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Carbohydrate oxidation coupled to Fe(III) reduction, a novel form of anaerobic metabolism

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An isolate, designated GC-29, that could incompletely oxidize glucose to acetate and carbon dioxide with Fe(III) serving as the electron acceptor was recovered from freshwater sediments of the Potomac River, Maryland. This metabolism yielded energy to support cell growth. Strain GC-29 is a facultatively anaerobic, Gram-negative motile rod which, in addition to glucose, also used sucrose, lactate, pyruvate, yeast extract, casamino acids or H_2 as alternative electron donors for Fe(III) reduction. Stain GC-29 could reduce NO^-_3, Mn(IV), U(VI), fumarate, malate, $S_2O_3^{2-}$, and colloidal S^0 as well as the humics analog, 2,6-anthraquinone disulfonate. Analysis of the almost complete 16S rRNA sequence indicated that strain GC-29 belongs in the *Shewanella* genus in the epsilon subdivision of the *Proteobacteria*. The name *Shewanella saccharophilia* is proposed. *Shewanella saccharophilia* differs from previously described fermentative microorganisms that metabolize glucose with the reduction of Fe(III) because it transfers significantly more electron equivalents to Fe(III); acetate and carbon dioxide are the only products of glucose metabolism; energy is conserved from Fe(III) reduction; and glucose is not metabolized in the absence of Fe(III). The metabolism of organisms like *S. saccharophilia* may account for the fact that glucose is metabolized primarily to acetate and carbon dioxide in a variety of sediments in which Fe(III) reduction is the terminal electron accepting process.

Introduction

Microbial Fe(III) reduction is an important mechanism for the oxidation of both natural and anthropogenic organic matter in a broad diversity of environments [1,2]. Over 30 organisms which can conserve energy to support growth from the oxidation of organic compounds coupled to the reduction of Fe(III) have been characterized [3]. Electron acceptors for Fe(III) reduction in these organisms include: fatty acids, ethanol, the amino acid proline, various monoaromatics, and H_2. However, the only Fe(III) reducers previously known to metabolize sugars are fermentative microorganisms in which Fe(III) reduction is a

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minor side reaction to their main fermentative metabolism. These organisms typically transfer less than 5% of the reducing equivalents from sugars metabolized to Fe(III) and Fe(III) reduction by these organisms is not essential to support growth [1,4].

The complete oxidation of glucose to carbon dioxide with Fe(III) serving as the sole electron acceptor is energetically favorable and thus could potentially support the growth of Fe(III)-reducers [5]. However, attempts to isolate such organisms have been unsuccessful. Furthermore, studies on the metabolism of [^{14}C]glucose in a variety of sediments in which Fe(III) reduction was the terminal electron-accepting process demonstrated that glucose is not directly oxidized completely to carbon dioxide, but rather is first converted primarily to acetate and carbon dioxide [5]. This indicated that even if there are microorganisms which, by themselves, can completely oxidize glucose to carbon dioxide with Fe(III) serving as the sole electron acceptor, they do not account for a significant fraction of the glucose metabolism in Fe(III)-reducing sediments. Previously described fermentative microorganisms which use Fe(III) reduction as a trivial electron sink cannot account for the metabolism of glucose to primarily acetate in Fe(III)-reducing sediments because these organisms produce a variety of fermentation acids and alcohols, even when Fe(III) is in abundance [4].

Here we report on a novel metabolism in which glucose is oxidized to acetate and carbon dioxide with Fe(III) serving as the sole electron acceptor. This metabolism provides a potential pure culture model for the major pathway of glucose metabolism that has been observed in sediments in which Fe(III) reduction is the primary terminal electron-accepting process.

Materials and Methods

Source of organism

Sediment cores were collected with a 5-cm diameter plastic core tube near the previously described [6] site in the Potomac River, MD. The top 2.5 cm of the cores were sectioned and stored in a glass canning jar for transport to the laboratory. All subsequent manipulations were carried out with strict anaerobic technique [7,8]. An anaerobic freshwater medium was dispensed into anaerobic pressure tubes as previously described [9]. H_2 (101 kPa) was the electron donor, and ferric iron chelated with nitrilotriacetic acid (Fe(III)-NTA) (10 mM) was the potential electron acceptor. Acetate (0.1 mM) was added as a carbon source. Serial 10-fold dilutions of the sediment were made in triplicate and then each dilution tube was amended with 3 ml of 4% molten noble agar (Difco) which had been washed prior to use as previously described [10]. The tubes were immediately plunged into an ice bucket after gentle mixing. The agar dilution sets were incubated inverted at 25°C in the dark. As described in the Results, isolated colonies from these dilutions were studied further.

Electron donors and acceptors

Electron donors and acceptors were added from sterile anoxic aqueous stocks as previously outlined [9] with the exception of benzene and toluene which were directly added (1 µl) to 10 ml volumes of the growth media. Soluble Fe(III) was supplied as Fe(III)-citrate (50 mM) [11], Fe(III)-NTA (10 mM) [12], or Fe(III)-pyrophosphate (10 mM) [13]. When noted, Fe(III) was also supplied as poorly crystalline amorphous Fe(III)-oxide (100 mM) which was prepared as previously described [6]. Mn(IV) was provided as synthetic MnO_2 [11] to give a final concentration of 10–30 mmol per liter. U(VI) was added as UO_2Cl_2. S^c was supplied as a polysulfide solution prepared as previously outlined [10]. All other electron acceptors were added as the sodium salts to give final concentrations of 10 mM. All incubations of the isolate were at 30°C.

Analytical techniques

Fe(II) was measured with the ferrozine assay after HCl extraction as previously described [11]. Sulfide concentrations was assayed with methylene blue as previously described [14]. U(VI) concentrations were determined under anaerobic conditions with a kinetic phosphorescence analyzer (KPA-10) as previously described [15]. Reduction of the humics analog, anthraquinone-2,6-disulfonate, was monitored as the production of anthrahydroquinone-2,6-disulfonate as previously described [16]. Cell growth was determined by direct cell counts with epiflourescent microscopy with acridine orange stain [17] or by increase in absorbance at 600 nm (A_{600}). Cytochrome content of whole-cell suspensions was identified by dithionite-reduced minus air-oxidized difference spectra on acetate-fumarate grown cultures as previously described [18]. Glucose was measured colorimetrically with a glucose-oxidase enzymatic kit (Sigma Chemicals, MO). Acetate concentrations were measured by gas chromatography with flame ionization detection. Volatile fatty acids were separated with a 2 m × 2 mm ID glass column packed with 80/100 Carbopack C/0.2% Carbowax packing. The column temperature was set at 120°C with injector and detector temperatures at 180°C.

16S rRNA gene sequencing and phylogenetic analysis

The nearly complete 16S rRNA gene of strain GC-29 was amplified and sequenced from a 1:10 dilution of a liquid culture using primers 8F and 1492R [19,20] as described previously [21]. Sequencing was performed at the Michigan State University Sequencing Facility.

16S rRNA sequences related to the 16S rDNA sequence of strain GC-29 were identified with the BlastN program [22]. The 16S rDNA sequence of strain GC-29 was manually aligned against closely related aligned sequences obtained from the Ribosomal Database Project [23]. A distance matrix tree was constructed with the least-squares algorithm of De Soete [24], with evolutionary distances computed by the method of Jukes and Cantor [25].

The GenBank and EMBL accession numbers for the sequences used in the phylogenetic analysis are as follows: *C. pasteurianum*, M29390; *D. palmitatis*, U28172; *F. balearica*, X93021; *G. chapelleii*, U41561; *G. metallireducens*, LO7834; *S. alga*, X81622; *S. benthica*, X82131; *S. hanedai*, X82132; *S. putrefaciens*, X81623. The aligned 16S rRNA sequence of *E. coli* was obtained from the Ribosomal Database Project [23]. The 16S rDNA sequence of strain GC-29 (accession number AF033028) has been submitted to GenBank.

DNA–DNA hybridization

DNA–DNA hybridizations were performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) using the spectroscopic method of De Ley [26] with modifications by Huss *et al.* [27] as previously described [28].

Results

Isolation

Small colonies were apparent in the Fe(III)-NTA agar dilution tubes after 1 month of incubation. The colonies ranged from 0.5 to 2 mm in diameter and were black, white or pink. Several of the pink colonies were surrounded by zones of clearing in the orange-colored agar and were selected for further purification. Colonies were picked from the 10^{-4} and 10^{-5} dilution tubes and transferred into fresh media amended with Fe(III)-NTA (10 mM), H_2 (101 kPa) and acetate (0.1 mM). Three morphologically identical isolates were obtained and one, strain GC-29, was selected for further characterization.

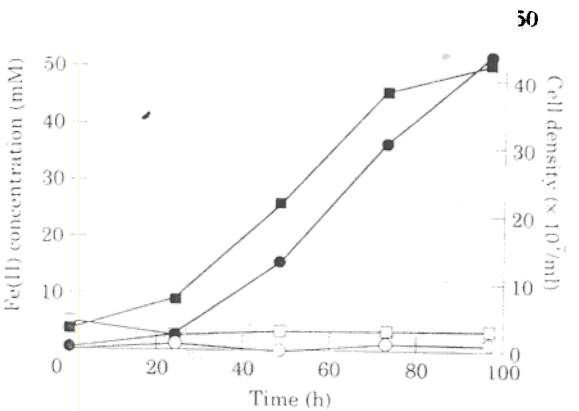

Figure 1. Growth of strain GC-29 with glucose (10 mM) as the sole electron donor and Fe(III)-citrate (50 mM) as the sole electron acceptor. The results are means of triplicate determinations. (■), Fe(II), 10 mM glucose; (□), Fe(II), no glucose; (●), cells, 10 mM glucose; (○), cells, no glucose.

Morphology

Strain GC-29 is a facultatively anaerobic, Gramnegative, motile, non-sporeforming, non-fermenting rod, 0.5 by 2.0–2.5 μm. When growing aerobically on tryptic soy broth agar plates, colonies are pink, smooth, and domed, approximately 4 to 5 mm in diameter. A dithionite-reduced minus air-oxidized spectrum of a strain GC-29 cell suspension had absorption maxima at 423, 523, and 552 nm which is indicative of the presence of *c*-type cytochrome(s). Strain GC-29 grew in a temperature range of 25 to 35°C with an optimum between 30 and 35°C and at salinities of up to 4% NaCl.

Glucose oxidation coupled to Fe(III) reduction

Strain GC-29 grew anaerobically with glucose as the electron donor and Fe(III) as the electron acceptor (Figure 1). There was no growth on glucose in the absence of Fe(III). Glucose oxidation coupled to Fe(III) reduction resulted in the accumulation of acetate. The ratio of Fe(II) and acetate produced to glucose consumed was 7.53 ± 0.25 and 1.73 ± 0.22 mmol, respectively (mean ± standard deviation, n=3) per mmol of glucose consumed which, when probable incorporation of carbon into the cells is considered, is consistent with the reaction:

$$C_6H_{12}O_6 + 2H_2O + 8Fe^{3+} \rightarrow 2CH_3COOH + 2CO_2 + 8Fe^{2+} + 8H^+$$

Aerobically, strain GC-29 completely oxidized glucose to CO_2 with no production of acetate.

Table 1. Compounds tested as electron donors in the presence of Fe(III)-NTA (10 mM) concentrations are given in parentheses

Utilized
Pyruvate (10 mM), lactate (10 mM), formate (10 mM), H_2 (101 kPa), yeast extract (1g/l), casamino acids (1 g/l), glucose (10 mM), sucrose (10 mM)
Not utilized
Acetate (10 mM), propionate (5 mM), butyrate (5 mM), palmitate (1 mM), benzoate (0.5 mM), succinate (1 mM), fumarate (10 mM), citrate (10 mM), methanol (10 mM), ethanol (10 mM), phenol (0.5 mM), methane (101 kPa), toluene (1 mM), benzene (1 mM), cyclohexane (1 mM), octane (1 mM), eicosine (0.35 mM), nitrilotriacetic acid (5 mM), arabinose (10 mM), xylose (5 mM), fructose (5 mM), starch (0.2%).

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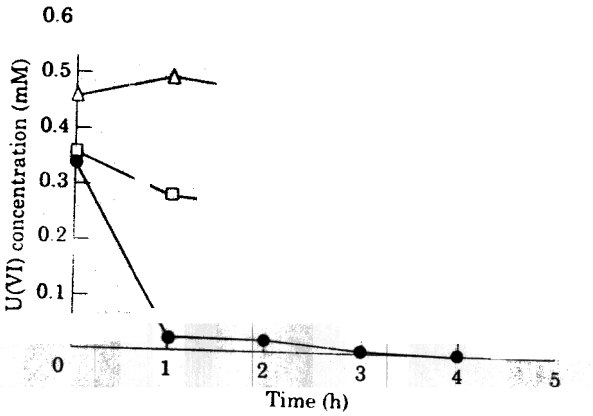

Figure 2. U(VI) reduction by strain GC-29 with H_2 as the sol electron donor. (●), U(VI), 101 kPa hydrogen; (□), U(VI), n hydrogen; (○), no cells.

Other electron donors for Fe(III) reduction included sucrose, H_2, formate, lactate, and pyruvate (Table 1). Further investigation of lactate metabolism indicated that it was incompletely oxidized to acetate. Yeast extract, or casamino acids also served as electron donors for Fe(III) reduction, but strain GC-29 did not grow in a defined medium with Fe(III)-NTA as the electron acceptor when the twenty amino acids were supplied at a final concentration of 1 mM in each of the following combinations; (i) glycine, cysteine, lysine, phenylalanine, arginine; (ii) serine, methionine, glutamine, valine, tyrosine; (iii) leucine, asparagine, histidine, proline, glutamic acid, alanine; (iv) aspartic acid, tryptophan, isoleucine, cystine, threonine. A variety of other potential electron donors were also not utilized (Table 1).

In addition to Fe(III)-NTA, strain GC-29 could grow with other Fe(III) forms such as Fe(III)-citrate, Fe(III)-pyrophosphate, Fe(III)-EDTA, and poorly crystalline Fe(III)-oxide. NO_3^-, Mn(IV), fumarate, malate, 2,6-anthraquinone disulfonate, thiosulfate, and elemental sulfur also served as electron acceptors with lactate as the electron donor. There was no growth when sulfate or sulfite were provided as potential electron acceptors.

Cell suspensions of strain GC-29 reduced U(VI) to U(IV) with H_2 as the electron donor (Figure 2). U(VI) reduction resulted in the formation of a precipitate, presumably the U(IV) mineral uraninite [29].

Phylogenetic analysis and DNA–DNA hybridization

Comparative analysis of the almost complete 16S rDNA sequence of strain GC-29 indicated that it belongs to the genus *Shewanella* in the gamma subdivision of the *Proteobacteria* (Figure 3). DNA–DNA hybridization analysis of strain GC-29, *Shewanella putrefaciens*, and *Shewanella alga* demonstrated that these organisms shared less than 40% sequence homology (Table 2) which indicates that each is a separate species [30].

Discussion

These results document the first microorganism known to conserve energy to support growth by coupling the oxidation of glucose to the reduction of Fe(III). Although it was previously suggested that some strains of *Shewanella putrefaciens* might use glucose it was not clear whether this was associated with Fe(III) reduction and no data on glucose utilization nor a description of the strains was provided [31]. The only other organisms previously shown to transfer electrons from glucose to Fe(III)

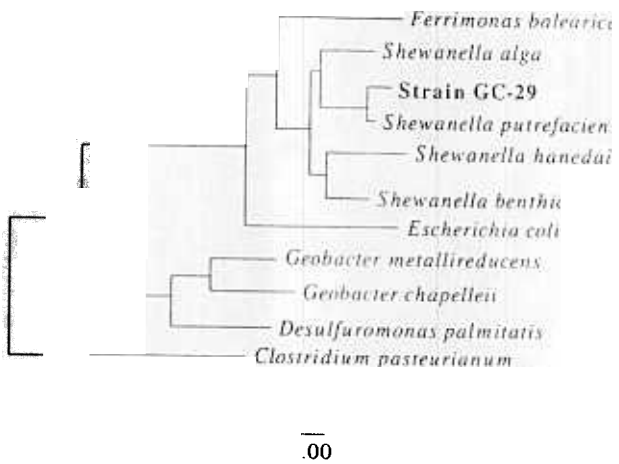

Figure 3. Phylogenetic tree inferred from 16S rDNA sequences showing the placement of strain GC-29 within the genus *Shewanella*. The tree was inferred by the distance matrix method from 1126 positions. The bar represents one evolutionary distance unit.

Table 2. DNA–DNA hybridization results of strain GC-29 with other *Shewanella* species

		Homolog	
pecies	S. *putrefacien* strain 8071	S. *alga* strain BrY	strain GC-29
putrefaciens rain 8071			
alga strain BrY			

were fermentative bacteria in which Fe(III) reduction is only a trivial electron sink in the overall glucose metabolism. Fermentative bacteria typically transfer less than 5% of the electron equivalents available in fermentable substrates to Fe(III) [4]. In contrast, strain GC-29 transfers a third of the electron equivalents in glucose to Fe(III). Acetate is the sole organic end product of glucose metabolism by strain GC-29 under Fe(III)-reducing conditions whereas fermentative bacteria which use Fe(III) reduction as a minor side reaction produce a variety of organic fermentation products. Thus, strain GC-29's metabolism represents a newly recognized pathway for processing glucose in anaerobic environments.

Potential environmental significance

The metabolism of strain GC-29 provides a potential model for the previous observation [5] that glucose is primarily metabolized to acetate and carbon dioxide in sediments in which Fe(III) reduction is the terminal electron-accepting process. Although glucose metabolism in these sediments also resulted in the production of other fermentation acids such as lactate and propionate, they were produced in lower relative proportions than in methanogenic sediments in which typical fermentative bacteria were presumably responsible for glucose metabolism. This result is consistent with microorganisms with a metabolism like strain GC-29 coupling the oxidation of glucose to acetate and carbon dioxide with the reduction of Fe(III).

If metabolism like that of strain GC-29 accounts for a significant proportion of the processing of fermentable sugars in Fe(III)-reducing sediments, then this could have implications for the importance of H_2 as an intermediate in electron flow in Fe(III)-reducing environments. Previous models for carbon and electron flow in Fe(III)-reducing environments have assumed that the formation of acetate and other fermentation acids is associated with concomitant H_2 production [1,2,5]. Thus, H_2 consumption by H_2-oxidizing Fe(III) reducers was considered to be an important pathway for Fe(III) reduction. However, if Fe(III)-reducing microorganisms such as strain GC-29 can convert sugars to acetate and carbon dioxide without the production of H_2 in sediments, then H_2 may be only a minor electron donor for Fe(III) reduction. The fact that Fe(III)-reducers can directly oxidize a variety of other compounds such as fatty acids, aromatic compounds, and at least one amino acid to carbon dioxide without the production of H_2 [3] suggests that there may be few sources of H_2 in Fe(III)-reducing environments. If so, this further emphasizes that microorganisms such as those found in the family *Geobacteraceae* [21], that are capable of completely oxidizing acetate and other organics to carbon dioxide are likely to account for most of the Fe(III) reduction in sedimentary environments [1,2].

Comparison of strain GC-29 with other Fe(III)-reducers

With the exception of its ability to utilize glucose, strain GC-29 shares many physiological characteristics with previously described Fe(III)-reducers. Like other *Shewanella* species [3], it incompletely oxidizes a limited number of multicarbon substrates to acetate, can oxidize H_2, contains *c*-type cytochromes, and can reduce a wide variety of electron acceptors including the toxic metal U(VI) and the humics analog 2,6-anthraquinone disulfonate. Microbial reduction of 2,6-anthraquinone disulfonate has been shown to serve as a good indicator for the ability to grow via reduction of humic substances [16,32].

Shewanella species are differentiated into two main clusters, the *Shewanella alga* group and the *Shewanella putrefaciens* group [33]. The growth temperature range of 25–35°C and an NaCl tolerance of up to 4% suggests that strain GC-29 falls into the *Shewanella putrefaciens* cluster. Based on DNA–DNA hybridization strain GC-29 is representative of a new species. The name *Shewanella saccharophilia* is proposed.

Acknowledgements

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