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NOTES

Functional Expression of *Desulfovibrio vulgaris* Hildenborough Cytochrome c_3 in *Desulfovibrio desulfuricans* G200 after Conjugational Gene Transfer from *Escherichia coli*

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Plasmid pJRDC800-1, containing the *cyc* gene encoding cytochrome c_3 from *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough, was transferred by conjugation from *Escherichia coli* DH5α to *Desulfovibrio desulfuricans* G200. The G200 strain produced an acidic cytochrome c_3 (pI = 5.8), which could be readily separated from the Hildenborough cytochrome c_3 (pI = 10.5). The latter was indistinguishable from cytochrome c_3 produced by *D. vulgaris* subsp. *vulgaris* Hildenborough with respect to a number of chemical and physical criteria.

Cytochrome c_3 is a periplasmic electron carrier that is found throughout and is perhaps diagnostic for the genus *Desulfovibrio*, a strictly anaerobic class of sulfate-reducing bacteria (20). The amino acid sequence (107 residues) of mature cytochrome c_3 from *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough (referred to hereafter as *D. vulgaris* Hildenborough) (26, 30; R. P. Ambler, Biochem. J. **109**:47p, 1968) is preceded by an NH_2-terminal signal sequence of 21 amino acid residues, which functions in the export of this cytochrome to the periplasm (19). The formation of periplasmic holo-cytochrome c_3 also requires the insertion of four *c*-type hemes, which are covalently bound to four pairs of cysteine residues in the protein (13, 18). Attempts to express cytochrome c_3 in functional form in *Escherichia coli* under aerobic or anaerobic growth conditions were unsuccessful because of the inability of *E. coli* to insert the hemes (19). *E. coli* does express multiheme *c*-type cytochromes (e.g., a hexahemoprotein [nitrite reductase] is expressed when nitrate is the terminal oxidant [14]), and its inability to produce holo-cytochrome c_3 under these same conditions points to a possible specificity of the heme insertion system. It is therefore appropriate to test more closely related hosts (e.g., *D. desulfuricans* G200 [8]) for their potential to produce holo-cytochrome c_3.

Conjugation of *E. coli* HB101 and *Desulfovibrio desulfuricans* G200. Plasmid pJRD215 (Table 1) was isolated from *E. coli* MM294(pJRD215) grown in LC medium (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract, and 5 g of NaCl per liter) with 50 μg of kanamycin per ml as described previously (7). Purified pJRD215 was doubly digested with restriction enzymes *Eco*RI and *Hin*dIII and ligated to pJ800 (Table 1), which had been digested with these same two enzymes. The ligation mixtures were transformed into *E. coli* TG2 and spread onto TY-kanamycin plates (15 g of agar, 8 g of NaCl, 10 g of tryptone, 5 g of yeast extract, and 50 mg of kanamycin per liter), and plasmid pJRDC800-1, containing the *cyc*-gene, was isolated (Table 1). Plasmids pJRDC800-1 and pJRD215 were transformed into *E. coli* DH5α with selection for resistance to 50 μg of neomycin per ml. Either of these donors and *E. coli* HB101, containing the helper plasmid pRK2073, were grown in LC medium to early stationary phase and mixed 1:1. Serial dilutions of these mixtures were then transferred to an anaerobic chamber and mixed 1:1 with a mid-exponential-phase culture of *D. desulfuricans* G200 grown in LS medium (22). Four 40-μl drops of the mating mixtures were spotted onto the surfaces of well-dried LS plates. After 4 h, the spots were spread and nalidixic acid (200 μg/ml, final concentration) was added, followed about 2 h later by the addition of 175 μg of neomycin per ml. Exconjugants of *D. desulfuricans* G200 were obtained routinely at frequencies of 10^{-5} to 10^{-4} per *E. coli* donor cell. Southern analysis was used to confirm the presence of the plasmids in *D. desulfuricans* G200. The 640-base-pair (bp) radiolabeled insert of plasmid pJ800, containing the *cyc* gene, hybridized strongly with the homologous 640-bp *Hin*dIII-*Eco*RI fragment in the exconjugant containing pJRDC800-1 (Fig. 1, lane 5). The gel purified probe (Fig. 1, lane 2) and doubly digested pJRDC800-1 (Fig. 1, lane 6) were also strongly positive, as expected. Weaker but distinct hybridization of DNA from *D. desulfuricans* G200 with the *cyc* probe indicated, upon longer exposure, the presence of a gene for cytochrome c_3 on a 10.0-kilobase-pair *Hin*dIII-*Eco*RI fragment in this bacterium (not shown).

Purification of cytochromes c_3. Approximately 300 g (wet weight) of cells of either *D. desulfuricans* G200(pJRD215), *D. desulfuricans* G200(pJRDC800-1), or *D. vulgaris* Hildenborough, obtained from 300-liter fermentations in Starkey medium (24) with 0.15 g of kanamycin per liter, was harvested as described elsewhere (16). The cells were suspended in 1,100 ml of 50 mM Tris hydrochloride–50 mM EDTA (pH 9.0), stirred for 15 to 30 min at 37°C, and then

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TABLE 1. Bacterial strains and DNA vectors used

Strain or vector	Genotype, comments, and reference
D. vulgaris Hildenborough	NCIMB 8303; isolated from clay soil near Hildenborough, Kent, United Kingdom (20); source of the *cyc* gene, encoding cytochrome c_3 (30)
D. desulfuricans G200	Spontaneous Nal^r derivative of *D. desulfuricans* G100A (32)
E. coli TG2[a]	Δ(*lac-pro*) *supE thi hsdM hsdR recA*(F′ *traD36 proAB*$^+$ *lacZ*ΔM15*I*^{q}); from T. J. Gibson
E. coli DH5α	φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1* λ$^-$ *gyrA96 relA1* F′Δ(*lacZA-argF*) *U169* (12)
E. coli HB101	λ$^-$ *recAB proA2 leu lacY1 galK2 xyl-5 mtl-1 ara-14* F$^-$ (*hsdS20*) (r_B^- m_B^-) (5)
E. coli MM294	*thr endA hsdR pro* (3)
pUC8	29
pJ800	Contains the *cyc* gene of *D. vulgaris* Hildenborough on a 640-bp *Eco*RI-*Hin*dIII insert in pUC8 (19)
pJRD215	IncQ group, broad-host-range cloning vector; Km^r Sm^r (7)
pJRDC800-1	Contains 640-bp insert of pJ800 in pJRD215; this study
pRK2073	Conjugational helper plasmid; Tc^r (15)

[a] Constructed from *E. coli* JM101 by T. J. Gibson and M. D. Biggin at the Laboratory of Molecular Biology, MRC Centre, Cambridge, United Kingdom.

removed from the suspension by centrifugation (20 min at 10,000 × *g*). The supernatant, containing the periplasmic proteins (27), was further processed by column chromatography. The Hildenborough cytochrome c_3 was purified in two simple steps, irrespective of whether the periplasmic fraction from *D. vulgaris* Hildenborough or *D. desulfuricans* G200(pJRDC800-1) was processed (final yields of 12 and 60 mg/300 g of cells, respectively). In the first step, the periplasmic fraction was loaded onto a hydroxyapatite column (3 by 15 cm) equilibrated with 10 mM Tris hydrochloride (pH 7.6), and the fraction containing the cytochromes was eluted with 0.5 M potassium phosphate buffer (pH 7.6). After dialysis of this fraction against 10 mM Tris hydrochloride (pH 7.6), it was loaded onto a column (3.5 by 2.5 cm) packed with carboxymethyl cellulose (Whatman CM 52) and equilibrated with the same buffer. The column was developed with a gradient of 50 to 500 mM Tris hydrochloride (pH 7.6). The two cytochromes c_3 present in extracts from *D. desulfuricans* G200(pJRDC800-1) were quantitatively separated in this step because they have different isoelectric points, pI = 5.8 and pI = 10.5 for the G200 and Hildenborough proteins, respectively (Table 2). The Hildenborough cytochrome c_3, eluting at 200 mM Tris hydrochloride (pH 7.6), was found to be pure by sodium dodecyl sulfate-gel electrophoresis (not shown). Purification of the G200 cytochrome c_3, which eluted at the start of the gradient used to develop the carboxymethyl cellulose column, required an additional step. The pooled G200 cytochrome c_3 fraction was again dialyzed against 10 mM Tris hydrochloride (pH 7.6) and then loaded onto a column (2 by 3 cm) packed with DEAE-cellulose (Whatman DE52). Pure G200 cytochrome c_3 (5 mg/300 g of cells) was obtained by elution of the column with 0.1 M Tris hydrochloride (pH 7.6). Expression of the plasmid-encoded *cyc* gene in *D. desulfuricans* G200(pJRDC800-1) apparently exceeded that of the G200 chromosomal *cyc* gene by a factor of 12. This exconjugant also produced five times more Hildenborough cytochrome c_3 than does *D. vulgaris* Hildenborough itself, probably because of the higher copy number of the plasmid-encoded *cyc* gene.

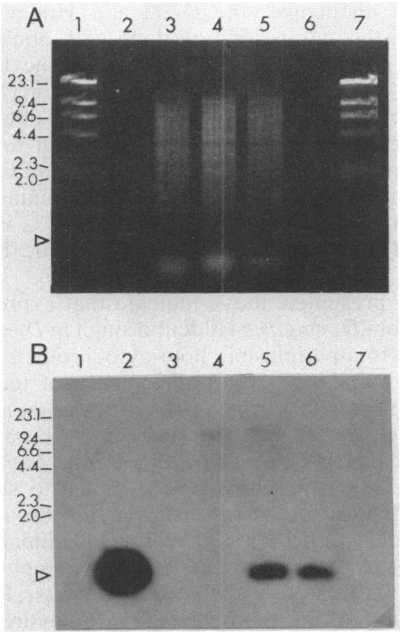

FIG. 1. Southern blotting of total (chromosomal and plasmid) DNA from *D. desulfuricans* G200 exconjugants. DNA was isolated with the minipreparation procedure of Ausubel et al. (2). All samples were doubly digested with *Eco*RI and *Hin*dIII. After electrophoresis through agarose, the gel was blotted onto a Zeta-Probe blotting membrane, using the alternative protocol described by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). The blot was incubated with the insert of plasmid pJ800, labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; 10 mCi/ml), using the oligolabeling kit (U.S. Biochemical Corp., Cleveland, Ohio). (A) Ethidium bromide-stained gel; (B) Southern blot analysis of panel A. Lanes: 1 and 7, *Hin*dIII-digested λ DNA; 2, gel-purified probe; 3, G200 total DNA; 4, G200(pJRD215) DNA; 5, G200(pJRDC800-1) DNA; 6, purified pJRDC800-1 plasmid DNA.

Physical and chemical properties of the isolated cytochromes c_3. The objectives of the characterization of the three cytochromes c_3 were (i) to define the properties of the G200 cytochrome c_3, which has not been purified before, and

TABLE 2. Comparison of physical properties of the three cytochromes c_3 purified in this study

Source of cytochrome c_3	pI	Redox potentials[a] (mV)	ε_{553} (M^{-1} cm^{-1})
G200	5.8	−284, −320, −356, −380	101,200
Recombinant Hildenborough	10.5	−244, −332, −336, −344	116,400
Native Hildenborough	10.5	−260, −326, −340, −362	115,000

[a] Experimental error, ±10 mV.

TABLE 3. Comparison of amino acid composition of the three cytochromes c_3 purified in this study

Amino acid	No. of residues in cytochrome c_3 from:		
	G200	Recombinant Hildenborough	Native Hildenborough[a]
Lys	13	20	20
His	8	9	9
Arg	1	1	1
Asp	11	12	12
Thr	5	5	5
Ser	8	6	6
Glu	6	5	5
Pro	6	4	4
Gly	6	9	9
Ala	10	10	10
Cys	8	8	8
Val	5	8	8
Met	3	3	3
Ile	3	0	0
Leu	5	2	2
Tyr	3	3	3
Phe	2	2	2
Total	103	107	107

[a] Derived from the known sequence (25, 30; Ambler, Biochem. J. **109**:47p, 1968).

(ii) to determine whether the Hildenborough cytochrome c_3 produced by the G200 strain (the recombinant protein) was the same as that synthesized by *D. vulgaris* Hildenborough (the native protein). All three isolated proteins migrated as single polypeptides with apparent molecular weights of 14 kilodaltons during sodium dodecyl sulfate-gel electrophoresis (not shown). Isoelectric focusing using a PHAST GEL apparatus from Pharmacia LKB Biotechnology (11) with Phast Gel IEF 3-9 and a Pharmacia Broad Range pI Calibration Kit, containing proteins with isoelectric points ranging from 3 to 10, indicated a pI of 5.8 for the G200 protein (Table 2). The recombinant Hildenborough cytochrome c_3 migrated similarly to the native Hildenborough protein, indicating it to have the same pI (10.5 [20]).

The amino acid compositions of the G200 and the recombinant Hildenborough apoproteins, prepared and isolated as described elsewhere (1, 6, 28), are shown in Table 3. The latter is in complete agreement with the amino acid composition derived from the sequence (25, 30; Ambler, Biochem. J. **109**:47p, 1968) (Table 3). The G200 protein contains only 13 lysine residues, whereas 20 lysines are found in Hildenborough cytochrome c_3. The lower pI for the G200 protein (Table 2) must originate from this lower lysine content. The NH_2-terminal sequence of recombinant Hildenborough cytochrome c_3, determined as described elsewhere (28), was found to be APKAP, identical to that of the native protein (25, 30; Ambler, Biochem. J. **109**:47p, 1968), indicating that the Hildenborough polypeptide is identically processed in *D. desulfuricans* G200 during transport to the periplasm. Duplicate analyses of both the unmodified and the carboxymethylated G200 apoprotein by automated Edman degradation gave the NH_2-terminal sequence of the G200 cytochrome c_3 indicated in Table 4. Comparison of this sequence with that of the Hildenborough protein indicates a sequence identity of 15 out of 21 residues.

TABLE 4. Comparison of the NH_2-terminal sequences of G200 and Hildenborough cytochromes c_3

Source of cytochrome c_3	Sequence
	10 20
G200	AEAPADGLKMENTKMPVIFNK
	********* ** ** **
Hildenborough	APKAPADGLKMEATKQPVVFNH
	10 20

Visible and UV absorption spectra were determined with a Philips PU8820 spectrophotometer. Molar extinction coefficients at the absorption maxima were obtained from these spectra, using protein concentrations derived from amino acid analysis data. All three purified cytochromes c_3 had absorption maxima at 553 nm in the fully reduced form, with the molar extinction coefficients as listed in Table 2. There was no absorption band at 695 nm (not shown). This result indicates the hemes in these cytochromes to be bis-histidine coordinated. The G200 cytochrome c_3 has eight histidines, which is sufficient for the coordination of the four hemes (Table 3).

Midpoint redox potentials of the four hemes of each of the three cytochromes were determined relative to that of the normal hydrogen electrode in 0.5 M Tris hydrochloride (pH 7.6), which also served as the supporting electrolyte, using the equipment and theoretical analysis described previously (4). The hemes of all three purified cytochromes had midpoint redox potentials in the range of −200 to −400 mV. These values are similar to those found for other cytochromes c_3 by cyclic voltammetry and other methods (9, 23). Whereas the electrochemical measurements are not suitable to distinguish the G200 and Hildenborough cytochromes, this can readily be done with nuclear magnetic resonance spectroscopy. Resonances of the heme methyl groups can be observed in the low-field region (10 to 35 ppm) of the spectrum (9, 10, 17). The spectrum obtained for the G200 cytochrome c_3 (Fig. 2A) confirmed it to be different from the Hildenborough protein (Fig. 2B and C), in view of the different positions of heme methyl resonances, whereas the similarity of the spectra in Fig. 2B and C indicates that authentic Hildenborough protein was made by the G200 strain.

The data presented above indicate that expression of the *cyc* gene from *D. vulgaris* Hildenborough in *D. desulfuricans* G200 leads to a periplasmic holo-cytochrome c_3. This work was made possible by the development of technology for conjugating *Desulfovibrio* and the other gram-negative bacteria. Powell et al. (21) transferred the broad-host-range IncQ plasmid R300B to two different *Desulfovibrio* species, which subsequently expressed resistance to streptomycin. Van den Berg et al. (26) transferred the broad-host-range IncQ plasmid pSUP104 to *D. vulgaris* Hildenborough and showed that the plasmid-encoded chloramphenicol resistance was expressed by the exconjugants. Recombinant plasmids carrying the genes encoding [Fe] hydrogenase from *D. vulgaris* Hildenborough (31) were also transferred, and this was shown to lead to a 2- to 10-fold-increased synthesis of hydrogenase subunits but not to a proportional increase in [Fe] hydrogenase activity. As discussed by the authors (26), this could mean that other functions, e.g., those active in the insertion of iron-sulfur clusters, are limiting. Such difficulties were not encountered in this study, in which yet another IncQ plasmid, pJRD215 (7), and its derivative pJRDC800-1 were transferred to *D. desulfuricans* G200. A fivefold-increased expression of fully functional Hildenborough cytochrome c_3 was observed in the *D. desulfuricans* G200 (pJRDC800-1) exconjugant, which could be readily purified and characterized. This work represents, therefore, the first

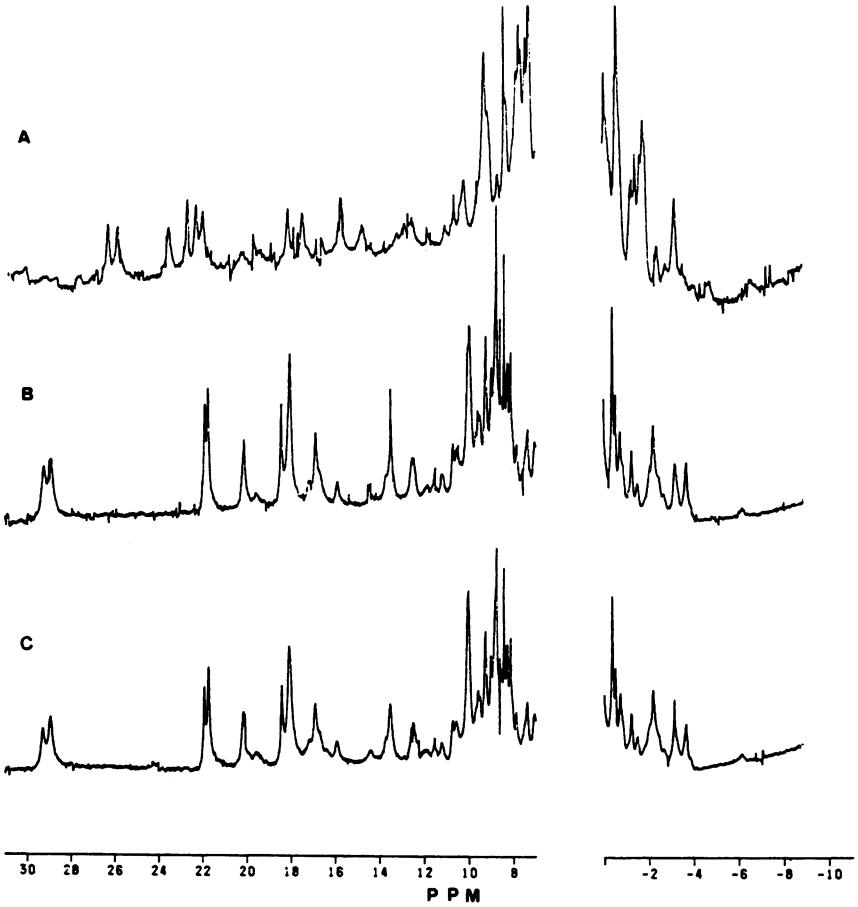

FIG. 2. ^1H nuclear magnetic resonance spectra of oxidized cytochromes c_3. Samples were lyophilized twice after proton exchange in D_2O for 3 h at 45°C and then dissolved in D_2O containing 0.1 M potassium phosphate (pH 7.6) at a protein concentration of 1 mM. Spectra were recorded in the Fourier mode on a Bruker AM 200 spectrophotometer at 35°C (10). Sample concentration, 1 mM in 0.1 M phosphate (pH 7.6). (A) G200 cytochrome c_3; (B) recombinant Hildenborough cytochrome c_3; (C) native Hildenborough cytochrome c_3.

successful application of *Desulfovibrio* species as a host for the expression of an introduced gene other than an antibiotic resistance marker.

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