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# Role of Iron Homeostasis in the Multispecies Biofilm Formation

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**Abstract**—Iron acquisition is of fundamental importance for microorganisms, since this metal is generally poorly bioavailable under natural conditions. Fe is mostly present as a ferric form in soils, which strongly limits its bioavailability, while most soil bacteria are tightly packed within multicellular communities named biofilms. This research showed that biofilm formation by a gram-positive bacterium *Bacillus subtilis* during the interactions with other microbial species was both essential to ensure Fe uptake from the environment and to maintain the cellular Fe homeostasis. The biofilm matrix appeared to play an important role, favoring the efficient usage of siderophores. Taken together, these results demonstrate a close link between biofilm formation and iron acquisition in *B. subtilis* and *Escherichia coli*, allowing a better comprehension of how bacteria can cope with metal limitation under environmental conditions.

**Keywords:** *Bacillus subtilis*, biofilm, iron homeostasis, multispecies biofilms

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## INTRODUCTION

Iron (Fe) is an essential nutrient required in trace amounts for plant growth as well as for human and animal health (Neilands, 1995). It is necessary for maintaining the life processes and occurs naturally in soils (Rajkumar et al., 2010). While sixteen distinct iron (hydr)oxide phases are known to exist on the Earth's surface, goethite and hematite are the most common of these in moderately to strongly oxidizing environments (Brinza et al., 2015). Iron availability in the rhizosphere is limited by the very low solubility and slow dissolution rates of inorganic iron compounds, which are beneath those required for plant and microbial growth (Guelke et al., 2010). Changes between iron redox states (ferrous or ferric) drive numerous reactions involving electron transfer, which are important for plants. However, there is great variation in the availability of iron in the soil, and starvation or excess can cause severe nutritional disorders, which significantly affect the physiology of the plants (Geisler and Venema, 2011). Although abundant in mineral soils, most of Fe exists as ferric iron [Fe(III)], which is sparingly soluble in the physiological pH range under oxic conditions. Therefore, an important abiotic stress in agriculture worldwide arises from low Fe availability due to high soil pH, with 30% of arable land too alkaline for optimal crop production (Römheld et al., 1986).

Iron is essential for microorganisms due to its involvement in multiple metabolic processes, including respiration ([Fe–S]-containing ferredoxins, heme-containing cytochromes) and key enzymatic

reactions (e.g. [Fe–S] proteins, such as fumarase and aconitase of the TCA cycle). However, iron can also present a hazard to organisms because of the Fe<sup>2+</sup>-triggered Fenton/Haber-Weiss reaction which produces harmful reactive oxygen species (ROS) such as superoxide (O<sub>2</sub><sup>-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and the highly destructive hydroxyl radical (·OH) (Cornelis et al., 2011). The small RNA (sRNA) RyhB has been shown to be a key actor of iron homeostasis regulation in bacteria. Through multiple molecular mechanisms, RyhB represses expendable iron-utilizing proteins, promotes siderophore production, and coordinates Fe–S cluster cofactor biogenesis, thereby establishing a so-called iron-sparing response (Chareyre and Mandin, 2018). Commonly, bacteria acquire iron by secretion of low-molecular mass iron chelators referred to as siderophores which have high association constants for complexing iron. Most of the siderophores are water-soluble and may be divided into extracellular siderophores and intracellular siderophores. Generally, rhizobacteria vary regarding to the siderophore cross-utilizing ability; some are proficient in using siderophores of the same genus (homologous siderophores) while others could utilize those produced by other rhizobacteria of different genera (heterologous siderophores) (Ahemad and Kibret, 2014). Suppression of the development of diseases caused by soilborne pathogens by competition for iron is often inconsistent, since iron availability varies in time and space and can be affected by the utilization of heterologous siderophores by other organisms or degradation of the siderophore-iron complex (Doornbos et al.,

2012). Siderophores produced by soil microorganisms can promote the dissolution of insoluble mineral phases (Sokolova et al., 2010), provide plants with Fe nutrition to enhance their growth when the bioavailability of Fe is low (Yadav et al., 2011; Beneduzi et al., 2012), and play an important role in disease control of fish by limiting Fe (Sugita et al., 2012).

Another important feature of most soil microorganisms, similar to other microorganisms, including those causing various diseases, involved in the remediation of waste waters and polluted soils etc., is their ability to form biofilms, multicellular communities embedded in a self-secreted extracellular matrix (Liu et al., 2014). Bacteria physically interact with surfaces to form complex multicellular and often multispecies assemblies, including biofilms and microcolonies (Doornbos et al., 2012) that are mediated by adhesins including polysaccharides and surface proteins, with initial contact often mediated by active motility (Romanova and Gintsburg, 2011). There is growing appreciation that the intensity, duration, and outcome of plant-microbe interactions are significantly influenced by the formation of adherent microbial populations (Doornbos et al., 2012). Plant-associated bacteria attach to and form biofilms on different tissues, including leaves, stems, vasculature, seeds, and roots (Doornbos et al., 2012; Castiblanco and Sundin, 2016) during pathogenesis and symbiosis, and in commensal relationships (Ramey et al., 2004). Biofilms are complex bacterial assemblages with a defined three-dimensional architecture, attached to solid surfaces and surrounded by a self-produced matrix generally composed of exopolysaccharides, proteins, lipids, and extracellular DNA. Biofilm formation has evolved as an adaptive strategy of bacteria to cope with harsh environmental conditions as well as to establish antagonistic or beneficial interactions with their host (Castiblanco and Sundin, 2016). Bacteria in biofilms are able to exchange signals and display coordinated activity that is inherent to multicellular organisms. Formation of biofilm communities turned out to be one of the main survival strategies of bacteria in their ecological niche. Bacteria in attached condition in biofilms are protected from the environmental damaging factors and effects of antibacterial substances in the environment (Romanova and Gintsburg, 2011).

Biofilms formed by a large variety of bacteria, such as *Bacillus subtilis* (Zhu et al., 2018), *Pseudomonas aeruginosa* (Soldano et al., 2020), and *Mycobacterium tuberculosis* (Pandey et al., 2018), were shown to be regulated by the environmental concentration and chemical form of Fe. In *B. subtilis*, an increased concentration of extracellular  $\text{FeCl}_3$  strongly promoted biofilm formation (Pelchovich et al., 2013), and deletion of the genes encoding the biosynthesis of the siderophore bacillibactin affected complex colony development (Zhu et al., 2018). Within biofilms, several different cell types coexisted, including subpopulations of motile cells, matrix-producing cells, and dor-

mant spores (Romero et al., 2010). The production of these large macromolecules involves strict transcriptional control of the genes required for synthesis of matrix components. Phosphorylation, and thus activation, of the transcription factor Spo0A is central to biofilm initiation (Branda et al., 2001). Spo0A can be activated by various environmental signals that allow the cell to tune its behaviour to the local environment. At threshold levels of Spo0A phosphate (Spo0A~P), two parallel pathways of anti-repression are triggered to allow transcription of the operons critical for biofilm matrix production. The concentration of Spo0A~P is determined by the activity of at least four kinases (KinA, KinB, KinC and KinD) that either act directly on Spo0A or indirectly via a phosphorelay and govern the regulatory pathway for matrix gene expression by influencing the activity of the master regulator SinR, a repressor of the *epsA-O* and the *tapA* operons (Vlamakis et al., 2013). The ability of siderophores to affect cellular differentiation in *B. subtilis* suggests that complex microbial interspecies interactions are likely in environments such as soil and intestine. Because *B. subtilis* possesses a diverse range of siderophore acquisition systems, it has a striking ability to pirate other species' siderophores. Recently, the presence of exogenous siderophores was reported to promote sporulation in *B. subtilis*, a cellular pathway controlled by the same transcriptional regulator as biofilm formation (Grandchamp et al., 2017). Most natural biofilms are multispecies in composition, but the mechanism of the regulation of multispecies biofilm development and amount of the biofilm matrix and its properties remains unclear.

In the study by Oh et al. (2018) biofilm formation by *Campylobacter jejuni* strains under conditions of oxidative stress was stimulated by  $40 \mu\text{M Fe}^{2+}$  or by  $20 \mu\text{M Fe}^{3+}$ . *B. subtilis* strains isolated from the rhizosphere of different types of soil had several beneficial attributes, which included biocontrol, plant growth promotion, sulfur oxidation, phosphorus solubilization and production of industrially important enzymes (amylase and cellulase), as well as endospores, which warrant the prevalence of *Bacillus* under different environmental conditions, its long-term storage and easy development of reliable formulations. These features have led to the increased devising and implementation of antimicrobial active biological products based on *Bacillus* species as agents for plant disease control under loading of ferric or ferrous ions and organic wastes of the soils. The neutral or alkaline soil pH and free Fe/Al (hydro) oxides, high contents of nutrients and moisture are important explanatory factors for *E. coli* survival in the soils. The strains can invade into the soil through sewage irrigation and is always considered as a fecal indicator bacterium, that can form biofilms in the presence of ferric or ferrous ions in the soil and during their interaction with other microbial species. Information on their association with *B. subtilis* strains, that serve as biocontrol agents,

and in the presence of heavy metal ions such as iron also remain poorly studied and justify the extension of studies on biofilm formation by the co-cultivation of the two strains.

The goal of the present work was to study the effects of ferrous ions on the development of *B. subtilis* biofilms during their interactions with *Escherichia coli* strain K-12 1655. Effects of minerals on bacterial biofilm formation and the relationships between both analyzed strains in their structures were analyzed by the crystal violet staining method combined with culture-based analysis and confocal laser scanning microscopy.

## MATERIALS AND METHODS

### *Experimental Design*

In the first part of this research, a dual-species model biofilm consisting of *Bacillus subtilis* and *Escherichia coli* was grown and used for subsequent experiments. In order to obtain a strongly adherent and mature model biofilm, different medium composition ( $\text{Fe}^{2+}$  concentrations) were altered. The amount of biofilm material at the experiment conditions was quantified by means of crystal violet staining and subsequent optical density (OD) measurements. To determine the cell density/maturity of the biofilm, viable plate counts were used. Luria Bertani agar (LB agar, National Centre of Infectious and Parasitic Diseases, Sofia, NCIPD, Sofia) were applied to determine the total biofilm cell density, and selective medium MacConkey agar (NCIPD, Sofia) was used to determine the contribution of colony-forming units (CFU) of *E. coli* 1655 strain to this total cell density.

### *Bacterial Strains and Culture Media*

In this research, *B. subtilis* 170 ( $\text{trpC}^{2+}$ , ability to form of surfactin and iturin), *B. subtilis* 168 ( $\text{trpC}^{2-}$ , transformation of 168 *his trpC2* with W23 DNA, unable to form of surfactin and iturin) and *E. coli* 1655 ( $\text{F lac}^{+}\text{str}^{-\text{s}}\text{met}^{-}$ ), both acquired from the bacteria collection of National Bank of Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria, and the “Stephan Angeloff” Institute of Microbiology in Sofia, were used. Stock cultures of both strains were stored at  $-80^{\circ}\text{C}$  in Luria Bertani Broth (LB, National Centre of Infectious and Parasitic Diseases, Sofia, NCIPD, Sofia), supplemented with 20% (v/v) glycerol (NCIPD, Sofia). For every experiment, a purity plate was prepared by spreading a loopful of stock culture onto a LB agar plate (Plate Count Agar (NCIPD, Sofia)) and MacConkey agar (NCIPD, Sofia). The purity plates for *B. subtilis* and *E. coli* were incubated for 24 h at  $37^{\circ}\text{C}$ .

Starting from the purity plates, pre-cultures were prepared by transferring one colony into an Erlenmeyer flask containing 100 mL of LB medium. *B. sub-*

*tilis* and *E. coli* pre-cultures were incubated for 24 h at  $37^{\circ}\text{C}$ . Following this incubation period, stationary phase cultures with a cell density of  $\sim 10^9$  CFU/mL were obtained.

### *Biofilm Development Conditions*

The stationary phase pre-cultures were used to develop a 100-fold diluted inoculum with a cell density of  $\sim 10^7$  CFU/mL. The investigated pre-culture ratios (*B. subtilis* and *E. coli*) were 1 : 1 and the growth medium was Luria Bertani broth, which proved to be the optimal media for single-species and multispecies biofilm development by both strains.

To develop the biofilms, 1.2 mL of the inoculum was transferred to a polystyrene petri dish (50 mm diameter, 9 mm height, Simport, Canada). After inoculation, the dishes were closed and gently shaken to make sure the inoculum covered the entire surface. At the incubation conditions, described above, the dishes were incubated for 24 h at  $20^{\circ}\text{C}$  onto a LB agar plate (Plate Count Agar (NCIPD, Sofia)) and MacConkey agar (NCIPD, Sofia), which was the optimal temperature for *B. subtilis* and *E. coli* single-species and multispecies biofilm formation (Ganchev et al., 2019).

### *Crystal Violet Assay*

Bacterial biofilms were grown in 96-well microtiter plates (Greiner Bio-One, Austria) with 100  $\mu\text{L}$  of bacteria in post-exponential growth phase in M63 (0.02 M  $\text{KH}_2\text{PO}_4$ , 0.04 M  $\text{K}_2\text{HPO}_4$ , 0.02 M  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 mM  $\text{MgSO}_4$ , and 0.04 M glucose) liquid medium per well. The final  $\text{OD}_{600}$  was 0.4. After 24 h of growth in static conditions at  $20^{\circ}\text{C}$ , the plates were washed thrice with NaCl (0.85%) and dried for 30 min at room temperature. Biofilms were stained for 15 min with 200  $\mu\text{L}$  of Crystal Violet at 0.01% (w/v) and rinsed thrice with NaCl (36 g  $\text{L}^{-1}$ ) to fully eliminate of rest of the dye, that was loosely adsorbed on the surface of the wells, unoccupied by biofilms structures, and dried for 10 min. The stain was extracted from the biofilm with absolute ethanol for 10 min at  $20^{\circ}\text{C}$ . Absorbance of the ethanol extract ( $\text{OD}_{540}$ ) was then measured at 540 nm on ELTA 90 M reader.

### *Quantification of Colonies*

The material from 6 wells was pooled together in a centrifuge tube containing 4.5 mL of sterile physiological solution, and these tubes were vortexed for 1 min to detach the biofilms from the samples (the material of the wells). After this, aliquots of 25  $\mu\text{L}$  of serial dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ , and  $10^{-4}$ ) were plated in duplicate on Plate Count Agar (NCIPD, Sofia) and MacConkey Agar (NCIPD, Sofia) for the identification of *B. subtilis* and *E. coli*, respectively. Red and pink colonies grown on MacConkey agar were presumptively

identified as *E. coli*. After incubation at 37°C for 24 h, the colony-forming unit per milliliter (CFU/mL) count was determined and log-transformed (log 10). The number of spores within the structure of the investigated biofilms was determined by pasteurizing the initial suspension of the biofilms, scraped with a sterile knife, at 75°C for 15 min to eliminate the vegetative cells. After bringing the temperature of the samples to 20°C, serial dilutions were performed according to the Koch method, with plating on solid medium Mesopeptone agar, incubation of the samples at 37°C for 24 h and counting the number of germinated colonies as spore-forming units.

### *Multispecies Biofilm Formation*

For the dual-species biofilm, bacteria in the post-exponential growth phase were suspended in 0.85% NaCl solution and inoculated in 24-well plates (Corning Incorporated Costar®, United States) to a final OD<sub>600</sub> of 0.3 (0.15 per strain). For the biofilms involving two bacterial strains, the final OD<sub>600</sub> was 0.3 or 0.4 (0.1 per strain). Controls included single-species biofilms formed at the same inoculum concentrations and conditions as the multispecies biofilm. For the purposes of the task, 18 h cultures of *B. subtilis* 170, *B. subtilis* 168 (trpC<sup>-</sup>, transformation of 168 *his trpC2* with W23 DNA) and *E. coli* K-12 1655 (F lac<sup>+</sup>str<sup>-s</sup> met<sup>-</sup>) strains were prepared in advance in a medium broth (Laboratory “BulBio”-Sofia). The liquid cultures (50 µL) were inoculated into 5 cm<sup>3</sup> of M63 liquid medium, to which FeSO<sub>4</sub>·7H<sub>2</sub>O was added to the final Fe<sup>2+</sup> concentrations of 5, 50, and 100 µM. The control sample was cultured in M63 medium without addition of FeSO<sub>4</sub>·7H<sub>2</sub>O. For each factor, the experiment was carried out in five test tubes, in the first one only 50 µL of the liquid culture of *B. subtilis* 170 was seeded, in the second—*B. subtilis* 168, in the third—*E. coli* K-12 1655, in the fourth tube the inoculum was 50 µL of the liquid culture of *B. subtilis* 170 and 50 µL of the liquid culture of *E. coli* K-12 1655, in the fifth place the inoculum was 50 µL of the liquid culture of *B. subtilis* 168 and 50 µL of the liquid culture culture of *E. coli* K-12 1655. Fresh cultures were dispensed into 96-well plates. Each fresh culture was dispensed into 12 wells, placing 150 µL of the liquid fresh culture in each well. Distilled water (150 µL) was dripped into the final unseeded wells. The first plate was incubated at 20°C. Cultivation of the biofilms on the plates was carried out for 24 h. After that, the cells of the biofilms were separated from each well after washing three times with saline (0.85% NaCl), i.m. 150 µL of saline was placed in half of the wells, and the biofilm was peeled off using a sterile knife and for each variant of the experiment, the suspension of 6 wells was collected into one Eppendorf tube. The absence of precipitates of high molecular weight compounds of the biofilm matrix served as a control check for the release of via-

ble cells within the biofilm structure in the present study.

### *Matrix Components Staining*

For the matrix staining, the biofilm was grown for 48 h under static conditions. Each biofilm was stained with DAPI at 5 µg/mL (Sigma-Aldrich, Germany) and one of the matrix dyes listed below. Exopolysaccharides were stained with the Wheat Germ Agglutinin (WGA) associated with the Alexa Fluor™ 555 conjugate (Thermo Fisher Scientific, United States) at 100 µg/mL to label N-acetyl-glucosamine. After 30 min of incubation of each probe, each coverslip was washed 3 times in PBS 1×. Finally, the coverslips were mounted with a drop of Prolong™ Diamond Antifade before observation under a Leica TCS SPE confocal laser scanning microscope at wavelength of 595 nm.

### *Data Extraction from Images and Statistics*

At least three replicates and five pictures per replicate were performed and used for data extraction. The pictures have been acquired by epifluorescence microscopy or by CLSM. The percentages of recovery of epifluorescence microscopy were determined using an algorithmic method with RStudio 0.98.1025 (RStudio, United States), where the brightness pixel was determined by threshold's definition of a small area of the picture around the pixel and against the background. The biovolume, the average thickness and the evaluation of the maximum coverage in the CLSM pictures were determined with the COMSTAT software developed in MATLAB R2015a (MathWorks, United States) as previously performed (Heydorn et al., 2000).

To test for statistically significant differences ( $P < 0.05$ ) between two conditions, a *t*-test was performed and between different time points, a two-way analysis of variance including the Bonferroni post-test were performed using SPSS 13.0 (IBM, Armonk, NY, United States).

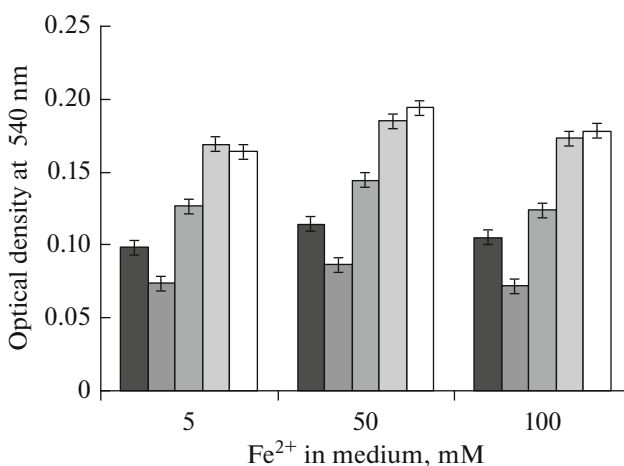
## RESULTS AND DISCUSSION

The antagonistic activity of *B. subtilis* against bacterial and fungal pathogens in the rhizosphere has been shown to be achieved by virtue of the production of surfactin and iturin A, which are lipopeptides, that contain a hydroxy fatty acid connected by an ester peptide linkage to a cyclic heptapeptide, and by ability to form biofilms on root surface during their interaction with other microbial species (Zhu et al., 2018), that was strongly promoted by increased concentration of extracellular Fe<sup>2+</sup> (Pelchovich et al., 2013). *B. subtilis* 170 was the subject of the present study because of its ability to form surfactin, which predetermines its future importance as a seed protectant and antifungal agent, and because of the fact that the root

microbiota of some agricultural crops is dominated by *B. subtilis* strains, which form abundant biofilms under conditions of soil contamination due to irrigation leading to the emergence of *E. coli* strains, as well in the presence of ferrous and ferric ions. Nevertheless, there is a lack of sufficient data on the importance of ferrous and ferric ions in the formation of multispecies biofilms, which determined the option of the present study, related to the investigation of the process of biofilm formation by the co-cultivation of *B. subtilis* and *E. coli* strains in the presence of  $\text{Fe}^{2+}$ .

Iron is a microelement, the content of which affected the nature of the relationships between *B. subtilis* and *E. coli* strains during the process of biofilm formation (Fig. 1). The change in the content of ferrous ions in the medium in the range from 5 to 50  $\mu\text{M}$  was accompanied by an increase in the biomass of mixed biofilms, as the optical density at 540 nm measured after staining with 1% crystal violet solution changed in the range of  $0.163 \pm 0.21$  to  $0.184 \pm 0.57$  as a result of the interaction between *B. subtilis* 170 and *E. coli* K-12 1655 and from  $0.145 \pm 0.96$  to  $0.198 \pm 0.78$  for the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655 strains. Their values were close to the biomass in the single-species biofilms of *B. subtilis* 170, *B. subtilis* 168, and *E. coli* K-12 1655 strains and were significantly lower in the absence of ferrous ions in the medium containing glucose as the only carbon source ( $p < 0.05$ ). Increasing its concentration to 100  $\mu\text{M}$  was accompanied by a decrease in the value of the optical density of the formed mixed biofilms.

The concentration of bivalent metal ions determines the direction of a number of cellular processes of *B. subtilis* (López et al., 2010) and *E. coli* (DePas et al., 2013), among which the process of biofilm formation stands out (López et al., 2010). The inhibitory effect of  $\text{Mg}^{2+}$  ions on the process of biofilm formation by *B. subtilis* strain NCIB3610 is determined by their concentration in the culture medium, and their influence is most pronounced at 25 and 100 mM according to the results of Oknin et al. (2015). A similar dependence was found for *E. coli* strain M1655 in the study of Wei et al. (2007), which was associated with the low activity of the Fe–S centers. The development of *E. coli* strains to form biofilms in an attached state under the influence of variable concentrations of ferric ions in the medium is regulated by the regulatory protein RpoS, which is activated under conditions of oxidative stress (Mika and Hengge, 2014). The increase in its intracellular level ensures the activation of both genes responsible for the response of cells to oxidative stress (Adnan et al., 2017), and ensures the transcription of genes for the development of biofilms (Adnan et al., 2017). According to another study, RyhB is a low-molecular-weight RNA that inhibits the biosynthesis of iron-containing proteins at a low content of ferric ions in the medium (Massé and Gottesman, 2002), which may explain the significantly lower value



**Fig. 1.** The influence of content of  $\text{Fe}^{2+}$  in the culture medium on the biofilm growth: ■ *B. subtilis* 170; ■ *B. subtilis* 168; ■ *E. coli* 1655; □ *B. subtilis* 170 + *E. coli* 1655; □ *B. subtilis* 168 + *E. coli* 1655. The biofilms were grown in 96-well plates for 24 h at 20°C in a medium M63 with  $\text{Fe}^{2+}$  concentrations of 5, 50, and 100  $\mu\text{M}$ . The optical density of the ethanol extract was measured OD540 after staining of biofilms with 0.1% crystal violet solution.  
 $y = -0.0128x^2 + 0.0548x + 0.0562$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 170;  
 $y = -0.0139x^2 + 0.0547x + 0.327$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 168;  
 $y = -0.0192x^2 + 0.0752x + 0.0707$ ,  $r^2 = 1$ ,  $p < 0.05$ —*E. coli* K-12 1655;  
 $y = -0.014x^2 + 0.0582x + 0.1248$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 170—*E. coli* K-12 1655;  
 $y = -0.0229x^2 + 0.0986x + 0.0883$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 168—*E. coli* K-12 1655.

of the optical density at 540 nm in the biofilms of *E. coli* K-12 1655 and in the mixed biofilms in its association with *B. subtilis* strains at a ferric ion concentration of 5  $\mu\text{M}$  compared to the control sample. The induction of oxidative processes explains the slight decrease in the biomass of single-species biofilms of *B. subtilis* 170, *B. subtilis* 168 and *E. coli* K-12 1655, when the concentration of ferric ions changed in the range from 100 to 200  $\mu\text{M}$ .

The dependence between the concentration of ferrous ions in the culture medium and the optical density at 540 nm in mixed biofilms had a different nature, which was probably attributed to the presence of mechanisms that ensure the competitive advantage of one strain in the process of biofilm formation on polystyrene plates. The quorum-sensing system is a mechanism of interaction between cells of two or more microbial species that affects the process of biofilm formation under conditions of oxidative stress (Abisado et al., 2018). In the study of Gambino et al. (2015), cells of a *B. subtilis* strain exposed to oxidative stress in the presence of ferric ions in the medium were characterized by a high level of expression of the genes encoding the formation of oxidative stress proteins AhpC, SufD, and thioredoxin, and increased quorum

**Table 1.** The impact of ferrous ions in the medium on CFU numbers of *B. subtilis* 168 and *E. coli* 1655 strains in the structures of single-species and dual-species biofilms

Fe <sup>2+</sup> , μM	Colony-forming units in biofilms of <i>B. subtilis</i> 168, CFU/cm <sup>3</sup>	Colony-forming units in biofilms of <i>E. coli</i> K-12 1655, CFU/cm <sup>3</sup>	Colony-forming units in dual-species biofilms, CFU/cm <sup>3</sup>	
			<i>B. subtilis</i> 168	<i>E. coli</i> K-12 1655
0	$(0.53 \pm 0.06) \times 10^6$	$(5.1 \pm 0.10) \times 10^6$	$(10.7 \pm 0.25) \times 10^6$	$(10.36 \pm 0.32) \times 10^6$
5	$(4.53 \pm 0.30) \times 10^3$	$(4.83 \pm 0.30) \times 10^3$	$(30.53 \pm 0.15) \times 10^3$	$(4.7 \pm 0.26) \times 10^3$
50	$(56.03 \pm 0.25) \times 10^3$	$(19.46 \pm 0.21) \times 10^3$	$(38.93 \pm 0.11) \times 10^3$	$(33.73 \pm 0.21) \times 10^3$
100	$(5.1 \pm 0.1) \times 10^3$	$(5.43 \pm 0.32) \times 10^3$	$(37.7 \pm 0.2) \times 10^3$	$(9.66 \pm 0.35) \times 10^3$

sensing activity-sensing system (DegU, OppF, CotE and SrfAB), which affected the transcription of genes for biofilm formation. DegU is a major regulator in *B. subtilis* strains, which takes part in the genetic control of a number of processes such as competence, cell motility and the secretion of hydrolytic enzymes (Verhamme et al., 2009).

The structure of the microbial population in the process of cultivation of mixed biofilms in the medium containing glucose as a carbon source was dominated by *B. subtilis* strain 170 and 168 in the number of  $(15.3 \pm 0.16) \times 10^6$  CFU/cm<sup>3</sup>, which was close to the number of colonies of *E. coli* K-12 1655 ( $p > 0.05$ ) and exceeded its value in the respective single-species biofilms ( $p < 0.05$ ). The inclusion of ferric ions in the nutrient medium changed the course of the development of bacterial strains from the structure of biofilms, associated with a significant decrease in the number of colonies for the studied pairs of strains ( $p < 0.05$ ) (Tables 1). Their number at 50 μM Fe(II) was  $(42.96 \pm 0.40) \times 10^3$  CFU/cm<sup>3</sup> for *B. subtilis* 170 (the data are presented in the research article by Ganchev et al. (2019)) and  $(5.23 \pm 0.15) \times 10^3$  CFU/cm<sup>3</sup> for *E. coli* K-12 1655 in the structure of mixed biofilms, which significantly exceeded their abundance in the respective single-species biofilms. A similar nature in the change in the abundance of the biofilm microbial populations was found in the joint cultivation of the strains *B. subtilis* 168 and *E. coli* K-12 1655. The number of colonies of the studied pair of strains in the biofilm structures reached the maximum value with increase in Fe<sup>2+</sup> concentration up to 50 μM. The population structure included the same number of colonies of *B. subtilis* 170 and *E. coli* K-12 1655 and of *B. subtilis* 168 and *E. coli* K-12 1655, which values decreased when the content of Fe<sup>2+</sup> was 100 μM. The degree of reduction was most pronounced in *E. coli* K-12 1655 in the structure of mixed biofilms in their interaction with *B. subtilis* strains 170 and 168, whose colony numbers decreased statistically insignificantly to values of  $(52.93 \pm 0.29) \times 10^3$  CFU/cm<sup>3</sup> and  $(37.7 \pm 0.2) \times 10^3$  CFU/cm<sup>3</sup>.

The basis of the antagonistic effect of *B. subtilis* strains in the process of biofilm formation in their

association with gram-negative bacterial species lies in their ability to produce surface-active compounds (Singh et al., 2014) of a lipoprotein nature (Stein, 2005), whose biosynthesis is initiated by ferric ions (in the form of ferric sulfate) at concentration of 1.7 mM up to a value of 300 mM (Wei et al., 2004). Mn<sup>2+</sup> in the form of manganese sulfate at the 0.01 mM concentration ensured the biosynthesis of surfactin by *B. subtilis* ATCC 21332 strain at concentrations of 0.33 to 2.6 g/dm<sup>3</sup> the surfactant according to the study of Wei and Chu (2007). The absence of Mn<sup>2+</sup> and Fe<sup>2+</sup> did not affect surfactin biosynthesis by *Bacillus subtilis* ATCC strain 21332, but slowed its growth and development (Wei et al., 2007). The formation of surfactin at Fe<sup>2+</sup> concentrations from 50 to 100 μM explains the significant increase in population numbers of *B. subtilis* 170 in the structure of mixed biofilms in their interaction with *E. coli* strain K-12 1655, whose CFU numbers reached the maximum value of  $(37.7 \pm 0.2) \times 10^3$  CFU/cm<sup>3</sup> at a Fe<sup>2+</sup> concentration of 100 μM and strongly decreased when it increased to 150 μM. The smaller number of colonies of *B. subtilis* 168 in the structure of biofilms in its interaction with *E. coli* K-12 1655 was determined by the mutations in the *sfp* gene, encoding the biosynthesis of enzymes for the formation of surfactin (McLoon et al., 2011), and gives reason to assume that the Fe<sup>2+</sup> content stimulates the formation of antimicrobial peptides of *B. subtilis* strains with an antagonistic effect by another mechanism.

The relatively equal number of colonies in the structure of biofilms formed as a result of co-cultivation of the pairs of *B. subtilis* 170 + *E. coli* K-12 1655 and *B. subtilis* 168 + *E. coli* K-12 1655 strains, may be attributed to the course of another signaling mechanism that determines the mutualistic relationships between them in the study. According to Tonati et al. (1995), suppression of the activity of the cytoplasmic membrane transport system by the introduction of chelating agents into the medium is accompanied by an increase in the resistance of *E. coli* K-12 strains to oxidative and metallic stress. The role of such compounds capable of forming complexes with ferric ions is performed by catechol amides, which are secreted by *B. subtilis* strains according to the study of Li et al. (2017), where the number of its population is close to

**Table 2.** Morphometric features of dual-species biofilms of *Bacillus subtilis* 170 and *Escherichia coli* K-12 1655, *Bacillus subtilis* 168 and *Escherichia coli* K-12 1655 strains, depending on the concentration of Fe<sup>2+</sup> in medium

Fe <sup>2+</sup> , μM	Mean thickness, μm*	Coefficient of unevenness	Relative area, μm <sup>2**</sup>	Relationship area/volume, μm <sup>2</sup> μm <sup>-3***</sup>
Dual-species biofilms of <i>Bacillus subtilis</i> 170 and <i>Escherichia coli</i> K-12 1655 strains				
0	10.35 ± 0.41	0.02 ± 0.05	0.99 ± 0.02	0.099 ± 0.006
5	8.66 ± 0.15	0.08 ± 0.02	0.82 ± 0.03	0.115 ± 0.003
50	9.16 ± 0.34	0.03 ± 0.05	0.89 ± 0.07	0.110 ± 0.007
100	8.18 ± 0.20	0.02 ± 0.03	0.78 ± 0.02	0.123 ± 0.003
Dual-species biofilms of <i>Bacillus subtilis</i> 168 and <i>Escherichia coli</i> K-12 1655 strains				
0	10.78 ± 0.37	0.03 ± 0.04	0.99 ± 0.01	0.093 ± 0.003
5	8.16 ± 0.27	0.02 ± 0.04	0.76 ± 0.02	0.123 ± 0.002
50	9.15 ± 0.36	0.02 ± 0.05	0.84 ± 0.02	0.110 ± 0.002
100	7.67 ± 0.44	0.05 ± 0.01	0.80 ± 0.03	0.126 ± 0.001

\*  $y = -0.74x^2 + 2.72x + 6.68$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 170—*E. coli* K-12 1655.

$y = -1.235x^2 + 4.695x + 4.7$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 168—*E. coli* K-12 1655.

\*\*  $y = -0.009x^2 + 0.34x + 0.57$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 170—*E. coli* K-12 1655.

$y = -0.06x^2 + 0.26x + 0.56$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 168—*E. coli* K-12 1655.

\*\*\*  $y = 0.009x^2 - 0.032x + 0.138$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 170—*E. coli* K-12 1655.

$y = 0.0145x^2 - 0.0565x + 0.165$ ,  $r^2 = 1$ ,  $p < 0.05$ —*B. subtilis* 168—*E. coli* K-12 1655.

*E. coli* strain K-12 1655 in the structure of the formed mixed biofilms at a Fe<sup>2+</sup> content of 100 μM in the present study.

Static cultivation of the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 strains on slides resulted in formation of biofilms, whose average thickness varied in the range from 8.66 ± 0.15 to 9.16 ± 0.34 μm when the Fe<sup>2+</sup> content in the culture medium changed from 5 to 100 μM, approaching its control value ( $p > 0.05$ ). The change in its value in the coculture of *B. subtilis* 168 and *E. coli* K-12 1655 in the course of the study was similar. The coefficient of unevenness of the formed structures fluctuated within a narrow range ( $p > 0.05$ ), while the ratio of their relative area of distribution to volume changed exponentially with increasing content of Fe<sup>2+</sup> in the medium, as a maximum value of 0.123 ± 0.003 μm<sup>2</sup> μm<sup>-3</sup> for the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 and 0.126 ± 0.001 μm<sup>2</sup> μm<sup>-3</sup> for the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655 were achieved at 150 μM of Fe<sup>2+</sup>, which increased the size of the formed structures after their cultivation in the medium without Fe<sup>2+</sup> (Table 2).

In the base of the adverse effect of Mg<sup>2+</sup> ions on the formation of biofilms by *B. subtilis* strains is the inhibition of the *epsA-O* and *tapA* operons (Oknin et al., 2015), responsible for the biosynthesis of extracellular polysaccharides and amyloid-like fibers of the matrix composition of the structures attached to different surfaces (Branda et al., 2006; Chu et al., 2006). According to other studies, Fe<sup>2+</sup> at a concentration of up to 100 μM stimulated cell motility and development of biofilm formation by *Serratia marcescens* strains (Lin et al., 2017). The content of polysaccha-

rides decreased in the structure of biofilms of an *E. coli* strain with an increase in the concentration of bivalent ions in the culture medium (DeDas et al., 2013). Inhibition of their synthesis in the process of formation of biofilms by a *B. subtilis* strain occurred at Mg<sup>2+</sup> concentrations below 5 mM and above 25 mM (Oknin et al., 2015), and a similar dependence was found in the co-cultures of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains in the present study. The obtained results were due to the activation of the histidine kinase KinC through the regulatory pathway YwcC-SlrA under the influence of bivalent ions from the composition of the medium in *B. subtilis* strains (Shemesh and Chai, 2013), which positively affected the intracellular level of the phosphorylated form of the regulatory protein Spo0A (Spo0A~P) (Chai et al., 2009), which negatively affected the expression of genes for the biosynthesis of exocellular polysaccharides and proteins of biofilm matrix composition (Branda et al., 2006). According to Gambino et al. (2015), the increase in exocellular polysaccharide content in the matrix of biofilms of *B. subtilis* strains was observed upon the occurrence of oxidative stress. In *E. coli* strains, the effect of ferric ions created conditions for an increase in the intracellular level of free radicals, which ensured the development of biofilms, consisting of subpopulations of matrix-forming and non-biofilm cells (DePas et al., 2013). Their incubation in a medium containing bivalent ions determines the development of distinct cell subpopulations, distinguished by their sensitivity to the effects of antibiotic compounds and development to biofilms (Wu et al., 2012).



The relative area of distribution of the formed structures in the process of their static cultivation on the surface layer of the slides in the study changed exponentially in the pair of *B. subtilis* 170 and *E. coli* K-12 1655, and in the joint development of *B. subtilis* 168 and *E. coli* K-12 1655. The maximum value was observed at 100  $\mu\text{M}$  of  $\text{Fe}^{2+}$  in the medium; at higher concentrations it decreased to  $0.78 \pm 0.01 \mu\text{m}^2$  in the co-culture of *B. subtilis* 170 and *E. coli* K-12 1655 and to  $0.80 \pm 0.02 \mu\text{m}^2$  in the co-culture of *B. subtilis* 168 and *E. coli* K-12 1655, when reaching the  $\text{Fe}^{2+}$  concentration of 150  $\mu\text{M}$ .

Bivalent ions from the composition of the nutrient medium affect the electrostatic forces of attraction between the bacterial surface and the surface layer of the substrate (Stanley and Lazazzera, 2004) and at low concentrations negatively affect the process of adhesion of bacterial cells to various abiotic and biotic surfaces (Somerton et al., 2013). The results of the present study regarding the dependence of the relative area of biofilms formed as a result of cultivation of the co-cultures of *E. coli* K-12 1655 with *B. subtilis* 170 or *B. subtilis* 168 at the tested concentrations of  $\text{Fe}^{2+}$  in the medium are in agreement with previous studies, leading to the conclusion that low and high concentrations of bivalent ions create conditions for the inhibition of microbial development in the attached state on the surface layer of the substrate.

In the study by Oknin et al. (2015) the concentrations of  $\text{Mg}^{2+}$  below 5 mM and above 25 mM in the culture medium leads to repression of the *epsA-O* and *tapA* operons responsible for the biosynthesis of exocellular polysaccharides and amyloid-like protein fibers that make up the matrix of the structures formed (Chai et al., 2009; Romero et al., 2010) and ensuring their adhesion and spreading on the surface layer of the substrate (Branda et al., 2006), as well as by stimulating the formation of poly- $\gamma$ -glutamic acid (McLoon et al., 2011) and surfactin (Abisado et al., 2018). The activation of the two-component regulatory system DegS-DegU in the presence of bivalent ions in the medium creates conditions for the transcription of the *bslA* gene (Mhatre et al., 2017), which stimulates the spread of biofilms of *B. subtilis* strains on the surface layer of the substrate (Kobayashi et al., 2012; Hobley et al., 2013). The increase in the level of transcription of the *csgD* gene of *E. coli* strain UTI89, which encodes the formation of the main regulator CsgD of the process of biofilm formation, occurs at  $\text{Fe}^{2+}$  content in the medium in the range of 2 to 10  $\mu\text{M}$  (DeDas et al., 2013). The regulatory protein affects the process of formation of these structures through the induction of the *csgBAC* operon, responsible for formation of the curly fringes (Ogasawara et al., 2011), which are amyloid-like fibers included in the composition of their matrix (Chapman et al., 2002). Together with cellulose and poly-N-acetylglucosamine, it takes part in the occurrence of intercellular contacts and cell

adhesion of *E. coli* strains on various abiotic and biotic surfaces during their development to the formation of biofilms (Oknin et al., 2015). The decreased expression of the *tapA* operon in *B. subtilis* strains, responsible for the biosynthesis of amyloid protein fibers from the composition of the matrix, is more pronounced compared to the transcription of the *eps* operon when the content of bivalent ions in the medium increases to a value of 10 mM in the study by Oknin et al. (2015). The presence of similar regulatory mechanisms in *B. subtilis* and *E. coli* strains explains the nature of the change in the value of the relative spread area of the mixed biofilms when the concentration of  $\text{Fe}^{2+}$  in the culture medium varied within the range from 5 to 150  $\mu\text{M}$  in the present study, characterized by a maximum value when its concentration reached 100  $\mu\text{M}$  for co-cultures of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655.

The number of spores in the structure of biofilms varied in a narrow range in *B. subtilis* strains 170 and 168, when the concentration of  $\text{Fe}^{2+}$  changed within the range from 5 to 100  $\mu\text{M}$ . The increase in its value to 150  $\mu\text{M}$  was accompanied by a statistically significant increase in the number of spores in the formed structures, which exceeded their number in a medium without the inhibitory agent. The established dependence between the number of spores on the concentration of  $\text{Fe}^{2+}$  in the structure of the single-species biofilms of *B. subtilis* strains 170 and 168 was logarithmic (Table 3). The increase in the activity of the histidine kinases KinA, KinB, KinC, and KinD (located in the cytoplasm and cytoplasmic membrane) due to the increased content of bivalent ions in the medium provided phosphorylation of the regulatory protein Spo0A (Spo0A~P) for biomass formation processes. The increase in the level of transcription of the kinases KinA and KinB leads to the accumulation of Spo0A~P in the cells of *B. subtilis* strains, which creates conditions for their differentiation into spores (Devi et al., 2015) and may explain the present study of dependences on the number of spores in the structure of biofilms of *B. subtilis* 170 and *B. subtilis* 168. Heterogeneity of the cell population during the formation of biofilms is expressed in the formation of matrix-forming cells and cells, differentiating to spore formation (Gambino et al., 2015).

The increase in the content of  $\text{Fe}^{2+}$  in the culture medium within the range from 5 to 100  $\mu\text{M}$  was accompanied by a significant increase in the number of spores in the structure of biofilms, formed as a result of joint development of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains, and reaching a value of 150  $\mu\text{M}$  did not lead to a statistically significant increase in their colony numbers. Their number was significantly higher than their value in single-species biofilms and exceeded their value in the absence of  $\text{Fe}^{+2}$  in the culture medium (Table 3). This pattern is consistent with data from previous



**Table 3.** The impact of ferrous ions in the cultural medium on spore-forming units of *B. subtilis* 170, *B. subtilis* 168 strains in the structures of single-species and dual-species biofilms

Fe <sup>2+</sup> , μM	Spore-forming units in biofilms of <i>B. subtilis</i> 170, CFU/cm <sup>3</sup>	Spore-forming units in biofilms of <i>B. subtilis</i> 168, CFU/cm <sup>3</sup>	Spore-forming units in dual-species biofilms, CFU/cm <sup>3</sup>	
			<i>B. subtilis</i> 170 + <i>E. coli</i> 1655	<i>B. subtilis</i> 168 + <i>E. coli</i> 1655
0	$(6.66 \pm 0.5) \times 10^3$	$(10 \pm 0.1) \times 10^3$	$(2.54 \pm 0.47) \times 10^2$	$(2.66 \pm 0.5) \times 10^3$
5	$(0.2 \pm 0.05) \times 10^2$	$(0.4 \pm 0.02) \times 10^2$	$(0.12 \pm 0.44) \times 10^2$	$(0.3 \pm 0.30) \times 10^2$
50	$(5.66 \pm 0.21) \times 10^2$	$(5.74 \pm 0.12) \times 10^2$	$(0.56 \pm 0.57) \times 10^2$	$(1.3 \pm 0.46) \times 10^2$
100	$(6.01 \pm 0.29) \times 10^2$	$(6.12 \pm 0.17) \times 10^2$	$(2.96 \pm 0.26) \times 10^2$	$(4.0 \pm 0.07) \times 10^2$

$y = 5.181\ln(x) + 0.572$ ,  $r^2 = 0.952$ ,  $p < 0.05$ —*B. subtilis* 170—*E. coli* K-12 1655.

$y = 4.810\ln(x) + 0.960$ ,  $r^2 = 0.949$ ,  $p < 0.05$ —*B. subtilis* 168—*E. coli* K-12 1655.

studies, which reduce the activating effect of the amounts of *E. coli* strain formed in the medium (Rendueles et al., 2014) on the regulator AbrB, which leads to increased levels of Spo0A~P and the formation of disputes (Lopez et al., 2009).

In the presence of Fe<sup>2+</sup> at the concentration of 50 μM, the bacterial growth rates of single-species biofilms of *B. subtilis* strains and biofilms resulting from their interaction with *E. coli* strains were similar and determined the mutualism between two strains in the structure of biofilms, but the concentrations of 5 and 100 μM lead to the impeded growth and affected adversely the process of biofilm formation by the participation of *B. subtilis* 170 and *E. coli* K-12 1655, *B. subtilis* 168 and *E. coli* K-12 1655 strains, as a result of which a decrease in the value of the optical density, average thickness, and relative spreading area and an inversely proportional increase in the ratio of the spreading area of the structures to their volume, but stimulates spore formation. Many studies on Fe homeostasis by soil-inhabiting bacteria of *Bacillus subtilis* strains have been performed in shaken cultures.

The importance of the interplay between extracellular matrix production and efficient Fe acquisition and Fe homeostasis might have been overlooked. Considering that static growth in a biofilm is a highly relevant growing state for many soil bacteria, more research on metal acquisition and homeostasis by model soil bacteria in static cultures is required to draw a more comprehensive picture of the role of biofilm production in siderophore use efficiency and metal homeostasis.

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