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Granular activated carbon (GAC) is added to methanogenic digesters to enhance conversion of wastes to methane, but the mechanism(s) for GAC's stimulatory effect are poorly understood. GAC has high electrical conductivity and thus it was hypothesized that one mechanism for GAC stimulation of methanogenesis might be to facilitate direct interspecies electron transfer (DIET) between bacteria and methanogens. Metabolism was substantially accelerated when GAC was added to co-cultures of *Geobacter metallireducens* and *Geobacter sulfurreducens* grown under conditions previously shown to require DIET. Cells were attached to GAC, but did not aggregate as they do when making biological electrical connections between cells. Studies with a series of gene deletion mutants eliminated the possibility that GAC promoted electron exchange *via* interspecies hydrogen or formate transfer and demonstrated that DIET in the presence of GAC did not require the electrically conductive pili and associated *c*-type cytochrome involved in biological interspecies electrical connections. GAC also greatly stimulated ethanol metabolism and methane production in co-cultures of *G. metallireducens* and *Methanosarcina barkeri*. Cells were attached to GAC, but not closely aggregated, suggesting little opportunity for biological electrical contacts between the species. GAC also enhanced methane production in samples from a methanogenic digester in which *Methanosaeta* were the predominant methanogens. The results demonstrate that GAC can promote DIET and suggest that stimulation of metabolism in methanogenic digesters can be attributed, at least in part, to the high conductivity of GAC providing better interspecies electrical connections than those that can be forged biologically.

Introduction

Direct interspecies electron transfer (DIET) may be a more effective mechanism for interspecies electron exchange under anaerobic conditions than interspecies electron transfer *via* reduced molecules such as hydrogen and formate.^{1–3} Thus, promoting DIET might be a good approach for accelerating microbial metabolism in bioenergy strategies that require inter-

species electron exchange. DIET has been studied most intensively in adaptively evolved co-cultures of *Geobacter metallireducens* and *Geobacter sulfurreducens*,⁴ but it also appeared that DIET was an important process for interspecies electron exchange in multi-species aggregates from a methanogenic digester in which *Geobacter* and *Methanosaeta* species predominated.⁵ Furthermore, it has been proposed that the semi-conductive minerals hematite and magnetite can promote electron exchange between *Geobacter* and *Methanosarcina* species, based on the predominance of these organisms in enrichment cultures in which the addition of hematite or magnetite stimulated methane production from a paddy soil inoculum.⁶

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Broader context

Conversion of biomass and organic wastes to methane is a proven bioenergy strategy. Amendments of granular activated carbon (GAC) can accelerate the initiation of methanogenesis in reactor start up or recovery from metabolic balances resulting from operator error. The findings presented here suggest that GAC promotes methanogenesis by providing an electrical connection between bacteria involved in the degradation of organic compounds and methane-producing bacteria. This understanding makes GAC addition a less empirical process. Furthermore, the more general concept that conductive materials can promote interspecies electron transfer may lead to other strategies for improving methanogenic digestion and has implications for methane production in anaerobic soils and sediments, which are important sources of this potent greenhouse gas.

Methane production is the most successful worldwide bio-energy strategy.⁷ It is commonly employed in small-scale, decentralized systems to harvest energy from organic wastes in rural environments^{8–11} as well as in industrial-scale systems for treating wastewater.^{12,13} Methanogenic digesters are an attractive option for treating the significant amount of organic-containing wastewater generated during conversion of organic substrates to biofuels and commodities.¹⁴ If methanogenic systems could be optimized they might prove to be simpler and more cost-effective strategies for converting biomass to fuel than many other options.¹⁵

The microbial communities in methanogenic digesters are generally resilient and stable, but disruptions in the syntrophic associations between bacteria and methanogens may lead to reactor instabilities.⁷ Granular activated carbon (GAC) is sometimes added to methanogenic digesters to overcome these metabolic disruptions inhibiting methane production or to accelerate the initiation of methanogenesis in digester start-up.¹⁶ Understanding how GAC stimulates methanogenesis under these circumstances might lead to more cost-effective strategies for methanogenic digester operation.

One potential benefit of GAC is its ability to absorb toxic organic compounds that might otherwise inhibit microorganisms involved in anaerobic digestion.¹⁷ Furthermore, GAC can provide a high-surface area substrate for microbial attachment, which may promote microbial growth, as well as aid in retention of biomass in some reactor designs.¹⁸

However, additional properties of GAC are its high electrical conductivity¹⁹ and the possibility that it can serve as an electron acceptor for anaerobic respiration.²⁰ For example, GAC is an effective electrode in microbial fuel cells because microorganisms can transfer electrons to GAC serving as anode.²¹ Negatively poised graphite electrodes can serve as an electron donor for a diversity of anaerobes,^{22–24} suggesting that once reduced, GAC might be able to serve as an electron donor for microbial respiration. Electrons stored in microbially reduced GAC could abiotically reduce Fe(III) or azo dyes.²⁰ Although this electron storage and shuttling *via* GAC was attributed to quinone moieties in GAC,²⁰ mechanistic studies have shown that the electron storage capacity of GACs does not arise from the redox surface functional groups but rather due to the charging of electrical double layer present at the interface of conductive GACs and the electrolyte.^{19,25,26}

The considerations that DIET can be an important mode of electron exchange in methanogenic digesters⁵ and that GAC is an electrically conductive material with which microorganisms can exchange electrons led us to investigate whether GAC might promote DIET. Multiple lines of evidence suggest that GAC facilitates DIET better than the electrical connections that microorganisms can generate themselves.

Results and discussion

Stimulation of metabolism in *Geobacter* co-cultures with GAC

The potential for GAC to stimulate DIET was first evaluated with co-cultures of *G. metallireducens* and *G. sulfurreducens* because it is known that this co-culture is capable of DIET⁴ and the co-culture members can be genetically manipulated,^{27,28}

facilitating mechanistic studies. When *G. metallireducens* and *G. sulfurreducens* were inoculated into a medium with ethanol as the sole electron donor and fumarate as the sole electron acceptor, ethanol was only slowly metabolized (Fig. 1A), consistent with previous observations that it took more than 30 days for the co-culture to begin to adapt for rapid ethanol metabolism *via* DIET.⁴

Addition of GAC to *G. metallireducens*/*G. sulfurreducens* co-cultures stimulated ethanol metabolism with a coincident accumulation of succinate, indicating that *G. metallireducens* and *G. sulfurreducens* were cooperating to oxidize ethanol with the reduction of fumarate (Fig. 1B). Rates of ethanol oxidation coupled to the reduction of fumarate to succinate accelerated with increased addition of GAC, up to 25 g L⁻¹ (Fig. 1), adding more GAC did not further accelerate ethanol metabolism (data not shown).

The co-cultures could readily be propagated with continued transfer (5% inoculum) in the presence of GAC. At no time did the GAC-amended cultures form large red (>1 mm diameter)

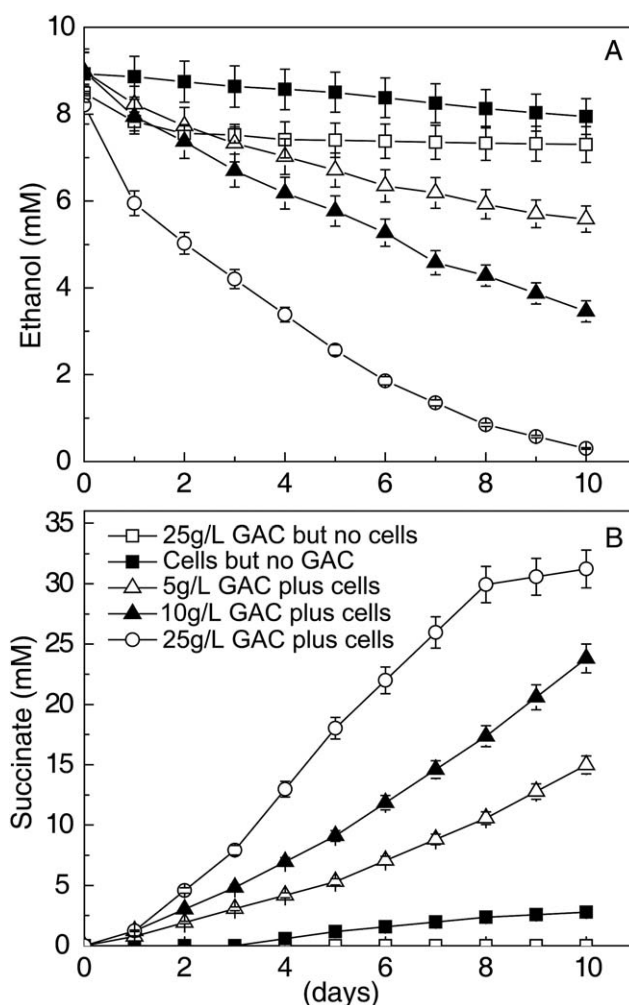


Fig. 1 Ethanol consumption (A) and succinate produced from fumarate reduction (B) by a syntrophic co-culture of *G. metallireducens* and *G. sulfurreducens* with amendments of different concentrations of GAC. The error bars represent standard deviations of the mean for triplicate cultures.

aggregates similar to those previously observed⁴ after long-term adaptation in the absence of GAC. Analysis of cell protein demonstrated that there was significant cell growth over a 10 day incubation period in the presence of GAC (Fig. 2). 80% of the cells were associated with GAC (Fig. 2).

Terminal restriction enzyme fragment length polymorphism (T-RFLP) targeting 16S rRNA gene sequences demonstrated that *Geobacter sulfurreducens* became the dominant member ($63 \pm 3\%$; mean \pm standard deviation, $n = 3$) in the co-cultures initiated with equivalent numbers of both species. This is similar to the proportions of *G. metallireducens* and *G. sulfurreducens* in the previously reported⁴ aggregates of the two organisms in which electrons were exchanged *via* DIET and is consistent with the concept that *G. metallireducens* metabolizes ethanol to acetate and carbon dioxide with the release of electrons and that *G. sulfurreducens* utilizes the electrons and the acetate for fumarate reduction. The stoichiometry of metabolism was also in accordance with this model. For example, in the presence of 25 g L^{-1} GAC (Fig. 1), 1.2 mM ethanol was removed due to absorption, and thus biological metabolism could account for the removal of 6.8 mM ethanol. Acetate, formate and hydrogen did not accumulate during the incubations. Each mole of ethanol oxidized to carbon dioxide coupled to the reduction of fumarate results in the production of six moles of succinate:



Thus, the 33 mM of succinate produced accounted for 81% of the electrons expected from ethanol oxidation. Considering that some of the ethanol substrate must be incorporated for biosynthesis, this stoichiometry is in accordance with the stoichiometry expected if fumarate was the sole electron acceptor for metabolism.²⁹

Evidence for interspecies electron conduction *via* GAC

One potential explanation for the ability of GAC to stimulate co-culture metabolism was that it provided a surface for cells to attach, thus promoting electron exchange either *via* the more

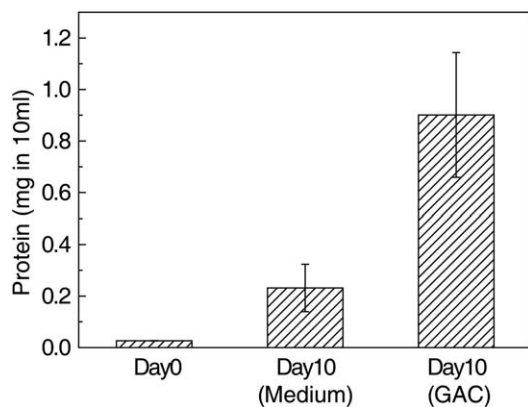


Fig. 2 Protein in 10 mL of bulk medium on day 0 and protein in the planktonic phase or associated with GAC in 10 mL cultures after 10 days of incubation. The error bars represent standard deviations of the mean for triplicate cultures.

traditional interspecies hydrogen/formate transfer or the biological electrical connections associated with DIET. However, providing glass beads with various diameters (0.1–1.5 mm) as a nonconductive surface for attachment did not stimulate interspecies electron transfer (1.5 mm diameter, Fig. 3A, other

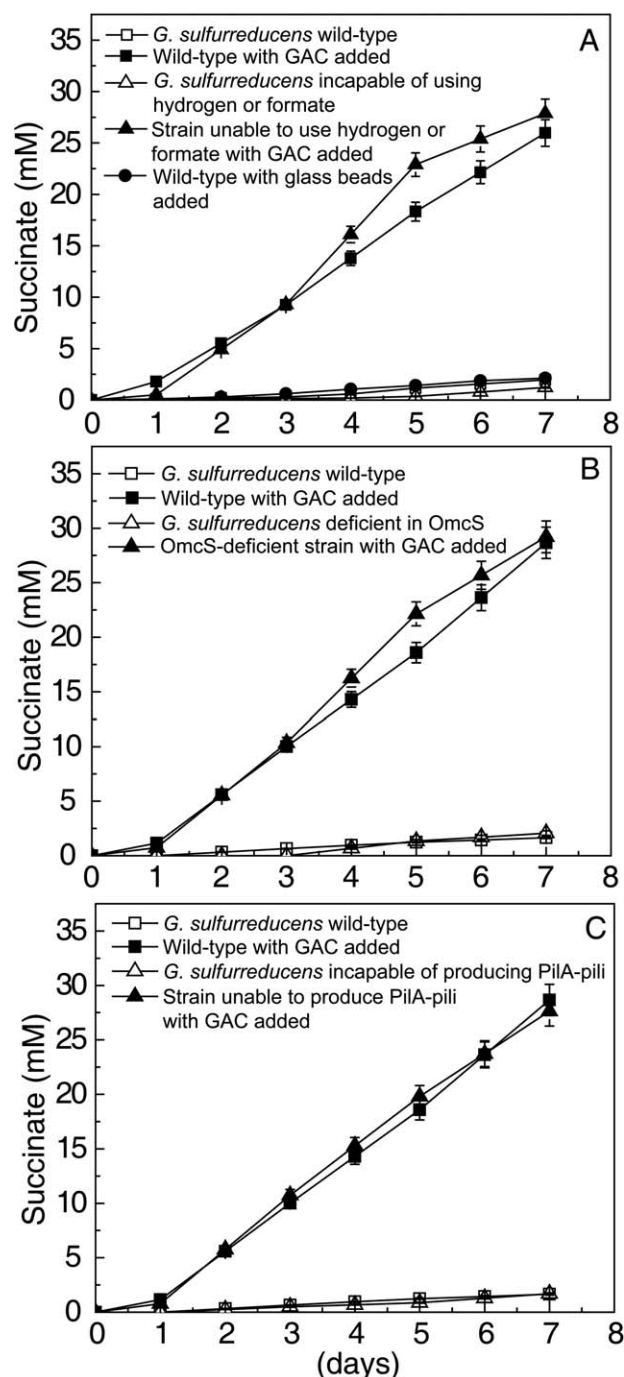


Fig. 3 Succinate produced from fumarate reduction in *G. metallireducens*/*G. sulfurreducens* co-cultures initiated with different strains of *G. sulfurreducens*, including: wild-type; a strain unable to use either hydrogen or formate as an electron donor (A); a strain deficient in the pilin-associated cytochrome, OmcS (B); or a strain that cannot produce type IV pili (C). Where noted, the co-cultures were amended with 25 g L^{-1} GAC or glass beads (1.5 mm). The error bars represent standard deviations of the mean for triplicate cultures.

diameters not shown). Furthermore, when GAC was added to co-cultures initiated with a strain of *G. sulfurreducens*, which previous studies³⁰ have demonstrated cannot metabolize hydrogen or formate, ethanol was readily metabolized with the reduction of fumarate, demonstrating that interspecies hydrogen or formate transfer was not the mechanism for interspecies electron transfer (Fig. 3A).

In previous studies in which *G. metallireducens* and *G. sulfurreducens* did form direct electrical connections,⁴ DIET was dependent on the ability of *G. sulfurreducens* to produce electrically conductive^{31,32} pili as well as the multi-heme *c*-type cytochrome OmcS,^{33,34} which is associated with the pili.⁴ In fact, DIET was promoted by a mutation that enhanced OmcS production.⁴ In contrast, in the presence of GAC, a co-culture initiated with a strain of *G. sulfurreducens* in which *omcS* had been deleted readily metabolized ethanol with the reduction of fumarate (Fig. 3B). A co-culture that functioned as well as those containing wild-type *G. sulfurreducens* could also be established with a strain in which the gene for PilA, the structural pilin protein, was deleted (Fig. 3C). These results indicated that the mechanism for electron transfer between *G. metallireducens* and *G. sulfurreducens* was different in the presence of GAC than in previous studies in which electron transfer between the species was established in the absence of GAC. These findings are consistent with the previous finding that *G. sulfurreducens* does not require PilA or OmcS for electron transfer from graphite cathodes to support fumarate reduction.³⁵ Analysis of gene expression patterns and a range of gene-deletion mutant strains have suggested that the mechanisms for electron transfer from graphite to cells are significantly different than those for electron transfer to graphite.³⁵

Furthermore, scanning electron microscopy of GAC-supplemented co-cultures revealed that cells were not in close physical contact, in contrast to what would be expected if there were biological electrical connections between the cells (Fig. 4). Rather, the cells were tightly associated with the GAC as would be expected if the cells were making an electrical connection with the GAC.

GAC stimulation of DIET with *Methanosarcina barkeri*

When *Methanosarcina barkeri* was substituted for *G. sulfurreducens* as the potential electron-accepting partner in co-culture

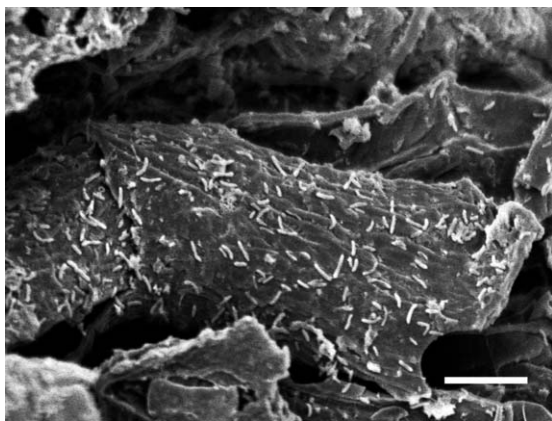


Fig. 4 Scanning electron micrograph GAC-amended *G. metallireducens*/*G. sulfurreducens* co-culture. The size bar of this photograph corresponds to 10 μm .

with *G. metallireducens*, ethanol was only slowly metabolized, if at all, over 2 months (Fig. 5A). However, in the presence of GAC, ethanol was metabolized with the production of methane, with little or no lag period (Fig. 5B). Unlike, the *Geobacter* co-culture studies, there was significant adsorption of ethanol (around 50 μmol) by GAC in abiotic controls, which might be attributed to the different media conditions or the fact that twice as much ethanol was added for the co-culture with *M. barkeri*. Therefore, 65 μmol of the ethanol loss in the co-culture could be attributed to microbial metabolism. Acetate transiently accumulated, but was further metabolized, and thus methane was expected to be the primary product of metabolism. The 75 μmol of methane produced compared well with the 97.5 μmol of methane expected from complete conversion of ethanol to methane when it is considered that some substrate must go toward biomass formation. Scanning electron microscopy confirmed that the cells were tightly associated with the surface of GAC, but were not in close physical contact that would be required for biological electrical connections between cells (Fig. 6).

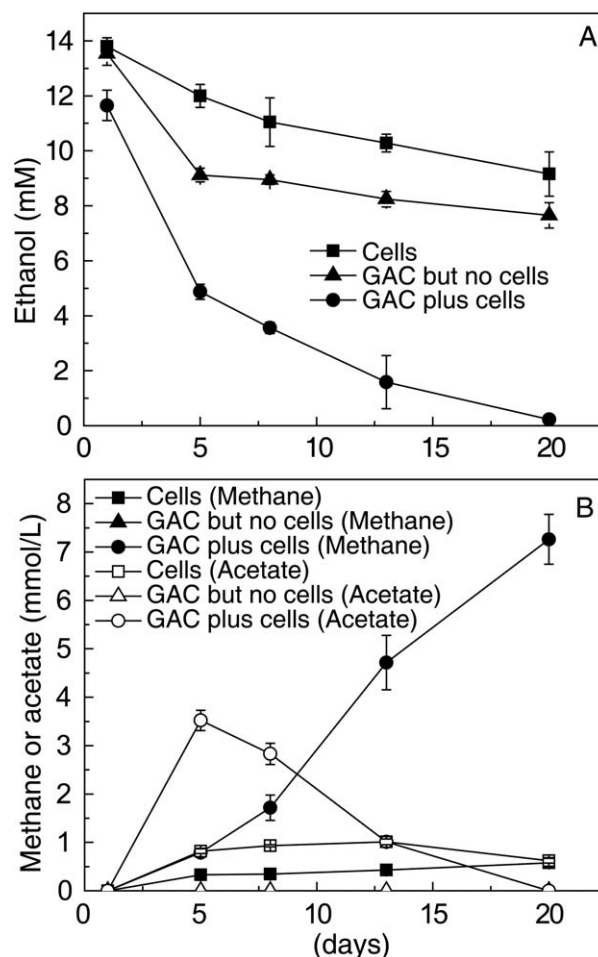


Fig. 5 Ethanol consumption (A) and methane and acetate production (B) by a syntrophic co-culture of *G. metallireducens* and *M. barkeri* in the presence of GAC (25 g L^{-1}) or in its absence. The designation mmol L^{-1} means the amount of methane produced per liter of medium. The error bars represent standard deviations of the mean for triplicate cultures.

Failure of quinone to replicate GAC stimulation of methanogenesis

It was previously suggested that the ability of GAC to stimulate the reduction of azo dyes in anaerobic digestion could be attributed to GAC functioning similar to the electron shuttle anthraquinone-2,6-disulfonate (AQDS).²⁰ In this model quinone moieties in the GAC accept electrons from microorganisms and the hydroquinones produced reduce the dyes.²⁰ AQDS functions as an electron shuttle between *Geobacter* species and a number of extracellular electron acceptors^{36–38} and was also shown to enhance interspecies electron transfer in short-term cell suspension studies in which acetate was oxidized with the reduction of fumarate by a co-culture of *G. metallireducens* and *Wolinella succinogenes*.³⁹

AQDS stimulated ethanol metabolism with the reduction of succinate in *G. metallireducens*/*G. sulfurreducens* co-cultures (Fig. 7A), compared with controls without AQDS (Fig. 1). However, AQDS did not promote ethanol metabolism to methane in *G. metallireducens*/*M. barkeri* co-cultures (Fig. 7B). The inability of AQDS to stimulate interspecies electron transfer in the co-culture with *M. barkeri* might be attributed to the relatively high mid-point potential of the AQDS/AHQDS redox couple ($E_0' = -184$ mV) which is low enough to drive fumarate reduction (E_0' of fumarate/succinate couple = 30 mV) but too high for the reduction of carbon dioxide to methane (E_0' of CO_2 /methane couple = -240 mV). AQDS is not toxic to *M. barkeri*.⁴⁰ These results indicate that AQDS is not a GAC equivalent under methanogenic conditions and suggest that in GAC electron carriers other than quinone moieties are responsible for promoting interspecies electron transfer, at least for methanogenesis.

GAC stimulation of aggregates in which DIET was already functional

As noted above, after a long lag period *G. metallireducens*/*G. sulfurreducens* co-cultures can adapt to effectively metabolize ethanol with the reduction of fumarate. This is associated with the formation of large (>1 mm diameter) electrically conductive

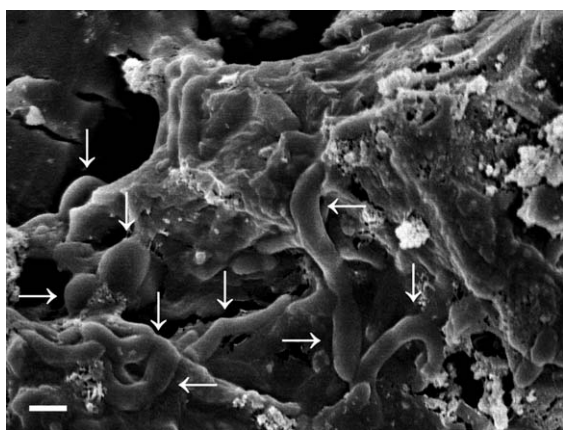


Fig. 6 Scanning electron micrograph of GAC-amended co-culture of *G. metallireducens* (rods) and *M. barkeri* (spheres). The white arrows point to representative cells. Scale bar, 1 μm .

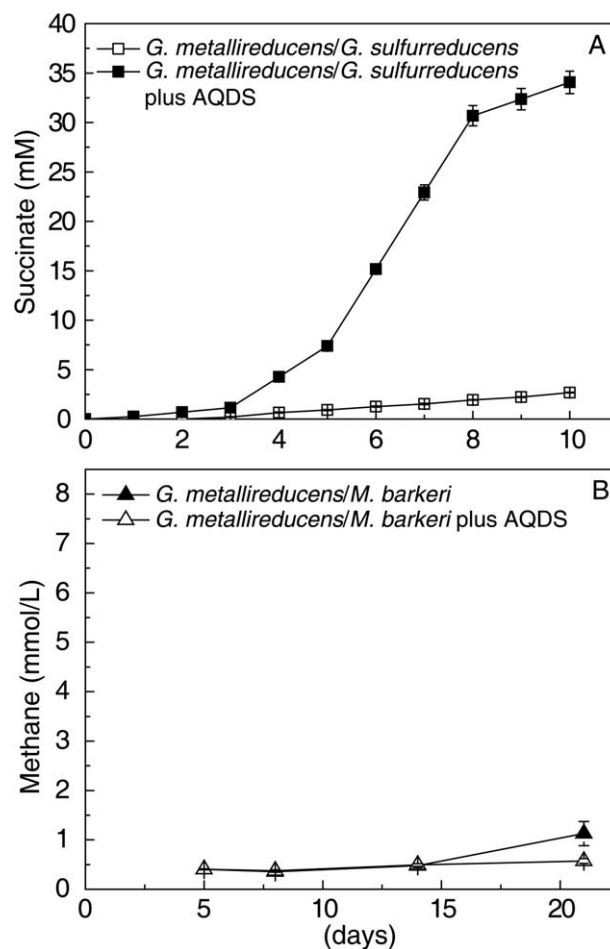


Fig. 7 AQDS (50 μM) stimulation of ethanol metabolism with the reduction of fumarate as evidenced by succinate accumulation in *G. metallireducens*/*G. sulfurreducens* co-cultures (A) and lack of AQDS impact on methane production in *G. metallireducens*/*M. barkeri* co-cultures (B). The error bars represent standard deviations of the mean for triplicate cultures.

aggregates. Therefore, to determine whether GAC could enhance interspecies electron transfer in systems in which electrons were already being transferred *via* other mechanisms, GAC was added to the aggregates of *G. metallireducens* and *G. sulfurreducens* adapted for ethanol metabolism. GAC stimulated ethanol metabolism with a rate during the linear phase of metabolism that was 40% faster than the rate of metabolism in aggregates without GAC (Fig. 8A).

The impact of GAC on metabolism was more substantial with aggregates from the methanogenic digester (Fig. 8B), which previous studies have suggested to produce methane *via* DIET between *Geobacter* and *Methanseta* species.⁵ Addition of GAC stimulated methane production with rates that were 2.5-fold faster than in controls without GAC (Fig. 8B).

One potential explanation for the ability of GAC to stimulate syntrophic metabolism even in systems in which DIET was already established with biological connections is that GAC provides higher conductivity between cells than is possible with biologically produced electrical connections. The conductivity of GAC measured with the same two-electrode system previously

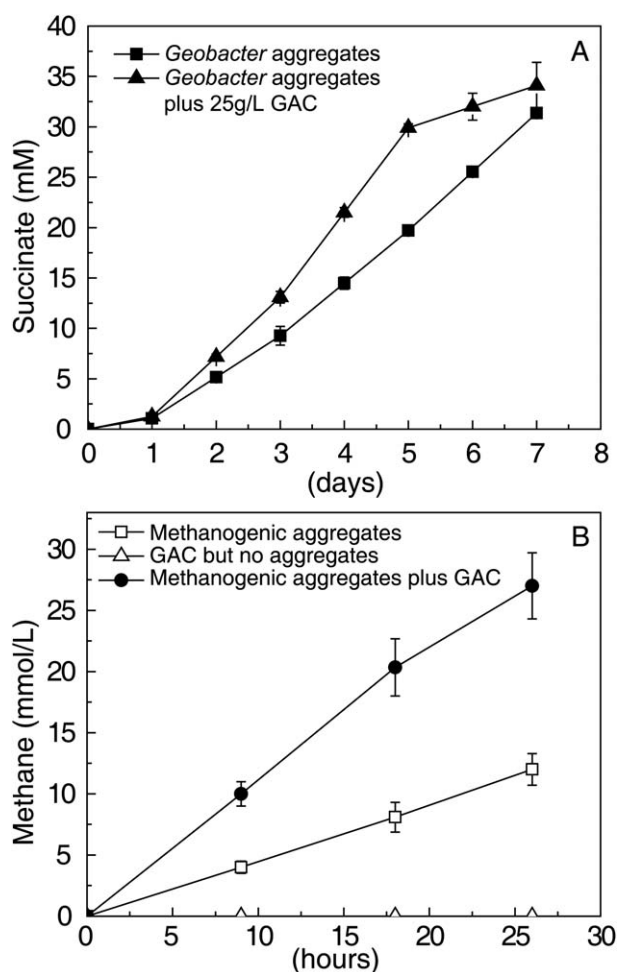


Fig. 8 Impact of GAC amendments (25 g L^{-1}) on the metabolism of syntrophic microbial aggregates that had formed biological electrical connections in the absence of GAC. (A) Succinate produced from fumarate reduction in adaptively evolved co-cultures of *G. metallireducens* and *G. sulfurreducens* forming large ($>1 \text{ mm}$ diameter) aggregates. (B) Methane production by natural methanogenic aggregates from a methanogenic digester. The error bars represent standard deviations of the mean for triplicate incubations of each treatment.

used to measure the conductivity of microbial aggregates^{4,31} was $3000 \pm 327 \mu\text{S cm}^{-1}$ (mean \pm standard deviation, $n = 3$), consistent with previous reports of GAC conductivity.¹⁹ This is substantially higher than the conductivity of $2\text{--}20 \mu\text{S cm}^{-1}$ for *Geobacter* co-culture aggregates or aggregates from methanogenic digesters.^{4,5,41}

Experimental

Microorganisms, media and growth conditions

Geobacter sulfurreducens strain DL1 (ATCC 51573)^{42,43} and *Geobacter metallireducens* strain GS-15 (ATCC 53774)^{44,45} were obtained from our laboratory culture collection. In some instances, co-cultures were also initiated with previously described strains of *G. sulfurreducens* in which genes for one of the following proteins had been deleted including: (1) Pila,³² (2) OmcS,³⁴ or (3) HybL and FdnG in a double mutant.³⁰

Methanosarcina barkeri strain DSM 800 (ATCC 43569)⁴⁶ was obtained from DSMZ (Braunschweig, Germany).

All culturing and sampling was performed under strict anaerobic conditions⁴⁷ with a gas phase of $\text{N}_2\text{--CO}_2$ (80 : 20). Inocula for co-cultures were developed by growing *G. metallireducens* in FC medium,⁴⁵ with 20 mM ethanol as the sole electron donor and 55 mM ferric citrate as the electron acceptor. *G. sulfurreducens* inocula were grown in NBF medium,²⁷ with 10 mM acetate as the sole electron donor and 40 mM fumarate as the electron acceptor. Co-cultures were initiated with equal amounts of both organisms in anaerobic pressure tubes containing 10 mL of NBF medium, with 10 mM ethanol as the sole electron donor and 40 mM fumarate as the electron acceptor. The cysteine that was sometimes added to the medium as a reductant in other studies was omitted to eliminate the possibility of a cysteine/cystine electron shuttle⁴⁸ between the organisms. Studies also were conducted with the previously described⁴ preestablished co-cultures of *G. metallireducens* and *G. sulfurreducens* which had adapted for effective ethanol oxidation with the reduction of fumarate by forming large, electrically conductive aggregates. The incubation temperature for all *G. metallireducens/G. sulfurreducens* co-cultures was 30°C .

For co-cultures of *G. metallireducens* and *M. barkeri*, *G. metallireducens* was grown in DSMZ methanogenic medium 120⁴⁹ with 20 mM ethanol as the electron donor and nitrate (10 mM) as the electron acceptor. *M. barkeri* was grown in the same medium with 50 mM acetate as the substrate. Co-cultures were grown in medium 120 with ethanol (20 mM) as the electron donor. Methane production by the previously described⁵ upflow anaerobic sludge blanket (USAB) digester aggregates was investigated in a similar manner with 0.25 g of aggregates added to each culture tube. The incubation temperature for all methanogenic studies was 37°C .

When noted, granular activated carbon (GAC, 8–20 mesh, Sigma-Aldrich, St Louis, MO, USA) was added into the medium before autoclaving. Additions of anthraquinone-2,6-disulphonate (AQDS) were made from a concentrated stock to provide a final concentration of $50 \mu\text{M}$.⁴⁰

Analytical techniques

Organic acids were monitored with high performance liquid chromatography as previously described.⁵⁰ Changes in ethanol concentration and methane production over time were monitored by gas chromatography as previously described.⁵

The total protein was determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard. Planktonic cells in the liquid phase were anaerobically separated from those attached to GAC by sterile syringe without centrifugation. 0.5 mL liquid sample and 0.25 g GAC were treated with 0.5 N NaOH respectively, and followed the standard method.⁵¹

Scanning electron microscopy

Scanning electron micrographs were taken of GAC granules and associated microorganisms, prepared with hexamethyldisilazane (Sigma Aldrich, St Louis, MO, USA) as previously described.⁵²

T-RFLP

DNA from the triplicate 10 mL samples was extracted with a slight modification of the previously described method.⁵³ Briefly, cell lysis was performed in a FastPrep® Instrument (MoBio Laboratories, Carlsbad, CA, USA) using 3 m s⁻¹ speed. Total nucleic acid thus obtained was digested using RNase A (Ambion, USA) and isolated pure DNA was used for terminal restriction length polymorphism (T-RFLP).

T-RFLP was carried out in triplicate as described earlier.⁵⁴ Briefly, PCR amplification of the 16S rRNA gene was performed using fam labeled 9f and unlabeled 1100r up to 25 PCR cycles. Then the PCR was purified using gel based PCR Purification Kit (Qiagen, Valencia, CA, USA); the purified PCR product was digested using *MspI*. The length of fluorescently labeled T-RFs was determined by comparison with the internal standard LIZ1200 using GeneScan software (Applied Biosystems, Foster City, CA, USA). The relative abundance of T-RFs was calculated as previously described.⁵⁴

Conclusions

These results demonstrate that GAC can promote interspecies electron transfer and suggest that this may be why GAC is such an effective stimulant of metabolism in methanogenic wastewater digesters. The most likely mechanism for GAC stimulation of syntrophic metabolism is its high conductivity, permitting electrical connections between microorganisms that are more conductive than those that can be formed biologically. Electrical connections between cells established with GAC may alleviate the necessity for cells to invest metabolic energy in producing conductive pili and the additional cytochromes that are required for the DIET in the absence of GAC.⁴

There is growing evidence that *Geobacter* species can form syntrophic associations with *Methanosaeta* or *Methanosarcina* species that function via DIET.^{5,6} *Geobacter* are important constituents of some methanogenic digesters⁵ and *Methanosaeta* or *Methanosarcina* species are often the predominant methanogens.^{5,9} The results presented here suggest that enhancing electron transfer to *Methanosaeta* or *Methanosarcina* species is a major factor in the ability of GAC to stimulate the conversion of organic wastes to methane.

A better understanding of the mechanisms by which microorganisms in general, and methanogens in particular, accept electrons from conductive materials like GAC or semi-conductive minerals⁶ might aid in the better design of anaerobic digesters and reveal molecular signatures that can be used to diagnose this form of metabolism, not only in waste digestion, but also in natural methanogenic environments.

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