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Influence of Divalent Metal Ions on Biofilm Formation by Bacillus subtilis

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Abstract
The wild strain of *B. subtilis* 168, the protease-deficient strain of *B. subtilis* BRB14, and the regulatory mutants of *B. subtilis* 168 degU and *B. subtilis* 168 ccpA were examined for their ability to form biofilms under stress conditions—with elevated concentrations of divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}), as well as in the presence of ethylenediaminetetraacetic acid (EDTA). It was shown that regulatory mutants are more resistant to the addition of divalent metal ions than wild and protease-deficient strains. At the same time, the introduction of EDTA reduces the level of biofilm formation by all strains on average by 50%.

Keywords *Bacillus subtilis* · Biofilms · Stress factors · Regulatory mutants · Metal ions · EDTA · DegU · CcpA

1 Introduction

The existence of bacteria in the structure of biofilms provides populations with protection from stressful environmental factors, such as various oxidants, toxins, and antibiotics [1, 2]. Infections caused by such bacteria are difficult to treat because of the ability of biofilms to withstand a wide range of external factors [3, 4]. Of particular interest is the identification of regulatory systems involved in the creation of biofilms [5, 6]. The non-pathogenic motile gram-positive spore-forming soil bacterium *Bacillus subtilis* is a widely used model for biofilm formation studying [7, 8]. The involvement of the Deg regulatory system in the biofilm formation in bacilli has been studied in considerable detail over the past years [9–12]. It was shown that low level of DegU~P activates transcription of flagellar genes, thereby inhibiting the synthesis of biofilm matrix [11]. Intermediate level of DegU~P stimulates the biofilm formation [13]. High level of DegU~P inhibits the biofilm matrix formation [9, 12]. Activation of DegU (DegU~P) leads to a subpopulation of cells that specialize in the secretion of exoproteases and promote the degradation of large biopolymers into smaller and more nutritive peptides for the community [14]. The catabolite control protein CcpA of *B. subtilis* is a transcriptional regulator that mediates catabolite repression of many genes in *B. subtilis* in response to glucose and fructose [15]. CcpA gene expression inhibited biofilm formation [16]. In addition to exopolysaccharides, the structure of the biofilm also contains proteins [17, 18], this means that the process of matrix formation can be influenced by the own secreted bacillus proteinases.

Obviously, such a complex regulatory mechanism of biofilm formation allows bacilli to respond most adequately to environmental changes, i.e., to stress. The aim of our work was to study the difference in the stress response of a full value and defective *B. subtilis* strains. To do this, we studied the dynamics of biofilm formation under stress in natural isolate *B. subtilis* 168, mutant strains with knockout regulatory protein genes: DegU and CcpA, and also in protease-deficient *Bacillus subtilis* BRB14 strain with deleted genes of nine extracellular and two membrane-bound proteinases. As a stress factor, we took an increased content in the medium of ions of such bivalent metals as calcium, magnesium, manganese, and zinc, as well as EDTA.

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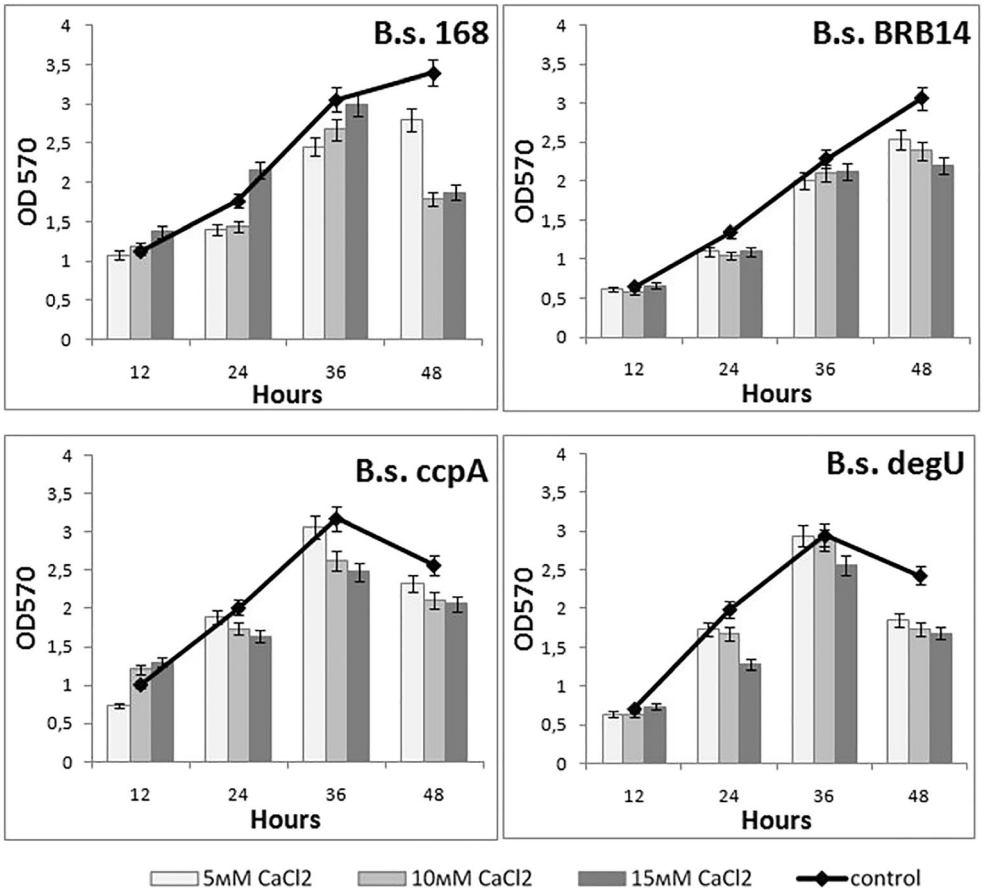

Fig. 1 Effect of calcium ions on the level of biofilm formation by *B. subtilis* strains

2 Materials and Methods

The strains used in the study:

Bacillus subtilis 168—natural isolate, wild-type (Professor J. Stuelke, University of Göttingen, Germany);
Bacillus subtilis 168 degU—degU gene knockout (two-component response regulator of signal transduction system DegU-DegU) (Dr. Jan Maarten van Dijl, University of Groningen, Netherlands);
Bacillus subtilis 168 ccpA—ccpA gene knockout (global regulator of carbohydrate metabolism) (Professor D. Zeigler, Ohio University, USA);
Bacillus subtilis 168 BRB14—deleted genes of extracellular proteases (trpC2, nprB, aprB, epr, bpr, nprE, mpr, vpr, wprA) and membrane-bound proteases (htrA and hrt) (Cobra Biologics, Keele, UK).

Cultivation of the strains was performed within 48 h at 37 °C on a synthetic-E medium, whose composition is described in [19]. Seed served 16-h inoculum (1% *v/v*).

Metal ions in the form of salts of $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$, as well as the solution of EDTA (Sigma-Aldrich, USA) were sterilized separately and introduced into the medium in final concentrations of 5, 10, and 15 mM before inoculation.

Biofilm formation defined by the method set incubation with crystal violet (CV) [20] with modification [21]. For the analysis of experimental data, Microsoft Excel program was used. Error bars denote the standard error of the mean as obtained from four individual samples. The results were considered statistically significant with the standard error less than 10%.

3 Results and Discussion

The addition of calcium ions in the range of concentrations of 5–15 mM affects the formation of biofilms by all strains only by the 48th hour of growth. Wild-type exhibits the highest sensitivity: the maximum reduction in the formation of biofilms is 50% with 15 mM of calcium in the medium (Fig. 1). The protease-deficient strain is slightly more

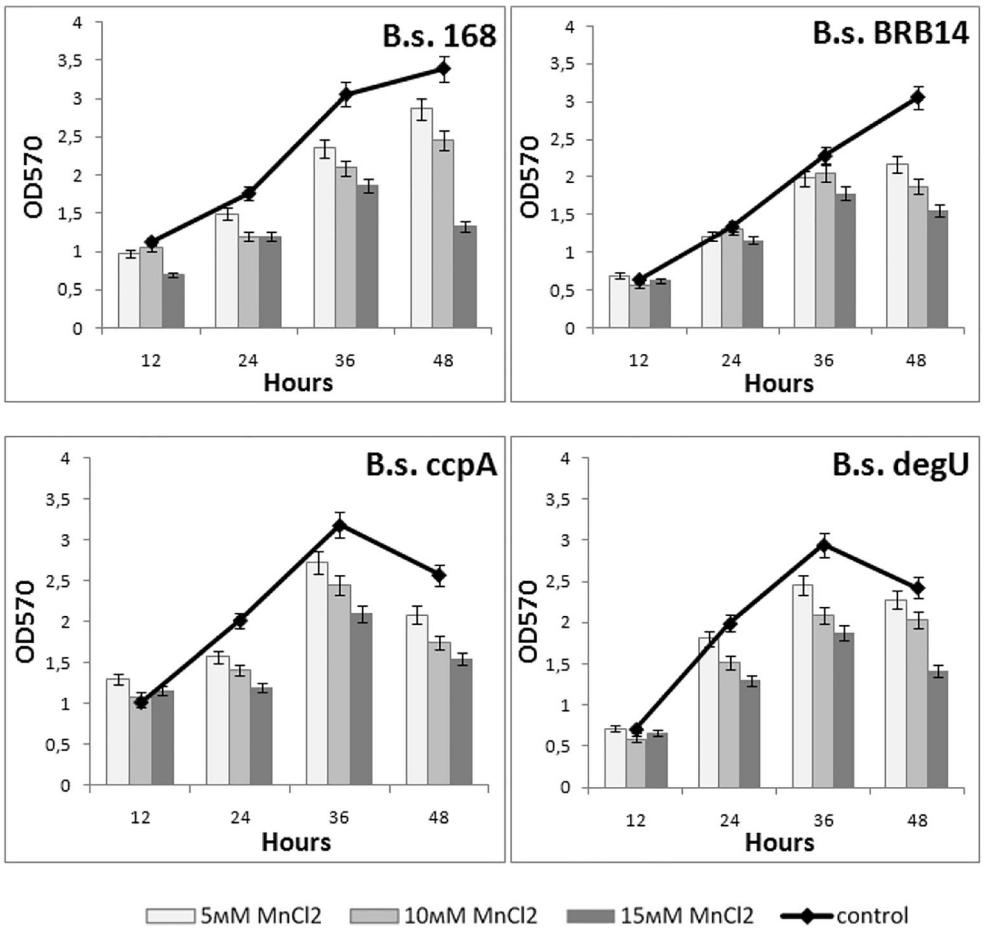

Fig. 2 Effect of manganese ions on the level of formation of biofilms by *B. subtilis* strains

resistant—at 15 mM at the 48th hour of growth, the decrease in the level of biofilm formation does not exceed 30%. Regulatory mutants are most resistant to the presence of calcium ions in the medium, even at the maximum concentration of 15 mM—by the 48th hour of growth, the decrease in the level of biofilm formation is from 10% to 20% (Fig. 1).

The introduction of manganese into the medium has an effect on all strains already with a minimum concentration of ions in the medium. The greatest decrease in the level of biofilm formation is observed for the wild strain with 15 mM manganese in the medium at the 48th hour of growth—60%. At the same time, a decrease in the level of biofilm formation can be clearly traced throughout the growth of the culture (from 12 to 48 h) with the entire range of concentrations (5–15 mM) (Fig. 2). The protease-deficient strain up to 36 h of growth is practically not sensitive to manganese, but for the 48th hour, even 5 mM manganese causes a decrease in the level of biofilm formation by 30% and 15 mM—by 50%. Regulatory mutants in the presence of manganese as well as with calcium are less sensitive. In the spectrum of concentrations from 5 to 15 mM for 48 h of growth, the decrease in the level of biofilm formation is from 10% to 30%, respectively (Fig. 2).

Regulatory mutants react similarly to the presence of magnesium in the medium (Fig. 3). The protease-deficient strain practically does not reduce the level of biofilm formation at all, even with 15 mM magnesium in the medium, only by the 48th hour of growth the reduction reaches 30%. The wild strain demonstrates the highest sensitivity—by the 36th hour of growth, the reduction in the biofilm formation for all concentrations ranges from 20% to 40%, and by the 48th hour—60% (Fig. 3).

The greatest influence on the level of biofilm formation by all strains is exerted by zinc ions (Fig. 4). However, as in previous experiments, regulatory mutants show less sensitivity to zinc: a strain with a *ccpA* gene knockout over 48 h of growth shows a decrease in the biofilm formation with 5 mM zinc by a maximum of 10%, a strain with a *degU* gene knockout—on 25%. The wild and protease-deficient strains with the entire spectrum of zinc ion concentrations up to 24 h of culture growth practically do not reduce the level of biofilm formation (10%), but with subsequent

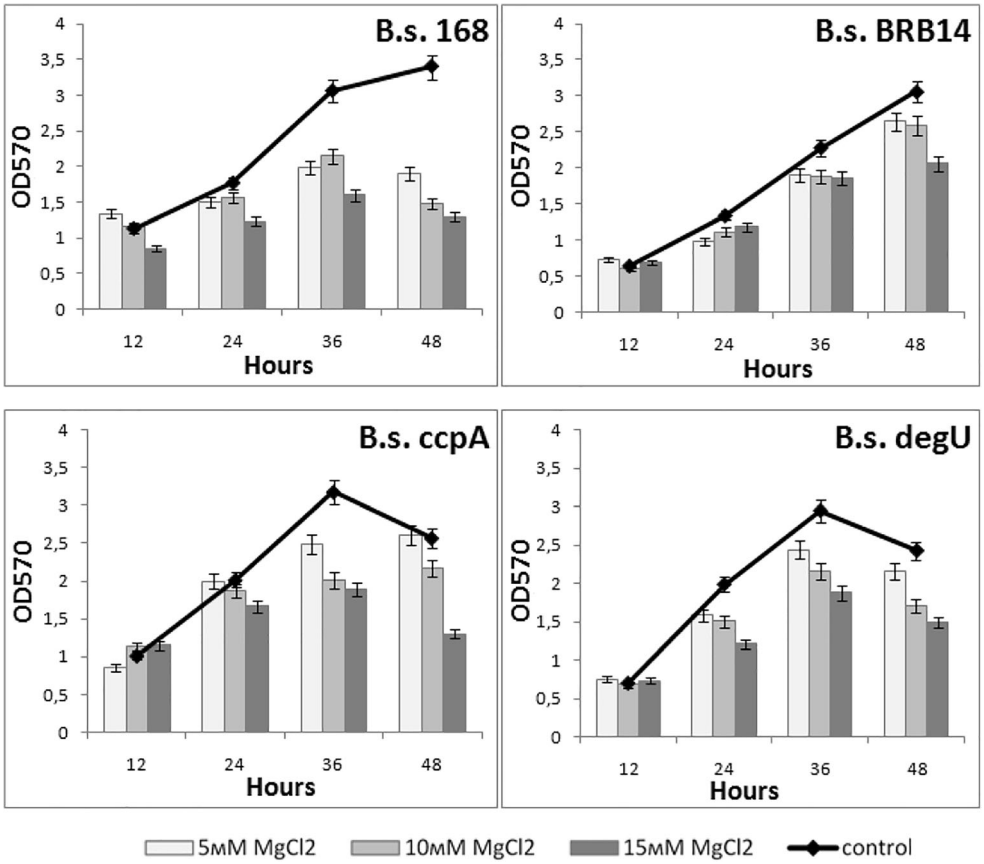

Fig. 3 The effect of magnesium ions on the level of biofilm formation by *B. subtilis* strains

growth up to 48 h the reduction of biofilm formation for all concentrations reaches 80% for the wild strain and 50% for protease deficiency strain (Fig. 4).

When added into the culture medium of EDTA at a concentration of 10 mM, all strains exhibit a similar picture: before 24 h of growth, the decrease in the level of biofilm formation does not exceed 15%; however, in the next 24 h, the level decreases by at least 50% (Fig. 5).

4 Conclusion

In general, recombinant strains with disturbances in the regulation systems for the transport of substances are relatively less sensitive to the presence of metal ions in the culture medium than wild and protease-deficient strains. At the moment, we cannot be sure to explain this effect. Since deleted regulatory proteins are multifunctional, it is likely that the observed increased resistance to the presence of divalent metal ions may be due to the inclusion of another regulatory network that compensates for the defective one. In addition, all metals used in the work are important trace elements and are involved in a wide range of cell physiological processes, including the biofilm structure formation [22–25]. Perhaps in the mutant strains, metal ions become more accessible to other important biochemical pathways, which in turn affects the relative increase in the biofilm formation. It is shown that metal ions are able to integrate into the structure of the matrix, increasing its resistance to external factors (matrix reinforcement) [26]. It is possible that this effect may be enhanced in the regulatory-deficient strains.

The absence of 11 extracellular proteinases has practically no effect on the reaction of *B. subtilis* BRB14 culture on the introduction of metal ions into the medium. This indicates that the deleted proteases are not involved in both the processes of substance transport and biofilm formation. It is also obvious that proteinases deleted in the *B. subtilis* BRB14 strain do not participate in the cleavage of the protein component of the matrix. Otherwise, the level of biofilm formation for this strain would be higher than the wild type one. The fact that the protease-deficient strain, like the regulatory mutants, is more resistant to divalent metal ions than the wild type may indicate that the absence of the need to spend energy and resources on the synthesis of proteinases allows the strain to withstand stress more effectively.

The presence in the culture medium of EDTA equally decreases the production of biofilms for all strains. Presumably, EDTA blocks the contained in the original media metal ions necessary for the synthesis of a biofilm. In this case, the biofilm formation does not stop, but only significantly decrease.

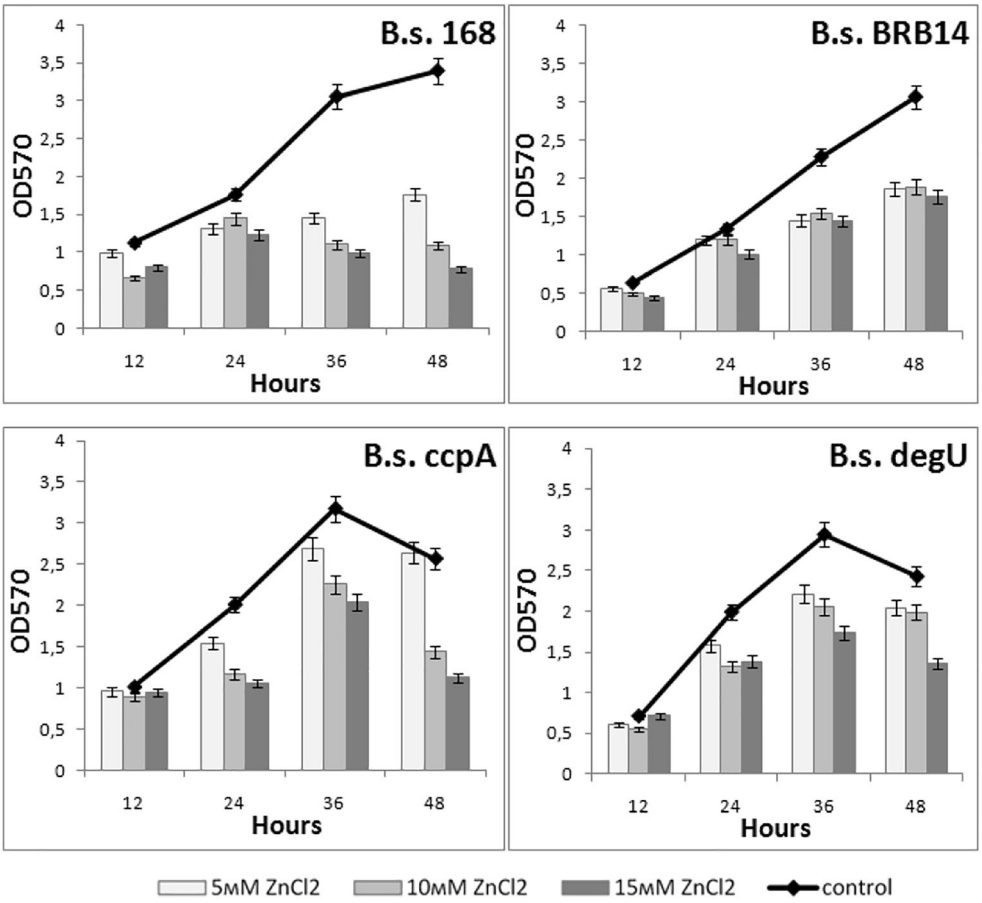

Fig. 4 The effect of zinc ions on the level of biofilm formation by *B. subtilis* strains

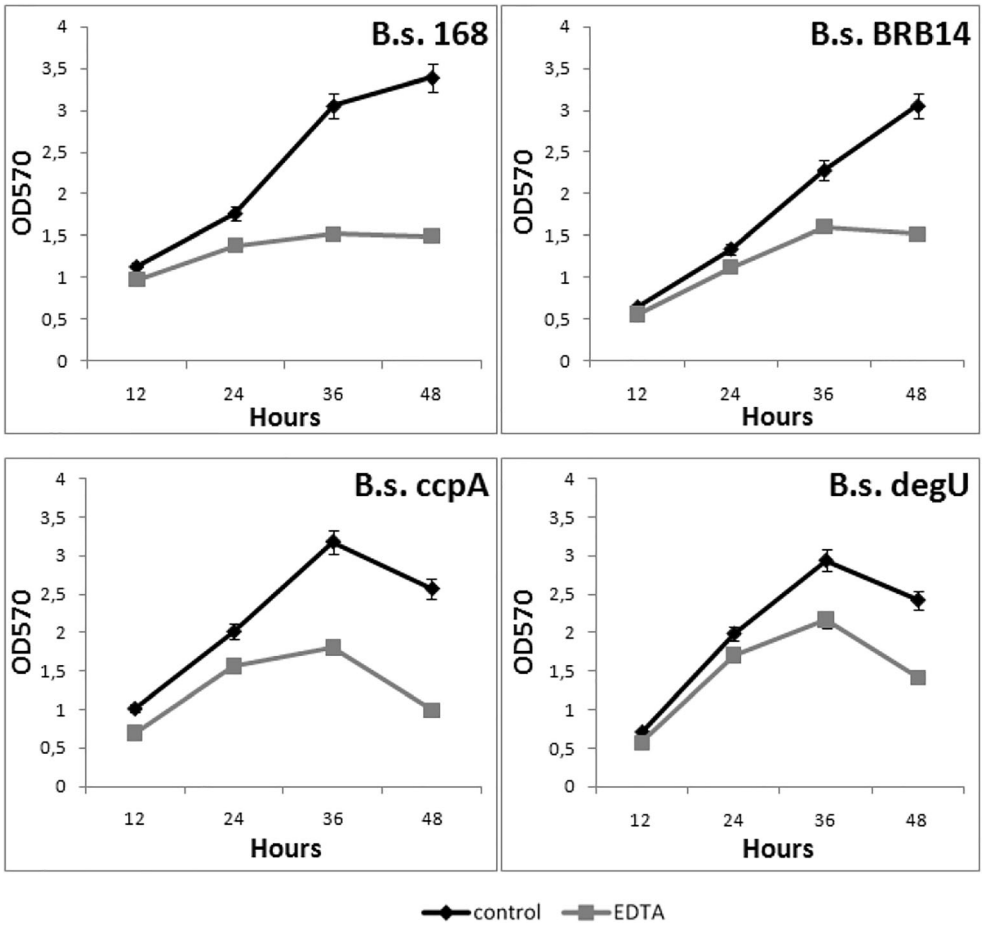

Fig. 5 The effect of EDTA (10 mM) on the level of biofilm formation by *B. subtilis* strains

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