Note

Role of Nitric Oxide-Detoxifying Enzymes in the Virulence of *Pseudomonas aeruginosa* against the Silkworm, *Bombyx mori*

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*Pseudomonas aeruginosa* has two nitric oxide (NO)-detoxification enzymes, NO reductase and flavohemoglobin, which catalyze the reduction and oxygenation of NO respectively. In this study, the NO reductase-deficient mutant showed decreased virulence against the silkworm *Bombyx mori*, but the flavohemoglobin-deficient mutant did not, indicating that NO-reduction is important to the full virulence of *P. aeruginosa* against *B. mori*.

Key words: denitrification; nitric oxide reductase; flavohemoglobin; *Pseudomonas aeruginosa*; *Bombyx mori*

*Pseudomonas aeruginosa* is a ubiquitously distributed opportunistic pathogen responsible for severe nosocomial infections in immunocompromised patients. It infects not only humans, but also a wide variety of animals, insects, and plants. *P. aeruginosa* has at least two enzymes that detoxify the bactericidal free-radical gas nitric oxide (NO). One is NO reductase (NOR), which catalyzes the reduction of NO to nitrous oxide. NOR is a denitrification enzyme that is involved in the anaerobic growth of *P. aeruginosa* by dissimilatory nitrate respiration.1) The other is flavohemoglobin (Fhp), which catalyzes the oxygenation of NO to nitrite.2) We have reported that NOR and Fhp are responsible for the detoxification of NO under anaerobic and aerobic conditions respectively.3) NO is produced by macrophages of the host immune system to counteract bacterial infections. Hence the ability to cope with the toxicity of NO is expected to contribute to the pathogenicity of *P. aeruginosa*.

The silkworm, *Bombyx mori*, has been found to be a good animal model to study bacterial infection.3) *P. aeruginosa* is lethal to *B. mori* larvae, and the effects of the production of such toxins and enzymes as exotoxin A, pyocyanin, and superoxide dismutase on the virulence of *P. aeruginosa* against *B. mori* have been successfully investigated.4–8) *B. mori* has NO synthase (NOS), which produces NO from L-arginine. NOS is induced by lipopolysaccharide in the larval fat body of *B. mori*, suggesting that NO is involved in insect immunity.9,10) To determine the role of the NO-resistance of *P. aeruginosa* in its pathogenicity, we investigated the virulence of NOR- and Fhp-deficient mutants of *P. aeruginosa* against *B. mori*.

NOR of *P. aeruginosa* is a cytochrome *bc* type complex. The *norC* and *norB* genes, encoding the cytochrome *c* and cytochrome *b* subunits respectively, are clustered with the *norD* gene, which is required for the expression of the active NOR enzyme.1) Fhp is encoded by the *fhp* gene.2) *P. aeruginosa* strain PAO1ut1) was used as the wild-type in this study. PAO1ut, its isogenetic *norCBD* mutant RM495,12) and fhp mutant FHP1 were injected into *B. mori*. Strain FHP1 was constructed by in-frame deletion using plasmid pEX18-Ap-Δfhp, which was constructed by tandem insertion of the upstream 1.2-kb SacI-KpnI and downstream 1.2-kb KpnI-XbaI fragments of *fhp*, which were amplified by PCR using primer sets fhpA (ggcggagctcgaggaacagg-ctgcc)-fhpB (ctcgggtacctgcagagcggctgcc) and fhpC (gacgcgtacggaactctactctc)-fhpD (acacttctagccgcatgca) respectively into the SacII-XbaI sites of *Escherichia coli* S17-114) was transformed with pEX18Ap-Δfhp, and then the plasmid was transferred to PAO1ut by conjugation. A single-crossover recombinant that carried pEX18Ap-Δfhp on the chromosome was selected on *Pseudomonas Isolation Agar* (Becton Dickinson, Sparks, MD) containing 300 μg/mL of carbenicillin. A second-crossover mutant that lost the *sacB*-containing vector region was selected on an LB plate containing 5% sucrose. An in-frame *fhp* deletion mutant was selected by PCR analysis, and was designated FHP1.

The virulence of the *P. aeruginosa* strains toward *B. mori* (Kinshu × Showa) was estimated by a method described previously.4) Bacterial cells were cultivated in LB medium at 37°C to an optical density at 660 nm of 0.5, and diluted to appropriate concentrations with sterilized water. Ten μL of a diluted bacterial suspension was injected with a syringe into the hemocoel of fourth-instar larvae. Control larvae were injected with sterilized water. The inoculated larvae were kept in an incubator at 25°C. The mortality of the inoculated larvae was monitored for 10 d. Five larvae were injected per dilution, and three replicates per trial were performed.

The results of the mortality assay are shown in Figs. 1 and 2. All the control larvae were viable through the experimental period. *norCBD* mutant strain RM495 was...
The low virulence of the conditions as one of the steps in the denitrification phosphorylation under low oxygen and under anaerobic is also involved in energy conservation by oxidative detoxification, reduction of NO to nitrous oxide by NOR regulator DNR at the transcriptional level in by oxygen-sensing regulator ANR and NO-sensing hemocoel of the larvae. Denitrifying growth is advantageous for survival in the These results strongly suggest that reduction of NO by norCBD gene product, NOR, contributes to the Virulence of nor-Deficient P. aeruginosa against Silkworm 199. ANR induces the expression of DNR under low oxygen concentration in the hemocoel of fourth-instar larvae determined using a micro-oxygen electrode (Microox TX3, Taitec, Koshigaya, Japan) was 0.681 ± 0.026% (n = 5), which is low enough to induce NOR. An NOR-deficient mutant of P. aeruginosa has been reported to show low survival ability in lipopolysaccharide-activated murine macrophages. The flavu-bredoxin-type NOR of enterohemorrhagic E. coli was also recently reported to be involved in survival in macrophages. These reports suggest that the ability to detoxify NO is effective for the survival of pathogenic bacteria in macrophages that produce NO. In the present study, reduced virulence of the norCBD mutant was observed only when a low amount (<10³) of cells was injected. The mutant was not less virulent than the wild-type strain (Fig. 1). No difference in mortality was found when 10⁵ cells were injected (Fig. 1A). Mortality at 120 h after injection was 80% when 10⁵ wild-type cells were used, whereas it was 40% when the norCBD mutant was used (Fig. 1B). The difference was more significant when 10³ cells were injected (Fig. 1C). Only 20% of the larvae died up to 240 h after injection of 10² cells of the norCBD mutant, whereas 73% of them died when the wild type was used. These results strongly suggest that reduction of NO by the norCBD gene product, NOR, contributes to the virulence of P. aeruginosa. In addition to the role of NO detoxification, reduction of NO to nitrous oxide by NOR is also involved in energy conservation by oxidative phosphorylation under low oxygen and under anaerobic conditions as one of the steps in the denitrification pathway. The low virulence of the norCBD mutant also indicates the possibility that a capacity for denitrifying growth is advantageous for survival in the hemocoel of the larvae. The expression of NOR is subject to dual control by oxygen-sensing regulator ANR and NO-sensing regulator DNR at the transcriptional level in P. aeruginosa. ANR induces the expression of DNR under low oxygen conditions, and DNR directly activates the norC promoter in the presence of NO. The oxygen concentration in the hemocoel of fourth-instar larvae determined using a micro-oxygen electrode (Microox TX3, Taitec, Koshigaya, Japan) was 0.681 ± 0.026% (n = 5), which is low enough to induce NOR. An NOR-deficient mutant of P. aeruginosa has been reported to show low survival ability in lipopolysaccharide-activated murine macrophages. The flavu-bredoxin-type NOR of enterohemorrhagic E. coli was also recently reported to be involved in survival in macrophages. These reports suggest that the ability to detoxify NO is effective for the survival of pathogenic bacteria in macrophages that produce NO. In the present study, reduced virulence of the norCBD mutant was observed only when a low amount (<10³) of cells was injected. The mutant was not less virulent when more than 10⁵ cells were injected (Fig. 1A). This is in contrast to the findings for the superoxide dismutase (sodM, sodB)-deficient and the exotoxin A (toxA)-deficient mutant strains, which were clearly less virulent at a dose of 10⁶ cells. This difference is probably due to the fact that NOR was effective for the survival of P. aeruginosa only after phagocytosis or nodule formation in the larval immune system. fhp mutant strain FHP1 showed slightly higher virulence than the wild-type strain when a low amount of cells (10³ or 10²) was injected (Fig. 2). The mutant
killed 100% of the larvae during the experimental period when $10^2$ or $10^3$ cells were injected. These results clearly indicate that oxygenation of NO does not contribute to the virulence of *P. aeruginosa*.

Expression of Fhp is controlled by another NO-sensing regulator, FhpR, regardless of the oxygen concentration. NO is oxygenated to nitrate by Fhp in the presence of oxygen, but nitrate is immediately reduced to NO by denitrification enzymes, nitrate reductase and nitrite reductase, under low oxygen conditions. Thus Fhp might not work as an NO scavenger in a closed, low-oxygen system. The metabolic burden caused by the useless cycle of NO-oxygenation and nitrate reduction was probably one of the reasons for the lower virulence of the wild type.

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**References**