

Succinate dehydrogenase functioning by a reverse redox loop mechanism and fumarate reductase in sulphate-reducing bacteria

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Sulphate- or sulphur-reducing bacteria with known or draft genome sequences (*Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans* G20, *Desulfobacterium autotrophicum* [draft], *Desulfotalea psychrophila* and *Geobacter sulfurreducens*) all contain *sdhCAB* or *frdCAB* gene clusters encoding succinate : quinone oxidoreductases. *frdD* or *sdhD* genes are missing. The presence and function of succinate dehydrogenase versus fumarate reductase was studied. *Desulfovibrio desulfuricans* (strain Essex 6) grew by fumarate respiration or by fumarate disproportionation, and contained fumarate reductase activity. *Desulfovibrio vulgaris* lacked fumarate respiration and contained succinate dehydrogenase activity. Succinate oxidation by the menaquinone analogue 2,3-dimethyl-1,4-naphthoquinone depended on a proton potential, and the activity was lost after degradation of the proton potential. The membrane anchor SdhC contains four conserved His residues which are known as the ligands for two haem B residues. The properties are very similar to succinate dehydrogenase of the Gram-positive (menaquinone-containing) *Bacillus subtilis*, which uses a reverse redox loop mechanism in succinate : menaquinone reduction. It is concluded that succinate dehydrogenases from menaquinone-containing bacteria generally require a proton potential to drive the endergonic succinate oxidation. Sequence comparison shows that the SdhC subunit of this type lacks a Glu residue in transmembrane helix IV, which is part of the uncoupling E-pathway in most non-electrogenic FrdABC enzymes.

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INTRODUCTION

Succinate : quinone oxidoreductases (SQR) from bacteria function either as succinate : quinone reductase (succinate dehydrogenase, or Sdh) in succinate oxidation, or as quinol : fumarate reductase (fumarate reductase, or Frd) in fumarate respiration (Hederstedt 2002; Kröger *et al.*, 2002; Cecchini *et al.*, 2002). *Bacillus subtilis*, which contains menaquinone as the only respiratory quinone, has been shown to contain a specific type of succinate dehydrogenase (Schirawski & Uden, 1998; Schnorpfel *et al.*, 2001). In contrast to the classical succinate dehydrogenase consisting of four subunits (SdhABCD), the enzyme from *B. subtilis* is

composed of three subunits (SdhABC). Catalytic subunit A and the Fe–S cluster subunit B are exposed to the cytoplasm. Subunit C serves as the membrane anchor and contains the active site for menaquinone reduction. SdhC of the *B. subtilis* enzyme carries two haem B molecules in a trans-membrane arrangement: one of the haem B molecules is close to the outer, the other to the inner aspect of the membrane (Hägerhäll & Hederstedt 1996; Hägerhäll *et al.*, 1995; Körtner *et al.*, 1990; Lancaster *et al.*, 1999; Simon *et al.*, 1998). The haem B molecules are bound by four conserved His residues.

Succinate oxidation by menaquinone is endergonic, since succinate ($E_0 = +30$ mV) is more electropositive than the acceptor menaquinone ($E_0 = -80$ mV). The reaction requires a transmembrane H^+ -potential (Δp) for function (Schirawski & Uden, 1998). Succinate oxidation (succinate \rightarrow fumarate + $2H^+$ + $2e^-$) takes place in the cytoplasm, whereas menaquinone reduction ($MK + 2H^+ + 2e^- \rightarrow MKH_2$) is believed to occur close to the outer

Abbreviations: BV⁺, reduced benzyl viologen; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCPIP, dichlorophenol indophenol; DMN, 2,3-dimethyl-1,4-naphthoquinone; TRAP, tripartite ATP-independent periplasmic.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is DQ643793.

aspect of the membrane (Schirawski & Uden, 1998). An active site for menaquinone close to the outer aspect is supported by the presence of a conserved Glu residue which is homologous to Glu66 in subunit FrdC of *Wolinella succinogenes* fumarate reductase. This residue is at the active site for menaquinol oxidation (Lancaster *et al.*, 2000). Location of the oxidative and reductive half-reactions on opposite sides of the membrane requires a transmembrane electron transfer, which is effected by the two haem B molecules in subunit C. The overall reaction according to this scheme consumes and releases protons on opposite sides of the membrane, and the enzyme functions by a reverse (Δp -driven) redox loop mechanism (Schirawski & Uden, 1998). Sdh of this type is active only in intact (energized) cells, and is inactivated by addition of an uncoupler, or in membrane preparations (Lemma *et al.*, 1990; Schirawski & Uden 1998; Schnorpfeil *et al.*, 2001). The reaction of ubiquinone ($E_0 = +110$ mV)-dependent succinate dehydrogenase, on the other hand, is exergonic. Bacterial and mitochondrial enzymes of this type retain activity with the quinones in the presence of uncouplers and in membrane preparations, and contain only one haem (with two conserved His residues) close to the inner aspect of the membrane. In addition, the membrane anchor consists of two subunits (subunits CD) which are smaller in size (for an overview see Hagerhall, 1997; Lancaster, 2002b).

Sulphate-reducing bacteria contain only menaquinone as the respiratory quinone (Collins & Widdel, 1986). The anaerobic Gram-negative bacteria are physiologically and phylogenetically largely distant from the (facultatively) aerobic Gram-positive genus *Bacillus*, raising the question whether succinate dehydrogenase is of the same type as in the genus *Bacillus*. Genomic sequences of several sulphate- and sulphur-reducing bacteria are available, and the genomes contain gene clusters similar to the *sdhCAB* genes of *B. subtilis*. The gene cluster is similar to the *frdCAB* genes encoding fumarate reductase in *W. succinogenes* (see Kroger *et al.*, 2002, Lancaster & Simon, 2002 for a review). Fumarate reductase from *W. succinogenes* is a three-subunit enzyme (FrdABC) and catalyses fumarate reduction by menaquinol (Uden *et al.*, 1980; Kroger *et al.*, 2002; Biel *et al.*, 2002). FrdC of *W. succinogenes* carries two haem B molecules with a transmembrane arrangement similar to SdhC of *B. subtilis* (Kortner *et al.*, 1990; Simon *et al.*, 1998). The SdhABC and FrdABC enzymes are similar in sequence and identical in the prosthetic groups.

To identify the role of the enzymes encoded by the *sdh/frd* genes, their function was determined for selected strains. *Desulfovibrio desulfuricans* (strain Essex 6) used the enzyme for fumarate respiration and a new form of fumarate disproportionation. In *Desulfovibrio vulgaris* on the other hand, the enzyme functions as succinate dehydrogenase. Succinate dehydrogenase of *D. vulgaris* had characteristic properties of the *B. subtilis* succinate dehydrogenase and required the proton potential for function, which appears to be a general property of menaquinone-dependent succinate dehydrogenases.

METHODS

Bacteria and growth. *Desulfovibrio desulfuricans* (strain Essex 6, DSMZ no. 642) and *Desulfovibrio vulgaris* (strain Hildenborough, DSMZ no. 644) were used. Subcultures of the bacteria were grown in modified *Desulfovibrio* medium 63 plus lactate (DSM, 1993) in Sovirell tubes under an atmosphere of N_2 at 37 °C. Modified medium 63 consists of 0.5 g K_2HPO_4 l^{-1} , 1.0 g NH_4Cl l^{-1} , 0.1 g $CaCl_2 \cdot 2H_2O$ l^{-1} , 1.0 g yeast extract l^{-1} (Serva no. 24540), 1.0 mg resazurin l^{-1} , 0.6 g trisodium citrate l^{-1} , 50 mg EDTA l^{-1} , 2.5 mg $FeSO_4 \cdot 7H_2O$ l^{-1} , 0.1 g sodium thioglycolate l^{-1} and 0.1 g ascorbic acid l^{-1} at pH 7.8. Sterile medium (400 ml) in infusion bottles (500 ml) was made anoxic by repeated degassing under vacuum and gassing with N_2 before inoculation. For growth on sulphate, the medium contained in addition sulphate (25 mM disodium sulphate, 25 mM magnesium sulphate) plus lactate (50 mM sodium DL-lactate) or succinate (50 mM disodium succinate) as indicated in the individual experiments. For growth on fumarate or formate + fumarate (50 mM disodium fumarate without or with 50 mM sodium formate), the disodium/magnesium sulphate was replaced by 17 mM NaCl and 21 mM $MgCl_2$. The media (400 ml) were inoculated with 7 ml of a subculture grown with lactate plus sulphate for 24 h, and incubated for about 24 h.

Cell suspensions and cell-free extract for measurement of enzyme activities. The bacteria were sedimented from the growth media by centrifugation for 30 min at 10 000 g, and washed three times with anoxic potassium phosphate (50 mM at pH 7.8). To prepare the cell suspension, the bacteria were suspended in the same buffer at 400 mg wet weight of bacteria per l. For preparing the cell-free extract, the sedimented and washed bacteria (100 mg wet weight) were mixed with 180 μ l of the same buffer and 0.5 g glass beads (0.13 mm in diameter, Zirkonia-silica, Roth). The suspension was swirled rapidly on a mixer for 30 s followed by incubation on ice for 5 min. The procedure was repeated six times; the debris and glass beads were sedimented by centrifugation. The glass beads were extracted once with the anoxic buffer, centrifuged, and the supernatants were combined.

Enzyme activities. Fumarate reductase was measured with reduced benzylviologen (BV[•]) as the electron donor (Lemma *et al.*, 1990; Schirawski & Uden, 1998; Schnorpfeil *et al.*, 2001). Measurement of succinate dehydrogenase activity with the dye dichlorophenol indophenol (DCPIP) or the menaquinone analogue 2,3-dimethyl-1,4-naphthoquinone (DMN) was as described previously (Schirawski & Uden, 1998; Schnorpfeil *et al.*, 2001). All enzyme tests were performed at 37 °C under anoxic conditions in cuvettes sealed with rubber stoppers, and 1 unit corresponds to the consumption of 1 μ mol fumarate or succinate min^{-1} (g protein)⁻¹. Protein concentrations of cell suspensions were determined by the biuret method with KCN (Bode *et al.*, 1968), and those of cell-free extracts by the Bradford assay (Bradford, 1976). The uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone, 10 μ M final concentration, stock solution 1 mM in ethanol) was added as indicated.

Fumarate disproportionation in cell suspensions and growing bacteria. *D. vulgaris* and *D. desulfuricans* were grown in modified medium 63 with lactate + sulphate and harvested in the late exponential growth phase. Cells were sedimented by centrifugation, washed twice and resuspended at OD₅₇₈ 8–10 in anoxic MOPS buffer (100 mM, pH 7.8). Cells were transferred into rubber-stoppered bottles and degassed and gassed by N_2 for three cycles. Growth was started by the addition of anoxic fumarate solution (5 mM final concn). Samples were withdrawn by syringes as required and centrifuged at 10 000 g for 5 min. The supernatants were used for HPLC analysis of the substrates and of the fermentation products (Richter *et al.*, 2001).

For measurement of the products of fumarate disproportionation by growing bacteria, samples were withdrawn from the anaerobic cultures in the serum bottles. After removal of bacteria and particles by centrifugation, the medium or buffer was used for quantitative measurement of the substrates and products by HPLC using a Aminex HPX 87H column (Richter *et al.*, 2001).

Amplification of the *sdhC* fragment of *D. desulfuricans* (strain Essex 6). For amplification of *sdhC*, genomic DNA of *D. desulfuricans* (Essex 6) was amplified with primers CGCAAGAT-GCCCTTCCGCA and TCG GAN CCT TTG ACS GTR TC (N = A/C/G/T, S = G/C; R = A/G). The primers are derived from the sequences of SdhC of *D. desulfuricans* G20 and *D. vulgaris* encoding the predicted loop between transmembrane helices 2 and 3, and from a conserved sequence in SdhA of *D. desulfuricans* G20, *D. vulgaris* and *Desulfobacterium autotrophicum*. PCR products of the expected size (645 bp) were amplified in a second step using the same primers and sequenced after purification.

Phylogenetic analysis of protein sequences. Sequences of TRAP genes were obtained from the ERGO (Overbeek *et al.*, 2003), NCBI and UniProt databases. The contigs from the *Rhodobacter capsulatus* genome sequence were analysed using the Artemis package (Berriman & Rutherford, 2003). For phylogenetic analyses, sequences were aligned in CLUSTAL X (Thompson *et al.*, 1997) and the output file used in PHYLIP (Felsenstein, 1989) to produce a bootstrapped distance matrix tree, which was viewed in TREEVIEW (Page, 1996).

RESULTS

sdhCAB/frdCAB gene clusters in sulphate-reducing bacteria and properties of subunit C

Genomic sequences from sulphate- and sulphur-reducing bacteria (*Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans* G20, *Desulfobacterium autotrophicum*, *Desulfotalea psychrophila* and *Geobacter sulfurreducens*) were screened for the presence of *sdh* or *frd* genes (<http://www.jgi.doe.gov/>; www.tigr.org/; http://www.regx.de/m_status.php). Each of the genomes contained one copy of a *sdh/frdCAB* gene cluster. The clusters contained no *sdhD/frdD* genes, and the predicted SdhC or FrdC proteins were of a size (23 to 30 kDa) characteristic for Sdh/FrdC proteins from enzymes lacking subunit D. Sdh/FrdC proteins from enzymes containing SdhD/FrdD in addition, are distinctly smaller (13 to 18 kDa, respectively) (Hägerhäll, 1997).

The proteins encoded by the *sdhA/frdA* and *sdhB/frdB* homologues showed 27 to 66% sequence identity for subunit A, and 22 to 61% identity for subunit B to the corresponding subunits of the *B. subtilis* and *W. succinogenes* enzymes. The FAD-binding sites in subunit A and the cysteine clusters in subunit B which bind the Fe-S clusters were conserved. The sequences allowed no prediction whether the enzyme from the sulphate-reducing bacteria represents Frd or Sdh. The SdhC/FrdC-subunits show a lower degree of sequence identity to FrdC of *W. succinogenes* (15.4 to 44.9% identical residues), and to SdhC of *B. subtilis* (18.3 to 24.8% identical residues) (Fig. 1). By the TMHMM program (www.cbs.dtu.dk/services/TMHMM/) five transmembrane helices are predicted for the C subunits, similar to the corresponding proteins from *B. subtilis* and *W. succinogenes* (Hägerhäll

et al., 1995; Gross *et al.*, 1998; Lancaster, 2002b; Lancaster & Simon, 2002; Hägerhäll, 1997). The four His ligands for the haem B molecules in Sdh/FrdC of *B. subtilis* and *W. succinogenes* are conserved in subunits C of the sulphate-reducing bacteria, suggesting a similar structure and topology. Some of the subunits contain also a Glu residue which is homologous to Glu180 of the E-pathway in the FrdC subunit of *W. succinogenes* (Lancaster, 2002a). *D. desulfuricans* strain G20, for which the genomic sequence is available, is not closely related to strain Essex 6, which was used for the physiological and biochemical experiments (Pires *et al.*, 2003). Therefore the sequence of part of FrdC from *D. desulfuricans* (Essex 6) was determined (Fig. 1). The sequence comprised the fragment with the third and fourth of the His residues which represent one of the ligands to each of the two haem B groups (Hägerhäll & Hederstedt, 1996; Simon *et al.*, 1998). It can be concluded that both haem groups are present in FrdC of *D. desulfuricans* (strain Essex). The Glu residue homologous to Glu180 from the E-pathway, however, was not conserved. Glu66 of *W. succinogenes*, which is at the menaquinone/menaquinol site close to the outer aspect of the membrane (Lancaster *et al.*, 2000), is found in all SdhC/FrdC sequences of the sulphate-reducing bacteria. The gene and sequence properties therefore suggest that the sulphate and sulphur reducers contain Sdh or Frd enzymes of the SdhABC or FrdABC type.

Growth of sulphate reducers by fumarate respiration versus succinate oxidation

It is not possible to decide from the sequence whether the enzymes encoded by *sdh/frd* function as succinate dehydrogenases or as fumarate reductases *in vivo*. Growth by fumarate respiration and succinate production on the other hand is a direct indication of the presence of fumarate reductase. To differentiate between the two types of enzymes, *D. desulfuricans* (strain Essex 6) and *D. vulgaris* were tested for growth with fumarate as an electron acceptor versus succinate as an electron donor in sulphate respiration (Fig. 2, Table 1). *D. desulfuricans* (Essex 6) was able to grow on fumarate when H₂ or formate were supplied as electron donors at rates comparable to growth on sulphate. Succinate on the other hand stimulated growth on sulphate only marginally. *D. vulgaris* showed the best growth on lactate plus sulphate, whereas growth on fumarate was very low. The growth experiments therefore suggest that the enzyme of *D. desulfuricans* (Essex 6) functions as fumarate reductase, and that of *D. vulgaris* as succinate dehydrogenase.

The growth yields of *D. desulfuricans* (strain Essex 6) by fumarate respiration with formate or H₂ were close to 5 g dry weight per mol fumarate (Table 1), corresponding to approx. 0.5 ATP/fumarate, which is comparable to the growth yield by fumarate respiration of *Escherichia coli* and *W. succinogenes* (Kröger *et al.*, 2002; Mell *et al.*, 1982; Bernhard & Gottschalk, 1978).

The major product of fumarate respiration was succinate, but in addition acetate (and CO₂) were produced (Table 2).

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D.v. 21N-VQMLSGAF-LALFVCM-HLVFLSSVLF-GAGAMDGLSALF-AV--HAEAVG-G---AI-----VVAAFVAH-IVA
D.d.G20 22N-LQMLSGVL-LILFLWA-HLLLVSSVIL-SPRLMNAIAWFF AT--FMAQIG-G---PV-----IFALMLFH-FILA
D.d.Ess
G.s. 25N-LMAITGQF-MVLFVIV-HMLGNSSIFIPG--GINAYA-EHLHALPPLVWAFR----L-V-----MLVAAGIH-HLFG
D.a. 21N-AQSGTGLV-LGLFMWV-HMLLVASILL-GKGSFNFVAKTMS-LS--FLSSTGHGYPSAV-FIAVFCVPTLFIH-HAALG
D.p. 15N-VMAFTGLL-LILFLCV-HAAGNAIFF-GS-AFLTYAHALHAVPLVVVLFSSG--LAV-----VLLA---HICFG
W.s. 28N-WQSATGLF-LGLFM-IGHMFFVSTILL-GDNVMLWVTKKFLD--FIFE-G-GKPIVVSFLAAF-VFAVFIHAFLA
B.s. 11N-LHSLGVIPIVGI FLIQ-HLVVNQFAAR-GAEAFNSAAHFMSLSP-FRYALE----IFI-----IFLPLIYH-AVYG

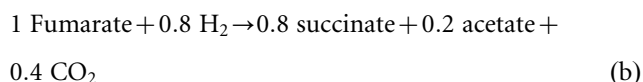
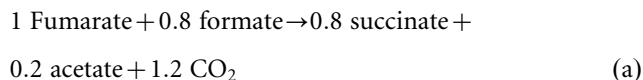
D.v. VGRKIPFRMAESIVMYRHARLLHHD T--WRVVQVLTALVLLMVTVHWEWEG-FMV-MPVAAGT--SAV-R--VQHG--
D.d.G20 --GRKMPFRTEQRAFLRHAGMLRHKDT--WMVVAQVVTAIIILVMASIMYE--VLTDLPITAQK--SAA-R--IQHG--
D.d.Ess RANELGVFVQHSKGLKEVD T--WLWLQVFTAIVILAGAFYHLYS--VMTDLPINVAG--SAK-R--L-HS--
G.s. --IQLSLENRAANPDT-YAVK-NYKKAT-MGSLSMYTGLLLLSFIIVYHLLH--FTIRATPDIKLGVDSLGRFDVFGMVT
D.a. --VRKFPITWKQHTIIRSQMGMNHGDTNLWYW--QVVTGFIMFFLGSVHLFI--MLT-HPGQIDPYLSSD-R--VFS---
D.p. --LYLFFENRTE-DSRYAVSVRTVKYS-LASKTMHWSGLFIFLFLIHLAV--FTFGGK-DIP--ISTLVE-ERLG---
W.s. --MRKFPINRYQLTFKTHKDLMRHGDTTLW-WI-QAMTGFAMFFLGSVHLYI--MMT-QPQTIGP-VSSSFR--MVS---
B.s. --VYIAFTAKNNA--GQYSYM-RN-----WLFVLQRVTGIITLIFVSWH/WETRIAAQMGAEVN--FDMAN--ILSS--

D.v. --PWGF-----FYLALLPVLVLAIGVWRLSVKYGALAS----AARPK-ARRWER-WFIGAFLGLA-L-LT-L-N8
D.d.G20 --GW-LS----FYLILLPMAEIHVGVGFYRIGVKYGFITR----ENR-KWYQR TENAMMIG-FIAVG-L-LT-L-N9
D.d.Ess --GW-LA----FYVFFLPCVILHFGIGVYRLAVKFGVCVK----ATRP AW-RKWTWIVMGC-YLLG-A-AA-L-N7
G.s. NSFSGHIIAFIYIAAMVVLFLHLSHG IQSFFQTMGWNND----KSLPV-FSKIGMIAAVLLLLGYATIPFV-I-N7
D.a. --SFMWP----LYLVLLVCVLEHGTIGLYRLCMKWGFGGKDPVQSRKK-LQLFKK-QATIFFLSIGGLALVVF-N84
D.p. D-FSFG----IYLISFAVLALHLSHGFWMSLQTFGVNHP----RYNDL-IHNLT YIVPLFFFVIFGGVTLY-F-N8
W.s. --EWMWP----LYLVLLFAVLEHGSVGLYRLAVKWGFWDGETPKTRAN-LKCLKT-LMSAFLIVLG-L-LT-F-N28
B.s. --PAMLG----FYIVGVLSLTIHHSNGLWSFAVTWGITVT-----PR-S--QRISTYVTLIIFVALS-Y-VG--N8

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Fig. 1. Alignment of FrdC/SdhC sequences of sulphate-reducing bacteria, *Wolinella succinogenes* and *Bacillus subtilis*. The conserved His residues (H, supposed haem-binding sites) and the Glu (E) residues corresponding to E66 of *W. succinogenes* and *B. subtilis*, and to E180 of *W. succinogenes*, are boxed and outlined in black, respectively. Transmembrane helices predicted by the TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM>) are marked in grey. The sequences were compared by CLUSTAL W in the protein database ExPASy (<http://www.ebi.ac.uk/clustalw/index.html>). The alignment contains 9 residues identical to all sequences, 23 conserved and 15 semiconserved substitutions according to CLUSTAL W (matrix, blosum; gap open, 1; gap extension, 0.05; end gaps, 10; other settings, default). B.s., *B. subtilis* (NCBI accession number CAB14805), D.v., *Desulfovibrio vulgaris* (AE017285); D.d., *Desulfovibrio desulfuricans* strains G20 (NC_007519) and Essex 6 (partial sequence; this study); D.a., *Desulfobacterium autotrophicum* (unpublished); D.p., *Desulfotalea psychrophila* (CR522870); G.s., *Geobacter sulfurreducens* (AE017180); W.s., *W. succinogenes* (CAE09943).

The (idealized) growth reactions (a, b) suggest that *D. desulfuricans* (Essex 6) uses about 70 to 80 % of the fumarate for fumarate reduction when formate or H₂ is present. Acetate production indicates oxidation of some fumarate.



Fumarate disproportionation

Fumarate supported growth of *D. desulfuricans* (Essex 6) and *D. vulgaris* also in the absence of formate or H₂ (Fig. 3). Growth was slower than by fumarate respiration (Table 1), but for *D. desulfuricans* the final cell densities were comparable. The type of products changed depending on the growth phase (Fig. 3, Table 2). In the early growth phase of *D. vulgaris* most of the fumarate was converted to malate and small amounts of acetate. In the second phase, the

production of acetate and succinate increased at the expense of malate. Overall, about two-thirds of the fumarate was excreted as succinate, the residue as acetate when malate is not considered (Table 2). Product formation from fumarate by *D. desulfuricans* was also biphasic, but the differences between the first phase with and the second phase without malate production were less pronounced. Malate production was much lower than for *D. vulgaris* (Fig. 3a). In the stationary phase about two-thirds of the fumarate was converted to succinate and one third to acetate (Table 2).

Fumarate disproportionation was studied in cell suspensions for a more detailed analysis (Table 3). In the early phase, *D. vulgaris* excreted mainly malate. In the second phase succinate was the major product and acetate the only further product. *D. desulfuricans* converted fumarate at much higher rates than *D. vulgaris*. Succinate and acetate were the main products, with small amounts of malate. In the late phase only succinate and acetate were produced, in an approximate ratio of 2 : 1. Malate seems to be an intermediate of fumarate fermentation and accumulates in the early phase due to limitation in subsequent metabolic steps.

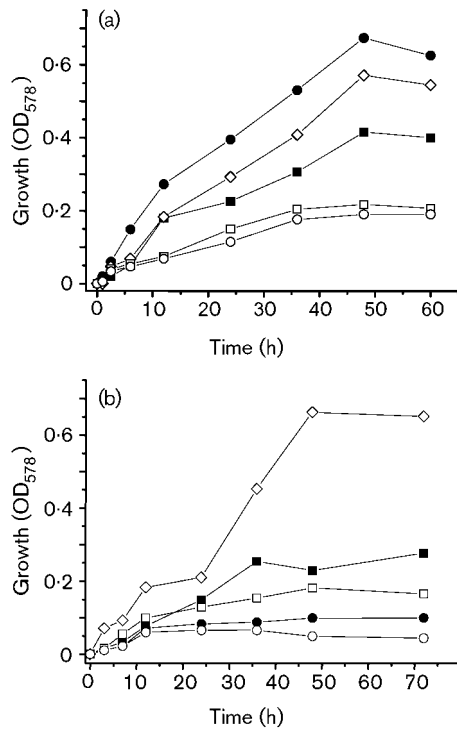


Fig. 2. Growth of *D. desulfuricans* (strain Essex 6) (a) and *D. vulgaris* (b) on fumarate (filled symbols) and sulphate (open symbols). Growth was performed under anoxic conditions in modified medium 63 containing H₂+fumarate (●), formate+fumarate (■), succinate+sulphate (□), lactate+sulphate (◇) and sulphate (○) as the substrates for growth.

The limitation was significant in *D. vulgaris* and in cell suspensions.

Succinate:menaquinone reductase from *D. vulgaris* requires Δp for function

Cell-free extracts of *D. desulfuricans* (Essex 6) and *D. vulgaris* were tested for fumarate reductase and succinate dehydrogenase activities with artificial electron donors or acceptors (Table 4). The activities of *D. vulgaris* were low and showed only a small variation (factor <3) after growth by fumarate

Table 2. Fermentation reactions and products for growth of *D. desulfuricans* (strain Essex 6) and *D. vulgaris* on fumarate

In all experiments >38 mM fumarate was metabolized. CO₂ formation was calculated by assuming that formate oxidation provides 1 CO₂/formate and oxidation of fumarate 2 CO₂/fumarate. For growth on H₂+fumarate it is assumed that 1 H₂ is consumed per succinate formed, and that 4 [H] are formed per acetate produced (see Fig. 5).

Substrates	Products [mol (mol fumarate) ⁻¹]				% C
	Succ	Mal	Acetate	CO ₂	
<i>D. desulfuricans</i>					
1 Fumarate + 0.69 formate	0.91	<0.02	0.24	1.17	112
1 Fumarate + 0.54 H ₂	0.84	<0.02	0.15	0.30	99
1 Fumarate*	0.67	<0.02	0.37	0.74	105
<i>D. vulgaris</i>					
1 Fumarate†‡	<0.02	0.85	0.13	0.26	163
1 Fumarate*	0.55	0.33	0.27	0.54	115

*Stationary growth phase.

†Early growth phase.

‡In addition formate was excreted.

or sulphate respiration, or in the presence of succinate. Fumarate reductase activities of *D. desulfuricans* were two orders of magnitude higher than those of *D. vulgaris*, and also showed no strong variation in response to the growth substrates. The ratio of fumarate reductase vs succinate dehydrogenase activities was significantly higher for *D. desulfuricans* than for *D. vulgaris* (mean value 25 versus 4), suggesting that the enzyme of *D. desulfuricans* resembles a fumarate reductase, and that of *D. vulgaris* a succinate dehydrogenase.

Intact cells of *D. vulgaris* were capable of menaquinone reduction by succinate (Fig. 4, Table 5) after growth on sulphate or fumarate. DMN is very similar to menaquinone in structure and midpoint potential ($E'_0 = -80$ mV) and

Table 1. Growth parameters of *D. desulfuricans* (strain Essex 6) for growth on fumarate and sulphate

Condition	μ (h ⁻¹)	Δ Fumarate* (mM)	Cell mass (g dw l ⁻¹)	Y_{Fum} [g dw (mol Fum) ⁻¹]
Formate + fumarate	0.09	39	192	4.9
H ₂ + fumarate	0.16	41	198	4.8
Fumarate	0.1	42	170	3.9
Lactate + sulphate	0.14	NA	NA	NA

NA, Not applicable.

*Fumarate consumption.

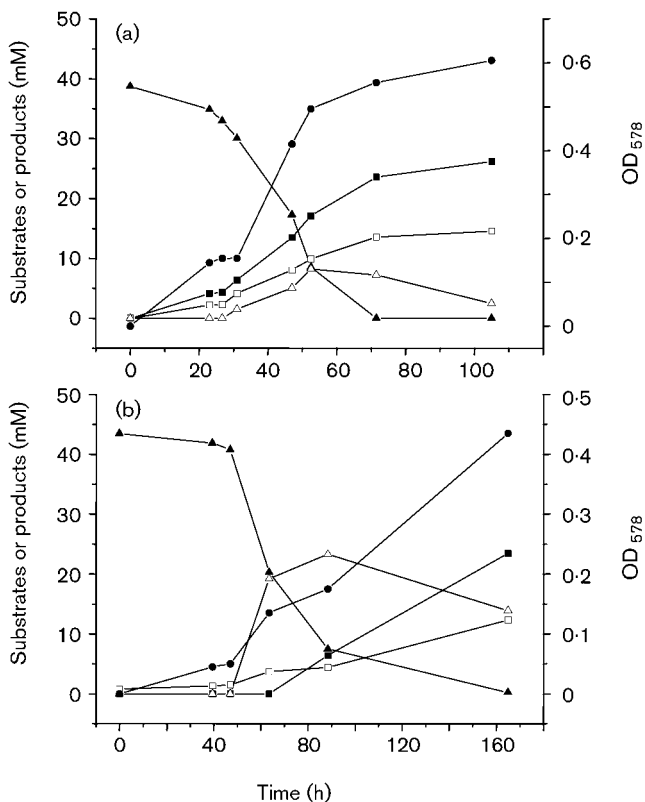


Fig. 3. Growth of *D. desulfuricans* (strain Essex 6) (a) and of *D. vulgaris* (b) on fumarate, and excretion of malate, succinate and acetate. The bacteria were grown (●, OD₅₇₈) in modified medium 63 with 50 mM fumarate. Fumarate (▲) and the end products [succinate (■), malate (△) and acetate (□)] were determined in the supernatant by HPLC.

can be used as a water-soluble analogue of menaquinone. The activity with DMN was lost after addition of the uncoupler CCCP to the bacterial cells (Fig. 4, Table 5), or breaking the cells and preparing cell homogenates (Table 5), presumably due to dissipation of the proton potential,

which was shown earlier as the driving force for DMN (or menaquinone) : succinate oxidoreductase in *B. subtilis* and other Gram-positive bacteria (Schirawski & Unden, 1998; Schnorpfeil *et al.*, 2001). In contrast, high activity of succinate dehydrogenase with the artificial electron acceptor DCPIP was retained in the cell homogenate [75 U (g protein)⁻¹]. It is concluded that this activity is, in contrast to succinate : DMN reductase, not sensitive to breaking the cell membrane.

D. desulfuricans (Essex 6) showed similar activities of DMN reduction by succinate as *D. vulgaris*, and the activity was also sensitive to the presence of uncoupler (not shown). Due to unstable activities the response to the uncoupler was not studied in detail. Future experiments will have to show whether the Sdh/Frd enzymes of other sulphate-reducing bacteria respond in the same way to uncoupler and membrane disintegration as in *D. vulgaris*.

DISCUSSION

Fumarate respiration and disproportionation by sulphate-reducing bacteria

All sulphate-reducing bacteria with known genome sequences (or drafts) contain one set of genes for succinate : quinone oxidoreductases of the FrdABC/SdhABC-type. In *D. desulfuricans* (strain Essex 6), the enzyme functions as fumarate reductase and the bacteria are able to grow by fumarate respiration or by fumarate disproportionation. Fumarate respiration and fumarate reductase have also been demonstrated for *Desulfovibrio gigas* and *Desulfovibrio multispirans* (Odom & Peck, 1981; He *et al.*, 1986; Lemos *et al.*, 2002), for which, however, no genome, gene or protein sequences are available. Fumarate reductase of the sulphate- and sulphur-reducing bacteria therefore is of the same type (FrdABC with two haem B groups in subunit C) as in other ϵ -proteobacteria (Lancaster & Simon, 2002).

Fumarate disproportionation by the bacteria allows balanced growth by oxidation of 1 mol fumarate to acetate

Table 3. Products of fumarate fermentation by cell suspensions of *D. desulfuricans* (Essex 6) and *D. vulgaris*

Cell suspensions (OD₅₇₈ 8–9; OD 1 corresponds to 300 mg dry weight l⁻¹) of the bacteria were incubated with 5 mM fumarate under anoxic conditions for 60 min (*D. desulfuricans*) and 720 min (*D. vulgaris*). Substrates and products were determined by HPLC for the indicated periods of time; the product concentrations shown represent the difference between the beginning and the end of the respective period.

Bacterium	Time (min)	Turnover [U (g dw) ⁻¹]	Products [mol (mol fumarate) ⁻¹]				%C
			Succinate	Malate	Acetate	CO ₂	
<i>D. vulgaris</i>	0–480	3.0	0.13	0.79	0.35	0.7	127
	480–720	5.3	0.77	0	0.41	0.82	117
<i>D. desulfuricans</i>	0–30	46.5	0.59	0.21	0.41	0.82	121
	30–60	15.7	0.76	0	0.36	0.72	112

Table 4. Fumarate and succinate dehydrogenase activities of *D. desulfuricans* (strain Essex 6) and *D. vulgaris* on various substrates

Specific activities were measured in cell-free homogenates under anoxic conditions with BV⁺ or DCPIP. One unit (U) corresponds to the consumption of 1 μmol fumarate or succinate min⁻¹ (g protein)⁻¹.

Bacterium	Growth	BV ⁺ →Fum (U)	Succ→DCPIP (U)	Frd/Sdh activity
<i>D. vulgaris</i>	Lactate + sulphate	117	31	3.8
	Formate + fumarate	77	38	2.0
	Succinate + sulphate	193	30	6.4
<i>D. desulfuricans</i>	Lactate + sulphate	13 690	385	36
	Formate + fumarate	7 770	449	17
	Lactate + sulphate	10 297	452	23

and reduction of 2 mol fumarate to succinate (reaction (c)) (Fig. 5). The [H] for succinate production is derived from pyruvate oxidation (Fd_{red}, H₂ or formate).

3 Fumarate → 2 succinate + 2 CO₂ + acetate (c)

The structural genes for the enzymes of the pathway are found in the genomes of *D. desulfuricans* G20 and *D. vulgaris* (Fig. 5) (www.jgi.doe.gov; www.tigr.org/), and presumably also in *D. desulfuricans* (strain Essex 6) due to the similar fermentation pattern. Malic enzyme, fumarase and acetate kinase were demonstrated also by their enzymic activities (Lewis & Miller, 1977; Brown & Akagi, 1966). Malate, which was suggested earlier as a product of fumarate disproportionation (Miller & Wakerley, 1966), apparently is only an intermediate. A similar form of fumarate disproportionation with succinate, acetate and CO₂ as the products was described for *Clostridium formicoaceticum* (Dorn *et al.*, 1978), whereas fumarate disproportionation by *Proteus*

rettgeri produced only succinate but no acetate (Kröger, 1974).

Succinate:menaquinone reductase of *D. vulgaris*: a reversed redox loop mechanism

The succinate:quinone oxidoreductase of *D. vulgaris* is a succinate dehydrogenase due to the lack of fumarate respiration and the high ratio of Sdh/Frd activity. *D. vulgaris* is not capable of complete oxidation of organic matter (Postgate, 1984) and does not encode all enzymes of the citric acid cycle and of related pathways (www.jgi.doe.gov; <http://www.genome.jp/kegg/>; www.tigr.org/; <http://www.microbesonline.org/>). Therefore succinate dehydrogenase activity might be important for specific metabolic reactions, but not for degradation of citric acid cycle intermediates, which would explain their poor growth on succinate. The sensitivity of succinate:DMN (but not succinate:DCPIP) reductase activity of *D. vulgaris* to dissipation of the proton potential is reminiscent of succinate dehydrogenase of *B. subtilis* and other aerobic Gram-positive bacteria (Schirawski & Unden, 1998). Therefore the need for a proton potential is common

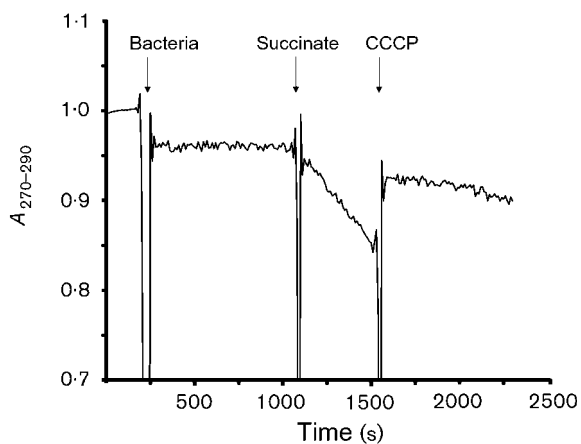


Fig. 4. Succinate:DMN reductase activity of *D. vulgaris* and effect of the uncoupler CCCP. The activity (DMN reduction by succinate) was measured with cells of the bacteria (6.5 mg protein ml⁻¹) by recording the reduction of DMN spectroscopically at 270 nm and 290 nm (reference wavelength). CCCP (arrow) was added at 10 μM.

Table 5. Specific activities of succinate:DMN reductase in cells of *D. desulfuricans* (Essex 6) and *D. vulgaris*, and response to the uncoupler CCCP (10 μM) and to cell disintegration (cell homogenate preparation)

The bacteria were grown in modified medium M63 with 50 mM formate + 50 mM fumarate. Similar activities were measured after growth of the bacteria on lactate + sulphate (*D. desulfuricans*) or succinate + sulphate (*D. vulgaris*). One unit corresponds to the consumption of 1 μmol succinate min⁻¹ (g protein)⁻¹.

Bacterium	Preparation	Suc → DMN (U)
<i>D. vulgaris</i>	Cells	15.3
	Cells + CCCP	1.4
	Cell homogenate	1.0
<i>D. desulfuricans</i>	Cells	19.2
	Cells + CCCP	0.3
	Cell homogenate	<0.5

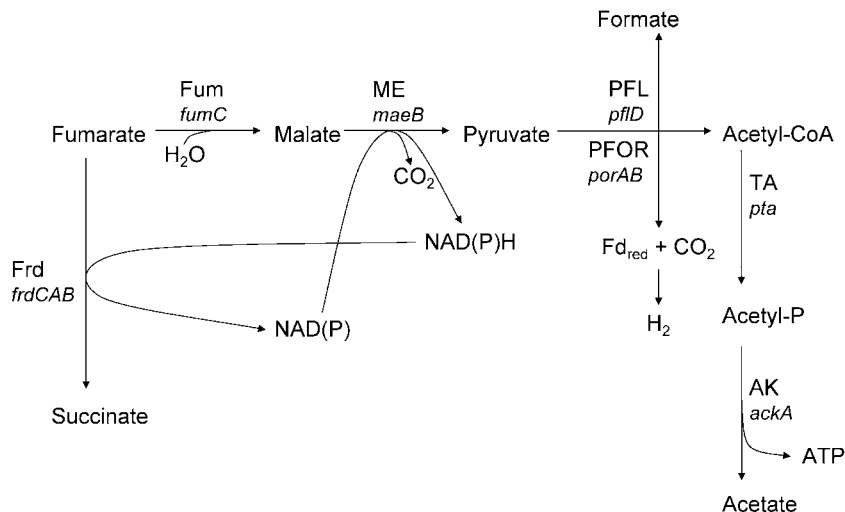


Fig. 5. Fermentation pathways for fumarate disproportionation by sulphate-reducing bacteria. The pathway shows the oxidation of fumarate to acetate and the reduction of fumarate to succinate. Fumarate oxidation supplies NAD(P)H and reduced ferredoxin/H₂ (or formate), which are reoxidized by two molecules of fumarate (fermentation balance: 3 Fum → 1 acetate + 2 succinate + 2 CO₂).

to menaquinone-dependent succinate dehydrogenases from aerobic Gram-positive and anaerobic Gram-negative bacteria. The same type of succinate dehydrogenase (SdhABC), the His ligands for transmembrane arrangement of two haem B groups and the conserved Glu residue of the external menaquinone site suggest that these phylogenetically and physiologically different bacteria use the same principle of a reverse redox loop mechanism to drive the reduction of menaquinone by succinate. Enzymes with an established structure constituting a redox loop are represented by formate dehydrogenase and nitrate reductase from *E. coli* (Jormakka *et al.*, 2002, 2003). Both enzymes generate Δp by the release of H⁺ at the periplasmic side of the membrane during oxidation of one substrate and consumption of H⁺ in the cytoplasm during reduction of the second substrate.

General presence of the 'E-pathway' in fumarate reductases of the FrdABC type

Fumarate reductase from *W. succinogenes* shows a transmembrane arrangement of the two haem B groups in FrdC (Körtner *et al.*, 1990; Gross *et al.*, 1998; Lancaster *et al.*, 1999). The enzyme, however, does not generate a proton potential by menaquinol:fumarate reduction (Biel *et al.*, 2002; Kröger *et al.*, 2002). Glu180 in transmembrane helix IV of *W. succinogenes* FrdC is part of a H⁺ shortcut ('E-pathway') which dissipates the proton potential generated by fumarate reductase (Lancaster, 2002a; Lancaster *et al.*, 2005; Haas *et al.*, 2005). Glu180 is conserved in the enzyme of *D. desulfuricans* G20 (and of *D. autotrophicum*), which is in agreement with the function of the enzyme as fumarate reductases. The enzyme from *D. vulgaris* (and similarly those from *B. subtilis*, *G. sulfurreducens* and *Desulfotalea psychrophila*) lacks the 'uncoupling' Glu180 residue, which is in accordance with their role as a 'coupling' succinate dehydrogenase.

D. desulfuricans (strain Essex 6), which was used for the biochemical and physiological experiments, is not closely

related to strain G20 (Pires, *et al.* 2003), which is reflected by the different sequences of the FrdC subunits. FrdC of strain Essex 6 lacks the Glu180 homologue, which is in agreement with the Δp -dependence of succinate:DMN oxidoreductase activity as determined experimentally. Further experiments will have to show how these properties are compatible with the physiological function of the enzyme of strain Essex 6 in fumarate respiration.

Lack of DcuB-type fumarate/succinate antiporter in *D. desulfuricans*

The genome of *D. desulfuricans* G20 contains no genes for anaerobic fumarate/succinate antiporters DcuB, DcuA or DcuC (Engel *et al.*, 1994; Six *et al.*, 1994; Zientz *et al.*, 1999; Jausch *et al.*, 2002; Golby *et al.*, 1998; Uden & Kleefeld, 2004). DcuB catalyses electroneutral fumarate/succinate antiport. DcuB is typical for bacteria growing by fumarate respiration and can be replaced by the homologues DcuA and DcuC.

D. desulfuricans contains genes for five putative periplasmic solute-binding proteins, four of which are clustered with homologues of the *dctQ* and/or *dctM* genes encoding membrane-bound components of TRAP carriers (tripartite ATP-independent periplasmic carriers). In *D. vulgaris*, genes for three solute-binding proteins and homologues of *dctQ* and *dctM* genes are found. TRAP transporters represent secondary carriers which are driven by a H⁺ gradient and use a periplasmic binding protein to recruit the solute (Kelly & Thomas, 2001; Forward *et al.*, 1997). Using CLUSTAL X-aligned sequences and PHYLIP analysis the solute-binding proteins of TRAP transporters have been used for constructing a phylogenetic tree, which showed clustering of C₄-dicarboxylate-binding proteins (Thomas *et al.*, 2006). By the same method the DctP homologues of *D. desulfuricans* and *D. vulgaris* were used to construct an unrooted bootstrapped tree with sequences from those TRAP binding proteins, where biochemical evidence for the identity of the

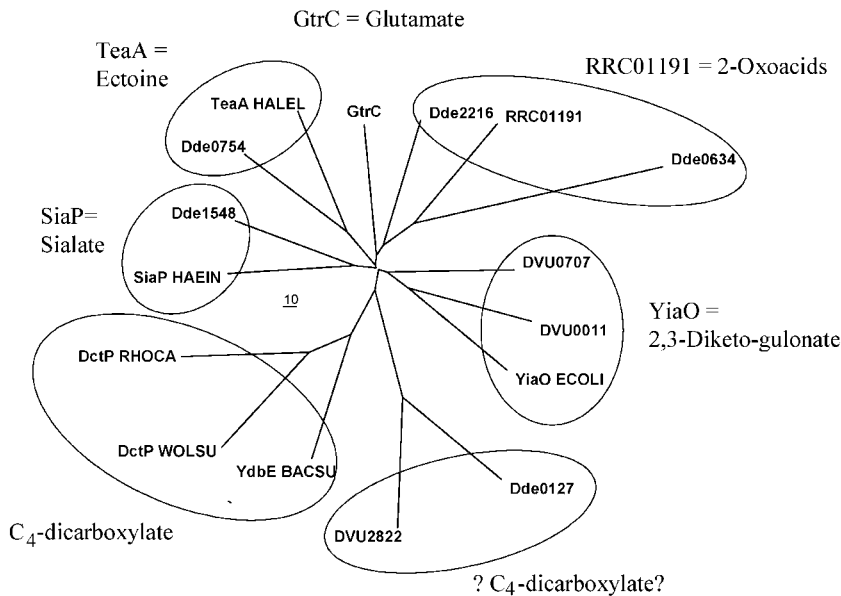


Fig. 6. Unrooted bootstrapped tree of the DctP homologues from *D. desulfuricans* G20 (Dde proteins) and *D. vulgaris* (DVU proteins). The tree was obtained by analysis of the CLUSTAL X aligned sequences by PHYLIP and is shown in TREEVIEW. As reference proteins, TRAP solute-binding proteins were used for which the ligands have previously been identified by biochemical analyses (for details see Kelly & Thomas, 2001; Thomas *et al.*, 2006). Proteins used: YdbE from *Bacillus subtilis* (YdbE BACSU), DctP from *Wolinella succinogenes* (DctP WOLSU), DctP from *Rhodobacter capsulatus* (DctP RHOCA), SiaP from *Haemophilus influenzae* (SiaP HAEIN), TeaA from *Halomonas elongata* (TeaA HALEL), GtrC from *Synechocystis* sp. (GtrC), RRC01191 from *R. capsulatus* (RRC01191) and YiaO from *E. coli* (YiaO).

ligand bound is available (Fig. 6). The proteins of *D. vulgaris* and *D. desulfuricans* cluster into six distinct groups. The clustering is significant since the known C₄-dicarboxylate-binding proteins from phylogenetically distinct bacteria cluster together. The *D. vulgaris* and *D. desulfuricans* proteins DVU2822 and Dde0127, respectively, are closely related to known C₄-dicarboxylate-binding proteins, and are good candidates for C₄-dicarboxylate-binding proteins. The other TRAP solute-binding proteins of *D. desulfuricans* and *D. vulgaris* cluster with binding proteins for 2,3-diketogulonate, 2-oxoacids, ectoine or sialate (Fig. 6) (see Kelly & Thomas, 2001; Severi *et al.*, 2005; Thomas *et al.*, 2006). *W. succinogenes* encodes a DctPQM TRAP carrier in addition to DcuB and DcuA. DctPQM catalysed fumarate uptake, but did not support growth by fumarate respiration (Ullmann *et al.*, 2000). The TRAP carriers could be involved in C₄-dicarboxylate uptake during aerobic growth, but in fumarate respiration the function of alternative secondary carriers is more likely. The genes Dde0326, 0693, 0808, 1204 and 1252 of *D. desulfuricans* encode secondary carriers similar to the di-/tricarboxylate carrier CitT or the Na⁺/H⁺ dicarboxylate symporter GltP (Pos *et al.*, 1998; <http://www.jgi.doe.gov/>). Carriers of the CitT family can be used for C₄-dicarboxylate antiport (O. B. Kim & G. Unden, unpublished). The glutamate carrier GltP (Tolner *et al.*, 1995) is homologous to C₄-dicarboxylate carriers and the homologues in the sulphate-reducing bacteria represent candidates for C₄-dicarboxylate transport. The genome of *D. vulgaris* encodes carriers with similarity to malonate carriers (predicted protein DVU1401) or an oxalate/formate antiporter (DVU1163) (<http://www.membranetransport.org>). Thus both *Desulfovibrio* strains contain candidate genes different from the *dcu* genes which could function in C₄-dicarboxylate transport.

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