Mutagenesis of Hydroxylamine Oxidoreductase in Nitrosomonas europaea by Transformation and Recombination

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Mutagenesis of Nitrosomonas europaea was achieved by electroporation and recombination. To demonstrate this, an aminoglycoside 3′-phosphotransferase (kan) gene was specifically inserted into each of the three gene copies of hao individually. Southern hybridizations and PCR analysis showed the incorporation of the kan gene at the chosen genetic loci. The isolation of mutant strains was achieved in 7 to 14 days when the strains were grown on solid medium. The induced mutations were stable even in the absence of kanamycin-selective pressure for periods of up to 45 days in culture. The mutant strains did not show an observable phenotype different from that of the wild type when grown under the same conditions.

Nitrosomonas europaea is an aerobic chemolithoautotrophic soil bacterium (25). As such, N. europaea must derive its energy by transforming NH₃ to NO₂⁻ and its carbon for growth from CO₂. Interest in N. europaea stems from its role in nitrification and its ability to cometabolize many halogenated and aromatic hydrocarbon compounds (11–16, 20–22, 25). Genetic information on N. europaea has expanded rapidly in the last few years. Many of the genes coding for the proteins known to be involved in the nitrification pathway have been cloned and sequenced (1, 2, 9, 17, 24). One unusual genetic feature of N. europaea is that at least five of the nitrification genes are present in more than one copy in the genome. Duplications exist for genes encoding ammonia monoxygenase (AMOa and AMOb) (2, 17), hydroxylamine oxidoreductase (HAO) (18, 24), and cytochrome c₅₅₄ (1, 9, 18) and a gene coding for an as yet uncharacterized c-type cytochrome (1). The genes amoA and amoB are adjacent to each other and are present in two copies (17). One copy of amoA and amoB has been cloned and sequenced (2, 17). The gene for HAO is present in three copies (1, 18, 24) (Fig. 1a). One copy of hao has been cloned, and the sequence of the gene and its flanking regions has been obtained (24). Southern hybridization experiments show that probes made for one copy hybridize with all three copies (18, 24). The gene encoding cytochrome c₅₅₄ (cycA; also called hcy) is also present in three copies (1, 18), one copy lying approximately 1.2 kb downstream from each copy of hao (18, 24) (Fig. 1a). Two copies of this gene have been cloned and sequenced (1, 9) and have a high degree of similarity. There are two copies of the uncharacterized c-type cytochrome which are immediately downstream of two of the three copies of the gene coding for cytochrome c₅₅₄ (1) (Fig. 1a). One copy of this gene has been cloned and sequenced (1). Another gene involved in nitrification, cyp, encoding cytochrome P-460, is present as a single copy (18) which has been cloned and sequenced (1). The reason why N. europaea has multiple copies of some genes remains unclear. It is not known if all copies of these genes are expressed or if they are differentially regulated.

In other bacteria, gene expression studies have often made use of insertional inactivations or the introduction of reporter genes into the organism. However, these and other potential genetic experiments with N. europaea have been hampered by the inability to transform the bacterium. To our knowledge, this is the first mutagenesis study of N. europaea. Transformation experiments with N. europaea can now be designed to investigate the roles of genes present in multiple copies. Using these techniques, we have investigated if each of the three copies of hao is essential.

MATERIALS AND METHODS

Strains and cell cultures. Strains of N. europaea and Escherichia coli used are described in Table 1. E. coli cells were grown in LB medium as described previously (23). N. europaea cells were grown in liquid medium (4) and on solid medium. The solid medium for N. europaea was similar to the liquid medium except that it contained 1% Bacto Agar (Difco Laboratories, Detroit, Mich.) and 50 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) buffer (pH 7.8) was substituted for the phosphate buffer. The growth plates were prepared by placing an autoclaved Nitran membrane (6 by 6 cm Schleicher & Schuell, Keene, N.H.) on the solid medium. The N. europaea cells were then streaked on the membrane and incubated at 30°C. Inhibition of cell growth as a result of metabolic by-products was avoided by transferring the membrane to fresh plates weekly. Individual colonies could be distinguished after a 7- to 14-day incubation, after which they were transferred to liquid culture. For long-term storage, cells from liquid cultures were frozen in 7% dimethyl sulfoxide at −80°C. Cells stored in this way are viable for at least 4 years. The NO₂⁻ accumulation, which is correlated with growth, was measured colorimetrically (5). Alternatively, growth was measured as cell density by light scattering at 600 nm in a Beckman (Palo Alto, Calif.) DU7 spectrophotometer. AMO and HAO specific activities in liquid cultures were determined as the rate of O₂ consumption with a Clark-type oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) as described previously (10).

DNA manipulation. Genomic and plasmid DNA preparations, DNA restriction digestions, and Southern hybridizations were done as described previously (23). DNA probes were labeled by random priming, using a Prime-a-Gene kit (Promega Corp., Madison, Wis.) with [α-³²P]dCTP (3,000 Ci/mmol; DuPont NEN Products, Wilmington, Del.) as instructed by the manufacturers. Restriction and DNA-modifying enzymes were from Promega and United States Biochemical (Cleveland, Ohio) except for AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.).

hao gene nomenclature and divergence of loci. N. europaea contains three copies of hao which are distinguishable by Southern hybridization. A KpnI digest of N. europaea genomic DNA probed with an hao probe (pRH [Fig. 1b]) detects three DNA fragments (Fig. 2A, lane 4). Each of the three fragments is specific to one of the three copies of hao. In this report, the hao copies contained on the three large fragments, small to large, are named haoA, haoB, and haoC. One hao gene has been cloned and fully sequenced (24); however, it is not clear if this clone is represented by the KpnI fragment containing haoB or haoC. In addition, another clone containing 0.7 kb of the 5′ end of haoC and 1.3 kb of upstream flanking DNA has been obtained and partially sequenced (unpublished data). These two clones showed great similarity in the hao coding region but showed...
and prN) are shown. Horizontal arrows indicate the direction of transcription. The map scales for panels a and b are the same.

Pst I; Sma I; S, (a) Location of DNA fragments NHB1 and NHC1, into which frame 2 (orf 2) are boxed. Because only a limited amount of the DNA flanking these genes has been sequenced, there may be other open reading frames in this area.

divergence in the 5′ flanking region in both their restriction sites (Fig. 1a) and their DNA sequences. On the basis of Southern hybridizations, some restriction sites (EcoRI, BamHI, and KpnI [Fig. 1a]) appear to be conserved in all three copies of hao (18).

DNA amplifications and constructs. PCRs were performed on an Ericomp (San Diego, Calif.) EasyCycler instrument in a 25-μl reaction volume with the following program: cycle A (94°C, 2 min) once and cycle B (94°C, 1 min; 50°C, 1 min; 72°C, 1.5 min) 40 times. The DNA probes used in the hybridizations were a 1.1-kb BamHI fragment (prN) containing kan which was isolated from pUC4 KSAC (Pharmacia, Piscataway, N.J.) (Fig. 1b) and a 0.5-kb amplified DNA fragment containing the 5′ portion of hao (prH [Fig. 1b]). The hao probe prH was amplified by using the primers PH4 (5′-CGGCAACTTCACGCGGCG) and PH5 (5′-GGGACGATGGGAGATTACTGGG) from genomic DNA (Fig. 1b). The kan probe prN was amplified from plasmid pUC4 KSAC with the primers NPT1 (5′-ATGAGCCATATTCAACGGGAAACG) and NPT2 (5′-ACGGGCCAATTCACCCGCGG) and NPT2 (5′-CTGCGAGCCGGGGGGGCGGCTG) (Fig. 1b).

The DNA constructs are described in Table 1. Two amplified DNA fragments containing portions of hao were used in the transformation experiments. The fragments were obtained by PCR using N. europaea genomic DNA as the template. First, a DNA fragment containing 0.8 kb of the 5′ half of hao A and 1.2 kb of upstream flanking DNA (NHC1 [Fig. 1a]) was amplified by using a single degenerate oligonucleotide primer, F80 (5′-ACYTCUCCATATCCCGCATT) (8). This PCR amplified a 1.9-kb fragment which was cloned into the pCRII vector (pNHC1). Second, a 2.1-kb DNA fragment (NHB1 [Fig. 1a]) containing about half of the second sequenced copy of hao and 1.1 kb of upstream flanking DNA was amplified by using the oligonucleotide primers PH90n (5′-GGNACRAANGGKRTARTNGCCCA) and PH6 (5′-CTTGTGTGCTGAAAACGC)CGCCG). This fragment was cloned into the pCRII vector (pNHB1). To take advantage of the BamHI site in the cloned fragments as an insertion site for the genetic marker kan, the amplified hao fragments were transferred to the cloning vector pRL139 (5). The hao fragments were cut out of pCRII gel purified, ligated into the EcoRI site of pRL139 (pNHB2 and pNHC2), and transformed into E. coli DH5α cells.

A 1.2-kb DNA fragment containing kan, including its promoter, was cut from plasmid pUC4 KSAC with BamHI and gel purified. The kan gene is derived from the transposon Tn903 (19), which encodes the enzyme aminoglycoside 3′-phosphotransferase and confers kanamycin resistance upon cells containing it. The 1.2-kb fragment was inserted into the BamHI site in the N. europaea hao target sequences (Fig. 1a). The resulting clones (pLJ101 and pNHC3) were selected for kanamycin resistance. Plasmids pLJ101 and pNHC3 were used for transformation of N. europaea.

Cell transformation by electroporation. Several conditions for transformation by electroporation were tested. Initially, the transformation parameters were determined by using those electroporation conditions which resulted in about 50% mortality of N. europaea cells as measured by rates of O2 uptake. Electroporation at 1,200 V, 25 μF, and infinite resistance in a 1-mm-gap electroporation cuvette provided the desired survival ratio. These conditions were used for all transformations. While a systematic program of optimized electroporation parameters was not attempted, it was found that reducing the resistance value or increasing the voltage was not as successful in transforming N. europaea.

N. europaea cells (0.5 liter) from an early-stationary-phase liquid culture (A600 ∼ 0.1) were harvested by centrifugation and washed three times with sterile H2O. The sediments were resuspended in 1.5 ml of H2O and kept on ice until use. Cell transformation was done by electroporation in an ElectroPorator (Invitrogen, San Diego, Calif.) in 1-mm-gap cuvettes (Invitrogen). Electroporation parameters tested were between 750 and 1,800 V at various settings (25 to 71 μF).
TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td><em>E. coli</em></td>
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<tr>
<td>DH5α</td>
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<tr>
<td>InvαF</td>
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<td><em>N. europaea</em></td>
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<td>ATCC 19178</td>
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<td>American Type Culture Collection</td>
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<td>HA1</td>
<td><em>N. europaea</em> strain containing the haoA1::kan mutation at the BamHI site</td>
<td>This study</td>
</tr>
<tr>
<td>HBI</td>
<td><em>N. europaea</em> strain containing the haoB1::kan mutation at the BamHI site</td>
<td>This study</td>
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<tr>
<td>HC1</td>
<td><em>N. europaea</em> strain containing the haoC1::kan mutation at the BamHI site</td>
<td>This study</td>
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<tr>
<td>Plasmids</td>
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<td>pNHC1</td>
<td>F60 fragment NHCl from haoA cloned into pCR1 vector</td>
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</tr>
<tr>
<td>pNHC2</td>
<td>Fragment NHCl from pNHC1 inserted into EcoRI site of pRL139</td>
<td>This study</td>
</tr>
<tr>
<td>pNHC3</td>
<td>pNHC2 with kan from pUC4 KSAC inserted into BamHI site</td>
<td>This study</td>
</tr>
<tr>
<td>pNHB1</td>
<td>Fragment NHBI from haoB cloned into pCR1 vector</td>
<td>This study</td>
</tr>
<tr>
<td>pNHB2</td>
<td>Fragment NHBI from pNHB1 inserted into EcoRI site of pRL139</td>
<td>This study</td>
</tr>
<tr>
<td>pLJ101</td>
<td>pNHB2 with kan from pUC4 KSAC inserted into BamHI site</td>
<td>This study</td>
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<td>pUC11 KSAC</td>
<td>Plasmid containing the kan cassette from Tn903</td>
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<td>pCR1</td>
<td>TA PCR cloning vector (Kan‘, Amp’, lacZΔM, φ1 ori, ColE1 ori)</td>
<td>Invitrogen</td>
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<td>pBluescript SKI+</td>
<td>Cloning vector (lacZ, ColE1 ori, Amp’, φ1 ori)</td>
<td>Stratagene</td>
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<tr>
<td>pRL139</td>
<td>pUC19 with a symmetrical multiple cloning site</td>
<td>3</td>
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and 500 ohms to infinite resistance). In a prechilled cuvette, 120 μl of cells was mixed with 1 μg of DNA (1 μg/μl) and pulsed. The cells were transferred immediately to 0.5 liter of fresh medium and allowed to grow for 24 h under nonselective conditions at 30 °C while shaking. Kanamycin was then added to a final concentration of 10 μg/ml. Cell growth was monitored by the accumulation of NO₂⁻. Transformed *N. europaea* cells from these liquid cultures were plated on solid nutrient medium as described above but containing 10 μg of kanamycin sulfate per ml.

RESULTS

Transformations by electroporation. We investigated the possibility of using electroporation and homologous recombination as a method for transforming *N. europaea*. The hao loci were chosen as target sites in the genome. The insertion of kan should prevent active HAO being produced from the affected locus. Plasmids containing fragments of hao with kan inserted into them were used in the electroporations of *N. europaea* and should allow kan to recombine into the genomic copies of hao. Attempts were made to specifically target insertions into haoA and the other hao copy for which DNA sequence was available (either haoB of haoC) by using a conserved BamHI site at the 5’ end of the hao coding region as the insertion site. This site is close to upstream flanking sequences which were the basis for discriminating between haoA and the other sequenced hao copy.

*N. europaea* cells were sensitive to kanamycin at concentrations above 1 μg/ml. When *N. europaea* cells were electroporated with pLJ101 or pNHC3, kanamycin-resistant growth was evident in 10 days. After 10 days, NO₂⁻ accumulation in the putative transformed cultures increased to rates comparable to those of wild-type cultures. No growth was detected in control cultures subjected to electroporation without DNA even after 30 days of incubation in the presence of kanamycin. Transformed suspension cultures grew in the presence of up to 200 μg of kanamycin per ml.

To confirm that the antibiotic resistance of the cultures was not due to maintenance of the plasmid vector, plasmid preparations were performed on kanamycin-resistant cultures obtained by transformation with pLJ101 and pNHC3. These preparations did not yield any detectable plasmid. Additionally, the Southern hybridization patterns with probes for hao or kan, using genomic DNA digested with KpnI or EcoRI, did not correspond with the predicted patterns for the presence of a plasmid vector in the cells (not shown).

Because the initial transformed *N. europaea* suspension cultures potentially contained mixed populations of cells, there was a need to obtain clonal lines of transformed cells. Typically with other bacteria this can be done by plating liquid cultures on solid nutrient agar plates and picking individual colonies. *N. europaea* has been, until now, cultivated only on silica-based solid media (7). On silica media, cell growth is marginal, requiring 3 to 4 weeks for colonies to become visible. Plating *N. europaea* directly on agar plates containing Luria-Bertani medium (23) or standard *N. europaea* medium (4) at best resulted in the formation of microcolonies too small to practically isolate, even after 30-day incubations. We designed an improved method for growing clones of *N. europaea* on solid medium plates. Using plates with the described *N. europaea* medium, cells were spread on a square of a sterile Nytran membrane laid on the agar surface. In this way, large colonies grew in 7 to 14 days, allowing the isolation of single clones. The numbers of colonies on the plates were generally low. Cells growing on
plates remained viable for more than a month when the membrane was transferred to fresh plates weekly.

Identification of transformants by Southern hybridization and by PCR. To corroborate that the kanamycin resistance was due to the insertion of the kan gene at specific hao loci, the cultures were analyzed by Southern hybridization and PCR. Cultures of newly transformed batch cultures and clonal cell lines were prepared for genomic DNA isolation. The DNA was digested with restriction enzymes, fractionated by gel electrophoresis, blotted onto a Nytran membrane, and probed with pPH (hao) and pPrN (kan). In all cases, DNA from cells transformed with constructs targeted to hao showed that one of the hao DNA fragments had altered mobility compared with DNA from wild-type cells (Fig. 2A, lanes 1 to 3 compared with lane 4). Plasmids pNHC3 and pLJ101 were intended to recombine into haoA and the second sequenced copy of hao (either haoB or haoC), respectively. However, transformation with pLJ101 produced mutant cells with an insertion into either haoB or haoC (Fig. 2A, lanes 2 and 3). The DNA fragments hybridizing to the hao probe (pPH) had increased in size by about 1.2 kb, the size of the kan cassette. The DNA from the newly transformed batch cultures often showed more than one displaced hao DNA fragment, indicating that the batch cultures contained either double insertions or mixed populations of singly transformed cells. However, in clonal cell lines derived from the initial batch cultures, only single insertions into hao were found (Fig. 2A, lanes 1 to 3). When DNA from transformed cultures was probed with pPrN (kan), only DNA fragments comigrating with the displaced hao DNA fragments were highlighted (Fig. 2B, lanes 1 to 3). DNA from wild-type cells did not hybridize to the probe for kan (Fig. 2B, lane 4). When DNA from transformed cells was digested with SmalI, which cuts kan in two, the corresponding hao DNA fragment was also cut into two fragments of the expected size which were highlighted by pPH (data not shown). Therefore, three N. europaea mutant strains in which one of each of the three hao DNA fragments was displaced singly (Fig. 2A, lanes 1 to 3) as a result of an insertion into the BamHI site were isolated. These mutant strains were named H1A, H1B, and H1C.

As an alternative to Southern hybridizations, PCR DNA amplification was used to confirm the presence of the kan gene in the hao loci. The amplifications were performed between primers located inside the kan cassette (NPT1 and NPT2) and a primer located in hao (PH8) (Fig. 1). The primer PH8 is located outside the hao fragments used in the original transforming plasmid constructs. An amplified product will result only if the primers for the kan and hao genes are in proximity and in the proper orientation. DNA fragments of the expected sizes were amplified in these reactions, demonstrating that kan is present in hao and oriented in the downstream direction in mutants H1A, H1B, and H1C (Fig. 3, lanes 2 to 4). No amplification products of the proper size were detected in the wild-type strain (Fig. 3, lane 1). Likewise, PCR with the primers PH8 and NPT2 failed to amplify any product of the proper size (not shown). The hybridization and amplification results confirm that the site of insertion of kan in the genome was in the targeted regions of hao.

Cell growth. To assess if the mutations in the hao loci resulted in any observable growth phenotype, liquid cultures of the mutant and wild-type strains were compared. The specific activities for AMO and HAO were determined (10). Cell growth was monitored by NO−_2 production and cell density monitored at 600 nm. Wild-type N. europaea cells grew with a doubling time of about 8 h. The cultures were compared both during exponential growth and during stationary phase. In the stationary-phase experiment, wild-type and mutant stock cultures were maintained in stationary phase, the latter in the presence of kanamycin, for up to 2 weeks. From these cultures, at 2-day intervals starting at 7 days, aliquots from these cultures were inoculated into fresh medium, with kanamycin added as necessary, and the lag period prior to the onset of active growth rates was monitored. A difference in growth rate might indicate a reduced ability of the mutant to produce HAO. In these experiments, cultures of the mutant and wild-type strains did not show significant differences with respect to growth rates or specific activities of HAO and AMO, regardless of the hao locus affected (data not shown).

The stability of the kan insertion in the mutant strains in the absence of selective pressure was tested. A wild-type and two kanamycin-resistant cultures were grown in medium lacking kanamycin. Cells from these cultures were transferred to fresh media (with and without 10 μg of kanamycin per ml) weekly for about 7 weeks. The mutant strains transferred into kanamycin grew at the same rate as mutants strains without kanamycin or the wild-type strain without kanamycin. The growth of the mutant strains did not show any discernible lag compared with growth rates of the wild-type strain. The growth of the wild-type strain was consistently inhibited by the presence of kanamycin (data not shown). These results attest to the stability of the kan insertions.

DISCUSSION

We have shown that it is possible to transform cultures of N. europaea. By means of electroporation and recombination, we produced and isolated pure cultures of N. europaea with mutations in hao. The cultures contained specific insertions of the kan reporter gene in each one of the three putative copies of the genes coding for HAO.

By making an insertional mutation into a specific hao locus, one could potentially determine the cell’s requirement for that particular copy. The targeting of the kan insertion into haoA and the second sequenced copy of hao was based on the observation that the DNA sequences upstream of these two loci were different. By choosing an insertion site that was flanked on one side by DNA specific to each of the two sequenced copies of hao, insertion of the kan cassette into that particular copy should be favored. This strategy was successful when pNHC3 was used to insert kan into haoA. The hao construct pLJ101, however, resulted in kan insertions into either haoB or haoC. This may be indicative of similarities in the DNA sequences upstream of these genes. Alternatively, recombination may have occurred in the region immediately upstream of the BamHI site which is still within the coding region of hao and
presumably common to all three copies of hao. These results show that recombination can be used effectively to mutagenize genetic loci in N. europaea.

The three hao mutants were used to explore the function of the three copies of hao in N. europaea. The reason for having three copies of hao in N. europaea or whether all three copies are expressed is as yet unknown. Because there are differences in the region upstream of the ribosome-binding site of haoA and the second sequenced copy of hao, it is possible that they differ with respect to transcriptional regulation. However, in experiments comparing the hao mutant strains and wild-type N. europaea cells, the mutation of any one of the three copies does not noticeably affect the growth characteristics of the cells under the experimental conditions described. Apparently, when one copy of hao is mutagenized, the remaining intact copies of hao are capable of producing sufficient active HAO enzyme. The results suggest that no single copy of hao is essential to the cell. Thus, the question of why N. europaea has three copies of hao remains unresolved.

The insertion of the kan cassette into the hao gene was intended to preclude the synthesis of an active enzyme from that locus. The insertion disrupts the translational reading frame of the target hao copy. Furthermore, an examination of the DNA sequence of the kan cassette and of hao does not reveal any viable alternative translational start sites. Therefore, it is unlikely that the mutagenized copies of hao could produce an active HAO. However, since the orientation of kan in the BamHI insertions is in the same direction as hao itself, transcriptional readthrough from kan into the downstream portion hao could possibly occur.

These experiments demonstrate the transformation of N. europaea with exogenous DNA by electroporation, homologous recombination, and the isolation of pure mutant strains on solid medium. These techniques open up a number of experimental possibilities with respect to N. europaea, including mutagenesis and expression studies which have proved so powerful in other bacterial systems.

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