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Biochimica et Biophysica Acta 1557 (2003) 125–131
BIOCHIMICA ET BIOPHYSICA ACTA
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Short sequence-paper

QcrCAB operon of a nocardia-form actinomycete *Rhodococcus rhodochrous* encodes cytochrome reductase complex with diheme cytochrome *cc* subunit☆

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Received 26 August 2002; received in revised form 12 November 2002; accepted 26 November 2002

Abstract

Structural genes encoding quinol-cytochrome *c* reductase (QcR) were cloned and sequenced from nocardia-form actinomycete *Rhodococcus rhodochrous*. *QcrC* and *qcrA* encode diheme cytochrome *cc* and the Rieske Fe–S protein, respectively, while the *qcrB* product is a diheme cytochrome *b*. These amino acid sequences are similar to those of *Corynebacterium* and *Mycobacterium*, the members of high G+C content firmicutes. The presence of diheme cytochrome *cc* subunit as a sole *c*-type cytochrome in these organisms suggests the direct elecron transfer to cytochrome *c* oxidase. The N-terminal half of the Rieske Fe–S proteins of these bacteria has a unique structure with three transmembrane helices, while the C-terminal half sequence is conserved. A phylogenetic tree using the latter region showed that high G+C firmicutes form a clear clade with *Thermus*, but not with low G+C firmicutes.

Keywords: Quinol-cytochrome *c* reductase; Cytochrome *cc*; Rieske Fe–S protein; Cytochrome *b*; *Rhodococcus rhodochrous*

The quinol-cytochrome *c* reductases (QcR) constitute a superfamily of enzymes found in mitochondrial/bacterial respiratory chains, and in plastidal/bacterial photosynthetic electron transfer chains. These enzymes translocate protons across membranes with electron transfer from quinol to cytochrome *c*, probably by a mechanism known as the proton motive Q cycle [1–3]. The functional core of the enzymes is composed of cytochrome b/b_6, the Rieske Fe–S protein and cytochrome c_1/f. Cytochrome b/b_6 has two protohemes for facilitating electron transfer across the membrane and plays a central role in quinol oxidation and reduction [3,4]. Cytochrome b_6, found in plastids and cyanobacteria, is much shorter than cytochrome *b*, but cytochrome b_6 (PetB) and subunit IV (PetD) correspond almost entirely to the cytochrome *b* in mitochondria and proteobacteria [5,6]. In the case of peripheral membrane proteins, only the C-terminal half of the Rieske iron–sulfur cluster are conserved, but other parts are not homologous in Rieske Fe–S proteins [7]. In most aerobic bacteria, QcR plays a role as the central segment of the respiratory chain, oxidizing quinol [4,8]. Our previous research indicates that Gram-positive bacilli have QcR with a small cytochrome b_6-type diheme cytochrome as the central membrane-bound enzyme to oxidize menaquinol [9–12]. They contain the Rieske Fe–S protein and a unique cytochrome c_1, which is composed of subunit IV with a kind of cytochrome c_6-like protein at the C-termini [11]. Recently, we studied the respiratory chain of *Corynebacterium glutamicum* [13–15], another type of Gram-positive bacterium other than *Bacillus* which is important in producing amino acids for nutritious supplement to food and feed. Its *qcrCAB* operon encodes for a 28-kDa cytochrome containing two C heme-binding sites, a Rieske Fe–S protein of 40 kDa with three transmembrane helices and a diheme cytochrome *b* with seven transmembrane helices. Protein analysis indicated that cytochrome *cc* (QcrC) is the only covalently bound hemoprotein and the bacterium does not contain any other *c*-type

Abbreviations: QcR, quinol:cytochrome *c* oxidoreductase [EC 1.10.2.2]; PCR, polymerase chain reaction; PS, photosystem; SDS-PAGE, sodium dodecylsulfate-polyacrylamide electrophoresis

☆ The nucleotide sequences reported in this paper have been submitted to the DDBJ/Gene Bank/EMBL Data Bank with accession number AB092698.

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0005-2728/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2728(02)00394-8

cytochrome [14,16]. The phylogenetic-tree analyses of cytochromes b/b_6 showed that *C. glutamicum* cytochrome *b* is closely related to other high G+C Gram-positive bacteria such as *Mycobacterium* and mycelial bacteria, but rather different from the *Bacillus* counterparts [14,16]. This indicates that there is a fourth group of bacteria in addition to the three clades: proteobacterial cytochrome *b*, cyanobacterial b_6 and green sulfur-low G+C Gram-positive bacteria such as Bacilli. Thus, two groups of Gram-positive bacteria use different types of QcR.

Nocardia group bacteria, such as *Rhodococcus rhodochrous* which are Gram-positive high G+C aerobes, are known as very active soil bacteria; they are rich in lipases, esterases, hydroxylases and nitrilase [17,18]. In this study, the genes encoding for the QcR of *R. rhodochrous* have been cloned and the deduced protein sequences reveal interesting features of the QcR in this bacterium. The presence of diheme cytochrome *cc*, a unique Fe–S protein with three transmembrane helices at the N-terminal half, and cytochrome *b* with 549 amino acid residues are shown. The evolution of QcR in bacteria is also discussed.

R. rhodochrous DMS 43202 was kindly donated by Dr. K. Nagata of Hyogo College of Medicine. It was grown aerobically at 30 °C in a Luria broth and the cells (about 3 g wet weight) were broken by grinding with sea sand. For the cloning of *R. rhodochrous*, *qcrCAB*, two sets of primers were designed: 5′-ACCGGYGTSTACCTSACSCTSTTC-TTY-3′ and 5′-GAASAKSAGSGCNGCCCAGTGKTG-3′ targetting TGVTLTLFF- and HHWAALMF- of consensus amino acid sequence of cytochrome *b*; and 5′-TTCTACC-TSACSACSGGYTTCCAYG-3′ and 5′-CCAGAC-SAYGTCSACRAARTGCCA-3′ targetting LDISFVRG- and QIQHWA- of cytochrome oxidase subunit III (ctaE), which is located at the 5′-upstream region of the *qcrC* gene in *C. glutamicum* [13]. The PCR products (95 °C 60 s/54 °C 60 s/65 °C 90 s for 35 cycles), about 170 and 130 bp, were ligated into pUC118, sequenced to confirm the validity of the clones, and named Rrh11 and cta11, respectively, as shown in Fig. 1. Since Rrh11 did not cover the C-terminal part of *qcrB*, we firstly tried to obtain the larger fragment, named qcr 11 using the inverse PCR method [19]. For this purpose, *Bgl*II-cut total DNA was self-ligated at a low concentration, and then PCR was carried out using this DNA as the template in the presence of the set of primers, pr-qb2 and pr-qb3. The resultant PCR product was about 1.2 kb, and was shown to be the correct gene. Qcr21 was prepared by PCR using a sense primer of pr-ox3 and an antisense primer of pr-qbr. In order to obtain the whole region of *qcrB*, we also cloned qcr41 using the cassette PCR method [20] with pr-qbf for the first PCR and *Pst*I cassette-ligated *Eco*T22I-digested *R. rhodochrous* DNA as the template. The second PCR was carried out using pr-qb31, which has a sequence in the 3′-downstream part of qcr31, formerly obtained by a similar cassette PCR method. The sequences of these primers are as follows: pr-qb2, 5′-GTGGGACGCGCCGAGTGCTACAGC-3′; pr-qb3, 5′-TTCGTCCGGCAGATCCAACACTGG-3′; pr-ox3, 5′-ACCGCAGCGATCGTCGTCTCCTATTACTGGC-3′; pr-qar, 5′-CGACATGCCTGTCGTCTGGTTGGCGTAG-3′; pr-qbf, 5′-TCGCGGTGCTGGCCCTGATGA-3′; and pr-qb31, 5′-GAAGAAGTTCACCAAGGACG-3′. The DNA sequencing was carried out using the dye primer method with pUC118 in a Shimazu DNA sequencer (DSQ-1000). General gene manipulations were carried out according to methods described by Sambrook et al. [21]. The sequence data were analyzed with a Genetyx (Tokyo)

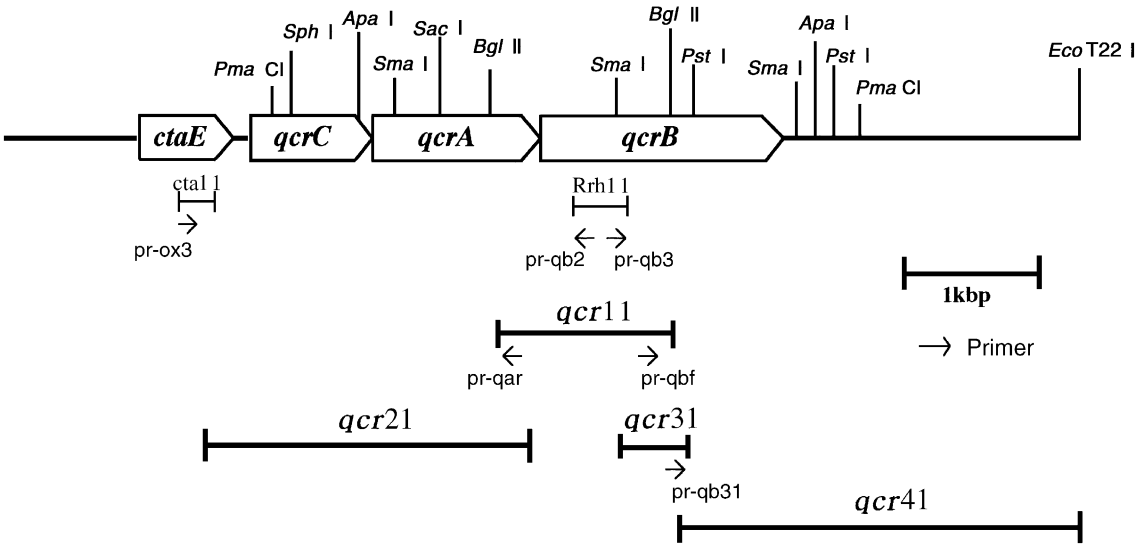


Fig. 1. A map of *R. rhodochrous* DNA around the *qcrCAB* gene cluster encoding cytochrome *cc*, the Rieske protein and cytochrome *b*, and sequence strategy. The six DNA fragments were cloned as described in the text. The name of the restriction enzymes used for subcloning in sequencing are shown in the top part of the figure. Primers (→) used for gene walking by inverse PCR and cassette PCR methods are also shown.

software program (ver. 7.1). The multiple alignment and phylogenetic tree was constructed by Clustal W [22].

Fig. 1 shows a map of the *R. rhodochrous qcrCAB* gene cluster, which is found just downstream of *ctaE* encoding for subunit III of the aa_3-type cytochrome oxidase. In fact, the predicted 2.3 kb DNA, containing the sequences encoding QcrC and QcrA, was amplified by PCR with a sense primer targetting C-terminal part of CtaE (pr-ox3) and an antisense primer targetting the C-teminal region of QcrA (pr-qar), which is shown as qcr21 in the figure. The deduced amino acid sequence of *qcrC* shows a presence of a unique cytochrome c_1-like subunit containing two heme C motifs in 277 amino acid residues. *QcrA*, the second gene of the operon, encodes a polypeptide composed of 373 amino acid residues containing the Rieske Fe–S protein motifs. The last gene, *qcrB*, encodes cytochrome *b*, which is composed of 549 amino acid residues with 4 His residues for two protoheme-binding sequences, and is followed by a terminator structure with inverted repeats. Since the *qcrCAB* cluster of *R. rhodochrous* is similar to those of *M. tuberculosis* [23], *C. glutamicum* [14,16] and *S. coelicolor* [24], the gene structure found in *R. rhodochrous* seems to be common in high G+C Gram-positive bacteria.

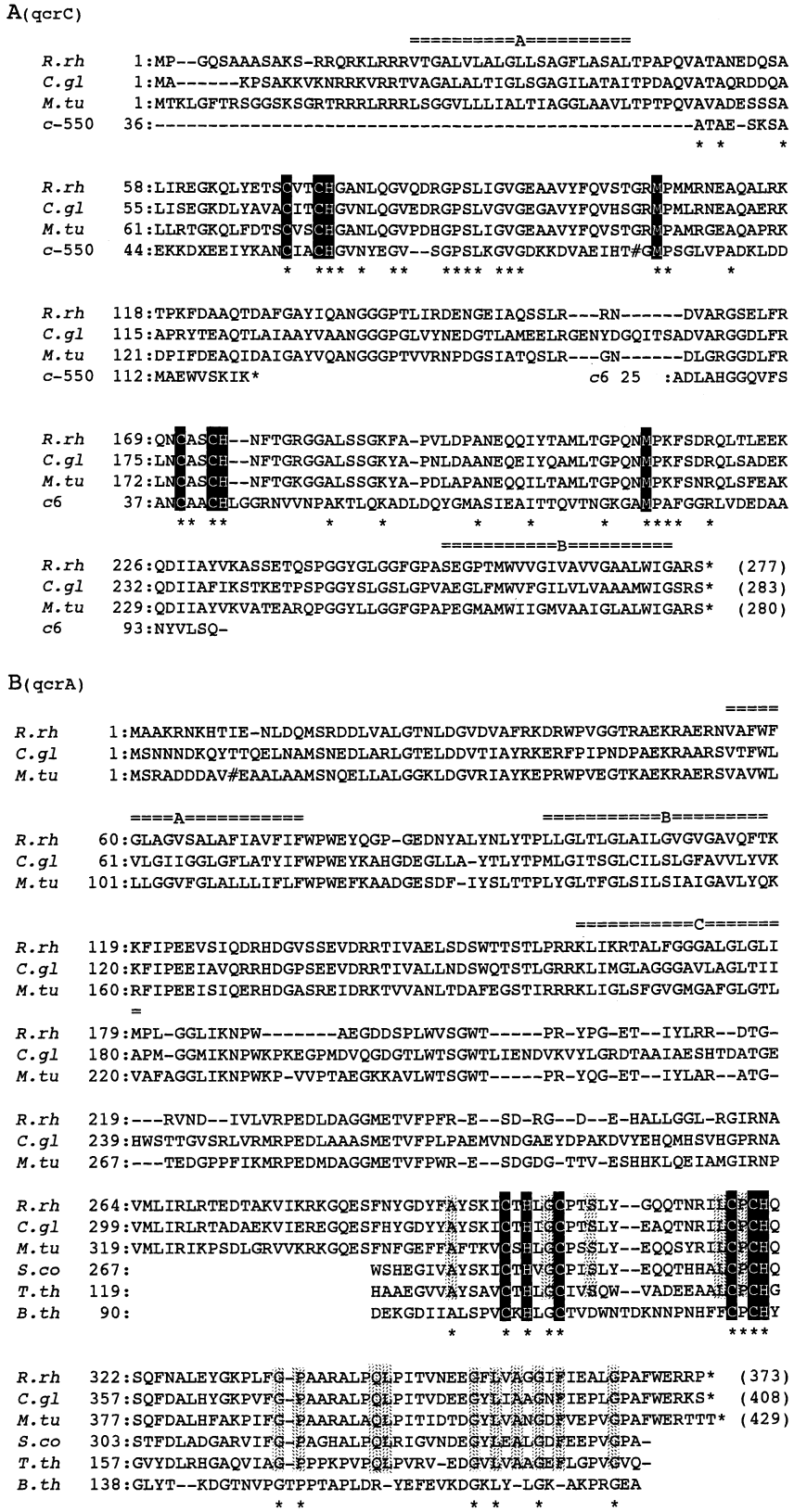
Fig. 2A shows an alignment of the QcrC of *R. rhodochrous*, *C. glutamicum*, [14,16] and *M. tuberculosis* [23]. The *S. coelicolor* sequence [24] is also homologous (not shown), although its amino acid identity is 46.7%, a little less than those of *C. glutamicum* and *M. tuberculosis*, which are 63.6% and 69.5%, respectively. Of the two hydrophobic regions, the N-terminal one (V19 to L35, A) may be a part of the signal sequence because the molecular mass of the mature cytochrome of *R. rhodochrous* is 26 kDa by SDS-PAGE (not shown), a little smaller than that expected from the DNA sequence (29,841 Da). It is possible to regard this 26-kDa diheme protein as a tandem product of two class I cytochromes *c* as in the case of *C. glutamicum* QcrC [14]. A homology search (Blast) with the protein data bank showed that the first N-terminal cytochrome *c* closely resembles the *Bacillus* small cytochromes *c* [25], especially *B. subtilis* cytochrome *c*-550 (CccA) [26]. On the other hand, the C-terminal cytochrome *c* resembles the cytochrome c_6 group of cyanobacteria.

The alignment of cytochrome c_6 of *Synecococcus* 6803 with the C-terminal half of the QcrC of *C. glutamicum* was also shown in a previous report [14]. It is worth mentioning that the *Bacillus* QcR contains a unique cytochrome c_1, named IVc, in which the *Bacillus* small *c*-like cytochrome is attached to the C-terminal side of subunit IV by gene fusion [11,12]. In the QcR of high G+C content Gram-positive bacteria, another type of unique diheme *c*-type cytochrome seems to be active, as the sole *c*-type cytochrome in the cells. *R. rhodochrous* membrane fraction was electrophoresed in the presence of SDS, and we found that the 26-kDa band only shows peroxidase activily (not shown) as in the case of *C. glutamicum* and *M. tuberculosis* [13]. It is thus likely that one of two hemes, probably the N-terminal one similar to the *Bacillus* small *c*, may directly donate electrons to the aa_3-type terminal oxidase, as in the QcR-caa3-type cytochrome *c* oxidase supercomplex of the thermophilic *Bacillus* species [27]. It is worth pointing out that *c*-type cytochromes in Gram-positive bacteria should be membrane-bound because they lack an outer membrane. Cytochrome *c*, mediating the electron transfer between QcR and cytochrome oxidase, seems to fuse to the cytochrome *c* subunit of QcR in the high G+C Gram-positive bacteria, while the cytochrome *c* of *Bacillus* is found in subunit II of caa_3-type cytochrome *c* oxidase [28]. The heme C in the C-terminal domain, which is similar to cytochrome c_6, on the contrary, may accept electrons from the Fe–S protein because cytochrome c_6 is the electron acceptor from QcR. It is also worth noting that the heme-region sequence of mono-heme QcrC from *Deinococcus* shows 32% identity with the corresponding C-terminal half of *R. rhodochrous* QcrC, suggesting that the C-terminal part plays a role for cytochrome c_1.

Fig. 2B shows an alignment of the Rieske Fe–S protein of *R. rhodochrous* with those of *C. glutamicum* and *M. tuberculosis*, which are composed of three N-terminal transmembrane helices and a C-terminal region with the characteristic Rieske Fe–S motif (CTHXGCP-12X-CPCH). The N-terminal part of these high G+C firmicutes is quite different from those of cyanobacteria/plastids and proteobacteria/mitochondria groups, which contains one transmembrane helix, although they are not homologous [7,29,30]. Conversely, the C-terminal part containing the Fe–S motif is conserved in the sequences of various species. In the figure, QcrAs of *S. coelicolor*, *T. thermophilus* and *B. thermodenitrificans* are also aligned. Identity in this region indicates that the *R. rhodochrous* sequence is more similar to that of *C. glutamicum* than that of *M. tuberculosis* in the high G+C group, or that of *T. thermophilus* in the out-of-group because the percent identities with *R. rhodochrous* were 82.9 (*C.gl*), 67.1 (*M.tu*), 62.7(*S.co*), 41.1(*T.th*) and 25.7(*B.th*).

Fig. 2C shows an alignment of *R. rhodochrous* cytochrome *b* with those from *C. glutamicum* and *M. tuberchrousis.* These cytochromes *b* are composed of 539–549 amino acid residues and 7 transmembrane helices. Two pairs of heme-ligating His residues are conserved in the transmembrane helices B and D and several residues in the vicinity are highly conserved. In general, many residues are conserved among high G+C Gram-positive bacteria and out of this group cytochrome *b* from *T. thermophilus* shows a close similarity (not shown). The percent identity between the N-terminal half, including helices A–D of *R. rhodochrous* cytochrome *b* and those of *C. glutamicum*, *M. tuberchrousis*, *S. coelicolor*, *T. thermophilus* and *B. thermodenitrificans* were 72.2%, 81.1%, 60.8%, 41.7% and 32.0%, respectively. It is also worth mentioning that the two His residues in helix D of these bacteria are separated by 14 residues, as in cytochrome b_6, and not by 13 residues as in the cytochromes *b* of proteobacteria [7]. The 13- residue separation seems to be specific for proteobacte-

rial cytochrome *b*, and is not found in other counterparts. Two His residues in helix B of high G+C Gram-positives are separated by 13 residues in almost all cytochrome b/b_6 [7]. The total length of cytochrome *b* from high G+C Gram-positives is 539–549, which is longer than proteobacterial/mitochondrial cytochrome *b*. The main reason for this is the longer extension after the transmembrane helix G, which contains hydrophobic sections, but is not sufficiently clus-



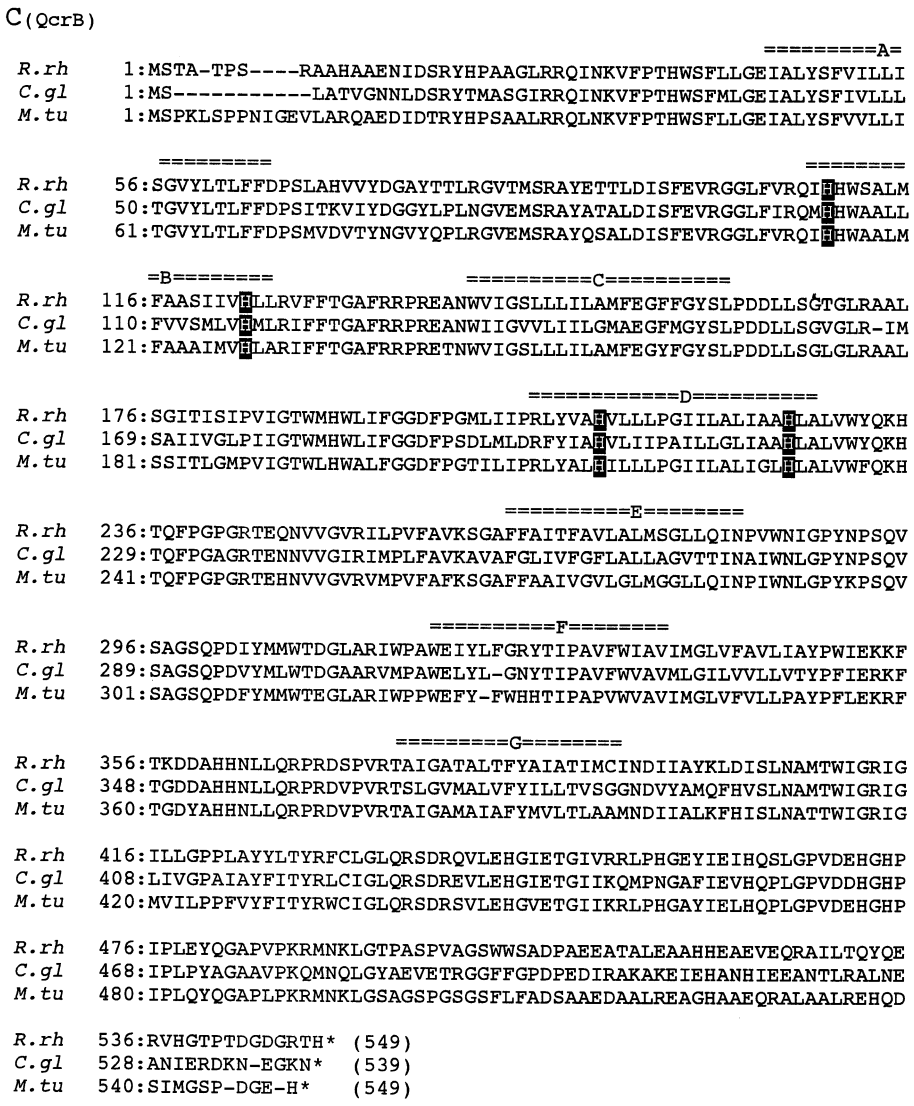


Fig. 2. Multiple alignment of the three subunit proteins of quinol cytochrome *c* reductases. Residues identical in all sequences are shown (*), while gaps are indicated by (-). The residues conserved only in *R. rhodochrous* (*R.rh*), *C. glutamicum* (*C.gl*) and *M. tuberculosis* (*M.tu*) are not marked, but the residues conserved in the high G+C firmicutes (the above three species and *S. coelicolor* (*S.co*) and *T. thermophilus* (*T.th*) are shadowed. The putative trans-membrane regions are marked and numbered (===). The residues that may ligate metal centers or heme C are shaded in black. (A) Cytochrome *cc* are compared with *B. subtilis* cytochrome *c*-550 (cccA). X and # in the sequence of *c*-550 denote ANASP and KIEKG, respectively. (B) Rieske Fe–S proteins of three high G+C Gram-positive bacteria are compared. At the C-terminal third region, where the sequences of many species are conserved, several sequences including those of *T. thermophilus* (*T.th*) and *B. thermodenitrificans* K1041(*B.th*) are also aligned. (C) Cytochromes *b* of three high G+C firmicutes are compared.

tered to be transmembranous. Hydropathy analyses indicate that seven to eight hydrophobic domains exist, except the fourth amphipathic alpha helix part between the transmembrane helices C and D. Cytochrome *b* of this group also has a longer insertion section before the transmembrane helix A than that of *T. thermophilus*.

The N-terminal half of the Rieske Fe–S proteins of high G+C Gram positives have a unique structure with three transmembrane helices, as shown in Fig. 2B, while the C-terminal half sequences are conserved. Using the latter region, we constructed a phylogenetic tree using the neighbor-joining method [31]. Fig. 3 shows that the high G+C firmicutes form a clear clade with *Thermus* and *Deinococus*. On the other hand, Gram-positive bacteria with low G+C content are closer to those containing cyanobacterial cytochrome b_6. A phylogenetic tree based on 16S rRNA showed the closest relationship between Gram-positive bacteria, high G+C and low G+C groups [32]. However, the present results showed that the old core genes of *qcrA* and *qcrB* in high G+C Gram-positive bacteria are not closely related to those of low G+C firmicutes, but form a clear clade with radio-resistant cocci. Although phototrophs are not found in either group, the variation and origin of QcR are worth noting. It is important and fruitful to study the molecular evolution of this superfamily common in respiration and photosynthesis.

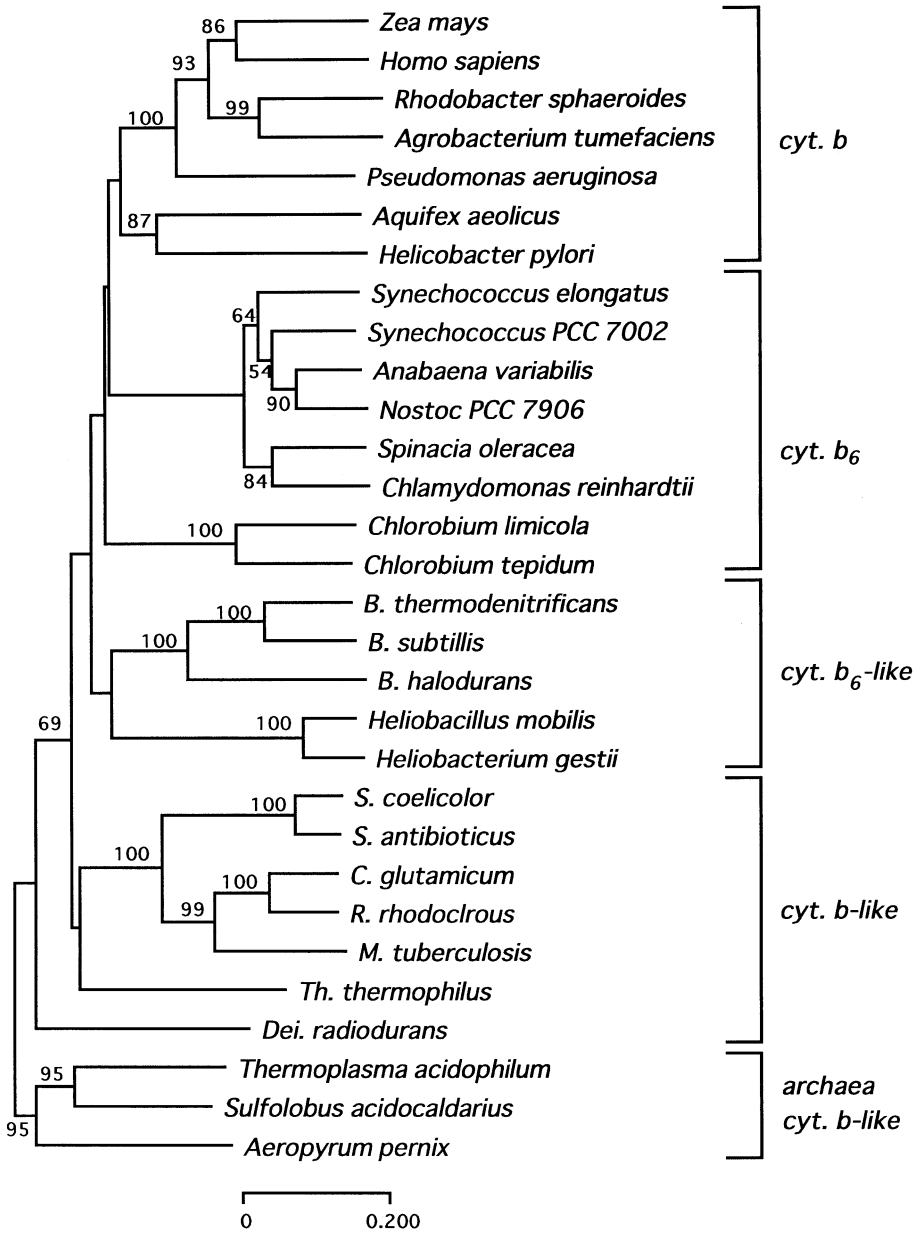


Fig. 3. A phylogenetic tree for the C-terminal one third of the Fe–S protein. Most sequences are obtained from the DDBJ data bank except those of *C. limicola* (x73628), *A. aeolicus* (ae000671), *C. glutamicum* [14], *R. rhodochrous* (the present work), *T. thermophilus* (N. Sone et al, unpublished observation), *B. thermodenitrificans* K1041 [11], *A. pernix* (AP 000062-168), and *S. acidocaldarius* SoxC and SoxG (z48338). Figures represent bootstrap confidence levels from 1000 bootstrap samples for the grouping. The scale bar represents a distance of 20%.

We constructed a phylogenetic tree using cytochrome b_6-corresponding sequences when the *C. glutamicum qcrCAB* genes were sequenced [14]. This tree also clearly showed the presence of a fourth clade of high G+C firmicutes plus *Deinococcus–Thermus* group (cytochrome *b*-like group) in addition to the proteobacterial cytochrome *b* and cyanobacterial cytochrome b_6 groups and the cytochrome b_6-like group of *Heliobacillus* and *Bacillus* [33]. The analysis of the sequences of the same region, including the datum of *R. rhodochrous* did not change this conclusion. Another tree, a little different from that described here, was reported by Schutz et al. [34], in which the *D. radiodurans* sequence locates close to the *Bacillus* group without using the sequences of the high G+C content firmicutes. However, if several sequences of newly elucidated high G+C content firmicutes are also aligned, a new clade including the high G+C content firmicutes and *Deinococcus–Thermus* group is clearly identified.

The present data and their comparison with those from other species show the following facts: (1) the C-terminal part of the Fe–S protein including the Fe–S cluster and cytochrome *b* (b_6+subunit IV) are the central core of QcR; (2) the N-terminal parts of the Fe–S proteins originate from different proteins depending on the group, and (3) electron-

accepting *c*-type cytochromes also differ from group to group. Furthermore, their positions in the operons are sometimes at the 5′-upstream side of the *qcrA–qcrB* cluster in some group, and at the 3′ -downstream side in the others. The proteobacterial ancestor had the anchor segment in the 5′-upstream end of the Fe–S gene and c_1 gene at the 3′-downstream side of the cytochrome *b* gene, while high G+C firmicutes had a novel cytochrome *cc* protein and three transmembrane sections in the N-terminal part of the Fe–S protein. In contrast, the ancestor of another group of bacteria had a prototype protein as the N-terminal half of the Fe–S protein. In that case, a stop codon was inserted into the cytochrome *b* of the cyanobacterial ancestor to divide it into cytochrome b_6 and subunit IV, and the gene for the Fe–S protein moved to a different place and the cytochrome *f* gene was inserted into 3′ -downstream. *Chlorobium* ancestry must precede these changes because it does not have a stop codon in cytochrome *b*. *Heliobacillus* and *Bacillus* also have split-type cytochromes *b*, i.e. b_6 + subunit IV. The *Heliobacillus petCBD* subcluster encoding for the Fe–S protein, cyt. b_6 and subunit IV is located in the midst of the large *pet* gene cluster, which also includes the monoheme and diheme *c*-type cytochromes [32], while *Bacillus* cytochrome c_1 is present as the C-terminal half with subunit IV [9]. It is tempting to speculate that the three types of QcR are correlated to the three types of bacterial photosynthesis; the proteobacterial PSII-type, the PSI-type of green sulfur and Gram-positive (low G+C content) bacteria, and the cyanobacterial oxygenic PSI+PSII type. Alternatively, bacteria that do not include any phototroph, such as the *Deinococcus–Thermus* group and the high G+C firmicutes, may conserve the prototype QcR gene, even if *c*-type cytochromes, and the N-terminal half portion of the Fe–S protein have been subsequently added.

Acknowledgements

We thank Dr. K. Nagata of Hyogo Medical College for donation of the strain of *R. rhodochrous*. We thank also Dr. W. Nitschke of Institut de Biologie Structurale et Microbiologie, Marseille for valuable information.

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