A cytochrome bc-type complex of *Roseobacter denitrificans* OCh114 was thought to be a novel cytochrome *c* oxidase. To determine its function, we deleted the genes encoding the complex. The mutant grew normally by aerobic respiration, but failed to grow by denitrification and lacked nitric oxide reductase activity, indicating that the physiological function of the gene product is nitric oxide reduction.

**Key words:** cytochrome *c* oxidase; denitrification; genetic manipulation; nitric oxide reductase; *Roseobacter denitrificans*

Bacterial denitrification is a type of anaerobic respiration that uses nitrogen oxides in place of oxygen as terminal electron acceptors. The reduction of nitrate to dinitrogen gas is catalyzed by nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR), and nitrous oxide reductase (N2OR). Since nitric oxide (NO) is a toxic intermediate of denitrification, NOR plays an important role in this pathway. Two major types of bacterial NOR have been identified to date: cytochrome *bc*-type complexes, which consist of cytochrome *b* (NorB) and cytochrome *c* (NorC) subunits that receive electrons from soluble *c*-type cytochromes, and enzymes consisting only of the cytochrome *b* subunit that receive electrons directly from quinones. As the cytochrome *b* subunits of NOR are homologous to the catalytic subunit of cytochrome *c* oxidase (CcO) of the heme-copper oxidase family, which contains a heme/copper binuclear center for the reduction of oxygen, NOR is thought to be the evolutionary ancestor of the terminal oxidases involved in aerobic respiration. Although NOR is a member of the heme-copper oxidase superfamily, it contains a heme/non-heme iron active site in place of the heme/copper binuclear center. Thus the presence of copper or non-heme iron in the binuclear center might be one of the factors determining the catalytic activity of the enzyme towards O2 and towards NO.

The aerobic anoxygenic photosynthetic bacterium *Roseobacter denitrificans* OCh114 has been reported to produce a cytochrome *bc*-type complex with CcO activity under aerobic conditions. Although the genes encoding the cytochrome *c* and cytochrome *b* subunits of the complex were found to be highly similar to the NorC and NorB subunit genes of cytochrome *bc*-type NOR, the purified complex contained a heme/copper binuclear center and had no NOR activity. Based on these properties, the complex of strain OCh114 was considered to be a novel type of CcO, and it attracted interest due to its unique evolutionary position in the heme-copper oxidase superfamily. However, the complex exhibited low specific CcO activity and its physiological function remained to be investigated. We have designated the genes encoding the cytochrome *c* and cytochrome *b* subunits of the complex *norC* and *norB* respectively, on their high similarity to the genes of cytochrome *bc*-type NOR. A genome analysis of OCh114 revealed that the *norCB* genes are clustered with the *nit* and *nos* genes, encoding denitrification enzymes NIR and N2OR respectively. The genes encoding the *aa3*-type and *cbb3*-type cytochrome *c* oxidases, the *bd*-type quinol oxidase, and an alternative oxidase were identified in the genome. However, as no genes encoding potential NOR enzymes were identified in the OCh114 genome other than *norCB*, we assumed that the *in-vivo* function of the *norCB* gene product is NO reduction. We attempted in this study to determine the physiological function of the *norCB* genes in OCh114, but gene manipulation techniques for *in-vivo* functional analyses of *R. denitrificans* genes had never been reported, and hence first we developed an effective gene disruption system for *R. denitrificans* and constructed a *norCB*-knockout mutant.

*R. denitrificans* OCh114 (NBRC15277) and a derivative mutant were cultured at 30°C in glycerol medium supplemented, when required, with 20 mM Na2CO3, 60 μg/mL of kanamycin (Km), or 25 μg/mL of streptomycin (Sm) and 25 μg/mL of spectinomycin (Sp). *Escherichia coli* strains were cultured in Luria–Bertani (LB) broth at 37°C with continuous shaking at 200 rpm or on LB plates supplemented, when required, with an antibiotic: 100 μg/mL of ampicillin, 40 μg/mL of Km, or 15 μg/mL of Sm. The introduction of DNA into *R. denitrificans* was carried out by transconjugation with *E. coli* strain S17-1. PrimeSTAR (Takara, Kyoto, Japan) was used for polymerase chain reactions (PCRs). The *norCB*-knockout mutant of strain OCh114, designated dNOR1, was constructed by homologous recombination using plasmid pLoDNOR2Sm*, which was constructed by tandem insertion of the upstream XbaI-
norC2
norB2
not grow under anaerobic denitrifying conditions identical to that of OCh114 (Fig. 2A), but dNOR1 did (Fig. 2). The aerobic growth of dNOR1 was nearly cultivated under aerobic or anaerobic conditions genes on cell growth, OCh114 and dNOR1 were

SacI and downstream SacI-SalI fragments of norCB, which were PCR amplified using primer sets norC1-norC2 and norB1-norB2 respectively, and the SacI fragment of the ΩSm'/Sp' cassette into the Xbal-BamHI ends of the upstream fragment and

The SalI and BamHI ends of the upstream fragment and

One double-crossover mutant was selected and desig-

SacI fragment of the norC2 and norB1-norB2 respectively, and the SacI

which were PCR amplified using primer sets norC1-

norC1
norC2
norB1
norB2

were digested with NdeI and ClaI and then blotted onto a nylon membrane. The blots were hybridized with probes specific for the norCB genes (lanes 1 and 2) or the Sm'/Sp' gene (lanes 3 and 4). Molecular size markers are indicated to the left of the blots. A DIG DNA Labeling and Detection kit (Roche, Madison, WI) was used for analysis.

SacI fragment of the ΩSm'/Sp' cassette of the plasmid indicate regions homologous to the chromosomal DNA of OCh114. The arrows indicate the binding sites of the primers used for PCR: norC1 (5'-GGCTTCTAGAAACCCACGCCTGCTGGC-3'), norC2 (5'-CATGTGACGCTGAGAAGTCACGCACTGAC-3'), norB1 (5'-TGGGGAAGCTTGCTTCATCGCGGTG-3'), and norB2 (5'-CGAAAGATCCGCGGTCAAGCTGCCCT-3'). Restriction sites are underlined. The fragment used as probe for detection of the norCB genes by Southern hybridization is indicated by a gray box. B, Southern blot analysis for confirmation of gene disruption. Genomic DNA extracted from wild-type strain OCh114 (lanes 1 and 3) and norCB-mutant strain dNOR1 (lanes 2 and 4) were digested with SalI and Clal and then blotted onto a nylon membrane. The blots were hybridized with probes specific for the norCB genes (lanes 1 and 2) or the Sm'/Sp' gene (lanes 3 and 4). Molecular size markers are indicated to the left of the blots. A DIG DNA Labeling and Detection kit (Roche, Madison, WI) was used for analysis.

Fig. 2. Effect of the norCB Mutation on the Growth of R. denitrificans under Aerobic (A) and Anaerobic Denitrifying (B) Conditions.

Hollow and solid circles indicate wild-type strain OCh114 and its isogenic norCB mutant strain dNOR1 respectively. Growth curves are representative of at least three independent cultures. Aerobic growth conditions were achieved by continuous shaking at 150 rpm with a culture volume of 4 mL in a 30-mL test tube. Denitrification conditions were created by culturing cells without shaking in 4 mL of glycerol medium supplemented with 20 mM NaNO3 in a 30-mL test tube. After inoculation, the test tube was sealed with a butyl rubber septum, and the headspace was replaced with argon by flushing the gas through a needle inserted into the septum for 5 min.

Table 1. Activities of the Denitrification Enzymes in Strain OCh114 and the norCB Mutant Strain dNOR1

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<thead>
<tr>
<th>Strain</th>
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<td>OCh114</td>
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<td>dNOR1</td>
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<td>31.9 ± 0.0</td>
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*Mean ± standard deviations for triplicate assays are shown. One unit of NAR or NIR activity was defined as the amount of enzyme that produces and that consumes, respectively, 1 μmole of nitrite per min. One unit of NOR activity was defined as the amount of enzyme that consumes 1 nmole of NO per min. The reactions were performed at 30 °C. The protein concentration of the cell suspension was determined by the Bradford method using a Bio-Rad Protein Assay kit.

ND, not detected.

(Fig. 2B). These results clearly indicate that the norCB genes are essential for denitrification by OCh114.

To clarify the in-vivo function of the norCB gene product and to determine why dNOR1 did not grow under anaerobic denitrifying conditions, the activities of denitrification enzymes NAR, NIR, and NOR in OCh114 and dNOR1 were measured (Table 1). Enzyme assays were performed at 30 °C by methods described previously, with minor modifications.15,16 The assay reaction mixtures consisted of 342 mM NaCl, 50 mM sodium phosphate (pH 7.5), 10 mM sodium succinate, and appropriate amounts of nitrogen oxides and OCh114 or dNOR1 cell suspensions. Aerobically grown cells

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were used in the assays, because OCh114 expresses denitrification enzymes even under aerobic conditions.\(^{15}\) Although both the OCh114 and the dNOR cells displayed nearly equal NIR activities (Table 1), NAR activity in dNOR1 was reduced by approximately 50\% as compared to the wild-type strain. In addition, relatively high NO-consumption activity was observed in the OCh114 cells, but no such activity was detected in dNOR1. Taken together, these results indicate that the inability of dNOR1 to grow by denitrification might have been due to an accumulation of toxic NO under anaerobic conditions in the presence of nitrate or to a deficiency in energy production by anaerobic respiration using NO as electron acceptor. The results also strongly suggest that the norCB gene product possesses \textit{in-vivo} NOR activity, a finding that contradicts a previous study, which reported that the purified norCB gene product lacked NOR activity.\(^6\)

Since the norCB gene product of OCh114 exhibits NOR activity, it must contain a heme/non-heme iron binuclear center, but the previously reported norCB gene product with C\(_c\)O activity in this strain was found to have a heme/copper binuclear center.\(^6\) A plausible explanation of this apparent contradiction is that the norCB genes produce two types of cytochrome bc-type complexes, one containing a heme/copper binuclear center with C\(_c\)O activity, and the other a heme/non-heme iron center with NOR activity. The complex with C\(_c\)O activity is probably produced by the spontaneous incorporation of copper into the binuclear center under aerobic, non-denitrification conditions, under which NOR enzymatic activity is not required. Although the physiological function of the cytochrome bc-type complex with C\(_c\)O activity in OCh114, if it has any, remains uncertain, the major function of the norCB gene product appears to be NO reduction, because the norCB mutant grew normally under aerobic conditions but was unable to grow anaerobically by denitrification. Future studies examining the metal in the binuclear center of the purified cytochrome bc-type complex with NOR activity and the proportion of the two types of cytochrome bc-type complexes should be conducted to clarify the enzymatic properties and physiological significance of the norCB gene products in OCh114.

**Acknowledgment**

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**References**