Applied and Environmental Microbiology

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Appl. Environ. Microbiol. **2013, 79(8):2818. DOI: 10.1128/AEM.03872-12.**
Published Ahead of Print 8 February 2013.

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Shewanella spp. Use Acetate as an Electron Donor for Denitrification but Not Ferric Iron or Fumarate Reduction

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Lactate but not acetate oxidation was reported to support electron acceptor reduction by *Shewanella* spp. under anoxic conditions. We demonstrate that the denitrifiers *Shewanella loihica* strain PV-4 and *Shewanella denitrificans* OS217 utilize acetate as an electron donor for denitrification but not for fumarate or ferric iron reduction.

Members of the genus *Shewanella* are distributed in diverse habitats, including soil, freshwater, marine, and estuarine environments (1–3). *Shewanella* spp. are recognized for their metabolic versatility and couple growth to a broad range of electron acceptors, including oxygen (O_2), nitrate, nitrite, fumarate, ferric iron, manganese(IV) oxide, thiosulfate, elemental sulfur, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO), and anthraquinone-2,6-disulfonate (AQDS) (1, 3, 4). A variety of electron donors have been reported to support anaerobic growth of *Shewanella* spp., including lactate, pyruvate, and hydrogen (3–5), but not acetate, which *Shewanella* spp. apparently only use under oxic conditions (6–9). Acetate utilization under anoxic conditions has been regarded as a distinguishing feature between *Shewanella* spp. and other well-known metal reducers, such as *Geobacter* spp. (9). In many anoxic environments, acetate is the key electron donor to support reductive processes, and microorganisms capable of acetate oxidation have a selective advantage. This has been demonstrated at the Rifle aquifer, where acetate injections generated conditions that caused *Geobacter* blooms (10). Here, we demonstrate that at least some denitrifying *Shewanella* spp. utilize acetate as an electron donor, thus expanding our understanding of *Shewanella* ecophysiology.

S. loihica strain PV-4 was isolated from a deep sea hydrothermal vent in Loihi Seamount and found not to utilize nitrate (NO_3^-) or nitrite (NO_2^-) as electron acceptors (1). Unexpectedly, genome sequencing revealed that strain PV-4 possesses the complete suite of denitrification genes, including *napEDABC*, *nirK*, *norB*, and *nosZ*, comparable to the genes of the characterized denitrifier *S. denitrificans* strain OS-217 (2). We demonstrate that strain PV-4 grows via NO_3^-/NO_2^- denitrification coupled to acetate oxidation. Experiments with the known denitrifier *Shewanella denitrificans* corroborated acetate utilization under denitrifying conditions. Contrary to the common perception that *Shewanella* spp. cannot oxidize acetate under anoxic conditions, these findings suggest that denitrifying *Shewanella* spp. utilize acetate as an electron donor.

S. loihica strain PV-4 was grown in 160-ml glass serum bottles containing 100 ml of phosphate-buffered basal salts medium adjusted to pH 7.0, which contained (in g liter^{-1}) NaCl (20), NH_4Cl (0.00535), KH_2PO_4 (0.233), K_2HPO_4 (0.57), and 2 ml of a trace metal solution (11). O_2 was removed by boiling and cooling under a stream of O_2-free dinitrogen (N_2) (12). The Hungate technique was used to dispense medium into N_2-flushed serum bottles, which were sealed with black butyl stoppers and autoclaved (12). Electron donors and electron acceptors were added from autoclaved, anoxic stock solutions, and vitamins were added from a filter-sterilized stock (13). After inoculation with 0.5 ml of a strain PV-4 culture grown with 2.0 mM lactate and 1.0 mM NO_3^-, all batch culture vessels (160 ml, total volume) had a total liquid volume of 103.1 ml. The culture bottles received acetylene (10% [vol/vol] of the headspace) to inhibit nitrous oxide (N_2O) reductase and allow quantification of N_2O as a measure of denitrification activity (14). NO_3^- and NO_2^- were analyzed using ion chromatography (15, 16). N_2O was quantified with a MicroGC 3000A apparatus (Agilent Technologies, Palo Alto, CA). Lactate, acetate, fumarate, and succinate concentrations were determined by high-performance liquid chromatography (HPLC) (17). A dimensionless Henry's constant for N_2O of 1.812 was calculated from data in the published literature for the incubation temperature of 23°C and was corrected for the effect of dissolved ions (18–20). Control incubations monitored lactate, acetate, NO_3^-, NO_2^-, and N_2O concentrations in culture bottles that received no inoculum.

In contrast to the original characterization of *S. loihica* strain PV-4 (1), a turbidity increase (i.e., growth) occurred in medium amended with lactate and NO_3^-, but no growth occurred if either substrate was omitted. With 0.47 ± 0.01 mM (48.4 ± 0.8 μmol) lactate and 1.01 ± 0.01 mM (103.0 ± 1.6 μmol) NO_3^- (means ± standard deviations), lactate was completely oxidized within 38 h after inoculation (Fig. 1A). Substantial N_2O production (12.9 ± 4.3 μmol) occurred following lactate depletion (hours 38 to 44) and was accompanied by acetate consumption (5.98 ± 2.55 μmol), suggesting that acetate generated from incomplete lactate oxidation served as an electron donor. In addition, the acetate concentration remaining at the end of the incubation period was much lower than expected from carbon assimilation into biomass, as only 4.93 ± 1.03 μmol remained, when >40 μmol would be expected if reducing equivalents were derived from incomplete lactate oxidation only. Consistent with previous observations in *S. oneidensis* strain MR-1 cultures (6),

Received 14 December 2012 **Accepted** 30 January 2013
Published ahead of print 8 February 2013
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doi:10.1128/AEM.03872-12

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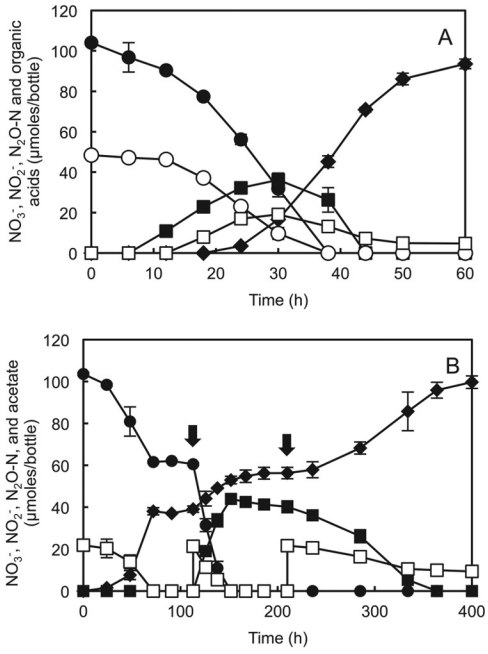

FIG 1 Denitrification in cultures of *S. loihica* strain PV-4. All vessels received acetylene to prevent N_2O reduction to N_2. (A) Cultures initially amended with 1.0 mM NO_3^- as the electron acceptor and 0.5 mM lactate as the electron donor. Substrate consumption and product formation were monitored. Error bars represent the standard deviations of triplicate cultures. (B) Cultures initially amended with 1.0 mM NO_3^- as the electron acceptor and 0.2 mM acetate as the electron donor. Additional feedings of 0.2 mM acetate occurred after 113 and 210 h, as indicated by the arrows. The data points represent the averages of triplicate cultures, with error bars showing the standard deviations. ○, lactate; □, acetate; ●, NO_3^-; ■, NO_2^-; ◆, N_2O-N.

small amounts of hydrogen (<3 μmol) were formed during lactate utilization; however, hydrogen was not consumed following lactate consumption (i.e., did not serve as an electron donor for NO_3^-/NO_2^- reduction).

To verify that *S. loihica* strain PV-4 utilized acetate as an electron donor, culture bottles amended with 1.01 ± 0.01 mM (103.6 ± 0.7 μmol) NO_3^- and a limiting amount of 0.21 ± 0.01 mM (21.3 ± 0.6 μmol) acetate as the sole electron donor were monitored. Following inoculation, the initial amount of acetate was depleted within 72 h, and 43.0 ± 1.0 μmol of NO_3^- was stoichiometrically reduced to 19.0 ± 0.9 μmol of N_2O without buildup of nitrite (Fig. 1B). Reduction of NO_3^- halted when acetate had been consumed and did not resume until additional acetate was supplied. Following another feeding of 21.2 ± 0.8 μmol of acetate, 40.2 ± 2.8 μmol of NO_3^- was reduced to nitrite within 73 h and 8.6 ± 1.6 μmol of N_2O was produced. Following another acetate feeding and 210 h of incubation, the remaining nitrite was reduced to stoichiometric amounts of N_2O.

A mass balance approach further supported acetate utilization coupled to denitrification. Triplicate cultures that received 208.1 ± 1.1 μmol of acetate and 103.8 ± 1.3 μmol of NO_3^- were incubated for 108 h to obtain nitrogen and carbon balances. To measure the amount of biomass produced, cells were enumerated by determining the *nrfA* gene (Shew_0844) copy number by using quantitative real-time PCR (qPCR) and SYBR green detection chemistry. Since strain PV-4 contains one *nrfA* gene per genome, the *nrfA* gene copy number equals the cell number. A primer set (slnrfA1083fwd, 5′-GGATATC CGTCACGCTCAAT-3′; slnrfA1308rev, 5′-GTCCATACCCAAT GCAGCTT-3′) specifically targeting a 226-bp region of this gene was designed using Primer3 software (21). qPCR was performed with an ABI ViiA7 real-time PCR system equipped with ViiA 7 software v1.1 with 2× Power SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA). Calibration curves were constructed with the same 226-bp *nrfA* fragment cloned into plasmid PCR2.1 and diluted over a range of 10^8 to 10^3 copies. The standard curve had a slope of −3.38, a *y* intercept of 33.4, and an R^2 value of 0.998, yielding 97.6% amplification efficiency. Triplicate qPCR assays were performed for each of the three replicate culture vessels. To estimate the dry weight of an individual *S. loihica* strain PV-4 cell, biomass was collected from early-stationary-phase cultures (i.e., optical density at 600 nm [OD_{600}] of 0.1) grown with 10 mM lactate and 10 mM fumarate as the substrates to yield sufficient biomass. The cell suspensions (5 ml each) were filtered onto preweighed 0.22-μm membrane filters (Millipore, Billerica, MA). The dry weight of the biomass on each membrane was determined after drying at 100°C for 24 h and cooling in a desiccator. An aliquot (1.5 ml) of the same cell suspension was centrifuged at 16,000 × *g* for 10 min, and DNA was extracted immediately using a DNeasy blood and tissue kit (Qiagen, Santa Clarita, CA) following the manufacturer's protocol. The analysis of three individual *S. loihica* strain PV-4 cultures yielded 0.26 ± 0.01 mg (dry weight) of biomass ml^{-1} and (9.35 ± 0.11) × 10^8 *nrfA* gene copies ml^{-1}. From these values, the dry weight of a single cell was estimated to be 2.77 × 10^{-13} g. The average *nrfA* copy number measured in the triplicate cultures amended with 208.1 ± 1.1 μmol of acetate and 103.8 ± 1.3 μmol of NO_3^- was 4.17 × 10^7 ± 3.48 × 10^6 ml^{-1}. From these values, an average biomass yield of 10.5 ± 0.9 μmol of cells with the formula $C_5H_7O_2N$ was calculated.

To establish a nitrogen balance, the amount of nitrogen input (as NO_3^- and NH_4^+) was compared with the amounts of products generated (i.e., N_2O and biomass) and the remaining amount of NH_4^+ (Table 1). From 114 ± 1 μmol of total nitrogen added to the cultures, 111 ± 7 μmol was recovered, resulting in 97.5 ±

TABLE 1 Mass balance of *Shewanella loihica* strain PV-4 grown with 2.0 mM acetate as electron donor and 1.0 mM nitrate as electron acceptor

Substrates consumed (μmol)[a]			Products (μmol)[a]		Yield (g of biomass [dry wt]/mol substrate consumed)[a]		f_s value, calculated using[c]:		
NO_3^-	NH_4^+	Acetate	N_2O	Biomass[b]	Acetate	NO_3^-	Eq. 1	Eq. 2	Theor.
103.8 (1.3)	6.58 (0.36)	74.7 (13.4)	97.0 (7.2)	10.5 (0.9)	15.9 (3.2)	11.4 (1.0)	0.35	0.42	0.38

[a] The values represent the averages of triplicate cultures, and the numbers in parentheses are standard deviations.
[b] Biomass was determined based on the empirical formula $C_5H_7O_2N$, with a molecular weight of 113.
[c] f_s values were calculated based on equation 1 or 2 (see text) or the theoretical maximum value for f_s (calculated as described previously [22]). Theor., theoretical.

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6.3% nitrogen recovery. The amount of acetate consumed was 74.7 ± 13.4 μmol, which is close to the theoretical electron donor demand (i.e., 48.5 μmol of acetate to yield 97.0 μmol of N_2O-N) and carbon demand (i.e., 26.3 μmol of acetate for the synthesis of 10.5 μmol of biomass). Values for f_s, the fraction of electrons consumed in biomass synthesis, were calculated based on mass balance and growth yield data (Table 1). From the amount of acetate consumed and N_2O generated, an f_e value of 0.65, and thus an f_s value of 0.35, was calculated according to equation 1. A slightly higher f_s value of 0.42 was determined based on the growth yield data (equation 2), assuming that one-half of the biomass N originated from ammonium and the other half from NO_3^-.

$$f_s = 1 - f_e = 1 - [(4\ e^-\ \text{equivalents/mol}) \times (\text{mol of nitrate reduced to } N_2O)]/[(8\ e^-\ \text{equivalents/mol}) \times (\text{mol of acetate consumed})] \quad (1)$$

$$f_s = [0.5 \times (\text{mol of biomass}) \times (20\ e^-\ \text{equivalents/mol} + 28\ e^-\ \text{equivalents/mol})]/[(\text{mol of acetate consumed}) \times (8\ e^-\ \text{equivalents/mol})] \quad (2)$$

These experimentally determined f_s values were very close to the theoretical maximum f_s value of 0.38, calculated for acetate-driven denitrification to N_2O under standard conditions at pH 7.0 (22). In summary, the mass balance data corroborated the observation that NO_3^--to-N_2O reduction occurs with acetate as the sole source of reducing equivalents.

Additional experiments explored if *S. loihica* strain PV-4 used acetate for the reduction of other electron acceptors. Oxic incubations used 50 ml of medium and 110 ml of air headspace. Anoxic cultures (100 ml of medium and 60 ml N_2 headspace) received 2 mM fumarate or 5 mM ferric citrate as the electron acceptor. All cultures received 2 mM acetate or 2 mM lactate as the electron donor. Contrary to a previous report (1), *S. loihica* strain PV-4 completely oxidized acetate with oxygen as electron acceptor. No acetate oxidation or electron acceptor consumption occurred in anoxic cultures that received fumarate or ferric iron as the electron acceptor. In positive-control cultures amended with 2 mM lactate and 2 mM fumarate, 1.14 ± 0.06 mM lactate was oxidized and 1.10 ± 0.01 mM acetate was produced. In cultures amended with 2 mM lactate and 2 mM ferric citrate, 0.55 ± 0.03 mM lactate was oxidized to 0.50 ± 0.02 mM acetate. Apparently, *S. loihica* utilizes acetate as an electron donor under oxic and denitrifying conditions, but acetate does not support the reduction of fumarate or ferric iron.

To explore if acetate utilization coupled to NO_3^- reduction is a shared feature among *Shewanella* spp., *S. oneidensis* strain MR-1 was grown with 2.0 mM acetate or 2.0 mM lactate and 1.0 mM NO_3^- in the same medium as *S. loihica*, except that L-glutamic acid, L-arginine, DL-serine (20 mg/liter each), and 0.1 g/liter Casamino Acids (Difco, Detroit, MI) were added to meet strain MR-1's growth requirements (5). Strain MR-1 has been reported to use acetate as an electron donor under oxic conditions, but not when coupled with the reduction of other electron acceptors, such as fumarate and ferric iron (6, 8, 17). Strain MR-1 performs dissimilatory nitrate reduction to ammonium (DNRA) but cannot denitrify (5). Consistent with previous findings, strain MR-1 coupled incomplete lactate oxidation with DNRA (5); however, strain MR-1 was unable to oxidize acetate under the same incubation conditions. The only other verified denitrifier in the *Shewanella* genus is *S. denitrificans* strain OS-217, which lacks lactate dehydrogenase and cannot utilize lactate (2, 23). *S. denitrificans* strain OS217 requires supplements (i.e., amino acids and Casamino Acids) for growth, which were added to the medium at the concentrations indicated above. Since strain OS217 grew with these supplements alone, cultures were grown to mid-exponential phase (OD_{600}, 0.6) in lysogeny broth (LB) with agitation (120 rpm) under oxic conditions at 23°C, before biomass from 40 ml of the cell suspension was harvested by centrifugation at 4,800 × *g* for 10 min. The pellets were washed twice with fresh phosphate-buffered basal salts medium without supplements (i.e., amino acids and Casamino Acids) and suspended in 6 ml of the same medium. Aliquots (1 ml) of the cell suspensions were each transferred to 160-ml glass vessels containing 100 ml of fresh medium without supplements. Then, triplicate vessels were amended with 194.9 ± 5.1 μmol (1.87 ± 0.05 mM) of sodium acetate and 101.0 ± 4.0 μmol (0.97 ± 0.04 mM) of sodium NO_3^-. The concentrations of nitrogen oxides and acetate were monitored in triplicate incubation vessels. Acetate utilization began after 30 h of incubation (Fig. 2), and acetate was consumed at a constant rate of 0.260 ± 0.004 μmol h^{-1}. Concomitant with acetate utilization, NO_3^- was consumed at constant rate of 0.63 ± 0.03 μmol h^{-1}, and N_2O was produced at a rate of 0.32 ± 0.01 μmol h^{-1}. Acetate oxidation stopped after NO_3^- had been consumed, indicating that acetate served as an electron donor for NO_3^- reduction to N_2O in strain OS217 cell suspensions. In the negative-control incubations lacking electron donors, 12.9 ± 3.8 μmol of NO_3^- was reduced to N_2O, but after 30 h no significant NO_3^- reduction occurred.

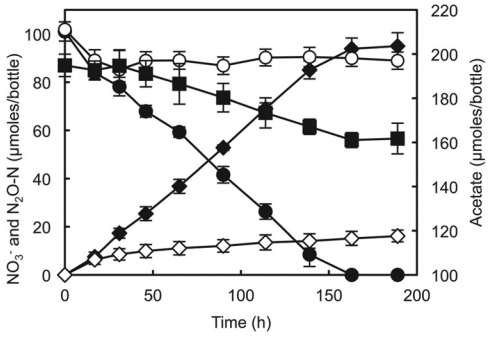

FIG 2 Acetate-driven denitrification in cultures of *Shewanella denitrificans* strain OS217. Pregrown cells were suspended in vessels containing basal salts medium amended with 2.0 mM acetate and 1.0 mM nitrate (see Materials and Methods for details). Acetylene was added to inhibit N_2O-to-N_2 reduction, and substrate consumption and product formation were monitored over a 189-hour incubation period. Negligible NO_3^- (○) consumption and N_2O (◇) formation were observed in cultures that did not receive an external electron donor. The data points represent the averages of triplicate cultures, with error bars showing the standard deviations. ■, acetate; ●, NO_3^-; ◆, N_2O-N.

These findings demonstrate that *Shewanella loihica* strain PV-4 and *Shewanella denitrificans* strain OS217 utilize acetate as an electron donor for denitrification, which may be a shared feature among denitrifying *Shewanella* spp. The reasons why strain PV-4 did not couple acetate oxidation to fumarate and ferric iron reduction are unclear but may be related to energetic constraints, as denitrification is thermodynamically a more favorable process than fumarate or ferric iron respiration ($\Delta G^{0'}$ values were calculated according to the methods described in reference 24):

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$CH_3COO^- + 2\,NO_3^- + 3\,H^+ \rightarrow 2\,CO_2 + N_2O + 3\,H_2O$ resulted in a $\Delta G^{0'}$ of -684.6 kJ mol^{-1} acetate, and $CH_3COO^- + 4$ fumarate$^{2-} + H^+ + 2\,H_2O \rightarrow 4$ succinate$^{2-} + 2\,CO_2$ resulted in a $\Delta G^{0'}$ of -249.3 kJ mol^{-1} acetate.

The free energy change associated with acetate oxidation coupled with DNRA (i.e., $NO_3^- \rightarrow NH_4^+$; $\Delta G^{0'}$ of -505.1 kJ mol acetate^{-1}) is closer to the energy yield associated with denitrification (i.e., $NO_3^- \rightarrow N_2O$), yet acetate did not support DNRA in *S. oneidensis* strain MR-1. Both denitrification and DNRA involve stepwise reactions that share the NO_3^--to-NO_2^- reduction step with a standard reduction potential of 0.43 V; however, the reduction potentials of the NO_2^-/N_2O and NO_2^-/NH_4^+ redox couples vary (i.e., 0.76 versus 0.36 V, respectively). Hence, energetics may explain acetate utilization as an electron donor by *Shewanella* spp., and growth experiments in fuel cells with potentiostat-controlled cathodic redox potentials should be conducted to further characterize the conditions that allow energetically favorable acetate oxidation.

Several sulfate-reducing (e.g., *Desulfobacter* spp.) and metal-reducing (e.g., *Geobacter* spp., *Anaeromyxobacter* spp.) bacteria utilize the tricarboxylic acid cycle to oxidize acetate under anoxic conditions (16, 17, 25). *Desulfobacter postgatei*, for example, grows with acetate by reducing sulfate to elemental sulfur ($\Delta G^{0'}$, -63 kJ mol acetate^{-1}). Biochemical studies and genome sequence information demonstrated that these species share an acetate oxidation pathway that is dissimilar to that of *Shewanella* spp. (26–28). The most prominent difference is that these acetate oxidizers activate acetate to acetyl coenzyme A (CoA) by using a succinyl-CoA:acetate-CoA transferase through direct transfer of CoA from succinyl-CoA to acetate, thus eliminating the ATP requirement to activate acetate (28–30). Although not yet verified, such differences in acetate metabolism may explain why *Shewanella* and other facultative anaerobic heterotrophs are unable to oxidize acetate under energy-constrained conditions (4, 27, 31). In conclusion, these results demonstrate that acetate utilization by *Shewanella* spp. is not restricted to oxic environments and can occur under anoxic conditions when coupled with energetically favorable reactions, such as denitrification.

ACKNOWLEDGMENT

This research was supported by the U.S. Department of Energy, Office of Biological and Environmental Research, Genomic Science Program, award DE-SC0006662.

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