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Biogenesis of Respiratory Cytochromes in Bacteria

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INTRODUCTION

The assembly of proteins into biologically functional structures or enzymes is a fascinating topic of research. How are cofactors incorporated into proteins? How are multisubunit enzymes assembled? Do these molecules oligomerize more or less randomly, or is the biogenesis of mature, proteinaceous complexes predetermined in a precise maturation pathway?

Different aspects of the posttranslational maturation of any enzyme and their interrelationship are illustrated in Fig. 1. A simple case of protein maturation is the conversion of the primary gene product (apoprotein) into the mature form (holoprotein) by insertion of a prosthetic group. When examined more closely, it becomes clear that incorporation of the cofactor is inevitably connected to the folding of the polypeptide: either the polypeptide folds first into a conformation that is suitable for cofactor binding, or the combining of the cofactor with the polypeptide promotes a certain way of folding. In the case of multiprotein complexes, the maturation process is complicated by the assembly of the polypeptide subunits. It is not clear a priori whether subunit assembly and cofactor incorporation occur independently. A further level of complexity must be taken into account when the subcellular sites of protein and cofactor synthesis differ from that of holoprotein function. Translocation of both polypeptide and cofactor into or through compartment membranes is then required and is expected to be tightly coupled with the other steps of holoprotein formation. Finally, proteolytic processing and modification of polypeptide side chains may also occur as posttranslational events in the process of maturation.

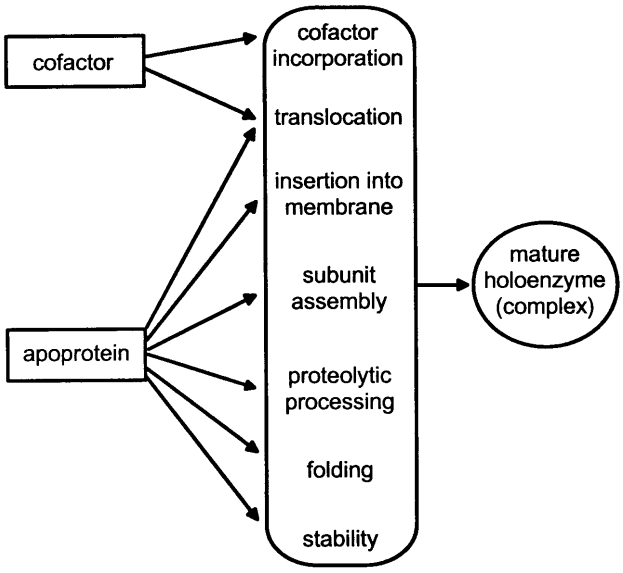


FIG. 1. General scheme of steps required for maturation of holoenzyme complexes. The order and interdependence of individual steps are not predictable.

This review is an attempt to compile and discuss the current knowledge of the posttranslational maturation processes that apply for a certain class of bacterial proteins, the respiratory cytochromes. All of the considerations mentioned above may hold true when the posttranslational maturation of this group of proteins is more fully understood.

Cytochromes are electron transfer proteins that carry heme as a prosthetic group. Their redox function is intimately related to the valence change of heme iron (156, 387). Most bacterial cytochromes function either in photosynthetic electron transport or in aerobic and anaerobic respiration, whereby ATP formation is coupled to the oxidation of reduced substrates such as organic substances, hydrogen, reduced sulfur, or metals. Since respiration takes place in the cytoplasmic membrane, cytochromes are often localized in this compartment. In addition, they are found in the periplasmic space, where their electron transfer function is connected with that of membrane-bound cytochromes. In many cases, cytochromes are assembled in multisubunit enzyme complexes that may contain additional nonheme cofactors. However, they can also occur solitarily, e.g. when they function as mobile, periplasmic electron shuttles between other membrane-bound respiratory complexes and their reaction partners.

As the name indicates, cytochromes are cellular pigments that absorb light at specific wavelengths via their heme cofactor. Since heme can be detected easily by spectroscopic methods, cytochromes are ideal proteins to use in the study of the general question of polypeptide-cofactor association. This is probably the reason why a relatively large amount of data on the biogenesis of cytochromes and cytochrome complexes has accumulated in recent years. Multiple facets of cytochrome assembly and maturation have been recognized. Although the biogenesis of cytochromes has become a topic of growing research interest, few review articles that either cover the entire area or treat certain aspects of this rather complex subject have appeared (90, 116, 149, 184, 341, 342). In this paper, an attempt is made to define, compile, and compare the individual reactions needed for the maturation of cytochromes and to connect them in a logical way. This review may contribute to a general understanding of how the biogenesis of proteinaceous complexes occurs in nature.

This article is divided into three major parts. First, the general characteristics and functions of bacterial cytochromes are discussed briefly, with emphasis on cytochromes that are involved in respiratory processes. Next, individual steps of cytochrome maturation are discussed separately and connected to other, related cellular processes. Finally, hypothetical complete pathways are presented for the maturation of the most extensively studied cytochromes, including cytochromes of the *c* type in general, the bc_1 complex as a somewhat special case, and several terminal oxidoreductases. Although the main focus of this review is bacterial redox proteins, knowledge of the biogenesis of eukaryotic cytochromes will occasionally be included for comparison.

CYTOCHROMES INVOLVED IN BACTERIAL RESPIRATION

General Features of Cytochromes

The term "cytochrome," as it is used in this article, refers to hemoproteins that are constituents of electron transport chains. Not included are a variety of hemoproteins with functions other than one-electron transfer, such as catalase, peroxidase, bacterioferritin, assimilatory sulfite reductases, hemoglobin-like proteins, cytochrome P-450, and heme-containing regulatory proteins (52, 53, 84, 100, 112, 209, 227, 229, 263, 267, 320, 361, 369, 373, 389). Cytochromes of the photosynthetic electron transport chain are discussed only when they also function in respiratory electron transport.

Cytochromes exhibit certain spectral characteristics, depending on the type of heme with which they are associated and on the local environments of the heme binding sites in the protein (107). The reduced or ferrous form of a cytochrome gives rise to three main absorption peaks in the visible to UV spectrum; these are designated the α, β, and γ (Soret) peaks. With few exceptions, the peak in the α region (>550 nm) can be used to classify a specific cytochrome: *c*-type cytochromes have a maximum between 550 and 557 nm, *b*- and *o*-type cytochromes have a maximum between 555 and 565 nm, *a*-type cytochromes have a maximum around 600 nm, and *d*-type cytochromes have a maximum around 630 nm (156). Accordingly, the cytochrome nomenclature often includes the α-band absorption maximum of a given species with a subscript: cytochrome b_{558}, for example, is a *b*-type cytochrome with an absorption maximum of the α band at 558 nm.

The heme iron of cytochromes can occur in either low-spin or high-spin conformation with paired or unpaired electrons, respectively (151). In the former, the heme cofactor has two axial strong-field ligands regardless of the oxidation state of iron, i.e., in a hemochrome linkage. In the latter, only one of the axial coordination places is occupied, and the component can react with other molecules such as O_2, CO, or cyanide.

Heme in *c*-type cytochromes is almost always attached covalently by two thioether bonds between its vinyl side chains and two conserved cysteine residues of the apoprotein; in addition, there are one or two axial ligands to the heme iron, of which one is always a histidine. By contrast, in *a*-, *b*-, *d*- and *o*-type cytochromes, the cofactor is bound noncovalently to the protein. While the prosthetic group of *b*- and *c*-type cytochromes is protoheme IX (heme B or C), the hemes of the other cytochromes are modified: hemes O and A both carry a farnesylhydroxyethyl side chain, but heme A differs from heme O by an additional formyl group at position 8 of the tetrapyrrole ring. In heme D, the C ring of the tetrapyrrole is saturated. Heme D_1 and siroheme have unconjugated A and B rings. Figure 2 shows the different types of heme found in bacterial cytochromes.

Bacterial Respiratory Chains

Bacterial respiratory chains are composed of a variety of electron transport constituents, such as flavoproteins, iron-sulfur proteins, quinones, and cytochromes. The differential transport of electrons and protons through the cytoplasmic membrane leads to the formation across the membrane of a proton gradient that can be used to drive ATP formation via the F_1F_0 ATPase. The electrons are passed through a number of redox carriers to oxygen during aerobic respiration or to alternative terminal electron acceptors when oxygen is not available, as depicted in Fig. 3. Bacterial electron transport chains are often branched. They can be divided roughly into quinone-reducing and quinol-oxidizing branches. The reductive parts are responsible for the electron transfer from a variety of substrates, such as NADH, hydrogen, succinate, lactate, and formate, to the quinones and include several reductases. The oxidative branches include redox systems that are able to oxidize quinol, cytochrome *c*, and the terminal oxidoreductases. Terminal oxidases are oxidoreductases which are capable of O_2 reduction. Alternative terminal oxidoreductases use electron acceptors other than O_2 and thus support anaerobic respiration. Typically, the quinol-oxidizing branch of electron transport chains is rich in cytochromes. Quite often, there is another mediator between the quinol oxidase and the terminal oxidoreductase, usually a *c*-type cytochrome.

Constituents of bacterial respiratory chains are part of or are associated with the cytoplasmic membrane across which the charge separation that is used for energy production takes place. However, there is one report of cytochromes being located in the outer membrane of certain gram-negative bacteria, which may link respiratory proton translocation to the reduction of extracellular, insoluble metal oxides (230). Since outer membrane cytochromes have not been shown to participate in respiratory electron transport, they will not be discussed further here.

A complete survey of different bacterial cytochromes and their functions is beyond the scope of this article, and the available information has been compiled by others (107, 133, 156, 164, 257, 262, 387). The cytochromes and cytochrome complexes which have been most extensively studied with respect to their assembly are described in more detail in the following sections. Some of them are depicted in Fig. 4 to show their subunit and cofactor composition and their orientation in or at the cytoplasmic membrane.

Cytochromes of the Quinone-Reducing Branch of the Respiratory Chain

Succinate dehydrogenase. Succinate dehydrogenase (SDH), which, by analogy with its mitochondrial homolog, is also known as complex II, catalyzes electron transfer from succinate to ubiquinone, whereby succinate is oxidized to fumarate. Thus, the enzyme catalyzes a reaction of the tricarboxylic acid cycle. However, SDH is not an energy-coupling site in the respiratory chain. It is a multimeric membrane protein complex containing heme B as one of several different prosthetic groups.

SDH is known best from work done with *Escherichia coli* (109) and *Bacillus subtilis* (139). In both organisms, two large subunits are involved in succinate oxidation (Fig. 4): SdhA carries a covalently attached flavin cofactor (flavin adenine dinucleotide [FAD]), and SdhB is an iron-sulfur (FeS) protein. These two subunits are located on the cytoplasmic side of the membrane, to which they are bound by the membrane-integral cytochrome *b*, which is the reductant of quinone. In *E. coli*, two membrane-integral polypeptides appear to share a B-type heme (181), whereas in *B. subtilis*, a single polypeptide is associated with two B-type hemes (126).

In *E. coli*, all four subunits are encoded in the *sdhCDAB* operon. The first two gene products contain cytochrome b_{556}, whereas *sdhA* and *sdhB* code for the FAD and FeS center-carrying subunits, respectively (64, 381). The *Paracoccus denitrificans* SDH was also shown to contain cytochrome *b* (255), and the nucleotide sequence of the *sdhCDAB* genes revealed a similar gene arrangement as in *E. coli* (74). In *B. subtilis*, *sdhA* and *sdhB* code for the cytoplasmic SDH subunits whereas *sdhC*

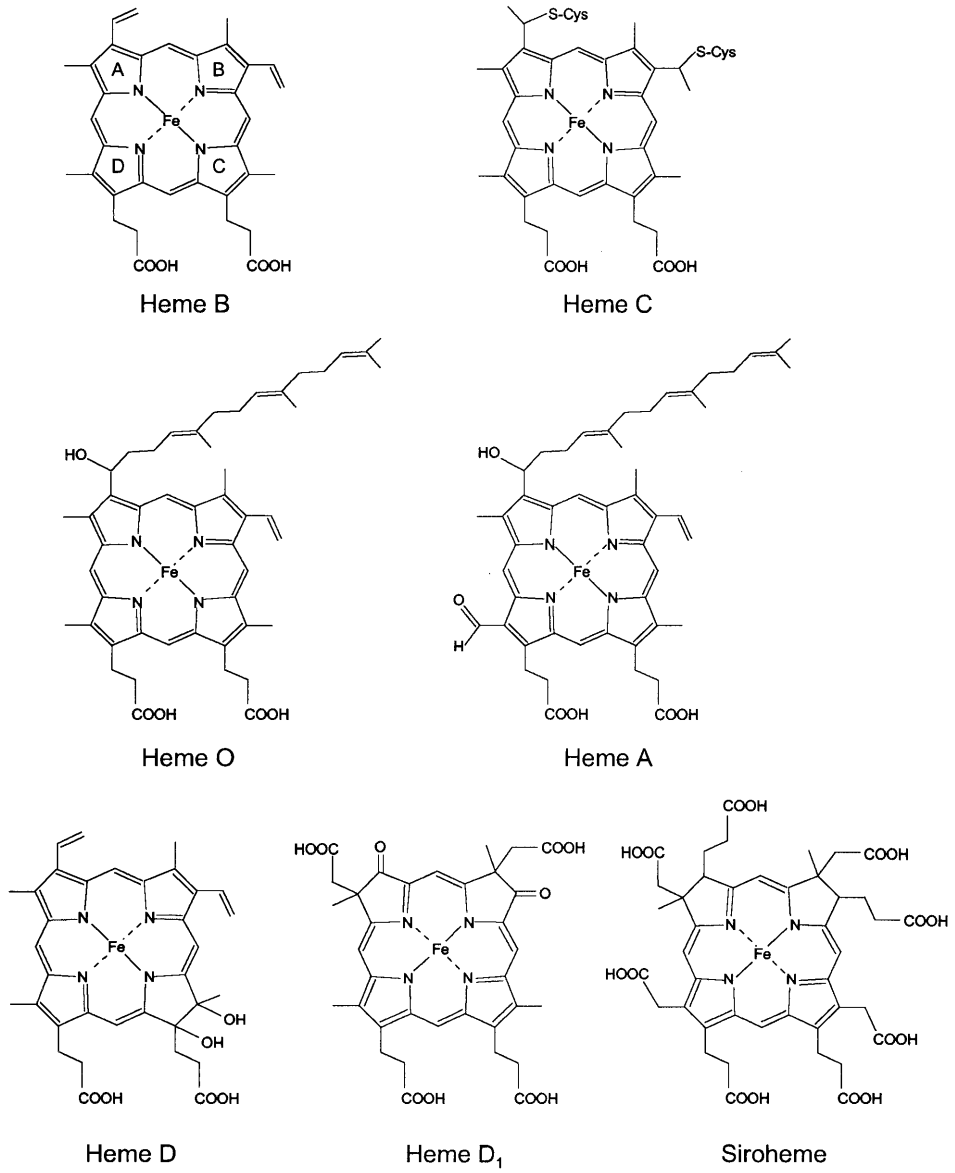


FIG. 2. Heme cofactors of bacterial cytochromes. In reduced heme, Fe is present as Fe^{2+}; in oxidized heme, it is present as Fe^{3+}. Note that heme C is bound covalently by two thioether bonds to cysteinyl residues of the polypeptide. The nomenclature of porphyrin rings A to D is indicated for heme B.

is the structural gene for the membrane-integral cytochrome b_{558} (201, 259).

Formate dehydrogenase. Formate can be oxidized to CO_2 in an anaerobic respiratory process with exogenous electron acceptors (109). When nitrate serves this function, formate dehydrogenase N (FDN) is induced. In *E. coli*, this enzyme is encoded by the *fdnGHI* operon (23) and contains three subunits: the large FdnG, which is a selenomolybdoprotein, a smaller FeS protein (FdnH), and a *b*-type cytochrome (FdnI) (82).

Another formate dehydrogenase containing cytochrome *b* has been found in *Wolinella succinogenes*. It is encoded by the *fdhABC* operon. FdhA is the molybdoprotein, FdhB is the FeS protein, and FdhC is a hydrophobic membrane protein that is presumed to carry a single heme B cofactor (30).

Hydrogenase. The *W. succinogenes* Ni/Fe hydrogenase (HYD) is the only member of the bacterial hydrogenases for which the presence of a heme cofactor has been shown. The membrane-bound enzyme mediates electron transfer from H_2 to quinone and consists of three polypeptides, HydA, HydB, and HydC. HydA and HydB are hydrophilic proteins and show similarity to the small and large subunits, respectively, of periplasmic Ni hydrogenases that bind FeS centers and nickel. HydC is an integral membrane protein that is thought to carry a heme B molecule liganded by two histidines (77). Since the putative heme ligands are conserved among the five known HydC sequences, it is possible that hydrogenases from other bacteria also contain a heme cofactor.

Cytochromes of the Quinol-Oxidizing Branch of the Respiratory Chain

Ubiquinol:cytochrome *c* oxidoreductase. The cytochrome bc_1 complex (which by analogy to the mitochondrial respiratory chain is also called complex III) is a central redox carrier

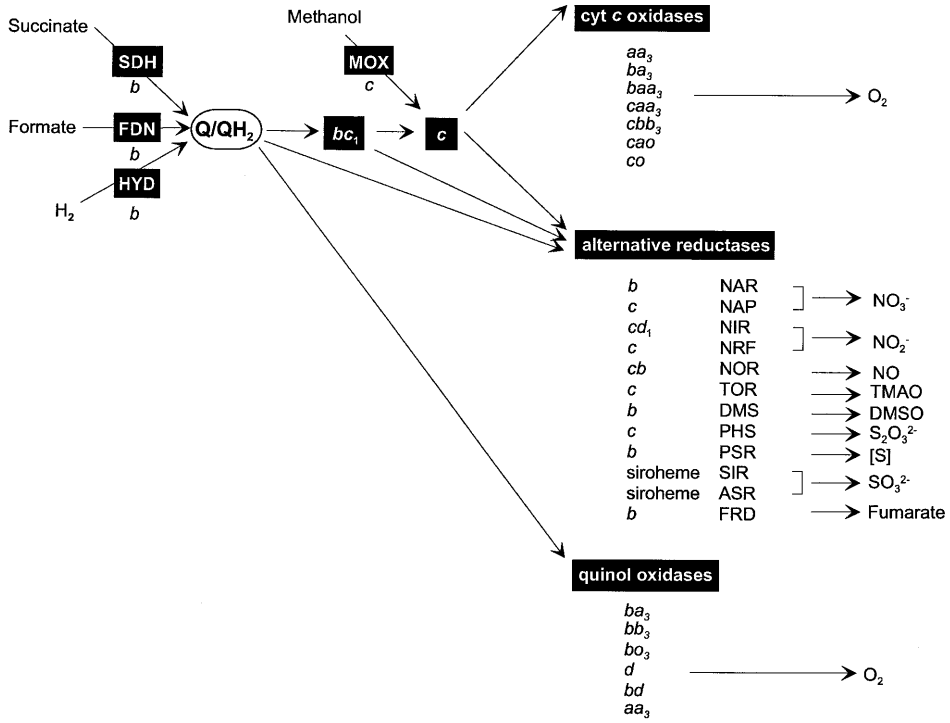


FIG. 3. Bacterial respiratory chains containing cytochromes. On the left, the quinone (Q = ubiquinone or menaquinone)-reducing branches are shown. On the right, the quinol-oxidizing branches with terminal oxidases reducing either O_2 or alternative electron acceptors are shown. Oxidoreductases are highlighted, and the type of cytochrome is indicated below. Q/QH_2 represents the quinone/quinol pool in the membrane. The case of hydrogenase containing a cytochrome *b* is an exception (see the text). Polysulfides are indicated by [S]. Respiratory chains without cytochromes are not shown. For abbreviations and comments, see the text.

in many bacterial respiratory chains. It oxidizes quinols and reduces metalloprotein electron carriers, which are in most cases *c*-type cytochromes. At the same time, it conducts vectorial proton translocation, leading to an electrochemical proton gradient across the membrane. The crystal structure of the beef heart mitochondrial cytochrome bc_1 complex was solved recently at 3.4-Å resolution (391). The bc_1 complex is found in gram-negative as well as in gram-positive bacteria. In phototrophic bacteria, the complex functions in both respiratory and photosynthetic electron transport. Notably, *E. coli* does not possess a bc_1 complex. Detailed information on bacterial bc_1 complexes can be obtained from several comprehensive reviews (118, 169, 332, 346, 347).

The classical, bacterial bc_1 complex is a membrane protein complex containing three subunits with four redox centers (346) (Fig. 4). The Rieske iron-sulfur protein carries a 2Fe-2S cluster that is bound to conserved cysteines and histidines. The protein is anchored to the cytoplasmic membrane by an N-terminal, hydrophobic sequence that seems to be used for export of the C-terminal, hydrophilic domain. Cytochrome *b* is an integral membrane protein with nine predicted hydrophobic α-helices, of which eight appear to span the membrane bilayer, with two heme molecules bound noncovalently to conserved histidines in transmembrane helices II and IV. The last three transmembrane helices represent a protein domain that in chloroplast or cyanobacterial bc_1-like complexes (b_6f) occurs as a separate subunit IV (see below). Cytochrome c_1 (the subscript in cytochrome *c* nomenclature is used to distinguish different classes of *c*-type cytochromes [257]) contains covalently bound heme C and is located on the periplasmic side of the membrane, to which it is anchored by a hydrophobic segment at the C terminus. Cytochrome c_1 is synthesized as a precursor whose N-terminal signal peptide is cleaved after membrane translocation.

A protein domain swapping in complex III in *B. subtilis* and *Bacillus stearothermophilus* has been recognized recently (Fig. 5) (316, 392). The cytochrome *b* homolog is of split character: its N-terminal part is encoded by a separate gene (*qcrB*) and contains the heme B groups, whereas the C-terminal part, i.e., the domain corresponding to cyanobacterial subunit IV (see below), is fused to the *c*-type cytochrome in a single protein (QcrC). The *c*-type cytochrome does not resemble cytochrome c_1 but, rather, shares similarity with small cytochromes *c* such as the *B. subtilis* cytochrome c_{550} (392).

There is even one case of a bacterial bc_1 complex in which three protein domains are fused: in *Bradyrhizobium japonicum*, a cytochrome *b*-cytochrome c_1 polyprotein precursor is synthesized from a single gene (Fig. 5) (336, 338, 343). This precursor is cleaved during maturation into classical types of cytochrome *b* and c_1 moieties.

The bc_1 complexes from *Rhodobacter* species copurify with a low-molecular-weight polypeptide whose identity and function are not clear (194), and it remains to be shown whether this protein is a true subunit of the complex.

The cyanobacterial cytochrome b_6f complex is structurally and functionally homologous to the bc_1 complex but more closely resembles the chloroplast b_6f complex (169). It differs from the bc_1 complex mainly in two characteristics. First, its membrane-integral subunit is split into an α subunit and a β subunit homologous to the N- and C-terminal halves of cytochrome *b*, respectively (Fig. 5). The α subunit comprises the first five transmembrane segments of cytochrome *b* and contains both heme B groups. The β subunit, also referred to as subunit IV (SUIV), is similar to the C-terminal domain of

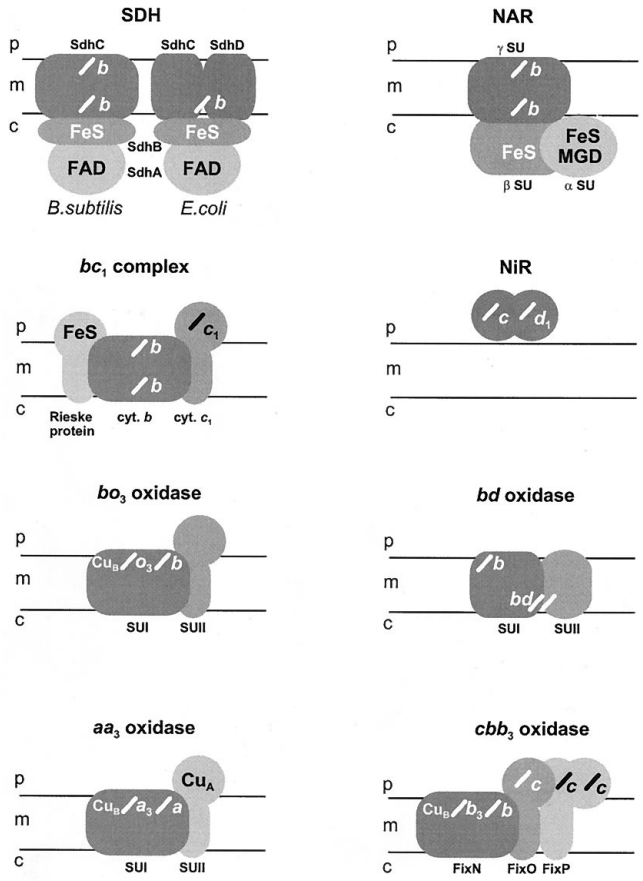


FIG. 4. Topological models of bacterial respiratory complexes. Only the catalytic subunits are shown. Noncovalently bound hemes are indicated in white, and covalently bound hemes C are indicated in black. Additional cofactors are also indicated. p, periplasm; m, cytoplasmic membrane; c, cytoplasm. For other abbreviations, see the text.

classical cytochrome *b* of complex III and does not contain a redox-active cofactor. Furthermore, the third subunit of the complex is more closely related to cytochrome *f*, which is also a *c*-type cytochrome, than to cytochrome c_1, as judged from amino acid sequence comparisons.

The genes encoding bacterial bc_1 complexes have been cloned and sequenced from a variety of species (34, 67, 103, 170, 187, 203, 306, 343, 362, 377, 393). They are called *fbc*, *pet*, or *qcr* genes (Fig. 5). Unfortunately, the *petA* and *petC* genes from *Rhodobacter capsulatus* code for the FeS protein and cytochrome c_1, respectively, whereas in cyanobacteria *petA* encodes cytochrome *f* and *petC* encodes the Rieske FeS protein, according to the chloroplast gene nomenclature. Cyanobacterial cytochrome b_6 is encoded by *petB*, and SUIV is encoded by *petD*. The bacterial complex III genes are organized in one operon or, in cyanobacteria, two operons, and they are present in different combinations (Fig. 5).

Cytochrome *c*. Cytochromes of the *c*-type (cytochromes *c*) are more or less mobile electron transfer proteins on the periplasmic side of the membrane. The heme cofactor is bound covalently via thioether bonds to the cysteines of the heme binding motif CXXCH. The histidine in this motif serves as an axial ligand of the heme iron. When heme is in a low-spin state, it is complexed by a second axial ligand, which is usually a methionine or a histidine located at a considerable distance from the heme binding site in the primary amino acid sequence (257).

Cytochromes *c* function in respiratory chains most often by passing electrons from the bc_1 complex to terminal oxidoreductases. They occur in two forms: either as soluble periplasmic proteins or bound to the membrane with N-terminal, hydrophobic extensions. The soluble forms are usually small, 8- to 14-kDa polypeptides which are also called class I cytochromes *c* and are homologous to the mitochondrial cytochrome *c* (223). A large variety of small, soluble *c*-type cytochromes have been isolated from diverse bacteria, and their structures and functions in electron transport have been discussed in several comprehensive reviews (200, 213, 257). Soluble *c*-type cytochromes do not always move electrons from the bc_1 complex to terminal oxidases (Fig. 3). For example, in gram-negative methylotrophic bacteria, methanol is oxidized to formaldehyde by a periplasmic methanol dehydrogenase (MOX), and the electrons are passed via a *c*-type cytochrome to the terminal oxidase (6, 117, 213). In sulfate-reducing bacteria, a tetraheme cytochrome c_3, which in some species has unusual heme C binding sites (see below), plays a role in the electron transport between hydrogenase and thiosulfate reductase (15).

Membrane-bound *c*-type cytochromes involved in respiratory electron transport have been identified from several species. They contain an N-terminal, hydrophobic extension that acts as an anchor for the otherwise periplasmically oriented, hydrophilic protein. Examples are the *B. subtilis* cytochrome c_{550} whose precise function is unknown (370, 371); the *Bradyrhizobium japonicum*, *Paracoccus denitrificans*, and *Rhizobium leguminosarum* CycM protein that transfers electrons from the bc_1 complex to the cytochrome aa_3 terminal oxidase (33, 349, 382); and the *Rhodobacter capsulatus* cytochrome c_y, which serves as an electron donor either to the photosynthetic reaction center (162, 163) or to the bc_1 complex (145). A diheme cytochrome *c*, the *cycG* gene product, has been characterized recently in *Rhodobacter sphaeroides*, but no involvement in respiration has been shown (94).

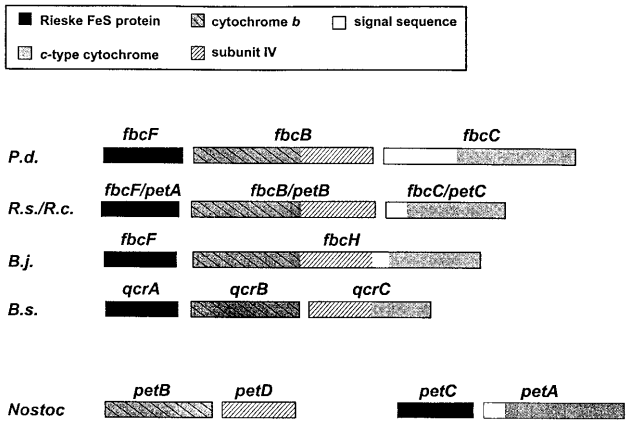


FIG. 5. Subunit and domain structure of precursors of different bacterial bc_1 complexes. The order of the represented precursor polypeptides from left to right reflects the direction of transcription of the corresponding genes, which are indicated on top of each polypeptide. The *Bradyrhizobium japonicum fbcH* gene product is synthesized as a bc_1 precursor and is cleaved posttranslationally into a cytochrome *b* and a cytochrome c_1 polypeptide. The *Bacillus subtilis qcrC* gene product is a fused polypeptide comprising an N-terminal domain homologous to the cyanobacterial subunit IV and a C-terminal domain that is more similar to soluble cytochrome *c* than to cytochrome c_1. The cyanobacterial (*Nostoc*) b_6f complex comprises four subunits that are encoded in two operons. Abbreviations: *B.j.*, *B. japonicum*; *B.s.*, *B. subtilis*; *P.d.*, *Paracoccus denitrificans*; *R.c.*, *Rhodobacter capsulatus*; *R.s.*, *R. sphaeroides.*

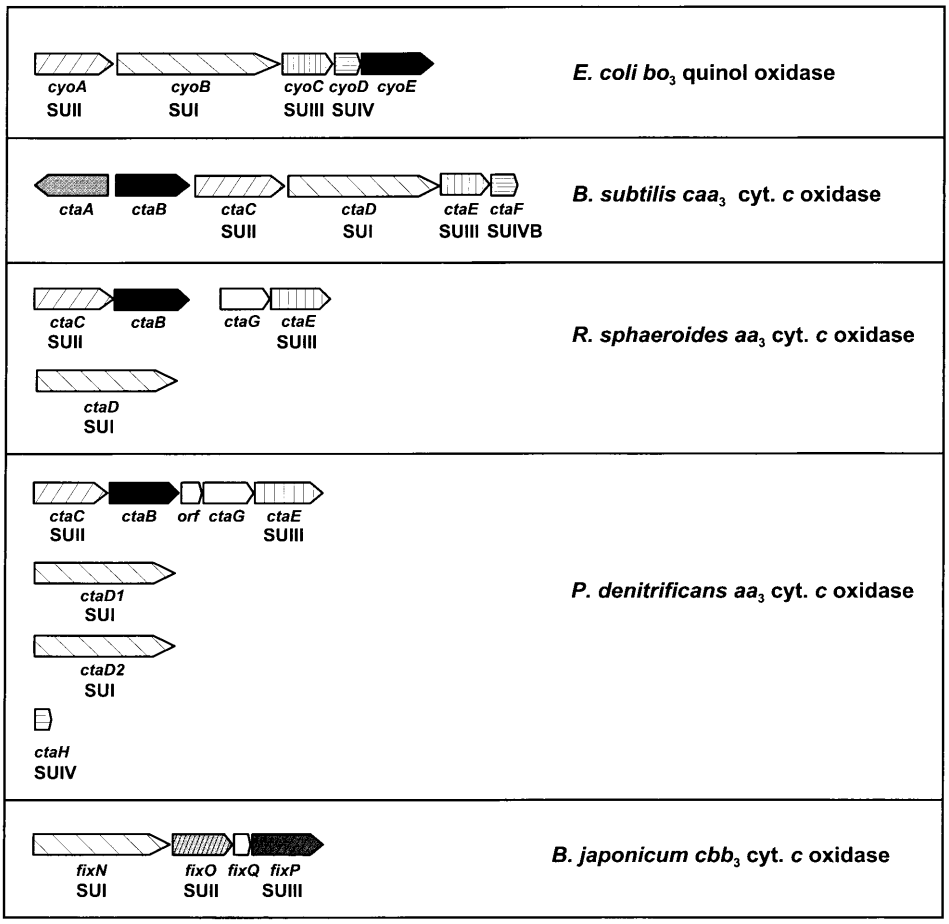


FIG. 6. Organization of genes encoding various bacterial heme-copper oxidases which have been studied with respect to subunit assembly. Below the structural genes are indicated the respective subunits. Genes involved in the production of heme O (*cyoE*/*ctaB*) and heme A (*ctaA*) are drawn with dark shading; genes coding for assembly factors or with unknown functions are white. SUI of *Paracoccus denitrificans* is encoded by two isogenes (*ctaD1* and *ctaD2*). The information on the *ctaH* gene encoding SUIV was provided by B. Ludwig (380). The *Rhodobacter capsulatus*, *R. sphaeroides*, and *P. denitrificans ccoNOQP* genes are organized in an operon similar to the homologous rhizobial *fixNOQP* genes (for references, see the text). More detailed information on the different gene clusters is presented in references 105 and 297.

Terminal oxidases involved in O_2 reduction. (i) Cytochrome *c* oxidases. The terminal oxidases which accept electrons from cytochrome *c* and transfer them in reactions coupled to H^+ transfer to oxygen to produce H_2O are called cytochrome *c* oxidases. They all belong to the family of heme-copper oxidases because they contain heme and copper as cofactors (105, 297). The types and combinations of heme cofactors can vary in these enzymes (Fig. 3).

Many bacteria contain a cytochrome *c* oxidase that is homologous to the mitochondrial aa_3 type of oxidase (298). This type of cytochrome *c* oxidase has been studied best in *Paracoccus denitrificans*, and the three-dimensional structure of the enzyme in this species has been solved recently (158). It and the corresponding and homologous bovine mitochondrial enzyme complex (348) were the first respiratory membrane protein complexes whose crystal structures were solved. The *P. denitrificans* enzyme contains four subunits, of which two carry the heme and copper redox centers (Fig. 4). Subunit I (SUI) is an integral membrane protein with 12 transmembrane helices. It binds a low-spin and a high-spin heme A, the latter being associated with Cu_B in a bimetallic Fe-Cu center, also referred to as the binuclear center, at which molecular oxygen is reduced to H_2O. Concomitantly with electron transport in O_2 reduction, the protein translocates protons. Subunit II (SUII) carries an N-terminal periplasmic loop between two transmembrane segments and a C-terminal globular domain, facing the periplasm, which binds a bimetallic Cu_A and probably also provides the cytochrome *c* binding site. Subunit III (SUIII) is a hydrophobic protein whose function is still unknown. It consists of seven transmembrane helices and contains no cofactors but does contain a firmly bound lipid (158). A fourth subunit (SUIV) was discovered in the crystallized complex. It forms a single transmembrane helix that is in contact with all other subunits. The function of this polypeptide is unknown.

This type of cytochrome *c* oxidase is widely distributed in bacteria, although there are certain variations. These include the type of heme associated with SUI (Fig. 3) and the arrangement of subunits, as summarized in a recent review (95). For example, a cytochrome *c* in *Thermus thermophilus* was found to be fused with the periplasmic domain of SUII, giving rise to a caa_3-type oxidase in which the electron-donating heme C was closely complexed with the Cu_A electron acceptor site (41). Furthermore, SUIII is fused to SUI (207).

The genes encoding aa_3-type cytochrome *c* oxidases are highly conserved in different organisms and organized in one or two operons (Fig. 6) (105). In *P. denitrificans*, the cyto-

chrome *c* oxidase SUII and SUIII are encoded by *ctaC* and *ctaE*, respectively, in the *ctaCBGE* operon, which contains additional genes required for biogenesis of the oxidase such as a gene for the biosynthesis of heme A (275, 319) (see below). SUI occurs as two isoenzymes encoded by *ctaD1* and *ctaD2* (70, 275). The gene encoding SUIV has been identified recently on a separate locus and was named *ctaH* (380). *Rhodobacter sphaeroides* contains two similar operons, one with the corresponding genes *ctaCBGE* (47, 105) and one expressing SUI from a single *ctaD* gene (307). In *B. japonicum*, the *coxB* gene encoding SUII precedes *coxA*, which encodes SUI, and the genes are probably also organized in an operon (287).

A different type of heme-copper oxidase that can use cytochrome *c* as electron donor was discovered first in *Sinorhizobium meliloti* and *B. japonicum* (167, 268). In rhizobia, the oxidase is required for microaerobic respiration during root nodule symbiosis and meets the energetic demands of nitrogen fixation. This type of terminal oxidase is now also known to be present in other bacterial species including *R. sphaeroides*, *R. capsulatus*, *P. denitrificans*, *Azorhizobium caulinodans*, *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Magnetospirillum magnetotacticum*, *Helicobacter pylori*, and *Thiobacillus* sp. strain W5, from which the proteins have been purified (71, 106, 120, 176, 232, 270, 333, 364) and/or the corresponding genes have been identified (71, 205, 268, 303, 335, 396). The oxidase is encoded by the *fixNOQP* (Fig. 6) or *ccoNOQP* operon, with the *fixN*/*ccoN* gene coding for a typical heme-copper oxidase SUI, the *fixO*/*ccoO* and *fixP*/*ccoP* genes coding for membrane-bound *c*-type cytochromes, and the *fixQ*/*ccoQ* gene coding for a small membrane protein of unknown function (400). Thus, this oxidase lacks the classical SUII and SUIII and contains *c*-type cytochromes instead. SUI is of the bb_3 type, and in the *R. sphaeroides* enzyme complex the presence of Cu_B was demonstrated (106). This type of heme-copper oxidase, referred to as cbb_3-type oxidase, was shown to translocate protons through the membrane (70, 71).

(ii) Quinol oxidases. An alternative route of electron transport from quinols to oxygen other than via a bc_1 complex is provided by the quinol oxidases, which are capable of accepting electrons directly from quinol and using them for reduction of molecular oxygen. As the bc_1 complex is an additional coupling site of the respiratory chain and drives net proton translocation, the bc_1-*c*-aa_3 branch is more effective in energy production than the branch conducting a one-step quinol:O_2 oxidation-reduction, because the quinol oxidase is the only proton-translocating complex.

Quinol oxidases contain cytochromes of various types (Fig. 3) and can be divided into heme-copper oxidases and cytochrome *d*-containing oxidases. Both types of quinol oxidases are present in *E. coli*, an organism that lacks cytochrome *c* oxidases for aerobic respiration, and have been well characterized (107, 109, 262).

The ubiquinol oxidase from *E. coli* operates at high oxygen tensions and is a heme-copper oxidase of the bo_3 type (Fig. 4). Its subunits are homologous to those of the aa_3-type oxidase described above. SUI carries a low-spin heme B and a high-spin heme O-Cu_B binuclear center. The main difference between the quinol- and cytochrome *c*-oxidizing heme-copper oxidases is the absence of Cu_A in SUII of the quinol oxidase (216). As quinol oxidases interact with quinol and not with cytochrome *c*, they lack a cytochrome *c* binding site, and two quinol binding sites have been identified (301). SUIII, whose function is not clear, is also present in the quinol oxidase, and an additional small SUIV has been shown to be part of the purified enzyme (216). This subunit has recently been proposed to assist copper incorporation into SUI during maturation (291). The genes encoding the bo_3-type oxidase are organized in the *cyoABCDE* operon (Fig. 6). The first gene, *cyoA*, codes for SUII, *cyoB* codes for SUI, *cyoC* codes for SUIII, and *cyoD* codes for SUIV. The *cyoE* gene is homologous to *ctaB* of the *P. denitrificans* cytochrome *c* oxidase operon and encodes heme O synthase (see below) (222). Corresponding genes encoding heme-copper-type quinol oxidases have been identified in many other bacteria (for references, see reference 105).

A completely different type of terminal quinol oxidase is found when *E. coli* cells are grown under conditions of low aeration: a high-affinity *bd* oxidase (Fig. 4). This enzyme complex is a heterodimer encoded by the *cydAB* genes (121, 122). A similar oxidase has also been identified in the N_2-fixing *Azotobacter vinelandii* (177, 224), where it is supposed to support extremely high respiration rates and thus be involved in respiratory protection of nitrogenase. Both subunits of the *bd*-type oxidase are integral membrane proteins with seven and eight putative transmembrane helices, respectively, as revealed by membrane topology studies (239). SUI (CydA) contains the low-spin heme B (cytochrome b_{558}), which is thought to be the quinol oxidation site (87, 175). Heme D and a high-spin heme B (cytochrome b_{595}) are shared between the two subunits and are believed to form a bimetallic center where oxygen is reduced to H_2O (75, 144).

Under microaerobic conditions, *E. coli* synthesizes a third terminal oxidase, again of the *bd* type. It is encoded by the *cyxAB* genes (66, 326), but its physiological function is not clear.

Oxidoreductases using alternative electron acceptors. (i) Nitrate reductase. Nitrate reductase, the enzyme involved in anaerobic nitrate respiration, couples respiratory phosphorylation to the reduction of nitrate in the absence of O_2. *E. coli* expresses two different membrane-bound nitrate reductase isoenzymes (NAR) and a periplasmic nitrate reductase (NAP) (31, 54, 109). Similar enzymes are also present in various denitrifiers (89).

The two membrane-bound nitrate reductases (Fig. 4) are alternative sets of homologous proteins encoded by the *narGHJI* or *narZYWV* operons. NarG and its isoenzyme NarZ (α SU) are the terminal nitrate reductases and contain a molybdopterin cofactor; NarH and NarY (β SU) carry four FeS centers. The α and β subunits can be purified as a soluble complex from the cytoplasm (9, 200); however, in the cell, the subunits are anchored to the membrane by a cytochrome *b* subunit (γ SU) that is a membrane-integral protein with five putative transmembrane helices and two B-type hemes (24, 85, 313). The γ subunit is the quinol oxidase and transfers electrons from ubiquinol to the β subunit. A fourth polypeptide is encoded by *narJ*/*narW*; however, this protein appears to be an assembly factor rather than a subunit of nitrate reductase (24, 28, 79).

A third type of nitrate reductase is found in various bacteria including enterobacteria, aerobic denitrifiers, and nonsulfur photosynthetic bacteria (26, 124, 280, 310): the periplasmic nitrate reductase (NAP) with two subunits, NapA and NapB, forming a biologically active complex. NapA is the catalytic subunit with a molybdopterin cofactor and a 4Fe-4S center. NapB is a diheme *c*-type cytochrome. Both subunits are synthesized as precursors with N-terminal signal sequences that are cleaved after translocation across the cytoplasmic membrane. Thus, NAP is a soluble, periplasmic enzyme complex. It is believed that the NapAB complex receives electrons from a tetraheme cytochrome *c*, the *napC* gene product, that is required for in vivo nitrate reductase activity and thus may be considered a further subunit of the complex (280). This protein is periplasmic, although it is anchored to the membrane with an N-terminal hydrophobic segment. The *nap* gene cluster contains seven genes in *E. coli*. Apart from *napA* and *napB*,

which encode the active complex, and *napC*, which encodes the tetraheme cytochrome *c*, other genes, which may code for additional subunits of the NAP complex, have been identified by nucleotide sequencing (124). Some of them, namely, *napD* and *napE*, have also been identified in *Thiosphaera pantotropha* (25).

(ii) Nitrite reductase. Two types of nitrite reductases contain heme as prosthetic group. The first type is the so-called cd_1 nitrite reductase (NIR), which is involved in denitrification in many bacterial species (38) (Fig. 4). The enzyme is a soluble periplasmic homodimer, which carries one heme C and one heme D_1 per monomer as prosthetic groups (5). The *T. pantotropha* cytochrome cd_1 crystal structure has been solved and shows that the two heme groups are localized in separate domains of the protein (101). The protein catalyzes two electron transfer reactions: first, the reduction of NO_2^- to NO and H_2O, and second, the reduction of O_2 to H_2O. Both reactions are thought to take place successively at the high-spin heme D_1 iron, whereas the low-spin heme C was proposed to donate electrons to the catalytic site (101). The structural gene *nirS*, encoding the nitrite reductase, has been cloned and sequenced from several species (69, 166, 312, 402). It is found in clusters of other *nir* genes coding for *c*-type cytochromes, an enzyme involved in siroheme synthesis, and other putative assembly factors (69).

The second type of heme-containing nitrite reductase is represented by the formate-dependent nitrite reductase (NRF) of *E. coli*. NrfA is a periplasmic tetraheme cytochrome c_{552} (65). The enzyme uses electrons that are donated from formate to quinone and subsequently to NrfB, a pentaheme *c*-type cytochrome (1, 154). NRF is encoded by the first two genes of the *nrfABCDEFGH* operon, which also encodes a FeS protein (NrfC), a hydrophobic membrane protein (NrfD), and putative cytochrome *c* biogenesis proteins (NrfE and NrfF) (154). All of these proteins are required for formate-dependent nitrite reduction.

(iii) NO reductase. Nitric oxide reductase (NOR) is the enzyme that converts NO to N_2O during denitrification. It is best characterized from *Pseudomonas stutzeri*, from which it has been purified as a two-subunit cytochrome *bc* complex (141). The subunits are encoded by the *norCB* operon. The gene *norB* codes for a *b*-type cytochrome with 12 putative transmembrane helices, which resembles SUI of the cbb_3-type heme-copper oxidases except that it is thought to carry a binuclear Fe-Fe center rather than a Fe-Cu center (356). The *norC* gene encodes a membrane-anchored *c*-type cytochrome (403).

(iv) TMAO reductase. In *E. coli*, the TMAO reductase (TOR) complex is able to reduce trimethylamine *N*-oxide (TMAO) under anaerobic conditions. It consists of two periplasmic subunits, a large molybdoprotein (TorA) and a *c*-type cytochrome (TorC) that appears to be anchored to the membrane by an N-terminal hydrophobic sequence (157), and a presumed intrinsic membrane protein (TorD) (210). The *torCAD* genes have been sequenced and shown to be organized in an operon (210).

(v) DMSO reductase. *R. capsulatus* dimethyl sulfoxide (DMSO) reductase (DMS) is anchored to the membrane by a *b*-type cytochrome (208). The amino acid sequence of the product of the corresponding *E. coli* DMSO reductase gene *dmsC* contains two histidines that have been postulated to be potential heme binding ligands (72); however, experimental support for this hypothesis is missing. *R. sphaeroides* possesses a DMSO reductase system involving the membrane-bound pentaheme cytochrome *c* DmsC, the transmembrane protein DmsB, and the periplasmic DMSO reductase DmsA, which are very similar to the subunits of the *E. coli* TMAO reductase system (352).

(vi) Reductases of sulfurous compounds. Several enzymes known to be involved in the respiratory reduction of sulfurous compounds may contain hemoprotein subunits. The *Salmonella typhimurium* PhsABC enzyme is a thiosulfate reductase (PHS) with three subunits: PhsA is a molybdoprotein, PhsB is an FeS protein, and PhsC is an intrinsic membrane protein that has been speculated to be a cytochrome, because thiosulfate reduction requires heme (140). The operon containing the *phsABC* genes carries additional genes, *phsDEF*, of which the first, *phsD*, codes for a *c*-type cytochrome with similarity to the periplasmic nitrate reductase NapB subunit (4). *Wolinella succinogenes* synthesizes a homologous polysulfide reductase (PSR) enzyme composed of the subunits PsrA, PsrB, and PsrC, which may contain heme B in PsrC (183).

A special type of hemoprotein is represented by the dissimilatory sulfite reductases (SIR) of sulfate-reducing bacteria, which contain as many as four 4Fe-4S clusters and probably two sirohemes in their α subunit (173). The composition of the enzyme was described first as an $\alpha_2\beta_2$ tetramer and later as an $\alpha_2\beta_2\gamma_2$ complex (3, 173, 260). The subunits are encoded by the *dsvAB* and *dsvC* operons (172, 173).

Unlike *E. coli*, *S. typhimurium* also possesses a dissimilatory sulfite reductase for anaerobic sulfite respiration (ASR), which is encoded by the *asrABC* genes. AsrA appears to be a ferredoxin-like protein, AsrB is a flavoprotein, and AsrC is similar to a subunit of *Desulfovibrio vulgaris* sulfite reductase in that it contains 4Fe-4S cluster binding sites and siroheme binding sites (150).

(vii) Fumarate reductase. Fumarate can be used as terminal electron acceptor of an electron transport chain through fumarate reductase (FRD), whereby succinate is formed. In *W. succinogenes*, the enzyme is membrane anchored by a *b*-type cytochrome, the *frdC* gene product. FrdA and FrdB are the catalytic subunits containing an FAD cofactor and at least one FeS center (353). Although the homologous *E. coli* enzyme has a similar subunit composition, it contains two hydrophobic subunits lacking a heme cofactor (192).

INDIVIDUAL STEPS OF CYTOCHROME BIOGENESIS

During maturation of cytochromes, a series of steps can be postulated in which transport, processing, and combination of the different subunits and cofactors result in a mature enzyme or enzyme complexes. These steps are discussed separately in the following sections.

Protein Targeting

Cytochromes involved in electron transport reactions that are accompanied by the establishment of a proton motive force are localized either in the cytoplasmic membrane or in the periplasm. After the protein component of a cytochrome has been synthesized in the cytoplasm, it must reach its final cellular location by translocation into or through the cytoplasmic membrane.

An interesting question in this context is whether cytochromes use the general secretory (*sec*) pathway, which is relatively well characterized in bacteria (165, 228, 272), or whether special transport systems are required. In the general secretory pathway, a secretion-specific chaperone (SecB) is used in combination with a number of membrane proteins termed SecA, SecY, SecE, SecG, SecD, and SecF, which are subunits of, or which interact with, the preprotein translocase. SecA is an ATPase that is thought to provide energy for translocation.

Directed by SecA, the precursor protein that is kept translocation competent by SecB inserts first with its N-terminal, hydrophobic signal sequence into the membrane. Subsequently, its C-terminal portion is exported by the SecY/E translocase. SecD and SecF are believed to function at a late step of translocation, perhaps in triggering the release of the translocated protein from the secretion complex. Precursors of soluble periplasmic proteins are released from the membrane by proteolytic cleavage of the signal sequence, which is achieved by the LepB signal peptidase. In contrast, membrane-bound proteins with a hydrophilic, C-terminal domain exposed to the periplasm are released from the translocation apparatus in an unknown way, with their noncleaved signal peptide anchoring them in the lipid bilayer. Although the general secretory pathway is used for protein export, certain bacterial proteins are known to cross the membranes in a *sec*-independent way (272, 375). However, these do not possess the typical N-terminal signal sequences.

Two different types of cytochromes can be distinguished with respect to their cellular location and consequently their needs for translocation: the periplasmic and the membrane-integral cytochromes.

Periplasmic cytochromes. The periplasmic cytochromes or cytochrome moieties are predominantly of the *c* type; i.e., they carry heme that is bound covalently and have an α band of 550 to 557 nm. This fact leads to a central question of cytochrome *c* biogenesis that was answered only recently: is the protein translocated in the apoform or in the holoform? Proteins are generally believed to be threaded through the membrane in a locally unfolded state, using the general protein secretion machinery (Sec) of the bacterial cell (273). It is difficult to imagine that a polypeptide with a covalently attached heme group of ca. 616 Da could cross the membrane by using the Sec apparatus. Perhaps such a holoprotein might be exported through a specific transport system. Since a number of bacterial mutants unable to synthesize holocytochrome *c* have been associated with genes encoding an ATP binding cassette (ABC) transporter whose precise role during cytochrome *c* maturation is unknown, the possibility that this special transport system is required for a nonthreaded translocation of relatively bulky holocytochrome *c* molecules formed in the cytoplasm has been considered (342).

The following circumstantial evidence suggests that *c*-type cytochromes traverse the cytoplasmic membrane as preapoproteins in a *sec*-dependent pathway. (i) All *c*-type cytochromes are synthesized as precursors with N-terminal signal sequences. These are thought to be recognized by the SecB and SecA proteins for initiation of translocation (228, 272). (ii) Signal sequences of *c*-type cytochromes, when translationally fused to alkaline phosphatase, are capable of directing the hybrid protein through the membrane (21, 36, 86, 292, 371). (iii) Normally, holocytochrome *c* is not detectable in the bacterial cytoplasm, even if the protein is overproduced and thus accumulates. (iv) Precursor apoproteins have been identified in the periplasm under conditions in which heme incorporation was not possible (249, 295, 340). (v) Deletion of the signal sequence prevents export of cytochrome *c* into the periplasm (296, 340). (vi) Mutants affected in several periplasmic proteins or protein domains that are required for maturation of *c*-type cytochromes (see below) do not impede apocytochrome *c* translocation (249, 339). Support of *sec*-dependent cytochrome *c* translocation was obtained recently, when the fate of the *Bradyrhizobium japonicum* cytochrome c_{550} in secretion-defective *E. coli* mutants was analyzed (339). The production of holocytochrome *c* was clearly affected, and precursor apocytochrome accumulated in the cytoplasmic membrane, suggesting that the secretion pathway interferes with cytochrome *c* maturation. However, mutants with mutations in genes encoding the ABC transporter specifically involved in cytochrome *c* maturation were tested for cytochrome *c* export, and cytochrome *c*-PhoA fusions were efficiently translocated to the periplasm of *Rhodobacter capsulatus*, *B. japonicum*, and *E. coli* ABC translocator mutants (21, 337, 339). Moreover, cleaved apocytochrome was detected by immunoprecipitation in the periplasm of such *E. coli* mutants in similar amounts to those in the wild type (339). These results revealed that the ABC transporter is not responsible for apoprotein translocation.

Few arguments can be made to challenge the view of a *sec*-dependent cytochrome translocation. For example, there is the exceptional case of *Hydrogenobacter thermophilus* cytochrome c_{552}, which, under certain circumstances, can be produced in the cytoplasm (see below). Thus, in principle, holocytochrome *c* can be formed in the cytoplasm, but a *sec*-dependent holocytochrome *c* translocation seems highly unlikely. A surprising result was found when the entire signal sequence (C2Δsig) of *R. sphaeroides* cytochrome c_2 was deleted: neither export of the protein nor covalent attachment of heme was prevented (35). This finding suggests either that cytochrome c_2 possesses additional export signals that can be recognized by the Sec apparatus or that it can reach the periplasm in a *sec*-independent manner. However, the fact that the C2Δsig mutant grew more slowly may indicate an interference of export-defective protein with components of the secretion pathway, thus blocking translocation or assembly of other essential proteins (35).

Export of membrane-bound, periplasmic *c*-type cytochromes such as CycM or c_y has been studied indirectly by analysis of PhoA fusions. It was shown that these proteins are anchored to the membrane with an N-terminal, signal sequence-like portion that is uncleaved (231, 285). If these proteins are also secreted by the general secretion pathway, they must be released from the translocation channel by lateral diffusion in the lipid bilayer (368), as was proposed for other bitopic membrane proteins (272).

In conclusion, the involvement of the general secretion system in apocytochrome *c* translocation has been supported experimentally in quite a convincing way.

Polytopic membrane proteins. Cytochromes of the *a*, *b*, *d*, and *o* types have different requirements for translocation because they are polytopic membrane proteins. They span the lipid bilayer several times with hydrophobic segments of approximately 20 amino acids and contain cytoplasmic and periplasmic loops. The heme groups are usually bound in a noncovalent way to specific ligands, which are most often histidines at conserved positions in the hydrophobic transmembrane segments. Thus, translocation of these cytochromes involves three main steps: (i) protection of the newly synthesized cytoplasmic protein from proteolytic degradation and co- or posttranslational targeting of the polypeptide to the cytoplasmic membrane, (ii) insertion of transmembrane segments into the membrane, and (iii) extrusion of periplasmic loops. Again, these steps may be dependent on the general secretory pathway or may occur in a *sec*-independent manner (367, 368). When polytopic membrane proteins are threaded through the membrane in a *sec*-dependent way, their hydrophobic domains are recognized as signal sequences and are used to drive the adjacent hydrophilic portions through the membrane one by one (272). Translocation of a segment is halted by "stop-transfer" signals, presumably positively charged amino acids. Subsequent segments can then be exported by reinitiation of translocation depending on the protein-internal signal sequences. An indication for such a mechanism of cytochrome translocation is the fact that certain polytopic cytochromes have C-

terminal domains containing heme C that must be exported completely. Such an example is SUII of the caa_3-type cytochrome *c* oxidase, in which a *c*-type cytochrome domain is fused C terminally to the periplasmic Cu_A domain (105). In addition to the cleaved signal sequence of SUII, two hydrophobic segments anchor the protein in the membrane; the more C-terminal of these segments probably acts as an internal export segment for the C-terminal bulk of the protein. Another example is found in the *B. japonicum fbcH* gene product, which is synthesized as a polyprotein precursor for cytochromes *b* and c_1. Cytochrome *b* carries an unusual ninth transmembrane helix that contains all the characteristics of, and functions as, a protein-internal signal sequence for the adjacent cytochrome c_1 moiety (338, 343) (see below). This signal sequence is cleaved during maturation.

Although in the past the question of how polytopic cytochromes are assembled in the membrane had not been addressed experimentally, it seems logical that the heme cofactors can be incorporated only after the polypeptide has adopted a certain three-dimensional structure in the membrane. The relative arrangement of transmembrane helices, which in most cases bind heme by pairs of histidines, is likely to be a prerequisite for this kind of cofactor incorporation. This is of particular importance as there are no catalysts known to assist in heme binding to transmembrane helices (see below).

Subunit Assembly

Contribution of individual subunits. Many of the respiratory cytochromes are assembled into oligomeric membrane protein complexes. The subunits themselves can be critical for their successful assembly.

It is not always clear what makes up a true subunit. From a biochemical point of view, a subunit is a polypeptide that copurifies with the enzymatic activity in stoichiometric amounts. On the one hand, it is sometimes difficult to distinguish between true subunits and impurities of the enzyme preparations. On the other hand, subunits can be lost during purification. If a polypeptide can be removed from a respiratory complex without loss of enzymatic activity, its function as a subunit is questionable. There are often genetic hints whether a polypeptide might be a subunit. Many of the bacterial respiratory enzyme complexes are encoded by genes organized in operons; i.e., the biosynthesis of the corresponding subunits is transcriptionally and often also translationally coupled. Therefore, subunits have also been discovered on the basis of gene organization in several cases.

A good example of the difficulties that can arise in subunit assignment is the *Paracoccus denitrificans* aa_3-type cytochrome *c* oxidase. This enzyme was initially purified as a two-subunit complex (199). Only later was SUIII identified due to the discovery of its gene (300); subsequently, a three-subunit enzyme preparation was obtained (129). Since SUIII does not contain any redox-active cofactors and can be removed from the complex without drastic alterations in oxidase function (128), its role in the complex has been disputed, and it has been suggested to be important for assembly of the oxidase. The crystal structure of cytochrome *c* oxidase revealed that this complex actually consists of four subunits, the fourth polypeptide being too small to be detected by normal sodium dodecyl sulfate-polyacrylamide gel electrophoresis (158). Deletion of the *ctaH* gene encoding SUIV had no effect on the integrity and enzymatic function of the complex (380). Thus, the function of SUIV also remains unknown.

One way of characterizing the requirement of subunits for complex assembly is to express or overexpress single subunits. This can lead to incorporation of the polypeptide into the membrane and sometimes also to cofactor binding, such that the protein is stably maintained and can be detected under steady-state conditions. Alternatively, the polypeptide may not assemble correctly and is subjected to rapid proteolytic degradation. SUI and SUII of the *E. coli* bo_3-type quinol oxidase were expressed independently and inserted stably into the membrane (234). SUI even incorporated both hemes although at decreased levels, whereas SUII did not contain any heme. The *B. subtilis* caa_3-type cytochrome *c* oxidase SUII (COII) was shown to be expressed and incorporated in the membrane as a holocytochrome in the absence of SUI (COI) (358). The Rieske FeS protein of the *R. sphaeroides* bc_1 complex could be expressed at low levels in the absence of the other subunits and contained the 2Fe-2S center (359). In an *E. coli* expression system which principally allows for the biosynthesis of *c*-type cytochromes, expression of the FixP subunit of the *B. japonicum* cbb_3-type oxidase led to an extremely unstable product that was not inserted into the membrane (399).

A good approach to study the effect of subunits on the assembly of enzyme complexes is the construction of mutations in the individual structural genes. Since most respiratory complexes are encoded by operons, it is often easy to knock out the last gene in the operon without affecting the expression of the other genes, but it is difficult to mutate the promoter-proximal genes without generating polarity effects. Therefore, for most respiration enzymes, a systematic removal of each individual subunit has not been achieved. Table 1 shows a survey of single-gene mutants of respiratory enzymes and their phenotypes with respect to subunit assembly. The best test of whether the apoproteins insert into membranes in such mutants requires that antiserum specifically recognizing the individual subunits be available. Unfortunately, in several cases where single-gene mutants have been described in the literature, the phenotypic characterization has not included immunological detection of the subunits. For example, mutants with deletions in SUI of the *R. sphaeroides* and *P. denitrificans* aa_3-type oxidase were shown to lack spectroscopically detectable oxidase, but the presence of SUII polypeptide in the membranes was not investigated (70, 307). In an *E. coli cyoD* mutant lacking SUIV of the bo_3-type oxidase, only the presence of SUI was confirmed; that of SUII was not analyzed. A systematic characterization of the contribution of subunit polypeptides to complex assembly was reported for the *B. japonicum* cbb_3-type oxidase. The structural genes *fixN*, *fixO*, *fixQ*, and *fixP* were deleted one by one, and the in-frame deletions were shown to be nonpolar (400). These mutants served as tools to characterize the biogenesis pathway of this oxidase more precisely (see below), and the requirement of FixN and FixO, but not of FixQ, for oxidase assembly was deduced.

Various respiratory complexes consist of cytoplasmically active enzymes that are anchored to the membrane by one or two subunits which often are *b*-type cytochromes. These subunits connect the cytoplasmic redox reactions with electron transport to or from the quinone and quinol pool in the membrane. Examples of this type of oxidoreductase are the SDH and FDN and the NAR-type nitrate reductase (Fig. 4). These enzymes can form subcomplexes of the cytoplasmic subunits even in the absence of the membrane anchor and usually retain oxidoreductase activity on their specific substrate, which is, however, uncoupled from electron transfer to or from quinone and quinol (Table 1). Thus, the attachment of these enzymes to the membrane by their cytochrome *b* subunits seems to be the last step of complex assembly and perhaps has evolved to direct the electron flux of such reactions into the respiratory chain.

Another dimension of respiratory complexes present in bac-

TABLE 1. Involvement of subunits in assembly of respiratory cytochrome complexes[a]

Enzyme	Organism	Affected subunit	Genotype	Phenotype	Reference(s)
SDH	*E. coli*	cyt b_{556} large SU	*sdhC*	No membrane insertion; no heme; soluble SDH activity	235
SDH	*E. coli*	cyt b_{556} small SU	*sdhD*	No membrane insertion; no heme; soluble SDH activity	235
SDH	*B. subtilis*	cyt b_{558}	*sdhC*	No cyt *b* polypeptide; soluble SDH	99, 138
bc_1 complex	*P. denitrificans*	cyt c_1	*fbcC*	No membrane insertion of cyt *b* and FeS protein	111
bc_1 complex	*R. sphaeroides*	Truncated cyt c_1	*fbcC′*	Soluble holocyt c_1; assembly defect of cyt *b*; FeS protein present	182
bc_1 complex	*R. capsulatus*	Truncated cyt c_1	*fbcC′*	cyt c_1 absent; small amounts of cyt *b* and FeS protein	68, 119
bc_1 complex	*R. capsulatus*	FeS protein	*fbcF*	Assembly of a bc_1 subcomplex	68
bc_1 complex	*B. japonicum*	cyt c_1	*fbcH*	All subunits absent	343
aa_3 oxidase	*P. denitrificans*	SUI	*ctaD1* *ctaD2*	No spectrally detectable oxidase	70
aa_3 oxidase	*P. denitrificans*	SUII	*ctaC*	No SUI present	318
aa_3 oxidase	*P. denitrificans*	SUIII	*ctaE*	Cofactors present; assembly defect	128
aa_3 oxidase	*R. sphaeroides*	SUI	*ctaD*	No spectroscopically detectable oxidase; presence of SUII ND	307
aa_3 oxidase	*B. japonicum*	SUI	*coxA*	SUI and SUII absent	195, 287
caa_3 oxidase	*B. subtilis*	SUI	*ctaD*	SUII present in membranes	358
cbb_3 oxidase	*P. denitrificans*	SUI, SUII	*ccoNO*	No CcoP	71
cbb_3 oxidase	*R. capsulatus*	SUI, SUII	*ccoNO*	No CcoP	335
cbb_3 oxidase	*B. japonicum*	FixN (SUI)	*fixN*	FixN, FixO, and FixP absent	400
cbb_3 oxidase	*B. japonicum*	FixO (SUII)	*fixO*	FixN, FixO, and FixP absent	400
cbb_3 oxidase	*B. japonicum*	FixP (SUIII)	*fixP*	FixNO complex present	400
cbb_3 oxidase	*B. japonicum*	FixQ	*fixQ*	FixNOP normally assembled	400
cbb_3 oxidase	*A. caulinodans*	FixN, FixO	*fixNO*	CcoP absent; polarity effect?	205
bo_3 oxidase	*E. coli*	SUII	*cyoA*	SUI present	234
bo_3 oxidase	*E. coli*	SUI, SUIII, SUIV	*cyoBCDE*	SUII present	234
bo_3 oxidase	*E. coli*	SUIV	*cyoD*	SUI present, SUII ND	291
aa_3 oxidase	*B. subtilis*	SUIII	*qoxC*	Defective cyt aa_3 assembly	363
aa_3 oxidase	*B. subtilis*	SUIV	*qoxD*	Normal assembly	363
bd oxidase	*E. coli*	SUI	*cydA*	SUI and SUII absent; no hemes; polarity effect?	121
bd oxidase	*E. coli*	SUII	*cydB*	SUI present; no heme D	121
NAR; NarGHI	*E. coli*	NarI	*narI*	No formate-linked membrane-bound enzyme; soluble NAR activity	28, 79
TMAO reductase	*E. coli*	TorA molybdoprotein	*torA*	TorC *c*-type cyt present	210
NAP	*R. sphaeroides*	Membrane-bound cyt *c*	*napC*	Soluble NapAB complex; no in vivo activity, but active with artificial electron donor	280
NO reductase	*P. stutzeri*	SUI	*norB*	NorC amount decreased	403

[a] ND, not determined; cyt, cytochrome.

terial membranes is found in the so-called supercomplexes that are often formed between respiratory complexes and their mediators, usually *c*-type cytochromes. For example, a bc_1-cytochrome *c*-aa_3 complex has been solubilized from membranes of *Paracoccus denitrificans* and the thermophilic bacterium PS3 (27, 315). This complex oxidizes ubiquinol and reduces molecular oxygen in a complete electron transfer system. The *c*-type cytochrome linking complexes III and IV is a membrane-anchored species that has been called cytochrome c_{552} and is encoded by the *cycM* gene (349). Similar membrane-bound *c*-type cytochromes, CycM and cytochrome c_y, connect complex III and IV of *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*, respectively. In *B. japonicum*, a deletion of *cycM* resulted in the loss not only of CycM but also of spectroscopically detectable aa_3-type oxidase (32). Mutations in the bc_1 complex genes caused the lack of complex III, CycM and aa_3-type oxidase (32). On the other hand, a *coxA* mutant affecting SUI of the aa_3-type oxidase retained the bc_1 complex and CycM (32). These results imply that the formation of a supercomplex involves a tight interaction of the cytochrome bc_1 complex with CycM followed by association of the aa_3-type oxidase. The stability of the last two cytochromes (CycM and aa_3) depends on an intact bc_1 complex, whereas the bc_1 complex per se is stable in the membrane. It has also been established that the bc_1 complex of *B. japonicum* can interact with a different, microaerobic respiratory chain, namely, the FixNOP oxidase (268, 343, 400). In this case, FixP may fill a position similar to CycM by being a membrane-bound mediator between the bc_1 complex and the terminal oxidase (FixNO complex). In a preparation of the cbb_3-type oxidase from *B. japonicum* bacteroids, subunits of the bc_1 complex were copurified with the oxidase, suggesting that a tightly associated cytochrome bc_1-cbb_3-supercomplex may exist (176). However, the presence of the bc_1 complex is not required for the stable maintenance of the cbb_3-type oxidase in membranes, since FixN, FixO, and FixP are detected at normal levels in bc_1 mutants (400).

Other assembly factors. A number of additional proteinaceous assembly factors that are not constituents of the mature complex have been identified for different respiratory complexes. In most cases, the precise function of these proteins during biogenesis is not clear, and they can affect either the correct assembly of the subunits or the processing and incorporation of cofactors. Such assembly factors are often encoded in operons containing the structural genes for the respiratory enzymes; i.e., these are genes whose derived polypeptides have not been demonstrated to be subunits of the corresponding complexes. Their involvement in subunit assembly can be tested by introducing mutations into these genes and analyzing the effect on the formation of spectroscopically normal and

TABLE 2. Putative assembly factors of respiratory cytochrome complexes

Gene locus	Organism	Subcellular location	Postulated or demonstrated function	Reference(s)
ctaA	*B. subtilis*/*B. firmus*	Membrane integral	Heme A synthase	225, 328–330
ctaB	*B. subtilis*/*B. firmus*	Membrane integral	Heme O synthase	22, 32, 46, 51, 204, 274, 275, 299
ctaB	*P. denitrificans*			
cyoE	*E. coli*			
cyoE	*N. winogradskyi*			
coxD	*Synechocystis* sp.			
orf1	*B. japonicum*/*R. sphaeroides*			
ctaG (*orf3*)	*P. denitrificans*	Membrane integral	Cytochrome aa_3 assembly	275, 318
tlpA	*B. japonicum*	Membrane bound periplasmic	Thioredoxin for cytochrome aa_3 assembly	195
senC/*prrC*	*R. capsulatus*/*R. sphaeroides*	Membrane bound periplasmic	Assembly of *cb*-type oxidase	40, 83
fixGHIS	*S. meliloti*/*B. japonicum*/*A. caulinodans*	Membrane complex	Cu transport and Cu_B assembly into FixN?	168, 206, 269
cydC	*E. coli*	Membrane integral	ABC transporter for *bd*-oxidase assembly	110
cydD	*E. coli*	Membrane integral	ABC transporter for *bd*-oxidase assembly	19, 264–266
napD	*T. pantotropha*	Cytoplasmic	NapAB maturation	25
narJ/*narW*	*E. coli*	Hydrophilic, not detectable in subcellular fractions	Required for assembly of membrane-bound, active NarGHI/NarZYV	28, 79, 313
nirC	*P. fluorescens*	Periplasmic	Assembly of active cd_1 nitrite reductase	390
nirECF	*P. denitrificans*	Cytoplasmic/periplasmic/cytoplasmic	Heme D_1 biosynthesis	69
nirD	*P. stutzeri*	Cytoplasmic	Heme D_1 biosynthesis	253
nirF	*P. aeruginosa*	Cytoplasmic	Heme D_1 biosynthesis	174

biologically active enzyme complexes. In such analyses, a negative effect on assembly may be indirect, for example, due to a defect in the biosynthesis of a cofactor.

Table 2 summarizes the various putative assembly factors that have been found in connection with bacterial respiratory enzymes. For example, mutations in *B. subtilis ctaA* or *ctaB* cause a defect in assembly of the cytochrome *c* oxidase because the mutants are blocked in heme A biosynthesis (222, 330). Other genes likely to be involved in cofactor biosynthesis or processing are *cydC* and *cydD*, which were hypothesized to be subunits of a heme transporter (238), and the *nirCDEF* genes, which appear to play a role in heme D_1 synthesis for the cd_1-type NIR (references are given in Table 2). A role of *P. denitrificans ctaG* in a late step of cytochrome aa_3 assembly has been postulated (318). More recently, the analysis of a yeast mutant affected in the *cox11* gene, a homolog of *ctaG* (350), suggested that the derived gene product may be another heme A biosynthetic enzyme involved in forming the formyl group at position 8 of the porphyrin ring (351).

The correct incorporation of cofactors may also be directed by specific proteins. The *B. japonicum tlpA* gene product, a membrane-bound, periplasmic thioredoxin, is thought to function as a periplasmic reductant during cytochrome aa_3 maturation (195, 198). Mutations in *tlpA* caused an assembly defect of the aa_3-type oxidase in that spectroscopically detectable cytochrome aa_3 was absent in mutant membranes whereas SUI was immunologically detectable, albeit at decreased levels (195). It was speculated that TlpA may keep the two cysteines of the Cu_A binding site of SUII of the aa_3-type oxidase reduced in the oxidative environment of the periplasm to allow subsequent incorporation of the copper cofactor (198).

The incorporation of Cu_B into the cbb_3-type oxidase SUI of the same organism has been suggested to depend on the function of the *fixGHIS* gene products. This hypothesis is based on the facts that (i) FixI appears to be similar to the P-type ATPases CopA and CopB of *Enterococcus hirae* (247), which are involved in copper uptake and export, respectively, and (ii) FixG seems to be a ferredoxin (168). Since a *fixGHI* mutant exhibited strongly reduced levels of immunologically detectable cbb_3 oxidase subunits of an otherwise inactive enzyme (269), the translocation of copper through the cytoplasmic membrane may be a requirement for correct assembly of the oxidase (see below).

Other putative assembly factors are characterized only by mutations of the corresponding genes that lead to incorrectly assembled complexes. The *narJ* gene product is necessary for the formation of a membrane-bound, active nitrate reductase (28, 79). In a *narJ* mutant, the *b*-type cytochrome NarI was not affected; however, low levels of cytosolic α and β SU, which contained the molybdopterin cofactor and FeS centers and retained low levels of enzyme activity, were present. The NarG and NarH polypeptides were partially degraded. Thus, NarJ appears not to be involved in the processing or incorporation of cofactors but, rather, is involved in assembly of the αβγ complex. The absence of NarJ causes accumulation of a cytoplasmic, immature αβ complex, which is susceptible to proteo-

lytic degradation. NarJ may therefore protect such an intermediate from proteolysis and facilitate the binding of the complex to the membrane-bound γ SU.

Among several nucleus-encoded assembly factors of yeast cytochrome aa_3 is the mitochondrial inner membrane protein SCO1, which is required for the posttranslational maturation of cytochrome *c* oxidase (304, 305). In the absence of SCO1, SUI, SUII, and, to a lesser extent, SUIII are rapidly degraded, as shown by pulse-chase experiments (185). SCO1-homologous counterparts have been found in bacteria. In *Rhodobacter sphaeroides* and *R. capsulatus*, *prrC* and *senC*, respectively, have been identified on the basis of sequence similarity of their derived gene products to SCO1 (approximately 30% identical and 50% similar amino acids) (40, 83). Based on alkaline phosphatase activities of a PrrC-PhoA fusion, PrrC was described to be a periplasmic protein that is anchored to the membrane (83). A *senC* mutant of *R. capsulatus* had strongly decreased *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity, which in this organism depends solely on the presence of an intact cbb_3-type oxidase. If these homologous proteins have a similar function in the assembly of terminal heme-copper oxidases to that in yeast, this function would not discriminate between aa_3- and cbb_3-type oxidases and therefore would be unlikely to be associated with heme incorporation. However, copper insertion is a common step in maturation of both types of oxidases and may require the same auxiliary protein. Another possibility is that the protein facilitates assembly of the individual subunits into a membrane protein complex. Unfortunately, the presence or absence of oxidase subunits and cofactors in the *prrC* and *senC* mutants has not been investigated. Alternatively, a role as an inhibitor of a periplasmic protease cannot be excluded for this type of assembly factor.

Heme Cofactor

Biosynthesis of the heme cofactor. The biosynthesis of hemes has been described in a number of comprehensive reviews (17, 18, 61, 245, 376) and is represented schematically in Fig. 7.

The first committed heme precursor that is universally used for tetrapyrrole synthesis is δ-aminolevulinate (ALA). This molecule can be synthesized by two different routes in bacteria. Members of the alpha proteobacteria (e.g., *Rhodobacter*, *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium*) condense ALA from glycine and succinyl-coenzyme A by using the enzyme ALA synthase, whereas most other bacteria (e.g., *E. coli*, *B. subtilis*, and cyanobacteria) use the C_5 pathway, by which glutamate serves as a precursor and is converted to ALA in a three-step reaction involving glutamyl-tRNA (161). Unfortunately, for historical reasons, the genes encoding ALA synthase and glutamyl-tRNA reductase are both called *hemA*. For the C_5 pathway, the genes *gltX*, encoding glutamyl-tRNA synthetase, and *hemL*, encoding glutamate-1-semialdehyde aminotransferase, are required in addition to *hemA*.

Three subsequent reactions are necessary to make the cyclic intermediate uroporphyrinogen III from ALA. First, two molecules of ALA are condensed by ALA dehydratase (*hemB* gene product) to yield porphobilinogen (PBG). Next, four molecules of PBG are transformed into uroporphyrinogen III: PBG is deaminated to hydroxymethylbilane by PBG deaminase (*hemC* gene product), and finally the formation of the macrocycle is carried out by uroporphyrinogen III cosynthase, the *hemD* gene product. At this point in heme biosynthesis, a side pathway for the synthesis of siroheme and heme D_1 branches off. This metabolic route is also known from vitamin

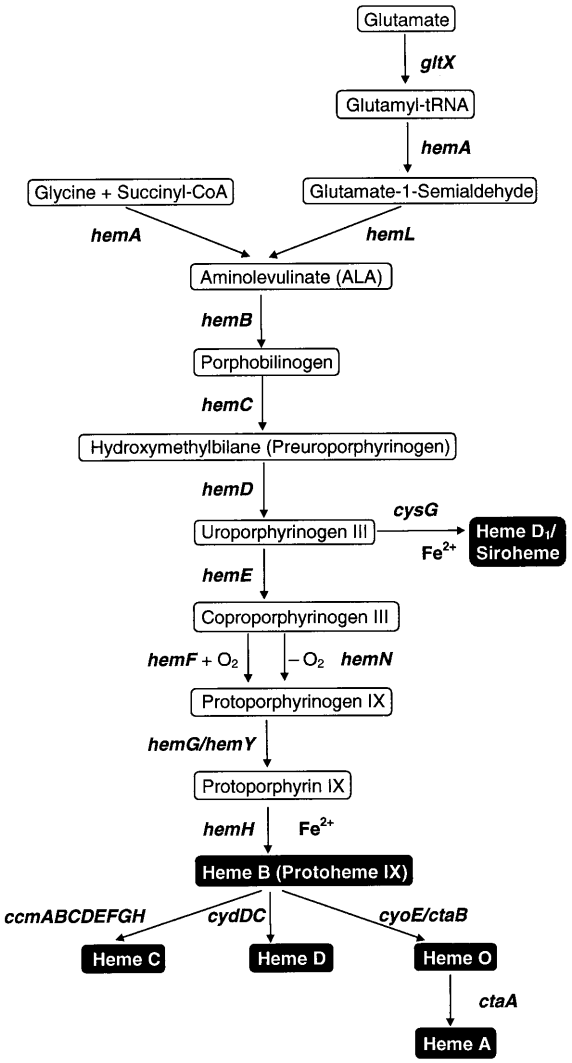


FIG. 7. Schematic representation of heme biosynthetic pathways. The genes encoding the different biosynthetic steps are indicated next to the arrows. Substrates and intermediates are framed; heme products are highlighted.

B_{12} biosynthesis. The only gene known to be involved in siroheme synthesis is *cysG*, and its product appears to be a multifunctional enzyme catalyzing all steps required for the formation of siroheme (18). As already mentioned, the *nirCDEF* genes of pseudomonads are likely to encode enzymes required for heme D_1 formation (Table 2). It is not known how iron is incorporated into these heme types.

The late steps of heme biosynthesis require uroporphyrinogen III decarboxylase (*hemE* gene product), coproporphyrinogen III oxidase (*hemF* or *hemN* gene product), protoporphyrinogen IX oxidase (*hemG* or *hemY* gene product), and ferrochelatase (*hemH* gene product). The last two enzymes are generally localized in the cytoplasmic membrane, with the exception of the *B. subtilis* ferrochelatase, which is soluble (18, 62, 131, 132). With regard to the biogenesis of *b*- and *c*-type cytochromes, the localization of ferrochelatase, the last enzyme of protoheme IX (heme B) synthesis which inserts iron into the porphyrin, is of particular interest. The hydrophobicity profile of ferrochelatase suggests that it is a peripheral membrane protein. Combined with the fact that ferrochelatase in

B. subtilis is a cytoplasmic, soluble protein (132), it is tempting to conclude that the membrane-bound enzyme faces the cytoplasmic side of the membrane. This is most probably where heme B is released to be incorporated into cytochromes and other hemoproteins.

Heme B not only is incorporated directly into respiratory enzymes but also serves as a precursor for the formation of hemes C, D, O, and A. Heme C biosynthesis involves the covalent attachment of the heme molecule to an apocytochrome polypeptide, which will be discussed in detail below. Heme D is most probably derived from protoporphyrin IX or protoheme by hydroxylation; however, it is not known whether this conversion is enzymatically catalyzed (18). Also, no genes involved specifically in heme D formation have been described in the literature.

The biosyntheses of heme O and heme A are coupled because heme O is an intermediate product of the heme A synthesis pathway (222). Heme O is synthesized from heme B by the addition of a 2-farnesylethyl group. The gene *cyoE* was shown to encode a farnesyl transferase in *E. coli* (289, 290). Heme A synthesis is best known in *B. subtilis* (328–330). In a first step, heme O is synthesized by the CyoE homolog CtaB. Heme O is then converted to heme A by the CtaA protein, a heme A synthase. This latter reaction includes a monoxygenation and an oxidation of the methyl group on the tetrapyrrole ring D, leading to a formyl group. CyoE/CtaB is a cytoplasmic membrane protein (290) with seven transmembrane helices (50). The majority of the conserved amino acids among the CyoE homologs are found in cytoplasmic loop domains, suggesting that this is the location where heme is bound and modified. Heme A synthase (CtaA) is also an integral membrane protein, has eight transmembrane segments, and carries heme B as a cofactor; it is thus a *b*-type cytochrome (329). Four histidines that are conserved in *B. subtilis* and *Bacillus firmus* OF4 CtaA are on the periplasmic side of transmembrane helices II, IV, VI, and VIII and may be involved in heme binding. The current model is that CtaA binds one molecule of heme B that is thought to function in electron transport for the monoxygenase reaction of CtaA and that the second heme binding site is the substrate binding site for heme O, which is converted to heme A (328).

Heme transport. The lipophilic molecule heme (defined as ferro-protoporphyrin) contains Fe^{2+} but possesses no net charge. Free heme in solution is rapidly oxidized to hemin (ferri-protoporphyrin), which has one net positive charge and forms salts (311). Heme is toxic to cells, since it promotes peroxidation of cell membranes (355) and tends to polymerize at neutral pH. This is probably why free heme usually does not occur in the cell but, rather, is bound to carrier proteins, such as albumin or hemopexin in the blood plasma of mammals. The intracellular transport routes for heme, and in particular translocation through membranes, are barely characterized. Most of our knowledge stems from pathogenic microorganisms that use heme as an iron source and thus must be able to take it up. In this case, however, the heme receptors and transporters are involved in translocating the substrate molecule from the outside into the cell. It is still an enigma how heme crosses the cytoplasmic membrane from the inside to outside. Membrane-bound cytochromes can contain the heme cofactor on either side of the membrane. For periplasmic cytochromes in general and for all *c*-type cytochromes in particular, a step involving translocation of heme through the membrane from the cytoplasmic side, where the last step of heme synthesis takes place (see above), to the periplasmic side, where the holoprotein resides, can be postulated. Heme translocation is likely to be achieved by the use of an energy-dependent transporter. Alternatively, passive diffusion may be a mechanism of how heme reaches the periplasmic side of the membrane. Indirect evidence points to the existence of specific heme transporters used during maturation of periplasmic cytochromes. (i) Heme-utilizing bacteria require specific uptake systems for heme translocation through both the outer membrane and the cytoplasmic membrane (55, 56, 130, 142, 143, 190, 214, 321–324, 386). In particular, heme passes through the cytoplasmic membrane by a specific periplasmic binding protein-dependent transport system (322). In the absence of these systems, heme cannot be utilized, which argues against a mechanism of heme uptake by diffusion. It is therefore difficult to imagine that heme can be translocated through bacterial membranes in the other direction simply by diffusion. (ii) Genes encoding the subunits of two different types of ABC transporter (76, 88, 155) are necessary for maturation of *c*-type cytochromes.

The members of the first type share quite extensive sequence similarity among themselves but are not very similar to other ABC transporters (2, 21, 93, 124, 252, 277, 337). They consist of an ATP-binding protein (CycV, HelA, or CcmA) and two integral membrane proteins (CycW, HelB, or CcmB and CycZ, HelC, or CcmC), which are thought to form the translocation pore, each containing six putative transmembrane helices characteristic for this class of permeases. The subunit structure $(CcmA)_2$-CcmB-CcmC with four membrane-associated protein domains typical for bacterial ABC transporters was proposed recently based on sequence comparisons of the subunits of six Ccm-like transporters (252). In *Rhodobacter capsulatus*, the ABC transporter subunits HelA, HelB, and HelC interact directly and form a complex including the small HelD polypeptide (113).

A heme-translocating ABC transporter is expected to have some sort of heme binding site. The subunits of the CcmABC subfamily of transporters for cytochrome *c* maturation are aligned in Fig. 8. Sequence comparisons revealed three features: (i) the peripheral ATP binding subunit (CcmA) does not contain any conserved motifs except those generally found in this class of proteins, i.e., the ABC with the Walker motifs of the nucleotide binding site (Fig. 8A) (374); (ii) in the first hydrophobic subunit (CcmB), many of the conserved amino acids reside in putative transmembrane helices, and the most prominent conserved sequences, DGSL and AHWXXXGLPL, are found in a predicted cytoplasmic loop connecting helices II and III and at the beginning of helix III, respectively (Fig. 8B); and (iii) the second hydrophobic subunit (CcmC) contains a tryptophan-rich motif, WGXPMWGTXWDXRLT, in a predicted periplasmic loop (Fig. 8C). Interestingly, a closely related motif has been identified in one of the subunits of the putative cytochrome *c* heme lyase (see below) and has therefore been suggested to be a possible heme binding site (149, 184, 342). Notably, a conserved histidine in each of the two hydrophobic subunits of the ABC transporter resides at the beginning, i.e., on the cytoplasmic side of transmembrane helices II. Since cytochrome hemes are often bound by axial histidine ligands that are located close to the end of transmembrane helices, one might speculate that the two histidines are part of a heme binding site. The relatively strong conservation of hydrophobic amino acid residues of these transmembrane helices points to the quality of a hydrophobic rather than a hydrophilic translocation pore, which may be expected for a heme transporter.

There is experimental evidence that the *ccmABC*-encoded proteins may not represent simply a general heme exporter. First, *E. coli ccm* mutants that are unable to synthesize holocytochromes *c* produced overexpressed periplasmic cytochrome b_{562}, which can be considered a heme reporter (114,

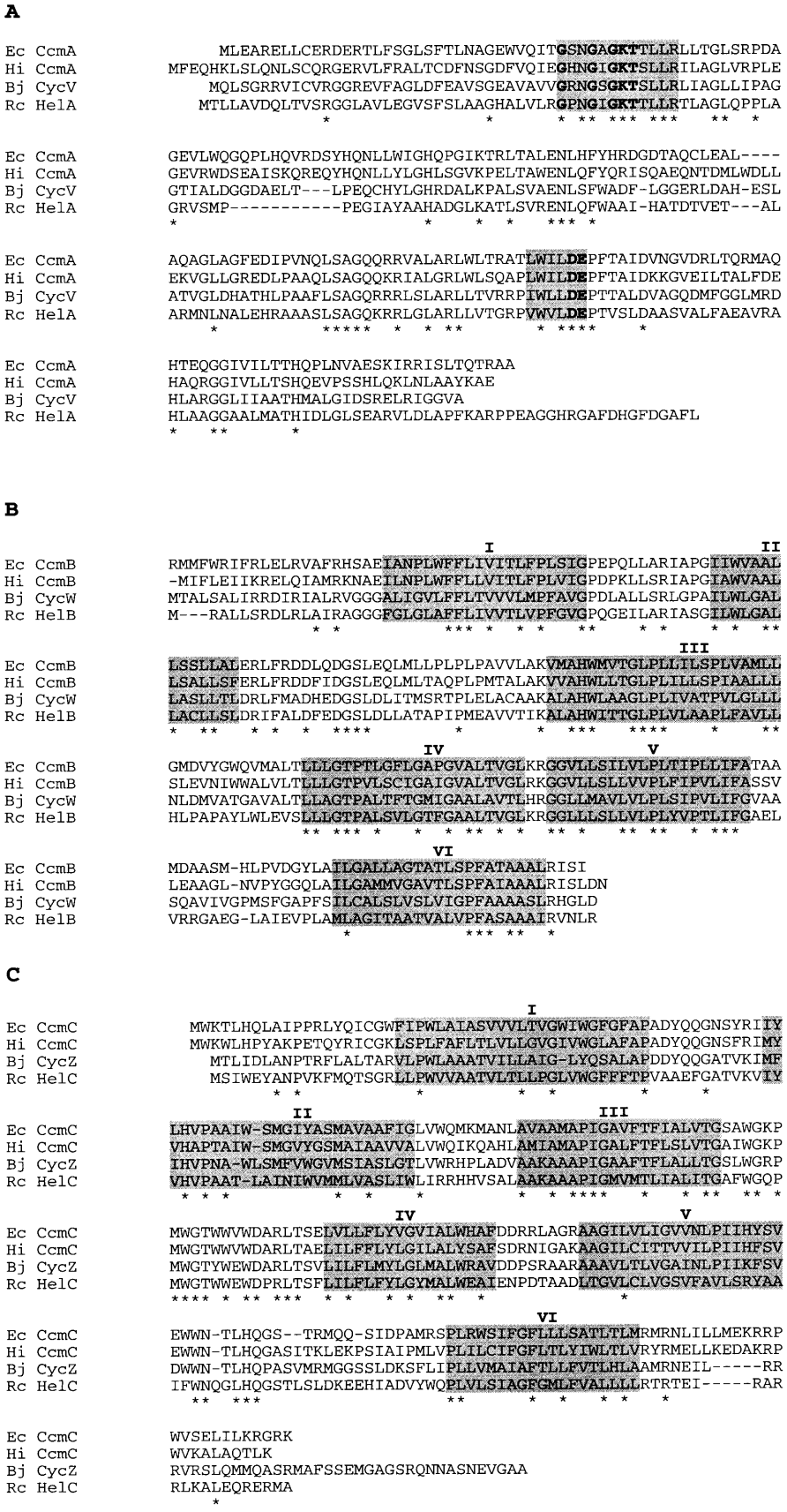


FIG. 8. Alignments of subunits of an ABC transporter involved in cytochrome *c* maturation. Identical amino acids are underscored with asterisks. (A) ATP-binding subunit. The predicted nucleotide binding sites (Walker motifs) are shaded, with the amino acids generally conserved in Walker motifs indicated by boldface type. (B and C) Membrane-integral subunits. The putative membrane-spanning helices (I to VI) are shaded. The tryptophan-rich motif WGXXWXWD in the loop between helices III and IV of the second hydrophobic subunit (C) is emphasized by boldface type.

344). Assuming that heme B is incorporated into the apocytochrome b_{562} in the periplasm, it must be translocated in these mutants in a *ccm*-independent way. Second, *P. denitrificans ccmA* and *ccmB* mutants could not be complemented for holocytochrome *c* formation by extracellular addition of heme, even though elevated levels of other soluble hemoproteins and membrane-bound *b*-type cytochromes were obtained (252).

The second type of a putative heme transporter has been identified in *E. coli* and is encoded by the *cydDC* genes, which play a role in biogenesis of the *bd*-type terminal oxidase as well as in the biosynthesis of *c*-type cytochromes (264, 265). This has been concluded from the experimental evidence that the sequences of CydC and CydD are similar to those of certain ABC transporters and that *cydDC* mutants can synthesize neither holocytochrome *bd* nor *c*-type cytochromes (264, 265). The CydC and CydD proteins have an N-terminal hydrophobic domain with six presumptive transmembrane segments and a C-terminal hydrophilic domain containing a putative nucleotide binding site, suggesting that this type of ABC transporter is heterodimeric. Since there are no known homologs of CydC and CydD in other bacteria, it is difficult to speculate about possible heme binding sites. Again, studies with periplasmic heme reporter proteins indicate a role in redox control rather than in heme export for this ABC transporter (114, 115). However, at present, the possibility that both the CcmABC and the CydCD transporters deliver heme to specific maturation systems for cytochromes *c* or *bd*, respectively, cannot be excluded.

The amino acid sequences of the hydrophobic CcmB and CcmC subunits were compared with those of other putative heme transporters or heme binding proteins to search for significant common motifs: (i) *E. coli* CydC and CydD (see above); (ii) the *Yersinia enterocolitica* HemU subunit of an ABC transporter for heme uptake (322); and (iii) heme receptors like *Y. enterocolitica* HemR (321), *Vibrio cholerae* HutA (142) and *Neisseria meningitidis* HmbR (323). However, such motifs were not identified.

Covalent attachment of heme to *c*-type cytochromes. Most *c*-type cytochromes known to date share the motif CXXCH, which comprises the heme binding site. Heme is bound covalently by two thioether bonds to the α-carbon of the vinyl side chains at pyrrole rings A and B (Fig. 2). Exceptional heme binding sites have been described for cytochromes *c* and c_1 of *Euglena* and *Crithidia* mitochondria (223, 226, 271), which lack the first of the two conserved cysteines, resulting in the formation of a single covalent bond. In the heme binding site of *Desulfovibrio vulgaris* cytochrome c_3, the spacing between the two conserved cysteines is different, i.e., CXXXXCH (372).

It is generally thought that covalent attachment of heme to the apocytochrome is catalyzed by the enzyme cytochrome *c* heme lyase (CCHL), because proteins with such an activity have been isolated and characterized from yeast and *Neurospora crassa* mitochondria and the corresponding genes have been cloned (78, 81, 398). However, an enzyme with CCHL activity has not yet been isolated from bacteria, although it is expected to exist, unless heme ligation can occur spontaneously.

It has been suggested that in certain cytochromes which fold to give a stable apoprotein and subsequently bind heme noncovalently, a suitably positioned cysteine thiol will spontaneously react with a heme vinyl group, giving rise to the thioether bond characteristic of *c*-type cytochromes (13, 293). In an approach to transmutate the *E. coli* cytochrome b_{562} to a *c*-type cytochrome by inserting a heme C binding site at the appropriate position of the polypeptide, a cytochrome with covalently bound heme was obtained. However, it could not be determined whether this was the product of an enzyme-catalyzed reaction (14).

A possible mode of CCHL function may be to position the heme and apocytochrome relative to each other so as to facilitate their ligation. Enzymatic assistance of heme attachment may be particularly important under circumstances when the local concentrations of the two reaction partners are low, as expected in the periplasm. Moreover, since the electrophilic addition of the cysteine thiols across the double bonds of the heme vinyl groups is a stereospecific reaction, CCHL may participate directly in this reaction, thus controlling the stereochemistry at the chiral center.

To date, it is relatively well established that heme binding occurs on the periplasmic side of the membrane, i.e., after translocation of apoprotein and heme moieties. In *Paracoccus denitrificans*, apocytochromes c_{550} and cd_1 were detected immunologically in the periplasm of a mutant that was affected in cytochrome *c* maturation or when the heme binding site was altered (248, 249, 295). When the *Bradyrhizobium japonicum* cytochrome c_{550} was overexpressed in *E. coli*, apoprotein was detected in the periplasm of mutants defective in heme attachment (340). The *Desulfovibrio vulgaris* cytochrome c_3 expressed in *E. coli* was exported to the periplasm, although holocytochrome c_3 was not formed (261). In light of the compelling evidence supporting the hypothesis that CCHL is a periplasmic enzyme (see below), the case of *Hydrogenobacter thermophilus* cytochrome c_{552}, which can be produced as a holoprotein in the cytoplasm of *E. coli*, appears to be a peculiarity of a cytochrome that can fold rapidly into a stable conformation (296).

Among the many bacterial cytochrome *c*-negative mutants that have been described in the literature (for a review, see reference 342), several contain mutations that map to genes whose products have been postulated to function in heme ligation: *ccl1/2* and *cycH* of *Rhodobacter capsulatus* (21, 189); *cycHJKL* of *B. japonicum* (285, 286), *Sinorhizobium meliloti* (178), and *Rhizobium leguminosarum* (73); and *ccmEFH* of *E. coli* (124, 154, 337) and *cycH* of *P. denitrificans* (250). These genes may encode a total of four subunits of a bacterial CCHL complex that (i) recognizes or selects and releases apocytochrome *c*, (ii) binds and releases heme, (iii) positions apoprotein and cofactor close to each other with the correct geometry for the addition reaction to proceed, and (iv) catalyzes the formation of the thioether bonds. For heme attachment on the periplasmic side of the membrane, the subunits of a bacterial CCHL are expected to be periplasmically oriented. In fact, membrane topology predictions and analysis of these proteins indicate that either they are exported through the membrane to which they remain anchored by noncleaved signal sequences or they are integral membrane proteins with conserved and thus potentially functional periplasmic loops. The characteristics of the predicted CCHL subunits and their hypothetical functions are summarized in Table 3. Note that homologous sequences are also available from *Haemophilus influenzae* and *Pseudomonas fluorescens* (93, 102). *E. coli* seems to have a second set of three of these genes, *nrfE*, *nrfF*, and *nrfG*, which are homologous to CcmF and the N- and C-terminal domains of CcmH, respectively, and are required for the production of the formate-dependent nitrite reductase (124, 154).

The least highly conserved of the four putative heme ligase/lyase polypeptides is CycH from the alpha proteobacteria (Fig. 9): it shares some similarity with part of the C-terminal half of *E. coli* CcmH and very little similarity with *E. coli* NrfG (250). A clearly conserved amino acid sequence is not detectable; however, the membrane topology of this protein shows an N-terminal segment comprising two putative transmembrane helices and a cytoplasmic loop, and a periplasmic C-terminal

TABLE 3. Conserved motifs of putative CCHL subunits

Protein[a]	Membrane topology	Conserved motif(s)	Hypothetical function	References
Bj CycH Sm CycH Rl CycH Re CycH Rc CycH Pd CycH Ec $CcmH_C$ Hi CcmH Pf CycH/CytG	One or two N-terminal transmembrane helices; C terminus periplasmic	**EQHLEKNPTDGRGWNVLA** **ENHLALNPQDGAGWDLLA** **ERHLAEKPDDGKGWDVLA** **ERHLAENPDDGKG**CRPGA RQAV**A**AR**PNDPQGLALLS** AEAVQ**KNPNDPRGLELLA** RTQ**LQKNPGDIEGWIMLG** RID**LQKNPTDAKKWWMLG** **ERAAAAQPDSAEGLYFLG**	Selection/discrimination of specific apocytochromes *c*; N-terminal domain can select cytochrome c_1	73, 93, 102, 178, 189, 250, 281, 285, 331, 337, 388
Bj CycJ Sm CycJ Rl CycJ Ec CcmE Hi CcmE Pf CycJ/Cyt3	N-terminal, noncleaved signal peptide; C terminus periplasmic	**YXGILPDLFRXGQGXV** and **VLAKHDE**X**YXP**	Heme binding and/or reducing	73, 93, 102, 178, 281, 286, 331, 337, 388
Bj CycK Sm CycK Rl CycK Rc Ccll Ec CcmF Hi CcmF Pf CycK/CytD Cv CcmF	Membrane integral; ca. 12 putative transmembrane helices; large periplasmic loops	**WAYYELGWGGXWFWDPVEN**	Heme binding and/or ligation	21, 49, 73, 93, 102, 178, 281, 286, 337, 388
Bj CycL Sm CycL Rl CycL Rc Ccl2 Ec $CcmH_N$ Hi CcmL Pf CycL/CytF	N-terminal, noncleaved signal peptide; C terminus periplasmic	**LRCXXC**	Direct or indirect reduction of apocytochrome *c* heme binding site, possibly interacting with CcmG/CycY/HelX	21, 73, 93, 102, 178, 281, 286, 337, 388

[a] Abbreviations of bacterial species: Bj, *B. japonicum*; Ec, *E. coli*; Hi, *H. influenzae*; Pd, *P. denitrificans*; Pf, *P. fluorescens*; Rc, *R. capsulatus*; Re, *R. etli*; Rl, *R. leguminosarum*; Sm, *S. meliloti*; Cv, *C. vinosum.*

segment (286). All of the *cycH* mutants described fail to assemble mature soluble *c*-type cytochromes. In mutants that lack only the C-terminal domain of the protein, significant levels of apocytochrome c_1 and holocytochrome c_1 can be detected, indicating that the N-terminal domain is required and sufficient for cytochrome c_1 formation, possibly by guiding the secreted, C-terminally membrane-bound cytochrome toward the heme lyase machinery (189). All other *c*-type cytochromes require the presence of the C-terminal periplasmic extension of CycH. Thus, CycH may be regarded as an assembly factor that is able to select specific apocytochromes and discriminate others for heme attachment. Such a situation is reminiscent of the eukaryotic cytochrome *c* and c_1 heme lyases, which are disparate, nonrelated enzymes (78, 81, 398).

In *E. coli*, CcmH appears to be a bifunctional protein. Its N-terminal half ($CcmH_N$) is homologous to CycL (see below), whereas its C-terminal half ($CcmH_C$) corresponds to a CycH lacking the N-terminal domain. Figure 9 shows the domain structure, as derived from translated nucleotide sequences, and the putative membrane topology of CycH and *E. coli* CcmH. In *E. coli* $CcmH_C$, the N-terminal transmembrane helix and subsequent cytoplasmic loop present in CycH are absent, and the C-terminal domain with its single transmembrane helix is fused to the C-terminal transmembrane helix of the CycL-homologous $CcmH_N$ domain. The fused protein lacks only the CycH portion that is required for biogenesis of cytochrome c_1. This can be rationalized in the sense that *E. coli* does not possess a cytochrome bc_1 complex. Interestingly, the $CcmH_C$ domain was shown not to be essential for cytochrome *c* maturation in *E. coli* (123). In addition, *E. coli* can synthesize a second CycH-like protein, NrfG, that may be specifically required for biogenesis of the NrfA and NrfB *c*-type cytochromes encoded in the same operon as NrfG (154). This protein is not used in the maturation of other *c*-type cytochromes such as NapB and NapC, which may be assisted in heme attachment by CcmH.

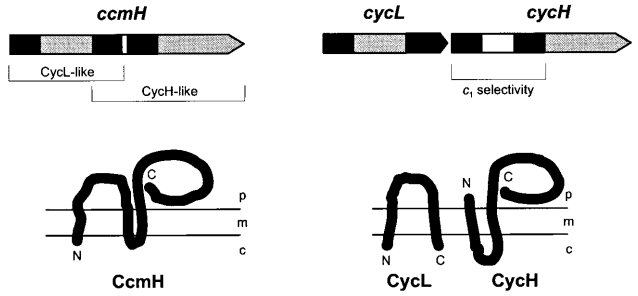


FIG. 9. Domain structure and topological model of *E. coli* CcmH and rhizobial CycL/CycH proteins. The corresponding genes which encode the functional domains are shown at the top as follows: black, hydrophobic (transmembrane) segments; gray, periplasmic domains; white, cytoplasmic loops. The topological models are presented below. In the case of CycH, the topological model has been supported experimentally, except that it is not clear whether the N-terminal hydrophobic segment of CycH is membrane spanning. c, cytoplasm; m, cytoplasmic membrane; p, periplasm.

CycJ/CcmE is a periplasmic protein that is N-terminally anchored to the membrane. The sequences from different organisms comprise two stretches of strong amino acid conservation (Table 3), of which one contains a strictly conserved histidine that may be involved in heme binding. This protein could possibly act as a periplasmic heme carrier, preventing the aggregation of heme released from a heme exporter. In addition, in vitro studies with eukaryotic CCHL have shown that only ferrous heme can be covalently attached to apocytochrome *c* (242). Reduction of the heme iron in the oxidative periplasmic space may thus be another possible function of CycJ.

The largest subunit of the postulated bacterial CCHL was identified first in *Rhodobacter capsulatus* and was named Ccl1. Its rhizobial homologues CycK and the *E. coli* CcmF protein are well conserved and contain 12 to 15 putative transmembrane helices. Applying the "positive inside rule" (366) to predict its topology in the membrane, several large periplasmic loops (approximately 30 to 60 amino acids) oppose to rather small cytoplasmic loops (12 to 17 amino acids). One of the former contains a tryptophan-rich segment: this segment, shown in an extended version in Table 3, represents the best-conserved region of the protein and has also been found in *E. coli* NrfE and in several mitochondrial and chloroplast proteins, which may have similar functions in cytochrome *c* biogenesis. It is also very similar to the tryptophan-rich segment that is present in a postulated periplasmic loop of the CcmC/CycZ/HelC subunit of the putative heme transporter (Fig. 8C). The general consensus among all of the tryptophan-rich segments is WGXXWXWD. The high proportion of aromatic amino acids in this sequence suggests that this motif might allow interaction with the hydrophobic porphyrin ring of heme. Interestingly, four histidines appear to be strictly conserved in the Ccl1/CycK/CcmF family of proteins. Two of them are located close to the periplasmic end of putative transmembrane helices, similar to certain membrane-integral *b*-type cytochromes.

Finally, Ccl2/CycL is also a periplasmic, membrane-anchored protein with the conserved motif LRCXXC. The precise sequence LRCPVC of *R. capsulatus* Ccl2 corresponds very well to the sequence CPV that is present in the eukaryotic CCHL and CC_1HL. For the yeast CC_1HL, evidence has been obtained that this sequence is involved directly in heme binding (317). Moreover, the sequence RCVP is found in the yeast transcriptional activator HAP1, where it most probably serves as a heme binding site (58, 258). Heme was demonstrated to bind specifically to a synthetic decapeptide with the sequence AK**RCPV**DHTM (397). Hence, Ccl2/CycL was postulated to be a periplasmic heme binding protein. However, as more homologous protein sequences are now available for comparison, it seems that the consensus characteristic for this protein family is LRCXXC. The spacing of the two cysteines is typical for (i) ferredoxins, (ii) apocytochromes *c*, and (iii) proteins of the thioredoxin/protein disulfide isomerase (PDI) family. In a periplasmic situation, it is likely that the cysteines form either intra- or intermolecular disulfide bonds and thus could interact with either apocytochrome *c* or a periplasmic thioredoxin (see below).

In vitro heme ligation has been demonstrated only for yeast and *N. crassa* CCHL and yeast CC_1HL (16, 240, 243). Unfortunately, attempts in different laboratories to set up an in vitro assay for the ligation of heme to bacterial apocytochromes *c* have failed so far. It is possible that heme attachment catalyzed by mitochondrial CCHL can be obtained in a cell-free system because the enzyme is soluble (81), whereas in bacteria the situation is more complicated, with up to four membrane-bound polypeptides that are thought to form a CCHL complex. The precise function of the putative CCHL subunits will probably be defined only once a reliable in vitro assay for bacterial CCHL is available.

Noncovalent binding of heme. All cytochromes except those of the *c*-type contain heme that is bound noncovalently, usually by axial ligation of the heme iron with one or two nucleophilic residues of amino acid side chains in the polypeptide. Sequence comparisons, site-directed mutagenesis, and X-ray crystallography have led to the identification of many axial heme ligands in a variety of bacterial cytochromes, as shown in Table 4. With few exceptions, these ligands are histidines, whose nonprotonated nitrogen of the imidazol ring provides electrons for binding of the heme iron.

It is not known whether heme insertion is spontaneous or requires the assistance of other proteins. It can be assumed that spontaneous heme insertion can occur only when the protein is already folded in the membrane. Heme may then slip into a predestined cleft in the protein, where it becomes fixed by the relatively strong interaction with the axial ligand(s). Alternatively, heme binding may be catalyzed by auxiliary proteins, which could happen in either the unfolded or partially folded protein and perhaps would trigger the specific conformation of certain domains. If the latter were true, mutation of heme binding residues to non-heme binding ones should lead to instability of the polypeptide, because folding is not induced. Site-directed mutagenesis of putative heme binding histidines has resulted in fully assembled but inactive cytochromes lacking individual heme cofactors and in overall assembly defects of the respective proteins. For example, mutation of H102 and H421 of the *Rhodobacter sphaeroides* CtaD subunit led to an unstable oxidase lacking the low-spin heme A, whereas mutation of the high-spin heme ligand H419 resulted in an apparently stable oxidase that could be purified, although as an inactive enzyme (308). Therefore, it is not possible to draw general conclusions about the relationship between noncovalent heme binding and protein folding.

So far, not a single protein has been shown to catalyze noncovalent heme insertion. Assembly factors such as CtaG or NarJ (cf. Table 2) may facilitate heme binding. Unfortunately, not enough data are available on these proteins or the corresponding mutants to specify their precise function.

Heme binding histidines are usually found in or next to transmembrane segments, suggesting that heme can reside near both the cytoplasmic and the periplasmic surfaces of the membrane (Fig. 4). It would be interesting to know how heme reaches its destination within the membrane protein, in particular when its location is more toward the periplasmic side: is a specific translocation of heme through the membrane required in this case, such that heme insertion can occur from the periplasmic side, or, alternatively, does heme cross the lipid bilayer autonomously? If heme binding occurs before the protein is assembled correctly in the membrane, its translocation could happen together with translocation of the respective hydrophobic segments. The CydCD proteins have been postulated to be involved in heme translocation through the membrane to allow assembly of the *E. coli bd*-type oxidase (see above) (264). As cytochrome b_{558} of the *bd* complex is thought to be on the periplasmic side of the membrane (87), translocation of heme B would be required. Interestingly, a *cydD* mutant was not only impaired in biosynthesis of the *bd*-type oxidase but also in that of *c*-type cytochromes. Lack of heme export in the *cydD* mutant would also explain such a cytochrome *c* negative phenotype.

It is possible that heme stabilizes a certain tertiary structure of the cytochrome apoprotein. In several cytochrome complexes, the lack of heme causes an assembly defect or instabil-

TABLE 4. Axial ligands of heme cofactors in respiratory cytochrome complexes

Enzyme	Organism	Amino acid(s)	Subunit	Cofactor	Reference(s)
SDH	*E. coli*	H84 H71	SdhC SdhD	b_{556} b_{556}	235, 256
SDH	*B. subtilis*	H70 H155 H28 H113	SdhC	b_H b_H b_L b_L	98, 127
bc_1 complex	*R. sphaeroides*/*R. capsulatus*	H97 H198 H111 H212	cytochrome *b*	b_L b_L b_H b_H	108, 394
NAR[a]	*E. coli*/*T. pantotropha*	H56/H53 H205/H204 H66/H63 H187/H186	NarI	b_H b_H b_L b_L	24, 313
bd oxidase	*E. coli*	M393 H186 H19	SUI	b_{558} b_{558} b_{595}/*d*	87, 175
bo_3 oxidase	*E. coli*	H106 H421 H419	SUI	b_{562} b_{562} o_3	87, 148, 175, 191, 215
aa_3 oxidase	*P. denitrificans*	H94 H413 H411	SUI	*a* *a* a_3	146, 158, 275
aa_3 oxidase	*R. sphaeroides*	H102 H421 H419	SUI	*a* *a* a_3	45, 308
cbb_3 oxidase	*B. japonicum*	H131 H420 H418	FixN	*b* *b* b_3	399, 401
cd_1 NiR	*T. pantotropha*	Y25 H200	NirS	d_1 d_1	101
SiR	*E. coli*	C483	SiRHP	Siroheme	57

[a] Histidines were assigned as putative heme ligands based on sequence comparison. No experimental support.

ity of the subunits. In *B. subtilis*, heme incorporation appeared to be critical for assembly of an active, membrane-bound SDH complex (138). However, there are other examples where heme binding is a late step in cytochrome biogenesis and is not required for assembly of the subunits into the membrane. In the case of *Paracoccus denitrificans* and *R. sphaeroides* aa_3-type oxidases, *E. coli* bo_3 oxidase, and *E. coli bd*-type oxidase, SUI and SUII can be integrated into the membrane in the absence of heme association (191, 238, 279, 308, 318).

Nonheme Cofactors

Many of the cytochrome complexes discussed here contain additional nonheme cofactors. Among them are metal ions such as Fe, Cu, and Mo, which are sometimes associated with other cofactors: iron is found most often in FeS clusters, and Mo occurs as molybdopterin guanosine dinucleotide. In addition, flavin can be a cofactor of cytochrome complexes. Specific uptake systems for the metal cofactors must exist, and biosynthetic pathways for the pterin and flavin cofactors are required. Similar to heme cofactors, these nonheme prosthetic groups usually bind to specific binding sites in the protein, which again may be located on either side of the membrane. Cofactors of periplasmically oriented subunits must therefore be translocated to this compartment. It can also be assumed that cofactors insert preferentially under defined redox conditions into the protein. Taking all these requirements into account, it can be postulated that a relatively large number of hitherto unidentified genes is responsible for proper processing and assembly of these cofactors in addition to the genes required for heme insertion.

Iron. The *Pseudomonas stutzeri* NorB subunit of the nitric oxide reductase contains nonheme iron as a cofactor (141). Nothing is known about metal insertion into this enzyme.

In addition to heme, the most abundant cofactor of cytochrome complexes is found in the form of 2Fe-2S-, 3Fe-4S-, or 4Fe-4S-type iron sulfur clusters. The corresponding binding sites in the proteins contain sequences of conserved arrangements of cysteines and histidines that serve as iron complexing ligands (109). Site-directed mutagenesis of the Rieske FeS protein in *R. sphaeroides* and *R. capsulatus* helped identify the liganding amino acids as two cysteines and two histidines in highly conserved motifs (118). It is not clear whether the for-

mation of FeS clusters requires additional enzymes. When the Rieske protein of the *R. sphaeroides* bc_1 complex was expressed in *E. coli*, an FeS cluster with an unusual electron paramagnetic resonance signal was assembled, whereas expression of the same protein in the homologous host resulted in formation of a normal Rieske-type FeS cluster (359). This suggested that an additional factor may be required for correct assembly of the FeS cluster of the Rieske protein. The BCS1 protein in yeast mitochondria has been speculated to have such a function because a *bcs1* mutant showed a selective loss of the Rieske FeS protein, but not of cytochromes *b* and c_1 (244). As *E. coli* does not possess a bc_1 complex, it is unlikely that it would synthesize such a factor. Alternatively, if the formation of a 2Fe-2S cluster happened spontaneously, it would be difficult to explain why the electron paramagnetic resonance signal of a Rieske protein expressed in *E. coli* is not normal. One possibility is that the formation of intramolecular disulfide bonds is required for correct folding of the protein and that this step is catalyzed by a specific protein, disulfide isomerase. In fact, the conserved FeS cluster binding motifs contain two additional cysteines that have been proposed to be essential for stabilizing the FeS cluster of the Rieske protein (68). The presence of such an intramolecular disulfide bridge was confirmed recently when the structure of a water-soluble fragment of the bovine heart mitochondrial Rieske protein was solved (159).

Another interesting question, which, however, has not been addressed, is in which subcellular compartment the FeS cluster is assembled. Since the Rieske protein is translocated to the periplasmic side of the membrane, where it is anchored by its N-terminal hydrophobic sequence, assembly of the holoprotein is likely to occur after translocation, similar to the case of cytochrome *c* maturation. It is not clear how iron and sulfur atoms would reach the periplasm to become associated with the protein.

Copper. Copper serves as cofactor in heme-copper terminal oxidases, where it occurs in a Cu_A redox center containing two copper atoms in a mixed-valence [Cu(1.5)-Cu(1.5)] configuration and in a binuclear high-spin heme-Cu_B center (7, 158, 357) (Fig. 4). Cu_B resides in SUI of the oxidase and is liganded by three strictly conserved histidines (39). Cu_A is the redox center of SUII in aa_3-type cytochrome *c* oxidases and is complexed in the periplasmic domain of the protein by conserved cysteines, histidines, a methionine, and the carbonyl oxygen of a glutamate (158). Most of these ligands belong to the highly conserved sequence YGQCSEICGXXHXXMPIV (106). This sequence is missing in SUII of the structurally related quinol heme-copper oxidases such as the bo_3-type oxidase of *E. coli*, in which, however, a Cu_A binding site could be restored artificially by alterations of the corresponding amino acids into metal binding residues (357). A cytoplasmically produced SUII domain containing the restored copper binding site was successfully loaded with copper during metal affinity chromatography, suggesting that insertion of the Cu_A cofactor can occur spontaneously and that the folding of the protein into its tertiary structure occurs before Cu_A insertion.

Incorporation of Cu_B into SUI of heme-copper oxidases is likely to be a late step in oxidase assembly. Site-directed mutagenesis of the putative copper ligands of the *R. sphaeroides* aa_3- and *E. coli* bo_3-type oxidases led to proteins with severe disruption of the aa_3-Cu_B binuclear center (reviewed in reference 148).

As discussed above, putative assembly factors were postulated to be involved in copper processing and insertion. The periplasmic *B. japonicum* thioredoxin TlpA is thought to interact with SUII of the aa_3 type oxidase to assist Cu_A insertion (198). As SUII extrudes into the relatively oxidative environment of the periplasm, it is likely that the cysteines of the copper binding site are oxidized, unless a specific reductant, perhaps TlpA, is active. TlpA homologs have not yet been described in other bacteria containing aa_3-type oxidases. Their existence in other species would certainly be expected if SUII were a direct target for TlpA function.

The possible involvement of the *B. japonicum fixGHIS* gene products in copper transfer to the cbb_3-type oxidase is based on the observation that mutations in these genes lead to an almost complete loss of the cbb_3 oxidase polypeptides (269). The structural genes for this oxidase are coregulated with *fixGHIS* and expressed in a FixK-dependent manner only under microaerobic and anaerobic conditions (92). A role of FixI as a specific Cu^+ uptake system under microaerobic conditions, combined with a role of FixG as a cytoplasmic copper oxidant, has been proposed to explain how copper may be inserted as a bivalent cation into the oxidase (269). Although this is an appealing theory, the question remains how under these circumstances Cu^{2+} is transferred to the binuclear center at the periplasmic periphery of the membrane. An alternative theory predicts that FixG reduces cytoplasmic Cu^{2+} to produce transport-competent Cu^+ and the FixI ATPase functions in copper export to the periplasm, where insertion into the binuclear center can take place more easily. This is supported by the finding that the FixG homolog RdxA of *R. sphaeroides* has a reductive function in the reduction of tellurite to tellurium (237). It should be emphasized that it is not known whether copper is inserted into the binuclear center in the Cu^+ or the Cu^{2+} form, because this metal changes its valence during the catalytic cycle of O_2 reduction (44).

Another interesting aspect of copper metabolism and cytochrome biogenesis has been described recently in the context of cytochrome *c* maturation in *P. fluorescens* (388). It was found that mutations in *helC*, *cycJ*, *cycK*, *tipB*, *cycL*, and *cycH* (see below) affected cytochrome *c*-dependent TMPD oxidase activity and copper resistance. Similarly, the *E. coli* gene *cutA2* that was isolated as the locus to which copper-sensitive mutations mapped (95), was identified independently as *dipZ*, encoding a protein disulfide isomerase essential for formation of mature *c*-type cytochromes (59). Thus, a link between cytochrome *c* maturation and copper metabolism must exist, perhaps involving a dual role of periplasmic, thioredoxin-like proteins.

Other cofactors. Not much is known about processing and incorporation of other cofactors into cytochrome complexes. Specific transport systems exist for metals like molybdenum and nickel (135, 276). Nickel insertion into hydrogenase has been studied most extensively in *E. coli*, and several genes (*hypA* through *hypF*) involved in formation of active hydrogenase rather than in synthesis of the polypeptides have been identified (for a review, see reference 29). Particularly interesting is the *hypB* gene product, a nickel and GTP binding protein that appears to function in delivery of nickel to the hydrogenase apoproteins (202).

Molybdenum is bound in a molybdopterin cofactor, whose synthesis in *E. coli* requires genes organized in at least five different operons (*moaABC*, *mobAB*, *modABCD*, *moeB*, and *mogA* [276]). Whereas the molybdopterin-containing subunit NarG (or NarZ) is located on the cytoplasmic side of the membrane in the membrane-bound nitrate reductase, other oxidoreductases such as the periplasmic nitrate reductase, TMAO reductase, and DMSO reductase have periplasmic molybdoproteins. Very little is known about how transfer and insertion of the metal is accomplished in these cases. In *E. coli*

TMAO reductase, the *torB* gene product is required for molybdenum cofactor processing (385).

In SDH, FAD is bound covalently to the soluble SdhA polypeptide before it associates with SdhB and SdhC into a membrane-bound complex (136). *Bacillus subtilis* SdhA expressed in *E. coli* does not bind flavin (137). It is not known whether FAD attachment depends on additional protein factors.

Protein Folding and Stability

The acquisition of thermodynamically stable tertiary structure is an important step during cytochrome biogenesis but has not been studied extensively. Although cytochrome *c* has been widely used as a model system for folding (179, 236), most studies were restricted to reversible denaturation experiments of holocytochrome *c*, where the unfolded state still represents a protein with covalently bound heme. A single linear pathway for in vitro cytochrome *c* folding was recently proposed based on the identification of major folding intermediates and cooperative folding units of 15 to 27 amino acids (8). However, not much is known about how cytochrome *c* in vivo reaches a conformation which allows the covalent ligation of heme and whether partial folding of the apocytochrome takes place. In the absence of heme, mitochondrial apocytochrome *c* precursor is unfolded; however, noncovalent binding of heme induces the formation of a compact structure of the polypeptide (80). As illustrated in Fig. 1, it seems that folding of cytochrome polypeptides is tightly connected with cofactor binding and in cytochrome complexes with oligomerization of subunits, i.e., the formation of a tertiary structure, and there are many open questions. Does protein folding influence cofactor incorporation and oligomerization in multisubunit complexes and vice versa? Are general or specific chaperones or folding catalysts required for cytochrome biogenesis? What is the role of cellular proteases in the maturation of cytochromes? Several aspects of protein folding and stability are discussed in the following sections.

Chaperones. As most cytochromes are either membrane bound or periplasmic, they must be kept in a conformation that protects them from cytoplasmic proteases before they reach the membrane, where they are either inserted into or transported through the membrane. Alternatively, they may be able to adopt a proteinase-resistant structure before they reach their destination. It is likely that cytoplasmic chaperones accomplish the task of protecting apocytochromes from cytoplasmic degradation. Generally, molecular chaperones can stabilize proteins in statu nascendi and newly synthesized proteins by keeping them in an appropriate conformation and preventing incorrect interactions within and between nonnative polypeptides in an ATP-dependent manner (134). So far, no cytochrome-specific chaperones have been discovered, and it is likely that at least one of the well-known cytoplasmic chaperones such as GroEL, DnaK, or SecB plays a role in cytochrome biogenesis. GroEL and SecB have been implicated in maintaining a translocation-competent conformation of proteins to be exported (186). However, their specific function in cytochrome maturation has not been addressed.

Periplasmic chaperones have been discovered only in recent years. They are involved in specific pathways: (i) assembly of cell surface structures such as pili, fimbriae, or flagella (152, 153), (ii) assembly of outer membrane proteins (48), or (iii) secretion of proteins into the medium (160). These proteins assist polypeptide folding in the periplasm, most probably in an ATP-independent manner, since the existence of ATP in this compartment is highly improbable (383). It is possible that certain periplasmic proteins involved in cytochrome maturation have chaperone-like functions. This hypothesis is particularly attractive in view of the many cytochrome *c* biogenesis proteins for which precise functions have not yet been elucidated. Among them are CcmE/CycJ and CcmH/CycL, which may well be candidates for periplasmic, cytochrome *c*-specific chaperones.

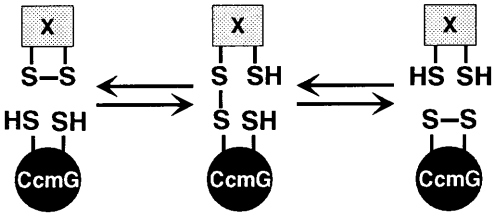
Folding catalysts. Folding catalysts are enzymes that catalyze defined steps in protein folding, as opposed to molecular chaperones, which prevent incorrect protein-protein interactions rather than actively catalyze folding. The best known folding catalysts are peptidylprolyl *cis-trans* isomerases (PPIs) (91, 104, 188, 193, 288, 325, 345, 383) and PDIs (10, 11, 96, 97, 197), which can also be found in the periplasm (217, 218).

Folded protein requires a unique proline conformer for each proline-accommodating site in the tertiary structure, in which the *trans* form is much more common (236). The spontaneous *cis-trans* isomerization of prolyl peptide bonds is a slow process, but PPI-folding catalysts accelerate folding reactions by allowing rapid rotation around the prolyl-peptidyl bond. This may be favorable during cytochrome biogenesis because the nonnative conformations of cytochromes (and their precursors) can be extremely unstable, e.g., in the case of the *B. japonicum* cytochrome c_{550}, whose preapoprotein and apoprotein precursors have half-lives of 20 to 30 and 90 to 105 s, respectively (340). PPIs have been discovered in all cellular compartments, and so far three families of these proteins are known in which the amino acid sequences within each family, but not between them, are conserved (288). However, no cytochrome biosynthesis accessory protein has been described so far that belongs to one of these PPI families.

In contrast, PDI-folding catalysts play quite an important role in cytochrome biosynthesis. PDIs catalyze the formation or cleavage of disulfide bonds (Fig. 10A). Both cytoplasmic and periplasmic PDIs have been found in bacteria (10, 197). The cytoplasmic thioredoxin and thioredoxin reductase are reducing proteins which prevent disulfide bond formation in cytoplasmic polypeptides. Since many periplasmic proteins contain intra- or intermolecular disulfide bonds, and since the spontaneous formation of disulfide bonds in proteins is slow, there is a general need for periplasmic PDIs with dithiol-oxidizing function. During translocation of periplasmic proteins through the membrane the polypeptides are unfolded; i.e., they contain dithiols. The Dsb (disulfide bond formation) proteins form a network of redox-active PDIs which exquisitely control disulfide bond formation in periplasmic proteins (10, 218). DsbA and DsbB are strong oxidants that facilitate the formation of disulfide bonds in a variety of proteins (12, 60, 171, 220, 384, 395), whereas DsbD/DipZ appears to be a thioredoxin-like protein with the capacity to reduce disulfide bonds (59, 221). DsbC was suggested to be required for the isomerization of disulfide bonds as part of a periplasmic redox system (219, 282, 309, 314). Since many cytochrome components are periplasmic and often contain conserved cysteines, it is not surprising to find that various periplasmic PDIs are involved in maturation of these proteins. Interestingly, both an oxidizing type (DsbA and DsbB) and a reducing type (DsbD/DipZ) of periplasmic PDIs affect the maturation of *c*-type cytochromes (59, 211, 212, 294), indicating that the redox state of the periplasm is critical for this process.

A number of bacterial PDIs which appear to be involved in a more specific way in cytochrome biogenesis have recently been identified. The first thioredoxin-like protein found to be required for the formation of an active *B. japonicum* aa_3-type cytochrome *c* oxidase was TlpA. This protein is membrane bound, faces the periplasm, and contains the CXXC sequence

A



B

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Ec CCMG                                          MKRKVLLIPLIIFLAIAAALLWQLARNAEGDDP
Hi CCMG                                           MKKKLLVPLILFLSITIAFLVQLKRNAQGED
Bj CYCY                                  MSEQSTSANPQRRTFLMVLPLIAFIGLALLFWFRLGSGDPSRIPS
Rl CYCY                                          MGRYTLALLPLIVFGGIAHGAKMLYDQDFHGKN
Rc HELX                                               MAKPLMFLPLLVMAGFVGAGYFAMQQND
Pf CYTE                                                MKRWLMVLPLALFLLVAVFLYRGLYL
Cv CCMG                                               MNTSAKRALIPLGSFWPWALLFYGLQLD
Bj TLPA                  MLDTKPSATRRIPLVIATVAVGGLAGFAALYGLGLSRAPTGDPACRAAVATAQKIAPLAH
Bs RESA                                              MDNEEKKAFIHSDRHPSRFNLRTRLYHLQ

                                                                    ___________________
Ec CCMG                  TNLESALIGKPVPKFRLESLDNPGQFYQADVLTQGKPVLLNVWATWCPTCRAEHQYLNQL
Hi CCMG                  IKALESALVGKPVPAKNLTELFENKTYTNELFQQGEPVLLNVWATWCPTCYAEHQYLNKL
Bj CYCY                  ALIGRPAPQTALPPLEGLQADNVQVPGLDPAAFKGKVSLVNVWASWCVPCHDEAPLLTEL
Rl CYCY                  IAEIPSALSHQGADAEPAAARRATLPALTDAAIKGKLTLVNVFASWCLPCRDEHPVLKEL
Rc HELX                  PNAMPTALAGKEAPAVRLEPLGAEAPFTDADLRDGKIKLVNFWASWCAPCRVEHPNLIGL
Pf CYTE                  DPAELPSAMIGKPFPAFSLPTVQGDKTLTQADLIGKPALVNVWATWCISCRVEHPVLNKL
Cv CCMG                  PRKIPSPLVDKPAPEFSLPDLKDPNQTLTRDILIGQVSLVNVWASWCPSCRQEHAELMRI
Bj TLPA                  GEVAALTMASAPLKLPDLAFEDADGKPKKLSDFRGKTLLVNLWATWCVPCRKEMPALDEL
Bs RESA                  RCICRQREYIRRSDAPNFVLEDTNGKRIELSDLKGKGVFLNFWGTWCEPCKKEFPYMANQ
Ec TRXA                                MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEI
                                                                    **  *

Ec CCMG                  SA-QGIRVVGMNYKDDRQKAISWLKELGNPYALSLFDGDGMLGLDLGVYGAPETFLIDGN
Hi CCMG                  AK-EGVRIIGLDYKDESPKAMKWLKDLGNPYQVVLKDEKGSFGLDLGVYGAPETFIVDGK
Bj CYCY                  GKDKRFQLVGINYKDAADNARRFLGRYGNPFGRVGVDANGRASIEWGVYGVPETFVVGRE
Rl CYCY                  AKDGRLNIVAINYKDQSDNALRFLGELGNPYQAIGIDPNGKAAIDWGVYGIPESYLVGAD
Rc HELX                  -KQDGIEIMGVNWKDTPDQAQGFLAEMGSPYTRLGADPGNKMGLDWGVAGVPETFVVDGA
Pf CYTE                  A-EKGVVIYGINYKDDNAAALKWLAEFHNPYQLDVRDEDGNLGLNLGVYGAPETFFIDAK
Cv CCMG                  AREHGVRVIGFNWKDTRPEALALLQRYGDPYTVSLYDPDNKAGIDWGVYGAPETFIVDAE
                                       **        *     *     *          ** * **

Bj TLPA                  QGKLSGPNFEVVAINIDTRDPEKPKTFLKEANLTRLGYFNDQKAKVFQDLKAIGRALGMP
Bs RESA                  YKHFKSQGVEIVAVNVGESKIAVHNFMKSYGVNFPVVLDTDRQVLDAYDVSPLPTTFLIN
Ec TRXA                  ADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVASATKVGALSKGQLKEFLD

Ec CCMG                  GIIRYRHAGDLNPRVWEEEIKPLWEKYSKEAAQ
Hi CCMG                  GVIHYRYAGDVNEKVWTQTLKPIYDKLSEQQ
Bj CYCY                  GTIVYKLVGPITPDNLRSVLLPQMEKALK
Rl CYCY                  GTILYKRVGPSTNISLKEGLVPAMEKALGKPVS
Rc HELX                  GRILTRIAGPLTEDVITKKIDPLLAGTAD
Pf CYT5                  GVIRDKFVGVIDEVVWREQLAAKYQALVDEAKP
Cv CCMG                  GIIRHKRVGPIDAQVWA
                         * *     *
Bj TLPA                  TSVLVDPQGCEIATIAGPAEWASEDALKLIRAATGKAAAAL
Bs RESA                  PEGKVVKVVTGTMTESMIHDYMNLIKPGETSG
Ec TRXA                  ANLA
```

FIG. 10. Proposed function and alignment of bacterial thioredoxin-like proteins. (A) CcmG serves as an example to show how a thioredoxin catalyzes the reversible reduction of an intramolecular disulfide bond in its target protein X. The intermediate that is formed between the thioredoxin and its substrate is called a mixed disulfide. The postulated mechanism involves the presence of a CXXC motif in the target protein, because it also occurs in the heme binding site of *c*-type cytochromes. (B) The primary gene products of periplasmic thioredoxins involved in cytochrome *c* maturation from *E. coli* (Ec), *Haemophilus influenzae* (Hi), *Bradyrhizobium japonicum* (Bj), *Rhizobium leguminosarum* (Rl), *Rhodobacter capsulatus* (Rc), *Pseudomonas fluorescens* (Pf), and *Chromatium vinosum* (Cv) are shown in the first seven lines. They comprise several identical amino acids (designated by asterisks) in the C-terminal domain, distinguishing them from other thioredoxin-like proteins such as the *B. japonicum* TlpA, the *Bacillus subtilis* (Bs) ResA protein, and *E. coli* thioredoxin (Trx), which are shown in the last three lines. Putative N-terminal signal sequences and transmembrane segments are shown in italics. Note that in ResA, as in thioredoxin, a signal sequence is missing, indicating a cytoplasmic location of these proteins. The active site that is relatively well conserved in all thioredoxin-like proteins is overscored by a horizontal line. For references, see Table 5.

motif typical for the active site of PDIs (Fig. 10B) (195). TlpA was shown to have PDI activity and redox properties comparable to those of cytoplasmic thioredoxin (196, 198), suggesting that its in vivo function is to produce and maintain reduced cysteines in a particular polypeptide. Since cytochrome oxidase SUI contains only one cysteine that is not conserved in other heme-copper oxidases, the best candidate target protein of TlpA is SUII (CoxB) of cytochrome *c* oxidase: this subunit contains two conserved cysteines that are thought to be required in the reduced form for Cu_A liganding. However, since direct protein-protein interactions between CoxB and TlpA have not been demonstrated, there are alternative possible target molecules, perhaps other assembly factors required for cytochrome aa_3 maturation.

A growing number of periplasmic thioredoxins belong to a family of PDIs required for cytochrome *c* biogenesis (Fig. 10B). The first-characterized member of this family is HelX, a periplasmic protein which is essential for cytochrome *c* maturation. Other members of this family for which an involvement in cytochrome *c* maturation has been shown experimentally include the *Rhizobium leguminosarum* and *Bradyrhizobium japonicum* CycY proteins (86, 360) and the *E. coli* CcmG protein (212). The latter appears to be identical to DsbE (Swissprot accession no. P33926) (212). The *Pseudomonas fluorescens* TipB/CytE (102, 388), *Haemophilus influenzae* CcmG (93), *Paracoccus denitrificans* CcmG (251), and *Chromatium vinosum* CcmG (49) proteins are postulated to have an analogous function because they are encoded by genes located in similar cytochrome *c* biogenesis gene clusters and clearly show more sequence identity to HelX than to other bacterial thioredoxins such as TlpA and TrxA (Fig. 10B). In the case of *B. subtilis* ResA, an involvement in cytochrome *c* biogenesis is not clear: *resA* seems to be an essential gene that is located in an operon together with genes encoding regulators of respiration and a hydrophobic protein with the sequence WGXXWWXWD resembling the putative heme binding motifs of CcmC and CcmF but otherwise not homologous to these proteins (327). The C-terminal portions of TlpA, ResA and TrxA are homologous neither to each other nor to the C-terminal portions of members of the CcmG family. For the latter family a second, conserved segment represented by the *E. coli* CcmG sequence LDLGVYGAPETFLI is present that may serve as a substrate binding site.

Except for ResA and TrxA, the proteins shown in Fig. 10B contain hydrophobic segments at their N termini that were either shown or can be predicted to act as translocation signal sequences (20, 86, 195, 360). ResA does not contain such a sequence and is more similar to cytoplasmic thioredoxin (TrxA) in this respect (327). The *B. japonicum* CycY protein was shown to be membrane anchored. The soluble domain of CycY was purified and characterized biochemically (86). Although it was not possible with this protein to show thiol:disulfide oxidoreductase activity by the classical insulin reduction assay (147), the standard redox potential of −217 mV measured for this protein indicates a function in dithiol formation rather than in disulfide bond formation (86). The lack of insulin-reducing activity generally found in PDIs indicates that CycY may interact only with very specific reaction partners. Two types of proteins can be proposed as putative substrates for reduction by CycY and its relatives in vivo: (i) apocytochrome *c*, whose heme binding cysteines are expected to be reduced before heme attachment can occur, and (ii) CycL, the only other cytochrome *c* biogenesis protein, which contains a CXXC motif and whose function is not clear. Upon reduction of its unknown substrate, CycY itself becomes oxidized and obviously must be reduced to be recycled in an active form (Fig. 10A shows the situation for the homologous CcmG). Such a reduction may arise from an interaction with a DipZ-like protein, a more general producer of reduction power in the periplasm (59, 221, 282, 292).

The mechanism of disulfide bond formation catalyzed by PDIs includes the formation of mixed disulfides between the folding catalyst and its target (Fig. 10A). It has been possible to detect these in the case of DsbA-DsbB interaction (125, 180). It should be interesting to test whether mixed disulfides of periplasmic thioredoxins such as CycY or TlpA and their cognate reaction partner(s) can be obtained.

Proteases. Many of the cytochrome subunits discussed here are periplasmic, soluble proteins; i.e., their signal sequence is cleaved after translocation. Signal sequences are approximately 20 amino acids in length and contain a positively charged N terminus preceding a hydrophobic core and some primary sequence constraints at positions −3 (Ala, Gly, Ser, Val, Ile) and −1 (Ala, Gly, Ser) relative to the cleavage site, with alanine found especially frequently (366). These amino acids constitute the recognition site of signal peptidase I (or leader peptidase), which in *E. coli* is the LepB protein (63).

For cytochrome biogenesis, it is interesting to define at which point of holoenzyme maturation the signal peptidase exerts its cleavage function and whether processing is essential for the formation of holoprotein complexes.

For the biogenesis of mature mitochondrial cytochrome c_1, cleavage of the translocated precursor occurs by the mitochondrial inner membrane protease 1, a homolog of *E. coli* LepB. This processing seems to be a late step of mitochondrial cytochrome c_1 maturation, since it takes place after, and depends on, heme attachment (243). A similar conclusion was reached for maturation of *B. japonicum* cytochrome c_1, which can occur in the absence of signal sequence cleavage, as shown in a mutant lacking the signal peptidase cleavage site: the mutant produced uncleaved *b*-c_1 polyprotein precursor that carried covalently bound heme and was biologically active (338). Since cytochrome c_1 is bound to the membrane by its hydrophobic C terminus, lack of signal sequence cleavage seems neither to interfere with heme ligation nor to disturb the folding of the polypeptide into an enzymatically active conformation. In principle, ligation of heme to an unprocessed apocytochrome seems possible for *c*-type cytochromes (339). An opposite finding was described recently for the *P. denitrificans* soluble cytochrome c_{550}, whose noncleaved precursor did not contain detectable levels of covalently bound heme. Obviously, in this case heme can be bound to the apoprotein only after signal sequence cleavage (295).

The actual involvement of leader peptidase in cytochrome *c* maturation was shown in an in vitro system in *Rhodobacter capsulatus*, in which the transcribed and translated apocytochrome c_2 precursor was efficiently cleaved (379). The *lepB* gene was recently cloned and sequenced from this organism (278). Moreover, expression of *B. japonicum* cytochrome c_{550} in a temperature-sensitive *E. coli* strain [*lepB*(Ts)] led to accumulation of a precursor polypeptide in the membrane (339).

Since in bacteria, as in yeast, different routes of soluble cytochrome *c* and membrane-bound cytochrome *c* and c_1 maturation may exist, the requirement for signal peptidase to cleave a precursor before heme binding occurs may vary (Fig. 11). It is possible that the heme binding machinery discriminates between membrane-bound and soluble apoproteins. This point may be clarified as different types of bacterial cytochromes are investigated with respect to how signal sequence cleavage and heme attachment depend on each other.

Proteases often come into play when cytochromes cannot

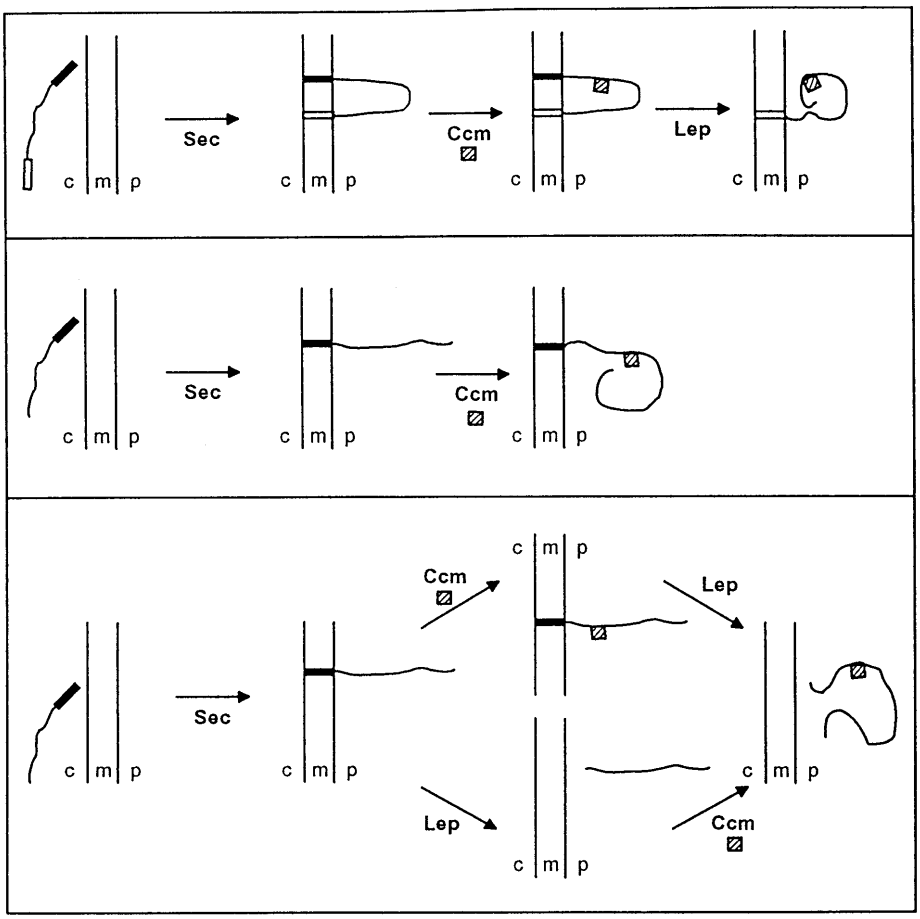

FIG. 11. Model of cytochrome *c* maturation. Maturation pathways are proposed based on the characterization of specific intermediates for cytochrome c_1 (top), membrane-bound *c*-type cytochromes (middle), and soluble *c*-type cytochromes (bottom). Black bars represent N-terminal signal sequences, white bars represent C-terminal membrane anchors, and hatched squares represent the heme. The type of gene products required for each step in the pathway is indicated next to the arrows. Abbreviations: Sec, subunits of the general secretion apparatus; Ccm, subunits of a cytochrome *c*-specific maturase complex; Lep, leader peptidase for cleavage of the signal sequence; c, cytoplasm; m, cytoplasmic membrane; p, periplasm.

proceed through the entire maturation pathway. For example, a yeast ATP- and Zn^{2+}-dependent protease machinery was described based on its capacity to degrade unassembled cytochrome *c* oxidase subunits (233, 254). Many bacterial cytochromes are unstable when maturation cannot be completed. This was shown with *B. japonicum* cytochrome c_{550}, which is extremely unstable when it is expressed as a cytoplasmic protein lacking a signal sequence (340), probably as a result of proteolysis. In contrast, it was possible to express the *Hydrogenobacter thermophilus* cytochrome c_{552} in the cytoplasm of *E. coli* as a holoprotein: the cytoplasmic apoprotein was thought to adopt a stable, protease-resistant conformation, allowing the incorporation of reduced heme (293). Although the existence of cellular proteases degrading cytochrome apoproteins is indisputable, these proteases may not be specific but may simply recognize unfolded or misfolded polypeptides as substrates. So far, no proteolytic enzymes are known to be specifically involved in controlling the degradation or stability of cytochrome subunits, depending on their stage of assembly. If proteases with a high specificity for cytochromes can be defined, this will be extremely useful to optimize the overproduction of cytochrome subunits by expressing their genes in the corresponding mutant backgrounds.

INTEGRATED VIEW OF SELECTED COMPLETE MATURATION PATHWAYS

Cytochrome *c* Maturation

Maturation of *c*-type cytochromes has become a topic of quite intense research in recent years, leading to the hypothesis that a pathway for cytochrome *c* biogenesis exists (for reviews, see references 90, 116, 117, 149, 184, 341, and 342). Here, the present state of knowledge is summarized briefly, with emphasis on new aspects that have not been covered previously.

The current picture of cytochrome *c* biogenesis is based on several different lines of evidence. (i) Bacterial mutants whose phenotype suggests they are affected in posttranslational steps of cytochrome *c* biosynthesis (Cyc^-) have been isolated and characterized (reference 342 and references therein). (ii) The genes mutated in Cyc^- mutants were cloned, and derived sequences of the gene products were used in database searches to identify similar proteins with known biochemical functions. This led to the identification of genes encoding an ABC transporter, a thioredoxin, and a putative CCHL with up to four subunits (20, 21, 277). In addition, homologs to the cytochrome *c* biogenesis genes that had been discovered in other contexts emerged from these searches (12, 49, 59, 93, 102, 211, 281,

388). For many of these, a function in cytochrome *c* biogenesis has not yet been confirmed by mutational analysis. The physical arrangement of these genes in clusters (see Fig. 12) and the conservation of specific sequence motifs (Fig. 8 and 10B; Table 3) of the gene products is of particular interest. (iii) Computer-assisted predictions of membrane-spanning helices combined with LacZ and PhoA fusion analyses of the membrane topology of cytochrome *c* biogenesis proteins revealed that many of the membrane-bound subunits of the cytochrome *c* maturation machinery contain large segments that seem to face the periplasmic side of the membrane, suggesting that important reactions of the pathway occur on this side (2, 20, 21, 73, 86, 102, 189, 250, 252, 277, 285, 286, 331, 360). (iv) Site-directed mutagenesis of cytochrome *c* polypeptides has helped identify regions of importance in the apoprotein (35, 36, 293, 295, 338, 340, 343). (v) Expression of *c*-type cytochromes in heterologous hosts, most often *E. coli*, has been used as an approach to define specific requirements for maturation (295, 340, 342). (vi) First attempts to obtain cytochrome *c* maturation in vitro or at least individual steps of a pathway have been partially successful: translocation of precytochrome c_2 and c_{550} into cytoplasmic membrane vesicles of *R. capsulatus* and *E. coli*, respectively, has been performed (283, 378), and a reductive function of bacterial thioredoxins has been shown to be involved in cytochrome *c* formation (86, 221). Unfortunately, neither heme translocation nor heme attachment to apocytochrome *c* has been demonstrated in vitro in any bacterial system, although the assay for holocytochrome *c* detection reported from mitochondrial systems (241, 243) has been reproduced with bacterial cytochrome *c* (284). (vii) Intermediates formed during cytochrome *c* biosynthesis have been trapped in time course (pulse-chase) radiolabelling experiments in an attempt to dissect specific steps of the cytochrome *c* maturation pathway (339, 340). All of these approaches have been used either alone or in combination to provide more insight into bacterial cytochrome *c* maturation.

Cytochrome *c* is formed in a linear maturation pathway. The current knowledge includes some aspects that were postulated before and now are supported experimentally, as well as others that are still hypothetical. Cytochrome *c* biogenesis is proposed to follow a linear pathway of events (presented in the following summary) which is in agreement with all findings reported (see also Fig. 11 for a simpler version that shows maturation pathways of three different types of cytochromes *c*).

(i) Cytochrome *c* is synthesized initially as a preapoprotein with an N-terminal signal sequence.

(ii) The apoprotein is then translocated as an unfolded polypeptide through the membrane in a *sec*-dependent pathway.

(iii) As the heme binding-site cysteines move into the periplasm, they become rapidly oxidized by the DsbA/DsbB system and form disulfides.

(iv) Intramolecular cysteines are not accessible for heme ligation and therefore must be subsequently rereduced by a periplasmic thioredoxin, most probably CcmG. DsbD has been suggested to be a periplasmic reductant and may be involved in regenerating reduced CcmG.

(v) The last step of heme biosynthesis occurs on the cytoplasmic side of the membrane, but heme C ligation takes place on the periplasmic side of the membrane. Thus, heme must cross the lipid bilayer, most probably by active transport. Two different ABC transporters have been postulated to translocate heme; one of them is encoded in the cytochrome *c* biogenesis gene cluster.

(vi) Heme may be released from the transporter by a periplasmic heme binding protein.

(vii) In the oxidative environment of the periplasmic compartment, heme is likely to oxidize rapidly, which may need to be prevented before ligation to apocytochrome takes place. A heme-iron-reducing protein can therefore be postulated.

(viii) Apocytochrome and heme must be presented to each other in a sterically favorable conformation such that covalent linkage is possible. CCHL is thought to be responsible for the presentation of the two reaction partners and the ligation reaction. The enzyme may be able to discriminate between cytochromes *c* and c_1.

(ix) Holocytochrome *c* is released from CCHL and folds into an active tertiary structure. It is now ready for oligomerization with other proteins.

(x) Soluble cytochrome *c* is released into the periplasm by leader peptidase. This step is independent from heme ligation, and vice versa, and thus may occur before heme is ligated.

Although genetic experiments have contributed to the above view of cytochrome *c* maturation, the postulated biochemical functions have not been experimentally amenable. This is partly due to the lack of specific assays for the individual reactions.

The most speculative step in this pathway concerns the function of the cytochrome *c* biogenesis-specific ABC transporter. Although it is attractive to speculate that it is a heme exporter, there is no experimental evidence to support this. The possibility that this ATP-dependent permease translocates another as yet unidentified substrate cannot be ruled out. For example, in *Pseudomonas fluorescens*, the cytochrome *c* maturation genes are involved in the production or uptake of pyoverdine, an iron-chelating compound. Although it is not clear how pyoverdine uptake and cytochrome *c* biogenesis are connected (if at all), one can imagine that additional molecules might have to be transported to the periplasm for cytochrome *c* maturation, either as a source of reductant or perhaps as a CCHL cofactor.

Another speculative aspect is the composition and function of the postulated CCHL. Although the existence of a bacterial CCHL seems certain and although it is well established that heme ligation occurs on the periplasmic side of the membrane (at least in *Paracoccus denitrificans* and *E. coli* [248, 249, 295, 339, 340]), the suggestion that CCHL is encoded by some of the known cytochrome *c* maturation genes remains to be investigated.

A difficulty in analyzing the cytochrome *c* maturation pathway step by step has become evident as a result of the observation that mutations in different cytochrome *c* biogenesis genes always lead to a single phenotype, i.e., the lack of apo- and holocytochrome *c*, thus not allowing a genetic dissection of individual steps. A possible reason for this complication may be the existence of a multimeric maturase complex, as discussed below.

Cytochrome *c* biogenesis genes. Eight or nine genes in different gram-negative bacterial species have been assigned to specific functions in a general cytochrome *c* maturation pathway, based on mutations which block cytochrome synthesis at a posttranslational level without affecting aerobic cellular growth. As more homologous genes are identified, the gene nomenclature becomes more confusing. A survey of the different names for cytochrome *c* biogenesis genes and the postulated functions of the derived proteins is presented in Table 5. To simplify further discussions the *ccm* nomenclature (for cytochrome *c* maturation) of the *E. coli* genes is used whenever possible.

The organization of *ccm* genes in different organisms is only partly conserved and partly different. The various combinations of different genes in clusters give rise to speculations about the function of the respective products. Figure 12 shows

the gene clusters of 10 different organisms in which *ccm*-like genes have been identified. Several points are noteworthy. (i) The *ccm* genes are organized in one (*E. coli*, *Haemophilus influenzae*, and *Pseudomonas fluorescens*), two (*Bradyrhizobium japonicum* and probably *Rhizobium leguminosarum*), or at least three (*Rhodobacter capsulatus*) clusters, most probably in operons. (ii) The genetic organization of the *ccmABC* genes is, where known, entirely conserved, suggesting that the derived polypeptides are functionally related. This is in agreement with their postulated function as subunits of an ABC transporter. In most cases, *ccmD* is also clustered with this unit; however, its function is unknown (see below). (iii) The *ccmEFL* genes are clustered, although *ccmG* can be inserted between *ccmF* and *ccmL*. In *R. capsulatus*, a *ccmE* homolog has not been identified; if it exists, it is located in yet a different locus, because the genes upstream and downstream of *helABCD*, *ccl1/2*, and *cycH* are known (184, 189). The CcmE, CcmF, and CcmL proteins may interact as the subunits of a CCHL (see above). (iv) *ccmH* is either adjacent to *ccmEFL* or not linked to any other *ccm* genes (*R. capsulatus* and *Paracoccus denitrificans*). CycH may be an additional CCHL subunit. (v) The *E. coli ccmH* gene is special in that it codes for a fused protein (Fig. 9): its N-terminal portion is similar to CycL, and its C-terminal portion is similar to CycH. (vi) The position of *ccmG* is variable and reflects the phylogenetic relationships among different groups of bacteria: in alpha proteobacteria, it is linked to *ccmABCD*, whereas in gamma proteobacteria, it is downstream of *ccmF*.

Membrane topology of the *ccm* gene products. Most of the Ccm proteins, whose postulated functions are summarized in Table 5, appear to be membrane bound. Predictions for membrane topologies have been supported experimentally for different Ccm-like proteins in *B. japonicum* (86, 284–286), *R. capsulatus* (20, 21, 189), *P. fluorescens* (102), and *P. denitrificans* (252). CcmA is thought to be a cytoplasmically oriented, peripheral membrane protein associated in a $(CcmA)_2$-CcmB-CcmC complex with the membrane-integral CcmB and CcmC subunits of the ABC transporter (252) (Fig. 8). CcmC is thought to contain a functionally important periplasmic loop in which a conserved tryptophan-rich motif resides. Experimental support for this hypothesis comes from an active PhoA fusion inserted at amino acid 125 in the middle of the *P. fluorescens* CytA (102). The topology of CcmD in the membrane is unknown: CcmD homologs are small proteins of 52 to 69 amino acids with a hydrophobic N-terminal domain and a basic C-terminal domain (net positive charge of +3 to +5). However, the N-terminal domain does not resemble a typical signal sequence with a positive net charge preceding it (data not shown). One might therefore predict that CcmD is anchored in the membrane facing the cytoplasm. Indeed, C-terminal LacZ fusions to the *R. capsulatus* homologs of CcmA, CcmB, CcmC, and CcmD were active, supporting the predicted topologies of these proteins (113). CcmE is thought to be N-terminally attached to the membrane by a noncleaved signal sequence, and PhoA fusion analysis showed that the hydrophilic domain of the protein in *B. japonicum* is exposed to the periplasm (286). CcmF appears to be a polytopic membrane protein with at least 12 transmembrane helices. Following the positive inside rule (365), the tryptophan-rich segment would reside in the periplasm. Indeed, a PhoA fusion at position 392 of the *R. capsulatus* Ccl1 protein was active, indicating the periplasmic location of that segment, which is in accordance with a predicted periplasmic loop (21). The CcmG homologs of *R. capsulatus* and *B. japonicum* were shown to reside predominantly in the periplasm. PhoA fusion analysis of the *R. capsulatus* HelX expressed in *E. coli* suggested that it was a soluble protein (20), whereas the *B. japonicum* CycY was shown im-

TABLE 5. Cytochrome *c* biogenesis genes (*ccm* homologs) identified in bacteria

Gene in:												Putative function	References
E. coli	*H. influenzae*	*B. japonicum*	*S. meliloti*	*R. leguminosarum*	*R. etli*	*R. capsulatus*	*P. denitrificans*	*P. fluorescens* 09906	*P. fluorescens* ATCC 17400	*C. vinosum*			
ccmA	*ccmA*	*cycV*			*ccmA*	*helA*	*ccmA*					ABC transporter	21, 93, 277, 281, 252
ccmB	*ccmB*	*cycW*			*ccmB*	*helB*	*ccmB*					ABC transporter	21, 93, 277, 281, 252
ccmC	*ccmC*	*cycZ*				*helC*	*ccmC*	*helC*	*cytA*			ABC transporter	21, 93, 277, 281, 252, 337
ccmD	*ccmD*	*cycX*		*cycX*		*helD*	*ccmD*		*cytB*			maturase assembly	21, 93, 102, 252, 277, 281, 360, 388
ccmE	*ccmE*	*cycI*	*cycJ*	*cycJ*	*cycJ*			*cycJ*	*cytC*			CCHL subunit, chaperone	73, 93, 102, 178, 281, 286, 331, 388
ccmF	*ccmF*	*cycK*	*cycK*	*cycK*		*ccl1*		*cycK*	*cytD*		*ccmF*	CCHL catalytic subunit	21, 49, 73, 93, 102, 178, 281, 286, 388
ccmG	*ccmG*	*cycY*		*cycY*		*helX*	*ccmG*	*tipB*	*cytE*		*ccmG*	thioredoxin	20, 49, 86, 93, 102, 251, 277, 281, 360, 388
$ccmH_N$[a]	*ccmL*	*cycL*	*cycL*	*cycL*		*ccl2*		*cycL*	*cytF*			CCHL subunit, reducing?	21, 73, 93, 102, 178, 189, 281, 286, 331, 337, 388
$ccmH_C$[b]	*ccmH*	*cycH*	*cycH*	*cycH*	*ccmH*	*cycH*	*cycH*	*cycH*	*cytG*			CCHL subunit, specificity	73, 93, 102, 178, 189, 250, 281, 285, 331, 337, 388

[a] 5′-half of the gene, coding for the N-terminal domain of CcmH.
[b] 3′-half of the gene, coding for the C-terminal domain of CcmH.

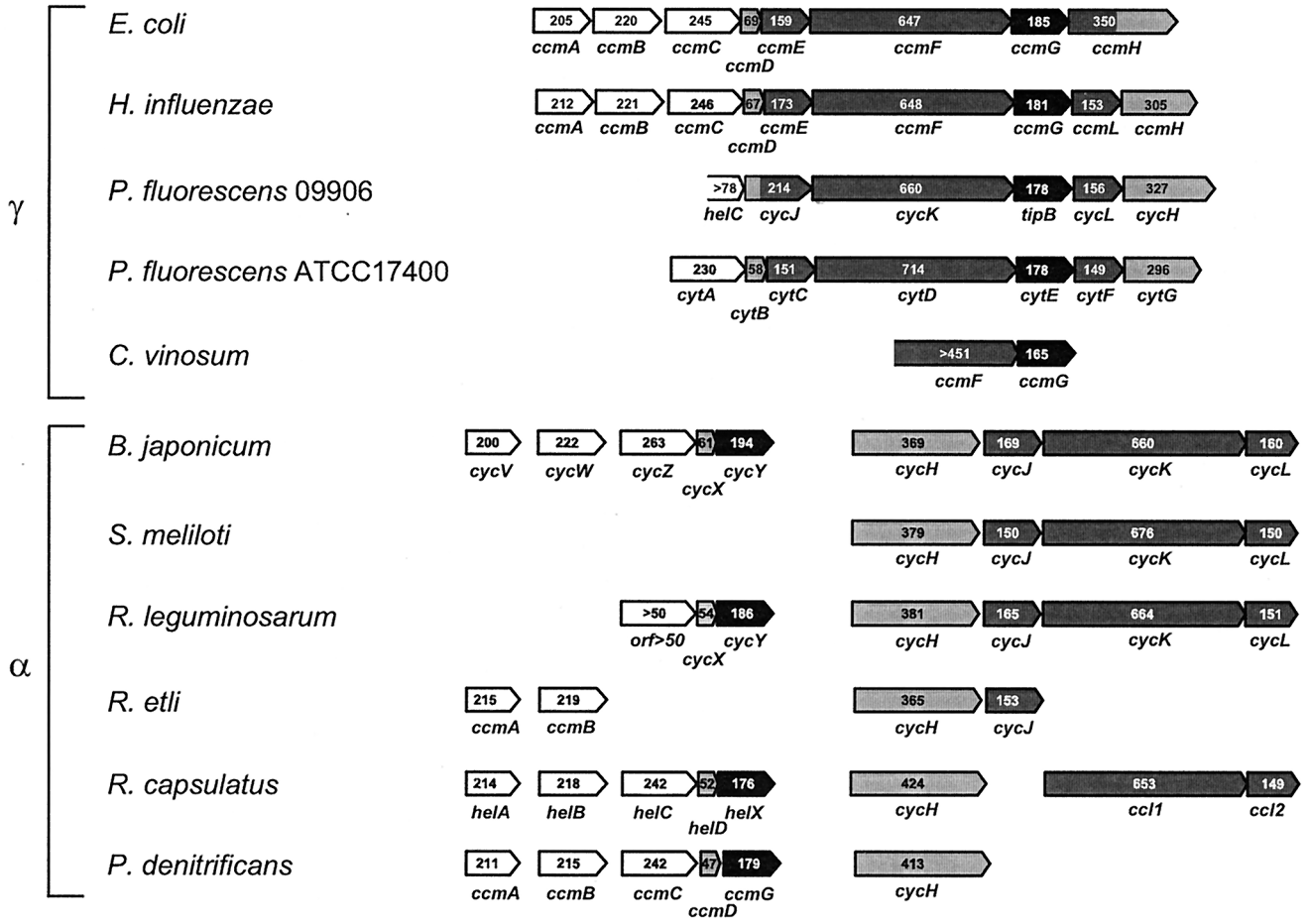

FIG. 12. Bacterial cytochrome *c* maturation genes. The *ccm* genes and their homologs from 10 different bacterial species are organized in one (gamma proteobacteria), two (rhizobia), or at least three (*Rhodobacter capsulatus* and *Paracoccus denitrificans*) gene clusters. For *Pseudomonas fluorescens*, from which *ccm* gene clusters of two different strains have been published, the gene cluster of strain ATCC 17400 is shown. Different groups of genes, i.e., genes coding for an ABC transporter, genes encoding putative CCHL subunits, genes coding for the thioredoxin-like protein, and the small gene with unknown function are indicated by different degrees of shading. The number of amino acids of the derived polypeptides is indicated for each gene. α and γ refers to the subgroups of proteobacteria.

munologically to be membrane bound, with a periplasmic orientation of its hydrophilic domain (86). The presumed membrane topology of CcmH (*E. coli*) was discussed above (Fig. 9). The topology of the corresponding CycH protein has been experimentally supported (102, 189, 285), as opposed to that of CycL, whose C-terminal domain has been postulated to be periplasmic due to the presence of an N-terminal signal sequence (286).

Maturase complex for cytochrome *c* biogenesis? It seems reasonable to suggest that CcmABC and CcmEFLH form oligomeric complexes for the translocation of a substrate, perhaps heme, and for cytochrome *c* heme ligation, respectively. If CcmG functions by keeping the heme binding site of apocytochrome *c* reduced before heme attachment occurs, it is likely to be in physical contact with the CCHL complex, and it may even be regarded as a CCHL subunit. The fact that all *ccm* genes are clustered in the gamma proteobacteria led to speculation that the *ccm* gene products may function in a "maturase" supercomplex, in which export of heme or another molecule required for cytochrome *c* maturation and the binding of heme to secreted apocytochrome occur in a coordinated way (337). A possible function for CcmD in such a supercomplex could be either to facilitate assembly of the oligomeric structure or to physically connect the ABC transporter and CCHL apparatus. Such roles have also been attributed to other small proteins with hydrophobic anchors that are found in multisubunit membrane complexes, for example SUIV of the aa_3- or *bo*-type oxidase or FixQ of the cbb_3-type oxidase (291, 380, 400). Figure 13 shows a model of how a cytochrome *c* maturase supercomplex might be assembled in the membrane.

The hypothesis that the *ccm* genes might code for subunits of a maturase supercomplex can be tested experimentally. For example, antibodies directed against *B. japonicum* CycJ (the

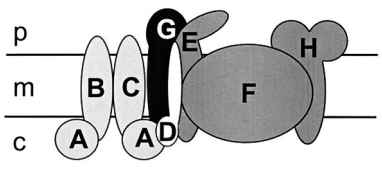

FIG. 13. A hypothetical maturase complex for cytochrome *c* biogenesis. The subunits are drawn with the predicted or established orientations in the cytoplasmic membrane. The different types of functions to which the subunits are assigned (Table 5) are represented by different shadings. The letters correlate with the Ccm protein nomenclature used in *E. coli*. CcmH is represented as a bifunctional subunit with two periplasmic domains, one of them corresponding to rhizobial CycL. c, cytoplasm; m, cytoplasmic membrane; p, periplasm.

CcmE homolog) did not react with membrane proteins prepared from ABC transporter- or thioredoxin-defective mutants, although the corresponding mutations were not polar on the expression of the *cycJ* gene (284). This result suggests that the presence of the ABC transporter and the thioredoxin is essential for stable maintenance of CycJ. To test all *ccm* gene products for a possible involvement in supercomplex formation, nonpolar mutations in each *ccm* gene and antibodies specifically recognizing the respective gene products should now be used in a combinatorial, systematic approach. In addition, cross-linking experiments are expected to help define the precise interactions between the individual subunits of such a putative complex.

Cytochrome *c* maturation in gram-positive bacteria. Most of the *ccm* genes identified so far from either mutant analysis or gene sequences are from gram-negative bacteria. However, in *Bacillus subtilis*, genes with putative roles in cytochrome *c* maturation have also been identified. The ResA and ResC gene products have low but significant sequence similarities to certain domains of proteins that are known to be involved in cytochrome *c* maturation. ResA has a thioredoxin-like sequence WCEPC (Fig. 10), and ResC has a tryptophan-rich WGXXWXWD motif that is found in the CcmC and CcmF homologs (327). The construction of null mutants defective in *resA* and *resC* failed, suggesting that these genes may be essential for survival. In a screening procedure for tetramethyl-*p*-phenylenediamine-negative mutants, the *ccdA* gene was identified and shown to be required for the biosynthesis of *c*-type cytochromes. The *ccdA* gene product is a membrane-integral protein that shares sequence similarity with the central part of DipZ/DsbD. The sequence CXXC, which is common to both proteins, differs from the thioredoxin motif WCXXC, which is also present in the C-terminal part of DipZ. The cysteines in CdcA have been proposed either to be functionally active in a redox reaction or to serve as ligands to a prosthetic group (302). Since *B. subtilis* synthesizes a variety of different *c*-type cytochromes but other *ccm*-like genes do not seem to be present, it may be speculated that gram-positive bacteria use a different mechanism to synthesize holocytochrome *c*.

Maturation of the bc_1 Complex

A tentative model for maturation of bacterial bc_1 complexes is proposed in Fig. 14. It predicts that formation of holocytochrome c_1 is an early requirement for the assembly of a three-subunit complex. First, cytochromes *b* and c_1 form a protease-resistant primary complex, which then permits association of the Rieske FeS protein with the complex. Although there is no direct evidence for such a pathway, the model presented in Fig. 14 is in agreement with all experimental findings discussed in the following paragraphs.

Mutations in the Rieske protein subunit had no consequence on the assembly of cytochromes *b* and c_1 in the membrane (68, 359). Based on site-directed mutagenesis and structural analysis, it was proposed that the stability of the Rieske protein depends on the formation of the FeS cluster and probably also on the formation of an intramolecular disulfide bond (68, 159).

Deletion mutations in the cytochrome c_1 gene caused degradation of the Rieske protein and of cytochrome *b* (111, 119, 343). Covalent attachment of heme C to cytochrome c_1 appears to be an early and essential step of bc_1 complex biogenesis: heme binding-site-defective mutants or mutants with mutations of cytochrome *c* biogenesis genes had no immunologically detectable bc_1-polypeptides (277, 338). However, residue M183 in the *Rhodobacter capsulatus* cytochrome c_1 could

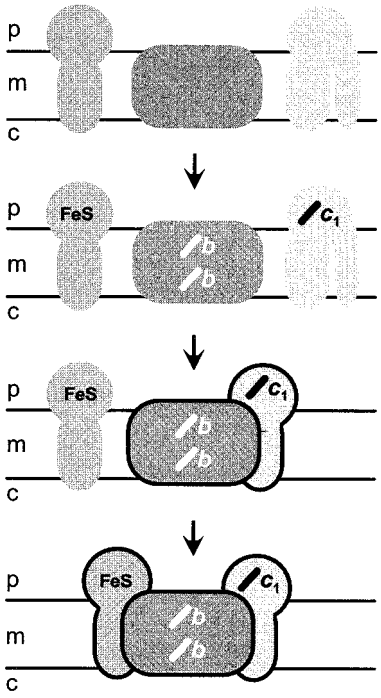

FIG. 14. Model of a pathway for maturation of the bc_1 complex. On the left, the Rieske SU is shown; in the middle, cytochrome *b* is shown; and on the right, cytochrome c_1, which is synthesized as a precursor, is shown. Subunits in framed (sub)complexes are detected in membranes as stable polypeptides. Cofactors are as in Fig. 4. c, cytoplasm; m, cytoplasmic membrane; p, periplasm.

be altered without causing a bc_1 assembly defect but was essential for the biological activity of the complex, probably because it is the sixth ligand of the heme C iron (119). The adoption of the proper tertiary structure of cytochrome c_1 occurs independently of complex formation and may be a late step in the maturation of an active bc_1 complex. Deletion of the hydrophobic, C-terminal membrane anchor from the *R. sphaeroides* cytochrome c_1 resulted in small amounts of soluble holocytochrome c_1 in the periplasm, indicating that heme ligation occurs before or independently of C-terminal membrane insertion (182). This deletion mutant was also severely disturbed in cytochrome *b* and FeS protein assembly. Cleavage of the signal sequence was found not to be essential for maturation of the *B. japonicum* cytochrome bc_1 complex, since mutational alteration or deletion of the signal peptidase cleavage site led to an active complex consisting of a Rieske subunit and a cytochrome bc_1 polyprotein (338, 339). Thus, cleavage of the cytochrome c_1 signal sequence takes place either after (Fig. 14) or independently from heme C attachment.

Unfortunately, no bacterial mutant lacking solely the gene for cytochrome *b* has been described in an attempt to address whether cytochrome *b* is essential for the assembly and/or stability of cytochrome c_1 and the Rieske protein. A kanamycin resistance cassette insertion in *R. capsulatus petB* was constructed; however, its orientation relative to the *pet* operon, which may affect the expression of the downstream *petC* gene (Fig. 5), is not known, and the presence of cytochrome c_1 and Rieske protein in the membrane has not been tested. The best mutant available is a Δ*fbcFB*::*kan* deletion-insertion in which *fbcC* encoding cytochrome c_1 should be expressed from the promoter in the kanamycin resistance cassette. This mutant lacks all three subunits of the complex, as determined immunologically (393). Since the Rieske protein is not essential for

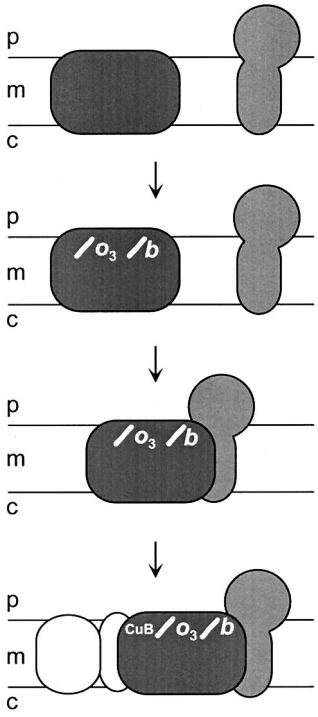

FIG. 15. Model for maturation of the bo_3-type quinol oxidase from *E. coli*. SUI (left) and SUII (right) are drawn with dark shading; SUIII and IV seem to be involved, if at all, in a late step of assembly. The framing of the subunits indicates their stability in the membrane. Cofactors are as in Fig. 4. c, cytoplasm; m, cytoplasmic membrane; p, periplasm.

cytochrome c_1 assembly, one might conclude that cytochrome *b* is required and that cytochromes *b* and c_1 form a primary subcomplex during maturation. Construction and analysis of nonpolar cytochrome *b* mutants would be desirable to clarify this point. Most mutational alterations of heme B binding histidines of cytochrome *b* have been shown to result in a normally assembled but inactive bc_1 complex (37). The noncovalent binding of heme does not seem to be needed for oligomerization. The model presented in Fig. 14 shows an assembly pathway for cytochrome bc_1 complex formation in which heme B is inserted early, i.e., as soon as the cytochrome *b* polypeptide is folded in the membrane. It is also possible that heme B incorporation is the last step of maturation. Based on current knowledge, it cannot be decided whether heme B insertion occurs at a specific step during maturation or is independent of oligomerization.

Maturation of Terminal Oxidases

***E. coli* cytochrome bo_3.** *E. coli* cytochrome bo_3 is one of the best-studied oxidases with respect to site-directed mutagenesis: characterizations of a large number of different mutants, especially of SUI, have provided some insight into how protein oligomerization and cofactor insertion might be coordinated.

Site-directed mutagenesis has been applied extensively to the characterization of the different cofactor ligands (42, 43, 45, 144, 191, 215, 334, 354). These studies also revealed that mutations of heme B ligands lead to defective Cu_B incorporation by causing a lower affinity of the oxidase for the metal cofactor (215). Mutants with mutations in either of two Cu_B ligands (H333 and H334) contain normal amounts of heme B and heme O but lack Cu_B (191, 215), suggesting that heme insertion occurs before or independently of Cu_B binding (Fig. 15). Formation of a functional heme B-Cu_B binuclear center requires the H284 Cu_B ligand as well as the H419 heme O ligand (215). Heme B ligand mutants (H106 and H421) show decreased levels of CO-reactive heme O (215).

Both catalytic subunits of the bo_3-type oxidase can insert autonomously into the membrane and can persist (234). The insertion of the low- and high-spin hemes B and O into SUI occurs also in the absence of SUII, whereas the insertion of Cu_B has not been investigated. The role of SUIII in the assembly or function of the bo_3 oxidase is unknown. Recently, a study on the function of SUIV has provided evidence that this subunit is not required for stability of the catalytic enzyme complex, although it may be needed for its biogenesis by assisting copper insertion (291). Once the enzyme and its cofactors are assembled, SUIV seems to be dispensable, because it can be removed in vitro without a loss of enzymatic activity (291). A model of cytochrome *bo* biogenesis that can explain the available experimental findings is shown in Fig. 15.

***E. coli* cytochrome *bd*.** A relatively straightforward model can be postulated for the biosynthesis of the *E. coli bd*-type quinol oxidase (Fig. 16). The two subunits can insert into the membrane in the absence of heme cofactors (238). SUI, when expressed in the absence of SUII, contains cytochrome b_{558}. On the other hand, SUII has never been observed in the absence of SUI (121), which may be due to a polarity effect of Δ*cydA* mutations in the *cydAB* operon. Site-directed mutagenesis of heme-liganding residues in SUI allows membrane assembly of both subunits (87). Mutation of H19, a ligand of the *bd* center, results in an oxidase with only heme b_{558}. Mutation of the heme b_{558} ligand H186 leads to specific elimination of cytochrome b_{558}, but not of cytochrome *bd*. These results suggest that insertion of the low-spin heme B and of the heme BD binuclear center occur independently from each other, but formation of the binuclear center requires the presence of both

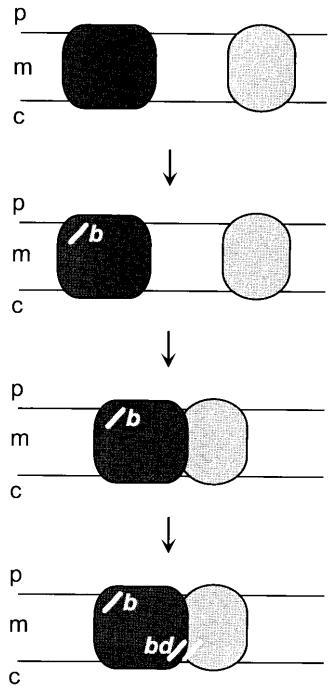

FIG. 16. Model for maturation of the *bd*-type quinol oxidase from *E. coli*. SUI (left) and SUII (right) are drawn with dark shading and are framed to indicate their stability in the membrane. Cofactors are as in Fig. 4. c, cytoplasm; m, cytoplasmic membrane; p, periplasm.

subunits. It has been suggested that SUI may interact with SUII to form an interface in which the heme BD cofactors bind (246).

Cytochrome aa_3. Biogenesis of the aa_3-type cytochrome *c* oxidase has not been studied systematically. It is not possible to arrive at a pathway for aa_3 oxidase maturation by combining pieces of information: too few questions have been addressed in this context, or there are uncertainties about how the data may be interpreted.

The aa_3-type oxidase is best studied in *Paracoccus denitrificans*. Deletion of SUI (Δ*ctaD1* Δ*ctaD2*) results in the loss of spectroscopically detectable oxidase; however, the presence or absence of the SUI and SUII polypeptides in membranes has not been analyzed (70). On the other hand, deletion of SUII (Δ*ctaC*) causes the loss of SUI (318). In this case, it seems likely that the Δ*ctaC* mutation has a polar effect on the downstream genes (Fig. 6). When this mutant was complemented with plasmid-borne *ctaC*, SUII was immunologically detectable, but SUI, which is not encoded in the same operon, was absent. This indicated that genes downstream of *ctaC* were involved in SUI assembly. When the mutant was complemented with *ctaCB*, both subunits were assembled, but the heme cofactors were not detected spectroscopically. The *ctaB* gene is known to encode a heme O synthase. Thus, the presence of SUII in the membranes appears to depend on heme O synthesis. This makes sense in view of the fact that heme O is a heme A precursor and suggests that heme A incorporation may be required for SUI assembly. Why, then, does complementation with *ctaBC* not lead to a spectroscopically detectable complex? This discrepancy can be explained by suggesting that another downstream gene, possibly *ctaG*, is necessary for heme A incorporation and that the presence of heme A may indirectly regulate either expression of SUI or its insertion into the membrane. Clearly, similar investigations should be done with nonpolar mutants. A model for the assembly of the *P. denitrificans* aa_3-type oxidase with respect to the role of SUIII, in which SUIII is regarded as a catalyst of assembly at a late stage of maturation, when the cofactors are already inserted and a SUI/SUII subcomplex is assembled, has been proposed (128). This view is based on the finding that all metal centers of the oxidase can be formed in the absence of SUIII, but at the same time a population of defectively assembled oxidases can be detected (128).

A *Rhodobacter sphaeroides ctaD* mutant was shown to lack spectroscopically detectable aa_3-type cytochrome, but the presence of SUII has not been investigated (307). Site-directed mutagenesis of heme- and Cu_B-liganding histidines resulted in the formation of inactive oxidases, in which SUI was present in the membranes (308). As some of these mutant oxidases could be purified, it must be assumed that SUII is also present; however, this has not been reported explicitly. At least SUI can be assembled into the membrane in the absence of heme A or Cu_B cofactors, suggesting that cofactor incorporation occurs after membrane insertion.

The *Bradyrhizobium japonicum* cytochrome aa_3 oxidase appears to behave differently regarding its assembly: SUI is essential for membrane association of SUII but the reverse is not the case (195, 287). In contrast, the *Bacillus subtilis* SUII, which is fused to cytochrome *c*, can be expressed and incorporated into the membrane as a heme C-containing protein in the absence of COI; however, it has not been shown whether the Cu_A center is present in this protein (358).

cbb_3-type oxidase. A systematic analysis of cytochrome complex formation has been done with the *B. japonicum* cbb_3-type oxidase (Fig. 17). Nonpolar in-frame deletion mutations in each gene of the *fixNOQP* operon were constructed, and the mutants were assayed for activity and complex formation (400). The small *fixQ* gene product was required neither for the function of the oxidase nor for its assembly. Deletion of FixP did not influence the stability of the other subunits, suggesting that a FixNO subcomplex can be formed. This is corroborated by the isolation of a two-subunit *cb*-type oxidase complex from *P. denitrificans*, consisting of the FixN homolog CcoN and the FixO homolog CcoO and containing spectroscopically detectable *b*- and *c*-type cytochromes (71). As this subcomplex is active both in cytochrome *c* reduction and in proton pumping, it can be assumed that Cu_B is also inserted at this stage. In a proposed last step, FixP associates with the FixNO subcomplex to form the mature cbb_3-type oxidase, consisting of the subunits FixN, FixO, and FixP. Deletion of either the *fixN* or *fixO* gene leads to rapid degradation of all three subunits, confirming that the FixN and FixO polypeptides are essential at an early stage of cbb_3 oxidase biogenesis (400).

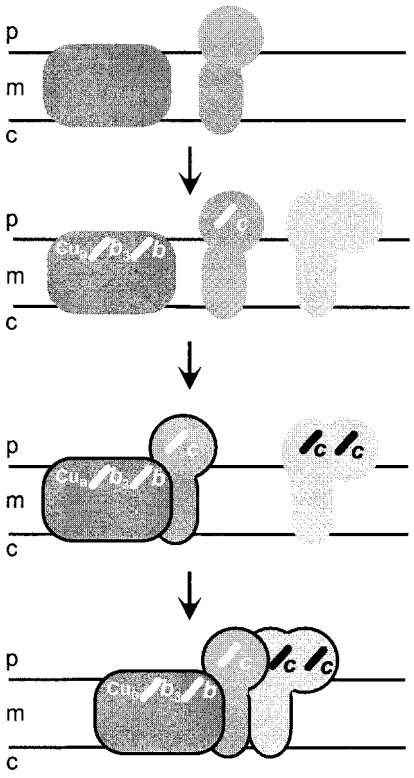

FIG. 17. Model for maturation of the cbb_3-type heme-copper oxidase (FixNOP). SUI (FixN) is shown on the left, SUII (FixO) is shown in the middle, and SUIII (FixP) is shown on the right. Subunits in framed (sub)complexes are detected in membranes as stable polypeptides. Cofactors are indicated as in Fig. 4. c, cytoplasm; m, cytoplasmic membrane; p, periplasm.

Another important question is at which stage in the pathway the heme cofactors are inserted into the apoproteins. It has become clear that insertion of heme C is critical for the stability of the subunits because mutants with altered heme binding sites in either FixO or FixP have an equally defective phenotype as the respective gene deletion mutants (399). In the diheme cytochrome *c* FixP, each of the two heme binding sites was mutated separately to test whether such mutants could produce monoheme FixP protein; however, this was not the case, since FixP was unstable in these mutants. Also, in mutants defective in cytochrome *c* biogenesis, in which all three genes, *fixN*, *fixO*, and *fixP*, are expressed normally under

microaerobic conditions, no apocytochrome polypeptides can be detected immunologically (86). From these results, it can be concluded that covalent heme binding takes place before oligomerization occurs, as suggested in the model shown in Fig. 17.

CONCLUSIONS AND FUTURE PERSPECTIVES

Review of the known details of cytochrome biogenesis reveals a complex process. While Fig. 1 illustrates important aspects of posttranslational cytochrome formation, it also indicates that no generally applicable conclusions about an order of events or the causality between different steps can be proposed. Cytochrome biogenesis does not appear to follow a universal maturation pathway; i.e., the way subunits and cofactors are assembled in complexes differs among the disparate types of oxidoreductases.

Few systematic studies that address directly the question of cytochrome complex formation have been undertaken. Many of the results that have contributed to a better understanding of cytochrome complex formation have emerged as by-products of studies on the composition and function of respiratory complexes. However, many tools to perform the required experiments are now available. For example, there are various mutants of structural genes encoding subunits of cytochrome complexes that could be analyzed for the presence or absence of other subunits. If different laboratories would share mutant strains and subunit-specific antibodies, much more information on cytochrome biogenesis might be obtained in such a collaborative effort.

During the maturation of several respiratory cytochrome complexes, heme appears to be incorporated relatively early, i.e., before or independently of subunit assembly. This is a common feature in the biogenesis of multisubunit complexes such as the bc_1 complex or the heme-copper oxidases, and it has proven to be particularly important in the case of the heme C-containing subunits. It is therefore possible that covalent attachment of heme triggers the correct folding of the holoprotein subunit, thereby protecting it from proteolysis and making it accessible for oligomerization.

In cases where immature subunits are unstable, mutational blocks in the maturation pathway lead to "all-or-nothing" phenotypes, if one analyzes proteins isolated from cells under steady-state conditions. It is often difficult to decide whether a protein reaches a certain level of maturation and is then degraded or whether maturation does not progress at all. Thus, maturation pathways proposed for different cytochrome complexes (Fig. 14 to 17) must be interpreted carefully: they represent one of several possible routes which lead to certain experimentally detectable intermediates. However, the temporal order of, and the causality between, the different steps of maturation are not always known. Pulse-chase kinetic experiments would help to resolve these uncertainties.

The genetic analysis of various cytochrome-containing oxidoreductases is progressing rapidly in many different laboratories and will yield important insights into cytochrome complex assembly. Unfortunately, this approach is complicated by the fact that many of the genes of interest are organized in operons, so that polar effects in the mutants often cannot be ruled out. A more appropriate, although technically more difficult and time-consuming, way of avoiding this problem is the construction of nonpolar translationally in-frame deletions. This has been proven to be an efficient way to analyze biogenesis of the cbb_3-type oxidase (400).

So far, Western blot analysis has been used mostly to demonstrate the presence or absence of specific subunits. In contrast, pulse-chase experiments would allow the detection of transiently formed subcomplexes that are not stable under steady-state conditions. To successfully apply this method, it is best if antibodies that recognize and precipitate precursor forms of immature cytochromes are available.

The best-studied cytochrome biogenesis pathway known to date is that for *c*-type cytochromes. Type *c* cytochromes seem to be synthesized in a linear pathway for which up to 10 specific steps can be predicted. It is now rather well established where these steps occur within the bacterial cell. Moreover, 16 different bacterial genes (*ccmABCDEFGHL*, *ccdA*, *dsbA*, *dsbD*, *cydD*, *secA*, *secY*, and *lepB*) have been shown by mutant analysis to be involved in cytochrome *c* maturation. The hypothetical assignment of specific biogenetic functions to the predicted gene products has led to a model of a cytochrome *c* maturation pathway. However, since no biochemical assays are available for many of these postulated reactions, the gap between the predicted gene products and their actual role in maturation has not yet been filled, and parts of the model remain completely speculative. True progress in the field of cytochrome *c* biogenesis will be made only when the functions of the postulated proteins can be shown in vitro. A first step in this direction has been taken with the thioredoxin-like protein, which has been purified and whose redox potential clearly supports its role as a periplasmic reductant (86). It will be interesting to identify the cognate reaction partners of this protein.

Cytochrome biogenesis has become an attractive research topic, and a lot of useful information accumulates each year, expanding our knowledge of this process. Nevertheless, we are still far from being able to assemble multisubunit cytochrome complexes in cell-free systems, which is probably one of the ultimate goals of research in this field. The challenges to ask and answer important questions in connection with cytochrome assembly are sufficiently manifold as to indicate that we are just beginning to comprehend this complex biological problem.

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REFERENCES

1. **Abou-Jaoudé, A., M.-C. Pascal, and M. Chippaux.** 1979. Formate-nitrite reduction in *Escherichia coli* K-12. 2. Identification of components involved in electron transfer. Eur. J. Biochem. **95:**315–321.
2. **Aguilar, G. R., and M. Soberon.** 1996. Cloning and sequence analysis of the *Rhizobium etli ccmA* and *ccmB* genes involved in *c*-type cytochrome biogenesis. Gene **182:**129–135.
3. **Akagi, J. N.** 1995. Respiratory sulfate reduction, p. 89–111. *In* L. L. Barton (ed.), Sulfate-reducing bacteria. Plenum Press, New York, N.Y.
4. **Alami, N., and P. C. Hallenbeck.** 1995. Cloning and characterization of a gene cluster, *phsBCDEF*, necessary for the production of hydrogen sulfide from thiosulfate by *Salmonella typhimurium*. Gene **156:**53–57.
5. **Alefounder, P. R., and S. J. Ferguson.** 1980. The location of dissimilatory nitrite reductase and the control of dissimilatory nitrate reductase by oxygen. Biochem. J. **192:**231–240.
6. **Anthony, C.** 1992. The *c*-type cytochromes of methylotrophic bacteria. Biochim. Biophys. Acta **1099:**1–15.
7. **Babcock, G. T., and M. Wikström.** 1992. Oxygen activation and the conservation of energy in cell respiration. Nature **356:**301–303.
8. **Bai, Y., T. R. Sosnick, L. Mayne, and S. W. Englander.** 1995. Protein folding intermediates: native-state hydrogen exchange. Science **269:**192–197.

9. **Ballard, A. L., and S. J. Ferguson.** 1988. Respiratory nitrate reductase from *Paracoccus denitrificans*. Eur. J. Biochem. **174:**207–212.
10. **Bardwell, J. C. A.** 1994. Building bridges: disulphide bond formation in the cell. Mol. Microbiol. **14:**199–205.
11. **Bardwell, J. C. A., and J. Beckwith.** 1993. The bonds that tie: catalyzed disulfide bond formation. Cell **74:**769–771.
12. **Bardwell, J. C. A., K. McGovern, and J. Beckwith.** 1991. Identification of a protein required for disulphide bond formation *in vivo*. Cell **67:**581–589.
13. **Barker, P. D., J. C. Ferrer, M. Mylrajan, T. M. Loehr, R. Feng, Y. Konishi, W. D. Funk, R. T. A. MacGillivray, and A. G. Mauk.** 1993. Transmutation of a heme protein. Proc. Natl. Acad. Sci. USA **90:**6542–6546.
14. **Barker, P. D., E. P. Nerou, S. M. V. Freund, and M. Fearnley.** 1995. Conversion of cytochrome b_{562} to *c*-type cytochromes. Biochemistry **34:** 15191–15203.
15. **Barrett, E. L., and M. A. Clark.** 1987. Tetrathionate reduction and production of hydrogen sulfide from thiosulfate. Microbiol. Rev. **51:**192–205.
16. **Basile, G., C. Di Bello, and H. Taniuchi.** 1980. Formation of an Iso-1-cytochrome *c*-like species containing a covalently bonded heme group from the apoprotein by a yeast cell-free system in the presence of hemin. J. Biol. Chem. **255:**7181–7191.
17. **Beale, S. I.** 1993. Biosynthesis of cyanobacterial tetrapyrrole pigments: hemes, chlorophylls, and phycobilins, p. 519–558. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
18. **Beale, S. I.** 1996. Biosynthesis of hemes, p. 731–748. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
19. **Bebbington, K. J., and H. D. Williams.** 1993. Investigation of the role of the *cydD* gene product in production of a functional cytochrome *d* oxidase in *Escherichia coli*. FEMS Microbiol. Lett. **112:**19–24.
20. **Beckman, D. L., and R. G. Kranz.** 1993. Cytochromes *c* biogenesis in a photosynthetic bacterium requires a periplasmic thioredoxin-like protein. Proc. Natl. Acad. Sci. USA **90:**2179–2183.
21. **Beckman, D. L., D. R. Trawick, and R. G. Kranz.** 1992. Bacterial cytochromes *c* biogenesis. Genes Dev. **6:**268–283.
22. **Berben, G.** 1996. *Nitrobacter winogradskyi* cytochrome *c* oxidase genes are organized in a repeated gene cluster. Antonie Leeuwenhoek **69:**305–315.
23. **Berg, B. L., J. Li, J. Heider, and V. Stewart.** 1991. Nitrate-inducible formate dehydrogenase in *Escherichia coli* K-12. I. Nucleotide sequence of the *fdnGHI* operon and evidence that opal (UGA) encodes selenocysteine. J. Biol. Chem. **266:**22380–22385.
24. **Berks, B. C., M. D. Page, D. J. Richardson, A. Reilly, A. Cavill, F. Outen, and S. J. Ferguson.** 1995. Sequence analysis of subunits of the membrane-bound nitrate reductase from a denitrifying bacterium: the integral membrane subunit provides a prototype for the dihaem electron-carrying arm of a redox loop. Mol. Microbiol. **15:**319–331.
25. **Berks, B. C., D. J. Richardson, A. Reilly, A. C. Willis, and S. J. Ferguson.** 1995. The *napEDABC* gene cluster encoding the periplasmic nitrate reductase system of *Thiosphaera pantotropha*. Biochem. J. **309:**983–992.
26. **Berks, B. C., D. J. Richardson, C. Robinson, A. Reilly, R. T. Aplin, and S. J. Ferguson.** 1994. Purification and characterization of the periplasmic nitrate reductase from *Thiosphaera pantotropha*. Eur. J. Biochem. **220:**117–124.
27. **Berry, E. A., and B. L. Trumpower.** 1985. Isolation of ubiquinol oxidase from *Paracoccus denitrificans* and resolution into cytochrome bc_1 and cytochrome *c*-aa_3 complexes. J. Biol. Chem. **260:**2458–2467.
28. **Blasco, F., J. Pommier, V. Augier, M. Chippaux, and G. Giordano.** 1992. Involvement of the *narJ* or *narW* gene product in the formation of active nitrate reductase in *Escherichia coli*. Mol. Microbiol. **6:**221–230.
29. **Böck, A., and G. Sawers.** 1996. Fermentation, p. 262–282. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
30. **Bokranz, M., M. Gutmann, C. Körtner, E. Kojro, F. Fahrenholz, F. Lauterbach, and A. Kröger.** 1991. Cloning and nucleotide sequence of the structural genes encoding the formate dehydrogenase of *Wolinella succinogenes*. Arch. Microbiol. **156:**119–128.
31. **Bonnefoy, V., and J. A. DeMoss.** 1994. Nitrate reductases in *Escherichia coli*. Antonie Leeuwenhoek **66:**47–56.
32. **Bott, M., M. Bolliger, and H. Hennecke.** 1990. Genetic analysis of the cytochrome *c*-aa_3 branch of the *Bradyrhizobium japonicum* respiratory chain. Mol. Microbiol. **4:**2147–2157.
33. **Bott, M., D. Ritz, and H. Hennecke.** 1991. The *Bradyrhizobium japonicum cycM* gene encodes a membrane-anchored homolog of mitochondrial cytochrome *c*. J. Bacteriol. **173:**6766–6772.
34. **Brand, S. N., X. Tan, and W. R. Widger.** 1992. Cloning and sequencing of the *petBD* operon from the cyanobacterium *Synechococcus* sp. PCC 7002. Plant Mol. Biol. **20:**481–491.
35. **Brandner, J. P., and T. J. Donohue.** 1994. The *Rhodobacter sphaeroides* cytochrome c_2 signal peptide is not necessary for export and heme attachment. J. Bacteriol. **176:**602–609.
36. **Brandner, J. P., E. V. Stabb, R. Temme, and T. J. Donohue.** 1991. Regions of *Rhodobacter sphaeroides* cytochrome c_2 required for export, heme attachment, and function. J. Bacteriol. **173:**3958–3965.
37. **Brasseur, G., A. Saribas, and F. Daldal.** 1996. A compilation of mutations located in the cytochrome *b* subunit of the bacterial and mitochondrial bc_1 complex. Biochim. Biophys. Acta **1275:**61–69.
38. **Brittain, T., R. Blackmore, C. Greenwood, and A. J. Thomson.** 1992. Bacterial nitrite-reducing enzymes. Eur. J. Biochem. **209:**793–802.
39. **Brown, S., A. J. Moody, R. Mitchell, and P. R. Rich.** 1993. Binuclear centre structure of terminal protonmotive oxidases. FEBS Lett. **316:**216–223.
40. **Buggy, J., and C. E. Bauer.** 1995. Cloning and characterization of *senC*, a gene involved in both aerobic respiration and photosynthesis gene expression in *Rhodobacter capsulatus*. J. Bacteriol. **177:**6958–6965.
41. **Buse, G., S. Hensel, and J. A. Fee.** 1989. Evidence for cytochrome oxidase subunit I and a cytochrome *c*-subunit II fused protein in the cytochrome c_1aa_3 of *Thermus thermophilus*. Eur. J. Biochem. **181:**261–268.
42. **Calhoun, M., J. J. Hill, L. J. Lemieux, W. J. Ingledew, J. O. Alben, and R. B. Gennis.** 1993. Site-directed mutants of the cytochrome *bo* ubiquinol oxidase of *Escherichia coli*: amino acid substitutions for two histidines that are putative Cu_B ligands. Biochemistry **32:**11524–11529.
43. **Calhoun, M., L. J. Lemieux, J. A. García-Horsman, J. W. Thomas, J. O. Alben, and R. B. Gennis.** 1995. The highly conserved methionine of subunit I of the heme-copper oxidases is not at the heme-copper dinuclear center: mutagenesis of M110 in subunit I of cytochrome bo_3-type ubiquinol oxidase from *Escherichia coli*. FEBS Lett. **368:**523–525.
44. **Calhoun, M., J. W. Thomas, and R. B. Gennis.** 1994. The cytochrome oxidase superfamily of redox-driven proton pumps. Trends Biochem. Sci. **19:**325–330.
45. **Calhoun, M. W., J. W. Thomas, J. J. Hill, J. P. Hosler, J. P. Shapleigh, M. M. Tecklenburg, S. Ferguson-Miller, G. T. Babcock, J. O. Alben, and R. B. Gennis.** 1993. Identity of the axial ligand of the high-spin heme in cytochrome oxidase: spectroscopic characterization of mutants in the *bo*-type oxidase of *Escherichia coli* and the aa_3-type oxidase of *Rhodobacter sphaeroides*. Biochemistry **32:**10905–10911.
46. **Cao, J., J. Hosler, J. Shapleigh, A. Revzin, and S. Ferguson-Miller.** 1992. Cytochrome aa_3 of *Rhodobacter sphaeroides* as a model for mitochondrial cytochrome *c* oxidase. J. Biol. Chem. **267:**24273–24278.
47. **Cao, J., J. Shapleigh, R. B. Gennis, A. Revzin, and S. Ferguson-Miller.** 1991. The gene encoding cytochrome *c* oxidase subunit II from *Rhodobacter sphaeroides*: comparison of the deduced amino acid sequence with sequences of corresponding peptides from other species. Gene **101:**133–137.
48. **Chen, R., and U. Henning.** 1996. A periplasmic protein (Skp) of *Escherichia coli* selectively binds a class of outer membrane proteins. Mol. Microbiol. **19:**1287–1294.
49. **Chen, Y. L., and D. B. Knaff.** 1996. Partial *ccmF* and complete *ccmG* sequences of *Chromatium vinosum*. GenBank accession no. L78437.
50. **Chepuri, V., and R. B. Gennis.** 1990. The use of gene fusions to determine the topology of all of the subunits of the cytochrome *o* terminal oxidase complex of *Escherichia coli*. J. Biol. Chem. **265:**12978–12986.
51. **Chepuri, V., L. J. Lemieux, D. C.-T. Au, and R. B. Gennis.** 1990. The sequence of the *cyo* operon indicates substantial structural similarities between the cytochrome *o* ubiquinol oxidase of *Escherichia coli* and the aa_3-type family of the cytochrome *c* oxidases. J. Biol. Chem. **265:**11185–11192.
52. **Claiborne, A., and I. Fridovich.** 1979. Purification of the *o*-dianisidine peroxidase from *Escherichia coli* B. J. Biol. Chem. **254:**4245–4252.
53. **Claiborne, A., D. P. Malinowski, and I. Fridovich.** 1979. Purification and characterization of hydroperoxidase II of *Escherichia coli* B. J. Biol. Chem. **254:**11664–11668.
54. **Cole, J.** 1996. Nitrate reduction to ammonia by enteric bacteria: redundancy, or a strategy for survival during oxygen starvation? FEMS Microbiol. Lett. **136:**1–11.
55. **Cope, L. D., S. E. Thomas, J. L. Latimer, C. A. Slaughter, U. Müller-Eberhard, and E. J. Hansen.** 1994. The 100 kDa haem:haemopexin-binding protein of *Haemophilus influenzae*: structure and localization. Mol. Microbiol. **13:**863–873.
56. **Cope, L. D., R. Yogev, U. Müller-Eberhard, and E. J. Hansen.** 1995. A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. J. Bacteriol. **177:**2644–2653.
57. **Crane, B. R., L. W. Siegel, and E. D. Getzoff.** 1995. Sulfite reductase structure at 1.6A: evolution and catalysis for reduction of inorganic anions. Science **270:**59–67.
58. **Creusot, F., J. Verdière, M. Gaisne, and P. P. Slonimski.** 1988. CYP1 (HAP1) regulator of oxygen-dependent gene expression in yeast. I. Overall organization of the protein sequence displays several novel structural domains. J. Mol. Biol. **204:**263–267.
59. **Crooke, H., and J. Cole.** 1995. The biogenesis of *c*-type cytochromes in *Escherichia coli* requires a membrane-bound protein, DipZ, with a protein disulphide isomerase-like domain. Mol. Microbiol. **15:**1139–1150.
60. **Dailey, F. E., and H. C. Berg.** 1993. Mutants in disulfide bond formation

that disrupt flagellar assembly in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **90:**1043–1047.
61. **Dailey, H. A.** 1990. Biosynthesis of heme and chlorophylls. McGraw-Hill Publishing Co., New York, N.Y.
62. **Dailey, T. A., P. Meissner, and H. A. Dailey.** 1994. Expression of a cloned protoporphyrinogen oxidase. J. Biol. Chem. **269:**813–815.
63. **Dalbey, R. E., and G. von Heijne.** 1992. Signal peptidases in prokaryotes and eukaryotes. Trends Biochem. Sci. **17:**474–478.
64. **Darlison, M. G., and J. R. Guest.** 1984. Nucleotide sequence encoding the iron-sulphur protein subunit of the succinate dehydrogenase of *Escherichia coli*. Biochem. J. **223:**507–517.
65. **Darwin, A., H. Hussain, L. Griffiths, J. Grove, Y. Sambongi, S. Busby, and J. Cole.** 1993. Regulation and sequence of the structural gene for cytochrome c_{552} from *Escherichia coli*: not a hexahaem but a 50 kDa tetrahaem nitrite reductase. Mol. Microbiol. **9:**1255–1265.
66. **Dassa, J., H. Fsihi, C. Marck, M. Dion, M. Kieffer-Bontemps, and P. L. Boquet.** 1991. A new oxygen-regulated operon in *Escherichia coli* comprises genes for a putative third cytochrome oxidase and for pH 2.5 acid phosphatase (*appA*). Mol. Gen. Genet. **229:**341–352.
67. **Davidson, E., and F. Daldal.** 1987. Primary structure of the bc_1 complex of *Rhodopseudomonas capsulata*. Nucleotide sequence of the *pet* operon encoding the Rieske, cytochrome *b* and cytochrome c_1 apoproteins. J. Mol. Biol. **195:**13–24.
68. **Davidson, E., T. Ohnishi, M. Tokito, and F. Daldal.** 1992. *Rhodobacter capsulatus* mutants lacking the Rieske FeS protein from a stable cytochrome bc_1 subcomplex with an intact quinone reduction site. Biochemistry **31:**3351–3358.
69. **de Boer, A. P. N., W. N. M. Reijnders, J. G. Kuenen, A. H. Stouthamer, and R. J. M. van Spanning.** 1994. Isolation, sequencing and mutational analysis of a gene cluster involved in nitrite reduction in *Paracoccus denitrificans*. Antonie Leeuwenhoek **66:**111–127.
70. **de Gier, J.-W. L., M. Lübben, W. N. M. Reijnders, C. Tipker, R. J. M. van Spanning, A. H. Stouthamer, and J. van der Oost.** 1994. The terminal oxidases of *Paracoccus denitrificans*. Mol. Microbiol. **13:**183–196.
71. **de Gier, J.-W. L., M. Schepper, W. N. M. Reijnders, S. J. van Dyck, D. J. Slotboom, A. Warne, M. Saraste, K. Krab, M. Finel, A. H. Stouthamer, R. J. M. van Spanning, and J. van der Oost.** 1996. Structural and functional analysis of aa_3-type and cbb_3-type cytochrome *c* oxidases of *Paracoccus denitrificans* reveals significant differences in proton-pump design. Mol. Microbiol. **20:**1247–1260.
72. **Degli Esposti, M.** 1989. Prediction and comparison of the haem-binding sites in membrane haemoproteins. EMBO J. **77:**249–265.
73. **Delgado, M.-J., K. H. Yeoman, G. Wu, C. Vargas, A. Davies, R. K. Poole, A. W. B. Johnston, and J. A. Downie.** 1995. Characterization of the *cycHJKL* genes involved in cytochrome *c* biogenesis and symbiotic nitrogen fixation in *Rhizobium leguminosarum*. J. Bacteriol. **177:**4927–4934.
74. **Dickins, M. A., T. Dhawan, R. P. Gunsalus, I. Schroeder, and G. Cecchini.** 1995. Cloning, sequencing, and expression of the succinate-ubiquinone oxidoreductase (SdhCDAB) operon from *Paracoccus denitrificans*. GenBank accession no. U31902.
75. **D'Mello, R., S. Hill, and R. K. Poole.** 1996. The cytochrome *bd* quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity *in vivo* by oxygen inhibition. Microbiology **142:**755–763.
76. **Doige, C. A., and G. Ferro-Luzzi Ames.** 1993. ATP-dependent transport systems in bacteria and humans: relevance to cystic fibrosis and multidrug resistance. Annu. Rev. Microbiol. **47:**291–319.
77. **Dross, F., V. Geisler, R. Lenger, F. Theis, T. Krafft, F. Fahrenholz, E. Kojro, A. Duchene, D. Triper, K. Juvenal, and A. Kröger.** 1992. The quinone-reactive Ni/Fe-hydrogenase of *Wolinella succinogenes*. Eur. J. Biochem. **206:**93–102.
78. **Drygas, M. E., A. M. Lambowitz, and F. E. Nargang.** 1989. Cloning and analysis of the *Neurospora crassa* cytochrome *c* heme lyase. J. Biol. Chem. **264:**17897–17906.
79. **Dubourdieu, M., and J. A. DeMoss.** 1992. The *narJ* gene product is required for biogenesis of respiratory nitrate reductase in *Escherichia coli*. J. Bacteriol. **174:**867–872.
80. **Dumont, M. E., A. F. Corin, and G. A. Campbell.** 1994. Noncovalent binding of heme induces a compact apocytochrome *c* structure. Biochemistry **33:**7368–7378.
81. **Dumont, M. E., J. F. Ernst, D. M. Hampsey, and F. Sherman.** 1987. Identification and sequence of the gene encoding cytochrome *c* heme lyase in the yeast *Saccharomyces cerevisiae*. EMBO J. **6:**235–241.
82. **Enoch, H. G., and R. L. Lester.** 1975. The purification and properties of formate dehydrogenase and nitrate reductase from *Escherichia coli*. J. Biol. Chem. **250:**6693–6705.
83. **Eraso, J. M., and S. Kaplan.** 1995. Oxygen-insensitive synthesis of the photosynthetic membranes of *Rhodobacter sphaeroides*: a mutant histidine kinase. J. Bacteriol. **177:**2695–2706.
84. **Ermler, U., R. A. Siddiqui, R. Cramm, and B. Friedrich.** 1995. Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 Å resolution. EMBO J. **14:**6067–6077.
85. **Esposti, M. D.** 1989. Prediction and comparison of the haem-binding sites in membrane haemoproteins. Biochim. Biophys. Acta **77:**249–265.
86. **Fabianek, R. A., M. Huber-Wunderlich, R. Glockshuber, P. Künzler, H. Hennecke, and L. Thöny-Meyer.** 1997. Characterization of the *Bradyrhizobium japonicum* CycY protein, a membrane-anchored periplasmic thioredoxin that may play a role as a reductant in the biogenesis of *c*-type cytochromes. J. Biol. Chem. **272:**4467–4473.
87. **Fang, H., R.-J. Lin, and R. B. Gennis.** 1989. Location of heme axial ligands in the cytochrome *d* terminal oxidase complex of *Escherichia coli* determined by site-directed mutagenesis. J. Biol. Chem. **264:**8026–8032.
88. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial transporters. Microbiol. Rev. **57:**995–1017.
89. **Ferguson, S. J.** 1994. Denitrification and its control. Antonie Leeuwenhoek **66:**89–110.
90. **Ferguson, S. J.** 1991. The functions and synthesis of bacterial *c*-type cytochromes with particular reference to *Paracoccus denitrificans* and *Rhodobacter capsulatus*. Biochim. Biophys. Acta **1058:**17–20.
91. **Fischer, G., H. Bang, and C. Mech.** 1984. Determination of enzymatic catalysis for the cis-trans-isomerization of the peptide bond in proline-containing peptides. Biomed. Biochim. Acta **43:**1101–1111.
92. **Fischer, H.-M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
93. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. A. Fields, J. D. Gocayne, J. D. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
94. **Flory, J., and T. J. Donohue.** 1995. Organization and expression of the *Rhodobacter sphaeroides cycFG* operon. J. Bacteriol. **177:**4311–4320.
95. **Fong, S.-T., J. Camakaris, and B. T. O. Lee.** 1995. Molecular genetics of a chromosomal locus involved in copper tolerance in *Escherichia coli* K-12. Mol. Microbiol. **15:**1127–1137.
96. **Freedman, R. B.** 1994. Folding helpers and unhelpful folders. Curr. Biol. **4:**933–935.
97. **Freedman, R. B., T. R. Hirst, and M. F. Tuite.** 1994. Protein disulphide isomerase: building bridges in protein folding. Trends Biochem. Sci. **19:** 331–336.
98. **Fridén, H., and L. Hederstedt.** 1990. Role of His residues in *Bacillus subtilis* cytochrome b_{558} for haem binding and assembly of succinate:quinone oxidoreductase (complex II). Mol. Microbiol. **4:**1045–1056.
99. **Fridén, H., L. Rutberg, K. Magnusson, and L. Hederstedt.** 1987. Genetic and biochemical characterization of *Bacillus subtilis* mutants defective in expression and function of cytochrome *b*-558. Eur. J. Biochem. **168:**695–701.
100. **Fu, R., J. D. Wall, and G. Voordouw.** 1994. DcrA, a *c*-type heme-containing methyl-accepting protein from *Desulfovibrio vulgaris* Hildenborough, senses the oxygen concentration or redox potential of the environment. J. Bacteriol. **176:**344–350.
101. **Fülöp, V., J. W. B. Moir, S. J. Ferguson, and J. Hajdu.** 1995. The anatomy of a bifunctional enzyme: structural basis for reduction of oxygen to water and synthesis of nitric oxide by cytochrome cd_1. Cell **81:**369–377.
102. **Gaballa, A., N. Koedam, and P. Cornelis.** 1996. A cytochrome *c* biogenesis gene involved in pyoverdine production in *Pseudomonas fluorescens* ATCC 17400. Mol. Microbiol. **21:**777–785.
103. **Gabellini, N., and W. Sebald.** 1986. Nucleotide sequence and transcription of the *fbc* operon from *Rhodopseudomonas sphaeroides*. Eur. J. Biochem. **154:**569–579.
104. **Galat, A.** 1993. Peptidylproline cis-trans-isomerases: immunophilins. Eur. J. Biochem. **216:**689–707.
105. **García-Horsman, J. A., B. Barquera, J. Rumbley, J. Ma, and R. B. Gennis.** 1994. The superfamily of heme-copper respiratory oxidases. J. Bacteriol. **176:**5587–5600.
106. **García-Horsman, J. A., E. Berry, J. P. Shapleigh, J. O. Alben, and R. B. Gennis.** 1994. A novel cytochrome *c* oxidase from *Rhodobacter sphaeroides* that lacks Cu_A. Biochemistry **33:**3113–3119.
107. **Gennis, R. B.** 1987. The cytochromes of *Escherichia coli*. FEMS Microbiol. Rev. **46:**387–399.
108. **Gennis, R. B., B. Barquera, B. Hacker, S. R. van Doren, S. Arnaud, A. R. Crofts, E. Davidson, K. A. Gray, and F. Daldal.** 1993. The bc_1 complex of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. J. Bioenerg. Biomembr. **25:**195–209.
109. **Gennis, R. B., and V. Stewart.** 1996. Respiration, p. 217–261. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
110. **Georgiou, C. D., H. Fang, and R. B. Gennis.** 1987. Identification of the *cydC*

locus required for expression of the functional form of the cytochrome *d* terminal oxidase complex in *Escherichia coli*. J. Bacteriol. **169:**2107–2112.
111. **Gerhus, E., P. Steinrücke, and B. Ludwig.** 1990. *Paracoccus denitrificans* cytochrome c_1 gene replacement mutants. J. Bacteriol. **172:**2392–2400.
112. **Gilles-Gonzalez, M. A., G. Gonzalez, M. F. Perutz, L. Kiger, M. C. Marden, and C. Poyart.** 1994. Heme-based sensors, exemplified by the kinase FixL, are a new class of heme protein with distinctive ligand binding and autoxidation. Biochemistry **33:**8067–8073.
113. **Goldman, B. S., D. L. Beckman, A. Bali, E. M. Monika, K. K. Gabbert, and R. G. Kranz.** 1997. Molecular and immunological analysis of an ABC transporter complex required for cytochrome *c* biogenesis. J. Mol. Biol. **268:**724–738.
114. **Goldman, B. S., K. K. Gabbert, and R. G. Kranz.** 1996. Use of heme reporters for studies of cytochrome biosynthesis and heme transport. J. Bacteriol. **178:**6338–6347.
115. **Goldman, B. S., K. K. Gabbert, and R. G. Kranz.** 1996. The temperature sensitive growth and survival phenotypes of *Escherichia coli cydDC* and *cydAB* strains are due to deficiencies in cytochrome *d* oxidase and are corrected by exogenous catalase and reducing agents. J. Bacteriol. **178:** 6348–6351.
116. **Gonzales, D. H., and W. Neupert.** 1990. Biogenesis of mitochondrial *c*-type cytochromes. J. Bioenerg. Biomembr. **22:**753–768.
117. **Goodwin, P. M., and C. Anthony.** 1995. The biosynthesis of periplasmic electron transport proteins in methylotrophic bacteria. Microbiology **141:** 1051–1064.
118. **Gray, K. A., and F. Daldal.** 1995. Mutational studies of the cytochrome bc_1 complexes, p. 747–774. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
119. **Gray, K. A., E. Davidson, and F. Daldal.** 1992. Mutagenesis of methionine-183 drastically affects the physicochemical properties of cytochrome c_1 of the bc_1 complex of *Rhodobacter capsulatus*. Biochemistry **31:**11864–11873.
120. **Gray, K. A., M. Grooms, H. Myllykallio, C. Moomaw, C. Slaughter, and F. Daldal.** 1994. *Rhodobacter capsulatus* contains a novel cytochrome *c* oxidase without a Cu_A center. Biochemistry **33:**3120–3127.
121. **Green, G. N., H. Fang, R. J. Lin, G. Newton, M. Mather, C. D. Georgiou, and R. B. Gennis.** 1988. The nucleotide sequence of the *cyd* locus encoding the two subunits of the cytochrome *d* terminal oxidase complex in *Escherichia coli*. J. Biol. Chem. **263:**13138–13143.
122. **Green, G. N., J. E. Kranz, and R. B. Gennis.** 1984. Cloning of the *cyd* gene locus coding for the cytochrome *d* complex of *Escherichia coli*. Gene **32:** 99–106.
123. **Grove, J., S. Busby, and J. Cole.** 1996. The role of the genes *nrfEFG* and *ccmFH* in cytochrome *c* biosynthesis in *Escherichia coli*. Mol. Gen. Genet. **252:**332–341.
124. **Grove, J., S. Tanapongpipat, G. Thomas, L. Griffiths, H. Crooke, and J. Cole.** 1996. *Escherichia coli* K-12 genes essential for the synthesis of *c*-type cytochromes and a third nitrate reductase located in the periplasm. Mol. Microbiol. **19:**467–481.
125. **Guilhot, C., G. Jander, L. N. Martin, and J. Beckwith.** 1995. Evidence that the pathway of disulphide bond formation in *Escherichia coli* involves interactions between the cysteines of DsbB and DsbA. Proc. Natl. Acad. Sci. USA **92:**9895–9899.
126. **Hägerhäll, C., R. Aasa, C. von Wachenfeldt, and L. Hederstedt.** 1992. Two hemes in *Bacillus subtilis* succinate:menaquinone oxidoreductase (complex II). Biochemistry **31:**7411–7421.
127. **Hägerhäll, C., and L. Hederstedt.** 1996. A structural model for the membrane-integral domain of succinate:quinone oxidoreductase. FEBS Lett. **389:**25–31.
128. **Haltia, T., M. Finel, N. Harms, T. Nakari, M. Raitio, M. Wikström, and M. Saraste.** 1989. Deletion of the gene for subunit III leads to defective assembly of bacterial cytochrome oxidase. EMBO J. **8:**3571–3579.
129. **Haltia, T., A. Puustinen, and M. Finel.** 1988. The *Paracoccus denitrificans* cytochrome aa_3 has a third subunit. Eur. J. Biochem. **172:**543–546.
130. **Hanson, M. S., S. E. Pelzel, J. Latimer, U. Müller-Eberhard, and E. J. Hansen.** 1992. Identification of a genetic locus of *Haemophilus influenzae* type b necessary for the binding and utilization of heme bound to human hemopexin. Proc. Natl. Acad. Sci. USA **89:**1973–1977.
131. **Hansson, M., and L. Hederstedt.** 1994. *Bacillus subtilis* HemY is a peripheral membrane protein essential for protoheme IX synthesis which can oxidize coproporphyrinogen III and protoporphyrinogen IX. J. Bacteriol. **176:**5962–5970.
132. **Hansson, M., and L. Hederstedt.** 1994. Purification and characterisation of a water-soluble ferrochelatase from *Bacillus subtilis*. Eur. J. Biochem. **220:** 201–208.
133. **Harold, F. M.** 1986. The vital force: a study of bioenergetics. W. H. Freeman & Co., New York, N.Y.
134. **Hartl, F. U.** 1996. Molecular chaperones in cellular protein folding. Nature **381:**571–580.
135. **Hausinger, R. P.** 1993. Biochemistry of nickel. Plenum Press, New York, N.Y.
136. **Hederstedt, L.** 1986. Molecular properties, genetics and biosynthesis of *Bacillus subtilis* succinate dehydrogenase complex. Methods Enzymol. **126:** 399–414.
137. **Hederstedt, L., T. Bergman, and H. Jörnvall.** 1987. Processing of *Bacillus subtilis* succinate dehydrogenase and cytochrome b_{558} polypeptides. FEBS Lett. **213:**385–390.
138. **Hederstedt, L., and L. Rutberg.** 1980. Biosynthesis and membrane binding of succinate dehydrogenase in *Bacillus subtilis*. J. Bacteriol. **144:**941–951.
139. **Hederstedt, L., and L. Rutberg.** 1981. Succinate dehydrogenase—a comparative review. Microbiol. Rev. **45:**542–555.
140. **Heinzinger, N. K., S. Y. Fujimoto, M. A. Clark, M. S. Moreno, and E. L. Barrett.** 1995. Sequence analysis of the *phs* operon in *Salmonella typhimurium* and the contribution of thiosulfate reduction to anaerobic energy metabolism. J. Bacteriol. **177:**2813–2820.
141. **Heiss, B., K. Frunzke, and W. G. Zumft.** 1989. Formation of the N-N- bond from nitric oxide by a membrane-bound cytochrome *bc* complex of nitrate-respiring (denitrifying) *Pseudomonas stutzeri*. J. Bacteriol. **171:**3288–3297.
142. **Henderson, D. P., and S. M. Payne.** 1994. Characterization of the *Vibrio cholerae* outer membrane heme transport protein HutA: sequence of the gene, regulation of expression, and homology to the family of *tonB*-dependent proteins. J. Bacteriol. **176:**3269–3277.
143. **Henderson, D. P., and S. M. Payne.** 1993. Cloning and characterization of the *Vibrio cholerae* genes encoding the utilization of iron from haemin and haemoglobin. Mol. Microbiol. **7:**461–469.
144. **Hill, J. J., J. O. Alben, and R. B. Gennis.** 1993. Spectroscopic evidence for a heme-heme binuclear center in the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. Proc. Natl. Acad. Sci. USA **90:**5863–5867.
145. **Hochkoeppler, A., F. E. Jenney, S. E. Lang, D. Zannoni, and F. Daldal.** 1995. Membrane-associated cytochrome c_y of *Rhodobacter capsulatus* is an electron carrier from the cytochrome bc_1 complex to the cytochrome *c* oxidase during respiration. J. Bacteriol. **177:**608–613.
146. **Holm, L., M. Saraste, and M. Wikström.** 1987. Structural models of the redox centres in cytochrome oxidase. EMBO J. **6:**2819–2823.
147. **Holmgren, A.** 1979. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J. Biol. Chem. **254:**9627–9632.
148. **Hosler, J. P., S. Ferguson-Miller, M. W. Calhoun, J. W. Thomas, J. Hill, L. Lemieux, J. Ma, C. Georgiou, J. Fetter, J. Shapleigh, M. M. J. Tecklenburg, G. T. Babcock, and R. B. Gennis.** 1993. Insight into the active-site structure and function of cytochrome oxidase by analysis of site-directed mutants of bacterial cytochrome aa_3 and cytochrome *bo*. J. Bioenerg. Biomembr. **25:** 121–136.
149. **Howe, G., and S. Merchant.** 1994. The biosynthesis of bacterial and plastid *c*-type cytochromes. Photosynth. Res. **40:**147–165.
150. **Huang, C. J., and E. L. Barrett.** 1991. Sequence analysis and expression of the *Salmonella typhimurium asr* operon encoding production of hydrogen sulfide from sulfite. J. Bacteriol. **173:**1544–1553.
151. **Hughes, M. N., and R. K. Poole.** 1989. Metals and micro-organisms. Chapman & Hall, Ltd., London, United Kingdom.
152. **Hultgren, S. J., S. Abraham, M. Caparon, P. Falk, J. W. St. Geme III, and S. Normark.** 1993. Pilus and non-pilus bacterial adhesins: assembly and function in cell recognition. Cell **73:**887–901.
153. **Hultgren, S. J., and C. H. Jones.** 1995. Utility of the immunoglobulin-like fold of chaperones in shaping organelles of attachment in pathogenic bacteria. ASM News **61:**457–464.
154. **Hussain, H., J. Grove, L. Griffiths, S. Busby, and J. Cole.** 1994. A seven-gene operon essential for formate-dependent nitrite reduction to ammonia by enteric bacteria. Mol. Microbiol. **12:**153–163.
155. **Hyde, S. C., P. Emsley, M. J. Hartshorn, M. M. Mimmack, U. Gileadi, S. R. Pearce, M. P. Gallagher, D. R. Gill, R. E. Hubbard, and C. F. Higgins.** 1990. Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature **346:**362–365.
156. **International Union of Biochemistry.** 1992. Nomenclature of electron-transfer proteins. Recommendations (1989) of the Nomenclature Committee of the International Union of Biochemistry. J. Biol. Chem. **267:**665–677.
157. **Iobbi-Nivol, C., H. Crooke, L. Griffith, J. Grove, H. Hussain, J. Pommier, V. Mejean, and J. A. Cole.** 1994. A reassessment of the range of *c*-type cytochromes synthesized by *Escherichia coli* K-12. FEMS Microbiol. Lett. **119:**89–94.
158. **Iwata, S., C. Ostermeier, B. Ludwig, and H. Michel.** 1995. Structure at 2.8 Å resolution of cytochrome *c* oxidase from *Paracoccus denitrificans*. Nature **376:**660–669.
159. **Iwata, S., M. Saynovits, T. A. Link, and H. Michel.** 1996. Structure of a water soluble fragment of the 'Rieske' iron-sulfur protein of the bovine heart mitochondrial cytochrome bc1 complex determined by MAD phasing at 1.5 Å resolution. Structure **4:**567–579.
160. **Jacobs, M., J. B. Andersen, V. Kontintn, and M. Sarvas.** 1993. *Bacillus subtilis* PrsA is required *in vivo* as an extracytoplasmic chaperone for secretion of active enzymes synthesized either with or without prosequences. Mol. Microbiol. **8:**957–966.
161. **Jahn, D., E. Verkamp, and D. Söll.** 1991. Glutamyl transfer RNA: a precursor of heme and chlorophyll biosynthesis. Trends Biochem. Sci. **17:**215–218.
162. **Jenney, F. E., Jr., and F. Daldal.** 1993. A novel membrane-associated *c*-type

cytochrome, cyt c_y, can mediate the photosynthetic growth of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. EMBO J. **12:**1283–1292.
163. **Jenney, F. E. J., R. C. Prince, and F. Daldal.** 1996. The membrane-bound cytochrome c_y of *Rhodobacter capsulatus* can serve as an electron donor to the photosynthetic reaction center of *Rhodobacter sphaeroides*. Biochim. Biophys. Acta **1273:**159–164.
164. **Jones, C. W.** 1988. Membrane-associated energy conservation in bacteria: a general introduction, p. 1–82. *In* C. Anthony (ed.), Bacterial energy transduction. Academic Press, Ltd., London, United Kingdom.
165. **Jungnickel, B., T. A. Rapoport, and E. Hartmann.** 1994. Protein translocation: common themes from bacteria to man. FEBS Lett. **346:**73–77.
166. **Jüngst, A., S. Wakabayashi, H. Matsubara, and W. G. Zumft.** 1991. The *nirSTBM* region coding for cytochrome cd_1-dependent nitrite respiration of *Pseudomonas stutzeri* consists of a cluster of mono-, di-, and tetraheme proteins. FEBS Lett. **279:**205–209.
167. **Kahn, D., J. Batut, M. L. Daveran, and J. Fourment.** 1993. Structure and regulation of the *fixNOQP* operon of *Rhizobium meliloti*, p. 474. *In* R. Palacios, J. Mora, and W. E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic Publishers, Dordrecht, The Netherlands.
168. **Kahn, D., M. David, O. Domergue, M.-L. Daveran, J. Ghai, P. R. Hirsch, and J. Batut.** 1989. *Rhizobium meliloti fixGHI* sequence predicts involvement of a specific cation pump in symbiotic nitrogen fixation. J. Bacteriol. **171:**929–939.
169. **Kallas, T.** 1993. The cytochrome b_6f complex, p. 259–317. *In* D. A. Bryant (ed.), The molecular biology of cyanobacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
170. **Kallas, T., S. Spiller, and R. Malkin.** 1988. Primary structure of cotranscribed genes encoding Rieske Fe-S and cytochrome *f* proteins of the cyanobacterium *Nostoc* PCC 7906. Proc. Natl. Acad. Sci. USA **85:**5794–5798.
171. **Kamitani, S., Y. Akiyama, and K. Ito.** 1992. Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. EMBO J. **11:**57–62.
172. **Karkhoff-Schweizer, R. R., M. Bruschi, and G. Voordouw.** 1993. Expression of the γ-subunit gene of desulfoviridin-type dissimilatory sulfite reductase and of the α- and β-subunit genes is not coordinately regulated. Eur. J. Biochem. **211:**501–507.
173. **Karkhoff-Schweizer, R. R., D. P. Huber, and G. Voordouw.** 1995. Conservation of the genes for dissimilatory sulfite reductase from *Desulfovibrio vulgaris* and *Archaeglobus fulgidus* allows their detection by PCR. Appl. Environ. Microbiol. **61:**290–296.
174. **Kawasaki, S., H. Arai, Y. Igarashi, and T. Kodama.** 1995. Sequencing and characterization of the downstream region of the genes encoding nitrite reductase and cytochrome *c*-551 (*nirSM*) from *Pseudomonas aeruginosa*: identification of the gene necessary for biosynthesis of heme d_1. Gene **167:**87–91.
175. **Kaysser, T. M., J. B. Ghaim, C. Georgiou, and R. B. Gennis.** 1995. Methionine-393 is an axial ligand of the heme b_{558} component of the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. Biochemistry **34:**13491–13501.
176. **Keefe, K. G., and R. J. Maier.** 1993. Purification and characterization of an O_2-utilizing cytochrome *c* oxidase complex from *Bradyrhizobium japonicum* bacteroid membranes. Biochim. Biophys. Acta **1183:**91–104.
177. **Kelly, M. J. S., R. K. Poole, M. G. Yates, and C. Kennedy.** 1990. Cloning and mutagenesis of genes encoding the cytochrome *bd* terminal oxidase complex in *Azotobacter vinelandii*: mutants deficient in the cytochrome *d* complex are unable to fix nitrogen in air. J. Bacteriol. **172:**6010–6019.
178. **Kereszt, A., K. Slaska-Kiss, P. Putnoky, Z. Banfalvi, and A. Kondorosi.** 1995. *Rhizobium meliloti cycHJKL* genes involved in cytochrome *c* biogenesis are required for "respiratory" nitrate reduction *ex planta* and for nitrogen fixation in symbiosis. Mol. Gen. Genet. **247:**39–47.
179. **Kim, P. S., and R. L. Baldwin.** 1982. Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. Annu. Rev. Biochem. **51:**459–489.
180. **Kishigami, S., E. Kanaya, M. Kikuchi, and K. Ito.** 1995. DsbA-DsbB interaction through their active-site cysteines. J. Biol. Chem. **270:**17072–17074.
181. **Kita, K., C. R. T. Vibat, S. Meinhardt, J. R. Guest, and R. B. Gennis.** 1989. One step purification from *Escherichia coli* of complex II (succinate:ubiquinone oxidoreductase) associated with succinate reducible cytochrome b_{556}. J. Biol. Chem. **264:**2672–2677.
182. **Konishi, K., S. R. van Doren, D. M. Kramer, A. R. Crofts, and R. B. Gennis.** 1991. Preparation and characterization of the water-soluble heme-binding domain of cytochrome c_1 from the *Rhodobacter sphaeroides* bc_1 complex. J. Biol. Chem. **266:**14270–14276.
183. **Krafft, T., M. Bokranz, O. Klimmek, I. Schröder, F. Fahrenholz, E. Kojro, and A. Kröger.** 1992. Cloning and nucleotide sequence of the *psrA* gene of *Wolinella succinogenes* polysulphide reductase. Eur. J. Biochem. **206:**503–510.
184. **Kranz, R. G., and D. L. Beckman.** 1995. Cytochrome biogenesis, p. 709–723. *In* R. E. Blankenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
185. **Krummeck, G., and G. Rödel.** 1990. Yeast SCO1 protein is required for a post-translational step in the accumulation of mitochondrial cytochrome *c* oxidase subunits I and II. Curr. Genet. **18:**13–15.
186. **Kumamoto, C. A.** 1991. Molecular chaperones and protein translocation across the *Escherichia coli* inner membrane. Mol. Microbiol. **5:**19–22.
187. **Kurowski, B., and B. Ludwig.** 1987. The genes of the *Paracoccus denitrificans* bc_1 complex. Nucleotide sequence and homologies between bacterial and mitochondrial subunits. J. Biol. Chem. **263:**13805–13811.
188. **Lang, K., F. X. Schmid, and G. Fischer.** 1987. Catalysis of protein folding by prolyl isomerase. Nature **329:**268–270.
189. **Lang, S. E., F. E. J. Jenney, and F. Daldal.** 1996. *Rhodobacter capsulatus* CycH: a bipartite gene product with pleiotropic effects on the biogenesis of structurally different *c*-type cytochromes. J. Bacteriol. **178:**5279–5290.
190. **Lee, B. C.** 1995. Quelling the red menace: haem capture by bacteria. Mol. Microbiol. **18:**383–390.
191. **Lemieux, L. J., M. W. Calhoun, J. W. Thomas, W. J. Ingledew, and R. B. Gennis.** 1992. Determination of the ligands of the low-spin heme of the cytochrome *o* ubiquinol oxidase complex using site-directed mutagenesis. J. Biol. Chem. **267:**2105–2113.
192. **Lemire, B. D., J. J. Robinson, and J. H. Weiner.** 1982. Identification of membrane anchor polypeptides of *Escherichia coli* fumarate reductase. J. Bacteriol. **152:**1126–1131.
193. **Liu, J., and C. T. Walsh.** 1990. Peptidyl-prolyl cis-trans-isomerase from *Escherichia coli*: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin. Proc. Natl. Acad. Sci. USA **87:**4028–4032.
194. **Ljungdahl, P. O., J. D. Pennoyer, D. E. Robertson, and B. L. Trumpower.** 1987. Purification of highly active cytochrome bc_1 complexes from phylogenetically diverse species by a single chromatographic procedure. Biochim. Biophys. Acta **891:**227–241.
195. **Loferer, H., M. Bott, and H. Hennecke.** 1993. *Bradyrhizobium japonicum* TlpA, a novel membrane-anchored thioredoxin-like protein involved in the biogenesis of cytochrome aa_3 and development of symbiosis. EMBO J. **12:**3373–3383.
196. **Loferer, H., and H. Hennecke.** 1994. Expression, purification and functional properties of a soluble form of *Bradyrhizobium japonicum* TlpA, a thioredoxin-like protein. Eur. J. Biochem. **223:**339–344.
197. **Loferer, H., and H. Hennecke.** 1994. Protein disulphide oxidoreductases in bacteria. Trends Biochem. Sci. **19:**169–171.
198. **Loferer, H., M. Wunderlich, H. Hennecke, and R. Glockshuber.** 1995. A bacterial thioredoxin-like protein that is exposed to the periplasm has redox properties comparable with those of cytoplasmic thioredoxins. J. Biol. Chem. **270:**26178–26183.
199. **Ludwig, B., and G. Schatz.** 1980. A two-subunit cytochrome *c* oxidase (cytochrome aa_3) from *Paracoccus denitrificans*. Proc. Natl. Acad. Sci. USA **77:**196–200.
200. **Lund, K., and J. DeMoss.** 1976. Association-dissociation behavior and subunit structure of heat-released nitrate reductase from *Escherichia coli*. J. Biol. Chem. **251:**2207–2216.
201. **Magnusson, K., M. K. Philips, J. R. Guest, and L. Rutberg.** 1986. Nucleotide sequence of the gene for cytochrome b_{558} of the *Bacillus subtilis* succinate dehydrogenase complex. J. Bacteriol. **166:**1067–1071.
202. **Maier, T., A. Jacobi, M. Sauter, and A. Böck.** 1993. The product of the *hypB* gene, which is required for nickel incorporation into hydrogenases, is a novel guanine nucleotide-binding protein. J. Bacteriol. **175:**630–635.
203. **Majewski, C., and A. Trebst.** 1990. The *pet* genes of *Rhodospirillum rubrum*: cloning and sequencing of the genes for the cytochrome bc_1 complex. Mol. Gen. Genet. **224:**373–382.
204. **Malakhov, M., H. Wada, D. Los, and N. Murata.** 1996. The *coxD* gene for heme O synthase in *Synechocystis*. Biochim. Biophys. Acta **1273:**84–86.
205. **Mandon, K., P. A. Kaminski, and C. Elmerich.** 1994. Functional analysis of the *fixNOQP* region of *Azorhizobium caulinodans*. J. Bacteriol. **176:**2560–2568.
206. **Mandon, K., P. A. Kaminski, C. Mougel, N. Desnoues, B. Dreyfus, and C. Elmerich.** 1993. Role of the *fixGHI* region of *Azorhizobium caulinodans* in free-living and symbiotic nitrogen fixation. FEMS Microbiol. Lett. **114:**185–190.
207. **Mather, M. W., P. Springer, S. Hensel, G. Buse, and J. A. Fee.** 1993. Cytochrome oxidase genes from *Thermus thermophilus*: nucleotide sequence of the fused gene and analysis of the deduced primary structures for subunits I and III of cytochrome caa_3. J. Biol. Chem. **268:**5395–5408.
208. **McEwan, A. G., D. J. Richardson, H. Hüdig, S. J. Ferguson, and J. B. Jackson.** 1989. Identification of cytochromes involved in electron transport to trimethylamine *N*-oxide/dimethylsulphoxide reductase in *Rhodobacter capsulatus*. Biochim. Biophys. Acta **73:**308–314.
209. **McRee, D. E., D. C. Richardson, J. S. Richardson, and L. M. Siegel.** 1986. The heme and Fe_4S_4 cluster in the crystallographic structure of *Escherichia coli* sulfite reductase. J. Biol. Chem. **261:**10277–10281.
210. **Méjean, V., C. Iobbi-Nivol, M. Lepelletier, G. Giordano, M. Chippaux, and M.-C. Pascal.** 1994. TMAO anaerobic respiration in *Escherichia coli*: involvement of the *tor* operon. Mol. Microbiol. **11:**1169–1179.
211. **Metheringham, R., L. Griffith, H. Crooke, S. Forsythe, and J. Cole.** 1995. An essential role for DsbA in cytochrome *c* synthesis and formate-dependent nitrite reduction by *E. coli* K-12. Arch. Microbiol. **164:**301–307.

212. **Metheringham, R., K. L. Tyson, H. Crooke, D. Missiakas, and S. Raina.** 1996. Effects of mutations in genes for proteins involved in disulphide bond formation in the periplasm on the activities of anaerobically induced electron transfer chains in *Escherichia coli* K12. Mol. Gen. Genet. **253:**95–102.
213. **Meyer, T. E.** 1996. Evolution and classification of *c*-type cytochromes, p. 33–99. *In* R. A. Scott and A. G. Mauk (ed.), Cytochrome *c*. A multidisciplinary approach. University Science Books, Sausalito, Calif.
214. **Mills, M., and S. M. Payne.** 1995. Genetics and regulation of heme iron transport in *Shigella dysenteriae* and detection of an analogous system in *Escherichia coli* O157:H7. J. Bacteriol. **177:**3004–3009.
215. **Minagawa, J., T. Mogi, R. B. Gennis, and Y. Anraku.** 1992. Identification of heme and copper ligands in subunit I of the cytochrome *bo* complex in *Escherichia coli*. J. Biol. Chem. **267:**2096–2104.
216. **Minghetti, K. C., V. C. Goswitz, N. E. Gabriel, J. J. Hill, C. Barassi, C. D. Georgiou, S. I. Chan, and R. B. Gennis.** 1992. Modified, large-scale purification of the cytochrome *o* complex of *Escherichia coli* yields a two heme/one copper terminal oxidase with high specific activity. Biochemistry **31:** 6917–6924.
217. **Missiakas, D., J.-M. Betton, and S. Raina.** 1996. New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. Mol. Microbiol. **21:**871–884.
218. **Missiakas, D., and S. Raina.** 1997. Protein folding in the bacterial periplasm. J. Bacteriol. **179:**2465–2471.
219. **Missiakas, D., C. Georgopoulos, and S. Raina.** 1994. The *Escherichia coli dsbC* (*xprA*) gene encodes a periplasmic protein involved in disulfide bond formation. EMBO J. **13:**2013–2020.
220. **Missiakas, D., C. Georgopoulos, and S. Raina.** 1993. Identification and characterization of the *Escherichia coli* gene *dsbB*, whose product is involved in the formation of disulfide bonds *in vivo*. Proc. Natl. Acad. Sci. USA **90:**7084–7088.
221. **Missiakas, D., F. Schwager, and S. Raina.** 1995. Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*. EMBO J. **14:**3415–3424.
222. **Mogi, T., K. Saiki, and Y. Anraku.** 1994. Biosynthesis and functional role of haem O and haem A. Mol. Microbiol. **14:**391–398.
223. **Moore, G. R., and G. W. Pettigrew.** 1990. Cytochromes *c*. Evolutionary, structural and physicochemical aspects. Springer-Verlag KG, Berlin, Germany.
224. **Moshiri, F., A. Chawla, and R. J. Maier.** 1991. Cloning, characterization, and expression in *E. coli* of the genes encoding the cytochrome *d* oxidase complex from *Azotobacter vinelandii*. J. Bacteriol. **173:**6230–6241.
225. **Mueller, J. P., and H. W. Taber.** 1989. Isolation and sequence of *ctaA*, a gene required for cytochrome aa_3 biosynthesis and sporulation in *Bacillus subtilis*. J. Bacteriol. **171:**4967–4978.
226. **Mukai, K., S. Wakabayashi, and H. Matsubara.** 1989. Molecular cloning and nucleotide sequence of a cDNA encoding *Euglena gracilis* cytochrome c_1. J. Biochem. **106:**479–482.
227. **Munro, A. W., and J. G. Lindsay.** 1996. Bacterial cytochromes P-450. Mol. Microbiol. **20:**1115–1125.
228. **Murphy, C. K., and J. Beckwith.** 1996. Export of proteins through the cell envelope in *Escherichia coli*, p. 967–978. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
229. **Murphy, M. J., L. M. Siegel, H. Kamin, D. V. DerVartanian, J. Lee, J. LeGall, and H. D. Peck.** 1973. An iron tetrahydroporphyrin prosthetic group common to both assimilatory and dissimilatory sulfite reductases. Biochem. Biophys. Res. Commun. **54:**82–88.
230. **Myers, C. R., and J. M. Myers.** 1992. Localization of cytochromes to the outer membrane of anaerobically grown *Shewanella putrefaciens* MR-1. J. Bacteriol. **174:**3429–3438.
231. **Myllykallio, H., F. E. Jenney, Jr., C. R. Moomaw, C. A. Slaughter, and F. Daldal.** 1997. Cytochrome c_y of *Rhodobacter capsulatus* is attached to the cytoplasmic membrane by an uncleaved signal sequence-like anchor. J. Bacteriol. **179:**2623–2631.
232. **Nagata, K., S. Tsukita, T. Tamura, and N. Sone.** 1996. A *cb*-type cytochrome oxidase terminates the respiratory chain in *Helicobacter pylori*. Microbiology **142:**1757–1763.
233. **Nakai, T., T. Yasuhara, Y. Fujiki, and A. Ohashi.** 1995. Multiple genes, including a member of the AAA family, are essential for degradation of unassembled subunit 2 of cytochrome *c* oxidase in yeast mitochondria. Mol. Cell. Biol. **15:**4441–4452.
234. **Nakamura, H., I. Yamato, Y. Anraku, L. Lemieux, and R. B. Gennis.** 1990. Expression of *cyoA* and *cyoB* demonstrates that the CO-binding heme component of the *Escherichia coli* cytochrome *o* complex is in subunit I. J. Biol. Chem. **265:**11193–11197.
235. **Nakamura, K., M. Yamaki, M. Sarada, S. Nakayama, C. R. T. Vibat, R. B. Gennis, T. Nakayashiki, H. Inokuchi, S. Kojiama, and K. Kita.** 1996. Two hydrophobic subunits are essential for the heme *b* ligation and functional assembly of complex II (succinate-ubiquinone oxidoreductase) from *Escherichia coli*. J. Biol. Chem. **271:**521–527.
236. **Nall, B. T.** 1996. Cytochrome *c* folding and stability, p. 176–200. *In* R. A. Scott and A. G. Mauk (ed.), Cytochrome *c*. A multidisciplinary approach. University Science Books, Sausalito, Calif.
237. **Neidle, E. L., and S. Kaplan.** 1992. *Rhodobacter sphaeroides rdxA*, a homolog of *Rhizobium meliloti fixG*, encodes a membrane protein which may bind cytoplasmic [4Fe-4S] clusters. J. Bacteriol. **174:**6444–6454.
238. **Newton, G., and R. B. Gennis.** 1991. *In vivo* assembly of the cytochrome *d* terminal oxidase complex of *Escherichia coli* from genes encoding the two subunits expressed on separate plasmids. Biochim. Biophys. Acta **1089:**8–12.
239. **Newton, G., C.-H. Yun, and R. B. Gennis.** 1991. Analysis of the topology of the cytochrome *d* terminal oxidase complex of *Escherichia coli* by alkaline phosphatase fusions. Mol. Microbiol. **5:**2511–2518.
240. **Nicholson, D. W., C. Hergersberg, and W. Neupert.** 1988. Role of cytochrome *c* heme lyase in the import of cytochrome *c* into mitochondria. J. Biol. Chem. **263:**19034–19042.
241. **Nicholson, D. W., H. Köhler, and W. Neupert.** 1987. Import of cytochrome *c* into mitochondria. Cytochrome *c* heme lyase. Eur. J. Biochem. **164:**147–157.
242. **Nicholson, D. W., and W. Neupert.** 1989. Import of cytochrome *c* into mitochondria: reduction of heme, mediated by NADH and flavin nucleotides, is obligatory for its covalent linkage to apocytochrome *c*. Proc. Natl. Acad. Sci. USA **86:**4340–4344.
243. **Nicholson, D. W., R. A. Stuart, and W. Neupert.** 1989. Role of cytochrome c_1 heme lyase and of the two proteolytic processing steps during import into mitochondria. J. Biol. Chem. **264:**10156–10168.
244. **Nobrega, F. G., M. P. Nobrega, and A. Tzagoloff.** 1992. *BCS1*, a novel gene required for the expression of functional Rieske iron-sulfur protein in *Saccharomyces cerevisiae*. EMBO J. **11:**3821–3829.
245. **O'Brian, M. R.** 1996. Heme synthesis in the rhizobium-legume symbiosis: a palette for bacterial and eukaryotic pigments. J. Bacteriol. **178:**2471–2478.
246. **Oden, K. L., and R. B. Gennis.** 1991. Isolation and characterization of a new class of cytochrome *d* terminal oxidase mutants of *Escherichia coli*. J. Bacteriol. **173:**6174–6183.
247. **Odermatt, A., H. Suter, R. Krapf, and M. Solioz.** 1993. Primary structure of two P-type ATPases involved in copper homeostasis in *Enterococcus hirae*. J. Biol. Chem. **268:**12775–12779.
248. **Page, M. D., and S. J. Ferguson.** 1990. Apo forms of cytochrome c_{550} and cytochrome cd_1 are translocated to the periplasm of *Paracoccus denitrificans* in the absence of haem incorporation caused by either mutation or inhibition of haem synthesis. Mol. Microbiol. **4:**1181–1192.
249. **Page, M. D., and S. J. Ferguson.** 1989. A bacterial *c*-type cytochrome can be translocated to the periplasm as an apo form; the biosynthesis of cytochrome cd_1 (nitrite reductase) from *Paracoccus denitrificans*. Mol. Microbiol. **3:**653–661.
250. **Page, M. D., and S. J. Ferguson.** 1995. Cloning and sequence analysis of *cycH* gene from *Paracoccus denitrificans*: the *cycH* gene product is required for assembly of all *c*-type cytochromes, including cytochrome c_1. Mol. Microbiol. **15:**307–318.
251. **Page, M. D., and S. J. Ferguson.** 1997. *Paracoccus denitrificans* CcmG is a periplasmic protein-disulfide oxidoreductase required for *c*- and aa_3-type cytochrome biogenesis; evidence for a reductase role *in vivo*. Mol. Microbiol. **24:**977–990.
252. **Page, M. D., D. A. Pearce, H. A. C. Norris, and S. J. Ferguson.** 1997. The *Paracoccus denitrificans ccmA*, *ccmB* and *ccmC* genes: cloning and sequencing, and analysis of the potential of their products to form a haem or apo-*c*-type cytochrome transporter. Microbiology **143:**563–576.
253. **Palmedo, G., P. Seither, H. Körner, J. C. Matthews, R. S. Burkhalter, R. Timkovich, and W. G. Zumft.** 1995. Resolution of the *nirD* locus for heme d_1 synthesis of cytochrome cd_1 (respiratory nitrite reductase) from *Pseudomonas stutzeri*. Eur. J. Biochem. **232:**737–746.
254. **Pearce, D. A., and F. Sherman.** 1995. Degradation of cytochrome oxidase subunits in mutants of yeast lacking cytochrome *c* and suppression of the degradation by mutation of *yme1*. J. Biol. Chem. **270:**20879–20882.
255. **Pennoyer, J. D., T. Ohnishi, and B. L. Trumpower.** 1988. Purification and properties of succinate-ubiquinone oxidoreductase complex from *Paracoccus denitrificans*. Biochim. Biophys. Acta **935:**195–207.
256. **Peterson, J., C. Vibat, and R. B. Gennis.** 1994. Identification of the axial heme ligands of cytochrome b_{556} in succinate:ubiquinone oxidoreductase from *Escherichia coli*. FEBS Lett. **355:**155–156.
257. **Pettigrew, G. W., and G. R. Moore.** 1987. Cytochromes *c*. Biological aspects. Springer-Verlag KG, Berlin, Germany.
258. **Pfeifer, K., K.-S. Kim, S. Kogan, and L. Guarente.** 1989. Functional dissection and sequence of yeast HAP1 activator. Cell **56:**291–301.
259. **Phillips, M. K., L. Hederstedt, S. Hasnain, L. Rutberg, and J. R. Guest.** 1987. Nucleotide sequence encoding the flavoprotein and iron-sulfur protein subunits of the *Bacillus subtilis* PY79 succinate dehydrogenase complex. J. Bacteriol. **169:**864–873.
260. **Pierik, A. J., M. G. Duyvis, J. M. L. M. van Helvoort, R. B. G. Wolbert, and W. R. Hagen.** 1992. The third subunit of desulfoviridin-type dissimilatory sulfite reductase. Eur. J. Biochem. **205:**111–115.
261. **Pollock, W. B. R., P. J. Chemerika, M. E. Forrest, J. T. Beatty, and G.**

Voordouw. 1989. Expression of the gene encoding cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough) in *Escherichia coli*: export and processing of the apoprotein. J. Gen. Microbiol. **135:**2319–2328.
262. **Poole, R. K.** 1988. Bacterial cytochrome oxidases, p. 231–291. *In* C. Anthony (ed.), Bacterial energy transduction. Academic Press, Ltd., London, United Kingdom.
263. **Poole, R. K., B. S. Baines, and C. A. Appleby.** 1986. Haemoprotein b_{590} (*Escherichia coli*), a reducible catalase and peroxidase: evidence for its close relationship to hydroperoxidase I and a 'cytochrome a_1' preparation. J. Gen. Microbiol. **132:**1525–1539.
264. **Poole, R. K., F. Gibson, and G. Wu.** 1994. The *cydD* gene product, component of a heterodimeric ABC transporter, is required for assembly of periplasmic cytochrome *c* and of cytochrome *bd* in *Escherichia coli*. FEMS Microbiol. Lett. **117:**217–224.
265. **Poole, R. K., L. Hatch, M. W. J. Cleeter, F. Gibson, G. B. Cox, and G. Wu.** 1993. Cytochrome *bd* biosynthesis in *Escherichia coli*: the sequences of the *cydC* and *cydD* genes suggest that they encode the components of an ABC membrane transporter. Mol. Microbiol. **10:**421–430.
266. **Poole, R. K., H. D. Williams, J. A. Downie, and F. Gibson.** 1989. Mutations affecting the cytochrome *d*-containing oxidase complex of *Escherichia coli* K12: identification and mapping of a fourth locus, *cydD*. J. Gen. Microbiol. **135:**1865–1874.
267. **Poulos, T. L., B. C. Finzel, I. C. Gunsalus, G. C. Wagner, and J. Kraut.** 1985. The 2.6 Å crystal structure of *Pseudomonas putida* cytochrome P-450. J. Biol. Chem. **260:**16122–16130.
268. **Preisig, O., D. Anthamatten, and H. Hennecke.** 1993. Genes for a microaerobically induced oxidase complex in *Bradyrhizobium japonicum* are essential for a nitrogen fixing endosymbiosis. Proc. Natl. Acad. Sci. USA **90:**3309–3313.
269. **Preisig, O., R. Zufferey, and H. Hennecke.** 1996. The *Bradyrhizobium japonicum fixGHIS* genes are required for the formation of the high-affinity cbb_3-type cytochrome oxidase. Arch. Microbiol. **165:**297–305.
270. **Preisig, O., R. Zufferey, L. Thöny-Meyer, C. A. Appleby, and H. Hennecke.** 1996. A high-affinity cbb_3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of *Bradyrhizobium japonicum*. J. Bacteriol. **178:**1532–1538.
271. **Priest, J. W., and S. L. Hajduk.** 1992. Cytochrome *c* reductase purified from *Crithidia fasiculata* contains an atypical cytochrome c_1. J. Biol. Chem. **267:** 20188–20195.
272. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
273. **Pugsley, A. P.** 1989. Protein targeting. Academic Press, Inc., San Diego, Calif.
274. **Quirk, P. G., D. B. Hicks, and T. A. Krulwich.** 1993. Cloning of the *cta* operon from the alkalophilic *Bacillus firmus* OF4 and characterization of the pH-regulated cytochrome aa_3 oxidase it encodes. J. Biol. Chem. **268:** 678–685.
275. **Raitio, M., T. Jalli, and M. Saraste.** 1987. Isolation and analysis of the genes for cytochrome *c* oxidase in *Paracoccus denitrificans*. EMBO J. **6:** 2825–2833.
276. **Rajagopalan, K. V.** 1996. Biosynthesis of the molybdenum cofactor, p. 674–679. *In* F. W. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
277. **Ramseier, T. M., H. V. Winteler, and H. Hennecke.** 1991. Discovery and sequence analysis of bacterial genes involved in the biogenesis of *c*-type cytochromes. J. Biol. Chem. **266:**7793–7803.
278. **Rauhut, R., A. Jäger, C. Conrad, and G. Klug.** 1996. Identification and analysis of the *rnc* gene for RNase III in *Rhodobacter capsulatus*. Nucleic Acids Res. **24:**1246–1251.
279. **Reid, G. A., B. A. Haddock, and W. J. Ingledew.** 1981. Assembly of functional *b*-type cytochromes in membranes from a 5-aminolaevulinic acid-requiring mutant of *Escherichia coli*. FEBS Lett. **131:**346–350.
280. **Reyes, F., M. D. Roldan, W. Klipp, F. Castillo, and C. Moreno-Vivian.** 1996. Isolation of periplasmic nitrate reductase genes from *Rhodobacter sphaeroides* DSM 158: structural and functional differences among prokaryotic nitrate reductases. Mol. Microbiol. **19:**1307–1318.
281. **Richterich, P., N. Lakey, G. Gryan, L. Jaehn, L. Mintz, K. Robison, and G. M. Church.** 1993. Centisome 49 region of *Escherichia coli* K-12 BHB2600. GenBank accession no. U00008.
282. **Rietsch, A., D. Belin, N. Martin, and J. Beckwith.** 1996. An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **93:**13048–13053.
283. **Ritz, D.** Unpublished result.
284. **Ritz, D.** 1995. Genetic and biochemical studies on the proteins encoded by the *cycHJKL* gene cluster, essential for the biogenesis of *c*-type cytochromes in *Bradyrhizobium japonicum*. Ph.D. dissertation ETH 11284. Eidgenössische Technische Hochschule, Zürich, Switzerland.
285. **Ritz, D., M. Bott, and H. Hennecke.** 1993. Formation of several bacterial *c*-type cytochromes requires a novel membrane-anchored protein that faces the periplasm. Mol. Microbiol. **9:**729–740.
286. **Ritz, D., L. Thöny-Meyer, and H. Hennecke.** 1995. The *cycHJKL* gene cluster plays an essential role in the biogenesis of *c*-type cytochromes in *Bradyrhizobium japonicum*. Mol. Gen. Genet. **247:**27–38.
287. **Rossmann, R.** Unpublished result.
288. **Rudd, K. E., H. J. Sofia, E. V. Koonin, G. Plunkett, S. Lazar, and P. E. Rouviere.** 1995. A new family of peptidyl-prolyl isomerases. Trends Biochem. Sci. **20:**12–14.
289. **Saiki, K., T. Mogi, and Y. Anraku.** 1992. Heme O biosynthesis in *Escherichia coli*: the *cyoE* gene in the cytochrome *bo* operon encodes a protoheme IX farnesyltransferase. Biochem. Biophys. Res. Commun. **189:**1491–1497.
290. **Saiki, K., T. Mogi, K. Ogura, and Y. Anraku.** 1993. *In vitro* heme O synthesis by the *cyoE* gene product from *Escherichia coli*. J. Biol. Chem. **268:**26041–26044.
291. **Saiki, K., H. Nakamura, T. Mogi, and Y. Anraku.** 1996. Probing a role of subunit IV of the *Escherichia coli bo*-type ubiquinol oxidase by deletion and cross-linking analysis. J. Biol. Chem. **271:**15336–15340.
292. **Sambongi, Y., and S. J. Ferguson.** 1994. Specific thiol compounds complement deficiency in *c*-type cytochrome biogenesis in *Escherichia coli* carrying a mutation in a membrane-bound disulphide isomerase-like protein. FEBS Lett. **353:**235–238.
293. **Sambongi, Y., and S. J. Ferguson.** 1994. Synthesis of holo *Paracoccus denitrificans* cytochrome c_{550} requires targeting to the periplasm whereas that of holo *Hydrogenobacter thermophilus* cytochrome c_{552} does not. FEBS Lett. **340:**65–70.
294. **Sambongi, Y., and S. J. Ferguson.** 1996. Mutants of *Escherichia coli* lacking disulphide oxidoreductases DsbA and DsbB cannot synthesise an exogenous monohaem *c*-type cytochrome except in the presence of disulphide compounds. FEBS Lett. **398:**265–268.
295. **Sambongi, Y., R. Stoll, and S. J. Ferguson.** 1996. Alteration of haem-attachment and signal-cleavage sites for *Paracoccus denitrificans* cytochrome c_{550} probes pathway of *c*-type cytochrome biogenesis in *Escherichia coli*. Mol. Microbiol. **19:**1193–1204.
296. **Sambongi, Y., J.-H. Yang, Y. Igarashi, and T. Kodama.** 1991. Cloning, nucleotide sequence and expression of the cytochrome *c*-552 gene from *Hydrogenobacter thermophilus*. Eur. J. Biochem. **198:**7–12.
297. **Saraste, M., L. Holm, L. Lemieux, M. Lübben, and J. van der Oost.** 1991. The happy family of cytochrome oxidases. Biochem. Soc. Trans. **19:**608–612.
298. **Saraste, M.** 1990. Structural features of cytochrome oxidase. Q. Rev. Biophys. **23:**331–366.
299. **Saraste, M., T. Metso, T. Nakari, T. Jalli, M. Laureus, and J. van der Oost.** 1991. The *Bacillus subtilis* cytochrome *c* oxidase: variations on a conserved protein theme. Eur. J. Biochem. **195:**517–525.
300. **Saraste, M., M. Raitio, T. Jalli, and A. Perämaa.** 1986. A gene in *Paracoccus* for subunit III of cytochrome oxidase. FEBS Lett. **206:**154–156.
301. **Sato-Watanabe, M., T. Mogi, T. Ogura, H. Kitagawa, H. Miyoshi, H. Iwamura, and Y. Anraku.** 1994. Identification of a novel quinone binding site in the cytochrome *bo* complex from *Escherichia coli*. J. Biol. Chem. **269:**28908–28912.
302. **Schiött, T., C. von Wachenfeldt, and L. Hederstedt.** 1997. Identification and characterization of the *ccdA* gene, required for cytochrome *c* synthesis in *Bacillus subtilis*. J. Bacteriol. **179:**1962–1973.
303. **Schlüter, A., S. Rüberg, M. Krämer, S. Weidner, and U. B. Priefer.** 1995. A homolog of the *Rhizobium meliloti* nitrogen fixation gene *fixN* is involved in the production of a microaerobically induced oxidase activity in the phytopathogenic bacterium *Agrobacterium tumefaciens*. Mol. Gen. Genet. **247:** 206–215.
304. **Schulze, M., and G. Rödel.** 1989. Accumulation of the cytochrome *c* oxidase subunits I and II in yeast requires a mitochondrial membrane-associated protein, encoded by the nuclear *SCO1* gene. Mol. Gen. Genet. **216:**37–43.
305. **Schulze, M., and G. Rödel.** 1988. *SCO1*, a yeast nuclear gene essential for accumulation of mitochondrial cytochrome *c* oxidase subunit II. Mol. Gen. Genet. **211:**492–498.
306. **Shankar, S., S. Güner, F. Daldal, M. K. Tokito, C. Moomaw, D. B. Knaff, and J. G. Harman.** 1992. Characterization of the *pet* operon of *Rhodospirillum rubrum*. Photosynth. Res. **32:**79–94.
307. **Shapleigh, J. P., and R. B. Gennis.** 1992. Cloning, sequencing and deletion from the chromosome of the gene encoding subunit I of the aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides*. Mol. Microbiol. **6:**635–642.
308. **Shapleigh, J. P., J. P. Hosler, M. M. J. Tecklenburg, Y. Kim, G. T. Babcock, R. B. Gennis, and S. Ferguson-Miller.** 1992. Definition of the catalytic site of cytochrome *c* oxidase: specific ligands of heme *a* and the heme a_3-Cu_B center. Proc. Natl. Acad. Sci. USA **89:**4786–4790.
309. **Shevchik, V. E., G. Condemine, and J. Robert-Baudouy.** 1994. Characterization of DsbC, a periplasmic protein of *Erwinia chrysanthemi* and *Escherichia coli* with disulfide isomerase activity. EMBO J. **13:**2007–2012.
310. **Siddiqui, R. A., U. Warnecke-Eberz, A. Hengsberger, B. Schneider, S. Kostka, and B. Friedrich.** 1993. Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. J. Bacteriol. **175:**5867–5876.
311. **Smith, A.** 1990. Transport of tetrapyrroles: mechanisms and biological and

regulatory consequences, p. 435–490. *In* H. A. Dailey (ed.), Biosynthesis of heme and chlorophylls. McGraw-Hill Book Co., New York, N.Y.
312. **Smith, G. B., and J. M. Tiedje.** 1992. Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria. Appl. Environ. Microbiol. **58:**376–384.
313. **Sodergren, E., and J. DeMoss.** 1988. *narI* region of the *Escherichia coli* nitrate reductase (*nar*) operon contains two genes. J. Bacteriol. **170:**1721–1729.
314. **Sone, M., Y. Akiyama, and K. Ito.** 1997. Differential *in vivo* roles played by DsbA and DsbC in the formation of protein disulfide bonds. J. Biol. Chem. **272:**10349–10352.
315. **Sone, N., M. Sekimachi, and E. Kutoh.** 1987. Identification and properties of a quinol oxidase super-complex composed of a bc_1 complex and cytochrome oxidase in the thermophilic bacterium PS3. J. Biol. Chem. **262:** 15386–15391.
316. **Sone, N., N. Tsuchiya, M. Inoue, and S. Noguchi.** 1996. *Bacillus stearothermophilus qcr* operon encoding Rieske FeS protein, cytochrome b_6, and a novel-type cytochrome c_1 of quinol-cytochrome *c* reductase. J. Biol. Chem. **271:**12457–12462.
317. **Steiner, H., G. Kispal, A. Zollner, A. Haid, W. Neupert, and R. Lill.** 1996. Heme binding to a conserved Cys-Pro-Val motif is crucial for the catalytic function of mitochondrial heme lyases. J. Biol. Chem. **271:**32605–32611.
318. **Steinrücke, P., E. Gerhus, and B. Ludwig.** 1991. *Paracoccus denitrificans* mutants deleted in the gene for subunit II of cytochrome *c* oxidase also lack subunit I. J. Biol. Chem. **266:**7676–7681.
319. **Steinrücke, P., G. C. Steffens, G. Pankus, and B. Ludwig.** 1987. Subunit II of cytochrome *c* oxidase in *Paracoccus denitrificans*—DNA sequence, gene expression and the protein. Eur. J. Biochem. **167:**431–439.
320. **Stiefel, E. I., and G. D. Watt.** 1979. *Azotobacter* cytochrome *b*-557.5 is a bacterioferritin. Nature **279:**81–83.
321. **Stojiljkovic, I., and K. Hantke.** 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in Gram-negative bacteria. EMBO J. **11:**4359–4367.
322. **Stojiljkovic, I., and K. Hantke.** 1994. Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersinia enterocolitica*. Mol. Microbiol. **13:** 719–732.
323. **Stojiljkovic, I., V. Hwa, L. de Saint Martin, P. O'Gaora, X. Nassif, F. Heffron, and M. So.** 1995. The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. Mol. Microbiol. **15:**531–541.
324. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic *Neisseria* spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:**4670–4678.
325. **Stoller, G., K. P. Rücknagel, K. H. Nierhaus, F. X. Schmid, G. Fischer, and J.-U. Rahfeld.** 1995. A ribosome-associated peptidyl-prolyl *cis/trans* isomerase identified as the trigger factor. EMBO J. **14:**4939–4948.
326. **Sturr, M. G., T. A. Krulwich, and D. B. Hicks.** 1996. Purification of a cytochrome *bd* terminal oxidase encoded by the *Escherichia coli app* locus from a Δ*cyo*Δ*cyd* strain complemented by genes from *Bacillus firmus* OF4. J. Bacteriol. **176:**1742–1749.
327. **Sun, G., E. Sharkova, R. Chesnut, S. Birkey, M. F. Duggan, A. Sorokin, P. Pujic, S. D. Ehrlich, and F. M. Hulett.** 1996. Regulators of aerobic and anaerobic respiration in *Bacillus subtilis*. J. Bacteriol. **178:**1374–1385.
328. **Svensson, B., K. K. Andersson, and L. Hederstedt.** 1996. Low-spin heme A in the heme A biosynthetic protein CtaA from *Bacillus subtilis*. Eur. J. Biochem. **238:**287–295.
329. **Svensson, B., and L. Hederstedt.** 1994. *Bacillus subtilis* CtaA is a heme-containing membrane protein involved in heme A biosynthesis. J. Bacteriol. **176:**6663–6671.
330. **Svensson, B., M. Lübben, and L. Hederstedt.** 1993. *Bacillus subtilis* CtaA and CtaB function in haem A biosynthesis. Mol. Microbiol. **10:**193–201.
331. **Tabche, M. L., E. G. Garcia, J. Miranda, E. Escamilla, and M. Soberon.** 1996. Cloning and sequence analysis of the *Rhizobium etli cycH* gene and its role in the establishment of symbiosis. GenBank accession no. U45318.
332. **Taber, H. W.** 1993. Respiratory chains, p. 199–212. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria. ASM Press, Washington, D.C.
333. **Tamegai, H., and Y. Fukumori.** 1994. Purification, and some molecular and enzymatic features of a novel *ccb*-type cytochrome *c* oxidase from a microaerobic denitrifier, *Magnetospirillum magnetotacticum*. FEBS Lett. **347:** 22–26.
334. **Thomas, J. W., M. W. Calhoun, L. J. Lemieux, A. Puustinen, M. Wikström, J. O. Alben, and R. B. Gennis.** 1994. Site-directed mutagenesis of residues within helix VI in subunit I of the bytochrome bo_3 ubiquinol oxidase from *Escherichia coli* suggests that tyrosine 288 may be a Cu_B ligand. Biochemistry **33:**13013–13021.
335. **Thöny-Meyer, L., C. Beck, O. Preisig, and H. Hennecke.** 1994. The *ccoNOQP* gene cluster codes for a *cb*-type cytochrome oxidase that functions in aerobic respiration of *Rhodobacter capsulatus*. Mol. Microbiol. **14:**705–716.
336. **Thöny-Meyer, L., A. Böck, and H. Hennecke.** 1992. Prokaryotic polyprotein precursors. FEBS Lett. **307:**62–65.
337. **Thöny-Meyer, L., F. Fischer, P. Künzler, D. Ritz, and H. Hennecke.** 1995. *Escherichia coli* genes required for cytochrome *c* maturation. J. Bacteriol. **177:**4321–4326.
338. **Thöny-Meyer, L., P. James, and H. Hennecke.** 1991. From one gene to two proteins: the biogenesis of cytochromes *b* and c_1 in *Bradyrhizobium japonicum*. Proc. Natl. Acad. Sci. USA **88:**5001–5005.
339. **Thöny-Meyer, L., and P. Künzler.** 1997. Translocation to the periplasm and signal sequence cleavage of preapocytochrome *c* depend on *sec* and *lep*, but not on the *ccm* gene products. Eur. J. Biochem. **246:**794–799.
340. **Thöny-Meyer, L., P. Künzler, and H. Hennecke.** 1996. Requirements for maturation of *Bradyrhizobium japonicum* cytochrome c_{550} in *Escherichia coli*. Eur. J. Biochem. **235:**754–761.
341. **Thöny-Meyer, L., H. Loferer, D. Ritz, and H. Hennecke.** 1994. Bacterial genes and proteins involved in the biogenesis of *c*-type cytochromes and terminal oxidases. Biochim. Biophys. Acta **1187:**260–263.
342. **Thöny-Meyer, L., D. Ritz, and H. Hennecke.** 1994. Cytochrome *c* biogenesis in bacteria: a possible pathway begins to emerge. Mol. Microbiol. **12:**1–9.
343. **Thöny-Meyer, L., D. Stax, and H. Hennecke.** 1989. An unusual gene cluster for the cytochrome bc_1 complex in *Bradyrhizobium japonicum* and its requirement for effective root nodule symbiosis. Cell **57:**683–697.
344. **Throne-Holst, M., L. Thöny-Meyer, and L. Hederstedt.** 1997. *Escherichia coli ccm* in-frame deletion mutants can produce periplasmic cytochrome *b* but not cytochrome *c*. FEBS Lett. **410:**351–355.
345. **Trandinh, C. C., G. M. Pao, and M. H. Saier.** 1992. Structural and evolutionary relationships among the immunophilins—2 ubiquitous families of peptidyl-prolyl cis-trans isomerases. FASEB J. **6:**3410–3420.
346. **Trumpower, B. L.** 1990. Cytochrome bc_1 complexes of microorganisms. Microbiol. Rev. **54:**101–129.
347. **Trumpower, B. L., and R. B. Gennis.** 1994. Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: the enzymology of coupling electron transfer reactions to transmembrane proton translocation. Annu. Rev. Biochem. **63:**675–716.
348. **Tsukihara, T., H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, and S. Yoshikawa.** 1996. The whole structure of the 13-subunit oxidized cytochrome *c* oxidase at 2.8 Å. Science **272:**1136–1144.
349. **Turba, A., M. Jetzek, and B. Ludwig.** 1995. Purification of *Paracoccus denitrificans* cytochrome c_{552} and sequence analysis of the gene. Eur. J. Biochem. **231:**259–265.
350. **Tzagoloff, A., N. Capitanio, M. P. Nobrega, and D. Gatti.** 1990. Cytochrome oxidase assembly in yeast requires the product of *COX11*, a homolog of the *P. denitrificans* protein encoded by ORF3. EMBO J. **9:**2759–2764.
351. **Tzagoloff, A., M. Nobrega, N. Gorman, and P. Sinclair.** 1993. On the functions of the yeast COX10 and COX11 gene products. Biochem. Mol. Biol. Int. **31:**593–598.
352. **Ujiiye, T., I. Yamamoto, H. Nakama, A. Okubo, S. Yamazaki, and T. Satoh.** 1996. Nucleotide sequence of the genes, encoding the pentaheme cytochrome (*dmsC*) and the transmembrane protein (*dmsB*), involved in dimethyl sulfoxide respiration from *Rhodobacter sphaeroides* f. sp. *denitrificans*. Biochim. Biophys. Acta **1277:**1–5.
353. **Unden, G., H. Hackenberg, and A. Kröger.** 1980. Isolation and functional aspects of the fumarate reductase involved in the phosphorylative electron transport of *Vibrio succinogenes*. Biochim. Biophys. Acta **591:**275–288.
354. **Uno, T., T. Mogi, M. Tsubaki, Y. Nishimura, and Y. Anraku.** 1994. Resonance Raman and Fourier transform infrared studies on the subunit I histidine mutants of the cytochrome *bo* complex in *Escherichia coli*. J. Biol. Chem. **269:**11912–11920.
355. **van der Berg, J. J. M., F. A. Kuypers, J. H. Qju, D. Chiu, B. Lubin, B. Roelofsen, and J. A. F. Op den Kamp.** 1988. The use of *cis*-parinaric acid to determine lipid peroxidation in human erythrocyte membranes. Comparison of normal and sickle erythrocyte membranes. Biochim. Biophys. Acta **944:**29–39.
356. **van der Oost, J., P. N. de Boer, J.-W. L. de Gier, W. G. Zumft, A. H. Stouthamer, and R. J. M. van Spanning.** 1994. The heme-copper oxidase family consists of three distinct types of terminal oxidases and is related to nitric oxide reductase. FEMS Microbiol. Lett. **121:**1–9.
357. **van der Oost, J., P. Lappalainen, A. Musacchio, A. Warne, L. Lemieux, J. Rumbley, R. B. Gennis, R. Aasa, T. Pascher, B. G. Malmström, and M. Saraste.** 1992. Restoration of a lost metal-binding site: construction of two different copper sites into a subunit of the *E. coli* cytochrome *o* quinol oxidase complex. EMBO J. **11:**3209–3217.
358. **van der Oost, J., C. von Wachenfeldt, L. Hederstedt, and M. Saraste.** 1991. *Bacillus subtilis* cytochrome oxidase mutants: biochemical analysis and genetic evidence for two aa_3-type oxidases. Mol. Microbiol. **5:**2063–2072.
359. **van Doren, S. R., C.-H. Yun, A. R. Crofts, and R. B. Gennis.** 1993. Assembly of the Rieske iron-sulfur subunit of the cytochrome bc_1 complex in the *Escherichia coli* and *Rhodobacter sphaeroides* membranes independent of the cytochrome *b* and c_1 subunits. Biochemistry **32:**628–636.
360. **Vargas, C., G. Wu, A. E. Davies, and J. A. Downie.** 1994. Identification of a gene encoding a thioredoxin-like product necessary for cytochrome *c* bio-

synthesis and symbiotic nitrogen fixation in *Rhizobium leguminosarum*. J. Bacteriol. **176:**4117–4123.
361. **Vasudevan, S. G., W. L. Armarego, D. C. Shaw, P. E. Lilley, N. E. Dixon, and R. K. Poole.** 1991. Isolation and nucleotide sequence of the *hmp* gene that encodes a haemoglobin-like protein in *Escherichia coli* K-12. Mol. Gen. Genet. **226:**49–58.
362. **Verbist, J., F. Lang, N. Gabellini, and D. Oesterhelt.** 1989. Cloning and sequencing of the *fbcF*, *B* and *C* genes encoding the cytochrome b/c_1 complex from *Rhodopseudomonas viridis*. Mol. Gen. Genet. **219:**445–452.
363. **Villani, G., M. Tattoli, N. Capitanio, P. Glaser, S. Papa, and A. Danchin.** 1995. Functional analysis of subunits III and IV of *Bacillus subtilis* aa_3-600 quinol oxidase by *in vitro* mutagenesis and gene replacement. Biochim. Biophys. Acta **1232:**67–74.
364. **Visser, J. M., G. A. H. de Jong, S. de Vries, L. A. Robertson, and J. G. Kuenen.** 1997. cbb_3-type cytochrome oxidase in the obligately chemolithoautotrophic *Thiobacillus* sp. W5. FEMS Microbiol. Lett. **147:**127–132.
365. **von Heijne, G.** 1992. Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. **225:**487–494.
366. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14:**4683–4690.
367. **von Heijne, G.** 1994. *Sec*-independent protein insertion into the inner *E. coli* membrane. A phenomenon in search of an explanation. FEBS Lett. **346:** 69–72.
368. **von Heijne, G.** 1997. Getting greasy: how transmembrane polypeptide segments integrate into the lipid bilayer. Mol. Microbiol. **27:**249–253.
369. **von Ossowski, I., M. R. Mulvey, P. A. Leco, A. Borys, and P. C. Loewen.** 1991. Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. J. Bacteriol. **173:**514–520.
370. **von Wachenfeldt, C., and L. Hederstedt.** 1990. *Bacillus subtilis* 13-kilodalton cytochrome c_{550} encoded by *cccA* consists of a membrane anchor and a heme domain. J. Biol. Chem. **265:**13939–13948.
371. **von Wachenfeldt, C., and L. Hederstedt.** 1990. *Bacillus subtilis* holo-cytochrome *c*-550 can be synthesised in aerobic *Escherichia coli*. FEBS Lett. **270:**147–151.
372. **Voordouw, G., and S. Brenner.** 1986. Cloning and sequencing of the gene encoding cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough). Eur. J. Biochem. **159:**347–351.
373. **Wakabayashi, S., H. Matsubara, and D. A. Webster.** 1986. Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla*. Nature **322:** 481–483.
374. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **8:**945–951.
375. **Wandersman, C.** 1996. Secretion across the bacterial outer membrane, p. 955–966. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
376. **Warren, M. J., and A. I. Scott.** 1990. Tetrapyrrole assembly and modification into the ligands of biologically functional cofactors. Trends Biochem. Sci. **15:**486–491.
377. **Widger, W. R.** 1991. The cloning and sequencing of *Synechococcus* sp. PCC 7002 *petCA* operon: implications for the cytochrome *c*-553 binding domain of cytochrome *f*. Photosynth. Res. **30:**71–84.
378. **Wieseler, B., and M. Müller.** 1993. Translocation of precytochrome c_2 into intracytoplasmic membrane vesicles of *Rhodobacter capsulatus* requires a peripheral membrane protein. Mol. Microbiol. **7:**167–176.
379. **Wieseler, B., E. Schiltz, and M. Müller.** 1992. Identification and solubilization of a signal peptidase from the phototrophic bacterium *Rhodobacter capsulatus*. FEBS Lett. **298:**273–276.
380. **Witt, H., and B. Ludwig.** 1997. Isolation, analysis, and deletion of the gene coding for subunit IV of cytochrome *c* oxidase in *Paracoccus denitrificans*. J. Biol. Chem. **272:**5514–5517.
381. **Wood, P. M., Darlison, M. G., R. J. Wilde, and J. R. Guest.** 1984. Nucleotide sequence encoding the flavoprotein and hydrophobic subunits of the succinate dehydrogenase of *Escherichia coli*. Biochem. J. **222:**519–534.
382. **Wu, G., M.-J. Delgado, C. Vargas, A. E. Davies, R. K. Poole, and J. A. Downie.** 1996. The cytochrome bc_1 complex but not CycM is necessary for symbiotic nitrogen fixation by *Rhizobium leguminosarum*. Microbiology **142:** 3381–3388.
383. **Wülfing, C., and A. Plückthun.** 1994. Protein folding in the periplasm of *Escherichia coli*. Mol. Microbiol. **12:**685–692.
384. **Wunderlich, M., A. Otto, R. Seckler, and R. Glockshuber.** 1993. Bacterial protein disulfide isomerase: efficient catalysis of oxidative protein folding at acidic pH. Biochemistry **32:**12251–12256.
385. **Yamamoto, I., M. Hohmura, and M. Ishimoto.** 1989. A novel gene, *torB*, for trimethylamine *N*-oxide reductase in *Escherichia coli*. J. Gen. Appl. Microbiol. **35:**95–105.
386. **Yamamoto, Y., Y. Hara, K. Tomochika, and S. Shinoda.** 1995. Utilization of hemin and hemoglobin as iron sources by *Vibrio parahaemolyticus* and identification of an iron-repressible hemin-binding protein. FEMS Microbiol. Lett. **128:**195–200.
387. **Yamanaka, T.** 1992. The biochemistry of bacterial cytochromes. Japan Scientific Societies Press, Tokyo.
388. **Yang, C.-H., H. R. Azad, and D. A. Cooksey.** 1996. A chromosomal locus required for copper resistance, competitive fitness, and cytochrome *c* biogenesis in *Pseudomonas fluorescens*. Proc. Natl. Acad. Sci. USA **93:**7315–7320.
389. **Yariv, J., A. J. Kalb, R. Sperling, E. R. Bauminger, S. G. Cohen, and S. Ofer.** 1981. The composition and the structure of bacterioferritin of *Escherichia coli*. Biochem. J. **197:**171–175.
390. **Ye, R. W., A. Arunakumari, B. A. Averill, and J. M. Tiedje.** 1992. Mutants of *Pseudomonas fluorescens* deficient in dissimilatory nitrite reduction are also altered in nitric oxide reduction. J. Bacteriol. **174:**2560–2564.
391. **Yu, C.-A., J.-Z. Xia, A. M. Kachurin, L. Yu, D. Xia, H. Kim, and J. Deisenhofer.** 1996. Crystallization and preliminary structure of beef heart mitochondrial cytochrome-bc_1 complex. Biochim. Biophys. Acta **1275:**47–53.
392. **Yu, J., L. Hederstedt, and P. J. Piggot.** 1995. The cytochrome *bc* complex (menaquinone:cytochrome *c* reductase) in *Bacillus subtilis* has a nontraditional subunit organization. J. Bacteriol. **177:**6751–6760.
393. **Yun, C.-H., R. Beci, A. R. Crofts, S. Kaplan, and R. B. Gennis.** 1990. Cloning and DNA sequencing of the *fbc* operon encoding the bc_1 complex from *Rhodobacter sphaeroides*. Eur. J. Biochem. **194:**399–411.
394. **Yun, C.-H., A. R. Crofts, and R. B. Gennis.** 1991. Assignment of the histidine axial ligands to the cytochrome b_H and cytochrome b_L components of the bc_1 complex from *Rhodobacter sphaeroides* by site-directed mutagenesis. Biochemistry **30:**6747–6754.
395. **Zapun, A., J. C. Bardwell, and T. E. Creighton.** 1993. The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation *in vivo*. Biochemistry **32:**5083–5092.
396. **Zeilstra-Ryalls, J. H., and S. Kaplan.** 1995. Aerobic and anaerobic regulation in *Rhodobacter sphaeroides* 2.4.1: the role of the *fnrL* gene. J. Bacteriol. **177:**6422–6431.
397. **Zhang, L., and L. Guarente.** 1995. Heme binds to a short sequence that serves a regulatory function in diverse proteins. EMBO J. **14:**313–320.
398. **Zollner, A., G. Rödel, and A. Haid.** 1992. Molecular cloning and characterization of the *Saccharomyces cerevisiae CYT2* gene encoding cytochrome *c* haem lyase. Eur. J. Biochem. **207:**1093–1100.
399. **Zufferey, R., H. Hennecke, and L. Thöny-Meyer.** Heme C incorporation into the *c*-type cytochromes FixO and FixP is essential for assembly of the *Bradyrhizobium japonicum* cbb_3-type oxidase. FEBS Lett., in press.
400. **Zufferey, R., O. Preisig, H. Hennecke, and L. Thöny-Meyer.** 1996. Assembly and function of the cytochrome cbb_3 oxidase subunits in *Bradyrhizobium japonicum*. J. Biol. Chem. **271:**9114–9119.
401. **Zufferey, R., L. Thöny-Meyer, and H. Hennecke.** 1996. Histidine 131, not histidine 43, of the *Bradyrhizobium japonicum* FixN protein is exposed towards the periplasm and essential for the function of the cbb_3-type cytochrome oxidase. FEBS Lett. **394:**349–352.
402. **Zumft, W. G.** 1993. The biological role of nitric oxide in bacteria. Arch. Microbiol. **160:**253–264.
403. **Zumft, W. G., C. Braun, and H. Cuypers.** 1994. Nitric oxide reductase from *Pseudomonas stutzeri*. Eur. J. Biochem. **219:**481–490.