PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

rstb.royalsocietypublishing.org

Research

Cite this article: Lohner ST, Spormann AM. 2013 Identification of a reductive tetrachloroethene dehalogenase in *Shewanella sediminis*. Phil Trans R Soc B 368: 20120326. http://dx.doi.org/10.1098/rstb.2012.0326

One contribution of 12 to a Theme Issue 'Organohalide respiration: using halogenated hydrocarbons as the terminal electron acceptor'.

Subject Areas:
microbiology

Keywords:
Shewanella, tetrachloroethene, trichloroethene, reductive dehalogenase, marine sediment

Author for correspondence:
Alfred M. Spormann
e-mail: spormann@stanford.edu

Identification of a reductive tetrachloroethene dehalogenase in *Shewanella sediminis*

Svenja T. Lohner[2] and Alfred M. Spormann[1,2]

[1]Department of Chemical Engineering, and [2]Civil and Environmental Engineering, Stanford University, Stanford, CA 94305, USA

The genome sequence of psychrophilic *Shewanella sediminis* revealed the presence of five putative reductive dehalogenases (Rdhs). We found that cell extracts of pyruvate/fumarate-grown *S. sediminis* cells catalysed reduced methyl viologen-dependent reductive dechlorination of tetrachloroethene (PCE) to trichloroethene (TCE) at a specific activity of approximately 1 nmol TCE min^{-1} (mg protein)$^{-1}$. Dechlorination of PCE followed Michaelis–Menten kinetics with an apparent K_m of 120 μM PCE. No PCE dechlorination was observed with heat-denatured extract or when cyanocobalamin was omitted from the growth medium; however, the presence of PCE in the growth medium increased PCE transformation rates. Analysis of mutants carrying in-frame deletions of all five Rdhs encoding genes showed that only deletion of Ssed_3769 resulted in the loss of PCE dechlorination activity suggesting that Ssed_3769 is a functional Rdh. This is the first study to show reductive dechlorination activity of PCE in a sediment-dwelling *Shewanella* species that may be important for linking the flux of organohalogens to organic carbon via reductive dehalogenation in marine sediments.

1. Introduction

Microbial reductive dehalogenation is a biochemically and metabolically intriguing activity found as co-metabolic process in a variety of micro-organisms such as methanogens or homoacetogens and more importantly as a catabolic process in phylogenetically diverse groups of bacteria including *Desulfitobacterium*, *Sulfurospirillum*, *Desulfomonile*, *Desulfovibrio*, *Anaeromyxobacter*, *Desulfuromonas*, *Dehalobacter*, *Dehalogenimonas* and *Dehalococcoides* [1,2]. The latter two genera have been of special interest as their catabolism is restricted to organohalide respiration [3,4]. Most of the so far isolated organohalide-respiring bacteria have been isolated based on their catabolic activity to dechlorinate anthropogenic chloroaliphatic or chloroaromatic compounds [5,6]. In all cases, the material source for the isolation of microbes was derived from terrestrial environments, such as chloroethene-contaminated aquifers, rivers or soil sediments as well as sewage sludge [2,7]. However, putative reductive dehalogenase (Rdh) genes (*rdh*) have been also found in uncontaminated sediments [8], including marine deep sea sediments [9]. These observations, in conjunction with the discovery of site-specific mobilization of *rdh* genes as an important mode for migration of these genes within *Dehalococcoides* populations [10], raise the question about the role of *rdh* genes and Rdh activities in these environments, especially in marine deep sea sediments, as well as their evolutionary history.

Reductive dehalogenation activity is associated with a monomeric RdhA enzyme encoded by *rdhA*. RdhA contains two iron–sulfur clusters, and many studies have shown the presence of a corrinoid cofactor involved in its activity. It is speculated that RdhA is associated *in vivo* to the outside of the cyptoplasmic membrane via a putative small integrated membrane protein RdhB [11–13]. Reductive dechlorination of PCE and other chloroethenes requires *in vitro* a low-redox potential electron donor such as reduced methyl viologen. The *in vivo* electron donor is typically molecular hydrogen for most of the catabolic

Royal Society Publishing
Informing the science of the future

Table 1. Strains and plasmids used in this study.

strain	relevant genotype and phenotype	reference
Escherichia coli		
DH5-λpir	host used for mating with *MR-1. ϕ80dlacZΔM15 Δ(lacZYA-argF)U196 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1/λpir*	[22]
WM3064-λpir	host used for mating with *MR-1. thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-1360 Δ(araBAD) 567ΔdapA 1341::[erm pir(wt)]*	[23]
S17-λpir	host used for mating with *MR-1. thi pro recA hsdR [RP4-2Tc::Mu-Km::Tn7]λpir* Tp^r Sm^r	[24]
Shewanella sediminis		
AS1028	*Shewanella sediminis* strain HAW-EB3, wild-type (WT)	[20]
AS1029	in-frame deletion of *Ssed_4120* in AS (WT), Δ*Ssed_4120*	this study
AS1030	in-frame deletion of *Ssed_3769* in AS (WT), Δ*Ssed_3769*	this study
AS1031	in-frame deletion of *Ssed_2100* in AS (WT), Δ*Ssed_2100*	this study
AS1032	in-frame deletion of *Ssed_2103* in AS (WT), Δ*Ssed_2103*	this study
AS1033	in-frame deletion of *Ssed_1729* in AS (WT), Δ*Ssed_1729*	this study
AS1034	AS1030 complemented with *Ssed_3769* by knock-in, *Ssed_3769*$^+$	this study

plasmid	description	reference
pDS3.0	suicide plasmid for constructing in-frame deletions; Gm^r	[25]
pDS132	suicide plasmid for constructing in-frame deletions; Cm^r	[26]
pDS132_Δ*ssed_4120*	*Ssed_4120* in-frame deletion fragment in pDS132; Cm^r	this study
pDS3.0_Δ*Ssed_3769*	*Ssed_3769* in-frame deletion fragment in pDS3.0; Gm^r	this study
pDS3.0_Δ*Ssed_2100*	*Ssed_2100* in-frame deletion fragment in pDS3.0; Gm^r	this study
pDS3.0_Δ*Ssed_2103*	*Ssed_2103* in-frame deletion fragment in pDS3.0; Gm^r	this study
pDS3.0_Δ*Ssed_1729*	*Ssed_1729* in-frame deletion fragment in pDS3.0; Gm^r	this study
pDS132_*Ssed_3769*	Plasmid for replacing WT *Ssed_3769* allele into Δ*Ssed_3769*; Cm^r	this study

organohalide-respiring bacteria, especially *Dehalococcoides* [13]. The cellular electron transfer pathway from hydrogen via membrane-bound hydrogenase(s) to the Rdh is unknown. In this overall catabolic process, the exergonic oxidation of hydrogen with organohalogens is coupled to energy conservation most likely via a chemiosmotic mechanism [12,14].

Analysis of *rdh* genes from many different microorganisms revealed common and unique characteristics [15,16]. Both *rdh* genes are often linked, and experimental evidence has shown that, if tested, they are co-transcribed [13]. Whole genome sequence analyses of several *Dehalococcoides mccartyi* strains revealed that some genomes can carry as many as 36 (strain VS) full-length, non-identical Rdh homologous genes [4,16,17]. Despite the presence of this unusually high number of Rdhs in some organohalide-respiring bacteria, only a few Rdhs have been characterized biochemically. These biochemical studies collectively have shown that each Rdh seems to be substrate-specific and structurally related halogenated compounds have been observed to be transformed at rates that are orders of magnitudes lower than the primary halogenated substrate [13,18,19].

Interestingly, the genome sequence of the marine sediment-dwelling *Shewanella* species strain HAW-EB3, named *Shewanella sediminis* (*S. sediminis*) [20], revealed the presence of five Rdh homologue genes. Bacteria of this genus are known for their versatile electron-accepting capabilities using a complex electron transfer network composed mainly of *c*-type cytochromes as well as iron–sulfur proteins [21]. The goal of this study was to gain insights into the function of the putative Rdhs in *S. sediminis* and to shed light on the function and evolution of these genes in marine sediment environments.

2. Material and methods

(a) Growth conditions and media (growth curves)

All strains and plasmids used in this study are described in table 1. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth medium at 37°C and *S. sedimins* strains were grown at 10°C in LB with addition of 1% (wt/vol) NaCl or minimal media (4 M) with the following composition (per litre): 1 mM $CaCl_2 \cdot 2H_2O$, 5 µM $CoCl_2$, 0.2 µM $CuSO_4 \cdot 5H_2O$, 5.4 µM $FeCl_2 \cdot 2H_2O$, 57 µM H_3BO_3, 5.7 mM K_2HPO_4, 3.3 mM KH_2PO_4, 1.0 mM $MgSO_4 \cdot 7H_2O$, 1.3 µM $MnSO_4$, 67.2 µM Na_2EDTA, 3.9 µM $Na_2MoO_4 \cdot 2H_2O$, 1.5 µM Na_2SeO_4, 342 mM NaCl, 2 mM $NaHCO_3$, 5 µM $NiCl_2 \cdot 6H_2O$, 1 µM $ZnSO_4$ and 9 mM $(NH_4)_2SO_4$ and 0.1% (wt/vol) casamino acids, pH 7.4). After autoclaving and before inoculation, a filter-sterilized vitamin solution was added to the medium to reach final concentrations of: 4.5 nM folic acid, 13.5 nM riboflavin, 24 nM DL-6,8-thioctic acid, 41 nM biotin, 365 nM 4-aminobenzoic acid, 46 nM pantothenate, 1.5 µM pyridoxamine, 812 nM nicotinic acid, 66 nM thiamine, 18 nM cyanocobalamin. Where necessary, medium was solidified by 1.5% (wt/vol) agar and supplemented with 10 µg ml^{-1} gentamycin or 10 µg ml^{-1} chloramphenicol. For growth under anoxic conditions, 30 mM fumarate was added as terminal electron acceptor and 40 mM pyruvate as electron

donor. Anaerobic cultures were either prepared in 150 ml serum vials or 1 l Schott bottles sealed with butyl rubber stoppers. Oxygen was removed from the medium by repeatedly flushing the headspace of each vial for 1 min with nitrogen (99.9% purity; Praxair, Santa Clara, CA) followed by a 1 min application of vacuum for at least 20 cycles. Alternatively, the medium was autoclaved and subsequently cooled down under a nitrogen stream while rigorous stirring. Mineral medium was inoculated (1% inoculum) to a starting OD of 0.02 from *S. sediminis* stationary phase LB + 1% NaCl cultures.

(b) Mutant construction and cloning in *Shewanella sediminis*

All genetic work was carried out according to standard protocols. Kits for isolation and/or purification of DNA were obtained from Promega (Madison, WI, USA), and enzymes were purchased from New England Biolabs (NEB, Ipswich, MA, USA). Ssed_1729, Ssed_2100, Ssed_2103, Ssed_3769 and Ssed_4120 knockout mutants were constructed via homologous recombination, resulting in a mutant lacking the entire open reading frame except for the start and stop codon. Briefly, approximately 750 bp upstream and downstream fragments of the target gene were PCR-amplified from wild-type (AS1028) genomic DNA and subsequently joined via a complementary tag that was added to the 5′-end of each inner primer (table 2). The fusion products of Ssed_1729, Ssed_2100, Ssed_2103 and Ssed_3769 were ligated into pDS3.0 via the *Sma*I restriction site, whereas for Ssed_4120, the fused fragment was ligated into the *Sac*I site of pDS132 after digestion of an introduced *Sac*I restriction site. *Escherichia coli* DH5α-λpir or *E. coli* S17-λpir strains were transformed with the ligation mixture and plated on LB containing 10 $\mu g\ ml^{-1}$ gentamycin (pDS3.0) or 10 $\mu g\ ml^{-1}$ chloramphenicol (pDS132). The resulting plasmids pDS132_Δ*Ssed_4120*, pDS3.0_Δ*Ssed_3769*, pDS3.0_Δ*Ssed_2100*, pDS3.0_Δ*Ssed_2103* and pDS3.0_Δ*Ssed_1729* (table 1) were verified by sequencing and transformed into the WT (AS1028) through bi-parental mating using *E. coli* WM3064 as conjugal donor. After 24 h incubation at room temperature, the mating mix was resuspended in LB + 1%NaCl and subsequently plated on LB + 1%NaCl agar plates containing 10 $\mu g\ ml^{-1}$ gentamycin or 10 $\mu g\ ml^{-1}$ chloramphenicol. Colonies were screened for single crossover events using PCR primers flanking the recombination region. Resolution of the integrated vector by a second crossover event was performed with a confirmed first crossover strain. This strain was grown in LB + 1%NaCl medium without selection and plated onto solid LB + 1%NaCl medium containing 10 per cent sucrose. Deletion events were verified by PCR using primer rdhX-F and primer rdhX-R and DNA sequencing of the resolved mutant strain.

To complement the mutant of Ssed_3769 (AS1030), the wild-type gene was reintroduced into the Ssed_3769 locus by gene replacement. This was carried out similar to the earlier mentioned method, except that the Ssed_3769 wild-type gene, and its flanking regions were cloned into pDS132 resulting in pDS132_ Ssed_3769. The mating was performed using *E. coli* WM3064-λpir and the ΔSsed_3769 (AS1030) strain instead of the wild-type strain of *S. sediminis*.

(c) RNA purification and cDNA synthesis

Total RNA was isolated from triplicate samples using the Trizol reagent protocol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA samples were treated with DNase I amplification grade (Invitrogen) according to the manufacturer's instructions to remove genomic DNA, with subsequent purification performed with an RNeasy minikit (Qiagen, Valencia, CA, USA). Electrophoretic analysis was performed

Table 2. Sequences of primers used in this study.

primer name	primer sequence
knockout primers	
rdh1_5o_*SacI*	GACTACTTGCGAGCTCGATGAGCAGAAAGTCAGTTATGG
rdh1_5i	TGAAGTTCATGTCACGGATCTCATAATGCTTAATTCTCTTCA
rdh1_3o_*SacI*	GACTACTTGCGAGCTCAAAGTACGGCATGAACTAAAT
rdh1_3i	AGATCCGTGACATGAACTTCATAACATATAGGAGAA AGAGTG
rdh1_Fo	GCAGCTTGCATACATTTACAT
rdh1_Ro	AAGCGAAGTTGAAGCACCTCT
rdh2_5o	TTACTGCAAACTATCGACAGC
rdh2_5i	AGATAGCTCCTTCAGATCCTTCATAGGGTTACCTTAATTAAC
rdh2_3o	GAGCCCCTTGGTGCAAGGCC
rdh2_3i	AAGGATCTGAAGGAGCTATCTTAGCAGGCAATGTCG GAGGCG
rdh2_Fo	TGCAGCACAGTCTGCAACTTAT
rdh2_Ro	AGGGCGACCATCAGGGCTTCA
rdh3_5o	TATAAACTTGACCTGTAACGA
rdh3_5i	CGTCATGGGTTTACAGCCGTTCATTTTCACCCTTATTACGTT
rdh3_3o	ATCGTGCAATTCAGCTTGGTT
rdh3_3i	AACGGCTGTAAACCCATGACGTAAATCTTTCTCTCCTTGCTC
rdh3_Fo	CTCACCGATGCAGGAATAGAG
rdh3_Ro	AGTTTAGTTGTTAACGTTTTC
rdh4_5o	TAAATGCTTTACAGAATGTAC
rdh4_5i	AGCATACTAGCTGTCATCACTCATTGATTACCTCTGACTATT
rdh4_3o	GGATCCCCATTGTCGATGATG
rdh4_3i	AGTGATGACAGCTAGTATGCTTAACGCAATATCCCCCGGTGT
rdh4_Fo	TAGCCTTCTATAACGCCATCA
rdh4_Ro	GGGACAGAGAGTATTGCAATA
rdh5_5o	CATTATTATCAGTCAGGGTAT
rdh5_5i	AGTAATCACTCGTTCCCAGTTCATACTTATCCCTACATCTTT
rdh5_3o	CCAAGACCTGCTTTAGCAGGC
rdh5_3i	AACTGGGAACGAGTGATTACTTGATCTCGTAAGATT GCGGCT
rdh5_Fo	GACGTTATAGCCCCAGTTTGC
rdh5_Ro	GCCTTGGACGATAACGCTTCG
qPCR primers	
rdh1_F	GCAAGCCATCTTACCCATGT
rdh1_R	CCGTGTCGGTATCGCTAAAT
rdh2_F	GTTCCAGCGTGGATCGTTAT
rdh2_R	CCCGGTTACCAGCAGATAGA
rdh3_F	GTCCCAGCGTTTATCACGTT
rdh3_R	TTGCTGGCATGGAATGAATA
rdh4_F	CGCGGTTGGTTATTCAACTT
rdh4_R	CACTTCTGGCGTCATCAAGA
rdh5_F	GGGTCCGATTAAGCCGGGCG
rdh5_R	CATCGGCGCCATCCGGTTCA
gyrA_F	CACGCAGCCCACTGATGCCT
gyrA_R	GTGGCCGAGCTGTCATGCGT

with an Agilent 2100 bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA), to assess RNA integrity. Absence of PCR amplification of a genomic region of 100 bp using primers and Phire polymerase (NEB) was determined for verification of the lack of genomic contamination. cDNA synthesis from total RNA was carried out using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Reverse transcription control reactions were performed with and without reverse transcriptase enzyme.

(d) Quantitative PCR

Quantitative PCR (qPCR) was performed with the IQ SYBR green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and iCycler iQ RT-PCR detection system (Bio-Rad Laboratories), using equal amounts of cDNA (added in 10 µl volume to a 25 µl reaction) for amplification of each gene region (*ca* 200 bp). Triplicate reactions were run for each sample with the following programme: 95.0°C for 3 min, 50 cycles of 95.0°C for 30 s, 59.0°C for 30 s, and 72.0°C, and a melt curve to determine primer specificity: 95.0 for 1 min, 55.0°C for 1 min, and 80 cycles of a stepwise increase by 0.5°C starting at 55.0°C for 10 s. Each RT-PCR was performed in triplicate based on three independent RNA extractions. A 200 bp fragment from the *gyrA* gene was amplified and used for derivation of the standard curve. Expression ratios are given as the $\log_2$-fold difference in the quantity of product from the experimental sample (WT + PCE) versus that from the control sample (WT − PCE). Data analysis was performed as previously described [27], and normalization of all expression ratios was conducted using *gyrA*.

(e) PCR amplification of putative rdh targets from DNA and cDNA

Specific primers were used to amplify an approximately 200 bp fragment of Ssed_4120, SSed_3768, Ssed_2100, Ssed_2103 and Ssed_1729 from *S. sediminis*. The PCR was performed with Dreamtaq Mastermix (Fermentas, Glen Burnie, MD, USA) using the following conditions: an initial incubation at 95°C for 5 min, followed by 30 cycles of 30 s at 95°C, 45 s at 59°C, 10 s at 72°C and a final extension of 10 min at 72°C. Genomic DNA extracted from *S. sediminis* served as positive control.

(f) Cell suspension assays

Shewanella sediminis wild-type was grown anaerobically in 30 mM fumarate and 40 mM pyruvate 4 M mineral medium as described earlier and harvested in early stationary phase (approx. 72 h). Cells were washed in phosphate buffer (5.7 mM K_2HPO_4, 3.3 mM KH_2PO_4, 2 mM $NaHCO_3$, 342 mM NaCl, pH 7.4) and resuspended in 100 mM Tris–HCl (pH 8) to reach an OD of approximately 1. Pyruvate (2.5 mM), hydrogen (0.6 bar) and reduced methyl viologen (2.5 mM) were tested as electron donors for dechlorination of PCE (2.5 mM were added to reach a saturated solution). In additional vials, fumarate (2.5 mM) was added as competitive electron acceptor. Headspace volume (500 µl) was withdrawn at different time points to monitor TCE formation via gas chromatography. Each cell suspension experiment was carried out twice with biological duplicates each time.

(g) Preparation of cell extracts

Preparation of cell extracts was performed in an anaerobic glovebox (Coy Laboratory Products, Inc., MI, USA) supplied with a gas mix of nitrogen and hydrogen (95 : 5). Anaerobically grown *S. sediminis* cells were harvested in early stationary phase (*ca* 72 h) by centrifugation for 10 min at 5000*g* at 4°C. The supernatant was removed, and the cell pellet was washed with phosphate buffer (5.7 mM K_2HPO_4, 3.3 mM KH_2PO_4, 2 mM $NaHCO_3$, 342 mM NaCl, pH 7.4) before being resuspended in 0.5 or 1 ml of anaerobic resuspension buffer (100 mM Tris–HCl, pH 8). Cells were lysed by bead beating on a vortex 10 times for 3 s with 30 s incubation periods on ice in between, and the lysis mixture was clarified by centrifugation at 122 500*g* for 50 min at 4°C. The supernatant was designated as cell-free extract and stored under nitrogen at −80°C in a butyl rubber stoppered serum vial until used.

(h) Reductive dechlorination assays

All cell extract assays were carried out in an anaerobic glove box with a N_2–H_2 (95 : 5) atmosphere using anoxic stock solutions. Dehalogenation assays were performed in 5 ml vials sealed with polytetrafluoroethylene Mininert valves. The standard assay mixture contained in a total volume of 1 ml, 100 mM Tris HCl, pH 8, 5 mM Ti(III)–NTA, 2.5 mM methyl viologen and 2.5 mM PCE were added to reach saturation concentrations. Ti(III)–NTA solution was prepared according to Moench & Zeikus [28]. The reaction was started by adding 100 µl cell extract corresponding to 300–500 µg protein. The reaction vials were incubated at room temperature in the glove box and stirred with a magnetic stirrer. Chlorinated compounds were monitored over time by taking 500 µl headspace samples with a gas-tight syringe for analysis via gas chromatography. All data represent the mean of at least two independent experiments with biological duplicates.

(i) Analytical methods

Analysis of chloroethenes was from a 500 µl headspace sample using a gas chromatograph from Hewlett–Packard (series II 5890) equipped with flame ionization detector (FID). Separation was accomplished in a DB-624 capillary column (30 m length, 0.530 µM ID, 3 µM film thickness). Helium was used as carrier gas at a flow rate of 5.1 ml min^{-1}. The injector and detector temperatures were 200°C and 280°C (FID), respectively, and analyses were isothermal at 100°C. Compounds were identified by comparison of their retention times with the retention times of external standards. Quantification of PCE and TCE was based on 16-point external calibration curves. Standards were prepared by adding appropriate ethanol stock solutions to sterilized assay buffer in vials having the same headspace : liquid ratio as the vials used in the assays.

Protein concentrations in the cell extract were determined with the Bradford assay using the Bio-Rad reagent (Bio-Rad Laboratories). Bovine serum albumin was used as the standard.

(j) Sequence analysis

Rdh gene sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov). All sequence analysis including translation of nucleotide sequences, alignments of amino acid sequences (using the ClustalW algorithm, default settings) and generation of neighbour-joining phylogenetic trees (Jukes–Cantor model, default settings) was performed with the software Geneious Pro v. 5.6.3.

3. Results

(a) Evolutionary relationship of reductive dehalogenases in *Shewanella sediminis*

Five putative Rdhs genes, Ssed_1729, Ssed_2100, Ssed_2103, Ssed_3769 and Ssed_4120 were identified in the genome of *Shewanella sediminis*, a cold temperature-adapted γ-proteobacterium isolated from marine sediments near Halifax Harbour

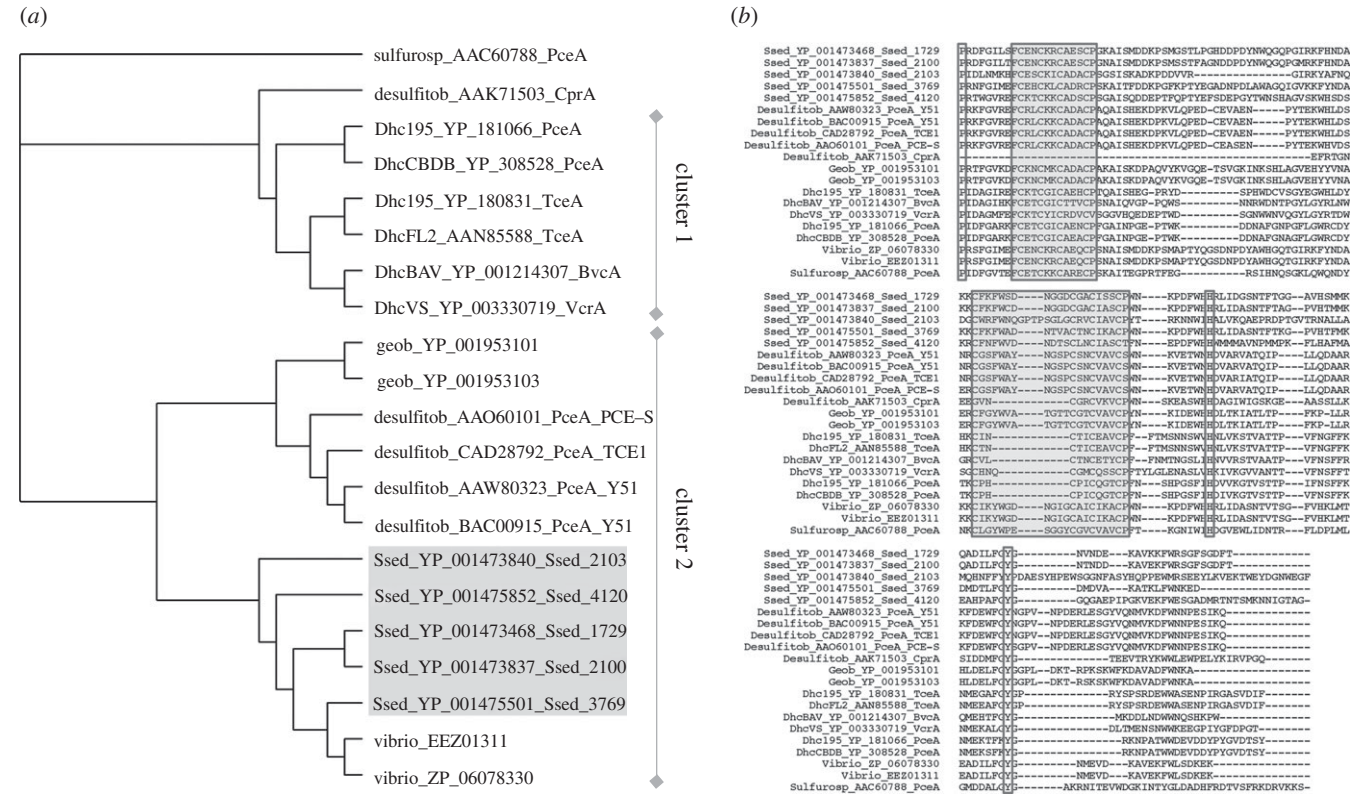

Figure 1. (*a*) Neighbour-Joining consensus tree of the deduced amino acid sequence from *S. sediminis* and a selection of organohalide-respiring bacteria. RdhA are labelled with the following abbreviations: Dhc, *Dehalococcoides*; Desulfitob, *Desulfitobacterium*; Sulfurosp, *Sulfurospirillum* and Ssed, *Shewanella*. (*b*) Multiple alignment of the C-terminal region of deduced amino acid sequences of reductive dehalogenase genes from *S. sediminis* and a range of known organohalide-respiring organisms. Consensus motifs for the iron sulfur clusters are highlighted in grey, other shared residues are marked with boxes.

(NCIMB 14036^T, DSM 17055^T, GenBank accession no. CP000821; [20]). The predicted amino acid sequences encoded by these genes were aligned with each other as well as with several putative dehalogenases identified in genomes of organohalide-respiring micro-organisms and compared. Although the predicted amino acid sequence identity among the five putative *S. sediminis* Rdhs (except for Ssed_2103) varied between 36 per cent and 47 per cent, identities to characterized Rdhs from *Dehalococcoides* sp., *Sulfurospirillum multivorans* and *Desulfitobacterium* were 12.5–17.5%, 19.5–22.5% and 23.5–26.5%, respectively (figure 1*a*). Similar to characterized Rdhs, the encoded amino acid sequences of Ssed_1729, Ssed_2100, Ssed_2103, Ssed_3769 and Ssed_4120 contained two iron sulfur binding motifs in the C-terminal region (figure 1*b*). Both motifs are equivalent to the conserved ferredoxin-type 4Fe4S consensus sequence characteristic for bacterial ferredoxins [29], with the first one being $CX_2CX_2CX_3XP$, whereas the second one exhibits a region of 10 residues between the first two cysteines (Ssed_1729, Ssed_2100, Ssed_3769, Ssed_4120Ssed) and 14 for Ssed_2103. The presence of an N-terminal twin-arginine signal peptide sequence as previously described in Rdhs was also observed in all predicted *S. sediminis* Rdhs, indicating that the mature enzymes are most likely periplasmatic and translocated as assembled holoenzymes from the cytoplasm via the TAT pathway [30,31]. A conserved amino acid sequence motif for corrinoid cofactor binding as previously derived from other corrinoid-dependent enzymes ($DXHX_2G$) could not be identified [32]. Other highly conserved amino acid residues include proline, histidine and tyrosine (position 480, 579 and 608 of the consensus sequence), whose functions are unknown so far [33,34]. Another feature common to all *S. sediminis* putative Rdhs, with the exception of Ssed_1729, is the location of a second open reading frame (putative *rdhB*) 0–15 bp downstream of the *rdhA* gene. Collectively, this sequence analysis strongly suggests that the identified putative Rdhs in *S. sediminis* represent true homologues of previously biochemically characterized Rdhs.

(b) Reductive tetrachloroethene dechlorination activity in whole cells and cell-free extract of *Shewanella sediminis*

Most of the putative Rdhs in *S. sediminis* (Ssed_1729, Ssed_2100, Ssed_3769 and Ssed_4120) were annotated as putative tetrachloroethene (PCE) Rdhs, which motivated us to initiate experiments in cell suspensions of wild-type cells to test for PCE dechlorination activity. Pyruvate, hydrogen or reduced methyl viologen was used as low-redox potential electron donors in these experiments. When reduced methyl viologen was provided as electron donor, the formation of TCE was observed that increased linearly over time, whereas neither hydrogen nor pyruvate could serve as electron donor for PCE dechlorination in whole cell suspensions. When fumarate was added in addition to PCE as competitive electron acceptor, no significant TCE was produced as the electrons from reduced methyl viologen were apparently directed towards fumarate reduction to succinate (data not shown).

In a subsequent batch culture screen, we tested whether anaerobically grown cells (10 mM fumarate, 10 mM pyruvate, 0.1% casamino acids) carry reductive dehalogenation activity towards organohalogen compounds other than PCE. Mineral medium batch cultures of *S. sediminis* (4 M) were

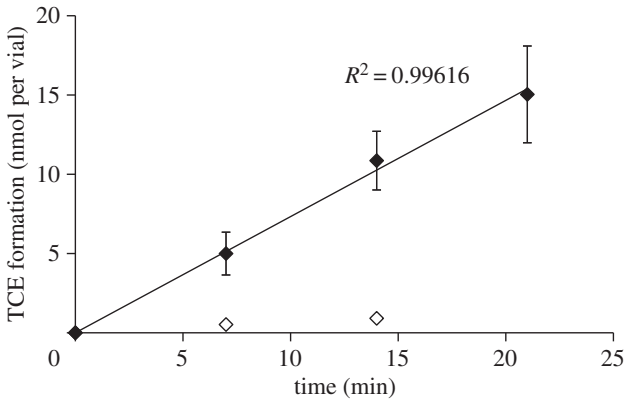

Figure 2. Product (TCE) formation from PCE by cell extracts of *Shewanella sediminis* in a standard dechlorination assay using (filled diamonds) 100 µl cell extract and (open diamonds) 100 µl heat-treated cell extract. The reaction mixture contained 100 mM Tris HCl buffer at pH 8, 2.5 mM methyl viologen, 5 mM Ti(III)–NTA and 2.5 mM PCE were added to reach saturation concentrations.

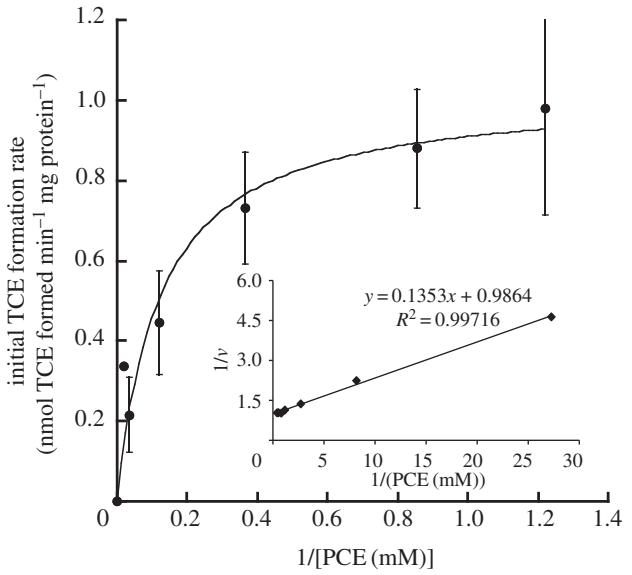

Figure 3. Kinetics of PCE dechlorination in cell extracts of *Shewanella sediminis*. Points represent initial dechlorination rates determined from individual dechlorination assays with PCE as substrate. The line represents the best fit of the data to the kinetic Michaelis–Menten model. The inset shows the Lineweaver–Burk plot. Values are means of results of at least duplicate biological experiments ± s.d.

amended with the halogenated compounds tetrachloroethene, trichloroethene, *cis*-dichloroethene, 1,1-dichloroethene, 1,1,2-trichloroethane, 1,1-dichloroethane, 1,2-dichloroethane, bromoethane, 1,2-dichloropropane, 2,2-dichloropropane, 1,2,3-trichloropropane, 2-bromo-1-chloropropane, 1,2-dibromo-3-chloropropane, chloroform, 2-chlorobutane, 2,2-dichloroethanol at concentrations ranging between 0.2 and 2.3 mM, and hydrogen as electron donor. Under these conditions, except for an abiotic 2,2-dichloropropane transformation, no catabolic dechlorination activity was observed nor was growth detected for all substrates over a period of six months (data not shown). No significant TCE formation from PCE was observed with hydrogen and pyruvate as *in vivo* electron donor as it was the case in the cell suspension experiments.

As we had identified PCE Rdh activity in whole cells, we assayed cell-free extracts of *S. sediminis* for PCE dehalogenation activity. Extracts of early stationary phase (OD approx. 0.45) *S. sediminis* cells that were grown on 30 mM fumarate and 40 mM pyruvate catalysed the reductive dechlorination of PCE to TCE with TCE being the only metabolite at a constant rate (figure 2). PCE dechlorination activity followed Michaelis–Menten kinetics as shown in figure 3. Fitting the Michaelis–Menten kinetic model to the data revealed an apparent K_m of 120 µM PCE and a v_{max} of approximately 1 nmol TCE produced min^{-1} (mg protein)$^{-1}$. In control assays without cell extract or with growth medium alone, no dechlorination activity was observed. To test whether the observed dehalogenation activity might have been due to dehalogenase-independent dechlorination by cellular corrinoid(s) alone and not to a corrinoid-containing Rdh, we tested heat-treated cell extract (10 min at 95°C) for Rdh activity. Heat-treated cell extract resulted in an at least 10-fold lower apparent activity than the non-heat-treated cell extract (figure 2). These data collectively show that the observed PCE dechlorination activity is due to a cellular Rdh activity and not to an abiotic reductive dehalogenation of PCE.

Dechlorination activity of *S. sediminis* required that vitamin B_{12} was present in the growth medium of the cells used for cell-free extract experiments as cell extracts from cells grown without cyanocobalamin in the growth medium did not show any TCE formation (data not shown). In addition, dechlorination activity occurred only in cell extracts from anaerobically grown cultures (data not shown).

(c) Induction of putative reductive dehalogenases gene expression

Although we showed above that PCE is not a catabolic substrate for *S. sediminis*, we tested whether PCE can induce Rdh activity by assaying for PCE dehalogenase activity *in vitro* in cell extracts of cells grown in a pyruvate/fumarate minimal medium that was amended with PCE. Extracts of cells grown in the presence of 0.1 mM PCE carried a 65 per cent higher specific activity than cells recovered from unamended media (1.82 ± 0.18 versus 1.19 ± 0.1 nmol min^{-1} (mg protein)$^{-1}$, respectively; figure 4).

In order to examine whether this difference in specific reductive dehalogenation activity was due to increased *rdh* gene transcription or to some post-transcriptional regulation, we prepared RNA from both PCE-amended and non-amended cultures, and tested for the presence of transcripts of Ssed_4120, Ssed_3769, Ssed_2100, Ssed_2103 and Ssed_1729 by qRT-PCR using *gyrA* as control gene for normalization. No differential regulation was seen for any of the putative Rdh genes expressed under both conditions (figure 5). Using more sensitive RT-PCR assays, we found that the transcript numbers in general were too low to obtain conclusive data from the qPCR assays. This apparent low level of *rdh* gene transcription is consistent with the low dechlorination activity we observed for PCE.

(d) Identification of the PCE reductive dehalogenase gene

In order to determine which, if any, of the five putative *rdh* genes in *S. sediminis* are associated with the PCE dehalogenation activity, we constructed markerless in-frame deletion mutations in each putative *rdh* in *S. sediminis* wild-type.

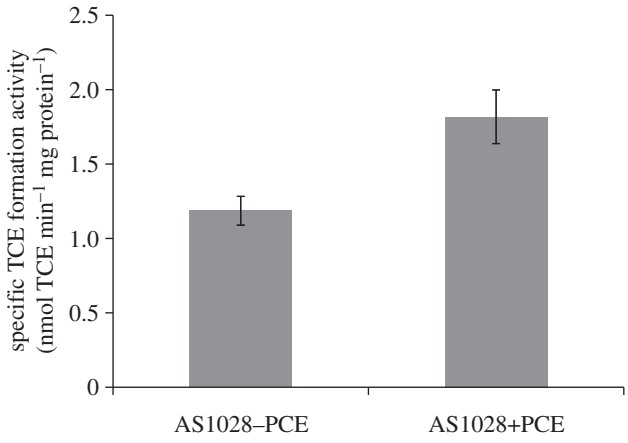

Figure 4. Specific PCE dechlorination activities in wild-type *Shewanella sediminis* cell extracts from cultures grown on 30 mM fumarate, 40 mM pyruvate and with or without addition of PCE (*ca* 0.1 mM). The assay mixtures contained 100 mM Tris–HCl buffer at pH 8, 2.5 mM methyl viologen and 5 mM Ti(III)–NTA and 2.5 mM PCE were added to reach saturation concentrations. Values are means of results of at least duplicate biological experiments $\pm$ s.d.

The resulting mutants, designated AS1029–AS1033, were tested *in vitro* for PCE dechlorination activity. Growth rate and final optical density of all mutant strains when grown on pyruvate and fumarate was indistinguishable from wild-type (figure 6*a*). Figure 6*b* shows that of all strains tested, only deletion of Ssed_3769 resulted in a significantly reduced TCE formation rate, which was about 10 times less than wild-type activity. Interestingly, although all other mutant strains catalysed PCE reduction at rates similar to wild-type, ΔSsed_2100 showed a slightly increased PCE transformation rate (1.4 ± 0.2 nmol min^{-1} (mg protein)$^{-1}$). To show unambiguously that the loss of activity in this mutant was not due to a secondary mutation, we complemented strain AS1030 (ΔSsed_3769) by introducing the wild-type Ssed_3769 allele at the chromosomal locus by homologous recombination ('knock-in'). The *in vitro* PCE reduction rate of this complemented mutant (AS1034) was restored to wild-type level (1.1 ± 0.03 nmol min^{-1} (mg protein)$^{-1}$; figure 6*b*). From these results, we concluded that the gene product of Ssed_3769 is necessary and sufficient for the *in vivo* PCE dehalogenation activity, and constitutes a functional Rdh.

4. Discussion

Previous work on reductively dehalogenating bacteria showed that a phylogenetically heterogeneous group of micro-organisms can catalyse reductive dechlorination of PCE catabolically or co-metabolically under anaerobic conditions [2]. In this study, we report the identification of an Rdh in *S. sediminis*, a marine psychrophilic γ-proteobacteria, that was isolated from an unexploded-ordnance-dumping site in the Baltic Sea, based on its ability to degrade hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) [20]. *Shewanella sediminis* clusters phylogenetically to other psychrophilic marine *Shewanella* strains that were all retrieved from deeper parts of marine ecosystems. Costal sediments, especially those close to the shore, are often a sink for anthropogenic pollutants, and Halifax Harbour has been shown to exhibit high levels of hydrocarbon and metal contamination [35]. All putative *S. sediminis rdh* sequences revealed the presence of features functionally similar to those of characterized Rdhs, and grouped into cluster 2 of the two major phylogenetic clades of RdhA sequences, which contains mainly non-*Dehalococcoides rdhA* genes [16]. Based on their amino acid sequence, they were most similar (46–58%) to a putative Rdh gene of *Vibrio* sp. RC586, another marine sediment micro-organism, isolated from Chesapeake Bay, MD, about 800 miles south of Halifax Harbour [36].

Using cell-free extract assays, we demonstrated, for the first time, reductive dechlorination of PCE to TCE in a *Shewanella* species, and were able to link this dechlorination activity to Ssed_3769, one of the putative Rdhs in *S. sediminis*. Similar to previous observations, transformation of PCE in *S. sediminis* was B_{12}-dependent based on growth experiments, which is of particular interest as most Rdhs characterized so far are corrinoid-dependent enzymes [12,13,18]. Moreover, many organohalide-respiring microbes require the addition of cyanocobalamin or B_{12} in the medium for successful growth [37]. Although there are many genes present in *S. sediminis* that are involved in the anaerobic and aerobic de novo B_{12} biosynthesis, according to BioCyc (v. 16.1) some of them are missing or not yet identified, such as *cbiK*, *cbiX*, *cbiD*, *cbiT or cobF*, *cobI*, *cobG*, *cobK* and *cobNST*, which would explain the necessity of cyanocobalamin supplementation to the growth medium for PCE dechlorination. In contrast to many catabolic organohalide-respiring bacteria, molecular hydrogen could not serve as electron donor to support *in vivo* dechlorination, in catabolic organohalide-respiring *Dehalococcides*, the HupL hydrogenase has been postulated to be the key hydrogenase coupling reductive dehalogenation of hydrogen consumption. *Shewanella sediminis* however does not contain a HupL-type hydrogenase but rather a Ni–Fe hydrogenase (Ssed_1907) that, although containing matching PROSITE patterns to an uptake hydrogenase, is only 37 per cent identical to *Dehalococcoides* HupL at the amino acid level. Moreover, under the conditions tested, PCE reduction could not support growth suggesting that PCE dechlorination is a co-metabolic process in *S. sediminis.* Co-metabolic dechlorination is mostly mediated by protein-bound tetrapyrrole cofactors such as iron(II)porphyrins, corrinoids or factor F_{430} [38]. Compared with metabolic dehalogenation, co-metabolic dechlorination processes have been shown to be ubiquitous in anaerobic bacteria such as homoacetogens or methanogens, and to proceed at much lower rates [2,39,40]. Anaerobic microbes such as *Methanosarcina* sp., and *Acetobacterium woodii* were shown to dechlorinate PCE co-metabolically at rates of 5.8×10^{-4} and 6×10^{-2} nmol min^{-1} (mg protein)$^{-1}$, respectively [41]. Metabolic PCE dechlorination rates in cell extracts of organohalide respirers range from 0.05 (PceA, *Desulfitobacterium*, strain Y51) to 1.5 μmol min^{-1} (mg protein)$^{-1}$ (PceA, *S. multivorans*) [12,14,42]. Interestingly, another *Shewanella* strain, *Shewanella oneidensis* MR1, was shown to co-metabolically dechlorinate tetrachloromethane (CT) to trichloromethane (CF) using *c*-type cytochromes produced during microaerophilic growth [43,44]. However, PCE could not be degraded by this strain, and the gene required for CT dehalogenation was not identified. Although for *S. sediminis* the v_{max} for PCE dehalogenation of about 1 nmol min^{-1} (mg protein)$^{-1}$ was about two to three orders of magnitude lower than previously reported PCE dechlorination activities in *Dehalococoides* or *Desulfitobacterium*, it was still significantly higher than the observed rates for co-metabolic PCE transformation in

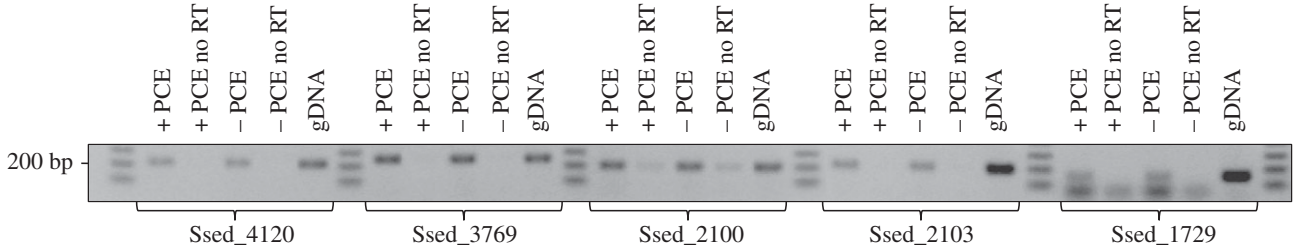

Figure 5. Agarose gel picture of RT-PCR products obtained with Ssed_4120, Ssed_3769, Ssed_2100, Ssed_2103 and Ssed_1729 specific primers from *Shewanella sediminis* cultures with and without supplementation of PCE to the growth medium. The approximate size of the respective amplicons according to a marker is given on the left. The same amount of cDNA was added to each reaction. Genomic DNA (gDNA) of *S sediminis* served as positive control.

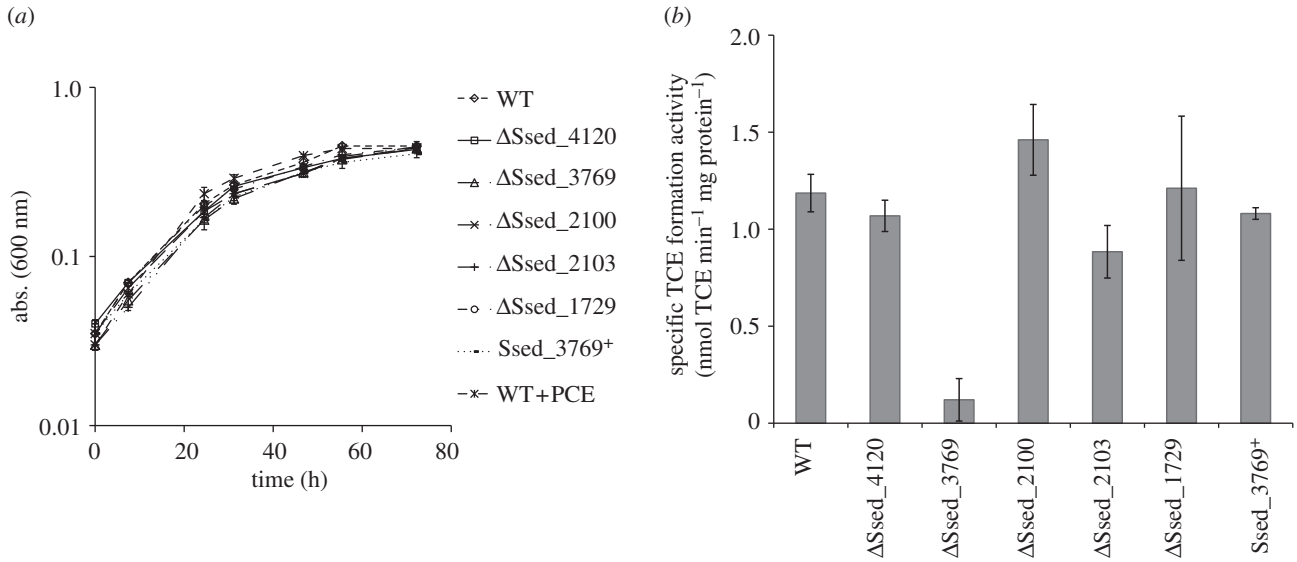

Figure 6. Effect of ΔSsed_4120, ΔSsed_3769, ΔSsed_2100, ΔSsed_2103 and ΔSsed_1729 deletions on (*a*) growth in 4 M minimal medium with 30 mM fumarate as electron acceptor and 40 mM pyruvate as electron donor and (*b*) PCE dechlorination activity in cell extracts from cells harvested at early stationary phase. The assay mixtures contained 100 mM Tris HCl buffer at pH 8, 2.5 mM methyl viologen and 5 mM Ti(III)–NTA and 2.5 mM PCE were added to reach saturation concentrations. Growth was measured by optical density at 600 nm (abs. (600 nm)). Values are means of results of at least duplicate biological experiments ± s.d.

methanogens or homoacetogens. The relatively high apparent K_m for PCE suggests that PCE may not be the natural substrate for Ssed_3769. Thus, other yet unidentified organohalogens in the sediment might be the primary substrate for Ssed_3769, and perhaps the other putative Rdhs, although we have not shown yet that they are functional.

While the organohalogens tested here as substrates for reductive dehalogenation in *S. sediminis* structurally resemble the most common anthropogenic halogenated compounds, there are many naturally occurring organohalogens that are not commercially available and therefore difficult to test as substrates for these Rdhs. This explains partially the so far limited spectrum of substrates identified for the catabolic organohalogen-respiring bacteria such as *Dehalococcoides mccartyi* or other *Dehalococcoides*-like *Chloroflexi* present in pristine environments [8,9]. According to Gribble [45,46], marine life is the largest source of naturally occurring organohalogen compounds. However, many organohalogens characterized from pristine marine environments are more complex, aromatic compounds, including brominated compounds such as indoles, phenols or pyrroles [45–47], which we did not include in our dehalogenation screen due to a lack in availability. On the other hand, PCE and TCE have been shown to be produced in various organisms such as algae, plants, bacteria or mammals [47,48]. In a recent study, it has been demonstrated that a cocktail of enzymatically generated chlorinated compounds from organic soil matter supported growth of *Dehalococcoides*-like *Chloroflexi* [8]; these undefined chlorinated organic compounds may function as terminal electron acceptors for these micro-organisms.

Dechlorination processes in sediments, especially in deep sea sediments, not only play an important role in the natural chlorine cycle but also have implications for the organic carbon flux in these environments. Organohalogens are organic carbon compounds that are typically inaccessible for most known heterotrophic micro-organisms because they are unable of reductive dechlorination. Yet, the vast majority of the members of deep sea microbial communities most likely depend on catabolizing organic compounds. Microbes such as *S. sediminis* may represent a group of micro-organisms that can uniquely access organic carbon resources locked as organohalogens in sediments that may not be available to non-dehalogenating heterotrophic microbes. Thus, *S. sediminis* might use the dehalogenated organic compounds eventually as catabolic electron donor rather than the organohalogens as catabolic electron acceptor. A variety of simple and complex organic compound degradation pathways such as for ethanol, glycerol, aldehyde or

phenylacetate degradation present in its genome, might be involved in the utilization of these dehalogenated organic compounds anaerobically, fermentative or under metal reducing conditions.

This work was supported by the Strategic Environmental Research Defense Project (SERDP) grant no. ER-1588 and an ENI grant to A.M.S. S.T.L. was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft), fellowship no. Lo 1597/1-1. We thank Frank E. Löffler for helpful discussions.

References

1. Smidt H. 2004 Anaerobic microbial dehalogenation. *Annu. Rev. Microbiol.* **58**, 43–73. (doi:10.1146/annurev.micro.58.030603.123600)
2. Damborský J. 1999 Tetrachloroethene-dehalogenating bacteria. *Folia Microbiol.* **144**, 247–262. (doi:10.1007/BF02818543)
3. Shivakumara S *et al*. 2012 Complete genome sequence of *Dehalogenimonas lykanthroporepellens* type strain (BL-DC-9T) and comparison to '*Dehalococcoides*' strains. *Standards Genomic Sci.* **6**, 251–264. (doi:10.4056/sigs.2806097)
4. Seshadri R, Adrian L, Fouts D, Eisen J. 2005 Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*. *Science* **307**, 105–108. (doi:10.1126/science.1102226)
5. Mohn W, Tiedje J. 1992 Microbial reductive dehalogenation. *Microbiol. Rev.* **56**, 482–507.
6. Bhatt P, Kumar MS, Mudliar S, Chakrabarti T. 2007 Biodegradation of chlorinated compounds: a review. *Crit. Rev. Environ. Sci. Technol.* **37**, 165–198. (doi:10.1080/10643380600776130)
7. Tas N, van Eekert MHA, de Vos WM, Smidt H. 2010 The little bacteria that can—diversity, genomics and ecophysiology of '*Dehalococcoides*' spp. in contaminated environments. *Microbial Biotechnol.* **3**, 389–402. (doi:10.1111/j.1751-7915.2009.00147.x)
8. Krzmarzick MJ, Crary BB, Harding JJ, Oyerinde OO, Leri AC, Myneni SCB, Novak PJ. 2012 Natural niche for organohalide-respiring *Chloroflexi*. *Appl. Environ. Microbiol.* **78**, 393–401. (doi:10.1128/AEM.06510-11)
9. Futagami T, Morono Y, Terada T, Kaksonen AH, Inagaki F. 2009 Dehalogenation activities and distribution of reductive dehalogenase homologous genes in marine subsurface sediments. *Appl. Environ. Microbiol.* **75**, 6905–6909. (doi:10.1128/AEM.01124-09)
10. McMurdie PJ, Hug LA, Edwards EA, Holmes S, Spormann AM. 2011 Site-specific mobilization of vinyl chloride respiration islands by a mechanism common in *Dehalococcoides*. *BMC Genomics* **12**, 287. (doi:10.1186/1471-2164-12-287)
11. Smidt H, Akkermans A, Van Der Oost J, de Vos W. 2001 Molecular characterisation of key enzymes in halorespiration. In *Biotechnology for the environment: strategy and fundamentals* (eds S Agathos, W Reineke), pp. 23–46. Dordrecht, The Netherlands: Kluwer Academic Publishers.
12. Wohlfarth G, Diekert G. 1999 Reductive dehalogenases. In *Chemistry and biochemistry of B_{12}* (ed. R Banerjee), pp. 871–893. New York, NY: John Wiley & Sons Inc.
13. Müller JA, Rosner BM, Abendroth von G, Meshulam-Simon G, Mccarty PL, Spormann AM. 2004 Molecular identification of the catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. *Appl. Environ. Microbiol.* **70**, 4880–4888. (doi:10.1128/AEM.70.8.4880-4888.2004)
14. Holliger C, Wohlfarth G, Diekert G. 1998 Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiol. Rev.* **22**, 383–398. (doi:10.1111/j.1574-6976.1998.tb00377.x)
15. Smidt H, Akkermans ADL, Van Der Oost J, de Vos WM. 2007 Halorespiring bacteria—molecular characterization and detection. *Enzyme Microbial Technol.* **27**, 812–820. (doi:10.1016/S0141-0229(00)00316-1)
16. McMurdie PJ *et al*. 2009 Localized plasticity in the streamlined genomes of vinyl chloride respiring *Dehalococcoides*. *PLoS Genet.* **5**, e1000714. (doi:10.1371/journal.pgen.1000714)
17. Kube M, Beck A, Zinder S, Kuhl H, Reinhardt R, Adrian L. 2005 Genome sequence of the chlorinated compound respiring bacterium *Dehalococcoides* species strain CBDB1. *Nat. Biotechnol.* **23**, 1269–1273. (doi:10.1038/nbt1131)
18. Adrian L, Rahnenfuhrer J, Gobom J, Hölscher T. 2007 Identification of a chlorobenzene reductive dehalogenase in *Dehalococcoides* sp. strain CBDB1. *Appl. Environ. Microbiol.* **73**, 7717–7724. (doi:10.1128/AEM.01649-07)
19. Waller AS, Krajmalnik-Brown R, Löffler FE, Edwards EA. 2005 Multiple reductive-dehalogenase-homologous genes are simultaneously transcribed during dechlorination by *Dehalococcoides*-containing cultures. *Appl. Environ. Microbiol.* **71**, 8257–8264. (doi:10.1128/AEM.71.12.8257-8264.2005)
20. Zhao J-S, Manno D, Beaulieu C, Paquet L, Hawari J. 2005 *Shewanella sediminis* sp. nov., a novel Na^+-requiring and hexahydro-1,3,5-trinitro-1,3,5-triazine-degrading bacterium from marine sediment. *Int. J. Syst. Evol. Microbiol.* **55**, 1511–1520. (doi:10.1099/ijs.0.63604-0)
21. Cordova CD, Schicklberger MFR, Yu Y, Spormann AM. 2011 Partial functional replacement of CymA by SirCD in *Shewanella oneidensis* MR-1. *J. Bacteriol.* **193**, 2312–2321. (doi:10.1128/JB.01355-10)
22. Miller VL, Mekalanos JJ. 1988 A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. *J. Bacteriol.* **170**, 2575–2583.
23. Saltikov CW, Newman DK. 2003 Genetic identification of a respiratory arsenate reductase. *Proc. Natl Acad. Sci. USA* **100**, 10 983–10 988. (doi:10.1073/pnas.1834303100)
24. Simon R, Priefer UA, Puhler A. 1983 A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1**, 784–791. (doi:10.1038/nbt1183-784)
25. Wan XF *et al*. 2004 Transcriptomic and proteomic characterization of the Fur modulon in the metal-reducing bacterium *Shewanella oneidensis*. *J. Bacteriol.* **186**, 8385–8400. (doi:10.1128/JB.186.24.8385-8400.2004)
26. Philippe N, Alcaraz J-P, Coursange E, Geiselmann J, Schneider D. 2004 Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. *Plasmid* **51**, 246–255. (doi:10.1016/j.plasmid.2004.02.003)
27. Livak K, Schmittgen T. 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method. *Methods* **25**, 402–408. (doi:10.1006/meth.2001.1262)
28. Moench TT, Zeikus J. 1983 An improved preparation method for a titanium (III) media reductant. *J. Microbiol. Methods* **1**, 199–202. (doi:10.1016/0167-7012(83)90024-6)
29. Bruschi M, Guerlesquin F. 1988 Structure, function and evolution of bacterial ferredoxins. *FEMS Microbiol. Rev.* **54**, 155–176. (doi:10.1111/j.1574-6968.1988.tb02741.x)
30. Palmer T, Sargent F, Berks B. 2005 Export of complex cofactor-containing proteins by the bacterial Tat pathway. *Trends Microbiol.* **13**, 175–180. (doi:10.1016/j.tim.2005.02.002)
31. Futagami T, Goto M, Furukawa K. 2008 Biochemical and genetic bases of dehalorespiration. *Chem. Rec.* **8**, 1–12. (doi:10.1002/tcr.20134)
32. Ludwig ML, Matthews RG. 1997 Structure-based perspective on B_{12}-dependent enzymes. *Annu. Rev. Biochem.* **66**, 269–313. (doi:10.1146/annurev.biochem.66.1.269)
33. Marsh A, Ferguson D. 1997 Knowledge-based modeling of a bacterial dichloromethane dehalogenase. *Proteins* **28**, 217–226. (doi:10.1002/(SICI)1097-0134(199706)28:2<217::AID-PROT10>3.0.CO;2-L)
34. Damborský J, Koča J. 1999 Analysis of the reaction mechanism and substrate specificity of haloalkane dehalogenases by sequential and structural comparisons. *Protein Eng.* **12**, 989–998. (doi:10.1093/protein/12.11.989)
35. Gearing J, Buckley D, Smith J. 1991 Hydrocarbon and metal contents in a sediment core from Halifax Harbor: a chronology of contamination.

Can. J. Fish Aquat. Sci. **48**, 2344–2354. (doi:10.1139/f91-275)

36. Haley BJ *et al*. 2010 Comparative genomic analysis reveals evidence of two novel *Vibrio* species closely related to *V. cholerae*. *BMC Microbiol.* **10**, 154. (doi:10.1186/1471-2180-10-154)
37. He J, Holmes VF, Lee PKH, Alvarez-Cohen L. 2007 Influence of vitamin B_{12} and cocultures on the growth of *Dehalococcoides* isolates in defined medium. *Appl. Environ. Microbiol.* **73**, 2847–2853. (doi:10.1128/AEM.02574-06)
38. Gantzer CJ, Wackett LP. 1991 Reductive dechlorination catalyzed by bacterial transition-metal coenzymes. *Environ. Sci. Technol.* **25**, 715–722. (doi:10.1021/es00016a017)
39. Terzenbach DP, Blaut M. 1994 Transformation of tetrachloroethylene to trichloroethylene by homoacetogenic bacteria. *FEMS Microbiol. Lett.* **123**, 213–218. (doi:10.1111/j.1574-6968.1994.tb07224.x)
40. Fathepure BZ, Nengu J, Boyd SA. 1987 Anaerobic bacteria that dechlorinate perchloroethene. *Appl. Environ. Microbiol.* **53**, 2671–2674.
41. Fetzner S. 1998 Bacterial dehalogenation. *Appl. Microbiol. Biotechnol.* **50**, 633–657. (doi:10.1007/s002530051346)
42. Fetzner S, Lingens S. 1994 Bacterial dehalogenases: biochemistry, genetics, and biotechnical applications. *Microbiol. Rev.* **58**, 641–685.
43. Picardal F, Arnold R, Couch H, Little A, Smith M. 1993 Involvement of cytochromes in the anaerobic biotransformation of tetrachloromethane by *Shewanella putrefaciens* 200. *Appl. Environ. Microbiol.* **59**, 3763–3770.
44. Ward MJ, Fu QS, Rhoads KR, Yeung CHJ, Spormann AM, Criddle CS. 2004 A derivative of the menaquinone precursor 1,4-dihydroxy-2-naphthoate is involved in the reductive transformation of carbon tetrachloride by aerobically grown *Shewanella oneidensis* MR-1. *Appl. Microbiol. Biotechnol.* **63**, 571–577. (doi:10.1007/s00253-003-1407-3)
45. Gribble GW. 1998 Naturally occurring organohalogen compounds. *Acc. Chem. Res.* **31**, 141–152. (doi:10.1021/ar9701777)
46. Gribble GW. 1992 Naturally occurring organohalogen compounds: a survey. *J. Nat. Prod.* **55**, 1353–1395. (doi:10.1021/np50088a001)
47. Vetter W, Gribble G. 2007 Anthropogenic persistent organic pollutants: lessons to learn from halogenated natural products. *Environ. Toxicol. Chem.* **26**, 2249–2252. (doi:10.1897/06-615R.1)
48. Cabrita MT, Vale C, Rauter AP. 2010 Halogenated compounds from marine algae. *Mar. Drugs* **8**, 2301–2317. (doi:10.3390/md8082301)