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Hanuszkiewicz, A., Pittock, P., Humphries, F., Moll, H., Rosales, A. R., Molinaro, A., ... Valvano, M. A. (2014). Identification of the Flagellin Glycosylation System in Burkholderia cenocepacia and the Contribution of Glycosylated Flagellin to Evasion of Human Innate Immune Responses. Journal of Biological Chemistry, 289, 19231-19244. DOI: 10.1074/jbc.M114.562603

Published in:
Journal of Biological Chemistry

Document Version:
Peer reviewed version

Identification of the Flagellin Glycosylation System in *Burkholderia cenocepacia* and the Contribution of Glycosylated Flagellin to Evasion of Human Innate Immune Responses

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Running title: Flagellin Glycosylation in *B. cenocepacia*

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Keywords: flagellin, glycosylation, cystic fibrosis, inflammation, TLR5

Background: The role of flagellin glycosylation is not well understood.
Results: The *Burkholderia cenocepacia* flagellin is glycosylated on at least ten different sites.
Conclusion: The presence of glycan in flagellin significantly impaired the inflammatory response of epithelial cells.
Significance: Flagellin glycosylation reduces recognition of flagellin by host TLR5, providing an evasive strategy to infecting bacteria.

ABSTRACT

***Burkholderia cenocepacia* is an opportunistic pathogen threatening patients with cystic fibrosis. Flagella are required for biofilm formation, as well as adhesion to and invasion of epithelial cells. Recognition of flagellin via the Toll-like receptor 5 (TLR5) contributes to exacerbate *B. cenocepacia*-induced lung epithelial inflammatory responses. In this study, we report that *B. cenocepacia* flagellin is glycosylated on at least ten different sites with a single sugar, 4,6-dideoxy-4-(3-hydroxybutanoylamino)-D-glucose [D-Qui4N(3HOBut)]. We have identified key genes that are required for flagellin glycosylation including a predicted glycosyltransferase gene that is linked to the flagellin biosynthesis cluster, and a putative acetyltransferase gene located within the O-antigen lipopolysaccharide cluster. Another O-antigen cluster gene, *rmlB*, which is required for flagellin glycan and O-antigen biosynthesis, was essential for bacterial viability, uncovering a novel target against**

***Burkholderia* infections. Using glycosylated and non-glycosylated purified flagellin and a cell reporter system to assess TLR5-mediated responses, we also show that the presence of glycan in flagellin significantly impair the inflammatory response of epithelial cells. We therefore suggest that flagellin glycosylation reduces recognition of flagellin by host TLR5, providing an evasive strategy to infecting bacteria.**

Burkholderia cenocepacia is a Gram-negative bacterium belonging to the *Burkholderia cepacia* complex (Bcc). This group of opportunistic pathogens poses a health threat to patients with cystic fibrosis (1,2). Chronic airway infection of these patients with the Bcc bacteria, particularly *B. cenocepacia*, accelerates the decay of lung function and in some cases leads to a lethal necrotizing pneumonia known as "cepacia syndrome" (3). Bcc infections have also been reported in nosocomial outbreaks not related to cystic fibrosis (4-7). Together with *B. multivorans*, *B. cenocepacia* accounts for the majority of Bcc infections in cystic fibrosis patients (8,9). *B. cenocepacia* encompasses at least four phylogenetic lineages, IIIA to IIID, but most of the CF isolates belong to lineage IIIA and IIIB (10,11). The clonal lineage ET12 belongs to the IIIA group and these bacteria were responsible for most of the deaths related to "cepacia syndrome" in early 1980s (3,12,13).

B. cenocepacia K56-2 is an ET12 strain that carries various virulence factors including lipopolysaccharide (LPS) and flagella. The LPS from K56-2 has been intensively studied in our laboratory (14-18) and consists of lipid A, core oligosaccharide, and polymeric O antigen (19). The K56-2 O antigen is a polymer of a trisaccharide-repeating unit containing rhamnose and two *N*-acetylgalactosamine residues (15). In general, LPS is a potent proinflammatory molecule, and the K56-2 O antigen influences phagocytosis by human macrophages and interferes with bacterial adherence to bronchial epithelial cells (18,20).

Flagella are organelles for bacterial motility, but they are also involved in pathogenicity (21) such as adhesion to and invasion of epithelial cells, and biofilm formation (22-26). Flagella consist of a basal body, flagellar hook, and a filament built of flagellin monomers, which are specifically recognized by the innate immune system via the Toll-like receptor 5 (TLR5) (26,27). Toll-like receptors are membrane-bound pattern-recognition receptors in epithelial and immune cells, which play an essential role in initiating innate immune responses (28). TLRs recognize pathogen-derived microbial molecules (pathogen-associated molecular patterns) like LPS (TLR4) or flagellin (TLR5). Engagement of TLR by its specific ligand initiates an intracellular signaling cascade leading to the activation of nuclear factor κB (NF-κB) and members of the mitogen-activated protein (MAP) kinase family. These signaling pathways subsequently activate transcription of pro-inflammatory cytokines like interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor α (TNF-α). The TLR5 signalling pathway plays a pivotal role in exacerbating lung inflammation in cystic fibrosis (29) and it is responsible for *B. cenocepacia*-induced lung epithelial inflammatory response (30). Furthermore, a mutation leading to reduced activating capacity of the TLR5 was associated with reduced organ failure and improved survival in patients infected with *B. pseudomallei*, another important pathogen of the genus *Burkholderia* (31), underscoring the critical role of TLR5 and its ligand in human infection.

B. cenocepacia strains produce two types of flagellin, type I and II, distinguished by the molecular size of the protein and restriction fragment length polymorphism analyses (32). Flagellin in *B. cenocepacia* K56-2 belongs to type II and these bacteria carry a single, long polar flagellum that contributes to virulence in a mouse infection model and induces host immune responses via TLR5 (26). *B. pseudomallei* and *B. thailandensis* produce glycosylated flagellin (33), but the glycosylation status of flagellin in *B. cenocepacia* is unknown. In this work, we report that *B. cenocepacia* flagellin filaments are posttranslationally modified by glycosylation at multiple sites with a single glycan residue and identify the key genes responsible for this modification. We also demonstrate that flagellin glycosylation reduces the ability of this protein to trigger TLR5-mediated inflammatory responses in epithelial cells.

EXPERIMENTAL PROCEDURES

Strains and Chemicals–The strains used in this study are listed in Table 1. Bacteria were grown either on 1.5% agar plates or in LB Broth (Lennox) at 37°C. When required, antibiotics were added as follows: trimethoprim, 50 µg ml^{-1} for *E. coli* and 100 µg ml^{-1} for *B. cenocepacia*; tetracycline, 20 µg ml^{-1} for *E. coli* and 100 µg ml^{-1} for *B. cenocepacia*; kanamycin, 40 µg ml^{-1} for *E. coli*; chloramphenicol, 30 µg ml^{-1} for *E. coli* and 150 µg ml^{-1} for *B. cenocepacia*. Ampicillin at 200 µg ml^{-1} was used during triparental mating to selectively eliminate donor and helper *E. coli* strains. When required, rhamnose was added to a final concentration of 0.4% (w/v). Sucrose plates for the final curing of deletion mutants were prepared with 10 g l^{-1} of tryptone, 5 g l^{-1} of yeast extract and 50 g l^{-1} of sucrose in 1.5% agar. Antibiotics and chemicals were purchased from Sigma Chemicals (St Louis, MO, USA). Growth media were purchased from Becton, Dickinson and Company (Sparks, MD, USA). Restriction enzymes, Antarctic phosphatase and T4 ligase were purchased from New England Biolabs (Ipswich, MA, USA). HEK293-TLR5 cells expressing human TLR5 were purchased from Invivogen (San Diego, USA) and p-P65, p-ERK, ERK, p-P38, P38, p-JNK and JNK antibodies from Cell Signalling (Danvers, MA, USA). P65 was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and β-actin antibody from Sigma-Aldrich (St Louis, MO, USA).

Isolation of Flagellin–Flagella were isolated as in Brett *et al* (34) with some modifications. Briefly, bacteria were grown for 18 h in 400 ml LB with antibiotics and/or rhamnose as required, centrifuged, and the bacterial pellets frozen at -20°C overnight. Thawed pellets were next resuspended in 20 ml PBS and flagella were sheared off with a homogenizer (low speed setting for 4 min on ice). Cell debris was removed by centrifugation (6,000 x g, 10 min, 4°C) and flagella were precipitated overnight from the supernatant with ammonium sulphate (end concentration 5%). The precipitate was centrifuged (12,000 x g, 30 min, 4°C) and the supernatant discarded. The pellet, containing flagella, was dissolved in 750 µl PBS of which 250 µl were stored at -20°C for SDS PAGE analysis (crude flagellar filaments fraction) and the remaining 500 µl were centrifuged again (16,900 x g, 10 min, 4°C). Flagellar filaments in the sediment were solubilized with 8 M urea; insoluble debris was removed by centrifugation (10,000 x g, 1 min) and the solubilized flagellin was desalted on a HiTrap ÄKTA FPLC column (GE Healthcare) using either 25 mM ammonium bicarbonate (prior to structural analyses) or PBS (for biological tests) as eluents. Soluble and purified flagellin was either stored at -20°C or lyophilized.

SDS-PAGE and Western Blot–The purity and the molecular mass of flagellin were assessed in 14% SDS-PAGE gels stained with PageBlue protein staining solution (Thermo Scientific). BioRad Precision Plus Dual Color Protein Standard was used as a molecular weight marker. To visualize glycosylated proteins, the Pro-Q Emerald glycoprotein stain kit was used accordingly to the manufacturer's manual (Molecular Probes). Flagellin was detected on Western blots with primary polyclonal antibody RFFL/ARP42986_P050 (http://www.avivasysbio.com/rffl-antibody-middle-region-arp42986-p050.html) provided by AVIVA Systems Biology; San Diego, USA and with secondary goat anti-rabbit IgG-HRP secondary antibody. The blots were developed with Western Lightning ECL Pro (Perkin-Elmer).

Mass Spectrometry and Enzymatic Digestion–Flagellin was in-gel digested with trypsin, chymotrypsin, AspN and a mixture of AspN and trypsin. LC MS/MS mass spectrometry analyses were performed on a Waters QTof Global mass spectrometer equipped with a Z-spray (ESI) source and run in positive ion mode (the instrument was run in DDA mode) in combination with a Waters nanoAcquity UPLC, and the results were confirmed with a Thermo Scientific Orbitrap Elite MS (LC-MS/MS). The Peaks software (Bioinformatics Solutions Inc.) was used to analyse the digested samples. Waters QTof Micro with Waters MassLynx 4.1 was used for whole protein analyses. Flagellin was analysed as an intact protein in 25 mM ammonium bicarbonate. Lyophilized, digested samples were

reconstituted in 20 µl of 0.2% formic acid in water and 10 µl were injected.

Chemical Deglycosylation of Flagellin– Desalted and lyophilized protein (1.5 mg) was chemically deglycosylated by trifluoromethanesulfonic acid (TFMS) (35). Briefly, 100 µl of a 10% toluene/TFMS mixture was slowly added to the sample in a glass vial placed in a dry ice/ethanol bath. After 2 h, the mixture was carefully neutralized with 300 µl pyridine solution (pyridine: methanol: water at a ratio of 3:1:1 v:v:v) for 5 min in a dry ice/ethanol bath, and the sample was transferred to wet ice for another 15 min. The mixture was transferred into a plastic 1.5 ml vial and 400 µl of 25 mM ammonium bicarbonate was added to precipitate the deglycosylated flagellin. After centrifugation (16,900 x g, 10 min), the supernatant was discarded and the pellet dissolved in 8 M urea. Further desalting in 25 mM ammonium bicarbonate was performed on a HiTrap column as described above and the sample was used directly for MS analysis.

GC/MS Analyses and β-Elimination– Methanolysis was used to analyze the glycan moiety of flagellin. Briefly, 400 µg of the lyophilized sample was treated with 0.5 M methanolic HCl (weak methanolysis), peracetylated and an aliquot was used to record GC/MS spectra. Next, the same sample was treated with 2 M methanolic HCl (strong methanolysis), peracetylated and analyzed again. To determine the character of bound glycosyl residue, another 400 µg of lyophilized flagellin was used for β-elimination. Briefly, 400 µg of lyophilized sample were treated with 0.1 M NaOH containing 0.8 M $NaBH_4$ for 8 h at 37°C in the dark. Next, the mixture was dried under nitrogen, peracetylated and analyzed. To confirm the conformation of the sugar, ions detected in GC/MS spectra from *B. cenocepacia* FliC were compared with GC/MS of the O antigen sample from *Providencia stuartii* O43 (kindly provided by J. Knirel and O. Ovchinnikova). The D-configuration of the sugar was determined by octanolysis (36). Mass spectrometric measurements were performed with Agilent Technologies 5975 inert XL MSD equipped with split/splitless injector system with EI under autotune conditions at 70 eV.

General Molecular Techniques and Genetic Manipulation of B. cenocepacia–Plasmid vectors and primers are listed in Table 1 and Table 2, respectively. DNA manipulations and cloning were performed as previously described (37). PCR reactions were performed with HotStar HiFidelity DNA Polymerase (Qiagen). Plasmid and genomic DNA were isolated using QiaPrep Spin kit and DNeasy Blood and Tissue kit (Qiagen), respectively. PCR products were purified using a QIAquick PCR purification kit or a QIAquick gel extraction kit (Qiagen). Freshly prepared chemically competent *E. coli* GT115 cells were transformed by the calcium chloride method. Plasmids were mobilized into *B. cenocepacia* by triparental mating (14,38).

Cloning of B. cenocepacia *K56-2* fliC–The *fliC* gene (BCAL0114) was amplified from *B. cenocepacia* K56-2 genomic DNA with the primer pair 6093/6094 and sequenced at the Core Molecular Biology Facility, York University, Toronto, Canada. The *B. cenocepacia* K56-2 *fliC* sequence was submitted to GenBank and is available under submission number KC763156.

Construction of Mutants in B. cenocepacia– Unmarked deletion mutants were constructed as described previously (14,38). Briefly, the target genes were deleted by allelic exchange using the pGPI-SceI-2 plasmid containing the corresponding upstream and downstream fragments. The resulting deletion plasmids were introduced into *B. cenocepacia* by triparental mating. Upstream fragments for deletion of the *vioA* homologue in the O-antigen cluster (BCAL3129), *flmQ* (BCAL0111), the *vioA* homologue in the *fliC* cluster (BCAL0110), the *rmlD* homologue (BCAS0105), the O-antigen cluster between *wbiI* and *wzm* (BCAL3119-BCAL3131), and *wbxC*/*wbxD* (BCAL3123-BCAL3124) (15) were amplified with primer pairs 6165/6166, 5235/5236, L0110 US XbaI/L0110 US NotI, 5922/5923, 5852/5853, and L3123 US BglII/L3123 US NotI, and downstream fragments by 6167/6168, 5237/5238, L0110 DS NotI/L0110 DS BglII, 5924/5925, 5888/5889, Q38/Q39, respectively (Table 2). The insertional inactivation of *rmlD* (BCAL3132) was achieved by cloning ~ 300 bp internal fragments from BCAL3132 (amplified using primers pair 5685/5686; Table 2) into

pGPΩTp. The resulting mutagenesis plasmid pGPΩTp/*rmlD* was mobilized into *B. cenocepacia* (39). Conditional mutants in *rmlB* (BCAL3135), *rmlC* (BCAL3133), *rmlD* (BCAL3132) and *flmQ* (BCAL0111) were constructed using pSC200 (17). The primers used to amplify DNA fragments were as follows: 6021/6022 (*rmlB*), Q92/Q91 (*rmlC*), 6023/6024 (*rmlD*) and Q89/Q90 (*flmQ*; Table 2). Each amplicon contained the NdeI restriction site in the starting codon of each gene to facilitate cloning into pSC200.

Rhamnose Depletion Assays–Conditional mutants were grown overnight in 5 ml LB with trimethoprim (100 μg ml^{-1}) and 0.4% rhamnose. The next day, 1 ml of each strain was centrifuged and washed thrice with LB without rhamnose. The optical density (OD_{600}) was adjusted to 1.0 in LB without rhamnose and 3 μl of each dilution of 10^{-1} to 10^{-6} were incubated at 37°C on LB agar with trimethoprim with or without 0.4% rhamnose for 24 h. The essentiality of each respective gene was also assessed in broth. For this, overnight cultures grown in 5 ml LB with trimethoprim (100 μg ml^{-1}) and 0.4% rhamnose were centrifuged and washed thrice in LB without rhamnose. Each strain was diluted to OD_{600} 0.03 in LB/trimethoprim with or without rhamnose and triplicates of 300 μl were incubated for 4 h in honeycomb plates at 37°C with shaking using a Bioscreen (Oy Growth Curves, Finland). Next, 3 μl of each dilution were transferred to fresh medium with or without rhamnose and incubated for additional 19 h. The OD_{600} was measured every 30 min. Strains XOA10 (*B. cenocepacia* K56-2 pSC200/BCAL1928; non-lethal conditional mutant) and XOA11 (*B. cenocepacia* K56-2 pSC200/arnT; lethal mutation) were used as controls (17).

Complementation experiments–Plasmid pIN62 (encoding chloramphenicol resistance; (40)) was used to complement BCAL3123, which was cloned from *B. cenocepacia* K56-2 genomic DNA using the L3123 XbaI/L3123 NdeI primer pair (Table 2). The plasmid and PCR product were digested with XbaI and NdeI at 37°C for 16 h. The digested plasmid DNA was subsequently dephosphorylated using Antarctic phosphatase (37°C, 30 min), which was then deactivated at 65°C (2 min). Ligation was performed at 16°C for 16 h using T4 DNA ligase. Transformation and triparental mating were performed as described previously (see text above). The resulting plasmid pIN62/BCAL3123 (as confirmed by sequencing) was introduced into the appropriate *B. cenocepacia* strains via triparental mating.

Whole Cell Lysates and LPS Staining–To determine the presence of O antigen, whole cell lysates were prepared and resolved on 14% SDS-polyacrylamide gels and LPS was visualized by silver staining as described previously (41), except that instead of citric acid, a mixture of 2.5% sodium carbonate (wt/v) with 0.05% formaldehyde (v/v) in water heated to 60°C was used as developing solution.

Motility Assays and Biofilm Formation–Bacterial motility was analyzed on soft agar plates (1% Bacto tryptone in 0.3% agar). The OD_{600} of overnight cultures was adjusted to 1.0 and 2 μl of culture were inoculated in the centre of agar plate. The growth zone diameter was measured after 24h of incubation at 37°C. Biofilm mass was quantified by the crystal violet protocol as described previously (42).

Biological Assays–Flagellin from *B. cenocepacia* parental strain and the BCAL0111 deletion mutant was purified in PBS as described above. The concentration of FliC was confirmed densitometrically. THP1 cells or HEK293-TLR5 cells were seeded (2 x 10^5 cells ml^{-1}; 2ml) in 12-well plates and stimulated with the indicated concentrations of WT and non-glycosylated flagellin for 24 h. Conditioned medium was then measured for levels of TNF-α, IL-6, IL-8 and IL-1β (DuoSet kits; R&D Systems) according to the manufacturer's protocol. For luciferase reporter assays, HEK293 TLR5 cells were seeded (1.5×10^5 cells ml^{-1}; 200 μl) in 96-well plates and transfected with constructs encoding NFκB-regulated firefly luciferase (80 ng) and the TK *Renilla* luciferase reporter construct (phRL-TK; 20 ng; Promega Biosciences). Cells were treated as indicated and cell lysates assayed for firefly luciferase activity and normalized for transfection efficiency using TK *Renilla* luciferase activity. Cell extracts were also assayed for phosphorylated and total levels of p65 and p38, JNK and ERK MAP kinases by Western blotting.

RESULTS

B. cenocepacia *Flagellin is Glycosylated with 4,6-dideoxy-4-(3-hydroxybutanoylamino)-D-glucose*–Flagella were sheared off *B. cenocepacia* cells and solubilized with 8 M urea, as described in Experimental Procedures (Fig. 1*A*). Mass spectrometric analyses of tryptic digests confirmed the identity of the flagellin monomer (FliC). Further MS analyses of native FliC revealed one major molecular ion at 40,836 *m/z* and minor ions at 40,605 *m/z*, 40,374 *m/z*, 40,143 *m/z*, and 41,067 *m/z* (Fig. 2*A*). These masses were compared with the theoretical mass of FliC from *B. cenocepacia* J2315, which is 38,779.79 Da. Strains J2315 and K56-2 belong to the ET12 clone, but J2315 was the only ET12 strain sequenced at the time of these experiments (43). Thus, the observed mass of the major molecular ion was 2,057 Da larger than expected from the theoretical amino acid sequence. Moreover, the molecular ions differed from each other by 231 *m/z*, suggesting the presence of at least five modifications. In SDS-PAGE gels, FliC was visualized by Coomassie blue staining and also reacted with Pro-Q Emerald glycoprotein stain, suggesting that the observed modifications were due to glycosylation. FliC was also detected on Western blot with the primary antibody RFFL/ARP42986_P050 (Fig. 1*B-C*).

To accurately determine the molecular mass of FliC, purified flagellin was chemically deglycosylated, as indicated in Experimental Procedures. The deglycosylation method was optimized to specifically cleave glycosidic bonds without damaging the peptide backbone (35). The MS analysis of the deglycosylated protein showed a single molecular ion of 38,756.90 *m/z* (Fig. 2*B*). This result provided additional evidence that FliC was modified by a glycan. Furthermore, MS of the tryptic digest confirmed the identity of the deglycosylated protein as FliC, except that it was 23 Da smaller than expected from the theoretical mass of the J2315 FliC (38,779.79 Da). This suggested that FliC proteins from K56-2 and J2315 were not completely identical. DNA sequencing of the *fliC* (BCAL0114) gene from K56-2 revealed a single C to A substitution at 1072 bp, resulting in a histidine to asparagine replacement at position 358 in the K56-2 FliC (H358N) giving a 23 Da difference in molecular mass. The difference in mass between native and chemically deglycosylated FliC was also reflected in SDS-PAGE analyses by Coomassie blue staining (Fig. 1*D*). However, deglycosylated FliC still reacted with Pro-Q Emerald, indicating that this stain was not specific for the *B. cenocepacia* FliC glycan.

Since trypsin digestion alone did not provide sufficient peptide coverage spanning the entire FliC protein, additional digestions were performed with chymotrypsin, AspN, and a mixture of AspN and trypsin. Mass spectra were recorded for all four digested samples separately and the combined data were analysed, giving 100% sequence coverage. This strategy allowed us to identify ions matching the peptides with one or two 231 *m/z* modifications (Table 3). Thus, the localization of single modifications was assigned to peptides ^{159}DLSQSMSAAK^{168}, ^{177}GQTVGTVTGLSLDNNGAYTGSGATITAINVLSDGK^{211}, and ^{287}DISTVSGANVAMVSIDNALQTVNNVQAALGAAQNR^{321}, while peptides ^{212}GGYTFTDQNGGAISQTVAQSVFGAN^{233}, ^{234}GANATTGTGTAVGNLTLQ^{251} and ^{252}SGATGAGTSAAQQTAITNAIAQINAVNKPATLVSNL^{286} carried two modifications. From these combined results, we could clearly identify nine out of ten possible modification sites (as determined by MS of the entire FliC (Figs. 2*A* and 2*D*, and Table 3). The exact position of the modifications in each peptide was not determined.

To identify the nature of the FliC glycan, flagellin was analysed by GC/MS. Combined data collected from GC/MS spectra after weak and strong methanolysis identified a 4,6-dideoxy-4-(3-hydroxybutanoylamino)-hexose. Comparison with GC/MS spectra obtained after similar treatment of *Providencia stuartii* O43 O-antigen samples (44), confirmed that the sugar possessed the *gluco* configuration, representing viosamine with 3-hydroxybutyric acid substituting amino group at C4, referred to as D-Qui4N(3HOBut) (Fig. 3 and 4). We used β-elimination to establish the character of the glycosidic bond between glycan and the FliC peptide backbone. The β-elimination releases glycans that form *O*-glycosidic bonds with

serine or threonine, leaving *N*-glycosidic bonds intact. D-Qui4N(3HOBut) was the only sugar identified by GC/MS analysis of the sample after β-elimination (Fig. 4), demonstrating that *B. cenocepacia* FliC was *O*-glycosylated. The structure of the glycan was also consistent with the measured mass difference of 231 Da (theor. MW 249.1212 - H_2O = 231.1106; Fig. 3).

Identification of the Genes Involved in FliC Glycosylation–The flagellin gene *fliC* (BCAL0114) lies upstream of *fliD* (BCAL0113), *fliT* (BCAL0112), BCAL0111 and BCAL0110. The *fliD* and *fliT* encode the flagellar hook associated protein and a flagellar chaperone, respectively. BCAL0110 encodes a putative VioA aminotransferase homologue (aminotransferase involved in synthesis of Qui4N; Fig. 5*A*), while BCAL0111 encodes a predicted protein with homology to the group 1 superfamily of glycosyltransferases and also containing four tetratricopeptide repeats. *In silico* analysis of BCAL0111 with HHpred (http://toolkit.tuebingen.mpg.de/hhpred) revealed a C-terminal domain 360 amino acids that is structurally homologous to several well characterized glycosyltransferases including the PimB mannosyltransferase from *Corynebacterium glutamicum* (45), the human UDP-*N*-acetylglucosamine-peptide *N*-acetylglucosamine transferase (46), and WaaG lipid A-core biosynthesis glycosyltransferase (47). To investigate whether BCAL0111 plays a role in FliC glycosylation, we constructed a ΔBCAL0111 deletion mutant and analysed its purified flagellin. Coomassie stained SDS-PAGE of FliC from ΔBCAL0111 showed a downshift in apparent molecular size (Fig. 1*C*), which was also evident by Western blotting with the RFFL/ARP42986_P050 antibody (Fig. 1*B*). Together, these results demonstrated that flagellin biosynthesis can proceed in the absence of glycosylation and that the antibody was specific for *B. cenocepacia* flagellin regardless of its glycosylation status. The MS spectrum of purified FliC from ΔBCAL0111 confirmed the loss of the glycan, as only a single molecular ion of 38,756.90 *m/z* corresponding to non-glycosylated flagellin could be detected (Fig. 2*C*). To confirm that BCAL0111 is required for FliC glycosylation, we placed BCAL0111 under the control of a rhamnose-inducible promoter (Fig. 5*A*). FliC purified from a culture in rhamnose-containing medium showed the same molecular weight in MS analysis and Coomassie staining as the parental strain. In contrast, flagellin isolated from a culture grown without rhamnose was present only in its non-glycosylated state (Fig. 6, *B*-*C*). Hence, we concluded that BCAL0111 is the FliC glycosyltransferase and designated the gene as *flmQ* for flagellin modifying protein that transfers D-Qui4N(3OHBut). The deletion of BCAL0110 (*vioA* homolog) did not cause any detectable defect in FliC glycosylation (see below).

The *B. cenocepacia* K56-2 LPS contains O antigen. Glycans from the O antigen were detected in our sugar analyses. Therefore, we sought to delete the O-antigen genes to avoid this contamination. Repeated attempts to delete genes between *wbiI* (BCAL3119) and *rmlB* (BCAL3135; Fig. 5*B* (15)) failed (see also below). However, a deletion including *wbiI* and *wzm* (BCAL3131) was obtained and confirmed by PCR and SDS-PAGE analyses of LPS profile of the mutant strain (Fig. 7). Analyses of FliC in the Δ*wbiI-wzm* mutant showed the loss of the flagellin glycan (Fig. 6*A*). Thus, we concluded that FliC glycosylation requires one or more components of the O-antigen cluster. Genes in the O-antigen cluster that could be involved in the biosynthesis pathway of the FliC glycan are *vioA* (BCAL3129), a nucleotide sugar aminotransferase from dTDP-D-Qui4N biosynthesis pathway (48) and *wbxC* (BCAL3123), a putative acetyltransferase. No differences in flagellin glycosylation were detected in ΔBCAL3129 compared to the parental isolate (data not shown). Attempts to generate a single *wbxC* deletion failed, but it was possible to delete this gene together with the neighbouring glycosyltransferase *wbxD* (BCAL3124). While the single *wbxD* deletion did not affect FliC glycosylation (Fig. 2*D*), MS and SDS-PAGE analyses of Δ*wbxCD* revealed loss of glycosylation (Fig. 6*D*). Introducing a functional *wbxC* on a plasmid (pIN62/*wbxC*) into Δ*wbxCD* restored FliC glycosylation (Fig. 6*E*). From these results we concluded that *wbxC* is involved in the biosynthesis of dTDP-D-Qui4N(3HOBut), possibly by catalysing an acetyltransferase step prior to the formation of

the 3-hydroxybutyric acid side chain. This interpretation is consistent with the high degree of homology in the primary amino acid sequence of WbxC and the *Acinetobacter baumannii* WeeI protein, which is an acetyltransferase involved in the biosynthesis of UDP-N,N'-diacetylbacillosamine (49,50). We did not succeed in any attempts to construct a double deletion mutant eliminating *vioA* (BCAL3129) and its putative homologue in the *fliC* region (BCAL0110) despite using the same mutagenic plasmids that were employed to delete both genes separately. However, it was possible to delete BCAL0110 in the Δ*wbiI-wzm* background and conversely, to delete the *wbiI-wzm* region in the ΔBCAL0110 strain. These results demonstrate that both *vioA* and its BCAL0110 homologue are non-essential genes (Fig. 5).

We also investigated the conservation of the genetic organization of the *fliC* region in other *Burkholderia* species. A similar gene organization as in J2315, with a putative *flmQ* (BCAL0111) homologue placed downstream of *fliCDT*, was observed in *B. pseudomallei*, *B. mallei*, *B. glumae*, *B. xenovorans*, *B. vietnamiensis* and *B. multivorans* (Fig. 8*A*). *B. thailandensis* carries 11 additional genes inserted between the *flmQ* homologue and *fliT* (Fig. 8*B*). In all these clusters the putative *flmQ* was placed downstream from putative *fliT* and upstream from putative *vioA* gene, in each case a homologue of BCAL0110, and had no homologues elsewhere in the genome. In *B. cepacia*, the flagellin cluster has a unique organization (Fig. 8*C*), where *fliT* is followed by a gene encoding a glycosyltransferase (GEM_0145) and the *flmQ* homologue (GEM_0144), but in the reverse orientation. Also in *B. cepacia*, the only BCAL0110 aminotransferase homologue (GEM_1565) is located outside of the flagellin cluster. Despite the variations among different species, the presence of homologous glycosyltransferase and aminotransferase genes in their flagellin clusters suggest that flagellin glycosylation is common in multiple species of the *Burkholderia* genus. Indeed, it was reported that *B. pseudomallei* and *B. thailandensis* produce glycosylated flagellin, but the glycan described in these strains is different than the one identified here (33).

RmlB is an Essential Gene in B. cenocepacia–RmlB (dTDP-D-glucose 4,6-dehydratase), one of the enzymes encoded by the *B. cenocepacia* O-antigen cluster, is needed for the synthesis of dTDP-L-rhamnose, which in turn is required for the assembly of the O-antigen repeating unit (15) (Fig. 5*B*). RmlB is also responsible for producing the precursor for biosynthesis of dTDP-D-Qui4N (51). In the course of these studies, we noticed that *rmlB* (BCAL3135) could not be deleted, suggesting the possibility that this gene is essential. To evaluate this notion, we constructed a conditional mutant by placing the rhamnose-inducible promoter upstream from *rmlB*. All tested strains including the control strains XOA10 (P_{rha}::BCAL1928; non-lethal conditional mutant) and XOA11 (P_{rha}::*arnT*; lethal conditional mutant) (17) grew well when incubated on LB agar plates with rhamnose. In contrast, only XOA10 grew well in the absence of rhamnose whereas XOA11 and the P_{rha}::*rmlB* strains grew very poorly (data not shown). The effect of rhamnose depletion was much more dramatic in liquid cultures (Fig. 9). The rhamnose inducible vector was also inserted upstream from *rmlC* (BCAL3133) and *rmlD* (BCAL3132), which are downstream from *rmlB* to examine their possible essentiality in *B. cenocepacia*, but rhamnose depletion did not cause any growth alteration in these strains (Fig. 9). Because BCAS0105, a gene located in the third chromosome of *B. cenocepacia*, encodes a putative RmlD homologue rhamnose depletion experiments were also performed in a ΔBCAS0105 strain carrying P_{rha}::*rmlD*. These experiments indicated that ΔBCAS0105/P_{rha}::*rmlD* is viable under rhamnose free conditions (Fig. 9), ruling out the possibility that BCAS0105 might have supplied the function of *rmlD* when this gene was placed under the control of the rhamnose-inducible promoter. Together, these results provide experimental evidence that *rmlB* is essential in *B. cenocepacia* K56-2.

Role of FliC Glycosylation on Bacterial Motility and Biofilm Formation–In natural environments, flagella are bacterial motility organelles. To examine the influence of flagellin glycosylation on *B. cenocepacia* motility, we tested the motility of the deletion mutants on

soft agar by measuring the diameter of bacterial growth after 24 h incubation at 37°C. The strain RSF44, which lacks flagella (38), did not migrate from the inoculation spot providing a negative control. Strain ΔBCAL0111 lacking the putative D-Qui4N(3HOBut) transferase *flmQ*, showed a slight alteration in motility when compared to the parental isolate (Fig. 10*A*), while Δ*wbxCD*, missing the putative acetyltransferase and an O-antigen glycosyltransferase, had a much stronger effect on motility. The Δ*wbiI-wzm* mutant, which causes complete loss of O antigen and the FliC glycan led to ~ 50% decrease in motility. Therefore, we conclude from these results that flagellin glycosylation and a complete O antigen are required for normal motility of *B. cenocepacia*. Flagella also contribute to biofilm production. When compared with the parental strain, production of biofilm by ΔBCAL0111 was at a similar level as the flagella lacking strain RSF44 (Fig. 10*B*), suggesting that the presence of glycosylation and not the flagella alone is required for normal biofilm formation.

FliC Glycosylation Reduces TLR5-mediated Responses–To examine the biological consequence of flagellin glycosylation in innate immune responses, human THP1 monocyte cells were stimulated with purified flagellins obtained from the parental strain (glycosylated FliC) and the ΔBCAL0111 mutant (non-glycosylated FliC). Stimulation of THP1 cells with both proteins resulted in production of the pro-inflammatory cytokines IL-1β (Fig. 11*A*), TNF-α (Fig. 11*B*) and IL-6 (Fig. 11*C*). However, non-glycosylated FliC was significantly more efficacious than the glycosylated counterpart in inducing IL-1β, TNF-α and IL-6. To eliminate the possibility that LPS contamination in the flagellin preparations could confound these results, additional experiments were performed in HEK293 cells stably expressing TLR5 (HEK293 cells normally lack Toll-like receptors (52,53)), which specifically recognizes flagellin. Again, the non-glycosylated FliC was more effective in inducing pro-inflammatory cytokine production in TLR5 cells as indicated by increased levels of IL-8 (Fig. 12*A*). We then looked at intracellular signalling and showed that non-glycosylated FliC is also more effective at activating NFκB (as measured by induction of a transfected NFκB-regulated luciferase reporter gene, Fig. 12*B*) and the phosphorylation of the NFκB subunit p65 (Fig. 12*C*). Also, non-glycosylated FliC mediated stronger phosphorylation of p38 MAPK (Fig. 12*D*). Together, these studies consistently show that non-glycosylated FliC is more effective than the glycosylated protein to stimulate pro-inflammatory signalling by TLR5.

DISCUSSION

Despite the previously described roles for flagella in *B. cenocepacia* pathogenicity (25,26), this is the first report describing flagellin glycosylation in this bacterium and identifying the genes involved in the biosynthesis of the glycan. We showed that the *B. cenocepacia* flagellin is modified with a viosamine (Qui4N) derivative, D-Qui4N(3HOBut), on at least ten glycosylation sites within the protein. A sugar similar to D-Qui4N(3HOBut) but carrying an additional methyl group at C2 (54) was previously identified in glycosylated flagellin from *P. syringae* pv. *tabaci* (54,55), while Qui4N itself is a component of the flagellin glycan in *P. aeruginosa* PAK (56). The biosynthesis of dTDP-viosamine requires three enzymatic steps: (i) conversion of D-glucose-1-phosphate into dTDP-D-glucose, catalyzed by RmlA; (ii) formation of dTDP-4-dehydro-6-deoxy-D-glucose, catalyzed by RmlB; and (iii) an amination step catalyzed by the dTDP-4-dehydro-6-deoxy-D-glucose aminotransferase encoded by the *vioA* gene (57,58). An additional step involves the acetylation of dTDP-viosamine to yield dTDP-*N*-acetylviosamine. Homologues of *vioA* and *vioB*, encoding the dTDP-viosamine acetyltransferase, have been identified in *P. syringae* pv. *tabaci* (54) and *P. aeruginosa* PAK (56), and both genes are required for the biosynthesis of the modified viosamine in *P. syringae* pv. *tabaci*. Despite that in *B. cenocepacia* there are two *vioA* homologues (BCAL0110 and BCAL3129), we could not identify a *vioB* homologue. Instead, we discovered that BCAL3123, encoding a putative acetyltransferase, is necessary for biosynthesis of D-Qui4N(3HOBut). Further experiments are necessary to provide evidence whether BCAL3123 encodes an enzyme catalyzing the

direct transfer of 3OHBut or if there are additional steps with BCAL3123 acting as an N-acetyltransferase prior to the formation of the 3OHBut side chain.

In particular, our results point to a complex link between O-antigen biosynthesis and the biosynthesis of the flagellin glycan. Two genes required for flagellin biosynthesis are located in the *fliC* gene cluster while the other genes are present in the O-antigen cluster. The flagellin gene cluster contains a *vioA* homolog that we show to be functionally redundant, and the *flmQ* glycosyltransferase gene, which is essential for FliC glycosylation. VioT, the flagellin glycosyltransferase in *P. syringae* pv. *tabaci*, has no homologues in *B. cenocepacia* and conversely, *P. syringae* pv. *tabaci* has no FlmQ homologues. Therefore, despite that both species use similar sugars for flagellin glycosylation, the specific glycosyltransferases involved are unique to each system, perhaps reflecting differences in the FliC acceptor protein in each species. Comparison of *fliC* biosynthesis clusters in other *Burkholderia* species indicated the presence of *flmQ* and *vioA* homologues just downstream from *fliC*, with only a few exceptions. Flagellins from *B. pseudomallei* and *B. thailandensis* were previously found to be glycosylated by a single glycan (33). Although the structures of the glycans are unknown their molecular masses are 291 Da and 342 Da for the *B. pseudomallei* and *B. thailandensis*, respectively, suggesting a different sugar than D-Qui4N(3HOBut). Therefore, we conclude that despite a common *fliC* gene cluster organization in most *Burkholderia* species the glycan structure and glycosylation pattern of flagellin is likely species specific.

The discovery that *rmlB* is an essential gene in *B. cenocepacia* was unexpected. In a previous study, Juhas *et al.* (59) reported 84 candidate essential genes in *B. cenocepacia* that were not previously described as essential in any other bacteria. One of these genes was *rmlD* (BCAL3132), located within the O-antigen cluster, but these authors did not report any experimental verification of *rmlD* essentiality. In our study, we conclusively demonstrate that *rmlB* (BCAL3135), not *rmlD*, is essential for *B. cenocepacia* viability. The *B. cenocepacia* dTDP-L-rhamnose biosynthesis genes (*rmlBACD*) form one transcriptional unit with the first 10 genes of O-antigen cluster (15). RmlB is a dTDP-D-glucose 4,6-dehydratase and its function is required for the biosynthesis of nucleotide sugars like dTDP-D-fucose, dTDP-L-rhamnose, dTDP-D-Qui4N and several other metabolites (48,51,60,61). In *B. cenocepacia*, *rmlB* is involved in the synthesis of O antigen, which contains rhamnose in its repeating unit (15), and in the synthesis of the D-Qui4N(3HOBut) flagellin glycan, as we show here. However, O-antigen production and flagellin glycosylation are not required for *B. cenocepacia* viability. To our knowledge, RmlB has not been reported as an essential in other bacteria. The *rlmB* gene could not be deleted in *B. thailandensis*, but its deletion was possible in *B. pseudomallei* (33), suggesting it may be essential for at least another *Burkholderia* species. We speculate that the RmlB function may be required for the synthesis of another sugar nucleotide that may play an essential role in an as yet unidentified metabolic pathway, perhaps becoming a novel attractive candidate for antimicrobial development.

While the flagellum is important for bacterial motility, colonization and virulence (21,62,63), the functional role of glycosylation in host-bacteria interactions is less clear, and has only been investigated in a handful of bacterial species. For example, non-glycosylated flagellin mutants of the plant pathogen *Pseudomonas syringae* pv. *tabaci* are much less virulent on tobacco leaves than the wild-type strain (54,64,65). In contrast, lack of flagellin glycosylation does not affect the pathogenicity of starfruit pathogen *P. syringae* pv. *averrhoi* (66), while glycosylated flagellin of *Acidovorax avenae* elicits a strong immune response in cultured rice cells (67).

Contradictory results have also been reported for *P. aeruginosa* glycosylated flagellins in their ability to modulate innate immune responses in human epithelial cells (68,69). Two notorious human pathogens, *Campylobacter jejuni* and *Helicobacter pylori*, cannot assemble flagella without glycosylation and lack of flagella in both strains significantly reduces their virulence (70,71). It is also not clear whether flagellin glycosylation modulates TLR5 responses. The glycosylated flagellin

from *C. jejuni* is unique in that it fails to stimulate TLR5 (72). Reconstituting a functional TLR5 binding site in the *C. jejuni* flagellin resulted in the expression of glycosylated flagellin that induces a potent TLR5 response, ruling out a role for flagellin glycosylation in *C. jejuni* evasion of TLR5 detection (72). The elucidation of the flagellin glycosylation pathway in *B. cenocepacia* provided us with the opportunity to directly test the role of glycosylation in TLR5/flagellin-mediated inflammatory responses. We show that non-glycosylated flagellin was more pro-inflammatory than its fully glycosylated form. We also demonstrate that glycosylation of flagellin was associated with reduced efficacy with respect to stimulating TLR5-mediated signal transduction and gene expression. These results suggest that the presence of the glycan may alter to some extent flagellin detection by TLR5, although this was not directly examined here. We conclude that flagellin glycosylation could provide *B. cenocepacia* a strategy to reduce recognition by the innate immune system. However, further experiments are required to assess *in vivo* the role of flagellin glycosylation in the ability of these bacteria to cause chronic infection in cystic fibrosis patients.

Acknowledgements–We thank Cristina L. Marolda for technical assistance, and Olga Ovchinnikova and Yuriy Knirel for providing us with the D-Qui4N standard.

REFERENCES

1. Mahenthiralingam, E., Baldwin, A., and Dowson, C. G. (2008) *Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology. *J Appl Microbiol* **104**, 1539-1551
2. Mahenthiralingam, E., Urban, T. A., and Goldberg, J. B. (2005) The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat Rev Microbiol* **3**, 144-156
3. Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P., and Levison, H. (1984) *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J Pediatr* **104**, 206-210
4. Graindorge, A., Menard, A., Neto, M., Bouvet, C., Miollan, R., Gaillard, S., de Montclos, H., Laurent, F., and Cournoyer, B. (2010) Epidemiology and molecular characterization of a clone of *Burkholderia cenocepacia* responsible for nosocomial pulmonary tract infections in a French intensive care unit. *Diagn Microbiol Infect Dis* **66**, 29-40
5. Katsiari, M., Roussou, Z., Tryfinopoulou, K., Vatopoulos, A., Platsouka, E., and Maguina, A. (2012) *Burkholderia cenocepacia* bacteremia without respiratory colonization in an adult intensive care unit: epidemiological and molecular investigation of an outbreak. *Hippokratia* **16**, 317-323
6. Lee, S., Han, S. W., Kim, G., Song, D. Y., Lee, J. C., and Kwon, K. T. (2013) An outbreak of *Burkholderia cenocepacia* associated with contaminated chlorhexidine solutions prepared in the hospital. *Am J Infect Control*
7. Satpute, M. G., Telang, N. V., Dhakephalkar, P. K., Niphadkar, K. B., and Joshi, S. G. (2011) Isolation of *Burkholderia cenocepacia* J 2315 from non-cystic fibrosis pediatric patients in India. *Am J Infect Control* **39**, e21-23
8. McDowell, A., Mahenthiralingam, E., Dunbar, K. E., Moore, J. E., Crowe, M., and Elborn, J. S. (2004) Epidemiology of *Burkholderia cepacia* complex species recovered from cystic fibrosis patients: issues related to patient segregation. *J Med Microbiol* **53**, 663-668
9. Novotny, L. A., Amer, A. O., Brockson, M. E., Goodman, S. D., and Bakaletz, L. O. (2013) Structural stability of *Burkholderia cenocepacia* biofilms is reliant on eDNA structure and presence of a bacterial nucleic acid binding protein. *PLoS One* **8**, e67629
10. Vandamme, P., Holmes, B., Coenye, T., Goris, J., Mahenthiralingam, E., LiPuma, J. J., and Govan, J. R. (2003) *Burkholderia cenocepacia* sp. *nov*.--a new twist to an old story. *Res Microbiol* **154**, 91-96
11. Vandamme, P., and Mahenthiralingam, E. (2003) Strains from the *Burkholderia cepacia* Complex: Relationship to Opportunistic Pathogens. *J Nematol* **35**, 208-211
12. De Soyza, A., Morris, K., McDowell, A., Doherty, C., Archer, L., Perry, J., Govan, J. R., Corris, P. A., and Gould, K. (2004) Prevalence and clonality of *Burkholderia cepacia* complex genomovars in UK patients with cystic fibrosis referred for lung transplantation. *Thorax* **59**, 526-528
13. Drevinek, P., and Mahenthiralingam, E. (2010) *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. *Clin Microbiol Infect* **16**, 821-830
14. Hamad, M. A., Di Lorenzo, F., Molinaro, A., and Valvano, M. A. (2012) Aminoarabinose is essential for lipopolysaccharide export and intrinsic antimicrobial peptide resistance in *Burkholderia cenocepacia*. *Mol Microbiol* **85**, 962-974
15. Ortega, X., Hunt, T. A., Loutet, S., Vinion-Dubiel, A. D., Datta, A., Choudhury, B., Goldberg, J. B., Carlson, R., and Valvano, M. A. (2005) Reconstitution of O-specific lipopolysaccharide expression in *Burkholderia cenocepacia* strain J2315, which is associated with transmissible infections in patients with cystic fibrosis. *J Bacteriol* **187**, 1324-1333
16. Ortega, X., Silipo, A., Saldias, M. S., Bates, C. C., Molinaro, A., and Valvano, M. A. (2009) Biosynthesis and structure of the *Burkholderia cenocepacia* K56-2 lipopolysaccharide core

oligosaccharide: truncation of the core oligosaccharide leads to increased binding and sensitivity to polymyxin B. *J Biol Chem* **284**, 21738-21751
17. Ortega, X. P., Cardona, S. T., Brown, A. R., Loutet, S. A., Flannagan, R. S., Campopiano, D. J., Govan, J. R., and Valvano, M. A. (2007) A putative gene cluster for aminoarabinose biosynthesis is essential for *Burkholderia cenocepacia* viability. *J Bacteriol* **189**, 3639-3644
18. Saldias, M. S., Ortega, X., and Valvano, M. A. (2009) *Burkholderia cenocepacia* O-antigen lipopolysaccharide prevents phagocytosis by macrophages and adhesion to epithelial cells. *J Med Microbiol* **58**, 1542-1548
19. Raetz, C. R., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**, 635-700
20. Kotrange, S., Kopp, B., Akhter, A., Abdelaziz, D., Abu Khweek, A., Caution, K., Abdulrahman, B., Wewers, M. D., McCoy, K., Marsh, C., Loutet, S. A., Ortega, X., Valvano, M. A., and Amer, A. O. (2011) *Burkholderia cenocepacia* O polysaccharide chain contributes to caspase-1-dependent IL-1β production in macrophages. *J Leukoc Biol* **89**, 481-488
21. Erhardt, M., Namba, K., and Hughes, K. T. (2010) Bacterial nanomachines: the flagellum and type III injectisome. *Cold Spring Harb Perspect Biol* **2**, a000299
22. Drake, D., and Montie, T. C. (1988) Flagella, motility and invasive virulence of *Pseudomonas aeruginosa*. *J Gen Microbiol* **134**, 43-52
23. Eaves-Pyles, T., Murthy, K., Liaudet, L., Virag, L., Ross, G., Soriano, F. G., Szabo, C., and Salzman, A. L. (2001) Flagellin, a novel mediator of *Salmonella*-induced epithelial activation and systemic inflammation: IκBα degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J Immunol* **166**, 1248-1260
24. Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., and Prince, A. (1998) Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* **66**, 43-51
25. Tomich, M., Herfst, C. A., Golden, J. W., and Mohr, C. D. (2002) Role of flagella in host cell invasion by *Burkholderia cepacia*. *Infect Immun* **70**, 1799-1806
26. Urban, T. A., Griffith, A., Torok, A. M., Smolkin, M. E., Burns, J. L., and Goldberg, J. B. (2004) Contribution of *Burkholderia cenocepacia* flagella to infectivity and inflammation. *Infect Immun* **72**, 5126-5134
27. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Aderem, A. (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**, 1099-1103
28. Strober, W., Murray, P. J., Kitani, A., and Watanabe, T. (2006) Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* **6**, 9-20
29. Blohmke, C. J., Victor, R. E., Hirschfeld, A. F., Elias, I. M., Hancock, D. G., Lane, C. R., Davidson, A. G., Wilcox, P. G., Smith, K. D., Overhage, J., Hancock, R. E., and Turvey, S. E. (2008) Innate immunity mediated by TLR5 as a novel antiinflammatory target for cystic fibrosis lung disease. *J Immunol* **180**, 7764-7773
30. de, C. V. G. M., Le Goffic, R., Balloy, V., Plotkowski, M. C., Chignard, M., and Si-Tahar, M. (2008) TLR 5, but neither TLR2 nor TLR4, is involved in lung epithelial cell response to *Burkholderia cenocepacia*. *FEMS Immunol Med Microbiol* **54**, 37-44
31. West, T. E., Chantratita, N., Chierakul, W., Limmathurotsakul, D., Wuthiekanun, V., Myers, N. D., Emond, M. J., Wurfel, M. M., Hawn, T. R., Peacock, S. J., and Skerrett, S. J. (2013) Impaired TLR5 functionality is associated with survival in melioidosis. *J Immunol* **190**, 3373-3379
32. Seo, S. T., and Tsuchiya, K. (2005) Genotypic characterization of *Burkholderia cenocepacia* strains by rep-PCR and PCR-RFLP of the *fliC* gene. *FEMS Microbiol Lett* **245**, 19-24
33. Scott, A. E., Twine, S. M., Fulton, K. M., Titball, R. W., Essex-Lopresti, A. E., Atkins, T. P., and Prior, J. L. (2011) Flagellar glycosylation in *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *J Bacteriol* **193**, 3577-3587

34. Brett, P. J., Mah, D. C., and Woods, D. E. (1994) Isolation and characterization of *Pseudomonas pseudomallei* flagellin proteins. *Infect Immun* **62**, 1914-1919
35. Edge, A. S., Faltynek, C. R., Hof, L., Reichert, L. E., Jr., and Weber, P. (1981) Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal Biochem* **118**, 131-137
36. De Castro, C., Parrilli, M., Holst, O., and Molinaro, A. (2010) Microbe-associated molecular patterns in innate immunity: Extraction and chemical analysis of gram-negative bacterial lipopolysaccharides. *Methods Enzymol* **480**, 89-115
37. Sambrook, R., and Russell, D. (2001) *Molecular Cloning: a Laboratory Manual.*, 3rd ed., Cold Spring Harbor, NY, Cold Spring Harbor Laboratory
38. Flannagan, R. S., Linn, T., and Valvano, M. A. (2008) A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. *Environ Microbiol* **10**, 1652-1660
39. Flannagan, R. S., Aubert, D., Kooi, C., Sokol, P. A., and Valvano, M. A. (2007) *Burkholderia cenocepacia* requires a periplasmic HtrA protease for growth under thermal and osmotic stress and for survival *in vivo*. *Infect Immun* **75**, 1679-1689
40. Vergunst, A. C., Meijer, A. H., Renshaw, S. A., and O'Callaghan, D. (2010) *Burkholderia cenocepacia* creates an intramacrophage replication niche in zebrafish embryos, followed by bacterial dissemination and establishment of systemic infection. *Infect Immun* **78**, 1495-1508
41. Marolda, C. L., Welsh, J., Dafoe, L., and Valvano, M. A. (1990) Genetic analysis of the O7-polysaccharide biosynthesis region from the *Escherichia coli* O7:K1 strain VW187. *J Bacteriol* **172**, 3590-3599
42. Saldias, M. S., Lamothe, J., Wu, R., and Valvano, M. A. (2008) *Burkholderia cenocepacia* requires the RpoN sigma factor for biofilm formation and intracellular trafficking within macrophages. *Infect Immun* **76**, 1059-1067
43. Holden, M. T., Seth-Smith, H. M., Crossman, L. C., Sebaihia, M., Bentley, S. D., Cerdeno-Tarraga, A. M., Thomson, N. R., Bason, N., Quail, M. A., Sharp, S., Cherevach, I., Churcher, C., Goodhead, I., Hauser, H., Holroyd, N., Mungall, K., Scott, P., Walker, D., White, B., Rose, H., Iversen, P., Mil-Homens, D., Rocha, E. P., Fialho, A. M., Baldwin, A., Dowson, C., Barrell, B. G., Govan, J. R., Vandamme, P., Hart, C. A., Mahenthiralingam, E., and Parkhill, J. (2009) The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J Bacteriol* **191**, 261-277
44. Ovchinnikova, O. G., Bushmarinov, I. S., Kocharova, N. A., Toukach, F. V., Wykrota, M., Shashkov, A. S., Knirel, Y. A., and Rozalski, A. (2007) New structure for the O-polysaccharide of *Providencia alcalifaciens* O27 and revised structure for the O-polysaccharide of *Providencia stuartii* O43. *Carbohydr Res* **342**, 1116-1121
45. Batt, S. M., Jabeen, T., Mishra, A. K., Veerapen, N., Krumbach, K., Eggeling, L., Besra, G. S., and Fütterer, K. (2010) Acceptor substrate discrimination in phosphatidyl-myo-inositol mannoside synthesis: structural and mutational analysis of mannosyltransferase *Corynebacterium glutamicum* PimB'. *J Biol Chem* **285**, 37741-37752
46. Lazarus, M. B., Jiang, J., Gloster, T. M., Zandberg, W. F., Whitworth, G. E., Vocadlo, D. J., and Walker, S. (2012) Structural snapshots of the reaction coordinate for O-GlcNAc transferase. *Nat Chem Biol* **8**, 966-968
47. Martinez-Fleites, C., Proctor, M., Roberts, S., Bolam, D. N., Gilbert, H. J., and Davies, G. J. (2006) Insights into the synthesis of lipopolysaccharide and antibiotics through the structures of two retaining glycosyltransferases from family GT4. *Chem Biol* **13**, 1143-1152
48. Hao, Y., and Lam, J. (2011) Pathways for the Biosynthesis of NDP Sugars. in *Bacterial Lipopolysaccharides*, Springer Vienna. pp 195-235
49. Morrison, M. J., and Imperiali, B. (2013) Biochemical analysis and structure determination of bacterial acetyltransferases responsible for the biosynthesis of UDP-N,N'-diacetylbacillosamine. *J Biol Chem* **288**, 32248-32260

50. Morrison, M. J., and Imperiali, B. (2013) Biosynthesis of UDP-*N,N'*-diacetylbacillosamine in *Acinetobacter baumannii*: Biochemical characterization and correlation to existing pathways. *Arch Biochem Biophys* **536**, 72-80
51. Giraud, M. F., and Naismith, J. H. (2000) The rhamnose pathway. *Curr Opin Struct Biol* **10**, 687-696
52. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem* **274**, 10689-10692
53. Kumar Pachathundikandi, S., Brandt, S., Madassery, J., and Backert, S. (2011) Induction of TLR-2 and TLR-5 expression by *Helicobacter pylori* switches *cag*PAI-dependent signalling leading to the secretion of IL-8 and TNF-alpha. *PLoS One* **6**, e19614
54. Nguyen, L. C., Yamamoto, M., Ohnishi-Kameyama, M., Andi, S., Taguchi, F., Iwaki, M., Yoshida, M., Ishii, T., Konishi, T., Tsunemi, K., and Ichinose, Y. (2009) Genetic analysis of genes involved in synthesis of modified 4-amino-4,6-dideoxyglucose in flagellin of *Pseudomonas syringae* pv. *tabaci*. *Mol Genet Genomics* **282**, 595-605
55. Toguchi, A., Siano, M., Burkart, M., and Harshey, R. M. (2000) Genetics of swarming motility in *Salmonella enterica* serovar *typhimurium*: critical role for lipopolysaccharide. *J Bacteriol* **182**, 6308-6321
56. Schirm, M., Arora, S. K., Verma, A., Vinogradov, E., Thibault, P., Ramphal, R., and Logan, S. M. (2004) Structural and genetic characterization of glycosylation of type a flagellin in *Pseudomonas aeruginosa*. *J Bacteriol* **186**, 2523-2531
57. Marolda, C. L., Feldman, M. F., and Valvano, M. A. (1999) Genetic organization of the O7-specific lipopolysaccharide biosynthesis cluster of *Escherichia coli* VW187 (O7:K1). *Microbiology* **145 (Pt 9)**, 2485-2495
58. Wang, Y., Xu, Y., Perepelov, A. V., Qi, Y., Knirel, Y. A., Wang, L., and Feng, L. (2007) Biochemical characterization of dTDP-D-Qui4N and dTDP-D-Qui4NAc biosynthetic pathways in *Shigella dysenteriae* type 7 and *Escherichia coli* O7. *J Bacteriol* **189**, 8626-8635
59. Juhas, M., Stark, M., von Mering, C., Lumjiaktase, P., Crook, D. W., Valvano, M. A., and Eberl, L. (2012) High confidence prediction of essential genes in *Burkholderia cenocepacia*. *PLoS One* **7**, e40064
60. Liu, H. W., and Thorson, J. S. (1994) Pathways and mechanisms in the biogenesis of novel deoxysugars by bacteria. *Annu Rev Microbiol* **48**, 223-256
61. Thibodeaux, C. J., Melancon, C. E., 3rd, and Liu, H. W. (2008) Natural-product sugar biosynthesis and enzymatic glycodiversification. *Angew Chem Int Ed Engl* **47**, 9814-9859
62. Hitchen, P. G., Twigger, K., Valiente, E., Langdon, R. H., Wren, B. W., and Dell, A. (2010) Glycoproteomics: a powerful tool for characterizing the diverse glycoforms of bacterial pilins and flagellins. *Biochem Soc Trans* **38**, 1307-1313
63. Logan, S. M. (2006) Flagellar glycosylation - a new component of the motility repertoire? *Microbiology* **152**, 1249-1262
64. Taguchi, F., Takeuchi, K., Katoh, E., Murata, K., Suzuki, T., Marutani, M., Kawasaki, T., Eguchi, M., Katoh, S., Kaku, H., Yasuda, C., Inagaki, Y., Toyoda, K., Shiraishi, T., and Ichinose, Y. (2006) Identification of glycosylation genes and glycosylated amino acids of flagellin in *Pseudomonas syringae* pv. *tabaci*. *Cell Microbiol* **8**, 923-938
65. Taguchi, F., Yamamoto, M., Ohnishi-Kameyama, M., Iwaki, M., Yoshida, M., Ishii, T., Konishi, T., and Ichinose, Y. (2010) Defects in flagellin glycosylation affect the virulence of *Pseudomonas syringae* pv. *tabaci* 6605. *Microbiology* **156**, 72-80
66. Wei, C. F., Hsu, S. T., Deng, W. L., Wen, Y. D., and Huang, H. C. (2012) Plant innate immunity induced by flagellin suppresses the hypersensitive response in non-host plants elicited by *Pseudomonas syringae* pv. *averrhoi*. *PLoS One* **7**, e41056

67. Hirai, H., Takai, R., Iwano, M., Nakai, M., Kondo, M., Takayama, S., Isogai, A., and Che, F. S. (2011) Glycosylation regulates specific induction of rice immune responses by *Acidovorax avenae* flagellin. *J Biol Chem* **286**, 25519-25530
68. Shanks, K. K., Guang, W., Kim, K. C., and Lillehoj, E. P. (2010) Interleukin-8 production by human airway epithelial cells in response to *Pseudomonas aeruginosa* clinical isolates expressing type a or type b flagellins. *Clin Vaccine Immunol* **17**, 1196-1202
69. Verma, A., Arora, S. K., Kuravi, S. K., and Ramphal, R. (2005) Roles of specific amino acids in the N terminus of *Pseudomonas aeruginosa* flagellin and of flagellin glycosylation in the innate immune response. *Infect Immun* **73**, 8237-8246
70. Guerry, P. (2007) *Campylobacter* flagella: not just for motility. *Trends Microbiol* **15**, 456-461
71. Schirm, M., Soo, E. C., Aubry, A. J., Austin, J., Thibault, P., and Logan, S. M. (2003) Structural, genetic and functional characterization of the flagellin glycosylation process in *Helicobacter pylori*. *Mol Microbiol* **48**, 1579-1592
72. de Zoete, M. R., Keestra, A. M., Wagenaar, J. A., and van Putten, J. P. (2010) Reconstitution of a functional Toll-like receptor 5 binding site in *Campylobacter jejuni* flagellin. *J Biol Chem* **285**, 12149-12158
73. Mahenthiralingam, E., and Vandamme, P. (2005) Taxonomy and pathogenesis of the *Burkholderia cepacia* complex. *Chron Respir Dis* **2**, 209-217
74. Hamad, M. A., Skeldon, A. M., and Valvano, M. A. (2010) Construction of aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in studies of intracellular bacteria with the gentamicin protection assay. *Appl Environ Microbiol* **76**, 3170-3176
75. Miller, V. L., and Mekalanos, J. J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J Bacteriol* **170**, 2575-2583
76. Figurski, D. H., and Helinski, D. R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* **76**, 1648-1652

FOOTNOTES

[+] This work was supported by supported by grants from the Canadian Institutes of Health Research and the UK Cystic Fibrosis Trust (to M.A.V.) and COST action BM1003 "Microbial cell surface determinants of virulence as targets for new therapeutics in cystic fibrosis" (to A.M. and M.A.V.)

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[2] The abbreviations used are: Bcc: *Burkholderia cepacia* complex; LPS, lipopolysaccharide; TLR, Toll-like receptor;

TABLE 1. Strains and plasmids used in this study.

Name	Description	Source
Strains		
Burkholderia cenocepacia		
K56-2	ET12 clone related to J2315, cystic fibrosis clinical isolate	BCRRC,[1] (2,73)
RSF44	K56-2, Δ*fliCD*	(38)
MH1K	K56-2, Δ*amrABC* (BCAL1674–1676); Gm^s	(74)
ΔBCAL3119-3131	MH1K, Δ*wbiI-wzm*	This study
ΔBCAL3123-3124	MH1K, Δ*wbxCD*	This study
ΔBCAL0111	MH1K, Δ*flmQ* (flagellin glycan glycosyltransferase)	This study
ΔBCAL0110	MH1K, ΔBCAL0110 (*vioA* paralog in the *fliC* gene cluster)	This study
ΔBCAS0105	MH1K, ΔBCAS0105 (*rmlD* paralog in chromosome 3)	This study
ΔBCAS0105 pSC200/*rmlD*	MH1K, ΔBCAS0105, containing P_{rha}::*rmlD*	This study
ΔBCAL3129	MH1K, Δ*vioA*	This study
XOA10	K56-2, P_{rha}::BCAL1928	(17)
XOA11	K56-2, P_{rha}::*arnT*	(17)
MH43	MH1K, Δ*wbxD* (BCAL3124)	M. Hamad
MH1K pSC200/*rmlD*	MH1K, P_{rha}::*rmlD*	This study
MH1K pSC200/*rmlB*	MH1K, P_{rha}::*rmlB*	This study
MH1K pSC200/*rmlC*	MH1K, P_{rha}::*rmlC*	This study
MH1K pSC200/BCAL0111	MH1K, P_{rha}::BCAL0111	This study
ΔBCAL3123-3124 pIN62/BCAL3123	MH1K, Δ*wbxCD*; *wbxC*$^+$	This study
E. coli		
GT115	F- *mcrA* Δ*(mrr-hsdRMS-mcrBC)* φ*80lacZ*Δ*M15* Δ*lacX74 recA1 rpsL endA1* Δ*dcm uidA(*Δ*MluI)::pir-116* Δ*sbcC-sbcD*; used as donor strain	Laboratory stock
SY327	*araD* Δ(*lac pro*) *argE*(Am) *recA56 nalA* λ *pir*; Rif^r; used as helper strain	(75)
Plasmids		
pRK2013	Helper plasmid used for bacterial conjugation; Kan^r	(76)
pGPI-SceI-2	Suicide vector used for genetic manipulation of *B. cenocepacia*; Tp^r	(14)
pDAI-SceI-SacB	Replicative vector expressing I-SceI homing endonuclease; Tet^r	(74)
pIN62	Broad host range replicative vector expressing DSRed, Cm^r	(40)
pGPΩTp	Suicide vector used for genetic manipulation of *B. cenocepacia*; Tp^r	(39)
pSC200	Rhamnose inducible vector used for depletion experiments; Tp^r	(17)
pGPI-SceI-2/BCAL3119-3131	Suicide vector used to delete O-antigen cluster	This study
pGPI-SceI-2/BCAL3123-3124	Suicide vector used to delete *wbxC* and *wbxD*	This study
pGPI-SceI-2/BCAL0111	Suicide vector used to delete BCAL0111	This study
pGPI-SceI-2/BCAL0110	Suicide vector used to delete BCAL0110	This study
pGPI-SceI-2/BCAS0105	Suicide vector used to delete BCAS0105	This study
pGPI-SceI-2/BCAL3129	Suicide vector used to delete *vioA* homologue in O-antigen cluster	This study
pGPΩTp/*rmlD*	Vector used to create gene disruption in *rmlD* (BCAL3132)	This study
pSC200/*rmlB*	P_{rha}::*rmlB* (BCAL3135)	This study
pSC200/*rmlC*	P_{rha}::*rmlC* (BCAL3133)	This study
pSC200/*rmlD*	P_{rha}::*rmlD* (BCAL3132)	This study
pSC200/BCAL0111	P_{rha}::BCAL0111	This study
pIN62/BCAL3123	Vector used for complementation of *wbxC* (BCAL3123)	This study

[1.] BCRRC, *B. cepacia* Research and Referral Repository for Canadian CF Clinics; Cm^r, chloramphenicol resistance; Tp^r, trimethoprim resistance; Tet^r, tetracycline resistance; Kan^r, kanamycin resistance; DSRed, red fluorescent protein from *Discosoma sp.*

TABLE 2. Primers used in this study (restriction sites are italicized).

Name	Sequence (5'- 3')	Restriction site
5235	gattgat*gcggccgc*gaagccgccatcggcgcgaacccg	NotI
5236	gcacctaa*gatct*gccagcatgcgccgtcttgcggg	BglII
5237	tagctg*agatct*ggcgcaatcggcaatgagggcgaccag	BglII
5238	aacgtg*tctaga*agtgtggtggtgtcgctgctgagc	XbaI
5685	cgtagt*gaattc*gacggcagcaagcaggcaccttattcgga	EcoR1
5686	atcata*tctaga*ccggcacgccgttccgcgagggacttc	XbaI
5852	aatga*agatct*cgccgccgtgccccatgctcgacgcctg	BglII
5853	catat*gcggccgc*ctacaagcacgtgccgctgatggaag	NotI
5888	gatcgat*gcggccgc*acttgaaagacgatcattcccacg	NotI
5889	attgc*tctaga*cgttttgatgaacgtttcggact	XbaI
5922	gcacctaa*gatct*ctaccgaaggggcaggccggggctgtt	BglII
5923	gtagtc*gcggccgc*cgagtcgaggacgtcgagttcggcg	NotI
5924	cagtac*tctaga*gtcgtcggacgggggatacggtggtc	XbaI
5925	gtagtc*gcggccgc*ccgttacccgacctacacgcccgacgtc	NotI
6021	tagcta*catatg*atcctggttacgggcggcgcggg	NdeI
6022	taacg*tctaga*gaacgtgccgaccacgttggtctggac	XbaI
6023	tagctg*catatg*cgtgaggcaacgatgagctggaaaccg	NdeI
6024	atatg*tctaga*cgagccgcgcgctgcggcaacgcgtgcc	XbaI
6093	cgggtgatccgggaagttctggatgaagacctggcggc	n/a
6094	aatgaacgagtgcttccgccgacgccaaaacggctttcc	n/a
6165	catag*cggccgc*cttctgcccaccattcgtcaaccacgc	NotI
6166	cgact*agatct*atctaagcatcggtcaggtcgacacatg	BglII
6167	catag*cggccgc*aagcagttcaacgtattcgcgcgtcgc	NotI
6168	gtca*tctaga*gctgagcgccgtgttgtatgcggcacatg	XbaI
Q38	tca*tctaga*gctcgtcgatttgatcggtacgcgccatac	XbaI
Q39	ccttttg*cggccgc*aatgcccgtattgcgcgcgccagac	NotI
Q89	tagctg*catatg*atgttctcgaccgaactgcccgccac	NdeI
Q90	taacg*tctaga*ccgtttgcccggtgcgatgcagcg	XbaI
Q91	tagcta*catatg*atggccatccaagtaacggtgacagc	NdeI
Q92	taacg*tctag*atcgtccgacaggacaacccccacccac	XbaI
L3123 US BglII	gact*agatct*ccgtggccattcgtgccacaggcatcc	BglII
L3123 US NotI	atta*gcggccgc*atcgcgatgctctggcgagacgagcg	NotI
L0110 US XbaI	agtca*tctaga*ttgcgtgcacgctgctcagcgtccgcgg	XbaI
L0110 US NotI	catag*cggccgc*gcaagggtgccgttcgcgaacagcgac	NotI
L0110 DS NotI	catag*cggccgc*gtcgcgaaccacgcgtatttcccgatc	NotI
L0110 DS BglII	acgcgttc*agatct*ttcgagttcgacaacagcgcgatgg	BglII
L3123 XbaI	tagtca*tctaga*ttaggccgaccgtttcatcaatggcac	XbaI
L3123 NdeI	acgct*catatg*gattggagtgaatgatggagcgaatcgc	NdeI

TABLE 3. Peptide ions identified after combining MS/MS data from tryptic, chymotryptic and AspN/tryptic digests of *B. cenocepacia* FliC. Representative unmodified and modified ions are presented. Ions were confirmed in QTof and Orbitrap Elite analyses; (+231) refers to glycan modification [Qui4N(3HOBut)]; oxidation refers to methionine (+16 Da).

Start-End	MW			Oxidation (+16)	Sequence
	Observed (*m/z*)	Calculated	Expected		
1 - 36	954.2421	3812.9393	3812.9915	yes	MLGINSNINSLVAQQNLNGSQNALSQAITRLSSGKR
37 - 52	773.3992	1544.7838	1544.7794	no	INSAADDAAGLAISTR
53 - 90	992.7329	3966.9025	3966.9011	yes (2 x)	MQTQINGLNQGVSNANDGVSMIQTASSALSSLTNSLQR
91 - 106	840.9565	1679.8984	1679.8512	yes	IRQLAVQASTGTMSTT
107 - 137	1154.2551	3459.7435	3459.7230	no	DQAALQQEVSQQIQEVNRIASQTTYNGTNIL
138 - 158	1010.5225	2019.0304	2019.0273	no	DGSAGIVSFQVGANVGQTISL
159 - 168	519.2255	1036.4364	1036.485	no	DLSQSMSAAK
159 - 168	527.2358	1052.4570	1052.4808	yes	DLSQSMSAAK
159 - 168	642.8051	1283.5956	1283.5677	yes	DLSQSMSAAK **(+231)***
169 - 176	386.2400	770.4654	770.4650	no	IGGGLVQK
177 - 211	1118.2345	3351.6817	3351.6794	no	GQTVGTVTGLSLDNNGAYTGSGATITAINVLSDGK
177 - 211	1195.2588	3582.7546	3582.7663	no	GQTVGTVTGLSLDNNGAYTGSGATITAINVLSDGK **(+231)**
187 - 206	1085.5468	2169.0790	2169.0199	no	SLDNNGAYTGSGATITAINV **(+231)**
187 - 211	813.7294	2438.1664	2438.1925	no	SLDNNGAYTGSGATITAINVLSDGK
187 - 211	890.7588	2669.2546	2669.2794	no	SLDNNGAYTGSGATITAINVLSDGK **(+231)**
189 - 208	969.9615	1937.9084	1937.9330	no	DNNGAYTGSGATITAINVLS
189 - 208	724.0175	2169.0307	2169.0199	no	DNNGAYTGSGATITAINVLS **(+231)**
189 - 217	1032.8304	3095.4694	3095.4333	no	DNNGAYTGSGATITAINVLSDGKGGYTFT **(+231)**
212-233	904.7971	2709.2692	2709.2659	no	GGYTFTDQNGGAISQTVAQSVF **(2 x 231)#**
234 - 251	1055.0292	2108.0438	2108.0009	no	GANATTGTGTAVGNLTLQ **(2 x 231)***
252 - 286	1258.6530	3772.9372	3772.9331	no	SGATGAGTSAAQQTAITNAIAQINAVNKPATVSNL **(2 x 231)**
287 - 321	1176.9285	3527.7637	3527.7637	yes	DISTVSGANVAMVSIDNALQTVNNVQAALGAAQNR
287 - 321	1253.9667	3758.8745	3758.8745	yes	DISTVSGANVAMVSIDNALQTVNNVQAALGAAQNR **(+231)**
290 - 321	1148.9210	3443.7412	3443.7076	yes	TVSGANVAMVSIDNALQTVNNVQAALGAAQNR **(+231)**
322 - 357	952.9465	3807.7569	3807.7381	yes	FTAIATSQQAESTDLSSAQSQITDANFAQETANMSK
359 - 382	849.4679	2545.3819	2545.4592	no	QVLQQAGISVLAQANSLPQQVLKL
371 - 384	790.4595	1578.9044	1578.9093	no	QANSLPQQVLKLLQ

***** data obtained with QTof only

data obtained with Orbitrap Elite only

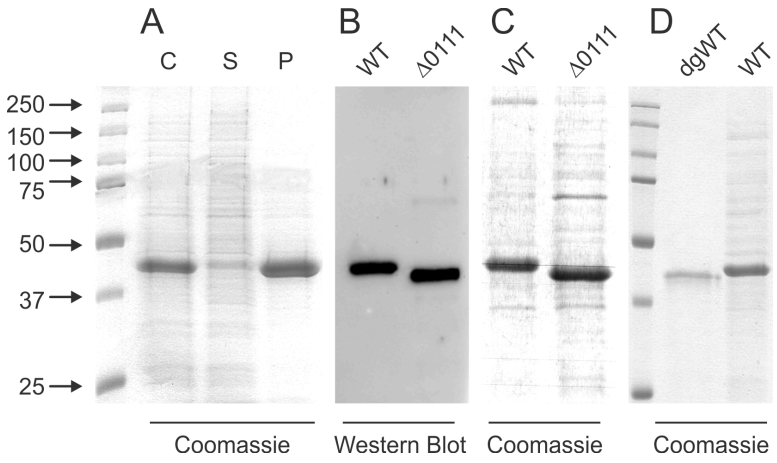

FIGURE 1**. SDS-PAGE and Western blot analyses of *B. cenocepacia* FliC.** *A*, Coomassie stained SDS-PAGE showing crude flagellar filaments (C), supernatant obtained after insoluble flagella were sedimented at 16,000 xg for 10 min (S), and purified flagellin after solubilization with 8 M urea and desalting (P). *B*, Crude flagellar filaments from the *B. cenocepacia* parental strain (WT) and ΔBCAL0111 (Δ0111) were analysed by Western blot with the AVIVA RFFL/ARP42986_P050 antibody. *C*, Coomassie-blue stained SDS-PAGE of crude flagellar filaments from *B. cenocepacia* parental strain (WT) and ΔBCAL0111 (Δ0111) from the same preparation used in panel B. *D*, Coomassie blue stained SDS-PAGE of chemically deglycosylated (dgWT) and native (WT) flagellin. Arrows indicate the corresponding molecular masses of the protein standards in kDa.

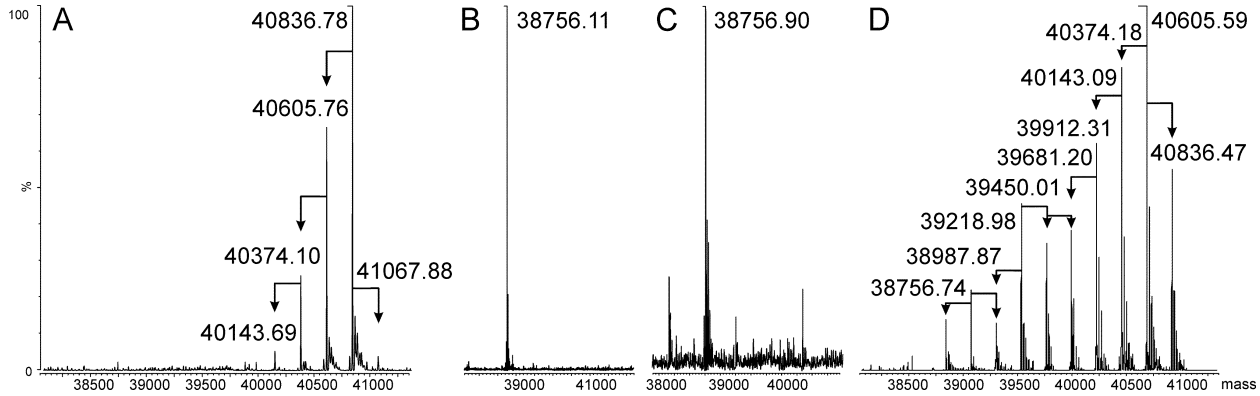

FIGURE 2. **Mass spectra of purified flagellin preparations.** *A, B. cenocepacia* flagellin. *B*, chemically deglycosylated flagellin. *C*, non-glycosylated flagellin purified from the ΔBCAL0111 mutant strain. *D*, flagellin purified from strain MH43 (Δ*wbxD*). Arrows indicate the difference of 231 *m/z* between ions.

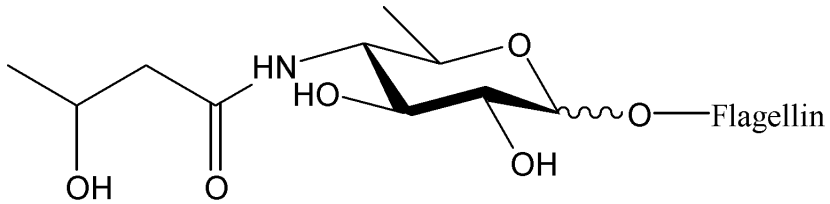

FIGURE 3. **Structure of the *B. cenocepacia* FliC glycan** [4,6-dideoxy-4-(3-hydroxybutanoylamino)-D-glucose, D-Qui4N(3HOBut)].

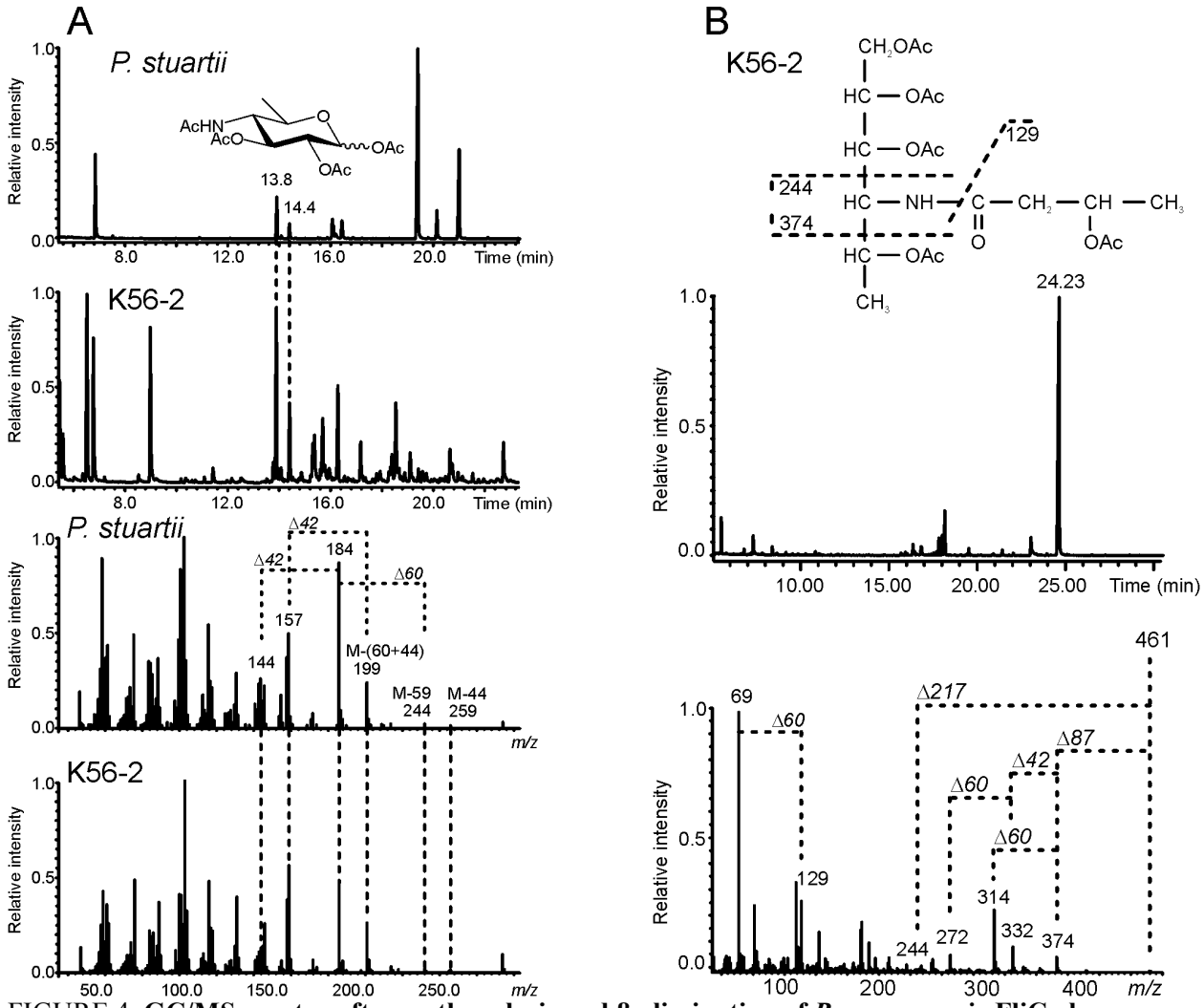

FIGURE 4. **GC/MS spectra after methanolysis and β-elimination of *B. cenocepacia* FliC glycan (K56-2) and control sample (O antigen of *P. stuartii* O43).** *A*, the top two graphs correspond to an overview of entire spectra for *P. stuartii* O43 O antigen and *B. cenocepacia* K56-2 FliC samples. Qui4N peaks at 13.8 and 14.4 (representing α and β-configured derivatives) are indicated. Additional peaks detected in the O43 spectrum represent other sugars from the O antigen (44). Additional peaks in the FliC spectrum represent derivatized amino acids released from the FliC protein during methanolysis. The lower two spectra show the characteristic fragmentation pattern of ions at 13.8 min (fragmentation pattern of ion at 14.4 min was identical). M corresponds to molecular weight of derivatized Qui4N (303 Da). *B*, the top graph shows an overview of the GC spectrum of the glycan released from FliC during β-elimination. Insert shows the derivatized glycan (461 Da) with the characteristic fragmentation pattern of the sugar and 3-hydroxy butyric acid. Lower graph shows the MS/MS fragmentation spectrum of the ion at 24.23 min. Differences between fragment ions (Δ) correspond to CH_2CO (Δ42), CH_3CHO (Δ44), CH_3COO^- (Δ59), and CH_3COOH (Δ60).

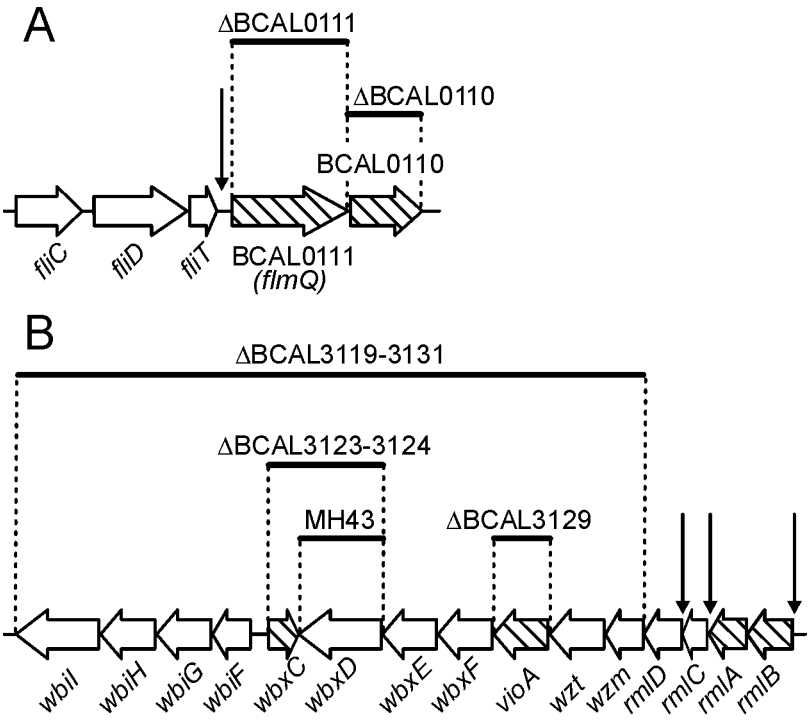

FIGURE 5. **Gene organization of the *fliC* region (*A*) and the O-antigen cluster (*B*) in *B. cenocepacia*.** Deletion mutants are indicated by thick bars. Vertical arrows indicate insertion sites of the rhamnose inducible pSC200 vector. Genes showed as striped arrows encode the predicted enzymes required for FliC glycosylation.

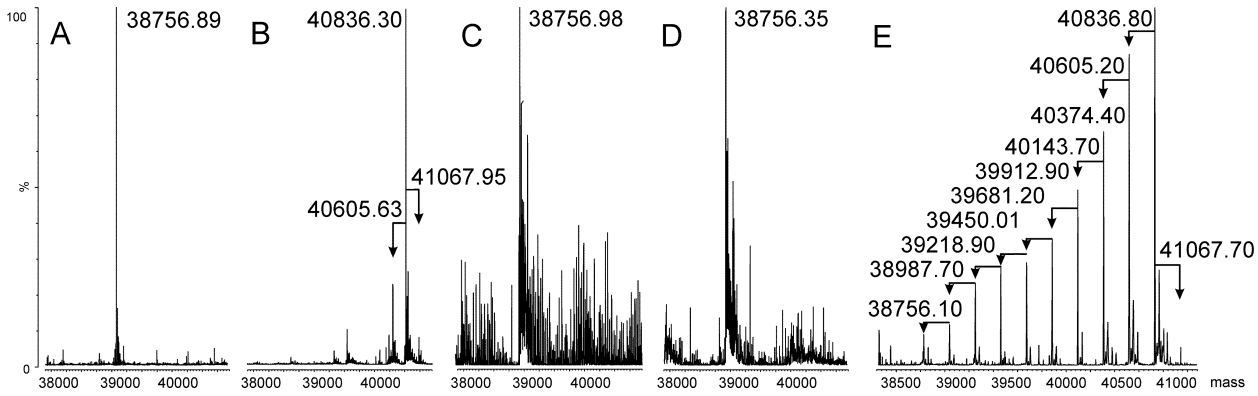

FIGURE 6. **Mass spectra of flagellin from various *B. cenocepacia* mutant strains.** *A*, ΔBCAL3119-3131; *B*, MH1K pSC200/BCAL0111 grown in the presence of rhamnose; *C*, MH1K pSC200/BCAL0111 grown without rhamnose; *D*, ΔBCAL3123-3124; *E*, ΔBCAL3123-3124 pIN62/BCAL3123. Arrows indicate the ΔMW of 231 Da.

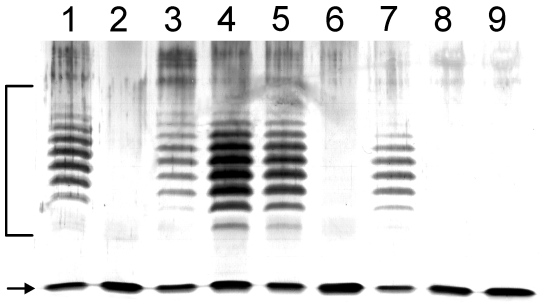

FIGURE 7. **Silver stained 14% SDS-PAGE of whole cell lysates of *B. cenocepacia***. Whole cell lysates from *B. cenocepacia* mutants were analyzed in silver stained 14% SDS-PAGE. The strains used were: MH1K (lane 1), ΔBCAL3119-3131 (lane 2), ΔBCAL3129 (lane 3), ΔBCAL0110 (lane 4), ΔBCAL0111 (lane 5), ΔBCAL3123-24 (lane 6), ΔBCAS0105 (lane 7), ΔBCAS0105 pGPΩTp/*rmlD* (lane 8), and MH1K pGPΩTp/*rmlD* (insertional mutant inactivating the last enzymatic step in dTDP-rhamnose biosynthesis; lane 9). Ladder-like bands (bracket) correspond to LPS containing lipid A-core covalently linked to O-antigen polysaccharides of varying length. Single bands in the low molecular weight region (arrow) correspond to lipid A-core molecules without O antigen.

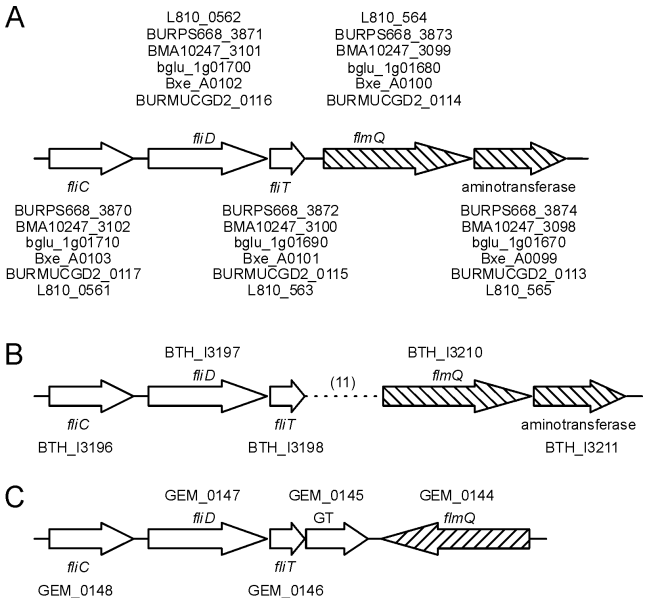

FIGURE 8. **Gene organization in *fliC* clusters of other *Burkholderia* species.** The identity to *flmQ* is indicated in parenthesis. *A*, *B. pseudomallei* 668 (BURPS668; 49%), *B. mallei* NCTC 10247 (BMA10247; 49%), *B. glumae* (bglu_1g; 47%), *B. xenovorans* LB400 (Bxe_A; 49%), *B. multivorans* CGD2 (BURMUCGD2; 80%), *B. vietnamiensis* AU4i (L810; 89%). *B*, *B. thailandensis* E264, dotted line represents eleven genes inserted between the putative *fliT* and *flmQ* (BCAL0111) homologues. *C*, *B. cepacia* GG4. Genes showed as striped arrows represent BCAL0111 (*flmQ*) homologue, and aminotransferase, a BCAL0110 homologue. GT, glycosyltransferase.

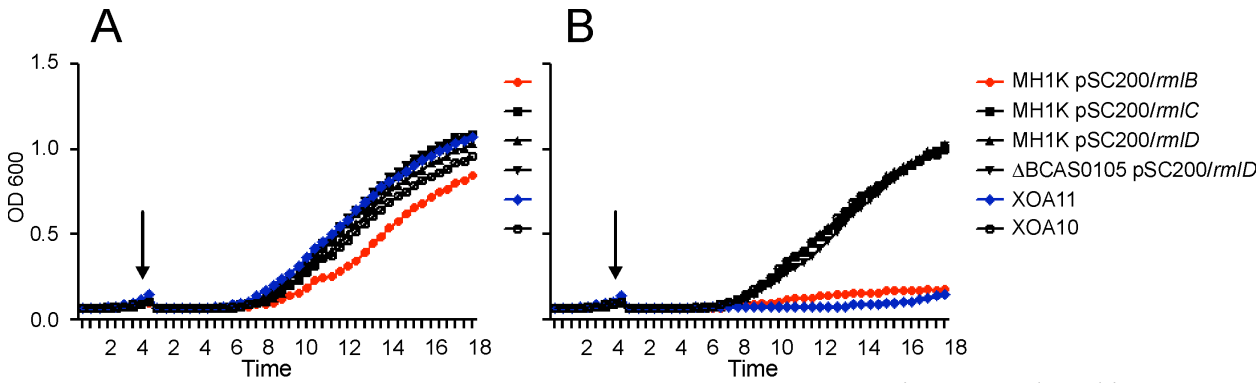

FIGURE 9. **Conditional lethal phenotypes of *B. cenocepacia* strains**. Strains were cultured in LB supplemented with 0.5% (wt/vol) rhamnose (*A*) or without rhamnose (*B*). After initial growth for 4 h (arrow), cultures were diluted 1:100 in fresh medium and incubated for 18 h.

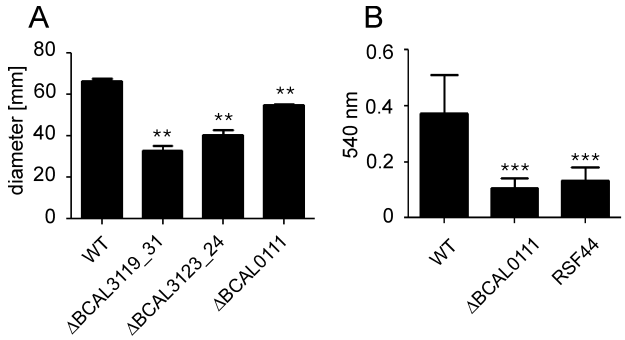

FIGURE 10. **Motility on soft LB agar plates (*A*) and biofilm formation (*B*) of *B. cenocepacia* strains.** Data are representative of three independent experiments. Statistical analysis was performed by paired *t*-test using two-tailed P-values. Significant differences in comparison with *B. cenocepacia* parental strain (WT) as control are indicated by ** ($P < 0.01$) or *** ($P < 0.005$).

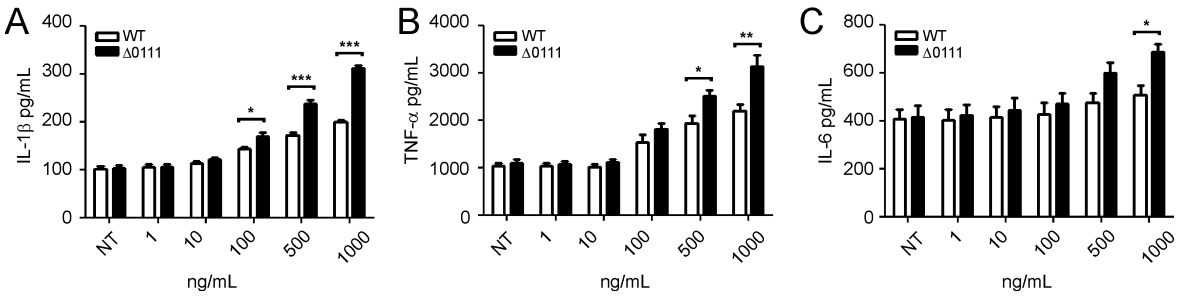

FIGURE 11. **Regulation of pro-inflammatory gene expression in THP1 cells by glycosylated and non-glycosylated forms of flagellin.** THP1 cells were stimulated for 24 h in the absence (NT, non-treated) or presence of varying concentrations of fully glycosylated wild type (WT) or non-glycosylated (Δ0111) forms of flagellin, purified from the *B. cenocepacia* parental or ΔBCAL0111 strains respectively. Conditioned media were assayed for expression levels of (*A*) IL-1β, (*B*) TNF-α and (*C*) IL-6. Data are representative of three independent experiments. Statistical analysis was performed by paired *t*-test using two-tailed P-values. Significant differences between samples from WT and Δ0111-treated cells are indicated by * ($P<0.05$), ** ($P < 0.01$) or *** ($P < 0.001$).

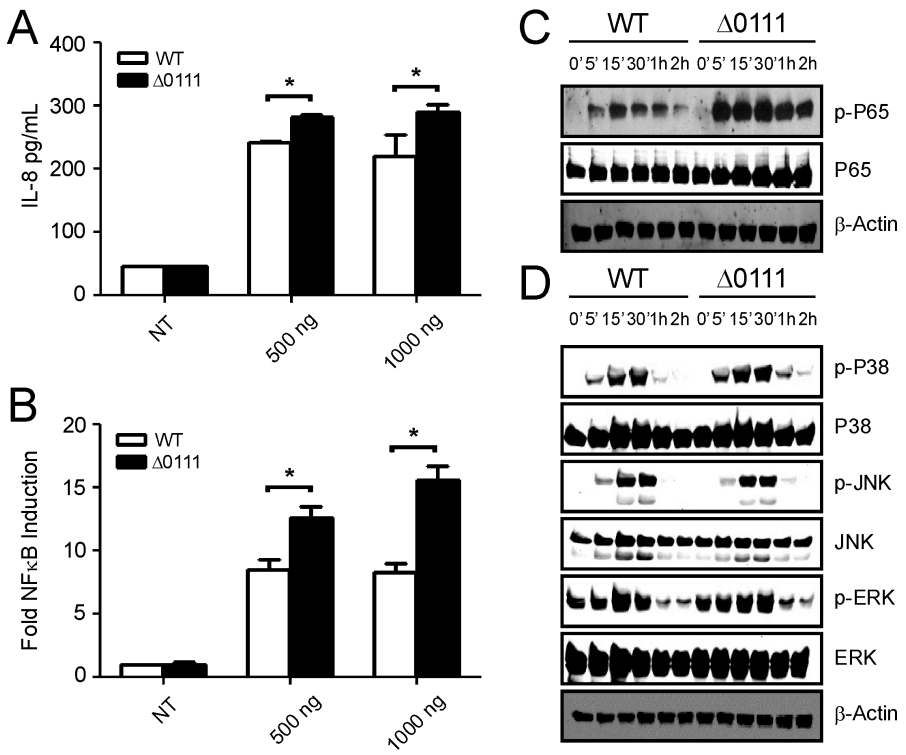

FIGURE 12. **Differential stimulation of TLR5 signalling by glycosylated and non-glycosylated forms of flagellin.** *A*, HEK293 cells, stably expressing TLR5 were stimulated for 24 h in the absence (NT, non-treated) or presence of varying concentrations of fully glycosylated wild type (WT) or non-glycosylated (Δ0111) forms of flagellin purified from the *B. cenocepacia* parental or ΔBCAL0111 strains respectively. Conditioned medium was assayed for expression levels of IL-8. *B*, HEK293 cells, stably expressing TLR5, were transfected with a NFκB-regulated luciferase reporter gene and stimulated for 24 h as indicated above. Cell lysates were assayed for NFκB-regulated firefly luciferase activity and fold induction levels of NFκB-regulated luciferase are expressed relative to non-treated (NT) cells. Data are representative of three independent experiments. Statistical analysis was performed by paired *t*-test using two-tailed P-values. Significant differences between samples from WT and Δ0111-treated cells are indicated by * ($P<0.05$). HEK293 cells, stably expressing TLR5 were stimulated for indicated times with WT and Δ0111 flagellin (500 ng/ml). Cell lysates were immunoblotted for phosphorylated (p-) and total levels of p65 (*C*) and p38 (*D*), JNK and ERK MAP kinases. β-Actin was used as a loading control.