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Participation of nitric oxide reductase in survival of *Pseudomonas aeruginosa* in LPS-activated macrophages

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Abstract

Nitric oxide (NO) plays a crucial role in the antimicrobial activity of host defense systems. We investigated the function of *Pseudomonas aeruginosa* NO reductase as a detoxifying enzyme in phagocytes. We found that the growth of the NO reductase-deficient mutant of *P. aeruginosa* under a microaerobic condition was inhibited by the exogenous NO. Furthermore, the intracellular survival assay within the NO-producing RAW 264.7 macrophages revealed that the wild-type strain survived longer than the NO reductase-deficient mutant. These results suggest that the *P. aeruginosa* NO reductase may contribute to the intracellular survival by acting as a counter component against the host's defense systems.

Keywords: *Pseudomonas aeruginosa*; Nitric oxide reductase; Denitrification; Nitrosative stress; Intracellular survival

Nitric oxide reductase (NOR) is a widely distributed in bacteria [1]. The enzyme is known as a respiratory enzyme in denitrification pathway in denitrifying bacteria [2]. Interestingly, studies of the nitrosative stress on *Brucella* [3], *Escherichia* [4–7], *Mycobacterium* [8,9], *Neisseria* [10–12], *Salmonella* [13,14], *Staphylococcus* [15], and *Synechocystis* [16] showed the presence of NO detoxification mechanisms such as flavohemoglobin, flavorubredoxin, and NORs. Thus, the NORs can function not only as a respiratory enzyme, but also as a detoxifying enzyme. In other words, the enzyme seems to make the bacteria survive when they are exposed to exogenous NO around their natural habitats and within their hosts.

Pseudomonas aeruginosa is well known to be one of the major opportunistic pathogens that causes acute and chronic infections. These infections are particularly severe in immunocompromised patients and cystic fibrosis (CF) patients [17,18]. Recent data were revealed that the microbial environment of the CF lung is anaerobic [19], and nitrate and nitrite are present in CF airway surface liquid [20] and sputum [21]. Furthermore, *P. aeruginosa* forms robust biofilms under anaerobic conditions [22]. Therefore, the anaerobic respirations of *P. aeruginosa* seem to be important when considering how to treat these infections. In this study, we compared the viability of the wild-type with that of the NOR-deficient mutant in NO-producing RAW 264.7 macrophages and found that the wild-type strain enhances the viability when compared with the NOR-deficient mutant. These results suggest that *P. aeruginosa* NOR functions as a detoxifying enzyme in the activated macrophages and contributes to the intracellular survival against the host's defense systems.

Materials and methods

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cells were routinely grown in L-broth (10 g of tryptone, 5 g of yeast extract, and 5 g

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Table 1
Bacterial strains and plasmids

Strains or plasmids	Relevant characteristics[a]	Reference or origin
Strains		
Pseudomonas aeruginosa		
PAO1	Wild-type	ATCC 47085
PAOKS30	*norCBD*::Gm^R	This study
Escherichia coli		
XL-1 Blue MRF'	Host for DNA cloning	Stratagene [35]
Plasmids		
pUC119	Ap^R, cloning vector	[36]
pNOR2	pUC119 derivative *norCBD* carrying plasmid, *Sac*I site-deleted	This study
pGMΩ1	Gm^R, source of Ω gentamicin cassette	[37]
pUCGm	pUC119 derivative Gm^R carrying plasmid	This study
pNOR2Gm	pNOR2 derivative *norCBD*::Gm^R	This study

[a] Ap^R, Ampicillin resistance; Gm^R, gentamicin resistance.

of NaCl per liter, pH 7.4) or L-agar (L-broth containing 1.5% (w/v) agar) under aerobic conditions at 37 °C. When necessary, antibiotics were supplemented to the medium as follows: ampicillin, 50 μg/ml (*Escherichia coli*); gentamicin, 10 μg/ml (*E. coli and P. aeruginosa*); carbenicillin, 200 μg/ml (*P. aeruginosa*).

Construction of P. aeruginosa norCBD::Gm^R mutant strain. For DNA manipulations, the standard protocols [23] or the manufacturers' recommendations of commercial products were followed. The genome sequence of *P. aeruginosa* PAO1 was obtained from the database of the Pseudomonas Genome Project (www.pseudomonas.com) [24]. The *norCBD* deficient mutant strain (PAOKS30) was constructed based on the method by Arai et al. [25]. As shown in Fig. 1, the *norCBD* was disrupted by homologous recombination with gentamicin resistance cassette (Gm^R). A 6.5-kb fragment containing *norCBD* genes with flanking regions was amplified with KODplus DNA polymerase (Toyobo, Osaka, Japan) and the primers nor5 (CTTTCATGTCGTCCTTGGCGTGAGC) and nor2 (ATCATCGCGAGCCTTCCCAGCATGG). The amplified fragment was excised with *Eco*RI and inserted into the *Eco*RI site of the *Sac*I site-deleted pUC119 (pNOR2). The *Sac*I site of pUC119 was deleted by *Sac*I-digestion followed by blunting and blunt end ligation. pUCGm was constructed by insertion of *Bam*HI fragment of gentamicin resistance cassette of pGMΩ1 into the *Bam*HI site of pUC119. pNOR2Gm was constructed by replacement of an internal *Sac*I–*Xho*I fragment of pNOR2 with *Sac*I–*Sal*I fragment of pUCGm. The pNOR2Gm was transformed into *P. aeruginosa* PAO1 by electroporation. The electroporated cells were plated on L-agar plates, and then the gentamicin-resistant and carbenicillin-sensitive colonies were selected as the *norCBD* deficient mutant, and designated PAOKS30. The mutation was confirmed by PCR and restriction patterns (data not shown).

NO reductase assay. NOR-activity was measured by the method previously described [26]. The electron-donating system involving the horse cytochrome *c* (Sigma–Aldrich, St. Louis, MO) was used. The protein contents were determined by the bicinchoninic acid method (BCA Protein Assay Kit (Pierce, Rockford, IL)) with bovine serum albumin as a standard. The membrane fractions were prepared from PAO1 and PAOKS30 grown in the presence of 50 mM nitrate in 1000 ml L-broth for 36 h under microaerobic condition. The cells (5.81 g and 2.64 g for PAO1 and PAOKS30, respectively [in wet weight]) were harvested using centrifugation at 8000*g* for 10 min, suspended in 30 ml of 10 mM Tris–HCl buffer (pH 8.5) and disrupted with an ultrasonic oscillator, Branson model 450 (20 kHz; 80 W), for 20 min with a-10 min interruption every 10 min. To remove the unbroken cells, the suspension was centrifuged at 8000*g* for 15 min. The supernatant was centrifuged at 104,000*g* for 1 h and the pellet obtained was suspended to be 10 mg protein/ml (final concentration) with 20 mM potassium phosphate buffer (pH 7.0) and used as the membrane fraction. All purification steps were conducted at 4 °C.

Effect of exogenous NO on growth. To investigate the effect of exogenous NO, *P. aeruginosa* strains were grown aerobically overnight in L-broth at 37 °C, with shaking at 130 rpm. Thirty microliters of the aerobic overnight cultures were inoculated into about 4 ml glass cuvettes containing 3 ml of fresh L-broth, and then the cuvettes were fitted with rubber septum and incubated statically at 37 °C. One hundred microliters pure NO gas was injected into the cultures in the cuvettes by a sterilized syringe and the growth was followed by measuring the absorbance at 600 nm.

Cell culture. RAW 264.7 cells, a murine macrophage cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (hiFBS), penicillin G (100 U), streptomycin (100 μg/ml) at 37 °C in 95% (v/v) air, 5% (v/v) CO_2.

Intracellular survival assay. Experimental procedures of the intracellular survival assay were established on the basis of the methods as described previously [27]. The early-stationary phase of *P. aeruginosa* strains were suspended into phosphate-buffered saline (PBS) to make 1×10^9 CFU/ml. Approximately 1×10^6 cells of RAW 264.7 cells per well were prepared in 24-well multi-well plates containing 300 μl of DMEM with 10% hiFBS, penicillin and streptomycin. When indicated, RAW 264.7 cells were treated with 10 μg/ml lipopolysaccharide (LPS) from *Salmonella enteritidis* (Sigma–Aldrich, St. Louis, MO) 24 h prior to experiments. The medium was exchanged with 300 μl of fresh DMEM with 10% hiFBS, penicillin and streptomycin just prior to experiments. One hundred microliters of PBS containing 1×10^8 CFU of *P. aeruginosa*

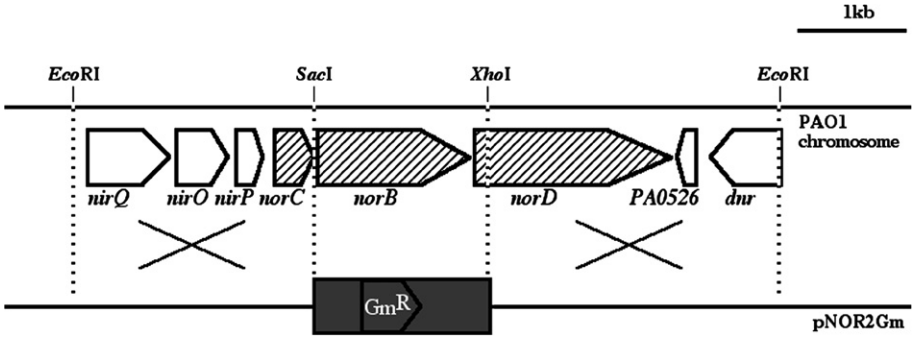

Fig. 1. Construction of *norCBD* mutant strain (PAOKS30) of *P. aeruginosa*. The Gentamicin-resistance casette was inserted into the *norCBD* region by homologous recombination which resulted in total deletion of *norB* and partial deletions of *norC* and *norD*. Restriction sites used for the construction are shown. Gm^R, gentamicin resistance.