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NOTES

Multiple Copies of Genes Coding for Electron Transport Proteins in the Bacterium *Nitrosomonas europaea*

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The genome of *Nitrosomonas europaea* contains at least three copies each of the genes coding for hydroxylamine oxidoreductase (HAO) and cytochrome c_{554}. A copy of an HAO gene is always located within 2.7 kb of a copy of a cytochrome c_{554} gene. Cytochrome P-460, a protein that shares very unusual spectral features with HAO, was found to be encoded by a gene separate from the HAO genes.

Multiple copies of protein-encoding genes in prokaryotes are unusual. Among the relatively few examples of such genes are the genes coding for the D1 and D2 polypeptides of the photosystem II core complex in cyanobacteria (8, 10, 11, 20, 26), elongation factor Tu (1, 16, 27) and ornithine transcarbamylase (5, 9, 25) in *Escherichia coli*, and phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase in *Alcaligenes eutrophus* (6, 17) and *Rhodobacter sphaeroides* 2.4.1 (12, 23, 24).

In this paper we report on the use of Southern blotting to probe the organization of the genes coding for hydroxylamine oxidoreductase (HAO), cytochrome c_{554}, and cytochrome P-460 in the bacterium *Nitrosomonas europaea*. We found that HAO and cytochrome c_{554} are encoded by multiple genes.

N. europaea is a slowly growing, obligately chemolithotrophic organism that acquires energy by oxidizing ammonia to nitrite. Ammonia is first oxidized to hydroxylamine by the integral membrane enzyme ammonia monoxygenase (13):

$$NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O$$

Next, hydroxylamine is oxidized to nitrite in a dehydrogenase reaction (2) in the periplasm, which is catalyzed by the multiheme enzyme HAO (15) in concert with the tetraheme electron acceptor cytochrome c_{554} (3, 4):

$$NH_2OH + H_2O \rightarrow HNO_2 + 4e^- + 4H^+$$

Another protein whose gene was probed in this study is cytochrome P-460 (7, 19). Cytochrome P-460 contains an unusual chromophore with an absorption maximum in the reduced state at 460 nm. Since a similar chromophore is present in HAO at the protein's active site, it has been speculated that cytochrome P-460, which has a molecular weight of 18,000, is a proteolytic fragment of the 63,000-molecular-weight HAO polypeptide. Immunological evidence, however, suggests that cytochrome P-460 is a distinct protein (19). In this study we resolved this question by determining that cytochrome P-460 is encoded by a gene separate from the genes coding for HAO.

N. europaea was grown and harvested and DNA was prepared as described by McTavish et al. (18). HAO (4), cytochrome c_{554} (3), and cytochrome P-460 (19) were purified as described previously. Each of the proteins was further purified by reverse-phase high-performance liquid chromatography (HPLC) on a C4 or C8 column with a trifluoroacetic acid-acetonitrile-water solvent system before the protein was sequenced. Agarose gel electrophoresis, Southern blotting, radioactive end labelling of oligonucleotides, and hybridization of oligonucleotide probes to Southern blots were performed by standard techniques (22). After hybridization, Southern blots were washed in high-stringency buffer (15 mM NaCl, 1 mM sodium phosphate [pH 7.4], 0.2% sodium dodecyl sulfate) at different temperatures to remove nonspecifically bound probe.

HAO and cytochrome c_{554} were each digested with trypsin (0.05 mg/ml) at approximately 10 μM heme in 100 mM Tris acetate (pH 7.6). The resulting peptides were separated by reverse-phase HPLC on a C8 column with a trifluoroacetic acid-acetonitrile-water solvent system. A heme-containing peptide from HAO with the sequence GGNAPTCAACHMEYEGEYTHNIT was isolated. A degenerate oligonucleotide corresponding to the underlined portion of this sequence was synthesized and used to probe a Southern blot of *N. europaea* genomic DNA digested with various restriction enzymes (Fig. 1). This oligonucleotide hybridized to three bands in the *Sma*I digest, as was previously found with *N. europaea* DNA digested with *Xho*I, *Sal*I, and *Bcl*I (14). This indicates there are at least three copies of the gene coding for HAO. The HAO oligonucleotide hybridized to only one band in the *Bam*HI and *Eco*RI digests and to two bands in the *Kpn*I digest. This indicates that the flanking restriction sites are conserved in all three copies of the gene in the cases of *Bam*HI and *Eco*RI and in two copies in the case of *Kpn*I. A second oligonucleotide, made to correspond to the amino-terminal sequence of the HAO polypeptide, hybridized to the same three bands on Southern blots of DNA digested with *Sma*I, *Xho*I, *Sal*I, *Bcl*I, and *Pst*I (data not shown), confirming that the results obtained with the first oligonucle-

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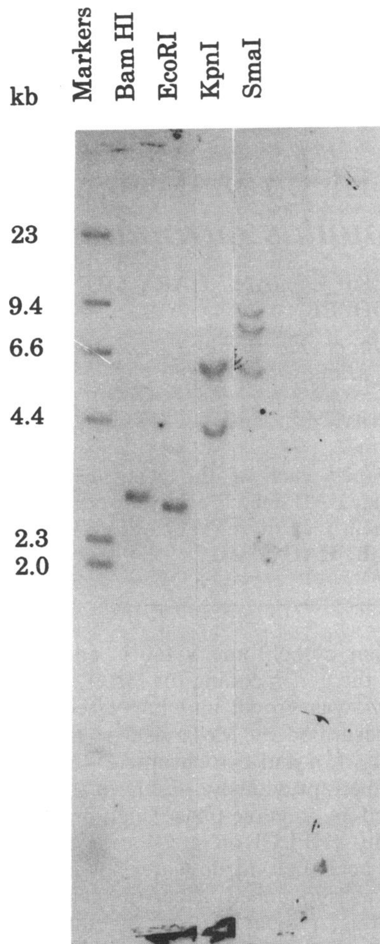

FIG. 1. Autoradiograph of a Southern blot of *N. europaea* DNA cut with four different restriction enzymes, hybridized with radiolabelled HAO oligonucleotide [5'-ATG-GA(A,G)-TA(T,C)-GA(A,G)-GGI-GA(A,G)-TA(T,C)-ACI-CA(T,C)-AA-3'], and washed in high-stringency buffer at 42°C for 30 min.

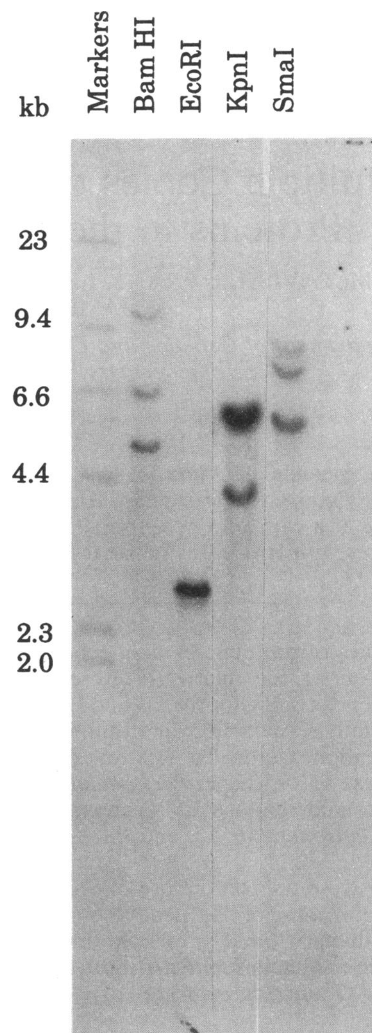

FIG. 2. Autoradiograph of the same Southern blot filter used in the experiment shown in Fig. 1, hybridized with radiolabelled cytochrome c_{554} oligonucleotide [5'-GA(A,G)-TGG-GA(A,G)-(T,C)TI-GTI-(C,T)TI-CA(T,C)-GTI-TGG-GCI-AA-3'], and washed in high-stringency buffer at 32°C for 30 min.

otide were not due to fortuitous hybridization of the oligonucleotide to two additional, unrelated, segments of the genome.

A trypsin-generated heme-containing peptide was also isolated from cytochrome c_{554}, and the amino acid sequence, VLSEGEWELVLHVWAK, was determined. An oligonucleotide corresponding to the underlined portion of the sequence was used to probe the same Southern blot filter used in the experiment shown in Fig. 1 (Fig. 2). Like the HAO oligonucleotide, this oligonucleotide hybridized to multiple bands (three bands in the *Bam*HI and *Sma*I lanes, two bands in the *Kpn*I lane, and one band in the *Eco*RI lane). Furthermore, in the *Sma*I, *Kpn*I, and *Eco*RI lanes it appeared to hybridize to the same bands as the HAO oligonucleotide. The HAO and cytochrome c_{554} oligonucleotides also appeared to hybridize to the same three bands on Southern blots of DNA digested with *Xho*I, *Sal*I, or *Pst*I (data not shown). These oligonucleotides hybridized to different bands when DNA was digested with *Bam*HI (Fig. 1 and 2) or *Bcl*I (data not shown). These results indicate that the genes encoding HAO and cytochrome c_{554} are near each other in all three copies of the genes. They must be within at most 2.7 kb of each other in all three copies since both oligonucleotides hybridized to just one 2.7-kb *Eco*RI band (Fig. 1 and 2).

Cytochrome P-460 from *N. europaea* was also isolated, and its N-terminal sequence was determined. The sequence, AGVAEFNDKGELLLPKNYREWV, differed from the sequence described in a previous report (21) only in having Lys instead of Ile at position 9. A degenerate oligonucleotide corresponding to the underlined portion of the amino acid sequence was synthesized and used to probe Southern blots of *N. europaea* DNA. With DNA digested by any of the restriction enzymes tested (*Bam*HI, *Eco*RI, *Kpn*I, *Sma*I, *Xho*I, *Sal*I, *Bcl*I, *Pst*I, and *Hin*dIII) the cytochrome P-460 probe was found to hybridize to only one band, and that band was different from any of the bands identified by the HAO or cytochrome c_{554} oligonucleotides (data not shown). Thus, cytochrome P-460 is a separate gene product from HAO and is not a proteolytic fragment of HAO or a truncated product of the HAO gene.

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