*Microbiology* (2001), **147**, 2659–2669 Printed in Great Britain

# Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa*

Heather Feltman,[1] Grant Schulert,[1] Salman Khan,[1] Manu Jain,[2] Lance Peterson[3] and Alan R. Hauser[1,2]

Author for correspondence: Alan R. Hauser. Tel: +1 312 503 1044. Fax: +1 312 503 1339. e-mail: ahauser@northwestern.edu

Departments of Microbiology/Immunology[1], Medicine[2] and Pathology[3], Northwestern University, Chicago, IL 60611, USA

**The type III secretion system of *Pseudomonas aeruginosa* transports four known effector proteins: ExoS, ExoT, ExoU and ExoY. However, the prevalence of the type III secretion system genes or the effector-encoding genes in clinical and environmental isolates of *P. aeruginosa* has not been well studied. Southern hybridization analyses and PCR were performed on over 100 *P. aeruginosa* isolates to determine the distribution of these genes. Clinical isolates were obtained from urine, endotracheal, blood and wound specimens, from the sputum of cystic fibrosis (CF) patients, and from non-hospital environmental sites. The *popB* gene was used as a marker for the presence of the large chromosomal locus encoding the type III secretion machinery proteins. Each isolate contained the *popB* gene, indicating that at least a portion of this large chromosomal locus was present in all isolates. Likewise, each isolate contained *exoT*-like sequences. In contrast, the *exoS*, *exoU* and *exoY* genes were variable traits. Overall, 72% of examined isolates contained the *exoS* gene, 28% contained the *exoU* gene, and 89% contained the *exoY* gene. Interestingly, an inverse correlation was noted between the presence of the *exoS* and *exoU* genes in that all isolates except two contained either *exoS* or *exoU* but not both. No significant difference in *exoS*, *exoU* or *exoY* prevalence was observed between clinical and environmental isolates or between isolates cultured from different disease sites except for CF respiratory isolates. CF isolates harboured the *exoU* gene less frequently and the *exoS* gene more frequently than did isolates from some of the other sites of infection, including the respiratory tract of patients without CF. These results suggest that the *P. aeruginosa* type III secretion system is present in nearly all clinical and environmental isolates but that individual isolates and populations of isolates from distinct disease sites differ in their effector genotypes. The ubiquity of type III secretion genes in clinical isolates is consistent with an important role for this system in human disease.**

**Keywords:** ExoU, ExoY, ExoS, ExoT, cystic fibrosis

## INTRODUCTION

*Pseudomonas aeruginosa* is a Gram-negative bacterium that causes a variety of diseases in compromised hosts. For example, this organism first colonizes the lungs of children with cystic fibrosis (CF) between 5 and 9 years of age (Pedersen *et al*., 1986) and can subsequently be cultured from the sputum of approximately 80% of adults over the age of 25 (Fitzsimmons, 1993). Once established within the lungs, *P. aeruginosa* usually causes episodic bouts of pneumonia that lead to progressive irreversible lung injury and ultimately death. Another group of individuals particularly prone to infections by this organism is hospitalized patients. In the United States, it is estimated that *P. aeruginosa* is responsible for 17% of nosocomial pneumonias, 11% of nosocomial urinary tract infections, 8% of surgical

**Abbreviation:** CF, cystic fibrosis.

wound infections and 3% of central-line-associated bloodstream infections (National Nosocomial Infections Surveillance System, 1996). Given the frequency of *P. aeruginosa* infections and the increasing resistance of this organism to conventional antibiotics (Fluit *et al.*, 2000; Hanberger *et al.*, 1999), it is not surprising that *P. aeruginosa* illnesses are associated with significant morbidity and mortality. For example, *P. aeruginosa* nosocomial pneumonia is associated with crude mortality rates as high as 70% overall (Pennington, 1995) and as high as 90% in mechanically ventilated patients (Fagon *et al.*, 1989); attributable mortality rates are approximately 40% (Fagon *et al.*, 1993).

To cause such severe disease, *P. aeruginosa* utilizes a large number of secreted and cell-associated virulence factors. These include exotoxin A, phospholipase, alkaline protease, elastase, pyocyanin, pili, flagella and lipopolysaccharide (Salyers & Whitt, 1994). Many of these factors are variable traits – they are produced by some clinical isolates but not by others. An important and recently recognized virulence determinant of *P. aeruginosa* is the type III secretion system (Kang *et al.*, 1997; Yahr *et al.*, 1996b). The genes encoding the secretion, translocation and regulatory machinery of this system are clustered together in the 55 min region of the *P. aeruginosa* chromosome and have been designated *psc*, *pcr*, *exs* and *pop* genes (reviewed by Frank, 1997). The *psc* and *pcr* genes primarily encode components of the bacterial secretion apparatus whereas the *exs* genes and their products are involved in regulation of this complex system. The two *pop* genes encode proteins that are essential for the translocation of effector proteins into host cells. The *popB* gene encodes PopB (also known as PepB) and the *popD* gene encodes PopD (also known as PepD).

In contrast to the clustered genes encoding the type III transport machinery, the genes encoding the type III effector proteins appear to be scattered throughout the chromosome (Stover *et al.*, 2000; the Pseudomonas Genome Project, http://www.pseudomonas.com). To date, four effector proteins have been identified: ExoS, ExoT, ExoU (also called PepA) and ExoY. ExoS possesses two distinct functional domains. The amino-terminus contains a GTPase-activating domain for Rho GTPases and results in disruption of actin microfilaments and subsequent cell rounding (Frithz-Lindsten *et al.*, 1997; Goehring *et al.*, 1999; Pederson *et al.*, 1999; Vallis *et al.*, 1999). The carboxyl-terminus has ADP-ribosyltransferase activity directed towards Ras and other proteins and is cytotoxic to eukaryotic cells (Frithz-Lindsten *et al.*, 1997; Henriksson *et al.*, 2000; Olson *et al.*, 1997, 1999; Vincent *et al.*, 1999). In addition, ExoS may play a role in modulating bacterial internalization by eukaryotic cells (Cowell *et al.*, 2000; Fleiszig *et al.*, 1997; Frithz-Lindsten *et al.*, 1997). ExoT is also an ADP-ribosyltransferase but has only 0·2% of the catalytic activity of ExoS (Yahr *et al.*, 1996a). Like ExoS, it is a GTPase-activating protein for Rho GTPases and inhibits bacterial internalization by eukaryotic cells (Cowell *et al.*, 2000; Garrity-Ryan *et al.*, 2000; Krall *et al.*, 2000). Although the mechanism of action of the third effector protein, ExoU, is unclear, laboratory studies suggest that this factor is quite important in disease. ExoU mediates killing of a variety of mammalian cell types *in vitro*, including macrophages, epithelial cells and fibroblasts (Coburn & Frank, 1999; Finck-Barbancon *et al.*, 1997; Fleiszig *et al.*, 1997; Hauser & Engel, 1999; Hauser *et al.*, 1998; Vallis *et al.*, 1999). Furthermore, isogenic mutants that do not produce or secrete ExoU are defective in virulence in a mouse model of pneumonia (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). Unlike the wild-type strain, an isogenic mutant defective in secretion of ExoU and ExoT did not cause sepsis in a rabbit model of pneumonia (Kurahashi *et al.*, 1999). The fourth effector, ExoY, is an adenylate cyclase that elevates the intracellular cAMP levels in eukaryotic cells and causes rounding of certain cell types (Vallis *et al.*, 1999; Yahr *et al.*, 1998).

Interestingly, the genes encoding some *P. aeruginosa* type III effector proteins are variable traits (i.e. they are found in some isolates but not in others) (Finck-Barbancon *et al.*, 1997; Fleiszig *et al.*, 1997; Hauser *et al.*, 1998). The distribution of these genes amongst clinical isolates of *P. aeruginosa* remains to be elucidated. Furthermore, the prevalence of effector genes in populations of isolates from different disease sites has not been systematically and thoroughly explored. In this report, we investigate the prevalence of type III effector genes in populations of *P. aeruginosa* isolates obtained from the blood, wounds, respiratory tracts and urinary tracts of patients as well as from non-hospital environmental sources.

## METHODS

**Bacterial strains and growth conditions.** An initial collection of 95 *P. aeruginosa* clinical isolates was obtained from patients at Northwestern Memorial Hospital in Chicago between March 1999 and December 2000. Consecutive isolates were collected and analysed using a protocol approved by the Northwestern University Institutional Review Board. Isolates were identified as *P. aeruginosa* by the Clinical Microbiology Laboratory at Northwestern Memorial Hospital using standard protocols (Gilardi, 1991). To prevent overrepresentation of certain strains, only one isolate per patient was included in the analysis. A second collection of 20 consecutive CF respiratory isolates and 20 consecutive non-CF respiratory isolates was analysed to further examine the distribution of *exoU* and *exoS*. These isolates had been previously collected from patients at Moffitt-Long Hospital at the University of California, San Francisco, between November 1997 and October 1998 and stored as frozen stocks. The origins of the *P. aeruginosa* environmental isolates are described in Table 1. Isolates were minimally passaged on Vogel–Bonner minimal media plates (Vogel & Bonner, 1956) or Luria–Bertani (LB) agar plates and were stored at −70 °C in 25% glycerol.

*P. aeruginosa* strain PA103, which contains *exoU*, *exoT* and *exoY* genes (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998; data not shown), and strain 388, which contains *exoS*, *exoT* and *exoY* genes (Yahr *et al.*, 1996a, 1998), were used as controls in Southern hybridization experiments.

***Table 1.*** Environmental isolates used in this study

| Isolate | Original designation | Origin | Source |
|---|---|---|---|
| EV1 | Env 1 | Green onion | David P. Speert, University of British Columbia |
| EV2 | Env 11 | Potato | David P. Speert |
| EV3 | Env 12 | Green pepper | David P. Speert |
| EV4 | Env 14 | Green pepper | David P. Speert |
| EV5 | Env 16 | Tomato | David P. Speert |
| EV6 | Env 32 | Zucchini | David P. Speert |
| EV7 | Env 37 | River isolate | David P. Speert |
| EV8 | Env 47 | River isolate | David P. Speert |
| EV9 | Env 58 | River isolate | David P. Speert |
| EV10 | Env 62 | River isolate | David P. Speert |
| EV11 | 15524 | Soil | ATCC |
| EV12 | 21472 | Soil from oilfield | ATCC |
| EV13 | 15523 | Soil | ATCC |
| EV14 | 7700 | Well water | ATCC |
| EV15 | 7701 | Well water | ATCC |
| EV16 | 14886 | Soil | ATCC |
| EV17 | 21776 | Soil | ATCC |
| EV18 | LPL5 | Lake | Robert V. Miller, Oklahoma State University |
| EV19 | LLPA10 | Lake | Robert V. Miller |
| EV20 | JB2 | Polychlorinated biphenyl-contaminated soil | William J. Hickey, University of Wisconsin |

***Table 2.*** Primers used for detection of type III secretion genes

| | Primers for amplification of probes for Southern hybridization assays* | Primers for PCR-based detection assays |
|---|---|---|
| *exoU* | 5′-AGCGTTAGTGACGTGCG-3′ | 5′-AGCGTTAGTGACGTGCG-3′ |
| | 5′-GCGCTCGCTCTTCAACGG-3′ | 5′-GCGCATGGCATCGAGTAACTG-3′ |
| *exoS*† | 5′-TTTGGATCCATGCATATTCAATCGCTTCAGCAGA-3′ | 5′-TCAGGTACCCGGCATTCACTACGCGG-3′ |
| | 5′-CCCCTTAAGTCAGGCCAGGTCGAGGCCGCG-3′ | 5′-TCACTGCAGGTTCGTGACGTCTTTCTTTTA-3′ |
| *exoT*† | 5′-TTTGGATCCATGCATATTCAATCGCTTCAGCAGA-3′ | 5′-TCACTGCAGTTCCGCGTGCTCCGACG-3′ |
| | 5′-CCCCTTAAGTCAGGCCAGGTCGAGGCCGCG-3′ | 5′-TCAGGTACCTGCTGGTACTCGCCGTT-3′ |
| *exoY* | 5′-TCCAAGCTTATGCGTATCGACGGTCATC-3′ | 5′-TCCAAGCTTATGCGTATCGACGGTCATC-3′ |
| | 5′-CCCTCTAGATCAGACCTTACGTTGGAAAAAG-3′ | 5′-CGTATCGATCCGAGGGGGGTGTATCTGACC-3′ |
| *popB* | 5′-TTTGGATCCATGAATCCGATAACGCTTG-3′ | 5′-TTTGGATCCATGAATCCGATAACGCTTG-3′ |
| | 5′-TTTGAATTCTCAGATCGCTGCCGGTCG-3′ | 5′-TTTGAATTCTCAGATCGCTGCCGGTCG-3′ |

* 5′-primers are listed first and 3′-primers are listed last.

† Since the *exoS* and *exoT* genes are 80% identical in nucleic acid sequence, the same primers were used with different templates to generate probes for both genes.

**Construction of gene-specific labelled probes for Southern hybridization analyses.** Digoxigenin-labelled probes for Southern hybridization analyses were generated using PCR with upstream and downstream primers listed in Table 2. PCR was performed in the presence of digoxigenin-11-dUTP (Roche Molecular Biochemicals). In this way, internal probes of the following sizes were generated: *exoU* gene, 1·1 kb; *exoS* gene, 1·4 kb; *exoT* gene, 1·4 kb; *exoY* gene, 1·1 kb; *popB* gene, 1·2 kb. Cloned or chromosomal copies of each gene were used as templates. The following protocol was used to amplify the desired DNA products: 94 °C for 3 min, then 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min, then a final extension at 72 °C for 5 min. Amplified and labelled DNA fragments were electrophoresed through agarose gels (0·8%, w/v), and the appropriately sized fragments were extracted and used as probes in Southern hybridization experiments.

**Southern hybridization analysis.** Southern hybridizations were performed using the DIG System (Roche Molecular Biochemicals) in accordance with the manufacturer's instructions. Chromosomal DNA from clinical isolates was purified by the method of Chen & Kuo (1993). DNA was digested with *Bam*HI, electrophoresed through agarose gels (0·8 %, w/v), and transferred to nylon membranes (Hybond-N; Amersham). Following hybridization with labelled probes, membranes were washed twice in 2 × SSC (SSC is 0·15 M NaCl, 0·015 M sodium citrate, pH 7·0), 0·1 % (w/v) SDS for 15 min at room temperature followed by two washes in 0·5 × SSC, 0·1 % (w/v) SDS for 15 min at 68 °C. Membranes were then soaked in blocking buffer followed by incubation with anti-DIG-POD Fab fragments (Roche Molecular Biochemicals). Membranes were next washed twice in washing buffer (0·3 % Tween 20 in 0·1 M maleic acid, 0·15 M NaCl, pH 7·5) for 15 min at room temperature. Labelled DNA fragments were visualized by incubating the membranes in 225 µM coumaric acid (Sigma), 1·25 mM 3-aminophthalhydrazide (Sigma) and 0·009 % hydrogen peroxide (Fisher Scientific) in 100 mM Tris, pH 8·5, for 1 min and then exposing the membranes to autoradiography film. Each membrane was sequentially exposed to multiple probes such that at least one probe hybridized to the DNA in each lane. In this manner, it was verified that sufficient DNA was present in each lane for gene detection.

**Gene detection by PCR.** PCR assays for detection of the *exoU*, *exoS*, *exoT*, *exoY* and *popB* genes were performed using intact *P. aeruginosa* bacteria as a source of template chromosomal DNA. *P. aeruginosa* isolates were grown as individual colonies on LB agar plates. Toothpicks were used to directly inoculate a single colony into a tube containing pre-mixed PCR amplification reagents. Amplification was performed in the presence of the primers listed in Table 2. Samples were immediately placed into a thermocycler, and PCR amplification was performed using the following protocol: 94 °C for 3 min, then 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, followed by 72 °C for 5 min. PCR products were electrophoresed through agarose gels (0·8 %, w/v) containing ethidium bromide (0·5 µg $ml^{-1}$) and visualized using UV radiation.

**DNA fingerprint analysis.** The clonality of *P. aeruginosa* isolates was determined using the enterobacterial-repetitive intergenic consensus (ERIC)-PCR method (Liu *et al*., 1996; Louws *et al*., 1994; Versalovic *et al*., 1991). ERIC2 primers (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were synthesized and used in PCR amplifications. Intact bacteria were used as a source of template chromosomal DNA. The following protocol was used: 94 °C for 3 min, then 45 cycles of 1 min at 94 °C, 1 min at 45 °C and 1 min at 74 °C. After the last cycle, samples were maintained at 74 °C for 10 min. The resulting amplified DNA fragments were electrophoresed through agarose gels (0·8 %, w/v) containing ethidium bromide (0·5 µg $ml^{-1}$) and visualized using UV radiation. All fingerprint patterns contained a minimum of eight bands. A modification of the method of Liu *et al*. (1996) was used to interpret DNA fingerprints whereby fingerprints were considered distinct if they differed by at least three bands.

A second method of random amplified polymorphic DNA (RAPD) PCR fingerprinting, described by Mahenthiralingam *et al*. (1996) and Campbell *et al*. (2000), was used to confirm these results. This method utilizes sequence 208 (5′-ACGGCCGACC-3′) as a primer. Samples were amplified using the same protocol as described for ERIC-PCR fingerprinting except that the initial 94 °C for 3 min step was omitted. Fingerprints were considered distinct if they differed by at least three bands.

**Statistical methods.** The prevalence of effector genes amongst the different sets of clinical isolates was compared using the chi-squared test or the Fisher's exact test when appropriate.

## RESULTS AND DISCUSSION

### Strain collection

To investigate the distribution and prevalence of *P. aeruginosa* type III secretion genes, a large panel of clinical isolates was collected and grouped by the body site from which they were cultured. Twenty isolates (labelled UR) were obtained from the urine of patients, 20 isolates from endotracheal tube aspirates of mechanically ventilated non-CF patients (ET), 20 isolates from the sputum of patients with cystic fibrosis (CF), 20 isolates from blood (BL) and 15 isolates from wounds (WD). For comparison, 20 isolates cultured from non-hospital environmental sites (EV) were included in the analysis. In all, 115 isolates were examined.

*P. aeruginosa* frequently causes hospital epidemics that result in dissemination of a single clone to multiple patients and sites (Holder, 1977; Kropec *et al*., 1993). Such outbreaks may bias studies such as ours in that the epidemic clone may be overrepresented in the study sample. To eliminate this possibility, PCR-based DNA fingerprinting using ERIC2 primers was performed on all isolates (Fig. 1). Twenty (100 %) of the 20 urine isolates, 18 (90 %) of the 20 endotracheal isolates, 17 (85 %) of the 20 CF respiratory isolates, 18 (90 %) of the 20 blood isolates, 15 (100 %) of the 15 wound isolates and 16 (80 %) of the 20 environmental isolates were non-clonal (data not shown). These findings were confirmed using a RAPD fingerprinting primer, sequence 208 (see Methods), which yielded results similar to those obtained using the ERIC2 primer (data not shown). Therefore, the great majority of these isolates represent independent strains that can be used to accurately determine the prevalence of type III secretion genes in *P. aeruginosa*. The absence of clonality among the majority of isolates indicates that most of the *P. aeruginosa* disease during the period of collection was endemic and not epidemic, and that endemic infections were due to non-clonal strains of this organism. These findings are in agreement with those of two recent studies that have concluded that isolates from the majority of endemic *P. aeruginosa* nosocomial infections are non-clonal (Bonten *et al*., 1999; Ruimy *et al*., 2001).

### The *popB* gene

We wished to determine the percentage of clinical and environmental isolates of *P. aeruginosa* that harboured the large cluster of type III secretion genes which encodes the conserved secretion machinery. We used the *popB* gene as a marker for the presence of the large *psc-pcr-exs-pop* gene cluster (Frank, 1997). The *popB* gene is located near the middle of this cluster and encodes a

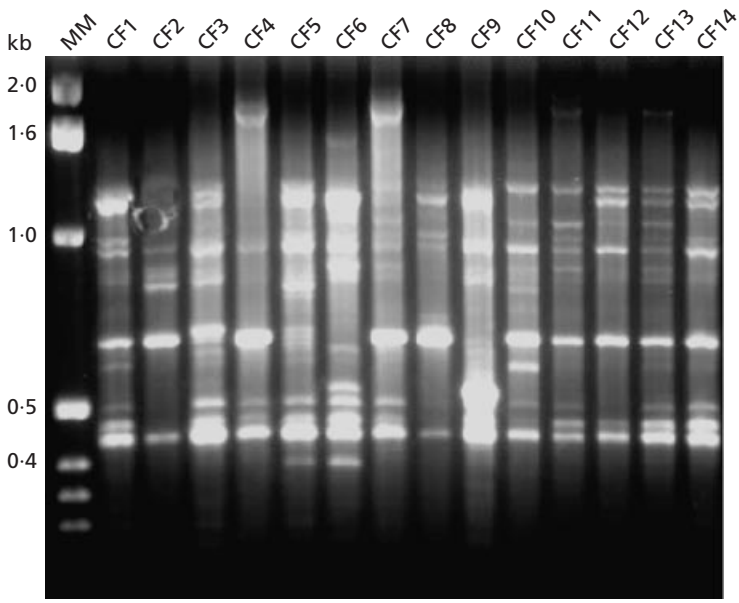


***Fig. 1.*** Representative ERIC-PCR fingerprinting results of 14 consecutive respiratory isolates from patients with CF. Molecular mass standards are listed to the left of the panel. Differences of three or more bands between any two isolates indicated that these two isolates were non-clonal.

protein essential for proper translocation of effector proteins (Frithz-Lindsten *et al*., 1998). An internal probe that hybridized to the *popB* gene was used to detect *popB*-like sequences in the chromosomal DNA of *P. aeruginosa* isolates (Fig. 2). All examined clinical isolates contained a single *Bam*HI–*Bam*HI DNA fragment that hybridized to the *popB* probe (Table 3). Likewise, all 20 of the examined environmental isolates harboured a single *Bam*HI–*Bam*HI DNA fragment that hybridized to this probe (Table 3). In total, 100% of 115 examined isolates contained *popB*-like sequences. These results were confirmed using a PCR-based detection assay that utilized primers specific for *popB* (data not shown). The presence of *popB* sequences in each of the tested isolates suggests that at least part of the *psc-pcr-exs-popB* locus is present in all or nearly all *P. aeruginosa* isolates. These conclusions are supported by several previous reports. Alonso *et al*. (1999) showed that each of seven examined environmental isolates contained the *pscJ* gene, which is also located in the large type III secretion gene cluster. Dacheux *et al*. (2000) noted that the *exsA* gene, another member of this gene cluster, was present in 28 (97%) of 29 examined CF isolates.

### The *exoT* and *exoS* genes

An internal probe constructed from the *exoT* gene was used to detect this gene in the chromosomal DNA of each isolate (Fig. 2). In addition to hybridizing to the *exoT* gene, this probe also occasionally yielded faint bands corresponding to the *exoS* gene due to the high degree of identity (80%; Yahr *et al*., 1996a) between these two genes. Corresponding experiments using an *exoS* probe demonstrated that this probe hybridized strongly to the *exoS* gene and also occasionally yielded faint bands corresponding to the *exoT* gene. In these situations, the faint bands due to cross-hybridization were not scored. The PCR-based gene detection assay was used to confirm the presence or absence of *exoT*- and *exoS*-hybridizing sequences in all isolates. One of the two PCR primers was designed to recognize DNA sequences internal to *exoT* while the second primer was designed to recognize unique DNA sequences flanking the *exoT* gene, thus ensuring specific amplification of this gene. A similar strategy was employed to uniquely amplify the *exoS* gene. In each case, the results of PCR amplification experiments agreed with the interpretation of Southern hybridization experiments (data not shown).

*exoT*-like sequences were found in 115 (100%) of 115 examined isolates, suggesting that this gene is not a variable trait (Table 3). In each case, a single *Bam*HI–*Bam*HI DNA fragment hybridized to the *exoT* probe. These results agree with previous observations made by Fleiszig *et al*. (1997), who noted that each of 14 examined isolates contained the *exoT* gene. Interestingly, the presence of this gene in all examined environmental isolates suggests that there may be selection for the *exoT* gene in the non-hospital environments of *P. aeruginosa*.

Unlike *exoT*, *exoS*-like sequences were not found in all clinical isolates (Fig. 2). Sixty-seven (71%) of 95 examined clinical isolates contained the *exoS* gene (Table 3). This finding is in agreement with previous reports, which had suggested that the *exoS* gene was not found in all *P. aeruginosa* clinical isolates. However, estimates of the actual prevalence of this gene or of ExoS production differed markedly. Woods *et al*. (1986) reported that of 132 tested burn, wound, urine, CF, acute pneumonia and blood isolates, 120 (91%) secreted ExoS as measured by an immunoblot assay. Rumbaugh *et al*. (1999), who examined a collection of 25 urinary, wound and tracheal isolates, found that 24 (96%) harboured the *exoS* gene. Fleiszig and co-workers

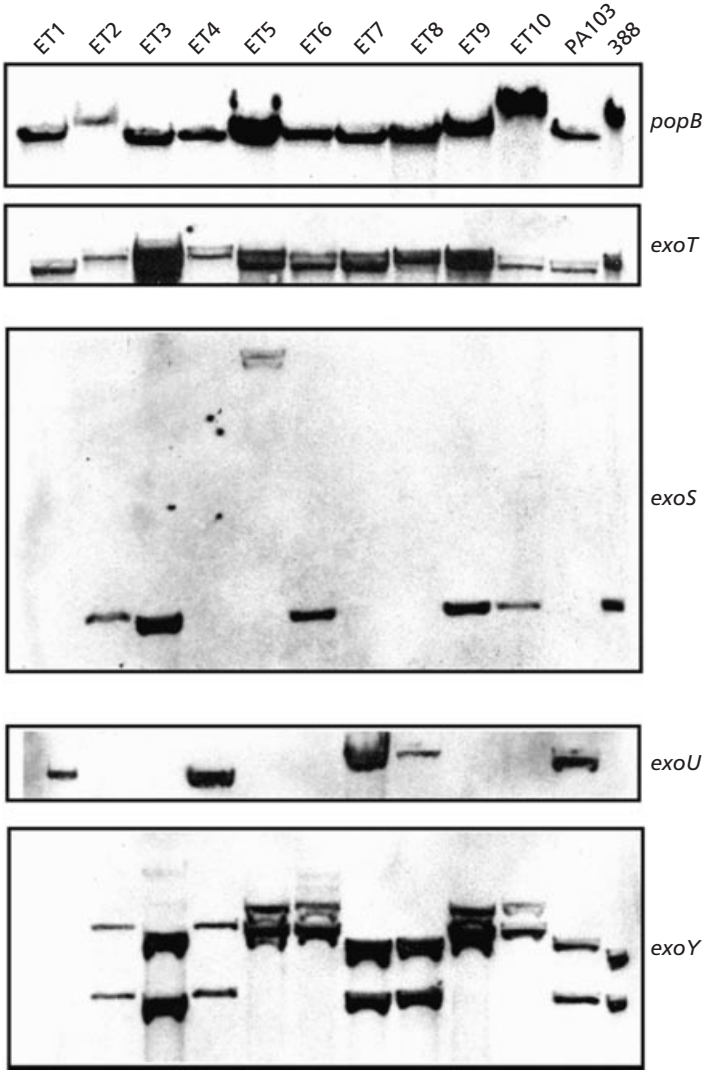


***Fig. 2.*** Representative Southern hybridization analyses of 10 consecutive endotracheal isolates using probes for the *popB*, *exoT*, *exoS*, *exoU* and *exoY* genes. Chromosomal DNA was digested with *Bam*HI, electrophoresed, transferred to membrane, and exposed to labelled probes for each of the indicated genes. *Bam*HI cuts once within the *exoY* gene, so two distinct DNA fragments hybridize with this probe in isolates that harbour a single copy of the *exoY* gene. PA103 and 388 are laboratory control strains. PA103 contains the *exoU*, *exoT* and *exoY* genes; 388 contains the *exoS*, *exoT* and *exoY* genes.

***Table 3.*** Prevalence of type III secretion genes in *P. aeruginosa* isolates

Values are the number of isolates with the percentage of the total in parentheses.

| Source | No. of isolates examined | *exoU* | *exoS* | *exoT* | *exoY* | *popB* |
|---|---|---|---|---|---|---|
| Urine | 20 | 7 (35) | 14 (70) | 20 (100) | 14 (70) | 20 (100) |
| Endotracheal | 20 | 5 (25) | 15 (75) | 20 (100) | 19 (95) | 20 (100) |
| Sputum (cystic fibrosis) | 20 | 2 (10) | 17 (85) | 20 (100) | 18 (90) | 20 (100) |
| Blood | 20 | 8 (40) | 12 (60) | 20 (100) | 18 (90) | 20 (100) |
| Wound | 15 | 6 (40) | 9 (60) | 15 (100) | 14 (93) | 15 (100) |
| Environment (non-hospital) | 20 | 4 (20) | 16 (80) | 20 (100) | 19 (95) | 20 (100) |
| **Total** | **115** | **32 (28)** | **83 (72)** | **115 (100)** | **102 (89)** | **115 (100)** |

(Finck-Barbancon *et al*., 1997; Fleiszig *et al*., 1996) noted that only 2 of 10 (20%) examined corneal isolates contained the *exoS* gene, although it is not clear if these isolates were randomly chosen or selected for certain phenotypic traits. The conflicting results of these studies may be due to differences in the sources of the isolates or

to the inability of the assays to differentiate the ExoS gene or protein from the ExoT gene or protein. In any case, our findings confirm that not all isolates have the ability to produce ExoS and suggest that the actual overall prevalence of the *exoS* gene in clinical isolates is approximately 70%.

The fraction of isolates harbouring *exoS*-like sequences differed somewhat between the various disease categories (Table 3). For example, whereas only 12 (60%) of 20 examined blood isolates contained *exoS*-like sequences, 17 (85%) of 20 CF respiratory isolates contained these sequences ($P = 0{\cdot}16$). Dacheux *et al.* (2000) also observed a high prevalence of the *exoS* gene in CF respiratory isolates. They found that 28 (97%) of 29 examined CF isolates contained the *exoS* gene. Clinical isolates from other sources had intermediate *exoS* prevalence values (Table 3). For example, 15 (75%) of 20 non-CF endotracheal isolates contained these sequences. None of these differences were statistically significant. Environmental isolates were similar to clinical isolates with respect to the prevalence of the *exoS* gene. Sixteen (80%) of 20 environmental isolates contained *exoS*-like sequences, suggesting that ExoS is not essential for survival of *P. aeruginosa* in nature. Again, no isolates contained more than a single *Bam*HI–*Bam*HI DNA fragment that hybridized to the *exoS* probe. When both clinical and environmental isolates were combined, a total of 83 (72%) of 115 examined isolates contained *exoS*-like sequences.

### The *exoU* gene

*exoU*-hybridizing sequences were found in a minority of isolates (Table 3; Fig. 2). In total, 32 (28%) of 115 examined clinical and environmental isolates harboured *exoU*-like sequences by Southern hybridization analysis. In each case, these results were confirmed using the PCR-based gene detection assay (data not shown). The prevalence of *exoU*-like sequences varied from 40% in wound and blood isolates, 35% in urine isolates, 25% in endotracheal isolates and 10% in CF respiratory isolates (Table 3). The difference in *exoU* prevalence between the blood or wound isolates and the CF isolates showed a trend towards significance ($P = 0{\cdot}06$). In comparison, 20% of environmental isolates harboured this gene. No isolate contained more than a single *Bam*HI–*Bam*HI DNA fragment that hybridized to the *exoU* probe. Dacheux *et al.* (2000) reported a similar value for the prevalence of the *exoU* gene in CF isolates. They detected the *exoU* gene in 3 (10%) of 29 examined CF isolates. Other investigators have reported significantly different values for the prevalence of the *exoU* gene among bacteraemia isolates. For example, Hirakata *et al.* (2000) detected the *exoU* gene in 4 (13%) of 32 bacteraemia isolates whereas Allewelt *et al.* (2000) detected the *exoU* gene in 9 (64%) of 14 bacteraemia isolates. It may be that these isolates were not randomly selected or that bacteraemia isolates from patients with different underlying clinical conditions (e.g. neutropenic cancer patients vs burn patients) harbour *P. aeruginosa* isolates with different population genotypes. Fleiszig and colleagues detected the *exoU* gene in 8 (80%) of 10 corneal isolates (Finck-Barbancon *et al.*, 1997; Fleiszig *et al.*, 1997), although it is unclear whether these isolates were randomly selected or chosen because of phenotypic characteristics. Corneal isolates were not examined in this study. In summary, our findings indicate that the *exoU* gene is present in approximately 28% of *P. aeruginosa* isolates, with the possible exception of CF isolates, which may harbour this gene less frequently.

Interestingly, *exoU* and *exoS* genes were not randomly distributed among isolates. Rather, nearly every isolate that harboured *exoS*-like sequences did not contain *exoU*-like sequences and vice versa. In fact, of the 115 *P. aeruginosa* clinical and environmental isolates examined in this study, 82 contained the *exoS* but not the *exoU* gene, 31 contained the *exoU* but not the *exoS* gene, a single isolate (UR16) contained both genes, and a single isolate (CF16) contained neither of these genes (data not shown). Similar findings were reported by Fleiszig and colleagues, who noted that each of 14 *P. aeruginosa* isolates contained either the *exoS* gene or the *exoU* gene (Finck-Barbancon *et al.*, 1997; Fleiszig *et al.*, 1997). Our results indicate that this is a near universal property of *P. aeruginosa* isolates. The reason for this unusual relationship is unclear. One possibility is that ExoU and ExoS fulfil important but redundant functions. However, ExoS and ExoU share no sequence similarity beyond the first 6 amino acids. Furthermore, whereas ExoS disrupts cell cytoskeletal elements and inhibits DNA replication (Frithz-Lindsten *et al.*, 1997; Goehring *et al.*, 1999; Henriksson *et al.*, 2000; Olson *et al.*, 1997, 1999; Pederson *et al.*, 1999; Vallis *et al.*, 1999; Vincent *et al.*, 1999), ExoU is a potent cytotoxin that rapidly induces death in many cell types (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). Therefore, any functional redundancy between these proteins is not obvious. Of note, *exoU*, like many of the genes that encode type III secretion effector proteins, may have been acquired by *P. aeruginosa* through horizontal transfer from another bacterial species. The G + C content of the *exoU* genes is 59 mol% (Hauser *et al.*, 1998) compared to 67·2 mol% for the *P. aeruginosa* chromosome as a whole (Palleroni, 1984). Also consistent with this hypothesis is that the *exoU* gene is located immediately adjacent to an insertion-sequence-like element (Finck-Barbancon *et al.*, 1997; Hauser *et al.*, 1998). Therefore, an alternative explanation for the inverse correlation between the presence of the *exoU* and *exoS* genes is that they occupy the same chromosomal locus and that the presence of *exoS* precludes the acquisition of *exoU* and vice versa. Two lines of evidence make this hypothesis unlikely. First, analysis of the genome sequence of strain PAO1 (Stover *et al.*, 2000; the Pseudomonas Genome Project, http://www.pseudomonas.com) suggests that the *exoU* and *exoS* genes occupy distinct loci. Although the chromosome of strain PAO1 contains the *exoS* gene but not the *exoU* gene, this strain does harbour sequences identical to those flanking the *exoU* gene, allowing the placement of the *exoU* gene at approximately 1 Mb in the chromosome. The *exoS* gene is located at 4·3 Mb.

Secondly, one of the isolates examined in this study, UR16, does contain both the *exoS* and *exoU* genes, indicating that a single strain may harbour both genes. An alternative explanation is that each of these proteins may be important for *P. aeruginosa* survival in a specific but different environmental niche. The genotype of an infecting isolate may simply be indicative of the particular environmental reservoir of that isolate. Interestingly, the only isolate in this study that contained neither the *exoS* nor the *exoU* gene (CF16) was cultured from the sputum of a 23-year-old CF patient. Once colonized, CF patients often remain colonized with the same strain for many years (Romling *et al.*, 1994). Therefore it is conceivable that either *exoS* or *exoU* was deleted from this isolate after many years of growth within the CF lung. Finally, it is possible that the *exoS* and *exoU* gene products in some way antagonize each other, resulting in a selective pressure upon *P. aeruginosa* strains to delete one or the other. Such a situation would be similar to the 'black holes' described by Maurelli *et al.* (1998) in *Shigella* spp. and enteroinvasive *Escherichia coli*.

### The *exoY* gene

Southern hybridization assays indicated that 102 (89%) of the 115 examined isolates contained *exoY*-hybridizing sequences, including 19 (95%) of 20 environmental isolates (Table 3; Fig. 2). The PCR-based gene detection assay yielded identical findings (data not shown). These results indicate that the *exoY* gene is present in the vast majority of *P. aeruginosa* clinical and environmental isolates and agree with those of Dacheux *et al.* (2000), who detected the *exoY* gene in 28 (97%) of 29 CF respiratory isolates. Interestingly, there was a trend towards a significant difference between the *exoY* gene prevalence in the urine isolates (70%) and the endotracheal or environmental isolates (95%) ($P = 0{\cdot}09$; Table 3). The implications of this difference are unclear.

### *exoS* and *exoU* gene prevalence in CF and non-CF respiratory isolates

Our results showed a trend towards a lower prevalence of the *exoU* gene and a higher prevalence of the *exoS* gene in CF respiratory isolates relative to non-CF isolates. That CF respiratory isolates should differ from non-CF respiratory isolates was particularly unexpected, since both sets of isolates were cultured from the same anatomical location. To further investigate this phenomenon, we analysed 20 additional respiratory isolates from patients with CF and 20 additional respiratory isolates from patients without CF. Whereas our initial set of 95 clinical isolates was collected in Chicago, Illinois, the additional isolates were obtained from patients in San Francisco, California, to ensure that *exoS* and *exoU* gene prevalence data did not vary with geographical location. PCR-DNA fingerprint analysis was performed on all 40 of these respiratory isolates and indicated that 80% of the CF isolates were non-clonal and that 100% of the non-CF respiratory isolates were non-clonal (data not shown). Thus the majority of these isolates are non-clonal and represent a suitable sample for determining the prevalence of the *exoS* and *exoU* genes.

Southern hybridization analysis indicated that 14 (70%) of 20 non-CF isolates and 19 (95%) of 20 CF isolates contained the *exoS* gene whereas 5 (25%) of the 20 non-CF isolates and 1 (5%) of 20 CF isolates harboured the *exoU* gene. These results, which were confirmed using the PCR-based gene detection assay (data not shown), are similar to those obtained using isolates from Chicago. Together, 29 (73%) of 40 non-CF respiratory isolates contained the *exoS* gene compared to 36 (90%) of 40 CF respiratory isolates. This difference was statistically significant ($P < 0{\cdot}05$). [The difference between *exoS* prevalence in CF isolates and blood or wound isolates was also significant ($P < 0{\cdot}05$).] Southern hybridization and PCR-based assays were also used to determine the prevalence of the *exoU* gene in these isolates. Ten (25%) of 40 non-CF respiratory isolates contained the *exoU* gene compared to 3 (8%) of 40 CF respiratory isolates, a statistically significant difference ($P < 0{\cdot}05$). Thus as a population, *P. aeruginosa* strains that infect CF patients differ in their type III secretion genotypes from those that infect the respiratory tract of non-CF patients. The difference in *exoU* prevalence between CF isolates and isolates from each of the other tested disease categories was also statistically significant ($P < 0{\cdot}05$). The decreased prevalence of the *exoU* gene in populations of CF isolates is unlikely to result from deletion of the *exoU* gene over time because most of these isolates also harbour the *exoS* gene. The presence of the *exoS* gene suggests that these isolates had not previously harboured the *exoU* gene, since both these genes are seldom found in the same isolate. It is possible that there is selection against ExoU-producing strains in the CF lung. Alternatively, production of ExoS may provide *P. aeruginosa* isolates with an advantage in colonizing or persisting in the CF lung. Finally, *exoS* may be genetically linked to a second factor that is important in the pathogenesis of *P. aeruginosa* pulmonary colonization and infection in CF patients.

## Conclusions

Taken together, our findings suggest that all *P. aeruginosa* isolates harbour at least some of the genes encoding the type III secretion apparatus. In contrast, three of the four genes encoding known *P. aeruginosa* type III effector proteins are variable traits. The *exoT* gene was detected in all examined isolates, but the *exoY* gene was present in only 89%, the *exoS* gene in 72%, and the *exoU* gene in 28%. Interestingly, the vast majority of isolates contained either the *exoS* or the *exoU* genes but not both. In general, clinical isolates did not differ from environmental isolates in the distribution of type III effector genes. The prevalence of genes encoding effector proteins was independent of the site of infection, with the exception of CF respiratory isolates and perhaps urine isolates. CF respiratory isolates

harboured the *exoU* gene less frequently and the *exoS* gene more frequently than did isolates from patients without CF. Urine isolates showed a trend towards a decreased prevalence of the *exoY* gene.

*P. aeruginosa* isolates from two cities were examined in this study and yielded very similar findings, suggesting that our results are not unique to *P. aeruginosa* populations from specific geographical locations. In addition, we have examined a collection of 35 *P. aeruginosa* isolates from patients in Spain and found similar prevalences: the *popB* and *exoT* genes were present in 100% of isolates, the *exoY* gene in 89%, the *exoS* gene in 66% and the *exoU* gene in 34% (A. R. Hauser, E. Cobb, M. Bodí, D. Mariscal, J. Vallés, J. N. Engel & J. Rello, unpublished results). Additional studies are in progress to determine if these values are representative of *P. aeruginosa* isolates from other geographical locations, anatomical sites and host conditions.

It should be noted that this study examined only genotype, and that isolates harbouring these genes may not be competent for secretion of functional effector proteins. However, assays for *in vivo* secretion are cumbersome, and it remains unclear if *in vitro* secretion accurately predicts the ability to secrete *in vivo*. Therefore, these results represent an upper limit for the percentage of clinical isolates capable of secreting each type III effector protein.

We conclude that type III secretion genes are part of the virulence arsenals of nearly all *P. aeruginosa* clinical isolates. The universal distribution of these genes in clinical isolates and their importance in animal models of infection suggest that they are relevant to human disease. Future studies to determine the percentage of clinical isolates capable of secreting specific effector proteins and whether secretion correlates with disease outcomes will further elucidate the role of type III secretion in *P. aeruginosa* pathogenesis.

## ACKNOWLEDGEMENTS

We thank David P. Speert, Robert V. Miller and William J. Hickey for generously supplying many of the *P. aeruginosa* environmental isolates and G. F. Brooks for facilitating collection of the Moffitt-Long Hospital isolates. We thank the technicians in the Northwestern Memorial Hospital Clinical Microbiology Laboratory for their help in collecting clinical isolates and Hank Seifert for reviewing the manuscript. We thank Barbara Kazmierczak for supplying the protocol for performing PCR directly on bacterial colonies.

This work was supported by Public Health Service grant KO8-AI01524, by a Career Development Award from the Schweppe Foundation, and by an Intramural Research Grant from Northwestern Memorial Hospital.

## REFERENCES

**Allewelt, M., Coleman, F. T., Grout, M., Priebe, G. P. & Pier, G. B. (2000).** Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. *Infect Immun* **68**, 3998–4004.

**Alonso, A., Rojo, F. & Martinez, J. L. (1999).** Environmental and clinical isolates of *Pseudomonas aeruginosa* show pathogenic and biodegradative properties irrespective of their origin. *Environ Microbiol* **1**, 421–430.

**Bonten, M. J. M., Bergmans, C. J. J., Speijer, H. & Stobberingh, E. E. (1999).** Characteristics of polyclonal endemicity of *Pseudomonas aeruginosa* colonization in intensive care units. *Am J Respir Crit Care Med* **160**, 1212–1219.

**Campbell, M., Mahenthiralingam, E. & Speert, D. P. (2000).** Evaluation of random amplified polymorphic DNA typing of *Pseudomonas aeruginosa*. *J Clin Microbiol* **38**, 4614–4615.

**Chen, W.-P. & Kuo, T.-T. (1993).** A simple and rapid method for the preparation of gram-negative bacterial genomic DNA. *Nucleic Acids Res* **21**, 2260.

**Coburn, J. & Frank, D. (1999).** Macrophages and epithelial cells respond differently to the *Pseudomonas aeruginosa* type III secretion system. *Infect Immun* **67**, 3151–3154.

**Cowell, B. A., Chen, D. Y., Frank, D. W., Vallis, A. J. & Fleiszig, S. M. J. (2000).** ExoT of cytotoxic *Pseudomonas aeruginosa* prevents uptake by corneal epithelial cells. *Infect Immun* **68**, 403–406.

**Dacheux, D., Toussaint, B., Richard, M., Brochier, G., Croize, J. & Attree, I. (2000).** *Pseudomonas aeruginosa* cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. *Infect Immun* **68**, 2916–2924.

**Fagon, J. Y., Chastre, J., Domart, Y., Trouillet, J. L., Pierre, J., Carne, C. & Gibert, C. (1989).** Nosocomial pneumonia in patients receiving continuous mechanical ventilation. *Am Rev Respir Dis* **139**, 877–884.

**Fagon, J.-Y., Chastre, J., Hance, A. J., Montravers, P., Novara, A. & Gibert, C. (1993).** Nosocomial pneumonia in ventilated patients: a cohort study evaluating attributable mortality and hospital stay. *Am J Med* **94**, 281–288.

**Finck-Barbancon, V., Goranson, J., Zhu, L., Sawa, T., Wiener-Kronish, J. P., Fleiszig, S. M. J., Wu, C., Mende-Mueller, L. & Frank, D. (1997).** ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Mol Microbiol* **25**, 547–557.

**Fitzsimmons, S. C. (1993).** The changing epidemiology of cystic fibrosis. *J Pediatr* **122**, 1–9.

**Fleiszig, S. M. J., Zaidi, T. S., Preston, M. J., Grout, M., Evans, D. J. & Pier, G. B. (1996).** Relationship between cytotoxicity and corneal epithelial cell invasion by clinical isolates of *Pseudomonas aeruginosa*. *Infect Immun* **64**, 2288–2294.

**Fleiszig, S. M. J., Wiener-Kronish, J. P., Miyazaki, H., Vallas, V., Mostov, K., Kanada, D., Sawa, T., Yen, T. S. B. & Frank, D. (1997).** *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. *Infect Immun* **65**, 579–586.

**Fluit, A. C., Verhoef, J. & Schmitz, F. J. (2000).** Antimicrobial resistance in European isolates of *Pseudomonas aeruginosa*. European SENTRY participants. *Eur J Clin Microbiol Infect Dis* **19**, 370–374.

**Frank, D. (1997).** The exoenzyme S regulon of *Pseudomonas aeruginosa*. *Mol Microbiol* **26**, 621–629.

**Frithz-Lindsten, E., Du, Y., Rosqvist, R. & Forsberg, A. (1997).** Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. *Mol Microbiol* **25**, 1125–1139.

**Frithz-Lindsten, E., Holmstrom, A., Jacobsson, L., Soltani, M., Olsson, J., Rosqvist, R. & Forsberg, A. (1998).** Functional conservation of the effector protein translocators PopB/YopB and PopD/YopD of *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*. *Mol Microbiol* **29**, 1155–1165.

**Garrity-Ryan, L., Kazmierczak, B., Kowal, R., Comolli, J., Hauser, A. & Engel, J. N. (2000).** The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of *Pseudomonas aeruginosa* by epithelial cells and macrophages. *Infect Immun* **68**, 7100–7113.

**Gilardi, G. L. (1991).** *Pseudomonas* and related genera. In *Manual of Clinical Microbiology*, pp. 429–441. Edited by A. Balows, W. J. Hausler, Jr, K. L. Herrmann, H. D. Isenberg & H. J. Shadomy. Washington, DC: American Society for Microbiology.

**Goehring, U.-M., Schmidt, G., Pederson, K. J., Aktories, K. & Barbieri, J. T. (1999).** The N-terminal domain of *Pseudomonas aeruginosa* exoenzyme S is a GTPase-activating protein for Rho GTPases. *J Biol Chem* **274**, 36369–36372.

**Hanberger, H., Garcia-Rodriguez, J.-A., Gobernado, M., Goossens, H., Nilsson, L. E. & Struelens, M. J. (1999).** Antibiotic susceptibility among aerobic gram-negative bacilli in intensive care units in 5 European countries. *JAMA* (*J Am Med Assoc*) **281**, 67–71.

**Hauser, A. R. & Engel, J. N. (1999).** *Pseudomonas aeruginosa* induces type III secretion-mediated apoptosis of macrophages and epithelial cells. *Infect Immun* **67**, 5530–5537.

**Hauser, A. R., Kang, P. J. & Engel, J. (1998).** PepA, a novel secreted protein of *Pseudomonas aeruginosa*, is necessary for cytotoxicity and virulence. *Mol Microbiol* **27**, 807–818.

**Henriksson, M. L., Rosqvist, R., Telepnev, M., Wolf-Watz, H. & Hallberg, B. (2000).** Ras effector pathway activation by epidermal growth factor is inhibited *in vivo* by exoenzyme S ADP-ribosylation of Ras. *Biochem J* **347**, 217–222.

**Hirakata, Y., Finlay, B. B., Simpson, D. A., Kohno, S., Kamihira, S. & Speert, D. P. (2000).** Penetration of clinical isolates of *Pseudomonas aeruginosa* through MDCK epithelial cell monolayers. *J Infect Dis* **181**, 765–769.

**Holder, I. A. (1977).** Epidemiology of *Pseudomonas aeruginosa* in a burns hospital. In *Pseudomonas aeruginosa: Ecological Aspects and Patient Colonization*, pp. 77–95. Edited by V. M. Young. New York: Raven Press.

**Kang, P. J., Hauser, A. R., Apodaca, G., Fleiszig, S., Wiener-Kronish, J., Mostov, K. & Engel, J. N. (1997).** Identification of *Pseudomonas aeruginosa* genes required for epithelial cell injury. *Mol Microbiol* **24**, 1249–1262.

**Krall, R., Schmidt, G., Aktories, K. & Barbieri, J. T. (2000).** *Pseudomonas aeruginosa* ExoT is a Rho GTPase-activating protein. *Infect Immun* **68**, 6066–6068.

**Kropec, A., Huebner, J., Riffel, M., Bayer, U., Benzing, A., Geiger, K. & Daschner, F. D. (1993).** Exogenous or endogenous reservoirs of nosocomial *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections in a surgical intensive care unit. *Intensive Care Med* **19**, 161–165.

**Kurahashi, K., Kajikawa, O., Sawa, T., Ohara, M., Gropper, M. A., Frank, D. W., Martin, T. R. & Wiener-Kronish, J. P. (1999).** Pathogenesis of septic shock in *Pseudomonas aeruginosa* pneumonia. *J Clin Invest* **104**, 743–750.

**Liu, Y., Davin-Regli, A., Bosi, C., Charrel, R. N. & Bollet, C. (1996).** Epidemiological investigation of *Pseudomonas aeruginosa* nosocomial bacteremia isolates by PCR-based DNA fingerprinting analysis. *J Med Microbiol* **45**, 359–365.

**Louws, F. J., Fulbright, D. W., Stephens, C. T. & de Bruijn, F. J. (1994).** Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Appl Environ Microbiol* **60**, 2286–2295.

**Mahenthiralingam, E., Campbell, M. E., Foster, J., Lam, J. S. & Speert, D. P. (1996).** Random amplified polymorphic DNA typing of *Pseudomonas aeruginosa* isolates recovered from patients with cystic fibrosis. *J Clin Microbiol* **34**, 1129–1135.

**Maurelli, A. T., Fernandez, R. E., Bloch, C. A., Rode, C. K. & Fasano, A. (1998).** "Black holes" and bacterial pathogenicity: a large genomic deletion that enhances the virulence of *Shigella* spp. and enteroinvasive *Escherichia coli*. *Proc Natl Acad Sci U S A* **95**, 3943–3948.

**National Nosocomial Infections Surveillance System (1996).** National Nosocomial Infections Surveillance report, data summary from October 1986–April 1996, issued May 1996. *Am J Infect Control* **24**, 380–388.

**Olson, J. C., McGuffie, E. M. & Frank, D. W. (1997).** Effects of differential expression of the 49-kilodalton exoenzyme S by *Pseudomonas aeruginosa* on cultured eukaryotic cells. *Infect Immun* **65**, 248–256.

**Olson, J. C., Fraylick, J. E., McGuffie, E. M., Dolan, K. M., Yahr, T. L., Frank, D. W. & Vincent, T. S. (1999).** Interruption of multiple cellular processes in HT-29 epithelial cells by *Pseudomonas aeruginosa* exoenzyme S. *Infect Immun* **67**, 2847–2854.

**Palleroni, N. J. (1984).** Pseudomonadaceae. In *Bergey's Manual of Systematic Bacteriology*, pp. 141–219. Edited by N. R. Kreig & J. G. Holt. Baltimore: Williams & Wilkins.

**Pedersen, S. S., Jensen, T., Pressler, T., Hoiby, N. & Rosendal, K. (1986).** Does centralized treatment of cystic fibrosis increase the risk of *Pseudomonas aeruginosa* infection? *Acta Paediatr Scand* **75**, 840–845.

**Pederson, K. J., Vallis, A. J., Aktories, K., Frank, D. W. & Barbieri, J. T. (1999).** The amino-terminal domain of *Pseudomonas aeruginosa* ExoS disrupts actin filaments via small-molecular-weight GTP-binding proteins. *Mol Microbiol* **32**, 393–401.

**Pennington, J. E. (1995).** Nosocomial respiratory infections. In *Principles and Practice of Infectious Diseases*, pp. 2599–2607. Edited by G. L. Mandell, J. E. Bennett & R. Dolin. New York: Churchill Livingstone.

**Romling, U., Fiedler, B., Bosshammer, J., Grothues, D., Greipel, J., von der Hardt, H. & Tummler, B. (1994).** Epidemiology of chronic *Pseudomonas aeruginosa* infection in cystic fibrosis. *J Infect Dis* **170**, 1616–1621.

**Ruimy, R., Genauzeau, E., Barnabe, C., Beaulieu, A., Tibayrenc, M., Andremont, A. & The *P. aeruginosa* Study Group (2001).** Genetic diversity of *Pseudomonas aeruginosa* strains isolated from ventilated patients with nosocomial pneumonia, cancer patients with bacteremia, and environmental water. *Infect Immun* **69**, 584–588.

**Rumbaugh, K. P., Hamood, A. N. & Griswold, J. A. (1999).** Analysis of *Pseudomonas aeruginosa* clinical isolates for possible variations within the virulence genes exotoxin A and exoenzyme S. *J Surg Res* **82**, 95–105.

**Salyers, A. A. & Whitt, D. D. (1994).** *Bacterial Pathogenesis: a Molecular Approach*. Washington, DC: American Society for Microbiology.

**Stover, C. K., Pham, X. Q., Erwin, A. L. & 28 other authors (2000).** Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959–964.

**Vallis, A. J., Finck-Barbancon, V., Yahr, T. L. & Frank, D. W. (1999).** Biological effects of *Pseudomonas aeruginosa* type III-secreted proteins on CHO cells. *Infect Immun* **67**, 2040–2044.

**Versalovic, J., Koeuth, T. & Lupske, J. R. (1991).** Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823–6831.

**Vincent, T. S., Fraylick, J. E., McGuffie, E. M. & Olson, J. C. (1999).** ADP-ribosylation of oncogenic Ras proteins by *Pseudomonas aeruginosa* exoenzyme S *in vivo*. *Mol Microbiol* **32**, 1054–1064.

**Vogel, H. J. & Bonner, D. M. (1956).** Acetylornithinase of *Escherichia coli* partial purification and some properties. *J Biol Chem* **218**, 97–106.

**Woods, D. E., Schaffer, M. S., Rabin, H. R., Campbell, G. D. & Sokol, P. A. (1986).** Phenotypic comparison of *Pseudomonas aeruginosa* strains isolated from a variety of clinical sites. *J Clin Microbiol* **24**, 260–264.

**Yahr, T. L., Barbieri, J. T. & Frank, D. W. (1996a).** Genetic relationship between the 53- and 49-kilodalton forms of exoenzyme S from *Pseudomonas aeruginosa*. *J Bacteriol* **178**, 1412–1419.

**Yahr, T. L., Goranson, J. & Frank, D. W. (1996b).** Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III secretion pathway. *Mol Microbiol* **22**, 991–1003.

**Yahr, T. L., Vallis, A. J., Hancock, M. K., Barbieri, J. T. & Frank, D. W. (1998).** ExoY, an adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. *Proc Natl Acad Sci U S A* **95**, 13899–13904.

Received 6 March 2001; revised 15 May 2001; accepted 12 June 2001.