Production of the *Pseudomonas aeruginosa* Neuraminidase Is Increased under Hyperosmolar Conditions and Is Regulated by Genes Involved in Alginate Expression

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Abstract

The pathogenesis of *Pseudomonas aeruginosa* infection in cystic fibrosis (CF) is a complex process attributed to specific characteristics of both the host and the infecting organism. In this study, the properties of the PAO1 neuraminidase were examined to determine its potential role in facilitating *Pseudomonas* colonization of the respiratory epithelium. The PAO1 neuraminidase was 1000-fold more active than the *Clostridium perfringens* enzyme in releasing sialic acid from respiratory epithelial cells. This effect correlated with increased adherence of PAO1 to epithelial cells after exposure to PAO1 neuraminidase and was consistent with in vitro studies demonstrating *Pseudomonas* adherence to asialoganglioside receptors. The regulation of the neuraminidase gene nanA was examined in *Pseudomonas* and as cloned and expressed in *Escherichia coli*. In hyperosmolar conditions neuraminidase expression was increased by 50% ($P < 0.0004$), an effect which was OmpR dependent in *E. coli*. In *Pseudomonas* the osmotic regulation of neuraminidase production was dependent upon algR1 and algR2, genes involved in the transcriptional activation of algD, which is responsible for the mucoid phenotype of *Pseudomonas* and pathognomonic for chronic infection in CF. Under the hyperosmolar conditions postulated to exist in the CF lung, nanA is likely to be expressed to facilitate the initial adherence of *Pseudomonas* to the respiratory tract. (*J. Clin. Invest.* 1992. 89:1866–1874.) Key words: cystic fibrosis • neuraminidase • *Pseudomonas aeruginosa*

Introduction

Pseudomonas aeruginosa is a common human pathogen which is particularly important as a cause of respiratory disease in patients with cystic fibrosis (CF).[1] The molecular basis for the unusual association of *Pseudomonas* and the CF lung is unknown, but is likely to involve the expression of one or more of the many exoproducts and/or virulence factors produced by *Pseudomonas*. Among the *Pseudomonas* virulence factors are several enzymes which modify eukaryotic proteins and matrix components (1), such as alkaline protease (2), elastase (3, 4), the ADP ribosylating enzymes, exotoxins A (5) and S (6), and the phospholipases, PLC-H (hemolytic) and PLC-N (nonhemolytic) (7). These exoproducts can have a direct toxic effect on host tissue or may interfere with host immunologic defense mechanisms. The production of the antiphagocytic exopolysaccharide, alginate, is a distinctive *Pseudomonas* phenotype pathognomonic for the mucoid *Pseudomonas* strains associated with the chronic infection of the CF lung (8). The genes that regulate alginate production can be activated by high osmolarity (9) or dehydration (10), environmental conditions present in CF owing to diminished chloride, and consequently water transport (11, 12) across the epithelial surface.

The *P. aeruginosa* strains that initiate infection in the CF patient have less obvious phenotypes than the grossly mucoid strains. Serological studies have demonstrated that antibodies to specific *Pseudomonas* exoproducts are present well before organisms can be recovered from cultures of the sputum or nasopharynx (13), suggesting a role for the in vivo production of exoproducts very early in the colonization process. However, the specific gene products required for colonization of the human epithelium have not been defined, nor is it clear why this colonization process is so specific to the CF, but not the normal respiratory tract.

Intact epithelial cells are relatively resistant to *Pseudomonas* attachment. Bacterial binding can be substantially increased by modification of the epithelial surface by acid (14), trypsin (15), influenza infection, trauma (16), or the effect of the accumulated *P. aeruginosa* exoproducts in culture supernatants (17). The GalNAcβ1-4Gal sequence present in asialylated gangliosides can function as a receptor for several pathogens of the respiratory tract including *P. aeruginosa* (18). A potential role for bacterial exoproducts, particularly a neuraminidase, would be to expose sequestered binding sites within the usually sialylated membrane glycolipids and facilitate attachment to the epithelial surface.

Although the role of neuraminidase in the pathogenesis of infection has not been clearly defined, these enzymes have long been postulated to facilitate interactions between prokaryotes and mammalian hosts (19). Commensal flora such as capnophilic bacteria and anaerobes (20), streptococcal species (21), as well as pathogens such as *Streptococcus pneumoniae* (22, 23) and *Vibrio cholerae* (24), express these enzymes. *P. aeruginosa* was first noted to produce neuraminidase by Shilo (25). Leprat and Michel-Briand (26) further characterized the neuraminidase produced by a clinical strain of *P. aeruginosa* isolated from a child with cystic fibrosis and suggested a role for the enzyme in the pathogenesis of infection. Unlike *Escherichia coli*, which can metabolize the free sialic acid generated by the action of neuraminidases (27), *P. aeruginosa* can utilize only a limited number of carbohydrate substrates and must have another function for the production of neuraminidase.

In the studies described the biochemical properties of the neuraminidase produced by *P. aeruginosa* PAO1 were deter-

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Received for publication 4 November 1991.

1. *Abbreviations used in this paper: CF, cystic fibrosis; IEF, isoelectric focusing.*

J. Clin. Invest.

0021-9738/92/06/1866/09 $2.00
Volume 89, June 1992, 1866–1874

mined, and the effect of this enzyme on human respiratory epithelial cells was examined. The environmental conditions optimal for the expression of the neuraminidase structural gene, *nanA*, were defined and the involvement of several central regulatory genes examined. Neuraminidase production was found to be increased under hyperosmolar conditions. This effect was dependent upon the response regulators AlgR1 or OmpR, suggesting that neuraminidase, like alginate, may be specifically expressed under the conditions expected to be present in the CF lung.

Methods

Bacterial strains and plasmids

P. aeruginosa PAO1 H103 and a PAO1 genomic library cloned in the cosmid vector pLAFR were generously provided by R. E. W. Hancock (University of British Columbia, Vancouver, BC). PAK and PAK N1, an isogenic RpoN$^-$ mutant constructed by insertion of Tc^R cartridge (28), were obtained from S. Lory (University of Washington, Seattle); PA103 and PA103-29, a ToxR$^-$ mutant (29), were obtained from B. Iglewski (University of Rochester, Rochester, NY); *P. aeruginosa* 8830, a stable constitutive mucoid strain, 8852 (*algR1*) and 8882 (*algR2*) (30) were obtained from A. Chakrabarty (University of Illinois, Chicago). The *E. coli* strains used included LE392, a neuraminidase-negative host and LE392pCVD364 containing the cloned *V. cholerae nanH* gene (31) obtained from E. Vimr (University of Illinois, Champaign-Urbana). MC4100 (Nal^R *recA* F$^-$ araD139, Δ(*agrF-lac*) U169 *rpsL*150 *relA1 fibB25 ptsF25 deo-1*), MH225.101 [*MC4100*Φ(*ompC'-lac*$^+$) 10–25 *ompR*101] and pFR29 which contains *ompR/envZ* cloned on pBR-322 (32) were obtained from T. Silhavy (Princeton University, Princeton, NJ).

Culture conditions

The *E. coli* strains were grown in L-broth (33) with tetracycline 30 μg/ml. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise specified. The *P. aeruginosa* strains were grown with aeration at 37°C in M9 media containing 10 μg/ml $FeCl_2$ unless otherwise indicated. PAK N1 was supplemented with 0.2% glutamine. For nitrogen regulation studies media containing K_2HPO_4 (10.5 g/liter), KH_2PO_4 (4.5 g/liter), 10 mM $MgSO_4$, 35 mM sodium citrate, 0.002% thiamine, 0.02 mM arginine, 0.2 mM uracil, 0.2 mM hypoxanthine was used, supplemented with 0.2% glutamine (high N_2) or 0.01% glutamine for conditions of nitrogen limitation. Desferroxylated M9 media was prepared using plastic containers and flasks with 0.05 μg/ml $FeCl_2$ (low iron) or 10 μg/ml $FeCl_2$ (high iron) (34).

Pseudomonas culture supernatants were prepared from cultures grown to log-phase OD_{600} 0.8 and harvested by centrifugation at 7,000 rpm (model RC5C, DuPont-Sorvall, Newtown, CT) for 10 min at 4°C in a model SS34 rotor (DuPont-Sorvall). For cultures grown in high salt the cultures were standardized by colony-forming units (CFU) per milliliter. The supernatants were sterilized by the addition of gentamicin 40 μg/ml and concentrated 20-fold with polyethylene glycol (mol wt 20,000) at 4°C in dialysis tubing.

Neuraminidase assays

To quantify neuraminidase activity from *Pseudomonas* culture supernatants, the exoproducts obtained above were further concentrated and desalted by extensive dialysis vs. 70 mM $NaPO_4$ buffer, pH 6.3. Neuraminidase activity was measured using a modification of the technique of Meyers et al. (35). The fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNeuNAc) was added to a final concentration of 25 μM (4 μl of a 1.25 mM solution in 50 mM NaAc pH 4.9) to one of duplicate wells of a 96-well microtiter plate containing 200 μl per well. Fluorescence (Δ*F*) was measured in a Titertek Fluoroskan II (Flow Laboratories, Inc., McLean, VA) correcting for background fluorescence from the control wells without substrate. Activity from LE392pCVD364 was used as the positive control. The specific activity was determined as the Δ*F* per milligram of protein. Protein concentration was measured using a commercially obtained kit (Bio-Rad Laboratories, Richmond, CA) based on the method of Bradford (36). Hydrolysis of MUNeuNAc was shown to be linear for the range of protein concentrations used for these determinations. All assays were performed in duplicate.

The neuraminidase activity of *E. coli* strains was determined by measuring the Δ*F* per milligram of protein from cell extracts using MUNeuNAc as a substrate. Cultures (40 ml) were harvested after 18 h of incubation with shaking at 37°C, washed, and resuspended in $NaPO_4$ buffer, pH 5.6. The cells were treated with lysosyme, 0.1 ml of a 10 mg/ml solution, incubated on ice for 10 min, and sonicated using a Branson sonifier at 40% power for 20 pulses. Cellular debris was removed by centrifugation; 16,000 rpm for 30 min in a model SS34 rotor (DuPont-Sorvall). The supernatants were concentrated and tested for neuraminidase activity.

The isoelectric point of the cloned neuraminidase present in the *E. coli* sonicates was determined by applying aliquots of the cell extracts to an acrylamide-ampholyte isoelectric focusing (IEF) gel (Pharmacia-LKB, Piscataway, NJ), pH 3.5–9, and focusing for 90 min. Neuraminidase activity was identified using a MuNeuNAc overlay and visualization with an ultraviolet (UV) light source.

Cloning procedures

Cloning of nanA in E. coli LE392. The gene encoding the PAO1 neuraminidase, *nanA*, was isolated from a genomic library of PAO1 H103 cloned on the cosmid vector pLAFR and expressed in *E. coli* LE392. The initial screening for clones expressing neuraminidase activity was done by plating clones on M9 media containing MUNeuNAc and examining under a UV light source for fluorescence. Individual clones were then inoculated into a 96-well microtiter plate containing Luria-Bertani media with tetracycline (30 μg/ml), incubated overnight at 37°C, and lysed with lysosyme, 2 μl of 10 mg/ml solution. MUNeuNAc was added to a final concentration of 25 μM and fluorescence measured.

DNA manipulations were carried out using standard methods (33). Restriction endonucleases and T4 ligase were obtained commercially and used according to the suggestions of their manufacturers.

Determination of molecular weight. A partial purification of the PAO1 neuraminidase was done using a combination of chromatographic and IEF techniques. An aliquot of the concentrated PAO1 supernatant was applied to a DE52 anion exchange column (Whatman, Inc., Clifton, NJ). Neuraminidase activity was found in the unbound flow through material. The proteins remaining in the unbound fraction were separated by electrophoresis on an acrylamide IEF gel and neuraminidase activity was identified by using an overlay of 2.5 mM MuNeuNAc. The band of fluorescence was cut out of the IEF gel, and the protein was precipitated and applied to a 12% SDS polyacrylamide gel allowing the identification of the protein band associated with neuraminidase activity.

Partial purification of the PAO1 neuraminidase. PAO1 was grown in M9 + 10 μg/ml $FeCl_2$ to OD_{600} 0.6- and 800-ml culture supernatants harvested by centrifugation at 8,000 rpm for 15 minutes a 4°C in a model RC5C SS34 rotor (DuPont Sorvall). The supernatants were concentrated twenty times using polyethylene glycol, mol wt 20,000, then dialyzed exhaustively against 70 mM $NaPO_4$, pH 5.75. This was applied to DE52 in a batch method. Unbound material was removed after 2 h at 4°C and applied to a CM52 column (Whatman, Inc.). Proteins were eluted using a stepwise gradient 0.105–0.245 M $NaPO_4$ buffer. Fractions were tested for neuraminidase activity using the microtiter assay above. The appropriate fractions were pooled, concentrated and adjusted to pH 6.0.

Analysis of neuraminidase activity and substrate profile. A modification of the thiobarbituric acid assay (37) was used to measure the release of sialic acid from a variety of substrates; 0.5% sialyllactose (*N*-acetylneuramin-lactose) in 35 mM $NaPO_4$ buffer, pH 7.0, fetuin, 100 mg/ml in H_2O, 1% mucin from bovine submaxillary glands, 0.5% *N*-

acetyllactosamine in H_2O. These assays were performed by adding 100 μl of substrate and 100 μl of the partially purified PAO1 neuraminidase, or commercially obtained neuraminidase from *C. perfringens* as a control, and incubating for 24 h at 37°C. The thiobarbituric assay was then performed (37). After cooling the reactions to room temperature, a 1-ml aliquot was extracted with 1 ml of cyclohexanone. the layers were separated by centrifugation at 700 rpm for 5 min in a model RC5C SS34 rotor. OD_{549} of the upper cyclohexanone phase was read and millimolars of sialic acid were quantified by comparison to a standard curve obtained using dilutions of sialic acid (*N*-acetylneuraminic acid) 0.1–1.0 mg.

Effect of neuraminidase on human epithelial cells. The release of sialic acid from human epithelial monolayers was measured by adding 5.16 μg of the partially purified PAO1 enzyme (6.0 ml) or 2.5 U (2.27 mg) of the *C. perfringens* enzyme in the same $NaPO_4$ buffer, or buffer alone as a control, to a T75 flask containing a confluent monolayer of primary respiratory epithelial cells obtained from normal human nasal polyps. The characteristics of these monolayers have been previously described in detail (17). After incubation for 18 h at 37°C, the supernatants were harvested and cellular debris removed. The supernatants were lyophilized and resuspended in 0.2 ml of 70 mM $NaPO_4$ buffer, pH 5.75, and assayed for sialic acid content as above.

The effect of neuraminidase treatment of the epithelial monolayers on the adherence of PAO1 was determined using the adherence assay previously described (17). In brief, epithelial cells isolated from nasal polyps were grown to confluence in 24-well tissue culture plates. Triplicate wells were treated with either PBS, pH 6.5, *C. perfringens* neuraminidase 0.25 U per well in 0.3 ml of PBS, pH 6.5, or the partially purified PAO1 neuraminidase 0.3 ml per well, for 60 min at 37°C. The plates were rinsed three times with PBS and incubated with ^{35}S-labeled PAO1 for 2 h at 37°C. The epithelial cells and adherent bacteria were solubilized in 0.5 ml of 2% sodium dodecyl sulfate and scintillations were counted (Tricarb liquid scintillation spectrophotometer, Packard Instrument Co., Inc., Downers Grove, IL). The scintillations associated with a 100-μl aliquot of the initial 5×10^8 CFU/ml PAO1 suspension were counted to determine the CFU/cpm. A mean, standard deviation (SD), and standard error (SE) were determined. Statistical analysis was performed using StatView software (Abacus Concepts, Inc., Berkeley, CA).

Results

Identification and partial purification of the neuraminidase activity in PAO1 culture supernatants. The neuraminidase isolated from late log-phase M9 culture supernatants was found to have an approximate molecular mass of 51 kD as estimated from its electrophoretic mobility on SDS-polyacrylamide gels (Fig. 1) and was isoelectric at pH 8.14.

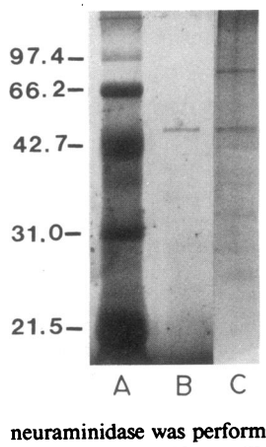

Figure 1. Isolation of the PAO1 neuraminidase from culture supernatants. (*A*) Molecular weight standards (*B*) Protein associated with neuraminidase activity. The proteins not bound to DE52 were electrophoresed on a preparative IEF gel, pH gradient 3.5–9.0. The neuraminidase activity was identified using a MUNeuNAc overlay and the corresponding band cut from the IEF gel. The protein in this gel slice was precipitated with trichloroacetic acid, electrophoresed on 12% polyacrylamide gel, and visualized with silver staining. (*C*) Partially purified neuraminidase preparation. A semiquantitative isolation of the neuraminidase was performed by applying the supernatant proteins not bound to DE52 to a CM52 column. The fractions eluted with 0.1 M $NaPO_4$ were associated with the neuraminidase activity and contained the proteins visualized here with silver staining.

Table I. Partial Purification of PAO1 Neuraminidase

	Protein concentration	ΔF*	ΔF/ml	ΔF/mg protein	Fold purification
	μm/ml		nm		
Supernatant	103.00	376	1,880	18,252	
DE52 unbound	22.50	256	1,280	56,889	3×
CM52 eluent	0.86	289	1,445	1,680,232	92×

* As determined by MUNeuNAc microtiter plate assay in 200 μl per well.

More quantitative yields of the enzyme were isolated using a combination of anion and cation exchange column chromatography (Table I). In addition to the 51×10^3 M_r protein corresponding to the neuraminidase activity identified using an overlay of the fluorescent substrate MUNeuNAc, a second protein with an 86×10^3 M_r copurified with the neuraminidase (Fig. 1). Separation of the high molecular weight protein by HPLC was performed and there was no neuraminidase activity associated with this protein (data not shown).

The activity of preparations containing the partially purified neuraminidase, visualized in Fig. 1, in releasing sialic acid from a number of substrates was determined (Table II). The specific activity of the partially purified enzyme was two orders of magnitude greater than that of the *C. perfringens* enzyme against substrates expected to contain sialic acid primarily in an α2,3 configuration such as sialyllactose. The *Pseudomonas* enzyme had even greater specific activity (three orders of magnitude) in releasing sialic acid residues from human epithelial cells, which contain membrane gangliosides usually sialylated in an α2,6 configuration.

Inhibitors of the partially purified PAO1 neuraminidase included 2,3 dehydro-2-deoxy-*N*-acetylneuraminic acid, sialic acid, $CaCl_2$, and EDTA (Table III). When incubated for 48 h activity was inhibited by each of these compounds by > 90%. Using the hydrolysis of MUNeuNAc to detect neuraminidase activity, the PAO1 enzyme continued to be active at 37°C for up to 72 h.

Modification of epithelial cells by the PAO1 neuraminidase. The effect of the PAO1 neuraminidase on primary cultures of human respiratory epithelial cells was tested by measuring the adherence of ^{35}S-labeled PAO1 to confluent monolayers under control conditions and after exposure to PAO1 neuraminidase. If asialogangliosides function as receptors in this model system, we would expect that neuraminidase-treated monolayers should bind increased numbers of *Pseudomonas*. As shown in Fig. 2, PAO1 adherence to epithelial cells was increased by 37.5% after the monolayers were incubated with partially purified PAO1 neuraminidase ($P < 0.0019$), and 18.6% after treatment with *C. perfringens* neuraminidase as compared to the PBS-treated control ($P < 0.02$). These effects were seen with very small amounts of neuraminidase; one-tenth the amount of *C. perfringens* enzyme used to quantitate the sialic acid released (Table II), as was used in the adherence assays; for the PAO1 enzyme the increase in adherence was associated with ~ 1/100 of the Warren assays. Larger amounts of the PAO1 neuraminidase disrupted the integrity of the monolayer and caused peeling from the collagen-coated plastic. Although this

Table II. Release of Sialic Acid (NANA) by Neuraminidase

Substrate	Neuraminidase	
	C. perfringens	*P. aeruginosa* PAO1
	mM NANA released/mg protein	
N-Acetylneuraminlactose	14.6*	1255.8‡
Fetuin	14.8*	3814.0‡
Bovine mucin	15.8*	4162.8‡
Normal human nasal polyp	0.028§	24.0‖
N-Acetyllactosamine	ND*¶	ND‡

* 45 μg = 0.05 U used. ‡ 86 ng used. § 2.27 mg = 2.5 U used. ‖ 5.16 μg used. ¶ ND, none detected.

did not interfere with the Warren assay, it did limit the amount of enzyme which could be used in the adherence assays.

Regulation of neuraminidase expression in PAO1. Central regulatory genes are often involved in the coordinate expression of the virulence factors (38). Specific environmental conditions can activate the expression of these regulatory systems. To determine if any of the previously characterized *Pseudomonas* regulatory genes might also control *nanA,* neuraminidase expression was quantified in isogenic pairs of strains containing mutations in specific regulatory genes. The strains were grown under conditions expected to maximize expression of the virulence determinant to be tested. The genetic elements screened included: *toxR* (*regA*) associated with Fe^{++}-dependent exotoxin A expression (29, 34), *rpoN,* involved in pilus and flagellar expression and nitrogen metabolism (28), and *algR,* involved in the osmoregulation of alginate production (39).

Neuraminidase activity in supernatants produced by PA103, and the $ToxR^-$ PA103-29 under conditions of limited Fe^{++} was equivalent (Fig. 3 *A*). In the presence of 10 μg/ml Fe^{++} neuraminidase production was increased in both of these strains during stationary phase of growth, a pattern of regulation which is not typical for ToxR-regulated genes (40). Neuraminidase production was not dependent upon the expression of *rpoN* as both PAK and PAK N1 produced neuraminidase in amounts dependent upon the availability of nitrogen and overall growth rate (Fig. 3 *B*).

As increased osmolarity has been an important environmental factor in the expression of the CF specific *Pseudomonas* virulence factor alginate, the effect of high salt was tested on the expression of the PAO1 neuraminidase (Fig. 4). There was a 50% increase in neuraminidase activity in supernatants from PAO1 grown in M9 + 400 mM NaCl (1,038 mosmol) as compared to control cultures grown in M9 (238 mosmol) ($P < 0.0004$). This effect could be completely negated by the addition of the osmoprotectant glycine betaine. Control assays to determine the effect of 400 mM salt on the activity of the enzyme were performed. PAO1 supernatant was associated with a $\Delta F - 280$; in the presence of 400 mM NaCl, $\Delta F - 185$, a 34% decrease. Thus, NaCl by itself was not responsible for the enhanced hydrolysis of MUNeuNAc. The increase in neuraminidase activity in response to high salt was not limited to strain PAO1. Strain PAK demonstrated a similar increase. PAK N1 with a fourfold increase had an exaggerated response to hyperosmolar growth conditions.

AlgR1 and AlgR2 are required for the osmoregulation of neuraminidase expression. High osmolarity is one of the signals involved in the transcriptional activation of *algD* which encodes GDP mannose dehydrogenase, the enzyme critical for the production of alginate characteristic of the mucoid phenotype of *Pseudomonas* present in CF (9, 41, 42). The expression of algD can be regulated by AlgR1 (AlgR), a member of the two component family of response regulators (39, 43) and by AlgR2 (AlgQ) (41, 44).

Table III. Inhibitors of PAO1 Neuraminidase Activity

	ΔF	Percent decrease
	nm	
PAO1 partially purified neuraminidase	510	—
+1 mM 2,3 dehydro-2-deoxy-NANA*	251	51
+10 mM 2,3 dehydro-2-deoxy-NANA	187	63
+10 mM NANA	213	58
+25 mM NANA	145	72
+50 mM NANA	96	81
+10 mM sucrose	426	16
+50 mM sucrose	430	16
+1 mM $CaCl_2$	353	23
+10 mM $CaCl_2$	268	42
+0.025 mM EDTA	220	52
+0.050 mM EDTA	218	53

* NANA, *N*-Acetylneuraminic acid.

To determine if mutations in *algR1* or *algR2* might affect the osmo-dependent regulation of *nanA,* the specific neuraminidase activity in *P. aeruginosa* strains 8830 (constitutive mucoid), 8852 (*algR1*) and 8882 (*algR2*) were compared after growth under normal and conditions of increased osmolarity (Fig. 5). The expression of neuraminidase by 8830 was increased by 50% in the presence on 400 mM NaCl ($P < 0.006$). Both *algR* mutants had decreased neuraminidase production

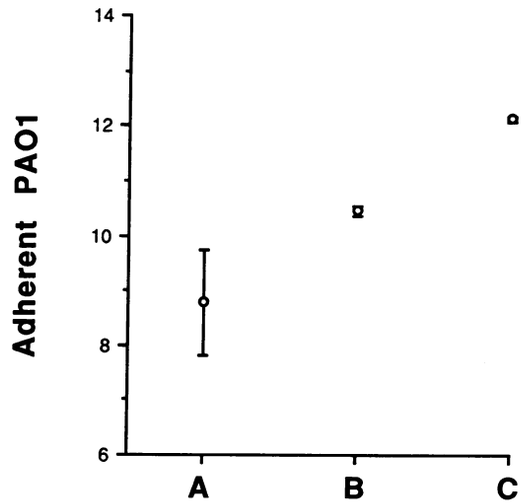

Figure 2. The effect of neuraminidase on PAO1 adherence to respiratory epithelial cells. The number of ^{35}S-labeled PAO1 adherent to confluent epithelial monolayers is plotted on the *y*-axis. The monolayers were preincubated with for 60 min at 37°C with (*A*) control PBS pH 6.5, (*B*) *C. perfringens* neuraminidase 0.25 U per well in PBS, pH 6.5, (*C*) partially purified PAO1 neuraminidase in PBS, pH 6.5. The data are expressed as the mean±SE ($n + 3$) × 10^6 CFU/ml. The SE of some data points is contained within the symbol of the mean.

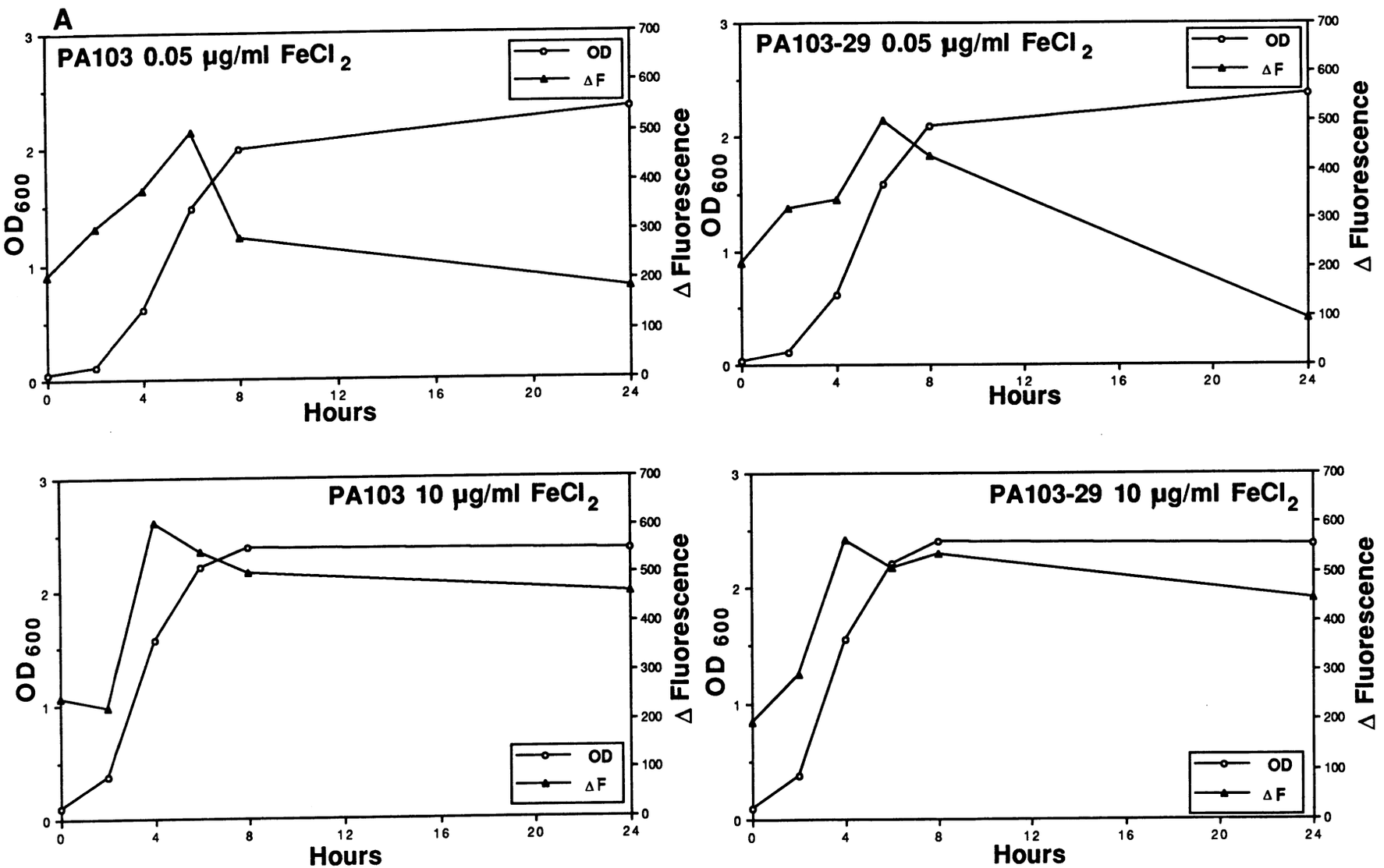

Figure 3. The production of neuraminidase as a function of growth in strains: (*A*) PA103 and PA103-29 in Fe^{++}-limited (0.05 µg/ml) and Fe^{++}-replete (10 µg/ml) conditions; and (*B*) PAK and PAK N1 in high and low N_2. Growth (*OD*) and the neuraminidase activity in aliquots of supernatant as determined by measuring the hydrolysis of MUNeuNAc (Δ *Fluorescence*) are plotted on the *y*-axes as a function of time.

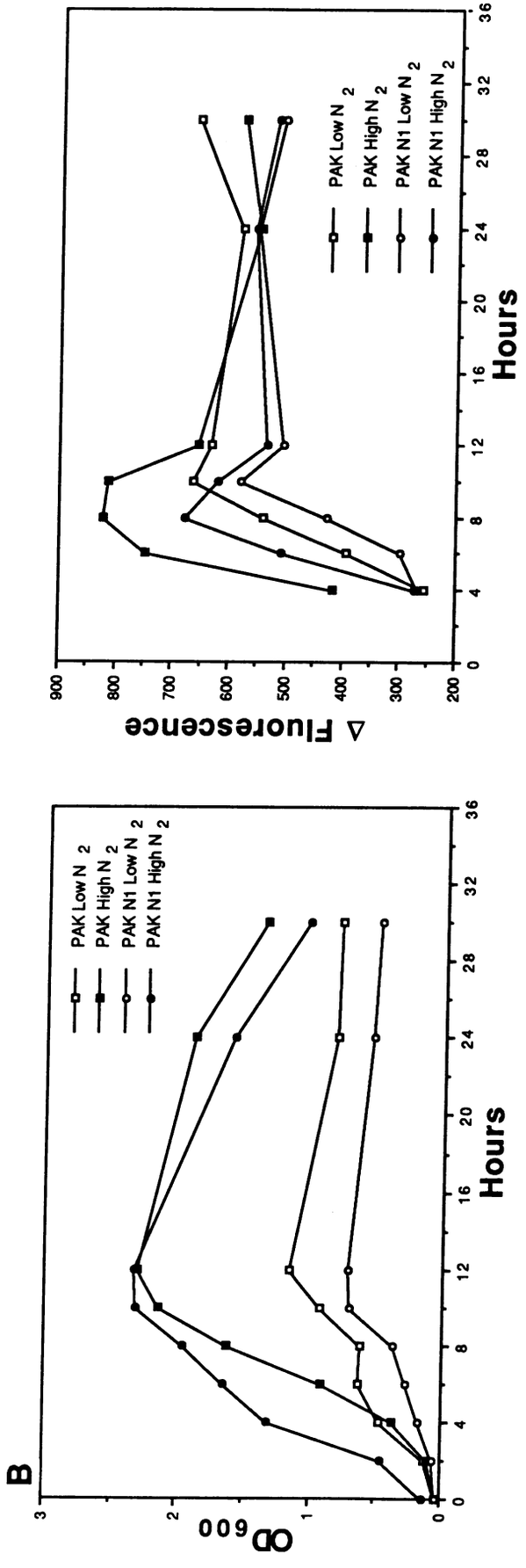

Figure 3 (Continued)

under control conditions. The *algR1* mutant had a diminished response to high salt. The *algR2* mutant had an actual decrease in neuraminidase production under hyperosmolar conditions. Thus, similar osmoregulatory systems may be involved in the expression of both alginate and neuraminidase.

Osmoregulation of nanA expressed in E. coli. To further analyze the regulation of neuraminidase expression, the structural gene for the PAO1 enzyme, *nanA* was cloned and neuraminidase production quantified in an *E. coli* background. The *nanA* cosmid, pASP101, contained a 7.5-kb *EcoR*1 fragment associated with the production of a neuraminidase with the same isoelectric point as that present in sonicates of PAO1 (Fig. 6). The 7.5-kb DNA fragment was verified by Southern hybridization to have originated from PAO1 (data not shown).

The expression of neuraminidase by *E. coli* MC4100-pASP101 was osmoregulated (Fig. 7). There was a 2.6-fold increase in activity in response to 400 mM NaCl (1,697 mosmol) ($P < 0.004$), a 2.4-fold increase in response to 400 mM KCl (1697 mosmol), and a 2.2-fold increase in response to 20% sucrose (881 mosmol). In the presence of an osmoprotectant, 2.5 mM glycine betaine, the effect of high salt was completely abolished.

The osmotic regulation of *nanA* in *E. coli* was dependent upon OmpR (Fig. 8). In the OmpR$^-$ mutant MH225.101 *nanA* expression was equivalent in cultures grown under control conditions or in 400 mM NaCl. When the *ompR* mutation was complemented by genes expressed on the plasmid pFR29, osmodependent *nanA* expression was restored. This is analogous to the transcriptional activation of *algD* by OmpR when measured in an *E. coli* background. OmpR can activate *algD* under conditions of high osmolarity functionally replacing AlgR1 (39).

Discussion

Although the production of neuraminidase by *Pseudomonas* was originally reported over 30 years ago (25) the function of

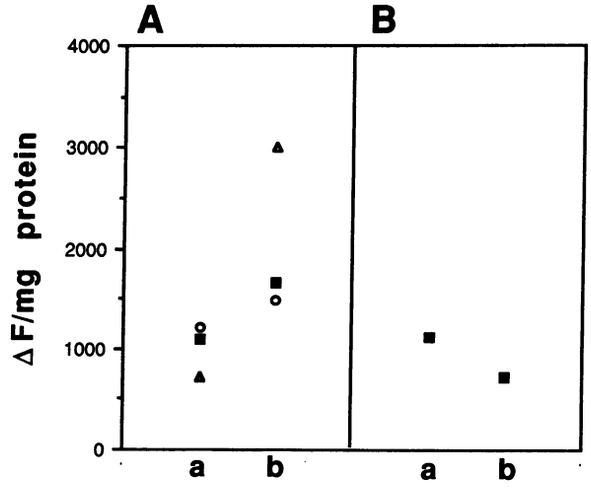

Figure 4. The effect of high osmolarity on the production of neuraminidase. (*A*) The neuraminidase specific activity of late log culture supernatants of strains PAO1 (■), PAK (○), and PAK N1 (△) are shown. Cultures were grown in (*a*) M9 (238 mosmol), (*b*) M9 + 400 mM NaCl (1038 mosmol) (*B*) The effect of an osmoprotectant. PAO1 cultures were grown in (*a*) M9 media + 1 mM glycine betaine and (*b*) M9 + 400 mM NaCl + 1 mM glycine betaine.

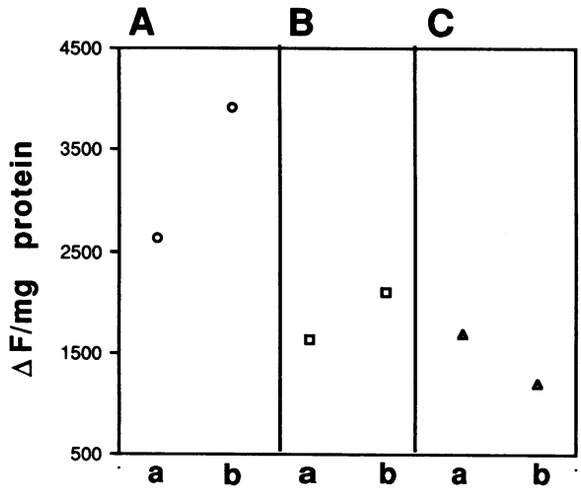

Figure 5. The effect of mutations in *algR1* and *algR2* on the production of neuraminidase. The neuraminidase specific activity in supernatants harvested from late log-phase cultures grown in (*a*) M9 media and (*b*) M9 + 400 mM NaCl was determined for P. aeruginosa strains: (*A*) 8830 (○), (*B*) 8852 (□) (*algR1*), (*C*) 8882 (△) (*algR2*).

this enzyme in the physiology of *P. aeruginosa* or its role in the pathogenesis of infection remains to be established. In the respiratory tract there are several potential substrates for the action of neuraminidase. The glycoproteins which comprise respiratory mucins are highly sialylated and epithelial membranes have abundant sialylated ganglioside and other glycoconjugate components (45). The PAO1 neuraminidase was active against a range of substrates expected to be present in the respiratory tract, including α2,3-linked sialic acids as found in sialyllactose as well as the sialic acid residues present on epithelial cell surfaces.

The properties of the PAO1 neuraminidase were quite similar to those of other bacterial neuraminidases in general (19), as well as to the *P. aeruginosa* neuraminidase originally characterized by Leprat and Michel-Briand (26). Although the purification procedure used did not allow a definitive estimation of the M_r, the data obtained is consistent with previously described neuraminidases. The 86,000-D protein found in all supernatant fractions with neuraminidase activity is close in size to the *V. cholerae* neuraminidase, M_r 90,000 (31), and that of *C. perfringens*, M_r 88,000 (46). The active enzyme identified on a nondenaturing ampholyte-acrylamide IEF gel was associated with a reduced and denatured protein band with M_r 51,000 which is closer in size to some of the viral neuraminidases (19). However, this may represent a product or subunit structure of the 86-kD protein. Subcloning and DNA sequencing studies will clarify this question in the future. The substrate profile, stability, and inhibition by $CaCl_2$, 2,3-dehydro-2-deoxy *N*-acetylneuraminic acid and by *N*-acetylneuraminic acid were quite similar to the reported properties of the *V. cholerae* enzyme (47), with which it shares an isoelectric point, and DNA homology (A. Prince, unpublished data).

The activity of the PAO1 neuraminidase differed from the *C. perfringens* enzyme most significantly, by its 1,000-fold greater activity in releasing sialic acid from the epithelial monolayers. As previous studies suggested that much of the superficial sialic acid available for lectin binding on these epithelial monolayers is present in an α2,6 configuration (Saiman, L., et al., manuscript submitted), this may represent an increased activity particularly against the multiply sialylated gangliosides typical of membrane glycolipids (48, 49).

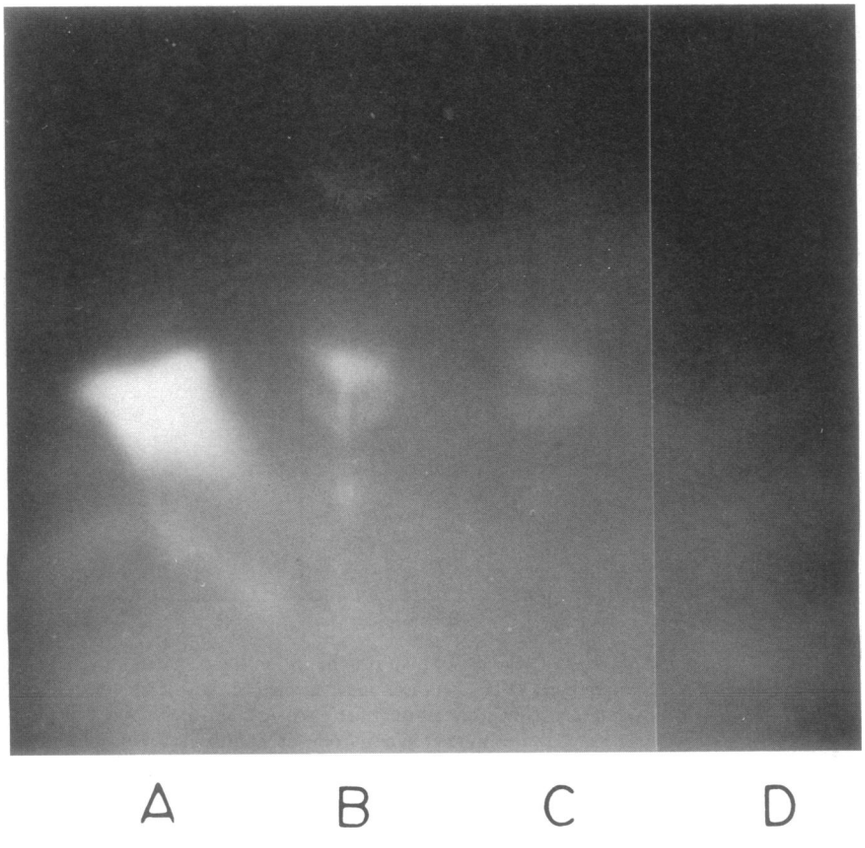

Figure 6. Expression of the cloned PAO1 neuraminidase in *E. coli* as detected by a MUNeuNAc overlay of an IEF gel. Cell sonicates from (*A*) LE392pCVD364, (*B*) PAO1, (*C*) LE392pASP101, and (*D*) LE392pLAFR.

Asialogangliosides that contain a GalNAcβ1-4Gal sequence have been shown to act as receptors for at least two discrete *Pseudomonas* adhesins, pilin (50), and exoenzyme S (51). Their common GalNAcβ1-4Gal receptor is a constituent of many gangliosides, but is not a sequence typical for mucin glycoproteins. Inasmuch as recent studies have failed to demonstrate specific receptor sequences for *P. aeruginosa* in human mucin glycoproteins (52), these asialoganglioside receptors appear to be of major importance in mediating *Pseudomonas* attachment to the respiratory tract. The activity of the neuraminidase in modifying membrane glycolipids and exposing these potential receptors is consistent with the observation that neuraminidase treatment of the epithelial monolayers increased bacterial attachment, and correlated with the release of sialic acid from the monolayers.

Many respiratory pathogens recognize these or similar GalNAcβ1-4Gal carbohydrate binding sites in vitro (18), yet do not selectively colonize CF patients in vivo. Thus, these receptors must not be equally accessible to all potential pathogens. Because *Pseudomonas* does not colonize the normal respiratory tree, but is peculiar to patients with cystic fibrosis, environmental factors specific to cystic fibrosis such as increased osmolarity may be important in triggering the expression of specific genes that facilitate colonization. Although the osmolarity of the uninfected CF lung has not been directly measured, current understanding of the consequences of the abnormal CF transmembrane conductance regulator suggests that this milieu is hyperosmolar (11, 52). Under these conditions, *nanA* is likely to be expressed.

It seems improbable that the modest biological effect of neuraminidase on the epithelial cell surface could account for the virtually universal colonization of CF patients with *P. aeruginosa*. However, the milieu of the CF, but not the normal lung, may specifically activate the expression of several genes, including *nanA*, which initiate colonization and facilitate long-term infection. The potentiation of neuraminidase activity by proteases may further facilitate this process (45).

The osmoregulation of *nanA* expression appears to share several features with the genes associated with the expression of the mucoid phenotype pathognomonic for chronic *Pseudomonas* infection in CF. The control of alginate biosynthesis is

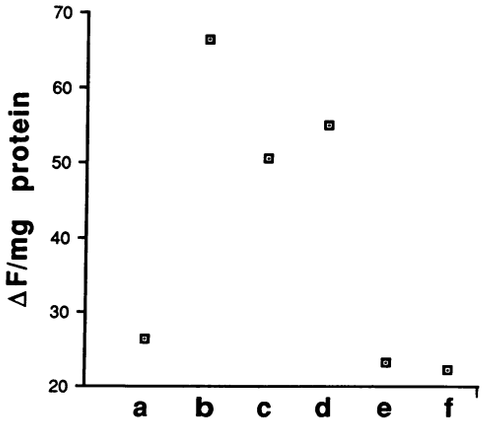

Figure 7. The expression of *nanA* in *E. coli* MC4100. The neuraminidase specific activity in sonicates of MC4100pASP101 grown under the following conditions is plotted: (*a*) M9; (*b*) M9 + 400 mM NaCl; (*c*) M9 + 20% sucrose; (*d*) M9 + 400 mM KCl; (*e*) M9 + 2.5 mM glycine betaine; and (*f*) M9 + 400 mM NaCl + 2.5 mM glycine betaine.

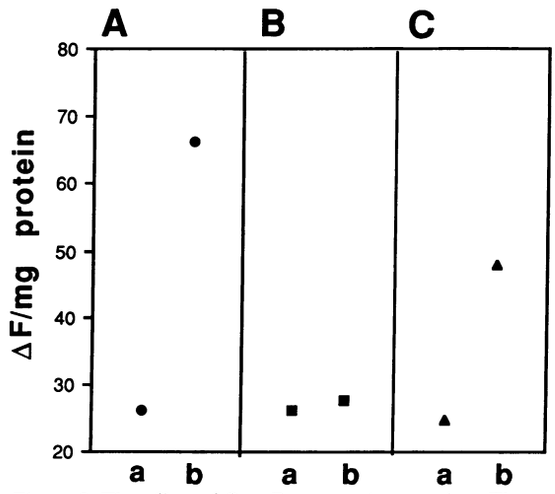

Figure 8. The effect of OmpR on *nanA* expression. The neuraminidase specific activity in sonicates of cultures of (*A*) MC4100pASP101, (*B*) MH225.101pASP101, and (*C*) MH225.101pASP101/pFR29 grown in (*a*) M9 and (*b*) M9 + 400 mM NaCl are shown.

highly complex involving several separate regulatory elements, some of which respond to increased osmolarity. *Pseudomonas* analogue of the *ompR-enzV* system (39), *ntrC*-type regulators (53), and rpoN dependent genes (42) have been implicated. The studies presented exclude a major role for *rpoN*-dependent genes in the expression of *nanA*. Nor was the regulatory gene *toxR* implicated, despite its central role in the regulation of the classic *Pseudomonas* virulence factors (40). However, the osmodependent expression of neuraminidase required both *algR1* and *algR2*, which are similarly involved in the osmodependent expression of algD, the critical enzyme in alginate synthesis (41, 43). The osmoregulation of *nanA* by the *E. coli* response regulator OmpR, suggests that neuraminidase also belongs to the "two-component" family of environmentally regulated proteins (38). This demonstration that "cross-talk" is responsible for the activation of *nanA* by OmpR, is further evidence that transcription of this gene is similarly regulated by an analogous two-component system of signal transduction. Thus, key elements of the genetic regulation of alginate and neuraminidase expression are shared.

These studies suggest that specific environmental conditions and corresponding regulatory genes may be involved in both the initial selection of strains, which can modify the epithelial surface and adhere and the eventual selection of the alginate-producing strains, which elude the normal host defense mechanisms to establish a chronic infection. Interruption of the activation of these genes in vivo might be accomplished by elimination of the environmental signal, high osmolarity. Therapeutic strategies to increase hydration within the CF lung (54) may be a practical strategy to prevent the expression of the *Pseudomonas* genes that allow colonization of the respiratory tract to occur.

Acknowledgments

We thank Nicholas Cacalano for assistance in the purification of the neuraminidase.

This work was supported by The Cystic Fibrosis Foundation and by U. S. Public Health Service grant DK-39693 to Dr. Prince. Dr. Kays was the recipient of a postdoctoral research fellowship from the Cystic Fibrosis Foundation–Research Development Program at Columbia University.

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