Biosynthesis of glucosaminyl phosphatidylglycerol in *Pseudomonas aeruginosa*

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Abstract

Glucosaminyl phosphatidylglycerol (GlcN-PG) was first identified in bacteria in the 1960s and was recently reported in *Pseudomonas aeruginosa*. Despite the important implications in altering membrane charge (by the modification of anionic PG with cationic glucosamine), the biosynthesis and functions of GlcN-PG have remained uncharacterized. Using bioinformatic and lipidomic analysis, we identified a 3-gene operon, renamed as *gpgSDF*, that is responsible for the biosynthesis and potential transport of GlcN-PG in *P. aeruginosa*: *gpgS* encodes a novel glycotransferase that is responsible for the modification of phosphatidylglycerol (PG) with *N*-acetylglucosamine (GlcNAc) to produce GlcNAc-PG, and *gpgD* encodes a novel deacetylase that removes the acetyl group from GlcNAc-PG to produce GlcN-PG. The third gene in the operon, *gpgF*, is predicated to encode a flippase whose activity remains to be experimentally verified. As expected, the heterologous expression of the *gpgSDF* operon in *Escherichia coli* resulted in production of both GlcNAc-PG and GlcN-PG. The identification of the biosynthetic genes of GlcN-PG paves the way for the investigation of its biological and pathological functions, which has significant implications in our understanding of the unique membrane physiology, pathogenesis and antimicrobial resistance of *P. aeruginosa.*

Introduction

Pseudomonas aeruginosa is a major Gram-negative pathogen widely responsible for pneumonia, surgical infection, bacteremia, and other life-threatening infections in immunocompromised individuals with underlying diseases such as cystic fibrosis (CF) (1, 2) and cancer (3). *P. aeruginosa* possesses an unusual ability to colonize diverse environments and rapidly develop antibiotic resistance. Multi-drug-resistant (MDR) and extreme drug-resistant (XDR) high-risk strains are widespread in healthcare settings, making the treatment of certain *P. aeruginosa* infections extremely challenging (4-6).

An important mechanism for bacteria to cope with antimicrobial stresses and develop antibiotic resistance is by altering the charge of their membrane lipids. A highly conserved and perhaps most studied enzyme responsible for modifying lipids in bacteria is MprF (multiple peptide resistance factor), which modifies lipids via the transfer of amino acids from charged tRNAs to the head groups of anionic phosphatidylglycerol (PG) and cardiolipin (CL) (7, 8), as well as to neutral glycolipids (9, 10). *P. aeruginosa* encodes MprF and produces alanine-modified PG (11). Additionally in *P. aeruginosa*, modifications of the phosphate groups of lipid A (the lipid anchor of lipopolysaccharide) with positively charged moieties such as aminoarabinose and phosphoethanolamine are critical for conferring resistance to cationic antimicrobial peptides and polymyxin (2, 12-16).

The modification of PG with cationic glucosamine, producing zwitterionic glucosaminyl phosphatidylglycerol (GlcN-PG), was recently reported in *P. aeruginosa* (17). Despite the obvious implications in altering membrane charge, and the fact that GlcN-PG was first observed in various bacteria in the 1960s (18-21), the biosynthesis and functions of GlcN-PG have remained uncharacterized.

In this study, by combining bioinformatic, genetic, and lipidomic approaches, we identified the biosynthetic genes required for GlcN-PG synthesis in *P. aeruginosa*. Using the PA14 strain as a model, we show that a 3-gene operon (consisting of PA14_56030, PA14_56040 and PA14_56050) is required for the synthesis and possible transport of GlcN-PG. We have thus renamed this operon as *gpgSDF* (GlcN-PG synthase, deacetylase and flippase). Since *gpgSDF* is conserved in all sequenced strains of *P. aeruginosa*, and many other bacteria, this work has significant implications for understanding the mechanisms of physiology and pathogenesis of *P. aeruginosa* as well as many other pathogenic bacteria.

Experimental Procedures

Bacterial Strains and Growth Conditions

The bacterial strains used in the study are described in Table 1. For lipidomic analysis, bacterial cells were grown in Luria Bertani (LB) broth (10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) at 37 °C with shaking at 225 rpm for ~20 hours. The optical density (OD) was measured in a disposable cuvette (Thermo Fisher Scientific, Waltham, MA) as absorption at 600 nm by a spectrophotometer (Thermo Scientific Genesys 30).

The *P. aeruginosa* PA14 MAR2xT7 transposon mutants carrying a gentamicin resistance cassette were previously reported (22) and were grown in LB broth containing 15 μg/ml gentamicin. *Escherichia coli* DH5*α* (Thermo Fisher Scientific) with the plasmid pGEM-T (Promega PR-A3600) was grown in LB broth containing 50 μg/ml ampicillin.

Plasmid Construction

The *gpgSDF* operon was amplified by PCR using specific primers (Table S1) and cloned into pGEM-T (23) by TA cloning per the manufacturer's instructions. The newly assembled pWO-GEM-T plasmid was confirmed through PCR reactions with T7 and SP6 and sequencing primer pairs with agarose gel electrophoresis analysis of products. The plasmid was transformed into *E. coli* DH5α and maintained by growth in the presence of 50 μg/ml of ampicillin.

Acidic Bligh-Dyer Lipid Extraction

The acidic Bligh-Dyer lipid extractions were performed as previously described (9, 24). Briefly, bacterial cells were grown in 20 mL of LB broth for ~20 hours; 19 mL of each culture was pelleted at 7000 rpm for 10 min. The cell pellet was washed twice with 1X phosphate buffer saline (PBS, Sigma-Aldrich, St. Louis, MO). Each cell pellet was resuspended in 0.8 mL of 1X PBS and transferred to a 17-mL glass tube with a Teflon-lined cap (Corning Pyrex™, VWR, Radnor, PA). Afterwards, 1 mL of chloroform and 2 mL of methanol were added to generate a single-phase Bligh-Dyer solution. This solution was incubated for 20 min at room temperature with intermittent mixing. After centrifugation at 1700 rpm for 10 min, the supernatant was transferred to a new glass tube, which was followed by the addition of 100 μl HCl (37%), 0.9 mL 1X PBS and 1 mL chloroform to generate a two-phase Bligh-Dyer solution. After mixing by a vortex, the solution was separated into two phases by centrifugation at 1700 rpm for 5 min at room temperature. The

lower phase was recovered and dried under a stream of nitrogen gas before being stored at −80˚C prior to lipidomic analysis.

Normal Phase LC/MS/MS Analysis

Lipidomic analysis by normal phase LC/MS/MS was described previously (9, 24). Briefly, normal phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 μm, 25 cm x 2.1 mm (Sigma-Aldrich, St. Louis, MO). Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v); mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v); mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The LC eluent (with a total flow rate of 300 μl/min) was introduced into the ESI source of a high resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). The instrumental settings for negative ion ESI/MS and MS/MS analysis of lipid species were as follows: IS = -4500 V; CUR = 20 psi; GSI = 20 psi; DP = -55 V; and FP = -150 V. The MS/MS analysis used nitrogen as the collision gas. Analyst TF1.5 software (Sciex, Framingham, MA) was used for data analysis.

Results

Identification of GlcN-PG in *P. aeruginosa* by LC/MS/MS

The lipid extract of *P. aeruginosa* strain PA14 cells was analyzed by normal phase LC/MS in both negative ion and positive ion modes. As shown by the negative total ion chromatogram (TIC) (Fig. 1A), the major detected lipids include alkyl quinolones (AQs), diacylglycerol (DAG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidylcholine (PC). These common lipids and their biosynthesis have been extensively studied in *P. aeruginosa* and other bacteria (25-27).

An unknown lipid with a major $[M-H]^-$ molecular ion at *m/z* 908.5 is observed at ~18.5 min (Fig.1B). Further analysis by tandem mass spectrometry (MS/MS) (Fig. 1C) and exact mass

measurement allowed us to propose this unknown lipid to be aminohexosyl-PG. A literature search found that our high-resolution MS measurement and MS/MS data are consistent with those of GlcN-PG recently reported in *P. aeruginosa* (17). GlcN-PG was previously found in other bacteria (18, 19, 21, 28). To our knowledge, there have been no reports on the studies of the biosynthesis and functions of GlcN-PG in *P. aeruginosa* or any other bacteria.

Identification of the biosynthetic genes of GlcN-PG

Enzymatically, GlcN-PG is most likely produced from PG via the covalent modification with GlcN. Indeed, this was supported by the observation that during the pellicle growth of *P. aeruginosa*, the increase of GlcN-PG was accompanied by the decrease of PG (17). Previously it was also observed in *Bacillus megaterium* that GlcN-PG was increased, while PG was decreased, when cells were grown in acidic conditions (28).

Given the biochemical mechanisms of other amino sugar modifications (29, 30), we hypothesize that GlcN-PG is likely produced from its immediate precursor, GlcNAc-PG, by *N*-deacetylation, an enzymatic process that is known to be involved in several biosynthetic pathways. For examples, LpxC is the UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase required for the biosynthesis of lipid A, the lipid anchor of lipopolysaccharide (LPS) in Gram-negative bacteria (31-33). Deacetylation mediated by carbohydrate esterase family 4 proteins (34) plays a vital role in exopolysaccharide processing in different bacterial species, including *P. aeruginosa*.

The potential involvement of deacetylation in the biosynthetic process of GlcN-PG prompted us to carefully examine the PA14 lipidomic data and identify a low level of GlcNAc-PG. The $[M-H]^-$ ion of GlcNAc-PG is observed at *m/z* 950.59, corresponding to the addition of an acetyl group (42 Da) to GlcN-PG (*m/z* 908.58). The detection of GlcNAc-PG provides an important clue for the existence of a deacetylase that converts GlcNAc-PG to GlcN-PG.

To search for the GlcNAc-PG deacetylase, we queried the PA14 genomic database and found six genes annotated as deacetylases. Among them, four have been well characterized with identified substrates, including LpxC (31, 32). However, the other two putative deacetylases, with locus ID PA14_44830 and PA14_56040 (*gpgD*), have not been experimentally characterized and their substrates have remained unknown.

To determine whether GlcNAc-PG is a substrate of PA14_44830 and/or PA14_56040, we performed lipidomic analysis of their respective transposon mutants. As shown in Fig. 2B, PA14_44830 mutant contains both GlcN-PG and GlcNAc-PG, with their relative levels similar to the wild-type (WT), suggesting that PA14_44830 is not a GlcNAc-PG deacetylase. By sharp contrast, the PA14_56040 (*gpgD*) mutant lacks GlcN-PG, but contains drastically elevated GlcNAc-PG (Fig. 2C), strongly supporting that GlcNAc-PG and GlcN-PG are the substrate and product of GpgD, respectively. The identification of the accumulated GlcNAc-PG (Fig. 3A) is further confirmed by MS/MS (Fig. 3B). The complete depletion of GlcN-PG in the PA14_56040 (*gpgD*) mutant also indicates that GpgD is the sole deacetylase responsible for the conversion of GlcNAc-PG to GlcN-PG.

PA14_56030 (*gpgS*) is adjacent to and in the same operon as *gpgD* and is predicted to be a glycosyltransferase. Its predicated function and genomic position strongly suggest that GpgS is likely the glycotransferase responsible for transferring the GlcNAc group onto PG to produce GlcNAc-PG. Indeed our lipidomic analysis shows that the PA14_56030 (*gpgS)*::Mar2xT7 mutant lacks both GlcNAc-PG and GlcN-PG (Fig. 2D).

Overall, the lipidomic analysis of the *gpgD* and *gpgS* transposon mutants reveals that GlcN-PG biosynthesis involves two steps: GpgS first transfers the GlcNAc group to PG to form GlcNAc-PG, which is then deacetylated by GpgD to produce GlcN-PG (Fig. 4). Although not experimentally verified, UDP-GlcNAc is likely the GlcNAc donor based on its utilization in other GlcNAc-modifications (35, 36).

PA14_56050 (renamed *gpgF*), predicted to be a flippase, is downstream of and co-transcribed under the same operon with *gpgS* and *gpgD*. To experimentally demonstrate its flippase function will require the development of an assay that can monitor the membrane translocation of GlcN-PG (37, 38).

The function of *gpgSDF* operon in PA14 is analogous to the function of *hexSDF* operon in *C. difficile*, which is involved in the synthesis of a novel glycolipid, aminohexosyl-hexosyldiradylglycerol (HNHDRG) (30, 39).

The *gpgSDF* operon is sufficient for the synthesis of GlcNAc-PG and GlcN-PG

E. coli contains PG as one of its most abundant phospholipids but does not have the *gpgSDF* operon and does not produce GlcNAc-PG or GlcN-PG (24, 27, 40). To confirm the

biochemical activities of the *gpgSDF* operon, the plasmid pWO-GEM-T with the entire *gpgSDF* operon of PA14 was constructed from pGEM-T vector and was then transformed into *E. coli* DH5α (Fig. 5A). Lipidomic analysis detected both GlcNAc-PG and GlcN-PG (Fig. 5B-D) in the transformed *E. coli*, confirming that the *gpgSDF* operon possesses the expected glycotransferase and deacetylase activities. The identification of GlcNA-PG and GlcN-PG produced in *E. coli* are supported by exact measurement and MS/MS. It is worth noting that the acyl chain profiles of the GlcNAc-PG and GlcN-PG molecular species heterogeneously produced in *E. coli* are slightly shorter (about 2 carbon atoms less) than those in *P. aeruginosa.*

GlcN-PG is much less abundant than GlcNAc-PG in the transformed *E. coli* (Fig. 5B-D), indicating the deacetylase activity from the expression of the whole *gpgSDF* operon is relatively weak, which may be optimized by expressing the GpgD deacetylase gene individually.

Identification of GlcN-PG in other *P. aeruginosa* strains

To assess the prevalence of GlcN-PG, we analyzed three other laboratory and clinical stains of *P. aeruginosa* and detected GlcN-PG in all of them (Fig. 6 and Table 2). The genomes of these strains all contain the *gpgSDF* operon (Table 2).

Discussion

Using a combination of lipidomic and bioinformatic approaches, we identified two biosynthetic enzymes that are involved in the GlcN-PG synthesis in *P. aeruginosa*. GpgS is a novel glycotransferase that catalyzes the modification of phosphatidylglycerol (PG) with *N*-acetylglucosamine (GlcNAc) to produce GlcNAc-PG, and GpgD is a novel deacetylase that removes the acetyl group of GlcNAc-PG to produce GlcN-PG. The functions of these enzymes were further confirmed by the synthesis of GlcNAc-G and GlcN-PG from the heterologous expression of the *gpgSDF* operon in *E. coli*.

Our bioinformatic analysis indicates that the *gpgSDF* operon is conserved in all sequenced strains of *P. aeruginosa* and the *gpgSDF*-like operon is potentially widespread in bacteria. Supporting this notion, our lipidomic analysis detected GlcN-PG in several other strains of *P. aeruginosa*.

The membrane distribution of GlcN-PG remains to be determined. The third gene in the *gpgSDF* operon, *gpgF*, is predicated to be a flippase, hinting that GlcN-PG is likely synthesized

in the cytoplasmic side of the inner membrane and then translocated to the periplasmic side of inner membrane. Thus, the biochemical activities mediated by the *gpgSDF* operon are analogous to MprF which catalyzes both Lys-PG synthesis and subsequent translocation to the outer leaflet of the bacterial membrane (8).

The identification of the GlcN-PG biosynthetic genes will enable the study of the biological and pathological functions of GlcN-PG in *P. aeruginosa*. Previously it was observed that GlcN-PG was dramatically increased under acidic conditions (28) and at stationary phase (17), suggesting the production of GlcN-PG is associated with coping with environmental and nutritional stresses.

The replacement of anionic PG with zwitterionic GlcN-PG is expected to lower the net negative charge of the bacterial membrane, and thus may help confer resistance to cationic antimicrobials. In gram-positive *C. difficile*, GlcN-modification of a glycolipid impacts resistance to cationic antibiotics including daptomycin and bacitracin (30). It is plausible that GlcN-PG plays a similar role in defending the cationic compounds that are toxic to *P. aeruginosa*.

Remarkably, the lipidome of *P. aeruginosa* is more diverse than *E. coli* which has been the model organism for studying lipid composition and metabolism in Gram-negative bacteria. In particular, *P. aeruginosa* contains a multitude of zwitterionic phospholipids, including GlcN-PG, while *E. coli* has only one zwitterionic phospholipid (PE) in addition to two major anionic phospholipids (PG and CL) (24, 27, 40) (Fig. 7). The lipid diversity of *P. aeruginosa* reflects its relatively large genome size (41, 42) and likely contributes to its unusual metabolic flexibility and membrane adaptability.

The elucidation of the functions of GlcN-PG will help shed light on the biological significance of the redundancy of zwitterionic phospholipids in *P. aeruginosa* and is important for fully understanding the molecular mechanisms underlying the unique membrane properties, the ability in colonization in diverse environments and high propensity in developing antibiotic resistance of *P. aeruginosa*.

Acknowledgements

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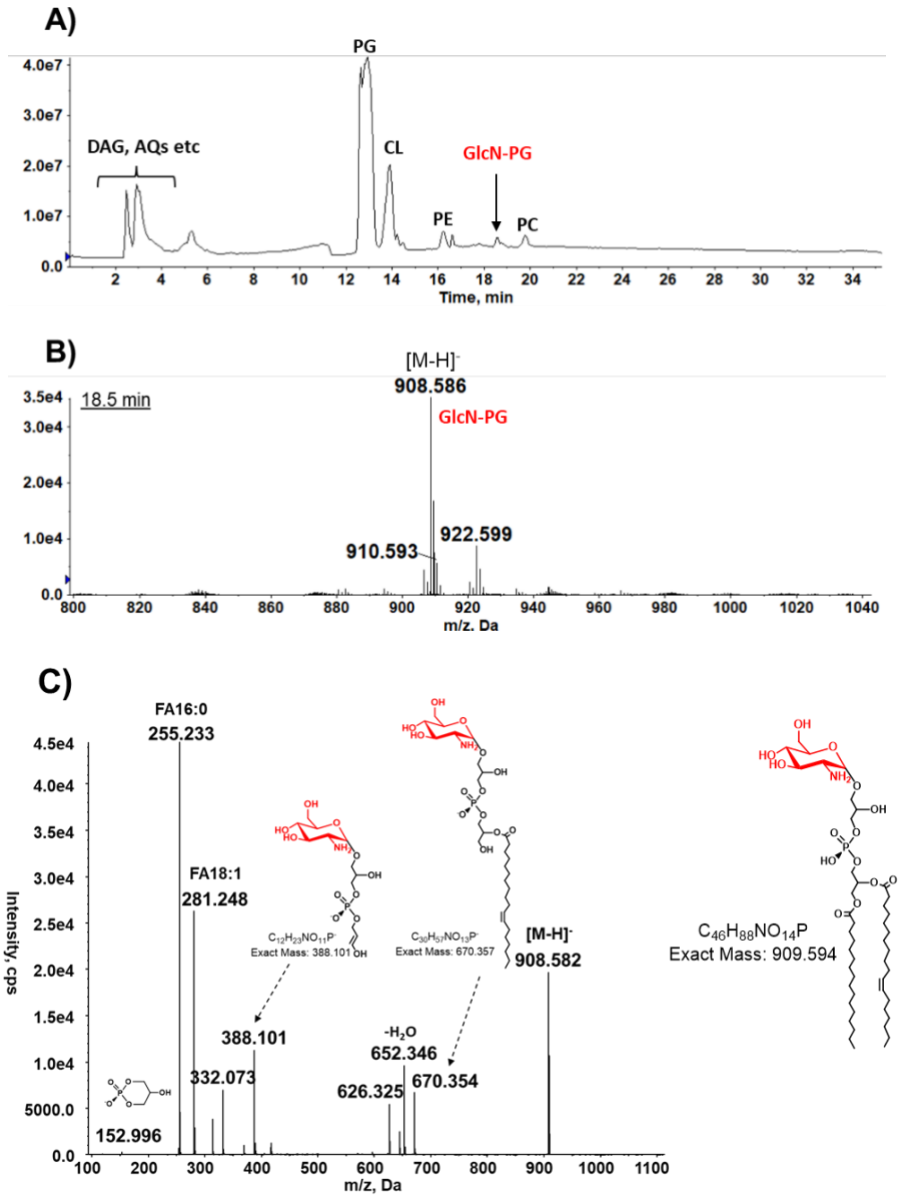

Figure 1. Identification of GlcN-PG in *P. aeruginosa* PA14 by LC/MS/MS

The lipid extract of PA14 strain is analyzed by normal phase LC/MS/MS, with GlcN-PG being identified by exact mass measurement and MS/MS. A) Negative total ion chromatogram (TIC) of normal phase LC/MS of the lipid extract of *P. aeruginosa* strain PA14. B) Negative ion ESI mass spectrum (at ~18.5 min) showing the $[M-H]^-$ ions of GlcN-PG. The observed exact mass (908.586) matches with the predicted exact mass (908.587) for the $[M-H]^-$ ion of GlcN-PG (16:0/18:1). C) MS/MS of $[M-H]^-$ ion at *m/z* 908.5 produces fragment ions consistent with GlcN-PG (16:0/18:1) whose chemical structure is shown.

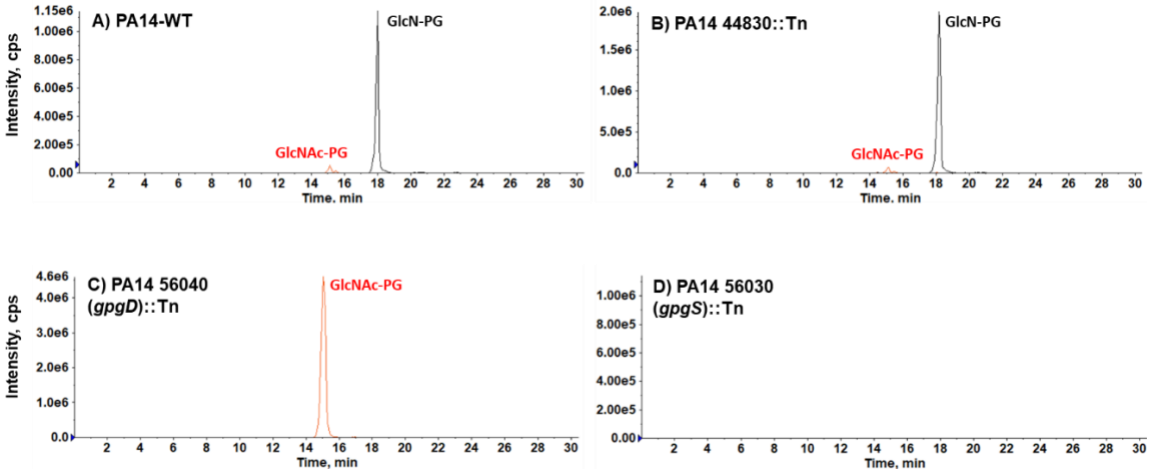

Figure 2. PA14_56030 (*gpgS*) and PA14_56040 (*gpgD*) genes are required for the synthesis of GlcNAc-PG and GlcN-PG in *P. aeruginosa*.

LC/MS analysis of the transposon mutants of PA14_56030 (*gpgS*, putative glycotransferase) and PA14_56040 (*gpgD*, putative deacetylase) confirm that they are responsible for the synthesis of GlcNAc-PG and GlcN-PG in *P. aeruginosa*, respectively. A, B) GlcN-PG and a low level of GlcNAc-PG are present in the wild-type PA14 and PA14_44830::Tn mutant. C) GlcN-PG is depleted, and GlcNAc-PG is drastically accumulated in the PA14_56040::Tn mutant. D) Both GlcN-PG and GlcNAc-PG are absent in the PA14_56030::Tn mutant.

Shown are the extracted ion chromatograms (EICs) of NPLC/MS in the negative ion mode.

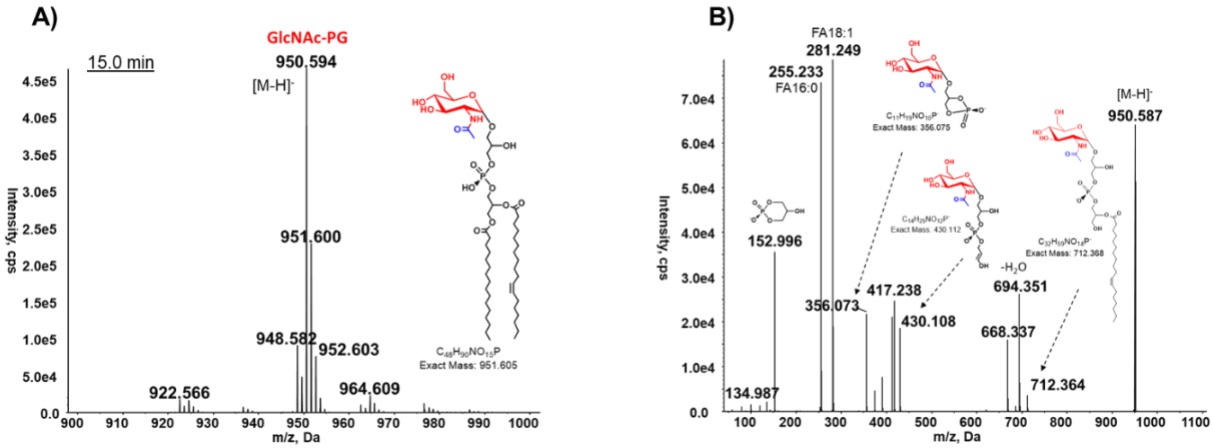

Figure 3. MS analysis of GlcNAc-PG accumulated in the PA14_56040 (*gpgD*)::Tn mutant. A) Negative ion mass spectrum of $[M-H]^-$ ion at *m/z* 950.6 for GlcNAc-PG (16:0/18:1). B) MS/MS of $[M-H]^-$ ion at *m/z* 950.6 produces fragment ions consistent with GlcNAc-PG (16:0/18:1)

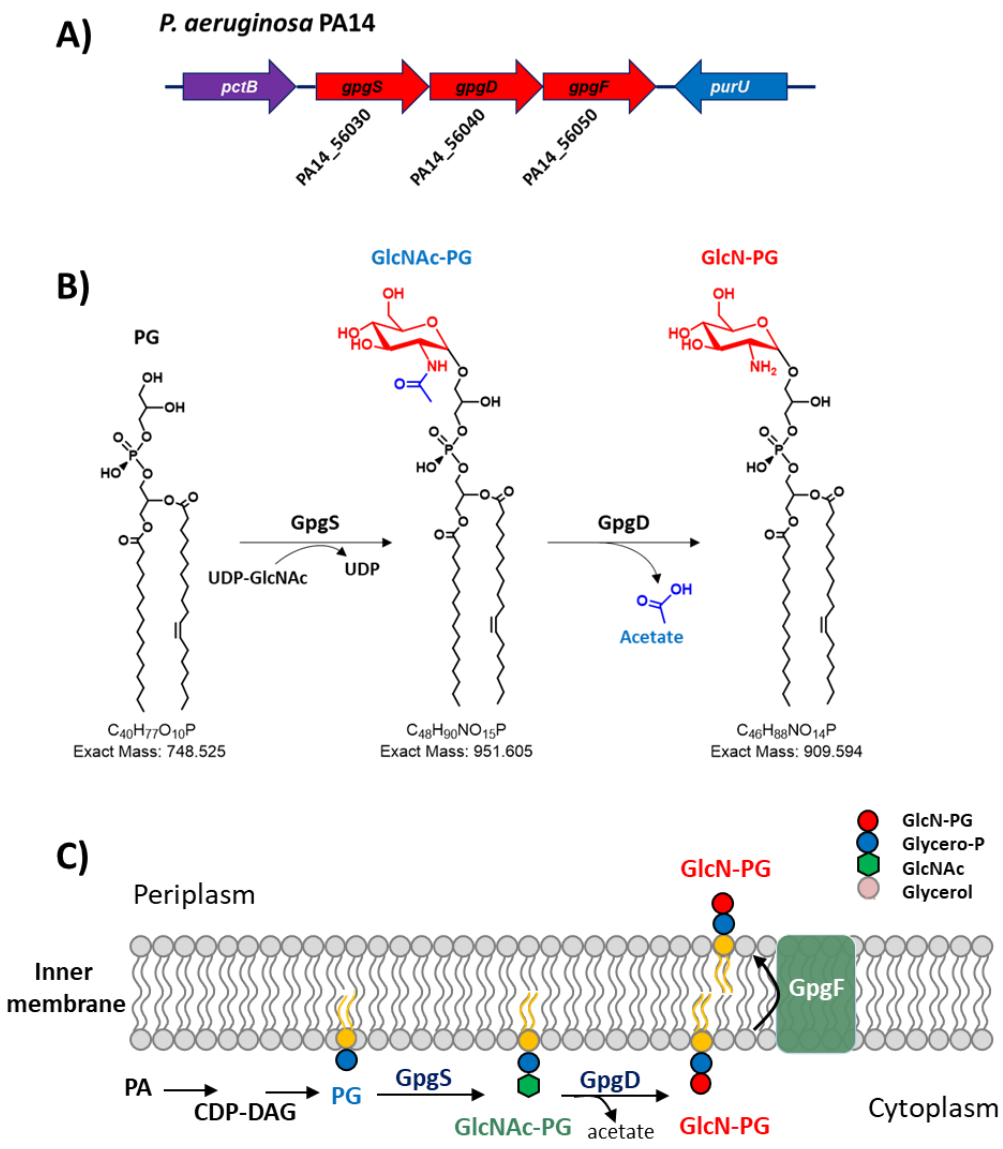

Figure 4. Proposed biosynthesis and translocation of GlcN-PG in *P. aeruginosa*.

A) Diagram of the *gpgSDF* operon and its neighboring genes in *P. aeruginosa* PA14. B) The biosynthesis of GlcN-PG from PG involves two enzymatic steps. First, PG is modified by GlcNAc by a glycosyltransferase (PA14_56030, GpgS), presumably using UDP-GlcNAc as the sugar donor. Second, GlcNAc-PG is de-acetylated by a deacetylase (PA14_56040, GpsD) to produce GlcN-PG. C) Cartoon illustration of the proposed biosynthesis and membrane translocation of GlcN-PG in *P. aeruginosa*.

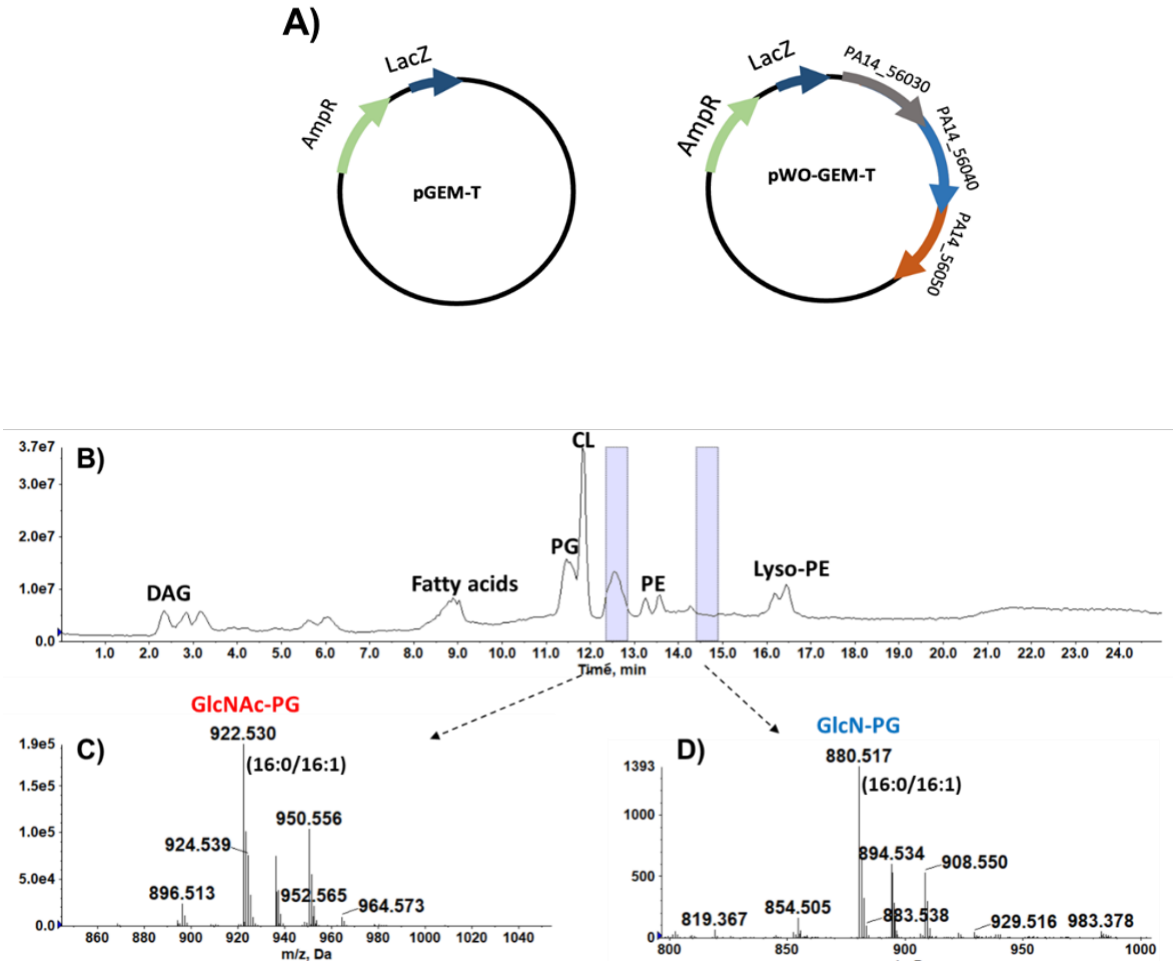

Figure 5. The heterologous expression of the *gpgSDF* operon of PA14 strain produces GlcNAc-PG and GlcN-PG in *E. coli*.

A) The plasmid pWO-GEM-T with the *gpgSDF* operon of PA14 was constructed from pGEM-T vector and was then transformed into *E. coli* DH5α. B) Total ion chromatogram of the normal phase LC/MS in the negative ion mode of the lipid extract of *E. coli* expressing the pWO-GMT-T. C) Negative ion mass spectrum of the $[M-H]^-$ ions of GlcNAc-PG species (appearing at ~12.5-13.0 min). D) Negative ion ass spectrum of the $[M-H]^-$ ions of GlcN-PG species (appearing at ~ 14.5-15.0 min). The identification of GlcNAc-PG (16:0/16:1) and GlcN-PG (16:0/16:1) is confirmed by MS/MS.

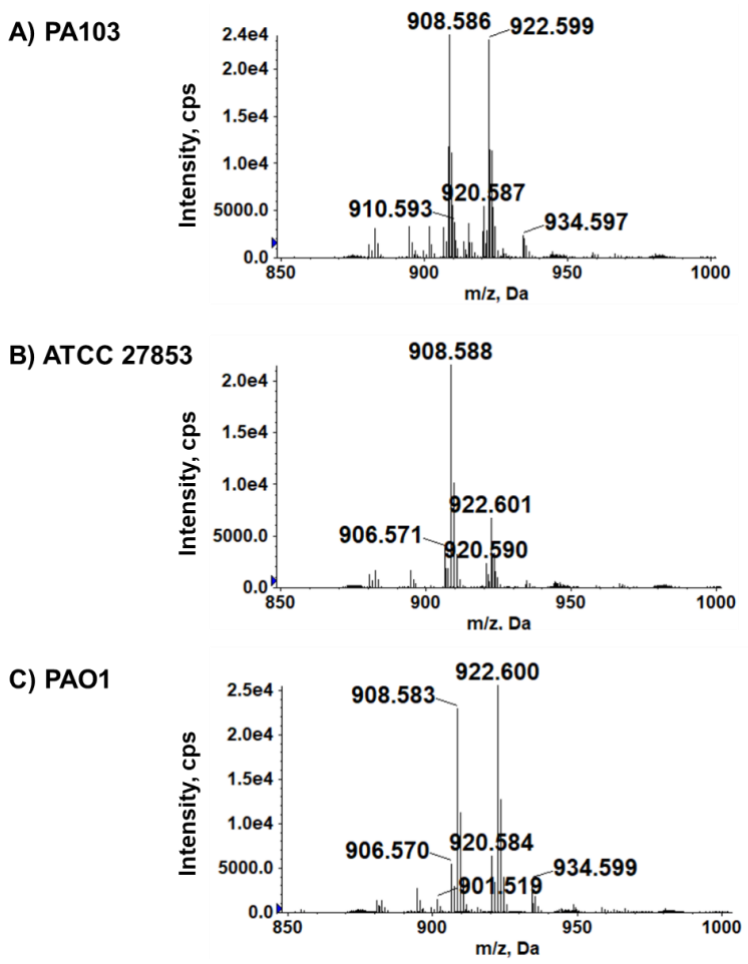

Figure 6. Detection of GlcN-PG in various *P. aeruginosa* laboratory and clinical strains by LC/MS.

Shown are the negative ion mass spectra of the $[M-H]^-$ ions of GlcN-PG species in A) PA103, B) ATCC 27853, C) PAO1 (Table 2). The identification of GlcN-PG molecular species is confirmed by exact mass measurement and MS/MS.

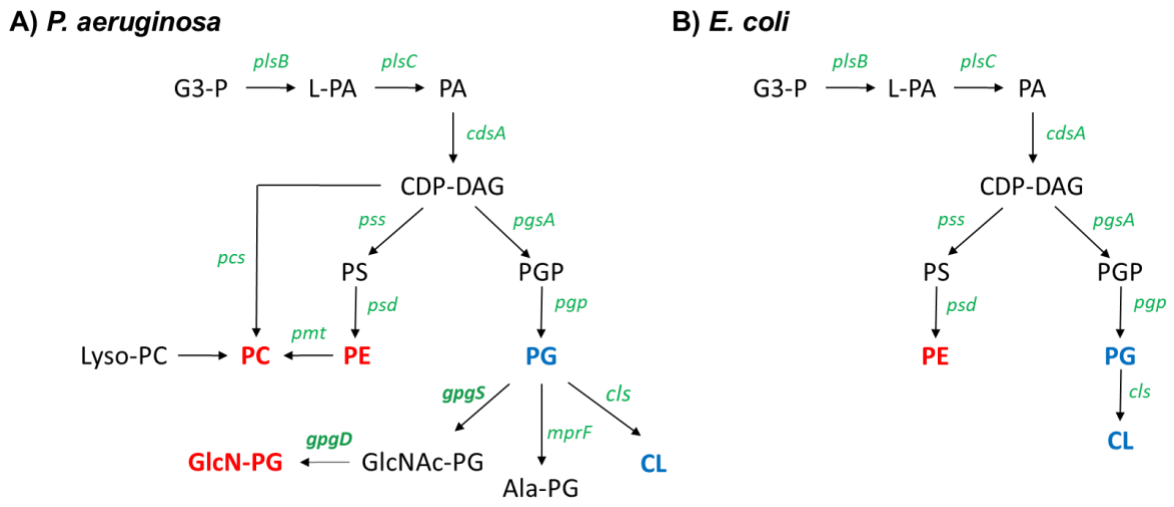

Figure 7. Comparison of phospholipid biosynthetic pathways in *P. aeruginosa* and *E. coli.*

P. aeruginosa possesses more complex phospholipids than *E coli*. Major zwitterionic phospholipids (red) and anionic phospholipids (blue) are in bold fonts. Their biosynthetic genes are green. The synthesis of GlcN-PG from PG in *P. aeruginosa* (the subject of this study) consists of two enzymatic steps catalyzed by GpgS and GpgD.

Abbreviations: G3-P, glycerol-3-phosphate; L-PA, lyso-phosphatidic acid; PA, phosphatidic acid; CDP-DAG, CDP-diacylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PGP, phosphatidylglycerol-3-P; PG, phosphatidylglycerol; Ala-PG, alanyl-phosphatidylglycerol; CL, cardiolipin.

Table 1. Bacterial Stains and Plasmid Used in This Study

Species and strains	Description	Source or reference
P. aeruginosa PAO1	Wild type (first isolated in 1954 from a wound in Melbourne, Australia)	(43, 44)
PA14	Wild type (first isolated in early 1970s from the blood of a burn patient at Mercy Hospital in Pittsburg, PA, USA)	(22)
PA103 (ATCC 29260)	Wild type	(45)
ATCC 27853_NP446	Wild type (isolated in 1971 from a blood specimen in Peter Bent Brigham Hospital, Boston, USA)	(46)
PA14_*56030::*Mar2xT7	PA14 with Mar2xT7 Tn insertion at nucleotide (nt) position 896 of 1116 nt PA14_56030 locus	(22)
PA14_*56040::*Mar2xT7	PA14 with Mar2xT7 Tn insertion at nt position 226 of 762 nt PA14_56030 locus	(22)
E. coli DH5α	Cloning host strain	Thermo Fisher Scientific
DH5α (pWO-GEM-T)	pWO-GEM-T with *gpgSDF* operon	This work

Table 2. Genomic and lipidomic analysis of GlcN-PG in *P. aeruginosa* stains

Strains	***gpgSDF* operon (Accession numbers shown for each)**			**GlcN-PG**
	gpgS	*gpgD*	*gpgF*	
PAO1	PA4311 (NP_253001.1)	PA4312 (NP_253002.1)	PA4313 (NP_253003.1)	✓
PA14	PA14_56030 (YP_792656.1)	PA14_56040 (YP_792657.1)	PA14_56050 (YP_792658.1)	✓
PA103	PA103_5903 (WP_003110035.1)	PA103_5904 (WP_003112786.1)	PA103_5905 (WP_019485776.1)	✓
ATCC 27853	RS24835 (WP_003101181.1)	RS24840 (WP_003101179.1)	RS24845 (WP_016852096.1)	✓

Table S1: List of primers

	Primer name	**Sequence (5' – 3')**	**Primer pair and use**
1	Screening_1 operon forward primer	GTGCACATCGCCGATATGACCAT	screening pair (with no. 2)
2	Screening_1 operon reverse primer	TCAGCCATAGCGCGGGAAC	screening pair (with no. 1)
3	Screening_2 operon forward primer	GCGCGAACTGTTCAGCCAC	screening pair (with no. 4)
4	Screening_2 operon reverse primer	GAACATCAGCAGCAGGCTGTC	screening pair (with no. 3)
5	Screening_3 operon forward primer	TGGAGCGCCACCTCGCCCTGGAC	screening pair (with no. X)
6	Forward primer with native promoter of 56030 operon	GCACTGCCCGAGCCGTAG	PCR amplified 56030 operon
7	Reverse primer for 56030 operon	TCAGGAAGAAGCCTCGCGACTG	PCR amplified 56030 operon + screening pair (with no. 5)
8	T7 forward	TAATACGACTCACTATAGGG	pGEM-T vector
9	SP6 reverse	GCT ATT TAG GTG ACA CTA TAG	pGEM-T vector

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