

Metabolism of H₂ by *Desulfovibrio alaskensis* G20 during syntrophic growth on lactate

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Syntrophic growth involves the oxidation of organic compounds and subsequent transfer of electrons to an H₂- or formate-consuming micro-organism. In order to identify genes involved specifically in syntrophic growth, a mutant library of *Desulfovibrio alaskensis* G20 was screened for loss of the ability to grow syntrophically with *Methanospirillum hungatei* JF-1. A collection of 20 mutants with an impaired ability to grow syntrophically was obtained. All 20 mutants grew in pure culture on lactate under sulfidogenic conditions at a rate and to a maximum OD₆₀₀ similar to those of the parental strain. The largest number of mutations that affected syntrophic growth with lactate was in genes encoding proteins involved in H₂ oxidation, electron transfer, hydrogenase post-translational modification, pyruvate degradation and signal transduction. The *qrcB* gene, encoding a quinone reductase complex (Qrc), and *cycA*, encoding the periplasmic tetrahaem cytochrome c₃ (Tplc₃), were required by G20 to grow syntrophically with lactate. A mutant in the *hydA* gene, encoding an Fe-only hydrogenase (Hyd), is also impaired in syntrophic growth with lactate. The other mutants grew more slowly than the parental strain in syntrophic culture with *M. hungatei* JF-1. *qrcB* and *cycA* were shown previously to be required for growth of G20 pure cultures with H₂ and sulfate. Washed cells of the parental strain produced H₂ from either lactate or pyruvate, but washed cells of *qrcB*, *cycA* and *hydA* mutants produced H₂ at rates similar to the parental strain from pyruvate and did not produce significant amounts of H₂ from lactate. Real-time quantitative PCR assays showed increases in expression of the above three genes during syntrophic growth compared with pure-culture growth with lactate and sulfate. Our work shows that Hyd, Qrc and Tplc₃ are involved in H₂ production during syntrophic lactate metabolism by *D. alaskensis* G20 and emphasizes the importance of H₂ production for syntrophic lactate metabolism in this strain.

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INTRODUCTION

Anaerobic degradation of many organic compounds, e.g. alcohols and fatty acids, is thermodynamically unfavourable when protons are used as the electron acceptor, unless H₂ can be maintained at very low levels. Under methanogenic conditions, complete degradation of organic matter therefore requires a microbial consortium composed of two or

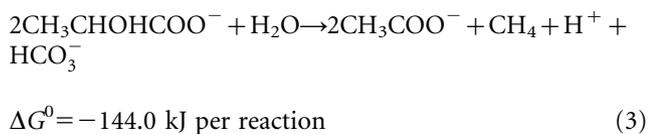
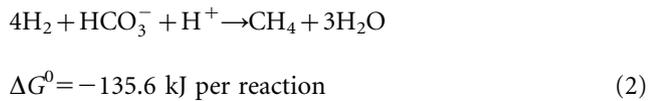
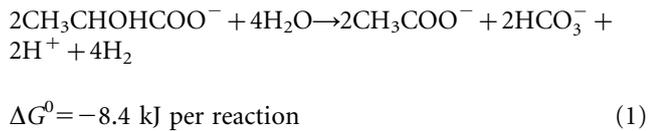
more microbial species (McInerney *et al.*, 2007, 2008; Schink & Friedrich, 1994; Stams, 1994). This synergistic interaction, termed syntrophy, was originally described (Bryant *et al.*, 1967) to involve syntrophic partners cooperating by transferring electrons from one species to the other using H₂ or formate (interspecies hydrogen/formate transfer) and maintaining H₂ at low levels through hydrogenotrophic methanogenesis, so that the overall reactions are exergonic.

Syntrophic interactions between sulfate-reducing microbes and methanogens occur commonly in nature and in man-made anaerobic environments (Bryant *et al.*, 1977; McInerney *et al.*, 1981; Oude Elferink *et al.*, 1998; Traore *et al.*, 1983). Members of the genus *Desulfovibrio* are sulfate-reducing bacteria that derive energy from the dissimilatory reduction of sulfate coupled to the oxidation of H₂ or organic substrates such as lactate. In the absence

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Abbreviations: CCCP, carbonyl cyanide chlorophenylhydrazine; COG, cluster of orthologous groups; Coq, carbon monoxide-induced hydrogenase; Hmc, transmembrane high-molecular-mass cytochrome c; Hyd, Fe-only hydrogenase; Hyn, NiFe hydrogenase; PMF, proton-motive force; Qrc, quinone reductase complex; Tplc₃, tetrahaem periplasmic cytochrome c₃.

of sulfate, *Desulfovibrio* alone cannot grow on lactate. However, when paired with a methanogen, *Desulfovibrio* gains energy, producing acetate, HCO₃⁻ and H₂ (equation 1); the latter two products are used by the methanogen for CH₄ production (equation 2) (McInerney & Bryant, 1981; Pankhania *et al.*, 1988; Stoljar *et al.*, 2007).



During lactate oxidation, lactate is oxidized first to pyruvate, which is then oxidized further to acetate. The oxidation of lactate to pyruvate ($E_0' = -190 \text{ mV}$) with protons as electron acceptors ($E_0' = -420 \text{ mV}$) is highly endergonic under standard conditions (Pankhania *et al.*, 1988). Since the complete oxidation of lactate to acetate coupled to the production of methane is an energy-yielding process (equation 3), some of the energy released during the conversion of pyruvate to acetate must be used to drive lactate oxidation. Through the use of protonophores, H₂ formation from lactate was shown to require a proton-motive force (PMF) (Pankhania *et al.*, 1988). However, the mechanism for harnessing the PMF to the production of H₂ has not been described.

Syntrophic growth probably requires functions that are not apparent when cells are growing in pure culture, as indicated by studies showing significant changes in gene expression between respiratory and syntrophic growth of *Desulfovibrio* (Plugge *et al.*, 2010; Walker *et al.*, 2009). Notably, microarray analyses of *Desulfovibrio vulgaris* Hildenborough showed that transcription of genes encoding certain hydrogenases and membrane-associated electron-transfer functions was elevated significantly during syntrophy (Walker *et al.*, 2009). Mutations in some of these genes [encoding *Coo* (carbon monoxide-induced hydrogenase), *Hmc* (transmembrane high-molecular-mass cytochrome *c*), *Hyd* and *Hyn*] impaired or severely limited syntrophic growth, but had little effect on growth via sulfate respiration.

The availability of genomes of several *Desulfovibrio* strains now provides a framework to investigate the mechanistic basis of syntrophic growth through comparative genetic and physiological studies. Some of the genes implicated in syntrophic growth are present in all of the available *Desulfovibrio* genomes. However, the genes encoding *Coo*, required for syntrophic growth of *D. vulgaris* Hildenborough,

are absent in *Desulfovibrio alaskensis* G20, suggesting that there may not be a common mechanism for syntrophy. Thus, although most *Desulfovibrio* species are capable of syntrophic growth, very little is known about the type and diversity of molecular mechanisms that allow this phylogenetically diverse group of sulfate-reducing micro-organisms to recover energy through association with hydrogenotrophic methanogens. One could therefore ask whether there are both common and specific biochemical pathways for maintenance of syntrophic systems.

In a previous study, we described a transposon mutant library in *D. alaskensis* G20, created using a mini-Tn10 transposon-bearing plasmid (Groh *et al.*, 2005). The library contains 5760 mutants and was used here to identify genes that are important for syntrophic growth of strain G20 with methanogens. In this report, 20 mutants were identified that grew poorly in the syntrophic relationship. Also, several genes, including those encoding a quinone reductase complex (*Qrc*) and tetrahaem periplasmic cytochrome *c*₃ (*TpIc*₃), known to be involved specifically in H₂/formate metabolism, were shown to be important for syntrophic growth of *D. alaskensis* G20.

METHODS

Strains, media and culture methods. *D. alaskensis* G20 and *M. hungatei* JF-1 (ATCC 27890) were used to establish syntrophic cultures with lactate as substrate for mutant-screening experiments. Strain G20 is a spontaneous nalidixic acid-resistant derivative of the wild-type strain G100A that was isolated from an oil-well corrosion site (Weimer *et al.*, 1988). A G20 mutant library with 5760 mutants was constructed in our laboratory using a mini-Tn10 transposon-bearing plasmid, pBSL180, for mutagenesis (Groh *et al.*, 2005), which therefore provides about 1.5-fold coverage of the 3775 candidate protein-encoding genes found in the G20 genome. Mutants were assembled into 96-well plates and stored at $-80 \text{ }^\circ\text{C}$.

Lactate/sulfate (LS) medium was prepared as described previously (Groh *et al.*, 2005) for maintenance of the mutant library and for growth of strain G20 on solid medium. Yeast extract (0.1%) was added as a carbon source. Kanamycin was added to solid ($175 \mu\text{g ml}^{-1}$) or liquid ($1050 \mu\text{g ml}^{-1}$) medium when growing pure mutant cultures. For growth experiments with pyruvate, sodium pyruvate (50 mM) was substituted for lactate. For all other experiments, a mineral salts (MS) medium (Li *et al.*, 2009) was used to grow G20, *M. hungatei* JF-1 and the syntrophic co-culture. Sulfate (usually 50 mM) was included in media used to grow pure cultures of strain G20 except where indicated. When H₂ or formate was used as an electron donor, acetate (10 mM) was included as a carbon source. For growth of G20 mutant cultures with lactate, formate or syntrophically, the headspace was flushed with N₂/CO₂ (80/20, v/v). Typically, 45 mM lactate was used in the medium, and was diluted further after inoculation of strain JF-1. Pure cultures of strains G20 and JF-1 grown on H₂ were flushed with H₂/CO₂ [80/20 (v/v) at 138 kPa] and strain JF-1 cultures were refushed every other day. Syntrophic cultures of strain G20 and strain JF-1 contained lactate but no sulfate. All cultures with H₂ added were shaken at 80 r.p.m. and others were stationary. All cultures were incubated at 37 °C.

Screening of mutants in syntrophic co-cultures. Syntrophic co-cultures were established by inoculation of early stationary-phase cultures of JF-1 ($\text{OD}_{600} = 0.5\text{--}0.7$) and individual strain G20 mutants

(0.1 ml, OD₆₀₀=0.7) into 5 ml MS –lactate medium in a serum tube (23 ml). Usually 1.0–1.5 ml JF-1 was inoculated to keep the initial OD₆₀₀ higher than 0.1. OD₆₀₀ was measured routinely to monitor growth. Syntrophic co-cultures containing the parental strain G20 reached a maximum OD₆₀₀ within 4–5 days. Mutants that grew significantly slower than the parental strain (i.e. required at least 2 additional days to reach the maximum OD₆₀₀) or non-growing mutants were identified as potential targets for further testing. All putative syntrophy-defective mutants were rescreened to verify that they were defective for syntrophic growth. Individual cells within co-cultures were quantified by direct counting of cells (both strains JF-1 and G20) with a haemocytometer using a phase-contrast microscope or by total viable counts involving plating on solid medium (for strain G20) (Groh *et al.*, 2005).

Identification of interrupted genes. Genomic DNA was purified with an Easy DNA kit (Invitrogen). In order to identify the gene interrupted by the transposon, transposon-insertion sites were determined by using a two-round arbitrarily primed PCR method as described previously (Das *et al.*, 2005). For the first round (50 µl reaction mixture), primers Tn10ext (5'-GTGTTCCGCTTCCTTTA-GCAGC-3') and Arb1 (5'-GGCCACGCGTCGACTAGTCANNNNNN-NNNNNTGAAC-3') were used. Reactions included 1 × PCR buffer, 0.2 mM each dNTP, 1.5 mM MgCl₂, 0.2 µM primer Tn10ext, 0.5 µM primer Arb1, 1.0 U Platinum *Taq* DNA polymerase (Invitrogen) and 10 ng genomic DNA as template. Parameters were: (i) 95 °C for 5 min; (ii) six cycles of 95 °C for 30 s, 30 °C for 30 s and 72 °C for 1.5 min; (iii) 30 cycles of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 2 min; (iv) 72 °C for 4 min. The first-round PCR product was purified by using a GenCatch PCR purification kit (Epoch Biolabs) and 2 µl was used for the second round of PCR (50 µl reaction mixture) with primers Tn10seq (5'-GTGACGGTATCGATAAGCTTG-3') and Arb2 (5'-GGCCACGCGTCGACTAGTCA-3'). The reaction mixture was similar to that used in the first-round PCR except that both primer concentrations were 0.2 µM. PCR parameters were: (i) 95 °C for 1 min; (ii) 30 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 2 min; (iii) 72 °C for 4 min. PCR products were loaded onto 1% agarose gels for electrophoresis and stained with ethidium bromide. The brightest bands were excised and purified by using a GenCatch gel extraction kit (Epoch Biolabs) and sequenced directly using the Tn10seq primer. The sequence was compared with those in GenBank by using BLASTN and also specifically with the *D. alaskensis* G20 genome sequence available in GenBank (accession no. CP000112).

Real-time quantitative RT-PCR (RT-qPCR) and enzyme assays.

D. alaskensis G20 cultures were grown with lactate (45 mM), H₂ [80/20 (v/v) H₂/CO₂] or formate (50 mM) with sulfate (50 mM), or under syntrophic conditions. Cells were collected during the early part of the exponential phase (OD₆₀₀=0.15–0.20). Growing cultures were centrifuged at 8000 r.p.m. and washed twice with 50 mM NH₄HCO₃ buffer (pH 7.5) in an anaerobic chamber.

Total RNA was extracted from the cells by using an Aurum total RNA mini kit (Bio-Rad) with on-column DNase digestion. RNA concentration, purity and integrity were checked with a Nanodrop ND-1000 spectrophotometer and gel electrophoresis. cDNA was synthesized by using a First Strand cDNA synthesis kit (Fermentas). The cDNA (10 ng) was used for RT-qPCR using Maxima SYBR Green qPCR master mix (Fermentas) with a Bio-Rad MyIQ Cycler. Quantitative PCR in the absence of reverse transcriptase was conducted as negative control to determine possible DNA contamination. Gene-specific primers were designed to generate 100 bp amplicons with the following amplification conditions: 95 °C for 10 min; 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Relative mRNA expression was calculated by using the method of Pfaffl (2001), with ratio = $(E_{\text{target}})^{\Delta C_{t,\text{target}}(\text{control}-\text{treated})} / (E_{\text{reference}})^{\Delta C_{t,\text{reference}}(\text{control}-\text{treated})}$. The

16S rRNA gene was used as a reference. The following primers were used: 16S rRNA gene, forward/reverse (5'→3'), AGCTAAT-CAGACGCGGACTC/ACGGTTGGAAACGACTGCTA; *Qrc* gene (*qrcB*, Dde_2933), CTGAACATAGGCACCAGCAAC/CGTTCATC-ATCACATAAACTCGT; cytochrome *c*₃ (*cycA*, Dde_3182), AAG-GAAAATCTTGCCAAAGTGTG/TTCTGTCTGGATGATTTTGTA-GT; Fe hydrogenase (Fe-hyd, *hydB*, Dde_0082), ATCAGT-TTCTGTATGCCCTGAGAC/AAACTGTACAAGGACTGGCTTGA; NiFeSe hydrogenase (NiFeSe-hyd, Dde_2135), CGTCTCAGATTG-TACAGCGTATCT/GTGGTCACTTTACACCGAAA; NiFe hydrogenase (NiFe-hyd, Dde_2137), ATAAGTCCCCAAAATCAAGTTC/CTCATGGCATCCAGAAATC.

Cells of strains G20 and JF-1 were isolated from syntrophic cultures and used to measure hydrogenase and formate dehydrogenase activity by using the procedures described previously (Li *et al.*, 2009). For syntrophic co-cultures, the Percoll gradient centrifugation method was used to separate strains G20 and JF-1 from syntrophic cultures according to the protocol of the manufacturer (GE Healthcare). Briefly, 49.5 ml Percoll was mixed with 5.5 ml 1.5 M NaCl to make the stock isotonic Percoll (SIP) solution; then the working solution was prepared with 70% SIP and 30% 0.15 M NaCl. A syntrophically grown culture (5 ml) was washed twice using 50 mM NH₄HCO₃ buffer (pH 7.5) and resuspended in 0.2 ml of the same buffer. Cells were added to 4.5 ml Percoll working solution, loaded into 4.9 ml ultracentrifuge tubes and centrifuged at 30 000 *g* for 15 min. Typically, two cell layers were formed, with strain G20 in the upper layer. Cells were then removed carefully with a syringe and purity was verified by using phase-contrast microscopy. The isolated cells were used for further experiments only if contamination with the other cells was <1%. Cells were then washed twice with 50 mM NH₄HCO₃ buffer (5 vols buffer : 1 vol. Percoll cell suspension). All solutions were bubbled with N₂, and manipulations were conducted in the anaerobic chamber.

Washed-cell experiments. Washed cells were used to measure H₂ production as described previously (Pankhania *et al.*, 1988). Briefly, a 100 ml culture grown to mid-exponential phase was harvested by centrifugation, washed twice in buffer containing 50 mM MOPS (pH 7.2), 5 mM MgCl₂ and 5 mM dithiothreitol (DTT), and resuspended in the same buffer. All buffers were flushed with N₂ for 30 min. Assays were carried out in serum tubes containing 2 ml buffer/cell mixture, incubated at 37 °C and shaken at 300 r.p.m. The assay mixture contained the washing buffer and either 25 mM DL-lactate or 50 mM sodium pyruvate. Lactate oxidation reactions included 5 mM DTT, whereas pyruvate-containing reactions contained DTT at a final concentration of 0.3 mM. Cells were grown on lactate or pyruvate to measure H₂ production from lactate or pyruvate, respectively. Between 50 and 200 µg cell protein was added to each tube. After addition of all components to the reaction, tubes were pre-incubated for 30 min, then flushed with N₂ and 0.5 ml gas samples were taken at 30 and 60 min for H₂ analysis. Assays were linear with time and protein.

Chemical analyses. H₂ was measured with a reduced gas analyser (Trace Analytical, Inc.). Methane was measured using a GC with a 1.8 m stainless steel column packed with Porapak Q resin and a flame-ionization detector.

RESULTS

Syntrophic co-culture growth

Neither *D. alaskensis* G20 nor *M. hungatei* JF-1 grew to any significant extent in pure culture in MS medium with lactate in the absence of sulfate (Fig. 1). However, a

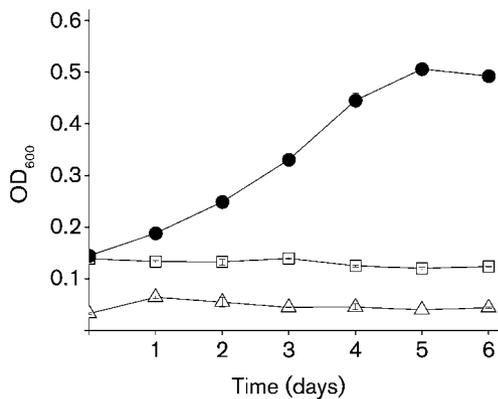


Fig. 1. Growth of *D. alaskensis* G20 parental strain (Δ) and *M. hungatei* JF-1 (□) monocultures and co-culture (●) in MS medium with lactate and no sulfate.

syntrophic co-culture of *D. alaskensis* G20 and *M. hungatei* JF-1 was established rapidly when both organisms were inoculated into this medium. The OD₆₀₀ of this syntrophic co-culture increased by 0.30–0.35 units after 4–5 days at 37 °C (Fig. 1), while consuming lactate completely with almost-stoichiometric production of acetate and CH₄ based on equation 3. Direct counts (strains G20 and JF-1) and viable plate counts (strain G20) showed that the cell numbers of both strains G20 and JF-1 increased during syntrophic growth (data not shown).

Mutant screening

We screened the strain G20 mutant library for mutants deficient in syntrophic growth by incubating each strain G20 mutant individually with *M. hungatei* JF-1. A collection of 20 mutants with an impaired ability to grow syntrophically was obtained (Table 1). All 20 mutants grew in pure culture under sulfidogenic conditions at a rate and to a maximum OD₆₀₀ similar to those of the parental strain (Table 1; Fig. 2). Two of these mutants, with insertions in genes Dde_2933 (*qrcB*) and Dde_3182 (*cycA*), did not grow syntrophically or produce significant amounts of methane [≤ 0.2 mmol (l culture)⁻¹] in MS medium with lactate in co-culture with *M. hungatei* JF-1 (Fig. 2). The other 18 mutants grew significantly slower than the parental strain in syntrophic culture with *M. hungatei* JF-1 (Table 1); these mutants reached a maximum OD₆₀₀ similar to that of the parental strain, but at least 2 days later. The mutant with an insertion in Dde_0082 (*hydB*) was more severely impaired and required 8 days to reach a final OD₆₀₀ that was lower than that of the parental strain (Fig. 2). All mutants except those with insertions in Dde_2933 (*qrcB*), Dde_3182 (*cycA*) and Dde_0082 (*hydB*) eventually oxidized all of the lactate that was provided and produced similar amounts of CH₄ in the headspace [8–12 mmol (l culture)⁻¹] to the parental strain. However, the rate of CH₄ production (day⁻¹) was lower than that of the

parental strain (Fig. 2). Generally, CH₄-production profiles were similar to growth profiles as measured by OD₆₀₀ for the mutants that eventually grew syntrophically, indicating that growth of the methanogen was coupled directly to syntrophic lactate degradation. Therefore, the growth rate of mutants was probably limited by their relative ability to oxidize lactate and provide the reducing equivalents to the methanogen.

Classification of the mutations

Clusters of orthologous groups (COGs) analysis grouped the mutations into the following categories: energy production and conservation (six genes); post-translational modification (five genes); signal transduction (two genes); cell wall/membrane biogenesis (one gene); general function (three genes). Four genes were not categorized in the G20 genome database, including TpIc₃ (*cycA*). However, *cycA* should be classified with the energy production and conservation genes.

The two mutants that did not grow syntrophically had mutations that grouped them into the energy production and conservation COG. One had an insertion in *qrcB* (Dde_2933), which encodes the putative Qrc subunit and is one of four genes in the *qrc* operon (Dde_2932–2935). These genes encode a periplasm-facing integral membrane protein with clear homology to alternative complex III (Li *et al.*, 2009; Venceslau *et al.*, 2010). This complex was described as Mop in a previous publication (Li *et al.*, 2009), but the complex has been recently characterized biochemically from *D. vulgaris* and renamed Qrc (Venceslau *et al.*, 2010). The other mutant had a lesion in Dde_3182 (*cycA*), predicted to encode the TpIc₃ (Table 1), a single-subunit protein. The TpIc₃ is thought to be involved in transferring electrons to a variety of membrane-associated cytochrome complexes (Pereira *et al.*, 2007). Each of the *qrcB* and *cycA* mutants has been complemented by using a vector containing an insert with the gene or operon described above to establish that the observed phenotype of the mutant resulted from the loss of those specific genes (Li *et al.*, 2009). In both cases, complementation restored syntrophic growth (data not shown).

Another mutant in the energy production and conservation COG had a mutation in *hydB* (Dde_0082) (Table 1) and grew slowly under syntrophic conditions (Fig. 2). The *hydB* gene is located in a predicted operon with another gene; together, they encode the periplasmic Fe-only hydrogenase. The *hydB* mutant was shown previously to grow more slowly than the parental strain in pure culture with H₂ (or formate) and sulfate, and the *qrcB* and *cycA* mutants did not grow at all with those substrates (Li *et al.*, 2009). Thus, the data suggest that these three genes encode proteins involved in certain pathways related to both syntrophy and H₂ oxidation.

Other mutants grouped in the energy production and conservation COG include those with an insertion in a

Table 1. Characteristics of attenuated *D. alaskensis* G20 mutants deficient in syntrophic growth

For, Formate; Lac, lactate; Pyr, pyruvate; ND, not determined.

COG/locus of mutated gene	Predicted protein	Growth*				Motility†	<i>D. vulgaris</i> syntrophy expression‡	
		Syntrophy (Lac, no SO ₄ ²⁻)	Lac/SO ₄ ²⁻	H ₂ /SO ₄ ²⁻	For/SO ₄ ²⁻			Pyr/SO ₄ ²⁻
Parental strain (wild-type)		+	+	+	+	+	+++	
Energy metabolism								
Dde_2933	Quinone reductase complex (<i>qrcB</i>)	-	+	-	-	±	+	2.0
Dde_0082	Iron hydrogenase small subunit (<i>hydB</i>)	±	+	±	-	±	++	2.8
Dde_3182	TpIc ₃ (<i>cycA</i>)	-	+	-	-	±	+	1.2
Dde_3282	Formate C-acetyltransferase (<i>pfl</i>)	±	+	+	+	+	++	2.0
Dde_1074	L-Lactate transport	±	+	+	+	±	++	2.6
Dde_3238	L-Lactate transport	±	+	+	+	+	++	2.3
Post-translational modification								
Dde_0364	Hydrogenase expression/formation protein HypD	±	+	+	+	+	+++	ND
Dde_0363	Hydrogenase expression/formation protein HypE	±	+	+	+	+	+++	ND
Dde_0555	(NiFe) hydrogenase maturation protein HypF	±	+	+	+	+	+++	1.0
Dde_1738	Small HspC2 heat-shock protein	±	+	+	+	+	+++	ND
Dde_3281	Radical-activating enzyme	±	+	±	+	+	+++	ND
Signal transduction								
Dde_3074	Methyl-accepting chemotaxis sensory transducer (flagellum structure and biogenesis)	±	+	±	±	+	++	ND
Dde_3234	Two-component transcriptional regulator	±	+	+	+	+	+	3.0
Cell wall/membrane biogenesis								
Dde_1682	Uncharacterized protein involved in outer membrane biogenesis-like	±	+	+	+	+	+	ND
General function prediction								
Dde_1972	tRNA nucleotidyl transferase	±	+	+	±	+	+	ND
Dde_0978	Metal-dependent phosphohydrolase	±	+	+	+	+	++	ND
Dde_3699	Membrane protein	±	+	+	+	±	+++	1.0
Uncategorized								
Dde_2849	ISPg7, transposase	±	+	±	+	+	++	ND
Dde_1612	Conserved hypothetical protein	±	+	±	±	+	++	1.0
Dde_3634	Hypothetical protein	±	+	+	+	+	+	ND

*+, Grows similarly to parental strain; ±, grows slower than parental strain; -, no growth within a growth period.

†+, +, + indicate motility relative to the parental strain, with +++ being equivalent to the parental strain's motility.

‡*D. vulgaris* expression data (Walker *et al.*, 2009) showing relative change during syntrophic growth. Data are ratios of expression in co-culture with *Methanococcus maripaludis*/*D. vulgaris* monoculture.

gene predicted to encode a formate C-acetyltransferase (EC 2.3.1.54), also known as pyruvate formate-lyase (*pfl*) (Dde_3282), and in the adjacent gene (Dde_3281), predicted to encode a radical-activating enzyme, perhaps a (formate C-acetyltransferase)-activating enzyme (Table 1). Pyruvate formate-lyase catalyses the reversible reaction pyruvate + CoA ↔ acetyl CoA + formate. A third gene in

this operon, Dde_3283, encodes a putative acetaldehyde dehydrogenase. The *pfl* mutant grew similarly to the parental strain in pure culture with LS and pyruvate/sulfate media (Table 1).

Other mutants in the energy production and conservation COG include those with insertions in Dde_1074 and

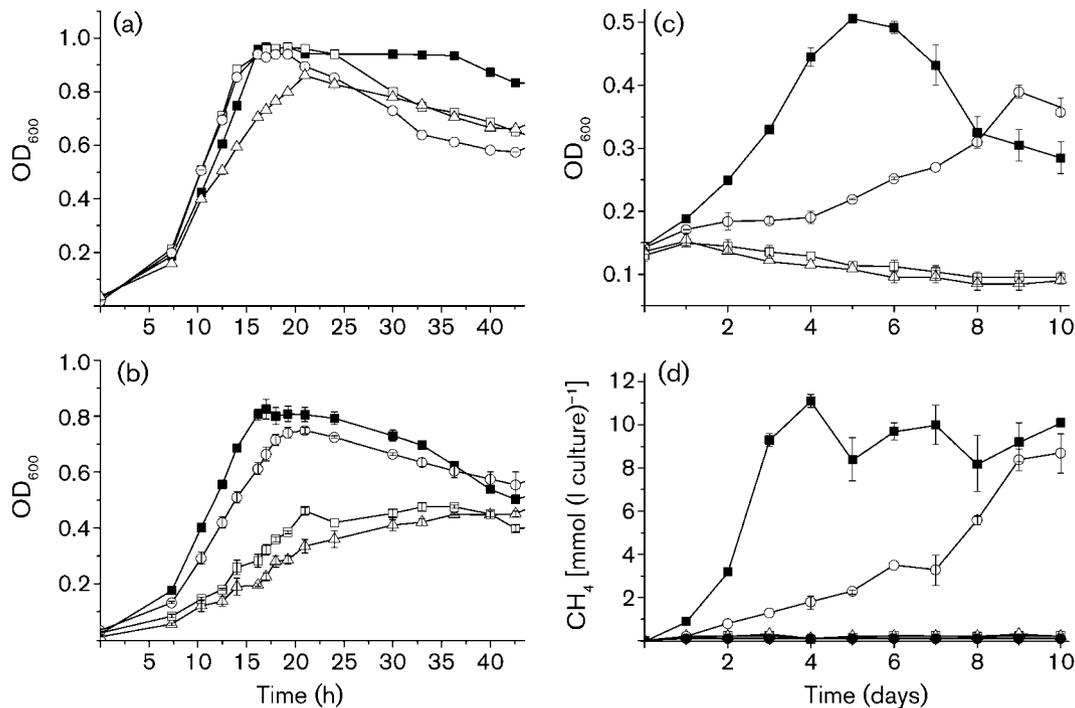


Fig. 2. Growth of the parental strain (wild-type) and mutants in *qrcB*, *cycA* and *hydB* (see full names in Table 1), as well as CH₄ production by syntrophic cultures over time. (a) OD₆₀₀ of pure cultures in LS medium; (b) OD₆₀₀ of pure cultures in pyruvate/sulfate medium; (c) OD₆₀₀ of syntrophic co-cultures with *M. hungatei* JF-1 in lactate medium; (d) CH₄ production from the co-cultures. ■, Parental strain; □, *qrcB* mutant; ○, *hydB* mutant; △, *cycA* mutant; ●, *M. hungatei* JF-1 in pure culture with no electron donor.

Dde_3238 (Table 1), which are both predicted to be L-lactate transporters. The gene Dde_3238 is located in a 23 kb putative operon, an 'organic acid oxidation region' (Pereira *et al.*, 2007) whose genes are believed to encode a number of enzymes critical for lactate or pyruvate oxidation. The genes in this region include Dde_3238, a putative L-lactate transporter; Dde_3237, encoding pyruvate:ferredoxin (flavodoxin) oxidoreductase involved in pyruvate oxidation to acetyl CoA and CO₂; Dde_3242, encoding acetate kinase, and Dde_3241, encoding phosphate acetyltransferase, both key enzymes in substrate-level phosphorylation. Dde_3239–3240 are proposed as likely candidate genes for lactate dehydrogenase (Pereira *et al.*, 2007). Also, the operon encodes two sensor proteins: Dde_3233, PAS/PAC sensor hybrid histidine kinase, and Dde_3246, a methyl-accepting chemotaxis sensory transducer; and a two-component Fis family transcriptional regulator (Dde_3234), which may be involved in controlling the expression of this operon. A mutant with an insertion in the gene Dde_3234 was also identified in this study. Because of potential downstream effects of an insertion, it is difficult to pinpoint the exact gene causing the phenotype.

Three other mutants that grew poorly under syntrophic conditions (Dde_0364, Dde_0363 and Dde_0555) had insertions in genes believed to be involved in the post-translational modification of hydrogenases (*hypD*, *hypE*,

hypF) (Table 1). The maturation protein genes are annotated as hydrogenase pleiotropic (Hyp), indicating that a mutation in one of these genes causes defects in the biosynthesis of several hydrogenases (Agrawal *et al.*, 2006). Given the similarities in structure of the NiFe and the NiFeSe hydrogenases (Fontecilla-Camps *et al.*, 2007) and the fact that they are located adjacent to each other on the chromosomes of strain G20 and other sulfate reducers, it is likely that these genes are involved in the maturation of both Ni-containing hydrogenases. The three mutants grew similarly to the parental strain with sulfate in pure culture with lactate, pyruvate, H₂ or formate as electron donors (Table 1); however, their syntrophic growth rates and CH₄-production rates were much lower than those of the parental strain. To determine whether other hydrogenases play a role in syntrophic growth, we obtained a G20 transposon mutant with an insertion in the NiFeSe hydrogenase gene (*hysA*) (Dde_2134). This mutant grew similarly to the parental strain under syntrophic conditions (data not shown). Unfortunately, we do not have a strain G20 mutant with a mutation in the NiFe hydrogenase. A mutation in the orthologue in *D. vulgaris* (*hynA*) grew more slowly than the parental strain in syntrophic culture (Walker *et al.*, 2009). Therefore, it is possible that the NiFe hydrogenases, as well as the Fe hydrogenase, are used by strain G20 during syntrophic growth.

Defects in H₂ production

The impaired growth of *qrcB*, *cycA* and *hydB* mutants with H₂ or formate under sulfidogenic conditions (Li *et al.*, 2009) and syntrophically with lactate suggests that mutated genes function not only in H₂ oxidation, but also in H₂ production. We found previously that these three mutants produced H₂ in MS medium with lactate in the presence or absence of sulfate (Li *et al.*, 2009). However, MS medium contains yeast extract and cysteine, both of which could be sources of electrons for H₂ production through an alternative pathway. To exclude this possibility, H₂ production from lactate and pyruvate by the parental and mutant strains was measured using cells suspended in buffer with no carbon sources other than DTT as the reductant. Washed cell suspensions of the parental strain produced H₂ from either lactate or pyruvate (Fig. 3). The above three mutants produced H₂ at rates similar to the parental strain from pyruvate, but none of the mutants produced significant amounts of H₂ from lactate (Fig. 3). The oxidation of lactate to pyruvate and H₂ requires energy input, and a previous study (Pankhania *et al.*, 1988) demonstrated the requirement for a PMF for H₂ production during lactate oxidation. These results were confirmed here with the addition of the protonophore carbonyl cyanide chlorophenylhydrazone (CCCP), which prevented H₂ production from lactate by the parental strain (Fig. 3). The results from the washed-cell experiments suggest that the Hyd–T_pIc₃–Qrc group of proteins may be involved both in H₂ production from lactate during syntrophic growth and in H₂ uptake during sulfidogenic growth (Li *et al.*, 2009).

Gene expression and enzyme activity

We isolated cells of strains G20 and JF-1 from syntrophic cultures using Percoll gradient centrifugation under anaerobic conditions. Significant levels of hydrogenase

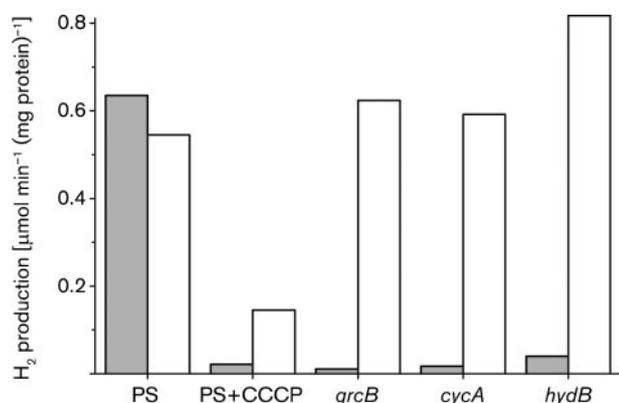


Fig. 3. H₂-production activity by washed cells of *D. alaskensis* G20 parental strain (PS) or mutants incubated with lactate (shaded bars) or pyruvate (empty bars). PS+CCCP indicates PS incubated in the presence of 50 μM CCCP.

activity were present in cells of strain G20 at 1.5 ± 0.6 and $1.85 \pm 1.25 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ with methyl and benzyl viologen as electron acceptors, respectively, whilst hydrogenase activities in *M. hungatei* cells were 0.28 ± 0.24 and $0.18 \pm 0.31 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ with methyl and benzyl viologen, respectively. RT-qPCR results showed that the expression of all three hydrogenases, *hydB* (Fe hydrogenase), Dde_2137 (NiFe hydrogenase) and Dde_2135 (NiFeSe hydrogenase), was upregulated during syntrophic growth compared with pure cultures grown with lactate/sulfate (Table 2). The *qrcB* and *cycA* genes were also upregulated when G20 was grown syntrophically or in pure culture with H₂/sulfate or formate/sulfate relative to pure cultures grown with lactate/sulfate (Table 2), further implicating the *qrcB* and *cycA* genes in syntrophic metabolism.

DISCUSSION

Genetic analysis showed that *qrcB*, *cycA* and *hydB* encode proteins involved in certain biochemical processes related to syntrophic growth of G20 with lactate (Table 1; Fig. 2). This was corroborated by the RT-qPCR results, which showed that all three genes were upregulated during syntrophic growth compared with pure-culture growth with lactate/sulfate (Table 2). Genetic analysis showed that *qrcB* and *cycA* are required for syntrophic growth of G20 with lactate, whilst a mutation in *hydB* impaired syntrophic growth (Table 1; Fig. 2). Based on the above H₂-production experiments (Fig. 3), it seems likely that the proteins encoded by these genes are involved either directly or indirectly in H₂ production.

When *D. vulgaris* was grown under syntrophic conditions, three of the four genes in the *qrc* operon were also shown to be upregulated by about 2-fold relative to pure culture-grown cells (Walker *et al.*, 2009) (Table 1). Genes in this operon have orthologues in all other sequenced strains of *Desulfovibrio*, as well as in many other organisms including the syntrophic propionate degrader *Syntrophobacter fumaroxidans*. *cycA* encodes the T_pIc₃, which has been suggested to act as electron acceptor for periplasmic hydrogenases and formate dehydrogenases in *Desulfovibrio* (Heidelberg *et al.*, 2004; LeGall & Fauque, 1988; Matias *et al.*, 2005) and to interact with and mediate electron transfer from the Fe hydrogenase to a transmembrane high-molecular-mass cytochrome *c* (Hmc) (Pereira *et al.*, 1998). Based on pure-culture experiments, T_pIc₃ and QrcABCD were proposed to interact to shuttle electrons from H₂ oxidation in the periplasm to the menaquinone pool in the inner membrane, after which electrons are ultimately used for sulfate reduction in the cytoplasm (Li *et al.*, 2009; Venceslau *et al.*, 2010). Another group has generated a mutation in the same *cycA* gene by plasmid insertion. However, that mutant was able to grow in hydrogen/sulfate medium (Rapp-Giles *et al.*, 2000) and syntrophically with strain JF-1 (unpublished data). The reasons are not clear, although that insertion may be less stable than those described here.

Table 2. Gene expression ratios under different growth conditions compared with the G20 parental strain grown in LS medium using RT-qPCR

The 16S rRNA gene was used as a reference. Values are means \pm SD of triplicate measurements.

Culture	<i>qrcB</i> (Dde_2933)	<i>cycA</i> (Dde_3182)	Fe hydrogenase (<i>hydB</i> , Dde_0082)	NiFe hydrogenase (Dde_2137)	NiFeSe hydrogenase (Dde_2135)
H ₂ /sulfate	2.7 \pm 1.7	2.9 \pm 0.5	2.2 \pm 2.2	7.8 \pm 0.2	4.1 \pm 2.0
Formate/sulfate	20.0 \pm 8.1	9.2 \pm 3.0	3.3 \pm 2.2	51.2 \pm 16.4	49.8 \pm 8.0
Pyruvate/sulfate	0.5 \pm 0.3	1.9 \pm 0.6	9.3 \pm 9.2	10.1 \pm 6.0	4.9 \pm 3.6
Syntrophic culture	11.1 \pm 5.3	2.8 \pm 1.5	6.3 \pm 4.8	4.0 \pm 2.5	12.2 \pm 4.6

The orthologue of the G20 Qrc was recently purified from *D. vulgaris* Hildenborough and was shown to be composed of four subunits present at a 1:1:1:1 ratio, forming a transmembrane complex with periplasmic components (Venceslau *et al.*, 2010). The purified Qrc was shown to be reduced by H₂ (or formate) in the presence of the TplC₃ and hydrogenase (or formate dehydrogenase). The purified complex also served to transfer electrons from the TplC₃ to menaquinone, of which the latter was suggested to provide electrons to another membrane complex (perhaps Qmo), which would then transfer the electrons to a cytoplasmic protein for reduction of sulfate (Venceslau *et al.*, 2010). Ultimately, the cycling of menaquinone provides this system with the capability to generate a PMF. We suggest here that a PMF generated during pyruvate oxidation to acetate could be harnessed to provide the energy needed for lactate oxidation to pyruvate and H₂ through the cycling of menaquinone coupled to the reversal of the electron-transfer system described for the Qrc (Li *et al.*, 2009; Venceslau *et al.*, 2010). This system may involve electrons from lactate oxidation in the cytoplasm being transferred to menaquinone, which could then be shuttled to hydrogenase via Qrc and TplC₃.

Three classes of periplasmic hydrogenase are present in *Desulfovibrio*, i.e. NiFe hydrogenase, NiFeSe hydrogenase and Fe-only hydrogenase (Lissolo *et al.*, 1986; Prickril *et al.*, 1987; Voordouw *et al.*, 1990), and it is therefore likely that multiple hydrogenases are involved in H₂ metabolism. Hydrogenase-activity data showed that hydrogenases (production or uptake) are more active during syntrophic growth. Similarly, there is higher gene expression of three hydrogenases in syntrophic co-culture compared with lactate/sulfate-grown cells. Both of these results provide evidence for the importance of hydrogenases during syntrophic growth. Identification of the *hyd* mutant as being deficient in syntrophic growth highlights the similarity in enzyme machinery used for syntrophic growth between different strains of *Desulfovibrio*, as a *hyd* mutant in *D. vulgaris* was also shown to be deficient in syntrophic growth (Walker *et al.*, 2009). Both genes in the *hyd* operon of *D. vulgaris* were upregulated (about 4-fold) during syntrophic growth relative to pure-culture growth (Walker *et al.*, 2009). Mutants in *hyd* have also previously been shown to grow poorly on H₂ (Caffrey *et al.*, 2007; Pohorelic

et al., 2002). It has been suggested that the Fe hydrogenase is most important when H₂ is present at high concentrations or during lactate growth, whilst with low intracellular H₂, the lower-activity, higher-affinity NiFeSe hydrogenase is used for H₂ oxidation (Caffrey *et al.*, 2007). During syntrophic growth, H₂ was measured at <900 Pa, and RT-qPCR results indicated that the NiFeSe hydrogenase was upregulated more strongly than the Fe hydrogenase during syntrophic growth conditions (Table 2). Similar experiments with *D. vulgaris* showed a very slight increase (log₂=0.5) in expression of the NiFeSe hydrogenase during syntrophic growth (Walker *et al.*, 2009).

Whilst syntrophic lactate metabolism in G20 and *D. vulgaris* involves an Fe hydrogenase (HydAB) (Table 1), significant differences exist between the two syntrophic growth systems. Genes in *D. vulgaris* reported to be required for or to impair syntrophic growth include *hyd* and *hyn* (encoding the periplasmic Fe-only and NiFe hydrogenases, respectively), *hmc*, encoding the high-molecular-mass cytochrome (Hmc), and *coolL*, encoding a putative membrane-bound carbon monoxide-induced hydrogenase (Walker *et al.*, 2009). These genes were among the most highly expressed and upregulated genes during syntrophic lactate growth of *D. vulgaris*. However, none of these genes other than *hyd* were identified in our study as being important for syntrophic growth with lactate. In another transcriptomic analysis with *D. vulgaris*, during which growth conditions were changed from syntrophy with *Methanosarcina barkeri* to sulfidogenic, no change in gene expression was observed for *coo*, *hydAB* or *hynAB-1* (Plugge *et al.*, 2010). To some extent, these differences may be due to methodology. Both Walker *et al.* (2009) and Plugge *et al.* (2010) used transcriptional profiling to detect genes of interest, whereas we used a comprehensive mutant screening. Also, the *coolL* gene has not been detected in the G20 genome, suggesting that physiological differences exist between the two species.

The oxidation of lactate to acetate and H₂ under syntrophic conditions is an exergonic reaction; however, the first step, the oxidation of lactate to pyruvate and H₂, is endergonic [e.g. $\Delta G > 0$ kJ mol⁻¹ at 295 Pa H₂, based on pyruvate at 1 mM (Pankhania *et al.*, 1988) and lactate at 37 mM]. Previous work suggested that a proton gradient, generated by the hydrolysis of ATP or by oxidative phosphorylation, is

required for the initial oxidation of lactate to pyruvate and H₂ (Pankhania *et al.*, 1988). Under syntrophic conditions, when H₂ is produced, sulfate respiration does not occur, and some of the energy generated during pyruvate oxidation to acetate would be required to generate this proton gradient. This type of mechanism, which uses a proton gradient to reduce the redox potential of electrons (reverse electron transport), has previously been postulated to occur during syntrophic growth (McInerney *et al.*, 2007). A model to understand these results as they apply to syntrophic cultures would involve electron flow from lactate in the cytoplasm through a cytoplasmic, membrane-associated complex and then through menaquinone to the Qrc–T_{PI}c₃ complex using a hydrogenase to generate H₂ in the periplasm. The energy needed to drive these reactions may come from a proton gradient coupled to quinone cycling, as has been described for the reduction of NAD⁺ by the NADH:quinone oxidoreductase (NDH-1) of bacteria including *Paracoccus denitrificans* (Kotlyar & Borovok, 2002) and *Acidithiobacillus ferrooxidans* (Elbehti *et al.*, 2000).

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