Dynamic Features of a Heme Delivery System for Cytochrome c Maturation*

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In *Escherichia coli*, heme is delivered to cytochrome c in a process involving eight proteins encoded by the *ccmABCDEFGH* operon. Heme is transferred to the periplasmic heme chaperone CcmE by CcmC and from there to apocytochrome c. The role of CcmC was investigated by random as well as site-directed mutagenesis. Important amino acids were all located in periplasmic domains of the CcmC protein that has six membrane-spanning helices. Besides the tryptophan-rich motif and two conserved histidines, new residues were identified as functionally important. Mutants G111S and H184Y had a clear defect in CcmC-CcmE interaction, did not transfer heme to CcmE, and lacked c-type cytochromes. Conversely, mutants D47N, R55P, and S176Y were affected neither in interaction with nor in delivery of heme to CcmE but produced less than 10% c-type cytochromes. A strain carrying a CcmCE fusion had a similar phenotype, suggesting that CcmC is important not only for heme transfer to CcmE but also for its delivery to cytochrome c. Co-immunoprecipitation of CcmE with CcmF was not detectable although CcmE co-precipitated individually with CcmC and CcmF. This contradicts the idea of CcmCEF supercomplex formation. Our results favor a model that predicts CemC to shuttle between CcmC and CcmF for heme delivery.

The covalent attachment of heme to the CXXCH signature motif of apocytochromes is a critical step during cytochrome c biosynthesis. The process of cytochrome c maturation involves the formation of two thioester bonds within the vinyl groups of heme and the cysteinyl residues of apocytochrome c. Three distinct systems for the biogenesis of c-type cytochromes have evolved in different classes of organisms, which are annotated as systems I, II, and III (reviewed in Refs. 1–4). System I is present in α- and γ-proteobacteria, *Deinococcus*, archaea, and protozoa and plant mitochondria. System II prevails in Gram-positive bacteria, cyanobacteria, some β-, δ-, and ε-proteobacteria, and plant and algal chloroplasts. System III cytochrome c maturation operates in fungal and metazoan mitochondria. The γ-proteobacterium *Escherichia coli* requires eight cytoplasmic membrane proteins encoded by the *ccmABCDEFGH* operon (5, 6). These proteins catalyze the covalent attachment of heme to the conserved CXXCH motifs of apocytochromes c. In *E. coli*, c-type cytochromes are synthesized exclusively under anaerobic respiratory conditions, under which the expression of the *ccm* genes is also induced (7–9). One of the key steps in the maturation pathway is the transfer and covalent attachment of heme to the cytochrome c maturation-specific heme chaperone, CcmE (10); subsequently, heme is transferred from the holo-CcmE intermediate to apocytochrome c. It has been shown earlier that covalent binding of heme to an essential histidine (His130) of apo-CcmE requires the activity of the CcmC maturation factor (11). CcmC is an integral membrane protein whose topology was derived from that of its homologues in *Rhodobacter capsulatus* and Pseudomonas fluorescens (12, 13). *E. coli* CcmC shares 41% identity and 58% similarity with its *R. capsulatus* homologue HelC and 52% identity and 70% similarity with its *P. fluorescens* homologue. According to the experimentally established membrane topology of these proteins, CcmC contains six transmembrane domains, separated by two cytoplasmic and three periplasmic domains (14) (Fig. 1).

An *E. coli ccmC* in-frame deletion mutant is deficient in the production of c-type cytochromes (15), most likely because heme cannot be transferred to CcmE (11). Several *ccmC* mutants from different organisms have been described, which were not only affected in cytochrome c maturation but also exhibited other phenotypes. Particularly interesting was the analysis of *P. fluorescens ccmC* mutants, which showed that CcmC is required for the production of pyoverdines, a fluorescent siderophore (13, 16). In *Paracoccus denitrificans*, a *ccmC* mutant in addition to deficiencies in cytochrome c maturation and siderophore production showed intolerance to rich medium (17). The connection between these processes is not known. In the pathogenic bacterium *Legionella pneumophila*, CcmC was found to be required for cytochrome c production, for growth in low iron conditions, and for at least some forms of intracellular infection of eukaryotic hosts (18).

CcmC contains a conserved tryptophan-rich signature motif (WGXXWXXWDXRXL, where ϕ represents an aromatic amino acid residue) (1, 3, 19, 20) that resides in the second periplasmic domain (Fig. 1) and shares a high degree of similarity with the tryptophan-rich motif of CcmF, Nre, and the CesA homologues of type II cytochrome c maturation (21). In addition, two absolutely conserved histidines are present in the first and third periplasmic domains of CcmC. Mutational analysis of these motifs of the *E. coli* CcmC protein revealed that they are functionally important (22). The accumulation of hydrophobic residues within the tryptophan-rich motif was initially thought to provide a platform for the binding of heme with the two conserved histidines serving as axial heme ligands (12). Only recently it was shown that CcmC can bind heme and that the tryptophan-rich motif is involved in a direct interaction with CcmE (14). Apart from the amino acids in this motif, CcmC contains several other highly conserved residues, which have never been studied so far in regard to their role in heme binding and/or delivery during cytochrome c maturation. In this study, we asked whether additional domains or amino acids...
acids are required for the function of CcmC. This is important because CcmC undergoes protein-protein interaction with at least CcmE but most likely also with CcmD, a small cytoplasmic-oriented membrane protein (22), and perhaps also with the ABC transporter subunits CcmAB (23). Although CcmC seems to bind heme (14), no mutants with defects in heme binding have been described so far. If CcmC is involved in heme transport, one might find residues with membrane-oriented membrane protein (22), and perhaps also with the ABC transporter subunits CcmAB (23). Although CcmC seems to bind heme (14), no mutants with defects in heme binding have been described so far. If CcmC is involved in heme transport, one might find residues with membrane-oriented membrane protein (22), and perhaps also with the ABC transporter subunits CcmAB (23). Although CcmC seems to bind heme (14), no mutants with defects in heme binding have been described so far. If CcmC is involved in heme transport, one might find residues with membrane-oriented membrane protein (22), and perhaps also with the ABC transporter subunits CcmAB (23). Although CcmC seems to bind heme (14), no mutants with defects in heme binding have been described so far. If CcmC is involved in heme transport, one might find residues with membrane-oriented membrane protein (22), and perhaps also with the ABC transporter subunits CcmAB (23). Although CcmC seems to bind heme (14), no mutants with defects in heme binding have been described so far. If CcmC is involved in heme transport, one might find residues with membrane-
with CcmD and CcmE was constructed by the QuikChange™ site-directed mutagenesis (Stratagene) using pEC406 as a template. For the construction of a plasmid expressing CcmF, the ccmF gene was amplified by PCR using a forward primer containing the NdeI site and a reverse primer with an EcoRI site. A 2-kb NdeI-EcoRI fragment containing ccmF was then cloned behind the arabinosine promoter of pISC2 (30), resulting in pEC825. Plasmid pEC826 expressing the CcmCE fusion protein was constructed by QuikChange™ site-directed mutagenesis with a primer pair that eliminates the ccmC stop codon in pEC809 by creation of an adenine nucleotide. In plasmid pEC409, the ccmC stop codon overlaps with start codon of ccmE. The plasmids pEC803, pEC804, pEC805, pEC806, pEC807, pEC808, pEC811, and pEC824 expressing CcmC with V177G, D47N, R55P, R128A, and L82A were used as starting points for mutagenesis studies.

**Random Mutagenesis by Using a Repair-deficient Strain**—Random mutagenesis of the ccmC gene was carried out in the Stratagene XL1-Red strain. Plasmid pEC486 containing wild-type ccmC was transformed into E. coli XL1-Red. This strain lacks three key enzymes for DNA repair, which leads to a 5000-fold increase in the mutation rate. DNA extracted from approximately 1000 colonies was extracted and electroporated into the EC28/H9262 strain. DNA extracted and precipitated with 5 M dIATP, dCTP, dGTP, and 180 M dTTP for 25 cycles. The PCR products were digested with NcoI and NdeI and ligated into NdeI- and NcoI-digested pEC486. The ligated product was then used to complement pEC821. The protein concentrations were determined using the Bradford assay (Bio-Rad) and bovine serum albumin as a standard. Holo-cytochrome c550 and holo-CcmE formation was analyzed qualitatively by immunoblot and heme staining (22). Soluble cytochrome c550 was quantitatively determined by a difference spectroscopy using a 5-mM [Fe(His)6]2+ standard. The proteins were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antisera against the tetra-His tag (for detection of other antigens) conjugated to alkaline phosphatase (Bio-Rad) as secondary antibodies and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate.
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**RESULTS**

**Rationale of the Screen**—In the ccmC in frame deletion mutant EC28Δdms (ΔA63–G156; ΔdmsABC::kan), the predicted trans-membrane helices II, III, and IV of CcmC together with the periplasmic domain II containing the tryptophan-rich motif are deleted. A ΔccmCΔdmsABC double mutant was constructed by P1 transduction of a ΔdmsABC::kan allele from a mutant kindly provided by Dr. J. Weiner. DmsABC constitutes an Me₂SO reductase that catalyzes the reduction of Me₂SO and TMAO under anaerobic conditions. The dms mutant cannot grow anaerobically on minimal medium with glycerol and 52064 dms TMAO under anaerobic conditions. The growth phenotype observed on TMAO plates under anaerobic conditions is indicative of cytochrome c maturation (ccm). The organism can facilitate heme delivery for cytochrome c maturation (ccm) using the NapABC system, and also from particularly strong signals in the immunoblot (data not shown). These mutants were generated from the double mutant was constructed by P1 transduction of a ΔdmsABC::kan allele from a mutant kindly provided by Dr. J. Weiner. DmsABC constitutes an Me₂SO reductase that catalyzes the reduction of Me₂SO and TMAO under anaerobic conditions. The dms mutant cannot grow anaerobically on minimal medium with glycerol and Me₂SO (26). However, the TMAO reductase allows *E. coli* to grow by anaerobic respiration with TMAO as a terminal electron acceptor. TMAO reductase is a hetrodimeric enzyme composed of TorA, a periplasmic reductase containing molybdenum as cofactor and TorC, a membrane-bound pentaheme c-type cytochrome. During anaerobic TMAO respiration, electrons are transferred from the menaquinone pool to TorC and then delivered to TorA, which in turn reduces TMAO to trimethylamine (31). TorC requires the cytochrome c maturation (ccm) system composed of the CcmABCDEGH proteins to insert the heme covalently. Any block of TorC maturation is expected to abolish anaerobic growth on TMAO, when the alternative pathway for electron transfer via Me₂SO reductase is excluded. Hence, the ΔccmCΔdms mutant had a strong anaerobic growth phenotype; in contrast to the wild type, which formed large red colonies on TMAO plates, the mutant grew poorly and was white. The remaining growth capability of this mutant may be due to yet another Me₂SO reductase encoded by a second set of dmsABC genes that are expressed constitutively (32). When the mutant was transformed with a plasmid expressing ccmC-H₆ encoding wild-type, His-tagged CcmC, the cells were red and formed normal size colonies, but when an empty vector was used as control, they were small and white. This phenotype was the basis for a color screen for ccmC mutants.

**Generation and Analysis of Random ccmC Mutants**—Random mutations were introduced into plasmid-borne ccmC described above by (i) error-prone PCR and (ii) the use of a repair-deficient strain for plasmid propagation. Based on the red/white phenotypes, we screened 14,905 colonies. 241 white candidates were tested for expression of full-length CcmC polypeptide by immunoblot using anti-His tag antibodies. 35 mutants were found to produce normal levels of the protein. This step allowed the exclusion of all mutants in which stop codons had been introduced accidentally or that produced unstable protein. The plasmids were isolated and sequenced. We found 14 single, 10 double, and four triple mutants. The following single mutations were identified (Table III): D47N, R55P (twice), G111S (twice), W125I (twice), S176Y (three times), L183P, and H184Y. Despite the high number of redundant hits at individual amino acids from independent experiments, some residues that had been altered previously were not mutated, suggesting that we had not reached saturation. Redundant mutants were found for both mutagenesis procedures. Surprisingly, in seven cases a wild-type sequence was found along with a high plasmid copy number, as was evident from the increased amount of plasmid DNA and also from particularly strong signals in the immunoblot (data not shown). These mutants were generated from the repair-deficient strain. It is possible that the mutant phenotype imparted to the strains harboring high copy plasmids was due to a dominant negative effect exerted by overexpressed CcmC on cytochrome c maturation. Such mutants were not pursued for further analysis. However, when wild-type ccmC was overexpressed from the pACYC184 vector relative to the other chromosomally expressed ccm genes, a dominant negative effect was not observed (data not shown).

Eight single mutants (Table III) were selected for a detailed analysis; among them, all except one (H184Y) mapped to residues that had not been investigated previously. H184Y was included in our analysis for a comparison with the previously described mutant H184A (22). The seven newly identified, important amino acids Asp⁴⁷, Arg⁵⁰, Gly¹¹¹, Trp¹¹⁹, Ser¹⁷⁶, Val¹⁷⁷, and Leu¹⁸³ were all located in periplasmic domains of CcmC (Fig. 1). To test the mutants for loss of cytochrome c maturation, they were grown anaerobically with nitrate as the sole electron acceptor. *E. coli* has multiple respiratory nitrate reductases (33–35), and one of them, the NarGHI enzyme, does not contain c-type cytochromes. Hence, the organism can facultatively produce holo-cytochrome c as part of an electron transport system to the periplasmic nitrate reductase, the NapABC system, and ccm mutants can grow without suffering from a selective pressure when cytochrome c maturation is blocked. This implies that formation of revertants or second site suppressors is not enforced. In our complementation experiments, we overexpressed exogenous *Bradyrhizobium japonicum* cytochrome c₅₅₀ to better monitor the effect of a mutation in CcmC. The ΔccmCΔdmsABC mutant strain was co-

| Class | Method | Mutant | Amino acid identity* | Level of CcmC* | Level of CcmC* | Co-immunoprecipitation* | Holo-CcmC* | Colony phenotype* |
|-------|--------|--------|----------------------|----------------|----------------|-------------------------|-----------|-----------------|
| Wild type | Random | G111S | + + + | ++ + | ++ + | ++ + | + (100) | Red |
| Class I | Random | W119R | + + + | + + | + (0.31) | White |
| Site-directed | D126A | + + + | ++ + | + (8.2) | White |
| Random | V177G | 96 | ++ + | ++ + | (1.2) | White |
| Random | L183P | 96 | ++ + | ++ + | White |
| Random | H184Y | 100 | ++ + | -- | -- | White |
| Class II | Random | D47N | 92 | ++ + | ++ + | + (6.9) | White |
| Random | R55P | 84 | ++ + | ++ + | + (6) | White |
| Site-directed | W114A | 100 | ++ + | ++ + | ++ + | (40) | Pink |
| Site-directed | W125I | 100 | ++ + | ++ + | ++ + | (18.5) | Pink |
| Site-directed | R128A | 100 | ++ + | ++ + | (13.7) | Pink |
| Random | S176Y | 88 | ++ + | ++ | -- | White |
| Class III | Site-directed | L82A | 96 | ++ + | ++ + | ++ + | (103) | Red |
| Site-directed | Y139A | 100 | ++ + | ++ + | ++ + | (84) | Red |
| Site-directed | W180A | 100 | ++ + | ++ + | ++ + | (61.5) | Red |

* Identity based on the sequences derived from the organisms listed in the legend of Fig. 1.  
* Levels were established from the signal intensities in the immunoblots and the heme stains presented in Figs. 2–4.  
* Spectroscopically determined concentration of soluble c-type cytochromes as described under “Experimental Procedures.”  
* Growth phenotype observed on TMAO plates under anaerobic conditions.
transformed with a plasmid expressing a His-tagged *B. japonicum* cytochrome *c*$_{550}$ plus a plasmid encoding either the His-tagged wild-type or mutant CcmC protein (Fig. 2A). Cytochrome *c* formation was assayed by heme staining (Fig. 2A, upper panel) and immunoblot (lower panel) of periplasmic fractions. The apocytochrome is degraded rapidly when heme is not incorporated (30). Quantification of *c*-type cytochromes was done by absorption difference spectrosopy. None of the mutants produced wild-type levels of *c*-type cytochromes. The mutants D47N (lane 3) and R55P (lane 4) localized in the first and V177G (lane 8) in the third periplasmic domain were the least affected in the cytochrome *c* formation when compared with the other random mutants. Mutant W119R (lane 6) and mutant L183P (lane 9) produced extremely low but detectable levels of holocytochrome *c*. Mutants G111S, S176Y, and H184Y and the double mutant D126G/H184Y lacked detectable amounts of cytochrome *c* (lanes 5, 7, 10, and 11). The H184Y phenotype matched that of the previously described mutant H184A (22). In contrast, in comparison with the D126G/H184Y double mutant, the single change of D126A in the tryptophan-rich motif had resulted in functional CcmC (22). The reduced minus oxidized spectra were recorded for the quantification of soluble type *c* cytochromes. The value for the wild type was assigned 100%, and relative values of the various *ccmC* mutants were calculated accordingly. Compared with the wild-type cytochrome *c* level (100%), mutants D47N, R55P, W119R, and V177G produced only 6.9, 6, 0.3, and 1.2% cytochrome *c*, respectively, whereas cytochrome *c* formed in mutant L183P was below the limit of detection. Since the random mutants affected cytochrome *c* maturation in varying degrees, it was necessary to investigate whether or not the mutations had (i) destabilized the protein in the membrane, (ii) affected CcmC-CcmE interaction, or (iii) affected the ability of CcmC to bind heme. The known function of CcmC is the delivery of heme to the heme chaperone CcmE, from where heme then is transferred in a second step to the apocytochrome. Hence, we isolated membranes from cells deleted in the entire *ccm* operon (EC06) and containing the plasmid pEc799 encoding CcmD and CcmE plus a plasmid expressing either the wild-type or a mutant *ccmC* allele. The levels of the CcmC (Fig. 2B, first panel) and CcmE polypeptides (Fig. 2B, second panel) were probed by immunoblot analysis and found to be the same for all strains; the control strain containing only CcmE but no CcmC (lane 2) showed no reaction. Holo-CcmE formation determined

**Fig. 1. Proposed topology model for E. coli CcmC.** The topology model was constructed by using the program HMMTOP (40). It predicts six transmembrane helices, four cytoplasmic domains, and three periplasmic domains. Conserved histidines and the Trp-rich motif are located in the periplasm. Amino acid residues characterized in this study are boxed. Black boxes represent amino acids for which a new phenotype was found (class II mutants). Gray boxes represent amino acid residues important for heme delivery to CcmE. Open boxes represent amino acid residues that were altered without an effect. Residues shown in *boldface type* are 100% conserved in CcmC homologues from the following species, derived from whole genome sequences deposited at NCBI: *E. coli*, *Helophilus influenzae*, *Yersinia pestis*, *Sinorhizobium meliloti*, *Agrobacterium tumefaciens*, *Rickettsia conorii*, *Rickettsia prowazekii*, *Pasteurella multiscida*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Salmonella typhimurium*, *Pantoaea citrea*, *Paracoccus denitrificans*, *B. japonicum*, *R. capsulatus*, *Pseudomonas putida*, *Shewanella putrefaciens*, *Rhodobacter sphaeroides*, *Vibrio cholerae*, *Brucella melitensis*, *Mesorhizobium loti*, *Caulobacter crescentus*, *Xylella fastidiosa*, *Deinococcus radiodurans*, and *Arabidopsis thaliana*. Dynamic Heme Delivery for Cytochrome c Maturation 52065
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Fig. 2. Functional analysis of random ccmC mutants. A, the ΔccmCdms double mutant EC28 dmsABC::kan was co-transformed with plasmid pRJ3290 (encoding B. japonicum cytochrome c550[His]) and pEC486 (wild-type CcmC-His6; lane 1), pACYC184 (empty vector; lane 2), pEC804 (D47N; lane 3), pEC805 (R55P; lane 4), pEC824 (G111S; lane 5), pEC806 (W119R; lane 6), pEC807 (S176Y; lane 7), pEC803 (V177G; lane 8), pEC808 (L183P; lane 9), pEC815 (H184Y; lane 10) or pEC815 (D126G/H184Y; lane 11). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel, periplasmic proteins (200 µg/lane) were separated by 15% SDS-PAGE and stained with Coomassie brilliant blue (CCM). Lower panel, immunoblot of trichloroacetic acid-precipitated periplasmic protein (50 µg/lane) probed with antisera against the His tag of cytochrome c550. NapB is a native periplasmic soluble c-type cytochrome expressed under these conditions. The positions of NapB and cytochrome c550 are indicated on the right. B, the ΔccmCA-H1 mutant EC06 was co-transformed with plasmid pEC799 (encoding CcmDE) and the same plasmids as in A. Cells were grown anaerobically in the presence of 5 mM sodium nitrate, and membranes were prepared. Proteins were separated by 15% SDS-PAGE. First panel, immunoblot of membrane proteins (100 µg/lane) probed with antisera against the His tag. Second panel, immunoblot of membrane proteins (100 µg/lane) probed with antisera against CcmE. Third panel, heme stain of the same membrane proteins (100 µg/lane). Fourth panel, the same membrane proteins (500 µg/lane) were immunoprecipitated with tetra-His antibodies, and co-precipitating CcmE was detected with anti-CcmE serum. The positions of CcmC and apo- and holo-CcmE are indicated on the right.

by heme stains (Fig. 2B, third panel) was normal in mutants D47N (lane 3) and R55P (lane 4), whereas mutants W119R, S176Y, V177G, and L183P (lanes 6–9) contained less holo-CcmE than the wild type (lane 1). Mutants G111S (lane 5) and H184Y (lane 10) and the double mutant D126G/H184Y (lane 11) lacked holo-CcmE completely. We also investigated the effect of the mutations on the physical interaction between CcmC and CcmE (Fig. 2B, bottom panel). A co-immunoprecipitation experiment revealed that mutants D47N (lane 3) and R55P (lane 4) co-precipitated CcmC and CcmE similar to the wild type (lane 1). Mutants S176Y, V177G, and L183P (lanes 7–9) showed slightly reduced and mutant W119R drastically reduced levels of co-precipitated CcmE. For mutants G111S (lane 5) and H184Y (lane 10) and the double mutant D126G/H184Y (lane 11), no CcmC-CcmE co-precipitation was found. We also tested the effect of the mutations on affinity binding to hemin-agarose, since it has been described previously (14), but no significant differences were observed (data not shown).

Analysis of ccmC Site-directed Mutants—CcmC contains a number of highly conserved residues, of which those localized in the core of the tryptophan-rich motif (Trp114-Gly119-Trp134-Xaa-Xaa-Trp134-Xaa-Trp134-Asp180) and the histidines at positions 60 and 184 have been altered previously (22). Here, we focused on additional conserved residues of the CcmC protein that had failed to be picked up in our random screen; the conserved amino acids Leu62 and Tyr149 in transmembrane segment II and IV, respectively, Trp114 and Arg228 in an extension of the Trp-rich motif, and Trp180 in the third periplasmic domain were changed to alanine. In addition, the C-terminal 23 residues were deleted to test the role of this cytoplasmic domain. The ΔccmCdmsABC mutant strain was complemented for cytochrome c biosynthesis in the presence of plasmid pRJ3290 encoding the B. japonicum cytochrome c550 plus a plasmid encoding either the wild-type or a mutant ccmC allele, and cytochrome c formation was tested by heme staining, immunoblot (Fig. 3A), and absorption difference spectra (Table III). Most affected in cytochrome c maturation were the mutants R128A (lane 5) and W114A (lane 4) on either side of the Trp-rich motif, whereas the other mutants in residues predicted to be in transmembrane domains (lanes 3 and 6) were almost like the wild type. When the C-terminal 23 residues were deleted, a drastic effect on cytochrome c formation was observed (lane 8). In the next experiment, the presence of CcmC and CcmE in the membrane, their interaction, and the efficiency of heme delivery to CcmE were investigated as described for the random mutants. The levels of the CcmC and CcmE polypeptides in the membrane and the formation of holo-CcmE in cells deleted in the entire ccm operon (EC06) and containing the plasmid pEC799 expressing ccmDE plus a plasmid with either the wild-type or a ccmC-His8 mutant allele were determined. The results presented in Fig. 3B (first and second panels) show that all mutants produced normal levels of CcmC and CcmE except for the C-terminal CcmC deletion, in which the levels of CcmC but not CcmE were decreased. Although this cytoplasmic domain is not well conserved, it seems to be important for the stability of the protein. Mutants L82A (Fig. 3B, third panel, lane 3), W114A (lane 4), R128A (lane 5), and W180A (lane 7) produced normal (wild type) levels (lane 1), whereas mutant Y139A (lane 6) produced less holo-CcmE. The C-terminal deletion mutant was strongly affected in heme delivery, since very little holo-CcmE was detected, most likely due to the small amount of CcmC polypeptide.

The physical interaction between CcmC and CcmE was also investigated (Fig. 3B, bottom panel). None of the site-directed mutants except for the C-terminal deletion mutant was affected in the physical interaction between the two proteins. We also tried with a 2-fold higher concentration of the C-terminally truncated CcmC mutant protein to co-precipitate CcmE but failed to detect it (data not shown).

Next we investigated the effect of the mutations on the affinity of CcmC to hemin-agarose. No significant differences between the wild-type and the mutants were observed (data not shown). We also detected some heme binding of the truncated CcmC, but the signal was comparatively weak, which could be due to the instability of the protein. In summary, cytochrome c maturation of the site-directed mutants was not
affected drastically in any case, and only the C-terminal deletion mutant had a clear deficiency. The site-directed mutant with the strongest effect on cytochrome c maturation (R128A) still produced more cytochrome c than the least affected random mutant (D47N). This could explain why mutations in the strongly conserved residues had not been picked up in our screen. Thus, our screen preferentially detects mutants severely affected in cytochrome c maturation. In fact, when the site-directed mutants were grown under the conditions of our screening procedure, none of them were white, confirming that they produced intermediate levels of c-type cytochromes.

Analysis of ccmC Mutants in the Tryptophan-rich Motif—The importance of the tryptophan-rich motif for CcmC function has been well documented. It is involved in the physical interaction between CcmC and CcmE (14). In previous work, mutants in residues Trp119, Trp125, and Asp126 had affected the cytochrome c formation slightly, whereas mutants in Gly119, Thr121, Trp122, and Trp125 had produced wild-type levels of cytochromes c (22). In this work, we were interested in investigating the individual contribution of the conserved amino acids in the extended Trp-rich motif to cytochrome c maturation. We thus included the new mutants W114A, W119R, and R128A and compared them with W125I and D126A in a detailed analysis. The mutants were used to complement cytochrome c biosynthesis in the ΔccmCAΔdmsABC strain (Fig. 4A). Whereas the changes W114A and W125I affected cytochrome c maturation only slightly, the R128A, D126A, and in particular W119R had more severe consequences, with W119R producing less than 1% of the c-type cytochromes seen in the wild type (Table III). The influence of the mutation on the presence of the CcmC and CcmE polypeptides in the membrane as well as the ability of the mutants to interact with CcmE and to deliver heme was investigated (Fig. 4B). We analyzed the levels of CcmC peptide, CcmE peptide, and holo-CcmE in cells deleted in the entire ccm operon (EC06) and containing the plasmid pEC799 expressing ccmDE plus a plasmid encoding either wild-type or a mutant CcmC-His6. The results (Fig. 4) show that all mutants produced normal levels of CcmC and CcmE. Mutants W119R and D126A (lanes 3 and 5, respectively) produced lower levels of holo-CcmE, although the polypeptide was synthesized normally, a finding that is in agreement with an earlier study (22). However, in a co-precipitation experiment, the interaction of CcmE with mutant W119R is clearly disturbed (Fig. 4B, bottom panel, lane 3).

Analysis of ccmC Mutants in the Absence of ccmD—In all of our experiments, holo-CcmE formation was investigated in the presence of CcmD. CcmD is a small monomeric membrane protein, which had been shown to influence heme ligation to apocytochrome c (22). In particular, it had been observed that the presence of CcmD can suppress a ccmC mutant phenotype prevailing in the absence of CcmD. Here, we investigated the ccmC mutants that had affected cytochrome c maturation drastically without apparently affecting holo-CcmE formation. We analyzed levels of CcmC polypeptide, CcmE polypeptide, and holo-CcmE in cells deleted in the entire ccm operon (strain EC06) and containing the plasmid pEC412 encoding CcmE plus a plasmid encoding either the wild-type or a mutant CcmC-His6. The results (Fig. 4) show that all mutants produced normal levels of CcmC and CcmE. Mutants W119R and D126A (lanes 3 and 5, respectively) produced lower levels of holo-CcmE, although the polypeptide was synthesized normally, a finding that is in agreement with an earlier study (22). However, in a co-precipitation experiment, the interaction of CcmE with mutant W119R is clearly disturbed (Fig. 4B, bottom panel, lane 3).

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CcmC Does Not Bind CcmF—One of the most striking findings of this mutant analysis was the discovery of a new phenotype of mutants D47N, R55P, and S176Y with a deficiency in cytochrome c formation despite normal levels of apo- and holo-CcmE and CcmC-CcmE interaction (Table III). One plausible explanation for this might be a direct interaction with another factor catalyzing the detachment of heme from holo-CcmE and its subsequent transfer to apocytochrome c. CmF had previously been shown to directly interact with CcmE, and it was...
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**Fig. 4. Functional analysis of ccmC mutants in tryptophan-rich motif.** A, the ΔccmCΔdms double mutant EC28 Δdms was co-transformed with plasmid pRJ3290 (encoding His-tagged B. japonicum cytochrome c550) and pEC486 (wild-type CcmC-His, lane 1), pEC816 (W114A; lane 2), pEC806 (W119R; lane 3), pEC828 (W125I; lane 4), pEC829 (D126A; lane 5), pEC817 (R128A; lane 6). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Upper panel, periplasmic proteins (200 µg/lane) were separated by 15% SDS-PAGE and stained for covalently bound heme. Lower panel, immunoblot of trichloroacetic acid-precipitated periplasmic proteins (50 µg/lane) probed with antiserum against the His tag. The positions of NapB and cytochrome c550 are indicated on the right. B, the ΔccmA-H mutant EC06 was co-transformed with plasmids pEC799 (encoding CcmDE) and the same plasmids as in A. Cells were grown anaerobically in the presence of 5 mM sodium nitrate. First panel, immunoblot of membrane proteins (100 µg/lane) probed with antiserum against CcmE. Third panel, heme stain of the same membrane proteins (100 µg/lane) after separation by 15% SDS-PAGE. Fourth panel, membrane proteins (500 µg/lane) from the indicated strains were immunoprecipitated with trichloroacetic acid, separated by 15% SDS-PAGE, and immunodetected with anti-CcmE serum. The positions of CcmC, CcmE, and holo-CcmE are indicated on the right.

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**Fig. 5. Functional analysis of ccmC mutants in absence of ccmD.** The ΔccmA-H mutant EC06 was co-transformed with plasmid pEC799 (encoding CcmDE) and pEC486 (wild-type CcmC-His, lane 1), pEC816 (W114A; lane 2), pEC806 (W119R; lane 3), pEC828 (W125I; lane 4), pEC829 (D126A; lane 5), pEC817 (R128A; lane 6), pEC806 (W119R; lane 7), pEC804 (D47N; lane 8), pEC805 (R55P; lane 9), or pEC807 (S176Y; lane 10). Cells were grown anaerobically in the presence of 5 mM sodium nitrate. Proteins were separated by 15% SDS-PAGE. First panel, immunoblot of membrane proteins (100 µg/lane) probed with antisera against the His tag. Second panel, immunoblot of membrane proteins (100 µg/lane) probed with antisera against CcmE. Third panel, heme stain of the same membrane proteins (100 µg/lane). The positions of CcmC and apo- and holo-CcmE are indicated on the right.

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**DISCUSSION**

Heme delivery is an essential step of cytochrome c maturation. In _E. coli_, the heme chaperone CcmE plays a crucial role in this process by binding heme transiently in a covalent manner that is still not understood in detail. However, it is known that CcmC, the subject of the present study, is needed for heme delivery. In _Burkholderia japonicum_, the heme chaperone CcmE plays a crucial role in the formation of holo-CcmE. Since CcmE shuttles between CcmC and CcmF and that CcmC mutants might prevent dissociation of CcmE from CcmC, we genetically fused the N terminus of CcmE to the C terminus of CcmC. We expected to obtain a functional fusion protein, because both termini are predicted to be in the cytoplasm. We thus tested whether we could find the same phenotype as we had obtained in mutants with a defect in the cytochrome c maturation but not in the formation of holo-CcmE. We first tested heme delivery to CcmE in the fusion protein. The CcmE fusion protein was charged with heme, but in addition, accumulation of a degradation product corresponding in size to the wild-type holo-CcmE was also observed (Fig. 6A). Additionally, we found that heme was preferentially delivered to apo-CcmE rather than to the apo-CcmE fusion protein (lane 2). To assess cytochrome c formation, ΔccmC (EC28) and ΔccmE (EC65) cells were complemented with the plasmid pEC826 encoding the CcmCE fusion or plasmid pEC409 encoding CcmC and CcmE individually. We included plasmid pRJ3290 encoding exogenous _B. japonicum_ cytochrome c550 for a better detection of low amounts of holocytochrome c. The fusion protein could efficiently complement neither the ΔccmC nor the ΔccmE mutant background as compared with the controls where CcmC and CcmE were expressed individually in the respective backgrounds. The low amounts of holocytochrome c formation in the mutant strains expressing the fusion protein might be attributed either to a low activity of the fusion protein or to the activity exerted by the cleaved product obtained from the fusion protein (Fig. 6B). Since the CcmCE fusion protein cannot efficiently complement cytochrome c maturation, this represents another line of support for the idea that CcmE must be mobile for efficient heme delivery.
transfer to CcmE at the periplasmic side of the membrane. The signature motif WGXXXXWD of CcmC and the two conserved histidines His60 and His184 have been shown to play an important role for the interaction with CcmE (14). However, the number of additional highly conserved residues within the 245-amino acid polypeptide was too high for a rational selection of specific residues for site-directed mutagenesis. Strikingly, many of the best conserved residues map to the three periplasmic loops, whereas the six-transmembrane helices are generally hydrophobic, and the small cytoplasmic parts are the least conserved. The genetic and biochemical analysis of CcmC has confirmed the role of the protein in heme delivery to CcmE, but it is still unclear whether CcmC transports heme across the membrane or picks it up on the periplasmic side of the membrane, either from the outer leaflet or from a heme transporter, for docking it specifically to the heme chaperone CcmE. A random mutagenesis of ccmC was initiated to answer two questions. (i) Can cytoplasmic or membrane-integral amino acids be identified as essential structures for CcmC function? If CcmC were a heme permease, an inward-oriented heme binding site might be identified. In our study, we could not identify essential residues in these domains. Site-directed mutations of Leu82 on the cytoplasmic side of the transmembrane helix II and Tyr139 in the middle of transmembrane helix IV to alanines had no effect on CcmC function. A deletion of the C-terminal cytoplasmic extension led to an assembly or stability problem, and only low levels of truncated CcmC were obtained. However, the protein was useful for heme transfer to CcmE but not for cytochrome c maturation. (ii) Are additional, not strictly conserved amino acids essential for CcmC function? Expected mutant phenotypes ranged from a complete block in heme binding, heme delivery, and CcmC-CcmE interaction to partially functioning proteins. For example, it was possible to obtain a mutant that interacted with heme and CcmE but was unable to transfer heme to CcmE? This could indicate an active participation of CcmC in the chemistry of heme attachment to CcmE. This phenotype was not found among our mutants.

The genetic screen applied to learn more about CcmC was designed such that it can be used later for other ccm genes as well. The combination of a dms with a ccm mutant led to a colony phenotype that could be easily distinguished from the wild type. In fact, the colony phenotype in our screen proved to be a very sensitive way to test cytochrome c maturation.

An important finding of this study is that all mutants with a cytochrome c-negative phenotype mapped to periplasmic domains. New residues were shown to be functionally important: in domain I by mutants D47N and R55P, in domain II by mutant G111S, and in domain III by mutants S176Y, V177G, and L183P. Other residues had previously been changed to alanine by site-directed mutagenesis (22). One of the important histidines, His184, and Trp119 within the Trp-rich motif were changed to Tyr and Arg, respectively, during the random mutagenesis. In addition, some highly conserved residues that had not been hit by the random mutagenesis were changed to alanine; W114A and R128A, representing CcmC-specific extensions of the Trp-rich motif, turned pink, and L82A close to the cytoplasmic side of the membrane and Y139A in the middle of transmembrane helix IV formed red colonies like the wild type. Truncation of the C-terminal, cytoplasmic domain led to low levels of CcmC protein in the membrane. The truncated protein was able to transfer some heme to CcmE and affected cytochrome c maturation even more. The physical interaction with CcmE was not detectable, perhaps due to the low level of protein.

G111S and H184Y were the only mutants that lacked interaction with CcmE. An interaction between CcmC and CcmE, to which residues in the periplasmic domains II and III contrib-
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However, we could not find signals in co-precipitation experiments between CcmC and CcmF. This finding implies that CcmC does not interact with CcmF, neither in the complex with CcmF was tested and rejected because co-precipitation between CcmC and CcmF was not observed, although CcmE precipitated with CcmC and CcmF individually. Our findings imply that CcmC does not interact with CcmF, neither directly nor indirectly via CcmE, and that CcmC and CcmF are involved in different, physically separable steps of cytochrome c maturation. Formally, it is also possible that CcmC interacts not only with CcmE but also with apocytochrome c directly. However, we could not find signals in co-precipitation experiments between CcmC and apocytochrome c (data not shown). From this work, we have no evidence for a "maturase supercomplex" present in E. coli membranes as was hypothesized previously (1). As a more likely alternative explaining the cytochrome c deficiency of heme delivery-positive ccm mutants, we consider CcmE to shuttle between CcmC and CcmF for heme transfer to apocytochrome c (Fig. 7). Subsequent dissociation of CcmC from CcmE may fail in these mutants, thereby inhibiting a proficient follow-up reaction of CcmE with CcmF.

If a dynamic movement of CcmE between other components of the cytochrome c maturation machinery is essential, the irreversible binding of the two proteins in a CcmE fusion polypeptide might stop the process. Therefore, a CcmE fusion protein was constructed and functionally characterized. Heme delivery to the CcmE part of the fusion proteins was quite efficient, but a wild-type CcmE protein was the preferred heme acceptor. Unfortunately, the fusion protein produced a degradation product approximately the size of wild-type CcmE, suggesting that a protease-sensitive site close to the fusion site was present. Nevertheless, it was obvious that the fusion protein was less efficient in cytochrome c formation than two separate molecules, again supporting the idea of CcmE shuttling between CcmC and CmF.

It had been noted previously that the small membrane protein CcmD is involved in heme transfer to CcmE by CcmF. It was not surprising to see that our mutants were all defective in heme delivery in the absence of CcmD. How CcmD influences the efficiency of heme transfer is unclear. We speculate that a direct interaction of CcmD with CcmC, CcmE, or both takes place. The role of CcmD will be addressed in future work.

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