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Variation of the adhesion to polystyrene of phenotypic mutants of *Pseudomonas aeruginosa* ATCC 27853 during starvation conditions

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SUMMARY

The aim of this work was to analyse the effects of different growth conditions (phosphate and contemporary carbon-phosphate starvation) on polystyrene adhesion of a strain of *Pseudomonas aeruginosa* ATCC 27853 and its four phenotypic mutants during experimental growth in starvation conditions. Bacterial adhesion was measured at 20, 40, 60 and 720 min. Data obtained showed that growth conditions are an important factor for the capacity of initial adhesion to inanimate surfaces. The analyses of adhesion of two phenotypic mutants (Mut-P-01 and Mut-P-02) isolated during growth on phosphate starvation is interesting. This kind of experiment yields important information on the prevention of nosocomial infections.

KEY WORDS: Pseudomonas aeruginosa ATCC 27853, Phenotypic mutants, Polystyrene, Adhesion, Starvation

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Pseudomonas aeruginosa is the epitome of an opportunistic pathogen of humans (Vanhrecke *et al*., 1990). This bacterium is frequently cultured as transient flora from hospitalized patients, but is also able to cause urinary tract infections, ulcerlike keratitis, respiratory system infections, cystic fibrosis, nosocomial infections and a wide range of severe and sometimes fatal diseases particularly in patients with severe burns, cancer and AIDS (Bodey *et al*., 1983; Briandet *et al*., 1999).
Adhesion of *Pseudomonas aeruginosa* to biomaterial surfaces is an essential step in the pathogenesis of these infections. The *Pseudomonas* infection may be separated into three distinct stages:

1) bacterial attachment and colonization of the implants;
2) local invasion;
3) disseminated systemic disease or destruction of the above mentioned implants (Jucker *et al*., 1996).

Numerous studies (McEldowney *et al*., 1986; Jucker *et al*., 1996; Van Schie *et al*., 1999; Donlan, 2002) show us that bacterial adhesion is a complicated process and it is generally described as a two-step process (Marshall *et al*. 1971). In the first step, the micro-organisms come close enough to the surface to be weakly held by electrostatic and superficial tension forces. In the second step the attached microorganisms are more difficult to remove from the surface and the bacteria produce exopolysaccharides that eventually form the biofilm matrix which is firmly adherent to the substrate (Zottola 1991).
In the first step of adhesion, the hydrophobicity of the bacterial cell surface was identified as the most important factor governing the initial mechanism of bacterial adhesion (Donlan, 2002; Vesterlund *et al*., 2005).

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The environmental factors (nutrients concentration, temperature, time of exposure, pH), the chemical composition of the material surface and physiological status of bacteria (shape, dimension, metabolic status) were factors influencing the process of adhesion.
Bacteria in the natural environment are often found under nutrient-limiting conditions (Peterson *et al*., 2005) and the colonization of solid surfaces has been described as a basic and natural bacterial stratagem in a wide variety of environment and the tendency to adhere to surfaces as a bacterial survival mechanism (Kjelleberg *et al*., 1983; Jana *et al*., 2000; Hunt *et al*., 2004).
These considerations are very important to explain the pathogenesis of any bacterium to avoid any implication of bacterial adhesion to medical devices.
This study analyzed the influence of different growth conditions (phosphate and contemporary carbon-phosphate starvation) on the capacity of initial adhesion to polystyrene of an strain of *Pseudomonas aeruginosa* ATCC 27853 (Vanhaecke *et al*., 1990) and its four phenotypic mutants isolated during experimental growth in starvation conditions.
Pseudomonas aeruginosa ATCC 27853 (ATCC Corp., Manassas, Va., USA) and phenotypic mutants Mut-P-01, Mut-P-02, Mut-CP-03 and Mut-CP-04 were used in all the experiments.
Strains Mut-P-01, Mut-P-02 were phenotypic mutants isolated during growth in phosphate starvation of a wild-type *Pseudomonas aeruginosa* ATCC 27853 respectively at the 7^{th} and 8^{th} days of experimentation. Phenotypic mutants Mut-CP-03 and Mut-CP-04 were isolated at the 6^{th} and 10^{th} days of cultivation of *Pseudomonas aeruginosa* ATCC 27853 in concomitant carbon and phosphate starvation. During growth in *Pseudomonas* agar base, Mut-P-01 and Mut-CP-03 present small mucous colonies, Mut-P-02 and Mut-CP-04 present small colonies.
To study the initial adhesion to polystyrene, cultures were prepared in M9 minimum salt medium (Sigma-Aldrich S.r.l. Milan, Italy).
In control cultures M9 medium was supplemented with 4% glucose as carbon and energy source.
Phosphate starvation was realized by changing the phosphate sources present in M9 buffer salts with an equivalent concentration of KCl and NaCl. Concomitant carbon and phosphate starvation was realized by inoculating bacteria in M9 minimum salt medium without glucose source and with M9 buffer salts modified.
The bacterial strains were incubated, in the media described, at 30°C with shaking (Certomat IS B. Braun Biothec International, 80 rpm) for 24 hours.
The cells grown were harvested by centrifugation at 11250 x g for 10 min, washed twice with PBS buffer (pH =7.4) at a final concentration of 3 x 10^8 cell ml^{-1} (A_{600nm} =0.3). The suspended cells were put in a polystyrene Petri plate (60 mm) and incubated at 30°C without shaking.
At regular intervals of 20, 40, 60 and 720 min the capacity of polystyrene adhesion was analysed such as percentage of hydrophobicity (%HP).
Cell surface hydrophobicity was determined by partitioning cell suspensions into a polystyrene plate and aqueous phases after incubation as previously described (Cappello *et al*., 2006). The percentage of hydrophobicity (%HP) of the experimental bacterial suspension was calculated using the following equation:

$$\% \text{ HP} = (OD_{INIT} - OD_{EXP}) \times 100 / OD_{INIT}$$

where OD_{INIT} was the optical density reading at 600nm (Beckman Spectrophotometer DU-640, Beckman Coulter Inc., Fullerton, Calif., USA) of the suspension before incubation in the polystyrene plate, and OD_{EXP} was the optical density of the bacterial suspension after incubation.
All the experiments were repeated three times and two parallels with the same conditions were analysed for each experiment. Statistically significant differences between experiments were detected by the analysis of variance (ANOVA).
The quantitative estimation of the initial adhesion to polystyrene measured as percentage of hydrophobicity (%HP) to *Pseudomonas aeruginosa* ATCC 27853 and its phenotypic mutant is reported in Figure 1.
The results obtained showed that difference growth condition may influence the capacity of adhesion of *Pseudomonas aeruginosa* ATCC 27853 and of phenotypic mutants Mut-P-01, Mut-P-02, Mut-PC-03 and Mut-PC-04.
Although it has been widely demonstrate that, in nature, the bacteria live in limited growth conditions, study of the adhesion ability in starvation

conditions is fundamental to analyze the real pathogenicity and behaviour of the bacterial cells in situations that best mirror the environmental conditions in which bacteria normally are found. During growth in M9 minimum salt medium supplemented with 4% glucose the analysis of the percentage of hydrophobicity (%HP) of *Pseudomonas aeruginosa* ATCC 27853 showed, in the first 720 min, an increment with values going from %HP = 24 to %HP = 62. The curve of cellular hydrophobicity presented a similar course for all bacterial strains characterized by a rapid in-

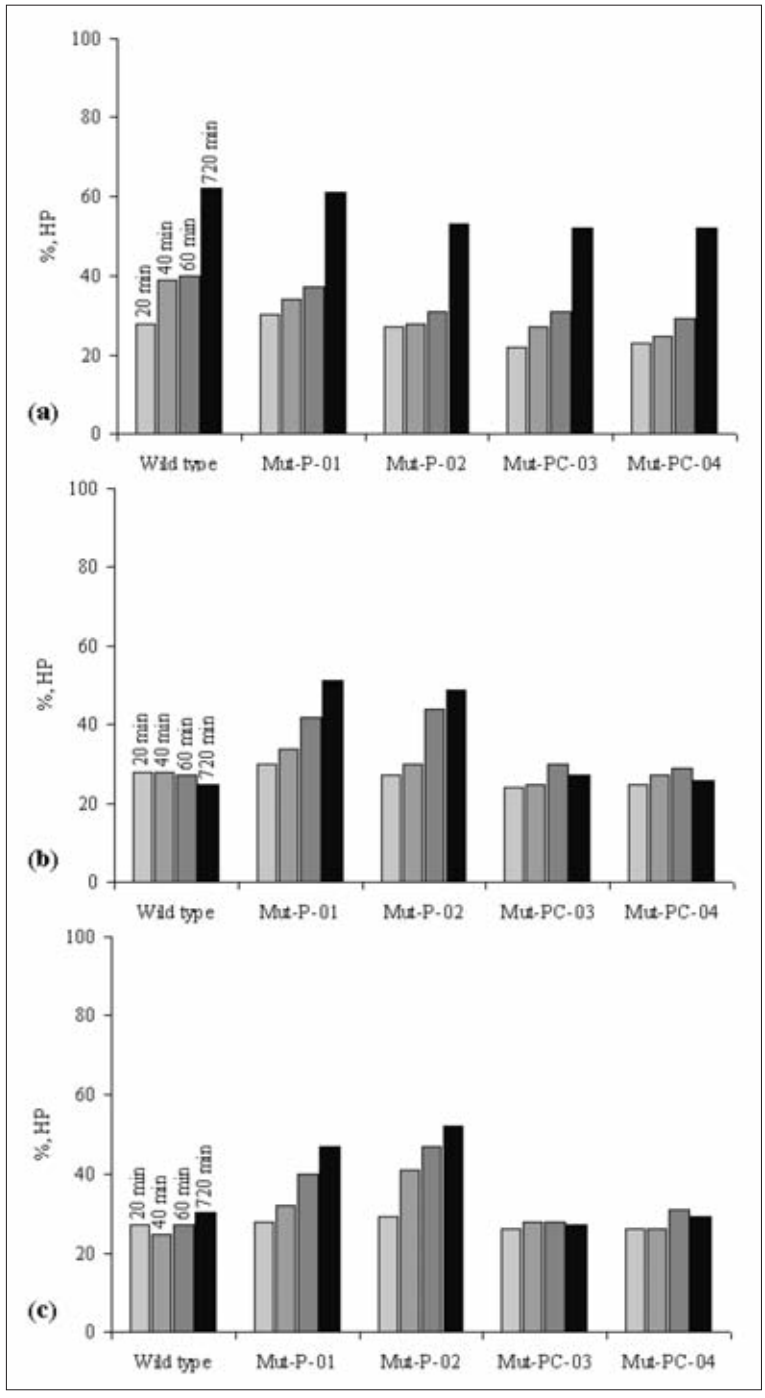

FIGURE 1 - *Variation of percentage of hydrophobicity (%HP) of Pseudomonas aeruginosa ATCC 27853 (wild type) and its phenotypic mutant Mut-P-01, Mut-P-02, Mut-PC-03 and Mut-PC-04 to polystyrene during cultivation in M9 minimum salt medium supplemented with 4% glucose (a), phosphate starvation (b) and concomitant carbon and phosphate starvation (c).*

crease in values of %HP after 60 min of experimentation. During phosphate starvation *Pseudomonas aeruginosa* ATCC 27853 and strains Mut-PC-03 and Mut-PC-04 showed a general decrement on percentage of hydrophobicity, with medium values of %HP =25. Conversely, in strains Mut-P-01, Mut-P-02 the values of adhesion increased from the starting time to the 60th min with the peaks, respectively, of %HP =52 and 56 after 720 min of experimentation. During concomitant carbon and phosphate starvation throughout the experiment the curve of percentage of hydrophobicity (%HP) of *Pseudomonas aeruginosa* ATCC 27853, Mut-PC-03 and Mut-PC-04 presented a similar course like that observed during the cultivation without phosphate. Also in this experiment strains Mut-P-01, Mut-P-02 presented greater values of hydrophobicity respect the others strains analyzed; after 60 min of incubation the percentage of hydrophobicity (%HP) increased, reaching maximum peaks after 720 min (%HP = 47 and 52, respectively).

Results obtained are in according with data present in literature and showed that growth conditions influence more or less negatively the initial hydrophobicity of *Pseudomonas aeruginosa* ATCC 27853 with a more or less marked effects depending on the stress applied.

Studies on the physiology of nondifferentiating bacteria have emphasized that to survive prolonged periods of starvation, many bacteria have evolved sophisticated stress response mechanisms to combat nutrient limitation and to enable them to persist in the environment until conditions become favourable for growth (Watson *et al*., 1998).

During phosphate starvation, variations of bacterial adhesion could be caused by the reutilization of the cellular phosphate, that may allow the synthesis of structures implied in the cellular adhesiveness such as the fimbriae. To end the certificated release of vesicle of external membrane during the phase of increase (Kadurugamuwa *et al*., 1999) which could be carried to their inside structures such as LPS of B-type (Kadurugamuwa *et al*., 1995; 1997) could determine a different distribution of the superficial charges in the external surface of the plasmatic membrane (Makin *et al*., 1996) consequently determining temporary variations in adhesiveness. In this case, the capacity of adhesion to inanimate surfaces of phenotypic mutants Mut-P-01, Mut-P-02, appeared particularly important showing elevated values of adhesion also in starvation conditions.

Strains Mut-P-01 and Mut-P-04 can be considered the adaptive mutants selected by the situation of induced stress (phosphate deficiency) characterized *in primis* by variations in adhesion ability and, presumably, in those structures (ex. fibriae, pili) involved in processes of colonization. With reference to results obtained, the ongoing study on these bacterial strains will prove particularly interesting. Analyses of external membrane proteins, variation in pilation, analysis of possible genomic mutations were steps fundamental of this research. In conclusion out experiments designed to estimate the dynamics of superficial adhesion of *Pseudomonas aeruginosa* and mutant strains generated in stress conditions open a important experimental window on the understanding of the natural phenomena of adhesion and the results appear particularly important if related to the health problems caused by the colonization of Pseudomonadacee and particularly by *Pseudomonas aeruginosa* on inanimate supports.

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