Cytochrome *c* Maturation and the Physiological Role of *c*-Type Cytochromes in *Vibrio cholerae*

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Vibrio cholerae lives in different habitats, varying from aquatic ecosystems to the human intestinal tract. The organism has acquired a set of electron transport pathways for aerobic and anaerobic respiration that enable adaptation to the various environmental conditions. We have inactivated the *V. cholerae* *ccmE* gene, which is required for cytochrome *c* biogenesis. The resulting strain is deficient of all *c*-type cytochromes and allows us to characterize the physiological role of these proteins. Under aerobic conditions in rich medium, *V. cholerae* produces at least six *c*-type cytochromes, none of which is required for growth. Wild-type *V. cholerae* produces active fumarate reductase, trimethylamine *N*-oxide reductase, *cbb*₃ oxidase, and nitrate reductase, of which only the fumarate reductase does not require maturation of *c*-type cytochromes. The reduction of nitrate in the medium resulted in the accumulation of nitrite, which is toxic for the cells. This suggests that *V. cholerae* is able to scavenge nitrate from the environment only in the presence of other nitrite-reducing organisms. The phenotypes of cytochrome *c*-deficient *V. cholerae* were used in a transposon mutagenesis screening to search for additional genes required for cytochrome *c* maturation. Over 55,000 mutants were analyzed for nitrate reductase and *cbb*₃ oxidase activity. No transposon insertions other than those within the *ccm* genes for cytochrome *c* maturation and the *dsbD* gene, which encodes a disulphide bond reductase, were found. In addition, the role of a novel CcdA-like protein in *cbb*₃ oxidase assembly is discussed.

The gram-negative bacterium *Vibrio cholerae* naturally inhabits aquatic ecosystems and is part of the normal flora of brackish water. While the vast majority of environmental isolates are nonpathogenic, some strains are able to cause cholera in humans. The disease is endemic in much of South Asia, Africa, and Latin America (49). Within the natural aquatic habitats, *V. cholerae* has been proposed to live in five distinctive stages, as follows: an independent, free-swimming form; a symbiont of phytoplankton; a commensal of zooplankton; a viable but not culturable state; and a biofilm attached to abiotic or chitinous surfaces (37). These changes in habitat require the bacterium to adapt to a broad range of growth conditions. Major factors that influence growth are the sources of nitrogen and carbon and the presence or absence of oxygen. The genome of *V. cholerae* has been sequenced and published (17), and various metabolic pathways can be predicted using this information. For example, the *cbb*₃-type heme copper oxidase has previously been shown to have a high affinity for dioxygen in *Bradyrhizobium japonicum* (32) and to retain the ability to conserve the energy liberated from the oxygen reduction reaction. These properties have been suggested to allow organisms harboring the corresponding genes to colonize microaerobic environments (9, 31), e.g., the small intestine, and thus these genes may be required for virulence.

Aerobic and anaerobic respiration require membrane-associated electron transport systems that acquire electrons from an electron donor and transfer them to an electron acceptor (e.g., oxygen), thereby conserving energy for ATP synthesis.

Several types of oxidation-reduction enzymes are involved in such electron transport. All respiratory electron transport chains comprise heme-containing proteins called cytochromes. Cytochromes are classified according to the nature of heme binding. Cytochromes of the *c* type bind heme covalently via two thioether bonds to the two cysteines in the sequence motif Cys-Xaa-Xaa-Cys-His located in the periplasmic domain of the cytochrome. Whether the cell produces a certain *c*-type cytochrome depends on the transcriptional and translational regulation of the corresponding gene, on posttranslational targeting of the protein to the periplasm, and on subsequent heme attachment. In this work, a single mutation inactivating the cytochrome *c* maturation pathway that enables the biochemical characterization of a subset of the predicted electron transport chains is described (Fig. 1).

MATERIALS AND METHODS

Strains and growth conditions. Bacterial strains used in this work are listed in Table 1. *Escherichia coli* strain DH5 α (16) was used for cloning S17 λ pir (43) for conjugation with *V. cholerae*. Media were prepared as described by Silhavy et al. (42). *E. coli* strains were grown aerobically in Luria-Bertani broth (LB). *V. cholerae* 2740-80 is a nontoxicogenic El Tor isolate from the Gulf Coast of the United States (1). Derivatives of this strain were used throughout the study. If not stated otherwise, *V. cholerae* samples were grown aerobically in LB. For anaerobic growth under argon of *V. cholerae*, M63 minimal medium was supplemented with trace elements (10 mg liter⁻¹ H₃BO₃, 1 mg liter⁻¹ ZnSO₄ · 7 H₂O, 0.5 mg liter⁻¹ CuSO₄ · 5 H₂O, 0.1 mg liter⁻¹ Na₂MoO₄ · 2 H₂O, 0.1 mg liter⁻¹ MnCl₂ · 4 H₂O, and 0.2 mg liter⁻¹ FeCl₃) and electron acceptors to final concentrations of 40 mM for sodium fumarate, 10 mM for trimethylamine *N*-oxide (TMAO), 14 mM for dimethyl sulfoxide (DMSO), and 5 mM for KNO₃. Antibiotics were used at the following concentrations in both LB and M63 media: for ampicillin, 200 μ g ml⁻¹; for kanamycin, 50 μ g ml⁻¹; for chloramphenicol, 20 μ g ml⁻¹; and for rifampin, 40 μ g ml⁻¹. If not otherwise stated, 0.1% arabinose was added for gene expression from the arabinose promoter P_{ara}. Cultures were grown at 37°C.

Sequence analyses and databases used. *V. cholerae* sequences were retrieved from the published genome (17) on the website of the Institute for Genomic

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FIG. 1. Predicted respiratory electron transport chains of *V. cholerae*. Electrons are transported from the quinol pool (QH₂) to the terminal electron acceptors indicated. Arrows with dashed lines show electron transport chains with *c*-type cytochrome subunits. BSO, biotin sulfoxide.

Research (<http://www.tigr.org/>; Rockville, MD). Other sequences were retrieved from the GenBank (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD) and EMBL (European Bioinformatics Institute, Cambridge, United Kingdom) databases. Protein sequences were analyzed using programs available at the Expasy Molecular Biology website (<http://www.expasy.ch/>; Swiss Institute of Bioinformatics, Epalinges and Geneva, Switzerland).

Cloning of *V. cholerae* *ccm* genes. Plasmids used and constructed in this work are shown in Table 1. The gene encoding CcmE of *V. cholerae* was amplified by PCR with primer pair VCEN (5' GGAGAATTCATATGAACCAAGACG 3') and VCEC (5' CGGGATCCTCATTGGTCGCTTCCTTG 3') using genomic DNA of *V. cholerae* 2740-80 as template. The PCR product was digested with BamHI, blunt ended with T4 DNA polymerase, restricted with NdeI, and cloned into NdeI- and SmaI-digested pISC-2 vector. The resulting plasmid, pVC11, has *ccmE* under the tight control of the *P_{ara}* promoter (19).

A DNA fragment encoding CcmC, CcmD, and CcmE of *V. cholerae* was

amplified by PCR using *Taq* polymerase, primer pair VCCN (5' GGAGAATT CATATGTGGAAATGGCTTCATCCC 3') and VCEC, and genomic DNA of *V. cholerae* 2740-80 as template. The PCR product was blunt ended with T4 DNA polymerase and cloned into the EcoRV site of pACYC184, resulting in plasmid pVC5.

To obtain expression of *ccmA-E* of *V. cholerae*, genomic DNA of the *ccmE*-mutant VC595 and the vector pISC-2 (19) were digested with PstI and BamHI. The DNA fragments were ligated, and clones containing a fragment harboring *ccmA-E* with *ccmE::kan* were obtained by selection on kanamycin, followed by screening for ampicillin sensitivity. The resulting plasmid (pVC31) was digested with AccIII and religated, thereby deleting one of the SacI sites, to obtain pVC50. Next, the 1,902-bp PstI-Mph11031 fragment of pACYC177 (Table 1) was cloned into the PstI site of pVC50, and selection on ampicillin plates resulted in pVC60, harboring *ccmA* to *ccmF'* with *ccmE::kan*. pVC60 was digested further with SacI and religated, thereby deleting the DNA fragment encoding the kanamycin resistance (*Km^r*) and reconstituting the gene *ccmE*. The resulting plasmid, pVC70, is a pBR322 derivative and harbors the 4,242-bp PstI-BamHI fragment encoding wild-type CcmA-E.

Construction of *V. cholerae* *ccmE* *kan* mutants. A DNA fragment harboring the *kan* gene encoding aminoglycoside 3'-phosphotransferase conferring kanamycin resistance (*Km^r*) was amplified by PCR using *Taq* polymerase, the primer pair Kan5'SacI (5' TAAGAGCTCGATCCTTCAACTCAGCAAAAG 3') and Kan3'SacI (5' TAAGAGCTCAATTCTGATTAGAAAACTC 3'), and pACYC177 as template. The PCR product was digested with SacI and cloned into the SacI site of pVC5, thereby disrupting the *ccmE* gene. Kanamycin-resistant clones were analyzed for the orientation of *kan* with respect to the *ccm* genes. In pVC20, *kan* is oriented in the same direction as the *ccm* genes, and in pVC21, it is oriented in the opposite direction (Table 1). In both cases, the BamHI-XbaI fragment harboring *ccmC*, *ccmD*, and *ccmE::kan* was isolated and cloned into the BglII-NheI site of the suicide vector pGS284 (Table 1). The *E. coli* strain CC118λpir was transformed with the ligation products. Kanamycin-resistant strains harboring *kan* in the same or opposite orientation as the *ccm*

TABLE 1. Strains and plasmids used in this study^a

Strain or plasmid	Genotype or relevant characteristic	Origin or reference
<i>E. coli</i> strain		
MC1061	<i>hsdR mcrB araD139 Δ(araABC-leu)7679ΔlacX74 galU galK rpsL thi</i>	25
DH5α	<i>supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	16
S17λpir	Expression of the π protein for replication of pCVD-based suicide vector	43
EC06	Δ <i>ccmA-H</i> derivative of MC1061; <i>Km^r</i>	47
EC65	Δ <i>ccmE</i> derivative of MC1061	39
<i>V. cholerae</i> strain		
2740-80	Nontoxigenic El Tor isolate from the Gulf Coast of the United States	1, 30
VC593	Spontaneous Rif ^r -resistant derivative of <i>V. cholerae</i> 2740-80	This study
VC595	VC593 <i>ccmE::kan</i> ; <i>kan</i> in same orientation as <i>ccmE</i>	This study
VC596	VC593 <i>ccmE::kan</i> ; <i>kan</i> in opposite orientation as <i>ccmE</i>	This study
Plasmid		
pGS284	Derivative of pCVD442 <i>SacB bla oriR6K RP4oriT</i>	44
pISC-2	Arabinose-inducible expression plasmid; Ap ^r	19
pACYC184	Cm ^r , Tc ^r	NEB
pACYC177	Ap ^r , Km ^r	NEB
pNJ17	TnAraOut, Km ^r	20
pRJ3291	<i>B. japonicum cycA</i> cloned into pISC-2; Km ^r	38
pEC86	<i>Ec ccmABCDEFGH</i> cloned into pACYC184; Cm ^r	2
pEC406	<i>Ec ccmCDE</i> cloned into pACYC184; Cm ^r	38
pEC412	<i>Ec ccmE</i> cloned into pISC-2; Ap ^r	39
pVC5	<i>Vc ccmCDE</i> cloned into pACYC184; Cm ^r	This study
pVC11	<i>Vc ccmE</i> cloned into pISC-2; Ap ^r	This study
pVC12	<i>Vc ccmE::kan</i> on pGS284 as in pVC20; Ap ^r , Km ^r	This study
pVC13	<i>Vc ccmE::kan</i> on pGS284 as in pVC21; Ap ^r , Km ^r	This study
pVC20	pVC5 <i>ccmE::kan</i> ; <i>kan</i> in same orientation as <i>ccmE</i>	This study
pVC21	pVC5 <i>ccmE::kan</i> ; <i>kan</i> in opposite orientation to <i>ccmE</i>	This study
pVC60	<i>Vc ccmA-CcmF' ccmE::kan</i> on pBR322; Ap ^r , Km ^r	This study
pVC70	<i>Vc ccmA-CcmF'</i> on pBR322; Ap ^r	This study

^a Ap^r, Cm^r, Km^r, Tc^r, and Rif^r, ampicillin, chloramphenicol, kanamycin, tetracycline, and rifampicin resistance, respectively. *Ec*, *E. coli*; *Vc*, *V. cholerae*; NEB, New England BioLabs.

TABLE 2. Predicted heme *c* binding proteins of *V. cholerae*^a

Protein	Function/electron transport	Locus	Location	MW of mat. prot. (Da)	No. of CXXCH motifs
TorC	TMAO reduction	VC1693	IM	44,381	5
YecK (TorY)	BSO reduction	VC1951	IM	40,815	5
PetC (Cyt. <i>c</i> ₁)	<i>bc</i> ₁ complex SU	VC0575	IM	27,689	1
CcoP	<i>cbb</i> ₃ oxidase SU	VC1439	IM	35,853	2
CcoO	<i>cbb</i> ₃ oxidase SU	VC1441	IM	23,629	1
NapB	Nitrate reduction	VCA0679	PP	14,610	2
NapC	Nitrate reduction	VCA0680	IM	21,404	4
YhjA (Cyt. <i>c</i> 551)	Cyt. <i>c</i> peroxidase	VC0089	IM	36,040	2
CycA (Cyt. <i>c</i> ₄)	Unknown	VC0112	PP	19,791	2
CycB (Cyt. <i>c</i> ₅)	Unknown	VC0168	PP	11,479	1
Cyt <i>c</i> 554	Unknown	VCA0766	PP	9,079	1
Cyt <i>c</i> 554	Unknown	VC2241	PP	8,480	1
	Unknown	VC1265	PP	48,221	2
	Unknown, chemotaxis	VC1298	IM	69,758	1

^a SU, subunit; BSO, biotin sulfoxide; Cyt., cytochrome; MW, molecular weight; IM, inner membrane; PP, periplasm; mat. prot., mature, protein.

genes were designated pVC12 or pVC13, respectively. *E. coli* S17λpir was transformed with these plasmids, and the resulting strains MB570 and MB571 were used as donor strains in biparental matings with *V. cholerae*. To obtain a marker of the recipient, the *V. cholerae* strain 2780-40 was plated on LB plates containing 40 µg ml⁻¹ rifampin. Rifampin-resistant (Rif^r) colonies occurred at a frequency of 8×10^{-8} cells. A Rif^r colony was isolated, analyzed for wild-type *ccmC-E* by PCR with the primer pair VCCN-VCEC, and designated VC593. Strain VC593 was cross-streaked with strains MB570 and MB571 for conjugation on LB plates and incubated at 37°C overnight. Selection for conjugal transfer of pVC12 and pVC13 was done by plating dilutions of the cross-streaks on LB plates supplemented with kanamycin and rifampin. Over 500 colonies were obtained per mating experiment. Two colonies of each mating were purified and grown for 6 h in 5 ml of LB supplemented with kanamycin. Fifty µl per culture was plated on LB in the absence of NaCl but containing 5% (wt/vol) sucrose at 30°C, thereby selecting for loss of *sacB* (35). Sucrose-resistant colonies were further screened for kanamycin resistance and ampicillin sensitivity. Colony PCR with primer pair VCEN-VCEC was used to verify the presence of *ccmE::kan* and the absence of wild-type *ccmE*. Strains VC595 and VC596 have *kan* insertions in *ccmE*. For VC595, *kan* is oriented in the same direction as the *ccm* genes, whereas for VC596, *kan* is oriented in the opposite direction.

Transformation with plasmid DNA and transposon mutagenesis of *V. cholerae*. Transformation of *V. cholerae* with plasmid DNA was performed by electroporation as previously described by Marcus et al. (24).

Plasmid pNJ17 (20) was transformed into the *E. coli* donor strain S17λpir and used for transposon mutagenesis after conjugal transfer into either the pVC70-carrying strain VC593 or VC593. Colonies were screened for nitrate reductase deficiency as follows. They were overlaid with 5 ml of 50°C top agar (42) containing 10 mM KNO₃. Plates were incubated for 15 min at room temperature. Equal volumes of Griess reagent (18) and melted 1.5% agarose in H₂O were mixed, and 5 ml of the mixture was poured onto the plates. Nitrate reductase-positive colonies turned pink, while nitrate reductase-negative colonies remained white. Negative colonies were streaked to single colonies for purification and tested for *cbb*₃ oxidase activity by overlaying them with a saturated solution of NNN'-tetramethyl-1,4-phenylene-diamine (TMPD) in 10% ethanol. Strains positive for *cbb*₃ oxidase activity turned blue immediately, while negative strains remained white for about 1 min. DNA from mutants of interest was extracted by using a QIAamp DNA mini kit (QIAGEN) as described by the manufacturer. Arbitrary PCR was used to amplify the DNA flanking the transposon with primers pBADout2 (5'-CTGACGCTTTTATCGCAAC-3') and ARB6 as described by O'Toole et al. (27) for the first round of PCR. A nested PCR was performed with primer pair pBADout3 (5'-CTCTCTACTGTTTCTCCATAC-3') and ARB2 (27), and PCR products were analyzed on and extracted from 1% agarose gels using NucleoSpin Extract (Macherey-Nagel, Oensingen, Switzerland). Subsequently, they were reamplified by PCR with primer pair pBADout3 and ARB2 and sequenced with primer pBADout3 by Microsynth (Balgach, Switzerland).

Cell fractionation. Periplasmic fractions were extracted with polymyxin B sulfate as described previously (12). Membrane fractions were purified from 10-ml cell cultures lysed by osmotic shock and subsequent centrifugation over a

two-step sucrose gradient (200 µl of 65% sucrose over 600 µl of 25% sucrose) as previously described (5). Larger quantities of membranes were obtained from 200-ml cultures of *V. cholerae* lysed by three passages through a French pressure cell (12). DNase and RNase were added to 5 mg ml⁻¹ in either case before centrifugation to get rid of some of the viscosity. Protein concentrations were determined with Coomassie Brilliant Blue G250 assay reagent (Interchim, Montluçon, France) using bovine serum albumin as the protein standard.

Determination of nitrate reductase activity and nitrite concentrations. Pre-cultures were grown aerobically in LB at 37°C for 8 h. They were diluted 1:50 in 2 ml of fresh M63 medium supplemented with 0.2% glucose and 0.2% glycerol. Cultures were grown aerobically overnight in the absence or presence of 0.5 and 10 mM KNO₃. Optical densities at 600 nm were determined, and supernatants were used for the determination of NO₂⁻ concentrations using fresh Griess reagent as described previously (18). Concentrations were calculated using a standard curve generated with solutions of known concentrations of NaNO₂.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and heme stain. Protein samples were suspended in SDS loading dye and loaded without boiling onto SDS-15% polyacrylamide gels (22). Heme-binding proteins were detected in gels using their intrinsic peroxidase activity with *o*-dianisidine (Sigma Chemical Co., St. Louis, MO) and H₂O₂ as substrates as previously described (40).

Spectroscopic methods. Membrane fractions containing 5 mg protein were diluted in 1 ml Tris, pH 7.5, containing 1% Triton X-100. To eliminate insoluble precipitate, the solution was centrifuged for 1 min at full speed in a microcentrifuge. Spectra of samples containing 5 mg ml⁻¹ protein and either oxidized by air or reduced with 1 mM sodium dithionite were recorded from 350 to 650 nm in 10-mm light path cuvettes with a Hitachi model U-3300 spectrophotometer in double-beam mode. Data were exported to Microsoft Excel, and difference spectra were calculated and saved as reduced-minus-oxidized spectra. Double difference spectra were calculated by subtracting the values of mutant difference spectra from those of the wild-type difference spectra.

RESULTS

Predicted *c*-type cytochromes of *V. cholerae*. The minimal sequence requirement for the conversion of apo- to holo-cytochrome *c* in vivo has been determined to be Cys-Xaa-Xaa-Cys-His (CXXCH) heme binding site following a signal sequence, which is required for targeting to the cell envelope (4, 6). The published sequences of both *V. cholerae* chromosomes and their derived proteomes (17) were analyzed for the existence of these motifs. A total of 48 CXXCH motifs were found in 31 proteins. Of these, 14 were predicted to have a cleavable or noncleavable N-terminal signal sequence and thus be targeted to the cell envelope (Table 2). Some of these are predicted to be components of respiratory chains using O₂, NO₃⁻,

TMAO, and biotin sulfoxide as electron acceptors (Fig. 1). YhjA is predicted to be a peroxidase that may be required to overcome oxidative stress. The remaining six *c*-type cytochromes have significant homologues in other bacteria; however, their function is unknown.

Cytochrome *c* maturation in *V. cholerae*. The genes *ccmA* to *ccmI* (VC2057 to VC2049) are clustered on the large chromosome of *V. cholerae* and were expected to be required for cytochrome *c* maturation. The *ccm* genes of *V. cholerae* are arranged on the chromosome like those of *E. coli*, whereby the genes *ccmI* and *ccmH* of *V. cholerae* encode separate domains of the *E. coli* protein CcmH, which is probably encoded by a fused gene (46).

The heme chaperone CcmE is a key player in cytochrome *c* maturation and is required for heme delivery to cytochrome *c* (39). Like *c*-type cytochromes, it binds heme covalently, but at a single histidine residue rather than at a CXXCH motif and only transiently (39). We reasoned that by constructing a mutant deficient in cytochrome *c* maturation, it would be possible to block all of the cytochrome *c*-dependent respiratory chains of *V. cholerae* (Fig. 1) in one single step. Two mutants were constructed in the *V. cholerae ccmE* gene. A kanamycin-resistance cassette (*kan*) was ligated into the *SacI* restriction site of the *ccmE* gene that had been cloned from genomic DNA of *V. cholerae* 2740-80 into a suicide vector. Two constructs with *kan* oriented in either direction with respect to the *ccmE* gene were obtained. These constructs were then used for the disruption of the genomic *ccmE* gene by homologous recombination. As a result, two mutant strains that differed with regard to the orientation of the kanamycin-resistance gene were obtained. The strain with the *kan* gene insertion in the same orientation as *ccmE* was named VC595, while the one with the *kan* cassette in the opposite orientation was named VC596. These strains showed no growth defects in nutrient-rich LB medium, although the cell pellets were white, unlike those of the wild type, which were reddish. For further characterization, membrane fractions of the aerobically grown wild-type strain and the two *ccmE* mutants were analyzed for the presence of cytochromes *c*. Proteins were separated on a 15% denaturing SDS-polyacrylamide gel, and covalently bound heme was subsequently stained. In the wild-type strain VC593, a typical profile of at least six distinguishable heme-staining bands between 16 to 35 kDa was obtained in the membrane fraction, and one was obtained in the soluble protein fraction (Fig. 2A, lanes 1 and 4). By contrast, in the *ccmE*-mutant strains VC595 and VC596, no heme-staining bands were visible when the same amount of protein was loaded, indicating the lack of *c*-type cytochromes in the strain. Additionally, reduced-minus-oxidized difference spectra of the membrane fractions were analyzed (Fig. 2B). In the wild type, peaks typical for cytochromes were found at 428.5 nm (γ peak), 523.5 nm (β peak), and 553.5 nm (α peak), with a shoulder at 560 nm. This is characteristic for membranes containing both *b*- and *c*-type cytochromes, because the former have maxima in the range of 560 nm, whereas the latter have maxima at $\sim$ 550 nm. The maxima found for the γ (431.5 nm), β (534 nm), and α (562.5 nm) absorption peaks of the mutant strain were typical for *b*-type cytochromes. To assess the difference between the wild type and the *ccmE* mutant, the difference spectrum of the mutant strain was subtracted from that of the wild type, resulting in a

FIG. 2. CcmE is required for cytochrome *c* maturation in *V. cholerae*. (A) Heme stain of membrane (lanes 1 to 3) and soluble (lanes 4 to 6) proteins (100 μ g per lane) of wild-type strain VC593 (lanes 1 and 4) and *ccmE*-mutant strains VC595 (lanes 2 and 5) and VC596 (lanes 3 and 6) separated by SDS-15% PAGE. Molecular mass marker bands (in kilodaltons) are indicated on the left. (B) Optical difference spectra of membrane proteins (5 mg ml⁻¹) in 50 mM Tris, pH 7.5. Dithionite-reduced minus air-oxidized spectra of the wild-type strain VC593 and the *ccmE*-mutant VC595 are shown. The difference spectrum of VC595 was subtracted from that of VC593, resulting in the double difference spectrum (dd). Inset, the α/β region at a higher magnification.

“double difference spectrum” with γ (422 nm), β (522.5 nm), and α (552.5 nm) peaks typical of *c*-type cytochromes (Fig. 2B, inset). In conclusion, *ccmE* mutants are able to synthesize *b*-type but not *c*-type cytochromes.

Complementation of the *ccmE* mutant and heme-binding properties of CcmE. The *ccmE* mutants VC595 and VC596 were complemented with a plasmid from which either *V. cholerae* or *E. coli ccmE* was expressed. Complementation was fully successful for VC595 with the *V. cholerae ccmE*, and at low levels even with the *E. coli ccmE*, whereas it was not possible for VC596. This indicated that the cassette insertion in VC596 had a polar effect on the downstream genes *ccmFGH* and *ccmI* (data not shown).

We also tested whether CcmE binds heme, as has been previously described for CcmE of *B. japonicum* (41) and *Arabidopsis thaliana* (45) expressed in *E. coli*. For this purpose, plasmids pVC60 and pVC70 harboring *ccmA-D* with *ccmE::kan* and wild-type *ccmA-E* were constructed. *E. coli* strain EC06, which has a deletion of the entire *ccm* operon, was transformed with pVC11 (*ccmE*), pVC5 (*ccmCDE*), pVC60 (*ccmA-D*), pVC70 (*ccmA-E*), and, as a control, pEC406 (*E. coli ccmCDE*), and membranes were analyzed for

FIG. 3. CcmE of *V. cholerae* binds heme in the presence of CcmA-D. *E. coli* EC06 ($\Delta ccmA-H$) was transformed with plasmids encoding the *V. cholerae* (Vc) proteins CcmE (pVC11, lane 1), CcmA-D (pVC60, lane 2), CcmA-E (pVC70, lane 3), and CcmC-E (pVC5, lane 4) or the *E. coli* (Ec) proteins CcmC-E (pEC406, lane 5) as the control.

proteins containing covalently bound heme. As with *E. coli* CcmE (38) (Fig. 3, lane 5), heme binding to *V. cholerae* CcmE was possible only when CcmABCD were coexpressed (Fig. 3, lane 3) and, more specifically, when CcmCD were present (lane 4). The characteristics of *V. cholerae* CcmE thus are very similar to the ones known for *E. coli* CcmE.

Physiological characteristics of cytochrome *c*-deficient *V. cholerae*. The *V. cholerae* genome comprises a whole set of genes encoding proteins involved in respiratory electron transfer. Some of these proteins are *c*-type cytochromes (Fig. 1 and Table 2) whose biosynthesis depends on a functional cytochrome *c* maturation system.

To determine which respiratory chains are active, *V. cholerae* VC593 (wild type) and VC596 (*ccmE::kan*) were grown under anaerobic conditions in minimal medium containing glycerol as a nonfermentable carbon source in the presence of a single electron acceptor. Cultures were grown in triplicate in 10-ml sealed glass tubes, and growth was monitored by measuring the optical density at 600 nm. Growth of the wild-type strain VC593 in the presence of fumarate as electron acceptor, which does not require electron transport via *c*-type cytochromes, was set at 100% (Fig. 4A). As a negative control, strains were incubated in the absence of electron acceptors, and, as expected, no growth was detected for these cultures. In the presence of DMSO, TMAO, or fumarate, the growth phenotypes of wild-type and *ccmE*-mutant *V. cholerae* corresponded well with the predicted phenotypes (Fig. 1 and 4A; Table 2).

Despite the presence of the *napABCDE* genes (Table 2), which encode a periplasmic nitrate reductase, none of the strains was able to grow on nitrate (NO_3^-) (Fig. 4A). A closer look at the genome revealed the lack of genes for a classic nitrite (NO_2^-) reductase. This suggested that nitrite, which is toxic, may accumulate as the product of the Nap-catalyzed reduction of nitrate and prevent further growth. To test whether nitrite is toxic, the wild-type strain was grown aerobically for 8 h in liquid M63 medium (42) in the presence of 0.2% glucose and 0.2% glycerol containing from 0.078 to 5 mM concentrations of nitrite. A toxic effect was visible at nitrite concentrations above 0.3 mM, and growth diminished completely at a nitrite concentration of 2.5 mM (data not shown). Nitrite accumulation was determined for VC593 and VC596 grown aerobically overnight in M63 medium supplemented with 0.2% glucose and 0.2% glycerol in the presence and ab-

FIG. 4. Respiratory chains present in *V. cholerae*. (A) Anaerobic growth of the wild-type strain VC593 (open bars) and *ccmE*-mutant strain VC596 (black bars) in M63 containing 0.2% glycerol and the indicated final electron acceptors (blank, no electron acceptor; Fum, fumarate). Growth of the wild-type strain in the presence of fumarate was taken as 100%. The average growth of three independent cultures is shown, and standard deviations are indicated. (B) Nitrite accumulation. Wild-type VC593 (open bars) and *ccmE*-mutant VC596 (black bars) strains were grown aerobically in M63 medium containing 0.2% glucose and 0.2% glycerol in the presence of the KNO_3 concentrations indicated. Nitrite accumulation was determined after overnight growth at 37°C.

sence of KNO_3 (0.5 and 10 mM). Nitrite concentrations were determined with the Griess reaction (18). While no nitrite accumulated in the *ccmE*-mutant VC596 (or VC595; data not shown), nitrite concentrations of up to 6 mM accumulated during growth of the wild type (Fig. 4B). Hence, in the presence of nitrate, a cytochrome *c*-deficient mutant survives because it does not accumulate nitrite.

V. cholerae is predicted to have at least three oxidases (one *cbh*₃- and two *bd*-type oxidases) that are able to use O_2 as the terminal electron acceptor (Fig. 1). Of these oxidases, only the high-affinity *cbh*₃-type heme copper oxidase contains *c*-type cytochrome subunits. The *cbh*₃-type oxidase consists of the proteins CcoNOQP, with the *c*-type cytochromes CcoO and CcoP. This oxidase transports electrons from the *bc*₁ complex, which also contains the *c*-type cytochrome *c*₁, to O_2 (21). However, it is unknown whether another *c*-type cytochrome shuttles electrons from cytochrome *c*₁ to the CcoP and CcoO subunits of the heme copper oxidase. Apparently, NNN'-tetramethyl-1,4-phenylene-diamine (TMPD) is able to substitute the *bc*₁ complex in this reaction and acts as an electron donor to the *cbh*₃-type oxidase (21). Oxidation of TMPD results in a deep blue color due to the resulting indophenol that can be seen in colonies treated with TMPD. While the wild-type strain VC593 turned blue when exposed to a saturated solution of TMPD in 10% EtOH (TMPD-positive phenotype), the *ccmE*-mutant strains VC595 and VC596 remained white (TMPD-negative phenotype).

Screening for cytochrome *c*-deficient *V. cholerae*. After having shown that the *c*-type cytochrome subunits of the *cbh*₃ oxidase and the periplasmic nitrate reductase depend on the cytochrome *c* maturation proteins, we next attempted to find new factors involved in cytochrome *c* maturation by using a random mutagenesis approach. *V. cholerae* wild-type strain VC593 was randomly mutagenized with the transposon TnAraOut. TnAraOut has previously been used to identify essential genes of *V. cholerae* by transcriptionally fusing them

TABLE 3. Representatives of nitrate reductase and TMPD oxidase negative strains generated by TnAraOut insertions

TIGR locus name ^a	Distance from TA to ATG	Encoded protein	Insertion sequence ^b	Strain ^c	Phenotype ^d	
					TMPD	NO ₃ ⁻
VC2057	-5	CcmA	ACCTGTT TA AGCATGTTAGAAGTTAAAAAT	TNV81	Neg.	Neg.
VC2056	84	CcmB	ACCTGTT TA AAAAACACAGAGGGTTAAAC	TNV73	Neg.	Neg.
VC2055	110	CcmC	ACCTGTT TA CNTGANNCGAAACTGTCCCC	TNV75	Neg.	Neg.
VC2053	-1	CcmE	ACCTGTT TA TGAACCCAAGACGTAAAAAGC	TNV78	Neg.	Neg.
VC2052	1308	CcmF	ACCTGTT TA GTGTTGGCAGCGTTGATGATG	TNV86	Neg.	Neg.
VC2051	395	CcmG (DsbE)	ACCTGTT TA TGTGGGGCTGGATCTTGGGG	TNV90	Neg.	Neg.
VC2701	94	DsbD	ACCTGTT TA ACACAGGCACTGCAAGCTTTG	TNV74	Neg.	Neg.
VC1435	127	CcdA like	ACCTGTT TA CTTTATTACAATCTAGGTCGC	TNV92	Neg.	Pos.
VC1437	522	CcoI	ACCTGTT TA CTTGTATCGTCTTGGTATCGC	TNV93	Neg.	Pos.
VC1441	104	CcoO	ACCTGTT TA ATCTCAACGACAGCACCCCAA	TNV97	Neg.	Pos.
VC1024	112	MoaA	ACCTGTT TA CCTGATGGTTACAAGCCATCA	TNV103	Pos.	Neg.

^a As published on the TIGR website, <http://www.tigr.org>.

^b Sequence to the left of the boldface TA is the end of TnAraOut; sequence to the right is *V. cholerae* genomic sequence. Predicted start codons are underlined.

^c Strains TNV88 to TNV103 additionally harbor pVC70 encoding CcmA-E.

^d TMPD oxidase and NO₃⁻ reductase activity. Neg., negative; Pos., positive.

to an outward-facing, arabinose-inducible promoter, P_{BAD}, located at one end of the transposon (20). Over 55,000 transposon mutants were screened for deficiency in nitrate reductase and TMPD oxidase. In summary, a total of 25 strains that were negative for both nitrate reductase and TMPD oxidase activity were isolated. One representative mutant per identified gene is shown in Table 3. The majority of these mutants had transposon insertions within genes previously known to be required for cytochrome *c* biogenesis, i.e., the *ccm* genes and *dsbD* (10, 47). Interestingly, the transposon insertion found 5 bp upstream of *ccmA* resulted in an arabinose-dependent phenotype. In the presence of 0.01% arabinose, nitrate reductase activity was restored.

In addition to mutants with both nitrate reductase and TMPD oxidase deficiencies, some mutants which had either nitrate reductase (TNV103) or TMPD oxidase (TNV92, TNV93, and TNV97) deficiencies also were isolated. Table 3 shows that they mapped to VC1024 (*moaA*) or VC1435, VC1437 (*ccoI*), and VC1441 (*ccoO*), respectively. VC1435 is an unknown membrane protein.

We also took advantage of some of the obtained mutants to identify some of the *c*-type cytochromes visible in the membranes after separation by SDS-PAGE and heme staining. The profiles of *c*-type cytochromes detected in membranes from the strains were compared with those of the wild-type strain VC593 (Fig. 5). For the wild type, at least six distinguishable bands were visible. Two of these bands were missing in the *ccoO*-mutant TNV97. The bands correspond to the expected size of the *c*-type cytochromes CcoP (35.8 kDa) and CcoO (23.6 kDa). The *ccoO* insertion in TNV97 is expected to be polar on the downstream gene VC1439 (*ccoP*). Therefore, the *c*-type cytochrome encoded by *ccoP* is also missing in this mutant. In contrast, the transposon insertions of strains TNV93 and TNV92 are in genes located further downstream; their cytochrome *c* profiles are identical to that of the wild type. This indicates that the proteins encoded by VC1437 and VC1435 are required for *cbh*₃ oxidase activity but not for maturation of the *c*-type cytochromes CcoP and CcoO.

In the *ccmF* mutant, no heme staining band was visible, i.e., all *c*-type cytochromes were absent. The CcmE heme chaperone was not detectable, indicating that chromosomal *ccmE*

expression was too low for the gene product to be detectable. No bands were visible in the *ccmE*-mutant VC595 or in the *dsbD*-mutant TNV74. These genes thus are essential for the maturation of all *c*-type cytochromes detected.

The *moaA*-mutant TNV103 was missing the largest *c*-type cytochrome visible on the gel (Fig. 5). This band may correspond to YecK (40.8 kDa) which together with BisZ forms the biotin sulfoxide reductase. BisZ shares homology with the DMSO reductase family of the molybdoenzymes, has a twin arginine (Tat) signal sequence, and is expected to be translocated through the cytoplasmic membrane via the Tat translocon (15). The defect in molybdopterin biogenesis in TNV103 is expected to disable BisZ maturation and secretion to the cell envelope (29). In the absence of BisZ, YecK may be degraded, or expression of YecK may be repressed. Besides the lack of this *c*-type cytochrome in strain TNV103, two other *c*-type cytochromes, running at about 20 to 23 kDa, were induced.

FIG. 5. Type *c* cytochromes of mutant *V. cholerae* strains. Membrane extracts containing 200 µg protein of the indicated strains were separated by SDS-PAGE in a 15% polyacrylamide gel. The gel was treated with *o*-dianisidine and H₂O₂ to identify polypeptides possessing covalently bound heme groups. The electrophoretic mobility of marker proteins is indicated on the left. The positions of CcoP and CcoO are indicated on the right.

The identities of these are unknown. Predicted *c*-type cytochromes of the expected sizes are CcoO (23.6 kDa), NapC (21.4 kDa), and CycA (19.8 kDa) (Table 3).

DISCUSSION

CcmE is a heme chaperone required for biogenesis of *c*-type cytochromes. CcmE of *E. coli* binds heme covalently at the β -carbon of a heme vinyl group via a strictly conserved histidine (23, 39). From CcmE, heme is then delivered to the *c*-type cytochromes. Here, we show that *V. cholerae* CcmE is similar to *E. coli* CcmE in that it also binds heme covalently and is required for cytochrome *c* maturation. Also, the CcmCD proteins are essential for the unusual binding of heme by CcmE. Furthermore, CcmE of *E. coli* is able to complement a *ccmE*-mutant *V. cholerae* for cytochrome *c* maturation, albeit not at 100%. This indicates that CcmE of *E. coli* can interact with CcmC and CcmF of *V. cholerae* to transfer heme to the *c*-type cytochromes, as was proposed for the *E. coli* cytochrome *c* maturation system (33, 34).

The regulation of cytochrome *c* biogenesis in *V. cholerae* is different from that of *E. coli*. *E. coli* produces *c*-type cytochromes only under anaerobic growth conditions, while wild-type *V. cholerae* produces at least six *c*-type cytochromes when grown aerobically in nutrient-rich medium. The *V. cholerae* *ccmE* mutants lack all *c*-type cytochromes. The physiological role of the *V. cholerae* *c*-type cytochromes was assessed by a comparison of the wild-type strain with the *ccmE* mutant. When both the wild type and the mutant were grown in LB, no difference in growth was observed. In accordance with the protein sequences predicted from the translated genome, the wild-type was able to use fumarate and TMAO as electron acceptors during respiration in the absence of O_2 . By contrast, the mutant strain VC596 was unable to grow on TMAO, probably because neither the TorC nor the YecK (TorY) *c*-type cytochrome was matured. Unlike *E. coli*, which is known to produce a *c*-type cytochrome-independent TMAO reductase (3), *V. cholerae* does not harbor the genes for this type of *c*-type cytochrome-independent TMAO reductase. Thus, in *V. cholerae*, *c*-type cytochromes are essential for respiration on TMAO.

V. cholerae harbors the genes encoding the periplasmic nitrate reductase NapABCFGH, but it lacks genes for a NarGHI-like nitrate reductase (7). We found that *V. cholerae* was not able to grow on NO_3^- as the sole electron acceptor and interpreted this to be the result of accumulation of NO_2^- . NO_2^- is toxic to *V. cholerae*, because the organism also lacks the genes for nitrite reductase, which are present in *E. coli* (7). Of the bacterial genomes sequenced to date and published on the TIGR homepage (<http://www.tigr.org>), the pathogens *Bordetella bronchiseptica* and *Bordetella parapertussis* are the only ones like *V. cholerae*, which harbors genes encoding a periplasmic nitrate reductase but lack genes for nitrite reductases. This implies that these bacteria are not able to use nitrate as a source of nitrogen. Additionally, some *Rhodobacter sphaeroides* strains have been reported to produce a Nap-type nitrate reductase but to lack a nitrite reductase (13). For these strains, the Nap system was shown to be involved in redox balancing during metabolic conditions, such as phototrophic growth on reduced carbon substrates, thereby generating an excess of

reductant (13). The function of the periplasmic nitrate reductase of *V. cholerae* is unclear, but perhaps it allows scavenging nitrate from nitrogen-limiting environments in the presence of other (micro-) organisms which are able to detoxify nitrite by reduction to ammonia.

The cytochrome *c* maturation pathway is not yet understood completely. While apo-cytochrome *c* has been shown to cross the cytoplasmic membrane via the Sec translocon (48), it is still unknown how heme reaches the periplasm. We have confirmed in this work that in *V. cholerae*, as in *E. coli*, the Ccm proteins are required for cytochrome *c* maturation. Two of the Ccm proteins, CcmA and CcmB, are predicted to form an ABC transporter, the substrate of which still is unknown, but there is circumstantial evidence that the transported substrate is not heme (8, 14). As it is not certain that all genes required directly or indirectly for cytochrome *c* biogenesis are already known, we combined two screening procedures, using nitrate reductase and *cbb*₃ oxidase activity that can be detected by colony staining of *V. cholerae*. These activities have a common requirement for *c*-type cytochromes. The wild-type strain VC593 was mutagenized with the transposon TnAraOut (20), and ~55,000 colonies were screened initially for nitrate reductase activity and then for *cbb*₃ oxidase activity or vice versa. Mutations inactivating genes that are required only for one of these activities, e.g., the structural genes of nitrate reductase, are excluded in the combined test. This increases the chance of finding genes required for cytochrome *c* maturation. DNA flanking the transposon insertion of candidates was sequenced. The mutagenesis seemed to be slightly biased; *ccmE* was hit four times while *ccmH* was never hit. Besides finding transposon insertions upstream of *ccmA*, within *ccmB*, *ccmC*, *ccmE*, *ccmF*, and *ccmG*, we also found mutants with transposon insertions within *dsbD*. DsbD and its homologues are capable of transporting disulfide bonds across the membrane and have previously been shown to be required for cytochrome *c* maturation in gram-negative bacteria (26, 28). No new genes specifically required for cytochrome *c* maturation were found in our screening. This may be due to the incomplete randomness of the transposon insertions, or the already identified cytochrome *c* maturation factors may represent the complete set of proteins specifically needed for this process. Alternatively, if an as-yet-unknown heme transporter exists for the transfer of heme from the cytoplasm to the periplasm (14), the same transporter may also be used to translocate heme to the outer leaflet of the cytoplasmic membrane before the cofactor is inserted into respiratory enzymes, such as the *bc*₁ complex, the *cbb*₃ oxidase subunit I, or the *bd* oxidases. Such a transporter would then be essential for aerobic growth, and mutants would be obtainable only as conditional ones. The selection of the transposon TnAraOut for mutagenesis would allow the isolation of conditional mutants when mutants are plated on medium containing arabinose.

Our screening revealed one novel gene, VC1435. It is located towards the 3' end of the gene cluster containing the structural and accessory genes for the *cbb*₃ oxidase. The encoded protein shares low but apparently significant similarity with one domain of DsbD and CcdA (<http://www.expasy.ch>), the latter being a cytochrome *c* biogenesis factor that was first identified in *Bacillus subtilis* and later in *Rhodobacter capsulatus* and is not present in *E. coli* (11, 36). It is interesting that in

V. cholerae, DsbD is essential for maturation of all *c*-type cytochromes, whereas VC1435 apparently is needed only for assembly of the *cbb*₃ oxidase. However, in this mutant, heme is still incorporated into the *c*-type cytochrome subunits CcoO and CcoP. Hence, VC1435 must have another role in biogenesis or assembly of the *cbb*₃-type heme copper oxidase, perhaps at the step of introducing the copper cofactor into the subunit I (CcoN).

In summary, electron transport chains that use O₂, fumarate, TMAO, and nitrate as terminal electron acceptors are active in *V. cholerae*. CcoO and CcoP are two of the major *c*-type cytochromes expressed under aerobic conditions in nutrient-rich medium. While *V. cholerae* has both cytochrome *c*-dependent and -independent electron transport chains required for O₂ respiration, respiration on fumarate does not, and respiration on TMAO does require *c*-type cytochromes. Respiration on nitrate requires *c*-type cytochromes; the resulting nitrite, however, cannot be further metabolized, and its toxic effect prevents further growth. Increasing knowledge of the physiology will result in a better understanding of how *V. cholerae* can adapt to the diverse environmental conditions it thrives in.

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