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Virulence determinants in *Pseudomonas aeruginosa* strains from urinary tract infections

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SUMMARY

A total of 121 uropathogenic *Pseudomonas aeruginosa* strains were examined for production of several virulence-related factors. These strains were distributed in five predominant O-serotypes, i.e. O 4, O 12, O 11, O 6 and O 5, which accounted respectively for 23·9, 23·1, 12·3, 8·2 and 5·7% of isolates. Pyochelin and pyoverdin siderophores were produced by most of the isolates, defective variants occurring at very low frequency (2·4% for pyochelin and 7·4% for pyoverdin). Adherence to uroepithelial cells and production of cytotoxins was demonstrated in 52·8 and 67·7% of the strains, respectively, with higher frequencies for epidemiologically related strains belonging to serotypes O 4 and O 12. Titration of total proteases, elastase and phospholipase C revealed a high degree of heterogeneity among isolates. However, examination of individual O-serotypes by exoenzyme production showed that elevated levels of total proteases and elastase were characteristics of serotypes of minor numerical importance, i.e. O 1, O 10, O 11 and O 17, whilst low levels of elastase were produced by strains belonging to the predominant serotypes, namely O 4 and O 12. Moreover, epidemiologically related strains belonging to serotypes O 4 and O 12 appeared more homogeneous than the whole serogroup, when compared with other groups on the basis of exoenzyme levels.

INTRODUCTION

During the last two decades *Pseudomonas aeruginosa* has emerged as a major opportunistic pathogen accounting for about 11% of all nosocomial infections, mainly of the lower respiratory tract, urinary tract and surgical wounds [1]. A prerequisite for *P. aeruginosa* infections is an impairment of the patient's local anti-infectious defences, for example the integrity of mucosa or skin or a general condition of immunodepression [2].

Particularly in urinary tract infections (UTIs) there is a number of predisposing factors, including instrumentation and catheterization, which enhance susceptibility to *P. aeruginosa* colonization and infection [2]. These factors play a relevant role in hospitalized patients, which are often catheterized because of neurological diseases or during the course of major surgery [3–5].

Pseudomonas aeruginosa seldom causes uncomplicated UTIs and no specific clinical characteristics differentiate pseudomonas infections from other types of

urinary infections [2]. Their pathogenesis could be related to the production of several cell-associated and extracellular virulence factors including adhesins [6–9], alginate [10, 11], endotoxin [12], exotoxin A [13–16], exoenzyme S [17–19], elastase [20–22], alkaline protease [23, 24], phospholipase C [25, 26] and the two siderophores pyochelin [27, 28] and pyoverdin [29, 30]. However, a detailed analysis of the virulence determinants in the most frequent epidemiological types of uropathogenic *P. aeruginosa* strains has not been carried out so far.

In view of these considerations, we have in previous research investigated the major epidemiological traits of uropathogenic *P. aeruginosa* strains and we have demonstrated the prevalence of particular serotypes, namely O 4 and O 12, in UTIs [31]. Moreover, the remarkable epidemiological relationships observed for some strains belonging to serotypes O 4 and O 12 raised the possibility of their clonal origin and strengthened the evidence for the existence of a common epidemic serotype O 12 strain in Europe [31, 32].

The aim of the present study was to compare the *in vitro* production of virulence-related factors among different epidemiological types of uropathogenic *P. aeruginosa* strains, and to investigate whether some peculiar bacterial phenotypes may relate to the pathogenesis of UTIs caused by this bacterium. For this purpose a set of 121 uropathogenic *P. aeruginosa* strains, representative of the most frequent types responsible for UTIs in patients hospitalized in Rome, has been analysed for most relevant virulence characteristics (adhesive properties and synthesis of exoenzymes, cytotoxins and siderophores).

MATERIALS AND METHODS

Patients and strains

One hundred and twenty-one strains were selected from positive monomicrobic urine cultures of symptomatic patients from the urological, surgical, medical and neurological clinics of Policlinico Umberto I (Rome) during the years 1984–9, and the first *P. aeruginosa* isolate from each patient was successively studied. The basic criterion for UTIs in non-catheterized patients was bacterial counts $> 10^5$ colony forming units (cfu)/ml of urine and this minimum value was decreased to 10^4 cfu/ml in catheter urines [33]. After the primary isolation on blood agar and MacConkey agar medium, single colonies were subcultured on cetrimide–agar plates (Pseudogel agar, BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md, USA) for identification. Isolates were first checked for growth at 42 °C and successively identified by the Sceptor (Becton Dickinson, Div. Johnston Laboratories, Towson, Md, USA) and the API 20NE (Bio-Mérieux, Charbonnières-Les-Bains, France) systems. Strains were stored at −80 °C in 15% glycerol. At the time of the experiment an aliquot of frozen culture was inoculated on cetrimide–agar plates; colonies were suspended in double-distilled water at 0·2 A_{620} and 0·1 ml aliquots used to inoculate 50 ml of medium for the assay of extracellular products.

Epidemiological typing

O-serotyping was performed by bacterial agglutination according to the scheme proposed by Liu and co-workers [34] using commercial antisera (Difco Laboratories, Detroit, Mich, USA) to the IATS serotypes.

Pyocin typing was carried out according to the method of Fyfe and co-workers [35] which identifies 105 main types and 25 subtypes. For all mucoid strains and for strains not clearly typable by the former method, the technique proposed by Govan [36] was also used.

Bacteriophage typing was performed according to Asheshov [37] whereas the determination of the lysogenic state was carried out according to Pitt and colleagues [32]. The mnemonic code of Farmer was used to represent the lysogenic state of the strains [38].

Data on the major epidemiological traits of the 121 uropathogenic *P. aeruginosa* strains have been reported in detail elsewhere [31], and are summarized in Table 1.

In vitro *adherence assay*

The adherence assay was performed with *P*-phenotype uroepithelial cells collected by centrifugation (2000 g, 10 min, 4 °C) from fresh morning urine of a single female healthy donor and washed three times in phosphate buffer saline (PBS) pH 7·2. The number of epithelial cells per ml was calculated by counting in a Burker haemocytometer and adjusted to a concentration of about 2×10^5 cells/ml with PBS. The adherence of *P. aeruginosa* to uroepithelial cells was examined by mixing together 0·5 ml samples of a standard bacterial suspensions in PBS (10^8 cfu/ml) and 0·5 ml of epithelial cells (10^5 cells/ml) in a water bath for 45 min at 37 °C. Cells were separated from unattached bacteria by continuous vacuum washing with PBS through 8 μm Nucleopore filters (Nucleopore Corporation, Pleasanton, Ca, USA). The filter membranes were gently pressed against glass slides which had previously been coated with a thin albumin layer. Slides were fixed in absolute methanol and stained with Giemsa. The total adherent bacteria per epithelial cell were microscopically determined. In each experiment 40 epithelial cells were examined and duplicate determinations were performed. Strains showing more than 30 attached bacteria per epithelial cell were classified as adhesive. Whenever this procedure was used, control epithelial cell preparations without exogenous bacteria were treated in the above manner to detect any indigenous organism which may have been present.

Assay of extracellular enzymes

Strains were cultivated for 20 h at 37 °C under vigorous agitation in 50 ml of an iron-poor medium (DCAA, total iron $< 0{\cdot}1$ μM, as determined by atomic absorption spectrophotometry) obtained after treatment of 5 g/l casaminoacids (Difco Laboratories, Detroit, Mich, USA) with the iron-chelating resin Chelex 100 (Biorad Laboratories, Richmond, Ca, USA) in deionized, double-distilled water. To compare enzyme activities among different strains, the A_{620} was measured for each culture and adjusted to 1 with sterile medium. Enzyme activities were determined in culture supernatants of organisms grown for 20 h in DCAA, after filtration through a 0·2 μm pore size Millipore filter (Millipore SpA, Rome, Italy). Assays were carried out in duplicate on two independent cultures grown under identical conditions. The assay was repeated a third time for defective strains and for those strains showing a variability in the quantitative determination of a single character higher than 20% and the mean value was reported in all cases.

Total proteolytic activity was quantified by using azoalbumin (Sigma Chemical

Company, St Louis, Mo, USA) as substrate according to Tomarelli and colleagues [39]. Two ml of a 5 mg/ml azoalbumin solution in 20 mM Tris-HCl pH 7·7 were added to 2 ml of culture filtrate and the mixtures were incubated at 37 °C for up to 10 h while being shaken. Aliquots were collected at different times and the non-hydrolysed substrate was precipitated by the addition of 0·25 volumes of 50% trichloroacetic acid. The precipitate was removed by centrifugation (3000 g, 10 min) and the protease activity spectrophotometrically determined by reading supernatants at 440 nm. The blank containing azoalbumin in Tris buffer was processed identically. The proteolytic activity was expressed in units (U) per litre; one unit was defined as the amount of enzyme hydrolysing 1 μg of azoalbumin in 1 min at 37 °C and calculated from the $A^{1\%}_{440} = 34$ for azoalbumin in Tris buffer.

Elastolytic activity was measured using elastin-congo red (Sigma Chemical Company, St Louis, Mo, USA) as insoluble substrate. Five mg of elastin-congo red were suspended in 1 ml of 100 mM Tris–succinate buffer pH 7·0, 1 mM-$CaCl_2$, supplemented with 1 ml of culture filtrate and incubated at 37 °C for up to 1 h under vigorous stirring. The reaction was stopped by adding 1 ml of 0·7 M sodium phosphate buffer pH 6. Unsolubilized elastin-congo red was removed by centrifugation (3000 g, 10 min) and elastase activity was measured by reading the A_{495} of supernatants. One unit enzyme was defined as the amount causing an optical density change of 1·0 unit in 1 h at 37 °C according to Bjorn and co-workers [40].

Phospholipase C activity was determined according to the method of Berka and Vasil [25] using *p*-nitrophenylphosphorylcholine (PNPC, Sigma Chemical Company, St Louis, Mo, USA) as substrate. Two ml of 10 mM-PNPC in 250 mM Tris-HCl, 1 μM-$ZnCl_2$, 60% glycerol pH 7·2 were added to 0·25 ml of culture supernatants and incubated for 1 h at 37 °C. Enzyme activity was measured spectrophotometrically at 405 nm wavelength and expressed in units; one unit of enzyme corresponded to 1·0 A_{405} according to Woods and colleagues [41].

Cytotoxic activity assay

To study cell–toxin interaction Chinese hamster ovary (CHO) cells were used according to a minor modification of the method by Iglewsky and Sadoff [42], which allows detection of 0·5 ng exotoxin A and 1·5 μg exoenzyme S as the lower limit [43]. CHO cells were propagated in Eagle's MEM, supplemented with 10% fetal calf serum, 1% L-glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% non-essential aminoacids. Cells (about 2×10^4) were cultivated in flat-bottom microtitre plates (Flow Laboratories, Gruppo Flow SpA, Opera, Italy), at 37 °C in 5% CO_2 atmosphere. Culture supernatants were directly diluted ten- and hundred-fold in the fresh cell growth medium supplemented with 1 mM nitrilotriacetic acid to minimize protease activity which could interfere in the assay. Incubation was carried out at 37 °C for up to 48 h and cytotoxicity was assessed by the characteristic morphological alterations of CHO cells during the first 24 h and by the unchanged colour of phenol-red indictor in the medium after extended incubation (48–72 h).

Extraction and characterization of siderophores

Minor modifications of the methods proposed by Cox and Graham [44] and Meyer and Abdallah [45] were adopted for the determination of pyochelin and

pyoverdin, respectively. The supernatants obtained after centrifugation (5000 g, 30 min, 4 °C) of iron-depleted cultures in DCAA were adjusted to pH 1·5–2 with HCl and extracted with 0·4 volumes of ethyl acetate. After evaporation of the organic phase the dry residue was resuspended in a small volume of methanol and applied to a silica gel G thin layer chromatography (TLC) in chloroform : acetic acid : ethanol (90 : 5 : 2·5) as the development solvent. Pyochelin from TLC plates was characterized by chromatographic mobility, iron binding capacity, fluorescence emission and chemical reactivity of thiazolidine rings according to Cox and Graham [44]. Pyoverdin was determined by a tenfold dilution of the culture supernatants in 100 mM Tris–HCl pH 7·0 and its concentration estimated by fluorimetric measurements at 460 nm after excitation at 400 nm, using a standard curve calibrated with known quantities of pure pyoverdin.

Statistical analysis

The chi-square exact probability test was used to assess the significance of the apparent association between selected characteristics.

RESULTS

Adherence of P. aeruginosa *to uroepithelial cells*

Adherence to uroepithelial cells was demonstrated for 64 of the 121 *P. aeruginosa* strains examined. Analysis of adhesiveness with respect to individual O-serotypes did not reveal any consistent association for this character, neither was it possible to associate adhesiveness with any other single epidemiological marker. It was interesting to notice that 13 of 18 serotype O 12 adhesive strains (72·2%) appeared to be epidemiologically related being characterized by: (i) multiple antibiotic resistance; (ii) pyocin type 1, 10 or 33 (subtype h); (iii) phage untypability or sensitivity to phages 68 or 119X; (iv) lysogenic profiles 3123151, 5123121, 5125121, 5165121 or 5425121.

Production of exoenzymes

Experiments carried out to quantify the production of exoenzymes revealed a high degree of heterogeneity for the 121 uropathogenic *P. aeruginosa* strains tested. Total protease and elastase levels ranged respectively from 25 to 1651 U/l (mean = 405; standard deviation = 374) and from 0 to 306 U/l (mean = 34·5; standard deviation = 57·2); similarly, phospholipase C production varied from 0 to 631 U/l (mean = 80·3; standard deviation = 95·9). Due to such a high variability in the levels of virulence-related exoenzymes observed for the whole strain collection, we decided to compare the exoproduct levels among strains belonging to different O-serotypes with the purpose of associating a peculiar virulence-phenotype with a major epidemiological marker. Data concerning the levels of exoproducts synthesized by strains belonging to most common uropathogenic O-serotypes are reported in Table 2, which shows that mean exoenzyme levels in strains belonging to serotypes O 4 and O 12 were lower than those found in other serotypes, particularly in serotype O 11. As a consequence of the high heterogeneity observed also for isolates belonging to the same O-serotype, strains were further subdivided by the definition of appropriate exoenzyme production categories. In this way it was possible to reduce the variation in

Table 1. *Relationships between most frequent O-serotypes, pyocin types and phage sensitivity patterns in 121* Pseudomonas aeruginosa *isolates from UTIs*

O-serotype	No. of strains	Pyocin type					Phage sensitivity				
		1	10	33	105	Others*	68	119x	188/1	NT†	Others‡
O 4	29	2	13	1	4	9	3	6		16	4
O 5	7	2		1		4		1	1	1	4
O 6	10			3		7				2	8
O 11	15	1	4	1	1	8			1	10	4
O 12	28	3	10	2		13	9	6		10	3
Others§	32	5	12	1	1	13	2	2	2	16	10
Total	121	13	39	9	6	54	14	15	4	55	33

* Distributed in 32 main types represented by 1–4 isolates, plus 4 not typable strains.
† Not typable.
‡ Distributed in 26 different sensitivity patterns represented by one or more isolates.
§ Distributed in 9 O-serotypes plus not typable, polyagglutinable and autoagglutinable strains.

Table 2. *Comparison of* in vitro *production of pyoverdin, total proteases, elastase and phospholipase C by most frequent* Pseudomonas aeruginosa *serotypes in UTIs*

O-serotype	No. strains	Exoproduct*			
		Pyoverdin (μM)	Total proteases (U/l)	Elastase (U/l)	Phospholipase C (U/l)
O 4	29	309·8 ± 103·6	247·8 ± 253·5	35·1 ± 84·2	67·1 ± 50·2
O 5	7	411·2 ± 91·6	421·4 ± 297·9	29·6 ± 38·3	75·6 ± 79·5
O 6	10	357·5 ± 116·1	592·4 ± 504·4	57·1 ± 85·1	148·3 ± 219·5
O 11	15	321·1 ± 84·1	814·4 ± 289·3	69·2 ± 29·7	110·2 ± 115·0
O 12	28	219·3 ± 82·3	244·5 ± 147·7	16·2 ± 32·6	48·1 ± 39·6

* Values represent means ± standard deviation.

exoenzyme levels for strains belonging to the same category (Table 3). Analysis of the distribution of strains belonging to the most frequent O-serotypes within exoenzyme production categories also allowed the establishment of some significant associations between O-serotype and exoproduct levels: in fact, high levels of total proteases and elastase were characteristic of strains belonging to serotype O 11 (chi-square = 23·25, $P < 0·01$ for total proteases and chi-square = 22·23, $P < 0·01$ for elastase) while strains belonging to serotypes O 4 and O 12 produced low levels of elastase (chi-square = 10·21, $P < 0·01$ and chi-square = 8·46, $0·02 < P < 0·01$, respectively). Moreover, serotype O 12 strains produced intermediate levels of total proteases and phospholipase C (chi-square = 21·14, $P < 0·01$ for total proteases and chi-square = 10·12, $P < 0·01$ for phospholipase C). Finally, strains belonging to O-serotypes of minor numerical importance (O 1, O 10, and O 17) were all high producers of the three exoenzymes tested.

Production of cytotoxins

Cytotoxic activity towards CHO cells was detectable in culture supernatants of 82 of 121 uropathogenic isolates tested (67·7%); in 59 strains the cytotoxic activity was observed at 100-fold dilution of culture supernatants whereas in 23

Table 3. *Distribution of most frequent uropathogenic* Pseudomonas aeruginosa *serotypes in exoenzyme production categories**

		Number of strains for each category of exoenzyme production								
		Total proteases†			Elastase‡			Phospholipase C§		
O-serotype	No. strains	Low	Inter-mediate	High	Low	Inter-mediate	High	Low	Inter-mediate	High
O 4	29	13	12	4	15	9	5	6	14	9
O 5	7	1	3	3	2	3	2	2	2	3
O 6	10	2	4	4	1	6	3	3	4	3
O 11	15	0	2	13	1	1	13	3	4	8
O 12	28	6	19	3	14	9	5	7	17	4

* From the distribution of exoenzyme values, ranges were arbitrarily selected which divided the 121 strains into low, intermediate and high producers. About one third of strains are included into each category for each enzyme.

† Low production (in 32% of isolates) = U/l < 150; mean (±S.D.) = 86·1 (±39·4) U/l. Intermediate production (in 36% of isolates) = 150 < U/l < 500; mean (±S.D.) = 256·2 (±71·7) U/l. High production (in 32% of isolates) = U/l > 500; mean (±S.D.) = 895·7 (±252·7) U/l.

‡ Low production (in 29% of isolates) = U/l < 5; mean (±S.D.) = 2·2 (±1·5) U/l. Intermediate production (in 38% of isolates) = 5 < U/l < 25; mean (±S.D.) = 8·7 (±3·9) U/l. High production (in 33% of isolates) = U/l > 125; mean (±S.D.) = 92·9 (±69·8) U/l.

§ Low production (in 31% of isolates) = U/l < 30; mean (±S.D.) = 11·7 (±9·6) U/l. Intermediate production (in 36% of isolates); mean (±S.D.) = 47·5 (±11·5) U/l. High production (in 33% of isolates) = U/l > 80; mean (±S.D.) = 166·6 (±115·0) U/l.

strains this activity was shown at tenfold dilution. Except for strains belonging to serotypes O 11 and O 17, all characterized by high cytotoxic activity, which was detectable at 100-fold dilution of culture supernatants, no significant association between cytotoxicity and individual epidemiological markers (i.e. O-serotype, pyocin type or phage type) or exoenzyme levels could be demonstrated. Nevertheless, a higher frequency of cytotoxic strains was observed for epidemiologically related clones belonging to serotypes O 4 and O 12, as compared to the whole serogroup or to the whole strain collection (data not shown).

Production of siderophores

When cultivated under conditions of limited iron *P. aeruginosa* is known to produce two different siderophores: pyochelin and pyoverdin. Pyochelin was produced by 118 out of 121 uropathogenic strains tested, resulting in three defective variants (2·47%). Two of these, although belonging to serotype O 5, were different in pyocin type (18x and 1n), antibiogram and production of exoenzymes and were thus epidemiologically unrelated. Pyoverdin production occurred in 112 of the 121 isolates and defective variants accounted for 7·43% of all the strains, distributed in 7 different types (serotypes O 1, O 4, O 5, O 11, O 12, O 15 and not typable). The mean concentration of pyoverdin produced by the 121 strains after 20-h growth in iron-poor medium was 298·3 μM (standard deviation = 55·6), ranging between 200 and 300 μM for most of the strains (54·5%). It is of note that no double-defective variants for siderophore synthesis were detected.

Table 4. *Comparative analysis of virulence determinants in epidemiologically related, uropathogenic* Pseudomonas aeruginosa *strains belonging to serotypes O 4 and O 12*

O-serotype	No. related strains	No. adhesive strains (%)	No. cytotoxic strains (%)	Levels (mean U/l ± S.D.) of			No. (%) strains producing	
				Total proteases	Elastase	Phospholipase C	Pyochelin	Pyoverdin
O 4	21	12 (57·1)	17* (80·9)	147·8 ± 57·2	4·7 ± 1·2	52·2 ± 16·4	21 (100)	21 (100)
O 12	16	13 (81·2)	16† (100)	235·5 ± 51·5	5·7 ± 1·8	60·0 ± 17·6	16 (100)	15 (93·7)
All types‡		64 (52·8)	82§ (67·7)	405·7 ± 374·2	34·5 ± 57·2	80·3 ± 95·9	118 (97·5)	112 (92·5)

* In 10 strains the cytotoxic activity was detectable at 100-fold dilution of the culture supernatant whereas in 7 strains the activity was detectable only at tenfold dilution.

† In all 16 strains the cytotoxic activity was detectable at 100-fold dilution of the culture supernatants.

‡ Representative of 121 uropathogenic strains distributed in 14 serogroups, plus not typable, polyagglutinable and autoagglutinable isolates.

§ In 59 strains the cytotoxic activity was detectable at 100-fold dilution of culture supernatants, whereas in 23 strains this activity was shown at ten-fold dilution.

For all the strains the addition of 100 μM iron to the medium strongly repressed the synthesis of both siderophores.

Virulence determinants in epidemiologically related serotype O 4 and O 12 strains

As the last step of our investigation we analysed the data concerning the adhesiveness and production of cytotoxins, exoenzymes and siderophores with respect to the epidemiological type for two groups of related isolates belonging to serotypes O 4 and O 12. The results (Table 4) indicate that the frequency of siderophore production and the levels of exoenzymes in serotype O 12 strains did not substantially differ from those derived by the analysis of the whole serotype but was markedly lower than those determined for the whole collection of strains; by contrast, adhesiveness and cytotoxicity were more frequent in this group of epidemiologically related clones than in unrelated strains belonging to the same or other serotypes (chi-square = 8·75, $P < 0{\cdot}01$).

Although statistically insignificant, a more frequent production of cytotoxins was also shown for epidemiologically related serotype O 4 strains; this group of strains appeared also to produce lower levels of the three exoenzymes tested by comparison with other strains belonging to the same or other serotypes. Finally, a markedly higher homogeneity in the levels of the three exoenzymes was shown for both groups of epidemiologically related isolates, by comparison with the total of strains.

DISCUSSION

During the last two decades several investigations pointed to a high incidence of particular epidemiological types of *P. aeruginosa* in specific infection episodes [31–33, 46–53]. Evidence has also been reported indicating the prevalence of *P. aeruginosa* serotypes O 4, O 6, O 11 and O 12 in hospital-acquired UTIs [31, 33, 50–54]. In addition, by combining multiple typing techniques, consistent epidemiological correlations have been established, leading to the hypothesis of a possible clonal origin for some uropathogenic strains belonging to serotypes O 4 and O 12 [31, 32]. It appears, therefore, that as with uropathogenic *E. coli* [55], only a few serogroups of *P. aeruginosa* cause the major proportion of UTIs. Consequently it can be supposed that a phenotype of particular virulence associated with these serogroups might enhance their ability to colonize the urinary tract and cause infection. Therefore, we carried out an investigation designed to compare the production of virulence-related factors in 121 strains of *P. aeruginosa* from hospital-acquired UTIs, as a similar experimental approach has been previously used to assess the virulence potential of strains isolated from different body sites [41] or at different stages of the disease in cystic fibrosis [56].

The first character to be studied was adhesion to uroepithelial cells, as the attachment of bacteria to the mucosal epithelium represents the initial step in the colonization of the urinary tract. Adhesiveness was demonstrated in 52·5% of *P. aeruginosa* isolates but no consistent association between this property and the O-serotype could be demonstrated. It was noted, however, that epidemiologically related uropathogenic clones belonging to serotype O 12 showed a higher incidence of the adhesive phenotype by comparison with strains of the same or other serogroups; these adhesive uropathogenic serotype O 12 strains were also

characterized by a typing profile very similar to that observed for epidemic and multiresistant serotype O 12 strains isolated in central Europe [32].

We then compared the levels of virulence-related exoproducts among urinary isolates of *P. aeruginosa*. Contrary to the reports of others [41], we observed a high variability of expression of these factors by uropathogenic strains, which did not allow the association of a particular virulence phenotype with a specific O-serotype. In fact, previous data on frequency and levels of virulence-related exoenzymes in urinary isolates of *P. aeruginosa* are equivocal. Janda and Bottone [57] reported a very high frequency (69%) of elastase defective variants and very poor production of this enzyme by *P. aeruginosa* strains from UTIs, whilst Woods and co-workers [41] showed that elastase and proteases were produced in large amounts by strains from UTIs as compared with isolates from other infections. Similarly, phospholipase C production by *P. aeruginosa* strains from UTIs was reported to occur at a low level in 63% of isolates examined by Janda and Bottone [57], while 37% of strains were defective. However, this enzyme was produced at very high levels in 100% of strains studied by Woods and co-workers [41] and by Berka and colleagues [26]. In view of the relatively limited number of strains examined by these authors, such discrepancies could be ascribed to the heterogeneity in the levels of extracellular enzymes produced by *P. aeruginosa*. It should also be pointed out that production of proteases, phospholipase C and cytotoxins is influenced by several factors including cultural conditions, growth stage, cation concentration and complexity of the growth medium [13, 15, 17, 18, 25, 40, 42, 58], which makes comparisons among studies carried out in different laboratories difficult. It is therefore crucial that *in vitro* conditions should approximate the *in vivo* growth environment in the urinary tract. This was accomplished in our study by the use of an iron-deprived medium where only trace elements and essential aminoacids were present. We showed that uropathogenic strains belonging to serotypes O 4 and O 12 produced statistically significantly lower amounts of elastase than other O-serotypes. Moreover, elastase production by epidemiologically related, uropathogenic *P. aeruginosa* strains belonging to serotypes O 4 and O 12 occurred at an even lower level when compared with strains belonging to other serogroups or with unrelated strains of the same serogroup. These observations are in agreement with the well-established concept that high elastase production is typical of systemic isolates, this enzyme being an important virulence factor of invasive strains recovered from disseminating infections [41]. Conversely, strains belonging to serotype O 11 produced very high levels of elastase, total proteases and cytotoxins which could explain previous observations on the high virulence of this serotype which is less frequent in UTIs than in systemic infections, and predominates in epidemic rather than in endemic situations [51, Visca and colleagues, unpublished observations]. It was also interesting to note that all strains belonging to some serotypes of minor numerical importance such as O 1, O 10 and O 17 produced high levels of total proteases, elastase, phospholipase C and cytotoxins.

Further reasons for choice of DCAA as preferential growth medium for *in vitro* assessment of exoenzyme production arise from previous observations that bacteria harvested from urines of patients with UTIs express high affinity iron transport system (i.e. siderophores and their cognate receptors) such as when

grown in iron restricted environments [59] and that low iron levels enhance exoenzyme production by *P. aeruginosa* [40]. Although relatively large amounts of iron are known to be excreted in normal urines [60], its concentration drastically decreases during infection, so that infecting bacteria need to scavenge this essential metal from the iron binding proteins of the host (transferrin and lactoferrin). Our results indicate that *P. aeruginosa* strains from UTIs produce at least one type of siderophore, i.e. pyochelin and/or pyoverdin. However, not all the uropathogenic strains produced both siderophores although pyochelin and pyoverdin defective variants were found at very low frequency (2·47 and 7·43%, respectively). In any case, the identification of at least one siderophore in supernatants of iron-deprived *P. aeruginosa* cultures points to a relevant role for these iron chelating agents in the pathogenesis of UTIs by this bacterium.

Clinical and environmental isolates of *P. aeruginosa* are known to produce also two distinct ADP-ribosyl-transferase enzymes, called exotoxin A and exoenzyme S. These two toxins have been shown to be pathogenic determinants in various experimental animal infections, burn wounds and chronic lung infections [14–16, 18, 19]. The results of our investigation indicate that about 68% of *P. aeruginosa* strains from UTIs excrete detectable amounts of cytotoxins when cultivated in DCAA. Although such an overall frequency is lower than that reported in the literature [13, 15, 17, 18, 42], it should be pointed out that it is comparable, or even higher, in the case of epidemiologically related strains belonging to serotypes O 4 and O 12. It must also be considered that the cultural conditions used in our study are not optimal for exotoxin production, since iron concentrations lower than 0·18 μM are known to reduce exotoxin A yields [42]. However, a stringent iron-deficiency has been shown to take place during the infection [59], so that exotoxin production in DCAA might better relate to the *in vivo* situation. Moreover, it may be possible that the nitrilotriacetic acid concentration present in the CHO cell assay was too low to neutralize the proteolytic effect so that toxins could have been degraded prior to detection. Thus it is likely that some *P. aeruginosa* strains which did not produce exotoxins as detected by our method did not represent true negatives.

In conclusion, our results do not allow the unequivocal association of any particular virulence determinant with the pathogenesis of *P. aeruginosa* in UTIs and strengthen the concept of a multifactorial virulence potential for this bacterium. In fact, we have shown that uropathogenic *P. aeruginosa* strains are very heterogeneous on the basis of their production of virulence-related factors. Nevertheless, examination of individual serotypes by exoenzyme levels revealed a certain homogeneity for epidemiologically related strains belonging to the major serotypes, namely O 4 and O 12.

Finally, it could be speculated that in spite of the overall low levels of exoenzymes produced, the virulence potential of epidemiologically related serotype O 12 and, to a certain extent, of serotype O 4 strains, might be ascribed to their ability to adhere to the urinary epithelium, to excrete siderophores and cytotoxins and to be resistant to several anti-pseudomonas antibiotics [31]. All these factors might have contributed to the epidemic spread of serotype O 12 strains in several European countries and their persistence in hospital environment [31, 32, 54, 62–65].

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