BIOLOGICAL LETT. 2012, 49(1): 59–64
Available online at: http:/www.versita.com/science/lifesciences/bl/ DOI: 10.2478/v10120-012-0011-6

Occurrence of the *nan1* gene and adhesion of *Pseudomonas aeruginosa* isolates to human buccal epithelial cells

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(*Received on 27 May 2011; Accepted on 16 June 2012)*

Abstract: This study shows an association between the frequency of the *nan1* gene (encoding neuraminidase) among 62 clinical *Pseudomonas aeruginosa* isolates and adhesion of these bacteria to human buccal epithelial cells. The 52 strains in which the gene was present (83.9%) were characterized by a higher adhesiveness (the mean number of adhering bacteria was 23.51 per cell) than strains in which the gene was not detected (16.23 per cell) and the difference was significant ($P = 0.009$, Mann-Whitney U test). Thus we found that the *nan1* gene may play a role in the binding of clinical *P. aeruginosa* strains to buccal cells.

Keywords: adherence, bacteria, virulence genes, *Pseudomonas aeruginosa*, *nan1* gene

INTRODUCTION

Pseudomonas aeruginosa is a major opportunistic pathogen, an important cause of nosocomial respiratory tract infections, and the chief cause of lung infection in cystic fibrosis (CF) (Saiman et al. 1992). Bacterial adherence to mucosal surfaces is a significant aetiological factor in infections. Several in vitro and in vivo studies find poor adherence of *P. aeruginosa* to functional and intact respiratory epithelia. In contrast, this bacterium is shown to avidly bind to injured epithelia (Woods et al. 1980 a, b, 1981; Saiman et al. 1992; De Bentzman et al. 1996). Elevated salivary protease

levels in CF patients may modify various cell surface components, such as fibronectin, or expose cryptic receptors for *P. aeruginosa* (Woods et al. 1981). The ability of *P. aeruginosa* to persist in the respiratory tract is correlated with the organism's ability to adhere to buccal epithelial cells (BECs) (Woods et al. 1980 a, b; Saiman et al. 1992). These bacteria adhere readily to trypsinized BECs from healthy individuals, suggesting a similarity between trypsinized BECs and those obtained from CF patients (Woods et al. 1980 a).

P. aeruginosa exoproducts, particularly proteinases and neuraminidase, are noted in many studies to enhance bacterial adherence and to act as virulence factors (Pastoriza & Hulen 2006; Soong et al. 2006). The importance of proteinases in facilitating attachment is substantiated by Saiman et al. (1990). They reported that PAO1 and P1 strains, with detectable proteinases during adherence assay, have the greatest adherence. The increased number of receptors in CF patients who have already been infected by *P. aeruginosa* might relate to the fact that their cells have been previously exposed in vivo to *P. aeruginosa* neuraminidase (Saiman et al. 1992). The milieu of CF, especially hyperosmolarity, may specifically activate the expression of several genes (e.g. the neuraminidase *nan1* gene) that initiate colonization and facilitate long-term infection (Cacalano et al. 1992; Lanotte et al. 2003). *P. aeruginosa* cells adhere to BECs *via* pili, which recognize a GalNAcβ1-4 disaccharide exposed in asialylated glycolipids, such as asialo-GM1 (Woods et al. 1980 b; Saiman & Prince 1993; De Bentzman et al. 1996). Several arguments suggest that the *nan1* gene encodes neuraminidase, an enzyme able to release terminal sialic acid residues from sialylated gangliosides, thus increasing the amount of potential bacterial receptors and consequently enhancing adhesion (Saiman & Prince 1993; Lanotte et al. 2003, 2004; Pastoriza & Hulen 2006). The removal of sialic acid residues from cell surface proteins also results in higher cell-to-cell contact, presumably by enhancing protein-protein interactions through reduction of the net negative surface charge of one of the distinct features of sialic acid (Sun et al. 2001).

In the present study, the *nan1* gene encoding neuraminidase was investigated for its role in the adhesion of clinical *P. aeruginosa* strains to human BECs.

MATERIAL AND METHODS

Bacterial strains

A total of 62 strains of *P. aeruginosa* were originally isolated from a variety of clinical specimens: faeces (26), urine (12), wound swabs (9), bronchial washings (8), and individual strains obtained from blood, sputum, throat swab, ulceration swab, swab from skin around tracheotomy, and from ear. The bacteria originated from 62 patients from various wards of the municipal hospital, main hospital, and outpatients' department in Siedlce (Poland), between December 2005 and March 2006. The strains were identified as *P. aeruginosa* on the basis of typical morphology by Gram-negative staining, a positive oxidase reaction, growth at 42°C, and using the Api 20NE system (bioMérieux, France).

Identification of the nan1 gene

The frequency of the virulence gene encoding neuraminidase (*nan1*) was determined by PCR (LANOTTE et al. 2004). Amplification was performed with specific primers *nan1*-F (5′-ACGCTCCGTCCAGCCGGA-3′) and *nan1*-R (5′-GTCTGGACGACGGCGGCA-3′), yielding a 221-bp product. The PCR mixture contained PCR buffer (10 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 200 µmol of each dNTP (Boehringer, Germany), 0.0125 mmol of each primer, DMSO at a final concentration of 4%, 1 U of Ampli*Taq* DNA polymerase (Perkin Elmer, USA), and 25 ng of DNA template. The DNA was amplified in PTC-100 Programmable ThermoController (MJ Research, USA) using the following protocol: 94°C for 3 min, 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min, and finally 72°C for 5 min. PCR products were separated in 1% agarose gel for 1 h at 100 V, stained with ethidium bromide, and detected by UV transillumination.

P. aeruginosa adherence to human BECs

Adherence was assayed by the method of Woods et al. (1980 a). BECs were obtained by vigorous swabbing of the buccal mucosa of healthy, non-smoking people, with a sterile, cotton-tipped swab. The cells were suspended in phosphate-buffered saline (PBS), pH 7.4, and washed 3 times by centrifugation (10 min at 150 *g*) to remove unattached bacteria. A total cell count was then determined employing a haemocytometer. The concentration of BECs was adjusted to 2.0×10^5 cells per ml. Bacteria were grown at 37°C in tripticase soy broth (TSB) (Difco, USA) overnight, pelleted by centrifugation, twice washed in PBS, pH 7.2, and resuspended to a concentration of 10^8 colony-forming units/ml. The BEC suspension and bacterial suspension were mixed and incubated at 37°C for 30 min. After incubation, bacteria and BECs were again washed 3 times with PBS. Smears were made, air-dried, fixed in methanol, and stained with Giemsa staining solution. The number of bacteria adhering to BECs was counted under a light microscope. In each experiment, the first 30 well-defined epithelial cells were observed. Three independent trials were used to calculate the mean number of bacteria adhering to cells in each experiment.

Statistical analysis

The frequency of the *nan1* gene among *P. aeruginosa* strains isolated from different biological materials was examined statistically using chi-square test ($P \leq 0.05$). The association between the frequency of the *nan1* gene among *P. aeruginosa* isolates and adhesion of these bacteria to human BECs was studied by the Mann-Whitney *U* test ($P \leq 0.05$).

RESULTS AND DISCUSSION

The *nan1* gene is one of many virulence factors that can play an important role in the pathogenesis of *P. aeruginosa* infection (Antonov et al. 2010). Endimiani et al. (2006) reported that the *nan1* gene was detected in 57.9% of *P. aeruginosa* isolates, causing bloodstream infections. Our study confirms the observations of these authors: 52 (83.9%) from 62 of hospital *P. aeruginosa* strains carried the *nan1* gene.

There was no significant difference in the frequency of this gene among *P. aeruginosa* strains isolated from different sources ($P = 0.053$). The *nan1* gene was detected in 100% of strains isolated from faeces, 50% of strains from bronchial washings, 88.9% of strains from wound swabs, 66.7% of strains from urine, and 85.7% of single strains from other sources. The analysis showed that there is a significant difference in adhesion to BECs between the group of *P. aeruginosa* strains in which the *nan1* gene was present and the group of strains in which the gene was not detected ($P = 0.009$). Significantly higher adhesion was observed when the gene was present (a mean value of adhesion was 23.51) than when the gene was absent (Fig. 1). Lanotte et al. (2004)

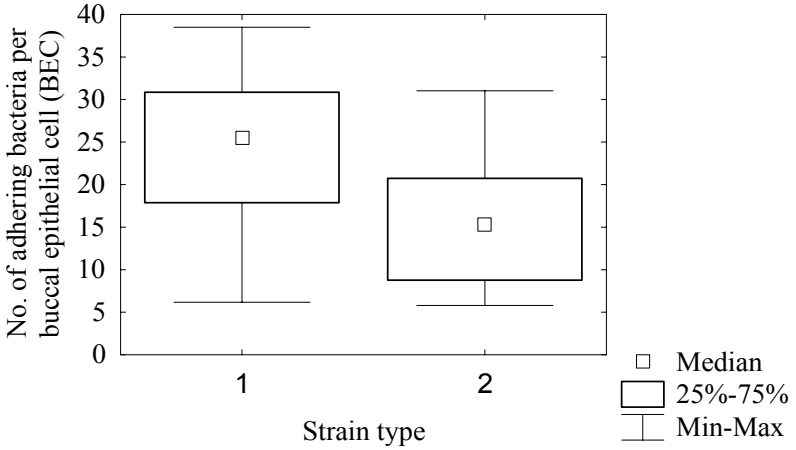

Fig. 1. Adhesion of *P. aeruginosa* strains to human buccal epithelial cells (assessed in 3 replications of 30 cells each), depending on the presence (1) or absence (2) of the *nan1* gene

demonstrated that the *nan1* gene was present in 61.7% of CF isolates and 44.4% of non-CF isolates. The frequency of this gene in CF isolates tended to increase as the clinical status worsened. Indeed, *nan1* was detected in 57% of isolates from patients with an excellent or good clinical status, in 63% of isolates from patients with a moderate status, and in 71% of isolates from patients with a poor or weak clinical status. The frequency of *nan1* in Bulgarian CF *P. aeruginosa* isolates in the study of Strateva et al. (2009) was significantly lower (38.2%) than that among French CF *P. aeruginosa* isolates. This correlates with the predominantly good clinical status of Bulgarian patients investigated. Pastoriza & Hulen (2006) observed that the presence of free sialic acid in culture media induces the secretion of neuraminidase by *P. aeruginosa*. As shown with A549 lung cells, this specific hydrolysis increases bacterial adhesion, probably by unmasking new binding sites on the cell surface. Incubation of BECs with the *Clostridium perfringens* neuraminidase in vitro significantly increased adherence of *P. aeruginosa* strains, since the mean number of

bacteria adhering to cells amounted then to 19.62±9.20, compared to 7.54±5.86 in the control group (Wolska et al. 2006). Cacalano et al. (1992) demonstrated that the *P. aeruginosa* neuraminidase was 1000-fold more active than the *C. perfringens* enzyme in releasing sialic acid from respiratory epithelial cells. This effect correlated with the increased adherence of PAO1 cells to epithelial cells after exposure to PAO1 neuraminidase. Our previous study (Wolska et al. 2005) demonstrated that the binding of *P. aeruginosa* strains isolated from humans and different animals to BECs was significantly reduced in vitro in the presence of the neuraminidase inhibitor, 2,2-dehydro-2-deoxy-N-acetyl-neuraminic acid (DANA). The greatest degree of inhibition by DANA was seen on the samples with the highest levels of adherence of control, suggesting that this high control may have been a result of neuraminidase-induced exposure of increased asialo receptors. Soong et al. (2006) reported that the PA2794 neuraminidase locus (*Delta 2794*) mutant of *P. aeruginosa* PAO1 was unable to colonize the respiratory tract. They suggest that inhibition of bacterial neuraminidase could provide a novel mechanism to prevent colonization of the respiratory tract by this important pathogen. A recent report showed that when the gene encoding neuraminidase is knocked out, it leads to a reduction in biofilm production in the lungs of mice, and it was suggested that the enzyme recognizes pseudaminic acid, a sialic acid analogue (Xu et al. 2009).

The results of this study show that *Pseudomonas aeruginosa* strains adhere much better to BECs carrying the *nan1* gene than to BECs lacking the gene. This suggests an important role of the *nan1* gene in the pathogenesis of *P. aeruginosa* respiratory infections.

Acknowledgements: We sincerely thank Mrs. Irena Lataczfor checking the text in terms of language.

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