ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 4, pp. 423–427. © Pleiades Publishing, Ltd., 2013.
Original Russian Text © D.R. Yarullina, L.V. Vakatova, A.V. Krivoruchko, E.V. Rubtsova, O.N. Ilinskaya, 2013, published in Mikrobiologiya, 2013, Vol. 82, No. 4, pp. 417–421.

EXPERIMENTAL ARTICLES

Effect of Exogenous and Endogenous Nitric Oxide on Biofilm Formation by *Lactobacillus plantarum*

D. R. Yarullina[a, 1], L. V. Vakatova[a], A. V. Krivoruchko[b], E. V. Rubtsova[b], and O. N. Ilinskaya[a]

[a] *Kazan (Volga Region) Federal University, ul. Kremlevskaya 18, Kazan, 420008 Russia*

[b] *Perm State National Research University, ul. Bukireva 15, Perm, 614990 Russia*

Received December 10, 2012

Abstract—Biofilms are a widespread form of occurrence of microorganisms in nature, and understanding the mechanism of regulation of their formation is of unquestionable practical significance for medicine and biotechnology. In the present work, the effect of nitric oxide (NO) on biofilm formation by *Lactobacillus plantarum* was investigated and the micromolar concentrations of exogenous NO were shown to have a negative effect on this process due to its toxic effect on the cells. However, the decrease in the level of endogenous NO in bacteria in the presence of a nitric oxide scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) impaired the characteristics of the forming biofilms, as was evident from the decrease in their size.

Keywords: nitric oxide, lactobacilli, biofilms

DOI: 10.1134/S0026261713040140

Bacteria of the genus *Lactobacillus* are an important component of the human intestinal microbiota and are extensively used in the microflora-normalizing (probiotic) preparations. The mechanisms of probiotic activity of lactobacilli include the production of bacteriocins and lactic and acetic acids [1]. Apart from these substances, some lactobacilli were shown to be capable of synthesizing nitric oxide (NO) [2, 3], which is probably also a factor of probiotic activity [4]. Intestinal colonization by biofilms of lactobacilli—agglomerations of microbial colonies immersed in the extracellular matrix and attached to the surface—is required for these microorganisms to reveal their beneficial properties [5]. Biofilm formation by lactobacilli was shown to be affected by bile, mucins, acidity, osmotic pressure, and composition of the nutrient medium [6], in particular, availability of the carbon sources [5]. The products of the following genes are involved in biofilm formation in lactobacilli: *wzb*, *dltD*, *luxS* [5], *mabA* [7], *ftsH* [8], *ftm*, and *ccpA* [9]. Participation of the LuxS genes in biofilm formation indicates the quorum-dependent nature of this process in lactobacilli [5]. Moreover, the correlation between bacterial culture density and biofilm formation was established [6].

It was recently found out that NO, while binding to the H-NOX (*h*eme-*n*itric oxide/*ox*ygen-binding) protein and changing the concentration of the secondary messenger cyclic diguanosine monophosphate (c-di-GMP), regulates biofilm formation in *Legionella pneumophila*, *Shewanella woodyi*, *Shewanella oneidensis*, and *Pseudoalteromonas atlantica* [10–13]. The ability of nitric oxide to inhibit biofilm formation in *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Candida albicans* was proposed to be used in the treatment for bacterial and fungal infections [14–16].

This study was performed to elucidate the role of nitric oxide in biofilm formation by the bacteria *L. plantarum*. Since these microorganisms are able to synthesize NO [2, 3], the effects of both exogenous nitric oxide evolving from sodium nitroprusside (SNP) and endogenous NO synthesized by the bacteria via the NO synthase pathway from L-arginine were assayed.

MATERIALS AND METHODS

The subjects of research were the strains *Lactobacillus plantarum* 8P-A3 isolated from the Lactobacterin Dry (Perm Scientific and Production Association Biomed) [3] and *L. plantarum* 52 from the collection of the Research Institute of Agricultural Microbiology, Russian Academy of Agricultural Sciences (St. Petersburg). The bacteria were grown at 37°C on the MRS medium (Merck). The NO synthase substrate L-arginine [17], the NO donor sodium nitroprusside, SNP [18], and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt, cPTIO [19] (Sigma-Aldrich) were used. The effects of these substances on biofilm formation were analyzed in 96-well plates (Greiner Bio-One CELLSTAR, no. 655180). The bacterial cul-

[1] Corresponding author; e-mail: kasfes@gmail.com

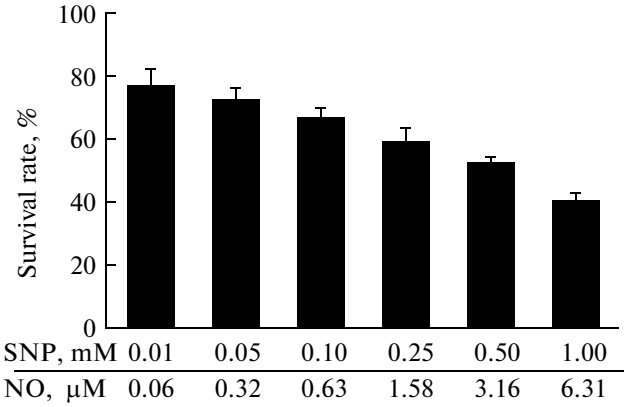

Fig. 1. Effect of exogenous NO evolved by sodium nitroprusside (SNP) on the viability of *L. plantarum* 8P-A3. Cell viability on the medium without SNP is taken as 100%.

ture (200 μL) with a density of 3×10^7 CFU/mL in the MRS medium containing different concentrations of the compounds under study was added into the wells and incubated for 72 h. The biofilms were assayed as follows: the contents of the wells was removed, washed three times with the 1× PBS buffer, stained with 0.1% aqueous solution of gentian violet (Sigma-Aldrich) (100 μL/well) for 1 h, and washed again with the buffer. Then 96% ethyl alcohol was added (100 μL/well) and the absorption was measured at 570 nm in a Tecan infinite 200 Pro microplate reader (Switzerland). The intensity of solution staining correlated with the biofilm size.

The nitric oxide concentration in SNP solutions was calculated from the content of NO oxidation products (nitrites) detected by spectrophotometry with the Griess reagent (Sigma-Aldrich). The calibration curve was plotted using fresh $NaNO_2$ solutions (Sigma-Aldrich) at concentrations from 0 to 100 μM.

In order to determine the effect of SNP on bacterial viability, lactobacilli were plated on solid MRS medium in the presence of different SNP concentrations. Their toxic effects were assessed by bacterial survival expressed in percentage of the total number of colonies grown on the medium without the additives. The viability of bacteria was also accessed by staining the cells with the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes) according to the manufacturer's instructions. This reagent comprises fluorescent dyes SYTO 9 and propidium iodide (PI); hence, the viable cells and the cells with damaged membranes exhibit green and red fluorescence, respectively. For the study of NO biosynthesis by lactobacilli, the cells grown to the stationary growth phase (48 h) were collected by centrifugation, washed three times with the HBSS buffer (1× Hanks' Balanced Salt Solution with calcium and magnesium salts, without phenol red, PAA Laboratories GmbH, Austria), stained for 1 h with the fluorescent NO indicator 1,2-diaminoanthraquinone sulfate (DAA) (Molecular Probes) at the concentration of 50 μg/mL, and washed three times from the dye with the same buffer [20]. The preparations were analyzed under a Leica DM 6000B epifluorescence microscope (Germany). The fluorescent images were obtained and fluorescence intensity was measured using the Leica FW4000 software package. The results were statistically processed in Microsoft Excel. The reliability of the differences was determined using the Mann–Whitney criterion; the probability criterion of $p \le 0.05$ was taken as a criterion of significance of the differences between the two groups of data.

RESULTS AND DISCUSSION

Effect of exogenous NO on the biofilms. In recent years, nitric oxide was hypothesized to act as a universal regulator of biofilm formation in bacteria [10–13]. SNP was added to the medium to elucidate the effect of exogenous NO on the biofilms of lactobacilli. The presence of this nitric oxide donor in the samples resulted in NO emission and a decrease in cell viability in proportion to the increase in SNP concentration (Fig. 1). With 1 mM SNP added to the growth medium, NO was found at the concentration of 6.3 μM and the loss of viability of lactobacilli by almost 60% compared to their growth without SNP was recorded (Fig. 1). At the same time, biofilm formation was inhibited in both *L. plantarum* strains under study (Fig. 2e).

Effect of endogenous NO on biofilms. Since some lactobacilli synthesize nitric oxide via the NO synthase (NOS) pathway [2, 3], we investigated the possibility of induction of nitric oxide synthesis in *L. plantarum* 8P-A3 and *L. plantarum* 52 from the NOS substrate L-arginine. It was shown that the viability of lactobacilli grown with and without the amino acid was similar (Fig. 3a). In accordance with the "L-arginine paradox", the addition of exogenous arginine, independent of its intracellular concentration, resulted in enhanced NO production [17]. We have previously demonstrated the possibility of NO determination in a bacterial culture using DAA fluorescent staining [20]. The addition of 100 μM L-arginine to the growth medium resulted in the enhancement of the fluorescent signal of *L. plantarum* 8P-A3 cells more than twofold compared to the cells incubated without arginine, while *L. plantarum* 52 did not activate NO synthesis in the presence of L-arginine (Figs. 3b, 3c), which is evidence of the strain differences in the level of NOS activity or the rate of metabolism of L-arginine as a substrate.

Addition of L-arginine to the growth medium did not alter the biofilm formation, irrespective of whether NO level in the sample was elevated, as in the case of *L. plantarum* 8P-A3, or remained basal, as in the case of *L. plantarum* 52 (Figs. 2a–2c).

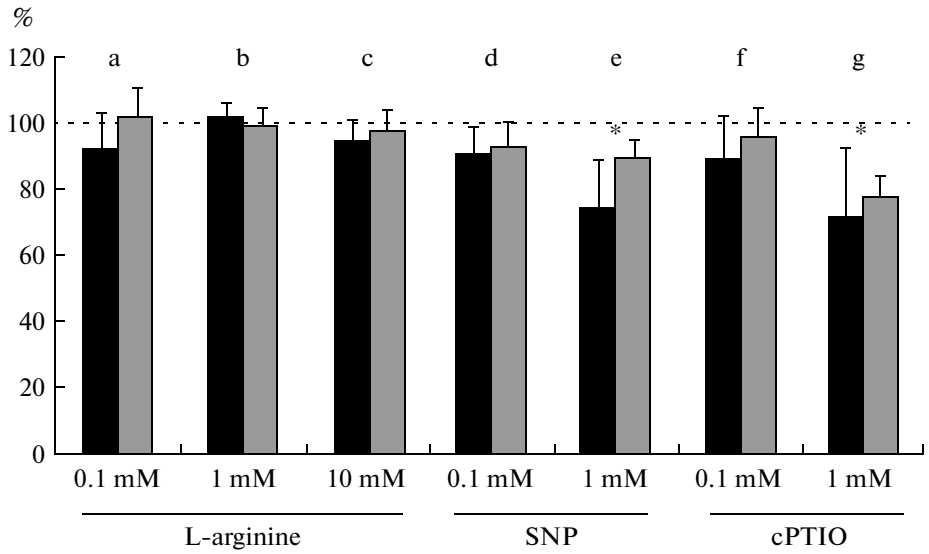

Fig. 2. Effect of L-arginine (a–c), sodium nitroprusside (SNP) (d, e) and NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) (f, g) on biofilm formation by *L. plantarum* 8P-A3 (dark columns) and *L. plantarum* 52 (light columns). Biofilm formation during the growth on MRS medium without the additives was taken as 100%. *, reliably differs from 100%.

Since we had recorded the basal NO level in the cells of lactobacilli, it seemed important to determine the influence of its decrease on biofilm formation. To this effect, the NO scavenger cPTIO was added to the medium. In the presence of 1 mM cPTIO, both strains showed lower levels of biofilm formation (Figs. 2f, 2g), which indicated the necessity of the basal level of endogenous NO for biofilm formL:ation in *L. plantarum*.

While physiological concentrations of NO are known to exert a regulatory effect on bacterial biofilms [10–13], the values of these concentrations and the nature of their influence vary significantly in different species. In *Pseudomonas aeruginosa*, millimolar SNP concentrations (25–100 mM) contributed to biofilm formation; in the presence of nano- and micromolar SNP concentrations, no biofilm formation occurred and the already formed bacterial microcolonies were destroyed [14]. Exogenous NO evolved by the nitric oxide donor DETA NONOat (200 μM) increased biofilm formation in *Shewanella oneidensis* [12] and inhibited this process in *Shewanella woodyi* [11]. Since it is known that the cells within biofilms are less susceptible to the action of antibiotics, toxins, bacterio-

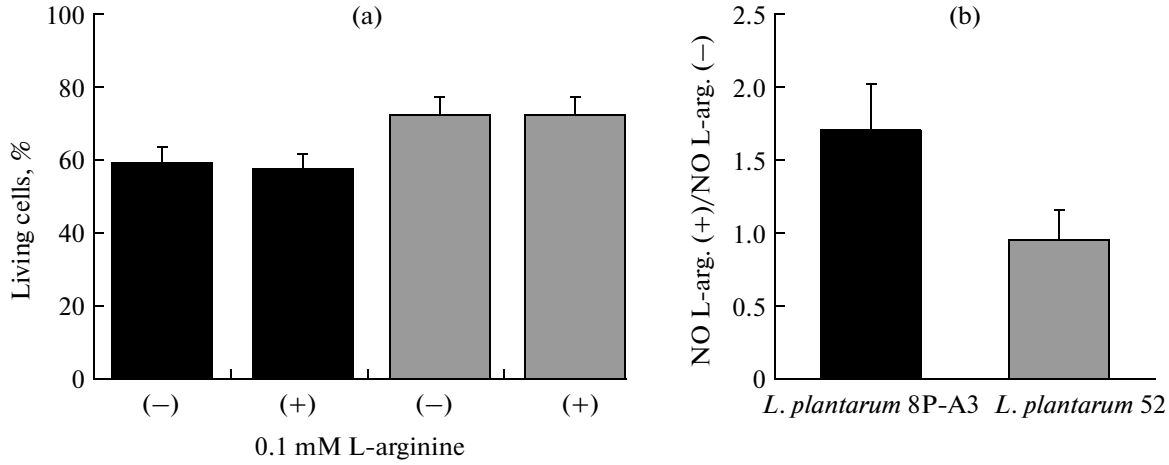

Fig. 3. Effect of L-arginine on viability of *L. plantarum* 8P-A3 (dark columns) and *L. plantarum* 52 (light columns) measured using the LIVE/DEAD reagent (a) and biosynthesis of nitric oxide measured by DAA (b). The total cell number in a sample was taken as 100%.

cins, and other antimicrobial agents [21], induction of biofilm formation by the high (milli- and micromolar) NO concentrations was also explained by the defense strategy of bacteria [12]. However, in spite of the higher toxicant resistance of microorganisms within biofilms compared to free cells [21], we demonstrated the toxic effect of exogenous NO on lactobacilli even at nanomolar concentrations (Fig. 1b), which had a negative influence on biofilm formation (Figs. 2d, 2e). Taking into consideration the functional activity of NO in the nonspecific immune system [22], the revealed sensitivity of lactobacilli to exogenous NO is important in the context of their symbiotic position in the human gastrointestinal tract.

We found that endogenous NO did not reach the toxic level (cell viability did not change during NOS activation, Fig. 3a) and was crucially important for biofilm formation. It is known that the involvement of nitric oxide in the regulation of biofilm formation by bacteria is mediated by c-di-GMP [10–13] and that the quantity of the latter in a cell depends on a number of factors apart from NO and is therefore not constant. Nitric oxide, being a "ubiquitous" molecule, has a dual effect on c-di-GMP level, depending on the enzymes and proteins (diguanylate cyclase/phosphodiesterase or histidine kinase) it interacts with in the cells, which can account for the multidirectional influence of addition of NO donors on the *Shewanella oneidensis* and *S. woodyi* biofilms [11, 12]. It would be erroneous to explain the stimulating/inhibitory effects of NO on biofilms only by the concentration of the effector, without taking into account the level of endogenous NO, as well as the inter-species and strain differences in manifestation of NO effects (including the toxic ones) in different microorganisms. Our research is the first demonstration of the involvement of nitric oxide in the regulation of biofilm formation in lactobacilli, where the toxic potential of nanomolar concentrations of exogenous NO and significance of the basal level of endogenous NO for this process were established.

ACKNOWLEDGMENTS

The work was supported by the Ministry of Education and Science of the Russian Federation in the framework of Federal Targeted Program "Scientific and Research–Pedagogical Personnel of Innovative Russia" (Agreement no. 14.A18.21.0185 of July 24, 2012), Russian Foundation for Basic Research (no. 12-04-01226_a) and the "Budget 12-03" project.

REFERENCES

1. Giraffa, G., Chanishvili, N., and Widyastuti, Y., Importance of lactobacilli in food and feed biotechnology, *Res. Microbiol.*, 2010, vol. 161, no. 6, pp. 480–487.
2. Adawi, D., Kasravi, F.B., Molin, G., and Jeppsson, B., Effect of *Lactobacillus* supplementation with and without arginine on liver damage and bacterial translocation in an acute liver injury model in the rat, *Hepatology*, 1997, vol. 25, no. 3, pp. 642–647.
3. Yarullina, D.R., Il'inskaya, O.N., Aganov, A.V., Silkin, N.I., and Zverev, D.G., Alternative pathways of nitric oxide formation in lactobacilli: evidence for nitric oxide synthase activity by EPR, *Microbiology*, 2006, vol. 75, no. 6, pp. 634–638.
4. Jones, M.L., Ganopolsky, J.G., Labbe, A., and Prakash, S., A novel nitric oxide producing probiotic patch and its antimicrobial efficacy: preparation and in vitro analysis, *Appl. Microbiol. Biotechnol.,* 2010, vol. 87, no. 2, pp. 509–516.
5. Lebeer, S., Verhoeven, T.L.A., Velez, M.P., Vanderleyden, J., and De Keersmaecker, S.C.J., Impact of environmental and genetic factors on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG, *Appl. Environ. Microbiol.*, 2007, vol. 73, no. 21, pp. 6768–6775.
6. Terraf, M.C., Juárez Tomás, M.S., Nader-Macías, M.E., and Silva, C. Screening of biofilm formation by beneficial vaginal lactobacilli and influence of culture media components, *J. Appl. Microbiol.*, 2012, vol. 113, no. 6, pp. 1517–1529.
7. Velez, M.P., Petrova, M.I., Lebeer, S., Verhoeven, T.L., Claes, I., Lambrichts, I., Tynkkynen, S., Vanderleyden, J., and De Keersmaecker, S.C., Characterization of Mab A, a modulator of *Lactobacillus rhamnosus* GG adhesion and biofilm formation, *FEMS Immunol. Med. Microbiol.*, 2010, vol. 59, no. 3, pp. 386–398.
8. Bove, P., Capozzi, V., Garofalo, C., Rieu, A., Spano, G., and Fiocco, D., Inactivation of the *ftsH* gene of *Lactobacillus plantarum* WCFS1: effects on growth, stress tolerance, cell surface properties and biofilm formation, *Microbiol. Res.*, 2012, vol. 167, no. 4, pp. 187–193.
9. Muscariello, L., Marino, C., Capri, U., Vastano, V., Marasco, R., and Sacco, M., Ccpa and three newly identified proteins are involved in biofilm development in *Lactobacillus plantarum, J. Bas. Microbiol.*, 2012. doi: 10.1002/jobm.201100456
10. Carlson, H.K., Vance, R.E., and Marletta, M.A., H-NOX regulation of c-di-GMP metabolism and biofilm formation in *Legionella pneumophila, Mol. Microbiol.*, 2010, vol. 77, pp. 930–942.
11. Liu, N., Xu, Y., Hossain, S., Huang, N., Coursolle, D., Gralnick, J.A., and Boon, E.M., Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi, Biochemistry*, 2012, vol. 51, no. 10, pp. 2087–2099.
12. Plate, L. and Marletta, M.A., Nitric oxide modulates bacterial biofilm formation through a multicomponent cyclic-di-GMP signaling network, *Mol. Cell*, 2012, vol. 46, no. 4, pp. 449–460.
13. Arora, D.P. and Boon, E.M., Nitric oxide regulated two-component signaling in *Pseudoalteromonas atlantica, Biochem. Biophys. Res. Comm.,* 2012, vol. 421, no. 3, pp. 521–526.
14. Barraud, N., Hassett, D.J., Hwang, S.-H., Rice, S.A., Kjelleberg, S., and Webb, J.S., Involvement of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa, J. Bacteriol.*, 2006, vol. 188, no. 21, pp. 7344–7353.

15. Privett, B.J., Nutz, S.T., and Schoenfisch, M.H., Efficacy of surface-generated nitric oxide against *Candida albicans* adhesion and biofilm formation, *Biofouling*, 2010, vol. 26, pp. 973–983.

16. Hetrick, E.M., Shin, J.H., Paul, H.S., and Schoenfisch, M.H., Anti-biofilm efficacy of nitric oxide-releasing silica nanoparticles, *Biomaterials*, 2009, vol. 30, pp. 2782–2789.

17. Tsikas, D., Boger, R.H., Sandmann, J., Bode-Boger, S.M., and Frolich, J.C., Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox, *FEBS Lett.*, 2000, vol. 478, pp. 1–3.

18. Yamamoto, T. and Bing, R.J., Nitric oxide donors, *Proc. Soc. Exp. Biol. Med.*, 2000, vol. 225, no. 3, pp. 200–206.

19. Yoshioka, T., Iwamoto, N., and Ito, K., An application of electron paramagnetic resonance to evaluate nitric oxide and its quenchers, *J. Am. Soc. Nephrol.*, 1996, vol. 7, no. 6, pp. 961–965.

20. Yarullina, D.R. and Il'inskaya, O.N., Detection of NO-synthase activity of lactobacilli by fluorescent staining, *Microbiology*, 2007, vol. 76, no. 4, pp. 503–505.

21. Donlan, R.M. and Costerton, J.W., Biofilms: survival mechanisms of clinically relevant microorganisms, *Clin. Microbiol. Rev.*, 2002, vol. 15, pp. 167–193.

22. MacMicking, J., Xie, Q.W., and Nathan, C., Nitric oxide and macrophage function, *Annu. Rev. Immunol.*, 1997, vol. 15, pp. 323–350.

Translated by E. Makeeva