Comparison of virulence between clinical and environmental Pseudomonas aeruginosa isolates

Summary. New strains of Pseudomonas aeruginosa were isolated from clinical and environmental settings in order to characterize the virulence properties of this opportunistic pathogen. P. aeruginosa was frequently recovered from oil-contaminated samples but not from non-oil-contaminated soils. The virulence of five environmental and five clinical strains of P. aeruginosa was tested using two different models, Drosophila melanogaster and Lactuca sativa var. capitata L. There was no difference in the virulence between the two groups of isolates in either of the models. Since environmental P. aeruginosa strains are used for bioaugmentation in bioremediation programs, the results presented here should be taken into account in the future design of degradative consortia and/or in establishing containment measures. [Int Microbiol 2006; 9(4):247-252]

Key words: Pseudomonas aeruginosa · pathogenicity test · biosafety · bioaugmentation · bioremediation

Introduction

Pseudomonas aeruginosa is a ubiquitous and remarkably versatile bacterium. Some strains have been reported as plant-growth-promoting rhizobacteria [3,6], and other can degrade environmental pollutants [9,20,26]. P. aeruginosa is also an important opportunistic pathogen and a major cause of serious nosocomial infections [5,25,38], especially in immunocompromised patients or patients with a predisposing condition [25,44]. Nevertheless, P. aeruginosa also affects healthy individuals and is an etiological agent to consider in the differential diagnosis of rapidly progressing community-acquired pneumonia [21,25]. Even though this bacterium is not known to be a primary pathogen for non-cystic fibrosis (CF) patients, McCallum et al. reported a case in which a CF patient chronically colonized with an epidemic P. aeruginosa strain cross-infected her non-CF parents [32]. These observations and many others show that P. aeruginosa is capable of also affecting healthy immunocompetent individuals.

Environmental P. aeruginosa isolates have been considered as potential biological control agents or inducers of systemic acquired resistance [3,6,14], in bioremediation programs [9,35], and in other applications [11,20]. Over the last 15 years our group has developed several bacterial consortia for the bioremediation of oil-derived hydrocarbons. Taking advantage of the degradative properties of native microbiota, those consortia have been used successfully in different contaminated regions of Colombia (data not published) in which strains of P. aeruginosa are naturally present. Several lines of evidence strongly suggest that there are no major differences in virulence between clinical and environmental isolates: clone and pilin-type distributions of P. aeruginosa isolates were shown to be the same in a group of strains from the environment as in strains obtained from CF patients [29,40]. No differences were found in selected pathogenicity determinants such as type-IV pilin genes [42], flagellin genes [34], genes for multidrug efflux systems and for the type III secretion system, the porin gene oprD [36], hemolytic and proteolytic activities, and invasion of epithelial cells [1]. Additionally, recent studies showed that the genomes of clinical and environmental strains are highly conserved [33,46]. All P. aeruginosa strains used for bioremediation were isolated from the environment, but the virulence of environmental P. aeruginosa strains has not yet been tested in plant or animal models.
In the present study, we compared the virulence of clinical and environmental \textit{P. aeruginosa} isolates as determined in two model hosts, the fruit fly \textit{Drosophila melanogaster} (animal model reported to be suitable to detect virulence differences in \textit{P. aeruginosa} strains) [13] and the plant \textit{Lactuca sativa} var. \textit{capitata} L. (plant model widely accepted as a test for bacterial pathogenicity) [15].

It is well-known that \textit{P. aeruginosa} infections in humans occur mainly in immunocompromised patients [25] and in patients with a pre-existing disease or other predisposing conditions [4,44]. However, reports of \textit{P. aeruginosa} affecting healthy individuals create additional concern about the possible effects of bioaugmentation with \textit{P. aeruginosa} populations. In a literature survey of infections caused by \textit{P. aeruginosa} in healthy individuals, we found reports of \textit{P. aeruginosa} having caused an epidemic folliculitis in 117 people [17]; dermatitis in a healthy 27-year old man and two other persons that used the same bathtub [10]; septicemia in healthy children [47]; ecthyma gangrenosum and infection in a child without known risk factors [39]; liver abscess in a healthy child [31]; community-acquired sacro-iliitis in a young, healthy man [8]; spread of \textit{P. aeruginosa} from a CF patient to healthy relatives [32]; community-acquired fatal bacteremia in two previously healthy patients [24], and community-acquired pneumonia [21].

**Materials and methods**

**Isolation and characterization of \textit{P. aeruginosa} strains.** Clinical \textit{P. aeruginosa} isolates were obtained from hospitals and medical institutions from several cities in Colombia, South America (Table 1). Environmental \textit{P. aeruginosa} isolates were recovered from soil and water samples by dilution and plating on cetrimide agar (Difco). Soil and water samples were taken from locations considered to have a low risk of hospital specimen contamination in order to ensure the non-clinical origin of the environmental isolates. Samples were processed as follows: water samples were serially diluted and plated, and an undiluted 0.1 ml aliquot was also plated. For soils, 50-g soil samples were mixed with 50 ml of sterile water and shaken for 30 min. The resulting suspension was allowed to settle for 30 min and the supernatant was serially diluted and plated. An undiluted 0.1 ml aliquot of the supernatant was plated as well. Plates were incubated at 30°C for 48 h.

Isolates received from hospitals and those recovered on cetrimide plates were maintained frozen at -80°C with glycerol. The 16S rRNA genes from all the isolates were amplified by PCR (primer forward AGAGTTT GATYMTGCG and reverse TACGGYACCTTGTTACGA) [18]. The reaction mixture contained 1.5 mM MgCl$_2$, 0.2 mM dNTP, 0.1 mg of each primer per ml, 2.5 U Taq polymerase, and 3 µl of crude extract obtained from an overnight culture as the DNA source. Reaction conditions were 94°C for 3 min, 25 cycles of 94°C for 45 s, 50°C for 45 s, 72°C for 45 s, and a final extension of 72°C for 7 min. Amplification products were purified and sequenced by the Micro Core Facility at Harvard Medical School, Boston, MA, USA.

**Pathogenesis assay in \textit{D. melanogaster}**. \textit{P. aeruginosa} strains were grown overnight on nutrient agar plates at 30°C. For each strain, a bacterial suspension was prepared in sterile saline solution (NaCl 0.85%) and adjusted to a McFarland turbidity standard of 0.5 (1 × 10$^8$ colony forming units [cfu]/ml) [23]. Five adult female wild-type flies (supplied by Marina Ordóñez, Instituto de Genética de Poblaciones, Los Andes University) 2–4 days old were pricked in the dorsal thorax with a sterile needle that had been dipped into a \textit{P. aeruginosa} isolate suspension [13]. Flies were returned to standard fly culture vials with food, and survival percentage at 16, 22, 25, 28, 30, 32 and 40 h after inoculation was recorded. The experiments consisted of three replicas per strain and were repeated twice. Negative controls for all experiments were done by pricking the flies with a needle dipped into sterile saline solution; an additional negative control was done by pricking the flies with a needle dipped into a suspension of \textit{P. putida} strain M2A (isolated in this study) prepared in the same way as described for the \textit{P. aeruginosa} strains. Controls were cultured as described above.

**Table 1.** Clinical (C) and environmental (E) \textit{Pseudomonas aeruginosa} isolates used in pathogenesis assays

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin*</th>
<th>NCBI database 16S rRNA (&gt;99% similarity)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1C</td>
<td>Post-operative infection (Clin. del Prado, Santa Marta)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
<tr>
<td>2C</td>
<td>Urinary-tract infection (Fund. Hosp. de la Misericordia, Bogotá)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
<tr>
<td>3C</td>
<td>Catheter (Hosp. Kennedy, Bogotá)</td>
<td>\textit{P. aeruginosa} SCD-1</td>
<td>AF448036</td>
</tr>
<tr>
<td>4C</td>
<td>Post-operative infection (Hosp. Federico Lleras Acosta, Ibagué)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
<tr>
<td>5C</td>
<td>Septicemia (Hosp. Federico Lleras Acosta, Ibagué)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
<tr>
<td>6E</td>
<td>Soil (Villeta, Cundinamarca)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
<tr>
<td>7E</td>
<td>Oil-contaminated water (Caño Limón, Arauca)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
<tr>
<td>8E</td>
<td>Oil-contaminated water (Caño Limón, Arauca)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
<tr>
<td>9E</td>
<td>Oil-contaminated water (Caño Limón, Arauca)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
<tr>
<td>10E</td>
<td>Oil-contaminated soil (Capachos, Arauca)</td>
<td>\textit{P. aeruginosa} SCD-13</td>
<td>AF448036</td>
</tr>
</tbody>
</table>

*All locations are in Colombia.
Pathogenesis assay in *Lactuca sativa var. capitata* L. Each *P. aeruginosa* strain was grown on LB broth to 2 × 10^9 cfu/ml. From the culture, a 1-ml sample was withdrawn, centrifuged twice, and resuspended in sterile saline solution. Serial dilutions of the final suspension were plated to determine viable counts, and 5 μl was inoculated into leaf segments of healthy plants of *L. sativa var. capitata* L acquired in local supermarkets. Leaves were detached and disinfected by washing them sequentially with tap water, 1% sodium hypochlorite, sterile distilled water, 70% ethanol, and sterile distilled water. Once disinfected, circular 2.5-cm leaf segments were cut out under sterile conditions. Individual segments were placed onto sterile Petri dishes and inoculated with 5 μl of the bacterial suspension, prepared as described before. Inoculated leaf segments were incubated at 30°C for 3 days in a humid chamber to avoid desiccation of the segments [15]. Lower doses of 1 × 10^6, 1 × 10^4, and 1 × 10^2 cfu were also tested. The diameter of the lesion was recorded daily. The experiments, consisting of two replicates per strain, were repeated three times. Negative controls for all experiments were done by inoculating leaf segments with sterile saline solution; additional negative controls consisted of leaf segments inoculated with a 5-μl suspension of *P. putida* strain M2A prepared as described for the *P. aeruginosa* strains. Controls were cultured in the same conditions as the inoculated experimental samples.

**Statistical analysis.** The normal distribution of the resulting data from the pathogenicity assays, with both the fruit fly and the lettuce model, was tested with the Shapiro-Wilk normality test, a standard test for small sample sizes. For this test, a small *P*-value indicates a non-normal distribution of the data with high significance. Results obtained from the clinical and environmental isolates were compared using the nonparametric Wilcoxon rank sum and Kruskal-Wallis tests [41]. These tests evaluate whether unpaired samples come from the same population (null hypothesis), with the null hypothesis rejected at *P* < 0.05. All tests were carried out using Statistix 8.0 or SPSS 7.5 software. Distribution of the data was diagramed with SPSS 7.5.

**Results**

**Isolation of *P. aeruginosa* from environmental samples.** Of the 38 water and soil samples analyzed (9 oil-contaminated and 29 non-oil-contaminated), 50 isolates were recovered on cetrimide plates. Of these, 19 were identified as *P. aeruginosa*, corresponding to 38% of the total isolates. The other 31 isolates belonged to different pseudomonads species (*P. putida, P. fluorescens, P. stutzeri*) or to other genera of gram-negative bacteria. Note that of the 19 *P. aeruginosa* isolates, 16 were found in samples contaminated with oil hydrocarbons; *P. aeruginosa* was recovered from all nine oil-contaminated samples, but only from three non-oil-contaminated samples (corresponding to 10.3%).

Parsimony analysis using biochemical and antimicrobial susceptibility characteristics did not group the strains according to the source (data not shown), indicating that environmental and clinical strains cannot be differentiated by means of these characteristics.

**Pathogenesis in the *D. melanogaster* model testing clinical and environmental *P. aeruginosa* isolates.** Both groups of isolates killed the flies irrespective of the source of the bacteria. The distribution of the data for the time points 16, 25, 30 and 40 h post-inoculation is shown in Fig. 1. Only one strain, isolate 6E, was particularly less virulent than any of the other strains (data not shown). The results did not show a normal distribution for the different time points evaluated (non-normal distribution accepted at *P* < 0.05; Shapiro-Wilk test at 16 h after inoculation *N* = 54, *W* = 0.5422, *P* < 0.001; at 22 h after inoculation *N*60, *W* = 0.7655, *P* < 0.001; 25 h after inoculation *N*60, *W* = 0.8775, *P* < 0.001; 28 h after inoculation *N*54, *W* = 0.9160, *P* < 0.001; 30 h after inoculation *N*45, *W* = 0.9176, *P* = 0.0035; 32 h after inoculation *N*60, *W* = 0.8522, *P* < 0.001; 40 h after inoculation *N*60, *W* = 0.6090, *P* < 0.001). The survival of animals at the end of the experiment (40 h post-inoculation) was the same for both groups of strains, clinical and environmental (Fig. 1; Wilcoxon rank sum test at 40 h after inoculation *N*60, two-tailed *P*-value 0.7145; Kruskal-Wallis test 40 h after inoculation *N*60, *KW* = 0.1402, *P* = 0.7081). The results at different times after inoculation were analyzed to detect possible differences in the course of infection between the two groups of strains, but again no significant difference in fly survival was found (Wilcoxon rank sum test at 16 h after inoculation [ai] *N*54, two-tailed [tt] *P*-value 0.9648; at 22 h ai *N*60, tt *P*-value 0.4531; at 25 h ai *N*60, tt *P*-value 0.7173; at 28 h ai *N*54, tt *P*-value 0.5487; at 30 h ai *N*45, tt *P*-value 0.7321; at 32 h ai *N*60, tt *P*-value 0.9757. Kruskal-Wallis test at 16 h ai *N*54, *KW* = 0.003, *P* = 0.956; 22 h ai *N*60, *KW* = 0.5747, *P* = 0.4484; 25 h ai *N*60, *KW* = 0.1366, *P* = 0.7116; 28 h ai *N*54, *KW* = 0.3703, *P* = 0.5428; 30 h ai *N*45, *KW* = 0.1258, *P* = 0.7229; 32 h ai *N*60, *KW* = 0.0014, *P* = 0.9697). The survival of controls inoculated with either *P. putida* M2A or sterile saline was 93–100% for all experiments.

**Pathogenesis in *L. sativa var. capitata* L. for clinical and environmental *P. aeruginosa* isolates.** Both groups of isolates produced similar necrotic lesions irrespective of their origin (Fig. 2) with the exception of strain 6E, which produced smaller lesions than all other strains. The results did not show a normal distribution (Shapiro-Wilk test at 72 h after inoculation *N* = 52, *W* = 0.8986, *P* < 0.0003). No significant differences were found in the diameter of the lesions of the two groups (Wilcoxon rank sum test *N*52, two-tailed *P*-value: 0.1078; Kruskal-Wallis test *N*52, *KW* = 2.6165, *P* = 0.1058). Leaf segments inoculated with *P. putida* M2A.1 showed neither lesions or a small yellow zone at the inoculation site. Lesions did not form on any of the leaf segments inoculated with sterile saline solution. At lower doses (1 × 10^6, 1 × 10^4 and 1 × 10^2 cfu) no differences were found in the diameters of any of the lesions between the two groups of isolates, clinical and environmental (for 1 × 10^6 cfu dose Kruskal-Wallis test [dKWt] *N*28: 0.105, *P* = 0.746; for 1 × 10^4 cfu dKWt *N*27: 0.144, *P* = 0.705; for 1 × 10^2 cfu dKWt *N*27: 0.204, *P* = 0.651).
Discussion

P. aeruginosa strains were recovered from all oil-contaminated samples, but only from 10.3% of the non-oil-contaminated ones. This result was surprising, since P. aeruginosa is considered to be ubiquitous [20,27]. Our results showing that P. aeruginosa thrives in oil-contaminated niches agree with previous reports regarding its degradative capabilities [9,20]. However, the bacterium has fewer growth advantages in non-oil-contaminated ecosystems. Thus, it is possible that P. aeruginosa populations for the 23 P. aeruginosa-negative samples were present but not detectable by the dilution technique.

P. aeruginosa strains have been previously isolated from environmental samples. Green et al. [19] isolated P. aeruginosa strains from 14 of 58 (24%) agricultural soil samples. Jacobsen et al. [22] reported an initially non-detectable population of P. aeruginosa (<100 cells per g soil) in a trichloroethylene-contaminated soil sample from Denmark. Kimata et al. [27] found that P. aeruginosa is part of the indigenous microbiota of the seawater in Tokyo Bay. They found important differences between the two enumeration techniques used to detect the bacterium. The direct viable count-fluorescent antibody technique yielded $10^2$–$10^4$ cells/ml whereas culture counts were 0.17–0.72 cells/ml, indicating that P. aeruginosa can survive in a viable but not culturable state. This observation could explain, at least partially, the low recovery efficiency of P. aeruginosa from soil and water. However, the low counts of P. aeruginosa can quickly increase when an external factor affects the environment and the bacterium has an advantage over the microbial population, a survival strategy known as zymogenous [30].

This was the case in the Danish study mentioned above, in which the initial count of <100 cells P. aeruginosa/g soil increased dramatically to $10^5$ cells/g after steam treatment but decreased rapidly afterwards [22]. Although “zymogenous” refers to the stimulation of microbial activity by substrates, other conditions, such as a reduction of competitors can also stimulate proliferation [22].

P. aeruginosa has been described as a common inhabitant of sewage and large populations persist even after anaerobic treatment of the sewage. Benatti et al. [7] reported P. aeruginosa populations of $10^5$ most probable number (MPN) per ml in the influent sludge of a municipal anaerobic digestor, and $10^4$ MPN/ml in the effluent sludge. Curran et al. [12] found even higher numbers on the surface of mushrooms sampled at supermarkets in five European countries ($10^4$–$10^7$ cfu/g); some samples of the compost used for growing the mushrooms also carried $10^7$ cfu of P. aeruginosa/g. A natural source of P. aeruginosa could be mammalian feces, but previous work showed that a low percentage (3–6%) of the healthy human population carries the bacterium fecally [28]. More recently, Pirnay et al. [37] reported a positive relationship between the extent of pollution in a Belgian river and the prevalence of P. aeruginosa. Our results indicating that P. aeruginosa is more successful in oil-contaminated environments suggest that this species is a poor competitor in undisturbed environments, but thrives and is able to rapidly grow when the appropriate nutrients become available or conditions are otherwise favorable (e.g., reduced microbiota).
Results from the two models used in this study did not differ with respect to the virulence of clinical and environmental isolates of P. aeruginosa. In addition, the antibiotic resistance profiles were almost exactly the same for all strains tested (MAR index: 0.5–0.6, data not shown). This finding clearly indicates that it is a mistake to consider environmental strains safer than clinical ones. For bioremediation purposes, environmental isolates are augmented and reintroduced into the polluted region, in a practice known as bioaugmentation [43], in which the bacterial population is increased 100- to 1000-fold in order to enhance bioremediation. According to the evidence presented here, this practice involving P. aeruginosa strains poses a serious threat to susceptible hosts, since P. aeruginosa is known to affect a wide variety of organisms, such as insects [13], nematodes [38], plants [38,45], birds [5], and mammals including humans [25]. With this broad range of potential hosts, the introduction of high populations of P. aeruginosa into the environment may represent a putative risk to wild life inhabiting locations where bioremediation will be applied. Moreover, it may be hazardous to people who might, unknowingly, enter the zone under treatment. Additional studies in other animal models (i.e., mice, rats, rabbits) are necessary to reach more definitive conclusions about possible threats to humans and animals.

The results of the two models chosen for this study were similar, but the assay with lettuce leaves is faster and easier. Only strains proven to be less virulent in the pathogenicity assay should be applied for bioremediation. If this is not possible, containment measures should be taken and a complete set of industrial safety regulations implemented for the appropriate use of virulent but nonetheless useful strains.

Acknowledgements. This research was funded by Fondo de Investigaciones de la Facultad de Ciencias, Universidad de los Andes, and Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología “Francisco José de Caldas” (CaliCiencias project code 1204-05-1360). We thank Roberto Kolter for his support, critical comments, and helpful discussions, Adolfo Amézquita for his invaluable assistance with statistical analysis of the data, and Rodrigo Cubillos and Maria T. Botero for their help with the initial isolation attempts. We also thank Gloria Uribe, Claudia Echeverri, Josefina Flores, Alexandria Acuña, and their institutions, which provided the clinical strains used for this work; Fundación Hospital de la Misericordia, Bogotá, Hospital Federico Lleras Ibagué, Clínica del Prado Santa Marta, and Investigaciones de la Facultad de Ciencias, Universidad de los Andes, and Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología “Francisco José de Caldas” (Colciencias project code 1204-05-1363) for their support.

References
Comparación de la virulencia entre aislados clínicos y ambientales de *Pseudomonas aeruginosa*

**Resumen.** Para caracterizar la virulencia del patógeno oportunista *Pseudomonas aeruginosa*, se aislaron nuevas cepas a partir de muestras clínicas y ambientales. *P. aeruginosa* se recuperó con una elevada frecuencia a partir de muestras contaminadas con petróleo, mientras que la frecuencia de recuperación a partir de muestras no contaminadas fue muy baja. La virulencia de cinco cepas clínicas y cinco cepas ambientales de *P. aeruginosa* se evaluó usando dos modelos diferentes, *Drosophila melanogaster* y *Lactuca sativa* var. *capitata* L. No se encontraron diferencias en la virulencia de los dos grupos de aislados con ninguno de los modelos. Dado que en procesos de biorremediación y bioaumento se usan cepas ambientales de *P. aeruginosa*, estos resultados deberían tenerse en cuenta en el futuro diseño de consorcios degradadores y para establecer las medidas de seguridad necesarias para su utilización. [Int Microbiol 2006; 9(4):247-252]

**Palabras clave:** *Pseudomonas aeruginosa* · prueba de patogenicidad · bioseguridad · bioaumento · biorremediación

Comparación de la virulencia entre aislados clínicos y ambientales de *Pseudomonas aeruginosa*

**Resumo.** Para caracterizar a virulência do patógeno oportunista *Pseudomonas aeruginosa*, foram isoladas novas cepas a partir de amostras clínicas e ambientais. *P. aeruginosa* foi recuperada a frequências elevadas a partir de amostras contaminadas com petróleo e lodos oleosos, enquanto a frequência de recuperação a partir de amostras não contaminadas foi muito baixa. Avaliou-se a virulência de cinco linhagens clínicas e cinco linhagens ambientais de *P. aeruginosa* usando dois modelos diferentes, *Drosophila melanogaster* e *Lactuca sativa* var. *capitata* L. Os resultados não mostraram diferenças na virulência dos dois grupos de isolados com nenhum dos modelos. Dado que em processos de biorremediação e bioaumento se usam linhagens ambientais de *P. aeruginosa*, deveriam ter-se em conta estes resultados no futuro desenho de consórcios degradadores e nas medidas de segurança necessárias para sua utilização. [Int Microbiol 2006; 9(4):247-252]

**Palavras clave:** *Pseudomonas aeruginosa* · prova de patogenicidade · biossegurança · bioaumento · biorremediado