Redundancy and specificity of multiple trigger factor chaperones in *Desulfitobacter*

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The ribosome-bound trigger factor (TF) chaperone assists folding of newly synthesized polypeptides and participates in the assembly of macromolecular complexes. In the present study we showed that multiple distinct TF paralogues are present in genomes of *Desulfitobacter*, a bacterial genus known for its ability to grow using organohalide respiration. Two full-length TF chaperones and at least one truncated TF (lacking the N-terminal ribosome-binding domain) were identified, the latter being systematically linked to clusters of reductive dehalogenase genes encoding the key enzymes in organohalide respiration. Using a well-characterized heterologous chaperone-deficient *Escherichia coli* strain lacking both TF and DnaK chaperones, we demonstrated that all three TF chaperones were functional *in vivo*, as judged by their ability to partially suppress bacterial growth defects and protein aggregation in the absence of both major *E. coli* chaperones. Next, we found that the N-terminal truncated TF-like protein PceT functions as a dedicated chaperone for the cognate reductive dehalogenase PceA by solubilizing and stabilizing it in the heterologous system. Finally, we showed that PceT specifically interacts with the twin-arginine signal peptide of PceA. Taken together, our data define PceT (and more generally the new RdhT family) as a class of TF-like chaperones involved in the maturation of proteins secreted by the twin-arginine translocation pathway.

Received 27 April 2011

Revised 24 May 2011

Accepted 25 May 2011

INTRODUCTION

Folding of newly synthesized polypeptides in the cytosol is assisted by molecular chaperones (Hartl & Hayer-Hartl, 2009). In bacteria, the ribosome-bound chaperone trigger factor (TF) plays a major role in this process. In *Escherichia coli*, it is believed that most nascent polypeptides emerging from the ribosome interact with TF before completing their folding (Hoffmann *et al.*, 2010; Kramer *et al.*, 2009). TF chaperone specifically binds to L23 protein at the polypeptide exit tunnel of active ribosomes with a 1:1 stoichiometry (Ferbitz *et al.*, 2004; Hesterkamp *et al.*, 1996; Kramer *et al.*, 2002b; Patzelt *et al.*, 2001). Remarkably, it has been recently shown that TF cycles the ribosome on and off and that ribosome-free TF may stay bound to

elongating polypeptides for a prolonged period (Kaiser *et al.*, 2006; Rutkowska *et al.*, 2008).

In agreement with such a property, it has recently been proposed that TF participates in the post-translational assembly of large protein complexes in the cytosol (Martinez-Hackert & Hendrickson, 2009). The TF chaperone is composed of three distinct protein domains, which have been functionally and structurally determined. The N-terminal domain (NTD) consists of a ribosome-binding domain containing a conserved motif responsible for binding to ribosomal proteins (Hesterkamp *et al.*, 1997; Kramer *et al.*, 2002a, 2004). The central module in the TF structure corresponds to the C-terminal domain (CTD) and harbours the core of the chaperone activity (Genevaux *et al.*, 2004; Kramer *et al.*, 2004). Finally the domain encoded at the centre of the *tig* gene exhibits a peptidyl-prolyl *cis/trans* isomerase (PPIase) activity dispensable for *in vivo* TF chaperone function (Genevaux *et al.*, 2004). *In vivo* investigation of TF functions has revealed that a single *tig* mutant strain has no observable effect on bacterial growth. In this case it has been proposed that the multifunctional DnaK (Hsp70) chaperone compensates for the lack of TF, thus highlighting the complex interplay between molecular chaperones (Deuerling *et al.*, 1999;

Abbreviations: BTH, bacterial two-hybrid; CTD, C-terminal domain; NTD, N-terminal domain; PCE, perchloroethene/tetrachloroethene; Tat, twin-arginine translocation; TF, trigger factor.

The GenBank/EMBL/DDBJ accession number for the trigger factors 1 and 2 (*tig1* and -2) sequences of *Desulfitobacterium hafniense* strain TCEI are GU136801 and GU136802, respectively.

Supplementary methods, three supplementary figures and a supplementary table are available with the online version of this paper.

Genevaux *et al.*, 2004; Liu *et al.*, 2005; Ullers *et al.*, 2007). In agreement with such a hypothesis, the double *tig dnaK* deletion strain shows a very narrow temperature range of growth and accumulates high levels of aggregated proteins (Deuerling *et al.*, 1999; Genevaux *et al.*, 2004; Teter *et al.*, 1999).

Remarkably we found that several genome sequences of *Desulfitobacteria* possess two complete TF chaperones and at least one additional TF-like protein that lacks the NTD (J. Maillard, unpublished results, and Morita *et al.*, 2009). *Desulfitobacteria* belong to the Firmicutes and are obligate anaerobic Gram-positive bacteria known for their metabolic versatility (for a review see Villemur *et al.*, 2006). Pure cultures of *Desulfitobacteria* have been mostly obtained from environments polluted with chlorinated organic compounds, which they can use as a terminal electron acceptor in a process called organohalide respiration (previously named dehalo- or halo-respiration). The key enzyme in this anaerobic respiration is the reductive dehalogenase (RdhA) which builds a large and diverse family of complex redox enzymes harbouring a corrinoid and iron-sulfur clusters as cofactors. RdhAs have been shown to be translocated across the cytoplasmic membrane (John *et al.*, 2006; Suyama *et al.*, 2002), probably via the twin-arginine translocation (Tat) pathway (Sargent, 2007a), strongly indicating that RdhAs need to be fully folded prior to translocation. Our model organism for tetrachloroethene [perchloroethene (PCE)] anaerobic respiration is *Desulfitobacterium hafniense* strain TCE1 (*DhaTCE1*) which harbours a conserved four-gene cluster, *pceABCT*, encoding the PCE reductive dehalogenase (PceA) (Maillard *et al.*, 2005, 2003). The *pceT* gene encodes a predicted protein of 316 amino acids with sequence homology to CprT which belongs to the chlorophenol reductive dehalogenase (*rdh*) operon of *Desulfitobacterium dehalogenans* and which has been shown to share clear sequence homology with the TF (Smidt *et al.*, 2000). Recently a study by Morita *et al.* (2009) has investigated the PPLase and chaperone activity of PceT from *D. hafniense* strain Y51 using an *in vitro* approach. Additionally co-immunoprecipitation data have indicated that PceT binds to PceA. Our goal in the present study was to shed light on the functionality of the multiple TF and TF-like chaperones found in *Desulfitobacteria* with emphasis on the peculiar TF-like protein, PceT, which lacks the NTD. We present the first, to our knowledge, *in vivo* functional investigation of all three *Desulfitobacteria* TFs by the use of heterologous complementation in *E. coli*. Moreover, we provide the evidence on the specific action of PceT in the folding process of PceA. Additionally protein-protein interaction revealed binding of PceT to the Tat signal peptide of PceA. Therefore we propose that PceT and its homologues in other *rdh* operons represent a class of chaperones dedicated to the family of Tat-dependent reductive dehalogenases.

METHODS

Bacterial strains and growth conditions. *D. hafniense* strain TCE1 (here abbreviated *DhaTCE1*, DSMZ strain 12704) was cultivated in

liquid medium in strictly anaerobic conditions using the DSM medium 717 with some modifications: no yeast extract was added and both vitamin solutions (DSM 141 and 503) were combined into a single one. PCE or thiosulfate was added alternatively as terminal electron acceptor. The *E. coli* strains used in this study are listed in Table 1. *E. coli* was routinely cultivated in liquid or solid Luria-Bertani medium containing the necessary antibiotics: 100 µg ampicillin ml⁻¹ (for p29SEN or pUT18 plasmid derivatives), 50 µg kanamycin ml⁻¹ (for *E. coli* W3110Δ*tigDnaKJ* and for pMPM-K5Ω plasmid derivatives) or 30 µg chloramphenicol ml⁻¹ (for pT25 plasmid derivatives). In contrast with other *E. coli* strains, the temperature-sensitive strain W3110Δ*tigDnaKJ* was routinely cultivated at 22 °C. Heat shock transformation of *E. coli* was applied. Competent cells of strain W3110Δ*tigDnaKJ* were prepared differently. An overnight pre-culture was diluted 50 × in fresh LB medium containing kanamycin and 10 mM MgSO₄ and was cultivated to OD₆₀₀ 0.4. Cells were collected by centrifugation, washed first in one-fifth of the initial volume of RF1 solution [30 mM potassium acetate, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM MnCl₂, 15 % (v/v) glycerol, pH corrected to 5.8 with acetic acid] and incubated for 20 min on ice before centrifugation. The cell pellet was resuspended in 1/25 of the initial volume of RF2 solution [10 mM MOPS, 10 mM RbCl₂, 75 mM CaCl₂, 15 % (v/v) glycerol, pH corrected to 6.5 with KOH]. Heat shock transformation of strain W3110Δ*tigDnaKJ* was performed for 2 min at 30 °C.

Nucleic acid extraction and amplification. Genomic DNA from *DhaTCE1* was extracted using the DNA Tissue kit (Qiagen) and quantified with the Nanodrop ND-1000 apparatus (Thermo Scientific). Total RNA was extracted from *DhaTCE1* as described previously (Prat, 2009) (see Supplementary Methods, available with the online version of this paper, for details). A standard 50 µl PCR contained 10 µl GoTaq 5 × PCR buffer (Promega), 1.5 µl 2.5 mM dNTPs, 2.5 µl 10 µM forward and reverse primer solution and 2.5 U GoTaq polymerase (Promega). Reverse transcription of RNA was achieved after 90 min at 42 °C in a 30 µl reaction containing 500 ng RNA, 1.5 µg random hexamer (Microsynth), 6 µl M-LMV RT 5 × buffer, 1.25 mM dNTPs (Microsynth) and 300 U M-LMV reverse transcriptase (Promega). Complementary DNA template was diluted between 500- and 5000-fold in order to amplify gene targets in the linear range. These products were finally analysed by the Bioanalyzer (Agilent Technologies) and their intensity was expressed as arbitrary units.

Sequence analysis. Nucleotide and protein sequence analysis was routinely performed with the Lasergene package (DNASTAR). Sequence alignment was performed with CLUSTAL_X (Thompson *et al.*, 1997) and homology trees built with MEGA4 (Tamura *et al.*, 2007).

Nucleotide sequence accession no. The sequence of *DhaTCE1 pceT* has been previously published (Maillard *et al.*, 2005) and is accessible with the GenBank accession no. AJ439608. Nucleotide sequences of *DhaTCE1* full-length trigger factors (*tig1* and -2) were submitted to GenBank with the accession nos GU136801 and GU136802, respectively.

Plasmid construction. Plasmids used in this study are summarized in Table 1. Oligonucleotides (Microsynth) and the corresponding restriction enzymes used for cloning are given in Supplementary Table S1 (available with the online version of this paper). Restriction enzymes were used according to the manufacturer's instructions (Promega). Ligation was performed with T4 DNA ligase according to the manufacturer's instructions (Roche). Plasmids were extracted with the QIAprep spin miniprep kit (Qiagen). All constructs were verified by in-house sequencing using the Big Dye Terminator mix v3.1 and the ABI Prism 3100 genetic analyser (Applied Biosystems). Derivatives of plasmid p29SEN (Genevaux *et al.*, 2004) were

Table 1. Strains and plasmids used in this study

Strains or plasmid	Strains	Description	Source or reference
<i>D. hafniense</i> strain TCE1		Strictly anaerobic low-GC Gram-positive	DSM 12704
<i>E. coli</i> DH5a		F ⁻ <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i> ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169, <i>hsdR17</i> (r _K m _K ⁻), λ ⁻	Laboratory strain
<i>E. coli</i> W3110		F ⁻ λ ⁻ <i>rph-1 INV(rrnD, rrnE)</i>	Bachmann (1972)
<i>E. coli</i> W3110 Δ <i>tig</i>		<i>E. coli</i> W3110 Δ <i>tig</i> (Cm ^S)	Genevax <i>et al.</i> (2004)
<i>E. coli</i> W3110 Δ <i>tig</i> Δ <i>dnaKJ</i>		<i>E. coli</i> W3110 Δ <i>tig</i> (Cm ^S) Δ <i>dnaKdnaJ::Kan</i> ^R	Genevax <i>et al.</i> (2004)
<i>E. coli</i> BTH101		F ⁻ , <i>cya-99, araD139 gale15 galK16 rpsL1</i> (Str ^r), <i>hsdR2 mcrA1 mcrB1</i>	Karimova <i>et al.</i> (1998)
Plasmids			
p29SEN		Low-copy-number vector, <i>Plac</i> promoter, pSC101 <i>ori</i>	Genevax <i>et al.</i> (2004)
p29- <i>EcoTF</i>		<i>E. coli</i> <i>tig</i> gene cloned into p29SEN	Genevax <i>et al.</i> (2004)
p29-PceT		<i>DhaTCE1 pceT</i> gene cloned into p29SEN	This study
p29-TF1		<i>DhaTCE1 tig1</i> gene cloned into p29SEN	This study
p29-TF2		<i>DhaTCE1 tig2</i> gene cloned into p29SEN	This study
p29- <i>EcoTF</i> _N ::PceT		NTD of <i>EcoTF</i> (1–115) fused to <i>DhaTCE1 PceT</i> (5–316), cloned into p29SEN	This study
p29 S- <i>EcoTF</i>		N-terminal Strep version of p29- <i>EcoTF</i>	This study
p29 S-PceT		N-terminal Strep version of p29-PceT	This study
p29 S-TF1		N-terminal Strep version of p29-TF1	This study
p29 S-TF2		N-terminal Strep version of p29-TF2	This study
p29 S- <i>EcoTF</i> _N ::PceT		N-terminal Strep version of p29- <i>EcoTF</i> _N ::PceT	This study
pMPM-K5 Ω		–	Mayer (1995)
pMPM-PceA		C-terminal His-tag version of pMPM-K5 Ω	This study
pUT18		BTH plasmid for X::Cya18 fusion	Karimova <i>et al.</i> (1998)
pT25		BTH plasmid for Cya25::X fusion	Karimova <i>et al.</i> (1998)
pUT18-PceT		BTH plasmid for PceT::Cya18 fusion	This study
pT25-PceT		BTH plasmid for Cya25::PceT fusion	This study
pUT18-prePceA		BTH plasmid for prePceA::Cya18 fusion	This study
pT25-prePceA		BTH plasmid for Cya25::prePceA fusion	This study
pUT18-PceA		As pUT18-PceA (processed form)	This study
pT25-PceA		As pT25-PceA (processed form)	This study
pUT18-PSP		BTH plasmid for PceA-signal-peptide::Cya18 fusion	This study
pT25-PSP		BTH plasmid for Cya25::PceA-signal-peptide fusion	This study
pUT18-TF1		BTH plasmid for TF1::Cya18 fusion	This study
pT25-TF1		BTH plasmid for Cya25::TF1 fusion	This study
pUT18-TF2		BTH plasmid for TF2::Cya18 fusion	This study
pT25-TF2		BTH plasmid for Cya25::TF2 fusion	This study

constructed to express *DhaTCE1* TFs. The same constructs were also prepared including an N-terminal Strep(II) epitope (MASWSHPQFEKIEGR). An additional TF plasmid expressing the hybrid protein Φ *EcoTF*_N::PceT was constructed by fusing the NTD of *E. coli* TF (TF_{1–115}, designated *EcoTF*_N) to PceT (PceT_{5–316}) using a PCR fusion method. The native *pceA* gene including the Tat signal peptide encoding region was cloned into plasmid pMPM-K5 Ω including the coding sequence for a C-terminal 6 $\times$ His tag, giving the plasmid pMPM-PceA. Plasmid constructs for the bacterial two-hybrid (BTH) system were constructed in pUT18 and pT25 vectors, from which proteins of interest are expressed as fusion to the T18- and T25-fragments of *Bordetella petrusis* adenylate cyclase (Karimova *et al.*, 1998).

Temperature-sensitive complementation assay of *E. coli* W3110 Δ *tig* Δ *dnaKJ*. Fresh transformants of *E. coli* W3110 Δ *tig* Δ *dnaKJ* harbouring p29-TF derivatives were cultivated at 22 °C for 16 h. The cultures were then serially diluted to 10⁻⁶ from which 5 μ l was spotted on plates containing from 0 to 100 μ M IPTG. Replica plates were incubated at various temperatures from 22 °C to 37 °C. The toxicity of *DhaTCE1* TFs for *E. coli* was tested in strain W3110 Δ *tig* by spotting the corresponding cultures on plates in the same way.

Isolation of aggregated proteins. Aggregated proteins were isolated from *E. coli* as described by Tomoyasu *et al.* (2001). Briefly pre-cultures of *E. coli* W3110 Δ *tig* Δ *dnaKJ* harbouring p29-TF derivatives were diluted 50 $\times$ in fresh medium and cultivated at 20 °C until OD₆₀₀ 0.6 was reached. TF expression was induced after 60 min by 10, 100 or 500 μ M IPTG. Cultures were then shifted to 37 °C for 2 h. Cell density was recorded and a culture volume corresponding to 16 OD₆₀₀ units was collected and centrifuged for 10 min at 4000 g. Cell pellets were resuspended in 120 μ l freshly prepared buffer A [10 mM potassium phosphate (pH 6.5), 1 mM EDTA, 20 % sucrose, 2 mg lysozyme ml⁻¹] and incubated on ice for 30 min. Then 1080 μ l buffer B [10 mM potassium phosphate (pH 6.5), 1 mM EDTA] was added and cells were disrupted by sonication (2 $\times$ 10 pulses of 1 s at 30 W). Cell debris were removed by 15 min centrifugation at 2000 g. The crude extract was centrifuged for 25 min at 14 000 g and the soluble protein fraction was discarded. Pellets were frozen at -80 °C and thawed again, then resuspended in 1 ml buffer B. A new sonication and centrifugation round was done. The pellet was resuspended in 960 μ l buffer B, sonicated, mixed with 240 μ l of 10 % Nonidet-P40 and centrifuged again. This extraction of membrane protein was repeated once and the resulting pellet considered as the fraction of aggregated proteins. Samples were

finally run on 12 % SDS gels and stained with Coomassie blue following standard procedures.

Isolation of TF associated with *E. coli* ribosome. This experiment was performed according to the method of Hesterkamp *et al.* (1997). *E. coli* W3110Δ*tig* harbouring the p29-TF Strep(II) derivatives were cultivated at 30 °C to OD₆₀₀ 0.5. TF expression was induced by 10 μM IPTG for 90 min. The cell density was recorded and cultures were kept on ice for 30 min. Cells were harvested by centrifugation for 10 min at 4000 g and the pellets were resuspended in a volume of lysis buffer [50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 10 mM MgCl₂, 15 % sucrose, 2 mg lysozyme ml⁻¹] corresponding to half of the OD₆₀₀ value. After 30 min on ice, samples were frozen at -80 °C and thawed. Four volumes of double-distilled water (ddH₂O) were added and cells were disrupted by sonication (3 × 10 pulses of 1 s). Cell debris were removed by centrifugation at 15 000 g for 15 min and the lysate was collected. In 3 ml ultracentrifugation tubes (Beckman), 0.75 ml lysate was added to 2.25 ml sucrose cushion solution [20 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 20 % sucrose, 0.1 or 0.5 M KCl, 0.035 % β-mercaptoethanol]. After 1 h of ultracentrifugation at 213 000 g the soluble protein fraction (S) was carefully collected. Ribosome pellets (P) were resuspended in 50 μl SDS loading buffer. Protein samples were separated by electrophoresis with a ratio of 1 : 5 (S : P) and the TFs detected by Western blotting with anti-Strep(II) antibody.

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were performed following standard procedures (Sambrook *et al.*, 1989). Proteins were then either stained with Coomassie R250 brilliant blue, or transferred onto PVDF membrane using the trans-blot semi-dry cell according to the manufacturer's instructions (Bio-Rad). Strep(II)-tagged TF derivatives were incubated with anti-Strep(II) antibody (Qiagen) and His-tagged PceA with Penta-His antibody (Qiagen), followed by detection with anti-mouse horseradish peroxidase-conjugated secondary antibody (Promega). Standard enhanced chemiluminescence (ECL) detection was performed according to the manufacturer's instructions (GE Healthcare) and revealed on Hyperfilm ECL by a Curix 60 apparatus (AGFA).

Heterologous co-expression of recombinant PceA with TF derivatives in *E. coli* W3110Δ*tig*. *E. coli* W3110Δ*tig* harbouring the pMPM-PceA plasmid was transformed with all p29-TF derivatives. Cells were cultivated at 37 °C in LB to OD₆₀₀ 0.5, and then simultaneous induction of PceA and individual TFs was initiated by adding 0.05 % arabinose and 20 μM IPTG, respectively. Induction temperature and times were double-distilled water: 90 min at 37 °C, 2 h at 30 °C, 6 h at 20 °C. Soluble and insoluble protein fractions were prepared using a simplified version of the protocol for the isolation of aggregated proteins. Briefly, the volume of induced cells corresponding to 2 OD₆₀₀ units was pelleted. Cells were incubated for 30 min in 60 μl buffer A (see above), after which 340 μl buffer B was added. Sonication was performed, followed by removal of cell debris. A 300 μl volume of supernatant was centrifuged for 25 min at 14 000 g at 4 °C. The soluble protein fraction (S) was carefully removed and the pellet (insoluble fraction, I) was resuspended in 300 μl of 10 mM Tris/HCl buffer (pH 7.5). Finally PceA and Strep(II)-tagged TF-derivatives were detected by Western blotting as described above.

Protein-protein interaction by the BTH system. After transforming the strain BTH101 with combinations of pT25- and pUT18-derivatives, single colonies were picked and cultivated overnight in liquid medium. The next day, 5 ml fresh medium was inoculated with the overnight cultures and cultivated at 30 °C to OD₆₀₀ 0.5. From there the following two interaction reporter assays were performed.

Colorimetric detection of protein interaction on MacConkey-maltose plates. MacConkey agar (Difco) was prepared as recommended by the manufacturer. While pouring plates, sterile-filtered

maltose was added to a final concentration of 1 %, in addition to the necessary antibiotics. For each BTH101 derivative, 5 μl of the daily culture was spotted in duplicate on MacConkey-maltose plates and incubated for at least 16 h at 30 °C.

Quantitative measurement of protein interaction by the β-galactosidase assay. OD₆₀₀ of daily cultures was recorded and 1 ml of each culture was treated as follows. Cells were permeabilized by the addition of 50 μl toluene, vortexed for 30 s followed by 15 min incubation on ice. To 450 μl Z buffer [1 l of Z buffer contains 8.52 g Na₂HPO₄ (anhydrous), 6.24 g NaH₂PO₄·2H₂O, 0.75 g KCl, 0.25 g MgSO₄·7H₂O and 0.7 ml freshly added β-mercaptoethanol], 50 μl of permeabilized cells was added and equilibrated at 28 °C for 5 min. Addition of 100 μl ONPG (4 mg ml⁻¹) initiated the enzymic reaction. After a sufficient yellow colour was developed (or after a maximum of 2 h), the reaction was stopped by the addition of 250 μl 1 M Na₂CO₃. Both start and stop times were carefully recorded. After rapid centrifugation to remove cell debris, the A₄₂₀ value of the supernatant was recorded and the β-galactosidase activity (Miller units) calculated as (A₄₂₀ × 103)/(time × volume × OD₆₀₀). Time and volume of cells were given in min and ml, respectively.

RESULTS

Redundancy of TFs in *Desulfitobacter*

A single copy of the TF-encoding gene (*tig*) was identified in classical bacterial models such as *E. coli* (Hesterkamp & Bukau, 1996; Stoller *et al.*, 1995) and *Bacillus subtilis* (Göthel *et al.*, 1997). In the genome sequence of *Desulfitobacter*, however, at least two TF paralogues were identified (see Fig. 1 and Supplementary Fig. S1, available with the online version of this paper). While multiple TFs have been recently reported for *D. hafniense* strain Y51 (Morita *et al.*, 2009), this trend has not yet been recognized as a general feature of the genus *Desulfitobacterium*. Two full-length three-domain TFs are encoded in *D. hafniense* strain Y51 (TF1, DSY3205; TF2, DSY3514), strain DCB-2 (TF1, Dhaf_4378; TF2, Dhaf_1894) and *Desulfitobacterium metallireducens* (TF1, DesmeDRAFT_0860; TF2, DesmeDRAFT_2807). Specific primers designed based on both *tig* genes from strain DCB-2 allowed the identification of the corresponding genes from our model organism, *DhaTCE1*. Both TF paralogues share a high degree of protein sequence identity with those found in the two other strains (100 and 90 %, respectively). Only one full-length TF was identified in the draft genomes of *D. dehalogenans* (DesdeDRAFT_0273) and *Desulfitobacterium dichloroeliminans* (DesdiDRAFT_1514), indicating that TF2 is not conserved in all *Desulfitobacter* and suggesting that this latter protein is not an essential chaperone. Beside the full-length TFs in *Desulfitobacter*, several shorter TFs paralogues were also identified in *Desulfitobacter* and other organohalide-respiring bacteria such as *Dehalobacter restrictus* (Maillard *et al.*, 2005) and *Geobacter lovleyi* as part of gene clusters involved in reductive dechlorination of various chlorinated compounds (see Supplementary Fig. S1 for a complete set). Within this new class of truncated TFs, PceT is encoded in the *pce* gene cluster of *DhaTCE1* and is composed of two of the three classical TF domains, namely the peptidyl-prolyl *cis/trans*

isomerase domain and the C-terminal chaperone domain. The N-terminal ribosome-binding domain (*E. coli* TF₁₋₁₁₈) is completely missing in this class of TFs; however, the linker region that has been shown to stabilize the CTD (Merz *et al.*, 2006) is present and located at the N-terminal end (PceT₁₋₂₂). A sequence alignment of all *Dha*TCE1 TFs is given in Fig. 1 along with *E. coli* TF and its secondary structure. Gene expression analysis of TFs in *Dha*TCE1 revealed that under standard anaerobic conditions all three TFs are transcribed at a steady state level with a clear prevalence of TF1 over TF2 (by 100-fold; see Supplementary Fig. S2, available with the online version of this paper).

***Desulfitobacterium* TFs are partially functional in *E. coli* and exhibit different levels of functionality**

Since no genetic tool is available to investigate the *in vivo* gene function in *Desulfitobacterium*, functional complementation was realized by heterologously expressing TFs of *Dha*TCE1 in the well-characterized *E. coli* triple mutant strain W3110Δ*tig*Δ*dnaKJ*, in which the genes encoding TF (*tig*) and both *dnaK* and *dnaJ* have been deleted (Genevax *et al.*, 2004). The strain lacking both TF and DnaK chaperones exhibits a strong temperature-sensitive phenotype (*Ts*) and accumulates high levels of aggregated cytosolic proteins. This strain is considered to be a very

sensitive tool to assess TF chaperone function heterologously. For this purpose, all three *Dha*TCE1 TFs encoding genes were cloned into p29SEN as native genes or with an additional N-terminal Strep(II)-tag to visualize protein expression using anti-strep-tag antibodies. In addition a hybrid protein consisting of the NTD of *E. coli* TF attached to PceT (Φ*Eco*-TF_N::PceT) was prepared to simulate a full-length TF version of PceT. The plasmid p29-*Eco*TF expressing *E. coli* TF (*Eco*-TF) was included as a positive control throughout.

TF-mediated bacterial growth at high temperature.

In order to characterize the TF function in *E. coli* W3110Δ*tig*Δ*dnaKJ*, all three *Dha* TFs were expressed at increasing IPTG concentrations and the cultures were incubated at permissive and non-permissive temperatures of growth (Fig. 2a). On one hand full-length TFs of *Desulfitobacterium* (Fig. 2a, lanes 3 and 4) gave the best *Ts* complementation, although not at the level of *E. coli* TF. While *Dha*-TF1 exhibited some toxicity, similarly to *Eco*-TF (Genevax *et al.*, 2004; Guthrie & Wickner, 1990), *Dha*-TF2 was not toxic in the limit of experimental conditions and complemented well up to 33 °C. *Dha*-PceT on the other hand showed a much weaker complementation and at less stringent temperatures. However no toxicity was observed. Remarkably, the hybrid protein Φ*Eco*-TF_N::PceT

Fig. 1. Protein sequence alignment of *Desulfitobacterium* TFs. Full-length TFs (*Dha*-TF1 and -TF2) and PceT of *Dha*TCE1 were aligned with *E. coli* TF by using CLUSTAL_x. The secondary structure of *Eco*-TF is also indicated: α -helices (spirals), β -strands (arrows) and the conserved motif for ribosome binding (hatched box).

Fig. 2. TF complementation. (a) Complementation of temperature-sensitive phenotype of *E. coli* W3110ΔtigΔdnaKJ. (b) Strep(II)-tagged TFs expression level in *E. coli* W3110ΔtigΔdnaKJ after 1 h induction at 10 μM IPTG at 20 °C. (c) TF-mediated toxicity in *E. coli* W3110Δtig. Cultures expressing the different TFs were spotted on plates under the conditions and at the concentrations indicated. (d) Strep(II)-tagged TF expression levels in *E. coli* W3110Δtig after 1 h induction at 10 mM IPTG at 20 °C. Sample numbers: 1, vector control; 2, *Eco*-TF; 3, *Dha*-TF1; 4, *Dha*-TF2; 5, *Dha*-PceT; 6, Φ *Eco*-TF_N::PceT.

was highly toxic and thus did not complement the *Ts* phenotype. No complementation was obtained at 35 °C or above. Very similar results were observed with the Strep-tagged TFs, which enabled visualization of their expression. With the exception of the hybrid protein all other TFs exhibited a rather similar expression level (Fig. 2b). The slight increased expression of *Dha*-TF2 might explain why this chaperone could still complement the *Ts* phenotype at 33 °C. The toxicity of the hybrid protein Φ *Eco*-TF_N::PceT was further demonstrated by expressing it in the *E. coli* W3110Δtig background, a strain that does not show the *Ts* phenotype (Fig. 2c). In this case, the protein was also

detrimental at low expression levels but only at the low temperature of 20 °C. Indeed, toxicity was fully relieved at 37 °C. Protein expression was also confirmed under these growth conditions (Fig. 2d).

Protein aggregation in *E. coli* W3110ΔtigΔdnaKJ. *E. coli* without TF and DnaK chaperones accumulates aggregated proteins when exposed to non-permissive temperature (Genevaux *et al.*, 2004). Complementation of *E. coli* chaperone mutant strain by *Dha*TFE1 TFs was also investigated for their ability to relieve the protein aggregation phenotype (Fig. 3). After 1 h of TF induction with 100 μM IPTG at permissive temperature, the cultures were incubated for 2 h at 37 °C. Protein aggregates were isolated which revealed that *Dha*-TF1 fully relieved this phenotype compared with the vector control, while *Dha*-TF2 had only a slight effect. In contrast, *Dha*-PceT did not prevent aggregation (Fig. 3b). When *Dha*-TF1 was expressed at a

Fig. 3. TF-relieved phenotype of protein aggregation in *E. coli* W3110ΔtigΔdnaKJ. *E. coli* and *Desulfitobacterium* TFs were expressed in the *E. coli* deletion strain and protein aggregation was induced by a shift from permissive temperature to 37 °C. (a) Coomassie-stained gel with whole-cell extracts (control) of *E. coli* expressing the TF indicated above. (b) Gel depicting the protein aggregates isolated from the same cells. The asterisk indicates the Strep-tagged *Dha*-PceT detected by Western blotting in the protein aggregates.

lower level (10 μ M), a significantly lower relief was observed, suggesting that a higher level is necessary for *Dha*-TF1 compared with *E. coli* TF (data not shown).

TF-mediated secretion delay. Overexpression of *E. coli* TF has been reported to cause a general secretion delay by sequestering Sec-dependent proteins (Lee & Bernstein, 2002; Ullers *et al.*, 2007). Such a property of TF was dependent on efficient binding to the ribosome (Ullers *et al.*, 2007). The capacity of *Dha*-TFs to retard protein export was tested by simultaneous expression of the TFs and the secreted maltose-binding protein (MBP) in *E. coli* W3110 Δ *tig* strain. Secretion delay was observed as an accumulation of the precursor of MBP (upper band in Supplementary Fig. S3, available with the online version of this paper). In contrast with the *E. coli* TF no secretion delay was observed here for all three *Dha*-TFs under the conditions used. Only the hybrid Φ *Eco*-TF_N::PceT, which is expected to strongly bind to ribosomes as it carries *E. coli* TF NTD, was capable of retarding MBP export.

*Dha*TCE1 TFs bind differently to *E. coli* ribosomes

To further characterize the behaviour of TFs from *Dha*TCE1 in *E. coli*, their association with ribosomes was investigated using the protocol established by Hesterkamp *et al.* (1997). Remarkably, the two full-length TFs harbouring a conserved NTD did not behave similarly (Fig. 4). While *Dha*-TF1 was found as soluble protein as well as associated with the ribosome fraction, showing some KCl-dependent dissociation, *Dha*-TF2, in contrast, was mostly seen in the soluble fraction. Its slight KCl-dependent dissociation suggested, however, that it binds to *E. coli* ribosomes with a severely reduced affinity. Almost no *Dha*-PceT was found in the pelleted ribosome fraction. The weak signal observed there did not show any KCl-dependent dissociation and might rather represent some aggregated PceT as observed in Fig. 3(b). In contrast, the hybrid PceT protein with the *E. coli* NTD was strongly retained at the ribosomes similarly to *Eco*-TF.

Fig. 4. TF-ribosome interaction. Strep(II)-tagged TFs were induced in *E. coli* W3110 Δ *tig*, then the whole-cell extracts were incubated with 100 or 500 mM KCl, followed by the separation of the soluble protein fraction (S) and the ribosome pellets (P). All tagged TFs were finally detected by Western blotting using anti-Strep antibody.

PceT is a dedicated molecular chaperone for the reductive dehalogenase PceA

In a first attempt to show the dedication of the TF-like protein PceT toward the reductive dehalogenase PceA, gene transcription analysis was investigated in *Dha*TCE1 following a pulse of PCE in a culture respiring thiosulfate. Samples for RNA extraction were collected at time intervals of 1, 2, 4 and 8 h and the transcription of *pceA*, *pceT* and *rpoB* genes was analysed semiquantitatively by reverse transcription, PCR amplification of serially diluted cDNA and the quantification of PCR products using the Bioanalyzer (Fig. 5). This approach showed first that the

Fig. 5. Gene transcription level of *pceA* and *pceT* upon addition of PCE. (a) Time-course of *Dha*TCE1 growth on thiosulfate without (control, $\circ$) and with ($\blacksquare$) a PCE pulse. The evolution of chloride release is also depicted for the culture on thiosulfate without PCE ($\triangle$) and after PCE addition ($\blacklozenge$). (b) Gene expression level analysed by combined RT-PCR on serially diluted cDNA and quantification with the Bioanalyzer. Gene transcription is expressed as the ratio of the targeted gene to constitutively expressed *rpoB*. White bars, expression in *Dha*TCE1 cultivated on thiosulfate (control); black bars, expression in *Dha*TCE1 cultivated on thiosulfate after a pulse of PCE (at time 0). Error bars indicate SD.

reductive dehalogenase (PceA) is active under thiosulfate respiration as chloride release was observed readily after addition of PCE to the culture (Fig. 5a). Moreover, the transcription level of *pceA* and *pceT* increased approximately twofold within a few hours of PCE addition compared with the constitutively expressed *rpoB* gene.

Previous attempts to produce recombinant reductive dehalogenases in *E. coli* have shown that the complex redox enzymes are produced as inactive aggregates (A. Duret & J. Maillard, unpublished data; Kimoto *et al.*, 2010; Neumann *et al.*, 1998; Suyama *et al.*, 2002). Here, we investigated whether PceT is involved in the maturation process of PceA by using the *E. coli* heterologous system in two independent approaches.

TF-mediated solubilization of the reductive dehalogenase PceA. Heterologous co-expression of a His-tagged pre-protein version of PceA together with all TFs was conducted at different induction temperatures in *E. coli* strain W3110Δ*tig*. Soluble (S) and insoluble (I) protein fractions were isolated and assayed for the presence of PceA. The results presented in Fig. 6(a) clearly demonstrated that both *Eco*-TF and *Dha*-PceT contributed positively to solubilize recombinant PceA at 37 °C. Indeed percentile signal quantification of soluble versus insoluble PceA (S:I) revealed that PceT led to a 57:43 ratio, while a ratio of 45:55 was calculated for *Eco*-TF (Fig. 6a, left panel). Inducing proteins at 30 °C dramatically shifted PceA toward the soluble fraction. The vector control already showed a clear shift, but co-expression of the chaperones had an even greater effect, with PceT showing the highest S:I ratio (97:3) (Fig. 6a, central panel). Further decreasing the induction temperature to 20 °C had an additional effect as the overall PceA signal was almost absent for the vector control and for *Dha*-TF1 and -TF2 and only slightly present in the soluble fraction of cells expressing *Eco*-TF. PceT and, to some extent, also the hybrid protein were still producing large amounts of soluble PceA, suggesting that at 20 °C PceT is not only solubilizing PceA but also preventing its degradation (Fig. 6a, right panel). To further investigate the effect of PceA expression on *E. coli* viability, strain W3110Δ*tig* co-expressing PceA and the chaperones were first cultivated in the absence of inducer then spotted on plates containing different combinations of inducers and incubated at 37 °C (Fig. 6b) and at 30 and 20 °C (data not shown). The expression of PceA affected the growth of *E. coli* at 37 °C, a phenotype which was completely relieved with the co-expression of PceT (Fig. 6b, panel iv, lane 5). No PceA-dependent toxicity was observed at a lower temperature, suggesting, together with the partition of PceA in the soluble and insoluble fractions, that PceA aggregation might lead to toxicity towards *E. coli* growth.

PceA-chaperone interaction revealed by the BTH system. In a second approach, all three *Dha*TCE1 TF coding sequences, and also the sequences corresponding to

Fig. 6. Heterologous co-expression of PceA together with the different TFs in *E. coli* W3110Δ*tig*. (a) Effect of TF expression on the solubility of PceA. TFs were induced at 20 μM IPTG, PceA at 0.05 % arabinose. Induction temperatures were 37, 30 and 20 °C. PceA was detected by Western blotting after separating the soluble (S) and insoluble (I) proteins. (b) Effect of PceA expression on the viability of *E. coli* W3110Δ*tig* by spotting the cultures on plates at 37 °C without any inducer (i), with 10 μM IPTG (ii), with 0.1 % arabinose (iii) or with both inducers (iv). Sample numbering: 1, vector control; 2, *Eco*-TF; 3, *Dha*-TF1; 4, *Dha*-TF2; 5, *Dha*-PceT; 6, Φ *Eco*-TF_N::PceT.

PceA pre-protein (prePceA, 551 amino acids), its processed form (PceA, 512 aa) and PceA Tat signal peptide (PSP, 36 aa, without the cleavage site), were cloned into BTH vectors pUT18 and pT25. Double transformants of *E. coli* BTH101 carrying all possible combinations of pUT18 and pT25 derivatives were first spotted on MacConkey-maltose plates to detect protein–protein interaction as red colonies (Fig. 7a). After a stringent incubation of 16 h only two spots were clearly red, namely (Φ PceT::T18/ Φ T25::PceT) and (Φ PceT::T18/ Φ T25::PSP). The reciprocal proteins (Φ PSP::T18 and Φ T25::PceT) did not interact, possibly due to the conformation of individual fusion proteins. Similarly *Dha*-PceT did not interact with prePceA harbouring the signal peptide suggesting that the Tat

Fig. 7. TF–PceA interaction by the BTH system. (a) *E. coli* BTH101 expressing a combination of TFs and various forms of PceA were cultivated on MacConkey-maltose plates. Protein interaction was detected as a red coloration of the colonies (shown here as darker spots). The plasmids expressing the T18- and T25-fusion proteins are given horizontally and vertically, respectively. (b) β -Galactosidase assay as a reporter for protein–protein interaction. The name of the interacting proteins are given with the T18-fusion first, followed by the T25-fusion.

signal peptide moiety in the fusion protein Φ T25::prePceA is not accessible to PceT. Nevertheless the positive results obtained here suggested that as for *E. coli* TF, PceT forms a dimer and that it seems to recognize the Tat signal peptide of PceA. No interaction was observed between the two full-length *Dha*-TFs and any form of PceA (data not shown), highlighting the specific nature of PceT–PceA interaction. This qualitative approach was confirmed by assaying the β -galactosidase activity of cells harbouring all plasmid combinations. A selection of the data obtained is presented in Fig. 7(b).

DISCUSSION

Multiple TFs in *Desulfitobacter*

Desulfitobacter is the first genus where multiple paralogues of TF were identified. The analysis of five genome sequences of *Desulfitobacter* revealed from two to five TF paralogues, all of them harbouring a conserved full-length TF (TF1) likely to be the general TF chaperone. The genetic context of TF1 contains the gene *clpX* in analogy to the context around the *tig* gene in *E. coli*. TF2 is encoded by the last gene in a cluster resembling an anaerobic oxidoreductase with unknown function in *D. hafniense* strain Y51 and DCB-2 and part of a molybdoenzyme gene cluster in *D. metallireducens*, none of these predicted proteins harbouring a Tat signal peptide. In *DhaTCE1* the very low gene transcription level observed for *tig2* compared with *tig1* strongly supports the view that TF1 plays the role of the general molecular chaperone, whereas TF2 might be involved in the maturation of specific proteins under as-yet unknown conditions.

Functionality of *Desulfitobacter* full-length TFs

The *Ts* phenotype of the *E. coli* chaperone mutant was significantly relieved by both full-length *Dha* TFs. However *Dha*-TF1 allowed *Ts* complementation to a lower extent than TF2, an observation we can partially explain by its higher affinity for the ribosomes and thus a possible toxicity starting from a relatively lower induction level.

Compared with the conserved ribosome-binding peptide motif in *E. coli* ($G_{43}FRKGKVP_{50}$), each *Dha* TF only differs by a single residue; TF1 harbours an alanine (A_{49}), while TF2 has an asparagine residue (N_{48}). Although TF-ribosome structures do not show the direct involvement of *E. coli* TF-K₄₈ in the interaction with the ribosome (Baram *et al.*, 2005; Ferbitz *et al.*, 2004), this residue could well participate in stabilizing the complex, thus explaining the apparent higher affinity of TF1 for *E. coli* ribosomes. Protein aggregation was also differently relieved by the two full-length TFs with *Dha*-TF1 showing a relief similar to *E. coli* TF when expressed at high levels, thus supporting the bona fide general chaperone activity of TF1 *in vivo*. The weaker complementation exhibited by *Dha*-TF2 correlates well with the weak ribosome-binding of *Dha*-TF2. Similarly *E. coli* TF variants in NTD produce also higher amounts of protein aggregates (Genevaux *et al.*, 2004). This observation further exemplifies the general nature of the *Dha*-TF1 chaperone.

TF functionality of *Dha*-PceT

TF complementation by *Dha*-PceT and the hybrid protein were more contrasting. Although lacking the full N-terminal ribosome-binding domain PceT showed a weak generic TF function with respect to the *Ts* phenotype. This is in agreement with the contribution of TF CTD to chaperone activity (Kramer *et al.*, 2004; Merz *et al.*, 2006). Nevertheless, our data support the view of PceT as a remarkable TF-like chaperone with a specific substrate. Indeed, transcription of both *pceA* and *pceT* genes was induced by PCE although they are produced as distinct mRNA transcripts, as it has been shown for other *rdh* gene clusters (Futagami *et al.*, 2006; Smidt *et al.*, 2000). Moreover, we demonstrated that PceT acts efficiently in the solubilization of PceA, revealing the prevalence of this unique TF-like chaperone during post-translational folding over the generic action of a housekeeping ribosome-bound TF. PceT retains a functional PPIase activity (Morita *et al.*, 2009) whose contribution remains enigmatic in *E. coli* TF. In the case of PceT, the PPIase may play a critical role as a folding catalyst as PceA shows a high proline content, i.e. 35 prolines representing 6.4 % of PceA residues, while all

coding sequences of *D. hafniense* strain Y51 contain an average of 4.1% prolines. Some general chaperones harbouring a PPIase domain have been shown to interact with the Tat signal peptide of the cofactor-less SufI in *E. coli* (Holzapfel *et al.*, 2009), further arguing towards the involvement of PceT in chaperoning of Tat substrates. With our co-expression data we also show that at a low temperature PceT prevents the degradation of PceA, which highlights a possible role in quality control. This temperature effect is consistent with observations made in psychrophilic bacteria (Kawamoto *et al.*, 2007; Piette *et al.*, 2010; Qiu *et al.*, 2006), but also in *E. coli* (Kandror & Goldberg, 1997), where cold shock experiments have pinpointed the importance of the TF chaperone.

Dedicated chaperone function of Dha-PceT

In our study, protein interaction data suggest that PceT forms a dimer, an observation that is consistent with several studies on TF dimer formation (Kristensen & Gajhede, 2003; Liu *et al.*, 2005; Ludlam *et al.*, 2004; Martinez-Hackert & Hendrickson, 2007, 2009; Patzelt *et al.*, 2002). Both NTD and CTD of TF have been shown to participate in dimerization, however, with conflicting orientations (Kristensen & Gajhede, 2003; Martinez-Hackert & Hendrickson, 2007; Patzelt *et al.*, 2002). As PceT has no NTD, it is remarkable that it is able to form dimers. The presence in PceT of the linker region connecting TF NTD to the PPIase domain (Martinez-Hackert & Hendrickson, 2009; Yao *et al.*, 2008) suggests that together with the CTD, the linker region represents the minimal structure allowing dimerization. In a recent report on the participation of the TF dimer in the assembly of large protein complexes it is proposed that TF is not only involved in co-translational protein folding but may also participate in assembly of large molecular complexes (Martinez-Hackert & Hendrickson, 2009). The PceT dimer could thus serve for similar purpose to the benefit of the complex redox enzyme PceA.

The mode of action of PceT resembles that of other chaperones involved in the maturation of Tat-dependent proteins such as TorD (Genest *et al.*, 2009; Sargent, 2007b), DmsD (Guymer *et al.*, 2009; Li *et al.*, 2010) or NapD proteins (Kern *et al.*, 2007; Maillard *et al.*, 2007; Potter & Cole, 1999). Although belonging to very distinct protein families, these chaperones specifically recognize the signal peptide of their cognate Tat-dependent protein and participate in their maturation process. Likewise, PceT binds to the Tat signal peptide of PceA and thus probably retards its translocation, allowing both folding and cofactor assembly of the reductive dehalogenase. Whether additional chaperones are required for complete maturation of PceA is not yet known. Considering PceT and its homologues in other reductive dehalogenase gene clusters, we propose to give the general name of RdhT to this class of dedicated TF-like Tat chaperones. Not only do they represent a new chaperone family in Tat proofreading, but

they also indicate a subtle evolutionary strategy developed by organohalide-respiring bacteria to transform a general TF chaperone into a dedicated one by losing its N-terminal ribosome-binding domain to avoid interference with the generic ribosome-bound TF and by evolving toward a specific substrate.

Conclusions

The functional redundancy of TFs in *Desulfitobacteria* is revealed to be only apparent as each TF seems to have its own role in the metabolism, although all of them share some general chaperone functionalities. While Dha-TF1 represents the general TF chaperone in *Desulfitobacteria*, no metabolic function could yet be assigned to TF2. Finally we can consider PceT as the paradigmatic member of a new class of Tat chaperones harbouring strong similarities with TF, which confirms that several evolutionary strategies have converged toward a unified goal of dedicated chaperones in the Tat machinery (Sargent, 2007a), namely to allow quality control of the folding state of Tat substrates prior to their translocation across the cytoplasmic membrane.

ACKNOWLEDGEMENTS

This study was partly made possible thanks to a short-term fellowship from the European Molecular Biology Organization (EMBO) (ASTF 80.00-2008) allowing J. M. to travel to and work in P. G.'s laboratory. P. G. is supported by Action Temporaire Incitative Sur Programme-Centre National de la Recherche Scientifique (ATIP-CNRS) Microbiology and the Region Midi-Pyrénées. We would like to thank Marion Blondeau for technical assistance and Nevenka Adler for critical reading of the manuscript.

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Edited by: F. Sargent