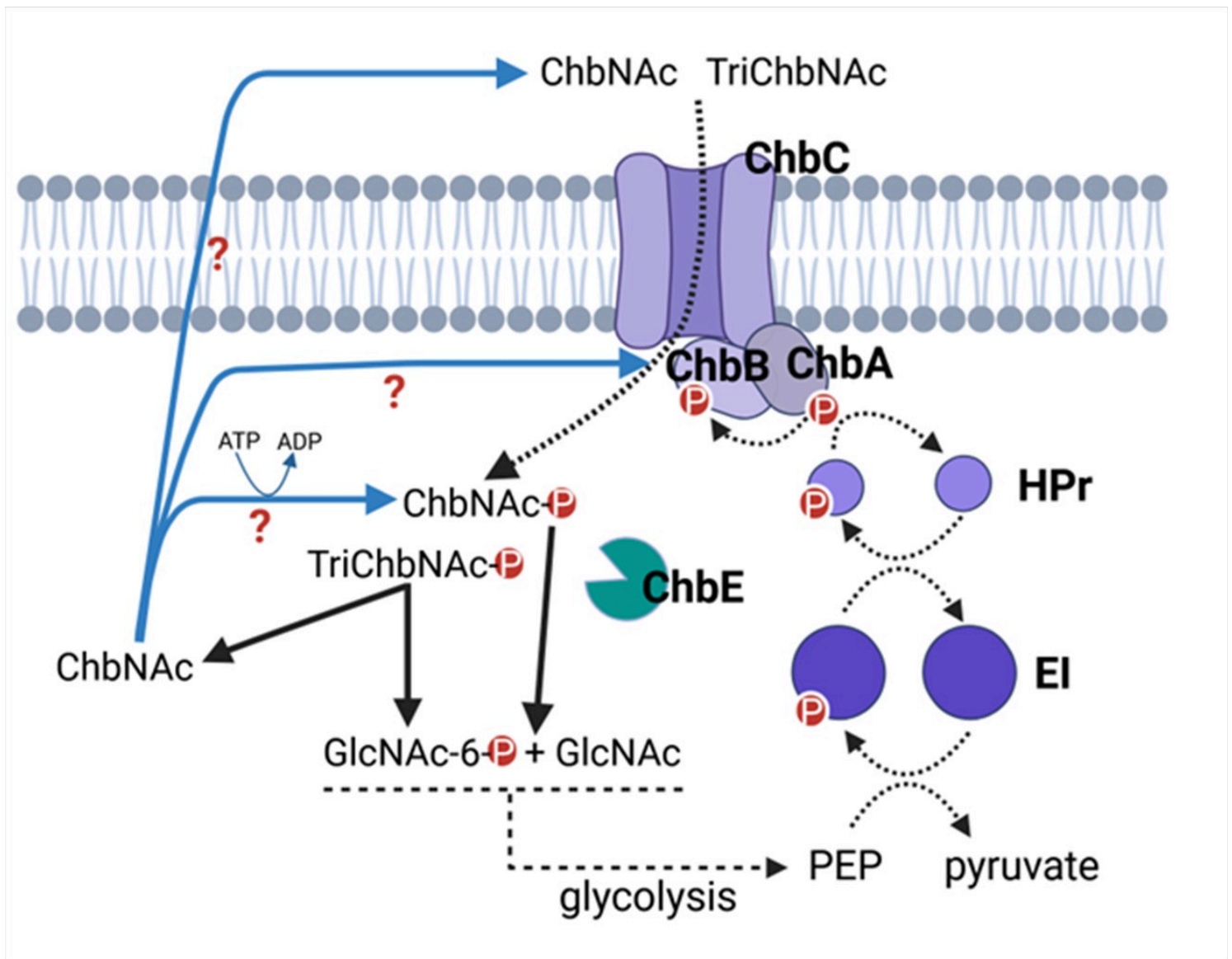


Figure 5

Schematic representation of *N,N'*-diacetylchitobiose (ChbNAc) and *N,N',N''*-triacetylchitotriose (TriChbNAc) metabolism in *L. paracasei* BL23. ChbNAc and TriChbNAc are transported and phosphorylated by the PTS^{chb}. Phosphorylated di- and trisaccharides are hydrolyzed by ChbE, which generates GlcNAc-6-phosphate plus GlcNAc or GlcNAc-6-phosphate plus ChbNAc, respectively. The monosaccharides are channeled through glycolysis, while intracellular ChbNAc can follow different alternative pathways (labelled with question marks): phosphorylation by a sugar kinase, intracellular phosphorylation by the PTS^{chb} or leakage from the cells and translocation and phosphorylation by the PTS^{chb}. In these three cases, ChbNAc-P would be obtained that could then be hydrolyzed by ChbE. ChbC, ChbB and ChbA are enzyme II domains of the cellobiose-type phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS); HPr, Histidin-containing protein of the PTS; EI, enzyme I of the PTS; PEP, phosphoenolpyruvate; GlcNAc, *N*-acetylglucosamine; ChbE, phospho-*b*-*N*-acetylglucosaminidase.

Supplementary Files

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