doi:10.1006/jmbi.2000.3915 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2000) 300, 663–675

JMB

REVIEW ARTICLE

Early Evolution of Cytochrome *bc* Complexes

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Primary structures, functional characteristics and phylogenetic relationships of subunits of cytochrome *bc* complexes from phylogenetically diverse bacterial and archaeal species were analysed. A single case of lateral gene transfer, i.e. the import of an ε-proteobacterial cytochrome bc_1 complex into Aquificales, was identified. For the enzyme in the remainder of the species studied, the obtained phylogenies were globally in line with small subunit rRNA trees. The distribution of a few key phylogenetic markers, such as contiguousness of cytochrome *b*, nature of the *c*-type subunit or spacing between *b*-heme ligands, are discussed. A localised modification of previous tree topologies is proposed on the basis of the obtained data. The comparison of extant enzymes furthermore allowed us to define the minimal functional and evolutionary core of the enzyme. The data furthermore suggest that the ancestral enzyme was put together from subunits that previously had played a role in other electron transfer chains.

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Keywords: bc complexes; bioenergetics; evolution; photosynthesis; respiration

Introduction

Energy conversion based on the chemiosmotic coupling of membrane-bound electron transfer chains to ATP synthases is almost ubiquitous in living organisms from Archaea through Bacteria to Eukarya. Whereas the ATP synthases in the various organisms form a relatively homogeneous group, the electron transfer chains building up the chemiosmotic potential are astonishingly diverse, comprising anaerobic and aerobic respiration as well as photosynthesis. Until recently, it was tacitly assumed that oxygenic photosynthesis had to evolutionarily precede aerobic respiration, since the latter requires a substrate, O_2, produced by the first. Phylogenetic analyses of the oxidase superfamily, however, demonstrated that electron transfer chains using this or a closely related enzyme as terminal electron acceptor must have existed in the common ancestor of Bacteria and Archaea, and must therefore have been present prior to the appearance of photosynthetic mechanisms (Castresana *et al.*, 1994, 1995; Castresana & Saraste, 1995; Castresana & Moreira, 1999). This, in turn, has far-reaching implications for the possible scenarios of the origin and evolution of photosynthesis (Nitschke *et al.*, 1998).

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Abbreviations used: SSU, small subunit; ORF, open reading frame; ISP, iron-sulphur protein; SUIV, subunit IV; cyt, cytochrome; UQ, ubiquinone; MK, menaquinone; PQ, plastoquinone; CQ, caldariellaquinone; RC, photosynthetic reaction centre.

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0022-2836/00/040663–13 $35.00/0

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Cytochrome *bc* complexes represent the only enzyme common to almost all respiratory (aerobic as well as anaerobic) and photosynthetic electron transfer chains. The evolutionary history of this common segment of energy-conserving electron transport chains can therefore be expected to contain valuable information with respect to the evolution of bioenergetic mechanisms in general. Until recently, however, detailed molecular information concerning *bc* complexes was restricted to rather limited areas of the phylogenetic tree of species, i.e. to proteobacteria, cyanobacteria, Firmicutes and one member of the Archaea.

This lack of information became remedied in the course of 1998/1999 by the genome sequencing projects on the phylogenetically diverse species *Aquifex* (*A.*) *aeolicus*, *Aeropyrum (A.) pernix*, *Chlorobium* (*C.*) *tepidum*, *Deinococcus* (*D.*) *radiodurans*, *Helicobacter* (*H.*) *pylori* and *Thiobacillus* (*T.*) *ferrooxidans*. In the following, we will combine the information content of these genomes with biochemical/biophysical data recently gathered in our laboratories. Association of these data sets yields a picture of the early stages of evolution of the *bc*-type complexes and allows us to derive conclusions concerning the evolution of bioenergetic chains in general.

A Compilation of Presently Available Data

Proteobacteria

Proteobacteria segregate into α, β, γ, δ and ε-subdivisions. The evolutionary origins of the mitochondrial genome were recently traced back to the α-subdivision and, consequently, the principal subunits of the mitochondrial bc_1 complex strongly resemble their counterparts in α-proteobacteria. The proteobacterial cytochrome bc_1 complex consists minimally of three subunits, encoded within a transcriptional unit called the fbc-operon (Figure 1), which contains genes for the Rieske iron-sulphur protein (FbcF or PetA), cytochrome *b* (FbcB or PetB) and cytochrome c_1 (FbcC or PetC) (Gray & Daldal, 1995). The amino acid sequences of the three subunits from previously sequenced representatives of the α, β and γ-proteobacteria are well conserved and have been compiled in several articles (e.g. Furbacher *et al*., 1996). In line with the presence of ubiquinone ($E_{m,7}$ of about +100mV) as pool quinone in species belonging to the α, β or γ-subdivision, the Rieske proteins in these complexes show the characteristic sequence signatures (Denke *et al*., 1998; Schröter *et al*., 1998) of high-potential *bc* complexes, i.e. the presence of the residues Ser and Tyr in specific positions (see Figure 2(a)) engaging in hydrogen bonding interactions with the iron-sulphur cluster (for a more detailed discussion, see Link, 1999; Schoepp *et al*., 1999).

The recently studied enzyme from the proteobacterium *T. ferrooxidans* deviates in a few respects from the bulk of α, β and γ-proteobacterial complexes. We will therefore discuss the complex from this organism in more detail.

Thiobacillus ferrooxidans, first hints to properties of the ancestors

T. ferrooxidans is an acidophilic, respiring chemolithotroph able to use reduced iron complexes as electron donor, a capacity that is exploited for biomining purposes. Taxonomic groupings of *T. ferrooxidans* into either the β or the γ-subdivision of the proteobacteria can be found in the literature.

The *Thiobacillus* cytochrome bc_1 complex was characterised in membranes (Elbehti *et al*., 1999; Brugna *et al*., 1999) and in partially purified samples (P.T. & D.L.-M., unpublished results). Although the complex shows a number of particularities with respect to electrochemical and functional properties, some of which seem to be related to the acidophilic and chemolithotrophic growth mode of the parent organism (as discussed by Elbehti *et al*., 1999), it nevertheless appears to strongly resemble the ''classic'' representatives of the proteobacterial phylum with respect to subunit composition. The sequences of all three subunits are closely related to those from the γ-proteobacterium *Allochromatium vinosum* (Figure 2), strongly indicating that *T. ferrooxidans* indeed belongs to the γ-subdivision (Figure 3).

An interesting sequence particularity of the *Thiobacillus* cytochrome *b*, however, consists in the presence of 14 rather than 13 amino acid residues between the heme-binding histidine residues in transmembrane helix IV (see Figure 2(b)). This feature has been considered to be characteristic for cytochrome b_6 from cytochrome b_6f complexes (Widger *et al*., 1984; Schütz *et al*., 1994; Hauska *et al*., 1996; Furbacher *et al*., 1996).

Helicobacter pylori, not yet running on ubiquinone

H. pylori belongs to the ε-subdivision of the proteobacteria. Interest in genome sequencing of two different *H. pylori* strains (Alm *et al*., 1999) was fuelled by the biomedical impact of this organism, which is involved in the induction of gastric ulcer in humans. Apart from *Helicobacter* species, the ε-subdivision contains only very few representatives so far. Data with respect to bioenergetic mechanisms in this subdivision are scarce.

The operon coding for the cytochrome *bc* complex in *H. pylori* is again organised in the way typical for proteobacteria (Figure 1). Three differences with respect to the bulk of proteobacterial cytochrome bc_1 complexes, however, are remarkable.

(i) Just as *T. ferrooxidans*, the *Helicobacter* cytochrome *b* contains 14 rather than 13 amino acid residues between the heme-ligating histidine residues in helix IV.

(ii) According to the genome data, the cytochrome *c* subunit in *Helicobacter* is a diheme cytochrome with a C-terminal transmembrane helix. A

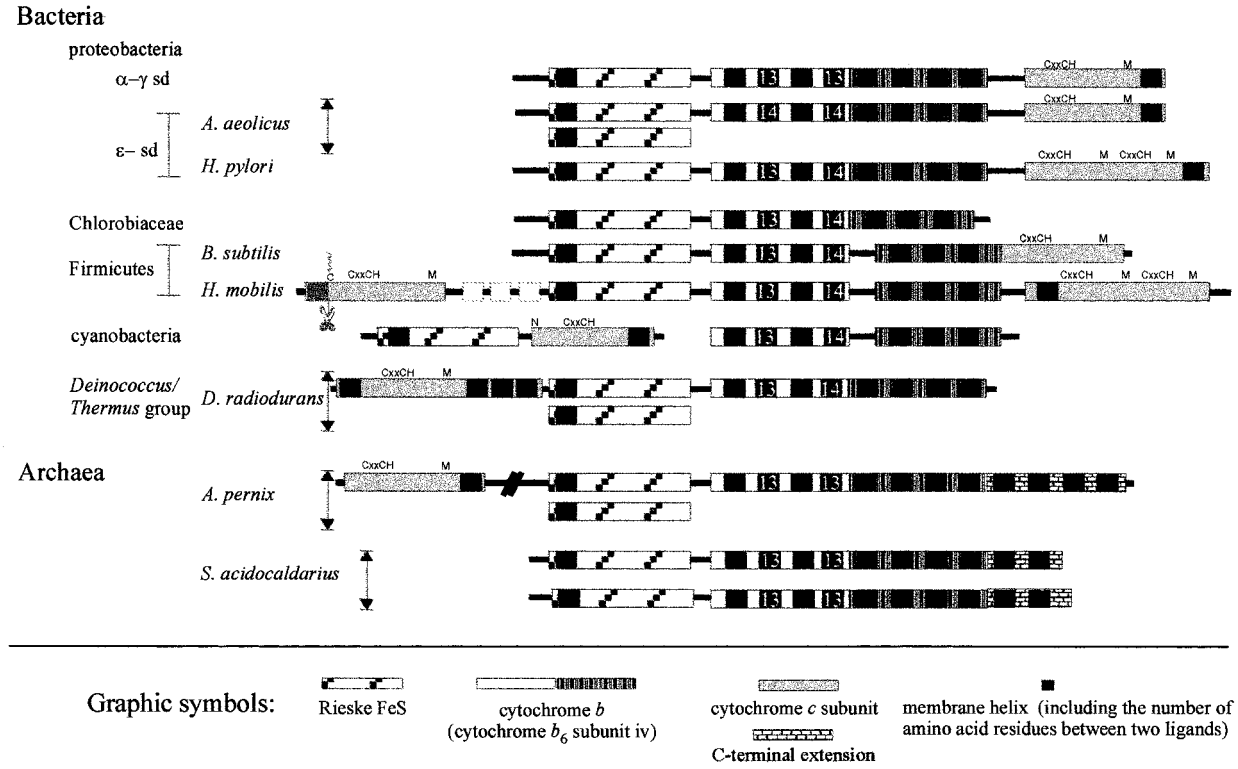

Figure 1. Physical organisation of the *bc* complex structural genes. The genes for the Rieske ISPs, cytochromes *b* or cytochromes b_6/subunits IV and of cytochromes *c* subunits are depicted as marked in the bottom line of the Figure. Clustering of genes is indicated by connecting lines. The barred line for *A. pernix* indicates that the genes are separated by only a few ORFs. The three genes drawn with dotted borders (*H. mobilis*) might be part of the *bc* complex. Black boxes denote putative transmembrane helices, the numbers inscribed in helices two and four of the cytochrome b/b_6 subunits indicate the number of amino acid residues between heme-ligating histidine residues. The positions of heme-binding motifs (CxxCH) and the putative sixth ligands (M for methionine and N for the amino terminus in the case of cytochrome *f*) are shown for the cases of the cytochrome *c* subunits. The putative position of a lipid anchor in the *H. mobilis* cytochrome is marked. *C. tepidum* and *T. ferrooxidans* are close-to-finished genome sequencing projects at TIGR. The genomes of *A. pernix* (Kawarabayasi *et al.*, 1999), *A. aeolicus* (Deckert *et al.*, 1998), *D. radiodurans* (White *et al.*, 1999) and *H. pylori* (Alm *et al.*, 1999) have been published and were accessed *via* the NCBI server. The sequences from *S. solfataricus* were retrieved *via* the home page of the *S. solfataricus* P2 genome project (http://niji.imb.nrc.ca/sulfhome/). The operon for the *bc* complex of *H. mobilis* is part of the photosynthetic gene cluster reported recently (Xiong *et al.*, 1998). The sequence of the Rieske protein in *T. thermophilus* was published recently (Gatti *et al.*, 1998). Database searches were performed using BLASTP (Altschul *et al.*, 1990) on the amino acid sequence database at the National Center for Biotechnology Information (NCBI), Washington, DC. Preliminary sequence data were obtained from The Institute for Genomic Research (TIGR) website at http://www.tigr.org

diheme cytochrome in this position in the operon for the *bc* complex is also present in the Firmicutis *Heliobacillus mobilis* (see below). The overall sequence similarity to the heliobacterial diheme cytochrome, however, is small.

(iii) In contrast to all other proteobacterial examples studied so far, the Rieske ISP in *Helicobacter* shows the sequence signatures typical for low-potential (menaquinol-oxidising) *bc* complexes (Figure 2; see Link *et al.*, 1999; and see the section on proteobacteria).

Aquifex aeolicus; a proteobacterial complex far from home

The most hyperthermophilic Bacteria known to date are members of the genus *Aquifex* (Huber *et al.*, 1992). According to small subunit (SSU) rRNA phylogenetic trees, the Aquificales form the lowest branching order of the domain Bacteria (Olsen *et al.*, 1994). The full genome of a hyperthermophilic representative, *Aquifex aeolicus*, has been sequenced recently (Deckert *et al.*, 1998).

We have characterised the cytochrome *bc* complex from *A. aeolicus* by biochemical and biophysical methods. A detailed description of the respective results will be published elsewhere. In summary, our data demonstrate that this *bc* complex belongs to the group of (low-potential) menaquinol-oxidizing enzymes. Astonishingly, however, a number of properties of the complex were strikingly reminiscent of those observed in the proteobacterial and mitochondrial complexes.

These findings are rationalised by the analysis of the primary structure of the enzyme encoded by the open reading frames (ORFs) Aq42, Aq44 and

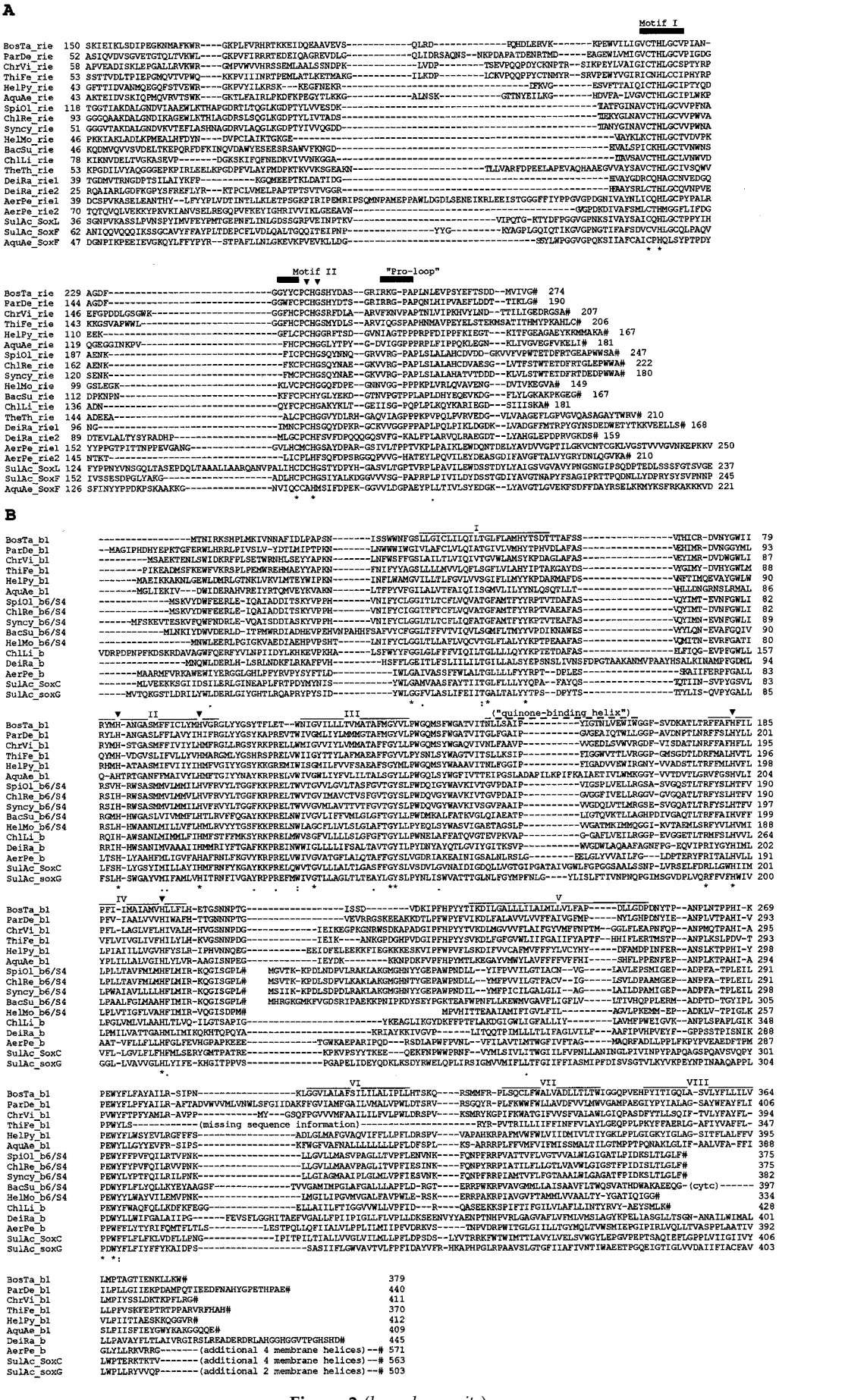

Figure 2 (*legend opposite*)

Aq45 in the *Aquifex* genome. Aq45 and Aq44 code for the Rieske protein and cytochrome *b*, respectively. As can be seen in the sequence alignment (Figure 2) and the phylogenetic analysis (Figure 3), both proteins are strongly related to the proteobacterial counterparts and in particular to those from the ε-proteobacterium *H. pylori*. In line with the electrochemical data on the *Aquifex* enzyme, the Rieske cluster binding site shows the characteristic signatures of low-potential Rieske proteins (Figure 2(a)) just like *Helicobacter* does.

It is worth mentioning that *Aquifex* is the only species found so far where the histidine residues serving as axial ligands to the *b*-hemes are spaced by 14 amino acid residues both in helix II and in helix IV (Figure 2(b)).

Since the complexes in all phyla other than proteobacteria have cytochrome *c* subunits substantially different from cytochrome c_1 (see below), the presence of a typical c_1 cytochrome in Aquificales further corroborates the strong similarity between the *Aquifex* enzyme and the proteobacterial bc_1 complex.

Two different scenarios may be invoked to rationalise these findings. (a) The phylogenetic positioning of the Aquificales as an early branching order is inadequate and the Aquificales should rather be classed within the proteobacteria. A respective scenario has been proposed based on the analysis of selected proteins (e.g. Klenk *et al*., 1999). More recent studies taking into account global genome signatures, however, support the original positioning based on SSU rRNA sequences (Snel *et al*., 1999). (b) The presence of the genes encoding the cytochrome bc_1 complex in Aquificales is the result of lateral gene transfer.

We strongly favour the latter possibility for the following reason. Phylogenetic analysis of the ORFs surrounding the operon of the bc_1 complex reveal the presence of a contiguous stretch of DNA where all ORFs bearing homologies to ORFs in other species show greatest similarities to proteobacterial and mostly *H. pylori* sequences. This stretch of DNA extends from ORF Aq31 to ORF Aq63. Upstream and downstream of these two limiting ORFs, the greatest resemblance to proteins/ORFs from *Thermotoga maritima* and *Thermus thermophilus* is obtained with occasional strong similarities to Archaeal species or Gram-positive bacteria. The genome part between ORFs Aq31 and Aq63 therefore probably represents a stretch that has been imported from an ε-proteobacterium *via* lateral gene transfer. The *Aquifex* genome in fact contains several such contiguous stretches, which mostly map back to *H. pylori*. A detailed analysis of the mosaic structure of the *Aquifex* genome will be published elsewhere.

In summary, these data suggest that Aquificales indeed represent a very early branching order close to the Thermotogales and the *Thermus*/*Deinococcus* group but that they have experienced extensive lateral gene transfers with an ε-proteobacterium as a prominent donor in the course of their subsequent differentiation. In this context, it is of note that the early branching orders appear to have been particularily exposed to frequent and extensive lateral gene transfer events, as recently demonstrated for the Thermotogales (Nelson *et al*., 1999).

In addition to the above-mentioned gene for the Rieske iron-sulphur protein (ISP), a second ORF, designated *sox*F (Aq234), might encode a Rieske-type ISP in *A. aeolicus*. The exchange of one of the two residues known to influence the redox potential (i.e. tyrosine for phenylalanine, see Figure 2(a)) indicates that SoxF probably is a moderately high-potential Rieske protein, in line with our biophysical data showing the presence of two electrochemically differing Rieske clusters in *A. aeolicus* membranes, one with a redox potential typical for ISPs from low-potential *bc* complexes and the other with a significantly higher potential (our unpublished results) incompatible with a functional role in menaquinol-oxidation. The physiological function of SoxF is unknown.

Figure 2. Sequence alignment of (a) Rieske ISPs and (b) of cytochromes *b* or cytochromes b_6/subunits IV. (a) Sequences are represented from the end of the ISPs' flexible linker regions onto the C-terminal end (indicated by #). The [2Fe-2S]-cluster binding regions (Motif I and Motif II), and the polyproline loop (Pro-loop) are indicated by bars above the sequences. The sequence positions influencing the redox potential of the cluster are marked by triangles. The presence of the (hydrogen-bonding) residues serine and tyrosine in these two positions results in relatively high midpoint potentials characteristic for ISPs working in UQ/PQ or CQ chains. The presence of non-hydrogen bonding residues is typical for members of the MK-group. (b) Membrane-spanning helices are indicated by straight lines above sequences. The amphipathic ''quinone-binding helix'' is marked in broken lines. Triangles indicate positions of heme-ligating histidine residues. The sequences of the N-terminal extensions of the *Chlorobium* cytochrome *b*, the sequences of the C-terminal extensions of the cytochromes *b* in Archaea and the sequence of the cytochrome *c* (cyt c) which is fused to subunit IV in *B. subtilis* are not shown. # marks the C-terminal end of the sequences. * and : below the alignments designate residues that are conserved or similar among all sequences. The following abbreviations are used: rie, Rieske ISP; bl, cytochrome b_1; b6/S4, cytochrome b_6 and subunit IV; b, unsplit cytochrome *b*; AerPe, *A. pernix*; AquAe, *A. aeolicus*; BacSu, *B. subtilis*; BosTa, bovine; ChlLi, *C. limicola* f.sp. *thiosulfatophilum*; ChlRe, *Chlamydomonas reinhardtii*; ChrVi, *Chromatium vinosum*; DeiRa, *D. radiodurans*; HelMo, *H. mobilis*; HelPy, *H. pylori*; ParDe, *Paracoccus denitrificans*; SpiOl, spinach; SulAc, *S. acidocaldarius*; Syncy, *Synechocystis* PCC6803; TheTh, *T. thermophilus*; and ThiFe, *T. ferrooxidans*. Amino acid sequences were aligned with the help of the program CLUSTAL (Higgins & Sharp, 1989) using the Blossom matrix.

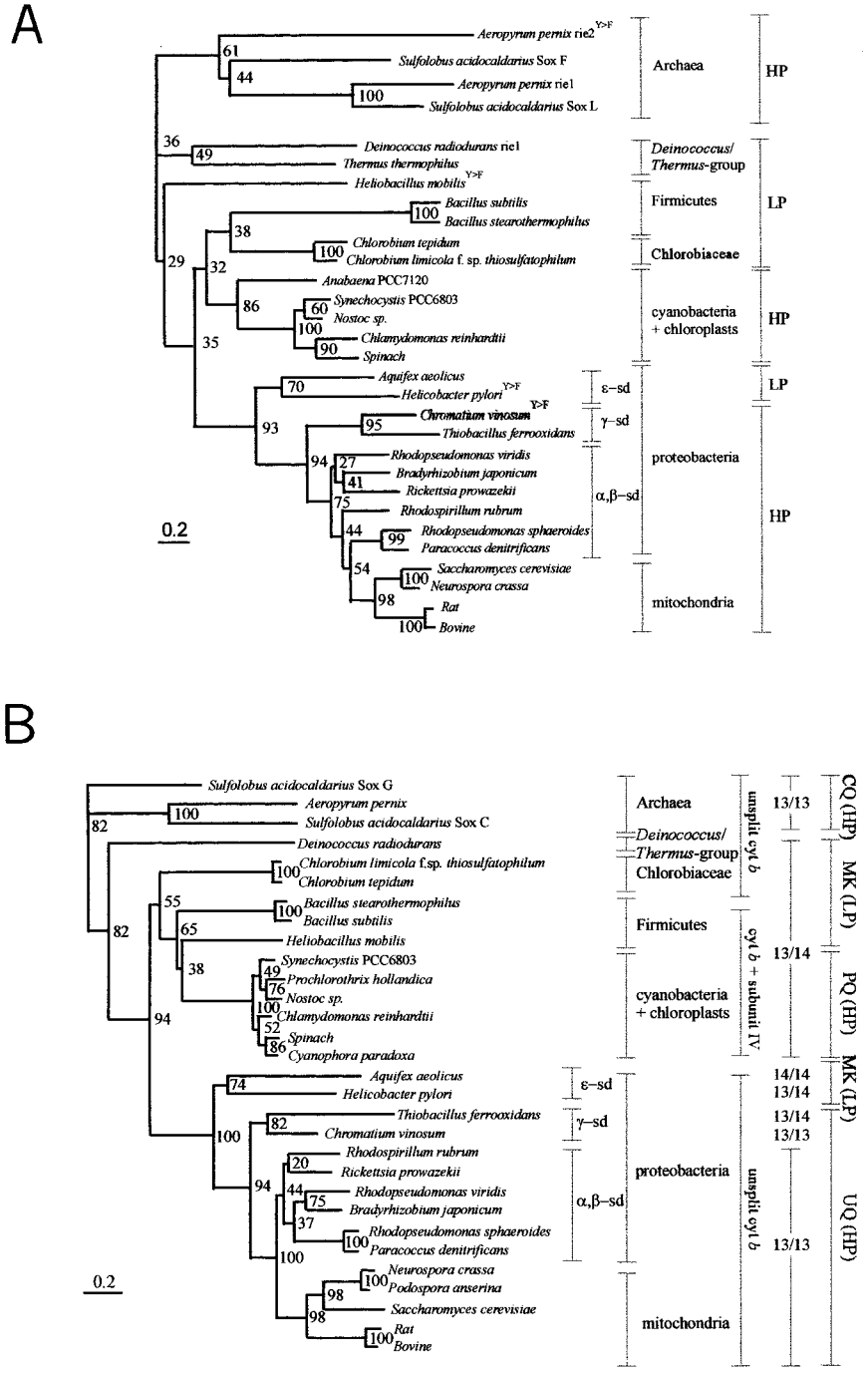

Figure 3. Phylograms of (a) Rieske ISPs as well as (b) cytochromes *b* and cytochromes b_6/subunits IV. (a) The following abbreviations are used: HP/LP, high/low potential Rieske ISP; sd, subdivision; rie, Rieske ISP. Y > F denotes proteins that contain phenylalanine instead of the hydrogen-bond forming tyrosine as discussed in the text. (b) Abbreviations are as follows: CQ, MK, PQ and UQ denote caldariella-, mena-, plasto- and ubiquinone, respectively. HP/LP, high/low potential; cyt *b*, cytochrome *b*; sd, subdivision. The numbers of amino acid residues between the two heme-ligating histidine residues in helix II and IV are indicated (see Figure 2(b)). The phylograms were constructed using the neighbour-joining method of Saitou and Nei as well as Kimura's correction for multiple substitutions. Gap positions were excluded. The archaebacterial proteins were defined as outgroups. Numbers on nodes correspond to the frequency of occurence of nodes in 5000 bootstrap replicates.

Cyanobacteria

The cyanobacterial/plastidic complex has been studied for several decades and has been dealt with in several recent reviews (Kallas, 1994; Hauska *et al*., 1996; Furbacher *et al*., 1996). We will therefore only summarise some of its main characteristics.

The complex contains a Rieske ISP belonging to the high-potential class (in line with the presence of the high-potential plastoquinone as pool quinone), a cytochrome *b* that is present as two separate protein subunits, i.e. four-helix cytochrome b_6 and three-helix subunit IV (SUIV), cytochrome *f* as well as at least three small subunits. Cytochrome *f* was found to have no structural homology to other known *c*-type cytochromes (Martinez *et al*., 1994). The structure of cytochrome *f* is well conserved between cyanobacteria and chloroplasts (Carrell *et al*., 1999).

Cyanobacteria are the only case where the ''typical'' organisation of the operon structure is broken. The unit ISP/cyt*b* is disrupted and the two entities are on distant loci of the physical map of the genome. The gene for cytochrome *f* is found directly downstream of that coding for the ISP (Figure 1).

In cyanobacteria, the complex operates both in oxygenic photosynthesis (transferring electrons from PS2 towards PS1) and in respiration.

In addition to the ISP found in the cyanobacterial cytochrome b_6f complex (sll1316), two more genes encoding Rieske proteins (sll1185 and sll1182) were detected in the *Synechocystis* genome. The latter appears to correspond to a low-potential Rieske ISP as judged by the mentioned sequence signatures. sll1185 shows strong similarities to the Rieske ISP of more distantly related cyanobacteria like *Anabena*. However, the expression and the functionality of these additional genes remains to be shown.

Firmicutes

Heliobacillus mobilis

According to SSU rRNA analyses, the Heliobacteria represent the lowest branching order of the phylum of the Firmicutes (formerly called Gram-positive bacteria and relatives). Heliobacteria are strictly anaerobic photosynthetic organisms. The functional characteristics in photosynthetic electron transfer as well as the electrochemical properties of the heliobacterial cytochrome *bc* complex have been reported and the enzyme was found to belong to the class of low-potential complexes (Kramer *et al*., 1997) in line with the sole presence of menaquinone in this organism (Hiraishi, 1998) and with the distinguishing sequence signatures of the Rieske protein (Xiong *et al*., 1998). Genes coding for subunits of *bc* complexes are found to be part of the photosynthetic gene cluster in *Heliobacillus mobilis* (Xiong *et al*., 1998). Unambiguous components of such a complex are the Rieske protein, cytochrome b_6 and SUIV (see Figures 1 and 2(a) and (b)). Cytochrome b_6 and SUIV correspond to the N and C-terminal halves of the enzyme's *b*-type cytochrome, similar to the situation found in the b_6f complexes from cyanobacteria and chloroplasts. Upstream the Rieske protein, several ORFs coding for small proteins (about 4 kDa) are present, one of which bears resemblance to the SSU petL from b_6f complexes. Further upstream, the gene (petJ) for the monoheme cytochrome c_{551}, which has been purified (Lee *et al*., 1997), is found. Downstream from SUIV, there is a gene encoding a diheme cytochrome, denoted petA. PetJ and PetA have been tentatively attributed to an RC-associated heme protein and the *c*-type cytochrome subunit of the *bc* complex, respectively (Lee *et al*., 1997; Xiong *et al*., 1998). This attribution appears plausible in view of the analogy to the situation in green sulphur bacteria but still needs to be verified by biochemical data.

Bacilli

The genes coding for the complex have been sequenced in *Bacillus subtilis* and in *Bacillus stearothermophilus* (Sone *et al*., 1996) and the complex has been studied with respect to biochemical and biophysical properties (Kutoh & Sone, 1988; Liebl *et al*., 1992). In all Bacilli, the enzyme was found to belong to the class of MK-oxidising complexes. The operon shows ORFs for the Rieske protein, cytochrome b_6, as well as a fused protein consisting of SUIV in its N-terminal part and a small monoheme *c*-type cytochrome at the C terminus.

Chlorobiaceae

Chlorobiaceae (or green sulphur bacteria) are strictly anaerobic and obligately phototrophic bacteria with an [Fe-S] (or RCI) type reaction centre. In the SSU rRNA tree reported by Olsen *et al*. (1994), they are positioned in a lineage separate from the Firmicutes, cyanobacteria and proteobacteria. In line with the fact that menaquinone is a major quinone in green sulphur bacterial membranes, the *bc* complex exhibits the characteristics of low-potential complexes (Brugna *et al*., 1998; Zirngibl *et al*., 1992) as discussed above (Figure 2(a)). Cytochrome *b* is unsplit and the heme-ligating histidine residues in helix II and IV are separated by 13 and 14 amino acid residues (Schütz *et al*., 1994). However, in contrast to the proteins from proteobacteria and the *Thermus/Deinococcus* group, which have eight membrane-helices, cytochrome *b* from *Chlorobium* is predicted to have only seven membrane-helices, similar to the b_6/SUIV unit of Firmicutes, cyanobacteria and chloroplasts.

The genes for the two proteins form an ISP-cytochrome *b* unit (Schütz *et al*., 1994). No *c*-type cytochrome-encoding ORF was found directly upstream or downstream of this unit.

The *Thermus/Deinococcus* group

Following the Aquificales and the Thermotogales (which apparently do not contain *bc*-type complexes), this group represents the third lineage branching-off within the domain of the Bacteria in

most SSU rRNA trees. So far, only biochemical and sequence data concerning a Rieske ISP in *T. thermophilus* have been published (Kuila & Fee, 1986; Gatti *et al*., 1998). Recent electron paramagnetic resonance (EPR) studies on membranes from *T. thermophilus* have demonstrated the presence of a membrane-attached, low-potential Rieske protein performing the pivoting conformational movement characteristic for the ISP-subunit of *bc* complexes (M.B., unpublished results). The previously studied Rieske protein therefore most probably represents the ISP-subunit of this enzyme.

In the genome of *Deinococcus radiodurans*, a cluster of three consecutive ORFs was found encoding a *c*-type cytochrome, the Rieske ISP and cytochrome *b* (Figure 1). The iron-sulphur cluster binding motifs of the protein encoded by the second ORF exhibit the characteristics of low-potential Rieske ISPs. The third ORF encodes an unsplit cytochrome *b* with eight membrane helices, and the heme-ligating histidine residues in helix II and IV are separated by 13 and 14 amino acid residues, respectively. The first ORF of the cluster encodes a *c*-type cytochrome with one N-terminal and three C-terminal putative membrane-helices.

The available biochemical and sequence data therefore suggest that the *bc* complex of the *Deinococcus/Thermus* group belongs to the low-potential, menaquinone-type, in line with the predominance of menaquinone in these species (Collins & Jones, 1981).

Reminiscent of the situation encountered in *Aquifex* and cyanobacteria, a second ORF encoding a Rieske ISP is present within the genome of *D. radiodurans*. The deduced amino acid sequence again exhibits the signature of a moderately high-potential Rieske ISP. As in *Aquifex* and cyanobacteria, this gene is not associated with a gene encoding cytochrome *b*.

Archaea

Only a limited amount of information is available concerning the occurence of *bc* complex analogues in Archaea. Within the Euryarchaeota, no ORF showing homology to a subunit of the *bc* complex was found in the genome of species for which data are accessible (*A. fulgidus*, *M. jannaschii*, *M. thermoautotrophicum*, *P. horikoshii*). Biochemical evidence for the presence of this enzyme, however, has been reported for *Halobacterium salinarum* (Sreeramulu *et al*., 1998). Within the Crenarchaeota, detailed infomation concerning *bc*-type enzymes is available only for *Sulfolobus acidocaldarius* (Schäfer, 1996) and *Sulfolobus* sp. strain 7 (Iwasaki *et al*., 1995). A Rieske ISP from *Pyrobaculum aerophilum* was described recently (Henninger *et al*, 1999).

Sulfolobus acidocaldarius

The so-called SoxM oxidase complex from the acidophilic obligate aerobe *S. acidocaldarius* resembles a supercomplex made up from a ''classic'' *bc* complex (however, lacking a *c*-type cytochrome subunit) and a terminal ba_3-type oxidase. The enzyme oxidises the high-potential (+100 mV; Schäfer, 1996) caldariellaquinol, the predominant quinone species in membranes of *Sulfolobus* (Collins & Jones, 1981). Its ''*bc*-part'' consists of SoxF and SoxG. SoxF is a high-potential Rieske ISP, as evidenced by sequence comparisons and redox titration experiments (Schmidt *et al*., 1997). SoxG has strong sequence similarity to apo-cytochrome *b* (Schäfer, 1996). It consists of ten membrane-helices with the histidine residues ligating two A_s-type hemes separated by 13 amino acid residues in helix II and IV. *Sulfolobus* does not dispose of *c*-type cytochromes and it has been suggested that sulfocyanin encoded by soxE might function in electron transfer from the ''*bc*''-moiety to the oxidase moiety of SoxM (Schäfer, 1996). The soxF and soxG genes, which are part of the *soxFGHI* operon within the SoxM complex gene cluster, are arranged in a way similar to the genomic organisation of the cytochrome *bc* genes of Bacteria (Figure 1).

Another quinol-oxidase supercomplex of *S. acidocaldarius*, the soxABCD oxidase, was seen to contain a subunit, SoxC, with significant homology to cytochrome *b* from *bc* complexes (Lübben *et al*., 1992). SoxC, however, also shows a number of biochemical (e.g. His-Met ligation of heme) and electrochemical (i.e. unusually high redox potential) differences with respect to typical cytochrome *b* from *bc* complexes (Schäfer, 1996; Iwasaki *et al*., 1995). This, together with the absence of an ISP-subunit in the operon, suggests that the soxABCD oxidase may not perform enzymatic reactions resembling those of *bc* complexes. We therefore tend to exclude the soxABCD oxidase from our present analysis.

A second Rieske ISP, encoded by the soxL gene, was isolated and characterized from *S. acidocaldarius*. Redox titrations and sequence comparisons revealed that SoxL belongs to the high-potential Rieske proteins (Schmidt *et al*., 1996; Schäfer *et al*., 2000). Its function within the respiratory chain remains to be established. However, the discovery of a new gene encoding an apo-cytochrome *b* (tentatively named soxN) (C.S. *et al*., unpublished results) immediately downstream of soxL suggests that SoxL and SoxN are part of a cytochrome *bc*-homologous complex.

It is noteworthy that genes homologous to soxL and soxN have been seen in the unfinished genome of *S. solfataricus*, whereas the *soxFGHI* operon has not been found in this organism so far.

Aeropyrum pernix

The hyperthermophilic aerobic strain *Aeropyrum pernix* forms a lineage within the Crenarchaeota distinct from the Sulfolobales (Olsen *et al*., 1994). Within the genome, two consecutive ORFs, APE1724 and APE1725, were identified that are

phylogenetically most closely related to SoxL and SoxN of *S. acidocaldarius* (see above, Figure 1). Noteworthily, five ORFs upstream from the Rieske ISP, an ORF is present that may encode a c_1-type cytochrome (APE1719). The protein encoded by the ORF downstream from APE1719 (APE1720) shows significant similarity to subunit II of cytochrome c oxidases.

These sequence data therefore suggest that a high-potential cytochrome *bc* complex consisting of an ISP, cytochrome *b* and possibly a *c*-type cytochrome subunit, functions in the respiratory electron transport chain of *A. pernix*.

Besides APE1724, the ORF APE2563 (i.e. a completely different locus on the physical map of the genome) may encode a second Rieske ISP (Figure 2(a)), showing once more the characteristic signatures of moderately high-potential Rieske proteins.

Conclusions

Horizontal gene transfer does not significantly blur the picture

The phylograms obtained using subunits of the *bc* complex are globally rather similar to trees based on other phylogenetic markers showing that lateral gene transfer events are rare for the case of the species and enzymes studied above. The only clearcut example for lateral gene transfer is the import of an ε-proteobacterial bc_1 complex into the *Aquifex* genome. A second phylum with an ambiguous phylogenetic position is that of the Chlorobiaceae. Whereas our data concerning the *bc* complex (see below) as well as results obtained previously for photosynthetic reaction centres (Nitschke *et al.*, 1995) favour a proximity to cyanobacteria and Firmicutes, SSU rRNA trees feature green sulphur bacteria at a multitude of different positions (Woese, 1987; Olsen *et al.*, 1994; Gruber & Bryant, 1997; Pace, 1987; López-García, 1999) some of which are close to that on the tree proposed in Figure 4. Whereas these ambiguities may be due to limitations of the tree-building algorithms, they may indicate a composite nature of the green sulphur bacterial genome.

Phylogenetic markers

In the past, a set of conspicuous differences between proteobacterial/mitochondrial cytochrome bc_1 complexes and cyanobacterial/plastidic cytochrome b_6f complexes has been used for classification of enzymes from phylogenetically different species. Consequently, new *bc*-type enzymes have generally been discussed with respect to their bc_1 or b_6f-like nature (e.g. Kutoh & Sone, 1988; Schütz *et al.*, 1994). The amount of data available today allows a more objective assay of the quality of the respective differences between bc_1 and b_6f complexes as phylogenetic markers.

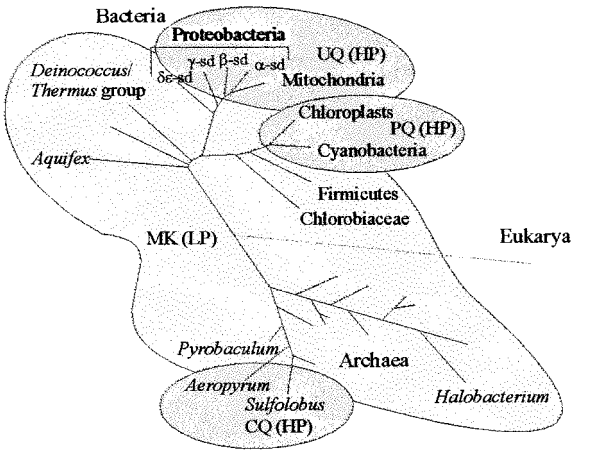

Figure 4. A schematic phylogenetic tree representing the positioning of the species considered in this work. The tree is based mainly on SSU rRNA-sequences. The topology of the branch containing Chlorobiaceae, Firmicutes and cyanobacteria derives from the data discussed in the text. Shaded areas denote species using different kinds of quinones, as marked in the Figure. Abbreviations are as indicated in the legend to Figure 3.

Spacing between heme-ligands in cytochrome b

The 13/14 pattern observed in cytochrome b_6f complexes as opposed to the 13/13 pattern in proteobacterial/mitochondrial complexes was initially considered as a peculiarity of the *bf* complex (Widger *et al.*, 1984) and both structural and functional modifications due to this difference were proposed. Inspection of Figure 3(b) together with available functional data dismiss this hypothesis. The 13/14 pattern is found to be predominant in the domain of the Bacteria and the representatives of the 13/14 group studied in sufficient detail appear to function very much like their proteobacterial counterparts (Kutoh & Sone, 1988; Kramer *et al.*, 1997). Moreover, even among the proteobacteria, representatives of the 13/14 class are found within the ε (*H. pylori*) and γ (*T. ferrooxidans*) subdivisions. This indicates that the 13/13 pattern indeed has appeared only recently within the γ-subdivision of the proteobacteria. With its 14/14 pattern, *A. aeolicus* stands out as a complete outsider.

In the Archaea studied so far, the 13/13 pattern is observed. This leaves an ambiguity, whether the ancestral cytochrome *b* belonged to the 13/13 or the 13/14 group.

The cytochrome c-subunit

As will be discussed in more detail below, the nature of the cytochrome *c* subunit varies significantly between phyla. Nevertheless, with respect to shorter evolutionary distances, this parameter reflects phylogeny sufficiently well. Most proteobacterial complexes as well as the enzyme from

Aquifex contain a typical c_1 cytochrome. The presence of a different *c*-type cytochrome seems to be a common characteristic of Gram-positive Bacilli and probably the *Deinococcus/Thermus* group. The lineage performing oxygenic photosynthesis, i.e. the cyanobacteria, invariably contains cytochrome *f*. Primary and 3D structures of cytochrome *f* are well conserved from cyanobacteria to chloroplasts (Furbacher *et al*., 1996; Carrell *et al*., 1999). It has been argued that structural details of this hemeprotein must therefore have been conserved over as much as three billion years (Carrell *et al*., 1999). The available evidence, however, concerns only cytochromes *f* formed after the radiation of the cyanobacteria, which probably has occurred in the range of one to two billion years ago. This time-range corresponds to the evolutionary distance between the (comparatively well conserved) cytochromes c_1 and c_2/c from proteobacteria and mitochondria.

"Long" versus "split" cytochrome b

The majority of cytochrome *b* proteins in Bacteria consist of a common ''core'' of eight (N-terminal) membrane-spanning helices. In the archaeal representatives, between two and four additional putative membrane-spanning regions are fused to the C-terminal end of this common core. In Chlorobiaceae, cytochrome *b* appears to contain only seven membrane-spanning stretches. Both cyanobacteria and Firmicutes contain two separate proteins, cytochrome b_6 and SUIV, corresponding to the N and C-terminal parts of the ''long'' cytochrome *b*, respectively (in a few Firmicutes, SUIV is fused to a cytochrome subunit; see above). Remarkably, in both cyanobacteria and Firmicutes, cytochrome b_6 and SUIV together contain seven membrane-helices, just as is the case for the Chlorobiaceae.

Based on the presently available sequence information and on the global topology of the SSU rRNA phylogenetic tree, the long-standing question of whether long cytochrome *b* and cytochrome b_6/SUIV are related to each other by fusion or cleavage of genes, appears to be solved in favour of the latter hypothesis (in line with Castresana *et al*., 1995). Some of the previously proposed SSU rRNA trees, albeit positioning Firmicutes and cyanobacteria close to each other, present these two phyla on separate branches of the tree. This would suggest that the cleavage event has occurred twice during the evolution of *bc* complexes. However, as will be detailed below, we believe that there is strong circumstantial evidence for a common branch containing cyanobacteria, Firmicutes and possibly also Chlorobiaceae. In this scenario, an eight-helix cytochrome *b* would first have lost a single C-terminal membrane-helix, yielding the green sulphur complex and have then cleaved into the four-helix cytochrome b_6 and the three-helix SUIV, resulting in the common ancestor of the cyanobacterial/Firmicutes proteins. Later species of the phylum of the Firmicutes would then have fused a *c*-type cytochrome to their SUIV. If a *Chlorobium*-like complex was indeed the ancestor to the ''split-*b*-lineage'', the absence of a *c*-type cytochrome in the corresponding operon might also rationalise the divergence of (secondarily added) *c*-type cytochromes in cyanobacteria and Firmicutes.

A case for a common branch containing cyanobacteria, Firmicutes and possibly Chlorobiaceae

The phylogenetic tree reported in 1994 by Olsen *et al*. frequently serves as the ''tree of reference'' for phylogenetic relationships based on SSU rRNA. In this tree, cyanobacteria and Firmicutes are positioned on separate branches of the main tree (Olsen *et al*., 1994). The previously proposed tree (based also on SSU rRNA but taking fewer species into consideration), by contrast suggested a common branching-off for these two phyla (Woese, 1987). Several recently published SSU rRNA trees also arrive at a common branch for the cyanobacteria and Firmicutes (Gruber & Bryant, 1997; Pace, 1997). The phylogram obtained using cytochrome *b* sequences (Figure 3(b)) strongly supports such a grouping together of cyanobacteria and Firmicutes. Globally, this is also true for the tree based on Rieske ISP sequences, although the relatively poor bootstrap values indicate that the tree topology may be less reliable in the crucial region of the tree. Corresponding topologies, although based on a smaller sample of species, have been reported for cytochrome *b*, the ISP and the cox2-gene of cytochrome oxidase (Castresana *et al*., 1995).

Apart from sequence signatures, the ensemble of phylogenetic markers related to the cytochrome *bc* complexes and to photosynthetic mechanisms further support a clustering of cyanobacteria, Firmicutes and Chlorobiaceae. (a) As discussed above, the distribution of cleaved cytochrome *b* would be rationalised more easily in this scenario. (b) These three phyla are the only ones that contain seven-helix cytochrome *b* units. (c) The three phyla are the only ones known to contain RCI-type photosystems (Nitschke *et al*., 1995). Phylogenetic trees based on sequences from RCIs show a closer relationship between the Firmicutis- and the cyanobacterial photosystems and suggest the chlorobial RC as the ancestral one (Nitschke *et al*., 1998; Xiong *et al*., 1998). The pathways of RCI evolution are enigmatic against the background of the tree proposed by Olsen *et al*. (1994) but become almost straightforward if the three phyla under consideration were grouped together.

Features of the ancestor

The evolutionary core of so-called bc complexes is an ISP/cytb complex

Both in Bacteria and Archaea, cytochrome *bc* complexes contain the Rieske ISP and cytochrome *b*, the latter either in its contiguous form or split

into two separate subunits after the fourth transmembrane helix. Apart from one exceptional case, i.e. the cyanobacteria (and consequently the chloroplasts), these two entities are coded within a transcriptional unit showing a conserved arrangement Rieske ISP-cytochrome *b* (Figure 1).

By contrast, although the majority of these enzymes furthermore contain a *c*-type cytochrome subunit, this subunit is highly divergent and certainly does not have a monophyletic origin. It rather seems as if the evolutionary ISP/cyt*b* core had captured available *c*-type cytochromes regardless of their detailed structure. Whereas most proteobacterial complexes contain cytochrome c_1, the ε-proteobacterium *H. pylori* and possibly also the Firmicutis *H. mobilis* contain diheme cytochromes related to cytochrome c_4. Other monoheme cytochromes are observed in Bacilli and in the *Deinococcus/Thermus* group. Most strikingly, cytochrome *f* in cyanobacteria is a structurally completely unrelated hemeprotein containing predominantly β-sheet structural elements.

The structural genes for almost all of these cytochromes are located either downstream or upstream of the ISP/cyt*b* core and only in the case of the cyanobacteria, where the gene for the ISP is not adjacent to that of cytochrome *b*, cytochrome *f* is situated downstream of the ISP (Figure 1).

The complexes in the Archaeon *S. acidocaldarius* and possibly in green sulphur bacteria are completely devoid of cytochrome *c* subunits, probably resembling the evolutionary ancestor of this class of enzymes (as already proposed by Castresana *et al*., 1995).

These data show that the historical name cytochrome *bc* complexes is in fact misleading. The functional as well as the evolutionary core of these enzymes is the unit ISP/cyt*b*. It is of note, however, that this unit may have never existed as an isolated enzyme. According to the recent discovery of the pivoting movement of the ISP being intimately involved in the functional mechanism of the complex (Zhang *et al*., 1998), the existence of the ISP/cyt*b* core devoid of a structurally fixed electron acceptor does not seem to make sense. This acceptor, however, can be a *c* or *b*-type cytochrome, a copper protein or another ISP. In *S. acidocaldarius*, the acceptor of one of the two ISP/cyt*b* enzymes appears to be the copper subunit of cytochrome oxidase, i.e. the ISP/cyt*b* core would be part of an ISP/cyt*b*/oxidase supercomplex (Schäfer, 1996). Correspondingly, if indeed no *c*-type cytochrome should be present in the *Chlorobium* enzyme, we would strongly suspect that the ISP/cyt*b* core forms a complex with another enzyme, possibly the photosynthetic reaction centre.

The urenzyme-oxidised menaquinol

Figure 4 shows a representation of the topology of the phylogenetic tree of species relevant to the evolution of the ISP/cyt*b* unit. As is evident from this scheme, the complexes in the vast majority of species and, in particular, in those close to the divergence of domains belong to the low-potential, menaquinol-oxidising class. This is in line with the significantly lower oxygen tensions (i.e. lower ambient potentials) in the atmosphere of the early earth (Knoll, 1992).

Adaptations to the rise in ambient potential induced by the action of oxygenic photosynthesis obviously entailed a change in the chemical nature of the quinone in at least three regions of the phylogenetic tree. In these three regions, the electrochemical potential of the pool quinone was increased from about −70 mV (menaquinone) towards about +100 mV. Interestingly, three chemically different solutions were developed in order to arrive at a pool-quinone having $E_{m,7}$ of about +100 mV. These solutions are ubiquinone (proteobacteria), plastoquinone (cyanobacteria) and caldariellaquinone (Sulfolobales). Within the proteobacteria, the transition from mena- to ubiquinone appears to have occurred in the lineage leading to α, β, and γ-proteobacteria. The γ-proteobacterium *Escherichia coli* is able to use both UQ and MK as pool quinone, but does not contain *bc* complexes. It is of note, that in the strictly anaerobic δ-proteobacteria, none of the ''classic'' respiratory mechanisms appears to be operative.

What was there prior to the ancestor?

It appears striking to us that in all phylogenetically old lineages (such as Aquificales, cyanobacteria, *Thermus/Deinococcus*, Archaea) genes for Rieske-type proteins are present that are not located in a *bc* operon context and that almost certainly have redox midpoint potentials (according to sequence signatures) making them unsuitable for oxidising menaquinones. In *Aquifex*, this second Rieske-type protein was found to be expressed and the observed electrochemical potential corresponds nicely to that expected from the amino acid sequence (our unpublished results). We would therefore tend to speculate that the ancestor of the *bc*-ISP had a task in one or several rather different electron transfer mechanisms and was, in a kind of evolutionary construction kit, combined with an ancestral cytochrome *b* giving birth to the functional unit of the *bc* complexes. A target for future studies will therefore be to elucidate the functional role of these ''non-*bc*'' Rieske ISPs in the electron transfer chains of the respective organisms.

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Edited by G. Von Heijne

(Received 2 March 2000; received in revised form 31 May 2000; accepted 2 June 2000)

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