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Genome and proteome analyses show the gaseous alkane degrader *Desulfosarcina* sp. strain BuS5 as an extreme metabolic specialist

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Summary

The metabolic potential of the sulfate-reducing bacterium *Desulfosarcina* sp. strain BuS5, currently the only pure culture able to oxidize the volatile alkanes propane and butane without oxygen, was investigated via genomics, proteomics and physiology assays. Complete genome sequencing revealed that strain BuS5 encodes a single alkyl-succinate synthase, an enzyme which apparently initiates oxidation of both propane and butane. The formed alkyl-succinates are oxidized to CO_2 via beta oxidation and the oxidative Wood–Ljungdahl pathways as shown by proteogenomics analyses. Strain BuS5 conserves energy via the canonical sulfate reduction pathway and electron bifurcation. An ability to utilize long-chain fatty acids, mannose and oligopeptides, suggested by automated annotation pipelines, was not supported by physiology assays and in-depth analyses of the corresponding genetic systems. Consistently, comparative genomics revealed a streamlined BuS5 genome with a remarkable paucity of catabolic modules. These results establish strain BuS5 as an exceptional metabolic specialist, able to grow only with propane and butane, for which we propose the name *Desulfosarcina aeriophaga* BuS5. This highly restrictive lifestyle, most likely the result of habitat-driven evolutionary gene loss, may provide *D. aeriophaga* BuS5 a competitive edge in sediments impacted by natural gas seeps.

Etymology: *Desulfosarcina aeriophaga*, *aério* (Greek): gas; *phágos* (Greek): eater; *D. aeriophaga*: a gas eating or gas feeding *Desulfosarcina*.

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Introduction

The short-chain volatile alkanes ethane, propane and butane are major components of natural gas and gas oils, accounting for up to 40% of the gas composition (Claypool and Kvenvolden, 1983; Milkov, 2005). Volatile alkanes are largely produced by the decomposition of fossil biomass at high temperature and pressure (thermogenic origin) (Tissot and Welte, 1984; Song *et al*., 2021). Geochemical data and experimental assays with marine sediments indicate that at least ethane and propane may have also a biological origin (Oremland *et al*., 1988; Hinrichs *et al*., 2006; Xie *et al*., 2013), although microorganisms and underlying biochemical mechanisms for their formation are yet to be uncovered. In marine sediments impacted by natural gas and crude oil seepage, volatile alkanes are important carbon and energy sources for aerobic and anaerobic microorganisms. Their anaerobic oxidation has been so far demonstrated only with sulfate as terminal electron acceptor. Microorganisms involved are either sulfate-reducing bacteria (SRB) or archaea, the latter forming close associations with SRB which in these cases act as electron sinks (Kniemeyer *et al*., 2007; Jaekel *et al*., 2013; Laso-Pérez *et al*., 2016; Chen *et al*., 2019; Hahn *et al*., 2020).

To date, the only known pure culture able to oxidize volatile alkanes is *Desulfosarcina* sp. strain BuS5 (Kniemeyer *et al*., 2007). Isolated from hydrothermal sediments around hydrocarbons seeps in the Guaymas Basin, strain BuS5 is a mesophile, with an optimum

growth temperature around 30°C (Jaekel *et al*., 2013), and can oxidize propane and butane, but not shorter (methane or ethane) or longer-chain alkanes (pentane or hexane) (Kniemeyer *et al*., 2007). Phylogenetically it belongs to the metabolically versatile *Desulfosarcina–Desulfococcus* cluster of the *Deltaproteobacteria*, a clade known to include other hydrocarbon-degrading SRB (Widdel *et al*., 2007). Strain BuS5 initiates oxidation of propane and butane by homolytic cleavage of the C-H bond of a subterminal (secondary) carbon atom, followed by addition of the resulting radical to fumarate (Kniemeyer *et al*., 2007). Products are isopropylsuccinate and (1-methylpropyl)succinate. This mechanism of activation has been documented with other alkane-oxidizing anaerobes, including SRB oxidizing long-chain alkanes (Rabus *et al*., 2001; Callaghan *et al*., 2006; Davidova *et al*., 2006; Jarling *et al*., 2012). In these strains, the activation reaction is catalysed by methylalkyl- or alkylsuccinate synthases (Mas or Ass) (Callaghan *et al*., 2008; Grundmann *et al*., 2008), which are glycyl-radical enzymes similar to the well-characterized benzylsuccinate synthase (Bss) involved in activation of alkyl-aromatic hydrocarbons (Leuthner *et al*., 1998; Heider *et al*., 2016). Strain BuS5 is expected to encode a similar enzyme (Musat, 2015). A peculiarity of strain BuS5 is the apparent ability to form 1-propylsuccinate, indicating an additional activation of propane at a terminal (primary) carbon atom (Kniemeyer *et al*., 2007). The presence of this route of activation, consolidated by bulk and position-specific stable isotope analyses, suggested that strain BuS5 may encode multiple *mas*/*ass* operons (Jaekel *et al*., 2014; Musat, 2015; Gilbert *et al*., 2019).

SRB related to strain BuS5 have been identified as the propane or butane oxidizers in enrichment cultures obtained from Gulf of Mexico and Hydrate Ridge seep sediments (Jaekel *et al*., 2013). In addition, phylotypes related to strain BuS5 have been retrieved from enrichment cultures or sediment incubations with alkanes, including short- and medium-chain alkanes and cycloalkanes (Savage *et al*., 2010; Bose *et al*., 2013; Jaekel *et al*., 2015), and are frequently encountered in marine sediments at gas seeps or hydrothermal vents (Case *et al*., 2015; Thompson *et al*., 2017). With a demonstrated *in situ* role in the oxidation of volatile alkanes (Kleindienst *et al*., 2014), strain BuS5 and related organisms may have a high impact on the sulfur and carbon cycles at seep sites where sulfate reduction rates cannot be explained by other dissimilatory processes, like the anaerobic oxidation of methane (Orcutt *et al*., 2005; Omoregie *et al*., 2009; Bowles *et al*., 2011).

The present study was undertaken to explore the metabolic potential of *Desulfosarcina* sp. strain BuS5, as the only cultivated representative of SRB able to oxidize gaseous alkanes. The complete genome of strain BuS5 was obtained and analysed with a focus on catabolic and energy conservation pathways. Genome-based predicted metabolic features were further tested by proteomics analyses and physiology assays. Finally, the metabolic potential of strain BuS5 was evaluated by comparative genomics. The goal of this integrative approach was to reconstruct a comprehensive model for the anaerobic metabolism of gaseous alkanes, and to gain insights into how environmental conditions may shape the lifestyle of alkane oxidizers in their natural habitats.

Results and discussion

Genome properties

The complete genome of *Desulfosarcina* sp. strain BuS5 consists of a circular chromosome of 4 066 517 bp, and an extrachromosomal circular genetic element, named BuS5-Plasmid 1, of 108 571 bp. The chromosome, whose general properties are summarized in Table 1, contains 4004 predicted genes of which 3951 encode proteins, three encode ribosomal RNA and 50 encode tRNA. Strain BuS5 genome contains genes encoding proteins likely involved in oxygen reduction and detoxification mechanisms, including two cytochrome bd quinol oxidase subunit I (*cydA*) and II (*cydB*), one bifunctional catalase/peroxidase, four rubredoxins, five rubrerythrins, one selenocysteine-containing peroxiredoxin and one desulfoferredoxin. Interestingly, over 70 genes related to flagellum structure and assembly, chemotactic signal

Table 1. General features of *Desulfosarcina* sp. strain BuS5 genome.

	Chromosome	Plasmid
Topology	Circular	Circular
Genome size (base pairs)	4 066 517	108 571
Number of scaffolds/contigs	1	1
G + C content (%)	41.1	39.9
Genome completeness[a] (%)	99.0*; 99.7†; 99.5¶;100[b]	–
Contamination[a] (%)	0.80*; 0.53†; 0.46¶	–
Duplication of single copy genes[a]	1	–
Strain heterogeneity[a] (%)	0	–
Number of ORF[c]	4004	106
Predicted protein-coding genes (CDS)	3951	106
Number of rRNA	3	0
Number of tRNA	50	0
Scaffold N50 (bp)	4 066 517	108 571
Coding density (%)	84.6	86.4
CRISPR loci	8	0
IS transposons	263	5

[a]Based on lineage-specific marker genes of Deltaproteobacteria (*), Desulfobacterales (†) and Desulfobacteraceae (¶) using CheckM.
[b]Based on bacterial-specific single-copy genes using AMPHORA2.
[c]ORF: open reading frames.

transduction, and pilus assembly were detected in the genome. In addition, all genes required for the selenocysteine metabolism were identified, including *selA* (L-seryl-rRNA selenium transferase), *selB* (selenocysteine-specific translation elongation factor) and *selD* (selenophosphate synthase). The plasmid contains 106 predicted open reading frames, including 13 *tra* genes typically involved in the conjugative transfer of the plasmid, and six genes encoding transposases or putative transposases. The *tra* genes are most closely related to similar genes of *Deltaproteobacteria* (Table S1), suggesting vertical transfer within SRB. The plasmid does not encode any recognizable essential metabolic feature; its function is presently unclear. The genome was further analysed with a focus on alkane oxidation, energy metabolism and other potential catabolic functions of strain BuS5.

Alkane activation and complete oxidation pathway

Desulfosarcina sp. strain BuS5 can oxidize both propane and butane (Kniemeyer *et al*., 2007). Analyses of metabolites identified propylsuccinate isomers in cultures grown on propane, and (1-methylpropyl)succinate in cultures grown on butane (Kniemeyer *et al*., 2007). These findings indicate that strain BuS5 activates propane and butane by homolytic cleavage of the C-H bond of a secondary (subterminal) carbon atom, followed by addition of the resulting radical to the double bond of fumarate, similar to other anaerobes oxidizing higher alkanes (Kropp *et al*., 2000; Rabus *et al*., 2001; Callaghan, 2013). The finding of 1-propylsuccinate as a metabolite indicates that, exceptionally, strain BuS5 activates propane also at a primary (terminal) carbon atom (Kniemeyer *et al*., 2007). The presence of this route of activation, so far not observed for the oxidation of C_{4+} alkanes (Musat, 2015), was further consolidated by bulk and position-specific isotope analyses (Jaekel *et al*., 2014; Gilbert *et al*., 2019). In anaerobic bacteria oxidizing medium- and long-chain alkanes, activation by addition to fumarate is catalysed by (methyl)alkylsuccinate synthases (Mas/Ass) (Callaghan *et al*., 2008; Grundmann *et al*., 2008). The genome of strain BuS5 contains a single predicted *mas*/*ass* operon, containing one gene encoding for the alpha, or the catalytic subunit (*masD*/*assA*), two putative genes encoding for the beta subunit (*masE*/*assB*), and one putative gene encoding for the gamma subunit (*masC*/*assC*) (Fig. 1C).

The predicted MasD was detected in protein extracts of strain BuS5 grown with both propane and butane (Table S2). Amino acid alignment showed that MasD contains the conserved Cys and Gly residues involved in radical formation and alkane activation, and shares over 40% sequence identity with similar proteins from other anaerobic alkane degraders (Fig. 1B and C). Phylogenetically, it is most closely related to AssA of *Peptococcaceae* from methanogenic cultures oxidizing C_6–C_{10} alkanes (Tan *et al*., 2013; Tan *et al*., 2014) (Fig. 1A). The predicted MasE are small, Cys-rich proteins similar to the beta subunit of benzylsuccinate synthase (Leuthner *et al*., 1998), and were not detected in protein extracts. MasC was detected in protein extracts of both propane- and butane-grown cells (Table S2). Two genes with high similarity to pyruvate-formate lyase activating enzymes (*pflA*/*masG*, Mas activating enzymes) were identified upstream and downstream of the *mas* operon respectively (Fig. 1C, Table S2). These findings suggest that strain BuS5 encodes a single functional Mas/Ass which is responsible for the activation of both propane and butane, the former at both primary and secondary carbon atoms.

Based on metabolite analyses, fatty acids profiles and analogy to pathways for the oxidation of higher alkanes, three distinct pathways have been proposed for the downstream oxidation of (methyl)alkyl-succinates (Kniemeyer *et al*., 2007). These involve conversion of alkylsuccinates to their coenzyme A thioester counterparts, carbon-skeleton re-arrangement, decarboxylation, and beta-oxidation, leading to isobutyryl-CoA, 2-methylbutyryl-CoA and acetyl-CoA (Fig. 2). The genome of strain BuS5 contains multiple genes encoding AMP-dependent acyl-CoA synthetases (*fadD*), propionyl-CoA carboxylases/methylmalonyl-CoA decarboxylases and methylmalonyl-CoA mutases (Table S2). In addition, multiple genes encoding all enzymes required for a complete beta-oxidation pathway were found, including acyl-CoA dehydrogenases (seven copies), 3-hydroxyacyl-CoA dehydrogenases (eight copies) and acetyl-CoA acetyltransferases (six copies) (Table S2). However, since strain BuS5 cannot utilize exogenously added fatty acids (Kniemeyer *et al*., 2007) (thereby preventing differential proteomics), and the beta-oxidation enzymes detected in protein extracts were (with few exceptions) found in cells grown with both propane and butane, these enzymes could not be unambiguously assigned to specific reactions (Fig. 2).

Isobutyryl-CoA could be oxidized via methylmalonic semialdehyde to propionyl-CoA, as proposed for *Desulfococcus multivorans* (Stieb and Schink, 1989; Dorries *et al*., 2016). Oxidation of 2-methylbutyryl-CoA could proceed via beta-oxidation, yielding propionyl-CoA and acetyl-CoA. Propionyl-CoA, a product of both propane and butane oxidation could be further converted to fumarate (regeneration of fumarate for the activation reaction) via the methylmalonyl-CoA pathway (Table S2, Fig. 2, Figs S1–S2).

Like other hydrocarbon-oxidizing SRB, strain BuS5 encodes a Wood–Ljungdahl pathway for the complete oxidation of acetyl-CoA to CO_2. The genome contains

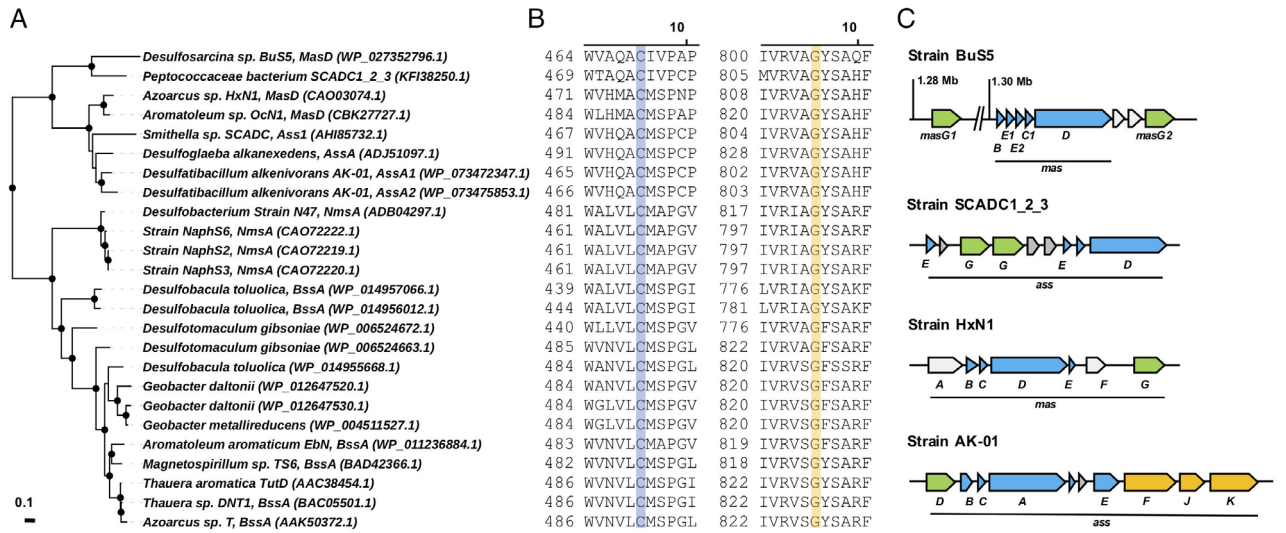

Fig. 1. Analysis of the methylalkylsuccinate synthase (*mas*/*ass*) operon of *Desulfosarcina* sp. strain BuS5.
A. Phylogenetic tree showing the similarity of the catalytic subunit (MasD/AssA) of strain BuS5 to homologues from other hydrocarbon degraders. The phylogenetic tree was constructed based on maximum likelihood algorithm considering 630 amino acids positions. Branches with bootstrap values over 90% are indicated by black dots. Scale bar = amino acid substitutions per site.
B. Sequence alignment of MasD/AssA of strain BuS5 with alpha subunits of alkylsuccinate synthase and benzylsuccinate synthase from other hydrocarbon degraders. Amino acids surrounding the conserved Cys and Gly residues of glycyl radical enzymes are shown. The numbers next to the alignment indicate residue positions of the corresponding protein sequences.
C. Genome organization of *mas*/*ass* and surrounding genes for *Desulfosarcina* sp. BuS5, *Smithella* sp. SCADC, *Azoarcus* sp. HxN1 and *Desufatibacillus alkenivorans* AK-01.

all genes for acetyl-CoA decarbonylase/synthase, methylenetetrahydrofolate reductase (MetFV), methylenetetrahydrofolate dehydrogenase (FolD), and formate dehydrogenase (Fdh), and all corresponding proteins were detected in extracts of strain BuS5 cells grown with both propane and butane (Table S2, Fig. 2).

Energy metabolism

The genome of strain BuS5 encodes all enzymes needed for the dissimilatory sulfate reduction pathway, including sulfate adenylyltransferase (Sat), adenosine phosphosulfate reductase (AprAB) and dissimilatory sulfite reductase (DsrAB) (Fig. 2). The coupling of dissimilatory sulfate reduction to energy metabolism may occur through the activity of multiple transmembrane redox complexes (Fig. 2). Strain BuS5 encodes three membrane-bound electron carriers associated with sulfate respiration, including QmoABC, DsrMKJOP and QrcABCD. Qmo and Dsr complexes are conserved among SRB and have been proposed to transfer electrons from menaquinone to AprAB and DsrAB respectively (Pereira *et al*., 2011). The reduced menaquinone pool could be replenished by Qrc, which is shown to accept electrons from periplasmic cytochrome c_3 and acts as a cytochrome c_3: menaquinone oxidoreductase (Venceslau *et al*., 2010; Venceslau *et al*., 2011). Qmo, Dsr and Qrc complexes may form energy-conserving redox loops via electron transfer to and from the menaquinone pool, creating proton motive force during sulfidogenesis. Additional electron source for membrane electron carriers may derive from beta-oxidation via electron transfer flavoprotein complexes (ETF). Two sets of *etfAB* gene clusters have been detected in strain BuS5 genome. One is adjacent to a gene encoding a membrane integral ETF:ubiquinone oxidoreductase (ETF-QO), and the second one is adjacent to a gene for a membrane-bound Fe-S oxidoreductase. ETF-QO catalyses quinone reduction by ETF, which typically harvests electrons from the acyl-CoA dehydrogenase (Watmough and Frerman, 2010). Thereby, in strain BuS5 ETF-QO and ETF together may link oxidation of butane- or propane-derived acyl-CoA to membrane circuits. ETF-associated Fe-S oxidoreductase has been hypothesized to channel electrons from beta-oxidation to membrane redox carriers in *Syntrophomonas wolfei* (Sieber *et al*., 2010). Sequence homology and gene arrangement imply a similar function of strain BuS5 Fe-S oxidoreductase with that in *S*. *wolfei*. The proposed role of these membrane-bound complexes in the energy metabolism of strain BuS5 is supported by their detection in protein extracts from cells grown with either propane or butane (Table S3).

Strain BuS5 lacks genes for periplasmic hydrogenases or formate dehydrogenases. This suggests that strain BuS5 is not able to couple energy conservation to hydrogen or formate cycling, a mechanism encountered in SRB like *Desulfovibrio* spp. (Heidelberg *et al*., 2004).

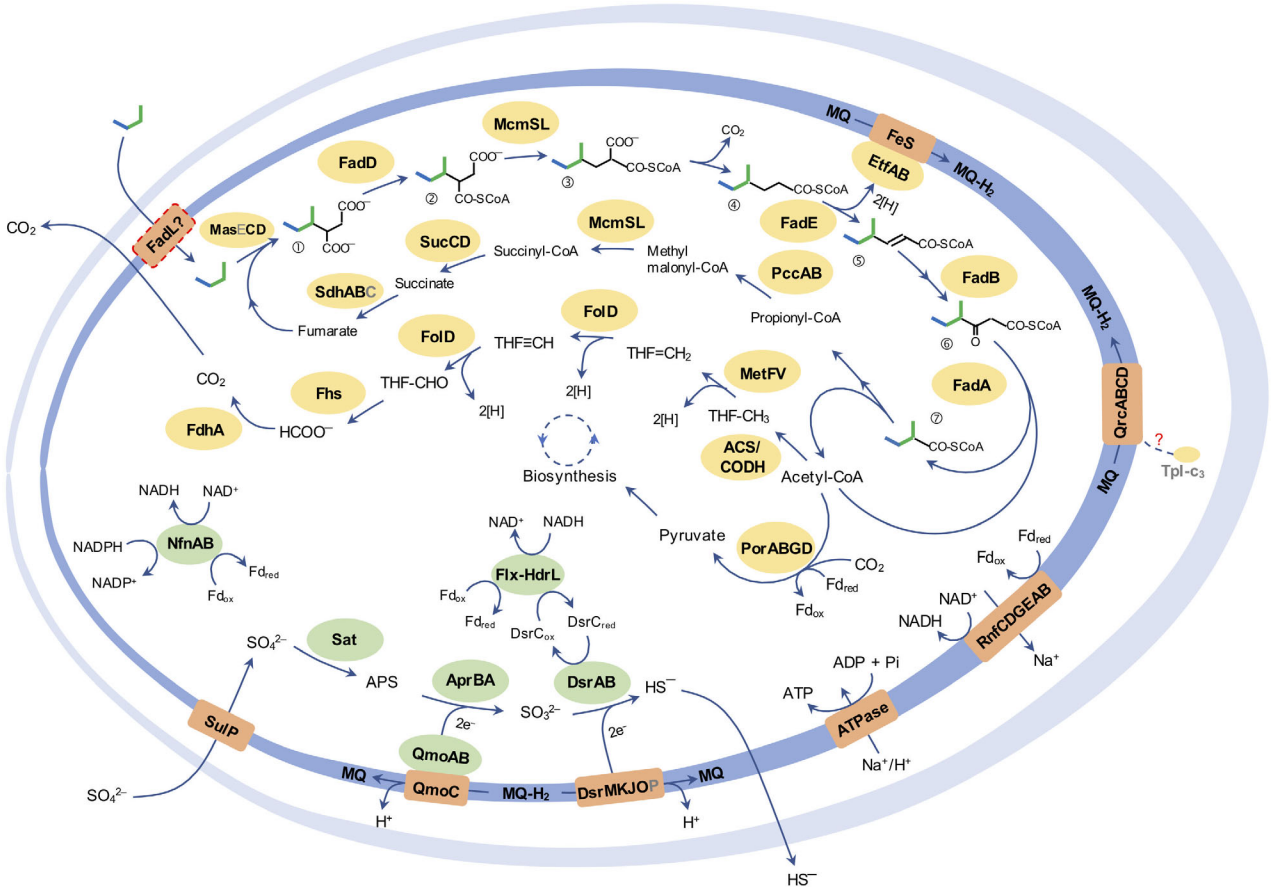

Fig. 2. Core metabolic network of *Desulfosarcina* sp. BuS5 according to genome, proteome and physiology analyses. Yellow: enzymes involved in alkane oxidation, including beta-oxidation and the Wood–Ljungdahl pathway; green: enzymes of the sulfate reduction pathway; orange: proteins involved in energy conservation. Enzymes and subunits detected in protein extracts are indicated in bold, black font; grey font: subunits not detected in protein extracts. Enzyme abbreviations are the same as in Tables S2–S5. Propane (green structure) and butane (blue-green structure) share similar pathways until intermediate 7. Further oxidation of isobutyryl-CoA (intermediate 7 of the propane oxidation pathway), and the oxidation of propane starting with activation at the terminal carbon atom are shown in Figs S1–S2. Pathway intermediates (propane/butane): (1) 2-isopropylsuccinate/(1-methylpropyl)succinate; (2) 2-isopropylsuccinyl-CoA/(1-methylpropyl)succinyl-CoA; (3) 2-carboxy-4-methylpentanoyl-CoA/2-carboxy-4-methylhexanoyl-CoA; (4) 4-methylpentanoyl-CoA/4-methylhexanoyl-CoA; (5) 4-methylpent-2-enoyl-CoA/4-methylhex-2-enoyl-CoA; (6) 3-keto-4-methylpentanoyl-CoA/3-keto-4-methylhexanoyl-CoA; (7) isobutyryl-CoA/2-methylbutyryl-CoA.

Instead, strain BuS5 encodes and expresses a number of enzyme complexes indicating the ability to perform flavin-based electron bifurcation (FBEB) (Fig. 2). These include a FlxABCD-HdrA complex which corresponds to an NADH dehydrogenase/heterodisulfide reductase (flavin oxidoreductases family), a ferredoxin-dependent transhydrogenase (NfnAB), and two membrane-bound ferredoxin:NAD^+ oxidoreductases (Rnf complexes). A *dsrC* gene is found downstream of the *flx-hdr* gene cluster (Table S3), suggesting that the FlxABCD-HdrA complex may couple NADH oxidation with reduction of both ferredoxin and DsrC; the latter, which is an electron donor for the DsrAB sulfite reductase (Venceslau *et al*., 2014), is one of the most abundant energy metabolism proteins detected in strain BuS5 protein extracts. Detection of NfnAB in protein extracts suggests the capacity of strain BuS5 to bifurcate electrons from NADPH to both NAD^+ and ferredoxin (Demmer *et al*., 2015). The presence and expression of both *rnf* operons indicate that strain BuS5 could generate the electrochemical H^+ or Na^+ potential needed for ATP synthesis using FBEB-derived ferredoxin (Westphal *et al*., 2018). In addition, the low-potential ferredoxin generated from Flx and Nfn complexes could drive endergonic reactions needed for biomass synthesis (Müller *et al*., 2018).

Limited metabolic potential for utilizing other substrates

The annotated genome was used to search for genes that would allow strain BuS5 to utilize growth substrates other than propane and butane. A remarkably low number of such genes were identified: a putative long-chain

fatty acids transporter (encoding FadL, an outer membrane protein essential for fatty acids transport); a genetic system for the uptake of hexoses and nitrogenous compounds, including a putative mannose-specific phosphopyruvate transfer system (PTS); and an oligopeptide transport system (Table S4). Although growth has not been observed with low molecular mass fatty acids (Kniemeyer *et al*., 2007), these findings suggested that strain BuS5 may be able to utilize long-chain fatty acids, mannose or oligopeptides as growth substrates. This was somewhat supported by the presence of metabolic modules required for the complete oxidation of such substrates, including genes for beta-oxidation, glycolysis and proteolysis (Table S4). These genome-based predictions were tested experimentally in physiology experiments. In incubations with saturated fatty acids (acetate and C_8 to C_{18} fatty acids), mannose, peptone or casein added as single growth substrates, substrate-dependent sulfate reduction was not observed; strain BuS5 was able to grow only when butane was provided as growth substrate (Fig. 3). To exclude possible inhibitory effects of the added substrates, butane was added to all cultures after 25 days of incubation. This led to an immediate increase in the formed sulfide (Fig. 3). The lack of growth with the predicted substrates prompted detailed analyses of their putative genetic systems. Sequence alignment showed that FadL has less than 35% amino acid sequence similarity with functionally characterized counterparts (Lepore *et al*., 2011) (Fig. 3). Instead, in phylogenetic analyses FadL of strain BuS5 (BuS5_00488) was branching with an alkane uptake protein from the hydrocarbon degrader *Marinobacter hydrocarbonoclasticus* (Mounier *et al*., 2018). Since FadL appeared abundant in protein extracts of strain BuS5 grown with both propane and butane (Table S2), a potential role in facilitating propane and butane uptake rather than in fatty acids transport is suggested (Fig. 3). PFAM-based domain analysis of the putative mannose uptake system (ManX; BuS5_02648) showed that this protein lacks the PTS system IIB domain, essential for the transport and phosphorylation of mannose (Erni, 2006). Finally, the putative peptide transport system of strain BuS5 lacks the transmembrane permease domain encoded by *ddpC*, which is essential in transporting oligopeptides across cell membranes (Lessard and Walsh, 1999). These findings show that the corresponding genetic systems are impaired or not functional, corroborating the outcome of the physiology experiments. In a wider context, these results indicate that caution needs to be applied when predicting function solely based on sequence homology, particularly when the assigned proteins show limited similarity to enzymes with confirmed function.

Consistent with the physiology experiments, comparative genomic analyses of strain BuS5 and of 12 related species, including other hydrocarbon-degrading SRB, revealed a streamlined BuS5 genome with a remarkable paucity of catabolic genes (Fig. 4; Table S5). Hydrogenases that would allow chemolithoautotrophic growth on H_2 and CO_2 are absent; periplasmic formate dehydrogenases and/or formate transporters needed to oxidize external formate are also lacking. The only formate dehydrogenase identified is putatively located in the cytoplasm, and may be involved in processing intracellular formate. Among the 13 genomes analysed, strain BuS5 has the smallest set of genes for fatty acids transport and beta-oxidation; directly supported by physiology experiments (Fig. 3), this indicates an inability to utilize fatty acids, which is exceptional among known *Desulfobacteraceae* (Table S6). Consistent with the inability of strain BuS5 to grow with alcohols or lactate (Kniemeyer *et al*., 2007), genes encoding homologues of alcohol dehydrogenase, lactate dehydrogenase and lactate permease were not found in the genome. The lack of growth with pyruvate is probably due to the absence of a pyruvate transporter, since strain BuS5 appears able to completely oxidize cytoplasmic pyruvate via a pyruvate: ferredoxin oxidoreductase and the oxidative Wood–Ljungdahl pathway (Table S4). Strain BuS5 encodes only one complete set of tripartite ATP-independent periplasmic transporter (DctPQM). This contrasts with over 10 DctPQM transport system sets in metabolically versatile *Desulfobacteraceae* species such as *D*. *ovata* and *D*. *alkanivorans* AK-01. DctPQM transporters are responsible for translocation of dicarboxylic acids (e.g. fumarate, malate and succinate) across the cell envelope. A limited number of DctPQM transporters suggest hampered capacity in the uptake of dicarboxylic acids. In addition, the absence of nearly half of the enzymes involved in the tricarboxylic acid cycle may preclude strain BuS5 from using the dicarboxylic acids as exogenous substrates.

Environmental implications

The genomic and comparative genomic analyses, along with current and former (Kniemeyer *et al*., 2007) substrate tests, show strain BuS5 as a true catabolic specialist, able to use only propane and butane as growth substrates. We therefore propose the name *Desulfosarcina aeriophaga* BuS5. Unlike most known members of *Desulfobacteraceae*, *D. aeriophaga* BuS5 shows a remarkable paucity of catabolic genetic modules (Fig. 4). A possible explanation is evolutionary gene loss following long-term exposure to relatively constant fluxes of hydrocarbons. Strain BuS5 was obtained from alkane-rich sediments where simple fermentation end-products like H_2, alcohols and organic acids are usually scarce due to high turnover rates (Ijiri *et al*., 2018; Dong *et al*., 2019). Physiological adaptations to such

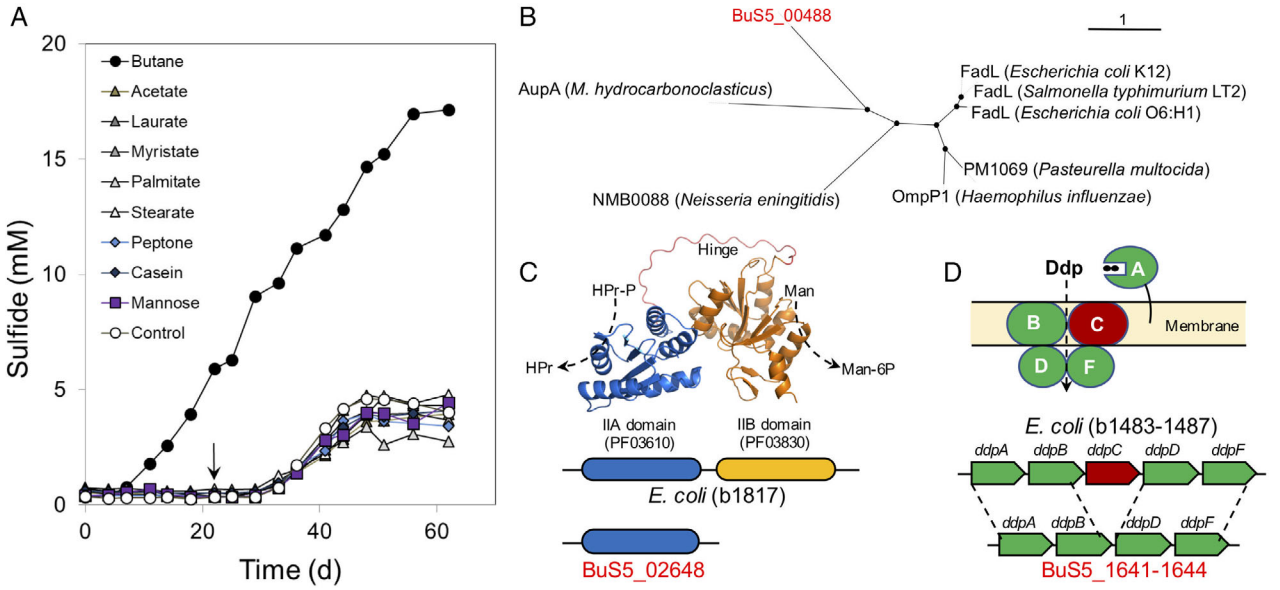

Fig. 3. Physiology assays and genetic analyses to test genome-predicted metabolic functions of strain BuS5.
A. Substrate-dependent sulfide formation during incubations with fatty acids, peptone, casein and mannose. Growth with butane (●) and without addition of substrate (○) were used as controls. Arrow indicates addition of butane to all cultures, including to the substrate-free control. Substrate tests with caprate and caprylate are shown in Fig. S3.
B. Phylogeny of FadL homologues. Filled circles on branches indicate bootstrap values >70%; scale bar, amino acid substitutions per site. The FadL homologue of strain BuS5 (BuS5_00488) is highlighted in red. *M. hydrocarbonoclasticus*: *Marinobacter hydrocarbonoclasticus*. FadL: long-chain fatty acid transport protein; OmpP1: outer membrane protein P1; AupA: alkane uptake protein A; NMB0088 and PM1069 are putative outer membrane proteins.
C. Domain composition of ManX (b1817) of *E. coli* and the putative ManX of strain BuS5 (BuS_02648). The PFAM id of the constituent domains is shown in parentheses. Man: Mannose; HPr: histidine phosphocarrier protein HPr.
D. Organization of genes encoding the peptide transport system in *E. coli* and strain BuS5. The locus tag of each gene is shown above (*E. coli*) or below (strain BuS5) the gene arrows. Ddp: dipeptide. Strain BuS5 lacks the *ddpC* gene.

environments may have caused relaxed selection of enzymes for fermentation-generated substrates, leading eventually to their partial loss from the genome. Gene loss as a consequence of niche adaptation, or genome streamlining, may offer *D. aeriophaga* a selective advantage by saving cellular maintenance energy. Conversely, catabolic specialization may have constrained the conservation of flagella and chemotaxis genes (Lever *et al*., 2015). Although a rather energetically expensive phenotype feature, motility could allow strain BuS5 to respond to *in situ* shifting alkane gradients.

Phylotypes related to *D. aeriophaga* BuS5 enriched from geographically distinct hydrocarbon seeps feature similarly restricted substrate ranges, at least with respect to oxidation of hydrocarbons, with propane and butane serving as the only growth substrates (Jaekel *et al*., 2013). Additional strain BuS5-related phylotypes have been largely confined to hydrocarbon-impacted sediments, like gas seeps and associated authigenic carbonates (Kleindienst *et al*., 2012; Case *et al*., 2015), hydrothermal sediments (Adams *et al*., 2013) and mud volcanoes (Kleindienst *et al*., 2014). Since these strains have not been obtained in pure culture and their genomes are not available, their metabolic capabilities cannot be presently assessed. Assuming similar metabolic specialization as shown here for *D. aeriophaga* BuS5, one may interpret the detection of BuS5-related sequences as indicators of short-chain alkane seepage. It remains for future studies to determine if metabolic specialization is common among anaerobic gaseous alkane oxidizers or an exceptional metabolic feature of strain BuS5.

Experimental procedures

Chemicals, organisms, culture media and cultivation techniques

Propane (purity >99.95%) and butane (purity >99.95%) were purchased from Air Liquide (Germany). Peptone, casein, mannose and sodium salts of fatty acids used for substrate tests, including acetic acid, octanoic (caprylic) acid, decanoic (capric) acid, dodecanoic (lauric) acid, tetradecanoic (palmitic) acid and hexadecanoic (stearic) acid, were purchased from Sigma Aldrich (Germany).

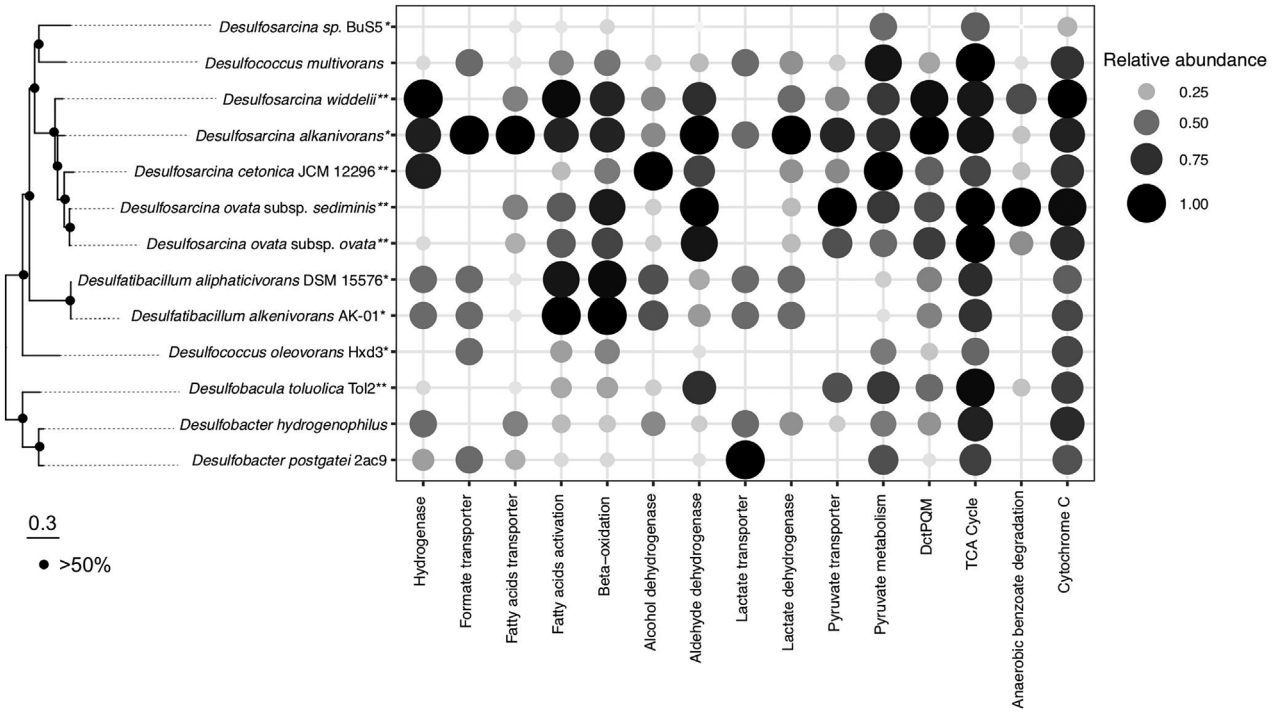

Fig. 4. Comparative genome analyses showing the abundance of functional modules of *Desulfosarcina* sp. strain BuS5 and of related strains, including other hydrocarbon oxidizers. Functional modules detected in each strain are based on KEGG and PFAM annotation. Total number of genes per functional module in each strain was normalized to the strain with the maximum number of genes in each module (relative abundance). The genes included in each functional module and the number of gene copies found in each strain are listed in Table S5. Degraders of alkanes (*) and aromatic hydrocarbons (**) are marked. The phylogenetic tree was built based on a concatenated alignment of 122 bacterial-specific marker genes. Branches with bootstrap values >70% are indicated by black circles. Scale bar = number of amino acid substitutions per site.

Desulfosarcina sp. strain BuS5 was routinely grown in artificial seawater medium with butane as growth substrate, as previously described (Kniemeyer *et al*., 2007). Preparation of anoxic culture media was done following established protocols (Laso-Pérez *et al*., 2018). Substrate tests were conducted in 60-ml serum bottles provided with 27 ml artificial seawater medium and inoculated with 3 ml of a butane-grown culture. The bottles were flushed with N_2:CO_2 (9:1 vol./vol.) and sealed with butyl rubber stoppers. Tested substrates were added with N_2-flushed syringes from anoxic stock solutions at the following working concentrations: stearate (1 mM), palmitate (1 mM), myristate (1 mM), laurate (1 mM), caprate (1 mM), caprylate (2 mM), acetate (10 mM), peptone (0.1% wt./vol.), casein (0.1% wt./wt.) and mannose (5.6 mM). Substrate-free controls and cultures with butane as the sole growth substrate were prepared in a similar way. The growth of strain BuS5 was monitored by measuring the substrate-dependent reduction of sulfate to sulfide. Sulfide concentrations were determined by photometric measurements of colloidal CuS (λ = 480 nm), as described elsewhere (Cord-Ruwisch, 1985).

Nucleic acids extraction and sequencing

For DNA extraction, cells were harvested from 40 ml of butane-grown *Desulfosarcina* sp. strain BuS5 cultures via centrifugation (16 000*g* for 15 min; ROTINA 380R, Hettich). Genomic DNA was extracted using NucleoSpin Microbial DNA kit (Takara Bio USA) according to the manufacturer's protocol for Gram-negative bacteria. Paired-end library was prepared from DNA fragments of about 500 bp and sequenced on a MiSeq sequencing platform. The short reads obtained were quality trimmed using Trimmomatic v.0.39 (minimum read length: 36). Long reads were generated from 1 μg genomic DNA using the SQK-LSK 109 Ligation Sequencing Kit and sequenced on a MinION Mk1B instrument with an R9.4.1 flow cell (Oxford Nanopore Technologies). Base-calling was performed using Albacore v2.3.1, yielding 65 312 reads (mean length 6547 bp, N50 length 13,095 bp). Adaptors in long reads were trimmed using Porechop v.0.2.3 and only reads longer than 1 kb were kept for subsequent analyses. Hybrid assembly using both Illumina MiSeq and Nanopore 1D reads was done with Unicycler v0.4.8-beta (Wick *et al*., 2017). The complete genome of strain BuS5 (one chromosome and one

plasmid) was deposited at the NCBI Genome database (Accession: PRJNA780815).

Genome annotation

The protein-coding genes were called using Prodigal 2.6.3 with the default settings and ATG, GTG and TTG as potential start codons (Hyatt *et al*., 2010). RNAmmer and tRNAscan-SE were executed to predict structural rRNAs and tRNAs (Lagesen *et al*., 2007; Chan and Lowe, 2019). All protein translations were searched against the NCBI reference sequence database (RefSeq), PFAM (Mistry *et al*., 2021), KEGG (Kanehisa and Goto, 2000) and eggNOGs (Huerta-Cepas *et al*., 2019) using BLASTP (Camacho *et al*., 2009) or HMMER v3.2 (Mistry *et al*., 2013). Functions were assigned with the annotation pipelines provided by the corresponding database. To identify putative pathways involved in butane or propane oxidation, proteins with curated function were used as query to search against the BuS5 genome using BLASTP with an *E*-value of 0.1. The resulting hits were checked gene-by-gene on the basis of protein length, presence of conserved domain or motif and annotations from GenBank's non-redundant database (NR) or UniProt/Swiss-Prot (The UniProt, 2019). Manually corrections to automated annotations were made whenever necessary. Further manual correction of automated annotation was done by pairwise sequence comparison between proteins of strain BuS5 and homologues from other anaerobic hydrocarbon-degrading bacteria using the EMBOSS needle program (Rice *et al*., 2000). Additionally, CRISPR loci and IS transposon were detected using CRISPRCasFinder (Couvin *et al*., 2018) and ISEScan (Xie and Tang, 2017) respectively.

Shotgun proteome analyses

Proteomics analyses were done for strain BuS5 cultures grown with propane or butane. For each growth substrate, cells from 30-ml culture volumes were harvested by centrifugation (16 000*g*, 4°C) and suspended in 30 μl ammonium bicarbonate buffer (50 mM). Cell lysis was achieved through three cycles of freezing in liquid nitrogen (−196°C) and thawing on a water bath (37°C). Protein extracts were reduced with dithiothreitol (50 mM; incubation for 1 h at 30°C), alkylated with iodoacetamide (200 mM; incubation for 1 h at room temperature) and digested with trypsin (0.6 μg; incubation for 12 h at 37°C) (Yang *et al*., 2015). The resulting peptides were desalted using ZipTip-C18 columns (Millipore) and analysed by an LTQ-Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled with a nanoUPLC system (nanoAquity, Waters) (Chen *et al*., 2019).

The acquired MS/MS spectra were searched against the complete genome of *Desulfosarcina* sp. BuS5 using the Sequest HT and Amanda algorithm implemented in Proteome Discoverer (v2.4, Thermo Fisher Scientific). Mass tolerance of 3 ppm and 0.1 Da were allowed for precursor- and fragment-ion respectively. Oxidation at methionine was selected as variable modifications and carbamidomethylation at cysteines as static modifications. Peptides and proteins were identified at a false discovery rate of 0.05. Precursor-ion intensity of peptides was used to calculate the relative abundance of proteins as previously described (Chen *et al*., 2019).

Phylogenetic analyses of AssA/MasD

Homologues longer than 500 amino acids of the alpha subunit of methyl-(alkylsuccinate)synthase (AssA/MasD), benzylsuccinate synthase (BssA), naphtylmethylsuccinate synthase (Nms) and pyruvate formate lyase (Pfl) were downloaded from the UniProt database. The retrieved protein sequences together with MasD of strain BuS5 were aligned by MAFFT v7.310 with L-INS-I mode (Katoh and Standley, 2013). Low-quality regions of the alignment were removed by TrimAl with option *-automated1* (Capella-Gutierrez *et al*., 2009). A maximum-likelihood tree was calculated using RAxML v8.2.9 under PROTGAMMALG mode (Stamatakis, 2014), which is the best-fitting condition according to both Akaian and Bayesian information criterion implemented in RAxML. A number of 100 bootstrap replicates were applied to calculate the support value for branches of the phylogenetic tree.

Comparative genome analyses

The genomic potential of strain BuS5 was compared to 12 cultivated species within the family *Desulfobacteraceae*. These include *Desulfococcus multivorans* (Dorries *et al*., 2016), *Desulfococcus oleovorans* Hxd3 (Aeckersberg *et al*., 1991), *Desulfosarcina cetonica* JCM 12296 (Galushko and Rozanova, 1991), *Desulfosarcina alkanivorans* (Watanabe *et al*., 2017), *Desulfosarcina widdelii* (Watanabe *et al*., 2017), *Desulfosarcina ovata* subsp. *sediminis* (Watanabe *et al*., 2020), *Desulfosarcina ovata* subsp. *ovata* (Harms *et al*., 1999), *Desulfatibacillum aliphaticivorans* AK-01 (So and Young, 1999), *Desulfatibacillum aliphaticivorans* DSM 15576 (Cravo-Laureau *et al*., 2004), *Desulfobacula toluolica* Tol2 (Wohlbrand *et al*., 2013), *Desulfobacter hydrogenophilus* (Marietou *et al*., 2020) and *Desulfobacter postgatei* 2ac9 (Widdel and Pfennig, 1981). The microbial genomes were downloaded from the NCBI Assembly database, and re-annotated with KEGG and PfamA (database release 33) using the GhostKoala tool

(Kanehisa *et al*., 2017) and pfam_scan.pl (Mistry *et al*., 2021) respectively. Key genes involved in the metabolism of H_2, formate, alcohols, fatty acids, lactate, dicarboxylic acids (e.g. fumarate, succinate, or malate) and benzoate were retrieved from genomes based on their KEGG Orthology number. Additionally, a more sensitive, Hidden Markov Model (HMM) based method was used to identify homologues of formate dehydrogenase (alpha subunit), acyl-CoA synthetase, acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, acetyl-CoA acetyltransferase, iron-containing alcohol dehydrogenase and tripartite ATP-independent periplasmic transporters. HMM profiles downloaded from the PFAM database and/or the TIGRFAMs database were queried against genomes using *hmmsearch* program (HMMER v3.2) with model-specific cutoff (--cut_ga option) (Mistry *et al*., 2013). The retrieved homologues were further inspected and curated based on domain structure. Genes identified by KO and PFAM analyses were populated into a gene count table, and visualized as bubble plot using the ggplot2 package in R software (v3.6). To infer phylogenetic relationships of the SRB, 122 bacteria-specific marker genes were identified from each species and aligned using the gtdbtk program (Chaumeil *et al*., 2020). A maximum-likelihood tree was calculated from a concatenated alignment of marker proteins using RAxML (v8.2.9) with PROTGAMMALG mode (Stamatakis, 2014). The RAxML inference involved 149 bootstrap analyses according to extended major rule-based bootstopping criterion.

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Supporting Information

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

Appendix S1: Supporting Information.