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Physiological Adaptation of *Desulfitobacterium hafniense* Strain TCE1 to Tetrachloroethene Respiration▽†

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***Desulfitobacterium* spp. are ubiquitous organisms with a broad metabolic versatility, and some isolates have the ability to use tetrachloroethene (PCE) as terminal electron acceptor. In order to identify proteins involved in this organohalide respiration process, a comparative proteomic analysis was performed. Soluble and membrane-associated proteins obtained from cells of *Desulfitobacterium hafniense* strain TCE1 that were growing on different combinations of the electron donors lactate and hydrogen and the electron acceptors PCE and fumarate were analyzed. Among proteins increasingly expressed in the presence of PCE compared to fumarate as electron acceptor, a total of 57 proteins were identified by mass spectrometry analysis, revealing proteins involved in stress response and associated regulation pathways, such as PspA, GroEL, and CodY, and also proteins potentially participating in carbon and energy metabolism, such as proteins of the Wood-Ljungdahl pathway and electron transfer flavoproteins. These proteomic results suggest that *D. hafniense* strain TCE1 adapts its physiology to face the relative unfavorable growth conditions during an apparent opportunistic organohalide respiration.**

The first members of the genus *Desulfitobacterium* have been isolated as organohalide-respiring bacteria able to use chlorinated aliphatic (chloroethenes and -ethanes) and/or aromatic compounds as terminal electron acceptor (6, 19, 39). The variety of environments from which *Desulfitobacterium* strains have been isolated suggests that they are ubiquitous organisms (for a review, see reference 34). Their ability to use such toxic chlorinated compounds as electron acceptors has probably allowed them to colonize a number of particular environmental niches. However, they survive probably thanks to their metabolic versatility using various nonchlorinated electron acceptors, such as fumarate, nitrate, sulfite, thiosulfate, humic acids, and metals (reviewed in reference 39).

The recently sequenced genomes of *Desulfitobacterium hafniense* strains Y51 (NCBI reference strain NC_007907) (23) and DCB-2 (NCBI reference strain NC_011830) (DOE Joint Genome Institute) have unraveled additional aspects of the metabolic versatility of this genus. A total of 59 members of the conserved iron-sulfur molybdoenzyme (CISM) family (28), previously known as the dimethyl sulfoxide (DMSO) reductase family, have been identified in the genome of strain Y51, among which are enzymes with a possible role in anaerobic respiration of DMSO, trimethylamine *N*-oxide, polysulfide, selenate, and arsenate. This genome also contains 18 paralogues of the fumarate reductase, indicating a gene reservoir far beyond the recognized physiological capabilities of *Desulfitobacterium* spp., especially with respect to their already considerable respiratory flexibility.

Anaerobic respiration implies the establishment of a structured electron transport chain within the cytoplasmic membrane enabling energy conservation via the proton motive force, and the electron transport chains can sometimes be rather simple in structure (33). From a variety of organohalide respirers, including *Desulfitobacterium*, *Dehalobacter*, *Dehalococcoides*, and *Sulfurospirillum*, the terminal reductase, the reductive dehalogenase, has been identified and characterized on the protein level (39). However, many aspects of the chloroethene respiration chain still remain to be elucidated. Only little biochemical information is available for additional components of the respiratory chain and for additional proteins supporting this anaerobic respiration pathway (31) (12, 30, 37). Furthermore, the fact that tetrachloroethene (PCE)-respiring bacteria are growing only at concentrations below saturation indicates a toxic effect of PCE, such as potential detrimental effects on the cell envelope. This implies that these microorganisms have developed still unknown physiological strategies to face the associated stress.

One way to investigate and identify metabolic pathways and physiological adaptations involved in a specific biological process is the comparative proteomic analysis by two-dimensional (2D) gel electrophoresis (GE), followed by protein identification by mass spectrometry (MS) and bioinformatics. This approach has been promoted and facilitated by the release of the genome sequence of *D. hafniense* strain Y51, a strain very closely related to *D. hafniense* strain TCE1, the model organism used in this study. The gel-based proteomic analysis represents a powerful tool to study bacterial physiology from a complex protein mixture, but it remains a limited approach to

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detect regulatory proteins due to their low abundance and membrane proteins because of their high hydrophobicity (2, 25, 29). Thus, the analysis and separation of integral membrane proteins, especially those containing several α-helical transmembrane domains, are not efficient with the gel-based electrophoretic method.

Despite the fact that some subfractions of proteins such as hydrophobic membrane proteins will not be visualized by the proteomic approach, 2D-GE analysis was performed on soluble and membrane-associated proteins obtained from cells of *D. hafniense* strain TCE1 that were growing on different combinations of the electron donors lactate and hydrogen and the electron acceptors PCE and fumarate. The 2D-GE analysis indicated a stress response to the toxicity of PCE and showed the protein pool associated with PCE respiration, highlighting pathways supporting growth on and allowing degradation of PCE.

MATERIALS AND METHODS

Cultivation of *Desulfitobacterium hafniense* strain TCE1. *Desulfitobacterium hafniense* strain TCE1 (DSM 12704, formerly *Desulfitobacterium frappieri* strain TCE1) was cultivated anaerobically in liquid batch cultures consisting of medium 717, recommended by the Deutsche Sammlung für Mikroorganismen (DSMZ; Braunschweig, Germany), with few modifications. Both vitamin solutions (DSMZ, media 141 and 503) were combined, and the concentration of vitamin B_{12} was increased 2.5-fold. The trace element solution was taken from the *Dehalobacter restrictus* medium (DSMZ, solution SL-10, medium 320). As electron donor, either lactate was added to a final concentration of 45 mM or hydrogen in the gas phase (80% H_2/20% CO_2) was added with 2 mM acetate as the carbon source. As terminal electron acceptor, the medium was amended either with 20 mM fumarate or with PCE by adding 10 ml/liter of a 2 M stock solution in hexadecane, resulting in an organic/aqueous biphasic system ensuring a continuous supply of 0.4 mM PCE to the aqueous phase. The different growth conditions were obtained by combining electron donors and acceptors as follows: hydrogen-fumarate (HF), hydrogen-PCE (HP), lactate-fumarate (LF), and lactate-PCE (LP). In addition, the effect of hexadecane was tested by cultivating strain TCE1 on fumarate in the presence of hexadecane. For each condition, three independent batch cultures were prepared. Fresh medium was inoculated with 1% (vol/vol) of cultures cultivated on the respective substrates for many transfers (more than a year of weekly transfers) and incubated in the dark at 30°C with gentle agitation (rotary shaker at 80 rpm).

Sample preparation and 2D-GE. *D. hafniense* strain TCE1 cells were harvested in exponential growth phase by centrifugation at 5,000 × *g* and 4°C for 15 min. Cell pellets were washed in 20 mM Tris-HCl buffer (pH 7.5) and resuspended in the same buffer containing a protease inhibitor cocktail (Complete; Roche) and a few crystals of DNase I (Roche). Cells were disrupted by sonication with 10 bursts of 5 s at 40 W and with 5 to 10 min incubation on ice after each sonication cycle to minimize heating. Unbroken cells and debris were removed by centrifugation at 4,000 × *g* and at 4°C for 10 min. The supernatant was collected and ultracentrifuged for 1 h at 200,000 × *g* and 4°C. The supernatant was considered the soluble protein fraction (SF). The membrane protein fraction was obtained by resuspending and stirring the pellet in membrane extraction buffer. Different combinations of detergents and chaotropes, including Triton X-100, Nonidet P-40, urea, thiourea, and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), were tested. The optimized membrane extraction buffer finally contained 7 M urea, 2 M thiourea, 4% (wt/vol) CHAPS, and 1% Triton X-100, similar to the buffer described previously (26). After a second ultracentrifugation step, the supernatant was collected and considered the membrane protein fraction (MF). SF and MF were subsequently analyzed in separate 2D gels. The protein concentration was determined by the Bradford assay (Bio-Rad) using bovine serum albumin as the standard.

For the first dimension, isoelectric focusing (IEF) was performed using 24-cm immobilized strips with a nonlinear pH 3 to 11 gradient (GE Healthcare). A total protein extract of 450 μg was mixed with 60 mM dithiothreitol (DTT) and 0.5% Immobiline pH gradient (IPG) buffer containing carrier ampholytes (GE Healthcare). Protein samples were centrifuged at 8,000 × *g* for 5 min. The supernatant was loaded onto IPG strips in the strip holder (IPGphor; GE Healthcare). IPG dry strips were first rehydrated for 12 h at 50 V in the presence of the protein sample. IEF was performed in three steps: (i) 1 h at 500 V, (ii) 1 h at 1,000 V, and (iii) 8.3 h at 8,000 V. Prior to the second electrophoresis dimension, IPG gel strips were equilibrated for 10 min in 10 ml equilibration buffer (50 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 65 mM DTT). IPG strips were transferred to a 12.5% SDS-polyacrylamide gel (18 by 24 cm). The strips were sealed with 0.5% agarose in electrophoresis buffer (25 mM Tris-HCl buffer [pH 8.3], 192 mM glycine, and 0.1% SDS). Second-dimension gels were run overnight at 2 W/gel. Proteins were visualized colorimetrically with Coomassie blue G250 or by fluorescence with Deep Purple protein stain, according to the manufacturer's instructions (GE Healthcare), and images were captured by using an Agfa imaging system and Fotolook 32 software (version 3.60.0; Agfa) and a Typhoon scanner (GE Healthcare), respectively.

Image analysis. Image analysis was performed using the software ImageMaster 2D Platinum (version 6.0; GE Healthcare). After automatic protein detection and matching, the gels were manually corrected to remove wrongly assigned or duplicated spots and image artifacts. Differential protein expression patterns were obtained by matching and comparing individual protein spots from all growth conditions. Two replicate gels from three independent cultures were run for each growth condition (see Fig. S1 in the supplemental material). After comparison of the protein patterns obtained with Coomassie blue staining, both protein subfractions of two cultures, for each growth condition, were chosen for fluorescence staining and quantification. The quantification was performed on one gel, revealing the higher number of proteins for each condition. The normalized protein amount for each protein spot was defined as the fraction of its relative volume (% vol) corresponding to the percentile intensity of the spot to the total volume of all spots present in the same gel. Spots having a volume lower than 0.1% within a gel were not considered in the analysis. Furthermore proteins were taken to be differentially expressed between two culture conditions if their % volumes differed more than 4-fold.

Protein identification by LC-IT-MS/MS. MS analysis was performed according to the protocol developed by the Protein Core Facilities (PCF; EPFL, Lausanne, Switzerland). Protein identification was obtained by MS/MS analysis. Briefly, each protein spot was excised, washed twice with 50% ethanol in 50 mM NH_4HCO_3 (pH 8.4), and dried with a SpeedVac concentrator. Gel pieces were covered with 25 to 30 μl of a trypsin solution at 12.5 ng · μl^{-1} in 50 mM NH_4HCO_3 (pH 8.4) containing 10 mM $CaCl_2$, incubated for 20 min on ice, covered again with 50 mM NH_4HCO_3 (pH 8.4), and digested overnight at 37°C. Peptides were extracted by two washes with 5% formic acid and 70% ethanol. After concentration, samples were resuspended in 100% acetonitrile and dried completely. Samples were finally resuspended in 20 μl of 2% acetonitrile and 20% formic acid and injected in a reverse-phase liquid chromatography (LC) apparatus for peptide separation at a flow rate of 400 nl · min^{-1} on an Agilent Chip C_{18} capillary column (75 μm [inner diameter] by 43 mm). MS analysis was performed on a Bruker HCT ion-trap (IT) instrument. MS/MS spectra were acquired in data-dependent acquisition mode. Each full mass spectrum was followed by one MS/MS spectrum of the two ions with the highest intensity. The peptide mass list generated was submitted to the Mascot search engine (version 2.2.01) using a dedicated *D. hafniense* strain Y51 protein database. All samples were identified with a good level of confidence with a minimum number of two matched peptides and with 15% minimal protein coverage.

RESULTS AND DISCUSSION

2D-gel electrophoresis was performed on the proteome of *D. hafniense* strain TCE1 to identify proteins increasingly expressed on PCE compared to the alternative electron acceptor fumarate. Lactate and hydrogen were used as alternative electron donors. For the ease of analysis, the proteome of *D. hafniense* strain TCE1 was divided into soluble and membrane proteins. The extraction of very hydrophobic proteins remains, however, a great challenge when a buffer is kept compatible with proteomic analysis and has still not really been solved (2, 25). The membrane fraction therefore contained only proteins with at most one transmembrane helix.

Overview of increasingly expressed proteins in the presence of PCE. Fluorescence staining revealed a total of 302 ± 22 and 305 ± 32 spots per gel for soluble and membrane proteins, respectively, hence, a total of approximately 600 proteins (see

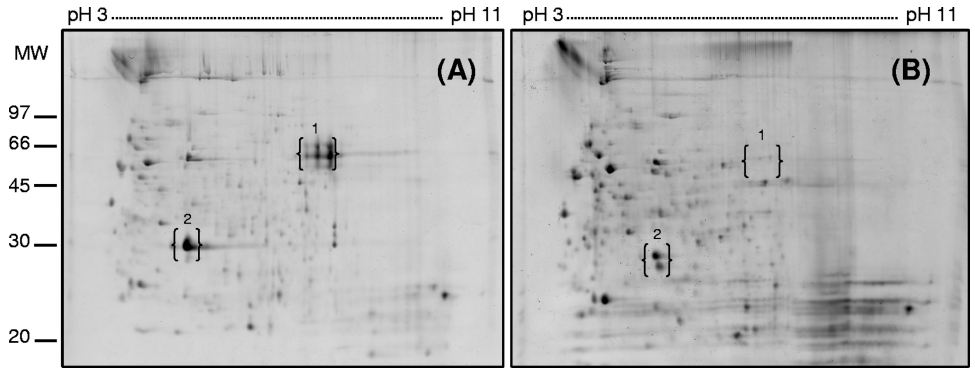

FIG. 1. Representative 2D gels of membrane protein fractions from *D. hafniense* strain TCE1 cultivated on lactate-PCE (A) and lactate-fumarate (B). The numbers in braces indicate the position of the isoforms of PCE reductive dehalogenase (PceA) ({1}) and a sulfur transferase ({2}), two examples of proteins increasingly expressed in the presence of PCE. MW, molecular weight in thousands.

Fig. S2 in the supplemental material), representing about 15% of the theoretical proteome (5,060 proteins). However, according to a virtual 2D protein gel realized with the *D. hafniense* strain Y51 database and JVirGel software (10), a maximum of 2,423 proteins could be visualized in a pH and size range of 3 to 11 and 15 to 200 kDa, respectively. Two-thirds of these proteins correspond to membrane proteins, with 699 proteins containing two or more transmembrane helices. This indicates that the gels obtained here reached a coverage of at least 35% of the theoretically detectable protein pool.

In the following, the LP gel (Fig. 1A) was compared to the LF gel (Fig. 1B), and similarly, the HP gel was compared to the HF gel (see Fig. S2 in the supplemental material). According to the criteria chosen for comparative analysis, 72 and 93 membrane protein spots and 49 and 12 soluble protein spots with increased intensity compared to their fumarate counterparts were observed on LP and HP, respectively. Gels performed with the proteome of a culture cultivated on lactate and fumarate in the presence of hexadecane showed the same protein pattern as LF gels, indicating that hexadecane had no dramatic effect on the proteome of strain TCE1 (see Fig. S1 in the supplemental material). Since some of the proteins were recovered as increasingly expressed proteins in the soluble as well as the membrane protein fractions, the results of the analysis of both fractions were combined and are presented below. Proteins downregulated in the presence of PCE were not considered here.

A total of 57 distinct proteins with at least 4-fold increased expression on PCE compared to that on fumarate as the electron acceptor were identified (Table 1). The distribution of these proteins into functional categories showed that most of them are involved in carbon metabolism (11) and energy production (9), followed by proteins involved in transcription and translation, secretion and transport, and cell wall and membrane metabolism (Fig. 2).

The key enzymes in PCE respiration. The PCE reductive dehalogenase PceA, the key enzyme in PCE respiration, represents the enzyme with the most striking difference in the overall proteome of cells growing on PCE instead of fumarate. Indeed, PceA consisted, on average, of 10 to 17% of total extracted membrane proteins and showed a 70- to 100-fold increased expression level. Although this result was intuitively expected, it was unforeseen, as the identified promoter upstream of the *pceA* gene is partly encoded in the insertion sequence of the composite transposon *Tn*-Dha1 in strain TCE1 and does not exhibit any obvious regulatory element (20). The absence of regulation of *pceA* at the mRNA level after a pulse of PCE in a continuous culture of strain TCE1 with fumarate as electron acceptor corroborated this hypothesis (see Fig. S3 in the supplemental material). The strong difference in PceA expression observed in proteomic gels was therefore probably the consequence of the long-term cultivation of strain TCE1 in the absence of PCE, resulting in a decrease of *pceA* gene copy number within the total TCE1 population. The long lag phase observed in a batch culture on PCE of a long-term fumarate-adapted inoculum compared with that of a PCE-adapted inoculum and the apparent increase in *pceA* gene copy number also suggested that the long-term fumarate-adapted inoculum contained only a small subpopulation where the *pceA* gene was still present (Fig. 3). Only this subpopulation is able to grow with PCE and was predominant within the overall *D. hafniense* population upon cultivation with this electron acceptor.

PceA was detected in at least seven different isoforms on 2D gels with variations in both charge (pI; horizontal axis in Fig. 1) and molecular mass (vertical axis in Fig. 1). Posttranslational modifications (PTMs), such as phosphorylation and glycosylation, could be considered for these isoforms. However, there is no evidence yet of the occurrence of PTMs on PceA.

None of the other products of the *pce* gene cluster was identified to be preferentially expressed on the gels of PCE-grown cells. The predicted high hydrophobicity of PceB and PceC probably explains their absence. Since PceT seems to function as a dedicated molecular chaperon for PceA (21), it does not need to be present in stoichiometric amounts and was therefore probably present in amounts too small to be detected.

Proteins involved in stress response and regulatory pathways. Five distinct increasingly expressed proteins in cells cultivated on PCE belong to functional categories linked to stress response and/or regulatory pathways (categories replication and repair, transcription and translation, cell wall and membrane metabolism, and posttranslational modification and chaperones in Table 1 and Fig. 2). Indeed, a DEAD/DEAH box RNA helicase (DSY1954) and the homologue of the well-

TABLE 1. Proteins from *Desulfitobacterium hafniense* strain TCE1 cells identified in 2D gels with an increase in PCE/fumarate relative volume ratio of at least 4-fold

Functional category[a]	LP/LF ratio (LP % vol)[b]	HP/HF ratio (HP % vol)[c]	*D. hafniense* Y51 locus	Predicted protein function	Predicted TMH[d]
EC	20 (0.87) 69 (17.69)[e]	31 (0.77) 102 (10.29)[e]	DSY2839	PCE reductive dehalogenase (PceA)	0
TT	26 (0.76)	27 (0.79)	DSY2548	Transcription regulator (CodY)	1
ST	4 (4.76) 5 (13.71)[e]	1 (1.43)[f]	DSY3892	Sulfur transferase	1
CM	33 (1.9)	23 (0.10)	DSY2133	Flotillin-like protein	0
CM	8 (0.26)	5 (0.49)	DSY2137	Phage shock protein A (PspA-like)	0
AA	16 (0.22)	25 (0.36)	DSY4462	Tryptophan synthase (β subunit)	0
PC	12 (0.18)	4 (0.29)	DSY3960	Chaperonin (GroEL)	0
RR	44 (0.63)	104 (1.50)	DSY1954	DEAD/DEAH box RNA helicase	0
UF	26 (0.76)	13 (0.38)	DSY2819	Putative hydrolase	0
CC	24 (0.34)		DSY1570	Acyl-CoA:acetate/acetoacetate CoA-transferase (β subunit)	1
CC	23 (0.33)		DSY4938	Transaldolase	1
CC	14 (0.20)		DSY1568	Acyl-CoA dehydrogenase	0
CC	9 (0.86)		DSY1647	Methyltetrahydrofolate:corrinoid/iron-sulfur protein methyltransferase	0
CC	4 (0.23)		DSY1569	Acyl-CoA:acetate/acetoacetate CoA-transferase (α subunit)	0
EC	19 (1.46)		DSY0246	Succinyl-CoA synthetase (α subunit)	1
EC	16 (0.23)		DSY1627	Flavoprotein oxidoreductase (FixC)	1
EC	11 (0.15)		DSY2668	Acetate kinase	0
TT	10 (0.28)		DSY0469	Elongation factor (Tu)	0
TT	5 (0.96)		DSY0742	50S ribosomal protein (L25)	0
ST	23 (0.33)		DSY3475	Uncharacterized ABC transport system (ATPase component)	1
ST	9 (0.13)		DSY1764	Cobalamin/Fe ABC-type transport system (periplasmic component)	1
ST	7 (0.62)		DSY4870	Sec preprotein translocase (SecA)	0
CM	25 (0.36)		DSY3323	Cell wall protein	0
CM	17 (0.24)		DSY2498	Dihydrodipicolinate synthase/*N*-acetylneuraminate lyase	0
CM	11 (0.33)		DSY4851	Cell wall protein	0
PC	19 (0.28)		DSY2426	Cysteine desulfurylase (IscS)	1
RR	17 (0.24)		DSY4393	Phage-related replication protein	0
CO	9 (0.13)		DSY2114	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (CobT)	1
DM	6 (0.89)		DSY1012	Catalase/peroxidase	0
CC		44 (0.64)	DSY4838	Enolase	0
CC		42 (0.60)	DSY2356	5,10-Methylenetetrahydrofolate dehydrogenase (FolD)	0
CC		13 (0.18)	DSY1709	Enoyl-CoA hydratase/isomerase	1
CC		12 (0.17)	DSY1717	3-Hydroxyacyl-CoA dehydrogenase	1
EC		43 (0.62)	DSY1629	Electron transfer flavoproteins (β subunit, FixA)	0
EC		36 (0.51)	DSY1715	Electron transfer flavoproteins (β subunit)	1
EC		35 (2.98)	DSY0138	5,10-Methylenetetrahydrofolate reductase (MetF)	0
EC		23 (0.34)	DSY1869	Assimilatory sulfite reductase	0
EC		22 (0.32)	DSY1714	Electron transfer flavoproteins (α subunit)	1
EC		19 (0.27)	DSY1651	CO dehydrogenase–acetyl-CoA synthase (γ subunit)	0
EC		17 (0.50)	DSY1653	CO dehydrogenase–acetyl-CoA synthase (β subunit)	0
TT		112 (1.61)	DSY0457	50S ribosomal protein (L10)	0
TT		30 (0.43)	DSY0483	50S ribosomal protein (L5)	0
TT		18 (0.26)	DSY4946	Arginyl-tRNA synthetase (ArgS)	0
TT		15 (0.22)	DSY2275	Transcriptional regulator (GntR)	0
ST		22 (0.32)	DSY3918	Methionine ABC-type transport system (ATPase component)	0
ST		20 (0.28)	DSY2736	Bicarbonate ABC-type transport system (periplasmic component)	1
ST		20 (0.28)	DSY5026	Branched-chain amino acid ABC-type transport system (periplasmic component)	1
AA		32 (0.41)	DSY1364	Branched-chain amino acid aminotransferase	0
AA		17 (0.24)	DSY4319	Serine/threonine dehydratase	0
AA		16 (0.23)	DSY4927	Serine/glycine hydroxymethyltransferase	1
PC		27 (0.39)	DSY1649	CO dehydrogenase maturation factor (CooC)	0
PC		13 (0.19)	DSY1654	CO dehydrogenase maturation factor (CooC)	0
RR		19 (0.27)	DSY1943	Recombinase (RecA)	1
RR		14 (0.20)	DSY0001	Chromosomal replication initiation protein (DnaA)	0
UF		23 (0.34)	DSY2662	2-Nitropropane dioxygenase	1
UF		17 (0.25)	DSY2771	CRISPR-associated protein	1
NT		18 (0.25)	DSY0437	ATP:guanido phosphotransferase	0

[a] Functional protein categories: carbon metabolism and carbohydrate transport (CC), energy production and conversion (EC), transcription and translation (TT), secretion and transport (ST), cell wall and membrane metabolism (CM), amino acid metabolism (AA), posttranslational modification and chaperones (PC), replication and repair (RR), undefined function (UF), nucleotide metabolism (NT), coenzyme metabolism (CO), and defense mechanism (DM).
[b] LP/LF ratio, protein identified in gel LP with the corresponding volume ratio between LP and LF gels and relative volume (% vol) obtained.
[c] HP/HF ratio, protein identified in gel HP with the corresponding volume ratio between HP and HF gels and relative volume (% vol) obtained.
[d] TMH, number of predicted transmembrane helices.
[e] Two values indicate that the corresponding protein was identified from both soluble (upper) and membrane (lower) fractions.
[f] Although not increasingly expressed under HP growth conditions, the value given as the % vol is significantly high.

studied *Bacillus subtilis* CodY transcription regulator (DSY2548) were detected with up to 104- and 27-fold increases, respectively. Both proteins are thought to be involved in processes at the RNA level, with the former possibly being involved in the management of mRNA and ribosome assembly (14) and the latter being a global transcriptional repressor protein.

The global regulator CodY. In *B. subtilis*, CodY is known to control, directly or indirectly, nearly 200 genes, many of which are involved in adaptive mechanisms allowing the bacterium to

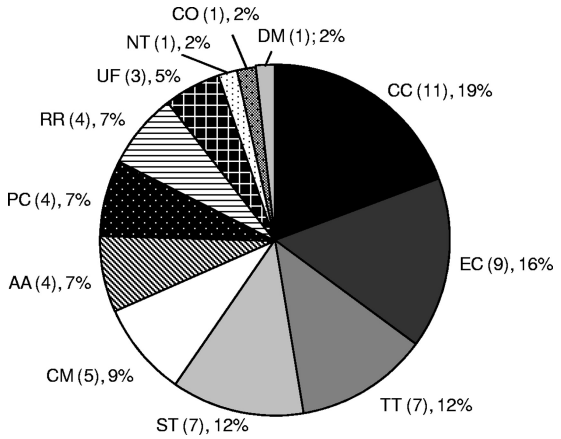

FIG. 2. Distribution of proteins whose expression was increased by PCE on the basis of the following functional categories: carbon metabolism and carbohydrate transport (CC), energy production and conversion (EC), transcription and translation (TT), secretion and transport (ST), cell wall and membrane metabolism (CM), amino acid metabolism (AA), posttranslational modification and chaperones (PC), replication and repair (RR), undefined function (UF), nucleotide metabolism (NT), coenzyme metabolism (CO), and defense mechanism (DM).

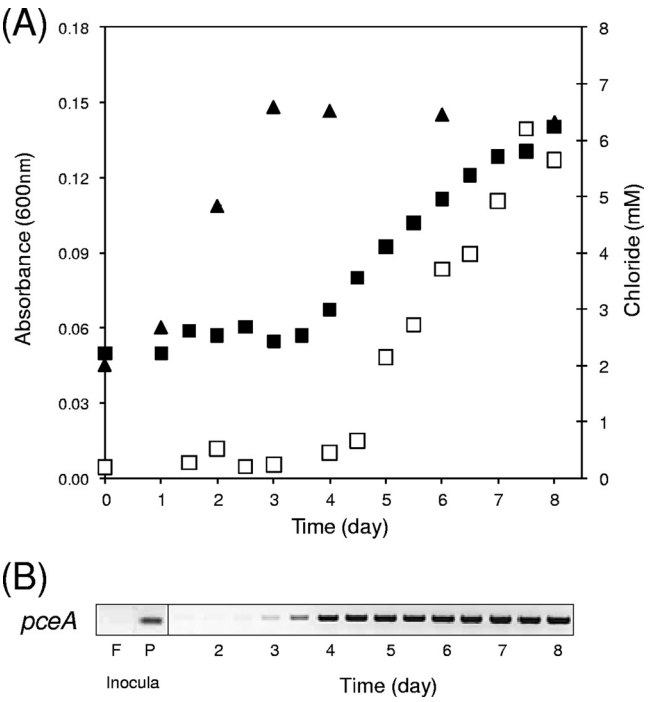

FIG. 3. PCE dechlorination recovery of *D. hafniense* strain TCE1 after long-term cultivation on fumarate. (A) Growth and chloride release during growth on PCE; black triangles, absorbance at 600 nm of a culture routinely cultivated on PCE; black squares, absorbance at 600 nm of the recovering culture on PCE; white squares, concentration of chloride released in the recovering culture. (B) PCR product targeting the *pceA* gene amplified from 0.2 ng of DNA extracted from 4-day cultures routinely cultivated on fumarate and PCE and used as inocula (left panel) and from the recovering culture on PCE over time (right panel).

adjust its metabolism to nutrient limitations (8). Two domains constitute the CodY protein, with the N-terminal domain being the binding site for at least two different effectors (GTP and branched-chain amino acids [BCAAs]), while the C-terminal domain contains a helix-turn-helix (HTH) motif that binds to DNA promoter regions (1, 8, 18). The CodY homologue of *D. hafniense* shares 55% identity with *B. subtilis* CodY and up to 74% identity in the DNA-binding domain, and the two proteins have fully identical HTH motifs. A 15-bp palindromic DNA sequence, the so-called CodY box (5′-AATTTTCNGA AAATT-3′), has been identified to be a consensus motif recognized by CodY in *Lactococcus lactis* and *B. subtilis*, respectively (1, 7). Using this pattern and allowing a 3-mismatch tolerance, more than 100 matches were identified in intergenic sequences of the genome of *D. hafniense* strain Y51 (see Table S1 in the supplemental material). In this list we can find the promoter regions of six gene clusters from which the gene products were identified in the proteomic analysis as being induced by PCE, such as the electron-transferring flavoprotein (ETF) complex DSY1718 to DSY1709, the gene cluster DSY1654 to DSY1647 encoding proteins involved in the Wood-Ljungdahl (WL) pathway, and the operon for CodY itself. The last observation suggests a negative-feedback loop, as has already been observed for CodY autoregulation in *L. lactis* (7). An increased amount of CodY protein might be explained by unfavorable growth conditions during PCE respiration. Thus, CodY is thought to help exploit all the nutritional resources such as CO_2 fixation, the use of ETF complexes, and other pathways (see below).

The chaperonin GroEL. PCE also triggered a slight increase (4- to 12-fold) of the general chaperonin protein GroEL (DSY3960), which is important for correct protein folding and repair, suggesting that PCE might disturb protein folding directly or might contribute to increasing the level of stress on *Desulfitobacterium* metabolism. Interestingly, in the *cpr* operon of *D. dehalogenans*, two additional homologues of GroEL (CprD and CprE) are present in the gene cluster and possibly contribute specifically to the folding of CprA, the chlorophenol reductive dehalogenase (35). No such proteins, however, are encoded in the *pce* gene cluster, suggesting that only the trigger factor-like protein PceT is specifically involved in the maturation of PceA (21).

Additional stress-related proteins. Two proteins corresponding to the loci DSY2133 and DSY2137 were clearly highlighted with up to 33- and 8-fold increased levels of expression, respectively. While DSY2133 shows strong homology to *Bacillus subtilis* YuaG and the eukaryotic flotillin-like protein, DSY2137 resembles the widespread family of *Escherichia coli* phage-shock protein A (PspA). Both proteins might participate in the PCE-dependent stress response at the cytoplasmic membrane. The presence of hydrophobic PCE might have a certain impact on cell membrane stability; therefore, these two proteins are suggested to support PCE respiration by maintaining the integrity of the bacterial envelope by guaranteeing the establishment of the proton motive force, as already suggested (15, 16), or by ensuring the Tat-dependent translocation of PceA (5, 40).

Finally, membranes of cells cultivated on lactate and PCE contained an additional protein (DSY3892) with a low but significant PCE/fumarate volume ratio of 5, representing, how-

ever, almost 14% of the analyzed membrane protein fraction. Sequence analysis clearly assigned a sulfur transferase function to this protein belonging to a large family of so-called rhodanese proteins. While their function often remains unclear, examples of this very diverse protein family are highlighted under various detrimental growth conditions such as oxidative stress (3) and cyanide toxicity (4). DSY3892 is present in a six-gene operon, among which DSY3896 shows strong homology with the complex CISM family (28) and, more precisely, with thiosulfate/polysulfide reductases (PhsA/PsrA [11]). Although the rhodanese was not increasingly expressed when hydrogen instead of lactate was given as electron donor, it was significantly present in membranes of cells cultivated under all conditions. This protein could be linked to the general sulfur metabolism in *Desulfitobacterium* and possibly to a certain stress caused by relatively high sulfide concentrations.

Proteins of the Wood-Ljungdahl pathway. Although not all proteins involved in the probably rather simple electron transport chain of organohalide respiration were detected by the 2D gel approach, possibly due to their high hydrophobicity, numerous other enzymes involved in general carbon and/or energy metabolism were preferentially detected in cells cultivated on PCE. Cultivation of strain TCE1 on hydrogen and PCE revealed an increased expression of several enzymes involved in the WL pathway of CO_2 fixation. Among them, proteins homologous to DSY2356 (FolD), DSY0138 (MetF), and DSY1651 and DSY1653 (γ and β subunits of CO dehydrogenase–acetyl coenzyme A [acetyl-CoA] synthase complex) were identified, representing 0.2 to 3.0% of total membrane proteins and showing 13- to 42-fold increased expression compared to cells cultivated on hydrogen and fumarate. All these proteins were not identified in LP, LF, and HF cultures, suggesting that the combination of H_2 and PCE in the presence of CO_2 did indeed trigger the WL pathway in PCE-respiring strain TCE1 cells (see Table S2 in the supplemental material). A 16S rRNA gene-based phylogenetic analysis of well-characterized homoacetogens and their close neighbors has highlighted that *D. hafniense* is part of them, and the comparison of the WL pathway gene clusters revealed the presence of a complete operon in this bacterium (24). The WL pathway serves two functions in homoacetogens: an electron-accepting energy-conserving function and a carbon assimilation function (27). Recently, a study by Kreher and coworkers has reported the involvement of enzymes of the WL pathway in two *Desulfitobacterium* strains during growth on phenyl methyl ether electron donors such as vanillate (17). There, the methyl branch of the WL pathway has been proposed to function in the reverse direction to oxidize the methyl group of the methoxylated substrate to CO_2 in order to obtain reducing equivalents. However, these strains were not able to grow only on vanillate in the presence of CO_2 like other homoacetogens (22), but they needed the presence of another terminal electron acceptor. This indicates that in desulfitobacteria CO_2 is not involved in energy metabolism. Under the growth conditions applied in our study (H_2, CO_2, PCE, acetate, and yeast extract), it is not clear whether the enzymes of the WL pathway serve any purpose or whether the WL pathway is simply induced under these conditions without providing a real benefit for growth. One could argue, though, that this pathway is used by strain TCE1 to assimilate CO_2. Heterotrophic CO_2 fixation has been shown to occur for the PCE-respiring bacterium *Dehalobacter restrictus* and for *Desulfitobacterium dehalogenans* (13, 38). However, the WL pathway is most probably not involved in this kind of CO_2 fixation. Replacing yeast extract by a defined amino acid solution allowed cultivation of strain TCE1 in the absence of acetate (see the supplemental material and see Table S4 in the supplemental material). However, when the amended amino acid concentration was decreased by a factor of 10, no growth was observed, indicating that strain TCE1 is not capable of autotrophic CO_2 fixation via the WL pathway. Therefore, the increased expression of the WL pathway might be the result of a pleiotropic effect due to growth under these specific conditions.

Electron-transferring flavoproteins. Cultivation of strain TCE1 on PCE highlighted the overexpression of two enzymatic complexes belonging to the functionally divergent family of ETFs. Two subunits of the four-gene operon DSY1629 to DSY1626 were identified alternatively with significantly increased PCE/fumarate volume ratios on lactate (DSY1627) and hydrogen (DSY1629) (Table 1), respectively, suggesting that this operon is generally required for PCE organohalide respiration. Sequence analysis of this operon indicates its affiliation with the group II ETF subfamily (36), showing an overall homology with the *fixABCX* gene cluster of *Bradyrhizobium japonicum* (41). ETF complexes of this type are known to replenish the NADH pool by providing electrons via ferredoxins to Rnf-type NADH ferredoxin oxidoreductases (9). As no Rnf complex could be identified in *Desulfitobacterium* genomes, this ETF complex might not contribute directly to energy conservation but might provide low-redox-potential electrons to the organohalide respiration or other metabolic pathways.

When strain TCE1 was cultivated on hydrogen, an additional ETF complex was clearly overexpressed on PCE. This ETF complex is part of a 10-gene cluster (DSY1718 to DSY1709) from which four proteins were overexpressed (Table 1; see Table S3 in the supplemental material), suggesting that the whole operon was being used by strain TCE1 during PCE respiration on hydrogen. This second ETF operon shows striking similarity to the recently described metabolic pathway of *Clostridium kluyveri* that is involved in the conversion of two acetate molecules into butyrate (32). Interestingly, a ferredoxin protein (DSY1713) is present in the gene cluster and might be considered an electron sink for the ETF-Bcd complex. Since the proteins of the DSY1718 to DSY1709 gene cluster were mainly found in cells cultivated on H_2, it is possible that these proteins are not involved in electron transfer to PceA but are involved in other metabolic pathways with hydrogen as electron donor. A detailed characterization of the metabolites present under these growth conditions (e.g., butyrate) would be useful to further investigate the function of the ETF complexes.

In conclusion, the proteomic results suggested that *D. hafniense* strain TCE1 adapts its physiology to face the relatively unfavorable growth conditions during PCE respiration. The increasing number of studies on *Desulfitobacterium* as well as the two available genome sequences highlights the great metabolic versatility of this genus. The ability of PCE respiration was very likely acquired by a recent horizontal gene transfer in strain TCE1, which could explain the low level of metabolic

dedication toward the use of chlorinated compounds. The preferential use of alternative electron acceptors is an additional indication for a yet incomplete adaptation to PCE respiration. It would certainly be of a great interest to investigate how *Dehalobacter restrictus* has evolved its metabolism toward organohalide respiration, as *D. restrictus* contains a *pce* gene cluster identical to that of *D. hafniense* strain TCE1; however, it is firmly integrated into the chromosome (20) and the organism is restricted to growth with hydrogen and PCE.

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