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Review Article

REVIEW ARTICLE

Syntrophic propionate-oxidizing bacteria in methanogenic systems

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One sentence summary: This review summarizes discoveries in syntrophic propionate degradation research and reveals intriguing metabolic capabilities, mechanisms of cooperation and environmentally driven kinetics by taxonomically distinct microorganisms that are important for biotechnological applications and biogenic methane emissions.

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ABSTRACT

The mutual nutritional cooperation underpinning syntrophic propionate degradation provides a scant amount of energy for the microorganisms involved, so propionate degradation often acts as a bottleneck in methanogenic systems. Understanding the ecology, physiology and metabolic capacities of syntrophic propionate-oxidizing bacteria (SPOB) is of interest in both engineered and natural ecosystems, as it offers prospects to guide further development of technologies for biogas production and biomass-derived chemicals, and is important in forecasting contributions by biogenic methane emissions to climate change. SPOB are distributed across different phyla. They can exhibit broad metabolic capabilities in addition to syntrophy (e.g. fermentative, sulfidogenic and acetogenic metabolism) and demonstrate variations in interplay with cooperating partners, indicating nuances in their syntrophic lifestyle. In this review, we discuss distinctions in gene repertoire and organization for the methylmalonyl-CoA pathway, hydrogenases and formate dehydrogenases, and emerging facets of (formate/hydrogen/direct) electron transfer mechanisms. We also use information from cultivations, thermodynamic calculations and omic analyses as the basis for identifying environmental conditions governing propionate oxidation in various ecosystems. Overall, this review improves basic and applied understanding of SPOB and highlights knowledge gaps, hopefully encouraging future research and engineering on propionate metabolism in biotechnological processes.

Keywords: propionic acid; syntrophy; methanogenesis; methylmalonyl-CoA pathway; interspecies electron transfer; anaerobic digestion

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INTRODUCTION: PROPIONATE—A KEY INTERMEDIATE IN ANAEROBIC DEGRADATION

Propionate is an important intermediate in anaerobic degradation and a significant precursor for biomethane production in engineered production systems (Mah *et al.* 1990; Ahring, Sandberg and Angelidaki 1995). Research on anaerobic zones of ecosystems has also revealed potential importance of propionate conversion to methane, resulting in emissions of methane as a potent greenhouse gas (Glissmann *et al.* 2004; Lueders, Pommerenke and Friedrich 2004; Schmidt *et al.* 2015). In biogas-producing anaerobic degradation systems and anaerobic environments such as rice fields, sediments and oil reservoirs, propionate arises as a product of fermentation and acidogenesis (Fig. 1). The dominant propionate formation routes vary in different habitats. The main sources in anaerobic digesters, dark fermentation processes and sediments are degradation of odd-numbered fatty acids, carbohydrates, amino acids, aromatic compounds or lactate (Laanbroek *et al.* 1983; Gallert and Winter 2005; Sanchez *et al.* 2021). In oil reservoirs, propionate is formed in metabolism of oil hydrocarbons and carbohydrates (Yang *et al.* 2017), whereas in rice fields propionate is produced by bacteria in the rhizosphere that ferment saccharides and lactate excreted from plant roots, but interestingly also from carbon dioxide (CO_2) and acetate (Conrad and Klose 1999, 2000). In the digestive system of animals, propionate is produced by breakdown of dietary fiber and further fermentation of sugars, amino acids (derived from proteins) and lactate (Koh *et al.* 2016; Louis and Flint 2017).

The fate of propionate, thereafter is governed by the availability of electron acceptors in the anaerobic ecosystem. For example, in environments containing sulfur compounds, the availability of an electron acceptor (e.g. sulfate) will benefit sulfate-reducing bacteria using propionate and acetate as a carbon and energy source. As these oxidized sulfur species are energetically more favorable electron acceptors than CO_2, methanogens will be outcompeted. In habitats with restricted availability of electron acceptors other than CO_2, such as biogas reactors, rice fields, peatlands and oil reservoirs, propionate will instead be converted to methane (Kaspar and Wuhrmann 1978; Schmidt *et al.* 2016; Chen *et al.* 2020; Jin *et al.* 2021). In these methanogenic ecosystems, propionate degradation proceeds through a closely interlinked multispecies cooperation between syntrophic propionate-oxidizing bacteria (SPOB) and hydrogen (H_2)/formate- and acetate-utilizing methanogens (Stams 1994; Fig. 1). In bioreactors treating protein-rich waste, the ammonia (NH_3) concentration readily reaches levels that inhibit aceticlastic (acetate-utilizing) methanogens and, under these conditions, propionate is converted by ammonia-tolerant SPOB and acetate removal has instead been shown to be accomplished by syntrophic acetate-oxidizing bacteria (SAOB) in cooperation with hydrogenotrophic (H_2-utilizing) methanogens (Singh, Schnürer and Westerholm 2021; Fig. 1). In both high and low ammonia conditions, the interplay with cooperating H_2- and acetate/formate-utilizing partners is an important factor, as propionate degradation is energetically unfavorable in the presence of reaction products, mainly H_2, formate and acetate (Müller *et al.* 2010). In engineered biogas systems, propionate degradation is of particular importance since propionate build-up, often in combination with high acetate levels, is a common consequence of disturbance in the process and can cause a severe decrease in productivity (Hill, Cobb and Bolte 1987; Ma *et al.* 2009; Westerholm *et al.* 2015). This calls for strategies to overcome restraints involved in propionate degradation in such systems.

Syntrophic bacteria have fascinated microbiologists for decades, as these organisms habitually thrive at the limits of what is considered energetically possible. Their adaptation to inter-species cooperation and diverse capability to switch between syntrophic and non-syntrophic lifestyles are other intriguing aspects highlighted in relevant research (Morris *et al.* 2013). Concerted efforts over the years have advanced understanding of SPOB and their taxonomic distribution and metabolic characteristics. With regard to metabolic functioning, four routes for syntrophic propionate oxidation have been proposed, *viz.* the methylmalonyl-CoA (mmc), lactate, hydroxypropionyl and dismutating pathways (Patón, Hernández and Rodríguez 2020). At present, the mmc pathway is the most investigated (Kato, Kosaka and Watanabe 2009; Sedano-Núñez *et al.* 2018) and, even in that case, many aspects remain to be elucidated with regard to gene identity and organization and enzymatic activities. Recently, iterative community-level functional investigations and reclassifications have been undertaken to capture the range of microbial taxa involved in conversion of propionate to methane, and many hypotheses on their functionality have been generated. However, further research is needed into both basic and applied questions, in order to underpin the use of anaerobic degradation systems that represent biotechnological solutions to generate renewable energy (biogas, H_2), manage waste and recover nutrients by using the anaerobic digestion residue as biofertilizer. Further progress in syntrophic propionate oxidation research would also benefit modeling work on anaerobic microbiomes in anoxic environments that cause greenhouse gas emissions, contributing to global warming.

In this review, we aim to provide a comprehensive and structured description of current knowledge on syntrophic bacteria involved in propionate oxidation in methanogenic environments. We compile and discuss recent and earlier advances that laid the foundation for understanding SPOB taxonomy, habitats, metabolism, kinetics and interspecies networking, and highlight current knowledge gaps. Our objective is to inspire future research and stimulate cross-disciplinary discussion that can further define the intriguing syntrophic associations involved in propionate degradation.

PHYLOGENY OF SPOB

Isolated or co-cultivated bacteria that exhibit syntrophic propionate oxidation traits in methanogenic cultures are broadly distributed throughout two phyla, Firmicutes and Deltaproteobacteria. They include species within the genera *Desulfofundulus* and *Pelotomaculum* (phylum Firmicutes), *Smithella*, *Syntrophobacter* and *Syntrophobacterium* (Deltaproteobacteria; Table 1). Biochemical, genomic and transcriptomic studies have demonstrated that all SPOB characterized to date use the mmc pathway with the exception of species in the genus *Smithella*, which convert propionate through the so-called dismutating pathway (Harmsen *et al.* 1998; de Bok *et al.* 2001; Imachi *et al.* 2002; Hidalgo-Ahumada *et al.* 2018). Brief descriptions of each of these genera are provided below. Their habitats, associations with operating conditions in anaerobic degradation systems and their genomic and metabolic features are discussed in more detail in subsequent sections.

Desulfofundulus

Desulfofundulus species (family Peptococcaceae) previously belonged to the genus *Desulfotomaculum*. However, a phylogenetic analysis in 2018 demonstrated segregation within the

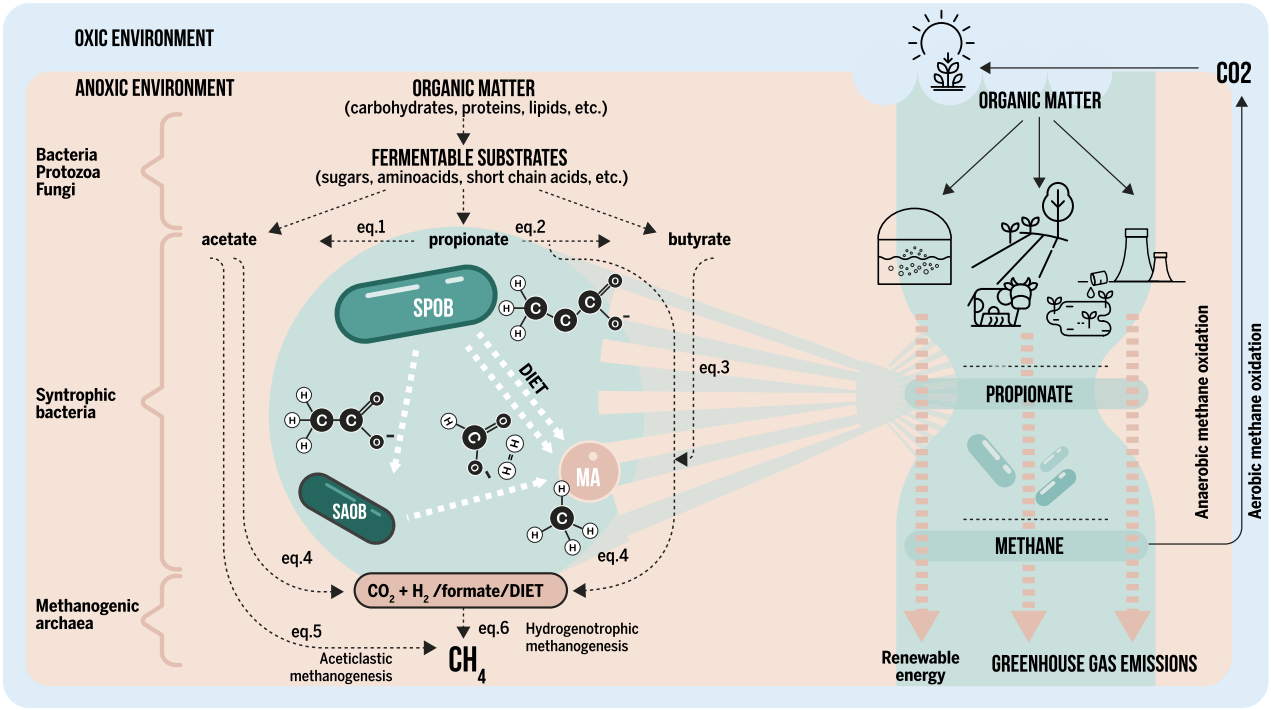

Figure 1. Overview of the main anaerobic process of organic biomass degradation (natural and engineered) with the focus on syntrophic propionate oxidation. Equations 1–6 correspond to the equations listed in the main text. SPOB—syntrophic propionate-oxidizing bacterium, SAOB—syntrophic acetate-oxidizing bacterium, MA—methanogenic archaeon and DIET—direct interspecies electron transfer.

genus and led to reclassification of the two thermophilic SPOB into the genus *Desulfofundulus* (Watanabe, Kojima and Fukui 2018). *Desulfofundulus thermocisternus* is the only SPOB isolated from a natural system (90°C oil reservoir water), while all other SPOB isolates originate from bioreactors (Table 1). As the genus epithet implies, its members characteristically use sulfate as an electron acceptor, coupled to oxidation of propionate. *D. thermocisternus* can also use sulfite and thiosulfate as electron acceptors. In pure culture, *Desulfofundulus* species grow within a thermophilic temperature range on substrates commonly used by syntrophic bacteria in pure culture, such as pyruvate and lactate (Table 1). Less common substrates for syntrophs, including benzoate and medium-chain fatty acids, are also used by the *Desulfofundulus* species. Another feature that differentiates *Desulfofundulus* from other characterized SPOB is their capability for autotrophic growth on H_2/CO_2, with production of acetate. Accordingly, searches using dedicated databases, i.e. AcetoBase and AcetoPath (Singh *et al.* 2019; Singh 2021), have revealed presence of a complete set of genes encoding the enzymes involved in the Wood–Ljungdahl pathway, including the key enzyme formyltetrahydrofolate synthetase, in the genome of the two *Desulfofundulus* SPOB. This suggests their assignment as acetogens which, in addition to their diversification between fermentative-type, sulfate reduction and capacity for syntrophic growth on propionate, indicate high metabolic versatility, more so than in other known SPOB.

Pelotomaculum

In the same family (Peptococcaceae) as the abovementioned SPOB is the genus *Pelotomaculum* (Rainey 2015), comprising two mesophilic and one thermophilic species (Table 1). The mesophilic species (*Pelotomaculum schinkii* and *Pelotomaculum propionicicum*) are the only SPOB that show obligate syntrophic characteristics, as they cannot be cultivated in pure culture (Boone and Bryant 1980; de Bok *et al.* 2005). However, pure cultivation of the thermophilic species *Pelotomaculum thermopropionicum* on pyruvate and fumarate has proven possible. A salient feature is that, in addition to propionate, this thermophilic SPOB can also degrade various alcohols and lactate in co-cultivation with a hydrogenotrophic methanogen (Imachi *et al.* 2002). A distinct characteristic compared with other SPOB is the inability of *Pelotomaculum* species to use sulfur compounds as electron acceptors (Table 1), which agrees with the finding that the genome of *P. thermopropionicum* lacks the necessary genes for dissimilatory sulfate reduction (Kosaka *et al.* 2008).

Syntrophobacter

The genus *Syntrophobacter* currently includes one mesophilic SPOB, *Syntrophobacter wolinii* (Galushko and Kuever 2019). *S. wolinii* was the first ever organism to be isolated as a syntroph in co-culture with a hydrogenotrophic methanogen (Boone and Bryant 1980). In pure culture, *Syntrophobacter* can grow fermentatively on pyruvate, fumarate and malate (Table 1). Propionate can be incompletely oxidized to acetate and CO_2 in the presence of sulfate, with sulfide formation.

Syntrophobacterium

Syntrophobacterium pfennigii, *Syntrophobacterium fumaroxidans* and *Syntrophobacterium sulfatireducens* previously belonged to the genus *Syntrophobacterium* (Wallrabenstein, Hauschild and Schink 1995; Harmsen *et al.* 1998; Chen, Liu and Dong 2005), but their low phylogenetic relatedness to members of that genus motivated the formation of a novel genus, *Syntrophobacterium* (Galushko and Kuever 2021). In pure culture, *Syntrophobacterium*

Table 1. Characterized bacteria grown in pure or co-culture with ability to degrade propionate degradation in syntrophic association with a methanogen.

Genus (Family)[1]	Species	Temperature optimum [°C] (range)	Salt tolerance	Isolation source[2]	Metabolic pathway[3]	pH optimum (range)	Substrate		Electron acceptor in pure culture	References
							Pure culture	Co-culture		
Desulfofundulus (Peptococcaceae)	*D. thermocisternus*	62 (41–75)	0.8 M NaCl	Oil reservoir	mmc	6.7 (6.2–8.9)	H_2/CO_2, lactate, pyruvate, ethanol, propanol, butanol and medium-chain fatty acids (e.g. pentanoate-decanoate)	Propionate	Sulfate, sulfite and thiosulfate[4]	Nilsen, Torsvik and Lien (1996); Watanabe, Kojima and Fukui (2018), Table S3 (Supporting Information)
	D. thermobenzoicus subsp. *thermosyntrophicus*	55 (45–62)	nd	UASB fed mixture of VFAs	mmc	7.2 (6–8)	Benzoate, fumarate, H_2/CO_2, pyruvate, lactate and glycine	Propionate	Sulfate[5]	Plugge, Balk and Stams (2002); Watanabe, Kojima and Fukui (2018); Bertran, Ward and Johnston (2020)
Pelotomaculum (Peptococcaceae)	*P. propionicicum*	37 (25–45)	0.09 M NaCl	UASB fed sucrose, propionate, acetate and yeast	mmc	6.5–7.2 (6.5–7.5)	Obligate syntroph	Propionate	-	Imachi *et al.* (2007); Hidalgo-Ahumada *et al.* (2018)
	P. schinkii	37 (nd-44)	nd	UASB fed sugar beet wastewater	mmc	nd	Obligate syntroph	Propionate	-	de Bok *et al.* (2005); Hidalgo-Ahumada *et al.* (2018)
	P. thermopropionicum	55 (45–65)	0.07 M NaCl	UASB fed sucrose, propionate, acetate and yeast	mmc	7.0 (6.7–7.5)	Pyruvate and fumarate	Propionate, ethanol, lactate, butanol, pentanol, propanediol, propanol and ethylene glycol	Fumarate[6]	Imachi *et al.* (2002); Kato, Kosaka and Watanabe (2009)
Syntrophobacter (Syntrophobacteraceae)	*S. wolinii*	35 (25–40)	0.09 M NaCl or KCl	AD of municipal sewage	na	7.0 (6.0–7.5)	Pyruvate, fumarate and malate	Propionate	Sulfate[7]	Boone and Bryant (1980); Wallrabenstein, Hauschild and Schink (1994); Liu *et al.* (1999); Galushko and Kuever (2019)
Syntrophobacterium (Syntrophobacteraceae)	*S. pfennigii*	37 (20–37)	nd	AD of municipal sewage	na	7.0–7.3 (6.2–8.0)	nd	Propionate, lactate and propanol	Sulfate, sulfite and thiosulfate [8]	Wallrabenstein, Hauschild and Schink (1995); Galushko and Kuever (2021)
	S. fumaroxidans	37 (20–40)	nd	Anaerobic granular sludge	mmc	7.0–7.6 (6.8–8.0)	Fumarate, malate, aspartate and pyruvate	Propionate	Sulfate and fumarate[9]	Harmsen *et al.* (1998); Plugge *et al.* (2012)

Table 1. Continued

Genus (Family)[1]	Species	Temperature optimum [°C] (range)	Salt tolerance	Isolation source[2]	Metabolic pathway[3]	pH optimum (range)	Substrate		Electron acceptor in pure culture	References
							Pure culture	Co-culture		
	S. sulfatireducens	37 (20–48)	0.1 M NaCl	UASB fed brewery/bean curd production wastewater	na	7.0–7.6 (6.2–8.8)	Pyruvate	Propionate	Sulfate, sulfite and thiosulfate[8]	Chen, Liu and Dong (2005)
Smithella (Syntrophaceae)	*S. propionica*	35 (20–40)	0.2 M NaCl	Up-flow anaerobic filter fed propionate	Dis-mutating	6.5–7.5 (6.5–7.5)	Crotonate	Propionate, butyrate, malate, crotonate and fumarate	nd	Liu *et al.* (1999); de Bok *et al.* (2001)

[1]Taxonomy using the National Center for Biotechnology Information (NCBI) database: phylum Firmicutes, class Clostridia, order Clostridiales and family Peptococcaceae. Corresponding taxonomy when using the Genome Taxonomy Database (GTDB (Parks *et al.* 2018): phylum Firmicutes, class Desulfotomaculia, order Desulfotomaculales and family Pelotomaculaceae. nd—not determined. Validation of the genus *Syntrophobacterium* (Oren and Garrity 2021).
[2]UASB -upflow anaerobic sludge bed, VFA—volatile fatty acids.
[3]Confirmed metabolic pathway used in syntrophic propionate oxidation, mmc—methylmalonyl-CoA pathway, na—sequenced genome not available
[4]H_2/CO_2, lactate, pyruvate, ethanol, propanol, butanol C_3-C_{10} and C_{14}-C_{17} carboxylic acids as electron donor/carbon source.
[5]Propionate, lactate, pyruvate and H_2/CO_2 as electron donor/carbon source.
[6]Propionate, ethanol and lactate as electron donor.
[7]Lactate as electron donor.
[8]Propionate as electron donor.
[9]Propionate, formate, succinate and H_2 as electron donor.

species grow on some organics such as pyruvate (substrate utilization has not been determined for *S. pfennigii*), but they are also able to oxidize propionate to acetate and CO_2 if sulfate, sulfite, thiosulfate or fumarate serves as terminal electron acceptor (Table 1). For sulfate reduction, the genome of *S. fumaroxidans* encodes a full suite of genes required for dissimilatory sulfate reduction (Plugge *et al.* 2012).

Smithella

Smithella propionica is the only currently known SPOB within the *Smithella* genus. This species is also the only SPOB known to use a pathway that starts with dismutation of propionate to acetate and butyrate. Butyrate is then ß-oxidized to acetate and H_2 (Liu *et al.* 1999). Substrates used for growth in pure culture have not yet been fully identified, but it has been reported that only crotonate supports fermentative growth, out of 12 substrates tested (Liu *et al.* 1999).

Candidate SPOB identified by omics studies

Moving beyond descriptive growth studies, several recent investigations have provided genomic insights into metabolic potential of candidate SPOB that taxonomically diverge from previously known species (Table 2). These SPOB candidates have been identified either by recording transcriptomic responses by feeding propionate or using a selective propionate enrichment technique, where the continuous wash-out of non-fed microorganisms creates a minimal consortium out of a complex inoculum.

Ca. Propionivorax syntrophicum In a recent study, Hao *et al.* (2020) activated the propionate-degrading community in sludge from an 38°C anaerobic digester treating activated and primary sludge from a wastewater treatment plant by feeding propionate after a starvation period. Increased transcriptomic response of the mmc pathway by a bacterium given the provisional name *Ca.* Propionivorax syntrophicum was observed after feeding with propionate. One complete set of genes for sulfate reduction was present in the genome of this putative SPOB (Hao *et al.* 2020).

Ca. Syntrophopropionicum ammoniitolerans Ammonia-tolerant SPOB have been enriched and detected in a propionate-fed reactor operated under mesophilic (37°C) high ammonia conditions (Singh, Schnürer and Westerholm 2021). Through molecular analyses and comparison with acetate-fed reactors, the ammonia-tolerant putative SPOB *Ca.* Syntrophopropionicum ammoniitolerans belonging to the family Peptococcaceae has been identified. Genomic analyses of this candidatus indicate presence of most of the genes required for the mmc pathway but, as shown for members of *Pelotomaculum* whose genomes have been sequenced, genes indicating potential for sulfur metabolism have not been found. A distinct feature of this species is its tolerance to ammonium (> 5 g NH_4^+-N/L) and ammonia (> 0.9 g NH_3/L).

Ca. Cloacimonetes In a thermophilic (55°C) propionate-degrading enrichment culture, a representative of the candidate phylum Cloacimonetes has been found to make up the majority of the bacterial community (Dyksma and Gallert 2019). Genomic analysis of the species (given the provisional name *Ca.* Syntrophosphaera thermopropionivorans) suggested that propionate oxidation is driven *via* the mmc pathway, although a complete set of genes was not found (Dyksma and Gallert 2019). Genome properties and/or transcriptomics indicating syntrophic propionate-degrading capabilities of other members of *Ca.* Cloacimonetes have been reported, including *Ca.* Cloacamonas acidaminovorans from mesophilic (33°C) wastewater sludge digesters (Pelletier *et al.* 2008) and *Ca.* Cloacimonetes from biofilm in hypermesophilic (46–50°C) bioreactors (Nobu *et al.* 2015).

Other syntrophic propionate-oxidizing candidates identified in methanogenic ecosystems

The vast majority of currently isolated and genomically characterized SPOB originate from bioreactors (Table 1). In addition to the characterized and candidate SPOB from these systems, other putative SPOB have also been identified by employing integrated omics, labeling analyses, or enrichment followed by 16S rRNA gene profiling. In mesophilic systems, members belonging to the genera known to include SPOB, i.e. *Smithella*, *Syntrophobacterium*, *Syntrophobacter* and *Syntrophomonas*, are those most frequently suggested to be associated with propionate degradation. However, members of *Cryptoanaerobacter*, *Ca.* Cloacamonas, *Variovorax* and the family Syntrophobacteraceae have also been proposed as SPOB (Table S1, Supporting Information). Members of the phylum Atribacteria identified through genomic and transcriptomic analyses of biofilm from hypermesophilic (46–50°C) bioreactors have also been suggested to be able to perform syntrophic propionate oxidation *via* the mmc pathway (Nobu *et al.* 2015, 2016).

The ecophysiology of SPOB in natural environments is less well understood than that in biogas-producing systems. However, available data on propionate-degrading communities in environments such as rice fields and oil reservoirs point to similarities in affiliations of putative SPOB, with a majority belonging to *Syntrophobacter*, *Pelotomaculum* and *Smithella*. Additionally, members of the families Acidaminococcaceae Desulfobacteraceae, Heliobacteriaceae, Christensenellaceae, Symbiobacteriaceae, Ruminococcaceae and Thermoanaerobacteraceae, and of the phylum Fibrobacteres, have also been postulated as SPOB candidates in marine and lake sediments, peat soil, rice fields and oil reservoirs (Table S2, Supporting Information).

The gastrointestinal tract of animals, including humans and the rumen are yet other methanogenic ecosystems where propionate is formed (van Lingen *et al.* 2016). The gut microbiome has typically co-evolved in such a way that it benefits the host by producing compounds which have positive health effects. In humans, propionate is one of these compounds. It plays a role in preventing obesity and diabetes by regulating intestinal hormones, and it has been shown that short-chain fatty acids such as propionate also affect tissues and organs beyond the gut, through circulation in the blood (van der Hee and Wells 2021). However, not all propionate is taken up by the host. In the rumen, for example, propionate concentrations typically reach 20 mM and methane is produced, suggesting that SPOB can be active (van Lingen *et al.* 2016; Lu *et al.* 2020). It has also been shown that propionate-producing bacteria use part of the mmc pathway in the opposite direction (i.e. the succinate pathway) to convert pyruvate to propionate in the human gut (Reichardt *et al.* 2014), which indicates presence of bacteria with genomic potential for syntrophic growth on propionate when existing in proximity to methanogens. However, a systematic inventory of the presence and activity of SPOB in humans and animals, and across different environmental habitats, is lacking. Many questions regarding the ecological processes shaping the propionate-degrading community also remain unanswered.

Table 2. Candidate SPOB identified with omics studies.

Candidatus epithet	Detected	Taxonomic position[1]	Indicated metabolic pathway[2]	Indicated interspecies cooperation	Additional characteristic	Reference
Ca. Cloacamonas acidaminovorans	Full-scale AD wastewater treatment plant (33°C)	Uncultured phylum Cloacimonetes	mmc			Pelletier *et al.* (2008)
Ca. Propionivorax syntrophicum F70	Full-scale AD degrading municipal sewage sludge (38°C)	Family Peptococcaceae	mmc	Acetotrophic and hydrogenotrophic methanogens	Sulfate reduction (as indicated by genomic features)	Hao *et al.* (2020)
Ca. Syntrophopropionicum ammoniitolerans	Propionate enrichment of sludge from mesophilic (37°C) high-ammonia biogas digester	Family Peptococcaceae	mmc	Hydrogeno-trophic *Methanoculleus* sp. and SAOB	Tolerating ammonia levels of 0.8 g NH_3/L	Singh, Schnürer and Westerholm (2021)
Ca. Syntrophosphaera thermopropionivorans	Propionate enrichment of sludge from thermophilic (55°C) plug flow (full-scale) digester fed biowaste	Uncultured Cloacimonadaceae W5	mmc (not all genes were identified)	-		Dyksma and Gallert (2019)

[1]Taxonomy using the National Center for Biotechnology Information (NCBI) database: phylum Firmicutes, class Clostridia, order Clostridiales and family Peptococcaceae. Corresponding taxonomy when using the Genome Taxonomy Database (GTDB (Parks *et al.* 2018): phylum Firmicutes, class Desulfotomaculia, order Desulfotomaculales and family Pelotomaculaceae. [2]Metabolic pathway used in syntrophic propionate oxidation, based on genomic gene content and/or gene expression, mmc—methylmalonyl-CoA pathway.

METABOLIC TRAITS OF SPOB

The detailed descriptions of various syntrophic propionate oxidizers in the above sections demonstrate that they are metabolically versatile, with diverse capabilities to switch between syntrophic and non-syntrophic lifestyles. In pure culture, most species grow fermentatively, degrading organic compounds, and most possess the capability for respiration in the presence of sulfuric compounds or fumarate (Table 1). As mentioned previously, *Desulfofundulus* SPOB in particular exhibit high metabolic versatility considering their ability for acetogenic growth on H_2/CO_2, which is a feature of interest for producing platforms for bioproducts from CO_2 (Katsyv and Muller 2020).

Of the four proposed pathways for syntrophic propionate oxidation, only the mmc pathway (Equations 1, 2 and 5) has been investigated as regards gene organization and potential enzymatic activities. However, only a small number of the enzymes suggested to be involved in the mmc pathway have been biochemically characterized for SPOB. The dismutation pathway has to date only been established within the genus *Smithella* (Equations 4 and 5). The lactate and hydroxypropionyl pathways are highly speculative, as they are based solely on genomic potential and thermodynamic feasibility and have not been experimentally verified (Patón, Hernández and Rodríguez 2020).

Oxidation of propionate to acetate and hydrogen in the mmc pathway is highly endergonic under standard conditions (Equation 1), but the thermodynamic constraints can be bypassed through product removal by hydrogen- and acetate-utilizing microorganisms (Equations 4–6). Product removal makes the first reactions 'energy-giving' for the microorganisms performing the oxidation steps, while the acetate-degrading and methane-forming microorganisms obtain substrate and electrons to support growth (Müller *et al.* 2010).

Propionate to acetate and hydrogen

$$CH_3CH_2COO^- + 2H_2O \rightarrow CH_3COO^- + CO_2 + 3H_2 \quad (\Delta G^{\circ\prime} = +73.7\,kJ/mol). \quad (1)$$

Propionate to acetate and butyrate

$$2CH_3CH_2COO^- \rightarrow CH_3COO^- + CH_3CH_2CH_2COO^- \quad (\Delta G^{\circ\prime} = +2.8\,kJ/mol). \quad (2)$$

Butyrate to acetate and hydrogen

$$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H^+ \quad (\Delta G^{\circ\prime} = +49.8\,kJ/mol). \quad (3)$$

Acetate oxidation to carbon dioxide and hydrogen

$$CH_3COO^- + H^+ + 2H_2O \rightarrow 2CO_2 + 4H_2 \quad (\Delta G^{\circ\prime} = +94.9\,kJ/mol). \quad (4)$$

Aceticlastic methane formation

$$CH_3COO^- + H^+ \rightarrow CO_2 + CH_4 \quad (\Delta G^{\circ\prime} = -35.9\,kJ/mol). \quad (5)$$

Hydrogenotrophic methane formation

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (\Delta G^{\circ\prime} = -130.8\,kJ/mol). \quad (6)$$

The $\Delta G^{\circ\prime}$ and ΔH values (see Table S3, Supporting Information, for details) of the reactions are based on Hanselmann (1991) for inorganics and Shock and Helgeson (1990) for organics. In the dismutation pathway of *Smithella*, propionate is converted to acetate and butyrate (i.e. oxidized and reduced compounds are formed simultaneously), after which butyrate is syntrophically oxidized to acetate (Equations 2 and 3). In this route, only minor amounts of butyrate are formed during cultivation with methanogen(s) (Liu *et al.* 1999; de Bok *et al.* 2001).

Propionate transport across the cell membrane

Irrespective of the pathway used for propionate degradation, propionate needs to be transported across the cell membrane before its metabolism can begin. Until 2009, when Jolkver *et al.* (2009) described, for the first time, a transport system for acetate, pyruvate and propionate uptake by *Corynebacterium glutamicum*, simple diffusion was considered the main mechanism of propionate uptake (Kell *et al.* 1981). The new transporter identified by Jolkver *et al.* (2009), called MctC, was assigned to the family of monocarboxylic acid transporters (MCT), which are sodium solute symporters driven by electrochemical proton potential (also called solute:sodium symporters, SSS; Soares-Silva *et al.* 2020). MctC has been shown to transport acetate and pyruvate, and actively import propionate, only at very low external substrate concentrations. At higher substrate concentrations and neutral or acidic external pH, passive diffusion is suggested to be the prevailing transport means. The *mct*C gene constitutes an operon together with another gene encoding a small membrane protein of unknown function, both being encoded downstream to acetyl-CoA carboxylase in *C. glutamicum* (Jolkver *et al.* 2009). Delta-blast (domain-enhanced protein blast) analysis for this review revealed presence of the *mct*C gene sequence in the genome of all known SPOB except members of *Ca.* Cloacimonetes and *Ca.* Syntrophopropionicum ammoniitolerans (Table 3 and Fig. 2), indicating that this gene could encode an enzyme involved in propionate transport. The transporters identified share 15–24% amino acid identity with the *mct*C gene from *C. glutamicum* (Table 3). However, the capability of these enzymes to transport propionate in SPOB has not yet been experimentally validated.

Even though putative propionate transporter coding genes have not been identified in all sequenced SPOB genomes, propionate import through passive diffusion is rather unlikely under methanogenic conditions. The pK_a of propionic acid is 4.88, suggesting that in a methanogenic reactor, where pH oscillates around neutral values, its ionic form should prevail and ions cannot pass freely across the cell membrane. Moreover, uncontrolled influx of propionate would threaten cell pH homeostasis, leading to bacterial death. A cell equipped with a specific transporter would have an advantage over other cells in a methanogenic reactor, especially at lower external propionate concentrations. Inefficient or uncontrolled propionate transport across the cell membrane might thus be one of the factors influencing propionate conversion rates in a methanogenic reactor. Future studies relating to syntrophic propionate oxidation should focus on identifying and characterizing propionate transport systems and their regulation.

Organization of genes involved in the methylmalonyl-CoA pathway

As a majority of the identified SPOB use the methylmalonyl-CoA pathway for propionate oxidation, the organization of

Table 3. Identification of putative propionate transporters in sequenced genomes of known and candidate SPOB. Delta-blast homology search conducted using propionate transporter protein sequence (WP_011013917.1) of *C. glutamicum* as query.

	Putative propionate transporter protein				Adjacent small hypothetical protein
SPOB	NCBI ID	Size (aa)	Identity (%)	Query cover (%)	NCBI ID (size aa)
P. thermopropionicum					
	BAF61029.1	508	20.97	88	No
	BAF58673.1	466	16.31	84	BAF58674.1 (84 aa)
	BAF61032.1	466	16.81	85	No
P. propionicum					
	WP_134211979.1	466	16.71	78	No
P. schinkii					
	WP_190258698.1	497	20.08	93	WP_134220397.1 (92 aa)
	WP_190259573.1	470	15.8	87	WP_190259574.1 (67 aa)
					WP_190259598.1 (73 aa)
	WP_190258717.1	471	17.05	87	WP_190258716.1 (149 aa)
Ca. Syntrophopropionicum ammoniitolerans					
	– No homology found				
Ca. Propionivorax syntrophicum[1]					
	Accession number not available	306[2]	23.53	-	-
S. fumaroxidans					
	WP_011697168.1	1071	15.20	93	WP_011697169.1 (88 aa)
D. thermocisternus					
	WP_027355922.1	506	17.15	92	No
	WP_027357197.1	544	17.95	92	No
D. thermobenzoicus					
	WP_152946753.1	470	17.29	91	WP_152946751.1 (90 aa)
	WP_152947031.1	507	18.46	92	No
Ca. Cloacamonas acidaminovorans					
	– No homology found				
Ca. Syntrophosphaera thermopropionivorans					
	– No homology found				

[1]The metagenome assembled genome sequence for *Ca.* Propionivorax syntrophicum was kindly provided by Morten Simonsen Dueholm at the Department of Chemistry and Bioscience, Aalborg University, Denmark.
[2]The gene was truncated, no full-length sequence could be recovered.

genes involved in this pathway within SPOB genomes was compared in order to evaluate and discuss similarities and disparities between the different bacteria. In the genome of SPOB belonging to the Peptococcaceae, most of the genes coding for enzymes involved in the different steps are grouped together in one cluster (except genes for propionate-CoA transferase and succinate dehydrogenase). These clusters are conserved between the different species, and for Peptococcaceae have a highly similar gene organization to *P. thermopropionicum* (Kosaka *et al.* 2006; Fig. 3). Gene clustering is advantageous under the restricted energy yields obtained from propionate degradation, since coordinated expression of series of genes requires less transcriptional machinery (Kato, Kosaka and Watanabe 2009). Genomes of two sequenced *Desulfofundulus* SPOB contain an additional gene cluster encoding the methylmalonyl-CoA mutase and associated proteins (not shown in Fig. 3). However, it has not yet been determined whether this second enzyme is expressed and participates in conversion of (R) methylmalonyl-CoA to succinyl-CoA. In contrast to Peptococcaceae SPOB, a gene encoding a pyruvate:ferredoxin oxidoreductase (*mmcM*) is not present in *Desulfofundulus* SPOB (Fig. 3). Instead, members of *Desulfofundulus* (Bertran, Ward and Johnston 2020) have identical methylmalonyl-CoA gene cluster organization to the close relative *Desulfotomaculum kuznetsovii* (Visser *et al.* 2013). However, the latter does not grow in syntrophy with methanogens and its metabolism is coupled to reduction of sulfate. Thus, presence of the methylmalonyl-CoA cluster is not always an indication of syntrophic propionate oxidation metabolism, which should be considered when looking for candidate SPOB in metagenomic studies.

In contrast to SPOB of the phylum Firmicutes, methylmalonyl-CoA genes do not cluster together in one location in the genome of SPOB from the phyla Proteobacteria and *Ca.* Cloacimonetes (Fig. 3). In total, two copies of fumarases are present in the genome of *S. fumaroxidans*, but only one has been detected in cells grown in syntrophic association with methanogens (Sedano-Núñez *et al.* 2018). In the case of *Ca.* Syntrophosphaera thermopropionivorans, some crucial genes of the mmc pathway are missing, including succinate and malate dehydrogenases (Dyksma and Gallert 2019). By contrast, a complete set of genes of the mmc pathway has been reported for *Ca.* C. acidaminovorans (Pelletier *et al.* 2008), although in an additional homology BLAST-search performed within the scope of the present review the two above-mentioned genes could not be identified.

Propionate oxidation *via* the methylmalonyl-CoA pathway

The mmc pathway comprises 11 steps, forming intermediates such as methylmalonyl-CoA, malate and pyruvate (Fig. 2, Table S4, Supporting Information). Some energy-dependent

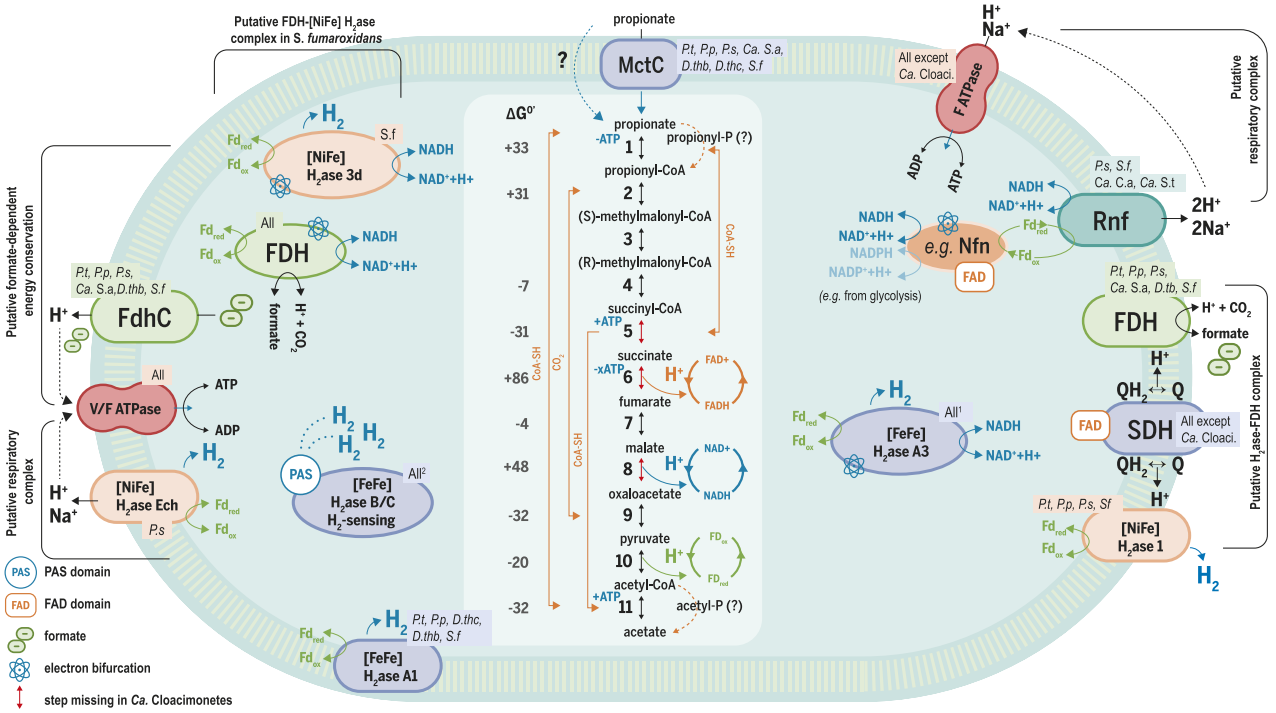

Figure 2. Generic illustration of the methylmalonyl-CoA pathway and main enzymatic complexes involved in the re-oxidation of reduced electron carriers, i.e. FADH, NADH and ferredoxin (Fd) and energy conservation in SPOB. Numbers 1–11 indicate the different steps of the pathways (see Table S4, Supporting Information, for details of the enzymes); (1) propionate-CoA transferase/acetate-CoA ligase; (2) methylmalonyl-CoA carboxyltransferase; (3) methylmalonyl-CoA epimerase; (4) methylmalonyl-CoA mutase; (5) succinyl-CoA synthetase; (6) succinate dehydrogenase; (7) fumarate hydratase; (8) malate dehydrogenase; (9) pyruvate carboxylase; (10) pyruvate-ferredoxin reductase and (11) acetate-CoA ligase Red arrows indicate steps putatively missing in *Ca.* Cloacimonetes. Dashed (orange and blue) arrows indicate putative reactions. Solid orange arrows indicate reactions that are putatively interconnected. [NiFe] and [FeFe] H_2ase refer to [NiFe] and [FeFe] hydrogenase, respectively. SDH—succinate dehydrogenase, FDH—formate dehydrogenase, FdhC—formate transporter, MctC—propionate transporter, Rnf—ferredoxin:NADH reductase, Nfn—ferredoxin-dependent transhydrogenase and PAS (Per-Arnt-Sim)—a signaling domain that acts as molecular sensor. $\Delta G^{0`}$ values were calculated for pH 7 (see Table S5, Supporting Information, for details of compounds involved in each reaction). Putative enzymatic complexes were identified based on the studies cited in the main text. To simplify the image, no separate subunits are visualized for the multimeric enzymes. *D.thc*—*D. thermocisternus*, *D.thb*—*D. thermobenzoicus* subsp. *thermosyntrophicus*, *P.p*—*P. propionicicum*, *P.s*—*P. schinkii*, *P.t*—*P. thermopropionicum*, *S.f*—*S. fumaroxidans*, *Ca.*C.a—*Ca.* Cloacamonas acidaminovorans, *Ca.*S.a—*Ca.* Syntrophopropionicum ammoniitolerans and Ca. S.t—*Ca.* Syntrophosphaera thermopropionivorans. [1]This enzyme is a putative non-bifurcating and NADH-dependent only [FeFe] hydrogenase in *Ca.* Cloacimonetes (Losey *et al.* 2020). [2]All syntrophic propionate-oxidising bacteria analysed possess in their genome genes encoding [FeFe] hydrogenases from the group B/C, but a PAS domian has been predicted only in the case of mesophilic bacteria belonging to the Peptococcaceae.

steps, e.g. propionate activation or propionyl-CoA conversion to methylmalonyl-CoA, are coupled to other energy-yielding reactions. For instance, the initial and energy-requiring propionate activation has been proposed to be conducted by (propionyl) CoA transferase (EC 2.8.3.1) and ADP-forming ligase (EC 6.2.1.13) that couple this step with the downstream and exergonic deactivation of acetyl-CoA (step 11; (Kosaka *et al.* 2006; Kato, Kosaka and Watanabe 2009). The corresponding genes are present in the genome of multiple SPOB (Table S4, Supporting Information), but these enzymes have not yet been characterized in any known SPOB. Alternatively, propionate activation has been proposed to be driven, as an autonomous reaction, by an AMP-generating acetyl-CoA synthetase (also called CoA ligase and identified by the code EC 6.2.1.1; Hao *et al.* 2020), and the corresponding enzyme has been purified and characterized in *P. thermopropionicum* (Tajima *et al.* 2016). Other steps that can be coupled in SPOB are steps 1 and 5 (Fig. 2), since (propionyl) CoA transferase (EC 2.8.3.1, present in several SPOB Table S4, Supporting Information) has been shown to act as a propionyl-CoA:succinate-CoA transferase in propionate-fermenting bacteria (Wang *et al.* 2015). It is also possible that the first step is a two-step reaction, comprising propionate phosphorylation catalyzed by a kinase (EC 2.7.2.1) followed by CoA addition catalyzed by an acetyl transferase (EC 2.3.1.8, Table S4, Supporting Information). Again, the corresponding genes are present in the genome of most SPOB, but a two-step reaction pathway of propionate activation in SPOB has not yet been discussed. The second step of the mmc pathway, i.e. endergonic carboxylation of propionyl-CoA to methylmalonyl-CoA, can be driven by the decarboxylation of oxaloacetate to pyruvate, further coupling steps 2 and 9 (Stams *et al.* 1993). Alternatively, a gene encoding a Na^+-transporting methylmalonyl-CoA decarboxylase is also present in the genome of SPOB, and in propionate-fermenting bacteria it couples the decarboxylation of S-methylmalonyl-CoA to the transport of sodium ions across the membrane, thus creating a sodium ion motive force used for ATP synthesis (Bott *et al.* 1997). Another suggestion for coupling of reaction steps has been made in the context of *P. thermopropionicum*, in which the activity of acetyl-CoA synthetase together with succinyl-CoA synthase could recycle CoA and generate ATP *via* substrate-level phosphorylation, with the prerequisite that a high AMP-to-ATP ratio is maintained in the cell (Liu and Lu 2018). However, this and most of the above-mentioned suggestions regarding coupling of reaction steps have to be experimentally confirmed. Insight in the coupling of energy-dependent and energy-yielding reactions in SPOB is highly relevant since it has potential practical

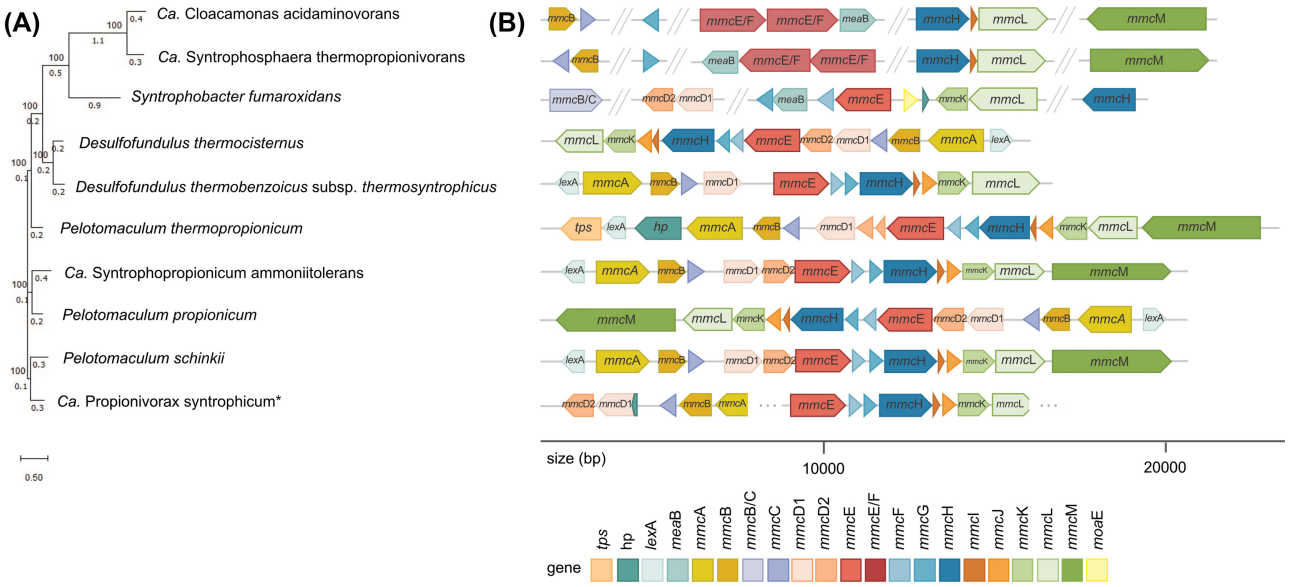

Figure 3. Phylogenetic characterization and gene organization of the methylmalonyl-CoA (mmc) cluster. **(A)**Maximum likelihood phylogeny of the SPOB genome, reconstructed by PhyloPhlAn 3.0 (Asnicar *et al.* 2020). Phylogenetic tree based on concatenated alignment of up to 400 ubiquitous conserved genes. **(B)** Corresponding gene organization of the methylmalonyl genomic cluster (most of the genes involved in propionate oxidation through the methylmalonyl-CoA pathway are clustered together in SPOB genomes). Gene content was determined directly from available genome sequences by homology search, using protein sequences of the mmc operon of *P. thermopropionicum* (Kosaka *et al.* 2006). Dotted line indicates contig edge. Genes are labeled according to the color code given below the image. Letter code refers to *tps*, transposase; *hp*, hypothetical protein; *lexA*, SOS-response transcriptional repressors; *mea*B, methylmalonyl Co-A mutase-associated GTPase; *mmc*A, transcriptional regulator; *mmc*B, fumarase, N-terminal domain; *mmc*C, fumarase, C-terminal domain; *mmc*B/C, fumarase single subunit; *mmc*D1, succinyl-CoA synthetase, beta subunit; *mmc*D2, succinyl-CoA synthetase, alpha subunit; *mmc*E, methylmalonyl-CoA mutase, N-terminal domain/subunit; *mmc*F, methylmalonyl-CoA mutase, C-terminal domain/subunit; *mmc*E/F, methylmalonyl-CoA mutase single subunit; *mmc*G, methylmalonyl-CoA epimerase; *mmc*H, methylmalonyl-CoA decarboxylase, alpha subunit; *mmc*I, methylmalonyl-CoA decarboxylase, epsilon subunit; *mmc*J, methylmalonyl-CoA decarboxylase, gamma subunit; *mmc*K, malate dehydrogenase; *mmc*L, transcarboxylase, 5S subunit; *mmc*M, pyruvate:ferredoxin oxidoreductase and *moa*E, molybdenum cofactor biosynthesis protein MoaE. *The metagenome assembled genome sequence for *Ca. Propionivorax syntrophicum* was kindly provided by Morten Simonsen Dueholm at the Department of Chemistry and Bioscience, Aalborg University, Denmark.

implications. An interesting theory postulated using a combination of genomics and proteomics is that the enzymes catalyzing the first two steps of the mmc pathway in *P. thermopropionicum* require downstream intermediate metabolites (e.g. pyruvate, acetyl-CoA; Kosaka *et al.* 2006). This is supported by the observation that addition of lactate shortens the lag phase before onset of syntrophic propionate degradation by *P. thermopropionicum*. Lactate is proposed to be taken up by this species and converted to pyruvate, with its successive conversion to acetate stimulating expression of enzymes and generation of downstream intermediate metabolites that in turn accelerate initiation of syntrophic propionate oxidation (Kato, Kosaka and Watanabe 2009).

Oxidation of succinate to fumarate by a membrane-bound succinate dehydrogenase (step 6 in Fig. 2) is the energetically most unfavorable step (Table S5, Supporting Information), requiring energy input from reverse electron transport (van Kuijk, Schlosser and Stams 1998; Kosaka *et al.* 2008; Plugge *et al.* 2012). A mechanism involving a quinone-dependent hydrogenase-formate dehydrogenase enzymatic complex has previously been proposed for *P. schinkii* (Hidalgo-Ahumada *et al.* 2018) and *S. fumaroxidans* (Sedano-Núñez *et al.* 2018). It is estimated that 0.66 ATP has to be invested to make this step energetically possible if the methanogen maintains hydrogen partial pressure and formate concentration below 1 Pa and 10 μM, respectively (Schink 1997). Succinyl-CoA synthetase and succinate dehydrogenase coding genes are missing from the *Ca.* Cloacimonetes genomes (Fig. 2). In the absence of cultivable representatives, future experimental validation using transcriptomic and proteomic approaches could be a way to bring clarity regarding the contribution of these candidate SPOB to syntrophic propionate oxidation. If this part of the propionate oxidation pathway can be resolved in *Ca.* Cloacimonetes through different steps (currently unknown), it could result in higher energy gain that could translate into higher growth yields and sometimes, very indirectly, into higher growth rates. Indeed, the abundance of *Ca.* Syntrophosphaera thermopropionivorans and other *Ca.* Cloacimonetes in anaerobic degradation systems largely exceeds the relative abundance of other known SPOB (Calusinska *et al.* 2018; Dyksma and Gallert 2019), suggesting that their growth rates might be higher. Another step not resolved in *Ca.* Cloacimonetes is the conversion of malate to oxaloacetate (step 8, Fig. 2). It is energetically difficult to reduce protons with NADH, which is essentially formed in this step (Stams and Plugge 2009), giving an energetic advantage to these putative SPOB if a different strategy has been developed. However, at this stage of current knowledge, existence of an alternative pathway in *Ca.* Cloacimonetes is still very speculative.

In general during propionate oxidation, pools of reduced electron carriers, i.e. FADH, NADH and ferredoxin, are formed at steps 6 (succinate dehydrogenation), 8 (oxidation of malate to oxaloacetate) and 10 (oxidation of pyruvate to acetyl-CoA; Fig. 2), respectively. Their re-oxidation is the key to syntrophy in methanogenic communities (Stams and Plugge 2009). SPOB rely on their hydrogenases and formate dehydrogenases, and use the reduced equivalents to reduce protons and CO_2 to hydrogen and formate, respectively. Further consumption of H_2 and formate by

methanogens makes the conversion exergonic, enabling energy conservation by SPOB (Stams and Plugge 2009).

Hydrogenases, formate dehydrogenases and energy conservation strategies

Multiple formate dehydrogenase and [NiFe] and [FeFe] hydrogenases have been reported in the different SPOB, but inconsistent naming conventions impede straightforward comparisons between SPOB species. Therefore, a comprehensive search for hydrogenases encoded in the sequenced genomes of SPOB representatives was performed in this review using automatic classification with a dedicated hydrogenase classifier (HydDB; Søndergaard, Pedersen and Greening 2016). Presence of conserved domains and other motifs typical for [FeFe] and [NiFe] hydrogenases (Calusinska *et al.* 2010) was then verified with the NCBI Conserved Domain Database (Marchler-Bauer *et al.* 2017). A summary consistent with the naming convention put forward by Meyer (2007) and Calusinska *et al.* (2010), further modified by Søndergaard *et al.* (2016), is provided in Fig. 4. A complete list of accession numbers of the amino acid sequences found in the different SPOB is given in Table S4 (Supporting Information). Considering the fact that the genome of most SPOB is still of draft quality, this analysis might not be exhaustive and other hydrogenases and formate dehydrogenases might still be present.

Hydrogenases and associated energy conservation strategies

The analysis demonstrated that only representatives of the genera *Pelotomaculum* and *Syntrophobacterium* possess [NiFe] hydrogenases, while all SPOB encode (multiple) [FeFe] hydrogenases (Figs 2 and 4; Table S4, Supporting Information). Dimeric [FeFe] hydrogenase of the group A1 is widespread across all analysed SPOB genera except *Desulfofundulus* and *Ca.* Cloacimonetes, and this enzyme was suggested to be localized in the periplasm in *S. fumaroxidans* (Sedano-Núñez *et al.* 2018). The A1 [FeFe] hydrogenases found in known SPOB (and in the non-SPOB *D. kuznetsovii*) differ from other characterized enzymes of this group by containing a small subunit comprising a thioredoxin-like domain typically found in group A3 of [FeFe] hydrogenases (Søndergaard, Pedersen and Greening 2016). All analysed SPOB possess at least one cytoplasmic, trimeric, putatively bifurcating [FeFe] hydrogenase classified to the group A3, confirming previous observations that electron bifurcation (the splitting of hydride electron pairs into one electron with a more positive and another with more negative reduction potential than that of the electron pair) is a common feature of syntrophic propionate degradation (Müller *et al.* 2010; Hidalgo-Ahumada *et al.* 2018). Logically, in SPOB a bifurcating hydrogenase will work in confurcating mode, stoichiometrically coupling endergonic formation of H_2 from NADH to its exergonic formation from reduced ferredoxin (Schut and Adams 2009; Buckel and Thauer 2018). Energetically, this strategy is more economical than ATP hydrolysis or reverse electron transfer as a driving force for endergonic reactions (Müller, Chowdhury and Basen 2018). This is particularly important for SPOB, which grow at the thermodynamic limit of life. Interestingly, a recent study indicated that the enzyme from *Ca.* C. acidaminovorans could actually be a non-bifurcating NADH-dependent [FeFe] hydrogenase (Losey *et al.* 2020). Based on the sequence homology of its catalytic subunit it is classified to group A3 of bifurcating enzymes, but the flavin-containing beta subunit shares a number of conserved residues with the beta subunit of non-bifurcating NADH-dependent enzymes. It has been suggested that this enzyme produces H_2 solely from NADH, without the need for a reduced ferredoxin (Losey *et al.* 2020).

Putative [FeFe] hydrogenases of the group B/C are present in all SPOB (Fig. 4). This type of hydrogenase is commonly found in Firmicutes (Calusinska *et al.* 2010; Søndergaard, Pedersen and Greening 2016), but its physiological function has not yet been discussed in SPOB. Hydrogenases of this group that contain a PAS domain (Per-Arnt-Sim signaling domains in proteins that act as molecular sensors; (Taylor and Zhulin 1999), are called 'sensing hydrogenases', and seem to be uniquely present in mesophilic SPOB belonging to the Peptococcaceae (Fig. 4). This type of hydrogenase performs regulatory functions by playing a role in transcriptional regulation of other hydrogenases, through detection of H_2 level in the cellular environment (Chongdar *et al.* 2018).

Periplasmic [NiFe] hydrogenases of group 1a with accessory cytochrome subunits are present only in *Pelotomaculum* spp. genomes. They are encoded in proximity to a periplasmic formate dehydrogenase, pointing to the existence of a specific hydrogenase–formate dehydrogenase complex (Hidalgo-Ahumada *et al.* 2018; Table S4, Supporting Information). Similarly, the [NiFe] hydrogenase of group 1b and periplasmic formate dehydrogenase have been proposed to be quinone-dependent in *S. fumaroxidans*, playing an important role in reverse electron transport associated with succinate oxidation (Sedano-Núñez *et al.* 2018). Only *P. schinkii* encodes multimeric [NiFe] hydrogenases of group 4, including Ech hydrogenase (Fig. 4). Ech hydrogenase is a Fd-dependent respiratory complex that utilizes the difference in potential between reduced Fd and protons for electrogenic and endergonic export of Na^+ or H^+. This results in an ion motive force across the cytoplasmic membrane that can be used for ATP formation (Müller, Chowdhury and Basen 2018). The second Fd-dependent respiratory enzyme found in SPOB is Rnf complex (ferredoxin:NADH reductase; Kuhns *et al.* 2020), which is present in *P. schinkii*, *S. fumaroxidans* and *Ca.* Cloacimonetes (Fig. 4). In contrast to Ech hydrogenase, Rnf complex utilizes NAD^+ instead of protons as the electron acceptor, which creates a greater difference in redox potential. It has been suggested that, while Ech hydrogenase can pump one proton per two electrons, Rnf can pump one proton per electron (Müller, Chowdhury and Basen 2018). This translates directly to a greater amount of conserved energy (*via* electron transport phosphorylation), which might explain why Rnf complex is more widely distributed in known SPOB than Ech hydrogenase. Furthermore, Rnf complex has been proposed to create a simple respiratory chain together with a flavin-based electron bifurcation enzyme (e.g. transhydrogenase Nfn; Fig. 2) and the Na^+-F_1F_O ATP synthase in the anaerobic bacterium *Thermotoga maritima* (Kuhns *et al.* 2020). A similar respiratory complex might be active in SPOB, as most species containing Rnf complex also possess F-type ATP synthase (Fig. 4). The only exception is *Ca.* Cloacimonetes, which possesses V-type ATP synthase, an enzyme mostly restricted to eukaryotes and archaea and present in only a few bacterial lineages (Mulkidjanian *et al.* 2007), including candidate butyrate-oxidizing bacteria (Hao *et al.* 2020).

Formate dehydrogenases and associated energy conservation strategies

Multiple (putative) cytoplasmic and/or extra-cytoplasmic (membrane-bound and periplasmic) formate dehydrogenases are encoded in the genome of all SPOB (Fig. 4). The two most

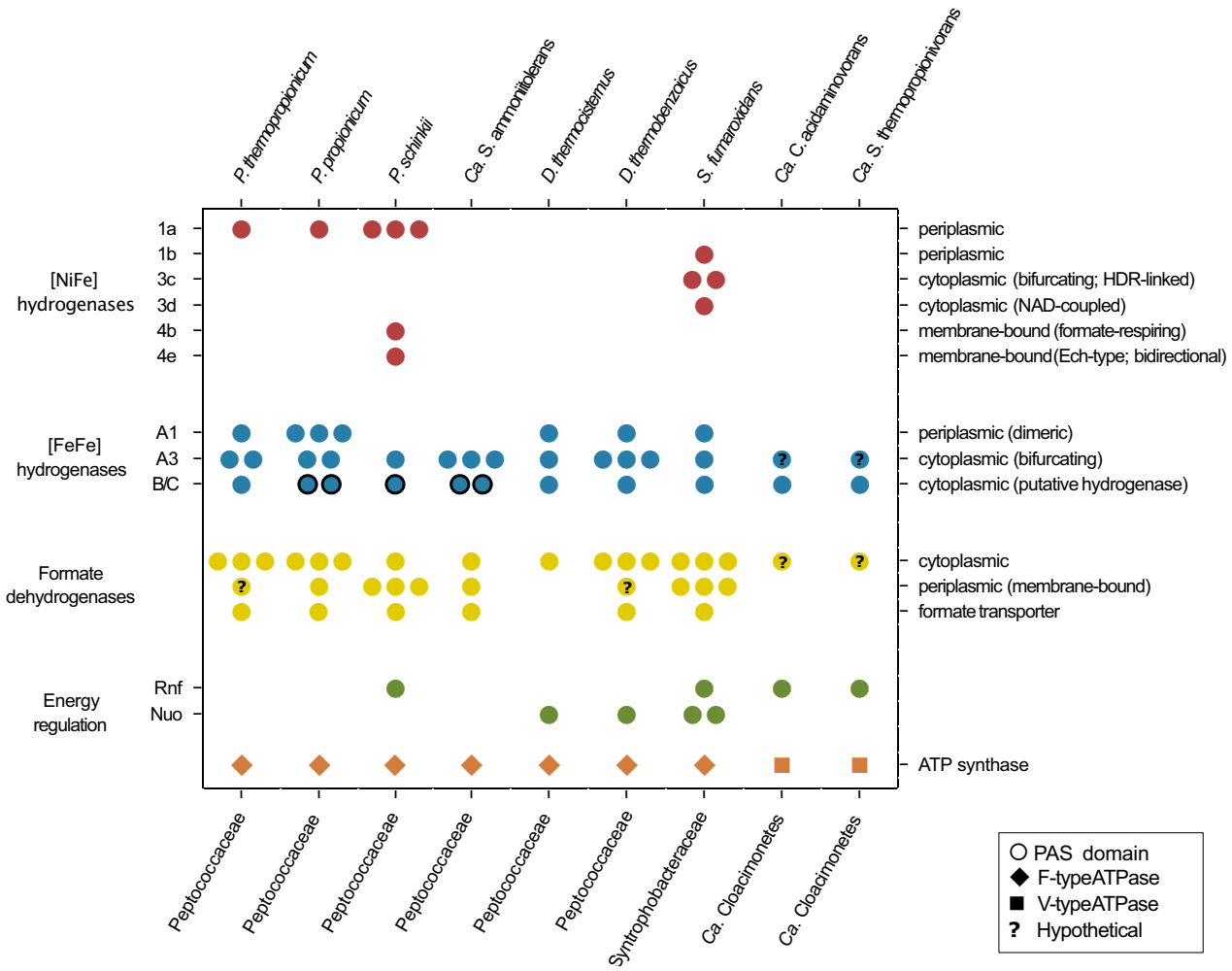

Figure 4. Presence of conserved domains and other motifs for hydrogenases or formate dehydrogenases in genomes of characterized and putative SPOB. The classification of hydrogenases to the different groups was done with the dedicated HydDB hydrogenase classifier (Søndergaard, Pedersen and Greening 2016), and further manually edited based on the presence of specific domains (Marchler-Bauer *et al.* 2017) and SPOB-related literature (cited throughout this paper). Information on the different formate hydrogenases, formate transporters and energy transduction mechanisms was retrieved from the SPOB-related literature (cited throughout this paper), further augmented with homology search for genomes when no literature information was available. The sequences of formate dehydrogenases identified in the study by Sedano-Núñez *et al.* (2018) were used as query for the blast search. Question mark indicates presence of a putative (partial) protein/protein complex that should be further verified. PAS—Per-Arnt-Sim signaling domain in proteins (that senses oxygen, redox potential and other stimuli). Only SPOB with sequenced genomes were used, and *Ca.* Propionivorax syntrophicum (Hao *et al.* 2020) was excluded from the analysis due to its highly fragmented genome status.

investigated SPOB species in this regard are *P. schinkii* (Hidalgo-Ahumada *et al.* 2018) and *S. fumaroxidans* (Sedano-Núñez *et al.* 2018). For the latter, two tungsten-containing formate dehydrogenases showing extremely high formate oxidation and CO_2 reduction rates have been isolated and biochemically characterized (de Bok *et al.* 2003). Previous comparative genomic analysis of several syntrophic and non-syntrophic short-chain fatty acid-degrading bacteria has indicated that extra-cytoplasmic formate dehydrogenases, present only in syntrophs, are essential for a syntrophic lifestyle (Worm *et al.* 2014). Availability of new SPOB genomes has allowed this early analysis to be extended to other genera, and genomic searches have accordingly demonstrated presence of genes encoding extra-cytoplasmic formate dehydrogenase in *Ca.* S. ammoniitolerans (Fig. 4; Table S6, Supporting Information). However, no extra-cytoplasmic formate dehydrogenase with sequence homology to any enzyme previously identified in SPOB was detected in the genome of *D. thermocisternus* and *Ca.* Cloacimonetes genomes in the present analysis (Fig. 4; Table S6, Supporting Information), contradicting the hypothesis that this enzyme is required for syntrophy. These SPOB also lack formate transporters (FdhC), while at least one putative transporter gene is encoded (often in proximity to a formate dehydrogenase) in the genome of all the other species (Table S6, Supporting Information). Formate is an ion that cannot freely cross the cell membrane and accumulates in the cytoplasm, forming a gradient from inside to outside. It has been hypothesized that FdhC may couple formate extrusion to the symport of protons, which would result in formation of ATP from the proton motive force generated (Fig. 2). This mechanism has recently been proposed as a new energy conservation strategy for syntrophic propionate degradation in *P. schinkii* (Hidalgo-Ahumada *et al.* 2018). A cytoplasmic formate dehydrogenase named Fdh1 and Hox hydrogenase (here classified as [NiFe] 3d) are suggested to be the main confurcating enzymes used for formate and hydrogen generation, respectively, in *S. fumaroxidans* (Sedano-Núñez *et al.* 2018). Moreover, both enzymes are encoded in proximity in the genome of *S. fumaroxidans*, suggesting the existence of a

putative enzymatic complex in this species (Fig. 4). The [NiFe] hydrogenase of group 3d is absent from the genome of other known SPOB.

Concluding remarks on genomic organization, hydrogenases and formate dehydrogenases

While there are some common patterns in the genomic organization of the mmc pathway and the content of hydrogenases and formate dehydrogenases, the number of observed differences suggests the existence of diverse strategies involved in the oxidative metabolism of known SPOB. Clearly, *Ca*. Cloacimonetes diverges from other SPOB, but the lack of cultivable representatives impedes further characterization of their involvement in the syntrophic propionate oxidation process. Moreover, to the best of our knowledge, only [NiFe] hydrogenase from *S. fumaroxidans* has yet been biochemically characterized to function in terminal reduction of protons (de Bok *et al*. 2002). The functions of hydrogenases are currently inferred through sequence homology to other characterized enzymes, although sometimes even structurally very similar enzymes can show distinct activities, as pointed out above for a presumably non-bifurcating [FeFe] A3 hydrogenase from *Ca*. Cloacimonetes (Losey *et al*. 2020). Consequently, it is likely that new strategies for propionate oxidation and energy conservation by SPOB will be uncovered in the future. Expanding the current understanding of how these steps are managed by the microbial community can help in formulating new strategies to overcome the problem of propionate accumulation in methanogenic reactors.

MECHANISMS OF COOPERATION WITH CO-METABOLIZING PARTNERS

Possible electron carrier compounds

As mentioned in the above sections, SPOB can generate both H_2 and formate for electron transfer to their cooperating partner, but the preferred electron carrier compound has been a topic of research for decades (Thiele and Zeikus 1988; de Bok, Plugge and Stams 2004; Stams *et al*. 2006; Schink *et al*. 2017). The redox potential of the CO_2/formate couple (−432 mV) is very close to that of the H^+/H_2 couple (−414 mV), rendering both electron acceptors energetically very similar (Schuchmann, Chowdhury and Mueller 2018). However, formate diffuses faster than H_2, which is one reason why formate transfer is kinetically more favorable than H_2 transfer (Boone, Johnson and Liu 1989). Formate concentrations also tend to be higher than hydrogen concentrations in propionate-degrading consortia (Fig. 5), which further increases the rate of diffusion. In addition to these kinetic advantages, there is also an energetic advantage, as gradients operating at higher concentrations imply lower energy losses for the organisms (Dolfing 1992). The findings that *S. fumaroxidans* cannot grow in co-culture with *Methanobrevibacter*, a methanogen that can only use H_2 and not formate, but that it can grow in co-culture with *Methanospirillum hungatei* or *Methanobacterium formicicum*, methanogens that use both H_2 and formate (Dong, Plugge and Stams 1994), are in line with these conceptual considerations. These findings also coincide with the reported presence of genes encoding a periplasmic formate dehydrogenase of importance for succinate oxidation in the mmc pathway in the genome of multiple SPOB (Fig. 4), as previously discussed for *S. fumaroxidans* (Sedano-Núñez *et al*. 2018) and *P. schinkii* (Hidalgo-Ahumada *et al*. 2018).

Although both the hydrogen and formate pools appear to be energetically feasible, at least in anaerobic digesters (Schink *et al*. 2017), recent transcriptomic and proteomic examinations have indicated temperature dependence for the preferred carrier compound. Under mesophilic (37°C) conditions, formate-based electron transfer is the most prevalent exchange mechanism during propionate degradation (Kato, Kosaka and Watanabe 2009; Hidalgo-Ahumada *et al*. 2018; Sedano-Núñez *et al*. 2018; Chen *et al*. 2020), although interspecies electron transfer proceeding with both H_2 and formate has also been observed (Hao *et al*. 2020). At higher temperatures (55°C), where the window of opportunity shifts towards permitting higher H_2 and formate concentration ranges compared with mesophilic temperature conditions (Fig. 5), dominance of both interspecies H_2 (Chen *et al*. 2020) and formate transfer (Liu and Lu 2018) has been demonstrated in *Pelotomaculum*-dominated communities. In low temperature-adapted Arctic peat soil, SPOB have showed a preference for formate-based electron transfer at temperatures below 7°C (Tveit *et al*. 2015). The study reported that at temperatures between 7 and 12°C propionate conversion to formate, H_2 and acetate was endergonic, whereas propionate conversion to acetate and H_2 was exergonic. This coincided with an observed change in the SPO community (dominated by Bacteroidetes) towards dominance of species relying on interspecies H_2 transfer at temperatures between 7 and 12°C (Tveit *et al*. 2015).

The dominance of H_2 versus formate will also be affected by bioavailability of the components required for enzymatic activity. This was exemplified in a study by Plugge *et al*. (2009), which demonstrated a more prominent role of H_2 as electron carrier when essential trace elements (tungstate/molybdate) for formate dehydrogenase were depleted. This is further discussed later in this review, in the section 'Trace element deficiency'.

Direct electron transfer

Although interspecies electron transfer *via* H_2 or formate is still considered an important mechanism, more recent observations suggest the potential occurrence and importance of direct interspecies electron transfer (DIET) in propionate-oxidizing syntrophic communities (Lovley 2017a; Martins *et al*. 2018). In DIET, electrically conductive pili (e-pili) and c-type cytochromes appear to play a central, if not essential, role (Liu *et al*. 2015; Lovley 2017b). Accordingly, it has been reported that addition of conductive materials, which are hypothesized to promote DIET, can increase the relative abundance of syntrophic propionate- and acetate-degrading species that possess genes for e-pili (Yin *et al*. 2020). Thus, the finding that genes for e-pili (type IV pili) are present in several genera of hydrogen/formate-producing syntrophs, including SPOB such as *Smithella* and *Ca*. Cloacimonetes, indicates the existence of DIET in SPOB communities (Dyksma and Gallert 2019; Walker *et al*. 2020; Yin *et al*. 2020). Another indication of preference for DIET by *Ca*. Cloacimonetes is their largely reduced genomic content of genes encoding hydrogenase and formate dehydrogenase, compared with other SPOB (Fig. 4).

On combining previous and more recent evidence, the hypothesis put forward by Jing and co-authors (2017) that DIET and interspecies hydrogen/formate transfer occur simultaneously in one and the same ecosystem appears logical. SPOB that transfer electrons in a mixed mode using hydrogen or/and formate as electron carriers and DIET in parallel would theoretically be more successful in getting rid of electrons from the oxidation process than bacteria which use only one channel to transfer

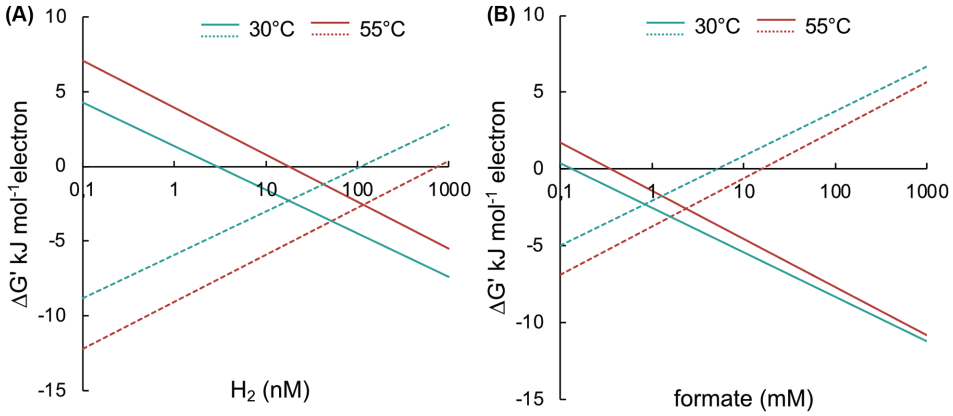

Figure 5. Window of opportunity for methanogenic oxidation of propionate through **(A)**interspecies hydrogen transfer and **(B)** interspecies formate transfer. Note the different units on the *x*-axis, the levels are the concentrations in solution. The diagram shows that (i) the molar concentrations of formate allowing interspecies electron transfer (IET)-based oxidation of propionate are higher than the molar concentrations of H_2 allowing IET-based propionate oxidation, and (ii) the window of opportunity shifts to a higher concentration range when the temperature increases. Calculations (Dolfing, Larter and Head 2008) based on G_f^0 and H_f° free energy values listed in Hanselmann (1991) and (Shock and Helgeson 1990) are for propionate oxidation *via* the methylmalonyl-CoA pathway, solute activities of propionate and acetate 10 mM, partial pressures of CH_4 and CO_2 at 1 atm, pH 7. Blue lines: temp = 30°C; red lines: temp = 55°C. Full lines: propionate oxidation; dotted lines: methanogenesis. The window of opportunity is the range of hydrogen or formate levels where propionate oxidation and hydrogenotrophic methanogenesis are both exergonic ($\Delta G' < 0$).

electrons. Gaining a more thorough understanding of these possible mechanisms underlying interspecies electron transfer is an intellectual challenge and has potential practical implications, for example it can help decrease the lag phase and increase the rate of propionate degradation in full-scale anaerobic digestion systems. It has been shown that enrichment with ethanol as co-substrate stimulates methanogenic communities to perform DIET, and that the resulting community degrades propionate at a higher rate and is no longer inhibited by high levels of H_2 (Zhao *et al.* 2016a). The actual reason for the boosted propionate degradation by ethanol addition warrants further investigation. It has been suggested that ethanol promotes dominance of *Geobacter* species that shift from ethanol to propionate degradation (Zhao *et al.* 2016b), but propionate degradation by *Geobacter* is rarely proposed in omics analyses or labeling experiments (Table S1, Supporting Information). Instead, it can be hypothesized that ethanol-degrading *Geobacter* act as drivers for establishment of e-pili networks that favor methanogens capable of DIET. The enrichment of DIET-capable methanogens could subsequently benefit SPOB that are able to use a similar electron transfer mechanism, thus speeding up propionate degradation compared with when a carrier compound is used by the SPOB. This raises interesting questions about potential drivers for DIET and bacterial and archaeal features required for use of this strategy. Furthermore, although DIET appears to facilitate interspecies electron transfer, the conditions for its establishment and occurrence might not be ideal in bioreactors and ecosystems, and a suite of conductive compounds have been evaluated (Martins *et al.* 2018). The observations made in microbial cultures so far argue in favor of accelerated propionate degradation and methane formation, potentially by improving DIET in the communities (Table S7, Supporting Information). This topic is considered further later in this review, in the section 'Addition of supportive material'.

Other mechanisms facilitating interspecies cooperation

Due to the small energy margin and the mutual requirement of the cooperating microorganisms to find each other, SPOB and their partners have most likely evolved specialized mechanisms to optimize the energetics and obtain spatial proximity that can facilitate interaction. Questions have been raised as to whether syntrophs follow special biochemical pathways to maintain their activity, supported by the finding that genes without functional assignment (e.g. hypothetical genes) are regulated differently during syntrophic growth compared with pure culture (Kato, Kosaka and Watanabe 2009; Sieber, McInerney and Gunsalus 2012). It has also been suggested that *Pelotomaculum* SPOB and the cooperating methanogen synchronize their biosynthesis of amino acids for the purpose of exchange of these between each other (Kato, Kosaka and Watanabe 2009; Hidalgo-Ahumada *et al.* 2018). This might explain why *P. thermopropionicum* requires yeast extract in pure culture, but not for co-culture growth (Kato, Kosaka and Watanabe 2009). Similarly, fructose and branched-chain amino acids synthesized by the methanogen may be used as an additional energy source by the SPOB (Hidalgo-Ahumada *et al.* 2018). Additional research on cultures growing in the absence of yeast extract could provide further insights on this issue. Past scientific discoveries also suggest that transfer of intermediates during syntrophic cooperation can be accelerated by reduced cell-to-cell distance and that cultivation with a self-aggregating methanogen improves the degradation of propionic acid by SPOB (Dolfing 1992; Ishii *et al.* 2005; Leng *et al.* 2018). Interaction *via* flagella could be helpful for the microbes, which would explain why *Pelotomaculum* species encode a complete flagellum biosynthesis machinery despite being immotile. It has even been speculated that the flagellum subunit which interacts with methanogens (the flagellar cap protein FliD) has a specificity or affinity to its methanogenic partner (Shimoyama *et al.* 2009; Hidalgo-Ahumada *et al.* 2018). This intriguing hypothesis, together with the other abovementioned issues, are research topics of considerable interest, as they promise clarity and further insights into the syntrophic lifestyle of SPOB.

ENVIRONMENTAL FACTORS GOVERNING SPOB

In every ecological system, microbial community structure and performance will be determined by the prevailing environmental conditions. In anaerobic digesters, the conditions for the microbiome are determined by the operating parameters and substrate characteristics. Given the myriad of interactions of chemical, biological and physical parameters, there are a variety

of causes of constrained growth of SPOB. Propionate build-up is often associated with stress imposed upon the microbial community, such as increased loading rate or presence of toxic compounds. Important drivers for propionate degradation and community assembly in anaerobic digesters include temperature, propionate concentration, feeding rate, pH and ammonia (Ariesyady *et al*. 2007; Worm *et al*. 2011; Li *et al*. 2018; Westerholm *et al*. 2018; Chen *et al*. 2020; Li *et al*. 2020; Singh, Schnürer and Westerholm 2021). Anaerobic digesters are generally operated in the pH range 7–8 and temperature ranges of 37–40°C or 50–55°C. Most of the known SPOB have been isolated from anaerobic digestion systems and, not surprisingly, reflect these optimum pH and temperature ranges (Table 1). However, studies of operational anaerobic degradation systems and investigations on natural ecosystems have revealed SPOB dynamics or propionate degradation capacities extending the ranges demonstrated in laboratory cultivations. All studies discussed in this section were performed before the validation of the genus *Syntrophobacterium* (Oren and Garrity 2021). Thus, *Syntrophobacter* may also include species currently belonging to *Syntrophobacterium*.

Temperature

Higher temperature increases the available energy of propionate oxidation (Guo *et al*. 2021b), with propionate-fed batch assays demonstrating nearly 3-fold higher maximum specific growth rate for microbial communities growing at 55°C than at 35°C (Li *et al*. 2020). However, the latter study also showed that the higher temperature considerably extended the lag prior to initiation of propionate degradation. For acetate, butyrate and valerate degradation, temperature had only a minor impact on the lag phase (Li *et al*. 2020).

Temperature is well-known to influence the overall microbial community structure in anaerobic systems, and species richness is often reported to decrease with increasing temperature (Moset *et al*. 2015; Westerholm *et al*. 2018). Likewise, studies of mesophilic anaerobic digesters often report of presence of several different genera responsible for syntrophic propionate oxidation, e.g. *Syntrophobacter*, *Smithella*, *Cryptanaerobacter* and *Pelotomaculum* (Table S1, Supporting Information). The identity of SPOB prevailing at thermophilic temperature is less well studied but, based on currently available knowledge, the diversity at species rank appears to be lower and primarily comprises *Pelotomaculum* species (Table S1, Supporting Information). It has also been shown that the prevailing SPOB abundance and the propionate degradation rate decrease on lowering the temperature below 35°C (Ban *et al*. 2013).

In natural ecosystems, temperature dependence in SPOB communities has been documented for various habitats, including peatland, rice fields and aquatic sediments (Table S2, Supporting Information). In one study on rice field soil, *Syntrophbacter* was the most active SPOB at 15°C, whereas at 30°C *Pelotomaculum* and *Smithella* were also involved in degradation of propionate (Gan *et al*. 2012). A biogeographical study spanning temperature zones demonstrated a correlation between higher temperature and higher propionate degradation rates and higher relative abundance of SPOB (Jin *et al*. 2021). In Arctic peat, syntrophic propionate oxidation has been identified as the rate-limiting step for methane production at temperatures below 7°C, whereas at higher temperatures the propionate pool is depleted at a higher rate (Tveit *et al*. 2015). Overall, the effects of temperature on propionate methanization in natural ecosystems and the correlation with additional factors driving SPOB activities, such as soil type, pH and periods of drought and flooding, are clearly understudied issues. They are also important issues, particularly when seeking to predict effects of changes in microbial activities and biogenic methane emissions in response to ongoing climate change.

Ammonia or sulfide toxicity

A build-up of acids (mainly propionate and acetate) commonly occurs in association with ammonia inhibition in anaerobic systems treating protein-rich materials (Westerholm, Moestedt and Schnurer). Ammonia nitrogen exists as the ammonium ion (NH_4^+) and ammonia (NH_3), where the latter is the most inhibitory form for anaerobic microorganisms (Sprott and Patel 1986). The NH_3/NH_4^+ ratio increases with temperature and pH, so the actual ammonia level that induces stress varies with operating conditions. Acetate and propionate accumulation is generally observed above 0.2 g NH_3-N/L (~2–3 g NH_4^+-N/L under mesophilic or thermophilic temperature conditions; Westerholm, Moestedt and Schnürer 2016; Bonk *et al*. 2018; Wang *et al*. 2019b). Regarding acetate, it is well known that the ammonia sensitivity of acetate-utilizing methanogens opens a window of opportunity for syntrophic acetate oxidation (Westerholm, Moestedt and Schnürer 2016). The reason for propionate accumulation is less well understood, but it may be triggered by direct ammonia inhibition of SPOB (Bonk *et al*. 2018) or it may be a consequence of product inhibition due to high acetate/formate/H_2 levels caused by restricted methanogenic activity. In addition to causing high ammonia concentrations, degradation of protein-rich material can give rise to high levels of sulfide, which precipitates metals. This restricts the bioavailability of essential trace elements (Westerholm, Moestedt and Schnürer 2016), so deficiency of trace elements required by SPOB could be a reason for higher propionate levels in high-ammonia systems. This is further addressed in the section 'Trace element deficiency'.

Under mesophilic temperature conditions, *Syntrophobacter* is inhibited above 1–2 g NH_4^+-N/L (Bonk *et al*. 2018; Zhang, Yuan and Lu 2018), whereas *Smithella* exhibits direct inhibition above 3 g NH_4^+-N/L (Zhang, Yuan and Lu 2018). This is consistent with absence of previously characterized SPOB in mesophilic systems exceeding 3 g NH_4^+-N/L (Westerholm *et al*. 2015; Bonk *et al*. 2018). Intrinsic disparities in SPOB taxonomy at high compared with low ammonia are further indicated by identification of a novel mesophilic SPOB candidate, *Ca*. Syntrophopropionicum ammoniitolerans, in a high-ammonia biogas system (Singh, Schnürer and Westerholm 2021). That SPOB candidate tolerated levels above 4 g NH_4^+-N/L (0.4–0.6 g NH_3-N/L), conditions under which it was suggested to cooperate with hydrogenotrophic *Methanoculleus* and SAOB. Absence of genes for ammonium transporters (causing redundant ammonium influx) and presence of genes for a transporter-complex that accumulates quaternary ammonium compounds inside the cell (protecting the cell under high osmotic stress) are suggested to be linked to tolerance to elevated ammonia by *Ca*. Syntrophopropionicum ammoniitolerans (Singh, Schnürer and Westerholm 2021).

Sulfide is another toxic compound for microorganisms that is known to be prevalent in sulfate-containing industrial wastewater. At high concentrations, free un-ionized hydrogen sulfide may cause microbial toxicity, as it can diffuse across the cell membrane and interfere with cell components. In such systems, addition of Fe is an effective management approach to keep the sulfide levels below the inhibition threshold, through FeS(s)

precipitation (Yekta *et al.* 2017). For *Smithella* and *Syntrophobacter*, inhibition by sulfide has been shown to occur above 0.1 g/L (Wang *et al.* 2019b). Further studies on both ammonia and sulfide inhibition in SPOB communities are strongly encouraged, to increase understanding of bottlenecks caused by deficiency in propionate degradation in processes operating with high levels of these compounds.

pH

Propionate oxidation can occur at a broad range of pH values (Table S8, Supporting Information). For instance, in acidic fens and upflow anaerobic sludge beds, methane formation from propionate has been reported at pH levels as low as 4.5 (Schmidt *et al.* 2016; Zhang *et al.* 2016), while in rice field soil presence of known SPOB and methane formation have been reported at pH 5.0 (Pan *et al.* 2021). However, at these low pH values syntrophic propionate oxidation and methane formation are generally quite low, due to restricted activity of many methanogens and acetogens. This can be advantageous in biotechnological applications where propionate is used as a building block for production of biobased chemicals. One such application is high-rate reactor systems, which at low pH (below 5.8) can achieve methanol-driven bacterial chain elongation resulting in the formation of valerate, a product that can be used as an additive in diesel fuel or for bioplastic production (de Smit *et al.* 2019).

The upper pH limit for propionate oxidation reported in the literature is pH 10, observed in enrichment from an hypersaline soda lake involving a member of the Syntrophobacteraceae (Sorokin *et al.* 2016). Pure cultures of some SPOB (*S. sulfatireducens*) demonstrate growth at pH 8.8 (Chen, Liu and Dong 2005; Table 1), while in high-ammonia anaerobic digesters propionate conversion by *Ca.* Syntrophopropionicum ammoniitolerans has been demonstrated at pH 8.1. In high-ammonia systems, it is also important to consider the pH effect on the NH_3/NH_4^+ ratio, as higher pH increases the proportion of NH_3, which is indicated to be most toxic for microorganisms (Singh, Schnürer and Westerholm 2021).

Trace element deficiency

Trace element availability can have a profound impact on the anaerobic degradation community, and sufficient availability of trace elements has been shown to lower the level of propionate accumulation in mesophilic (36–42°C) high-ammonia (Banks *et al.* 2012; Westerholm *et al.* 2015; Capson-Tojo *et al.* 2018; Molaey, Bayrakdar and Calli 2018) and thermophilic (52°C; Safaric *et al.* 2018) biogas digesters. However, as this response may be linked to improved activity of hydrogenotrophic methanogens and lower hydrogen levels, direct effects of trace element deficiency on propionate-degrading communities have yet to be demonstrated. The few research attempts made to date in this area have revealed importance of molybdate, tungstate and selenite, which are crucial for the function of formate dehydrogenases and hydrogenases (de Bok *et al.* 2003; Worm *et al.* 2011). In a defined co-culture containing *S. fumaroxidans* and *M. hungatei*, the propionate-degrading activity has been shown to decrease due to lack of molybdate and tungstate, but depletion of these trace elements has little effect on the SPOB when grown in pure culture on propionate and fumarate, indicating the importance of trace elements particularly for syntrophic activity (Plugge *et al.* 2009). In a propionate-fed upflow anaerobic sludge blanket (UASB) reactor, deficiency of molybdate, tungstate and selenite has been suggested to interfere with the competitive advantage of members of *Syntrophobacter* and instead favor *Pelotomaculum* and *Smithella* (Worm *et al.* 2009).

Propionate threshold and dilution rate

Substrate specificity is a key facet of microbial responses to conditions in their environment. However, the effect of the propionate level on SPOB activity is an issue that largely remains to be determined, particularly under thermophilic and high-ammonia conditions. In mesophilic, low-ammonia anaerobic degradation systems, *Smithella* is suggested to have higher affinity to propionate than *Syntrophobacter*, with *Smithella* coming to the fore below 0.5 mM propionate (Ariesyady *et al.* 2007; Ito *et al.* 2012). Further studies are needed to investigate the interplay between different SPOB at various propionate levels, preferably in combination with other parameters such as temperature, ammonia level and pH.

Build-up of high propionate levels in anaerobic digesters is a frequently reported side-effect of process imbalance. High propionate concentrations cause losses in methane yield and further instability, and it can be problematic to get rid of the high propionate levels once formed (Nielsen, Uellendahl and Ahring 2007; Gallert and Winter 2008; Khafipour *et al.* 2020). In order to develop strategies to counteract this, knowledge of the causes of the accumulation would be helpful and some suggestions have been proposed. One possible explanation relates to the small population size of SPOB as a result of the small energy gain in syntrophic propionate catabolism (Ito *et al.* 2012). An alternative reason is that high propionate levels inhibit methanogens (Barredo and Evison 1991; Dogan *et al.* 2005), with the ensuing shortcomings in cooperating network and high H_2/formate/acetate levels slowing down the propionate degradation rate. Once propionate has reached certain levels, direct toxicity by high propionate on SPOB is also plausible (Li *et al.* 2020). A pH dependency of the upper threshold for propionate degradation due to variation in ionization of propionate (as mentioned in the section 'Propionate transport across the cell membrane'), might also be of importance. Nevertheless, short exposure to extremely high propionate levels appears to be tolerated by certain SPOB, as indicated by process recovery of an anaerobic degradation reactor fed dairy manure after reaching 20 g propionate/L (Khafipour *et al.* 2020). In batch trials, it has been indicated that the lag phase before initiation of propionate conversion is extended when incubation occurs at higher propionate levels (Han, Green and Tao 2020). The reason for this remains to be determined, but it is an area worthy of further investigation as it holds potential for development of microbial-based strategies to overcome extended periods of propionate accumulation in anaerobic processes.

Dilution rate and retention time are other factors affecting the conditions for survival, particularly for the slow-growing syntrophs. In this context, higher susceptibility to increased dilution rate (from 0.025 to 0.05/day) in a thermophilic community compared with a mesophilic community has been reported (Chen *et al.* 2020). In a mesophilic propionate enrichment study, *Syntrophobacter* and *Smithella* were found to be favored at high dilution rate (0.15/day), while at lower rates (0.05/day) unclassified Syntrophaceae were more abundant (Wang *et al.* 2019a). Shortening the retention time from 10 to 4 h is reported to cause a shift in dominance from *Pelotomaculum* to *Syntrophobacter* and *Smithella* (Ban, Zhang and Li 2015).

In the rhizosphere and bulk soil of a rice field, it has been demonstrated that SAOB, methanogens and also SPOB increase in abundance during rice growth, with soil moisture,

carbon/nitrogen ratio, oxalate and succinate indicated to be the factors shaping the structure and diversity of syntrophic propionate-degrading communities (Pan *et al.* 2021). The threshold for commencement of syntrophic propionate oxidation remains to be elucidated, but in peat soil propionate degradation is reported to start at levels as low as 0.3 mM in a community including *Syntrophobacter* and *Smithella* (Schmidt *et al.* 2016).

Addition of supportive material

As mentioned in previous sections, there is potential to increase the propionate degradation rate by bringing SPOB into close proximity with methanogens (reducing the distance needed for H_2/formate diffusion) and/or to facilitate direct electron transfer with conductive material acting as electron conduits. Numerous reactor studies report reduced propionate and acetate accumulation in the presence of supportive materials, but few studies have specifically examined the impact on propionate degradation (Table S7, Supporting Information). Magnetite (Fe_3O_4) and granular activated carbon are the only conductive materials tested specifically for propionate degradation, with studies conducted at 20–55°C all reporting enhanced methane production rates following addition of these materials (Table S7, Supporting Information). Contrasting observations have been made in a propionate-degrading culture grown at 20°C, however, where granular activated carbon and magnetite had no impact on the methane production rate (Guo *et al.* 2021a).

The current incomplete state of knowledge demonstrates a need for progress within this area in order to answer open questions. First, the mode of action and actual promotion of DIET by conductive materials in propionate-degrading communities have yet to be established. Second, all studies to date have been performed in batch or batch-fed reactors, while the long-term effects of addition of the supportive material on propionate degradation and the microorganisms involved (both in terms of degradation rate and their ability to cope with inhibitions) in continuously-fed reactors have yet to be delineated. Finally, there are indications that environmental factors, such as ammonia levels, can influence the impact of the supportive material (Lee *et al.* 2019; Yan, Mukherjee and Zhou 2020). Thus, holistic studies that include synergetic effects of the supportive material and environmental conditions that screen the effectiveness of the material under the large span of different conditions that often prevail in biogas reactors would be beneficial. This would help in formulation of guidelines and facilitate choice of materials suitable for different biogas processes.

Product inhibition

The Gibbs free energy values associated with propionate degradation (Equations 1–5) imply that the process may be sensitive to product (H_2, acetate) inhibition. This has indeed been found in dispersed cultures of SPOB. Elevated levels of hydrogen and acetate inhibit propionate degradation under both mesophilic and thermophilic conditions (Smith 1980; Gorris *et al.* 1989; Fukuzaki *et al.* 1990; Schmidt and Ahring 1993; van Lier *et al.* 1993; Felchner-Zwirello, Winter and Gallert 2013; Guo *et al.* 2021a). In the dismutation pathway, one mole of H_2 is produced per mole of propionate degraded (Equations 2and3), compared with three moles of H_2 in the methylmalonyl-CoA pathway (Equation 1). This reduces the energetic and thermodynamic sensitivity of *Smithella* to elevated H_2 levels, and it has been hypothesized that *S. propionica* can stabilize methanogenic bioreactors in which propionate degradation is the bottleneck (Dolfing 2013). However, acetate frequently prevails in association with propionate accumulation in bioreactors, and thermodynamic calculations indicate that use of the dismutation pathway would involve higher sensitivity to high levels of acetate (Schmidt *et al.* 2016). In cultivation trials, propionate degradation by *Smithella* sp. has been shown to be completely inhibited in the presence of 40 mM acetate (Liu *et al.* 1999). On using a non-competitive inhibition model to analyse the kinetics of propionate degradation, Fukuzaki *et al.* (1990) observed that the inhibition constants for acetate and dissolved H_2 were in the range of 50–70 μM, suggesting that product removal is crucial to maintain efficient propionate degradation rates. However, propionate degradation by SPOB using the mmc pathway for propionate oxidation has been shown to occur in the presence of higher acetate levels (e.g. 17–20 mM) (Imachi *et al.* 2000; Plugge, Balk and Stams 2002). Consequently, the impact of acetate levels on propionate degradation represents one of the numerous knowledge gaps on SPOB activity, and research within this area may provide critical insights of value for biogas process operation.

One approach to provide insights into thermodynamic and energetic aspects underlying syntrophic propionate degradation is to perform chemostat studies with characterized SPOB for which preferred concentrations of acetate, propionate and H_2 have been reported (Scholten and Conrad 2000). In this regard, it is important to note that propionate degraders residing in flocs or biofilms are shielded from high hydrogen concentrations by close proximity of their hydrogenotrophic partners (Conrad, Phelps and Zeikus 1985; Schmidt and Ahring 1993). An addendum to this knowledge is provided by Zhao *et al.* (2016b), who report that propionate degradation is not affected by formate or high partial pressures of H_2 in cultures suggested to conduct DIET. Supplementing reactors with ethanol during start-up is a suggested strategy to promote DIET in anaerobic degradation systems, and it would be interesting to see whether propionate degradation is indeed more stable in such systems. Regardless of the electron-carrying product of propionate-degrading SPOB, close proximity between SPOB and their electron-consuming partners, sometimes referred to as juxtaposition, is consistently recommended as the best way to stabilize the degradation process and make it less sensitive to product inhibition (Conrad, Phelps and Zeikus 1985; Speece *et al.* 2006; Zhao *et al.* 2016b).

CO_2 is another product of propionate degradation and the impact on anaerobic degradation efficiency of increased CO_2 partial pressure (pCO_2) is of interest in development of high-pressure bioreactors operated to integrate digestion with *in situ* biogas upgrading. With increasing partial pressure, the higher solubility of CO_2 than of methane will raise the methane content of the gas phase, reaching methane levels suitable for direct injection into the gas grid or industrial processes. However, elevated pCO_2 (from 0.3 to 10 bar) has been shown to significantly impair propionate degradation rates and increase the lag phase (with 4–14 days) under mesophilic temperature (30–35°C) conditions (Lindeboom *et al.* 2013, 2016; Ceron-Chafla *et al.* 2020, 2021). One likely reason for the decrease in propionate conversion is the lowering of pH (generally down to pH 6 at a pressure of 5 bar) caused by formation of carbonic acid (H_2CO_3) from the dissolved CO_2. However, complementary culture-based studies indicate that decreases in pH do not solely explain the detrimental effect on propionate degradation, with the decrease in propionate oxidation rates instead suggested to be due to a concomitant effect of reduced thermodynamic feasibility, physiological effects associated with lowering of pH, and increased levels of undissociated propionate (Lindeboom *et al.* 2016; Ceron-Chafla *et al.* 2020, 2021), as mentioned in the section 'Propionate transport

across the cell membrane'. A possible strategy to counteract propionate accumulation under those conditions could be to increase pH_2 temporarily in order to enhance CO_2 removal *via* hydrogenotrophic methanogenesis (Lindeboom *et al.* 2016).

KINETICS PROVIDE A GLIMPSE INTO SPOB ACTIVITES IN COMPLEX COMMUNITIES

Information on the kinetics of syntrophic propionate oxidizers is scant, the data presented and quoted are representative for co-cultures, rather than for propionate oxidizers specifically, and unit conversions are frequently questionable (Paton and Rodriguez 2019; Junicke 2020). Based on chemostat experiments with a propionate-fed co-culture of *S. fumaroxidans and M. hungatei*, Scholten and Conrad (2000) reported maximum theoretical growth yield for the propionate oxidizer of 5.7 g [dw]/mol propionate in the absence of maintenance. Growth yield is substantially lower under conditions generally occurring in anaerobic digesters and other ecosystems where maintenance is part of the energy budget, even though the syntrophs appear to have evolved for low maintenance energy requirements. The observation that propionate-oxidizing syntrophs grow equally fast with or without sulfate as electron acceptor (Wallrabenstein, Hauschild and Schink 1995; Scholten and Conrad 2000) suggests that the sulfide produced exerts toxic effects and that the burden of synthesizing the enzymes needed for sulfate reduction barely outweighs the higher energy available with sulfate compared with a methanogenic partner as electron acceptor, even though sulfate reduction in *S. fumaroxidans* is poorly regulated (Sedano-Núñez *et al.* 2018).

Interestingly, different specific activities of SPOB at syntrophic versus non-syntrophic growth have been reported (Scholten and Conrad 2000), with maximum specific activity for *S. fumaroxidans* in co-culture with *M. hungatei* of 0.2 mol propionate/g [dw]/day, more than 10-fold higher than in pure culture with sulfate as electron acceptor. In chemostat culture, the specific activity also increases with increasing dilution rate (Scholten and Conrad 2000). This conforms with chemostat theory, yet has implications for specific activities in bioreactors where biomass is usually (self)-immobilized as granular sludge, on carrier material, or on membranes.

With the advent of molecular techniques, research is gradually reaching a point where it is possible to estimate specific activities of propionate oxidizers in bioreactors *in situ*, or at least in sub-samples taken from those reactors. It is not always clear which propionate oxidizers are present and which are active, but molecular techniques can allow cell numbers and/or copy numbers to be estimated, while omics approaches can help to identify propionate-degrading activities by not-yet characterized SPOB. Until now, 'specific' activities for propionate have been expressed per gram of reactor biomass (e.g. Dolfing and Bloemen 1984), yielding activities of up to 6 mmol propionate per gram of volatile suspended solids per day for methanogenic reactor biomass enriched on a mixture of acetate and propionate as the sole energy source. Comparing these values with maximum specific activities of known propionate oxidizers indicates that the fraction of propionate oxidizers in methanogenic ecosystems is low. Other members of the community are of course involved in hydrogenotrophic methanogenesis and conversion of acetate into methane, but there is increasing evidence that a substantial part of the biomass also consists of organisms thriving on cell debris (Nobu *et al.* 2015; Wang *et al.* 2019b). It may well be the case that bacteriophages are a force to reckon within these systems (Zhang *et al.* 2017).

CONCLUSIONS AND PERSPECTIVES

Over recent decades, research efforts within iterative cultivation experiments with pure and mixed cultures, thermodynamic calculations and omics approaches have increased understanding of syntrophic propionate oxidization. The results of these efforts have demonstrated dispersed taxonomic placement of SPOB and key SPOB traits, including ability to use methylmalonyl-CoA or the dismutating pathway for propionate degradation and capability to circumvent thermodynamic constraints by transferring reduced compounds (H_2, formate) or directly relocating electrons to a hydrogenotrophic methanogen.

The broad taxonomic heterogeneity of known SPOB, belonging to the two phyla Firmicutes and Deltaproteobacteria and indicatively even a third phylum, *Ca.* Cloacimonetes, brings many challenges in the research field, as it makes generalization of SPOB difficult. Mounting evidence obtained using combinations of enrichment and omic analyses indicates even wider taxonomic and metabolic versatility of SPOB. Identification of key functional gene(s) for syntrophic propionate degraders or gene expression related to specific SPOB activities (e.g. involved in their interspecies communication or activities carried out to come into close physical proximity with cooperating partners) would help overcome some of these limitations. The current progress within the field of SAOB and identification of key genes in the Wood–Ljungdahl pathway suitable as marker genes (Singh 2021) could be a source of inspiration. There is also a need for experimental analyses that span a larger range of anaerobic digestion systems and environments than hitherto studied, using a combination of cultivation and omic analyses to classify SPOB based on activity within a community, rather than based on their genotypes. It is known that just a few of the characterized SPOB specialize solely in syntrophic cooperation and that most have at least one alternative mode of electron disposal (e.g. sulfate reduction) or mode of growth (e.g. fermentative or autotrophic). Hence, syntrophic cooperation might only be a stopgap for many representatives of the SPOB. Still, identification of a functional gene encoded by all SPOB would facilitate identification of key players in more complex settings. This would allow information to be gathered on how the propionate-degrading capability of different SPOB relates to environmental conditions and would enable identification of biotic and abiotic drivers controlling their activity, especially with respect to the bottlenecks associated with propionate degradation in anaerobic digesters.

Critical SPOB traits have yet to be identified, although capability to operate one of the biochemical pathways (the methylmalonyl-CoA or the dismutating pathway) for propionate oxidation and acetate formation can be argued to be a unique SPOB feature. However, there are considerable variations in gene repertoire, gene organization and enzymatic activities between the species. It can be hypothesized that indirect and/or direct electron transfer is well-organized and system-integrated, but it might in fact be unpredictable and difficult to analyse and control, especially in engineered digester systems. Unique features for adaptation to a syntrophic lifestyle, such as synchronized amino acid biosynthesis and transport with a cooperating methanogen, or altered expression of chemotaxis genes in response to a methanogenic partner, have been found in SPOB. However, whether these are a characteristic shared by all SPOB and unique for SPOB (hence not found in other syntrophs)

remains to be determined, as little has been explored within the syntrophic world (Sieber, McInerney and Gunsalus 2012).

Important discoveries within recent research have revealed some intriguing metabolic capabilities and enzymatic activities of SPOB. We hope that this review will inspire research on key unknowns warranting further investigation, including enzymatic activities for translocation of propionate across the cell membrane, the connection between the first and the last step (i.e. propionate activation and acetate generation; steps 1 and 11 in Fig. 2) in the intracellular propionate degradation pathways of SPOB, and how *Candidatus* SPOB (such as Cloacimonetes) conduct the energetically unfavorable oxidation of succinate to fumarate. Continuing research relating to the possible division of labor to amino acid biosynthesis and amino acid or fructose exchange between syntrophic interacting strains (Kato, Kosaka and Watanabe 2009; Hidalgo-Ahumada *et al*. 2018), promotion of syntrophic propionate oxidation by intermediates such as succinate (Pan *et al*. 2021) and potential flagellum-mediated syntrophic interaction (Hidalgo-Ahumada *et al*. 2018) is highly important in this regard. Communications by exchanging quorum-sensing molecules and connections *via* membrane-derived nanotubes have been demonstrated in anaerobic cultures that interact nutritionally in tight cell-cell interactions (Pande *et al*. 2015; Ranava *et al*. 2021). This raises questions as to whether syntrophic bacteria, which rely on finding a suitable partner microorganism in order to conduct that metabolic activity, exhibit similar behavior. A scouting study on this topic revealed a positive correlation between enhanced abundance of species involved in acetate, propionate and ethanol degradation and presence of genes for quorum sensing (Yin *et al*. 2020). Another open question regarding the metabolic capabilities of SPOB is possible capability for bidirectional use of the methylmalonyl-CoA pathway. Propionate production *via* the methylmalonyl-CoA pathway is feasible from an enzymatic biochemical point of view. From an ecological perspective, bidirectional use of the pathway could benefit SPOB, especially in ecosystems with fluctuating hydrogen levels. Hypothetically, if the hydrogen concentrations become too high to sustain propionate oxidation *via* the methylmalonyl-CoA pathway, operating the pathway in a propionate-producing direction would help to counteract excessive hydrogen accumulation and enable SPOB to survive under adverse conditions. However, bacteria operating the methylmalonyl-CoA pathway in a propionate-producing direction ($\Delta G^{\circ\prime} = -74$ kJ/reaction) would not be able to compete for H_2 with hydrogenotrophic methanogens ($\Delta G^{\circ\prime} = -130.8$ kJ/reaction; Table S9, Supporting Information). Thus it can be argued that, in environments with high H_2 levels and no activity of hydrogenotrophic methanogens (e.g. due to low pH), bacteria could thrive on operating the methylmalonyl-CoA pathway in the 'reverse' propionate-producing direction. It can even be speculated that bacteria with bidirectional use of the methylmalonyl-CoA pathway could act as hydrogen scavengers for SPOB operating the dismutating pathway (Figures S1, S2 and Table S9, Supporting Information). In such a system, the SPOB would benefit from the hydrogen scavenging in two ways, through sustainably low hydrogen concentration and recycling of additional propionate. For this system to work for prolonged periods in practice, hydrogen would need to be produced continuously by other organisms, hydrogenotrophic methanogens would need to be inactive, and the acetate level would need to be lower than the propionate level. Hydrogen dark fermentation could be such a system but is currently merely a theory, so cultivation experiments including variations of SPOB are needed. Advanced knowledge in this area, coupled with insights on bidirectional use of the Wood-Ljungdahl pathway by SAOB when growing in syntrophy with methanogens (Müller, Sun and Schnürer 2012), could help to discern specific syntrophic attributes such as that hypothesized above.

With multiple process studies reporting lower propionate levels in bioreactors upon addition of selected supportive and conductive materials, biotechnological research is currently making strides towards the development of real, applicable and reactor management approaches. However, the myriad of interspecies interactions sustaining anaerobic degradation processes complicates identification of the actual mechanisms involved in microbe–material interactions in more complex settings. This calls for research focusing on the biochemistry, underpinning the observed effects on process function achieved by the supportive material, including synergistic effects of the ambient environment. Identification of potential drivers for establishment of direct electrical communication, i.e. DIET between SPOB and cooperating partners, is an area that raises interesting questions, such as how factors that at first glimpse do not seem to benefit SPOB activity (e.g. ethanol addition) can increase the rate of propionate degradation. The answer may lie in the suggestion that DIET-capable cooperating partners for SPOB are enriched by such addition, or in the highly speculative suggestion that an existing electron transfer network can function as a 'high voltage line' to which other species can connect their electron transport wire. Combined cultivation trials, conductive measurements, electronic microscopy analyses and omic approaches to evaluate the mechanisms of electron transfer, and the impact on the interspecies connection from addition of supportive and conductive materials to diverse SPOB communities, could help to answer these questions. To help predict the outcome of adding widely diverse materials to support syntrophic propionate-oxidizing communities, links between different environmental conditions (e.g. temperature and pH) and the effect on the specific material on microbial activity need to be established. Subsequent research should then examine whether the positive effects of the supportive material vary depending on the microbial species and its cooperating partner, or on its competitive advantage, and whether the material also provides the microbial community with higher resistance to fluctuations in environmental conditions such as ammonia, H_2, formate or acetate levels.

Within all the above areas, but in particularly regarding novel enzymatic activities, division of labor for biosynthesis, promotion of propionate degradation by intermediates and mediation of cooperating interactions between SPOB and methanogens, we strongly encourage further research to obtain fundamental knowledge on syntrophic traits. Given the current substantial interest in syntrophic microorganisms in anaerobic habitats, we are optimistic about future advances in answering unresolved fundamental questions about SPOB metabolism and the strategies and mechanisms these organisms use for interspecies cooperation. A more holistic understanding of syntrophic interactions would open up new avenues of innovation for future biotechnologies and approaches that can be implemented in engineered systems for more robust process control. Prediction of methane emissions from anaerobic soils/sediments and adaptation of syntrophic propionate oxidation communities to the reality of global changes in temperature is another research area of biogeochemical and practical significance.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSRE* online.

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