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Identification and Heterologous Expression of Genes Involved in Anaerobic Dissimilatory Phosphite Oxidation by *Desulfotignum phosphitoxidans*

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***Desulfotignum phosphitoxidans* is a strictly anaerobic, Gram-negative bacterium that utilizes phosphite as the sole electron source for homoacetogenic CO_2 reduction or sulfate reduction. A genomic library of *D. phosphitoxidans*, constructed using the fosmid vector pJK050, was screened for clones harboring the genes involved in phosphite oxidation via PCR using primers developed based on the amino acid sequences of phosphite-induced proteins. Sequence analysis of two positive clones revealed a putative operon of seven genes predicted to be involved in phosphite oxidation. Four of these genes (*ptxD-ptdFCG*) were cloned and heterologously expressed in *Desulfotignum balticum*, a related strain that cannot use phosphite as either an electron donor or as a phosphorus source. The *ptxD-ptdFCG* gene cluster was sufficient to confer phosphite uptake and oxidation ability to the *D. balticum* host strain but did not allow use of phosphite as an electron donor for chemolithotrophic growth. Phosphite oxidation activity was measured in cell extracts of *D. balticum* transconjugants, suggesting that all genes required for phosphite oxidation were cloned. Genes of the phosphite gene cluster were assigned putative functions on the basis of sequence analysis and enzyme assays.**

Phosphorus (P) is an important nutrient for all living organisms. The predominant forms of phosphorus in biological systems are inorganic phosphate and its organic esters and acid anhydrides in which P is at its highest oxidation state (+V). The P requirements of living cells can be fulfilled with phosphate in various forms, including reduced organic and inorganic phosphorus compounds (23). Several aerobic bacteria were shown to be able to oxidize hypophosphite (+I) and phosphite (+III) to phosphate (+V) and to incorporate the last into their biomass (5, 15–17, 31, 34). Phosphite can also be oxidized under anaerobic conditions, as shown for an anaerobic *Bacillus* strain (7) and for *Pseudomonas stutzeri* which can use phosphite under denitrifying conditions (17, 21). The only bacterium known to oxidize phosphite as the sole source of electrons in lithoautotrophic energy metabolism is *Desulfotignum phosphitoxidans* (24, 25).

Three different metabolic pathways for the use of phosphite as a single P source have been characterized so far. Two of them were discovered and characterized with *Escherichia coli* and one with *Pseudomonas stutzeri*. The first pathway in *E. coli* is mediated by the enzyme carbon phosphorus lyase (C-P lyase), and the second one by the alkaline phosphatase encoded by *phoA* (16, 34). This alkaline phosphatase not only hydrolyzes phosphate esters but also hydrolyzes phosphite to phosphate and molecular hydrogen (32). This is a particular property only of the *E. coli* alkaline phosphatase and is not observed with alkaline phosphatases of other bacteria. The third pathway is encoded by the *ptxABCDE* gene cluster in *P. stutzeri* (17). In this system, phosphite is transported into the cell by a binding protein-dependent phosphite transporter at the expense of ATP (PtxABC). Phosphite is oxidized by a phosphite:NAD^+ oxidoreductase (encoded by *ptxD*), a new member of the 2-hydroxy acid dehydrogenases (8). The *ptx* operon of *P. stutzeri* is regulated in response to phosphate starvation by the two-component regulatory system *phoBR* (28, 29). Furthermore, in *Alcaligenes faecalis* WM2072, another gene cluster involved in hypophosphite and phosphite uptake and oxidation was characterized: the *htxABCD-ptxDE* locus (31). The *htxABCD-ptxDE* genes and their products in *A. faecalis* WM 2072 have high nucleotide and amino acid sequence identities with those found in the *htx* and *ptx* operons in *P. stutzeri* WM88, which are required for the oxidation of hypophosphite and phosphite, respectively. This unique genetic arrangement of hypophosphite- and phosphite-oxidizing genes in *A. faecalis* WM2072 suggests a horizontal gene transfer and an ancient evolution of phosphite oxidation.

The diversity of pathways used for assimilatory phosphite oxidation and the fact that *D. phosphitoxidans* is so far the only bacterium known to use phosphite as an electron source caused us to investigate the phosphite uptake and oxidation gene cluster of this bacterium. The aims of our study were (i) to establish enzymatic assays for measurement of phosphite oxidation activity in cell extracts, (ii) to identify the genes involved in phosphite uptake and oxidation, and (iii) to characterize these genes physiologically.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and vectors used in this study are summarized in Table 1. *Desulfotignum phosphitoxidans* strain FiPS-3, *Desulfotignum balticum* (DSM 7044), and their transconjugants were grown anaerobically at 30°C in 100-ml infusion bottles

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, fosmid, or plasmid	Characteristics[a]	Reference or source
Strains		
D. phosphitoxidans FiPS-3 (DSM13687)	Wild type; Pt^+(phosphite)	25
D. balticum SaxT (DSM7044)	Wild type; Pt^- (phosphite)	13
E. coli WM3118	*mcrA endA1 recA1 deoR* Δ(*mrr-hsdRMS-mcrBC*) ϕ80(Δ*lac*M15) Δ*lacX74* Δ(*ara leu*)*7697 araD139 galU galK nupG rpsL* λ*attB*::pAMG27(*PrhaB*::*trfA33*)	6
E. coli NEB 10-beta	*araD139*Δ(*ara-leu*)*7697 fhuA lacX74 galK* (ϕ80Δ(*lacZ*)M15) *mcrA galU recA1 endA1 nupG rpsL* (Str^r) Δ(*mrr-hsdRMS-mrcBC*)	New England Biolabs
E. coli HB101	*supE44 hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*	22
Fosmids		
pJK050	Cm^r, copy control, fosmid cloning vector	3
pJK1043	Fosmid clone carrying the *D. phosphitoxidans* phosphite oxidation genes	This study
	Fosmid clone carrying the *D. phosphitoxidans* phosphite oxidation genes	This study
pJK1044	Mini-MuAE5 transposon vector	
Plasmids		
pAE4		3
pRK600	ColE1 Tra^+ Cm^r	9
pBBR1MCS-5	Broad-host-range cloning vector, Mob IncP Gm^r	12
pMB3A8	Gm^r, pBBR1MCS-5 containing 5-kbp phosphite oxidation gene cluster	This study
pDS2	Gm^r Km^r E *ptdC*::TN<KAN-2>; knockout mutant	This study
pDS3	Gm^r Km^r E *ptdG*::TN<KAN-2>; knockout mutant	This study
pDS4	Gm^r Km^r E *ptdF*::TN<KAN-2>; knockout mutant	This study
pDS17	Gm^r Km^r E *ptxD*::TN<KAN-2>; knockout mutant	This study

[a] Str^r, streptomycin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance.

containing 50 ml of mineral medium or in 22-ml screw-cap tubes with mineral medium (30), under a N_2-CO_2 (90:10 [vol/vol]) headspace. The medium was supplemented with 10 mM fumarate as an electron donor and carbon source and 10 mM sulfate as an electron acceptor. The genomic DNA library from *D. phosphitoxidans* chromosomal DNA was constructed in a dual-*cos* fosmid, pJK050 (3, 6), and transformed into a derivate strain of *E. coli* DH10B-WM3118. The pAE5 plasmid carries a mini-Mu transposon (mini-MuAE5) that was used for DNA sequencing.

For studies of phosphite uptake and oxidation, the mineral medium (23, 24) was prepared without any P source and supplemented with 0.1 to 1 mM phosphite as the only P source from sterile anoxic stock solutions. For biochemical studies, strains were cultured in 1-liter infusion bottles and harvested at an optical density at 578 nm (OD_{578}) of 0.30 to 0.35. Phosphite assimilation by *D. balticum* transconjugants containing the pMB3A8 or EZ::TN<Kan> transposon derivatives of pMB3A8 was performed under conditions identical to those described for *D. balticum* and *D. phosphitoxidans*. *Escherichia coli* NEB 10-beta (harboring pMB3A8 and its derivative plasmids) was used as a donor strain, and *E. coli* HB101(pRK600) as a helper strain in triparental matings. All *E. coli* strains were grown aerobically at 37°C in Luria-Bertani medium (22) supplemented with 100 μM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 50 μM IPTG (isopropyl β-D-1-thiogalactopyranoside) if appropriate. When needed, media were supplemented with antibiotics as follows: gentamicin (Gm), 20 μg ml^{-1}; chloramphenicol (Cm), 25 μg ml^{-1}; and kanamycin (Kan), 50 μg ml^{-1}.

Growth and analysis of phosphorus compounds. Samples were withdrawn at specific time points with a sterile plastic syringe that was preflushed with N_2-CO_2. Growth was monitored spectrophotometrically at OD_{578} in a Hitachi U-100 spectrophotometer in 1-cm light-path plastic cuvettes or in a Camspec M107 into which culture tubes could be directly inserted. For determination of phosphite and phosphate concentrations in culture supernatants, samples were centrifuged (5 min in a microcentrifuge, 16,000 × *g*, 4°C), and the supernatant was stored at −20°C. Phosphite and phosphate were analyzed by high-pressure liquid chromatography (HPLC) on an ion-exchange column (Aminex HPX-87H; Bio-Rad, Munich, Germany) operating at a 0.6-ml min^{-1} flow rate at 60°C with 5 mM sulfuric acid as the mobile phase. Phosphite was eluted after 7.5 min, and phosphate after 8.5 min. Both compounds were quantified against external standards with a refraction index detector type ERC-7512 (ERC, Inc., Tokyo, Japan) operating with polychromatic, visible light.

Identification and sequencing of the *D. phosphitoxidans ptx-ptd* gene cluster. For identification of the *D. phosphitoxidans ptx-ptd* gene cluster, individual clones from the *E. coli* DH10B-WM3118 genomic library were screened by PCR with the primers F3XF1 (CCCAAAAGCTTCTTGAGGAAA) and F3XR1 (ACTG TGTCTGTGCGCCCTTA). These were designed from the sequence of a 299-bp PCR product obtained by amplification of *D. phosphitoxidans* genomic DNA using degenerate primers. The degenerate primers were designed from the amino acid sequence of peptides obtained from the PtdF protein, which is induced only in the presence of phosphite (26). Two forward and two reverse degenerate primers were designed from each of the following three amino acid internal peptide sequences from PtdF: 59-LIEMGKDVTLFDNNEQHNMY, 62-VYGLPTIGLNIQNCYGAR, and 70-YYVHPPRPPLSVWLTPYIISK. Edman sequencing was performed by Kendrick Labs (Madison, WI) and the Columbia University Protein Core Facility (New York, NY). The screening reaction mix consisted of 1 μl LB-grown cell culture, 500 nM each primer, and *Taq* polymerase in Failsafe buffer G (Epicentre, Madison, WI) at an annealing temperature of 60°C. Two positive clones, pJK1043 and pJK1044, were chosen for DNA sequencing using pAE5 (mini-MuAE5) transposon insertions as mobile priming sites (6). Transposition reactions of BglII-digested pAE5 and either pJK1043 or pJK1044 were conducted *in vitro* using MuA transposase (MJ Research, Waltham, MA) per the manufacturer's instructions. Fosmid DNA was sequenced at the University of Illinois Urbana-Champaign Biotechnology Center using the primers seqAETnR (5′-TAGGAACTTCGGGATCCGTT-3′) and SEQAETnL (5′-TCGCCTTCTTGACGAGTTCT-3′). The sequence was assembled using Sequencher (Gene Codes Corp., Ann Arbor, MI).

Subcloning of the gene cluster involved in phosphite uptake and oxidation. A 4,998-bp-long fragment, *ptxD-ptdFCG*, was amplified from *D. phosphitoxidans* genomic DNA via long PCR with the long PCR enzyme mixture (Fermentas, Germany) and the following primers: F766 (5′-GAAAACCTGGGCATATCA ATTTGCGGAGAA-3′) and R5764 (5′-AGCACCTTGGCCCAATAAACACT TGTCAGG-3′). The amplified product was purified and cloned into the pGemT Easy vector (Promega, Madison, WI) and transformed into *E. coli* NEB 10-beta cells. Plasmids from several positive colonies were extracted and digested with a set of restriction endonucleases to check for the presence of the insert, and two

of them—pMB2 and pMB3 harboring the insert— were sequenced farther from both ends with M13-FP and M13-RP universal primers. pMB3 was digested with ApaI/SalI, purified, and ligated into the large fragment of pBBR1MCS-5 ApaI/SalI to generate the plasmid pMB3A8 (Table 1).

DNA methods. Genomic DNA was extracted with the genomic DNA purification kit Purgene (Gentra Systems, Minneapolis, MN). DNA digestion with restriction endonucleases and ligations were performed as described above. Agarose gel electrophoresis was performed via standard methods (22). Plasmids were prepared for sequencing and *in vitro* transposon mutagenesis with the plasmid purification kit Qiagen (Qiagen, Hilden, Germany) according to the manufacturer's instruction.

Genetic techniques/triparental mating. *D. balticum* strain SaxT was the recipient in triparental matings. It was grown into the late exponential phase (OD_{578} of 0.3 ± 0.02) in mineral medium supplemented with 10 mM fumarate plus 10 mM sulfate. The *E. coli* donor strains (NEB 10-beta, containing recombinant pMB3A8 (gentamicin resistant [Gm^r]) or EZ::TN<Kan> derivatives of this plasmid with kanamycin resistance [Kan^r]) and the helper strain (HB101, containing helper plasmid pRK600) were grown overnight in LB medium. For each mating reaction, 1 ml of the donor and 1 ml of the helper *E. coli* cultures were centrifuged at 13,000 × *g* for 3 min at room temperature, and 10 ml of the recipient strain was centrifuged at the same speed for 30 min at 4°C. Cell pellets were washed twice with 1 ml LB medium (*E. coli* strains) and once with 5 ml mineral medium (recipient strain). Pellets of all three cell types were suspended together in 50 to 60 μl LB and pipetted on sterile membrane filters (25-mm diameter, 0.22-μm pore size; Schleicher & Schuell, Dassel, Germany). The inoculated plates were incubated for 16 to 24 h in anoxic jars under N_2-CO_2 (95:5 [vol/vol]) at 30°C. On the next day, filters were transferred into 1.5-ml sterile tubes (Eppendorf, Germany), containing 1 ml of mineral medium. Cells were suspended by vortexing and transferred to a 22-ml tube containing 9 ml of anoxic mineral medium. One ml of each tube was inoculated immediately into a 100-ml infusion bottle containing 50 ml mineral medium supplemented with fumarate (10 mM) plus sulfate (10 mM) and the required antibiotic, according to the plasmid. Cultures were incubated for 30 days in the dark at 30°C without shaking. The ability to grow with phosphite as the sole P source was examined in mineral medium with 0.1 to 1 mM phosphite. The ability of these transconjugants to grow with phosphite was checked by phosphite depletion analysis.

Construction, isolation, and sequencing of transposon insertion mutants. The *ptxD-ptdFCG* genes cloned into pMB3A8 were knocked out by *in vitro* transposon mutagenesis with an EZ::TN<Kan> insertion kit (Epicenter, Oldendorf, Germany) per the manufacturer's instructions and then subjected to transformation in *E. coli* NEB 10-beta competent cells (New England BioLabs, GmbH, Germany). Positive clones were selected on LB plates containing gentamicin and kanamycin (pMB3A8 Gm^r, TN<KAN-2>). The clones carrying one single insert of the transposon into different positions were identified by restriction digestion with a selected set of restriction enzymes and sequenced with transposon-specific primers from the *in vitro* transposon mutagenesis kit (KAN-2 FP-1 and KAN-2 RP-1).The exact positions of the transposon inserts were mapped for each plasmid.

RT-PCR. Total RNAs were isolated from *D. phosphitoxidans* cultures grown to a mid-logarithmic OD_{578} of 0.25 (maximum growth of the strain at an OD_{578} of ca. 0.35) in minimal medium supplemented with phosphite. RNAs were isolated with an RNeasy minikit, containing RNAprotect bacterial reagent (Qiagen GmbH, Germany), per the manufacturer's instructions. The removal of contaminating chromosomal DNA was performed with the DNase I on-column digestion kit (Qiagen GmbH, Germany). DNase-treated RNA was used as a template for amplification in reverse transcriptase (RT) assays with SuperScript II reverse transcriptase, and resulting cDNAs were amplified with Platinum *Taq* DNA polymerase (Invitrogen) in single-step reactions, per the manufacturer's instructions. Both a positive control (only chromosomal DNA) and a negative control (RNA, without reverse transcriptase in the reaction tube) were run under identical amplification conditions. The primers used to amplify each of the *ptx-ptd* junctions are shown in Table 2.

Preparation of cell extracts and protein determination. *D. phosphitoxidans*, *D. balticum*, and a *D. balticum* transconjugant E strain harboring pMB3A8, were grown in 0.5 liters mineral medium with 10 mM phosphite or 10 mM fumarate as an electron donor plus 10 mM sulfate and harvested in the late-exponential growth phase. Phosphite-induced or noninduced cultures were further handled to obtain cell extracts as described elsewhere (26). Protein content in the preparations was determined spectrophotometrically with the bicinchoninic acid method (BCA protein assay kit; Pierce, IL), with bovine serum albumin as a standard.

Enzyme assays. A spectrophotometric assay was established for measurement of phosphate-oxidizing enzyme activity in cell extracts of cells grown with phosphite. Activity was detected with 0.5 to 1 mM NAD^+ or with 2 mM benzyl viologen (BV) as an electron acceptor in the presence of 1 to 10 mM phosphite as substrate in 10 mM Tris-HCl or Tris-2-(*N*-morpholino)ethanesulfonic acid-acetic acid (Tris-MES-acetic acid) buffer, pH 7.0 to 7.2, in the presence of 2 mM dithiothreitol (DTT) and 5 mM $MgCl_2$. Enzyme tests were performed anoxically under N_2 at 30°C, with 20 to 50 μl cell extract (cytoplasmic or membrane fraction) containing 3 to 5 mg protein · ml^{-1}. Specific enzyme activities were calculated on the basis of continuously monitored absorbance changes (NADH at 340 nm; ε = 6.22 $mM^{-1} \cdot cm^{-1}$; reduced BV at 578 nm; ε = 8.65 $mM^{-1} \cdot cm^{-1}$).

TABLE 2. Oligonucleotide primers used for amplification of *ptd* junction sequences

Junction amplified	Primer set	Predicted product size (bp)
ptxED	5′-AAGAGAAAATGCCAAATGTGATGA-3′ 5′-GCTTCCGATGGTATTTTGTGAGTA-3′	458
ptxD-ptdF	5′-TTTCTCGGCCAATTAATACTCTCC-3′ 5′-AGCTTTTGGGTTTCTTCATACAT-3′	550
ptdFC	5′-AATACGGCCACGGGGTTCTGTTGTC-3′ 5′-AAGTGGTTGGCTGTTGGTGGTC-3′	399
ptdCG	5′-AAGGGCGCACAGACACAGTTTA-3′ 5′-ACACATTCCCCAGTTGAGTCTTCC-3′	858
ptdGH	5′-CGAGCGCGGGTCTGAAG-3′ 5′-GTCCTGAATGCCTGAAAGTT-3′	327
ptdHI	5′-AAAACTTTCAGGCATTCAGGACTA-3′ 5′-TCCCCAGCCATAAATGCGAAAATC-3′	480

Sequence analysis. Nucleotide and amino acid sequences were analyzed with the National Centre for Biotechnology Information alignment tools (http://www.ncbi.nlm.nih.gov/BLAST) (1) and the ExPASy molecular biology server (http://www.expasy.org). Transmembrane helices in proteins were predicted with the TMHMM software, version 2.0, 3.0 (http://www.cbs.dtu.dk), TMpred (www.ch.embnet.org), and TopPred (http://mobyle.pasteur.fr) software (10, 27). Signal peptide cleavage sites were searched with SignalP software server 3.0 (http://www.cbs.dtu.dk) (4, 18) where appropriate.

Nucleotide sequence accession number. The nucleotide sequence of the phosphite oxidation cluster was deposited in the NCBI (GenBank) under accession number GU324300, submitted on 15 December 2009 at http://www.ncbi.nlm.nih.gov.

RESULTS

Identification of the gene cluster involved in phosphite metabolism by *D. phosphitoxidans*. In the present study, we focused on the identification of a group of genes (*ptxD-ptdFCG*) involved in phosphite uptake and oxidation by *Desulfotignum phosphitoxidans* which represent part of the phosphite operon of this strain. A genomic library of *D. phosphitoxidans* was constructed in *E. coli*, using fosmid vector pJK050. To screen the library for positive clones, we used a PCR approach. The PCR primers were developed on the basis of three internal peptide sequences from a protein (PtdF) found only if the strain was grown with phosphite (26). The two positive clones, pJK1043 and pJK1044, containing targeted genomic DNA of strain FiPS-3, were sequenced and gave a contig of 32,463 bp in length in which 25 genes were identified and grouped in three putative operons. The putative phosphite operon consists of 7 genes (*ptxED-ptdFCGHI*). Its structure is represented in Fig. 1 and in Table 3, in which the number of amino acids encoded by each new protein, the percent similarity, and the percent identity of the amino acid sequences toward corre-

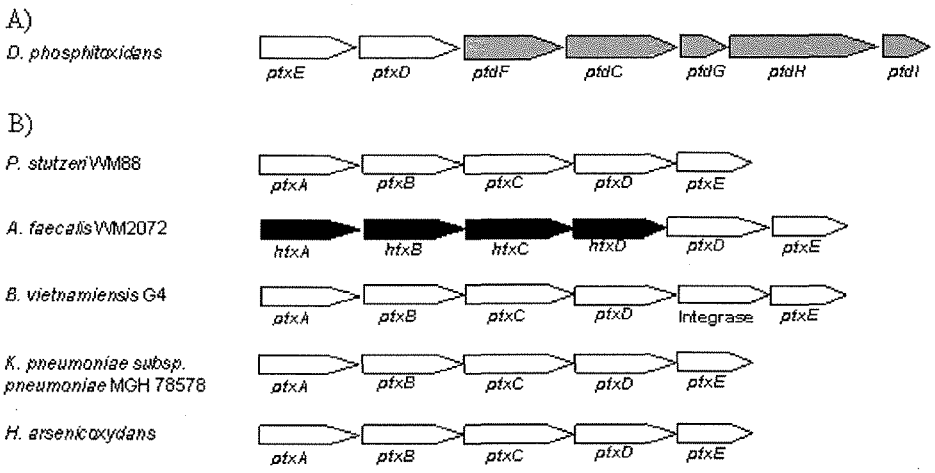

FIG. 1. Structure of the *ptd* region of *D. phosphitoxidans* strain FiPS-3 (A) and comparison with previously described regions involved in phosphite oxidation and uptake by other bacteria, including *Burkholderia vietnamiensis* G4, *Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578, and *Herminiimonas arsenicoxydans* (B). The unique genes and their arrangement found only in this operon are presented in gray color. Common genes, despite their diversity, are represented in white, and the specific arrangement of the previously described clusters is shown in black.

sponding proteins with known sequences in the databases are given.

Sequence analysis of the *D. phosphitoxidans ptx-ptd* operon. The newly discovered phosphite oxidation cluster in *D. phosphitoxidans* was named *ptx-ptd.* This locus encodes the *ptxE* and *ptxD* gene products which are similar to those of the previously described *ptxE* and *ptxD* genes in *P. stutzeri* WM88 or the *ptxE* and *ptxD* genes in the *ptx-htx* locus of *A. faecalis* WM2072. In this locus, five new genes, *ptdFCGHI*, were identified which do not show analogy in phosphite clusters described so far. The abbreviation "*pt*" stands for phosphite and the letter "d" is derived from the genus name of the strain in which the new genes were discovered. In Table 4, the amino acid coverage of the newly discovered proteins is compared to that of the *ptx* gene products of *P. stutzeri* WM88.

The gene *ptxE* encodes a member of the LysR family of regulatory proteins. This protein shows a low level of amino acid identity with the products of the *ptxE* genes of *P. stutzeri* and *A. faecalis*, whereas there were no identities with the genes at the nucleotide level. Nevertheless, the protein possesses a conserved helix-turn-helix domain at its N terminus, as does the PtxE protein of *P. stutzeri* WM88 (28). The product of the *ptxD* gene is similar to PtxD in *A. faecalis* and *P. stutzeri*. The identities between the amino acid residues of PtxD of *D. phosphitoxidans* and PtxD of *A. faecalis* and *P. stutzeri* were 40% and 39%, respectively. This structural similarity infers a function of this protein as an NAD:phosphite oxidoreductase in *D. phosphitoxidans*. Interestingly, the nucleotide sequence alignments of the three genes revealed a significant divergence between the *ptxD* gene of *D. phosphitoxidans* and the *ptxD* genes of *P. stutzeri* and *A. faecalis* (17, 31).

The predicted product of the *ptdF* gene is homologous to known NAD(P)-dependent epimerase/dehydratases or to short-chain dehydrogenases (26). The exact function of this protein in phosphite uptake and oxidation is still unclear. Nevertheless, this is the only readily identifiable protein that is

TABLE 3. Genes and gene products of the phosphite oxidation cluster of *D. phosphitoxidans*

Gene product	Gene range (positions)	Protein length (amino acids)	Corresponding protein				Closest homolog of predicted protein
			% Identity	% Similarity	E value	Reference no.	
PtxE	13715–14656	313	38	60	2e−53	gi 89211710 ref ZP_01190059.1	LysR regulatory protein (*Halothermothrix orenii* H 168)
PtxD	14763–15791	354	40	58	1e−67	gi 47231506 ref AAT12779	PtxD (*Alcaligenes faecalis* WM2027)
PtdF	16358–17311	322	31	49	1e−33	gi 251771726 ref EES52301	UDP-glucose 4-epimerase (*Leptospirillum ferrodiazotrophum*)
PtdC	17465–18784	439	28	49	4e−39	gi 3097811 ref AAC15510	2-Phophonopropionate transporter (*Pseudomonas fluorescens* 23F)
PtdG	18892–19338	148	36	55	2e−13	gi 16080976 ref NP_391804.1	UspA protein (*Bacillus subtilis* subsp. *subtilis* strain 168)
PtdH	19545–20924	459	29	49	1e−32	gi 31621273 ref AAP59031	BchE-anaerobic magnesium-protoporphyrin IX monomethyl ester cyclase (*Thiocapsa roseopersicina*)
PtdI	20914–22014	225	23		0.48	gi 150391227 ref YP_001321276.1	Hypothetical protein Amet_3489 (*Alkaliphilus metalliredigens* QYMF)

TABLE 4. Comparison of the phosphite oxidation *ptx-ptd* gene cluster products of *D. phosphitoxidans* with those from the phosphite oxidation *ptx* gene cluster of *P. stutzeri* WM88[a]

Gene	No. of amino acids with identity between *D. phosphitoxidans* and *P. stutzeri* WM88/total no.	No. of amino acid residues in *D. phosphitoxidans*/*P. stutzeri* WM88
ptxE	54/289	313/289
ptxD	131/336	354/336
ptdF	NA	322/NE
ptdC	NA	439/NE
ptdG	NA	148/NE
ptdH	NA	459/NE
ptdI	NA	225/NE

[a] NE, not existing in *ptx* gene cluster of *P. stutzeri*; NA, not available.

specifically induced in the presence of phosphite in *D. phosphitoxidans* and is found in four differently phosphorylated states in the proteome of the strain (26).

The *ptdC* product most probably functions as a permease, an inner-membrane protein involved in phosphite transport across the membrane. It is a monomer with 11 transmembrane helices. The N terminus points outside the cytoplasmic membrane, and the C terminus is oriented toward the cytoplasm as predicted with TMHMM, TopPred, and TMpred software. This protein is affiliated with the major facilitator superfamily (MFS) of proteins with regulatory functions, based on the identified conserved domains and amino acid residues of the UhpC and UhpT proteins of *E. coli*, which were shown to be involved in carbon metabolism. The UhpC is an organophosphate and inorganic phosphate-sensing protein, and UhpT plays a role in organophosphate:inorganic phosphate antiport (2, 11). The PtdC protein is also homologous to the GlpT protein, a monomeric antiporter protein which couples the import of glycerol-3-phosphate with the efflux of the phosphate ion across the cytoplasmic membrane (14). GlpT is a member of the same family of proteins as UhpC and UhpT, the organophosphate:inorganic phosphate antiporter (OPA) family within the MFS family (21). This protein showed 28% identity to PtdC, and its crystal structure was the only one showing similarity to the predicted structure of PtdC based on amino acid sequence.

PtdG was assigned as a cytoplasmic universal stress protein harboring an UspA-like domain. The PtdH protein is a member of the B_{12}-binding radical SAM family of proteins, the function of which is still unclear. The last gene in this operon, *ptdI*, encodes a hypothetical protein.

All open reading frames (ORFs) were preceded by putative Shine-Dalgarno ribosome-binding sites. These sequences were located 13 to 4 nucleotides upstream of the respective start codon. According to the ORF transcription direction and the intergenic spaces, we assumed that this coding region is organized as an operon (Fig. 1).

The genes of the *ptx-ptd* locus form a single transcriptional unit. The proximity or overlap of the ORFs within the *ptx-ptd* locus suggests that they form a single transcriptional unit. Of the seven ORFs identified, two have coding regions that overlap, and four ORFs are separated by at most 110 nucleotides. The exception are the *ptxD-ptdF* genes, which are separated by 567 nucleotides. We tested whether these genes are cotranscribed using RT-PCR, which allows determination of the presence of the junction sequences between each gene in total RNA isolated from phosphite-grown *D. phosphitoxidans* cells (Fig. 2). Amplification products were obtained for all intergenic regions between adjacent *ptx-ptd* genes, including the *ptdHI* genes that overlap by 7 bp. Therefore, the *ptxED-ptdFCGHI* genes almost certainly form one operon.

Phosphite oxidation in cell suspensions. In a previous study, we demonstrated that *D. phosphitoxidans* can grow autotrophically with phosphite as the sole electron donor (25). In the present study, *D. phosphitoxidans* was tested in addition for its ability to grow with 0.1 and 0.5 mM phosphite as the only P source. Results are shown in Fig. 3B. The specific physiological rate of phosphite oxidation by growing cells of *D. phosphitoxidans* was calculated to be 91 ± 4 mU · mg protein^{-1} (one unit of activity is equal to conversion of one μmol phosphate · min^{-1} · mg protein^{-1}). The phosphite oxidation rate of resting cells in 50 mM Tris-HCl buffer, pH 7.2, under N_2-CO_2, was found to be 21 ± 2 mU · mg protein^{-1}, which equals only one-fourth of the physiological activity of growing cells.

Desulfotignum balticum SaxT (DSM 7044) was chosen as a candidate for heterologous expression of the phosphite oxidation cluster of *D. phosphitoxidans*. It is unable to grow autotrophically with phosphite as the sole electron source or to use phosphite as its P source (Fig. 3A). No phosphite oxidation or phosphate formation was observed in experiments with dense suspensions of intact cells of *D. balticum*.

Both strains were tested also for resistance toward the antibiotics ampicillin, chloramphenicol, carbenicillin, gentamicin,

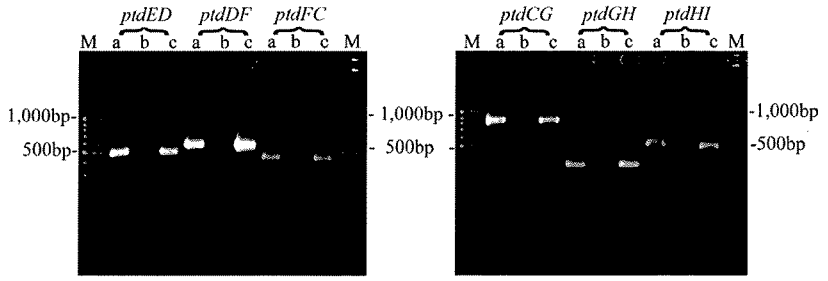

FIG. 2. Determination of the operon structure of *ptd* locus through RT-PCR with total RNA of *D. phosphitoxidans* grown with phosphite. Lanes: a, complete RT reactions; b, negative controls (reactions without reverse transcriptase in the assays); c, PCR positive controls (chromosomal DNA used as template); M,100-bp DNA ladder. The junction sequences amplified are indicated above each reaction set.

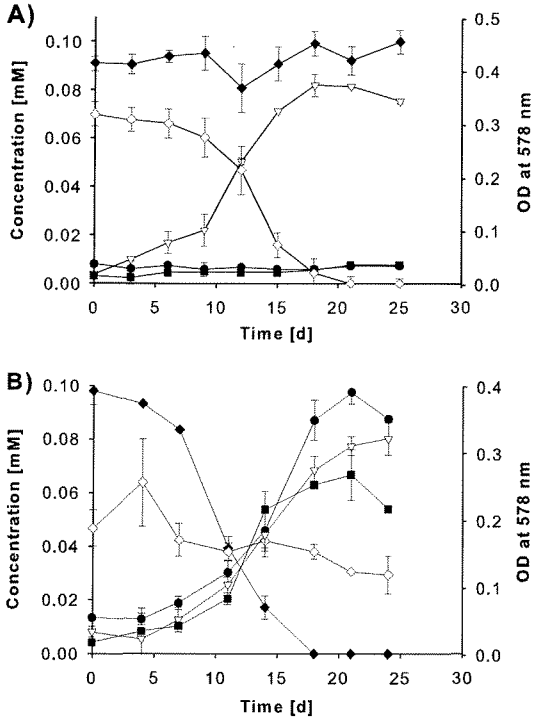

FIG. 3. Growth of *D. balticum* (A) and *D. phosphitoxidans* (B) under various conditions. Autotrophic growth with 10 mM phosphite as sole energy source in mineral medium (■); the graphic representation is scaled down 10:1. Heterotrophic growth with 10 mM fumarate plus 10 mM sulfate, supplemented with 0.1 mM phosphite (●) or phosphate (▽) as sole P source and concomitant depletion of phosphite (◆) or phosphate (◇).

kanamycin, nalidixic acid, streptomycin, and tetracycline. The tests were performed in liquid medium under autotrophic and heterotrophic growth conditions. Interestingly, *D. phosphitoxidans* was resistant toward all tested antibiotics under both growth conditions, except for chloramphenicol. On the contrary, *D. balticum* was sensitive to all tested antibiotics.

Phenotypic characterization of the phosphite oxidation cluster. *D. balticum* and *D. phosphitoxidans* belong to the *Deltaproteobacteria* and are able to grow either heterotrophically or autotrophically with sulfate as the electron acceptor (25). The *ptxD-ptdFCG* gene cluster of *D. phosphitoxidans* was cloned into the broad-host-range plasmid cloning vector pBBR1MCS-5 (Mob IncP Gm^r), to form pMB3A8, which was transferred to *D. balticum* strain SaxT via triparental mating. Transconjugants of *D. balticum* harboring the pMB3A8 were able to grow with 0.1 mM phosphite as a P source but could not utilize it as an energy source (Fig. 4). No phosphate was released into the medium during growth with phosphite as a P source. For further studies, including the ability of phosphite oxidation in cell extracts, we selected transconjugant E (harboring the *ptxD-ptdFCG* cluster), which showed the highest growth rate of all transconjugants obtained.

Enzyme activities in cell extracts. The phosphite-oxidizing enzyme activity was measured discontinuously in cell extracts of *D. phosphitoxidans* strain FiPS-3 with 1 mM NAD^+ or 1 mM

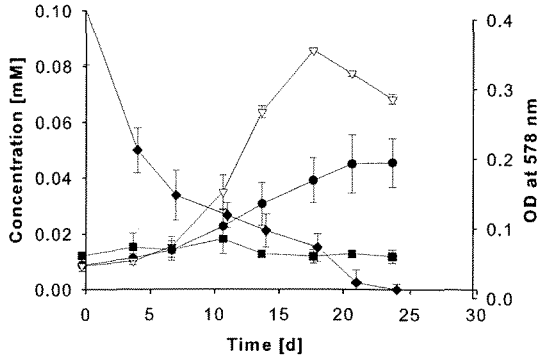

FIG. 4. Utilization of phosphite by transconjugant E (*D. balticum* harboring pMB3A8). Lithoautotrophic growth with 10 mM phosphite in mineral medium (■), heterotrophic growth with 10 mM fumarate plus 10 mM sulfate, with (i) phosphite (●) or depletion of phosphite (◆) or (ii) phosphate (▽) as sole P source.

$NADP^+$ as the electron acceptor upon addition of 1 mM phosphite. Activities were found to be 20 ± 3 mU · mg protein^{-1} with NAD^+ and 68 ± 2 mU · mg^{-1} with $NADP^+$ as the electron acceptor. With 2 mM benzyl viologen (BV) as the electron acceptor, the enzyme activity was in the range of 7 to 9 mU · mg protein^{-1} upon addition of 10 mM phosphite. The reaction rate with BV as the electron acceptor increased about 5-fold (45 ± 5 mU · mg protein^{-1}) in the presence of 2 μM cyanocobalamin as a coenzyme. In the absence of cell extract, no chemical reduction of electron acceptors with phosphite was measured. Phosphate production was assayed by measuring phosphate accumulation via ion-exchange HPLC. The activity of cell extracts according to phosphate formation in HPLC assays was calculated to be in the range of 20 to 47.2 ± 7 mU · mg protein^{-1}. Phosphite-dependent BV reduction in the presence of 2 μM cyanocobalamine was measured also in cell extracts of *D. balticum* and the transconjugant of *D. balticum* harboring pMB3A8 (Table 5).

Construction and phenotypic characterization of mutants carrying knockout in *ptxD-ptdFCG*. To analyze the pathway of phosphite uptake and oxidation by *D. phosphitoxidans* in greater detail, *in vitro* transposon mutagenesis was carried out with pMB3A8 using the EZ::TN<KAN-2> transposon. About 1,000 transposon mutants were generated in *E. coli*, 37 of which were isolated and sequenced. Fourteen out of the 37

TABLE 5. Specific activities of phosphite oxidation in cell extracts of *D. phosphitoxidans* and *D. balticum* strains[c]

Strain	Growth substrate	Activity (mU mg^{-1} protein)
D. phosphitoxidans FiPS-3	Phosphite	40 ± 7
	Fumarate	0
D. balticum SaxT (wild type)	Fumarate	0
D. balticum SaxT/pMB3A8	Fumarate[a,b]	11 ± 3[a]
		36 ± 2.5[b]

[a] Grown in the absence of phosphite.
[b] Grown in the presence of phosphite.
[c] Values are given with standard deviations of at least six measurements in every case.

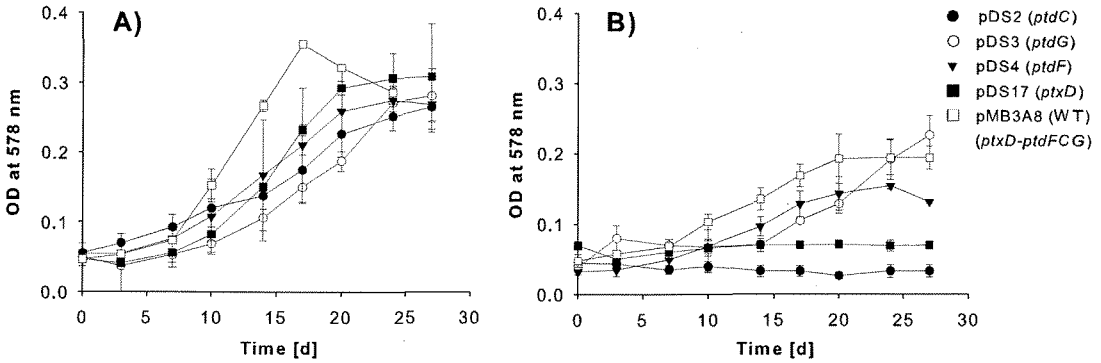

FIG. 5. Growth of *D. balticum* recombinant strains harboring transposon-inactivated genes from pMB3A8 in fumarate-sulfate medium with phosphate (A) or phosphite (B) as sole P source.

transposon insertional plasmids contained one transposon inserted in the cloned fragment of plasmid MB3A8. Further, for each transposon-inactivated gene of the cloned fragment, one plasmid was transferred into *D. balticum* via triparental mating, and the transconjugants were screened for their ability to grow with phosphite as a single P source (Table 1). Growth of these mutants was compared with those of the wild-type strains of *D. phosphitoxidans* and *D. balticum* and the transconjugants of *D. balticum* harboring pMB3A8 in phosphate-free mineral medium supplemented with 0.1 mM phosphite, 10 mM fumarate, and 10 mM sulfate. Growth controls were run in mineral medium supplemented with 1.5 mM phosphate, 10 mM fumarate, and 10 mM sulfate. All mutants displayed similar growth rates with phosphate as a P source (Fig. 5). The growth rate of the transconjugant harboring pMB3A8 with phosphite as a P source was the highest one among the mutants. The pDS3 mutant (*ptdG*::TN<KAN-2>) displayed the longest lag phase compared to the other mutants and *D. balticum* containing the WT plasmid when grown with phosphite.

In three separate experiments with at least three replicates, we found that the mutants harboring the plasmid pDS2 containing the transposon insert in *ptdC* were unable to grow with 0.1 mM phosphite as a single phosphorus source, whereas they grew well with phosphate. The same results were obtained with the mutants carrying pDS17 (*ptdD*::TN<KAN-2>). These mutants were unable to grow with 0.1 mM phosphite as a single P source, as shown on Fig. 5.

DISCUSSION

In the present report, we describe the phosphite oxidation gene cluster of *D. phosphitoxidans* strain FiPS-3, the only bacterium known to oxidize phosphite anaerobically as an electron donor in its energy metabolism. Until now, at least two metabolic pathways for uptake and oxidation of phosphite have been characterized at the genetic and the metabolic levels for *P. stutzeri* WM88 and for *A. faecalis* WM2072, respectively (17, 31). Both bacteria use hypophosphite or phosphite as a P source in the absence of phosphate. In addition, several genetic regions were assigned to be involved in phosphite uptake and oxidation in different strains, based on genomic data (Fig. 1). All these regions displayed similar structures, major components of which are the LysR-family regulatory protein (encoded by *ptxE*), a specific phosphite dehydrogenase (encoded by *ptxD*), and an ABC-type uptake system encoded by genes annotated as *ptxABC* in *P. stutzeri* or as *htxBCDE* in *A. faecalis*. The last two encode periplasmic substrate-binding proteins PtxB and HtxB, respectively. The phosphite uptake system encoded in the *ptx-ptd* locus of *D. phosphitoxidans* involves a new permease, PtdC, a member of the single-subunit MFS family of proteins, according to the identification scores obtained. This protein is most probably playing a role in the antiport of phosphite/phosphate across the cytoplasmic membrane of *D. phosphitoxidans* and is not homologous to PtxB and HtxB. In addition, there were no genes orthologous to *ptxA* and *ptxB* found in our gene cluster, suggesting that the phosphite uptake system of *D. phosphitoxidans* differs significantly from the ABC-type ATP-dependent transport systems described so far.

The *ptdF* gene codes for an NAD(P)-dependent epimerase/dehydratase which is a unique protein that is specifically expressed in the presence of phosphate, not only under starvation conditions (26). This protein could function either as a supplementary protein involved in phosphite uptake, or it might be part of the phosphate-sensing system. It is found specifically in *D. phosphitoxidans*. The gene coding for this protein is a part only of the gene cluster involved in phosphite oxidation found in *D. phosphitoxidans*.

The two genes *ptxE* and *ptxD*, coding for the LysR-type regulatory protein and for a phosphite:NAD oxidoreductase, are conserved among all known phosphite gene clusters (Fig. 1). The highest conservation at the amino acid level was found for the *ptxD* gene product. This protein contains the consensus sequence of a Rossman fold, including the GxxGxGxxG motif completely conserved. This motif is common among α-D-hydroxy acid dehydrogenases. Furthermore, the three catalytic amino acid residues involved in phosphite oxidation as described for the *P. stutzeri* phosphite dehydrogenase are as well completely conserved in the product of the *ptxD* gene (32, 33). Nevertheless, PtxD of *D. phosphitoxidans* was found to be 18 amino acids longer than the PtxD protein of *P. stutzeri* (Table 4). Another specific feature of the *D. phosphitoxidans* phosphite gene cluster is the inverted positioning of the *ptxE* gene toward the *ptxD* gene and of both genes toward the genes involved in phosphite uptake. This comparison is shown in Fig. 1A and B.

The *ptdG* gene product exhibited the highest identity with the proteins that contain a UspA-like domain. The UspA protein of *E. coli* is a serine and threonine phosphoprotein able to form homodimers, but also heterodimers with other proteins (20). It was found that the induction of the UspA protein in *E. coli* is *phoB* dependent and that UspA synthesis is induced by growth inhibition in a mineral medium (19). The phosphorylated forms of this protein become predominant under phosphate starvation. Therefore, the presence of *ptdG* in the *D. phosphitoxidans* phosphite oxidation gene cluster finds its proper explanation: this cluster is not only involved in phosphite oxidation and uptake in the energy metabolism of this strain but serves also a further function in supplying the bacterium with phosphate under phosphate limitation. The genes *ptdH* and *ptdI* are coding for two new proteins, the roles of which in this cluster and in phosphite oxidation are yet unclear.

Obviously, the gene cluster of *D. phosphitoxidans* is the first one to show divergence among the phosphite gene clusters of bacteria. In this locus, five new genes which are involved in phosphite oxidation were identified. This cluster differs from all phosphite oxidation loci so far described, in terms of structure and nucleotide sequence of the genes and also at the amino acid level of the conserved proteins. This leads to the conclusion that there should be a greater variation of proteins and gene clusters involved in sensing, transport, and either the utilization of phosphite as a single P source and/or its use as an electron donor in the energy metabolism of bacteria.

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