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Mechanism of bacterial co-aggregation between *Bacillus subtilis* and *Escherichia coli* K-12 strains

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This work aims at exploring the co-aggregation of Bacillus subtilis 170 and Bacillus subtilis 168 with Escherichia coli K-12 strains. The influence of different physical and chemical factors involved in this features was investigated. Co-aggregation was found to be dependent on various factors: pH, temperature. Treatment with trypsin, proteinase K and chelating agents such as EDTA (ethylene diamine tetra acetic acid), heat treatment was found to inhibit co-aggregation. Among the different kind of sugars, the co-inoculation of Bacillus subtilis 170 and Bacillus subtilis 168 with Escherichia coli K-12 strains inhibited in a higher degree in the presence of N-acetyl glucosamine as compared to the other monosaccharides. Therefore, co-aggregation between tested strains was mediated by the lectin – polysaccharide interactions.

Keywords: *Bacillus subtilis* 170, *Bacillus subtilis* 168, *Escherichia coli* K-12, co-aggregation, biofilms

INTRODUCTION

Microorganisms are found in a wide range of diverse ecosystems as highly structured, multispecies communities termed biofilms [1, 2, 3, 4, 5]. Actually, biofilms can develop on a wide range of surfaces of food industry plants: stainless steel surfaces of open or closed pieces of equipment, floor, belts, rubber seal and so on [6].

Co-aggregation is an integral process in the formation of mixed biofilms and is therefore ecologically important [7, 8, 9] is characterized as an intra- or inter-species interaction of bacteria [1, 2, 10]. It differs from autoaggregation, which is defined as the adherence of bacteria belonging to the same strain [11, 12].

Physical interactions between co-aggregating bacteria facilitate metabolic interactions, such as oxygen protection, cell–cell communication and genetic exchange between cells [13, 14, 15]. The co-aggregation interaction is a highly specific process mediated by the recognition of complementary lectin — carbohydrate molecules between the aggregating partners [13, 14, 15, 12, 9], specific host-like patterns within the hexa- and heptasaccharide repeating units of different receptor polysaccharides [16].

Many studies have described the mechanisms of oral biofilm formation [16]. Co-aggregation also occurs between members of the urogenital flora and between strains of Lactobacillus from chicken crops [17, 18], as well as on human enteropathogens [19], uropathogens and chronic periodontitis [20]. In addition, co-aggregation has also been shown to occur between bacteria derived from aquatic ecosystems [19, 21] and rhizosphere [22].

This article is a thorough study of impact of environmental factors on the degree of co-aggregation between of *B. subtilis* 168 and *B. subtilis* 170 strains at their interaction with of *E. coli* K-12 strains.

MATERIALS AND METHODS

Bacterial strains. This study used of *Bacillus subtilis*168, *Bacillus subtilis* 170 strains, *Escherichia coli* W1655, *Escherichia coli* 406, *Escherichia coli* 420, *Escherichia coli* 446 K-12 strains, deposited in the collection of National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria, and *E. coli* 1655 K-12 strain from the collection of Institute of Molecular Biotechnology, Jena, Germany. All strains were inoculated into 9.00 ml of a liquid culture medium (LB broth) and incubated at 37 ° C for a period of 18 hours before the beginning of each determination.

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Co-aggregation assay. 1.00 ml aliquots of B. subtilis and E.coli K-12 overnight cultures were mixed together in 10,00 ml co-aggregate buffer (0,01 mM $CaCl_2$, 0,01 mM $MgCl_2$, 0,15 M NaCl) and vortexed for 10 s. The mixture was incubated in a rotary shaker for three min and left undisturbed for 4 h. Then co-aggregation assay was investigated according to methods described from Rathi et co-workers [21].

Effect of proteinase, trypsin and heat treatment, pH and temperature, chelating agent EDTA and sugars on the co-aggregation. All experiments were investigated according to methods described by Rathi et co-workers [21].

Scanning electron microscope assays. Bacterial co-aggregates were prepared according to method described by Phuong et co-workers [19]. Observations were performed on a scanning electron microscope.

Confocal laser scanning microscope assays. Bacterial co-aggregates were staining with Live Bacterial Gram Stain Kit according to instructions of the manufacturing company Biotum.

Statistical analysis. To investigate differences in co-aggregation between various *B. subtilis* strains and *E.coli* K-12, a one-way ANOVA and a Student's t -test was performed for the comparison between strains. The level of significance for all statistical tests was set at $P < 0.05$.

RESULTS AND DUSCUSSION

Bacteria in multispecies biofilms are able to make physical contact each other [23]. Specific direct interactions are known as co-aggregation [24]. This process underlies formation of biofilms of different microbial species [1, 2] and is influenced by features of the cell surface, environmental factors and the presence of specific compounds, inhibitors of co-aggregation.

In the present investigation of *Bacillus subtilis* 170, and *Bacillus subtilis* 168, and *Escherichia coli* K-12 have been tested for co-aggregation capability as reported in Table 1. The combination of *Bacillus subtilis* 170 with *Escherichia coli* K-12 1655 and *Bacillus subtilis* 168 with *Escherichia coli* K-12 1655 resulted in the highest co-aggregation percentage (77,01 ± 0,63% and 79,21 ± 0,72%).

Reports regarding the co-aggregation between *B.subtilis* 170, *B.subtilis* 168, and *E.coli* K-12 strains were scarce. The scanning electron micrograph of co-aggregates (Fig. 1 and Fig. 2) shows intermingled cells of *B.subtilis* 170, and *B.subtilis* 168 (long rods with surface protrusion), and *E.coli* K-12 (smaller smooth-surfaced rods).

Table 1. Co-aggregation of *B.subtilis* 170 with *E.coli* K-12 strains.

№	Strains	Autoaggregation index, %	Co-aggregation index, % with	
			B.subtilis 170	*B.subtilis* 168
1.	*Bacillus subtilis* 170	54,32±0,58	-	-
2.	*Bacillus subtilis* 168	52,85±0,26	-	-
3.	*Escherichia coli* 1655	53,94±0,95	77,01 ± 0,63	79,21 ± 0,72
4.	*Escherichia coli* 406	44,52±0,06	76,10 ± 0,00	79,08 ± 0,23
5.	*Escherichia coli* 446	44,95±0,73	77,02 ± 0,08	78,50 ± 0,42
6.	*Escherichia coli* 420	43,97±0,21	73,57 ± 0,11	78,06 ± 0,61
7.	*Escherichia coli* W3110	41,53±0,51	71,04 ± 0,60	78,86 ± 0,21

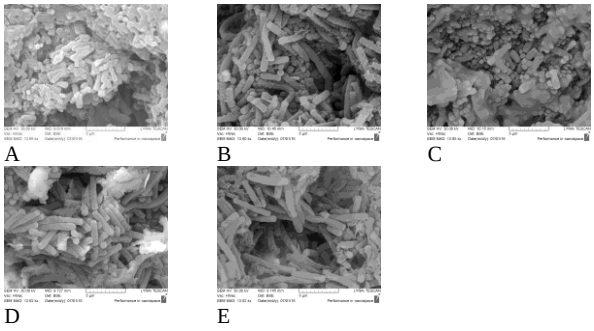


Fig. 1. SEM micrographs of the co-aggregates formed between Bacillus subtilis 170 strain and their co-aggregating partners: (A) Escherichia coli K-12 406 ; (B) Escherichia coli K-12 420; (C) Escherichia coli K-12 446; (D) Escherichia coli K-12 1655; (E) Escherichia coli K-12 W 3110.

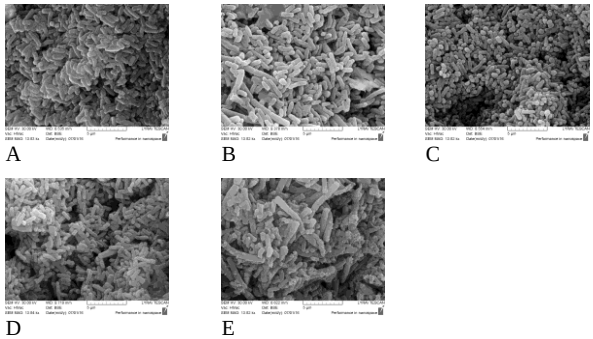


Fig. 2. SEM micrographs of the co-aggregates formed between Bacillus subtilis 168 strain and their co-aggregating partners: (A) Escherichia coli K-12 406 ; (B) Escherichia coli K-12 420; (C) Escherichia coli K-12 446; (D) Escherichia coli K-12 1655; (E) Escherichia coli K-12 W 3110.

The co-aggregation of *B. subtilis* 170 and *B. subtilis* 168 with *E. coli* K-12 strains at different pH levels is presented in graphical form inFig. 3 and Fig. 4. Co-aggregation percentage is higher at

lower and higher pH levels namely 5,0 and 8,0 irrespective of strains. However at a neutral pH of 7,0 the results show a slight decrease in the co-aggregation percentage for all tested pair strains.

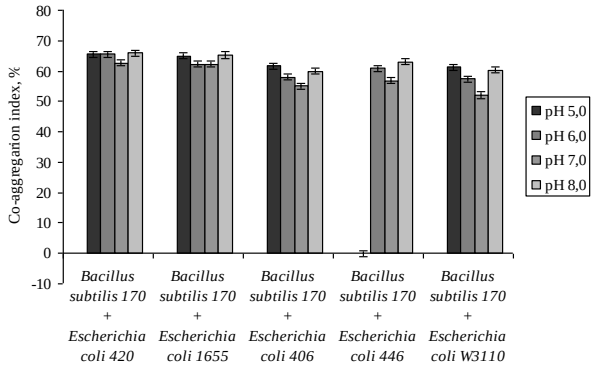


Fig. 3. Effect of different levels of pH on co-aggregation of *Bacillus subtilis* 170 with *Escherichia coli* K-12 strains

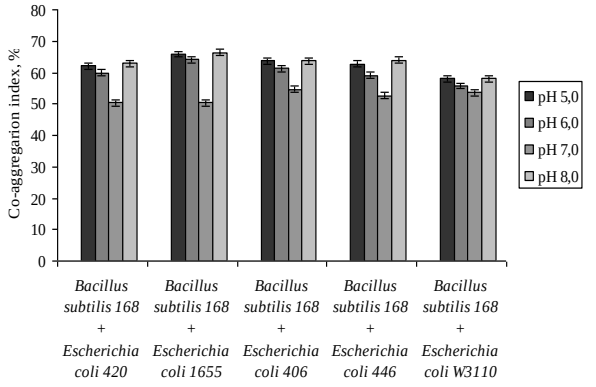


Fig. 4. Effect of different levels of pH on co-aggregation of *Bacillus subtilis* 168 with *Escherichia coli* K-12 strains

Rathi et co-workers [21], Burdman et co-workers [25, 26] reported that *Azospirillum* strain FAJ0204, *P. fluorescens* and *B. subtilis* strains displayed an increase in aggregation at a lower pH level. At acidic pH, negative ionized groups can be neutralized by protonation thus diminishing the strength of the repulsive forces between bacteria and leading to increased aggregation. After a decline in co-aggregation at pH 7.0, the increase observed in pH 8,0.

The effect of different temperature on the co-aggregation was studied. The increasing level of growth temperature showed an increase in co-aggregation percentage up to 35–40 °C, and beyond 40 °C was observed a reduction in co-aggregation index (Fig. 5 and Fig. 6). Burdman co-workers [26] reported the positive effect of growth temperature on co-aggregation of *A. brasilense* cells. A similar trend was also observed in *Azospirillum*, when co-aggregated with other PGPR strains.

In order to determine the nature of surface components, involved in cell–cell interaction leading to aggregation, the bacterial strains were treated with a variety of potential disaggregating reagents such as Tween, EDTA, Triton, protease, trypsin, and heat treatment.

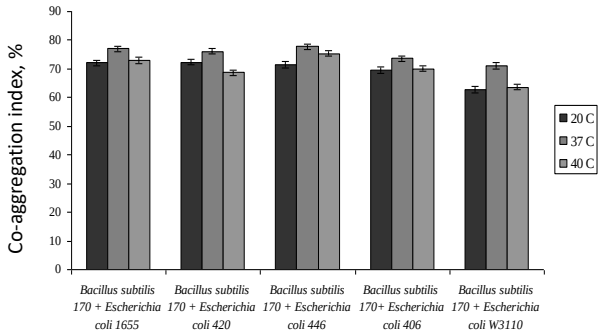


Fig. 5. Effect of different levels of temperature on co-aggregation of *Bacillus subtilis* 170 with *Escherichia coli* K-12 strains.

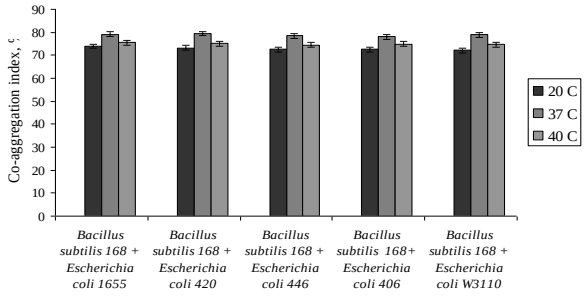


Fig. 6. Effect of different levels of temperature on co-aggregation of *Bacillus subtilis* 168 with *Escherichia coli* K-12 strains.

Table 2. Effect of EDTA, Tween и Triton on the co-aggregation of *B.subtilis* 170 with *E.coli* K-12 strains

№	Strains	Co-aggregation index, %			
		Control	EDTA	Tween	Triton
1.	*Bacillus subtilis* 170 + *Escherichia coli* 1655	77,01 ± 0,63	69,55 ± 0,75	57,05 ± 0,42	58,88 ± 0,21
2.	*Bacillus subtilis* 170 + *Escherichia coli* 420	76,10 ± 0,00	62,37 ± 0,42	59,06 ± 0,21	58,94 ± 0,42
3.	*Bacillus subtilis* 170 + *Escherichia coli* 446	77,82 ± 0,08	65,76 ± 0,13	55,76 ± 0,17	56,30 ± 0,39
4.	*Bacillus subtilis* 170+ *Escherichia coli* 406	73,57 ± 0,11	64,98± 0,27	59,49 ± 0,91	61,89 ± 0,33
5.	*Bacillus subtilis* 170 + *Escherichia coli* W3110	71,04 ± 0,60	58,92 ± 0,71	56,19 ± 0,23	57,20 ± 0,21

Table 3. Effect of EDTA, Tween и Triton on the co-aggregation of *B.subtilis* 168 with *E.coli* K-12 strains

№	Strains	Co-aggregation index, %			
		Control	EDTA	Tween	Triton
1.	*Bacillus subtilis* 168 + *Escherichia coli* 1655	79,21 ± 0,72	70,68 ± 0,28	55,84 ± 0,87	62,06 ± 0,49
2.	*Bacillus subtilis* 168 + *Escherichia coli* 420	79,48 ± 0,23	70,18 ± 0,32	55,76 ± 0,49	63,46 ± 0,48
3.	*Bacillus subtilis* 168 + *Escherichia coli* 446	78,50 ± 0,42	69,62 ± 0,43	61,03 ± 0,79	64,35 ± 0,50
4.	*Bacillus subtilis* 168+ *Escherichia coli* 406	78,06 ± 0,61	70,53± 0,30	60,11 ± 0,41	64,87 ± 0,36
5.	*Bacillus subtilis* 168 + *Escherichia coli* W3110	78,86 ± 0,21	71,33 ± 0,41	58,81 ± 0,40	66,42 ± 0,95

Results of Table 2 and 3 shows that with the greatest inhibitory activity against co-aggregation process differs Tween, the index of inhibition of co-aggregation process ranges from 19,12% to 28,35% at *B. subtilis* 170 and *E. coli* K-12 strains, while at the pair of *B. subtilis* 168 and *E. coli* K-12 strain its value varies in the range of 22,26% to 29,84%. The lower degree of reduction of co-aggregation between investigated strains is under effect of Triton, followed by EDTA.

The highest resistance to thermal impact at 80 ° C for 15 min. were featured pairs of *B.subtilis* 170 and *E.coli* K-12 1655, and *B.subtilis* 168 and *E.coli* K-12 1655 strains, followed by pairs of *B.subtilis* 170 and *E.coli* K-12 406, *B.subtilis* 168 and *E.coli* K-12 406 (Tables 4 and 5). In the base of thermal tolerance of flocs, formed by strains of *B. subtilis* and *E. coli* species is most likely standing increase of intracellular content of poly-β-butyrate [24, 11]. The reduction of value of the index of co-aggregation between tasted pair strains of the *B. subtilis* and *E. coli* species in the present study was more pronounced under the influence of the cells with proteinase K compared to trypsin (Table 4 and Table 5). Addition of proteinase K enzyme reduced the co-aggregation percentage from 72,45 to 62,69 % of *B. subtilis* 170 and *E. coli* K-12 strains, from 72,45 to 62,69 % at *B. subtilis* 168 and *E. coli* K-12 strains, as seen in Table 5. There results are in conformity with the earlier findings of Burdmanet and co-workers [26] which indicated significant reduction in the aggregation inducing *Azospirillum* cells on protease treatment. These results suggest that adhesive proteins are at least partially responsible for the aggregation-inducing activity.

Table 4. Co-aggregation index between *B. subtilis* 170 and *E. coli* K-12 strains after heat treatment at 80 °C, treatment with Trypsin and Proteinase K

Strains	Co-aggregation index, %			
	Control	Heat treatment	Trypsin	Proteinase K
B.subtilis 170+*E.coli* K-12 420	70,55±0,13	68,21±0,23	72,37±0,68	64,26±0,48
B.subtilis 170+*E.coli* K-12 1655	74,30±0,93	73,90±0,55	68,49±0,71	69,17±0,25
B.subtilis 170+*E.coli* K-12 446	70,55±0,12	70,38±0,41	70,33±0,15	68,47±0,33
B.subtilis 170+*E.coli* K-12 406	73,99±0,18	70,36±0,02	65,16±0,34	62,69±0,24
B.subtilis 170+*E.coli* K-12 W3110	70,92±0,18	69,38±0,46	67,98±0,64	63,17±0,44

Table 5. Co-aggregation index between *B. subtilis* 168 and *E. coli* K-12 strains after heat treatment at 80 °C, treatment with Trypsin and Proteinase K

Strains	Co-aggregation index, %			
	Control	Heat treatment	Trypsin	Proteinase K
B.subtilis 168+*E.coli* K-12 420	73,99±0,18	71,19±0,65	71,32±0,15	62,69±0,39
B.subtilis 168+*E.coli* K-12 1655	74,30±0,90	72,85±0,80	70,92±0,09	58,78±0,81
B.subtilis 168+*E.coli* K-12 446	70,55±0,13	68,66±0,45	71,57±0,45	69,36±0,52
B.subtilis 168+*E.coli* K-12 406	72,53±0,50	71,24±0,37	63,40±0,66	59,11±0,97
B.subtilis 168+*E.coli* K-12 W3110	70,92±0,88	70,55±0,32	68,56±0,47	63,55±0,55

The presence of methyl mannoside did not affect significantly on the co-aggregation capability of *B. subtilis* 170 and *E. coli* K-12 and *B. subtilis* 168 and *E. coli* K-12. However, the presence of N-acetyl galactosamine and N-acetyl glucosamine

significantly ($p<0,05$) inhibited the co-aggregation of *B. subtilis* 170 and *E. coli* K-12, *B. subtilis* 168 and *E. coli* K-12 (Table 6 and Table 7). Therefore

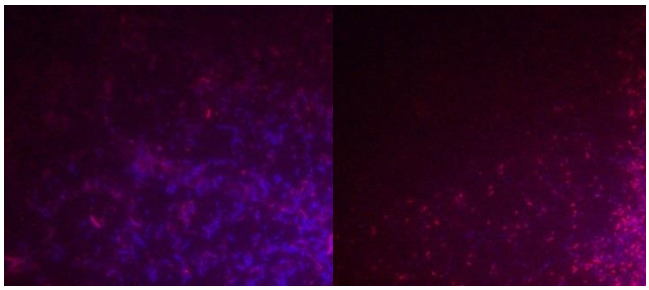
Table 6. Co-aggregation index between *B. subtilis* 170 and *E. coli* K-12 strains in the presence of N-acetylgalactosamine, N- acetylglucosamine and methylmanoside.

Strains	Co-aggregation index, %			
	Control	N-acetylgalactosamine	N-acetylglucosamine	Methyl mannoside
B.subtilis 170+*E.coli* K-12 420	70,55 ±0,13	67,28±0,21	68,03±0,21	65,49± 0,77
B.subtilis 170+*E.coli* K-12 1655	74,30 ±0,93	64,63±0,90	65,39±0,51	66,01± 0,84
B.subtilis 170+*E.coli* K-12 446	70,55 ±0,12	67,28±0,21	68,03±0,21	65,49± 0,77
B.subtilis 170+*E.coli* K-12 406	73,99 ±0,18	66,89±0,36	72,28±0,38	67,85± 0,46
B.subtilis 170+*E.coli* K-12 W3110	70,92 ±0,18	65,79±0,60	68,48±0,92	59,79± 0,86

Table 7. Co-aggregation index between *B. subtilis* 168 and *E. coli* K-12 strains in the presence of N-acetylgalactosamine,N- acetylglucosamine and methylmannoside

Strains	Co-aggregation index, %			
	Control	N-acetylgalactosamine	N-acetylglucosamine	Methyl mannoside
B.subtilis 168+*E.coli* K-12 420	73,99 ±0,18	66,89±0,36	65,40±0,66	71,88±0 ,71
B.subtilis 168+*E.coli* K-12 1655	74,30 ±0,90	64,63±0,90	66,42±0,16	72,31±0 ,37
B.subtilis 168+*E.coli* K-12 446	70,55 ±0,13	68,59±0,63	65,93±0,96	69,01±0 ,14
B.subtilis 168+*E.coli* K-12 406	72,53 ±0,50	66,52±0,91	68,86±0,45	70,95±0 ,86
B.subtilis 168+*E.coli* K-12 W3110	70,92 ±0,88	57,92±0,23	62,84±0,22	68,63±0 ,13

co-aggregation between tested strains was mediated by the lectin – polysaccharide interactions. This conclusion correlates with Ebisu and co-workers [27] on the formation of co-aggregates of *Eikenella corrodens* 1073 strain at his association with *Actinomyces viscosus* ATCC 19246, *A. viscosus* T14AV, *Streptococcus sanguis* 34 and*S. sanguis* ST160R strains. The protein adhesin was associated with *E. coli* K-12 strains, the complementary galactosamine- like sugar receptor was associated with *B. subtilis* 170 strain and *B. subtilis* 168 strain.



A B

Fig. 7. Confocal laser scanning micrographs of the coaggregates formed between (A) *Bacillus subtilis* 170 (red) and *Escherichia coli* K-12 1655 (blue) , (B) *Bacillus subtilis* 168 (red) and *Escherichia coli* K-12 1655 (blue)

By using confocal laser scanning microscopy are illustrated intercellular contacts in formed co-aggregates. The obtained results indicate that during a process of co-aggregation bacterial cells of *B. subtilis* 170 and *E. coli* K-12 strains, *B. subtilis* 168 and *E. coli* K-12 strains are viable (Fig. 7). In the study of Bradshaw et co-workers [24] it is concluded that the formation of flocs of bacterial cells ensure their protection against reactive oxygen species among the obligate and facultative anaerobe species.

CONCLUSIONS

The values of temperature and pH have an impact on the degree of co-aggregation between the *B. subtilis* 170 and *E. coli* K-12, *B. subtilis* 168 and *E. coli* K-12 strains, the maximum value of the index of co-aggregation is achieved in the alkaline (pH 8.0) and acidic (pH 5.0) medium and at a temperature of 37 ° C.

The high degree of inhibition of co-aggregation between strains of *B. subtilis* 170 and *E. coli* K-12 species at treatment of cells with Tween, Triton, and N-acetylglucosamine give grounds to assume that in the base of the process stands lectin-polysaccharide interactions.

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МЕХАНИЗЪМ НА КОАГРЕГАЦИЯ МЕЖДУ ЩАМОВЕ BACILLUS SUBTILIS AND ESCHERICHIA COLI K-12

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(Резюме)

Целта на проучването е да се изследва коагреацията между щамове Bacillus subtilis и Escherichia coli K-12. Проучено е влиянието на отделните физични и химични фактори. Коагрегацията се повлиява от pH и температурата на средата. Въздействието с трипсин, протеиназа К, етилендиаминотетраоцетна киселина, както и термичното въздействие води до инхибиране на процеса на коагрегация. N-ацетилглюкозаминът в най-голяма степен инхибира процеса на коагрегация между щамове *Bacillus subtilis* 170 и *Escherichia coli* K-12, *Bacillus subtilis* 168 и *Escherichia coli* K-12. Следователно, в основата на процеса стоят лектин-полизахаридни взаимодействия.