Carbon cloth stimulates direct interspecies electron transfer in syntrophic co-cultures

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Abstract

This study investigated the possibility that the electrical conductivity of carbon cloth accelerates direct interspecies electron transfer (DIET) in co-cultures. Carbon cloth accelerated metabolism of DIET co-cultures (*Geobacter metallireducens* - *Geobacter sulfurreducens* and *Geobacter metallireducens* - *Methanosarcina barkeri*) but did not promote metabolism of co-cultures performing interspecies H_2 transfer (*Desulfovibrio vulgaris – Geobacter sulfurreducens*). On the other hand, DIET co-cultures were not stimulated by poorly conductive cotton cloth. Mutant strains lacking electrically conductive pili, or pili-associated cytochromes participated in DIET only in the presence of carbon cloth. In co-cultures promoted by carbon cloth, cells were primarily associated with the cloth although the syntrophic partners were far apart for cell-to-cell biological electrical connections to be feasible. Carbon cloth seemingly mediated interspecies electron transfer between the distant syntrophic partners. These results suggest that the ability of carbon cloth to accelerate DIET should be considered in anaerobic digester designs that incorporate carbon cloth.

***Keywords*:** carbon cloth, cotton cloth, syntrophy, methanogenesis, direct interspecies electron transfer, *Geobacter*, *Methanosarcina*

1 Introduction

Materials that have the potential to support biofilm growth can enhance anaerobic digestion of organic wastes to methane (Adu-Gyamfi et al., 2012). One of the materials that has shown promise is carbon cloth (Sasaki et al., 2007; Sasaki et al., 2009; Sasaki et al., 2010; Tatara et al., 2008; Zhang et al., 2012; Zhao et al., 2013). In these studies the enhanced methane production in the presence of carbon cloth was attributed to its

ability to promote microbial attachment. However, another possibility is that the conductive properties of carbon cloth will impact electron exchange between microorganisms similar to other conductive materials which were used to mediate electron transfer between cells and electrodes or other cells (Chen et al., 2014; Cruz Viggi et al., 2014; Kato et al., 2012; Liu et al., 2012; Liu et al., 2014; Rotaru et al., 2014a). It has been recently discovered that some methanogens can receive electrons from an electron-generating microorganism either directly - using molecular electric connections (Chen et al., 2014; Liu et al., 2012; Rotaru et al., 2014a; Rotaru et al., 2014b), or indirectly - using conductive minerals (Chen et al., 2014; Kato et al., 2012; Liu et al., 2012; Liu et al., 2014).

DIET is an alternative to interspecies H_2/formate transfer for syntrophic electron exchange between microbial species (Rotaru et al., 2014a; Rotaru et al., 2014b; Summers et al., 2010). DIET was initially described in co-cultures of *Geobacter metallireducens* and *Geobacter sulfurreducens* growing in medium in which ethanol was the electron donor and fumarate was the electron acceptor (Summers et al., 2010). *G. metallireducens* can metabolize ethanol, but can not use fumarate as an electron acceptor (Lovley et al., 1993), whereas *G. sulfurreducens* can not metabolize ethanol, but can respire fumarate, which is then reduced to succinate (Caccavo et al., 1994). The co-culture adapted to metabolize ethanol with the reduction of fumarate (Summers et al., 2010). Multiple lines of evidence (Rotaru et al., 2012; Shrestha et al., 2013a; Shrestha et al., 2013b; Summers et al., 2010) suggested that the electron transfer between the species was via the *Geobacter* pili that have metallic-like conductivity (Malvankar et al., 2011; Reguera et al., 2005). The possibility of interspecies H_2/formate transfer was ruled out by the fact that *G. metallireducens* is unable to metabolize ethanol with the production of H_2 or formate (Rotaru et al., 2012; Shrestha

et al., 2013a; Shrestha et al., 2013b), and the fact that interspecies electron exchange remained effective when the co-cultures were initiated with a *G. sulfurreducens* strain incapable of H_2 and formate uptake, because the genes encoding formate dehydrogenase and an uptake hydrogenase were deleted (Rotaru et al., 2012).

Methanosaeta and *Methanosarcina* species, which are often abundant in anaerobic digesters (Angenent et al., 2004; De Vrieze et al., 2012; McMahon et al., 2004; Morita et al., 2011; Steinhaus et al., 2007), are also capable of receiving electrons via DIET (Rotaru et al., 2014a; Rotaru et al., 2014b). *Methanosaeta harundinacea* (Rotaru et al., 2014b) or *Methanosarcina barkeri* (Rotaru et al., 2014a) grew in defined co-cultures with ethanol-metabolizing *G. metallireducens*, but only with strains of *G. metallireducens* that could produce pili which are electrically conductive (Malvankar et al., 2011; Reguera et al., 2005) . Metatranscriptomic analysis, as well as an assessment of metabolic potential and granule conductivity suggested that *Methanosaeta* species in a digester treating simulated brewery wastes also reduced carbon dioxide to methane with electrons derived from DIET (Morita et al., 2011; Rotaru et al., 2014b).

Although the biological electrical connections necessary for DIET are sufficient for effective syntrophic metabolism, studies with granular activated carbon (GAC), biochar, or nano-magnetite minerals demonstrated that DIET could be promoted via the conductive materials (Chen et al., 2014; Liu et al., 2012; Liu et al., 2014). For example, amending *G. metallireducens* - *G. sulfurreducens* or *G. metallireducens* - *M. barkeri* co-cultures with GAC greatly accelerated the initial rate of interspecies electron exchange (Liu et al., 2012). In the presence of GAC, digester granules in which *Methanosaeta* species were the predominant methanogens produced methane 2.5-fold faster than in GAC-free controls (Liu et al., 2012). GAC was 3000-fold more conductive than the *Geobacter* pili and in the presence of GAC even pili-deficient

strains can participate in DIET (Liu et al., 2012; Rotaru et al., 2014a). Electron-donating and accepting cells attached onto GAC, which served as a conduit for electron transfer between species.

This study aimed to reveal if carbon cloth, often used in rector design, presumably because of its biomass retention properties (Sasaki et al., 2007; Sasaki et al., 2009; Sasaki et al., 2010; Tatara et al., 2008; Zhang et al., 2012; Zhao et al., 2013) would rather serve as an electrical conduit to promote DIET. As control we tested non-conductive cotton cloth with similar biomass retention properties. Additionally, we examined if the conductivity of carbon cloth affected interspecies H_2 transfer. Learning about the impact of carbon cloth on electron transfer mechanism will assist future reactor designs and improve methane production during anaerobic digestion.

2 Materials and Methods

2.1 Microorganisms, media and growth conditions

All pure cultures and co-cultures were incubated in 27 mL pressure tubes with 10 mL medium under anoxic conditions with a gas phase of 80:20 of N_2:CO_2. *Geobacter sulfurreducens* strain DL1 (ATCC 51573) and various mutant strains were transferred routinely on NBF medium with 10 mM acetate as the electron donor and 40 mM fumarate as the electron acceptor (Coppi et al., 2004). *Geobacter metallireducens* strain GS-15 (ATCC 53774) and mutant strains were transferred routinely on FC medium with 10 mM ethanol as electron donor and 55 mM ferric citrate as the electron acceptor (Lovley et al., 1993). Co-cultures of *G. sulfurreducens* and *G. metallireducens* were initiated with a 5% inoculum of each microorganism into NBF medium which contained 10 mM ethanol as the electron donor and 40 mM fumarate as the electron acceptor. To determine whether pure cultures of *G. sulfurreducens* and *G.*

metallireducens could grown in the same medium, each strain was also inoculated separately into NBF medium with 10 mM ethanol as the electron donor and 40 mM fumarate as the electron. The incubation temperature for the *Geobacter* co-culture studies was 30 °C.

To prepare co-cultures of *D. vulgaris* and *G. sulfurreducens* we inoculated NBF medium with 10 mM ethanol as electron donor with 5% of each strain and incubated at 30 °C. Prior to incubations, *D. vulgaris* was grown routinely in NB medium with 20 mM sulfate and 10 mM ethanol.

To prepare co-cultures of *G. metallireducens* and *M. barkeri*, *M. barkeri* type strain DSM 800 (ATCC 43569) was grown in a modified DSMZ methanogenic medium DSMZ medium 120 with 20 or 30 mM acetate as substrate as previously described (Rotaru et al., 2014a). The medium modifications were adopted to improve growth of *G. metallireducens* on this medium as well. Co-cultures of *G. metallireducens* and *M. barkeri* were initiated with 5% inoculum of each microorganism in the modified DSMZ methanogenic medium 120 with 10 mM ethanol as sole electron donor (Rotaru et al., 2014a). To determine whether the strains were capable of utilizing ethanol alone, *G. metallireducens* and *M. barkeri* were inoculated separately into the same medium. The incubation temperature for all studies with *M. barkeri* was 37 °C.

2.2 Culturing with Carbon cloth

Carbon cloth named (Zoroflex; buyactivatedcharcoal.com) was cut into strips of 1.5 cm × 4 cm or 1.5 cm × 2 cm in order to provide 0.2 g or 0.1 g per tube in 10 mL of medium, respectively. The carbon cloth strips were wet-sterilized by autoclaving in pressure tubes in 2 mL of the same NBF culture medium, under a N_2:CO_2 atmosphere for 30 minutes. Then 7.5 mL of NBF medium (for *Geobacter* cocultures) or 7.5 mL of

modified 120 media (for *Geobacter-Methanosarcina* cocultures) were added to the cloth-containing tubes under anaerobic conditions along with 10 mM final concentration ethanol. Cotton cloth, which served as a non-conductive control, was treated in a similar manner. The conductivities of carbon cloth and cotton cloth were measured with a voltmeter by connecting the negative and positive probes to the diagonal ends of 1.5 cm × 2 cm cloth sheets.. Co-cultures were initiated and incubated as described in the previous section.

2.2 Analytical techniques

Liquid and headspace samples were withdrawn with hypodermic needles and syringes under strict anaerobic conditions. Liquid samples were passed through 0.2 μm Acrodisc filters. Concentrations of volatile fatty acids (butyrate, propionate, succinate, malate, fumarate, acetate, formate) were analyzed with high performance liquid chromatography, ethanol and methane were analyzed with gas chromatography as previously described (Rotaru et al., 2014a).

To quantify protein in the planktonic phase, 0.5 mL of the culture medium was removed from early stationary cultures. To quantify the proteins attached to carbon cloth the entire carbon cloth was separated from the liquid with a tweezer and cell protein was extracted from the cloth using NaOH 0.5 mM as previously described (Liu et al., 2012) and additional bead beating with sterile glass beads for 30 seconds. The concentration of protein extracted was quantified with the bicinchoninic acid method (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as protein standard.

2.3 Scanning electron microscopy

To visualize cell attachment to carbon cloth, scanning electron micrographs of the carbon cloth were taken at the end of the co-culture growth (day 10 of the *G. metallireducens-G.sulfurreducens* cycle, and day 30 of the *G. metallireducens-M.*

barkeri). Samples were first fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for up to 12 hours at 4 °C, then washed 3 times in 0.1 M phosphate buffer at 4 °C for 10 minutes each, dehydrated further in an ethanol/water mixture of 50%, 70%, 80%, 90%, 95% and 100% for 10 minutes each (dehydration in 100% ethanol was done 3 times), and then immersed twice for 0.5 minutes in pure hexamethyldisilazane (Sigma Aldrich, St Louis, MO, USA) followed by 10 minutes of air-drying as previously described (Araujo et al., 2003). The samples were sputter coated with gold and were visualized with a Magellan 400 FESEM.

3 Results and Discussion

3.1 Carbon cloth stimulation of DIET in Geobacter co-cultures

The ability of carbon cloth to promote DIET was first examined with co-cultures of *G. metallireducens* and *G. sulfurreducens*, because the availability of a diversity of gene-deletion mutants facilitates analysis of electron transfer mechanisms in these co-cultures. Addition of carbon cloth to co-cultures of *G. metallireducens* and *G. sulfurreducens* in medium with ethanol as the electron donor and fumarate as the electron acceptor stimulated syntrophic metabolism of ethanol (Figure 1A) with the reduction of fumarate to succinate (Figure 1B) within two days. There was no ethanol metabolism or fumarate reduction in pure cultures with carbon cloth (data not shown). The rates of metabolism in the co-cultures increased when the amount of carbon cloth was doubled. As previously reported, in the absence of carbon cloth, ethanol was metabolized very slowly as it initially takes the *G. metallireducens*-*G. sulfurreducens* ca. 30 days to adapt to grow via DIET (Summers et al., 2010). In contrast, the addition of cotton cloth did not stimulate co-culture metabolism (Figure 2SM). The electrical resistance of the cotton cloth (774 ± 43 kΩ; mean ± standard deviation, n=6) was more

than four orders of magnitude greater than for carbon cloth (54 ± 6 Ω). These results suggested that the conductivity of the carbon cloth was an important feature promoting DIET.

Acetate did not accumulate in the co-cultures and thus each mole of ethanol oxidized to carbon dioxide coupled to the reduction of fumarate could be expected to result in the reduction of 6 moles of fumarate to succinate (Liu et al., 2012). The 3.82 ± 0.77 mM ethanol metabolized in the co-cultures amended with 0.1 g of carbon cloth was associated with the accumulation of 16.90 ± 1.42 mM of succinate, accounting for 74 % of the electrons expected from ethanol removal. The electron recovery for the cultures amended with 0.2 g of carbon cloth was 78 %. These electron recoveries are consistent with previous results (Chen et al., 2014; Liu et al., 2012; Liu et al., 2014) and reflect the incorporation of some of the substrate into biomass. Most of the co-culture biomass (86%) was attached to carbon cloth (1.99 ± 0.12 mg in attached to cloth and 0.33 ± 0.05 mg in suspension) at the end of the 10 days of incubation. Scanning electron microscopy revealed that that cells were dispersed, with each cell in direct contact with the carbon cloth (Figure 1SM), rather than forming multilayered biofilms as previously reported for *Geobacter* species producing electrical current (Franks et al., 2010). This is the expected arrangement for cells if carbon cloth functions as the conduit for electron transfer between cells.

3.2 Carbon cloth restores DIET in co-cultures lacking conductive pili or the pili-associated cytochrome, OmcS

In order to further evaluate the mechanisms for electron transfer in the presence of carbon cloth, the impact of several gene deletions was investigated. Deletion of the gene for PilA, the structural protein for the electrically conductive pili completely inhibits co-culture metabolism when only biological electrical connections are feasible

(Rotaru et al., 2014a; Rotaru et al., 2014b; Summers et al., 2010). However, in the presence of carbon cloth the co-cultures initiated with PilA-deficient strains performed as well (co-culture with the PilA-deficient *G. sulfurreducens* strain) or nearly as well (co-culture with the PilA-deficient *G. metallireducens* strain) as co-cultures initiated with wild-type strains of both species (Figure 2). After 10 days of incubation all of the cells were strictly attached onto carbon cloth, indicating that the absence of pili did not prevent attachment to the cloth. Low protein recoveries (ca. 1 mg total protein) in carbon cloth co-cultures with the PilA-deficient *Geobacter* strains as compared to wild type co-cultures (ca. 2.3 mg total protein), is consistent with the inferior metabolic rates in pili-deficient co-cultures amended with carbon cloth. In general we noticed ca. 40% less protein produced by co-cultures with the PilA-deficient *G. sulfurreducens* (1.32 ± 0.04 mg attached to cloth and no protein detected in suspension) and ca. 55% less protein by co-cultures with the PilA-deficient *G. metallireducens* (1.05 ± 0.09 mg attached onto cloth and no protein detected in suspension). The pili-associated multi-heme *c*-type cytochrome OmcS (Leang et al., 2010; Mehta et al., 2005) is also essential for biological electrical connections in co-cultures without cloth (Summers et al., 2010), but a co-culture initiated with an OmcS-deficient strain of *G. sulfurreducens* metabolized as well as co-cultures initiated with wild-type in the presence of carbon cloth (Figure 2). These results suggested that carbon cloth, rather than conductive pili and pili-associated cytochromes, was the conduit for electron transfer between *G. metallireducens* and *G. sulfurreducens*.

3.3 Carbon cloth does not affect interspecies H_2 transfer between Desulfovibrio vulgaris and Geobacter sulfurreducens.

Desulfovibrio species can grow syntrophically by producing H_2 or formate as interspecies electron carriers (Meyer et al., 2013; Walker et al., 2009). The addition of

carbon cloth did not accelerate the metabolism of a co-culture of *Desulfovibrio vulgaris* and *G. sulfurreducens*. Triplicate cultures with and without carbon cloth both metabolized ethanol with similar rates of ca. 0.8 mM ethanol day $^{-1}$. These results are consistent with the concept that carbon cloth promoted ethanol metabolism in the *G. metallireducens*-*G. sulfurreducens* co-cultures by facilitating DIET.

3.4 Carbon cloth stimulation of DIET between Geobacter metallireducens and Methanosarcina barkeri

In order to determine whether carbon cloth might also promote DIET with methanogens, the impact of carbon cloth on co-cultures of *G. metallireducens* and *M. barkeri* was investigated. Although *M. barkeri* has the ability to use H_2 as an electron donor, *G. metallireducens* is unable to metabolize ethanol with the production of H_2 (Rotaru et al., 2014b; Shrestha et al., 2013a; Shrestha et al., 2013b). Therefore, effective metabolism of ethanol to methane in *G. metallireducens* - *M. barkeri* co-cultures can be attributed to DIET (Rotaru et al., 2014a). *G. metallireducens* - *M. barkeri* co-cultures not amended with cloth require ca. 40 days before detectable ethanol metabolism and methanogenesis starts (Rotaru et al., 2014a). However, when co-cultures were initiated in the presence of carbon cloth ethanol metabolism began within 10 days (Figure 3) and in 30 days 9.9 mmol ethanol were consumed and 10.1 mmol methane were produced. This was comparable to results previously observed in GAC-amended co-cultures (Liu et al., 2012; Rotaru et al., 2014a). Similar to previous results (Rotaru et al., 2014a), there was no ethanol metabolism or methane production within 30 days in the absence of carbon cloth (Figure 3). Cultures of *M. barkeri* alone amended with carbon cloth did not metabolize ethanol (data not shown).

Non-conductive cotton cloth did not promote DIET (Figure 3). Most of the cellular protein (82%) of the co-cultures adhered to the carbon cloth (1.20 ± 0.07 mg attached

onto cloth and 0.26 ± 0.08 mg in suspension). Scanning electron microscopy revealed that *G. metallireducens* and *M. barkeri* attached to the carbon cloth, but were not closely associated with each other, suggesting that the electrical connections between the two species were through the carbon cloth rather than via cell-to-cell electron transfer (Figure 3SM).

4 Conclusions

These results demonstrate that carbon cloth can promote DIET. Carbon cloth may be particularly effective in accelerating the initial electron exchange between species, eliminating the need for biological connections via structures such as conductive pili. The selective attachment of *Methanosarcina* and *Methanosaeta* species to carbon cloth in methanogenic digesters is consistent with the finding that methanogens within this family are capable of DIET. Thus, the conductivity of carbon cloth should be considered as an important parameter when interpreting the impact of carbon cloth on methane production rates in anaerobic digesters and in the design of digesters that incorporate conductive cloth.

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7. Figure legends

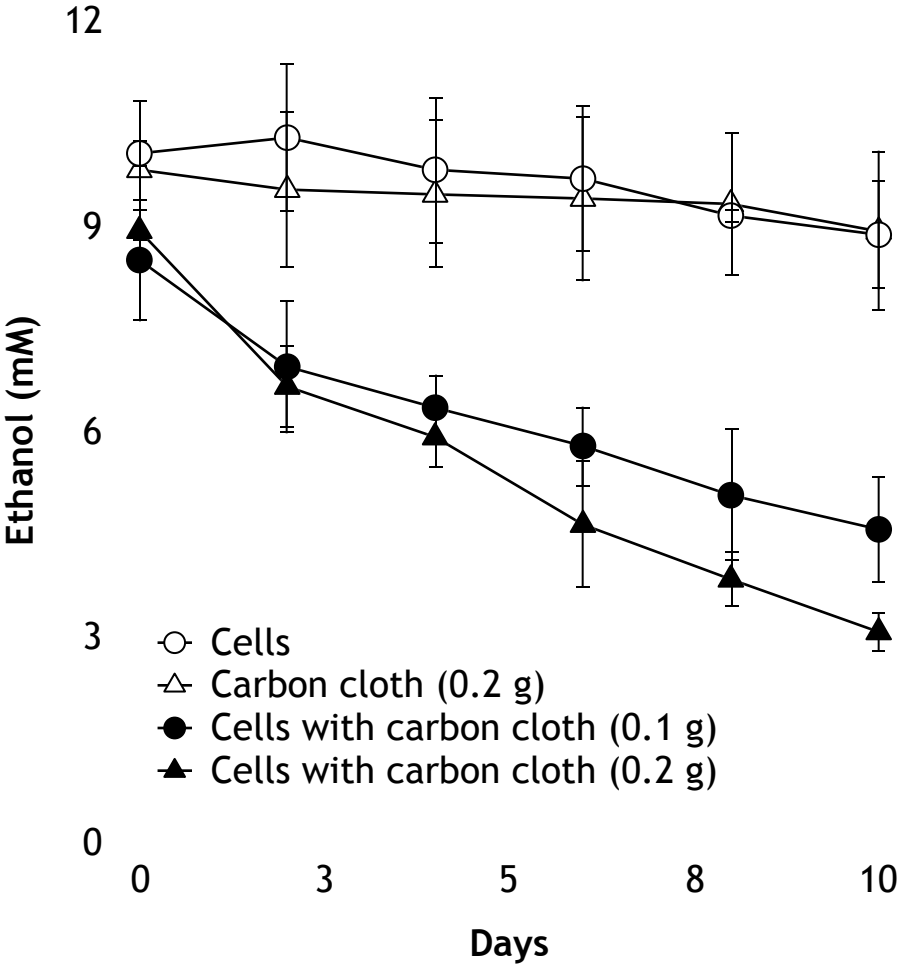
Figure 1.

The impact of carbon cloth on syntrophic electron exchange between *G. metallireducens* and *G. sulfurreducens*. a) Ethanol utilization by *G. metallireducens*–*G. sulfurreducens* co-cultures established with conductive carbon cloth. a) Succinate production by the same co-cultures.

Figure 2. Carbon cloth rescues DIET between strains lacking pili or the pili-associated cytochrome, OmcS. Succinate production in carbon cloth-amended co-cultures established with different gene deletion strains. Abbreviations used: G.m. (*G. metallireducens*), G.s. (*G. sulfurreducens*).

Figure 3. The impact of carbon cloth on syntrophic electron exchange between *G. metallireducens* and *M. barkeri*.

Ethanol consumption and methane production in carbon cloth-amended co-cultures (squares), compared to co-cultures without carbon cloth (triangles), and co-cultures with non-conductive cotton cloth (circles).


12
9
6
3
0
Ethanol (mM)
0
3
5
8
10
Days
Cells
Carbon cloth (0.2 g)
Cells with carbon cloth (0.1 g)
Cells with carbon cloth (0.2 g)

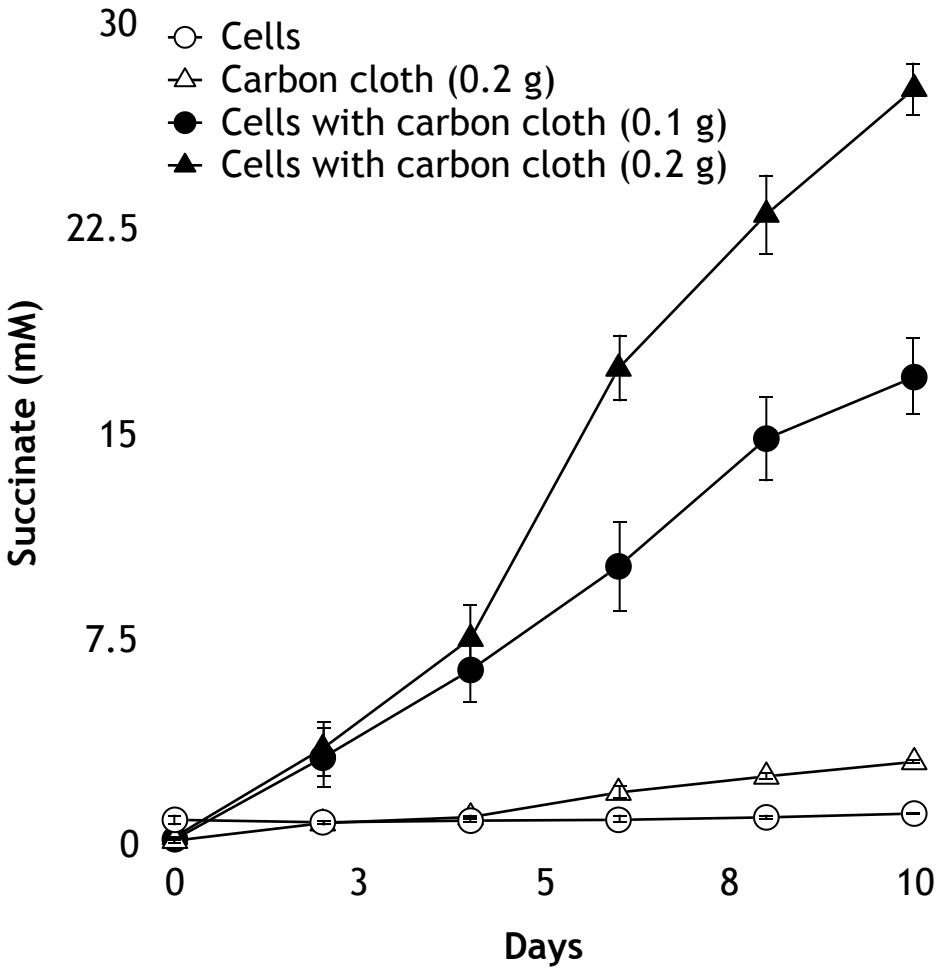

30
22.5
15
7.5
0
Succinate (mM)
0
3
5
8
10
Days
Cells
Carbon cloth (0.2 g)
Cells with carbon cloth (0.1 g)
Cells with carbon cloth (0.2 g)

Figure 1

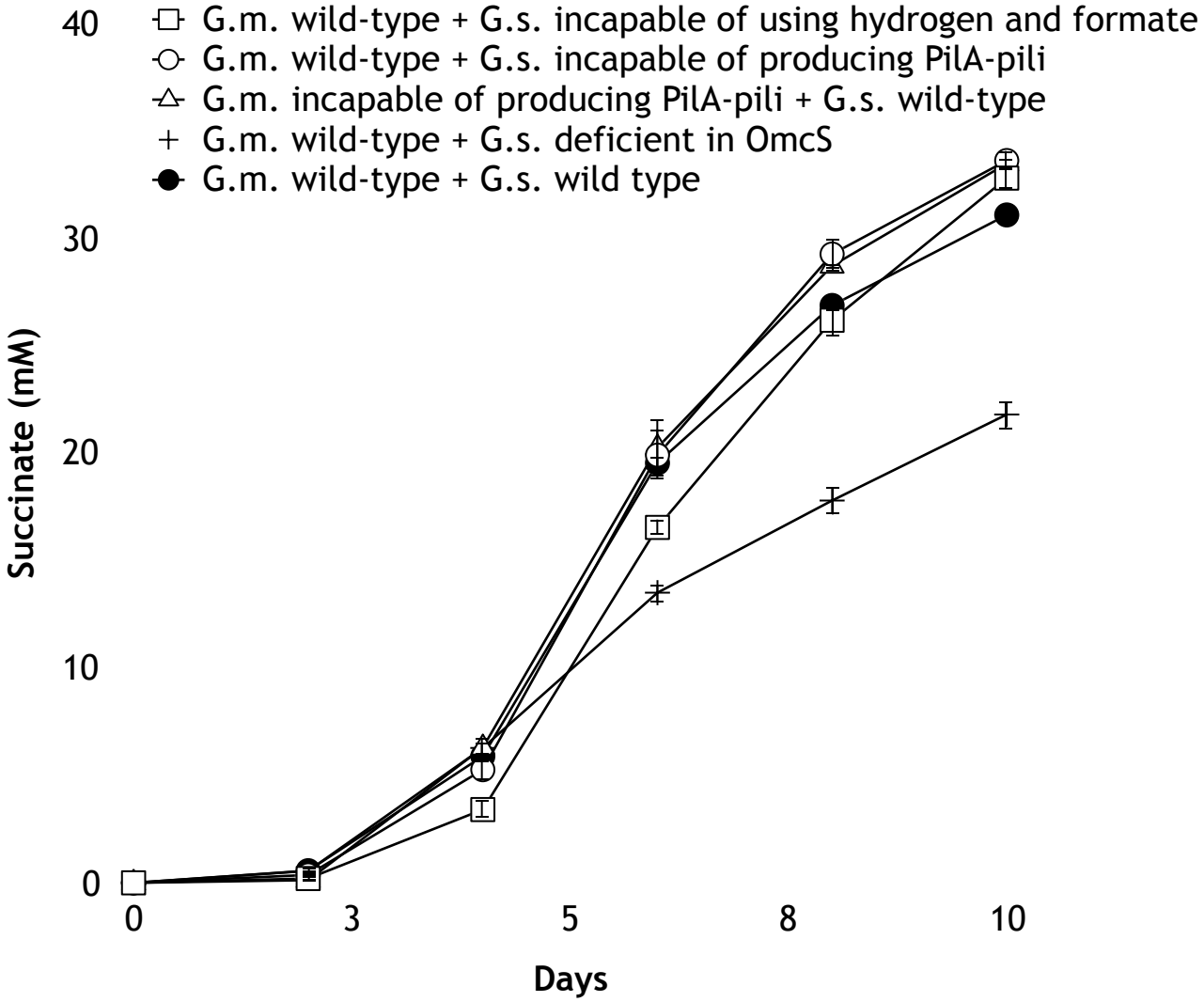
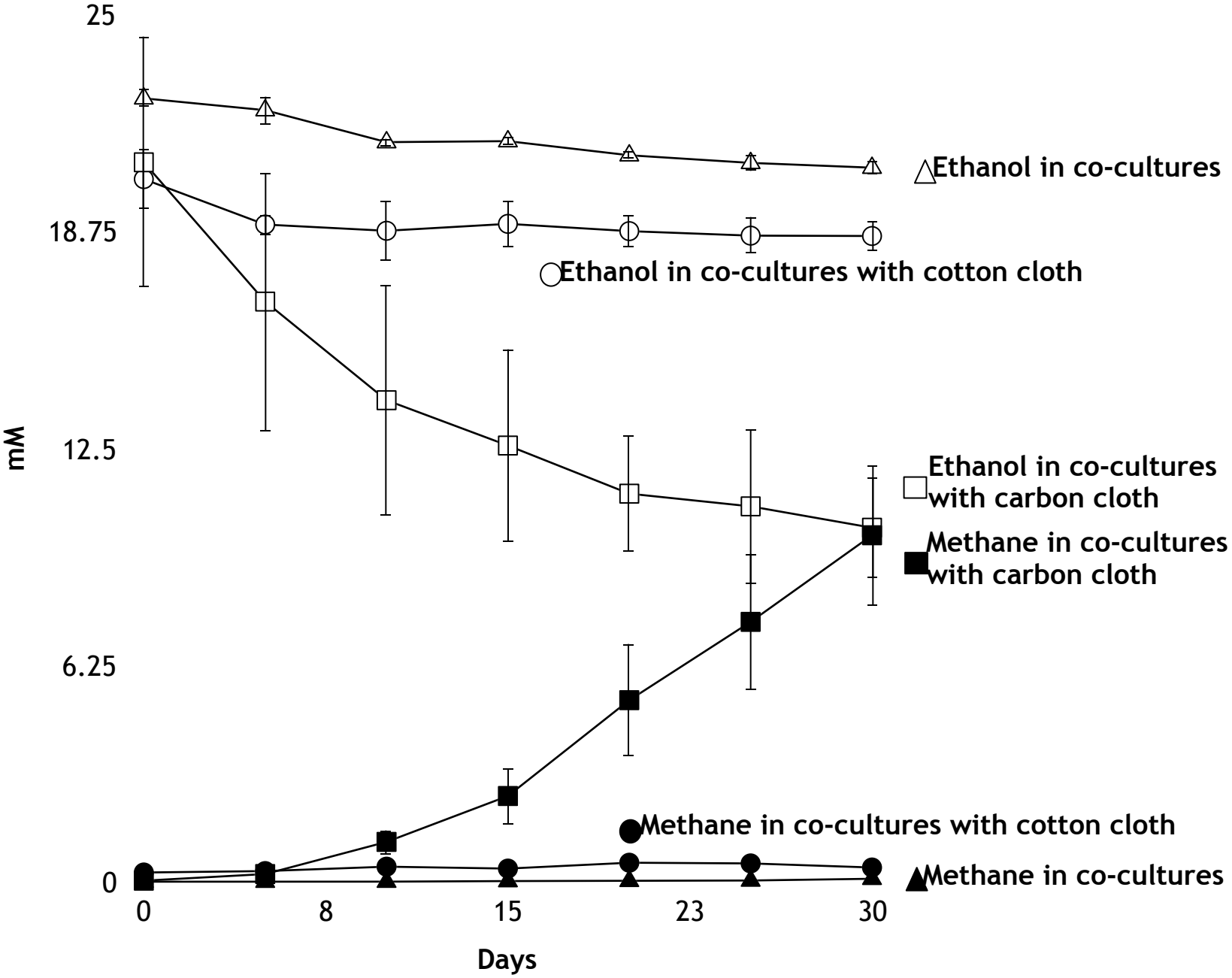

G.m. wild-type + G.s. incapable of using hydrogen and formate
G.m. wild-type + G.s. incapable of producing PilA-pili
G.m. incapable of producing PilA-pili + G.s. wild-type
G.m. wild-type + G.s. deficient in OmcS
G.m. wild-type + G.s. wild type
Succinate (mM)
40
30
20
10
0
0
3
5
8
10
Days

Figure 2

Figure 3


25
18.75
12.5
6.25
0
mM
0
8
15
23
30
Days
Ethanol in co-cultures
Ethanol in co-cultures with cotton cloth
Ethanol in co-cultures with carbon cloth
Methane in co-cultures with carbon cloth
Methane in co-cultures with cotton cloth
Methane in co-cultures