



**QUEEN'S
UNIVERSITY
BELFAST**

A general protein O-glycosylation machinery conserved in Burkholderia species improves bacterial fitness and elicits glycan immunogenicity in humans

Mohamed, Y. F., Scott, N. E., Molinaro, A., Creuzenet, C., Ortega, X., Lertmemongkolchai, G., Tunney, M. M., Green, H., Jones, A. M., DeShazer, D., Currie, B. J., Foster, L. J., Ingram, R., De Castro, C., & Valvano, M. A. (2019). A general protein O-glycosylation machinery conserved in Burkholderia species improves bacterial fitness and elicits glycan immunogenicity in humans. *The Journal of Biological Chemistry*, 294(36), 13248. Advance online publication. <https://doi.org/10.1074/jbc.RA119.009671>

Published in:

The Journal of Biological Chemistry

Document Version:

Peer reviewed version

Queen's University Belfast - Research Portal:

[Link to publication record in Queen's University Belfast Research Portal](#)

Publisher rights

© 2019 The Authors. This work is made available online in accordance with the publisher's policies. Please refer to any applicable terms of use of the publisher.

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Open Access

This research has been made openly available by Queen's academics and its Open Research team. We would love to hear how access to this research benefits you. – Share your feedback with us: <http://go.qub.ac.uk/oa-feedback>

A general protein *O*-glycosylation machinery conserved in *Burkholderia* species improves bacterial fitness and elicits glycan immunogenicity in humans†

Yasmine Fathy Mohamed^{1,2#}, Nichollas E. Scott^{3,4#}, Antonio Molinaro⁵, Carole Creuzenet⁶, Ximena Ortega⁶, Ganjana Lertmemongkolchai⁷, Michael M. Tunney⁸, Heather Green⁹, Andrew M. Jones⁹, David DeShazer¹⁰, Bart J. Currie¹¹, Leonard J. Foster⁴, Rebecca Ingram¹, Cristina De Castro¹² and Miguel A. Valvano^{1,6*}

From the ¹Wellcome-Wolfson Institute of Experimental Medicine, Queen's University Belfast, Belfast, BT97BL, United Kingdom; ²Department of Microbiology and Immunology, Faculty of Pharmacy, Alexandria University, Egypt; ³Department of Microbiology and Immunology, University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne 3000, Australia; ⁴Department of Biochemistry & Molecular Biology, University of British Columbia, Vancouver, British Columbia, V6T1Z4, Canada; ⁵Department of Chemical Sciences, University of Naples Federico II, Via Cintia 4, 80126 Napoli, Italy; ⁶Department of Microbiology and Immunology, University of Western Ontario, London, ON, N6A 5C1, Canada; ⁷Centre for Research and Development of Medical Diagnostic Laboratories, Mekong Health Sciences Research Institute, Khon Kaen University, Khon Kaen, Thailand; ⁸Halo Research Group, School of Pharmacy, Queen's University Belfast; ⁹Manchester Adult Cystic Fibrosis Centre, University Hospital of South Manchester NHS Foundation Trust, Manchester, M23 9LT, United Kingdom; ¹⁰Bacteriology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, United States; ¹¹Menzies School of Health Research and Infectious Diseases Department Royal Darwin Hospital, Darwin, Australia; ¹²Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055 Portici, Italy

Running title: *Protein O-glycosylation in Burkholderia*

equal contribution

* To whom correspondence should be addressed: Miguel A. Valvano: Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, BT9 7BL; m.valvano@qub.ac.uk; Tel. +44 289097-6025.

† This manuscript is dedicated to the memory of Professor Mary Jane Osborn, deceased on January 17, 2019, for her pioneering work in microbial glycobiology.

Keywords: protein glycosylation, *O*-glycosylation, cystic fibrosis, melioidosis, glanders, *Burkholderia*, bacterial virulence, glycobiology, glycosyltransferase

ABSTRACT

The *Burkholderia* genus encompasses many Gram-negative bacteria living in the rhizosphere. Some *Burkholderia* species can cause life-threatening human infections, highlighting the need for clinical interventions targeting specific *Burkholderia* proteins. *Burkholderia cenocepacia* *O*-linked protein glycosylation has been reported, but the chemical structure of the *O*-glycan and the machinery required for its biosynthesis are unknown and could reveal potential therapeutic targets. Here, using bioinformatics approaches, gene-knockout mutants, purified recombinant

proteins, LC-MS-based analyses of *O*-glycans, and NMR-based structural analyses, we identified a *B. cenocepacia* *O*-glycosylation (*ogc*) gene cluster in necessary for synthesis, assembly, and membrane translocation of a lipid-linked *O*-glycan, as well as its structure, which consists of a β -Gal-(1,3)- α -GalNAc-(1,3)- β -GalNAc trisaccharide. We demonstrate that the *ogc* cluster is conserved in the *Burkholderia* genus and confirm the production of glycoproteins with similar glycans in the *Burkholderia* species *B. thailandensis*, *B. gladioli*, and *B. pseudomallei*. Further, we show that absence of protein *O*-glycosylation severely affects bacterial fitness and accelerates

bacterial clearance in a *Galleria mellonella* larva infection model. Finally, our experiments revealed that patients infected with *B. cenocepacia*, *B. multivorans*, *B. pseudomallei*, or *B. mallei* develop O-glycan-specific antibodies. Together, these results highlight the importance of general protein O-glycosylation in the biology of the *Burkholderia* genus and its potential as a target for inhibition or immunotherapy approaches to control *Burkholderia* infections.

The *Burkholderia* genus encompasses widespread environmental Gram-negative bacteria adapted to survive in the rhizosphere and fresh water (1,2). Some *Burkholderia* species including those in the *B. cepacia* complex, *B. mallei*, and *B. pseudomallei* cause life-threatening infections in humans. The *B. cepacia* complex encompasses over 18 closely related species typically infecting patients with cystic fibrosis; some of these species, especially *B. cenocepacia*, cause the fatal cepacia syndrome (3). *B. mallei* and *B. pseudomallei* are potential biological warfare agents (4), which cause glanders and melioidosis, respectively. *B. mallei* typically infects horses, donkeys and mules, which are the primary reservoir of this mammalian-adapted pathogen that evolved from the environmental pathogen *B. pseudomallei* through gene loss (5). Glanders is characterized by pneumonia, often with systemic sepsis, or by skin and lymph node disease (Farcy). *B. pseudomallei* is endemic to southeast Asia and northern Australia and is being increasingly recognized in the tropics globally, including Africa and the Americas (6-8). Melioidosis can be acquired by inhalation or through skin abrasions or ingestion, causing a wide spectrum of symptoms including pneumonia and rapidly progressive systemic sepsis (7). *B. pseudomallei* can also remain latent and asymptomatic, with subsequent activation and presentation as melioidosis many years after initial exposure (9). Antibiotic treatment of *Burkholderia*-related infections is difficult due to the high-level intrinsic multidrug resistance of these bacteria (10), and there are no clinically approved vaccines for either melioidosis or glanders prevention in humans or animals (11).

Protein glycosylation is a post-translational modification that modulates protein physicochemical properties and

functions, and is common in bacteria (12-14). Glycans may be attached to the amide nitrogen of asparagine residues of a polypeptide (*N*-glycosylation) or to hydroxyl oxygen of serine or threonine residues (*O*-glycosylation) (15). In other cases, α -mannopyranose can be linked to tryptophan via a carbon-carbon link (*C*-mannosylation) (16) and glycans can also be attached to the sulfur of cysteine (*S*-linked glycosylation) (17). The *N*-linked general protein glycosylation pathway in bacteria was first elucidated in *Campylobacter jejuni* (18,19). This system includes the oligosaccharyltransferase (OTase) PglB (20), which is homologous to the eukaryotic Stt3, the catalytic subunit of the OTase complex responsible for *N*-glycosylation in the lumen of the endoplasmic reticulum.

O-glycosylation systems have also been noted in many different bacteria (12-15). The *O*-glycosylation of at least 23 proteins with an uncharacterized predicted trisaccharide glycan, mediated by the OTase PglL, was previously identified in *B. cenocepacia* (21). However, the genes and gene products for the *B. cenocepacia* glycosylation machinery and the chemical structure of the glycan moiety were not elucidated. In this study, we report the identification of the *Burkholderia* *O*-glycosylation (*ogc*) cluster, which includes genes in chromosome 1 of *B. cenocepacia* (BCAL3114 to BCAL3118) encoding the enzymes required for the stepwise assembly of the lipid-linked *O*-glycan in the cytoplasm and its flipping across the inner membrane. We also discovered these genes are present in all species of *Burkholderia* and confirm biochemically the presence of *O*-linked proteins in several species of *Burkholderia*, suggesting protein *O*-glycosylation is a conserved feature of this genus. To elucidate the biosynthesis of the *O*-linked glycan, we generated knockout mutants in the *ogc* genes characterizing their effect on glycosylation and assessing the function of the epimerase within this cluster. From these studies we have confirmed the predicted role of the *ogc* genes and also established the structure of the trisaccharide glycan. We also show that *O*-glycosylation is required for bacterial fitness *in vitro* and survival in a larvae infection model. Lastly, we demonstrate that individuals previously infected with *B. cenocepacia*, *B. multivorans*, *B. pseudomallei* and *B. mallei* develop anti-*O*-glycan serum antibodies, suggesting *Burkholderia* glycoproteins display

a common epitope perceived by the human immune system.

RESULTS

A predicted *B. cenocepacia* O-glycosylation gene cluster is conserved in the *Burkholderia* genus. Previous work identified PglL (BCAL0960) as the OTase catalyzing the addition of an uncharacterized trisaccharide O-glycan to at least 23 *B. cenocepacia* proteins (21). In OTase-dependent glycosylation pathways, the lipid-linked glycan precursor is assembled on the cytoplasmic side of the membrane, translocated across the membrane, and then transferred onto acceptor proteins at the periplasmic side (22). We therefore hypothesized that at least four genes encoding a phosphoglycosyl transferase (initiating enzyme that mediates the synthesis of a lipid-linked sugar) (23,24), two additional glycosyltransferases, and a flippase protein would be required for the synthesis and translocation of a *B. cenocepacia* lipid-linked trisaccharide glycan (22,24). The chromosome 1 of *B. cenocepacia* has a putative five-gene operon flanked by the O-antigen synthesis cluster and lipid A-core biosynthesis genes (25) (Fig. 1A), which could be involved in protein glycosylation. These genes were previously annotated, according to their predicted functions, as *wecA* (BCAL3118, phosphoglycosyl transferase), *galE* (BCAL3117, UDP-glucose epimerase), *wbxA* and *wxB* (BCAL3116 and BCAL3115, glycosyltransferases, GT2 family forming β -glycosidic bonds and GT4 family forming α -glycosidic bonds, respectively) and *wzx* (BCAL3114, flippase) (25). This putative operon was herein renamed *ogc* (for O-glycosylation) (see below). The last *ogc* gene, BCAL3118, which encodes a predicted UDP-N-acetylhexosamine-1-P transferase homologous to WecA-like initiating enzymes for O-antigen synthesis, was renamed *ogcI* (for initiating transferase). The immediately upstream gene, BCAL3117, encodes a protein similar to UDP-glucose-4-epimerases of the NAD-dependent epimerase/dehydratase family, and it was renamed *ogcE*. The two predicted glycosyltransferase encoding genes, BCAL3115 and BCAL3116, were renamed *ogcA* and *ogcB*, respectively. BCAL3114, renamed *ogcX*, encodes a predicted flippase protein belonging to the Wzx-like

polysaccharide transporter family. Therefore, the *ogc* cluster encodes proteins whose predicted functions fit well with the required enzymatic activities to assemble the lipid-linked trisaccharide glycan for protein glycosylation, according to the model depicted in Fig. 1B.

Using the SyntTax web server (26) and the *Burkholderia* genome database (27), we discovered the *ogc* cluster is conserved in the majority of *Burkholderia* and *Paraburkholderia* species whose genomes have been sequenced (Fig. 1C). Most species retain the five-gene cluster with the genes arranged as in *B. cenocepacia*. In a reduced number of species, *ogcXABE* remain together while *ogcI* appears as a single monocistronic gene separated from the cluster by a variable number of genes depending on the species examined (Fig. 1C). In *P. rhizoxinica*, *ogcB* is present as a monocistronic gene while the rest of the genes, *ogcXAEI*, form a putative operon (Fig. 1C). Therefore, the *ogc* cluster appears to be a highly conserved loci encoding the minimal set of gene products to produce the trisaccharide O-linked glycan observed in *B. cenocepacia*.

A deletion mutant of the *ogc* cluster cannot glycosylate proteins. To validate the *ogc* cluster's role in protein glycosylation, we constructed an unmarked deletion mutant (Δogc) in the *B. cenocepacia* strain K56-2, and a $\Delta pglL$ mutant as a control. We also generated the individual unmarked, non-polar deletion mutants $\Delta ogcX$, $\Delta ogcI$, $\Delta ogcAB$, and $\Delta ogcE$. In parallel, we constructed recombinant plasmids expressing His-tagged fusion forms of BCAL2640 and the DsbA1 protein from *Neisseria meningitidis*. BCAL2640 is a native glycosylated protein in *B. cenocepacia*, while the heterologously expressed DsbA1 is glycosylated by PglL (21) and also by several different OTases (28). Expression of BCAL2640 and DsbA1 was examined in the K56-2 parental strain and its isogenic Δogc and $\Delta pglL$ mutants. The purified BCAL2640 polypeptide obtained from Δogc and $\Delta pglL$ had increased relative mobility compared to that obtained from the parental strain, suggesting loss of glycosylation (Fig. 1D, left panel). Similarly, examination of DsbA1 mobility by immunoblot with anti-His tag antibodies also revealed DsbA1 polypeptides of lower apparent mass in samples purified from Δogc and $\Delta pglL$, as well as from $\Delta ogcX$, $\Delta ogcI$, $\Delta ogcAB$, and

$\Delta ogcE$ mutants (Fig. 1D, right panel). Conversely, no difference in protein mobility was observed in DsbA1 expressed in an O-antigen synthesis deletion mutant $\Delta BCAL3119$ - $\Delta BCAL3131$, ruling out any contribution of the O antigen genes to protein glycosylation (Fig 1D, right panel).

To directly monitor the glycosylation status of purified BCAL2640 expressed in strains K56-2, Δogc and $\Delta pglL$, the purified protein was digested with trypsin, and analyzed by liquid chromatography–mass spectrometry (LC-MS). The trypsin-derived BCAL2640 peptide

¹⁵²YAPPPAAVPVAATSGAQGGAAAAAAPAGTKPANAPR¹⁸⁷, previously shown to be glycosylated (at the underlined serine residue) (21), was readily observable. Tandem mass spectrometry (MS/MS) and fragmentation by collision-induced dissociation (CID) confirms the peptide is modified with at least two glycans corresponding to HexNAc-HexNAc-262 (Fig. 2A) and HexNAc-HexNAc-Hex (Fig. 2B), while higher-energy collisional dissociation (HCD) fragmentation confirmed the peptide sequence (Fig. 2C). The glycosylated form of this peptide was 15-fold more abundant than the unglycosylated form, indicating the majority of BCAL2640 was glycosylated (Fig. 2D). In contrast, this glycopeptide was absent in the purified protein obtained from $\Delta pglL$ or Δogc mutants (Fig. 2e and f). Therefore, deletion of the *ogc* cluster causes the same defect in protein glycosylation as the loss of the OTase PglL, demonstrating it contains the genes encoding functions for the synthesis and assembly of the protein glycan.

Structural characterization of the O-glycan. To determine the structure of the *B. cenocepacia* O-glycan, glycopeptides modified with the trisaccharide were generated from glycosylated DsbA1 by digestion with proteinase K and enriched by size exclusion chromatography. This glycopeptide fraction was identified by nuclear magnetic resonance (NMR) spectroscopy analysis by both homo- and heteronuclear two-dimensional NMR experiments (Fig. 3A and Table S1). The heteronuclear single quantum coherence (HSQC) spectrum (Fig. 3B) showed three main anomeric signals with ¹H/¹³C values at 5.07/94.5, 4.63/101.7, and 4.45/106.3 ppm (Fig. 3A), each labeled with a capital letter. For the **A** spin system, total correlation spectroscopy (TOCSY) displayed three

correlations that connected H-1 up to H-4 (Fig. 3c and Table S1). The attribution of these densities to the right proton in the sequence was inferred by analyzing the homonuclear correlation (COSY) spectrum, while the position of H-5 was identified by the H-4/H-5 correlation in the transverse rotating-frame Overhauser enhancement (T-ROESY) spectrum, which in turn defined the position of the two H-6 protons at 3.76 ppm. These results, together with those derived from the HSQC spectrum (Fig. 3B) and the H-2/C-2 values (4.38/51.0 ppm) along with the presence of an acetyl group in the proton spectrum, indicated that **A** was *N*-acetylgalactosamine. The ³J_{H1,H2} value (3.3 Hz) pointed to a α -configuration for the anomeric centre. In addition, the downfield displacement of C-3 (79.1 ppm) with respect to the standard value (72.3 ppm) (29) indicated a substitution at this position.

For **B** and **C** residues, their ³J_{H1,H2} values (8.4 and 8.0 Hz, respectively) disclosed their β configuration at the anomeric center. Using the same approach as described for residue **A**, residue **B** was identified as *N*-acetylgalactosamine (H-2/C-2 values at 4.11/52.0 ppm) and residue **C** as galactose (H2/C-2 values at 3.51/71.6 ppm). The comparison of the carbon chemical shifts of these two residues with those taken as reference (29) showed that C-3 of **B** (75.8 ppm) and C-6 of **C** (64.4 ppm) were shifted downfield with respect to the standard values (72.3 and 62.0 ppm, respectively), suggesting the presence of a substitution at these positions. The sequence of the three units was inferred by NOE contacts found in the T-ROESY spectrum among H-1 of **A** and both H-3 and H-4 of **B**, and H-1 of **C** with H-3 of **A** (Fig. 3c). Therefore, combining data from the nuclear Overhauser effects with the ¹³C chemical shift displacement observed allowed us to build the sequence C-(1→3)-A-(1→3)-**B**. Importantly, H-1 of **B** did not correlate with any sugar-related signal, but only with protons related to the peptide moiety, for which we conclude the glycan structure is: β -Gal-(1→3)- α -GalNAc-(1→3)- β -GalNAc-(1→) directly linked to the protein (Fig. 3B). The T-ROESY spectrum did not contain cross-peaks connecting to any of the two H-6 of **C** to the substituent at O-6, but the type of chemical displacement of C-6 and C-5, along with the ¹H chemical shift of both H-6s, suggested the presence of an acyl substituent that was

consistent with a succinylated hexose residue, observed as a mass of 262 Da by MS analysis, but remains to be structurally identified by NMR.

Dual role of OgcE in protein glycosylation and O-antigen synthesis. The predicted OgcE epimerase (Fig. 1A) could be required for the synthesis of UDP-GalNAc, UDP-Gal or both, which are the expected precursors for the assembly of the trisaccharide glycan. However, the O antigen in *B. cenocepacia* K56-2 is composed of a GalNAc-GalNAc-Rha repeating unit (25), suggesting UDP-GalNAc is a common precursor for both O-antigen and protein glycan trisaccharide synthesis in this strain. To determine if OgcE is also required for O-antigen synthesis, we examined the lipopolysaccharide (LPS) profile of K56-2, Δ *pglL*, Δ *ogc*, and various deletion mutants in *ogc* genes. Only the Δ *ogc* and Δ *ogcE* mutants produced truncated O antigen, as indicated by a strong band migrating above the band corresponding to lipid A-core and the loss of bands corresponding to polymeric O antigen (Fig. 4A). The O antigen in *B. cenocepacia* K56-2 is synthesized *via* the ABC export pathway (25,30). This requires an adaptor sugar, bound to undecaprenyl-PP, to which the repeating O-antigen units are attached, and this sugar in K56-2 is QuiNAc (31). Therefore, the truncated O-antigen band, in the absence of UDP-GalNAc due to the loss of *ogcE* (Fig. 4A, asterisk), was interpreted as lipid A-core plus Rha-WhoNAc disaccharide. We have previously shown that a similar band appears in a GalNAc transferase mutant (25). Complementation of Δ *ogc* with a plasmid expressing OgcE restored full O-antigen synthesis (Fig. 4A), but did not restore protein glycosylation (Fig. 4B). Therefore, OgcE (BCAL3117) participates in both the synthesis of O antigen and the protein glycan (Fig. 4A and B), while the remaining *ogc* genes are not involved in O-antigen synthesis.

OgcE is a UDP-Glc and UDP-GlcNAc epimerase. To elucidate the biochemical function of OgcE, we examined its enzymatic activity by capillary electrophoresis. Purified OgcE showed C4 epimerase activity on UDP-Gal and UDP-Glc (Fig. 5A and C), reaching a final equilibrium of 79 % UDP-Glc and 21% UDP-Gal, irrespective of the starting substrate. OgcE also had C4 epimerase activity on UDP-GalNAc and UDP-GlcNAc (Fig 5B and D), reaching a final equilibrium of 68 % UDP-

GlcNAc and 32 % UDP-GalNAc with either substrate. Identical results were obtained with both NAD⁺ (Fig. 5A and B) and NADP⁺ (Fig. 5C and D), suggesting the enzyme does not use any of these exogenously supplied cofactors. This conclusion is further supported by the unaltered amount of input cofactor after catalysis, the lack of release of reduced NADP⁺, and because catalysis can also occur in the absence of any cofactor (Fig. S1, top two traces). A small amount of NADH was observed in all enzyme-containing reactions irrespective of the type of cofactor exogenously added, but no NADPH was released. Since the NADH appearance occurs irrespective of the type of added substrate or exogenous cofactor and is not proportional to the amount of catalysis, we surmised that the detected NADH was released by the enzyme. Indeed, release of NADH was also observed in control reactions performed without substrate (Fig. S1, bottom trace). This suggests the OgcE preferentially binds NAD⁺ over NADP⁺ during overexpression in *E. coli* and explains why the bound cofactor alleviates the need for any exogenous cofactor. The release may occur due to progressive unfolding of the enzyme during lengthy incubations at 37 °C. To assess which type of substrate is most efficiently catalyzed, reactions were performed with serial dilutions of enzyme on UDP-Gal and UDP-GalNAc. These substrates were selected over UDP-Glc and UDP-GlcNAc since as high as 79 and 67% catalysis can be observed with UDP-Gal and UDP-GalNAc (versus only around 25-30% starting from their glucose counterparts). Enhanced catalysis of UDP-Gal versus UDP-GalNAc was observed at all enzyme dilutions and all time points tested (Fig. S2 and S3). Therefore, we conclude OgcE is an epimerase with dual specificity for the interconversion of UDP-Gal/UDP-Glc and UDP-GlcNAc/UDP-GalNAc, with slightly higher efficiency for non-acetylated substrates.

Predicted function of the remaining genes in the *ogc* cluster. To gain clues on the function of the remaining *ogc* genes we investigated the glycosylation status of the DsbA1 derived peptide ²³VQTSVPADSAPAASAAAAPAGLVEGQNYTVLANPIPQQQAGK⁶⁴ in the various *ogc* gene deletion mutants. MS spectra of the DsbA1 derived peptide in K56-2, Δ *pglL*, Δ *ogc*, Δ *ogcX*, Δ *ogcI*, Δ *ogcAB* and Δ *ogcE* are shown in Fig. S4. Using HCD fragmentation, four

glycoforms of this peptide were identified, with all glycopeptides showing nearly identical peptide fragmentation maps (Fig. 6A). The glycan attached to the four glycoforms corresponded to: HexNAc-HexNAc-Hex (Glycan A), HexNAc-HexNAc-262 (Glycan B), QuiNAc-Rha, and a single HexNAc (Fig. 6B). The area under the curve of the extracted ion chromatograms for all observed glycoforms of the peptide enabled us to semi-quantitatively compared each state (Fig. 6C and Table S2). The results show that glycoforms A and B were present in K56-2 with relative abundances of 44% and 52%, respectively, while the remainder 4% of the peptide was unmodified. In contrast, 100% and 84% of unmodified peptide was found in preparations obtained from Δ pglL and Δ ogc, respectively. The peak in Δ ogc with a relative abundance of 16% corresponded to a peptide modified by a glycan with the predicted mass of a QuiNAc-Rha disaccharide. As shown previously (Fig. 4A), deletion of the *ogc* cluster results in a truncated O antigen containing a terminal QuiNAc-Rha attached to the lipid A core (Fig. 4A, asterisk). Therefore, we interpreted this result as due to the incorporation of the QuiNAc-Rha disaccharide, which is assembled as Und-PP-QuiNAc-Rha by the O antigen synthesis machinery and likely transferred to the target protein by PglL. In contrast, the Δ ogcI mutant lacked glycosylated peptides, as the glycan assembly is blocked at its initiation step (Fig. 6C, Table S2 and Fig. S4). Both Δ ogcX and Δ ogcAB showed predominantly unmodified peptide with 90% and 65 % relative abundance, respectively. The remainder glycosylated peptide in these 2 mutants corresponded to an HexNAc glycan form (Fig. 6C, Table S2 and Fig. S4). The low abundance of a modified peptide with a single HexNAc in Δ ogcX, (9%) suggest the possibility that another flippase in the *B. cenocepacia* genome could mediate in part the membrane translocation of Und-PP-GalNAc (32). A single Δ ogcB mutant also resulted in the production of HexNAc decorated glycoproteins as in Δ ogcAB, supporting the assignment of OgcB as the enzyme responsible for the addition of the second GalNAc (Figure S5). Multiple attempts to delete *ogcA* were unsuccessful. It is possible that the absence of this gene may be deleterious for bacterial cell viability due to the accumulation of an Und-PP-linked GalNAc disaccharide that cannot be processed further, suggesting that OgcA is responsible for the

addition of the terminal Gal and explaining why Δ ogc, Δ ogcAB, and Δ ogcB are well tolerated.

O-glycosylation is common in other *Burkholderia* species. The gene-by-gene conservation of the *ogc* cluster in the *Burkholderia* genus (Fig. 1C) suggests that protein glycosylation with β -Gal-(1 \rightarrow 3)- α -GalNAc-(1 \rightarrow 3)- β -GalNAc-(1 \rightarrow is widespread within *Burkholderia* species. We tested this idea in two ways. First, we showed that glycosylated proteins in lysates from *B. cenocepacia*, *B. thailandensis* and two different strains of *B. gladioli* can be detected using the peanut agglutinin (Fig. S6). This lectin has specificity for Gal- β (1-3)-GalNAc terminal disaccharides. Second, bacterial glycopeptides were investigated by LC-MS in isolates representing several different *Burkholderia* species. Glycopeptides derived from five proteins were readily detectable from whole proteome samples of *B. thailandensis* E264 (Supplementary Data S1), such as the peptide ¹⁵⁹PAAASGAPAPAASGAAAH¹⁷⁶ of BTH_I3002ABC, which corresponds to the periplasmic substrate-binding protein of an ABC transporter (Fig. 7) and is modified with the expected HexNAc-HexNAc-Hex. As with *B. cenocepacia* glycopeptides (21), we also noted the presence of HexNAc-HexNAc-262 and an additional modified glycan corresponding to HexNAc-HexNAc-362 in *B. thailandensis* (Supplementary Data S1). Examination of whole cell lysates from two clinical isolates of *B. gladioli* revealed at least 14 glycosylated proteins (Supplementary Data S2 and S3). The analysis of one of the glycosylated peptides, ⁵⁹⁴AAHPGDIASEAAATGQPR⁶¹¹, of the *B. gladioli* bifunctional uroporphyrinogen-III synthetase/uroporphyrin-III is shown in Fig. 7. Analysis of eight clinical isolates of *B. pseudomallei* revealed three modified proteins, one of which was identified across all eight clinical isolates and corresponded to the known virulence factor Ecotin (33) (Fig. 7 and Supplementary Data S4). As in the other cases, the *B. pseudomallei* glycopeptides were also modified with either HexNAc-HexNAc-Hex or HexNAc-HexNAc-262. Collectively, our results demonstrate that protein glycosylation occurs in *Burkholderia* species outside *B. cenocepacia* and that the addition of the HexNAc-HexNAc-Hex glycan appears to be an invariant feature across members of this genus.

Loss of O-glycosylation is associated to growth defects under many different carbon sources as well as oxidative and osmotic stress. The conservation of the *O*-glycosylation pathway in the *Burkholderia* genus suggested it might be necessary for bacterial cell homeostasis. Therefore, we performed a global comparative analysis of carbon sources utilized by the K56-2 parental strain and $\Delta pgll$ using phenotypic microarrays (Biolog). The results show that $\Delta pgll$ utilized exogenous carbon sources less effectively than K56-2, especially sugar alcohols such as xylitol, monosaccharides (L-arabinose and D-mannose), deoxysugars (L-fucose and 2-deoxy-ribose), and various amino acids (D-alanine, L-alanine, D-serine, L-arginine, L-lysine and L-ornithine) (Fig. 8A and Supplementary Data S5). Also, $\Delta pgll$ could not utilize putrescine, which is produced from L-ornithine by ornithine decarboxylase (BCAL2641 and BCAM1111) or from L-arginine by arginine decarboxylase (BCAM1112) (34). Further, $\Delta pgll$ grew poorly in media with tweens, formic acid, butyric acid, D-glucosaminic acid and D-galacturonic acid. A subset of significant phenotypic microarray differences resulting in growth defects relative to the parental strain were independently validated (Fig. 8B). We also examined the sensitivity of $\Delta pgll$ towards oxidative stress under a range of H₂O₂ concentrations. The results showed that the mutant has higher sensitivity towards H₂O₂ than K56-2 (Fig. S7A). Because in long-term exposure experiments bacteria could adapt to H₂O₂ stress and since H₂O₂ is short-lived, we also performed a challenge experiment. In this case, bacteria were challenged with either 300 μ M or 1 mM of H₂O₂ with bacterial inoculum of OD₆₀₀ of 0.01 and 0.1 respectively for 1 h at 37°C. In both conditions, we found more than a 30% reduction in $\Delta pgll$ survival relative to K56-2 (Fig. 7SB). The $\Delta pgll$ mutant was also more susceptible to osmotic stress induced by high salt (4% NaCl) than K56-2 parental strain (Fig. S7C). We also noted that the $\Delta pgll$ mutant was more proteolytically active than the parental strain (Fig. 8C and D), a phenotype that was complemented by a plasmid carrying a functional *pgll* gene. Collectively, the results indicate that in the absence of protein glycosylation *B. cenocepacia* displays a global defect in utilization of multiple carbon sources, increased proteolytic activity, and reduced tolerance to stress conditions. We therefore

conclude that the general protein *O*-glycosylation pathway may be important for the metabolic fitness of *Burkholderia*.

Loss of protein glycosylation reduces bacterial growth in the *Galleria mellonella* infection model and results in a rapid activation of the larval antimicrobial response. $\Delta pgll$ was previously reported to be avirulent in the wax moth *G. mellonella* and the duckweed *Lemna minor*, which are widely used insect and plant infection models, respectively, based on end-point experiments (21). However, the mechanisms for the reduced virulence was not investigated. First, we compared the virulence of the *O*-glycan defective mutants and the bacterial loads recovered from the *G. mellonella* haemolymph over 72-h infections. Under our experimental conditions, the parental K56-2 strain caused death of all larvae at 48 h postinfection (Fig. 9A), while larvae infected with $\Delta pgll$ and Δogc had survival rates of 80% and 98%, respectively. We attributed the reduced virulence of Δogc (compared to $\Delta pgll$) to the cumulative effect caused by the simultaneous loss of protein glycosylation and O antigen synthesis due to absence of *ogcE* in Δogc (Fig. 4). Consistent with this, a strain in which Δogc was complemented by *OgcE* retained the same virulence level as $\Delta pgll$ at 48 and 72 h postinfection (Fig. 9A). Second, we examined whether decreased virulence is associated with poor growth of the *O*-glycosylation defective mutants in the infected larvae. Analysis of bacterial growth in larval haemolymph showed a 5-log increase in K56-2 (relative to the initial inoculum) at 48 h postinfection ($p < 0.01$; Fig. 9B). In contrast, $\Delta pgll$ and Δogc bacteria only showed 2-log CFU increase at 48 h and Δogc demonstrated poorer growth overall compared to $\Delta pgll$ despite the differences were not statistically significant.

Third, we determined whether the significantly reduced virulence and *in larvae* growth of the *O*-glycosylation defective mutants correlated with rapid killing mediated by the *G. mellonella*'s innate immune system. For this, we determined the expression pattern of innate immune genes in the infected larvae by RT-qPCR analysis. The results showed that the levels of cecropin and galliomyacin transcripts were 6 to 11-, and 4-fold higher, respectively, in larvae infected with $\Delta pgll$ and Δogc than in those infected with K56-2 at 8 h postinfection (Fig. 9C), while the levels of the

galleriomyacin transcripts did not differ at this time. By 18 h postinfection, the levels of cecropin and galleriomyacin transcripts increased dramatically in larvae infected with the three strains, but the increased expression was more significant in larvae infected with K56-2. Lower levels of cecropin gene expression in *Galleria* infected with Δogc at 18 h could be related to the rapid death of bacterial cells. For galliomyacin, the transcript levels did not increase much, although they were still significantly higher in larvae infected with $\Delta pglL$ and Δogc than in those infected with K56-2 (Fig. 9C). As infection in K56-2 was accompanied by increased pigmentation, we inferred the level of melanin formation by measuring the expression of the peptidoglycan recognition protein B (PGRP) gene. PGRP is a microbial pattern recognition molecule that recognizes bacterial cell fragments and mediates host responses towards bacterial infections through activation of prophenoloxidase cascade, responsible for melanization of pathogens and dead tissues, activation of Toll receptor and phagocytosis induction (35). We found that the level of PGRP gene transcription was similar for larvae infected with the three strains at 8 h postinfection, but at 18 h the increase in PGRP gene expression was significantly higher in larvae infected with the parental strain than in those infected with the *O*-glycosylation mutants (Fig. 9C). In contrast, transcript levels of the inducible metallo-protease inhibitor gene, which encodes a host inhibitor of bacterial proteases (36), remained similar but significantly higher in larvae infected with both K56-2 and $\Delta pglL$ than with Δogc . The expression of the gene encoding the cell wall degrading enzyme lysozyme (37) remained the similar in larvae infected with K56-2 or both *O*-glycosylation defective mutants, albeit their levels dramatically increased from 8 to 18 h postinfection. Collectively, these findings support the notion that the *G. mellonella* innate immune system responds early to infection by *O*-glycosylation defective bacteria, which compounded by reduced bacterial growth and reduced resistance to oxidative stress, could result in rapid and early killing of the mutant bacteria.

***Burkholderia* infected patients develop serum antibodies against the *Burkholderia O*-glycan.** Given the previous results and the conservation of the *O*-glycosylation pathway in

Burkholderia species, we examined if infected patients develop antibodies specifically recognizing *Burkholderia O*-glycoproteins. For these experiments, we deliberately chose the *N. meningitidis* DsbA1 as an heterologous glycosylation target expressed in *Burkholderia* to screen human sera because the assay was designed to reveal antibodies recognizing the *Burkholderia* glycan independently of the nature of the target protein. The *N. meningitidis* DsbA1 contains the sequence PAAASAAA with an invariable serine for *O*-glycosylation, which is similar to the glycosylation motif for the *Burkholderia* proteins. Convalescent sera collected from 16 patients infected with *B. cenocepacia*, 14 patients infected with *B. multivorans*, 20 patients infected with *B. pseudomallei*, and one patient infected with *B. mallei* were investigated for the presence of antibodies against purified glycosylated DsbA1 expressed in the K56-2 strain. Fourteen of the 16 samples isolated from *B. cenocepacia* infected patients contained antibodies against glycosylated DsbA1 protein, while only two serum samples reacted with non-glycosylated DsbA1 (Fig. 10A, thick arrows). For *B. multivorans* infected patients, 13 of the 14 serum samples tested gave positive reaction with glycosylated DsbA1 (Fig. 10B). Also, 16 of the 20 samples tested for patients with *B. pseudomallei* reacted with glycosylated DsbA1 and not with the non-glycosylated version (Fig. 10B). This amounts to 86% of the tested serum samples giving positive reaction in the ELISA assay with glycosylated DsbA1. To confirm that the serum samples contained *Burkholderia O*-glycan specific antibodies, positive samples were adsorbed against glycosylated DsbA1 by serial passage on microtiter plates containing bound glycosylated protein and the adsorbed serum samples were then retested by ELISA. The results showed that all the originally positive samples became negative after adsorption (Fig. 10B). Together, the results reveal that the majority of the convalescent serum samples investigated had antibodies toward the *Burkholderia* glycan, indicating that either the glycan itself or the glycosylated proteins are antigens recognized upon human infection with *Burkholderia* species. Also, the results indicate that the antibodies recognize the glycoprotein made in *B. cenocepacia* irrespective of the type of infection cause, providing additional evidence that the infecting bacteria produce the same *O*-glycan.

DISCUSSION

In this study, we have uncovered the gene cluster encoding the enzymes responsible for the synthesis and assembly of the lipid-linked glycan precursor for the general protein *O*-glycosylation pathway in *B. cenocepacia* and also elucidated the glycan structure as the trisaccharide β -Gal-(1-3)- α -GalNAc-(1-3)- β -GalNAc-peptide. As previously reported (21), we found two forms of the *O*-glycan, one of which corresponds to a modified trisaccharide. Our data suggest that the trisaccharide core moiety of both glycans derives from the *ogc* pathway, while the modification likely involves non-stoichiometric succinylation at the terminal Gal residue. Succinylation appears as a variable modification in glycans from *Rhizobium meliloti* (38) and several other Proteobacteria (39). Further studies are required to elucidate the structure of the modified glycan and identify the gene encoding the modifying enzyme.

Finding the *ogc* genes allowed us to predict gene functions, most of which were validated by single or double deletion mutagenesis followed by LC-MS analyses of the resulting glycopeptides. Thus, the OgcI protein was assigned as a member of the WecA family of phosphoglycosyltransferases, which catalyze the formation of undecaprenyl-diphosphate sugars typically utilizing UDP-GlcNAc or UDP-GalNAc as nucleotide sugar substrates (24). Our results support the assignment of OgcB as the glycosyltransferase adding the second GalNAc residue and predict OgcA is responsible for the addition of the terminal Gal. The inability to generate Δ *ogcA* suggests that absence of this gene may be deleterious for bacterial cell viability. This interpretation is consistent with previous Tn-Seq analysis showing that insertions are unrepresented within the *B. cenocepacia ogcA* (40) and with a report indicating the *B. pseudomallei* K96243 *ogcA* homologue (BPSL2668) is essential for survival (41). It is likely that without the terminal Gal the incomplete *O*-linked disaccharide glycan cannot be either effectively translocated across the membrane or processed by PgIL, leading in both cases to the accumulation of Und-PP that cannot be recycled thus becoming growth limiting. Further experiments are required to confirm the function of OgcA.

Both the *O*-glycan and the O antigen in *B. cenocepacia* share GalNAc residues (25). Therefore, the epimerization of UDP-Glc to form UDP-Gal would be needed for *O*-glycan synthesis. Our demonstration that the *ogcE* gene product catalyzes both reactions explains why the deletion of this gene results both in truncation of O-antigen and loss of protein glycosylation. This scenario is similar to that of *C. jejuni* GalE, which contributes to the biosynthesis of lipooligosaccharide, capsular polysaccharide, and *N*-linked glycosylation (42,43), and *N. meningitidis* GalE, which plays a role in pilin glycosylation and lipooligosaccharide synthesis (44). A closer homologue of OgcE is the UDP-GalNAc-4-epimerase of *Yersinia enterocolitica*, named Gne, which was reported to be specific for UDP-GalNAc with practically no activity on non-acetylated substrates (45). However, we provide conclusive biochemical evidence that while *B. cenocepacia* OgcE has a slight preference for non-acetylated substrate UDP-Gal it can also interconvert UDP-GalNAc efficiently.

We also demonstrate that the general *O*-glycosylation pathway is a conserved feature of the *Burkholderia* genus. This conclusion is supported by multiple evidence. First, the *ogc* genes are conserved and collinear in most of the *Burkholderia* species for which genomic sequencing is available, with only few exceptions where some of genes were in different genomic locations. Second, analysis of the *O*-glycosylation glycoproteome in a subset of *B. thailandensis*, *B. gladioli*, and *B. pseudomallei* isolates confirms the production of a similar trisaccharide structure in these species. Third, *B. cenocepacia*, *B. thailandensis* and *B. gladioli* protein lysates reveal polypeptides that react with the peanut agglutinin, a lectin that recognizes Gal- β (1-3)-GalNAc terminal disaccharide of the *O*-glycan moiety.

The conservation of the glycosylation pathway in *Burkholderia* raises the question of the role of protein glycosylation in the biology of these bacteria, which despite their ability to cause infection in humans and certain domestic animals are also widespread in the environment (1). We provide evidence that loss of protein glycosylation causes global metabolic defects in *B. cenocepacia* concerning the utilization of several different carbon sources and tolerance to osmotic and oxidative stress. Therefore,

general protein *O*-glycosylation is required for metabolic fitness, possibly because it is involved in the stability of its protein targets, as shown in *C. jejuni* (46,47). We also found that not only are glycosylation defective mutants less virulent in the *Galleria mellonella* infection model, but also that the pathogen-host interaction at the cellular level is different. Indeed, the level of expression of antimicrobial peptides is higher and temporally faster in larvae infected with the glycosylation defective mutants. Delayed expression of antimicrobial peptides towards infection with the wildtype strain could be due to the rapid replication of the bacterium inside the infection model, suggesting that *B. cenocepacia* may need to reach certain threshold to cause overwhelming induction of *G. mellonella* immune response, which leads to larval death. This can also be explained by the higher sensitivity of Δ *apgII* to oxidative stress, which is also induced upon *Galleria* infection. Further, a relation between defective transporters and growth in invertebrate cells was demonstrated for *Listeria monocytogenes* implying that certain nutritional sources are also important substrates for bacterial growth in host cells (48). Potentially, the *ApGII* defect in utilizing several carbon sources suggests that loss of glycosylation affects the function of proteins involved in nutrient transport across the periplasmic space, and could be another reason for the reduced virulence in *G. mellonella*. Therefore, loss of the general protein *O*-glycosylation system would make *Burkholderia* more susceptible to clearance by the host's innate immune, suggesting protein *O*-glycosylation could be considered as a novel target to develop inhibitors for possible clinical applications.

Another significant question arising from the conservation of the *Burkholderia* glycosylation pathway is whether *Burkholderia* glycoproteins are recognized by the human immune system. *Burkholderia* human infections represent a health risk to susceptible patient groups such as those with cystic fibrosis, as well as patients in endemic areas where *B. pseudomallei* prevails. Being multidrug resistant pathogens compounds this problem since *Burkholderia* infections are also difficult to treat (49). We show that the vast majority of serum samples from cystic fibrosis patients infected with *B. cenocepacia* and *B. multivorans*, as well as from patients with

melioidosis and glanders have antibodies that react with the *O*-glycosylated DsbA1 protein. This suggests that either the *Burkholderia* glycan or the combination of the glycan and the target protein at the glycosylation site are epitopes specifically recognized by the immune system, paving the way to future research to determine if these antibodies afford protection against infection.

In summary, this study has comprehensively characterized a conserved general protein *O*-glycosylation pathway in the *Burkholderia* genus demonstrating the relevance of protein glycosylation in the biology of *B. cenocepacia* and by extension in this group of bacteria widespread in multiple environmental niches and also responsible for opportunistic infections.

EXPERIMENTAL PROCEDURES

Strains and growth conditions. Strains and plasmids used in this study are listed in Table S3. Bacteria were grown at 37°C in Luria-Bertani (LB) medium. Antibiotics were used at the following final concentrations: 50 µg trimethoprim ml⁻¹ for *E. coli* and 100 µg ml⁻¹ for *B. cenocepacia*, 30 µg tetracycline ml⁻¹ for *E. coli* and 100 µg ml⁻¹ for *B. cenocepacia* and 40 µg kanamycin ml⁻¹ for *E. coli*. Ampicillin at 200 µg ml⁻¹ and polymyxin at 20 µg ml⁻¹ were used to select against donor and helper *E. coli* strains in triparental mating. Antibiotics and chemicals were purchased from Sigma-Aldrich (UK).

Recombinant DNA methods and deletion mutagenesis. The primers used are listed in Table 4. DNA ligations, restriction endonuclease digestions, and agarose gel electrophoresis were performed according to standard techniques (50) or by Gibson assembly (51). Restriction enzymes, Antarctic phosphatase and T4 DNA ligase were purchased from New England Bio labs (Ipswich, MA) and used as recommended by the manufacturer. *E. coli* GT115 and DH5α cells were transformed by the calcium chloride method (50). PCR amplifications were carried out using the HotStar HiFidelity polymerase (Qiagen). Colony-PCR was performed with Taq polymerase (Qiagen). Amplifications reactions were optimized for each primer pair. DNA sequencing was performed at the sequencing facility in GATC Biotech (London, UK). Unmarked, non-polar gene deletion mutants were constructed as previously

described (52), and verified by DNA sequencing of PCR amplicons spanning the deletion endpoints.

Purification of BCAL2640. A plasmid constitutively expressing the BCAL2640 protein was constructed by amplifying the gene with primers Q539 and Q540. The resulting amplicon was digested with *NdeI* and *XbaI* and ligated into a similarly digested pDA12 resulting in pDA12-BCAL2640, which was introduced into K56-2 wild type, Δ *pglL*, and Δ *ogc* by triparental mating (52). Exconjugants were isolated by plating on LB agar plates supplemented with 100 μ g tetracycline ml⁻¹, 200 μ g ampicillin ml⁻¹, 20 μ g polymyxin ml⁻¹. Cultures of pDA12-BCAL2640 expressed in wild type, Δ *pglL*, or Δ *ogc* were grown overnight at 37°C. Bacteria were harvested and lysed using a cell disrupter (Constant Systems Ltd., Northants, UK) at 18 kPsi. Supernatants containing soluble BCAL2640 protein were incubated with 0.2 M Ni²⁺-coated sepharose beads (GE Healthcare Life Sciences, UK) overnight at 4°C with mixing. The beads were washed and the His-tagged BCAL2640 was eluted twice with 250 mM imidazole. Purified BCAL2640 was run on 14% SDS-PAGE and stained by PageBlue Protein Staining Solution (Thermo Scientific, UK). Gel bands were excised and prepared for MS analysis.

Purification of DsbA1. Plasmid pMF22 (pMLBAD-DsbA1) was introduced into wild type *B. cenocepacia* K56-2, Δ *pglL*, Δ *ogc*, Δ *ogcX*, Δ *ogcI*, Δ *ogcAB*, Δ *ogcE*, and Δ BCAL3119-3131 by triparental mating (52). Exconjugants were selected on LB agar plates supplemented with 100 μ g trimethoprim ml⁻¹. For the purification of the lipoprotein DsbA1, stationary phase bacterial cultures were harvested at 12000 xg for 15 min at 4°C and resuspended in buffer A (1 μ g ml⁻¹ DNase I, complete EDTA-free protease inhibitor cocktail, 2% Triton X-114 in PBS). Bacteria were lysed with the cell disrupter, as described above. The supernatant was incubated at 37°C until phase separation was complete. The aqueous phase was removed after centrifugation at 10 000 xg for 10 min at 30°C. The remaining detergent layer containing the glycosylated lipoproteins was diluted to the original solution volume with buffer A without detergent for 1 h. Ten millimolar imidazole and 300 mM NaCl were added to the detergent phase prior to the addition of an appropriate volume of Ni²⁺ coated sepharose beads

equilibrated in buffer B (2% Triton X-114, and 30 mM NaCl in phosphate-buffered saline). This solution was allowed to mix overnight at 4°C. The beads were packed and then washed three times with buffer B containing 50 mM imidazole. The selected His-tagged DsbA1 was eluted twice, with two column volumes, with buffer B containing 300 mM imidazole. Purified DsbA1 was resolved on 14% SDS-PAGE and stained by PageBlue protein staining dye for MS analysis. Purified DsbA1 was either concentrated by TCA 10% precipitation overnight or passed on detergent removal spin column (ThermoScientific) prior to ELISA and NMR analysis respectively.

Protein manipulation and immunoblotting. Whole-cell lysates were prepared from overnight cultures of K56-2 and all mutants containing pAMF22 and induced by 0.2% arabinose overnight at 37°C in the presence of 100 μ g trimethoprim ml⁻¹. Two hundred μ l of cells with O.D₆₀₀ of 1 were pelleted, then resuspended in 1× sample buffer with 5% β -mercaptoethanol and boiled for 10 min. Protein separation was performed by 14% SDS-PAGE. His-tagged DsbA1 was revealed by immunoblotting using a 1:10,000 dilution of the mouse anti-His monoclonal antibody (GE Healthcare Life Sciences, UK). Proteins were visualized using a Licor Infrared Imaging System with Odyssey software. These experiments were replicated 3 times.

In-gel digestion of proteins. Gel separated proteins were processed as previously described (53) with minor modifications. Briefly, gel bands of interest were excised and destained in a 50:50 solution of 50 mM NH₄HCO₃ (pH 8.0) : 100% ethanol for 20 min at room temperature with shaking at 750 rpm. destained samples were then washed with 100% ethanol and vacuum-dried to dryness. Dried samples were then rehydrated in 10 mM DTT in 50 mM NH₄HCO₃ and reduced for 60 min at 56°C with shaking. Following reduction samples were washed twice in 100% ethanol for 10 min to ensure the complete removal of DTT and vacuum-dried to dryness. Reduced samples were rehydrated in 55 mM iodoacetamide in 50 mM NH₄HCO₃ in the dark for 45 min at room temperature. Following alkylation samples were washed twice with 100% ethanol and vacuum-dried. Dried alkylated samples were then rehydrated with 12 ng/ μ l trypsin (Promega, Madison WI) in 40 mM NH₄HCO₃ at 4°C for 1 h. Following

rehydration excess trypsin was removed, gel pieces covered in 40 mM NH_4HCO_3 and incubated overnight at 37°C. Peptides samples were extracted from gel sample twice using four gel volumes of 30% ethanol 3% Acetic acid followed by four gel volumes of 100% ethanol with the supernatant from each extraction pooled. The resulting peptide mixtures were dried down, desalted using C18 stage tips (54) and stored on tip at 4°C. Peptides were eluted in Buffer B (80% acetonitrile, 0.1% Formic acid) and dried down before analysis by LC-MS.

Generation of whole cell lysates for proteome analysis. *Burkholderia* strains of interest were grown overnight on confluent LB plates. Plates were flooded with 5 mL of pre-chilled sterile phosphate-buffered saline (PBS) and colonies removed with a cell scraper. Cells were washed 3 times in PBS and collected by centrifugation at 10,000 x g at 4°C then snap frozen. Frozen whole cell samples were resuspended in 4% SDS, 100mM Tris pH 8.0, 20mM DTT and boiled at 95°C at 2000rpm for 10 min. Samples clarified by centrifugation at 17,000 xg for 10 min, supernatant collected and protein concentration determined by bicinchoninic acid assay (Thermo Scientific Pierce). 200ug of protein from each sample was acetone precipitated by mixing 4 volumes of ice-cold acetone with one volume of sample. Samples were precipitated overnight at -20°C and then spun down at 16,000G for 10 min at 0°C. The precipitated protein pellets were resuspended with 80% ice-cold acetone and precipitated for an additional 4 hours at -20°C. Samples were centrifuged at 17,000 xg for 10 min at 0°C, the supernatant was discarded and excess acetone driven off at 65 °C for 5 min.

Digestion of complex protein lysates. Dried protein pellets were resuspended in 6 M urea, 2 M thiourea, 40 mM NH_4HCO_3 and reduced / alkylated prior to digestion with Lys-C (1/200 w/w) and trypsin (1/50 w/w) overnight (55). Digested samples were acidified to a final concentration of 0.5% formic acid and desalted with home-made high-capacity StageTips composed on 5mg Empore™ C18 material (3M, Maplewood, Minnesota) and 5mg of OLIGO R3 reverse phase resin (Thermo Fisher Scientific) according to the protocols of Ishihama and Rappsilber (56,57). Bound peptides were eluted with buffer B, dried and stored at -20 °C.

Liquid Chromatography and Mass Spectrometry analysis. Prior to LC-MS analysis, samples were re-suspended in 15 μL Buffer A (2% acetonitrile, 0.1% Formic acid). LC-MS was performed on either an Agilent 1290 Series HPLC (Agilent Technologies, Mississauga, ON) coupled to LTQ-Orbitrap Velos (Thermo Scientific, San Jose CA), a Dionex Ultimate 3000 UPLC (Thermo Scientific) coupled to an Orbitrap Elite or an EASY-nLC1000 system coupled to a Q-Exactive. For the LTQ-Orbitrap Velos and Q-Exactive, LC-MS was accomplished using a two column system in which samples were concentrated prior to separation on a 2-cm-long, 100- μm -inner diameter fused silica trap column containing 5.0- μm Aqua C-18 beads (Phenomenex) and then separated using an in-house packed C_{18} analytical 75 μm inner diameter, 360 μm outer diameter column composed of 35 cm ReproSil-Pur C18 AQ 1.9 μm (Dr. Maisch, Ammerbuch-Entringen, Germany) column for the EASY-nLC1000 system or a 20cm ReproSil-Pur C18 AQ 3.0 μm for the Agilent 1290 Series HPLC. Samples were concentrated onto the trap for 10 min using 100% Buffer A at 5 $\mu\text{l}/\text{min}$ after which the gradient was altered from 100% phase A to 40% Buffer B over 90 min at 250 nl/min with the eluting peptides infused directly into the mass spectrometers via nESI. For the Orbitrap Elite LC-MS was accomplished using a two-column chromatography set up comprising a PepMap100 C18 20 mm x 75 μm trap and a PepMap C18 500 mm x 75 μm analytical column (Thermo Scientific). Samples were concentrated onto the trap column at 5 $\mu\text{l}/\text{min}$ for 5 mins and infused into an Orbitrap Elite at 300 nl/min via the analytical column. One hundred and 80-min gradients were run altering the buffer composition from 1% buffer B to 28% B over 145 mins, then from 28% B to 40% B over 10 mins, then from 40% B to 100% B over 2 mins, the composition was held at 100% B for 3 mins, and then dropped to 3% B over 5 mins and held at 3% B for another 15 mins. All instruments were operated in a data-dependent manner using Xcalibur v2.2 (Thermo Scientific). For samples analyzed on the LTQ-Velos, one full precursor scan in the Orbitrap (resolution 60,000; 500-2,000 Th, AGC target of 1×10^6) was followed by the selection of the top four most intense, multiply charged ions above 5000 counts for collision-induced dissociation (CID, normalized collision energy

35, AGC of 4×10^4) followed by higher energy collisional activated dissociation (HCD, resolution 7500, normalized collision energy 40, ACG of 2×10^5) with 30 s dynamic exclusion enabled as previously described (55). For samples analyzed on the Orbitrap Elite, one full precursor scan in the Orbitrap (resolution 60,000; 500-2,000 Th, AGC target of 1×10^6) was followed by the selection of the five most intense, multiply charged ions above 10000 counts for collision-induced dissociation (CID, normalized collision energy 35, AGC of 4×10^4) followed by higher energy collisional activated dissociation (HCD, resolution 15000, normalized collision energy 40, ACG of 2×10^5) with 45 s dynamic exclusion enabled. For samples analyzed on the Q-Exactive, one full precursor scan (resolution 70,000; 350-2,000 m/z, AGC target of 3×10^6) was followed by 10 data-dependent HCD MS-MS events (resolution 35k, normalized collision energy of 25 with 50% stepping, AGC target of 1×10^6) with 25 s dynamic exclusion enabled. Area of the most intense charge state of each species was used to assess the relative levels / distribution of observed peptides.

Glycopeptide identification. Raw data files were processed with Proteome Discover version 1.4 (Thermo Scientific) to generate mgf files. The resulting mgf files were searched using MASCOT v2.4 (Matrix Science, provided by the *BC Proteomics Network* <http://www.proteincentre.com/mascot/>). Searches were carried out using semi-trypsin specificity, carbamidomethylation of cysteine as a fixed modification and Oxidation (M) as a variable modification. A precursor and product tolerance of 20ppm was used and *B. cenocepacia* data searched against the *B. cenocepacia* K56-2Valvano proteome (<http://www.uniprot.org/taxonomy/985076>, downloaded from NCBI February 15th 2013), while non-*B. cenocepacia* species were searched against the NCBI proteome database (Downloaded 1st of September 2016) with the taxonomy restricted to "Other Proteobacteria". Scan events that did not result in peptide identification were manually inspected and identified as possible glycopeptides based on the presence of the diagnostic oxonium ion 204.09 m/z of HexNAc or 188.09 m/z in the case of QuiNAc modified peptides. To facilitate glycopeptide assignments from HCD scans, the ions below the mass of the predicted deglycosylated peptides were extracted with

Xcalibur v2.2 using the Spectrum list function. Ions with a deconvoluted mass above that of the deglycosylated peptide and ions corresponding to known carbohydrate oxoniums were removed in a similar approach to post-spectral processing of ETD data (58) and searched as above with mascot. All spectra were searched with the decoy option enabled with all peptides passing a 1% FDR. Identified glycopeptide spectra were manually inspected and spectra annotated according to the nomenclature of Roepstorff and Fohlman (59) (Supplementary Data S1-S4). Glycopeptides derived from clinical *Burkholderia* strains were matched to either the *B. gladioli* reference strain BSR3 or the *B. pseudomallei* reference strain K96243.

Structural characterization of the protein glycan moiety. DsbA1, that was heterologously expressed in *B. cenocepacia*, obtained from TCA precipitation was dissolved in water (1 ml), and two aliquots of Proteinase K (0.3 mg each, Sigma P-2308) were added at 37°C, at 8 h interval, with last addition left overnight. The solution was directly loaded on a column packed with Bio-Gel P10 (d = 1.5 cm, h = 118 cm) using water as eluent (flow = 16 ml/h) and monitoring the eluate with a refractive index detector (Knauer K-2301). Fractions were pooled and monitored by ¹H NMR and the fraction enriched in the glycopeptide was eluted after approx. 40% of the total column volume. NMR analyses were performed on a Bruker 600 MHz equipped with a cryo-probe. Spectra were recorded at 15°C using acetone as internal standard (¹H 2.225 ppm, ¹³C 31.45 ppm). 2D spectra (DQF-COSY, TOCSY, TROESY, and gHSQC) were recorded using Bruker software (TopSpin 2.1). Homonuclear experiments were recorded using 512 FIDs of 2048 complex with 40 scans per FID, for TOCSY and TROESY spectra a mixing time of 100 and 300 ms was applied, respectively. HSQC spectrum was acquired with 512 FIDs of 2048 complex point and 60 scans per FID; spectra were processed and analyzed using Bruker TopSpin 3 program.

Extraction and analysis of *B. cenocepacia* lipopolysaccharide. Proteinase K-treated whole cell lysates were resolved by SDS-PAGE (16% v/v polyacrylamide) and visualized following silver staining (60). Complementation of O-antigen synthesis in Δogc was done by conjugating pXO23 (pAP20::*ogcE*) into the mutant by triparental mating and exconjugants were counterselected

on 100 µg chloramphenicol ml⁻¹, 200 µg ampicillin ml⁻¹ and 20 µg polymyxin ml⁻¹.

OgcE enzymatic assay. BCAL3117 was amplified by primers Q729 and Q728, cut by restriction enzymes *NdeI* and *EcoRI* and cloned in pET28 vector containing an N-terminal 6x His-tag that had been cut with the same restriction enzymes. Expression was induced by IPTG 0.5 mM for 3 h at 37°C for a 300 ml culture. The cells were harvested by centrifugation and lysed by passage in a cell disruptor after resuspension in buffer A: 50 mM Tris-HCl, 100 mM NaCl. OgcE was purified using a 3 ml Fast Flow chelating Sepharose column loaded with Ni²⁺ and equilibrated in buffer A. After extensive washing with buffer A, OgcE was eluted with increasing concentrations of imidazole (100-400 mM). The purified enzyme was preserved by addition of glycerol (25% final concentration) and frozen at -20°C. Reactions were set on 4 substrates: UDP-Gal, UDP-Glc, UDP-GalNAc and UDP-GlcNAc each with NADP⁺ or NAD⁺. Reactions were done in Tris 200 mM pH 8, with 0.1 mM substrate and 0.1 mM cofactor in a volume of 10 µl, with 7.3 µl enzyme fraction (comprising ~ 5 µg of enzyme) and incubated for 4h at 37°C unless stated otherwise in the legends to the figures. The reactions were analyzed by capillary electrophoresis (PACE MDQ, Beckman) using a bare silica capillary, 200 mM Borax buffer pH 9 and UV detection at 254 nm (61). The % of conversion was obtained by integrating the substrate and product peak surface areas using the 32Karat software.

Oxidative and osmolar susceptibility testing. Sensitivity towards different concentrations of hydrogen peroxide (Sigma) was assessed using the bioscreen. Bacterial cultures were diluted to an OD₆₀₀ of 0.01 in LB and dispensed in 100-well plates in 270 µl volumes. 30 ml aliquots of hydrogen peroxide in different concentrations were added to the bacterial cultures. Plates were incubated in a Bioscreen C automated growth curve reader for 16 h with OD₆₀₀ readings taken every 1 h. The same procedure was employed to determine the susceptibility towards high concentrations of NaCl using glucose as a carbon source.

Survival assay was also made after challenging bacterial cultures in LB with hydrogen peroxide at concentrations of either 300 µM with bacterial inoculum of 8x10⁶ cfu/ml or 1 mM with bacterial inoculum of

8x10⁷ for 1 h at 37°C with shaking. Samples were withdrawn and serially diluted in PBS. Then, 10 µl portions were dropped onto the surface of LB agar plates. The plates were incubated at 37°C for 24 h, the resulting colonies were counted and the % of survival was determined relative to parallel-untreated control samples.

Phenotypic microarray experiments.

The metabolic phenome of *B. cenocepacia* K56-2 and Δ *pglL* was assessed using pre-configure Biolog Phenotype MicroArrays (PMs) from Biolog Inc., Hayward, CA, USA. We used PM plates 1 and 2, which contain 190 of the most common carbon sources in 96-well arrangements composed of one negative control well and 95 wells prefilled with a given carbon source in a dried state. PM experiments were performed following the standard protocol (62). Briefly, *B. cenocepacia* strains were grown on LB agar plates for 36 h at 37°C. A cell suspension of 95 % transmittance in Biolog solution IF-0 was prepared by resuspending bacteria grown on LB agar plates. Dye A was then added and then 100 µl/well were transferred to each of the 96-well PM microplates (a set of 95 substrates and one blank well). The plates were incubated at 37 °C for 48 h. The optical density (OD₆₀₀) values were then measured using microtiter plate reader. Each experiment was performed as biological duplicates and growth differences were established by comparing the area under the growth curve (AUC). Growth in a given carbon source was considered positive when median AUC was more than 40% higher than negative control AUC. A cut-off value of 80% was used to determine a significant growth reduction of the mutant compared to wildtype. Hits ranged from reduction in growth from 80% up to less than 60% (Fig. 8A). Positive results were subsequently validated by the performing growth studies using the automated Bioscreen C in M9 minimal media to test the utilization of selected carbon sources compared to PM1 and PM2 plates. Bacterial cultures were diluted to approximately an OD₆₀₀ of 0.01 in M9 minimal media deprived of carbon sources and dispensed in 100-well plates in 270 µl volumes. Each required carbon source was tested individually by adding 30 µl of a stock solution to each to the 100-well Bioscreen plate to give final concentrations of 0.2% (W/V) in the wells. Plates were incubated in a Bioscreen C automated growth curve analyser for 48 h and

bacterial growth was assessed turbidimetrically at 600 nm every 2 h. Comparisons were also made by determining the AUC obtained over 4 biological replicas in triplicate.

Proteolytic activity. To determine protease activity, overnight cultures grown in LB were diluted to an OD₆₀₀ of 1, and 3 µl of this culture was spotted onto a nutrient agar plate containing 1.5% skim milk (63). The plates were incubated for 48 h at 37°C, after which the radii of the cleared zones surrounding the colonies were measured.

***Galleria mellonella* larvae Infection.** *G. mellonella* wax moth larvae were acquired from UK Waxworms Ltd. and stored in wood shavings in the dark at 16 °C prior to infection. Larvae of approximate weight of 250 to 350 mg were used. Bacteria were grown in 5 ml LB, harvested during exponential phase, resuspended in sterile PBS and serially diluted. The surface of the larvae was disinfected by ethanol (70%) and then larvae were injected with 10 µl of bacterial suspension, containing approximately 8x10⁴ CFU/ml of either K56-2 or the tested mutants, into the last right proleg by use of a Hamilton syringe with a 26-gauge needle. A group of 10 control larvae were injected with 10 µl of PBS in parallel. Larvae were kept at 37°C in the dark. Larval survival was monitored at 24 h intervals over a period of 72 h and was judged based on visual appearance and lack of movement in response to stimuli. Three independent experiments were performed.

Determination of *in vivo* bacterial loads in *G. mellonella*. Larvae were infected with approximately 8 x 10⁴ CFU of K56-2 or Δ *pglL* or Δ *ogc*. Groups of three insects were collected at 24 h and 48 h, and hemolymph samples from 3 larvae (approximately 100 µl) were collected in microcentrifuge tubes containing 10 µl of a saturated solution of N-phenylthiourea (Sigma-Aldrich, UK). Serial dilutions of the homogenate in PBS were plated on LB agar supplemented with spectinomycin 100 µg ml⁻¹, and colonies were counted after incubation at 37°C for 24 h. Three independent experiments were performed. No CFU were recovered from non-infected larvae in LB agar supplemented with spectinomycin.

RNA extraction and RT-PCR. Larvae were infected with approximately 1 x 10⁴ CFU of exponentially growing bacterial cultures. At 8 h and 18 h post infection, 3 larvae from either the control PBS group or the infected group

were homogenized on ice with 1ml of Tri-reagent (Ambion), using Stuart homogenizer SHM2 (Bibby Scientific Limited, Staffordshire, UK). Total RNA was purified by a standard chloroform-isopropyl alcohol protocol, and the obtained RNA was further purified using a Nucleospin RNAII kit (Macherey-Nagel) that included one step of on-column DNase treatment, following the manufacturer's instructions. The quantification of purified RNA samples was performed with a nanodrop (Nanovue Plus, GE Healthcare). cDNA was obtained from 1 µg total RNA by using a commercial Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen, UK). Real-time PCR (RT-PCR) analyses were performed with a Mx3005p qPCR system (Agilent Technologies, UK). One hundred nanograms of cDNA were used as the template in a 20-µl reaction mixture containing KapaSYBR Fast qPCR mix (Kapa Biosystems) and primer mix. Actin and 18S rRNA genes were amplified as housekeeping genes. The primers used are listed in Table S1. The thermocycling protocol was as follows: 95°C for 3 min for hot-start polymerase activation, followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s. SYBR green dye fluorescence was measured at 521 nm during the annealing phase. Fold changes in gene expression were calculated using the Livak method ($\Delta\Delta CT$) (64) with normalization to the actin gene (Supplementary Data S6).

ELISA. Antibodies against glycosylated and unglycosylated DsbA1 were detected by indirect ELISA. Ninety-six well Nunc MaxiSorp plates were coated with 50 µl of purified DsbA1 protein expressed in K56-2 WT (glycosylated) or in Δ *pglL* (non-glycosylated) and diluted in coating buffer (carbonate/bicarbonate, 100 mM, pH 9.6) to reach a final concentration of 2-4 µg/ml. Control wells contained only coating buffer. The plates were covered by plastic adhesive film and incubated at 4°C overnight. Coating solution was removed and plates were washed with 300 µl PBS/Tween20 (0.05%). Additional blocking was achieved by adding 300 µl of blocking buffer (BSA 5%). Plates were covered and incubated at room temperature for 1 h and then washed 3 times with PBS/Tween20. Fifty microliters of serum samples, diluted in half-strength blocking buffer, were added to the wells and incubated for 90 min at room temperature. Normal human serum from non-

infected individuals was used as a negative control. Plates were washed 4 times with PBS/Tween20. Fifty microliters of biotinylated rabbit anti-human IgG secondary antibody diluted to 1:20000 (1 μ l in 20 ml PBS) were added and incubated for 1 h at room temperature. Plates were washed 5 times with PBS/Tween20. Fifty microliters of Streptavidin-Horseradish Peroxidase diluted 1:300 in PBS were then added to the wells and incubated for 1 h in dark at room temperature. After washing 4 times with PBS/Tween20, 50 μ l of the substrate solution 3,3',5,5'-tetramethylbenzidine were added per well and incubated in the dark at room temperature. After sufficient color development, 30 μ l of stop solution (3M HCl) were added and the absorbance of each well was read with POLARstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) at 450nm. Three independent repeats were made for every sample, each done in duplicate. A positive ELISA test was defined by an absorbance reading exceeding the cut-off value computed by two standard deviations above the mean of the negative control. We also performed a confirmatory reverse ELISA was done, in which the glycan specific antibodies from positive serum samples were adsorbed using the glycosylated DsbA1 protein by incubating the samples with the glycosylated protein at a concentration of 100 μ g/ml for 2 h at room temperature and overnight at 4°C for 3 successive passages. Serum samples were then collected and used in a normal ELISA procedure as described above in parallel to the same samples without adsorption. Results are presented in ELISA units in which a cut-off of 2X standard deviation of the mean of the negative control was used to decide whether a serum sample is reactive towards the glycan or not.

Ethics. Patients with confirmed diagnosis of CF were recruited between 2010-2014 during routine outpatient appointments at the adult CF Centre in the Belfast Health and Social Care Trust (Belfast City Hospital) and provided written informed consent for the provision of serum samples. The study was approved by the Office for Research Ethics Committees Northern Ireland (10/NIR01/41) and co-sponsored by the Belfast Health and Social

Care Trust and Queen's University Belfast (10067SE-OPMS). Samples from patients attending the Manchester Adult CF Clinic were collected under the framework of the Manchester Respiratory and Allergy Biobank (ManRAB) (Full ethical approval was provided by the ManRAB ethics committee; REC reference: 10/H1010/7). Blood samples were collected from adult patients with melioidosis who had received written information before signing the consent form, approved by the Khon Kaen University Ethics Committee for Human Research Number HE561234. The convalescent human glanders serum was obtained under USAMRIID protocol FY00-15. The investigators adhered to the policies regarding the protection of human subjects as prescribed by 45 CFR 46 and 32 CFR 219 (Protection of Human Subjects). Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army or any of the granting agencies that supported this study.

ACKNOWLEDGEMENTS

We thank M. Feldman for the plasmid pAMF22, J. Torres Bustos for technical assistance with the Omnilog, B. Oickle and K. Patel for OgcE expression and purification, T. Stinear, B. Howden and S. Pidot for providing clinical *B. gladioli* strains, and M. Mayo, V. Theobald and M. Barnes for isolate selection, curation and sample preparation of genetically diverse clinical *B. pseudomallei* strains. This research was supported by a grant from the Canadian Institutes of Health Research (to M.A.V. and C.C.), and from the UK Medical Research Council Confidence in Concept project CD1617-CIC04 (to M.A.V.). Y.F.M. was supported by an International PhD Scholarship from Queen's University, Belfast. N.E.S was supported by National Health and Medical Research Council of Australia (NHMRC) project grant (APP1100164) and an Overseas (Biomedical) Fellowship (APP1037373).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

REFERENCES

1. Coenye, T., and Vandamme, P. (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ. Microbiol.* **5**, 719-729
2. Eberl, L., and Vandamme, P. (2016) Members of the genus *Burkholderia*: good and bad guys. *F1000Res* **5**
3. Scoffone, V. C., Chiarelli, L. R., Trespidi, G., Mentasti, M., Riccardi, G., and Buroni, S. (2017) *Burkholderia cenocepacia* Infections in Cystic Fibrosis Patients: Drug Resistance and Therapeutic Approaches. *Front Microbiol* **8**, 1592
4. Lipsitz, R., Garges, S., Aurigemma, R., Baccam, P., Blaney, D. D., Cheng, A. C., Currie, B. J., Dance, D., Gee, J. E., Larsen, J., Limmathurotsakul, D., Morrow, M. G., Norton, R., O'Mara, E., Peacock, S. J., Pesik, N., Rogers, L. P., Schweizer, H. P., Steinmetz, I., Tan, G., Tan, P., Wiersinga, W. J., Wuthiekanun, V., and Smith, T. L. (2012) Workshop on treatment of and postexposure prophylaxis for *Burkholderia pseudomallei* and *B. mallei* Infection, 2010. *Emerg Infect Dis* **18**, e2
5. Losada, L., Ronning, C. M., DeShazer, D., Woods, D., Fedorova, N., Kim, H. S., Shabalina, S. A., Pearson, T. R., Brinkac, L., Tan, P., Nandi, T., Crabtree, J., Badger, J., Beckstrom-Sternberg, S., Saqib, M., Schutzer, S. E., Keim, P., and Nierman, W. C. (2010) Continuing evolution of *Burkholderia mallei* through genome reduction and large-scale rearrangements. *Genome Biol Evol* **2**, 102-116
6. Limmathurotsakul, D., Golding, N., Dance, D. A. B., Messina, J. P., Pigott, D. M., Moyes, C. L., Rolim, D. B., Bertherat, E., Day, N. P. J., Peacock, S. P., and Hay, S. I. (2016) Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nature Microbiol* **15008**
7. Wiersinga, W. J., Currie, B. J., and Peacock, S. J. (2012) Melioidosis. *N Engl J Med* **367**, 1035-1044
8. Wiersinga, W. J., Virk, H. S., Torres, A. G., Currie, B. J., Peacock, S. J., Dance, D. A. B., and Limmathurotsakul, D. (2018) Melioidosis. *Nat Rev Dis Primers* **4**, 17107
9. Currie, B. J., Ward, L., and Cheng, A. C. (2010) The epidemiology and clinical spectrum of melioidosis: 540 cases from the 20 year Darwin prospective study. *PLoS Negl Trop Dis* **4**, e900
10. Rhodes, K. A., and Schweizer, H. P. (2016) Antibiotic resistance in *Burkholderia* species. *Drug Resist Updat* **28**, 82-90
11. Limmathurotsakul, D., Funnell, S. G., Torres, A. G., Morici, L. A., Brett, P. J., Dunachie, S., Atkins, T., Altmann, D. M., Bancroft, G., Peacock, S. J., and Steering Group on Melioidosis Vaccine, D. (2015) Consensus on the development of vaccines against naturally acquired melioidosis. *Emerg Infect Dis* **21**
12. Eichler, J., and Koomey, M. (2017) Sweet New Roles for Protein Glycosylation in Prokaryotes. *Trends Microbiol* **25**, 662-672
13. Schäffer, C., and Messner, P. (2017) Emerging facets of prokaryotic glycosylation. *FEMS Microbiol Rev* **41**, 49-91
14. Valguarnera, E., Kinsella, R. L., and Feldman, M. F. (2016) Sugar and Spice Make Bacteria Not Nice: Protein Glycosylation and Its Influence in Pathogenesis. *J Mol Biol* **428**, 3206-3220
15. Nothaft, H., and Szymanski, C. M. (2010) Protein glycosylation in bacteria: sweeter than ever. *Nat Rev Microbiol* **8**, 765-778
16. Spiro, R. G. (2002) Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* **12**, 43R-56R
17. Stepper, J., Shastri, S., Loo, T. S., Preston, J. C., Novak, P., Man, P., Moore, C. H., Havlicek, V., Patchett, M. L., and Norris, G. E. (2011) Cysteine S-glycosylation, a new post-translational modification found in glycopeptide bacteriocins. *FEBS Lett* **585**, 645-650
18. Szymanski, C. M., Yao, R., Ewing, C. P., Trust, T. J., and Guerry, P. (1999) Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. *Mol Microbiol* **32**, 1022-1030
19. Wacker, M., Linton, D., Hitchen, P. G., Nita-Lazar, M., Haslam, S. M., North, S. J., Panico, M., Morris, H. R., Dell, A., Wren, B., and Aebi, M. (2002) N-linked glycosylation in *Campylobacter jejuni* and its functional transfer into *E. coli*. *Science* **298**, 1790-1793
20. Lizak, C., Gerber, S., Numao, S., Aebi, M., and Locher, K. P. (2011) X-ray structure of a bacterial oligosaccharyltransferase. *Nature* **474**, 350-355
21. Lithgow, K. V., Scott, N. E., Iwashkiw, J. A., Thomson, E. L., Foster, L. J., Feldman, M. F., and Dennis, J. J. (2014) A general protein O-glycosylation system within the *Burkholderia cepacia* complex is involved in motility and virulence. *Mol Microbiol* **92**, 116-137

22. Hug, I., and Feldman, M. F. (2011) Analogies and homologies in lipopolysaccharide and glycoprotein biosynthesis in bacteria. *Glycobiology* **21**, 138-151
23. Lukose, V., Walvoort, M. T. C., and Imperiali, B. (2017) Bacterial phosphoglycosyl transferases: initiators of glycan biosynthesis at the membrane interface. *Glycobiology* **27**, 820-833
24. Valvano, M. A. (2011) Common Themes in Glycoconjugate Assembly Using the Biogenesis of O-Antigen Lipopolysaccharide as a Model System. *Biochemistry-Moscow* **76**, 729-735
25. 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 the *Burkholderia cenocepacia* strain J2315 that is associated with transmissible infections in patients with cystic fibrosis. *J. Bacteriol.* **187**, 1324-1333
26. Oberto, J. (2013) SyntTax: a web server linking synteny to prokaryotic taxonomy. *BMC Bioinformatics* **14**, 4
27. Winsor, G. L., Khaira, B., Van Rossum, T., Lo, R., Whiteside, M. D., and Brinkman, F. S. (2008) The *Burkholderia* Genome Database: facilitating flexible queries and comparative analyses. *Bioinformatics* **24**, 2803-2804
28. Gebhart, C., Ielmini, M. V., Reiz, B., Price, N. L., Aas, F. E., Koomey, M., and Feldman, M. F. (2012) Characterization of exogenous bacterial oligosaccharyltransferases in *Escherichia coli* reveals the potential for O-linked protein glycosylation in *Vibrio cholerae* and *Burkholderia thailandensis*. *Glycobiology* **22**, 962-974
29. Bock, K., and Pedersen, C. (1983) Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. *Adv Carbohydr Chem Biochem* **41**, 27-66
30. Cuthbertson, L., Kos, V., and Whitfield, C. (2010) ABC transporters involved in export of cell surface glycoconjugates. *Microbiol. Mol. Biol. Rev.* **74**, 341-362
31. 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
32. Feldman, M. F., Marolda, C. L., Monteiro, M. A., Perry, M. B., Parodi, A. J., and Valvano, M. A. (1999) The activity of a putative polyisoprenol-linked sugar translocase (Wzx) involved in *Escherichia coli* O antigen assembly is independent of the chemical structure of the O repeat. *J. Biol. Chem.* **274**, 35129-35138
33. Ireland, P. M., Marshall, L., Norville, I., and Sarkar-Tyson, M. (2014) The serine protease inhibitor Ecotin is required for full virulence of *Burkholderia pseudomallei*. *Microb Pathog* **67-68**, 55-58
34. El-Halfawy, O. M., and Valvano, M. A. (2014) Putrescine reduces antibiotic-induced oxidative stress as a mechanism of modulation of antibiotic resistance in *Burkholderia cenocepacia*. *Antimicrob Agents Chemother* **58**, 4162-4171
35. Royet, J., Gupta, D., and Dziarski, R. (2011) Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nat Rev Immunol* **11**, 837-851
36. Vertyporokh, L., and Wojda, I. (2017) Expression of the insect metalloproteinase inhibitor IMPI in the fat body of *Galleria mellonella* exposed to infection with *Beauveria bassiana*. *Acta Biochim Pol* **64**, 273-278
37. Andrejko, M., Mizerska-Dudka, M., and Jakubowicz, T. (2008) Changes in *Galleria mellonella* lysozyme level and activity during *Pseudomonas aeruginosa* infection. *Folia Microbiol (Praha)* **53**, 147-151
38. York, G. M., and Walker, G. C. (1998) The succinyl and acetyl modifications of succinoglycan influence susceptibility of succinoglycan to cleavage by the *Rhizobium meliloti* glycanases ExoK and ExsH. *J Bacteriol* **180**, 4184-4191
39. Bohin, J. P. (2000) Osmoregulated periplasmic glucans in Proteobacteria. *FEMS Microbiol Lett* **186**, 11-19
40. Gislason, A. S., Turner, K., Domaratzki, M., and Cardona, S. T. (2017) Comparative analysis of the *Burkholderia cenocepacia* K56-2 essential genome reveals cell envelope functions that are uniquely required for survival in species of the genus *Burkholderia*. *Microb Genom* **3**
41. Moule, M. G., Hemsley, C. M., Seet, Q., Guerra-Assuncao, J. A., Lim, J., Sarkar-Tyson, M., Clark, T. G., Tan, P. B., Titball, R. W., Cuccui, J., and Wren, B. W. (2014) Genome-wide saturation

- mutagenesis of *Burkholderia pseudomallei* K96243 predicts essential genes and novel targets for antimicrobial development. *MBio* **5**, e00926-00913
42. Bernatchez, S., Szymanski, C. M., Ishiyama, N., Li, J., Jarrell, H. C., Lau, P. C., Berghuis, A. M., Young, N. M., and Wakarchuk, W. W. (2005) A single bifunctional UDP-GlcNAc/Glc 4-epimerase supports the synthesis of three cell surface glycoconjugates in *Campylobacter jejuni*. *J Biol Chem* **280**, 4792-4802
 43. Fry, B. N., Feng, S., Chen, Y. Y., Newell, D. G., Coloe, P. J., and Korolik, V. (2000) The *galE* gene of *Campylobacter jejuni* is involved in lipopolysaccharide synthesis and virulence. *Infect Immun* **68**, 2594-2601
 44. Stimson, E., Virji, M., Makepeace, K., Dell, A., Morris, H. R., Payne, G., Saunders, J. R., Jennings, M. P., Barker, S., Panico, M., and et al. (1995) Meningococcal pilin: a glycoprotein substituted with digalactosyl 2,4-diacetamido-2,4,6-trideoxyhexose. *Mol. Microbiol.* **17**, 1201-1214
 45. Bengoechea, J. A., Pinta, E., Salminen, T., Oertelt, C., Holst, O., Radziejewska-Lebrecht, J., Piotrowska-Seget, Z., Venho, R., and Skurnik, M. (2002) Functional Characterization of Gne (UDP-N-Acetylglucosamine- 4-Epimerase), Wzz (Chain Length Determinant), and Wzy (O-Antigen Polymerase) of *Yersinia enterocolitica* Serotype O:8. *J. Bacteriol.* **184**, 4277-4287
 46. Larsen, J. C., Szymanski, C., and Guerry, P. (2004) N-linked protein glycosylation is required for full competence in *Campylobacter jejuni* 81-176. *J Bacteriol* **186**, 6508-6514
 47. Cain, J. A., Dale, A. L., Niewold, P., Klare, W. P., Man, L., White, M. Y., Scott, N. E., and Cordwell, S. J. (2019) Proteomics reveals multiple phenotypes associated with N-linked glycosylation in *Campylobacter jejuni*. *Mol Cell Proteomics*
 48. Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskas, A., and Chakraborty, T. (2010) *Galleria mellonella* as a model system for studying *Listeria* pathogenesis. *Appl Environ Microbiol* **76**, 310-317
 49. Regan, K. H., and Bhatt, J. (2016) Eradication therapy for *Burkholderia cepacia* complex in people with cystic fibrosis. *Cochrane Database Syst Rev* **11**, CD009876
 50. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1990) *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
 51. Gibson, D. G., Young, L., Chuan, R.-Y., Venter, J. C., Hutchinson, C. A., and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* **6**, 343-345.
 52. Aubert, D. F., Hamad, M. A., and Valvano, M. A. (2014) A markerless deletion method for genetic manipulation of *Burkholderia cenocepacia* and other multi antibiotic resistant Gram-negative bacteria. in *Host-bacteria interactions: Methods and Protocols* (Vergunst, A., and Callaghan, D. O. eds.), Springer Science+Business Media, New York. pp 311-327
 53. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* **1**, 2856-2860
 54. Rappsilber, J., Ishihama, Y., and Mann, M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal Chem* **75**, 663-670
 55. Scott, N. E., Parker, B. L., Connolly, A. M., Paulech, J., Edwards, A. V., Crossett, B., Falconer, L., Kolarich, D., Djordjevic, S. P., Hojrup, P., Packer, N. H., Larsen, M. R., and Cordwell, S. J. (2011) Simultaneous glycan-peptide characterization using hydrophilic interaction chromatography and parallel fragmentation by CID, higher energy collisional dissociation, and electron transfer dissociation MS applied to the N-linked glycoproteome of *Campylobacter jejuni*. *Mol Cell Proteomics* **10**, M000031-MCP000201
 56. Ishihama, Y., Rappsilber, J., and Mann, M. (2006) Modular stop and go extraction tips with stacked disks for parallel and multidimensional Peptide fractionation in proteomics. *J Proteome Res* **5**, 988-994
 57. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* **2**, 1896-1906
 58. Good, D. M., Wenger, C. D., and Coon, J. J. (2010) The effect of interfering ions on search algorithm performance for electron-transfer dissociation data. *Proteomics* **10**, 164-167
 59. Roepstorff, P., and Fohlman, J. (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed Mass Spectrom* **11**, 601

60. Marolda, C. L., Lahiry, P., Vinés, E., Saldías, S., and Valvano, M. A. (2006) Micromethods for the characterization of lipid A-core and O-antigen lipopolysaccharide *Methods Mol. Biol.* **347**, 237-252
61. Butty, F. D., Aucoin, M., Morrison, L., Ho, N., Shaw, G., and Creuzenet, C. (2009) Elucidating the formation of 6-deoxyheptose: biochemical characterization of the GDP-D-glycero-D-mannoheptose C6 dehydratase, DmhA, and its associated C4 reductase, DmhB. *Biochemistry* **48**, 7764-7775
62. Bochner, B. R. (2009) Global phenotypic characterization of bacteria. *FEMS Microbiol Rev* **33**, 191-205
63. Kazanas, N. (1968) Proteolytic activity of microorganisms isolated from freshwater fish. *Appl Microbiol* **16**, 128-132
64. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Methods* **25**, 402-408

Figure Legends

FIGURE 1. Identification of the *O*-glycosylation cluster (*ogc*) in *B. cenocepacia*. *A*, genetic organization of the *ogc* cluster (BCAL3114-BCAL3118), placed between the O-antigen and lipid A-core clusters. Names of ORFs indicated above the arrows are according to the original gene annotation (25). Names below are the ones assigned in the current study. *B*, proposed model for the general *O*-glycosylation assembly pathway in *B. cenocepacia*; OgcI: initiating enzyme, OgcE: UDP-glucose/galactose epimerase, OgcAB: glycosyltransferases, OgcX: flippase, and PglL: oligosaccharyltransferase. *C*, synteny of *ogc* genes in members of the *Burkholderia* genus. Genes indicated in bold are the ones used to search for synteny in the SyntTax server. The species showing similar arrangements are listed for each group. The synteny scores ranged from 55 to 96 (>30 is considered highly significant conservation) (26). *D*, relative gel mobility of BCAL2640 and DsbA1 polypeptides in $\Delta ogcX$ -*ogcI* and single deletion mutants. Similar results were obtained in 3 biological repeats. Left panel: Coomassie-stained 14% SDS-polyacrylamide gel of the purified *B. cenocepacia* BCAL2640 protein expressed in wildtype K56-2, $\Delta pglL$, and Δogc . Right panel Western blot of His-tagged DsbA1 acceptor protein expressed in the parental K56-2 strains and the mutants $\Delta pglL$, $\Delta ogcX$, $\Delta ogcI$, $\Delta ogcAB$, $\Delta ogcE$ and ΔO -antigen cluster (Δ BCAL3119-BCAL3131).

FIGURE 2. Identification of PglL and Ogc dependent glycosylation of BCAL2640. *A-C*, MS-MS analysis of detected glycopeptide, CID fragmentations confirms that the peptide is modified with at least two glycans corresponding to *A*, HexNAc-HexNAc-262 (m/z 959.97, +4 corresponding to 3835.868 Da) and *B*, HexNAc-HexNAc-Hex (m/z 934.97, +4 corresponding to 3735.859 Da). *C*, HCD fragmentation of the HexNAc-HexNAc-Hex modified peptide confirming the identity of the peptide sequence. *D-F*, extracted ion chromatograms of the major charge state, +3, of the glycosylated (modified with HexNAc-HexNAc-Hex) and non-glycosylated forms of $^{152}\text{YAPPPAAVPVAATSGAQGGAAAAAAPAGTKPANAPR}^{187}$. In K56-2 (wildtype), the BCAL2640 glycosylated peptide was >15-fold more abundant than the unglycosylated form. This peptide was not glycosylated in $\Delta pglL$ or $\Delta ogcX$ -*ogcI*. *denotes deglycosylated peptide resulting from in-source fragmentation.

FIGURE 3. NMR spectra of the glycopeptide. Representative regions of the NMR spectrum acquired at 600 MHz spectrometer, in D₂O at 15°C, for the glycopeptides from DsbA1 after Proteinase K digestion and chromatographic purification. *A*, zoom of ¹H NMR spectrum in the ring proton region, *B*, multiplicity edited ¹H-¹³C HSQC spectrum with signals in grey represent methylene carbon atoms; the inset shows a zoom of the downfield region with the three anomeric resonances of A-C residues. *C*, superimposition of ¹H-¹H TOCSY (grey) and ¹H-¹H T-ROESY (black) spectra. Attribution of most relevant cross peaks is indicated nearby the corresponding density, and labels reflect those reported in Table S1. The structure of the glycopeptide's glycan moiety, along with the labels used during spectra attribution, is shown as well panel *B*. Unattributed peaks are related to peptides signals.

FIGURE 4. Comparison of effects of single *ogc* mutants on LPS synthesis and protein glycosylation. Similar results were obtained in 3 biological repeats. *A*, proteinase K-treated whole cell lysates were resolved on 14% tricine gel and silver staining. Arrowheads indicate a lipid A-core band + truncated O antigen (see Results). *B*, western blot of His-tagged DsbA1 acceptor protein expressed in K56-2 WT, $\Delta pglL$ and Δogc + (OgcE).

FIGURE 5. OgcE enzymatic activity as UDP-glucose and UDP-GlcNAc epimerase. Capillary electrophoresis electrophoregrams showing the C4 epimerase activity of OgcE on (*A*) UDP-Glc, (*B*) UDP-GlcNAc, UDP-Gal (*C*), and UDP-GalNAc (*D*) and in the presence of NAD⁺ (*A* and *C*) or NADP⁺ (*B* and *D*). + and - signs indicate presence or absence of OgcE in the reaction. Reactions were analyzed at equilibrium after 4 h incubation at 37°C in the presence of 0.1 mM substrate, 0.1 mM cofactor and 5 μg enzyme in a total volume of 10 μl of Tris-HCl 200 mM pH8. Two sets of samples prepared with independent OgcE enzyme fractions and each including one reaction per condition were analyzed with 2 CE runs per sample. Both sets showed similar data at equilibrium and only 1 set is shown in the figure.

Activity assessment and peak assignments were also based on optimization at different enzyme / substrate ratios and various reaction times, and based on co-injections with standards.

FIGURE 6. Effect of *O*-glycan biosynthesis gene disruptions on protein glycosylation. *A*, DsbA1 derived glycopeptide ²³VQTSVPADSAPAASAAAAPAGLVEGQNYTVLANPIPQQQAGK⁶⁴ indicating the sites of HCD fragmentation. *B*, the four glycoforms observed corresponded to Glycan A (HexNAc-HexNAc-Hex, m/z 1543.10, +3, 4626.266Da), Glycan B (HexNAc-HexNAc-262, m/z 1576.44, +3), QuiNAc-Rha (m/z 1464.75, +3, 4391.226Da), and a single HexNAc (m/z 1421.39, +3, 4261.146Da). *C*, semi-quantitative comparison of each glycoform in different genetic backgrounds using the area under the curve of the extracted ion chromatograms for all observed glycoforms of the DsbA1 peptide.

FIGURE 7. Glycopeptides detected in *B. thailandensis*, *B. gladioli*, and *B. pseudomallei* are modified by an identical trisaccharide to that in *B. cenocepacia*. CID fragmentation (left panels) enabled the identification of the expected HexNAc-HexNAc-Hex glycan attached to peptides, while HCD fragmentation (right panels) confirmed the identity of the peptide sequences corresponding to the *B. thailandensis* ABC transporter, periplasmic substrate-binding protein (BTH_I3002, m/z 1007.466, +2, 2012.9161 Da), the *B. gladioli* Bifunctional uroporphyrinogen-III synthetase/uroporphyrin-III C-methyltransferase (bglA_1g31160, m/z 763.359, +3, 2779.3218 Da), and the *B. pseudomallei* serine protease inhibitor Ecotin (m/z 927.449, +3, 2779.3218 Da).

FIGURE 8. Metabolic alterations in the protein *O*-glycosylation mutant Δ pglL. *A*, heat map for carbon source phenotypes as determined by phenotypic microarrays using an Omnilog system (see Materials and Methods). Yellow represents wildtype growth. Blue shading denotes ranges of growth below 80% of the wildtype growth in the same carbon source, calculated by comparing the area under curve of wildtype and mutant strain (AUC). The complete results for all carbon sources tested are presented in Supplementary Data S5. *B*, selective carbon sources giving differences between K56-2 and Δ pglL were independently validated by generating growth curves measured by the automated bioscreen C using M9 minimal medium with the relevant carbon source under investigation. Statistical differences (mean \pm standard deviation) of the area under each growth curve were analyzed by multiple t-test comparisons with confidence interval of 95%. n=4 per carbon source. *, $p = 0.02$; **, $p < 0.01$; ***, $p < 0.001$. *C*, casein proteolytic activity of K56-2, Δ pglL, and the complemented mutant (Δ pglL + PglL) was assayed casein-containing nutrient agar plates. Plates shown are representatives of at least 2 experiments done in triplicates. *D*, quantification of the data in *C* by measuring the clear halo around the colonies after incubation at 37°C for 48 h. Values are expressed as mean \pm standard deviation in millimetres. Statistical differences were analyzed by one-way ANOVA with Tukey's multiple comparisons tests; $\alpha = 0.01$; ***, $p < 0.0001$.

FIGURE 9. Role of *O*-glycosylation in bacterial proliferation and *G. mellonella* larval mortality. *A*, larvae were injected with 8×10^4 CFU of parental and mutant strains and the percent survival was monitored over 72 h postinfection. Statistical differences (means \pm standard deviations) were examined by 2-way ANOVA with Tukey's multiple comparisons tests; $\alpha=0.05$. *B*, hemolymph from *B. cenocepacia*-infected larvae (n=3) was pooled and CFU were determined by viable count. Results are presented as a superimposed scatter plot and statistical differences (means \pm standard deviations) were examined by 2-way ANOVA as indicated above. *C*, transcriptional activation of immune-responsive genes following infection determined by quantitative real-time RT-PCR analysis. The graphs show the normalized fold expression levels of the target transcripts relative to those in PBS-injected animals, and represent means \pm standard deviations of 3 independent experiments, each with two technical replicas. Expression data were normalized to the expression of the housekeeping actin gene. PGRP, Peptidoglycan recognition protein; IMPI, inducible metallo-protease inhibitor. Data were statistically analyzed by two-way ANOVA with Tukey's multiple comparisons tests and $\alpha=0.05$. ns, non-significant. The calculated p values from individual comparisons are indicated in the figure.

FIGURE 10. **Immune reaction of *Burkholderia* O-glycan towards convalescent sera from *Burkholderia* infected patients.** *A*, Indirect ELISA results using as an antigen purified glycosylated DsbA1 protein produced in *B. cenocepacia* K56-2 and serum samples collected from patients infected with *B. cenocepacia*, *B. multivorans*, *B. mallei*, and *B. pseudomallei*. Non-glycosylated heterologously-expressed DsbA1 protein in $\Delta pgLL$ was used in the experiment. Thin arrows point at the serum samples that reacted with both glycosylated and unglycosylated DsbA1 heterologously expressed in K56-2 and $\Delta pgLL$, respectively. Three independent experiments were done, each in duplicate. Thick arrows point at the serum samples that gave negative results with glycosylated DsbA1. *B*, serum samples were adsorbed by incubation with the glycosylated DsbA1 protein for 3 successive passages prior to ELISA. Two independent experiments were done. Dotted blue lines in *A* and *B* indicate the negative cut-off OD₄₅₀ value for background color in the ELISA determined by running ELISA assays in normal human serum from 30 donors. Error bars represent standard deviation of the mean.

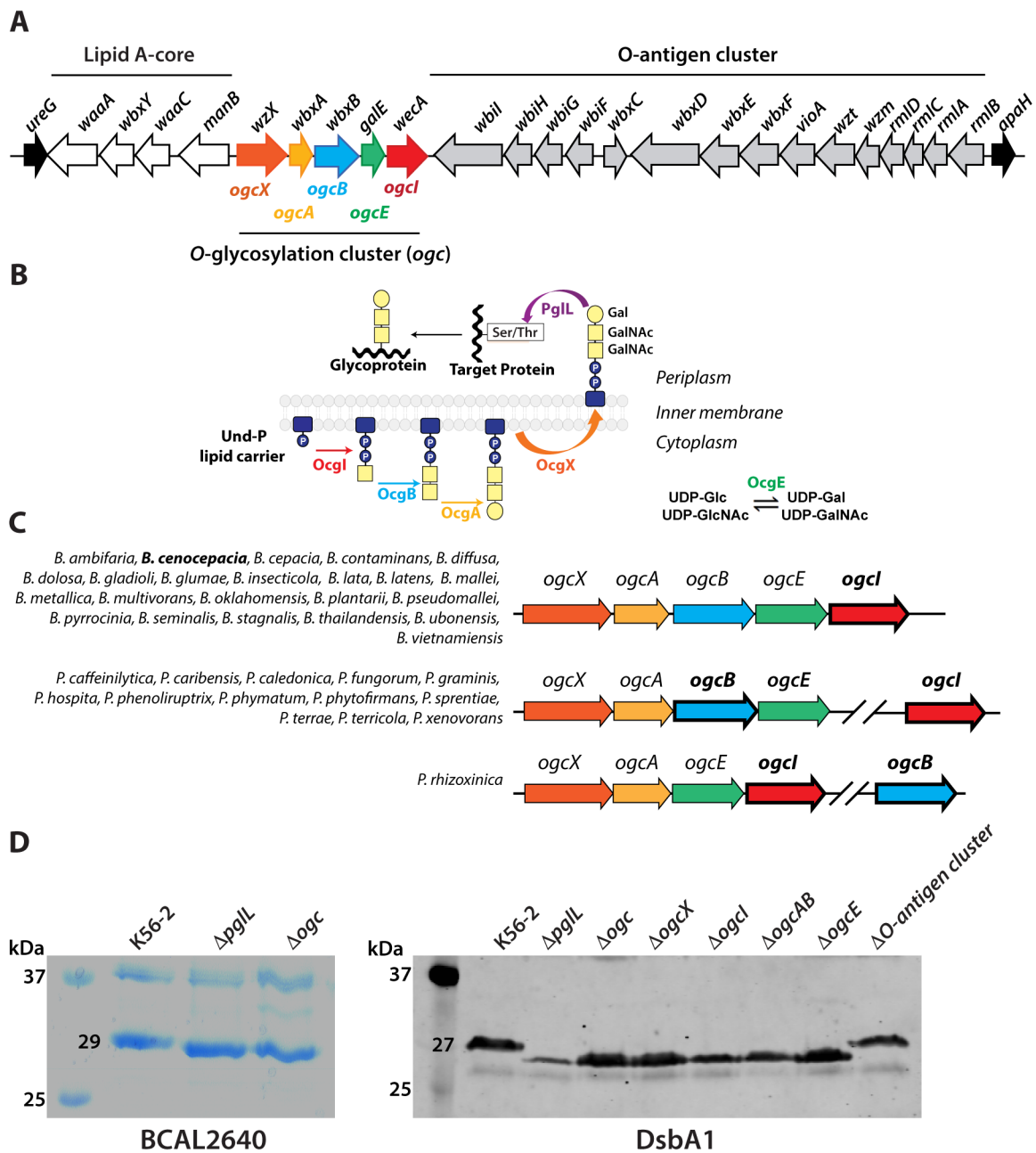


Fig. 1

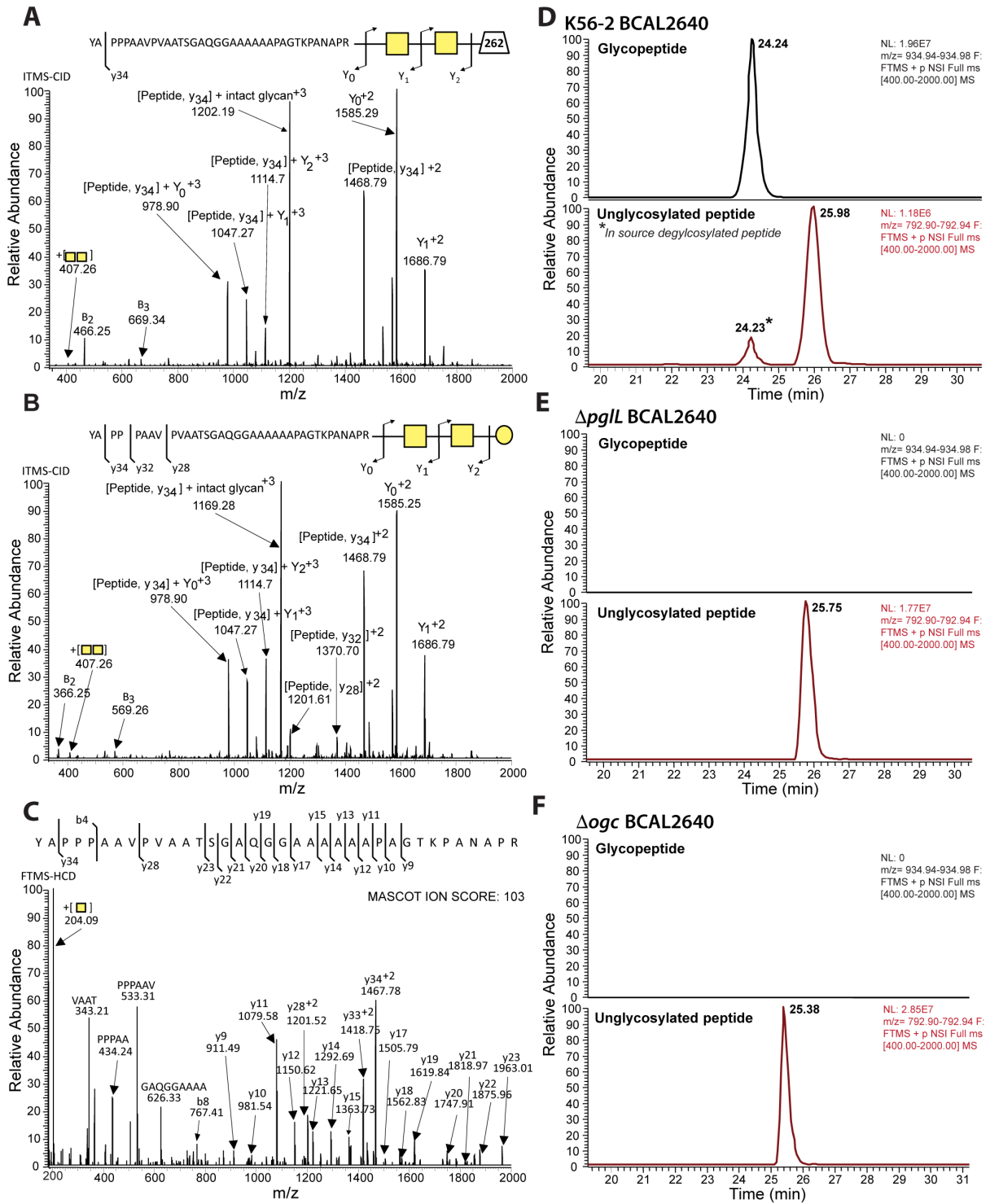


Fig. 2

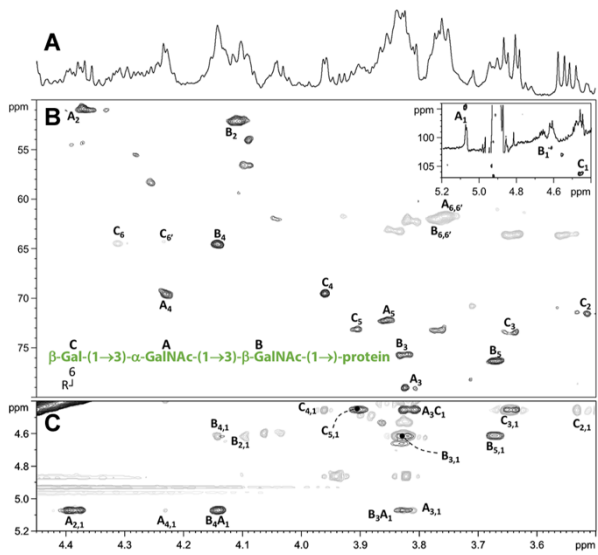


Fig. 3

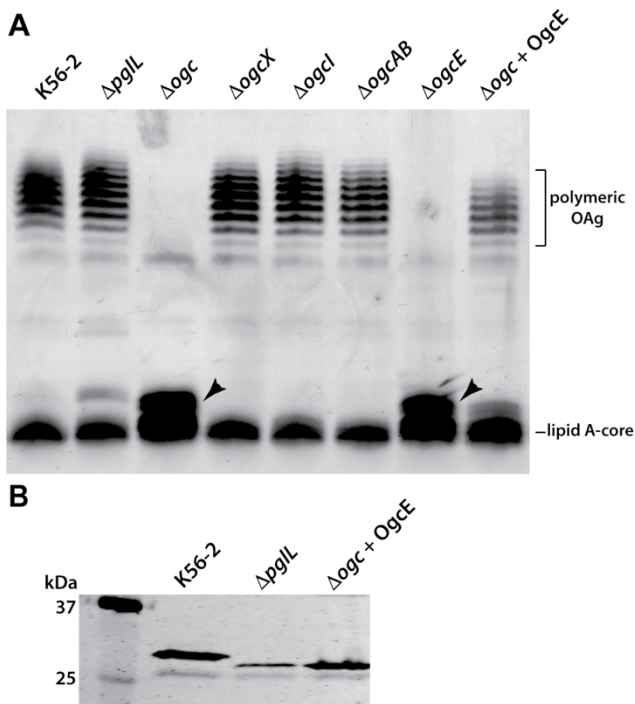


Fig. 4

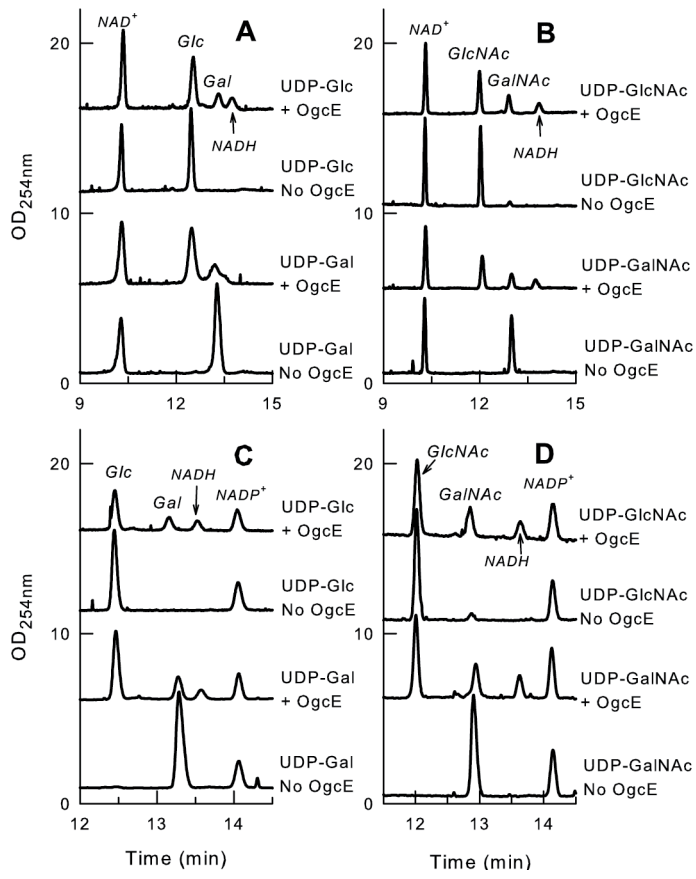


Fig. 5

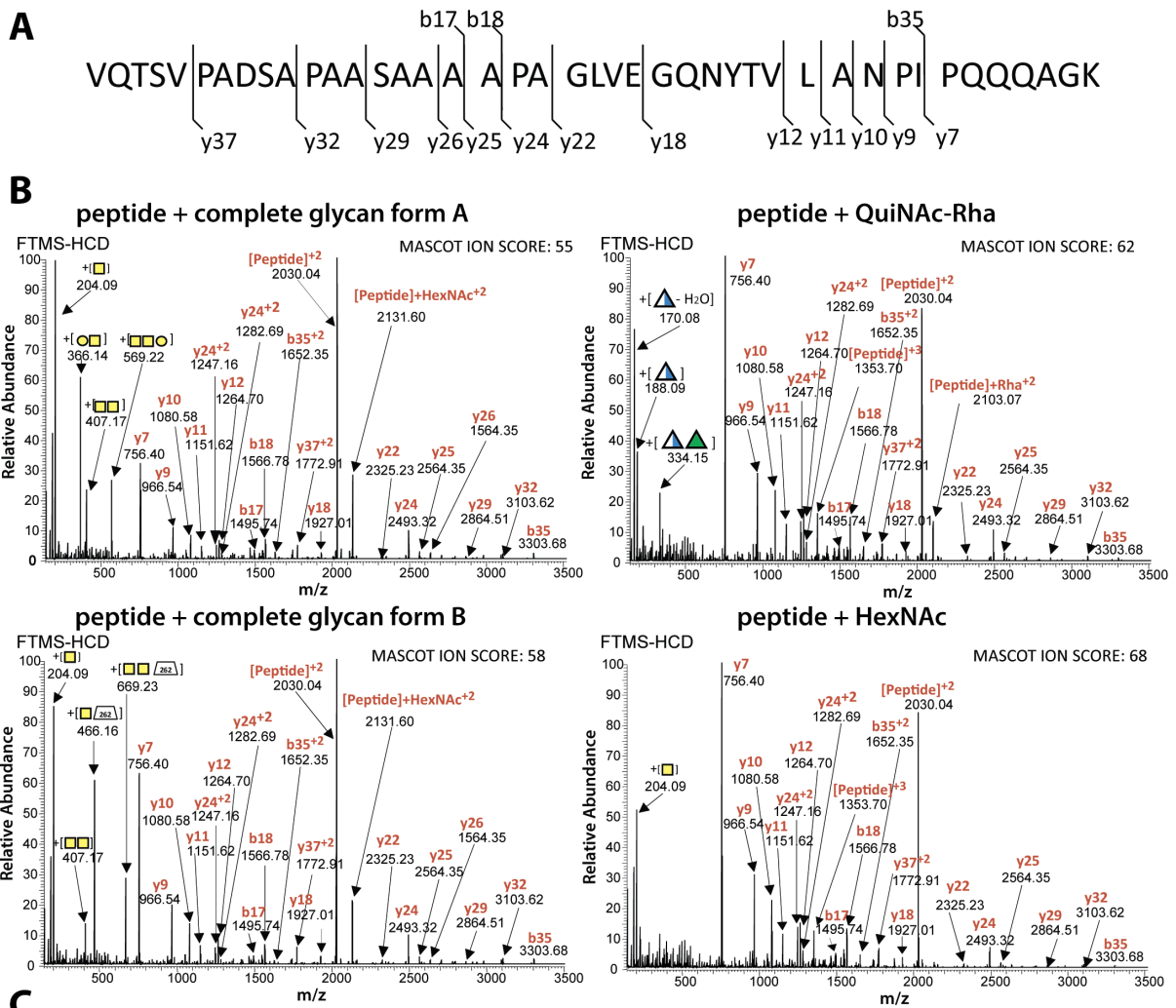
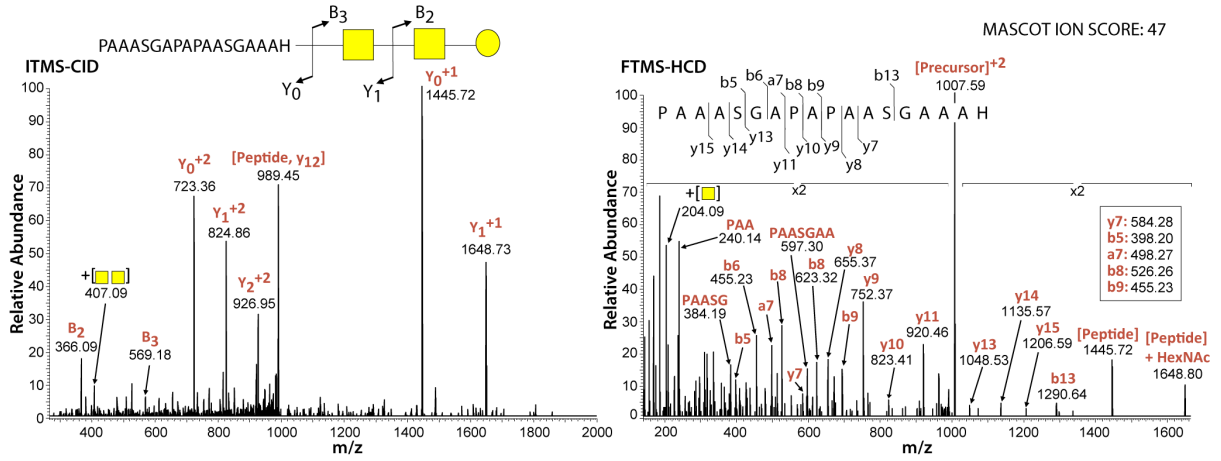
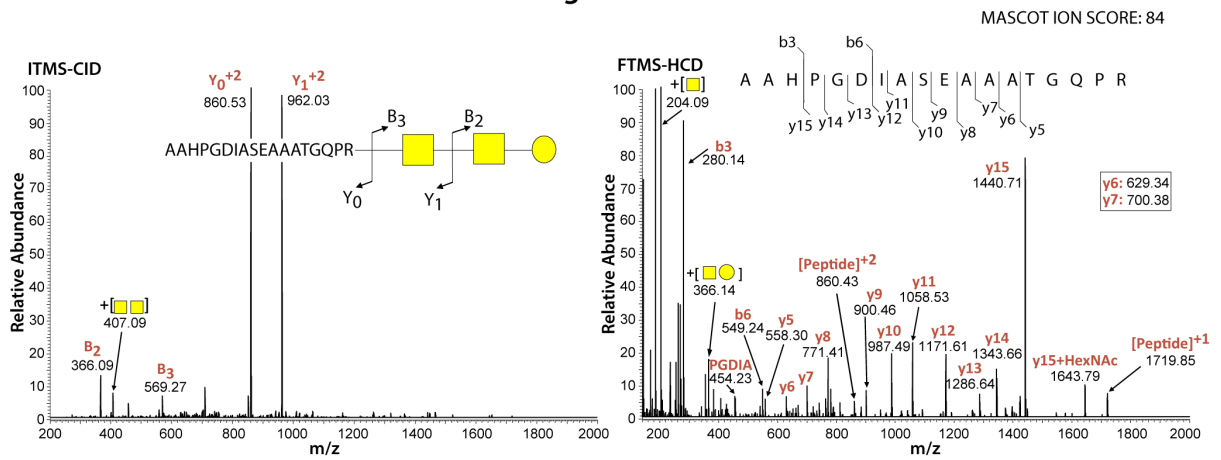


Fig. 6

B. thailandensis



B. gladioli



B. pseudomallei

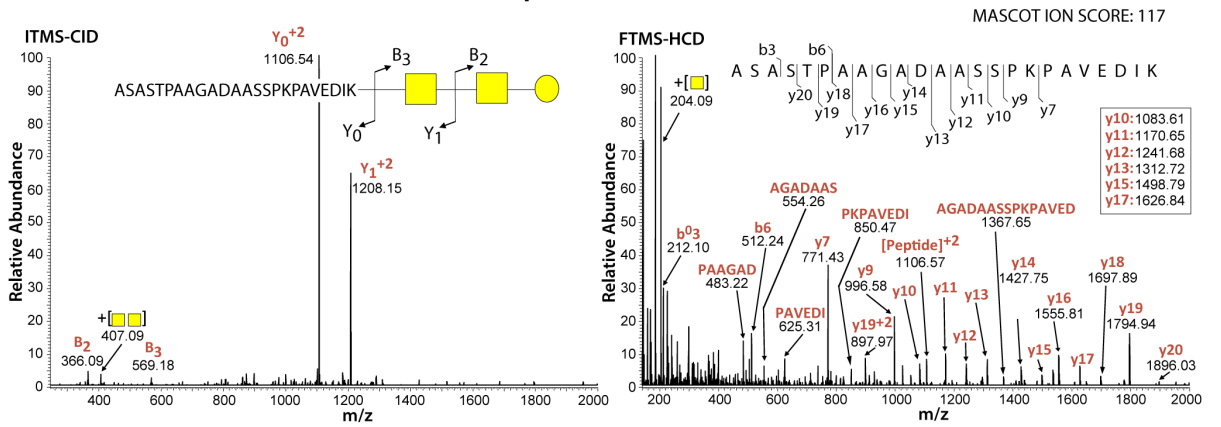


Fig. 7

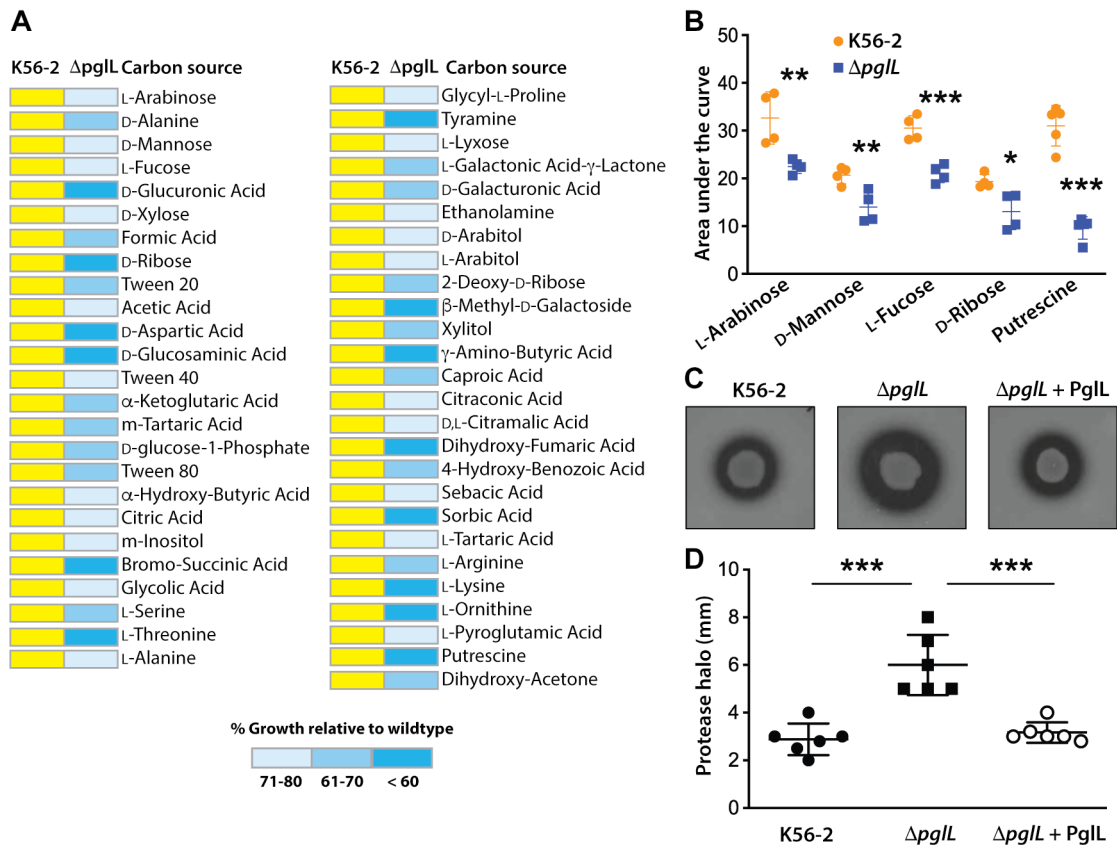


Fig. 8

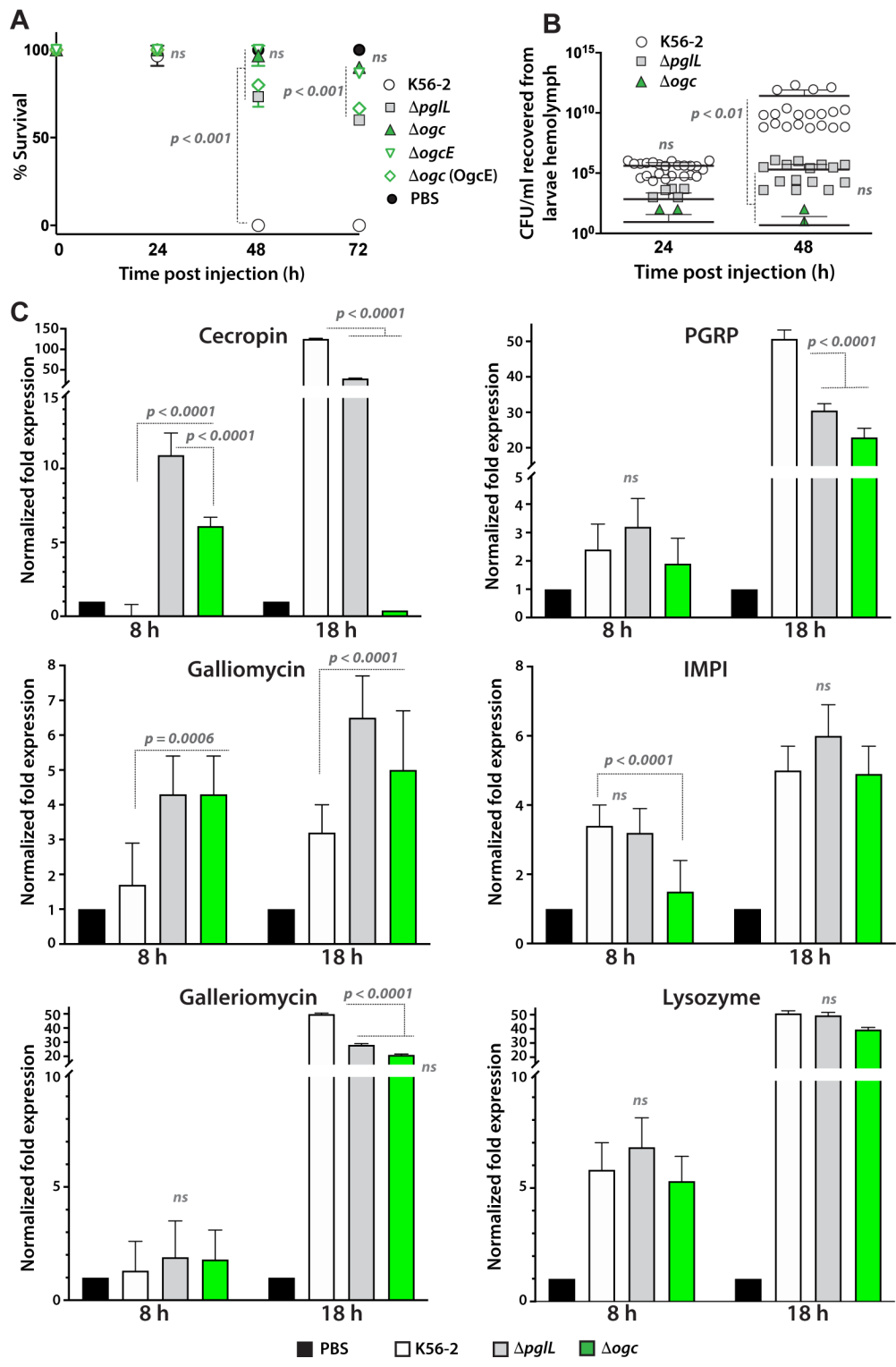


Fig. 9

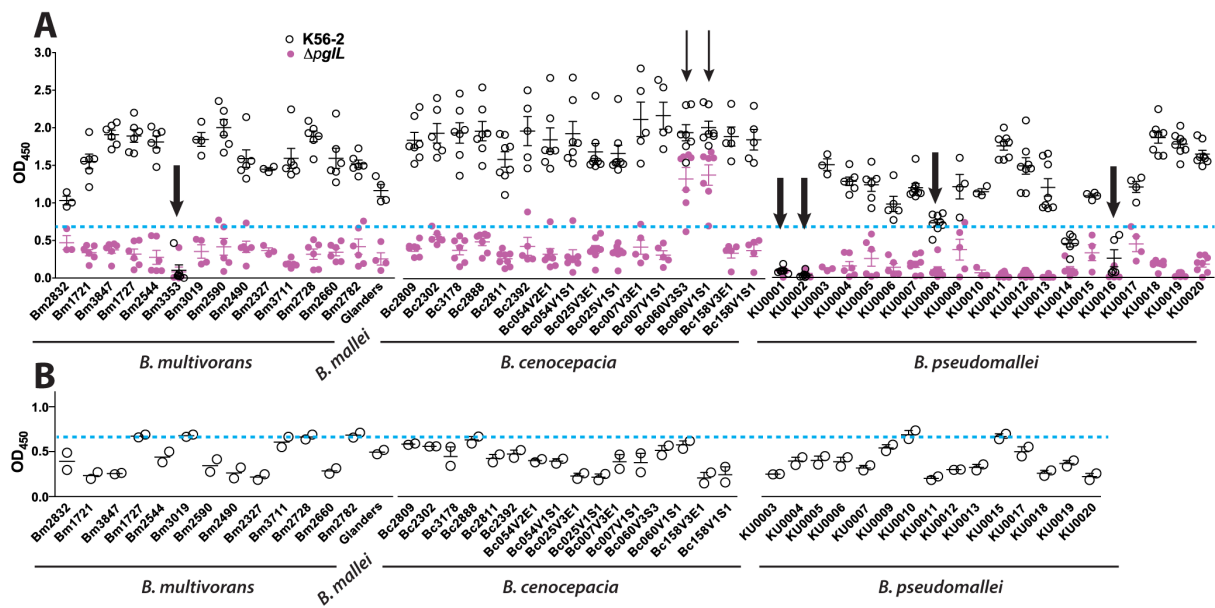


Fig. 10