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EXPERIMENTAL ARTICLES

Extracellular Factors Affecting the Adhesion of *Pseudomonas fluorescens* Cells to Glass Surfaces

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Abstract—Two factors affecting the adhesion of *Pseudomonas fluorescens* to glass surfaces were revealed in the culture liquid (CL) of this bacterium. One of these factors, adhesin, which is responsible for cell adhesion, was found to be a protein substance located both at the cell surface and in the CL. Bacterial cells grown in rich LB medium were less adhesive than cells grown in minimal M9 medium. The adhesive capacity of cells was independent of the growth phase. The other factor, antiadhesin (AA), which reduces cell adhesion, was found only in the CL. AA concentration in the CL increased with the culture age.

Key words: adhesion, *Pseudomonas fluorescens*, extracellular antiadhesin

The ability of bacterial cells to adhere to various surfaces is of vital importance for their adaptation to different ecotopes and unfavorable conditions [1]. The adhesion of bacteria to solid surfaces is even considered as a strategy of their life in aquatic habitats [2]. The attachment of bacterial cells to a surface modifies their metabolism and morphology and promotes their adaptation to unfavorable conditions [1]. The mechanisms of cell attachment to various surfaces are well studied. Cell adhesion was found to be promoted by some exopolymers, such as polysaccharides, lipopolysaccharides, glycoproteins, and proteins [3], as well as by an increased motility of cells [4]. On the other hand, reduced cell adhesion was observed when bacteria produced some specific exopolysaccharides [3, 5], enzymes capable of modifying adhesive polymers [6], and surfactants [7], as well as when cell motility was decreased [4]. It should be noted that researchers paid much attention to the factors promoting cell adhesion, whereas the mechanisms responsible for the inhibition of bacterial adhesion are yet insufficiently studied.

The aim of the present work was to search for extracellular compounds involved in the regulation of the adhesion of the soil bacterium *Pseudomonas fluorescens* to glass surfaces.

MATERIALS AND METHODS

The strain *Pseudomonas fluorescens* NCIMB 9046 was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) in Aberdeen, United Kingdom. The strain was grown in M9 medium [8] supplemented with glucose (0.2%) and trace elements (mg/l): $MgSO_4 \cdot 7H_2O$, 247, $CaCl_2$, 14.7, $MnSO_4 \cdot 4H_2O$, 0.4, $ZnSO_4 \cdot 7H_2O$, 0.4; and $FeCl_3 \cdot 6H_2O$, 1.0 (pH 7.0) [9]. The bacterium was cultivated in 100- or 250-ml flasks containing, respectively, 10 or 50 ml of medium, on a Lab-line orbital incubator shaker (180 rpm) at 30°C. The growth medium was inoculated with a stationary-phase culture (16 h of growth) at concentrations ranging from 2 to 5% and cultivated for 3–5 h to the exponential growth phase. Experiments were performed with three preparations: (a) exponential-phase culture; (b) washed cells; and (c) the culture liquid (CL) filtrate. To obtain washed cells and the CL filtrate, the CL was passed through a 0.45-μm-pore-size Millipore filter. The residue cells were washed with fresh nutrient medium using the same filter and resuspended either in the nondiluted nutrient medium or in the medium diluted twofold with the CL filtrate. In all the experiments, the initial optical density (OD) of cell suspensions ranged from 0.05 to 0.1 units. Culture growth and cell adhesion were followed by measuring the OD at 600 nm on a Pye-Unicam SP-450 UV/VIS spectrophotometer with a scale division value of 0.01 units. A drop in the optical density of cell suspensions, which was observed after inoculation of the medium, was explained as bacterial adhesion to the flask walls (evidence for this is given below).

To study the effect of material on cell adhesion, we used flasks made of glass, polypropylene, and polycarbonate (BDH, United Kingdom). In some experiments, glass flasks were covered from the inside with Teflon (GTI Limited, United Kingdom) or siliconized with Sigmacote (Sigma, United States) as recommended by the manufacturers.

Cell adhesion was characterized by two parameters: the adhesion value and the adhesion time (the time of cell occurrence in the attached state).

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The adhesion value, defined as the percentage of cells attached to the flask walls, was calculated by the following formula:

$$(OD_{ini} - OD_{min})/OD_{ini} \times 100\%, \quad (1)$$

where OD_{ini} is the optical density of culture immediately after inoculation and OD_{min} is the minimum value of the optical culture density corresponding to maximum cell adhesion.

Adhesion time was defined as the time elapsing from the moment of inoculation to the moment of cell detachment from the surface (the latter moment corresponded to a drastic increase in the optical culture density).

The concentration of antiadhesin (AA) in CL was expressed in arbitrary units defined as the amount of AA in 1 ml inducing a twofold decrease in cell adhesion under standard conditions (10 ml of culture in M9 medium with an optical density of 0.1 in a 100-ml flask). AA concentration was calculated by the formula:

$$AA = 2 \times (OD_{minCL} - OD_{min\ M9})/(OD_{ini} - OD_{min\ M9}), \quad (2)$$

where AA is the concentration of AA in the CL; $OD_{min\ CL}$ and $OD_{min\ M9}$ are the minimum optical culture densities in the CL and M9 medium, respectively; and OD_{ini} is the optical culture density at the moment of inoculation.

To elucidate whether adhesin is a protein, we compared cell adhesion in the CL treated with proteinase K (BDH, UK) at a concentration of 0.2 mg/ml for 2 h at 30°C with that in untreated CL.

All the experiments were replicated no fewer than five times; every data point was calculated from the results of two to three replicated measurements. Figures show the results of typical experiments. Statistical analysis (determination of arithmetic mean and standard deviation) was performed using the Statgraphics software package.

RESULTS AND DISCUSSION

The growth of *P. fluorescens* in glucose-containing minimal M9 medium was found to be dependent on the concentration of inoculum (Fig. 1). After inoculation with exponential-phase cells in amounts of 25–50%, the culture began to grow exponentially without a lag phase. As the inoculum concentration was reduced to 10% and less, there was a time period after the inoculation during which the optical density of the culture not only did not grow but even decreased, presumably, due to cell adsorption on the flask walls. The amount of adsorbed cells was inversely proportional to the inoculum concentration (Fig. 2). Typically, the optical density of culture after inoculation decreased by 20–80% for 30–60 min and then increased for 1–2 h at a rate exceeding the specific growth rate μ under given cultivation conditions by several times. During the following cultivation, the culture grew exponentially at μ 0.5 h^{-1} (Figs. 1, 3, and 5). In some experiments, almost all cells left the liquid phase, and the OD of the medium fell to nondetectable values (below 0.002 OD unit). Both the extent and the rate of OD lowering were dependent on the OD and concentration of inoculum, the proportion between the volumes of culture and the flask, and the flask material.

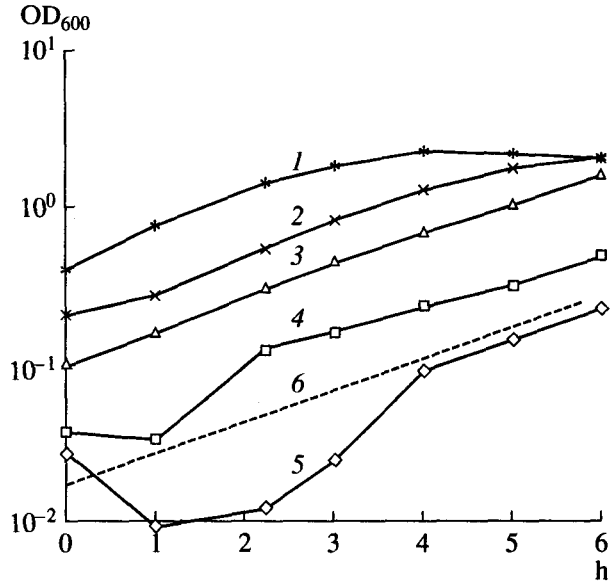

Fig. 1. Growth of *P. fluorescens* as a function of inoculum concentration (%): (*1*) 100, (*2*) 50, (*3*) 25, (*4*) 10, and (*5*) 5. Dashed line (*6*) indicates the extrapolation of the exponential region of the growth curve to the moment of inoculation.

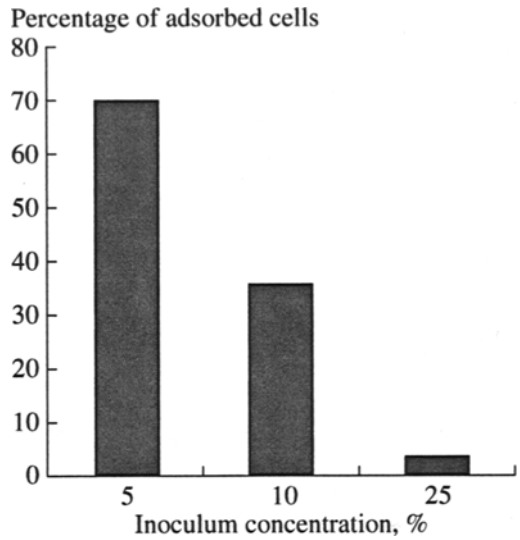

Fig. 2. Percentage of adsorbed cells as a function of inoculum concentration.

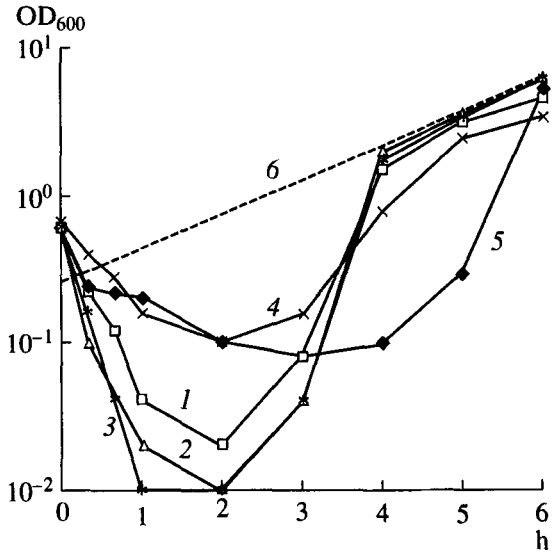

Fig. 3. Dependence of the growth of *P. fluorescens* on the flask material: (*1*) glass; (*2*) polycarbonate; (*3*) polypropylene; (*4*) Teflon-coated glass; and (*5*) siliconized glass. Dashed line (*6*) indicates the extrapolation of the exponential region of the growth curve to the moment of inoculation.

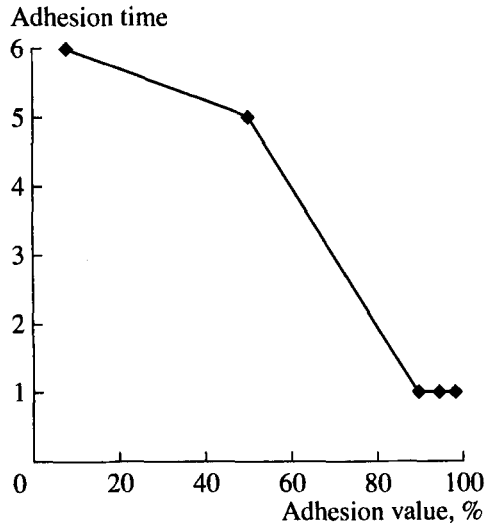

Fig. 4. Adhesion time versus adhesion value.

The assumption that the decrease in the OD of the culture following inoculation is due to cell adhesion to the flask walls was confirmed by the following observations. (a) After the removal of a culture from a flask, its walls were covered with a whitish deposit containing bacterial cells. (b) Attached cells could be desorbed from the flask walls to fresh medium by vigorously shaking the flask. The medium soon became turbid due to cell desorption from the flask walls. The OD of the thus-obtained cell suspension comprised 50–90% of the expected value calculated based on the assumption that all of the adsorbed cells were desorbed during the shaking. (c) When the culture in a flask was replaced with fresh medium, we observed a prolonged lag period followed by an exponential increase in the medium turbidity. The observed rate of the turbidity increase could not be accounted for by the growth of cells present in negligible amounts in the medium drops remaining on the flask walls, but could be explained by the growth of higher amounts of cells detached from the flask walls. (d) The amount of attached cells was found to be directly proportional to the area of the flask walls (in this experiment, equal amounts of culture (10 ml) were added to 50-, 100-, 250-, and 500-ml flasks (data not shown)). (e) The decrease in the OD after inoculation was always followed by a rapid increase in the culture turbidity up to the initial or even greater value lasting 1–2 h. The rate of this increase exceeded the maximum specific growth rate of the culture (0.5 h^{-1}) by several times. Therefore, the rapid increase in culture turbidity can only be explained by cell detachment from the flask walls. (f) Extrapolation of the exponential region of the growth curve in fresh media to the onset of the experiment (dashed lines in Figs. 1, 3, and 5) gives OD values close to the initial culture turbidity. Therefore, within the period of the culture turbidity drop, the cells, although bound to the flask walls, grew exponentially.

Similar changes in culture turbidity were observed when bacteria were cultivated in flasks made of (or covered with) weakly adhesive materials. In these experiments, 10 ml of medium in 250-ml flasks were inoculated with 0.2-ml inocula. The rate and the value of cell adhesion were maximum in polypropylene and polycarbonate flasks and minimum in the glass flasks covered from the inside with Teflon or siliconized with Sigmacote (Fig. 3).

The adhesive properties of bacterial cells taken from the exponential and early stationary growth phases were similar (data not shown). The composition of medium considerably influenced cell adhesion. For example, within one hour of inoculation, 80% of cells grown in M9 medium and only 30% of cells grown in LB medium were adsorbed to the flask walls. Therefore, the cultivation of bacteria in rich medium led to a two- to threefold decrease in cell adhesion.

Analysis of the data presented in Fig. 3 showed that the adhesion value and adhesion time were inversely related (Fig. 4).

The data presented above were interpreted through the accumulation of an extracellular compound reducing the adhesion of *P. fluorescens* cells to solid surfaces, which was named antiadhesin (AA). When the CL is diluted or replaced with fresh medium, the AA concentration decreases and bacterial cells adhere to the flask surface. This leads to the concentration of cells in a small volume of the near-wall liquid and, consequently, to the accumulation of AA. When the AA concentration in the near-wall CL becomes sufficiently high, cells detach from the flask surface, and the culture turbidity rapidly rises. This suggestion was tested in the experi-

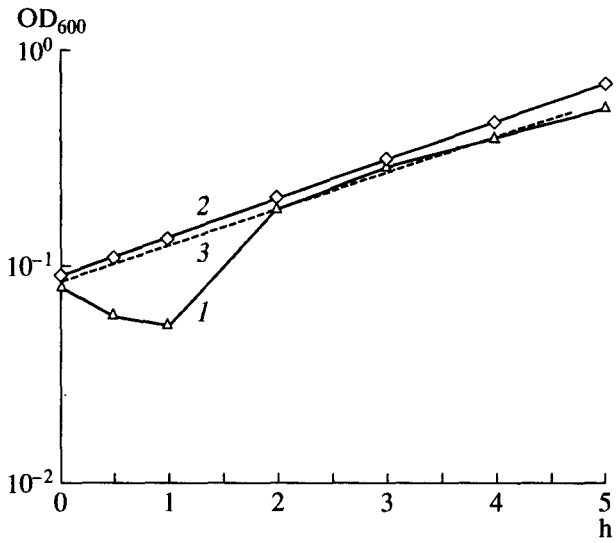

Fig. 5. Growth of *P. fluorescens* in (*1*) fresh medium and (*2*) fresh medium diluted twofold with the CL. Dashed line (*3*) indicates the extrapolation of the exponential region of the growth curve to the moment of inoculation.

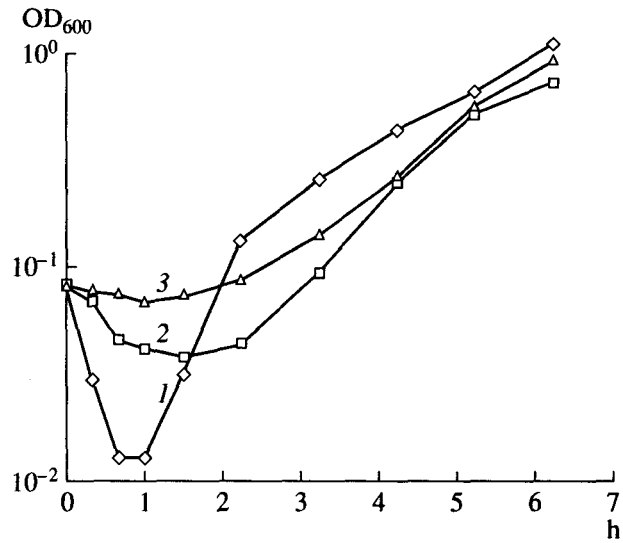

Fig. 6. Growth of *P. fluorescens* in (*1*) M9 medium; (2) M9 medium diluted with the untreated CL; and (*3*) M9 medium diluted with the CL treated with proteinase K.

ments described below. The twofold dilution of CL with fresh medium only slightly increased cell adsorption (Fig. 5), whereas adsorption in completely fresh medium was high (Fig. 6). The inhibitory effect of AA on cell adhesion increased in the course of the exponential growth of the culture. The AA concentration in the CL with a turbidity of 0.7 OD units was 2–3 AA units, with the maximum value of six units.

Another adhesion factor, adhesin, was believed to be located at the cell surface, since the washing of bacterial cells on filters did not make them incapable of adhesion. However, the treatment of CL with proteinase K considerably decreased cell adhesion (Fig. 6), indicating the degradation of an extracellular compound of protein nature responsible for cell adhesion.

Our data concerning a high-molecular exopolymer of protein nature responsible for cell adhesion are consistent with the relevant data available in the literature. In particular, the adhesion factor of *Pseudomonas* sp. is a protein-containing exopolysaccharide [3], and the adhesion factors of lactic acid bacteria and azospirillas are proteins [10, 11]. To the best of our knowledge, antiadhesin-like compounds have not yet been described in the literature.

The effect of the inoculum concentration on the growth characteristics (lag phase, biomass yield, etc.) of microbial cultures has long been known, but its influence on the cell adhesion value was first revealed in this study. In the case of common laboratory cultures (such as *Escherichia coli* and selected strains of other species), the transfer of exponentially growing cells into fresh medium results in the continuation of exponential growth. Conversely, *P. fluorescens* cells transferred to fresh medium mostly adhered to the glass surface. This fact is explained by the important role of cell adhesion in the life of aquatic and soil bacteria [2]. Microbiologists usually deal with irreversible, long-term, strong adhesion leading to the formation of biofilms and microcolonies as specific forms of bacterial life. Such adhesion considerably influences bacterial metabolism, growth rate, and cell resistance to unfavorable conditions [1]. Meanwhile, the adhesion discussed here is reversible in terms of the definition proposed by Marshall [3]: bacterial cells can be easily washed off of solid surfaces or detach themselves from surfaces in a natural way within 0.5–1 h of attachment. In our experiments, the reversibility of cell adhesion is proved by the following facts: (1) bacterial cells readily detached from the flask surface in the presence of AA; (2) the majority of cells (up to 90%) could be washed off by vigorous shaking of the flask; (3) the concentration of desorbed cells calculated by extrapolating the exponential region of the growth curve to the moment of inoculation was close to the amount of inoculated cells; and (4) attached cells continued their exponential growth at a rate typical of free-living cells. Therefore, reversible adhesion is a mechanism for the rapid (within one cell division cycle) adaptation of microbial cells to varying environmental conditions without considerable changes in the life strategy. A microorganism reversibly attached to a surface can choose between the possibility of returning to the free-living state, which provides for better growth but poorly protects it from unfavorable conditions, and the possibility of remaining in the attached state, which provides for poorer growth but better protects it from unfavorable conditions. The importance of such a mode of cell adaptation is evident from the very fact of the existence of a fine mechanism for its regulation involving adhesin and antiadhesin. The significance of reversible adhesion as the first stage of the

transition of bacterial cells from the free-living state to the state of irreversible attachment is also recognized in the literature [3].

The study of the properties of adhesin and antiadhesin is in progress.

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