Structural basis for alginate secretion across the bacterial outer membrane

John C. Whitney1,2, Iain D. Hay3, Canhui Li3, Paul D. W. Eckford4, Howard Robinson5, Maria F. Amaya6, Lynn F. Wood6, Dennis E. Ohman6, Christine E. Bear6,7, Bernd H. Rehm7, and P. Lynne Howell6,8,1

1Molecular Structure and Function, The Hospital for Sick Children, Toronto, ON, Canada M5G 1X8; 2Department of Biochemistry, University of Toronto, Toronto, ON, Canada M55 1A8; 3Institute of Molecular Biosciences, Massey University, Private Bag 11222, North Palmerston, New Zealand; 4Biotechnology Department, Brookhaven National Laboratory, Upton, NY 11973-5000; 5Department of Microbiology and Immunology, McGuire Veterans Affairs Medical Center, Virginia Commonwealth University Medical Center, Richmond, VA 23298-0678; and 6Department of Physiology, University of Toronto, Toronto, ON, Canada M5S 1A8

Edited by Bert van den Berg, UMass Medical School, Worcester, MA, and accepted by the Editorial Board June 23, 2011 (received for review March 31, 2011)

Pseudomonas aeruginosa is the predominant pathogen associated with chronic lung infection among cystic fibrosis patients. During colonization of the lung, P. aeruginosa converts to a mucoid phenotype characterized by the overproduction of the exopolysaccharide alginate. Secretion of newly synthesized alginate across the outer membrane is believed to occur through the outer membrane protein AlgE. Here we report the 2.3 Å crystal structure of AlgE, which reveals a monomeric 18-stranded β-barrel characterized by a highly electropositive pore constriction formed by an arginine-rich conduit that likely acts as a selectivity filter for the negatively charged alginate polymer. Interestingly, the pore constriction is occluded on either side by extracellular loop L2 and an unusually long periplasmic loop, T8. In halide efflux assays, deletion of loop T8 (ΔT8-AlgE) resulted in a threefold increase in anion flux compared to the wild-type or ΔL2-AlgE supporting the idea that AlgE forms a transport pathway through the membrane and suggesting that transport is regulated by T8. This model is further supported by in vivo experiments showing that complementation of an algE deletion mutant with ΔT8-AlgE impairs alginate production. Taken together, these studies support a mechanism for exopolysaccharide export across the outer membrane that is distinct from the Wza-mediated translocation observed in canonical capsular polysaccharide export systems.

biofilm | exopolysaccharide secretion | porin | virulence factor | uronic acids

Chronic infection by the opportunistic gram-negative bacterium Pseudomonas aeruginosa is the leading cause of morbidity and mortality among Cystic Fibrosis (CF) patients worldwide (1). The difficulty in eradicating P. aeruginosa infection arises in part from its high intrinsic resistance to antibiotics as well as its conversion to a mucoid phenotype (2). Mucoidy is a descriptive term for the production of copious amounts of the exopolysaccharide alginate, a phenotype that appears to be selected by the environment of the CF lung (3). Alginate contributes to the persistence of P. aeruginosa lung infection by facilitating microcolony formation during biofilm development (4), protecting the bacteria from opsonic phagocytosis (5) and limiting the toxicity of oxygen radicals produced by host macrophages (6).

Alginate is a linear copolymer of 1,4-linked β-D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G). Of the thirteen genes required for the biosynthesis and secretion of alginate by P. aeruginosa, twelve are located on the tightly regulated algD operon. The algC gene, located elsewhere in the genome, has additional roles in lipopolysaccharide (LPS) and rhamnolipid biosynthesis (7, 8). Biosynthesis of alginate starts with the production of the activated sugar-nucleotide precursor GDP-mannuronic acid from fructose-6-phosphate via the concerted actions of the AlgA, AlgC, and AlgD enzymes (reviewed in ref. 9). While this process has been fairly well characterized, the manner in which the activated monomer is polymerized, modified, and exported across the cell envelope is less well understood. The polymerization reaction requires the inner membrane proteins AlgG and Alg44 (10–13). Alg8, which contains multiple transmembrane domains and a large cytosolic domain that shares homology with family 2 glycosyl transferases, is the rate-limiting step in alginate production (12). In contrast, Alg44 possesses a single transmembrane domain that separates a cytoplasmic bis-(3′–5′)-cyclic-di-guanidine monophosphate (c-di-GMP) binding PiZ domain and a periplasmic domain that is predicted to resemble the membrane-fusion protein MexA from the MexAB-OprM multidrug efflux pump (10). Regulation of alginate polymerization requires the binding of the bacterial secondary messenger c-di-GMP to Alg44, thus it is speculated that the coordinated activities of Alg8 and Alg44 facilitate polymerization and export of alginate across the inner membrane (13). Once in the periplasm, the nascent polysaccharide chain is O-acetylated by the combined activities of AlgI, AlgL, and AlgF and selectively epimerized by the C-5 manuronan operase AlgG (14, 15). The periplasmic alginate lyase, AlgL, plays a dual role in guiding the polysaccharide across the periplasm and degrading free alginate that is not exported (16, 17). AlgX is a protein of unknown function thought to be part of a protein scaffold that protects the newly synthesized alginate as it traverses the periplasm (18). At the outer membrane, the lipoprotein AlgK facilitates the proper localization of the integral outer membrane protein AlgE (19). AlgK is an all α-helical protein with multiple copies of the tetra-copetide repeat (TPR) protein–protein interaction motif, which has been suggested to act as a scaffold protein that links the periplasmic components of the alginate biosynthetic machinery to the putative alginate export protein AlgE (19). Preliminary characterization of AlgE has shown that it can spontaneously incorporate into planar lipid bilayers and forms a highly anion specific channel—consistent with its proposed role in the export of the polyionic alginate polymer (20).

In the current study, we have determined the 2.3 Å crystal structure of AlgE, revealing the first structure of a β-barrel outer membrane protein involved in the export of a polysaccharide. In addition to providing insight into how alginate is exported across the outer membrane, the presence of an unusually long periplasmic loop in AlgE suggests a mechanism for complex assembly at the outer membrane. Interestingly, AlgE was found to be structurally homologous to the OprD family of substrate-specific


The authors declare no conflict of interest.

This article is a PNAS Direct Submission. B.V. is a guest editor invited by the Editorial Board.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB Code: 3RBH).

1To whom correspondence should be addressed. E-mail: howell@sickkids.ca.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1104984108/-/DCSupplemental.
nutrient uptake channels despite its unrelated function and the absence of sequence identity (<10%) to any known members of that family. A model for alginate export, which is distinct from the W2a-mediated translocation observed in canonical capsular polysaccharide export systems and the evolutionary implications for the divergence of the AlgE and OprD folds are discussed.

Results and Discussion
Structure Determination. For our structural studies we overexpressed P. aeruginosa AlgE into insoluble inclusion bodies and refolded the protein in a detergent-containing buffer (21). Correct refolding was confirmed by observation of the characteristic “heat-modifiability” property of β-barrel outer membrane proteins using SDS-PAGE (22). Selenomethionine-incorporated AlgE crystals were grown in the presence of the nonionic detergent C$_{16}$$E_{4}$ and the structure was solved using the single-wavelength anomalous dispersion (SAD) technique (Table S1). AlgE crystallized in the monoclinic space group C2 with four molecules in the asymmetric unit. Each monomer interacts laterally with two other monomers in a head-to-tail fashion resulting in upside down barrel-to-barrel associations. The electron density was generally of very good quality, but we were unable to model the His$_{6}$ tag or the first seven residues of the N terminus (Ala$^{33}$ - Thr$^{292}$) (Fig. S1). The final structure was refined to an R$_{work}$ of 22.1% and an R$_{free}$ of 25.9%.

AlgE forms a monomeric 18-stranded antiparallel β-barrel with the register of the β-strands being in reasonable agreement with previously reported topology models (20, 23) (Fig. S1). Notable structural features include a calcium ion that is found coordinated to extracellular loop L1. Given its distance from the pore constriction, this ion likely plays a role in loop stability as opposed to contributing directly to channel specificity. Also of interest, are β-strands S5 and S6, which appear to be too short to traverse the outer membrane (Fig. L4). A similar phenomenon has been observed in other outer membrane proteins that have crystallized as a monomer, including OprD and OpdK from P. aeruginosa (24, 25). Those proteins form labile trimers, and it is hypothesized that the short β-strands contribute to the interaction surface between monomers. However in the case of AlgE, in vivo cross-linking experiments suggest that this protein exists exclusively as a monomer in the outer membrane (20). Examination of the AlgE structure suggests that two conserved residues, Phe$^{187}$ and Tyr$^{190}$, which are unique to this protein may play a key role in governing its oligomeric state. These residues are located on extracellular loop L4 and extend the hydrophobic surface area above strands S6 and S5, respectively (Fig. S2). The existence of these aromatic residues provides sufficient hydrophobic surface area to allow AlgE to exist as a monomer in the outer membrane.

An Electropositive Pore Constriction Facilitates Alginate Export. Substrate-specific outer membrane proteins typically contain narrow pore constrictions whose properties dictate the substrate selectivity of the channel. For AlgE, this constriction is formed by extracellular loops L3 and L7, which fold into the barrel cavity and occupy the majority of the volume (Fig. 1B). However, unlike most other substrate-specific outer membrane proteins where a clear passage through the channel is observed, the constriction in AlgE is occluded by extracellular loop L2 and periplasmic loop T8 (Fig. 1C). The proposed conduit for alginate secretion is lined with highly conserved charged and polar residues that likely confer selectivity towards the polyanionic polymer. The electrostatic surface representation of AlgE with the occluding loops removed reveals a highly electropositive conduit with an ~8 Å constriction at its narrowest point (Fig. 2A and B). The conserved residues lining the constriction are predominantly located on loops L3 (Arg$^{152}$, Asp$^{162}$, and Asn$^{164}$) and L7 (Arg$^{353}$ and Arg$^{362}$) and strands S1 (Lys$^{47}$), S2 (Arg$^{74}$), S4 (Arg$^{129}$), S17 (Arg$^{459}$), and S18 (Asp$^{485}$) (Fig. 2C, Fig. S3). The abundance of arginine residues lining the pore constriction is reminiscent of the electropositive pore-like active site of the A1-III alginate lyase (PDB ID 3EVL) from Sphingomonas sp., which has been solved in complex with an alginate tetrasaccharide (Fig. S4). In this complex, the arginine residues of the active site interact with both the C6 carboxylate moiety and many of the hydroxyl groups of each monosaccharide. Presumably, the clustering of arginine residues found in the pore constriction of AlgE play a similar role in conferring specificity towards alginate. However, the diameter of the alginate lyase active site is narrower (~6 Å compared to ~8 Å) than the pore constriction of AlgE, possibly reflecting their contrasting functions as an enzyme and channel, respectively.

Periplasmic Loop T8 Is Conformationally Flexible. The crystal structure of AlgE suggests that it exists in a “closed” conformation with the electropositive pore constriction being occluded on either side by extracellular loop L2 and periplasmic loop T8. Therefore, to further examine the dynamics of these loops in a noncrystalline state, we performed anion (iodide) efflux experiments with purified AlgE, AlgEΔL2, and AlgEΔT8 protein reconstituted into liposomes. The use of this flux assay allows for the simultaneous assessment of the maximal rate of electrodiffusive anion flux of a population of AlgE molecules. In these experiments, AlgE and AlgEΔL2 showed similar levels of iodide efflux that were significantly above background suggesting that AlgE can adopt an “open” conformation and that the rate of anion flux through this conformation is not modulated by the presence or absence of loop L2 (Fig. 4A, Fig. S5). In contrast, the AlgEΔT8 mutant showed a threefold increase in iodide efflux.

![Fig. 1](https://example.com/fig1.png) Overall structure of AlgE. (A) Side view of AlgE shown as a cartoon representation. Conserved residues Phe$^{187}$ and Tyr$^{190}$ that extend the hydrophobic surface area above Glu$^{166}$ and Arg$^{150}$ of strands S6 and S5, respectively, are shown in blue. The calcium ion bound to loop L1 is shown in gray. (B) AlgE viewed from the extracellular space. Loops L2, L3, L7, and T8 are shown in cyan, black, blue, and orange, respectively. The asterisk indicates a region of L2 where no interpretable electron density is observed in the structure. (C) Slab view of AlgE showing occlusion of the pore constriction by loops L2 and T8.)
implying that this loop plays a role in regulating the passage of anions across the membrane. These data are consistent with the structural data regarding L2 and T8, wherein there is no electron density observed for most of L2, suggesting it is highly mobile—whereas T8 can be modeled in the structure. The higher than average B-factors found for the T8 loop support the iodide efflux experimental data that demonstrates this loop does exhibit some conformational flexibility. We subsequently wanted to test whether the observed iodide efflux could be attenuated in the presence of alginate. To this end, each sample was incubated with a large molar excess (100 : 1) of di-mannuronic acid and assayed for iodide efflux. For all three proteins, the level of iodide efflux was reduced to that of empty liposomes suggesting that under these experimental conditions, AlgE is able to form a pore through the membrane with which di-mannuronic acid can interact. These results are consistent with earlier planar lipid bilayer studies with AlgE that demonstrated an increase in mean closed-state lifetime with increasing concentrations of the alginate precursor, GDP-mannuronic acid (20). However, it should be noted that in the context of the alginate secretion apparatus, the energy for alginate export is believed to originate from the alginate polymerase activity of the putative family 2 glycosyl transferase, Alg8.

As AlgE is always found coexpressed with the other genes from the algD operon in vivo, we suspected that the observed binding of di-mannuronic acid to purified AlgE reflects the specificity of the channel towards its substrate, not the biological mechanism for alginate export.

Complementation of an algE deletion mutant with various forms of AlgE was used to study the role of the L2 and T8 loops in alginate biosynthesis (Table S2). As reported previously, the algE mutant was nonmucoid and produced no detectable levels of alginate (Fig. 4C) (23). When the algE gene was introduced on a plasmid, wild-type levels of alginate production were restored. When this algE deletion mutant was complemented with algEΔL8 and algEΔT2, although Western blot analysis of outer membrane

![Fig. 2. AlgE contains an electropositive pore constriction. (A and B) Electrostatic surface representation (contoured from +10 to −10 kT/e) with (left) and without (right) occluding loops L2 and T8 as seen from the extracellular space (A) and as a slab view from the plane of the outer membrane (B). Positive and negative charges are colored blue and red, respectively. (C) Stick representation of the pore-forming residues as viewed from the extracellular space. The 2F_o - F_c electron density map is contoured at 1.2σ and displayed as a gray mesh. Carbon, nitrogen, and oxygen atoms are colored yellow, blue, and red, respectively. (D) Side view of the pore constriction showing a surface representation of the interior of the pore generated using HOLLOW (45).

![Fig. 3. Periplasmic loop T8 occludes the pore constriction. (A) Molecular surface representation of AlgE, AlgEΔL2, and AlgEΔT8. (B) Quantification of halide efflux rates for AlgE, AlgEΔL2, and AlgEΔT8 in the absence or presence of 1 mM di-mannuronic acid (MM). All efflux rates are the mean of four independent measurements carried out from at least two separate reconstitutions of purified protein. Flux rates from empty (no protein) liposomes were subtracted from all measurements. (C) Overall amount of alginate production in P. aeruginosa PDO300(MCS-5), PDO300ΔalgE(MCS-5), PDO300ΔalgE(MCS-5:algE), PDO300ΔalgE(MCS-5:algEΔL2), and PDO300-ΔalgE(MCS-5:algEΔT8). The amount of alginate produced is the mean of three independent replicates. The error bars in (B) and (C) represent the standard errors (SEM).]
fractions revealed that the AlgEΔT8 and AlgEΔL2 proteins were expressed at the same level as wild-type protein (Fig. S6), complementation resulted in 48% and 60% of wild-type alginate production, respectively. Because deletion of loop L2 did not significantly affect halide efflux in vitro, the reduction of alginate secretion in vivo suggests the loop may play a role in preventing the surrounding LPS from interfering with efficient alginate secretion. Of the twenty-three residues that comprise L2, seven are acidic. These residues could potentially interact with LPS via the divalent cations present in the outer membrane that stabilize negatively charged LPS. In contrast, the functional implications of the T8 loop are intriguing given that it appears to partially block the pore constriction in vitro. T8 is unique in that it is significantly longer in length than most periplasmic turns found in β-barrel membrane proteins. As such, it is the most likely region within AlgE to facilitate its association with the other proteins involved in alginate secretion. The down and outward movement of T8, which would be required for AlgE to be in an “open” conformation, would also enable this loop to interact with another protein. Such an interaction would have the advantage of potentially locking AlgE into an open conformation, and hence facilitating alginate export. Thus, deletion of this loop may compromise the ability of AlgE to physically interact with the other components of the alginate secretion machinery and reduce its ability to secrete newly formed alginate polymers. The outer membrane lipoprotein AlgK is the most likely candidate to interact with AlgE given its structure, which contains multiple copies of the TPR protein–protein interaction motif, its subcellular localization in the outer membrane, and its role in the proper localization of AlgE (19). It has also been suggested that the putative periplasmic membrane-fusion protein (MFP) domain of Alg44 may also interact with AlgE (10, 13). However, because Alg44 localizes to the inner membrane, we speculate that its periplasmic domain may not be large enough to span the entire ∼20 nm of the periplasm to contact AlgE directly unless a conformational change is present, which narrows the periplasmic space (26).

AlgE Is Structurally Homologous to the OprD Family of Substrate-Specific Porins. Although AlgE has no significant sequence similarity to other outer membrane proteins of known structure, a remarkable structural similarity between AlgE and the OprD family proteins OprD and OpdK from P. aeruginosa and BenF from P. putida was observed (Fig. 4A) (24, 25, 27). AlgE superposes each of these proteins with an rms deviation of 2.7 Å, 2.9 Å, and 2.8 Å over 302, 316, and 323 equivalent Cx positions, respectively. Not only are these proteins comparable in overall β-barrel shape, they also all contain an extracellular loop L7 that is similarly much longer than the other loops and is always found to be folded into the barrel lumen, thereby creating a narrow pore constriction. This structural similarity was somewhat unexpected given that AlgE is classified as a member of the general diffusion porin family (28) and the divergent function of AlgE and OprD-family proteins, which are involved in import of small molecule substrates rather than exopolysaccharide export. Other carbohydrate transporters such as E. coli LamB and S. typhimurium ScrY also form 18-stranded β-barrels however aside from this similarity their overall barrel and loop structures are significantly different (29, 30) (Fig. S7).

As the outer membrane of P. aeruginosa is notoriously impermeable due to its lack of general diffusion porins, the observation that two unrelated proteins adopt a very similar topology suggests that this “OprD-like” fold may represent a common evolutionary framework from which many substrate-specific outer membrane β-barrels evolved. Specifically, extracellular loops L3 and L7 act to occlude the majority of the channel volume (Fig. 4B) and substrate selectivity is dictated by the constellation of side-chain residues that form the channel constriction. This arrangement allows for the relative impermeability of the outer membrane to be maintained while facilitating the specific transport of various substrates across the membrane. Further evidence that AlgE likely diverged from the OprD family of proteins comes from the observation that the algE gene is only found in bacteria with larger numbers of OprD-family members in their genome (between 10–29) (31). Needless to say, the number of available outer membrane proteins structures is still relatively small and as a result the potential diversity of this OprD-like fold is not yet understood. However, we have shown that two separate families of outer membrane proteins with no sequence identity, unrelated cellular function, and opposite transport directionality are structurally homologous; a finding that has consequences for the classification of porins in general.

Mechanism of Polysaccharide Secretion. It was recently proposed that the molecular mechanism for the export of exopolysaccharides such as alginate, cellulose, and poly β-1,6-GlcNAc (PGA) across the outer membrane likely differs from that of canonical capsular polysaccharide secretion systems (19, 32–34). The structure of AlgE presented herein confirms that there is very little similarity between the two systems (Fig. S8). The β-barrel fold observed for AlgE suggests an export mechanism that is more typical of the translocation of hydrophilic molecules across the bacterial outer membrane whereby transport is mediated by a single polypeptide chain folded in the membrane (35). In contrast, group 1 and 4 capsular polysaccharides are secreted through a Wza-like octameric transmembrane lipoprotein, which contains little in the way of conservation among the residues that line the interior of the translocation pathway and is much wider (17 Å at its narrowest point) than AlgE. As a result, a model has been proposed in which water molecules “lubricate” the polymer during an export process that appears to be relatively nonspecific (32, 36). In comparison, the majority of the residues located at the pore constriction (∼8 Å wide) within AlgE are invariant and
undoubtedly play a significant role in substrate selectivity given the charge complementarity between protein and polysaccharide.

Large protective domains that protrude into the periplasm are a common feature of not only Wza but also the outer membrane proteins of various other secretion systems. Given the phenotype of algK deletion and algK-C28S mutants, which produce dialyzable urchin acids, we have previously proposed that AlgK plays a protective role as the polymer passes through the periplasm (19). AlgE is also mislocalized in the algK mutants suggesting that these proteins may physically interact (19). Although demonstrating a direct interaction between purified AlgE and AlgK has proven refractory, it is not inconceivable that AlgK needs to be anchored to the outer membrane in close proximity to AlgE and/or that a conformational change in AlgK is required for the interaction to occur. Indeed, the four molecules of AlgK found in the asymmetric unit exhibit considerable conformational flexibility (19). Additional support that AlgK/AlgE interact and form a new type of secretin is provided by the bioinformatics analysis of PgaA and BcsC. These proteins, involved in the export of PGA and cellulose, respectively, appear to be a fusion of AlgK/AlgE as both are predicted to contain a large TPR domain and β-barrel porin domain (Fig. 5) (19, 33, 34). The finding that alginate secretion is regulated at the protein level by the binding of c-di-GMP to the PilZ domain of Alg44 (13) has led to speculation that Alg44 may not only activate Alg8 but may also exert an effect through its putative periplasmic MFP domain. The MFP domain has been suggested to interact with the outer membrane components of the alginate secretion machinery as a means to couple biosynthesis to export. Because AlgE lacks large periplasmic domains, the TPR motifs of AlgK may provide the scaffold that links AlgE to the inner membrane components of the alginate secretion apparatus.

The structure of AlgE sheds light on the export mechanism of one of the major virulence factors produced by P. aeruginosa. Moreover, the structure confirms previous speculation that the apparatus for alginate export is structurally distinct from those involved in capsular polysaccharide export. It remains to be seen if other less characterized exopolysaccharide secretion systems such as cellulose and PGA will use a similar mechanism. The high-resolution structures of the outer membrane proteins of these respective exopolysaccharide secretion systems will enable a unifying theme to be established.

Material and Methods

Protein Expression, Purification, and Structure Determination. The expression, refolding, and purification of selenomethionyl-incorporated AlgE was performed as described previously for the native protein with the following modifications (21): (i) Expression was carried out as per the protocol of Lee et al. using B834 Met+E. coli cells (Novagen) (37) and (ii) all purification buffers contained 1 mM tris(2-carboxyethyl)phosphine to prevent oxidation of selenomethionine. Purified selenomethionine-incorporated AlgE was concentrated to 8 mg/mL by Amicon ultrafiltration [50 kDa molecular weight cut-off (MWCO), Millipore] and screened for crystallization conditions using commercially available crystal screens. Diffraction quality crystals that belonged to the monoclinic space group C2 were obtained in 10% (w/v) PEG 3350, 0.2 M ammonium formate. After cryoprotection with the mother liquor supplemented with 20% (v/v) ethylene glycol, a 2.3 Å Se-SAD dataset was collected on beam line X29 at the NSLS (Brookhaven National Laboratory). The resulting data were indexed and scaled using HKL2000 (38). A total of fifteen (out of sixteen) selenium sites were located using HKL2MAP (39), and density modified phases were calculated using SOLVE/RESOLVE (40). The resulting electron density map was interpretable and allowed for manual model building in COOT (41). Refinement was carried out using PHENIX.REFINE (42) and the protocol monitored at each step by the reduction in R-factor and Rfree.

Halide Efflux Assays. The generation of the constructs required for expression of AlgEΔL2 and AlgEΔT8 and the protocol to reconstitute these proteins, and AlgE into liposomes is described in the SI Text. External iodide concentrations in the proteoliposome samples were monitored continuously using an iodide-selective electrode (Lazar Research Laboratories) interfaced to the Digidata 1320A Data Acquisition System and controlled by Clampex 8 software (Axon Instruments), as described previously (43). Valinomycin (20 nM) was added to prevent charge-buildup across the membrane that would inhibit significant I− movement. Control liposomes were prepared and treated identically, but in the absence of AlgE protein. For the alginate inhibition assays, freeze dried di-mannuronic acid was dissolved in the extraliposomal isoosmotic flux buffer to a final concentration of 1 mM prior to data acquisition. Vesicles were lysed by addition of 0.5% Triton X-100 at approximately 300 s after the initiation of electrodiffusion with valinomycin to verify that sufficient iodide was trapped in the proteoliposomes.

Alginic Acid Secretion Assay. The generation of plasmids for in vivo complementation is described in SI Text. Each P. aeruginosa strain was inoculated into 2 mL LB medium containing the appropriate antibiotics and grown overnight. The harvested cell pellets were then resuspended in saline, plated onto PIA medium (to promote alginate production) and incubated at 37 °C for 72 h. The bacteria were subsequently scraped off the agar plates using a sterile spatula and resuspended in saline. The resulting suspensions were pelleted to separate the alginate-containing supernatants from the cells. The cell pellets were freeze dried and the final weights were determined. The alginate was precipitated from the supernatants by the addition of an equal volume of ice-cold isopropanol and freeze dried. The alginate precipitates were then redissolved in 50 mM Tris-HCl pH 7.4, 10 mM MgCl2 to a final concentration of 0.5% (w/v) and incubated with 15 µg/mL DNase I and 15 µg/mL RNase I for 6 h at 37 °C followed by 20 µg/mL Pronase E for an additional 18 h at 37 °C. The solutions were dialyzed...
(3.5 kDa MWCO, Fisher) against 5 L of ultrapure H2O for 48 h. The purified alginate was precipitated with an equal volume of ice-cold isopropanol and freeze dried for subsequent quantification using uronic acid analysis [44] and SI Text.

Acknowledgments. The authors thank J. Weadge, L. Riley, F. Wolfram, Y. Lobasanov, L. Burrows, G.D. Smith, G. Prive, and T. Moreas for helpful discussions and P. Yip for technical assistance. This work was supported by the following grants: Canadian Institutes of Health Research (CIHR) (to P.L.H.) (number 13337) and C.E.B. (number 97954); Canadian Cystic Fibrosis Foundation (CCFF) (to C.E.B.); Public Health Service (AI-19146), National Institutes of Allergy and Infectious Disease, CF Foundation and Department of Veterans Affairs (Merit Award I01BX000477) (to D.E.O.); and Deutsche Forschungsgemeinschaft (Re 1097/6-1) and the Institute of Molecular Biosciences at Massey University (to B.H.R.). P.L.H. is the recipient of a Canada Research Chair; J.C.W. has been supported by graduate scholarships from the National Science and Engineering Research Council of Canada (NSERC), CCFF, the Ontario Graduate Scholarship Program, the Ontario Student Opportunities Trust Fund, and The Hospital for Sick Children Foundation Student Scholarship Program. I.D.H. is funded by a Massey University doctoral scholarship. P.D.W.E. is supported by a fellowship from the CCFF. Beam line X29 at NSLS is supported by the Department of Energy and the National Institutes of Health (NIH) National Center for Research Resources.


References: