This is the final draft of the contribution published as:

Türkowsky, D., **Lohmann, P.**, **Mühlenbrink, M.**, Schubert, T., **Adrian, L.**, Goris, T., **Jehmlich, N.**, **von Bergen, M.** (2019):
Thermal proteome profiling allows quantitative assessment of interactions between tetrachloroethene reductive dehalogenase and trichloroethene
J. Proteomics **192** , 10 – 17

The publisher's version is available at:

http://dx.doi.org/10.1016/j.jprot.2018.05.018

Thermal Proteome Profiling Allows Quantitative Assessment of Interactions between Tetrachloroethene Reductive Dehalogenase and Trichloroethene

Dominique Türkowsky[§,1], Patrick Lohmann[§,1], Marie Mühlenbrink[1], Torsten Schubert[2], Lorenz Adrian[3,4], Tobias Goris[2], Nico Jehmlich[1], Martin von Bergen[1,5]

[1]Department of Molecular Systems Biology, Helmholtz Centre for Environmental Research – UFZ, Permoserstraße 15, 04318 Leipzig, Germany

[2]Department of Applied and Ecological Microbiology, Institute of Microbiology, Friedrich Schiller University, 07743 Jena, Germany

[3]Department of Isotope Biogeochemistry, Helmholtz Centre for Environmental Research – UFZ, Permoserstraße 15, 04318 Leipzig, Germany

[4]Chair of Geobiotechnology, Technische Universität Berlin, Ackerstraße 76, 13355 Berlin

[5]Institute of Biochemistry, Faculty of Life Sciences, University of Leipzig, Brüderstraße 34, Germany

[§]These authors contributed equally to this work

Correspondence:

Prof. Dr. Martin von Bergen

E-mail: martin.vonbergen@ufz.de, Tel: +49-341-235-1211

Keywords: Proteome interaction; organohalide respiration; TCE; PCE; *Dehalococcoides mccartyi*; quantitative proteomics

Abstract

Thermal proteome profiling (TPP) is increasingly applied in eukaryotes to investigate protein-ligand binding through protein melting curve shifts induced by the presence of a ligand. In anaerobic bacteria, identification of protein-substrate interactions is a major challenge. We applied TPP to *Sulfurospirillum multivorans*, which is able to use trichloroethene as electron acceptor for growth, to investigate the interaction of its tetrachloroethene reductive dehalogenase PceA with trichloroethene. Several modifications in the protocol (e.g., incubation under anaerobic conditions; increasing the temperature range up to 97°C) extended the protein detection range and allowed the investigation of oxygen-sensitive proteins. Enzymatic reductive dehalogenation was prevented by omitting the electron donor during incubations. This enabled detecting the interaction of PceA with trichloroethene and confirmed that trichloroethene is a substrate of this enzyme. Interestingly, a putative response regulator showed a similar trend, which is the first biochemical hint for its proposed role in trichloroethene respiration. We proved that our TPP approach facilitates the identification of protein-substrate interactions of strictly anaerobic reductive dehalogenases and probably their regulators. This strategy can be used to identify yet unknown substrate specificities and possible signal-sensing proteins, and therefore has the potential to elucidate one of the unresolved fields in research on organohalide-respiring bacteria.

Significance

The assessment of enzyme-substrate or protein-ligand interactions in organohalide-respiring bacteria is a fundamental challenge. *Thermal proteome profiling* (TPP) allows elucidating proteome-wide thermal stability changes relying on the sensitivity of modern mass spectrometry. This gives access to the identification of interactions not detectable with other methods. In this TPP study, we demonstrate the interactions of a chlorinated substrate with a

45 reductive dehalogenase and potentially with a response regulator, thereby supporting the
46 response regulator's function in organohalide respiration. The strategy might also be applied
47 to identify yet unknown substrates of other enzymes in bacteria which are difficult to
48 investigate or for which only low amounts of biomass are available. The assessment of
49 enzyme-substrate interactions, which might enable conclusions about enzyme specificities,
50 represents a new application for TPP.

51 **Highlights**

52 - *Thermal proteome profiling* (TPP) was modified for analyzing bacterial oxygen-
53 sensitive enzymes

54 - Protein-trichloroethene interactions in organohalide-respiring *Sulfurospirillum*
55 *multivorans* were identified

56 - Interaction of the tetrachloroethene reductive dehalogenase with trichloroethene was
57 confirmed

58 - A first hint for the interaction of a response regulator and a chlorinated ethene was
59 provided

60

61 **Introduction**

62 Many organohalides are hazardous to human health and widely distributed in our
63 environment [1]. Many of them were prohibited decades ago [2-4] but had been used in
64 industry and agriculture for a long time and are recalcitrant against biodegradation [5].
65 Several anaerobic bacteria are capable of reductively dehalogenating organohalides, i.e.,
66 they use organohalides as a terminal electron acceptor during organohalide respiration [6, 7].
67 The catalyzing enzymes are the iron-sulfur cluster- and corrinoid-cofactor containing
68 reductive dehalogenases [8]. There are still many unanswered questions in the research on
69 organohalide-respiring bacteria, involving the functioning of reductive dehalogenases, their
70 substrate specificities and regulation [9].

71 *Sulfurospirillum multivorans*—an organohalide-respiring bacterium—produces the
72 tetrachloroethene reductive dehalogenase PceA, which dechlorinates tetra- and
73 trichloroethene (TCE) [10] but also brominated phenols [11]. A two-component regulatory
74 system encoded in close vicinity to *pceA* was predicted to be involved in the transcriptional
75 regulation of the *pceA* gene expression. However, this has not yet been biochemically proven
76 [12, 13]. In general, two-component regulatory systems involve a histidine protein kinase
77 detecting a chemical or physical signal from the environment and transducing this signal into
78 an intracellular signal cascade by phosphorylating a response regulator. The activated
79 response regulator usually binds to the DNA and induces or suppresses gene expression
80 [14].

81 Organohalide-respiring bacteria and their reductive dehalogenases are difficult to investigate
82 because many are extremely sensitive to oxygen. Additionally, many organohalide-respiring
83 bacteria grow slowly to low cell densities, and protocols for their genetic modification are not
84 yet available [15], which hinders, e.g., gene deletion studies. Of special interest for
85 biochemical investigations and bioremediation is the substrate specificity of reductive

86 dehalogenases. Due to the difficulties in heterologous expression and limited possibilities for
87 protein purification, approaches such as native polyacrylamide gel electrophoresis coupled to
88 enzymatic assays and mass spectrometry arose [16] but could not resolve reductive
89 dehalogenase substrate specificity in all cases [17, 18]. Here, we used *S. multivorans* as a
90 model organism to investigate substrate specificity of a reductive dehalogenase via *thermal*
91 *proteome profiling* (TPP), since PceA is a well-studied enzyme and *S. multivorans* one of the
92 few easier to handle organohalide-respiring bacteria, although genetic modification is
93 severely hampered also in this bacterium.
94 TPP is a further development of the cellular thermal shift assay on a proteome-wide scale. It
95 was established by Savitski *et al.* [19] in order to screen the whole proteome in an unbiased
96 way for potential targets of kinase inhibitors. Both techniques were mostly used for the
97 analysis of the mechanistic effects of drugs, usually inhibitors of enzymes, or of protein-
98 protein interactions in mammalian cells [20, 21]. The principle of TPP is that if a protein binds
99 to its ligand, higher temperatures are needed to denature the protein because part of the heat
100 energy dissociates the enzyme-ligand-complex. Consequently, the protein's melting
101 temperature (T_m), at which 50% of the protein is denatured and which can be calculated from
102 its melting curve, is shifted to higher temperatures.

103 In this study, we verified that the TPP method is suitable to analyze protein-TCE interactions
104 of *S. multivorans*. Due to the oxygen sensitivity of involved enzymes [22], oxygen was
105 excluded during the cultivation, protein extraction, substrate and temperature incubation. We
106 quantified the soluble protein fraction by a label-free approach instead of the isotopic labeling
107 as used by Savitski *et al.* [19], which opens up the TPP method to other applications.

108 Materials and Methods

109 Anaerobic cultivation and media composition

110 *Sulfurospirillum multivorans* was cultivated anaerobically with 40 mM pyruvate as an electron
111 donor and 10 mM PCE (nominal concentration, PCE was added from a 0.5 M stock solution
112 in hexadecane) as an electron acceptor at 30°C and 120 rpm in a defined mineral medium
113 [23]. In order to reduce the amount of chlorinated ethenes in the cultures to a minimum but to
114 obtain cells which still produce PceA, the organism was cultivated for three transfers with
115 40 mM fumarate as an electron acceptor and 40 mM pyruvate as an electron donor with 10%
116 inoculum each [24].

117 **Cell harvest and lysis**

118 The bacterial cells were harvested after 24 h in the late exponential phase. The culture was
119 centrifuged under anoxic conditions at 4,800 g for 20 min at 10°C for two times with a
120 washing step in between using 4 mM L-cysteine in phosphate buffered saline. The cell pellet
121 was dissolved in 4 mL of an anoxic lysis buffer ensuring preservation of PceA activity
122 (100 mM Tris-HCl, 4 mM ammonium sulfate, 1x MS-SAFE protease and phosphatase
123 inhibitor (Sigma-Aldrich, St. Louis, USA) and 2 mM L-cysteine) [10]. Cell lysis was performed
124 anaerobically by using a FRENCH® press (Thermo Fisher Scientific, Waltham, USA) with a
125 pressure of 1,000 psi. Cell debris was removed by centrifugation of the cell extract at
126 20,000 g for 10 min at 10°C. The protein concentration of the supernatant was determined by
127 using the Bradford assay (Sigma-Aldrich, St. Louis, USA). The quality of the sample
128 preparation was controlled by photometrical measuring the specific activity of the reductive
129 dehalogenase PceA in the crude extract on TCE using the synergy™ HT multi detection
130 microplate reader photometer (BioTek Instruments, Inc., Vermont, USA) under anoxic
131 conditions [23]. The microplate was sealed with a microseal 'B' film (Bio-Rad, CA, USA) and
132 measured in an anoxic chamber to avoid oxygen exposure.

133 **Preparation of cell extract for thermal proteome profiling**

134 While working in the anoxic chamber, the cell extract was split into two equal sets and
135 incubated with either 5 mM TCE in ethanol (final concentration) or with the same volume of
136 ethanol as a control. TCE as the substrate was added to the cell extract under anoxic
137 conditions by using an eVol xR glass pipette (SGE Analytical Science). Each sample per
138 condition was further divided into 30 aliquots and transferred into 0.6 mL micro bottles (lab
139 logistic group GmbH, Meckenheim, Germany) sealed with gas-tight caps. The 60 samples
140 were sequentially incubated for 3 min with one of ten temperatures between 43°C and 97°C
141 in a ThermoMixer (Thermo Fisher Scientific, Waltham, USA). The heated samples were
142 shock-frozen in liquid nitrogen. To separate native from denatured proteins, samples were
143 ultra-centrifuged at 100,000 g for 20 min at 4°C by using an Optima™ MAX-XP
144 ultracentrifuge and an ML-130 rotor (Beckman Coulter, Pasadena, USA). The supernatant
145 containing the soluble protein fraction was used for further analysis.

146 **SDS-PAGE, proteolytic digestion, and peptide extraction**

147 The SDS-PAGE was performed to remove contaminants from the samples, according to the
148 protocol in Franken *et al.* [25]. 25 µg protein of the lowest temperature point (43°C) and equal
149 volumes of the other samples were reduced in sample buffer (containing 50 mM dithiothreitol
150 and 1x lithium dodecyl sulfate, Sigma-Aldrich, St. Louis, USA) for 30 min in a ThermoMixer at
151 50°C and 700 rpm. Subsequently the samples were alkylated with 100 mM 2-iodoacetamide
152 for 30 min in the dark at room temperature [25]. After SDS-PAGE and staining with colloidal
153 Coomassie brilliant blue (Merck, Darmstadt, Germany) overnight, the gel band of each
154 temperature point containing all proteins was cut out, sliced into smaller gel pieces to
155 increase accessibility to the protease and destained according to Franken *et al.* [25]. In order
156 to reduce the number of missed cleavages, proteins in each band were proteolytically
157 digested using both, 0.6 µg lysyl endopeptidase (Wako Chemicals GmbH, Neuss, Germany)
158 at 37°C for 4 h and 0.5 µg trypsin (Sigma-Aldrich, St. Louis, USA) at 37°C, overnight [25].

159 Digestion was stopped by adding formic acid (FA) to a final concentration of 0.1%. After
160 peptide extraction [25, 26], the samples were lyophilized using the freeze-dryer alpha 2-4
161 LSC (Christ, Osterode, Germany) at 0.1 mbar vacuum and 1,650 mbar pressure (-40°C,
162 overnight). The extracted peptides were desalted using SOLAµ plates (Thermo Fischer
163 Scientific, Waltham, USA). Peptides were dissolved in 0.1% FA and injected into liquid
164 chromatography-mass spectrometry.

165 **LC-MS/MS analysis**

166 Samples were analyzed using a liquid chromatography (HPLC, Ultimate 3000 RSLCnano,
167 Dionex/Thermo Fisher Scientific, Idstein, Germany) coupled via a TriVersa NanoMate
168 (Advion, Ltd., Harlow, UK) source in LC chip coupling mode with an Orbitrap Fusion mass
169 spectrometer (Thermo Fisher Scientific, Waltham, USA). Samples (5 µL) were first loaded for
170 5 min on the precolumn (µ-pre-column, Acclaim PepMap C18, 2 cm, Thermo Scientific) at 4%
171 mobile phase B (80% acetonitrile in nanopure water with 0.08% formic acid) and 96% mobile
172 phase A (nanopure water with 0.1% formic acid) at a flow rate of 300 nl/min and at 35°C.
173 Then they were eluted from the analytical column (Acclaim PepMap C18 LC column, 25 cm,
174 Thermo Scientific) over a 100-min linear gradient of mobile phase B (4%–50%). The MS was
175 set on Top Speed for 3 s using the Orbitrap analyzer for MS and MS/MS scans with higher
176 energy collision dissoziation (HCD) fragmentation at normalized collision energy of 30%. MS
177 scans were measured at a resolution of 120,000 in the scan range of 400–1,600 *m/z*. The MS
178 ion count target was set to $4x10^5$ at an injection time of 60 ms. Most intense peaks (charge
179 state 2-7) were isolated for MS/MS scans by a quadrupole with an isolation window of 2 Da
180 and were measured with a resolution of 15,000. The dynamic exclusion was set to 30 s with a
181 +/-10 ppm tolerance. The automatic gain control target was set to $5x10^4$ with an injection time
182 of 150 ms

183 **Bioinformatical analysis**

184 *Protein identification and quantification*

185 Proteome Discoverer (v2.1, Thermo Fischer Scientific) was used for protein identification and
186 quantification (detailed workflow in Supplemental Methods). The MS/MS spectra (.raw files)
187 were searched by Sequest HT against a database containing 3,233 non-redundant protein-
188 coding sequence entries (downloaded January 2017 from NCBI GenBank, accession number
189 CP007201.1). A "common repository of adventitious proteins database" (cRAP) was
190 integrated to exclude contaminants. Trypsin was selected as protease and up to 2 missed
191 cleavages, 10 ppm precursor and 0.02 Da fragment mass tolerance were allowed. Peptides
192 with < 1% false discovery rate (FDR), XCorr ≥ 2, q-value and the posterior error probability
193 (PEP) ≤ 0.01 were considered as identified. Proteins were quantified using the average of top
194 three peptide MS1-areas, yielding raw protein abundances. The mass spectrometry
195 proteomics data (including the *.raw- and result-files) have been deposited to the
196 ProteomeXchange Consortium via the PRIDE (https://www.ebi.ac.uk/pride) partner repository
197 with the dataset identifier PXD009308.

198 *Melting curve fitting, melting point determination, and significance test*

199 Raw protein abundances of all quantified proteins were log transformed and scaled between
200 0 and 1 by subtracting the global minimum and normalizing to the abundance at the lowest
201 temperature of each protein to yield fold changes (Fig. S3). Proteins with at least two
202 abundance values in three replicates were considered as quantified. The average of these
203 two or, if available, all three replicates was calculated for each temperature point and
204 condition (criterion i). Furthermore, only proteins with an average quantitative value in at least
205 five temperature points (ii) were considered for the melting curve analysis by the adapted *R*
206 script TPP-TR [25, 26]. The melting curves were calculated using a sigmoidal fitting approach

207 with the *R* package *TPP*. This fitting was used to determine the melting point (T_m), which is
208 defined as the temperature at which half of the amount of proteins was denatured. The
209 melting point differences (ΔT_m) were calculated by subtracting the T_m with ethanol from the T_m
210 with TCE [25, 26]. The sigmoidal melting curves were quality-filtered according to the
211 following criteria [25, 26]: (iii) melting curves must reach a relative abundance plateau < 0.3
212 and (iv) the quality of the fit as expressed by the coefficient of determination (R^2) of both, the
213 TCE treated and control melting curves, must be > 0.8 in at least two of three replicates. The
214 statistical significance was calculated by using the non-parametric analysis of response
215 curves (NPARC) of the *R* package *TPP*, comparing the spline progression of the TCE-treated
216 condition and the ethanol-control [27]. The significance threshold was set to $p < 0.01$. This
217 estimation was further adjusted by the Benjamini-Hochberg correction to exclude potential
218 false positives [25, 26]. The quality-filtered melting curve data were used for figure generation
219 in *R* v3.4.2, by using the R packages *graphics*, *stats,* and *pheatmap*.

220 **Results**

221 We analyzed the melting proteome of *S. multivorans* in order to identify protein-substrate
222 interactions of the reductive dehalogenase PceA with the substrate TCE. First, we modified
223 the protocol [25] for analyzing oxygen-sensitive enzymes of bacteria. Therefore, cultivation of
224 bacteria, cell harvesting, protein extraction, substrate treatment, and temperature incubation
225 were performed under strictly anoxic conditions. A reductive dechlorination activity test of the
226 crude extract revealed an initial specific enzyme activity of 7.7±0.9 nkat/mg with TCE as
227 substrate, which decreased to 2.8±0.5 nkat/mg after cell lysis. This shows that the reductive
228 dehalogenase did retain enzyme activity after sample preparation.
229 The samples were treated with either TCE or ethanol for the negative control. Afterward, the
230 samples (each aliquot) were exposed to one out of ten different temperatures in the range of
231 43°C to 97°C. The denatured proteins were separated from the native proteins by

232 ultracentrifugation. After mass spectrometric analysis of the native fraction and statistical
233 analysis, the proteins were quality-filtered and fitted to protein melting curves. In total, 1,335
234 of the identified proteins were quantified, which comprises about 42% of the predicted
235 protein-coding sequences (Fig. 1).

236 In general, the protein abundances show that the native protein fractions decrease with
237 increasing temperatures (Fig. 2A). This allowed the calculation of protein melting curves by
238 sigmoidal curve fitting (Fig. 2B). After a stringent filtering procedure (Fig. S3), we obtained
239 highly valid sigmoidal melting curves of 435 proteins (Fig. 1, Tab. S1), with an average
240 standard error of 7%. Of all protein melting curves, 73% reached a plateau of zero at higher
241 temperatures (e.g., Fig. 2B). Of the organohalide respiratory core region [13], 31 gene
242 products were identified; for nine of them an average sigmoidal melting curve could be
243 calculated and a melting curve analysis could be conducted (Fig. S1). Melting curves for
244 proteins produced from the organohalide respiratory core region include the reductive
245 dehalogenase PceA (SMUL_1531, Fig. 3A), an IscU/NifU-like protein (SMUL_1533) which
246 might aid in PceA maturation, several proteins predicted to be involved in corrinoid synthesis
247 (SMUL_1544, 1545, 1547, 1548, 1551, 1559, 1560, 1562) and two flavin-containing proteins,
248 the FeS-cluster binding flavoprotein (SMUL_1573) and a putative flavin mononucleotide-
249 binding protein (SMUL_1575).

250 Melting temperatures (T_m) were defined as the temperature at which half of the protein
251 amount has been denatured (Tab. S1). The median T_m of all *S. multivorans* proteins was at
252 73°C (Fig. S2). In order to assess the reproducibility of the TPP, the melting temperatures of
253 the individual replicates were correlated to each other (Fig. 2C). The T_m values of two
254 replicates each were linear fitted, yielding coefficients of determination ranging from $R^2 = 0.58$
255 to 0.84.

256 To show the effect of TCE treatment on the stability of the proteome, the T_m shift (ΔT_m =
257 T_mTCE – $T_m$control) and their adjusted p-values after Benjamini-Hochberg correction are

258 displayed (Fig. 2D). 82 proteins (19%) have a mean ΔT_m outside the mean ±1 standard
259 deviation (ΔT_m < -1.8°C or ΔT_m > 4.1°C). Of the 435 protein melting curves, 20 were
260 significantly shifted, i.e., exhibit an adjusted $p < 0.01$ (Tab. 1). These are candidates for an
261 interaction with TCE. In total, five of the 20 significant protein melting curves fall outside both,
262 the ΔT_m and the significance threshold (Fig. 2D), including the reductive dehalogenase PceA
263 (ΔT_m = 5.5°C, adj. p-value = 0.0028, Fig. 3A). The melting curve shift of the reductive
264 dehalogenase was validated by western blot analysis (Fig. S4). In addition, the putative
265 response regulator SMUL_1539, most probably involved in the induction of PCE respiration,
266 showed a ΔT_m of 4.6°C, thus being outside the mean ΔT_m ±1 standard deviation threshold.
267 This, however, could not be statistically tested (Fig. 3A), because of too many missing values
268 in the curves (Tab. S1). The corresponding membrane-bound putative histidine kinase
269 (SMUL_1538) was not detectable.
270 Most proteins did not exhibit a significant melting curve shift (Fig. 2D). As representative
271 examples, the melting curves of three proteins of different functional classes, localizations,
272 and abundances (30-/40-/38-fold less abundant than PceA) are displayed (Fig. 3B). The
273 tryptophan synthase (SMUL_0559) is involved in the amino acid synthesis, the TetR family
274 protein (SMUL_1358) is a generic transcriptional regulator and the ATP synthase delta
275 subunit (SMUL_0684) is a membrane-associated representative of the energy metabolism.

276 Discussion

277 Direct detection of protein interactions in organohalide-respiring bacteria is a major challenge.
278 Therefore, we modified the TPP method to monitor changes in protein thermal stability across
279 the whole proteome of oxygen-sensitive bacterial cells and the substrate specificities of
280 reductive dehalogenases. In total, about 33% of the quantified proteins yielded two high-
281 quality average melting curves of at least two replicates each. Compared to Savitski *et al.*
282 [19], we have fewer identifications, which is due to our label-free approach. However, our

283 results are similar to other label-free studies [28] and highly valid and reproducible due to our
284 comprehensive statistical filtering procedure.

285 **Proof of enzyme-substrate interactions**

286 While the melting temperature of most background proteins was essentially unchanged by
287 exposure to TCE, the reductive dehalogenase PceA exceeded the stringent p-value and
288 melting temperature shift thresholds. The significant melting curve shift by TCE could be
289 confirmed by western blot analysis. In former studies, PceA was shown to be able to
290 dechlorinate TCE [10, 11]. The substrate specificity of PceA towards TCE was supported by
291 TPP, providing a proof-of-concept that TPP is a suitable instrument to study enzyme-
292 substrate interactions. The stabilization of an enzyme by its substrate first might sound
293 counterintuitive, since a substrate, unlike a ligand, is converted by the enzyme. However, our
294 data indicate that the enzyme-substrate complex endures long enough to induce a detectable
295 stability shift of the enzyme. We promoted the stability of the enzyme-substrate complex by
296 omitting the electron donor for the dehalogenating reaction, e.g., reduced methyl viologen,
297 thereby blocking the transformation of TCE. Furthermore, we provided TCE in excess to be
298 able to detect a PceA melting curve shift despite other electron donors in the cell lysate, e.g.,
299 reduced ferredoxin, which also might reduce TCE. The binding of TCE to PceA was shown to
300 occur mainly via van der Waals contacts in a hydrophobic active site pocket [30]. It is
301 therefore comparable to the binding of, e.g., the inhibitor methotrexate to the dihydrofolate
302 reductase, which occurs via intermolecular forces or, more specifically, ionic bonds [29] and
303 was detectable by TPP [21].

304 A protein with a melting curve shift above the ΔT_m threshold, which, however, did not pass the
305 applied stringent filter criteria and therefore could not be tested for significance, was a
306 putative response regulator (SMUL_1539). SMUL_1539 is part of the two-component system

307 presumably involved in regulating organohalide respiration [13]. According to the classical
308 two-component regulatory system model [14], the histidine kinase would be the protein
309 sensing the TCE and upon binding activating the response regulator by phosphorylation.
310 Hence, the stabilization of the response regulator by TCE should be indirect. Thermal stability
311 of a protein depends on bound ligands, posttranslational modifications, other proteins or
312 cofactors. Savitski *et al*. [19] demonstrated how down-stream effectors exhibit T_m shifts, even
313 if the effectors do not bind the ligand themselves: Kinase inhibitor treatment did not only
314 cause T_m shifts of kinases but also of a phosphatase and an adaptor protein binding
315 phosphorylated proteins. To date, the role of the two-component system in *S. multivorans* has
316 only been inferred from the localization of the respective genes in the genome, sequence
317 alignments and proteomic studies [12, 13]. More detailed biochemical analyses failed
318 because of the difficulties when working with organohalide-respiring bacteria, including the
319 unfeasible genetic manipulation and obstructed heterologous expression. Therefore, our data
320 on the indirect interaction of the putative response regulator SMUL_1539 with TCE offer a
321 valuable indication about the transmission of the signal from trichloroethene to expression of
322 the organohalide respiratory gene region gene region.

323 The unaltered melting curves of most proteins support the hypothesis that only a few proteins
324 directly interact with TCE, corresponding to the observation that most of the genes outside
325 the organohalide respiratory region have orthologs also in non-dehalogenating
326 *Sulfurospirillum* spp. [12].

327 **Other proteins affected by TCE**

328 Besides PceA, 19 further proteins with significant TCE-induced thermal stability changes
329 were identified. Those neither have any hypothesized relation to organohalide respiration nor
330 are encoded in any cluster supposed to be involved in organohalide respiration [12] nor were

331 specifically induced by chlorinated ethenes [13]. Therefore, we assume that these proteins
332 bind TCE unspecifically and do not have any physiological role in organohalide respiration of
333 *S. multivorans*. This can be promoted by the molecular size of the substrates of some of
334 these proteins, i.e., fumarate and pyruvate (fumarate reductase iron-sulfur protein,
335 SMUL_552; acetolactate synthase small subunit, SMUL_1644). In general, biophysical
336 methods such as ligand-binding studies using thermal shift assays on purified proteins are
337 considered to produce only a few false-positive results [21]. Our study is in line with that,
338 yielding 4.4% unspecific background binders.

339 **Protocol adaptations to TCE-tolerant, oxygen-sensitive bacteria**

340 Peng *et al*. [31] used *E. coli* protein extract and quantified the denatured protein fraction.
341 They observed that at their highest chosen temperature, 70°C, most proteins are still in
342 solution. We circumvented this challenge by increasing the maximum temperature from
343 approximately 70°C, which was also as the highest temperature used in most other studies
344 on mammalian cells [19, 32, 33], to 97°C. Thus, in our study, the melting curves of the
345 majority of proteins reached a base of zero at the highest temperatures. The median melting
346 point of the *S. multivorans* proteins (73°C) lies between the T_m-median of human cells, yeast
347 or *E. coli* (51-59°C) and *Thermus thermophilus* (81°C) [19, 26, 28]. Since *S. multivorans* is a
348 mesophilic organism, the elevated median melting point might hint at an adaptation of the
349 proteins towards solvent stress, to which proteins might develop similar strategies as towards
350 heat, such as inflexibility, compactness and core hydrophobicity [34]. However, further TPP
351 studies with other bacteria are required to prove the reason for the specific protein melting
352 points.

353 Important to note is that we modified our protocol in order to exclude oxygen during cell
354 harvesting, protein extraction, substrate, and temperature incubation. All working steps were

355 accomplished in an anoxic chamber or in gas-tight tubes and by adding cysteine to all used
356 buffers. These modifications were preceded by an elaborate optimization procedure. Another
357 challenge of using TPP for bacterial applications is that protein extraction is not as efficient
358 and reproducible as in mammalian cells [35]. As a consequence, instead of using intact cells
359 for substrate and temperature incubation, we used aliquoted cell lysate.

360 **Future implications**

361 The benefit of TPP is that protein-protein and protein-substrate interactions can be
362 investigated under nearly physiological conditions. TPP is therefore highly suitable for
363 organohalide-respiring bacteria because related studies suffer from high oxygen sensitivity of
364 their enzymes, poor biomass yields, impeded enzyme purification and missing heterologous
365 expression systems. As we have shown in our study, TPP is a favorable screening method to
366 reduce the list of potential proteins interacting with halogenated compounds for subsequent
367 molecular biochemical validations.

368 In the future, TPP could resolve substrate specificities of reductive dehalogenases with
369 unresolved substrate spectrum, e.g., of *Dehalococcoides mccartyi*, *Dehalobacter restrictus* or
370 *Desulfitobacterium spp*., which harbor several reductive dehalogenases [15, 36-38]. It might
371 also serve to elucidate the association of reductive dehalogenases with the respiration
372 complex by comparing the T_m values of different reductive dehalogenases. In *D. mccartyi*,
373 several studies indicate that electron transfer does not occur via a classical electron transport
374 chain involving quinones but within a large multiprotein complex, the composition of which is
375 not completely resolved [17, 18]. The suitability of TPP for studying protein complexes was
376 demonstrated by Savitski *et al.* [19] and Bai *et al.* [39].

377 **Conclusion**

378 The protein interaction of a reductive dehalogenase with its specific substrate was
379 demonstrated using the TPP method. Additionally, we found indications that the response
380 regulator, at least indirectly, interacts with TCE. Our findings provide useful complementary
381 information on their protein stability. The TPP protocol is transferable to other bacteria, even
382 though the optimal temperature range needs to be defined for the organism and proteins of
383 interest. To test the protein binding to one substrate, approximately 3 mg protein amount is
384 required per replicate, with additional 1.5 mg per additional substrate. TPP will further help us
385 to resolve the specificities and regulatory circuits of reductive dehalogenases towards many
386 different substrates, which are among the most unresolved fields in research on
387 organohalide-respiring bacteria, but also to gain insights into the physiology of other slow-
388 growing or difficult to study bacteria.

389 **Acknowledgment**

390 This work was supported by the German Research Foundation (DFG), as part of the research
391 group FOR 1530. D.T. was also supported by the Helmholtz Interdisciplinary Graduate
392 School for Environmental Research (HIGRADE). The authors are grateful for the use of the
393 analytical facilities of the Centre for Chemical Microscopy (ProVIS) at the Helmholtz Centre
394 for Environmental Research, which is supported by European Regional Development Funds
395 (EFRE - Europe funds Saxony) and the Helmholtz Association. Benjamin Scheer is thankfully
396 acknowledged for maintenance of the Orbitrap Fusion mass spectrometer.

397 **References**

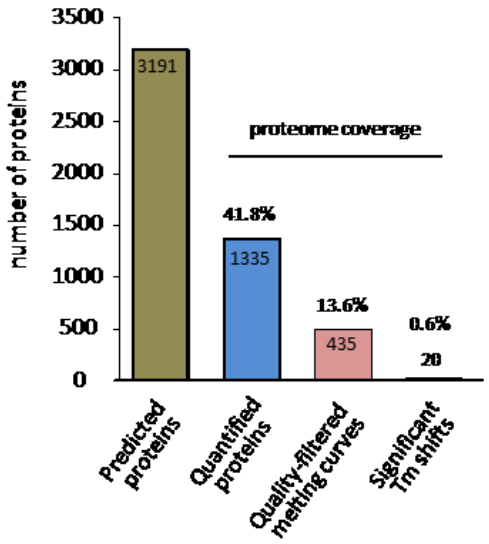
398 [1] ATSDR, Agency for toxic substances and disease registry's Substance Priority List, 2017.
399 [2] IPCS, International Programme for Chemical Safety, Environmental health criteria 195, 1997.
400 [3] IPCS, International Programme for Chemical Safety, Environmental health criteria 140, 1993.
401 [4] IPCS, International Programme for Chemical Safety, Environmental health criteria 71, 1987.
402 [5] J. Koenig, M. Lee, M. Manefield, Aliphatic organochlorine degradation in subsurface environments,
403 Rev. Environ. Sci. Biotechnol. 14(1) (2015) 49-71.

404 [6] D. Leys, L. Adrian, H. Smidt, Organohalide respiration: microbes breathing chlorinated molecules,
405 Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 368(1616) (2013).
406 [7] S. Atashgahi, Y. Lu, H. Smidt, Overview of Known Organohalide-Respiring Bacteria—Phylogenetic
407 Diversity and Environmental Distribution, in: L. Adrian, F.E. Löffler (Eds.), Organohalide-Respiring
408 Bacteria, Springer Berlin Heidelberg, Berlin, Heidelberg, 2016, pp. 63-105.
409 [8] H. Dobbek, D. Leys, Insights into Reductive Dehalogenase Function Obtained from Crystal
410 Structures, in: L. Adrian, F.E. Löffler (Eds.), Organohalide-Respiring Bacteria, Springer Berlin
411 Heidelberg, Berlin, Heidelberg, 2016, pp. 485-495.
412 [9] T. Schubert, L. Adrian, R.G. Sawers, G. Diekert, Organohalide respiratory chains: composition,
413 topology and key enzymes, FEMS Microbiol. Ecol. 94(4) (2018) fiy035.
414 [10] A. Neumann, G. Wohlfarth, G. Diekert, Purification and Characterization of Tetrachloroethene
415 Reductive Dehalogenase from *Dehalospirillum multivorans*, J. Biol. Chem. 271(28) (1996) 16515-
416 16519.
417 [11] C. Kunze, M. Bommer, W.R. Hagen, M. Uksa, H. Dobbek, T. Schubert, G. Diekert, Cobamide-
418 mediated enzymatic reductive dehalogenation via long-range electron transfer, Nat. Commun. 8
419 (2017) 15858.
420 [12] T. Goris, T. Schubert, J. Gadkari, T. Wubet, M. Tarkka, F. Buscot, L. Adrian, G. Diekert, Insights into
421 organohalide respiration and the versatile catabolism of *Sulfurospirillum multivorans* gained from
422 comparative genomics and physiological studies, Environ. Microbiol. 16(11) (2014) 3562-3580.
423 [13] T. Goris, C.L. Schiffmann, J. Gadkari, T. Schubert, J. Seifert, N. Jehmlich, M. von Bergen, G.
424 Diekert, Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum*
425 *multivorans* adapted to tetrachloroethene and other energy substrates, Sci. Rep. 5 (2015) 13794.
426 [14] E.A. Groisman, Feedback Control of Two-Component Regulatory Systems, Annu. Rev. Microbiol.
427 70(1) (2016) 103-124.
428 [15] D. Türkowsky, N. Jehmlich, G. Diekert, L. Adrian, M.v. Bergen, T. Goris, An integrative overview of
429 genomic, transcriptomic and proteomic analyses in organohalide respiration research, FEMS
430 Microbiol. Ecol. 94(3) (2018) fiy013.
431 [16] L. Adrian, J. Rahnenführer, J. Gobom, T. Hölscher, Identification of a Chlorobenzene Reductive
432 Dehalogenase in *Dehalococcoides* sp. Strain CBDB1, Appl. Environ. Microbiol. 73(23) (2007) 7717-
433 7724.
434 [17] A. Kublik, D. Deobald, S. Hartwig, C.L. Schiffmann, A. Andrades, M. von Bergen, R.G. Sawers, L.
435 Adrian, Identification of a multi-protein reductive dehalogenase complex in *Dehalococcoides mccartyi*
436 strain CBDB1 suggests a protein-dependent respiratory electron transport chain obviating quinone
437 involvement, Environ. Microbiol. 18(9) (2016) 3044-3056.
438 [18] S. Hartwig, N. Dragomirova, A. Kublik, D. Türkowsky, M. von Bergen, U. Lechner, L. Adrian, R.G.
439 Sawers, A H2-oxidizing, 1,2,3-trichlorobenzene-reducing multienzyme complex isolated from the
440 obligately organohalide-respiring bacterium *Dehalococcoides mccartyi* strain CBDB1, Environ.
441 Microbiol. Rep. 9(5) (2017) 618-625.
442 [19] M.M. Savitski, F.B. Reinhard, H. Franken, T. Werner, M.F. Savitski, D. Eberhard, D. Martinez
443 Molina, R. Jafari, R.B. Dovega, S. Klaeger, B. Kuster, P. Nordlund, M. Bantscheff, G. Drewes, Tracking
444 cancer drugs in living cells by thermal profiling of the proteome, Science 346(6205) (2014) 1255784.
445 [20] A. Mateus, T.A. Määttä, M.M. Savitski, Thermal proteome profiling: unbiased assessment of
446 protein state through heat-induced stability changes, Proteome Science 15 (2016) 13.
447 [21] D. Martinez Molina, R. Jafari, M. Ignatushchenko, T. Seki, E.A. Larsson, C. Dan, L. Sreekumar, Y.
448 Cao, P. Nordlund, Monitoring Drug Target Engagement in Cells and Tissues Using the Cellular Thermal
449 Shift Assay, Science 341(6141) (2013) 84-87.

450 [22] J. Gadkari, T. Goris, C.L. Schiffmann, R. Rubick, L. Adrian, T. Schubert, G. Diekert, Reductive
451 tetrachloroethene dehalogenation in the presence of oxygen by *Sulfurospirillum multivorans*:
452 physiological studies and proteome analysis, FEMS Microbiol. Ecol. 94(1) (2018) fix176-fix176.
453 [23] H. Scholz-Muramatsu, A. Neumann, M. Meßmer, E. Moore, G. Diekert, Isolation and
454 characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing,
455 strictly anaerobic bacterium, Arch. Microbiol. 163(1) (1995) 48-56.
456 [24] M. John, R. Rubick, R.P. Schmitz, J. Rakoczy, T. Schubert, G. Diekert, Retentive memory of
457 bacteria: Long-term regulation of dehalorespiration in *Sulfurospirillum multivorans*, J. Bacteriol.
458 191(5) (2009) 1650-1655.
459 [25] H. Franken, T. Mathieson, D. Childs, G.M.A. Sweetman, T. Werner, I. Tögel, C. Doce, S. Gade, M.
460 Bantscheff, G. Drewes, F.B.M. Reinhard, W. Huber, M.M. Savitski, Thermal proteome profiling for
461 unbiased identification of direct and indirect drug targets using multiplexed quantitative mass
462 spectrometry, Nat. Protoc. 10 (2015) 1567.
463 [26] F.B.M. Reinhard, D. Eberhard, T. Werner, H. Franken, D. Childs, C. Doce, M.F. Savitski, W. Huber,
464 M. Bantscheff, M.M. Savitski, G. Drewes, Thermal proteome profiling monitors ligand interactions
465 with cellular membrane proteins, Nat. Methods 12 (2015) 1129.
466 [27] D. Childs, N. Kurzawa, Introduction to the TPP package for analyzing Thermal Proteome Profiling
467 data: Temperature range (TR) or concentration compound range (CCR) experiments (2016).
468 [28] P. Leuenberger, S. Ganscha, A. Kahraman, V. Cappelletti, P.J. Boersema, C. von Mering, M.
469 Claassen, P. Picotti, Cell-wide analysis of protein thermal unfolding reveals determinants of
470 thermostability, Science 355(6327) (2017).
471 [29] H.B. Schlegel, M. Poe, K. Hoogsteen, Models for the Binding of Methotrexate to *Escherichia coli*
472 Dihydrofolate Reductase, Mol. Pharmacol. 20(1) (1981) 154.
473 [30] M. Bommer, C. Kunze, J. Fesseler, T. Schubert, G. Diekert, H. Dobbek, Structural basis for
474 organohalide respiration, Science 346(6208) (2014) 455-458.
475 [31] H. Peng, H. Guo, O. Pogoutse, C. Wan, L.Z. Hu, Z. Ni, A. Emili, An Unbiased Chemical Proteomics
476 Method Identifies FabI as the Primary Target of 6-OH-BDE-47, Environ. Sci. Technol. 50(20) (2016)
477 11329-11336.
478 [32] B.X. Tan, C.J. Brown, F.J. Ferrer, T.Y. Yuen, S.T. Quah, B.H. Chan, A.E. Jansson, H.L. Teo, P.
479 Nordlund, D.P. Lane, Assessing the Efficacy of Mdm2/Mdm4-Inhibiting Stapled Peptides Using Cellular
480 Thermal Shift Assays, Sci. Rep. 5 (2015) 12116.
481 [33] J.-J. Qin, W. Wang, S. Voruganti, H. Wang, W.-D. Zhang, R. Zhang, Identification of a new class of
482 natural product MDM2 inhibitor: *In vitro* and *in vivo* anti-breast cancer activities and target validation,
483 Oncotarget 6(5) (2015) 2623-2640.
484 [34] Y. Ding, Y. Cai, Y. Han, B. Zhao, Comparison of the structural basis for thermal stability between
485 archaeal and bacterial proteins, Extremophiles 16(1) (2012) 67-78.
486 [35] S.C. Tan, B.C. Yiap, DNA, RNA, and Protein Extraction: The Past and The Present, J. Biomed.
487 Biotechnol. 2009 (2009) 574398.
488 [36] L.A. Hug, F. Maphosa, D. Leys, F.E. Löffler, H. Smidt, E.A. Edwards, L. Adrian, Overview of
489 organohalide-respiring bacteria and a proposal for a classification system for reductive
490 dehalogenases, Philos. Trans. R. Soc. Lond., Ser. B: Biol. Sci. 368(1616) (2013) 20120322.
491 [37] T. Kruse, T. Goris, J. Maillard, T. Woyke, U. Lechner, W. de Vos, H. Smidt, Comparative genomics
492 of the genus *Desulfitobacterium*, FEMS Microbiol. Ecol. 93(12) (2017) fix135.
493 [38] T. Kruse, J. Maillard, L. Goodwin, T. Woyke, H. Teshima, D. Bruce, C. Detter, R. Tapia, C. Han, M.
494 Huntemann, C.-L. Wei, J. Han, A. Chen, N. Kyrpides, E. Szeto, V. Markowitz, N. Ivanova, I. Pagani, A.
495 Pati, S. Pitluck, M. Nolan, C. Holliger, H. Smidt, Complete genome sequence of *Dehalobacter restrictus*
496 PER-K23^{T}, Stand. Genomic Sci. 8(3) (2013) 375-388.

497 [39] L. Bai, J. Chen, D. McEachern, L. Liu, H. Zhou, A. Aguilar, S. Wang, BM-1197: A Novel and Specific
498 Bcl-2/Bcl-xL Inhibitor Inducing Complete and Long-Lasting Tumor Regression *In Vivo*, PLoS One 9(6)
499 (2014) e99404.

500 **Figures**

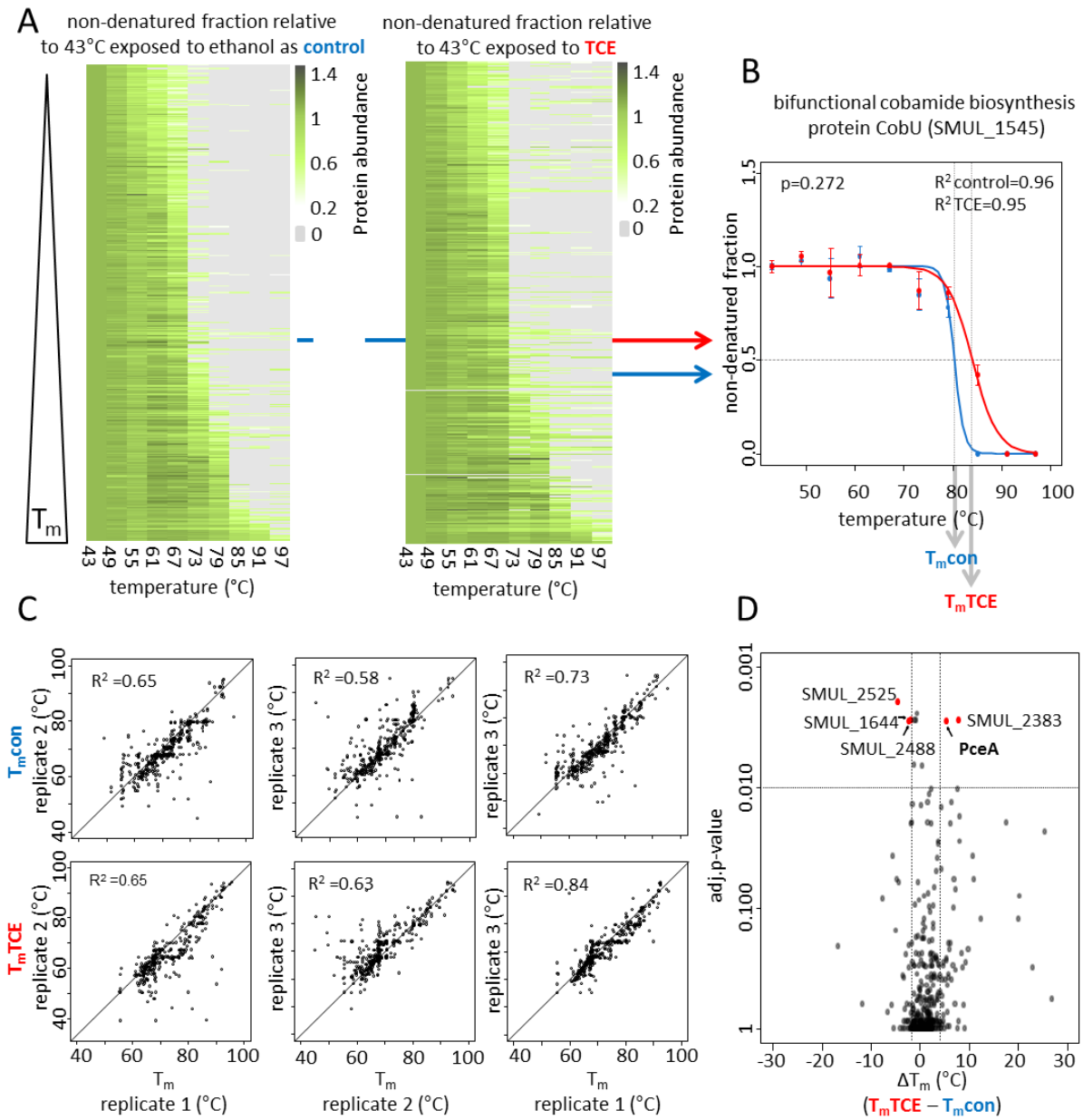


501

502 Fig. 1. Proteome coverage of *S. multivorans.* The predicted proteome is compared to the
503 number of quantified proteins, sigmoidal protein melting curves, and protein melting curves
504 with a significant shift.

505

506

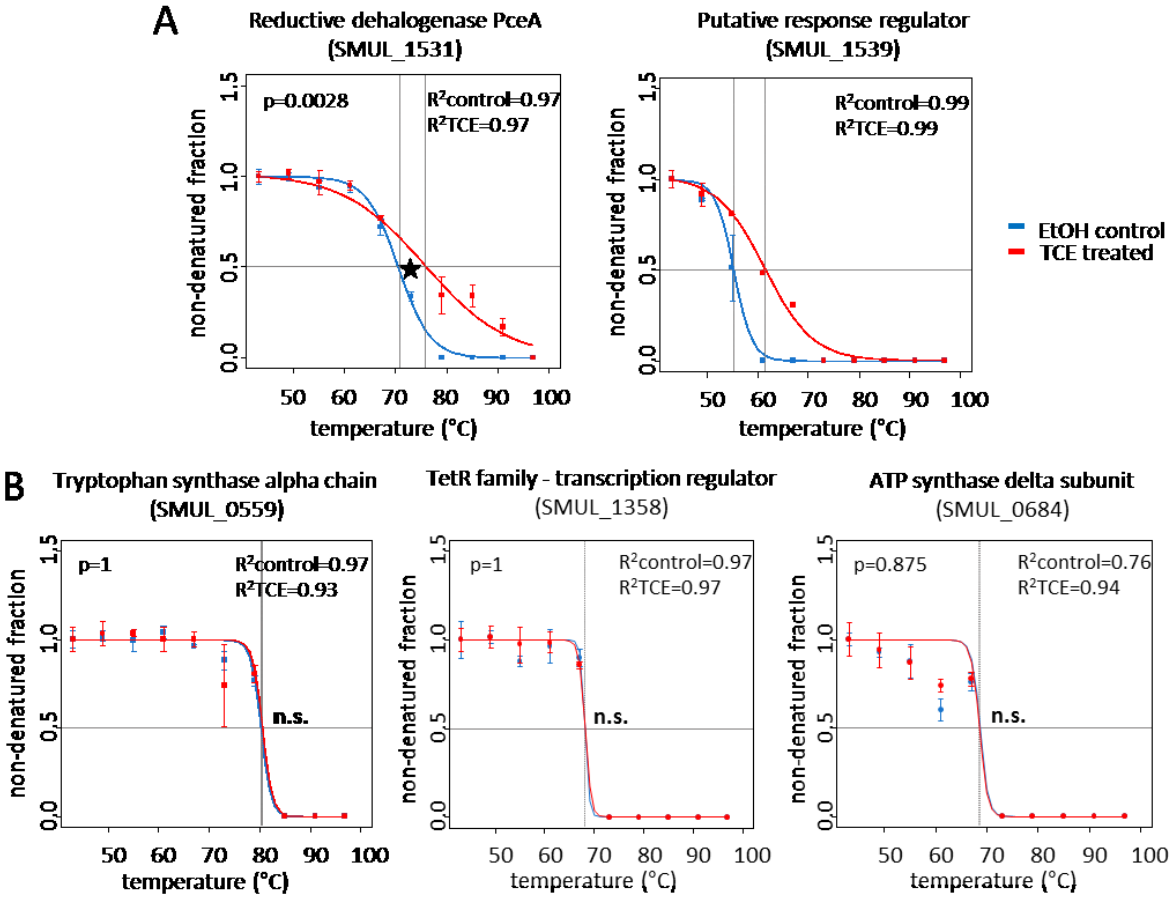


507

508 Fig. 2. (A) Heatmap of the thermal stability of all proteins exposed to trichloroethene (TCE,
509 right) or ethanol as a control (left). The colors indicate protein abundance levels of the non-
510 denatured protein fractions after incubation at one of ten temperatures. Shown are relative
511 abundances normalized to the abundance after incubation to the lowest temperature (43°C).
512 Each line represents the average of at least two replicates of a protein. (B) Two
513 representative protein melting curves with and without TCE treatment, calculated by a
514 sigmoidal fitting approach over the temperature range. The melting temperature (T_m)
515 represents the temperature at which half of the protein is denatured. (C) The reproducibility of

516 the thermal proteome profiling displayed by the coefficient of determination (R^2) of the T_m of
517 two replicates each. (D) Volcano plot of the melting temperature differences (ΔT_m) between
518 TCE-exposed and control-proteins and their Benjamini-Hochberg adjusted p-values. The
519 vertical and horizontal lines mark the threshold for adjusted p-value (< 0.01) and ΔT_m (mean ±
520 1 standard deviation). SMUL_2525, acetyl-coenzyme A; SMUL_1644, acetolactate synthase
521 α-subunit; SMUL_2488, phosphomannomutase/phosphoglucomutase; SMUL_2383,
522 hydrogenase-4 component A; PceA, reductive dehalogenase.

523



524

525 Fig. 3. Protein-substrate interactions of selected proteins. (A) Melting curves of PceA and the
526 response regulator (both are encoded in the organohalide respiratory gene region) showed a
527 melting temperature (T_m) shift towards higher temperatures upon trichloroethene (TCE)
528 treatment. (B) Three examples of protein melting curves with no significant T_m shifts after
529 TCE treatment. Y-axes give $\log_2$ fold changes of the non-denatured proteins relative

530 to 43°C. Error bars indicate standard errors of n=3 at ten different temperature points. A
531 significant protein melting curve shift (adj. $p < 0.01$) is represented by an asterisk. R^2 =
532 coefficient of determination.

533

534 Table 1. Proteins with a significant melting curve shifts. The p-values are adjusted according
535 to Benjamini-Hochberg and the reductive dehalogenase PceA is highlighted in red.

Accession no.	Protein name	adj. p-value	ΔTm (°C)
SMUL_2383_2340	hydrogenase-4 component A, iron-sulfur cluster containing subunit	3E-06	12.0
SMUL_0693_673	outer membrane lipoprotein omp16-like	3E-03	7.8
SMUL_1531_1502	tetrachloroethene reductive dehalogenase catalytic subunit PceA	3E-03	5.5
SMUL_0481_468	LSU ribosomal protein L11p (L12e)	7E-04	2.5
SMUL_0593_579	peptide chain release factor 2	2E-05	1.3
SMUL_2819_2768	L-asparaginase	4E-05	1.0
SMUL_2009_1975	phosphoribosylaminoimidazole-succinocarboxamide synthase	7E-03	0.5
SMUL_0563_549	translation elongation factor Ts	2E-03	-0.6
SMUL_2097_2063	hypothetical protein	3E-03	-0.8
SMUL_2912_2859	YceI family protein	3E-03	-1.1
SMUL_0552_538	fumarate reductase iron-sulfur protein	7E-03	-1.2
SMUL_2488_2444	phosphomannomutase / phosphoglucomutase	3E-03	-1.8
SMUL_1644_1613	acetolactate synthase small subunit	8E-03	-2.2
SMUL_2525_2481	acetyl-coenzyme A carboxyl transferase alpha chain	2E-03	-4.5
SMUL_1909_1875	hypothetical protein	6E-03	-
SMUL_2989_2936	hypothetical protein	1E-03	-
SMUL_0850_828	uridylate kinase	3E-03	-
SMUL_0273_266	molybdopterin oxidoreductase, chain B	6E-03	-
SMUL_1442_1414	isocitrate dehydrogenase [NADP]	3E-03	-
SMUL_1098_1075	single-stranded DNA-binding protein	2E-05	-

536

537