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ARTICLE

Syntrophic growth with direct interspecies electron transfer between pili-free *Geobacter* species

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Abstract

Direct interspecies electron transfer (DIET) may prevail in microbial communities that show methanogenesis and anaerobic methane oxidation and can be an electron source to support anaerobic photosynthesis. Previous mutagenic studies on cocultures of defined *Geobacter* species indicate that both conductive pili and extracellular cytochromes are essential for DIET. However, the actual functional role of the pili in DIET is uncertain, as the pilus mutation strategy used in these studies affected the extracellular cytochrome profile. Here we repressed the function of pili by deleting the pilus polymerization motor PilB in both *Geobacter* species. The PilB mutation inhibited the pilus assembly but did not alter the pattern of extracellular cytochromes. We report that the two pilus-free *Geobacter* species can form aggregates and grow syntrophically with DIET. The results demonstrate that the Gmet_2896 cytochrome of *Geobacter metallireducens* plays a key role in DIET and that conductive pili are not necessary to facilitate DIET in cocultures of *Geobacter* species, and they suggest cytochromes by themselves can meditate DIET, deepening the understanding of DIET.

Introduction

Direct interspecies electron transfer (DIET) was first demonstrated in the cocultures of *Geobacter metallireducens* with *Geobacter sulfurreducens* [1]. *G. metallireducens* cannot reduce fumarate, and *G. sulfurreducens* cannot oxidize ethanol. When these two species were inoculated in the medium with ethanol as electron donor and fumarate as electron acceptor, they formed a syntrophic partnership after aggregation with direct electrical connections [1]. Compared to mediated interspecies electron transfer (MIET), in which H_2 or formate usually acts as an electron carrier, DIET has unique advantages under some circumstances and its biogeochemical impact may be significant, such as: (i) DIET is the predominant form of interspecies electron transfer in some anaerobic methanogenic digesters and may be involved in the production of atmospheric methane in terrestrial wetlands [2, 3]. (ii) DIET has been proposed to control methane release [4–6]. (iii) A recent finding indicates that DIET can be electron sources to support anaerobic photosynthesis [7].

The knowledge of DIET mainly originates from the study of cocultures between *G. metallireducens* and *G. sulfurreducens*, owing to the availability of genetic operation systems for both species. However, present knowledge of DIET is limited to the identification of key elements in this process, including the outer-surface Gmet_2896 cytochrome, flagella, and conductive pili of *G. metallireducens* [8] and the extracellular hexaheme cytochrome OmcS and conductive pili of *G. sulfurreducens* [1]. The proposed mechanism of DIET for these defined *Geobacter* species cocultures is that conductive pili from both *Geobacter* species facilitate the interspecific transfer of electrons with the help of cytochromes, which emphasizes the importance of the high conductivity of pili during DIET [9]. The importance of cytochromes in DIET is clear, such as the following: (i) the mutation of the above-mentioned cytochromes inhibits the formation of cocultures; (ii) the DIET community, such as anaerobic bioreactors for treatment of brewery wastes [10], methanogenic digestion of wastewater [3] and rice paddy soils [11], always contains *Geobacter* species at high abundance, which take a hall-mark

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feature of abundance with *c*-type cytochromes [12]; (iii) The metagenome of proposed DIET-based consortia of anaerobic methane-oxidizing archaeal group 1 (ANME-1) methanotrophic archaea coupled to sulfate reduction contains abundant genes encoding multi-heme cytochromes [13]; (iv) in the DIET consortia of ANME-2 with its sulfate-reducing partner, regions with dense heme-staining in the multicellular assemblages are visualized indicating a cytochrome-based electron transfer between species [5]; and (v) the outer membrane surface cytochrome OmcB of *G. sulfurreducens* is necessary for syntrophic anaerobic photosynthesis [7].

However, the electrically conductive function of pili in DIET seems ambiguous, as the nonconductive pili of *Candidatus Desulfofervidus auxilii* can also participate in DIET with ANME-1 [6, 14]. Moreover, the direct deletion of conductive pilin gene *pilA* of *G. sulfurreducens* affects the distribution and content of extracellular cytochromes [15, 16], especially OmcS (Fig. 1a, b). Unfortunately, all conclusions related to DIET based on conductive pili come from studies of pilin-deficient strains [1, 8, 17]. A new mutation strategy is warranted to unambiguously specify the function of conductive pili in DIET.

The *pilA* determines the expression of conductive pili, while the type IV pilus biogenesis ATPase PilB powers the polymerization of *Geobacter* pilin [18]. The PilB-deficient strain of *G. sulfurreducens* demonstrates no measurable differences in expression of extracellular cytochromes compared to the wild-type strain [16]. Here, we constructed PilB-deficient strains of both *G. metallireducens* and *G. sulfurreducens*. We found that the PilB-deficient *Geobacter* species possess integrated profiles of outer-surface cytochromes but cannot assemble conductive pili. However, these pili-free *Geobacter* species form cocultures readily with DIET, and the Gmet_2896 cytochrome plays a key role in this process. Our findings indicate that cytochromes by themselves can mediate DIET and suggest that DIET may widespread in natural environment.

Materials and methods

Bacterial strains and cultivation condition

The bacterial strains used in this study are listed in Supplementary Table 1. The wild-type strains, *Geobacter sulfurreducens* PCA (ATCC-51573) and *Geobacter metallireducens* GS15 (ATCC-53774), were obtained from the ATCC and used for the construction of related mutants. All *Geobacter* strains were cultured at 30 °C under strict anaerobic conditions (80%/20% N_2/CO_2) and were regularly grown in bicarbonate-buffered medium with 20 mM acetate as the electron donor and either 40 mM fumarate or

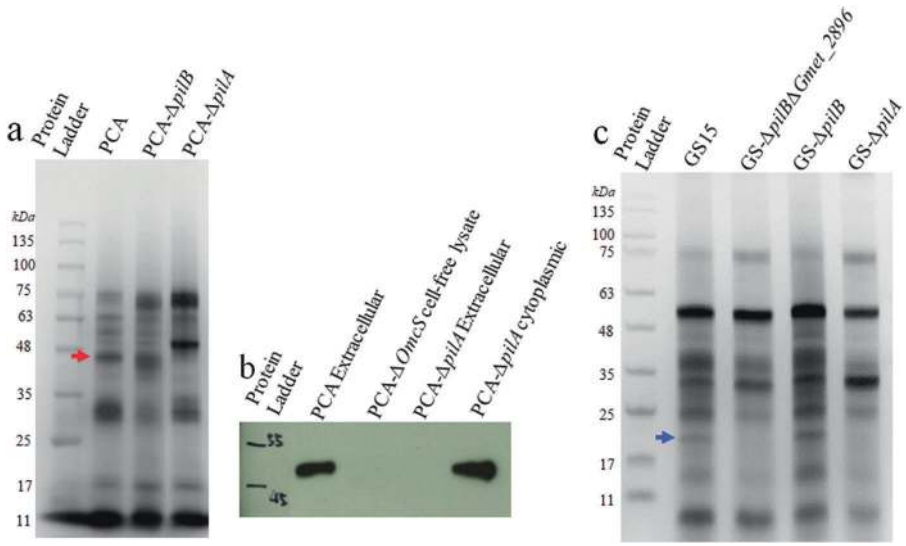

Fig. 1 Extracellular cytochromes profile. **a** Heme-stained SDS-PAGE gel of extracellular c-type cytochromes prepared from *G. sulfurreduencs* strain PCA, pili mutant (PCA-Δ*pilA*) and type IV pilus biogenesis ATPase deficient strain PCA-Δ*pilB*. **b** Western blot analysis of extracellular proteins prepared from *G. sulfurreducens* strains PCA and PCA-Δ*pilA*, and cell-free lysate from the OmcS mutant (PCA-*ΔOmcS*) of *G. sulfurreducens*, and cytoplasm from PCA-Δ*pilA*, using antibodies specific for OmcS. The deletion of *pilA* inhibited the excretion of OmcS in *G. sulfurreducens*. **c** Heme-stained SDS-PAGE gel of extracellular c-type cytochromes prepared from *G. metallireducens* strain GS15, pili mutant (GS-Δ*pilA*), type IV pilus biogenesis ATPase deficient strain GS-Δ*pilB* and the Gmet_2896 gene deletion strain of *G. metallireducens* GS-*ΔpilB* (strain GS-*ΔpilBΔGmet_2896*). The deletion of *pilA* inhibited the excretion of Gmet_2896 cytochrome in *G. metallireducnes*. The red and blue arrows indicate the OmcS and Gmet_2896 cytochrome bands, respectively, which are assigned based on molecular weight. 2 µg proteins were loaded on the gel

50 mM ferric citrate as the electron acceptor, as previously reported (Liu et al 2014b). Specially, H_2 (138 KPa) was complemented in the medium for the cultivation of a citrate synthase-deficient strain of *G. sulfurreducens*, strain PCA-*ΔpilBΔgltA*. When needed, agar (1.5%) and antibiotics were added to the medium for selection of all mutants on plates. For insoluble Fe(III) reduction analysis, 100 mM poorly crystalline Fe(III) oxide was used as the electron acceptor (Liu et al 2014b). The metabolism of Fe(III) was measured by using the FerroZine assay [19].

To coculture the mutants of *G. sulfurreducens* and *G. metallireducens*, 2% inoculum of each syntrophic partner was inoculated into a mineral-based medium with 20 mM ethanol as the electron donor and 40 mM fumarate as the electron acceptor, as previously described [20]. All cocultures were regularly transferred (2% inocula) under strict anaerobic conditions for at least six generations before monitoring growth. The growth of individual *Geobacter* strains and the cocultures containing the gene Gmet_2896 deletion strain of *G. metallireducens* (strain GS-*ΔpilBΔGmet_2896*) was analyzed during the initial inoculation because they cannot grow on ethanol. *Escherichia coli* strain DH5α was routinely used for cloning and was cultured at 37 °C in Luria-Bertani medium supplied with the appropriate antibiotics.

Generation of *G. sulfurreducens* and *G. metallireducens* mutants

All primers used for the construction of mutants and for the verification of mutant strains are listed in Supplementary Table 2. All *G. metallireducens* mutants were constructed following a previously published method [21]. To construct the *G. metallireducens* strain deficient in the type IV pilus biogenesis ATPase (PilB, GenBank locus Gmet_1393), GS-*ΔpilB*, three fragments were prepared: first, the primer pairs gsBupf/gsBupr and gsBdnf/gsBdnr were used to amplify the sequences 500 bp upstream and 500 bp downstream, respectively, of Gmet_1393, using *G. metallireducens* genomic DNA as template and the primer pair spef/sper was used to amplify the spectinomycin resistance cassette flanked by *loxP* sites from plasmid pRG5. These three fragments were connected and inserted into the In-Fusion Cloning site of linear plasmid pUC19 (included in the kit) using the In-Fusion HD Cloning Kit (Takara Biomedical Technology, Beijing, China), generating the plasmid pUC-GspilB. The plasmid was linearized with NcoI (New England BioLabs (NEB), Ipswich, MA, USA) and then electroporated into electrocompetent *G. metallireducens* GS15. The deletion of *pilB* was confirmed by PCR (Supplementary Fig. S1). To construct the *pilB* and Gmet_2896 double-deletion strain of *G. metallireducens*, named GS-*ΔpilBΔGmet_2896*, the spectinomycin resistance cassette (sp-*loxP*) carried by GS-*ΔpilB* was first excised from its chromosome by expressing the Cre recombinase from the plasmid pCM158 using a published procedure [21]. The excision of the antibiotic marker was confirmed by PCR with primer pairs verGmet1393F/verGmet1393R (Supplementary Fig. 1), generating the strain GS-*ΔpilB2*. Two fragments, the sequences 500 bp upstream and downstream of gene Gmet_2896, were amplified from the *G. metallireducens* genome using the primer pairs Gs2896upf/Gs2896upr and Gs2896dnf/Gs2896dnr, respectively, and then, together with the fragment of sp-*loxP*, they were inserted into the pUC19 In-Fusion Cloning site using the In-Fusion HD Cloning Kit, generating the plasmid pUC-GS2896. After linearizing with NcoI (NEB), the plasmid was electroporated into the electrocompetent *G. metallireducens* strain GS-*ΔpilB2*. The *pilA* (Gmet_1399) deletion strain of *G. metallireducens* was constructed following the same method, using the primer pairs GspilAupf/GspilAupr and GspilAdnf/GspilAdnr to amplify sequences 500 bp upstream and downstream of the gene Gmet_1399, and then connecting them with sp-*loxP* and the linearized plasmid pUC19, generating plasmid pUC-GspilA. The plasmid was linearized with NcoI (NEB) and then electroporated into electrocompetent *G. metallireducens*.

A previously published procedure was used to construct the *G. sulfurreducens* mutants [22]. To construct the *G. sulfurreducens* strain deficient in the type IV pilus biogenesis ATPase (PilB, GenBank locus GSU1491), PCA-*ΔpilB*, three fragments were prepared: first, the primer pairs ppilBupf/ppilBupr and ppilBdnf/ppilBdnr were used to amplify the sequences 500 bp upstream and downstream, respectively, of GSU1491 with *G. sulfurreducens* genomic DNA as the template, and then, the primer pair gmf/gmr was used to amplify the gentamycin-resistance cassette flanked by *loxP* sites (Gm-*loxP*) from the plasmid pCM351. These fragments were connected with the linearized plasmid pUC19 using In-Fusion HD Enzyme, generating the plasmid pUC-PCApilB. The plasmid was linearized with NcoI (NEB) and then electroporated into electrocompetent *G. sulfurreducens* PCA. To construct the formate dehydrogenase (*fdnG* gene, GSU0777), uptake hydrogenase (*hybL* gene, GSU0785) and PilB triple-deficient strain, as well as the citrate synthase (*gltA* gene, GSU1106) and PilB double-deficient strain, of *G. sulfurreducens*, strains PCA-*ΔpilBΔfdnGΔhybL* and PCA-*ΔpilBΔgltA*, respectively, the gentamycin-resistance cassette (Gm-*loxP*) carried in the chromosome of PCA-*ΔpilB* was excised by expressing the Cre recombinase from plasmid pCM158 following a published procedure [21]. The removal of Gm-*loxP* was confirmed by PCR with primer pairs verpilBf/verpilBr (Supplementary Fig. S1), generating strain PCA-*ΔpilB2*. The primer pairs hybLupf/hybLupr and hybLdnf/hybLdnr were used to amplify the

sequences 500 bp upstream and downstream of GSU0785, and the primer pairs gltAupf/gltAupr and gltAdnf/gltAdnr were used to amplify the corresponding sequences 500 bp upstream and downstream of GSU1106, each from the *G. sulfurreducens* genome. These fragments, together with Gm-*loxP*, were connected with the linearized plasmid pUC19 by In-Fusion HD Enzyme (Takara), generating the plasmids pUC-hybL and pUC-gltA, respectively. Plasmids were linearized by NcoI (NEB) and electroporated into the electrocompetent *G. sulfurreducens* strain PCA-*ΔpilB2*, generating the strains PCA-*Δ pilBΔhybL* and PCA-*Δ pilBΔgltA*, respectively. The PilA-deficient strain of *G. sulfurreducens*, PCA-*ΔpilA*, was constructed in a similar manner as a previous report [23] by in-frame deleting the *pilA* (GSU1496) and using the primer pairs pilAupf/pilAupr, pilAdnf/InpilAr and InpilAf/pilAdnr to amply the sequences last 500 bp of *pilR*, promoter of GSU1496 and downstream of GSU1496, respectively. To construct strain PCA-*ΔpilBΔfdnGΔhybL*, the sequences 500 bp upstream and downstream of GSU0777 were amplified from PCA genome with the primer pairs fdnGupf/fdnGupr and fdnGdnf/fdnGdnr, respectively, and a kanamycin resistance cassette was amplified from the plasmid pCM158 with the primer pair kanf/kanr. These three fragments were connected with pUC19, generating plasmid pUC-fdnG, and then digested with NcoI (NEB) and transformed into the electrocompetent *G. sulfurreducens* strain PCA-*ΔpilBΔhybL*. All mutants were verified by PCR (Supplementary Fig. S1) and Sanger sequencing.

SDS-PAGE gel analysis and Western blot

Extracellular proteins were collected from late-log-phase cells as previously described [24, 25] and quantified by using the bicinchoninic acid assay following the manufacturer's instructions (Micro BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein mixed with reducing SDS-PAGE loading buffer were loaded on 4–20% Mini-PROTEAN TGX Precast Gels (Bio-Rad Laboratories, Hercules, California, USA) and separated by SDS-PAGE. For heme-staining, non-reducing loading buffer was used. After electrophoresis, proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane with a semi-dry blotting system (Bio-Rad). The blotting membrane was sequentially incubated with ani-OmcS rabbit polyclonal antibodies and anti-rabbit secondary monoclonal antibody (Sangon Biotech, Shanghai, China) following a previously described method [26]. The OmcS bands were detected by chemiluminescence (SuperSignal West Pico kit, Thermo Scientific). Heme-staining was performed according to a previous study [27] by using N,N,N′,N′-tetramethylbenzidine to stain the hemes of *c*-type cytochromes.

Raising the anti-OmcS antibodies

The full-length of *omcS* gene (GSU2504) without the signal peptide sequences was amplified from *G. sulfurreducens* PCA genome with primer pairs OmcSndeI (5′-catatgTTCCACTCCGGCGGCGTT-3′) and OmcSxhoI (5′-ctcgagtGTCCTTGGCGTGGCACTTGT-3′). The PCR products were digested by *NdeI* (NEB) and *XhoI* (NEB), and were sub-cloned into pET-29a(+) plasmid between the *NdeI* and *XhoI* sites. The constructed vector carrying the OmcS-coding gene was sent out for protein overexpression and purification, and for antibody preparation (Genscript Biotech, Nanjing, China).

Analytical techniques

For monitoring the growth of cocultures, 500 µL of culture medium was sampled under strict anaerobic conditions and was filtered with a 0.2 µm sterilized filter. Samples were stored at 4 °C for a maximum of 1 week before testing. The organic acids were separated and quantified by high-performance liquid chromatography as described in a previous study [20] using an Aminex NPX-87H column (Bio-Rad) with 8 mM H_2SO_4 as the eluent under a flow rate of 0.6 mL min^{-1} and were detected at 210 nm by a UV detector (Thermo U-3000 HPLC, Thermo Fisher Scientific). Ethanol was analyzed by gas chromatography performed on an Agilent 7890 A (Agilent Technologies, Santa Clara, California, USA) equipped with a headspace automatic sampler and an FID. Separation of ethanol was achieved with a HP-INNOWAX (Agilent) column (30 m length, 0.530 mm inner diameter, 1-µm film thickness) using N_2 as the carrier gas and under the following conditions: 50 °C for 1 min, a ramp of 12 °C per minute to reach 200 °C, 1 min at 200 °C. The injector and front detector temperatures both were set at 200 °C.

Microscopy

For transmission electron microscopic imaging, a drop of culture was loaded on a 400-mesh carbon-coated copper grid and left at room temperature for 5 min before wiping with filter paper. Samples were negatively stained with 2% uranyl acetate and imaged under a Tecnai 12 transmission electron microscope operating at 100 kV. The coculture aggregates were visualized by phase-contrast microscopy on a Leica DMi8 microscope (Leica Microsystems Inc., Buffalo Grove, United States).

The formation of syntrophic partners and the distribution of the two strains in the cocultures were verified by fluorescence in situ hybridization with strain-specific probes as previously described [1] and were visualized on a Leica TCS SP8 confocal fluorescence microscope (Leica Microsystems Inc.) using consecutive line scanning. Briefly, the

cocultures were fixed by direct addition of paraformaldehyde and glutaraldehyde at final concentrations of 2% and 0.5%, respectively, followed by incubation at room temperature for 1 h. Aggregates were applied on a gelatin-coated slide, dried at 46 °C, and then dehydrated in 70% ethanol for 30 min at 4 °C. Slides were incubated at 46 °C for 2 h in hybridization buffer, which contained probe GEO1 (5′-[cy3]AGAATCCAAGGACTCCGT-3′), specific for *G. metallireducens*, and GEO2 (5′-[FAM]GAAGACA GGAGGCCCGAAA-3′, specific for *G. sulfurreducens*. After hybridization, slides were washed for 30 min in washing buffer at 48 °C, and then rinsed with Milli-Q water. Slides were mounted with ProLong Gold antifade reagent (Thermo Fisher Scientific) and then were ready for confocal microscopy.

Results and discussion

Similar profiles of extracellular *c*-type cytochromes in PilB-deficient strains of *Geobacter* species

The conductive pili of *G. sulfurreducens* are mainly composed of geopilin, PilA, who shares certain characteristics with those of type II secretion systems [28]. Direct knockout of the *pilA* from *G. sulfurreducens* (strain PCA-*ΔpilA*) altered its extracellular *c*-type cytochrome patterns (Fig. 1a), especially with regard to the *c*-type cytochrome OmcS, which disappeared from the extracellular matrix (Fig. 1a, b) and which is necessary during the direct interspecies electron transfer between *G. metallireducens* and *G. sulfurreducens* with ethanol as the only electron donor and fumarate as the only electron acceptor [1]. In contrast, deletion of the *pilB* from *G. sulfurreducens* (strain PCA-*ΔpilB*) did not alter the extracellular *c*-type cytochrome patterns (Fig. 1a) but inhibited pilus assembly [16]. Similarly, deletion of *pilA* from *G. metallireducens* (strain GS-*ΔpilA*) also affected its extracellular cytochrome profile (Fig. 1c), especially the Gmet_2896 cytochrome, which is necessary in DIET [8] and could not be detected in the extracellular matrix of GS-*ΔpilA*. On the contrary, the deletion of *pilB* from *G. metallireducens* (strain GS-*ΔpilB*) did not affect the extracellular *c*-type cytochrome profile (Fig. 1c).

Deletion of *pilB* from *G. metallireducens* inhibited pilus assembly and affected Fe(III) oxide reduction

The wild-type strain of *G. metallireducens* grew both pili and flagella (Fig. 2a) when Fe(III) citrate acted as an electron acceptor at 25 °C, a temperature inducing pilus synthesis in *Geobacteraceae* [21]. The motor ATPase PilB powers pilus assembly but not flagellar assembly. *G. metallireducens* strain GS-*ΔpilB* expressed normal flagella but could not assemble pili (Fig. 2b). Pili of *G. metallireducens* are necessary for the reduction of insoluble outer cellular electron acceptors. Deletion of *pilA* from *G. metallireducens* (strain GS-*ΔpilA*) inhibited the reduction of Fe(III) oxide but not reduction of soluble Fe(III) citrate [21]. *G. metallireducens* strain GS-*ΔpilB* displayed certain Fe(III)

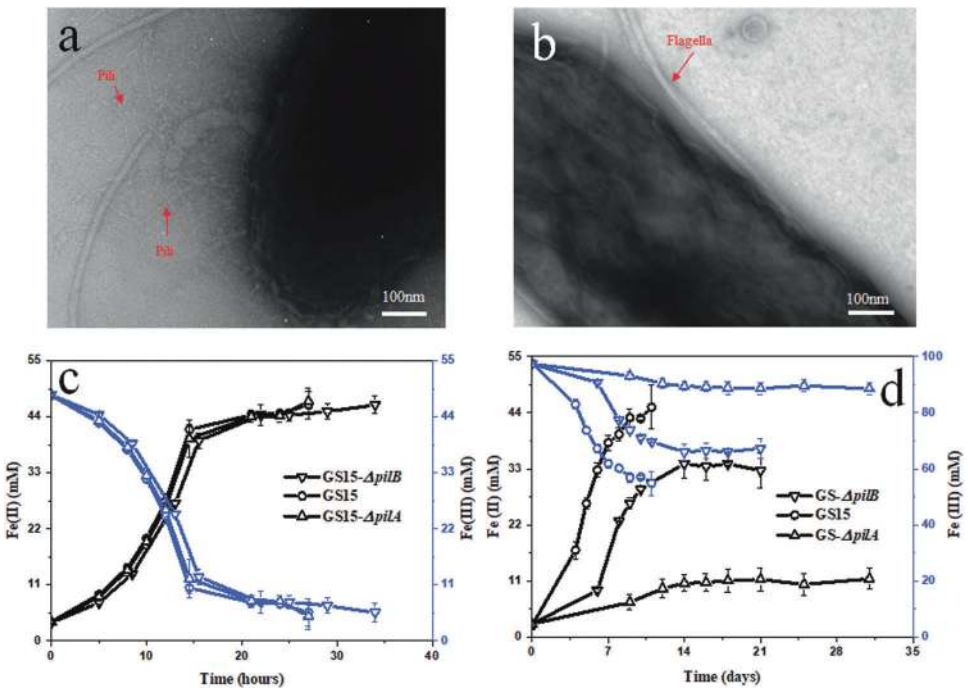

Fig. 2 Phenotypic characterization of *G. metallireducens* strains. Representative transmission electron micrographs of negatively stained (**a**) strain GS15 and (**b**) strain GS-*ΔpilB*. The deletion of *pilB* inhibited the growth of pili. Representative of 16 and 23 images obtained, respectively. Both strains were cultured at 25 °C with ferric citrate as electron acceptor. Fe (II) production from the reduction of (**c**) Fe (III) citrate or (**d**) Fe (III) oxide by strain GS15, GS-*ΔpilB* and GS-*ΔpilA*. The pili did not contribute the electrons transfer to the outer cell surface but affected the extracellular electron transfer. The results shown were the means ± s.d. for triplicate cultures

reduction phenotype similar to that of strain GS-*ΔpilA* and GS15, in which the reduction of soluble Fe(III) was unaffected (Fig. 2c), indicating that the deletion of *pilB* also did not affect electrons transfer to the outer cell surface of *G. metallireducens*, except that GS-*ΔpilB* only showed diminished Fe(III) oxide reduction with a much longer lagging-phase and the incomplete reduction of total Fe (III) oxide (Fig. 2d), unlike *G. metallireducens* strain GS15.

Direct interspecies electron transfer between GS-PilB and PCA-PilB in cocultures

G. metallireducens and *G. sulfurreducens* cocultures can form aggregates with DIET, a process that has been thought to require the participation of conductive pili from both species [1, 8]. When disable the expression of either pili of *G. metallireducens* or *G. sulfurreducens*, the cocultures cannot grow [1, 8]. Notably, in one study, it seems that the *G. metallireducens* pili are more important than *G. sulfurreducens* pili in DIET [29], as the magnetite can partially compensate the deficient of *G. sulfurreducens* pili in DIET cocultures but cannot compensate for the mutation of *G. metallireducens* pili. Either of the two pilus-assembly-deficient *Geobacter* species (*G. metallireducens* strain GS-*ΔpilB* and *G. sulfurreducens* strain PCA-*ΔpilB*) could not grow individually in coculture medium with ethanol as electron donor and fumarate as electron acceptor (Fig. 3a, b). Surprisingly, they formed cocultures in which the oxidation of ethanol to acetate and the reduction of fumarate to succinate occurred as fast as in cocultures initiated with wild-type *Geobacter* species (Fig. 3c, d). Contact between syntrophic partners is the defining characteristic of DIET-based cocultures [2]. The cocultures of *G. metallireducens* GS-*ΔpilB* and *G. sulfurreducens* PCA-*ΔpilB* could also form obvious large flocks by associations of individual cells (Fig. 4a, b). After hybridization with strain-specific oligo probes, this cocultures demonstrated a composition of mixed strains with the domination of *G. sulfurreducens* PCA-*ΔpilB* (Fig. 4c).

To eliminate the possibility of H_2 and/or formate facilitating interspecies electron transfer, cocultures were initiated with the *G. metallireducens* GS-*ΔpilB* and a *G. sulfurreducens* strain, PCA-*ΔpilBΔhybLΔfdnG*, which cannot assemble pili and metabolize H_2 and formate owing to the triple deletion of the *pilB* gene, the large subunit of the uptake hydrogenase (gene *hybL*) [30] and the catalytic subunit of formate dehydrogenase (gene *fdnG*) [20]. As expected, the cocultures also grew with the metabolism of ethanol and the reduction of fumarate to succinate (Fig. 3e). Here acetate also accumulated.

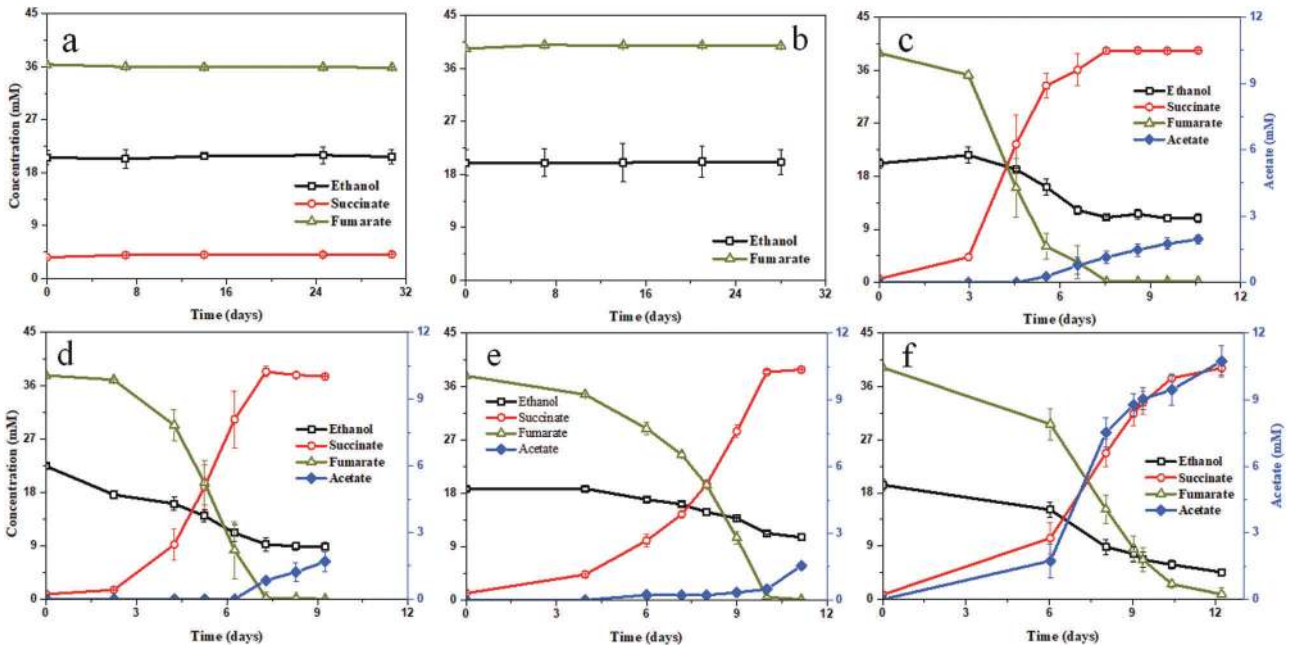

Fig. 3 Ethanol metabolism, acetate accumulation and succinate production from fumarate reduction of cocultures. **a** PilB-deficient strain PCA-Δ*pilB* of *G. sulfurreducens* could not metabolize ethanol. **b** PilB-deficient strain GS-*ΔpilB* of *G. metallireducens* could not reduce fumarate. **c** Cocultures of *G. metallireducnes* strain GS-*ΔpilB* and *G. sulfurreducens* strain PCA-*ΔpilB*. **d** Cocultures of wild-type strains of *G. metallireducens* and *G. sulfurreducens*. **e** Cocultures of *G. metallireducens* strain GS-*ΔpilB* and formate dehydrogenase, uptake hydrogenase, PilB triple-deficient strain PCA-*ΔpilBΔfdnGΔhybL* of *G. sulfurreducens*. **f** Cocultures of *G. metallireducnes* strain GS-*ΔpilB* and PilB, citrate synthase double deficient strain (PCA-*ΔpilBΔgltA*) of *G. sulfurreducens*. The pili-deficient strains of both *Geobacter* species were able to form syntrophic growth with direct interspecies electron transfer. The results are the means ± s.d. for triplicate cocultures

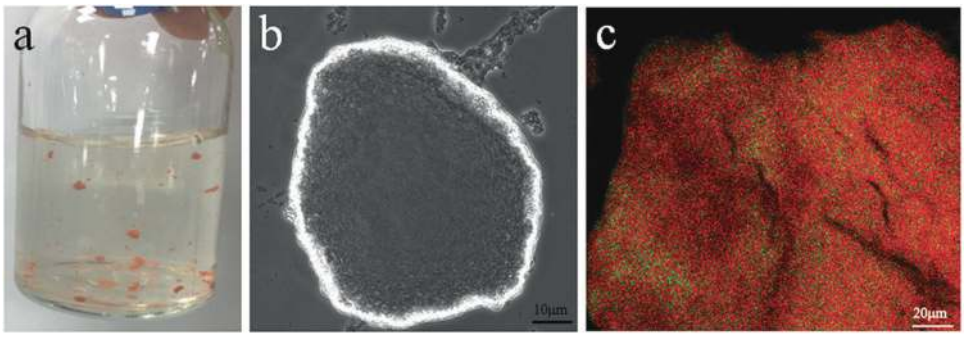

Fig. 4 Aggregates formed in the cocultures of *G. metallireducens* GS-Δ*pilB* and *G. sulfurreducens* PCA-*ΔpilB*. **a** Appearance of the aggregates. **b** Phase-contrast micrograph of aggregates. **c** Scanning laser confocal microscope image of aggregates after fluorescence in situ hybridization of strain-specific probes. The *G. metallireducens* strain GS-Δ*pilB* was labeled with a green probe, and the *G. sulfurreducens* strain PCA-*ΔpilB* was labeled with a red probe. Representative aggregates of 23 similar aggregates recorded

G. metallireducens catabolizes ethanol to acetate, and *G. sulfurreducens* oxidizes the acetate to reduce fumarate. To evaluate the possibility of metabolic coupling between these syntrophic species, cocultures were initiated with the *G. metallireducens* strain GS-*ΔpilB* and such a *G. sulfurreducens* strain that cannot assemble pili and catabolize acetate (deletion of the citrate synthase gene, *gltA*) [31], strain PCA-*ΔpilBΔgltA*. As previously reported for cocultures of *G. metallireducens* and citrate synthase-deficient *G. sulfurreducens* [32], the cocultures of *G. metallireducens* GS-*ΔpilB* with *G. sulfurreducens* PCA-*ΔpilBΔgltA* metabolized ethanol as fast as the cocultures of *G. metallireducens* GS-*ΔpilB* with *G. sulfurreducens* PCA-*ΔpilB* (Fig. 3f). Acetate accumulated continuously in the cocultures, with more ethanol oxidized and acetate generated from reduction of the same amount of fumarate than in the cocultures of *G. metallireducens* GS-*ΔpilB* with *G. sulfurreducens* PCA-*ΔpilB* (Fig. 3f). The much higher acetate accumulation in the cocultures of *G. metallireducens* GS-*ΔpilB* and *G. sulfurreducens* PCA-*ΔpilBΔgltA* than in the cocultures between *G. metallireducens* GS-*ΔpilB* and *G. sulfurreducens* PCA-*ΔpilB* implies that acetate produced by ethanol catabolism of *G. metallireducens* GS-*ΔpilB* can also be metabolized by *G. sulfurreducens* PCA-*ΔpilB* to provide electrons for the reduction of fumarate, which is the same case as in the wild-type cocultures of *G. metallireducens* with *G. sulfurreducens* [32].

The electrons derived from DIET cocultures can support the cell growth [32]. The stoichiometry of metabolism in *Geobacter* species cocultures was calculated based on the following formulas:

$$CH_3CH_2OH + 6CH_3CHCHCOOH + 3H_2O \rightarrow 2CO_2 + 6CH_3CH_2CH_2COOH \quad (1)$$

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + 4e^- + 5H^+ \quad (2)$$

$$CH_3COO^- + 2H_2O \rightarrow 2CO_2 + 8e^- + 7H^+ \quad (3)$$

$$CH_3CHCHCOOH + 2e^- + 2H^+ \rightarrow CH_3CH_2CH_2COOH \quad (4)$$

In theory, the electrons generated from the total oxidation of 1 mM ethanol and 1 mM acetate can reduce 6 mM fumarate producing 6 mM succinate (Reaction 1) and reduce 4 mM fumarate producing 4 mM succinate (Reaction 3, 4), respectively. When *G. metallireducens* GS-*ΔpilB* was cocultured with *G. sulfurreducens* PCA-*ΔpilB*, 9.34 mM ethanol was catabolized associated with the formation of 39.34 mM succinate and the accumulation of 1.95 mM acetate, accounting for electron recoveries of 81.6%. The ratio is consistent with previously reported *Geobacter* cocultures, in which partial metabolism of ethanol has been used for the biomass production [33].

The partial catabolism of 1 mM ethanol can generate 1 mM acetate and release 4 mM electrons (Reaction 2) reducing 2 mM fumarate to generate 2 mM succinate. However, in cocultures between *G. metallireducens* GS-*ΔpilB* and *G. sulfurreducens* PCA-*ΔpilBΔgltA*, the metabolism of 14.76 mM ethanol was associated with the generation of 39.14 mM succinate and the accumulation of 10.74 mM acetate. Considering acetate also can be the electron donor for *G. metallireducens* to grow in DIET cocultures [34] and *G. sulfurreducens* does not use H_2 and formate as electron donor in DIET cocultures (Fig. 3e) [32] and in addition, the transcription of both formate dehydrogenase and uptake hydrogenase of *G. sulfurreducens* in cocultures of *G. metallireducens* GS-*ΔpilB* and *G. sulfurreducens* PCA-*ΔpilBΔgltA* were comparable with those in cocultures of *G. metallireducens* GS15 and synthase-deficient strain of *G. sulfurreducens* (data not shown), the extra electrons used for the reduction of fumarate must come from the oxidation of acetate by *G. metallireducens* GS-*ΔpilB*. So the electron recoveries from the metabolism of ethanol were 85.83%, which is also consistent with previous reports of DIET cocultures [32, 35].

Gmet_2896 cytochrome is necessary for DIET between pili-free *Geobacter* species

The Gmet_2896 tetraheme cytochrome is located on the outer surface of *G. metallireducens* and is essential for insoluble Fe (III) oxide reduction but not for the reduction of soluble Fe (III) citrate [25], indicating a key role in extracellular electron transfer of *G. metallireducens*. A previous study showed that the Gmet_2896 mutant of *G. metallireducens* cannot form cocultures with *G. sulfurreducens* [8], which indicates that Gmet_2896 cytochrome also plays a critical role in the DIET. The same mutation was constructed in *G. metallireducens* strain GS-*ΔpilB*, generating strain GS-*ΔpilBΔGmet_2896*. Attempts to establish cocultures between *G. metallireducens* GS-*ΔpilBΔGmet_2896* and *G. sulfurreducens* PCA-*ΔpilB* were unsuccessful even after extended growth (Fig. 5a) when both species were grown in a medium containing ethanol as the electron donor and fumarate as the electron acceptor. Notably, the deletion of Gmet_2896 gene did not affected the distribution of extracellular cytochromes (Fig. 1c).

Conclusion

These results demonstrate that although conductive pili are necessary during extracellular electron transfer between *Geobacter* species to reduce extracellular insoluble electron acceptors, such as anodes [21, 36], they do not play a key role in the DIET between *G. metallireducens* and *G. sulfurreducens*. In constrast, the extracellular Gmet_2896 cytochrome of *G. metallireducens* is necessary for the DIET. The *pilA* determines the expression of PilA protein and affects the distribution of outer-surface cytochromes, while PilB just motors the assembly of type IV pili and deletion of *pilB* does not alter the distribution of extracellular cytochromes. The deletion of PilB should be a better choice to characterize the pilus related phenotype of *Geobacter* species.

Deletion of the *pilA* impaired the distribution of the extracellular cytochrome OmcZ [16] and OmcS (Fig. 1a, b), which plays a key role in DIET [1]. Considering that the extracellular OmcS is attached on pili [37] but not always associated [38], and that the strain *G. sulfurreducens* PCA-*ΔpilB* has pattern of extracellular cytochromes similar to that of *G. sulfurreducens* strain PCA, it can be speculated that the pilin of *G. sulfurreducens* can aid the excretion of OmcS. *G. sulfurreducens* pili are conductive [39, 40] and even *G. metallireducens* pili show much higher conductivity [41]. Conductive pili have been thought to be essential during DIET between different syntrophic species [1, 2]. However, in the syntrophic consortia of *G. metallireducens* with *G. sulfurreducens*, the pili of *G. sulfurreducens* is moderately expressed comparing with the cocultures with MIET [8]. Moreover, the nonconductive flagella [8] and nonconductive pili [6, 14] also play key roles in DIET. Integrating those reports with our study, it can be inferred that the growth of conductive pili should not be suggested to be a criterion for selecting cocultures with DIET and investigations to identify the nonconductive function of filament structure in cocultures with DIET are warranted.

Our study highlights the importance of extracellular cytochromes in DIET. Even the normal expression of conductive pili cannot compensate for the deficient expression of either OmcS or Gmet_2896 cytochrome in DIET [1, 8]. The cytochromes involved in DIET can be ubiquitous, as all published DIET cocultures contain abundant cytochromes [1, 8]. Notably, cytochrome-mediated DIET has been evidenced only between species with outer cell surfaces tightly connected [5, 7], and this does not yet exclude the function of conductive pili during this process. Previous studies indicat that in electroactive

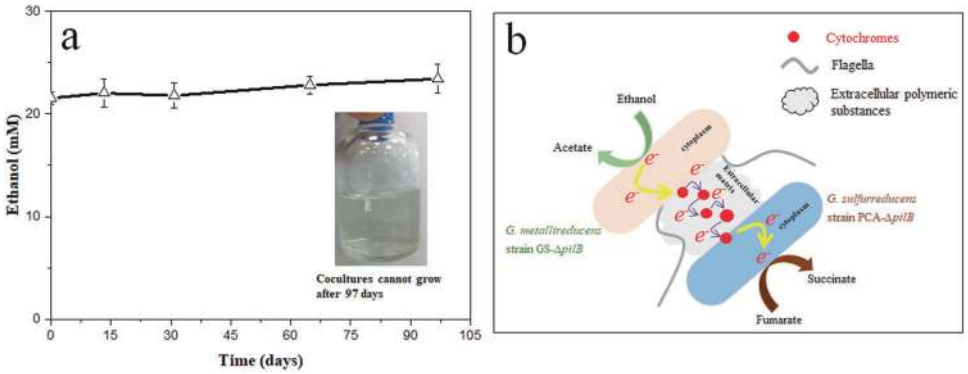

Fig. 5 Cocultures. **a** Ethanol metabolism of cocultures between the *G. metallireducens* double-mutant strain lacking the extracellular Gmet_2896 *c*-type cytochrome, and PilB (strain GS- *ΔpilBΔGmet_2896*) and strain PCA-*ΔpilB*. These two strains cannot form cocultures. The results are shown as the means ± standard deviations for triplicate cultures. **b** Model of long-range DIET mediated by cytochromes in cocultures between *G. metallireducens* GS-ΔpilB and *G. sulfurreducens* PCA-Δ*pilB*. The DIET was mediated via electron hopping between extracellular cytochromes

biofilms, the electrons can hop among cytochromes to transfer a long distance [42–44]. Considering that the two *Geobacter* species were clustered individually in long-range DIET cocultures (Fig. 4c) [1] and cells were not tight contact in *Geobacter* cocultures (Figure S2) [1], it can be speculated that cytochromes alone can mediate DIET not only between intimately contacted species but between spatially separated species (Fig. 5b).

Conductive pili are mainly restricted to the order of Desulfuromonadales [45], but cytochromes are widely distributed in microbial world. The discovery of conductive pili-free DIET solely mediated by the extracellular cytochromes suggests that DIET syntrophic consortia may be ubiquitous in natural environments. The abundance of cytochromes in the extracellular matrix can act as a criterion to identify syntrophic consortia with DIET. Cytochromes facilitate electrons to transfer along electron transport chains, to cross the cell membrane and to reduce extracellular electron acceptors (Fe (III) oxide, humic substances or in our case syntrophic partners et al.). Our findings reveal a general bio-electron transport way, involved by cytochromes, in the biological world that electrons hop between the redox active cofactors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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