Correlation between Alterations of the Penicillin-binding Protein 2 and Modifications of the Peptidoglycan Structure in Neisseria meningitidis with Reduced Susceptibility to Penicillin G*

Received for publication, May 2, 2003, and in revised form, June 10, 2003
Published, JBC Papers in Press, June 10, 2003, DOI 10.1074/jbc.M304607200

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Reduced susceptibility to penicillin G in Neisseria meningitidis is directly correlated with alterations in the penA gene, which encodes the penicillin-binding protein 2 (PBP2). Using purified PBP2s from different backgrounds, we confirmed that the reduced susceptibility to penicillin G is associated with a decreased affinity of altered PBP2s for penicillin G. Infrared spectroscopy analysis using isogenic penicillin-susceptible strains and strains with reduced susceptibility to penicillin G suggested that the meningococcal cell wall is also modified in a penA-dependent manner. Moreover, reverse-phase high pressure liquid chromatography and mass spectrometry analysis of these meningococcal strains confirmed the modifications of peptidoglycan components and showed an increase in the peaks corresponding to pentapeptide-containing muropeptides. These results suggest that the D,D-transpeptidase and/or D,D-carboxypeptidase activities of PBP2 are modified by the changes in penA gene.

Neisseria meningitidis is an exclusive human bacterium that usually infects the nasopharynx. It occasionally provokes invasive infections such as septicaemia, meningitis, and arthritis. Meningococcal infections are still a major public health concern due to periodic epidemics of meningitis in Africa and local outbreaks in developed countries. This concern is heightened by the increasing prevalence of meningococcal strains with reduced susceptibility to penicillin G (PenI), one of the major antibiotics used for the treatment of meningococcal infections.

Penicillin G binds to penicillin-binding proteins (PBP). SDS-PAGE analysis showed that N. meningitidis contains three PBPs (1, 2), PBP1 encoded by penA, and PBP2 encoded by penB. Two other putative PBPs have also been identified from the complete genomic sequences of a serogroup B strain (3) and a serogroup A strain (4) of N. meningitidis. Meningococcal strains that are highly resistant to penicillin G (MIC > 1 μg/ml) due to the production of a plasmid-encoded β-lactamase have been detected in some countries (5–8). However, meningococcal strains with reduced susceptibility to penicillin G (PenI) (MICs between 0.125 and 1 μg/ml) have been reported worldwide (9), and this reduction in susceptibility is at least partly due to the decreased affinity of PBP2 encoded by an altered penA gene (10, 11). N. gonorrhoeae also shows increased resistance to penicillin G due to the decreased affinity of both PBP1 and PBP2 for penicillin G combined with alterations of the gonococcal porin and the overexpression of the MtrCDE efflux pump (12). N. meningitidis is naturally competent for transformation, which enables the horizontal transfer of DNA between strains, resulting in mosaic structures at genetic loci and a highly diverse meningococcal population (13, 14). The penA gene is altered as a result of DNA transfer between meningococcal strains or from commensal Neisseria species (15–18). We previously developed a rapid approach to define meningococcal penA alleles based on restriction fragment length polymorphism (RFLP). Penicillin-susceptible (PenS) strains harbor highly related penA alleles regardless of their genotype. Conversely, PenI strains harbor different penA alleles, and the PenI phenotype is directly related to alterations in the penA gene in N. meningitidis (19). The penA gene encodes PBP2, a 60-kDa protein, which is homologous to high molecular weight class B PBPs. By analogy with PBP3 from Escherichia coli, PBP2 probably catalyzes a transpeptidation reaction that is necessary for the cross-linking of peptidoglycan in the meningococcal cell wall. Alterations of penA gene mostly affect the 3′ transpeptidase-encoding region. The active serine residue SXXK, the SXX, and the KTG motifs, which are all located in this region, are usually conserved, but a high degree of polymorphism is observed in the surrounding sequences (19).

Because penicillin binds covalently to PBP2, the alterations around the active site-encoding domain probably reduce the affinity of PBP2 for penicillin G, thus accounting for the PenI phenotype. However, the effects of penA alterations on the enzymatic activity of PBP2 (particularly the transpeptidation reaction involved in peptidoglycan biosynthesis) and the involvement of such modifications in the PenI phenotype have not been addressed. The lack of information concerning the structure of the N. meningitidis peptidoglycan has hindered this...
Meningococcal Peptidoglycan and Penicillin G Susceptibility

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Growth Conditions**—The meningococcal strains used in this study are listed in Table I. *N. meningitidis* was grown for 18 h on GCB medium (Difco) containing Kellogg supplements (20). *E. coli* TG1 was used as a host for cloning experiments, and *E. coli* BL21(DE3) pLysS was used as an expression system (21). *E. coli* cultures were grown in Luria-Bertani medium supplemented with 40 μg/ml kanamycin and 15 μg/ml chloramphenicol when necessary. An aliquot of cells and a 20-μl aliquot of purified *N. meningitidis* chromosome was amplified with oligonucleotides AA-13 (with a 5′-NdeI adaptor) and AA-14 (with a 5′-SacI adaptor) and AA-2 (with 5′-SacI and 3′-NdeI adaptors) as previously described (19). Amplicons were then digested with HpaII, XhoI, and SacI and ligated into the NcoI and XhoI sites of pET28b (Novagen) to create genes encoding PBP2as with six C-terminal histidine residues (His10-tagged PBP2a) expressed under the control of the T7 bacteriophage promoter. These recombinant plasmids were expressed in *E. coli* BL21(DE3) pLysS, and protein purification was performed using a nickel nitrotriacetic acid-agarose column (Qiagen) as previously described (29). The purified protein was dialyzed against phosphate-buffered saline, adjusted to 20% glycerol (w/v), and stored at −20 °C.

**Penicillin Binding Assays**—Total meningococcal membrane extracts were prepared as previously described (24). The crude membranes (30 μg of protein) were incubated for 30 min at 37 °C in the presence of increasing concentrations of [3H]benzylpenicillin (20 Ci/mmol, 1 mCi/ml, Amersham Biosciences). The reactions were stopped by adding 5 μl of unlabeled penicillin G (25 mg/ml) for 20 min at room temperature. The binding affinity of penicillin G to purified PBP2as was assayed by competition experiments with [3H]benzylpenicillin. The purified PBP2as (2 μg of protein) were first incubated with increasing concentrations of penicillin G, then with saturating concentrations of [3H]benzylpenicillin and processed as described above. After SDS-PAGE analysis, the gels were fixed for 30 min in 40% ethanol and 10% acetic acid, washed for 10 min with water, and placed in Amplify (Amersham Biosciences) for 30 min. The gels were dried and used to expose Hyperfilm-MP (Amersham Biosciences) at −80 °C. The signals were quantified using the ImageQuant program (Molecular Dynamics).

**Infrared Spectroscopy**—Cells that had been grown for 18 h were harvested by centrifugation and resuspended in distilled water. The optical densities of the suspensions were adjusted to yield optimal infrared spectra. A 35-μl aliquot of cells and a 20-μl aliquot of purified peptidoglycan preparations were transferred to a ZnSe optical plate (sample holder), vacuum-dried, and then used directly for Fourier-transform infrared spectroscopy. Each sample was measured in five independent assays. All spectra were recorded between wave numbers 4000 and 500 cm⁻¹ with an IFS 28/B Fourier-transform infrared spectrometer (Bruker, Karlsruhe, Germany). The OPUS software, version 3.02 (Bruker), was used to process the data.

**Determination of Peptidoglycan Structure**—Peptidoglycan preparations, reverse-phase HPLC analysis, desalting of muropeptides, and mass spectrometry were performed as previously described (57). Briefly, peptidoglycans were isolated by an adapted version of the method developed for *E. coli* (25) with boiling sodium dodecyl sulfate, digested with muramidase from Streptomyces globisporus, and reduced with sodium borohydride. Muropeptides were separated and desalted by HPLC on a Hypersil ODS column (Thermosil-Keystone) and submitted to mass spectrometry analysis. All matrix-assisted laser desorption ionization mass spectrometry and post source decay experiments were carried out on a Voyager DE STR instrument (Applied Biosystems Inc., Framingham, MA) as previously described (57).

**Molecular Typing**—Strains were typed by multilocus DNA fingerprinting using five genes (pilA, pilD, crgA, regF, and iga) as previously described (26–28). Polymorphisms of the penA and ponA genes were analyzed by RFLP as previously described (19). The ponA gene was amplified by PCR using oligonucleotides AA-6 and AA-7 (Table II). Amplicons were then digested with HpaII and analyzed on polyacrylamide gels. The nucleotide sequences of the penA gene were analyzed as previously described (19).

**Purification of Recombinant PBP2s**—penA′ codes for a water-soluble form of PBP2 lacking the N-terminal transmembrane domain. penA′ from strains with different penA alleles (LNP8013, LNP16519, LNP17041, LNP16454, LNP17123, and LNP18425) was PCR-amplified with oligonucleotides AA-4 (with 5′ BsoI adonp) and AA-2 (with 5′ XhoI adaptor) (Table II). These resulting fragments were digested with BsoI and XhoI and ligated into the NcoI and XhoI sites of pET28b (Novagen) to create genes encoding PBP2s with six C-terminal histidine residues (His10-tagged PBP2a) expressed under the control of the T7 bacteriophage promoter. These recombinant plasmids were expressed in *E. coli* BL21(DE3) pLysS, and protein purification was performed using a nickel nitrotriacetic acid-agarose column (Qiagen) as previously described (29). The purified protein was dialyzed against phosphate-buffered saline, adjusted to 20% glycerol (w/v), and stored at −20 °C.

**Construction of penA-lacZ Transcriptional Fusions**—The entire penA coding sequence from strain LNP8013 (penA′ allele) was amplified with oligonucleotides 99-1 and 99-2 (Table II) and cloned into pGEM-T Easy (Promega) to obtain the recombinant plasmid pAA3. A 400-bp fragment located immediately downstream from the penA gene on the meningococcal chromosome was amplified with oligonucleotides AA-13 (with a 5′ NdeI adaptor) and AA-14 (with a 5′ SacI adaptor) (Table II). The resulting fragment was cloned between the NdeI and SacI sites located downstream from the penA gene in pAA3. A promoterless lacZ gene (30)

| Strain    | Serogroup/serotype:serotype: | MIC (μg/ml) | MLDF<sup>a</sup> | RFLP<sup>b</sup> |
|-----------|-------------------------------|------------|------------------|-----------------|
| LNP8013   | C:NT:NST                      | 0.994      | 0.094            | 0.006           |
| TH-41     | B:NT, Pil10                   | 0.38       | 0.75             | 0.023           |
| LNP16544  | C:2a:P1                       | 1.01       | 1                | 0.012           |
| LNP16504  | B:NT, Pil1                    | 0.25       | 0.38             | 0.008           |
| LNP17041  | B:1, Pil1                     | 0.5        | 0.75             | 0.012           |
| LNP16519  | C:2a, Pil1                    | 0.28       | 0.5              | 0.008           |
| TR-41     | C:NT:10                       | 0.25       | 0.75             | 0.004           |
| TR-17723  | C:NT:10                       | 0.75       | 0.75             | 0.032           |
| TR-14654  | C:NT:10                       | 0.5        | 0.75             | 0.006           |
| TR-17041  | C:NT:10                       | 0.38       | 0.75             | 0.006           |
| TR-16519  | C:NT:10                       | 0.5        | 0.75             | 0.012           |
| TR-14654  | C:NT:10                       | 0.5        | 0.75             | 0.006           |
| TR-17723  | C:NT:10                       | 0.75       | 0.75             | 0.012           |
| TR-18425  | C:NT:10                       | 0.75       | 0.75             | 0.012           |

<sup>a</sup> NT, nontypable; NST, nonsubtypable.
<sup>b</sup> MICs: susceptible <0.125 μg/ml; resistant >1 μg/ml.
<sup>c</sup> MLDF: multilocus DNA fingerprinting.
penA gene. The aph-3’ gene, encoding kanamycin resistance (31), was amplified with oligonucleotides KM-6 and KM-7 (Table II) and inserted into the blunt-ended pAA8 plasmid, harboring penta-lacZ-aph-3’ operon (Fig. 1A) and was used to transform meningococcal Pen S strain LNP8013 (penA1) and Pen I strains LNP16969 (penA21) and LNP16635 (penA11) (Table I). Upon transformation, the recombinant plasmid pAA8 was maintained, and no double combination occurs. PCR and RFLP analysis were used to select transformants in which lacZ and aph-3’ genes had been integrated correctly but in which the original penA allele in the chromosome had been preserved in each strain. β-Galactosidase assays were performed as previously described (32).

RESULTS

Characterization of Meningococcal Strains—We first selected a subset of well defined meningococcal clinical isolates including ten Pen S strains and one Pen I strain (Table I). We determined the serogroup, serotype, and serosubtype of each strain. The MICs of penicillin G, amoxicillin, and cefotaxime were determined by the Etest method. The multilocus DNA fingerprinting approach using five genes (pilA, pilD, crgA, regF, and gca) showed that strains belonged to different genetic lineages (Table I). RFLP analysis showed that the penA allele of all Pen S strains differed from that of the strain LNP8013 (Pen S) (Table I). Because alterations of both PBPs and PBPs1 are associated with an increased level of penicillin resistance in N. gonorrhoeae (12), we also used RFLP to analyze the penA polymorphism in 66 Pen S and Pen I meningococcal strains. Several restriction enzymes were tested, and only digestion with HpaII gave four highly related penA alleles, but no association was observed between penA alleles and Pen S/Pen I phenotypes (Table I and data not shown). It has previously been shown that the disappearance of PstI site in the penA gene is correlated with penicillin resistance in gonococci (12). However, this polymorphism was not found in any of our meningococcal strains (data not shown).

We next transformed PCR-amplified penA alleles from eight Pen S strains into the Pen S strain LNP8013 to generate a set of isogenic meningococcal variants that differed only by penA alleles. All the transformants (TR) acquired the penA allele from the donor strain and showed reduced susceptibility to penicillin G, with an MIC greater than 0.125 μg/ml (Table I).

Effects of penA Alterations on Its Own Expression—We studied the expression of different penA alleles by analyzing the expression of penA-lacZ transcriptional fusions. The penA coding sequence from strain LNP8013 was cloned upstream from a promoterless lacZ gene. The resulting recombinant plasmid, pAA8, also harbored the aph-3’ gene, encoding resistance to kanamycin and the region of the meningococcal chromosome located immediately downstream from the penA gene (Fig. 1A). Transformation and allelic replacement into the Pen S strain, LNP8013, and two Pen I strains, LNP16969 and LNP16635, allowed us to construct strains expressing lacZ under the control of the promoter region of the chromosomal penA, corresponding to the two types of strains, even in strains with higher MICs (saturation at 0.5 and 0.05 μg/ml [3H]benzylpenicillin, respectively) (Fig. 2). There was no apparent difference in the amount of [3H]benzylpenicillin bound to the other PBPs (PBP1 and PBP3) between the two types of strains, even in strains with higher MICs (saturation at 0.5 and 0.05 μg/ml [3H]benzylpenicillin for PBP1 and PBP3, respectively). In transformants, the acquisition of altered penA genes and reduced susceptibility to penicillin G were accompanied by changes in the binding of [3H]benzylpenicillin to PBP2s as in the donor strains but not to PBP1 and PBP3 (Fig. 2).

We next studied the binding of penicillin G to purified PBP2s from Pen S and Pen I strains. penA alleles lacking the transmembrane-coding region (penA') were amplified and cloned into pET28b (Novagen), creating genes coding for water-soluble forms of PBP2s with six C-terminal histidine residues. His6-tagged PBP2s were overproduced and purified to greater than 95% purity (data not shown). We then determined the binding affinity of the purified PBP2s for penicillin G in competition experiments with [3H]benzylpenicillin. We tested purified PBP2s from one Pen S (S-PBP2) and five Pen I (R-PBP2s) strains with different penicillin G MICs. The binding was scored by determining the concentration of non-radioactive penicillin G that inhibited the binding of saturating concentrations of [3H]benzylpenicillin by 50% (inhibition concentration 50, I50). R-PBP2s bound less penicillin G than S-PBP2. The I50 values and the penicillin G MICs of the corresponding Pen S transformants matched closely. However, no strict correlation was found between the I50 values and the penicillin G MICs of the corresponding clinical Pen S strains (Fig. 3). These data strongly suggest that alterations in the penA genes resulted in PBP2s with decreased affinity for penicillin G. However, the decreased
affinity of PBPs for penicillin G cannot solely explain the different penicillin G MICs of the clinical PenI isolates.

Effects of penA Alterations on the Structure of Meningococcal PBP2—We sequenced the region of the penA gene that encodes the transpeptidase domain of PBP2 of a large collection of PenI clinical isolates obtained in France, England, and Spain as previously described (19). The deduced amino acid sequences of PBP2 (amino acids 298–581) were aligned, and meningococcal PenI strains showed 8.5–14.4% divergence (data not shown). Eight positions were modified in almost all PenI strains (Fig. 4). These altered positions were located around the conserved KTG motif. The corresponding residues in PBP2x from Streptococcus pneumoniae are part of the structure that forms the active site (33), particularly the β-sheets 3 and 4 (Fig. 4). This suggests that these amino acid substitutions are important in determining reduced affinity of PBP2 for penicillin G. We transformed the PenS strain LNP8013 with a PCR-amplified DNA fragment containing the penA-lacZ-aph3' transcriptional fusion and recombination events with the homologous locus on the meningococcal chromosome. A, oligonucleotides are represented by small arrows, white boxes correspond to DNA of the vector pGEM-T easy, black boxes represent meningococcal penA gene, and hatched boxes stand for the meningococcal DNA fragment located downstream from the penA gene. The recombinant plasmid pAA8 was used to transform N. meningitidis strains LNP8013 (PenS), LNP16969 (PenI), and LNP16635 (PenI). lacZ was integrated by double-crossing-over (dotted lines) downstream from each penA allele, creating three transcriptional fusions, penA1-lacZ (strain LNP8013), penA21-lacZ (strain LNP16969), and penA11-lacZ (strain LNP16635). B, β-galactosidase activity assays. β-Galactosidase activities, which reflect the expression level of the three corresponding penA alleles were assayed after 2 and 6 h of growth.

Binding affinity of purified S-PBP2 (from PenS strain LNP8013) and R-PBP2s (from PenI strains LNP16519, LNP17041, LNP16454, LNP17723, and LNP18425) for penicillin G in competition experiments with [3H]benzylpenicillin. Purified PBP2s (2 μg of protein) were incubated with the indicated concentrations of [3H]benzylpenicillin. After SDS-PAGE analysis, the gels were fixed, treated with Amplify (Amersham Biosciences), and then autoradiographed. The positions of the three PBPs (PBP1, PBP2, and PBP3) and the penicillin G MICs of each strain are indicated on the left.
genic Pen\(^s\) derivatives clustered together and separately from the Pen\(^s\) parental strain (Fig. 5). Identical results were obtained when we compared purified peptidoglycan preparations from these strains (data not shown). These data suggest that penA alterations affect the meningooccal cell wall and possibly the peptidoglycan.

**Peptidoglycan Structure in Meningococcal Strains**—We recently described the structure of the peptidoglycan of *N. meningitidis*, which consists of disaccharide subunits of N-acetylglucosamine and N-acetylmuramic acid carrying a pentapeptide (\(\text{L-Ala-D-Glu-meso-diaminopimelate-d-Ala-d-Ala}\)) (57). To determine the nature of the peptidoglycan structural modifications that could be induced by penA alterations, we compared the muropeptide composition of the Pen\(^s\) strain LNP8013 and its isogenic Pen\(^s\) derivatives (Table I). We analyzed purified peptidoglycan preparations digested with muramidase by HPLC and mass spectrometry. We determined the structures of 28 different muropeptides species (57) (Fig. 6 and Table III). Although no new peaks were detected, differences in the amplitudes of preexisting peaks were observed between the Pen\(^s\) strain and its seven isogenic Pen\(^s\) derivatives. These mostly involved an increase in pentapeptide-containing muropeptides (Fig. 6 and Table III). The amplitude of peak 5, which corresponds to the disaccharide pentapeptide N-acetylglucosamine-N-acetylmuramic acid-L-Ala-d-Glu-meso-diaminopimelate-d-Ala-d-Ala, was significantly higher in all the isogenic Pen\(^s\) derivatives clustered together and separately from the Pen\(^s\) parental strain (Fig. 5). Identical results were obtained when we compared purified peptidoglycan preparations from these strains (data not shown). These data suggest that penA alterations affect the meningooccal cell wall and possibly the peptidoglycan.

**DISCUSSION**

Susceptibility to \(\beta\)-lactams antibiotics can be mediated by three major mechanisms, (i) decreased binding of antibiotics to PBP\(_s\), (ii) antibiotic inactivation by \(\beta\)-lactamase, and (iii) reduced membrane permeability. In *N. meningitidis*, the first mechanism is usually incriminated. SDS-PAGE analysis revealed the presence of three PBP\(_s\) in this bacterium, but other PBP\(_s\) may also exist, as suggested from the in silico analysis of the genome of two completely sequenced meningoococal strains (3, 4). We have previously reported that penA alterations are directly linked to the Pen\(^s\) phenotype (19). Moreover in this study, we showed that the polymorphism in the penA gene, encoding PBP1, is not associated with the Pen\(^s\) phenotype. Our results confirm that alterations of the penA gene, encoding PBP2, decrease the binding affinity of PBP2 for penicillin G. However, this decreased affinity for penicillin G may not be the only mechanism accounting for the increased MIC of this antibiotic, as the binding of penicillin G to purified PBP2s from different clinical Pen\(^s\) strains was not directly correlated with penicillin G MIC levels (Fig. 3). Penicillin G binds covalently to PBP\(_s\) in the active site of PBPs (35), therefore, we expected that the ability of altered PBP\(_s\) to bind penicillin would be affected because alterations of PBP\(_s\) are usually located near to the active site of the transpeptidase moiety of PBP2. Moreover, the eight residues modified in most Pen\(^s\) isolates were in the region of the meningoococal PBP2 that is homologous to the active site of PBP2x from *S. pneumoniae* (33) and seem to play a major role in the decreased affinity of PBP2 for penicillin G.
modifications of several PBPs (41, 42), and it has been suggested that changes in peptidoglycan structure are closely related with resistance to /H9252-lactams (43–45). The peptidoglycan of non-/H9252-lactamase-mediated antibiotic resistant strains of Haemophilus influenzae has been shown to contain more trippeptides than susceptible strains (46). This may be due to an increase in the activity of aD,D-carboxypeptidase that would generate more tripeptides (46). Moreover, the cell wall of H. influenzae-resistant strains showed enhanced inflammatory activity in an animal model of meningitis (47). We recently developed a murine model that should make it possible to study the effect of modifications in N. meningitidis peptidoglycan structure on the induction of inflammatory response as well as on meningococcal virulence (48).

It has been suggested that the degree of cross-linking differs between PenS and PenI strains of N. gonorrhoeae (49, 50), which is closely related to N. meningitidis. However, isogenic derivatives were not used in this work, and HPLC peaks were not characterized (51). We have shown (57) that the reduced susceptibility to penicillin G of meningococcal PenI clinical isolates was associated with changes in peptidoglycan structure (accumulation of pentapeptides). Here, we studied the

![FIG. 6. Reverse-phase HPLC analysis of N. meningitidis muramidase-digested peptidoglycan from the PenS strain LNP8013. Arrows indicate the peaks that differ between the PenS strain LNP8013 and the seven PenI isogenic strains. The amplitudes of peaks 5, 9, and 18 were increased in PenI isogenic strains, whereas the amplitude of peak 2 was decreased. The structures of the muropeptides corresponding to these peaks that differ quantitatively between the strain LNP8013 (PenS) and its isogenic PenI derivatives are presented in Table III.](image)

### Table III

| Muropeptide composition of meningococcal PenS strain LNP8013 and its isogenic PenI derivatives |
|---------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| HPLC peak | LNP8013 | TR 16454 | TR 16504 | TR 16519 | TR 17041 | TR 17723 | TR 18425 | TR W-39 | Observed m/z | Muropeptide structure |
|-----------|------------|----------|----------|----------|----------|----------|----------|----------|----------------|----------------------|
| 1         | 1.61       | 1.42     | 1.62     | 2.21     | 2.18     | 2.07     | 2.30     | 1.62     | 893.3633 | Tri                   |
| 2         | 12.99      | 10.45    | 10.97    | 11.22    | 12.09    | 10.08    | 11.38    | 12.07    | 964.4686 | Tetra                |
| 5         | 1.86       | 3.76     | 3.68     | 2.4      | 3.47     | 2.57     | 2.23     | 2.98     | 1035.5927 | Penta                |
| 9         | 3.78       | 4.98     | 5.15     | 4.49     | 4.03     | 4.00     | 3.39     | 4.39     | 19558.8453 | Tetra-penta          |
| 13        | 0.96       | 1.3      | 1.28     | 1.66     | 2.04     | 0.82     | 0.9      | 1.72     | 2882.4990 | Tetra-tetra-penta   |
|           |            |          |          |          |          |          |          |          |              | OAc                   |
| 14        | 0.88       | 2.38     | 2.13     | 0.83     | 1.92     | 1.8      | 1.55     | 1.87     | 1077.2378 | Penta OAc           |
| 18        | 5.97       | 7.89     | 6.94     | 6.13     | 4.88     | 7.92     | 7.39     | 6.78     | 2043.1358 | Tetra-penta OAc      |
|           |            |          |          |          |          |          |          |          |              |                       |
|           |            |          |          |          |          |          |          |          | 1971.8306 | Tetra-tetra-di-OAc   |

% Cross-linking

|            | LNP8013 | TR 16454 | TR 16504 | TR 16519 | TR 17041 | TR 17723 | TR 18425 | TR W-39 |
|------------|---------|----------|----------|----------|----------|----------|----------|---------|
|            | 38      | 40       | 38       | 38       | 36       | 40       | 39       | 38      |

% OAc per disaccharide

|            | LNP8013 | TR 16454 | TR 16504 | TR 16519 | TR 17041 | TR 17723 | TR 18425 | TR W-39 |
|------------|---------|----------|----------|----------|----------|----------|----------|---------|
|            | 37      | 40       | 38       | 37       | 35       | 41       | 38       | 37      |

\[a\] Determined by mass spectrometry (MALDI-MS).

\[b\] Determined as previously described (57). Tri, disaccharide tripeptide; Tetra, disaccharide tetrapeptide; Penta, disaccharide pentapeptide (disaccharide = N-acetylglucosamine-N-acetylmuramic acid); OAc, O-acetylation on N-acetylmuramic acid.

\[c\] Numbers are as in Fig. 6. Peaks that were quantitatively different between the strain LNP8013 (PenS) and its isogenic PenI derivatives are presented as well as the peak 1 (unchanged) for comparison. Each percentage corresponds to the peak UV area over the total muropeptide UV peak area.

\[d\] These HPLC peak corresponds to a mixture of two muropeptides.
peptidoglycan structure of several meningococcal isogenic Pen1 strains obtained by transformation of the parental susceptible strain with PCR-amplified altered penA genes. We did not observe any new peak in the HPLC profiles. However, relative changes in the amplitudes of preexisting peaks were observed. As previously observed in clinical Pen1 isolates (57), our data indicated an increase in muropeptides with pentapeptide chains. As we found isogenic strains, these modifications in peptidoglycan structure are directly linked to the alterations in penA gene, and changes in another locus cannot be involved, suggesting that the modified PBP2 in Pen1 transformants may have an altered d,l-transpeptidase activity. However, the increase in muropeptides carrying pentapeptide chains may reflect a decrease in d,l-carboxypeptidase activity on newly synthesized peptidoglycan chains. This in turn suggests that the meningococcal PBP2 rather has d,l-carboxypeptidase activity. Most of the modifications affecting PBP2s from Pen1 strains were located within a region that is homologous to the active-site region of Penl transformants, these modifications in penA gene, at least as the amino acid modifications are all predicted to be less likely as the amino acid modifications are all predicted to be involved, suggesting that the modified PBP2 in Pen1 transformants may have an altered d,l-transpeptidase activity. Most of the modifications affecting PBP2s from Pen1 strains were located within a region that is homologous to the active-site region of Penl transformants, these modifications in penA gene, at least as the amino acid modifications are all predicted to be less likely as the amino acid modifications are all predicted to be involved, suggesting that the modified PBP2 in Pen1 transformants may have an altered d,l-transpeptidase activity. Most of the modifications affecting PBP2s from Pen1 strains were located within a region that is homologous to the active-site region of Penl transformants, these modifications in penA gene, at least as the amino acid modifications are all predicted to be less likely as the amino acid modifications are all predicted to be involved, suggesting that the modified PBP2 in Pen1 transformants may have an altered d,l-transpeptidase activity.
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*J. Biol. Chem.*, 2003, 278:31529-31535. doi: 10.1074/jbc.M304607200 originally published online June 10, 2003

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