Microbiology, Vol. 72, No. 4, 2003, pp. 466–471. Translated from Mikrobiologiya, Vol. 72, No. 4, 2003, pp. 521–527.

EXPERIMENTAL ARTICLES

Effect of Cultivation Conditions on Growth and Adhesion of *Bacillus licheniformis*

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Received October 9, 2001; in final form, January 16, 2003

Abstract—Adhesion to glass of actively growing cells of the thermophilic *Bacillus licheniformis*, isolated from the Medyaginskaya test borehole (Yaroslavl' oblast), was studied. The reversible adhesion (RA) manifests itself in a decline of cell density (without cell lysis) in the liquid culture over the first 20–40 min of growth followed by normal exponential growth. The RA is minimal under favorable growth conditions but increases when cells are transferred to a new medium, especially one with a pH, temperature, salinity, or concentration of Ca^{2+} ions nonoptimal for the given species. Under unfavorable growth conditions, the adhesion becomes irreversible. The obtained data suggest that RA represents an adaptation mechanism important for population survival.

Key words: reversible adhesion, *Bacillus licheniformis.*

Adhesion of microorganisms to solid surfaces and formation of biofilms and colonies are typical and universal features of microorganisms inhabiting natural environments. Two phases of adhesion are to be distinguished: a reversible and irreversible one [1–4]. The first and faster phase is thought to do mostly with physicochemical mechanisms and is reversible, meaning that cells can readily pass from the attached to the free-swimming state [2, 4, 5]. The reversible adhesion (RA) of bacterial cells is much less studied than the irreversible one. The latter is the subject of a number of surveys and monographs [6–8]. It was noted in several works that the attachment of bacterial cells to a surface remains reversible over a few first minutes, but this behavior was never investigated in detail [4, 9]. The RA of *Pseudomonas fluorescens* was studied in a recent series of papers [10, 11]. It was established that the adhesion of cells to glass walls of the fermentor could be readily and promptly estimated from the decrease in the optical density of the culture during the first 1–3 h after a fresh medium is inoculated with a small amount of inoculum (less than 10 vol %). The RA is controlled by compounds present in the culture liquid, and the number of attached cells can be drastically reduced or adhesion totally suppressed by adding to the medium culture liquid together with washed cells. Today we know the chemical nature of several antiadhesins of *P. fluorescens*, represented by high-molecular paraffins [10] and extracellular protease [11].

The RA of bacteria occurring in biofilms is of special interest. Microorganisms able to colonize solid surfaces by forming mucous biofilms were isolated from a mixture of stratal water and drilling fluid obtained from a test borehole producing water and identified as *Bacillus licheniformis*. The purpose of this work was to investigate the reversible adhesion of this bacterium and, specifically, the dependence of RA upon cultivation conditions (the temperature and pH of the medium, and the concentrations of NaCl and Ca^{2+}).

MATERIALS AND METHODS

Strain 603 used in this work was isolated from a mixture of drilling fluid (DF) and stratal water (SW) obtained from a depth of 2086–2095 m of the Medyaginskaya test borehole (Kuznechikha, Yaroslavl' oblast). The isolation procedure followed Kholodnyi's method [12] using glass slides and agarized tap water with 2% sucrose. The bacteria were selected according to their mucus-forming capacity and strong adhesion to glass. Based on physiological and biochemical features, culture properties, cytological, morphological and genetics features (results of 16S rRNA sequence analysis and DNA–DNA hybridization), the isolated organism was assigned to the genus *Bacillus licheniformis*.

The pure culture of *B. licheniformis* 603 was maintained on agarized tap water with sucrose (20 g/l) or on LB medium [13]. In studies of growth kinetics and RA of *Bacillus licheniformis* 603, medium M9 was used, composed of solution A (200 ml), solution B (2 ml), solutions C and D (0.1 ml each), solution E (10 ml), and sterile distilled water added to the final volume of 1 l. Solution A contained (g/l distilled water) Na_2HPO_4, 34.0; KH_2PO_4, 15.0; NaCl, 2.5; and NH_4Cl, 5.0; solution B was composed of $MgSO_4 \cdot 7H_2O$, 246.5 g/l; solution C contained $CaCl_2$, 111.0 g/l; solution D contained (g/l 0.1 N HCl) $MnSO_4 \cdot 4H_2O$, 4.0; $ZnSO_4 \cdot 7H_2O$, 4.0;

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and $FeCl_3 \cdot 6H_2O$, 10.0; and solution E contained glucose, 20% [13]. Solutions A, B, and C were sterilized by autoclaving at 1 bar, and solutions D and E at 0.5 bar.

The effect of temperature, pH, NaCl, and Ca^{2+} on the specific growth rate (SGR) and adhesion was studied in an exponential bacterial culture. Cells were separated from the culture liquid by filtration (Millipore, pore size = 0.45 μm) and resuspended in a fresh medium with the given temperature, pH, and concentrations of NaCl and Ca^{2+} in such a way that the initial optical density (OD) of the resulting suspension was 0.05–0.1. The cultivation was performed in 250-ml shake flasks (100 rpm) containing 10 ml of the cell suspension. The pH value of the medium and concentrations of NaCl and Ca^{2+} were changed by adding 98.0% H_3PO_4 and 0.1 M KOH, dry sterile NaCl, and sterile 1 M $CaCl_2$. In experiments involving Ca^{2+}, medium M9 was used with its phosphate content reduced 20-fold to preclude precipitation of calcium phosphate. In the course of tests, the optical density of the cell suspension was measured at 540 nm (OD_{540}) on a Specol spectrophotometer (Carl Zeiss, Jena). Microscopic examinations were performed as follows: a glass flask containing a suspension of washed cells and immersed microscope slides was placed on a temperature-controlled shaker. During cultivation, every 5 to 10 min, the OD_{540} of the bacterial suspension was determined and the slides were taken out, rinsed in sterile M9 medium, and dried. Preparations of cells attached to the slides were flame-fixed, stained with a solution of fuchsin [14], and examined under a Docuval microscope (Carl Zeiss, Jena, Germany). Bacterial cells were counted in 50 microscopic fields. Protein in the culture liquid was determined by the Lowry method [14]. Viable free-swimming cells and attached cells (CFU) were enumerated by plating their 10-fold dilutions on a solid LB medium. Before plating, cells were desorbed from the flask surface. This was accomplished by putting glass beads (d = 1–2 mm), sterilized in dry heat, in a flask with the culture and shaking it vigorously on a shaker for 1 min. This procedure also led to greater homogeneity of free-floating cells. In all tests, replicated at least four times, the cultivation period was 1.5–2 h. The SGR and the degree of adhesion as a percent of the initial OD_{540} were calculated using the synthetic chemostat model [15] and an empirical model of bacterial adhesion. The parameter values of mathematical models were determined by fitting the model to the obtained data and minimizing the mean square error of the fit with the aid of Solver, Excel, and Microsoft Office 2000. The parameter values were subject to additional constraints based on their biological meaning and on preliminary kinetic observations.

RESULTS AND DISCUSSION

Our growth studies of *B. licheniformis* revealed curious dynamics of OD_{540} that was different under different culture conditions. The optical density of a suspension of cells washed free of culture liquid and placed in a fresh medium of the same composition but at nonoptimal temperature, e.g., 30°C (Fig. 1a, curve *1*), declined rapidly (for 15–20 min) and then grew up fast to the initial or a somewhat higher level. After that, a regular exponential growth was observed. Such a decrease of OD_{540} failed to occur if a certain quantity of culture liquid (50%) was introduced in the medium together with the cells. In this case, the initial phase of growth looked like a short lag phase or a phase of accelerated growth (Fig. 1a, curve *2*). It was also noted that the decrease of OD_{540} of the suspension of bacteria washed from culture liquid was accompanied by an increase in the quantity of cells attached to the flask glass walls (Fig. 1b). Thus, the microorganisms under study showed the ability to reversible adhesion [4, 10, 11].

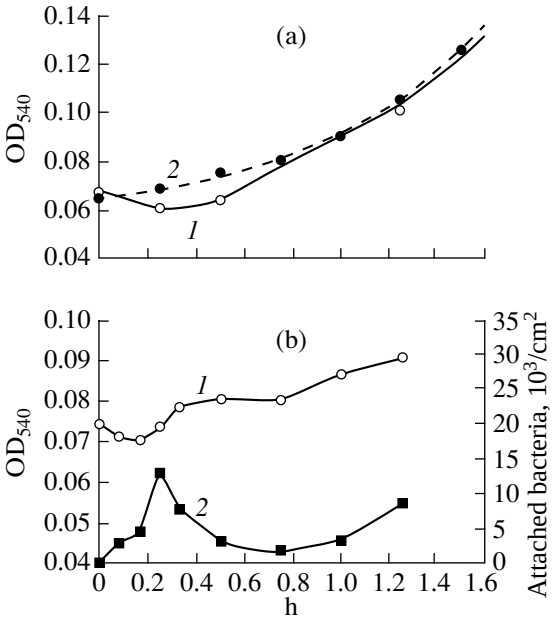

Fig. 1. (a) Dynamics of the optical density of a suspension of cells of *B. licheniformis* 603 grown at 45°C, washed free of culture liquid, and resuspended (*1*) in fresh medium or (*2*) in fresh medium supplemented with 50% of culture luquid filtrate at 30°C. The curves were calculated using equation (1) and the SCM. (b) Biomass dynamics of (*1*) free-swimming cells and (*2*) cells attached to glass. A suspension of cells of *B. licheniformis* 603 washed free of culture liquid was used as inoculum.

When the difference between the actual medium temperature and its optimal value was far greater, for example, when cells were transferred to patently unfavorable conditions (62°C), an irreversible decrease of the suspension OD_{540} occurred. This dynamics of OD_{540} was in accord with the observed decrease in the CFU number for the whole population, indicating a large-scale loss of viable cells (their death), which, however, as evidenced by the concentration dynamics of protein in the culture liquid, was not accompanied by fast cell lysis (Fig. 2). A similar pattern was observed in

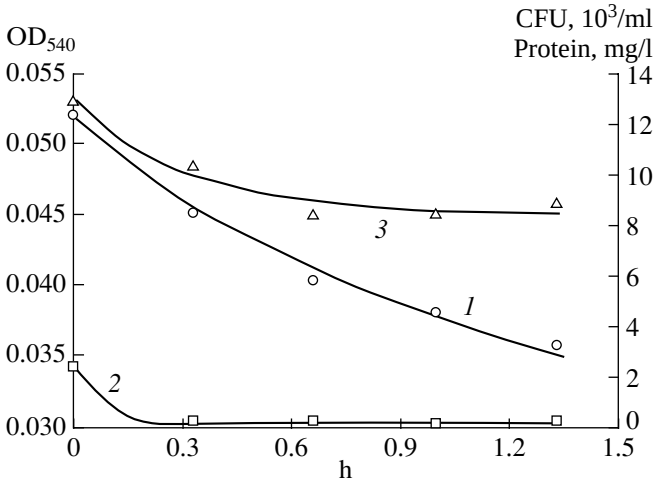

Fig. 2. Survival dynamics of *B. licheniformis* 603 at 62°C: (*1*) OD_{540}, (*2*) CFU, and (*3*) the concentration of protein in the culture liquid.

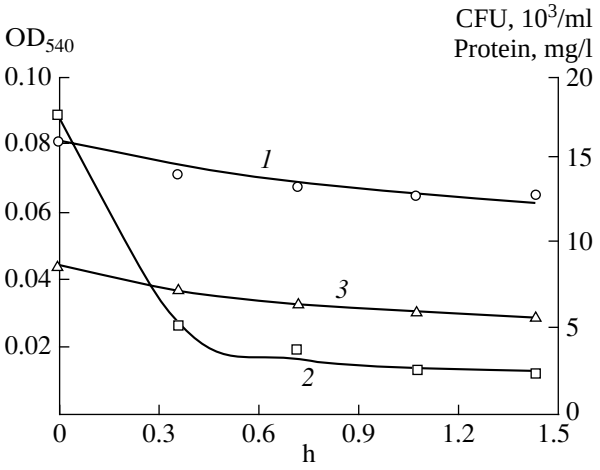

Fig. 3. Survival dynamics of *B. licheniformis* 603 at pH 4.1: (*1*) OD_{540}, (*2*) CFU, and (*3*) the concentration of protein in the culture liquid.

an experiment with a pH value of 4.11, extremely low for the given microorganism (Fig. 3). It follows that the decrease in OD_{540} of the bacterial suspension as the culture starts to die-off under the action of extreme environmental factors is likely to be caused mostly by the aggregation and adhesion of cells (viable or dead) to flask walls.

The number of attached cells and the SGR of the bacterial population was evaluated using the following empirical equation that expresses the number of attached cells both during active growth and in the decline phase as a function of time,

$$x = x_0 e^{\mu t} - \alpha(t/(\beta + t + \chi t^5)). \qquad (1)$$

Here, x is the number of free-swimming cells, $x_0 e^{\mu t}$ is the total number of cells in the population, $\alpha(t/(\beta + t + \chi t^5))$ is the number of attached cells, x_0 is the initial number of cells (at $t = 0$), μ is the specific growth rate (SGR), and α, β, and χ are empirical constants. The parameter α determines the potential maximum adhesion value, i.e., the number of attached cells in the absence of the inhibition term χ. With nonvanishing χ, the observed adhesion maximum is less than α, as seen from the expression for the adhesion maximum (*AM*). The parameters β and χ determine the shape of the adhesion graph as a function of time. The steepness of the valley increases with decreasing β, while both its spread and depth decrease with increasing χ. The second term on the right-hand side of equation (1) describes a single-humped curve of the reversible adhesion of cells as a function of time. Having identified the four parameter values (μ, α, β, and χ), one can determine the true specific growth rate of microorganisms μ (which is masked by the process of cell attachment to flask walls and their subsequent liberation); the time T of AM, $T = \sqrt[5]{\beta/3\chi}$; and its height (in optical density units, as a percent of the initial optical density, or in any other units used to measure the amount of biomass) $AM = \alpha T/(\beta + T + \chi T^5)$.

In order to describe the dynamics of growth with a lag phase and to determine the corresponding parameter values (SGR, lag phase, and the adhesion strength), we employed the synthetic chemostat model SCM (see [15] for details).

Effect of temperature on growth and adhesion of *B. licheniformis* 603. The specific growth rate of the studied microorganism as a function of temperature is plotted in Fig. 4. Growth of bacteria was observed in the temperature interval of 20 to 60°C. The effect of temperature on the specific growth rate is demonstrated by curve *1* obtained by fitting the data points with the equation

$$\mu_m = A \exp \lambda T / \left[1 + \exp B\left(1 - \frac{C}{T + 273}\right)\right], \qquad (2)$$

where T is the temperature, °C; μ is the specific growth rate; and A, B, and C are empirical constants. A is equal to the growth rate at 0°C; λ and B characterize, respectively the steepness of temperature-induced growth acceleration and inactivation; and C is numerically equal to the temperature at which 50% of cells are thermally inactivated (in practice, the value of C is close to the point of the temperature maximum). The numerical values of these parameters for the bacteria studied are listed in the table.

Parameter	Numerical value	Unit
A	0.0132	h^{-1}
B	133.99	Dimensionless
C	52.63	°C
λ	0.0926	$(°C)^{-1}$

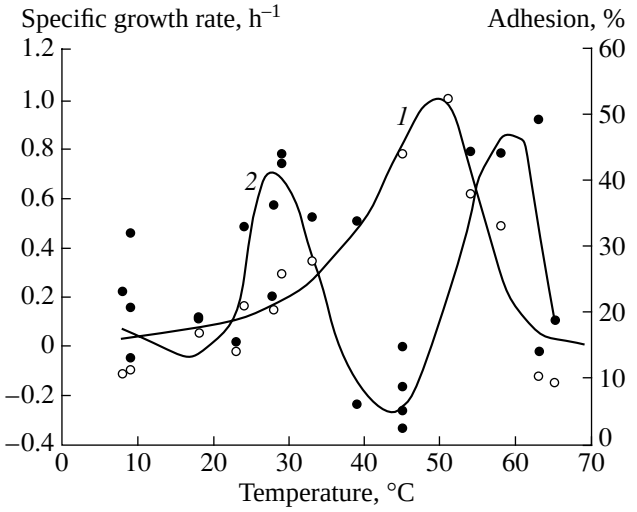

Fig. 4. Effect of temperature on (*1*) the specific growth rate and (*2*) adhesion of *B. licheniformis* 603.

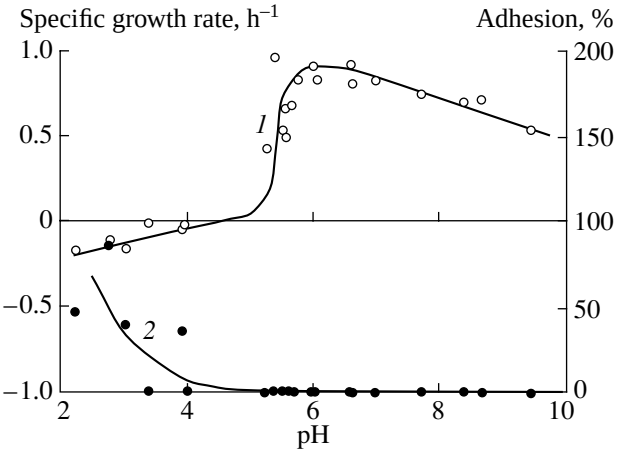

Fig. 5. Effect of the pH of the medium on (*1*) the specific growth rate and (*2*) adhesion of *B. licheniformis* 603.

The maximum specific growth rate of h^{-1} was observed at 48–51°C, corresponding to a generation time of 41.59 min. This organism is, therefore, a moderate thermophile.

The dependence of the fraction of reversibly attached cells upon the cultivation temperature is shown in Fig. 4 (curve *2*). In the zone of optimum growth (37–52°C), the cells washed free of culture liquid and resuspended in a fresh medium showed minimal adhesion to glass. The number of attached cells increased, however, with the deviation of temperature from its optimum value. The adhesion vs. temperature graph has two peaks at 25–32 and 54–63°C. The adhesion of cells at temperatures near the interval boundaries was irreversible.

Effect of the pH of the medium on growth and adhesion of *B. licheniformis* 603. The dependence of the specific growth rate of a culture of *B. licheniformis* upon the medium pH is plotted in Fig. 5 (curve *1*). The data points for the SGR and the number of attached cells in the pH range 2.2–4.0 were calculated by equation (1). In the pH range of 5.2–5.4, a short phase of sluggish growth was observed. No adhesion-related decline of OD_{540} or lag phase were noted in neutral or alkalescent media. The SGR as a function of pH was represented by a single-humped nonsymmetric curve with a gentle slope, showing that the growth rate remained high in a broad pH range of 5.7–8.5. Moreover, the transition from zero-growth pH values to the optimum pH was markedly more abrupt in the acid rather than alkaline region of the pH profile. It follows that what we see, in passing from low pH values to a neutral medium, is not a smooth acceleration of growth but rather a step-like transition from no growth at pH < 5 to maximum growth at pH > 5.5. The transition on the alkaline descending branch of the pH curve was more smeared, with the culture responding to medium alkalization by a more gradual SGR fall-off. Strong adhesion was noted only in acidic media, dropping down to zero in the region where growth started. The physicochemical mechanisms giving rise to such pH profiles are well-known: the glass surface and cells in neutral to alkaline media are negatively charged and adhesion is suppressed by electrostatic repulsion forces. In an acid medium, many ionogenic groups undergo protonation on glass and cells, thereby reducing the negative charge and facilitating cell attachment [1–3, 16].

Effect of NaCl on growth and adhesion of *B. licheniformis* 603. The influence of NaCl on the specific growth rate is displayed in Fig. 6 (curve *1*). The maximum SGR (0.94 h^{-1}) in this series of tests was observed at NaCl concentrations close to 0.5 g/l. At concentrations above 70 g/l, the SGR for *B. licheniformis* 603 was vanishing. The effect of salinity on adhesion is also shown in this figure. At low concentrations of NaCl (up to 50 g/l), the adhesion was minimal, increasing sharply with salinity and leveling at 18–22%.

Effect of bivalent cations on growth and adhesion of cells of *B. licheniformis* 603. The effect of bivalent cations on cell adhesion in an exponentially growing culture of *B. licheniformis* 603 was studied for Ca^{2+}, because stratal water from the Medyaginskaya borehole contained 0.438 M Ca^{2+}. It can be seen from the variation of SGR with the concentration of Ca^{2+} plotted as curve *1* in Fig. 7 that this cation had a strong inhibiting influence on the growth of the microorganism studied. Growth of *B. licheniformis* 603 on medium M9 was almost totally suppressed by 0.1 M Ca^{2+}. The dependence between the number of reversibly immobilized cells and the concentration of Ca^{2+} (Fig. 7, curve *2*) followed the Michaelis–Menten kinetics and can be described by the equation,

$$v = Vs/(K_m + s), \quad (3)$$

where K_m = 0.006 M and V = 98.9%.

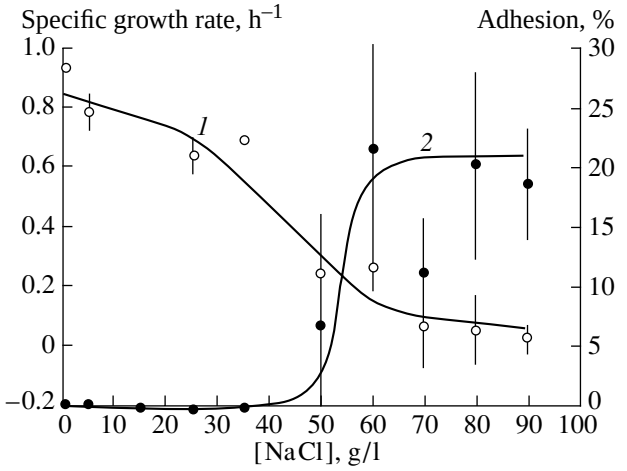

Fig. 6. Effect of NaCl on (*1*) the specific growth rate and (*2*) adhesion of *B. licheniformis* 603.

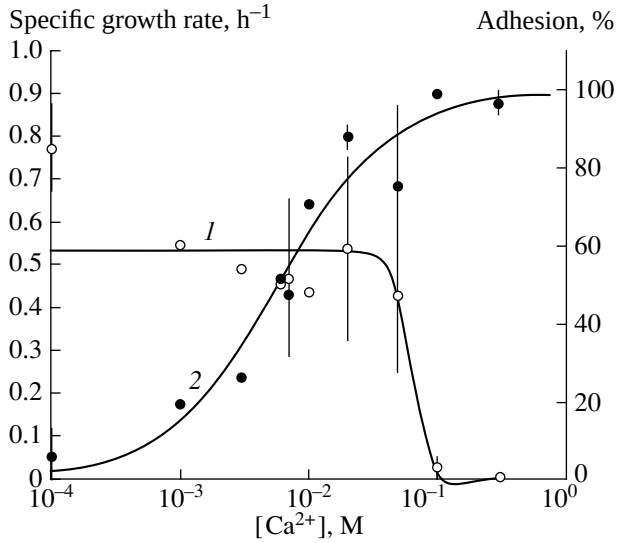

Fig. 7. Effect of Ca^{2+} on (*1*) the specific growth rate and (*2*) adhesion of *B. licheniformis* 603.

Given that this equation finds many applications in describing enzymatic reactions, it can be supposed that Ca^{2+} either activates protein systems responsible for adhesion or derepresses synthesis of adhesins. Ca^{2+} could also directly affect adhesion. The presence of bi- and trivalent cations in the medium (as well as increased solution ionic strength) is known to decrease the width of the double charged layers formed in aqueous media on interacting surfaces. This effectively reduces electrostatic repulsion forces in the cell–solid surface system and, in doing so, facilitates cell attachment [1, 4, 16, 17]. In addition, the presence of bivalent cations makes possible formation of coordination bonds between the cell and the solid surface [17].

It follows that adhesion (including reversible attachment) of *B. licheniformis* 603 arises only under conditions unfavorable for growth of this microorganism. This pattern is particularly clear-cut in the range of nonoptimal temperatures: overcooling as well as overheating of the bacterial suspension cause a notable increase in the number of attached cells (both reversibly and irreversibly) in the initial period of growth. The medium composition and its ionic strength also play an important part in the interaction of microbial cells with the phase interface. According to colloid chemistry principles, adhesion of suspended particles, including bacterial cells, must increase with the ionic strength of the solution [1, 3, 4]. In real experiments, this is true only when the ionic strength of the solution is sufficiently low (in solutions with NaCl concentration under 0.1 M). At higher values of this parameter, various deviations from this prediction derived from colloid chemistry principles are possible. For example, the adhesion of *Vibrio alginolyticus* cells was found to increase as the concentration of NaCl increased to 0.1 M and decrease beyond this point [18]. In our experiments, cell attachment of *B. licheniformis* 603 cells increased regularly with medium salinity. Ca^{2+} had a much stronger inhibiting effect on the growth of bacilli under study than Na^{+} did and, at the same time, much more effectively increased their adhesion. A similar effect of increased medium osmolarity was established earlier in experiments with *Staphylococcus epidermidis* [19] and with the anaerobic hyperthermophilic bacterium *Archaeoglobus fulgidus* [20]. The effect of pH on the adhesion of *B. licheniformis* 603 is least significant compared to the action of other factors studied and is observed only at pH under 4. Growth of microorganisms in hand under such conditions is impossible and bacterial cells die-off. Therefore, adhesion at low pH values of the medium can be explained mostly by physicochemical processes, and the contribution of adaptive metabolic reactions is bound to be insignificant.

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