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The cbb_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides*, a proton-pumping heme-copper oxidase

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Abstract

Rhodobacter sphaeroides expresses a bb_3-type quinol oxidase, and two cytochrome *c* oxidases: cytochrome aa_3 and cytochrome cbb_3. We report here the characterization of the genes encoding this latter oxidase. The *ccoNOQP* gene cluster of *R. sphaeroides* contains four open reading frames with high similarity to all *ccoNOQP*/*fixNOQP* gene clusters reported so far. CcoN has the six highly conserved histidines proposed to be involved in binding the low spin heme, and the binuclear center metals. *ccoO* and *ccoP* code for membrane bound mono- and diheme cytochromes *c*. *ccoQ* codes for a small hydrophobic protein of unknown function. Upstream from the cluster there is a conserved *Fnr*/*FixK*-like box which may regulate its expression. Analysis of a *R. sphaeroides* mutant in which the *ccoNOQP* gene cluster was inactivated confirms that this cluster encodes the cbb_3-type oxidase previously purified. Analysis of proton translocation in several strains shows that cytochrome cbb_3 is a proton pump. We also conclude that cytochromes cbb_3 and aa_3 are the only cytochrome *c* oxidases in the respiratory chain of *R. sphaeroides*.

Keywords: Cytochrome oxidase; Cytochrome cbb_3; Proton pumping; *ccoNOQP* operon; Respiratory system; (*Rhodobacter sphaeroides*)

1. Introduction

Rhodobacter sphaeroides is a Gram-negative facultative bacterium. It can grow aerobically, anaerobically in the dark, or photosynthetically. As in many bacteria, these growing abilities are mainly due to the expression of a branched respiratory system.

It has been shown previously that *R. sphaeroides* possesses at least two cytochrome *c* oxidases and one quinol oxidase [1–3]. The aa_3-type cytochrome *c* oxidase predominates when the cells are grown aerobically [4], while the cbb_3-type alternative cytochrome *c* oxidase is expressed mainly under microaerobic or photosynthetic conditions, or in strains which lack the aa_3-type oxidase [2]. On the other hand, the presence of a quinol oxidase was also previously confirmed with the generation of a bc_1-deficient mutant capable to grow in aerobic conditions [3].

The *R. sphaeroides* alternative cytochrome *c* oxidase was purified and characterized as a novel member of the heme-copper oxidase superfamily, and was identified as a cbb_3-type oxidase [1], also detected in

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several other bacteria [5–11]. This type of oxidase shows features quite different from the main family of heme-copper oxidases [12]. One of these characteristics is the presence of a B-type heme in the binuclear center. Analysis of bacterial [13] and mitochondrial [14,15] cytochrome *c* oxidase crystal structures shows that the hydroxyl group of the heme a_3 hydroxyethylfarnesyl side chain may be involved in a hydrogen-bond path, which in turn might be conducting protons to the binuclear center [13–15]. Moreover, a strict requirement for a hydroxyethylfarnesyl side chain of the high spin heme for activity has been pointed out for the *Paracoccus denitrificans* cytochrome ba_3 [16], and for *Escherichia coli* cytochrome bo_3 [17,18]; the corresponding bb_3-type enzymes in these organisms are inactive.

It has been shown that the cbb_3-type oxidases play important roles in low-oxygen growth conditions. In *Bradyrhizobium japonicum* the cbb_3-type oxidase is essential for nitrogen fixation [19]. In these bacteria this oxidase is expressed under microaerophilic growth conditions and shows high affinity for oxygen [8]. Additionally, cbb_3-type oxidases can also play important roles in the aerobic growth, as in *Rhodobacter capsulatus*, where cytochrome cbb_3 is the sole cytochrome *c* oxidase present [6].

In this study we have characterized the *R. sphaeroides* chromosomal region that encodes cytochrome cbb_3, and described the complete *ccoNOQP* gene cluster sequence. Based on sequence comparisons among all cbb_3 sequences known so far and the crystal structure for cytochrome aa_3 published recently, a two-dimensional model of the CcoN subunit is proposed. We also provide evidence that the cbb_3-type oxidase is a proton pump.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this work are summarized in Table 1. *E. coli* strains were grown at 37°C in L broth in the presence of the appropriate antibiotic concentrations according to the strain and plasmid hosted (ampicillin 100 μg/ml; kanamycin 50 μg/ml; tetracycline 20 μg/ml). *R. sphaeroides* strains were grown aerobically or photosynthetically in Siström's medium at 32°C as reported previously [3]. Antibiotics were added when required (streptomycin/spectinomycin 50 μg/ml; tetracycline 4 μg/ml; kanamycin 20 μg/ml).

2.2. Sequencing and DNA manipulation

DNA cloning techniques were performed according to standard protocols [20]. Southern hybridization was performed using a non-radioactive DNA labeling and detection system (DIG-System non-radioactive nucleic acid detection system, Boehringer Mannheim). DNA sequencing was carried out using an automated sequencer (Applied Biosystems) at the Biotechnology Center, University of Illinois at Urbana. Nucleotide sequences were analyzed with computer programs available at ExPASy Molecular Biology Server at the University of Geneva (http://expasy.hcuge.ch/www/expasy-top.html) or at the Virtual Genome Center at the University of Minnesota (http://alces.med.umn.edu/JGC.html). The nucleotide sequence reported here is available in the GenBank (accession No. U58092).

The *ccoNOQP* insertion mutant (MT101) was made by the introduction of a blunt-ended *Pst*I-cleaved kanamycin resistance cartridge into the unique *Sal*I site in pT7-1943 (Fig. 1; Table 1). The kanamycin cartridge was inserted in the 3′ to 5′ orientation with respect to the *ccoN* gene (pT71943Kan in Table 1; Fig. 1). The *Pst*I-*Eco*RI fragment from pT71943Kan was then cloned into the ampicillin- and chloramphenicol resistance loci of pSUP202. This construct was designated pSUP1943Kan. This vector was used to transform *E. coli* S17-1. The resulting strain was used as donor to mobilize the plasmid into *R. sphaeroides*.

The deletion mutant (ME127) was made by replacing part of the *ccoN* and *O* genes by a kanamycin resistant cartridge (Table 1). For this purpose we cloned a 1.7 kbp *Pst*I-*Sal*I fragment from pUI1957 to the pT7-T319α plasmid, generating pT7PS1.7. Another 1.7 kbp *Sal*I-*Eco*RI fragment from pUI1957 was subcloned into this vector, resulting in the plasmid designated as pT7PSE3.4. In this,

an *Eco*RI restriction site was introduced into the *Hin*dIII site by the linker EcoLINK (5′-AGC CAA GCT TGA ATT CAA GCT TGG CT-3′), generating now the plasmid pT7EPSE3.4. A *Sal*I cleaved kanamycin cartridge was ligated (with the 3′ to 5′ orientation with respect of the *ccoN* gene) into the *Sal*I site of pT7EPSE3.4, generating pT7EPSE-Kan (Table 1). Finally, the *Eco*RI-*Eco*RI fragment from this last construction was subcloned into the chloramphenicol resistance locus of pSUP202 to generate pSUP-EPSE-Kan. This was again used to transform *E. coli* S17-1 for using it as a donor to mobilize the plasmid into *R. sphaeroides*.

Conjugation between *E. coli* S17-1 and *R. sphaeroides* strains was carried out as described before [21].

Complementation of the cytochrome cbb_3 deficient mutants (MT101 or ME127) was made by introducing a plasmid (pRK415) containing a *Bam*HI-*Eco*RI 5.5 kbp insert which contains the whole *ccoNOQP* operon. The strains were called MT101C and ME127C.

2.3. Biochemical methods

2.3.1. Membrane preparation

Aerobically grown cells were washed and treated with lysozyme as described by Raitio and Wikström [22]. Osmotically shocked cells were treated with DNase I (Sigma) and membranes were recovered and washed by centrifugation.

2.3.2. Activity measurements

Oxygen uptake was measured polarographically as described by Raitio and Wikström [22], in 50 mM Tris-HCl pH 7.5, 50 mM KCl and 0.5 mM EDTA. Ascorbate-*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine (TMPD)-cytochrome *c* oxidase activity was assayed in the presence of sodium ascorbate (5 mM), TMPD (10 μM), horse heart cytochrome *c* (15 μM) and antimycin A, or myxothiazol (2.8 μg/ml). 1,4-Dithiothreitol (DTT; 4.4 mM) and ubiquinone-1 (Q_1; 0.2 mM) were used to assay quinol oxidase activity in the presence or absence of myxothiazol.

Proton translocation measurements were performed by the oxygen-pulse method using intact cells washed and resuspended in 200 mM KCl as reported previously [23–25].

3. Results

3.1. Cloning and sequencing of the ccoNOQP cluster from R. sphaeroides

From studies on the environmental regulation of the *R. sphaeroides* gene expression, Zeilstra-Ryalls and Kaplan found a cosmid (pUI8180) in their *R. sphaeroides* genomic library which contained sequences homologous to the *fixN* gene of *B. japonicum* [26,27]. pUI1957 (kindly provided by Zeilstra-Ryalls and Kaplan), a *Bam*HI subclone in pRK415-1 of pUI8180, was used here to sequence the complete *ccoNOQP* gene cluster (Fig. 1). This cluster maps at around 443 kbp on the physical map of the *R. sphaeroides* 2.4.1 chromosome I [26].

The sequence strategy is outlined in Fig. 1. Different DNA fragments from pUI1957 were cloned into pT7T319α and nucleotide sequences of both strands were obtained using standard M13/pUC forward and reverse primers, or internal primers (sequences shown in Fig. 2).

The nucleotide sequence of the region reported here comprises 4258 bp. 3395 bp correspond to the *ccoNOQP* predicted coding region. The sequences of the *ccoNOQP* cluster 5′ and 3′ flanking regions of 365 bp and 461 bp respectively are shown in Fig. 2. The entire sequence showed a high G+C content (63.4%) which is typical for the *R. sphaeroides* genome. The cluster comprises four open reading frames (ORFs) in close physical proximity: *ccoN*, *ccoO*, *ccoQ* and *ccoP*. One potential transcription termination site was found with a stem-loop structure, located 16 bp downstream from the *ccoP* gene

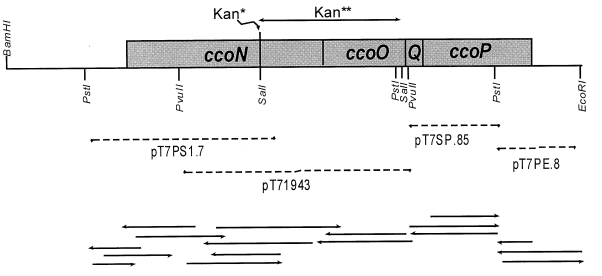

Fig. 1. Physical map of the *R. sphaeroides ccoNOQP* cluster and sequence strategy. Broken lines indicate the fragments subcloned for sequencing. Arrows indicate sequences obtained. The places where the kanamycin resistance gene was inserted (Kan*) or replaced for internal sequence (Kan**) are shown. See text for details.

Table 1
Strains and plasmids used in this work

Strain/plasmid	Relevant characteristics	Ref./source
Strain		
E. coli		
JM101	*SupE*, *thi*, Δ(*lac-proAB*), [F′, *traD*36, *proAB*, *lacI*^{q}*Z* ΔM15]	[42]
NM522	*hsd*Δ5Δ[*lac.pro*]F′*lacI*^{q} *lacZ*ΔM15Pro$^+$	[43]
S-17-1	Sm^r. Modified RP4 plasmid integrated into the genome of *E. coli* 294. *RecA*	[44]
R. sphaeroides		
Ga	2.4.1 derivative with a mutation in carotenoid biosynthesis	[45]
JS100	Ga derivative, Δ*ctaD*::*sm*/*sp*	[21]
MT101	JS100 derivative, *ccoN*::Kan	This work
ME127	JS100 derivative, Δ*ccoNO*::Kan	This work
Plasmid		
pUI1957	pRK-415 derivative containing an 8–9 kbp *Bam*HI *R. sphaeroides* DNA fragment including the complete *ccoNOQP* cluster and surrounding sequences	[26]
pUI1943	pBluescript II SK+/− derivative containing a 2.2 kbp *Pvu*II *R. sphaeroides* DNA fragment that includes the *ccoO* gene and a fragment of *ccoN*	[26]
pT7PS1.7	pT7T319 derivative containing a 1.7 kbp *Pst*I-*Sal*I *R. sphaeroides* DNA fragment that includes the 5′ region of *ccoN*	This work
pT7SP0.85	pT7T319 derivative containing a 0.85 kbp *Sal*I-*Pst*I *R. sphaeroides* DNA fragment that includes *ccoQ* and a fragment of *ccoP*	This work
pT7PE0.8	pT7T319 derivative containing a 0.8 kbp *Pst*I-*Eco*RI *R. sphaeroides* DNA fragment that includes the 3′ region of *ccoP*	This work
pRK-415	Tc^r, *oriT lacZ*	[46]
pSUP202	*mob*, Cm^r, Amp^r, Tc^r	[44]
pUC4K	pUC18 derivative Amp^r, Kn^r, source of kanamycin resistant gene	[47]
pBluescript II SK+/−	Amp^r, sequencing vector with T3 and T7 promoters	Stratagene (La Jolla, CA, USA)
pT7-1943	pT7T319 derivative containing the *Pst*I-*Pvu*II fragment derived from pUI1943	This work
pT7-1943Kan	pT7-1943 derivative where the kanamycin gene was introduced in the *Sal*I site of *ccoN* gene (see Fig. 1)	This work
pSUP1943Kan	pSUP202 derivative containing the *Pst*I-*Eco*RI fragment derived from pT7-1943Kan	This work
pT7PSE3.4	pT7PS1.7 derivative containing a 1.7 kbp *Sal*I-*Eco*RI fragment that includes the *ccoQ* and *P* genes from *R. sphaeroides*	This work
pT7EPSE3.4	pT7PSE3.4 derivative where an *Eco*RI site was introduced by an oligonucleotide	This work
pT7EPSE-Kan	pT7EPSE3.4 derivative where the kanamycin cartridge was introduced into the *Sal*I site	This work
pSUP-EPSE-Kan	pSUP202 derivative where the 4.6 kbp *Eco*RI-*Eco*RI fragment from pT7EPSE-Kan was subcloned	This work

(Fig. 2). The proposed start codons of all four genes are 5–8 bases downstream from putative Shine-Dalgarno sequences. A potential binding site for an *Fnr*/*FixK*-like protein was found located at positions −133 and −101 upstream from the start codon of *ccoN*. This sequence is almost identical to the 'anaerobox' found upstream from the *ccoNOQP* cluster from *P. denitrificans* [28], and that of *Azorhizobium*

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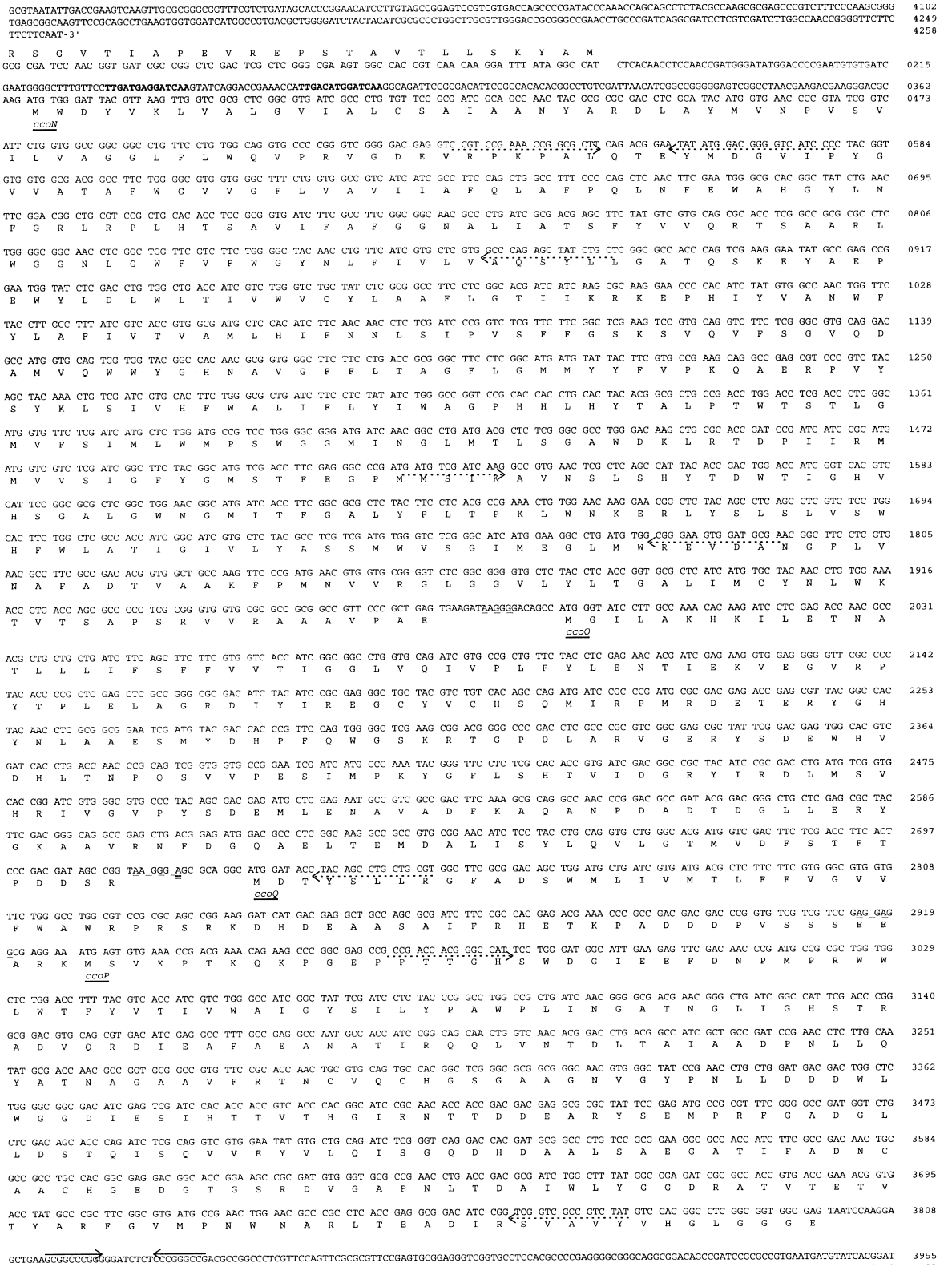
Fig. 2. The complete nucleotide sequence of the *ccoNOQP* cluster of *R. sphaeroides*. The deduced amino acid sequence from the four ORFs (corresponding to *ccoN*, *O*, *Q* and *P* genes) is shown, as well as the partial sequence of Rs-ORF277, which is transcribed in opposite direction to the *ccoNOQP* cluster. Bold letters indicate a potential binding site for an *Fnr*/*FixK*-like protein. The opposite arrows indicate a putative factor-independent transcription terminator at the end of *ccoP*. Possible Shine-Dalgarno ribosome-binding sites (SD) are shown upstream from each ORF by dotted underline. Internal sequencing primer sequences are indicated by broken arrows.



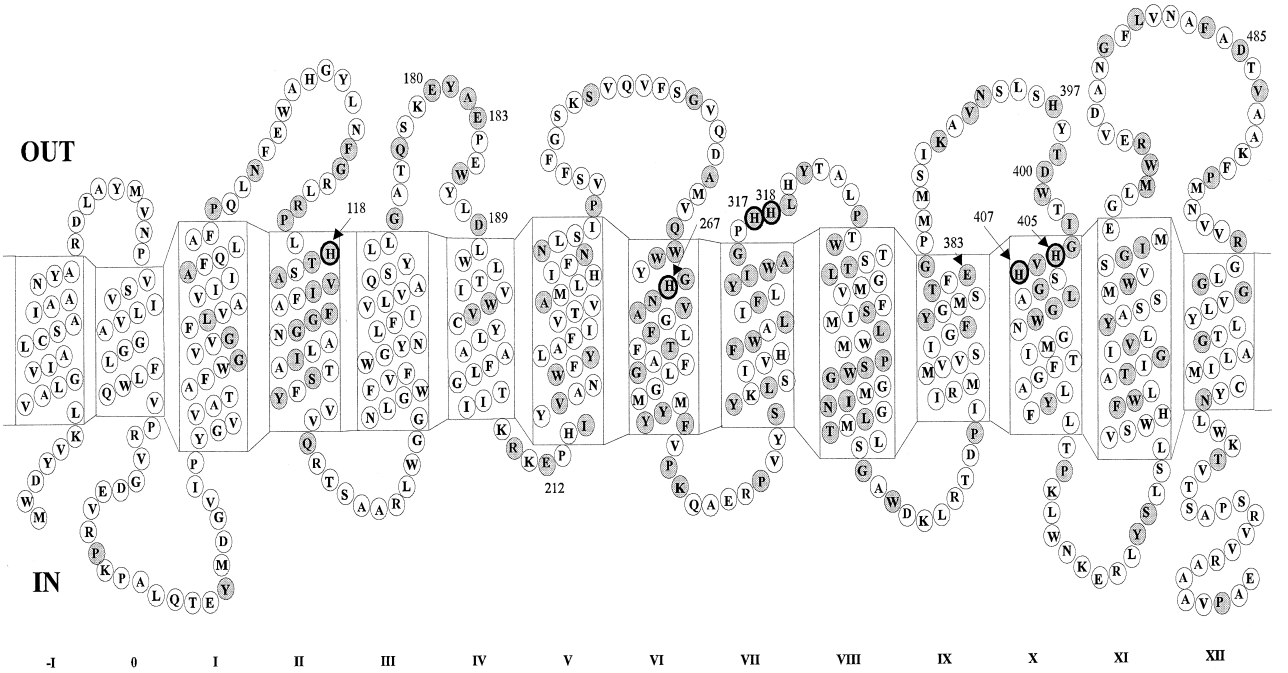


Fig. 3. Proposed two-dimensional model of the *R. sphaeroides* CcoN subunit. The model was derived from hydropathy plots of the CcoN/FixN consensus sequence, and from the inspection of the crystal structure of cytochrome aa_3. Totally conserved residues among CcoN/FixN sequences are shaded. Sequence numbers are indicated for the totally conserved histidines and acidic residues. Putative metal ligands are indicated by bold circles.

caulinodans [29]. It has been observed that the cbb_3-like oxidase is preferentially expressed in low-oxygen tensions [2,19], and a FNR-type gene expression regulatory system has been described in *R. sphaeroides*. Zeilstra-Ryalls and Kaplan [26] reported that the *fnrL* gene product is involved in the regulation of gene expression in response of environmental changes. De Gier et al. [28] also found an ORF that potentially codes for a *fnrP* transcription regulator. This is located in the flanking region of the *ccoNOQP* operon of *P. denitrificans*. Accordingly, this *fnrL* or *fnrP* gene seems to be the regulator for the oxygen-regulated expression of the cbb_3-type oxidase through binding to the *Fnr*/*FixK*-like site.

Around 190 bp upstream from the *ccoN* start codon, an ORF was found with an orientation opposite to the *ccoNOQP* operon. This ORF corresponds to Rs-ORF277 identified by Zeilstra-Ryalls and Kaplan [26], which is homologous to the *B. japonicum* ORF 277 of unknown function [19].

3.1.1. ccoN

This gene encodes a basic (pI = 8.97) membrane protein of 535 amino acid residues, with a predicted molecular mass of 60 060 Da. The corresponding polypeptide in the purified enzyme showed a relative molecular mass of 45 000 on SDS-PAGE [1]. This discrepancy may be due to anomalous electrophoretic migration and/or possible protein processing. Hydropathy analysis predicts 14 transmembrane segments with both N and C termini facing the cytoplasmic side (see Fig. 3). This subunit is predicted to bind the low spin heme *b*, the high spin heme b_3 and the Cu_B, which have been spectroscopically detected [1,30]. From 13 histidine residues in the sequence only seven are conserved in all the CcoN/FixN sequences reported (Fig. 4). Hydropathy analysis predicts that only four conserved histidines (His^{118}, His^{267}, His^{405}, and His^{407}) are within transmembrane α-helices while three (His^{317}, His^{318}, and His^{397}) are probably located on periplasmic loops. By comparison with the location of the histidine ligands in cytochrome aa_3 crystal structure, it is most likely that His^{118} and His^{407} are the ligands for the low spin heme *b*, and His^{405} the axial ligand for the high spin heme b_3. His^{267}, His^{317} and His^{318} are probably lig-

CcoN/FixN

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Agrobacterium tumefaciens MNYTLETADR-ALGA FPALLGAAFAH---- ----------DSLFA AHMWVLFFTLVVSTL LLLRRV--SFLPPVA GPPC---RRTEYFDE  70
Rhizobium meliloti        MKHTVEMVVL-SVGA FLALVGAGLAQ---- ----------DRLFG AHMWVLFFALLAGTL VLMRRV---DFRPAV AGHPG--RRREYFDE  70
Paracoccus denitrificans  ---MLDTIKLIALGT IAVLAAIAANYARP- ----------DDLAY LVNALIIMLAAGIMF LRVLRQMGNE-QPAL EPHP----ETQYMDD  71
Rhodobacter capsulatus    ---MWDYVKLVALGV VAAIAAYAASQAR-- ----------DLPYM VNMVEVALAAVIALI WVLRTMG--DPKPSK ---------DEYFDG  64
Rhodobacter sphaeroides   ---MWDYVKLVALGV IALCSAIAANYAR-- ----------DLAYM VNPVSVILVAGGLFL WQVPRVG-DEVRPKP AL------QTEYMDG  68
Azorhizobium caulinodans  MSI-VQTPAKRMTGG ELGLILVFAALGFFS IVVAAKAYTPE--YA FHAYLFAAASIATVF VIGNRYM--D-RPAE LPPQTIDGKPNYNMA  84
Bradyrhizobium japonicum  MS-QPSISKSMTIGE SGLAVVFAATAFLCV IAAAKAL---DAPFA FHAALSAAASVAAVF CIVNRYF--E-RPAA LPPAEINGRPNYNMG  83
Rhizobium etli            MNYTETMVIAV-AAF LALLAAAFAH----- ----------DHLFA VHMGILCLCLVAGTL LLVRNA---EFSPTG QQRKTEL--TGYCDE  69
Rhizobium leguminosarum   MNYTTETMVI-AVAA FLALLGAAFAH---- ----------DHLFA VHMGILCFCLAVGAA LLVRNV---DFSPQP T-AESRYI--SYFDE  69
Helicobacter pylori       --------------- --------------- --------------- --------------- ----MQ---ENVP-- ---------LSYDYS  12
                                     -I                                          0

Agrobacterium tumefaciens VVKYGVMATVFWGVV GFLVGVVVALQLAFP DLNIAPY----FNFG RMRPLHTSAVIFAF- -GGNALIATS-FY-V VQRTCRARLFGG---  149
Rhizobium meliloti        VVKYGVVATVFWGVV GFLVGVVVALQLAFP ELNVEPW----FNFG RVRPLHTSAVIFAF- -GGNALIATS-FY-V VQRTSRARLFGG---  149
Paracoccus denitrificans  VVRAGVIATAFWGVV GFLVGVVIAFQLAFP ALNLSDITMGYTNFG KLRPLHTSAVIFAF- -GGNGLIATS-FY-V VQRTSAARLWGG---  154
Rhodobacter capsulatus    VIRAGVIATTFWGIV GFLVAVVIAFQLAFP ALNLEFG-NGMLNFG RLRPLHTSAVIFAF- -GGNALIA-SAFY-V VQRTSAARLFGGT--  147
Rhodobacter sphaeroides   VIPYGVVATAFWGVV GFLVAVIIAFQLAFP QLNFEWA-HGYLNFG RLRPLHTSAVIFAF- -GGNALIATS-FY-V VQRTSAARLWGG---  150
Azorhizobium caulinodans  PVKVGTLLAVFWGIA GFLIGVIIALQMAYP LFNFDLP---WISFG RLRPLHTSAVIFAF- -GGNVLIATS-FY-V VQRTSHARLAGY---  164
Bradyrhizobium japonicum  PIKFSSFMAMFWGIA GFLVGLIIASQLAWP ALNFDLP---WISFG RLRPLHTSAVIFAF- -GGNVLIATS-FY-V VQKSCRVRLAGD---  163
Rhizobium etli            VIRYGLIATVFWGVV GFLVGVIIALQLAFP DLNIAPY----WNFG RLRPVHTSAVIFAF- -GGNALIMTS-FY-V VQRTCRARLFGG---  148
Rhizobium leguminosarum   VIRYGLIATVFWGVV GFLVGVVIALQLAYP DLNIAPY    LNFG RLRPVHTSAVIFAF- -GGNALIMTS-FY-V VQRTCRARLFGG---  148
Helicobacter pylori       ISKLFLYAMVGFGIV GMLIGIVLAFELSFP NLNY--IAGEYGIFG RLRPLHTNAVIYGFT LGG---IWAS-WYYI GQRVLKITYHQHPFL   96
                                          I                                   *           II

Agrobacterium tumefaciens -NLGWFVFWGYNLFI IMAATGYLLGITQGR EYAEPEWYVDLWLTI VWVAYLATFLGTILT RKEPHISVANWFYLS FIVTIAMLHIVNNLA  238
Rhizobium meliloti        -DLGWFVFWGYQLFI VLAASGYLLGITQSR EYAEPEWYVDLWLTI VWVAYLVAFLGTIMK RKEPHIYVANWFYLA FIVTIAMLHVVNNLA  238
Paracoccus denitrificans  -NAAWFVFWGYQLFI VLAATGYILGATQSK EYAEPEWYVDWWLTV VWVVYLAVFLGTILK RKEPHIYVANWFYLS FIVTIAMLHIVNNLA  243
Rhodobacter capsulatus    -ALGWFVFWGWQLII VTAATSYLLGGSQGK EYAELNWHLDILVAV VWVAYLIAFLGTIFK RKEPHIYVANWFYLS FIVTIAMLHIVNNLA  236
Rhodobacter sphaeroides   -NLGWFVFWGYNLFI VLVAQSYLLGATQSK EYAEPEWYLDLWLTI VWVCYLAAFLGTIIK RKEPHIYVANWFYLA FIVTVAMLHIFNNLS  239
Azorhizobium caulinodans  -LAPWFVVLGYNFFI VIAGTGYLLGITQGK EYAEPEWYADLWLTI VWVTYFLVFLGTVLK RKEPHIYVANWFYLA FILTIAVLHLGNNAA  253
Bradyrhizobium japonicum  -LAPWFVVVGYNFFI LVAGTGYLLGVTQSK EYAEPEWYADLWLTI VWVVYLLVFLATIIK RKEPHIFVANWFYLA FIVTIAVLHLGNNPA  252
Rhizobium etli            -TLAWFVFWGYQLFI VMAATGYVIGINQSR EYAEPEWYVDLWLTI VWVAYLAVYLGTILK RKEPHIYVANWFYLS FIVTIAMLHVVNNLA  237
Rhizobium leguminosarum   -SLAWFVFWGYQLFI VMAATGYVLGITQAR EYAEPEWYVDLWLTI VWVAYLAVYLGTILK RKEPHIYVANWFYLS FIVTIAMLHVVNNLA  237
Helicobacter pylori       KIVGLLHFWLWIILL ILGVISLFAGLTQSK EYAELMWPLDIIVVV AWVLWGVNMFGSMSV RRENTIYVSLWYYIA TYVGIAVMYIFNNLS  186
                                   III                                 IV                          V

Agrobacterium tumefaciens VPVS-FLGVK----- SYSAFSGVQAALTQW WYGHNAVGFFLTAGF LGMMYYFIPKQVNRP VYSYRLSIIHFWALI FMYIWAGPHHLHYTA  322
Rhizobium meliloti        VPVS-FLGSK----- SYSAFSGVQDALTQW WYGHNAVGFFLTAGF LAMMYYFIPKQVNRP VYSYRLSIIHFWAII FMYIWAGPHHLHYTA  322
Paracoccus denitrificans  IPVSLFG-SK----- SVQLFSGVQDAMTQW WYGHNAVGFFLTAGF LGMMYYFIPKQAERP VYSYKLSIIHFWALI FLYIWAGPHHLHYTA  327
Rhodobacter capsulatus    VPVSIFG-TK----- SVQLMAGVQDAMTQW WYGHNAVGFFLTAGF LGMMYYFVPKQAERP VYSYKLSIVHFWALI FLYIWAGPHHLHYTA  320
Rhodobacter sphaeroides   IPVS-FFGSK----- SVQVFSGVQDAMVQW WYGHNAVGFFLTAGF LGMMYYFVPKQAERP VYSYKLSIVHFWALI FLYIWAGPHHLHYTA  323
Azorhizobium caulinodans  IPVSVFS-PK----- SYIVWSGVQDAMVQW WYGHNAVGFFLTAGF LALMYYFIPKRADKP VYSYRLSIVHFWALI FLYIWAGPHHLHYTA  337
Bradyrhizobium japonicum  LPVSAFG-SK----- SYVAWGGIQDAMFQW WYGHNAVGFFLTAGF LAIMYYFIPKRAERP IYSYRLSIIHFWALI FLYIWAGPHHLHYTA  336
Rhizobium etli            VPAS-FLGSK----- SYSLFSGVQDALTQW WYGHNAVGFFLTAGF LGMMYYFVPKQANRP VYSYRLSIIHFWALI FMYIWAGPHHLHYTA  321
Rhizobium leguminosarum   VPAS-FLGSK----- SYSVSSGVQDALTQW WYGHNAVGFFLTAGF LGMMYYFVPKQANRP VYSYRLSIIHFWALI FMYIWAGPHHLHYTA  321
Helicobacter pylori       VPTY-FVADMGSVWH SISMYSGSNDALIQW WWGHNAVAFVFTSGV IGTIYYFLPKESGQP IFSYKLTLFSFWSLM FVYIWAGGHHLIYST  275
                                                          *    VI                               VII        **

Agrobacterium tumefaciens LPDWAQTLGMVFSIM LWMPSWGGMINGLMT LSGAWDKIRTDPIVR MMVMAVAFYGMATFE GPMMSIKAVNSLSHY TDWTIGHVHSGALGW  412
Rhizobium meliloti        LPDWAQTLGMVFSIM LWMPSWGGMINGLMT LSGAWDKIRTDPVVR MMVMAVAFYGMATFE GPMMSIKTVNSLSHY TDWTIGHVHSGALGW  412
Paracoccus denitrificans  LPDWASTLGMVFSII LWMPSWGGMINGLMT LSGAWDKLRTDPIIR MMVVAVGFYGMATFE GPMMSIKAVNFVSHY TDWTIGHVHSGALGW  417
Rhodobacter capsulatus    LPDWASTLGMVMSVI LWMPSWGGMINGLMT LSGAWDKLRTDPVIR MMVVSIGFYGMSTFE GPMMSIKAVNSLSHY TDWTIGHVHSGALGW  410
Rhodobacter sphaeroides   LPTWTSTLGMVFSIM LWMPSWGGMINGLMT LSGAWDKLRTDPIIR MMVVSIGFYGMSTFE GPMMSIKAVNSLSHY TDWTIGHVHSGALGW  413
Azorhizobium caulinodans  LPDWAQTLGMTFSIM LWMPSWGGMINGLMT LSGAWDKLRTDPIIR MMVVAVAFYGMATFE GPMMSVKSVNSLSHY TEWGIGHVHSGALGW  427
Bradyrhizobium japonicum  LPDWTQTLGMTFSIM LWMPSWGGMINGLMT LSGAWDKLRTDPVLR MLVVSVAFYGMSTFE GPMMSIKVVNSLSHY TDWTIGHVHSGALGW  426
Rhizobium etli            LPDWAQTLGMVFSVM LWMPSWGGMINGLMT LSGAWDKIRTDPIIR MIIVAIAFYGMSTFE GPMMSIKAVNSLSHY TEWQIGHVHSGALGW  411
Rhizobium leguminosarum   LPDWAQTLGMVFSIM LWMPSWGGMINGLMT LSGAWDKIRTDPIIR MMIVAIAFYGMSTFE GPMMSVKTVNSLSHY TEWTIGHVHSGALGW  411
Helicobacter pylori       VPDWVQTLSSVFSVV LILPSWGTAINMLLT MRGQWHQLKESPLIK FLVLASTFYMLSTLE GSIQAIKSVNALAHF TDWIIGHVHDGVLGW  365
                                  VIII                                         IX                        * *   X

Agrobacterium tumefaciens NGMITFGAIYYLTPK LWGRDRLYSLQLVNWH FWLATLGIVVYAAVM WVAGIQQALMWREY DSQGFLVYSFAESVA ALFPYYVMRALGGLM  502
Rhizobium meliloti        NGLITFGAIYYLVPK LWNRERLYSVRMVNWH FWLATLGIVVYAAVM WVAGIQQGLMWREY DDQGFLVYSFAETVA AMFPYYVMRAAGGAL  502
Paracoccus denitrificans  NGMITFGALYYLVPR LWGRERLYSTGLVSWH FWLATIGLVLYAASM WVSGIMEGLMWREV DAQGFLVNAFADTVA AKFPMNVVRALGGVL  507
Rhodobacter capsulatus    NGMITFGMLYFLTPR LWGRSGLYSLKLVSWH FWLATIGIVLYASAM WVTGIMEGLMWREV DAQGFLVNAFADTVA AKFPMYVVRGVGGVL  500
Rhodobacter sphaeroides   NGMITFGALYFLTPK LWNKERLYSLSLVSWH FWLATIGIVLYASSM WVSGIMEGLMWREV DANGFLVNAFADTVA AKFPMNVVRGLGGVL  503
Azorhizobium caulinodans  VAYISFGAIYCLIPW LWNKREMYSMKAIEWH FWVSTLGIVLYICAM WVAGILQGLMWRAY TALGFLEYSFIETVE AMHPLYVIRAIGGIL  517
Bradyrhizobium japonicum  VGFVSFGALYCLVPW AWNRKGLYSLKLVNWH FWVATLGIVLYISAM WVSGILQGLMWRAY TSLGFLEYSFIETVE AMHPFYIIRAAGGGL  516
Rhizobium etli            VGMITFGAIYYLTPK LWGRERLYSLRMVNWH FWLATLGIVIYAAVL WVAGIQQGLMWREY NSQGFLVYSFAETVA AMIPYYVLRAVGGAL  501
Rhizobium leguminosarum   VGMITFGAIYYLTPK LWGRERLYSLRMVNWH FWLATFGIVVYAAVL WVAGIQQGLMWREY NSQGFLVYSFAETVA AMFPYYVLRAVGGTL  501
Helicobacter pylori       VGFTLIASMYHMTPR LFKR-EIYSGRLVDFQ FWIMTLGIVLYFSSM WIAGITQGMMWRDV DQYGNLTYQFIDTVK VLIPYYNIRGVGGLM  454
                                                                  XI                                             XII
```

Fig. 4. Amino acid sequence alignments for the CcoN/FixN, CcoO/FixO, CcoQ/FixQ and CcoP/FixP subunits. Fully conserved residues are bold faced. Potential transmembrane helices are underlined. Putative histidine ligands for the low spin heme and the binuclear center metals are indicated by asterisks. Amino acid residues probably involved in the binding of the hemes *c* in CcoO/FixO and CcoP/FixP are also indicated by asterisks. GenBank accession Nos.: *Agrobacterium tumefaciens*, Z46239; *R. capsulatus*, X80134; *B. japonicum*, L07487; *A. caulinodans*, X74410; *Rhizobium meliloti*, Z21854; *R. etli*, U76906; *R. leguminosarum*, Z70305; *P. denitrificans*, U34353; *R. sphaeroides*, U58092; *H. pylori*, AE000536).

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Agrobacterium tumefaciens FLSGALIMAYNVTMT ILGHQREEGAS-KGA APSLQPAE-   539
Rhizobium meliloti        FLAGALLMAFNVTMT ILGRVRDEEPI-FGA APLPAPAE-   539
Paracoccus denitrificans  YLGGALIMCYNLWAT VAKQPK------TQS TAAAVPAE-   539
Rhodobacter capsulatus    YLLGGLIMAYNLWAT VAKQPK------TAN LAVAVPAE-   532
Rhodobacter sphaeroides   YLTGALIMCYNLWKT VTSAPS------RVV RAAAVPAE-   535
Azorhizobium caulinodans  FLAGSLIMAWNVFMT ITRAETVS----QPS GAALAPAE-   551
Bradyrhizobium japonicum  FLIGALIMAYNLWMT VRVGEAEV----QMP -VALQPAE-   549
Rhizobium etli            YLAGGFVMAWNVFMT IRGHLRDEAPIPTSL VPQAQPAE-   539
Rhizobium leguminosarum   YLAGGLVMAWNVFMT IRGHLRDEAAIPTTF VPQAQPAE-   539
Helicobacter pylori       YFIGFIIFAYNIFMT ITAGKKLE-----RE PNYATPMSR   488
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CcoO/FixO

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Agrobacterium tumefaciens ---MSILDKHGVIER NATLLLVGSLLVVSI GGIVEIAPLFYLENT IEKVEGMRPYSPMEL AGRNIYIREGCYVCH SQMIRPFRDEVERYG   87
Rhizobium meliloti        ---MSILDKHAILER NATLLLIGSLLVVSI GGIVEIAPLFYLENT IEKVEGMRPYSPLEL AGRDIYIREGCYVCH SQMIRPFRDEVERYG   87
Paracoccus denitrificans  ---MAILEKHKVLEK NATLLLVFSFLVVTI GGIVEIAPLFYLQNT IEKVQGMRPYTPLEL KGRDIYVREGCYVCH SQMIRPMRDEVERYG   87
Rhodobacter sphaeroides   ---MGILAKHKILET NATLLLIFSFFVVTI GGLVQIVPLFYLENT IEKVEGVRPYTPLEL AGRDIYIREGCYVCH SQMIRPMRDETERYG   87
Rhodobacter capsulatus    ---MSIMDKHHVLEK NATLLLIFAFLVVTI GGIVEIAPLFYLENT IEKVEGMRPYTPLEL TGRDIYIREGCYVCH SQMIRPMRDEVERYG   87
Azorhizobium caulinodans  MSATSIWSKHAIFEK HSILLLLGVLIVISI GGLVEIVPLFYLKST IEKVDGVRPYTPLEL AGRNIYLREGCYLCH SQMIRPLRDEVERYG   90
Bradyrhizobium japonicum  ---MSFWTRHQVFEK NSIILIVGILLVIAI GGLVEITPLFYLKST IEKVDGVRPYTPLEL AGRNVYVREGCYLCH SQMIRPLRDEVERYG   87
Rhizobium leguminosarum   --MASILDKHQILEK NATLLLVGSLLVVSI GGIVEIAPLFYLQNT IEKVEGIRPYTPLEL AGRNIYIREGCYLCH SQMIRPFRDEVERYG   88
Helicobacter pylori       --------MFSFLEK NPFFFTLAFIFVFAI AGLVEILPNFFKS-- ARPIEGLRPYTVLET AGRQIYIQEGCYHCH SQLIRPFQAEVDRYG   80
                                                                                                          *  **

Agrobacterium tumefaciens HYTLAAESMYDHPFQ WGSKRTGPDLARVGD RYSNEWHVQHLANPR SVVPESIMPSYAFLK TTP-LKITDVSMELK ANRAVGVPYSDEMIE   176
Rhizobium meliloti        HYSLAAESMYDHPFQ WGSKRTGPDLARVGD RYSNEWHVQHMIEPR SVVPESVMPSYAFLK ETP-LEVKNVAMSLE ANRAVGVPYTDEMIG   176
Paracoccus denitrificans  HYSLAAESMYDHPFQ WGSKRTGPDLARVGG RYSDEWHLDHLVDPQ AVVPESIMPKYGFLL NRQ-VDASNMQQRLK TDALGGVPYDDAMIA   176
Rhodobacter sphaeroides   HYNLAAESMYDHPFQ WGSKRTGPDLARVGE RYSDEWHVDHLTNPQ SVVPESIMPKYGFLS HTV-IDGRYIRDLMS VHRIVGVPYSDEMLE   176
Rhodobacter capsulatus    HYSLAAESMYDRPFQ WGSKRTGPDLARVGG RYSDAWHVEHLINPQ SVVPESVMPSYGYLA KVP-LDSTWIEDRVS TDAFVGVPYTSEMIA   176
Azorhizobium caulinodans  HYSLAAESMYDHPFQ WGSKRTGPDLARVGG KYSDLWQLEHLNNPR AVVPASIMPAYPWLA KTP-LQAKHIADDMK VLRAEGVPYTDEMIA   179
Bradyrhizobium japonicum  HFSLAAESMFDHPFQ WGSKRTGPDLARVGA KYSDDWHVTHLTNPR AIVPQSVMPGYPFLS ATE-VDPDTIADHMR TLRTVGVPYTDDQIA   176
Rhizobium leguminosarum   HYSLAAESMYDHPFQ WGSKRTGPDLARVGA RYSNEWHVQHLADPR AVVPESIMPSYAFLK EQR-VTVKDVGMDLK ANEDVGVPYDDDMLA   177
Helicobacter pylori       AYSLSGEYAYDRPFL WGSKRIGPDLHRVGD YRTTDWHEKHMFDPK SVVPHSIMPAYKHLF TKKSDFDTAYAEALT QKKVFGVPYDTENGV   170
                                                                                  **

Agrobacterium tumefaciens KSATDLHAQADP-NA DGAELLERYP-KAKV GDFDGDRP------- ---------------- ----------   212
Rhizobium meliloti        NAAADLKAQADP-NA DGSGVEARYP-KAKL GDFDGD-PQRLTEMD ALVAYLQMLGTLVDF STYDDAAGYR   243
Paracoccus denitrificans  AAGEDFRVQAAP-DA DASGLEERYP-GAQQ RNFDRR--PGVSEMD ALIAYLQVLGTMVDF STFEPDPNR-   241
Rhodobacter sphaeroides   NAVADFKAQANP-DA DTDGLLERYG-KAAV RNFDGQ--AELTEMD ALISYLQVLGTMVDF STFQPVASR-   241
Rhodobacter capsulatus    SAKADFVAQADP-NA DSTTLLAGYGEKVNI RNFDGQ--PGLTEMD ALVAYLQVLGTMVDF KLYDNKANVR   243
Azorhizobium caulinodans  SAQDDLKLQATP-EA DADALQKRYP-KAQA RDFDGN-PGELTEAD ALIAYLQQLGTQVDF KIYNEKANLR   246
Bradyrhizobium japonicum  NASADLKAQADPDNA GADAFNKRYA-KAVV RNFDGK-TGTPTEMD ALIAYLQMLGTLVDF ----------   234
Rhizobium leguminosarum   NAEADMKAQADP-NA DTTALLARYP-KAKT GDFDGD-PAALTEMD ALVSYLQMLGTLVDF STYDDATGYR   244
Helicobacter pylori       KLGSVEEAKKAY-LE EAKKITADMKDKRVL EAIERG---EVLEIV ALIAYLNSLGNSRIN ANQNAK----   232
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CcoQ/FixQ

```
Rhizobium meliloti        ----METYTAMRHFA DSWGLLAMTLFFLGV VFFIFRPGAKNAAAQ ASVIPLKED------ ---------------- ---   50
Paracoccus denitrificans  ----MDRYSFLRELA DSWVLLLLVVFFLGT IVFAFRPGFAAAASR RGRKHLP-------- ---------------- ---   48
Rhodobacter sphaeroides   ----MDTYSLLRGPA DSWMLIVMTLFFVGV VFWAWRPRSRKDHDE AASAIFRHETKPADD DPVSSSDDARK---- ---   67
Rhodobacter capsulatus    -----MDYHILREFA DSWAALALLLTFIGA VIWAFRPGSSKVHDD IANIPFRHEDKPADH GRG------------ ---   58
Azorhizobium caulinodans  ---MRETYMQLAGFA QTWGLVYFVGAFLCV CAYAYWPSKKKSFNE AAQIPLKED------ ---------------- ---   51
Bradyrhizobium japonicum  MKAILTLDNLASGLV TTIWTPVFVAIFLAI IAYAFWPRNKAAFDE AAHLPLREE------ ---------------- ---   54
Rhizobium leguminosarum   ----METYTAMRHFA DSWALLAMAAFFVGV VVFTLRPGSKQTAKE AADIPLKDD------ ---------------- ---   50
Helicobacter pylori       -----MMDLESLRGF AYAFFTILFTLFLYA YIFSMYRKQKKGIMD YERYGYLALNDALED ELIEPRHKKVHDNGI KES   73
```

CcoP/FixP

```
Rhizobium meliloti        ---------------- ---------------- -----------MADK HKHVDEVS------- ---------GVETTG HEWDGIRELNNPMPR   33
Paracoccus denitrificans  MADTDDEHASPQNPD NRIELERQAADEAHK AKILAHPPEAGGDPL HPPVTPRPGATRVVR DRKGGRRVVEVPSTG HSWDGIEEYDNPLPR   90
Rhodobacter capsulatus    ---------------- ---------------- ---------------- ---MSKKP------- -----TTKKEVQTTG HQWDGIEELNTPLPR   30
Rhodobacter sphaeroides   ---------------- ---------------- ---------------- ---MSVKP-----TK QKPG-----EPPTTG HSWDGIEEFDNPMPR   32
Azorhizobium caulinodans  ---------------- ---------------- -------MSTSHESH HAPVDGAG------- ---------GPSTTG HEWDGIQELNNPLPR   37
Bradyrhizobium japonicum  ---------------- ---------------- ------------MTD HSEFDSVS------- ---------GKTTTG HEWDGIKELNTPLPR   32
Helicobacter pylori       ----------M DFLNDHINVFGLIAA LVILVLTIYESSSLI KEMRDSKS------- -------QGELVENG HLIDGIGEFANNVPV   62
```

Fig. 4 (continued).

ands of Cu_B. The His^{397} might be equivalent to the conserved His^{403} (*P. denitrificans* cytochrome aa_3) which is a ligand for the magnesium ion. Otherwise, alignments of the CcoN sequences with subunit I of the main heme-copper oxidase family indicate that there is little homology between them. For example,

```
Rhizobium meliloti        WWVYSFYATIIWAIG YAIAYPSWPMLTE-A TKGMLGYSSRAEVSV ELAAAKAAQAGNLEQ IASSSVEEIIANPQL QQFAVSAGASAFKVN   122
Paracoccus denitrificans  WWLWTFYATIVWGVL YLIAYPAIPLVNG-A TQGLLGQNYRSDVAA EIQRFNEANAPIQAK LVETPLEEIAADPEL ANYTANAGAAIFRTW   179
Rhodobacter capsulatus    WWLWTFYATIIWGVA YSIAMPAWPIFSDKA TPGLLGSSTRADVEK DIAKFAEMNKAVEEK LVATDLTAIAADPEL VTYTRNAGAAVFRTW   120
Rhodobacter sphaeroides   WWLWTFYVTIVWAIG YSILYPAWPLING-A TNGLIGHSTRADVQR DIEAFAEANATIRQQ LVNTDLTAIAADPNL LQYATNAGAAVFRTN   121
Azorhizobium caulinodans  WWLWTFYATIIWAFG YWVAYPAWPLVSN-Y TSGVLGWNSRSAVVE QISDLQKLRAASSAK LANVPLEDIEKNPEL LSLARAEGKVAFADN   126
Bradyrhizobium japonicum  WWVICFYLTIVWAIG YWIVYPAWPLISS-N TTGLFGYSSRADVAV ELANLEKIRGDKMAA LGAASLADVEKDPAL LALARAKGKTVFGDN   121
Helicobacter pylori       GWIASFMCTIVWAFW YFFFG--YPLNSF-- --SQIGQYN-EEVKA HNQKFEAK----WKH LGQKELVDM------ -------GQGIFLVH   128

Rhizobium meliloti        CAQCHGSGAAGGQGF PNLNDDDWLWGGKPQ EIYQTIAHGVRHAPD -GETRVSEMPPFG-- DMLTPELMQQTAAYV VSLTQAP-SQPHLVQ   208
Paracoccus denitrificans  CAQCHGSGAGGATGY PSLLDNDWLWGGTLE EIHTTVMHGIRDPKD -ADTRYSEMPRFGID GLLENAQISQVVNHV LELGGLP-HDAALAA   267
Rhodobacter capsulatus    CAQCHGAGAGGNTGF PSLLDGDWLHGGAIE TIYTNVKHGIRDPLD PDTLLVANMPAHLTD ELLEPAQIDEVVQYV LQISGQP-ADEVKAT   209
Rhodobacter sphaeroides   CVQCHGSGAAGNVGY PNLLDDDWLWGGDIE SIHTTVTHGIRNTTD -DEARYSEMPRFGAD GLLDSTQISQVVEYV LQISGQD-HDAALSA   209
Azorhizobium caulinodans  CAPCHGAGGGGAKGF PNLNDDDWLWGGTLA QIQQTITHGIRSGDD --EGHQGNMLAFG-- SILSKADISNVADYV RSLSGAAPGDTPAAK   212
Bradyrhizobium japonicum  CAPCHGSGGAGAKGF PNLNDDDWLWGGTLD QIMQTIQFGARSGHA --KTHEGQMLAFGKD GVLKGDEIVTVANYV RSLSGLPTRKGYDAA   209
Helicobacter pylori       CSQCHGITAEGLHGS AQNLVR---WG-KEE GIMDTIKHGSK-GMD ---YLAGEMPAMELD EKDAKAIASYVMAEL SSVKKTK--NPQLID   208
                          *  **                                              *

Rhizobium meliloti        QGKQVFADN-CASCH GADAKGNREMGAPNL ADAIWLKGEG--EQA VITQMKTPKHGVMPA WL-------PRLGDD TVKQLAVFVHSLGGG   288
Paracoccus denitrificans  EGVEVFADN-CSSCH AEDGTGDRAQGAPDL TDAVWLYGSD--PAT ITRIVRDGPFGVMPA WT-------GRLSEA DIVAVAAYVHSLGGG   347
Rhodobacter capsulatus    AGQQIFAEN-CASCH GEDAKGLVEMGAPNL TDGIWLYGGD--VAT LTSTIQYGRGGVMPS WSWAADGAKPRLSEA QIRAVASYVHSLGGG   296
Rhodobacter sphaeroides   EGATIFADN-CAACH GEDGTGSRDVGAPNL TDAIWLYGGD--RAT VTETVTYARFGVMPN WN-------ARLTEA DIRSVAVYVHGLGGG   289
Azorhizobium caulinodans  KGAEIFAAN-CATCH GENGKGNQELGSKNL TDGIWLYGGD--KAT IVQTITNGRGGVMPA WG-------PRLSPT TIKALTVYVHTLGGG   292
Bradyrhizobium japonicum  KGEKIFVEN-CVACH GDGGKGNQEMGAPNL TDKIWLYGSD--EAA LIETISQGRAGVMPA WE-------GRLDPS TIKAMAVYVHSLGGG   289
Helicobacter pylori       KGKELFESMGCTGCH GNDGKGLQEN--QVF AADLTAYGTENFLRN ILTHGKKGNIGHMPS FK------YKNFSDL QVKALLNLSNR----   286
                                *  **                                              **

Rhizobium meliloti        E   289
Paracoccus denitrificans  E   348
Rhodobacter capsulatus    Q   297
Rhodobacter sphaeroides   E   290
Azorhizobium caulinodans  Q   293
Bradyrhizobium japonicum  K   290
Helicobacter pylori       -   286
```

Fig. 4 (continued).

residues suggested to be involved in H^+ conduction (Asp^{124}, Glu^{278}, Lys^{354}, Leu^{393} and Asp^{399} in *P. denitrificans* cytochrome aa_3), or in H bonding to the Cu_B histidine ligands (Thr^{344} and Tyr^{280}) are missing in the cytochrome cbb_3 sequences, or placed in other positions (but see below). A suggested two-dimensional model for this protein is shown in Fig. 3. The high degree of hydrophobicity for the first two helices of CcoN (helices −I and 0 in Fig. 3) contributes to our prediction of them being transmembrane. However, Zufferey et al. [31] showed evidence which suggests that these two helices in *B. japonicum* FixN may be peripheral facing the cytoplasm. These two helices might not have a role in the function/structure of the oxidase since they are missing in the *Helicobacter pylori ccoN* sequence, in which the predicted protein starts at the level of the third transmembrane segment of the rest of the sequences.

3.1.2. *ccoO*

ccoO gene encodes for a 241 amino acid polypeptide with a calculated molecular mass of 27 376 Da, which is predicted to be a *c*-type membrane bound cytochrome. The corresponding polypeptide in the purified preparation shows a M_r of 29 000. Furthermore, the identity of the CcoO subunit in the protein preparation is now here confirmed since the N-terminal amino acid sequence reported before [1] corresponds to the deduced sequence from the *ccoO* gene (the first methionine residue is missing from the peptide sequence, but this should be due to post-translational processing).

Hydropathy analysis shows that CcoO probably has a single membrane-spanning domain near the N terminus, and a hydrophilic domain, which is most probably exposed to the periplasmic side where heme C is attached. A predicted totally conserved heme C binding site (CXXCH-//-MP) motif is found in this region (C_{68}-Y_{69}-L_{70}-C_{71}-H_{72}, and M_{140}-P_{141}; Fig. 4).

3.1.3. *ccoQ*

The *ccoQ* gene codes for a 67 amino acid acidic ($pI = 5.52$) polypeptide with a calculated molecular mass of 7778 Da (Fig. 4). It has a high hydrophobic amino acid stretch near the N terminus, and a charged motif at the C terminus as previously suggested [10]. The corresponding polypeptide is apparently not present in the purified preparation [1] and its role remains unknown.

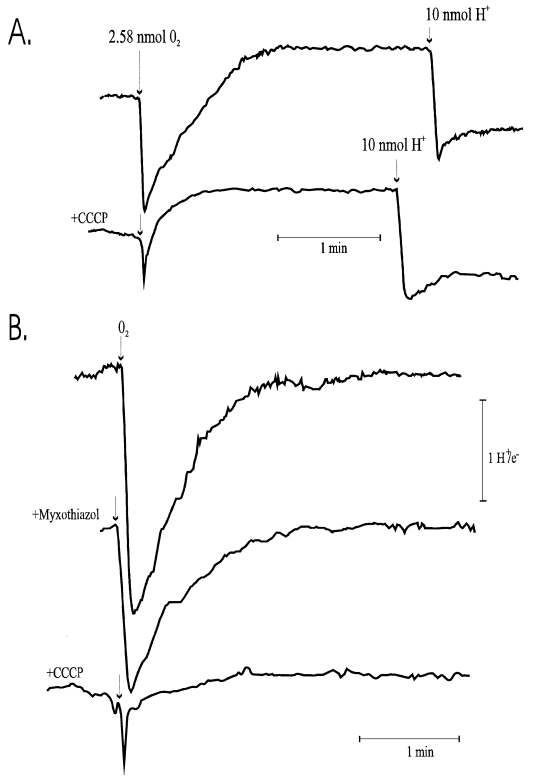

Fig. 5. Proton translocation in *R. sphaeroides* JS100 whole cells. Washed and starved cells were incubated to anaerobiosis in a medium containing 100 mM KSCN, 100 mM KCl, 15 μM rotenone, and 10 μM TMPD/5 mM K^+-ascorbate (A), or 15 mM succinate (B). Arrows indicate the addition of oxygen saturated water (O_2) or anaerobic HCl solution (H^+). Myxothiazol (2 μM) was added to the incubation medium (A) or when indicated (B). CCCP was added to 6 μM.

3.1.4. *ccoP*

The product of the *ccoP* gene codes for an acidic membrane protein (p*I* = 4.54) of 290 amino acids and a calculated M_r of 31 361. This product corresponds to the 35 kDa subunit on the SDS-PAGE analysis of the purified enzyme [1]. Hydropathy profiles predict two main domains: a hydrophobic transmembrane domain near to the N terminus, and a hydrophilic domain most probably facing the periplasmic side (Fig. 4). Two heme C binding motifs can be found in this domain (-C_{124}-V_{125}-Q_{126}-C_{127}-H_{128}—//—M_{176}-P_{177}; and C_{220}-A_{221}-A_{222}-C_{223}-H_{224}—//—M_{265}-P_{266}).

Considering that only CcoN, CcoO, and CcoP are part of the functional cytochrome *cbb*$_3$, the total mass calculated from the sequence accounts for 118 797 Da, which is very close to the mass electrophoretically estimated before using electrophoresis [1].

3.2. Deactivation of the *ccoNOQP* gene cluster and trans-complementation

In order to confirm that the *ccoNOQP* cluster encodes for the *cbb*$_3$-type cytochrome *c* oxidase, we constructed two mutants, one in which the cluster was inactivated by inserting a kanamycin resistance gene and another in which most of the sequences coding for the *ccoN* and *ccoO* genes were substituted by the kanamycin cartridge (see Section 2).

During the mating of *R. sphaeroides* JS100 (Δ*aa*$_3$) with *E. coli* S17-1/pSUP1943Kan (*ccoNOQP* Kan-interrupted vector), kanamycin resistant colonies were obtained only when the plates were incubated under photosynthetic conditions. Approx. 100 colonies were picked and were able to grow aerobically. Three of them were sensitive to tetracycline, which indicated that the double crossover event had taken place. One of these colonies (designated here as MT101) was grown and its respiratory activity tested. A similar strategy was employed for obtaining ME127, which is a mutant in which a deletion of part of *ccoN* and *ccoO* was filled in with the kanamycin resistance gene, also using the JS100 strain as parent. Location of the kanamycin resistance gene was confirmed by Southern blot hybridization (data not shown).

Membranes isolated from MT101 and ME127 showed no detectable cytochrome *c* or TMPD oxidase activity, but they could oxidize ubiquinol at high rates, in a myxothiazol resistant way. Succinate and NADH dehydrogenase activities, as well as ubiquinol:cytochrome *c* oxidoreductase activity, were

Table 2
Proton translocation in *R. sphaeroides* cells

Substrate	H^+/e^- [a]	
	Ga (wild type)	JS100 (Δ*aa*$_3$)
Succinate	2.6–3.1	2.7–3.1
Succinate+myxothiazol	1.7–1.9	1.8–2.0
Ascorbate/TMPD[b]	0.9–1.2	0.6–1.1

See legend to Fig. 5 and Section 2 for experimental details.
[a]Range obtained from four to six independent experiments.
[b]Corrected for the release of 0.5 H^+/e^- by ascorbate oxidation.

comparable with these activities measured in the parent strain JS100.

Cytochrome *c* oxidase activity was complemented in *R. sphaeroides* MT101 or ME127 by the introduction of a plasmid encoded *ccoNOQP* operon (see Section 2 for details). The resulting strain (MT101C, or ME127C) had cytochrome *c* oxidase activity at comparable levels as our wild type strain (JS100), and the spectroscopic cytochrome *c* levels were enhanced in comparison with MT101 or ME127 (data not shown).

3.3. Proton translocation

Since genetic inactivation of the cbb_3-type cytochrome *c* oxidase produces strains (MT101 and ME127) that do not show detectable cytochrome *c* or TMPD oxidase activity, we conclude that cytochrome cbb_3 is the sole cytochrome *c* oxidase in the parent strain (JS100, Δaa_3). Washed and starved *R. sphaeroides* JS100 cells were incubated in a non-buffered medium in the presence of a reductant (TMPD/ascorbate or succinate). After anaerobiosis, proton translocation following a small pulse of O_2 was determined by previously reported methods [23–25].

Fig. 5A shows an experiment with TMPD/ascorbate as electron donor. The addition of O_2 first induces fast release of protons to the extent of 1.1–1.6 H^+/e^- (Table 2). On subsequent anaerobiosis there is a slow decay of the pH trace to an equilibrium, which corresponds to an overall consumption of 0.5 H^+/e^- in the reaction. In the presence of the uncoupling agent carbonylcyanide *m*-chlorophenylhydrazone (CCCP) the decay is much faster, but the equilibrium position is unchanged. In this type of experiment the O_2 pulse might activate oxidation of endogenous substrate by cytochrome bb_3-type quinol oxidase despite starvation of the cells. However, the observation of an overall consumption of 0.5 H^+/e^- excludes significant contribution from quinol oxidase activity, showing that ascorbate is the electron donor. We conclude therefore, that this experiment measures the activity of the cbb_3 enzyme alone. After subtraction of the proton release due to ascorbate oxidation from the extent of proton ejection after the O_2 pulse, we arrive at an observed proton translocation quotient of 0.6–1.1 H^+/e^-. This result strongly suggests that the cbb_3-type oxidase from *Rhodobacter sphaeroides* functions as a proton pump much like the major enzymes of the heme-copper oxidase family.

In similar experiments with succinate as substrate (Fig. 5B, Table 2), we observed H^+/e^- ratios of proton ejection in the range of 2.7–3.1 H^+/e^-. This ratio fell to values below 2.0 in the presence of the cytochrome bc_1 complex inhibitor myxothiazol. Succinate is exclusively oxidized via the quinol oxidase with myxothiazol present, and the expected H^+/e^- ratio is 2 [22,32]. In the absence of myxothiazol virtually all electron flux occurs via the bc_1 complex (and cytochrome cbb_3), as in the case of *P. denitrificans* [22,32]. This is possibly due to a higher affinity of the bc_1 complex than the quinol oxidase for ubiquinol. Since the H^+/e^- quotient of proton release is 2 for the bc_1 complex, we may conclude that cytochrome cbb_3 pumps protons with an H^+/e^- ratio close to unity.

4. Discussion

The *ccoNOQP* gene cluster from *R. sphaeroides* was cloned and sequenced and in the current work is demonstrated to encode the cbb_3-type cytochrome *c* oxidase that has been observed spectroscopically in photosynthetically grown cells, and purified from a strain from which the aa_3-type oxidase has been deleted [1]. Many of the features predicted from the sequence correspond with those previously found by biochemical characterization of the purified enzyme.

The cluster seems to be organized as an operon based on the following criteria: (1) close physical proximity of the ORFs, (2) the presence of just one potential transcription termination site at the end of *ccoP*, (3) the loss of cytochrome cbb_3 expression by genetic interruption of the gene cluster, and (4) the fact that *fixNOQP* or *ccoNOQP* clusters from other bacteria appear to be regulated as operons [10,19,31,33].

Alignments of all the cbb_3-type oxidase sequences reported so far (Fig. 4) indicate that the subunits with a substantial degree of conservation are those encoded by the *ccoN*/*fixN* and *ccoO*/*fixO* genes (25% and 24%, respectively). The degree of conservation among the CcoP subunits is lower (9.7%) and the

CcoQ subunits are the least conserved (1.3%). These values seem to reflect the significance of each subunit. Zufferey et al. [31] have shown that the CcoN and CcoO polypeptides are essential for the assembly and function of the oxidase complex. In that study, an in-frame deletion of the *ccoP* gene gave rise to the assembly of a partially active CcoN-CcoO complex. The CcoQ subunit seems not to be important for the assembly or function of the oxidase complex [31]. This agrees with the high activity found for the purified cbb_3-type enzyme of *R. sphaeroides*, which appears to lack the subunit encoded by *ccoQ* [1]. However, it cannot be excluded that subunit CcoQ could be part of the oxidase in sub-stoichiometric amounts, as previously suggested [31], or that it could have a role in the expression of the complex, or in activity regulation.

The ability of the cbb_3-type oxidases to pump protons has been subject of discussion. It has been reported that maximal rates of oxidative phosphorylation in *P. denitrificans* were found only in the presence of cytochrome aa_3 [34,35]. Both growth yield [36] and some proton translocation measurements [5,37,38] were interpreted to suggest that *P. denitrificans* alternative cytochrome *c* oxidase (i.e. of cbb_3-type) is not a proton pump.

However, Raitio and Wikström [22] have found that the cbb_3 oxidase in *P. denitrificans* does pump protons during oxidation of succinate, while proton translocation linked to oxidation of TMPD/ascorbate appears very poor. More recently, De Gier et al. [28] reported very similar results. In agreement with these reports, we show here that the *R. sphaeroides* cytochrome cbb_3 readily translocates protons during succinate oxidation. We also found that, in this organism, proton translocation is also observed with TMPD/ascorbate as substrate. The reason for this difference is presently unknown. It could be due to the presence of another O_2-consuming enzyme in *P. denitrificans* that accepts electrons from TMPD/ascorbate system, but not from succinate [22]. Alternatively, it could be due to a more subtle phenomenon related to the mechanism of proton translocation in the cbb_3-type enzymes. One may speculate that the proton translocation mechanism in some of these enzymes could be unusually sensitive to controlled redox potential at the donor site. Sufficient control may be achieved only if electrons are fed into the enzyme via the cytochrome bc_1 complex, but not when fed via the TMPD/ascorbate system. On the other hand, our present finding of proton translocation with TMPD/ascorbate in the *R. sphaeroides* cbb_3 enzyme, and its strong homology to the enzyme from *P. denitrificans*, does not seem to favor explanations of this kind.

Both the hydropathy profile and the conservation of the histidine residues that serve as ligands to heme, iron, and Cu_B in subunit I suggest that the general architecture of the cbb_3-type oxidases is similar to that of the more traditional cytochrome *c* oxidases, despite the low level of sequence homology. This subunit (CcoN) therefore probably contains the low spin heme *b* as well as the heme b_3-Cu_B binuclear center. The Cu_A center of the traditional cytochrome *c* oxidases is probably replaced functionally by the membrane bound *c*-type cytochromes, which are likely to transfer electrons to the binuclear site via the low spin heme *b*.

More interesting, perhaps, is that the cbb_3-type oxidases lack several residues that have been shown to be key components of the proton translocating mechanism in the more conventional heme-copper oxidases. For example, none of the otherwise very highly conserved residues of subunit I, K319, Y244, D91 and E242 (bovine oxidase numbering) are conserved in the cbb_3-type enzymes. Yet, the present work clearly confirms the proton translocating property of the cbb_3-type enzyme. Two different scenarios may be considered to explain this problem. On one hand, many of the mentioned residues have been implicated in passive proton transfer pathways, either leading to the binuclear center or to the molecular pump machinery, which is still elusive. Hence, the basic molecular mechanism of the proton pump could still be the same in all heme-copper oxidases, while the structural support from the protein for passive proton transfer pathways could differ. There are already reports suggesting that bound water molecules may be essential in such pathways [39,40]. This scenario would lead to a direct role of the metal cofactors and their ligands in a universal proton-pumping mechanism [41]. The other extreme scenario is, of course, that the mechanistic principle is different in the cbb_3-type enzymes. It is for this reason that both functional and structural research on the cbb_3-type oxidases should be encouraged.

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