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SHORT COMMUNICATIONS

Detection of NO-Synthase Activity of Lactobacilli by Fluorescent Staining

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Nitric oxide (NO) is continuously produced in animal and human organisms enzymatically from *L*-arginine, and functions as one of the universal regulators of cell and tissue metabolism. It also functions as the main cytotoxic/cytostatic effector of the cellular immunity system. In mammalian cells, NO production is catalyzed by the family of NO synthase (NOS) isoenzymes [1]. The process of bacterial NO synthesis, in contrast to the eukaryotic one, is still far from being fully understood. An NO synthase mechanism of nitric-oxide production alternative to denitrification was initially revealed in the microorganisms of the genus *Nocardia* [2]. Later, genes that encode proteins homologous to the mammalian NOS oxygenase domain were identified in the genomes of a number of gram-positive bacteria [3]. As yet, there are few works on the isolation, purification, and study of the properties of bacterial NO synthases [4–6].

NO synthase is supposed to be present in *Lactobacillus plantarum* [7]. Previously, we have applied EPR (electron paramagnetic resonance) to establish the capability of *L. plantarum* 8P-A3 to synthesize nitric oxide. This strain was shown not to produce NO via denitrification but to have a NO-synthase activity, like mammalian cells [8].

This work presents the first evidence of NO-synthase nitric-oxide production in *L. plantarum* obtained by the method of fluorescent staining.

The culture of *L. plantarum* 8P-A3 [8] isolated from the Lactobacterin Dried preparation (Biomed, Russia) was a test subject. MRS medium of known composition [8] was used for bacterial growth. One ml of 18-h culture was added into 50-ml test tubes containing 30 ml of the medium with or without *L*-arginine (100 μM) and incubated without mixing at 37°C. The growth kinetics was determined at 590 nm on a Lambda 35 double-beam spectrophotometer, Perkin Elmer Instruments (USA). For fluorescent detection of nitric oxide, 30 ml of the culture from the stationary growth phase (48 h) was precipitated by centrifugation at 4000 *g* for 20 min in an Avanti™ J-20XP centrifuge (United States). The cells were washed three times, resuspended in Hanks' buffer with calcium and magnesium (PAA Laboratories, GmbH, Austria) supplemented with 50 μg/ml of the fluorescent dye 1,2-diaminoanthraquinone (DAA) (Molecular Probes, Germany), and incubated for 1 h. In the control variant, the microorganisms were incubated in the buffer without the dye. At the end of the incubation period, the cells were washed from the dye with the buffer and preparations were made, which were then examined using a Leica DM 6000B fluorescent microscope (Germany) with a CCD camera connected to a computer. The fluorescent signal images were analyzed using the Leica FW4000 software package. The results were statistically processed using Microsoft Excel.

The induction of NO-synthase activity in *L. plantarum* 8P-A3 by *L*-arginine was studied. *L*-Arginine is known to be a NOS substrate, since the oxidation of its guanidine group results in the release of NO [1]. In accordance with the phenomenon known as the "*L-arginine* paradox" (exogenous *L*-arginine stimulates NO synthesis independently of its intracellular concentration) [9], one might expect that the addition of *L*-arginine to *L. plantarum* culture medium will contribute to the increase of the NO level. We were the first to use the method of staining with an NO-sensitive fluorescent dye DAA for nitric oxide registration in lactobacilli (Fig. 1). Since the complex of DAA with nitric oxide is water insoluble [10], this dye makes it possible to visualize the real NO distribution in native bacterial cells. Although NO molecules have high permeability through the membranes of cells and subcellular structures due to their small sizes and the absence of charge [11], the registered NO localization was exclusively intracellular. Both background NO production from endogenous arginine (Fig. 1a) and NO synthesis induced by exogenous arginine (Fig. 1b) were recorded; the introduction of *L*-arginine into the growth medium increased the fluorescent signal intensity as compared with the cells incubated without arginine; i.e., it significantly activated NO synthesis by the microorganisms under study.

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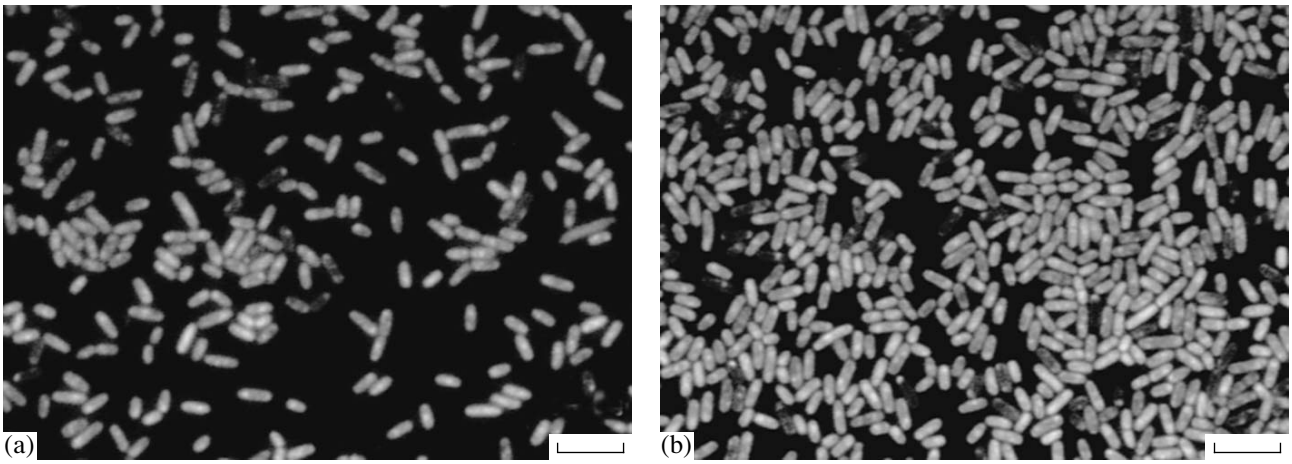

Fig. 1. Visualization of nitrogen oxide in the culture of *Lactobacillus plantarum* 8P-A3 incubated for 48 h in the absence (a) and presence (b) of 10 μM L-arginine by fluorescent staining with 1,2-diaminoanthraquinone (DAA). The quantity of microorganisms on slides (a) and (b) is equivalent. Scale bar is 5 μm.

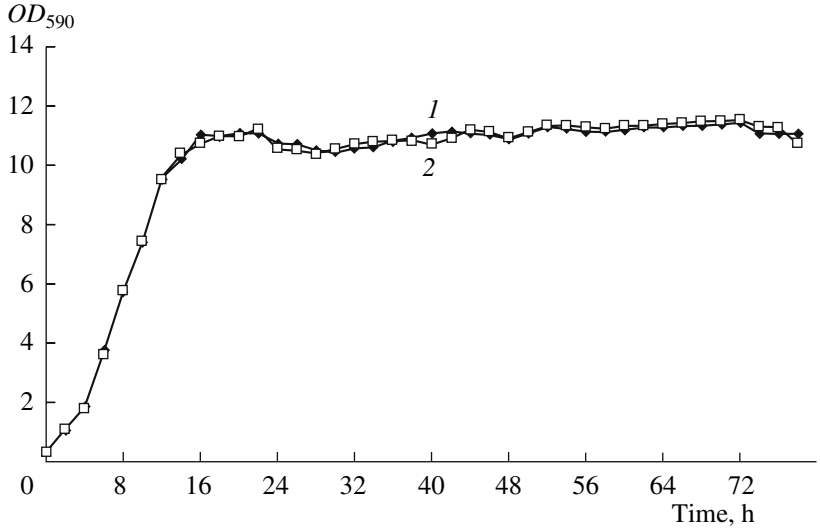

Fig. 2. The growth dynamics of *Lactobacillus plantarum* 8P-A3 culture incubated in the absence (a) and presence (b) of 100 μM *L*-arginine.

Being a free radical, nitric oxide can have a cytotoxic effect on *L. plantarum* 8P-A3 cells; this effect is based primarily on the inhibition of cell division and energy metabolism [12]. On the other hand, bacterial growth may intensify due to the addition of arginine as a supplementary nutrient source. However, comparison of the dynamics of *L. plantarum* growth in the absence and presence of *L*-arginine revealed that the latter had no effect on growth (Fig. 2). Thus, the change of the fluorescent signal intensity (Fig. 1) is not associated with the influence of this amino acid on the culture growth. We have shown previously that *L. plantarum* 8P-A3 is unable to produce NO via denitrification [8], and the results of the present study indicate that nitric oxide production increases in the cells of *L. plantarum* 8P-A3, like in mammalian cells, under the influence of exogenous *L*-arginine (the *L*-arginine paradox). Hence, it is possible to draw inferences about the NO-synthase mechanism of NO production by the lactobacillus strain under study.

Thus, the presence of NO synthase activity in lactobacilli has been confirmed for the first time by the method of fluorescent staining. This finding seems to be particularly important due to the high commercial significance of this group and the need for development of fundamental techniques for practical manipulation of intracellular regulators at the molecular level.

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