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The intensity of biofilm formation by heterotrophic bacteria isolated from soil ferrosphere

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Abstract. The intensity of biofilm formation by heterotrophic bacteria possessed ammonifying ability (*Bacillus simplex* ChNPU F1, *Streptomyces canus* NUChC F2, *Streptomyces gardneri* ChNPU F3), ammonifying and iron-reducing ability (*Fictibacillus* sp. ChNPU ZVB1) previously isolated from soil ferrosphere was studied. Methods used: indirect measurement of the biomass of the bacterial biofilm using the adsorption/desorption of crystal violet, the aggregation test (to determine aggregation properties), the salt aggregation test (to determine hydrophobicity). The correlation analysis between the intensity of biofilm formation and aggregation of strains showed a significant positive correlation. The studied strains of microorganisms did not show high adhesive properties, they were moderately-adhesive (*B. simplex*, *S. canus* and *S. gardneri*) and weakly-adhesive (*Fictibacillus* sp.). It is supposed that the role these bacteria in the microbial damage of materials is determined preferably by bioelectrochemical reactions (iron-reducing bacteria) and the production of corrosive and/or antimicrobial metabolites (ammonifying and iron-reducing bacteria), but not by the biofilms formation. The prospect of further research is to analyze the antagonistic properties and biofilm formation of heterotrophic bacteria under co-cultivation conditions, in particular, with sulfate-reducing bacteria.

Keywords: Aggregation, ammonifying bacteria, biofilm, hydrophobicity, iron-reducing bacteria, ferrosphere.

1. Introduction

In the soil directly adjacent to the underground metal construction, there is a zone of development of a group of corrosive microorganisms, which is called "ferrosphere" (Andreyuk et al., 2002). This community is characterized by the stability of the qualitative composition of the associates: sulfate-reducing (SRB), thionic, denitrifying, ammonifying (AMB), hydrocarbon-oxidizing and iron-reducing (IRB) bacteria. They attach to the surface of a metal or protective coating, create a biofilm and cause microbiologically influenced corrosion (MIC), the rate of which is determined by the interaction of microorganisms in the community with the metal (Andreyuk et al., 2005).

Considerable attention is paid to SRB (Sungur et al., 2010; Chen et al., 2021; Tripathi et al., 2021), however, the conditions for their development are created by heterotrophic bacteria, in particular, AMB (Purish & Asaulenko, 2007; Purish et al., 2009). MIC is considered as a bioelectrochemical process that takes place in a biofilm - a highly organized, selfregulating biological system, the operation of which is aimed at optimizing their vital functions (Andreyuk et al., 2005). At the same time, the participation of AMB, IRB of ferrosphere in MIC processes, in particular their ability to form biofilms, remains a topical issue.

Previously, we isolated from the ferrosphere and identified some predominant representatives of heterotrophic bacteria with ammonifying ability (*Bacillus simplex, Streptomyces gardneri, Streptomyces canus*), as well as with ammonifying and iron-reducing ability of *Fictibacillus* sp. (Tkachuk & Zelena, 2021a), the property of which to form biofilms has not been studied. Therefore, the aim of this research was to investigate the intensity of biofilm formation of heterotrophic AMB and IRB, previously isolated from the soil ferrosphere.

2. Materials and methods

2.1. Microorganisms and growing conditions

Five-day pure cultures of *B. simplex* ChNPU F1 (KX349220 in the GenBank), *Fictibacillus* sp. ChNPU ZVB1 (KX349222 in the GenBank), *S. canus* NUChC F2 (MG924748 and MG924855 in the GenBank) and *S. gardneri* ChNPU F3 (KX349221 in the GenBank) isolated from the sulfidogenic microbial community of soil ferrosphere (Tkachuk & Zelena, 2021a) were used for the research. Suspensions from the studied monocultures with an optical density of 0.5 McFarland were prepared in solution of sterile isotonic NaCl. Then these suspensions were added to a sterile meat-peptone broth (MPB) in a ratio of 1:1, therefore, the calculated initial concentration of bacterial cells in MPB was approximately 7.5×10^7 cells/ml. Bacterial cultures were grown in glass tubes in a thermostat (static conditions) at 29 ± 2°C for 6 days.

2.2. Strain aggregation study

The strains aggregation was investigated according to the aggregation test proposed by Del Re et al. (2000). Aggregation was calculated by the formula (Eq.1):

$$\left(1-\left(\frac{A_0}{A}\right)\right)\times 100\,\%$$

where A0 is an optical density of the upper layer of the bacterial suspension after incubation for 2 hours at 30°C;

A is the optical density before incubation.

2.3. Investigation of the hydrophobicity of the strains

The salt aggregation test was used to determine the hydrophobicity of the strains. This test is based on the formation of aggregates by bacteria in the presence of ammonium sulfate at concentrations from 0.2 mol/L (M) to 4.0 M (Nwanyanwu & Abu, 2013). We used the following scale for assessing the bacterial hydrophobicity: high (< 1.0 M), moderate (1.0 – 2.0 M), low (> 2.0 M).

2.4. A biofilm assay

Used indirect measurement of the biomass of the bacterial biofilm using the adsorption/desorption of crystal violet according to the method described by Stepanović et al. (2000). Staining of the formed biofilms was performed with a 0.1% aqueous solution of crystal violet at 30°C for 60 minutes.

2.5. Statistical analysis of experimental data

Statistical processing of the obtained results was carried out using PAST 2.17 (Hammer et al., 2001). We used descriptive statistics methods to calculate the arithmetic mean and standard error of the arithmetic mean. The Student's significance criterion was calculated, and the 95% probability of differences ($p \leq 0.05$) was considered statistically significant. Correlation analysis according to Spearman was carried out.

3. Results and Discussion

3.1. Aggregation and hydrophobicity of the studied strains

Bacterial cell surface hydrophobicity is one of the most important factors that influence bacterial adhesion (Zita & Hermansson, 1997; Furuhata et al., 2009). The modern view of the model of biofilm formation is based on the inclusion of the role of aggregates in biofilm initiation (Kragh et al., 2016). Therefore, we evaluated the ability of the studied strains of heterotrophic bacteria to aggregation and their hydrophobicity. The results are shown in Table 1.

Table 1. Aggregation (by the aggregation test) and hydrophobicity (by salt aggregation test) of the studied strains on the 6th day of cultivation

No.	A variant of the experiment	The aggregation, %	Hydrophobicity	
			M	Characteristic
1.	*B. simplex* ChNPU F1	8.89 ± 0.14	> 2	Low
2.	*S. canus* NUChC F2	–	> 2	Low
3.	*S. gardneri* ChNPU F3	7.12 ± 0.25	< 1	High
4.	*Fictibacillus* sp. ChNPU ZVB1	0	1-2	Moderate

Note: – the experiment was not carried out; M - mol/L.

Slight aggregation was determined for bacteria of *B. simplex* strain ChNPU F1 and *S. gardneri* strain ChNPU F3: 8.89 ± 0.14% and 7.12 ± 0.25%, respectively (Table 1). At the same time, these strains differed in hydrophobicity (Table 1). Thus, *B. simplex* strain ChNPU F1 showed low hydrophobicity, and *S. gardneri* strain ChNPU F3 high hydrophobicity. Low hydrophobicity was also observed for *S. canus* strain NUChC F2 (Table 1). Bacteria *Fictibacillus* sp. strain ChNPU ZVB1 was characterized by the absence of aggregation and moderate hydrophobicity (Table 1).

3.2. Biofilm formation of heterotrophic bacteria isolated from soil ferrosphere

Biofilms are a model for the growth of microorganisms on surfaces and are being actively studied (Jardak et al., 2017; Ogawa et al., 2020). The "gold standard" for determining the total biomass of bacterial biofilms is the method of crystal violet staining (Haney et al., 2018). This method is simple and affordable, used in a number of modern studies of biofilm formation of microorganisms (Jardak et al., 2017; Ogawa et al., 2020). Therefore, it was chosen to determine the biomass of biofilms formed by the studied heterotrophic bacteria. The results of the study are presented in Table 2.

Table 2. Intensity of biofilm formation of heterotrophic bacteria isolated from soil ferrosphere, by absorption of crystal violet by formed biofilms

No.	A variant of the experiment	Mass of crystal violet sorbed by biofilm, mg/L	Strain adhesion category
1.	Control (MPB without bacteria)	5,36 ± 0,21	Negative control
2.	*B. simplex* ChNPU F1	13,17 ± 0,40*	Moderately adhesive
3.	*S. canus* NUChC F2	18,14 ± 1,61*	Moderately adhesive
4.	*S. gardneri* ChNPU F3	14,14 ± 0,51*	Moderately adhesive
5.	*Fictibacillus* sp. ChNPU ZVB1	8,43 ± 0,83*	Weakly adhesive

Note: * the differences are significant compared to the control at $p \leq 0.05$

It is now known that ammonifying bacteria produce a significant amount of exopolymers and provide anaerobic conditions on metals that are favorable for the further development of bacteria of other groups (Costerton et al., 1995; Purish & Asaulenko, 2007). However, for the studied ammonifying bacteria *Fictibacillus* sp. ChNPU ZVB1 noted weakly adhesive property, and other strains showed moderately adhesive ability (Table 2). The correlation analysis between the intensity of biofilm formation and aggregation of strains showed a significant positive correlation (coefficient of correlation = 0.69016, r^2 = 0.012986). However, no significant correlation was found between the intensity of biofilm formation and the hydrophobicity of the strains.

Since the strains did not show high adhesive properties, they can be expected to participate in corrosion processes due to the influence of ammonium (a product of AMB metabolism), which is considered a corrosive compound (Maruthamuthu et al., 2009; Guo et al., 2022). IRB, similar to SRB, is capable of hydrogen consumption and electrode depolarization (De Windt et al., 2003; Hernández-Santana, 2021), destruction of mineral films of carbon steel surface due to iron-reducing activity, which increases the speed of corrosion processes (Valencia-Cantero & Pena-Cabriales, 2014).

The trigger for the conversion of a natural microbial community into a corrosive one is an excess of Fe (II) ions, which are formed as a result of bacterial iron reduction (Hamilton, 2003; Andreyuk et al., 2005). However, the role of IRB in microbial corrosion processes is ambiguous (Videla et al., 2008; Herrera & Videla, 2009; Zaidi et al., 2021). It has been reported that microorganisms capable of reducing Fe (III) in aqueous media can inhibit corrosion processes (Potekhina et al., 1999; Dubiel et al., 2002).

Currently, the use of some microorganisms to suppress others is the basis of an eco-friendly approach to prevention, biocontrol of microbial damage (Lin & Ballim, 2012; Tripathi et al., 2021). Today the question of the application of biological control for corrosion protection is debated (Little et al., 2020). In particular, it is known that the formation of SRB biofilm is inhibited by metabolites of *S. lunalinharesii* 235 (Rosa et al., 2016), *B. velezensis* NUChC C1 and NUChC C2b (Tkachuk et al., 2021). *S. lunalinharesii* belong to actinobacteria, as well as the studied *S. canus* NUChC F2 and *S. gardneri* ChNPU F3. The publications discuss the participation of actinobacteria in the MIC process by the formation of biofilms, antimicrobials or corrosive substances (Li et al., 2010; Pacheco da Rosa et al., 2013; Winn et al., 2014; Rosa et al., 2016). The participation of bacteria of the genus *Bacillus* in the processes of biodamage of materials examines in the review (Tkachuk & Zelena, 2021b). In particular, it noted that compounds of bacilli - antimicrobial (antibiotics, lipopeptides, poly-γ-glutamate, biosurfactants) and antibiofilm-forming (siderophores at high concentrations, compounds with the property of destroying acylhomoserinlactone) are the part of the biocontrol agents in mechanisms against damaging of metals and plastics (Tkachuk & Zelena, 2021b). Therefore, to understand the mechanisms of MIC, the participation of individual species and ecological groups of microorganisms should investigate the antagonistic properties of heterotrophs, their ability to form biofilms under co-cultivation.

4. Conclusions

Thus, the studied bacteria were characterized as moderately- and weakly-adhesive. Therefore, their participation in microbial damage to materials will primarily be determined not by the biofilms formation, but by bioelectrochemical reactions (IRB), production of corrosive and/or antimicrobial metabolites (AMB and IRB). The prospect of further research is to determine the antagonistic properties and biofilm formation of heterotrophic bacteria under conditions of co-cultivation, in particular, with SRB.

References

Andreyuk E.I., Kozlova I.A., Kopteva Zh.P., Pilyashenko-Novokhatnyy A.I., Zanina V.V. & Purish L.M., 2002, Ferrosfera - zona formirovaniya korrozionno-aktivnogo soobshchestva mikroorganizmov [Ferrosphere – the zone of formation of a corrosion-active community of microorganisms]. Dopovidi NAN Ukrainy 3: 157–161 (in Russian).

Andreyuk K.I., Kozlova I.P., Koptieva Zh.P., Piliashenko-Novokhatnyi A.I., Zanina V.V. & Purish L.M., 2005, Mikrobna koroziia pidzemnykh sporud [Microbial Corrosion of Underground Structures]. Naukova Dumka: 258, Kyiv (in Ukrainian).

Chen L., Wei B. & Xu X., 2021, Effect of Sulfate-Reducing Bacteria (SRB) on the Corrosion of Buried Pipe Steel in Acidic Soil Solution. Coatings 11: 625. doi: 10.3390/coatings11060625

Costerton J.W., Lewandowski Z., Caldwell D.E., Korber D.R. & Lappin-Scott H.M., 1995, Microbial Biofilms, Annual Review of Microbiology 49: 711–745. doi: 10.1146/annurev.mi.49.100195.003431

Del Re B., Sgorbati B., Miglidi M. & Palenzona D., 2000, Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. Letters in Applied Microbiology 31: 438–442. doi: 10.1046/j.1365-2672.2000.00845.x

De Windt W., Boon N., Siciliano S.D. & Verstraete W., 2003, Cell density related H2 consumption in relation to anoxic Fe(0) corrosion and precipitation of corrosion products by *Shewanella ondensis* MR-1, Environmental Microbiology 5(11): 1192–1202. doi: 10.1046/j.1462–2920.2003.00527.x

Dubiel M., Hsu C.H., Chien C.C., Mansfeld F. & Newman D.K., 2002, Microbial iron respiration can protect steel from corrosion, Applied and Environmental Microbiology 68(3): 1440–1445. doi: 10.1128/AEM.68.3.1440-1445.2002

Furuhata K., Kato Y., Goto K., Hara M. & Fukuyama M., 2009, Diversity of heterotrophic bacteria isolated from biofilm samples and cell surface hydrophobicity, The Journal of General and Applied Microbiology 55(1): 69–74. doi: 10.2323/jgam.55.69

Guo Zh., Ruan Q., Liu T., Mao X., Chai Z., Guo N. & Dong L., 2022, Microbiologically influenced corrosion of Cu by marine ammonifying *Alcaligenes aquatilis* bacterium. Bioelectrochemistry, 108052. doi: 10.1016/j.bioelechem.2022.108052

Hamilton W.A., 2003, Microbial influenced corrosion as model system for the study of metal microbe interaction: a unifying electron transfer hypothesis. Biofouling 19(1): 65–76. doi: 10.1080/0892701021000041078

Hammer Ø., Harper D.A.T. & Ryan P.D., 2001, PAST: Paleontological Statistics Software Package for Education and Data Analysis. Palaeontologia Electronica 4(1) 4: 9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm

Haney E.F., Trimble M.J., Cheng J.T., Vallé Q. & Hancock R.E.W., 2018, Critical Assessment of Methods to Quantify Biofilm Growth and Evaluate Antibiofilm Activity of Host Defence Peptides. Biomolecules 8(2): 29. doi: 10.3390/biom8020029

Hernández-Santana M.A., 2021, The role of iron-reducing bacteria in the corrosion of carbon steel: a new microbiologically influenced corrosion mechanism, A Dissertation submitted to the graduate faculty. Norman, Oklahoma, 113 pp. https://shareok.org/handle/ 11244/332337

Herrera L.K. & Videla H.A., 2009, Role of iron-reducing bacteria in corrosion and protection of carbon steel. International Biodeterioration & Biodegradation 63(7): 891–895. doi: 10.1016/j.ibiod.2009.06.003

Jardak M., Abdelli F., Laadhar R., Lami R., Stien D., Aifa S. & Mnif S., 2017, Evaluation of biofilm-forming ability of bacterial strains isolated from the roof of an old house. Journal of General and Applied Microbiology 63(3): 186–194. doi: 10.2323/jgam.2016.10.005

Kragh K.N., Hutchison J.B., Melaugh G., Rodesney C., Roberts A.E., Irie Y., Jensen P.Ø., Diggle S.P., Allen R.J., Gordon V. & Bjarnsholt T., 2016, Role of Multicellular Aggregates in Biofilm Formation. Mbio 7(2): e00237-16. doi: 10.1128/mBio.00237-16

Li S.-M., Zhang Y.-Y., Du J., Liu J.-H. & Xu M., 2010, Influence of *streptomyces* on the Corrosion Behavior of Steel A3 in *Thiobacillus ferrooxidans* Media. Acta Chimica Sinica 68(01): 67–74.

Lin J. & Ballim R., 2012, Biocorrosion control: Current strategies and promising alternatives. African Journal of Biotechnology 11: 15736–15747. doi: 10.5897/AJB12.2479

Little B.J., Blackwood D.J., Hinks J., Lauro F.M., Marsili E., Okamoto A., Rice S.A., Wade S.A., Flemming H.-C., 2020, Microbially influenced corrosion – Any progress? Corrosion Science 170: 108641. doi: 10.1016/j.corsci.2020.108641

Maruthamuthu S., Dhandapani P., Ponmariappan S., Bae Jeong-Hyo, Palaniswamy N., Rahman Pattanathu K.S.M., 2009, Impact of ammonia producing *Bacillus* sp. on corrosion of cupronickel alloy 90:10. Metals and Materials International 15: 409–419. doi: 10.1007/s12540-009-0409-9

Nwanyanwu C.E. & Abu G.O., 2013, Influence of growth media on hydrophobicity of phenol-utilizing bacteria found in petroleum refinery effluent. International Research Journal of Biological Sciences 2(10): 6–11. www.isca.in

Ogawa A., Takakura K., Hirai N., Kanematsu H., Kuroda D., Kougo T., Sano K. & Terada S., 2020, Biofilm Formation Plays a Crucial Rule in the Initial Step of Carbon Steel Corrosion in Air and Water Environments. Materials 13(4): 923. doi: 10.3390/ma13040923

Pacheco da Rosa J., Korenblum E., Franco-Cirigliano M. N., Abreu F., Lins U., Soares R.M., Macrae A., Seldin L. & Coelho R.R., 2013, *Streptomyces lunalinharesii* strain 235 shows the potential to inhibit bacteria involved in biocorrosion processes BioMed Research International, 309769. doi: 10.1155/2013/309769

Potekhina J.S., Sherisheva N.G., Povetkina L.P., Pospelov A.P., Rakitina T.A., Warnecke F. & Gottschalk G., 1999, Role of microorganisms in corrosion inhibition of metals in aquatic habitats. Applied Microbiology and Biotechnology 52(5): 639–646. doi: 10.1007/s002530051571

Purish L.M. & Asaulenko L.H., 2007, Dynamika suktsesiinykh zmin u sulfidohennii mikrobnii asotsiatsii za umov formuvannia bioplivky na poverkhni stali [Dynamics of successional changes in sulfidogenic microbial association under conditions of biofilm formation on steel surface]. Mikrobiolohichnyi Zhurnal 69(6): 19–25 (in Ukrainian).

Purish L.M., Asaulenko L.H. & Ostapchuk A.M., 2009, Osoblyvosti rozvytku mono- y asotsiatyvnykh kultur sulfatvidnovliuvalnykh bakterii ta utvorennia ekzopolimernoho kompleksu [Features of development of mono- and associative cultures of sulfate-reducing bacteria and formation of exopolymer complex]. Mikrobiolohichnyi Zhurnal 71(2): 20–26 (in Ukrainian).

Rosa J.P., Tibúrcio S.R., Marques J.M., Seldin L. & Coelho R.R., 2016, *Streptomyces lunalinharesii* 235 prevents the formation of a sulfate-reducing bacterial biofilm. Brazilian Journal of Microbiology 47(3): 603–609. doi: 10.1016/j.bjm.2016.04.013

Stepanović S., Vuković D., Dakić I., Savić B. & Švabić-Vlahović M., 2000, A modified microtiter-plate test for quantification of staphylococcal biofilm formation Journal of Microbiological Methods 40: 175–179. doi: 10.1016/s0167–7012(00)00122–6

Sungur E.I., Türetgen I., Javaherdashti R. & Çotuk A., 2010, Monitoring and disinfection of biofilm-associated sulfate reducing bacteria on different substrata in a simulated recirculating cooling tower system. Turkish Journal of Biology 34(4): 389–397. doi: 10.3906/biy-0902-8

Tkachuk N. & Zelena L., 2021a, Some corrosive bacteria isolated from the technogenic soil ecosystem in Chernihiv city (Ukraine). Studia Quaternaria 38(2): 101–108. doi: 10.24425/sq.2021.136826

Tkachuk N. & Zelena L., 2021b, The impact of bacteria of the genus *Bacillus* upon the biodamage/biodegradation of some metals and extensively used petroleum-based plastics. Corrosion and Materials Degradation 2: 531–553. doi: 10.3390/cmd2040028

Tkachuk N., Zelena L., Lukash O. & Mazur P., 2021, Microbiological and genetic characteristics of *Bacillus velezensis* bacillibactin-producing strains and their effect on the sulfate-reducing bacteria biofilms on the poly(ethylene terephthalate) surface. Ecological Questions 32(2): 119–129. doi: 10.12775/EQ.2021.019

Tripathi A.K., Thakur P., Saxena P., Rauniyar S., Gopalakrishnan V., Singh R.N., Gadhamshetty V., Gnimpieba E.Z., Jasthi B.K. & Sani R.K., 2021, Gene sets and mechanisms of sulfate-reducing bacteria biofilm formation and quorum sensing with impact on corrosion. Frontiers in microbiology 12: 754140. doi: 10.3389/fmicb.2021.754140

Valencia-Cantero E. & Pena-Cabriales J.J., 2014, Effects of iron-reducing bacteria on carbon steel corrosion induced by thermophilic sulfate-reducing consortia. Journal of Microbiology and Biotechnology 24(2): 280–286. doi: 10.4014/jmb.1310.10002

Videla H., Borgne S., Panter C. & Raman R., 2008, MIC of Steels by Iron Reducing Bacteria NACE Store. 10 pp. http://www.nace.org/cstm/Store/Product.aspx?id=58302510-ab52-dd11–889d-0017a446694e

Winn M., Casey E., Habimana O. & Murphy C.D., 2014, Characteristics of *Streptomyces griseus* biofilms in continuous flow tubular reactors. FEMS Microbiology Letters 352: 157–164. doi: 10.1111/1574-6968.12378

Zaidi M.A.F.M., Masri M.N. & Kew W.S., 2021, Microbiologically Influenced Corrosion of Iron by Nitrate Reducing *Bacillus* sp. Journal of Physics: Conference Series 2129: 012066. https://iopscience.iop.org/article/10.1088/1742-6596/2129/1/012066/pdf

Zita A. & Hermansson M., 1997, Determination of bacterial cell surface hydrophobicity of single cells in cultures and in wastewater in situ. FEMS Microbiology Letters 152(2): 299–306. doi: 10.1111/j.1574-6968.1997.tb10443.x