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Escherichia coli Genes Required for Cytochrome *c* Maturation

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The so-called *aeg-46.5* region of *Escherichia coli* contains genes whose expression is induced under anaerobic growth conditions in the presence of nitrate or nitrite as the terminal electron acceptor. In this work, we have examined more closely several genes of this cluster, here designated *ccmABCDEFGH*, that are homologous to two separate *Bradyrhizobium japonicum* gene clusters required for the biogenesis of *c*-type cytochromes. A deletion mutant of *E. coli* which lacked all of these genes was constructed. Maturation of indigenous *c*-type cytochromes synthesized under anaerobic respiratory conditions, with nitrite, nitrate, or trimethylamine *N*-oxide as the electron acceptor, was found to be defective in the mutant. The biogenesis of foreign cytochromes, such as the soluble *B. japonicum* cytochrome c_{550} and the membrane-bound *Bacillus subtilis* cytochrome c_{550}, was also investigated. None of these cytochromes was synthesized in its mature form when expressed in the mutant, as opposed to the situation in the wild type. The results suggest that the *E. coli ccm* gene cluster present in the *aeg-46.5* region is required for a general pathway involved in cytochrome *c* maturation.

Type *c* cytochromes are electron transfer proteins of pro- and eukaryotic respiratory chains which contain covalently bound heme (heme C) as their prosthetic group. Although they represent an extremely well-studied class of proteins (21, 25), their biogenesis is not understood in detail. Bacterial cytochrome *c* biogenesis, in particular, has only recently become a field of more intensive research (for reviews, see references 15 and 39).

Bacterial *c*-type cytochromes are located on the periplasmic side of the cytoplasmic membrane. Their posttranslational maturation pathway includes the presumptive transport of heme and the secretion of the apoprotein through the cytoplasmic membrane and the covalent attachment of heme to the apoprotein. Genes that are thought to encode such functions have been identified in several bacterial species, most comprehensively in *Bradyrhizobium japonicum* (29, 31, 32, 39) and *Rhodobacter capsulatus* (2, 3).

Studies devoted to cytochrome *c* biogenesis in *Escherichia coli* have been done only recently (10, 16, 33, 34), perhaps because this organism does not synthesize detectable amounts of cytochromes *c* under aerobic growth conditions. However, anaerobic, nonfermentative growth leads to induction of the synthesis of several *c*-type cytochromes, depending on the electron acceptor that is provided (17). This implies that *E. coli* ought to possess a specific biochemical pathway for cytochrome *c* maturation, possibly similar to the one that has been proposed for other gram-negative bacteria.

In the course of the *E. coli* genome sequencing project, Richterich et al. (30) identified a chromosomal gene cluster in the centisome 49 region that contains several open reading frames (ORFs) with strong similarity to the cytochrome *c* biosynthesis genes of *B. japonicum* and *R. capsulatus*. These ORFs (*yejWVUTSRQP*) appear to represent the 3′ half of the putative *aeg-46.5* operon, whose expression was shown to be induced under anaerobic growth conditions in the presence of nitrate and nitrite (9, 28). The 5′ half of the *aeg-46.5* cluster contains additional genes: *yojG*, *yojB*, *yojA*, and *yejZ*, encoding ferredoxin-like proteins; *yoiC*, which is homologous to a gene (*napA*) coding for a periplasmic nitrate reductase identified in *Alcaligenes eutrophus* (37); and *yejY* and *yejX*, which both code for proteins with conserved heme-binding sites (Cys-X-X-Cys-His) typically found in *c*-type cytochromes. The latter show high similarity to the *Thiosphaera pantotropha napB* and *napC* gene products, which are *c*-type cytochromes involved in the periplasmic nitrite reductase electron transport pathway (4, 5).

In this study, we compared in greater detail the genes in the 3′ half of the putative *E. coli aeg-46.5* operon with the *B. japonicum cycVWZXY* and *cycHJKL* genes. To test their predicted function in cytochrome *c* maturation, we constructed a deletion mutation that removed all of the relevant genes and analyzed the ability of the corresponding *E. coli* mutant to synthesize native and foreign *c*-type cytochromes in their mature form.

MATERIALS AND METHODS

***E. coli* strains and growth conditions.** *E. coli* K-12 strain DH5α (14) was used as the host for clonings, and strain MC1061 (21) was used for cytochrome *c* expression and analysis. Cells were grown either in LB medium (22) or in M9 minimal medium supplemented as described in reference 17. For selective growth, ampicillin was added at 50 to 100 μg ml^{-1}, and kanamycin was added at 30 μg ml^{-1}.

Construction of *ccmABCDEFGH* deletion mutant. A 9.5-kb *Nco*I-*Eco*RI fragment present in the λ phage 371 (19) was cloned into pUCBM20 (Boehringer, Mannheim, Germany), resulting in plasmid pEC2. This plasmid was digested with *Bam*HI, and the three internal *Bam*HI fragments were deleted by replacing them with a 1.7-kb kanamycin resistance cassette from pUC4-KIXX (New England Biolabs, Schwalbach, Germany) that had been digested with *Bam*HI. The resulting recombinant plasmid, pEC6, was linearized with *Eco*RI and *Nde*I, and 1 μg thereof was used to transform *E. coli* MC1061 cells for marker exchange mutagenesis. Southern hybridization analysis was used to identify mutant EC06, in which 5.4 kb of DNA between the *Bam*HI sites in *ccmA* and *ccmH* is deleted. The standard protocols of Sambrook et al. (35) were used for recombinant DNA techniques.

Sequence analysis. To repeat the sequencing of a critical position where we presumed the presence of a frameshift error that was probably caused by a sequence run showing a strong compression, a 364-bp *ccmC*-internal *Nsi*I-*Cla*I fragment was cloned into pUC19 and sequenced by the dideoxy nucleotide chain

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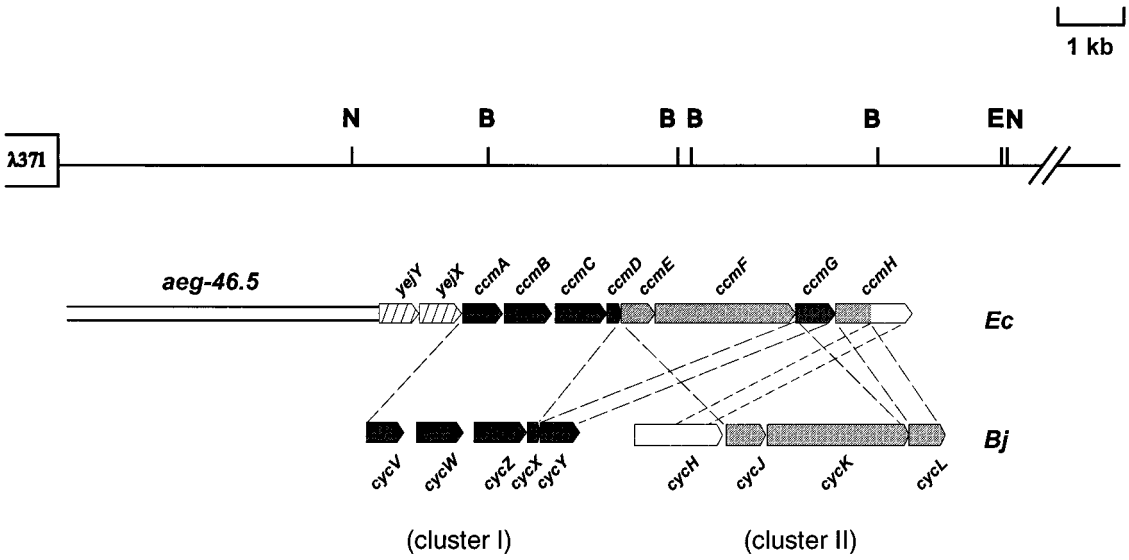

FIG. 1. Comparison of the *E. coli* and *B. japonicum* cytochrome *c* biogenesis gene clusters. On top, a physical map of part of λ clone 371 which contains all of the relevant genes is shown. Below, the 3′ half of the *aeg-46.5* cluster of *E. coli* (Ec) containing the *ccm* genes and the two *cyc* gene clusters of *B. japonicum* (Bj) are shown. The 5′ extension of the *aeg-46.5* cluster has been omitted. Hatched arrows represent genes coding for *c*-type cytochromes. Genes of or homologs to the *B. japonicum* clusters I and II that are 2,850 kb apart (20) are distinguished by dark and light shading, respectively. Open arrows represent genes with questionable homology (see Discussion). The initial designations of the *E. coli* ORFs (*yej* and *yoj* nomenclature) are given in Table 1. Restriction sites: B, *Bam*HI; E, *Eco*RI; N, *Nco*I.

termination method (36) on both strands. The synthetic oligonucleotide primer yejut1 (Microsynth, Balgach, Switzerland) having the sequence 5′-CGTCTG ACTTCTGAACTGG-3′ was used to resolve the compression on one DNA strand. Amino acid sequence comparisons of the *ccm* gene products were done with the TFASTA and GAP programs from the University of Wisconsin Genetics Computer Group (Madison, Wis.).

Enzyme activity. Alkaline phosphatase activity from strains transformed with pCPC20 (41) was determined by the method of Brickman and Beckwith (7). One unit of activity is defined as 1 μmol of *o*-nitrophenol per min per A_{600} unit of cells.

Analysis of *c*-type cytochromes. Extracts of anaerobically grown wild-type and mutant cells were prepared by the protocol described in reference 17. Plasmids pRJ3268, which directs expression of the *B. japonicum cycA* gene (6), coding for cytochrome c_{550} from the arabinose-inducible *araB* promoter (38), and pLUW1954, which contains the *Bacillus subtilis cccA* gene (plus promoter), coding for a membrane-bound cytochrome c_{550} (provided by L. Hederstedt, Lund, Sweden [41]), were transformed into *E. coli* MC1061 and mutant EC06. The transformants were induced for cytochrome *c* production and analyzed as described before (38, 41). Cell fractionation, preparation of extracts, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), heme staining, Western (immunoblot) analysis, and visual absorption difference spectroscopy were done as described previously (38, 40). Antibodies directed against soluble *B. japonicum* cytochrome c_{550} were used at a 1:1,000 dilution. Antibodies against membrane-bound *B. subtilis* cytochrome c_{550} were provided by L. Hederstedt, Lund, Sweden. Both antisera were preadsorbed against *E. coli* MC1061 acetone powder before use in immunoblots.

Nucleotide sequence accession number. The sequence of the corrected *E. coli ccmC* gene in the *aeg-46.5* cluster (accession number U00008) was reported to the GenBank database.

RESULTS

Comparison of genes present in the *E. coli aeg-46.5* cluster with those of *B. japonicum* cytochrome *c* biogenesis gene clusters. The putative translation products of the *E. coli aeg-46.5* cluster downstream of *yejX* were compared with the gene products of the *B. japonicum* cytochrome *c* biogenesis gene clusters *cycVWZXY* and *cycHJKL* (Fig. 1). Homologs to almost all of these genes were clearly identified, the only ambiguous exception being the *cycH* gene (see below). The degree of amino acid sequence identity between the individual *E. coli* ORFs and the *B. japonicum cyc* gene products ranged from 32 to 48%, as shown in Table 1. The *yejU* and *yejT* ORFs, as derived from the previously established DNA sequence (30), appeared to contain a frameshift error. When we tentatively fused the N-terminal 153 amino acids of YejU in frame to the C-terminal 91 amino acids of YejT by adding a single nucleotide in the DNA sequence to create an additional amino acid at the fusion site, a 245-amino-acid gene product with strong similarity to the entire *B. japonicum cycZ* gene product was obtained. The postulated frameshift error was then confirmed by resequencing the DNA encompassing the questionable region on both strands. An additional G was indeed found 405 nucleotides downstream of the *yejU* start codon, resulting in a frameshift that now perfectly fused the *yejU* and *yejT* sequences to make them homologous to *B. japonicum cycZ*. Furthermore, a small ORF (*yojM*) between *yejUT* and *yejS* that had not been included in the list of putative coding regions by Richterich et al. (30) encodes a 69-amino-acid peptide showing 50% similarity with the *B. japonicum cycX* product (Fig. 1 and Table 1). Finally, a CycL-homologous polypeptide (160 amino acids in *B. japonicum*) was predicted from the 5′ half of *yejP*; the entire *yejP* gene, however, codes for 350 amino acids. At first glance, no convincing homology was found for the *B. japonicum cycH*

TABLE 1. Similarity between the *E. coli ccm* and the *B. japonicum cyc* gene products

Gene[a]	*E. coli* gene product (no. of residues)	Amino acids	
		% Similar	% Identical
ccmA/*cycV* (*yejW*)	CcmA (205)	59	39
ccmB/*cycW* (*yejV*)	CcmB (219)	67	42
ccmC/*cycZ* (*yejTU*)	CcmC (245)	71	48
ccmD/*cycX* (*yoiM*)	CcmD (69)	50	25
ccmE/*cycJ* (*yejS*)	CcmE (159)	62	45
ccmF/*cycK* (*yejR*)	CcmF (647)	64	46
ccmG/*cycY* (*yejQ*)	CcmG (185)	61	35
ccmH/*cycL* (*yejP*)	CcmH (350)	56	32

[a] Genes are shown as *E. coli*/*B. japonicum* (*E. coli*). The initial designation of the *E. coli* ORFs submitted to the GenBank database by Richterich et al. (accession number U00008) is given in parentheses.

gene product, whose C-terminal 260 amino acids form a protein domain that is exposed to the periplasm (31). When the sequence of this domain was compared with the sequences of gene products of the entire centisome 49 sequence containing the *aeg-46.5* cluster, the best similarity was obtained with the C-terminal half of YejP (19% identity over 136 amino acids). The C-terminal 190 amino acids of YejP were then optimally aligned with CycH, resulting in a 46% similarity score, which is still somewhat ambiguous. Recently, *cycH* homologs were sequenced from *Paracoccus denitrificans* (26) and *Rhizobium meliloti* (18), for which a certain similarity to the *yejP* ORF was suggested. It is thus possible that the *E. coli yejP* ORF codes for both CycL- and CycH-related functional domains. Hence, all of the *B. japonicum* cytochrome *c* biogenesis genes have a homologous, corresponding gene in the *E. coli aeg-46.5* region. Therefore, and for reasons given below, we changed the initially used *yej* and *yoj* gene designations for the *E. coli* genes and named them *ccm*, for cytochrome *c* maturation (Table 1 and Fig. 1), thus keeping the *cyc* gene nomenclature reserved for structural genes encoding *c*-type cytochromes.

Notably, the order of the *E. coli ccmABCDEFGH* genes differs from that in *B. japonicum* in that all genes are clustered in a single locus and the *cycY* homolog (*ccmG*) is not adjacent to the *ccmABCD* genes but lies between the two genes *ccmF* and *ccmH*.

Cloning of the *E. coli ccm* genes and construction of a *ccmABCDEFGH* deletion mutant. The λ phage 20F6 of the Kohara library (clone 371 of the miniset [19]) was used to clone a 9.5-kb *Nco*I-*Eco*RI fragment (Fig. 1) into pUCBM20, resulting in plasmid pEC2. This plasmid contains the complete coding regions for the two *c*-type cytochromes of the *aeg-46.5* cluster (*yejY* and *yejX*), as well as the *ccmABCDEFGH* genes (Fig. 1).

A 5.36-kb DNA region between the *Bam*HI site in *ccmA* and the *Bam*HI site in *ccmH* was deleted and replaced with a kanamycin resistance cassette, yielding plasmid pEC6. This plasmid was linearized with *Eco*RI and *Nde*I and subsequently transformed into *E. coli* MC1061 for marker replacement mutagenesis. The genomic structure of kanamycin-resistant, ampicillin-sensitive transformants was verified by appropriate Southern blot hybridization experiments; we tested both the absence of the deleted fragments and the presence of the expected new border fragments created by fusion with the kanamycin cassette. The *E. coli* mutant was named EC06.

Requirement for the *ccm* genes for cytochrome *c* formation in *E. coli*. Under nonfermentative, anaerobic growth conditions with nitrate, nitrite, or trimethylamine *N*-oxide (TMAO) as the terminal electron acceptor, *E. coli* synthesizes detectable levels of five different *c*-type cytochromes (17). Wild-type and Δ*ccmABCDEFGH* mutant cells were grown under these conditions, and their content of *c*-type cytochromes was then determined and compared in soluble and membrane fractions. Difference spectra of soluble extracts from the wild type and mutant were first recorded to determine the approximate levels of total cellular *c*-type cytochromes. Figure 2 shows an analysis of soluble extracts prepared from cells grown anaerobically in the presence of fumarate and nitrite. In the ascorbate-reduced minus ammonium persulfate-oxidized spectrum, the *c*-type cytochromes produced a peak at 552 nm that was only detectable in the wild type. Ascorbate plus dithionite-reduced minus ammonium persulfate-oxidized difference spectra of the wild type showed an additional shoulder at 558 nm, indicative of the presence of *b*-type cytochromes. In the mutant, a symmetric peak was observed at 558 nm, suggesting the presence of normal levels of *b*-type cytochromes, whereas the peak at 552 nm was absent (data not shown). Similar results were obtained when extracts from cells grown in the presence of nitrate or TMAO were analyzed (data not shown). Figure 3 shows heme stains specific for *c*-type cytochromes of membranes from wild-type and mutant cells grown in the presence of nitrite and fumarate. None of the heme-staining polypeptides detected in the wild type were visible in the mutant. In summary, the results indicate that, in contrast to the wild type, mutant EC06 is not able to produce mature *c*-type cytochromes under anaerobic nonfermentative growth conditions.

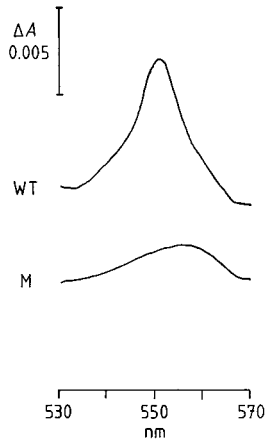

FIG. 2. Ascorbate-reduced minus ammonium persulfate-oxidized difference spectra of soluble fractions from wild-type (WT; 17 mg/ml) and EC06 mutant (M; 15.8 mg/ml) cells. Cells were grown anaerobically in the presence of fumarate and nitrite.

Maturation of foreign *c*-type cytochromes is affected in the Δ*ccm* mutant EC06. To strengthen our hypothesis that the *aeg-46.5* region contains genes involved in a general pathway of cytochrome *c* maturation, we tested the ability of mutant EC06 to produce mature *c*-type cytochromes derived from two other bacterial species.

First, the soluble *B. japonicum* cytochrome c_{550} was expressed in the *E. coli* wild-type and EC06 mutant strains from plasmid pRJ3268, which carries the arabinose-inducible promoter of the *araB* gene in front of *cycA*, the gene encoding apocytochrome c_{550}. We had previously shown that the *B. japonicum* cytochrome c_{550} could be produced as a periplasmic, mature holocytochrome *c* (with covalently bound heme) in *E. coli* MC1061 under anaerobic growth conditions in the presence of nitrate (38). In contrast to the wild type, mutant EC06 lacked mature cytochrome c_{550}, as shown by heme staining of cell extracts (Fig. 4A) and by spectrophotometric analysis of the cytochrome c_{550} content in the periplasmic fractions (data not shown). Western blot analysis with antiserum directed against a synthetic peptide of *B. japonicum* cytochrome

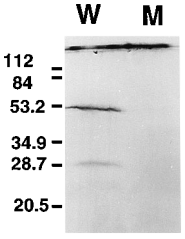

FIG. 3. Heme stain of *E. coli* membrane proteins separated by SDS-PAGE. Wild-type (W) and EC06 mutant (M) membranes were prepared from cells grown anaerobically in the presence of fumarate and nitrite. Equal amounts of protein were loaded (200 μg per lane). The left margin shows the positions of marker proteins, with molecular masses given in kilodaltons.

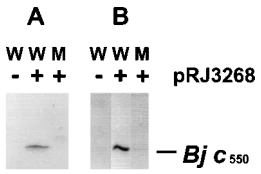

FIG. 4. Expression of *B. japonicum* cytochrome c_{550} in *E. coli* wild-type (W) and Δ*ccm* mutant (M) backgrounds. (A) Heme stain of cell extracts prepared from 1.5 ml of cells with (+) and without (−) the expression plasmid pRJ3268. After heme staining, the proteins were stained with Coomassie brilliant blue to confirm that comparable amounts had been loaded in each lane (not shown). (B) Western blot of periplasmic fractions (approx. 4 μg of protein per lane) from the same strains as in panel A. Antibodies directed against a cytochrome c_{550}-specific synthetic peptide were used. The lanes originated from the same gel. The position of the *B. japonicum* (Bj) cytochrome c_{550} is indicated in the right margin.

c_{550} showed that the apoprotein was completely absent from the mutant cells, whereas the wild type showed a strong cross-reaction (Fig. 4B). Our results are in agreement with the previous observation that apocytochrome c_{550} is rapidly degraded upon arrest of the maturation process (38).

To test whether the deficiency in cytochrome *c* biogenesis in the mutant is protein specific or perhaps more general, expression of a second foreign *c*-type cytochrome from another bacterial source was analyzed. It had been shown previously that the membrane-anchored *B. subtilis* cytochrome c_{550}, the *cccA* gene product, could be synthesized under both aerobic and anaerobic growth conditions in *E. coli* (41). Thus, it was particularly interesting to see whether the genes of the anaerobically induced *aeg-46.5* cluster had an influence on cytochrome *c* maturation in this special case. Plasmid pLUW1954 (41), carrying the entire *cccA* gene together with its *B. subtilis* control region, was transformed into the *E. coli* MC1061 and mutant EC06 strains. Membranes from aerobically grown cells were isolated and analyzed for *B. subtilis* cytochrome c_{550} content by difference spectroscopy (data not shown), heme stains (Fig. 5A), and Western blot analysis (Fig. 5B). The complete absence of cytochrome c_{550} in the mutant clearly demonstrates that the *ccm* deletion affects genes required for a cytochrome *c* biogenesis pathway which does not discriminate between soluble and membrane-bound *c*-type cytochromes. Although expression of the *aeg-46.5* operon is known to be induced under anaerobic growth conditions (9), the "aerobic" shake-flask growth conditions that we used apparently allowed sufficient expression of the *ccm* genes to support cytochrome *c* maturation.

Under no circumstances were apocytochrome *c* precursors

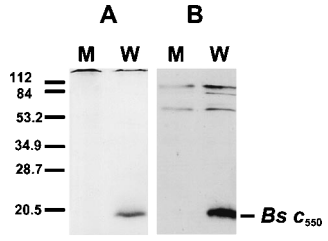

FIG. 5. Expression of *B. subtilis* cytochrome c_{550} in *E. coli* wild-type (W) and Δ*ccm* mutant (M) backgrounds. (A) Heme stain of membrane protein factions separated by SDS-PAGE. Proteins (30 to 35 μg per lane) from aerobically grown cells carrying plasmid pLUW1954 (containing the *B. subtilis cccA* gene) were used. The left margin shows the positions of marker proteins, with molecular masses given in kilodaltons. (B) Western blot of membrane protein fractions from the same strains as in panel A. Antiserum directed against *B. subtilis* cytochrome c_{550} was used. The position of the *B. subtilis* (Bs) cytochrome c_{550} is indicated in the right margin.

TABLE 2. Alkaline phosphatase activity in wild-type and Δ*ccm* mutant backgrounds

Strain/plasmid	*phoA* fusion	PhoA activity (U)
MC1061	None	5.7 ± 0.3
EC06	None	5.2 ± 0.3
MC1061/pCPC20	*cccA'-'phoA*	80 ± 2.2
EC06/pCPC20	*cccA'-'phoA*	89 ± 3.6

detected, probably because of an extreme instability of such intermediates, as suggested previously (38). In the case of the *B. subtilis cccA* gene, which can be expressed from its own promoter in *E. coli*, we tested whether the defect in cytochrome *c* biogenesis in the mutant was posttranslational. A translational *cccA'-'phoA* fusion that had previously been shown to produce active alkaline phosphatase (41) was used to compare PhoA activity in the wild type with that in the mutant (Table 2). We found that PhoA expression in the Δ*ccm* mutant was not altered, indicating that normal levels of translation products were synthesized.

DISCUSSION

The discovery by Richterich et al. (30) of an *E. coli* DNA sequence homologous to the two functionally related *B. japonicum cyc* gene clusters in a single chromosomal locus prompted us to speculate that *E. coli* contains the same set of genes required for the maturation of *c*-type cytochromes. We compared the amino acid sequence of each of the *B. japonicum cyc* gene products with the ORFs of the *aeg-46.5* cluster and assigned the corresponding *ccm* genes as shown in Fig. 1 and Table 1. The *B. japonicum* genes *cycV*, *cycW*, *cycZ*, *cycX*, *cycY*, *cycJ*, and *cycK* have homologs of about the same lengths in *E. coli*, which show 50 to 67% similarity at the level of amino acid sequences. The *B. japonicum* genes *cycL* and *cycH* appear to be fused in *E. coli* in the *ccmH* gene; however, the similarity between the *cycH* gene product and the C-terminal portion of CcmH is not very prominent.

The *E. coli ccm* genes are located immediately downstream of two genes, *yejY* and *yejX*, coding for putative *c*-type cytochromes homologous to the *napB*- and *napC*-encoded *c*-type cytochromes of *T. pantotropha*. A combination of structural and biosynthetic genes for cytochromes within the same operon has been found before in *E. coli*, e.g., in the *cyoABCDE* operon, required for the synthesis of the cytochrome *o* quinol oxidase (8), and in the *nrfABCDE* operon, for the formate-dependent nitrite reductase (16). The latter operon contains two genes encoding *c*-type cytochromes (*nrfA* and *nrfB*) followed by biosynthesis genes (*nrfEFG*).

Can the distribution and order of the different cytochrome *c* biogenesis genes tell us anything about the way that they functionally belong together? In *B. japonicum* and *R. capsulatus*, those genes are located in two separate gene clusters (*cycVW ZXY*/*helABCDX* and *cycHJKL*/*ccl1ccl2*), whereas they are combined in a single locus in *E. coli*. Moreover, the order of the genes is slightly different; in particular, the *ccmG* gene, whose homologs in *B. japonicum* and *R. capsulatus* are linked to the *cycVWZX* and *helABCD* gene clusters, respectively (2, 3), is located between *ccmF* and *ccmH* in *E. coli*. Thus, the CcmG protein may have a more independent function, being compellingly associated neither with the *ccmABCD*-encoded functions nor with those encoded by *ccmEFH*. The clustering and arrangement of the *ccm* genes in *E. coli*, compared with those in other species, make it attractive to speculate that they all encode subunits of a cytochrome *c* maturation supercomplex,

in which heme export and the covalent ligation of heme to apocytochrome *c* can occur in a coordinated manner.

To test the involvement of the *E. coli ccm* genes in cytochrome *c* maturation, we constructed a null mutant (Δ*ccmABCDEFGH*) and analyzed its phenotype with respect to the maturation of both indigenous and foreign *c*-type cytochromes. None of the indigenous *c*-type cytochromes synthesized in the wild type during nitrite, nitrate, or TMAO respiration were detected in the *ccm* deletion mutant EC06. Likewise, maturation of the soluble *B. japonicum* cytochrome c_{550} and the membrane-bound *B. subtilis* cytochrome c_{550} was completely abolished in the mutant. In the case of the *B. subtilis* cytochrome c_{550}, we made sure that this was not due to a block in transcription and translation of its gene (*cccA*). This suggests that the lack of detection of cytochrome *c* in the mutant is due to a posttranslational effect. Since the mutant discriminated neither between membrane-bound and soluble nor between indigenous and foreign *c*-type cytochromes, we argue that the requirement for the *E. coli ccm* genes for cytochrome *c* maturation can be generalized.

E. coli seems to possess two sets of certain cytochrome *c* biogenesis genes. Homologs of *ccmF* and *ccmH*, for instance, are the *nrfE* and *nrfF* genes in the *nrf* operon, required for formate-dependent nitrite reduction (16). In addition, some similarity of the CycH-homologous portion of CcmH with the *nfrG* gene product was observed (26). It is therefore possible that certain cytochrome *c* biogenesis genes are specific for certain *c*-type cytochromes as substrates and thus exist as different variants. For example, the heme-apoprotein ligation reaction may be substrate specific, as has been described for mitochondria, in which different cytochrome *c* heme lyases are used for either cytochrome *c* or cytochrome c_1 (11, 12, 25). In *B. japonicum*, *cycH* may confer some specificity for cytochrome *c* maturation, because mutation of *cycH* causes lack of all *c*-type cytochromes except cytochrome c_1, and there is circumstantial evidence for a second *cycH*-like gene in this organism (24, 31).

Another redundancy may be brought about by CcmG and DipZ, which are both thioredoxin-like proteins required for cytochrome *c* maturation (10, 13, 34). The *dipZ* gene is the only other *E. coli* gene outside the *aeg-46.5* region that has been described as being involved more generally in cytochrome *c* biogenesis (33, 34), yet it may also affect other processes, such as the production of active human placental alkaline phosphatase in *E. coli* (1). Thus, DipZ protein may contribute to the control of the redox state in the periplasm rather than being part of the primary cytochrome *c* maturation complex.

No redundant occurrence of the *ccmABC* genes has been observed so far. They are presumed to encode a heme exporter (3, 29, 39). This would explain why maturation of all of the *c*-type cytochromes is affected in mutant EC06. The construction of separate mutations in each of the *ccm* genes may help in the future to distinguish between specific and general functions in cytochrome *c* maturation.

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ADDENDUM IN PROOF

While this article was being typeset, we learned that a similar Δ*ccm* mutant of *E. coli* was also constructed in the laboratory of J. Cole (University of Birmingham, Birmingham, United Kingdom). Apart from having a defect in cytochrome *c* biogenesis, this strain was also defective in formate-dependent nitrite reduction (Nrf^- phenotype; J. Cole, personal communication).

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