

Clinical populations of *Pseudomonas aeruginosa* isolated from acute infections show a wide virulence range partially correlated with population structure and virulence gene expression

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Pseudomonas aeruginosa is a ubiquitous environmental bacterium responsible for a variety of infections in humans, as well as in animal hosts. While the evolution of virulence in *P. aeruginosa* strains isolated from chronic lung infection in cystic fibrosis (CF) patients has been extensively studied, the virulence phenotype of *P. aeruginosa* isolated from other infection types or from the environment is currently not well characterized. Here we report an extensive analysis of the virulence of *P. aeruginosa* strains isolated from acute infections compared with population structure. Virulence profiles of individual strains were also compared with the expression levels of the *rhlR* gene, the transcriptional regulator of the *rhl* quorum-sensing system, and the gene encoding *Crc*, a global regulator controlling catabolite repression and carbon metabolism. Additionally, the presence/absence of the two mutually exclusive genes, *exoU* and *exoS*, encoding effectors of the type III secretion system, was assessed. In order to capture the widest range of genetic variability, a collection of 120 clinical strains was initially characterized by repetitive element-based PCR genotyping, and a selection of 27 strains belonging to different clonal lineages was subsequently tested using three different virulence assays, including two *Dictyostelium discoideum* assays on different growth media, and a *Caenorhabditis elegans* fast-killing assay. We show that the parallel application of virulence assays can be used to quantitatively assess this complex, multifactorial phenotypic trait. We observed a wide spectrum of virulence phenotypes ranging from weakly to highly aggressive, indicating that clinical strains isolated from acute infections can present a reduced or altered virulence phenotype. Genotypic associations only partially correlated with virulence profiles and virulence gene expression, whereas the presence of either *exoU* or *exoS* was not significantly correlated with virulence. Interestingly, the expression of *rhlR* showed a significant and positive correlation with the virulence profiles obtained with the three assays, while the expression of *crc* was either negatively or not correlated with virulence, depending on the assay.

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen and one of the main causes of nosocomial infections, including pneumonia, urinary tract infections, surgical wound infections and bloodstream infections. It is frequently isolated from immunocompromised individuals and intubated patients, and causes chronic lung infections in cystic fibrosis (CF) patients, as well as in adults with

bronchiectasis and chronic obstructive pulmonary disease (Valderrey *et al.*, 2010).

Understanding bacterial virulence and, more generally, the host–pathogen relationship at the cellular and molecular level is essential to identify new targets and develop new strategies to fight infection. Molecular analysis of host–pathogen interactions benefits from the use of model systems, allowing a systematic study of the factors involved. In this regard the social amoeba *Dictyostelium discoideum* has been extensively used in recent virulence studies of various pathogenic bacteria, among which are *Salmonella typhimurium* (Sillo *et al.*, 2011), *Streptococcus suis* (Bonifait

Abbreviations: CF, cystic fibrosis; rep-PCR, repetitive element-based PCR.

et al., 2011), *Vibrio cholerae* (Miyata *et al.*, 2011), *Burkholderia pseudomallei* (Hasselbring *et al.*, 2011), *Legionella pneumophila* (Shevchuk & Steinert, 2009), *Klebsiella pneumoniae* (Pan *et al.*, 2011) and *P. aeruginosa* (Alibaud *et al.*, 2008). *Dictyostelium* cells are typically used as a screening system to determine the role of individual genes in virulence, by comparing the virulence phenotype of wild-type and mutant bacterial strains. In *P. aeruginosa*, such an approach has led to the identification of a number of virulence genes involved in various processes, among which are quorum-sensing (Pukatzki *et al.*, 2002; Cosson *et al.*, 2002), induction of the type III secretion system (Alibaud *et al.*, 2008) and global metabolic regulation (Linares *et al.*, 2010).

The microevolution of *P. aeruginosa* during the course of infection in CF patients has been extensively studied and typically leads to the selection of diversely virulent variants, based on mutations or expression changes in many virulence genes, including type III secretion, quorum sensing and iron acquisition (Hogardt & Heesemann, 2010; Lelong *et al.*, 2011; Kesarwani *et al.*, 2011). While early infection CF isolates are more virulent and more likely to cause acute infections than late isolates, the latter have been shown to maintain their ability to cause chronic infection and inflammation (Bragonzi *et al.*, 2009). Late CF isolates therefore exhibit an altered virulence pattern with respect to acute infection isolates, as recently confirmed by the comparison of transcriptomes of isogenic early acute infection versus chronic infection isolates (Naughton *et al.*, 2011). In contrast to CF isolates, very little is known about the virulence phenotype of *P. aeruginosa* isolated from other infection types or from the environment; a recent study (Bradbury *et al.*, 2011) showed that CF strains are globally less virulent against *D. discoideum* than those isolated from other sources. However, the experimental settings used by Bradbury and co-workers allowed the study of the virulence range of CF strains only, since only one non-CF strain supported growth of *D. discoideum*.

In the present study, we performed an extensive analysis of the virulence range of *P. aeruginosa* strains isolated from acute infections and compared it with the genotypic population structure. Additionally, we measured in a selection of strains the expression level of the *rhlR* gene, the transcriptional regulator of the *rhl* quorum-sensing system, and of the gene encoding Crc, a global regulator controlling catabolite repression and carbon metabolism in *P. aeruginosa*. Both genes have been shown to play a role in the virulence phenotype of *P. aeruginosa*, in particular against *D. discoideum* (Cosson *et al.*, 2002; Linares *et al.*, 2010). Furthermore, the presence/absence of the two mutually exclusive genes *exoU* and *exoS* (Wareham & Curtis, 2007), encoding effectors of the type III secretion system, was also assessed. In order to capture the widest range of genetic variability, a collection of 120 clinical strains was initially characterized by repetitive element-based PCR (rep-PCR) genotyping (Syrmis *et al.*, 2004), and a selection of 27 strains belonging to different clonal lineages was subsequently

characterized using three different virulence assays, including two *D. discoideum* assays on SM and HL5 growth media (Froquet *et al.*, 2009), and a *Caenorhabditis elegans* fast-killing assay (Tan *et al.*, 1999). In a previous study (Fumanelli *et al.*, 2011), we showed the importance of determining the correct experimental parameters for the application of *Dictyostelium* virulence assays to bacterial species or strains of unknown aggressiveness. In order to quantitatively estimate the level of virulence associated with each strain and to make results directly comparable, a virulence score was defined for each assay. A surprisingly high range of virulence phenotypes was observed. Correlations between clonal lineages and virulence profiles of individual strains were examined by mapping virulence scores, virulence gene expression levels, and *exoU/exoS* genotype on a phylogenetic tree derived from rep-PCR genotyping data.

METHODS

Strains and growth media. A total of 120 strains of *P. aeruginosa* were isolated from patients at Santa Chiara Hospital (Trento, Italy) affected by lung infections, urinary tract infections or skin ulcers. These strains were initially grown on MacConkey agar and stored at -80°C in 20% (v/v) glycerol. *D. discoideum* NC4 strain (DBS0304666) was obtained from the Dicty Stock Center (Northwestern University, IL, USA). *C. elegans* strain N2 was obtained from the Caenorhabditis Genetic Center (University of Minnesota). *D. discoideum* NC4 was grown on SM broth using *Klebsiella aerogenes* as a food source, whereas *C. elegans* N2 was grown on NGM (nematode growth medium) agar plates with *E. coli* OP50 as a carbon source.

Rep-PCR-based genotyping assays. ERIC-PCR and BOX-PCR assays were performed as previously described (Syrmis *et al.*, 2004) on the whole *P. aeruginosa* collection, consisting of 120 clinical strains isolated from acute infections, and on reference strains PA14, PAO1, PA2192 and LESB58. The reaction mixture contained 6 mM MgCl_2 , $1\times$ PCR buffer, 200 μM each dNTP, 1 μM each ERIC primer and 1.2 μM BOX primer, 2.5 U *Taq*, 0.2% (v/v) glycerol and 100 ng genomic DNA. The final reaction volume was adjusted to 50 μl with PCR grade water. PCR amplification cycling was an initial denaturation step at 94°C for 7 min, followed by 30 cycles with a denaturation step of 1 min at 94°C , an annealing step of 1 min at 53°C for BOX and 55°C for ERIC primers, and an extension step of 2 min at 72°C , followed by a final extension step of 10 min at 72°C . Ten microlitres of each amplicon was loaded on a 2% agarose gel made in $1\times$ Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide. The gels were visualized under UV light using a BioDoc-It gel documentation system (UVP). ERIC and BOX electrophoretic profiles were analysed and transformed into binary matrices using Cross Checker software, available at http://www.plantbreeding.wur.nl/UK/software_crosschecker.html.

Phylogenetic analyses. Phylogenetic analyses were initially performed on the whole collection of clinical strains (120) by merging BOX and ERIC band patterns in a single binary matrix. Cluster analyses were generated using the unweighted pair group method using arithmetic averages (UPGMA) and the Dice similarity coefficient was calculated using TREECON software (Van de Peer & De Wachter, 1994). The criterion for defining clonal lineages was taken as profiles with 85% or more similar bands. At least one clinical strain per clonal lineage was selected for further virulence assays and correlation analyses. The resulting tree was represented as a radial cladogram using

an in-house tool (Segata *et al.*, 2011), and virulence profiles, virulence gene expression and *exoU/exoS* genotype of each taxon were mapped on the tree.

Virulence assays using *D. discoideum* NC4. Two *D. discoideum* virulence assays were applied, the standard SM agar assay (Cosson *et al.*, 2002) and the HL5 diluted medium assay (Froquet *et al.*, 2009). Both assays offer the opportunity to distinguish among different degrees of virulence of pathogenic bacteria. The HL5 assay proved to be more suitable to quantify virulence in bacterial strains with extreme virulence phenotypes, such as ‘super-virulent’ or ‘non-virulent’ strains (Froquet *et al.*, 2009). For the SM agar assay, *P. aeruginosa* cells were harvested during exponential phase and resuspended into SM broth at a final OD₆₀₀ of 1, diluted into 5 ml SM broth and spread on SM agar plates to make a homogeneous bacterial lawn. *D. discoideum* NC4 was grown in SM broth with *K. aerogenes* as a food source. The cells were harvested after 2–3 days of incubation at 20 °C, and resuspended into HL5 medium and 1 × Sorensen phosphate buffer. Cell concentration was determined using a Countess Automated Cell Counter (Invitrogen). Nine 5 µl droplets consisting of serial dilutions of *D. discoideum* cells were spotted on the bacterial lawn. The dilution factor was threefold, and the approximate number of cells in each droplet was 20 000, 6600, 2200, 750, 250, 90, 30, 10 and 3, respectively. Each assay was run in triplicate. A control plate was obtained using non-pathogenic *E. coli* DH5α instead of *P. aeruginosa* strains. The plates were incubated for 6 days at 19 °C before examining the growth pattern of *D. discoideum*.

For the diluted HL5 medium assay, *P. aeruginosa* strains were harvested during the exponential phase and diluted into 2.5 ml SM broth. A suspension of 500 µl of bacterial cells at a final OD₆₀₀ of 1 was deposited into 12-well Corning Costar cell culture plates (Sigma-Aldrich) containing serially diluted (twofold) HL5 agar at the following concentrations: 100, 50, 25, 12.5, 6.25, 3.1, 1.5, 0.75, 0.37, 0.18, 0.09, 0.04 and 0.02 %. The plates with the bacterial lawn were initially dried at room temperature for 1 h before adding to *D. discoideum* cells. *D. discoideum* NC4 was grown in SM broth with *K. aerogenes* at 20 °C as a food source and resuspended into SM broth diluted 1:1 with 1 × Sorensen buffer. Two microlitres of *D. discoideum* containing approximately 7000 cells was spotted on a *P. aeruginosa* lawn plated on HL5 medium at different concentrations. The plates were incubated at 19 °C for 6 days before examining the growth pattern of *D. discoideum*. Each assay was run in triplicate. As in the assay above, a plate incubated with *E. coli* DH5α was used as a control.

Fast-killing virulence assays using *C. elegans* N2. The *C. elegans* N2 strain was grown on NGM (nematode growth medium) agar plates with *E. coli* OP50 as a food source. The *C. elegans* fast-killing assay was performed as described elsewhere (Tan *et al.*, 1999). *P. aeruginosa* cells were grown in peptone glucose (PG) medium, harvested during exponential phase and resuspended into PG broth at a final OD₆₀₀ of 1, and 200 µl was spread on PG agar plates. The plates were incubated at room temperature for 18 h. On average, 60–70 *C. elegans* individuals were picked and placed on PG plates, and incubated at 22 °C for 4 h. The viability/mortality of the worms was observed under a microscope using a ×10 lens and monitored with a CCD camera. The percentage killing of *C. elegans* was calculated by determining the number of dead worms out of the total worms deposited on each plate. Each assay was run in triplicate. As in the assays above, *E. coli* DH5α was used as a control.

Gene expression analyses. Two replicates of each *P. aeruginosa* strain were harvested during exponential phase at OD₆₀₀ 0.6 and pelleted by centrifugation (6000 g for 5 min at 4 °C). Total RNA was isolated from bacterial pellets by using the TRIzol Max Bacterial Isolation kit (Life Technologies) as described by the manufacturer.

Approximately 10 µg of the total RNA preparation was treated twice with the RNase-free DNase set (Qiagen) to remove genomic DNA contamination and was subsequently cleaned up with the RNeasy Mini kit (Qiagen) following the manufacturer’s instructions. RNA concentration and purity were determined by UV absorption (260:280 nm) using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and 0.8 % agarose gels stained with ethidium bromide. One microgram of DNase-treated RNA was reverse-transcribed into cDNA using a First Strand cDNA Synthesis kit (Fermentas). cDNAs were amplified by real-time PCR using Kapa Sybr Fast qPCR Mastermix (KapaBiosystems) and a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). PCR conditions were as follows: 95 °C for 3 min, 40 cycles of 3 s at 95 °C and 30 s at 60 °C, with a final melting curve analysis from 72 to 95 °C, with increments of 1 °C every 5 s. Real-time PCR amplifications were performed with two experimental replicates for each sample.

Primers for the *rhlR*, *crc*, *exoU* and *exoS* genes were designed using Primer3 software (Rozen & Skaletsky, 2000) to produce amplicons ranging from 80 to 220 bp. Primers sequences are reported in Table 1. Each primer pair was controlled for dimer formation by melting curve analysis, and PCR efficiency was calculated over a sixfold 2 × dilution series. The *rpoD* and *rplS* genes were used as housekeeping genes (Savli *et al.*, 2003; Llanes *et al.*, 2004), and gene expression values were further normalized to those obtained with PSUR28, the strain displaying the lowest score in virulence assays. Amplification profiles were analysed using Bio-Rad Manager Software and cycle threshold (C_t) values for each target gene were normalized to the geometric mean of the C_t of *rpoD* and *rplS* amplified from the corresponding sample. The fold change of target genes for each strain with respect to the PSUR28 control strain was calculated using the $\Delta\Delta C_t$ method.

Virulence scores and correlation analyses. In order to quantitatively estimate the level of virulence associated with each strain and to make them directly comparable, a virulence score was defined for each assay. For the SM agar assay using *D. discoideum*, the scores were attributed according to the most concentrated droplet for which no growth was observed, as follows: virulence score of 9 for inhibition of *D. discoideum* growth with 20 000 cells; score of 8 for inhibition with 6600 cells, and so on, down to a score of 0 for absence of growth inhibition, even at the lowest number of cells spotted (Table 2). For the diluted HL5 assay the same principle was applied: a virulence score of 9 was attributed to strains inhibiting *D. discoideum* growth at 0.02 % HL5 medium concentration; a score of 8 for growth inhibition at 0.04 % concentration, and so on, down to a score of 0 for absence of growth inhibition, even for the most concentrated HL5 medium used (Table 2). For the *C. elegans* assay, the scores were attributed as follows: a virulence score of 9 for ≥90 % killing; a score of 8 for killing percentages in the range 80–90 %, and so on, down to a score of 0 for killing percentages lower than 10 % (Table 2).

Pearson correlation analysis (producing correlation coefficients and *P* values) was applied on the virulence scores after averaging the replicates to check the consistency of the three virulence assays. The same approach was also employed to test the interdependency between the virulence profiles and the expression profiles of *rhlR* and *crc* genes, and to study the relationship between their expression patterns. For testing the hypothetical dependence of the virulence profiles with respect to the mutually exclusive presence of the *exoU* and *exoS* genes, we adopted the Wilcoxon test.

We investigated the correlation between genotype and virulence profiles by estimating the genetic versus virulence pairwise distances among strains and by comparing them using Pearson correlation. For the genotype profiles, the distance matrix was generated by computing the all-versus-all minimum branch length distance among leaf nodes in the phylogenetic tree. For the virulence profiles based on

Table 1. Sequences of primers (5'–3') used in this study

Primers were designed for gene expression analysis of *rhIR* and *crc* using *rpoD* and *rplS* as housekeeping genes, and for determining *exoU/exoS* genotype.

Gene	Forward primer	Reverse primer
Target genes		
<i>rhIR</i>	TCCTCGGAAATGGTGGTCTG	CGCTCGAAGCTGGAGATGTT
<i>crc</i>	TCCTTCCAAGTGGACGGCTA	AGCAGGGTGGCGATACTCAC
<i>exoU</i>	GGAGTATCTGCCAGCGCATC	GAACCCGGAGATCACAGACG
<i>exoS</i>	CTTCGGCGTCACTGTGGATG	AGGTCAGCAGAGTATCGGC
Housekeeping genes		
<i>rpoD</i>	GCTGCTGTCGTCGCTTTCTT	AGCATCCTGGCCGACTACAA
<i>rplS</i>	ACCCGCGTATACACCACCAC	CACACCCGGAAGGTCCTTTA

the three assays described above, the standard Euclidean distance was employed.

RESULTS AND DISCUSSION

Rep-PCR-based genotyping assays and phylogenetic analyses

A total of 31 and 26 different electrophoretic bands were generated by BOX and ERIC PCR amplifications, respectively, and binary data extracted from both genotyping techniques were merged for subsequent phylogenetic reconstruction. The 120 clinical strains analysed were clustered in a total of 27 different clonal lineages with a Dice coefficient of >15 %. Each clonal lineage included up to seven strains. In order to comprehensively represent the genetic diversity of the clinical strain collection, one strain from each clonal lineage was selected for further phylogenetic analyses and virulence assays. In order to determine

the intra-clonal variability of virulence profiles, up to three strains from each clonal lineage were tested with the SM agar assay. Hierarchically clustering the genotyping profiles, a phylogenetic tree depicting the relationships between 31 strains (27 clinical strains and four reference strains) was reconstructed (Fig. 1), as described in Methods. Several pairs of strains showed very similar genotyping patterns and were thus tightly clustered in the tree. The three pairs with highest similarity were Hpu43/Hpu45 (eight synapomorphic characters), PSUR28/VRSP32 (seven) and LESB58/Hpu106 (seven). On the other hand, several pairs of strains had no synapomorphic characters (13 % of pairs) or a single one (41 %).

Virulence assays using *D. discoideum* NC4

In the first assay on SM agar plates, 5 µl droplets of *D. discoideum* culture were applied on a lawn of *P. aeruginosa*, with each droplet containing a number of *Dictyostelium*

Table 2. Parameters used for defining virulence scores for the three assays

Virulence score	Model organism		
	<i>D. discoideum</i>		<i>C. elegans</i>
	SM agar assay*	HL5 medium assay†	Fast-killing assay‡
0	3	12.5	<10 %
1	10	6.25	10–20 %
2	30	3.1	20–30 %
3	90	1.5	30–40 %
4	250	0.75	40–50 %
5	750	0.37	50–60 %
6	2200	0.18	60–70 %
7	6600	0.09	70–80 %
8	20 000	0.04	80–90 %
9	>20 000	0.02	>90 %

*Lowest number of *D. discoideum* cells spotted for which growth was inhibited.

†Concentration of HL5 medium (%) for which *D. discoideum* growth was inhibited.

‡Percentage of *C. elegans* individuals killed.

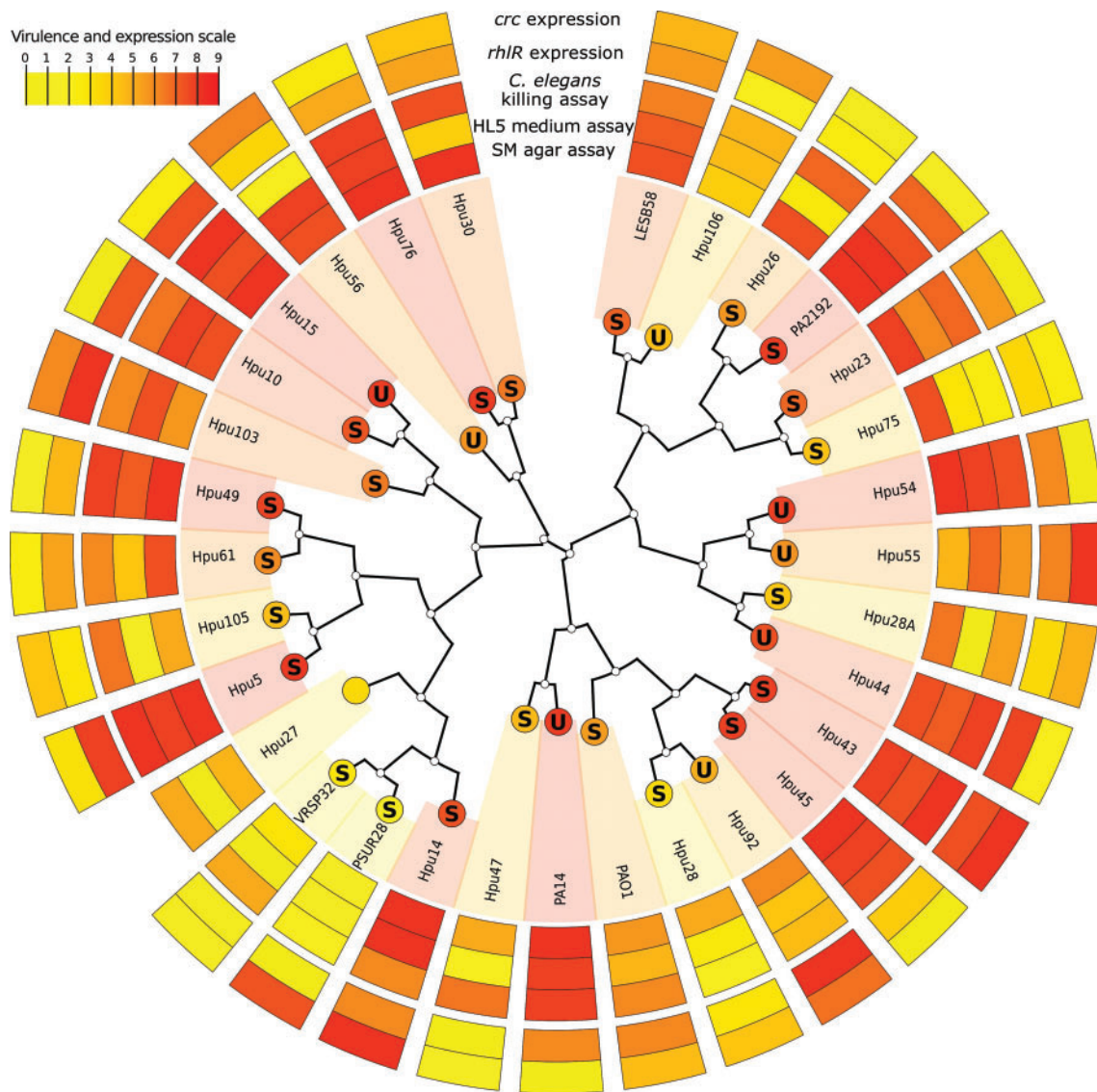


Fig. 1. Population structure of *P. aeruginosa* clinical strains according to rep-PCR genotyping, virulence phenotypes, virulence gene expression and *exoU/exoS* genotype. Names of strains are indicated on terminal branches. The inner cladogram represents the phylogenetic relationships between the 31 strains characterized genotypically. The first three inner coloured rings report the three virulence scores (from 0 to 9) for SM agar, HL5 medium and *C. elegans* killing assay, while the two external rings report the expression levels of *rhIR* and *crc* genes relative to the less virulent strain PSUR28. Log-ratio gene expression levels were normalized on a 0–9 scale, winsorizing the highest and the lowest values. The *exoU*⁺/*exoS*[−] or *exoU*[−]/*exoS*⁺ genotype found on individual strains is indicated on leaf spots by 'U' or 'S', respectively. The colour of spots and of the radial background represents the mean of the three virulence scores.

cells ranging from 3 to 20 000 (Fig. 2). Under these conditions, even 20 000 *Dictyostelium* cells failed to create a phagocytic plaque in a lawn of the most virulent *P. aeruginosa* clinical strains (Table 3). Plaques instead appeared around *D. discoideum* cells spotted on moderately or weakly virulent strains. The most permissive *P. aeruginosa* strains, such as PSUR28 and VRPS32, allowed *D. discoideum* growth and radial expansion, even starting from a very limited number of cells (<250) (Table 3). Moderately virulent strains allowed *D. discoideum* growth

when at least several hundred cells were deposited on the bacterial lawn. The mean virulence scores obtained with this assay on 43 *P. aeruginosa* strains ranged from 2.0 to 9.0, with a mean value of 7.14.

In the second virulence assay on diluted HL5 medium, the number of *D. discoideum* cells spotted on the bacterial lawn was constant (about 6000 cells in 2 µl). The serial dilution of HL5 medium is aimed at reducing the growth capacity of the strains, presumably rendering them less aggressive,

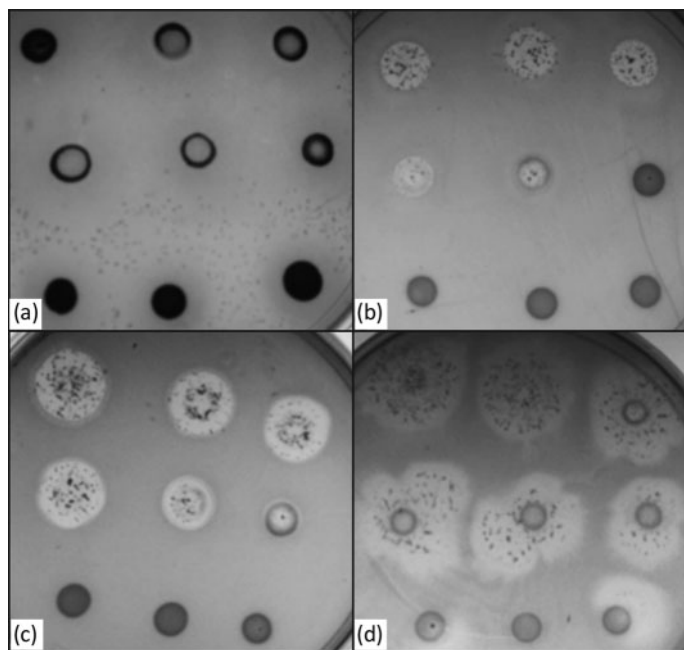


Fig. 2. Quantitative assessment of *D. discoideum* growth using SM agar assays in the presence of *P. aeruginosa*. For each bacterial strain, the most concentrated *Dictyostelium* spot is at the top left; concentration decreases serially to the bottom-left spot. (a) Highly virulent strain PA14 (mean virulence score 9.0); (b, c) moderately virulent strains Hpu105 and Hpu106 (mean virulence scores 5.7 and 4.3, respectively); (d) weakly virulent strain PSUR28 (mean virulence score 2.0).

and consequently diminishing their ability to resist phagocytosis by *D. discoideum*. Preliminary experiments showed that the majority of *P. aeruginosa* strains, including most of the weakly virulent ones, inhibited *D. discoideum* growth on HL5 dilutions ranging from 100 to 25% (data not shown). We therefore started the dilution range at 12.5% and decreased it serially (twofold dilutions) to 0.02%. The minimal HL5 medium concentration at which a given bacterial strain allowed *D. discoideum* growth was used to define the virulence score of individual strains, as described in Methods. The HL5 medium virulence scores were globally consistent with those obtained with the assays on SM agar (in more than half of the cases the difference was smaller than 1 unit of virulence score values) and with a significant correlation (P value 9.7×10^{-5}), although in four cases a difference of more than 4 virulence score units was observed. The HL5 assay was more discriminating and showed less saturation than the SM agar assay for highly virulent strains, since only a few of the strains with a mean virulence score of 9 in the SM agar assay (nine strains) also displayed this value for the HL5 assay (two strains) (Table 3). The virulence scores obtained with this assay on 31 *P. aeruginosa* strains ranged from 0.3 to 9.0, with a mean value of 5.9.

Fast-killing virulence assays using *C. elegans* N2

We used an additional virulence and pathogenesis assay that consists of measuring killing percentages of the soil nematode *C. elegans* by *P. aeruginosa*. Previous studies using *C. elegans* as a virulence model have shown that, depending on the growth medium, *P. aeruginosa* causes different outcomes: slow or fast killing, lethal paralysis and red death (Tan *et al.*, 1999). We tested all *P. aeruginosa*

clinical strains selected in the present study for their ability to kill *C. elegans* using the fast-killing assay, as described in Methods. As for the *D. discoideum* assays, a wide range of virulence phenotypes was observed (Table 3). Ten out of 31 *P. aeruginosa* strains were found to be highly aggressive, killing more than 80% of the worms in 4 h (virulence score >8). The lowest percentage of killing was observed with strains Hpu28, Hpu56 and PSUR28 (20–30% killing, mean virulence score of 2.3). The virulence scores obtained with this assay on 31 *P. aeruginosa* strains ranged from 2.3 to 9.0, with a mean value of 6.8.

Virulence gene expression and assessment of *exoU/exoS* genotype

The expression of *rhlR* and *crc* genes in 26 clinical strains and four reference strains of *P. aeruginosa* was analysed and is reported in Fig. 1 and Table 3. A single strain (Hpu27) was not analysed for gene expression as good quality RNA could not be obtained. The PSUR28 strain, which on average presented the lowest score for the three virulence assays (Table 3), was selected as a reference to evaluate the fold change in the other strains. For *rhlR*, all tested strains ($n=30$) showed a much higher level of expression compared with strain PSUR28. The fold change values (expressed as \log_{10} values) obtained for this gene ranged from 3.04 in strain Hpu47 to 4.55 in strain Hpu103 (Table 3). In contrast, most of the tested strains presented a lower expression level (negative fold change value) of the *crc* gene with respect to PSUR28. The fold change values for this gene ranged from -0.43 (in Hpu45) to 0.21 (in Hpu55) (Table 3).

In addition, the presence in individual strains of genes encoding the type III secretion system effectors ExoU and

Table 3. Virulence assay scores using *D. discoideum* or *C. elegans* expressed as the mean of three replicates, *rhIR* and *crc* gene expression values (mean \pm SD, values expressed as \log_{10}) relative to PSUR28 strain values, and *exoU*⁺/*exoS*[−] (U) or *exoU*[−]/*exoS*⁺ (S) genotype

Data were obtained from 27 clinical *P. aeruginosa* strains belonging to different clonal lineages and from reference strains PAO1, PA14, LESB58 and PA2192. Non-pathogenic *E. coli* DH5 α was used as a control strain for virulence assays. ND, Not determined.

Bacterial strain	<i>D. discoideum</i> SM agar	<i>D. discoideum</i> HL5 medium	<i>C. elegans</i> fast-killing	<i>rhIR</i> expression	<i>crc</i> expression	<i>exoU/exoS</i> genotype
Hpu5	9.0	8.7	9.0	4.35 \pm 0.08	−0.20 \pm 0.07	S
Hpu10	8.3	8.7	7.3	4.27 \pm 0.03	−0.32 \pm 0.02	S
Hpu14	9.0	9.0	6.7	4.05 \pm 0.12	0.17 \pm 0.03	S
Hpu15	9.0	8.3	9.0	4.30 \pm 0.02	−0.23 \pm 0.02	U
Hpu23	8.7	6.7	7.7	3.98 \pm 0.03	−0.34 \pm 0.00	S
Hpu26	8.3	3.0	7.7	3.45 \pm 0.05	−0.31 \pm 0.08	S
Hpu27	5.3	0.3	5.7	ND	ND	ND
Hpu28A	7.3	0.3	6.0	3.62 \pm 0.10	−0.11 \pm 0.07	S
Hpu28	5.7	3.0	2.3	3.52 \pm 0.01	−0.15 \pm 0.05	S
Hpu30	9.0	4.3	8.3	3.93 \pm 0.01	−0.14 \pm 0.03	S
Hpu43	8.7	8.3	9.0	4.29 \pm 0.03	0.03 \pm 0.09	S
Hpu44	8.3	8.0	8.7	4.33 \pm 0.02	−0.24 \pm 0.04	U
Hpu45	9.0	8.7	8.3	3.71 \pm 0.06	−0.43 \pm 0.04	S
Hpu47	5.7	2.3	7.3	3.04 \pm 0.01	−0.28 \pm 0.12	S
Hpu49	9.0	8.0	8.7	3.83 \pm 0.03	−0.27 \pm 0.01	S
Hpu54	9.0	8.7	8.3	4.06 \pm 0.07	−0.32 \pm 0.02	U
Hpu55	5.3	7.7	6.3	4.08 \pm 0.06	0.21 \pm 0.14	U
Hpu56	8.3	8.7	2.3	3.64 \pm 0.04	−0.05 \pm 0.02	U
Hpu61	8.3	4.7	6.7	3.93 \pm 0.04	−0.23 \pm 0.12	S
Hpu75	8.3	3.3	2.7	3.63 \pm 0.00	−0.23 \pm 0.00	S
Hpu76	9.0	8.7	8.7	3.89 \pm 0.10	−0.22 \pm 0.11	S
Hpu92	6.7	4.7	5.0	4.40 \pm 0.02	−0.03 \pm 0.00	U
Hpu103	6.3	8.3	6.7	4.55 \pm 0.05	−0.06 \pm 0.07	S
Hpu105	5.7	1.7	7.3	3.50 \pm 0.19	−0.15 \pm 0.12	S
Hpu106	4.3	5.0	5.3	3.41 \pm 0.01	−0.09 \pm 0.11	U
VRSP32	3.3	0.3	5.7	3.15 \pm 0.02	−0.28 \pm 0.07	S
PSUR28	2.0	1.7	2.3	0.00 \pm 0.00	0.00 \pm 0.00	S
PA2192	9.0	9.0	8.3	4.21 \pm 0.02	−0.27 \pm 0.02	S
PAO1	6.3	5.3	6.7	4.06 \pm 0.08	−0.13 \pm 0.04	S
PA14	9.0	8.7	8.7	4.05 \pm 0.05	−0.33 \pm 0.06	U
LESB58	8.3	8.0	7.0	3.98 \pm 0.04	−0.12 \pm 0.05	S
<i>E. coli</i> DH5 α	0.0	0.0	0.0	—	—	—

ExoS was evaluated by PCR amplification. Our results confirm that both genes are mutually exclusive. The *exoU*⁺/*exoS*[−] genotype was less prevalent (found in 26.7 % of the strains) than the *exoU*[−]/*exoS*⁺ genotype (73.3 %), as reported earlier (Feltman *et al.*, 2001; Wareham & Curtis, 2007) (Fig. 1, Table 3).

Virulence score consistency and virulence range

All three virulence profiling approaches were statistically significantly correlated. In particular, the two *D. discoideum* NC4 assays were strongly correlated ($r=0.66$, P value = 3.9×10^{-5}), and both showed a slightly lower correlation with the *C. elegans* N2 assay ($r=0.58$ and P value = 0.0005 for the SM agar assay, and $r=0.48$, P value = 0.006 for the HL5 medium assay). Both assays using *D. discoideum* as a model

organism showed saturation for highly virulent strains. These strains completely inhibited *D. discoideum* growth, even at high concentrations (20 000 cells per droplet) in the SM agar assay, whereas some of them also prevented *D. discoideum* growth at the lowest HL5 dilution (0.02 %). We noted that it was not possible to perform the HL5 assay by further decreasing the concentration of the medium below 0.02 %, as in these conditions the availability of nutrients was apparently insufficient to support bacterial growth, and consequently the formation of the lawn did not occur. Likewise, most of the strains also killed 90–100 % of the population using the *C. elegans* fast-killing assay. This implies that the assays cannot accurately quantify eventual differences between highly and ‘extremely virulent’ strains. Conversely, the virulence assays worked remarkably well in determining and quantifying the virulence phenotype of

weakly and moderately virulent bacterial strains. Globally, a surprisingly high virulence range was noted for the clinical strains, indicating that *P. aeruginosa* strains showing reduced or altered virulence are not exclusively a characteristic of chronic infections.

Correlation between virulence profiles and rep-PCR genotyping

By comparing genotyping with virulence profiling (Fig. 1), it can be noted that two pairs of genotypically more similar strains, Hpu43/Hpu45 and PSUR28/VRSP32, showed almost identical virulence patterns: the former is highly virulent (values higher or equal to 8.3 for all six mean scores), whereas the latter has much lower virulence values (means of the three scores of 2.0 and 3.1, respectively). Several other patterns of convergence between virulence and genotyping profiling were observed for the pairs Hpu92/Hpu28 and Hpu10/Hu15. In other cases, however, virulence was clearly uncoupled from genetic similarity, with the most striking examples being the pairs Hpu28A/Hpu44, Hpu47/PA14, Hpu5/Hpu105 and LESB58/Hpu106.

Intra-clonal virulence variation was investigated for eight distinct lineages, testing multiple strains for each lineage with the SM agar assay. The three lineages represented by LESB58, VRSP32 and Hpu75 showed a marked virulence consistency, with a maximum variation lower than 1 virulence score unit (ranges 8.3–9.0, 2.7–3.3 and 8.0–9.0, respectively). In the other cases with three clonal strains tested, one clone with reduced virulence was detected; specifically, these cases were Hpu40 with score 2.0 in the Hpu44 lineage (score 8.3), Hpu105 with a smaller score (5.7) than its clonal counterparts Hpu6 and Hpu9, and Hpu39 (score 3.0), which was much less virulent than Hpu35 (8.7) and Hpu45 (9.0). Markedly different virulence phenotypes also characterized the two tested lineages with two clonal variants, as Hpu33 (score 2.3) and Hpu106 (score 4.3) were less virulent than their clonal counterparts Hpu43 and Hpu101, respectively (both 8.7). These data indicate that even closely related strains can present marked differences in their virulence profiles. Although more clonal strains should be investigated to test this hypothesis, it seems that variation of the virulence phenotype is due to the loss of virulence in a fraction of strains belonging to a given clonal lineage.

The global correlation between virulence profiles and genotyping distance was not statistically significant, but this appeared to be mainly due to genotypically distant strains. Given that few or no informative genotypic characters are shared by the most distantly related strains, precise long-range branching relations cannot be reliably estimated. For these reasons, we compared short-range genotyping distance (Euclidean distance below 1.0) with virulence distance. Under these conditions, a clear correlation ($r=0.68$, P value 6.5×10^{-5}) between virulence profiles and genotyping distance was found. The observation that some pairs of strains presented quite different virulence patterns

despite their genetic closeness can have multiple explanations. Firstly, divergent genotypes are not necessarily also divergent in the virulence phenotype, as multiple virulence determinants may have evolved independently or have been transmitted horizontally. Additionally, rep-PCR genotyping targets specific repeated regions in the core genome and does not take into consideration either the variability in the accessory genome or mutations in virulence genes from the core genomes that could affect the virulence phenotype. The marked ability of *P. aeruginosa* to acquire or discard genes and genomic segments is considered to be the main factor explaining its capacity to colonize and survive in different host environments. While most known virulence factors located in the core genome of *P. aeruginosa* show a high degree of conservation (Wolfgang *et al.*, 2003), this bacterium possesses a large accessory genome consisting of blocks of genes distributed in several dozen regions of genomic plasticity (Mathee *et al.*, 2008). As a consequence, one may hypothesize that the genotypically closely related strains analysed in the present study that showed very different virulence patterns likely either differ in the composition of their accessory genome, as a consequence of the acquisition or deletion of virulence determinants, or have accumulated mutations in virulence genes located in the core genome.

Correlation between virulence profiles and virulence gene expression

We observed that the expression of the *rhlR* gene strongly and significantly correlated with all three virulence assays (correlations of 0.60, 0.73 and 0.50 for SM agar, HL5 medium and *C. elegans* assays, respectively; P values of 4.1×10^{-3} , 5.3×10^{-6} and 4.8×10^{-3}), confirming that quorum sensing, and in particular the *rhl* system, plays an important role in *P. aeruginosa* virulence (Cosson *et al.*, 2002). Such a strong correlation indicates that as an alternative to virulence assays, *rhlR* gene expression experiments can provide a reliable estimation of the aggressiveness of individual strains of *P. aeruginosa*.

In contrast, only a weak and negative correlation between *crc* expression and virulence was observable (correlations of 0.39, 0.68 and 0.42 for SM agar, HL5 medium and *C. elegans* assays, respectively; all P values >0.01). Accordingly, no significant patterns of co-expression could be detected for the *rhlR* and *crc* genes. The *C. elegans* assay seems to capture a partially distinct virulence phenotype, as it is more strongly correlated with *rhlR* expression than *D. discoideum* assays and is completely uncoupled from *crc* expression. It has been reported that a *P. aeruginosa* strain lacking the *Crc* regulator shows defects in type III secretion, motility and expression of quorum sensing-regulated virulence factors, and is less virulent against *D. discoideum* (Linares *et al.*, 2010). *Crc* is a global metabolic regulator controlling different cellular pathways, and its deletion apparently globally weakens physiological performance. Our results indicate that *crc* expression is either not correlated or negatively correlated with virulence,

and therefore do not support the hypothesis of a direct role of Crc in the aggressiveness of *P. aeruginosa*.

While the relative prevalence of *exoU* and *exoS* genes encoding type III secretion effectors has been shown to be associated with infection type (chronic infections in CF patients versus blood infections) (Wareham & Curtis, 2007) or even with specific hospital departments (Bradbury *et al.*, 2010), we showed here that the presence of either *exoU* or *exoS* was not associated with specific virulence patterns, which is not surprising. While almost no strain encodes or secretes all four known type III secretion effectors, the commonly found combinations of ExoU/ExoT or ExoS/ExoT provide redundant and failsafe mechanisms to cause mucosal barrier injury, and inhibit many arms of the innate immune response (Engel & Balachandran, 2009). Although ExoU has been shown to have a slightly greater impact on virulence than ExoS in the mouse lung (Shaver & Hauser, 2004), it is generally accepted that the secretion of different combinations of type III effectors does not translate into a synergistically significant enhancement of disease severity (Shaver & Hauser, 2006).

In the present study, we have shown that the parallel application of virulence assays can be used to quantitatively assess this complex, multifactorial phenotypic trait. A wide range of virulence phenotypes was observed, from weakly to highly aggressive, indicating that clinical strains isolated from acute infections can present a reduced or altered virulence phenotype, as known for chronic *P. aeruginosa* infections in CF patients (Bragonzi *et al.*, 2009; Lelong *et al.*, 2011). However, the time required by the host to exert selection pressure for mutations and reduced expression in CF patients is not applicable to *P. aeruginosa* isolates from acute infections. The low virulence of some of the strains analysed here may be due instead to their intrinsic reduced aggressiveness. Alternatively, they may have been previously involved in chronic infections. Further studies of *P. aeruginosa* clinical populations at the genomic, transcriptomic and proteomic levels are needed to better understand the molecular determinants and mechanisms underlying the wide virulence range of *P. aeruginosa* in acute infections.

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