ISOLATION AND CHARACTERIZATION OF A NOVEL As(V) REDUCING BACTERIUM: IMPLICATIONS FOR ARSENIC MOBILIZATION AND THE GENUS *DESULFITOBACTERIUM*

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Allison Niggemyer

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Major Professor: R. Frank Rosenzweig, Ph.D.

AUTHORIZATION TO SUBMIT

THESIS

This thesis of Allison M. Niggemyer, submitted for the degree of Master of Science with a major in Environmental Science and titled "Isolation and characterization of a novel As(V) reducing bacterium: Implications for arsenic mobilization and the genus *Desulfitobacterium*," has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor _____ Date May 8, 2001
R. Frank Rosenzweig

Committee Members _____ Date 5-8-01
Donald Crawford

_____ Date 5-8-01
Joel Hamilton

_____ Date 5/8/01
Matthew Morra

Department Administrator _____ Date 5/14/01
Margrit von Braun

Dean of College of Letters and Science _____ Date 5-16-01
Kurt Olsson

Final Approval and Acceptance by the College of Graduate Studies

_____ Date 5/16/2001
Charles R. Hatch

ABSTRACT

Dissimilatory arsenate reducing bacteria have been implicated in the mobilization of arsenic from arsenic-enriched sediments. An As(V)-reducing bacterium, designated strain GBFH, was isolated from arsenic-contaminated sediments of Lake Coeur d'Alene, Idaho. Strain GBFH is capable of reducing As(V), Fe(III), Se(VI), Mn(IV) and a variety of oxidized sulfur species. 16s rDNA sequence comparisons reveal that strain GBFH is closely related to *Desulfitobacterium hafniense* and *Desulfitobacterium frappieri*. Comparative physiological studies illustrate that *Desulfitobacterium hafniense* and *Desulfitobacterium frappieri*, known for reductively dechlorinating chlorophenols, are also capable of toxic metal(loid) respiration. DNA:DNA hybridization and comparative physiological studies suggest that *D. hafniense D. frappieri*, and strain GBFH should be united into one species. The isolation of an Fe(III) and As(V)-reducing bacterium from Lake Coeur d'Alene suggests a mechanism for arsenic mobilization in these contaminated sediments while the discovery of metal(loid) respiration in the genus *Desulfitobacterium* has implications for environments co-contaminated with arsenous and chlorophenolic compounds.

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Introduction

Arsenic is the 20th most abundant element in the earth's crust (62) and is widely distributed throughout nature as a result of weathering, dissolution, fire, volcanic activity, and anthropogenic input (13). Anthropogenic inputs are caused by the use of arsenic in pesticides, herbicides, wood preservatives and dye stuffs as well as by production of arsenic-containing wastes during smelting and mining operations (62). The occurrence and fate of arsenic in aquatic environments is of particular interest due to its toxic effects on microbes, plants, and animals (62). The toxicity of arsenic is dependent upon the oxidation state, with arsenite (As(III)) being more toxic than arsenate (As(V)) (84, 85). As(V) is a structural analogue of phosphate and can substitute for inorganic phosphate during ATP synthesis. The substitution of arsenate for phosphate results in an unstable arsenate ester that is spontaneously and rapidly hydrolyzed, representing a loss of potentially energy-yielding ATP. As(III) reacts with the sulfhydryl groups of cellular enzymes, rendering them dysfunctional (85). In arsenic-enriched environments, there is concern about the mobilization and transport of this toxic element to groundwater and drinking water supplies. Recent human exposure to arsenic in drinking water wells in Bangladesh has resulted in 7,600 cases of arsenicosis, with an estimated 24 million people exposed to arsenic through contaminated water wells (1). This incident serves as an unfortunate reminder of the toxic consequences of arsenic mobilization (66). Factors controlling the solubility and mobility of arsenic must be clearly understood in order to predict and prevent the transport of arsenic into aquatic systems that supply drinking water.

Arsenic exists in the +5, +3, 0 and –3 oxidation states in aquatic systems, but at the Eh and pH of most natural waters As(V) and As(III) are the only stable dissolved arsenic species (22). Figure 1, a Porbaix stability diagram, illustrates the effect of pH and Eh on the degree of protonation and the redox state of dissolved arsenic. The oxidation state of arsenic influences not only toxicity, but also the mobility of arsenic in aqueous environments (13, 40). As(V) strongly adsorbs to the surface of iron oxyhydroxide minerals (17, 21, 57, 68) and this sorption is frequently used to explain the sequestration of arsenic in aquatic sediments (2, 8, 17, 29, 41, 56). The occurrence of arsenic in sediment pore waters, anoxic

groundwaters and hypolimnetic waters is often attributed to the reductive dissolution of solid phase iron oxyhydroxides and subsequent arsenic release to the aqueous phase (2, 5, 8, 17, 58, 66). As(III) is commonly released from anoxic sediments following reductive dissolution of iron oxyhydroxides (2, 8, 57, 58). This observation has been attributed to the reduced ability of As(III) to sorb iron oxyhydroxides (17, 68) and is consistent with the idea that As(III) is more mobile than As(V) in aquatic environments (40). Recent evidence, however, suggests that As(III) exhibits strong sorption to iron oxyhydroxides and that at circumneutral pH, As(III), in fact, may sorb more strongly to such minerals than As(V) (55, 69). Discrepancies between earlier studies (68) and later studies (55, 69) with respect to the sorption characteristics of arsenite on ferrihydrite have been attributed to differing experimental conditions, including iron to arsenic ratios, ionic strength, and pH maintenance throughout the experiment (69).

In anoxic waters and sediments, the reductive dissolution of iron oxyhydroxides is principally catalyzed by the action of dissimilatory iron reducing bacteria (DIRB) (45). Our laboratory has recently demonstrated that an iron-reducing bacterium, *Shewanella alga* strain BrY, is capable of mobilizing arsenate from the solid-phase ferric arsenate mineral scorodite to the aqueous-phase by the reduction of Fe(III) to Fe(II) (14). It had been previously postulated that the elevated aqueous As(III) concentrations observed under anaerobic conditions (2, 8, 57, 58) resulted from As(V) release following DIRB-mediated dissolution of iron oxyhydroxides and subsequent conversion of As(V) to As(III) (14, 71). While As(V) reduction is not a prerequisite to arsenic solubilization from $FeO(OH)_x$, it is also possible that direct As(V) reduction plays a role in mobilizing arsenic sorbed to iron minerals.

Reduction of As(V) to As(III) occurs both chemically and biotically. Chemical reduction of As(V) to As(III) by sulfide occurs very slowly at circumneutral pH (11, 64, 70). One research group has reported a second-order rate constant for reduction of As(V) by sulfide of $K_{pH6.8} = 1.04\ M^{-1}h^{-1}$ at sulfide:arsenate molar ratios ranging from 0.06-0.6 (64). Another group observed that even at sulfide:arsenate ratios of 100:1, essentially no chemical arsenate reduction occurs at pH 7 over 7 days (70). Although chemically mediated As(V) reduction by sulfide occurs slowly at pH 7, it proceeds much more rapidly under acidic conditions (11,

70). As(V) reduction by sulfide at pH 4 occurs 300 times more rapidly than at pH 7, with a second-order rate constant of $K_{pH4} = 320\ M^{-1}h^{-1}$ (70). Additionally, at sulfide:arsenate ratios of 10^4:1, As(V) was nearly completely converted to As(III) at pH 4 over a 33 hour interval; only 10% was reduced over the same time period at pH 7 (11). In light of the slow kinetics of As(V) reduction by sulfide in the pH range of most natural waters, researchers have begun to investigate the importance of microbially catalyzed reduction of As(V) to As(III) as a potential mechanism for arsenic mobilization from iron oxyhydroxide surfaces (4, 18, 24, 29).

Microbial reduction of As(V) occurs by two metabolically distinct mechanisms: reductive detoxification and dissimilatory arsenic reduction. Reductive detoxification is a mechanism which couples the reduction of As(V) to As(III) with export of As(III) from the cell. The proteins responsible for these activities are encoded within the *ars* operon; energy does not appear to be conserved for growth by the reduction detoxification of As(V) (87). While many bacteria, fungi and microbial consortia have been observed to reduce aqueous As(V) to As(III) (13), to date, only one study specifically investigates the role microbial As(V) detoxification plays in arsenic cycling in aquatic systems (42). This study utilizes a glucose-fermenting organism that reduces aqueous As(V) by reductive detoxification. Although this organism contributes to reduction of soluble As(V), it is neither capable of reducing As(V) sorbed to mineral phases nor capable of reducing the Fe(III) of iron oxyhydroxides and causing subsequent increases in aqueous As(V) concentrations. The authors conclude that reductive detoxification is unlikely to play a major role in the mobilization of As(V) sorbed to iron oxyhydroxides (34).

Dissimilatory arsenic reducing bacteria (DAsRB) are organisms that utilize As(V) as a terminal electron acceptor (TEA). DAsRB couple the reduction of As(V) to the oxidation of an organic compound or H_2 and conserve energy for growth in the process. In contrast to investigations involving reductively detoxifying bacteria (42), studies of dissimilatory arsenic reduction suggest that DAsRB very likely play a role in mobilizing arsenic from iron oxyhydroxides (4, 18, 29, 88). In one such study (18) researchers amended marine and fresh water sediments with 5-10 mM As(V) and found that under anoxic conditions As(V) was

reduced to As(III). Addition of respiratory inhibitors and uncouplers to these microcosms prevented the reduction of As(V) to As(III), indicating that the reduction was coupled to respiration rather than reductive detoxification. This study provided the first experimental evidence that dissimilatory As(V) reduction may help explain elevated aqueous As(III) concentrations observed in anoxic sediments. Additionally, our laboratory has recently shown that heavy metal(loid)-contaminated sediments of Lake Coeur D'Alene, ID, are capable of biotic As(V) reduction (29). In this study, live sediment microcosms amended with aqueous 10 mM As(V) reduced 21% of the As(V) to As(III), while formalin-killed sediment microcosms reduced only 5% of the added As(V) to As(III). Sediment microcosms which received additional organic acids reduced just over 50% of the As(V) added. Additionally, most probable number analysis of Lake Coeur d'Alene sediments revealed the presence of As(V) reducers at 10^3 to 10^5 cells per g wet weight sediment. These data indicate that active DAsRB are present in Lake Coeur d'Alene, possibly in sufficient numbers to play an important role in arsenic cycling, and that biotic As(V) reduction is likely to contribute to diagenesis of arsenic in these contaminated sediments.

Two additional studies have investigated the contributions of specific DAsRB to arsenic cycling in sediment systems. One research group inoculated sterile sediments with a pure culture of the dissimilatory arsenic-reducing bacteria *Sulfurospirillum arsenophilum* strain MIT-13 (4). A pure culture of MIT-13 was capable of increasing the aqueous concentration of As(III) when provided with ferrous arsenate mineral or contaminated sediments. This study provided the first experimental evidence that a specific dissimilatory As(V) reducing bacterium can mobilizate arsenic from the solid phase to the aqueous phase. A study using DAsRB *Sulfurospirillum barnesii* strain SES-3 (88), illustrated that As(V) reduction occurs when As(V) is coprecipitated with or adsorbed to poorly crystalline iron oxyhydroxide minerals. In this investigation, more than half of the resulting As(III) remained sorbed to the solid phase iron, while less than half the total As(III) was released into aqueous solution. This study illustrates that DAsRB can reduce the sorbed and co-precipitated As(V), which are surrogates for forms of As(V) likely found in natural environments. It is clear that dissimilatory As(V) reducing bacteria are capable of reducing As(V) in either aqueous or

solid phase and that this reduction can result in mobilization of As(III) from the solid to the aqueous phase.

Currently, eight dissimilatory As(V) reducing bacteria have been isolated in pure culture (3, 6, 34, 44, 51, 52, 64). Detailed physiological characterization of these bacteria is essential for understanding the role DAsRB play in natural environments. Metabolic versatility, including the ability to utilize various electron donors and electron acceptors, the optimum temperature and pH for growth, and the rate of growth will determine the extent to which DAsRB are capable of mediating environmentally significant arsenic transformations in specific environments. Table 1 summarizes relevant physiological characteristics of the eight known DAsRB and reveals that the physiological capabilities of these organisms vary widely. As(V)-reducing bacteria can obtain energy from the oxidation of a variety of carbon compounds including organic acids, sugars and alcohols. Additionally, oxidation of H_2 provides energy for chemolithoautotrophic growth coupled to As(V) reduction for two of the eight DAsRB. Arsenate reducing bacteria are also capable of coupling the oxidation of organic matter to the reduction of a range of oxidized sulfur, selenium, and nitrogen species, as well as iron(III), manganese(IV) and fumarate. Individual DAsRB exhibit unique electron donor and electron acceptor combinations. For example, BAL-1 utilizes only As(V), NO_3^-, NO_2^- as TEAs, but it is capable of oxidizing a number of different carbon compounds, while MLS10 can oxidize relatively few carbon compounds but can use a wider range of TEAs. Additionally, As(V) reducers are capable of growth at extreme temperatures and pH. Strains E1H and MLS10 are alkaliphilic and can grow at pH as high as 10 while strain PZ6 grows optimally at 95°C. Considered as a group, DAsRB are metabolically versatile and can be expected to flourish under a wide range of environmental conditions.

Considering their physiological diversity, it is perhaps not surprising that DAsRB are phylogenetically diverse (Table 2). The eight known As(V) reducers include members of the Bacteria and Archaea. Within the Bacteria, several phylogenetic classes are represented including low GC gram positive and the δ and ε-subclass of the proteobacteria. *Chrysiogenes arsenatis* provides an example of a deep-branching lineage with no cultivated close relatives. The diverse phylogenetic affiliations and wide range of physiological

capabilities of DAsRB suggest that the capability to couple growth to As(V) reduction may be more widespread than originally estimated. If further investigation shows that this ability is in fact widespread among members of the Bacteria and Archaea and that such organisms are abundant and active, it follows that DAsRB may play a major role in mediating the reductive portion of the arsenic cycle and for the mobilizing arsenic in aquatic environments.

Isolation and characterization of a novel As(V) reducing bacterium: Implications for arsenic mobilization and the genus *Desulfitobacterium*

INTRODUCTION

Coeur d'Alene Lake (CDAL) is the second largest lake in Idaho and its waters feed six community water supplies (86). As a result of a century of mining along the Coeur d'Alene River, one of the two rivers feeding CDAL, the sediments of the lake are highly enriched in trace metals including Ag, As, Cu, Cd, Hg, Pb, Sb and Zn (31). An estimated 75 million metric tons of trace element enriched sediments have been carried down the Coeur d'Alene River and deposited in the lakebed of CDAL (32). Extensive sampling of the lakebed reveals that 85% of the sediments are enriched in heavy metals and trace elements (32). Arsenic concentrations in CDAL sediments range from 3.5 to 845 ppm with a mean value of 103 ppm, while lead concentrations range from 12 to 275,000 ppm with an average concentration of 3200 ppm (32). The pore waters of these sediments are also enriched in metal(loid) contaminants, with the mean arsenic concentration in the interstitial waters measuring 160 μg/L and the mean lead concentration measuring 250 μg/L (29). Nevertheless, the concentrations of contaminating trace elements in CDAL surface waters comply with Federal Drinking Water Standards. Since the residents of Northern Idaho use these waters for recreation, for fishing and for their water supply (86), there is concern over the possibility that contaminants could be mobilized from the sediment to the water column. Clearly it is important to have an understanding of the physical and biological factors that dictate the fate and stability of metal(loid)s in this environment.

Iron is the dominant trace element contaminant in CDAL sediments, constituting approximately 10% of the sediments by dry weight (14, 28). We have observed that the maxima for both arsenic and iron in these sediments generally occur less than 15 cm below the sediment-water interface, in contrast with lead and zinc, whose maxima generally exceed 25 cm below the sediment-water interface (28). Moreover, the distribution of Fe and As is strongly correlated ($r = 0.82$), as is the distribution of lead and zinc ($r = 0.90$) (28). Other researchers have recently observed similar distributions of arsenic, lead and zinc in the

sediments of the lateral lakes adjacent to the CDAR (76). The observation that As accumulates near the redox boundary in CDAL sediments is consistent with similar observations in other sediment systems and water columns (24, 74, 75). Our laboratory has recently demonstrated that DIRB are both present and active in the CDAL sediments (15). We have also demonstrated that DIRB are capable of mobilizing arsenate from both synthetic scorodite and CDAL sediments (14). Altogether these observations suggest diagenesis of iron and arsenic. Specifically, we have hypothesized that arsenic is subject to post-depositional mobilization under conditions that favor reductive transformations of iron (14, 15). Under this hypothesis, desorbed arsenic diffuses down its concentration gradient towards the sediment-water interface until resorbed by freshly deposited iron oxyhydroxides in the oxidized surficial environment (14, 15).

Direct reductive transformations may also affect arsenic speciation and mobilization. Indeed, we have demonstrated that the sediments of CDAL support biotic generation of As(III) (29). In this study, As(V) reduction was neither limited to nor exclusive to sulfate-reducing bacteria, indicating that multiple microbial taxa in CDAL are capable of As(V) reduction. Herein we report the enrichment, isolation and characterization of novel DAsRB from these arsenic-contaminated sediments. We report the discovery of an organism, designated strain GBFH, which is capable of coupling the oxidation of formate to the reduction of As(V), conserving energy for growth in the process. Strain GBFH clusters phylogenetically with members of the genus *Desulfitobacterium*. Comparative physiological studies with strain GBFH and its two closest relatives, *D. hafniense* and *D. frappieri*, reveal that all three organisms are capable of utilizing a range of metal(loid)s as TEAs, including As(V), Se(VI), Mn(IV) and Fe(III). Carbon source utilization profiles, TEA utilization profiles, and other physiological measurements such as growth rate and temperature optima also suggest that the three organisms are similar phenotypically. Lastly, molecular genetic analyses using 16s rDNA sequences and DNA:DNA hybridization reveal a degree of relatedness that supports union of these three organisms in one species, *D. hafniense*, resulting in emendation of the genus *Desulfitobacterium*.

The isolation of DAsRB from CDAL sediments is significant because it suggests an alternative mechanism by which arsenic can be mobilized from iron oxyhydroxides and because the product of this transformation (As(III)) is 60 times more toxic than the reactant (As(V)) (40). The isolation of strain GBFH confirms that organisms capable of both dissimilatory Fe(III) reduction (15) and dissimilatory As(V) reduction exist in this sediment system. Moreover, our study of this organism's use of terminal electron acceptors reveals that strain GBFH is capable of *both* dissimilatory As(V) and Fe(III) reduction. Strain GBFH is also the first example of an organism capable of utilizing formate as the carbon source and electron donor for As(V) reduction. Additionally, members of the genus *Desulfitobacterium* are known principally for their ability to reductively dechlorinate a range of chlorinated phenols and have not previously been reported to utilize toxic metal(loid)s as TEAs (7, 12, 72, 80). Herein we demonstrate that previously characterized *Desulfitobacterium* spp. are also capable of reducing metal(loid)s. This finding has important implications for the bioremediation of environments co-contaminated with arsenous and chlorophenolic wastes.

METHODS AND MATERIALS

Enrichment and isolation: Strain GBFH was isolated from an enrichment culture inoculated with sediment obtained from the Coeur d'Alene River delta, Lake Coeur d'Alene, Idaho. Sediments were collected using a gravity coring device (61) fitted with PVC pipe. Sediment cores were immediately capped with butyl rubber stoppers to maintain anoxic conditions and were stored at 4°C until return to the laboratory. In the laboratory, cores were maintained at 4°C under flowing nitrogen gas until processed. Cores were extruded and sub-sampled in a LabConco anaerobic chamber containing an atmosphere of N_2:CO_2:H_2 (75:15:10). 30 cm cores were split into two 15 cm sections and homogenized using sterile, anaerobic lake water. The resulting slurry served as inoculum for enrichment of As(V) reducing bacteria. 1 mL of sediment slurry was added to 9 mL of media and the enrichments were incubated at 25°C in the dark.

Strict anaerobic technique (60) was used in the preparation of media and manipulation of cultures. The media used for enrichment of strain GBFH contained the following (per L):

$NaHCO_3$ (2.5 g), NH_4Cl (1.5 g), KH_2PO_4 (0.6 g), KCl (0.1 g), Wolfe's vitamin solution (10 mL) and modified Wolfe's mineral solution (10 mL). Components of the vitamin and mineral solutions can be found in ATCC medium #1957. Salts, trace elements and vitamins were combined, heated, cooled under O_2-free N_2:CO_2:H_2 (75:15:10), dispensed into degassed 20-mL Balch tubes or 120 mL serum vials, sealed with butyl rubber stoppers and autoclaved. Cysteine-HCl (1 mM), formate (10 mM) and As(V) (10 mM) were added separately from sterile, anaerobic stocks. The pH of the complete media was 7.0.

Enrichments were analyzed for accumulation of As(III) (see analytical techniques) and observed microscopically. The enrichment culture chosen for continued investigation rapidly accumulated As(III) and consisted mostly of rods with few cocci. This enrichment culture was passaged seven times through terminal dilution series in liquid media, at which point the culture appeared to contain only one type of morphologically distinct bacterium, a long curved rod, designated strain GBFH. Strain GBFH was then passaged three times through agar shake tube dilutions (67). Media for the agar shake tubes contained the same components as the enrichment media, with the addition of 1% Bacto-agar (Difco) and 0.1 g/L yeast extract (Difco). Small, white, round colonies formed within one week and were picked using a flame-sterilized Pasteur pipet for transfer into the next dilution series. Microscopy confirmed that the bacteria comprising the colonies in the shake tubes possessed the same morphology as the bacteria observed in liquid media. Culture purity was determined by microscopy and DGGE. Strain GBFH will be deposited in the American Type Culture Collection.

Media, cultivation, and strains: The medium used for growth of strain GBFH during all physiological experiments differed in two ways from the enrichment media. Yeast extract (0.1 g/L) was added to the media and the headspace was changed to N_2:CO_2 (4:1), as hydrogen was found to be mildly inhibitory (see results - temperature optimum determination). Unless otherwise specified, this slightly modified medium was used for the maintenance of GBFH and all growth experiments following isolation. During growth experiments, strain GBFH was incubated in the dark at 37°C.

Desulfitobacterium dehalogenans JW/IU-DC1 (DSMZ 9161) (80), *D. hafniense* DCB-2 (DSMZ 10664) (12), and *D. frappieri* PCP-1 (DSMZ 12420) (7) were graciously donated by Erko Stackbrandt (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany)). *D. dehalogenans* was enriched from freshwater lake sediments, while *D. hafniense* and *D. frappieri* were enriched from sewage sludge (7, 12, 80). All three were originally cultured on the recommended DSMZ media. All three previously characterized *Desulfitobacterium* species were easily cultured on the media used for physiological experiments with GBFH, and therefore this media was used for all comparative growth experiments. Unless otherwise stated, all experiments were conducted at 37°C in the dark.

Electron Microscopy: Samples for scanning electron microscopy were pipetted onto a poly-lysine coated nucleopore filter and/or coverslip and fixed with 2.5% glutaraldehyde for 30 minutes. After fixation, samples were washed with 0.1M sodium cacodylate buffer 2 times. Samples were post-fixed with 1% buffered osmium tetroxide for 30 minutes and washed 2 times with buffer. After dehydration in a graded ethanol series, samples were dried with HMDS. Samples were mounted on aluminum stubs and viewed on a Hitachi S-4000 scanning electron microscope and digital images captured with SEMages software (Advanced Database Systems).

Samples for transmission electron microscopy were fixed with 2% glutaraldehyde and washed with 0.1M sodium cacodylate buffer 3 times. Samples were post-fixed with 1% buffered osmium tetroxide for 1 hour and washed several times with double distilled water. Samples were dehydrated in a graded ethanol series followed by acetone. Finally the samples were infiltrated in a graded epoxy resin and polymerized at 60°C for two days. Seventy nanometer sections were collected on 100-mesh copper grids and post stained with 2% uranyl acetate followed by Reynold's lead citrate. Sections were viewed in a Hitachi H-7000 transmission electron microscope and digital images captured with Gatan Digital Micrograph 3.3.1 (Gatan, Inc.). Samples for negative staining received no pretreatment and were stained for ten minutes using either 2% uranyl acetate or 2% Phosphotungstic acid (PTA).

PCR and DGGE (denaturing gradient gel electrophoresis): Each 50 μL PCR reaction contained the following (stock concentrations are in parenthesis): 5 μL 10X PCR buffer (GibcoBRL), 1.25 μL dNTPs (10 mM each) (GibcoBRL), 1 μL bovine serum albumin (20 mg/mL) (Boehringer-Mannheim), 3 μL $MgCl_2$ (50 mM) (GibcoBRL), 2 μL forward primer (12.5 μM) (GibcoBRL), 2 μL reverse primer (12.5 μM) (GibcoBRL), 0.25 2 μL Taq DNA polymerase (5U/μL) (GibcoBRL), 1 μL DNA template, and 33.5 μL water (HPLC grade, Aldrich). Whole cells of strain GBFH from liquid media were used as the DNA template. Additionally, cells of strain E4, an As(V) reducing culture which was enriched independently from Lake Coeur d'Alene using the same procedure and media used for the enrichment of strain GBFH, were also used as DNA template for PCR. The forward primer, EUB338F, contained a GC-rich clamp for DGGE (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGACTCCTACGGGAGGCAGC-3'). The reverse primer was EUB907R and contained no GC-clamp (5'-CCGTCAATTCMTTTRAGTTT-3'). Numbers associated with primers represent 16s rDNA gene position using *E. coli* numbering. PCR reactions began with an initial denaturation at 94°C for 4 minutes. This was followed by 35 cycles of 94°C (30 seconds) for denaturation, touchdown from 65°C to 55°C in 0.5°C units over 20 cycles (30 seconds) for primer annealing, and 72°C (30 seconds) for template extension. After 35 cycles, 3 minutes of 72°C extension was allowed for final product extension. Reaction products were visualized on 1.5% agarose gels prior to analysis by DGGE. DGGE analysis was performed using the D Gene System (Bio-Rad). Acrylamide gels contained a gradient of denaturants from 40 – 60%, with 100% denaturant defined as 7M urea and 40% formamide. Gels ran for 15 hours at 60°C and at 65V before being stained for 15 minutes in ethidium bromide solution (100 μg/L) and de-strained for 15 minutes in de-ionized water. Gels were visualized using UV light and photographed digitally using Molecular Analyst Software (Bio-Rad).

Oxidation of formate coupled to the reduction of As(V): Mid-log phase formate (10 mM) and As(V) (10 mM) grown cells of strain GBFH or *D. frappieri* were inoculated into fresh medium (10% inoculum) containing 10 mM formate and 10 mM As(V) and adjusted to pH 7.5. Triplicate cultures were inoculated for each of the following 5 experimental conditions:

(1) live cells inoculated into complete medium, (2) live cells inoculated into medium containing no formate, (3) live cells inoculated into media containing no As(V), (4) heat-killed cells added to complete media, and (5) uninoculated complete media. Culture tubes were subsampled for determination of cell number, As(V), As(III) and formate.

Determination of Temperature and pH Optima: For temperature and pH determinations, strain GBFH was grown in media containing 10 mM formate and 10 mM As(V). Cultures used for inoculum were also grown under these conditions. Temperatures tested included 4, 15, 25, 32, 37, 39, 42 and 45°C. Temperature optimum determinations were made at pH 7.0. For pH media, bicarbonate was eliminated and replaced with a buffer system consisting of 25 mM each Hepes, Pipes, Mes and Tris Base. The initial pH was set using sterile, anoxic 2N HCl or 2 N NaOH. pH values tested included 6.0, 6.5, 7.0, 7.5 and 8.0. Because As(V) respiration with formate as the carbon and electron source is proton consuming (see discussion), pH increased during growth. However, the pH increase during log phase was not more than 0.3 pH units. pH optimum determinations were made at 37°C. All experiments were carried out in triplicate. Growth rates were calculated using the linear portion of the semi-logarithmic plot of optical density (OD_{420nm}) versus time.

Heat-resistant spore formation: To test for the ability of strain GBFH to form heat resistant spores, triplicate cultures of GBFH were grown on 10 mM formate and 10 mM As(V). Cultures were grown until they reached stationary phase, and the presence of spores was confirmed by microscopy. Three spore-containing cultures were incubated at 85°C for 30 minutes, and then each culture was transferred to fresh media and incubated at 37°C. Growth was monitored daily by change in absorbance at 420 nm. As(III) was quantified after inoculation of cultures into fresh media and after 8 days of incubation at 37°C.

Carbon source and electron donor survey: Strain GBFH, *D. hafniense*, and *D. frappieri* were grown to mid-log phase on 10 mM lactate and 10 mM As(V). Each organism was inoculated onto each carbon source in triplicate using a 10% inoculum. Carbon sources were added from sterile, anaerobic stock solutions to yield concentrations given in Table 3. All carbon sources were tested with As(V) supplied as the terminal electron acceptor, except for

cultures grown under fermentative conditions which were given no As(V). Hydrogen was tested as a potential electron donor by the injection of 10 mL of hydrogen to the headspace of culture tubes using a sterile syringe. Cultures with hydrogen in the headspace were incubated with shaking 125 rpm. Growth was monitored daily by optical density measurements and As(III) was quantified once growth was completed. If growth occurred, the culture was passaged two additional times on the carbon source in question. Organisms were considered positive for carbon source utilization if an average of 2.5-3 doublings occurred during the third passage and if more than 80% of the supplied As(V) was respired to As(III) after the third passage. Controls were inoculated, but contained no carbon source.

Terminal electron acceptor (TEA) survey: To investigate the potential for strain GBFH, *D. hafniense*, and *D. frappieri* to utilize a variety of terminal electron acceptors, cells were pre-grown on either 10 mM lactate and 10 mM As(V) or fermentatively on 11.3 mM pyruvate. A 10% inoculum of log-phase cells was used to inoculate triplicate cultures for each electron acceptor tested. Freshly inoculated cultures contained approximately 10^7 cells per mL. Electron acceptors were tested with 10 mM lactate serving as the electron donor and carbon source. If growth occurred, the culture was passaged two additional times on the terminal electron acceptor in question. Organisms were considered positive for TEA utilization if an average of 2.5-3 doublings occurred during the third passage and if reduction of the TEA had occurred (see below) after the third passage. Control tubes were inoculated but contained no TEA.

Sulfate, sulfite, thiosulfate, nitrate, nitrite, fumarate, selenate, selenite, arsenate, arsenite, ferric pyrophosphate and 3-Cl-4-OHPA were added to media from sterile anaerobic stock solutions to final concentrations listed in Table 4. Elemental sulfur (0.0032g) and MnO_2 (0.0059g) were added as powder to Balch tubes containing 10 mL media before autoclaving. Elemental sulfur was obtained from Fisher Chemicals and MnO_2 was synthesized by the method of Burdige (9). Fe(III) gel was prepared according to the method of Lovley and Phillips (46). To test oxygen (100% air) as the TEA, cultures were grown in media that was not boiled and contained no cysteine-HCl. Additionally, 5% air was tested as a potential TEA by boiling and cooling media under N_2:CO_2 and adding sterile air to 5% of the

headspace volume to each Balch tube. No reductant was added during the testing of 5% air as the TEA and culture tubes containing air were incubated with shaking at 120 rmp.

Reduction of sulfate, sulfite, thiosulfate, and elemental sulfur was tested by the addition of 0.1% $FeSO_4$ to culture tubes. Formation of black FeS indicated the presence of sulfide resulting from the reduction of the oxidized sulfur compounds. The reduction of MnO_2 was assessed by a color change from black MnO_2 to white $MnCO_3$. Precipitation of red elemental selenium was indicative of Se(VI) and Se(IV) reduction. Reduction of Fe(III) pyrophosphate was determined by the color change from yellow to clear with siderite ($FeCO_3$) formation, while reduction of Fe(III) gel was assessed by monitoring color change from rust to black. Cell growth was measured spectrophotometrically. Direct cell counts were used to confirm growth on selenate, selenite, sulfate, and manganese.

Oxygen sensitivity: Strain GBFH was grown to mid-log phase on 10 mM lactate and 10 mM As(V) and a 10% inoculum was used to inoculate experimental tubes. Each O_2 level was tested in triplicate. Sterile air was added to final concentrations of 0, 1, 2, 5, and 10% air by volume in the Balch tube headspace. Media was prepared as before but no reductant was added to any of the experimental tubes. To test whether strain GBFH could resume growth after being exposed to 10% air, cells from the 10% air treatment were sub-sampled after 24 hours of incubation and were re-inoculated into 0% air tubes. Cultures were shaken at 120 rpm. Growth was monitored spectrophotometrically and accumulation of As(III) was quantified once growth was evident. Controls consisted of autoclaved cells.

***Desulfitobacterium dehalogenans* inhibition by As(V):** To determine if growth of *Desulfitobacterium dehalogenans* was inhibited by the presence of As(V), *D. dehalogenans* cultures pre-grown on pyruvate (11.3 mM) under fermentative conditions were inoculated in duplicate into the following 8 treatments: (1) 11.3 mM pyruvate, (2) 11.3 mM pyruvate + 10 mM As(V), (3) 10 mM lactate + 10 mM 3-Cl-4-OHPA, (4) 10 mM lactate + 10 mM 3-Cl-4-OHPA + As(V), (5) 10 mM lactate + 10 mM nitrate, (6) 10 mM lactate + 10 mM nitrate + 10 mM As(V), (7) 10 mM lactate and (8) 10 mM lactate + 10 mM As(V). The same experiment was carried out with *D. hafniense* in order to compare the response of *D. dehalogenans* to

that of an organism capable of reducing As(V). To determine if *Desulfitobacterium dehalogenans* could survive exposure to As(V), cultures of *D. dehalogenans* (treatments 2, 4, and 6) were harvested after one week by filtration through a sterile 0.22 μm syringe filter in an anaerobic chamber. The filter was placed into anaerobic salts medium lacking electron donor and acceptor and vortexed to release cells from the filter. This cell wash was used to inoculate media lacking As(V) and containing the electron donor-TEA pair present in the original treatment. Cell growth was measured spectrophotometrically and arsenic was quantified and speciated at the end of the experiment.

Analytical techniques: Cell densities were determined by one of two methods. Where cell densities are reported as optical density, Balch tubes were directly inserted into a Spectronic 20 D+ spectrophotometer and the absorbance at 420 nm (77) was recorded. Uninoculated media served as the blank. Where cell densities are reported as number of cells/mL, cells were stained with 4,6-diamidino-2-phenylindole (DAPI) and counted directly under epiflourscence (30) using a Zeiss Axioskop microscope.

As(III) (arsenite) was quantified as previously described (14) using a modification of Pilson and Johnson's (39) spectrophotometric method. Because of the presence of phosphate in the growth media, a reduction step was necessary to quantify As(V) in the same vessel. Briefly, unoxidized samples were prepared by acidifying 300 μL sample with 100 μL 25 mM HCl, and oxidized samples were prepared by oxidizing 300 μL sample with 100 μL KIO_3 solution (5 mM KIO_3 in 50 mM HCl). Reduced samples were prepared by reducing 300 μL sample with 100 μL cold reducing solution (1 part 1.4% sodium thiosulfate, 2 parts 14% sodium metabisulfite, and 2 parts 3.5 N sulfuric acid) (38). All treatments were incubated at room temperature for 10 minutes. 600 μL of molybdenum-containing reaction mixture (79) was then added to each treatment. Samples were immediately incubated at 78°C for 10 minutes, followed by a 5-minute incubation on ice. Absorbance was measured at 865 nm. Arsenite concentrations were determined by subtracting the oxidized sample absorbance from the unoxidized sample absorbance. Arsenate concentrations were determined by subtracting the unoxidized sample absorbance from the reduced samples. Acidified arsenate standards were used to generate a standard curve from which sample arsenite and arsenate concentrations

were extrapolated. Mixtures of known concentrations of arsenite and arsenate illustrated that all As(V) was reduced by treatment with the reducing solution and all As(III) was oxidized by treatment with KIO_3.

Formate was quantified enzymatically using a modified spectrophotometric assay for formate dehydrogenase (33). Briefly, 800 uL phosphate buffer, 130 uL ddH_2O, 30 uL sample, 20 uL NAD solution, and 20 uL formate dehydrogenase were added to a 1 mL cuvette, mixed and incubated in the dark for 1 hour. Absorbance was read at 340 nm. To quantify formate, sample optical densities were compared to a standard curve of formate concentration versus A_{340nm}.

Phylogeny: Phylogenetic analysis and sequence similarity comparisons were performed by Dr. Stephan Spring (DSMZ (Braunschweig, Germany)). Cells of strain GBFH, *D. hafniense*, *D. frappieri*, and *D. dehalogenans* were used directly for PCR amplification of almost full-length bacterial 16S rRNA gene fragments (82). The resulting PCR product was purified using the Prep-A-Gene DNA purification kit (Bio-Rad, Munich, Germany) and sequenced using a LICOR automated sequencer (MWG Biotech, Ebersberg, Germany). Cycle sequencing protocols based upon the chain termination technique were applied using the Thermo Sequenase flourescent labeled primer cycle sequencing kit of Amersham (Braunschweig, Germany). The new sequence was added to an alignment of about 3,800 published and unpublished homologous primary structures from bacteria using the alignment tool of the ARB program package (49). Phylogenetic analysis was performed by applying maximum parsimony (ARB, PHYLIP), distance matrix (ARB, PHYLIP, 20), and maximum likelihood methods (fastDNAml, 54) on different data sets. Maximum parsimony analyses were performed using the complete data set of bacterial 16S rRNA sequences, whereas distance matrix and maximum likelihood analyses were based on a subset of the database comprising sequences of representatives belonging to the low GC Gram positive clade. The data sets varied with regard to alignment position included. Highly variable positions were successively removed from the data set after determining positional variabilities of the individual alignment positions using the ARB package. The accession number for the 16s rDNA sequence of strain GBFH is AJ3047028.

DNA-DNA hybridization and G+C content of genomic DNA: DNA:DNA hybridization experiments were preformed at the DSMZ (Braunschweig, Germany) in collaboration with the laboratory of Dr. Erko Stackebrandt. DNA was extracted by the method of Cashion (10). G+C contents were determined by HPLC (59). DNA-DNA hybridization were carried out using the thermal renaturation method (16) with modifications according to Huss (36) and Escara and Hutton (19) using a Gilford System 2600 Spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter. Renaturation rates were computed by the Program TRANSFER.BAS (37).

RESULTS

Morphology of strain GBFH: Cells of strain GBFH were curved rods measuring 2 – 4 uM in length and 0.3 – 0.5 uM in width (Figure 2). Strain GBFH possessed 2-5 laterally attached flagella (Figure 3), and transmission electron micrographs revealed a typical Gram positive cell wall structure (Figure 4). Additionally, strain GBFH formed endospores after entering stationary phase (Figure 5). When grown on plates or shake tubes containing 1% agar, colonies of strain GBFH were white with rounded edges.

Denaturing gradient gel electrophoresis: Denaturing gradient gel electrophoresis (DGGE) analysis of strain GBFH revealed three distinct bands resulting from PCR amplification of 16s rDNA gene sequences (Figure 6, lanes 1 and 3). The same result was obtained with two independent PCR amplifications (Figure 6, lanes 1 and 3). Strain E4, an As(V)-reducing organism isolated independently of strain GBFH from Lake Coeur d'Alene, also exhibited the same DGGE banding pattern as strain GBFH (see Figure 6, lane 2).

Oxidation of formate coupled to the reduction of As(V): Strain GBFH was capable of coupling the oxidation of 10 mM formate to the reduction of 10 mM As(V) while conserving energy for growth (Figure 7). Figure 7 shows concomitant increase in cell number, consumption of formate, depletion of As(V), and accumulation of As(III). *D. frappieri* is also capable of coupling the reduction of As(V) to the oxidation of formate and can conserve

energy in the process (Figure 8). During growth of both *D. frappieri* and strain GBFH, As(V) and formate consumption were tightly coupled, and As(III) accumulation occurred concomitantly with cell growth (Figures 7 and 8). Both organisms utilized As(V) and formate in an approximately 1:1 ratio (Figures 7 and 8). Controls treatments included media lacking formate, media lacking As(V), heat-killed cells, and uninoculated media. No increase in cell number, depletion of formate, or reduction of As(V) to As(III) was detected in any of the control treatments for strain GBFH or *D. frappieri* (data not shown).

Temperature and pH Optima: Strain GBFH grew optimally at 37°C, with a maximum growth rate (μ_{max}) of 0.12 h^{-1}(Figure 9) at pH 7.0. Strain GBFH was capable of growth and As(V) respiration at temperatures ranging from 15 to 42°C. Growth was not observed at 4°C or at 45 °C, but at both temperatures small amounts of As(V) respiration (2mM) were observed. Inclusion of 10% hydrogen in the headspace of culture tubes completely inhibited the growth of GBFH at 42°C; H_2 lowered growth rates at all other temperatures (data not shown). The optimal pH for growth of strain GBFH was 7.5, with a maximum growth rate of (μ_{max}) = 0.313 h^{-1} (Figure 10). The growth rate at pH 7.5 (0.313 h^{-1}) is almost three-fold higher than at pH 7.0 (0.12 h^{-1}) and two-fold higher than at pH 8 (0.167 h^{-1}). GBFH was capable of growth and As(V) respiration from pH 6.0 to pH 8.0.

Heat-resistant spore formation: After 8 days of incubation at 37°C in fresh culture medium, all three heat-treated cultures of strain GBFH exhibited a log increase in cell number and respired all provided (10 mM) As(V) (data not shown).

Carbon source and electron donor survey: Carbon source and electron donor utilization profiles are summarized in Table 3. *D. hafniense* and *D. frappieri* exhibited identical carbon source utilization patterns. *D. hafniense*, *D. frappieri* and strain GBFH were all capable of utilizing pyruvate, lactate, formate and fumarate. When grown on formate and As(V) or fumarate and As(V), cell yield for all three organisms was lower ($OD_{420nm} \approx 0.08$-0.1) than when grown on lactate and As(V) or pyruvate and As(V) ($OD_{420nm} \approx 0.15$-2.0). Only *D. hafniense* and *D. frappieri* were able to utilized butyrate, succinate, malate and ethanol. Growth of *D. hafniense* and *D. frappieri* on malate and succinate was slow, with cells

requiring one to two weeks to reach densities equivalent to those reached in only two to three days when lactate was provided as the carbon source. Neither As(V) respiration nor growth was observed when *D. hafniense*, *D. frappieri* or strain GBFH was supplied with glycerol, methanol, citrate, benzoate, H_2, H_2 and actate, propionate, glucose or galactose. After three passages, *D. hafniense*, *D. frappieri* and strain GBFH showed an increase in cell number when acetate was provided as the carbon source, but in all cases cells did not achieve sufficient densities to be considered positive for utilization of acetate (see methods). Despite poor cell yields ($OD_{420nm} \approx 0.03$), all three organisms reduced 8 – 10 mM As(V) in the presence of acetate, even after three consecutive transfers. Pyruvate supported fermentative growth of *D. hafniense*, *D. frappieri* and strain GBFH, while lactate and butyrate did not support fermentative growth of any of these organisms. Inoculated controls containing no carbon source showed no increase in cell number and no reduction of As(V).

Terminal electron acceptor survey: Results of the TEA survey are summarized in Table 4. As in the carbon source utilization profile, *D. hafniense* and *D. frappieri* exhibited identical TEA utilization patterns. The inability of strain GBFH to utilize nitrate represents the singular difference between strain GBFH and *D. hafniense* and *D. frappieri* with respect to the terminal electron accepting processes tested. Strain GBFH, *D. hafniense* and *D. frappieri* experienced a lag phase of approximately two weeks when lactate and As(V) grown cells were inoculated in media containing fumarate, Se(VI), Fe(III) gel, elemental sulfur, MnO_2, thiosulfate or sulfite, while no such lag phase was observed when ferric pyrophosphate, As(V) or nitrate was provided as the TEA. Reduction of MnO_2 resulted in a change in color from black to white, which presumably represents the formation of rhodochrosite, $MnCO_3$ (47). Reduction of Se(VI) resulted in the precipitation of red elemental selenium. *D. hafniense* grew noticably more slowly on Se(VI) than strain GBFH and *D. frappieri*. Cell growth on both Se(VI) and MnO_2 was confirmed by direct cell count because the color changes in the media prevented the use of the spectrophotometer for determining cell densities.

Cells grown on elemental sulfur, thiosulfate and sulfite produced sulfide as determined by the precipitation of FeS upon addition of $FeSO_4$. Strain GBFH, *D. hafniense* and *D. frappieri*

cultures grown on 10 mM As(V) and transferred to media containing either sulfur, thiosulfate or sulfite accumulated a bright yellow precipitate presumed to be orpiment (As_2S_3) (65) or realgar (As_2S_2) (34). The occurrence of yellow precipitate varied based on the organism and sulfur compound provided. Strain GBFH and *D. hafniense* cultures precipitated the yellow substance when grown on elemental sulfur and sulfite, but not on thiosulfate, while *D. frappieri* cultures formed precipitate when grown on all three sulfur species. In cultures with yellow precipitate formation, sulfide was generally not detectable although cell growth occurred. Once these cultures were passaged a second time, sulfide was detectable by FeS precipitation, and sulfide was evolved on the third transfer as well. Cultures grown on sulfate never contained detectable amounts of sulfide and direct cell counts confirmed that no growth was occurring when sulfate was provided as the TEA. In cultures where no TEA was provided, no growth occurred, reconfirming that an exogenous TEA must be provided for growth on lactate by strain GBFH, *D. hafniense* and *D. frappieri*.

Oxygen sensitivity: Strain GBFH was capable of growth and As(V) respiration when 0%, 1% or 2% air was present in the headspace of culture tubes, but no cell growth occurred in cultures containing 5% or 10% air (data not shown). After 24 hours of exposure to 10% air, strain GBFH resumed growth and As(V) reduction when re-inoculated into tubes containing no air (data not shown).

***Desulfitobacterium dehalogenans* inhibition by As(V):** *D. dehalogenans* was unable to grow by respiration of As(V) when lactate was supplied as the electron donor (Figure 11), and no reduction of As(V) to As(III) was observed (data not shown). In contrast, *D. hafniense* grew when lactate was supplied as the electron donor and As(V) was provided as the respiratory TEA (Figure 12), and As(V) was completely converted to As(III) (data not shown). Neither *D. dehalogenans* nor *D. hafniense* were capable of fermentative growth on lactate (Figures 11 and 12). Both *D. dehalogenans* and *D. hafniense* increased in cell number when pyruvate was supplied for fermentation (Figures 11 and 12). When As(V) was added as a potential respiratory TEA to media containing pyruvate, growth of *D. dehalogenans* was completely inhibited (Figure 11). However, *D. hafniense* grew more quickly and to higher densities when As(V) was added to pyruvate than when pyruvate was supplied with no

additional TEA (Figure 12). Both *D. dehalogenans* and *D. hafniense* were capable of growth on lactate when nitrate was supplied as a respiratory TEA (Figures 11 and 12). The addition of As(V) to media containing lactate and nitrate caused complete inhibition of the growth of *D. dehalogenans* but did not cause severe inhibition of the growth of *D. hafniense* (Figures 11 and 12). Even in the presence of nitrate, *D. hafniense* reduced 9.5 mM As(V) to As(III) (data not shown). When lactate and 3-Cl-4-OHPA were provided, *D. dehalogenans* increased in cell number (Figure 11) but *D. hafniense* did not (Figure 12). As noted with other treatments, when As(V) was added to media containing lactate and 3-Cl-4-OHPA, growth of *D. dehalogenans* was completely inhibited (Figure 11). *D. hafniense*, however, was capable of growth on media containing lactate, 3-Cl-4-OHPA and As(V) (Figure 12), and all provided As(V) was reduced to As(III) (data not shown).

Cultures of *D. dehalogenans* containing As(V) which were washed and re-inoculated into As(V)-free media differed in their response to prolonged exposure to As(V). When cultures containing As(V) and pyruvate were washed and re-inoculated into pyruvate-only media, growth resumed after 1 to 2 weeks, with cultures reaching maximum densities ($O.D._{420}$ = 0.19) that were slightly lower than the maximum density reached when cells were grown on pyruvate with no prior exposure to As(V) ($O.D._{420}$ = 0.26) (Figure 11). Neither cultures originally containing As(V), lactate and nitrate nor cultures originally containing As(V), lactate and 3-Cl-4-OHPA resumed growth after more than one month of incubation in As(V)-free media (data not shown).

Phylogenetic analysis and DNA:DNA hybridization: Phylogenetic analysis of 16s rDNA gene sequences resulted in the assignment of strain GBFH to the genus *Desulfitobacterium*. Members of the genus *Desulfitobacterium* are tightly grouped in the low GC Gram + clade (Figure 13). Construction of a phylogenetic tree showed that strain GBFH clustered most closely to *D. hafniense* and *D. frappieri* (Figure 14). 16S rDNA sequence analysis supported phylogenetic analysis revealing that GBFH had 98% sequence similarity to *D. hafniense* and 97% sequence similarity to *D. frappieri*. DNA:DNA hybridization experiments revealed that strain GBFH exhibited 81.2% DNA:DNA re-association with *D. frappieri* and 79.7% DNA:DNA hybridization with *D. hafniense*. *D. frappieri* and *D. hafniense* had 88.7%

DNA:DNA re-association. DNA:DNA reassociation between these three strains and *D. dehalogenans* was substantially lower, ranging between 41 and 46% (Table 5).

DISCUSSION

This report describes the isolation of strain GBFH, the first As(V)-reducing organism capable of conserving energy for growth by coupling the reduction of As(V) to the oxidation of formate when formate is supplied as the sole electron donor and carbon source (Figure 7). Controls illustrate that formate alone cannot support growth in the absence of an exogenous terminal electron acceptor such as As(V). Additionally, yeast extract (0.1 g/L) did not serve as a carbon and energy source as no growth or As(V) reduction occurred in the absence of formate. Comparative physiological studies reveal that *D. frappieri* (Figure 8) and *D. hafniense* (Table 3) are also capable of coupling the reduction of As(V) to the oxidation of formate, conserving energy for growth in the process. Calculations using published thermodynamic values (27, 81) reveal that the oxidation of formate coupled to the reduction of As(V) is an energy yielding and proton consuming reaction.

$$HCO_2^- + 3/2\ H^+ + 1/2\ HAsO_4^{2-} + 1/2\ H_2AsO_4^- \rightarrow H_3AsO_3 + HCO_3^-$$

($\Delta G^{0'}_{pH=7}$ = -82 kJ/mole formate)

$$C_3H_5O_3^- + 2\ H^+ + HAsO_4^{2-} + H_2AsO_4^- \rightarrow 2\ H_3AsO_3 + HCO_3^- + C_2H_3O_2^-$$

($\Delta G^{0'}_{pH=7}$ = -172 kJ/mole lactate)

The oxidation of formate yields approximately half the energy produced by the oxidation of lactate (-172 kJ/mole lactate) per mole electron donor, which is consistent with the higher cell densities observed when *Desulfitobacterium* species were grown on lactate and As(V) compared to formate and As(V). Theoretical considerations predict a 1:1 ratio of formate consumed to As(V) reduced. Analysis of growth of strain GBFH shows that formate and As(V) are consumed in a ratio of approximately 1:1 (Figures 7 and 8). Experimental data are therefore in relatively good agreement with theoretical predictions. Because the oxidation of

formate does not support ATP generation through substrate level phosphorylation, it can be inferred that ATP generation occurs via electron transport phosphorylation.

Although formate serves as the electron donor for As(V) respiration by *Sulfurospirillum arsenophilum* and *Sulfurospirillum barnesii*, these organisms are not capable of assimilating carbon for cell growth when formate is supplied as the carbon source. *S. arsenophilum* and *S. barnesii* require the addition of acetate as the carbon source in order for the oxidation of formate to support growth (63). Formate is a characteristic end product of mixed acid fermentation (73) and may be an important end product in other anaerobic fermentations (23, 35). Organisms with the capability to utilize formate as the electron donor and carbon source for growth coupled to As(V) reduction may have a competitive advantage over those which are unable to utilize formate in carbon-limited environments. The discovery of organisms with the ability to grow by As(V) reduction with formate as the sole carbon and electron source is perhaps not surprising as formate serves as both the carbon course and electron acceptor for other types of anaerobic respiration, including Fe(III) and Mn(IV) reduction by *Shewanella putrefaciens* (48) and reductive dechlorination by *Desulfitobacterium dehalogenans* (50, 80) and *Desulfitobacterium* sp. strain PCE1 (26).

Comparative physiological studies reveal that strain GBFH is very similar phenotypically to its two closest relatives, *D. frappieri* and *D. hafniense*. Temperature optima for all three organisms are nearly identical, with *D. frappieri* growing optimally at 38°C (7) and *D. hafniense* (12) and GBFH (Figure 9) growing optimally at 37°C. The pH optimum for *D. frappieri* (7) and strain GBFH (Figure 10) is 7.5. No pH optimum has been reported for *D. hafniense* (12). The maximum growth rate reported for *D. frappieri* is 0.33 h^{-1} (7), which is nearly identical to that observed for GBFH (Figure 10). The maximum growth rate reported for *D. hafniense* when grown fermentatively on 11.3 mM pyruvate was 0.025 h^{-1} (generation time of approximately 40 hours) (12). This reported rate is considerably slower than that observed when *D. hafniense* was grown fermentatively on 11.3 mM pyruvate on the media described here. Although the growth rate was not determined, Figure 12 (pyruvate only) illustrates that cells of *D. hafniense* underwent nearly 4 doublings within 42 hours when grown on 11.3 mM pyruvate. During culture maintenance and growth experiments, *D.*

hafniense grew at rates similar to those of strain GBFH and *D. frappieri*. *D. hafniense* and *D. frappieri* were both enriched after incubation of enrichment cultures at 80°C for 30 – 60 minutes in an effort to recover spore-forming bacteria (7, 53). Although heat-treatment was not a selective pressure applied to the enrichment of strain GBFH, this strain is also capable of heat-resistant spore formation. Strain GBFH is also capable of tolerating oxygen at low levels, with growth and As(V) reduction occurring at concentrations of 2% air and lower. Additionally, although 10% air inhibits growth of strain GBFH, exposure to 10% air for 24 hours did not prevent growth and As(V) reduction by strain GBFH once anaerobic conditions were re-established. Similar oxygen tolerance was reported for *D. hafniense* (53).

Carbon source utilization profiles are identical for *D. hafniense* and *D. frappieri* with respect to the carbon sources tested. The results presented here are in good agreement with previously reported carbon utilization patterns (7, 12, 25). The only difference between this and other carbon usage investigations is the report of formate serving as both a respiratory electron donor and as a carbon source for growth of *D. hafniense* and *D. frappieri*. To the best of our knowledge, formate has never been tested as a potential electron donor or carbon source for respiration or fermentation by *D. frappieri*; it was not included in the donors tested in the original characterization (7). The original characterization of *D. hafniense* reports that neither formate nor lactate supports the growth of this organism (12). Later reports describe the use of lactate by *D. hafniense* (25), and this report details the use of formate. The ability of *D. hafniense* and *D. frappieri* to use butyrate, succinate, malate and ethanol distinguishes them from strain GBFH from with respect to the carbon sources tested. This difference in carbon utilization may be attributable to the natural habitat of each organism. Strain GBFH was isolated from an oligotrophic-mesotrophic (86) lake whose sediments are low in carbon, containing an average of 2.1% total organic carbon (32). Enrichment media for strain GBFH contained formate as the sole carbon source. *D. hafniense* and *D. frappieri* were both isolated from dechlorinating consortia enriched from carbon-rich sewage sludge (7, 53).

The consortia from which *D. hafniense* and *D. frappieri* were isolated contained high concentrations of complex carbon sources such as glucose (7, 53). Because *Clostridia* ferment these complex carbon compounds under anaerobic conditions (73), it is likely that

they were present in the consortia from which *D. hafniense* and *D. frappieri* were isolated. *Clostridia* produce butyrate, succinate and ethanol during glucose fermentation (73). Thus, we may reasonably infer that *D. hafniense* and *D. frappieri* were exposed to greater selective pressure for the utilization of butyrate, succinate and ethanol in their original habitat and than strain GBFH. These factors may explain the ability of *D. hafniense* and *D. frappieri* to utilize a wider variety of carbon sources than strain GBFH. Although growth of all three organisms on acetate did not produce enough cell mass to be considered positive for growth, respiration of 9.5 mM As(V) after three passages suggests that acetate can be used an as electron donor, but not as a source of carbon for assimilation into cell mass.

A comparison of the terminal electron accepting capabilities of *D. hafniense*, *D. frappieri* and strain GBFH reveals that these three organisms exhibit almost identical profiles (Table 4). As with the carbon source utilization profile, the terminal electron accepting capabilities of *D. hafniense* and *D. frappieri* are identical with respect to the TEAs tested in this study. The single difference between the TEA profiles of *D. hafniense* and *D. frappieri* and strain GBFH is the inability of strain GBFH to utilize nitrate. Again, this difference may be understood when the original habitats of the three organisms are considered. Sewage sludge is highly enriched in nitrogen compounds and such an environment is likely to exert a stronger selective pressure for the utilization of nitrate than the sediments of an oligotrophic-mesotrophic lake.

The ability of members of the genus *Desulfitobacterium* to conserve energy for growth by reducing toxic metal(loid)s such as As(V) and Se(VI) is documented here for the first time. The members of the genus *Desulfitobacterium* have previously been enriched for their ability to reductively dechlorinate chlorinated ethenes as well as a wide variety of chlorinated phenols (7, 12, 25, 26, 53, 80). Indeed, to the best of our knowledge, GBFH is the first member of the genus *Desulfitobacterium* not to be enriched on the basis of its ability to reductively dechlorinate hydrocarbons. *D. hafniense D. frappieri*, and strain GBFH all grow by coupling the reduction of As(V) and Se(VI) to the oxidation of lactate and formate (Table 4). Additionally, these organisms can mediate the precipitation of arsenic-sulfide minerals during concomitant of As(V) and oxidized sulfur species. The ability of *D. hafniense* and *D.*

frappieri to reduce toxic metalloids and to reductively dechlorinate phenols implies that these organisms could be useful in remediation of co-contaminated environments. It is possible that *D. hafniense* and *D. frappieri* could mediate the formation of arsenic sulfides, decrease aqueous concentrations of selenate by reductive formation of elemental selenium, and reductively dechlorinate a range of phenols simultaneously. The ability of strain GBFH to reductively dechlorinate phenols and aliphatic compounds remains to be investigated.

Not all members of the genus *Desulfitobacterium* are capable of As(V) reduction. Figure 11 illustrates that *D. dehalogenans* is incapable of growing by coupling the oxidation of pyruvate or lactate to the reduction of As(V). Additionally, the presence of As(V) completely inhibited fermentative growth on pyruvate and respiratory growth on nitrate and 3-Cl-4-OHPA (Figure 11). *D. dehalogenans* is therefore not a good choice for potential remediation of co-contaminated environments because the presence of As(V) inhibits both respiratory and fermentative growth. The presence of As(V) appears to have different effects of fermentative and respiratory metabolism of *D. dehalogenans*. When *D. dehalogenans* was grown with pyruvate and As(V), washed in donor-free and acceptor-free media, and re-inoculated into pyruvate only media, growth resumed within 1 to 2 weeks. Prolonged exposure to As(V) does not appear to effect the ability of *D. dehalogenans* to resume fermentative growth once As(V) was eliminated. Cultures of *D. dehalogenans* that were grown with lactate, nitrate and As(V) or lactate, 3-Cl-4-OHPA and As(V) did not resume growth in media containing lactate and nitrate or lactate and 3-Cl-4-OHPA. This implies that prolonged exposure to As(V) permanently inhibits respiratory function. As(V) has been shown to uncouple oxidative phosphorylation (62). These results suggest that, in *D. dehalogenans*, enzymes responsible for substrate-level phosphorylation are not rendered permanently functionless by As(V), but that ATPases responsible for oxidative phosphorylation are permanently damaged by prolonged exposure to As(V). Similar experiments with *D. hafniense* (Figure 12) suggest that growth of *Desulfitobacterium* species capable of reducing arsenic is not inhibited by As(V), as is growth of *D. dehalogenans*. In fact, *D. hafniense* grows faster and to higher densities when coupling the oxidation of pyruvate to the reduction of As(V) than when fermenting pyruvate (Figure 12).

Strain GBFH is phenotypically similar to *D. hafniense* and *D. frappieri*. The DGGE banding pattern of strain GBFH yielded 3 bands (Figure 6), which is consistent with the number of DGGE bands observed in other *Desulfitobacterium* species (S. Spring, personal communication). Phylogenetic analysis shows that strain GBFH is related to the low GC Gram positive bacteria (Figure 13) and clusters most closely with *D. hafniense* and *D. frappieri* (Figure 14). Because of the high levels of sequence similarity (> 97%), DNA:DNA hybridization was undertaken to determine if strain GBFH constituted a unique species in the genus *Desulfitobacterium*. Determination of genomic similarities by DNA:DNA reassociation revealed that *D. hafniense* DSM 10664^T and *D. frappieri* DSM 12420^T share 89% similarity (Table 5). Interpreting reassociation values of higher than 70% as indicative of species affiliation (83) and considering their similar physiologies, the two species should be united. Strain GBFH should also be considered a member of this species as DNA:DNA reassociation values obtained for strain GBFH and *D. hafniense* DSM 10664^T and *D. frappieri* DSM 12420^T range between 78 and 81% (Table 5). Differences in DNA sequence may be sufficient to account for strain-specific differences in morphology and physiology, such as motility, the ability to utilize various electron acceptors and donors, and the ability to dechlorinate various chlorophenols. The degree of DNA similarity between these three strains and *D. dehalogenans* (80) is significantly lower, ranging between 41 and 46% (Table 5). The international Code of Nomenclature of Bacteria, governing the rules of nomenclatures, describes in rule 62 (43) that in cases where species are united, the oldest legitimate epithet is retained. Though *D. hafniense* (12), and *D. frappieri* (7) are both described in the same journal in 1996, *D. hafniense* has priority over *D. frappieri* because of the earlier date of its description (i.e., page 446 versus page 1012 of the International Journal of Systematic Bacteriology).

The isolation of an As(V)-respiring strain GBFH from Coeur d'Alene Lake sediments is significant because it suggests a mechanism for the mobilization of arsenic as As(III) from sediments. Biotic generation of As(III) by CDAL sediments has been previously demonstrated by our laboratory (29). The isolation of strain GBFH confirms the existence of microorganisms capable of this transformation in CDAL sediments. Additionally, physiological characterization of strain GBFH reveals that this organism is capable of Fe(III)

reduction. Our laboratory has also demonstrated that microbial Fe(III) reduction can mobilize As(V) from sediments of CDAL (14). Strain GBFH therefore could potentially contribute to mobilization of arsenic as As(V) from sediments by mediating the reductive dissolution of Fe(III) oxyhydroxides. Additionally, strain GBFH could contribute to mobilization of arsenic as As(III) by direct reduction of solid-phase As(V) to aqueous As(III), or by reduction of aqueous As(V) to As(III). DAsRB strain SES-3 also reduces Fe(III) and has been shown to mediate As(III) solubilization using synthetic iron minerals with As(V) sorbed or co-precipitated on the mineral surface (88). Although specific experiments designed to test the contribution of strain GBFH to arsenic mobilization in CDAL sediments have not been performed, the isolation of an organism capable of both Fe(III) and As(V) reduction suggests that strain GBFH may contribute to arsenic and iron diagenesis in this system. Additionally, the independent enrichment of an organism with a 16s rDNA DGGE banding pattern identical to that of strain GBFH, strain E4, (Figure 6) suggests that strain GBFH or organisms similar to strain GBFH are sufficiently abundant to be recovered multiple times from these sediments.

In this report we detail the isolation and characterization of the first organism capable of coupling growth to As(V) reduction while utilizing formate as the sole carbon source and electron donor. Additionally, this paper contains the first description of the ability of members of the genus *Desulfitobacterium* to utilize toxic metal(loid)s as terminal electron acceptors. The ability of *Desulfitobacterium* species to reductively dechlorinate phenols, reduce metal(loids) and mediate precipitation of arsenic sulfides makes certain members of this genus excellent choices for bioremediation of co-contaminated environments. Physiological and genomic comparisons suggest the genus *Desulfitobacterium* be emended by collapsing strain GBFH, strain PCP-1 (formerly *D. frappieri*) and strain DCB-2 (*D. hafniense*) together under the name *Desulfitobacterium hafniense*.

Emended description of *Desulfitobacterium hafniense*: This emendation is based on the description of *Desufitobacterium hafniense* DSM 10664^T (12), *D. frappieri* DSM 12420^T (7), the current report detailing the description of strain GBFH, and comparative physiological and genomic studies conducted in this study. Cells rod shaped, Gram negative, occurring

singly, in pairs, and in short chains. Dimensions vary from 2.0 to 4.0 μm in length and 0.6 to 0.7 μm width to 3.3 to 6.0 μm in length and 0.3 to 0.5 μm in width. Motile by one or two terminal flagella (DSM 10664),or 2-5 laterally attached flagella (strain GBFH), or non-motile (DSM 12420). Heat-resistant spores are formed. Spores terminal. Obligate anaerobe with slight oxygen tolerance. Optimum pH is 7.5 and optimum growth temperature is 37°C to 38°C. Pyruvate, lactate, formate and fumarate (10 mM each) support growth. Growth of strains DSM 10664 and DSM 12420 was also supported by butyrate, succinate, malate (10 mM each), and ethanol (5mM). Tryptophane and yeast extract may support growth in the presence of inorganic electron acceptors. Reduces nitrate to ammonia and nitrite. Elemental sulfur, thiosulfate, sulfite, Fe(III) and Fe (III) gel, MnO_2, fumarate, As (V), Se(VI) and Fe-pyrophosphate are reduced in the presence of lactate. Does not reduce nitrite, Mn(IV), Se(IV) As(III), or sulfate in the presence of lactate. DSM 10664 can conserve energy for growth by reduction of 3-Cl-4-OPHA in the presence of pyruvate. Reductively dechlorinates pentachlorophenol, 2,4,5-trichlorophenol (TCP), 2,4,6-TCP, 2,4-dichlorophenol (DCP), 3,5-DCP and 3-Cl-4-OHPA. Does not dechlorinate 3,4,5- TCP, 3,4-DCP, 2-chlorophenol, 3-chlorophenol, and 4-chlorophenol. The following compounds may be degraded by individual strains: 2,3,4,5-TeCP, 2,3,5,6-TeCP, 2,3,4-TCP, 2,3,5-TCP, 2,3,6-TCP, 3,4,5-TCP and 2,6-DCP. Contains cytochrome c but no desulfoviridin. Indole positive and catalase negative. Does not liquify gelatin. The DNA G+C content is between 46 and 47 mol%. The type strain has been isolated from municipal sludge. Type strain: *Desulfitobacterium hafniense* DSM 10664^T (strain DCB-2^T).

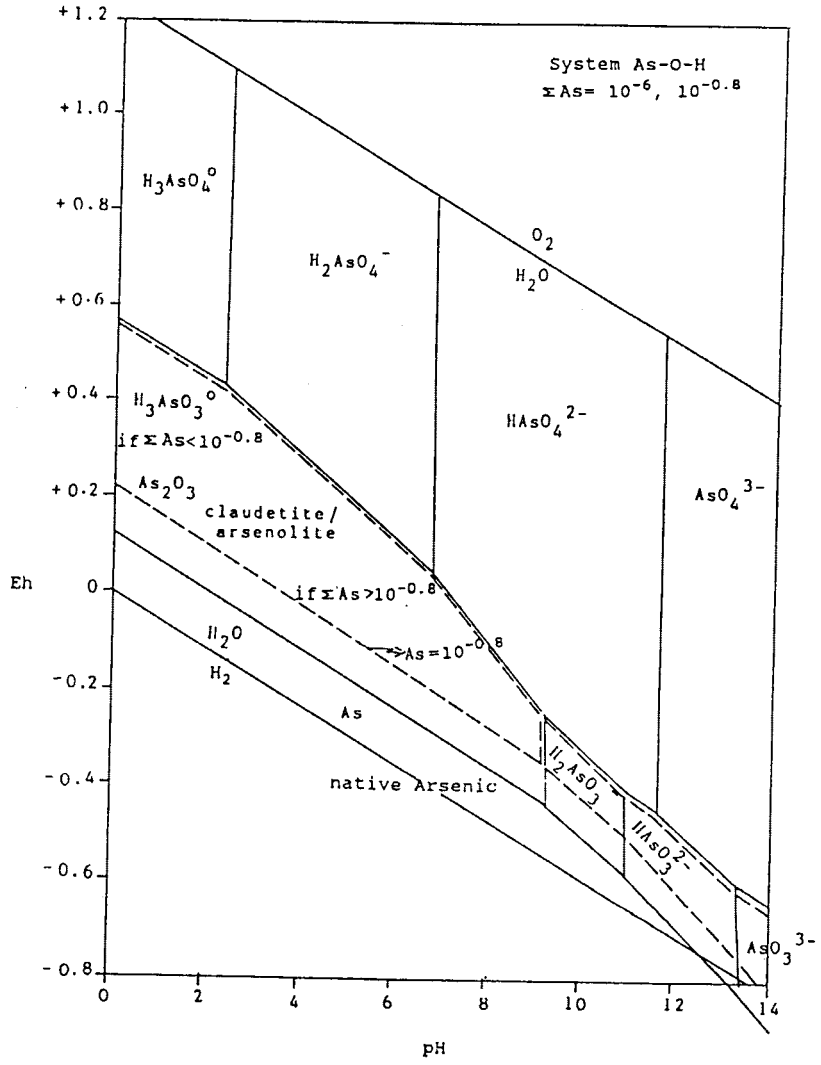

Figure 1. Eh-pH diagram for the system As-O-H. Activities of $\Sigma As = 10^{-6}$ M, contoured for $10^{-0.8}$ M. Taken from (81).

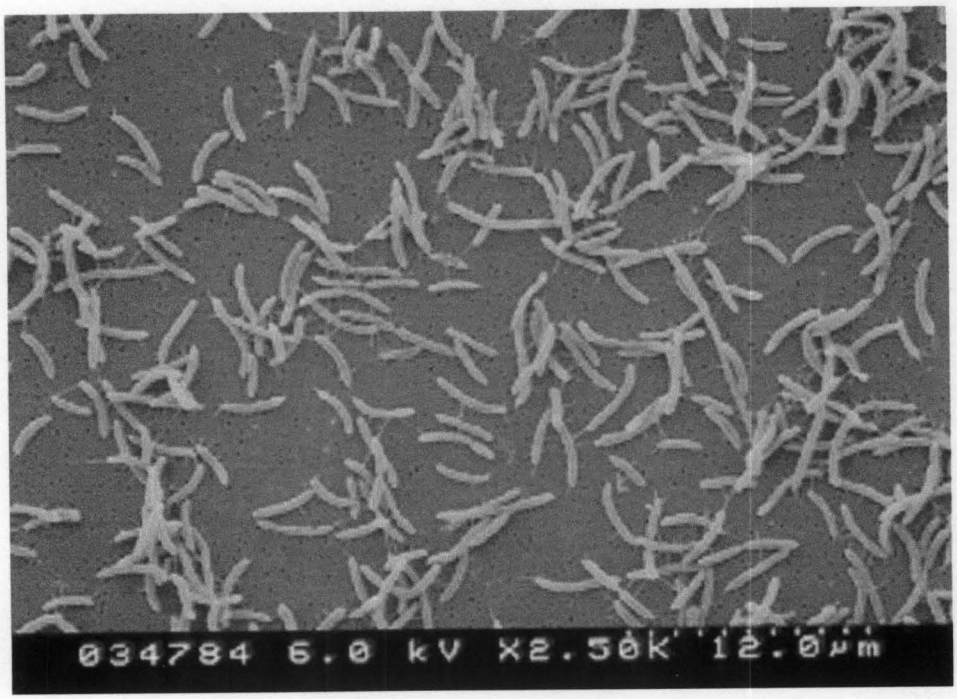

Figure 2. Scanning electron micrograph illustrating the cells of strain GBFH are curved rods measuring 2 – 4 uM in length and 0.3 – 0.5 uM in width.

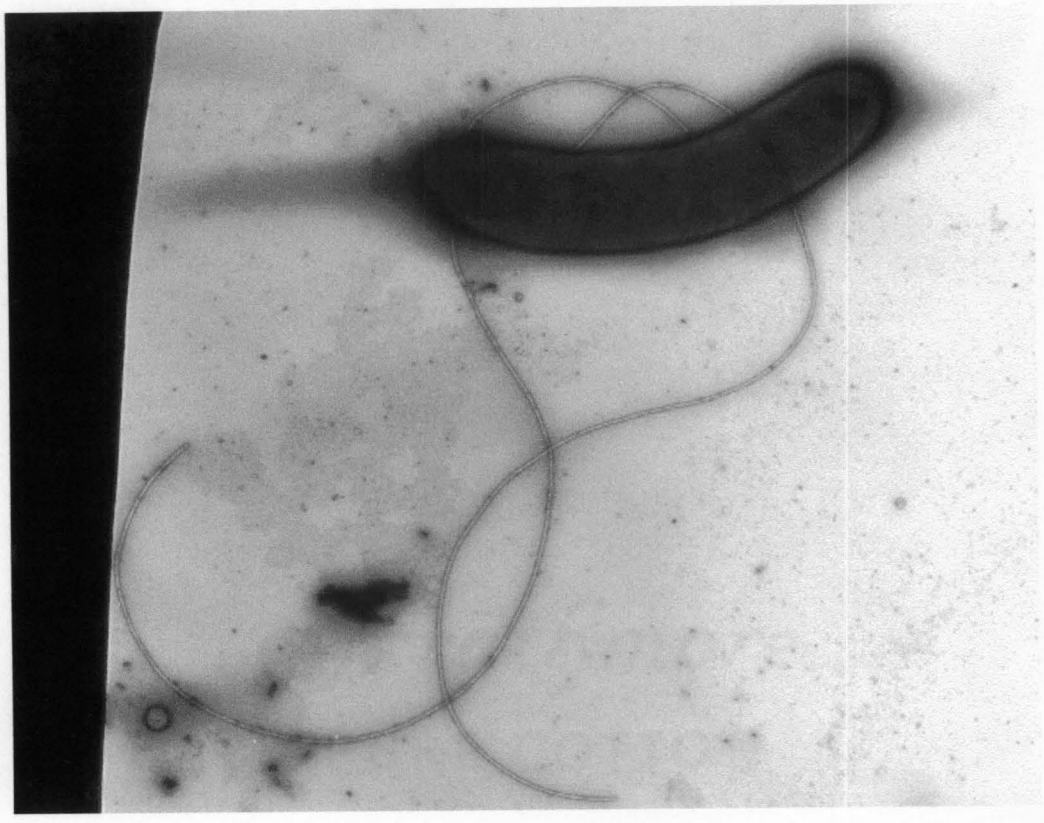

Figure 3. Transmission electron micrograph of strain GBFH illustrating cell morphology and two laterally attached flagella. The sample was negatively stained with 2% uranyl acetate.

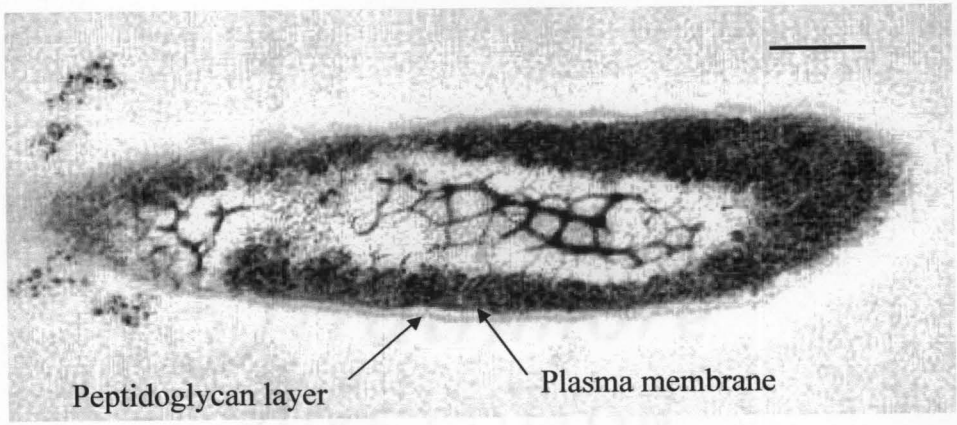

Figure 4. Transmission electron micrograph showing a thin-section of strain GBFH illustrating a typical Gram positive cell wall structure, including peptidoglycan and plasma membrane. Bar = 200nm.

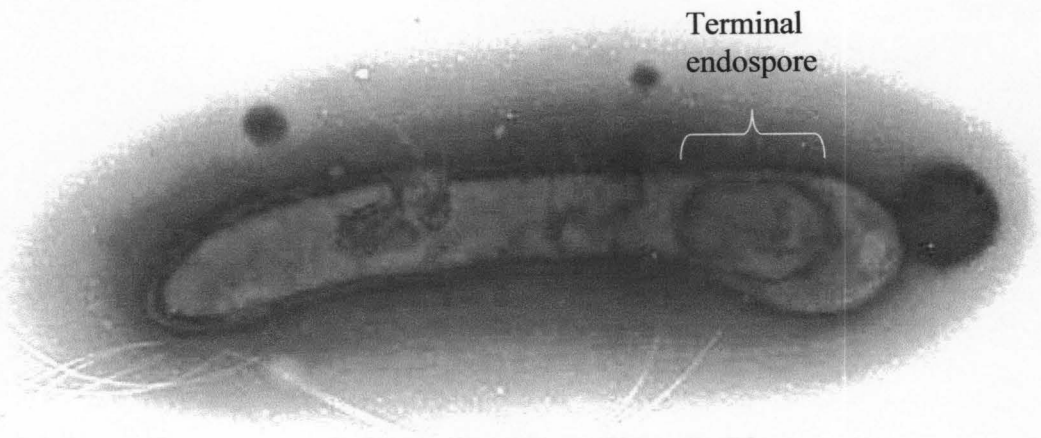

Figure 5. Transmission electron micrograph of endospore formation by strain GBFH. Note the five lateral flagella. The sample was negatively stained with 2% PTA.

Figure 6. DGGE showing banding patterns resulting from amplification of 16s rDNA of strain GBFH (lanes 1 and 3) and strain E4 (lane 2). Strain E4 was enriched for As(V) reducing ability independently from strain GBFH, but using identical enrichment methods. Arrows indicate three distinct bands migrating at the same position are visible in all three lanes.

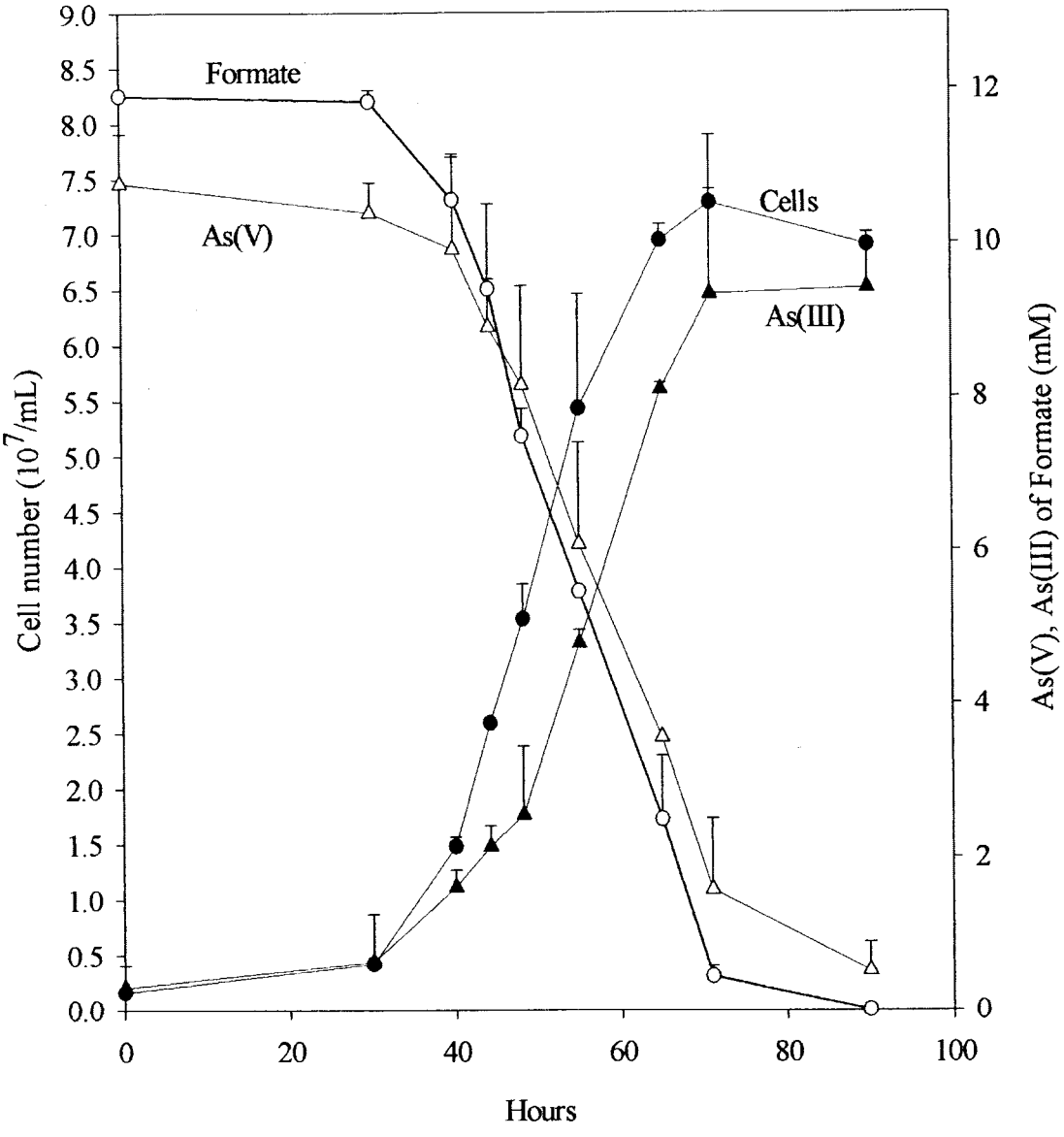

Figure 7. Growth curve of strain GBFH at pH 7.5 illustrating an increase in cell number (●), increase in As(III) (▲), decrease in As(V) (△), and decrease in formate (○). Experiments were performed in triplicate and error bars represent standard deviations.

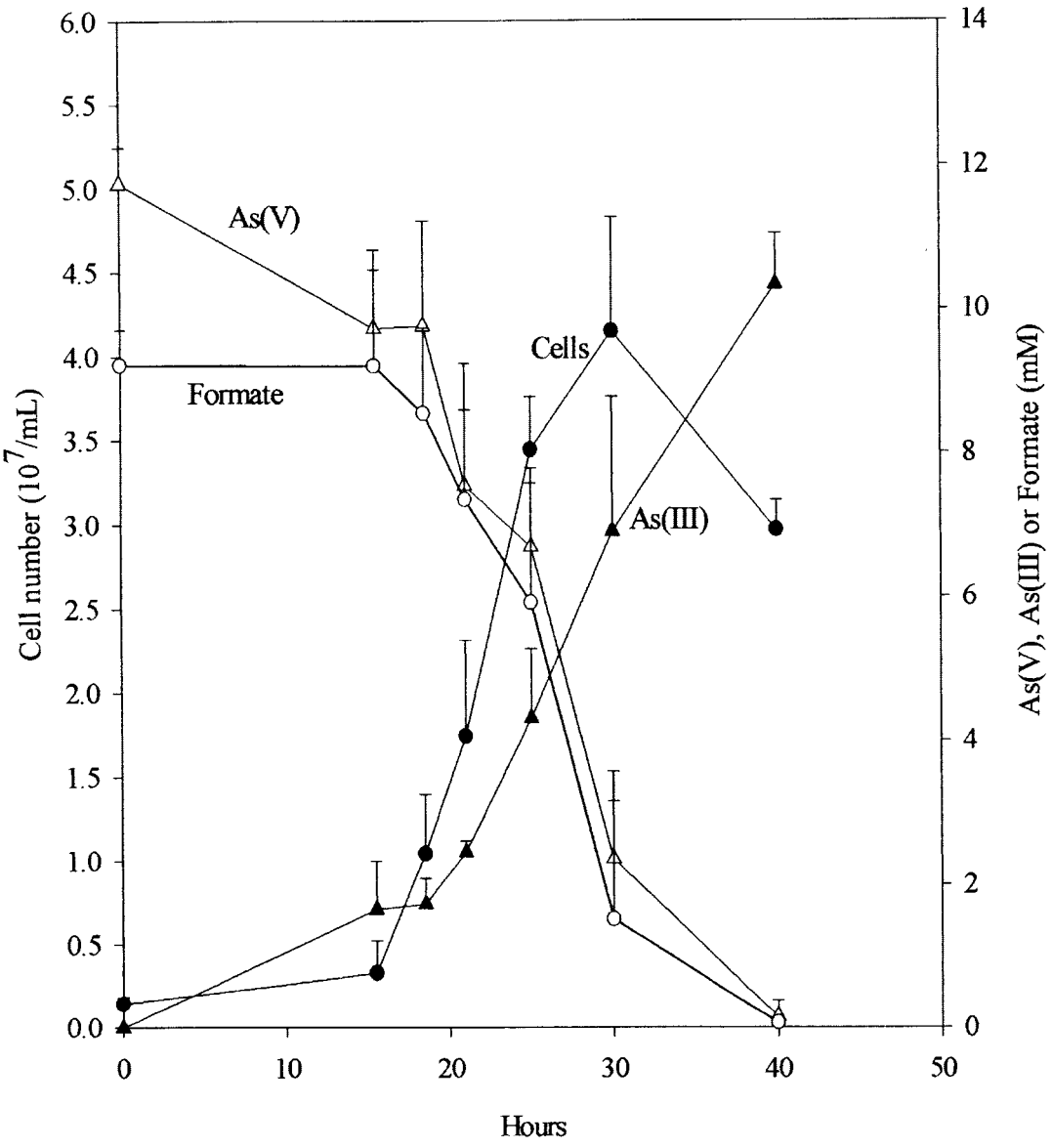

Figure 8. Growth curve of *D. frappieri* at pH 7.5 illustrating an increase in cell number (●), increase in As(III) (▲), decrease in As(V) (△), and decrease in formate (○). Experiments were performed in triplicate and error bars represent standard deviations.

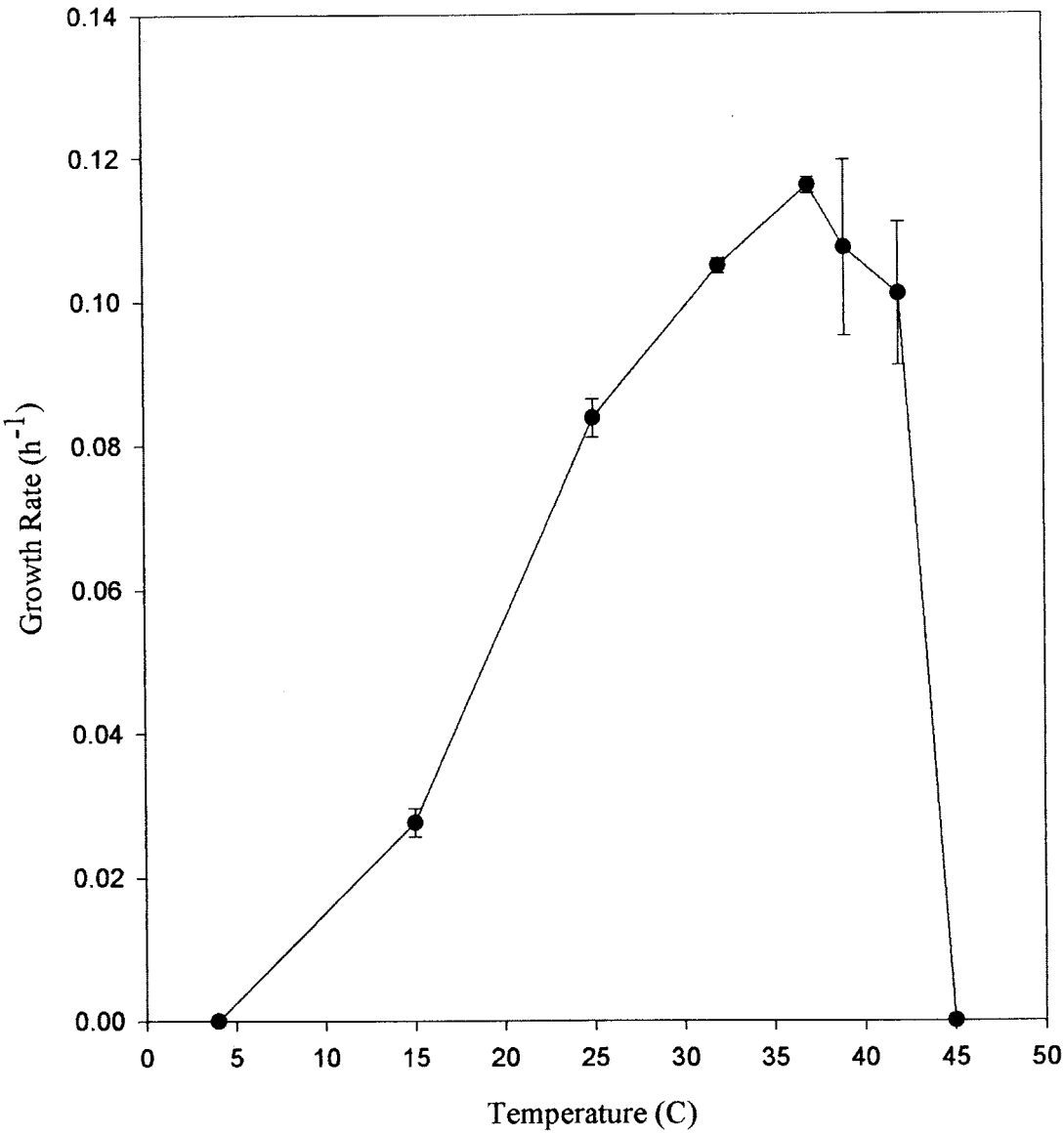

Figure 9. The effects of temperature on the specific growth rate (h^{-1}) of strain GBFH grown on 10 mM formate and 10 mM As(V). The pH of the media was 7.0. Experiments were performed in triplicate and error bars represent standard deviations.

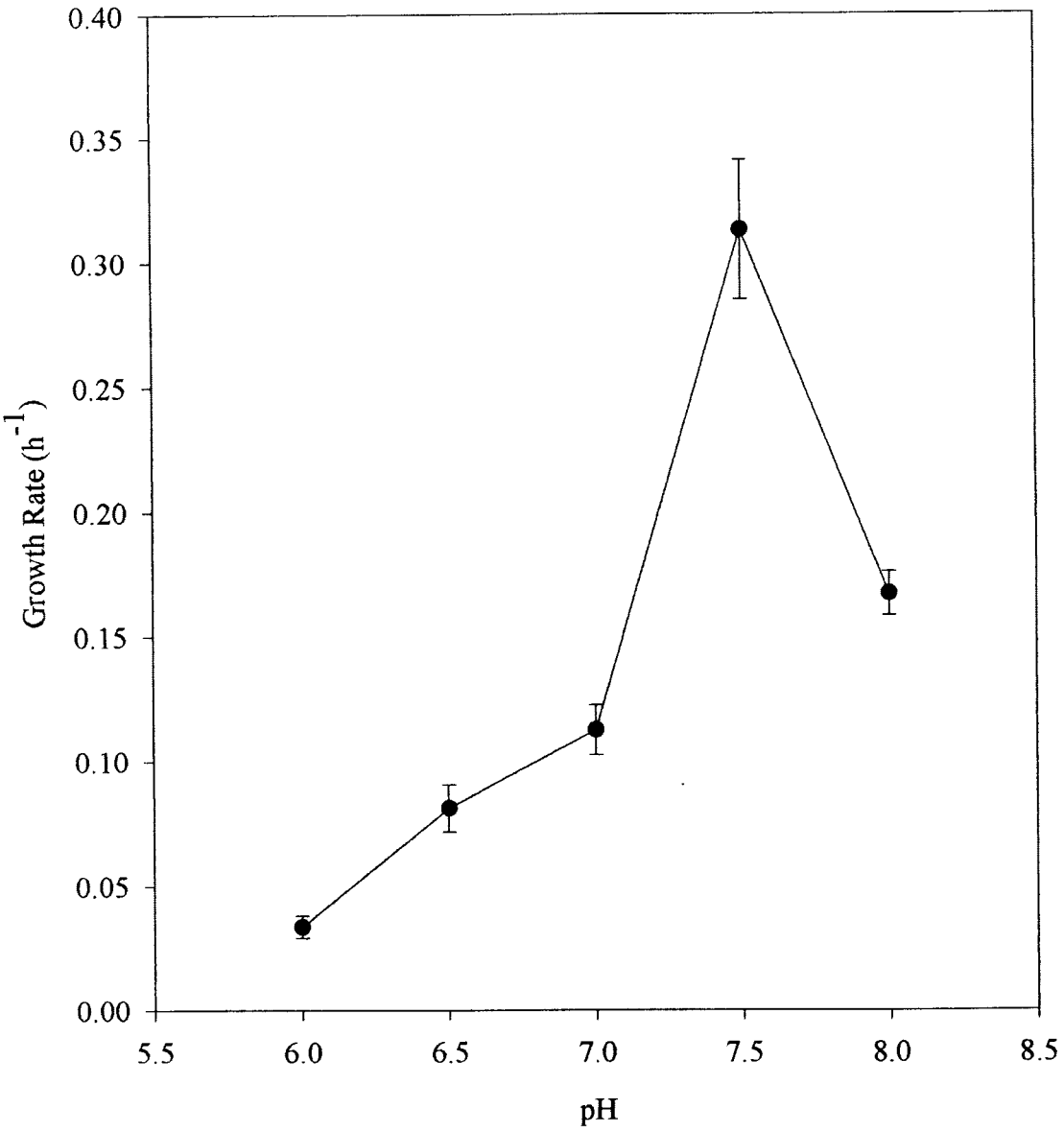

Figure 10. The effect of pH on the specific growth rate (h^{-1}) of strain GBFH grown on 10 mM formate and 10 mM As(V) at 37°C. Error bars represent standard deviations of triplicate cultures.

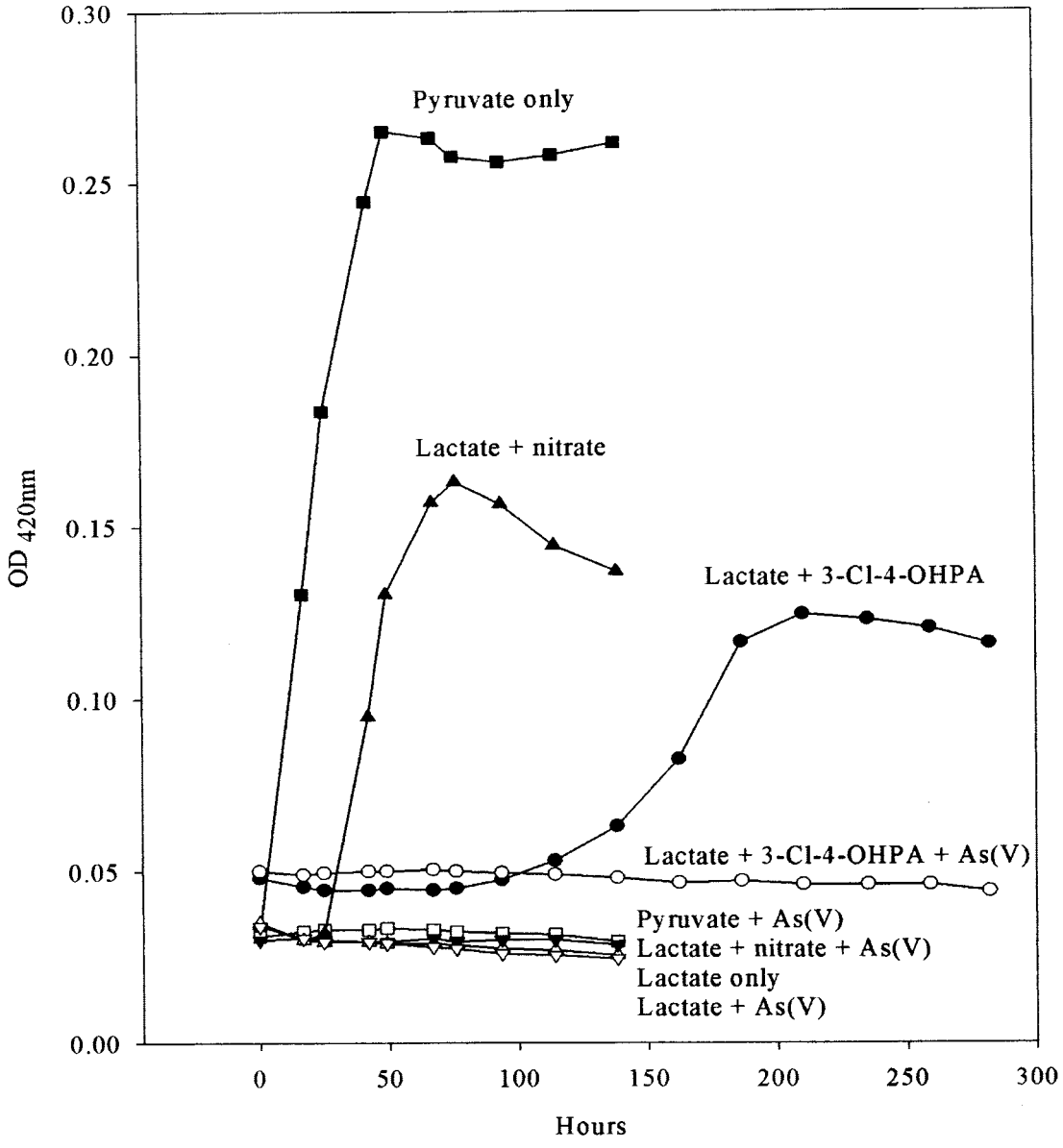

Figure 11. The growth of *D. dehalogenans* in the presence of As(V) and other electron acceptors. Cells grown fermentatively on pyruvate were inoculated into one of the following 8 treatments: (■) pyruvate only, (□) pyruvate + As(V), (●) lactate + 3-Cl-4-OHPA, (○) lactate + 3-Cl-4-OHPA + As(V), (▲) lactate + nitrate, (△) lactate + nitrate + As(V), (▼) lactate alone, (▽) lactate + As(V). Experiments were performed in duplicate and symbols represent average values.

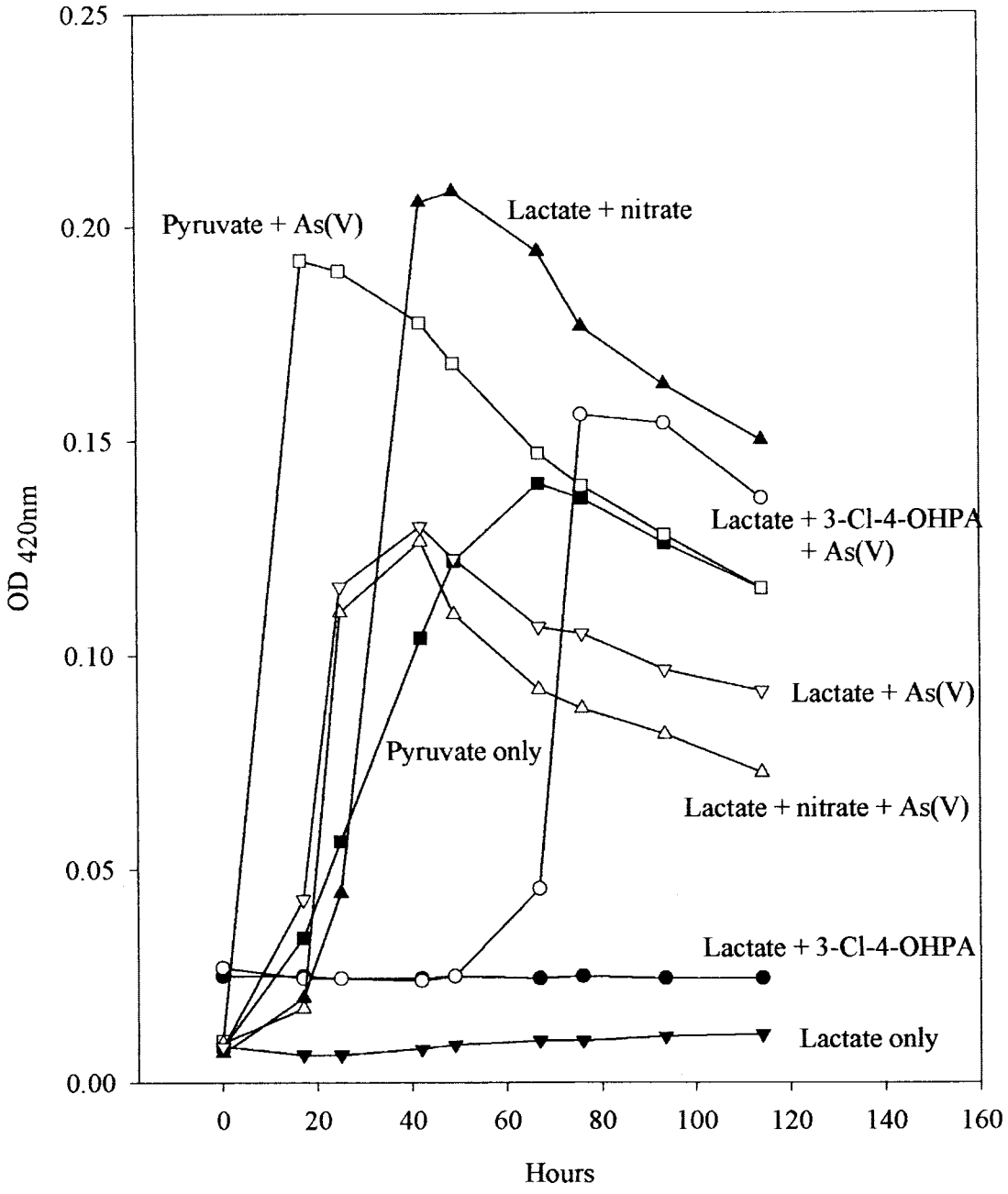

Figure 12. The growth of *D. hafniense* in the presence of As(V) and other electron acceptors. Cells grown fermentatively on pyruvate were inoculated intc one of the following 8 treatments: (■) pyruvate only, (□) pyruvate + As(V), (●) lactate + 3-Cl-4-OHPA, (○) lactate + 3-Cl-4-OHPA + As(V), (▲) lactate + nitrate, (△) lactate + nitrate + As(V), (▼) lactate alone, (▽) lactate + As(V). Experiments were performed in duplicate and symbols represent averave values.

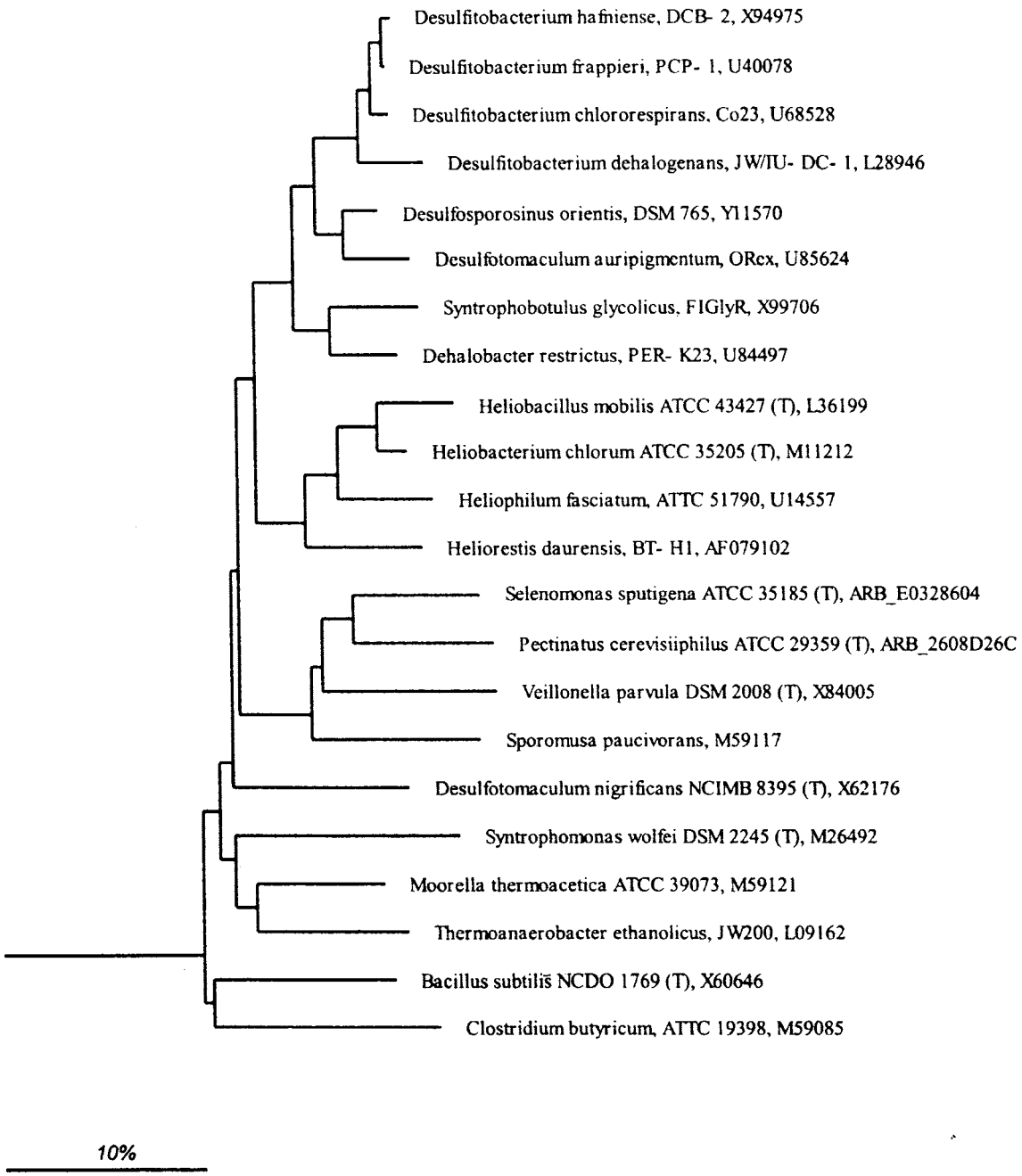

Figure 13. Phylogenetic tree illustrating placement of members of the genus *Desulfitobacterium* into low GC Gram positive clade.

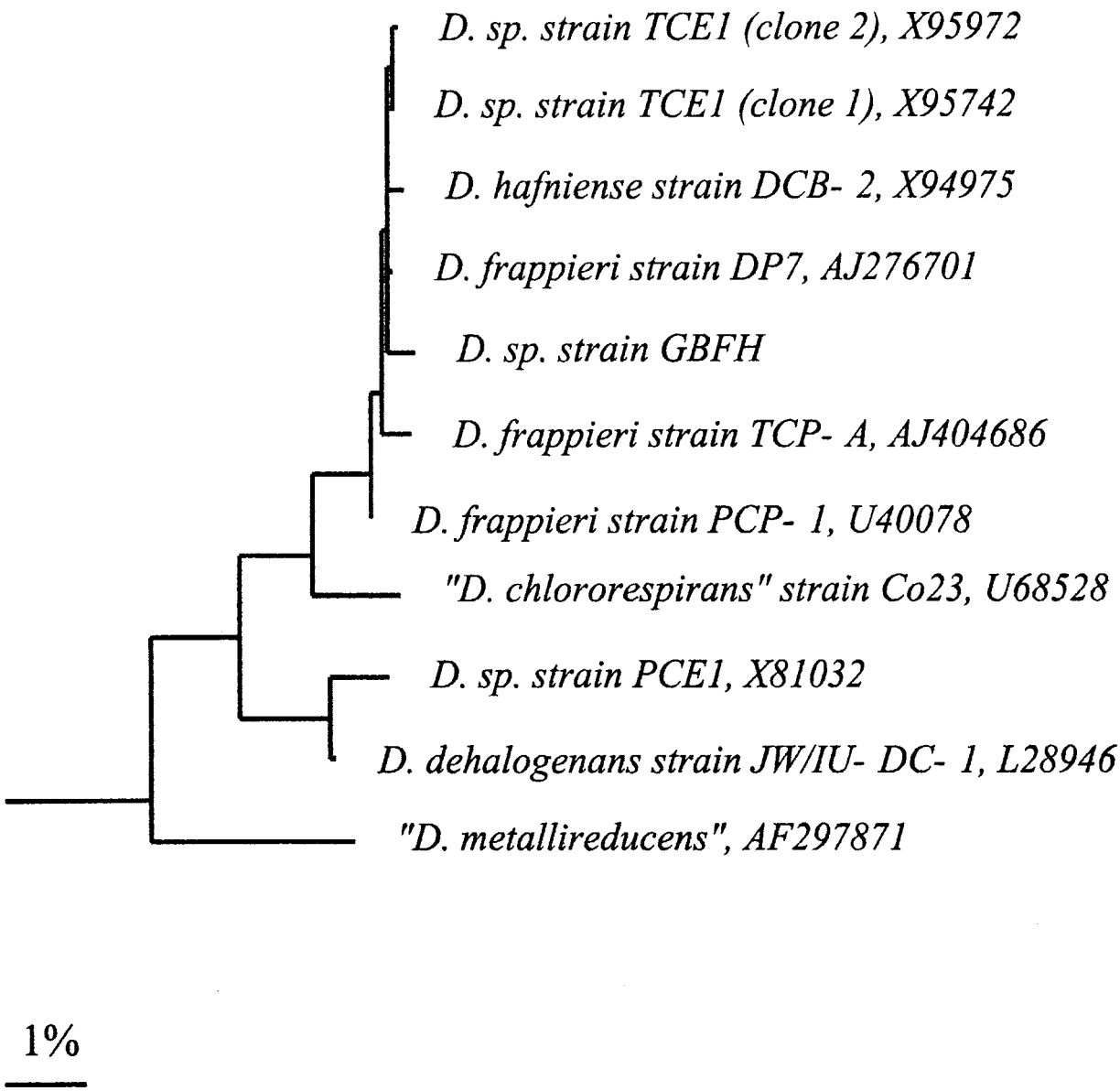

Figure 14. Phylogenetic tree illustrating relatedness of strain GBFH to other members of the genus *Desulfitobacterium*.

	MIT-13	SES-3	BAL-1	OREX-4	E1H	MLS10	BEN-RB[a]	PZ6
Electron donors								
Lactate	+	+	+	+	+	+	+	
Pyruvate	+	+	+	+	–	+		
H_2 + acetate	+	+	+	+	–	–		
Fumarate	+	+	+	–				
Malate			+	+	+	–		
Succinate		+	+	–	–	–		
Citrate		+	–	–	+	–		
Butyrate	+[b]			+				
H_2	–	–	–	+				+
Formate	+[b]	+[b]	–	–	–	–		
Acetate	–	–	+	–	–	–		–
Glucose			–	–	–	+		
Glycerol				+				
Ethanol				+	–	–		
Galactose					–	+[c]		
Methanol			–	–	–	–		
Electron acceptors								
As(V)	+	+	+	+	+	+	+	+
Nitrate	+	+	+	–	+	+		–
Nitrite		+	+		–	+		
Se(VI)		+	–		+	–		+
Se(IV)	–	–		–	–	+		–
Sulfate	–	–	–	+	–	–	+	
Thiosulfate		+	–	+	–	–		+
Sulfite		–		+	–	–		
S_0	–	+						+
Fe(III)		+	–	–	+	–		
Mn(IV)	–	+		–				
Fumarate	+	+		+	+	+		
Oxygen	–	–[d]	–	–	–	+[d]		–
Physiological optima								
Temperature optimum	20°C	33°C	27°C	25-30°C				95°C
pH optimum	7.5	7.5		6.4-7.0	8.5	9.5		
Doubling time	14 h	5 h	4 h				9 h	1.3 h

Table 1. A summary of relevant physiological characteristics of previously published dissimilatory As(V) reducing bacteria. Blank cells indicate that no data is available with regard to the specific variable; + growth is supported; – growth is not supported.

Data for MIT-13 are from (3, 63, 78); Data for SES-3 are from (44, 63, 78); Data fro BAL-1 are from (52); Data for OREX-4 are from (64); Data for E1H and MLS10 are from (6); Data for Ben-RB are from (51); Data for PZ6 are from (34).

[a]Physiological characterization is minimal with no formal survey of electron donors or acceptors; [b]Growth occurs only in the presence of acetate as a carbon source; [c]Growth is fermentative rather than repiratory; [d]Growth is microaerophilic.

Strain	Genus species designation	Major phylogenetic group (Domain)
MIT-13	*Sulfurospirillum arsenophilum*	ε-Proteobacteria (Bacteria)
SES-3	*Sulfurospirillum barnesii*	ε-Proteobacteria (Bacteria)
BAL-1	*Chrysiogenes arsenatis*	Deep branching near *Flexistipes* (Bacteria)
OREX-4	*Desulfotomaculum auripigmentum*	Low GC/Gram positive (Bacteria)
E1H	*Bacillus arsenicoselenatis*	Low GC/Gram positive (Bacteria)
MLS10	*Bacillus selenitireducens*	Low GC/Gram positive (Bacteria)
BEN-RB	*Desulfomicrobium* spp.	δ-Proteobacteria (Bacteria)
PZ6	*Pyrobaculum arsenaticum*	Chrenarchaeota (Archaea)

Table 2. Phylogenetic assignments of known DAsRB.

Data for MIT-13 are from (78); Data for SES-3 are from (78); Data from BAL-1 are from (52); Data for OREX-4 are from (64); Data for E1H and MLS10 are from (6); Data for Ben-RB are from (51); Data for PZ6 are from (34).

Carbon source (final concentration)	GBFH	*D. hafniense*	*D. frappieri*
Pyruvate (10 mM)	+	+	+
Pyruvate (10 mM – fermentative)	+	+	+
Lactate (10 mM)	+	+	+
Formate (10 mM)	+	+	+
Fumarate (10 mM)	+	+	+
Butyrate (10 mM)	-	+	+
Succinate (10 mM)	-	+	+
Malate (10 mM)	-	+	+
EtOH (5 mM)	-	+	+
Acetate (10 mM)	-	-	-
Glycerol (5 mM)	-	-	-
MeOH (5 mM)	-	-	-
Citrate (10 mM)	-	-	-
Benzoate (5 mM)	-	-	-
H_2 (10 mL)	-	-	-
H_2 + acetate (10 mL + 10 mM)	-	-	-
Propionate (10 mM)	-	-	-
Glucose (10 mM)	-	-	-
Galactose (10 mM)	-	-	-
No donor	-	-	-
Lactate (10 mM – fermentative)	-	-	-
Butyrate (5 mM - fermentative)	-	-	-

Table 3. Carbon source and electron donor utilization profiles for strain GBFH, *D. hafniense*, and *D. frappieri*. All carbon sources were tested in triplicate using 10 mM As(V) as the terminal electron acceptor under an atmosphere of 80:20 (N_2:CO_2). Cultures grown under fermentative conditions were not provided with As(V). +, growth was supported (see methods); - growth was not supported.

TEA (final concentration)	GBFH	*D. hafniense*	*D. frappieri*
Elemental S (10 mM)	+	+	+
Sulfite (5 mM)	+	+	+
Thiosulfate (10 mM)	+	+	+
Fumarate (10 mM)	+	+	+
MnO_2 (10 mM)	+	+	+
As(V) (10 mM)	+	+	+
Fe(III) gel (50 mM)	+	+	+
Fe-pyrophosphate (3 g/L)	+	+	+
Se(VI) (10 mM)	+	+	+
Nitrate (10 mM)	-	+	+
Nitrite (10 mM)	-	-	-
Sulfate (10 mM)	-	-	-
100% air	-	-	-
5% air	-	-	-
Se(IV) (10 mM)	-	-	-
As(III) (10 mM)	-	-	-
3-Cl-4-OHPA (10 mM)	-	-	-
No TEA	-	-	-

Table 4. Terminal electron accepting utilization profiles for strain GBFH, *D. hafniense*, and *D. frappieri*. All TEAs were tested in triplicate using 10 mM lactate as the carbon source and electron donor under an atmosphere of 80:20 (N_2:CO_2). +, growth was supported (see methods); - growth was not supported.

Strain	*D. frappieri* DSM 12420^T	*D. hafniense* DSM 10664^T	Strain GBFH
D. frappieri DSM 12420^T	X		
D. hafniense DSM 10664^T	88.7	X	
Strain GBFH	81.2	79.7	X
D. dehalogenans DSM 916^T	45.9	43.9	40.6

Table 5. DNA:DNA reassociation values of type strains of *Desulfitobacterium* species and strain GBFH.

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