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Effects of Differential Expression of the 49-Kilodalton Exoenzyme S by *Pseudomonas aeruginosa* on Cultured Eukaryotic Cells

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Production of the ADP-ribosylating enzyme exoenzyme S (ExoS) by *Pseudomonas aeruginosa* has been associated with increased virulence. Previous studies, however, have been unable to confirm an effect of soluble ExoS in cell culture or animal model systems. To determine if bacteria must come in contact with target cells in order for an effect of ExoS to be observed, coculture systems were developed to compare the effects of ExoS- and non-ExoS-producing bacteria on eukaryotic cell function. The two *P. aeruginosa* strains used in these studies, 388 and 388Δ*exoS*, maintained genetic identity, with the exception that strain 388Δ*exoS* lacked production of the 49-kDa form of ExoS. When bacteria were cocultured with Detroit 532 fibroblastic cells, ExoS-producing 388 bacteria caused a significant decrease in DNA synthesis and viability compared to the decrease caused by non-ExoS-producing 388Δ*exoS* bacteria. Maximal differences between the two strains were observed when 10^4 to 10^7 CFU of bacteria/ml were cocultured with Detroit cells for 4 or 6 h. Both strains were effective in eliminating Detroit cell DNA synthesis after a 20-h coculture period. Secreted ExoS had no effect on Detroit cell growth and viability, indicating that bacteria must have contact with target cells for the effect of ExoS on cellular function to be observed. Similar effects on cell proliferation and viability were observed when the two strains were cocultured with the KB epithelioid cell line. ExoS-associated decreases in eukaryotic cell viability were not found to be mediated by an inhibition of protein synthesis. These studies confirm that the 49-kDa ExoS contributes to the cellular pathogenesis of *P. aeruginosa* by interfering with eukaryotic cell growth and viability. In addition, the coculture system developed which recognizes this effect should provide a means for defining the function of ExoS in vivo.

The opportunistic pathogen *Pseudomonas aeruginosa* produces multiple cell-associated and extracellular proteins which contribute to its virulence. Two secreted enzymes, exotoxin A (ETA) and exoenzyme S (ExoS), catalyze the transfer of ADP-ribose from NAD to eukaryotic protein substrates (15, 16). While the substrate specificity and cytotoxic effects of ETA have been defined (15), the action of ExoS is less well understood.

ExoS was originally defined and characterized for *P. aeruginosa* 388 (14). The protein is isolated from culture supernatants as a high-molecular-weight aggregate which resolves on sodium dodecyl sulfate (SDS)-polyacrylamide gels as two proteins of 49 and 53 kDa (19, 24). These proteins are immunologically cross-reactive (19, 24), have a common amino-terminal sequence (16), and yield common peptides subsequent to cleavage with trypsin, chymotrypsin, and cyanogen bromide (14). Despite their high degree of similarity, recent studies confirmed that the 49- and 53-kDa forms of ExoS are encoded by separate genes in *P. aeruginosa* 388 (21). Primary amino acid sequence comparisons of the 49-kDa ExoS and the 53-kDa ExoS, now referred to as ExoT, found the two proteins to be 75% identical (39), with ExoS having higher in vitro enzymatic activity than ExoT (19, 24, 38). Functional analysis of the 49-kDa cloned gene product localized the aggregation properties of ExoS to the first 99 amino acids, while the ADP-ribosyltransferase activity localized to the carboxyl-terminal 222 amino acids (17).

ExoS production has been related to increased *P. aeruginosa* virulence in burn wounds and chronic lung infections (3, 24, 26, 34) and to increased cell injury (2, 18). By in vitro assays, the active 49-kDa form of ExoS was found to modify multiple, functionally diverse proteins in mammalian cell lysates. A eukaryotic protein cofactor termed FAS (factor activating ExoS) is required for ExoS activity in vitro (9). This protein is a member of the 14-3-3 protein family (12), which is a large group of proteins that appear to function as scaffolds in diverse cellular processes (36). Despite considerable in vitro studies, the substrate specificity of ExoS in vivo has not been identified, nor are the effects of ExoS on cell function known. The soluble protein purified from *P. aeruginosa* 388 culture supernatants has not been found to be toxic in cell culture or animal model systems (6, 25). Consistent with this lack of effect, it has been difficult to confirm that soluble ExoS binds to and becomes internalized in eukaryotic cells in a manner similar to that of other bacterial toxins.

Recent studies suggest that difficulties in identifying the function of soluble ExoS in vivo might relate to its mechanism of secretion from the bacterial cell. Elucidation of the amino acid sequence of ExoS showed that it lacked a characteristic signal sequence and that its amino-terminal sequence was not cleaved during secretion (20). Further support for the lack of use of the general secretory pathway by ExoS came with the recognition of sequence homology between proteins involved in ExoS production and those involved in the production of the Yop virulence determinants by *Yersinia* spp. Homology was identified between the *P. aeruginosa trans*-regulatory locus,

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which regulates production and secretion of ExoS, and proteins involved in the regulation and secretion of Yops (1, 11, 37, 39). Homology was also observed between the amino terminus of ExoS and the *Yersinia* protein YopE (37). Translocation of YopE into eukaryotic cells appears to require direct contact between the bacterium and the target cell, which then initiates the polarized transfer of YopE at the site of contact (30). The similarities between YopE and ExoS have led to the speculation that direct contact between the *Pseudomonas* bacterium and the target cell is required for the efficient translocation of ExoS into eukaryotic cells.

In this paper we describe the development of a coculture system which allows bacteria to interact with eukaryotic cells and allows us to examine an effect of ExoS on cell function. Using this system, we report that production of the 49-kDa ExoS by *P. aeruginosa* 388 is associated with a significant decrease in the rate of eukaryotic cell DNA synthesis and viability.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Parental *P. aeruginosa* 388 (3, 15) and construction of the 388Δ*exoS* mutant (21) have been previously described. The 388Δ*exoS* mutant is genetically identical to the parent 388 strain with the exception that it lacks the production of the 49-kDa ExoS due to genetic replacement of the majority of the 49-kDa structural gene with a selectable tetracycline gene cartridge (21). *P. aeruginosa* PA103, a high-level ETA(−), low-level-protease-producing strain (23), was provided by Barbara Iglewski. All bacterial strains were stored as bacterial stocks at −70°C in 10% sterile skim milk solutions. For studies examining the effects of ExoS, bacterial stocks were inoculated into a chelated dialysate of Trypticase soy broth supplemented with 10 mM nitrilotriacetic acid (Sigma Chemical Co., St. Louis, Mo.), 1% glycerol, and 100 mM monosodium glutamate (TSBD-N medium) (15). Cultures were grown in a shaking water bath at 37°C for the times indicated in the figures. To compare the growth rates and ExoS production by strains 388 and 388Δ*exoS*, 2-ml primary cultures of both bacteria were grown overnight. A 1:300 dilution of these cultures was used to inoculate secondary cultures, and culture aliquots were removed at defined times throughout a 24-h growth period. The optical density at 590 nm (OD_{590}) of each aliquot was determined prior to centrifugation at 8,500 × *g* for 10 min. Culture supernatants were then aliquoted and stored at −70°C for future protein and enzymatic assays. In order to quantify the number of bacteria being added to eukaryotic cell cultures, serial dilutions of bacterial cultures at different ODs were plated and used to calculate a CFU per OD_{590} factor.

To examine the effect of ExoS production by bacteria on eukaryotic cell function, bacteria were grown in TSBD-N medium, as described above, with a 1:1,000 dilution to inoculate secondary cultures, which were harvested after 18 h. The culture OD_{590} was determined, and then 0.5 ml of culture was removed and centrifuged at 8,500 × *g* for 10 min; the pellet was washed once with RPMI 1640 containing L-glutamine and 25 mM HEPES (Gibco BRL, Gaithersburg, Md.) and supplemented with 0.6% (wt/vol) bovine serum albumin (RPMI-BSA). Following centrifugation, bacteria were diluted in RPMI-BSA to the number of CFU indicated in the figures based on the calculated factor of 1.5×10^9 CFU/OD_{590}. The actual number of CFU added to eukaryotic cultures was subsequently confirmed to be of the calculated magnitude by plating diluted bacteria.

Eukaryotic cell culture. The Detroit 532 fibroblastic cell line (ATCC CLL 54) and KB epidermoid cell line (ATCC CLL 17) were kindly provided by Mark Willingham in the Department of Pathology and Laboratory Medicine at the Medical University of South Carolina. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco BRL), 100 U of penicillin/ml, and 100 μg of streptomycin/ml (RPMI-FBS-PS) at 37°C in 5% CO_2–95% air. Cells were routinely maintained in T-75 culture flasks, split 1:60 (Detroit cells) and 1:30 (KB cells), and passaged every 5 days.

Analysis of effect of bacterial production of ExoS on eukaryotic cell function. (i) Culture system development. Detroit cells were seeded at a concentration of 10^5 cells per well (500-μl volume) in 48-well tissue culture plates (Costar, Cambridge, Mass.) and allowed to grow for 48 h to obtain 80 to 90% monolayer confluency. Culture supernatants were then removed and replaced with 500 μl of RPMI-BSA containing 0 to 10^7 CFU of 388 or 388Δ*exoS* bacteria/ml. Bacteria were cocultured with Detroit cells for 0.5 to 20 h, as indicated in the figures, and then either Detroit cell morphology was examined by phase-contrast microscopy or Detroit cell functional assays were performed as described below. All comparative studies were performed on cells cultured in the same 48-well plate and used bacteria which were grown simultaneously under identical culturing conditions. KB cell culture studies were performed by procedures identical to those used for the Detroit cell line.

(ii) Quantification of DNA synthesis. Following incubation with bacteria, culture supernatants containing bacteria were removed and replaced with 200 μl of pulsing medium (RPMI-FBS-PS) containing 1 μCi of [*methyl*-^{3}H]thymidine (20 Ci/mmole; New England Nuclear Research Products, Wilmington, Del.) per ml. After 20 h, the pulsing medium was removed and 500 μl of ice-cold 5% trichloracetic acid (TCA) was added to each well. Plates were then placed at 4°C for 15 min, and precipitated cell material was washed once with 500 μl of ice-cold 5% TCA and then once with 500 μl of ice-cold phosphate-buffered saline. Precipitated material was dissolved in 250 μl of 0.3 N NaOH containing 1% SDS. Following solubilization, 200 μl of sample was added to scintillation fluid (ScintiVerse; Fisher, Pittsburgh, Pa.) acidified with 52 μl of 0.1 N HCl and then the samples were counted in a scintillation counter.

(iii) Quantification of protein synthesis. The assay used to quantify protein synthesis in cell cultures was developed from procedures described by Carroll and Collier (5) and standardized with ETA and diphtheria toxin. In these studies, culture supernatants containing bacteria were removed following the coculturing periods indicated in the figures and replaced with leucine-deficient RPMI pulsing medium (Gibco BRL) supplemented with 10% FBS, 100 U of penicillin/ml, and 100 μg of streptomycin/ml and containing 1 to 5 μCi of L-[4,5-[^{3}H]leucine (130 Ci/mmole; Amersham Life Sciences, Arlington Heights, Ill.) per ml. Following a 2-h pulse, the amount of [^{3}H]leucine incorporated into TCA-precipitable material was determined as described for the DNA synthesis assay mentioned above.

(iv) Analysis of cell viability. To quantify eukaryotic cell viability following exposure to bacteria, culture supernatants were removed and 100 μl of 0.25% trypsin–1 mM EDTA (trypsin-EDTA; Gibco BRL) was added to each well. Tissue culture plates were incubated at 37°C for 10 min. The cells were then removed from wells, added to 300 μl of RPMI-FBS-PS, and gently mixed to inactivate the trypsin. A 100-μl volume of the cell mixture was added to 100 μl of 0.4% trypan blue, and the number of viable cells was counted with a hemacytometer.

Measurement of ExoS enzymatic activity. ExoS ADP-ribosyltransferase activity was measured as the incorporation of radiolabelled ADP-ribose into the substrate, soybean trypsin inhibitor (SBTI; Sigma), as previously described (19). Briefly, 40-μl reaction mixtures contained 0.2 M sodium acetate (pH 6.0), 1 μM nicotinamide [U-^{14}C]adenine dinucleotide (250 mCi/mM; Amersham), 100 μM SBTI, 40 nM FAS (Upstate Biotechnology, Inc., Lake Placid, N.Y.), and a 10-μl volume of culture supernatant. Reaction mixtures were incubated at 25°C for 30 min and then stopped by the addition of 40 μl of ice-cold 20% TCA. Mixtures were spotted onto 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. Radiolabel incorporation was quantified by scintillation counting and reported as picomoles of ADP-ribose transferred to SBTI per milligram of supernatant protein. Protein concentrations in supernatant fractions were determined with bicinchoninic acid protein assay reagent with BSA as a standard (Pierce, Rockford, Ill.).

Analysis of exoproduct production by strains 388 and 388Δ*exoS*. Exoproduct production by strains 388 and 388Δ*exoS* bacteria was compared by SDS-polyacrylamide gel electrophoresis and immunoblot analyses. As different *P. aeruginosa* exoproducts require different culture conditions for expression, product expression was examined under growth conditions that enhanced production of each product. Specifically, ExoS and ETA production were examined in a cation-deficient medium, TSBD (27), with 10 mM nitrilotriacetic acid added for ExoS production (TSBD-N) (32). Elastase and alkaline protease production were examined in TSBD-N medium and a cation-containing 2YT medium (1% yeast extract, 1.6% Bacto Tryptone, 1% sodium chloride) (Difco Laboratories, Detroit, Mich.) (29). Exoproduct production by the two strains in the two tissue culture media used in this study, RPMI-FBS-PS and RPMI-BSA, was also compared. All cultures were grown in a shaking water bath at 37°C, with product analysis performed on secondary cultures harvested at 18 h.

For analysis of protein content, unconcentrated or 10× concentrated culture supernatants were separated by the method of Laemmli (22) on a 4.5% stacking gel and a 10 to 20% gradient resolving gel (56:1, acrylamide to *N′N*-methylenebis acrylamide; Bio-Rad, Hercules, Calif.). To detect specific proteins in culture supernatants, immunoblot analyses were performed according to the method of Towbin et al. (33). Polyclonal antisera against purified ExoS, ETA, elastase, and alkaline protease used in immunoblots were produced as previously described (28, 29). Secondary antisera, either peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) or peroxidase-conjugated affinity-isolated goat anti-rat immunoglobulin G (Sigma), were used at a 1:2,000 dilution.

Statistical analysis. Statistical analysis was performed with SigmaStat (Jandel Corporation, San Rafael, Calif.).

RESULTS

Comparison of growth and exoproduct production by strains 388 and 388Δ*exoS*. Recent studies suggest that difficulties in identifying an effect of ExoS on eukaryotic cell function might relate to the requirement for contact between bacteria and target cells for efficient internalization of ExoS. To examine this, cell culture systems which allow ExoS- or non-ExoS-producing bacteria to interact with eukaryotic cells were developed and their effect on eukaryotic cell function was

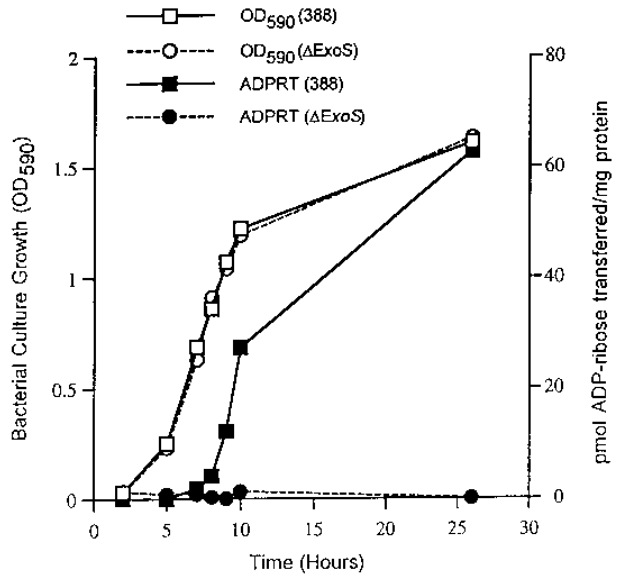

FIG. 1. Comparison of growth and ExoS production of *P. aeruginosa* 388 and 388Δ*exoS* during growth in ExoS induction medium. Growth rates are plotted as culture OD_{590} versus time. Levels of ExoS enzymatic activity are plotted as picomoles of ADP-ribose transferred (ADPRT) to soybean trypsin inhibitor per milligram of protein in culture supernatants versus culture time. Medium background has been subtracted from the ADP-ribosyltransferase activity reported.

assessed. The two bacterial strains used in these studies were *P. aeruginosa* 388, which produces high levels of enzymatically active ExoS, and its genetically derived mutant, strain 388Δ*exoS*, which lacks production of the 49-kDa form of ExoS.

As these studies required the addition of growing bacteria to cultured eukaryotic cells, it was essential to confirm that loss of the ExoS structural gene had no secondary effects on bacterial growth rate or exoproduct expression during in vitro culture conditions. The growth rates of the two strains were found to be identical in all media tested, including RPMI-BSA used in bacterial-eukaryotic coculture studies. Neither strain was able to grow when inoculated directly into the RPMI-FBS-PS tissue culture medium used during the radiolabelling pulsing periods. The calculated CFU/OD_{590} factor of 1.5×10^9, used in determining the number of bacteria being added to eukaryotic cell culture systems, was also found to be the same for both strains. Figure 1 compares the growth rates and the production of enzymatically active ExoS by strains 388 and 388Δ*exoS* when grown in the ExoS induction medium TSBD-N. Enzymatically active ExoS was detected in culture supernatants during the mid-log phase of growth of strain 388, with its concentration increasing throughout the culture period. ExoS ADP-ribosyltransferase activity in culture supernatants of the 388Δ*exoS* mutant strain remained low during the culture period, but slight activity above the medium background was observed.

When the protein contents of 10×-concentrated culture supernatants of 388 and 388Δ*exoS* were compared following 18 h of growth in TSBD-N medium, the only apparent difference in secreted products was the production of 49-kDa ExoS by strain 388 (Fig. 2A). When bacteria were grown in cation-containing 2YT medium, a different electrophoretic pattern of exoproduct expression was observed but no differences were apparent between the two strains, nor were the 49- and 53-kDa ExoS bands apparent. When specific exoproduct expression was examined by immunoblot analysis (Fig. 2B), the 53-kDa form of ExoS was detected in culture supernatants of both 388 and 388Δ*exoS* bacteria grown in TSBD-N but the 49-kDa form was detected only in strain 388 culture supernatants. ExoS was not detected in culture supernatants grown for 18 h of either bacterial strain when grown in 2YT medium or RPMI-BSA medium (data not shown). When the ETA contents of 10× culture supernatants were examined, neither strain produced detectable levels of ETA by immunoblot analysis. In comparison, high levels of ETA were detected in strain PA103 culture supernatants, which were obtained and processed at the same time and included as a positive control in these experiments. As Zn^{2+} and Ca^{2+} are known to facilitate expression of *P. aeruginosa* elastase (4, 29), comparisons of protease production by the two strains were made following growth in both the cation-containing 2YT medium and the cation-deficient TSBD-N medium. Elastase and alkaline protease were detected at comparable levels in supernatants of both strains when grown in 2YT medium and, conversely, were not detected in culture supernatants of either strain when grown in TSBD-N medium.

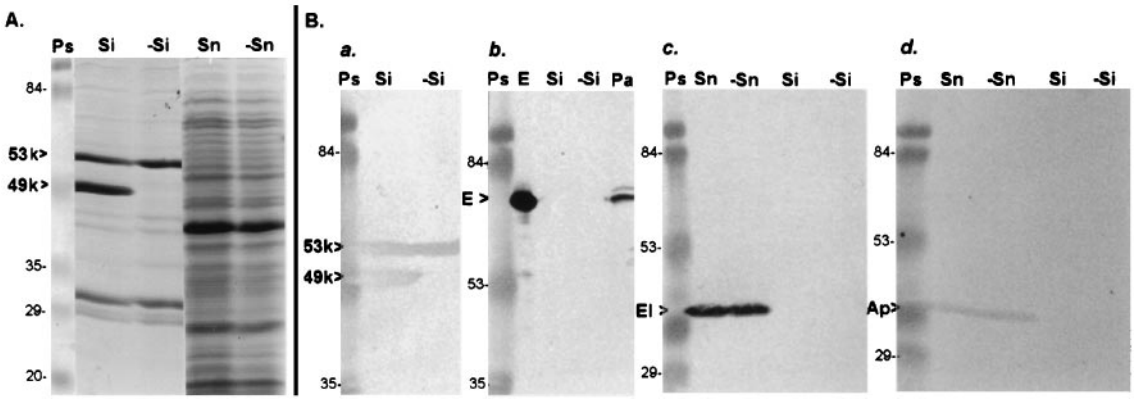

FIG. 2. Comparison of exoproduct production by strains 388 and 388Δ*exoS* following 18 h of culture. (A) Coomassie blue-stained SDS-polyacrylamide gels of 10× culture supernatants of 388 (S) and 388Δ*exoS* (−S) strains grown in ExoS induction medium (i) or in 2YT noninduction medium (n). (B) Immunoblot analysis of specific *P. aeruginosa* exoproducts in 388 (S) or 388Δ*exoS* (−S) culture supernatants. Gel *a*, ExoS antiserum reactivity with 10× supernatants produced in TSBD-N medium; gel *b*, ETA antiserum reactivity with 10× supernatants produced in TSBD medium; E, 0.5 μg of purified ETA; Pa, 10× culture supernatant of strain PA103, a hyper-ETA-producing strain; gel *c*, *Pseudomonas* elastase (El) antiserum reactivity with 1× supernatants of bacteria grown in 2YT medium (n) or TSBD-N medium (i); gel *d*, *Pseudomonas* alkaline protease (Ap) antiserum reactivity in 1× supernatants produced in 2YT medium (n) or TSBD-N medium (i). Specific products are identified relative to prestained molecular mass markers (Ps). k, kDa.

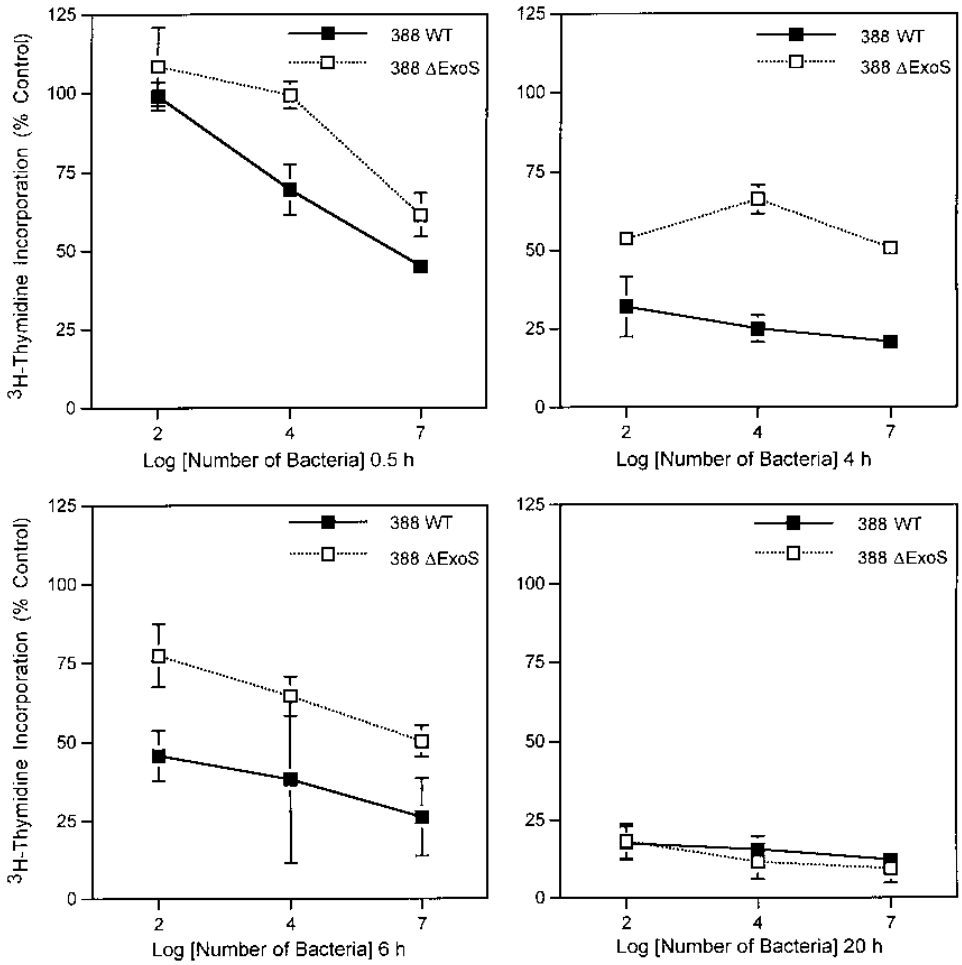

FIG. 3. Effect of differential bacterial production of the 49-kDa ExoS on DNA synthesis of Detroit fibroblastic cells. Detroit 532 cells were seeded at a concentration of 10^5 cells/well in 48-well tissue culture plates and incubated for 48 h to obtain 80 to 90% cellular confluency. At this time, culture medium was removed and replaced with RPMI-BSA and the indicated number of 388 wild-type (WT) or 388Δ*exoS* bacteria. Incubation with bacteria proceeded for 0.5, 4, 6, or 20 h, as indicated in each panel. After these periods, culture supernatants containing the bacteria were removed and replaced with RPMI-FBS-PS containing 1 μCi of [^{3}H]thymidine/ml. DNA synthesis after a 20-h pulse was determined and is expressed as percentages of non-bacterium-treated control monolayer DNA synthesis. Each symbol represents the mean and standard deviation of duplicate samples.

Development of cell culture systems to examine the effect of bacterial production of ExoS. In initial attempts to identify bacterial-eukaryotic coculture conditions that might allow detection of an effect of ExoS on eukaryotic cell function, a range of 10^2 to 10^7 CFU of strain 388 or 388Δ*exoS* bacteria/ml were added to wells containing Detroit 532 monolayers for 0.5 to 20 h. This range of concentration of bacteria resulted in a multiplicity of infection (MOI) range of 1:5,000 to 20:1 bacterial to eukaryotic cells. When Detroit cell morphology was examined by phase-contrast microscopy, both bacterial strains were found to cause alterations in cellular morphology, the severity of which increased with bacterial cell number and incubation time. Only minor differential effects, however, were apparent between the two strains, with parent strain 388 causing slightly more Detroit cell rounding and detachment from the wells (data not shown).

Effect of ExoS production on Detroit cell DNA synthesis. To determine if the slight differences in Detroit cell morphology observed following exposure to wild-type ExoS-producing bacteria reflected a quantifiable alteration of eukaryotic cell function, the effects of 388 or 388Δ*exoS* bacteria on cellular proliferation were examined. Under the coculture conditions described above, supernatants containing bacteria were removed following a 0.5- to 20-h coculture period and replaced with medium containing [^{3}H]thymidine and antibiotics. As shown in Fig. 3, a consistent decrease in DNA synthesis was observed when Detroit cells were incubated with strain 388 compared to that with strain 388Δ*exoS*. Maximal differences between the effects of these two strains were observed after 4 or 6 h of exposure to 10^4 and 10^7 CFU/ml. After a 20-h exposure period, both bacteria proved effective in eliminating DNA synthesis.

To confirm the statistical significance of an effect of ExoS production on DNA synthesis, cell culture studies were repeated, focusing on culture conditions that produced maximal differences. In these studies, Detroit cells were exposed to either 0, 10^4, or 10^7 CFU of 388 or 388Δ*exoS* bacteria (MOI, 1:50 or 20:1, respectively) per ml for 4 h, with each culture condition being applied to six separate wells in the same 48-well tissue culture plate. A significant decrease ($P < 0.001$) in [^{3}H]thymidine incorporation was caused by both concentrations of 388 ExoS-producing bacteria when compared to that of 388Δ*exoS* non-ExoS-producing bacteria (Fig. 4). Levels of [^{3}H]thymidine incorporation directly related to Detroit cell

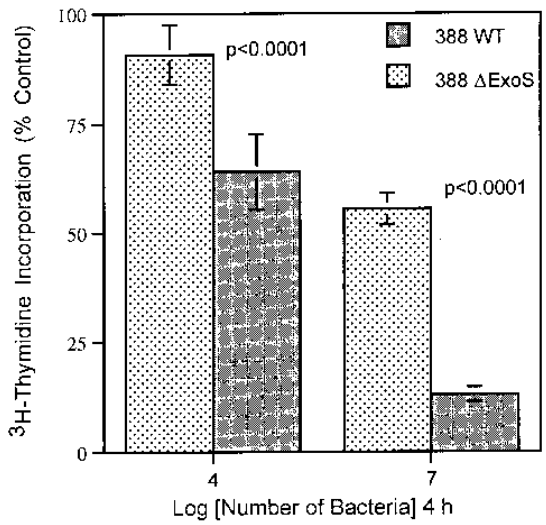

FIG. 4. Confirmation of the statistical significance of the effect of ExoS production by bacteria on DNA synthesis of Detroit cells. By coculture methods recognized to produce maximal effects on DNA synthesis, as shown in Fig. 3, either 0, 10^4, or 10^7 388 wild-type (WT) or 388Δ*exoS* bacteria were incubated with Detroit cells for 4 h and [^{3}H]thymidine incorporation after a 20-h pulse was determined. Each bar represents the mean and standard deviation of six identically treated wells, with results expressed as percentages of the non-bacterium-treated control monolayer response. Statistical differences between wild-type 388 and mutant 388Δ*exoS*-treated cells were calculated by the Student *t* test.

viability as determined by trypan blue exclusion assays performed at the end of the DNA synthesis study. As a negative control, bacteria were added to wells which lacked Detroit cell monolayers and then pulsed with [^{3}H]thymidine for 20 h. No TCA-precipitable [^{3}H]thymidine incorporation was detected. Antibiotics included during the 20-h pulse period were found to have no differential effects on the survival rates of strain 388 or 388Δ*exoS* bacteria.

Direct effect of ExoS production on Detroit cell viability. To quantify the direct effect of ExoS production on Detroit cell viability, Detroit cells were assayed for trypan blue exclusion following exposure to 10^4 or 10^7 388 or 388Δ*exoS* bacteria for 6 or 25 h. After 6 h with bacteria, Detroit cells cultured with 388Δ*exoS* mutant bacteria showed no loss of viability compared to the viability of untreated, control cells (Fig. 5). In contrast, a significant loss of viability was observed between control cells and Detroit cells cultured with either 10^4 or 10^7 CFU of 388 ExoS-producing bacteria ($P < 0.003$) per ml. Following a 25-h exposure to bacteria, a significant decrease in Detroit cell viability was caused by both strains at both concentrations of bacteria compared to the viability of untreated control cells ($P < 0.007$). Under all culture conditions, 388 ExoS-producing bacteria caused a significantly greater decrease in Detroit cell viability than 388Δ*exoS* bacteria, with strain 388 causing the death of almost all Detroit cells after 25 h.

Comparison of the effect of soluble ExoS and ExoS-producing bacteria on Detroit cell DNA synthesis. To determine if the secreted form of ExoS could affect DNA synthesis in the above-described cell culture studies, supernatants obtained from Detroit cell cultures following a 4-h exposure to 10^7 CFU of 388 or 388Δ*exoS* bacteria/ml were filter sterilized to remove bacteria and then added to fresh Detroit cell monolayers for 4 h. DNA synthesis of Detroit cells exposed to culture supernatants was then compared with that of Detroit cells originally in contact with the bacteria. As shown in Fig. 6, the coculture of ExoS-producing bacteria with Detroit cells caused a significant inhibition of DNA synthesis. In comparison, culturing Detroit cells in the presence of culture supernatants containing soluble ExoS had no effect on DNA synthesis. The supernatant obtained from Detroit cells cultured with 388 bacteria was found to have an ExoS enzymatic activity of 25.9 pmol/mg of protein. This compared with levels of ADP-ribosyltransferase activity of <1.5 pmol/mg of protein in supernatants of Detroit cells cultured with 388Δ*exoS* bacteria or no bacteria.

Effect of ExoS production on Detroit cell protein synthesis. ETA, another ADP-ribosyltransferase produced by *P. aeruginosa*, is known to cause cell death by inhibiting eukaryotic cell protein synthesis. To determine if the observed effects of ExoS production on Detroit cell viability were related to the direct or

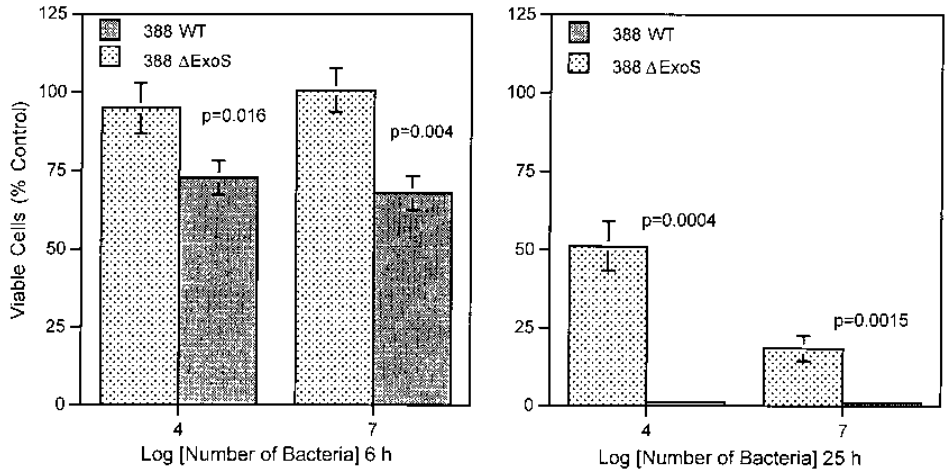

FIG. 5. Direct effect of differential production of the 49-kDa ExoS by bacteria on Detroit cell viability. Detroit cells were seeded and incubated with 0, 10^4, or 10^7 388 wild-type (WT) or 388Δ*exoS* bacteria, as described in the legend to Fig. 3. Following 6 or 25 h of exposure to bacteria, culture supernatants were removed and then Detroit cells were removed from wells by treatment with trypsin-EDTA. Detroit cell viability was determined by counting the number of cells excluding trypan blue dye with a hemacytometer. Each bar represents the mean and standard deviation of three identically treated wells, with results expressed as percentages of non-bacterium-treated control monolayer viability. Statistical analyses were performed by the Student *t* test, with the statistical difference between effects of 388 or 388Δ*exoS* bacteria on Detroit cell viability indicated.

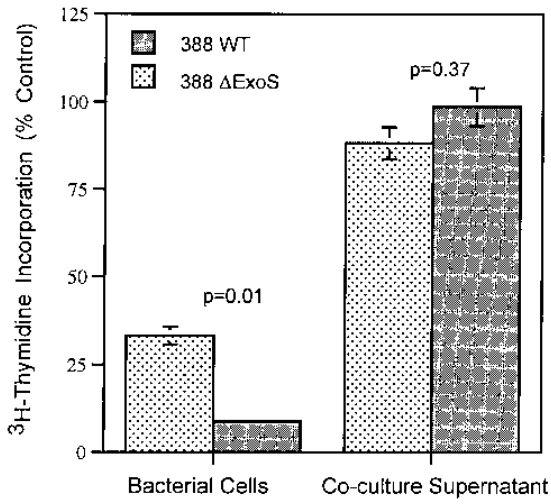

FIG. 6. Effect of ExoS-producing bacteria versus that of secreted ExoS on Detroit cell DNA synthesis. Supernatants obtained from Detroit cells following a 4-h coculture period with 0 or 10^7 CFU of 388 wild-type (WT) or 388Δ*exoS* bacteria/ml were filter sterilized to remove live bacteria and then added to fresh Detroit cell monolayers for 4 h. DNA synthesis of Detroit cells exposed to culture supernatants was then compared with that of Detroit cells originally in contact with the bacteria for 4 h. Supernatants obtained from Detroit cells cultured with 388 bacteria, 388Δ*exoS* bacteria, or no bacteria contained 25.9, 0.7, or 1.5 pmol of ExoS enzymatic activity/mg, respectively. Each bar represents the mean and standard deviation of duplicate samples, with results expressed as percentages of the non-bacterium-treated controls. A one-way analysis of variance on ranks was used to determine the statistical difference between results with control monolayers and those with treatment groups incubated with either bacterial cells or culture supernatants.

indirect inhibition of protein synthesis, Detroit cells were assayed for alterations in [^{3}H]leucine incorporation under coculture conditions known to affect DNA synthesis. In these assays, no consistent differences in Detroit cell protein synthesis were observed following exposure to 388 or 388Δ*exoS* bacteria, except at the highest concentration of 388 bacteria (10^7 CFU/ml) (data not shown). Exposure to this concentration of 388 ExoS-producing bacteria for 6 h, however, was also found to have pronounced effects on Detroit cell viability (Fig. 5). The lack of a direct relationship between the effect of 388 bacteria on Detroit cell DNA synthesis and protein synthesis suggests that mechanisms other than inhibition of protein synthesis were involved in mediating the effect of ExoS on DNA synthesis.

Effect of ExoS-producing bacteria on the KB epithelial cell line. Previous studies have implicated ExoS production in the damaging effects exerted by *P. aeruginosa* on Madin-Darby canine kidney (MDCK) cell epithelial monolayers (2). These results were obtained by comparing the effects of wild-type *P. aeruginosa* strains with those of strains having a mutation in the *trans*-regulatory locus, *exsA*. This mutation causes bacteria to be deficient in the production of ExoS and possibly other coregulated products. To determine if ExoS-associated effects on epithelial cell function could be recognized and quantified in the eukaryotic coculturing system described above, the effect of 388 and 388Δ*exoS* bacteria on KB epithelial cell monolayers was examined. By procedures identical to those developed for Detroit cells, when KB cells were exposed to 388 or 388Δ*exoS* bacteria, both strains caused alterations in cellular morphology when examined by phase-contrast microscopy. However, little difference was apparent when the effects of the two strains were compared. To determine the effects of 388 and 388Δ*exoS* bacteria on KB cell DNA and protein synthesis, [^{3}H]thymidine or [^{3}H]leucine incorporation was assayed following exposure to bacterial concentrations of 10^2 to 10^7 CFU/ml (MOI, 1:7,000 to 14:1) for 4 h. Although the KB cell line was found to be, in general, more sensitive to both strains of *P. aeruginosa* bacteria than Detroit cells, the effects of ExoS production on KB cell function were similar to those observed for Detroit cells (Fig. 7). Exposure to ExoS-producing bacteria caused a significant decrease in DNA synthesis but no consistent differential effect on KB cell protein synthesis. KB cell viability assays performed in parallel with [^{3}H]thymidine incorporation studies confirmed that the observed decrease in DNA synthesis directly correlated with decreased KB cell viability (data not shown).

DISCUSSION

Although many studies implicate ExoS in *P. aeruginosa* virulence, the effect of ExoS on eukaryotic cell function remains

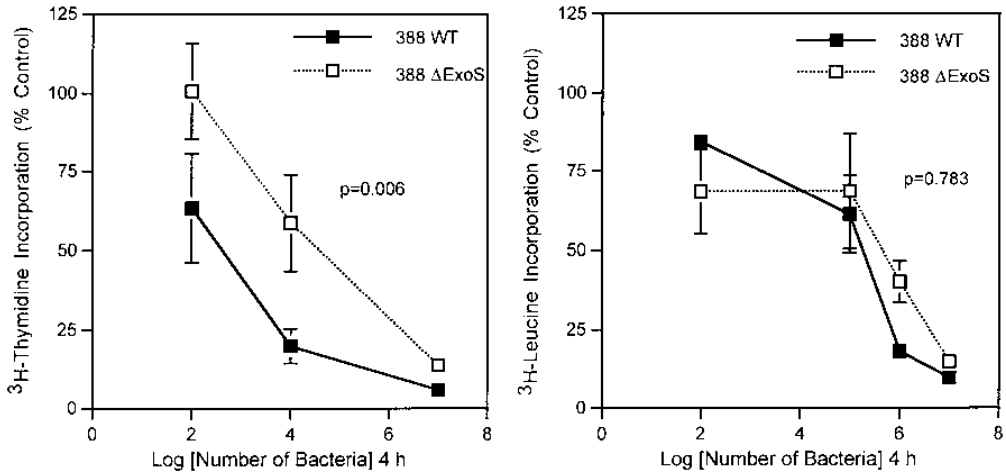

FIG. 7. Effect of ExoS production by bacteria on DNA and protein synthesis of the KB epithelioid cell line. By procedures identical to those described in the legend to Fig. 3 for Detroit cells, 10^5 KB cells/well were seeded in 48-well tissue culture plates and incubated for 48 h and then increasing concentrations of 388 wild-type (WT) or 388Δ*exoS* bacteria were added for 4 h and levels of [^{3}H]thymidine or [^{3}H]leucine incorporation were measured. Each symbol represents the mean and standard deviation of duplicate samples, with results expressed as percentages of the non-bacterium-treated control monolayers. Statistical analyses using a paired Student *t* test are indicated.

unknown. Many factors have contributed to difficulties in identifying the in vivo effect of ExoS. Initial attempts at purifying ExoS were complicated by properties of the protein itself, which caused it to form high-molecular-weight aggregates (17). The significance of these homo- or heteroaggregates to the enzymatic function of ExoS was difficult to interpret until the ExoS gene was cloned. Development of in vitro assays for ExoS was also complicated by the absolute requirement for a eukaryotic 14-3-3 cofactor for detection of ExoS enzymatic activity (9). Awareness of such a factor emphasized the integral relationship between ExoS activity and eukaryotic cell function. In vitro analyses of ExoS substrate specificity found the enzyme to ADP-ribosylate a number of diverse proteins, including low-molecular-weight GTP-binding proteins (8, 10), vimentin (7), and soybean trypsin inhibitor (19). This diversity obscured the recognition of the actual substrate or substrates being modified by ExoS in vivo.

A further difficulty in defining the function of ExoS in vivo related to an apparent inability of secreted ExoS to efficiently enter eukaryotic cells. Early studies noting a lack of toxic effects of ExoS secreted from *P. aeruginosa* 388 (14, 25) were a first indication that secreted ExoS might not function in a manner analogous to other secreted bacterial toxins. The in vivo toxicity of ExoS remained controversial, however, for when ExoS was purified from *P. aeruginosa* DG1 culture supernatants, it was found to be toxic to mice and several tissue culture cell lines (35). Other differences were noted between ExoS isolated from strains DG1 and 388 when ExoS was subsequently purified and cloned from strain 388. These include differences in the specific ADP-ribosyltransferase activities of the two isolated ExoS proteins, differences in their amino acid compositions, and the absence of an internal DG1 ExoS peptide sequence from either the 49- or 53-kDa strain 388 ExoS sequences (13). Although an understanding of the direct relationship between ExoS as defined for strain 388 and ExoS isolated from strain DG1 awaits publication of the cloned DG1 protein sequence, current evidence supports the finding that discrepancies in the toxicities of the two secreted proteins reflects the fact that distinctly different proteins are being compared.

Studies described in this paper confirm that production of the 49-kDa ExoS by strain 388 does have a toxic effect on eukaryotic cells. This toxic effect, however, was not recognized with the secreted form of ExoS but was recognized only when bacteria were allowed direct contact with eukaryotic cells. Since *P. aeruginosa* produces multiple cell-associated and secreted factors that affect eukaryotic cell function, examination of a function of ExoS when bacteria are in contact with eukaryotic cells required the development of a coculture system that would differentiate the effect of ExoS from that of the other *Pseudomonas* factors. For this, two *P. aeruginosa* strains differing only in their production of the 49-kDa ExoS were compared. The two strains used were strain 388 and a recently constructed mutant strain, 388Δ*exoS*, which maintains genetic identity with the parent strain except for loss of the 49-kDa ExoS structural gene. Prior to the initiation of cell culture studies, it was confirmed that deletion of the 49-kDa ExoS had no secondary effects on bacterial growth rates nor on the production of other *Pseudomonas* extracellular proteins. Both strains were found to regulate and produce elastase, alkaline protease, and the 53-kDa form of ExoS in identical manners. ETA was not detected immunologically in 10×-concentrated culture supernatants of either strain, which is consistent with previous reports of the lack of detectable ETA production by strain 388 (26).

Since in vitro bacterial culture studies supported the phenotypic identity of strains 388 and 388Δ*exoS*, with the exception of production of the 49-kDa ExoS, the differential effects of these two strains on eukaryotic cell function should reflect the activity of the 49-kDa ExoS. In bacterial-eukaryotic coculture studies, ExoS-producing strain 388 bacteria were found to cause a significant decrease in Detroit cell DNA synthesis compared with that of the 388Δ*exoS* mutant strain. Maximal differential effects between the two strains were observed after 4 or 6 h of exposure to 10^4 or 10^7 CFU of bacteria/ml. Measurement of Detroit cell viability at the end of [^{3}H]thymidine incorporation assays confirmed that the decrease in DNA synthesis directly reflected a decrease in Detroit cell viability. Similar results were observed when 388 or mutant 388Δ*exoS* bacteria were cocultured with KB epithelioid cell monolayers, indicating that the effect of ExoS is not specific for the Detroit fibroblastic cell line. When protein synthesis was examined under coculture conditions which allowed detection of differential effects on DNA synthesis, no significant differences were observed. These results are supportive of cellular processes other than protein synthesis mediating the ExoS interference of DNA synthesis.

Consistent with recent studies linking ExoS secretion to the type III secretory process (39), only bacteria producing ExoS, not ExoS-containing supernatants, caused an effect in the cell coculture system. The induction and secretion of proteins via the type III process appear to require the bacterium to sense a junction between itself and a target cell, and then proteins are translocated across the eukaryotic cell membrane at this contact site. Supernatants used to examine the role of secreted ExoS in the cell culture system were obtained from bacterial-eukaryotic cocultures in which a significant effect on DNA synthesis was caused by the ExoS-producing bacteria. Analysis of enzymatic activity in this culture supernatant confirmed the presence of ExoS ADP-ribosyltransferase activity. Production of ExoS enzymatic activity during the coculturing period was notable, since no ExoS production was detected in culture supernatants grown for 18 h when 388 bacteria were directly inoculated into RPMI-BSA coculture medium. In coculturing studies, bacteria were first grown in ExoS induction medium and then resuspended in RPMI-BSA and added to Detroit monolayers. Under these conditions, it appeared that ExoS production was able to continue in the noninducing RPMI-BSA medium, reaching increasing concentrations of 25.9, 47.8, and 155.8 pmol/mg of protein after 4, 6, and 20 h of coculture, respectively. The detection of ExoS enzymatic activity in supernatants, even after 20 h of coculture, indicated that the lack of an effect of ExoS in culture supernatants did not relate to its proteolytic degradation. Furthermore, it is unlikely that the lack of recognition of a cellular effect of soluble ExoS related to a synergistic requirement with other *P. aeruginosa* exoproducts, since supernatants used in these studies included all products produced by strain 388 during the coculture period. These results corroborate an increasing amount of evidence that soluble ExoS, as originally defined in 388 culture supernatants, has no detectable effect on eukaryotic cell function. This remains a curiosity in view of the high concentrations of enzymatically active ExoS released by *P. aeruginosa* into culture supernatants. It is possible that the release of ExoS into culture medium reflects the bacterium's requirement to maintain low steady-state amounts of ExoS on the bacterial surface, as has been observed for specific proteins in the Yops system (31).

Although ExoS production caused a significant decrease in eukaryotic cell proliferation, during prolonged coculture studies it became apparant that ExoS was not the only factor produced by *P. aeruginosa* that could cause such an effect. Following a 20-h exposure to bacteria, a significant decrease in

Detroit cell viability and DNA synthesis was observed, regardless of the production of the 49-kDa ExoS. These results reflect the multifactorial nature of *P. aeruginosa* infections and the contribution of other factors to *Pseudomonas* virulence. In this regard, strain 388 has provided an investigative advantage in dissociating ExoS activity from other virulence factors, for under cation-deficient conditions, ExoS is the predominant exoproduct detected in culture supernatants and it is produced in the absence of detectable ETA ADP-ribosyltransferase activity. Although mechanisms regulating the production of virulence factors by *P. aeruginosa* when in contact with eukaryotic cells remain unknown, it is likely that the preferential production of ExoS by strain 388 also occurs in this environment. Consequently, an effect of ExoS production on eukaryotic cell function may not be as easily recognized or may be different if other strains, particularly those producing higher levels of ETA, are examined in cell culture studies. A true understanding of the function of ExoS will therefore require similar analyses of other *P. aeruginosa* strains.

We conclude from our studies that the 49-kDa ExoS, as originally defined for *P. aeruginosa* 388, causes a significant inhibition of eukaryotic cell growth and viability. In contrast to other *Pseudomonas* exoproducts, the effect of ExoS can be recognized only when bacteria are allowed direct contact with the target cells. It is currently unknown whether the 49-kDa ExoS directly inhibits eukaryotic cell growth or whether ExoS indirectly facilitates the activities of other *Pseudomonas* virulence factors. The former explanation, however, is consistent with the ability of ExoS to modify Ras proteins in vitro. The fact that the 49-kDa form of ExoS alone is able to exert a significant effect on eukaryotic cell function is notable in light of recent findings which also implicate the 53-kDa form of ExoS in *P. aeruginosa* pathogenesis (2, 18). As the 388Δ*exoS* mutant still produces the 53-kDa form of ExoS, it is possible that the residual virulence of this mutant relates to its production of the 53-kDa ExoS. Such evaluations will require functional analyses of the 53-kDa mutant and the 49- and 53-kDa double-mutant *P. aeruginosa* strains.

The significance of the study described here relates to the identification of coculture systems which allow the confirmation and quantification of ExoS-associated effects on eukaryotic cell function. With this comes a means of identifying the in vivo activity of ExoS. Further studies should allow assessment of the effects of the 49-kDa ExoS on bacterial cell adhesion, eukaryotic membrane integrity, and eukaryotic cell signaling processes, all of which are implicated as targets of ExoS activity. These findings, coupled with the construction of *P. aeruginosa* mutant bacteria which produce ExoS in the absence of other virulence factors, should lead to an understanding of the site of activity of ExoS in vivo and determine whether ExoS works alone or facilitates the activity of other virulence factors in altering eukaryotic cell growth and viability.

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