A broad spectrum protein glycosylation system influences type II protein secretion and associated phenotypes in Vibrio cholerae

Dina Vorkapic

Mario F Feldman

et al

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs
A Broad Spectrum Protein Glycosylation System Influences Type II Protein Secretion and Associated Phenotypes in Vibrio cholerae

Dina Vorkapic¹, Fabian Mitterer¹, Katharina Pressler¹, Deborah R. Leitner¹, Jan Haug Anonsen², Laura Liesinger³, Lisa-Maria Mauerhofer¹, Torben Kuehnast¹, Manuela Toeglhofer¹, Adina Schulze¹, Franz G. Zingl¹, Mario F. Feldman⁵, Joachim Reidl¹,⁶, Ruth Birner-Gruenberger³,⁴,⁷, Michael Koomey² and Stefan Schild¹,⁶

¹ Institute of Molecular Biosciences, University of Graz, Graz, Austria, ² Centre for Ecological and Evolutionary Synthesis, Department of Biosciences, University of Oslo, Oslo, Norway, ³ Institute of Pathology, Medical University of Graz, Graz, Austria, ⁴ Omics Center Graz, BioTechMed-Graz, Graz, Austria, ⁵ Department of Molecular Microbiology, Washington University School of Medicine in St. Louis, St. Louis, MO, United States, ⁶ BioTechMed-Graz, Graz, Austria, ⁷ Institute of Chemical Technologies and Analytics, Vienna University of Technology, Vienna, Austria

Protein secretion plays a crucial role for bacterial pathogens, exemplified by facultative human-pathogen Vibrio cholerae, which secretes various proteinaceous effectors at different stages of its lifecycle. Accordingly, the identification of factors impacting on protein secretion is important to understand the bacterial pathophysiology. PglL_Vc, a predicted oligosaccharyltransferase of V. cholerae, has been recently shown to exhibit O-linked protein glycosylation activity with relaxed glycan specificity in an engineered Escherichia coli system. By engineering V. cholerae strains to express a defined, undecaprenyl diphosphate-linked glycoform precursor, we confirmed functional O-linked protein glycosylation activity of PglL_Vc in V. cholerae. We demonstrate that PglL_Vc is required for the glycosylation of multiple V. cholerae proteins, including periplasmic chaperones such as DegP, that are required for efficient type II-dependent secretion. Moreover, defined deletion mutants and complementation strains provided first insights into the physiological role of O-linked protein glycosylation in V. cholerae. RbmD, a protein with structural similarities to PglL_Vc and other established oligosaccharyltransferases (OTases), was also included in this phenotypical characterization. Remarkably, presence or absence of PglL_Vc and RbmD impacts the secretion of proteins via the type II secretion system (T2SS). This is highlighted by altered cholera toxin (CT) secretion, chitin utilization and biofilm formation observed in ΔpglL_Vc and ΔrbmD single or double mutants. This work thus establishes a unique connection between broad spectrum O-linked protein glycosylation and the efficacy of type II-dependent protein secretion critical to the pathogen's lifecycle.

Keywords: post-translational modification, O-OTase, Vibrio cholerae, biofilm, virulence, chaperone
INTRODUCTION

The Gram-negative pathogen *Vibrio cholerae*, the causative agent of the severe secretory diarrheal disease cholera, transits between two dissimilar habitats along its life cycle. *V. cholerae* efficiently colonizes the human gastrointestinal tract upon oral ingestion of contaminated food or water, but also persists as a natural inhabitant in aquatic ecosystems during inter-epidemic periods (Schild et al., 2008; Nelson et al., 2009). *V. cholerae* employs a number of strategies to quickly adapt to the differential conditions faced along its life cycle, which ensures survival even under unfavorable conditions.

A hallmark of environmental survival and transmission of *V. cholerae* is the ability to form biofilms on chitinous surfaces in the aquatic reservoirs. Ubiquitous chitin particles in the nutrient-limited aquatic reservoir not only act as an attachment platform for subsequent biofilm formation, but also serve as a carbon and nitrogen source. Accordingly, *V. cholerae* expresses a complex chitin utilization program including several secreted chitinases (Meibom et al., 2004). Once ingested by humans, *V. cholerae* passes the acidic barrier in the stomach and reaches the small intestine, the primary site of colonization. During this passage, *V. cholerae* activates a complex regulatory cascade to induce the expression of virulence factors and achieve full colonization fitness (Childers and Klose, 2007). The pathology of cholera is mainly due to the activity of the secreted cholera toxin (CT), which induces a massive efflux of chloride ions and water into the intestinal lumen resulting in a secretory diarrhea (Childers and Klose, 2007).

Several proteins crucial for survival fitness along the different stages of the life cycle are secreted via the type II secretion system (T2SS). These include CT, the biofilm matrix proteins (RbmA, RbmC, and Bap1), and chitinases along with complementary proteins involved in chitin metabolism (Sikora et al., 2011; Johnson et al., 2014). Adaptation to different conditions has so far been mainly investigated through transcriptional profiling, although there is growing evidence that *V. cholerae* also takes advantage of post-translational regulatory strategies. These are exemplified by proteolysis control of transcription factors TcpP and ToxR affecting virulence factor expression, RpoS responsible for the mucosal escape response and FliA, which inversely controls flagella gene transcription and virulence gene expression (Matson and DiRita, 2005; Nielsen et al., 2006; Almagro-Moreno et al., 2015; Pressler et al., 2016; Rogers et al., 2016; Wurm et al., 2017).

O-linked protein glycosylation appears to be widely distributed in bacteria and represents a post-translational modification whereby oligosaccharyltransferases (OTases) transfer a pre-assembled glycan onto target proteins (Iwashkiw et al., 2013). Based on current models for Gram-negative bacteria, the glycan is synthesized on an undecaprenyl pyrophosphate (Und-PP) lipid carrier in the cytoplasm, flipped across the inner membrane and utilized by O-OTases to glycosylate serine or threonine residues of target proteins in the periplasm (Nothaft and Szymanski, 2010). Probably the best studied O-glycosylation system of Gram-negative bacteria is the protein glycosylation (*pgl*) in *Neisseria gonorrhoeae*. Briefly, PglB, PglC, and PglD are required for synthesis of Und-PP linked di-N-acetyl bacillosamine (diNAcBac), the proximal sugar in the oligosaccharide (Aas et al., 2007; Hartley et al., 2011). The fully synthesized glycan is flipped to the periplasmic space by the flipase PglF and subsequently transferred by the OTase PgL/O on diverse target proteins via en block transfer (Aas et al., 2007; Vik et al., 2009).

Intrigued by a potential, intrinsic *V. cholerae* post-translational modification system (Gebhart et al., 2012), we constructed and characterized a *pglL*Vc null mutant. Using a reverse glycoengineering strategy, we found that PgLVc is required for the glycosylation of multiple *V. cholerae* proteins. In addition, the *pglL*Vc null mutant showed altered biofilm features and a trend toward enhanced type II substrate secretion. Remarkably, these phenotypes were significantly augmented in a background simultaneously lacking RbmD, a protein of unknown function with limited structural similarities to PgLVc and other established oligosaccharyltransferases (Supplementary Figure S1). Thus, this work reveals genetic interactions between *pglL*Vc and *rbmD*, and establishes clear connections between broad spectrum O-linked protein glycosylation and secretion processes important in diverse pathogen life styles.

MATERIALS AND METHODS

Strain Construction and Growth Conditions

Bacterial strains and plasmids or oligonucleotides used in this study are listed in Tables 1, 2, respectively. The clinical isolate *V. cholerae* O1 El Tor C6709 was used as WT strain in all experiments. *Escherichia coli* strains DH5αλpir and SM10λpir were used for genetic manipulations. Unless stated otherwise, all strains were grown with aeration in lysogeny broth (LB, 1% tryptone; 1% NaCl; 0.5% yeast extract) on 37°C, on LB agar plates at 37°C, or for biofilm formation under static conditions at 24°C. To assess CT expression and secretion, *V. cholerae* strains were grown under virulence gene inducing conditions at 37°C for 4 h anaerobically using AK1 broth, followed by 4 h aerobic growth with shaking at 180 rpm (Iwanaga and Yamamoto, 1985; Iwanaga et al., 1986). Minimal media M9 was prepared according to standard recipe (Miller, 1972), and is indicated as M9-X, whereby X represents the sole carbon source used. Antibiotics and other supplements were used in the following final concentrations: streptomycin (Sm, 100 µg/ml), ampicillin (Ap, 50 µg/ml in combination with other antibiotics, otherwise 100 µg/ml), kanamycin (Km, 50 µg/ml), chloramphenicol (Cm, 20 µg/ml for *E. coli* strains or 2 µg/ml for *V. cholerae* strains), sucrose (10%), glucose (Glc, 0.2%), chitin (0.2%, Sigma-Aldrich), N-acetylglucosamine (GlcNAc, 0.2%).

DNA Manipulations, Construction of Suicide Plasmids, Reporter Fusions, Mutant Strains, and Expression Plasmids

Qiagen® Gel extraction and Qiagen® PCR Purification kits (Qiagen) were used for purifying PCR products and digested
### TABLE 1 | Strains and plasmids used in this study.

| Strain description | References |
|-------------------|-------------|
| **E. coli** | |
| DH5α::pir | F-ΔlacZYA-argF U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1::pir | Kolter et al., 1978 |
| SM10::pir | thi- recA1 thy- leu- tonA1 lacY1 supE44 23-54 Tc- Muc8::pir | Miller and Mekalanos, 1988 |
| CLM24 | Constructed from E. coli W3110 (ΔmmDΔmE1 rph-1), waaL mutant | Feldman et al., 2005 |
| **V. cholerae** | |
| WT | C6709, O1 El Tor Inaba, clinical isolate, 1991 Peru, tcpA+ ctxAB+ hapR+, spontaneous Sm′ | Roberts et al., 1992 |
| C6709lacZ | C6709, lacZ::neo-sacB::res | Tamayo et al., 2008 |
| ΔpglVc | Deletion of pglVc (VC0393) in C6709, Sm′ | This work |
| ΔrbmA | Deletion of rbmA (VC0928) in C6709, Sm′ | This work |
| ΔpglVcΔrbmA | Deletion of pglVc and rbmA in C6709, Sm′ | This work |
| ΔpglVcΔrbmAΔdegPcat | Deletion of pglVc, rbmA and degP in C6709, Sm′, Cm′ | This work |
| ΔepsC-N | Deletion of epsC-N (VC2734-VC2723) in C6709, Sm′ | This work |
| ΔhapR | Deletion of hapR in C6709, Sm′ | Seper et al., 2011 |
| vpsA::phoA | Insertion of pGP::phoA in vpsA of C6709, Sm′, Ap′ | Seper et al., 2011 |
| ΔhapRvpsA::phoA | Insertion of pGP::phoA in vpsA of ΔhapR, Sm′, Ap′ | Seper et al., 2011 |
| ΔpglVcΔrbmA vpsA::phoA | Insertion of pGP::phoA in vpsA of ΔpglVcΔrbmA, Sm′, Ap′ | This work |
| ΔlacZ::gfp | Insertion of gfp in the lacZ of C6709, Sm′ | This work |
| ΔpglVcΔlacZ::gfp | Insertion of gfp in the lacZ of ΔpglVc, Sm′ | This work |
| ΔrbmAΔlacZ::gfp | Insertion of gfp in the lacZ of ΔrbmA, Sm′ | This work |
| ΔpglVcΔrbmAΔlacZ::gfp | Insertion of gfp in the lacZ of ΔpglVcΔrbmA, Sm′ | This work |
| Δvps-l::Km | Deletion of vps-l cluster (VC0917-VC0927) in C6709 by exchange with a kanamycin cassette, Sm′, Km′ | This work |
| Δvps-l | Deletion of kanamycin cassette in Δvps-l::Km, Sm′ | This work |
| ΔpglVcΔrbmAΔvps-l::Km | Deletion of vps-l cluster (VC0917-VC0927) in ΔpglVcΔrbmA by exchange with a kanamycin cassette, Sm′, Km′ | This work |
| ΔpglVcΔrbmAΔvps-l | Deletion of kanamycin cassette in ΔpglVcΔrbmAΔvps-l::Km, Sm′ | This work |
| Δrbf::Km | Deletion of rfb to rbfU (VC0241-VC0260) in C6709 by exchange with a kanamycin cassette, Sm′, Km′ | This work |
| Δrfb | Deletion of kanamycin cassette in Δrfb::Km, Sm′ | This work |
| **Plasmids** | |
| pCVD442 | onlK mobRP4 sacB, Ap′ | Donnenberg and Kaper, 1991 |
| pJZ111 | Plac-gfp-lacZ in pCVD442, Ap′ | Seper et al., 2014 |
| p | pMMB67EH, IncQ broad-host-range low-copy-number cloning vector, IPTG inducible, Ap′ | Morales et al., 1991 |
| pMMBneo | pMMB67EH-based plasmid, IncQ broad-host-range low-copy-number cloning vector, IPTG inducible, Kan′ | Tamayo et al., 2008 |
| pQE60 | C-terminal His-tag expression plasmid, Ap′ | Qiagen |
| pAC1000 | Cm′ | Hava et al., 2003 |
| pACYC184 | Cloning and expression vector, p15A ori, IPTG inducible, derived from pACT3, Cm′ | Chang and Cohen, 1978 |
| pACYC38FBCD | pACYC184-based plasmid containing the pgfBCD locus from N. gonorrhoeae, Cm′ | Egge-Jacobsen et al., 2011 |
| pGPlphaAPS | pGP::phaA with “phaA” fragment of C6709, Ap′ | Seper et al., 2011 |
| pCVD::pglVc | pCVD442 with up- and downstream fragments of pglVc, Ap′ | This work |
| pCVD::rbmA | pCVD442 with up- and downstream fragments of rbmA, Ap′ | This work |
| pCVD::rbmA | pCVD442 with up- and downstream fragments of rbmA, Ap′ | This work |
| pCVD::degPcat | pCVD442 with up- and downstream fragments of degP flanking a cat cassette, Ap′, Cm′ | This work |
| pCVD::epsC-N::cat | pCVD442 with upstream fragment of epsC and downstream fragment of epsN flanking a cat cassette, Ap′, Cm′ | This work |
| p::pglVc | pglVc of V. cholerae in pMMB, Ap′ | This work |
| p::rbmA | rbmA of V. cholerae in pMMB, Ap′ | This work |
| pMMBneo-p::pglVc | pglVc of V. cholerae in pMMBneo, Km′ | This work |
| pMMBneo::rbmA | rbmA of V. cholerae in pMMBneo, Km′ | This work |

(Continued)
plasmid DNA. PCR reactions for subcloning were carried out using the Q5® High-Fidelity DNA Polymerase (NEB), whereas Taq DNA Polymerase (NEB) was used for all other PCRs. Plasmid DNA was isolated using plasmid DNA purification kits (Qiagen).

Constructions of in-frame deletion mutants were carried out as described by Donnenberg and Kaper (1991). 800 bp long DNA fragments flanking the gene of interest were amplified using the oligonucleotide pairs x_y_1 and x_y_2 or x_y_3 and x_y_4, where x represents the gene and y the restriction site/enzyme used (Table 2). For the construction of pCVDΔdegP:cat the cat-fragment was additionally amplified using pAC1000 as template and the oligonucleotide pairs cat_EcoRI_zw and cat_EcoRI_yx. After digestion with the appropriate restriction enzyme, indirect cloning was performed using the plasmid pACYC177, consisting of about two-thirds of its plasmid pACYC177.) This was constructed by amplifying PCR fragments using the oligonucleotide pairs vpsA_EcoRI-fw and vpsA_EcoRI-rev. After digestion with the appropriate restriction enzyme, indirect cloning was performed using the plasmid pACYC177, consisting of about two-thirds of its plasmid pACYC177.

After digestion with the appropriate restriction enzyme, indirect cloning was performed using the plasmid pACYC177, consisting of about two-thirds of its plasmid pACYC177.) This was constructed by amplifying PCR fragments using the oligonucleotide pairs vpsA_EcoRI-fw and vpsA_EcoRI-rev. After digestion with the appropriate restriction enzyme, indirect cloning was performed using the plasmid pACYC177, consisting of about two-thirds of its plasmid pACYC177.

TABLE 1 | Continued

| Strain description | References |
|--------------------|------------|
| pCVDΔvpsA-KanI     | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |
| pCVDΔvpsA-KanII    | This work |

The ΔrBf deletion mutant was generated as described above (see Δvps-A deletion mutant construction for details) and comprises a deletion spanning from rBfA to rBfU (VC0241-VC0260). This required the pCVDΔrBf-A:Kan (Schild et al., 2005) and pGPrfBfU:KanII, which was constructed by amplifying PCR fragments using the oligonucleotide pairs vpsK_SacI and pCVDΔvpsA-K, which was constructed by amplifying PCR fragments using the oligonucleotide pairs vpsA_SacI and vpsA_NcoI as well as vpsK_NcoI and vpsK_XbaI, digested with XbaI/NcoI or NcoI/SacI, and ligated into a SacI/XbaI-digested pGP704, resulting in pGPVpsK-KanII. The correct chromosomal deletion of genes vpsA-K was confirmed by PCR.

Construction of Δvps-C-N mutant was done as previously described by Sikora et al. (2007) using the appropriate oligonucleotide pairs listed in the Table 2.

All expression plasmids were constructed in a similar manner. PCR fragments of the respective genes containing their own ribosomal binding sites were generated using
the oligonucleotide pairs x.y.z fwd and x.y.z rev, where x represents the gene, y the restriction site/ enzyme used and z the respective expression plasmid (Table 2). PCR fragments digested with the respective restriction enzymes were ligated into the similarly digested IPTG-inducible expression vectors pMMB67EH, pMMBneo or pQE60. Expression constructs were transformed into DH5αλpir, and Ap™ colonies were characterized by colony PCR.

**Immunoprecipitation**

Immunoprecipitation was performed using Dynabeads® Protein G Immunoprecipitation Kit (Invitrogen) according to manufacturer's manual. 4 µl of npg1 antibody was incubated with 196 µl of binding and washing buffer and subsequently bound to the Dynabeads. Periplasmic protein fractions of respective strains were precipitated using ammonium sulfate and 200 µg of protein in 500 µl sample buffer was used as antigen. Proteins in the immunoprecipitations were incubated in 5 × Laemmli buffer for 10 min at 100°C, separated with SDS-PAGE and analyzed by mass spectrometry.

**SDS-PAGE and Immunoblot Analysis**

To separate proteins the standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure was used with 12 or 15% gels and the Prestained Protein Marker Broad Range (New England Biolabs) as a molecular mass standard. Proteins were visualized by conventional colloidal Coomassie staining according to Kang et al. (2002) or transferred to a nitrocellulose membrane (Amersham) for immunoblot analysis using mouse anti-His antisera (Qiagen, 1:2,000 diluted in 3% BSA) for detection of His-tagged proteins, anti-CT antiserum (Sigma-Aldrich, 1:5,000 diluted in 10% skim milk) as primary antibodies. Peroxidase-conjugated goat anti-mouse (Dianova GmbH, diluted 1:7,500 in 10% skim milk) or peroxidase conjugated goat anti-rabbit (Dianova GmbH, 1:7,500 diluted in 10% skim milk) or rabbit anti-BSA antiserum (Sigma-Aldrich, 1:5,000 diluted in 3% BSA) were used as secondary antibodies. Loading of equal amounts proteins was achieved by Bradford quantification and verified by Kang staining of SDS-PAGE gels in parallel to the immunoblot analysis. Chemiluminescence detection was performed by incubating the membrane in an ECL solution (Bio-Rad Laboratories) according to manufacturer's manual for batch purification. Briefly, appropriate amounts of V. cholerae strains were precipitated using ammonium sulfate and 200 µg to the Dynabeads. Periplasmic protein fractions of respective strains were precipitated using ammonium sulfate and 200 µg of protein in 500 µl sample buffer was used as antigen. Proteins in the immunoprecipitations were incubated in 5 × Laemmli buffer for 10 min at 100°C, separated with SDS-PAGE and analyzed by mass spectrometry.

**His-Purification of Proteins Using Immobilized Metal Ion Affinity Chromatography**

Proteins with C-terminal His-tag were purified using a Ni Sepharose™ Fast Flow (GE Healthcare) according to the manufacturer's manual for batch purification. Briefly, appropriate amounts of V. cholerae cultures grown over night (ON) were inoculated in fresh LB to an OD600 of 0.1, grown to and OD600 of 0.5 following induction with IPTG (1 mM) for 6 h at 37°C. Cells were pelleted for 30 min at 17,000 × g and pellets were stored at −20°C. On the following day, pellets were thawed in 5 ml lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% Triton-X) with the addition of protease inhibitor cocktail tablet (cComplete™, Roche). After
sonification to lyse the cells, the soluble fraction was collected by centrifugation (30 min, 17,000 × g) and filtered through a syringe filter (0.45 μm). The resin was washed once with ddH₂O and twice with binding and washing buffer pH 7.4 (81 mM Na₂HPO₄, 19 mM NaH₂PO₄, 0.5 mM NaCl, 30 mM imidazole). Finally, 1 ml 50% Ni Sepharose slurry was added to 5 ml sample and incubated 1 h at 4°C with rotation. To wash the unbound protein, Ni Sepharose/sample was incubated three times 2 min with 1 ml binding and washing buffer on a rocker. Each time, Ni Sepharose/sample was collected by centrifugation 5 min at 500 × g. His-tagged protein was eluted stepwise with 500 μL elution buffer containing 100 mM, 200, 300 or 500 mM imidazole, respectively. Each elution step was performed for 30 min at 4°C with rotation. All fractions were collected in the separate tubes and subjected to SDS-PAGE and/or immunoblot analyses.

**Protein Analysis by Mass Spectrometry**

Protein bands excised from the SDS gel were reduced, alkylated and digested with Promega modified trypsin according to the method of Shevchenko et al. (1996). Peptide extracts were dissolved in 0.1% formic acid (FA), 5% acetonitrile (ACN) and separated by nano-HPLC (Dionex Ultimate 3000) equipped with an enrichment column (C18, 5 μm, 100 Å, 5 × 0.3 mm) and an Acclaim PepMap RSLC nanocolumn (C18, 2 μm, 100 Å, 500 × 0.075 mm) (all Thermo Fisher Scientific, Vienna, Austria). Samples were concentrated on the enrichment column for 6 min at a flow rate of 5 μL/min with 0.1% formic acid as isocratic solvent. Separation was carried out on the nanocolumn at a flow rate of 250 nl/min at 60°C using the following gradient, where solvent A is 0.1% formic acid in water and solvent B is acetonitrile containing 0.1% formic acid: 0–6 min: 4% B; 6–94 min: 4–25% B; 94–99 min: 25–95% B, 99–109 min: 95% B; 109.1–124 min: 4% B; The sample was ionized in the nanospray source equipped with stainless steel emitters (ES528, Thermo Fisher Scientific, Vienna, Austria) and analyzed in an Orbitrap Velos pro mass spectrometer (Thermo Fisher Scientific, Waltham, MA, United States) operated in positive ion mode applying alternating full scan MS (m/z 200–2000) at a resolution of 70000 (at m/z 400) and MS/MS by HCD with NCE@28 of the 12 most intense peaks at a resolution of 17500 (at m/z 400) with dynamic exclusion for 30 s. Generated mass spectra were analyzed with the DeGInaX software. Glycopeptides mass spectra were extracted based on the presence of the three diNAcBac reporter ions at m/z 229.118, at m/z 211.107 and at m/z 169.096 using Thermo Xcalibur (version 3.0.63) and manually assigned to DeGInaX peptide sequences.

**PTM Analysis by Mass Spectrometry**

Kang stained protein bands of affinity purified DeGInaX were washed, de-stained and digested with both trypsin and GluC as previously described (Anonsen et al., 2012). Dried samples were redissolved in 0.1% FA prior to LC-MS/MS analysis. The LC-MS/MS analysis was performed as for protein analysis by MS with the following modification: Solvent B was changed to 90% ACN/9.9% H₂O containing 0.1% FA. The column temperature was set at 40°C. The separation gradient was modified to, 0–3 min: 3% B; 3–53 min: 3–55% B; 55–59 min: 80% B, 60.3–63.3 min: 3% B. The sample was ionized in the nanospray source and analyzed in a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, United States). Mass spectra were acquired in the positive ion mode applying alternating full scan MS (m/z 200–2000) at a resolution of 70000 (at m/z 400) and MS/MS by HCD with NCE@28 of the 12 most intense peaks at a resolution of 17500 (at m/z 400) with dynamic exclusion for 30 s. Generated mass spectra were analyzed with the DeGInaX software. Glycopeptides mass spectra were extracted based on the presence of the three diNAcBac reporter ions at m/z 229.118, at m/z 211.107 and at m/z 169.096 using Thermo Xcalibur (version 3.0.63) and manually assigned to DeGInaX peptide sequences.

**Alkaline Phosphatase Assays**

To determine the enzymatic activities for the transcriptional phoA fusions, alkaline phosphatase assays were performed as described previously (Mannoil, 1991). Bacterial cultures were grown to mid-log phase (~OD₆₀₀ = 0.5) in LB media at 24°C. The activities were expressed in Miller units, given by (A⁴₀₅ × 1,000)/(A₆₀₀ × ml × min).

**Static Biofilm Assay With Crystal Violet Staining**

Biofilm assays under static conditions were essentially performed as previously described (Seper et al., 2011). Briefly, the respective strains were grown ON in LB-Sm or LB-Ap/Glc liquid media (for plasmid containing strains). On the following day, a fresh culture LB-Sm or LB-Ap/ IPTG (in case of strains harboring pMMB67EH or its derivatives), was adjusted to an OD₆₀₀ = 0.01 and inoculated in a 96 well microtiter plate (U bottom, Sterilin). Biofilm was grown in a 24°C climate chamber for 24, 48 or 72 h. Wells were subsequently rinsed using a microplate washer (anths Mikrosysteme GmbH, Fluido2). Biofilm was stained with 0.1% crystal violet, solubilized in 96% ethanol and the OD₅₉₃ was measured (microplate reader: BMG Labtech SPECTROstarNano) to quantify the amount of biofilm.

**Flow Cell Biofilm Experiments**

For visualization and quantification of dynamically formed biofilm, a three channel flow cell system using 2% LB-Sm broth (24 h, RT) was used as described previously (Seper et al., 2011, 2014; Pressler et al., 2016). The respective GFP expressing *V. cholerae* strains were used for biofilm formation to allow acquisition of fluorescent images with confocal laser scanning microscopy. A coverslip 24 × 50 mm (Menzel-Glaeser) was used as substratum for biofilm growth. The respective ON cultures...
were adjusted to \(OD_{600} = 0.1\) using 2% LB. Approximately 300 \(\mu l\) of the dilutions were inoculated per channel. After static incubation for 2 h at RT, initial attachment of cells to the abiotic surface was analyzed in the attachment assay. For analysis of mature biofilm, the static incubation for 2 h at RT was followed by a constant flow of 2% LB at the rate of 3 \(ml h^{-1}\) for 22 h. Images of attached bacteria or biofilms were recorded by confocal laser scanning microscopy.

### Confocal Laser Scanning Microscopy

Images of biofilms were acquired using a Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) with spectral detection and a Leica HCX PL APO CS 63x water objective (NA 1.2). Optical sectioning performed in 0.13 \(\mu m\) steps. GFP was excited at 488 nm, fluorescence emission was detected between 500 and 560 nm, and images were recorded without differential interference contrast (DIC) optics. For visualization and processing of image data the Leica LAS AF Lite and ImageJ 1.46 software was used. Quantification and morphological analysis of image stacks was performed using the computer program COMSTAT\(^1\) (Heydorn et al., 2000; Vorregaard, 2008).

### Cell Fractionation

A cell fractionation was performed essentially as described by Neu and Heppel (1965). Briefly, *V. cholerae* cultures grown ON were inoculated in fresh LB to an \(OD_{600}\) of 0.1, grown until \(OD_{600}\) of 0.5 and subsequently induced with 1 mM IPTG for 4 h. Supernatant (SUP) of the culture was collected by centrifugation 10 min, 5,000 \(\times\) g and filter-sterilized using a 0.22 \(\mu m\) syringe filter. In parallel, cell equivalents reflecting 1 l of a culture with \(OD_{600}\) of 1 were pelleted by centrifugation (10 min, 5,000 \(\times\) g) and washed in buffer 20 mM Tris–HCl pH 8. After another round of centrifugation, cells were resuspended in 20% sucrose and 1 mM Na-EDTA and incubated for 10 min. Cells were then centrifuged for 10 min, 5,000 \(\times\) g at 4\(^\circ\)C and resuspended in ice cold 0.5 mM Mg\(_2\)SO\(_4\) by slow pipetting. The combination of cold temperature and hypotonic solution caused the burst of the bacterial outer membrane and release of the periplasmic fraction (PF) in the supernatant. After incubation for ~5 min, samples were centrifuged for 5 min, 10,000 \(\times\) g at 4\(^\circ\)C to remove cell debris and intact cells. The supernatant was recovered as the periplasmic fraction. Periplasmic fractions were directly used for CT quantification by ELISA, while in all other cases proteins in the collected SUP and PF were either precipitated using trichloroacetic acid (TCA)/acetone for immunoblot analyses or ammonium sulfate in case of immunoprecipitation. In case of ELISA and immunoblot analyses, periplasmic fractions were collected for cytoplasmic contamination and cell lysis via dot blot analysis detecting RpoA, the \(\alpha\)-subunit of the RNA-Polymerase (for details see section “Dot Blot Analysis”). A representative dot blot is shown in Supplementary Figure S4A. In general, similar intensities for periplasmic fractions used for ELISA were observed. Moreover, intensities in all periplasmic fractions were magnitudes lower compared to whole cell extracts (WCE). This indicates equally low levels of cytoplasmic contamination for all periplasmic fractions used for ELISA.

### Dot Blot Analysis

Dot blot analyses were essentially performed as described recently using a 96-well Whatman Minifold 1 Dot-Blot System (GE Healthcare) (Roier et al., 2016). Briefly, equal protein amounts (according to Bradford) of periplasmic fractions or dilutions (3-fold steps) thereof were spotted onto a nitrocellulose membrane. Dilutions (3-fold steps) of a whole cell extract derived from the WT served as positive control (for details see section “Generation of Whole Cell Extracts”). Then, each membrane was dried, incubated in TBS (20 mM Tris/HCl, pH 7.5, 150 mM NaCl) for 2 min, and blocked in 10% skim milk in TBS for 2 h. Afterward, the membrane was incubated with a monoclonal antibody to the \(\alpha\)-subunit of the RNA polymerase (RpoA, NeoClone Biotechnology, diluted 1:2,000 in 10% skim milk) overnight. The next day, membranes were washed three times in TBS for 10 min, incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG antibody from Dianova, diluted 1:7,500 in 10% skim milk in TBS) for 2 h, washed once in TBS-T (20 mM Tris/HCl, pH 7.5, 250 mM NaCl, 0.05% Tween-20), and twice in TBS for 10 min each. Finally, chemiluminescence detection was performed as described above (for details see section “SDS-PAGE and Immunoblot Analysis”).

### Precipitation of Proteins Using TCA/Acetone

Protein precipitation using TCA and acetone was essentially performed as described by Link and LaBaer (2011), with following modifications. Samples were spiked with BSA (0.1 \(\mu g/ml\)), before a solution of 100% TCA was added to the protein samples (SUP or the PF) to the final TCA concentration of 20%. Samples were mixed and stored ON at ~20\(^\circ\)C. On the next day, samples were thawed and centrifuged 30,000 \(\times\) g for 1 h. Supernatant was carefully decanted and 20 ml of acetone was added to the pellet. Samples were again centrifuged 30,000 \(\times\) g for 1 h. A second washing step with 20 ml of acetone was performed with the final centrifugation step of 50,000 \(\times\) g. After decanting the supernatant, pellets were air-dried for 30 min and subsequently suspended in 200 \(\mu l\) of PBS buffer. In attempt to completely dissolve the pellet, samples were placed in a sonification bath for 30 min. Undissolved particles were removed by short centrifugation. Concentration of proteins was determined with Bradford assay (BioRad). Similar efficiency of protein precipitation using TCA was controlled by immunoblot analyses detecting BSA (for details see section “SDS-PAGE and Immunoblot Analysis”).

### Precipitation of Proteins Using Ammonium Sulfate

Periplasmic protein content was also subjected to precipitation using ammonium sulfate, which yields a relatively soluble protein extract essential for immunoprecipitation. Following cell fractionation, ammonium sulfate was added to the periplasmic

\(^1\)http://www.comstat.dk
fraction to final concentration of 50% (Wood, 1976) and stirred for 1 h at RT. Sample was then subjected to centrifugation 10,000 \( \times g \) for 30 min at RT. After decanting supernatant, pellet was suspended in 500 \( \mu l \) PBS buffer. Sample was dialyzed ON against PBS in a dialysis tube with pore size 3.5 (Roth).

**Growth Assays**

Growth kinetics were performed as previously described in M9-chitin or M9-GlcNAc with following modifications (Schild et al., 2007). Briefly, \( \Delta pglL_{Vc} \Delta rbmD \) or WT were grown in a M9-Glc pre-culture ON and inoculated in M9-chitin or M9-GlcNAc to a starting OD\(_{600} = 0.025\). To determine the exact CFUs appropriate dilutions were plated on LB plates at 0, 24, 48, and 72 h. Before plating each culture was rigorously mixed by vortexing. The cultures were incubated at 37\( ^{\circ} \)C with aeration and samples were collected after 24, 48, and 72 h. Results are given as CFU/ml normalized to a starting concentration of 10\(^7\) CFU/ml at \( t = 0 \) h.

**Generation of Whole Cell Extracts**

Whole cell extracts were generated by collecting the pellet of 20 ml culture by centrifugation 5 min at 5,000 \( \times g \). Pellet was suspended in 1 ml of PBS buffer and transferred to a cryotube. Glass beads (0.5 mm in diameter, Roth) were added to the sample and lysed using the PowerLyser\textsuperscript{TM} Bench Top bead-Base Homogenizer (MO BIO Laboratories, Inc.) 3 times 1 min, with 1 min incubation steps on ice after each round of lysis. Samples were centrifuged 2 min at 10,000 \( \times g \) to remove cell debris and unbroken cells. Supernatant was separated from glass beads and used as WCE.

**CT ELISA**

Cholera toxin production in culture supernatants or periplasmatic fractions was determined by the ganglioside GM\(_1\) ELISA (Pressler et al., 2016). \( V. \) cholerae strains were grown ON in LB medium supplemented with 5% glucose, which is beneficial for growth of the \( \Delta pspC-N \) (Sikora et al., 2007). On the following day, strains were shifted to AKI broth with 5% glucose, which represents virulence gene inducing conditions. Strains were grown at 37\( ^{\circ} \)C for 4 h anaerobically, followed by 4 h aerobic growth with shaking at 180 rpm (Iwana et al., 1986). For complementation strains, which have been grown and induced with IPTG on 37\( ^{\circ} \)C, neither LB medium nor AKI broth contained additional glucose to avoid interference with \( p_{lac} \) promoter expression. Subsequently, cells were removed from CT containing supernatants by centrifugation and supernatants were stored at \(-20\)\(^{\circ} \)C. Periplasmatic fractions were obtained from the cell pellet as described above (see section “Cell Fractionation”) and adjusted to protein equivalents (Bradford). ELISA plates (BRANDplates\textsuperscript{®}, 96-well, immunoGrade\textsuperscript{TM}) were coated with 10 \( \mu g/ml \) GM\(_1\) ganglioside (Calbiochem) in 60 mM Na\(_2\)CO\(_3\) ON at 37\( ^{\circ} \)C and washed four times with PBS pH 7.4 and 0.5% Tween (PBS-T). Free binding sites were blocked with 4 mg/ml BSA in PBS (BSA-PBS) for 1 h at RT. After washing as described above, CT containing supernatants were diluted in PBS and added to the plate. Additionally, purified CT in PBS (Sigma-Aldrich) was inoculated in separate wells to generate a standard curve. ELISA plates were incubated with supernatants and purified CT for 1 h at RT and were washed again as described above. After incubation with the primary antibody (Sigma-Aldrich, anti-CT antibody), diluted 1:10,000 in BSA-PBS for 1 h at RT, ELISA plates were washed four times with PBS-T. After incubation with the secondary antibody (Dianova GmbH, peroxidase conjugated goat anti-rabbit), diluted 1:2,000 in BSA-PBS for 1 h at RT and subsequent washing, the ELISA plates were incubated with TMB Substrate Reagent Set (BioLegend, Vienna) for detection of CT. The reaction was stopped by the addition of 1 M H\(_3\)PO\(_4\) and ELISA plates were measured at OD\(_{450}\) by using SPECTROstar\textsuperscript{Nano} microplate reader (BMG Labtech).

**Statistical Analysis**

Data were analyzed using the Mann–Whitney \( U \) test or a Kruskal–Wallis test followed by \textit{post hoc} Dunn’s multiple comparisons. Differences were considered significant at \( P\)-values of \(<0.05\). For all statistical analyses, GraphPad Prism version 6 was used.

**RESULTS**

**Reverse Glycoengineering Reveals That PglL\(_{Vc}\) Glycosylates Multiple \( V. \) cholerae Proteins**

The activity of PglL\(_{Vc}\) has only been shown in a heterologous \( E. \) coli system via simultaneous expression of PglL\(_{Vc}\) along with a defined glycan and protein acceptors for \( O\)-linked protein glycosylation from foreign bacteria (Gebhart et al., 2012). Although this demonstrated functionality of PglL\(_{Vc}\) with relaxed glycan specificity, \( O\)-linked protein glycosylation has not been documented in \( V. \) cholerae. Hence, neither the transferred glycan structure nor glycosylated proteins of \( V. \) cholerae are known. Along this study we focused on the latter and exploited the relaxed glycan specificity of PglL\(_{Vc}\) along with a well-characterized glycan biosynthesis locus from \( N. \) gonorrhoeae to identify a first set of glycosylated proteins in \( V. \) cholerae. Therefore, we introduced a plasmid harboring the core pgfBFCD glycosylation locus from \( N. \) gonorrhoeae into \( V. \) cholerae to express the lipid-linked monosaccharide precursor Und-PP-diNAcBac. Proteins glycosylated with diNACBac monosaccharide could then be readily detected using the specific monoclonal antibody npgl (Aas et al., 2007; Faridmoayer et al., 2007; Egge-Jacobsen et al., 2011; Anonsen et al., 2015). Immunoblotting of WCE revealed a number of distinct npgl1-reactive proteins whose detection was dependent on the presence of both PglL\(_{Vc}\) and the \( N. \) gonorrhoeae pgf plasmid (Figure 1A). Moreover, these findings were independent on the presence or absence of RbmD. This indicates that PglL\(_{Vc}\), in contrast to RbmD, can utilize the diNACBac glycan to modify a defined subset of \( V. \) cholerae proteins. Hence, we focused on the identification of proteins glycosylated via PglL\(_{Vc}\).

To identify \( V. \) cholerae proteins bearing the diNACBac monosaccharide, immunoaffinity purification was employed as
Vorkapic et al.

**Vibrio cholerae O-Glycosylation System**

FIGURE 1 | Expression of the diNAcBac glycan in *V.* cholerae results in multiple glycosylated proteins in a PglL<sub>Vc</sub>-dependent manner. (A) Shown is a representative immunoblot using the anti-diNAcBac antibody (npg1) to detect diNAcBac-glycosylated proteins and the corresponding Kang-stained SDS-gel of whole cell extracts derived from Δpgl<sub>Vc</sub>Δrbm<sub>D</sub> with or without *in trans* expression of respective O-OTases PglL<sub>Vc</sub> or Rbm<sub>D</sub> (+ = pMMBneo-pglL<sub>Vc</sub> or pMMBneo-rbmD; – = pMMBneo) as well as the diNAcBac glycan (+ = pACYCpglFBCD; – = pACYC184). Only the presence of PglL<sub>Vc</sub> and diNAcBAc yielded in the detection of multiple bands. (B) Shown is an immunoblot using the npg1-antibody to detect diNAcBac-glycosylated proteins and the corresponding Kang-stained SDS-gel of immunoprecipitated samples of Δpgl<sub>Vc</sub>Δrbm<sub>D</sub> expressing the diNAcBac glycan (pACYCpglFBCD) in the presence (+ = pMMBneo-pglL<sub>Vc</sub>) or absence (– = pMMBneo) of PglL<sub>Vc</sub>. Immunoprecipitation was performed using npg1 immobilized onto Dynabeads coupled with protein G in combination with membrane and periplasmic enriched protein samples from the respective strains. Selected protein bands, which appear to be enriched upon presence of PglL<sub>Vc</sub> on the SDS-gel and resulted in a decent signal on the immunoblot, were excised and subjected to MS. Identified proteins are indicated on the right with their respective position on the gel, protein identities and accession numbers. (A,B) Lines to the left indicate the molecular masses of the protein standards in kDa.

previously described (Anonsen et al., 2012) using samples enriched for membrane and periplasmic proteins from various backgrounds as prey (Figure 1B). Immunoblotting using the npg1 monoclonal antibody of immunoprecipitated samples revealed a number of proteins specifically detected in the presence of both PglL<sub>Vc</sub> and the *N. gonorrhoeae* pgl plasmid. Besides several bands with low intensity the immunoblot shows two intense bands at approximately 50 and 25 kDa. The 50 kDa band is likely a cross-reacting band as it also detected in absence of PglL<sub>Vc</sub>. In contrast, the 25 kDa band only appears in presence of PglL<sub>Vc</sub>, but its exact nature remains to be elucidated.

Robust signals obtained by immunoblot indicating immunoaffinity purified proteins or complexes thereof, were aligned to protein bands on a conventionally stained gel that were subsequently excised and identified by mass spectrometric (MS) techniques. The analyses revealed several proteins, such as abundant outer membrane proteins OmpA and OmpU as well as periplasmic chaperones, i.e., the peptidyl-prolyl *cis-trans* isomerases (SurA-like and FkpA) and the chaperone/protease DegP (Figure 1B).

**DegP Glycosylation Can Be Reconstituted in an E. coli Background**

In order to further validate the glycosylation status of the proteins identified above, the ability of DegP as a representative candidate to be glycosylated in a heterologous system was examined. Plasmids individually expressing PglL<sub>Vc</sub>, a C-terminal His-tagged DegP and Und-PP-diNAcBac were introduced into the glycosylation-deficient *E. coli* strain CLM24. Following a His-tag purification, samples were subjected to immunoblot analyses using penta-His or npg1 antibodies as well as conventional gel staining (Figure 2A). Co-expression of PglL<sub>Vc</sub> and Und-PP-diNACBac was both necessary and sufficient to establish reactivity of DegP with npg1, indicative of diNACBac modification (Figure 2A). In addition, a cross-reactive band (∼20 kDa) was detected by the npg1 antibody upon expression of the
diNACBac. It could be speculated that this band reflects the lipid-linked monosaccharide precursor Und-PP-diNACBac (Und-PP-diNACBac), but the exact origin remains to be elucidated.

Gel slices bearing His-tagged DegP were excised and processed for MS analyses to detect glycan attachment sites. Utilizing LC-MS/MS employing higher-energy collision dissociation in conjunction with in-gel digestion with trypsin and GluC (HCD) (Anonsen et al., 2012) peptides covering 98% of DegP including two glycopeptides were identified (Supplementary Figure S2A). Specifically, the MS/MS
spectrum of the doubly charged precursor ion at m/z 720.917 (charge adjusted mass 1440.827 [M+H+]) corresponds to the peptide 47KVTPAVSIAVE58 (theoretical monoisotopic mass 1212.720 [M+H+]) modified with a single diNAcBac moiety (228.111 Da) (Figure 2B). Similarly, the MS/MS spectrum of the doubly charged precursor ion at m/z 613.803 (charge adjusted mass 1226.599 [M+H+]) corresponds to the peptide 371SLHQGLSGAE380 (theoretical monoisotopic mass 998.490 [M+H+]) with a single diNAcBac modification (Figure 2C).

In addition to extensive y- and b-ions series that were detected in both MS/MS spectra enabling the accurate amino acid sequence determination for both peptides, the diNAcBac specific reporter ions at m/z 229.118, at m/z 211.107 and at m/z 169.096 (Anonsen et al., 2012) was detected in the low mass area. The MS results therefore clearly establish both the 47KVTPAVSIAVE58 and the 371SLHQGLSGAE380 peptides as modified by a diNAcBac moiety at a single site (Figures 2B, C and Supplementary Figures S2B, C).

Interestingly, both DegP glycopeptides are located within defined domain structures. The 47KVTPAVSIAVE58 glycopeptide is localized within the protease domain whereas the 371SLHQGLSGAE380 glycopeptide is positioned in the carbohydrate binding loop of the PDZ2 domain (Figure 2D), which is suggested to be involved in substrate binding (Krojer et al., 2002). Thus, if identical sites of glycan attachment occur in V. cholerae, DegP activities might be altered.

**PglL_Vc and RbmD Status Affect Secretion of Cholera Toxin**

Based on the confirmed O-glycosylation of DegP, the identification of other periplasmic chaperones to be targets for O-glycosylation in V. cholerae (Figure 1B) and a recent report implying that DegP affects type II-dependent secretion in V. cholerae (Altindis et al., 2014), we sought to examine if PglL_Vc influences T2SS-associated phenotypes. Given the structural similarities of RbmD to PglL_Vc and other established oligosaccharyltransferases (Supplementary Figure S1) we also examined the potential effects of RbmD, although the O-Tase activity of RbmD remains to be confirmed. Thus, respective single and double mutants (i.e., ΔpglL_Vc, Δarbmd, and ΔpglL_VcΔrbmD) as well as complementation strains for both gene products were included in the following phenotypic analyses.

V. cholerae secretes several important T2SS effectors along its lifecycle (Sikora, 2013). The most prominent example is probably CT, which is predominantly responsible for the secretory diarrhea during human colonization. To investigate whether secretion of CT is affected in the context of the PglL_Vc, and RbmD status, secretion levels of CT were compared for WT, ΔdegP, ΔpglL_Vc, Δarbmd, ΔpglL_VcΔarbmd, and ΔpglL_VcΔarbmdΔdegP. As a control, CT secretion was measured in a ΔepsC-N background that lacks essential parts of the T2SS machinery and is therefore severely impaired for secretion. Of note, CT expression remained equal in all strains tested as indicated by comparable signal intensities in the WCE of respective strains (Supplementary Figure S3A). Quantification of secreted CT revealed significant higher levels in the supernatants of ΔpglL_Vc, Δarbmd, and ΔpglL_VcΔarbmd backgrounds compared to WT (Figure 3A).

Restoration of PglL_Vc expression significantly lowered the CT secretion compared to the ΔpglL_VcΔarbmd with an empty vector control, while with restoration of RbmD expression, only a mild reduction was observed (Figure 3B). In addition, the CT levels in the periplasm were quantified for these strains showing an inverse pattern compared to secreted CT. Periplasmic CT levels were significantly lower for ΔpglL_VcΔarbmd with an empty vector compared to WT with empty vector. Moreover, PglL_Vc or RbmD expression significantly increased periplasmic CT levels compared to ΔpglL_VcΔarbmd with an empty vector. Again, equal CT expression was observed in these strains with plasmids indicated by comparable signal intensities in the respective WCE (Supplementary Figure S3B). In comparison to the WT a marked decrease of CT secretion for the T2SS-mutant ΔepsC-N and ΔdegP was observed (Figure 3A). Moreover, deletion of degP in the ΔpglL_VcΔarbmd background (ΔpglL_VcΔarbmdΔdegP) also reduced CT secretion compared to ΔpglL_VcΔarbmd. Thus, degP is epistatic to pglL_Vc and rbmD. In summary, CT is more efficiently secreted in the absence of PglL_Vc and RbmD, while presence of PglL_Vc and RbmD is associated with reduced translocation into the supernatant resulting in accumulation of CT in the periplasm.

Next, we tried to pinpoint the potential origin of an endogenous protein glycosylation by assuming that a defect in endogenous glycan availability might phenocopy pglL_Vc- and rbmD-mutants. In principle, V. cholerae encodes two major glycan biosynthesis pathways, represented by the vps- (Vibrio exopolysaccharide, VPS) and rfb- (O-antigen glycan) gene clusters (Manning et al., 1995). To assess the potential influence of these two pathways, CT secretion was quantified for strains lacking either the rfb or vps genes. Similar to ΔpglL_Vc, Δarbmd, and ΔpglL_VcΔarbmd, mutants lacking the entire vps-I gene cluster (Δvps-I and ΔpglL_VcΔarbmdΔvps-I) showed increased CT levels in the supernatant compared to WT, whereas deletion of rfb genes (Δrfb) had no effect (Figure 3A). Deletion of pglL_Vc and rbmD in addition to the vps-I gene cluster did not further enhance the secreted CT levels. Moreover, Δvps-I and ΔpglL_VcΔarbmd showed a similar secretion efficacy of CT with almost no detectable CT amounts in the periplasm and high levels in the supernatant (Figure 3B). Notably, expression of PglL_Vc and RbmD in Δvps-I (Δvps-I p-pglL_Vc and Δvps-I p-rbmD) did not restore detectable CT levels in the periplasm (Figure 3B). These phenotypes provide a first hint that the vps cluster could be involved in providing an endogenous glycan.

**PglL_Vc and RbmD Affect Growth on Chitin**

In the aquatic lifestyle, V. cholerae secretes several proteins of the chitin utilization program via the T2SS (Sikora et al., 2011). As PglL_Vc and RbmD may generally affect secretion of T2SS-dependent substrates including chitinases, mutants thereof might exhibit altered growth on chitin. Therefore, we compared growth dynamics of ΔpglL_Vc, Δarbmd, and ΔpglL_VcΔarbmd to the WT in minimal media supplemented with chitin or its corresponding monomer GlcNAc as sole carbon sources,
respectively (Figures 4A,B). All strains showed similar growth on GlcNAC demonstrating equal uptake and metabolic usage of the monosaccharide (Figure 4B). In contrast, the ΔpgLLVc, ΔrbmD and especially the ΔpgLLVc ΔrbmD mutant showed enhanced growth on chitin that gradually increased over the course of 72 h growth period compared to the WT (Figure 4A). Similar to ΔpgLLVc ΔrbmD mutant, the Δvps-I mutant also showed enhanced growth on chitin, but similar growth on GlcNAC, if compared to the WT (Figures 4A,B). In trans expression of both PgLVC and RbmD in the double mutant did not affect growth on GlcNAC, but significantly reduced growth on chitin and allowed (partial) restoration of the WT phenotypes at 48 and 72 h (Figures 4C,D). In conclusion, single and double mutants of PgLVC and RbmD have a fitness advantage compared to WT while growing on chitin, consistent with the notion of a more efficient secretion of chitinases as T2SS substrates.

PgLVC and RbmD Affect Biofilm Formation

Both, PgLVC and rbmD, have been previously identified in a screen for genes up-regulated during dynamic biofilm formation (Seper et al., 2014). In that report, they were excluded from subsequent characterization as they did not meet the stringent criteria for bona fide in biofilm induced and were at time only annotated with unknown function. Of note, rbmD is part of the rbm-gene cluster (rugosity and biofilm structure modulators) that harbors the biofilm matrix protein genes rbmA and rbmC (Fong et al., 2006; Fong and Yildiz, 2007). Based on this, we analyzed the pgLLVc and rbmD mutants for alterations in biofilm formation. First, we focused on static biofilm formation after 24 and 48 h, representing two time points frequently used for biofilm analyses in V. cholerae (Seper et al., 2011). In general, altered biofilm formation can already be observed at 24 h (Supplementary Figure S5A), but phenotypes are more pronounced after 48 h (Figure 5A). Here, the ΔpgLLVc mutant showed a slight, but significant increase in static biofilm formation, while the ΔrbmD mutant and especially the ΔpgLLVc ΔrbmD double mutant exhibited pronounced increases in biofilm formation compared to the WT (Figure 5A). In trans expression of both PgLVC and RbmD in the double mutant significantly reduced biofilm formation and allowed partial restoration of the WT phenotypes at both time points (Figure 5B and Supplementary Figure S5B).

Three-dimensional biofilm morphology was microscopically analyzed in a flow cell system using GFP-expressing backgrounds (Seper et al., 2014). The ΔpgLLVc mutant and the ΔpgLLVc, ΔrbmD double mutant exhibited a significant increased attachment capability, while the surface coverage of the ΔrbmD mutant was unaltered compared to the WT (Figures 6A,B). In mature biofilms, distinct differences could be observed upon deletion of pgLLVc and rbmD (Figure 6C). Most pronounced phenotypes in comparison to the WT were the increased biomass in case of ΔpgLLVc biofilms and the enhanced maximum thickness and higher roughness coefficient for ΔrbmD biofilms (Figure 6D). Biofilm formation of the double mutant ΔpgLLVc ΔrbmD combines characteristics of both single mutants with an increase in attachment, biomass and maximum thickness (Figure 6D). V. cholerae mutants exhibiting increased biofilm formation can be frequently correlated with enhanced expression of vps genes encoding proteins for the V. cholerae exopolysaccharide (VPS) matrix synthesis and secretion (Yildiz and Schoolnik, 1999). To assess whether the enhanced biofilm formation was related to enhanced vps expression here, chromosomal transcriptional fusions of a promoterless phoA reporter gene to vpsI representing one of the first genes in the vps-I locus, were constructed in the WT and mutant backgrounds, as well
Deletion of O-OTases affects chitin utilization. (A,B) Shown is growth (CFU/ml) at indicated time points of WT, ΔpglVc, ΔrbmD, ΔpglVcΔrbmD, and Δvps-I in minimal media M9 supplemented with chitin (A) or supplemented with GlcNAc (B) as a sole carbon source. (C,D) Shown is growth (CFU/ml) at indicated time points of WT with empty vector (WT p), ΔpglVcΔrbmD with empty vector (ΔpglVcΔrbmD p), ΔpglVcΔrbmD p-ΔpglVc, and ΔpglVcΔrbmD p-rbmD in minimal media M9 supplemented with chitin (C) or supplemented with GlcNAc (D) as a sole carbon source. (A–D) The data is given as median with interquartile range (n ≥ 8). Asterisks highlight significant differences between respective data sets (*P < 0.05 Kruskal–Wallis test followed by post hoc Dunn’s multiple comparisons).

RbmD Restricts RbmA Secretion in a VPS Dependent Manner

As vps expression was unaltered in the ΔpglVcΔrbmD mutant, we hypothesized that the alterations in biofilm amounts and architecture could be due to differential secretion of the T2SS substrates RbmA, RbmC or Bap1, which represent three major adhesive protein components of the V. cholerae biofilm matrix (Absalon et al., 2011; Berk et al., 2012). We therefore deleted rbmA, rbmC and bap1 independently in the WT, ΔpglVc, ΔrbmD, and ΔpglVcΔrbmD backgrounds and tested biofilm formation. Individual mutants ΔrbmA, ΔrbmC, and Δbap1 exhibit similar biofilm levels compared to the WT in the static biofilm assay (Figure 7A), which is concordant with a previous report as single deletion of each of the three matrix proteins does not impact biofilm formation (Fong et al., 2006). The absence of PglVc and RbmD still resulted in increased biofilm formation in the ΔrbmC and ΔhapA backgrounds (Figure 7A). Similarly, ΔpglVc and ΔrbmAΔpglVc showed equally enhanced biofilm levels compared to the WT. Deletion of rbmA in the ΔrbmD or ΔpglVcΔrbmD background negated the RbmD-dependent biofilm phenotypes and resulted in significant lower biofilm amounts compared to the parental ΔrbmD or ΔpglVcΔrbmD as in the ΔhapR mutant, which served as a positive control for de-repressed vpsA expression (Seper et al., 2011). Thus, PhoA activities reflect the transcription levels of vpsA in the respective strains. O-OTase mutants and WT exhibited comparable levels of PhoA activity indicating similar transcription levels of vpsA in these strains (Supplementary Figure SSC). Thus, the alterations in biofilms and attachment cannot be simply explained by elevated transcription of VPS genes.
strains (Figure 7A). This phenotypic analysis thus revealed synthetic genetic interactions between rbmA and rbmD.

To further investigate the relationships between RbmD and RbmA, we examined RbmA trafficking using strains expressing a His-tagged derivative allowing immunoblot analysis. In the absence of PgLNVc and RbmD, RbmA was relatively efficiently secreted into the supernatant with a reciprocal minority localized in the periplasmic fraction. Remarkably, expression of solely RbmD, but not PgLNVc, effectively reversed RbmA localization resulting in its relative accumulation in the periplasmic fraction and reduced translocation into the supernatant (Figure 7B). RbmD thus diminishes the secretion efficacy of RbmA, providing
FIGURE 7 | O-OTases impact RbmA secretion. (A) Deletion of rbmA, but not rbmC or bap1, negates the increased biofilm formation in O-OTase mutants. The biofilm formation capacity of the strains indicated on the x-axis were quantified after 48 h under static conditions by crystal violet staining and subsequent determination of the OD_{595} (n ≥ 24). (B–D) Shown are representative immunoblots detecting His-RbmA in whole cell extracts (WCE), periplasmic fractions (PF), and supernatants (SUP) of strain \( ΔpglL_{Vc}ΔrbmDΔrbmA \) (B), \( ΔpglL_{Vc}ΔrbmDΔrbmAΔvps-I \) (C), or \( ΔpglL_{Vc}ΔrbmDΔrbmAΔfb \) (D) expressing His-tagged RbmA in trans (pQE60-rbmA) in the presence or absence of RbmD (pMMBneo-rbmD or pMMBneo), respectively. Equal amounts of proteins for WCE, PF or SUP were loaded according to Bradford to allow direct comparison of the fractions. Semiquantitative densitometric evaluation of detected His-RbmA was performed with the Quantity One software (Bio-Rad Laboratories) and is indicated below the immunoblots as arbitrary intensity units [mean AIU with standard deviation (n ≥ 3)] normalized to WCE of the respective strain, which was always set to 1. Notably, TCA precipitation of PF and SUP was necessary to ensure stable detection. Similar efficiency of protein precipitation for PF and SUP was checked by spiking the samples with BSA prior to the precipitation and subsequent immunoblot analyses detecting BSA in the precipitated samples. A representative immunoblot is shown in Supplementary Figure S6, indicating equal precipitation efficiency in all samples. Moreover, PF obtained after TCA precipitation were controlled for differential cytoplasmic contamination and cell lysis via dot blot analyses detecting RpoA, the \( \alpha \)-subunit of the RNA-Polymerase (for details see section “Dot Blot Analysis”). A representative dot blot is shown in Supplementary Figure S4B, indicating equally low levels of cytoplasmic contamination for all periplasmic fractions.

an example for differential secretion of a T2SS substrate modulated by the status of RbmD.

Having revealed a RbmD-dependent periplasmic localization of RbmA, we tried to pinpoint the origin of the glycan potentially transferred by RbmD. Based on the differential secretion pattern of CT in presence or absence of the VPS, we focused on a strain lacking the vps-I gene cluster. In the \( Δvps-I \) background the RbmD-dependent periplasmic accumulation of His-tagged
RbmA was negated and a robust translocation of His-tagged RbmA was observed (Figure 7C). Concordant to the CT data, RbmD-dependent periplasmic accumulation of His-tagged RbmA was still observed in a Δrfb background (Figure 7D). This suggests that the vps cluster can provide a glycan precursor transferred by RbmD.

**DISCUSSION**

Here, we establish a clear association between O-linked protein glycosylation mediated by the oligosaccharyltransferase PgL_{Vc} and the efficacy of type II protein secretion-dependent processes in the human pathogen *V. cholerae*. These findings define a first connection between protein glycosylation and processes critical to *V. cholerae* fitness in and outside of its host, such as differential secretion of T2SS-dependent substrates including CT, chitinases and the biofilm matrix protein RbmA. Moreover, we show that mutants lacking RbmD, whose functions remain unknown but shares structural features with PgL_{Vc}, exhibit phenotypic perturbations analogous to those lacking PgL_{Vc}.

These findings raise the obvious question as to how O-linked protein glycosylation status mediated by PgL_{Vc} might impact on the translocation of type II protein secretion substrates across the outer membrane. Perhaps the simplest explanation would be that one or more of the glycoproteins targeted by PgL_{Vc} influences type II protein secretion proficiency and that the activity or functionality of that target protein is influenced by its glycosylation status. Here, obvious candidates for such glycoproteins would be DegP as well as other periplasmic chaperones such as SurA and FkpA acting alone or in concert. Nonetheless, we hypothesize that glycosylation of these and/or other proteins might diminish their intrinsic functionality or abundance. In this model, lack of glycosylation would thus enhance their activity or levels to foster the terminal steps of the T2SS. Confirmation of this model requires a more thorough characterization of the *V. cholerae* glycoproteome and the effects of O-linked protein glycosylation on glycoprotein abundance and functionality. Notably, **pgl**_{Vc} is located upstream of the msh-operon encoding a surface pilus termed the mannose-sensitive hemagglutinin (MSHA). Although not identified along our study, it is tempting to speculate that O-glycosylation also targets components of MSHA or affect their secretion as O-glycosylation of pili components has been demonstrated for other bacteria (Aas et al., 2007; Egge-Jacobsen et al., 2011). Notably, MSHA is important for the first steps in biofilm formation by facilitating adherence to abiotic and chitinous surfaces as well as for the pharyngeal colonization of nematodes (Watnick et al., 1999; Chiavelli et al., 2001; Meibom et al., 2004; List et al., 2018). Thus, it cannot be excluded that the biofilm phenotypes of **pgl**_{Vc} and **rbmD** mutants observed herein are at least partially associated to differential MSHA functionality.

An intriguing aspect of this work relates to role of RbmD whose mutation phenocopies that of the **pgl**_{Vc} null mutant. To our knowledge, this is one of the first reports of these phenotypes associated with the failure to express this protein in *V. cholerae* WT. Notably, *V. cholerae* can undergo phenotypic variation generating smooth and rugose variants. The later shows elevated VPS-production, increased biofilm formation and enhanced resistance to several environmental stresses. While the work herein is based on a smooth *V. cholerae* isolate, Fong and Yildiz (2007) analyzed biofilm formation of a **rbmD**-mutant in a rugose variant. In the rugose background deletion of **rbmD** resulted in less compact biofilms and larger cell aggregates compared to the parental rugose variant. However, direct comparison of the results from our study and the work by Fong et al. is difficult, due to the differential phenotypic background and different *V. cholerae* isolates used. Based on the results presented herein, we surmise that RbmD impacts on the same pathway as that modulated by PgL_{Vc} in a non-redundant fashion. Besides similarity PgL_{Vc}, RbmD is also related to WaaL-type O-antigen ligases and members of the SEDS (shape, elongation, division and sporulation) protein family members that serve as bacterial cell wall polymers (Meeske et al., 2016). The potential for RbmD to have a role other than in protein glycosylation is intriguing especially given its inability to use the UndPP-diNACBac donor that can be used by virtually all O-OTases tested to date.

Another obvious point of interest relates to the nature of the endogenous glycan used by PgL_{Vc} and potentially RbmD. Computational analyses of the Sequence Similarity DataBase revealed no obvious similarities of the entire gonococcal **pgl** gene cluster in *V. cholerae*. However, similarity searches for orthologs to the individual gonococcal **pgl** genes revealed several hits in two gene clusters (VC0240–VC0263 and VC917–VC927) encoding enzymes for the O-antigen biosynthesis and the *Vibrio* exopolsaccharide (VPS), respectively. Deletion of the **vps**-1 gene cluster (but not that for the O-antigen biosynthesis) phenocopied **pgl**_{Vc} and **rbmD** mutants in the enhanced secretion of both CT and RbmA, providing first evidence that the diverse sugars present in the VPS [i.e., α-GulNaAcA Gly, α-Glc p, β-Glc p, α-Gal p, and α-D-Glc Na c (Yildiz et al., 2014)] or combinations thereof may serve as at least one source for the transferred O-glycan in *V. cholerae*. Notably, the VPS oligosaccharide is notoriously recalcitrant to biochemical characterization as it is bound to a yet unidentified component, which gives it high viscosity and which would theoretically complicate its structural characterization by MS as a post-translational protein modification.

Further insights into the mechanisms operating here will undoubtedly require elucidation of the cognate glycoform utilized by PgL_{Vc} and potentially by RbmD. Along this line we tried to identify the glycan present in *V. cholerae*. Briefly, His-tagged DegP was isolated in absence and presence of PgL_{Vc} and/or RbmD and subjected to MS analysis, but no defined modification could be identified. It should be emphasized that detection and identification of a glycan with unknown composition is not straightforward and requires substantial expertise and instrumentation. A series of unfavorable limitations decreased the likelihood of glycan identification: (i) Overexpression of periplasmic His-tagged DegP reduced fitness of *V. cholerae*. Thus, the yield of purified protein was relatively low. (ii) The chemical composition of the natural glycan is not only unknown, but might exhibit heterogeneity. Thus, the glycosylated population may not represent a single peak in the MS. (iii) O-glycosylation might render protein stability.
ultimately impact on the efficacy of the on these limitations the identification of the cognate glycan seems should be noted, that such low modification rates could massively affect overall DegP chaperone activity in the periplasm as DegP monomers assemble from trimers and hexamers to multimeric polyhedral cages with up to 24 subunits (Krojer et al., 2002; Kim and Sauer, 2014). However, such modification rates further reduce the chances for identification of the natural glycan. Based on these limitations the identification of the cognate glycan seems to be a challenging task for the future.

In summary, we herein reveal multiple gene interactions that ultimately impact on the efficacy of the V. cholerae T2SS at the level of translocation of substrates from the periplasm to the extracellular milieu. T2SS modulation affects several stages of the pathogens’ lifecycle as highlighted by the diverse phenotypes reported herein. It is tempting to speculate that O-glycosylation provides a post-translational mechanism allowing bacterial cells to accumulate effector proteins, like RbmA, chitinases or CT in the periplasm to be readily available for release when the cells settle down on the new substratum for biofilm formation or at the colonization site in the gastrointestinal tract. Thus, O-glycosylation via PglL Vc and possibly RbmD could represent a fine-tuned feedback mechanism controlling release of T2SS effectors by modulation of secretion efficacy.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

REFERENCES

Aas, F. E., Vik, A., Vedde, J., Koomey, M., and Egge-Jacobsen, W. (2007). Neisseria gonorrhoeae O-linked pilin glycosylation: functional analyses define both the biosynthetic pathway and glycan structure. Mol. Microbiol. 65, 607–624. doi: 10.1111/j.1365-2958.2007.05806.x

Absalon, C., Van Dellen, K., and Watnick, P. I. (2011). A communal bacterial adhesin anchors biofilm and bystander cells to surfaces. PLoS Pathog. 7:e1002210. doi: 10.1371/journal.ppat.1002210

Almagro-Moreno, S., Kim, T. K., Skorupski, K., and Taylor, R. K. (2015). Proteolysis of virulence regulator ToxR is associated with entry of Vibrio cholerae into a dormant state. PLoS Genet. 11:e1005145. doi: 10.1371/journal.pgen.1005145

Altindis, E., Fu, Y., and Mekalanos, J. J. (2014). Proteomic analysis of Vibrio cholerae outer membrane vesicles. Proc. Natl. Acad. Sci. U.S.A. 111, E1548–E1556. doi: 10.1073/pnas.1403683111

Anonsen, J. H., Vik, A., Borud, B., Vibiriene, R., Aas, F. E., Kidd, S. W., et al. (2015). Characterization of a unique tetrasaccharide and distinct glycoproteome in the O-linked protein glycosylation system of Neisseria elongata subsp. glycolytica. J. Bacteriol. 198, 256–267. doi: 10.1128/JB.00383-11

Anonsen, J. H., Vik, A., Egge-Jacobsen, W., and Koomey, M. (2012). An extended spectrum of target proteins and modification sites in the general O-linked protein glycosylation system in Neisseria gonorrhoeae. J. Proteome Res. 11, 5781–5793. doi: 10.1021/pr300584x

Berku, V., Fong, J. C., Dempsey, G. T., Develioglu, O. N., Zhuang, X., Lhiphardt, J., et al. (2012). Molecular architecture and assembly principles of Vibrio cholerae biofilms. Science 337, 236–239. doi: 10.1126/science.1222981

AUTHOR CONTRIBUTIONS

DV, FM, KP, JA, MF, JR, MK, and SS designed the study. DV, FM, KP, DL, JA, LL, L-MM, TK, MT, AS, FZ, RB-G, JR, MK, and SS performed the experiments and analysis. DV, FM, KP, JA, FZ, MF, JR, MK, and SS contributed to the discussion and data evaluation. DV, FM, JR, MK, and SS wrote the manuscript.

FUNDING

This work was supported by the Austrian Science Fund (FWF) grants: W901 (DK Molecular Enzymology) to DV, FM, KP, FZ, and SS as well as P27654 to SS and KLI425 to RB-G. MK and JA were supported by the University of Oslo and its associated Center for Integrative Microbial Evolution. AS was supported by the Doctoral Academy Graz.

ACKNOWLEDGMENTS

We are grateful to M. Aebi and colleagues for the opportunity to test alternative bacterial glycosylation systems.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02780/full#supplementary-material

Chang, A. C., and Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134, 1141–1156.

Chiavelli, D. A., Marsh, J. W., and Taylor, R. K. (2001). The mannose-sensitive hemagglutinin of Vibrio cholerae promotes adherence to zooplankton. Appl. Environ. Microbiol. 67, 3220–3225. doi: 10.1128/aem.67.7.3220-3225.2001

Childers, B. M., and Klose, K. E. (2007). Regulation of virulence in Vibrio cholerae: the ToxR regulon. Future Microbiol. 2, 335–344. doi: 10.2217/17460913.2.3.335

Donnenberg, M. S., and Kaper, J. B. (1991). Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector. Infect. Immun. 59, 4310–4317.

Egge-Jacobsen, W., Salomonsson, E. N., Aas, F. E., Forslund, A. L., Winton-Larsen, H. C., Maier, J., et al. (2011). O-linked glycosylation of the PilA pilin protein of Francisella tularensis: identification of the endogenous protein-targeting oligosaccharyltransferase and characterization of the native oligosaccharide. J. Bacteriol. 193, 5487–5497. doi: 10.1128/JB.00383-11

Faridmoayer, A., Fentabil, M. A., Mills, D. C., Klassen, J. S., and Feldman, M. F. (2007). Functional characterization of bacterial oligosaccharyltransferases involved in O-linked protein glycosylation. J. Bacteriol. 189, 8088–8098. doi: 10.1128/JB.01318-13

Feldman, M. F., Wacker, M., Hernandez, M., Hitchen, P. G., Marolda, C. L., Kowari, M., et al. (2005). Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 102, 3016–3021. doi: 10.1073/pnas.0500044102

Fong, J. C., Karplus, K., Schoolnik, G. K., and Yildiz, F. H. (2006). Identification and characterization of RbmA, a novel protein required for the development of rugose colony morphology and biofilm structure in Vibrio cholerae. J. Bacteriol. 188, 1049–1059. doi: 10.1128/jb.188.3.1049-1059.2006

Frontiers in Microbiology | www.frontiersin.org
17 December 2019 | Volume 10 | Article 2780
Vorkapic et al.
Fong, J. C., and Yildiz, F. H. (2007). The rmbBCDEF gene cluster modulates development of rugose colony morphology and biofilm formation in Vibrio cholerae. J. Bacteriol. 189, 2319–2330. doi: 10.1128/jb.01569-06

Gebhart, C., Jelmini, M. V., Reiz, B., Price, N. L., Aas, F. E., Koomey, M., et al. (2012). Characterization of exogenous bacterial oligosaccharide transferases in Escherichia coli reveals the potential for O-linked protein glycosylation in Vibrio cholerae and Burkholderia thailandensis. Glycobiology 22, 962–974. doi: 10.1093/glycob/wcs059

Hartley, M. D., Morrison, M. J., Aas, F. E., Borud, B., Koomey, M., and Imperiali, B. (2011). Biochemical characterization of the O-linked glycosylation pathway in Neisseria gonorrhoeae responsible for biosynthesis of protein glycans containing N,N′-diacytethylbilisammonium. Biochemistry 50, 4936–4948. doi: 10.1021/bi2003372

Hava, D. L., Hemsley, C. J., and Camilli, A. (2003). Transcriptional regulation in the Streptococcus pneumoniae rfa-pathogenicity island by RlrA. J. Bacteriol. 185, 413–421. doi: 10.1128/jb.185.4.413-421.2003

Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Erbsoll, B. K., et al. (2000). Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology 146( Pt 10), 2395–2407. doi: 10.1099/00221287-146-10-2395

Iwanaga, M., and Yamamoto, K. (1985). New medium for the production of cholera toxin by Vibrio cholerae O1 biotype El Tor. J. Clin. Microbiol. 22, 405–408.

Iwanaga, M., Yamamoto, K., Higa, N., Ichinose, Y., Nakasone, N., and Tanabe, M. (1986). Culture Conditions for Stimulating Cholera Toxin Production by Vibrio cholerae O1 El Tor. Microbiol. Immunol. 30, 1075–1083. doi: 10.1111/j.1344-4246.1986.tb03073.x

Iwaskiw, J. A., Vozza, N. F., Kinsella, R. L., and Feldman, M. F. (2013). Pour some sugar on it: the expanding world of bacterial protein O-linked glycosylation. Mol. Microbiol. 89, 14–28. doi: 10.1111/mmi.12265

Johnson, T. L., Fong, J. C., Rule, C., Rogers, A., Yildiz, F. H., and Sandkvist, M. (2014). The Type II secretion system delivers matrix proteins for biofilm formation by Vibrio cholerae. J. Bacteriol. 196, 4425–4425. doi: 10.1128/JB.01944-1914

Kang, D., Gho, Y. S., Suh, M., and Kang, C. (2002). Highly sensitive and fast protein detection with coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bull. Kor. Chem. Soc. 23, 1511–1512. doi: 10.5012/bkcs.2002.23.11.1511

Kim, S., and Sauer, R. T. (2014). Distinct regulatory mechanisms balance DegP proteolysis to maintain cellular fitness during heat stress. Genes Dev. 28, 902–911. doi: 10.1101/gad.238394.114

Kolter, R., Inouzuka, M., and Helinski, D. R. (1978). Trans-complementation-dependent replication of a low molecular weight origin fragment from plasmid R6K. Cell 15, 1199–1208. doi: 10.1016/0008-6210(78)90046-6

Kroger, T., Garrido-Franco, M., Huber, R., Ehrmann, M., and Clausen, T. (2002). Crystal structure of DegP (HtrA) reveals a new protease-chaperone mechanism. Nature 416, 455–459. doi: 10.1038/4164455a

Link, A. J., and LaBaer, J. (2011). Trichloroacetic acid (TCA) precipitation of proteins. Cold Spring Harb. Protoc. 2011, 993–994. doi: 10.1101/pdb.prot5651

List, C., Gutatsch, A., Radler, C., Cakar, F., Zingl, F. G., Schild-Prufert, K., et al. (2018). Genes activated by Vibrio cholerae upon exposure to Caenorhabditis elegans reveal the mannose-sensitive hemagglutinin to be essential for colonization. mSphere 3, e00238-18. doi: 10.1128/mSphereDirect.00238-18

Manning, P. A., Stroehler, U. H., Karageorgos, L. E., and Morona, R. (1995). Putative O-antigen transport genes within the rfb region of Vibrio cholerae O1 are homologous to those for capsule transport. Gene 158, 1–7. doi: 10.1016/0378-1119(95)00124-0

Manoil, C. (1991). Analysis of membrane protein topology using alkaline phosphatase and beta-galactosidase gene fusions. Methods Cell Biol. 34, 61–75. doi: 10.1016/s0091-679x(08)61676-3

Matson, J. S., and DiRita, V. J. (2005). Degradation of the membrane-localized virulence activator TcpP by the YaeL protease in Vibrio cholerae. Proc. Natl. Acad. Sci. U.S.A. 102, 16403–16408. doi: 10.1073/pnas.0505818102

Meeks, A. J., Riley, E. P., Robins, W. P., Uehara, T., Mekalanos, J. J., Kahne, D., et al. (2016). SEDS proteins are a widespread family of bacterial cell wall polymerases. Nature 537, 634–638. doi: 10.1038/nature19331

Meibom, K. L., Li, X. B., Nielsen, A. T., Wu, C. Y., Roseman, S., and Schoolnik, G. K. (2004). The Vibrio cholerae chitin utilization program. Proc. Natl. Acad. Sci. U.S.A. 101, 2524–2529. doi: 10.1073/pnas.0308707101
in vivo-induced cyclic Di-GMP phosphodiesterase CdpA. *Infect. Immun.* 76, 1617–1627. doi: 10.1128/IAI.01337-07

Vik, A., Aas, F. E., Anonsen, J. H., Bilsborough, S., Schneider, A., Egge-Jacobsen, W., et al. (2009). Broad spectrum O-linked protein glycosylation in the human pathogen *Neisseria gonorrhoeae*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4447–4452. doi: 10.1073/pnas.0809504106

Vorregaard, M. (2008). **Comstat2 - a Modern 3D Image Analysis Environment for Biofilms.** Lyngby: Technical University of Denmark.

Watnick, P. I., Fullner, K. J., and Kolter, R. (1999). A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. *J. Bacteriol.* 181, 3606–3609.

Wood, W. I. (1976). Tables for the preparation of ammonium sulfate solutions. *Anal. Biochem.* 73, 250–257. doi: 10.1016/0003-2697(76)90165-2

Wurm, P., Tutz, S., Mutsam, B., Vorkapic, D., Heyne, B., Grabner, C., et al. (2017). Stringent factor and proteolysis control of sigma factor RpoS expression in *Vibrio cholerae*. *Int. J. Med. Microbiol.* 307, 154–165. doi: 10.1016/j.ijmm.2017.01.006

Yildiz, F., Fong, J., Sadovskaya, I., Grard, T., and Vinogradov, E. (2014). Structural characterization of the extracellular polysaccharide from *Vibrio cholerae* O1 El-Tor. *PLoS One* 9:e86751. doi: 10.1371/journal.pone.0086751

Yildiz, F. H., and Schoolnik, G. K. (1999). *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4028–4033. doi: 10.1073/pnas.96.7.4028

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Vorkapic, Mitterer, Presler, Leitner, Anonsen, Liesinger, Mauherhofer, Kuehnast, Toeglhofer, Schulze, Zingl, Feldman, Reidl, Birner-Gruenberger, Koomey and Schild. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.