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Surface charge of erythrocytes and lactobacilli *S. thermophilus* and their intercellular adhesion depend on the concentration of bivalent cations

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We propose a simple and accessible model of lactobacilli adhesion to human erythrocytes. The influence of bivalent cations on the surface charge of erythrocytes and lactobacilli *Streptococcus thermophilus* and their adhesive interaction has been investigated. We have shown that, despite the seemingly similar unidirectional effect of Ca^{2+} and Mg^{2+} cations, the cause of decreased adhesion lactobacilli *S. thermophilus* to human erythrocytes differ. While Ca^{2+} ions affect the surface charge of erythrocytes, not changing it in *S. thermophilus*, Mg^{2+} ions, conversely, affect the surface charge of lactobacilli and do not change it in erythrocytes. Our results confirm our proposition that in this case, the bivalent cations affect the second irreversible stage of adhesion process, but not the physical interactions during the first reversible stage.

Keywords: adhesion; bivalent cations; cell surface charge; erythrocyte; *S. thermophilus*

1. Introduction

From the physicochemical point of view, the initial instantaneous phase of microbial adhesion is mediated by nonspecific interactions characterized by long-range forces, including Lifshitz-van der Waals forces, electrostatic forces, acid-base, and hydrophobic interactions.[1–4] Bacteria possess multiple substrate specific adhesions, usually lectins and lectin-like proteins or carbohydrates, which constitute parts of surface polymer structures, including capsules, villi or pili. However, many studies have argued that these structural features are less important during the initial stages of adhesion than thermodynamic factors,[5,6] and a number of detailed studies have been undertaken to support this assertion.[7,8]

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Thus, electrostatic forces can affect the initial stage of bacterial adhesion, but they are also important for molecular recognition processes, including Ca^{2+}-binding adhesion and cell–cell adhesion. The role of bivalent cations, particularly calcium, in vitality is very complex and cannot be reduced to primitive unidirectional influence in a variety of processes. The Ca^{2+} regulates many biological processes through interaction with proteins, which have different conformational, dynamic, and metal-binding properties. In particular, it affects the multicellular behavior of different micro-organisms. A significant amount of large proteins involved in cell–surface and cell–cell interactions contain calcium-binding domains.[9,10] Calcium is associated with a variety of biological processes in bacteria, but its role, particularly in a biofilm development, is controversial. In Ref. [9] it was shown, for example, that calcium promotes more rapid biofilm formation in *P. putida*, as well as in other organisms, such as *Xyllela fastidiosa* [11] or *Vibrio vulnificus*.[12] Thus, calcium may regulate biofilm formation in opposite directions in different bacteria, strengthening, or weakening adhesion processes.

In Ref. [13] it was proposed that lactobacilli adhesion to intestinal surfaces is a result of weak bonds rather than specific interactions, as observed in the case of pathogenic organisms. Moreover, in Ref. [14] it is emphasized that the mechanism of adhesion of lactobacilli to human intestinal cells remains unknown. The authors suggest that adhesion of lactic acid bacteria to intestinal cells, accompanied by calcium bivalent cations, differ from adhesion that occurs in the absence of cations.

A wide variety of microbial pathogens and their toxins, as well as amicable bacteria often use the same glyco-compounds, proteins, or sialomucoproteins as a place of attachment to cell membranes elsewhere, but the target tissue or organ.[15] Erythrocytes display a huge variety of complex glycoproteins, glycosphingolipids, and gangliosides that are identical or similar to adhesion receptors on epithelial cells. Therefore, erythrocytes are a convenient source of mammalian cells, which have a large number of exposed complex carbohydrates of potentially related carbohydrate sequences for bacterial adhesions.[16] Here we propose a simple and accessible model of adhesion of lactobacilli to human erythrocytes, based on the fact that the same hydrocarbon parts of receptors as in target tissues are involved in adhesion.

Based on this model, we examined the influence of bivalent cations (Ca^{2+} and Mg^{2+}) on the surface charge of erythrocytes and lactic acid bacteria *Streptococcus thermophilus* and their adhesive interaction.

2. Materials and methods

Solutions were prepared using deionized bidistillate. All chemicals which are used to prepare the solutions were purchased from Sigma (USA). *S. thermophilus* bacterial cells were purchased from yogurt ferment VIVO (Kiev, Ukraine).

Erythrocytes were isolated from human donor blood, washed twice with 0.1 M phosphate-buffered saline (PBS composition: 11.5 g/l Na_2HPO_4 + 2.28 g/l NaH_2PO_{4+} 2.92 g/l NaCl), pH 7.4 and collected by centrifugation at 700 g. To examine the influence of the medium pH the precipitates of both cell types were resuspended in 1:2 ratio in buffered saline, pH 5.8, 6.6, 7.4, and 8.0. In experiments assessing the influence of ionic strength of the medium (I) on the adhesion index of bacterial cells to erythrocytes, and binding of AB to both cell types the medium ionic strength has been reduced by replacing electrolytes with sucrose to maintain the solution osmolality at a physiological level. The following solutions have been used in the experiment: 0.1 M sucrose + 0.1 M salts (I = 0.175); 0.2 M sucrose + 0.05 M salts (I = 0.087); 0.25 M

sucrose + 0.025 M salts ($I = 0.044$); blank test: a physiological buffered solution ($I = 0.312$). All the solutions were at pH 7.4.Ca^{2+} and Mg^{2+} concentrations in the solutions were varied by adding $CaCl_2$ and $MgCl_2 \cdot 6H_2O$.

Dried bacterial cells of *S. thermophilus* were suspended in PBS with the addition of 5% glucose and incubated at 37 °C for 30 min, washed in PBS, and harvested by centrifugation at 4000 g. The pellets of both cell types were resuspended in 1:2 ratio in PBS supplemented with varying concentrations of bivalent cations. We used the following concentration of cells suspensions in the adhesion experiment: 1.25×10^9 cells/ml of erythrocytes and 30×10^9 cells/ml of bacterial cells.

Both suspensions were mixed in 1:1 ratio, incubated at 37 °C for 30 min, and shaked every 5 min. Adhesion of bacterial cells to human erythrocytes was observed by means of the Axio Observer Z1 microscope (oil immersion lens ×63). The number of adherent bacteria was calculated in five fixed microscope fields after mechanical shaking of the sample to take into account only adherent lactobacilli in reality. The number of bacteria adherent to each erythrocyte was counted in each microscopic field and the average number of adherent bacteria per erythrocyte (adhesion index) was calculated.

The surface charge of erythrocytes was evaluated using Alcian blue cationic dye (Alcian blue 8GX) (AB) (50 mg/ml in 100% ethanol).[17] Dye solution was filtered through a filter paper and diluted 100 times in PBS or in PBS supplemented with varying concentrations of bivalent cations to obtain a final concentration of 1% ethanol. To determine the actual concentration of AB in the final solution after filtration, its losses on the walls of the sample tube and the losses of AB and the solvent (alcohol) were taken into account by weighing the sample tube and the filter before preparation and after filtration of the solution as well as the filter after drying. Absorbance of the solution was measured with a spectrophotometer (Pye Unicam SP 8000, UK) at 650 nm immeadiatly before use.

We used the same concentration of cells, as in Ref. [17] (1.25×10^9 cells/ml) for experimental evaluation of the surface charge of erythrocytes. In experiments on evaluation of the surface charge of lactobacilli *S. thermophilus* the optimum number of cells (10 mg/ml) was chosen to match their concentration of 5×10^9 cells/ml.

About 0.1 ml of the erythrocyte suspension (1.25×10^9 cells/ml) or bacterial cells (5×10^9 cells/ml) were mixed with 2 ml of the final AB solution. The mixture was incubated for 30 min at 37 °C. After cells removal by centrifugation the residual amount of AB was measured by its absorbance at 650 nm. The amount of bound AB per cell was calculated by the difference in absorbances of the initial AB solution and the supernatant and expressed in nanograms per 10^6 cells. Experiments were performed in five repetitions.

3. Results and discussion

Our experiments have shown that binding of cationic AB dye to erythrocytes did not differ significantly from blank test in the investigated pH and ionic strength range (Tables 1 and 2).

Instead, introducing physiological concentrations of Ca^{2+} cations led to a significant reduction of the AB bound by erythrocytes. The changes in this characteristic correlate with the changes in adhesion index (correlation coefficient $r = 0.935$) (Table 3, columns 2 and 3). At the same time, binding of AB to *S. thermophilus* cells did not change significantly in the investigated range of Ca^{2+} concentrations (Table 3, column 4).

Table 1. Adhesion index of *S. thermophilus* bacterial cells to human erythrocytes and binding of AB to both cell types dependent on the medium pH.

Solution pH	Adhesion index	Quantity of bound AB	
		By erythrocytes, ng/10^6 er	By lactobacilli, ng/10^6 *S. thermophilus*
5.8	0.71 ± 0.49[a]	218 ± 7.5	444.3 ± 11.7
6.6	1.67 ± 0.56[a]	211.1 ± 9.8	453.2 ± 11.3
7.4 (blank test)	2.21 ± 0.87	220.8 ± 4	444.1 ± 8.7
8.0	1.6 ± 0.74[a]	227.3 ± 6.9	449.8 ± 9.8

[a]Data significantly differ from data for blank test, $p < 0.01$.

Table 2. Adhesion index of *S. thermophilus* bacterial cells to human erythrocytes and binding of AB to both cell types dependent on the ionic strength of the medium.

Ionic strength (I) (Mol/l)	Adhesion index	Quantity of bound AB	
		By erythrocytes, ng/10^6 er	By lactobacilli, ng/10^6 *S. thermophilus*
0.312 (blank test)	2.21 ± 0.87	220.8 ± 4	444.1 ± 8.7
0.175	1.44 ± 0.94[a]	228.2 ± 7.7	438.4 ± 9.6
0.087	1.52 ± 0.85[a]	226.8 ± 9.8	427.3 ± 13.3
0.044	0.95 ± 0.63[a]	226.8 ± 9.3	438.4 ± 8.8

[a]Data significantly differ from data for blank test, $p < 0.01$.

Table 3. Adhesion index of *S. thermophilus* bacterial cells to human erythrocytes and binding of AB to both cell types dependent on the concentration of Ca^{2+} in the incubation medium.

Ca^{2+} concentration (%)	Adhesion index	Quantity of bound AB	
		By erythrocytes, ng/10^6 er	By lactobacilli, ng/10^6 *S. thermophilus*
0.00 (blank test)	2.21 ± 0.87	220.8 ± 4	444.1 ± 8.7
0.01	0.97 ± 0.84[a]	180.98 ± 11.5[b]	432 ± 10.8
0.02	1.57 ± 0.96[a]	195.1 ± 6.3[b]	435 ± 9.7
0.03	1.4 ± 0.84[a]	199.9 ± 9.7[b]	443 ± 11
0.04	1.17 ± 0.86[a]	196.3 ± 12.5[b]	428 ± 10.2

[a,b]Data significantly differ from data for blank test, $p < 0.01$.

We studied the influence of Mg^{2+} ions on the AB binding to both cell types-*S. thermophilus* and human erythrocytes. Our experiments showed that the introduction of Mg^{2+} ions did not change significantly the quantity of the dye binding by erythrocytes (Table 4, column 3), whereas *S. thermophilus* cells show significant reduction in the quantity of bound AB, which correlates with the changes in adhesion to erythrocytes with a correlation coefficient $r = 0.98$ (Table 4, columns 2 and 4). These results are consistent with the data presented in Ref. [17] where $MgCI_2$ does not change AB binding to erythrocytes.

Table 4. Adhesion index of *S. thermophilus* bacterial cells to human erythrocytes and binding of AB to both cell types dependent on the concentration of Mg^{2+} in the incubation medium.

Mg^{2+} concentration (%)	Adhesion index	Quantity of bound AB	
		By erythrocytes, ng/10^6 er	By lactobacilli, ng/10^6 *S. thermophilus*
0.00 (blank test)	2.21 ± 0.87	220.8 ± 4	444.1 ± 8.7
0.01	1.08 ± 0.82^a	214.5 ± 8.5	384 ± 12.9^b
0.02	1.22 ± 0.83^a	218.2 ± 7.7	385.8 ± 11.3^b
0.03	1.37 ± 0.85^a	209.3 ± 11.5	405.4 ± 8.4^b
0.04	1.62 ± 0.91^a	220.1 ± 6.9	419.8 ± 9.9^b

[a,b]Data significantly differ from data for blank test, $p < 0.01$.

Authors of Ref. [18] report on the different effects of calcium and magnesium on the adhesion of different lactobacilli lines. According to this work, the addition of Ca^{2+} resulted in a significant ($p < 0.05$) increase in the adhesion of *L. acidophilus* GK20, *L. paracasei* GK74, and *P. pentosaceus* MLK67 lines. The largest effect was observed for *L. acidophilus* GK20 and *L. paracasei* GK74 lines, for which adhesion increased up to 31.70% and 22.19%, respectively, compared with the blank test. However, the adhesion ability of *L. plantarum* GK81 and *L. brevis* MLK27 lines did not differ from the blank test when calcium is added. There was no significant change in adhesion for all the investigated lines when Mg^{2+} ions are added.

Different effects of Ca^{2+} and Mg^{2+} ions on various adhesion molecules may be caused by different ionic radius of these molecules. For example, the interaction of CD11b/CD18 integrin with physiological ligands was shown to be mainly dependent on monomodal binding of ligand carboxylate to the Mg^{2+} ion in metal ion-dependent adhesion site (MIDAS) in the A-domain of integrin. This interaction stabilizes the A-domain in the high-reactive state, which differs from a passive low-reactive state by adopting changes in the tertiary structure of the domain and leading to a cell adhesion. Out of the two bivalent cations (Mg^{2+} and Ca^{2+}), which are present in peripheral blood in sufficient quantity, octahedral environment at MIDAS perfectly meets the requirements for Mg^{2+} attaching. It has been shown that the adaptation of a larger Ca^{2+} ion (ionic radius 1.0 Å) compared with Mg^{2+} ion (ionic radius 0.72 Å) in octahedral environment at MIDAS is thermodynamically unfavorable and would lead to significant restructuring of the environment and to a reduction in reactivity for the natural ligands.[19]

Derjaguin–Landau–Verwey–Overbeek (DLVO) theory does not provide for any significant changes in the character of surface electrostatic potential distribution even at complete replacement of the monovalent cations to bivalent in electrolyte solutions at the same ionic strength. Only the value of the counterions diffusion layer $1/\chi$ decreases, where χ is inverse Debye length. In the case of 1–1 electrolyte $\chi^2 = 8\pi e^2 n_1/\varepsilon kT$; in the case of 2–1 electrolyte $\chi^2 = 24\pi e^2 n_2/\varepsilon kT$ (where ε is dielectric permeability of the medium, e is elementary charge, n_1 and n_2 are ion concentrations),[1] which would have led to adhesion facilitation in the second case. Taking into account that concentrations of bivalent Ca^{2+} and Mg^{2+} cations used in our experiments were close to physiological and significantly lower than the concentration of Na^+, the ionic strength of the solution and the length of the counterions diffusion layer were influenced only slightly. We can assume that the electrostatic component of disjoining pressure did not change

significantly. As for the structural component, the increase in the concentration of electrolytes causes the reduction in a radius of action of the repulsive structural forces and leads to their sharp weakening, which should also facilitate adhesion.

Nevertheless, obtained results showed a significant effect of bivalent cations on adhesion index toward its reduction. This result can be explained by the influence of bivalent cations on the second (irreversible) stage of adhesion process. A considerable number of large proteins involved in cell–surface and cell–cell interactions contain calcium-binding domains.[9,10] However, our results based on the influence of Ca^{2+} and Mg^{2+} cations on adhesion of *S. thermophilus* to human erythrocytes indicate, that adhesion molecules involved in this process, are not Ca^{2+} and Mg^{2+}-dependent, i.e. they are not activated by these cations. This is supported by the negative effect of both of these cations on adhesion. The obtained result, apparently, is the aftereffect of the influence of divalent cations to ligands and/or receptors and is not associated with their activation.

4. Conclusions

Our results indicate that, despite the seemingly similar unidirectional effect of Ca^{2+} and Mg^{2+} cations, the cause of adhesion of lactobacilli *S. thermophilus* to human erythrocytes differ. While Ca^{2+} ions affect the surface charge of erythrocytes, not changing it in *S. thermophilus*, Mg^{2+} ions, conversely, affect the surface charge of lactobacilli and do not change it in erythrocytes. This result confirms our proposition that in this case, the bivalent cations affect the second irreversible stage of adhesion process, but not the physical interactions during the first reversible stage.

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