Characterization of a Glucosamine/Glucosaminide N-Acetyltransferase of Clostridium acetobutylicum

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Many bacteria, in particular Gram-positive bacteria, contain high proportions of non-N-acetylated amino sugars, i.e., glucosamine (GlcN) and/or muramic acid, in the peptidoglycan of their cell wall, thereby acquiring resistance to lysozyme. However, muramidases with specificity for non-N-acetylated peptidoglycan have been characterized as part of autolytic systems such as of Clostridium acetobutylicum. We aim to elucidate the recovery pathway for non-N-acetylated peptidoglycan fragments and present here the identification and characterization of an acetyltransferase of novel specificity from C. acetobutylicum, named GlmA (for glucosamine/glucosaminide N-acetyltransferase). The enzyme catalyzes the specific transfer of an acetyl group from acetyl coenzyme A to the primary amino group of GlcN, thereby generating N-acetylg glucosamine. GlmA is also able to N-acetylate GlcN residues at the nonreducing end of glycosides such as (partially) non-N-acetylated peptidoglycan fragments and β-1,4-glycosidically linked chitosan oligomers. \(K_m\) values of 114, 64, and 39 \(\mu\)M were determined for GlcN, (GlcN)\(_2\), and (GlcN)\(_3\), respectively, and a 3- to 4-fold higher catalytic efficiency was determined for the di- and trisaccharides. GlmA is the first cloned and biochemically characterized glucosamine/glucosaminide N-acetyltransferase and a member of the large GCN5-related N-acetyltransferases (GNAT) superfamily of acetyltransferases. We suggest that GlmA is required for the recovery of non-N-acetylated muropeptides during cell wall rescue in C. acetobutylicum.

The glycan chains of the peptidoglycan of the bacterial cell wall are composed of alternating, β-1,4-glycosidically linked amino sugars N-acetylg glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) (30). The two glycosidic bonds of these glycans are targetted by muramidases, such as lysozymes, that hydrolyze the MurNAc-GlcNAc linkages, and by (endo-)N-acetylmuramidinidases that cleave the GlcNAc-MurNAc bonds. Many bacteria, in particular Gram-positive pathogens, have been reported to acquire resistance against cell lysis due to the action of lysozyme and N-acetylmuramidinidases by N-deacetylation of a great portion of the amino sugars of the peptidoglycan of their cell wall (29). Araki et al. showed that the majority of glucosamine residues of the peptidoglycans of Bacillus cereus, B. subtilis, and B. megaterium have free amino groups and that non-N-acetylated glucosamine residues account for the resistance of these strains to lysozyme (1, 2, 12). The peptidoglycan of Streptococcus pneumoniae, which contains 40 to 80% glucosamine (GlcN) and up to 10% muramic acid (19), is N-deacetylated at the GlcNAc residues in the peptidoglycan by the GlcNAc deacetylase PgdA, the first characterized peptidoglycan deacetylase (31). Clostridium acetobutylicum, which is an endospore-forming, anaerobic fimbriate that is closely related to bacilli, presumably also contains N-deacetylated peptidoglycan since orthologs of pgdA are present on its chromosome. Moreover, an autolytic muramidase has been identified in this organism that is active only on non-N-acetylated peptidoglycan, generating peptidoglycan fragments (muropeptides) that contain a GlcN residue at the nonreducing terminus (10).

We have been studying the peptidoglycan recycling and recognized a muropeptide rescue pathway in B. subtilis that involves the sequential hydrolysis of muropeptides (GlcNAc-MurNAc peptides) by N-acetylg glucosaminidase and N-acetylmuramic acid-1-Ala amidase in the wall compartment (16). We recognized that a muropeptide rescue pathway is likely also present in C. acetobutylicum, which, however, appears to display some distinct features. This organism contains an ortholog of the cytoplasmic MurNAc-6-phosphate etherase MurQ (13) that allows the conversion of MurNAc-6-phosphate to GlcNAc-6-phosphate, which subsequently can enter the nag pathway for further catabolism (32, 33). However, C. acetobutylicum apparently lacks a specific phosphotransferase system for MurNAc that is present in B. subtilis. In an accompanying paper (22), we showed that C. acetobutylicum instead possesses a cytoplasmic kinase that phosphorylates both GlcNAc and MurNAc. The cell wall sugars presumably are released from cell wall fragments in the cytoplasm by orthologs of the N-acetylg glucosaminidase NagZ and the N-acetylmuramyl-L-Ala amidase AmiE of B. subtilis (16), which apparently are not a secreted in C. acetobutylicum but cytoplasmic. We recently have investigated the structure-function relationship of the B. subtilis NagZ and revealed that this enzyme is highly specific for the N-acetyl group of the substrate (17). Hence, a proposed recovery of non-N-acetylated sugars would require the N-acetylation of GlcN residues at the nonreducing end of peptidoglycan fragments (GlcN-MurNAc peptides) prior to NagZ cleavage. We describe here the cloning and biochemical characterization of an enzyme from C. acetobutylicum that has a unique glucosamine/glucosaminide N-acetyltransferase activity.
Bacterial strains, plasmids, chemicals, and growth conditions. The Escherichia coli and B. subtilis strains used in the present study were grown aerobically at 37°C in LB medium (5) that was supplemented with an appropriate antibiotic for plasmid maintenance. Kanamycin and ampicillin were used at final concentrations of 50 and 100 μg/ml, respectively. All chemicals were obtained from Sigma-Aldrich, except as otherwise noted. MurNac was purchased from Bachem (Weil am Rhein, Germany), chitosan from Medac (Wedel, Germany), and [acetyl-1-14C]-coenzyme A (lacteyt-1-14C)-CoA from Hartmann Analytic (Wedel, Germany).

Plasmid construction. ORF184 of C. acetobutylicum ATCC 824 was amplified by PCR from chromosomal DNA kindly provided by G. Bennett (University Rice, Houston, TX) and Hubert Bahl (University of Rostock, Rostock, Germany) using the primers 5’-GGGGATCCCATGGATATTTAGTAGC and 5’-ATCTCGAGATCTCCCGACCTTTAATC (the recognition sites for the restriction endonucleases NcoI and BglII are underlined). The resulting 963-bp DNA fragment was cloned into SphI-digested, dried-in-gasphase pGEM-T (Promega) using the primer 5’-CTCGAGGATCC (Gael) to generate plasmid pGEMORF184, which encodes the putative acetyltransferase. Kanamycin resistance cassette [Kanr]; Qiagen) harboring pGlmA was cultivated under conditions of 50 mM Tris-HCl (pH 7.5) at 37°C. A contiguous time course was set up in methanol containing 2% (vol/vol) concentrated H2SO4, followed by 30 min at 20°C in LB medium (5) that was supplemented with an appropriate antibiotic for plasmid maintenance. Kanamycin and ampicillin were used at final concentrations of 50 and 100 μg/ml, respectively. All chemicals were obtained from Sigma-Aldrich, except as otherwise noted. MurNac was purchased from Bachem (Weil am Rhein, Germany), chitosan from Medac (Wedel, Germany), and [acetyl-1-14C]-coenzyme A (lacteyt-1-14C)-CoA from Hartmann Analytic (Wedel, Germany).

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the protein, GlmA, appeared as one band of high purity and with an apparent molecular mass of 35 to 40 kDa, which is in agreement with the calculated mass (M_r) of 38,447 of GlmA-His6 fusion protein. GlmA is an N-acetyltransferase with specificity for GlcN. In a first attempt to examine the role of GlmA in the N-acetylation of non-N-acetylated peptidoglycan fragments, we tested the amino sugar GlcN as a possible substrate. Purified GlmA generated a radioactively labeled product with GlcN using [acetyl-1-14C]CoA as the cosubstrate (Fig. 2, lanes 4 and 5). With MurK, the GlcNAc/MurNAc kinase of C. acetobutylicum (22), and ATP, the product of the acetylation reaction was converted to a sugar phosphate only when GlcN had been previously acetylated by GlmA (Fig. 2, lanes 6 and 7). This indicates that GlcNAc is generated from GlcN by the action of GlmA, which can be further processed to GlcNAc-6-phosphate by MurK. Thus, GlmA is a GlcN N-acetyltransferase.

We determined the substrate specificity of GlmA and tested whether MurN and other amino sugars are recognized as substrates, thereby applying a nonradioactive acetylation assay as described in Materials and Methods. GlmA only acetylated GlcN, yielding GlcNAc of the tested amino sugar substrates (Fig. 4A). Both the 4-enantiomer GalN and the 2-enantiomer ManN, as well as MurN, the 3-lactic acid derivative of glucosamine, were not acetylated by GlmA (Fig. 4B). These results were also confirmed by the applied more sensitive radioactive acetylation assay (see Fig. S2 in the supplemental material), showing that GlmA of C. acetobutylicum is a GlcN-specific N-acetyltransferase.

Acetylation of chitosan oligomers and determination of the acetylation site. If GlmA is required for muropeptide rescue in C. acetobutylicum, the enzyme should be able to acetylate not only GlcN but also terminal GlcN residues of glycosides, which could then be processed by NagZ. To investigate whether GlmA also converted di- or oligosaccharides, we tested commercially available dimeric to pentameric fragments of chitosan, a linear homoglycan of /beta-1,4-glycosidically linked GlcN residues, as substrates. We observed that GlmA could acetylate all tested chitosan oligomers. Subsequent treatment with N-acetyglucosaminidase NagZ(Na) released GlcNAc (Fig. 5A). To further examine the specific acetylation site in the chitosan glycan strand, we incubated GlmA in various buffers at different pH and activity was determined with GlcN and [acetyl-1-14C]CoA by applied TLC as described in Materials and Methods. The buffers used were phospho-citrate at pH 4.5 to 8.0, Tris-HCl at pH 7.5 to 9.0, and glycine-NaOH at pH 9.0 to 10.5. The data are means of four independent experiments; errors were <5%.

GlmA, which can be further processed to GlcNAc-6-phosphate by MurK. Thus, GlmA is a GlcN N-acetyltransferase. The pH dependency of GlmA was determined by quantification of the reaction product GlcNAc, as described in Materials and Methods. The relative activity was plotted against pH, which resulted in a bell-shaped curve (Fig. 3; see also Fig. S1 in the supplemental material). GlmA was fully active between pH 7 and pH 8, retained half-maximal activity at about pH 6, and was inactive below pH 5. GlmA was stable in the pH range 4.5 to 8. At greater than pH 8, the enzyme rapidly lost activity. To investigate whether GlmA requires divalent metal ions for its activity, the effect of EDTA was studied. GlmA activity was not inhibited by treatment with EDTA (data not shown) in either the presence or the absence of Mg^2+. Thus, GlmA activity apparently does not require divalent metal ions.

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we acetylated chitosan trimer with GlmA as before but inactivated GlmA prior to treatment with NagZBs. With this procedure, the remaining dimeric product (GlcN)₂ could be detected by radioactive acetylation only if GlmA was freshly added (Fig. 5B). This indicates that GlmA acetylated only the terminal nonreducing glucosamine residue of chitosan.

**GlmA acetylates non-N-acetylated muropeptides.** We used the radioactive acetylation assay to determine whether

![Diagram of GlcN, GalN, ManN, and Glc structures]

**FIG. 4.** GlmA is an N-acetyltransferase specific for GlcN. (A) Only GlcN (not GalN, ManN, or MurN amino sugars and not glucose (Glc)) was acetylated by GlmA using nonradioactive acetyl-CoA (AcCoA) as an acetyl donor. Lanes 1 to 3, standards as indicated. (B) Structures of the tested sugars.
GlmA can acetylate non-N-acetylated glucosamine residues in muropeptides derived from Gram-positive peptidoglycan. Cell wall preparations from *B. subtilis* were first degraded with the purified lytic enzyme mutanolysin (an N-deacetylated peptidoglycan). After treatment with mutanolysin and AmiD, an acetylated disaccharide product can be obtained upon GlnA acetylation. The radioactively labeled GlnNac of this product could be released after treatment with NagZ, which was added subsequent to the acetylation reaction. Lanes 1 to 3, standards as indicated. AcCoA, acetyl-CoA.

### DISCUSSION

Cell wall turnover is a catabolic pathway of bacteria by which up to 50% of the cell wall is turned over in one generation (20). Whether cell wall fragments released in this way are also recovered in Gram-positive bacteria is currently unclear (cf. our recent review [23]). The Gram-positive firmicute *C. acetobutylicum* is an important solvent producer strain and widely used for metabolic engineering. The initiation of the solvent production in this organism is coupled with endospore formation and associated with the thinning of the peptidoglycan layer, which apparently results in the increased resistance against the solvents (15). A better knowledge of cell wall modifications, turnover, and recycling is mandatory for optimization of solvent production in *C. acetobutylicum* by rational approaches.

In our effort to contribute to the understanding of cell wall catabolic processes in clostridia and Gram-positive bacteria in general, we cloned and characterized an N-acetyltransferase of *C. acetobutylicum*, GlnA, that is capable of acetylating GlcN and glucosaminides to GlnNac and N-acetylglucosaminides, respectively. GlnA belongs to the GNAT (GCN5-related N-acetyltransferases) superfamily of functionally diverse acetyltransferases that catalyze the transfer of an acetyl group from acetyl-CoA to the primary amine of a wide range of acceptor substrates (11). Members of this enzyme superfamily include aminoglycoside N-acetyltransferases, serotonin N-acetyltransferase, the glucosamine-6-phosphate N-acetyltransferase of yeast and mammals, the histone acetyltransferases, mycothiol synthase, and the Fem family of amino acyl transferases, among others (28). GlnA shows only very weak amino acid sequence homology with characterized enzymes of the GNAT family but exhibits an overall identity of up to 36% with predicted N-acetyltransferases of various species of the *Clostridium* and *Thermoanaerobacter* genera, including the two extremely thermophilic ethanol- and acetate-producing organisms *T. mathrani* strain A3 and *T. tengcongensis* strain MB4 (14, 34). GlcN N-acetyltransferase activity has been identified in crude pigeon liver extracts and membrane fractions isolated from oat coleoptile segments, which are so far the only descriptions of such an enzymatic activity (9, 21). This is thus the first report of a cloned and characterized GlcN N-acetyltransferase (EC 2.3.1.13). Our results indicate that the substrates of GlnA other than GlcN are GlcN-containing muropeptides, as well as GlcN glycosides such as β-1,4-linked chitosan oligomers. These substrates were acetylated by the enzyme at the terminal nonreducing GlcN. Kinetic analysis of GlnA showed that the catalytic efficiency of the enzyme is about 3- to 4-fold higher for

### Table 1. Kinetic parameters of GlnA<sup>a</sup>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( V_{\text{max}} ) (μmol/min/mg)</th>
<th>( K_m ) (μM)</th>
<th>( k_{\text{cat}} ) (1/s)</th>
<th>( k_{\text{cat}}/K_m ) (1/s/μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>2.11</td>
<td>114.3</td>
<td>1.35</td>
<td>11.8</td>
</tr>
<tr>
<td>(GlcN)₂</td>
<td>2.69</td>
<td>64.5</td>
<td>1.72</td>
<td>26.7</td>
</tr>
<tr>
<td>(GlcN)₃</td>
<td>2.60</td>
<td>39.2</td>
<td>1.66</td>
<td>42.4</td>
</tr>
<tr>
<td>AcCoA</td>
<td>2.10</td>
<td>46.4</td>
<td>1.34</td>
<td>28.9</td>
</tr>
</tbody>
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<sup>a</sup> Kinetic parameters were determined in 100 mM sodium phosphate (pH 7.27) at 30°C as described in Materials and Methods. Data represent the means of two independent experiments. Standard errors were <5%. AcCoA, acetyl-CoA.
chitosan oligomers (GlcN)$_2$-$3$ compared to the monomeric GlcN. This indicates that GlmA is primarily involved in the acetylation of di- or oligosaccharides that are transported into the cytoplasm. Similar observations have previously indicated that heparin/heparan sulfate-α-glucosamine N-acetyltransferases from purified lysosomal membrane fractions of human placenta and rat liver N-acetyl with high efficiency terminal α-linked GlcN residues of sulfated di- and tetraglycosaminoglycan chains derived from heparin and heparan sulfate (4, 18). De-N-acetylated GlcNAc residues are found primarily in the peptidoglycan of Gram-positive bacterial species (29), such as S. pneumoniae, B. cereus, B. subtilis, and L. monocytogenes, as well as in the Gram-negative bacterium Rhodopseudomonas viridis (25). Our data confirmed that GlcN residues are not present in the cell wall peptidoglycan of the Gram-negative bacterium C. coli.

GlmA from C. acetobutylicum apparently did not act on intact peptidoglycan isolated from B. subtilis, presumably because terminal nonreducing GlcN residues are not present. These, however, can be generated by the action of mutanoly- sin, which is a muramidase with broad specificity that acts on non-N-acetylated peptidoglycan similar to the lyc gene encoding autolytic lysozyme of C. acetobutylicum. The autolytic extracellular muramidase Lyc of C. acetobutylicum is active on nonacetylated cell wall peptidoglycan and generates GlcN-containing muropeptides such as GlcN-MurNAc-peptide compounds (10). NagZ-like N-acetylgalcosaminidases involved in muropeptide processing are highly specific for N-acetylated substrates (17), which is presumably also true for the NagZ ortholog of C. acetobutylicum. Therefore, N-acetylation of terminal GlcN residues is required for processing of muropeptides by NagZ-like glycosidases. In an accompanying study, we show that both amino sugars GlcNAc and MurNAc, presumably released by NagZ of C. acetobutylicum, are phosphorylated by the MurK kinase, yielding GlcNAc- and MurNAc-6-phosphate in the cytoplasm of C. acetobutylicum (22). We conclude therefore that GlmA is required for the recovery of de-N-acetylated muropeptides during putative cell wall recycling in C. acetobutylicum. Interestingly, a glucosamine/glucosaminide N-acetyltransferase should also be present in B. subtilis. The Bacillus enzyme is expected to be not cytoplasmic like GlmA but secreted, since muropeptide catabolism occurs in the wall compartment in this organism (16). However, no apparent ortholog of glmA was found on the chromosome of B. subtilis. Furthermore, both GlcN and MurN residues are present in the peptidoglycan of B. subtilis (3), suggesting that a MurN N-acetyltransferase may also be present in this organism. GlmA of C. acetobutylicum had no specificity for MurN. Further studies are required to clarify the role of GlmA in cell wall rescue in C. acetobutylicum.

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