Interspecies Mobility of Organohalide Respiration Gene Clusters Enables Genetic Bioaugmentation

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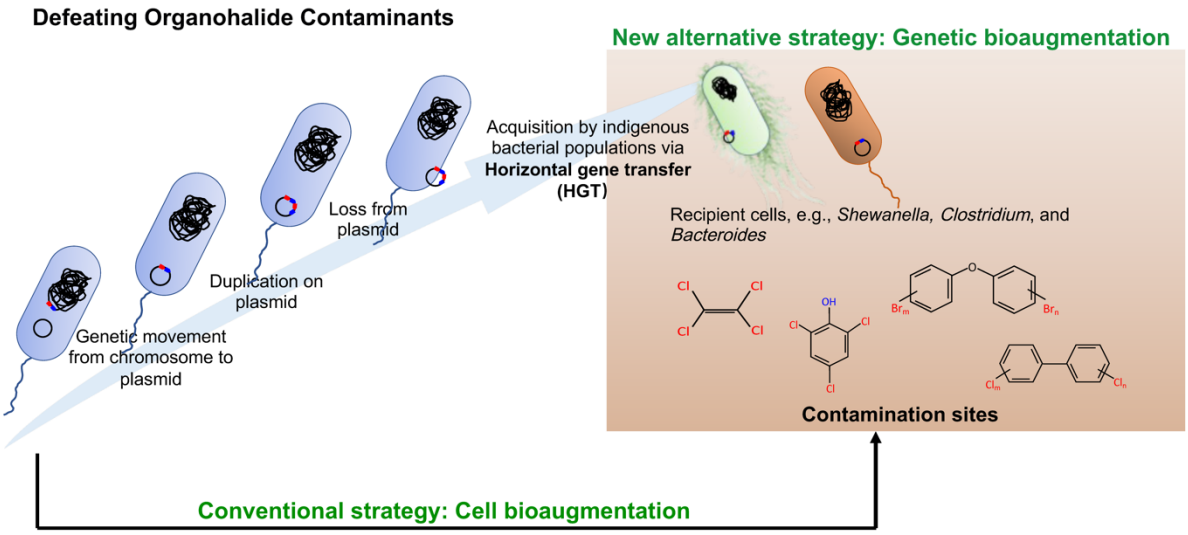
ABSTRACT

Anthropogenic organohalide pollutants poses a severe threat to public health and ecosystems. *In situ* bioremediation using organohalide respiring bacteria (OHRB) offers an environmental-friendly and cost-efficient strategy for decontaminating organohalide-polluted sites. The genomic structures of many OHRB suggest that dehalogenation traits can be horizontally transferred among microbial populations but its occurrence among anaerobic OHRB has not yet been demonstrated experimentally. This study isolates and characterizes a novel tetrachloroethene (PCE)-dechlorinating *Sulfurospirillum sp*. strain SP, distinguishing itself among anaerobic OHRB through showcasing a mechanism essential for horizontal dissemination of reductive dehalogenation capabilities within microbial populations. Its genetic characterization identifies a unique plasmid (pSULSP), harboring reductive dehalogenase and *de novo* corrinoid biosynthesis operons - functions critical to organohalide respiration, flanked by mobile elements. The active mobility of these elements was demonstrated through genetic analyses of spontaneously emerged non-dehalogenating variants of strain SP. More importantly, bioaugmentation of non-dehalogenating microcosms with pSULSP DNA triggered anaerobic PCE dechlorination in taxonomically diverse bacterial populations. Our results directly support the hypothesis that exposure to anthropogenic organohalide pollutants can drive the emergence of dehalogenating microbial populations via horizontal gene transfer and demonstrate a mechanism by which genetic bioaugmentation for remediation of organohalide pollutants could be achieved in anaerobic environments.

KEYWORDS:

Sulfurospirillum, organohalide respiring bacteria, reductive dehalogenation, horizontal gene transfer, genetic bioaugmentation

SYNOPSIS: Acquisition of reductive dehalogenase genes and the underlying genetic mechanisms contribute to developing novel strategy to remediate sites contaminated with organohalide pollutants via genetic bioaugmentation.



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INTRODUCTION

Historic industrial and commercial use of halogenated organic compounds has resulted in widespread environmental contamination.[1, 2] Many organohalide pollutants are resistant to environmental attenuation and accumulate in subsurface anaerobic environments where they may persist for decades. [3] Although the manufacture and use of many organohalides have been largely restricted due to their potential toxicity and tendency for bioaccumulation,[4] the persistence of legacy pollution presents an ongoing challenge to impacted ecosystems and human populations.[1, 5-7] After the discovery of microbial reductive dehalogenation in the 1980s,[8] a group of researchers have contributed to a growing understanding of organohalide respiring bacteria (OHRB) and identified bacterial enrichments and isolates capable of dehalogenating diverse persistent organohalide pollutants.[9-19] Field-scale trials have demonstrated the environmental and economic benefits of *in situ* bioremediation for chlorinated solvents in contaminated groundwaters by bioaugmentation with an array of dehalogenating microbial populations.[20-23] Yet, the development of viable microbial consortia for remediation of organohalide pollutants has progressed slowly, with a limited number of platforms currently available for commercial application. Further, the efficacy of dehalogenating cultures used for *in situ* remediation could also be potentially hindered by inhospitable environmental conditions, since some OHRB are notoriously fastidious and may struggle to gain a foothold among indigenous microbiota when subjected to suboptimal growth conditions (e.g., acidity[20, 24], presence of multiple dissimilar organohalide pollutants,[25] and elevated concentrations of toxic organohalides[26]) and resource competition with more metabolically versatile bacteria.[27, 28]

Genetic bioaugmentation seeks to achieve bioremediation goals by introducing and propagating the genetic potential for organohalide respiration (OHR) into the genetic complement of the indigenous microbiome of contaminated environments through horizontal gene transfer. Genetic bioaugmentation, which is considerably less studied than bioaugmentation with live cells, requires introduction of one or more reductive dehalogenases (RDase; enzymes that catalyse removal of halogens from organohalide compounds) into contaminated ecosystems. [29-31] Plasmid-mediated transfer of biodegradation of xenobiotics via mechanisms other than OHR has been observed in aerobic systems,[30, 32] although the mechanisms underlying the propagation of the phenotype within microbial communities and potential environmental consequences remain unclear. Genetic bioaugmentation of anaerobic microbiota has not been reported, though there is compelling genomic evidence that reductive dehalogenase homologous genes (*rdh*), which encode RDases, have historically been transferred among the genomes of some anaerobic OHRB.[33-35] However, attempts to induce and decipher the horizontal transfer of *rdh* have thus far been unsuccessful,[36] and the lack of model strains and genetic systems have hindered investigations into the mechanisms that mediate transfer of *rdh* gene clusters among anaerobic microbiota. Furthermore, potential delivery systems (e.g., mobile extrachromosomal genetic elements) must be identified before progress can be made towards development of genetic bioaugmentation technologies for *in situ* remediation of anaerobic environments contaminated by persistent organohalide pollutants.

This study describes transposon-mediated duplication, loss, and acquisition of plasmid - borne *rdh* and corrinoid biosynthesis gene clusters in a newly discovered *Sulfurospirillum* sp. strain SP, providing direct evidence and insight into naturally occurring genetic mechanisms that contribute to the distribution of OHR genes in the environment. The acquisition of an OHR

phenotype by non-dechlorinating bacterial population in environmental samples demonstrated the successful genetic bioaugmentation of non-dehalogenating anaerobic microbial communities, representing a pioneering step in the development of genetic bioaugmentation technologies and demonstrating a potential system for targeted delivery of the genetic capacity for OHR.

MATERIALS AND METHODS

Cultivation and Chemical Analyses

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany) at the highest purity available unless specified otherwise. A microcosm exhibiting PCE-dechlorinating capability was established with anaerobic sludge from a domestic wastewater treatment plant in a city located in Asia, and further enriched via consecutive 5% vol:vol subculturing. Cultures were grown in anaerobic serum bottles containing a bicarbonate-buffered mineral salts medium (DCB1) prepared as previously described;[37] cultures were amended with lactate as the sole carbon source and electron donor and PCE as the sole electron acceptor. All cultures and microcosms were incubated at 30 °C in the dark without shaking. Chloroethenes were measured using an Agilent gas chromatograph (GC7890) equipped with a flame ionization detector and a DB-5 column (10 m × 0.25 mm ×0.25 μm; J&W Scientific USA) via manual injection of headspace samples as previously described.[26] The temperature program was initiated at 50 ºC for 1 min, increased to 120 ºC at 15 ºC /min and held for 1 min.

Isolation and Characterization of a PCE-dechlorinating Microbial Population

A PCE-dechlorinating population in the sub-cultured enrichment was isolated by three iterations of serial dilution-to-extinction (10^{-1}–10^{-7}) in 20 ml serum bottles containing 10 ml

DCB1 medium spiked with 1 mM PCE. Colonies were selected from the lowest dilution and streaked onto DCB1 agar plates amended with 10 mM pyruvate and 1 mM PCE in an anaerobic chamber. After a two-week growth period, 10 colonies were picked and re-streaked onto fresh DCB1 agar plates to verify uniformity. Dechlorination of PCE to trichloroethene (TCE) was observed in colonies obtained from 3 of the 10 agar plates. The metabolic versatility of the isolated populations was evaluated to quantify the cell growth and to detect metabolites after amending cultures with either elemental sulfur, thiosulfate, sulfite, sulfate, nitrite, or nitrate (all 10 mM) as potential electron acceptors, with hydrogen or formate (10 mM) as potential electron donors, and with pyruvate, lactate (10 mM), or fumarate (all 10 mM) as potential carbon sources for fermentation. Cell growth was measured using real-time quantitative PCR as described below and metabolites were detected using ion chromatography (IC) and high-performance liquid chromatography (HPLC) as previously described.[38]

Emergence and Characterization of Non-PCE-dechlorinating Variants

Six replicate cultures of *Sulfurospirillum* sp. strain SP (designated as SP-1 to -6) were established concurrently in 20 ml serum bottles containing 10 ml DCB1 medium amended with 10 mM pyruvate but without addition of PCE. These six replicate cultures were sub-cultured (10% vol:vol) once each week over 12 weeks (designated as G1-G12). For example, SP-1-G2 indicates replicate 1 in 2nd subculturing. PCE dechlorination was assayed in each culture series (G0, G3, G5, G7, G9, and G11).

Molecular Analysis

Real-time quantitative PCR (qPCR) analysis. Cultures were grown in 160 ml serum bottles containing 100 ml DCB1 medium in triplicate. Genomic DNA (gDNA) was extracted

from 1 ml samples using the DNeasy Blood and Tissue Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. Cell growth was determined by qPCR targeting markers for all *Sulfurospirillum* (16S rRNA gene, primer pair Sulfur114F/421R),[39] *Sulfurospirillum* sp. strain SP (*pceA*, primer pair SulPceA-786F/833R designed for this study, Table S1), and total bacteria (16S rRNA gene, primer pair 338F/518R).[40] qPCR was conducted on an ABI 7500 Fast System using the SYBR green reporter (SensiFAST™ SYBR Lo-ROX Kit, BIOLINE, London, UK).[41, 42] The thermal cycler program for all primer pairs was 95 ºC for 3 min, followed by 40 cycles of 94 ºC for 15 s, 55–60 ºC for 30 s, depending on the annealing temperature of the primer pair, and 72 ºC for 10s followed by a final extension of 30s at 72 ºC after completion of last cycle. Target amplicons were quantified against a standard curve established from serial dilutions of known concentrations of plasmids carrying the target amplicon constructed using the pGEM-T Easy Vector system (Promega, Madison, WI, USA) according to the manufacturer's instruction and extracted using the QIAprep Spin Miniprep kit (QIAGEN® GmbH, Hilden, Germany).

Transcriptional analysis. Expression of *pceA* and 16S rRNA genes in *Sulfurospirillum* sp. strain SP were determined by reverse-transcription qPCR (RT-qPCR), as previously described.[43] Briefly, 1 ml samples were collected for RNA extraction at defined time points during cultivation. Samples were concentrated by centrifugation (12,000 × g, 10 min) and resuspended in TRIzol (150 µL; ThermoFisher Scientific, Waltham, MA, USA) before storage at -80 ºC for later processing. RNA was extracted and reverse transcribed to cDNA using the QIAGEN RNeasy and QuantiTect Reverse Transcription kits according to the manufacturer's instructions. Defined concentrations of luciferase control RNA (Promega) were added to samples before RNA extraction as an internal standard to normalize RNA loss during extraction.

Genome Sequencing and Comparative Analyses

Sulfurospirillum sp. strain SP and subcultures were harvested for gDNA extraction using the Qiagen Genomic-Tip 100/G (Qiagen, GmbH, Hilden, Germany). The quality and concentration of the extracted gDNA were assessed by Qubit and the availability of large DNA fragments (>25 kbp; required for the generation of PacBio CLR libraries) was determined by electrophoresis. Whole genome shotgun sequencing of gDNA from *Sulfurospirillum sp.* strain SP and subcultures was performed on an Illumina NovaSeq6000 platform by NovogeneAIT Genomics, Singapore. A PacBio SMRT library of gDNA from *Sulfurospirillum sp.* strain SP was prepared and sequenced on a PacBio Sequel system by the Beijing Genomics Institute (Beijing, China).

De novo assembly of the *Sulfurospirillum sp.* strain SP genome from the PacBio CLR subreads (135,158 reads; mean length 8,536 bp; N50 8,536 bp) and Illumina reads (11,909,473 reads; 150 bp) was done using both the St. Petersburg genome assembler (SPAdes v3.15.2)[44] and Canu v2.2[45]. The draft assemblies were polished using Pilon v1.24 and contigs were scaffolded using SSPACE-LongRead;[46] polished scaffolds from the two assemblers were reconciled with Metassembler v1.5.[47] The assembled genome of *Sulfurospirillum sp.* strain SP comprised two circular elements of 2,675,374 and 200,597 bp. ORFs were identified in the two circular elements using Prodigal v2.6.3 and translated CDS were annotated against the Swiss-Prot[48] and NCBI RefSeq[49] databases using BLAST v2.13.0+; rRNAs and tRNAs were identified in the sequence using RNAmmer v1.2[50] and tRNAscan-SE v2.0.12,[51] respectively. CRISPR and putative phage elements were identified using the CRISPRCasFinder online tool[52] and the PHAST web tool,[53] respectively. Illumina reads from the non-dechlorinating variants in subcultures were mapped to the assembled *Sulfurospirillum sp.* strain SP genome with bowtie2

v2.4.5;[54] calling and visualization of variants (i.e., SNPs and indels) was done with BCFtools v1.14.[55]

Average nucleotide identity (ANI) and tetrad analyses were used to compare the assembled genome of *Sulfurospirillum sp.* strain SP with representative publicly available *Sulfurospirillum* genomes using the JSpecies web server.[56] A 16S rRNA gene sequence of *Sulfurospirillum sp.* strain SP was aligned with 16S rRNA gene sequences of other *Sulfurospirillum* mined from Genbank using Muscle v5[57] and a neighbor-joining phylogeny supported by 1000 bootstrap replicates was inferred using MEGA11.[58]

Genetic Bioaugmentation with Extrachromosomal Genetic Elements of Strain SP

Extrachromosomal genetic elements were extracted from PCE-dechlorinating cultures of *Sulfurospirillum sp.* strain SP using a standard alkaline lysis-based protocol, which allows for recovery of larger intact fragments and increases the likelihood of obtaining circular plasmid DNA from the extraction. Briefly, 1 ml samples were collected from cultures after complete dechlorination of PCE and cells were harvested by centrifugation. Cell pellets were resuspended in 180 µl Tris-buffered EDTA and lysed by addition of 180 µl lysis buffer (NaOH and SDS) and incubation at 50 ºC for 10 min. The lysed preparation was neutralized with 3 M KAc and centrifuged at 12,000 × g for 10 min at 4 °C. The top layer containing the DNA was extracted and mixed gently with an equal volume of isopropanol. Precipitated DNA was collected by centrifugation and washed twice with 70% ethanol before resuspension in 100 µL Tris-buffered EDTA. Extracted DNA was quantified on a Nanodrop 2000 UV Visible Spectrophotometer (ThermoFisher Scientific) and visualized on a 1% agarose gel.

Genetic bioaugmentation was performed by amending extrachromosomal DNA extracted from *Sulfurospirillum sp.* strain SP to a series of environmental enrichment cultures. Fourteen non-PCE dechlorinating, enrichment cultures sub-cultured from microcosms established from soils and sediments collected at geographically distinct sites were selected for genetic bioaugmentation. The PCE dechlorinating capability of these fourteen enrichment cultures were assessed in two separate batches (with triplicates in each batch) over a three-month cultivation period. Each enrichment was inoculated (10% vol:vol) into 60 ml serum vials containing 30 ml DCB1 medium amended with 0.6 mM PCE and 10 mM lactate. DNA extracted from *Sulfurospirillum sp.* strain SP was amended to a final abundance of 1×10^5 *pceA* copies /ml, comparable to the empirically determined abundance of *pceA* in *Sulfurospirillum sp.* strain SP cultures after complete dechlorination of PCE. Biological controls were established by amending cultures with a corresponding volume of sterilized water. Abiotic controls were established in DCB1 medium amended with 0.6 mM PCE, 10 mM lactate and augmented with DNA extracted from *Sulfurospirillum sp.* strain SP but without addition of live bacterial cells. All cultures were established in triplicate (i.e., 9 cultures for each environment: 3 abiotic controls, 3 negative biological controls and 3 experimental cultures). Experimental cultures exhibiting PCE dechlorination activity that differed from respective biological controls were selected for further investigation; the biological control cultures from selected enrichments were used as the inoculum for subsequent studies. Four genes, Sul-*pceA*, transposase IS21(*istA*), replicate initiating gene (*rep*), and the *Sulfurospirillum* 16S rRNA gene (Sul-16S) were used to investigate the structure of putative horizontally transferred genetic elements. All primers used in this study are described in Table S1.

Data Availability

The assembled and annotated *Sulfurospirillum sp.* strain SP chromosome and plasmid (pSULSP) have been deposited in NCBI under BioProject PRJNA927133 and Genbank under accession number CP118374.

RESULTS

Metabolic Versatility of a Novel *Sulfurospirillum* Strain Capable of Dechlorinating PCE

A PCE-dechlorinating bacterium was isolated from an anaerobic sludge sample collected at a domestic wastewater treatment plant. Sequencing of the only sequence present in a 16S rRNA gene clone library identified the isolate as a member of the genus *Sulfurospirillum*, which was dubbed as *Sulfurospirillum* sp. strain SP. When provided pyruvate as the sole electron donor, the isolate dechlorinated 0.6 mM PCE to TCE within 3 d with an estimated doubling time of 6.8 h (Figure 1a). In contrast to the exclusive production of *cis*-DCE as the end-products of PCE dechlorination in other reported *Sulfurospirillum* strains, considerable amount (0.18 mM) of *trans*-DCEs was generated after depletion of PCE on day 4. TCE was gradually dechlorinated to *trans*- and *cis*-DCE after three months and eventually reaching a *trans*-DCE/*cis*-DCE ratio of 1.76. The ability of strain SP to convert PCE predominantly to *trans*-DCE is unique in organohalide-respiring *Sulfurospirillum* strains, which has been exclusively ascribed to members of *Dehalococcoides* in previous studies.[16, 59]

Like other members of *Sulfurospirillum*, strain SP exhibited facultative microaerophilic growth when oxygen (5% vol:vol) was present in the headspace of cultivation vials. Under anaerobic conditions, the isolate exhibited metabolic flexibility, being able to utilise a variety of

electron acceptors other than PCE, including elemental sulfur, thiosulfate, sulfite, sulfate, nitrite, and nitrate, as well as multiple electron donors, including hydrogen, pyruvate, lactate, formate, and acetate (Figure S1). Fermentative growth was also supported by pyruvate and lactate. Similar to other members within this genus, strain SP was able to dechlorinate PCE when cultivated without exogenous amendment of cobalamin, an essential co-factor for anaerobic OHR, indicating endogenous production of this cofactor. The demonstrated metabolic versatility of the newly isolated *Sulfurospirillum* sp. strain SP may facilitate its proliferation and involvement in halogen cycling in a variety of ecosystems.

Unique Genomic Characteristics of the Novel PCE-dechlorinating *Sulfurospirillum* Strain

To gain more genomic insights, the genome of strain SP was assembled using a hybrid assembly approach incorporating PacBio CLR and Illumina Novaseq 6000 reads. The assembled genome comprised a 2,676 kbp chromosome and a 201 kbp circular extrachromosomal element. The chromosome and plasmid (pSULSP) have GC content of 37.64 and 35.59%, respectively, and have 2,731 and 223 coding sequences, respectively (Table S2). The chromosome encodes 50 tRNA and contains four rRNA operons; no rRNAs or tRNAs are encoded in the plasmid sequence. A phylogeny constructed using the sequence of the three identical 16S rRNA genes in the assembled genome places strain SP within a cluster of *Sulfurospirillum* sp. strains 11S05485, UCH001, and RPFA-1 (Figure 1b), all of which share greater than 99% similarity. The members of this cluster have not been formally classed *Sulfurospirillum* and, other than strain SP, have not been reported to dehalogenate organohalides. The phylogenetic placement of the strain SP was supported by ANI analysis, revealing a 97.26% similarity with strain UCH001 and <90% with other strains from *Sulfurospirillum* (Figure S2). Based on these data, the isolated PCE-

dechlorinating bacterium was putatively identified within this unknown species in the *Sulfurospirillum* genus.

A distinctive attribute of *Sulfurospirillum* sp. strain SP is its possession of the substantial extrachromosomal element measuring 201 kbp (Figure 2a, Table S3). This plasmid stands out as the largest among the infrequently reported plasmids in OHRB-specifically, only *Geobacter lovleyi* SZ, *Sulfurospirillum* sp. ACSTCE and ACSDCE, with sizes ranging from 38.0 to 77.1 kbp, and encompassing a remarkable range within universal bacterial populations.[60, 61] The plasmid sequence contains a replication origin, replication initiation protein (SULFSP-027470), and parA/B (SULFSP-027470, SULFSP-027480), suggesting that the plasmid is self-replicating and heritable. Uniquely and for the first time, this plasmid contains core gene clusters necessary for OHR of PCE, both the *pceAB* and *de novo* corrinoid synthesis gene clusters (Figure 2, Figure S3). The *pceAB* gene clusters in all previously reported organohalide-respiring *Sulfurospirillum* are characteristically clustered on the chromosome and comprise two distinct *rdhA* sequences, one PCE RDase encoding gene (*pceA*) and one functionally uncharacterized *rdh* that encodes a protein with ~70% amino acid sequence identity to PceA. The OHR gene clusters on pSULSP of strain SP are arranged similarly to those on chromosome of other PCE-dechlorinating *Sulfurospirillum*, providing genetic evidence of capabilities in both PCE dechlorination and *de novo* corrinoid synthesis in strain SP (Figure 2b, Figure S3). [62, 63] However, the duplication of the complete OHR gene clusters evident on the plasmid in strain SP is also unique among currently reported organohalide-respiring *Sulfurospirillum* or any other OHRB.

The majority of the CDS on the plasmid sequence of strain SP are genetic elements associated with cellular defence and resistance to invasion by foreign genetic materials (i.e., CRISPR, toxin-antitoxin systems, and metal-responsive repression) as well as environmental

adaptation (i.e., transposes, integrases, recombinases). A 3.7 kbp CRISPR region on the plasmid contains 56 spacers separated by direct repeats of 30 bp. Seven type IB *cas* genes (SULFSP-027460, -029650, -029660, -029670, and -029680) are located downstream of the CRISPR region. The large number of spacers in this region is unusual for a genome in a member of the ε-Proteobacteria, which typically feature CRISPR regions with fewer than 10 spacers.[64] Similarly high numbers of spacers have been reported in other *Sulfurospirillum* [65] and the large number of spacers may indicate that strain SP has been attacked by different types of phage, which was also suggested by 64 inactive and one active phage candidates in the genome (Table S4). The genome of strain SP encodes six different categories of toxin-antitoxin system in 14 distinct clusters located on both the plasmid and chromosome. Almost all (13 of 14) of the toxin-antitoxin genes are located near indicators of genetic mobility (i.e., transposase, recombinases and integrases), suggesting that these systems may be involved in stabilisation and fitness of the mobile elements. The *Sulfurospirillum sp.* strain SP genome encodes a total of 92 transposases, 20 of which are present on the plasmid sequence (Table S5).

Transposon-mediated Mobility and Duplication of Gene Clusters Required for Organohalide Respiration

The presence of multiple direct and inverted repeats and mobile genetic elements flanking the OHR gene clusters on pSULSP provide putative evidence of mobility and horizontal transfer. The OHR gene clusters are flanked on both sides by two identical DDE IS21 transposases for insertion sequence IS408 with 12 bp terminal inverted repeats [TGAT(A)TTAGGCTG] and 5 bp flanking direct repeats [CG(C)GTT]. The terminal inverted and direct repeats are highly conserved, each with only 1 bp difference. The IS21 family DDE transposase is composed of an *istA* transposase (SULFSP-028100, -028640, -028660, and -

029200), for reactive junction pathways, and co-integrase *istB* (SULFSP-028110, -028650, -028670, and -029210), which has an ATP-binding motif that is thought to promote integration efficiency.[66] The IS21-408 sequence does not share a high degree of homology with other IS21 elements in *Sulfurospirillum* genomes; its closest affiliate belongs to an identified genome in the genus *Sulfurovum* (65.12% similarity, Figure 3a, Table S5). Like the IS21-408 sequence, the nearest phylogenetic neighbor of the plasmid pSULSP replicate initiation sequence (SULFSP-027470) is not derived from a *Sulfurospirillum* genome (Figure 3b, Table S6).

Initially identified following reconstruction of the strain SP genome, the presence of the duplicated region was also verified by sequencing PCR amplicons generated using a series of primers spanning different regions of the duplication (Figure S4a) and by identification of contiguous PacBio CLRs spanning portions of the duplicated region (Figure S4b). The transposon-flanked, duplicated OHR regions account for nearly half (43.2%) of the entire length of plasmid pSULSP. While the events that led to the duplication of this region on an extrachromosomal element remain unclear, the presence on the chromosome of an additional IS21 element with inverted repeats and flanking directs repeats identical to those of the IS21 elements on pSULSP may be an indication of past chromosomal excision (Figure S5). The genetic composition and structure of the OHR regions suggest that at least some portion of the plasmid pSULSP sequence may have been acquired through horizontal transfer and could indicate the involvement of IS21 transposable elements in the mobility of the OHR gene clusters in *Sulfurospirillum sp.* strain SP.

To further experimentally test the mobility of the OHR gene clusters, strain SP was continuously sub-cultured in growth medium without PCE. Strikingly, exclusion of PCE from the growth medium resulted in the emergence of non-dechlorinating variants of *Sulfurospirillum*

sp. strain SP within 4 weeks, colonies of which identified as *Sulfurospirillum* sp. failed to dechlorinate PCE after two weeks incubation (Figure S6). Factors contributing to the emergence of the non-dechlorinating variants were investigated by establishing six parallel cultures of strain SP in medium without PCE amendment. Each replicate culture of strain SP was transferred in parallel to fresh medium without PCE amendment once each week over 12 weeks. PCE dechlorination by cells extracted from the subcultures were assayed weekly. Five of the six replicate cultures had ceased to dechlorinate PCE by transfers 10-12 (Figure 4a). Whole genome shotgun sequencing of the five non-dechlorinating variants found similar deletions in a 19.6 kbp region on the chromosome (Figure S7) and two 40.6 kbp regions on the plasmid in the different variants (Figure 4b). The culture that retained PCE dechlorination activity (SP-4-G12) was genetically identical to the original culture. The deletion of the duplicated 40.6 kbp region of pSULSP, which encodes both the reductive dehalogenase and corrinoid biosynthesis gene clusters, was likely responsible for the inability of SP-1-G11, -2-G10, -3-G10, -5-G11 and -6-G12 to dechlorinate PCE. Spontaneous deletion of these regions could explain the irregular read coverage depth of the pSULSP sequence observed during the initial assembly of the genome of strain SP (Figure 2a). Notably, the regions of pSULSP that are absent from the non-dechlorinating variants of *Sulfurospirillum* sp. strain SP are enclosed by a transposable genetic element that is flanked by duplicated IS sequences (Figure 4b, Figure S8). All non-dechlorinating variants harbored similar deletions on the plasmid and chromosome, except for SP-5-G11, which did not have the chromosomal deletion. These findings collectively suggested that the OHR gene cluster in *Sulfurospirillum* sp. strain SP is mobile, which could be attributed to the presence of the transposable genetic element on the plasmid.

Acquisition of *pceA*-mediated PCE Dechlorination by Genetic Bioaugmentation

The validated mobility of OHR gene clusters on pSULSP in a short time promotes the hypothesis that augmentation of the mobile genetic elements might be an alternative approach to conventional bioaugmentation strategy using live cells. Therefore, genetic bioaugmentation of non-dechlorinating microcosms was attempted to investigate whether the OHR gene clusters could be transferred to other *Sulfurospirillum* populations or bacterial lineages in mixed microbial communities to enhance microbial reductive dechlorination of PCE. Extrachromosomal DNA was extracted from strain SP and spiked (10^4–10^5 copies /ml) into 14 enrichment cultures whose microbial communities contained no PCE-dechlorinating *Sulfurospirillum* (tested by PCR and quantitative PCR analysis using *Sulfurospirillum*-specific primers and *Sulfurospirillum pceA* primers). One (i.e., EN-11) of these 14 non-dechlorinating, enrichment cultures showed significantly enhanced PCE dechlorination following genetic augmentation and incubation for 2 weeks, which exhibited complete dechlorination of PCE to TCE (Figure 5a). EN-11 was previously shown to be dominated by a *Dehalogenimonas* population capable of debrominating polybrominated diphenyl ethers but had shown no PCE dechlorination after incubation for 3 months in two separate trials (Figure S9). No PCE dechlorination was observed in control cultures of EN-11 not amended with DNA from strain SP. A supplemental amendment of PCE (0.6 mM; day 21) following complete dechlorination of the initial PCE dose was also efficiently dechlorinated although at a slower rate than the initial dose (0.014 and 0.043 mM Cl^- released /d, respectively; Figure 5b). The number of copies of *pceA* in DNA extracted from the genetically augmented EN-11 culture varied by an order of magnitude during cultivation (3.43×10^4–6.32×10^5 copies /ml; Figure 5c). Transcription of *pceA* was not detected during dechlorination of the initial dose of PCE, likely because all PCE had

been completely dechlorinated before cells were harvested for mRNA quantitation. Nevertheless, *pceA* transcripts were detected at 2.20±0.46 $\times 10^7$ copies /ml and 6.24±0.56 $\times 10^7$ copies /ml during active dechlorination of the supplementary PCE amendment (days 28 and 42) but were below the detection limit again by day 56 when PCE dechlorination had ceased. Transcription of *pceA* during PCE dechlorination suggests that *pceA* was involved in, if not solely responsible for, the observed PCE dechlorination in EN-11. These results also indicate that the mobile gene clusters containing *pceA* on pSULSP have been successfully acquired by native microbes in the microbial communities.

To identify the bacterial population(s) in culture EN-11 responsible for acquiring and expressing the *pceA* detected during PCE dechlorination, culture EN-11 was spread anaerobically onto solid DCB1 medium agar plate containing PCE. DNA extracted from colonies arising on these plates after 3 d was assayed for the presence of genes associated with pSULSP (i.e., *pceA*, *dnaA*, *istA*) by end-point PCR using gene-specific primers (Table S1). Evidence of three gene sequences associated with pSULSP was found in 5 of 72 colonies identified as members of the genera *Shewanella*, *Clostridium* and *Bacteroides* via 16S rRNA gene sequencing (Figure S10a). Among these five colonies, One *Shewanella* colony harbored all three of the genes associated with pSULSP and did not contain any *Sulfurospirillum* 16S rRNA genes (Figure 5d), suggesting acquisition of the augmented DNA. The remaining colonies each lacked one or more of the three genes tested (Figure S10b). The presence of both *dnaA* and *ist* in the *Shewanella* colony that contained *pceA* suggests the presence of a replicating form of the plasmid rather than the transposable OHR gene cassettes alone, but further study is needed to determine factors that contribute to the maintenance and loss of this extrachromosomal element in this lineage.

DISCUSSION

We report the discovery of a novel PCE-dechlorinating *Sulfurospirillum* sp. strain SP which is distinguished from other known PCE-dechlorinating *Sulfurospirillum* or any other known OHRB by the presence of duplicated active composite transposons containing complete OHR gene clusters on a large extrachromosomal genetic element. The *rdh* and *de novo* corrinoid biosynthesis gene clusters on the plasmid are flanked by mobile genetic elements, comprising an active composite transposable element that could have contributed to the duplication of the OHR gene clusters on pSULSP and the emergence of non-dechlorinating variants of strain SP when cultivated in the absence of PCE. Leveraging the innate mobility of the *rdh* on pSULSP, we demonstrated the genesis of PCE dechlorination in a non-dehalogenating environmental microcosm by augmentation with DNA extracted from strain SP. Molecular analyses confirmed that PCE dehalogenation was mediated by the acquisition and expression of the *pceA* gene from pSULSP by a specific bacterial population in the microcosm, providing the first direct evidence of functional lateral transfer of a *rdh* gene cluster and demonstrating a mechanism by which the capacity for OHR can be shuttled among diverse bacterial genera in anaerobic systems. Our results provide long-sought evidence supporting the widely held hypothesis that exposure to novel anthropogenic organohalide pollutants in the environment can drive the emergence of dehalogenating microbial populations via the horizontal transfer of genetic elements.

The complete duplication of the OHR gene cluster on a large extrachromosomal element of strain SP is not present in the published genomes of any other organohalide-respiring *Sulfurospirillum* or any other OHRB. The events that led to the duplication of an ~50 kb region on the plasmid remain unclear, although such structures can arise via transposon-mediated spontaneous tandem duplication.[67] An explicit understanding of the mechanisms underlying the

apparent mobility of the OHR gene clusters on plasmid pSULSP will require further study. Nonetheless, the demonstrated utility of this naturally arising phenomenon for genetic bioaugmentation provides valuable insight into the roles of duplication and transposon-mediated transfer of genes among OHRB. Previous studies have reported how environmental conditions, such as cultivation without suitable organohalide substrates, can give rise to phenotypic variants through spontaneous[68] and transposon-mediated[69, 70] deletions of chromosomal regions in phylogenetically distinct OHRB. The transposon-mediated deletion causing loss of PCE dechlorination in *Desulfitobacterium* TCE1 and Y51 is comparable to that observed in *Sulfurospirillum* strain SP cultivated without PCE, suggesting a mechanistic similarity underlying the emergence of phenotypic variation in disparate OHRB.[69, 70] Although different families of transposases flank the OHR operons in each genus (i.e., IS21 in *Sulfurospirillum* sp. SP and IS256 in *Desulfitobacterium* TCE1 and Y51), the OHR-bearing transposons in *Sulfurospirillum* strain SP and *Desulfitobacterium* strains TCE1 and Y51 are each flanked by tandem schemes of insertion sequences, which can increase the efficiency and frequency of transposition[71] and may contribute to the observed genetic response to the absence of organohalide compounds in growth medium (Figure S5a & S5b). It is likely that the structure of the transposases flanking the OHR cassettes in strain SP explains the emergence of non-dechlorinating variants after prolonged cultivation without PCE. Further evidence for this hypothesis can be found in the stability of the genes proximal to the lone *IS21* element on plasmid pSULSP that is not flanked by a tandem scheme of *IS21* elements (Figure S5c). The completeness of the transposons that enclose the OHR gene clusters on plasmid pSULSP may also contribute to an increased frequency of transposition of these genes compared to those reported non-mobile OHR gene clusters in the genomes of *Sulfurospirillum* strains DSM12446

448 and ACSTCE,[61] which are downstream of lone transposases instead of tandem IS21 transposable
449 elements and have no indication of flanked repeats. The genomes of other PCE-respiring
450 *Sulfurospirillum* strains do not harbor transposases near the RDase or corrinoid biosynthesis gene
451 clusters[61] and there are no reports of loss of PCE dechlorination in these strains.[72, 73]

452 Bioaugmentation is considered to be a cost-effective and sustainable strategy for the
453 remediation of organohalide-contaminated ecosystems. Successful cell-based bioaugmentation is
454 dependent on the establishment and proliferation of inoculated OHRB within the autochthonous
455 microbial community.[74, 75] Genetic bioaugmentation is an alternative strategy by stimulating
456 bioremediation through the introduction of the genetic capacity to dehalogenate organohalide
457 pollutants into the indigenous gene pool, thereby increasing the biodegradation potential of the
458 microbial community *in situ*. Genetic bioaugmentation enables acquisition of metabolic
459 capabilities via horizontal gene transfer, bypassing the need to establish and sustain non-native
460 bacterial populations or manipulate environmental conditions (e.g., pH, soil composition, water
461 content). Genetic bioaugmentation by conjugational transfer of a plasmid carrying genes required
462 for OHR has been successfully demonstrated in aerobic systems,[27, 29, 64, 66] but genetic
463 bioaugmentation of anaerobic systems which eventually serve as the primary sinks for the
464 majority of the persistent organic pollutants has proven more challenging. Anaerobic OHR
465 requires the participation of two clusters of genes: the reductive dehalogenase operon,
466 comprising genes encoding a functional reductive dehalogenase, an anchoring protein, and
467 regulatory elements, and a corrinoid biosynthesis cluster, comprising genes required for
468 uroporphyrinogen III synthesis, corrinoid ring synthesis, and adenosylation. Versions of these
469 gene clusters are present in the genomes of anaerobic OHRB, but the current study is the first

report of both a reductive dehalogenase operon and a corrinoid biosynthesis cluster on a transferable plasmid in a naturally occurring OHRB.

The development of genetic bioaugmentation technologies has been largely impeded by a lack of tools to mediate the transfer of the genetic components required for anaerobic OHR. The discovery of a genetic system that could be used to introduce specific metabolic traits into anaerobic microbial ecosystems could broaden the scope of impacted sites that can be remediated using sustainable bioremediation technologies. Heterologous expression of RDases in engineered cells has been reported previously but requires the addition of extra elements, such as cobalamin precursors,[76-79] and thus far no transgenic OHRB has been developed for *in situ* bioremediation. In the current study, heterologous expression of PceA was achieved via horizontal transfer without any conventional genetic manipulation or construction of engineered cells. More importantly, the PceA expressed by recipient cells functioned without any addition of auxiliary substrates, which could be attributed either to synthesis by recipient cells via the corrinoid biosynthesis gene clusters originating from *Sulfurospirillum* sp. strain SP or to the scavenging of cobalamin produced by other populations in the enrichment. Future studies will investigate factors that could impact the feasibility of leveraging horizontal gene transfer for *in situ* bioremediation. The demonstration of horizontal acquisition of a naturally occurring plasmid and functional heterologous expression of *pceA* by diverse bacteria represents a proof of concept for genetic bioaugmentation of anaerobic environmental compartments.

ASSOCIATED CONTENT

Supporting Information

More details about genomic analyses and other biomolecular measurement are provided in supporting information.

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Figure legends

Figure 1. Identification and characterization of a novel *Sulfurospirillum sp.* strain SP. (a) Dechlorination of tetrachlorethene (PCE) to trichloroethene (TCE) and dichloroethenes (DCEs) coupled with growth of strain SP. **(b)** 16S rRNA gene-based phylogeny of strain SP (shown in red). *Sulfurospirillum* strains whose genomes are known to harbor reductive dehalogenase genes are shown in blue. Accession numbers of sequences used to construct the phylogeny are indicated.

Figure 2. Genomic insight of plasmid of strain SP. (a) Circular representation of pSULSP. From outer to inner: circle 1, protein coding sequences on positive (green) and negative strands (blue); circle 2, short read coverage; circle 3, reductive dehalogenase gene clusters (pink), corrinoid biosynthesis gene clusters (teal), CRISPR associated gene clusters (gray), toxin-antitoxin systems (purple), and genetic modification-associated genes (light blue); circle 4, codon bias; circle5, GC content (difference from mean – red < 0 and green > 0); circle 6, GC skew (purple < 0 and yellow > 0). **(b)** Alignment and comparison of organohalide respiration gene clusters in *Sulfurospirillum* genomes.

Figure 3. Phylogeny of **(a)** the amino acid sequences of transposases with the highest identity to the IstA-IS21 sequences in *Sulfurospirillum sp. s*train SP (dark blue) and transposes collocated with organohalide respiration (OHR) gene clusters in the genomes of organohalide respiring *Sulfurospirillum* and transposases (pink); and **(b)** the amino acid sequences of replication initiating protein (DnaA) on the plasmid and chromosome of *Sulfurospirillum sp.* strain SP. DnaA sequences from extrachromosomal elements are indicated in red color font. The maximum-likelihood trees are supported by 1000 bootstrap replicates.

Figure 4. Transposon loss leads to PCE dechlorination loss in variants of strain SP. (a) Dechlorination kinetics of strain SP and phenotypic variants that emerged spontaneously during cultivation in the absence of PCE for 10–12 successive transfers to new medium. **(b)** Structural scheme of single-copy *IS21* and duplicated $(IS21)_2$ on the plasmid of strain SP and the non-dechlorinating variants; **(c)** Sequence of the inverted repeats (IR) of *IS21*; (**d**) Sequences of *IS21* direct repeats (DR).

Figure 5. Genetic bioaugmentation of DNA from strain SP. (a) PCE removal in enrichments spiked with DNA extracted from *Sulfurospirillum* sp. strain SP. **(b)** PCE dechlorination profile in genetically augmented enrichment culture EN-11 and **(c)** *pceA* transcription in the genetically augmented enrichment culture EN-11. **(d)** The presence of genes associated with organohalide respiration derived from plasmid pSULSP in putative PCE-dechlorinating colonies from the genetically augmented culture EN-11 as determined by PCR using gene-specific primers. The red arrows in panel (**b**) indicate amendment of PCE.

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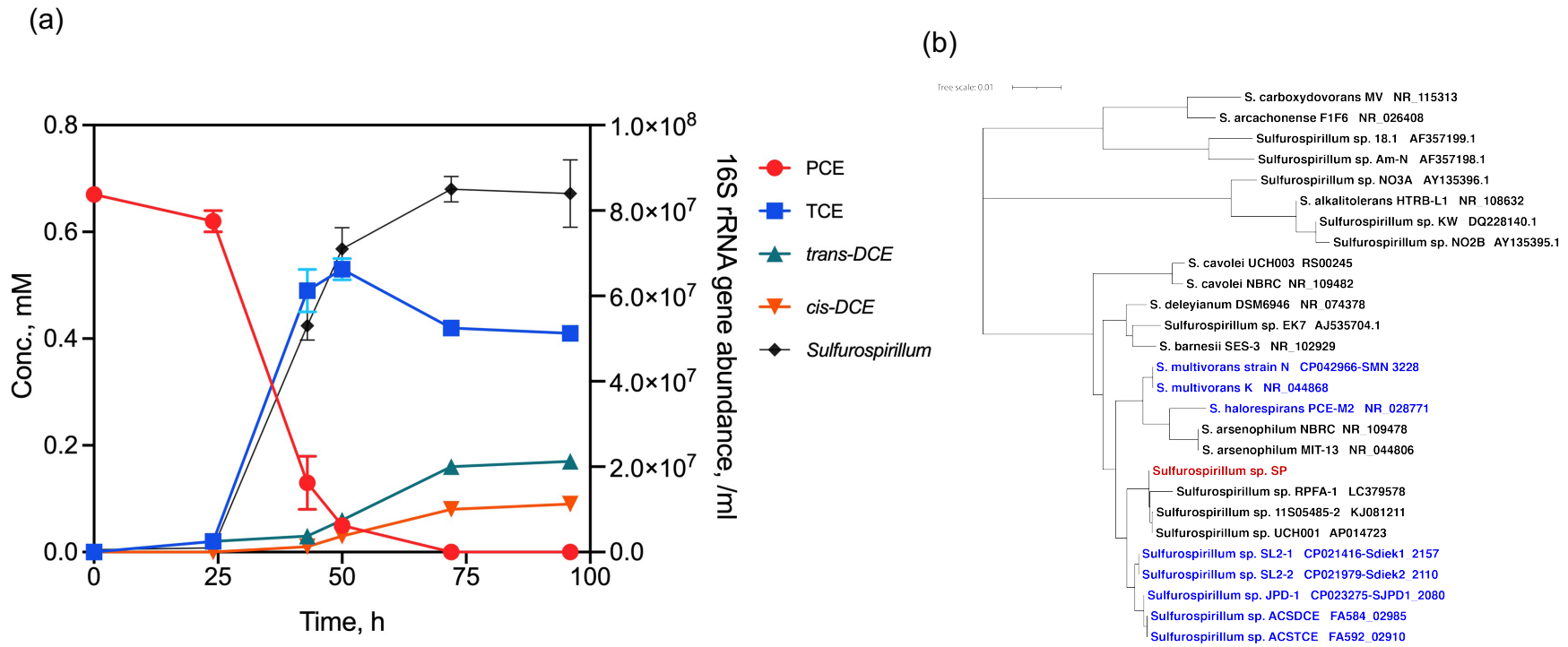

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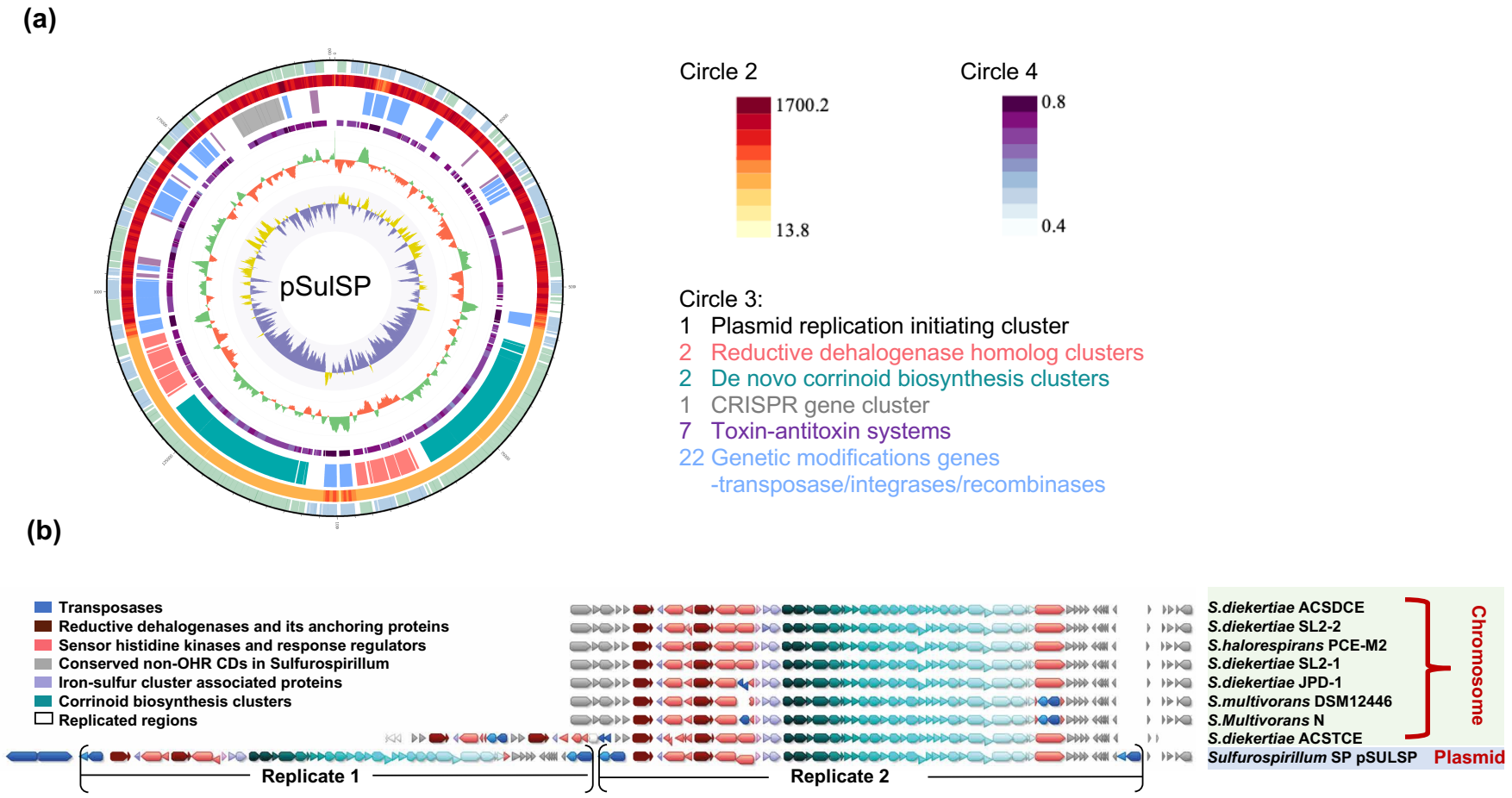

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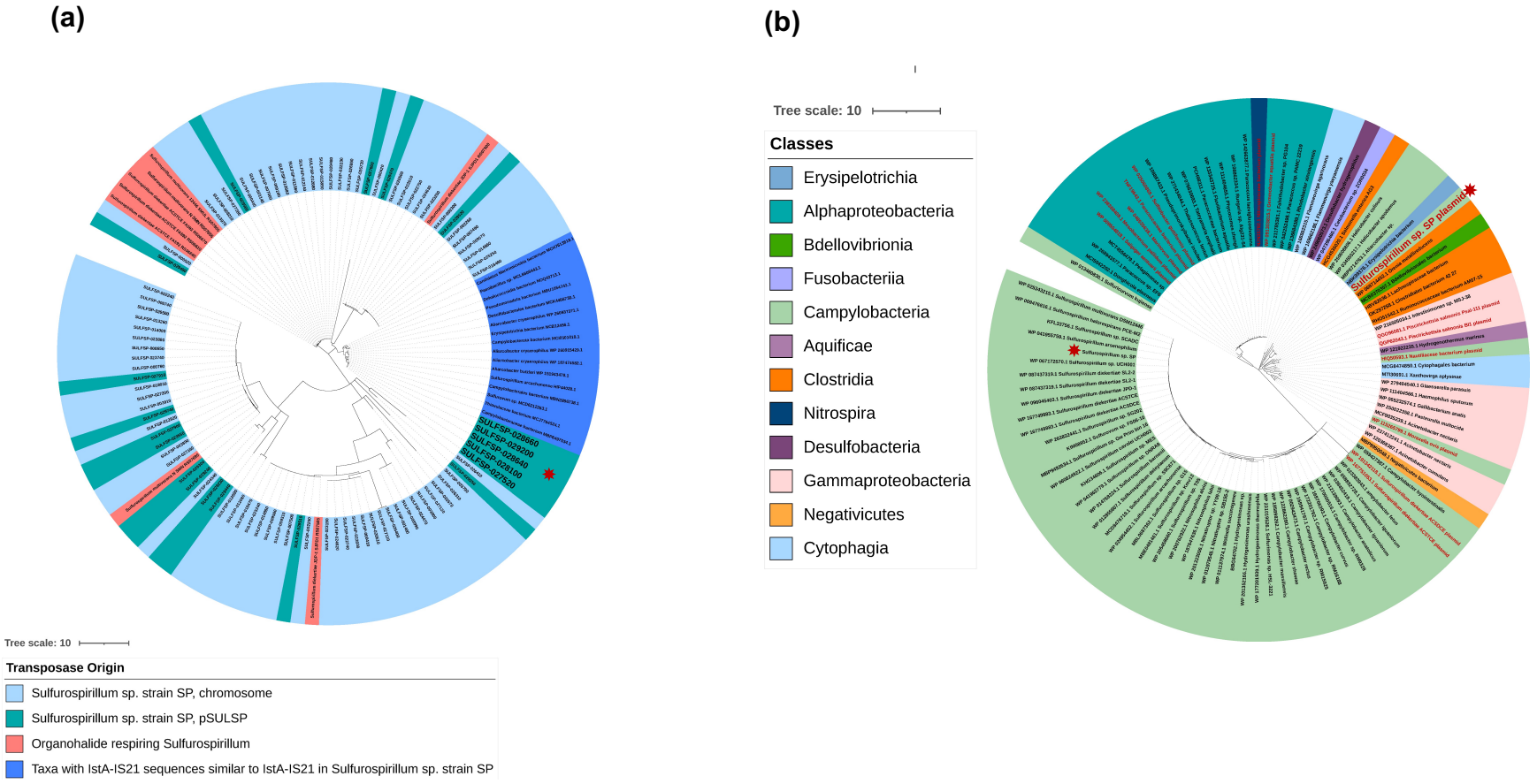

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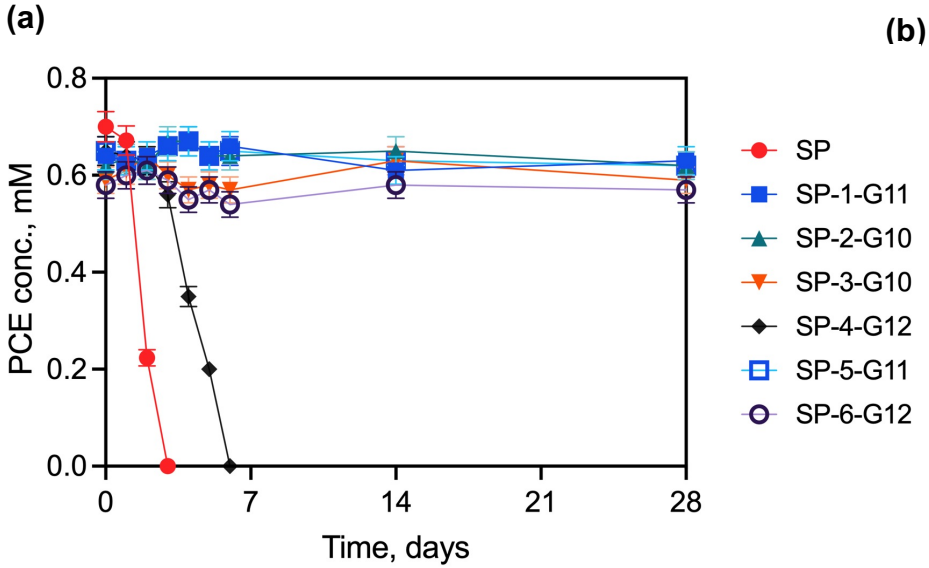

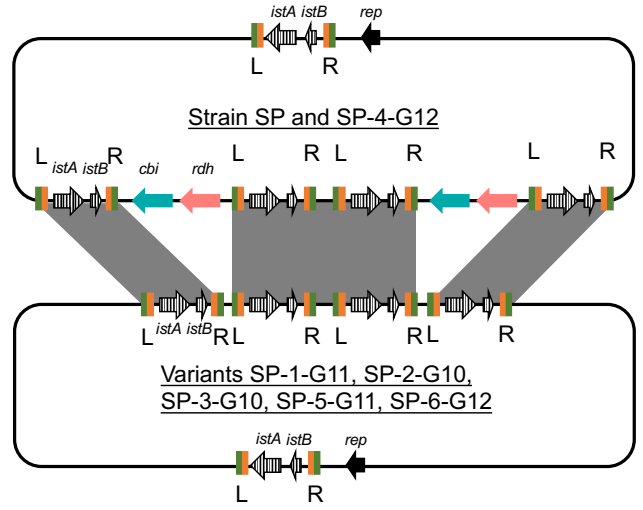

(c) IS21 IRs

IRL	5’-TGATTTAGGCTG-3’
IRR	5’-CAGCCTAATTCA -3’

(d) IS21 DRs

DRL	5’-CGGTT-3’
DRR	5’-CCGTT-3’

cbi, corrinoid synthesis gene cluster
rdh, reductive dehalogenase homolog cluster
rep, replication initiating cluster
direct repeats
inverted repeats

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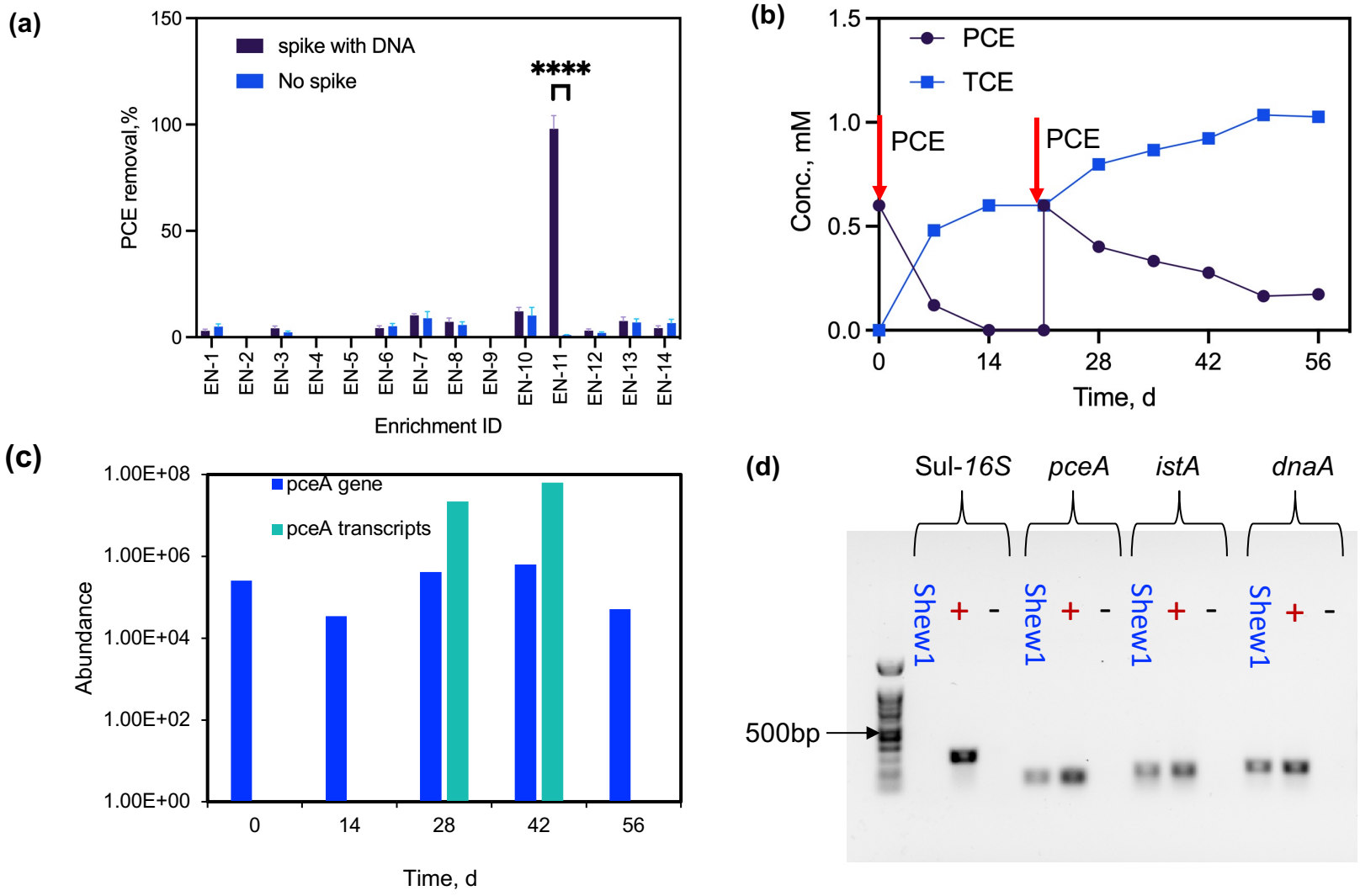

Supplementary Materials

Interspecies Mobility of Organohalide Respiration Gene Clusters Enables Genetic Bioaugmentation

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Number of pages: 14; number of tables: 2; number of figures: 10

Supplementary Tables

Table S1. Primers used in this study.

Target	Locus	Primer ID	Sequence 5′-3′	Product size (bp)	Tm	Reference
Sul-pceA	SULFSP-028630, SULFSP-029190	SulPceA-786F	TAT GAA CCG CGA AAT GAT GC	147	60	This study
		SulPceA-833R	CAG CAG CAG CAT TTG GTT AT			
Sul-rdhA2	SULFSP-028580, SULFSP-029140,	SulRdh2-764F	ACC GTG ATA TGA TGC AGT GT	148	60	This study
		SulRdh2-911R	CCA ACA CCA TTA CAG CTT GG			
istA	SULFSP-027520, SULFSP-028100, SULFSP-028640, SULFSP-028660, SULFSP-029200	IstA-1034F	GCC TCT ACA GTG TGC CTT AT	200	60	This study
		IstA-1234R	TTA GTA TTC GCC CAG GTG AC			
Sul-16S	SULFSP-rRNA005, SULFSP-rRNA006, SULFSP-rRNA007, SULFSP-rRNA008	Sulfuro114F	GCT AAC CTG CCC TTT AGT GG	308	58	[1, 2]
		Sulfuro421R	GTT TAC ACA CCG AAA TGC GT			
DnaA-pSULSP	SULFSP-027460	pSulSPDnaA-886F	GTT ATC GGT CCA CAA GGC TA	200	58	This study
		pSulSPDnaA -1007R	CAC GTC CAC ATC AAC ATA GC			

Table S2. Features of the *Sulfurospirillum* sp. strain SP genome.

	Chromosome	Plasmid (pSULSP)
Length (kbp)	2,676	201
GC content (%)	37.6 ± 0.03	35.6 ± 0.04
Mean coverage (Illumina NovaSeq6000)	1104.53 ± 117.32	676.911 ± 391.84
Mean coverage (PacBio Sequel)	984.13 ± 166.40	449.65 ± 249.80
CDS	2,731	223
16S rRNA genes	4	-
tRNA	50	-

Supplementary Figures

Figure S1. Versatile metabolic capabilities of *Sulfurospirillum* sp. strain SP. **(a)** Growth of strain SP with different electron acceptors (pyruvate as electron donor and carbon source), electron donors (nitrate as electron acceptors and pyruvate as carbon source), and fermentative substrates. Illustration of key pathways and putative genes in strain SP related to **(b)** nitrate reduction and **(c)** sulfur cycle.

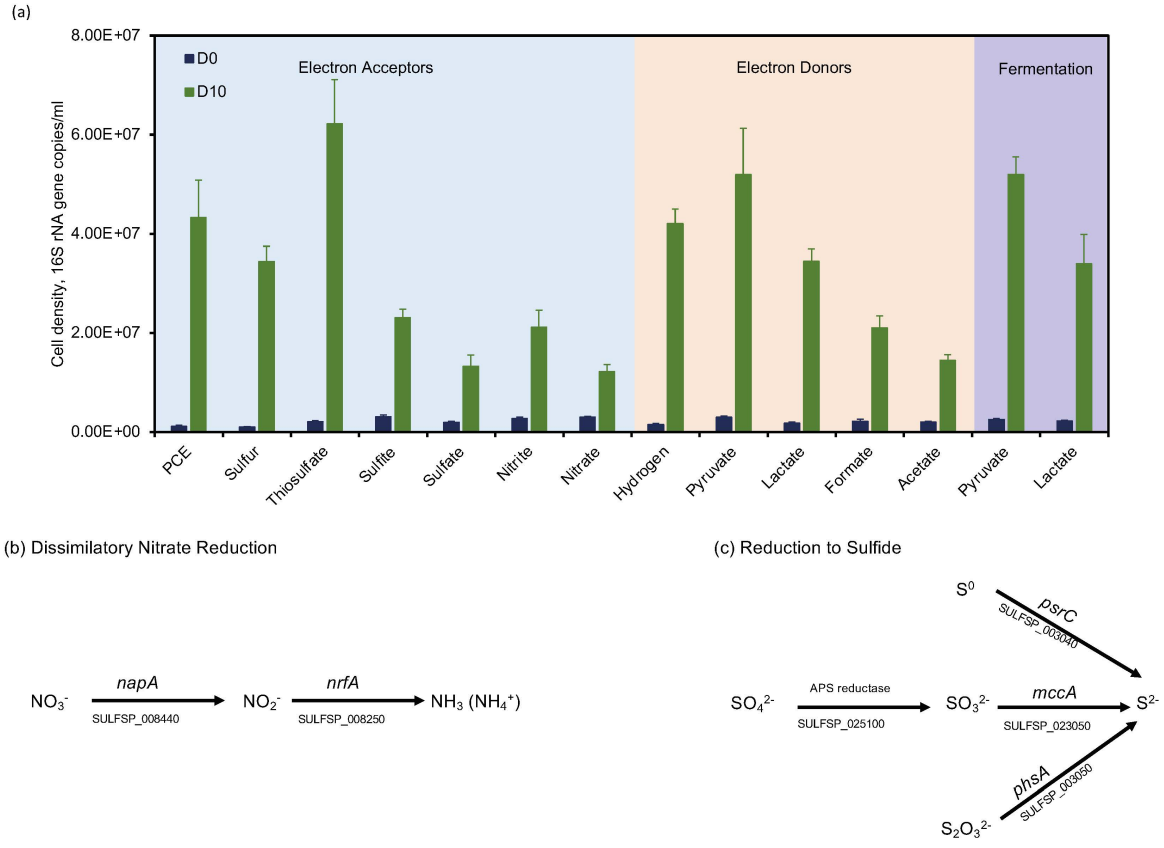


Figure S2. Phylogeny of *Sulfurospirillum* sp. strain SP inferred by average nucleotide identity (ANI). ANI was calculated with the JSpecies Web Service (https://jspecies.ribohost.com/jspeciesws/#home) using mummer.

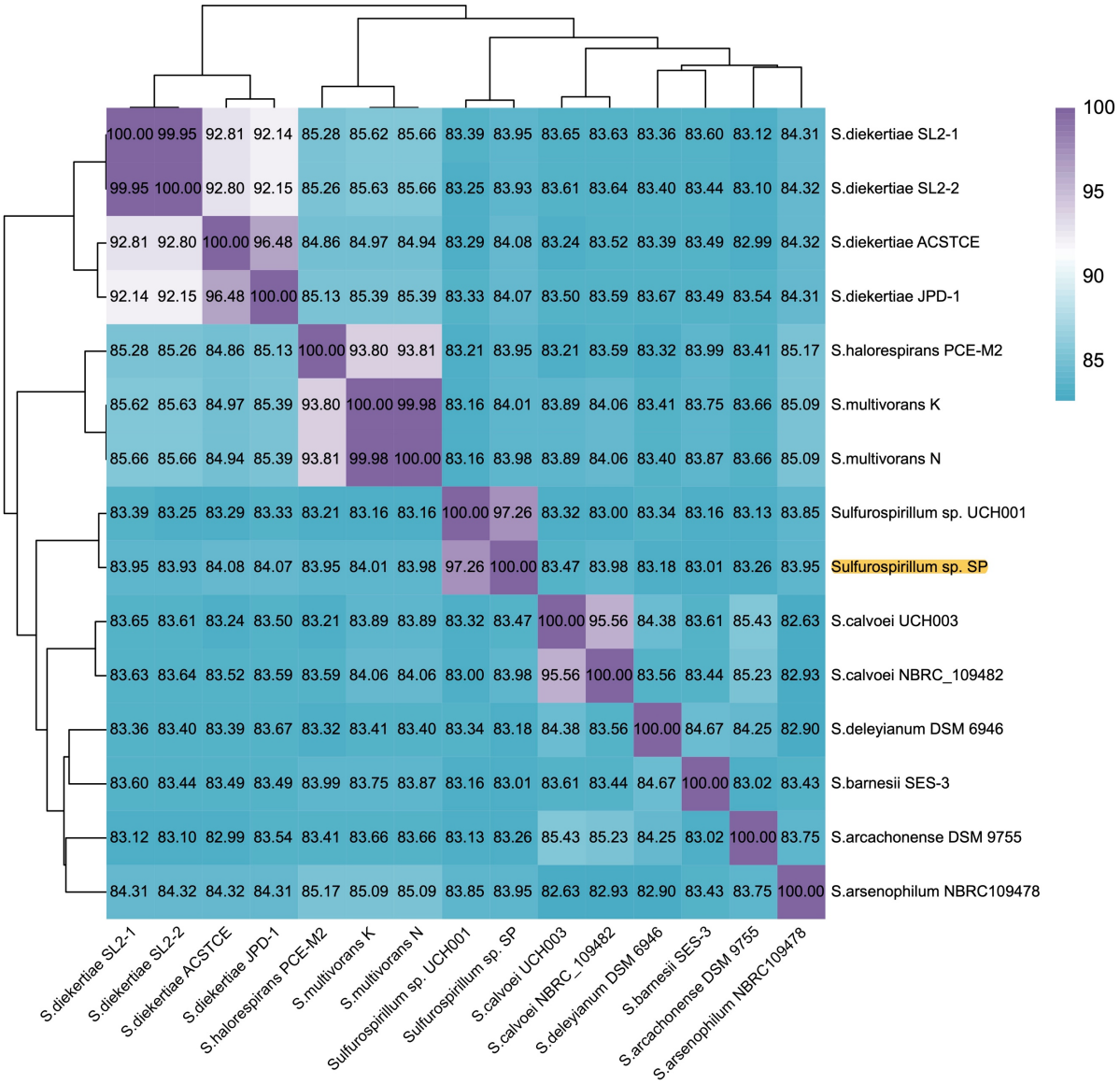


Figure S3. Illustration of Replicate 2 of OHR gene regions (gene ID: SULFSUP_002770-SULSP_002718) located on plasmid pSULSP in strain SP.

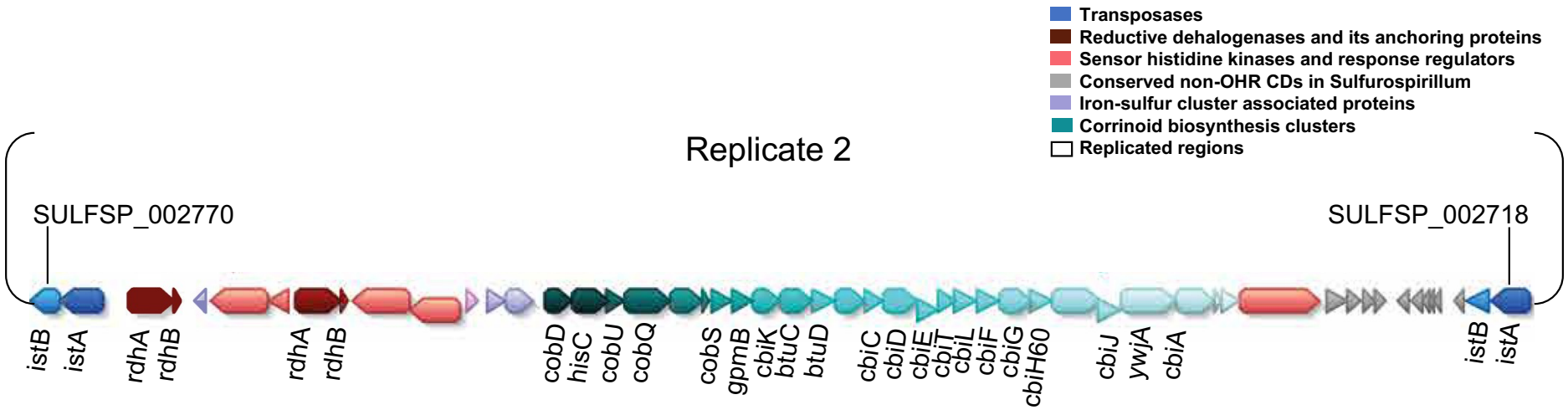
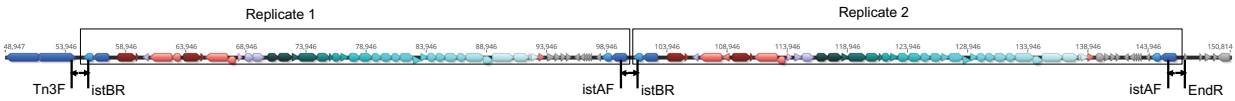


Figure S4. Verification of the duplicated OHR regions on pSULSP by **(a)** end-point PCR and by **(b)** contiguous Illumina short reads and PacBio long reads spanning portions.

(a)



Primer ID	Target Protein ID	Target	Primer sequence 5′-3′
Tn3F	SULFSP-029230	Tn3 family transposase	ATCGCCAACAACTGTAAACG
IstBR	SULFSP-028110 SULFSP-028650 SULFSP-028670 SULFSP-029210	IS21 helper ATPase	CACTTAAAGCCAGTGAAGCC
IstAF	SULFSP-028100, SULFSP-028640, SULFSP-028660, SULFSP-029200	IS21 family transposase	CATGCTTGGAGTCAGTTTGG
EndR	SULFSP-028090	Helix-turn-helix transcriptional regulator	TGACAGACGACGAGCAATAA

(b)

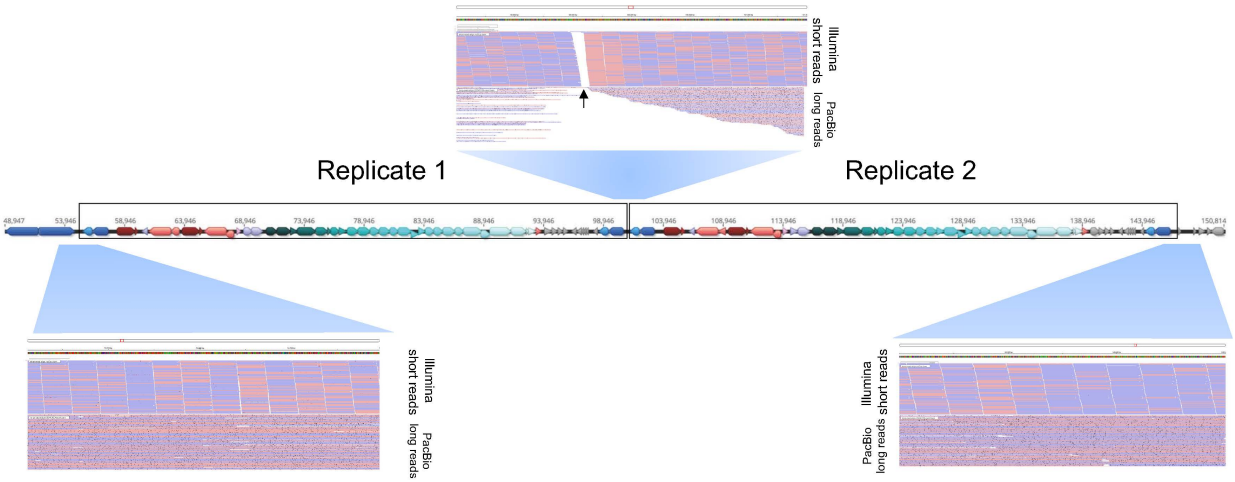


Figure S5. Putative models of transposition events that could lead to duplication of the organohalide respiration gene clusters in *Sulfurospirillum sp.* strain SP. **(a)** model 1 and **(b)** model 2 of formation of duplicated $(IS21)_2$ and **(c)** simple transposition of IS21 on plasmid.

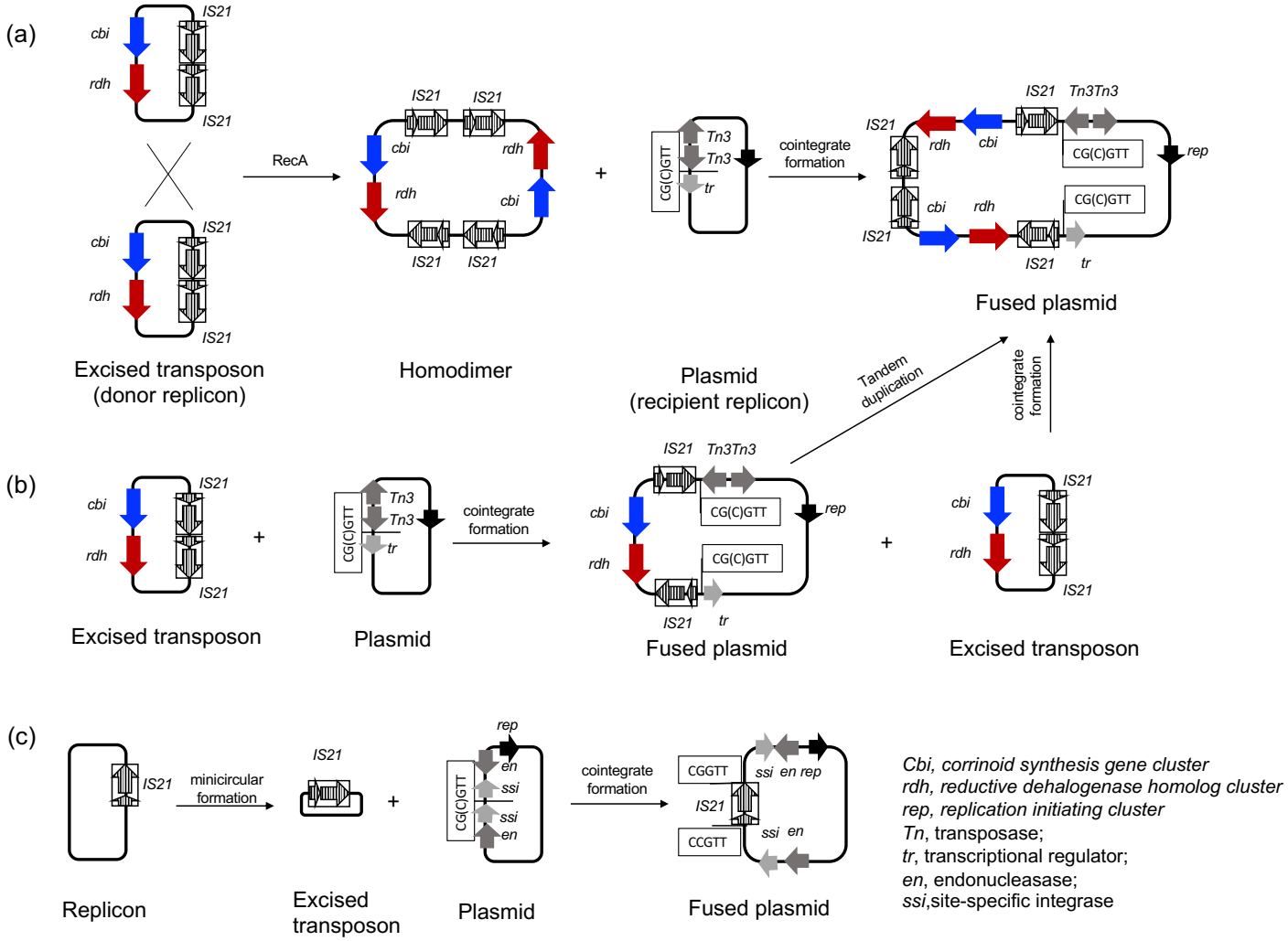


Figure S6. Emergence of non-dechlorinating variants of *Sulfurospirillum* sp. strain SP. **(a)** Experimental demonstration of discovery and **(b)** PCE dechlorination of emerged non-dechlorinating variants of *Sulfurospirillum* sp. strain SP.

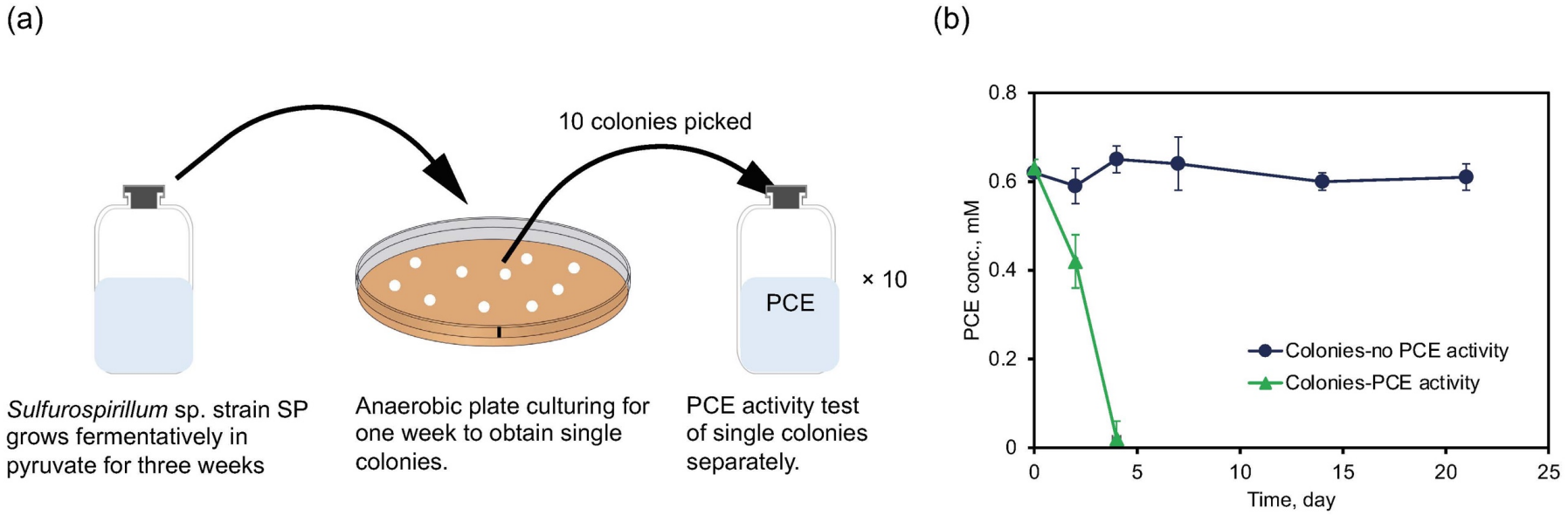


Figure S7. Structure of 19.6 kb deleted region in the chromosomes of non-dechlorinating variants of *Sulfurospirillum* sp. strain SP (SP-1-G11, SP-2-G10, SP-3-G10, and SP-6-G12).

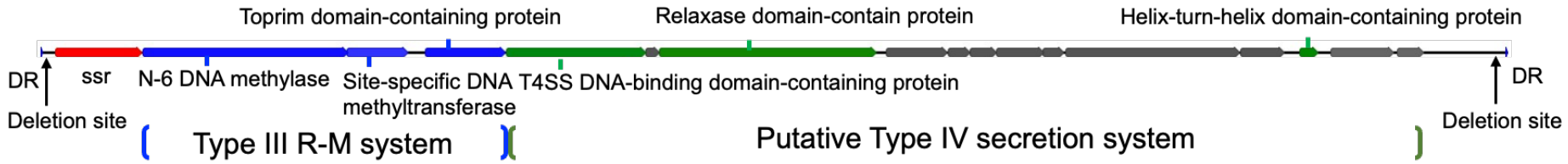


Figure S8. Tentative model of transposition events of **(a)** direct deletion by $(IS21)_2$ and **(b)** Tn-IS21 leading to deletions.

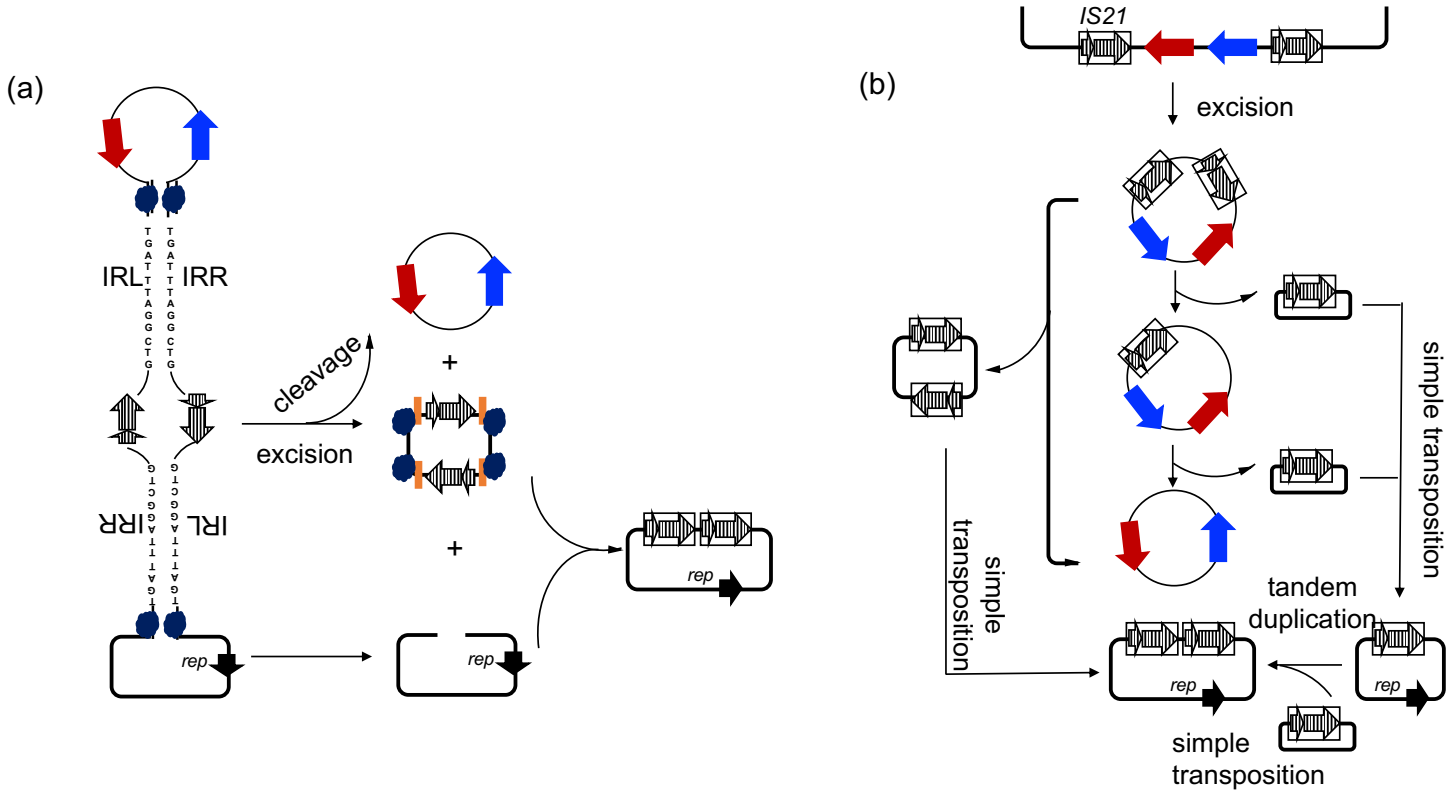


Figure S9. Dehalogenation performance by enrichment EN-11. **(a)** Debromination of a tetra- and penta-BDE mixture (BDE 47, 99, and 100) and **(b)** its coupled growth of *Dehalogenimonas* in enrichment EN-11; **(c)** lack of PCE dechlorinating activity in enrichment EN-11 within three-month incubation in two separate trials, each trial in triplicates.

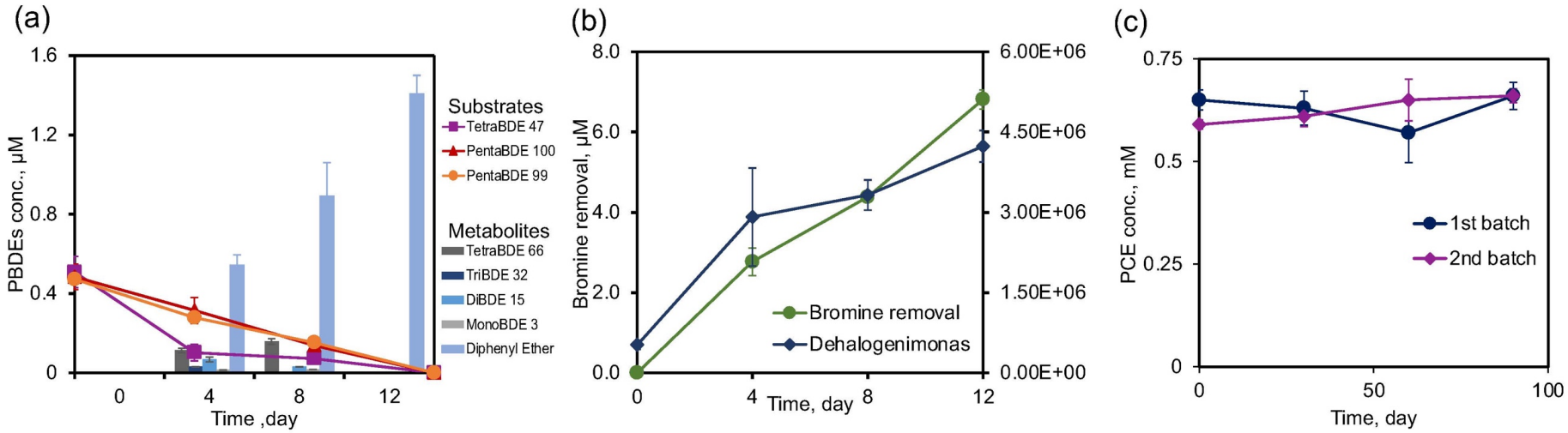
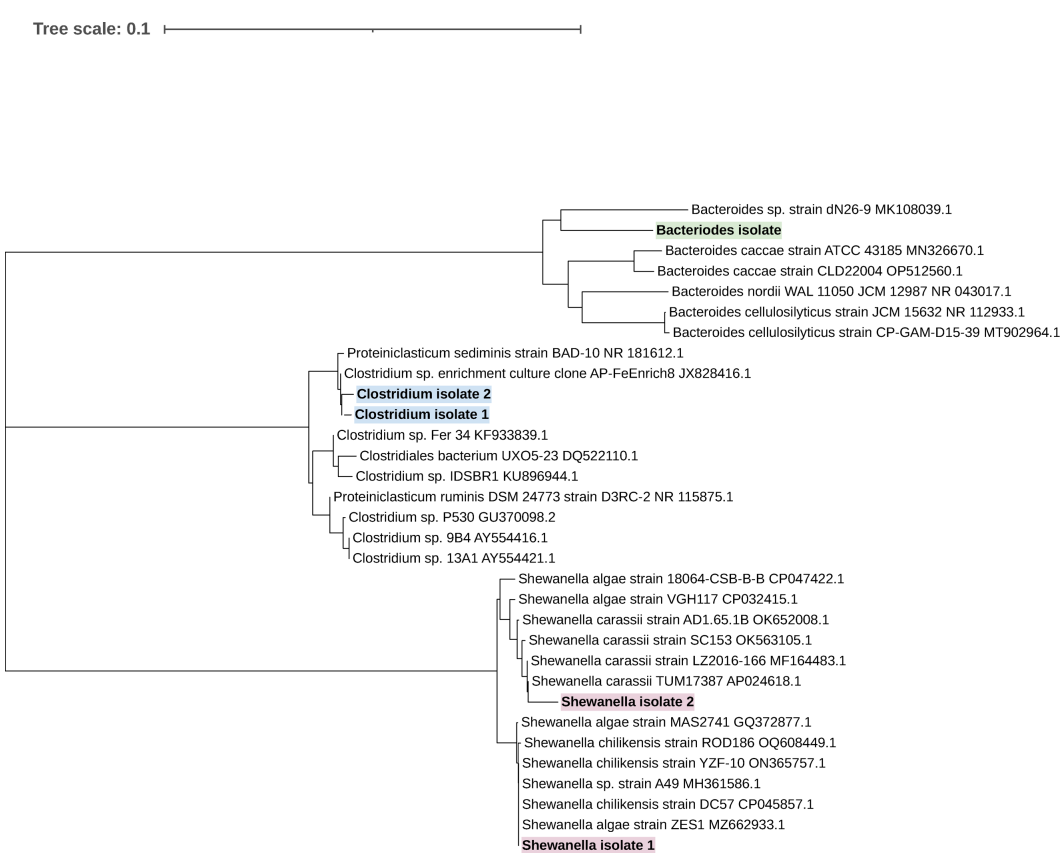
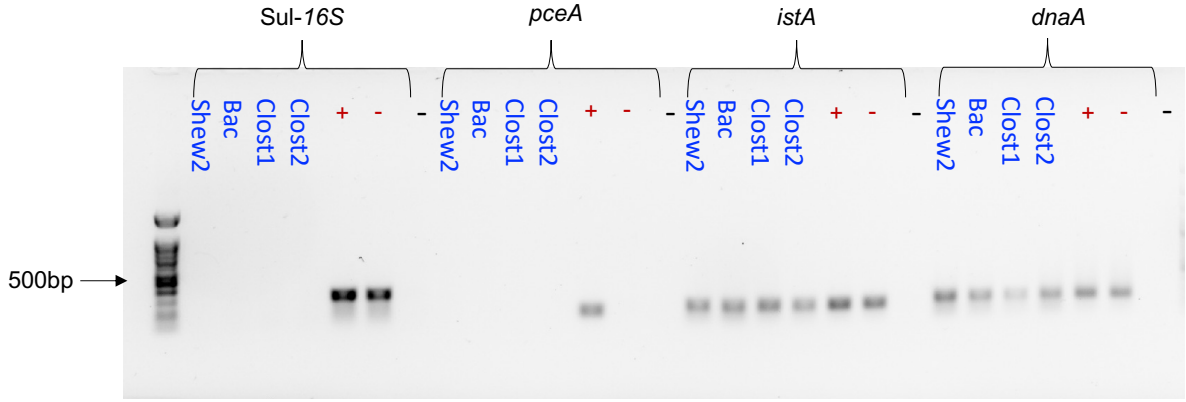


Figure S10. Acquisition of extracellular genetic elements of taxonomically diverse microbial populations in enrichment EN-11. **(a)** 16S rRNA gene-based phylogeny of putative PCE dechlorinating bacteria. **(b)** End-point PCR using gene-specific primers to determine the presence of genes associated with pSULSP in putative PCE-dechlorinating colonies from the genetically augmented culture EN-11. Shew1/2: *Shewanella* isolates #1 and 2; Bac: *Bacteroides* isolate; Clost1/2: *Clostridium* isolate #1 and #2. DNA extracted from strain SP and the non-dechlorinating variant SP-2-G10 was used for positive (+) and negative (–) DNA template controls for PCR, respectively. The neighbor-joining tree was constructed in MEGA11; the final tree is supported by 1000 bootstrap replicates.

(a)



(b)



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