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Identification of an anaerobic bacterium which reduces perchlorate and chlorate as *Wolinella succinogenes*

W Wallace[1], T Ward[2], A Breen[2] and H Attaway[3]

[1]*Applied Research Associates, Inc, Gulf Coast Division, PO Box 40128, Building 1142, Mississippi Rd, Tyndall AFB, FL 32403;* [2]*Department of Molecular Genetics, College of Medicine, University of Cincinnati, Cincinnati, OH 45267;* [3]*Man Tech Environmental Technology, Inc, Armstrong Laboratory/EQ, Tyndall Air Force Base, FL 32403-6001, USA*

A bacterium coded as strain HAP-1 was isolated from a municipal anaerobic digestor for its ability to reduce > 7000 ppm perchlorate in wastewaters. The organism is capable of the dissimilatory reduction of perchlorate or chlorate to chloride for energy and growth. It is a Gram-negative, non-sporeforming, obligately anaerobic, motile, thin rod. Antibiotic resistance, utilization of carbon substrates and utilization of electron acceptors by bacterium HAP-1 were similar to *Wolinella succinogenes*. The organism's 16S rRNA sequence was 0.75% different from that of the type strain of *W. succinogenes*. The fatty acid compositions of the two organisms are very similar. The morphological, physiological and 16S rRNA sequence data indicated that bacterium HAP-1 is a strain of *W. succinogenes* that can utilize perchlorate or chlorate as a terminal electron acceptor.

Keywords: bioreduction; perchlorate; chlorate: *Wolinella succinogenes*

Introduction

Perchlorate and chlorate salts are widely used by the chemical, aerospace and defense industries as oxidizers in propellant, explosives, and pyrotechnics [28]. Arms reduction treaties and aging missile inventories of the United States Air Force are requiring development of more efficient and environmentally acceptable methods of rocket propellant disposal. Hazard Class 1.1 rocket motor propellants typically contain 14% ammonium perchlorate, 14% HMX, 30% nitroglycerin, 20% aluminum fuel, and 22% nitrocellulose binder [28]. Hazard Class 1.3 rocket motor propellants typically contain 70% ammonium perchlorate, 17% aluminum fuel, and 13% PBAN binder. Current technology removes propellant from the motor casing with high pressure water. This method generates a 10–13% ammonium perchlorate waste stream which can be diluted further and treated biologically with a bacterium coded as strain HAP-1 to produce an innocuous chloride salt wastewater [2,3].

Some heterotrophic bacteria can reduce perchlorate and chlorate [10,11,24]. However, most studies suggest that perchlorate and chlorate reduction is catalyzed by nitrate reductase in these organisms. The amount of perchlorate reduced by the organisms was minimal and higher levels of perchlorate and chlorate were toxic. *Vibrio dechloraticans* Cuznesove B-1168 was reported to reduce perchlorate at concentrations up to 300 ppm in a continuous process [16,22]. Similar results were shown for chlorate with an unidentified helical bacterium [17]. Bacterium HAP-1 was shown to reduce perchlorate at concentrations as high as 7750 ppm to chlorate followed by stoichiometric production of chloride. We have demonstrated that the organism reduces perchlorate and chlorate irrespective of the presence of nitrate suggesting this is not a nitrate reductase system [29]. Inhibition, pH and mixed substrate studies demonstrate enzyme activity which preferentially reduces perchlorate prior to reducing nitrate [29].

Due to the high rate of perchlorate reduction by bacterium HAP-1, it has been incorporated into a treatment process for the removal of perchlorate from rocket motor wash water [2,3]. A better understanding of the organism is required to optimize reactor conditions. Biochemical, morphological, and physiological comparisons have been traditionally used to identify similar organisms. Recently, nucleic acid sequence comparisons have been shown to be an accurate, rapid and fundamental technology for identification of microorganisms [5,20,21]. Therefore, 16S rRNA sequencing was undertaken to identify bacterium HAP-1. This paper describes the genus and species characterization of the organism as *Wolinella succinogenes*.

Materials and methods

Bacterial isolation and maintenance

Bacterium HAP-1 was isolated from an anaerobic sewage enrichment culture [1] on the following isolation medium (g L^{-1}): K_2HPO_4, 6; Na_2HPO_4, 2; NH_4ClO_4 1.17; yeast extract (Difco Laboratories, Detroit, MI, USA), 10; Peptone (Difco), 10; resazurin, 0.001; and agar (Difco). The pH was adjusted to 7.5 with 1 N HCl. To ensure anaerobic conditions, the medium was flushed with nitrogen until the resazurin was reduced. Agar plates were poured, streaked with the enrichment culture, and incubated in an anaerobic chamber (Coy Laboratories, Midland, MI, USA) under an atmosphere of N_2: CO_2: H_2 (80 : 10 : 10). Plates were incubated at 37°C and within 48 h produced an assortment of colony types including transparent mucoid colonies with pink halos. This pink colony color was the result of the oxidation of resazurin in conjunction with the reduction of perchlorate and is a unique identifying characteristic of the

Correspondence: W Wallace, Applied Research Associates, Inc, PO Box 40128, Tyndall AFB, FL 32403-6001, USA
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organism. The pink color allowed easy colony differentiation from non-perchlorate reducing organisms present in the enrichment culture. A colony was picked and the organism was maintained on the above medium in pure form.

Wolinella succinogenes ATCC 29543 (previously named *Vibrio succinogenes* [27]) was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained on fumarate/formate medium containing: sodium formate 5 g L^{-1}; disodium fumarate 5 g L^{-1}; yeast extract (Difco) 10 g L^{-1}; peptone (Difco) 10 g L^{-1}; K_2HPO_4 6 g L^{-1}; Na_2HPO_4 2 g L^{-1}; resazurin, 0.001 g L^{-1}; and Bacto agar (Difco) 15 g L^{-1}, pH adjusted to 7.2 with 1 N HCl.

Fatty acid composition

Cellular fatty acids of bacterium HAP-1 and *Wolinella succinogenes* ATCC 29543 were determined and compared by workers at the American Type Culture Collection.

Growth substrates and electron acceptors

Growth substrates were tested by supplements to the following medium (g L^{-1}): yeast extract (Difco), 0.5; K_2HPO_4, 6; Na_2HPO_4, 2; resazurin, 0.001. The pH was adjusted to 7.2 with 1 N HCl. All substrates were added aseptically at a concentration of 5 g L^{-1} with the exception of ethanol, methanol and 1-propanol which were added at 0.5 g L^{-1}. Cultures were incubated in an anaerobic chamber at 37°C. Samples were observed after 24 and 48 h. Turbidity changes were measured spectrophotometrically at 600 nm.

Electron acceptors were determined by adding them (1 g L^{-1}) to the isolation medium without NH_4ClO_4 and agar. Cultures and sampling conditions were the same as in studies on growth substrates.

Antibiotic resistance

Bacterium HAP-1 and *W. succinogenes* were individually grown to mid-log phase and spread separately (0.1 ml) on fumarate/formate agar. Antibiotic-impregnated discs (BBL Microbiology Systems, Cockeysville, MD, USA) were placed on the agar surface immediately after inoculation. Cells were incubated anaerobically and examined after 24 and 48 h for inhibition of growth.

DNA isolation

Large scale genomic DNA isolation was performed according to Sambrook *et al* [23] with the following modifications. DNA spooled from the isopropanol precipitation was extracted four times with phenol/chloroform to remove proteins. The additional extractions removed sufficient protein to omit the cesium chloride centrifugation step. RNAse (Gibco BRL, Gaithersburg, MD, USA) was added to the DNA preparation at 10 μg ml^{-1}. The DNA was measured spectrophotometrically and protein contamination determined by the 260/280 ratio [23].

Polymerase chain reaction

The 27–321 base pair region of the 16S rRNA was amplified using the polymerase chain reaction (PCR) with universal primers [15] and standard reagents (Perkin Elmer Corp, Norwalk, CT, USA). The amplification protocol was as follows using a thermocycler (MJ Research, Watertown, MA, USA): initial denaturation 95°C 1 min, 30 cycles 94°C 1 min, 60°C 1.5 min, 72°C 1.5 min. Amplification products were run on low melt agarose and purified with Magic™ PCR Preps (Promega Corp, Madison, WI, USA).

DNA sequencing

Sequencing was conducted using an Exo(–) pfu Cyclist DNA sequencing kit (Stratagene, Lajolla, CA, USA) and BioRad sequencing apparatus (Hercules, CA, USA). Primers were end labeled using polynucleotide kinase (New England Biolabs, Boston, MA, USA) and ^{32}P-dATP (DuPont Corp, Boston, MA, USA) labeled in the gamma position. Sequencing reactions were loaded on 6.0% polyacrylamide gels. Both forward and reverse DNA strands were sequenced. Gels were read manually.

Sequence analysis

The sequence of DNA from bacterium HAP-1 was aligned with homologous regions of sequence obtained from the NCBI database (Bethesda, MD, USA) for *Wolinella succinogenes*, *Heliobacter pylori*, *Campylobacter jejuni* and *Campylobacter rectus*. The sequence alignment method [18] was performed using the Clustal V software package [14]. Aligned sequences were manually inspected and regions of ambiguity were discarded. Parsimony analysis (PAUP, v 3.0s [25]) was performed with transversions being weighed twice as much as transitions, using the exhaustive search option. Accelerated transformation [8,26] and delayed transformation [26] character state change optimizations were performed. Bootstrap resampling [7,9] with 2000 replications [13] was done to determine the stability of tree topologies. Mean percent pairwise divergences were also calculated (PAUP, v 3.0s [25]).

Results

Morphology

Bacterium HAP-1 is a Gram-negative rod, 0.5 μm wide and 2–8 μm in length. Cells are motile and do not form spores. Cells grow well on agar medium with colonies appearing pale yellow, circular and mucoid. The organism and *W. succinogenes* colonies form similar colonies when grown on fumarate/formate medium.

Fatty acid composition

The *W. succinogenes* strain was not included in the library database, but comparison of the gas chromatographs indicate that bacterium HAP-1 and *W. succinogenes* ATCC 29543 strongly resemble each other and have the same fatty acid composition.

Physiology

Bacterium HAP-1 is obligately anaerobic and catalase-negative. The temperature range for growth was 20 to 45°C with an optimum at 40°C. The pH range for growth was 6.5 to 8.0 with an optimum of 7.1. Antibiotic susceptibility profiles for bacterium HAP-1 and *W. succinogenes* matched (Table 1) although there were some slight variations in inhibition zone sizes. The absence of detectable plasmids suggests that resistance determinants are probably chromoso-

Table 1 Antibiotic susceptibility

	W. succinogenes HAP-1	*W. succinogenes* ATCC 29543
Ampicillin	s[a]	s
Carbenicillin	s	s
Chloramphenicol	s	s
Erythromycin	s	s
Kanamycin	s	s
Neomycin	r[b]	r
Novobiocin	r	s
Penicillin	r	r
Rifampicin	r	r
Streptomycin	s	s
Tetracycline	s	s
Vancomycin	r	r

[a]s = sensitive (inhibition zone > 8 mm).
[b]r = resistant (no inhibition zone).

mally encoded (data not shown). The nutritional characteristics of bacterium HAP-1 (Table 2) are similar to those of *W. succinogenes* [19,27,30] including the use of fumarate as both a carbon source and electron acceptor. The ability of malate and aspartate to serve as carbon sources and electron acceptors is probably due to their bioconversion to fumarate as observed in *W. succinogenes* [6]. Hydrogen or formate was required as electron donor for growth of the organism.

W. succinogenes used both nitrate and nitrite as electron acceptors with the final product being ammonia [4]. Bacterium HAP-1 reduced nitrate solely to nitrite and produced neither ammonia nor nitrogen gas. Perchlorate and chlorate were readily reduced to chloride and along with nitrate served as the only inorganic electron acceptors identified for the organism (Table 3).

Table 2 Substrate utilization

	Growth with:			
	H_2[a]	H_2 and ClO_4[b]	H_2	H_2 and ClO_4
	W. succinogenes HAP-1		*W. succinogenes* ATCC 29543	
Pyruvate	–	+	–	–
Succinate	–	+	–	–
Acetate	–	+	–	–
Aspartate	+	+	+	+
Fumarate	+	+	+	+
Malate	+	+	+	+
Whey powder	–	+	–	–
Peptone	–	+	–	–
Yeast extract	–	+	–	–
Brewers yeast	–	+	–	–
Casamino acids	–	+	–	–
Cottonseed protein	–	+	–	–

[a]Hydrogen was provided from gas mix in an anaerobic chamber.
[b]Sodium perchlorate was added to media at 1000 ppm.
Glucose, fructose, galactose, lactose, sucrose, butyrate, citrate, formate, propionate, benzoate, ethanol, methanol, 1-propanol and starch did not support growth for either *W. succinogenes* HAP-1 or *W. succinogenes* ATCC 29543.

Table 3 Inorganic electron acceptors examined

Compounds tested	*W. succinogenes* HAP-1	*W. succinogenes* ATCC 29543
	Growth	
Perchlorate	+	–
Chlorate	+	–
Chlorite	–	–
Nitrate	+	+
Nitrite	–	+

Sulfate, sulfite, thiosulfate, iron oxide, manganese dioxide and magnesium oxide were not used as electron acceptors by either *W. succinogenes* HAP-1 or *W. succinogenes* ATCC 29543.

Ribosomal RNA sequence

Based on the physiological data, the sequences from organisms in the delta-epsilon subdivision of the proteobacteria including *W. succinogenes* were compared to sequences from bacterium HAP-1 [20]. The single most parsimonious tree was obtained by weighted parsimony analysis of the five sequences, with both accelerated and delayed transformation character state change optimization (Figure 1). This tree had a total of 146 mutations and the distribution of all other possible trees showed this topology to be stable. An additional 12 steps were required to get a different tree topology. Bacterium HAP-1 was shown to be nearly identical to *W. succinogenes* as they were separated by a combined branch length of only two changes.

Percent divergence was calculated for all pairwise comparisons of the taxa in the parsimony analysis. In addition, percent divergences were calculated for pairwise comparisons of the five taxa and *Escherichia coli* in order to provide a recognizable benchmark (Table 4). The sequence divergence between bacterium HAP-1 and *W. succinogenes* was only 0.75%. All other values exceeded 15% (range = 15.5–80.5%).

Discussion

Physiological and morphological characteristics demonstrated that bacterium HAP-1 belongs to genus and species *W. succinogenes*. Fatty acid composition, antibiotic susceptibility profiles, and electron acceptors were all in agreement between the two organisms.

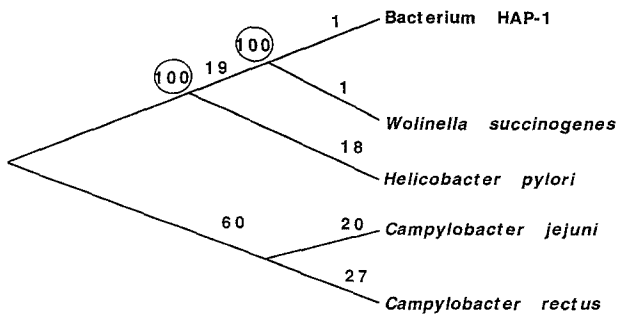

Figure 1 Single most parsimonious tree (146 mutations) found in exhaustive weighted parsimony search. Branch lengths are shown at the internodes and bootstrap replication scores are shown as the circled values at the nodes.

Table 4 Pairwise distances between taxa. Absolute distances are shown below the diagonal and mean distances (adjusted for missing data) are shown above the diagonal

	1	2	3	4	5	6
W. succinogenes HAP-1 (1)	–	0.0075	0.161	0.340	0.338	0.772
W. succinogenes (2)	2	–	0.155	0.337	0.332	0.770
H. pylori (3)	38	37	–	0.359	0.369	0.795
C. jejuni (4)	90	90	85	–	0.165	0.794
C. rectus (5)	89	88	87	46	–	0.805
E. coli (6)	173	174	159	185	186	–

Weighted parsimony analysis grouped bacterium HAP-1 with *W. succinogenes*. This same tree topology was found when the sequence was analyzed with equal weighting of transitions and transversions, and when transversions alone were considered. This result was further supported by a 2000-replication bootstrap resampling score of 100% and by the pairwise comparison value showing only 0.75% sequence divergence between bacterium HAP-1 and *W. succinogenes*. The DNA sequence for the 16S rRNA gene of the organism HAP-1 so closely matched the published sequence of *W. succinogenes* we felt it was unnecessary to sequence the entire 16S rRNA gene. This decision was further supported by the pairwise comparison data which showed a large amount of divergence between relatively closely related taxa.

The preponderance of data indicates that strain HAP-1 belongs to the genus and species *Wolinella succinogenes*, however the ability to utilize perchlorate and chlorate as terminal electron acceptors has not been reported for *Wolinella* species previously. We have examined *W. succinogenes* ATCC 29543 and determined it cannot reduce perchlorate or chlorate. Therefore, we suggest that bacterium HAP-1 has acquired the ability to reduce perchlorate and chlorate via a genetic event which allows this new phenotype. Based on the morphology, physiology and 16S rRNA sequence information we describe bacterium HAP-1 as a strain of *W. succinogenes*.

The incorporation of plasmid DNA provides a common method for phenotype alterations in bacteria. Attempts to identify the genetic modification in *W. succinogenes* HAP-1 which allows the reduction of perchlorate and chlorate have focused on plasmid isolation. To date we have been unable to isolate plasmid(s) with either the alkaline lysis [23] or Hansen–Olsen [12] procedures. Current studies are focusing on cloning the genes responsible for perchlorate reduction and hopefully providing information on the origin of perchlorate and chlorate reduction in this organism.

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