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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 (O₂), nitrate, nitrite, fumarate, ferric iron, manganese(IV) oxide, thiosulfate, elemental sulfur, trimethylenamine 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 (NO₃⁻) or nitrite (NO₂⁻) 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 NO₃⁻/NO₂⁻ 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), NH₄Cl (0.00535), KH₂PO₄ (0.233), K₂HPO₄ (0.57), and 2 ml of a trace metal solution (11). O₂ was removed by boiling and cooling under a stream of O₂-free dinitrogen (N₂) (12). The Hungate technique was used to dispense medium into N₂-flushed serum bottles, which were sealed with black butyl stoppers and autoclaved (12).
Acetate feeding and 210 h of incubation, the remaining nitrite was reduced to stoichiometric amounts of N₂O.

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 NO₃⁻ 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 (slnrfA1083fw, 5'-GGATATC CGTCACGCTCAAT-3'; slnrfA1308rev, 5'-GTCCATAACCCCAAT 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 2X 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 10⁶ to 10⁷ copies. The standard curve had a slope of −3.38, a y-intercept of 33.4, and an R² 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 [OD₆₀₀] 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 desicator. 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) × 10⁸ 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 NO₃⁻ was 4.17 × 10⁷ ± 3.48 × 10⁶ ml⁻¹. From these values, an average biomass yield of 10.5 ± 0.9 μmol of cells with the formula C₅H₇O₂N was calculated.

To establish a nitrogen balance, the amount of nitrogen input (as NO₃⁻ and NH₄⁺) was compared with the amounts of products generated (i.e., N₂O and biomass) and the remaining amount of NH₄⁺ (Table 1). From 114 ± 1 μmol of total nitrogen added to the cultures, 111 ± 7 μmol was recovered, resulting in 97.5 ±

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**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

<table>
<thead>
<tr>
<th>Substrates consumed (μmol)</th>
<th>Products (μmol)</th>
<th>Yield (g of biomass)</th>
<th>fᵢ value, calculated using:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[dry wt/ml]</td>
<td>Eq. 1</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>NH₄⁺</td>
<td>Acetate</td>
<td>N₂O</td>
</tr>
<tr>
<td>103.8 (1.3)</td>
<td>6.58 (0.36)</td>
<td>74.7 (13.4)</td>
<td>97.0 (7.2)</td>
</tr>
</tbody>
</table>

*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 C₅H₇O₂N, with a molecular weight of 113.

*c fᵢ values were calculated based on equation 1 or 2 (see text) or the theoretical maximum value for fᵢ (calculated as described previously [22]). Theor., theoretical.
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 N₂O-N) and carbon demand (i.e., 26.3 μmol of acetate for the synthesis of 10.5 μmol of biomass). Values for \( f_\text{s} \) 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 N₂O generated, an \( f_\text{s} \) value of 0.65, and thus an \( f_\text{e} \) value of 0.35, was calculated according to equation 1. A slightly higher \( f_\text{s} \) 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 NO₃⁻.

\[
\begin{align*}
\frac{f_\text{s} - f_\text{e}}{1} & = 1 - \left(\frac{4 \text{ e}^- \text{ equivalents/mol}}{8 \text{ e}^- \text{ equivalents/mol}}\right) \\
& \times \left(\frac{\text{mol of nitrate reduced to N}_2\text{O}}{\text{mol of acetate consumed}}\right) \\
\end{align*}
\]

These experimentally determined \( f_\text{s} \) values were very close to the theoretical maximum \( f_\text{s} \) value of 0.38, calculated for acetate-driven denitrification to N₂O under standard conditions at pH 7.0 (22). In summary, the mass balance data corroborated the observation that NO₃⁻-to-N₂O 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. Oxidic incubations used 50 ml of medium and 110 ml of air headspace. Anoxic cultures (100 ml of medium and 60 ml N₂ 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 anaerobic and denitrifying conditions, but acetate does not support the reduction of fumarate or ferric iron.

To explore if acetate utilization coupled to NO₃⁻ 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 NO₃⁻ in the same medium as S. loihica, except that D-glutamic acid, D-arginine, D-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 anaerobic 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 OS217, 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 (OD₆₀₀, 0.6) in lysogeny broth (LB) with agitation (120 rpm) under anaerobic 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 (1.67 ± 0.05 mM) of sodium acetate and 101.0 ± 4.0 μmol (0.97 ± 0.04 mM) of sodium NO₃⁻. The concentrations of nitrogen oxides and acetate were monitored in triplicate cultures. 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, NO₃⁻ was consumed at constant rate of 0.63 ± 0.03 μmol h⁻¹, and N₂O was produced at a rate of 0.32 ± 0.01 μmol h⁻¹. Acetate oxidation stopped after NO₃⁻ had been consumed, indicating that acetate served as an electron donor for NO₃⁻ reduction to N₂O in strain OS217 cell suspensions. In the negative-control incubations lacking electron donors, 12.9 ± 3.8 μmol of NO₃⁻ was reduced to N₂O, but after 30 h no significant NO₃⁻ reduction occurred.

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 (ΔG° values were calculated according to the methods described in reference 24):
CH\textsubscript{3}COO\textsuperscript{−} + 2 NO\textsubscript{3}\textsuperscript{−} + 3 H\textsuperscript{+} → 2 CO\textsubscript{2} + N\textsubscript{2}O + 3 H\textsubscript{2}O resulted in a ΔG\textsuperscript{o} of −684.6 kJ mol\textsuperscript{−1} acetate, and CH\textsubscript{3}COO\textsuperscript{−} + 4 fumarate\textsuperscript{2−} + H\textsuperscript{+} + 2 H\textsubscript{2}O → 4 succinate\textsuperscript{2−} + 2 CO\textsubscript{2} resulted in a ΔG\textsuperscript{o} of −249.3 kJ mol\textsuperscript{−1} acetate.

The free energy change associated with acetate oxidation coupled with DNRA (i.e., NO\textsubscript{3}− acetate) stepwise reactions that share the NO\textsubscript{3}− strain MR-1. Both denitrification and DNRA involve with a standard reduction potential of 0.43 V; however, the reduction potentials of the NO\textsubscript{3}−/N\textsubscript{2}O and NO\textsubscript{3}−/NH\textsubscript{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., Desulfbacter 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). Desulfbacter postgatei, for example, grows with acetate by reducing sulfate to elemental sulfur (ΔG\textsuperscript{o} = −63 kJ mol acetate\textsuperscript{−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.

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