Volume 261, number 2, 431–435 FEBS 08185 February 1990

Are there isoenzymes of cytochrome *c* oxidase in *Paracoccus denitrificans*?

Mirja Raitio, Johanna M. Pispa, Tuula Metso and Matti Saraste*

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10, 00170 Helsinki 17, Finland

Received 27 December 1989

We have used a gene replacement strategy to delete the previously isolated gene [(1987) EMBO J. 6, 2825–2833] for the cytochrome *c* oxidase subunit I from *Paracoccus denitrificans*. The resulting mutant was still able to synthesize active cytochrome *c* oxidase. This led us to look for another locus which could completely suppress the mutation. In this study we report the isolation of a second gene encoding subunit I. An open reading frame coding for cytochrome *c* 550 was found upstream from this gene. We suggest that there are isoenzymes of cytochrome *c* oxidase (cytochrome aa_3) in this bacterium.

Cytochrome *c* oxidase; Subunit I; Cytochrome *c* 550; Gene duplication; (*Paracoccus*)

1. INTRODUCTION

Aerobically grown *Paracoccus denitrificans* expresses a mitochondrial-type respiratory chain [1,2]. The terminal catalyst is a cytochrome aa_3 (cyt.aa_3) which transfers electrons from cytochrome *c* to oxygen and couples this to proton translocation across the membrane. The *P. denitrificans* cyt. aa_3 has at least three subunits (COI, COII and COIII) which all are homologous to the mitochondrial-coded polypeptides in the eukaryotes [3,4]. Four catalytically active redox centres, two haems and two coppers (Cu_A and Cu_B), are bound to this enzyme. Three of them, the haems *a* and a_3 and Cu_B, are probably located in COI [5,6] which appears to be the functionally most important subunit. COII is believed to bind Cu_A [5]. COIII was recently shown to have a role at least in the assembly of native cyt. aa_3 [7].

The bacterial genes coding for cytochrome *c* oxidase have been isolated and sequenced from *P. denitrificans* [3,8], the thermophilic bacillus PS3 [9] and *Bacillus subtilis* (see [10]). In *P. denitrificans* the gene coding for COI (we shall call it *cta* DI following the nomenclature of [11]) was found to reside in a different genomic region from the genes coding for COII and COIII which are clustered together in the same genomic locus with three open reading frames [3].

We have used a gene replacement strategy to remove the previously cloned COI gene [3]. The unchanged phenotype of the mutant led us to look for a second locus coding for COI. This paper reports the nucleotide sequence of *cta* DII that codes for another variant of the COI (COIβ). A gene coding for cytochrome *c* 550 (*cyc* A) is located upstream from the *cta* DII-gene. The *cyc* A-gene has also been isolated and sequenced independently by others at the same time [12].

2. MATERIALS AND METHODS

2.1. *Bacteria and growth conditions*

P. denitrificans strain 1222 [13] was used in deletion mutagenesis. Bacteria were routinely grown in Luria Broth. The final concentrations of antibiotics (Sigma) when used were ampicillin 50 μg/ml; kanamycin sulfate 50 μg/ml; streptomycin sulfate 25 μg/ml and rifampicin (in selection against *E. coli* 50 μg/ml), otherwise 20 μg/ml.

P. denitrificans was grown in 5 l batch cultures in a minimal medium [14] containing succinate (50 mM) for the isolation of cyt. aa_3-containing membranes. Spheroplasts and isolated membranes were prepared as described in [15] and suspended in 200 mM Hepes, pH 7.2, 2 mM EDTA. Membrane solubilizations were made with 10% Triton X-100 (Boehringer Mannheim) [7].

2.2. *DNA manipulations and sequencing*

The cloning procedures were performed by standard methods [16]. Genomic DNA of *P. denitrificans* was isolated as described in [17]. A *P. denitrificans*-MR3 genomic library was prepared with the EMBL3 λ-vector [18]. The 2 kb *SalI-XhoI* fragment isolated from the *cta* DI gene (solid bar in fig.1) was nick-translated and used as a hybridization probe in the library screening. Plasmid DNA was introduced into *P. denitrificans* via conjugation [7]; the *E. coli* strain S17.1 was used as a donor. The deletion mutagenesis was made using

Correspondence address: M. Raitio, Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10, 00170 Helsinki 17, Finland

* *Present address:* EMBL, Meyerhofstrasse 1, Postfach 10.2209, D-6900 Heidelberg, FRG

The nucleotide sequence(s) presented here has (have) been submitted to the EMBL/GenBank database under the accession number no.Y07533

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

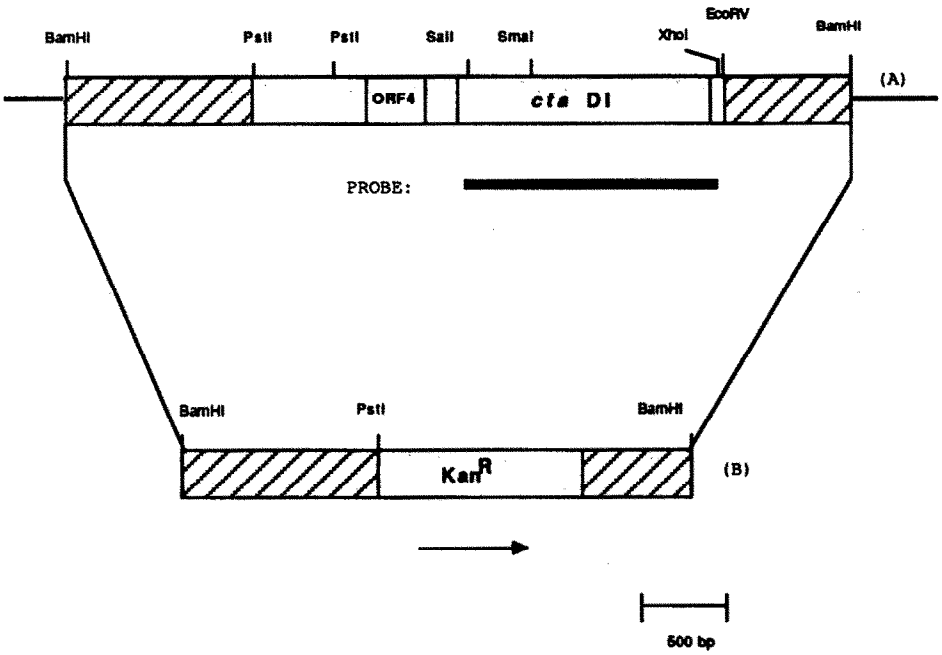

Fig.1. Restriction maps of the wild type (A) *cta* DI locus and the construction made for deletion mutagenesis (B). Dashed boxes are the flanking regions for gene replacement.

the pSUP202 plasmid [19] as a vector. A streptomycin resistance gene (from Tn1831 [13]) was first inserted into an *Eco*RI site of this plasmid. The kanamycin resistance (Kan^B) gene was obtained from pUC-4-KIXX (Pharmacia).

Overlapping restriction fragments from a *cta* DII-containing λ-clone were subcloned into M13mp18 and mp19. Both strands were sequenced by the dideoxy chain termination method [20] using the Sequenase kit (USB). Specific primers were synthesized with an Applied Biosystems Synthesizer 381A. Southern blots were developed with the Nonradioactive DNA labelling and Detection Kit (Boehringer Mannheim).

2.3. *Spectrophotometry and enzyme activity*

The cyt. aa_3 content of the membranes was determined from the difference spectra at about 605 nm of dithionite-reduced minus air-oxidized samples using the extinction coefficient 23.4 $mM^{-1}cm^{-1}$ [21]. The cytochrome oxidase activity was measured with a Clark-type oxygen electrode. NADH dehydrogenase was inhibited in all measurements by supplementing the buffer (50 mM Hepes, pH 7.2, 50 mM KCl) with 5 μM rotenone. Cyt. aa_3 activity was measured using ascorbate (15 mM) as reductant; 10 μM cyt *c* (horse heart type VI, Sigma) and 300 μM TMPD were added to mediate the electron transfer. Ubiquinol oxidation was determined with 41 μM ubiquinol (UQ-1, obtained from Hoffman-La Roche) which was reduced by 5 mM dithiothreitol. The activity due to the ubiquinol oxidase cytochrome b_0 was determined by inhibiting the cytochrome *c* reductase (cyt. bc_1-complex) with 6 μM myxothiazol. All reactions were stopped by 0.1 mM potassium cyanide, and the respiration rates were corrected for the cyanide insensitive oxygen consumption.

3. RESULTS AND DISCUSSION

Our aim is to study the functional role of the conserved amino acid residues in COI. Thus we wanted to delete the *cta* DI gene from the chromosome. The mutant was constructed by a site-specific deletion mutagenesis technique that is based on homologous recombination. The construction was made as follows. The 3.3 kb *Pst*I-*Eco*RV fragment containing the entire *cta* DI-gene and the small open reading frame (ORF4) upstream from it was replaced with the *Sma*I-fragment of the Kan^R marker. The flanking areas targeted to recombine with chromosomal DNA extended on both sides to the *Bam*HI-sites (see fig.1). This casette was inserted as a *Bam*HI-fragment into the pSUP 202 plasmid. A streptomycin-resistance gene was inserted into this plasmid (see section 2) in order to select against the integration of the entire plasmid that would be the result of single cross-overs. The constructed plasmid, pMKR-2, was mobilized from *E. coli* S17.1 to *P. denitrificans* 1222 by biparental matings as described in [7]. The donor *E. coli* cells were eliminated with rifampicin, and kanamycin was used in selection for the *P. denitrificans* which had integrated the vector. Integration occurred with a frequency of 1.4×10^{-6}. 10% of Kan^R-transconjugants had Sm-sensitive phenotype. Genomic Southern blots of the latter showed that in 12 transconjugants all but one were true double-cross-over recombinants. One of these, MR-3, was used for further studies. Fig.1 shows the map of the *cta* DI-region before and after the mutagenesis.

To our surprise the membranes isolated from the succinate-grown MR-3 contained spectroscopically normal cyt. aa_3 in the same amounts as the parent strain (fig.2A). In fact, the 'mutant enzyme' had the same activity as the wild-type oxidase (see table 1).

Southern analyses of the genomic DNA verified that the deletion of *cta* DI gene had succeeded, but they also showed that another genomic locus in *P. denitrificans* hybridizes strongly to the probe prepared from *cta* DI gene (figs 1 and 2B). We constructed a genomic library

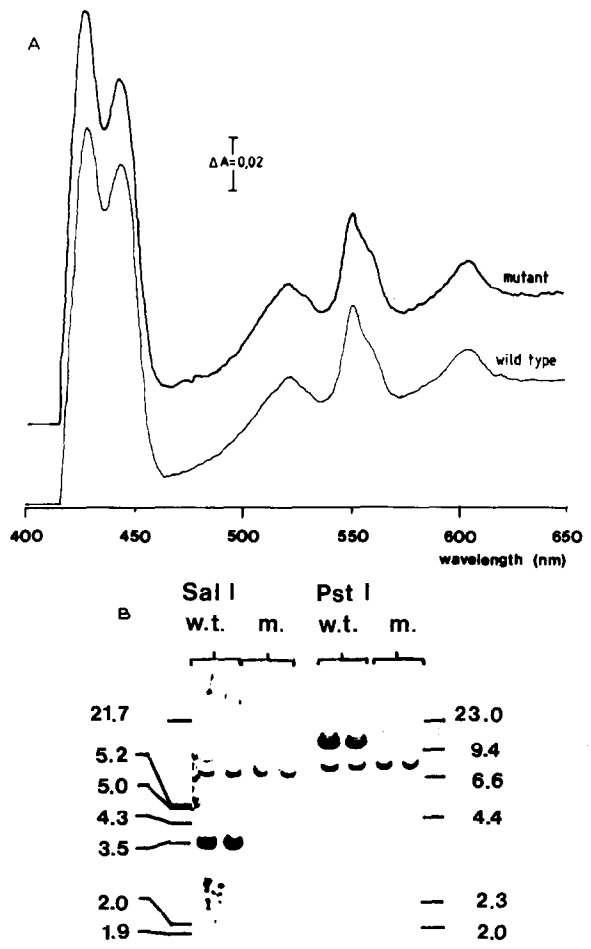

Fig.2. (A) Dithionite-reduced minus air-oxidized difference spectrum of solubilized membranes. (B) Southern analysis of chromosomal DNA from the mutant MR-3 (m) and wild-type (w.t.) digested with *SalI* and *PstI*. Molecular weight markers on the left and right (kb).

of MR-3 and isolated the latter chromosomal segment. The sequence analysis of this locus (figs 3 and 4) revealed another copy of the COI gene. Only single copies of the COII and COIII genes were detected by Southern analysis (data not shown).

The two genes coding for COIα (*cta* DI) and COIβ (*cta* DII) are strongly homologous (fig.5). The deduced amino acid sequences of COIα and COIβ are 89% identical, all amino acid substitutions are conservative and fall outside of the pattern of strictly invariant residues in COI [10]. The greatest difference between these polypeptides is at the N-terminus, where the first 12 residues in COIα are replaced by 15 different residues in COIβ (fig.4).

Table 1

Activity measurements of succinate-grown *P. denitrificans* membranes

	Asc. + TMPD e^-/s	UQ + DTT inhibition by myxothiazol (%)
Strain 1222(wt)	315	71
Strain MR-3	275	70

Values are means of three parallel measurements (see section 2); e^- means electron

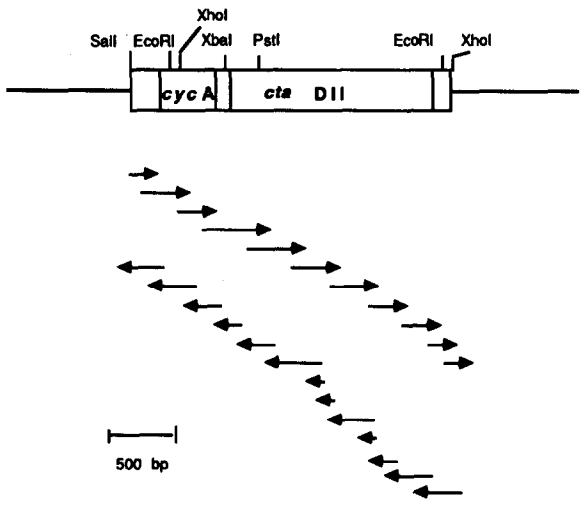

Fig.3. The restriction map and the sequencing strategy (below) of the *Paracoccus* chromosomal region containing the *cyc* A and *cta* DII genes. The direction and the length of sequencing reactions are indicated with arrows.

An open reading frame is present upstream from the *cta* DII gene. The amino acid sequence predicted from that is almost in perfect agreement with the known protein sequence of *P. denitrificans* cyt. *c* 550 [23,24]. The additional 20 residues between the probable initiator methionine and the known N-terminus of the mature cyt. *c* 550 protein are presumably the signal sequence of this periplasmic protein; the putative cleavage site is indicated with an arrow in fig.4. The extreme C-terminus differs from the previously sequenced cyt. *c* 550.

A strong hairpin (horizontal arrows starting from nucleotide 731 in fig.4) followed by a T-rich segment is found between *cyc* A and *cta* DII genes. It may be a stop signal for transcription. Thus it is likely that these two genes are not (always) cotranscribed. This is also supported by the fact that the synthesis of cyt. *c* 550 is regarded to be constitutive [25] whereas cyt. aa_3 is an inducible enzyme.

It is known that the *E. coli* cyt. b_0-complex is structurally related to cyt. aa_3. 37% of the amino acid residues in its largest subunit are identical to COIα [26]. Cyt. b_0 is a quinol oxidase, and the reduction of oxygen which it catalyses does not utilize the bc_1-complex or the cytochrome *c*-pool. It has recently been shown that cyt. b_0 from both *E. coli* and *P. denitrificans* are able to pump protons [27]. This functional similarity implies that the *P. denitrificans* cyt. b_0 may also be structurally related to cyt. aa_3. The electron transfer from ubiquinol to oxygen in the mutant membranes was in-

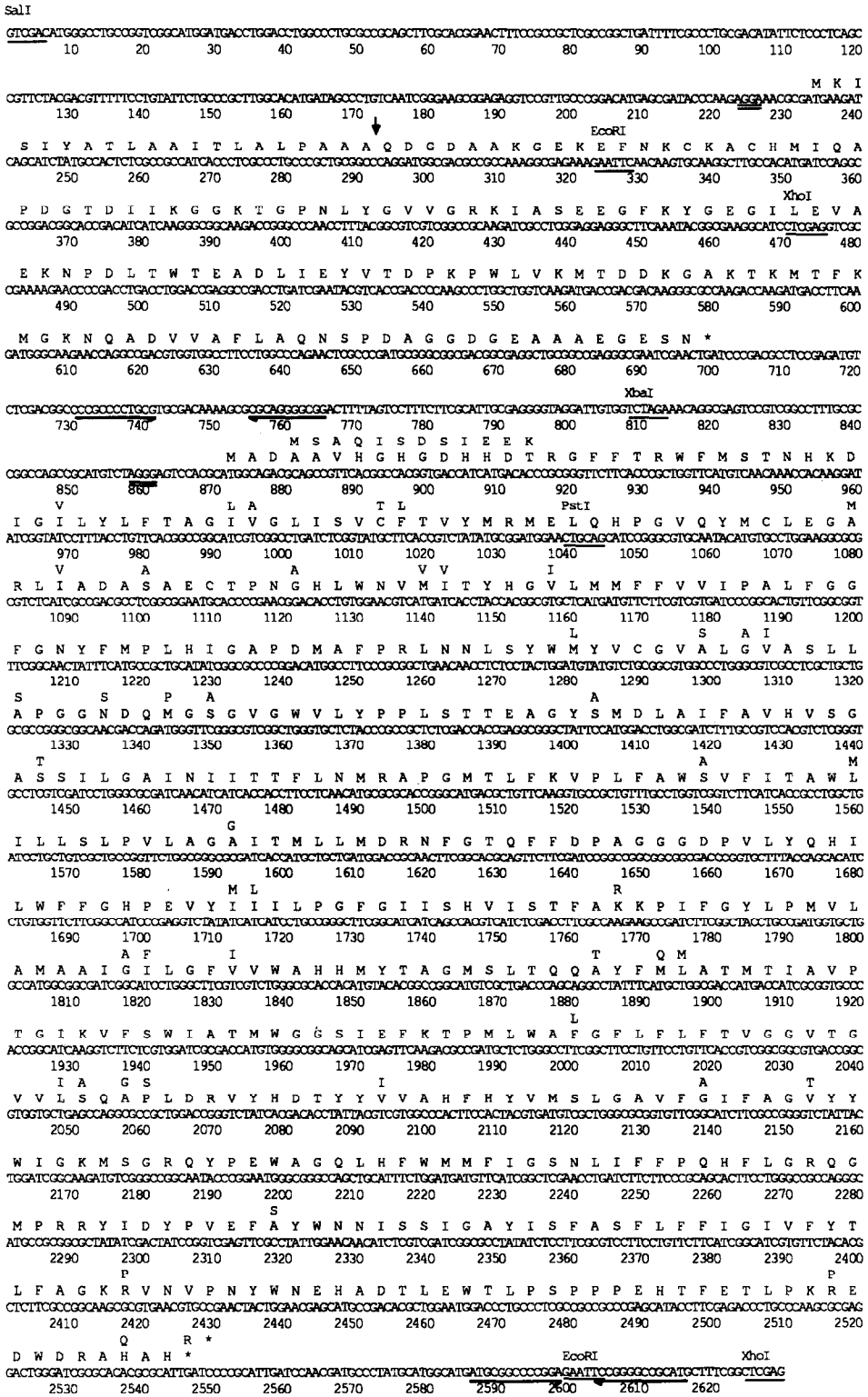

Fig.4. The nucleotide sequence and the deduced amino acid sequences of the *P. denitrificans cyc* A and *cta* DII genes. The putative ribosome-binding sites (double-underlined), termination loops (horizontal arrows) and some of the restriction sites (underlined) are indicated. A small vertical arrow shows the presumed cleavage site for the signal sequence of cyt *c* 550. Only the substituted amino acids in COIα [3] are written above the COIβ protein sequence.

hibited by cyanide but only partially blocked by myxothiazol, a specific inhibitor of the bc_1-complex. The myxothiazol-insensitive quinol oxidase activity was similar in the mutant and wild type (table 1). Therefore neither *cta* DI nor *cta* DII codes for cytochrome b_0 subunit.

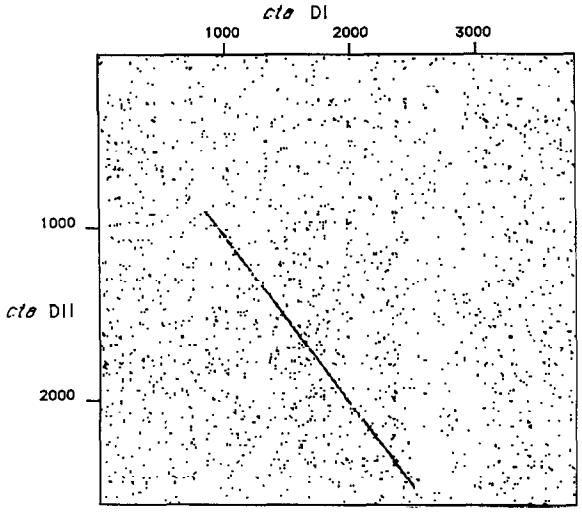

Fig.5. Comparison of the nucleotide sequences of the two *cta* D genes. The *cta* DI region begins at the *Pst*I site at the 5′ end of ORF4 (fig.1). The *cta* DII region is the sequence shown in fig.4. The numbers refer to the nucleotide residues. The computer program DIAGON [22] was used, and the window length was 11. The dots represent matches where 9 or more nucleotides out of 11 are identical. The diagonal line represents the coding region of the *cta* DI and *cta* DII genes. Note that the flanking regions contain no continuous homology.

The extensive homology between COIα and COIβ suggests that both *cta* D genes are active in vivo. It also suggests that the catalytic properties of these two polypeptides are basically the same. There might be regulatory reasons for this gene duplication as has been proposed for some other prokaryotic multigene families [28–31]. At the moment we do not know the promoter structures in the *cta*-loci. The expression of the subunit I genes might be regulated differentially under different growth conditions: it is possible that *P. denitrificans* has isoenzymes of cytochrome *c* oxidase.

Acknowledgements: This work was financially supported by the Academy of Finland (SCR) and the Sigrid Juselius Foundation. We are grateful to Dr Nellie Harms for providing the restriction-deficient *P. denitrificans* strain 1222. We thank Mirja Leppäkoski, Armi Pirinen and Hilkka Vuorenmaa for excellent technical assistance; Tuomas Haltia, Marko Lauraeus, Joel Morgan, Anne Puustinen and Mårten Wikström for advice and critical reading of the manuscript.

REFERENCES

[1] John, P. and Whatley, F.R. (1970) Nature 254, 495–498.
[2] Albracht, S.P.J., Van Verseveld, H.W. and Kalkman, M.L. (1980) Biochim. Biophys. Acta 593, 173–186.
[3] Raitio, M., Jalli, T. and Saraste, M. (1987) EMBO J. 6, 2825–2833.
[4] Steffens, G.C.M., Buse, G., Oppliger, W. and Ludwig, B. (1983) Biochim. Biophys. Acta 116, 335–340.
[5] Holm, L., Saraste, M. and Wikström, M. (1987) EMBO J. 6, 2819–2823.
[6] Müller, M., Schläpfer, B. and Azzi, A. (1988) Biochemistry 27, 7546–7551.
[7] Haltia, T., Finel, M., Harms, N., Nakari, T., Raitio, M., Wikström, M. and Saraste, M. (1989) EMBO J. 8, 3571–3579.
[8] Steinrücke, P., Steffens, G.C.M., Panskus, G., Buse, G. and Ludwig, B. (1987) Eur. J. Biochem. 167, 431–439.
[9] Sone, N., Yokoi, F., Fu, T., Ohta, S., Metso, T., Raitio, M. and Saraste, M. (1988) J. Biochem. 103, 606–610.
[10] Saraste, M. (1990) Q. Rev. Biophys., in press.
[11] Mueller, J.P. and Taber, H.W. (1989) J. Bacteriol 171, 4967–4978.
[12] Van Spanning, R., Wansell, J.M., Harms, N., Oltmann, L.F. and Stouthamer, A.H. (1990) J. Bacteriol., in press.
[13] Harms, N. (1988) Genetic and Physiological Studies on Methanol Metabolism in *Paracoccus denitrificans*, PhD Thesis, Free University, Amsterdam.
[14] Harms, N., De Vries, G.E., Maurer, K., Veltkamp, E. and Stouthamer, A.H. (1986) J. Bacteriol. 164, 1064–1070.
[15] Berry, E.A. and Trumpower, B.L. (1985) J. Biol. Chem. 260, 2458–2467.
[16] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) in: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[17] Harms, N., De Vries, G.E., Maurer, K., Hoogendijk, J. and Stouthamer, A.H. (1987) J. Bacteriol. 169, 3969–3975.
[18] Frischauf, A.-M., Lehrach, H., Poutska, A. and Murrey, N.J. (1983) J. Mol. Biol. 170, 827–842.
[19] Simon, R., Priefer, U. and Pühler, A. (1983) in: Molecular Genetics of the Bacteria-Plant Interactions (Pühler, A. ed.) pp.98–106, Springer, Berlin.
[20] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[21] Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 196–200.
[22] Staden, R. (1982) Nucleic Acids Res. 10, 2951–2961.
[23] Timkovich, R. and Dickerson, R.E. (1976) J. Biol. Chem. 251, 2197–2206.
[24] Ambler, R.P., Meyer, T.E., Kamen, M.D., Scichman, S.A. and Sawyer, L. (1981) J. Mol. Biol. 147, 351–356.
[25] Bosma, G., Braster, M., Stouthamer, A.H. and Van Verseveld, H.W. (1987) Eur. J. Biochem. 165, 665–670.
[26] Saraste, M., Raitio, M., Jalli, T., Chepuri, V., Lemieux, L. and Gennis, R. (1988) Ann. NY Acad. Sci. 550, 314–324.
[27] Puustinen, A., Finel, M., Virkki, M. and Wikström, M. (1989) FEBS Lett. 249, 163–167.
[28] Hulett, F.M., Wang, P.Z., Sussman, M. and Lee, J.K. (1985) Proc. Natl. Acad. Sci. USA 82, 1035–1039.
[29] Golden, S.S., Brusslan, J. and Haselkorn, R. (1986) EMBO J. 5, 2789–2798.
[30] Downard, J.S., Kupfer, D. and Zusman, D.R. (1984) J. Mol. Biol. 175, 469–492.
[31] Golden, S.S., Cho, D.-S.C. and Nalty, M.S. (1989) J. Bacteriol. 171, 4707–4713.