K-074 The physiological role of the cytoplasmic hydrogenases in *Desulfovibrio vulgaris*

S. Stolyar[1,3], N. Pinel[1], C. B. Walker[1,3], J. Wall[2,3], and D. A. Stahl[1,3]

[1] University of Washington, Seattle, WA; [2] University of Missouri-Columbia, Columbia, MO; [3] Virtual Institute of Microbial Stress and Survival.

ESPP2 Environmental Stress Pathway Project

DOE GENOMICS:GTL ACCELERATING DISCOVERY FOR ENERGY AND ENVIRONMENT — OFFICE OF SCIENCE U.S. DEPARTMENT OF ENERGY

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INTRODUCTION

The Gram-negative Deltaproteobacterium *D. vulgaris* Hildenborough is able to grow with sulfate, sulfite or thiosulfate as electron acceptors and in their absence via fermentation or syntrophic association with hydrogenotrophic organisms. Despite decades of research, the mechanism of energy generation by *D. vulgaris* is not well understood. Genome sequence revealed genes for at list six different hydrogenases, four periplasmic and two cytoplasmic. Although some of them have been characterized, their roles in *D. vulgaris* remain obscure. Three periplasmic hydrogenases have been mutated and the mutants demonstrated some changes in growth rate, biomass yield and hydrogen evolution although still able to grow under the all conditions tested (Pohorelic *et. al.*, 2002; Voordow 2002; Caffrey *et. al.*, 2007).

In this work, we have examined the consequences of mutations in two cytoplasmic hydrogenases on respiratory and syntrophic growth: 1) *echA* (DVU0434), the first gene in the operon for the Ech type NiFe- containing hydrogenase and 2) *cooL* (DVU2288), the third gene in the operon coding for the second cytoplasmic NiFe-containing hydrogenase Coo. Growth rate, cell yield, and metabolite production were characterized for three growth conditions: i) sulfate with lactate or pyruvate, ii) sulfate with acetate and hydrogen, and iii) in syntrophic association with a hydrogenotrophic methanogen.

MATERIALS and METHODS

D. vulgaris was grown on a B3 medium in 25 ml Balch tubes or 250 ml bottles with either a 80%N_2: 20%CO_2 or 80%H_2:20% CO_2 gas mixture in the headspace volume of approximately 15 ml. The basal B3 medium (pH 7.2) contained (per liter): 0.25g NaCl, 5.5 g $MgCl_2$•$6H_2O$, 0.1g $CaCl_2$•$2H_2O$, 0.5g NH_4Cl, 0.1g KCl, 1.4g Na_2SO_4, 25mM $NaHCO_3$, 5.75mM K_2HPO_4, 0.001g resazurine, 0.078g Na_2S · 9 H_2O, 1ml Thauer's vitamins of (containing per liter 0.02g biotin, 0.02g folic acid, 0.1g pyridoxine HCl, 0.05g thiamine HCl, 0.05g riboflavin, 0.05g nicotinic acid, 0.05g DL pantothenic acid, 0.05g *p*-aminobenzoic acid, 0.01g vitamin B12), 1ml of trace minerals (per liter: 1.0g $FeCl_2$•$4H_2O$, 0.5g $MnCl_2$•$4H_2O$, 0.3g $CoCl_2$•$4H_2O$, 0.2g $ZnCl_2$, 0.05g Na_2MoO_4•$4H_2O$, 0.02g H_3BO_3, 0.1g $NiSO_4$•$6H_2O$, 0.002g $CuCl_2$•$2H_2O$, 0.006g Na_2SeO_3•$5H_2O$, 0.008g Na_2WO_4•$2H_2O$). This basal medium was amended with lactate and sulfate for growth in mono-culture or with lactate only for co-culture. The concentration of organic acids and inorganic ions (sulfate, phosphate) in culture fluids were determined using a Dionex 500 system equipped with an AS11HC column. In some cases the concentration of organic acids was also measured on an HPLC equipped with a HPX 78 (Bio-Rad) column. Hydrogen concentrations were determined with a RGD2 Reduction Gas Detector (Trace Analytical) with 60/80 MOLE SIEVE 5A column (6' X 1/8") with N2 as carrier gas. The concentration of methane and carbon dioxide was measured on a GC equipped with a TCD and "80/100 HAYESEP Q" column (6' X 1/8") with helium as carrier gas.

Cell suspensions were prepared from cultures grown with lactate and sulfate in 1L bottles. Cells were harvested by centrifugation and resuspended in 10 ml of 50mM MOPS buffer (pH 7.2) and 5mM of DTT and MgCl2. Lactate or pyruvate at 5mM were used as substrate for hydrogen evolution experiments.

To measure hydrogen consumption activity in membrane fractions, cultures were grown in 800 mL B3 medium amended with 40 mM lactate and 20 mM sulfate at 37ºC to mid/late exponential phase (OD600 0.3). Cells were harvested by centrifugation at 10,000-12000 rcf/4 ºC, rinsed with suspension buffer (50 mM MOPS (pH 7.2)/5 mM dithiothreitol) and resuspended in 5 mL of the same buffer. Cells were ruptured on two passes through a french press at 20 kPa. Cell debris and metal sulfides were removed via centrifugation at 20,000 rcf/4ºC for 1 hour. Membrane fractions were collected by centrifugation of the crude extract at 200,000 rcf/4ºC for 1 h. The supernatant (cytoplasmic fraction) was collected, the pellet rinsed with 1 mL suspension buffer and resuspended in 2 mL of the same buffer. Protein concentration was measured by the Bradford method using commercial kits (Pierce, #23238). After recovery, extracts were flushed for at least 15 minutes with N_2, and maintained under anoxic conditions during analyses, all extracts were kept at 4ºC.

Hydrogenase activity was measured by the reduction of methyl viologen (MV) (ε578 = 9.8 mM-1 cm-1) with hydrogen as electron donor. The assay mixture contained 50 mM Tris-HCl, pH 8.5 and 5 mM MV in a ruber-stoppered quartz cuvette. The cuvette was flushed with hydrogen for ~5 minutes. The reaction was started by the addition of ~100 µg protein, and monitored spectrophotometrically at 578 nm.

Figure 1.

***cooL* Tn insertion mutant (JW3034)**

***echA* deletion mutant (JW380)**

RESULTS

Growth of *D. vulgaris* monocultures

Figure 2. With lactate and sulfate / With pyruvate and sulfate / With H_2, CO_2 and sulfate / Hydrogen in headspace during growth with lactate and sulfate

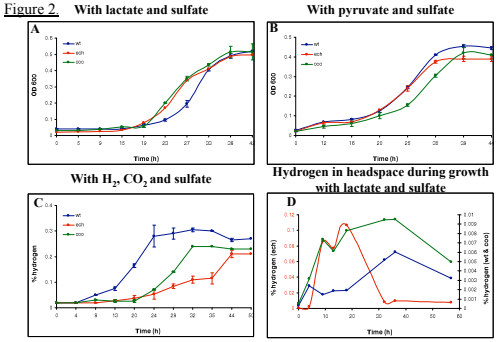
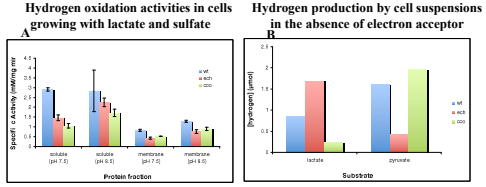


Figure 3. Hydrogen oxidation activities in cells growing with lactate and sulfate / Hydrogen production by cell suspensions in the absence of electron acceptor



Growth of *D. vulgaris echA* mutant in syntrophic association with *M. maripaludis* without sulfate.

Figure 4.

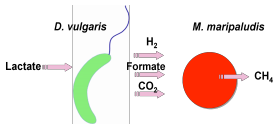
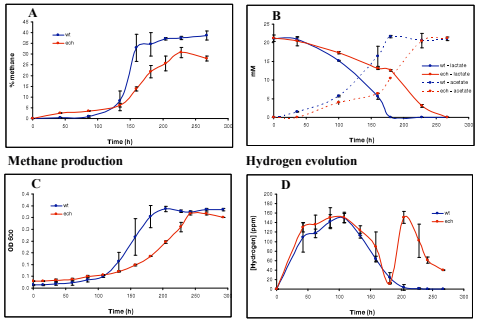


Figure 5. Growth / Lactate consumption and acetate production / Methane production / Hydrogen evolution



The *cooL* mutant did not grow in syntrophic association

SUMMARY and CONCLUSIONS

- Although growth rates of both mutants on sulfate with pyruvate or lactate were comparable to the wild type, hydrogen evolution was much greater for the *echA* mutant during growth in batch culture with lactate and sulfate (Figure 2A, B and D).
- Growth of the *cooL* mutant on hydrogen/CO_2 and sulfate was impaired to a lesser extent, relative to the wild type, than that of the *echA* mutant (Figure 2C).
- Syntrophic growth of the *cooL* mutant was severely impaired on lactate but not on pyruvate; *echA* mutation had little effect on syntrophic growth on either lactate or pyruvate (Figure 4).
- Hydrogen oxidation activities in soluble and membrane fractions of the mutants were lower, yet substantial compared to wild type (Figure 3A). In contrast, hydrogen evolution by *echA* cell suspension from pyruvate was substantially lower than from lactate. Conversely, hydrogen evolution by *cooL* cell suspension from lactate was substantially lower than from pyruvate (Figure 3B).

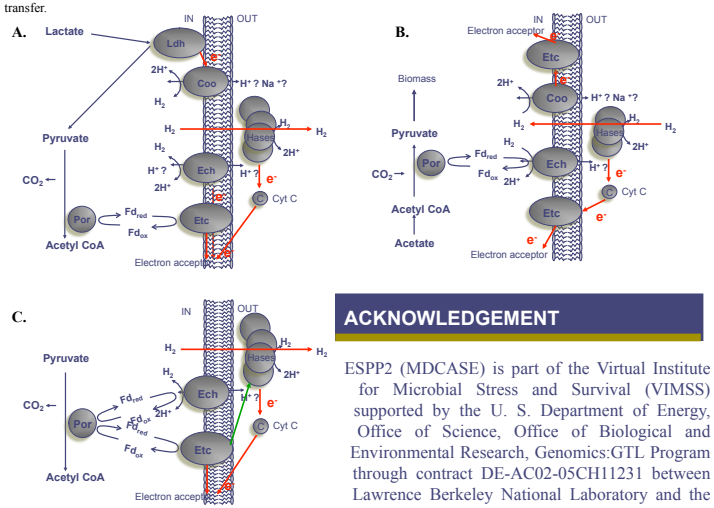
Based on these observations we concluded that :

i) The main role of the Coo hydrogenase is likely directly coupled to the oxidation of lactate to pyruvate, accepting electrons and reducing protons inside the cells. Also, Coo hydrogenase can oxidize hydrogen during growth on hydrogen/sulfate. This explains some of the effects of the *coo* mutation on growth under this condition (Figure 2C).

ii) The main role of the Ech hydrogenase is in hydrogen oxidation, likely coupled to pyruvate oxidation/reduction. Also, Ech hydrogenase might produce hydrogen from pyruvate as a great reduction in hydrogen production from pyruvate by *echA* cell suspension was observed (Figure 3B).

iii) Alternative electron transfer pathways are involved in all modes of *D.vulgaris* Hildenborough growth (Figure 6).

Figure 6. The hypothetical mechanism of a) lactate oxidation in the presence of an electron acceptor or in syntrophy; b) pyruvate oxidation in the presence of an electron acceptor or in syntrophy; c) hydrogen oxidation in the presence of an electron acceptor. Etc, electron transfer complex; Ldh, lactate dehydrogenase; Por, pyruvate oxidoreductase; CytC, cytochrome C. Arrows, electron transfer.



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