Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Zihuan (Rita) Wang April 10, 2020

Analysis of *wzz2* expression in different *Pseudomonas aeruginosa* serotypes and the correlation of resistance to serum and outer membrane stress

by

Zihuan (Rita) Wang

Joanna B. Goldberg, Ph.D.
Adviser

Department of Physics

Joanna B. Goldberg, Ph.D.

Adviser

Minsu Kim, Ph.D.

Committee Member

Laura Finzi, Ph.D.

Committee Member

2020

Analysis of *wzz2* expression in different *Pseudomonas aeruginosa* serotypes and the correlation of resistance to serum and outer membrane stress

By

Zihuan (Rita) Wang

Joanna B. Goldberg, Ph.D.

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Department of Physics

2020

Abstract

Analysis of *wzz2* expression in different *Pseudomonas aeruginosa* serotypes and the correlation of resistance to serum and outer membrane stress

By Zihuan (Rita) Wang

Pseudomonas aeruginosa is a Gram-negative opportunistic bacterial pathogen that can cause infections in immunocompromised people as well as lung infections in those living with cystic fibrosis (CF). This bacterium produces a virulence factor, lipopolysaccharide (LPS), which is a glycolipid molecule embedded in the bacterial outer membrane. The polysaccharide that extends from the distal portion of LPS is referred to as O antigen, the composition of which categorizes *P. aeruginosa* into 20 different serotypes. O antigen is further characterized as long or very long based on the number of sugar repeat units. The expression of long O antigen is controlled by the chain-length control protein Wzz1, while the expression of the very long is controlled by the chain-length control protein Wzz2. This study focuses on the production of Wzz2 and very long O antigen with the goal of understanding how they contribute to the fitness of different serotype strains in human serum and other outer membrane disrupting environments. Analysis of LPS in all 20 serotype strains revealed that each serotype produced varying amounts and lengths of O antigen. *wzz2* clean deletion mutants of serotype O1 and O8 confirmed that the mutation was responsible for the production of very long O antigen in both strains. Next, we determined whether the expression of very long O antigen contributed to serum resistance of individual serotype strains and found that the deletion of *wzz2* caused serotype O1 strain to become sensitive to normal human serum, but had little effect on the serum resistance of the serotype O5 control strain PAO1 or the serotype O8 strain. We also evaluated the relative fitness of individual serotype strains and their *wzz2* mutants under different osmotic stress and pH. Deletion of *wzz2* caused serotype O1 and O8 strains to grow poorly in low pH condition but the

mutation in *wzz2* caused PAO1 to grow better. Our experimental results suggest that the role of O antigen in the protection against outer membrane disrupting environments varies across different serotypes. Future experiments aim to elucidate the contribution of very long O antigen to serum resistance and antibiotic resistance across all serotypes.

Analysis of *wzz2* expression in different *Pseudomonas aeruginosa* serotypes and the correlation of resistance to serum and outer membrane stress

By

Zihuan (Rita) Wang

Joanna B. Goldberg, Ph.D.

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Science with Honors

Department of Physics

2020

Acknowledgements

I would like to thank my PI, Dr. Joanna Goldberg, and my graduate student mentor, Dr. Ashley Cross, for their excellent mentorship and guidance on my Honors Thesis project. They introduced me to the fascinating world of scientific research and have always encouraged me to think and solve problems like a real scientist. They are the best research mentors I could ask for. I could not have gone this far in my undergraduate research without their kind help and support.

I am grateful for having Dr. Minsu Kim, and Dr. Laura Finzi on my Honors Thesis Committee. Dr. Kim and Dr. Finzi are the professors who have influenced me the most in my study of biophysics at Emory University. I appreciate the attention and support they have provided me during the course of this project.

A special gratitude goes out to the wonderful group of scientists I have met during my time in the Goldberg Lab. Thank you for giving me the most amazing memories in my college years!

Table of Contents

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium commonly found in environments that are associated with human activity[1]. It has drawn considerable attention because of its ability to cause a variety of infections, especially in immunocompromised people as well as those living with cystic fibrosis (CF)[23]. *P. aeruginosa* produces a wide variety of virulence factors, among which is lipopolysaccharide (LPS), which contributes to the immune evasion of this bacterium[4]. LPS is a group of glycolipid molecules extending from the outer portion of the outer membrane of *P. aeruginosa*. The LPS molecule consists of three parts: the lipid A, the core oligosaccharide, and the O antigen[5]. O antigen is divided into specific and common O antigen with the specific O antigen categorizing *P. aeruginosa* into 20 different serotypes depending on the composition of the sugars in the O antigen molecule[6]. Specific O antigen is further characterized as long or very long based on the number of the sugar repeat units. The expression of long O antigen is controlled by a chain-length control protein named Wzz1, while the expression of the very long is controlled by chain-length control protein Wzz2[78]. Previous studies indicate that considerable amount of variation exists across serotypes in the nucleotide sequence of Wzz1, whereas Wzz2 seems to be conserved for all serotypes[68]. This study focuses on the expression of Wzz2 protein and the very long O antigen with the goal of understanding how they contribute to the fitness of different serotype strains in human serum and some other outer membrane disrupting environments.

In order to study Wzz2 and the very long O antigen in detail, our lab is in the progress of constructing clean *wzz2* deletion mutants in each of the 20 serotype strains available in our laboratory. With the time constraint of this project and the lengthy process of mutant construction, we chose to focus on serotype O1 and serotype O8 strains because of their clinical

relevance in cystic fibrosis research[9]. We used PAO1, a serotype O5 laboratory strain, as a reference strain since Wzz2 production and regulation have been well characterized in this strain[10]. Since it remains unknown whether Wzz2 similarly regulates the very long O antigen chain-lengths in all serotypes, we wanted to confirm that the deletion of *wzz2* in our serotype O1 and serotype O8 strains abrogated Wzz2 and very long O antigen production. To do so, we performed a serotype specific O antigen western immunoblot. We found that the deletion of *wzz2* decreases the production of very long O antigen in both serotype O1 and serotype O8 strains. In addition to O antigen production, we also examined whether the deletion of *wzz2* altered the LPS profile of each serotype strain using the Pro-Q Emerald 300 LPS staining technique. We found that the high molecular weight portion of LPS was also absent in the serotype O8 *wzz2* deletion strain. We did not observe the same change in the serotype O1 strain; however, we noticed an increased production of shorter O antigen chain lengths, which is not quite surprising since O antigen of both chain lengths are assembled onto the same pool of lipid A-core molecules.

Given the location of O antigen on the distal portion of LPS molecule, this polysaccharide plays an important role in *P. aeruginosa*'s initial interaction within a host. For example, O antigen forms a physical barrier to protect *P. aeruginosa* from complement-mediated killing launched by the innate immune system in human. Published experiments conducted in our lab on a serotype O11 strain suggest that the very long O antigen may not be important for serum resistance of this strain[4]. Therefore, we sought to determine if the very long O antigen chain-length confers resistance to human serum in each of our *P. aeruginosa* strains. To test if this finding holds true for other serotypes, we performed serum resistance assay on PAO1, serotype O1, serotype O8 strains with their respective *wzz2* mutants. We found that the deletion of *wzz2* caused serotype O1 strain to be very sensitive to human serum. However, deletion of *wzz2* did

not affect serum resistance in PAO1 or serotype O8 strain. We attributed such variability in survival to the sugar composition of serotype specific O antigen, since there was no intrinsic difference in growth across the different strains. The killing of serotype O1 *wzz2* mutant by human serum is unlikely to be caused by complement proteins, since it was also killed in heat-inactivated serum.

Apart from human serum, we also compared the growth of PAO1, serotype O1, and serotype O8 strains with their *wzz2* mutants in a series of osmotic and pH conditions using the Biolog™ Phenotype MicroArray Plates. Biolog™ Phenotype Microarray Technology measures and compares cell metabolism between strains based on cellular respiration. Deletion of *wzz2* caused serotype O1 and serotype O8 strains to grow poorly in low pH condition and interfered with their ability to use certain types of amino acids to neutralize pH. Surprisingly, PAO1 exhibited the opposite fitness pattern under the same experimental setup, with the *wzz2* mutant grown better than the wild type strain in a range of osmotic and pH conditions. With *wzz2* mutants constructed for all 20 serotypes in the near future, we will be able to determine what effects loss of Wzz2 have on O antigen production and the LPS profile of each serotype. We can also test the susceptibility of the other serotype strains and their respective *wzz2* mutants to antibiotics that have been proven to be effective to *P. aeruginosa*.

RESULTS AND DISCUSSION

Lipopolysaccharide (LPS) production of serotype O1-O20 *P. aeruginosa* strains.

Pro-Q Emerald 300 LPS staining was performed on all twenty serotypes for the purpose of analyzing the total structure of the LPS molecules produced by each strain. All of the strains

tested produced the lipid A plus core oligosaccharide portion of the LPS molecule (Fig. 2). However, serotype O7, O12, and O14-O16 strains did not produce O antigen as detected by this staining method (Fig. 2). Those serotypes that did produce detectable O antigen exhibited distinctive banding patterns, with different serotypes producing different portion and lengths of O antigen in various amounts.

There exist two possible explanations for the inadequate staining of O antigen in serotype O7, O12, and O14-O16 strains: these serotype strains may carry some mutations in their O antigen biosynthesis operon that prevent them from producing O antigen, or they are composed of sugar units that may not stain well using the Pro-Q LPS staining technique. Genetic studies on the O antigen biosynthetic locus in *P. aeruginosa* reported that serotype O15 strain has a much shorter O-antigen biosynthetic gene cluster that lacks the sequence necessary for the synthesis of specific O antigen[11]. Limitations associated with Pro-Q staining may have also caused the staining problem of O antigen, since past experiences from our lab suggest that this staining technique could be less reactive to some LPS, and may not stain all types of sugar equally well.

Deletion of *wzz2* ceases the production of Wzz2 protein in serotype O5, O1, and O8 *P. aeruginosa* strains. We next wanted to determine how loss of the chain-length control protein Wzz2 would affect O antigen production. First, clean deletion mutants of *wzz2* in serotype O1 (sO1) and serotype O8 (sO8) strains were confirmed. To confirm the effect of each mutant, western immunoblot was performed to determine the effect on the production of Wzz2 protein. We used our reference laboratory strain PAO1 and a PAO1 *wzz2* transposon mutant (PAO1 *wzz2::tet*) as a control since we know that this mutant does not produce Wzz2. Although the polyclonal antibody we used to detect Wzz2 led to some non-specific background banding, we

were able to observe the absence of Wzz2 protein in *wzz2* mutants of both sO1 and sO8 strains since these mutants had a missing band at around 48 kDa compared to their respective wild type strains (Fig. 3). Therefore, we confirmed that each *wzz2* mutant did not produce Wzz2. Further, we noticed that the Wzz2 band appeared to be the faintest and therefore more lowly expressed in wild type sO1 and the darkest and therefore most highly expressed in PAO1 (Fig. 3). Although *wzz2* is highly conserved across *P. aeruginosa* strains[8], there exists the possibility that each serotype strain produces a Wzz2 protein of slightly different sequence and structure. Therefore, it is possible that PAO1 might appear to have more prominent Wzz2 since the antibody used to detect Wzz2 protein was originally generated to PAO1 and might detect this protein better because of its higher similarity. To elucidate the potential variation of Wzz2 protein, we could sequence *wzz2* from each of our serotype strains and determine how different or similar each sequence is. Alternatively, the *wzz2* genes may be differentially expressed between different serotype strains, which led to the observed different reactivity in our western blots. To test if this is the case, we could sequence the upstream regions of the *wzz2* genes and identify the difference in the promoter sequences that control the expression of *wzz2*.

Deletion of *wzz2* decreases the production of very long O antigen in serotype O5, O1, and O8 *P. aeruginosa* strains; however, its impact on LPS varies across serotypes. To investigate the impact of *wzz2* deletion on seroypte-specific O antigen production for each strain we performed a western immunoblot using polyclonal antibody against each serotype specific O antigen. We hypothesized that the deletion of *wzz2* would result in altered or decreased production of the very long O antigen portion of the LPS molecule since these are the chain-lengths we know that are controlled by Wzz2 in PAO1. When analyzed, we saw that the deletion

of *wzz2* did in fact decreased the production of very long O antigen in both sO1 and sO8 strains, as evident from the missing higher molecular weight portion of the mutant bandings (Fig. 4A). We also observed that the deletion of *wzz2* in sO1 strain resulted in an increased production of shorter O antigen chain-lengths (Fig. 4A). Interestingly, different from the continuous length spectrum of the very long O antigen observed for PAO1 and sO8 strain, there appeared to be two dominant forms of the very long O antigen for sO1 strain, both of which were controlled by the Wzz2 protein (Fig. 4A). Next, we analyzed the LPS production of each wild type and mutant strain using Pro-Q staining. Since Pro-Q staining stains the entire LPS molecule, it allows for a broader view of what have changed as a result of *wzz2* deletion in all three components of LPS; however, Pro-Q staining may not reflect the change in O antigen production to the same resolution as allowed by the western immunoblot that targets serotype specific O antigen only. In support of this, when we visualized the LPS of each wild type and mutant by Pro-Q staining, the loss of very long O antigen was not clearly observed in the PAO1 and sO1 *wzz2* mutants (Fig. 4B). There was a visible loss in high molecular weight very long O antigen production in sO8 strain when *wzz2* was deleted, however (Fig. 4B). We did observe by Pro-Q staining that the deletion of *wzz2* resulted in a faint increase in the production of the shorter O antigen chain length in each of the *wzz2* mutants. The increase in shorter O antigen chain-lengths can be expected considering that more lipid-A core molecules are made available for the addition of these shorter chain-lengths in the absence of the longer chain-lengths.

Growth curves of PAO1, serotype O1, O8 and their respective *wzz2* mutants. We next wanted to determine if deletion of *wzz2* altered the growth of our strains. To do this, we monitored the growth of PAO1, sO1, sO8 and each respective *wzz2* mutants in lysogeny broth

(LB) for 20 hours. The growth rate of PAO1 was similar to that of its *wzz2::tet* transposon mutant in the first eight hours, during which the cells were in the exponential phase (Fig. 5). A sudden uplifting of the growth curve was observed in PAO1 as cells transitioning from the late exponential phase to the stationary phase, which could be attributed to the clump-forming tendency of the wild type PAO1 strain; however, this was not seen to be as pronounced in the *wzz2::tet* mutant, suggesting that the clumping of PAO1 could be attributed to the expression of very long O antigen (Fig. 5). The growth curve of sO1 strain and its *wzz2* clean deletion mutant projected in a very similar manner, with the mutant ended at a slightly higher OD_{600} measure (Fig. 5). As suggested from the difference in growth of PAO1, sO1 and each respective *wzz2* mutant, the lack of very long O antigen may allow cells to grow longer without clumping due to less electrostatic interaction between the cells. The degree of electrostatic interaction may vary across serotypes due to the different sugar composition and the length and amount of each O antigen. Previous studies reported that the presence of O antigen plays a role in the overall surface hydrophobicity of cells and affects their binding to charged materials[12]. The sO8 strain and its *wzz2* clean deletion mutant have very closely aligned growth curves (Fig. 5). Overall, the mutation in or deletion of *wzz2* does not seem to significantly alter the growth of any of the strains.

Deletion of *wzz2* in serotype O1 strain results in sensitivity to normal human serum. However, deletion of *wzz2* does not affect the serum resistance of PAO1 or the serotype O8 strain. Previous studies have shown that O antigen plays an important role in the serum resistance of Gram-negative bacteria[4]. We reasoned that the possession of very long O antigen should be able to provide *P. aeruginosa* better protection in human serum by blocking

complement proteins from approaching the cell surface and initiating complement-mediated killing. Previous experiments conducted in our lab indicate that the *wzz1* transposon mutant of PA103, a serotype O11 strain, exhibited reduced survival in human serum, but the *wzz2* transposon mutant exhibited similar survival compared to the wild type strain[4]. This result suggests that the very long O antigen may not be important for serum resistance of PA103. We are interested to know if this finding holds true for PAO1, sO1, and sO8 strains. We modified the traditional serum resistance experiment to replace colony forming units (CFUs) counting with the use of BacTiter-Glo reagent for the determination of cell viability post incubation in normal human serum.

We first confirmed that the BacTiter-Glo assay produced similar results when we plated for CFUs according to the traditional method. Both methods yielded similar survival trend for all strains tested (data not shown). In each experiment we included a PAO1 *galU* mutant (PAO1 *galU::aacC1*) as a control since this strain is very sensitive to normal human serum[13]. Next, we tested resistance to serum for PAO1, sO1, sO8 strains and each respective *wzz2* mutant in 20% normal human serum over the course of 2 hours. First, we observed that PAO1, sO1, and sO8 wild type strains are very serum resistant because each had a percent survival exceeding 100% (Fig. 6A). These strains continued to grow and proliferate in normal human serum (NHS) over the 2-hour duration of the experiment. Corroborating the published results described for strain PA103, deletion of *wzz2* did not alter survival of PAO1 or sO8 strain when grown in normal human serum. Conversely, the sO1 *wzz2* mutant became very sensitive to serum and was about 100-500 times more sensitive than the wild type strain (Fig. 6A). To determine the direct effects of complement-mediated killing by the serum, we also grew each strain in heat-inactivated normal human serum (HI), which inactivates the complement proteins. In heat-inactivated serum,

PAO1 *galU::aacC1* now survives to very high levels due to the loss of the complement-mediated killing pathway (Fig. 6B). Surprisingly, the sO1 *wzz2* mutant was still killed by the heat-inactivate serum (Fig. 6B). However, after 2 hours of growth this mutant was able to reach starting densities of about 100%. All the other strains were unaffected by growth in heat-inactivated serum (Fig. 6B).

As evident from our serum resistance experiment, the very long O antigen played a crucial role in the survival of sO1 strain in both normal human serum and heat-inactivated serum, but had little impact on the growth of PAO1 or sO8 strain in either type of serum. Given that there was no significant intrinsic growth difference across all six strains being tested, any observed difference in the percent survival across serotype strains or between the wild type and the mutant should be attributed to the interaction of cells with the serum. The fact that the *wzz2* mutant of sO1 strain was very susceptible to both NHS and HI suggests that the deletion of *wzz2* may have caused some major defects in the membrane. It seems that in addition to the complement proteins, there exist some heat-stable particles in human serum that are disrupting to the sO1 strain lacking the very long O antigen. Corroborating the results described for strain PA103, the contribution of very long O antigen to the serum resistance of *P. aeruginosa* varies across serotypes.

Deletion of *wzz2* alters resistance and sensitivity to different osmotic pressures and pHs, but this is dependent on serotype and strain background. Previous studies revealed that the sugar composition of O antigen contributes to the permeability properties of the outer bacterial membrane, with the lack of certain subset of sugars resulted in an increased permeability to hydrophobic agents[14]. We are interested to test whether the absence of the very

long O antigen would have an impact on the sensitivity of each serotype strain to varying osmotic stress and pH conditions. We compared the growth of PAO1, sO1, and sO8 strains with their *wzz2* mutants in varying osmotic and pH conditions using the Biolog™ Phenotype MicroArray Plates PM9 and PM10. The Biolog™ Phenotype MicroArrays (PMs) technique allows for the testing of cell survival in a wide array of environments. Cells prepared with pre-manufactured buffers are incubated in the OmniLog® machine, which measures cell metabolism based on the vigor of cellular respiration, which is, the amount of NADH produced from the cells[15]. Biolog™ MicroArray Plate PM9 and PM10 are 96-well plates pre-manufactured with a different osmotic stress (PM9) and pH condition (PM10) in each well. We were interested to see if the production of very long O antigen contributes to the resistance of different serotype strains to these outer-membrane disrupting conditions.

We grew PAO1, sO1, sO8 strains and each respective *wzz2* mutant in a total of 192 different osmotic and pH conditions available from the PM9 and PM10 Biolog™ 96-well MicroArray Plates and then determined whether the deletion of *wzz2* altered survival of each strain when compared to the wild type. Significant growth differences are shown in Table 1. Disrupting *wzz2* in PAO1 led to an increased growth compared to wild type PAO1 in a variety of osmolytes, such as sodium formate, 6% NaCl supplemented with organic compounds, and sodium lactate. The deletion of *wzz2* seems to have little impact on the growth of sO1 strain in any osmolytes, and caused sO8 strain to grow poorly only in sodium formate and sodium lactate. There were barely any overlaps between PAO1 and sO8 strain in terms of osmolytes (except for sodium formate and sodium lactate) that led to significant metabolic difference between the wild type and the mutant. Regarding pH conditions, disrupting *wzz2* in PAO1 led to an increased growth compared to the wild type PAO1 in pH 4.5 supplemented with a group of amino acids.

The deletion of *wzz2* caused sO1 and sO8 strains to grow poorly in a similar set of pH conditions, but with some variations in the type of amino acids that were supplemented.

We noticed that PAO1 exhibited a completely opposite survival pattern compared to the other two serotype stains: the *wzz2* transposon mutant outgrew the wild type strain in nearly every condition in which significant growth difference was identified. This result was unexpected since the production of very long O antigen was anticipated to provide protection, rather than compromising the survival of *P. aeruginosa* under different osmotic stress and pH conditions. One possible explanation for the uniqueness of PAO1 in this experiment is that this laboratory strain has been sub-cultured for years and has been adapted to grow in a laboratory setting. It is necessary to repeat this experiment to determine if PAO1 behaves differently than the other two serotype strains in a consistent way. We attribute the difference in the set of pH conditions identified for sO1 and sO8 strains to the variation in the sugar compositions of their O antigens (Fig. 1).

The only overlap across all three serotype strains we tested in terms of osmolytes and pH conditions is pH 4.5, which is described by the Biolog™ manufacturer as the "pH, decarboxylase control". Supplementing amino acids to this low pH condition was expected to help bacteria restore neutral pH and resume growth. We found that the *wzz2* mutant of each serotype strain grew relatively poorly compared the wild type strains in pH 4.5, which suggests that the very long O antigen may play a role in protecting *P. aeruginosa* in low pH conditions. The deletion of *wzz2* may also interfere with the ability of sO1 and sO8 strains to use certain types of amino acids to neutralize pH, since the mutants exhibited significantly less growth in a few conditions in which amino acids are supplemented to pH 4.5.

CONCLUSIONS AND FUTURE DIRECTIONS

With the use of *wzz2* clean deletion mutants, this project aims to understand how the loss of very long O antigen would affect the survival of different *P. aeruginosa* serotype strains in human serum and some other outer membrane disrupting environments. We found that the very long O antigen was only important to a subset of serotype strains in their resistance to human serum and certain osmolyte and pH conditions. We attributed this result partially to the variation in the sugar composition of very long O antigen across serotype O1, O5, and O8 (Fig. 1). In addition to the different monosaccharides that constitute the O antigen of each serotype, these monosaccharides are connected with either α or β glycosidic bonds at varying carbon positions[6], which leads to more variety and greater dissimilarity of O antigen across serotypes.

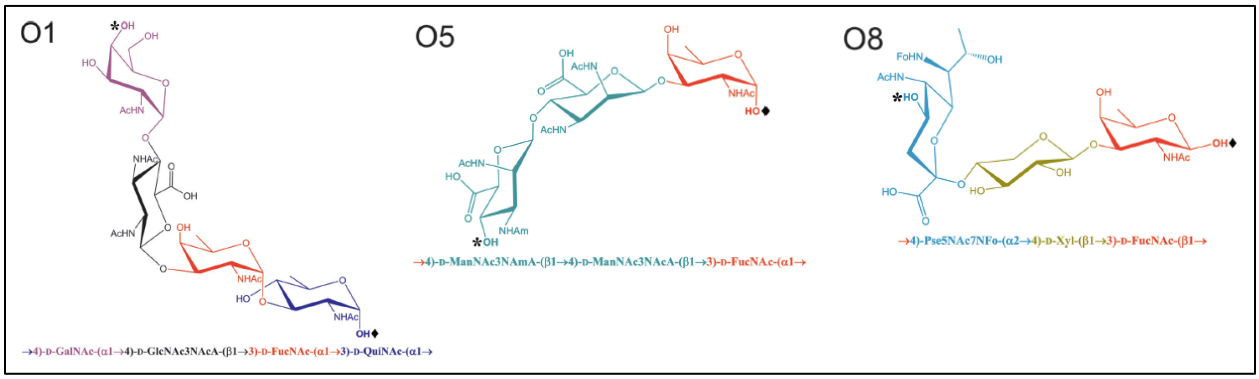

Figure 1. Sugar composition of serotype O1, O5, and O8 *Pseudomonas aeruginosa* strains. The hydroxyl group marked with (◆) links to the preceding O antigen subunit and the hydroxyl group marked with (*) links to the succeeding O antigen subunit. Monosaccharides that constitute the sugar repeat unit of each serotype are colored differently based on type: navy blue, quinovose (Qui); red, fucose (Fuc); black, glucose (Glc); purple, galactose (Gal); teal, mannose (Man); amber, xylose (Xyl); cyan, pseudaminic acid (Pse). Model credits to Islam and Lam[6].

It is worth addressing the limitations associated with the techniques and assays used in this study and proposing alternative candidates for future experiments. Considering the poor staining of O antigen from serotype O7, O12, and O14-O16 strains by the Pro-Q LPS staining kit, we reasoned that this staining technique may not be ideal for the purpose of determining the change in LPS profile when *wzz2* is deleted. Silver staining, despite being lengthier and of higher throughput, may allow for better visualization of O antigen compared to Pro-Q staining. With regard to the serum resistance experiment, we noticed that large variations often occurred in the same experiment across technical replicates and lack of consistency existed across experiments with biological replicates of the same strain. This suggests the need for further modification of our serum resistance assay, despite the great efforts we have put into modifying it over the course of this project. Taking into the account the clumping of wild type serotype strains, we may want to consider applying orbital shaking during the incubation of cells in serum to mix the cells more adequately with the serum. We could also plate cells onto agar plate post incubation and count CFU according to the traditional approach to check whether the BacTiter-Glo assay yields similar survival trend for every experiment. Plating for CFU would also allow us to better compare our data with those from previous experiments conducted in our lab.

The construction of *wzz2* clean deletion mutants is essential for this project yet it happens to be the most difficult and time-consuming process in this project. Due to the time constraints of this project, I was only able to test sO1 and sO8 stains with their *wzz2* clean deletion mutants. However, with more mutants being constructed in the near future, our lab will be able to repeat the serum resistance experiment and the Biolog experiment on a greater number of serotype strains and to compare the effect of *wzz2* deletion on survival. We will also be able to incorporate better control in many of the experiments conducted for this project with the use of *wzz2* clean

deletion mutant of PAO1 instead of the transposon mutant. Another experiment that is worth considering is the minimum inhibitory concentration (MIC) test of antibiotic susceptibility. We could test if the deletion of *wzz2* renders certain serotype strains more susceptible to the antibiotics that are known to have activity against *P. aeruginosa*.

We were unable to make any conclusion across different serotypes from the data collected in this project because each serotype strain has a different strain background. To address this problem, we could introduce the entire O antigen gene locus of each serotype to the same strain background via the transfer of a plasmid[16]. If all serotype specific O antigen can be expressed under the same strain background, we will be able to determine how the production of O antigen contributes to the survival of each respective serotype in isogenic strains.

Given that O antigen is a major virulence factor of *P. aeruginosa*, the study of very long O antigen, which locates on the outermost portion of the bacteria, may help develop strategies against infections caused by *P. aeruginosa* and provide a better outcome for people who are infected.

METHODS

Construction of pEXG2-*Δwzz2* deletion plasmid and generation of mutants. To generate pEXG2-Δ*wzz2* 1000 bps of the upstream and downstream regions of *wzz2* was PCR amplified from PAO1 genomic DNA using ck01/ck02 and ck03/ck04, respectively. These two fragments were then inserted into HindIII digest pEXG2 using isothermal assembly (Gibson Assembly Master Mix, New England BioLabs) following the manufacturers protocol. The reaction was then transformed by heat-shock into chemically competent DH5α and selected for on

gentamicin. Plasmids were extracted (Qiagen Miniprep Kit) from isolated colonies and PCR verified using oAC91/oAC92. Plasmid pEXG2-Δ*wzz2* was then transformed into chemically competent SM10 by heat-shock. SM10 containing pEXG2-Δ*wzz2* was conjugated with each of the serotype strains following the puddle-mating protocol, in a 3:1 ratio as previously described[17]. After sucrose counterselection, single colonies were patched onto LA and LA containing gentamicin. Gentamicin sensitive colonies were screened for loss of *wzz2* by single-colony PCR using primers ck05/ck06. A complete list of strains, plasmids, and primers is available in Table 3.

Protein and LPS isolation. Colonies from an overnight LA plate were resupsended in LB and normalized to an OD_{600} of 1.0 and then 1 ml was pelleted. Protein was prepared resuspending the pellet in 50 µl lysis stock buffer (20 mM Tris pH 7.5, 1 mM EDTA, 10 mM $MgCl_2$) containing 10 µg/mL DNase, 10 µg/mL RNase, and 1X ProBlock Protease Inhibitor (Goldbio) and incubated at 37°C for 10 min. Next, 50 µl of 2x Laemmli buffer (Biorad) was added and then the samples were boiled in a water bath for 5 min. Samples were stored at -20°C and then boiled for 5 mins before every use. LPS was prepared by resuspending pelleted bacteria were in 200 µl of 1x Laemmli buffer (Biorad). This suspension was boiled in a water bath for 15 minutes and allowed to cool to room temperature. Next, 10 µl of 10 mg/ml Proteinase K was added to each sample, incubated at 59°C for 3 hours, and then stored at -20°C. After storage, the sample was boiled for 5 mins before every use.

Pro-Q staining. 15 µl of sample was loaded into each well and then separated by SDS-PAGE in a 10% Mini-PROTEAN TGX gel (Bio-Rad) along with CandyCane glycoprotein ladder

(Thermo). LPS was then stained using Pro-Q 300 Glycoprotein Stain (Thermo) according to the manufacturer's instructions except that the initial fixation step and each wash step was repeated three times (instead of twice). All steps were performed at room temperature and excess volume of fix and wash solutions were used per gel in each step. In summary, the gel was immediately incubated, rocking in a large glass container, in ~100 ml of fix solution (50% methanol and 5% acetic acid in H_2O) for 45 min. This step was repeated two more times in fresh fix solution. Next, the gel was incubated in ~100 ml wash solution (3% glacial acetic acid in H_2O) for 20 min. This step was repeated two more times in fresh wash solution. Next, the gel was incubated in 25 ml of oxidizing solution (provided with kit) for 30 min and then washed again three times in wash solution as described above. To stain, the Pro-Q Emerald 300 stock solution was diluted 50-fold into Pro-Q Emerald 300 staining buffer (both provided with kit) and the gel was incubated in 25 ml of this stock/buffer solution, rocking in the dark (glass container was wrapped in aluminum foil), for 2 hrs. Finally, the gel was washed once in ~100 ml of wash solution for 20 min. The images were taken with a UV transilluminator.

Western immunoblot. 15 μl of LPS or 10 μl of protein was loaded into each well and then separated by SDS-PAGE in a 10% Mini-PROTEAN TGX gel (Bio-Rad) along with Precision Plus All Blue Protein ladder (Bio-Rad). The LPS was then transferred to a PVDF membrane and blocked for 1 hour, at room temperature, in PBS-T containing 5% milk. Next, specific primary antibody for each serotype (Table 2) was incubated in a 1:5,000 dilution in PBS-T containing 3% BSA overnight at 4°C. Secondary α-rabbit-HRP IgG (Sigma; α-mouse-HRP IgG was used for EF-Tu) was incubated in a 1:10,000 dilution in PBS-T containing 3% BSA for 1 hour at room

temperature. The blot was visualized using Pierce ECL Western Blotting Substrate (Thermo) according to the manufacturer's instructions.

Serum assay. Single colonies grown overnight in LB were back-diluted 1:20 into fresh LB and grown, rolling at 37ºC, for 2 hours. At the same time, the BacTiter-Glo (Promega) reagent was equilibrated to room temperature and the normal human serum (NHS) and heat-inactivated (HI) serum (inactivated by heating at 56°C for 1 hour) was thawed on ice. After 2 hrs, the subcultured bacteria were spun down (5 min at 4,500 x g) and the pellet was resuspended in 1 ml 1X PBS (filter-sterilized). The OD_{600} was taken of the cell suspension and then normalized to 5 x 10^6 bacteria, assuming that an OD_{600} of 1.0 is equivalent to 1 x 10^9. 80 μl of cell suspension was added to a 96-well V-bottom plate in triplicate. Next, either 20 μl PBS, 20 μl NHS, or 20 μl HI serum was added to each well and mixed gently and incubated at 37ºC for desired time. At each time point (0, 1, and 2 hrs, a replicate plate was prepared for each time point being tested), the plate was spun down for 5 mins at 4,000 xg. The supernatant was removed from the plate by turning the plate upside down over a waste container. Without turning the plate back over, the plate was gently set on a paper towel to remove the rest of the liquid. Next, the pellets in each well were resuspended in 100 μl PBS and mixed by pipetting up and down. Finally, 50 μl of BacTiter-Glo reagent was added to each well and was shaken for 5 min at room temperature in the Synergy-H1 microplate reader. Luminescence was then measured to obtain relative fluorescent units (RFUs). Relative survival was calculated as: [(final RFU in serum - final RFU in PBS) / (initial RFU in PBS)] *100.

Growth curves. Growth curves were performed in 3 ml LB at 37ºC, with continuous shaking, in a 6-well plate using the Synergy-H1 microplate reader.

Biolog phenotypic microplate assay. Bacteria were grown in LB, shaking at 37ºC, until turbid. Cell suspension was then added to IF-0 (provided by the manufacturer) to 42% T (transmittance). The suspension was then added to IF-0+dye (provided by the manufacturer) in a 1:5 dilution and gently mixed to avoid creating air bubbles. Next, this suspension was added to IF-10+dye (provided by the manufacturer) in a 1:200 dilution and mixed gently. 100 µl of final suspension was added to each well of Biolog plates PM9 and PM10. These plates were then incubated in the Biolog for 48 hrs at 37ºC. After run, the Kinetic Analysis Software and Parametric Software provided by Biorad were used to analyze the data.

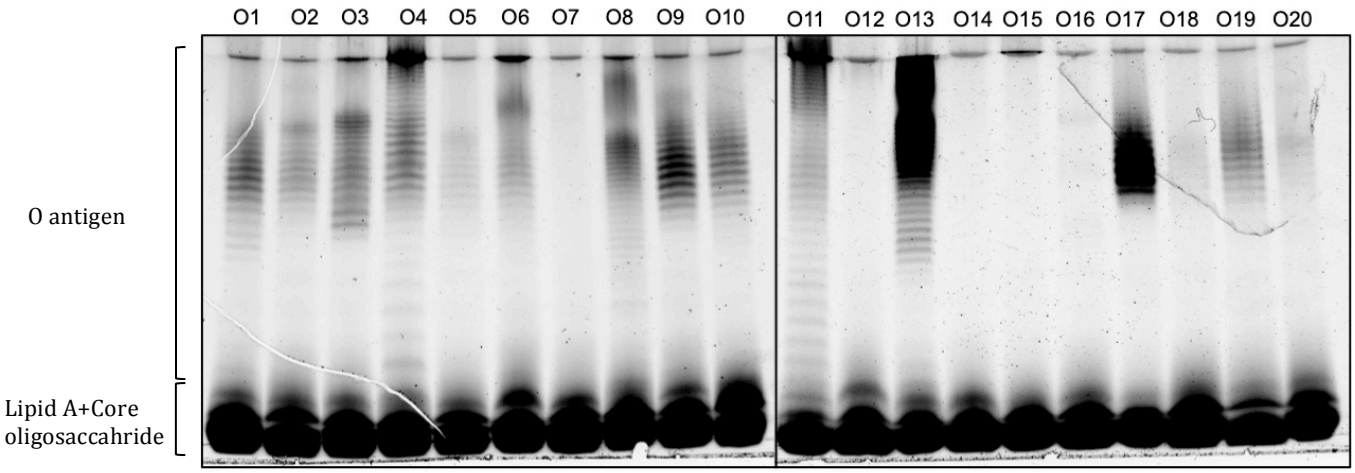

Figure 2. Lipopolysaccharide (LPS) production of serotype O1-O20 *P. aeruginosa* strains. Lipopolysaccharide (LPS) was isolated from whole cells grown in overnight cultures and separated by electrophoresis on a 12% SDS-PAGE gel. A Pro-Q Emerald 300 LPS stain kit was used to stain the entire LPS molecule, including the lipid A, the core, and the O antigen that is characterized based on the ladder pattern, which is distinctive of each serotype. The dark spots located at the bottom of the gel represent the lipid A plus core oligosaccharide portion of the LPS molecule, whereas each individual band in the lane represents an additional sugar repeat unit. Bands were visualized using an UV transilluminator. Each serotype is depicted above each lane.

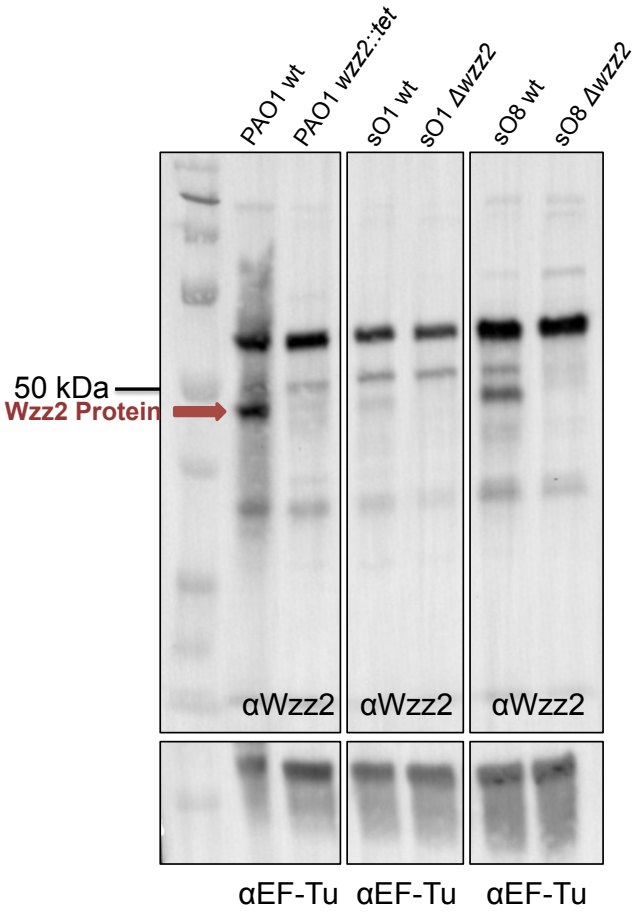

Figure 3. Deletion of *wzz2* ceases the production of Wzz2 protein in PAO1, serotype O1, and serotype O8 *P. aeruginosa* strains. All proteins were extracted from PAO1, serotype O1 (sO1), and serotype O8 (sO8) cells and their respective *wzz2* mutants and were separated by electrophoresis on a 10% SDS-PAGE gel. PAO1 *wzz2::tet* transposon mutant from the PAO1 library were used as a positive control since it is known that this strain does not produce Wzz2. These proteins were then transferred from the gel to a PVDF membrane, blocked, and then incubated with polyclonal antibody against Wzz2 protein. Bands were visualized using αRabbit-HRP IgG secondary antibody and chemiluminescence substrate. Wzz2 has a molecular weight of around 48 kDa. We confirmed the clean deletion of *wzz2* in sO1 and sO8 mutants since they do not produce Wzz2. EF-Tu is a highly conserved protein in bacteria, which is used here as the loading control.

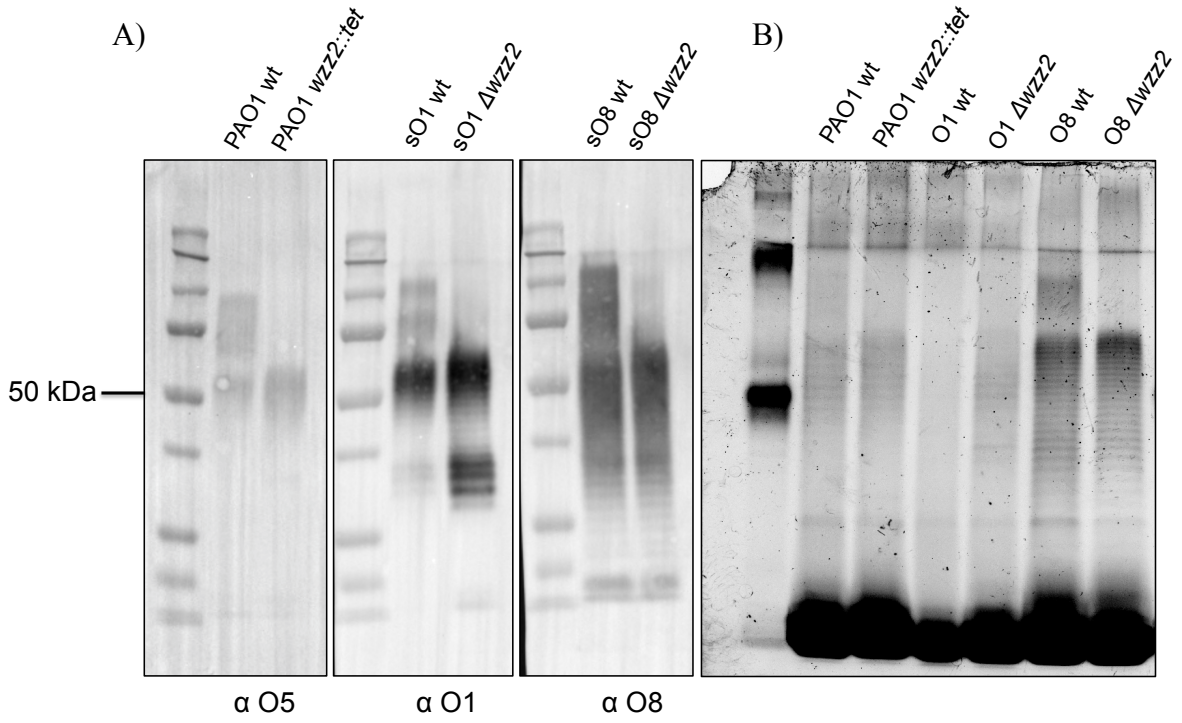

Figure 4. The effect of *wzz2* deletion on the production of serotype O antigen (A) and total LPS (B) in PAO1, serotype O1, and serotype O8 *P. aeruginosa* strains. A) A western immunoblot was performed on PAO1, serotype O1 (sO1), serotype O8 (sO8) wild type strains and their respective *wzz2* mutants as described in Figure 3, but with serotype specific antibodies being used. Again, PAO1 *wzz2::tet* transposon mutant from the PAO1 library was used as a positive control since it is known that this strain loses the expression of very long O antigen when *wzz2* is disrupted. B) Pro-Q staining was performed on the same set of wild type and mutant strains to examine the impact of *wzz2* deletion on the production of LPS. Loaded in the leftmost lane of the Pro-Q gel is the CandyCane glycoprotein molecular weight standards, with the top dark band weighted around 82 kDa and the bottom band weighted around 42 kDa.

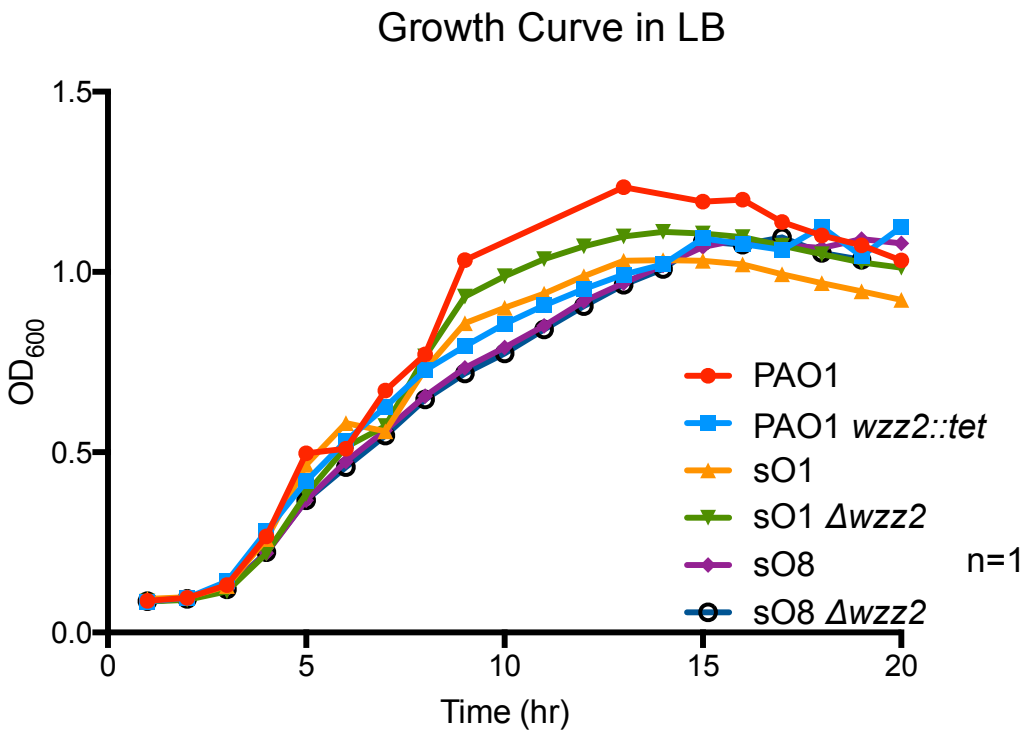

Figure 5. Growth curves of PAO1, serotype O1, and serotype O8 strains with their respective *wzz2* mutants in LB. Overnight cultures grown in LB were back-diluted to an OD_{600} of 0.01, and were then allowed to grow at 37°C for 20 hours in the Biotek Synergy H1 microplate reader with continuous orbital shaking. An OD_{600} measurement was taken by the plate reader every 20 minutes throughout the duration of the experiment. Data collected from individual strains are plotted in the same graph with respect to time. There was no significant difference in growth between wild type and mutant strains. This experiment represents a biological replicate on one.

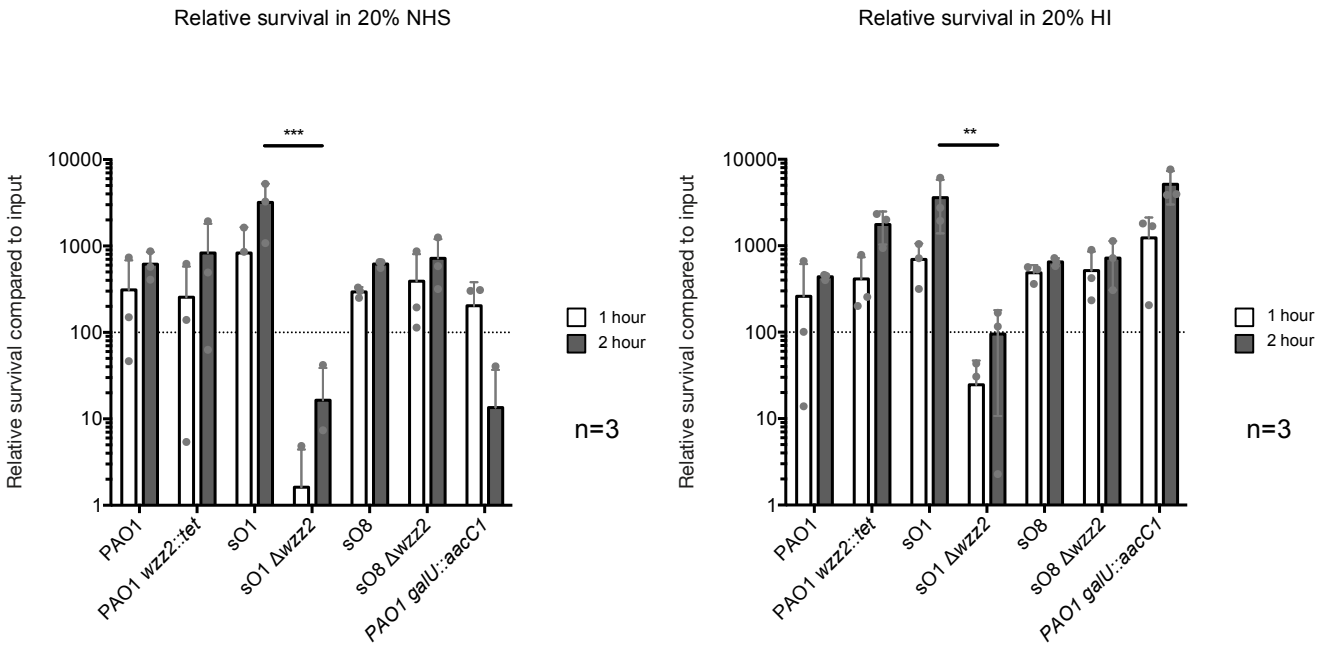

Figure 6. Deletion of *wzz2* in serotype O1 strain results in sensitivity to normal human serum. However, deletion of *wzz2* does not affect the serum resistance of PAO1 or the serotype O8 strain. Overnight cultures were back diluted into fresh LB media and allowed to grow at 37°C for 2 hours. Cells were pelleted from each back-diluted culture, with supernatants removed, and were then resuspended in 1X PBS. Cultures were normalized to the same number of bacteria per unit volume, and were allowed to grow in 20% of either (A) normal human serum (NHS) or (B) heat-inactivated serum (HI) for one or two hours as shown in the figure. BacTiter-Glo reagent was used to measure the viability of cells based on the amount of ATP released from each reaction. Relative survivals compared to the input are plotted on a base 10 logarithmic scale. The dotted line at 100% survival divides strains into two categories: serum sensitive (if relative survival fails to reach the line) and serum resistant (if relative survival exceeds the line). Three biological replicates are included for each strain, with individual replicate being represented as a gray dot. Error bars represent standard deviation of the mean. Significance was determined by a two-way ANOVA with Tukey's multiple comparisons analysis.

PM9 (Osmolytes)	PAO1 vs. PAO1 *wzz2::tet*	sO1 vs. sO1 *Δwzz2*	sO8 vs. sO8 *Δwzz2*
Conditions common to more than one serotypes	Sodium formate 4%		Sodium formate 4%
	Sodium formate 5%		Sodium formate 5%
	Sodium formate 6%		Sodium formate 6%
Conditions specific to each individual serotype	NaCl 5%		
	NaCl 5.5%		
	NaCl 6% + N-N Dimethyl Glycine		
	NaCl 6% + Sarcrosine		
	NaCl 6% + Dimethyl sulphonyl propionate		
	NaCl 6% + MOPS		
	NaCl 6% + Ectonine		
	NaCl 6% + Choline		
	NaCl 6% + Phosphoryl Choline		
	NaCl 6% + Creatine		
	NaCl 6% + L-Carnitine		
	NaCl 6% + N-Acetyl L-Glutamine		
	NaCl 6% + β-Glutamic Acid		
	NaCl 6% + γ-Amino-N-Butyric Acid		
	NaCl 6% + Glutathione		
	NaCl 6% + Glycerol		
	NaCl 6% + Trehalose		
	NaCl 6% + Trimethylamine-N-oxide		
	NaCl 6% + Trimethylamine		
	NaCl 6% + Octopine		
	Urea 5%		
	Urea 6%		
	Sodium Lactate 2%		
	Sodium Lactate 3%		
	Sodium Lactate 4%		Sodium Lactate 8%

PM10 (pH)	PAO1 vs. PAO1 *wzz2::tet*	sO1 vs. sO1 *Δwzz2*	sO8 vs. sO8 *Δwzz2*
Conditions common to more than one serotypes	pH 4.5	pH 4.5	pH 4.5
	pH 4.5 + L-Alanine	pH 4.5 + L-Alanine	
	pH 4.5 + L-Phenylalanine	pH 4.5 + L-Phenylalanine	
	pH 4.5 + L-Citrulline		pH 4.5 + L-Citrulline
	pH 4.5 + DL-a-Amino-n-Butyric acid		pH 4.5 + DL-a-Amino-n-Butyric acid
		pH 4.5 + L-Lysine	pH 4.5 + L-Lysine
		pH 4.5 + L-Proline	pH 4.5 + L-Proline
Conditions specific to each individual serotype	pH 4.5 + L-Serine	pH 4.5 + L-Aspartic acid	pH 4.5 + Urea
	pH 4.5 + L-Valine	pH 4.5 + L-Glutamic acid	pH 9.5 + Phenylethylamine
	pH 4.5 + Hydroxy-L-Proline	pH 4.5 + L-Glutamine	
	pH 4.5 + L-Cysteic acid	pH 4.5 + Glycine	
	pH 9.5 + L-Tryptophan	pH 4.5 + L-Arginine	
		pH 4.5 + L-Homoserine	
		pH 4.5 + 5-Hydroxy-L-Lysine	

Table 1. Deletion of *wzz2* alters resistance and sensitivity to different osmotic pressures and pHs, but this is dependent on serotype and strain background. Cells were prepared by back-diluting overnight cultures into manufacturer provided solutions following the manufacturers procedure described in the method section. The final suspension was divided equally to all wells in Biolog™ plates PM9 (containing differing osmolytes) and PM10 (containing differing pHs). Plates were incubated in the OmniLog® machine for 48 hours at 37°C. The growth of cells in each well was monitored by the OmniLog® machine over the duration of the experiment. Data were compared across each wild type strain and its respective mutant, with only the conditions showing a significant difference in metabolism summarized in this table. Conditions labeled in

red denote a higher metabolism of the mutant strain compared to the wild type, whereas conditions labeled in blue denote a higher metabolism of the wild type compared to the mutant strain. Conditions that are common to two or more serotypes are listed first in the upper half of the table and those specific to individual serotypes are listed in the lower half.

Serotype	Homma scheme	Polyvalent Groups
O1	I	1
O2	B	2
O3	A	1
O4	F	3
O5	B	2
O6	G	3
O7	C	1
O8	C	1
O9	D	3
O10	H	1
O11	E	3
O12	L	1
O13	K	2
O14	K	2
O15	J	2
O16	B	2
O17	N	3
O18	B	2
O19	H	1
O20	B	2
DENKA SEIKEN Key Scientific Products		
Accurate Chemical & Scientific. Westbury, N.Y.		

Table 2. Polyvalent and primary antibodies to detect serotype O antigen.

Name	Description	Reference
E. coli		
DH5a	plasmid maitenance	Invitrogen
SM10	bi-parental mating and conjugations	Simon et. al. 1983
P. aeruginosa		
PAO1		Hancock and Carey, 1979
PAO1 *wzz1::tet*	*wzz1* transposon mutant PW6291	Held et al. 2012, Jacobs et al. 2003
PAO1 *wzz2::tet*	*wzz2* transposon mutant PW2707	Held et al. 2012, Jacobs et al. 2003
PAO1 *galU::aacC1*	*galU* mutant disrupted by gentamicin resistant marker	Priebe et al. 2004
Pa. serotype 001	serotype O1 strain	ATCC
Pa. serotype 002	serotype O2 strain	ATCC
Pa. serotype 003	serotype O3 strain	ATCC
Pa. serotype 004	serotype O4 strain	ATCC
Pa. serotype 005	serotype O5 strain	ATCC
Pa. serotype 006	serotype O6 strain	ATCC
Pa. serotype 007	serotype O7 strain	ATCC
Pa. serotype 008	serotype O8 strain	ATCC
Pa. serotype 009	serotype O9 strain	ATCC
Pa. serotype 010	serotype O10 strain	ATCC
Pa. serotype 011	serotype O11 strain	ATCC
Pa. serotype 012	serotype O12 strain	ATCC
Pa. serotype 013	serotype O13 strain	ATCC
Pa. serotype 014	serotype O14 strain	ATCC
Pa. serotype 015	serotype O15 strain	ATCC
Pa. serotype 016	serotype O16 strain	ATCC
Pa. serotype 017	serotype O17 strain	ATCC
Pa. serotype 018	serotype O18 strain	ATCC
Pa. serotype 019	serotype O19 strain	ATCC
Pa. serotype 020	serotype O20 strain	ATCC
Pa. serotype O1 △*wzz2*	serotype O1 strain with *wzz2* deletion	This study
Pa. serotype O8 △*wzz2*	gentamicin resistant PAO1	This study
Plasmids		
pEXG2-△*wzz2*	*wzz2* deletion plasmid	This study
Primers		
ck01	GGAAGCATAAATGTAAAGCAAGGCCCACCAGAGTGACTTTTAC	This study
ck02	ACCTTTGATCACCAACGTCCTTTTCGATAGG	This study
ck03	GGACGTTGGTGATCAAAGGTGCGCCCCC	This study
ck04	AGAGTCGACCTGCAGAAGCTAAGTCGGTTCCTGCCCTG	This study
ck05	GTGGTGCTGCTCAACGTACA	This study
ck06	AATAGGCTGGATTTCGGTTG	This study
oAC091	CCACACATTATACGAGCCGGAAGC	This study
oAC092	GGTACCGAATTCGAGCTCGAGC	This study

Table 3. Strains, plasmids, and primers used in this study.

REFERENCES

1. Crone, S. *et al.* The environmental occurrence of Pseudomonas aeruginosa. *APMIS Acta Pathol. Microbiol. Immunol. Scand.* (2019) doi:10.1111/apm.13010.
2. Bauernfeind, A. *et al.* Qualitative and quantitative microbiological analysis of sputa of 102 patients with cystic fibrosis. *Infection* **15**, 270–277 (1987).
3. Jarvis, W. R. & Martone, W. J. Predominant pathogens in hospital infections. *J. Antimicrob. Chemother.* **29 Suppl A**, 19–24 (1992).
4. Kintz, E., Scarff, J. M., DiGiandomenico, A. & Goldberg, J. B. Lipopolysaccharide O-Antigen Chain Length Regulation in Pseudomonas aeruginosa Serogroup O11 Strain PA103. *J. Bacteriol.* **190**, 2709–2716 (2008).
5. Lam, J. S., Taylor, V. L., Islam, S. T., Hao, Y. & Kocíncová, D. Genetic and Functional Diversity of Pseudomonas aeruginosa Lipopolysaccharide. *Front. Microbiol.* **2**, (2011).
6. Islam, S. T. & Lam, J. S. Synthesis of bacterial polysaccharides via the Wzx/Wzy-dependent pathway. *Can. J. Microbiol.* **60**, 697–716 (2014).
7. Burrows, L. L., Chow, D. & Lam, J. S. Pseudomonas aeruginosa B-band O-antigen chain length is modulated by Wzz (Ro1). *J. Bacteriol.* **179**, 1482–1489 (1997).
8. Daniels, C., Griffiths, C., Cowles, B. & Lam, J. S. Pseudomonas aeruginosa O-antigen chain length is determined before ligation to lipid A core. *Environ. Microbiol.* **4**, 883–897 (2002).
9. Thrane, S. W., Taylor, V. L., Lund, O., Lam, J. S. & Jelsbak, L. Application of Whole-Genome Sequencing Data for O-Specific Antigen Analysis and In Silico Serotyping of Pseudomonas aeruginosa Isolates. *J. Clin. Microbiol.* **54**, 1782–1788 (2016).
10. Cross, A. R. & Goldberg, J. B. Remodeling of O Antigen in Mucoid Pseudomonas aeruginosa via Transcriptional Repression of wzz2. *mBio* **10**, (2019).

11. Raymond, C. K. *et al.* Genetic Variation at the O-Antigen Biosynthetic Locus in Pseudomonas aeruginosa. *J. Bacteriol.* **184**, 3614–3622 (2002).

12. Makin, S. A. & Beveridge, T. J. The influence of A-band and B-band lipopolysaccharide on the surface characteristics and adhesion of Pseudomonas aeruginosa to surfaces. *Microbiol. Read. Engl.* **142 (Pt 2)**, 299–307 (1996).

13. Priebe, G. P. *et al.* The galU Gene of Pseudomonas aeruginosa is required for corneal infection and efficient systemic spread following pneumonia but not for infection confined to the lung. *Infect. Immun.* **72**, 4224–4232 (2004).

14. Nikaido, H. & Vaara, M. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev.* **49**, 1–32 (1985).

15. Phenotype MicroArrays for Microbial Cells – Biolog. https://www.biolog.com/products-portfolio-overview/phenotype-microarrays-for-microbial-cells/.

16. Dean, C. R. & Goldberg, J. B. The wbpM gene in Pseudomonas aeruginosa serogroup O17 resides on a cryptic copy of the serogroup O11 O antigen gene locus. *FEMS Microbiol. Lett.* **187**, 59–63 (2000).

17. Hmelo, L. R. *et al.* Precision-engineering the Pseudomonas aeruginosa genome with two-step allelic exchange. *Nat. Protoc.* **10**, 1820–1841 (2015).