Escherichia coli ccm in-frame deletion mutants can produce periplasmic cytochrome *b* but not cytochrome *c*

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Abstract ***Escherichia coli*** **CcmA, CcmB and CcmC polypeptides are required for cytochrome *c* synthesis and are thought to constitute the subunits of an ABC-type transporter as judged from sequence data. Using a periplasmic reporter system based on *Bacillus subtilis* cytochrome *c*-550 and *E. coli* cytochrome *b*-562 we show that the synthesis of the *b*-type cytochrome in the periplasm is normal in *E. coli ccmA* and *ccmC* in-frame deletion mutants. Mutants deleted for *ccmF* or *ccmG* encoding a component of a putative cytochrome *c*-heme lyase and a membrane bound thioredoxin-like protein, respectively, have the same phenotype. The *ccm* mutants produce cytochrome *c*-550 polypeptide, but not holocytochrome *c*. Taken together the results demonstrate that heme can be transported to the periplasm by a *ccm*-independent mechanism.**

Key words: Cytochrome *c* synthesis; ABC-transporter; Cytochrome *b*-562; Heme C; *ccm* Gene

1. Introduction

It is not known how heme is transported from the cytoplasm to the outer side of the cytoplasmic membrane in bacteria. Such transport seems required for the synthesis of all known *c*-type and some *b*-type cytochromes that mature in the periplasm [1,2]. The main difference in the synthesis of cytochromes of *b*- and *c*-type is that in the case of the *c*-type cytochromes it involves covalent attachment of heme to the polypeptide (*b*-type cytochromes bind heme non-covalently).

Genes for two putative heme transporters have been identified in *Escherichia coli*:*cydDC* and *ccmABC* [3,4]. The CydDC and CcmABC polypeptides show no apparent sequence similarity except for the ATP-binding sequence motifs present in CydC, CydD, and CcmA. Mutants defective in *cydC* or *cydD* lack both cytochrome *bd*- and *c*-type cytochromes [4,5]. It has been shown that a *cydC* mutant is partially defective, but not blocked, in the synthesis of periplasmic *b*-type cytochromes [5]. Hence, if CydDC constitute a heme-transporter in the cytoplasmic membrane, it cannot be the only one in *E. coli*. The *ccmABC* genes are the first in a cluster of eight genes encoding proteins for cytochrome *c* maturation [3]. Genes for proteins homologous to the *E. coli ccm* gene products are present in other Gram-negative bacteria, e.g. *Bradyrhizobium japonicum* and *Rhodobacter capsulatus* [2]. The CcmA, -B, and -C polypeptides correspond to the CycV, -W, and -Z polypeptides of *B. japonicum* and the HelA, -B, and -C polypeptides of *R. capsulatus*, respectively [6,7].

To analyse whether CcmA (CycV/HelA) and CcmC (CycZ/HelC) function in heme-transport we have used *E. coli ccmA* and *ccmC* deletion mutants in combination with an engineered periplasmic heme-reporter protein based on *Bacillus subtilis* cytochrome *c*-550 and *E. coli* cytochrome *b*-562. Cytochrome *c*-550 is a membrane-bound 13 kDa protein encoded by the *cccA* gene [8]. The CccA polypeptide is composed of an N-terminal membrane-anchor and a C-terminal cytochrome *c* domain located on the outside of the cytoplasmic membrane. Cytochrome *b*-562 is a soluble 12 kDa monomeric protein found in the periplasm of some *E. coli* strains [9–11]. It is encoded by the *cybC* gene and contains one non-covalently bound heme. Both cytochromes have histidine/methionine axial ligation of the heme iron and have similar midpoint redox potentials at pH 7, i.e. +178 mV for cytochrome *c*-550 and +180 mV for cytochrome *b*-562 [12,13]. The reporter protein consists of the membrane-anchor domain of CccA (residues 1–32) fused to the CybC polypeptide without its native signal-peptide (residues 23–128), i.e. it is a membrane-bound cytochrome-like cytochrome *c*-550 but contains non-covalently bound protoheme IX (Fig. 1).

We predicted that the CccA–CybC fusion protein with heme incorporated, i.e. holocytochrome, would not be synthesised in *E. coli ccmA* and *ccmC* mutants if the proteins encoded by these genes are essential components of a general heme-transporter in the cytoplasmic membrane.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this work are presented in Table 1. Construction of the Δ*ccm* in-frame deletion mutants used in this work will be described elsewhere (Thöny-Meyer, Fabianek, Fischer, Künzler, Schulz and Hennecke, manuscript in preparation). The Δ*ccmA*, Δ*ccmC*, and Δ*ccmG* mutants contain three or four additional codons at the site of the deletion due to linker sequences used for the constructions (Table 1). The construction of pLUT193 will be described elsewhere (Schiött, Throne-Holst and Hederstedt, manuscript in preparation). The *cccA–cybC* hybrid gene in pLUT193 consists of bp 18–638 of the *B. subtilis cccA* DNA sequence [8] fused to bp 250–703 of *E. coli cybC* [9]. The hybrid gene is expressed from the *cccA* promoter.

2.2. Growth of bacteria and preparation of membranes

Bacteria were grown in 1 l of LB medium containing 100 mg/l ampicillin in 5 l indentated E-flasks at 37°C on a rotary shaker (200 rpm). About 1 h into stationary growth phase ($OD_{600} \approx 1.2$) the bacteria were harvested by centrifugation at 4400×*g* for 10 min at 4°C, washed in 40 ml of 30 mM Tris-HCl, pH 8.0, and suspended in the Tris buffer (1 ml/g w.wt. cells) containing 0.5 g/l lysozyme and 2 mM Na-EDTA. After 30 min at 30°C, one volume of 50 mM potassium phosphate buffer, pH 6.6, containing 5 mM $MgSO_4$ and 2 mg/l DNase was added. The suspension was passed twice through a pre-

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cooled French Press cell operated at 18000 psi. The resulting cell lysate was incubated at 30°C for 30 min before cell debris was removed by centrifugation at 5000×*g* for 10 min at 4°C. Membranes were then collected by centrifugation at 100000×*g* for 60 min at 4°C and washed in 30 ml 60 mM Tris-HCl, pH 8.0, containing 10 mM Na-EDTA (pH 7.4). The membranes were finally homogenised in 20 mM Na-MOPS/HCl buffer, pH 7.0, to give a final volume of 0.2 ml/g (w.wt.) starting cell material.

2.3. Isolation of the periplasmic cell fraction

Cells grown and harvested as described under Section 2.2 were washed in 30 ml of 0.5 M sucrose, 0.1 M Tris-HCl, 1 mM Na-EDTA, pH 8.0, and suspended in 10 ml of ice-cold 0.5 M sucrose, 0.1 M Tris-HCl, 1 mM Na-EDTA, pH 8.0. After 10 min incubation on an ice-bath, lysozyme was added to a final concentration of 16 mg/l and immediately thereafter 10 ml of ice-cold water was added. The suspension was mixed using a Wortex and incubated for 5 min in an ice-bath before $MgSO_4$ was added to a final concentration of 18 mM and the suspension centrifuged at 10000×*g* for 20 min at 4°C. The clear supernatant was taken as the periplasmic cell fraction. The pellet was suspended in 20 ml of 25 mM Tris-HCl, pH 8.0, and passed twice through a pre-cooled French Press cell operated at 18000 psi to obtain the total cell lysate. The extent of contamination of the periplasmic cell fraction by cytoplasmic material was estimated from the malate dehydrogenase activity in that fraction compared to the activity in the total cell lysate.

2.4. Other methods

Competent *E. coli* cells were prepared by $CaCl_2$ treatment. Protein concentrations were determined using the bicinchoninic acid protein assay (Pierce Chem. Co.) with bovine serum albumin as standard. Light absorption spectroscopy was carried out as described before [14]. Western blot analysis with antibodies against *B. subtilis* cytochrome *c*-550 was performed as described previously [3]. Malate dehydrogenase activity was determined as the oxaloacetate-dependent oxidation of NADH [15].

3. Results

Plasmid pLUT193 (encoding the CccA–CybC heme-reporter protein), pLUW1954 (encoding *B. subtilis* cytochrome *c*-550), and pUC19, were transformed into the *E. coli* strains EC21 (Δ*ccmA*), EC28 (Δ*ccmC*), EC29 (Δ*ccmG*), EC50 (Δ*ccmF*), and the parental strain MC1061. *E. coli* MC1061, like other K12 strains, has a defective *cybC* gene in the chromosome and therefore lacks cytochrome *b*-562 [10]. The *B. subtilis cccA* gene is known to be expressed in *E. coli* and results in membrane bound cytochrome *c*-550 with normal properties [14].

Membranes isolated from the different transformed strains were analysed by visible light absorption spectroscopy to determine the content of cytochromes *b* and *c*. Ascorbate was used to preferentially reduce the high potential heme in cytochrome *b*-562 and *c*-550. The spectra are presented in Fig. 2.

Membranes of EC21/pLUT193 and MC1061/pLUT193 contained cytochrome *b*-562 as judged from the absorption peak at 562 nm after reduction with ascorbate (Fig. 2, traces A and B) and the absence of such a peak in the spectra of EC21/pUC19 and MC1061/pUC19 (Fig. 2, traces E and F). We noted that membranes of the mutant consistently contained higher amounts of cytochrome *b*-562 than the wild type.

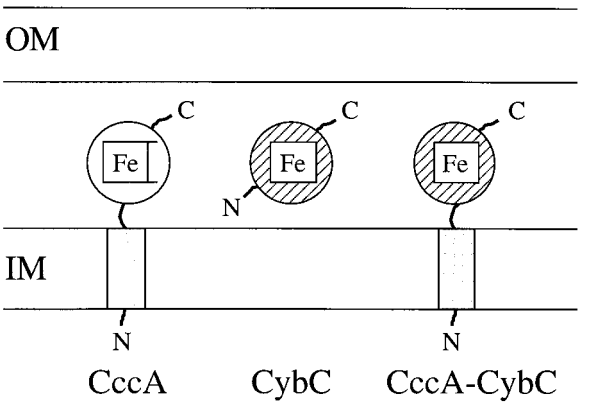

Fig. 1. Schematic illustration of membrane-bound CccA, periplasmic CybC and membrane-bound CccA–CybC reporter protein in the *E. coli* envelope. The cytochrome *c*-550 domain and membrane anchor of CccA are indicated in white and grey, respectively. CybC, cytochrome *b*-562, is hatched. Heme groups are indicated as boxes. IM and OM are the inner and outer membrane, respectively.

The membranes of strain MC1061/pLUW1954 contained cytochrome *c*-550 as judged from the absorption peak at 550 nm in ascorbate-reduced sample (Fig. 2, trace D). Essentially no cytochrome absorbing at 550 nm was present in ascorbate-reduced membranes from strain EC21/pLUW1954 (Fig. 2, trace C) as expected because of the *ccm* mutation. Spectra of ascorbate- and dithionite-reduced membranes from strains EC28, EC29 and EC50 containing pLUT193, pLUW1954 or pUC19 were very similar to those of strain EC21 containing these plasmids (spectra not shown). To demonstrate that the cytochrome *c* polypeptide is also synthesised and inserted into the membrane in a *ccm* negative background, we performed Western blot analysis with EC21/pLUW1954 membranes and anti-*B. subtilis* cytochrome *c*-550 serum (Fig. 3). As a control for background reaction MC1061/pUC19 was used (Fig. 3, lane 1). With MC1061/pLUW1954 (Fig. 3, lane 2) a strong signal was obtained at the position where holocytochrome *c*-550 migrates [3]. Weaker exposure of the blot allowed resolution of this signal into two bands (data not shown). In strain EC21/pLUW1954 (Fig. 3, lane 3) a faint signal corresponding to the upper band was detected. Since holocytochrome *c*-550 is not produced in EC21 we conclude that this form represents the apoprotein that is synthesised in the mutant. The same results were found for strains EC28/pLUW1954 and EC29/pLUW1954 (data not shown).

To confirm that cytochrome of the *b*-type can form in the periplasm in the absence of CcmA we also analysed strain EC21/pNS207. Plasmid pNS207 contains the native *E. coli cybC* gene which encodes soluble periplasmic cytochrome *b*-562 [9]. The periplasmic cell fractions of MC1061/pNS207 and

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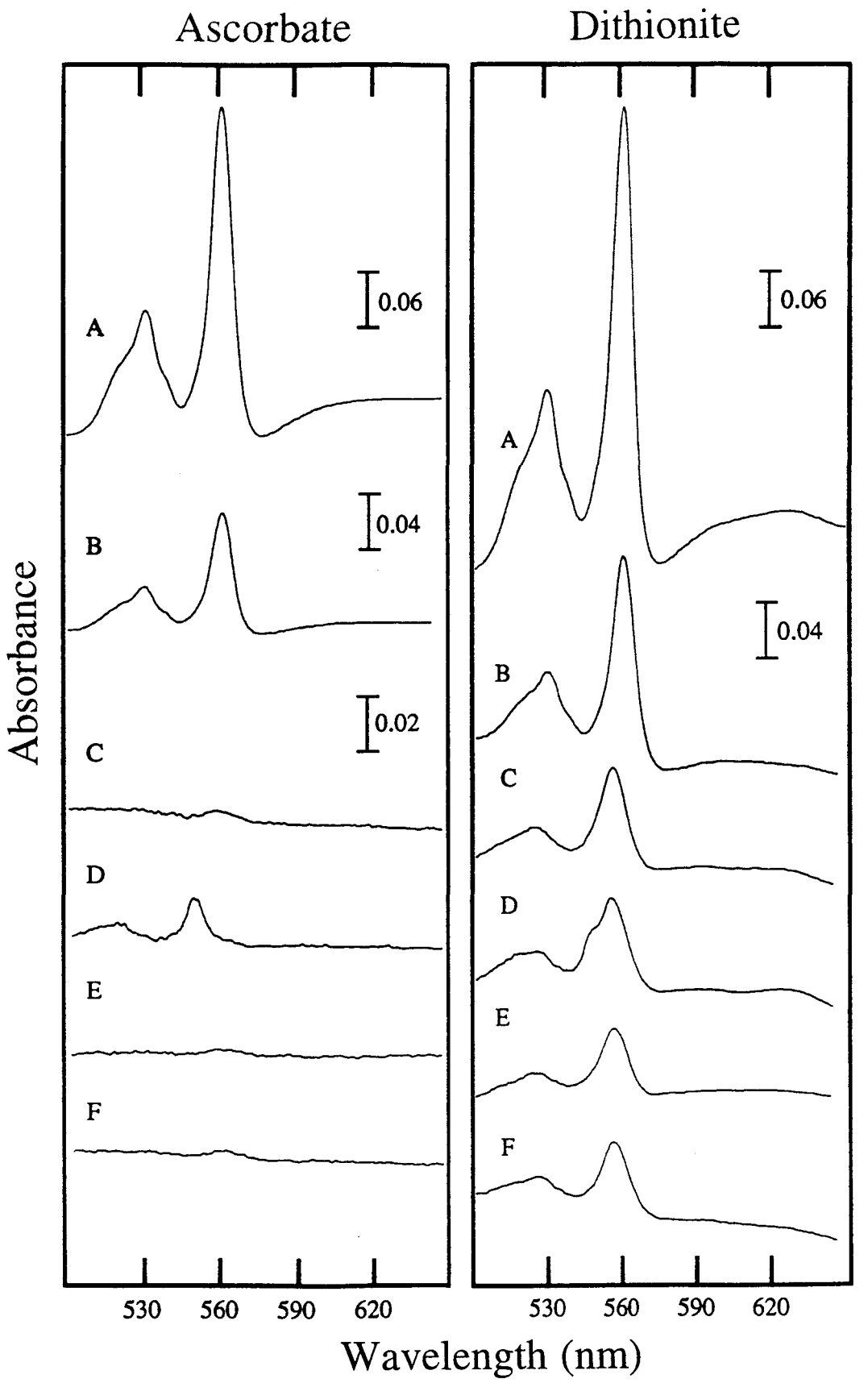
Fig. 2. Difference (reduced minus oxidised) light absorption spectra of isolated *E. coli* membranes. A, EC21/pLUT193; B, MC1061/pLUT193; C, EC21/pLUW1954; D, MC1061/pLUW1954; E, EC21/pUC19; F, MC1061/pUC19. The membranes, 4 mg protein/ml, were in 20 mM Na-MOPS/HCl, pH 7.0, containing 2.2 mM KCN. The content in the reference cuvette was oxidised with 1 mM $K_3Fe(CN)_6$. To the sample cuvette was first added 8 mM sodium ascorbate (pH = 6.3) to reduce high potential cytochromes (left-hand panel). A few grains of solid sodium dithionite was then added to reduce all cytochromes (right-hand panel). The absorbance scales are indicated by bars.


Ascorbate
Dithionite
Absorbance
0.06
0.04
0.02
A
B
C
D
E
F
530
560
590
620
Wavelength (nm)

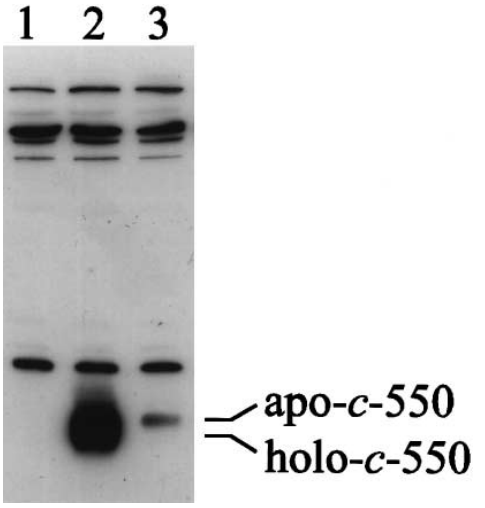


Fig. 3. Western blot analysis of *B. subtilis* cytochrome *c*-550 polypeptides expressed in *E. coli*. Membrane proteins (25 μg/lane) of MC1061/pUC19 (lane 1), MC1061/pLUW1954 (lane 2) and EC21/pLUW1954 (lane 3) were separated by 15% SDS PAGE, immunoblotted and analysed using antiserum directed against *B. subtilis* cytochrome *c*-550. The positions of apo- and holocytochrome *c*-550 are indicated on the right.

EC21/pNS207, and, as negative control, MC1061/pUC19, were isolated using osmotic shock and analysed by light absorption spectroscopy as above. The periplasmic fraction of both the wild type and the mutant contained cytochrome *b*-562, whereas that of the control strains with pUC19 lacked detectable amounts of cytochrome (spectra not shown). The contamination of the periplasmic cell fractions with cytoplasm was 14% and 19% for MC1061/pNS207 and EC21/pNS207, respectively, as determined using malate dehydrogenase as a marker enzyme for cytoplasm.

4. Discussion

Our experimental results demonstrate that membrane-bound and soluble periplasmic cytochrome of the *b*-type, but not cytochrome of the *c*-type, can be synthesised in *E. coli* mutants deleted for *ccmA*, *ccmC*, *ccmF* and *ccmG*, respectively.

The *E. coli ccmABCDEFGH* gene cluster is situated at 46.5 min on the *E. coli* chromosome immediately downstream of the *napFDAGHBC* cluster which encodes a periplasmic anaerobic nitrate reductase [3,16]. The *nap* and *ccm* genes consitute the '*aeg*46.5' operon which is transcribed from a Fnr-dependent, NarL- and NarP-regulated promoter located in front of *napF* [16]. Goldman et al. recently showed that periplasmic cytochrome of the *b*-type can be formed in an *E. coli* mutant with a kanamycin resistance gene inserted into the *aeg46.5*– promoter region [5]. From these results the authors made the same conclusion as we do in this work, i.e. that the *ccm* genes are not required for heme-transport to the periplasm. However, expression of the *ccm* gene cluster is not completely blocked in such a promoter-insertion mutant because *ccm* genes are also transcribed from promoters within the *nap* gene region [16]. In this work we used mutants deleted for *ccm* genes to unambiguously determine whether the respective gene specifically is required for cytochrome synthesis in the periplasm. It should be noted that the in-frame *ccm* gene deletion in the respective mutant most likely has no effect on the expression of the remaining seven *ccm* genes since transcriptional and translational control elements are not affected in this type of mutant. Moreover, the cytochrome *c* negative phenotype of the Δ*ccmA* mutant can be complemented by the *ccmA* gene provided in trans (L. Thöny-Meyer and P. Künzler, unpublished result).

CcmF, a predicted polytopic integral membrane protein, and CcmG, a membrane protein with a thioredoxin-motif, are proposed to be components of the periplasmic machinery that covalently binds heme to apocytochrome *c*. Such a role for these two proteins is consitent with the observation that synthesis of cytochrome *b*-562 is unaffected in the *E. coli ccmF* and *ccmG* deletion mutants.

CcmA is a peripheral membrane protein containing the 'Walker' ATP-binding sequence motif, –G–G–GK–, in a predicted loop on the cytoplasmic side of the membrane. CcmB and CcmC are also membrane proteins, each with six putative transmembrane alpha-helical segments. CcmC contains a motif, –WG–W–WD–, that is also present in CcmF and is thought to indicate heme-binding properties. Based on these features in the amino acid sequences and the presence of a *ccmABC* gene cluster in several Gram-negative bacteria, is the proposal that CcmA, CcmB and CcmC together constitute an

Table 1
Bacterial strains and plasmids

Strain or plasmid	Properties[a]	Source/reference
E. coli		
MC1061	*hsdR araD139 Δ(araABC-leu)7679 galU galK Δ(lac) ΦX74 rpsL thi*	[21]
EC21	MC1061 Δ*ccmA* (codons for amino acids Q31–W148 deleted, for YPG inserted)	Thöny-Meyer, Fischer and Hennecke, unpublished
EC28	MC1061 Δ*ccmC* (codons for amino acids A63–G156 deleted, for RRAC inserted)	Thöny-Meyer, Fischer and Hennecke, unpublished
EC29	MC1061 Δ*ccmG* (codons for amino acids Q23–S114 deleted, for GAP inserted)	Thöny-Meyer, Fischer and Hennecke, unpublished
EC50	MC1061 Δ*ccmF* (codons for amino acids P168–D243 deleted)	Thöny-Meyer, Fischer, Künzler and Hennecke, unpublished
Plasmids		
pUC19	Ap^r	[22]
pLUW1954	Ap^r, pUC19 with the *B. subtilis cccA* gene	[8]
pLUT193	Ap^r, pUC19 with the *cccA-cybC* fusion	Schiött, Throne-Holst and Hederstedt, unpublished
pNS207	Ap^r, pUC18 with the *E. coli cybC* gene	[9]

[a]Ap^r denotes ampicillin resistance.

ABC-type transporter in the cytoplasmic membrane. The putative transporter has been suggested to transport heme to apocytochromes in the periplasm because the *ccmABC* genes are required for cytochrome *c* synthesis, but not for the transport of apocytochrome *c* across the cytoplasmic membrane. This is supported by the detection of low levels of apocytochrome *c*-550 in membranes of Δ*ccm* mutants. The previous finding that a cytochrome *c*-550–PhoA hybrid is normally exported to the periplasmic side of the membrane in a mutant deleted for the entire *ccm* gene cluster implies that apoprotein translocation can proceed normally in the mutant [3]. It has been relatively well established for bacterial cytochrome *c* biosynthesis that heme is ligated to apocytochrome in the periplasm, i.e. after both, heme and apocytochrome have transversed the membrane [17,18]. In eukaryotic cells, heme is synthesised in the mitochondrial matrix and needs to be transported to other compartments in the cell. Genes encoding proteins very similar to CcmB and CcmC, but of unknown function, are present in the mitochondrial DNA of certain plants (cf. [19]). It has been shown that the *Pseudomonas fluorescens cytA*, which apparently corresponds to *E. coli ccmC*, is required for both cytochrome *c* synthesis and pyoverdine production suggesting other or additional functions for the putative CcmABC translocator [20]. The available experimental data show that the CcmA and CcmC proteins cannot be essential components of a general, non-redundant heme-transporter in the cytoplasmic membrane of *E. coli*. A remaining possibility is that CcmABC is a heme-transporter restricted to cytochrome *c* synthesis.

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