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A novel hydrophobic diheme *c*-type cytochrome. Purification from *Corynebacterium glutamicum* and analysis of the *QcrCBA* operon encoding three subunit proteins of a putative cytochrome reductase complex[1]

Nobuhito Sone [a,*], Kumiko Nagata [b], Haruka Kojima [a], Junnichi Tajima [a], Yoriko Kodera [a], Tomoe Kanamaru [a], Shunnsuke Noguchi [a], Junshi Sakamoto [a]

[a] *Department of Biochemical Engineering and Science, Kyushu Institute of Technology, 680-4 Kawazu, Iizuka, Fukuoka-ken 820-8502, Japan*
[b] *Department of Bacteriology, Hyogo college of Medicine, 1-1, Mukogawa-cho, Nishinomiya 663, Japan*

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Abstract

Electrophoresis of a *Corynebacterium glutamicum* membrane preparation in the presence of sodium dodecyl sulfate, followed by staining for peroxidase activity (heme staining), showed only one band at about 28 kDa. This 28 kDa protein was purified from *C. glutamicum* membranes by chromatography in the presence of decylglucoside using DEAE–Toyopearl and hydroxylapatite columns, as the sole *c*-type cytochrome in the bacterium. The cytochrome showed an alpha band at 551 nm, and its $E_{m,7}$ was about 210 mV. A *QcrCAB* operon encoding the subunits of a putative quinol cytochrome *c* reductase was found 3′-downstream of *ctaE* encoding subunit III of cytochrome aa_3 in the *C. glutamicum* genome. The deduced amino acid sequence of *qcrC*, composed of 283 amino acid residues, contained two heme C-binding motifs and was in agreement with partial peptide sequences obtained from the 28 kDa protein after V8 protease digestion. We propose to name this protein cytochrome *cc*. The presence of cytochrome *cc* is a common feature of high G+C content Gram-positive bacteria, since we could confirm this protein by electrophoresis; homologous *QcrCAB* operons are also known in *Mycobacterium* and *Streptomyces*. *QcrA* and *qcrB* of *C. glutamicum* encode the Rieske Fe–S protein and cytochrome *b*, respectively, although these proteins were not co-purified with cytochrome *cc*. The phylogenetic tree of cytochromes *b* and b_6 show that *C. glutamicum* cytochrome *b*, along with those of other bacteria in the high G+C group, is rather different from the *Bacillus* counterparts, but highly similar to the *Deinococci* and *Thermus* cytochromes. 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. © 2001 Published by Elsevier Science B.V.

Keywords: Quinol cytochrome *c* reductase; Cytochrome *cc*; Glutamate fermentation; (*Corynebacterium glutamicum*)

Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ORF, open reading frame; PCR, polymerase chain reaction; bp, base pairs

* Corresponding author. Fax: +81-948-297801; E-mail: sone@bse.kyutech.ac.jp

[1] The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank/EMBL Data Bank with accession number AB038380.

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1. Introduction

The quinol–cytochrome *c* reductases constitute a large superfamily of enzymes found in mitochondrial and bacterial respiratory chains, and in plastid and bacterial photosynthetic electron transfer chains. These enzymes translocate protons across the membranes coupled 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 iron–sulfur protein and cytochrome c_1/f. Cytochrome b/b_6, having two protohemes for facilitating electron transfer across the membrane, 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) almost correspond to the cytochrome *b* in mitochondria and proteobacteria [5,6]. Determining the extent of sequence similarity makes it possible to evaluate the molecular evolution of these proteins [7]. A recent analysis was made by constructing a phylogenetic tree including *Chlorobium limicola* cytochrome *b* [8], *Bacillus* cytochrome b_6 [9,10] and *Heliobacillus mobilis* cytochrome b_6 [11] in addition to various cytochromes b_6 and purple bacterial cytochromes *b*. The results indicate that these sequences form a new clade between proteobacterial cytochrome *b* and cyanobacterial cytochrome b_6, and suggest that this correlates with the development of non-oxygenic to oxygenic photosynthesis [11]. The Rieske iron–sulfur protein and a *c*-type cytochrome are peripheral membrane proteins necessary for the enzymes to transfer electrons to cytochrome *c*. The C-terminal half of the former has a non-heme iron-binding motif and is homologous to the corresponding subunit of quinol–cytochrome *c* reductase, but the N-terminal halves of proteobacterial and mitochondrial proteins are quite different from cyanobacterial and plastid proteins [12]. Cyanobacterial cytochrome *f* and proteobacterial cytochrome c_1 seem to develop from different parental proteins in terms of sequence similarity [12]. Recent cloning and sequence analysis of the gene for the *Thermus thermophilus* Rieske protein revealed that this protein, along with *Chlorobium* and *Bacillus* proteins cannot be included in either the proteobacterial and mitochondrial proteins group or the cyanobacterial and plastid protein group [13].

We have been involved in studying the respiratory chains of Gram-positive bacteria, such as the *Bacillus* PS3 and *B. stearothermophilus*, leading to the identification, characterization, and gene cloning of the respiratory complexes. In these thermophilic bacilli, cytochrome b_6c_1-type quinol–cytochrome *c* reductase [9,10,14] and caa_3-type cytochrome *c* oxidase [15–17] form a super-complex and plays a major role in the respiratory chain [18]. Cytochrome reductase purified from *Bacillus* PS3 contains four chromophores in a 29 kDa cytochrome c_1, a 23 kDa Rieske iron-sulfur protein, and a 21 kDa diheme cytochrome *b* [14]. The diheme *b*-type cytochrome is more similar to cyanobacterial-plastid cytochrome b_6, than to proteobacterial and mitochondrial cytochrome *b*. Cloning and sequencing of the structural genes of quinol–cytochrome *c* reductase subunits revealed that *qcrABC* encodes the iron-sulfur protein, cytochrome b_6 and cytochrome c_1 in this order [9,10,19], and thus its gene order is superficially similar to that of the proteobacterial gene, *fbc/petABC* operon [20,21], but different from the genes of cyanobacteria composed of *petAC* and *petBD* which are located in different sites [6]. *Bacillus* cytochrome c_1 has an unique structure, composed of subunit IV in the N-terminal and a type of class I cytochrome *c* (see [22]) at the C-terminus, and thus different from both proteobacterial and cyanobacterial counterparts [10,19]. The presence of this new type cytochrome c_1 may correlate with the fact that Gram-positive bacteria have no periplasmic space and all *c*-type cytochromes acting at or facing this space must be membrane-bound. In fact, c_2-type cytochrome *c*, as the substrate of the main terminal oxidase is fused to subunit II of the usual cytochrome aa_3 to form the 'IIc protein' of cytochrome caa_3 [16]. Also, a small cytochrome *c*-550 [23] and cytochrome *c*-551 [24,25] are membrane-bound because of the signal peptide (the former) or the diacyl glycerol moiety being attached to a N-terminal Cys residue after processing (the latter).

Gram-positive bacteria fall into two major subdivisions, namely high or low G+C content in the chromosomal DNA [26]. There are several aerobes such as *Corynebacterium*, *Mycobacterium* and mycerial bacteria belonging to the high G+C group, while *Bacillus* bacteria are known as typical members of

the aerobic low G+C content group. *Corynebacterium glutamicum* is of industrial importance in producing amino acids for nutritious supplement to food and feed, but to date, little has been reported on its respiratory chain, although the relationship between respiration ability and glutamate formation has been studied [27]. The presence of *a*-, *b*- and *c*-type cytochromes was spectroscopically identified in *C. glutamicum* (*Brevibacterium lactofermentum*), formerly called *Brevibacterium lactofermentum* [28]. Recently, the presence of superoxide-generating cyanide-resistant NADPH oxidation systems was identified in *C. glutamicum* in addition to the cyanide-sensitive respiratory chain with *a*-, *b*- and *c*-type cytochromes [29].

During a study on the respiratory chain of *C. glutamicum* we found a 28 kDa cytochrome containing two C hemes as the only protein with covalently bound heme C. We also cloned the genes for this *c*-type cytochrome with the Rieske protein and cytochrome *b* as *qcrCAB* operon of this bacterium. Here we report the characteristics of this protein, including the deduced amino acid sequence which is encoded in the *qcrC* gene in the *qcrCBA* operon for the putative quinol–cytochrome *c* reductase of this bacterium. The characteristics and phylogenetic positions of other proteins deduced from the *qcrCBA* operons of high G+C content Gram-positive bacteria such as *C. glutamicum*, *Mycobacterium tuberculosis* [30] and *Streptomyces coelicolor* [31] are also reported.

2. Materials and methods

2.1. Material

Dimethylaminoethyl–Toyopearl and hydroxylapatite were purchased from Tosoh (Tokyo), and Bio-Rad (Hercules), respectively. Polyvinylidene difluoride membranes for protein blotting were purchased from Millipore (Bedford). Decyl, α,β-glucoside (decylglucoside) were the product of Kao Co. (Tokyo). Other reagents were of analytical grade.

2.2. Bacterial strains and growth conditions

Corynebacterium glutamicum KY 9002 (ATCC 13032) and *Corynebacterium glutamicum* (*Brevibacterium lactofermentum*) (ATCC13869) were kindly donated by Professor K. Matsushita of Yamaguchi University and Dr Y. Kawahara of the Ajinomoto Central Research Institute, respectively. *C. glutamicum* KY 9002 was cultured as described previously [29]. *C. glutamicum* (*B. lactofermentum*) was grown aerobically at 30°C in a medium containing 50 g sucrose, 5 g polypeptone, 5 g urea, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4$, 0.01 g $MnCl_2 \cdot 4H_2O$, 5 mg nicotinamide, 0.2 mg thiamine and 0.05 mg biotin per liter at pH 7.5. Two-hundred-ml cultures were inoculated with a 6 ml starter culture and then used to inoculate a 6-l culture in a jar fermenter, model MBF-801 (Eyela, Tokyo). *Mycobacterium tuberculosis* strain H37Ra was cultured at 37°C in synthetic Sauton medium for 20 days [32]. The cultured cells were harvested by centrifugation (10 000 × *g* for 10 min at 4°C), washed with PBS (0.139 M NaCl; 3 mM KCl; 10 mM potassium phosphate) at pH 7.0, and stored as a cell pellet at −80°C. A 2.2-g (wet weight) cell pellet was suspended in a preparation medium containing 0.25 M sucrose, 10 mM KCl, 1 mM 2-mercaptoethanol, 0.1 mM EDTA, 20 mM Tris–HCl buffer (pH 6.5) and 10 mM potassium phosphate buffer (pH 6.8), and the suspension was ground with 2 g of alumina, type 305 (Sigma, St. Louis, MO) in a mortar. After centrifugation (10 000 × *g*, 20 min, 4°C) to remove cell debris, the supernatant was centrifuged (100 000 × *g*, 90 min, 4°C), and the pellet was suspended in the preparation medium and used as the membrane fraction. The protein concentration of the membrane fraction was 20 mg/ml. *S. azureus* was cultured aerobically at 30°C in L-broth, and harvested at the late log phase, and the membrane fraction was prepared as described avobe for *M. tuberculosis*.

2.3. Purification of cytochrome cc

The crude membranes, prepared from *C. glutamicum* cells as described previously [29], were washed at 10 mg protein/ml in 200 ml of 1% (w/v) sodium cholate with 0.5% sodium deoxycholate in the presence of 0.1 M NaCl and 10 mM Na–P_i buffer at pH 6.8. The intrinsic membrane proteins, sedimented at 100 000 × *g* for 45 min, were solubilized at 10 mg protein/ml in 1% (w/v) decylglucose in the presence of 0.05 M K–P_i buffer (pH 6.5) with brief sonication. The mixture was centrifuged at 100 000 × *g* for 30

min and the supernatant was dialyzed against 20 mM Tris–HCl buffer for 3 h. The dialyzed supernatant was then applied to a DEAE–Toyopearl column (1.6×8.0 cm), pre-equilibrated with 1% (w/v) decylglucoside containing 20 mM Tris–HCl buffer, pH 7.2. Proteins were eluted stepwise by increasing the NaCl concentration in 1% decylglucoside containing 20 mM Tris–HCl buffer, pH 7.2. The red colored peak fractions (60–80 mM NaCl) were combined and applied to a hydroxyapatite column (0.5×1.5 cm), and the column was washed with 1% octylglucoside containing 20 mM Tris–HCl buffer, pH 7.2. The *c*-type cytochrome was eluted with 1% (w/v) decylglucoside containing 5 mM sodium phosphate buffer, pH 6.8. This fraction was concentrated through a Centricon 30 (Amersham) and the concentrated preparation (0.1 ml) was fractionated with a gel filtration column (Toso G3000SW) by high-pressure liquid chromatography. The column was equilibrated with 10 mM Tris–HCl buffer (pH 7.2) containing 0.1 M NaCl and 0.05% decylglucoside. A concentrated aliquot (0.1 ml) from step 4 was injected at 0 time, and the flow rate was 0.8 ml/min. The peak fraction was used as it was or after being concentrated.

2.4. Cloning and sequencing of the gene

QcrCAB was cloned by colony hybridization of *Escherichia coli*×L1-b transformed with a library of *C. glutamicum (B. lactofermentum)* in pUC18 which was kindly donated by Dr Kawahara of the Central Research Institute of Ajinomoto [33]. For this a 700 bp DNA fragment of *C. glutamicum ctaC/caaA* (probe1) was labeled with digoxigenin following the manufacture's protocol (Boehringer Mannheim), and used as the probe. By selecting about 5000 colonies, one positive clone containing both *ctaC* and *qcrC* was obtained and named pBLC1. The Bl2 and Bl3 regions of the DNA were cloned in the gene-walking fashion using 154 bp DNA (probe 2) from *qcrC* in pBLC1, and 500 bp DNA from *qcrA* in pB12 (probe 3) as the probes, respectively (cf. Fig. 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 made according to methods described by Sambrook et al. [34].

2.5. Spectrophotometric measurement and potentiometric titration

Absorption spectra were recorded using a Beckman DU-70 spectrophotometer at room temperature. The spectra of air-oxidized enzymes were taken and then a few grains of solid sodium dithionite were added to obtain their reduced forms. Contents of heme C were determined as described previously [35]. In the case of membrane preparations, proteins were extracted with 5% (w/v) Triton X-100 before the redox spectra of the supernatant was measured. The redox potential was measured using a Pt electrode with a calomel reference (Toa Denpa PTS-5011C) as described previously [14]. The partially purified but spectrally pure cytochrome at 6.8 μM (without fractionation on the HPLC gel filtration column) was used in 0.1 M Na–P_i buffer (pH 7.0) containing 1% decylglucose, 10 mM ferrocyanide, 0.2 mM sodium ascorbate, 10 μM hydroquinone, 10 μM benzoquinone and 20 μM 2,6-dichlorophenol indophenol. The ambient redox potential was changed by adding a small aliquot of 0.1 M ferricyanide.

2.6. Miscellaneous methods

Protein concentration was determined by the method of Lowry et al. [36] after precipitation with 5% trichloroacetic acid in the presence of 0.05% sodium deoxycholate. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [37], except that the protein samples were not boiled. For sequence analysis, proteins obtained at step 4 and concentrated by Centricon 30 were separated by SDS–PAGE, and electro-transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was washed extensively with water to remove glycine, treated with 0.6 N HCl at room temperature for 24 h to release, a possible N-terminal formyl groups [38], and applied to a pulse-liquid peptide sequencer, model 477A (Applied Biosystems). Proteolytic fragments for sequencing were obtained using *Staphylococcus aureus* V8 protease as described previously [39]. The sequence data were analyzed with a Genetix (Tokyo) software program (ver. 7.1). The multiple alignment and phylogenetic tree was constructed by Clustal W [40].

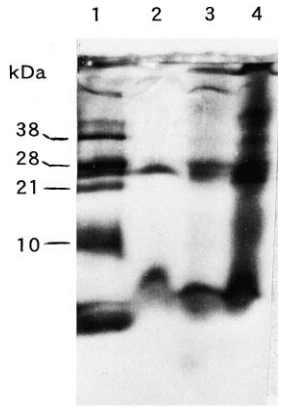

Fig. 1. SDS–PAGE pattern of membrane fractions showing cytochromes with covalently bound heme. The concentration of the gel was 13.5%, and it was stained for heme with *o*-toluidine in the presence of H_2O_2 [42]. Lane 1, *B. stearothermophilus* membranes; lane 2, *C. glutamicum* membranes; lane 3, *M. tuberculosis* membranes; lane 4, *S. azuleus* membranes.

3. Results and discussion

3.1. Detection of c-type cytochromes in high G+C Gram-positive bacteria

C-type cytochromes contain covalently bound heme C through thioether bonds at the motif –CXXCH–. It was demonstrated that one of heme B of cytochrome b_6 is covalently bound to the polypeptide [41], but this is probably a rare case. In order to determine how many kinds of *c*-type cytochromes are present and whether there is any covalently bound heme B, we analyzed the membrane fractions of high G+C content Gram-positive bacteria by SDS–PAGE, and stained for peroxidase activity on the gel, as shown in Fig. 1. Generally, one band was stained at about 28 kDa in lanes 2–4 for the membranes from *C. glutamicum*, *M. tuberculosis* and *S. azureus*, respectively. This is very clear in lane 2, but two bands around 28 kDa are seen in lane 3 and several unclear bands at high molecular mass regions in addition to the main band are seen in lane 4. Conversely, *B. stearothermophilus* membrane (lane 1) showed four bands which have been designed ctaB (38 kDa), qcrC (28 kDa), qcrB (22 kDa) and cccA (10 kDa).

3.2. Cytochrome cc from C. glutamicum

We then purified this 28 kDa heme C-containing protein from *C. glutamicum*. Cytochromes were solubilized by the addition of 1% decylglucoside, and chromatographed in the presence of this detergent; involving absorption to and release from DEAE–Toyopearl and hydroxylapatite and fractionation by gel filtration. The elution pattern of the final chromatography (step 5) showed that the *c*-type cytochrome was mainly found at around 35 kDa, which is almost proportional to the absorption at 280 nm. A small portion of the cytochrome eluted at the void volume with a higher ratio of proteins. Table 1 summarizes the results of a typical purification course. The final preparation contained 62 nmol heme C/mg protein, which was purified 885-fold from the crude membrane fraction.

Fig. 2A shows a typical result of SDS–PAGE analysis in which the final sample is almost pure at 28 kDa where the heme-stained band appeared (cyt. in Fig. 1). Since automated peptide sequencing of the 28 kDa polypeptide blotted on a sheet of PVDF membrane showed very low free N-terminal polypeptides, treatment of the 28 kDa band with V8 protease and succeeding gel electrophoresis was carried out. Automated peptide sequencing of the digested and electrophoresed peptides gave the following sequences: XQAERKAPRITEAQVLA- and LRGENYDGQITSADVARGGDLFRL-. Two residues in the first peptide sequence (I and V) were different from those deduced from the DNA sequence (Y and T), while the second sequence was completely identical

Table 1
Purification of *C. glutamicum* cytochrome *cc*

Purification step	Volume (ml)	Protein (mg)	Heme C (nmol)	Heme content (nmol/mg)
1. Membrane fraction	169	169	120	0.07
2. Decylglucoside extract	157	166	99	0.06
3. DEAE fraction	60	4.5	33	7.3
4. Hydroxyapatite fraction	10	0.45	12.7	29
5. Gel filtration	4	0.15	9.3	62

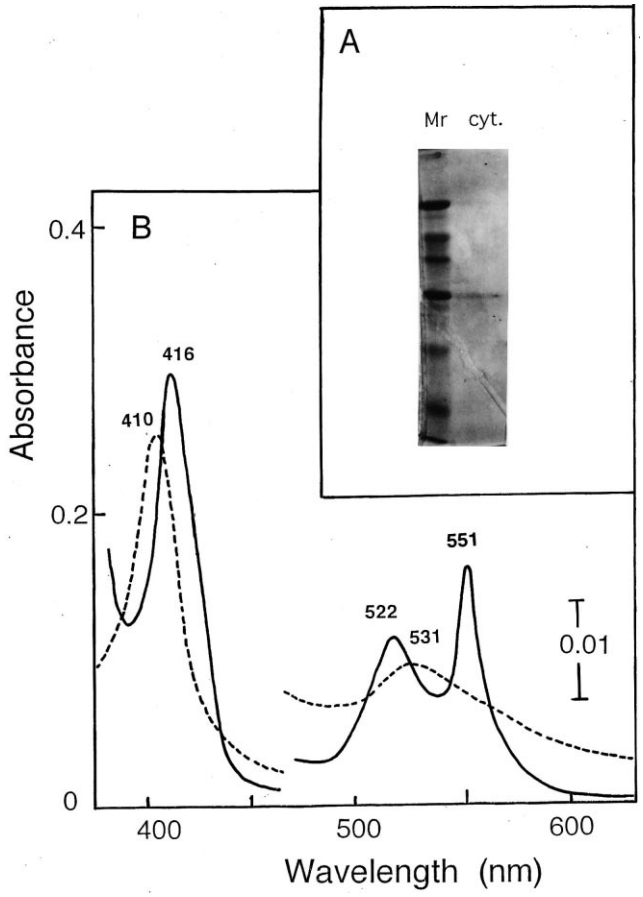

Fig. 2. SDS–PAGE pattern (A) and spectra of *C. glutamicum* cytochrome *cc* (B). (A) Marker proteins (Mr) are bovine serum albumin (66 kDa), ovalbumin (45 kDa), glycerol 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14 kDa). Cytochrome *cc* (cyt.) is the preparation from step 5 (0.57 μg). (B) $Na_2S_2O_4$-reduced form spectrum (continuous line) and, oxidized form spectrum as prepared (dotted line) are taken for the sample of 0.38 mg protein/ml.

with the corresponding sequence deduced from DNA. Since the phenylthiohydantoin derivative patterns of the cycle were clear, the difference is probably due to the use of different strains.

Fig. 2B shows the absorption spectra of the final preparation. It is noteworthy that cytochrome *b*, the other main cytochrome component of quinol–cytochrome *c* reductase, was not found in the final preparation. Although rough fractionation with DEAE–cellulose gave a fraction which contained both *c*-type cytochrome and cytochrome *b*-562 as previously described [29], and the *c*-type cytochrome-rich fraction from the DEAE–Toyopearl column in this study also contained a small amount of *b*-type cytochrome, this *b*-type cytochrome was separated from the *c*-type cytochrome on the hydroxyapatite column. The hydroxyapatite fraction for cytochrome *cc* contained no *b*-type cytochrome. Although the present double heme cytochrome in the fully reduced form showed an alpha-peak at 551 nm (Fig. 2B), there is an indication of the presence of two hemes C. The spectra, fully reduced minus half reduced, and half reduced minus oxidized, showed the peaks at 552 and 550.25 nm, respectively. indicating that the respective hemes C locate at the different places in the cytochrome. The average E_m value of the two hemes C was about 210 mV, and the E_m values of the individual hemes differed from this by not more than 40 mV; determination of more exact values is left as a future problem.

3.3. Cloning and gene structure of the QcrCAB operon

We have been trying to clone the genes encoding for cytochrome aa_3 in *Corynebacterium*. During this effort, a putative *qcrC* for the cytochrome c_1 subunit was found just 3′-downstream of *ctaC* for subunit II of cytochrome aa_3 (to be published). One clone selected from a library of *C. glutamicum* in pUC118 by hybridization with a DNA probe for the Cu_A-binding site of subunit II also contained a large part of *qcrC* downstream of the 4.5 kbp recombinant DNA (BLC1). We then selected two plasmids (pBl2 and pBl3) from a library of *C. glutamicum* (*B. lactofermentum*) which covered the whole *qcr* operon. Figs. 3 and 4 show a map of the *ctaCAB* region of this bacterium, and DNA and deduced amino acid sequences, respectively. The deduced amino acid sequence of *qcrC* tells us that qcrC, composed of 283 amino acid residues, contains two heme C motifs and two MP motifs, which probably form the sixth ligands for the Fe atoms. *QcrA*, the second gene of the operon, encodes a polypeptide composed of 392 amino acid residues containing the Rieske Fe–S protein motif. The last gene, *qcrB*, encodes cytochrome *b* which is composed of 539 amino acid residues with 4 His residues for two protoheme-binding sequences, and is followed by a terminator structure with inverted repeats. Since *qcrCAB* operons similar to that of *C. glutamicum* can be found in whole DNA

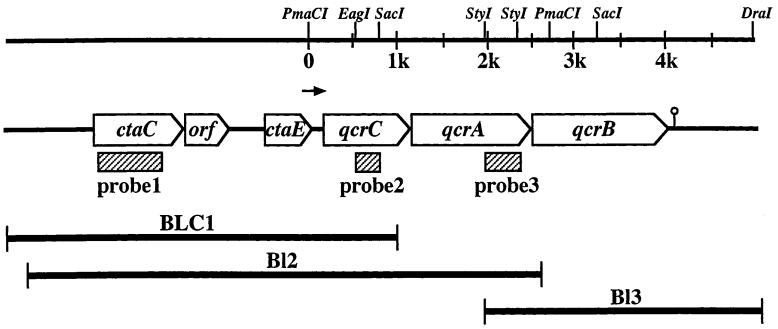

Fig. 3. A map of *C. glutamicum* (*B. lactofermentum*) DNA around the *qcrCAB* operon encoding cytochrome *cc*, the Rieske protein and cytochrome *b*, and sequence strategy. Three clones obtained cover the operon. Putative promoter (→) and terminator (♀) regions, and probes and several restriction sites used for cloning are shown.

sequences of *M. tuberculosis* [30] and *S. coelicolor* [31], the gene structure found in *Corynebacterium* seems to be common in high G+C Gram-positive bacteria.

3.4. Sequence characteristics of QcrC

Fig. 5A shows an alignment of the most part of the qcrC of *C. glutamicum*, *M. tuberculosis* [30] and *S. coelicolor* [31]. There is 54% identity between *C. glutamicum* and *M. tuberculosis*, and about 34% of the whole amino acid residues are identical among the three species. Out of two hydrophobic regions, the N-terminal one (V19 to L35, not underlined in Fig. 4) may be a part of the signal sequence, since the molecular mass of the mature cytochrome of *C. glutamicum,* 28 kDa (Figs. 1 and 2), is a little smaller than that expected from the DNA sequence (29,841 Da). No N-terminus was detected by Edman degradation even after HCl treatment [37].

It is possible to regard this 28 kDa diheme protein as a tandem product of two class I cytochromes *c*. A homology search (Fasta) with the protein data bank showed that the first N-terminal cytochrome *c* mostly resembles a *Bacillus* small cytochrome *c* such as the thermophilic *Bacillus* PS3 cytochrome *c*-551 [24] and *B. subtilis* cytochrome *c*-550 [23] which are in the class I cytochrome *c* group. On the other hand, the C-terminal cytochrome *c* resembles those found in the cytochrome c_6 group of cyanobacteria. respectively. However, since the best score of the N-terminal half was obtained with the C-terminal half of cco/fixP protein, a diheme protein found in FixN-type cytochrome oxidase, alignments are illustrated with homologous parts of ccoP of *R. capsulatus* and *Synechococcus* cytochrome c_6 in Fig. 5A. It is worth pointing out that *B. stearothermophilus* cytochrome *c* reductase contains a unique cytochrome c_1 subunit, named IVc, in which the *Bacillus* small *c*-like cytochrome is attached to subunit IV by gene fusion [10]. On the contrary, high G+C content Gram-positive bacteria possess unique diheme *c*-type cytochrome without having a small-size cytochrome *c*. We propose to call this novel protein hydrophobic cytochrome *cc*. The role of this cytochrome may be to accept electrons from the Rieske Fe–S center and to donate them to a terminal oxidase, since the diheme protein of fixN-type cytochrome *c* oxidase (cco/fixP), *Bacillus* small *c* and cytochrome c_6 having similarity in the sequences to the *C. glutamicum* cytochrome *cc* may play such roles.

3.5. Sequence characteristics of QcrA

Fig. 5B shows an alignment of the Rieske Fe–S sequence at the C-terminal region which includes the characteristic Rieske Fe–S motif (CTHXGCP–12X–CPCH) and its vicinity. Fewer amino acid residues are conserved in this region, however, except for Gly or Pro even among high G+C Gram-positive bacteria, suggesting that their main role is structural. The N-terminal halves of this protein are quite different in plastid-cyanobacterial and mitochondria-proteobacterial groups as pointed out previously [12]. The *C. glutamicum* protein showed a weak similarity to the former. The Fasta database search showed that the most similar protein out of high G+C Gram-positive bacteria is the *T. thermophilus* Rieske

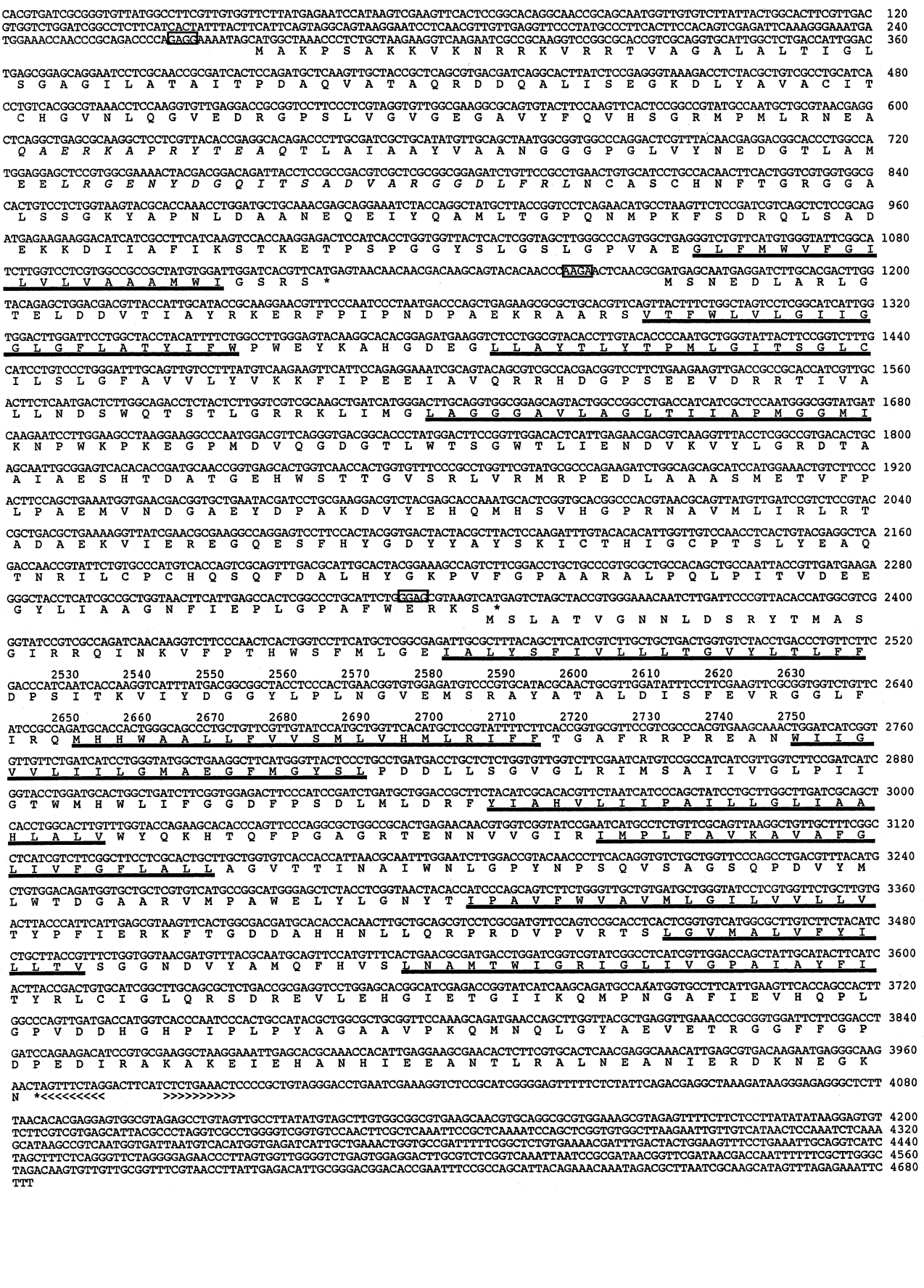

Fig. 4. DNA and deduced amino acid sequences of *C. glutamicum* (*B. lactofermentum*) DNA around the *qcrCAB* operon. The putative Shine–Dalgarno sequences are boxed. The arrows show a putative stem for the terminator of the gene. The residues found in peptide sequence analyses are shown in italic letters. The putative trans-membrane regions of each subunit are underlined.
←

Fe–S protein [13], rather than the *B. stearothermophilus* protein, which is clearly shown by the shadows in the figure.

3.6. Sequence characteristics of QcrB

An alignment of the N-terminal half from cytochrome *b*, including hydrophobic helices A and D (Fig. 5C) was constructed from sequences of cytochrome *b* from high G+C Gram-positive bacteria, cytochrome *b* from *T. thermophilus*, and cytochrome b_6 from *B. stearothermophilus*. Two pairs of heme-ligating His residues are conserved in 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 several residues are conserved in the enzymes from *T. thermophilus* cytochrome *b* or *B. stearothermophilus* cytochrome b_6 (shadowed). It is noteworthy that the sequences of the high G+C group are closer to those of *Thermus* and *Deinococcus* than those of *Bacillus* and *Clorobium*. The total length of cytochrome *b* from high G+C Gram-positive bacteria is 500–510, 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 (Fig. 4). Hydropathy analyses indicate that 7–8 hydrophobic domains exist, in addition to the fourth amphipathic alpha helix part between the transmembrane helices C and D [4]. Cytochromes *b* in this group also have a longer insertion section before the transmembrane helix A than that of *T. thermophilus* (Fig. 5C).

3.7. An analysis with a phylogenetic tree

A phylogenetic tree of cytochrome *b*, including proteobacterial-mitochondrial cytochrome *b*, cyanobacterial-plastidal cytochrome b_6, and soxG and soxC found in *S. acidocaldarius* quinol oxidase as an out group was constructed. *C. limicola*'s cytochrome *b* is isolated in the midst and the archaebacterial SoxG and SoxC form an out-group against the bacterial cytochromes [7]. After this analysis, the sequences of *C. limicola* cytochrome *b* [8], *Bacillus* cytochrome b_6 [9] and *Heliobacillus mobilis* cytochrome *b* [11] were revealed, and construction of a phylogenetic tree introduced the new idea that these sequences formed a new clade between proteobacterial cytochrome *b* and cyanobacterial cytochrome b_6, correlating with the development of non-oxygenic to oxygenic photosynthesis [11]. The present work on *C. glutamicum* and also the progress of whole genome sequencing have made it possible and meaningful to construct a new phylogenetic tree, because several cytochrome *b* sequences are available, which are not proteobacterial and cyanobacterial. Fig. 6 shows that the high G+C content group form a clear clade with radio-resistant cocci such as *Thermus* and *Deinococcus*, while the position of bacilli, Gram-positives with low G+C content, take a position between *Heliobacillus* and *Chlorobium*. A similar phylogenetic tree is also drawn using the Rieske Fe–S protein sequences (not shown). A phylogenetic tree based on 16S rRNA showed the closest relationship between Gram-positive bacteria, high G+C and low G+C groups [26]. The present result may be caused by horizontal gene transfer. However, it seems important and fruitful to study the molecular evolution of this superfamily common in respiration and photosynthesis for elucidation of their evolution.

3.8. Characteristics of cytochrome cc

The data presented here demonstrate that proteins of about 28 kDa are commonly observed as the major heme C-containing protein in high G+C Gram positive bacteria. This protein was partially purified from *C. glutamicum* membranes as a double heme cytochrome. Gram-positive bacteria are known to have no periplasmic space because of having no outer membrane. At least three types of cytochrome *c* have been recognized in bacilli (Fig. 1); cytochrome c_1 in cytochrome *c* reductase forms a polypeptide with subunit IV, cytochrome *c*, as the substrate of the main terminal oxidase, forms cytochrome caa_3 by

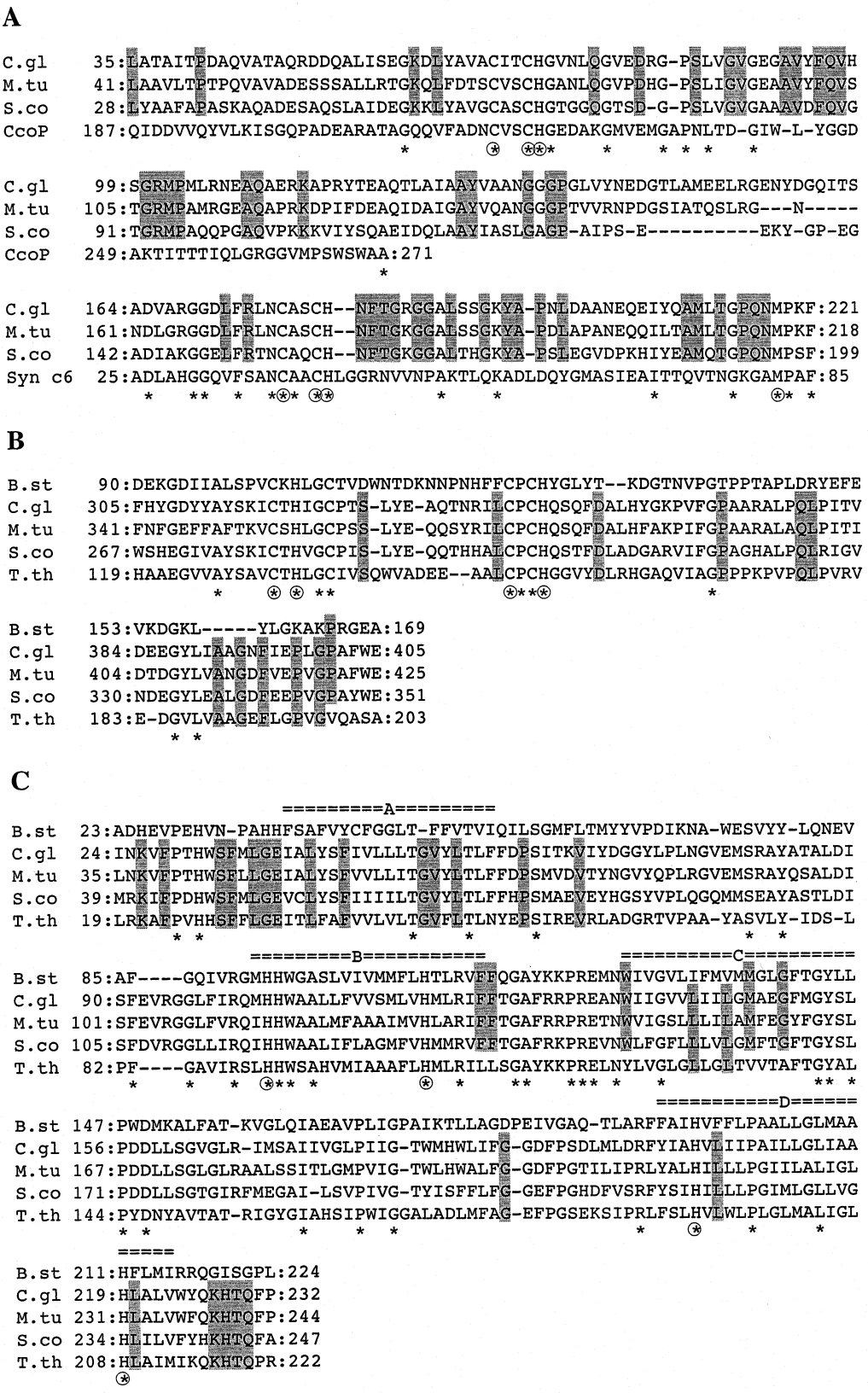
A
C.gl 35:LATAITPDAQVATAQRDDQALISEGKDLYAVACITCHGVNLQGVEDRG-PSLVGVGEGAVYFQVH
M.tu 41:LAAVLTPTPQVAVADESSSALLRTGKQLFDTSCVSCHGANLQGVPDHG-PSLIGVGEAAVYFQVS
S.co 28:LYAAFAPASKAQADESAQSLAIDEGKKLYAVGCASCHGTGGQGTSD-G-PSLVGVGAAAVDFQVG
CcoP 187:QIDDVVQYVLKISGQPADEARATAGQQVFADNCVSCHGEDAKGMVEMGAPNLTD-GIW-L-YGGD
C.gl 99:SGRMPMLRNEAQAERKAPRYTEAQTLAIAAYVAANGGGPGLVYNEDGTLAMEELRGENYDGQITS
M.tu 105:TGRMPAMRGEAQAPRKDPIFDEAQIDAIGAYVQANGGGPTVVRNPDGSIATQSLRG---N-----
S.co 91:TGRMPAQQPGAQVPKKKVIYSQAEIDQLAAYIASLGAGP-AIPS-E----------EKY-GP-EG
CcoP 249:AKTITTTIQLGRGGVMPSWSWAA:271
C.gl 164:ADVARGGDLFRLNCASCH--NFTGRGGALSSGKYA-PNLDAANEQEIYQAMLTGPQNMPKF:221
M.tu 161:NDLGRGGDLFRLNCASCH--NFTGKGGALSSGKYA-PDLAPANEQQILTAMLTGPQNMPKF:218
S.co 142:ADIAKGGELFRTNCAQCH--NFTGKGGALTHGKYA-PSLEGVDPKHIYEAMQTGPQNMPSF:199
Syn c6 25:ADLAHGGQVFSANCAACHLGGRNVVNPAKTLQKADLDQYGMASIEAITTQVTNGKGAMPAF:85
B
B.st 90:DEKGDIIALSPVCKHLGCTVDWNTDKNNPNHFFCPCHYGLYT--KDGTNVPGTPPTAPLDRYEFE
C.gl 305:FHYGDYYAYSKICTHIGCPTS-LYE-AQTNRILCPCHQSQFDALHYGKPVFGPAARALPQLPITV
M.tu 341:FNFGEFFAFTKVCSHLGCPSS-LYE-QQSYRILCPCHQSQFDALHFAKPIFGPAARALAQLPITI
S.co 267:WSHEGIVAYSKICTHVGCPIS-LYE-QQTHHALCPCHQSTFDLADGARVIFGPAGHALPQLRIGV
T.th 119:HAAEGVVAYSAVCTHLGCIVSQWVADEE--AALCPCHGGVYDLRHGAQVIAGPPPKPVPQLPVRV
B.st 153:VKDGKL-----YLGKAKPRGEA:169
C.gl 384:DEEGYLIAAGNFIEPLGPAFWE:405
M.tu 404:DTDGYLVANGDFVEPVGPAFWE:425
S.co 330:NDEGYLEALGDFEEPVGPAYWE:351
T.th 183:E-DGVLVAAGEFLGPVGVQASA:203
C
=========A=========
B.st 23:ADHEVPEHVN-PAHHFSAFVYCFGGLT-FFVTVIQILSGMFLTMYYVPDIKNA-WESVYY-LQNEV
C.gl 24:INKVFPTHWSFMLGEIALYSFIVLLLTGVYLTLFFDPSITKVIYDGGYLPLNGVEMSRAYATALDI
M.tu 35:LNKVFPTHWSFLLGEIALYSFVVLLITGVYLTLFFDPSMVDVTYNGVYQPLRGVEMSRAYQSALDI
S.co 39:MRKIFPDHWSFMLGEVCLYSFIIIILTGVYLTLFFHPSMAEVEYHGSYVPLQGQMMSEAYASTLDI
T.th 19:LRKAFPVHHSFFLGEITLFAFVVLVLTGVFLTLNYEPSIREVRLADGRTVPAA-YASVLY-IDS-L
=========B========== ==========C==========
B.st 85:AF----GQIVRGMHHWGASLVIVMMFLHTLRVFFQGAYKKPREMNWIVGVLIFMVMMGLGFTGYLL
C.gl 90:SFEVRGGLFIRQMHHWAALLFVVSMLVHMLRIFFTGAFRRPREANWIIGVVLIILGMAEGFMGYSL
M.tu 101:SFEVRGGLFVRQIHHWAALMFAAAIMVHLARIFFTGAFRRPRETNWVIGSLLLILAMFEGYFGYSL
S.co 105:SFDVRGGLLIRQIHHWAALIFLAGMFVHMMRVFFTGAFRKPREVNWLFGFLLLVLGMFTGFTGYSL
T.th 82:PF----GAVIRSLHHWSAHVMIAAAFLHMLRILLSGAYKKPRELNYLVGLGLLGLTVVTAFTGYAL
==========D======
B.st 147:PWDMKALFAT-KVGLQIAEAVPLIGPAIKTLLAGDPEIVGAQ-TLARFFAIHVFFLPAALLGLMAA
C.gl 156:PDDLLSGVGLR-IMSAIIVGLPIIG-TWMHWLIFG-GDFPSDLMLDRFYIAHVLIIPAILLGLIAA
M.tu 167:PDDLLSGLGLRAALSSITLGMPVIG-TWLHWALFG-GDFPGTILIPRLYALHILLLPGIILALIGL
S.co 171:PDDLLSGTGIRFMEGAI-LSVPIVG-TYISFFLFG-GEFPGHDFVSRFYSIHILLLPGIMLGLLVG
T.th 144:PYDNYAVTAT-RIGYGIAHSIPWIGGALADLMFAG-EFPGSEKSIPRLFSLHVLWLPLGLMALIGL
=====
B.st 211:HFLMIRRQGISGPL:224
C.gl 219:HLALVWYQKHTQFP:232
M.tu 231:HLALVWFQKHTQFP:244
S.co 234:HLILVFYHKHTQFA:247
T.th 208:HLAIMIKQKHTQPR:222

Fig. 5. Multiple alignment of the three subunit proteins of quinol cytochrome *c* reductases. Residues identical in all sequences are shown (asterisks), while gaps are indicated by dashes. Circled asterisks show the amino acid residues ligating metal centers or binding hemes covalently. The residues conserved only in the three high G+C Gram-positive bacteria, *C. glutamicum* (B.la), *M. tuberculosis* (M.tu) and *S. coelicolor* (S.co) are shadowed. The putative trans-membrane regions of cytochrome *b* subunits are marked and numbered (= = =). (A) Cytochrome *cc* compared with the C-terminal half of diheme subunit of *Rhodobacter capsulatus* cbb_3-type cytochrome *c* oxidase (ccoP) at the N-terminal region, and with *Synechococcus* cytochrome c_6 (Syn c6) at the C-terminal region. (B) Rieske Fe–S proteins of three high G+C Gram-positive bacteria are compared with those of *B. stearothermophilus* [10] and *T. thermophilus* [13]. (C) Cytochrome *b* of three high G+C Gram-positive bacteria are compared with those of *B. stearothermophilus* [9] and *T. thermophilus* (Sone et al., unpublished observation). In B and C, The residues conserved only in the four bacteria including the three high G+C Gram-positive bacteria are shadowed.
←

forming a IIc protein [15,16], a fusion protein of usual subunit II and cytochrome *c* (c_2-type). Finally, a small cytochrome *c*-550 and cytochrome *c*-551 are membrane-bound because of the signal peptide [23], or diacyl glycerylation of the N-terminal Cys residue [24], respectively. The cytochrome *cc* of high G+C Gram-positive bacteria identified here introduces a new type in which cytochrome *c* attaches to another class I cytochrome *c* protein by gene fusion.

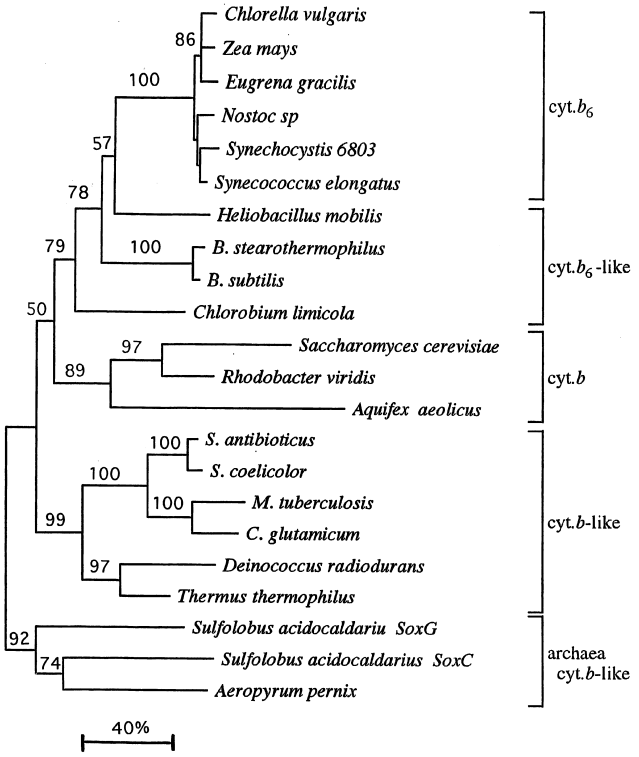

Fig. 6. A phylogenetic tree for cytochrome b/b_6. Most sequences are obtained from the DDBJ data bank except those of *C. limicola* (×73628) *A. aeolicus* (ae000671), *C. glutamicum* (present work). *T. thermophilus* (Sone et al, unpublished observation), *A. pernix* (http://www.mild.nite.go.jp/APEK1/index.html), and *S. acidocaldarius* SoxC and SoxG (z48338). Figures represent bootstrap confidence levels from 1000 bootstrap samples for the groupings; only values above 50% are shown. The scale bar represents a distance of 40%.

Another specific feature of this cytochrome *cc* is the fact that the protein was purified alone without cytochrome *b* and the Fe–S protein. In most cases qcrB (cytochrome *b*)/petBD (cytochrome b/b_6+ subunit IV) have been purified either as a complex (complex III) of cytochrome *b*, the Fe–S protein and cytochrome c_1 from proteobacteria and mitochondria or of cytochrome b_6, subunit IV, the Fe–S protein and cytochrome *f* from cyanobacteria and plastids. The reason why cytochrome *cc* from *C. glutamicum* was not copurified with cytochrome *b* is not known at present. Inactivation of the cytochrome *b* during the preparation may be possible, but the thermostable *T. thermophilus* complex has not been purified to date [13]. We suggest that the interaction between cytochrome *cc*, to cytochrome *b* and the Rieske protein is weak in some cytochrome *c* reductases. This may be a common characteristic in the cytochrome *c* reductases in this group.

4. Conclusion

A novel cytochrome which contained two C hemes in a 28 kDa protein was prepared from the solubilized membranes of *C. glutamicum*. The gene encoding this protein is the first gene in the *qcrCAB* operon for quinol–cytochrome *c* reductase, and homologous gene structure has been found in *M. tuberculosis* and *S. coelicolor*. This feature of *C. glutamicum,* therefore, seems to be a common characteristic in high G+C Gram-positive bacteria. A homology search showed that this double heme cytochrome may be regarded as the sum of two class I cytochromes *c* and we propose to call this protein hydrophobic cytochrome *cc*. A phylogenetic tree based on

the N-terminal half of cytochromes *b* and cytochrome b_6 showed that high G+C bacteria form a clear clade which includes radio-resistant cocci such as *Thermus* and *Deinococcus*, while the low G+C content bacteria contain cytochromes more similar to cyanobacterial cytochrome b_6.

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