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EXPERIMENTAL ARTICLES

Some Properties of the *Pseudomonas fluorescens* Adhesin and Antiadhesin

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Abstract—Some properties of the adhesion-modifying factors of *Pseudomonas fluorescens* are described. Adhesin, which promotes the adhesion of *P. fluorescens* cells, is a hydrophobic compound of a protein nature with a molecular mass of more than 10 kDa located either at the cell surface or in the medium. Antiadhesin, which suppresses the adhesion of *P. fluorescens* cells, is a thermolabile hydrophobic compound of a nonprotein nature with a molecular mass of less than 3 kDa. Heating makes antiadhesin hydrophilic. The role of adhesin and antiadhesin in the adhesion and adaptation of *P. fluorescens* cells is discussed.

Key words: adhesion, factors, properties, *Pseudomonas fluorescens*

The present study is a continuation of our investigation [1] of two adhesion-modifying factors of *Pseudomonas fluorescens*, adhesin and antiadhesin, with emphasis on their physicochemical properties.

MATERIALS AND METHODS

The strain *Pseudomonas fluorescens* NCIMB 9046 used in this study was obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) in Aberdeen (Scotland). Cultivation and experimental conditions were described in detail in the accompanying paper [1]. Experiments were carried out with exponential-phase cells separated from the culture liquid (CL) by filtration and washed. Adhesion was studied by adding cells to fresh cultivation medium or to medium diluted twofold (unless otherwise stated) with CL.

Cell adhesion was characterized by two parameters, adhesion value and adhesion time. 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 the culture at the moment of inoculation and OD_{min} is the minimum value of the optical density of culture corresponding to the maximum level of cell adhesion.

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

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

$$AA = 2 \times (OD_{min\ CL} - OD_{min\ M9})/(OD_{ini} - OD_{minM9}), \quad (2)$$

where AA is the concentration of AA; OD_{ini} is the optical density of the culture at the moment of inoculation; and $OD_{min\ M9}$ and $OD_{min\ CL}$ are the minimum optical densities of the cultures in, respectively, M9 medium and M9 medium to which CL was added. To judge the physicochemical properties of the adhesion factors, we tested how the following treatments affected the adhesion-modifying activity of the culture liquid:

the heating of CL in a water bath at 100°C for 20 min;

the incubation of CL with 0.2 mg/ml proteinase K (BDH, United Kingdom) at 30°C for 2 h;

the solid-phase extraction of CL compounds on a SepPak C18 column (Millipore, United States); the adsorbed material was eluted with 5 ml of methanol, evaporated to a volume of 1 ml, and tested for adhesion-modifying activity in an amount of 0.1 ml/10 ml M9 medium;

the ultrafiltration of CL through YM membranes (Millipore) with a molecular weight cut-off of 3 or 10 kDa; the 30-fold concentrate was tested for adhesion-modifying activity in an amount of 0.2 ml/10 ml M9 medium.

All the experiments were performed no fewer than five times. The results presented are the means of 2–3 replicated measurements of a typical experiment. Statistical parameters (arithmetic mean and standard deviation) were calculated using the Statgraphics software package.

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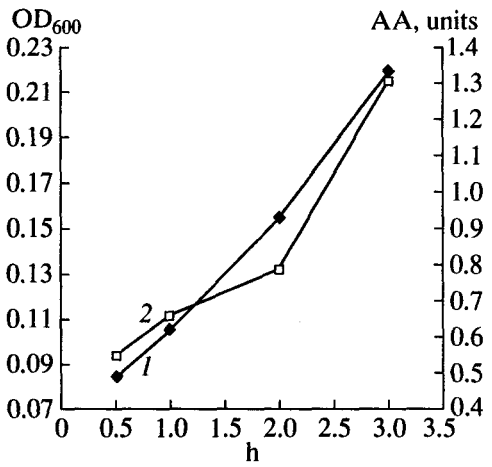

Fig. 1. Effect of culture age on the ability of CL to diminish the adhesion of *P. fluorescens* cells to glass: (*1*) growth; (2) concentration of antiadhesin in CL.

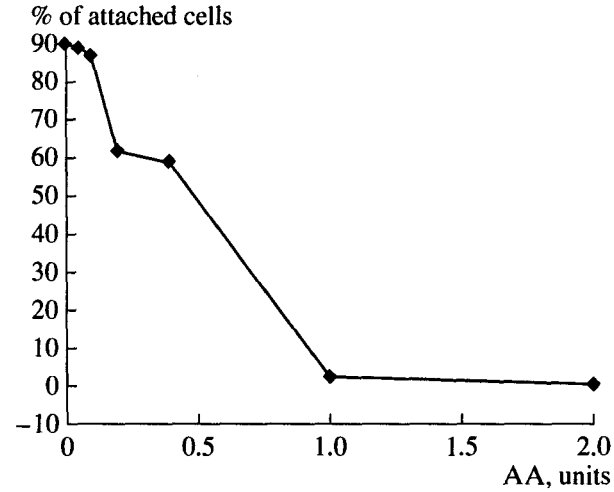

Fig. 2. Effect of antiadhesin as a function of its concentration.

RESULTS

Dynamics of AA during Culture Growth

Earlier, we found that AA tends to accumulate in the medium during culture growth [1]. A more detailed analysis showed that the concentrations of biomass and AA in the culture liquid grow in parallel (Fig. 1).

Identification of the AA Target

If the culture liquid with a high content of AA was shaken in a flask for 30 min and then removed, the subsequent incubation of cells in fresh M9 medium in this flask did not lead to the effect typical of AA. This suggests that AA interacts with a substance occurring in the medium or on the cell surface rather than with the glass surface.

The concentration dependence of the effect of AA was studied by adding CL to M9 medium in amounts up to 90%. As can be seen from the results presented in Fig. 2, the concentration dependence is described by a two-phase curve with saturation. Formula (2) was valid only for the region of the linear dependence of cell adhesion on the AA concentration. At concentrations exceeding 1 AA unit (saturation point, Fig. 2), AA concentrations were calculated based on the amount of the CL added and AA concentrations determined from the linear region of the concentration curve. Typically, the concentration of AA in the CL with optical densities exceeding 0.7 units was 2–6 AA units and, hence, corresponded to saturation AA concentrations. The two-phase behavior of the AA concentration curve suggests that adhesin, the possible target of AA, can be in two states, either free or bound to cells, which agrees with the data presented in the accompanying paper [1].

Properties of the adhesin and antiadhesin of *P. fluorescens*

Adhesion factor	Molecular mass, Da	Nature	Hydrophobicity
Adhesin	>10000	Protein	Hydrophobic
Antiadhesin	<3000	Nonprotein	Hydrophobic, but becomes hydrophilic after incubation at 36°C for several hours

Physicochemical Properties of AA

Determined as described in the *Materials and Methods* section, are summarized in the table. Based on some experimental observations, in particular, that AA is able to pass through 3-kDa cut-off membranes (Fig. 3), is adsorbed on the reversed-phase column but can be eluted from it with methanol (Fig. 4), and fails to be inactivated by proteinase K (Fig. 4), it can be concluded that AA is a hydrophobic nonprotein compound with a molecular mass of less than 3 kDa. AA is a rather labile substance. Indeed, the heating of CL at 100°C for 20 min or at 36°C for a short time brought about a decrease in the AA activity of the CL by 5 and 2–3 times, respectively (Fig. 5). The storage of CL at –18°C for 7 days resulted in a complete loss of AA activity. In the presence of 30 μM *N*-ethylmaleimide (Sigma, United States), AA activity rapidly decreased two- to threefold. Of much interest is the fact that the incubation of CL at 36°C for several hours made AA hydrophilic, so that it could pass through the reversed-phase column.

The Properties of Adhesin

The table also presents the properties of adhesin, a substance that promotes the adhesion of *P. fluorescens* cells. Adhesin was obviously located at the surface of the cells, since their washing and resuspending in fresh cultivation medium did not cause a loss in adhesive

properties. The treatment of CL with proteinase K brought about a decrease in cell adhesion (Fig. 4), indicating the protein nature of adhesin. The CL ultrafiltrate somewhat decreased cell adhesion (Fig. 3), while the addition of the CL ultraconcentrate enhanced it again, albeit not to the level observed in fresh cultivation medium. The incomplete restoration of the adhesive properties of the culture might be due to the fact that the ultraconcentrate contains AA, which exerts its antiadhesive effect at rather low concentrations (Fig. 2).

DISCUSSION

Because of the interfering effect of adhesin, which is readily inactivated by proteinase K, experiments with this proteinase cannot be convincing evidence for the nonprotein nature of AA. We think, however, that, even if adhesin in the medium is inactivated by proteinase K, the amount of cell-bound adhesin would have been sufficient to cause cell adhesion, as was the case with the CL preliminary passed through the reversed-phase column and, hence, lacking hydrophobic AA and extracellular adhesin (Fig. 4) and with the transfer of cells to fresh medium [1]. The short-term contact of the cells with proteinase K could hardly cause the inactivation of all adhesin molecules on the cell surface; therefore, there would have been a decrease in the optical density of the culture. Actually, however, this was not the case: OD remained constant during 1–1.5 h of incubation (Fig. 4), suggesting that AA is not of a protein nature.

It should be noted that our data on the protein nature of adhesin agree with the data available in the literature. In particular, it was shown that the adhesion factor of *Pseudomonas* sp. is a protein-containing exopolysaccharide [2] and that the adhesion of lactobacilli and azospirillas is mediated by proteins [3, 4].

On the other hand, almost all the factors known to diminish cell adhesion are extracellular high-molecular-weight compounds. These are the emulsifier of staphylococci [5], the alginate-like exopolymer of *Pseudomonas fluorescens* [6], the lactobacillar protein suppressing the adhesion of *E. coli* cells [7], and the alginate lyase which degrades the adhesive polymers of *Pseudomonas aeruginosa* [8]. Among low-molecular-weight substances with antiadhesive properties, there are detergents [9] and, presumably, chelating compounds [10]. In its properties, the low-molecular-weight AA described in this paper is close to detergents but has some properties atypical of detergents. In particular, it is inactivated at low and elevated temperatures and in the presence of oxidizers.

The assumption that antiadhesin interacts with the glass walls of incubation flasks was not confirmed in our experiments. On the other hand, the two-phase concentration dependence of AA action allowed us to suggest that AA interacts with two adhesin pools, extracellular and cell-bound.

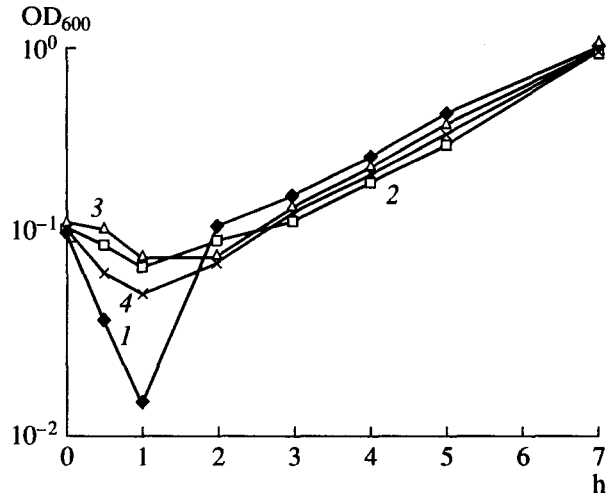

Fig. 3. Effect of ultrafiltration on the adhesion-modifying properties of CL: (*1*) M9 medium; (*2*) untreated CL; (*3*) ultrafiltrate of CL; and (*4*) ultraconcentrate of CL.

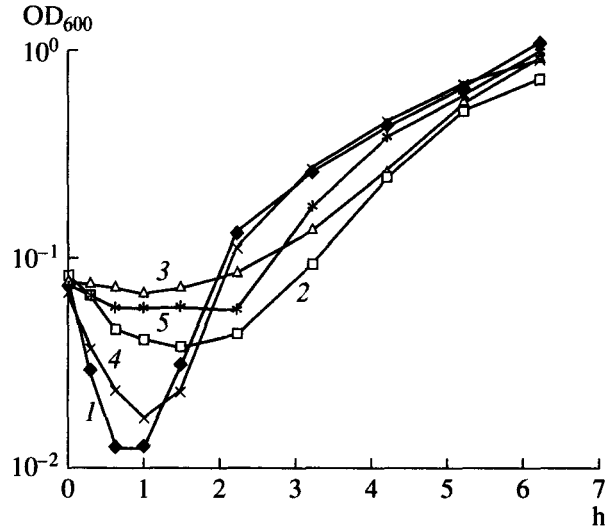

Fig. 4. Effect of proteinase K and extraction on a SepPak C18 column on the adhesion-modifying properties of CL: (*1*) M9 medium; (*2*) untreated CL; (*3*) CL treated with proteinase K; (*4*) CL passed through the SepPak column; and (*5*) M9 medium supplemented with methanol eluate from the SepPak column.

The significance of cell adhesion in bacterial life has now been recognized [2]. Based on the results of the experiments described in this paper, it is tempting to speculate on the possible relationship between the reversible attachment of *P. fluorescens* cells to solid surfaces mediated by adhesin and antiadhesin and the adaptation of this bacterium to unfavorable conditions.

Adhesin, a protein-containing polysaccharide occurring at the cell surface, promotes the attachment of bacterial cells to solid surfaces. Part of the cell-bound adhesin can be detached from the cell surface: the existence of free adhesin in the medium is evidenced by the

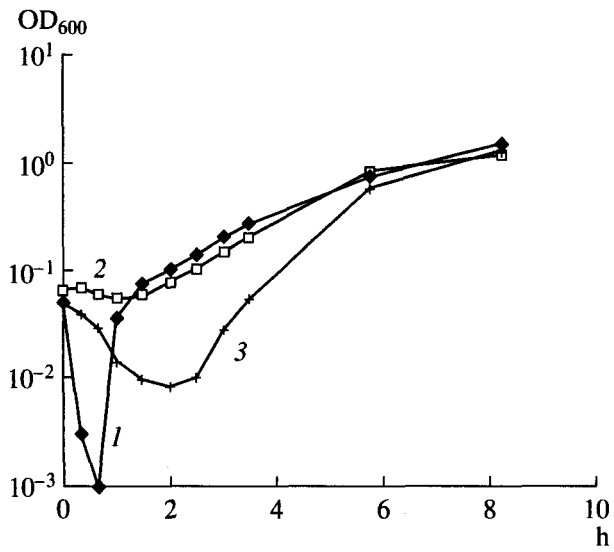

Fig. 5. Effect of heating at 100°C for 20 min on the adhesion-modifying properties of CL: (*1*) M9 medium; (2) untreated CL; and (*3*) heat-treated CL.

two-phase concentration dependence of the action of AA (an antagonist of adhesin) and by a decrease in total cell adhesion after the inactivation of free adhesin by proteinase K. AA neutralizes the action of adhesin and thus suppresses cell adhesion. It is the balance between adhesin and antiadhesin that determines the level of attachment of *P. fluorescens* cells to solid surfaces. Under favorable conditions, bacterial cells extensively secrete AA into the medium, due to which cells are free, grow well, and rapidly disseminate. In response to unfavorable conditions, such as the stress of an unknown medium (by which we mean a new or highly diluted medium), heating, or the action of the strong oxidative agent *N*-ethylmaleimide, the concentration or the antiadhesive activity of AA decreases, whereas cell attachment to solid surfaces increases. If new environmental conditions are beneficial for growth (as in the case of the dilution of the cultivation medium), cells remain metabolically active and continue to produce AA, as a result of which its concentration tends to increase, eventually reaching a value sufficient to block the action of adhesin and thus to cause the detachment of bacterial cells from the solid surface. Under stress conditions (elevated temperature or the presence of a poison, e.g., *N*-ethylmaleimide), cells are not very active, the concentration of AA is low, and cells remain attached to solid surfaces. The putative protective effect of reversible cell attachment can be explained as follows. The attachment of a great number of cells to a solid surface is equivalent to their concentration in a thin near-surface layer of the medium. In a first approximation, we can make use of the data of Characklis [11], according to which the thickness of the near-wall layer of the medium not involved in turbulent motion caused by flask shaking is about 40 μm. For our experimental conditions (100-ml flasks with 10 ml of medium shaken at a rotation speed of 100 rpm), the volume of the undisturbed near-wall medium is no more than 0.2 cm^3. Therefore, provided that all the cells in the flask are adsorbed, the concentration of the cell population will increase 50-fold. Reversible adhesion implies that cells may occur in both the free and attached states. The advantage of the free state is the possibility of unlimited material exchange between the cells and their environment, whereas the advantage of the attached state is that a high population density allows the cells to implement an efficient "collective defence" [15, 16] and cell-to-cell communications. The reversible adhesion can be considered a state of transient compartmentation, differing from the free or attached states. The volume of this compartment is rather high: the calculations based on the cell size and flask volume showed that this compartment can accommodate four times more cells than would theoretically grow under given conditions in a flask.

These speculations agree with the observations described above and in the accompanying paper [1]. If the rate of AA synthesis is proportional to the biomass (Fig. 1), the concentration of AA sufficient to suppress the activity of adhesin and to cause cell detachment will be attained faster the stronger the cell adhesion (Figs. 3 and 4 of [1]). However, the actual mechanism responsible for the cell-to-cell transfer of information about the attached state still remains unknown. In particular, such transfer may be mediated by homoserine lactones [12] or by as yet unidentified gaseous compounds (unpublished data). According to data available in the literature, the very attachment of cells to the solid surface can also cause some changes in cell metabolism [13] and stimulate the formation of exopolysaccharides [14]. As shown above, cell attachment can give rise to low-molecular-weight compounds.

Thus, we described the mechanisms of attachment of *P. fluorescens* cells to solid surfaces with the involvement of high-molecular adhesin and low-molecular antiadhesin and emphasized the protective significance of such an attachment. To gain closer insight into the mechanisms of cell adhesion, it is necessary to isolate and purify these adhesion-modifying factors. Work along this line is in progress.

ACKNOWLEDGMENTS

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