The pgdA Gene Encodes for a Peptidoglycan N-Acetylglucosamine Deacetylase in *Streptococcus pneumoniae*†

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Analytical work on the fractionation of the glycan strands of *Streptococcus pneumoniae* cell wall has led to the observation that an unusually high proportion of heptosamine units (over 80% of the glucosamine and 10% of the muramic acid residues) was not N-acetylated, explaining the resistance of the peptidoglycan to the hydrolytic action of lysozyme, a muramidase that cleaves in the glycan backbone. A gene, pgdA, was identified as encoding for the peptidoglycan N-acetylglucosamine deacetylase A with amino acid sequence similarity to fungal chitin deacetylases and rhizobial nodB chitooligosaccharide deacetylases. Pneumococci in which pgdA was inactivated by insertion duplication mutagenesis produced fully N-acetylated glycan and became hypersensitive to exogenous lysozyme in the stationary phase of growth. The pgdA gene may contribute to pneumococcal virulence by providing protection against host lysozyme, which is known to accumulate in high concentrations at infection sites.

The unusual complexity and diversity of the cell surface of *Streptococcus pneumoniae* is apparent both in the capsular polysaccharides (1) and in the cell wall. S. pneumoniae is capable of producing at least 90 chemically distinct capsular polysaccharides (1). The cell wall of this microorganism contains a teichoic acid of unusually complex structure (2, 3) the components of which include ribitol phosphate, galactosamine, trideoxyaminohexose, and covalently linked phosphocholine residues (4). The peptidoglycan of *S. pneumoniae* is also unusual because its stem peptides are cross-linked in both a direct and an indirect manner (5). Furthermore, the proportion of distinct linear and branched mucopeptides in the peptidoglycan is clonally related (6). The pneumococcal cell wall is a potential target for components of the first line host defense such as lysozyme. In addition, the peptidoglycan and teichoic acid may represent bacterial ligands recognized by the innate immune system of the host.

The recent introduction of high resolution analytical techniques and genetic approaches began to shed light on the determinants and biological functions of the pneumococcal cell wall. In this study we describe the identification of a genetic determinant, pgdA, of the first bacterial peptidoglycan GlcNAc deacetylase. We show that the innate activity of this enzyme is responsible for the high proportion of non-acetylated hexosamine residues in the peptidoglycan that appears to play a role in the resistance of *S. pneumoniae* to the activity of exogenous lysozyme, an enzyme that is known to accumulate in high concentrations at infection sites.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains, Plasmids, and Growth Media—Cultures of *S. pneumoniae* R36A, a non-encapsulated laboratory strain from the Rockefeller University collection, were grown in a casein-based semi-synthetic medium (C + Y) containing 1 mg/ml yeast extract (7) or in a chemically defined medium (Cden) (8) at 37 °C without aeration. Plasmid pJDC9 (9) was used for insertion duplication mutagenesis. *Escherichia coli* DH5α was grown in Luria broth medium at 37 °C with aeration. If necessary, erythromycin (Sigma) was added in the following concentrations: 1 mg/ml (*E. coli*) and 1 μg/ml (*S. pneumoniae*).

**Standard DNA Methods—**Routine methods were used for the isolation and manipulation of DNA (10, 11). Enzymes were purchased from New England Biolabs and were used as recommended by the manufacturer. Preliminary sequence data were obtained from the Institute for Genomic Research. Open reading frames were analyzed using DNASTAR software. Sequence comparisons were performed with the BLAST algorithm. CLUSTAL was used for multiple sequence alignments.

**Sequenceing of pgdA—**A DNA fragment (1801 base pairs) including the pgdA gene was amplified by PCR† from chromosomal DNA isolated from *S. pneumoniae* R36A with the primers TCTACAGATACGGATGT-3′ and CTATCTTGTGATTGCTTGACC using the GeneAmp PCR reagent kit (Perkin-Elmer). The following conditions were used for amplification: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 3 min, and one final extension step of 72 °C for 5 min. After purification (PCR purification kit, Promega) the DNA sequence was determined by the Rockefeller University Protein/DNA Technology Center with the *Taq* fluorescent dye terminator sequencing method by using a PE/ABI 377 automated sequencer.

**Inactivation of the pgdA Gene—**The gene encoding the peptidoglycan GlcNAc deacetylase was inactivated by insertion duplication mutagenesis. An internal fragment of the gene was amplified by PCR from chromosomal DNA isolated from *S. pneumoniae* R36A with the primers TCTACAGATACGGATGT-TGGG and CTATCTTGTGATTGCTTGACC using the GeneAmp PCR reagent kit (Perkin-Elmer) with the following primers: 5′-GGTGAGATTGGAGTGGCTTTAATCTGTTGATGTG-3′ and 5′-GGGGATCCGACAAACACACTAGACCCAGAGTATTG-3′. With these primers, EcoRI and BamHI restriction sites were introduced. The PCR reaction was performed in a total volume of 100 μl with 20 ng of template DNA, 40 pmol of primers, and 2.5 units of *Taq* polymerase using the following conditions: 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 3 min, and one final extension step at 72 °C for 5 min. After purification (Promega PCR purification kit) the PCR product and plasmid pJDC9 were restricted with EcoRI and BamHI. After purification (Promega DNA purification kit) the internal gene fragment and the plasmid were ligated, and the resulting vector pPGDA was transformed into *E. coli* DH5α with selection for erythromycin resistance. Next, pPGDA was isolated (Promega plasmid miniprep) and used as donor DNA to transform

† The abbreviations used are: PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography.

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form competent *S. pneumoniae* R36A. Competent bacteria were obtained by a published procedure (12) with addition of competence-stimulating peptide (13). Transformation was performed by 30 min of incubation at 30 °C followed by a phenotypic expression period of 2 h at 37 °C and growth on tryptic soy agar containing 3% sheep blood and 1 μg/ml penicillin (12). One transformant was picked, and the correct insertion of the plasmid into the chromosome was verified by PCR analysis (data not shown). The mutant was able to grow in 1 μg/ml erythromycin. However, because the growth rate was reduced, we included erythromycin only in the pre-cultures and not in the cultures used for the experiments in order to have growth conditions similar to that of the parental strain.

**Biothetic Labeling of Pneumococcal Cell Walls with [3H]GlcNAc—** The labeling of cell walls of strain R36A was as described previously (14). The bacteria were first grown in Cden synthetic medium and then transferred into Cden medium with a reduced concentration of glucose (0.1 mg/ml) containing 1.2 μCi/ml [3H]GlcNAc (Amersham Pharmacia Biotech). After three generations of growth the cells were harvested, and cell walls were isolated. The specific radioactivity was 3.4 × 10⁶ cpm/mg of cell wall with 70% of the label being present in cell wall teichoic acid and 30% in the peptidoglycan glycan strands (data not shown).

**Isolation of Cell Wall and Peptidoglycan—** Pneumococcal cell walls were prepared from cultures in exponential growth phase as described (15) with the modifications described in Ref. 16. Wall teichoic acid was removed by treatment with hydrofluoric acid (16) to obtain peptidoglycan.

**N-Acetylation of Peptidoglycan—** Cell walls were N-acetylated according to the method of Heymann et al. (17) with the following modifications. A suspension of 2 mg/ml cell walls in water was cooled with ice water, and subsequently 0.25 volume of saturated NaHCO₃ and 0.25 volume of freshly prepared 5% acetic anhydride were added. The mixture was stirred for 30 min at 0 °C. After a second aliquot of 0.25 volume of 5% acetic anhydride was added, the mixture was stirred for 30 min at 0 °C and for 1 h at 25 °C. The peptidoglycan was recovered by centrifugation at 50,000 × g for 30 min, washed three times with water, and resuspended in water.

**Assay for Murin Hydrolyase Activity—** [3H]GlcNAc-labeled peptidoglycan (7.5 μg, 14,000 cpm), either non-modified or N-acetylated in vitro, was incubated with different murin hydrolases in a total volume of 100 μl for 60 min at 37 °C. The enzyme concentrations and buffers were as follows: 50 mM sodium phosphate (pH 7.0) for affinity-purified LytA (10 μg/ml) (18), 25 mM sodium phosphate (pH 5.5) for chicken egg white lysozyme (Roche Molecular Biochemicals) (20 μg/ml) and for mutanolysin from Streptomyces globisporus (Sigma) (40 μg/ml). After addition of 100 μl of 1% cetyltrimethylammonium bromide and an incubation on ice for 30 min, the sample was centrifuged (10,000 × g, 20 min, 4 °C), and the radioactivity in 100 μl of supernatant was determined as described (19).

**Fluorodinitrophenylation of Peptidoglycan and Analysis of the Amino Sugars—** Free amino groups in the glycan strands of the peptidoglycan were derivatized by dinitrofluorobenzene according to a modified published method (20). Tritium-labeled peptidoglycan (see above) was incubated with 4% sodium phosphate (pH 5.5) and for mutanolysin from Streptomyces globisporus (Sigma) (40 μg/ml). After addition of 100 μl of 1% cetyltrimethylammonium bromide and an incubation on ice for 30 min, the sample was centrifuged (10,000 × g, 20 min, 4 °C), and the radioactivity in 100 μl of supernatant was determined as described (19).

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**Lysozyme Digestion of Pneumococcal Peptidoglycan—** In the course of studies on the characterization of cell wall glycan strands, we made the unexpected observation that the native peptidoglycan of this bacterium was a poor substrate for lysozyme; only 11% of the peptidoglycan was solubilized even after extensive treatment with this enzyme (Fig. 1). Re-testing lysozyme sensitivity with chemically acetylated peptidoglycan resulted in rapid and complete hydrolysis of the glycan chains, suggesting that the pneumococcal glycan strains may not be fully acetylated (Fig. 1). The LytA amidase had higher activity against the non-modified peptidoglycan, and the M1 muramidase showed similar activities toward both substrates. The mechanisms of these effects are not understood.

**Identification of N-Deacetylated Amino Sugars in the Pneu-**

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A. Severin, Z.-H. Huang, D. A. Gage, and A. Tomasz, unpublished results.
glucosamine-6-phosphate was described previously (22). More deacetylase activity in *E. coli* tosamine polymer produced by glucosamine-6-phosphate deacetylase involved in bases for proteins with homology to known also been described (24). For the identification of genes encoding peptidoglycan—To test whether GlcNAc, MurNAc, or both amino sugars of the pneumococcal peptidoglycan were non-acetylated, the free amino groups of [3H]labeled peptidoglycan by derivatization with 2,4-dinitrofluorobenzene. After total hydrolysis (12 h at 105 °C) the products were separated by HPLC and detected with a flow-through scintillation detector (C). The retention time of 2,4-dinitrophenylated glucosamine ([DNP-G]) was determined after hydrolysis of [3H]GlcNAc followed by dinitrophenylation (A). Hydrolysis of [3H]labeled peptidoglycan (label in GlcNAc and MurNAc) and derivatization of the products yielded 2,4-dinitrophenylated glucosamine (DNP-G) and muramic acid (DNP-M) (B). In C, the radioactivities in the DNP-G and DNP-M peaks represent 42% and 5% of the total radioactivity, respectively. The signals between 8 and 11 min are the non-derivatized amino sugars, which cannot be separated by this chromatographic system.

Pneumococcal Peptidoglycan—To test whether GlcNAc, MurNAc, or both amino sugars of the pneumococcal peptidoglycan were non-acetylated, the free amino groups of [3H]labeled peptidoglycan were derivatized with 2,4-dinitrofluorobenzene. After total hydrolysis the fragments were separated by reversed-phase HPLC, and the radioactive amino sugars and their derivatives were detected with a flow-through scintillation counter. As shown in Fig. 2, lack of acetylation was mainly the property of glucosamine residues. Assuming both amino sugars were [3H]labeled equally, it was estimated that 84% of GlcN and 10% of MurN residues are present in non-acetylated form in the pneumococcal peptidoglycan (Fig. 2).

Identification of the pgdA Gene—An N-acetylglucosamine deacetylase activity in *E. coli* that also deacetylates N-acetylgalactosamine-6-phosphate was described previously (22). More recently, the *E. coli* NagA was reported to be an N-acetylgalactosamine-6-phosphate deacetylase involved in N-acetylgalactosamine metabolism (23). Partly deacetylated hexosamine polymers or oligosaccharides such as the extracellular galactosamine polymer produced by *Aspergillus parasiticus* have also been described (24). For the identification of genes encoding putative peptidoglycan deacetylases we searched data bases for proteins with homology to known N-deacetylases of macromolecules with structures similar to peptidoglycan such as poly(β-1,4)-GlcNAc (chitin) or oligo(β-1,4)-GlcNAc (nodulation factors). In the unfinished nucleotide sequence of *S. pneumoniae* obtained from the Institute for Genomic Research, including hypothetical proteins in Streptococcus pyogenes, Enterococcus faecalis, and Clostridium difficile. It remains to be verified experimentally whether these proteins deacetylate the bacterial peptidoglycan or other macromolecules like chitin.

Inactivation of the Pneumococcal pgdA Gene by Insertion Duplication Mutagenesis—A 601-bp pair insert of the gene (from base 370 to 970) was amplified by PCR from chromosomal DNA of the laboratory strain R36A. This fragment was cloned into plasmid pJDC9, which carries an erythromycin resistance marker. Cloning was performed in *E. coli*. The resulting plasmid, pPGDA, was used to transform competent cells of *S. pneumoniae* R36A. Because the plasmid could not replicate in *S. pneumoniae*, resistance to erythromycin could only be acquired by a homologous recombination event yielding two truncated copies of the target gene on the chromosome, flanking the inserted plasmid with the erythromycin resistance
marker. One erythromycin-resistant clone with an inactivated pgdA gene was used in all subsequent studies.

Cell Wall Structure of the pgdA Mutant—The cell walls of the mutant strain as well as those of the parental strain R36A were isolated, and the peptidoglycan parts were analyzed in two different ways. First, the peptidoglycan was digested with a muramidase (mutanolysin), yielding disaccharides substituted by the peptide side chains (muropeptides), which were separated by HPLC. As shown in Fig. 4, compounds previously shown to represent partly N-deacetylated muropeptides were not present in the peptidoglycan of the pgdA mutant (arrows in Fig. 4). The absence of N-deacetylated muropeptides resulted in an overall simplification of the muropeptide pattern of the mutant cell wall as compared with that of R36A. In the parental strain, the region of higher cross-linked muropeptides (dimers to tetramers) between 35 and 70 min of the elution profile contained a large number of non-resolvable peaks presumably representing variants of N-deacetylated muropeptides carrying the same peptide side chains. In contrast, the muropeptide profile of the pgdA mutant showed fewer peaks, which were better resolved in this region.

In a second analytical approach the purified glycan strands free of the stem peptide side chains were analyzed. Stem peptides were released from peptidoglycan by the pneumococcal amidase LytA. Parental and mutant peptidoglycans were digested by the amidase with similar rates, whereas amidase activity was considerably slower with the chemically acetylated peptidoglycan. Glycan strands were purified by size-exclusion chromatography and were treated with lysozyme. The glycan strands of the parental strain were found to be partly N-deacetylated as indicated by the incompleteness of the lysozyme digestion (Fig. 5): in addition to the expected main products, the reduced form of GlcNacMurNAc and GlcNacMurNAc±, additional peaks appeared in the elution profile between 30 and 50 min. After chemical N-acetylation in vitro these peaks became better resolved and shifted toward higher retention times, indicating that the peaks represented glycan fragments with different N-deacetylation patterns.

In contrast to the glycan strands of the parental strain, the glycan purified from the pgdA-inactivated mutant was quantitatively digested by lysozyme to the disaccharide and the tetrasaccharide, and the lysozyme products showed no change in retention times after a chemical N-acetylation.

These findings allowed two conclusions. First, the data identified the mechanism of the resistance of pneumococcal peptidoglycan to lysozyme digestion (demonstrated in Fig. 1) as the poor hydrolytic activity of this enzyme against deacetylated glycan strands. Second, the results also suggest that the gene product of pgdA is the primary, if not the only, enzyme responsible for the deacetylation of the hexosamine residues of the peptidoglycan.

Lysozyme Sensitivity of the pgdA Mutant—R36A::pgdA and the parental strain R36A were grown in semi-synthetic medium (C + Y) at 37 °C. Either in exponential growth phase or shortly after entering the stationary phase, the cultures were divided, and to one part 80 µg/ml lysozyme was added (Fig. 6). Control cultures received no lysozyme. The parental strain R36A was not affected by the addition of lysozyme until about 2 h after the onset of stationary phase, when the cultures with lysozyme showed a slightly increased rate of lysis. In contrast, cultures of the mutant strain began to lyse rapidly upon entering the stationary phase. Neither the parental nor the mutant strain was affected by lysozyme during exponential growth. If lysozyme was added to the mutant culture at the onset of stationary phase, lysis started immediately.

**DISCUSSION**

During studies on the chemical structure of the pneumococcal peptidoglycan we noted the resistance of this macromolecule to lysozyme, suggesting the absence of N-acetyl substitu-
The presence of conserved blocks in the sequence of the catalytic tureres that resemble peptidoglycan glycan strands. Thus, the substrate of this enzyme is the polymerized peptido-
Gram-positive bacteria (31). Structural features of PgdA imply a secreted protein with an N-terminal signal peptide typical for one part (triangles and squares) 80 µg/ml lysozyme was added. × indicates control culture without lysozyme.

tions of hexosamine residues. The presence of such non-acetylated hexosamines was already observed earlier (30). More recently, mass differences of ~42 and ~84 Da were detected among different pneumococcal muropeptides that otherwise had the same amino acid and amino sugar composition, indicating the loss of acetyl groups from one or more of the N-acetyl amino sugars (21). It was speculated that this could be the result of an artificial deacetylation reaction during preparation of the peptidoglycan (21). Our observations do not confirm this speculation. Rather, our results indicate that the deacetylated hexosamines of the pneumococcal peptidoglycan are the products of an enzymatic reaction by a pneumococcal deacetylase encoded by the gene pgdA, which we describe in this study.

Two observations strongly suggest that pgdA is the structural gene for a deacetylase. Inactivation results in a virtually complete disappearance of deacetylated residues (see arrows in Fig. 4), and the relevant C-terminal domain shows 27% identity and 50% similarity to enzymes with similar catalytic activities.

The pgdA gene described here is the first bacterial determinant encoding an enzyme activity responsible for the loss of N-acetyl groups of the peptidoglycan hexosamine residues. The pgdA gene was identified through its sequence similarity with known deacetylases, the fungal chitin deacetylases and the rhizobial NodB proteins. The substrates of these enzymes are polymers or oligomers of β-1,4-linked GlcNac residues, structures that resemble peptidoglycan glycan strands. Thus, the presence of conserved blocks in the sequence of the catalytic domains of these proteins is not surprising.

The pgdA gene of S. pneumoniae encodes for a putative secreted protein with an N-terminal signal peptide typical for Gram-positive bacteria (31). Structural features of PgdA imply that the substrate of this enzyme is the polymerized peptidoglycan at some stage of its assembly. There is no cleavage site for any known leader peptidase. Most likely the protein is translocated across the cytoplasmic membrane by components of the general secretory pathway and remains anchored to the cytoplasmic membrane by its N-terminal membrane domain. Thereby it would face to the outside and would be able to reach its substrate, the peptidoglycan. By this location of the enzyme, the deacetylation reaction would be a secondary modification, which is in accordance with the known pathway of peptidoglycan synthesis that leads to a fully acetylated glycan backbone (32).

The sequence similarity of PgdA with other deacetylases covers only the C-terminal half of the protein. The function of the N-terminal part showing no homology to known proteins remains to be elucidated. It may be involved in substrate recognition and/or specificity or in interactions with other proteins.

The presence of non-acetylated aminosugars in the peptidoglycan is not limited to pneumococci (25, 26, 28, 29, 33, 34), and the increased activity of lysozyme after N-acetylation of partly deacetylated peptidoglycan was also reported in B. cereus (35, 36). The data search suggests that, similar to pneumococci in bacilli and other bacterial species, the presence of non-acetylated amino sugars in the peptidoglycan is related to the activity of PgdA-like enzymes.

The virtually normal growth rate of the pgdA insertion mutant shows that this gene is not essential for in vitro growth of S. pneumoniae. The mutant showed no differences in morphology, and the growth rate in exponential phase and the cell density of stationary cultures were only slightly lower as compared with the parental strain. Apparently, under in vitro laboratory conditions, the reduced number of positively charged amino groups in the peptidoglycan of the mutant seems to have no drastic effect on cell wall-related processes like binding and uptake of ions and nutrition or non-covalent binding of proteins.

It is conceivable that the PgdA protein plays a role in controlling the activity of pneumococcal cell wall-hydrolyzing enzymes, which may have different activities toward substrates with different levels of deacetylation. The LytB endo-β-N-acetylglucosaminidase is involved in daughter cell separation (37), whereas the LytC 1,4-β-N-acetylmuramidase increases the rate of autolysis at 30 °C (38). The deacetylation of the glycan strand GlcNac residues could be a way of controlling the activities of these potentially autolytic enzymes.

The most striking effect of the inactivation of pgdA was the appearance of hypersensitivity of the mutant bacteria to exogenous lysozyme. Lysozyme is part of the first defense of host organisms against bacterial invasion, including response to pneumococcal infection (39, 40). Large amounts of lysozyme accumulate both in the cerebrospinal fluid of the rabbit after inoculation with pneumococci in an animal model for bacterial meningitis (41) and in human meningeal disease (42). Our observations suggest that the pgdA gene may be part of the virulence mechanism of S. pneumoniae, providing increased resistance of the bacterium against the lysozyme of the human host.

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