Transcriptional control and essential roles of the *Escherichia coli* *ccm* gene products in formate-dependent nitrite reduction and cytochrome *c* synthesis

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The eight *ccm* genes located at minute 47 on the *Escherichia coli* chromosome, in the order *ccmABCDEFGH*, encode homologues of proteins which are essential for cytochrome *c* assembly in other bacteria. The *ccm* genes are immediately downstream from the *napFDAGHBC* genes encoding a periplasmic nitrate reductase. CcmH was previously shown to be essential for cytochrome *c* assembly. Deletion analysis and a two-plasmid strategy have now been used to demonstrate that CcmA, B, D, E, F and G are also essential for cytochrome *c* assembly, and hence for cytochrome-*c*-dependent nitrite reduction. The *ccm* genes are transcribed from a *ccmA* promoter located within the adjacent gene, *napC*, which is the structural gene for a 24 kDa membrane-bound *c*-type cytochrome, NapC. Transcription from this *ccmA* promoter is induced approximately 5-fold during anaerobic growth, independently of a functional Fnr protein: it is also not

regulated by the ArcB–ArcA two-component regulatory system. The *ccmA* promoter is an example of the ‘extended –10 sequence’ group of promoters with a TGX motif immediately upstream of the –10 sequence. Mutagenesis of the TG motif to TC, CT or CC resulted in loss of about 50% of the promoter activity. A weak second promoter is suggested to permit transcription of the downstream *ccmEFGH* genes in the absence of transcription readthrough from the upstream *napF* and *ccmA* promoters. The results are consistent with, but do not prove, the current view that CcmA, B, C and D are part of an essential haem transport mechanism, that CcmE, F and H are required for covalent haem attachment to cysteine-histidine motifs in cytochrome *c* apoproteins in the periplasm, and that CcmG is required for the reduction of cysteine residues on apocytochromes *c* in preparation for haem ligation.

INTRODUCTION

Enteric bacteria not only survive but grow well in both aerobic and anaerobic environments. In *Escherichia coli*, *c*-type cytochromes are found only during anaerobic growth. At least five *c*-type cytochromes are synthesized, depending on the terminal electron acceptor available [1]. The 43 kDa TorC cytochrome is produced only in the presence of trimethylamine-*N*-oxide (TMAO). Synthesis of this cytochrome, encoded by the *tor* operon located at minute 22 on the chromosome, is induced during anaerobic growth, independently of the transcription activator, Fnr, which is required for the synthesis of most of the other anaerobically induced enzymes involved in respiration. It is also unaffected by the presence of nitrite or nitrate [2]. Two further *c*-type cytochromes, the periplasmic 50 kDa nitrite reductase (cytochrome *c*₅₅₂) and an 18 kDa pentahaem cytochrome *c*, are encoded by the first two genes in the *nrf* operon (nitrite reduction by formate), *nrfA* and *nrfB* [3,4]. Expression of the *nrf* operon, which is located at minute 92 on the chromosome, is dependent on the Fnr protein. It is induced by nitrite but is repressed by nitrate [3–6]. The other two *c*-type cytochromes, a 16 kDa periplasmic cytochrome and a 24 kDa membrane-associated cytochrome [1], are encoded by the operon located at minute 47 on the chromosome originally designated ‘the *aeg46.5* operon’ (anaerobically expressed genes at minute 46.5 on the chromosome; see Figure 1) [7–9]. The expression of this complex operon, which has been renamed the *nap–ccm* operon [8], requires the functional Fnr protein and is induced more strongly by nitrite than by nitrate [6]. Phosphorylated NarP protein activates

transcription from the *napF* promoter; transcription activation is antagonized by nitrate-activated NarL [10].

The *nap–ccm* operon consists of fifteen genes. The first seven genes encode a periplasmic nitrate reductase complex (Nap) including the two *c*-type cytochromes encoded by *napB* and *napC* [8,9]. Downstream of the genes for two *c*-type cytochromes are eight genes which encode homologues of proteins essential for cytochrome *c* synthesis in *Bradyrhizobium japonicum*, *Rhodobacter capsulatus*, *Paracoccus denitrificans*, *Pseudomonas fluorescens*, *Rhizobium meliloti* and *Rhizobium leguminosarum* [11–18]. The first four genes, *ccmABCD*, are predicted to encode an ABC transporter (ATP-binding cassette family of membrane transport proteins), possibly for the transport of haem; *ccmE*, *ccmF* and *ccmH* probably encode a haem-lyase complex; and *ccmG* encodes a thioredoxin-like protein which is thought to maintain haem or cysteines of the cytochrome apoproteins in a reduced state (reviewed in [19]).

We and others have previously established that at least some of the *ccm* genes are essential for cytochrome *c* maturation in *E. coli* and that the essential genes include the 5'-end of the final gene, *ccmH* [8,9,20]. It has also been suggested that CydD and CydC, which have not yet been reported in other bacteria, might be essential for the transport into the periplasm of haem destined for ligation to cytochrome *c* apoproteins [21,22]. If so, either CcmA, B, C and D are unlikely to be essential for cytochrome assembly, or they fulfill a role other than haem transport. Furthermore, unlike most other bacteria, homologues of two of the final three *ccm* genes have been identified in the *E. coli* *nrf* operon. Two of these three genes, *nrfE* and *nrfG*, encode proteins

Abbreviations used: TMAO, trimethylamine-*N*-oxide; LB, Lennox broth.

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that are essential for cytochrome-*c*-dependent nitrite reduction, but are not required for the synthesis or targeting of *c*-type cytochromes to their functional locations in the membrane or periplasm [4,9]. The first aim of the work now described was therefore to establish whether *ccmABCD*, encoding a putative ABC-type traffic ATPase, the thioredoxin-like protein CcmG and the putative haem-binding proteins, CcmE and CcmF, are also essential for cytochrome *c* synthesis, and hence for formate-dependent nitrite reduction.

We have also presented preliminary evidence that the *ccm* genes can be transcribed from two weak promoters, independently of transcription from the much stronger, Fnr-dependent *napF* promoter [8]. This provided a possible explanation of how haem can be incorporated into the 43 kDa TorC cytochrome even in an *fnr* mutant [2]. Synthesis of a functional TorC was still inducible by TMAO, even in the absence of transcription readthrough from the *napF* promoter, but it was not clear whether any downstream *ccm* promoters are regulated by the availability of TMAO, or whether TMAO reduction was due to TorR–TorS-dependent activation of transcription of the structural genes for TMAO reductase, *torCAD* [23,24]. Further aims of the present work were therefore to locate these *ccm* promoters and determine how their activity is regulated, especially by oxygen and the availability of alternative electron acceptors such as nitrate, nitrite and TMAO.

MATERIALS AND METHODS

Bacterial strains, plasmids and primers used in this project

Strains and plasmids used in this work are listed in Tables 1 and 2. Details of the construction by standard recombinant DNA techniques [25] of plasmids encoding sections of the *ccm* region are shown in Figure 1. Bacteria for inocula were grown in Lennox broth (LB) and stored for short periods on nutrient agar plates. Antibiotics were used at 30 mg/l for chloramphenicol and 80 mg/l for ampicillin. Primers used for plasmid construction and site-directed mutagenesis are listed in Table 3.

In several experiments, it was necessary to transform a deletion mutant with two plasmids, one of which was derived from pBR322 (pJG numbers below 400). The second, compatible plasmid (the pJG70 series) was a derivative of the low-copy-number vector, pMAK705. To make the plasmid pJG774, which is compatible with the pBR322-based plasmid pJG73, pJG70 was digested with *SspI*, resulting in a 4 kb fragment containing

Table 1 *E. coli* K-12 strains used in this work

Strain	Description	Reference or source
ECL619	<i>arcB1 zgi::Tn10</i>	[25]
JCB601	<i>ccm::λplacMu</i> derivative of JCB387	[8]
JCB7120	JM712Δ <i>nir</i>	[3]
JCB71202	JCB7120Δ <i>ccmA-H</i>	[8]
JCB712028	Transduce JCB71202 to Kan ^R with RP7947-P1 [49]	This work
JM110	Dam ⁻	Stratagene, Cambridge, U.K.
M182	Δ <i>lac</i>	[48]
M1821	Δ <i>lac fnr</i>	[5]
M1822	Δ <i>lac arcB</i>	Transduce M182 to Tet ^R with phage P1 propagated on ECL619
M1823	Δ <i>lac fnr arcB</i>	Transduce M1821 to Tet ^R with phage P1 propagated on ECL619
RP7947	<i>pcnB1 zad-981::mini-kan</i>	[49]

Table 2 Plasmids used in this work

Plasmid	Description	Reference or source
pAA204p14112	pBR322 derivative with <i>gal</i> promoter	[26]
pAA182	Promoter probe vector	[41]
pAA182*	As pAA182, but <i>HindIII</i> site filled in	This work
pAA234	<i>BglII</i> and <i>SmaI</i> cloning sites in pAA204p14112	This work
pJG70	pBR322 derivative containing <i>ccmA-H</i>	[8]
pJG71	As pJG70, but with an in-frame deletion in <i>ccmG</i>	This work
pJG72	As pJG70, but with an in-frame deletion in <i>ccmB</i>	This work
pJG73	<i>BglII-SspI</i> fragment from pJG600 in pBR322	[8]
pJG74	<i>ccmFGH</i> in pBR322	[8]
pJG77	As pJG70, but with an in-frame deletion in <i>ccmF</i>	This work
pJG79	As pJG70, but with an in-frame deletion in <i>ccmD</i>	This work
pJG84	pJG70 lacking internal <i>HpaI</i> fragment	[8]
pJG86	pJG70 lacking <i>HpaI-Eco47III ccm</i> fragment	[8]
pJG93	' <i>napA-ccmG</i> ' in pBR322	This work
pJG351	<i>ccmA-H</i> expressed from a constitutive <i>gal</i> promoter	This work
pJG352	Exonuclease digestion from <i>NcoI</i> site of pJG70 into <i>ccmA</i>	This work
pJG450	<i>ccmA</i> promoter PCR fragment cloned into pAA182*	This work
pJG451	<i>ccmA</i> promoter PCR fragment cloned into pAA182	This work
pJG452	PCR fragment from <i>ccmA</i> in pAA182*	This work
pJG455	PCR fragment from <i>ccmC-ccmF</i> in pAA182	This work
pJG457	PCR fragment from <i>ccmD-ccmF</i> in pAA182	This work
pJG458	PCR fragment from <i>ccmC-ccmD</i> in pAA182	This work
pJG458*	As pJG458, but <i>HindIII</i> site filled in	This work
pJG465	4 kb <i>BglII-BamHI</i> from <i>napA-ccmA</i> in pAA182	[8]
pJG465*	As pJG465, but <i>HindIII</i> site filled in	[8]
pJG722	<i>ccmB</i> expressed from the pAA234 <i>gal</i> promoter	This work
pJG792	<i>ccmD</i> expressed from the pAA234 <i>gal</i> promoter	This work
pJG4511	As pJG451, but TG replaced by TC	This work
pJG4512	As pJG451, but TG replaced by CT	This work
pJG4513	As pJG451, but TG replaced by CC	This work
pJG774	Insert from pJG74 transferred into pMAK705	This work
pJG794	Insert from pJG94 transferred into pMAK705	This work
pMAK705	Low copy number vector; pBR322-compatible	[50]

Figure 1 DNA fragments from the centisome 47 region of the *E. coli* chromosome cloned into plasmids used in this work

The vectors were: for plasmids with pJG numbers below 100, pBR322; for the 3xx series, pAA234; for the 4xx series, pAA182; and for the 7xx series, pMAK705 (Table 2). Abbreviations for restriction sites are: A, *Afl*III; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*47III; Ec, *Eco*RI; H, *Hpa*II; R, *Rsr*II S, *Ssp*I; Sc, *Scal*; Sn, *Sna*BI.

Table 3 Oligonucleotide primers used for this work

In primer 3, S = 50% C and 50% G; R = 50% A and 50% G; + refers to the coding strand; – refers to the complementary strand.

Primer, location and strand	Sequence (5' → 3')
1 <i>napC</i> +	ACAGAATTCGGATTGTCACGTTCCG
2 <i>napC</i> –	AGTTATTGTCCTTCATGCGCGCCACTCAT
3 <i>napC</i> –	AACGCCAAAAATTTACSRACAGCTCTTTAC
4 <i>napC</i> –	ACTAAGCTTGCATACCCACGCAATAACCC
5 <i>ccmA</i> +	ACAGAATTCCTTATTTAGTGGCTTGTCATTAC
6 <i>ccmC</i> +	ACAGAATTCAGTGGCAGCGTTTATTGGC
7 <i>ccmC</i> –	CGGGATCCCGTGGTGGGATCGCCAG
8 <i>ccmD</i> +	ACAGAATTCGTTGGTGGTTTGGTCGTG
10 <i>ccmD</i> –	AGAGGATCCGCATACCCAGTGCACG
11 <i>ccmD</i> –	AAGAATTCAGGGGTCATTACGGC
12 <i>ccmF</i> +	TCTTTACCTCTAACCCG
13 <i>ccmF</i> –	TTGCGCTTCATACGGCC
14 <i>ccmG</i> –	CTCGGCGCGCCAAGAGTCAGCACATCCGCC
15 pAA182 –	GGCATTGGTCAGCGCG
16 pAA234 +	AGCTCCCGGAGATCTA (<i>Bgl</i> II– <i>Sma</i> I linker)
17 pAA234 –	AGCTTAGATCTCCCGGG (<i>Bgl</i> II– <i>Sma</i> I linker)

ccmFGH. The vector for cloning this *Ssp*I fragment was plasmid pAA234, which was constructed from pAA204p14112 [26]. This plasmid contains the *E. coli gal* operon regulatory region and the first six codons of *galE* fused in phase to the eighth codon of *lacZ*. It also contains the pBR322 origin of replication and an ampicillin-resistance gene. To make pAA234, *Sma*I and *Bgl*II cloning sites were inserted immediately before the *Hind*III site of pAA204p14112 using the complementary primers 16 and 17 (Table 3). The 4 kb *Ssp*I fragment was then cloned into pAA234 at the *Sma*I site to create plasmid pJG374. This plasmid was digested with *Pst*I and *Hind*III and the 5 kb fragment was cloned into pMAK705 so that *ccm* genes in the resulting plasmid, pJG774, can be expressed from the *gal* promoter of pAA234. Similarly, pJG794 was made by digesting pJG94 with *Pst*I and *Hind*III to give a 2.6 kb fragment containing *ccmH* which was cloned into pMAK705. This *ccmH* gene is transcribed from the *bla* promoter of pBR322 (Figure 1).

Construction of pJG79

To determine whether *ccmD* is essential for cytochrome *c* synthesis and Nrf activity, plasmid pJG79, containing an in-frame deletion within *ccmD*, was constructed from two PCR fragments generated with pJG70 DNA as the template. Two primers, 5 and 11 (Table 3), containing *Eco*RI sites at both ends, were used to create the 2 kb DNA fragment containing a region from the 5'-end of *ccmA* to the middle of *ccmD*. Similarly, the second fragment was made using primer 8, which also contains an *Eco*RI site, and primer 13 to create a 2.5 kb DNA fragment containing DNA from the middle of *ccmD* to the 5'-end of *ccmG*. These two fragments were then digested with *Eco*RI and ligated by T4 ligase. The ligated fragment was isolated and used as a template for the second round of PCR to create a 4.5 kb DNA fragment. The fragment with the deletion in *ccmD* was further digested with *Afl*III and *Sna*BI and the 3 kb fragment was cloned into pJG70 which had been linearized with *Afl*III and *Sna*BI and dephosphorylated.

Construction of plasmids pJG351 and pJG352

Plasmid pJG351 was made by three-way ligation. The first fragment was made using the PCR technique. Primer 1, with an

*Eco*RI site at the 5'-end, binds to the middle of *napC*. The second primer, 7, binds to the middle of *ccmC*. The 1.7 kb PCR fragment was then digested with *Eco*RI, the 5' overhanging ends were filled in with dNTPs and the Klenow fragment of DNA polymerase to give a blunt-ended fragment which was digested with *Afl*III to give a 1.4 kb DNA fragment. The second fragment was made by digesting pJG70 with *Afl*III and *Bcl*I to give a 6 kb DNA fragment. The third fragment was derived from the vector pAA234 by digestion with *Sma*I and *Bgl*II. All three fragments were isolated and then ligated by T4 ligase. The resulting plasmid, pJG351, contains all of the *ccm* genes that can be expressed from the *gal* promoter.

Plasmid pJG352, which expresses all of the *ccm* genes except *ccmA*, was constructed from the plasmid pJG70; because one of the restriction endonucleases used for digesting the DNA, *Bcl*II, is sensitive to *dam* methylation, pJG70 was isolated from the *Dam*[–] host, *E. coli* strain JM110. Plasmid DNA was linearized by digestion with *Nco*I and incubated with exonuclease III. Samples removed at intervals were transferred to tubes containing S1 nuclease to stop the nuclease digestion, and the 5' overhanging ends were filled in with the Klenow fragment of DNA polymerase and dNTPs. The DNA was digested with *Bcl*II and fragments of digested DNA of various sizes were isolated and ligated into pAA234 which had been digested with *Sma*I and *Bgl*II and dephosphorylated. The resulting plasmids were screened for the deletion extending into *ccmA*. One of these plasmids, pJG352, contained a deletion from the translation start site of *ccmA* to the *Bam*HI site in *ccmA*. Because the vector plasmid, pAA234, contains the *galE* promoter, all of the *ccm* genes except *ccmA* in the cloned fragment can be expressed under the control of this promoter.

Determination of the Nrf phenotypes and rates of nitrite reduction

The Nrf phenotype was scored by inoculating a test tube containing 1 ml of LB + 1 mM nitrite with the strain to be tested. After overnight growth without aeration, 3 ml of minimal salts containing 0.4% (v/v) glycerol and 40 mM fumarate was added. After a further 1 h at 37 °C without aeration, and at intervals thereafter, 50 µl samples of isogenic Nrf⁺ and Nrf[–] control cultures were spot-tested for nitrite [4]. When no nitrite remained in the Nrf⁺ control, 0.5 ml of 1% (w/v) sulphanilamide in 1 M HCl and 0.5 ml of 0.02% *N*-1-naphthylethylenediamine dihydrochloride were added to the test cultures. The formation of an intense pink colour within 30 s indicated the presence of nitrite and an Nrf[–] phenotype.

To determine rates of nitrite reduction by formate or glycerol, bacteria were grown anaerobically in 1 or 2 litres of minimal salts medium supplemented with 0.4% (v/v) glycerol, 40 mM fumarate, 5% (v/v) LB and 2.5 mM nitrite. Bacteria in the middle of the exponential phase of growth were harvested by centrifugation, washed in Na⁺/K⁺ phosphate buffer, pH 7.5 (prepared from a solution of 50 mM NaH₂PO₄, adjusted to pH 7.5 with a 5 M solution of KOH) and resuspended in phosphate buffer. The rates of nitrite reduction by formate or glycerol were measured [27].

β-Galactosidase assays

Cultures for β-galactosidase assays were inoculated with a fresh overnight culture of the strain to be tested. For anaerobic cultures, 300–600 µl of the overnight culture was used to inoculate 10 ml of medium, with or without 20 mM sodium nitrate or 2.5 mM sodium nitrite, in a test tube. The cultures were incubated at 37 °C without shaking until the absorbance at 650 nm was between 0.5 and 0.7. For aerobic cultures, 5 µl of inoculum was

Figure 2 DNA fragments synthesized by PCR and cloned into the promoter probe vector pAA182 to locate the two promoters that direct expression of the *ccm* genes

Top line: the relative positions of the *E. coli napFDAGHBC* and the *ccmABCDEFGH* genes in the centisome 47 region of the chromosome. Lower part: open lines show chromosomal DNA fragments synthesized by PCR and cloned into plasmid pAA182; filled lines show vector sequences adjacent to the *Hind*III site in pAA182. Half-arrows indicate the position of the primer sequences; the number above or below each arrow refers to the oligonucleotide primers listed in Table 3. Restriction sites are: A, *A*III; B, *B*amHI; Bg, *B*glII; E, *E*coRVIII; Ec, *E*coRI; H, *H*paI; R, *R*saI; S, *S*spI; Sc, *S*caI; Sn, *S*naBI.

transferred to 5 ml of medium in a 25 ml conical flask. The culture was then aerated vigorously. After permeabilizing the cells, the level of β -galactosidase activity was determined [28]. The data presented represent the average of at least two independent experiments, which differed by no more than 10%. Units are nmol of *o*-nitrophenol formed/min per mg of bacterial dry mass.

Cytochrome concentrations

Concentrations of *c*-type cytochrome in the soluble protein fraction (which includes both periplasmic and cytoplasmic proteins) of bacteria broken in the French press were determined spectroscopically as described [27], except that the oxidant was potassium ferricyanide rather than nitrite. The major cytochrome in these fractions is cytochrome c_{552} , the product of the *nrfA* gene.

Primer extension analysis

E. coli M182, transformed with pJG451 or pAA182, were grown aerobically or anaerobically in minimal salts medium with 0.4% (v/v) glycerol, 40 mM fumarate and 5% (v/v) LB. The transcription start point from the internal *ccmA* promoter was determined using the primer extension method and total RNA was extracted as described [29]. The RNA samples (50–150 μ g) were hybridized at 30 °C for 3 h in buffer containing 20 mM Hepes, 0.4 M NaCl and 80% formamide to the 30 bp primer (primer 2; Table 3), which had been labelled at the 5'-end with polynucleotide kinase and [γ ³²P]ATP. This primer anneals to the sequence in the *nap-ccm* operon between positions 24881 and 24911, which is within *napC* (the DNA sequence positions are numbered as in Richterich et al. [29]). The mixture was then

incubated with avian myeloblastosis virus reverse transcriptase and deoxynucleoside triphosphates at 37 °C for 1 h. The length of the extension product, deduced after gel electrophoresis, indicated the position of the transcription start point.

Unsuccessful S1 mapping and Northern blotting experiments were completed by standard methods [25]. Several commercial kits were also used to extract RNA from the slow-growing anaerobic cultures, but no clear evidence of hybridization with the probes was obtained.

Run-off transcription experiments

To locate the transcription start site of the *ccmA* promoter, the DNA fragment containing the promoter was constructed by PCR using primers 1 and 2. The purified 160 bp fragment [4 μ l of 20 nM fragment in 1 $\times$ binding buffer which contains 5% (v/v) glycerol, 20 mM Tris (pH 8.0), 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol and 50 μ g/ml BSA] was incubated with 4 μ l of 300 nM RNA polymerase in 1 $\times$ binding buffer. After 5 min at 37 °C, 4 μ l of 'hot nucleotide triphosphate + heparin' solution (200 μ M ATP/CTP/GTP, 10 μ M UTP, 1.5 μ Ci [α ³²P]UTP and 100 μ g/ml heparin) was added and the mixture was incubated at 37 °C for further 5 min. The reaction was stopped by adding 12 μ l of stop solution (20 mM EDTA, 80% de-ionized formamide, 0.1% xylene cyanol FF and 0.1% Bromophenol Blue). The samples were heated at 90 °C for 2 min and loaded onto the sequencing gel.

SDS/PAGE

For SDS/PAGE, an 18 cm square slab-gel apparatus was filled with a 15% (w/v) resolving and a 6% (w/v) stacking polyacrylamide gel. Cell fractions (10 μ l) were mixed with an equal

volume of sample buffer, frozen and then heated to 100 °C for 10 min. After electrophoresis for 17 h at 70 V, proteins with covalently bound haem were identified by their haem-dependent peroxidase activity [30].

Localization of the *ccmA* promoter

Two primers were used in the PCR to localize the promoter in plasmid pJG465* [10] (Figure 2, top part). The *EcoRI* linker primer, primer 1 (Table 3), annealed within *napC*. Primer 15 annealed downstream from the *HindIII* site of the pAA182 vector sequence in the template pJG465*. This PCR fragment was then digested with *EcoRI* and *BamHI* and cloned into the *EcoRI*–*BamHI* site of pAA182* to create the plasmid pJG450, which contains 800 bp from the 3'-end of *napC* to the *BamHI* site in *ccmA*.

Two further plasmids were constructed to localize the promoter in pJG450 more precisely. The first plasmid was made using primers 1 and 4 (Table 3 and Figure 2, top part). This fragment was then cloned into the *EcoRI*–*HindIII* site of pAA182 to create plasmid pJG451, which includes 360 bp from the middle of *napC* to the translation start site of *ccmA* (Figure 2, top part). Primers 5 and 15 were similarly used to create plasmid pJG452 containing 400 bp from the 5'-end of *ccmA* to the *BamHI* site in *ccmA* (Figure 2, top part).

Site-directed mutagenesis of the TG motif in the *ccmA* promoter

Site-directed mutagenesis was used to change the TG motif immediately upstream of the –10 sequence of the *ccmA* promoter to TC, CT and CC in two PCR steps in which the product of the first PCR was used as a mega-primer for the second PCR. The two primers used to generate the mega-primer in the first PCR reaction were primer 1, which binds to the middle of *napC* at position 25050 and contains an *EcoRI* site, and the mutagenic primer 3, which binds to the *napC* gene at position 24960. The template was pJG465* DNA and the TG bases in primer 3 were changed so that the PCR product would incorporate the desired mutation in this TG motif. This PCR product was then purified from the 7.5% acrylamide gel and used in the second PCR reaction with primer 4, which includes a terminal *HindIII* site at the 5'-end. The second PCR product was digested with *EcoRI* and *HindIII* and the 360 bp fragment was purified from a 7.5% (w/v) polyacrylamide gel. The digested fragment was cloned into the promoter probe vector, pAA182, which had been cut with *EcoRI* and *HindIII* and dephosphorylated. The resulting plasmids were transformed into the Lac[–] host strain M182, and white colonies were purified for screening. The presence of mutations in the TG motif was confirmed by DNA sequencing.

RESULTS

We have previously established that one or more, and perhaps all, of the *ccm* genes are essential for the assembly of functional *c*-type cytochromes in *E. coli* and that the essential genes include *ccmH* [8,9]. The first objective of the current work was to establish whether the genes *ccmABCD* encoding a putative ABC-type traffic ATPase, the putative haem-binding proteins CcmE and CcmF, and the thioredoxin-like protein CcmG, are also essential for cytochrome *c* synthesis and hence for formate-dependent nitrite reduction. This required the construction of a series of strains able to express all but one of the *ccm* genes and was achieved using two general strategies. Both strategies were based on the previously described plasmid pJG70 (Figure 1), which includes all of the *ccm* genes as well as the upstream

napGHBC genes encoding components of the periplasmic nitrate reductase, but not *napF*, *napD* or *napA* [8]. Although the *ccm* genes in plasmid pJG70 cannot be expressed by transcription readthrough from the strong, Fnr-regulated *napF* promoter, they are transcribed from at least one weaker, downstream promoter at a level sufficient to complement an Nrf[–] deletion mutant for Nrf activity [8]. In the first strategy, in-frame deletions were engineered into the *ccmB*, *ccmD*, *ccmF* and *ccmG* genes, either by exploiting convenient restriction sites, or by creating in-frame deletions by PCR, to generate single plasmids from which all of the remaining seven genes were expressed. The ability of the resulting plasmid to direct the synthesis of *c*-type cytochromes and to complement a mutant deleted for all of the *ccm* operon was then determined. The product of the *E. coli* *pcnB* gene is essential for maintaining the copy number of pBR322-based plasmids, which, in a *pcnB* host, is decreased to about one. The phenotypes of both *pcnB*⁺ and *pcnB* transformants were compared to check for any artefacts arising from the use of multi-copy plasmids. The second strategy involved the construction of pairs of compatible plasmids from which either all of the *ccm* genes, or all except one of the *ccm* genes, could be expressed. The phenotypes of the *pcnB*⁺ and *pcnB* deletion mutants after transformation with these pairs of plasmids were again compared.

In-phase deletion analysis to establish an essential role for the *ccmABCD*, *ccmF* and *ccmG* genes in cytochrome *c* assembly

Plasmid pJG72 containing an in-frame deletion of 342 bp from *ccmB* was transformed into the deletion mutant, strain JCB71202, and the phenotype of the transformant was compared with those of the untransformed deletion mutant and the mutant transformed with the starting plasmid pJG70. Only the pJG70 transformant was Nrf⁺. The same phenotypes were found, irrespective of whether the host strain was *pcnB* or *pcnB*⁺, so the failure of pJG72 to complement the deletion of the *ccm* genes in strain JCB71202 was not an artefact due to the use of multi-copy plasmids.

To confirm that CcmB, and hence the putative transport complex encoded by *ccmABCD*, is essential for cytochrome *c* synthesis, the deletion mutant and transformants were grown anaerobically under conditions optimal for cytochrome *c*₅₅₂ synthesis and expression of Nrf activity. The concentration of soluble *c*-type cytochromes in the resulting bacteria and their rates of nitrite reduction by formate or by the growth substrate, glycerol, were then determined (Table 4). No *c*-type cytochromes and only very low residual rates of nitrite reduction were detected in either the deletion mutant or the mutant transformed with pJG72. In contrast, JCB71202 transformed with pJG70 accumulated comparable quantities of *c*-type cytochromes and reduced nitrite as rapidly as the parental strain JCB7120. Furthermore, *c*-type cytochromes were clearly visible in haem-stained SDS/PAGE gels of proteins from strains JCB7120 and JCB71202 pJG70, but not in either the untransformed deletion mutant or in the mutant transformed with pJG72 (Figure 3).

Translation of the *ccmD* open reading frame results in a 69 amino acid polypeptide, which is unlike any other polypeptide in the current databases. However, genes for similar-sized polypeptides are located amongst genes for cytochrome *c* assembly in other bacteria, including *ccmD* genes of *Paracoccus denitrificans* [31] and *Haemophilus influenzae*, *cycX* of *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*, *cytB* of *Pseudomonas fluorescens* and *helD* of *Rhodobacter capsulatus* [19]. Each of these polypeptides is predicted to be associated with the membrane by a single, membrane-spanning, N-terminal α -helix. The hydrophilic C-terminus of CcmD is predicted to be located in the

Table 4 Effects of defects in *ccmE* and in-frame deletions in *ccmB*, *ccmD*, *ccmF* and *ccmG* on formate-dependent nitrite reduction and cytochrome *c* biosynthesis

Bacteria that had been grown anaerobically in minimal salts medium supplemented with 5% (v/v) LB, glycerol, fumarate and nitrite were harvested and the rates of nitrite reduction by washed bacteria in the presence of the growth substrate, glycerol, or the fermentation product, formate, were determined. Rates are given as nmol of nitrite reduced per min/mg of bacterial dry mass. Cytochrome concentrations are pmol/mg of soluble protein released by the French press (periplasmic and cytoplasmic proteins including both cytochrome *c*₅₅₀ and cytochrome *c*₅₅₂). Values are the average of two independent cultures.

Strain	Plasmid(s)	Nrf phenotype	Genes absent	Nitrite reduction by		Soluble cytochrome <i>c</i>
				Glycerol	Formate	
JCB7120	—	Nrf ⁺	None	73.6	33.0	324
JCB71202	—	Nrf ⁺	<i>ccmA-H</i>	2.4	0.8	< 10
	pJG70	Nrf ⁺	None	68.5	54.7	653
	pJG70 + pMAK705	Nrf ⁺	None	76.8	57.6	662
Use of plasmids with in-frame deletions	pJG72	Nrf ⁺	<i>ccmB</i>	0.6	0.6	< 10
	pJG72 + pJG722	Nrf ⁺	None	91.0	72.8	692
	pJG79	Nrf ⁺	<i>ccmD</i>	3.7	1.5	< 10
	pJG79 + pJG792	Nrf ⁺	None	78.6	56.7	696
	pJG77 + pMAK705	Nrf ⁺	<i>ccmF</i>	4.3	2.5	< 10
	pJG77 + pJG774	Nrf ⁺	None	54.5	24.8	506
	pJG71 + pMAK705	Nrf ⁺	<i>ccmG</i>	1.9	0.8	< 10
	pJG71 + pJG774	Nrf ⁺	None	50.0	35.2	850
Two-plasmid strategy	pJG73 + pJG774	Nrf ⁺	<i>ccmE</i>	2.2	1.1	< 10
	pJG93 + pJG794	Nrf ⁺	<i>ccmG</i>	2.1	0.7	< 10
	pJG93 + pJG774	Nrf ⁺	None	81.5	54	873
Controls: effects of plasmid copy number and the presence of a second plasmid vector	pJG70 + pMAK705	Nrf ⁺	None	38.6	31	371
	pJG70 + pJG774	Nrf ⁺	None	45.0	44.6	729
	pJG77 + pJG774	Nrf ⁺	None	42.4	27.6	634
	pJG71 + pJG774	Nrf ⁺	None	51.0	47.6	520
	pJG77 + pMAK705	Nrf ⁺	<i>ccmF</i>	4.2	1.0	< 10
	pJG71 + pMAK705	Nrf ⁺	<i>ccmG</i>	0.3	0.8	< 10

Figure 3 Effect of loss of expression of *ccmA*, *ccmB*, *ccmD*, *ccmE*, *ccmF* and *ccmG* on cytochrome *c* accumulation by anaerobic cultures of *E. coli*

Bacteria were grown anaerobically in minimal salts medium supplemented with glycerol, fumarate, nitrite, TMAO and 5% (v/v) LB. Samples loaded on to tracks 1, 4, 7, 10, 13 and 16 were the untransformed strain JCB71202; samples in tracks 2, 5, 8, 11, 14 and 17 were from cultures of JCB71202 transformed with plasmids encoding all genes except *ccmA* (pJG352), *ccmB* (pJG72), *ccmD* (pJG79), *ccmE* (pJG73 + pJG774), *ccmF* (pJG77) and *ccmG* (pJG71) respectively. Samples in tracks 3, 6, 9, 12, 15 and 18 were the positive controls for the preceding tracks, as listed in Table 4.

cytoplasm. Not one amino acid is conserved throughout these polypeptides. To determine whether CcmD is essential for cytochrome *c* assembly in *E. coli*, an in-frame deletion was introduced into the *E. coli ccmD* gene of pJG70 to generate plasmid pJG79. This plasmid was also unable to transform strain JCB71202 to Nrf⁺ or to restore cytochrome *c* synthesis (Table 4 and Figure 3). Both the CcmB and CcmD components for the

ABC-type of traffic ATPase are therefore essential for cytochrome *c* maturation.

As a further confirmation that the *ccmABCD* genes are essential, two further plasmids were constructed. Plasmid pJG351 encodes all of the *ccm* gene products, but pJG352 lacks *ccmA*. Only pJG351 transformed strain JCB71202 to Nrf⁺ and restored both cytochrome *c* synthesis (440 pmol/mg of soluble protein) and formate-dependent nitrite reduction (10.6 nmol of nitrite reduced/min per mg bacterial dry mass), confirming that CcmA is also essential for cytochrome *c* maturation.

PCR was also used to engineer in-frame deletions in the *ccmF* and *ccmG* genes of plasmid pJG70 (Table 3). The resulting plasmids (pJG77 and pJG71 respectively) failed to transform the deletion mutant to Nrf⁺. After anaerobic growth in the presence of nitrite, these transformants were unable to catalyse formate-dependent nitrite reduction and were devoid of *c*-type cytochromes (Table 4). Both nitrite reduction and cytochrome *c* synthesis were fully restored when JCB71202 pJG71 or JCB71202 pJG77 were transformed with a second, compatible plasmid encoding CcmF, G and H (Table 4 and Figure 3). We conclude that both CcmF and CcmG are essential for the synthesis of all *c*-type cytochromes in *E. coli*.

A two-plasmid strategy to establish that CcmE is essential for cytochrome *c* assembly

The CcmE polypeptide of *E. coli* is homologous to the CycJ product of *B. japonicum*, which has been shown to be essential for cytochrome *c* assembly in that organism [12]. Homologues essential for cytochrome *c* assembly have also been found in

Figure 4 Localization of the *ccmA* promoter in plasmid pJG465*

Bacteria (top and middle, strain M182; bottom, strain M1821) were grown aerobically (open bars), anaerobically in the presence of 40 mM fumarate as the only terminal electron acceptor (solid bars), anaerobically in the presence of 2.5 mM nitrite (bars with vertical lines), anaerobically with 20 mM nitrate (bars with slanting lines) or anaerobically in the presence of 20 mM TMAO (bars with horizontal lines). Error bars indicate the range of activities obtained from two or more replicate experiments with independently grown cultures on different days. Activities are expressed as nmol of *o*-nitrophenol formed/min per mg of bacterial dry mass. The asterisk in pJG465 indicates that, following its construction, plasmid pJG465 was opened with *Hind*III, the *Hind*III site was filled in with the Klenow fragment of DNA polymerase and dNTPs and religated to form pJG465* in which the phase in which ribosomes cross the linker cloning site into the *trp* DNA of pAA182 has been changed to allow greater expression of β -galactosidase.

other bacteria [15,16,18]. A two-plasmid strategy was used to establish that CcmE is essential for cytochrome *c* assembly in *E. coli*. The pBR322-based plasmid, pJG73, is a truncated derivative of pJG70, terminating at the *Ssp*I site within *ccmE*; pJG73 therefore encodes functional copies of *ccmA*, *B*, *C* and *D*. The compatible plasmid, pJG774, expresses *ccmF*, *G* and *H* under the control of a constitutive *gal* promoter. The deletion mutant remained Nrf^- when transformed either with each plasmid alone, or when doubly transformed with both plasmids. Furthermore, only very low rates of nitrite reduction and no spectroscopically detectable cytochrome *c* were found in the double transformant (Table 4). Two further plasmids, pJG93 and pJG794, were used to validate the double plasmid strategy. Plasmid pJG93 encodes CcmA, *B*, *C*, *D*, *E* and *F*; plasmid pJG794 encodes a functional *ccmH* gene under the control of the *gal* promoter of pMAK705 (Figure 1). As expected, the deletion mutant doubly transformed

with pJG93 and pJG794 remained Nrf^- and unable to catalyse nitrite reduction or to assemble *c*-type cytochromes, consistent with the essential role of CcmG in cytochrome assembly (see above). In contrast, full complementation was observed when the mutant was transformed with pJG93 and pJG774, a combination which encodes functional copies of all of the *ccm* genes (Table 4).

The phenotypes of both single and double transformants of the *penB* strain, JCB71208, were identical with those of the *penB*⁺ parent. Despite the greatly decreased copy number of the essential genes for the ABC-type transporter, rates of nitrite reduction and concentrations of *c*-type cytochromes accumulated were decreased by at most 2-fold in the *penB* host (Table 4). The conclusion from the two-plasmid strategy, that CcmE and CcmG are both essential for cytochrome *c* maturation, was therefore validated. Thus two strategies have been used successfully to establish that the putative ABC transporter encoded by *ccmABCD*, the thioredoxin-like protein CcmG, the putative haem-binding protein CcmE, and the putative haem lyase component CcmF, are all essential for cytochrome *c* assembly in *E. coli*.

Expression of the *ccm* genes from an internal *ccmA* promoter

The ability of plasmid pJG70 to complement the mutant JCB71202 and the $\text{Nrf}^- \text{Ccm}^-$ mutants to $\text{Nrf}^+ \text{Ccm}^+$ suggests that there is at least one promoter which can direct the transcription of these *ccm* genes in the absence of the *nap* promoter. Different parts of the *ccm* region were subcloned into the promoter probe vector pAA182, which includes a multiple cloning site immediately upstream from a promoterless *lacZ* gene. The resulting plasmids were transformed into the Lac^- host *E. coli* M182. One of these plasmids, pJG465*, which includes the last two *nap* genes and the 5'-end of *ccmA*, was promoter active [8]. Further plasmids generated by PCR were used to localize this promoter activity. Plasmids pJG450 (which contains 800 bp from the 3'-end of *napC* to the *Bam*HI site in *ccmA*) and pJG451 (which includes 360 bp from the middle of *napC* to the translation start site of *ccmA*) both transformed *E. coli* M182 into Lac^+ . In contrast, no promoter activity was detected in bacteria transformed with pJG452, which includes a 400 bp fragment of *ccmA* (Figure 2). The β -galactosidase activities of these transformants after aerobic or anaerobic growth were then determined (Figure 4). The promoter in these plasmids was more active during anaerobic than aerobic growth, but it was not significantly affected by the addition of nitrate, nitrite or TMAO to the growth medium. The slightly lower activities after growth in the presence of nitrate than nitrite or TMAO, though statistically insignificant, were reproducible. It can be concluded that there is a promoter for the *ccm* genes located between the middle of *napC* and the translation start site of *ccmA*. This is therefore designated the *ccmA* promoter.

Identification of the transcription start site by reverse transcriptase mapping and transcription run-off experiments

RNA was prepared from *E. coli* strain M182 pJG450 and from M182 pJG451 which had been grown aerobically or anaerobically. Attempts to detect *ccm* transcripts and locate the transcription start point from the *ccmA* promoter by S1 mapping were unsuccessful. A *ccmA* transcript initiated within the *napC* open reading frame was readily detected by reverse transcriptase mapping using primer 2 (Table 3) and RNA prepared from aerobically grown bacteria. Although this transcript was expected

Figure 5 Effect of an *arcB* mutation (strain M1822, top), or double mutations in *fnr* and *arcB* (strain M1823, bottom), on the activity of the *ccmA* promoter during aerobic (open bars) or anaerobic (filled bars) growth

to be more abundant in RNA from anaerobic cultures, RNA from these slow-growing cultures was too degraded to be used successfully in these experiments. Run-off transcription experiments with purified RNA polymerase and plasmid pJG451 DNA were therefore used to confirm that transcription begins at the site identified by reverse transcriptase mapping. These experiments revealed that the sequence of the *ccmA* promoter matches almost perfectly the consensus sequence for an extended -10 promoter with a TG motif located one base upstream of the TAAAT -10 sequence, but no recognizable -35 sequence.

Regulation of transcription from the *ccmA* promoter

Although the *ccmA* promoter was more active during anaerobic than during aerobic growth (Figure 4, top), inspection of the DNA sequence upstream of the transcription start point revealed no obvious binding site for the Fnr protein. In the absence of the *nap* promoter, the promoter located in pJG465* can transcribe at least some of the *ccm* genes, which are essential for the synthesis of all *c*-type cytochromes including *torC*, the expression of which is not dependent on the functional Fnr protein [2,32]. This raised the possibility that the regulation of the *ccmA* promoter might be Fnr-independent. The plasmids pJG465*, pJG450 and pJG451 were therefore transformed into the *fnr* derivative of strain *E. coli* M182, strain M1821. All three plasmids transformed the *E. coli* M1821 to Lac⁺, suggesting that the promoter located in these plasmids does not require a functional Fnr protein to activate transcription. The β -galactosidase activities of the three transformants were determined. The activities of the promoter found in pJG450 and pJG451 in the M1821 strain were similar to those in the *fnr*⁺ strain M182 (Figure 4, bottom). This established that a functional Fnr protein is not required for transcription from the *ccmA* promoter.

During anaerobic growth, many aerobically expressed genes are repressed by the two-component regulatory system, ArcB–ArcA [33]. However, ArcA has also been reported to activate transcription of some genes, for example, the *cydAB* operon of *E. coli* and the *cob* genes of *Salmonella typhimurium* [34,35]. Two

Figure 6 Effects of changing the TG motif in the extended -10 region of the *ccmA* promoter in plasmid pJG451 (Figure 3) to TC (plasmid pJG4511), CT (plasmid pJG4512) or CC (plasmid pJG4513)

Other details are as for Figure 4.

further derivatives of *E. coli* strain M182 were constructed to determine whether the *ccmA* promoter is regulated by ArcB–ArcA. Strain M1822 is the *arcB* derivative of M182; strain M1823 is defective for both *arcB* and *fnr*. Lac⁺ colonies were obtained when these strains were transformed with plasmids pJG465*, pJG450 and pJG451 and plated on to MacConkey–lactose agar. Purified transformants were grown either aerobically or anaerobically in minimal salts medium with glycerol and fumarate, and the β -galactosidase activities were determined. Higher activities were again found after anaerobic than after aerobic growth, confirming that the *ccmA* promoter is not regulated as part of the Arc modulon (Figure 5). Note, however, that lower β -galactosidase activities were detected in the *fnr arcB* double mutant than in the parental strain or in the single mutants (Figure 5).

The *ccmA* promoter belongs to the extended -10 region class of promoters

At the majority of promoters, RNA polymerase binds to two conserved sequences located 10 and 35 bases upstream of the transcription start. However, promoters located within structural genes often lack the conventional -35 sequence. Transcription at these promoters depends upon a TGX motif immediately upstream of the -10 hexamer [26,36]. Such a motif was found upstream of the -10 sequence of the *ccmA* promoter. To confirm that this feature contributes to transcription activation of the *ccm* genes, the TG motif in plasmid pJG451 was altered by site-directed mutagenesis to TC, CT and CC and the effects of the changes on rates of β -galactosidase expression in the *E. coli* host, M182, were determined. Rates of transcription were decreased by about 50 % by each of these mutations (Figure 6). We conclude that the *ccmA* promoter can be added to the growing list of extended -10 sequence promoters [37].

A second, weak promoter directs synthesis of the downstream *ccm* genes

Promoter activity was previously detected associated with a plasmid, pJG461, which contains the *Bam*HI fragment encoding the 3'-end of *ccmA*, *ccmBCDE* and the 5'-end of *ccmF* [8].

Table 5 β -Galactosidase activity after aerobic or anaerobic growth of *E. coli* strain M182 transformed with various plasmids to investigate the regulation of transcription from the second promoter in the *ccm* region

β -Galactosidase activities, expressed as nmol *o*-nitrophenol formed/min per mg of dry mass, are the average of two independent cultures which were assayed in duplicate. Replicates for a given culture were reproducible to within 10%; independent cultures were reproducible to within $\pm 20\%$.

Plasmid	Aerobic	Anaerobic	Anaerobic + nitrite	Anaerobic + nitrate	Anaerobic + TMAO
pAA182 (vector only)	69	220	230	160	220
pJG461	702	882	890	855	905
pJG455	419	609	568	410	658
pJG457	200	409	400	328	356
pJG458	99	328	322	249	346
pJG458*	375	719	684	555	673

Table 6 Complementation of a mutation in the downstream *ccm* genes in the *Nrf⁻ Ccm⁻* strain JCB601, by plasmids pJG86 and pJG84 which include or lack the *ccmA* promoter respectively

Results are the average values for two independent cultures. Individual values for all cultures were usually within $\pm 10\%$ of the mean. Bacteria were grown anaerobically in minimal salts medium supplemented with 5% (v/v) LB, glycerol, fumarate and nitrite. Cells were harvested and the rate of nitrite reduction by washed bacteria was determined. Rates are nmol nitrite reduced/min per mg of dry bacteria. Cytochrome concentrations are pmol/mg of protein in soluble, cytoplasmic and periplasmic proteins released by the French press.

Strain	Plasmids	Phenotype	Nitrite reduction by		Cytochrome <i>c</i>
			Glycerol	Formate	
JCB601	—	<i>Nrf⁻</i>	2.9	1.4	< 10
	pJG86	<i>Nrf⁺</i>	49.3	36.5	299
	pJG84	<i>Nrf⁺</i>	49.2	29.4	228

Attempts to identify by reverse transcriptase mapping transcripts originating from a promoter within this fragment, as well as transcription run-off experiments, were unsuccessful. Two further plasmids, pJG457 and pJG458*, were therefore constructed to validate the previous observations and localize promoter activity more precisely (Figure 2, lower part). Only pJG458* transformed *E. coli* strain M182 to Lac⁺, but the promoter activity was weak, resulting in pale red rather than deep red colonies on MacConkey–lactose agar. Measurements of the β -galactosidase activities of the various transformants after aerobic and anaerobic growth also yielded equivocal results, any promoter activity associated with plasmid pJG458* being only slightly above that of pJG457 (Table 5). The slightly higher activities after anaerobic rather than aerobic growth were typical of those seen for the pAA182 vector, which lacks promoter activity and can be ascribed to an increase in DNA supercoiling of these plasmids during anaerobic growth [38].

E. coli strain JCB601 is unable to synthesize *c*-type cytochromes due to a polar insertion mutation in either *ccmF* or *ccmG* [8]. Cytochrome *c* synthesis was restored by transformation with plasmid pJG74, encoding *ccmF*, *G* and *H* expressed from a vector promoter, but not by pJG73 which lacks promoter activity. Thus promoter activity is essential for plasmids encoding only *ccmFGH* to restore cytochrome *c* synthesis to these mutants. Plasmids pJG84 and pJG86 were used to confirm that some of the genes downstream of *ccmA* can be expressed from the weak second promoter. Both of these plasmids include an uninterrupted *ccmD* gene and therefore should include any weak downstream promoter activity located in this region (Figure 1).

In contrast, only pJG86 includes a functional *ccmA* promoter [8]. Both plasmids pJG84 and pJG86 restored cytochrome *c* synthesis to JCB601 and, furthermore, restored near normal activities of formate-dependent nitrite reduction (Table 6). These data suggest that there is a weak downstream promoter located within *ccmD* which directs transcription of the downstream *ccm* genes *ccmE*, *F*, *G* and *H*. The alternative possibility, that the ability of pJG84 to complement strain JCB601 is due to transcription outwards from a vector promoter, cannot be excluded. Furthermore, failure to detect the transcripts from the upstream *ccmA* promoter by Northern blotting prevented us from establishing whether there is also transcription readthrough from the upstream *napA* and *ccmA* promoters into these downstream *ccm* genes.

DISCUSSION

At the start of this project, two sets of genes had been described in *E. coli* that were candidates for encoding a mechanism for the transport of haem from the cytoplasm to the periplasm, and for the periplasmic ligation of haem to cytochrome *c* apoproteins. The availability of a strain deleted for all of the *ccm* genes except the 3'-end of *ccmH* has now enabled us to demonstrate that all of the products of the *ccm* genes are essential for cytochrome *c* maturation in *E. coli*. Consequently, if CcmA, B, C and D are components of a haem transporter, CydC and CydD cannot also be essential for the transport of haem destined for cytochrome *c* synthesis from the cytoplasm to the periplasm [21,22,39]. We have confirmed that deletion of *cydDC* results in a substantial decrease in cytochrome *c* accumulation in the periplasm, but a residual concentration is still detectable, and this is sufficient to support about 30% of the rate of formate-dependent nitrite reduction of the parental strain (H. Crooke, unpublished work). Our results support the conclusion that CydC and CydD play a subsidiary or indirect role in the assembly of cytochrome-*c*-dependent electron-transfer chains in the periplasm of enteric bacteria, for example, by disrupting the redox environment of the periplasm [39]. They are consistent with, but do not prove, the proposal that CcmA, B, C and D are components of a single, essential, ATP-dependent complex which transports haem from the cytoplasm to the periplasm [14,39].

We previously reported that a mutation in a gene originally called *dsbE* had little effect on cytochrome *c* accumulation and hence on the rate of formate-dependent nitrite reduction [40]. Subsequently, it was realized that the gene defective in this mutant was *ccmG* encoding a thioredoxin-like protein located in the periplasm. Two approaches have now been used to establish that cytochrome *c* synthesis is abolished in mutants totally defective in *ccmG*; this is consistent with an essential role for CcmG homologues in cytochrome *c* maturation by other bacteria

[11,13,17,18,31]. We conclude that the phenotype of the spontaneously generated mutant used previously, strain DM1479, is leaky. Our results also suggest possible roles for CcmG in cytochrome *c* maturation. Having established that DsbA–DsbB-dependent disulphide-bond formation is an essential step in cytochrome *c* assembly, our preferred view is that pairs of cysteine residues locked in disulphide bonds are presented to a haem–lyase complex in the periplasm, where they are reduced by electrons transferred to CcmG from the disulphide isomerase-like protein DipZ [41,42] prior to haem attachment, as proposed previously [43]. Alternative possible roles for CcmG include the reduction of haemin to haem, or the reduction of Ccm components, such as CcmF in the oxidizing environment of the periplasm, as proposed by Thöny-Meyer [19]. A further possibility, not seriously considered previously, is that the CXXC motifs in one or both CcmG and CcmF provide the initial site for the covalent attachment of haem, which is then transferred to the cytochrome *c* apoproteins (or possibly also to the preapoproteins, as haem ligation and leader peptide cleavage appear to be independent events [44]). This suggestion implies that, under certain conditions, haem might be detected covalently bound to CcmF or CcmG. In a strain defective for CcmF, a polypeptide of apparent mass 17.5 kDa with covalently bound haem was the only haem-stained band detected after SDS/PAGE (Figure 3). An alternative possibility is that CcmE binds haem non-covalently, passing it to CcmG which is the substrate for CcmF-dependent ligation of haem to the cytochrome apo- (or pre-apo-) proteins. Attempts to determine whether this 17.5 kDa band is CcmE or CcmG are in progress.

Although Northern analysis and S1 mapping experiments were unsuccessful, a promoter for transcription of the *ccm* genes independently of the *nap* promoter was detected by reverse transcriptase mapping. The transcription start determined by reverse transcriptase mapping was confirmed by *in vitro* run-off transcription mapping. Transcription from the *ccmA* promoter, cloned into the promoter probe vector pAA182, established that the *ccmA* promoter is expressed more during anaerobic growth than during aerobic growth, but is insensitive to the availability of nitrate, nitrite or TMAO. Transcription from the *ccmA* promoter is also independent of the transcription factor Fnr; this explains how TorC synthesis can continue either in an *fnr* mutant, or when transcription from the upstream *napF* promoter is repressed. No explanation can yet be offered for the anaerobic induction of the *ccmA* promoter. Furthermore, as the *ccmA* promoter does not respond specifically to TMAO, TMAO induction of TMAO reductase is due solely to regulation of *torCAD* by TorR–TorS [23,24].

The *ccmA* promoter is clearly another example of a promoter with an extended –10 sequence rather than a consensus –35 sequence. Such promoters are frequently found within the coding regions of upstream genes in a complex operon. The first examples of this type included the *cysG* promoter located within *nirC*, an open reading frame encoding a hydrophobic protein of unknown function [36]. The *cysG* gene is the final gene of the *nir* operon, which encodes the major NADH-dependent nitrite reductase [45].

It has been argued that three types of cytochrome *c* assembly complexes have evolved in different types of organisms [46]. System I, found in enteric bacteria, is the most complex and can be considered to consist of two sub-systems, a haem transport mechanism encoded by *ccmABCD*, and a haem presentation and ligation system encoded by *ccmEFGH*. It was therefore important to demonstrate whether there is also a second promoter that can direct transcription of the four downstream *ccm* genes, independently of either the *napF* or *ccmA* promoters. Although

attempts to detect the transcripts originating from this promoter were unsuccessful, two types of evidence were presented that such promoter activity, albeit weak, is present (Tables 5 and 6). *E. coli* is atypical in that it also expresses a second set of genes, *nrfEFG*, which are candidates for encoding components of a haem lyase. We recently provided direct evidence that these genes are essential for the attachment of haem to a novel CWSCK motif in NrfA, and that haem attached to this motif is the active site for nitrite reduction to ammonia [47]. Both of these observations add further credence to the two-component proposal [46].

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