INFECTION AND IMMUNITY, Apr. 2001, p. 2198–2210 Vol. 69, No. 4
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.4.2198–2210.2001

Comparison of the *exoS* Gene and Protein Expression in Soil and Clinical Isolates of *Pseudomonas aeruginosa*

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Received 2 August 2000/Returned for modification 6 October 2000/Accepted 28 December 2000

Exoenzyme S (ExoS) is translocated into eukaryotic cells by the type III secretory process and has been hypothesized to function in conjunction with other virulence factors in the pathogenesis of *Pseudomonas aeruginosa*. To gain further understanding of how ExoS might contribute to *P. aeruginosa* survival and virulence, ExoS expression and the structural gene sequence were determined in *P. aeruginosa* soil isolates and compared with ExoS of clinical isolates. Significantly higher levels of ExoS ADP-ribosyltransferase (ADPRT) activity were detected in culture supernatants of soil isolates compared to those of clinical isolates. The higher levels of ADPRT activity of soil isolates reflected both the increased production of ExoS and the production of ExoS having a higher specific activity. *ExoS* structural gene sequence comparisons found the gene to be highly conserved among soil and clinical isolates, with the greatest number of nonsynonymous substitutions occurring within the region of ExoS encoding GAP function. The lack of amino acid changes in the ADPRT region in association with a higher specific activity implies that other factors produced by *P. aeruginosa* or residues outside the ADPRT region are affecting ExoS ADPRT activity. The data are consistent with ExoS being integral to *P. aeruginosa* survival in the soil and suggest that, in the transition of *P. aeruginosa* from the soil to certain clinical settings, the loss of ExoS expression is favored.

Pseudomonas aeruginosa is an opportunistic pathogen that can produce severe infections or death when the person affected has a compromised immune system or severe tissue damage (2). While *P. aeruginosa* can readily adapt to growth within a human host if allowed access, its primary residence is within the environment, where it can be found in a number of habitats, including soil, water, plant and animal surfaces, and decaying organic matter (3, 31, 53, 55, 63). Although the precise ecological niche of *P. aeruginosa* is unknown, it likely functions, along with other pseudomonads, in decomposition and environmental recycling within the soil (21, 55, 57). Factors that contribute to the virulence of *P. aeruginosa* in the human host, such as adhesins, invasins, and resistance to desiccation, are the same as those that contribute to its adaptability in the environment.

Exoenzyme S (ExoS) has been implicated as a virulence factor of *P. aeruginosa*; however, its precise role in the pathogenicity of this organism remains unknown. ExoS is directly translocated into eukaryotic cells by the contact-dependent type III secretory process (61) and, as such, it provides the bacterium with a mechanism for manipulating the eukaryotic cells it encounters. In support of ExoS contributing to *P. aeruginosa* pathogenicity, bacterial translocation of ExoS into epithelial cells results in a general inactivation of cellular function, as recognized by the inhibition of DNA synthesis, loss of focal adhesion, cell rounding, and microvillus effacement (46). ExoS cellular toxicity has also been found to parallel the opportunistic nature of *P. aeruginosa* infections, with compromised epithelial cell monolayers being more sensitive to the effects of bacterially translocated ExoS than healthy confluent, polarized monolayers (12, 40). While the mechanism of action of ExoS leading to altered cell function is only partially understood, the evidence supports the view that both its amino-terminal GTPase-activating (GAP) activity (19) and carboxy-terminal ADP-ribosyltransferase (ADPRT) activity (30) contribute to these effects (17, 19, 39, 59). Notably, the ADPRT activity of ExoS has a strict requirement for a eukaryotic protein cofactor, 14-3-3 proteins (7), emphasizing a functional link between this activity and eukaryotic cells. The ability of ExoS to inactivate eukaryotic cell function, combined with its preferential toxicity for compromised epithelial cells, is consistent with ExoS providing the bacterium with a means of selectively targeting and interfering with the function of eukaryotic cells it encounters.

While an extensive number of studies have been performed to determine the contribution of ExoS to *P. aeruginosa* virulence in clinical settings, the role of ExoS in the survival of *P. aeruginosa* in the environment remains relatively unexplored. Our studies have focused on examining the role of ExoS in the fitness of *P. aeruginosa* in its natural environmental habitat, with the notion that this might provide insight into the function of ExoS in the *P. aeruginosa* infectious process. To assess the relevance of ExoS production to *P. aeruginosa* survival in the environment, we analyzed *P. aeruginosa* soil isolates for the production of ExoS and then compared both ExoS production and the *exoS* gene sequence of soil and clinical isolates. We detected the *exoS* gene in all soil isolates examined, a finding

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which is consistent with the *exoS* gene being more prevalent in *P. aeruginosa* within the soil than previously reported for clinical isolates (11). While the *exoS* gene sequence of soil and clinical isolates was found to be highly conserved, the ExoS ADPRT activity was significantly higher in culture supernatants of soil isolates compared to clinical isolates. The data support the idea that ExoS production is favored in *P. aeruginosa* soil isolates and, as such, may be more integral to *P. aeruginosa* survival in soil than in certain clinical habitats.

MATERIALS AND METHODS

Soil collection and *P. aeruginosa* isolation. Eight of the soil isolates examined in this study were obtained using a standard soil probe from soil samples taken from random locations throughout Horry and Georgetown Counties in South Carolina. Isolates were obtained by laying out 6-m transects across each location, and five samples that were 15-cm deep were taken at 1-m intervals across the transect. The samples were bulked in zip-lock bags and held on ice until processing. Following collection, the soil samples were processed as outlined by Green et al. (21). Fifty grams of each soil sample was placed in a flask containing 100 ml of sterile distilled water. The contents were shaken for 1 h at 300 rpm in a water bath at room temperature. A 0.5-ml aliquot of the soil solution was added to a capped tube with 4.5 ml of sterile salts plus acetamide (SA) broth (5 g of NaCl, 0.2 g of $MgSO_4$, 1 g of $NH_4H_2PO_4$, 1 g of K_2HPO_4, and 20 g of acetamide [Sigma Chemical Co., St. Louis, Mo.] per 1 liter of distilled H_2O) (22, 54) and incubated without shaking at 42°C for 24 to 48 h. A 0.1-ml aliquot of this culture was spread on plates of King's B plus cetrimide (hexadecyltrimethylammonium bromide; Sigma) (KBC) medium (4, 29) and incubated for 24 to 48 h at 42°C. Bacterial colonies that were fluorescent on KBC plates were streaked on plates of King's A medium (29) to check for pyocyanin production. Isolates that produced both fluorescein and pyocyanin, grew at 42°C, and had an odor characteristic of *P. aeruginosa* were subsequently verified as *P. aeruginosa* based on biochemical characteristics by the Clinical Microbiology Laboratory at the Medical University of South Carolina, Charleston. Two additional *P. aeruginosa* soil isolates, U1 and U3, were isolated from a creosote-contaminated site in Fairhope, Ala., and were provided by Pamela Morris.

Bacterial strains and growth conditions. The following clinical *P. aeruginosa* isolates were used in this study: strain 388, a burn wound isolate (1), and its derivatives 388ΔS and 388ΔT (33, 60) were provided by Dara Frank; PA01, a wound isolate (24); DG1, a cystic fibrosis (CF) lung isolate (5); PA103, a sputum isolate (38); FRD1 (45), its derivative, FRD2 (20), DO62, and DO249 are CF isolates provided by Dennis Ohman; strain ATCC 27853 is a blood isolate obtained from the American Type Culture Collection (ATCC); strain WR5 (49) was provided by Barbara lglewski; and PAK (ATCC 25102) is included among the clinical isolates, although its origin is uncertain. Soil isolates examined in this study include CCU1 to CCU6, CCU8, CCU9, U1, and U3. Stock cultures of all strains were maintained in 10% sterile skim milk at −70°C. For short-term laboratory maintenance, cultures were maintained on Luria-Bertani (LB) agar plates and stored at 4°C. To induce ExoS production in vitro, bacteria were cultured for 18 h at 37°C in a chelated dialysate of Trypticase soy broth supplemented with 10 mM nitrilotriacetic acid (Sigma), 1% glycerol, and 100 mM monosodium glutamate (TSBD-N) medium (27, 34). The optical density at 590 nm (OD_{590}) of cultures was determined at 18 h to compare the growth rates of the bacterial strains.

Measurement of ExoS enzyme activity. ExoS ADPRT activity was assayed and distinguished from that of exotoxin A (ETA) using a defined assay system which measured the incorporation of radiolabeled ADP-ribose into the artificial substrate soybean trypsin inhibitor (SBTI; Sigma) as previously described (34). In these analyses, each 40-μl reaction mixture contained 0.2 M sodium acetate (pH 6.0), 1 μM nicotinamide [U-^{14}C]adenine dinucleotide (252 Ci/mol; Amersham Life Sciences, Arlington Heights, Ill.), 100 μM SBTI, a 40 nM concentration of the 14-3-3ξ, co-factor (Upstate Biotechnology, Inc., Lake Placid, N.Y.), and 10 μl of culture supernatant, diluted as indicated. The reaction mixtures were incubated at 25°C for 30 or 40 min and stopped by the addition of 40 μl of ice-cold 20% trichloroacetic acid (TCA). The mixture was then spotted on 0.45-μm-pore-size HA filters (Millipore, Bedford, Mass.) on a vacuum manifold, washed twice with 5% TCA and once with ethanol, and dried. The incorporation of radiolabel was determined by scintillation counting and quantified as picomoles or femtomoles of ADP-ribose transferred per minute to SBTI per supernatant volume. The ExoS ADPRT activity of each isolate was calculated relative to the slope of the dilution curve within the linear range of the ADPRT assay. To relate ExoS ADPRT activity to total protein secretion, protein concentrations in culture supernatants were determined based on densitometry analysis of 10 μl of culture supernatant resolved on sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) gels and stained with Coomassie blue. This method of analysis both allowed a visualization of culture supernatant proteins and circumvented high background protein levels associated with bacterial medium. The specific activity of ExoS of each isolate was calculated as the ratio of ExoS ADPRT activity to ExoS protein concentrations in culture supernatants, the latter being determined based on immunoblot analyses (described below). Statistical analyses of quantified ExoS activity levels were performed using Sigma Stat Statistical Software version 2.0 and the indicated test.

Immunoblot analysis of ExoS in culture supernatants and cell extracts. Culture supernatants of *P. aeruginosa* growth in ExoS induction media were resolved on SDS–7.5% PAGE gels using the method of Laemmli (36). Immunoblots were performed according to the method of Towbin et al. (58) by transferring the resolved culture supernatants to polyvinylidene difluoride membranes (Millipore) and probing them with antisera, produced using previously described methods (47), against either native ExoS or ExoS reduced and denatured with 5% β-mercaptoethanol and 8 M urea, respectively, gel purified from strain DG1. Immunoblots were developed using a peroxidase-conjugated goat anti-rat immunoglobulin G (IgG; Sigma) and visualized by enhanced chemiluminescence (ECL; Amersham). Quantification of ExoS was performed on immunoblot images within the linear concentration range, and densitometry values were obtained using the NIH image version 1.6 program and related to a previously quantified ExoS standard.

Genomic DNA purification and amplification of the *exoS* structural gene. DNA was purified from overnight Luria broth cultures of *P. aeruginosa* strains using the salting-out procedure (41) of the PureGene DNA isolation kit (Gentra Systems, Inc., Minneapolis, Minn.) according to manufacturer's specifications for purifying DNA from gram-negative bacteria. Two sets of PCR primers, synthesized at the DNA synthesis facility at the Medical University of South Carolina, were used to isolate the *exoS* gene from *P. aeruginosa* genomic DNA. Primer set 1 consisted of the following: (upper) 5′-GTCAGCATATGCATATTCAATCGCT-3′, which included an *Nde*I restriction site (underlined), with the ATG start site of ExoS indicated in boldface, and (lower) 5′-CGAACCGAATTC**TCA**GGCCAGATCA-3′, which included an *Eco*RI site (underlined), with the TGA stop codon of ExoS in boldface. Primer Set 2 consisted of: (upper) 5′-GTCAGCAT**ATG**CATATTCAATCGCTTCAGCAG-3′, which extended beyond the upper primer of set 1, and (lower) 5′-GCATGGATCCGCTGCCGAGCCAAGAATC-3′, which is downstream of the *exoS* structural gene of strain 388 and includes a *Bam*HI site (underlined). PCRs were performed using a GeneAmp PCR System 9700 machine (PE Applied Biosystems, Foster City, Calif.). Each 50-μl reaction mixture contained 29.5 μl of distilled water, 10 μl of a 5× buffer solution containing 1.7 mM $MgCl_2$ (Gibco-BRL, Gaithersburg, Md.), 1 μl of each primer (25 μM), 1 μl of each deoxynucleoside triphosphate (10 μM; Gibco-BRL), 2.5 μl of dimethyl sulfoxide (Sigma), 1 μl of genomic DNA (100 ng/ml), and 1 μl of Elongase Enzyme Mix (Gibco-BRL). The Elongase Mix contains *Taq* polymerase and the proofreading *Pyrococcus* sp. strain GB-D polymerase and was used to reduce the possibility of sequence errors in the PCR amplification of ExoS. The polymerase Elongase was added after 5 min at 98°C as a "hot start." The PCR temperature cycles differed for the two primer sets. The cycle for primer set 1 was as follows: 98°C (5 min); 80°C (2 min); 20 cycles of 94°C (30 s), 60°C (30 s, ramped down 0.5°C lower each cycle, to an endpoint of 50°C), and 68°C (90 s); and then 20 cycles of 94°C (30 s), 50°C (30 s), and 68°C (90 s). The cycle for primer set 2 was as follows: 98°(5 min); 80°(2 min); 16 cycles of 94°C (30 s), 68°C (30 s ramped downed down 0.5°C lower every cycle, to an endpoint of 60°C), and 68°C (90 s); and then 24 cycles of 94°C (30 s), 60°C (30 s), and 68°C (90 s).

Preparation of *exoS* PCR products for sequencing. *exoS* PCR products were purified by excising the appropriate sized band from 1% agarose gels, following electrophoresis using TAE buffer (40 mM Tris-acetate, 1 mM EDTA; pH 8.0) for approximately 30 min at 100 V. The DNA was extracted from the agarose gel slices using a GeneClean kit, a silica-based DNA purification procedure (Bio 101, Vista, Calif.), according to the manufacturer's specifications. The *exoS* PCR products were sequenced directly without cloning at the DNA sequencing facility at the Medical University of South Carolina using an ABI 377 automated DNA sequencer.

Sequence comparison. Computer manipulations of sequence data were performed using the BLAST programs available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) and, for multiple sequence alignments, CLUSTALW 1.8, available at Baylor College of Medicine's Search Launcher Web Site (http://searchlauncher.bcm.tmc.edu/searchlauncher). Nucleotide sequences were aligned using the PileUp program of the Wisconsin Package of sequence analysis programs (GCG version 10; Madison, Wis.). The

mean number of synonymous nucleotide substitutions per potential synonymous site (d_S), the mean number of nonsynonymous substitutions per potential nonsynonymous site (d_N), and their variances were estimated from all pairwise comparisons of the sequences being analyzed by using established methods (42, 43). These nucleotide distances (proportion of nucleotide differences) were used to determine the form of natural selection operating at the protein level, where $d_S > d_N$ indicates purifying selection (selection against amino acid changes) and $d_N > d_S$ indicates positive selection (selection for amino acid changes) (25). The null hypothesis of no difference between d_S and d_N was tested using a two-tailed Z test (44). Evolutionary trees were constructed using the neighbor-joining method (52). When nucleotide distances between sequences corrected for multiple substitutions at a site (28) were ≤0.05, as for *exoS* sequences, the neighbor-joining method was applied to the distances. When nucleotide distances were much greater, such that d_S was greater than 0.5, indicating the saturation of synonymous sites, as in the case of distances between the ExoT sequence and the ExoS sequences, the neighbor-joining method was applied to amino acid distances (proportion of amino acid differences) between sequences (44). The level of confidence in internal branches of a tree (those separating clusters of sequences) was determined by running 1,000 bootstrap pseudoreplicates (44). Branch lengths of the ExoS tree in terms of the number of synonymous nucleotide substitutions per potential synonymous site (b_S) and the number of nonsynonymous substitutions per potential nonsynonymous site (b_N) were estimated from proportional synonymous (p_S) and nonsynonymous (p_N) distances between pairs of sequences (42) using Rzhetsky and Nei's (51) method of computing branch lengths (64).

RESULTS

Soil survey. A survey for the presence of *P. aeruginosa* in soils was conducted as part of an undergraduate research project (23). Approximately 16% of soils surveyed were positive for *P. aeruginosa*. These soils were predominantly sandy and acidic in nature, but individual soils were variable with respect to both percent sand fraction and pH. Nine isolates from this survey were obtained for further study and labeled CCU1 to CCU9. Culture CCU7 was lost early in the process and does not appear in these studies. The phenotypic characteristics of these soil isolates when grown under identical conditions were consistent with each isolate being different. Two other *P. aeruginosa* strains, U1 and U3, isolated from a different geographical region, were introduced into the study at a later time to evaluate whether the findings on ExoS production extended to a more distant geographical location.

Analyses of ExoS production by environmental isolates. Preliminary assays of soil isolates, CCU1 to CCU9, grown under ExoS induction conditions, found all but one strain, CCU1, to produce ExoS ADPRT activity in culture supernatants (Fig. 1). Further examination of the supernatants by immunoblot analyses using antibodies produced either against the native or the reduced-denatured form of ExoS detected variation in the amount of ExoS produced by the individual soil isolates (Fig. 2A). Variation was also detected in the levels of ExoS of the clinical strains, which were produced and analyzed in parallel for comparison. The antiserum produced against the native form of ExoS cross-reacted with the highly homologous protein ExoT (60), while that produced against the reduced-denatured form of ExoS favored specific reactivity with ExoS. The differential intensities of the ExoS banding patterns of the two antisera are consistent with their recognition of different epitopes on ExoS. Immunoblot analyses of cell extracts of the soil isolates found relative levels of ExoS to closely correspond to those observed in culture supernatants (Fig. 2B). This indicates that differences in ExoS production by these isolates occurred at the level of gene expression rather than secretion.

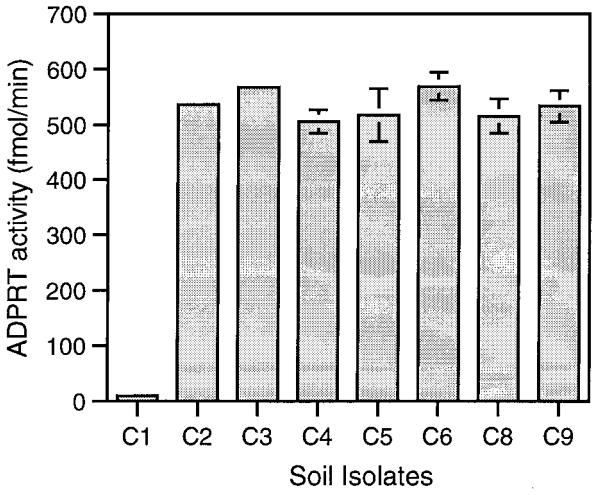


FIG. 1. ExoS ADPRT activity in culture supernatants of soil isolates. The eight CCU soil isolates, indicated by the respective designations C1 to C6, C8, and C9, were cultured under ExoS induction conditions for 18 h, and 10 μl of culture supernatant was assayed for ExoS ADPRT activity. The results are expressed as the femtomoles of ADP-ribose transferred per minute. The mean and standard error (SE) of assays performed in duplicate from cultures grown and analyzed in parallel in three independent studies are represented.

Quantification of ExoS ADPRT activity in culture supernatants of soil and clinical isolates. To further compare the properties of ExoS produced among soil and clinical isolates, ExoS ADPRT activity was quantified in culture supernatants of the strains grown, processed, and assayed for ADPRT enzymatic activity in parallel, under identical conditions (Fig. 3A and B). As shown in Fig. 3A, differences were observed in the levels of ExoS ADPRT activity among the soil isolates when

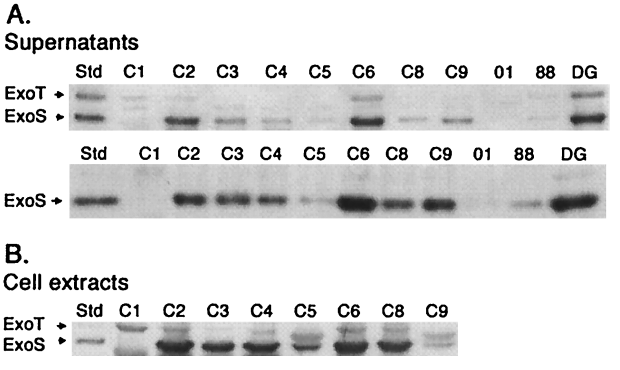


FIG. 2. Immunoblot analyses of ExoS produced by soil and clinical isolates. (A) Equal volumes (4 μl) of culture supernatants from soil and clinical isolates grown in parallel under ExoS induction conditions were resolved by SDS-PAGE on 7.5% polyacrylamide gels and immunoblotted with antisera produced against the native (upper blot) or the reduced-denatured form of ExoS (lower blot). (B) Cell extracts (4 μl of a twofold concentrate) were resolved as described above and immunoblotted with antisera produced against the native form of ExoS. ExoS proteins were detected using peroxidase-conjugated goat anti-rat IgG and ECL. The blots are representative of one of three independent studies performed. Std represents an ExoS or ExoT standard, previously quantified to have 20 ng of ExoS. Supernatants from CCU isolates are labeled C1 to C6, C8, and C9, and the clinical isolates are PAO1 (lane 01), 388 (lane 88), and DG1 (DG).

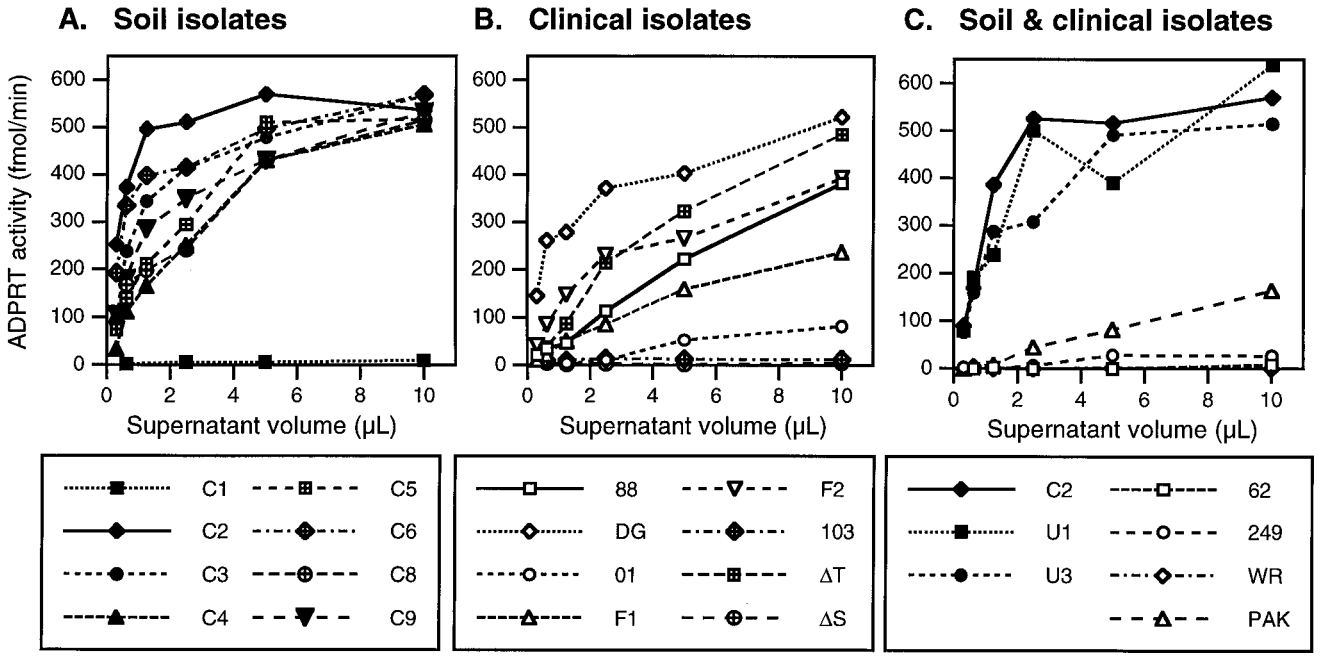


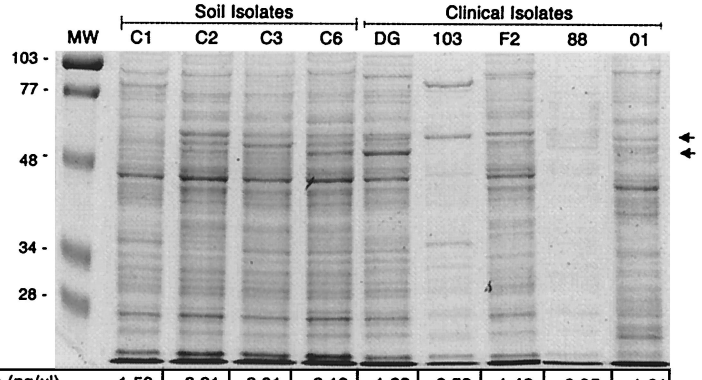
FIG. 3. Quantification of ExoS ADPRT activity in culture supernatants of soil and clinical isolates. Serial dilutions of culture supernatants from the indicated soil isolates (A) and clinical isolates (B) were assayed for ADPRT activity, and the mean values of analyses performed in parallel in two independent studies are represented. (C) ExoS ADPRT activities of soil isolates from a different geographical site and of additional clinical isolates were compared in a second independent study in cultures grown and assayed for ADPRT activity in parallel. Strain CCU2 was included in these studies as an internal culture control, and ADPRT results were normalized to those of panels A and B using an internal ADPRT assay control. The results are expressed as femtomoles of ADP-ribose transferred per minute, and values obtained in the linear range of the assay were used to quantify ExoS ADPRT of the individual isolates. Soil isolates are strains CCU1 to CCU6, CCU8, and CCU9 (C1 to C6, C8, and C9), U1, and U3. Clinical isolates are strains 388 (curve 88), DG1 (DG), PAO1 (O1), FRD1 (F1), FRD2 (F2), PA103 (103) 388ΔT (ΔT), 388ΔS (ΔS), DO62 (curve 62), DO249 (curve 249), WR5 (WR), and PAK.

serial dilutions of culture supernatants were assayed for activity. The highest levels of ExoS ADPRT activity were detected in culture supernatants of strains CCU2 and CCU6, with the lowest levels of activity produced by strains CCU4, CCU8, and CCU1. ExoS ADPRT activity of each isolate was quantified relative to the slope of the dilution curve within the linear range of the assay (see Fig. 4 and 5). The results from these studies are consistent with immunoblot analyses in supporting that differences exist in the amount of ExoS being produced by soil isolates.

More notable in comparisons of Fig. 3A and B were the general lower levels of ExoS production detected in culture supernatants of clinical isolates than in soil isolates. Strain DG1 showed the highest level of ExoS ADPRT activity among these clinical isolates, most closely approaching that of the soil isolates. Lower levels of ExoS ADPRT activity were detected in supernatants of strain 388, the prototype ExoS-producing strain and its 388 ΔT (non-ExoT-producing) derivative strain (60), the FRD1 and FRD2 related strains, and still lower levels were detected in strain PA01 supernatants, the latter previously recognized as a low-ExoS-producing strain (14). Baseline levels of ExoS ADPRT activity (~2 fmol min^{-1} ml^{-1}) were detected in culture supernatants of strain PA103, which produces high levels of the ADP-ribosylating toxin ETA but lacks the *exoS* structural gene (11), confirming the specificity of the ExoS ADPRT assay for ExoS. Similarly, baseline levels of ExoS ADPRT activity (~0.4 fmol min^{-1} ml^{-1}) were detected in supernatants of strain 388ΔS, a derivative of strain 388 that lacks the *exoS* structural gene (33).

To examine whether the differences detected in ExoS production by soil isolates and clinical isolates would be evident in soil isolates from a more distant geographical region and in other clinical isolates, a second study was performed in which two additional soil isolates, U1 and U3, and the clinical isolates WR5, DO62, DO249, and PAK were cultured and assayed in parallel for ExoS production (Fig. 3C). Soil isolate CCU2 was cultured and assayed in these studies as an internal culture control, and ADPRT results were normalized to those of previous studies using an internal ADPRT assay control. Again, these analyses found the soil isolates to produce higher levels of ExoS ADPRT than the clinical isolates, with strains WR5 and DO62 producing baseline levels of ExoS ADPRT activity. Statistical analyses of ExoS ADPRT activity in culture supernatants of the 10 soil and 9 clinical isolates shown in Fig. 3 found the soil isolates to produce significantly higher levels of ADPRT activity than the clinical isolates ($P = 0.002$), based on Student *t* test analysis, with the mean production levels of ExoS ADPRT activity of soil and clinical isolates being 209.1 ± 36.9 and 48.9 ± 24.8 fmol min^{-1} μl^{-1}, respectively.

Comparison of ExoS production with other bacterial culture characteristics. To evaluate how bacterial growth rates and levels of protein secretion contributed to differences in ExoS production by soil and clinical isolates, bacterial culture densities and culture supernatant protein concentrations were



Protein (ng/μl)	1.59	2.21	2.01	2.13	1.62	0.52	1.46	0.35	1.61
Culture OD	4.66	6.82	7.12	4.10	4.12	9.01	3.66	8.98	3.55
ExoS (ng/μl)	0.56	18.65	13.25	25.17	31.97	0.30	16.40	4.97	1.32
ADPRT (fmol/min μl)	1.7	687.5	315.0	566.0	403.5	1.9	123.0	87.1	9.4
ADPRT/Protein (fmol/min ng)	1.07	311.1	156.7	265.7	249.1	3.65	84.2	248.6	5.83

FIG. 4. Comparison of ExoS production with bacterial culture characteristics. Culture supernatant protein concentrations and bacterial growth rates were compared with ExoS protein and ExoS ADPRT activity of representative high- and low-ExoS-producing soil and clinical isolates. Secreted proteins in culture supernatants were compared and quantified based on an analysis of 10 μl of culture resolved by SDS–10% PAGE gels and stained with Coomassie blue. Bacterial growth rates were related to the OD_{590} of cultures after growth in TSBD-N medium for 18 h. ExoS protein concentrations were determined based on densitometry analysis of ExoS immunoblots of culture supernatants, performed as described in the legend to Fig. 2, and probed with antisera against both the native and reduced-denatured forms of ExoS. ExoS ADPRT activity was quantified as described in the legend to Fig. 3. The relative ratio of ExoS ADPRT activity to the concentration of total secreted protein for each isolate is indicated. Strains are labeled as in Fig. 3, and the approximate mobilities of ExoT and ExoS are indicated by arrows.

compared with the ExoS ADPRT activity of the isolates. Representative comparisons of these parameters in high- and low-ExoS-producing soil and clinical isolates are shown in Fig. 4. Calculations of ExoS ADPRT activity relative to protein levels in culture supernatants were found to parallel the rates of ExoS production in general, with the exception of strain 388. Only low levels of protein were secreted by strain 388, making the ratio of ExoS ADPRT activity to total supernatant protein high. No correlations were detected between bacterial growth rates or levels of secreted proteins and the production of ExoS in these analyses ($r = 0.386$ to 0.690) based on linear regression analyses. The data support the idea that ExoS production is regulated independently of bacterial growth rate or levels of protein secretion when bacteria are grown in ExoS induction medium.

To assess whether functional differences might exist in ExoS secreted by the different strains, the relative specific activity of ExoS in culture supernatants of soil and clinical isolates was calculated. The specific activity of ExoS was quantified as the ratio of ExoS ADPRT activity relative to the concentration of ExoS immunoreactive protein, using densitometry analyses of immunoblots probed with antisera to both the native and denatured forms of ExoS. Both antisera were used in ExoS quantification to help alleviate bias in detection due to antibody specificity. As shown in Fig. 5, the calculated specific activity of ExoS produced by the soil isolates was, in general, higher than that of clinical isolates, indicating that ExoS produced by soil isolates was functionally more active. The exceptions to this were CCU1, which lacked detectable ExoS production, and ExoS produced by CCU4, which had a lower specific activity, one closer to that of the clinical isolates.

Amplification of the *exoS* structural gene from soil isolates. Differences observed in the immunoreactivity and specific activity of ExoS produced among soil and clinical isolates suggested that variation might exist in the *exoS* structural gene sequence of *P. aeruginosa* within these two habitats. The *exoS* genes of two *P. aeruginosa* clinical isolates, strains 388 and PAO1, have previously been determined and were found to differ by only one amino acid residue, the former having a valine and the latter having a methionine at residue 62 (15, 35).

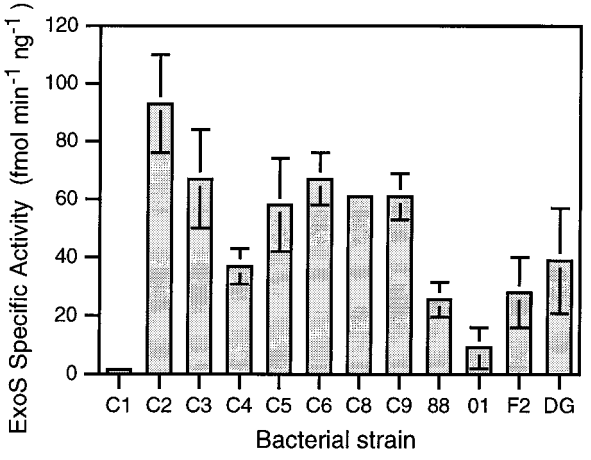


FIG. 5. Specific ADPRT activity of ExoS produced by soil and clinical isolates. The specific activity of ExoS in culture supernatants of soil and clinical isolates was calculated based on the ratio of quantified levels of ExoS ADPRT activity to ExoS protein concentrations, determined by immunoblot analyses, and related to an ExoS standard. The specific activity is expressed as femtomoles of ADP-ribose transferred per minute per nanogram of ExoS protein in the culture supernatants. The results represent the mean and SE of two independent studies. Soil and clinical isolates are labeled as in Fig. 3.

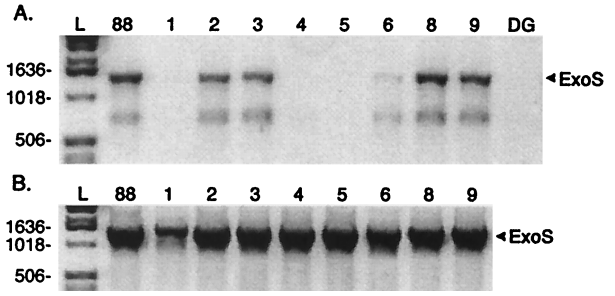


FIG. 6. Amplification of *exoS* structural genes from *P. aeruginosa* soil and clinical isolates. The *exoS* gene was amplified from the genomic DNA of the indicated bacterial strains using primer set 1 (A) or primer set 2 (B) (see Materials and Methods). Products were resolved on 1% agarose gels. "L" indicates the DNA ladder, the CCU soil isolates are numbered 1 to 6, 8, and 9, as indicated, and clinical strains 388 (lane 88) and DG1 (DG) are labeled.

A valine residue was, however, detected at position 62 in the *exoS* gene in the PAO1 genome sequence (56) (GenBank accession number AE004801). Since the degree of conservation of the *exoS* structural gene sequence between clinical and environmental *P. aeruginosa* isolates has not been previously examined, the complete nucleotide sequence of *exoS* from soil isolates and additional clinical isolates was determined from the respective PCR products.

Initial PCRs using primer set 1 allowed amplification of the appropriate sized product from some but not all of the CCU soil isolates (Fig. 6A). Primer set 2 amplified ExoS from all isolates (Fig. 6B). The two primer sets differed in that primer set 1 included shorter regions of hybridization, which defined the beginning and end of the *exoS* gene and resulted in the amplification of a second product. PCR products generated from primer set 2 were used to determine the *exoS* gene sequences of soil and clinical isolates examined this study.

Comparison of nucleotide sequences of soil and clinical isolates. The nucleotide sequences of the *exoS* genes determined from PCR products were compared, along with the *exoS* gene sequence of strain PAO1 (15) (GenBank accession number X99471) to that of the prototype *exoS* gene of strain 388 (35) (GenBank accession number L27629). Analyses of the *exoS* genes of soil isolates found that CCU1, which did not produce a detectable ExoS product based on ADPRT activity or immunoblot analyses, maintained an *exoS* structural gene identical to that of strain 388, as did clinical isolate ATCC 27853. The *exoS* genes of CCU6 and CCU9 were also found to be identical to each other. The *exoS* genes of CCU2, CCU6, CCU9, CCU8, and DG1 had only synonymous (silent) nucleotide substitutions when compared to *exoS* of strain 388 (Table 1), while those of CCU3, CCU4, CCU5, U1, U3, FRD1, and PAK had both synonymous and nonsynonymous substitutions (Table 1). The nonsynonymous nucleotide substitutions resulted in one to six amino acid changes in ExoS, depending on the isolate (Table 1). Nucleotide changes were further classified into transitions (purine-to-purine or pyrimidine-to-pyrimidine shifts) and transversions (purine-to-pyrimidine or pyrimidine-to-purine shifts) (42, 43), with transitions being typically more common than transversions. Table 2 summarizes the ExoS sequence data of all strains, with respect to strain 388 ExoS. Sixty-one percent of nucleotide substitutions were synonymous transitions in the third position, while 24% were synonymous transversions in the third position. Fifteen percent of the substitutions were nonsynonymous, with transitions occurring at frequencies of 4 and 2% in the first and second positions, respectively, and transversions occurring at frequencies of 3, 4, and 2% in the first, second, and third positions, respectively. Of interest in the comparison of the ExoS sequences is the clustering of synonymous substitutions in the region corresponding to residues 52 to 83 and the clustering of nonsynonymous substitutions in the region corresponding to residues 157 to 191. Notable in the sequence comparisons among soil and clinical isolates was the lack of nonsynonymous substitutions in the region of ExoS predicted to form the ADPRT active site cleft, thereby providing no direct explanation for the increased specific activity detected in ExoS produced by soil isolates. What was noticed instead was the clustering of amino acid substitutions in the region involved in GAP function.

The evolution of the *exoS* genes of clinical and environmental isolates examined in this study was analyzed and is displayed as a tree showing three major clades (Fig. 7). Statistical comparisons (two tailed) of the number of synonymous substitutions per synonymous site (d_S) and the number of nonsynonymous substitutions per nonsynonymous site (d_N) were performed to determine whether the *exoS* gene was under positive selection, favoring amino acid replacement ($d_N > d_S$), or under purifying selection, favoring amino acid stability ($d_S > d_N$). ExoS was analyzed in this manner relative to (i) the entire *exoS* gene, encoding amino acid residues 1 to 453; (ii) the N-terminal domain, residues 1 to 234; (iii) the C-terminal domain, residues 235 to 453; (iv) the type III signal sequence within residues 1 to 9 (61); (v) the aggregation region, residues 1 to 99, predicted to interact with the chaperone, Orf1 (13, 30); (vi) the GAP homology region, defined by residues 107 to 191; and (vii) the ADPRT active site cleft, defined by residues 316 to 403. When all ExoS sequences were compared, d_S was significantly greater than d_N for the entire *exoS* gene ($P < 0.001$), its N-terminal and C-terminal domains ($P < 0.01$), and within the N-terminal domain, the aggregation region ($P < 0.05$), indicating a strong purifying selection for ExoS generally and these regions specifically (Table 3). Similar results were obtained when ExoS sequences of the environmental and clinical isolates were compared (not shown).

When the most divergent *exoS* genes, those of CCU4 and PAK, were compared with all the other *exoS* genes, d_S was again significantly greater than d_N for the entire *exoS* gene ($P < 0.01$), a finding indicative of purifying selection (Table 3). However, within the GAP region of ExoS of CCU4 and PAK, which includes the nonsynonymous substitutions at residues 157, 159, and 162 in ExoS of CCU4, d_N was greater than d_S, albeit not statistically significantly so ($P > 0.05$). To further test the hypothesis of positive selection of the GAP domain in the evolution of CCU4 and PAK, the method of Zhang et al. (64) was used to estimate the rates of synonymous (b_S) and nonsynonymous (b_N) substitutions along the branches of the ExoS tree leading to CCU4 and PAK. This analysis showed a significant positive selection of GAP ($P < 0.05$) along the branch leading to CCU4 ($b_N = 0.1616 \pm 0.00910$; $b_S = 0.0 \pm 0.0$) using a one-tailed Z test with infinite degrees of freedom. The

TABLE 1. Synonymous and nonsynonymous substitutions in the *exoS* gene of *P. aeruginosa* environmental and clinical isolates compared to strain 388[a]

Substitution type and amino acid position	Nucleotide base position	Codon from 388[b]	Amino acid	Sequence of isolate no.:												
				CCU2	CCU3	CCU4	CCU5	CCU6	CCU8	CCU9	U1	U3	DG1	PAO1	FRD1	PAK
Synonymous																
52	156	GGG	Gly		GGT											
52	156	GGG	Gly												GGA	
58	174	GGC	Gly	GGT			GGT				GGT	GGT	GGT	GGT		
60	180	GCG	Ala	GCA	GCA		GCA				GCA	GCA	GCA	GCA		
64	192	CCG	Pro	CCC	CCC	CCC	CCC	CCC		CCC	CCC	CCC	CCC	CCC	CCC	CCC
74	222	AAA	Lys	AAG	AAG		AAG				AAG	AAG	AAG	AAG	AAG	
83	249	GGC	Gly			GGG		GGG		GGG						GGG
130	391	CTG	Leu								TTG					
160	480	CAG	Gln						CAA				CAA			
212	636	CAG	Gln			CAA										CAA
221	663	CGC	Arg		CGT											
288	864	CAG	Gln				CAA									
289	867	GAG	Glu				GAA									
303	909	GCG	Ala				GCC									
310	930	CAG	Gln				CAA									
329	987	GCA	Ala	GCC	GCC		GCC	GCC		GCC				GCC	GCC	
329	987	GCA	Ala			GCG					GCG	GCG				GCG
387	1161	GAA	Glu	GAG			GAG	GAG		GAG				GAG		
406	1218	GTT	Val	GTC			GTC	GTC		GTC				GTC		
436	1308	GGC	Gly			GGT					GGT	GGT	GGT			GGT
448	1344	CGC	Arg		CGT	CGT	CGT				CGT	CGT	CGT		CGT	CGT
450	1350	CTT	Leu		CTC	CTC	CTC				CTC	CTC	CTC		CTC	CTC
Nonsynonymous																
46	138	GAC	Asp			GAG/Glu										GAG/Glu
62	184	GTG	Val											ATG/Met		
121	362	AGC	Ser												AAC/Asn	
157	469	GCC	Ala			TCC/Ser										
159	476	AGC	Ser			AAC/Asn										
162	484	GAG	Glu			AAG/Lys										
170	505	CGC	Arg		TGC/Cys											
191	571	GCG	Ala								TCG/Ser	TCG/Ser				
266	796	GTG	Val				ATG/Met									
415	1244	CAG	Gln			CTG/Leu										CTG/Leu
434	1301	CGT	Arg			CCT/Pro										CCT/Pro

[a] Data from the previously published sequences of strains 388 (35) and PAO1 (15) are incorporated into this table.
[b] The sequences of CCU1 and ATCC 27853 are identical to that of strain 388.

TABLE 2. Type of nucleotide substitution for *exoS* genes of *P. aeruginosa* environmental and clinical isolates compared to *exoS* gene of strain 388[a]

Amino acid location	Codon from 388	Amino acid	Mutated codon	Resulting amino acid	Type of mutation		
					Type of nucleotide substitution	Syn or Nonsyn[b]	Codon position
46	GAC	Asp	GAG	Glu	Transversion	Nonsyn	3rd
52	GGG	Gly	GGT		Transversion	Syn	3rd
52	GGG	Gly	GGA		Transition	Syn	3rd
58	GGC	Gly	GGT		Transition	Syn	3rd
60	GCG	Ala	GCA		Transition	Syn	3rd
62	GTG	Val	ATG	Met	Transition	Nonsyn	1st
64	CCG	Pro	CCC		Transversion	Syn	3rd
74	AAA	Lys	AAG		Transition	Syn	3rd
83	GGC	Gly	GGG		Transversion	Syn	3rd
121	AGC	Ser	AAC	Asn	Transition	Nonsyn	2nd
130	CTG	Leu	TTG		Transition	Syn	1st
157	GCC	Ala	TCC	Ser	Transversion	Nonsyn	1st
159	AGC	Ser	AAC	Asn	Transition	Nonsyn	2nd
160	CAG	Gln	CAA		Transition	Syn	3rd
162	GAG	Glu	AAG	Lys	Transition	Nonsyn	1st
170	CGC	Arg	TGC	Cys	Transition	Nonsyn	1st
191	GCG	Ala	TCG	Ser	Transversion	Nonsyn	1st
212	CAG	Gln	CAA		Transition	Syn	3rd
221	CGC	Arg	CGT		Transition	Syn	3rd
266	GTG	Val	ATG	Met	Transition	Nonsyn	1st
288	CAG	Gln	CAA		Transition	Syn	3rd
289	GAG	Glu	GAA		Transition	Syn	3rd
303	GCG	Ala	GCC		Transversion	Syn	3rd
310	CAG	Gln	CAA		Transition	Syn	3rd
329	GCA	Ala	GCC		Transversion	Syn	3rd
329	GCA	Ala	GCG		Transition	Syn	3rd
387	GAA	Glu	GAG		Transition	Syn	3rd
406	GTT	Val	GTC		Transition	Syn	3rd
415	CAG	Gln	CTG	Leu	Transversion	Nonsyn	2nd
434	CGT	Arg	CCT	Pro	Transversion	Nonsyn	2nd
436	GGC	Gly	GGT		Transition	Syn	3rd
448	CGC	Arg	CGT		Transition	Syn	3rd
450	CTT	Leu	CTC		Transition	Syn	3rd

[a] Data from the previously published sequences of strains 388 (35) and PAO1 (15) are incorporated into this table.
[b] Nonsyn and Syn, nonsynonymous and synonymous, respectively.

same analyses detected no positive selection of GAP along any other branch, and there was no positive selection of either the aggregation domain or the ADPRT domain along any branch in the ExoS tree. While positive selection indicates that a protein sequence is still under selection pressure to change, it does not indicate the direction of change (i.e., indicating gain or loss of a particular function). The data support the notion that while the ADPRT domain is genetically stable, changes in the GAP region are being favored under certain conditions. This in turn suggests that the evolution of the ADPRT domain preceded the GAP region and that the GAP domain provides an additional adaptive advantage to *P. aeruginosa*.

A third analysis was performed comparing the ExoT amino acid sequence of strain 388 (60) (GenBank accession number L46800), which is highly homologous to ExoS, with all ExoS sequences, to gain further understanding of the evolutionary relationship between these two proteins. The comparison revealed strong purifying selection ($d_S > d_N$) for ExoT and functional regions within ExoT (Table 3). A tree constructed based on the ExoT protein sequence and all ExoS protein sequences shows the distant divergence of ExoT from ExoS, relative to the variation observed in ExoS sequences (Fig. 8). The tree also shows strong support (83% of the bootstrap

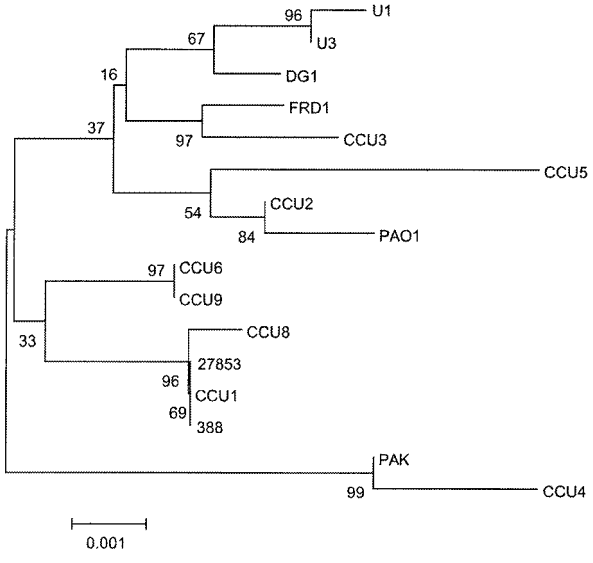


FIG. 7. Evolutionary relationship of *exoS* nucleotide sequences. The *exoS* tree was constructed using the neighbor-joining method (52) on Jukes-Cantor corrected nucleotide distances (28). The numbers on the tree are the percentages of 1,000 bootstrap pseudo-replicates supporting internal branches.

TABLE 3. Mean d_S and d_N per 100 sites as estimated from comparisons between ExoS sequences, between CCU4 plus PAK and other ExoS sequences, and between ExoT and ExoS sequences

Comparison	Domain	Amino acids	Mean $d_S \pm$ SE	Mean $d_N \pm$ SE	P^a
Between ExoS sequences	All	1–453	2.1 ± 0.4	0.2 ± 0.1	<0.001
	Type III signal	1–9	0.0 ± 0.0	0.0 ± 0.0	NS
	Aggregation	1–99	3.9 ± 1.6	0.2 ± 0.1	<0.05
	GAP homology	107–191	0.5 ± 0.4	0.5 ± 0.2	NS
	ADPRT homology	316–403	1.8 ± 1.1	0.0 ± 0.0	NS
Between CCU4 plus PAK and other ExoS sequences	All	1–453	2.4 ± 0.6	0.5 ± 0.2	<0.01
	Type III signal	1–9	0.0 ± 0.0	0.0 ± 0.0	NS
	Aggregation	1–99	4.1 ± 1.8	0.5 ± 0.5	NS
	GAP homology	107–191	0.3 ± 0.3	1.0 ± 0.5	NS
	ADPRT homology	316–403	1.9 ± 1.3	0.0 ± 0.0	NS
Between ExoT and ExoS sequences[b]	All	1–457	50.9 ± 5.0	16.2 ± 1.4	<0.001
	Type III signal	1–9	71.7 ± 64.8	14.6 ± 8.6	NS
	Aggregation	1–102	56.1 ± 11.5	16.9 ± 3.0	<0.01
	GAP homology	110–194	46.4 ± 10.4	14.4 ± 3.0	<0.01
	ADPRT homology	319–407	38.4 ± 9.4	14.4 ± 2.9	<0.05

[a] NS, not significant.
[b] Amino acid boundaries reflect the ExoT sequence.

samples) for the cluster of all ExoS proteins, excluding CCU4 and PAK, suggesting an early divergence of the latter among ExoS proteins. The purifying selection of ExoT and ExoS is further depicted in Fig. 8 (inset), which shows the distribution of nonsynonymous substitutions throughout ExoT and ExoS relative to strain 388 ExoS. The data support that during the evolutionary divergence of ExoS and ExoT, both proteins, and their respective functional regions, have undergone purifying selection. This does not exclude the possibility of positive selection associated with, or occurring shortly after, the divergence of these proteins.

DISCUSSION

The ubiquitous distribution of *P. aeruginosa* in nature and clinical settings indicates a close link between the environmental organism and the opportunistic pathogen. ExoS is one of multiple factors believed to contribute to the virulence of *P. aeruginosa* as an opportunistic pathogen. While it has been previously recognized that both environmental and clinical isolates produce ExoS (26), the genetic and functional relationship of ExoS produced in these two habitats has not been explored. The purpose of our studies was to examine ExoS produced by soil isolates and, through comparisons with clinical isolates, to gain an understanding of the conservation of the ExoS structural gene, as well as insight into the role of ExoS in *P. aeruginosa* virulence and survival.

In a cursory survey of the soils in Horry and Georgetown counties in South Carolina, *P. aeruginosa* was isolated from 16% of the samples, indicating that it is relatively common in soils in this region. Examination of ExoS production by these isolates found seven of eight strains to produce high levels of ExoS ADPRT activity. Variations were, however, detected in levels of ExoS cross-reactive protein among the isolates when culture supernatants were examined by immunoblot analyses. These variations were later confirmed in ADPRT activity assays when serial dilutions of culture supernatants were assayed for enzymatic activity and concentrations of ExoS ADPRT activity were determined within the linear range of the dilution curve. Of interest in comparisons of ExoS production of *P. aeruginosa* soil and clinical isolates were the overall higher levels of ExoS ADPRT activity produced by soil isolates. This difference was apparent in our initial comparison of eight soil and five clinical isolates and then in a second study comparing two soil isolates from a more distant geographical location and four additional clinical isolates. Statistical analysis of ExoS ADPRT activity of 10 soil and 9 clinical isolates found production by soil isolates to be significantly greater than that of clinical isolates ($P = 0.002$). When ExoS production was related to bacterial growth rates or levels of protein secretion, no significant correlations were detected, supporting the idea that ExoS production was regulated independently of these culture characteristics.

To gain further understanding of the structure-function relationship of ExoS produced by the different *P. aeruginosa* strains, the specific activity of ExoS in culture supernatants (ADPRT activity per nanogram of ExoS protein) was calculated for individual isolates. Considerable variation was detected in ExoS specific activity among the isolates, with soil isolates in general producing ExoS with a higher ADPRT activity than clinical isolates. When the *exoS* genes of soil and clinical strains were sequenced to gain further understanding of the molecular mechanism for the higher activity, no obvious explanation based on amino acid substitutions was evident. This points to the possibility that other factors produced by *P. aeruginosa* and/or amino acid substitutions outside the ADPRT domain may be modulating ExoS ADPRT activity.

There is precedence for the notion that ExoS function or ADPRT activity may be enhanced by either bacterial or eukaryotic factors. A chaperone, Orf1, coordinately regulated with ExoS production, facilitates the transport of ExoS from the prokaryotic cell and hence increases secreted ExoS activity (15, 62). Within the eukaryotic cell, the modulating effect of the 14-3-3 protein cofactor is required for maximal ADPRT activity (7, 16). The potential therefore remains for these

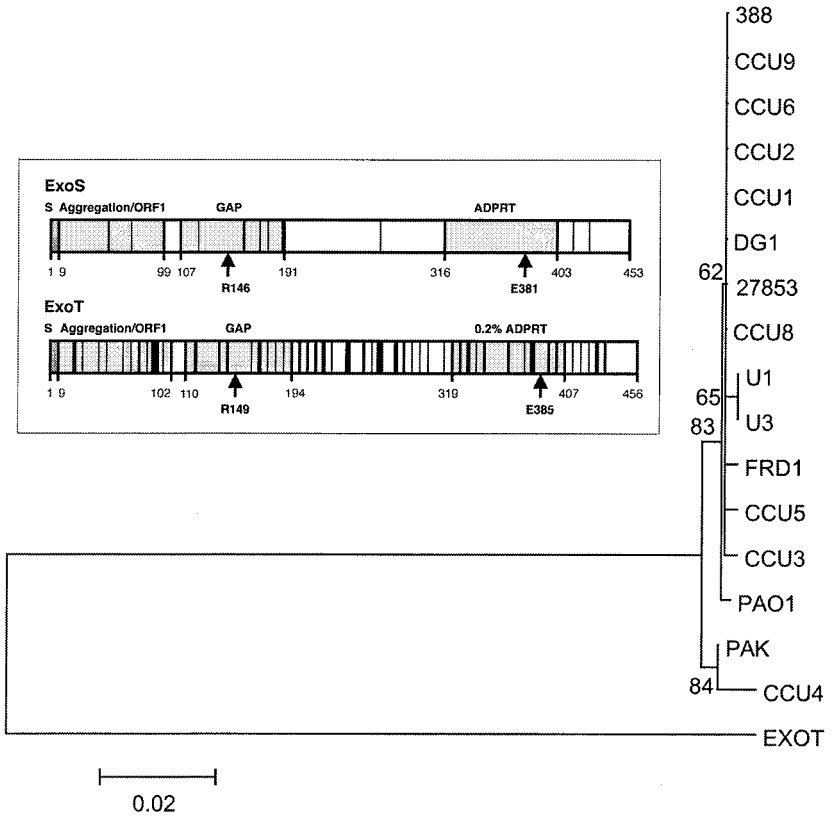


FIG. 8. Evolutionary relationship of ExoS and ExoT protein sequences. The ExoS-ExoT tree was constructed using all ExoS protein sequences and the strain 388 ExoT sequence (60) as described in the legend to Fig. 7, except that the neighbor-joining method was applied to amino acid distances between sequences. (Inset) Nonsynonymous substitutions within ExoT and ExoS. The positions of nonsynonymous substitutions within ExoS and ExoT, relative to ExoS of strain 388, are indicated by vertical lines within the linear map of the respective proteins, with the width of each line reflecting the number of substituted amino acids. Predicted functional regions within ExoS and ExoT are labeled and shaded. Numbers that define the amino acid boundaries of the type III signal sequence (S) and aggregation domain have been previously reported (61). The GAP and ADPRT regions are defined based on homology with other GAP or ADPRT proteins. The positions of residues integral to GAP function (R146 or R149 in ExoS and ExoT, respectively) (19, 32) and ADPRT activity in ExoS (E381) (37) and the homologous residue in ExoT (E385) are marked by arrows.

and/or other unidentified factors to act as inhibitors or potentiators of the ExoS ADPRT activity and contribute to the differences observed in activity in environmental and clinical isolates. In this regard, we found that ExoS produced by soil isolates, like clinical isolates, requires the 14-3-3 cofactor for ADPRT activity, indicating that if an enhancing factor is produced by soil isolates, it cannot replace the activity of the 14-3-3 cofactor. Another factor that may influence ExoS activity in soil isolates is the highly homologous and coordinately regulated protein ExoT. ExoT was detected in all of the soil isolates examined in this study, based either on immunoblot analyses or on PCR amplification of the ExoT structural gene (unpublished observation). ExoS exists in a high-molecular-weight complex with ExoT in culture supernatants (34) and, while there has been speculation as to the potential competitive or coordinated function of these two proteins, the specific effect of ExoT on ExoS function remains unknown.

Notably, relative to the possibility that residues outside the ADPRT domain might be able to modulate ADPRT activity is the clustering of amino acid substitutions in the GAP region of ExoS, sometimes in association with a decrease in ADPRT activity. While relatively few amino acid substitutions were detected in ExoS produced by soil and clinical isolates, 7 of the 15 substitutions resulting in amino acid changes occurred within the GAP homology region of ExoS. CCU4, which had a lower level of ExoS ADPRT activity than the other soil isolates, had the greatest number of amino acid substitutions, three of which were within the GAP region (Ala-157 to Ser, Ser-159 to Asn, and Glu-162 to Lys). The ExoS of FRD1, CCU3, U1, and U3 also had amino acid substitutions within the GAP region (Ser-121 to Asn, Arg-170 to Cys, and Ala-191 to Ser, respectively). While it remains to be proven whether the GAP region of ExoS is contributing to the differences in ADPRT activity detected among the isolates, the increased amino acid variation within this region identifies it as a candidate site for modulation of ExoS function.

Analysis of the sequence variability of the *exoS* genes of 10 soil and 5 clinical *P. aeruginosa* isolates, a group which includes the two previously sequenced *exoS* genes of strains 388 and PAO1, provided insight into the evolutionary relationship of ExoS produced in these two settings. The inter-relatedness of the ExoS proteins of the soil and clinical isolates is evident in the interspersing of the *exoS* genes among the clades in the tree shown in Fig. 7. While foci of silent or synonymous substitu-

tions were detected in soil isolates relative to strain 388, nucleotide substitutions at these sites in many instances were identical to those found in the other clinical isolates. The positioning of isolates producing either low or high levels of ExoS ADPRT activity on the same branch further highlights the lack of relationship between the *exoS* gene sequence and the levels of ExoS production. The data as a whole are consistent with the ExoS of clinical isolates representing a fairly accurate cross section of that produced in environmental settings. Comparisons of the variability of the ExoS protein sequences to the ExoT sequence also provided insight into the relatively distant divergence of these two proteins. The evidence of strong purifying selection for both ExoS and ExoT in these analyses, and their respective functional domains, supports the idea that both proteins maintain a stable, independent function within *P. aeruginosa*.

The increased rate of ExoS production and the prevalence of the *exoS* structural gene in soil isolates imply the importance of ExoS to survival of *P. aeruginosa* in the soil. These results are consistent with early studies of ExoS that found a high percentage of environmental isolates (91%) to produce ExoS (26). Conversely, the decreased rate of ExoS production by clinical isolates, as well as the reported absence of the *exoS* structural gene from cytotoxic corneal isolates (11) or the lack of ExoS secretion from CF isolates (9), indicates the possible lesser importance of ExoS in certain clinical settlings. The production of ExoS by clinical isolates, however, appears somewhat related to the site of infection, as is evident from the general higher levels of ExoS production in *P. aeruginosa* isolated from wound and urinary tract infections (50). When ExoS expression is examined relative to that of other type III effector proteins, a preferred pattern of expression emerges among *P. aeruginosa* strains. For example, the paired expression of ExoU-ExoT or ExoS-ExoT has been noted in *P. aeruginosa* clinical isolates in the absence of the paired expression of ExoU-ExoS or the expression of ExoS, ExoT, and ExoU (10). Although ExoU production was not examined in our studies, ExoS and ExoT expression or genes were detected in all soil isolates, favoring the paired expression of ExoS and ExoT in the soil. No conclusion can be drawn from our studies, however, as to the relative efficiency of ExoS and ExoT production by soil isolates, due to the method of detection of ExoT and the bias of the antisera used in immunoblot analyses for ExoS. All data obtained from these analyses, though, are consistent with ExoS production being favored and upregulated in association with *P. aeruginosa* growth in the soil.

Based on the high expression of ExoS in the soil and assuming that the soil is the point of origin for *P. aeruginosa*, it can be hypothesized that the evolution of ExoS occurred as a result of a selection pressure within this environment. The requirement for a eukaryotic cofactor for ExoS ADPRT activity presumes that ExoS coevolved with eukaryotic organisms. *P. aeruginosa* likely encounters a myriad of eukaryotic species in the soil, including plant roots, free-living nematodes, protozoa, and fungi. Possible relationships that might introduce selection pressures in this environment are parasitic or commensal relationships on plant roots, prey-predator relationships with protozoa and nematodes, and competition for substrates with fungi. As *P. aeruginosa* adapts to certain clinical settings, the selection pressure for ExoS production appears to be lessened, as indicated by decreased levels of ExoS production or the lack of the structural gene, rather than alterations in the gene sequence. While we can currently only speculate as to the different advantages ExoS might offer *P. aeruginosa* in its interaction with eukaryotes in the soil versus clinical environments, the cellular sensitivities to ExoS notably mimic the opportunistic lifestyle of *P. aeruginosa*. For example, relative to epithelial monolayers, confluent, polarized monolayers are resistant to the effects of ExoS-producing *P. aeruginosa*, whereas compromised or subconfluent monolayers are sensitive (12, 40). In addition, when ExoS is translocated into sensitive epithelial or fibroblastic cell lines, a general inactivation of cell function is recognized, rather than an immediate cytotoxic effect (40, 48). Similarly, the type III-mediated translocation of ExoS into macrophages has been reported to have little effect on viability (6) but can inhibit bacterial uptake (15). Consistent with the role of ExoS as an anti-invasive factor, both ExoS and ExoT have recently been found to interfere with the uptake of *P. aeruginosa* by epithelial cells and macrophages in an ADPRT-independent manner (8, 18). In contrast to the above cell types, the promyeloblastic HL-60 cell line appears to be resistant to the effects of ExoS (E. A. Rucks, T. S. Vincent, J. C. Olson, Abstr. 100th Gen. Meet. Am. Soc. Microbiol. 2000, abstr. B-329, p. 116, 2000). Based on these findings, ExoS may provide an advantage to *P. aeruginosa* survival in the soil through its ADPRT-dependent inactivation of eukaryotes it encounters and its ADPRT-independent interference with bacterial uptake. Alternatively in clinical settings, while the inactivation of host cell function and antiphagocytic properties of ExoS might be predicted to aid in the infectious process, the limited toxicity of ExoS, combined with its inefficient targeting of cells of lymphoid origin, may favor the production of more cytotoxic factors, such as ExoU and ETA, at certain sites of *P. aeruginosa* infection. Where ExoS is likely to come into play in the infectious process, however, is when *P. aeruginosa* encounters damaged epithelial tissue.

We conclude from these studies that ExoS is integral to the survival of *P. aeruginosa* in the soil but is less so in some clinical settings. In relating this finding to an understanding of the origin and evolution of ExoS as a virulence factor, our data suggest that ExoS existed prior to the evolutionary transition from the soil organism to the human pathogen. This, in turn, supports the view that the lower frequency of expression of ExoS in certain clinical settings relates to the loss of the gene or gene expression rather than to its limited acquisition.

ACKNOWLEDGMENTS

We thank Dara Frank and Joseph Barbieri for their advice and helpful discussions during these studies and Dennis Ohman, Dara Frank, Barbara Iglewski, and Pamela Morris for providing us with *P. aeruginosa* strains. We also appreciate the contribution of Kathy Dolan, Jennifer O'Brien, Eileen McGuffie, and Zachery Olson to these studies.

This work was supported by Public Health Service grants AI41180 (M.W.F.) and AI41694 (J.C.O.) from the National Institute of Allergy and Infectious Diseases.

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Editor: J. D. Clements