Studies on the function of nitric oxide reductase of pathogenic bacterium Pseudomonas aeruginosa

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Abstract: *Pseudomonas aeruginosa* is a pathogenic bacterium and its infections are commonly difficult to treat due to various virulence determinants such as biofilm-forming ability, drug efflux pumps, quorum-sensing system and so on. Detoxification of exogenous nitric oxide (NO) seems to be essential for survival of pathogens within their host because inducible NO synthase (iNOS)-derived NO is one of the major microbicidal molecules of the innate immune response. *P. aeruginosa* possesses NO reductase (NOR) as a respiratory enzyme, which reduces NO to N_2O in an anaerobic respiration pathway: denitrification. Until now, there are no reports on the contribution of *P. aeruginosa* NOR to the intracellular survival within phagocytes. In this study, I constructed the disruption mutatnt of the *norCBD*, encoding NOR, and compared the growth of the mutant to that of the wild-type. Under oxygen-limited conditions, nitrate in medium was used as a final electron acceptor of denitrification in the wild-type, but not in the NOR-deficient mutant. The sensitivity to exogenous NO was also compared by the growth curve assay. The results indicated that the presence of *norCBD* contributes to the detoxification of exogenous NO. Finally, I 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 than the NO reductase-deficient mutant. These results suggest that *P. aeruginosa* NOR functions as a detoxifying enzyme within macrophages and contributes to the intracellular survival against the host's defense systems.

Introduction: *Pseudomonas aeruginosa* is a ubiquitous bacterium that is capable of adapting to a wide variety of environmental conditions. Presently, this gram-negative bacterium is well known as one of the major opportunistic pathogens that cause both acute and chronic infections [1], and these infections are commonly difficult to treat. Recent data have revealed that the anaerobic growth of *P. aeruginosa* seems to be important when considering how to treat these infections [2-6]. Denitrification is an anaerobic respiration employed in *P. aeruginosa*. In this bacterium, an integral membrane protein NOR reduces NO to N_2O in the complete denitrification pathway. Interestingly, many reports have shown that several NO-detoxifying proteins involve intracellular survival within phagocytes, including NORs [7-20]. Thus, the enzyme seems to make the bacteria alive when they are exposed to exogenous NO around their natural habitats and within their hosts. However, until now, there are no reports on the contributions of *P. aeruginosa* NOR to the intracellular survival within phagocytes.

Materials and Methods:

Bacterial strains and growth conditions. *P. aeruginosa* PAO1 was used as a wild-type strain. *P. aeruginosa* PAOKS30 was constructed as described below and used as a *norCBD* deficient mutant strain. *Escherichia coli* XL-1 Blue MRF' (Stratagene, La Jolla, CA) was used as the host for cloning [21]. Bacterial cells were routinely grown in L-broth or L-agar (L-broth containing 1.5% (w/v) agar).

Construction of *P. aeruginosa norCBD*::Gm^R mutant strain. The *norCBD* deficient mutant strain (PAOKS30) was constructed based on the method by Arai *et al.* [22]. The genome sequence of *P. aeruginosa* PAO1 was obtained from the database of the Pseudomonas Genome Project <www.pseudomonas.com (Currently http://v2.pseudomonas.com)> [23]. Briefly, the *norCBD* genes were disrupted by homologous recombination with gentamicin resistance cassette (Gm^R). The disruption plasmid pNOR2Gm was transformed into *P. aeruginosa* PAO1 by electroporation performed by the method of Smith and Iglewski [24] with slight modifications. The mutation was confirmed by PCR and restriction patterns (data not shown).

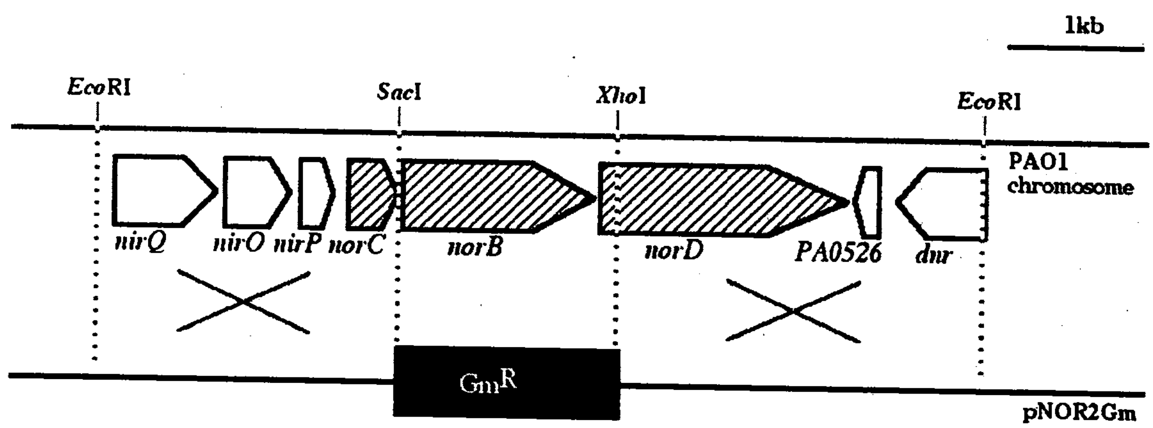

Fig. 1. Construction of *norCBD* mutant strain (PAOKS30) of *P. aeruginosa*.
The Gentamicin-resistance casette was intserted into the *norCBD* region by homologous recombination which resulted in total deletion of *norB* and partial deletions of *norC* and *norD*. Restriction sites used for the construction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

Microaerobic growth of *P. aeruginosa* and effect of exogenous NO on growth. *P. aeruginosa* strains were grown statically in L-broth in the presence or absence of 50 mM nitrate at 37 °C. Pure NO gas was injected into the cultures using a sterilized syringe and the growth was followed by measuring the absorbance at 600 nm.

Assay of NO produced by RAW 264.7 macrophages. NO production from RAW 264.7 cells was measured as its end product, nitrite, by the Griess reagent [25] with minor modifications. The cell culture supernatant was mixed with the Griess reagent and incubated at 30°C for 10 min, followed by the determination of the absorbance at 540 nm. Nitrite concentrations were calculated with the standard curve using sodium nitrite.

Intracellular survival assay. Experimental procedures of the intracellular survival assay were established on the basis of the methods as described by Rest and Speert [26]. A RAW 264.7 macrophage: bacteria ratio of 10^6 : 10^8 was chosen for this experiment. The macrophages were treated with 10 μg/ml LPS (*Salmonella enteritidis*) 24 h prior to experiments.

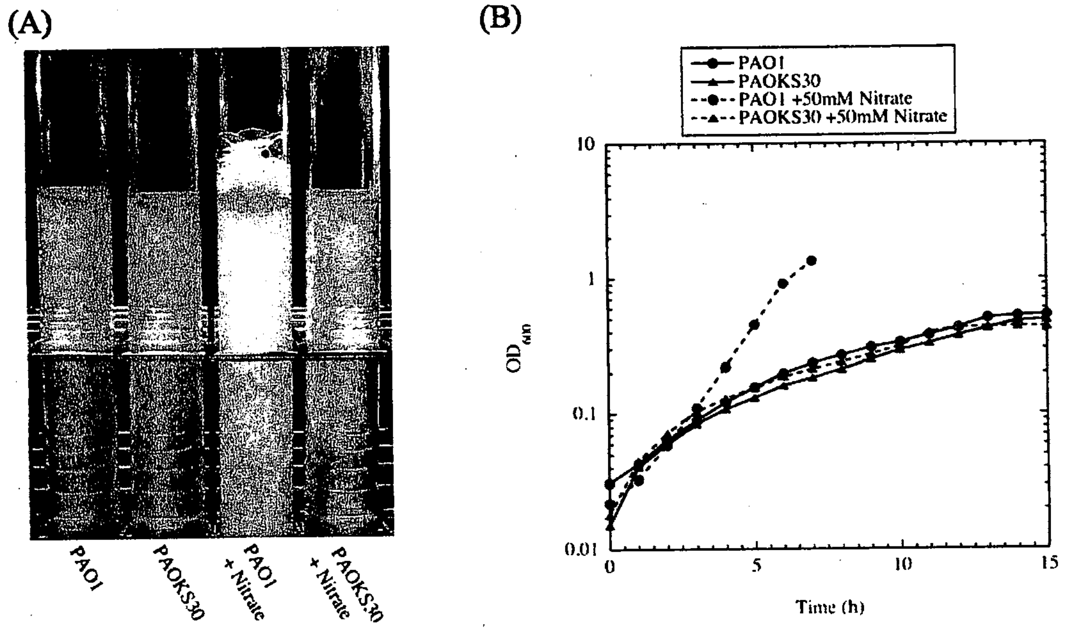

Fig. 2. Microaerobic growth of *P. aeruginosa* in the presence and absence of nitrate. The bacterial cultures were grown in the presence and absence of 50 mM KNO_3. (A) The wild-type strain PAO1 and the *norCBD*-deficient mutant strain PAOKS30 were cultivated statically in L-broth at 37°C. (B) The growth curves of PAO1 and PAOKS30 were performed in 3 ml glass cuvette with gas tight rubber septum containing 3 ml of L-broth. The OD_{600} of the cultures were determined at 1 h intervals.

Results and Discussion:

Microaerobic growth of *P. aeruginosa* and effects of NO on growth of PAO1 and PAOKS30. As shown in Fig. 1, the *norCBD*-deficient mutant strain was constructed. To investigate the effects of the deletion of *norCBD* on microaerobic growth, I cultivated two strains of PAO1 and PAOKS30 in L-broth supplemented with or without 50 mM KNO_3 under microaerobic conditions. As shown in Fig. 2A, no apparent differences were observed between the growth of PAO1 and PAOKS30 in the absence of nitrate. On the other hand, PAO1 showed an enhanced growth in the

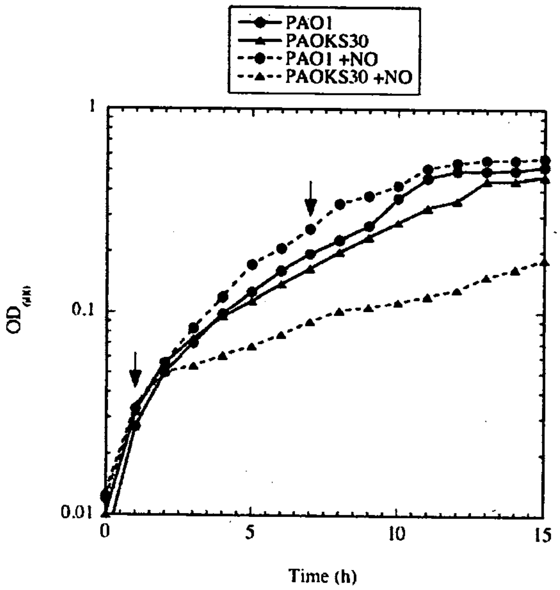

Fig. 3. Effects of exogenous NO on the growth of *P. aeruginosa*.
The growth curves of PAO1 and PAOKS30 were performed in 4 ml glass cuvette with gas tight rubber septum containing 3 ml of L-broth. *P. aeruginosa* strains were incubated statically in L-broth at 37°C. The bacterial cultures were exposed to 100 μl of pure NO gas at the times indicated by the arrows. The OD_{600} of the cultures were determined at 1 h intervals.

presence of nitrate, while the growth of PAOKS30 remained at a level comparable to its growth without nitrate. These results clearly show that although the *norCBD* mutant strain PAOKS30 utilizes oxygen as a final electron acceptor as same as PAO1 in the absence of nitrate, the wild-type strain preferentially utilizes nitrate even under microaerobic condition.

Secondly, the effects of the exogenous NO on the growth of PAO1 and PAOKS30 were examined. I cultivated the two strains in the absence of nitrate under microaerobic conditions using the L-broth (Fig. 3). Although the growth of PAO1 was not inhibited at all, the growth of PAOKS30 was strongly inhibited by the NO gas injection. These results suggest that PAO1 carrying NOR has the ability to detoxify the exogenous NO, while PAOKS30 can not do it.

Intracellular survival of *P. aeruginosa* in phagocytes. To investigate the intracellular survival of *P. aeruginosa* in phagocytes, I developed an intracellular survival assay. I first confirmed the NO production by the RAW 264.7 macrophages, which have been known to produce NO from iNOS several hours after exposure to LPS [27]. As shown in Fig. 4, the LPS-nonactivated RAW 264.7 macrophages did not produce NO, however, when the RAW 264.7 macrophages had been primed with LPS for 24 h, high amounts of NO were produced. The concentration was calculated to be about 9.92±0.57 μM

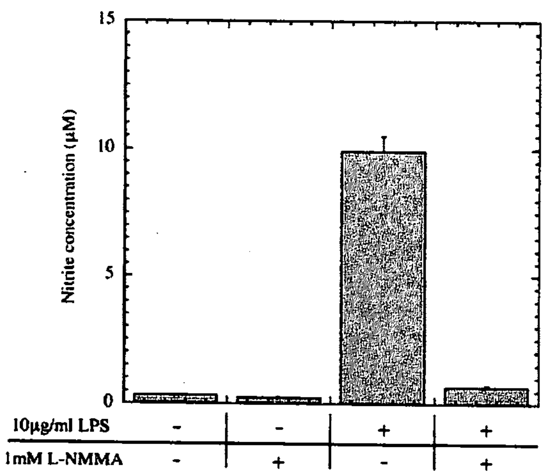

Fig. 4. NO production by RAW 264.7 macrophages.
Nitrite concentration in cell culture supernatants was measured by the Griess reagent and used as the index of NO production from macrophages. LPS from *Salmonella enteritidis* was used as the stimulus, inducing RAW 264.7 macrophages to produce NO. L-NMMA was used as a competitive iNOS inhibitor. Data are means±SEM from three independent experiments performed in triplicate.

(means±SEM, n=3 in triplicate).
Furthermore, the NO production with LPS in the macrophages was strongly repressed

by the competitive iNOS inhibitor, L-NMMA. Therefore, it is concluded that NO is produced by the iNOS of the RAW 264.7 macrophages primed with LPS, under the condition employed.

I compared the intracellular survival of PAO1 and PAOKS30 within the LPS-activated RAW 264.7 macrophages. As shown in Fig. 5A, although the number of viable bacteria was approximately equivalent in each strain at the end of the internalization period, the number of viable PAOKS30 was significantly decreased ($P < 0.02$) than that of PAO1 after the 30 min of killing period. This result indicates that PAOKS30 is killed more efficiently than PAO1 by LPS-activated macrophages. As already described, NO production from the LPS-activated RAW 264.7 macrophages was strongly inhibited by the competitive iNOS inhibitor, L-NMMA. To confirm this iNOS inhibitory effect on the survival of *P. aeruginosa* within the RAW 264.7 macrophages, the macrophages were incubated with L-NMMA during the killing period. As shown in Fig. 5B, the number of viable bacteria remained approximately equivalent level in each strain even after the 30 min of killing period when macrophages were treated by 1mM L-NMMA. Furthermore, no significant differences were observed between two strains. These results indicate that NO produced by iNOS is a major bactericide under the present experimental conditions and that PAOKS30 is more sensitive to the NO-mediated killing by macrophages.

(A) (B)

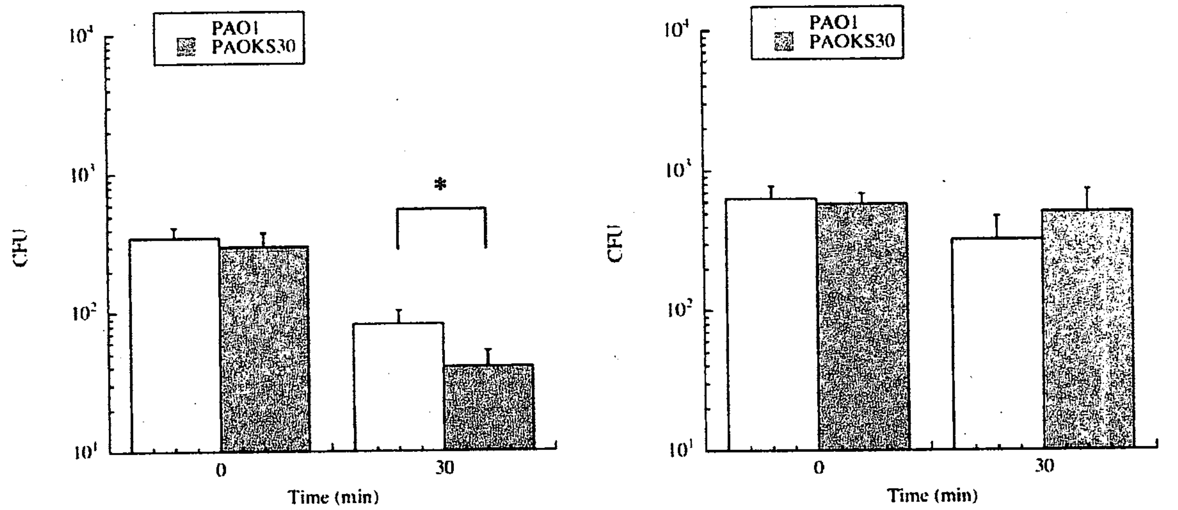

Fig. 5. Intracellular survival of internalized *P. aeruginosa*.
CFUs of bacteria per well at 0, 30 min are shown. (A) Bacteria were internalized by RAW 264.7 treated with LPS 24 h prior to the experiment. (B) iNOS inhibitor L-NMMA (1 mM) was added after wash to remove extracellular bacteria. Data are means±SEM from at least six (A) or three (B) independent experiments performed in triplicate. Significance is shown with asterisk (P <0.02) by Wilcoxon signed-rank test.

Conclusions:

i. I constructed the NOR-deficient mutant strain PAOKS30 of *P. aeruginosa*. The *norCBD* genes were disrupted by double-crossover homologous recombination with gentamicin resistance cassette (Gm^R).
ii. I observed that PAOKS30 could not utilize nitrate as a final electron acceptor under oxygen-limited conditions.
iii. I found that PAOKS30 was more sensitive to exogenous NO than the wild-type strain PAO1.
iv. I established the intracellular survival assay to compare the viability of the PAOKS30 with that of the wild-type strain PAO1 in NO-producing RAW 264.7 macrophages. The assay revealed that PAOKS30 is killed more efficiently than PAO1.

The studies presented here indicated that the presence of NOR not only enhances the anaerobic/microaerobic growth via denitrification, but also contributes to the survival of the bacteria exposed to the exogenous NO. Therefore, *P. aeruginosa* may utilize NOR as a detoxifying enzyme against the host's defense systems. The selective inhibition of NOR may provide novel insights into the important clinical goal of eliminating notorious *P. aeruginosa* infections.

References

1. Rev Infect Dis (1983) **5:** 279-313.
2. Arch Dis Child (1998) **78:** 49-53.
3. Pediatr Pulmonol (2000) **30:** 79-85.
4. Nature (2000) **407:** 762-764.
5. Dev Cell (2002) **3:** 593-603.
6. J Clin Investig (2002) **109:** 317–325.
7. J Bacteriol (2002) **184:** 2987-2993.
8. J Bacteriol (2005) **187:** 3960-3968.
9. J Biol Chem (1998) **273:** 12543-12547.
10. Science (2003) **302:** 1963-1966.
11. J Biol Chem (2002) **277:** 8166-8171.
12. J Biol Chem (2002) **277:** 8172–8177.
13. FEBS Lett (2006) **580:** 1817-1821.
14. Infect Immun (2000) **68:** 5241-5246.
15. J Biol Chem (2005) **280:** 2636-2643.
16. Infect Immun (2006) **74:** 1973-1976.
17. J Biol Chem (1999) **274:** 748-754.
18. Mol Microbiol (2002) **45:** 1303-1314.
19. Infect Immun (2005) **73:** 3322-3329.
20. Infect Immun (2002) **70:** 4399-4405.
21. Strategies Newsletter (1992) Vol. 5, pp. 81-83. Stratagene, La Jolla, California.
22. FEMS Microbiol Lett (1999) **170:** 19-24.
23. Nature (2000) **406:** 959-964.
24. Nucleic Acids Res (1989) **17:** 10509.
25. FASEB J (1993) **7:** 349-360.
26. Methods Enzymol (1994) **236:** 91-108.
27. Science (1992) **256:** 225-228.

学位論文審査結果の要旨

本論文は、緑膿菌 *Pseudomonas aeruginosa* の一酸化窒素還元酵素(NOR)がマクロファージ細胞内で iNOS により生成された外因性 NO を解毒する機能を果たし、本細菌のマクロファージ細胞内生存能に寄与していること明らかにした論文である。

緑膿菌は日和見感染性の病原菌であり、その感染症は本細菌のバイオフィルム形成能や薬剤排出ポンプ、クオラムセンシング機構等の存在により難治性となり、慢性化することが多い。緑膿菌は嫌気呼吸の一つである脱窒で機能する NOR をもつが、現在までに、緑膿菌 NOR が貪食細胞内での NO の解毒に関与するという報告はなされていない。そこで本研究では、まず緑膿菌 NOR 欠損株を作製し、嫌気または微好気条件下で野生株と生育を比較した。その結果、硝酸存在下では野生株は硝酸を最終電子受容体として利用して非常に良く増殖したのに対し、NOR 欠損株ではこれを利用することができなかった。さらに、NO ガス、NO 産生物質を用いて両株の外因性 NO への感受性について検討し、NOR 欠損株でその増殖が顕著に阻害されることを見いだした。最後に NO 産生マクロファージ内での細胞内生存について野生株と比較検討することにより、NOR 欠損株ではその生存率が野生株と比べて有意に低いことを明らかにした。

以上、本研究により、緑膿菌 NOR は外因性 NO 解毒酵素として機能していることを明らかにし、それらの知見は当該分野の研究発展に大いに寄与するものと考えられる。従って、審査委員会は、本研究が博士（理学）に値すると判断した。