



The low-molecular-mass, penicillin-binding proteins DacB and DacC combine to modify peptidoglycan cross-linking and allow stable Type IV pilus expression in *Neisseria gonorrhoeae*

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Summary

Neisseria gonorrhoeae is the causative agent of the sexually transmitted infection gonorrhea and is adapted to survive in humans, its only host. The *N. gonorrhoeae* cell wall is critical for maintaining envelope integrity, resisting immune cell killing and production of cytotoxic peptidoglycan (PG) fragments. Deletion of the *N. gonorrhoeae* strain FA1090 genes encoding two predicted low-molecular-mass, penicillin-binding proteins (LMM PBPs), DacB and DacC, substantially altered the PG cross-linking. Loss of the DacB peptidase resulted in global alterations to the PG composition, while loss of the DacC protein affected a much narrower subset of PG peptide components. A double $\Delta dacB/\Delta dacC$ mutant resembled the $\Delta dacB$ single mutant, but had an even greater level of cross-linked PG. While single $\Delta dacB$ or $\Delta dacC$ mutants did not show any major phenotypes, the $\Delta dacB/\Delta dacC$ mutant displayed an altered cellular morphology, decreased resistance to antibiotics and increased sensitivity to detergent-mediated death. Loss of the two proteins also drastically reduced the number of Type IV pili (Tfp), a critical virulence factor. The decreased piliation reduced transformation efficiency and correlated with increased

growth rate. While these two LMM PBPs differentially alter the PG composition, their overlapping effects are essential to proper envelope function and expression of factors critical for pathogenesis.

Introduction

The Gram-negative, diplococcal bacterium *Neisseria gonorrhoeae* is the etiologic agent of gonorrhea. This human-restricted pathogen causes an estimated 78 million infections annually worldwide (Francis Ndowa, 2012; Newman *et al.*, 2015). *N. gonorrhoeae* typically colonizes the genitourinary tract resulting in non-complicated urethritis and cervicitis in men and women respectively. Presumably due to the development of nucleic acid amplification tests, detection of rectal and pharyngeal gonorrhea, which often remains asymptomatic, has increased recently (Unemo and Shafer, 2014). Infection can, in rare cases, result in severe sequelae including pelvic inflammatory disease (PID), epididymitis and bacteremia (O'Brien *et al.*, 1983; Wasserheit, 1994; Swadlow *et al.*, 1996). Resistance to all recommended antibiotics has been observed in *N. gonorrhoeae*, although not yet in a single strain (Goire *et al.*, 2014). The observed resistance(s), coupled with the ability to undergo horizontal gene transfer, which can mediate spread of resistance, has led the Centers for Disease Control to label *N. gonorrhoeae* an urgent threat (Prevention, 2013). A greater understanding of the basic biology and fundamental virulence factors of this pathogen is of critical public health importance.

As a human restricted pathogen, *N. gonorrhoeae* has no known environmental reservoir and has evolved under constant pressure from the human immune system (Virji, 2009). This restricted environment has led to exquisite adaptation to its niche with several virulence factors capable of undergoing antigenic variation to maintain function while avoiding immune surveillance (Zelevska *et al.*, 2016). One such factor is the Type IV

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pilus (Tfp), an organelle widespread amongst Gram-negative bacteria and required for productive *N. gonorrhoeae* infection (Swanson *et al.*, 1987). Tfp mediate an array of functions including microcolony formation, cellular adherence, modulation of host-cell signaling and resistance to oxidative killing (Swanson, 1973; Dietrich *et al.*, 2011; Bottcher, 2011; Stohl *et al.*, 2013). The Tfp complex is also required for natural transformation, mediating at least the initial binding and uptake of DNA from the environment into the periplasmic space (Gangel *et al.*, 2014; Obergfell and Seifert, 2015). In many Gram-negative bacteria, transformation efficiency is highly correlated with the expression of Tfp, and piliated strains of *N. gonorrhoeae* can undergo transformation more than a million times more often than a non-piliated strain (Sparling, 1966; Long *et al.*, 2003; Craig *et al.*, 2004).

The Tfp is a dynamic structure, undergoing cycles of extension and retraction that can result in pili extended several microns from the cell surface (Stephens *et al.*, 1985; Craig *et al.*, 2004). While these fibers are composed of thousands of polymerized pilin (PilE) subunits and are only six nm in width, they can exert significant force (Maier *et al.*, 2002). Tfp are produced from an assembly complex that spans the bacterial envelope. The ATPases that drive extension and retraction (PilF and PilT respectively) trade off occupying the inner membrane complex consisting of PilG, PilM, PilN, PilO (Berry and Pelicic, 2015; Chang *et al.*, 2016). PilP spans the periplasmic space from the inner-membrane complex to the secretin PilQ. PilQ multimerizes to form a pore in the outer membrane through which the pilus extends. Both TsaP and PilQ mediate proper anchoring of the complex through interactions with the outer membrane and peptidoglycan (PG) layer (Siewering *et al.*, 2014). Mpg, a zinc-dependent carboxypeptidase and endopeptidase known to hydrolyze (PG) side chains, is required for stable pilus expression through an unknown mechanism (Stohl *et al.*, 2013).

The characteristic coccal shape of *N. gonorrhoeae* requires the cell-wall PG sacculus (Zapun *et al.*, 2008). In most bacteria, penicillin binding proteins (PBPs) polymerize and cross-link the cell-wall PG to form the large, macromolecular complex located in the periplasmic space (Typas *et al.*, 2011). Transglycosylases polymerize the long glycan chains while transpeptidases establish the peptide cross-linking between the glycan strands. Along with hydrolase modification by carboxypeptidases and endopeptidases, these enzymes build a macromolecule, strong enough to withstand the osmotic pressure differential yet flexible enough to allow for cell growth and division. During *N. gonorrhoeae* cell-wall formation and maturation, PG fragments are released into the extracellular space that are cytotoxic and stimulate

inflammation (Melly *et al.*, 1984; Cloud-Hansen *et al.*, 2008; Mavrogiorgos *et al.*, 2014). The *N. gonorrhoeae* cell wall also helps to protect the organism from neutrophil-mediated killing (Ragland *et al.*, 2017).

Similarly to other coccal bacteria, *N. gonorrhoeae* has a relatively small number of penicillin-binding proteins (PBPs) (Barbour, 1981; Zapun *et al.*, 2008). Of the four identified PBPs in *N. gonorrhoeae*, only the two high molecular mass (HMM) proteins, PBP1 and PBP2 are essential for cell viability (Barbour, 1981). HMM PBPs are largely responsible for the synthesis of new PG. PBP1 is a class A HMM PBP, homologous to *Escherichia coli* PBP1a, which is likely responsible for transglycosylation and transpeptidation (Ropp and Nicholas, 1997). PBP2 is a class B HMM PBP, homologous to *E. coli* PBP3, which also catalyzes transpeptidation reactions (Spratt and Cromie, 1988; Dowson *et al.*, 1989; Zhang and Spratt, 1989). Mutations in the *penA* gene encoding PBP2 account for the majority of the widespread resistance to β -lactams (Ameyama *et al.*, 2002; Unemo and Nicholas, 2012).

Low molecular mass (LMM) PBPs tend to play non-essential roles in PG modification, PG recycling and cell separation (Sauvage *et al.*, 2008). The functional outcome of activity of LMM PBPs is typically harder to determine as LMM PBPs tend to be more numerous and often have overlapping functions; however, only two LMM PBPs have been identified and characterized in *N. gonorrhoeae*. PBP3 binds radiolabeled penicillin and is homologous to *E. coli* (DacB) (Barbour, 1981; Stefanova *et al.*, 2003). DacB exhibits both carboxypeptidase activity and endopeptidase activity. *N. gonorrhoeae* PBP4 was identified through genetic analysis, is most similar to PBP7 of *E. coli* and does bind radiolabeled penicillin (Stefanova *et al.*, 2003, 2004). PBP4 is a DD-carboxypeptidase with a preference for N^c-acylated substrates. While initial observation of single knockout strains of PBP3 or PBP4 of *N. gonorrhoeae* revealed no gross abnormalities, loss of PBP3 and PBP4 together was reported to reduce cell growth and alter the cellular morphology (Stefanova *et al.*, 2003).

In this study, we mutated the *N. gonorrhoeae* genes encoding DacB (PBP3) and DacC, a third predicted LMM PBP not previously identified in radiolabeled penicillin binding assays in *N. gonorrhoeae*. Inactivation of *dacB* dramatically altered the PG cross-linking, while mutation of *dacC* had much subtler effects on the PG composition. Loss of the two proteins in tandem produced highly cross-linked PG and resulted in significant cellular abnormalities. As these abnormalities were largely absent in the single knockout strains, our data suggest these two proteins have overlapping effects on the *N. gonorrhoeae* cell wall despite uniquely altering the PG profile.

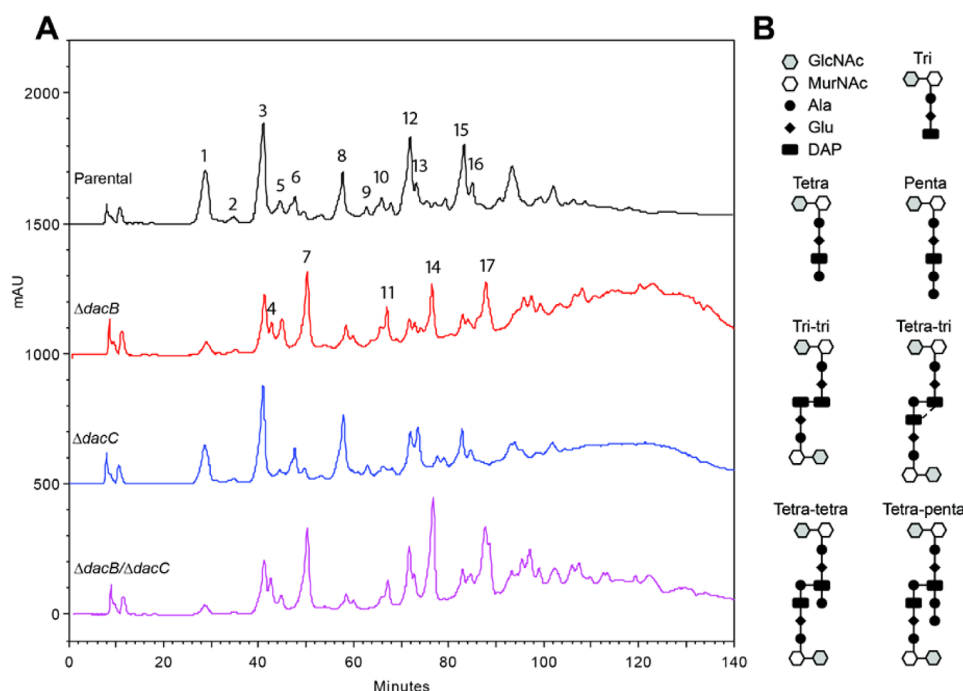


Fig. 1. Peptidoglycan profiling of $\Delta dacB$ and $\Delta dacC$ mutants. Purified sacculi were digested with mutanolysin and soluble fragments were separated by HPLC.

A. Representative PG profiles of the parental, $\Delta dacB$, $\Delta dacC$ or $\Delta dacB/\Delta dacC$ strains. The numbered peaks correspond to the identities listed in Table 1 as determined by mass spectrometry.

B. Representation of major peptidoglycan fragments identified. For simplicity, 1,6-anhydromuramic acid and *O*-acetylation of *N*-muramic acid species are not depicted. Dashed line indicates a possible minor DAP-DAP linked Tetra-tri fragment.

Results

$\Delta dacB$, $\Delta dacC$ and $\Delta dacB/\Delta dacC$ strains have altered cell wall PG profiles

While *N. gonorrhoeae* only has two identified LMM PBPs, a third predicted LMM PBP is expressed (Zielke *et al.*, 2014). Here, we investigated the role of *DacB* and the third predicted LMM PBP, encoded in locus NGO_0443 of *N. gonorrhoeae* FA1090. NGO_0443 protein sequence aligns fully to COG1686 (*DacC*) with an *E*-value of 4.36×10^{-128} and hereafter will be referred to as *dacC* (Altschul *et al.*, 1990; Marchler-Bauer *et al.*, 2017). *DacC* homologs are DD-carboxypeptidases and members of the S11 peptidase family (Kanehisa *et al.*, 2017). An alignment of *N. gonorrhoeae* *DacC* with *DacC* of *E. coli* shows that the three active site motifs (SxxK, SxN and KTG) are not conserved in *N. gonorrhoeae* (Supporting Information Fig. 1) (Chen *et al.*, 2009). As *DacB* and *DacC* are confirmed and predicted LMM PBPs respectively, we constructed loss-of-function mutations in each gene to determine their role in bacterial physiology. The coding sequence of each gene was deleted and replaced with an antibiotic resistance cassette. A double mutant was also constructed. Both single mutants were viable, as was the double mutant.

To determine whether the *DacB* and *DacC* modify the cell-wall PG, high performance liquid chromatography (HPLC) analysis of the PG was conducted. Cell-wall PG was extracted from the $\Delta dacB$, $\Delta dacC$ or $\Delta dacB/\Delta dacC$ mutants, digested by mutanolysin to cleave glycan

strands, and the resulting di-saccharide, peptide fragments were separated by HPLC (Fig. 1). Identities of the individual peaks were confirmed by mass spectrometry analysis and are consistent with those detailed by Antignac *et al.* (2003). Loss of *DacB* resulted in global changes to almost all identifiable peptide fragment peaks. The PG had fewer non-cross linked di, tri and tetra peptides and increased penta peptides and cross-linked tetra-tri and tetra-penta peptides (Table 1). These results confirm previous biochemical analysis characterization of *DacB* as a carboxypeptidase and endopeptidase (Stefanova *et al.*, 2003). Contrastingly, loss of *DacC* altered a smaller subset of PG peptides to a lesser degree. The mutant strain had fewer non-cross-linked peptides, an increase in cross-linked tetra-penta peptides and a large increase in the larger cross-linked fragments with retention times longer than 100 min. The PG composition of the double mutant strain closely resembles that of the single $\Delta dacB$ mutant. Loss of the enzymes together results in PG that is much more heavily cross-linked than that of the parental strain. Loss of *DacC* in conjunction with *DacB* shifted the cross-linked fragments from tetra-tri toward tetra-penta and even larger fragments.

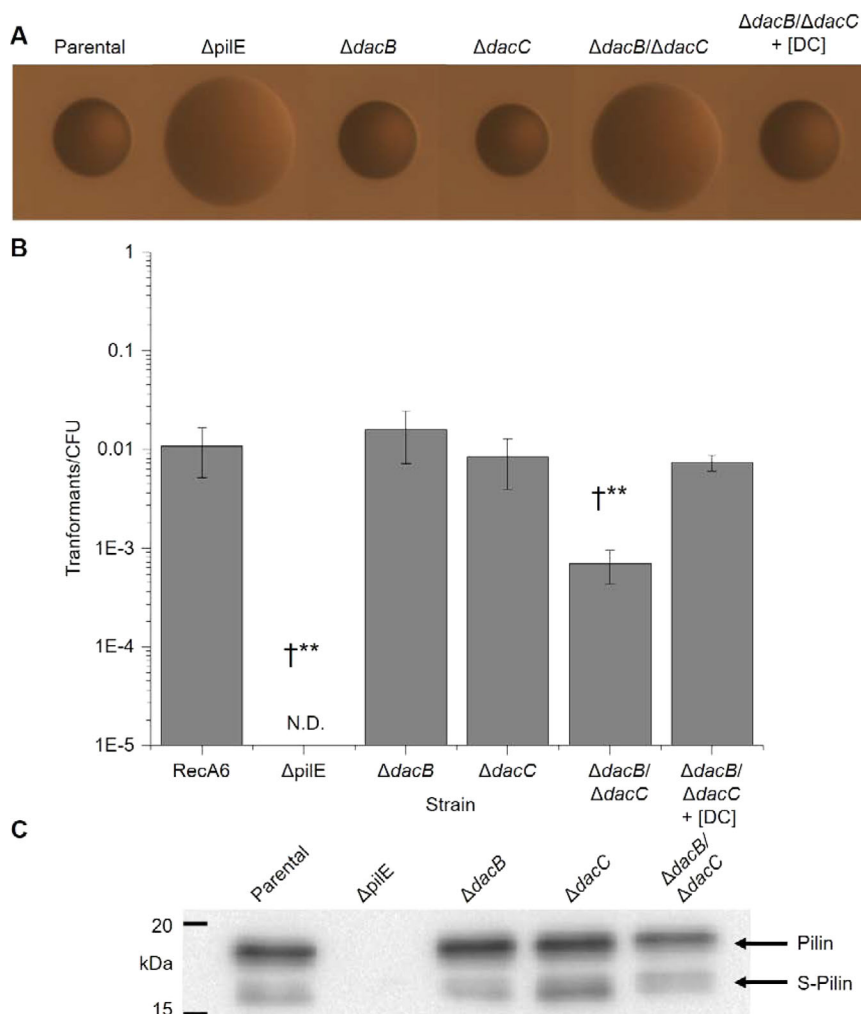
Mutation of *dacB* and *dacC* disrupts Type IV pilus expression

The $\Delta dacB/\Delta dacC$ double mutant strain exhibited a non-piliated colony morphology similar to a *pilE* deletion strain (Fig. 2A). Neither individual mutant displayed an

Table 1. Percent area of peptidoglycan fragments separated by HPLC

Peak	Identity	Parental	$\Delta dacB$	$\Delta dacC$	$\Delta dacB/\Delta dacC$
1	Tri	5.199 \pm 0.718	0.799 \pm 0.121	3.334 \pm 0.552	0.584 \pm 0.060
2	Tetra (Gly)	0.973 \pm 0.106	0.510 \pm 0.095	0.606 \pm 0.080	0.211 \pm 0.029
3	Tetra	9.279 \pm 0.397	3.473 \pm 0.281	7.209 \pm 0.792	2.924 \pm 0.204
4	Penta (Gly)	—	1.283 \pm 0.100	—	1.390 \pm 0.185
5	Di	2.768 \pm 0.307	1.850 \pm 0.300	1.162 \pm 0.104	1.149 \pm 0.178
6	Tri (OAc)	2.593 \pm 0.132	—	2.369 \pm 0.466	—
7	Penta	0.970 \pm 0.212	5.108 \pm 0.187	0.752 \pm 0.117	4.730 \pm 0.620
8	Tetra (OAc)	5.408 \pm 0.326	2.110 \pm 0.049	5.596 \pm 0.722	1.501 \pm 0.095
9	Tri-tri	1.301 \pm 0.321	0.427 \pm 0.063	1.065 \pm 0.101	0.236 \pm 0.094
10	Tetra-tri	2.480 \pm 0.172	3.282 \pm 0.440	1.340 \pm 0.201	1.951 \pm 0.112
11	Penta (OAc)	1.414 \pm 0.036	3.282 \pm 0.440	0.853 \pm 0.088	2.072 \pm 0.165
12	Tetra-tetra	6.395 \pm 1.809	1.480 \pm 0.174	3.936 \pm 0.281	2.811 \pm 1.118
13	Tri (Anh)	3.640 \pm 0.808	1.265 \pm 0.541	4.439 \pm 0.544	1.189 \pm 0.294
14	Tetra-penta	1.605 \pm 0.459	3.873 \pm 0.534	1.703 \pm 0.227	5.926 \pm 1.356
15	Tetra-tetra (OAc)	7.982 \pm 0.778	1.815 \pm 0.114	5.134 \pm 0.776	2.391 \pm 0.884
16	Tetra (Anh)	3.857 \pm 1.226	1.429 \pm 0.177	2.168 \pm 0.128	1.816 \pm 0.453
17	Tetra-penta (OAc)	—	6.249 \pm 0.543	—	7.146 \pm 1.408

Quantification of normalized percent area of peaks and standard deviation of peaks identified in Fig. 1. Reported area is the mean of three independent experiments. Di: disaccharide dipeptide (disaccharide: *N*-acetylglucosamine-*N*-acetylmuramic acid); Tri: disaccharide tripeptide; Tetra: disaccharide tetrapeptide; Penta: disaccharide pentapeptide; Gly: indicates the replacement of an alanine with a glycine at the fourth or fifth position of the peptide stem; OAc: *O*-acetylation on *N*-acetylmuramic acid. Anh: 1, 6-anhydromuramic acid.

**Fig. 2.** Analysis of piliation in $\Delta dacB/\Delta dacC$ *N. gonorrhoeae*.

A. Images of representative colonies grown on GCB solid media for 20 hr and imaged using a stereo microscope. Non-piliated colonies are larger in diameter and are flatter rather than domed, resulting in different refraction of the light source.

B. Transformation efficiencies of *dacB* and *dacC* mutant strains. Reported transformation efficiencies are the mean \pm standard deviation of at least four independent experiments.

N.D. = transformants not detected. † represents statistically significant difference by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test compared to the parental strain $**p < 0.001$. [DC] = Double complement.

C. PilE western blot of whole cell lysates of *dacB* and *dacC* mutant strains. Western blot analysis was performed using the K36 anti-pilin peptide antibody.

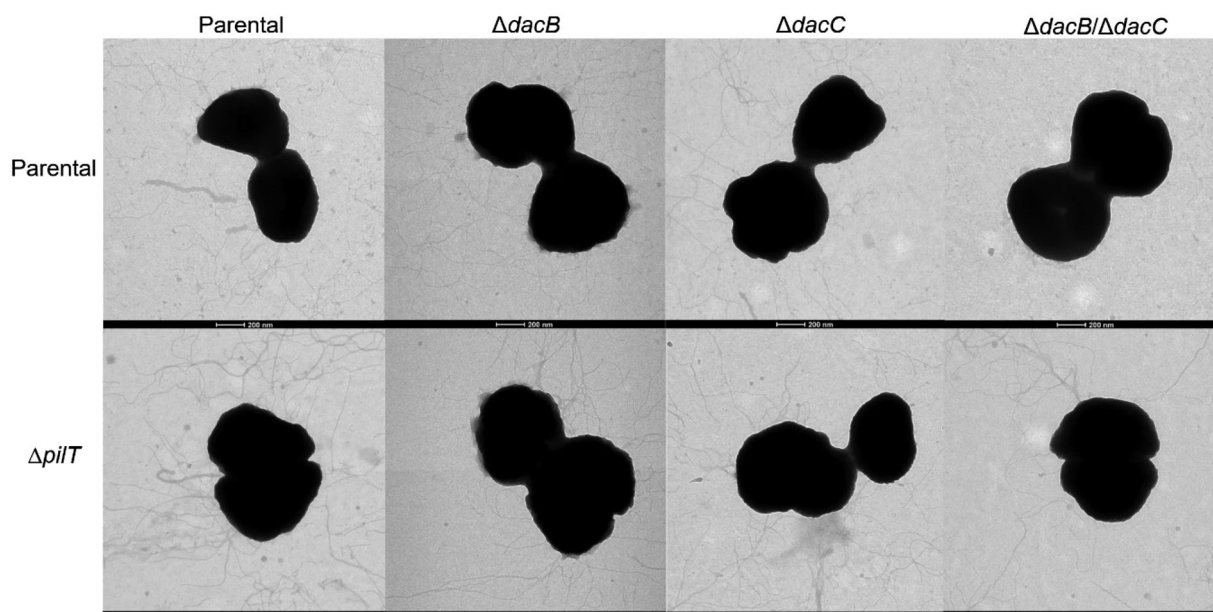


Fig. 3. Analysis of Piliation in $\Delta dacB$ and $\Delta dacC$ mutants.

Micrographs of *N. gonorrhoeae* strains grown for 20 hr on solid medium, negatively stained with uranyl acetate and imaged on a TEM. The top panel has images of the detailed parental strains while the lower panel images are of the detailed strains harboring a *pilT::erm* allele.

altered colony morphology, and complementation with *dacB* and *dacC* under control of their native promoters at an ectopic locus restored the piliated colony morphology. To determine the extent to which piliation was disrupted in the double mutant, we assayed the transformation efficiency of the strains. While loss of either *DacB* or *DacC* individually did not alter transformation efficiency, the double knockout strain exhibited a 94% reduction in transformation efficiency (Fig. 2B). Although this represents a significant drop in transformation efficiency, the double mutant still exhibits relatively high levels of competence compared to the $\Delta pilE$ strain, which is non-transformable. The double complement strain fully restored transformation efficiency. As previously reported, *N. gonorrhoeae* strains with very low levels of pilin expression retain significant levels of transformation competence (Gibbs *et al.*, 1989; Rudel *et al.*, 1995; Long *et al.*, 1998, 2003). Loss of piliated colony morphology and transformation efficiency was not due to different expression levels of PilE as there is only a slight reduction in protein levels in the double mutant compared to the parental strain (Fig. 2C) (Haas *et al.*, 1987). These data show that loss of *DacB* and *DacC* together, reduces piliation but does not completely abrogate pilus function.

To confirm the piliation status of the mutant strains, we imaged the strains using transmission electron microscopy (TEM). This technique allows qualitative analysis of the piliation state of the cell, but quantitative analysis is prevented by the inability to determine which

cell a pilus originates from as well as the inability to differentiate individual pili from different sized pilus bundles. TEM analysis confirmed that both single mutants still expressed numerous pili, and the double mutant had few detectable pili per cell (Fig. 3).

To determine whether loss of both *DacB* and *DacC* disrupts pilus biogenesis or stability, we introduced a *pilT* loss-of-function mutation into the single and double mutant strains. Pilus expression can be restored in many mutants that decrease the number of surface exposed pili by inactivating *pilT*, encoding the ATPase that is required for pilus retraction (Wolfgang *et al.*, 1998a, 1998b). Deletion of *pilT* resulted in increased piliation in all strains (Fig. 3). Piliation of the $\Delta dacB/\Delta dacC$ double mutant was restored to levels apparently similar to that of the parental strain, indicating that the loss of both *DacB* and *DacC* prevents stable pilus expression but that the pilus assembly process is not disrupted.

DacB and *DacC* alter cell fitness and morphology

To determine whether *DacB* and *DacC* play a role in general cell fitness, we assayed the growth of the mutant strains on solid medium by determining the colony forming units (CFU) per colony after 16 hr (Fig. 4A). While loss of *DacB* or *DacC* individually did not alter the growth, loss of both gene products resulted in approximately a twentyfold increase in the number of CFU/colony compared to the wild type strain. This increase in growth is the same magnitude as that displayed by a

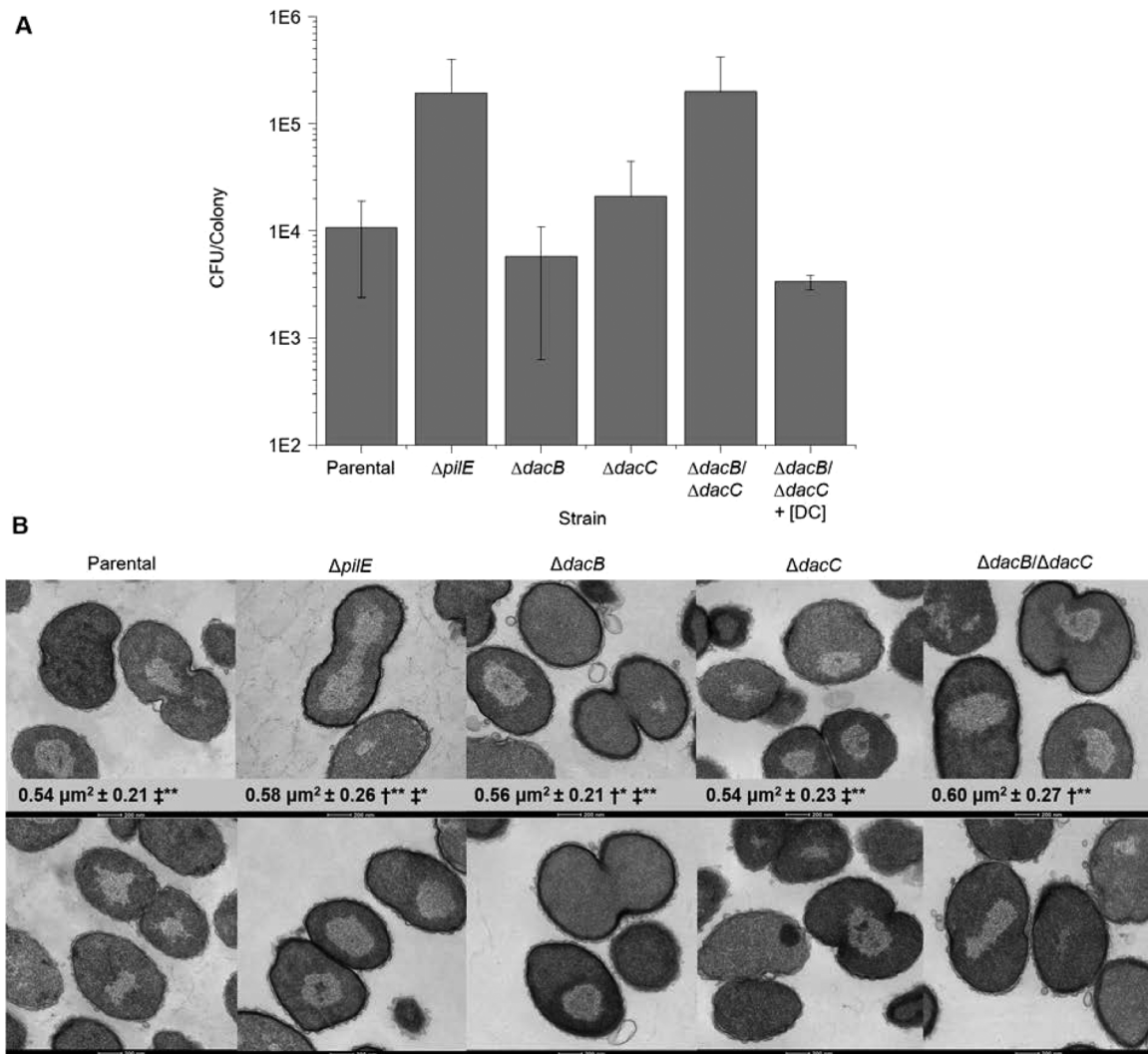


Fig. 4. Cell growth and morphology in $\Delta dacB$ and $\Delta dacC$ mutants.

A. Growth of *N. gonorrhoeae* strains grown on GCB solid media. At 16 hr post inoculation, six individual colonies per strain were picked from plates, diluted in liquid culture, and plated in triplicate to enumerate CFU/colony. Data points are the mean of at least three independent experiments. Error bars represent standard deviation. One-way analysis of variance (ANOVA) indicates statistically significant difference amongst the strains, but Tukey's multiple-comparison test shows no statistical differences between paired strains. [DC] = Double complement. B. Representative electron micrographs of thin sections of *N. gonorrhoeae* strains. Mean cell cross sectional area \pm standard deviation of at least 1,300 individual cells is shown on each micrograph. † ‡ symbols represent statistically significant difference by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test compared to the parental strain (†) and $\Delta dacB/\Delta dacC$ strain (‡). Asterisk indicate degree of significance * $p < 0.05$ ** $p < 0.001$.

$\Delta pilE$ strain, suggesting that rather than directly altering growth, the increased growth in the $\Delta dacB/\Delta dacC$ strain is likely due to the loss of stable pilus expression.

As the murein sacculus is the scaffold on which the cell is shaped, we analyzed cellular morphology using TEM of thin-sections of the parental, $\Delta pilE$, $\Delta dacB$, $\Delta dacC$ and $\Delta dacB/\Delta dacC$ strains (Fig. 4B). While loss of either protein did not result in any gross morphologic abnormalities, the micrographs show increased cross-sectional area of the $\Delta dacB/\Delta dacC$ cells. To analyze the change in cross-sectional area as a proxy for change in cell volume, we quantified the cross-sectional area of at least 1,300 cells

per strain using ImageJ particle analysis. The analysis was gated on cells with a cross-sectional area of at least of 0.3 μm^2 to prevent analysis of slices that transited near the edge of cells. While all cross-sections do not necessarily transect the thickest part of the cell, analyzing over 1,300 cells allows for an appropriate comparison of the average cellular volume. The $\Delta dacB/\Delta dacC$ strain exhibited a cross-sectional area 11% larger than the parental strain, which translates to a calculated 16% increase in total cell volume. While the $\Delta pilE$ strain also showed an increase in cross-sectional area, the magnitude of increase was less than that of the $\Delta dacB/\Delta dacC$ strain. The $\Delta dacB$ strain

Table 2. Antibiotic sensitivities of $\Delta dacB$ and $\Delta dacC$ mutant strains

Antibiotic	Parental	$\Delta pilE$	$\Delta dacB$	$\Delta dacC$	$\Delta dacB/\Delta dacC$	$\Delta dacB/\Delta dacC$ + [DC]
Ampicillin	0.19–0.25	0.125–0.25	0.125–0.38	0.19–0.38	0.064–0.125	0.19–0.25
Ceftazidime	0.064–0.125	0.047–0.094	0.047–0.064	0.064–0.125	0.016–0.032	0.064–0.094
Imipenem	0.064–0.094	0.064	0.064–0.094	0.064	0.023–0.032	0.064–0.094
Spectinomycin	12–16	12–16	12–16	12–24	12–16	16
Erythromycin	0.094–0.125	0.094	0.094–0.125	0.094–0.125	0.064–0.094	N.D.
Nalidixic Acid	0.25–0.38	0.19–0.38	0.19–0.38	0.25–0.38	0.19–0.38	0.25–0.38
Rifampicin	0.023–0.032	0.023–0.032	0.023–0.032	0.023–0.032	0.012–0.023	0.023–0.032
Polymyxin B	64–96	64–96	64	64–96	48–64	64–96

Minimum inhibitory concentrations (MIC) in $\mu\text{g ml}^{-1}$ for *N. gonorrhoeae* strains grown on solid media and exposed to antibiotic E-test strips. MICs are represented as a range of at least three independent experiments. N.D. Not determined due to erythromycin resistance gene present in this strain.

also showed a very small but statistically significant increase in cell size. Together these data demonstrated that PG modification mediated by *DacB* and *DacC* constrains the cell size in FA1090 independent of their effect on piliation.

Antibiotic sensitivity is increased in the $\Delta dacB/\Delta dacC$ strain

To determine the effect of inactivating *DacB* and *DacC* on antibiotic sensitivity, we determined the minimum inhibitory concentration of an array of antibiotics representing eight major classes (Table 2). Cell wall synthesis inhibitors included Ampicillin (Penicillins), Ceftazidime (Cephalosporins) and Imipenem (Carbapenems). Protein synthesis inhibitors included Spectinomycin (Aminoglycosides) and Erythromycin (Macrolides). Nalidixic acid (Fluoroquinolones) inhibits DNA gyrase. RNA synthesis was inhibited by Rifampicin and cell membranes structure was disrupted with Polymyxin B. Loss of *DacC* did not alter antibiotic sensitivity of *N. gonorrhoeae* to any of the tested antibiotics. However, loss of *DacB* slightly increased the susceptibility to the cell wall synthesis inhibitor, Ceftazidime. In contrast, the $\Delta dacB/\Delta dacC$ double mutant strain was slightly more susceptible to all three classes of cell wall synthesis inhibitors tested. These data demonstrate that PG modification by either *DacB* or *DacC* serves to make *N. gonorrhoeae* more resistant to antibiotics that target PG. Moreover, the $\Delta dacB/\Delta dacC$ double mutant strain was also slightly more sensitive to Erythromycin, Rifampicin and Polymyxin B. Complementation of the double mutant restored all antibiotic sensitivities to those displayed by the parental strain. Finally, the $\Delta dacB/\Delta dacC$ double mutant showed increased detergent sensitivity suggesting that it is the changes to the cell wall that provides the alterations in sensitivity (Fig. 5).

When performing western blot analysis, we observed there was less total protein present in lysates from the $\Delta dacB/\Delta dacC$ strain relative to the single mutants or the parental strain. This was despite normalizing the input

cells in each sample. We hypothesized that the increased cross-linking observed in the $\Delta dacB/\Delta dacC$ strain was interfering with SDS-mediated lysis in the bacterial cells. To test this hypothesis, lysozyme was added to the SDS lysis buffer to independently digest the PG cross-links. $\Delta dacB/\Delta dacC$ cells lysed in the absence of lysozyme resulted in 15% less total protein as determined by densitometry of the coomassie stained gels as compared to cells lysed in the presence of lysozyme (Fig. 5A and B). No significant difference was observed in the parental and single mutant strains between the two conditions, and the $\Delta dacB/\Delta dacC$ cells lysed in the presence of lysozyme resulted in the same total protein amounts as the other strains in both conditions.

To investigate if the observed resistance to SDS mediated lysis was biologically relevant, we assayed the sensitivity of the strains to SDS killing. When liquid cultures were exposed to 0.001% SDS for 1 hr, the $\Delta dacB/\Delta dacC$ strain was ten times more sensitive to SDS-mediated killing than the parental and $\Delta dacB$ strains (Fig. 5C). Contrastingly, the $\Delta pilE$ strain was not killed by SDS at this concentration, and the $\Delta dacC$ was killed less than the parental strain, although all strains could be fully killed by 0.01% SDS. Complementation restored resistance to SDS killing to parental levels. Taken together, these results demonstrate that while increased cross-linking of the PG can hold the proteinaceous content of the cell together when exposed to detergent, the increased cross-linking observed in a $\Delta dacB/\Delta dacC$ strain makes FA1090 more susceptible to killing by SDS.

Discussion

We have shown through loss-of-function mutations of *dacB* and *dacC* alone or in combination that mutants deficient for these proteins have distinct PG composition. Characterization of cell-wall PG from the single mutants revealed increased PG cross-linking. The decreased activity in the double mutant resulted in an

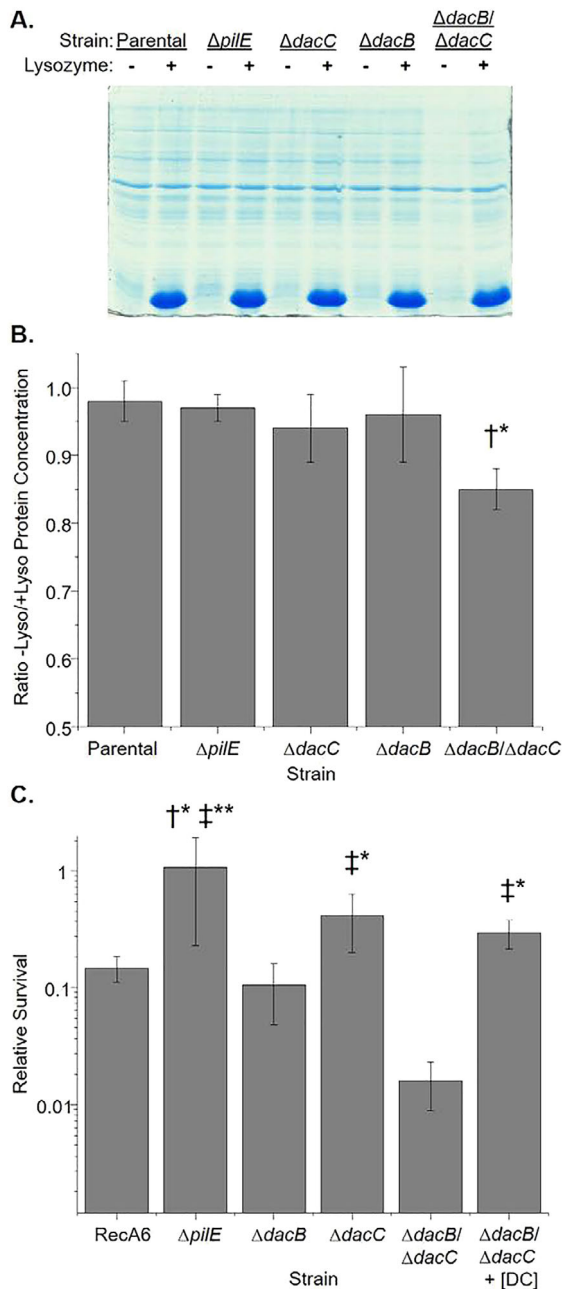


Fig. 5. Detergent sensitivity of $\Delta dacB$ and $\Delta dacC$ mutants.

A. Representative coomassie stained SDS-PAGE gel of protein samples of *dacB* and *dacC* mutants lysed by SDS sample buffer in the presence or absence of lysozyme.

B. Ratio of total protein in samples lysed in SDS sample buffer in the absence of lysozyme to samples lysed in the presence of lysozyme. Total protein was quantified using Image Studio to analyze total protein run in a lane of a SDS-PAGE gel and stained by Coomassie Brilliant Blue. Ratio represents the mean of at four independent experiments.

C. Relative survival of strains exposed to 0.001% SDS for 1 hr. The reported ratio represents the number of CFU enumerated from cultures treated with SDS to the number of CFU enumerated from cultures treated with vehicle alone. Ratio represents the mean of at least three independent experiments. †‡ symbols represent statistically significant difference by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test compared to the parental strain (†) and $\Delta dacB/\Delta dacC$ strain (‡).

even further cross-linked murein sacculus, confirming that these are not redundant activities. Deletion of each protein caused the loss of specific PG modification subsets. These results confirm that *DacB* acts as a carboxypeptidase and endopeptidase and show that even though *DacC* was not identified in radiolabeled penicillin binding assays, it should be classified as a LMM PBP.

The analysis of the $\Delta dacC$ mutant PG showed that while there were obvious changes in the PG cross-linking, the PG structure was more similar to that found with the parental PG. In contrast, the $\Delta dacB$ mutant PG showed extensive increases in PG cross-links. These results suggest that *DacB* acts as a global modifier of PG peptides while the presence of *DacC* results in the alteration of a more specific subset of peptides. The PG profile observed in the *DacB* mutant closely resembles the profile observed when *N. gonorrhoeae* is treated with sub-minimum-inhibitory-concentration doses of beta-lactams (Garcia-Bustos and Dougherty, 1987). While loss of *DacB* drives the majority of the characteristic changes observed in the double mutant PG, concomitant loss of *DacC* is necessary for any large phenotypic changes. This finding is notable as the lack of conserved catalytic residues in *N. gonorrhoeae* *DacC* suggests the protein should be enzymatically inactive (Supporting Information Fig. 1). Our data clearly demonstrate, however, that expression of the *dacC* gene product results in an alteration of the PG. If the protein is indeed catalytically inactive, it may modify the PG profile through direct interaction with PG, PG precursors or an additional PG modifying enzyme. Taken together these data demonstrate that *DacB* and *DacC* each have unique activities within the cell and result in the modification of specific subsets of PG peptides. However, they have largely overlapping effects on cellular biology, as loss of both is required for phenotypes that demonstrate the importance of proper PG processing for normal cellular activities.

Because the study of cell division and PG synthesis has been mainly performed in rod-shaped bacteria, mechanistic insight into coccal growth and cell division is still relatively scarce (Pinho *et al.*, 2013). Therefore, it is hard to predict how loss of *DacB* and *DacC* increases the overall cell volume, but it may involve regulation of the septal synthetic machinery at the division site (Zapun *et al.*, 2008). Consistent with *DacC* playing a specific role in coccal growth, an alignment of *DacC* from different *Neisseria* species shows that the rod-shaped *Neisseria* have *DacC* orthologues that retain the catalytic residues while most coccal *Neisseria* species have at least one defective active site motif (Supporting Information Fig. 2). If the coccal forms were derived from rod-shaped precursors through the modification of PG processing factors, then the loss of *DacC* peptidase

activity may have occurred during the transition from rod to coccus (Veyrier *et al.*, 2015). We cannot determine whether the remaining DacC activity in the coccal *Neisseria* is shared with the DacC from the rod-shaped members of this genus, or whether there were other mutations that altered DacC function in the coccal organisms. The sequence conservation of the NGO_0443 gene product with prototypical members of the DacC family, support continued labeling of the gene product as DacC, but the lack of conserved catalytic residues show that it is not a functional homolog. A detailed structure/function analysis will be required to determine exactly how the different DacC orthologues function in their representative cell types.

Alternatively, the increase in overall cell volume may be due to abnormal PG cross-linking interfering with tethering of the outer membrane to the PG. A recent study in *Salmonella enterica* demonstrated that outer-membrane lipoprotein tethering to the PG is important for proper periplasmic length, and loss of this tethering disrupted osmotic balance between the cytoplasm and periplasm (Cohen *et al.*, 2017). We did observe signs of outer membrane disruption and increased blebbing in thin section electron micrographs of the double mutant (Fig. 4B), although not to the extent observed in the lipoprotein mutant in *S. enterica*.

DacB and DacC expression is important for resistance to multiple classes of antibiotics. The increased susceptibility of the $\Delta dacB/\Delta dacC$ strain to cell wall targeting antibiotics is likely due to the $\Delta dacB/\Delta dacC$ induced cell wall perturbations making the cell less able to resist additional insults to the cell wall synthesis machinery. These data indicate that DacB and DacC play a functionally redundant role in protecting FA1090 (and possibly other gonococci) from beta-lactams. Of particular note is that the double mutant was also more sensitive to non-cell wall targeting antibiotics Erythromycin, Rifampicin and Polymixin B. We presume that the altered cell wall in the $\Delta dacB/\Delta dacC$ double mutant influences steady state levels of these compounds in the cell. The simplest explanation for an increase in cytoplasmic levels of antibiotics is that the barrier function of the PG sacculus is disrupted; However, if this explanation was correct, the sensitivity to most antibiotics should have increased. An alternative hypothesis is that expression or activity of outer membrane porins or efflux pumps is disrupted (Goire *et al.*, 2014). It is also possible that the expression or availability of the targets of these antibiotics is altered in the double mutant, but we consider this option less likely.

Our data demonstrate that pilus functions critical for productive gonococcal infection are dependent on proper expression of either DacB or DacC. These findings contrast to the recent observation that

Pseudomonas aeruginosa Tfp do not require PG-remodeling enzymes for proper pilus expression (Carter *et al.*, 2017). *Pseudomonas aeruginosa*, which is rod shaped and only has polar pili, targets and preinstalls the pilus complexes into nascent poles. As *N. gonorrhoeae* is coccoid and has peritrichous pili, a different strategy appears to be necessary for pilus elaboration. Rather than insertion of the complex into nascent poles prior to PG formation, our data suggest that the peritrichous nature of *N. gonorrhoeae* pili requires specific PG remodeling for proper pilus elaboration.

The $\Delta dacB/\Delta dacC$ strain exhibited both an increased cell volume and increased growth relative to the parental strain or either individual mutant. We presume these phenotypes are partially dependent on the loss of stable pilus expression as the $\Delta pilE$ strain also shows similar changes, but we do not presently have a direct link of the change in cell volume to the loss of pilus elaboration. One possibility to explain these phenotypes is that there is an increase in ATP availability when pili are lost. Assembly and retraction of the pilus requires the sequential insertion or removal of pilin subunits and each subunit step is predicted to require hydrolysis of two ATP molecules (Chang *et al.*, 2016; McCallum *et al.*, 2017). As pili consist of thousands of subunits per fiber, extension and retraction of large numbers of pili per cell would be energetically expensive. Hence, cells not having to waste energy on pilus dynamics could put more energy toward cell growth and division.

As the single mutants have no phenotype with respect to pilus expression, either DacB or DacC activity is sufficient to modify the cell wall to allow stable pilus expression. An additional *N. gonorrhoeae* carboxypeptidase/endopeptidase, Mpg (Stohl *et al.*, 2013), acts on PG cross-links and is also required for stable Tfp expression. This Zinc metalloprotease also acts on the PG side chains, but there is no change in bulk PG, suggesting the Mpg activity is localized to the pilus complex. As with the $\Delta dacB/\Delta dacC$ mutant, the piliation defect observed in the *mpg* mutant strain can also be rescued by mutation of the *pilT* gene. Pilus expression can be restored in a subset of pilus complex mutations that decrease the number of surface exposed pili by inactivating *pilT* (Wolfgang *et al.*, 1998b). As pili are undergoing frequent cycles of extension and retraction, deletion of the ATPase responsible for retraction can increase the number of pili per cell by locking all elaborated pili in the extended position. Non-stable pili are also retracted through the action of PilT, and thus, deletion of the ATPase prevents retraction of non-stable pilus fibers and results in an increased number of surface exposed pili. Taken together the data from these studies as well as the Mpg studies clearly demonstrate a critical

role for PG modification in pilus biogenesis in *N. gonorrhoeae*.

One possible explanation for the requirement for PG modification is that it is necessary to create holes in the PG layer through which the macromolecular Tfp complex can assemble. This would be consistent with the lack of PG-modifying enzymes required in *P. aeruginosa*, as the complex can form prior to cell wall formation (Carter *et al.*, 2017). However, as neither loss of Mpg cleavage nor loss of DacB and DacC activity abrogated all piliation and a large number of pili could be restored in $\Delta pilT$ strains, it is unlikely that the sole function of PG modification in pilus biogenesis by these peptidases is to cut holes in the cell wall to allow proper pilus complex assembly. While limiting the amount of cross-linking probably plays a role in allowing for type IV complexes to assemble through the cell wall, disruption of PG remodeling by loss of DacB and DacC as well as Mpg may alter the PG structure in a manner that disrupts anchoring to the cell wall. This loss of stability would result in fewer pili per cell due to increased PilT-mediated retraction and help account for the restoration of piliation in the *pilT* mutant. PilQ levels were not reduced in either single mutant or the double mutant; therefore PilQ assembly does not explain this phenotype (Supporting Information Fig. 3) (Helm *et al.*, 2007). Regardless of the mechanism, it is clear that PG modification is necessary for proper type IV complex expression. Taken together, these data demonstrate the critical role DacB and DacC mediated PG modification plays in normal *Neisseria* physiology. The fact that loss of both activities is necessary to produce an observable phenotype shows that a major shift in the PG cross-linking is required to alter the role(s) of the cell wall in cellular processes and underscores the importance of PG remodeling for maintaining cell wall architecture to allow for proper elaboration of fundamental cellular processes.

Experimental procedures

Bacterial strains and growth

All experiments were performed using derivatives of strain FA1090 with the 1–81-S2 *pilE* sequence and an IPTG inducible *recA6* allele (Seifert *et al.*, 1994; Seifert, 1997). The $\Delta pilE$ strain contains a deletion from the upstream silent copy through the coding region of *pilE* including the promoter and ribosome binding site (Chen *et al.*, 2004). The $\Delta pilT$ strains contain a *pilT::erm* allele described previously (Wolfgang *et al.*, 1998a). For *N. gonorrhoeae* growth, solid media was GC Medium Base (Difco) plus Kellogg supplements I and II (GCB) at 37°C with 5% CO₂, and liquid medium was GCB Liquid Broth (GCBL: 1.5% peptone protease no. 3 [Difco], 0.4% K₂HPO₄ [Fisher], 0.1% KH₂PO₄ [Fisher], 0.1% NaCl [Fisher]) plus Kellogg supplements I

and II and 0.042% sodium bicarbonate (GCBL+). Antibiotic resistant *N. gonorrhoeae* were selected on the following antibiotics and concentrations: Chloramphenicol (Cm) 1 µg ml⁻¹, Kanamycin (Kan) 50 µg ml⁻¹, Nalidixic Acid (Nal) 0.75 µg ml⁻¹ and Erythromycin (Erm) 2 µg ml⁻¹. *E. coli* were grown on Luria-Bertani (LB) agar or broth at 37°C. Plasmids were propagated in *E. coli* 10G ELITE electrocompetent *E. coli* (Lucigen) or BH10C containing a mutated *pcnB* gene for low plasmid copy number (Howell-Adams and Seifert, 2000). Antibiotic resistant *E. coli* were selected on the following concentrations: Kanamycin (Kan) 50 µg ml⁻¹, Ampicillin (Amp) 100 µg ml⁻¹, Chloramphenicol (Cm) 20 µg ml⁻¹.

Construction of $\Delta dacB$, $\Delta dacC$, $\Delta dacB/\Delta dacC$

PCR was used to amplify the upstream and downstream region of NGO_0443 (*dacC*) and NGO_0107(*dacB*) using primers KP264–267 and KP272–275 respectively. The 3' primer of the upstream region and 5' primer of the downstream region contained complementary sequence including a KpnI restriction site. This region of complementarity was used in a splicing overlap extension PCR (SOE-PCR) to combine the upstream and downstream regions with a KpnI restriction site in-between. This construct was cloned into pSMART LCamp (Lucigen) following manufacturer's instructions and introduced in *E. coli* by electroporation. Following confirmation by sequencing, the resulting plasmids (pKP152, 154) were isolated using the QIAprep Spin Miniprep Kit (Qiagen), digested with KpnI (NEB) and gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The Kan resistant *nptII* gene was digested from plasmid pBSL86 using KpnI and gel purified. The *nptII* allele was cloned into the KpnI digested plasmids using T4 DNA ligase (NEB), electroporated into *E. coli* and selected for on Amp and Kan. For use in the double mutant, a Cm resistance cassette was digested from plasmid (pKP133) using KpnI and cloned into the NGO_0443 upstream and downstream containing plasmid (pKP152) resulting in plasmid (pKP157). Sequencing confirmed the plasmid sequences and the *nptII* containing plasmids were used to transform the parental strain with selection on Kanamycin. Deletion was confirmed by PCR using primers KP293–294 ($\Delta dacC$) and KP297–298($\Delta dacB$). The double mutant was made by transforming the Kan resistant $\Delta dacB$ with the *dacC::cat* containing plasmid (pKP157).

Construction of $\Delta dacB/\Delta dacC$ double complement

NGO_0443(*dacC*) and the flanking region was PCR amplified using primers KP309 and KP310 containing an AatII and a SpeI restriction site respectively. NGO_0107(*dacB*) and the flanking region was PCR amplified using primers KP311 and KP312 containing a SpeI and a PacI restriction site respectively. Each amplicon was cloned separately into pSMART LCKan and electroporated into *E. coli*. The sequence confirmed *dacC* containing plasmid (pKP178) was digested with AatII and SpeI, the *dacB* containing plasmid (pKP179) was digested with SpeI and PacI. The *dacC* and *dacB* containing fragments were gel purified and

cloned into AatII and PacI cut pGCC2 in a single ligation reaction (Mehr *et al.*, 2000). The ligation reaction was electroporated into DH5 α *pcnB*. The resulting plasmid pKP180 was confirmed by PCR amplification and sequencing and was transformed into the $\Delta dacB/\Delta dacC$ strain with selection on Erm for correct chromosomal insertion between *lctP* and *aspC*. Transformants were verified by PCR amplification and sequencing.

Spot transformation of *N. gonorrhoeae*

Constructs were transformed into *N. gonorrhoeae* through coculture on solid media (spot transformation). Strains to be transformed were plated from frozen stocks on GCB solid media and grown for 18 hr. Several isolated colonies were streaked as lawns onto GCB solid media supplemented with 1 mM IPTG, 10 μ l of DNA containing solution was mixed with 10 μ l GCBL containing supplements I and II and 5 mM MgSO₄ and was immediately spotted onto the lawn. Following incubation for 20 hr at 37°C, the bacteria from the area where the DNA solution was spotted were suspended in GCBL and dilutions were plated on GCB solid media containing the proper selective antibiotics.

Cell wall PG characterization by HPLC and mass spectrometry

Triplicate cultures of each strain were grown to exponential phase in amended GCBL. PG was isolated as described previously (Dillard and Hackett, 2005). Briefly, cells were spun down at 3,800 g for 10 min at 4°C and washed twice with cold 25 mM sodium phosphate buffer pH6. Following resuspension in cold, 10 ml phosphate buffer, the cells were added drop wise to an equal volume of boiling 8% SDS solution, boiled for 1 hr and spun down for at 30,000 g for 30 min at 15°C. Boiling and centrifugation were repeated. The pellet was washed 5 times in 10 ml of phosphate buffer following centrifugation at 30,000 g for 30 min. PG was collected through ultracentrifugation for 30 min at 162,000 g with resuspension in 3 ml phosphate buffer. Cell wall PG was digested for 48 hr in 20 μ g ml⁻¹ mutanolysin (Sigma) and Amico-Ultra 10 kDa filters were used to eliminate insoluble fragments. Soluble PG was reduced with 10 mg ml⁻¹ sodium borohydride in 0.5 M borate buffer pH = 8 for 20 min with the reaction stopped by lowering sample pH to 2 using α -phosphoric acid. HPLC separation of soluble PG fragments was performed as previously described (Dougherty, 1985). Fractions of individual peaks were collected and desalted by rp-HPLC using an acetonitrile gradient. Fractions of interest were analyzed by positive ion electrospray ionization time-of-flight mass spectrometry at the University of Wisconsin Biotechnology Center.

Transformation assays

Strains were plated for lawn growth on GCB solid media and grown for 20 hr. Lawns were swabbed into 1 ml transformation media (GCBL, 1 mM IPTG, 5 mM MgSO₄ and Kellogg supplements I and II, pH7.2) using a sterile

polyester swab (Puritan) to an optical density (OD) at 550 nm of approximately 1.5. Twenty microliter of cell suspension was added to 200 μ l of transformation media containing 150 ng of pSY6, a plasmid containing the *gyrB1* allele conferring resistance to Nal (Stein *et al.*, 1991). The cell and DNA suspensions were incubated at 37°C for 20 min prior to dilution into 2 ml of 37°C transformation media. Cells were incubated for 4 hr at 37°C, 5% CO₂ before serial dilution. Ten microliter of serial dilutions were plated in triplicate on GCB solid media and GCB solid media containing Nal. Reported transformation efficiencies are the ratio of antibiotic resistant CFUs to total CFUs and are the mean of at least three replicate experiments.

Imaging of colony morphology

Strains were grown on GCB solid media for 20 hr and imaged using a Nikon DS-Fi1 camera attached to a Nikon SMZ-10A stereo microscope.

Measuring growth on solid media

Cells were plated on GCB from frozen stocks and grown for 20 hr. Several isolated colonies were suspended in GCBL and dilutions were plated on GCB for isolated colony growth. At 16 hr post inoculation, six single colonies per strain were picked using a sterile 6 mm filter disk (GE Healthcare) and dispersed in 500 μ l GCBL by vortexing. Serial dilutions of 10 μ l were spot plated in triplicate and the resulting colonies enumerated.

Transmission electron microscopy

Whole cell TEMs were taken from preparations as previously described (Oberfell and Seifert, 2016). Briefly, strains were grown on solid media and adhered to 300 mesh nickel grids. Cells were fixed with PFA and glutaraldehyde and negatively stained with uranyl acetate. Thin-section micrographs were taken from samples prepared by Northwestern's Center for Advanced Microscopy. Strains were plated for lawn growth on GCB solid media and grown for 20 hr. Two plates per strain were swabbed into 1 ml phosphate buffered saline pH7.4 (PBS) and pelleted at 14,000 g for 1 min. The pellet was suspended in 1% paraformaldehyde in PBS and kept at room temperature (RT) for 1 hr. Cell culture samples were fixed in 0.1 M sodium cacodylate buffer (pH 7.3) containing 2% paraformaldehyde and 2.5% glutaraldehyde and were postfixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer. They were then rinsed with distilled water, en bloc stained with 3% uranyl acetate, rinsed a second time with distilled water, dehydrated in ascending grades of ethanol, transitioned with propylene oxide, embedded in the resin mixture of the EMbed 812 kit and cured in a 60°C oven. Samples were sectioned on a Leica Ultracut UC6 ultramicrotome. Thin sections of 70 nm were collected on 200 mesh copper grids, post stained with 3% uranyl acetate and Reynolds lead citrate. All imaging was performed on a FEI Tecnai

Spirit G2 120-KV with digital images captured on FEI Eagle camera at Northwestern's Center for Advanced Microscopy.

Quantification of average *N. gonorrhoeae* cross-sectional area

Thin-section TEMs were imaged at 1,900–2,900 \times magnification. Images were imported into ImageJ, converted to binary format, inverted and particle size was analyzed with exclusion on the edges and gating on a cross-sectional area between 0.3 and 2.0 μm^2 to eliminate sections through the edges of cells and cell clusters. Reported cell cross-sectional area is the mean \pm standard deviation of at least 1,300 cells imaged.

Western blots

Protein was isolated from cell lysates of *N. gonorrhoeae* strains grown as lawns on GCB solid media for 20 hr. Bacteria was swabbed into 1 ml PBS, spun down at 4,000 g for 5 min and washed with 0.5 ml PBS. Cells were suspended in 550 μl PBS and 50 μl of the cell suspension was reserved for BCA analysis. SDS lysis buffer (60 mM Tris-HCl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue, pH6.8) and 1 mg ml⁻¹ chicken egg white lysozyme was added to the cell suspension and rotated for 2 hr at 4°C. Samples were passed through a needle to shear DNA and boiled for 5 min. Samples were loaded and run on SDS-Page gels and blotted as described previously (Obergfell and Seifert, 2016). K36 α -PilE peptide antibody (Forest *et al.*, 1996) was used at 1:50,000 dilution as the primary antibody and Peroxidase-conjugated AffinPure Gt α -Rabbit IgG (Jackson ImmunoResearch) was used as the secondary antibody at 1:10,000 dilution.

For the PilQ blot, protein samples were normalized by coomassie quantification and were run on a Mini-PROTEAN TGX 4–15% gel (BioRad). PilQ antibody was used at a dilution of 1:10,000 (graciously provided by C. E. Wilde, Indiana University School of Medicine) and Peroxidase-conjugated AffinPure Gt α -Rabbit IgG was again used as the secondary antibody. Quantification of blots was done using ImageJ.

Antibiotic sensitivity testing

Minimum inhibitory concentrations (MIC) were assayed using Etest strips (Biomérieux). *N. gonorrhoeae* was grown in lawn cultures for 20 hr and suspended in GCBL to an OD₅₅₀ of approximately 1.0. Cell suspensions were diluted 1:4 into 48°C GCB-Top agar (23.2 g GCB in a 1:5 dilution of GCBL). Four milliliter of GCB-Top-agar, cell suspensions were spread onto 37°C GCB agar plates and allowed to solidify at RT for 15 min. A single E-test strip was placed in the center of each plate. Following incubation at 37°C, 5% CO₂ for 20 hr the zone of clearing was used to determine the MIC for each strain and each antibiotic. Reported MICs are the mean of at least three independent experiments.

Assaying detergent mediated lysis

Lawn growths of strains were grown for 20 hr on GCB solid media and suspended in 1 ml PBS. Cells were centrifuged at 14,000 g and suspended in 1 ml PBS two times. A 50 μl aliquot was taken for each strain and used to determine the total protein concentration using a bicinchoninic acid assay (Peirce BCA Assay). Cells were then centrifuged at 14,000 g and resuspended to a normalized concentration based on the BCA results. Cells were then lysed in SDS lysis buffer \pm 1 mg ml⁻¹ chicken egg white lysozyme for 2 hr rotating at 4°C. Samples were then passed through a small bore needle to shear genomic DNA, boiled for 5 min and equal volumes were loaded and electrophoresed on 10% SDS-PAGE gels. Total protein was visualized using Coomassie Brilliant Blue and quantified using Image Studio (LiCor).

SDS sensitivity testing

Strains were grown for 22 hr on GCB solid media and 20 colonies were picked and struck for lawn growth on fresh GCB solid media. Lawns were grown for 8 hr, suspended in 6 ml amended GCBL, and incubated at 30°C overnight in a rotating drum. Liquid cultures were back diluted 1:2 in fresh amended GCBL and grown for 2.5 hr at 37°C in a rotating drum. Cultures were back diluted to an OD₅₅₀ of 0.07 and grown for 4 hr at 37°C in a rotating drum. Nine hundred microliter of culture from each strain was incubated with either 100 μl PBS or 0.001% SDS in PBS for 1 hr at 37°C in a rotating drum. Cultures were serially diluted in GCBL and 10 μl of serial dilutions were spotted on GCB solid media in triplicate. CFUs were enumerated and sensitivity was reported as the ratio of CFUs of SDS treated culture to CFUs of PBS treated cultures. The reported ratio represents the mean of at least three independent experiments.

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