Trehalose Biosynthesis Promotes Pseudomonas aeruginosa Pathogenicity in Plants

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Trehalose Biosynthesis Promotes *Pseudomonas aeruginosa* Pathogenicity in Plants

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**Abstract**

*Pseudomonas aeruginosa* strain PA14 is a multi-host pathogen that infects plants, nematodes, insects, and vertebrates. Many PA14 factors are required for virulence in more than one of these hosts. Noting that plants have a fundamentally different cellular architecture from animals, we sought to identify PA14 factors that are specifically required for plant pathogenesis. We show that synthesis by PA14 of the disaccharide trehalose is required for pathogenesis in Arabidopsis, but not in nematodes, insects, or mice. In-frame deletion of two closely-linked predicted trehalose biosynthetic operons, *treYZ* and *treS*, decreased growth in Arabidopsis leaves about 50 fold. Exogenously co-inoculated trehalose, ammonium, or nitrate, but not glucose, sulfate, or phosphate suppressed the phenotype of the double Δ*treYZΔtreS* mutant. Exogenous trehalose or ammonium nitrate does not suppress the growth defect of the double Δ*treYZΔtreS* mutant by suppressing the plant defense response. Trehalose also does not function intracellularly in *P. aeruginosa* to ameliorate a variety of stresses, but most likely functions extracellularly, because wild-type PA14 rescued the in vivo growth defect of the Δ*treYZΔtreS* in trans. Surprisingly, the growth defect of the double Δ*treYZΔtreS* double mutant was suppressed by various Arabidopsis cell wall mutants that affect xyloglucan synthesis, including an *xxt1xxt2* double mutant that completely lacks xyloglucan, even though xyloglucan mutants are not more susceptible to pathogens and respond like wild-type plants to immune elicitors. An explanation of our data is that trehalose functions to promote the acquisition of nitrogen-containing nutrients in a process that involves the xyloglucan component of the plant cell wall, thereby allowing *P. aeruginosa* to replicate in the intercellular spaces of a leaf. This work shows how *P. aeruginosa*, a multi-host opportunistic pathogen, has repurposed a highly conserved “house-keeping” anabolic pathway (trehalose biosynthesis) as a potent virulence factor that allows it to replicate in the intercellular environment of a leaf.


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**Introduction**

The ubiquitous bacterium *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that infects a wide diversity of hosts. For example, *P. aeruginosa* strain PA14 is infectious in several model genetic hosts including the plant *Arabidopsis thaliana* [1], the insect *Drosophila melanogaster* [2], and the nematode *Caenorhabditis elegans* [3]. Using these model hosts, we and others have sought to identify PA14 virulence-related factors that play key roles in pathogenesis with the goal of elucidating conserved mechanisms underlying the pathogenic process and to determine whether the spectrum of virulence-related genes in a multi-host opportunistic pathogen are distinct from the virulence genes in more specialized pathogens [1–7]. Our work to date suggests that PA14 virulence depends primarily on genes that are part of a conserved *P. aeruginosa* genome [8] rather than on an arsenal of host-specific virulence-related factors [8,9].

One of the most unusual and unexpected features of *P. aeruginosa* is its ability to infect both plants and animals. Because plant cells are distinguished from metazoan cells primarily by their rigid and tough cellulosic walls, we reasoned that *P. aeruginosa* pathogenesis in plants may rely on plant-specific virulence factors related to the plant cell walls. Presumably as a consequence of these tough plant cell walls, most bacterial foliar pathogens replicate extracellularly in intercellular spaces and subvert plant cellular processes such as sugar transporters to obtain nutrients from mesophyll cells rather
Author Summary

*Pseudomonas aeruginosa* is an opportunistic human bacterial pathogen that infects a wide range of plants and animals, including the model laboratory plant *Arabidopsis thaliana*. *P. aeruginosa* utilizes many of the same virulence-related factors to infect both plants and animals. However, because plants have fundamentally different cellular architecture than animals, we hypothesized that *P. aeruginosa* synthesizes specific factors required for infecting plants but not animals. We found that synthesis of the sugar molecule trehalose, an unusual dimer of glucose, is required for plant but not animal pathogenesis. Although *P. aeruginosa* mutants defective in trehalose synthesis are non-pathogenic in Arabidopsis, *Arabidopsis* mutants that lack the polysaccharide xyloglucan in their cell walls can be infected by *P. aeruginosa* trehalose mutants. Moreover, application of ammonium nitrate overcomes the requirement for trehalose for infecting an Arabidopsis leaf. Our data suggest that trehalose promotes the acquisition of nitrogen-containing nutrients, thereby allowing *P. aeruginosa* to replicate in the nutrient-poor intercellular spaces in a leaf. This work shows how an opportunistic pathogen has repurposed a highly conserved “house-keeping” function (trehalose biosynthesis) as a potent virulence factor.

determine whether canonical cell wall degrading enzymes including cellulases, xylanases, and pectinases are encoded in the genome. In susceptible ecotypes (wild accessions) of *Arabidopsis*, *P. aeruginosa* PA14 causes soft-rot symptoms [1], typically caused by pathogens that secrete pectinases and other hydrolytic cell wall degrading enzymes. Moreover, PA14 infection causes extensive degradation of Arabidopsis mesophyll cell walls including the generation of “holes” approximately the diameter of *P. aeruginosa* through which the bacteria enter host cells [11]. We thus expected that the PA14 genome would encode a variety of cell wall degrading enzymes (CWDEs). However, our survey of the PA14 genome identified only a single, candidate cellulase, identified ambiguously as “cellulase/peptidase” (PA14_36500). Although PA14_36500 was upregulated two and three days post-inoculation in * planta*, correlating with the development of disease symptoms (Figure S1A), a transposon insertion in PA14_36500 (PA14_36500::MAR2xT7), in-frame deletion of the cellulase/peptidase gene (ΔPA14_36500), or in-frame deletion of a putative cellulase/peptidase operon (ΔPA14_36480-36520) did not cause a significant attenuation in virulence in Arabidopsis leaves (Table S1).

Because PA14_36500, which encodes the putative cellulase/peptidase, was induced during plant infection and because genes are often functionally clustered on bacterial genomes, we sought to identify genes adjacent to PA14_36500 that are co-regulated with PA14_36500. This led to the identification of a set of 38 genes (42.23 kb region; PA14_36375 to PA14_36830) spanning the cellulase/peptidase gene that is coordinately down-regulated in an *msfR* (multiple virulence factor regulator) mutant grown under various culture conditions [12,13]. Importantly, the quorum sensing-associated transcriptional regulator *MvfR* is required for maximum PA14 virulence in Arabidopsis [7]. Consistent with the in vitro transcriptional profiling data, cellulase/peptidase PA14_36500 expression was significantly reduced in * planta* in an *msfR* mutant (Figure S1B).

Besides the putative cellulase/peptidase, the PA14_36375–36830 42.23 kb region encodes putative glucanolytic enzymes (PA14_36590, PA14_36630, PA14_36640) as well as two closely linked predicted operons (http://www.pseudomonas.com), PA14_36570-36630 consisting of six genes, and PA14_36710-36740 consisting of three genes, referred to hereafter as the “treYZ” and “treS” operons, respectively, that encode enzymes involved in two different trehalose biosynthetic pathways (Figure 1; Table S2). TreY and TreZ convert maltodextrins into trehalose in a two-step enzymatic reaction [14], whereas TreS catalyzes conversion of maltose into trehalose in a single reaction [15] (Figure S2). In addition to *treY* (PA14_36605) and *treZ* (PA14_36580), the predicted *treYZ* operon contains *glgA* (PA14_36570), *malQ* (PA14_36590), hypothetical gene (PA14_36620) and *glgX* (PA14_36630). *glgA*, *malQ*, *glgX* encode enzymes with a putative role in α,1,4-linked glucon synthesis (*glgA*) and degradation (*malQ*, *glgX*), that could serve as precursors for trehalose synthesis. In addition to *treS*, the *treS* operon contains a predicted α-amylase (PA14_36740), and *glgB* (PA14_36710), a predicted α,1,4-branching enzyme (Figure 1).

The 42.23 kb PA14_36375–36830 region containing 38 genes is highly conserved among several sequenced *P. aeruginosa* strains that were examined and the *treYZ* and *treS* operons are conserved among pseudomonads in general (Table S2).

We utilized a previously constructed non-redundant PA14 transposon insertion mutant library [16] to determine whether particular PA14 genes in the 38-gene region promote pathogenesis in Arabidopsis. Among 16 transposon insertions in 16 different genes that were available in the library, two were significantly
attenuated in virulence. These mutants, with insertions in \( \text{glgA} \) and \( \text{treZ} \), exhibited a decrease in virulence of 20 and 16 fold, respectively, as measured by \textit{in planta} growth (Table S1). \( \text{glgA} \) and \( \text{treZ} \) are the first two genes in the \( \text{treYZ} \) operon, pointing to an important role for trehalose in the infectious process.

To further investigate whether the trehalose operons and/or other genes in the 38-gene cluster are required for virulence, we constructed an in-frame deletion of the entire 42.23 kb region (referred to hereafter as \( \Delta \text{42} \)) by homologous recombination. In contrast to insertions in \( \text{glgA} \) and \( \text{treZ} \), which exhibited at most a 20 fold decrease in growth compared to wild-type, the \( \Delta \text{42} \) mutant exhibited severe attenuation in virulence, affecting growth of \( \text{PA14} \) infiltrated into Arabidopsis leaves about 120 fold and preventing the appearance of pathogenic symptoms (Figure 2A). Similar results were obtained with four independently constructed \( \Delta \text{42} \) mutants (data not shown), demonstrating that the non-pathogenic phenotype was caused by the deletion of the 42.23 kb region. Importantly, the \( \Delta \text{42} \) mutant does not appear to be slow growing or to be generally deficient in a variety of phenotypes associated with virulence in \( \text{P. aeruginosa} \). The \( \Delta \text{42} \) deletion mutant was not auxotrophic, grew at the same rate as wild-type \( \text{PA14} \) in a variety of minimal and rich media, and had no observable phenotypes with respect to the production of pyocyanin (Figure S3), motility, or biofilm formation (Table S3), and similar results were obtained

![Figure 1. Annotation of a 42.23 kb region of the \( \text{P. aeruginosa} \) PA14 genome encoding 38 genes (PA14_36375–36830) and schematic representation of transposon and deletion mutants used in this study. Colors depict trehalose biosynthetic genes (red), glucanolytic genes (blue) and glucan synthesis genes (yellow). Numbers below the genes correspond to PA14 gene locus tags (http://ausubellab.mgh.harvard.edu/pa14sequencing). On the top of the Figure, vertical arrows indicate the positions of MAR20x77 transposon insertions [16], and on the bottom, horizontal arrows denote the extent of in-frame deletion mutants. doi:10.1371/journal.ppat.1003217.g001](https://doi.org/10.1371/journal.ppat.1003217.g001)

![Figure 2. Attenuation of \( \Delta \text{42} \) and trehalose biosynthetic mutants in Arabidopsis leaves and suppression of attenuation of trehalose mutants with exogenous trehalose. (A) Columbia (Col-0) ecotype plants were infiltrated with \( \text{PA14} \) wild-type or \( \Delta \text{42} \). Leaves were harvested 3 days after infiltration and bacterial counts determined as described in Materials and Methods. Representative photographs of infected leaves were taken 3 days after infiltration. Four independently constructed \( \Delta \text{42} \) mutants exhibited the same dramatic non-pathogenic phenotype as \( \text{PA14} \) infiltrated into Arabidopsis leaves about 120 fold and preventing the appearance of pathogenic symptoms (Figure 2A). Similar results were obtained with four independently constructed \( \Delta \text{42} \) mutants (data not shown), demonstrating that the non-pathogenic phenotype was caused by the deletion of the 42.23 kb region. Importantly, the \( \Delta \text{42} \) mutant does not appear to be slow growing or to be generally deficient in a variety of phenotypes associated with virulence in \( \text{P. aeruginosa} \). The \( \Delta \text{42} \) deletion mutant was not auxotrophic, grew at the same rate as wild-type \( \text{PA14} \) in a variety of minimal and rich media, and had no observable phenotypes with respect to the production of pyocyanin (Figure S3), motility, or biofilm formation (Table S3), and similar results were obtained](https://doi.org/10.1371/journal.ppat.1003217.g002)
with a second independently-constructed Δ42 mutant (Figure S3; Table S3). Because independently-constructed Δ42 mutants exhibited the same phenotypes, one of the Δ42 mutants was chosen for subsequent experiments.

We next constructed several smaller deletions within the 42 kb region to determine which of the 38 encoded genes are primarily responsible for the severe avirulent phenotype of Δ42: ΔPA14_36570-36560 (sub-region I) contains a deletion of the cellulase/peptidase operon and several adjacent genes, and ΔPA14_36570-36700 (sub-region II) and ΔPA14_36710-36830 (sub-region III) contain deletions of the treZ operon, respectively. Deleting either the putative cellulase/peptidase operon or the treZ operon caused a 5.9 fold decrease in growth (Figure S4), whereas deletion of sub-region III that contains the treS operon caused a 5.9 fold decrease in growth (Figure S4). These experiments suggested that the treYZ operons play a significant role in PA14 pathogenesis in Arabidopsis.

Further evidence suggesting an important role for trehalose biosynthesis in plant pathogenesis was obtained by measuring the levels of trehalose synthesized in planta. To corroborate the involvement of the trehalose genes in plant pathogenesis we constructed ΔPA14_36570-36630 (ΔtreYZ) and ΔPA14_36710-36740 (ΔtreS) containing deletions of only the two putative operons containing the treYZ and treS genes, respectively, and ΔPA14_36570-36630:PA14_36710-36740 (ΔtreYZΔtreS) containing deletions of both of the trehalose isomerase-encoding operons (Figure 1). Deleting either the putative treZ or the treS operons (Figure 2B) had approximately the same effects as deleting the more extensive corresponding subregions II or III, respectively (Figure S4), and deleting both trehalose operons resulted in an approximately 50 fold decrease in virulence compared to the Δ42 mutant (Figure 2B). These data show that the treYZ and treS operons play a key role in pathogenesis in Arabidopsis leaves, but that genes in the 42 kb region in addition to those involved in trehalose biosynthesis also play a role in plant pathogenesis.

Further evidence suggesting an important role for trehalose biosynthesis in plant pathogenesis was obtained by measuring the levels of trehalose synthesized in vitro by PA14 wild-type and trehalose biosynthetic mutants. While wild-type PA14 synthesized readily detectable levels of trehalose, there was approximately 50% less trehalose in the ΔtreS mutant, and there were undetectable levels of trehalose in the ΔglgA, ΔtreZ, ΔtreYZ, ΔtreYZΔtreS, and ΔΔ42 mutants (Table 1). These data show that the treYZ and treS operons encode enzymes involved in trehalose biosynthesis. These data also suggest that treS operon may be dependent on treYZ for trehalose production, as reported previously [17]. When we compared the levels of trehalose synthesized in vitro (Table 1) and the extent of growth of the various strains in Arabidopsis leaves (Figure 2B; Table S1), we found an excellent positive correlation coefficient ($R^2 = 0.87$).

Importantly, we found that co-infection of the PA14 trehalose mutants and pure trehalose essentially completely suppressed the avirulent phenotypes of the ΔtreYZ ΔtreS and ΔtreYZΔtreS mutants and mostly suppressed the phenotype of the Δ42 mutant (Figures 2B and 2C). However, 0.25 mg/ml trehalose also rescued the Δ42 mutant almost as well as 2.5 mg/ml, and 0.025 mg/ml trehalose partially suppressed the growth defect of the Δ42 mutant (Figure 2C). These data indicated a requirement for trehalose for PA14 virulence in planta, potentially at physiologically relevant concentrations.

In summary, the data in this section shows that the ΔtreYZ ΔtreS, and ΔtreYZΔtreS mutants are less virulent in planta, that they either synthesize undetectable (ΔtreYZ and ΔtreYZΔtreS) or reduced (ΔtreS) levels of trehalose, that their level of virulence positively correlates with the level of trehalose they synthesize, and that their reduced virulence phenotype can be suppressed by exogenous trehalose. These data demonstrate that the virulence deficient phenotypes of the ΔtreYZ ΔtreS, and ΔtreYZΔtreS mutants are a consequence of the inability of these strains to synthesize trehalose, thereby correlating the genotype of these mutants with their avirulent phenotypes.

### Table 1. Trehalose levels in the trehalose mutants.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Trehalose (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. PA14_36570::MAR2xT7 (glgA)</td>
<td>not detected</td>
</tr>
<tr>
<td>2. PA14_36580::MAR2xT7 (treZ)</td>
<td>not detected</td>
</tr>
<tr>
<td>3. ΔPA14_36570-36630 (ΔtreYZ)</td>
<td>not detected</td>
</tr>
<tr>
<td>4. ΔPA14_36710-36740 (ΔtreS)</td>
<td>0.21±0.015</td>
</tr>
<tr>
<td>5. ΔPA14_36570-36630:PA14_36710-36740 (ΔtreYZΔtreS)</td>
<td>not detected</td>
</tr>
<tr>
<td>6. ΔPA14_36375-36830 (ΔA22)</td>
<td>not detected</td>
</tr>
<tr>
<td>7. WT</td>
<td>0.44±0.003</td>
</tr>
</tbody>
</table>

### Trehalose biosynthetic genes are not required for virulence in metazoans

As described in the Introduction, PA14 infection models have previously been established in *C. elegans* [3], *D. melanogaster* [18], and mice [19,20], as well as in other metazoans. Interestingly, the ΔtreYZΔtreS double trehalose mutant was not less virulent in a *C. elegans* killing model or in a murine acute pneumonia model. In fact, the ΔtreYZΔtreS appeared to be slightly more virulent in the metazoan hosts (Figure 3). Similar results were obtained with the ΔΔ42 mutant in these two models as well as in a *D. melanogaster* ingestion model and in a chronic oropharyngeal colonization model in transgenic mutant mice lacking the cystic fibrosis transmembrane conductance regulator protein (see Materials and Methods for the mutant description; Figure S5). These data suggest that trehalose appears to be specifically required for plant but not for metazoan pathogenesis. In the sections that follow, we considered several hypotheses concerning the role of trehalose in promoting the virulence of *P. aeruginosa* during the infectious process in plants but not in animals.

### Specific Arabidopsis cell wall mutants suppress the phenotype of PA14 trehalose mutants

Since a major difference between plant and animals cells is the plant celluloseic cell wall, we reasoned that trehalose may function in a process that involves the plant cell wall. Because PA14 infection in Arabidopsis leaves causes extensive degradation of mesophyll cell walls [11], we first investigated the possibility that trehalose enhances the activity of cell wall degrading enzymes (CWDs). We tested whether trehalose enhanced the activity of a variety of commercial CWDs to hydrolyze partially purified Arabidopsis cell walls in vitro to generate reducing sugars, which were measured using the Somogy-Nelson assay [21,22]. However, we were not able to conclusively demonstrate that trehalose enhanced the activity of the CWDs tested (data not shown).
We next reasoned that if trehalose interacts with the plant cell wall, specific Arabidopsis cell wall mutants might suppress the phenotype of the ΔtreYZtreS mutant. We tested the growth of wild-type PA14 and the ΔtreYZtreS mutant in several Arabidopsis cell wall mutants involved in xyloglucan (mur2-1, mur3-2, xxt1/xxt2), arabinose (mur4-1), or cellulose (mur10-2) synthesis. Remarkably, the ΔtreYZtreS mutant grew to the same titer as wild-type PA14 in an xxt1/xxt2 double mutant that completely lacks xyloglucan in its cell walls and in a mur4-1 mutant that has decreased levels of arabinose in pectins, xylans, and xyloglucans [23] (Figure 4). Similar results were obtained with the ΔI2 mutant; i.e., the Arabidopsis xxt1/xxt2 mutant completely suppressed and the mur4-1 mutant mostly suppressed the avirulent phenotype of the ΔI2 mutant (Figure S6).

We ruled out the possibility that the Arabidopsis cell wall mutants suppress the avirulent phenotype of the PA14 trehalose mutants simply because they are generally more susceptible to pathogen attack. As shown in Figure 5A, the cell wall mutants did not exhibit enhanced susceptibility to the P. syringae pv. tomato strain DC3000, a well-studied bona fide Arabidopsis pathogen. The Arabidopsis cell wall mutants were also not more susceptible to a DC3000 hrcC mutant (Figure 5B), which is greatly impaired in virulence, or to the bean pathogen P. syringae pv. phaseolicola strain 3121 (Figure 5C), which is not normally pathogenic in Arabidopsis. Consistent with these data, we also showed that the xxt1/xxt2 mutant, which exhibits the most severe cell wall defect of the Arabidopsis mutants tested, mounts a normal defense response when challenged with the flagellin peptide flg22 (Figure 5D). Flg22 elicits so-called “pattern triggered immunity” in Arabidopsis. When Arabidopsis leaves are pre-infiltrated with flg22, flg22 exerts a protective effect against subsequent infection with P. syringae DC3000 [24]. As shown in Figure 5D, flg22 elicits the same level of protection against P. syringae DC3000 in xxt1/xxt2 plants as in wild-type plants.

Ammonium and nitrate but not glucose or sucrose suppress the phenotype of trehalose mutants

As described in the Introduction, because bacterial plant pathogens primarily replicate in the intercellular spaces in a leaf, they need to acquire nutrients from plant mesophyll cells. We therefore tested whether trehalose may be involved in the acquisition of a variety of nutrient sources including carbon, nitrogen, sulfur and phosphorous. If this were the case, we reasoned that co-infiltration of particular nutrients with the ΔtreYZtreS or the ΔI2 mutant would suppress their non-pathogenic phenotypes.

Co-infiltration of the ΔtreYZtreS double mutant with glucose (Figure 6) or co-infiltration of the ΔI2 mutant with glucose or sucrose (Figure S7A) did not rescue the attenuated phenotype in the Arabidopsis leaf assay. These experiments showed that the

Figure 3. The ΔtreYZtreS mutant is more virulent than wild-type PA14 in nematodes and mice. (A) C. elegans are more susceptible to killing by ΔtreYZtreS than PA14 wild-type (P<0.0001). Mutant fer1/5fem1 C. elegans animals were exposed to P. aeruginosa strains and survival was determined as described in Materials and Methods. Data at each time point correspond to the average of three plates per strain, each with approximately 40 animals per plate, and are representative of two independent experiments. (B) The ΔI2 mutant is more virulent than wild-type PA14 in a murine acute lung infection model. See Materials and Methods for details of infection protocol. The median CFU/gram of lung tissue of mice infected with ΔtreYZtreS is 2-fold higher than with wild-type PA14 18 hours post intranasal infection (P<0.05, Mann-Whitney U test). Data are representative of two independent experiments.

doi:10.1371/journal.ppat.1003217.g003

Figure 4. The in planta growth defect of the PA14 ΔtreYZtreS mutant in Arabidopsis is suppressed by cell wall mutations. Growth of PA14 wild-type and the ΔtreYZtreS mutant 3 days post infiltration in Arabidopsis cell wall mutants mur2-1, mur3-2, mur4-1, mur10-2 and xxt1/xxt2. Data represent the mean of bacterial titers ± SE of six leaf disks excised from 6 leaves of 3 plants. Letters above bars denote statistically significant differences (P<0.05, Fisher’s PLSD test). The experiments were repeated at least two times.

doi:10.1371/journal.ppat.1003217.g004
The data in this section suggest that trehalose enhances access to nitrogen sources during an Arabidopsis infection. An alternative model is that ammonium nitrate (as well as trehalose) suppresses the avirulent phenotype of the PA14 trehalose mutants by suppressing the plant defense response. To test this possibility, we tested whether infiltration of leaves with trehalose or ammonium nitrate resulted in enhanced susceptibility to *P. syringae* DC3000 (Figure 7A), the DC3000 hrcC mutant (Figure 7B), or *P. syringae pv. phaseolicola* strain 3121 (Figure 7C); however, neither trehalose nor ammonium nitrate increased the susceptibility to any of these strains. Moreover, infiltration of trehalose or ammonium nitrate did not block the ability of flg22 to elicit protection against infection by *P. syringae* DC3000 (Figure 7A).

Trehalose does not appear to function as a stress-response molecule either *in vivo* or *in vitro*

Trehalose is well-studied as a so-called compatible solute, which is defined as a molecule that functions as an osmolyte and helps an organism survive osmotic stress. We therefore tested whether other di- and trisaccharide compatible solutes would suppress the avirulent phenotype of the Δ42 mutant. Indeed, as shown in Figure S9, both maltose and maltotriose functioned similarly to trehalose in allowing the Δ42 mutant to grow *in planta*, albeit somewhat less efficiently than did trehalose.
Figure 6. The in planta growth defect of the ΔtreYZ::treS double mutant is suppressed by ammonium or nitrate ions. Leaves of four-week-old Arabidopsis Col-0 plants were infiltrated with PA14 wild-type or with Δ42 co-inoculated with various solutions of phosphate, sulfate, nitrate, or ammonium salts at 1 mM. Suppression of the growth defect of Δ42 with 2.5 mg/ml trehalose (Tre) and 1.25 mg/ml glucose (Glc) were tested as positive and negative controls, respectively. Data represent the mean of bacterial titers (CFU) from six leaf disks excised from 6 leaves of 3 plants. Letters above bars denote statistically significant differences (P<0.05, Fisher’s PLSD test). The experiments were repeated at least two times. doi:10.1371/journal.ppat.1003217.g006

Given these results, we next considered the hypothesis that trehalose enhances the virulence of PA14 by ameliorating a variety of environmental stresses [17,25,26]. However, the Δ42 mutant was not more susceptible than wild-type PA14 to osmotic stress in response to 0.5 M NaCl (Figure 8A). As a positive control for the osmotic stress experiment, we constructed an in-frame deletion of a predicted (http://www.pseudomonas.com) three-gene operon (PA14_19350-19370) responsible for the synthesis of a major organic osmoprotectant in P. aeruginosa, N-acetylglutaminylglutamine amide (NAGGN) [27]. As expected, the ΔPA14_19350-19370 mutant (ΔNAGGN) was more susceptible to 0.5 M NaCl than wild-type PA14 or the Δ42 mutant (Figure 8A).

We further tested whether trehalose functions to protect PA14 from osmotic stress in vitro by comparing its ability to enhance growth in minimal medium supplemented with 0.5 M NaCl compared to the well-studied osmoprotectant molecule betaine [27]. In vitro, betaine rescued the growth of PA14, Δ42, and the ΔNAGGN mutant in 0.5 M NaCl whereas trehalose had no effect (Figure S10A). We also tested whether betaine would rescue the Δ42 mutant for in planta growth, similarly to trehalose. However, as shown in Figure 8B, betaine had no significant effect in rescuing Δ42 growth in planta, showing that the ability of trehalose to rescue Δ42 in planta is not likely due to the fact that it is functioning to protect Δ42 from osmotic stress. In contrast to Δ42, the ΔNAGGN mutant, which is very susceptible to osmotic stress in vitro, had no significant impairment in growth in planta (Table S1). These data show that the Δ42 mutant is not highly susceptible to osmotic stress and that trehalose does not play a major role as an osmoprotectant in PA14.

As an alternative to functioning as an osmoprotectant, we investigated whether trehalose protects PA14 from reactive oxygen-mediated stress generated as a consequence of the plant innate immune response. However, we found no significant difference between the Δ42 mutant and wild-type PA14 with respect to tolerance to paraquat or hydrogen peroxide (Figures 8C and 8D, respectively). Because a P. aeruginosa zwf mutant has been reported to be hyper-sensitive to paraquat-mediated killing [28], we also tested a PA14 zwf::MAR2xT7 mutant [16] as a positive control for determining the sensitivity of PA14 and Δ42 to paraquat. As shown in Figure 8C, the zwf mutant exhibited enhanced susceptibility to paraquat.

Figure 7. Trehalose or ammonium nitrate does not suppress the Arabidopsis flg22-mediated defense response and does not make Arabidopsis more susceptible to non-pathogenic P. syringae strains. (A) Growth of P. syringae pv. tomato strain DC3000 three days post infiltration of Col-0 plants pretreated for 24 hours with 1 μM flg22, 1 mM trehalose, 1 mM ammonium nitrate individually or with a mixture of flg22 with trehalose or ammonium nitrate. (B) P. syringae pv. tomato strain DC3000 hrcC or (C) P. syringae pv. phaseolicola strain 3121 were co-inoculated with 1 mM trehalose or 1 mM ammonium nitrate and bacterial counts determined 3 days post infiltration. Glucose or glucose plus ammonium nitrate were included as controls. Data represent the mean of bacterial titers ± SE of six leaf disks excised from 6 leaves of 3 plants. Letters above bars denote statistically significant differences (P<0.05, Fisher’s PLSD test). Absence of letters indicates no statistically significant differences. The experiments were repeated at least two times. doi:10.1371/journal.ppat.1003217.g007

Figure 8. Trehalose enhances virulence of P. aeruginosa. (A) Growth of PA14 (Δ42) and PA14 ΔtreYZ::treS (Δ42) in minimal medium supplemented with 0.5 M NaCl compared to the well-studied osmoprotectant molecule betaine [27]. In vitro, betaine rescued the growth of PA14, Δ42, and the ΔNAGGN mutant in 0.5 M NaCl whereas trehalose had no effect (Figure S10A). We also tested whether betaine would rescue the Δ42 mutant for in planta growth, similarly to trehalose. However, as shown in Figure 8B, betaine had no significant effect in rescuing Δ42 growth in planta, showing that the ability of trehalose to rescue Δ42 in planta is not likely due to the fact that it is functioning to protect Δ42 from osmotic stress. In contrast to Δ42, the ΔNAGGN mutant, which is very susceptible to osmotic stress in vitro, had no significant impairment in growth in planta (Table S1). These data show that the Δ42 mutant is not highly susceptible to osmotic stress and that trehalose does not play a major role as an osmoprotectant in PA14.

As an alternative to functioning as an osmoprotectant, we investigated whether trehalose protects PA14 from reactive oxygen-mediated stress generated as a consequence of the plant innate immune response. However, we found no significant difference between the Δ42 mutant and wild-type PA14 with respect to tolerance to paraquat or hydrogen peroxide (Figures 8C and 8D, respectively). Because a P. aeruginosa zwf mutant has been reported to be hyper-sensitive to paraquat-mediated killing [28], we also tested a PA14 zwf::MAR2xT7 mutant [16] as a positive control for determining the sensitivity of PA14 and Δ42 to paraquat. As shown in Figure 8C, the zwf mutant exhibited enhanced susceptibility to paraquat.
paraquat in vitro, but did not exhibit an impaired growth phenotype in planta (Table S1). These data show that it is unlikely that trehalose functions to protect PA14 from oxidative stress.

In addition to oxidative and osmotic stress, we also tested whether the Δ42 mutant is susceptible to pH or temperature stress, displayed a defect in biofilm formation under osmotic stress, or was deficient in the generation of persister cells in the presence of antibiotics. However, wild-type PA14 and the Δ42 mutant were indistinguishable in all of these tests (Figure S10B–E).

Trehalose functions extracellularly

The data in the previous section suggest that trehalose does not function intracellularly to protect PA14 from a variety of stresses during free-living growth. To provide evidence that trehalose functions extracellularly, we tested whether wild-type PA14 “complements” the growth defect of PA14 trehalose mutants in planta. Specifically, we co-inoculated Arabidopsis leaves with equal mixtures of wild-type PA14 and the ΔtreLΔtreS double trehalose mutant carrying plasmids that express GFP or DsRed, respectively (Figure 9A). Dramatically, co-inoculation of wild-type PA14 with ΔtreLΔtreS completely rescued the growth defect of ΔtreLΔtreS (Figure 9A), strongly suggesting that trehalose is most likely acting extracellularly and not internally within PA14 cells. Similar results were obtained when PA14 expressing GFP was mixed with the Δ42 mutant expressing DsRed (Figure 9B, left panel). In this latter experiment, to make sure that the expression of red or green fluorescent protein does not affect bacterial strain viability, we also carried out an experiment in which the plasmids expressing fluorescent proteins were switched in wild-type PA14 and the Δ42 mutant and obtained the same result (Figure 9B, right panel).

Discussion

In this study, we report that synthesis of the disaccharide trehalose by the multi-host opportunistic pathogen P. aeruginosa is required for plant pathogenesis, but not for pathogenesis in at least three metazoan hosts, mice, D. melanogaster or C. elegans. Trehalose has been extensively characterized as a stress-response molecule that protects cells from osmotic, oxidative, and other environmental stresses. Surprisingly, however, our data suggest that trehalose does not function internally in P. aeruginosa to alleviate a variety of stresses that P. aeruginosa might encounter in its interaction with a plant host. Trehalose also does not function as a major osmoprotectant molecule for P. aeruginosa. Instead, because nitrate and ammonium ions, but not glucose, sucrose, or betaine, suppress the non-pathogenic phenotype of trehalose mutants in planta, we propose that trehalose may function to promote the acquisition of nitrogen-containing nutrients, thereby allowing P. aeruginosa to replicate in the intercellular spaces in a leaf. Moreover, because Arabidopsis cell wall mutants also suppress the non-pathogenic phenotype of the trehalose mutants, it is possible that the plant cell wall normally functions directly or indirectly as a barrier to block nutrient uptake by extracellular bacteria.

Trehalose as a virulence factor for plant and animal pathogens

An important result from this work is that in contrast to many other bacteria and fungi, trehalose appears to have very little effect
on protecting *P. aeruginosa* PA14 from a variety of diverse stresses, including osmotic, oxidative, pH, antibiotic, and temperature stress, and yet trehalose mutants are highly impaired in virulence in *Arabidopsis*. Instead of trehalose, our data show that N-acetylgalactosaminylglycine amide (NAGGN) and glycine-betaine appear to be the primary stress response molecules in *P. aeruginosa*, in agreement with published data showing that osmotically stressed *P. aeruginosa* cultures accumulate NAGGN and glycine-betaine [27,29]. Specifically, we found that a ΔNAGGN mutant was highly impaired in growth under osmotic stress and that exogenously added glycine-betaine, but not trehalose, protected the strain, which conserves energy by not synthesizing trehalose. Models of infection may simply be the result of increased fitness of the strain, which conserves energy by not synthesizing trehalose.

**Role of plant cell walls in PA14 infection**

Because Arabidopsis cell wall mutants suppress the non-pathogenic phenotype of trehalose mutants, it seems likely that the virulence-enhancing role of trehalose is mediated through the plant cell wall. Can we attribute the lack of a particular plant cell wall polymer as playing a key role in the suppression of the non-pathogenic phenotype of the trehalose mutants? As shown in Figures 4 and S7, several Arabidopsis mutants that we tested either completely (xxt1/xxt2 and mur10-2) or partially (*mur2-1, mur3-2, and mur10-2*) suppressed the phenotype of the trehalose mutants. A common feature of all of the cell wall mutants that we tested (Figures 4 and S6) is that they exhibit alterations in xyloglucan, the most abundant hemicellulose in the walls of dicotyledonous plants. The xxt1/xxt2 mutant completely lacks xyloglucan [33], *mur2-1* and *mur3-2* display altered side chains in xyloglucan [34,35], *mur4-1* has decreased levels of arabinose in xyloglucan [23], and *mur10-2* exhibits alterations in xyloglucan remodeling throughout the plant.
Interestingly, wild-type PA14 grew significantly less in mur3-2 than in Col-0 plants, showing that mur3-2 is more resistant to PA14 than wild-type plants. Thus, the fact that the trehalose mutants grew to the same extent in mur3-2 as in Col-0 (Figures 4 and S6) suggests that mur3-2 also partially suppresses its growth defect. These data suggest that xylanoglanuc may be a key component of the cell wall that affects the virulence of P. aeruginosa.

At the mechanistic level, it is not necessarily the case that the rigid plant cell wall is functioning, for example, simply as a physical barrier that blocks the ability of P. aeruginosa to extract nutrients from the cytoplasm of mesophyll cells. If the primary role of trehalose is to facilitate nutrient uptake, the source of the nutrients could be the apoplastic fluid or even components of the cell wall itself, such as specific cell-wall associated proteins.

Importantly, the enhanced susceptibility of the Arabidopsis cell wall mutants to P. aeruginosa trehalose mutants is not simply a consequence of enhanced susceptibility to pathogens in general or the inability of the cell wall mutants to elicit an effective defense response. As shown in Figure 5, the cell wall mutants are not more susceptible to virulent or non-pathogenic P. syringae strains and appear to mount an effective innate immune response when challenged with the flagellin peptide flg22.

How does trehalose promote P. aeruginosa virulence?

Specialized bacterial foliar phytopathogens primarily replicate in the intercellular spaces between mesophyll cells. It is poorly understood which plant-derived nutrients are critical for bacterial growth in this environment as well as the mechanisms utilized by pathogens to obtain nutrients from their hosts. The majority of these pathogens utilize type III effectors not only to suppress the host innate immune response [37,38] but also to extract nutrients from mesophyll cells. Interestingly, however, the P. aeruginosa type III secretion system is not necessary for pathogenesis in plants [6] and it seems unlikely that a broad host range pathogen such as P. aeruginosa would encode host-specific effectors that subvert the Arabidopsis sugar export system analogously to the Xanthomonas effectors that activate glucose efflux in mesophyll cells [10]. Indeed, it also seems highly unlikely that any particular P. aeruginosa strain has extensively co-evolved with any particular host [4,9]. Instead, our finding that P. aeruginosa utilizes trehalose as a major virulence factor for plant pathogenesis is consistent with our studies with P. aeruginosa as a C. elegans pathogen, which have shown that the majority of virulence related factors required to infect nematodes correspond to genes that encode conserved global transcriptional regulators or “house-keeping” genes that encode enzymes involved in conserved metabolic processes [4,9].

Trehalose biosynthesis is highly conserved. All pseudomonads (Table S2) and at least 30% of sequenced prokaryotic genomes encode presumptive trehalose biosynthetic enzymes (J. Urbach and F. Ausubel, unpublished data). It appears that P. aeruginosa has capitalized on what is mostly likely an ancient biosynthetic pathway to promote plant pathogenesis.

How does trehalose promote pathogenesis in an Arabidopsis leaf? Trehalase can serve as a carbon and energy source for growth of many bacteria and fungi including P. aeruginosa [25,39]. However, we have shown that sucrose and glucose do not suppress the phenotype of P. aeruginosa trehalose mutants and co-inoculation of a double Δ2hlE/ΔMAR2xT7 mutant (which cannot metabolize trehalose) with trehalose rescues the non-pathogenic phenotype similarly as co-inoculation of the Δ42 mutant with trehalose (Figure S6). These data suggest that either the level of carbon is not limiting or that trehalase is not involved in carbon acquisition. In addition, experiments designed to determine whether trehalase promotes activity of CWDEs failed to provide evidence that trehalose plays a significant role in cell wall degradation, with the caveat, however, that the experiments we carried out were done in vitro with commercial CWDEs and that in our particular assay a relatively low level of trehalose – enhanced hydrolysis would have not been detected.

Does trehalose function as a general toxin to disrupt host cellular processes? A number of studies have shown that exogenously applied trehalose can have a major negative impact on seedling growth and development [40–42]. On the other hand, the concentrations of trehalose used in these seedling experiments (from 30 mM to 100 mM) were significantly higher than the levels that would be expected to be encountered under natural conditions. By way of contrast, in our experiments we used mature four-week old plants and substantially lower concentrations of trehalose (most often 1 mM). In mature plants, trehalose did not have a toxic effect as evidenced by the lack of any visible symptoms following trehalose (1 mM) infiltration (data not shown). Importantly, in our experiments, trehalose concentrations as low as 0.74 mM largely suppressed the non-pathogenic phenotype of the Δ42 mutant and 0.074 mM had a significant effect (Figure 2C). Taken together, our data indicate that trehalose does not have a toxic effect on mature plants in the P. aeruginosa - plant infection model.

Does trehalose upregulate PA14 virulence genes expression? If so, it would have to specifically upregulate genes required for plant pathogenesis because as shown in Figures 3 and S3, PA14 trehalose mutants are not less virulent in nematodes, flies, or mice. However, we do not favor this explanation. As shown in Figures 6, S8, and S9, nitrate, ammonium, maltose and maltotriose functioned similarly to trehalose in suppressing the inability of the PA14 trehalose mutants to grow in planta. It seems highly unlikely that all three sugars as well as nitrate and ammonium would function similarly to each other as signaling molecules.

Since the non-pathogenic phenotype of P. aeruginosa trehalose mutants can also be suppressed by ammonium nitrate, we propose that trehalose promotes the acquisition of nitrogenous compounds and that nitrogen is limiting in the intercellular environment. The intercellular spaces in leaves are mostly filled with air [43] and very little is known about the mechanisms that plant pathogens utilize to obtain nutrients in this dry environment. Nitrogen limitation during P. aeruginosa infection in plants has been reported previously [44,45]. One way that trehalose could promote nitrogen acquisition is by modulating host nitrogen metabolism, thereby diverting nitrogen-containing compounds to invading P. aeruginosa cells. Several in planta studies have shown that trehalose-6-phosphate (T6P) plays a key role in the regulation of carbon and nitrogen metabolism [41,46,47] and is associated with altered cell wall structure and starch accumulation [48–50]. In our study, preliminary transcriptional profiling analysis has shown that infiltration of trehalose into Arabidopsis leaves at a concentration that is effective in rescuing the trehalose mutants (1 mM) has only a very modest effect on Arabidopsis gene expression (S. Djonovic and F. Ausubel, unpublished data). In addition, we showed that trehalose or ammonium nitrate does not modulate plant defense responses, since infiltration of ammonium nitrate or trehalose into Arabidopsis leaves did not make them more susceptible or resistant to virulent or non-pathogenic P. syringae strains or interfere with their ability to mount an effective innate immune response when challenged with the flagellin peptide flg22 (Figure 7). Finally, another way that P. aeruginosa could use trehalose to promote nitrogen acquisition is by generating a high local concentration of trehalose to create an osmotic gradient that causes an efflux of nitrogen containing nutrients from neighboring plant cells, perhaps in conjunction with P. aeruginosa-encoded pore forming proteins.
toxins. The data in Figure 9, which shows that trehalose functions externally to *P. aeruginosa*, is consistent with these proposed models.

Conclusions

We have found that *P. aeruginosa*-synthesized trehalose plays a key role as a virulence factor during infection of plant leaves. Although the mechanistic details remain to be elucidated, our data suggest that a role of trehalose during the infectious process involves the procurement of nitrogen-containing molecules. In contrast to specialized plant pathogens that utilize highly evolved Type III virulence effectors to promote virulence, the multi-host opportunistic pathogen *P. aeruginosa*, which is not likely to have co-evolved with particular plant hosts, appears to have repurposed a highly conserved anabolic pathway (trehalose biosynthesis) as a potent virulence factor.

Materials and Methods

Ethics statement

Experiments with mice were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the Harvard Medical Area Institutional Animal Care and Use Committee (Permit Number: 404). All efforts were made to minimize suffering.

Bacterial strains and media

*P. aeruginosa* strain UCBPP-PA14 [1], *P. syringae* pv. *tspato* strain DC3000 [31], and *P. syringae* pv. *phaseolicola* strain 3121 [32] have been described. A nonpolar *hrcC* mutant of *P. syringae* strain DC3000 (CUCBP5112) was obtained from A. Collmer and B. Kvitklo, Cornell University. *Escherichia coli* strain SM10 λpir was used for triparental mating [53]. Strains were routinely maintained at 37°C on Luria-Bertani (LB) agar plates or cultured in LB broth supplemented with appropriate antibiotics as needed. The concentrations of antibiotics were: ampicillin or carbenicillin, supplemented with appropriate antibiotics as needed. The mutant lacking both trehalose operons was constructed by trans-poson insertion mutant [16]. We confirmed that transposon insertion mutant could not grow when provided trehalose as sole carbon source in diluted LB and that *treA*::MR2x7 could not hydrolyze trehalose to glucose using the Somogyi-Nelson assay [21,22]. All deletion mutants were confirmed by PCR analysis and sequencing.

Generation of fluorescently labeled bacterial strains

PA14 wild-type and the 

\[ \Delta \text{42} \]

mutant were transformed with pSMC2 carrying green fluorescent protein (GFP) [55]. To construct strains expressing red fluorescent protein (RFP), a variant of *DsRed2*, *DsRed.T3(DNT)* from *Vibrio fischeri* [56] was transferred (on a 719 bp *SphI* - *XhoI* fragment from pVSV206) into the *SphI* - *XhoI* sites of pUCP19 [57] generating pUCP19/ *DsRed.T3(DNT)*, which was designated pAA100. pAA100 was transformed into PA14 wild-type and \( \Delta \text{42} \) by electroporation.

Motility and growth assays

Twitching and swimming motility assays were performed as previously described [58]. To compare growth rates of wild-type and mutants, the cultures were grown at 37°C overnight in LB, centrifuged, washed and resuspended into minimal medium (M63). Bacterial growth was monitored in vitro by plating and counting CFU/ml at 3-, 6- and 9-hour time points. Growth rate (\( h^{-1} \)) was calculated using the equation for exponential growth:

\[
\mu = (\ln N_1 - \ln N_0)/(t_1 - t_0),
\]

where \( N_0 \) and \( N_1 \) equal bacterial abundance (CFU/ml) at the beginning \( (t_0) \) and end \( (t_1) \) of the exponential growth phase. Each experiment was repeated at least twice with similar results.

Plant material and growth of plants

Arabidopsis ecotypes Columbia (Col-0) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Plants were grown on 30-mm Jiffy-7 peat pellets (Jiffy Products, Shippagan, New Brunswick, Canada) in a Conviron E7/2 chamber (Winnipeg, Manitoba, Canada) set at a 23°C/20°C day/night regime with a 12-h photoperiod at a light intensity of 100 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) and 60% relative humidity. Arabidopsis cell wall mutants were obtained from the Arabidopsis Biological Resource Center: *mur2-1* (AT2G03220; CS6556), *mur3-2* (AT2G0370, CS5657), *mur4-1* (at1g30620, CS5864), *mur10-2* (at3g17420, CS5878), and an *xt/txt* double T-DNA insertion line (at3g6272; SALK_119658C/at4g02500; SALK_1013080) as previously published [33].

Arabidopsis pathogenicity assays

Plant infection assays were carried out as previously described [1] with some modifications. *P. aeruginosa* strains were grown in LB medium overnight, subcultured and grown to an OD\(_{600}\) of 2.5. Cells were centrifuged, washed and resuspended in 10 mM MgSO\(_4\). Leaves of four-week old plants were inoculated with a \( 1 \times 10^7 \) CFU/ml suspension of PA14 wild-type or various PA14 mutants, which corresponds to \( 1 \times 10^7 \) CFU/cm\(^2\) leaf area. Infected plants were incubated in a growth chamber at 28°C with a 12-h photoperiod at a light intensity of 60 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \) and 90% relative humidity. Six to eight leaves were harvested from three to four plants for CFU determination. Each experiment was repeated at least two to four times with similar results. Co-inoculation of bacteria with betaine, trehalose or sucrose (Sigma, St. Louis, MO) was performed as described above. Before inoculation of leaves, betaine, trehalose, glucose, or sucrose was added to bacterial suspensions at the indicated concentrations, or various phosphate, sulfate, nitrate, or ammonium salts were added at 1 mM. Trehalose was initially added at 2.5 mg/ml, but after we carried out dose response curves and found that 0.25 mg/ml
suspensions were prepared in PBS (OD600 = 0.5) after overnight bacterial suspension was applied to each nostril. Bacterial cycle. For infections assays, infec-
tions were carried out at 25°C, which were grown under non-crowded conditions on strains w[118] (Bloomington stock C3719) were obtained from the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/annotation/genome/pseudomonas_group/MultiHome.html).

To identify orthologs to PA14_36375-36830 genes, two criteria were used. First, putative ortholog pairs were required to be reciprocal best hits, with an e-value less than or equal to 0.0001 for best hits of the PA14 proteins against compared protein sets, and an e-value less than or equal to 0.001 for reciprocal best hits against the PA14 protein set. Secondly, the putative orthologs were required to align for at least 80 percent of their lengths and have less than a 20% difference in protein sequence lengths, thereby conserving overall domain structure. Of these constraints, the e-value and sequence length constraints are very permissive, whereas the requirement for alignment length is stringent.

Bioinformatic analysis of PA14_36375-36830 genes
To assign putative functions to genes within the block of PA14_36375 through PA14_36830, each protein in the 42 kb cluster was used as a query in a BLAST or PSI-BLAST homology search. In the process of assigning putative functions, several types of information were taken into account: homologous proteins with experimentally assigned function; homologous proteins with computationally predicted function; matches to HMMs from conserved domain databases; and the genomic/operon context of close homologs.

The protein and nucleotide sequences of prokaryotic genomes were obtained from NCBI (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/). Additionally, two P. aeruginosa genomes (P. aeruginosaa 2192; P. aeruginosa C3719) were obtained from the Broad Institute of Harvard and MIT (http://www.broadinstitute.org/annotation/genome/pseudomonas_group/MultiHome.html).

To identify orthologs to PA14_36375-36830 genes, two criteria were used. First, putative ortholog pairs were required to be reciprocal best hits, with an e-value less than or equal to 0.0001 for best hits of the PA14 proteins against compared protein sets, and an e-value less than or equal to 0.001 for reciprocal best hits against the PA14 protein set. Secondly, the putative orthologs were required to align for at least 80 percent of their lengths and have less than a 20% difference in protein sequence lengths, thereby conserving overall domain structure. Of these constraints, the e-value and sequence length constraints are very permissive, whereas the requirement for alignment length is stringent.

Trehalose quantification assay
P. aeruginosa strains were grown at 37°C in MinA medium with 0.5 M NaCl to an early stationary phase. Trehalose was extracted from a 19 ml culture by pelleting the cells, resuspending in 0.5 ml water, and heating at 95°C for 20 min [60]. The concentration of trehalose in the supernatants was determined using an enzymatic assay by converting trehalose to glucose with trehalase and then measuring the glucose using a trehalase assay kit (Megazyme International Ireland Limited). The pre-existing glucose in each sample was determined in a control reaction without trehalase and subtracted from the total glucose. The experiment was repeated at least twice with similar results.

Stress response, resistance and biofilm assays
Osmotic stress sensitivity: P. aeruginosa was grown at 37°C in MinA containing 17 mM glucose [39], washed and subcultured into MinA containing 0.5 M NaCl. Bacterial growth was monitored by plating CFU. Persistence assay: This assay was performed as previously described [61]. Briefly, persisters were determined by exposure of stationary cultures to antibiotics at concentrations exceeding the corresponding bacterial minimal inhibitory concentrations (MICs). The antibiotics were used at the following
concentrations: 6 μg/ml tobramycin, 2 μg/ml ciprofloxacin and 3 mg/ml carbenicillin. Oxidative stress resistance: Hydrogen peroxide was added directly to an overnight culture grown for 14 h in LB medium and the cells were incubated for 8 h at 37°C. The following concentrations of hydrogen peroxide were used: 1 M (non-lethal), 2 M (sub-lethal), and 3 M (lethal dose). To test sensitivity to paraquat (Sigma, 856177), overnight MinA cultures were diluted 100 fold in MinA containing different amounts of paraquat (0.1, 1 and 10 mM) and cultured for three days. pH shift: Cultures were grown to stationary phase in LB medium that had been titrated with HCl to pH 4, 5, 6, or 7. Thermotolerance: Small volumes of stationary phase cells were heated in Eppendorf tubes in a heating block at different temperatures and incubation times, then rapidly diluted and plated. Biofilm formation: Biofilm attachment assays were performed using wild-type PA14, ΔA2 and APA14_19350-19370 (ΔMAGGM) cultures grown in 96-well polystyrene (PVC) plates as described previously [62]. Overnight cultures were diluted 1/100 in MinA medium or MinA medium supplemented with 0.5 M or 0.75 M NaCl. Aliquots of 100 μL were dispensed into the wells of PVC microtiter plates and incubated at 37°C. Attachment was detected by staining with 1% crystal violet dissolved in water. Dye not associated with bacteria was removed by rinsing with water. Bacteria-associated dye was solubilized using 95% ethanol and absorbance was determined at 550 nm. Each experiment was repeated at least twice with similar results.

Statistical analysis

Statistical analyses in animal experiments were performed using GraphPad Prism 5 software (La Jolla, CA) and a log rank (Mantel-Cox) test to assess the significance of differential survival, and a Mann-Whitney U non-parametric test for significance of CFU data, which were not normally distributed. Statistics in all other experiments was performed using analysis of variance (ANOVA) and a Fisher’s PLSD test (Statview v. 5.0.1, SAS Institute, Cary, NC).

Supporting Information

Figure S1 Expression of PA14_36500 encoding a putative cellulase/peptidase in infected Arabidopsis leaves. Semiquantitative RT-PCR was carried out as described in Materials and Methods. (A) PA14_36500 transcript levels on various days post infiltration (dpi) with PA14 wild-type. (B) PA14_36500 expression in wild-type PA14 and a PA14 Δmfr mutant 2 days post-infiltration. P. aeruginosa PA14 ribosomal protein L21 (rplU) was used as a control for equal amounts of cDNA. The experiment was repeated at least two times with similar results. (TIF)

Figure S2 The 42 kb cluster encodes two independent pathways for trehalose biosynthesis. In the top pathway, gene product 36605 (a putative maltoligosyltrehalose synthase) alters the regiochemistry of the terminal sugar linkage from alpha-1,4 to alpha-1,1; the terminal disaccharide is subsequently cleaved by gene product 36580 (a putative maltoligosyltrehalose trehalohydrolase), releasing trehalose. In the bottom pathway, gene product 36740 (a putative alpha-amylose) cleaves the terminal disaccharide of the alpha-1,4-glucan, releasing maltose. The alpha-1,4 linkage of the maltose disaccharide is then isomerized to alpha-1,1 by gene product 36730 (a putative trehalose synthase), yielding trehalose. (TIF)

Figure S3 Pyocyanin production by ΔA2 and PA14 wild-type. Bacterial strains were streaked onto Pseudomonas agar P to assess pyocyanin production (see Materials and Methods) and incubated 20 h at 37°C. Sectors: ΔA2-1 and ΔA2-2 (two independent ΔA2 deletion constructs); phzM (phzM:MAR2xT7, negative control: a pyocyanin-defective mutant); WT (PA14 wild-type). Characteristic blue-green color indicates that the strain is proficient in pyocyanin production. (TIF)

Figure S4 Growth of P. aeruginosa ΔA2, three sub-region in-frame deletion mutants, and PA14 wild-type in Arabidopsis Col-0 leaves. Plants were inoculated and incubated as described in Materials and Methods. The leaves were harvested 3 days post-inoculation. Data represent the mean of bacterial titers ± SE of six leaf disks excised from 6 leaves of 3 plants. Different letters above bars denote statistically significant differences (P<0.05, Fisher’s PLSD test). See Figure 1 for a description of the mutants. (TIF)

Figure S5 The ΔA2 mutant is more virulent than wild-type PA14 in nematodes, insects, and mice. (A) C. elegans are more sensitive to killing by the ΔA2 mutant than PA14 wild-type (P<0.004). Mutant fer15;fen1 C. elegans animals were exposed to P. aeruginosa strains and survival was determined as described in Materials and Methods. Data at each time point correspond to the average of three plates per strain, each with approximately 40 animals per plate, and are representative of two independent experiments. (B) D. melanogaster infected with ΔA2 die faster than flies infected with PA14 wild-type (P<0.03). D. melanogaster strain Oregon R was infected with P. aeruginosa and approximately 25 flies per vial were scored several times a day for survival throughout the time course of infection. Data are representative of four independent experiments carried out with two different D. melanogaster lines (Oregon R and w[118]). (C) The ΔA2 mutant is more virulent in a murine acute lung infection model. See Materials and Methods for details of infection protocol. CFU/gram of lung tissue of mice infected with ΔA2 mutant is 3.8-fold higher than with wild-type PA14 18 hours post intranasal infection (P<0.01, Mann-Whitney U test). Data are representative of two independent experiments. (D) FABP-CFTR transgenic mice are more susceptible to killing by ΔA2 mutant than by PA14 wild-type after oropharyngeal colonization (P<0.04, log rank test). All mice in both groups (n = 9 for ΔA2; n = 8 for PA14 WT) had positive throat cultures for the duration of the experiment after initial colonization by exposure to bacteria in drinking water for one week. (TIF)

Figure S6 The in planta growth defect of the ΔA2 mutant is suppressed by Arabidopsis cell wall mutants. Growth of PA14 wild-type or ΔA2 3 days post infiltration in Arabidopsis cell wall mutants mun2-1, mun3-2, murl-1, murl10-2 and xst1/xst2. Data represent the mean of bacterial titers ± SE of six leaf disks excised from 6 leaves of 3 plants. Letters above bars denote statistically significant differences (P<0.05, Fisher’s PLSD test). The experiments were repeated at least two times. (TIF)

Figure S7 The growth of the ΔA2 mutant in planta is not limited by lack of a carbon source. (A) The growth of the ΔA2 mutant in planta is not suppressed by 1.25 mg/ml glucose or 2.5 mg/ml sucrose. (B) Suppression of the growth defect of ΔA2 treated with trehalose in Arabidopsis leaves. Plants were inoculated and incubated as described in Materials and Methods and leaves were harvested 3 days post-inoculation. In (A) and (B), data represent the mean of bacterial titers ± SE of six leaf disks excised from 6 leaves of 3 plants. Different letters above bars
denote statistically significant differences (P<0.05, Fisher’s PLSD test). The experiments were repeated at least two times.

**Figure S8** The in planta growth defect of the ΔΔ2 mutant is suppressed by ammonium or nitrate ions. Leaves of four-week-old Arabidopsis Col-0 plants were infiltrated with PA14 wild-type or ΔΔ2 as described in Materials and Methods except that the infiltration solution contained various phosphate, sulfate, nitrate, or ammonium salts at 1 mM. Suppression of the growth defect of ΔΔ2 with 2.5 mg/ml trehalose (Tre) and 1.25 mg/ml glucose (Glc) were tested as positive and negative controls, respectively. Data represent the mean of bacterial titers ± SE of six leaf disks excised from 6 leaves of 3 plants. Letters above bars denote statistically significant differences (P<0.05, Fisher’s PLSD test). The experiments were repeated at least two times.

**Figure S9** Suppression of attenuation of ΔΔ2 with exogenous maltose and maltotriose. Col-0 plants were infiltrated with PA14 wild-type or ΔΔ2 co-inoculated with maltose (M) or maltotriose (MT) at the indicated concentrations. Leaves were harvested 3 days post-infiltration. Data represent the mean of bacterial titers ± SE of six leaf disks excised from 6 leaves of 3 plants. Letters above bars denote statistically significant differences (P<0.05, Fisher’s PLSD test). The experiment was repeated at least two times.

**Figure S10** Response of ΔΔ2 to various stress conditions. (A) Rescue of ΔΔ2, ΔΔ4GGV and PA14 wild-type by betaine but not trehalose under osmotic stress in vitro. Cells were grown at 37°C in M63 medium with 0.5 M NaCl (squares), or with 0.5 M NaCl+1 mM betaine (Bet) (circles) or 1 mM trehalose (Tre) (triangles). Data represent the mean ± SE of 3 replicates. (B) Thermotolerance. Survival of stationary phase bacteria after a 30 minute exposure to 53°C. Temperatures below 53°C were non-lethal and above 56°C were 100% lethal. (C) Biofilm attachment under osmotic stress. Overnight cultures were diluted 1/100 in M63 medium supplemented with 0.5 or 0.75 M NaCl. Attachment assays were performed as described in Materials and Methods. (D) Growth under pH stress. Cultures were grown to stationary phase in LB medium adjusted to pH 4, 5, 6, or 7. (E) Persistence assay. Persisters were determined by exposure of stationary cultures (inoculum, time point zero on x axis) to 6 μg/ml tobramycin, 2 μg/ml ciprofloxacin or 3 mg/ml carbenicillin. The assay was performed as described in Materials and Methods. Based on analysis of variance (ANOVA) and Fisher’s PLSD test (P<0.05), there was no significant differences between ΔΔ2 and PA14 wild-type in any of the assays (A–E).

**Table S1** Growth of *P. aeruginosa* mutants in Arabidopsis Col-0 leaves. Plants were inoculated and incubated as described in Materials and Methods and leaves were harvested 3 days post-inoculation. Data represent the mean of bacterial titers ± SE of six leaf disks excised from 6 leaves of 3 plants. Different superscript letters denote statistically significant differences (P<0.05, Fisher’s PLSD test).

**Table S2** Predicted functions and orthologs of PA14_36375-36830 genes across sequenced *Pseudomonas* genomes. Putative functions of PA14_36375-36830 genes were assigned as described in Materials and Methods. Genes of the reference taxon PA14 are indicated in the left column (PA14 loci), and different pseudomonal genomes are indicated on the top row. The presence of an ortholog is indicated by a checkmark. Boxes of identical hue indicate that the genes are contiguous in a particular genome, with the lighter shades on the top strand, and darker shades on the bottom strand. Checks in white boxes indicate an ortholog that is not contiguous with other PA14_36375-36830 block orthologs.

**Table S3** Growth, biofilm formation and motility of ΔΔ2 and PA14 wild-type. Growth rate (h⁻¹) of *Pseudomonas* strains in minimal media (M63) was calculated by the equation for exponential growth [see Materials and Methods]. Biofilm formation was measured as attachment to polynivchlorylate plates in absorbance units (OD550); see Materials and Methods. Swimming and twitching motility are represented as a radius of a halo in cm (see Materials and Methods). Two independent ΔΔ2 deletion constructs were tested. Data represent the mean ± SE. Based on analysis of variance (ANOVA) and Fisher’s PLSD test (P<0.05), there was no significant differences between ΔΔ2 mutant and PA14 wild-type in any of the assays.

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**Author Contributions**

Conceived and designed the experiments: SD JMU ED RF CK MAF GPP FMA. Performed the experiments: SD JMU ED JB RF JLA DT MR CK. Analysis of the data: SD JMU ED MAF GPP FMA. Wrote the paper: SD JMU ED MAF GPP FMA.

**References**


