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Electron acceptor availability alters carbon and energy metabolism in a thermoacidophile

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Summary

The thermoacidophilic *Acidianus* strain DS80 displays versatility in its energy metabolism and can grow autotrophically and heterotrophically with elemental sulfur (S⁰), ferric iron (Fe³⁺) or oxygen (O₂) as electron acceptors. Here, we show that autotrophic and heterotrophic growth with S⁰ as the electron acceptor is obligately dependent on hydrogen (H₂) as electron donor; organic substrates such as acetate can only serve as a carbon source. In contrast, organic substrates such as acetate can serve as electron donor and carbon source for Fe³⁺ or O₂ grown cells. During growth on S⁰ or Fe³⁺ with H₂ as an electron donor, the amount of CO₂ assimilated into biomass decreased when cultures were provided with acetate. The addition of CO₂ to cultures decreased the amount of acetate mineralized and assimilated and increased cell production in H₂/Fe³⁺ grown cells but had no effect on H₂/S⁰ grown cells. In acetate/Fe³⁺ grown cells, the presence of H₂ decreased the amount of acetate mineralized as CO₂ in cultures compared to those without H₂. These results indicate that electron acceptor availability constrains the variety of carbon sources used by this strain. Addition of H₂ to cultures overcomes this limitation and alters heterotrophic metabolism.

Introduction

Hydrothermal environments can be dynamic and vary chemically or physically on time scales that range from

seconds (e.g., earthquakes) to days (e.g., temperature variations and diurnal cycling) to seasons (e.g., precipitation) (Hurwitz and Lowenstem, 2014). A common adaptive strategy for microorganisms that inhabit dynamic environments, such as hot springs, is to harbor flexibility in their metabolism (Kassen, 2002). Among characterized thermophilic organisms, crenarchaeotes may exhibit the most flexible of lifestyles as they relate to carbon and energy metabolism (Dworkin, 2006). For example, several members of the Sulfolobales, in particular those within the *Acidianus* genus, have been characterized as being facultatively anaerobic, facultatively autotrophic and capable of using a variety of complex organic and inorganic compounds to support their energy metabolism (Huber and Prangishvili, 2006; Huber and Stetter, 2006).

Common among all characterized members of the *Acidianus* genus is the ability to utilize elemental sulfur (S⁰) as an electron donor and/or acceptor, regardless of their sensitivity to O₂ and their mode of carbon metabolism (Huber and Stetter, 2015). Despite exhibiting an overall metabolically flexible lifestyle, however, several S⁰ metabolizing thermophiles exhibit characteristics during growth on certain substrates that are more reflective of specialization. For example, the crenarchaeotes *Acidianus ambivalens* (Sulfolobales; Zillig *et al.*, 1986; Laska *et al.*, 2003) and *Stetteria hydrogenophila* (Desulfurococcales; Jochimsen *et al.*, 1997) were shown to be incapable of organoheterotrophic (i.e., organic energy and carbon source) growth via reduction of S⁰, but exhibited an ability to grow autotrophically via reduction of S⁰. Autotrophic growth with S⁰ as oxidant was shown to be obligately dependent on hydrogen (H₂) as the electron donor. Potential reasons for the obligate requirement for H₂ during S⁰ reduction in these strains were not further explored.

Acidianus is common in acidic, high-temperature, sulfur-rich hot spring environments (Huber and Stetter, 2015), including those in Yellowstone National Park (YNP), Wyoming, USA (Seegerer *et al.*, 1986; Inskeep *et al.*, 2010; Hochstein *et al.*, 2016). We previously isolated an *Acidianus* strain (strain DS80) from a sulfur-rich, acidic hot spring in YNP (Amenabar *et al.*, 2017). Like other *Acidianus* strains (Seegerer *et al.*, 1986; Zillig *et al.*, 1986; Plumb *et al.*, 2007; Giaveno *et al.*, 2013), strain DS80 displayed versatility in its energy metabolism

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involving H_2 or S° as electron donors and S° or ferric iron (Fe^{3+}) as electron acceptors (Amenabar *et al.*, 2017). The strain was also shown to be capable of aerobic autotrophic growth with S° serving as electron donor and aerobic organoheterotrophic growth using a variety of substrates. Intriguingly, under anaerobic lithotrophic growth conditions, DS80 cells provided with $H_2/Fe^{3+}/S^\circ$ utilized the H_2/S° redox couple rather than the H_2/Fe^{3+} or S°/Fe^{3+} redox couples, despite the lower energy yield associated with the former (Amenabar *et al.*, 2017). We suggested that the use of the H_2/S° redox couple was due to more thermodynamically efficient coupling of electron transfer reactions in an evolutionarily optimized membrane-bound [NiFe]-hydrogenase and membrane-associated sulfur reductase, which co-purify as a complex in *A. ambivalens* (Laska *et al.*, 2003). Evolutionary optimization to efficiently couple electron transfer reactions between H_2 and S° in strain DS80, and potentially other strains (e.g., *A. ambivalens* and *S. hydrogenophila*), may lead to specificity in the source of reductant that can be used to reduce S° via the sulfur reductase complex. If true, this may explain why S° reducing *A. ambivalens* and *S. hydrogenophila* cells were shown to be obligately dependent on H_2 as electron donor. In turn, such specificity may affect the carbon metabolism of S° reducing cells, especially if organic compounds cannot be used as electron donors in energy metabolism.

In the present study, we aimed to test whether growth with S° as an oxidant is limited to H_2 as a reductant and, if so, what effect this might have on the carbon metabolism of the cell. To achieve this goal, we characterized the ability of alternative substrates (as carbon sources and/or electron donors) to support growth in DS80 cells provided with a variety of oxidants, including S° , Fe^{3+} , or O_2 . Second, we aimed to determine whether H_2 influenced the use of inorganic and organic carbon sources in cells grown with S° , Fe^{3+} or O_2 as oxidant. Putative inorganic and organic carbon sources capable of supporting growth in strain DS80 were identified using genome reconstruction followed by homology-based informatics approaches. Physiological analyses were then used to examine carbon metabolism in cells provided with S° , Fe^{3+} or O_2 in the presence or absence of H_2 . Results are discussed in the context of the role of electron acceptor availability in dictating the variety of electron donor and carbon sources that can support the growth of strain DS80. Moreover, the role of H_2 in allowing heterotrophic growth (chemolithoheterotrophy) in cells reducing S° is discussed, with an emphasis placed on biochemical mechanisms potentially explaining observed patterns of electron donor and carbon source usage with different electron acceptors in this and other thermophilic strains.

Results

Genomic prediction of electron donor, electron acceptor and carbon source usage

A partial genome for DS80 was reported previously (Amenabar *et al.*, 2017) and was shown to code for sulfur reductase (Sre) and pilin proteins that might be involved in reduction of S° and Fe^{3+} with H_2 as electron donor and CO_2 as a carbon source, respectively; c-type multiheme cytochromes putatively involved in Fe^{3+} reduction in bacteria (Myers and Myers, 1997; Leang *et al.*, 2003) were not detected in the genome. Thus, the exact mechanism used by strain DS80 to reduce Fe^{3+} is unknown. The partial genome of DS80 also encodes proteins with homology to those that allow use of a variety of single carbon compounds as putative electron donors or carbon sources (Fig. 1). For example, a complement of genes encoding the majority (15 out of 16) of the enzymes involved in the hydroxypropionate–hydroxybutyrate pathway of CO_2 fixation was identified in the DS80 genome, consistent with the ability of strain DS80 to grow with CO_2 as the sole carbon source (Amenabar *et al.*, 2017). The gene encoding for methylmalonyl-CoA epimerase was not detected in the genome. However, this enzyme is not required for this pathway to be functional (Berg *et al.*, 2010b). A full suite of genes encoding a Ni-dependent carbon monoxide dehydrogenase (CODH), an ‘H’-type formate dehydrogenase (FDH-H) and a membrane bound [NiFe]-hydrogenase (Hyn) were also identified in the genome of DS80 (Supporting Information Fig. S1). Synteny in the genes coding for Hyn and Sre in the genomes of strain DS80 and *A. ambivalens* (Laska *et al.*, 2003) suggest that a similar mechanism of coupling H_2 oxidation with S° reduction like that described for *A. ambivalens* (Laska *et al.*, 2003) could be operational in strain DS80 (Fig. 1).

The DS80 genome was shown to encode several other enzymes putatively involved in sulfur metabolism, including a cytoplasmic sulfur oxygenase reductase (SOR) that has been shown or hypothesized to function in S° oxidation in other members of the *Acidianus* genus such as *Acidianus brierleyi* (Emmel *et al.*, 1986), *A. ambivalens* (Kletzin, 1989) and *Acidianus tengchongensis* (He *et al.*, 2000). In addition to inorganic electron donors and carbon sources, the genome of DS80 coded for numerous sugar transporters and glycosidases suggesting an ability to utilize carbohydrates as carbon sources or energy substrates. Moreover, homologs of genes predicted to encode amino acid and oligopeptide transporters, as well as genes encoding proteolytic enzymes including endopeptidases, aminopeptidases and carboxypeptidases were identified in the DS80 genome, suggesting an ability for the cell to hydrolyze peptides and oxidize amino acids. Genes encoding a complete suite of homologs of

Fig. 1. Diagram of metabolic capabilities of *Acidianus* strain DS80.

Abbreviations: APH, acylphosphate phosphohydrolase; CODH/ACS, carbon monoxide dehydrogenase/acetyl-CoA synthase; CP, carbamoyl phosphate synthetase; FDH, formate dehydrogenase; FUM, fumarate hydratase; GDH, glutamate dehydrogenase; GNC, gluconeogenesis; GOGAT, glutamate synthase; GS, glutamine synthetase; HHC, hydroxypropionate-hydroxybutyrate cycle; KGOR, 2-oxoglutarate ferredoxin oxidoreductase; ME, malic enzyme; NDH, NADH dehydrogenase; PAT, phosphate acetyltransferase; PFOR, pyruvate:ferredoxin oxidoreductase; Q, quinone; redFd, reduced ferredoxin; SDH, succinate dehydrogenase; SOR, sulfur oxygenase reductase; TQO, thiosulfate quinone oxidoreductase; TTH, tetrathionate hydrolase. Extracellular electron transport via pili has been hypothesized to be involved in iron reduction in *Acidianus* strain DS80 previously (Amenabar et al., 2017). The hypothetical model of S^0 oxidation and reduction were modified from Kletzin and colleagues (2004). Only those metabolic reactions and pathways that are the focus of this article are indicated. [Colour figure can be viewed at wileyonlinelibrary.com]

the tricarboxylic acid (TCA) cycle, gluconeogenesis and the non-phosphorylative Entner–Doudoroff pathway (Fig. 1 and Supporting Information Fig. S2) were also detected in the DS80 genome, suggesting an ability for this strain to utilize organic substrates during organotrophic metabolism. A homolog of the pyruvate ferredoxin oxidoreductase (PFOR), putatively involved in the carboxylation of acetyl-CoA to pyruvate (Furdui and Ragsdale, 2000), was also detected in the DS80 genome, consistent with the ability of strain DS80 to assimilate acetate through the gluconeogenic pathway (see below for more details). Other genes encoding for homologs of enzymes putatively involved in acetate assimilation such

as those of the glyoxylate shunt (Kornberg and Krebs, 1957) or the ethylmalonyl-CoA pathway (Erb et al., 2007) were not detected in DS80 genome.

Physiological examination of carbon source and electron donor usage

To test genome-based predictions of the ability of DS80 to utilize a wide variety of substrates to support growth, cultures were provided with a range of inorganic (CO_2 , CO) and organic compounds (acetate, formate, glucose, fructose, galactose, mannose, lactose, maltose, sucrose, yeast extract, peptone and casamino acids) as carbon

sources (100% H₂ headspace) or as carbon and energy sources (without H₂ in the head space) with S⁰, Fe³⁺ and O₂ as electron acceptors. Importantly, the presence of H₂ in growth assays containing an organic carbon source does not necessarily guarantee that H₂ is used as the sole electron donor or that these organic substrates are used solely as a carbon source. However, the comparison of growth in medium supplied with and without H₂ helps to differentiate if these substrates can serve as a carbon source or a primary reductant. Substrate reduction and oxidation activities and the production of cells were monitored in these growth assays (Table 1).

Cells coupled the oxidation of numerous inorganic (i.e., CO) and organic carbon sources (i.e., acetate, formate, glucose, galactose, mannose, lactose, maltose, yeast extract, peptone and casamino acids) to the reduction of Fe³⁺ to sustain growth under anaerobic conditions, regardless of whether H₂ was provided to cultures. This indicates that these inorganic and organic substrates can be used as both a carbon source and as an electron donor. In contrast, when S⁰ served as electron acceptor (anaerobic conditions), these inorganic and organic carbon sources could only support growth if H₂ was also provided in what can be described as chemolithoheterotrophic metabolism (i.e., inorganic energy source with an organic compound as the source of carbon). This indicates that CO, formate, acetate, glucose, galactose, mannose, lactose, maltose, yeast extract, peptone and casamino acids can serve as a carbon source but not as the primary reductant during growth with S⁰ as electron acceptor. The addition of CO₂ to cultures provided with these carbon sources

did not allow growth under S⁰ reducing conditions in the absence of H₂ (Supporting Information Table S1), indicating that the lack of growth was not due to cells being obligate mixotrophs with respect to their carbon metabolism. Fructose and sucrose did not support growth of DS80 under any of the growth conditions tested. Most of the organic substrates tested could support growth under aerobic conditions, and growth under these conditions was not dependent on the presence of H₂ (Table 1).

Carbon assimilation and mineralization

Growth experiments indicating that DS80 could not couple organotrophic metabolism with S⁰ reduction but could couple organotrophic metabolism to Fe³⁺ and O₂ reduction prompted additional experiments using ¹⁴C radio-labeled acetate to determine the ability of strain DS80 to assimilate and/or oxidize (mineralize) acetate with different electron acceptors. In cultures provided with ¹⁴C-acetate/H₂/S⁰, the density of cells, the amount of acetate assimilated and the concentration of total sulfide increased concurrently (Fig. 2A). ¹⁴CO₂ was not detected above background levels in the headspace of these cultures suggesting that if any acetate was mineralized to CO₂, it was likely simultaneously assimilated into biomass. However, the data presented in Table 1 suggest that acetate is primarily used as a carbon source and not as the primary electron donor when S⁰ served as electron acceptor. Indeed, no evidence for acetate oxidation/mineralization or assimilation was observed in cultures without H₂ provided with S⁰ as the terminal electron acceptor and acetate as the sole carbon and electron source (data not shown), consistent with growth assays indicating that this cultivation condition did not support growth.

In cultures provided with ¹⁴C-acetate/H₂/Fe³⁺, the cell density, the amount of acetate assimilated and the concentration of total Fe²⁺ increased concurrently (Fig. 2B). ¹⁴CO₂ was detected in the headspace indicating that acetate was also being oxidized/mineralized under this condition. However, cultures provided with Fe³⁺ as the terminal electron acceptor and acetate as a carbon and electron source (no added H₂) had a significantly greater amount of ¹⁴CO₂ headspace than cultures provided with H₂ (Fig. 2C). This indicates that H₂ can serve as an additional source of reductant in Fe³⁺ reducing cells grown with acetate and/or that cells are simultaneously assimilating acetate and the CO₂ released from acetate mineralization.

The cell yield per mol of Fe³⁺ reduced, calculated during log phase (72–168 h), when grown with ¹⁴C-acetate/H₂/Fe³⁺, was 0.6-fold lower than when cells were grown with acetate as the sole carbon and energy source (1.1 versus 1.8 cells per fmol Fe³⁺ reduced, respectively) (*P* = 0.04). This suggests that cells were more efficient at coupling energy metabolism with biomass synthesis when acetate

Table 1. Growth of *Acidianus* strain DS80 in base salts medium (80°C; pH 3.0) with specified carbon sources when S⁰, Fe³⁺ or O₂ served as electron acceptor.

Carbon source	S ⁰		Fe ³⁺		O ₂
	H ₂ (+)	H ₂ (–)	H ₂ (+)	H ₂ (–)	H ₂ (–)
CO ₂	+	N/D	+	N/D	N/D
CO	+	–	+	+	N/D
Formate	+	–	+	+	+
Acetate	+	–	+	+	+
Glucose	+	–	+	+	+
Fructose	–	–	–	–	–
Galactose	+	–	+	+	+
Mannose	+	–	+	+	+
Lactose	+	–	+	+	+
Maltose	+	–	+	+	+
Sucrose	–	–	–	–	–
Yeast extract	+	–	+	+	+
Peptone	+	–	+	+	+
Casamino acids	+	–	+	+	+

The ability for each carbon source to support growth was tested in the presence (+) and absence (–) of H₂. +, growth [denotes whether a given growth condition supported a significant (*P* < 0.05) increase in the number of cells at any point during growth, when compared to the number of cells at time zero]; –, no growth; N/D, not determined.

Fig. 2. Growth and substrate transformation activities of cultures of *Acidianus* strain DS80 provided with H_2 , acetate (a portion of the acetate provided was enriched in $1\text{-}[^{14}\text{C}]$ acetate) and S^0 (A), H_2 , acetate (a portion of the acetate provided was enriched in $1\text{-}[^{14}\text{C}]$ acetate) and Fe^{3+} [provided as $\text{Fe}_2(\text{SO}_4)_3$] (B) or with acetate (a portion of the acetate provided was enriched in $1\text{-}[^{14}\text{C}]$ acetate) and Fe^{3+} [provided as $\text{Fe}_2(\text{SO}_4)_3$] (C). No increase in cell density, acetate mineralization/assimilation activity or S^0 reduction activity was observed when cultures were provided with only S^0 and acetate (data not shown). Error bars reflect the standard deviation of average measurements made on three replicate cultures.

served as both the energy and carbon source. The amount of C assimilated per cell in log phase cultures (72–168 h) when grown with ^{14}C -acetate/ $\text{H}_2/\text{Fe}^{3+}$

(0.3 ± 0.1 fmol C cell $^{-1}$) was not significantly different ($P = 0.36$) than the amount observed when grown without H_2 (0.5 ± 0.2 fmol C cell $^{-1}$). These data suggest that electrons recovered from H_2 are involved in less efficient respiratory chains in DS80 than electrons from acetate under Fe^{3+} reducing conditions. The production of $^{14}\text{CO}_2$ in the headspace of ^{14}C -acetate/ $\text{H}_2/\text{Fe}^{3+}$ grown cultures (Fig. 2B) suggests that this strain may be growing mixotrophically with respect to its energy metabolism when it is provided with acetate.

Influence of acetate on DIC assimilation

To explore the effects of acetate on DIC assimilation, DS80 cells were provided with $^{14}\text{CO}_2/\text{H}_2/\text{S}^0$ or $^{14}\text{CO}_2/\text{H}_2/\text{Fe}^{3+}$ and these cultures were amended with different concentrations of nonisotopically enriched acetate (up to 200 μM). In cultures provided with H_2/S^0 , the amount of DIC assimilated into biomass decreased systematically with higher concentrations of acetate, relative to unamended controls (Fig. 3A). However, the magnitude of the suppression of DIC assimilation was not proportional to the amount of acetate provided, in particular when cells were provided with 100 μM or greater acetate. This indicates that acetate was not limiting growth and that cells were assimilating DIC in the presence of acetate. Moreover, despite evidence indicating that acetate suppresses DIC assimilation in cells provided with H_2/S^0 (Fig. 3A), amendment of cultures with acetate had no effect on the production of cells (Fig. 3B). This indicates that acetate, when provided at concentrations of 200 μM or less, is not toxic to the cells due to internal acidification, as has been described for other thermoacidophiles (Urschel *et al.*, 2016). Together, these results indicate that cells provided with H_2/S^0 are simultaneously using DIC and acetate to meet their carbon demands in what can be termed mixotrophy with respect to their carbon metabolism.

Like cells provided with $^{14}\text{CO}_2/\text{H}_2/\text{S}^0$, rates of DIC assimilation in cells provided with $^{14}\text{CO}_2/\text{H}_2/\text{Fe}^{3+}$ decreased systematically by amendment with higher concentrations of acetate (Fig. 3C). The magnitude of the suppression of DIC assimilation was not proportional to the amount of acetate provided, and amendment with acetate also did not influence the production of cells (Fig. 3D). Together, these observations indicate that cells provided with $\text{H}_2/\text{Fe}^{3+}$ are simultaneously using DIC and acetate to meet their carbon demands in what can be described as mixotrophy with respect to both their carbon and their energy metabolism.

Influence of DIC on acetate assimilation and mineralization

To explore the effects of DIC on acetate assimilation, DS80 cells were provided with ^{14}C -acetate/ H_2/S^0 or

Fig. 3. DIC assimilation and the number of cells produced in cultures of *Acidianus* strain DS80 provided with H₂, S⁰ and CO₂ (a portion of the CO₂ provided was enriched in ¹⁴C) (A and B, respectively) or H₂, Fe³⁺ [provided as Fe₂(SO₄)₃] and CO₂ (a portion of the CO₂ provided was enriched in ¹⁴C) (C and D, respectively) amended with 0, 50, 100 or 200 μM acetate. Error bars reflect the standard deviation of average measurements made on three separate cultures.

¹⁴C-acetate/H₂/Fe³⁺ and these cultures were amended with nonisotopically enriched CO₂. The addition of CO₂ did not significantly affect the final number of cells produced at stationary phase in cultures provided with ¹⁴C-acetate/H₂/S⁰ (Fig. 4A), when compared to those not provided with CO₂ (3.93×10^6 versus 3.72×10^6 cells mL⁻¹, respectively) ($P = 0.28$) (Fig. 2A). In contrast, the addition of CO₂ to cultures provided with ¹⁴C-acetate/H₂/Fe³⁺ (Fig. 4B) resulted in a 311% increase in the number of cells produced at stationary phase, when compared to cells provided with only ¹⁴C-acetate/H₂/Fe³⁺ (9.72×10^6 versus 2.36×10^6 cells mL⁻¹, respectively) ($P < 0.01$) (Fig. 2B). At stationary phase, the ratio of acetate mineralized to that assimilated was 1:4 for ¹⁴C-acetate/H₂/Fe³⁺ grown cells in the absence of CO₂ (Fig. 2B) versus 3:4

for cells grown in the presence of CO₂ (Fig. 4B), which is likely due to a decreased reliance on acetate as the sole source of carbon (i.e., cells are fixing CO₂). Consistent with this interpretation, ¹⁴C-acetate/H₂/Fe³⁺ grown cells provided with CO₂ assimilated 0.7 fmol of acetate per cell at stationary phase when compared to 3.7 fmol of acetate per cell when ¹⁴C-acetate/H₂/Fe³⁺ grown cells were not provided with CO₂. These observations confirm that strain DS80 is mixotrophic with respect to both its carbon and energy metabolism, when Fe³⁺ is provided as an electron acceptor. Moreover, ¹⁴C-acetate/H₂/Fe³⁺ grown cells provided with CO₂ mineralized 25 fmol of acetate per cell at stationary phase when compared to 50 fmol of acetate mineralized per cell when ¹⁴C-acetate/H₂/Fe³⁺ grown cells were not provided with CO₂. This may

Fig. 4. Acetate assimilation and mineralization (a portion of the acetate provided was enriched in $1\text{-}[^{14}\text{C}]$ acetate) in the presence of CO_2 (headspace of 20/80%, vol/vol, CO_2/H_2) in cultures of *Acidianus* strain DS80 provided with H_2 , S° , CO_2 and 100 μM total acetate (A) or with H_2 , Fe^{3+} [provided as $\text{Fe}_2(\text{SO}_4)_3$], CO_2 and 100 μM acetate (B). Error bars reflect the standard deviation of average measurements made on three separate cultures. The concentration of acetate remaining in culture medium (residual acetate) was calculated by subtracting the total amount of acetate that was used (mineralized and assimilated) from the total amount of acetate added (mass balance).

suggest that a portion of the acetate oxidized in cells grown with ^{14}C -acetate/ $\text{H}_2/\text{Fe}^{3+}$ is to generate CO_2 as a substrate for gluconeogenic carboxylation reactions.

Discussion

Genomic and physiological characterization indicates that *Acidianus* strain DS80, like other *Acidianus* strains (Seeger *et al.*, 1986; Zillig *et al.*, 1986; Bettstetter *et al.*, 2003; He *et al.*, 2004; Yoshida *et al.*, 2006; Plumb *et al.*, 2007; Giaveno *et al.*, 2013), exhibits flexibility in its metabolism at the level of electron donors, electron acceptors and carbon sources capable of supporting its metabolism. However, flexibility in the energy and carbon metabolism of strain DS80 was shown to have its limitations. For example, strain DS80 was capable of growing mixotrophically with respect to its carbon metabolism

(simultaneous assimilation of CO_2 and organic carbon) and energy metabolism (simultaneous oxidation of H_2 and acetate) when Fe^{3+} served as electron acceptor; mixotrophic growth was not rigorously examined when O_2 served as electron acceptor. In contrast to growth with Fe^{3+} as electron acceptor, cells provided with S° as electron acceptor could only grow mixotrophically with respect to carbon metabolism (assimilation of CO_2 and acetate) and this activity was obligately dependent on H_2 as an electron donor. This observation is like those made for *S. hydrogenophila* and for *A. ambivalens*, both of which were shown to be obligately dependent on H_2 as electron donor during S° dependent growth (Zillig *et al.*, 1986; Jochimsen *et al.*, 1997).

Despite genomic data indicating an apparent ability to utilize a range of organic substrates as electron and carbon requirements, organic substrates cannot serve as the sole electron donor when S° served as electron acceptor. Rather, H_2 is required as an electron donor in acetate oxidizing, S° reducing cells. It is likely that cells grown with acetate/ $\text{H}_2/\text{S}^\circ$ or acetate/ $\text{H}_2/\text{Fe}^{3+}$ (or other organic compounds) are oxidizing at least a portion of the acetate, since CO_2 is needed to drive the carboxylation of acetyl CoA to pyruvate via the activity of PFOR prior to entry into the gluconeogenic pathway during chemolithoheterotrophic growth (Supporting Information Fig. S2). A low potential electron donor such as ferredoxin (Fd) is also required to drive the reductive carboxylation of acetyl-CoA to pyruvate by the PFOR enzyme (Furdui and Ragsdale, 2000). Since the redox potential of some Fds are close to that of the H_2 electrode (Buckel and Thauer, 2013), it is possible that Fd is reduced by the activity of the [NiFe]-hydrogenase encoded in the DS80 genome. Indeed, a protein encoded in an open reading frame [Integrated Microbial Genomes (IMG) gene ID: 2690452627] adjacent to the large subunit of [NiFe]-hydrogenase is homologous to a small Rieske Fd from *A. ambivalens* (Genbank accession number: CAC86888). Biochemical characterization of a recombinant form of this Fd from *A. ambivalens* indicates that the protein harbors a high potential (+170 mV) Rieske-like iron sulfur (FeS) cluster (Kletzin *et al.*, 2005). This indicates that the FeS cluster in this Fd may be reduced by H_2 (~ -420 mV; Buckel and Thauer, 2013) via [NiFe]-hydrogenase but is unlikely to be of low enough potential to carboxylate acetyl CoA to yield pyruvate (~ -500 mV) (Thauer *et al.*, 1977).

Other reactions have been shown to produce reduced Fd under chemo(litho)heterotrophic growth conditions to support PFOR activity. One of these involves the oxidative decarboxylation of 2-oxoglutarate to form succinyl-CoA, CO_2 and reduced Fd via the activity of 2-oxoglutarate ferredoxin oxidoreductase (KGOR) (Mai and Adams, 1996). Two copies of the alpha and beta subunits of the KGOR were detected in the genome of

A. hospitalis (You *et al.*, 2011) and in the partial genome of strain DS80 (IMG gene IDs: 2690452354/2690452355 and 2690452739/2690452738). The presence of genes encoding for homologs of the KGOR in DS80 could potentially explain how reduced Fd and CO₂ are being generated as substrates for PFOR during chemolithoheterotrophic growth with acetate/H₂/S⁰ or acetate/H₂/Fe³⁺ (or other organic compounds).

While reduced Fd is likely required during chemolithoheterotrophic growth in strain DS80, reduced Fd might not be required for autotrophic growth. This conjecture is based on previous reports suggesting that under autotrophic conditions, gluconeogenesis in aerobic Sulfolobales does not proceed via acetyl-CoA, since acetyl-CoA was shown to not be assimilated via the Fd-dependent carboxylation activity of pyruvate synthase (Berg *et al.*, 2010b; Estelmann *et al.*, 2011). Rather, succinyl-CoA, which is withdrawn from the carbon fixation cycle via oxidation to malate or oxaloacetate, serves as a precursor for biosynthesis/gluconeogenesis in aerobic Sulfolobales (Berg *et al.*, 2010b; Estelmann *et al.*, 2011). Pyruvate and phosphoenolpyruvate (PEP) formation likely proceeds from these two C4 compounds via the activities of the malic enzyme and PEP carboxykinase respectively (Berg *et al.*, 2010b; Estelmann *et al.*, 2011). While these pathways for Fd independent generation of pyruvate and PEP as substrates for gluconeogenesis have been described in aerobic Sulfolobales (Berg *et al.*, 2010b; Estelmann *et al.*, 2011), it has also been shown that under anaerobic autotrophic conditions other Sulfolobales such as *Stygiolobus azoricus* do not use PFOR to generate pyruvate from acetyl-CoA. Rather, these cells appear to use the malic enzyme to generate pyruvate from malate as input to the gluconeogenesis pathway (Berg *et al.*, 2010a) similar to what has been reported for Sulfolobales grown aerobically. Like *S. azoricus*, the genome of strain DS80 encodes for a homolog of the malic enzyme (IMG gene ID: 2690453198) suggesting potential involvement of this enzyme in Fd-independent pyruvate formation and gluconeogenesis during anaerobic, autotrophic growth.

The glyoxylate shunt (Kornberg and Krebs, 1957) or the ethylmalonyl-CoA (Erb *et al.*, 2007) pathways are not involved in acetate assimilation in DS80 since homologs of the enzymes comprising these pathways were not detected in the DS80 genome. Thus, we suggest that the lack of detectable CO₂ in cultures provided with acetate/H₂/S⁰ was due to its use as a substrate in carboxylation reactions (Fig. 2A). Evidence in potential support of this conjecture comes from the observation that cells are capable of simultaneously assimilating both acetate and CO₂ when S⁰ serves as oxidant (Fig. 3A). Since addition of CO₂ to acetate/S⁰ grown cultures also did not allow growth under what would be considered mixotrophic

conditions (Table S1), we conclude that acetate cannot serve as the primary electron donor in S⁰ grown cells. Rather, acetate is primarily used as a carbon source with H₂ serving as the primary electron donor to cells growing autotrophically or heterotrophically with S⁰ as oxidant. The dependence on H₂ as the primary electron donor during organotrophic growth with S⁰ as electron acceptor can be described as chemolithoheterotrophic metabolism, which has been shown (albeit not necessarily defined as such) in sulfur oxidizing Bacteria (King, 2007; Ghosh and Dam, 2009) and sulfur-reducing Archaea (Jochimsen *et al.*, 1997).

Several possible explanations exist for why members of the genus *Acidianus* may not be able to respire S⁰ using the organic compounds tested here. The first involves the cellular localization of the Sre complex, which may constrain delivery of electrons to the complex due to specificity of the Sre for electron carriers. Based on genomic inferences, respiration of S⁰ with H₂ in hyperthermophiles, including *Thermoproteus tenax* (Siebers *et al.*, 2011), *A. ambivalens* (Laska *et al.*, 2003) and presumably DS80 (see Supporting information) is mediated by a short electron transfer chain comprising a membrane-associated [NiFe]-hydrogenase and a membrane-associated (Sre1) complex linked by a quinone cycle (Laska *et al.*, 2003) (Fig. 5A). Respiration with S⁰ as electron acceptor in these strains is like the membrane-bound respiratory system found in mesophilic S⁰/polysulfide reducing bacteria such as *Wolinella succinogenes* (Hedderich *et al.*, 1998). The Sre1 operon in *T. tenax*, *A. ambivalens* and *Acidianus* strain DS80 codes for five subunits (ABCDE), with the alpha subunit (SreA) coding for a twin arginine motif indicating that it is localized on the external side of the plasma membrane. However, the genome of *T. tenax* also codes for a second Sre operon (Sre2) that includes only three subunits (ABC); none of the subunits code for twin-arginine motifs indicating a cytoplasmic orientation of this membrane bound complex (Siebers *et al.*, 2011) (Fig. 5B), which may influence the source of reductants that can be used to reduce this complex.

Unlike *A. ambivalens* and strain DS80, *T. tenax* can respire S⁰ using organic compounds, which are oxidized via the oxidative TCA cycle. During organotrophic growth, *T. tenax* conserves energy via a membrane-bound electron transport chain with S⁰ as the terminal electron acceptor (Siebers *et al.*, 2004, 2011). In addition to organotrophic growth coupled to S⁰ reduction, *Thermoproteus* strain CP80 can grow autotrophically with H₂ and S⁰ (Urschel *et al.*, 2016); the genome of CP80 encodes both cytoplasmic (Sre2) and extracellular oriented (Sre1) Sre complexes. We suggest that differences in the orientation of the Sre complex (extracellular versus cytoplasmic) allow the use of electron donors present in the cytosol by

Fig. 5. Diagrams of proposed pathways for S° reduction with H_2 or organic carbon substrates as sources of reductant. Abbreviations: Sre, sulfur reductase; Hyd, group 1 [NiFe]-hydrogenase; TCA, tricarboxylic acid cycle; O, outside of cell; M, membrane; C, cytosol. [Colour figure can be viewed at wileyonlinelibrary.com]

Sre2, including NAD(P)H produced from the TCA cycle. This enables electrons derived from oxidation of organic carbon to be coupled to the reduction of S° (Fig. 5B).

A second possible explanation for the difference in the ability to respire S° with organic electron donors in the Thermoproteales (i.e., *Thermoproteus* spp.) but not the Sulfolobales (i.e., *Acidianus* spp.) is in the types of co-factors involved in electron transfer to S° . For example, it has been suggested that succinate dehydrogenase (complex II) in *T. tenax* (Siebers *et al.*, 2004, 2011) is involved in the reduction of S° when organic compounds serve as electron donor. Succinate dehydrogenase is a membrane-bound component of the TCA cycle and can provide an entry point for electrons derived from the oxidation of succinate into the membrane quinone pool, which can then be used to reduce S° via the membrane bound Sre (Siebers *et al.*, 2004, 2011) (Fig. 5C). Like *T. tenax*, members of the Sulfolobales, including DS80 and *A. ambivalens*, code for succinate dehydrogenase (Lemos *et al.*, 2001; Schafer *et al.*, 2002). However, as-isolated succinate dehydrogenase from *A. ambivalens* was shown to contain bound caldariellaquinone (Lemos

et al., 2001; Schafer *et al.*, 2002), suggesting that electrons are transferred during succinate oxidation to this specific quinone pool. Biochemical studies indicate that caldariellaquinone cannot be used to reduce membrane-bound Sre in *A. ambivalens* (Laska *et al.*, 2003). Rather, the specific quinone that is used to reduce Sre in *A. ambivalens* is sulfolobusquinone (Laska *et al.*, 2003), which differs structurally from caldariellaquinone in that it lacks a mercaptan side chain (Collins and Langworthy, 1983; Thurl *et al.*, 1986; Elling *et al.*, 2016).

While caldariellaquinone is produced by *A. ambivalens*, it is usually only found in the membranes of aerobically grown cells and not S° grown cells (Laska *et al.*, 2003). Similarly, caldariellaquinone is the predominant quinone in aerobically grown *Sulfolobus solfataricus* cells; sulfolobusquinone is detected in greater amounts in *S. solfataricus* cells grown under progressively O_2 limited conditions (Nicolaus *et al.*, 1992). Thus, differences in the specificity of the membrane bound Sre complex for sulfolobusquinone versus caldariellaquinone may be another reason why DS80, and *Acidianus* strains in general, cannot link organic carbon oxidation to the reduction of S° . Further

evidence that this phenomenon is due to the specificity of Sre for sulfobolusquinone rather than for the [NiFe]-hydrogenase for specific quinones comes from the fact that electrons from H₂ (presumably generated via the sole [NiFe]-hydrogenase homolog encoded in the DS80 genome) can be used to reduce Fe³⁺, albeit with a low efficiency of energy conservation as indicated by a 0.6-fold reduction in cell yields when H₂ is provided to acetate/Fe³⁺ + grown cells when compared to those not provided H₂.

The ability of strain DS80 to grow with organic substrates as sole electron donors when respiring Fe³⁺ or O₂ (Table 1) but not with other oxidants (i.e., S⁰) is a potentially widespread phenomenon among hyperthermophiles. For example, *Ferroglobus placidus* cannot utilize acetate as the sole electron donor when either nitrate or thiosulfate were provided as electron acceptor (Hafenbradl *et al.*, 1996). However, acetate was shown to be capable of supporting growth when iron was provided as the electron acceptor (Tor *et al.*, 2001). These observations are consistent with those from DS80 and support the notion that the metabolic versatility of carbon metabolism in hyperthermophilic microorganisms may be expanded when iron is provided as an electron acceptor (Vargas *et al.*, 1998). These observations also suggest the possibility that the mechanisms for iron reduction in these taxa are not necessarily specific, which may allow for co-factors or oxidoreductase complexes and their associated co-factors to non-specifically reduce iron. The results shown here indicate that providing H₂ as electron donor can also allow for use of alternative electron acceptors (i.e., S⁰) when organic carbon sources are available to these cells. It is not known if the addition of H₂ would allow heterotrophic growth in nitrate or thiosulfate grown *F. placidus* cells.

The greater amount of acetate mineralized by Fe³⁺ reducing DS80 cells when acetate served as electron donor when compared to acetate/H₂ as electron donors suggests that the addition of H₂ decreases the flux of low potential electrons derived from acetate into electron transport chains and a potential diversion of a portion of this acetate away from oxidation via the TCA cycle and toward gluconeogenesis. Indeed, observed growth yields of DS80 with Fe³⁺ when acetate was used as carbon and energy source when compared with growth yields when both H₂ and acetate were present, were higher. Here, less efficient electron transfers involving the H₂/Fe³⁺ redox couple, additional energetic costs associated with reduction of acetate during gluconeogenesis pathways and additional energetic costs associated with synthesis of the enzymes necessary to metabolize H₂ and to perform gluconeogenesis could influence the overall efficiency of coupling energy metabolism to biomass synthesis (Furdui and Ragsdale, 2000; Amenabar *et al.*, 2017).

The metabolic flexibility displayed by strain DS80 appears to be a shared characteristic among members of the *Acidianus* genus (Table S2), which is comprised of physiologically versatile members that can grow as facultative anaerobes or facultative autotrophs with the ability to oxidize or reduce S⁰ depending on O₂ availability (Huber and Stetter, 2015). Like DS80 (Amenabar *et al.*, 2017), some *Acidianus* species have also been shown to respire Fe³⁺ with H₂ or S⁰ (Yoshida *et al.*, 2006; Giaveno *et al.*, 2013). This generalist strategy, in addition to the observation that H₂ enables the use of organic carbon sources in DS80 cells respiring S⁰ (chemolithoheterotrophic metabolism), may contribute to a broadening of ecological niche space, both spatially and temporally, and therefore provide a competitive advantage to these cells in dynamic environments where the availability of carbon sources, electron donors and electron acceptors varies (Hurwitz and Lowenstern, 2014). To this end, we suggest that electron acceptor availability alters the carbon and energy metabolism of strain DS80, with H₂ being obligate for growth when S⁰ serves as electron acceptor regardless if cells are growing autotrophically or heterotrophically. Based on work presented here, and the work of others (Jochimsen *et al.*, 1997; King, 2007; Ghosh and Dam, 2009), we suggest that chemolithoheterotrophic metabolism may be much more common in nature than is currently recognized.

In summary, the data presented here suggest that the use of electron donors and carbon sources can be influenced by the types of electron acceptors available (e.g., Fe³⁺ versus S⁰ reduction) and the presence or absence of H₂ (i.e., in the case of S⁰ reduction). These data also clearly indicate that predictions of the metabolism of a cell based on the presence or absence of genes or geochemical/thermodynamic data indicating energetic favorability of a given redox reaction may not play out in natural systems. We suggest the need to more completely characterize the physiology of microbial cells and to integrate this with genomic and thermodynamic data to more accurately predict the distribution of microorganisms and their metabolic activities in natural systems. Future studies are needed to investigate the metabolic capabilities of strain DS80 when grown chemolithoheterotrophically or mixotrophically under microaerophilic conditions.

Experimental procedures

Culture conditions

Acidianus strain DS80, previously isolated from 'Dragon Spring', Yellowstone National Park, Wyoming (Amenabar *et al.*, 2017), was cultivated in anoxic base salts medium containing NH₄Cl (0.33 g l⁻¹), KCl (0.33 g l⁻¹), CaCl₂·2H₂O (0.33 g l⁻¹), MgCl₂·6H₂O (0.33 g l⁻¹) and

KH_2PO_4 (0.33 g l^{-1}). The pH of the medium was adjusted to 3.0 with concentrated hydrochloric acid. Fifty-five mL of medium was dispensed into 160 mL serum bottles and was subjected to autoclave sterilization. Following autoclave sterilization and while still hot ($\sim 90^\circ\text{C}$), filter-sterilized Wolfe's vitamins (1 mL l^{-1} final concentration), filter sterilized SL-10 trace metals (1 mL l^{-1} final concentration) and S° (5 g l^{-1} final concentration; sterilized by baking at 100°C for 24 h) or filter sterilized ferric iron in the form of $\text{Fe}_2(\text{SO}_4)_3 \cdot \text{H}_2\text{O}$ (3.7 g l^{-1}) were added. The bottles and their contents were deoxygenated by purging with sterile nitrogen (N_2) gas passed over heated (210°C) and H_2 reduced copper shavings. The serum bottles were sealed with butyl rubber stoppers and heated to 80°C prior to the replacement of the headspace with 80:20% H_2 : CO_2 , unless otherwise specified. All growth experiments were conducted at 80°C in medium with a pH of 3.0.

Isolation of genomic DNA and sequencing

Biomass for DNA extraction was concentrated via centrifugation ($14,000 \times g$, 20 min, 4°C) from a single 55 mL autotrophically grown culture provided with H_2 and S° as electron donor and acceptor, respectively. The cell pellet was re-suspended in 1 mL of sterile base salts medium and carbon disulfide (CS_2) was added to solubilize and partition S° into the organic phase to improve the recovery of DNA, as has been described previously (Boyd *et al.*, 2007). CS_2 was added to a final concentration of 10% (vol/vol), and following phase separation, the aqueous phase was collected and concentrated by centrifugation ($14,000 \times g$, 20 min, 4°C). Genomic DNA was extracted from the cell pellet using a modified phenol-chloroform extraction procedure. Cells were first re-suspended in 150 mM Tris-HCl buffer (pH 7.5) containing 15 mM EDTA (pH 8.0) and then were subjected to three cycles of sonication (1 min each) on ice. Lysozyme was added to the extract to a final concentration of 1 mg mL^{-1} , and the extract was incubated at 37°C for 30 min. Following incubation, proteinase K (200 $\mu\text{g mL}^{-1}$ final concentration) and sodium dodecyl sulfate (1% final concentration) were added to the extract and the extract was incubated at 50°C for 30 min. Cell debris was removed by centrifugation ($9000 \times g$ for 20 min), and the resultant clarified phase was extracted with two volumes of phenol/chloroform and subjected to centrifugation ($14,000 \times g$, 10 min, 4°C). The soluble phase was again collected and extracted with two volumes of chloroform/isoamyl alcohol (24:1) and centrifuged ($14,000 \times g$, 10 min, 4°C). Following centrifugation, the soluble phase was collected and DNA was precipitated from this fraction by adding two volumes of 100% ethanol, followed by incubation at -20°C for 12 h and centrifugation ($14,000 \times g$, 10 min, 4°C) (Sambrook and Russell, 2001). The pellet was collected, dried in a laminar flow hood and re-suspended in 50 μl of

molecular grade water. The concentration of DNA in the extract was determined using a Qubit 2.0 Fluorimeter (Invitrogen, Carlsbad, CA, USA) and a Qubit dsDNA HS Assay kit (Molecular Probes, Eugene, OR, USA).

Total genomic DNA was sequenced at the Genomics Core Facility at the University of Wisconsin-Madison using the Illumina MiSeq platform and the 2×300 bp v3 sequencing kit. Paired-end DNA fragments were prepared according to the manufacturer's protocol. Illumina reads were processed, assembled and analysed using the protocols described in the supporting online information. Details of the genome assembly statistics, percent coverage and relationships with other available *Acidithiobacillus* genomes at the level of genome composition and phylogeny are provided in the supplemental online information.

Physiological characterization

Organic compounds that support growth of strain DS80 when S° or Fe^{3+} served as terminal electron acceptor were evaluated in anoxic base salts medium, prepared as described earlier. Putative carbon sources capable of supporting growth were identified based on analysis of the DS80 genome (supplemental online information). Glucose, fructose, galactose, mannose, lactose, maltose, sucrose, yeast extract, peptone and casamino acids were added to anoxic base salts medium to achieve a final concentration of 0.1% (wt/vol). Sodium formate and sodium acetate were added to base salts medium to achieve a final concentration of 200 μM , since previous studies have shown these organic acids to be toxic to acidophiles at concentrations higher than 200 μM (Urschel *et al.*, 2016). All carbon sources were tested for their ability to serve as a carbon source (100% H_2 headspace) (lithoheterotrophy) or as a carbon and electron donor (100% N_2 headspace) (organoheterotrophy). The ability of strain DS80 to grow with carbon monoxide (CO) as a carbon source (90:10% H_2 :CO headspace) or as a carbon and electron donor (90:10% N_2 :CO) was also examined. The ability of the specified organic compounds to support mixotrophic growth, with respect to carbon metabolism, was evaluated as described above using an 80:20% N_2 : CO_2 headspace. The ability of strain DS80 to grow organoheterotrophically with these different carbon sources when O_2 served as terminal electron acceptor (serum bottle headspace was air) was also evaluated in cultures grown on a rotary shaker (100 rpm) at 80°C . The convention used to report experimental conditions for each growth assay throughout the text is carbon source/electron donor/electron acceptor in that order.

Growth and activity of strain DS80 with different carbon sources in the presence of various electron donors and acceptors were quantified in terms of cell density and

production of total sulfide [(S²⁻); proxy for S⁰ reduction] or ferrous iron [(Fe²⁺); proxy for Fe³⁺ reduction], as previously described (Amenabar *et al.*, 2017). Aerobic cultures were monitored based only on cell density. Triplicate measurements (technical replicates) were made on each of the replicate cultures (biological replicates), and these were averaged for each culture. The results presented represent the average and standard deviation of the triplicate biological cultures

Carbon assimilation and mineralization

The amount of acetate assimilated into cellular biomass or mineralized and released as CO₂ was determined in cultures provided with S⁰ or Fe³⁺ as terminal electron acceptor, in the presence or absence of H₂. Briefly, 160 ml serum bottles containing 55 ml of base salts medium (described above) and S⁰ or Fe³⁺ with a 100% H₂ or 100% N₂ headspace was amended with acetate to a final concentration of 100 μM [45:1 ratio of unlabeled acetate to 1-[¹⁴C] sodium acetate (CH₃¹⁴COONa)]. Each assay received a total of 5 μCi of 1-[¹⁴C]-acetate. At each time point, a 2 ml subsample of culture and a 1 ml subsample of gas was removed from each vial for use in determining the amount of 1-[¹⁴C]-acetate assimilated into biomass or mineralized as CO₂, using scintillation counting as previously described (Urschel *et al.*, 2016). The concentration of CO₂ that was present in the headspace was measured. The concentration of dissolved CO₂ was assumed to be negligible due to the acidity of the medium. Three ml of sterile N₂ or H₂ (depending on the cultivation condition) was added to each vial at each sampling interval to maintain atmospheric pressure in the vials. Conversion of the assimilation or mineralization of 1-[¹⁴C]-labeled acetate to the assimilation or mineralization of total acetate (unlabeled plus labeled) was performed as previously described (Urschel *et al.*, 2016).

Influence of acetate on the assimilation of dissolved inorganic carbon (DIC)

The amount of DIC assimilated into cellular biomass was determined in cultures of DS80 provided with S⁰ or Fe³⁺ as terminal electron acceptor in the presence of different concentrations of acetate. One hundred sixty milliliter serum bottles containing 55 ml of base salts medium and S⁰ or Fe³⁺ with an 80:20% H₂:CO₂ headspace (described earlier) were amended with a total of 5 μCi of [¹⁴C]-bicarbonate and with 0, 50, 100 or 200 μM unlabeled acetate. Cultures were inoculated, and the influence of acetate on the assimilation of ¹⁴CO₂ into biomass was determined as previously described (Urschel *et al.*, 2016).

Influence of DIC on the assimilation and mineralization of acetate

The amount of acetate assimilated into cellular biomass was determined in cultures provided with S⁰ or Fe³⁺ as terminal electron acceptor in the presence or absence of added CO₂. One hundred sixty milliliter serum bottles containing 55 ml of base salts medium and S⁰ or Fe³⁺ with an 80:20% H₂:CO₂ headspace (described earlier) were amended with a total of 5 μCi of 1-[¹⁴C]-acetate and unlabeled acetate to achieve a final concentration of 100 μM. Cultures were inoculated, and the influence of CO₂ on the assimilation/mineralization of 1-[¹⁴C] acetate was determined as previously described (Urschel *et al.*, 2016).

Statistical analyses

Two tailed Student's *t*-tests were used to evaluate the statistical significance (*P* < 0.05) of differences between datasets and were conducted in Microsoft Excel.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Table S1. Growth of *Acidianus* strain DS80 in base salts medium (80°C; pH 3.0) with specified substrates as energy/ carbon sources when S⁰ served as electron acceptor. The ability for each carbon source to support growth was tested in the absence (–) of H₂ and in the presence (+) of 20% CO₂.

Table S2. Comparison of metabolic characteristics among characterized *Acidianus* strains, as compiled from the specified literature references.

Fig. S1. Gene clusters encoding the [NiFe]-hydrogenase (*hyn*) and the sulfur reductase complex (*sre*) in *Acidianus* strain DS80. The ruler demarcates base pair positions, arrows indicate ORFs, RR indicates a conserved twin-arginine motif and the *tat* cluster encodes proteins required for the twin-arginine protein translocation pathway (TAT).

Fig. S2. Gluconeogenesis and glucose degradation via a nonphosphorylative Entner–Doudoroff (ED) pathway.

DHAP, dihydroxyacetone phosphate; ENO, enolase; FBPA/ase, fructose biphosphate aldolase/phosphatase; GAD, gluconate dehydratase; GADH, glyceraldehyde dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDH, glucose dehydrogenase; GK, glycerate kinase; GLac, gluconolactonase; KDGA, 2-keto-3-deoxy-gluconate aldolase; PEPS, phosphoenolpyruvate synthetase; PFOR, pyruvate: ferredoxin oxidoreductase; PGAM, phosphoglycerate mutase; PGI, glucose-6-phosphate isomerase; PGK, phosphoglycerate kinase; PK, pyruvate kinase. Oxidative reactions are indicated in red, and reductive reactions are indicated in blue.

Fig. S3. Quality assessment via GC content of the *Acidianus* strain DS80 genome assembly.

Left panel shows average GC content of contigs calculated over a nonoverlapping sliding window of 5000 base pairs. The right panel shows the size of each genomic contig as a function of its deviation from the mean GC of the entire assembly. The red lines denote the expected deviation from the mean using a 95% confidence interval.

Fig. S4. Phylogenetic placement of *Acidianus* strain DS80 within the Sulfolobales order.

The Maximum Likelihood tree was constructed using a concatenation of 104 phylogenetic marker genes for a total of 30 archaeal genomes. All nodes shown exhibited bootstraps of 100% except for one node where the bootstrap value is indicated. Scale bar shows expected number of substitutions/site. *Pyrobaculum islandicum* was specified as an outgroup for the Sulfolobales genomes.

Fig. S5. Average amino acid identity (AAI) of proteins encoded in the *Acidianus* strain DS80 genome when compared to the genome of *Acidianus hospitalis* W1. The top panel represents the percent identity while the bottom panel represents the bit score distribution.

Fig. S6. Average nucleotide identity (ANI) of the genome of *Acidianus* strain DS80 when compared to the genome of *Acidianus hospitalis* W1. The top panel represents the percent identity while the bottom panel represents the bit score distribution.

Fig. S7. Complete genome mapping between the *Acidianus hospitalis* W1 genome and 133 contigs generated from the sequencing of the genome of *Acidianus* strain DS80. Here, the orange strip (right) represents the genome from *A. hospitalis* W1 while the green strip (left) represents the 133 contigs. Colored links represent regions of high similarity as obtained from BLASTn. The red links represent the regions that were > 90% identical, green links represent regions that were between 80 and 90% identical, and blue links represent regions that were between 70 and 80% identical.