Need for Cytochrome bc1 Complex for Dissimilatory Nitrite Reduction of Pseudomonas aeruginosa

Norio HASEGAWA, Hiroyuki ARAI, and Yasuo IGARASHI†

Department of Biotechnology, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

Received July 19, 2002; Accepted September 19, 2002

Pseudomonas aeruginosa strains deficient in the genes for cytochrome c1, a subunit of the cytochrome bc1 complex, or the tetraheme membrane protein NapC, which is similar to NirT of Pseudomonas stutzeri, were constructed and their growth was investigated. The cytochrome c1 mutant could not grow under anaerobic conditions with nitrite as an electron acceptor and did not reduce nitrite in spite of its producing active nitrite reductase. NirM (cytochrome c551) and azurin, which are the direct electron donors for nitrite reductase, were reduced by succinate in the presence of the membrane fraction from the wild-type strain as a mediator but not in the presence of that from the cytochrome c1 mutant. These results indicated that cytochrome bc1 complex was necessary for electron transfer from the membrane quinone pool to nitrite reductase. The NapC mutant grew anaerobically at the expense of nitrite, indicating that NapC was not necessary for nitrite reduction.

Key words: Pseudomonas aeruginosa; electron transport; cytochrome bc1; cytochrome c551; azurin

Denitrification is a kind of anaerobic respiration with N oxides used as terminal electron acceptors. Denitrifying bacteria reduce nitrate to N2 via nitrite, nitric oxide, and nitrous oxide through reactions catalyzed by four enzymes: nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N2OR). All denitrification bacteria are facultative anaerobes and use oxygen readily as a terminal electron acceptor. The electron pathway of denitrification seems to be shared with that of aerobic respiration, and denitrification enzymes act as alternative terminal oxidoreductases of the respiratory chain under anaerobic conditions.1,2 That is, denitrification enzymes receive electrons from the quinone pool or cytochrome bc1 complex (complex III) as do quinol oxidases or cytochrome oxidases of aerobic respiration. Electrons are transferred from cytochrome bc1 complex to denitrifying enzymes via periplasmic electron-carrying proteins such as c-type cytochromes or blue-copper proteins. In Paracoccus denitrificans, denitrification is inhibited by antimycin A, an inhibitor of cytochrome bc1 complex, indicating that cytochrome bc1 complex is necessary for denitrification.3,4

The NIR of Pseudomonas aeruginosa is cytochrome cd1, which contains heme c and heme d1 as cofactors and is located in the periplasm.5 The genes needed for nitrite reduction are clustered (nir gene cluster) on the genome of P. aeruginosa. The structural gene for NIR, nirS, is followed by the genes for two mono-heme cytochromes c, nirM and nirC.6,7) The gene product of nirM is cytochrome c551 (NirM), which is a physiological electron donor for NirM.6,8) The gene product of nirC (NirC) also acts as an electron donor for NIR.9) The nirSMC genes are followed by the genes needed for biosynthesis of heme d1.7,10) A blue-copper protein, azurin, which is not encoded in the nir gene cluster, also acts as an electron donor for NIR.5,10-12) We have previously reported that the reducing reaction of soluble cytochrome c by a P. aeruginosa membrane fraction is not inhibited by the inhibitor of cytochrome bc1 complex, antimycin A or n-heptylhydroxyquinoline-N-oxide when succinate is the electron donor.8) The results suggested the existence of an alternative electron pathway bypassing cytochrome bc1. The NapC/NirT family of membrane-bound tetraheme proteins has been discovered to mediate electron transport from the membrane quinone pool to soluble periplasmic oxidoreductases.11,12) NirT of Pseudomonas stutzeri is encoded in the nir gene cluster for cytochrome cd1-type NIR.13) NirT is involved in the electron transfer pathway of NIR, but whether NirT acts as a bypass of cytochrome bc1 complex is not known.14) Although NIR of P. aeruginosa is similar to that of P. stutzeri, NirT is not encoded in the nir gene cluster of P. aeruginosa. A protein corresponding to NirT is encoded by napC in the nap gene cluster for periplasmic nitrate reductase in P. aeruginosa,15) but the role of NapC in nitrite respiration has not been investigated. In this work, we investigated the role of cytochrome bc1 complex and NapC in the electron transference from the membrane fraction to NirM and azurin, which act as direct electron donors for NIR.

† To whom correspondence should be addressed. Fax: +81-3-5841-5272; E-mail: aigara@mail.ecc.u-tokyo.ac.jp
Materials and Methods

Bacterial strains and growth conditions. Escherichia coli JM109 was used as a host for the experiment on DNA manipulation. P. aeruginosa PAO1 was used as the wild type, and mutant strains were generated from PAO1. E. coli and P. aeruginosa strains were grown at 37°C in Luria-Bertani medium. The denitrification system of Pseudomonas strains was induced by addition of NaNO3 to 20 mM or NaNO2 to 5 mM. When necessary, antibiotics were added to the medium as follows: for E. coli, ampicillin to 150 μg/ml and tetracycline (Tc) to 12.5 μg/ml, and for P. aeruginosa, carbenicillin to 150 μg/ml and Tc to 200 μg/ml.

Anaerobic cultivation by denitrification was done in a 50-ml vial containing 30 ml of the Luria-Bertani medium containing 5 mM NaNO3. After the vial was sealed with a butyl rubber septum and an aluminum cap, the headspace gas was replaced with argon, and incubation was continued for 3 h more anaerobically.

Construction of mutant strains of P. aeruginosa. To generate mutant strains, marker exchange mutagenesis was done. The Tc resistance gene tet from pBR322 was used as the marker. Cytochrome b and cytochrome c1, subunits of the putative cytochrome bc1 complex, are encoded by PA4430 and PA4429, respectively, on the genome of P. aeruginosa. PA4429 has 60% sequence similarity to the gene for cytochrome c1 of Chromatium vinosum; no other gene similar to the gene for cytochrome c1 was found on the P. aeruginosa genome. PA4429 was cloned by PCR with the primers 5’ACTGGAAGGGCGGGCAATTCGCTTG3’ and 5’ATTCTCTTAGACCCAGGACCAGGCGGAT3’. (Recognition sites for restriction enzymes are underlined.) napC was cloned separately as two fragments with the primers 5’GGGTGTTGATGGATCCGAAGAATTCT3’ and 5’GGGTGTTGATGGATCCGAAGAATTCT3’, and the primers 5’GGGTGTTGATGGATCCGAAGAATTCT3’ and 5’GGGTGTTGATGGATCCGAAGAATTCT3’. (Fig. 1.) The two fragments were combined at the EcoRV or PmaCl site.

Preparation of cytochrome c and azurin. NirM (cytochrome c551) was purified by a method described previously. Purified azurin of P. aeruginosa was purchased from Sigma.

Reducing reaction by the membrane fraction. The preparation of the membrane fraction and reaction system was described previously. The reaction mixture (1.0 ml) contained 50 mM sodium phosphate (pH 7.4), membrane vesicles (0.24 mg of protein), and either 2 nmol of cytochrome c551 or 2.5 nmol of azurin. The reaction was started by the addition of sodium succinate to 3.2 mM. The spectrum was measured by a spectrophotometer (U-3210, Hitachi, Tokyo).

Western blotting and promoter assay. Preparation of a cell extract and western blotting with anti-NIR antibody were done as described previously. Broad-host-range plasmids pH351, pH352, and pH353, which carry the nirS::lacZ, nirQ::lacZ, and norC::lacZ transcriptional fusions, respectively, were used for a promoter assay. P. aeruginosa PAO1 and RM4429 were transformed with the plasmids. Transformants were cultivated under anaerobic conditions. The activity of β-galactosidase, the gene product of lacZ, was measured by a method described before.

Results and Discussion

Growth of P. aeruginosa mutant strains

RM4429 (cytochrome c1) grew poorly under aerobic conditions (Fig. 2(A)). The genome sequence of
Involvement of Cytochrome $bc_1$ in Nitrite Reduction

*P. aeruginosa* PAO1 shows that the strain has three cytochrome oxidases and two quinol oxidases. The aerobic growth of RM4429 must occur because the quinol oxidases transfer electrons directly from the quinone pool to oxygen. RM4429 could not grow under anaerobic conditions with nitrite as the electron acceptor (Fig. 2(B)), indicating that cytochrome $bc_1$ was necessary for growth on nitrite. Probably the electron pathway passing through cytochrome $bc_1$ is the only route to NIR.

*P. aeruginosa* has NapC, which is a putative electron donor for periplasmic nitrate reductase. NirT, structurally similar to NapC, is involved in the electron pathway to NIR in *P. stutzeri*. The NapC/NirT family of membrane-bound tetraheme proteins has been reported as an alternative electron pathway bypassing the cytochrome $bc_1$ complex. However, NapC could not replace the function of cytochrome $bc_1$ for nitrite reduction, because RM4429 did not reduce nitrite. Disruption of napC did not affect the growth of *P. aeruginosa* under aerobic conditions. Under anaerobic conditions, the growth of RM1172 (napC) was retarded, but the consumption rate of nitrite was the same as that of the wild type (Fig. 2(C)), suggesting that NapC was not necessary for nitrite reduction in *P. aeruginosa*. The reason for the slow growth under anaerobic conditions was not clear. Probably, anaerobic growth was stunted by the defect in electron flow through NapC to periplasmic nitrate reductase or other oxidoreductases.

**Reduction of electron donors for NIR**

NirM (cytochrome $c_{551}$) is a physiological electron donor for NIR. Succinate-dependent reduction of NirM by the membrane fraction was investigated in vitro. NirM was reduced by succinate when the membrane fraction from PAO1 was used as the mediator (Fig. 3). However, the succinate-dependent reduction of NirM was not observed when the membrane fraction from RM4429 was used. In our previous work, we found that the succinate-dependent reduction of NirM is not blocked by inhibitors of the cytochrome $bc_1$ complex. However, the results reported here suggested that no route other than cytochrome $bc_1$ exists for the electron transport from succinate dehydrogenase to NirM. Perhaps the inhibitors did not work under those assay conditions. A blue-copper protein, azurin, also acts as an electron donor for NIR, although its physiological function has not been identified. As for NirM, succinate-dependent reduction of azurin was observed when the membrane fraction from PAO1 was used as the mediator but not when that from RM4429 was used (Fig. 4). The results indicated that azurin also received electrons only from cytochrome $bc_1$. 

---

**Fig. 2.** Aerobic Growth (A), and Anaerobic Growth and Nitrite Consumption (B) of *P. aeruginosa* PAO1 (circles), RM1172 (triangles), and RM4429 (squares).

**Fig. 3.** Absorption Spectra of NirM with the Membrane Fractions Prepared from PAO1 (A) and RM4429 (B). Dotted lines, oxidized form; solid lines, after addition of succinate.
Expression of NIR

Western blotting and the NIR assay in vitro were done to investigate the expression of NIR in RM4429 (cytochrome c1). Nitrite was not reduced in RM4429, but western blotting showed that the NIR protein was expressed in RM4429 (Fig. 5). Moreover, the in vitro specific activity of NIR in the cell extract of RM4429 was higher (0.36 U/mg) than that of PAO1 (0.16 U/mg) when methylviologen was the artificial electron donor. These results confirmed that the deficiency in nitrite reduction of RM4429 was not caused by the lack of active NIR but by a defect in electron donation to NIR.

The transcriptional activity of nirS in the presence of nitrite is reduced by disruption of nirS. The transcriptional activities of nirQ, which is located upstream of nirS and is needed for activation of NOR, and norCB, the structural genes for NOR, were low in the nirS mutant. These results suggested that NIR, the gene product of nirS, is necessary for the transcription of these genes. NO produced from nitrite by NIR seems to be the signal molecule for induction of these genes. Transcription of the denitrification genes, including nirS, nirQ, and norC, were measured with the lacZ fusion plasmids pHAl531 (PnirS), pHAl532 (PnirQ), and pHAl533 (PnorC) (Fig. 6). The promoter activity of nirS in RM4429 was higher than that in the wild type. This result coincided with the NIR activity in vitro. The nirQ and norC promoter activities in RM4429 in the presence of nitrite were one third and one half, respectively, of those in PAO1. Because NO was not produced endogenously from nitrite in RM4429, NO seemed to be important for activation of the nirQ and norC promoters. The nirQ and norC promoter activities in RM4429 in the presence of nitrite were lower than those in the nirS mutant, in which the activities were one fifth and one tenth, respectively, of those in PAO1. Probably, because RM4429 produced active NIR enzyme, production of NO was not completely blocked. The nirS promoter activity in the nirS mutant in the presence of nitrite was one third of that in PAO1, but the activity in RM4429 was 50% higher than that in PAO1 (Fig. 6), indicating that regulation of the nirS promoter was different from that of nirQ or norC promoter. Nitrite rather than endogenous NO seemed to be important for activation of the nirS promoter in RM4429. The transcriptional activities of the three promoters in the absence of nitrite were higher in RM4429 than in PAO1. Such an increase in the basal expression level of respiratory enzymes is often observed when the respiratory chain is blocked. The promoter activity of nirS in RM4429 was especially high in the absence of nitrite. Nitrite produced from nitrogen compounds in the medium may have accumulated to a concentration above the threshold of activation of the nirS promoter.

Acknowledgments

We thank the Pseudomonas Genome Project and the Pseudomonas aeruginosa Community Annota-
Fig. 6. Transcriptional Activity of the nirS (pHA531), nirQ (pHA532), and norC (pHA533) Promoters. The data show means and SD from at least three independent experiments.

References

12) Vijgenboom, E., Busch, J. E., and Canters, G. W., In vivo studies disprove an obligatory role of azurin in denitrification in Pseudomonas aeruginosa and show that azu expression is under control of RpoS and ANR. Microbiology, 143, 2853–2863 (1997).
15) Jüngst, A., Wakahayashi, S., Matsubara, H., and Zumft, W. G., The nirSTBM region coding for cytochrome cd1-dependent nitrite respiration of


