New Class of Bacterial Membrane Oxidoreductases†

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ABSTRACT: A new class of bacterial multisubunit membrane-bound electron-transfer complexes has been identified based on biochemical and bioinformatic data. It contains subunits homologous to the three-subunit molybdopterin oxidoreductases and four additional subunits, two of which are *c*-type cytochromes. The complex was purified from the filamentous anoxygenic phototrophic bacterium *Chloroflexus aurantiacus*, and putative operons for similar complexes were identified in a wide range of bacteria. In most cases, the presence of the new complex is anticorrelated with the cytochrome *bc* or *bf* electron-transfer complex, suggesting that it replaces it functionally. This appears to be a widespread yet previously unrecognized protein complex involved in energy metabolism in bacteria.

Electron transport chains of many bacteria contain common features. Lipophilic quinones transfer reducing equivalents along the energy transducing membrane between membrane-embedded protein complexes. Simultaneously, they participate in transmembrane proton translocation, accepting protons from the cytoplasm upon reduction and releasing protons into the periplasm upon oxidation. Membrane-bound dehydrogenases reduce quinones, and some of them react with their substrates in the cytoplasm. These dehydrogenases include succinate-quinone oxidoreductase (*1*) and NADH-quinone oxidoreductase I (*2*), which are very widely distributed. The latter complex is able to translocate protons across the membrane independent of interacting with a quinone, acting as a conformational proton pump (*3*, *4*). The other dehydrogenases interact with substrates in the periplasm, like some formate dehydrogenases (*5*) and some hydrogenases (*6*). In these cases, primary proton translocation occurs.

In light-driven photosynthetic electron transport chains, quinones are reduced either directly by photosystem II of cyanobacteria (*7*) and reaction centers of purple (*8*) and green nonsulfur bacteria or indirectly with possible participation of NADH dehydrogenase in green sulfur bacteria and heliobacteria.

There are at least three pathways for the oxidation of quinols in respiratory chains. The simplest one is the reaction with integral membrane terminal oxidoreductases. In anaerobic respiratory chains, dependent upon the bacterial species and environmental conditions, this reaction is mediated by membrane molybdopterin-containing reductases of the dimethyl sulfoxide (DMSO)[1] reductase family [DMSO-, nitrate-, polysulfide-, thiosulfate-, and tetrathionate reductases (*9*−*12*)], fumarate reductases (*1*), nitrite reductases (*13*), and quinol-oxidizing NO reductases (*14*). In oxygen-utilizing respiratory chains, this reaction is mediated by terminal quinol oxidases (*15*, *16*). The second pathway includes small membrane cytochromes of NirT/NapC type (*12*, *17*, *18*), which transfer electrons to soluble periplasmic terminal reductases.

The most complex pathway is the combination of a quinol: cytochrome *c* oxidoreductase (bc_1 complex), a mobile periplasmic electron transporter, usually cytochrome *c*, and a terminal oxidoreductase. In anaerobic chains, such terminal oxidoreductases are cytochrome *c* oxidizing NO reductases (*14*) and a cd_1 type of nitrite reductase (*19*), while in aerobic chains, various kinds of cytochrome oxidases are found (*15*, *16*). Both quinol oxidases and cytochrome oxidases are able to translocate additional protons through membranes besides those released to the periplasm on quinol oxidation and consumed from the cytoplasm on oxygen reduction (*20*, *21*). Similar to NADH dehydrogenase I, they are conformational proton pumps.

Among the complexes that release protons to the periplasm on quinol oxidation, the bc_1 complex is unique in that it releases four protons per two electrons transferred to cytochrome *c*. This reaction, the so-called Q cycle (*22*, *23*), is due to the bifurcation of two-electron flow from quinol. One of the electrons is transferred to cytochrome *c* at the periplasmic side of the enzyme with simultaneous release of two protons to the periplasm. The second electron is transferred via two hemes *b* to quinone near the cytoplasmic side, thus producing semiquinone. The repetition of this action results in the net transfer of two electrons and translocation of four protons through the membrane from two pool quinols, one of which is replaced with the quinol reduced during the Q cycle. In the well-studied oxygen-

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[1] Abbreviations: DMSO, dimethyl sulfoxide; HiPIP, high-potential iron sulfur protein; FAP, filamentous anoxygenic phototroph; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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utilizing respiratory chains, the two ways with and without participation of the bc_1 complex are considered as alternatives. The only known exception is the case of *Rhodothermus marinus* where the periplasmic electron transporter high-potential iron sulfur protein (HiPIP) is reduced by an unusual cytochrome complex different from the normal bc_1 complex (*24*, *25*).

In light-driven electron transport chains, cytochrome bc_1 complexes or their homologues cytochrome b_6f complexes function as energy conservation and proton-pumping complexes. Mobile periplasmic e-transporters, *c*-type cytochromes, copper proteins, and HiPIPs, transfer electrons to photosystem I or to the oxidized side of bacterial reaction centers. Indeed, bc_1 (b_6f) complexes have been purified from cyanobacteria (*26*), nonsulfur (*27*, *28*), and sulfur (*29*) purple bacteria. Genes encoding subunits of a bc_1 complex were sequenced in the heliobacterium *Heliobacillus mobilis* (*30*) and in the green sulfur bacterium *Chlorobium tepidum* (*31*). Thus, bc_1 (b_6f) complexes are essential participants of electron transport chains of four of five photosynthetic lineages.

A study aimed to find a bc_1 complex in *Chloroflexus aurantiacus* detected no complex with the expected features (*32*). The bacterium *C. aurantiacus* is a representative of the fifth photosynthetic phylum, named the filamentous anoxygenic phototroph (FAP), previously known as green nonsulfur bacteria (*33*). The phylum occupies the lowest position among the photosynthetic phyla as measured by 16 S rRNA and also contains some nonphotosynthetic species. Two similar multisubunit membrane complexes were isolated from *C. aurantiacus*, each containing two cytochromes *c* as some of their subunits (*32*). The bacterium contains menaquinone as the sole quinone (*34*) and the globular copper proteins, auracyanins A and B (*35*, *36*), as soluble periplasmic electron carriers. When we take into account that there must be an oxidoreductase working between them, it was proposed that the novel complexes fulfill this function.

In this study, we have identified the genes encoding subunits of both the novel complexes using mass spectroscopy fingerprinting techniques and the partially sequenced *C. aurantiacus* genome (U.S. Department of Energy Joint Genome Institute, http://www.jgi.doe.gov/). Then, searching for their homologues in published bacterial genomes, we discovered a group of very similar operons presumably encoding a novel class of oxidoreductases. The generalized novel operon contains genes homologous to the three subunits of membrane-bound molybdopterin oxidoreductases and four additional genes. Besides the FAP, we found such operons in the following phyla: δ-proteobacteria, cytophaga-flexibacter-bacteroides group, planctomycetes, spirochaetes, and verrucomicrobia. In almost all cases, the presence of the operon is associated with the lack of the bc_1-complex genes and also with the presence of putative operons encoding a defined subgroup of cytochrome oxidases.

MATERIALS AND METHODS

Strain and Cultivation. C. aurantiacus strain J-10-fl was grown in the medium described (*32*) in a 16 L Lab-Line Bioengineering glass walled fermenter at 55 °C. The aerobic culture was grown in the dark during 12 h at bubbling of air (5 L per min). The photosynthetic culture was grown the same time in the fermenter flushed at the beginning with nitrogen and illuminated by a bank of 12 60-W incandescent lamps. In both cases, cultures were stirred at 150 rpm.

Isolation of Membranes and Cytochrome Complexes. Isolation of membranes and cytochrome complexes was carried out as described earlier (*32*) and modified for large volumes.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was carried out in 15% polyacrylamide gel (*37*); gels were stained for proteins (*38*) or for cytochromes *c* (*39*).

Trypsin Digestion and Determination of the Masses of the Products. Stained bands of the subunits were excised and subjected to trypsin treatment accordingly to Williams et al. (*40*). Mass spectral data were obtained using a Voyager DE STR MALDI−TOF mass spectrometer equipped with a nitrogen laser that produced 337 nm pulses of 3 ns duration at a repetition rate of 20 Hz. Mass spectra were acquired in the positive-ion mode using delayed extraction and a reflectron. Trypsin-digested protein samples were mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid dissolved in a mixture of 0.1% trifluoroacetic acid in water and acetonitrile (2× dilution) A total of 1 μL of this mixture was further mixed with 4 μL of the matrix solution (10× dilution). Samples (1 μL) of both dilutions were dried on a stainless steel sample plate, and mass spectra were acquired from the dilution giving the best results used for database searches. Each mass spectrum was the average of at least 100 laser shots. Calibration was performed using CalMix 2 from Applied Biosystems as a close external standard. CalMix 2 is a mixture of the following polypeptides (singly protonated monoisotopic masses in parentheses): angiotensin1 (1296.685), ACTH clip 1−17 (2093.087), ACTH clip 18−39 (2465.199), ACTH clip 7−38 (3657.929), and bovine insulin (5730.609). In some cases, fragments from porcine trypsin with monoisotopic masses of 842.509 and 2211.104 were identified in the samples and used as internal standards for more precise calibration. Monoisotopic masses obtained by this procedure are accurate to within 0.1 Da over the mass range of peptides examined in this work.

Virtual Trypsin Digestion. Virtual trypsin digestion of putative polypeptides encoded by found ORFs was performed online at http://prospector.ucsf.edu.

Bioinformatic Methods: Search for Homologies. We used the online BLAST program (*41*) at the National Center for Biotechnology Information (NCBI) WWW site (http://www.ncbi.nlm.nih.gov/) (http://www.ncbi.nlm.nih.gov/BLAST/) versus nonredundant protein DB of NCBI or versus microbial genomic DB of NCBI. In the case of annotated completed and annotated unfinished genomes, the protein versus the protein version of BLAST was used. In the case of not annotated unfinished genomes, the protein versus the DNA version of BLAST was used. ORFs found in homologous stretches of DNA were then determined by the ORF-finder online program at the NCBI site (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). At first, we found genes strictly homologous to the subunits of C_p and C_r complexes in the protein database and thereby defined those bacteria possessing corresponding operons. Then, we searched for homologous genes of related but different complexes in the microbial genomic database, restricting the search to completed ge-

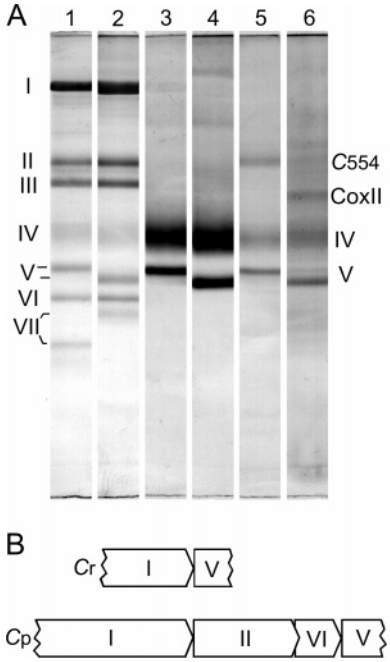

FIGURE 1: Expression of *C. aurantiacus* cytochrome complexes in different growth conditions and correspondence between the subunits of the complexes and the genes of putative operons. (A) SDS−PAGE in 15% polyacrylamide gel. Lanes 1 and 3, purified C_p complex; lanes 2 and 4, purified C_r complex; lane 5, membranes from photosynthetic anaerobic culture; lane 6, membranes of *C. aurantiacus* from aerobic dark culture; lanes 1 and 2, staining for protein; lanes 3−6, staining for hemes *c*. (B) Two contigs found in partially sequenced *C. aurantiacus* genome with ORF coding for the indicated subunits of the complexes.

nomes and only those unfinished genomes where C_p-like operons had already been detected. No C_p-like operons were found in Archaea.

Alignment and Phylogenetic Tree Construction. Alignment and phylogenetic tree construction were carried out by the online ClustalW program (*42*) at GenomeNet CLUSTALW Server of Kyoto University (http://clustalw.genome.jp). Default settings were used.

Transmembrane α *Helices and Signal Peptides.* Transmembrane α helices and signal peptides were predicted using online programs at the WWW site of The Center for Biological Sequence Analysis at the Technical University of Denmark (www.cbs.dtu.dk/services/).

RESULTS AND DISCUSSION

Two novel similar multisubunit membrane complexes containing cytochromes *c* were found earlier in batch phototrophic cultures of *C. aurantiacus* (*32*). In the conditions used in that work, dissolved oxygen in the culture medium was exhausted by the initial respiration of the cells after the addition of the inoculum. Now, we have found that one of the complexes is expressed in cells growing in anaerobic phototrophic conditions, while the other one is expressed in cells growing aerobically in darkness. We have therefore correspondingly renamed the complexes as C_p (photosynthetic) and C_r (respiratory). Pairs of heme *c*-containing bands characteristic for C_p and C_r complexes appeared on SDS−PAGE in lanes correspondingly loaded with membranes from cells grown under phototrophic and respiratory conditions (Figure 1A). A cytochrome *c* band characteristic for cytochrome *c*-554, the immediate electron donor to the photosynthetic reaction center (*43*), also appeared in the first case, serving as a marker of the light-driven electron transport chain. In the second lane, a band with a molecular mass of 36 kDa was observed. This band apparently belongs to the second subunit of cytochrome oxidase, whose amino acid sequence contains the cytochrome *c*-binding motif (see below). This distribution pattern suggests that the C_p complex is a component of the light-driven cyclic electron transport chain, while the C_r complex is a component of the respiratory chain of *C. aurantiacus*.

Table 1: Correspondence between the Results of the Trypsin Digestion of C_p and C_r Subunits and the Virtual Trypsin Digestion of Putative ORF Products

protein	fragments coincided	average error (ppm) and standard deviation	percent sequence coverage
C_p I, N part	7 (6)	-213 ± 25 (223 ± 2.4)	35.5 (129/363 residues)
C_p I, C part	17	-223 ± 10.9	39.1 (269/688)
C_p II	5	-42.0 ± 3.0	14.8 (72/486)
C_p III	5	-25.8 ± 5.5	20.7 (85/411)
C_p IV	2	-10.3 ± 0.3	25 (28/112)
C_p V	4	8.6 ± 3.9	33.1 (59/178)
C_p VI	4	-201 ± 10.7	35.3 (74/204)
C_p seventh	1	15.1	5.6 (6/107?)
C_r I, N part	8	-62.6 ± 5.5	18.5 (102/550)
C_r I, C part	7	19.4 ± 12.0	23.9 (96/401)
C_r V	3	11.5 ± 1.6	21.1 (40/190)

We identified the genes in the *C. aurantiacus* partial genome sequence encoding most of the subunits of the C_p and C_r complexes using protein mass spectroscopic fingerprinting. Subunits of the C_p and C_r complexes were separated by SDS−PAGE and digested by trypsin. Masses of the resulting oligopeptides were determined by MALDI−TOF mass spectrometry. The sets of the masses resulting from each subunit were then used for comparison with the sets of products of virtual trypsin dissection of the putative gene products. We first identified all possible ORFs putatively encoding cytochromes *c* in the partially sequenced *C. aurantiacus* genome (U.S. Department of Energy Joint Genome Institute, http://www.jgi.doe.gov/) using various bacterial cytochromes *c* as queries in BLAST searches and applied the virtual trypsin digestion procedure to them. We found reliable correspondences between subunits V of complexes C_p and C_r and ORFs encoding putative cytochromes *c* in two short contigs (Table 1). Then, we established the correspondences between the rest of the ORFs of these contigs and subunits I, II, and VI of the C_p complex and subunit I of the C_r complex (Table 1 and Figure 1B). We thus found two contigs in the *C. aurantiacus* genome sequence that partially cover putative operons encoding the C_p and C_r complexes.

Searching for homologies to these subunits in other organisms in the genomic database of NCBI, we found in several bacterial genomes highly homologous stretches of six ORFs, organized in the same order as in the putative operon encoding the C_p complex (Figure 2). Two genes, in addition to the previously identified four-gene stretch, were situated up- and downstream. Using these genes as queries, we found two contigs in the *C. aurantiacus* genome that contained the missing parts of the putative C_p operon and a contig containing the N part of the gene-encoding subunit I

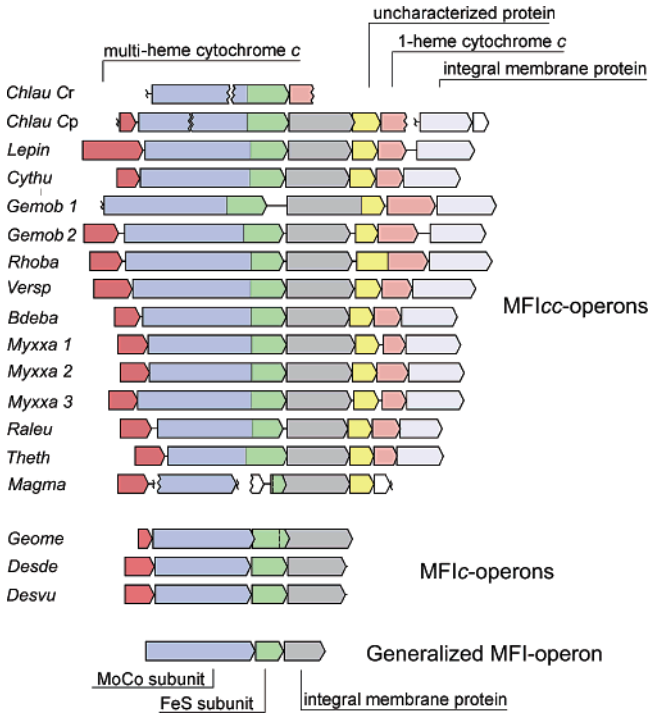

FIGURE 2: Alignment of stretches of genes encoding subunits of C_p and C_r complexes of *C. aurantiacus*, putative MFI*cc* and MFI*c* complexes, and three-subunit molybdopterin oxidoreductases. Broken lines stand for the borders of the contigs of uncompleted genomes that interrupt the putative operons. Abbreviation of the bacteria: *Cythu*, *Cytophaga hutchinsonii*; *Lepin*, *Leptospira interrogans*; *Gemob*, *Gemmata obscuriglobus*; *Rhoba*, *Rhodopirellula baltica*; *Versp*, *Verrucomicrobium spinosum*; *Bdeba*, *Bdellovibrio bacteriovorus*; *Myxxa*, *Myxococcus xantus*; *Raleu*, *Ralstonia eutropha*; *Theth*, *Thermus thermophilus*; *Magma*, *Magnetospirillum magnetotacticum*; *Geome*, *Geobacter metallireducens*; *Desde*, *D. desulfuricans*; *Desvu*, *Desulfovibrio vulgaris.*

of the C_r complex. The trypsin-digest test confirmed the correspondence between the genes and the subunits.

As of December 2004, similar putative operons were found in *Cytophaga hutchinsonii* (cytophaga-bacteroides-flexibacter group), *Leptospira interrogans* (spirochaetes), *Gemmata obscuriglobus* and *Rhodopirellula baltica* (planctomycetes), *Verrucomicrobium spinosum* (verrucomicrobia), *Bdellovibrio bacteriovorus* and *Myxococcus xantus* (δ-proteobacteria), *Ralstonia eutropha* (β-proteobacteria), *Thermus thermophilus* (thermus-deinococcus group), and *Magnetospirillum magnetotacticum* (α-proteobacteria). A putative operon encoding this complex can be described as follows.

The first gene encodes a five-heme cytochrome *c* and corresponds to subunit IV of the C_p complex of *C. aurantiacus*. The N- and C-terminal parts of the second gene and the third gene are correspondingly homologous to the three genes of membrane-bound molybdopterin oxidoreductases. This class of enzymes is broadly distributed in bacteria and archaea and includes formate dehydrogenases, DMSO, tetrathionate, polysulfide, and respiratory nitrate reductases. Subunit I of these oxidoreductases contains molybdopterin cofactor (MoCo); subunit II contains four iron−sulfur clusters; and subunit III is an integral membrane polypeptide containing quinone-binding sites. The class is a part of a more numerous group called the DMSO family of molybdopterin oxidoreductases, which also includes some soluble cytoplasmic and periplasmic oxidoreductases (*44*). The

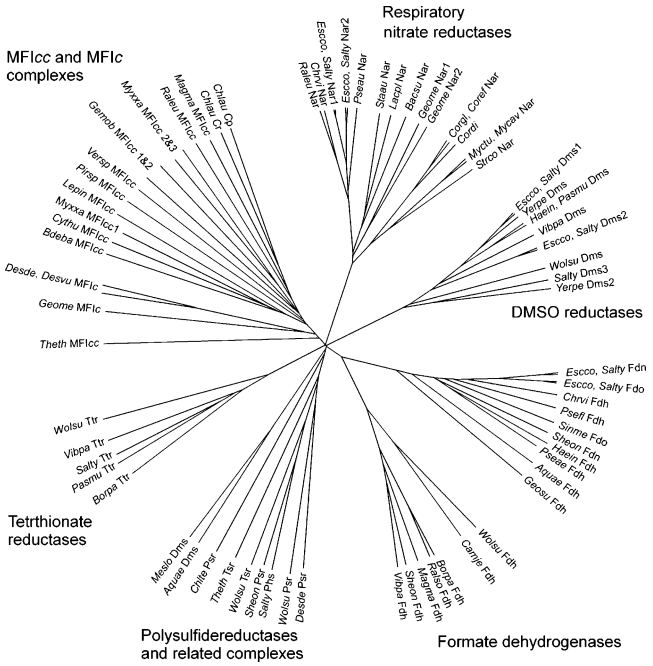

FIGURE 3: Dendrogram for MoCo subunit of three-subunit molybdopterin oxidoreductases and MFI*c* complexes and the corresponding N part of the main subunit of MFI*cc* complexes.

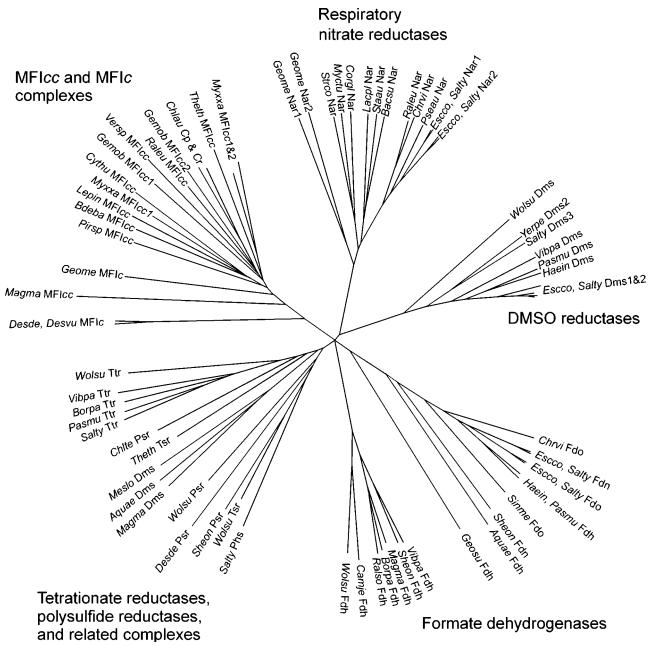

FIGURE 4: Dendrogram for FeS subunit of three-subunit molybdopterin oxidoreductases and MFI*c* complexes and the corresponding C part of the main subunit of MFI*cc* complexes.

second gene of the novel operon is a fusion of two genes and encodes the largest subunit of the complex. The third gene of the operon encodes an integral membrane polypeptide with 10 predicted transmembrane α helices.

The fourth gene of the putative novel operon encodes an unknown polypeptide with two transmembrane helices at the center of the polypeptide. There are no homologues to this gene in any published genome except the indicated range of bacteria. The fifth gene encodes a mono-heme cytochrome *c*, and the sixth one encodes the second integral membrane polypeptide with 10 transmembrane helices homologous to the third gene of the operon. More detailed descriptions of

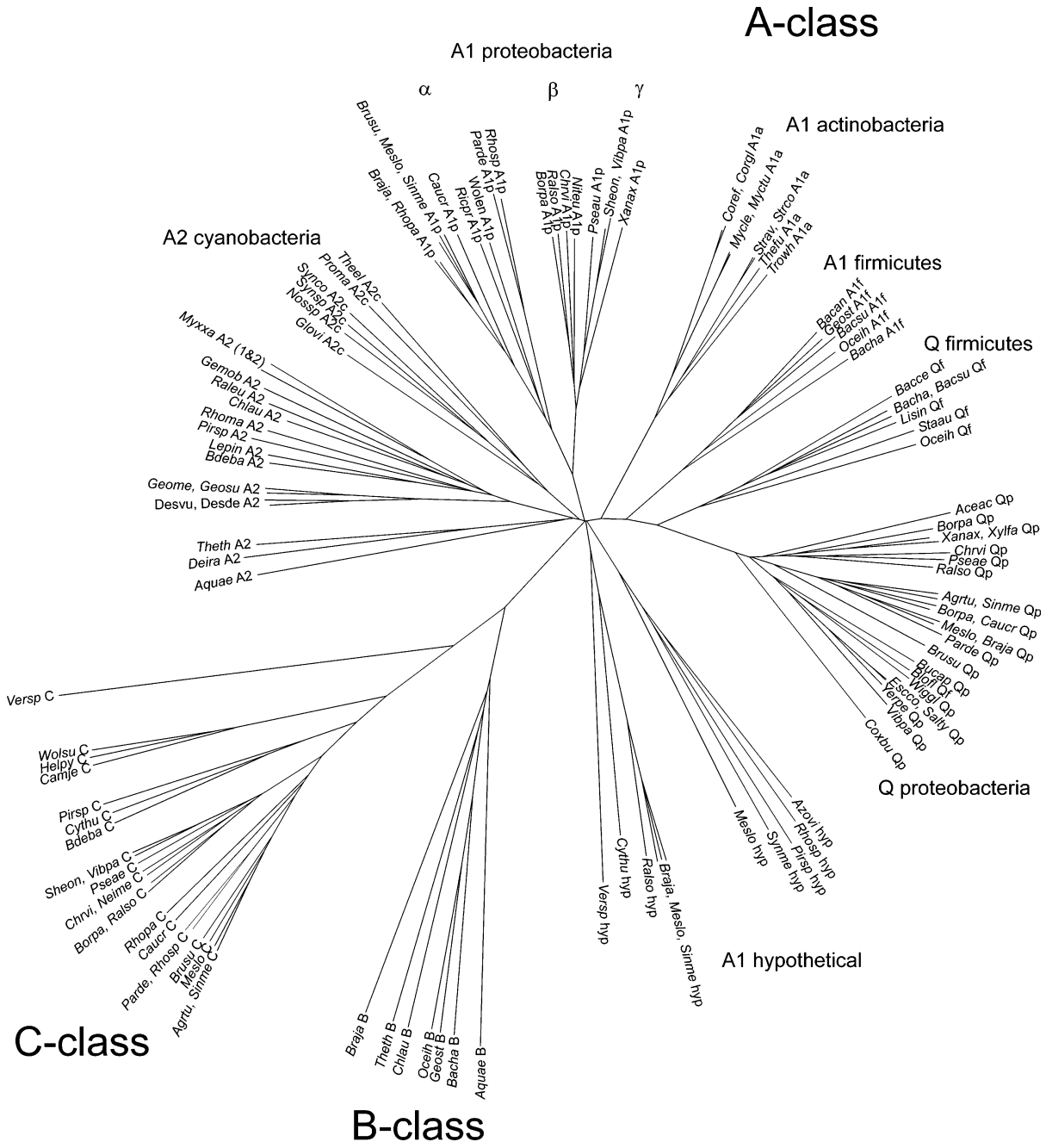

FIGURE 5: Dendrogram for subunit I of Cu−heme cytochrome and quinol oxidases. Abbreviations: A1a, A1f, A1p, and A2c, A-class cytochrome oxidases of actinobacteria, firmicutes, proteobacteria, and cyanobacteria, correspondingly; Qf and Qp, quinol oxidases of firmicutes and proteobacteria.

the genes with amino acid alignments are given in the Supporting Information.

We also found in the genomes of *Geobacter metallireducens*, *Desulfovibrio vulgaris*, and *D. desulfuricans* (all *δ*-proteobacteria) stretches of four genes, of which the first was homologous to the first gene of the putative novel operon and the next three were homologous to the genes of the three-subunit molybdopterin oxidoreductases. The distribution patterns suggest that a novel class of oxidoreductases arose from three-subunit molybdopterin oxidoreductases via an intermediate class by acquisition of additional subunits. We have named the initial class of oxidoreductases MFI (molybdopterin, FeS, and integral membrane subunits), with the intermediate one MFI*c* and the last one MFI*cc*. In the dendrograms for the MoCo and FeS subunits (Figures 3 and 4), the sequences belonging to the MFI*cc* class form separate clusters and sequences belonging to the MFI*c* subclass form subclusters deviating near the roots of the main clusters.

In the genomes of *G. obscuriglobus*, *R. baltica*, *M. xantus*, and *R. eutropha*, the genes of the MFI*cc* complexes are immediately followed by homologous stretches of genes each encoding a polypeptide involved in biogenesis of respiratory and photosynthetic systems (SCO1/SenC) (*45*) and subunits of a cytochrome oxidase. We found homologous stretches of genes in the genomes of all of the bacteria possessing the MFI*c* or MFI*cc* operons except the unfinished genome sequences of *C. hutchinsonii* and *V. spinosum*. These cytochrome oxidases form a separate cluster in the dendrogram of Cu−heme cytochrome (quinol) oxidases (Figure 5). Again, as in the case for MFI*c* complexes, the cytochrome

Table 2: Distribution of bc_1 and MFI*cc* Operons and Different Classes of Cu−Heme Cytochrome (Quinol) Oxidases among Eubacteria

phylum	MFI(*cc*) operon	*bc* (*bf*) operon	type of oxidase	examples
actinobacteria		*bc*	A1a	four genera
firmicutes		*bc*	A1f, Qf	three genera
			Qf	*Staphylococcus*, *Listeria*
cyanobacteria		*bf*	A2c	five genera
proteobacteria				
α		*bc*	A1p, C(±), Qp(±)	seven genera
	MFI*cc*	*bc*	A1p, C	*Magnetospirillum magnetotacticum*[a]
β		*bc*	A1p, C(±), Qp(±)	four genera
		bc	C	*Neisseria meningitidis*
	MFI*cc*	*bc*	A1p, A2	*Ralstpnia eutropha*
γ			Qp	six genera
		bc	A1p, C(±), Qp(±)	*Pseudomonas*, *Vibrio*
δ		*bc*	A2	*Geobacter sulfurreducens*
	MFI*c*	*bc*	A2	*Geobacter metallireducens*[a]
	MFI*c*		A2	*Desulfovibrio desulfuricans*[a]
	MFI*c*		A2	*Desulfovibrio vulgaris*[a]
	3MFI*cc*		2 A2	*Myxococcus xanthus*[a]
	MFI*cc*		A2	*Bdellovibrio bacteriovorus*
ϵ		*bc*	C	three genera
green nonsulfur	2MFI*cc*		A2	*Chloroflexus aurantiacus*[a]
planctomyces	MFI*cc*		A2, C	*Rhodopirellula baltica*
planctomyces	2MFI*cc*		A2	*Gemmata obscuriglobus*[a]
spirochaetes	MFI*cc*		A2	*Leptospira interogans*
CFB group	MFI*cc*		Ah, C	*Cytophaga hutchinsonii*[a]
verrucomicrobia	MFI*cc*		Ah, C	*Verrucomicrobium spinosum*[a]
aquificales		*bc*	A2	*Aquifex aeolicus*
TD group		*bc*	A2	*Deinococcus radiodurans*
TD group	MFI*cc*	*bc*	A2	*Thermus thermophilus*
green sulfur		*bc*		*Chlorobium tepidum*

[a] Uncompleted genomes.

oxidases of the genera *Geobacter* and *Desulfovibrio* form a subcluster situated closer to the node of this cluster. The cluster belongs to the A2 subclass of cytochrome oxidases defined by Pereira et al. (*16*), which is distinguished from the A1 subclass by differences in amino acid residues participating in proton channel D. The subclass also includes cytochrome oxidases of *Thermus thermophilus*, *Deinococcus radiodurans*, *Aquifex aeolicus*, and cyanobacteria. Subclass A1 includes the clusters of the cytochrome oxidases of many proteobacteria, actinomycetes, and firmicutes and the quinol oxidases of firmicutes and proteobacteria and also two unidentified small clusters. Two other main classes, B and C, are clearly resolved in the figure, as found earlier by Pereira et al. (*16*). Amino acid alignments of subunits I and II and some comments are given in the Supporting Information.

The bacteria simultaneously possessing MFI*cc* operons and operons of the A2 class of cytochrome oxidase belong to unrelated bacterial lineages (*46*, *47*), although these operons themselves form coherent clusters. This situation can be explained most plausibly by lateral gene transfer of the relatively recently "invented" MFI*cc* operon along with the attached operon of the cytochrome oxidase. The cases of the separate position of these two operons in some bacterial genomes should be considered as secondary events.

The apparently congruent clustering of the genes of MFI*c*(*c*) operons and the genes of cytochrome oxidases belonging to the same range of bacteria suggests a functional interaction between these two enzymes and their concatenate origin and evolution. Putative genes encoding subunit II of these oxidases contain the Cu- and the cytochrome *c*-binding signatures (see corresponding amino acid alignments in the Supporting Information), which is characteristic for terminal oxidases reacting with just periplasmic electron transporters and not with quinol (*15*, *16*). The simplest interpretation of these results is that MFI*cc* complexes are quinol:periplasmic electron transporter oxidoreductases. The distribution of the genes of MFI*cc* complexes, *bc* complexes, and different kinds of Cu−heme cytochrome (quinol) oxidases among completed and some incomplete genomes (Table 2) supports such a hypothesis. Most genomes possessing the operons of *bc* complexes contain the operons of cytochrome oxidases of class A. Sometimes these genomes additionally contain the operons of quinol oxidases of class A and/or cytochrome oxidases of classes B and C. The genomes of ϵ-proteobacteria and *Neisseria meningitidis* (*β*-proteobacteria) contain the cytochrome oxidases of class C only. The cases of the absence of the *bc* complex comprise only two categories. The absence of the *bc* complex in enterobacteria, some other members of *γ*-proteobacteria, and two genera of firmicutes is associated with the presence of the quinol oxidase only. The dendrogram shown in Figure 5 suggests that quinol oxidases are derived from the cytochrome oxidases of bacilliaceae as was noted earlier (*15*, *16*). The absence of *bc* complexes in these cases may be explained by the loss of the redundant enzyme. The other cases of the absence of the *bc* complex are associated with the presence of the MFI*c* or MFI*cc* complexes and the cytochrome oxidases of the A2 subclass. As we have already noted, these terminal oxidases almost certainly react with periplasmic electron transporters. In the absence of a *bc* complex, the only known class of quinol:periplasmic electron transporter oxidoreductases, the most plausible candidate for its role is the MFI*cc* complex.

The supposition given above implies that there has been a rearrangement of the active site of an ancestral enzyme. Indeed, all molybdopterin enzymes react with chemical

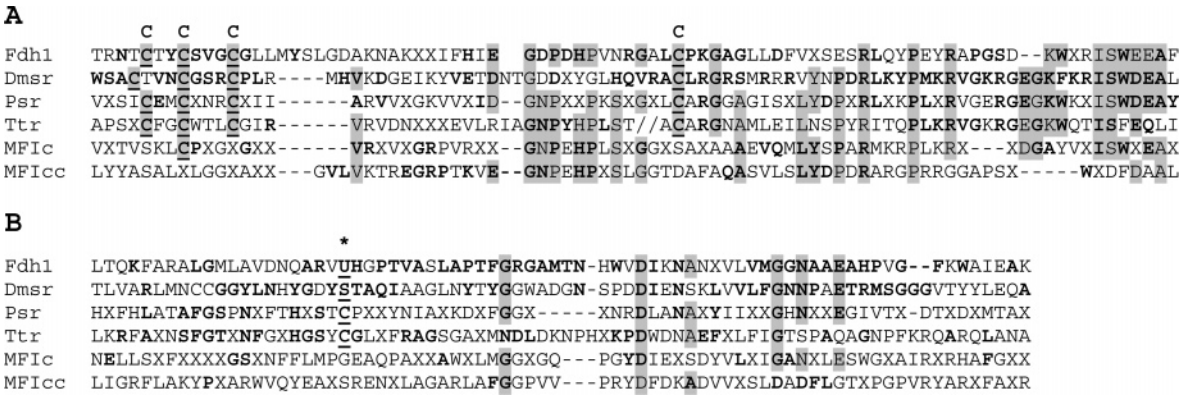

FIGURE 6: Alignment of the amino acid consensus sequences of the classes of membrane-bound molybdopterin oxidoreductases and MFI*c* and MFI*cc* complexes. The consensus sequences for each of the classes were determined by applying ClustalW to the sets indicated in Figure 3. The residues conserved in no less than $^2/_3$ of the cases within each class are indicated by bold letters. The residues conserved in no less than $^2/_3$ of the cases between the consensus sequences are indicated by a gray background. (A) Conservative region near the N terminus bearing the motif of four Cys (marked by a C above the alignment and underlined). (B) Region around amino acid ligands to molybdenum atom (marked by an asterisk above the alignment and underlined). Full amino acid alignments of the alignments are presented in the Supporting Information.

compounds, while according to its supposed role, the new oxidoreductase must react with an electron-transport protein. Such an arrangement could occur because of the acquisition of new subunits and the loss of the molybdopterin cofactor. We did not find molybdenum in three separate preparations of the *C*p complex of *C. aurantiacus* using atomic absorption spectroscopy, although the amounts of the enzyme in assays were enough for confident molybdenum detection if it is equimolar to the enzyme. We also applied standard procedures used for the oxidation of pterin and extraction of the fluorescent products from molybdopterin enzymes (*48*) to the C_p and C_r complexes. Although we used amounts of C_p and C_r complexes that were enough to detect the fluorescence if the cofactor was present, we did not observe the characteristic fluorescence spectra.

The amino acid alignment of MoCo subunits corroborates this finding (Figure 6). In comparison with MFI complexes, the MFI*c* and MFI*cc* complexes do not contain the motif of four Cys at the N terminus. These cysteines bind the FeS(0) cluster, which transfers electrons to the molybdopterin cofactor from the second subunit in nitrate reductases (*49*), formate dehydrogenases (*50*), and presumably other MFI complexes. Recent studies on the X-ray structure of molybdopterin enzymes of the DMSO-reductase family determined the amino acid residues that serve as ligands to molybdenum: Ser in DMSO reductase (*51*), Asp in respiratory nitrate reductase (*49*), and Se−Cys or Cys in formate dehydrogenase (*50*). In some formate dehydrogenases, tetrathionate reductases, and polysulfide reductases and related enzymes, Cys was proposed as a Mo ligand in the corresponding place by consideration based on homology (*52*). Although there is no apparent homology between classes in the regions around these residues, the degree of homology within each class is rather high as it is indicated by bold letters for conservative amino acid residues. In the MFI*c* and MFI*cc* complexes, there are no conserved amino acid residues that could serve as ligands for Mo in the corresponding regions (Ser in the MFI*cc* consensus is not conserved). Moreover, the regions themselves are variable within these two classes. We conclude that, despite the overall amino acid similarity of the MFI*c* and MFI*cc* complexes to the classic MFI complexes, the MFI*c* and MFI*cc* complexes do not contain Mo or pterin.

The genomes of *T. thermophilus*, *M. magnetotacticum*, and *R. eutropha* operons for *bc* complexes coexist with MFI*cc* operons. In the Supporting Information, we present data in favor that in theses cases the MFI*cc* operons were brought by LGT in addition to already existing bc_1 operons.

It was supposed earlier that the C_p and C_r complexes of *C. aurantiacus* are quinol:periplasmic e-transporter oxidoreductases (*32*). The supposition was based on the absence of any complex bearing features of the bc_1 complex in the membranes of actively grown *C. aurantiacus* and that the only candidates for this function were C_p and C_r complexes expressed in actively grown photosynthetic and respiratory cultures of the bacterium. We have not yet confirmed unambiguously the supposed activity in the *C. aurantiacus* complexes; thus, the statement remains a hypothesis. However, the present analytical study gives strong support in its favor, and the genomic data indicate that such complexes are widespread in bacteria. Experimental work documenting catalytic activity and additional physical and chemical characterization of the complexes is now in progress and will be published elsewhere.

SUPPORTING INFORMATION AVAILABLE

Alignments of amino acids of the putative products of MFI*c* and MFI*cc* operons, alignments of amino acid consensuses of different classes of MFI(*cc*) oxidoreducases, and alignments of amino acid consensuses of different clusters of class A cytochrome (quinol) oxidases. This material is available free of charge via the Internet at http://pubs.acs.org.

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