Changes in the Hydrophobic-Hydrophilic Cell Surface Character of Halomonas elongata in Response to NaCl

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Received 18 June 1987/Accepted 6 October 1987

Phase-partitioning studies of the euryhaline bacterium Halomonas elongata demonstrated that the hydrophobic-hydrophilic nature of the cell surface changed as the bacterium grew in different NaCl concentrations. Mid-log-phase cells grown in a high (3.4 M) NaCl concentration were more hydrophilic than were cells grown in a low (0.05 M) NaCl concentration. Mid-log-phase cells from defined medium containing 3.4 M NaCl normally produced a hydrophobicity reading of only 14 (hexadecane hydrophobicity = 100), while corresponding cells from defined medium containing 0.05 M NaCl gave a hydrophobicity reading of 90. Compared with cells grown in low salt concentrations, cells grown in high salt concentrations were more hydrophilic at all stages of growth. Rapid suspension of log-phase cells grown in 1.37 M NaCl into a 0.05 or 3.4 M NaCl solution produced no detectable rapid changes in surface hydrophobicity. These data suggest that as H. elongata adapts to different NaCl concentrations, it alters the affinity of its outermost cell surface to water.

Halomonas elongata is a euryhaline bacterium able to tolerate a wide range of NaCl concentrations (18). Vreeland and Martin (19) previously demonstrated that this requirement is directed to the sodium portion of the salt. Later, Martin et al. (4) showed that the sodium cation was necessary for α-aminoisobutyric acid uptake and suggested the presence of a Na⁺-amino acid transport mechanism. Recently, Vreeland et al. (17) demonstrated that adaptation of H. elongata to increasing salinity forces the bacterium to make several structural and biochemical modifications to its cell wall. Outer membrane blebs present on cells grown in low salinity were not observed on cells grown in higher NaCl concentrations. The nucleoplasm and ribosomes became densely packed, and freeze-fracture patterns suggested that the cell envelope was more coherent at elevated NaCl levels. Various researchers have reported the increased presence of charged fatty acid (2, 7–9) and phospholipid (16, 17) species in salt-tolerant bacteria after growth in high NaCl concentrations. This information has led to the development of several hypotheses concerning the water relations of euryhaline bacteria. This is particularly important since no study of euryhaline bacteria has as yet demonstrated that these microorganisms are in osmotic balance with their external environment (5, 6, 20). The cytoplasm of most of these bacteria has been found to be more dilute than the external milieu (20). The hypotheses have included a two-membrane, three-compartment model for moving water against its concentration gradient and the structuring of cell-associated water to slow or restrict water movement (16).

At present, there is no satisfactory explanation for the salt tolerance of Halomonas elongata. Therefore, it was of interest to study the relationship of water and the cell surface. The purpose of this investigation was to measure the relative hydrophobic-hydrophilic nature of the outer envelope of cells grown in a range of salinities.

MATERIALS AND METHODS

Culture. The organism used in these experiments was H. elongata 1H9 (ATCC 33173) (15, 18). Stock cultures were maintained on modified Abram and Gibbons medium (18, 19) and were stored at 4°C. The cultures used in this study were inoculated and grown by the methods of Vreeland and Martin (19) in the defined medium described by Vreeland et al. (17).

Hydrophobicity assay. Cell surface hydrophobicity was determined by a modified phase partitioning assay on the basis of techniques of Rosenberg et al. (13) as described by Olsson and Westergren (10). Cultures were grown in defined medium, harvested at 10,000 × g, and washed twice in a sterile basal salt solution containing an NaCl concentration equal to that of the growth medium (19). The pH of the basal salt solution was adjusted to 7.2 to 7.3 with 20% KOH before autoclaving. The final pellet was suspended to an optical density of 0.5 at 436 nm. Portions (4 ml) of the cell suspension were dispensed into acid-cleaned test tubes (13 by 100 mm). The optical density of each assay suspension was measured after brief mixing. Then, 0.13 ml of n-hexadecane was added, and the system was allowed to equilibrate at 30°C for 10 min. Each tube was then vortexed for 60 s. The phases were allowed to separate for 15 min, and the optical density of the lower aqueous phase was determined. The percent decrease in the optical density of the aqueous phase was used as the measure of cell surface hydrophobicity (14); the hydrophobicity of hexadecane is 100. In all graphs, higher values denote more-hydrophobic cell surfaces.

The hydrophobicity of log-phase H. elongata cells was measured by growing cells in defined medium containing NaCl at concentrations ranging from 0.05 to 3.4 M. Cells were harvested after an optical density between 0.6 and 0.8 at 600 nm had been reached. Six assay suspensions were prepared and tested at each NaCl concentration. To verify that the cell numbers in each aqueous phase were comparable, the numbers of viable cells in the 0.05, 1.37, and 3.4 M NaCl assays were determined with standard surface-spread plates containing complex media. Colonies were counted after 7 days of incubation at 30°C. The possibility that any observed differences in cell surface hydrophobicity were caused by instantaneous effects of the different NaCl concentrations was examined by rapidly suspending cells grown in 1.37 M NaCl into 0.05 or 3.4 M NaCl. The surface

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Hydrophobicity of the cells was determined 1 h after suspension.

Hydrophobicity changes during growth cycles were studied by growing cells in 3 liters of defined medium supplemented with either 0.05, 1.37, or 3.4 M NaCl. These large cultures were periodically sampled for optical density measurement at 600 nm, pH measurement, and hydrophobicity testing. Except for lag-phase samples in which low culture density permitted preparation of only a single assay suspension, the results recorded for these experiments were averages of three hydrophobicity assays per sample per NaCl concentration; standard deviations of the means are also given.

**RESULTS**

The hydrophobicity of log-phase cells decreased as growth medium salinity increased (Fig. 1). Cells grown in a low-salt medium (0.05 M NaCl) were at least six times more hydrophobic than those grown in a high-salt medium (3.4 M NaCl). The cell surface hydrophobicity initially decreased slowly from the high value of 90 ± 3 in cells grown in low-salt medium (0.05 M NaCl) to 79 ± 5 in cells grown in medium containing 1.37 M NaCl. The rate of decrease was much more rapid with NaCl concentrations above 1.37 M, dropping to the low value of 14 ± 3 in 3.4 M NaCl (Fig. 1). Light-microscopic examination revealed that the shapes and sizes common to the cells grown at various NaCl concentrations were not affected by the assay procedure. The results of the total viable counts showed that the assay suspensions from the different NaCl concentrations tested contained comparable numbers of viable cells (ca. 2.5 \times 10^8 CFU/ml).

Results of experiments conducted to follow changes in the surface hydrophobicity during growth corroborated the observation that cells grown in the presence of 3.4 M NaCl were more hydrophilic than cells grown in the presence of 0.05 M NaCl. The experiments also showed that the results obtained from this assay, while being consistent at any given time, were dependent on the physiological age of the cells (Fig. 2).

When the individual growth curves were examined, it was apparent that cells grown in low salt concentrations were always more hydrophobic than cells grown in higher NaCl concentrations. The hydrophobicity of low-salt-grown cells peaked early in the log phase and changed only slightly

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**FIG. 1.** Differences in the surface hydrophobicity of log-phase cells of *H. elongata* after growth in different NaCl concentrations. Bars indicate standard deviations of the means.

**FIG. 2.** Changes in hydrophobicity of *H. elongata* during growth in 0.05 M (A), 1.37 M (B), and 3.4 M (C) NaCl. Bars indicate standard deviations of the means.
during the rest of the experiment. Figures 2B and C display entirely different hydrophobicity and growth profiles. The surface hydrophobicity of cells grown in medium containing 1.37 or 3.4 M NaCl rose from hydrophilic values in the lag and early log phases to more hydrophobic values in the mid-log phase. Relatively constant hydrophobicity readings were obtained in the late log and stationary phases. The data in Table 1 indicate that the pH changes occurring in the medium during the growth of cells did not affect the measured hydrophobicity. It is also apparent (Fig. 3) that the hydrophobicity differences of these cells were probably due to some cell-mediated physiological changes and not due to any sudden osmotic changes or to salinity interference with hexadecane. When log-phase 1.37 M NaCl-grown cells were suspended in either 0.05 or 3.4 M NaCl, the cells still had a hydrophobicity of 79 ± 5 after 1 h of exposure to the new salt concentration (Fig. 3).

**DISCUSSION**

The experiments described here show a definite trend toward increasing hydrophilicity in cells grown in high NaCl concentrations (Fig. 1). These results can be attributed to a variety of factors. In addition to real physiological differences, several factors could cause the type of results described here. The measured cell surface hydrophobicity could be dependent on the growth phase or growth rate of the bacterium. The observed differences might also arise as artifacts caused by pH changes or by NaCl interference with either the phase separation or the ability of hydrophobic cells to bind to hexadecane.

The data shown in Fig. 2 indicate that the hydrophobicity-hydrophilicity of the cells does indeed change in relation to the growth phase of the cells. This was specifically true with 1.37 and 3.4 M NaCl, where the first assays actually yielded negative values. Similar negative values have been reported by other researchers (1, 10, 12), but the reasons for such results were not addressed. A series of cell-free tests conducted at the start of this research indicated that hexadecane alone caused approximately a 0.04-U increase in the absorbance of the aqueous phase, corresponding to a hydrophobicity of −8. This increase was consistent at all NaCl concentrations. Therefore, the negative hydrophobicity readings may actually reflect slightly imperfect phase separations in suspensions of predominantly hydrophilic cells.

Another obvious characteristic of the curves shown in Fig. 2B and C is that the surface hydrophobicity tended to increase as the cells approached the stationary phase of growth. Rosenberg et al. (14) have reported similar observations for *Acinetobacter calcoaceticus*. This rise in hydrophobicity could be due to several reasons. The most attractive of these would be starvation, since the available nutrients are slowly used up. Kjelleberg and Hermansson (3) have shown that several bacterial strains exhibit increased surface hydrophobicity upon the initiation of a starvation regimen. However, not all bacteria seem to respond in the same way to nutritional depletion, since Olson and Westergren (10) demonstrated that the surface hydrophobicity of *Streptococcus mutans* decreases with culture age. Olson and Westergren (10) have also suggested that pH changes during growth in batch culture might affect surface hydrophobicity. No such effect was found during this study (Table 1). Either the pH remained constant while surface hydrophobicity changed, or surface hydrophobicity remained constant as the pH fluctuated. At present, the available data are consistent with the conclusions of Kjelleberg and Hermansson (3). Regardless of the reason for the changes occurring during the growth of these bacteria, the fact is that the cells grown in 3.4 M NaCl were always more hydrophilic than those grown at other NaCl concentrations.

As stated above, it is conceivable that differences in cell hydrophobicity-hydrophilicity could be caused by differences in the growth rate of the bacteria under different conditions. The differences seen in this study, however, probably did not arise in that manner for the following reasons. Vreeland and Martin have shown that the growth rate of this bacterium is nearly equal with 0.375 to 1.37 M NaCl in the growth media (19), yet the hydrophobicities at these two NaCl concentrations were significantly different (Fig. 1). This becomes even more apparent at the extremes of tolerance (0.05 and 3.4 M NaCl), where very slowly growing cultures yielded opposite hydrophobicity measurements (Fig. 1 and Fig. 2A and C versus B).

One of the major problems in studies of NaCl adaptation in

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**TABLE 1. Effect of changes in medium pH on the measured hydrophobicity during growth of *H. elongata* in different NaCl concentrations**

<table>
<thead>
<tr>
<th>NaCl concn (M)</th>
<th>Time (h)</th>
<th>pH</th>
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* Hydrophobicity of hexadecane is 100.
euryhaline bacteria is the inherent need to perform all experiments in different NaCl concentrations. This can often lead to problems of NaCl interference in the assays. In this work, such interference could affect either the phase separation or the way that the cells bind to hexadecane. This interference could cause several types of erroneous results which could cause hydrophobic cells to appear hydrophilic. Alternatively, the NaCl could enhance the binding of cells to the hexadecane, causing hydrophilic cells to appear hydrophobic. This latter possibility was tested in two ways during the course of this work. First, the cell-free tests described above indicated that the presence of up to 3.4 M NaCl in the aqueous phase did not affect the phase separation process enough to explain the observed differences in the hydrophobicity/hydrophilicity of the cells grown in low- and high-salt media. A second and more rigorous test for NaCl interference was conducted by growing cells in 1.37 M NaCl (hydrophobicity = 79; Fig. 1) and then suspending the log-phase cells in basal salts solution containing 0.05 or 3.4 M NaCl and determining the hydrophobicity. The data from these experiments are shown in Fig. 3. After the 1 h required to run the assay, the 1.37 M NaCl-grown cells that had been suspended in 3.4 M NaCl still had a hydrophobicity of 79 ± 5 (Fig. 3). These data also show that lowering the NaCl concentration of the aqueous phase to 0.05 M did not significantly alter the apparent hydrophobicity of the 1.37 M NaCl-grown cells. If the amount of NaCl present in the aqueous phase of the assay did in fact cause the hydrophobicity values given in Fig. 1 and during the growth curve experiments (Fig. 2), we would have expected to see similar hydrophobic-hydrophilic values in Fig. 3. The fact that there were no significant changes suggests that the hydrophobic-hydrophilic values observed with H. elongata are due to actual physiological adjustments between cells from different salinities and that H. elongata cells are not subject to the types of NaCl-related artifacts seen with other bacteria (11).

Vreeland et al. (17) have suggested that the cell envelope of H. elongata becomes more tightly packed and coherent at higher salinity. The data presented here would be consistent with those conclusions if a loosened low-salt cell wall exposed hydrophobic molecules or moieties which could then attach to the hexadecane droplets. In contrast, a tighter packing of envelope molecules could shield such hydrophobic groups, producing a more hydrophilic surface at higher salinity. Furthermore, the fact that this bacterium produces more charged phospholipids in high NaCl concentrations (17) may also explain the increased hydrophilicity of the cell surface.

H. elongata appears to make physiological adjustments in the hydration state of its outer cell surface under the various NaCl concentrations in which it grows. At high salinity, a hydrophilic cell surface would make the cell more attractive to water molecules in a water-poor environment. Similarly, a hydrophobic cell would also help repel water in a water-rich situation. At high NaCl concentrations, a hydrated cell surface might help the cell obtain cytoplasmic water and thereby prevent desiccation.

ACKNOWLEDGMENTS

We thank Karen Meggs for her technical assistance and Myra Helmke and Janet Forbes for typing the manuscript.

LITERATURE CITED


