Pseudomonas aeruginosa virulence genes identified in a Dictyostelium host model

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Summary

The human pathogen Pseudomonas aeruginosa has been shown previously to use similar virulence factors when infecting mammalian hosts or Dictyostelium amoebae. Here we randomly mutagenized a clinical isolate of P. aeruginosa, and identified mutants with attenuated virulence towards Dictyostelium. These mutant strains also exhibited a strong decrease in virulence when infecting Drosophila and mice, confirming that P. aeruginosa makes use of similar virulence traits to confront these very different hosts. Further characterization of these bacterial mutants showed that TrpD is important for the induction of the quorum-sensing circuit, while PchH and PchI are involved in the induction of the type III secretion system. These results demonstrate the usefulness and the relevance of the Dictyostelium host model to identify and analyse new virulence genes in P. aeruginosa.

Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen and a major cause of nosocomial infections. This bacterium infects burn victims, intubated, immunocompromised and cystic fibrosis patients as well as users of soft contact lenses (Lyczak et al., 2000). Its pathogenicity has been linked to at least two major virulence pathways, the quorum-sensing (QS) system (Van Delden and Iglewski, 1998) and the type III secretion system (TTSS) (Yahr and Wolfgang, 2006). The las and rhl QS circuits as well as the Pseudomonas Quinolone Signal (PQS) control the production of numerous secreted virulence factors, including proteases and secondary metabolites, and are important for biofilm formation (Pesci et al., 1999; Rumbaugh et al., 2000). The QS system in P. aeruginosa is organized in a hierarchy, in which the las system exerts a positive control on the expression of the PQS (Wade et al., 2005), and on the rhl system (Latifi et al., 1996; Pesci et al., 1997). When the bacterial density reaches a certain threshold, the accumulation of signalling autoinducer molecules in the medium induces the QS pathways, leading to transcription of virulence genes. In particular, the rhl QS pathway controls the production of rhamnolipids, which are glycolipids with biosurfactant properties involved in bacterial virulence (Cosson et al., 2002; Zulianello et al., 2006). A distinct mechanism, making use of the ExsA transcription factor, regulates the expression of the TTSS, a crucial element required for bacterial cytotoxicity (Dacheux et al., 1999; Roy-Burman et al., 2001). The ExsA pathway is activated by a direct contact with the host cells, but can also be induced in vitro by chelation of extracellular calcium (Yahr et al., 1997).

A comprehensive study of the pathways controlling bacterial pathogenicity is essential in understanding the mechanisms by which P. aeruginosa establishes harmful infections. However, the assessment of bacterial pathogenicity in mammalian systems is limited by ethical and practical restrictions on animal experiments. Consequently, many investigators have studied P. aeruginosa virulence in non-mammalian hosts such as insects.
(D'Argenio et al., 2001; Fauvarque et al., 2002), nematodes (Sifri et al., 2005), plants (Rahme et al., 1997) and amoebae (Cosson et al., 2002; Pukatzki et al., 2002). Overall, these studies indicate that in different hosts, bacterial pathogenicity relies on a largely overlapping set of bacterial virulence genes (reviewed in Hilbi et al., 2007). For example, Dictyostelium amoebae can be used to assess the virulence of many different bacterial species, including Klebsiella (Benghezal et al., 2006), Aeromonas (Froquet et al., 2007) or Pseudomonas (Cosson et al., 2002; Pukatzki et al., 2002). While Klebsiella virulence against Dictyostelium relies mostly on its ability to resist intracellular killing (Benghezal et al., 2006), Pseudomonas virulence depends largely on secreted toxins that can kill Dictyostelium cells (Cosson et al., 2002; Pukatzki et al., 2002).

In this study, we identified three new bacterial genes, trpD, pchH and pchI, implicated in P. aeruginosa virulence towards Dictyostelium. These same genes are also implicated in virulence towards two animal hosts, Drosophila and mouse. While TrpD seems to be involved in the induction of the QS circuit, PchH and PchI are required for efficient induction of the TTSS system.

Results

Isolation of non-pathogenic P. aeruginosa mutants in a Dictyostelium host model

So far, studies of P. aeruginosa virulence have focused on a relatively limited number of strains. In order to increase our chances of identifying new virulence genes in this pathogen, we used the 22D10 strain, recently isolated from an intubated patient (Denervaud et al., 2004). This strain exhibits a deletion in the gene encoding the QS regulator LasR and, in consequence, fails to produce two QS-regulated virulence products, rhamnolipids and elastase (Denervaud et al., 2004). LasR mutants were shown recently to emerge spontaneously in cystic fibrosis patients, probably due to a growth advantage on certain carbon sources (D'Argenio et al., 2007). When tested in a Dictyostelium assay, the 22D10 strain was highly virulent, because it fully inhibited the ability of Dictyostelium cells to grow and to form a phagocytic plaque (Fig. 1). This suggested that strain 22D10 could be used to identify new virulence genes that are not under the control of the las QS circuit. For this, we mutagenized 22D10 by random insertion of a mini-Tn5 transposon, and tested the ability of Dictyostelium to grow on 2500 individual mutants. Dictyostelium was capable of growing on 17 of these mutants, which were further characterized in this study.

The virulence of these bacterial mutants was first quantified more precisely by determining the number of Dictyostelium cells necessary to create a phagocytic plaque in a P. aeruginosa bacterial lawn (Cosson et al., 2002). For this a droplet containing 10 000, 2000, 400, 80, 16 or 3 Dictyostelium cells was deposited on a bacterial lawn, and the Dictyostelium cells allowed to grow for 5 days at 25°C (Fig. 1A). On the basis of this assay, two types of P. aeruginosa mutants were distinguished (Fig. 1B): partially permissive mutants allowed growth of Dictyostelium only when a large number of cells (10 000) were deposited on the bacterial lawn, while fully permissive mutants supported the growth of even a single Dictyostelium cell. Of the 17 mutants analysed, four were fully permissive.
and the others, partially permissive (Table 1). When grown in Luria–Broth (LB) medium, all these mutant strains grew as well as the parental 22D10 strain (data not shown), indicating that their phenotype was not due to a non-specific growth defect. In SM medium, only DP5 (pchD) showed a slight growth retardation before reaching stationary phase (data not shown and see below).

The site of transposon insertion was determined for all 17 mutants (Table 1). Sequence analysis identified 15 different transposon insertions in the pchEFGHI gene cluster (Fig. 1C): six in pchE, six in pchF, two in pchH and one in pchl. Another mutant was created by an insertion in the pchDCBA operon (Serino et al., 1997) (pchD gene), localized in the same region. The fact that so many mutants were generated by a transposon insertion in the same region clearly indicated that this genomic region was a highly favoured site for transposon insertion. Finally, one insertion in the trpGDC operon (trpD gene) was also identified. Southern-blot experiments confirmed that each selected mutant contained a single mini-Tn5 insertion at the identified location in the genome (data not shown).

Several of the genes identified (pchE, F and D) are implicated in the synthesis of pyochelin, a siderophore involved in iron uptake by P. aeruginosa (Serino et al., 1997; Reimmann et al., 1998), and have been named accordingly (pch stands for pyochelin). The corresponding mutants showed only a slightly attenuated pathogenicity towards Dictyostelium (Table 1 and Fig. 1C). On the contrary, the name of the pchH and pchl genes is due to their position in the same pchEFGHI gene cluster, but PchH and Pchl are not necessary for pyochelin synthesis (Reimmann et al., 2001). The pchH and pchl genes encode proteins that are homologous to ABC transporters but their function is still unknown. Here we observed that insertions in pchH or pchl caused a drastic decrease in bacterial virulence against Dictyostelium. To our knowledge, our observation that PchH and Pchl might be involved in Pseudomonas virulence provides the first indication about their putative function in bacterial physiology.

The trpD mutant (trpGDC operon) was also completely permissive for Dictyostelium growth. The product of the trpD gene is implicated in the synthesis of tryptophan (Essar et al., 1990). We focused the rest of this study on the three most permissive mutants, DP5 (trpD), DP7 (pchH) and DP28 (pchH) (Table 1). We also studied, for comparison, one of the partially permissive mutants, DP35 (pchE).

As several insertions in the pchEFGHI gene cluster resulted in variable effects on bacterial virulence, we first verified by complementation that the loss of P. aeruginosa virulence was indeed caused by the inactivation of pchH and pchl. For this, we transformed these mutants with plasmids expressing either pchH, or pchEFGHI, and assessed their virulence towards Dictyostelium (Table 2). The relatively minor virulence defect seen in pchE mutants was apparently complemented by expression of pchEFGHI, but not by expression of pchH, suggesting that the loss of pyochelin synthesis led to the minor decrease in virulence in pchE mutants. However, as virulence is only subtly changed in pchE and the complemented strains, we cannot exclude the possibility of a polar effect of the pchE mutation on pchH and pchl. As expected, expression of pchEFGHI or of pchHI restored a fully virulent phenotype in the pchH mutant, indicating that inactivation of pchH or pchl caused a marked decrease in bacterial virulence towards Dictyostelium.

**Virulence of P. aeruginosa mutants towards metazoan hosts**

To determine whether the isolated mutants presented a reduced virulence in other host systems, they were used

| Strain   | Position of insertion* | Gene   | Virulence in Dictyostelium |
|----------|------------------------|--------|---------------------------|
| 22D10    | Parent                 |        | Virulent                  |
| DP5      | 704879                 | trpD   | Permissive                |
| DP7      | 4728195                | pchH   | Partially permissive      |
| DP9      | 4733839                | pchF-1 | Partially permissive      |
| DP18     | 4736008                | pchF-2 | Partially permissive      |
| DP20     | 4738360                | pchE-2 | Partially permissive      |
| DP21     | 4738997                | pchE-1 | Partially permissive      |
| DP22     | 4738079                | pchE-3 | Partially permissive      |
| DP23     | 4732532                | pchF-3 | Partially permissive      |
| DP27     | 4728907                | pchH-1 | Partially permissive      |
| DP28     | 4729607                | pchH-2 | Partially permissive      |
| DP29     | 4735541                | pchF-4 | Partially permissive      |
| DP30     | 4732534                | pchF-5 | Partially permissive      |
| DP32     | 4743957                | pchD   | Partially permissive      |
| DP33     | 4732334                | pchF-6 | Partially permissive      |
| DP34     | 4740344                | pchE-4 | Partially permissive      |
| DP35     | 4739825                | pchE-5 | Partially permissive      |
| DP36     | 4738352                | pchE-6 | Partially permissive      |

*Numbers refer to the PAO1 sequenced genome (http://www.pseudomonas.com).

| Strain   | Complementation of virulence defects in pch mutants. |
|----------|-----------------------------------------------------|
|          | DP5 (trpD) | DP28 (pchH) | DP35 (pchE) |
| Plasmid  | None       | 6           | 6           |
|          | pchH       | 6           | 0           |
|          | pchEFGHI   | 6           | 1           |

Depending on the degree of virulence of each bacterial strain, Dictyostelium growth was seen when a minimum of 10 000 (respectively 2000, 400, 80, 16, 3) cells were applied on the bacterial lawn (score 1, respectively: 2, 3, 4, 5 or 6). No growth of Dictyostelium is indicated by a 0. These results varied at most by one unit in repeated experiments.
to infect Drosophila flies, a well-characterized model for bacterial infections. In early infection experiments, flies were pricked with a needle previously dipped in a bacterial solution (D’Argenio et al., 2001; Fauvarque et al., 2002). More physiological methods of infection by P. aeruginosa have been developed more recently (Erickson et al., 2004; Vodovar et al., 2004; 2005), where flies are fed with a sucrose solution containing bacteria. When Drosophila flies were fed with a solution containing the parental 22D10 P. aeruginosa, or a pchE mutant, 90% of them died within 8 days (Fig. 2A). On the contrary, when pchH, pchl or trpD mutants were used, at least 90% of the Drosophila survived until the end of the experiment (Fig. 2A). This result indicated that these three mutant strains were less virulent towards Drosophila than the parental 22D10 strain in a feeding assay. In order to ascertain if lack of virulence reflected a decrease in bacterial viability or replication, we determined the number of viable bacteria extracted from live infected flies 48 h post infection. Very similar numbers of viable bacteria were found following infection with mutant or wild-type Pseudomonas strains (Fig. 2B). The most important difference seen (between 22D10 and DP28, 48 h post infection) was modest (55% difference) and not statistically significant (P = 0.3, three independent experiments) (Fig. 2B). Interestingly, a different result was obtained when Drosophila flies were infected with a needle: in this case the infection led to a very rapid death of the flies (within 24 h), and all mutants were as virulent as the parental strain (Fig. 2C).

In order to test directly the effect of these mutations on P. aeruginosa virulence in a mammalian host, a neutropenic mouse pneumonia model was used. Neutropenic mice infected with 22D10 or with a pchE mutant became severely ill, and died within 7 days (Fig. 3). In contrast, survival curves showed that trpD, pchH and pchl mutants were markedly less virulent than their parent strain (Fig. 3 and Fig. S1).

Together these experiments demonstrated an excellent correlation between P. aeruginosa virulence as measured using Dictyostelium amoebae, Drosophila flies, or mice, suggesting that P. aeruginosa uses largely similar sets of virulence genes to interact with these various hosts.

In vitro induction of the TTSS is altered in pchH and pchl mutants

The phenotype of avirulent mutants was investigated in more detail to determine how these mutations might interfere with P. aeruginosa virulence. The fact that trpD, pchH and pchl mutations affected virulence with no major effect on the number of viable bacteria in infected flies suggested that they might rather affect the production of virulence factors in Pseudomonas mutants. In order to
test this, we assessed the production of the TTSS and of QS-dependent effectors, the two main virulence traits characterized so far in *Pseudomonas*. Both TTSS and QS effectors were shown previously to be equally active against *Dictyostelium* amoebae, *Drosophila* flies and mammalian hosts (D’Argenio *et al*., 2001; Cosson *et al*., 2002; Fauvarque *et al*., 2002; Pukatzki *et al*., 2002).

In order to assess the ability of *P. aeruginosa* mutants to produce and secrete components of the TTSS, bacteria were grown in calcium-depleted medium to induce the expression of the TTSS (Yahr *et al*., 1997) and the secreted proteins were separated on polyacrylamide gels and revealed by silver staining. In the parent 22D10 strain, chelation of Ca\(^{2+}\) with EGTA induced the secretion of four main proteins (Fig. 4A), which presumably correspond to ExoS, ExoT, PopB and PopD, as previously described (Dacheux *et al*., 1999). Identical patterns of secreted proteins were observed with *trpD* and *pchE* mutants (Fig. 4A). The amount of secreted TTSS proteins was much lower in mutants affected in *pchH* (Fig. 4A) or in *pchI* (data not shown). This result was confirmed by immunodetection with antibodies directed to the ExoS exotoxin (Fig. 4B) and to proteins of the translocation apparatus (PopB, PopD and PcrV, data not shown). Immunodetection also demonstrated that the absence of TTSS proteins in the supernatant of *pchH* mutants was caused by a defect in their production, because no intracellular accumulation of the protein was seen in *pchH* mutants (Fig. 4B). In addition, plasmid-driven expression of *pchH* and *pchI* restored the ability of a *pchH* mutant to produce TTSS proteins (Fig. 4B). Together, these results indicated that the full induction of TTSS proteins requires the presence of the PchH and PchI proteins.
In order to characterize in more detail the role of PchH in TTSS induction, we performed real-time PCR to quantify the levels of exoT and popD mRNAs (Table 3). As the production of TTSS proteins is controlled by the inducible ExsA transcriptional activator, we also assessed the level of transcription of exsA. The amounts of exoT and popD mRNA were increased by a factor of 16 and 24, respectively, in 22D10 upon chelation of calcium (Table 3). In the same conditions, the level of exsA mRNA increased sixfold. When the pchH mutant was grown in LB, exsA, exoT and popD mRNA levels were slightly lower than the basal level seen in parental cells, and their increase upon calcium chelation was limited. These results suggested that PchH and PchI are primarily involved in the regulated induction of ExsA.

Table 3. Determination of TTSS expression in vitro by quantitative RT-PCR.

|                  | Non-induced/induced | Experiment 1 | Experiment 2 |
|------------------|---------------------|--------------|--------------|
| ExsA             | 22D10               | 100/582      | 100/416      |
| DP28 (pchH)      |                     | 48/96        | 67/47        |
| PopD             | 22D10               | 100/2480     | 100/2193     |
| DP28             |                     | 28/142       | 50/88        |
| ExoT             | 22D10               | 100/1734     | ND           |
| DP28             |                     | 39/104       | ND           |

A partial tryptophan depletion inhibits rhl QS

An inhibition of the production of TTSS proteins might account for the loss of virulence of pchH and pchI mutants, but no alteration was seen in trpD mutants. We therefore tested the production of the second major virulence factor in Pseudomonas, the QS system. As the parental 22D10 isolate exhibited a deletion in lasR, we focused our studies on the induction of the rhl QS system, which controls notably the induction of RhlA, a rhamnosyl-transferase involved in the production of rhamnolipids. Original observations suggested that the production of rhamnolipids is deficient in 22D10 (Denervaud et al., 2004), presumably due to the fact that the las QS system participates in the activation of the rhl QS system (Latifi et al., 1996). However, we observed that supernatants from 22D10 bacteria caused rapid lysis of amoebal cells, a property previously attributed to the presence of rhamnolipids (Cosson et al., 2002). Indeed, high amounts of rhamnolipids could be purified and titrated in 22D10 supernatant when the bacteria were grown in SM medium (Fig. 5A). This discrepancy with previously published results is presumably due to the fact that in previous experiments bacteria were grown in a medium that was optimal for induction of QS by PAO1, but not by 22D10. The SM medium used in this study seems therefore more appropriate to induce the rhl QS in 22D10. Indeed, we observed that the concentration of the rhl autoinducer (C4-HSL) was much higher in SM medium (1300 ± 90 nM in SM vs 18 ± 2 nM in LB). The supernatants produced by pchI, pchH and pchE mutants also caused rapid cell lysis and contained high amounts of rhamnolipids (Fig. 5A and data not shown). On the contrary, the supernatant of the trpD mutant did not lyse Dictyostelium cells and rhamnolipid concentration was strongly reduced (Fig. 5A). We evaluated the production of rhamnolipids by trpD and its 22D10 parent strain during their growth in SM medium, using a semiquantitative assay. No rhamnolipids were produced during the first 10 h (Fig. 5B). After 10 h, 22D10 began to secrete detectable concentrations of rhamnolipids. At the same time, a slight but reproducible growth defect was evident when the trpD mutant was compared with the parent strain (Fig. 5B), suggesting that the bacteria were confronted with a partial tryptophan depletion. This depletion was not sufficient to arrest completely the growth of the trpD mutant, but it fully obliterated rhamnolipid production in these bacteria (Fig. 5). This observation indicated that even a partial tryptophan depletion can lead to a marked effect on the expression of bacterial virulence factors, which might account for the loss of virulence of the trpD mutant. As expected, complementation with a plasmid-encoded trpD gene restored partially bacterial virulence against Dictyostelium of the DP5 mutant as well as production of rhamnolipids.
and mutant cells (data not shown). The number of viable bacteria was checked in parallel and was found to be strictly proportional to the optical density of the culture for both wild-type and mutant DP5 (Table 4). These results indicate that infecting bacteria encounter conditions in vivo in which trpD is essential for induction of the rhl QS system.

Discussion

Many studies have shown that *P. aeruginosa* uses a largely overlapping set of virulence genes to infect different multicellular hosts, ranging from plants to insects and animals (Rahme et al., 2000). Only two studies so far have analysed the virulence of *P. aeruginosa* towards *Dictyostelium* unicellular amoebae (Cosson et al., 2002; Pukatzki et al., 2002). They have shown that, like in other systems, the TTSS and the QS circuits are essential for the virulence of *P. aeruginosa* towards *Dictyostelium*. However, as *Dictyostelium* is the only unicellular host used to analyse *P. aeruginosa* virulence, it might conceivably present significant differences compared with multicellular hosts. In this study we used *Dictyostelium* amoebae as a host to assess virulence of *P. aeruginosa*, and as a tool to find new bacterial virulence genes. Our work led to the identification of three new virulence genes in *P. aeruginosa*: trpD, pchH and pchI. The genetic inactivation of any one of these genes resulted in a strong decrease in bacterial virulence measured in a *Dictyostelium* host model. This diminished virulence was also observed in two other models of infection, a feeding assay in *Drosophila* and a mouse pneumonia model. This demonstrates further that *Dictyostelium* represents a host system comparable to other more complex hosts, and can be used as a simple means to dissect *P. aeruginosa* virulence mechanisms.

Our results also question the relevance of various animal models and modes of infection to assess the virulence of *P. aeruginosa*. Indeed, several *P. aeruginosa* mutants were not virulent when ingested by *Drosophila* flies but were virulent when inoculated by pricking. Our results thus indicate that PchH, PchI and TrpD are required during the slow infectious process following ingestion, but dispensable during the very rapid infection initiated by pricking flies. These differences could reflect

![Fig. 5. Production of rhamnolipids is impaired in trpD mutant bacteria.](image)

**A** 

| amoeba lysis | rhamnolipids (μg ml⁻¹) |
|-------------|----------------------|
| 22D10       | yes                  |
| trpD        | no                   |
| pchH        | yes                  |

**B**

| Time (h) | OD 600 nm | 22D10 | trpD |
|----------|-----------|-------|------|
| 0        | -         | -     | -    |
| 3        | -         | -     | -    |
| 5        | -         | -     | -    |
| 6        | -         | -     | -    |
| 7        | -         | -     | -    |
| 9        | +         | +     | +    |
| 12       | +         | +     | +    |
| 13       | +         | +     | +    |
| 14       | +         | +     | +    |
| 15       | +         | +     | +    |
| 16       | +         | +     | +    |
| 17       | +         | +     | +    |
| 18       | +         | +     | +    |
| 19       | +         | +     | +    |
| 20       | +         | +     | +    |
| 21       | +         | +     | +    |
| 22       | +         | +     | +    |
| 23       | +         | +     | +    |
| 24       | +         | +     | +    |
| 25       | +         | +     | +    |
| 26       | +         | +     | +    |
| 27       | +         | +     | +    |

![Table 4. Evaluation of rhlA RNA levels (rhlA/rpsL ratio) in orally infected flies (t = 48 h).](image)

|          | Exp 1 | Exp 2 | Exp 3 |
|----------|-------|-------|-------|
| 22D10    | 127   | 8.41  | 118   |
| DP28 (pchH) | 151   | 8.61  | 82.5  |
| DP5 (trpD)| 0.01  | 0.26  | 3.26  |
qualitative differences between the two modes of infection, related for example to the fact that bacteria are initially introduced in very different environments. The two modes of infection might also differ due to their very different kinetics. We can, however, only speculate on this point, because it is not known precisely how ingested bacteria kill infected flies. They may escape the gut and spread to the whole body, or damage to the gut might be sufficient to cause death. As alternative infection models are usually required to mimic the results obtained in mammalian systems, the slow infection following ingestion seems preferable to evaluate the virulence of 22D10 P. aeruginosa. More generally, the use of several hosts (mouse, flies, amoebae) and modes of infection appears as a good strategy to circumvent the limitations inherent to any given model.

The fact that TrpD was necessary for virulence of P. aeruginosa in our in vitro assay indicates that bacteria encountered at least a partial tryptophan depletion. This was confirmed by assessing P. aeruginosa growth in the conditions of our assay (SM medium): a slight but reproducible decrease in bacterial growth was observed after 12 h of growth, indicating a partial depletion of tryptophan. Remarkably, this partial depletion was sufficient to completely inhibit the rhl-dependent production of rhamnolipids. As these observations were made in vitro, in conditions that were chosen largely arbitrarily, one must question their relevance to the situation in an infected animal. Two lines of evidence in the literature support the idea that bacteria infecting an animal host encounter conditions of at least partial tryptophan depletion. First, the ability to synthesize tryptophan has been shown to be essential for the establishment of a harmful infection in several instances: trpD mutants of Klebsiella pneumoniae or Mycobacterium tuberculosis are incapable of establishing a lethal pneumonia in mice (Smith et al., 2001; Benghezal et al., 2006), and Staphylococcus aureus trpD mutants are incapable of colonizing the mouse spleen (Mei et al., 1997). Second, it has been proposed that the activation of the immune system can cause a local tryptophan depletion, which might participate in the control of invading pathogens (Moffett and Namboodiri, 2003). Our results show that trpD mutants of P. aeruginosa are incapable of mounting a harmful pneumonia in mice, indicating that they did encounter at least a partial depletion of tryptophan in vivo. Indeed, expression of rhlA in the trpD mutant was strongly reduced during Drosophila infection, a result that directly demonstrates the requirement for TrpD in the induction of the rhl QS system in vivo. This observation is compatible with recent work showing that environmental stresses and nutritional factors affect the induction of QS systems in P. aeruginosa (Bazire et al., 2005; Soberon-Chavez et al., 2005). Another possibility is suggested by the fact that one of the precursors in tryptophan biosynthesis, anthranilate, is also a precursor for the synthesis of PQS, which can impact on the rhl QS system (McKnight et al., 2000). An alteration in tryptophan biosynthesis may thus alter induction of the rhl QS system via an effect on PQS synthesis.

Our observations also establish a link between the virulence of pchH and pchI mutants and their ability to induce the TTSS, another major virulence system in P. aeruginosa. In this case also, the conditions of induction of TTSS, although similar to those used by many other investigators, were arbitrarily established in vitro. The relevance of our observations to the situation in infected animals is proven mainly by the observation that the same mutants lost virulence towards Drosophila and mouse. However, it remains to be established if this loss of virulence was really caused by an effect on TTSS in vivo. Unfortunately, we were unable to measure reproducibly the expression of TTSS genes in vivo, probably due to lower expression levels (data not shown). Much more detailed studies will be necessary to determine the precise role played by PchH and PchI in the induction of TTSS proteins in vivo and, more generally, the function of these proteins during bacterial infections.

One clear limitation of this study was caused by the strong bias for transposon insertion, resulting in a high degree of redundancy among the mutants isolated. Consequently, only a few new virulence genes were identified. It is likely that the use of another P. aeruginosa strain or of another mutagenesis protocol would allow a more extensive identification of bacterial virulence genes. Our results so far suggest that this approach can lead to the identification of genes that are equally important during infection of mammalian hosts. The Dictyostelium host model might thus prove useful in the future for the systematic analysis of bacterial virulence.

**Experimental procedures**

**Cell culture and strains**

*Dictyostelium discoideum* DH1-10 cells (Caterina et al., 1994; Cornillon et al., 2000) were grown at 21°C in HL5 medium (Cornillon et al., 1998). To test Dictyostelium growth on bacteria, 50 μl of overnight bacterial cultures was diluted in 3 ml of LB and grown at 37°C for 7 h. Thirty microlitres of this culture was plated on SM-Agar (peptone 10 g l\(^{-1}\), yeast extract 1 g l\(^{-1}\), KH\(_2\)PO\(_4\) 2.2 g l\(^{-1}\), K\(_2\)HPO\(_4\) 1 g l\(^{-1}\), MgSO\(_4\).7H\(_2\)O 1 g l\(^{-1}\), Agar 20 g l\(^{-1}\)) (24 well plates, 2 ml SM-Agar per well), then Dictyostelium cells were deposited on the bacterial lawn. Amoebal growth created phagocytic plaques after 4–7 days of incubation at 25°C. For screening, a single test was performed for each mutant strain tested (5000 Dictyostelium deposited). To assess the virulence of a given strain more quantitatively, variable numbers of Dictyostelium cells (from 10 000 to 3) were deposited on the bacterial lawn.
**Mutagenesis of P. aeruginosa**

The *P. aeruginosa* clinical isolate 22D10 (Denervaud et al., 2004) was used for random miniTn5 mutagenesis (de Lorenzo et al., 1990). The 22D10 isolate has a 465 bp deletion in the lasR gene encompassing the promoter region and part of its 5′ end (Denervaud et al., 2004), ensuing in reduced elastase and rhamnolipid production. For random mutagenesis, the miniTn5uxAB cassette was transferred by conjugation from strain S17-1pir (de Lorenzo et al., 1990) into the recipient 22D10. Transconjugants were selected on LB agar plates supplemented with tetracycline (75 mg l⁻¹) and ampicillin (40 mg l⁻¹) to counterselect against the *Escherichia coli* donor strain. A total of 2500 transconjugants were tested for decreased virulence in the *D. discoideum* host system as described above. Mutants exhibiting decreased virulence were characterized by Southern blotting using the RP3040 kit (Amersham) and a labelled probe corresponding to the inserted transposon to verify that only one transposon copy was inserted in each mutant. To identify the site of insertion, the genomic DNA was digested with NotI and NcoI, ligated into pGESZ opened with NotI and NcoI, and used to transform DH10B bacteria. Only bacteria carrying the plasmid possessing the inserted fragment of the transposon (and the flanking region) were capable of growing on LB media supplemented with ampicillin (100 mg l⁻¹) and tetracycline (10 mg l⁻¹). The region located next to the tetracycline-resistance cassette, and corresponding to the genomic insertion site, was then sequenced. In order to ascertain that the transposon insertion was causing the phenotype observed, the mutation was transduced to the wild-type strain by using the phage E79 (Morgan, 1979). The strain obtained exhibited the same phenotype as the original mutants from which they were derived (data not shown).

For complementation experiments, the *pchEFGH* (respectively *pchH*) genes were excised from pME6490 (Reimmann et al., 2001) on a 14.9 kb BgIII-HindIII (respectively 4.4 kb KpnI-EcoRI) fragment and cloned into pUCP-SK (Watson et al., 1996) under the vector’s lac promoter. This pME7047 (respectively pME6826) plasmid confers resistance to carbenicillin (250 mg l⁻¹).

The trpD gene of *P. aeruginosa* strain 22D10 was amplified by PCR using the following primers: GGATCCGGATCCTGGACGCCAGGTTGACGCG. The resulting 1.3 kb DNA fragment was digested with BamHI-HindIII and ligated into BamHI-HindIII cleaved plasmid pUCP22, downstream of the vector encoded promoter. This pME7047 (respectively pME6826) plasmid confers resistance to carbenicillin (250 mg l⁻¹).

The trpD gene of *P. aeruginosa* strain 22D10 was amplified by PCR using the following primers: GGATCCGGATCCTGGACGCCAGGTTGACGCG. The resulting 1.3 kb DNA fragment was digested with BamHI-HindIII and ligated into BamHI-HindIII cleaved plasmid pUCP22, downstream of the vector encoded promoter. A trpD carrying clone, termed pTRPD5, as well as the vector control pUCP22, were introduced into the *P. aeruginosa* trpD mutant DP5 by electroporation. Growth complementation was tested on M9 salts based agar medium supplemented with 0.2% glucose and 1 mM MgSO₄ ≥ 0.02% tryptophan. Virulence of the transformants in the *Dictyostelium discoideum* assay was assessed as described above.

To express constitutively *exsA*, cells were transformed with the pDD2 vector (Dacheux et al., 1999) and selected in the presence of carbenicillin (250 mg l⁻¹).

A pchH mutant in the CHA strain (Dacheux et al., 1999) was constructed by transducing the mutant pchH from PAO6335 (Reimmann et al., 2001) using the lipopolysaccharide-specific phage E79 tv2 (Morgan, 1979) and a spectinomycin selection (1 g l⁻¹). The transduced clones were further characterized by PCR.

Drosophila survival upon *P. aeruginosa* infection

Flies were raised at 25°C in standard medium and infections were performed at 25°C. Flies used for infection are *w¹¹¹⁸* in a wild-type CantonS background (*w¹¹¹⁸* 10 outcroses with wild-type CantonS). *P. aeruginosa* strains were grown on LB overnight at 37°C. For infection by septic injury, 30 adult male flies (5–7 days old) were pricked into the upper part of the thorax with a thin needle previously dipped into a bacterial solution prepared from an exponential phase culture (OD₆₀₀ = 0.8) diluted twice in sterile phosphate buffer (final OD₆₀₀ = 0.4). In control experiments, flies were pricked with a clean needle dipped in sterile PBS, and 100% survival was observed after 10 days. Multiplicity of infection was determined by infecting 30 flies and immediately killing them. Flies were dipped 30 s in ethanol to kill external bacteria. The flies were then ground in PBS, the debris pelleted by centrifugation (1000 r.p.m., 1 min), and the bacteria recovered in the supernatant were plated on LB plates. The multiplicity of infection in these experiments was about 150 bacteria per fly (data not shown). The number of viable bacteria (colony-forming units) in infected flies was determined using a similar procedure. For infection by ingestion, an exponential growth phase culture of *P. aeruginosa* was diluted to a final OD₆₀₀ of 0.2 in 5% sucrose; 2 ml of which was dispatched on absorbent paper placed into a vial. Female flies were transferred onto vials containing fresh bacterial solution every 3 days. Survival experiments were carried out with three sets of 10 flies for each strain tested. Results are expressed as percentage of live infected flies at different time points following infection, given as the mean of the three sets. Experiments were repeated at least three times with results similar to those presented.

**Mouse pneumonia model**

A lethal pneumonia model was used to evaluate bacterial pathogenicity. This protocol was approved by the comité consultatif d’éthique of the Centre de Recherche du Service de Santé des Armées (number 2003/53). Six female SWISS/CERJ mice (8 weeks old) were infected to assess the virulence of each *P. aeruginosa* strain. Bacteria were grown overnight at 37°C in LB, washed and resuspended in 0.9% NaCl (7.5 x 10⁴ bacteria ml⁻¹). Mice were injected with cyclophosphamide (150 mg kg⁻¹) at days −3 and −1 before the infection. At day 0, mice were anaesthetized with ketamine (60 mg kg⁻¹) and xylazine (8 mg kg⁻¹) and infected with 1.5 x 10⁸ bacteria by nasal instillation of 20 μl. Body temperature and weight were measured twice a day for 10 days. A temperature below 26°C was used as humane end-point for euthanasia.

**Production of rhamnolipids**

*Pseudomonas aeruginosa* was inoculated in SM medium at OD₆₀₀ = 0.1, and grown for 20 h at 37°C. Bacteria were pelleted by centrifugation (5 min, 25°C, 10 000 g) and supernatants were recovered and filtered. The ability of supernatants to cause lysis of *Dictyostelium* cells was assessed by incubating *Dictyostelium* cells in a diluted supernatant (1:1 in SM medium) and by direct observation in a microscope as described previously (Cosson et al., 2002). To evaluate the presence of rhamnolipids in supernatants, a 5 μl drop of supernatant was deposited on a plastic
Petri dish, and the diameter of the drop determined. The concentration of rhhamnolipids is correlated to the diameter of the drop (Ochsner et al., 1994). To measure precisely the concentration of rhhamnolipids, they were extracted from the bacterial supernatants and their concentration determined by the orcinol assay (Chandrasekaran and Berniller, 1980). C4-HSL concentrations were determined after 15 h of growth in culture supernatants. Culture supernatants were extracted twice with ethyl acetate acidified by the addition of 0.01% acetic acid. After centrifugation, the supernatant was recovered after centrifugation. The identity of three secreted proteins (ExoS, PopD and PopB) secreted by the CHA strain, the four major proteins observed during TTSS induction, and grown at 37°C for 4 h. The supernatant was recovered after centrifugation (10 min, 10 000 g, 4°C), and 15 μl was loaded on a 10% SDS-polyacrylamide gel. After migration the proteins were visualized by silver staining (Switzer et al., 1979) or transferred to a nitrocellulose membrane and revealed with rabbit antiserum as indicated. When compared with the well-characterized proteins secreted by the CHA strain, the four major proteins observed migrated at the size of the ExoT, ExoS, PopB and PopD proteins.

To measure induction of *Pseudomonas* *rhlA* in infected flies, flies were collected at 48 h post infection and ingested bacteria recovered as described above. RNAs were extracted (RNAo+, MP Biomedicals) and analysed by real-time PCR as described below, using the following pair of primers: TCCGCAGCTCGGTGGT GATG and GGTGGCACGGCAAAGCATG.

**Induction of the TTSS**

To induce expression of TTSS proteins, bacteria were grown overnight in LB medium, then inoculated (OD600 = 0.1) in either LB medium (no TTSS induction) or LB medium supplemented with 5 mM EGTA and 20 mM MgCl2 (TTSS induction), and grown at 37°C for 4 h. The supernatant was recovered after centrifugation (10 min, 10 000 g, 4°C), and 15 μl was loaded on a 10% SDS-polyacrylamide gel. After migration the proteins were visualized by silver staining (Switzer et al., 1979) or transferred to a nitrocellulose membrane and revealed with rabbit antiserum as indicated. When compared with the well-characterized proteins secreted by the CHA strain, the four major proteins observed migrated at the size of the ExoT, ExoS, PopB and PopD proteins. The identity of three secreted proteins (ExoS, PopD and PopB) was confirmed using specific antibodies (data not shown).

To measure the abundance of mRNAs encoding TTSS proteins, RNA was extracted from 250 μl of bacteria using the RNeasy kit (Quiagen). The purified RNA was eluted in 50 μl of RNA-free water, treated with DNase, reverse-transcribed, and analysed by real-time PCR as described previously (Dumas et al., 2006) using the following pairs of primers:

CAAGGGAAAGGACAGCCGAATACTC and GCGGCGATAGCTCTGGTAGAATAG (for *exoS*), GTCGCCCAACAGCCT GTACTCCATGCTC and ATGAAAGCACCAGATGAAACCATCAT (for *popD*), ACGGAGGTAAGGGCCAGCTCTTGA and AGTGTGCCGG TACTCCGGTGTGTTGG (for *exoT*), GCAAGGCATGGCTCGA CAAGA and CGCTGTGCCTGTGGTGGTTGTA (for *rpsL*). The ribosomal *rpsL* gene was used as a control to normalize the expression levels of the target genes (Dumas et al., 2006).

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**References**

Bazire, A., Dheilly, A., Diab, F., Morin, D., Jebbar, M., Haras, D., and Dufour, A. (2005) Osmotic stress and phosphate limitation affect production of cell-to-cell signal molecules and rhhamnolipid biosurfactant by *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 253: 125–131.

Benghezal, M., Faouarque, M.O., Tournebize, R., Froquet, R., Marchetti, A., Bergeret, E., et al. (2006) Specific host genes required for the killing of *Klebsiella* bacteria by phagoctyes. *Cell Microbiol* 8: 139–148.

Caterina, M.J., Milne, J.L., and Devreotes, P.N. (1994) Mutation of the third intracellular loop of the cAMP receptor, cAR1, of *Dictyostelium* yields mutants impaired in multiple signaling pathways. *J Biol Chem* 269: 1523–1532.

Chandrasekaran, E.V., and Berniller, J.N. (1980) Constituent analysis of glycosaminoglycans. In *Methods in Carbohydrate Chemistry*. Whistler, R.L. (ed.). New York: Academic Press, pp. 89–96.

Dumillon, S., Olle, R.A., and Golstein, P. (1998) An insertional mutagenesis approach to *Dictyostelium* cell death. *Cell Death Differ* 5: 416–425.

Dumillon, S., Pech, E., Benghezal, M., Ravanel, K., Gaynor, E., Letournier, F., et al. (2000) Phg1p is a nine-transmembrane protein superfAMILY member involved in *dictyostelium* adhesion and phagocytosis. *J Biol Chem* 275: 34287–34292.

Cosson, P., Zulianello, L., Join-Lambert, O., Faurisson, F., Gebbie, L., Benghezal, M., et al. (2002) *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. *J Bacteriol* 184: 3027–3033.

D’Argenio, D.A., Gallagher, L.A., Berg, C.A., and Manoil, C. (2001) Drosophila as a model host for *Pseudomonas aeruginosa* infection. *J Bacteriol* 183: 1466–1471.

D’Argenio, D.A., Wu, M., Hoffman, L.R., Kulasekara, H.D., Deziel, E., Smith, E.E., et al. (2007) Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol* 64: 512–533.

Dacheux, D., Attree, I., Schneider, C., and Toussaint, B. (1999) Cell death of human polymorphonuclear neutrophils induced by a *Pseudomonas aeruginosa* cystic fibrosis isolate requires a functional type III secretion system. *Infect Immun* 67: 6164–6167.

van Delden, C., Comte, R., and Bally, A.M. (2001) Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. J *Bacteriol* 183: 5376–5384.

Denervaud, V., TuQuoc, P., Blanc, D., Favre-Bonte, S., Krishnapillai, V., Reimann, C., et al. (2004) Characterization of cell-to-cell signaling-deficient *Pseudomonas aeruginosa* strains colonizing intubated patients. *J Clin Microbiol* 42: 554–562.

Dumas, J.L., van Delden, C., Perron, K., and Kohler, T. (2006) Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. *FEMS Microbiol Lett* 254: 217–225.

Erickson, D.L., Lines, J.L., Pesci, E.C., Venturi, V., and Storey, D.G. (2004) *Pseudomonas aeruginosa* relA contributes to virulence in Drosophila melanogaster. *Infect Immun* 72: 5638–5645.

Essar, D.W., Eberly, L., Han, C.Y., and Crawford, I.P. (1990)
DNA sequences and characterization of four early genes of the tryptophan pathway in *Pseudomonas aeruginosa*. *J Bacteriol* 172: 853–866.

Fauvarque, M.O., Bergeret, E., Chabert, J., Dacheux, D., Satre, M., and Attree, I. (2002) Role and activation of type III secretion system genes in *Pseudomonas aeruginosa*-induced *Drosophila* killing. *Microb Pathog* 32: 287–295.

Froquet, R., Cherix, N., Burr, S., Frey, J., Vilches, S., Tomas, J.M., and Cosson, P. (2007) An alternative host model to evaluate Aeromonas virulence. *Appl Environ Microbiol* 73: 5657–5659.

Hilbi, H., Weber, S.S., Ragaz, C., Nyfeler, Y., and Uweryl, S. (2007) Environmental predators as models for bacterial pathogenesis. *Environ Microbiol* 9: 563–575.

Latifi, A., Fogliano, M., Tanaka, K., Williams, P., and Lazdunski, A. (1996) A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* 21: 1137–1146.

de Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K.N. (1999) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* 172: 6568–6572.

Lyczak, J.B., Cannon, C.L., and Pier, G.B. (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect* 2: 1051–1060.

McKnight, S.L., Igleswki, B.H., and Pesci, E.C. (2000) The *Pseudomonas* quinolone signal regulates rhl quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 182: 2702–2708.

Mei, J.M., Nourbakhsh, F., Ford, C.W., and Holden, D.W. (2017) Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteremia using signature-tagged mutagenesis. *Mol Microbiol* 26: 399–407.

Moffett, J.R., and Namboodiri, M.A. (2003) Tryptophan and the immune response. *Immunol Cell Biol* 81: 247–265.

Morgan, A.F. (1979) Transduction of *Pseudomonas aeruginosa* with a mutant of bacteriophage E79. *J Bacteriol* 139: 137–140.

Ochsner, U.A., Koch, A.K., Fiechter, A., and Reiser, J. (1994) Isolation and characterization of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* 176: 2044–2054.

Pesci, E.C., Milbank, J.B., Pearson, J.P., McKnight, S., Kende, A.S., Greenberg, E.P., and Igleswki, B.H. (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 96: 11229–11234.

Pesci, E.C., Pearson, J.P., Seed, P.C., and Igleswki, B.H. (1997) Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 179: 3127–3132.

Pukatzki, S., Kessin, R.H., and Mekalanos, J.J. (2002) The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. *Proc Natl Acad Sci USA* 99: 3159–3164.

Rahme, L.G., Ausubel, F.M., Cao, H., Drenkard, E., Gounnerov, B.C., Lau, G.W., et al. (2000) Plants and animals share functionally common bacterial virulence factors. *Proc Natl Acad Sci USA* 97: 8815–8821.

Rahme, L.G., Tan, M.W., Le, L., Wong, S.M., Tompkins, R.G., Calderwood, S.B., and Ausubel, F.M. (1997) Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. *Proc Natl Acad Sci USA* 94: 13245–13250.

Reimmann, C., Patel, H.M., Serino, L., Barone, M., Walsh, C.T., and Haas, D. (2001) Essential PchG-dependent reduction in pyochelin biosynthesis of *Pseudomonas aeruginosa*. *J Bacteriol* 183: 813–820.

Reimmann, C., Serino, L., Beyeler, M., and Haas, D. (1998) Dihydroaeruginoic acid synthetase and pyochelin synthetase, products of the pchEF genes, are induced by extracellular pyochelin in *Pseudomonas aeruginosa*. *Microbiology* 144 (Part 11): 3135–3148.

Roy-Burman, A., Savel, R.H., Racine, S., Swanson, B.L., Revadigar, N.S., Fujimoto, J., et al. (2001) Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis* 183: 1767–1774.

Rumbaugh, K.P., Griswold, J.A., and Hamood, A.N. (2000) The role of quorum sensing in the *in vivo* virulence of *Pseudomonas aeruginosa*. *Microbes Infect* 2: 1721–1731.

Serino, L., Reimmann, C., Visca, P., Beyeler, M., Chiesa, V.D., and Haas, D. (1997) Biosynthesis of pyochelin and dihydroaeruginoic acid requires the iron-regulated *pchDCBA* operon in *Pseudomonas aeruginosa*. *J Bacteriol* 179: 248–257.

Sifri, C.D., Begun, J., and Ausubel, F.M. (2005) The worm has turned – microbial virulence modeled in *Caenorhabditis elegans*. *Trends Microbiol* 13: 119–127.

Smith, D.A., Parish, T., Stoker, N.G., and Bancroft, G.J. (2001) Characterization of auxotrophic mutants of *Mycobacterium tuberculosis* and their potential as vaccine candidates. *Infect Immun* 69: 1142–1150.

Soberon-Chavez, G., Aguirre-Ramirez, M., and Ordonez, L. (2005) Is *Pseudomonas aeruginosa* only ‘sensing quorum’? *Crit Rev Microbiol* 31: 171–182.

Switzer, R.C., 3rd, Merril, C.R., and Shifrin, S. (1979) A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal Biochem* 98: 231–237.

Van Delden, C., and Igleswki, B.H. (1998) Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* 4: 551–560.

Vodovar, N., Vodovar, N., Vinals, M., Liehl, P., Basset, A., Degrouard, J., Spellman, P., et al. (2005) *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species. *Proc Natl Acad Sci USA* 102: 11414–11419.

Wade, D.S., Calfee, M.W., Rocha, E.R., Ling, E.A., Engstrom, E., Coleman, J.P., and Pesci, E.C. (2005) Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* 187: 4372–4380.

Watson, A.A., Alm, R.A., and Mattick, J.S. (1996) Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. *Gene* 172: 163–164.

Yahr, T.L., Mende-Mueller, L.M., Friese, M.B., and Frank, D.W. (1997) Identification of type III secreted products of
the *Pseudomonas aeruginosa* exoenzyme S regulon. *J Bacteriol* **179**: 7165–7168.

Yahr, T.L., and Wolfgang, M.C. (2006) Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Mol Microbiol* **62**: 631–640.

Zulianello, L., Canard, C., Kohler, T., Caille, D., Lacroix, J.S., and Meda, P. (2006) Rhamnolipids are virulence factors that promote early infiltration of primary human airway epithelia by *Pseudomonas aeruginosa*. *Infect Immun* **74**: 3134–3147.

**Supplementary material**

The following supplementary material is available for this article online:

**Fig. S1.** *P. aeruginosa* mutants are less virulent towards mice. A lethal mouse pneumonia infection model was used to assess the virulence of each *P. aeruginosa* strain as described in the legend to Fig. 3. *PchI* and *trpD* mutants (but not *pchE*) were less virulent than 22D10.

**Fig. S2.** Complementation of DP5 (*trpD*) mutant. DP5 mutant bacteria were transformed with a plasmid expressing (pTRPD5), or not (vector) the *trpD* gene.

A. The ability to grow on M9 medium was partially restored by *trpD* expression.

B. Complementation with *trpD* also restored partially virulence against *Dictyostelium* and production of rhamnolipids in SM medium.

**Table S1.** Evaluation of *rhlA* RNA levels (*rhlA/rpsL* ratio) *in vitro*.

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