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EXPERIMENTAL ARTICLES

Saturated C_{21}–C_{33} Hydrocarbons Are Involved in the Self-Regulation of *Pseudomonas fluorescens* Adhesion to a Glass Surface

Yu. A. Nikolaev*, N. S. Panikov*, S. M. Lukin, and G. A. Osipov****

**Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117811 Russia*
***Research Institute of Cardiovascular Surgery, Russian Academy of Medical Sciences, Moscow, Russia*
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Abstract—One of the two putative groups of antiadhesins was identified in *Pseudomonas fluorescens* by the method of gas chromatography–mass spectrometry. A mixture of high-molecular unbranched hydrocarbons (HC) with a chain length from 21 to 33 carbon atoms reduced cell adhesion to a glass surface. These HC accumulated in the culture liquid to a total concentration of 10–15 μg/l; the concentrations of individual HC ranged from 0.1 to 3.0 μg/l. After the addition of individual HC to the bacterial culture, the number of cells attached to the glass surface decreased. This decrease in cell adhesion was due to the enhanced aggregation of the bacterial cells, which promoted mechanical (hydrodynamic) cell detachment from the surface.

Key words: adhesion, antiadhesin, *n*-alkane, *Pseudomonas fluorescens*, R variant.

Microorganism adhesion to solid surfaces is vitally important for their adaptation to the adverse environmental conditions in aquatic ecotopes [1, 2]. The mechanisms of adhesion are fairly well studied. Such compounds as exopolysaccharides, glycoproteins (lectins), or specific proteins (adhesins) mediate cell attachment to surfaces [3, 4]. Conversely, a release of specific polymers [3] or mediator-modifying enzymes causes cell detachment [5]. However, cell desorption and blockage of adhesion are still insufficiently studied. In particular, no evidence is available about the low-molecular extracellular metabolites inhibiting microorganism adhesion.

We have previously studied the reversible adhesion of *Pseudomonas fluorescens* to glass surfaces and showed that the culture liquid of this microorganism contains an adhesion-suppressing factor that we designated antiadhesin (AA) [6, 7]. In addition, the R–S dissociation of *P. fluorescens* was shown to lead to changes in cell adhesion and culture growth: R variants were superior to S variants in their ability to attach to glass surfaces [8].

Since the *P. fluorescens* population proved to be heterogeneous, we chose the R variant of the bacterium to serve as the test strain to increase the sensitivity of AA biotesting.

In this study, we isolated and identified an extracellular compound suppressing the attachment of the *Pseudomonas fluorescens* R variants to the glass surface.

MATERIALS AND METHODS

The bacterial strain and cultivation conditions used in this study were described previously, as well as the procedures of biotesting of the culture liquid (CL), CL extracts, or pure hydrocarbon (HC) preparations [6, 7] and the method used to isolate the R variant of *P. fluorescens* [8].

A decrease in the culture optical density (OD) indicated the degree of cell adhesion [6, 7]. The amount of antiadhesin (AA) was expressed in arbitrary units (AU) as described in [6, 7]. The amount of AA that reduced cell attachment to the flask walls twofold as compared to control was defined as 1 AU. The ratio of maximal decrease in OD to the original OD was taken to indicate the degree of adhesion. To avoid errors in the calculation of the AA amount, the measurements were made under strictly defined conditions: each time, the same test culture (R variant) was used; flasks of a definite shape were made of the same glass; the medium volume and intensity of shaking were also faithfully reproduced.

To obtain an active concentrated antiadhesin preparation by solid-phase extraction, 50 ml of CL exhibiting AA activity were passed through a column with solid-phase hydrophobic adsorbent (Sep-Pak C18 cartridge, Waters), and then AA was eluted with 1 ml of nonpolar solvent. Four different solvents were tested preliminary (hexane, ethanol, methanol, and diethyl ether). The best results were obtained with hexane; therefore, it was used in the subsequent experiments. Intracellular HC were determined upon drying bacterial cells at 105°C followed by extraction with hexane.

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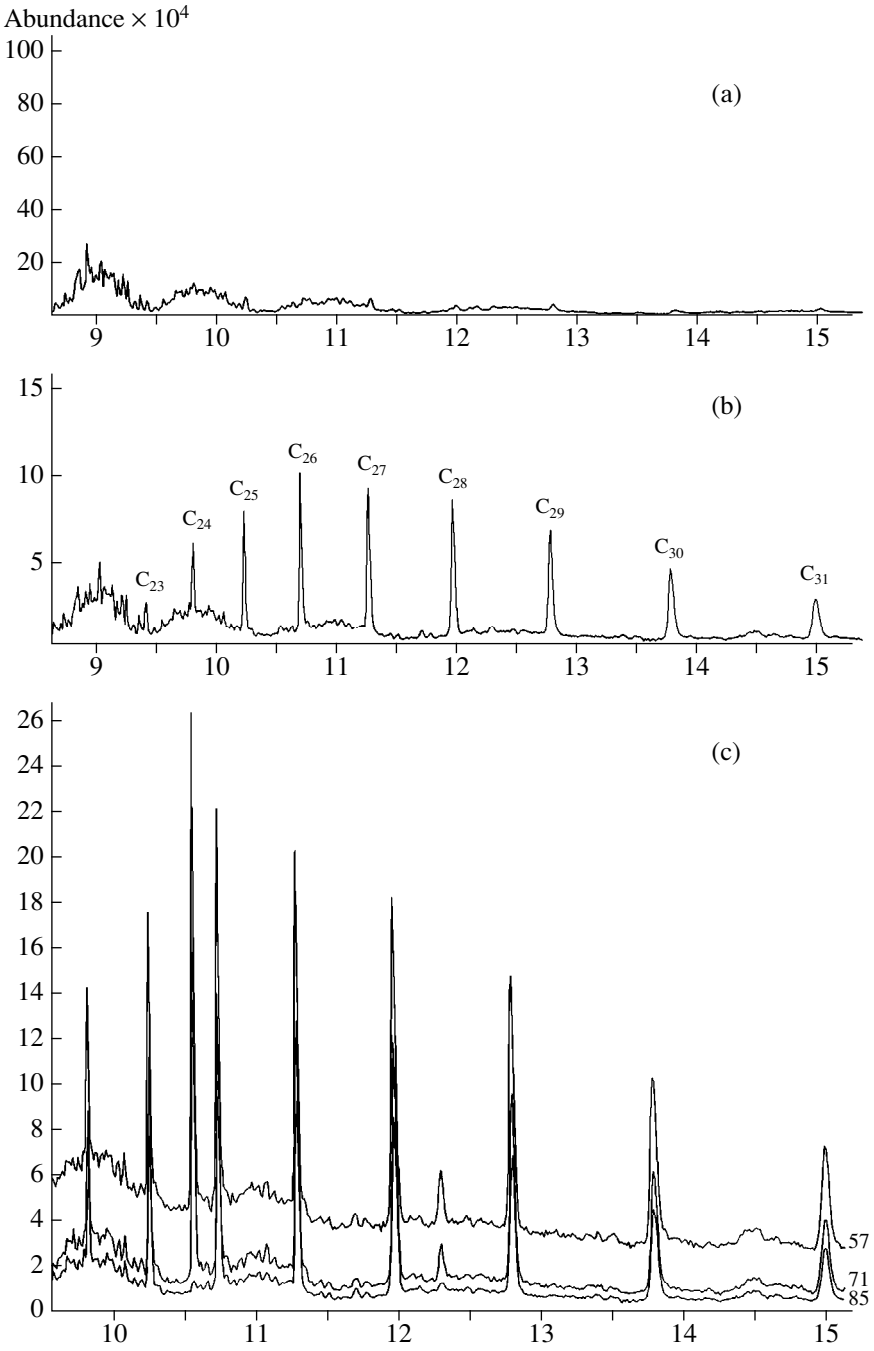

Fig. 1. Chromatograms of hexane extracts of original medium (a) and culture liquid (b and c). The abscissa shows the time of elution from the column (min); the ordinate shows ionic flow (arbitrary units) of ions 85 (a and b), 57, 71, and 85 (c). The peaks corresponding to *n*-alkanes $C_{23–31}$ are shown in Fig. 1b.

Sample analysis by gas chromatography–mass spectrometry (GCMS) was conducted as follows: CL (from 10 to 25 ml) was extracted with 2–5 ml of hexane, and the extract obtained was evaporated to a volume of 100 μl to be analyzed on a gas chromatograph (model 6890, Hewlett Packard) supplied with a mass-selective detector (model 5973, Hewlett Packard). Fractionation was performed on an HP-5ms column (Hewlett Packard) (30 m length, 0.25 mm inner diameter, and 0.25-mm-thick immobile phase). Carrier gas (He) flow was 1 ml/min. The temperature regime was as follows: 70°C isotherm for 1 min; programmed heating (25°C per minute) to 280°C; and then 10 min at 280°C. The sample volume was 1 μl. Registration of the spectrum of positive ions with a mass range from 29 to 600 was carried out upon ionization with an elec-

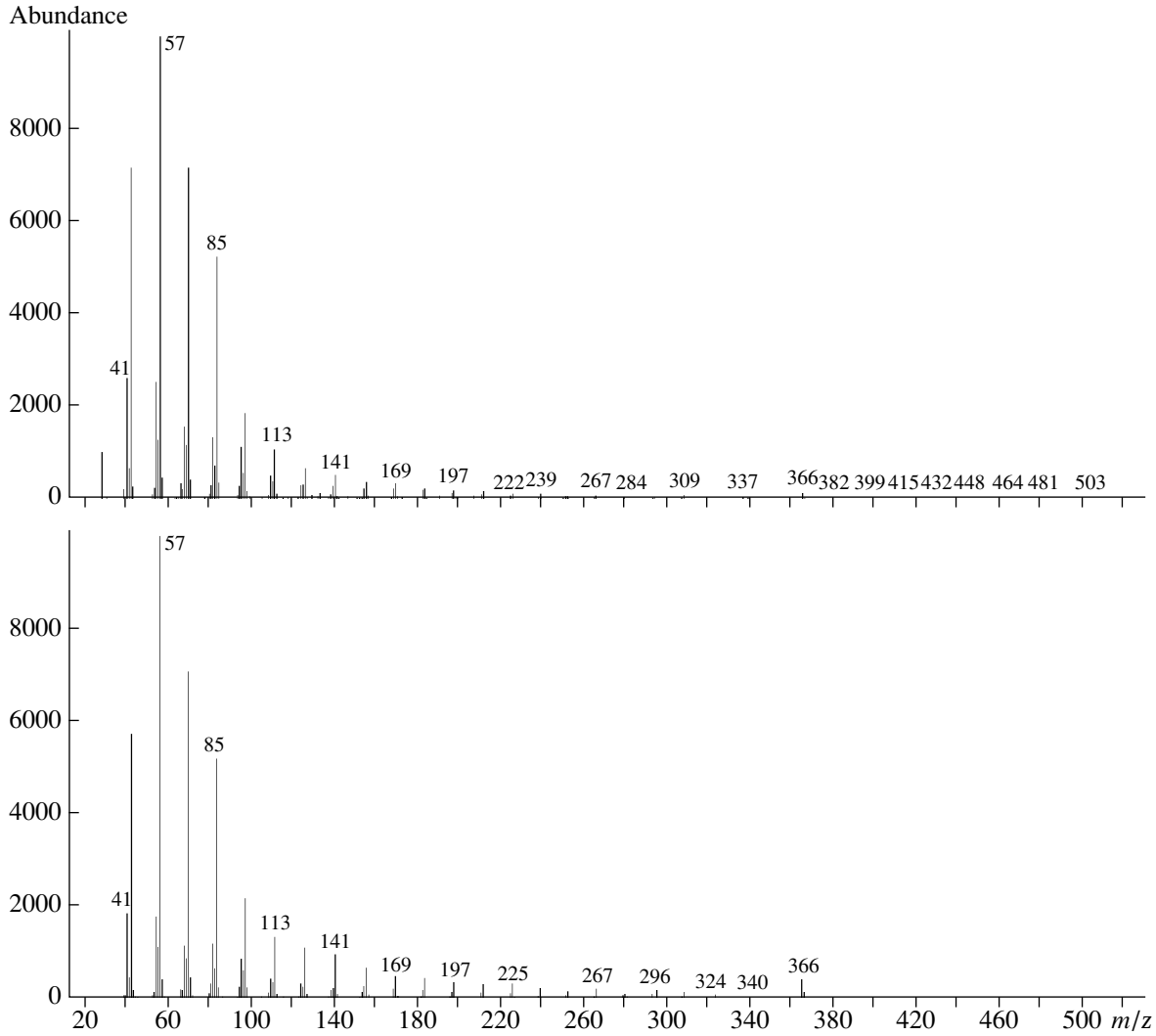

Fig. 2. Example of HC identification from their mass spectra: at the top is the mass spectrum of a compound with the retention time of 10.73 min (see Fig. 1b), which was identified as C_{26}-hexacosane; at the bottom is the mass spectrum of a pure hexacosane preparation.

tronic impact. The separated components were identified using the Willey275 and NTST98 spectral libraries and ChemStation software (Hewlett Packard).

The following hydrocarbon solutions in hexane were used: HC-1, which was a mixture of $C_{12–27}$ homologues (GCMS standard, Alltech), and HC-2, a mixture of $C_{20–32}$ paraffins (a solution prepared from a household candle).

To calculate HC solubility in water, the data on C_4–C_9 solubility in water [9] were extrapolated on longer HC.

RESULTS

Identification of HC by the GCMS method. After purification on C18 solid-phase adsorbent, the hexane extracts of the culture liquid exhibited antiadhesive activity. They were compared with control extracts of the original medium by the GCMS method.

Unlike the control medium, the bacterial culture liquid proved to contain saturated unbranched HC (paraffins) with the chain length from 21 to 33 carbon atoms. The concentration of each homologue ranged from 0.1 to 0.5 ng/ml of CL. As hexane treatment led to a more complete extraction of HC than solid-phase extraction, the data obtained with hexane extraction were analyzed.

The chromatogram regions corresponding to retention times from 9.5 to 16 min for ions with *m/z* 85 are shown in Fig. 1 to compare results obtained with the original medium and the bacterial culture liquid (Figs. 1a and 1b, respectively). The peaks corresponding to C_{23}–C_{31} HC are clearly discernible. Chromatograms of three ions with *m/z* 57, 71, and 85, characteristic of *n*-alkanes, are shown in Fig. 1c. The maxima of all three ions coincided in time with those revealed in the bacterial CL (cf. Figs. 1b and 1c), which suggests

that bacterial CL contains HC. By comparison of the mass spectra characteristic of the CL extracts with those given in the Willey275 database, the compounds studied were definitely identified. The spectrum of the compound with the retention time of 10.73 min (Fig. 2a) was quite similar to that of hexacosane (Fig. 2b). The degree of their identity calculated using ChemStation software was 95%. Analogous calculations were made for all other HC peaks, and the corresponding compounds were identified with an accuracy of no less than 90% (i.e., the expected error of identification is less than 10%).

The presence of the molecular ions is another strong argument in support of the fact that the compounds studied are *n*-alkanes rather than other hydrocarbons. Thus, the molecular ions with *m/z* 338, 352, 366 (Fig. 2a), 380, 394, 408, and 422 were revealed in the mass spectra of the most prominent HC peaks with retention times of 9.82, 10.25, 10.73, 11.30, 11.97, 12.79, and 13.77, respectively. These data correspond to empirical formulas of saturated hydrocarbons from *n*-tetracosane (C_{24}) to *n*-tricontane (C_{30}).

A strong correlation between the molecular mass of the HC identified in CL and their retention times on the column also confirms that these HC are *n*-alkanes rather than some other saturated hydrocarbons (e.g., branched hydrocarbons).

In the row of *n*-alkane homologues, the retention time is known to increase, at first, with an increment of 0.3 min per C atom (HC C_{20}–C_{22}) and, afterwards, with an increment linearly growing up to 1.9 min per C atom (HC C_{30}–C_{33}) (Table 1). Thus, the retention times in the row of *n*-alkanes follow a smooth curve approximating a polynomial of the third power. We observed in our experiments the same relationships between m/z and retention time, which would have been impossible if the contribution of the branched isomers were noticeable.

The results obtained are summarized in Table 1. The content of each individual HC in CL ranged from 0.1 to 3.0 ng/ml of CL, with a maximum for the C_{26} homologue, *n*-hexacosane (Fig. 1). HC concentrations never exceeded the near-saturation concentrations, although they approximated them.

Dynamics of AA formation. Distinctions between S and R variants. In the CL of the S variant, the amount of HC (from 0.05 to 0.6 ng/ml) was two to five times lower than in the CL of the R variant. These results are consistent with the fact that the CL of the S variant had a lower antiadhesive effect than the CL of the R variant. Thus, the higher adhesiveness of R bacterial forms was due to the higher affinity of these cells for the glass surface rather than to a reduced amount of AA in the CL.

In both S and R bacterial variants, the content of HC in the CL correlated with that in the dried cell biomass (DCB). The biomass of the R variant contained from 0.4 to 6 μg of individual HC per gram of DCB, whereas the S-variant biomass contained a two- to threefold lower amount of HC, i.e., from 0.2 to 2 μg per gram of DCB. The total content of HC in R cells was about 60 μg/g of DCB; in S cells, from 20 to 30 μg/g DCB. A question arises of whether or not the HC determined in

Table 1. Hydrocarbons (*n*-alkanes) identified in the culture liquid of *P. fluorescens*

*N**	Hydrocarbon	Concentration, ng/ml	Solubility in water, ng/ml	Retention time, min
21	*n*-Cheneicosane	0.9	16	8.80
22	*n*-Docosane	0.8	13	9.15
23	*n*-Tricosane	1.0	8.9	9.43
24	*n*-Tetracosane	1.4	6.3	9.82
25	*n*-Pentacosane	1.1	4.5	10.25
26	*n*-Hexacosane	3.0	3.8	10.73
27	*n*-Heptacosane	1.2	2.6	11.30
28	*n*-Octacosane	1.2	2.1	11.97
29	*n*-Nonacosane	1.0	1.6	12.78
30	*n*-Tricontane	0.8	1.3	13.77
31	*n*-Chentriacontane	0.5	1.0	15.00
32	*n*-Dotriacontane	0.2	0.8	16.50
33	*n*-Tritriacontane	0.1	0.6	18.40

* Number of carbon atoms in the molecule.

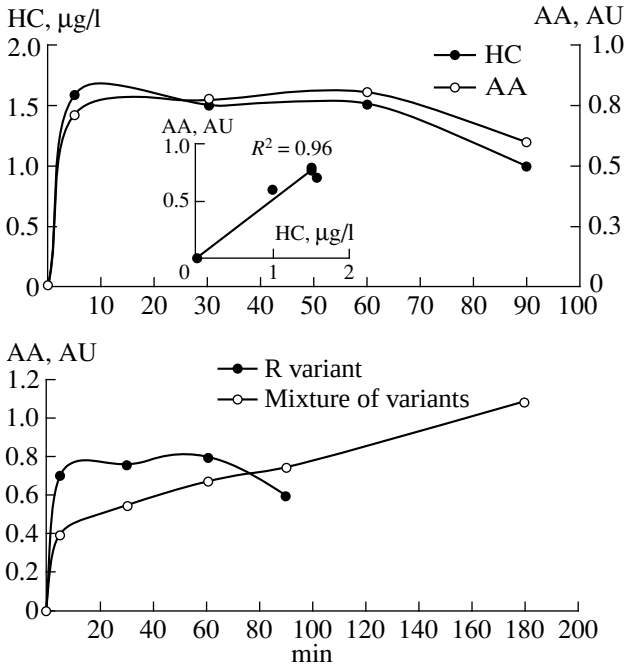

Fig. 3. Dynamics of HC and AA content in the culture liquid of *P. fluorescens*. At the top: comparison of HC and AA accumulation dynamics in the culture liquid of the R variant. The insertion shows the regression of AA content determined by biotesting versus HC content determined chromatographically. One arbitrary unit (AU) for AA corresponds to 2 μg HC/l. At the bottom: comparison of antiadhesin dynamics in the culture liquids of the R variant and mixed culture.

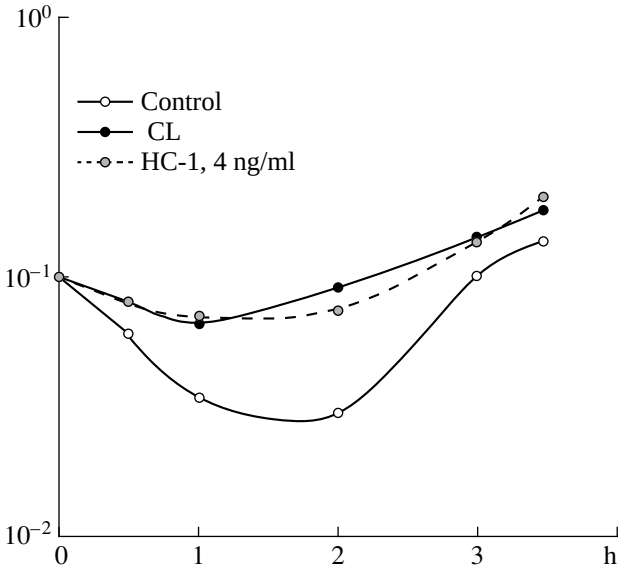

Fig. 4. *P. fluorescens* growth in the presence of culture liquid or HC (HC-1 preparation, 1.4 ng/ml).

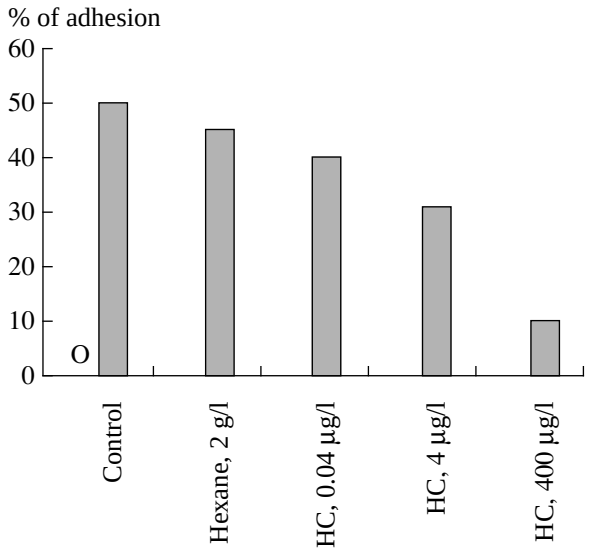

Fig. 5. Effect of HC concentration on *P. fluorescens* adhesion.

the CL were synthesized de novo or they were transferred to the solution from the cell wall. The second hypothesis seems more probable. It is supported by the dynamics of HC appearance in the culture liquid (Fig. 3). The maximum HC concentration in the CL was observed several minutes after the cells were washed and transferred into a fresh medium; i.e., the time needed for HC biosynthesis is greater than the time it takes HC to appear in CL. Accumulation of AA in *P. fluorescens* culture liquids (obtained from either R variants or from a mixed population containing both S and R bacterial forms) followed the curves shown in Fig. 3. The maximum AA concentration was reached in the CL of R bacterial forms a few minutes after they were transferred to a fresh medium, whereas AA accumulation in the CL of the original population did not reach a plateau for several hours.

Strong correlation between the HC and AA amounts in the CL of the R variant ($r^2 = 0.96$) suggests that HC exhibited antiadhesive properties.

The effect of individual HC added to the cultures. A mixture of HC added to the *P. fluorescens* culture completely reproduced the effect of the CL from the R variants of these bacteria: cell adhesion decreased similarly in the presence of either HC or the CL from the R bacterial forms (Fig. 4). In both cases, the degree of irreversible cell adhesion remained high, as judged from the absence of the phase when the OD began to increase rapidly. After a fall in OD for a definite time interval, the culture growth resumed at a normal specific rate, which indicates that no large-scale desorption of bacteria occurred.

The antiadhesive effect of HC (HC-1 preparation, C_{12}–C_{27}) on cell adhesion was studied at a wide range of concentrations, from 40 pg/ml to 400 ng/ml (Fig. 5); it was found to increase with the HC concentration. The HC-2 preparation was a hexane solution of a routine household candle. As determined in preliminary experiments, the HC composition of this preparation was similar to that of CL ($C_{20–32}$). The HC-2 effect resembled that of the HC-1 preparation.

The effects of different individual HC (C_{16}, C_{22}, and C_{34}) were studied in a concentration range from 4 to 400 ng/ml. In addition, an equivalent amount of hexane (C_6, 2 mg/ml) was introduced in all experiments. Hexane by itself inhibited adhesion by only 5 to 10%, as compared to the control (no hexane, no HC), even if its concentration was several orders of magnitude higher. Each of the three HC added at equal concentrations produced similar effects, which differed little from that of the HC-1 mixture added at the same concentration. On average, in a concentration range from 4 to 40 ng/ml, the adhesion decreased to a value of 30 ± 15%, which is only half of the 60% value obtained with fresh medium. Note that the results obtained varied considerably. At an HC concentration of 400 ng/ml, a decrease in adhesion reached 100%. Thus, the low-molecular HC (hexane) have a minor biological effect and only at extremely high concentrations. Comparison of C_{16}, C_{22}, and C_{34} revealed their high efficiency without a pronounced predominance of any single one. These results suggest that a mixture of high-molecular HC rather than an individual compound is responsible for the antiadhesive effect of CL.

Other properties of HC and AA. HC had no effect on the specific growth rate and cell yield. The content of HC in the CL remained unchanged upon boiling for 15 min or storing at –18°C for several days. Antiadhesive activity of the CL from the R variant of *P. fluorescens* was also resistant to such treatments, whereas that

Table 2. Properties of antiadhesins (AA) present in the culture liquids of the original (mixed) *P. fluorescens* culture and of its R variant

Property	AA of the mixed culture	AA of the R variant
Biological effect	Complete prevention of adhesion; the phase of rapid cell detachment is observed; complete desorption occurs	A lower effect; no phase of rapid cell detachment; incomplete cell desorption
Stability during boiling	Unstable	Stable
Stability during storage	Unstable	Stable
Time needed for manifestation of antiadhesive properties	Extended; the maximum is reached in 2–3 h	Instantaneously
In what populations found	Only in the mixed culture	In both mixed culture and R-variant culture

of the CL from the original strain (containing both R and S variants) showed great lability [7].

Phenomenology of the *P. fluorescens* adhesion. It is interesting to describe the specific features of pseudomonad adhesion and experimental nuances, because they provide an insight into the mechanisms underlying the antiadhesive HC effects.

Cell adhesion depended even on whether the HC solution was introduced with a microsyringe or a automatic pipette. When a syringe was used, a more pronounced antiadhesive effect was observed and the results were more reproducible. The antiadhesive effect of HC depended largely on the volume of the preparation (either 20 or 2 μl): when even a smaller amount of HC was introduced with a microsyringe in a greater volume, the antiadhesive effect was more pronounced. This indicates that the HC effect depended largely on the degree to which these compounds were dispersed.

After the addition of a considerable amount of HC (from 40 to 400 ng/ml), cell aggregation was observed concomitant with a decrease in adhesion. This interesting phenomenon was revealed both microscopically (the number of cell aggregates increased) and by direct visual observation of dense granules (one tenth of mm in size) formed by aggregated cells. The cells attached to each other and to the surface of the HC drops. In these experiments, when HC looked like nonwettable drops or flakes on the surface, the OD dropped down instantly by 20–30% in a few seconds upon HC addition. No further decrease in optical density was observed during the culture incubation; OD gradually increased due to culture growth. This was regarded as a 100% blockage of adhesion.

DISCUSSION

Thus, the chemical nature of AA was established based on the following arguments: (1) several high-molecular HC revealed by the GCMS in the bacterial culture liquid were absent from the original medium; (2) the biological effect of the same exogenous HC introduced into the culture (inhibition of bacterial cell adhesion to a glass surface) was similar to the effect of CL; (3) the properties of antiadhesins determined in CL from the R forms of bacteria (thermostability, resistance during prolonged storage, etc.) were similar to those of individual HC (Table 2).

Thus, these three groups of different and independent evidence are consistent with the hypothesis that antiadhesins revealed in CL are high-molecular HC. Identification of AA was simplified by the fact that they proved to be well-known compounds having specific mass spectra of their ions.

The mechanism underlying antiadhesive effect of HC is a challenging problem for further studies. Several suggestions can be nevertheless made. A decrease in adhesion may occur due to two reasons: either cell attachment to the surface may be suppressed or, conversely, cells already attached may be removed from the surface. Since HC are slightly soluble in water (wherein their mobility is low), their intercalation between the glass surface and an adherent cell is improbable. On the other hand, hydrophobic bonds and ionic interactions between a cell and glass are primarily involved in adhesion [3, 10]. Therefore, HC probably act as competitive inhibitors of adhesion, blocking hydrophobic sites on the cell surface. Accelerated cell desorption is also possible, because HC binding to an attached cell interact with other cells to form a heavy multicellular aggregate, which is readily detached from glass surface. Thus, cell aggregation induced by HC promote a decrease in cell adhesion, which was demonstrated in our direct experiments.

An assumption that HC may serve as AA falls into the following scheme of bacterial metabolism and ecology: (i) HC easily reach saturating concentrations in the aquatic medium and are efficient at a minor concentration; (ii) HC are synthesized by numerous organisms [11, 12] and may serve as indicators showing whether a given cenosis is sufficiently abundant and whether the habitat is favorable; (iii) HC are readily eliminated from the aquatic medium because of their adsorption on hydrophobic surfaces, which suggests that they may serve as biologically active regulatory compounds (inertialess elimination and easy restoration of the active concentration in the medium).

In addition, cell aggregation enhances the capacity of *P. fluorescens* to colonize new habitats, because a

large cell community in this case is superior to single cells.

The reversible adhesion, when cells can leave the surface under the influence of a flow of liquid [3], is not only the first stage of irreversible adhesion, but also an important adaptive phenomenon. We believe that this process is also important because it ensures compartmentalization and population structuring when the advantages of different ways of life (plankton and attached forms) are combined [6].

In this study of *P. fluorescens*, we have also shown that special attention should be given to the dissociation of microbial strains. We succeeded in our experiment only after the R and S bacterial variants were studied separately.

The mere fact that HC can be present in a bacterial culture liquid has already been described. As noted in manuals, the bacterial biomass [13, 14] and the biomass of many other microorganisms [11] contains HC. HC concentration in bacteria ranges from 0.003 to 2.69% of the dry biomass weight. We determined that the content of HC in *P. fluorescens* (0.006%) approximated the least value. In *Rhodopseudomonas spheroides*, *Rhodospirillum rubrum*, and *Escherichia coli*, the same parameter equals to 0.006, 0.005, and 0.003%, respectively. In CL of *Desulfovibrio desulfuricans*, the content of HC was reported to be as high as 3.5–3.8 mg/l [12], which is two orders of magnitude higher than that in pseudomonad CL. All the HC revealed in *Escherichia coli* were *n*-alkanes. However, the published data on the content of HC in the CL of bacteria are fragmentary.

The HC functions remain unclear. They were assumed to render cell walls hydrophobic and increase cell resistance. Our results suggest some new functions of these compounds. They can (1) regulate bacterial adhesion, (2) promote cell aggregation and, probably, cooperation, and (3) serve as an indicator of the presence of well-growing cells, i.e., an indicator of the quality of the environment.

Note that after HC addition to the test organism (the R form of *P. fluorescens*), the adhesion phase was not followed by the rapid removal of the attached cells from the glass. The degree of irreversible adhesion was rather high in our experiments. This was probably due to the fact that we used a model system consisting of only R variants of bacteria. The natural adhesion and culture growth patterns with correspondent changes in OD can probably be reconstructed with the natural populations, which commonly contain both R and S bacterial forms and wherein some additional group of AA might have a stronger effect on the attached cells.

The latter suggestion is supported by comparison of antiadhesive properties of CL from R bacterial forms and that from the mixed bacterial population (Table 2). These data suggest that one more antiadhesin is present in CL of the natural (mixed) population.

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