2022

# Genomic Analysis of Acetoanaerobium sp. VLB-1, an Anaerobic Bacterium Isolated from Nebraska's Eastern Saline Wetlands

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# Genomic analysis of *Acetoanaerobium* sp. VLB-1, an anaerobic bacterium isolated from Nebraska's Eastern Saline Wetlands

An Undergraduate Honors Thesis

Submitted in Partial Fulfillment of

University Honors Program Requirements

University of Nebraska-Lincoln

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March 14, 2022

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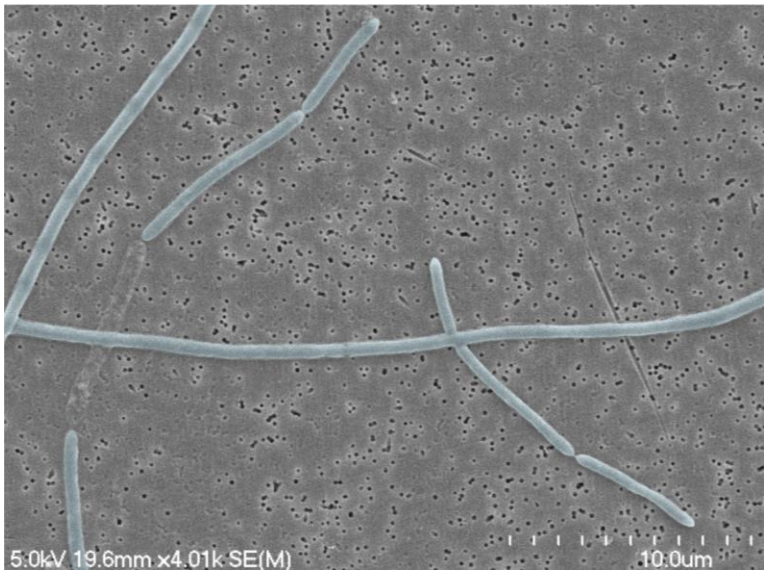


Figure 1: *Acetoanaerobium* sp. VLB-1 under scanning electron microscopy

## Abstract

Genomic and physiological characteristics of an anaerobic, environmental bacterial isolate, *Acetoanaerobium* sp. strain VLB-1, were determined from the assembled annotated 2.57 megabase-pair draft genome. Strain VLB-1 was isolated from an anaerobic, alkaline, saline methanogenic enrichment initiated from soils collected from the Eastern Saline Wetlands in Lincoln, NE. With this isolate, an investigation into elemental and amino acid cycling via the Stickland reaction and the Wood-Ljungdahl pathway was conducted to determine possible metabolic products. The Stickland reaction is a relatively newly discovered pathway, observed in the genus *Clostridium*. *A. sticklandii* is the main model for this method of anaerobic amino acid fermentation and the new way to generate energy. The genome of our isolate was sequenced, using long read sequencing techniques from Novogene with a goal to close the genome for a complete reading. The genome had a close relation to *Acetoanaerobium sticklandii* strain DSM 519 and *Acetoanaerobium noterae* strain NOT-3, each with a similarity of 98.48%. With the analysis of the genome performed, the organism appears to use the Stickland reaction to oxidize

amino acids and the Wood-Ljungdahl pathway to fix carbon, which is characteristic of many *Acetoanaerobium* and *Clostridium* species.

## Methods

*Acetoanaerobium* sp. strain VLB-1 was isolated from an enrichment initiated with soil from the Eastern Saline Wetlands in Lincoln, NE. The enrichment was done on minimal, semi-freshwater saline wetland culture medium (argon headspace with $H_2$ added via syringe, pH 8.3) with calcium carbonate as a sole source of carbon. To further enrich for the *Acetoanaerobium* species, an aliquot of enrichment culture was transferred into anaerobic (100% argon) DSMZ Medium 38 (without sulfide, pH 7.5), which enables Stickland fermentation, a metabolism specific to the *Acetoanaerobium* spp. vs. other organisms found in the carbonate enrichment. After enrichment of the Acetoanaerobium sp. on DSMZ Medium 38, a serial dilution was performed and the organism was cultured via the agar deep method. Five colonies were isolated from the agar, of which VLB-1 was one.

DNA was extracted from culture VLB-1 by harvesting cell mass mid log-phase and extracting nucleic acid using the Griffith's method (Griffiths *et al*. 2000). After fluorometric DNA quantification with a Qubit fluorometer, the DNA sample was sent for Illumina sequencing with Novogene. The raw sequence data was assembled with MEGAHIT, binned with CONCOCT, MetaBat, and MaxBin 2, and binning results were joined with DAS Tool. The resulting genome was estimated to be 98.46% complete with nine contigs and a size of 2,574,940 base pairs with only 1.40% contamination.

The Ribosomal Database Project (RDP) Classifier was used to verify the taxonomy of *Acetoanaerobium* sp. VLB-1 based on the 16S rRNA sequence. With a confidence threshold of

80%, VLB-1 is classified in the phylum Firmicutes, class Clostridia, order Clostridiales, family Peptostreptococcaceae, and genus *Acetoanaerobium* by 100%. Through using the Type (Strain) Genome Server which calculates the average nucleotide identity of the genome against other Acetoanaerobium species, sp. VLB-1 was identified as *Acetoanaerobium noterae* with a 97.65% percent similarity, followed by A. sticklandii with a 96.14% similarity.

Molecular Evolutionary Genetics Analysis (MEGAX) was used to construct a multiple sequence alignment of organisms with genetic similarity to strain VLB-1 (Kumar *et al*. 2018). The alignment was then used to generate into a phylogenetic tree to visualize taxonomic and ancestral relationships.

BlastKOALA (Kyoto Encyclopedia of Genes and Genomes) was then used to classify and analyze the individual genes present in metabolic pathways of interest, while also providing a list of functional genes (Kanehisa *et al*. 2016). The 1484 entries of the genome were annotated to 60.3%.

## Background

The Nebraska Saline Wetlands are a result of the floodplains of Salt Creek, Little Salt Creek, and Rock Creek (Nebraska Outdoor Legacy Project 2015). The salinity is created by underground salts from prehistoric oceans brought to the surface through groundwater flow (Nebraska Outdoor Legacy Project 2015). Vegetation consists of salt tolerant plants and salt tolerant microbiota. All this flora and fauna is unique to the only saline wetlands in Nebraska, however, over 90% of the original landscape has been lost (Nebraska Outdoor Legacy Project 2015). Unfortunately, these wetlands are host to some of the only known populations of organisms such as the Salt Creek Tiger Beetle and the only place in Nebraska that contains the

Saltwort (Athen *et al.* 2021). There are continued factors contributing to the diminishment of the wetlands such as livestock grazing practices, rural and urban development, the decline in groundwater levels, agricultural runoff, and invasive plant and insect species (Nebraska Outdoor Legacy Project 2015). With this change comes the possible loss of a unique microbiome that, from analysis and comparison to coastal salt marshes, have been prevalent for billions of years (Athen *et al.* 2021).

Metagenomic sequencing of the wetland's microbiome has shown that it is unexpectedly different from coastal salt marshes and contains bacteria that are found in cold, alkaline, saline environments, although only a little is known about the potential microbial diversity that this environment provides (Athen *et al.* 2021). One of the most prevalent species in this unique environment is a bacterium that had only previously been isolated from an Eastern Siberian lake (Athen *et al.* 2021). These organisms are responsible for biogeochemical cycling of sulfur, nitrogen, and carbon (Athen *et al.* 2021). Further analysis of the diverse metabolisms present can predict the possible effect that these organisms have on their environment and reveal possible outcomes for conservation efforts (Athen *et al.* 2021). The survival of the plant and animal species is reliant on a balanced microbial community to keep the ideal salinity, pH, and acetate and methane levels.

*Acetoanaerobium*, formerly identified as a *Clostridium* genus, are gram positive, anaerobic bacteria. A unique physiology of this organism is acetogenesis. Acetogenesis is defined as the synthesis of acetate, paired with the reduction of carbon dioxide and organic acids (Ragsdale 2008). Acetogenic species belong to two groups: hydrogen-producing species and obligate proton-reduction species. Among both the non-obligate and obligate proton-reduction species are *Acetoanaerobium* (Borja & Rincón 2016). The former prefers slightly acidic

conditions and to be paired with hydrogen sinks; the latter can only be cultured in an electron devoid environment (Borja & Rincón 2016). Their metabolism is directed to the production of acetate, resulting in the degradation of pyruvate, lactate, alcohols, and more (Fonknechten *et al.* 2010). In this sense, acetogens and methanogens make a perfect pair, a mutualistic culture. However, the homoacetogens also compete with methanogens for hydrogen, formate, and methanol (Fonknechten *et al.* 2010). The purpose of acetogenesis and the acetyl-CoA pathway is to use it as a way to reduce carbon dioxide to acetyl-CoA, use it as a terminal electron acceptor and therefore energy generation, or to use it for the cell's main source of carbon (Fonknechten *et al.* 2010).

Fermentation resulting from organic acids and alcohols are oxidized to acetate, and the electrons produced are transferred to hydrogen cations to produce $H_2$ or bicarbonate used in the synthesis of formate (Borja & Rincón 2016). Electrons are given to hydrogen or carbon dioxide; however, this transfer is energetically unfavorable (Borja & Rincón 2016). In fact, the organisms are limited in their energy production, so interspecies hydrogen transfer must be employed. A hydrogen sink, like a methanogen fixing hydrogen to produce methane, must be used in order to keep the level of these products low. Both methanogens and acetogens are reliant on each other to degrade fatty acids for growth, regulate growth rate, grow in thermodynamic equilibrium, and benefit from the resulting chemical energy.

Acetogens and methanogens in combination degrade organic material to $CH_4$, $CO_2$ and $H_2S$ via their interactions in a similar environment (Dar et al. 2008). Due to fermentation, carbon dioxide is reduced to acetate (acetogens) or methane (methanogens), along with the resulting reduction of sulfur (Dar *et al.* 2008). Acetogenesis creates products that can be used as reactants for acetate-and propionate-oxidizing sulfate reducing bacteria or methanogens depending on the

presence of sulfur in the environment (Dar *et al.* 2008). Acetogens, methanogens, and sulfur reducing bacteria compete for $H_2$ in their respective fermentation (Dar *et al.* 2008). However, in an environment with a limited supply of hydrogen, methanogens and sulfur reducing bacteria are typically favored due to the thermodynamic favorability of these metabolic processes (Dar *et al.* 2008).

## Metabolic Network Reconstruction and Analysis

The genome of strain VLB-1 is the most similar to *Acetoanaerobium sticklandii* strain DSM 519 and *Acetoanaerobium noterae* strain NOT-3, each with a similarity of 98.48% in their respective genomes.

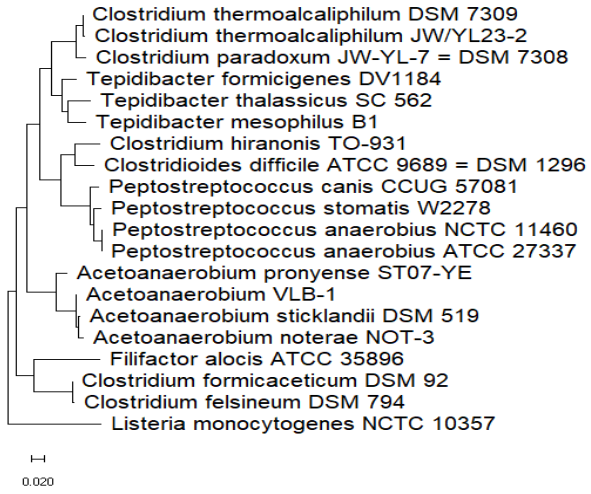


Figure 2: MEGAX constructed phylogenetic tree based on 16 rRNA gene sequences using MUSCLE alignment of DNA including strain VLB-1 and closely related taxa in the Family Clostridiales.
(*Listeria monocytogenes* was used as an outgroup.

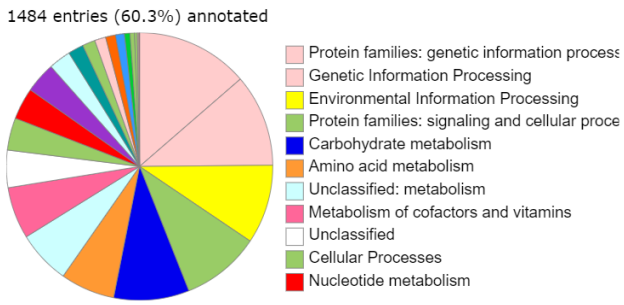


Figure 3: Proportion of genome annotated by BlastKOALA for VLB-1. The chart shows the functional category and abundance of genes in the DNA.

**Stickland Reaction**

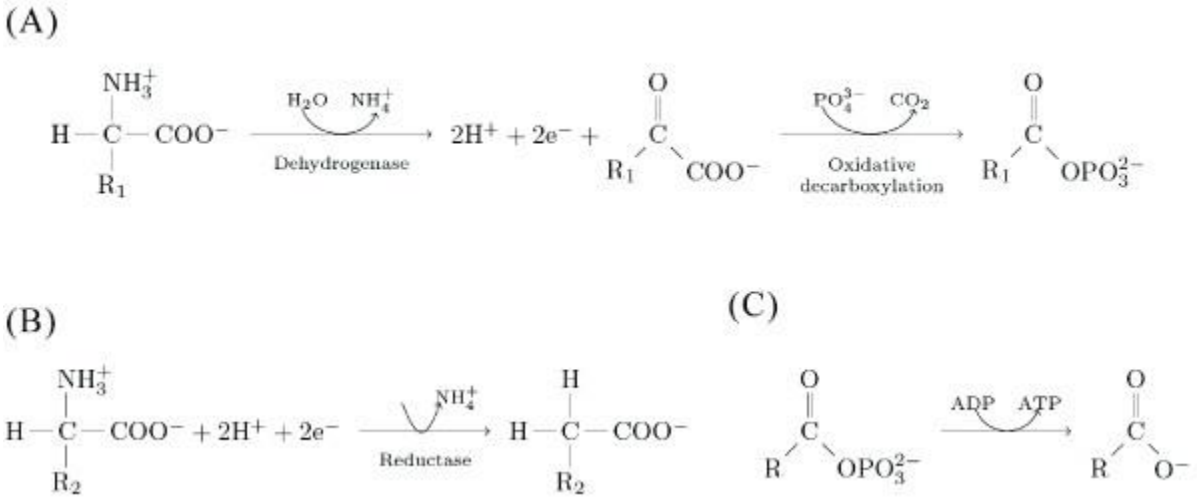


Figure 4: Stickland Reaction (de Vladar 2012). The reaction happens in a stepwise fashion, with the help of hydrogenases, reductases, and catabolism by kinases.

One common feature among *Acetoanaerobium* and the genus *Clostridium* is their special role in amino acid degradation with the Stickland reaction which preferentially oxidizes an amino acid, while reducing its pair (Fonknechten *et al.* 2010). However, not much is known about the Stickland reaction or its attributes to growth or providing energy to organisms. Amino acids are used as the main carbon and thus energy sources (Fonknechten *et al.* 2010). As one amino acid is oxidized, its pair is subsequently reduced. In this process, ATP is formed by substrate-level phosphorylation (Fonknechten *et al.* 2010). *A. sticklandii*, which is closely related to strain VLB-1, uses the amino acids threonine, arginine, lysine, and serine (Fonknechten *et al.* 2010). Along with these, aromatic and branched amino acids can also be degraded, however, the pathways and processes are still unknown (Fonknechten *et al.* 2010). Strain VLB-1 does contain the enzymes to metabolize all of these, including aromatic and branched amino acids. The benefit of this pathway is the availability of these amino acids and their pairs when synthesized via abiotic amino acid pathways and in protein-rich environments or when protein synthesis is inhibited (Fonknechten *et al.* 2010). In analysis of the genome of *Acetoanaerobium* sp. VLB-1, it has the capability to degrade arginine via the arginine succinyltransferase pathway and threonine, serine, and cysteine via their respective biosynthesis pathways. Cysteine biosynthesis is crucial for sulfur fixation for the synthesis of many vitamins and cellular components.

The Shikimate pathway is used to degrade aromatic amino acids, starting with phosphoenolpyruvate and erythrose-4P to be degraded into chorismate through a multistep reaction involving the dephosphorylation and dehydration of the compounds with a transferase. With many steps later, the result is chorismate and orthophosphate to be used in acetogenesis or as a carbon source. Threonine biosynthesis is made by aspartate first by using aspartate kinase

and aspartate-semialdehyde dehydrogenase for the conversion to homoserine. From there, homoserine is converted to threonine using the enzymes homoserine dehydrogenase, homoserine kinase, and threonine synthase. VLB-1 is able to synthesize leucine through leucine biosynthesis. In the steps preceding biosynthesis, 2-oxoisovalerate is converted to 2- oxoisocaproate through 2-isopropylmalate synthase, 3-isopropylmalate/(R)-2-methylmalate dehydratase large subunit and the small subunit in conjunction, and 3-isopropylmalate dehydrogenase.

Arginine is a source of carbon, energy, nitrogen, and a precedent to polyamine synthesis. It produces ammonia without moving nitrogen. Arginine is synthesized in two main steps: ornithine biosynthesis and arginine biosynthesis. In ornithine biosynthesis, glutamate is converted using a series of glutamate acetyltransferases and kinases to become L-ornithine. In the second step, ornithine is converted to arginine through the enzyme ornithine carbamoyltransferase catalyzing the reaction between carbamoyl phosphate and L-ornithine. The product, L-citrulline then reacts with L-aspartate with an input of energy and the enzyme argininosuccinate synthase, to result in the product of N-(L-arginino) succinate, which is then converted to arginine using argininosuccinate lyase.

There does appear to be multiple glycine reductase complex component B subunits, hinting at a possible way to reduce glycine, however, the pathway is unknown. In comparison, *A. sticklandii* has both the glycine synthase and reductase pathways and the Wood-Ljungdahl pathways, which is fairly uncommon.

**The Wood-Ljungdahl Pathway**

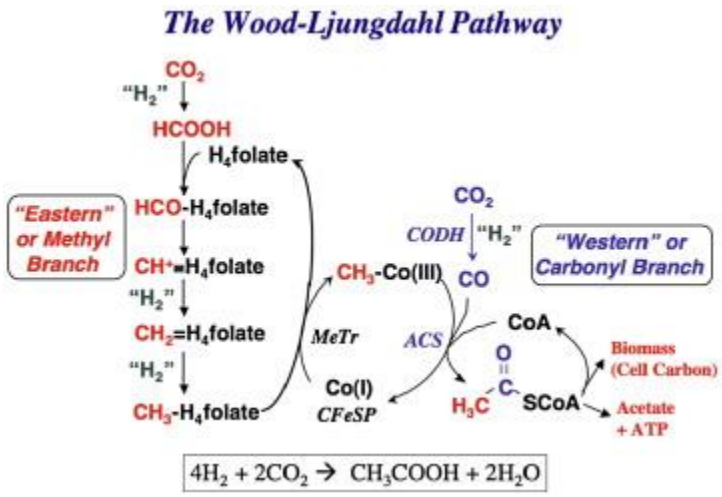


Figure 5: The Wood-Ljungdahl Pathway (Ragsdale & Pierce 2003). The Eastern and Western branches are shown. The Western branch is used by acetogens to create acetate and carbon.

The Wood-Ljungdahl pathway is also invaluable to many *Acetoanaerobium* spp. to produce acetic acid via acetogenesis. In this pathway, energy is conserved by converting carbon dioxide and carbon monoxide into acetyl-CoA which is then used for mass energy production in the citric acid cycle (Ragsdale 2008). All in all, they create around $10^{13}$ kg of acetic acid per year, far greater than the world's commercial production (Ragsdale 2008). Acetogens, with methanogens and sulfate reducers commonly use the Western branch of the pathway (Ragsdale 2008).

VLB-1 contains all genes for the metabolic conversion of pyruvate to CoA. Among the enzymes used, pyruvate ferredoxin reductase catalyzes the oxidative decarboxylation of pyruvate (Ragsdale 2008). The carbon dioxide electrons are then converted to carbon monoxide by carbon monoxide dehydrogenase (Ragsdale 2008). Carbon monoxide dehydrogenase commonly pairs with acetyl CoA synthase, in which the electrons released from carbon monoxide transfer to a mediator, which then pair to reduce nicotinamide adenine dinucleotide phosphate (NADPH) (Ragsdale 2008). .

Acetyl CoA then steps in to catalyze the condensation of carbon monoxide, CoA, and a methyl group of a methylate corrinoid iron-sulfur protein to create acetyl-CoA, which is then used a source of energy for the bacterium (Ragsdale 2008). However, one gene in this pathway is missing: K22015 or formate dehydrogenase, which is paired with iron hydrogenase HydA2 and the FeS- containing electron transfer protein. The supposed absence of this gene could be attributed to the less accurate nature of the draft genome. VLB-1 does contain the genes for the carbon monoxide dehydrogenase and acetyl CoA synthetase machinery: acsA/acsB, which are invaluable tools in this pathway. The unique trait of this pathway is the changed perception of the roles of metal ions in bacterial use (Ragsdale 2008). This study, chemical framework, and biophysical traits will likely also be applied to undiscovered metal enzyme systems. Strain VLB-1 could use the Wood-Ljungdahl pathway, for carbon fixation, concluding that this organism produces acetic acid as its main metabolic output, as do most acetogens

VLB-1 likely fixes most of its biosynthetic carbon via the phosphate acetyltransferase-acetate kinase pathway. The phosphate acetyltransferase-acetate kinase pathway is used to fix acetyl-CoA to acetate, the first step being acetyl-CoA and orthophosphate reacting due to the enzyme acetyl-CoA: phosphate acetyltransferase to create CoA and acetyl phosphate. The second step of this reaction involves the enzyme acetate kinase and input of energy to create acetate.

**Cysteine Biosynthesis**

VLB-1 also metabolizes sulfur via one pathway: cysteine biosynthesis. Sulfur incorporation is critical for the synthesis of methionine, vitamins, and iron-sulfur clusters (Wirtz & Hell 2003). Glycine, serine, and threonine, and methane are degraded to L-serine, which is

then either degraded into sulfide or L-cysteine (Wirtz & Hell 2003). This is also representative of its ability to metabolize certain amino acids via the Stickland reaction. Cysteine desulfurase is the crucial enzyme that causes the conversion of L-cysteine to L-alanine and sulfane sulfur via a protein-bound intermediate on a cysteine residue (Wirtz & Hell 2003). Cysteine is the source of sulfur for many catalytic activities. The production of cysteine is one of the only ways for sulfur to be introduced into cell metabolism. Along with many other pathways discussed here, the incorporation of sulfur is not well known (Wirtz & Hell 2003). Cysteine biosynthesis starts with the formation of O-acetylserine, catalyzed by serine acetyltransferase, which is regulated by a negative feedback loop with cysteine, resulting in the controlled regulation of cysteine in biological production. A free sulfide is added by catalysis of O-acetylserine lyase to O-acetylserine to produce L-Cysteine and acetate. Production of cysteine is regulated by intrinsic means, such as the cys-regulon, in addition to the possible cytotoxicity induced by large amounts (Wirtz & Hell 2003).

## Conclusion

*Acetoanaerobium sp.* strain VLB-1 metabolically resembles *A. sticklandii* and *A. notarae* closely with the metabolic potential for autotrophy or acetogenesis and amino acid fermentation via the Wood-Ljungdahl pathway and the Stickland reaction respectively. The Wood-Ljungdahl pathway is environmentally crucial in the balance between acetogenesis and methanogenesis, with methanogens acting as hydrogen sinks. Due to the unique environment this organism was found in, understanding elemental cycling and metabolism is crucial to understanding its role in the preservation in the Nebraska Saline Wetlands. The organism is known for its distinct pathways in amino acid degradation and acetogenesis. In the presence of methanogens,

acetogens create acetic acid while still keeping the alkaline environment of these wetlands intact. With an excess of acetogen metabolism comes the opportunity for microbial-induced corrosion of the environment. In the presence of methanogens, acetogenesis can be regulated and the alkaline environment is preserved (Palacios *et al.* 2021). Understanding the balance and the unique traits that these organisms have can help in understanding how to protect the valuable but sensitive ecosystem in the wetlands and prevent its further destruction by agricultural and livestock practices, as well as the further urban development around the Salt Creek area in Lincoln. In keeping with environmental practices, the biotechnological poterntial of these organisms could be used to generate acetic acid and possibly diminish methane production affecting climate change and microbial-induced corrosion.

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