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EXPERIMENTAL ARTICLES

Alternative Pathways of Nitric Oxide Formation in Lactobacilli: Evidence for Nitric Oxide Synthase Activity by EPR

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Abstract—The study of the ability of *Lactobacillus plantarum* 8P-A3 to synthesize nitric oxide (NO) showed that this strain lacks nitrite reductase. However, analysis by the EPR method revealed the presence of nitric oxide synthase activity in this strain. Like mammalian nitric oxide synthase, lactobacillar NO synthase is involved in the formation of nitric oxide from L-arginine. *L. plantarum* 8P-A3 does not produce NO in the denitrification process. The regulatory role of NO in symbiotic bacteria is emphasixed.

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Nitric oxide (NO) is one of the most important biological mediators involved in many physiological and pathophysiological processes, such as vasodilatation, neurotransmission, immune system functioning, thrombasthenia, regulation of smooth muscle contraction, memory processes, etc. In mammalian cells, NO is produced by NO synthase (NOS), which catalyzes the oxidation of L-arginine to citrulline and NO. There are three isoforms of NO synthase: neuronal (NOS-I or nNOS), inducible (NOS-II or iNOS), and endothelial (NOS-III or eNOS) [1].

Unlike nitric oxide produced in eukaryotes, bacterial nitric oxide produced in the denitrification process is formed from nitrite by nitrite reductase (NIR). Then nitric oxide is reduced to N_2O. Analysis of nitrite reductases isolated from various bacterial sources showed the existence of two classes of dissimilatory NIRs, one containing copper and the other containing hemi (Cu-NIR and cd_1NIR, respectively) as cofactors. None of these enzymes is structurally and functionally similar to mammalian NOS [2].

To avoid the toxic effect of reactive nitrogen species formed from NO (such as peroxinitrite), denitrifying bacteria implement successive reactions catalyzed by nitrite reductase and NO reductase. These reactions maintain the concentration of NO in bacterial cells at a very low level (of the order of nanomoles) [3].

Recent studies have shown that nitrite reductase is not the only NO-producing enzyme in prokaryotes. Bacterial NO synthases functionally homologous to mammalian NO synthases were first found and studied in bacteria of the genus *Nocardia* [4]. Later, genes encoding proteins homologous to the oxygenase domains of mammalian NO synthases were detected in *Bacillus subtilis*, S*taphylococcus aureus*, *Deinococcus radiodurans*, *B. halodurans*, and *B. anthracis* [5]. This finding is consistent with the earlier observation of NO synthase activity in bacterial cell extracts [4, 6].

Some researchers suggested the presence of NO synthase in *Lactobacillus plantarum* and *L. fermentum* based on the incorporation of the ^{15}N of L-arginine into the oxidized forms of nitrogen [7, 8]. However, without control performed for denitrification, this suggestion needs further evidence.

Since lactobacilli are extensively used to manufacture probiotic preparations, the problem of existence of NO synthases in these bacteria (which suggests an important regulatory function of NO) is of great significance.

The aim of this study was to detect nitric oxide produced by NO synthase in lactobacilli by the EPR method. The experiments were designed so as to exclude the detection of abiotically produced nitric oxide or that produced through nitrate reduction.

MATERIALS AND METHODS

Experiments were carried out with the strain *Lactobacillus plantarum* 8P-A3, which is the principle ingredient of the dry lactobacterin preparation produced by the Perm' Research and Production Association "Biomed." The contents of one flask were dissolved in

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25 ml of MRS medium containing (in g/l distilled water) yeast extract, 5; meat extract, 10; bactopeptone, 10; glucose, 20; ammonium citrate, 2; sodium acetate, 5; cysteine–HCl, 0.4; Twin-80, 1; K_2HPO_4, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.04; and sorbic acid, 0.4 (pH 6.2–6.6). Bacterial suspensions were incubated at 37°C for 24 h in 100-ml flasks with 25 ml of the medium and then plated on MRS agar. The inoculum was prepared using individual bacterial colonies.

To detect the capacity of bacterial cultures for denitrification, they were grown at 37°C for seven days in long culture tubes containing the MRS medium supplemented with 1% KNO_3. Nitrites in the culture liquid were detected spectrophotometrically by using the Griess reagent. Gaseous products of denitrification were detected by measuring these gases in a float. Nitrates were measured with diphenylamine and concentrated sulfuric acid [9].

For EPR studies, the bacterial strain was grown to the stationary growth phase in a medium containing (in g/l) glucose, 5; peptone, 10; and NaCl, 5. Cells harvested from 30 ml of the stationary-phase culture by centrifugation at 5000 *g* for 20 min were washed three times with 50 mM Tris–HCl buffer (pH 7.2) and concentrated to a volume of 0.1 ml by centrifugation. Aliquots of these concentrated suspensions were used to prepare suspensions of *L. plantarum* 8P-A3 cells of equal concentrations in 50 mM Tris–HCl buffer (pH 7.2). The suspensions were supplemented with spin traps and substrates for NO-synthesizing enzymes.

Ascorbic acid (AA) and a complex of Fe^{2+} and diethyldithiocarbamate ($Fe^{2+}(DETC)_2$) were used as spin traps. Interacting ascorbic acid and NO produce an EPR signal with a *g* factor equal to approximately 2.02 [10]. The complex of NO and $Fe^{2+}(DETC)_2$ produces an EPR signal with a *g* factor equal to 2.035 [11]. To study the ability of the iron–sulfur centers of lactobacillar proteins to form nitrosyl complexes with NO, we used bacterial suspensions without exogenously added spin traps (in fact, NO traps) [12].

Experimental bacterial suspensions for detecting NO synthase were supplemented with L-arginine and NADPH at concentrations of 20 mM each. In some experiments, NO synthase was inhibited by adding N-omega-nitro-L-arginine methyl ester (L-NAME) at a concentration of 100 mM. Experimental bacterial suspensions for detecting nitrite reductase contained KNO_3 at a concentration of 20 mM. After the addition of a spin trap and substrate for NO-synthesizing enzymes to suspensions of *L. plantarum* 8P-A3 cells, the reaction mixtures were incubated at 37°C for 0.5–1 h. To detect NO produced during denitrification, the reaction mixture was incubated anaerobically in a carbon dioxide atmosphere. After incubation, the suspensions were frozen in liquid nitrogen in the form of cylinders 3 mm in diameter and 30 mm in length. The EPR spectra of these cylinders were recorded using a Brucker ESP-300 spectrometer (Germany) at the temperature of liquid nitrogen (–196°C), a microwave power of 50 mW, and a modulating amplitude of 1 G. The control samples contained no bacterial cells.

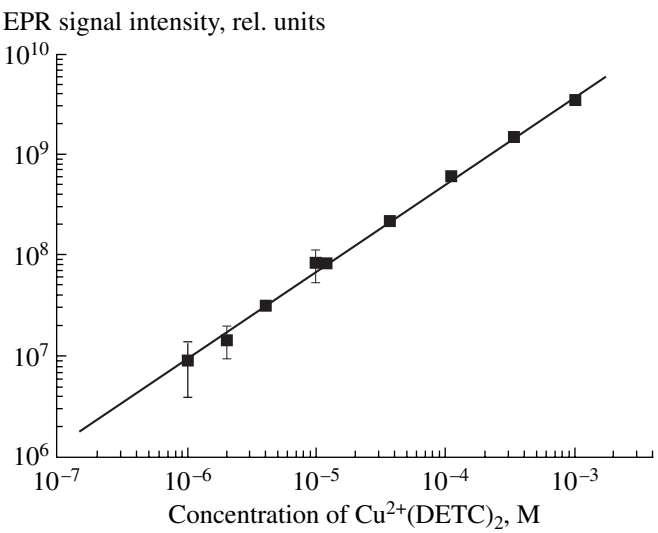

Fig. 1. Integral intensity of the EPR signal of the $Cu^{2+}(DETC)_2$ complex as a function of its concentration.

To evaluate the amount of the nitrosyl complex $(DETC)_2$-Fe^{2+}–NO, the integral intensity of the EPR signal of the model complex of *bis*-N,N-diethyldithiocarbamate with copper (II) ($Cu^{2+}(DETC)_2$) was determined versus the copper concentration. The latter complex was obtained by adding a slightly acidic solution of 25 mM $CuSO_4$ (25 ml) to a solution of 5 mM Et_4NCS_2Na (100 ml). The $CuSO_4$ solution was added in drops to the stirred mixture. The produced black precipitate was collected by filtration, washed with water, dried, and recrystallized from CCl_4. The resultant crystals collected by filtration were washed with CCl_4 and dried. To construct a calibration curve illustrating the dependence of the relative integral intensity of EPR signals on the concentration of copper (II), we prepared a series of solutions containing various concentrations of $Cu^{2+}(DETC)_2$ (from 1 μM to 1 mM) in toluene. Before use, this solvent was distilled, dried, and stored over a 4Å molecular sieve. The stock solution of $Cu^{2+}(DETC)_2$ was prepared through the long-term dissolution of an exact amount of $Cu^{2+}(DETC)_2$ in a certain volume of toluene kept in a hermetically sealed flask. Other $Cu^{2+}(DETC)_2$ solutions were prepared by serial dilutions of the stock solution and immediately frozen in liquid nitrogen in order to record their EPR spectra. The concentration dependence of the integral intensity of the EPR signals of $Cu^{2+}(DETC)_2$ with g = 2.005 was used to calculate the concentration of the $(DETC)_2$-Fe^{2+}-NO complex (Fig. 1).

The results were statistically processed using the Microsoft Excel program. The standard deviation of the results did not exceed 12%. Statistical significance was estimated for $p \le 0.05$.

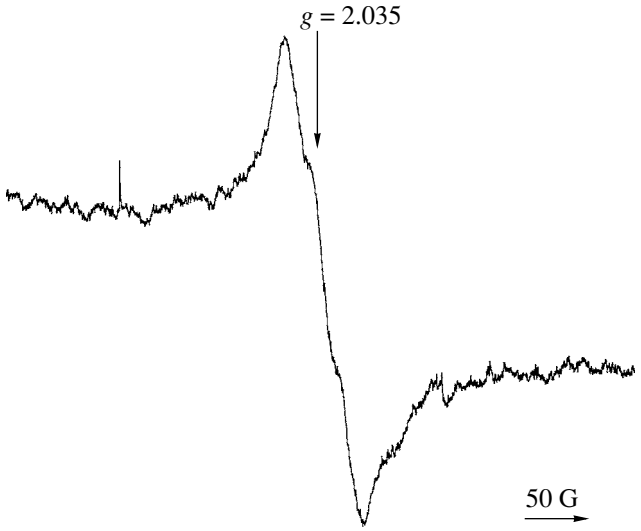

Fig. 2. The EPR spectrum of the nitrosyl complex $(DETC)_2$-Fe^{2+}-NO recorded in a suspension of *L. plantarum* 8P-A3 cells with $Fe^{2+}(DETC)_2$ as the spin trap of nitric oxide. The spectrum was recorded at the temperature of liquid nitrogen (–196°C) the frequency of microwave radiation of 9.4 GHz, a microwave power of 50 mW, and a modulating amplitude of 1 G.

RESULTS AND DISCUSSION

The main objective of this study was to estimate the amount of NO produced by lactobacilli. Ascorbic acid as a spin probe was not useful in detecting the EPR signal of the nitrosyl complex Fe^{2+}-AA-NO with $g = 2.02$. We also failed to detect EPR signals with $g = 2.03$, which are typical for the iron–sulfur centers of proteins, in lactobacillar suspensions containing no exogenous NO traps. At the same time, the preincubation of *L. plantarum* 8P-A3 cells with 30 mM DETC gave rise to a well-resolved triplet EPR signal with $g = 2.035$ for the central line (Fig. 2).

Thus, of all the spin traps studied (ascorbic acid, $Fe^{2+}(DETC)_2$, and the iron–sulfur centers of lactobacillar proteins), the $Fe^{2+}(DETC)_2$ complex was found to be the best trap of NO of bacterial origin. It should be noted that the disadvantage of this complex is its poor solubility in water [13]. However, the use of the water-soluble analogue of DETC, N-methyl-D-glucaminedithiocarbamate (MGD), would not be appropriate since the complex $Fe^{2+}(MGD)_2$ is not specific to NO and the complex $(MGD)_2$-Fe^{2+}-NO is much less stable than the complex $(DETC)_2$-Fe^{2+}-NO [11].

Our studies showed that lactobacilli are not capable of denitrification but can reduce nitrates to nitrites. This finding is in agreement with the data available in the literature that lactobacilli contain nitrate reductase [14]. After seven days of incubation, the culture liquid of *L. plantarum* 8P-A3 was found to contain 6–7 μg/l nitrites but no denitrification gases (N_2O or N_2). Nitric oxide, as an intermediate of denitrification, cannot accumulate in this gaseous mixture because of its high toxicity.

The absence of gaseous denitrification products does not exclude the possibility of the presence of nitrite reductase with low activity. To be sure that such reductase is absent, lactobacillar suspensions were incubated anaerobically in the presence of 20 mM KNO_3 and then subjected to EPR analysis with $(DETC)_2$-Fe^{2+}. These studies showed that extending the incubation time did not increase and even decreased the amount of detected paramagnetic centers. Moreover, the concentration of detected NO did not depend on the presence of lactobacillar cells in the samples (Fig. 3b). These data show that nitric oxide detected by EPR is of abiotic origin. As was reported earlier, NO can be produced chemically from nitrites in acidic media [15]. This process can probably explain the production of NO in samples analyzed for the activity of nitrite reductase.

Thus, *L. plantarum* 8P-A3 can only implement the first stage of denitrification, i.e., dissimilatory nitrate reduction. Under conditions favoring denitrification (oxygen deficiency and the presence of nitrates in the incubation medium), nitric oxide is not produced biotically in lactobacillar suspension, indicating the absence of nitrite reductase in lactobacilli.

At the same time, the EPR analysis of lactobacillar suspensions incubated in the presence of oxygen and L-arginine showed that lactobacilli contain NO synthase. Indeed, the intense EPR signal of the complex $(DETC)_2$-Fe^{2+}-NO indicated the presence of NO in the reaction mixture. When the incubation time was extended from 30 to 60 min, the concentration of NO increased from 37.2 to 95.8 mM (Fig. 3a). This type of dependence is typical of enzymatic reactions and confirms the biotic origin of detected NO.

It should be noted that NO was also detected in the control samples containing no lactobacillar cells, although in considerably smaller amounts than in the case of cell-containing samples (Fig. 3a). The nonenzymatic production of nitric oxide can probably be explained by the abiotic synthesis of NO from L-arginine and H_2O_2 [16]. The physiology of lactobacilli suggests their resistance to considerable concentrations of hydrogen peroxide [17].

Our studies showed that L-NAME, a classic inhibitor of mammalian NO synthase, does not inhibit the NO synthase activity of *L. plantarum* 8P-A3 cells at concentrations up to 100 mM. Similar data were obtained by other authors for *Staphylococcus aureus*. The isolated NO synthase of staphylococci is inhibited by L-NAME, like that of mammals [6]. However, the inhibitory effect of L-NAME in cell suspensions of *S. aureus* is not profound since this bacterium degrades L-NAME with the formation of methanol, which exerts a stimulatory effect on NO synthase [18]. We also observed an increase in the concentration of NO in the presence of L-NAME,

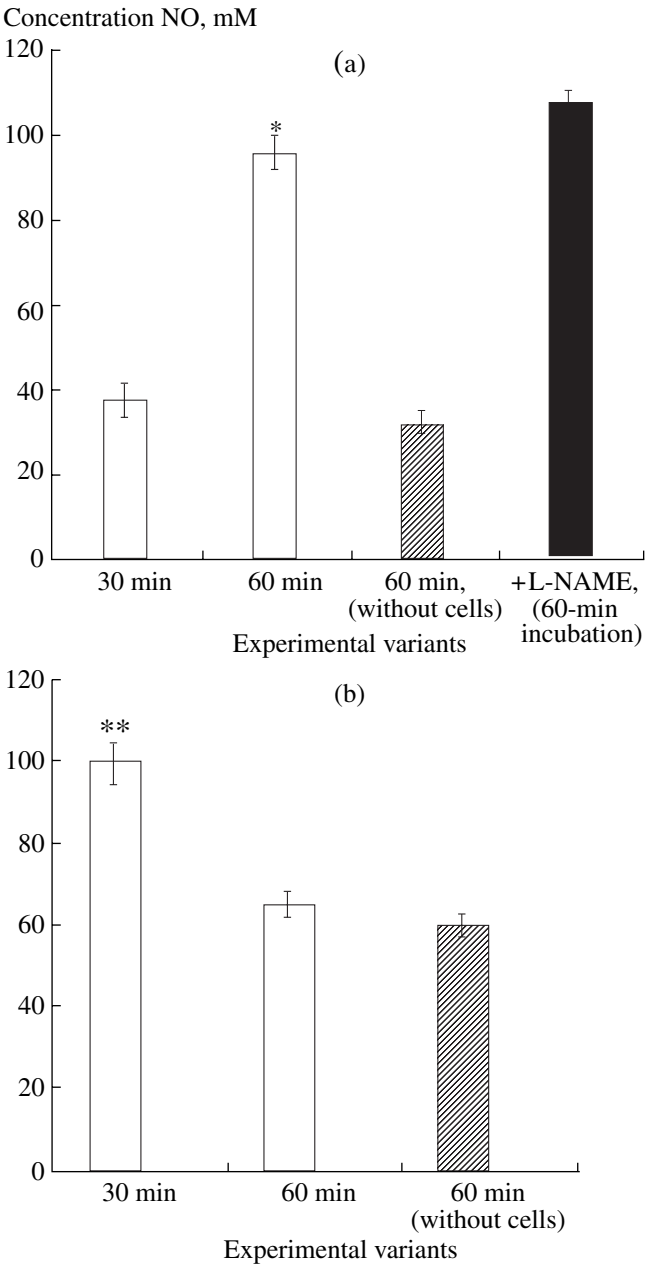

Fig. 3. Content of nitric oxide in suspensions of lactobacillar cells incubated for 30 or 60 min (a) aerobically with 20 mM L-arginine (open bars), with 20 mM L-arginine and 100 mM L-NAME (inhibitor of NO synthase) (dark bar), and without L-arginine (hatched bar), as well as anaerobically (b) in the presence of 20 mM KNO_3 (open bars) and without lactobacillar cells (hatched bar). The data marked by one asterisk (*) (aerobic incubation for 60 min) differ (in a statistically significant manner) from those obtained upon 30-min incubation and the data marked by two asterisks (**) (anaerobic incubation for 30 min) differ from those obtained upon 60-min incubation.

although this increase was statistically insignificant (Fig. 3a) and can be explained by the relatively short incubation time (60 min) or possible differences in the L-NAME metabolism in representatives of the *Staphylococcus* and *Lactobacillus* genera.

To conclude, *L. plantarum* 8P-A3 cells do not produce NO during denitrification but, like mammalian cells, can produce NO from L-arginine with the involvement of NO synthase. Taxonomically, lactobacilli and other NO synthase-containing prokaryotes belong to gram-positive bacteria, whereas denitrifying bacteria are mainly proteobacteria.

The biological function of prokaryotic NO synthase is far from being well-understood. It is known that the homologous NO synthase of *Streptomyces turgidiscabies, S. scabies*, and *S. acidiscabies* is involved in the nitration of a peptide phytotoxin, which is responsible for the phytopathogenicity of the host microorganism. This finding suggests the involvement of bacterial NO synthase in the biosynthesis of secondary metabolites [5]. Since NO is a pleiotropic regulator of cell functions in mammals, the NO synthesized by pathological microorganisms can play a crucial pathophysiological role in infectious processes [6]. The regulatory role of NO synthase in nocardia can mainly be related to the activation of guanylate cyclase by NO [19].

Lactobacilli are an important component of the natural microflora of human and animal intestinal and urogenital tracts, where they are involved in regeneration of the mucosal epithelium and exert an immunomodulating effect [20]. The extensive use of lactobacilli for manufacturing important probiotic preparations on an industrial scale attracts researchers' interest to the investigation of the regulatory function of nitric oxide in these specific microorganisms.

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