Evolution of *Pseudomonas aeruginosa* virulence in infected patients revealed in a *Dictyostelium discoideum* host model

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Abstract

Pseudomonas aeruginosa can cause acute lung infections in intubated patients or chronic infections in patients with cystic fibrosis (CF). In both situations, *P. aeruginosa* accumulates specific mutations, in particular in the *lasR* quorum-sensing regulator gene. Using a *Dictyostelium discoideum* amoeba model, we assessed whether these mutations affect bacterial virulence. Among a collection of clinical isolates from 16 CF patients, initial isolates were fully virulent in 15 patients, but for late isolates collected several years later, virulence was decreased in eight patients. No significant correlation between genetic inactivation of *lasR* and decreased virulence was observed. Among strains isolated from ten colonized intubated patients, all initial isolates were fully virulent. Despite the accumulation of *lasR*-inactivating mutations in strains collected over a 3-week period, no decrease in virulence was observed in eight of 10 patients. In one intubated patient, the virulent initial strain was replaced a few days later with a different, less virulent, strain. We observed a gradual decrease in bacterial virulence in only one intubated patient. We conclude that adaptation of *P. aeruginosa* to chronically infected CF patients can lead to a slow and gradual loss of virulence, as measured in a *Dictyostelium* model system. However, loss of virulence is not caused predominantly by mutations in *lasR*. During short-term colonization of intubated patients for up to 20 days, a decrease in virulence was exceptional, despite the accumulation of *lasR* mutations.

Keywords: Cystic fibrosis, *Dictyostelium discoideum*, lasR, *Pseudomonas aeruginosa*, quorum-sensing, virulence
Original Submission: 27 July 2010; **Revised Submission:** 29 October 2010; **Accepted:** 15 November 2010
Editor: G. Greub
Article published online: 22 November 2010
Clin Microbiol Infect 2011; **17:** 1415–1420
10.1111/j.1469-0691.2010.03431.x

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Introduction

Pseudomonas aeruginosa is a bacterial pathogen responsible for a wide variety of infections, in particular in hospitalized patients, or individuals with cystic fibrosis (CF) [1]. *P. aeruginosa* mutates and adapts rapidly to a changing environment. In a seminal study [2], two *P. aeruginosa* isolates collected from a CF patient with a 7.5-year interval were fully sequenced, and 68 mutations were identified in the later isolate. Thirteen of these mutations affected genes potentially implicated in virulence, including *lasR*, which encodes a central regulator of the quorum-sensing (QS) circuit, and *exsA*, which encodes the regulator of the type III secretion system (TTSS). These mutations were also found to be frequent in late *P. aeruginosa* isolates from CF patients [2]. Remarkably, in *P. aeruginosa* isolates colonizing intubated patients, *lasR*-inactivating mutations accumulate within days, suggesting that this evolution can be extremely rapid [3]. Although these observations suggest that airway persistence of *P. aeruginosa* may be linked to decreased virulence, the pathogenicity of these strains was not tested. Thus, whether bacterial virulence effectively changes during airway colonization and chronic infections remains an open question.

Changes in bacterial virulence in such clinical settings can be detected experimentally by systematically testing the virulence of sequential *P. aeruginosa* isolates. However, it is difficult to envisage such extensive analyses using rodent infection models, for both practical and ethical reasons. Non-mammalian host models do not suffer from these limitations, and many studies have shown that they provide an accurate measure of *P. aeruginosa* virulence [4]. Previous studies have established that *P. aeruginosa* uses essentially the

same virulence pathways, in particular those of the QS and the TTSS systems, to infect mice and *Dictyostelium discoideum* amoebae [4–6]. Indeed, amoebae are natural predators of *P. aeruginosa*, and the bacterial pathogenic mechanisms may have developed primarily as defence systems [7,8]. In the present study, we used this non-mammalian host model to assess the dynamics of *P. aeruginosa* virulence during colonization and chronic infection, as well as its link to genetic inactivation of *lasR*.

Materials and Methods

Cells and culture

P. aeruginosa strains isolated from CF patients [2] or intubated patients [3] have been described previously. Control strains were included in every test: PAO1 (PT5) and two isogenic QS mutants, PT462 (*rhlR*) and PT531 (*rhlR–lasR*) [4]. In agreement with previously published results, the virulence scores for these strains were 8, 2 and 0, respectively.

The *D. discoideum* cells used in this study were subclone DH1-10 of the DH1 strain [9]. *D. discoideum* cells were grown at 21°C in HL5 medium [10].

Bacterial virulence assay

Bacterial virulence was determined essentially as previously described [10]. Briefly, *P. aeruginosa* isolates were streaked on LB plates and grown at 37°C, and individual colonies were then grown overnight (18 h) in 3 mL of LB, at 37°C with shaking. The very few isolates (<1%) that did not reach high cell densities ($OD_{600\ nm}$ > 3) were not analysed further. Fifty microlitres of this culture was deposited on 2 mL of SM50%-Agar (5 g/L peptone, 0.5 g/L yeast extract, 1.1 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 5 g/L glucose, 20 g/L agar) in each well of a 24-well plate. Five microlitres of HL5 containing 10 000, 1000, 100 or ten *D. discoideum* cells was added to the bacterial lawn, and the plate was incubated at 25°C. *D. discoideum* growth was followed for 10 days.

Results

Evolution of virulence during chronic *P. aeruginosa* infections in CF patients

We first analysed the virulence of previously characterized isolates from 16 CF patients chronically infected with *P. aeruginosa* [2]. For this, we used *D. discoideum* as a model host as previously described [10], but with a few variations, described below, that provided a more quantitative measure of bacterial virulence. Growth of *D. discoideum* cells on bacteria was assessed at two different times (days 4 and 10) (Fig. 1). The results (from 0 to 4) of these two time points were added, and the virulence of *P. aeruginosa* strains was scored on a scale between 0 (avirulent) and 8 (highly virulent). In addition, as some clinical isolates are mixtures of different clones, we tested at least six individual clones for each clinical isolate.

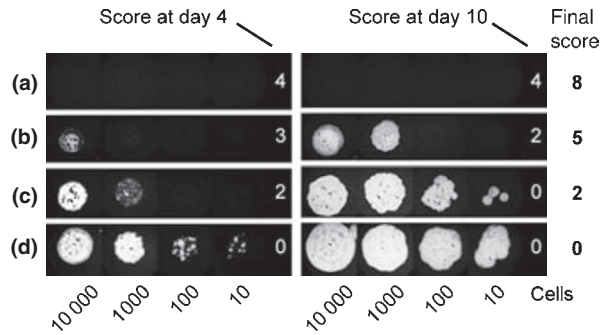

FIG. 1. Measuring the virulence of *Pseudomonas aeruginosa*. The virulence of bacteria was determined by their ability to prevent growth of *Dictyostelium discoideum*. *D. discoideum* cells (10 000, 1 000, 100 or 10) were deposited on a lawn of bacteria in a 24-well plate. *D. discoideum* cells grew on avirulent bacteria and created a phagocytic plaque (white). To score the virulence, we determined, at days 4 and 10, the number of wells in which growth of *D. discoideum* was inhibited. The final virulence score was obtained by adding up the scores for days 4 and 10, yielding a number ranging from 0 (avirulent) to 8 (fully virulent). (a) PT5. (b) isolate 4A from patient 15 101. (c) PT462. (d) PT531 [4].

With the exception of patient 14, all early isolates of *P. aeruginosa* from CF patients were virulent in our model (Fig. 2). For eight patients (patients 1, 4, 5, 6, 10, 11, 15 and 16), we observed no decrease in virulence in isolates collected at older ages (Fig. 2). We analysed the virulence of all 35 isolates available from patient 1, in which most of the initial genetic analysis was performed [2]. Although only a few of the results are shown in Fig. 2, all of these isolates were virulent, indicating that the mutations previously identified in this strain did not cause a significant loss of virulence in the *D. discoideum* host model.

For the seven other patients (patients 2, 3, 7, 8, 9, 12 and 13), at least one late isolate was significantly less virulent (Fig. 2). Although a few isolates (e.g. in patient 12) showed uniformly avirulent phenotypes, we often observed a wide range of intermediate virulence levels when different clones of the same isolate were tested. None of these heterogeneous isolates corresponded to a characterized mutator isolate [11], suggesting that these results reflected the heterogeneity of the original isolates. Indeed, upon retesting, the