Transcriptional regulation of tetrachloroethene respiration in *Sulfurospirillum* species

Dissertation

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Contents

Glossary

5′P/5′PPP/5′OH	5′monophosphate/5′triphosphate/5′hydroxyl
5-OH-Bza	5-hydroxybenzimidazolyl
A	adenine
α-CTD	α subunit C-terminal domain
AdeNCba	norpseudocobalamin
Asp~P	phosphorylated aspartic acid
asRNA	antisense ribonucleic acid
ATP	adenosine triphosphate
bp	base pairs
C	cytosine
CA	catalytic and ATP-binding domain
cAMP	cyclic adenosine monophosphate
CAP	cAMP receptor protein
Cba	cobamide, complete corrinoid
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
Co^{2+}	cobalt ion, includes the metabolically less relevant Co^{3+}
CRE	*cis*-regulatory element
DHp	dimerization and histidine phosphotransfer domain
DNA	deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH
EMSA	electrophoretic mobility shift assay
FeS	iron-sulfur cluster, 4Fe-4S
FMN	Flavin mononucleotide
GAF	cGMP-specific phosphodiesterases, adenylylcyclases, and FhlA domain
His~P	phosphorylated histidine
HK	histidine kinase
HTH	helix-turn-helix
K_a	association binding constant
LC-MS/MS	liquid chromatography – tandem mass spectrometer
lfc	$\log_2$-fold change

MQ	menaquinone pool
m/z	mass-to-charge ratio
N	any base (adenine, cytosine, guanine or thymine)
OD_{578}	optical density measured at a wavelength of 578 nm
OHR	organohalide respiration
ORF	open reading frame
padj	Benjamini Hochberg corrected p-value
PAGE	polyacrylamide gel electrophoresis
PAS	Per-Arnt-Sim domain
PCE	tetrachloroethene
PceA	PCE reductive dehalogenase
PceMN	putative components of a quinol dehydrogenase
poly d(I-C)	poly(deoxyinosinic-deoxycytidylic) acid sodium salt
PTM	posttranslational modification
RdhA	reductive dehalogenase
REC	receiver domain
RNA	ribonucleic acid
RNAP	DNA-directed RNA polymerase
(d)RNA-seq	(differential) RNA sequencing
RP-HPLC	reversed-phase high-performance liquid chromatography
RR	response regulator
rRNA	ribosomal ribonucleic acid
RT-(q)PCR	reverse transcription - (quantitative) polymerase chain reaction
sRNA	small ribonucleic acid
T	thymine
TCE	trichloroethene
TCS/TCS1/TCS2	two-component system/SHALO_1498+1499/SHALO_1502+1503
TetR	Tet Repressor protein
TEX	terminal exonuclease
tRNA	transfer ribonucleic acid
TSS	transcriptional start site
UTR	untranslated region
W	weak base (adenine or thymine)
wHTH	winged helix-turn-helix

Summary

Energy conservation via organohalide respiration (OHR), comprising the reductive dehalogenation of halogenated organic compounds, is an inducible process in OHR capable *Sulfurospirillum* species. Understanding the molecular details of the regulatory process will aid the application of organohalide-respiring bacteria in bioremediation attempts and will guide synthetic approaches for sensing and detoxification of organohalides. This work adds a first global RNA sequence of the well-studied *Sulfurospirillum multivorans*, a proteome and an acetylome of *Sulfurospirillum halorespirans* and two new *Sulfurospirillum* genomes that enlarge the repertoire of omics data allowing for future comparative analyses of organohalide-respiring bacteria. In this study, gene products involved in tetrachloroethene (PCE) signal transduction have been unambiguously identified *in vivo* and *in vitro*. Differential RNA sequencing allowed for the identification of eight transcriptional units forming the PCE regulon. The responsible OmpR-family regulator encoded in a two-component system (TCS) operon was functionally characterized. The results emphasize its role in promoting class I and class II transcriptional activation induced by binding a *cis*-regulatory element containing a consensus sequence identified as a direct CTATW repeat separated by 17 bp. In *S. multivorans*, OHR is also subject to a unique memory effect in terms of a long-term transcriptional downregulation of the genes involved in OHR. This retentive memory effect in OHR gene regulation is now identified in a second species suggesting a broader distribution of this regulatory phenomenon. The acetylome data indicate that both response regulator and histidine kinase of the TCS involved in induction of PCE respiration are highly acetylated during short-term cultivation in the absence of PCE. Additionally, this study focuses on the impact of Co^{2+} limitation and its consequences for OHR. Although it was shown that Co^{2+} limitation is a serious and frequent problem, the biosynthesis of the norcobamide cofactor of the PCE reductive dehalogenase exhibited no Co^{2+}-dependent transcriptional regulation. Both acetylation pattern of the TCS and the amount of bioavailable norcobamide cofactor were identified as potential factors affecting the memory effect. The first published acetylome of Campylobacterota might help to study other ecologically or medically important species of this clade. This study is also a big step forward in resolving the regulatory network controlling OHR gene expression in *Sulfurospirillum* spp. and might aid investigation of OHR regulation in other bacteria as well.

Zusammenfassung

Die an die Energiekonservierung gekoppelte Organohalidrespiration (OHR) ist ein induzierbarer Prozess in OHR-fähigen *Sulfurospirillum* spp. Die Erkenntnisse über die molekularen Details der regulatorischen Mechanismen können dabei helfen eine Anwendung von organohalidrespirierenden Bakterien zum Nachweis und biologischen Abbau der giftigen Organohalide zu ermöglichen. Diese Arbeit enthält eine erste globale RNA-Sequenz des Modellorganismus *Sulfurospirillum multivorans*, ein Proteom und ein Acetylom von *Sulfurospirillum halorespirans* sowie zwei neue *Sulfurospirillum*-Genome, die das Repertoire an Omics-Daten erweitern und zukünftige vergleichende Analysen ermöglichen. In dieser Studie wurden Proteine, die an der Signaltransduktion von Tetrachlorethen (PCE) beteiligt sind, *in vivo* und *in vitro* eindeutig identifiziert. Die differentielle RNA-Sequenzierung ermöglichte die Identifizierung von acht Operons, die das PCE-Regulon bilden. Der verantwortliche Regulator gehört zur OmpR-Familie, ist Bestandteil eines Zweikomponentensystems (TCS) und wurde funktional charakterisiert. Die Ergebnisse unterstreichen seine Rolle bei der Initiation der Transkription über Klasse I und II Mechanismen. Die Induktion erfolgt durch die Bindung eines *cis*-regulatorischen Elements. Dieses enthält eine Konsensussequenz, welche eine direkte Wiederholung von CTATW im Abstand von 17 bp enthält. In *S. multivorans* unterliegt die OHR einer generationenübergreifenden Herunterregulierung der Transkription der an der OHR beteiligten Gene. Jetzt wurde dieser „Erinnerungseffekt“ bei einer zweiten Art identifiziert. Dies deutet auf eine breitere Verteilung dieses regulatorischen Phänomens hin. Die Acetylomdaten zeigen, dass sowohl der Regulator als auch die Histidinkinase des TCS, welches an der Induktion der PCE-Atmung beteiligt ist, während einer kurzzeitigen Kultivierung in Abwesenheit von PCE, stark acetyliert sind. Darüber hinaus konzentriert sich diese Arbeit auf die Auswirkungen einer Co^{2+}-Limitation auf die OHR. Obwohl gezeigt wurde, dass Co^{2+}-Limitation ein ökologisch relevantes Problem darstellt, ist die Biosynthese des Norcobamid-Cofaktors der PCE-reduktiven Dehalogenase auf transkriptioneller Ebene nicht Co^{2+}-abhängig reguliert. Sowohl das Acetylierungsmuster des TCS als auch die Menge an bioverfügbarem Norcobamid-Cofaktor wurden als mögliche Faktoren identifiziert, die den „Erinnerungseffekt“ beeinflussen. Das erste veröffentlichte Acetylom von Campylobacterota kann helfen, andere ökologisch oder medizinisch wichtige Arten dieses Phylums zu untersuchen. Diese Arbeit liefert zudem einen signifikanten Beitrag zur Entschlüsselung des regulatorischen Netzwerks, das die OHR-Genexpression in *Sulfurospirillum* spp. kontrolliert und kann die Untersuchung der OHR-Regulation in anderen Bakterien unterstützen.

1 Introduction

1.1 Organohalide respiration

Organohalide respiration (OHR) is an anaerobic mode of energy conservation in bacteria that uses halogenated organic compounds as terminal electron acceptor in a respiratory chain. Although archaea have been found in association with organohalide-respiring bacteria in environmental samples, there are no axenic cultures of archaea that are associated with OHR assuming that the OHR capacity has been restricted to the bacterial domain of life (Atashgahi *et al.*, 2016). Organohalides are hazardous environmental pollutants, which are carcinogenic and toxic for humans. The toxicity rests upon its accumulation in the cell membrane and due to uncoupling the mitochondrial oxidative phosphorylation (Jeffrey and Koplan, 1999). The ongoing pollution, high environmental persistence, and the bioaccumulation of organohalides in food chains can cause severe detrimental effects on human health, including damage of the reproductive and developmental system as well as the hematopoietic, endocrine, nervous, and immune system (Henschler, 1994; Kelly *et al.*, 2007; Lucena *et al.*, 2007). The most environmental pollution with organohalides has an anthropogenic origin. Chlorinated hydrocarbons have been widely used as solvents, degreasing agents, dyes, chemical precursors, additives to polymers, as disinfectants and pesticides in different industrial processes, in agriculture, and private households (Mohn and Tiedje, 1992; Bunge *et al.*, 2007; Jugder *et al.*, 2016a). Despite their toxicity, these compounds have been released for many years and, due to their high persistence in the environment, polluted industrial sites like the industrial region of Bitterfeld-Wolfen still contaminate air and river systems (Jayachandran *et al.*, 2003; Barber *et al.*, 2005; Bunge *et al.*, 2007). Apart from anthropogenic pollution, halogenated hydrocarbons occur naturally. More than 5000 natural compounds have been identified (Gribble, 2003, 2012). Either they emerge from abiogenic sources like volcanic eruptions, wildfires, processes of soil humus formation, and decomposition of soil organic matter (Jordan *et al.*, 2000; Gribble, 2003; Leri *et al.*, 2007) or they are produced by lignin-degrading basidiomycete fungi, bacteria, lichens, insects, marine sponges, and algae (Gribble, 1992; Abrahamsson *et al.*, 1995; de Jong and Field, 1997; Swarts *et al.*, 1998; Giese *et al.*, 1999; Jordan *et al.*, 2000; Field and Wijnberg, 2003; Gribble, 2003; Harper and Hamilton, 2003; Cabrita *et al.*, 2010; Paul and Pohnert, 2011; Gribble, 2012; Walker and Chang, 2014). Released halogenated hydrocarbons accumulate mainly in soil, groundwater, ocean, and water sediments, but also escape into the atmosphere.

The degradation of these pollutants and remediation of contaminated sites are now of keen interest. While abiotic detoxification by ultraviolet irradiation, ozone or metals is very inefficient (Castro, 1998; Totten and Assaf-Anid, 2003; Tobiszewski and Namieśnik, 2012), dehalogenating microorganisms are more suitable for this purpose. In addition, bioremediation is mostly more cost-effective than invasive techniques (Steffan and Schaefer, 2016; Dolinová *et al.*, 2017; Qiao *et al.*, 2018).

Next to dehalogenation reactions in aerobic environments and cometabolic dehalogenation under denitrifying conditions proceeded by members of the α-, β- or γ-Proteobacteria and of Gram-positive bacteria with high G+C content (Fetzner and Lingens, 1994; Song *et al.*, 2000; Dijk *et al.*, 2003; Löffler *et al.*, 2003; Chen *et al.*, 2013; Kiel and Engesser, 2015), dehalogenation occurs also anaerobically in organohalide-respiring bacteria (Adrian and Löffler, 2016). These bacteria couple reductive dehalogenation of halogenated organic compounds to the generation of a proton motive force that drives adenosine triphosphate (ATP) synthesis (Fig. 1.1). The key enzyme in this process is called reductive dehalogenase. This enzyme harbors a cobalt-containing cobamide cofactor in its active site and two iron-sulfur clusters, which mediate the transfer of electrons to the organohalides, which serve as terminal electron acceptors (Hug *et al.*, 2013; Jugder *et al.*, 2015; Jugder *et al.*, 2016a; Schubert and Diekert, 2016; Fincker and Spormann, 2017). The enzyme is located at the exoplasmic site of the cytoplasmic membrane and associated to the membrane via a putative membrane anchor protein (Futagami *et al.*, 2008; Jugder *et al.*, 2015). Several hundred putative reductive dehalogenases have been identified in bacteria so far encompassing a broad substrate spectrum ranging from monohalogenated alkanes to polyhalogenated aromatic compounds (Hug *et al.*, 2013; Adrian and Löffler, 2016). Facultatively organohalide-respiring δ-Proteobacteria like *Desulfomonile* spp., *Sulfurospirillum* spp. as a member of the ε-Proteobacteria, which phylum name has been recently changed in Campylobacterota (Waite *et al.*, 2017; Parks *et al.*, 2018; Waite *et al.*, 2018), and Firmicutes such as *Desulfitobacterium* spp. harbor only a lower number of reductive dehalogenase genes. These species exhibit a versatile metabolism and utilize numerous electron acceptors, whereas obligately organohalide-respiring species belonging to the genera *Dehalobacter* (Firmicutes), *Dehalococcoides*, and *Dehalogenimonas* (both Chloroflexi) are exclusively restricted to organohalide respiration and encode for up to 36 reductive dehalogenases (Maphosa *et al.*, 2010; Hug *et al.*, 2013; Jugder *et al.*, 2015; Atashgahi *et al.*, 2016). Some of these bacteria, e.g. *Sulfurospirillum multivorans*, are able to synthesize the cobamide cofactor *de novo*, whereas others, e.g. *Dehalococcoides mccartyi*, depend on salvaging and remodeling of exogenous cobamides or precursors thereof (Holliger *et al.*, 1998; Löffler *et al.*,

2013; Rupakula *et al.*, 2013; Moore and Escalante-Semerena, 2016). In a dehalogenating bacterial community, the cobamide cofactors can be shared, as different co-culture experiments revealed an interspecies cobamide transfer (J. He *et al.*, 2007; Yan *et al.*, 2012; Yan *et al.*, 2013; Men *et al.*, 2014; S. Kruse *et al.*, 2018). Nevertheless, the bacterial community depends on the bioavailability of cobalt in order to ensure cobamide biosynthesis. Therefore, cobalt can be a limiting growth factor in the environment of sites contaminated with organohalides.

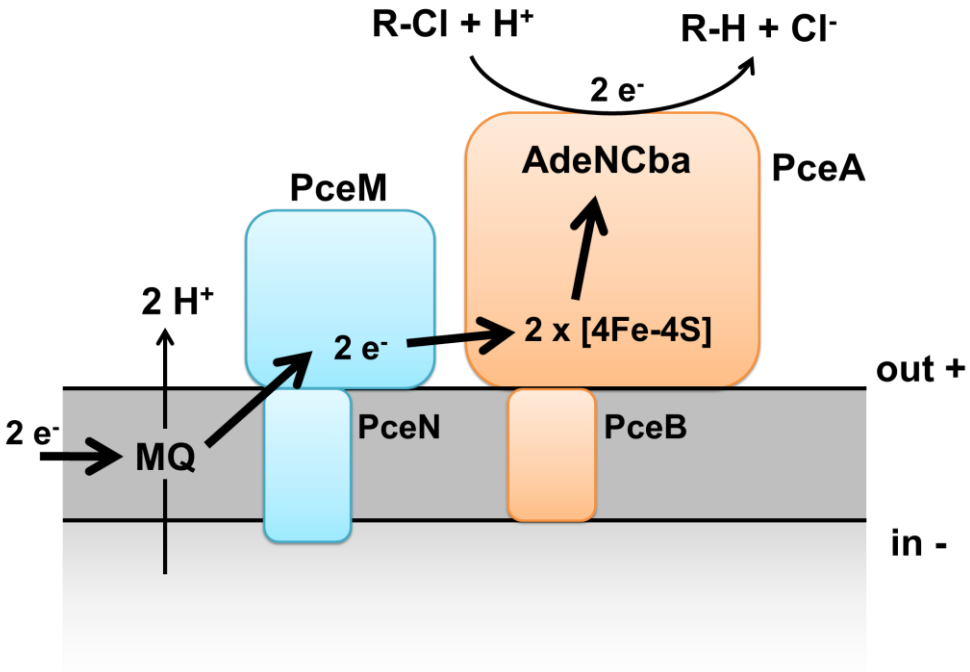

Fig. 1.1: Tentative scheme for the composition of a quinone-dependent organohalide respiratory chain. Electrons are predicted to pass the menaquinone pool (MQ), the putative components of a quinol dehydrogenase (PceMN, turquoise), and the tetrachloroethene reductive dehalogenase (PceA, orange) via two iron-sulfur clusters [4Fe-4S] and the cobalt-containing adeninyl-norcobamide (AdeNCba, synonym: norpseudo-B_{12}), which catalyzes the reductive dehalogenation. PceB is the putative membrane anchor (orange). The figure is adopted and modified from (Schubert and Diekert, 2016).

Organohalide respiration has been well studied in the model organism *S. multivorans*, which belongs to the gram-negative Campylobacterota. *S. multivorans* has been identified in marine and river sediments, groundwater aquifers, and soil all across the world (Scholz-Muramatsu *et al.*, 1995; von Wintzingerode *et al.*, 2001; Grabowski *et al.*, 2005; Duhamel and Edwards, 2006; Tomaras *et al.*, 2009; Y. H. Li *et al.*, 2010b; Dugat-Bony *et al.*, 2011; Y. H. Li *et al.*, 2011;

Maillard *et al.*, 2011; Srinivas *et al.*, 2011; Hubert *et al.*, 2012; C. Zhang *et al.*, 2012; Buttet *et al.*, 2013; Z. Li *et al.*, 2013; C. Zhang *et al.*, 2013; Z. Li *et al.*, 2014). The species is an anaerobic, facultatively organohalide-respiring bacterium capable of dehalogenating tetrachloroethene (PCE), trichloroethene (TCE) as well as brominated phenols and brominated ethenes using pyruvate, lactate, formate or hydrogen as electron donor (Neumann *et al.*, 1996; T. Goris and Diekert, 2016; Kunze *et al.*, 2017b). It grows faster than most organohalide-respiring bacteria and has only two genes encoding for reductive dehalogenases. Only one reductive dehalogenase has a known inductor allowing intensive investigations of the so-called PCE reductive dehalogenase PceA (Neumann *et al.*, 1994; Scholz-Muramatsu *et al.*, 1995; Miller *et al.*, 1996; Neumann *et al.*, 1996; Neumann *et al.*, 2002; Siebert *et al.*, 2002; John *et al.*, 2006; John *et al.*, 2009; Bommer *et al.*, 2014; Kunze *et al.*, 2017b). In addition, *S. multivorans* has a versatile metabolism allowing a comparison of gene expression under various dehalogenating and non-dehalogenating conditions (T. Goris *et al.*, 2014; T. Goris *et al.*, 2015a; T. Goris and Diekert, 2016). However, only little is known about the regulation of organohalide respiration in dehalogenating bacteria.

1.2 Transcriptional regulation of organohalide respiration

The facultatively organohalide-respiring *Desulfitobacterium* spp. strongly regulate individual *rdhA* genes in response to specific organohalides (Smidt *et al.*, 2000; Tsukagoshi *et al.*, 2006; Bisaillon *et al.*, 2011; Kim *et al.*, 2012; Mac Nelly *et al.*, 2014). As an exception, there are three *De. hafniense* isolates, TCE1, Y51, and PCE-S, which harbor a *pceA* gene under control of a constitutive promoter that belongs to a transposon (Maillard *et al.*, 2005; Futagami *et al.*, 2006). Obligately organohalide-respiring bacteria, such as *D. mccartyi* and *Dehalobacter restrictus*, seem to have a low but steady transcription of the majority of *rdhA* genes (Wagner *et al.*, 2009; Rupakula *et al.*, 2013; Wagner *et al.*, 2013). However, a few *rdhA* genes have been ascertained to be regulated substrate-dependently (Wagner *et al.*, 2009; Wagner *et al.*, 2013). Reductive dehalogenase genes are assumed to be regulated by nearby encoded transcription factors. The comparison of the genome sequences available suggests that the regulatory mechanisms of organohalide respiration differ accordingly to the phylogeny rather than the organism's status as facultatively or obligately organohalide-respiring bacterium. Thus, members of the Firmicutes including *Desulfitobacterium* and *Dehalobacter* encode for CRP/FNR family transcriptional regulators up- or downstream of the reductive dehalogenase genes (Gábor *et al.*, 2008; Fletcher *et al.*, 2011; Rupakula *et al.*, 2013). These regulators consist of a binding domain

for allosteric effector molecules and a DNA-binding domain allowing the response to a broad range of intracellular signals (Körner *et al.*, 2003). CprK was characterized in *Desulfitobacterium dehalogenans* and *Desulfitobacterium hafniense* as a 3-Chloro-4-hydroxy-phenylacetic acid-binding sensor that activates expression of the *cpr* genes including the *ortho*-chlorophenol reductive dehalogenase gene *cprA* (Pop *et al.*, 2004; Gábor *et al.*, 2006). Apart from that, members of the *Dehalococcoidia* class of the Chloroflexi mostly regulate the transcription of the reductive dehalogenases either via MarR-type repressors or two-component systems with a cytosolic histidine kinase (Kube *et al.*, 2005; Wagner *et al.*, 2013). Whether the regulators directly respond to chlorinated compounds or indirectly by sensing another signal, such as changes in the redox status, is still unknown, but the MarR-type regulator family was already described to regulate aromatic catabolic pathways, virulence, and the response to environmental stress (Wilkinson and Grove, 2006).

The OHR in *S. multivorans* is subject of a unique memory effect (John *et al.*, 2009). OHR is induced by both substrates, PCE and TCE, within one generation, whereas the transcription maintains for more than 100 generations once the substrate is depleted (John *et al.*, 2009). Such a long-term loss of OHR activity was also observed in *De. hafniense*, but due to a transposon-mediated loss of the *pce* gene cluster in the majority of the bacterial population after cultivation in the absence of PCE (Futagami *et al.*, 2006; Duret *et al.*, 2012; T. Goris *et al.*, 2015b). Contrary to this observation, *pceA* in *S. multivorans* was still functional and inducible by PCE (John *et al.*, 2009). This raises the question about the regulatory mechanism of organohalide respiration in this organism. The genomes of *S. multivorans* (T. Goris *et al.*, 2014) and three other dehalogenating *Sulfurospirillum* species have recently become available (T. Goris *et al.*, 2017; Buttet *et al.*, 2018). Comparative genomics allowed the definition of an almost 100% conserved gene region including almost all genes responsible for OHR (Fig. 1.2). Beside the genes encoding for the two reductive dehalogenases and their putative membrane anchors *pceAB* and *rdhAB*, all genes for the *de novo* biosynthesis of the cobamide derivative adeninyl-norcobamide (AdeNCba, synonym: norpseudo-B_{12}) are located in close vicinity downstream of genes encoding for components of a *napGH*-like putative quinol dehydrogenase (*pceMN*), whereas the genes encoding for the iron-sulfur cluster biosynthesis, *isc* and *suf*, are located elsewhere in the genome (T. Goris *et al.*, 2014). Within the AdeNCba biosynthesis gene cluster, an incomplete corrinoid ABC transporter BtuCDF is encoded missing the outer membrane vitamin B_{12} receptor BtuB. Downstream of these genes, there is a *tetR*-like repressor gene encoded. This gene is functional in *Sulfurospirillum halorespirans* and '*Candidatus* Sulfurospirillum diekertiae' strain SL2 but disrupted by a transposase in *S. multivorans* (T.

Goris *et al.*, 2014; T. Goris *et al.*, 2017; Buttet *et al.*, 2018). Both operons, *pceAB* and *rdhAB*, are closely associated with two two-component systems (TCSs) consisting of a membrane bound histidine kinase (HK) and a cytosolic response regulator (RR). The proteomic analyses of *S. multivorans* in the presence and absence of PCE allowed to further define the gene cluster, which is induced by PCE (T. Goris *et al.*, 2015a). Among others, *pceAB* and most norcobamide biosynthesis genes as well as the putative quinol dehydrogenase component genes *pceMN* are highly upregulated in the presence of PCE and mostly not detectable after approximately 200 bacterial generations in the absence of PCE. The second reductive dehalogenase gene *rdhA* and its putative membrane anchor encoding gene *rdhB* as well as the first TCS (TCS1) upstream of *rdhAB* were not induced by PCE. It might be possible that the gene products have a different substrate spectrum than PceA and the second TCS (TCS2). Since TCS1 was not detected, it is more likely that this sensing system is not functional. On the contrary, TCS2 was detectable in quantifiable amounts once PCE was depleted enabling the organism to putatively sense PCE in the periplasm.

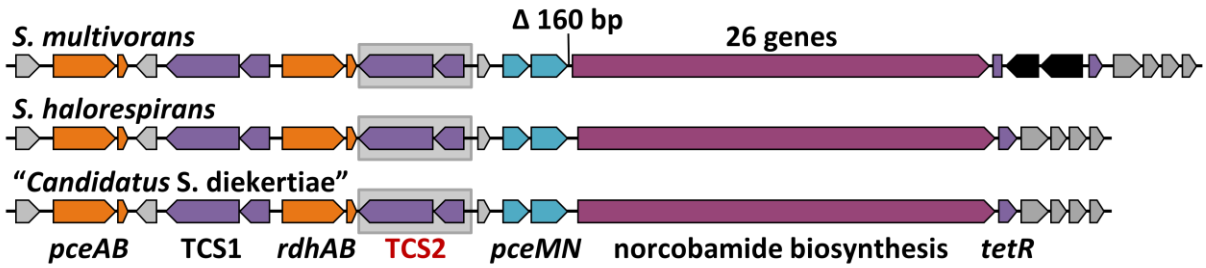

Fig. 1.2: Illustration of the OHR gene region. The dehalogenating *S. halorespirans* (GenBank: CP017111.1), *S. multivorans* (GenBank: CP007201.1) and '*Candidatus* S. diekertiae' strain SL2 populations (GenBank: CP021416.1 and CP021979.1) are represented. The gene region encodes for two reductive dehalogenases and their putative membrane anchors (orange), two TCSs as well as a TetR-like repressor (violet), two genes *pceMN*, which encode putative components of a quinol dehydrogenase (turquoise), and genes for the biosynthesis of the norcobamide cofactor (purple) with three genes for the incomplete cobamide transport system (pink). Some of the genes have a yet unknown function (gray). All species share > 99% sequence identity of the OHR gene cluster, except for *pceA* (95%). *S. multivorans tetR*-like repressor gene is disrupted by two transposase genes (black) and *S. multivorans* lacks a 106 bp sequence upstream of the AdeNCba biosynthesis genes.

1.3 Two-component systems

The TCS is a family of signal transduction proteins present in all domains of life (A. M. Stock *et al.*, 2000). In archaea and eukaryotes, TCSs play a minor role. Consequently, only one TCS has been identified in the yeast *Saccharomyces cerevisiae* and four in the plant *Arabidopsis thaliana* (J. F. Barrett and Hoch, 1998). TCSs are completely absent in the animal kingdom (Wolanin *et al.*, 2002). In bacteria, TCSs constitute one of three major forms of gene control in response to changes in environmental conditions. The most predominant form of bacterial signal transduction is a one-component system with a single protein harboring both an input and an output domain (Ulrich *et al.*, 2005). Another dominant signaling mechanism relies on extracytoplasmic function sigma factors and their cognate anti-sigma factors (Staroń *et al.*, 2009). The TCS protein family has been implicated in the adaptation to a variety of stress conditions (Groisman, 2001; Dubrac *et al.*, 2008; Kato and Groisman, 2008; Hsieh and Wanner, 2010; Freeman *et al.*, 2013; Raivio, 2014), in pathogenic (Groisman, 2001; Lin *et al.*, 2008; Ryndak *et al.*, 2008) and symbiotic (Raghavan and Groisman, 2010; Norsworthy and Visick, 2015) interactions with eukaryotic hosts, and in essential cellular pathways (Quon *et al.*, 1996; Dubrac *et al.*, 2008). A classical TCS consists of a HK that is often membrane-integrated responding to physical or chemical signals and a soluble RR mostly serving as a DNA-binding transcription factor adjusting the gene expression profile (Jung *et al.*, 2012) (Fig. 1.3). TCSs are highly homologous between organisms, i.e. the recognition surfaces that confer specificity between sensor and regulator as well as the biochemical properties of these proteins are largely conserved across systems and species (Grebe and Stock, 1999; Laub and Goulian, 2007). However, the number of HKs and RRs in a bacterial genome seems to correlate with the genome size (Beier and Gross, 2006) and the number of ecological niches (Alm *et al.*, 2006) and is usually not equivalent due to orphan and hybrid proteins (A. M. Stock *et al.*, 2000; Skerker *et al.*, 2005; Wuichet *et al.*, 2010; Capra and Laub, 2012). Thus, the amount of HK/RR ranges from zero in the intracellular living parasite *Mycoplasma genitalium*, which faces stable environmental conditions, to 136/127 in *Myxococcus xanthus*, which has one of the largest bacterial genomes (Capra and Laub, 2012). *Sulfurospirillum multivorans* encodes for 54 HKs, 63 RRs, and one hybrid TCS, which is about twice the amount of HK/RR compared to *Escherichia coli* with 30/32 or *Bacillus subtilis* with 36/34 (Heermann and Jung, 2010).

TCSs have been classified first in 1986 (Nixon *et al.*, 1986; Winans *et al.*, 1986; Ronson *et al.*, 1987). Histidine kinases are multidomain proteins with an N-terminal sensing and a C-terminal transmitter domain (Grebe and Stock, 1999). The sensing domain is very flexible and varies

with the input signals that are transduced by the systems. Depending on the location of the sensing domain either extracellular, periplasmic, transmembrane or intracellular signals can be recognized. Extracytoplasmic sensing HKs, such as the nitrate sensor NarX or the C_4-dicarboxylate sensor DcuS, contain a large extracellular sensing domain often flanked by two transmembrane helices (Mascher *et al.*, 2006; Krell *et al.*, 2010; Mascher, 2014). Transmembrane-sensing HKs have at least six transmembrane helices and very short extracellular loops. The stimulus is perceived directly from the membrane interface, as it is described for DesK in *B. subtilis*, or from a binding pocket within the membrane similar to the signal peptide sensing AgrC in *Staphylococcus aureus* (Mascher *et al.*, 2006). Intracellular stimuli sensing HKs harbor no transmembrane helices and are cytosolic proteins potentially membrane associated via accessory proteins (Mascher, 2014). So far, transmembrane domains with up to 13 transmembrane helices have been identified (Galperin, 2005). The C-terminal domain of HKs is located in the cytoplasm, largely conserved, and consist of a dimerization and histidine phosphotransfer domain (DHp, Pfam: HisKA) and a catalytic and ATP-binding domain (CA, Pfam: HATPase_c).

Upon receipt of a stimulus at the N-terminal sensing domain, the signal is transduced across the membrane to the C-terminal domain promoting an autophosphorylation with the γ-phosphate of ATP at a conserved histidine residue located in the DHp domain. In other words, when an organism experiences inducing conditions for a given TCS, the concentration of phosphorylated sensor elevates, which constitutes the usual substrate for the phosphorylation of a cognate RR. HKs can comprise additional domains located between the transmembrane region and the DHp domain (Mascher, 2014). One example is the HAMP domain, which is a common cytoplasmic coiled-coil linker domain of about 50 amino acids found in histidine kinases, adenosyl cyclases, methyl-accepting chemotaxis receptors, and phosphatases (Jin and Inouye, 1994; Aravind and Ponting, 1999). It mediates the signal conversion by a conformational change between two possible packing modes (Hulko *et al.*, 2006; Barakat *et al.*, 2010). Additional sensors for intracellular signals, such as the PAS (Per-Arnt-Sim) and GAF (found in cGMP-specific phosphodiesterases, adenylylcyclases, and FhlA) domains, can be located in the same region allowing a concerted response to changes in internal and external conditions (Henry and Crosson, 2011; Mascher, 2014). The PAS domain of ArcB for example responds to the redox state of the quinone pool and affects the autophosphorylation activity of the HK (Malpica *et al.*, 2004). Thus, the ArcA/ArcB TCS allows facultatively anaerobic bacteria to sense various respiratory growth conditions and adapt their gene expression accordingly (Sawers, 1999). HKs may also function as phosphatases and hydrolyze the cognate phosphorylated regulator. As a

consequence, the combined activities of signal-induced HK phosphorylation, phosphotransfer to the regulator, and the phosphatase activity contribute to the overall flux of phosphorylated RR (A. M. Stock *et al.*, 2000).

The RR consists of two domains. The conserved N-terminal receiver domain (REC) typically contains a conserved aspartate residue that is able to acquire the phosphoryl group of the His~P of the cognate HK. The phosphorylation may result not only from a HK that autophosphorylates continuously but also from a small-molecular-weight phosphoryl donor, such as acetyl phosphate (McCleary and Stock, 1994), the levels of which are modulated by the metabolic status of the bacterial cell (Xu *et al.*, 2010; Kosono *et al.*, 2015; Schilling *et al.*, 2015). The variable C-terminal effector domain gets typically activated by a conformational change initiated by the Asp~P (Zwir *et al.*, 2012; Zwir *et al.*, 2014; Gao and Stock, 2015). Commonly, the effector domain is a DNA-binding domain, which can be subdivided into three major families represented by the winged helix-turn-helix (wHTH) motif-containing OmpR, the helix-turn-helix (HTH) motif-containing NarL, and the ATPase domain and HTH motif-containing NtrC (J. B. Stock *et al.*, 1990; A. M. Stock *et al.*, 2000; Wolanin *et al.*, 2002). A few other regulators lack a DNA-binding domain but function as enzymes, such as the chemotaxis methylesterase CheB (Simms *et al.*, 1985), or exert their regulatory effects by establishing direct interactions with protein (Hengge, 2008) or RNA (Shu and Zhulin, 2002) targets.

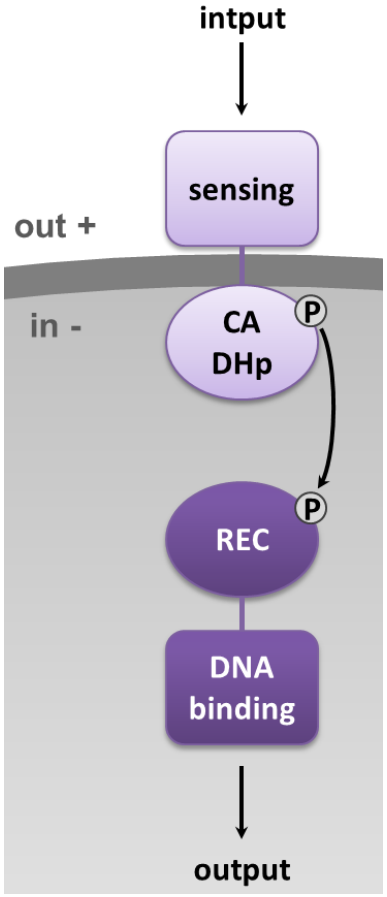

Fig. 1.3: Architecture of a classical two-component signal transduction system. The sensor (pale violet) harboring an extracytoplasmic sensor domain and a cytoplasmic catalytic and ATP-binding (CA) and a dimerization and histidine phosphotransfer (DHp) domain responds to an input by autophosphorylating a conserved histidine residue. The phosphorylated sensor serves as a phosphoryl donor for the regulator (violet) harboring a receiver (REC) domain and a DNA-binding output domain. The phosphorylated regulator generates the output. The figure is modified from (Groisman, 2016).

TCSs are usually autoregulated, i.e. the regulator binds the promoter of the TSC operon resulting in a positive feedback loop. This allows the organism to increase the amount of phosphorylated RRs necessary to upregulate all genes that are required to answer the environmental changes denoted by the signal. In addition, TCSs must have a constitutive promoter in order to function properly. It is possible that a single promoter serves as both constitutive and autoregulated promoter as it is the case for the *comD*/*comE* operon in *Streptococcus pneumoniae* (B. Martin *et al.*, 2010). Feedback control can additionally be guaranteed by effector molecules, which synthesis is controlled by the TCS. The effector may influence input access to the sensor (Kato *et al.*, 2012; S.-Y. Park and Groisman, 2014; Raghavan *et al.*, 2014), bind to the sensor modifying its enzyme activity (Raivio *et al.*, 1999; Lippa and Goulian, 2009; Jeong *et al.*, 2012; Schrecke *et al.*, 2013; Hörnschemeyer *et al.*, 2016) or modify the phosphorylation state of the regulator in a direct or indirect fashion (F.-F. Wang *et al.*, 2014a). Even more extrinsic mechanisms are conceivable.

1.4 Omics

Omic technologies aim at the collective characterization of data representing an entire set of biological molecules such as DNA (genomics), RNA (transcriptomics), proteins (proteomics), and metabolites (metabolomics) in a specific sample. Omic research provides quantitative datasets that are aligned to appropriate databases, which allow the characterization of an organism, its comparison to other organisms as well as the comparison of data sets gained under different growth conditions. It is especially advantageous for organisms that are not accessible for genetic manipulation, such as *Sulfurospirillum* species with only on mutant strain available (Kunze *et al.*, 2017b).

Genomics is the systematic study of the total DNA of an organism. Genes can be annotated and the information about the genetic equipment allows hypotheses about an organism's features, such as possible physiological pathways or putative regulatory networks. Comparative genomics unravels the relationship of genome structures and functions across different biological species and strains. It also provides a powerful method for studying evolutionary changes among organisms as well as identifying genes that are conserved among species or genes that are unique for a specific organism.

The transcriptome is the total RNA in an organism reflecting the genes that are expressed at any given moment. Previously, the microarray technology was used for transcriptomic analysis,

but RNA-sequencing (RNA-seq), which dissects complementary DNA (cDNA) molecules in parallel by high-throughput sequencing, is presently the method of choice to address the complexity of bacterial transcriptomes (Croucher and Thomson, 2010; van Vliet, 2010; Westermann *et al.*, 2012). It has a single-nucleotide resolution, a high dynamic range and sensitivity as well as a beneficial discriminatory power. Typically, total RNA is converted to a cDNA library and amplified (Fig. 1.4). The resulting short sequences also referred to as reads are obtained by deep sequencing and subsequently mapped onto a reference genome. In order to distinguish between sense and antisense transcription, different RNA-seq protocols are used, i.e. ligation of a 5′ RNA linker and 3′ poly(A)-tailing plus oligo-d(T) priming of cDNA or cDNA synthesis from a ligated 3′ linker (Borries, 2012; Passalacqua *et al.*, 2012). Differential RNA-seq (dRNA-seq) was introduced in 2010 (Sharma *et al.*, 2010). This approach aims to selectively sequence bacterial primary transcripts, which carry a 5′ triphosphate end (5′ PPP), and processed transcripts including the abundant ribosomal and transfer RNAs, which carry a 5′ monophosphate (5′ P) or the less abundant 5′ hydroxyl (5′ OH) group (Sharma *et al.*, 2010). The original RNA sample is divided into two fractions. One of which remains untreated in order to capture both 5′ PPP and 5′ P RNAs. The other fraction is treated with a 5′ P-dependent terminator exonuclease (TEX), which degrades processed 5′ P RNAs resulting in a relative enrichment of primary transcripts (Borries, 2012). Thereby, dRNA-seq is a powerful method enabling quantitative expression profiling, global mapping of transcriptional start sites (TSS), and identification of operon and regulon structures, previously unknown open reading frames (ORFs) as well as determination of 5′ untranslated region (5′ UTR) lengths and global regulatory small RNA (sRNA) identification (Sharma and Vogel, 2014). Global transcriptomic studies of organohalide-respiring bacteria are currently available only for *Dehalococcoides* spp., performed by microarrays (Johnson *et al.*, 2008; Men *et al.*, 2012; West *et al.*, 2013; Mansfeldt *et al.*, 2014; Men *et al.*, 2014; Mansfeldt *et al.*, 2016; Heavner *et al.*, 2018) and RNA-seq (S. Wang *et al.*, 2014b; Men *et al.*, 2017), and for *Desulfitobacterium* spp. performed by microarrays solely (Kim *et al.*, 2012; Peng *et al.*, 2012). Apart from these global analyses, the regulation of single *rdh* genes has been studied in *Dehalococcoides* spp., *Desulfitobacterium* spp., and *Dehalobceter* spp. by means of RT-PCR, RT-qPCR, and Northern blots (Maillard and Willemin, 2019).

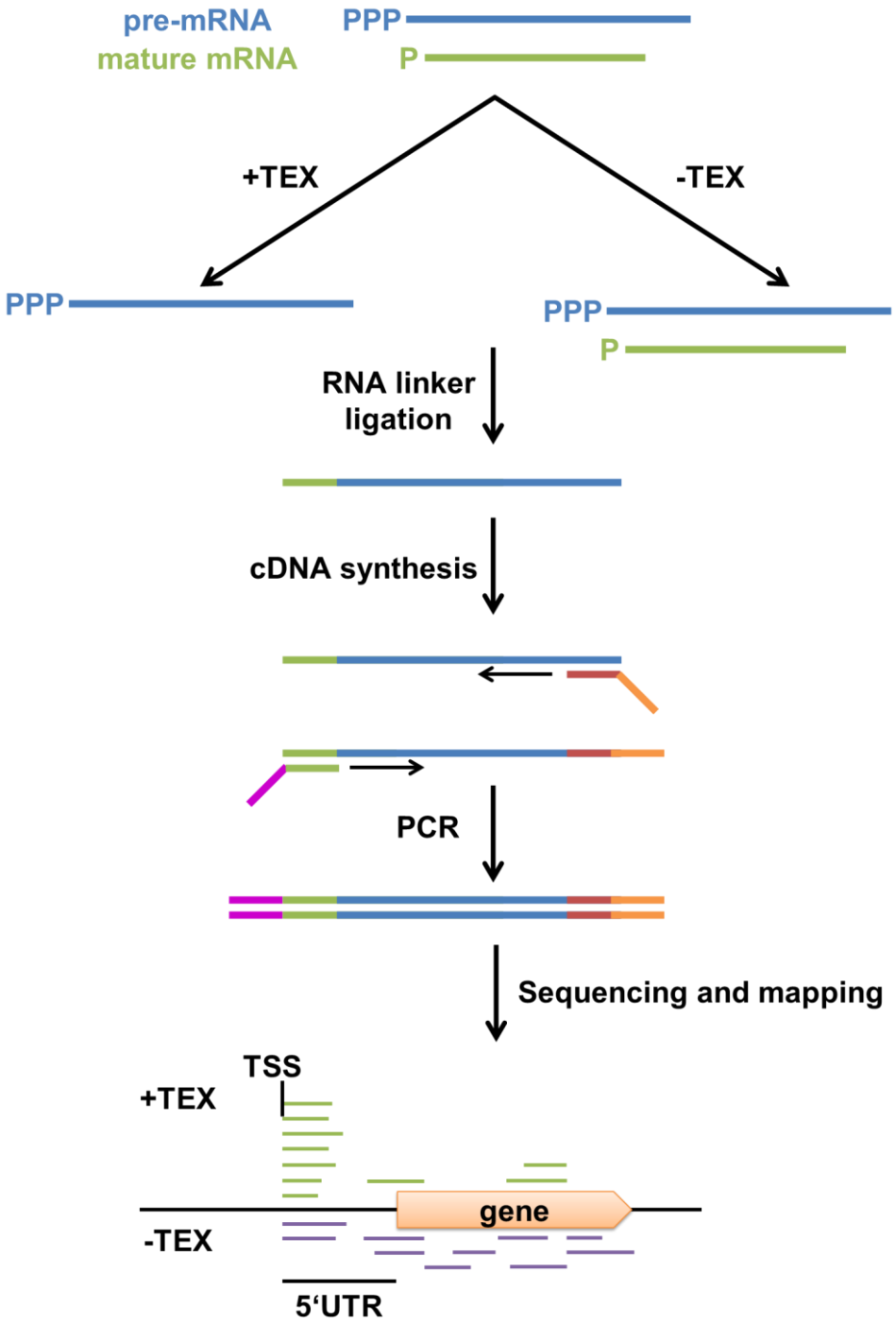

Fig. 1.4: Differential RNA-seq workflow. The RNA sample is split into two fractions. One of which remains untreated whereas the second is digested by using terminator 5′ monophosphate-dependent exonuclease (TEX) resulting in an enrichment of primary transcripts. This is followed by RNA linker ligation, cDNA synthesis, amplification, deep sequencing and the mapping onto a reference genome. Sequencing reads that start at the same nucleotide position denote the transcriptional start site (TSS) allowing for the definition of the 5′ untranslated region (5′ UTR). The figure is modified from (L. Martin *et al.*, 2013).

The proteome is the sum of all proteins expressed in an organism at a certain time point under given conditions. Its quantitative analysis enables information about the expression profile and the identification of genes co-regulated in *cis* (operons) and *trans* (regulons) on the protein level. The standard shotgun proteomics approach comprises a proteolytic digestion of the proteins followed by reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to mass spectrometry (MS) (Fig. 1.5). The RP-HPLC reduces the sample complexity and salt concentration. Ionized peptides are subsequently separated due to their mass-to-charge (*m/z*) ratios in the MS and the abundances are determined. The most abundant ions are isolated, fragmented at their peptide bonds into y- and b-ion series, which are again separated enabling peptide sequencing and the identification of abundant and stable post-translational modifications (PTMs) (Muddiman, 2018). In order to detect less abundant PTMs, they have to be enriched in an additional step. Protein acetylations for example can be captured from the protein digest by using specific antibodies. The acetylome is the sum of all acetylated proteins, which can be identified by a specific mass shift in a tandem MS. Next to phosphorylation, acetylation is one of the most abundant PTMs and can amount to 1-34% acetylated proteins in a proteome depending on the bacterial organism and its physiological state (Hentchel and Escalante-Semerena, 2015). In bacteria, acetylation mainly occurs at lysine residues, whereas the acetylation at the N-terminal amine residue of a protein or the hydroxyl group of serine and threonine is rare (Cain *et al.*, 2014; Ouidir *et al.*, 2016). Acetylations neutralize the lysine's positive charge and can alter local and global protein structures, which can affect protein stability, activity, localization, and intermolecular interactions (Cain *et al.*, 2014; Hentchel and Escalante-Semerena, 2015). In bacteria, protein lysine acetylations have been reported to play a role in the regulation of cell motility and shape, both gene expression and RNA degradation, stress response, DNA replication, and repair (Hentchel and Escalante-Semerena, 2015; Carabetta and Cristea, 2017). Indeed, some TCSs have been described to be acetylated as well. It causes inhibiting effects as it has been described for the global RR RcsB, which controls cell division, capsule and flagellum biosynthesis in many bacteria (Thao *et al.*, 2011; Hu *et al.*, 2013). Similar inhibiting effects have been described for the virulence regulating RR PhoP in *Salmonella enterica* serovar Typhimurium (Ren *et al.*, 2016) and the global nitrogen RR GlnR in *Streptomyces coelicolor* (Amin *et al.*, 2016). In addition, the chemotaxis RR CheY in *E. coli* has a repressed binding affinity to all its target proteins when it is acetylated (R. Li *et al.*, 2010a; Liarzi *et al.*, 2010). Comparable to phosphorylation, acetylation can occur specifically by the help of N-acetyltransferases (R. Li *et al.*, 2010a; Liarzi *et al.*, 2010; Thao *et al.*, 2011; Hu *et al.*, 2013; Amin *et al.*, 2016; Ren *et al.*, 2016) and Sir2 family deacetylases (R. Li *et al.*, 2010a;

Thao *et al.*, 2011; Amin *et al.*, 2016; Ren *et al.*, 2016) or non-specifically by small molecules, such as acetyl phosphate (Brian T. Weinert *et al.*, 2013). The acetylome of *S. halorespirans* published within this work (Türkowsky *et al.*, 2018b) is the only one available for organohalide-respiring bacteria so far, but there are proteomic analyses available for *Dehalococcoides* spp. (Morris *et al.*, 2006; Fung *et al.*, 2007; Rowe *et al.*, 2012; Padilla-Crespo *et al.*, 2014; Chau *et al.*, 2018), *Desulfitobacterium* spp. (Prat *et al.*, 2011; T. Kruse *et al.*, 2015), *Dehalobacter* spp. (Rupakula *et al.*, 2013; Jugder *et al.*, 2016b), and *Sulfurospirillum* spp. (T. Goris *et al.*, 2015a; Türkowsky *et al.*, 2018b). However, proteomes of dehalogenating δ-Proteobacteria have not been published yet. The merging of omics data provides comprehensive information that contribute to the understanding of gene regulation, particularly when knock out mutants are not available.

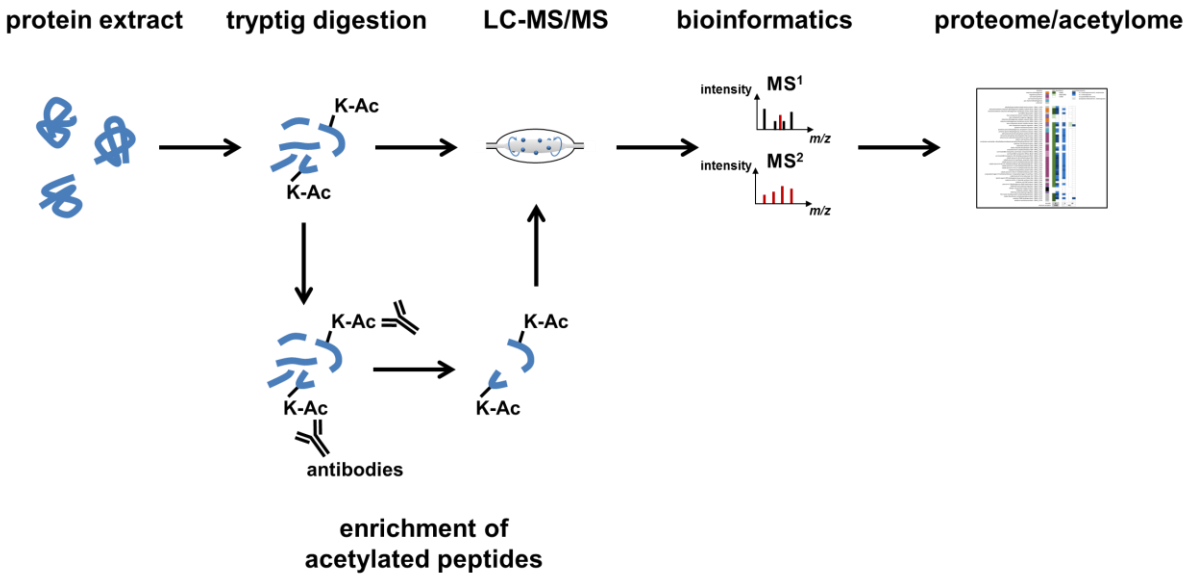

Fig. 1.5: Proteomics and acetylomics workflow. The protein sample is digested using trypsin. Acetylated peptides are immunologically enriched. Afterwards, non-modified and acetylated peptides are injected into a liquid chromatography – tandem mass spectrometer (LC-MS/MS). The proteins are identified by database alignments, quantified, and the posttranslational modifications are classified. The figure is modified from (Türkowsky, 2018).

1.5 Aim of this study

This work aims at a better understanding of the substrate-dependent regulation of OHR in *Sulfurospirillum* species. With the study focusing on the maintenance of the PCE-dechlorinating ability of *S. multivorans* in the presence and absence of PCE (John *et al.*, 2009), it was known that the reductive dehalogenase PceA is only expressed in the presence of its substrates. However, in the absence of the enzyme's substrate PCE and in the presence of an alternative electron acceptor (e.g. nitrate), the production of catalytically active PceA enzyme is declining but still detectable for more than 100 generations (John *et al.*, 2009). The genome of this species, which was published in 2014 (T. Goris *et al.*, 2014), gave a first hint pointing towards two TCSs that might be involved in the transcriptional regulation of OHR. This study focusses on both the induction of OHR and the long-term downregulation also referred to as retentive memory effect.

Since knockout mutants were not available for studying the induction of OHR expression in *Sulfurospirillum* spp., omic analyses were the methods of choice to answer most questions. In order to identify the signal transduction system responsible for PCE-induced OHR, comparative genomics introducing two new *Sulfurospirillum* species and differential transcriptomics were performed. Using these methods, TCS2 was identified as the essential signal transduction system and other transcription factors as well as sRNAs within the OHR gene region could be eliminated as potential transcriptional regulators. In addition, the differential RNA sequencing evaluated in this study allowed for the determination of the TCS2 regulon and identification of the promoter regions of the PCE-dependently regulated operons. For the structural and functional characterization of TCS2, *in silico* analyses and promoter binding studies were performed. The characterization was rounded off by the discovery of a direct repeat containing consensus sequence in the *cis*-regulatory element (CRE), which allowed for the prediction of the molecular binding mechanism. This part is summarized in the first manuscript.

While the transcriptome provides a more comprehensive picture of gene expression and information about promoter and untranslated regions, the proteome reflects the actual physiology of an organism more accurately. The publication of a new *Sulfurospirillum* proteome did not only enable the comparative analysis of *S. multivorans* and *S. halorespirans* but also complemented information about the PCE-dependent expression profile. In addition, the retentive memory effect was demonstrated to be a more universal regulatory phenomenon not unique to *S. multivorans*. In order to examine whether lysine acetylations might play a role in OHR regulation, the acetylome was analyzed in parallel. Whether the posttranslational acetylated

TCS is the sought effector protein, is discussed in this part summarized in the second manuscript.

Finally, the AdeNCba cofactor was discussed to play a role as effector molecule during the transcriptional regulation of OHR, since it is essential for the PceA enzyme activity. In the last part, the effect of cobalt limitation on dehalogenating *Sulfurospirillum* species and its potential role in the retentive memory effect was examined. The results are resumed in the last manuscript.

2 Manuscripts

2.1 Tetrachloroethene respiration in *Sulfurospirillum* species is regulated by a two-component system as unraveled by comparative genomics, transcriptomics, and regulator binding studies

Esken J*, Goris T*, Gadkari J, Bischler T, Förstner K U, Sharma C, Diekert G, Schubert T, *Applied and Environmental Microbiology* (in revision since March 17th, 2020)

2.2 A Retentive Memory of Tetrachloroethene Respiration in *Sulfurospirillum halorespirans* – involved Proteins and a possible link to Acetylation of a Two-Component Regulatory System

Türkowsky D*, Esken J*, Goris T, Schubert T, Diekert G, Jehmlich N, von Bergen M (2018), *J Proteomics* 181, 36-46

2.3 Cobalt - an essential micronutrient for organohalide respiration in dehalogenating *Sulfurospirillum* spp.

Esken J, Schubert T, Diekert G, *FEMS Microbiology Ecology* (in preparation for submission)

* Authors contributed equally to this work.

2.1 Tetrachloroethene respiration in *Sulfurospirillum* species is regulated by a two-component system as unraveled by comparative genomics, transcriptomics, and regulator binding studies

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Applied and Environmental Microbiology (in revision since March 17th, 2020)

* Authors contributed equally to this work.

The Manuscript includes genomes of two new *Sulfurospirillum* species and transcriptomes of *S. multivorans* cultivated under dehalogenating and non-dehalogenating conditions. The data support the assumption that TCS2 is solely responsible for the induction of OHR. Electrophoretic mobility shift assays (EMSAs) with RR2 prove its function as central regulator of the PCE regulon.

My own contribution to this manuscript covers about 70%.

Tobias Goris initiated the project and evaluated the genome sequences. Jennifer Gadkari performed cultivations and RNA isolation for the dRNA-seq, which was performed at the University of Würzburg in cooperation with Cynthia Sharma, Konrad U. Förstner, and Thorsten Bischler. I evaluated the RNA-seq data of the OHR gene region. I cultivated *S. multivorans*, isolated its RNA and conducted RT-PCR and RT-qPCR to examine the RNA-seq results. I conducted cultivations of *S. multivorans* strain N and *S.* sp. JPD-1. I did the conception and implementation of the functional characterization of the PCE regulator (RR2, PceP) including cloning, protein purification and EMSAs. Tobias Goris assisted me writing the manuscript. All coauthors reviewed the manuscript before submission.

For supplementary information see appendix, pp. i - xxii

Tetrachloroethene respiration in *Sulfurospirillum* species is regulated by a two-component system as unraveled by comparative genomics, transcriptomics, and regulator binding studies

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Abstract

Energy conservation via organohalide respiration (OHR) in dehalogenating *Sulfurospirillum* species, such as *S. multivorans*, is an inducible process. However, the gene products involved in tetrachloroethene (PCE) sensing and signal transduction have not been unambiguously identified. Here, genome sequencing of *Sulfurospirillum* strains defective in PCE respiration and comparative genomics, which included the PCE-respiring representatives of the genus, uncovered the inactivation of a two-component system (TCS) in the OHR gene region by transposon insertion in the natural mutants. The assumption that the TCS gene products serve as PCE sensor that initiates gene transcription was supported by the constitutive low-level expression of the TCS genes in fumarate adapted cells of *S. multivorans*. Via RNA sequencing, eight transcriptional units were identified in the OHR gene region, which includes the TCS operon, the PCE reductive dehalogenase operon, the gene cluster for norcobamide biosynthesis, and putative accessory genes with unknown functions. The OmpR-family regulator encoded in the TCS operon was functionally characterized by promoter binding assays. The results emphasized its role in promoting class I and class II transcriptional activation induced by binding a *cis*-regulatory element containing a consensus sequence uncovered as a direct repeat of CTATW separated by 17 bp. Sequence variations in the regulator binding sites identified in the OHR gene region were in accordance with differences in the transcript levels of the respective gene clusters forming the PCE regulon. The results indicate the presence of a fine-tuned regulatory network controlling PCE metabolism in dehalogenating *Sulfurospirillum* species, a group of metabolically versatile organohalide-respiring bacteria.

Importance

Organohalide respiration (OHR) is a mode of energy conservation in anaerobic bacteria that is of high environmental relevance. Unraveling the molecular details of this process will aid the application of organohalide-respiring bacteria in bioremediation attempts and improve our understanding of an essential part of the natural halogen cycle. Most organohalide-respiring bacteria need to sense organohalides in the environment in order to induce the production of the enzymatic machinery required for their utilization. Identification and characterization of biological sensors for halogenated organic compounds, such as the two-component system described here, could guide synthetic approaches for monitoring organohalides at contaminated sites. The presented integrative genomic, transcriptomic, and molecular biological approach allowed for a substantial step forward in resolving the regulatory network controlling OHR gene expression in *Sulfurospirillum* spp. and might help to study OHR regulation in other bacteria, which are difficult to manipulate genetically.

Introduction

Sulfurospirillum multivorans, which belongs to the Campylobacterota (formerly Epsilonproteobacteria) (1, 2), gains energy from respiring chlorinated and brominated ethenes, e.g. tetrachloroethene (PCE) (3, 4). The terminal reductase for the organohalide respiration (OHR) is the PCE reductive dehalogenase (PceA), which was structurally characterized before (5, 6). Genomic and proteomic analyses revealed the presence of a large gene region, the expression of which is induced upon cultivation with PCE as energy substrate (7, 8). This region, which we termed OHR gene region, includes *pceA* and *pceB* (encoding a putative membrane anchor) and an operon *rdhAB* for a second reductive dehalogenase, the latter without proven expression and thus with unknown function. Additionally, we detected genes encoding membrane proteins probably involved in electron transfer, proteins responsible for the biosynthesis of the norcobamide cofactor of PceA, and accessory proteins putatively involved in PceA maturation (7). Regulatory proteins are also encoded in this region. Histidine kinase and response regulator genes of two-component systems (TCSs) are located downstream of *pceAB* (TCS1) and *rdhAB* (TCS2). In general, the signaling pathway of TCSs relies on the transfer of a phosphoryl group from the sensor to the regulator protein (9). A gene for a TetR-like regulator is positioned downstream of the norcobamide biosynthesis gene cluster. While the *tetR* gene is disrupted by a transposase in *S. multivorans*, it is intact in *Sulfurospirillum halorespirans* (10) and '*Candidatus* Sulfurospirillum diekertiae' (11), which represent species containing an OHR gene region otherwise nearly identical to that of *S. multivorans*. The expression of *pceA* and other genes whose products are involved in OHR is under control of a long-term downregulation in *S. multivorans* (12). In the absence of PCE or trichloroethene (TCE), transcripts are still detectable for more than hundred generations before the expression stops. However, as shown for cultures derived from single cells with downregulated *pceA* transcription, OHR remains inducible by PCE within one generation, excluding a loss of function (12). The PCE-respiring *S. halorespirans* was shown to downregulate the OHR gene region in a similar way (13). TCS2 is conserved in both *Sulfurospirillum* species and was detected in the proteomes of OHR-downregulated cells and thus predicted to play a role in PCE-sensing and OHR induction. However, further evidence for the involvement of TCS2 in transcriptional regulation of the OHR gene region was missing. An acetylome study with *S. halorespirans* showed that this TCS was subject to protein acetylation (13). The molecular mechanisms responsible for the induction of OHR gene expression and the long-term downregulation in dehalogenating *Sulfurospirillum* species are still unknown. A similar regulatory long-term effect was not observed in other organohalide-respiring bacteria (14). A long-term decrease in OHR efficiency of *Desulfitobacterium hafniense* strains cultivated in the absence of halogenated growth substrates (15, 16) was caused by the irreversible loss of the OHR gene cluster via transposon excision (15, 17-19). Although the knowledge about regulatory mechanisms in organohalide-respiring bacteria is limited, it is assumed that different bacterial genera employ different regulatory systems for induction of OHR. Besides TCSs, the obligate organohalide-respiring *Dehalococcoides mccartyi* harbors MarR-type regulators (20, 21), while the OHR gene clusters of the Firmicutes *Desulfitobacterium* and

Dehalobacter are often encoding a CRP/FNR-type regulator (22-24) such as the functionally and structurally characterized CprK (25). In general, transcription of OHR-related genes is non-constitutive in versatile organohalide-respiring bacteria (24).

Since tools for the generation of defined single-gene knockouts in *Sulfurospirillum* spp. are not established, we used physiological experiments, comparative genomics, and gene expression studies to obtain further information on OHR in this genus (7, 10, 12). Further insights were derived from whole proteome analyses (8, 13). Apart from *S. multivorans* and *S. halorespirans*, two other *Sulfurospirillum* species were described to be capable of OHR, *S.* sp. JPD-1 (26, 27) and, with a genome sequence available, '*Candidatus* S. diekertiae' (11). *Sulfurospirillum* sp. JPD-1 (DSM 16452; *S. tacomaensis* BAA-971™ in the ATCC) was isolated in the US, close to Tacoma, Washington, as a psychrotrophic PCE to *cis*-dichloroethene (*c*DCE) dechlorinating bacterium physiologically similar to *S. multivorans* (27). The '*Candidatus* S. diekertiae*'* strains SL2-1 (dechlorinating PCE to TCE) and SL2-2 (dechlorinating PCE to *c*DCE) were enriched from a Dutch bioreactor loaded with PCE-contaminated water. In addition, the non-dechlorinating *S. multivorans* strain N was isolated from the same PCE-dechlorinating enrichment culture that contained the dechlorinating *S. multivorans*. Interestingly, strain N was shown to contain the *pceA* gene, but no PceA protein or norcobamide cofactor has been produced by this isolate (28).

A multi-level comparative analysis of these *Sulfurospirillum* isolates was designed to assist our efforts in uncovering the molecular basis of OHR gene regulation in PCE-respiring *Sulfurospirillum* spp. Therefore, we combined physiological studies and genome sequencing of *S.* sp. JPD-1 and *S. multivorans* strain N with comparative genomics and a whole transcriptome analysis (RNA-seq) of *S. multivorans* cultivated with or without PCE as electron acceptor. Based on the results, we were able to propose a detailed transcriptional map of dehalogenating *Sulfurospirillum* spp. In addition, we identified the regulator responsible for initiation of OHR gene transcription. DNA-binding studies performed with this regulator protein uncovered its dedicated binding box and indicated the presence of a PCE regulon in dehalogenating *Sulfurospirillum* spp.

Results

The genomes of *Sulfurospirillum multivorans* strain N and *S.* sp. JPD-1

In search for the genetic defect causing the inability of *S. multivorans* strain N to reductively dechlorinate PCE, we sequenced its genome (Tab. 1, Fig. 1A). The genome of strain N (DSM No.: 15119) is nearly identical (average nucleotide identity of 99.99% as calculated with ANI calculator (29)) to that of the dechlorinating *S. multivorans* (DSM No.: 12446^{T}). The most apparent difference between the two strains is the location of transposase genes, which are numerous in the genome of *S. multivorans* (7). The OHR gene region of both *S. multivorans* strains is identical with two exceptions. In strain N, a transposase

gene (SMN_1525) disrupts the gene encoding the response regulator (RR) of TCS2 (SMN_1524) and a short stretch (106 bp) of additional DNA sequence is present in the intergenic region upstream of the first gene (*cbiB*) of the norcobamide biosynthesis gene cluster (Fig. 2). These 106 bp have been detected before in the OHR gene regions of *S. halorespirans* and '*Candidatus* S. diekertiae' strains, too (10, 11).

Opposed to its original description (27), *S.* sp. JPD-1 was not able to dechlorinate PCE or TCE regardless of the electron donor (formate or pyruvate), presence or absence of vitamin B_{12} or yeast extract in the medium. Fumarate served as alternative electron acceptor (Fig. S1). To determine the reason for its inability to dechlorinate PCE, the genome of the species was sequenced (Tab. 1, Fig. 1B). Similar to the results obtained for strain N, a transposase, here comprising two genes, disrupted the RR gene of TCS2 of JPD-1. The total number of transposases in JPD-1 is much lower compared to *S. multivorans*. A conserved domain search revealed only 16 transposase genes in JPD-1, while *S. multivorans* and its non-dechlorinating counterpart strain N harbor about 80 genes related to transposable elements like transposases, integrases, and phage-dependent recombinases. The 106 bp additional DNA sequence in the intergenic region upstream of the *cbiB* gene are also present in JPD-1.

Tab. 1: Comparison of the five different *Sulfurospirillum* genomes carrying an OHR gene region.

	S. multivorans	***S. multivorans* strain N**	***S. halorespirans***	***S.* sp. JPD-1**	**'*Candidatus* S. diekertiae' SL2-1***
Strain designation	DSM 12446^T	DSM 15119	DSM 13726	DSM 16452	(11)
GenBank Acc. no.	CP007201.1	CP042966	CP017111.1	CP023275	CP021416.1
Genome size (Mbp)	3.18	3.18	3.03	2.81	2.88
GC content (%)	41	41	41	39	39
CRISPR regions	1	1	1	-	1
rRNA operons	2	3	2	3	3
tRNAs	45	43	41	33	33

The comparison is based on the automated NCBI RefSeq annotation. (*): '*Candidatus* S. diekertiae' includes two strains with a very similar genome, here the complete SL2-1 genome was chosen as representative.

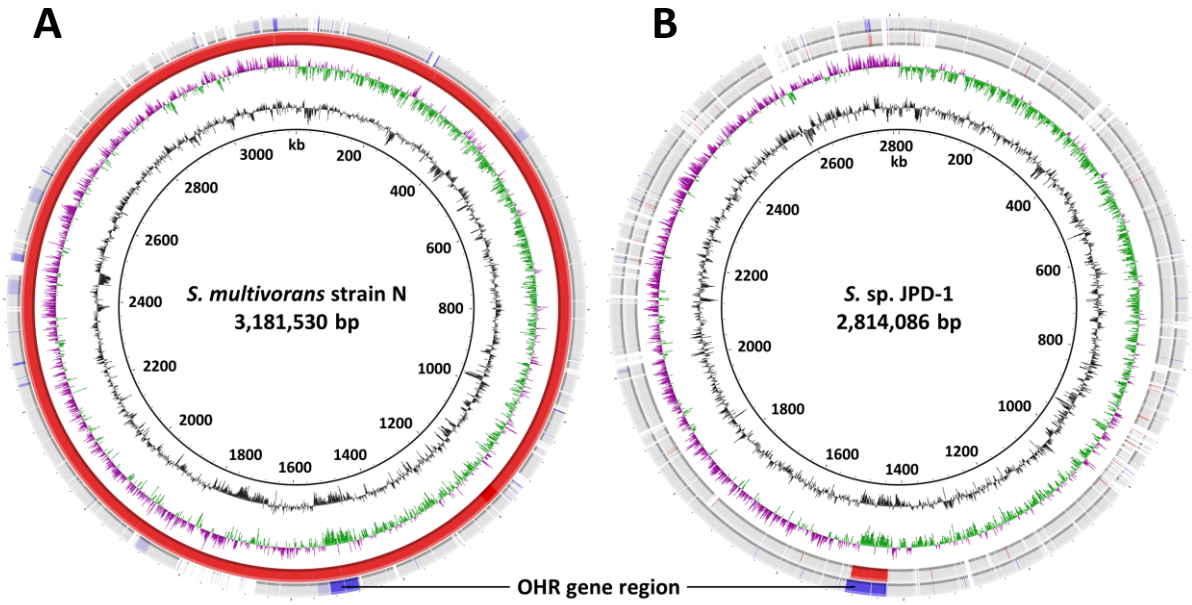


Fig. 1: Circular representations of the genomes of A) *S. multivorans* strain N (acc. no.: CP042699) and B) *S.* sp. JPD1 (acc. no.: CP023275) aligned with those of *S. halorespirans* (outer circle; acc. no.: CP017111.1) and *S. multivorans* (second circle from outside; acc. no.: CP007201.1). Blast hits with more than 99% nucleotide identity are colored in blue or red, respectively, hits with 95–99% identity are shown in pale color, respectively. The third circle shows the GC skew (green and purple) and the fourth circle shows the GC content (black). Positions are given at the inner circle. The images were generated with BRIG (30).

The OHR gene region is highly conserved with nearly 100% sequence identity in all *Sulfurospirillum* species and strains containing this region. The sequence identity exceeds those of the genes for ribosomal RNAs and proteins (98-99%). The OHR gene region contains 44 genes plus transposable elements and was described before in detail for *S. multivorans* (7) and *S. halorespirans* (10) (Fig. 2). In comparison to the other genes in the OHR gene region, the *pceA* gene displays the lowest sequence identity among all the species analyzed here (approximately 95%, Tab. S1). In contrast, the product of the second reductive dehalogenase gene *rdhA* is identical in all isolates. Neither an *rdhA* transcript in *S. multivorans* (7) nor the RdhA protein in *S. multivorans* (8) or *S. halorespirans* (13) were detected in previous studies.

The transposase genes disrupting the gene encoding the response regulator of TCS2 (RR2) in strain N and JPD-1 are located at different positions in the coding sequence and are not phylogenetically related to each other. The transposase gene disrupting the RR2 gene in strain N appeared in several copies in the genomes of *S. multivorans*, *S. halorespirans*, and strain N itself (Tab. S3). The transposable element disrupting RR2 in JPD-1 (SJPD1_1513 and SJPD1_1514) was also identified at other loci in JPD-1, *S. multivorans*, strain N, and '*Candidatus* S. diekertiae'. The TetR-like regulator encoded downstream of the norcobamide biosynthesis gene cluster is intact in JPD-1 and *S. halorespirans*, while it is disrupted by the same transposable element in both *S. multivorans* strains (Fig. 2).

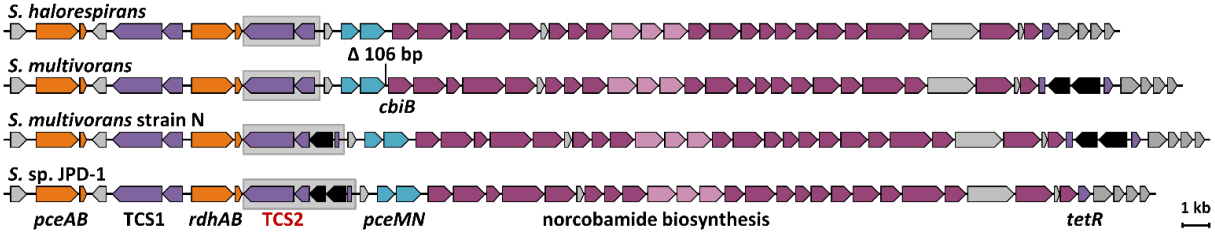


Fig. 2: Illustration of the organohalide respiration (OHR) gene region in *Sulfurospirillum* spp. The gene region encodes for two reductive dehalogenases and their putative membrane anchors (orange), two two-component systems (TCS1 and TCS2) as well as a TetR-like repressor (violet), two genes, which encode for components of a putative quinol dehydrogenase (turquoise, *pceMN*), and genes for the biosynthesis of the norcobamide cofactor (purple) including genes for an incomplete cobamide transport system (pink). Some of the genes have a yet unknown function (gray). Transposase genes are colored in black. *S. multivorans* lacks a 106 bp sequence in the intergenic region upstream of *cbiB*. '*Candidatus* S. diekertiae' strains SL2-1 and SL2-2 were not included, since the organization of their OHR gene region is identical to *S. halorespirans* (11). An overview of the locus tags is given in Tab. S2.

Transcriptome of *S. multivorans* cultivated with PCE or fumarate

To identify the genes whose transcription is PCE-dependent, a differential RNA sequencing (dRNA-seq) was performed. The PCE-respiring *S. multivorans* (DSM 12446^T) was cultivated with pyruvate as electron donor and either PCE (Py_PCE) or fumarate (Py_Fu) as electron acceptor. One cDNA library was generated from untreated total RNA (-TEX), whereas the second library was generated after treatment with terminator exonuclease (+TEX). TEX specifically degrades processed RNAs bearing a 5'-monophosphate (31, 32). Sequencing of these libraries leads to a characteristic enrichment of cDNA reads at the transcriptional start sites (TSSs) in the TEX-treated sample, which allows for an exact determination of TSSs in a given genome. Two biological replicates (A and B) for each condition were sequenced and showed a highly similar transcription pattern (Fig. 3 and Fig. S2). The total number of reads in all eight samples averaged to an amount of six million per library (Tab. S4). In the presence of PCE, the OHR gene region was transcribed, while in cells cultivated for more than 100 generations with fumarate instead of PCE most of the transcription stopped in this small section of the genome. The genes with the most notable changes in the transcript level were located in the OHR gene region (see next paragraph). Only two genes outside the OHR gene region displayed a positive log2-fold change (lfc) of at least 3.32 (corresponding to more than 10-fold higher transcript abundance in Py_PCE compared to Py_Fu): The gene encoding the heat shock protein Hsp20 (SMUL_0547) and the gene encoding the periplasmic-binding protein of a metal transporter (SMUL_0188) (Tab. S5). The three genes with the highest decrease in the transcript level form a single transcriptional unit and encode for a dicarboxylate transporter (SMUL_2818, lfc -8.75), an asparaginase (SMUL_2819, lfc -8.92), and an aspartate ammonia lyase (SMUL_2817, lfc -9.11), respectively. Also, genes encoding proteins involved in

fumarate utilization were substantially downregulated in the presence of PCE, the fumarate hydratase genes (SMUL_1679 and 1680; lfc -3.78 and -3.63, respectively) and an adjacent dicarboxylate transporter gene (SMUL_1681, lfc -4.00).

Transcription of the OHR gene region in *S. multivorans*

Most of the transcripts in the OHR gene region are significantly more abundant in the presence of PCE with an lfc > 3.32 (37 out of 44 genes, Fig. 3A, Tab. S6, suppl. data set). Exceptions are *rdhB* and the transcripts of the TCS1 histidine kinase and RR2, which are also more abundant in Py_PCE, but not significantly (lfc 0.34/1.65/0.89, Benjamini Hochberg-corrected p-values (padj) 0.96/0.15/0.093, respectively). However, the histidine kinase of TCS2, which shares a single transcript with RR2, is significantly more abundant (lfc 1.69, padj $1.5 \cdot 10^{-5}$) (Fig. 3B, Tab. S6, suppl. data set). In the absence of PCE, only TCS2 genes are transcribed in the OHR gene region (Fig 3B, Tab. S6, suppl. data set). The TSS of TCS2 is identical in cells cultivated with or without PCE indicating the absence of an alternative promoter for basal expression. The basal transcription was also detected in the non-dechlorinating strain N and JPD-1 using RT-PCR (Fig. S3A). In contrast to TCS2, the transcript abundance of TCS1 in *S. multivorans* was low under both conditions Py_PCE and Py_Fu (Fig. 3B, Tab. S6, suppl. data set).

The dRNAseq results allowed for the determination of eight TSSs in the OHR gene region of *S. multivorans* (Tab. S7). The first transcript covered a single gene encoding an alkylhydroperoxidase AhpD family protein (SMUL_1530), whose transcriptional level was comparably low. The second unit comprised *pceAB*. The third transcript encoded an IscU/NifU-like protein (SMUL_1533), followed by the transcripts of TCS1 and TCS2. As mentioned earlier, the *rdhAB* gene cluster showed no transcription. The sixth small transcript covered the gene encoding a putative membrane protein (SMUL_1540, 111 amino acids), which was not detected in any of the proteomes so far. It is predicted to contain three transmembrane helices (Fig. S4) and is related to bacterial cytochrome *b* of NiFe hydrogenases. It belongs to the DUF4405 (pfam14358) protein family, which contains two conserved histidine residues. The conserved histidine residues at position 37 and 40 are predicted to face the periplasm close to or at the beginning of the second transmembrane helix. Similar proteins (35 to 50% amino acid sequence identity) are found in different genomic contexts mainly in Proteobacteria and the flavobacterial genus *Lutibacter*. The seventh, very long transcriptional unit (29 genes, approximately 25 kb) contains genes encoding components of a putative quinol dehydrogenase protein complex (*pceMN*) (14) as well as genes for the *de novo* norcobamide biosynthesis. The location of both gene regions on a single large transcript was verified by RT-PCR for *S. halorespirans*, too (Fig. S3B). The *tetR* gene is also part of this long transcript (Fig. S3C). This was additionally proven via RT-qPCR in *S. halorespirans*, which harbors a functional *tetR* gene (Tab. S8). The transposases located within the *tetR* gene of *S. multivorans* were not transcribed, but we could detect a putative antisense RNA with an unknown function. The

eighth transcript included four genes encoding a putative iron-sulfur cluster-containing flavoprotein (SMUL_1573), a Rieske-like putative redox protein (SMUL_1574), a putative FMN-binding protein (SMUL_1575), and a putative membrane protein (SMUL_1576). This region was also transcribed in *S. halorespirans* (Fig. S3D).

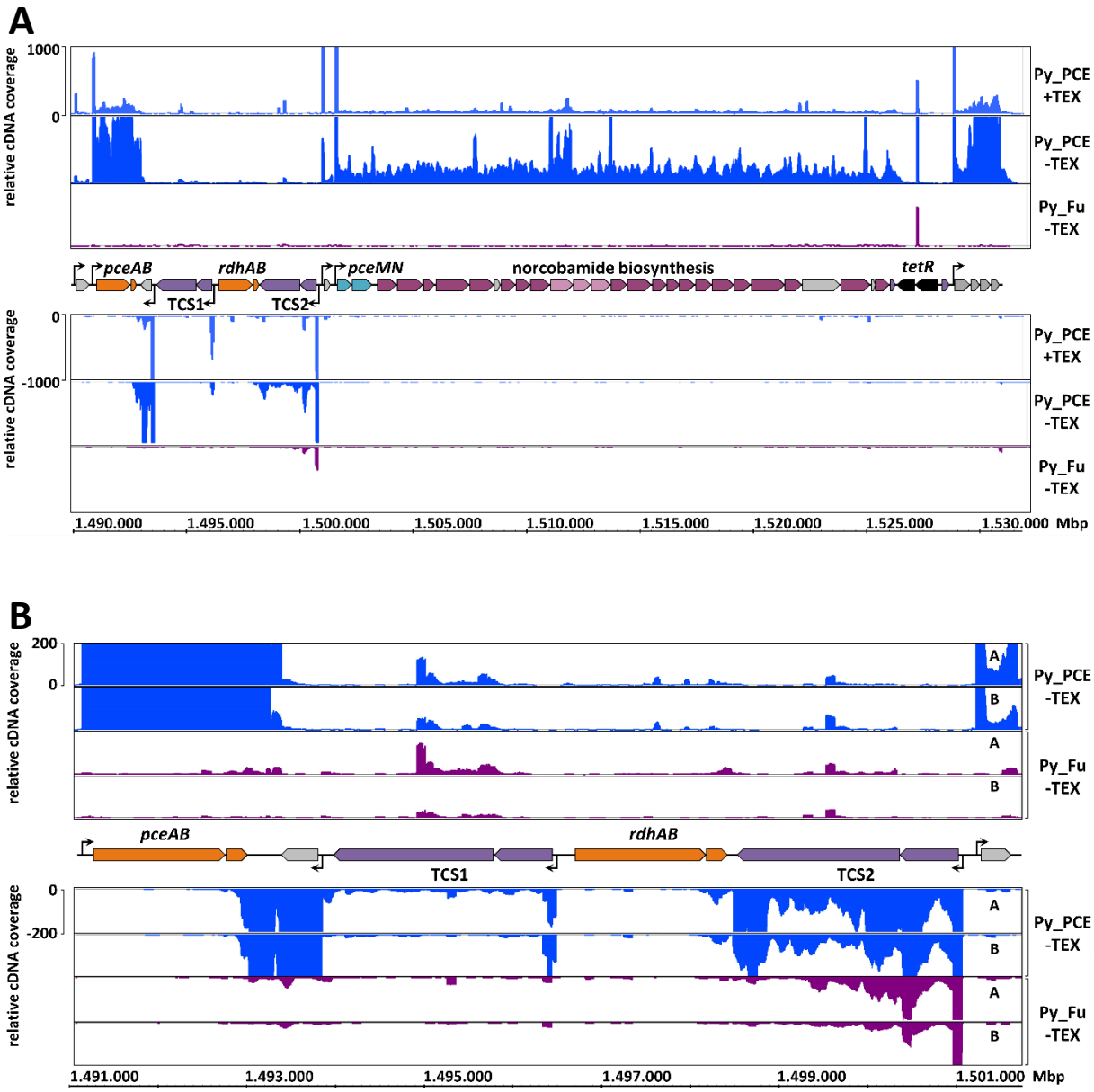


Fig. 3: Differential RNA sequencing (dRNA-Seq) results of the *S. multivorans* OHR gene region. A) Data for the complete OHR gene region. Only replicate B is shown. The analysis led to the annotation of global transcriptional start sites (TSS) marked by arrows. The "Integrated Genome Browser" (v. 9.0.1) was used for data evaluation (33). For the read alignment statistics please refer to Tab. S4. B) RNA sequencing results focusing on the genes encoding the reductive dehalogenases and the two TCSs in the OHR gene region. Both biological replicates (A and B) are displayed.

The promoter sequences of the OHR gene region differed from promoter sequences in the remaining *S. multivorans* genome (Fig. S5). The -35 box was identified as ACAA (Fig. S6). The -10 box sequence TANNAT displayed similarities to the sequence bound by the sigma factor RpoD of *Escherichia coli*

(TATAAT) (34, 35). RpoD is the primary sigma factor during exponential growth (36). From this observation one might conclude that PCE respiration in *S. multivorans* is regulated in dependence on the growth-phase. The alignment of 10 randomly chosen RpoD-dependent promoters in the genome showed a conserved -10 box, whereas the -35 box of this group of genes seemed to be less conserved and more similar to the consensus sequence of *Campylobacter jejuni* (TTTAAGTNTT) (37). The consensus sequence of the -35 box of the OHR gene region appeared to be unique for this set of transcriptional units. To further characterize this set of potentially co-regulated gene clusters, binding studies of the regulator protein RR2 and various promoter DNA sequences have been conducted.

Characterization of RR2 by DNA-binding assays

The molecular details of the OHR gene regulation and the role of TCS2 was further investigated in *S. halorespirans* because transposase genes are absent in its OHR gene region and the 106 nucleotides upstream of *cbiB* are present (Fig. 2). The locus tags for the OHR genes in *S. halorespirans* are given in Tab. S2. The promoter sequences within the OHR gene region were almost 100% conserved among the dehalogenating *Sulfurospirillum* spp. Because of the high conservation of the OHR gene region's regulatory elements among *Sulfurospirillum* spp., the results obtained for *S. halorespirans* are most likely transferable to the other species. Only the -10 box of the last transcriptional unit, which encodes the flavoproteins and proteins of unknown function, differs in both '*Candidatus* S. diekertiae' strains. Instead of TACAAT in *S. multivorans* and *S. halorespirans*, TAAAAT was identified.

The RR2 protein of *S. halorespirans* was heterologously produced in *E. coli* and purified via affinity chromatography (Fig. S7A). The concentrated RR2 sample (>25 pmol/µl) contained the monomeric as well as the dimeric form of the protein (Fig. S7B). Subsequently, the binding of the unphosphorylated regulator to the OHR promoter sites was analyzed in electrophoretic mobility shift assays (EMSAs) using fluorescently labeled DNA fragments containing the various promoter sequences. In order to ensure the integrity of every promoter sequence, the DNA fragments covered a region that spans from 150 bp upstream of the TSS to the start codon of the first gene of the transcriptional unit in every case. Therefore, amplified intergenic regions of TCS1 and *rdhAB* as well as those of TCS2 and SHALO_1504 overlapped, because these operons are located on neighboring and opposing open reading frames.

Using 2.5 pmol fluorescently labeled DNA, shifts were detected in the cases of *pceAB,* TCS2, and SHALO_1504 after adding different amounts of purified RR2 (Fig. 4A and B). The addition of the osmolyte triethylene glycol was necessary to stabilize protein-DNA complexes and to reduce complex dissociation during electrophoresis. A second DNA band appeared upon addition of triethylene glycol, but the intensity of this band did not change when RR2 was added. In general, between 8 and 60 pmol of RR2 were required to visualize complex formation with promoter regions of the OHR gene region (Fig. S8). For selected promoter regions (SHALO_1494, *rdhAB*, and TCS1), the protein-DNA complex

band was barely visible, although the DNA band disappeared at higher protein concentrations. After boiling, the protein-free DNA reappeared, which could be interpreted as a result of the dissociation of the RR2-DNA complex. A set of negative controls confirmed the specific binding of RR2 (Fig. 4C). DNA sequences amplified from the *pceA* structural gene, the 16S rRNA gene or the promoter sequence of a gene encoding for a phage CI repressor superfamily protein (SHALO_1552, located downstream of the OHR gene region) did not interact with RR2. All assays performed indicate that the regulator binds to all promoter sites of the OHR gene region that have been predicted by RNA sequencing before.

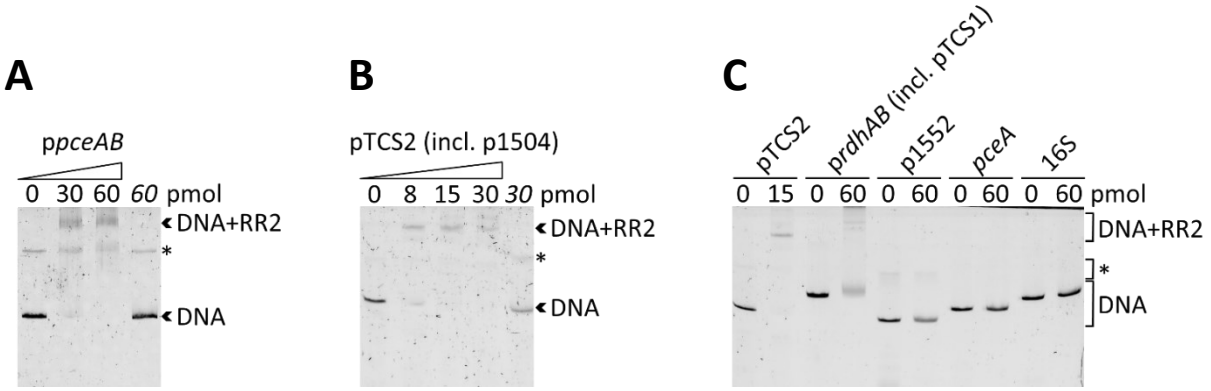


Fig. 4: Gel shift assays of *S. halorespirans* RR2-twin-Strep binding to the promoters of A) p*pceAB* and B) the intergenic region between TCS2 and SHALO_1504. As a negative control (last lane in both cases, the amount of protein is given in italics), the sample mixture was boiled for 5 min after the binding reaction. C) Control reactions for the gel shift assay with pTCS2 as positive control. The amplified intergenic region sequence of p*rdhAB* also contains pTCS1, which is transcribed on a minimal level. Promoter p1552 is located in the flanking region downstream of the OHR gene region and belongs to a gene encoding for a phage CI repressor superfamily protein. Short DNA stretches from the coding sequences of the *pceA* and 16S rRNA genes were used as negative controls. The amount of fluorescently labeled DNA was 2.5 pmol, whereas the protein amount was increased in each lane as indicated. A DNA artifact not targeted by RR2 is marked by an asterisk.

RR2 has a predicted OmpR-family domain structure (Fig. S9). Thus, it was assumed that an RR2 dimer pair binds a direct repeat. To identify and localize the *cis*-regulatory elements (CREs) bound by RR2, a screening of the promotor sequences with short overlapping DNA fragments was conducted. In the case of the intergenic region upstream of *pceAB*, which showed the highest upregulation in the dRNA-seq, the minimum binding sequence defined by EMSA was a 70 bp DNA fragment (Fig. 5A and C). This DNA fragment harbors direct repeats overlapping the -35 box. A sequence of CTATA is repeated twice with a gap of six nucleotides, respectively. While the central CTATA overlaps the -35 box and is probably bound by the σ^{70} factor, the first and last CTATA are available for binding an RR2 dimer.

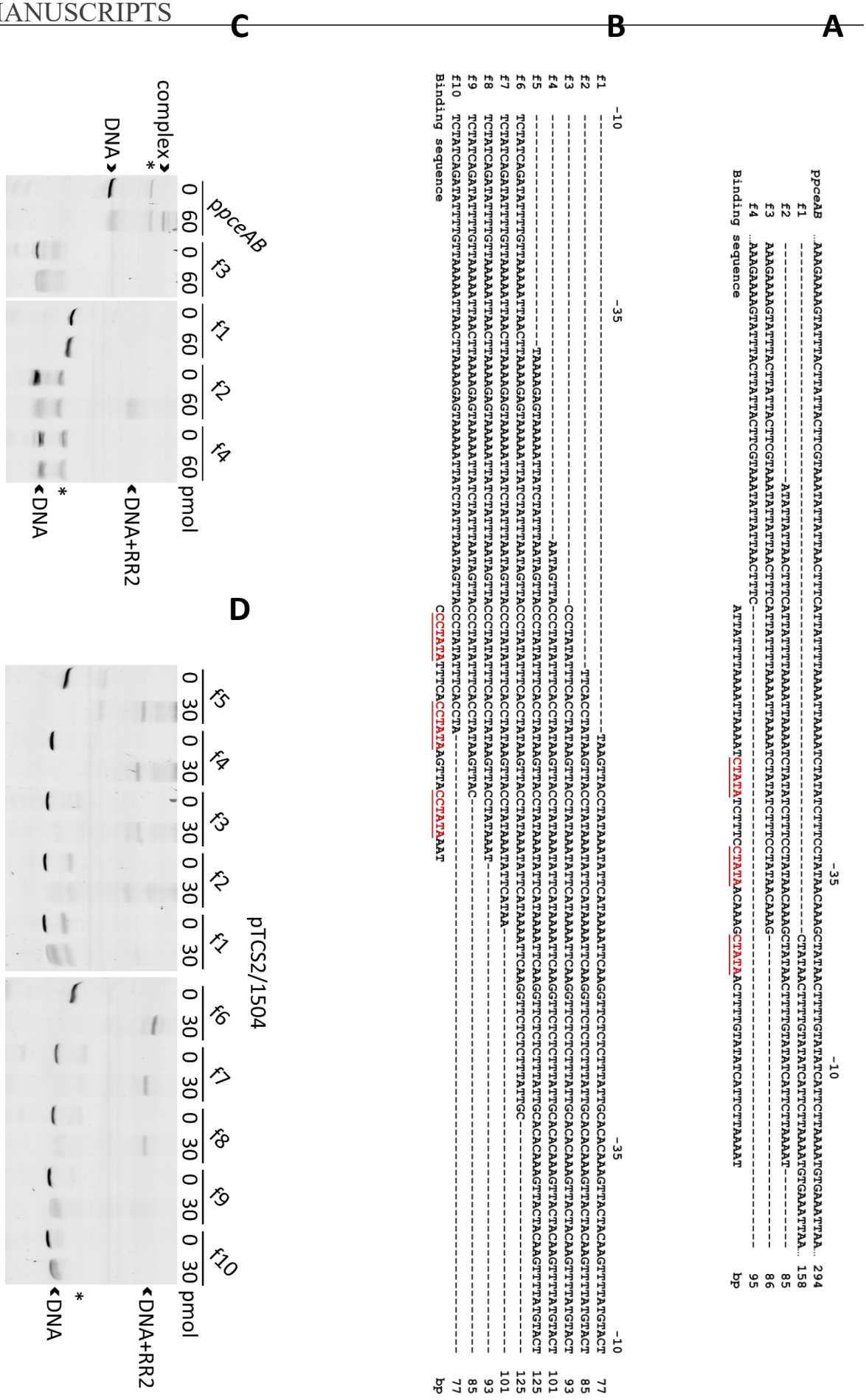


Fig. 5: Truncated promoter DNA sequences (f1-10) of A) p*pceAB* and B) pTCS2/1504 are shown together with the respective gel shift assays performed with RR2 and the DNA fragments of C) p*pceAB*

and D) pTCS2/1504. The direct repeat in the suggested binding sequence is underlined. The amount of 6Fam-labeled promoter DNA was 2.5 pmol, whereas the protein amount was 0, 30 or 60 pmol as indicated. A DNA artifact not targeted by RR2 is marked by an asterisk. See also Fig. S10 for further EMSA analyses of the promoter sequences of *pceMN*/B_{12}, SHALO_1534, SHALO_1494, SHALO_1497, and TCS1.

Except for the promoters of TCS2 and SHALO_1504, the CREs overlap the -35 box. These class II promoters (38) lost the capacity to be bound by RR2 when either repeat, up- or downstream of the -35 box, was absent (Fig. S10). A result that disfavors binding of monomeric RR2. The intergenic region between TCS2 and SHALO_1504 does not have two separate CREs, but a single CRE in the center of this intergenic region that is located 85 bp upstream of both TSSs (Fig. 5B and D). This region also contains the three repeats of the CTATA motif with an additional C at the 5' end, respectively. In contrast to the class II promoters, the central repeat will not be bound by the RNA polymerase and is therefore available for binding RR2. When the intergenic region between TCS2 and SHALO_1504 was tested, the gel shift was larger compared to the other DNA-RR2 complexes indicating that probably more than a single RR2 dimer binds this CRE and promotes class I transcriptional activation. This assumption was supported by the fact that the shift was remarkably lower when the first CCTATA sequence at the CRE's 5' end was incomplete (Fig. 5D, f2). The repeat closest to the SHALO_1504 TSS, is essential for RR binding at all (Fig. D5, f9). A sequence alignment of the CRE of the class I with the six CREs of the class II promoters revealed a CTATW consensus sequence, which is separated by a gap of 17 bp (Fig. 6). The promoter sequences of the genes with a comparably low transcript level showed a higher divergence in this repeat. The promoters of the *ahpD*-like gene, p1494, as well as p*rdhAB* showed the highest discrepancy, which might be the reason for the weak interaction with RR2 in the EMSA in both cases. It might further explain the weak upregulation of SHALO_1494 in dehalogenating *Sulfurospirillum* spp. cultivated with PCE and the absence of RdhA in such cells. Taken together these findings, the data strongly support the assumption of a PCE-dependent regulon for OHR in *Sulfurospirillum* spp.

Fig. 6: Sequence alignment of the CREs in the OHR gene region. The class I (labeled with an asterisk) and class II promoter DNA sequences of all transcriptional units and the putative promoter sequence of the *rdhAB* genes are listed. The -35 box with the consensus sequence for the binding of the σ^{70} factor (RpoD) is given below (gray background). The suggested consensus sequence of the CREs was determined to be CTATW-N_{17}-CTATW. Nucleotides that fit to the consensus sequence are printed in bold. Direct repeats (≥ 3 nucleotides) that match the consensus sequences are underlined.

Discussion

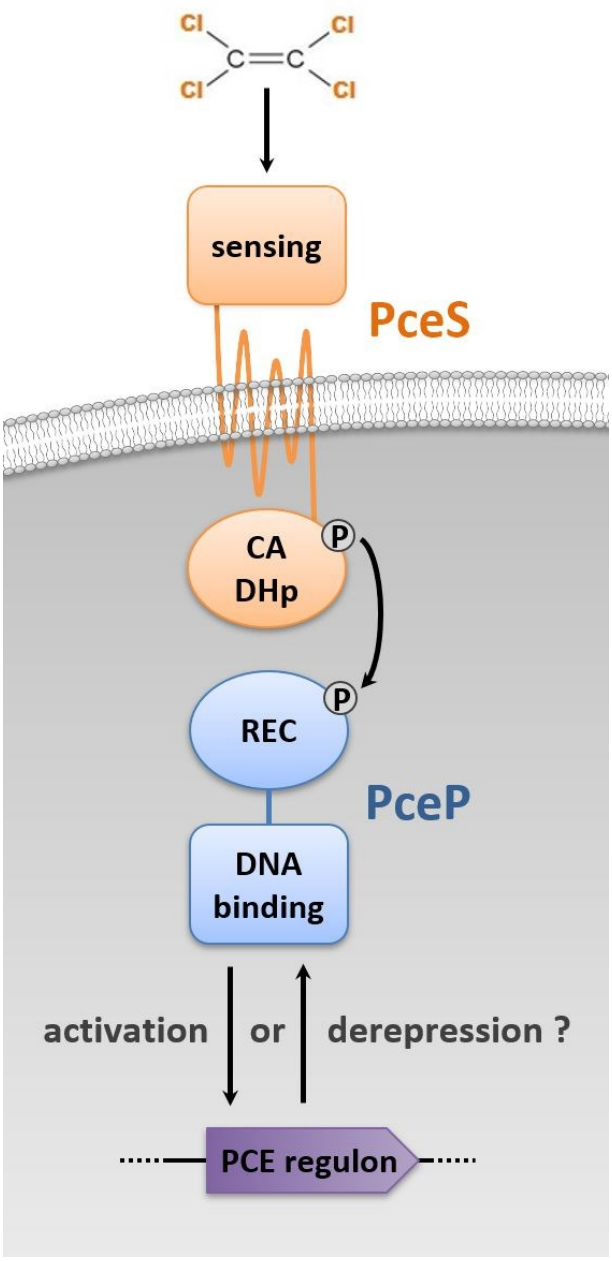
The expression of the OHR gene region in *Sulfurospirillum multivorans* is inducible by PCE or TCE (12). However, the cellular components responsible for PCE-sensing and regulation of OHR were not known. In this study, we used transcriptomics, comparative genomics, and molecular regulator binding studies to unravel the molecular details underlying PCE-dependent transcription initiation in *Sulfurospirillum* spp. In the genomes of *Sulfurospirillum multivorans* strain N and *S.* sp. JPD-1, which are unable to dechlorinate PCE, the response regulator gene of TCS2 was disrupted by transposase genes. While strain N was isolated as a non-dechlorinating strain, JPD-1 was initially characterized as PCE-dechlorinating (27), but might have lost this physiological trait during strain conservation. The insertion of transposase genes into RR2 might be a general evolutionary mechanism to shutdown PCE-dependent regulation in *Sulfurospirillum* spp., albeit more long-term population studies are necessary to confirm this hypothesis. The high conservation of the whole OHR gene region is unusual, but it is still difficult to postulate the underlying molecular mechanisms and to shed light on the reason for this invariability. A very recent horizontal gene transfer is ruled out by the geographical distance of the species' habitats, especially for JPD-1, which was isolated in North America, while the other *Sulfurospirillum* spp. were isolated in Europe. Also, the variability of *pceA* gene sequences among *Sulfurospirillum* spp. argue against a very recent gene transfer. This evolutionary hotspot might reflect an adaptation of the enzyme's substrate range to a certain ecophysiological role as observed in the two populations of '*Candidatus* S. diekertiae' (11). Small changes in the amino acid composition of reductive dehalogenases may lead to a change in the substrate preferences (39, 40).

Using dRNA-seq, eight transcriptional units were identified within the OHR gene region in the transcriptome of the PCE-respiring *S. multivorans*. The different transcript levels were in accordance with the protein levels detected in proteome analyses of *S. multivorans* (8) and *S. halorespirans* (13) with the PCE reductive dehalogenase among the most abundant transcripts and proteins. Only a few transcripts outside the OHR gene region showed substantial differences in the abundance under Py_PCE versus Py_Fu growth conditions, which was also similar to results obtained in previous proteomic studies (8, 13). One example is Hsp20, which was discussed as a stress response protein before. While the detection of small membrane-integral proteins remains difficult in proteomic studies, the transcription of the corresponding genes was detectable in the RNA-seq data. For example, the expression of the small putative membrane protein encoded by SMUL_1540 with an unknown function was observed for the first time.

In the absence of PCE, the transcription of the OHR genes stopped, while the TCS2 operon was still transcribed at low level. This observation is in accordance with the detection of the corresponding gene products in *S. multivorans* and *S. halorespirans* cells cultivated in the absence of PCE (8, 13). These data further support the assumption that TCS2 is crucial for the transcriptional response to PCE in *Sulfurospirillum* spp. There is no indication that the functional TetR in *S. halorespirans* affects gene

regulation of the OHR gene region. The TCS2 genes do not colocalize with the reductive dehalogenase gene, the expression of which is regulated by TCS2. Such a situation is uncommon in organohalide-respiring bacteria studied so far (24). Other organohalide-respiring bacteria harbor different signaling systems that sense environmental halogenated organic compounds (22-24, 41), with TCS genes encoding putative cytoplasmic sensor variants present only in Dehalococcoidia (20). Whether the presence of organohalides is directly sensed by Dehalococcoidia or indirectly monitored via other signals such as the redox state of the cell is unknown (20, 21, 42). OHR regulation via membrane-associated TCSs is found in *Sulfurospirillum* spp. and probably in a few *Desulfitobacterium* species, in which TCS genes are encoded adjacent to unstudied *rdh* genes (22). Outside the genus *Sulfurospirillum*, TCS2 exhibits the highest protein sequence identity (histidine kinase: 35%; RR: 46%) to a TCS in a gene cluster with unknown function in *Arcobacter ebronesis* (Campylobacterota).

In accordance to the nomenclature of OHR genes (14), RR2 and the cognate histidine kinase encoded in the OHR gene region of *Sulfurospirillum* spp. were designated as PceP and PceS, respectively. In order to derive a first model for PCE sensing in the dehalogenating *Sulfurospirillum* spp. the domain structures of PceP and PceS were predicted (Fig. 7). PceS, contains a putative periplasmic N-terminal sensing domain and an extensive transmembrane domain with seven transmembrane helices. One of the two domains might be involved in the interaction with the hydrophobic PCE. The cytoplasmic C-terminus of PceS consists of the dimerization and histidine phosphotransfer domain (DHp) and the catalytic and ATP-binding domain (CA). PceP belongs to the OmpR-family and contains a receiver (REC) domain and a winged helix-turn-helix (wHTH) motif-containing DNA-binding domain (Fig. 7). The REC domain contains a highly conserved aspartate residue that is most probably phosphorylated during signal transduction. Although phosphorylation of PceP was not necessary for dimerization and DNA-binding *in vitro*, phosphorylation might catalyze dimer formation *in vivo*. From the results presented here, one cannot exclude that in the cases of the CREs overlapping the -35 boxes phosphorylation of RR2 leads to a decrease in the DNA-binding efficiency and results in derepression of gene transcription rather than activation. However, the upregulation of TCS2 transcription in the presence of PCE and the increase of the RR2 level in such cells strongly support the assumption of a class II transcriptional activation. *S. halorespirans* PceP differs from those of *S. multivorans* and '*Candidatus* S. diekertiae' SL2-1 and SL2-2 only in a single amino acid ($V215_{Sh}/I215_{Sm}$, $Y85_{ShSm}/H85_{CSd}$). Both conservative replacements are predicted to be exposed to the surface of the protein and the respective amino acid residues face away from the DNA-binding wHTH motif or the dimerization site (Fig. S9). Hence, an influence on protein function is unlikely and the results obtained for PceP of *S. halorespirans* might also be valid for the corresponding RR2 proteins in the other dehalogenating *Sulfurospirillum* spp.



Unusual for two-component RRs, the OmpR-like regulator of TCS2 seems to promote both class I and class II transcriptional activation. In the case of pTCS2/p1504, when PceP binds the CRE 85 bp upstream of the TSSs, it is predicted to promote transcription initiation by recruiting the RNA polymerase comparable to other OmpR-family regulators (43). When it binds the CREs overlapping the -35 box, PceP is predicted to interact with the sigma factor and assist the isomerization of an initially closed into an open complex of RNA polymerase and promoter DNA. The DNA sequences adjacent to the -35 box of pTCS2, which are not involved in binding PceP, differ from the sequences of the class II promoters (Fig. S5). The basal transcription of the TCS2 operon might be a result of these promoter sequence variations. Further studies including the mutagenesis of the CRE sequences will help to understand the reason for the basal transcription of TCS2 and the role of the CRE sequence variations in fine-tuning OHR gene expression in the PCE regulon.

Fig. 7: Tentative scheme of the signal transduction by TCS2, which is essential for PCE-induced OHR in *Sulfurospirillum* spp.

Experimental Procedures

Cultivation of *S. multivorans*

S. multivorans (DSMZ 12446^T) was cultivated under anaerobic conditions at 28°C in a defined mineral medium (3) without vitamin B_{12} (cyanocobalamin) or yeast extract. Pyruvate (40 mM) was used as electron donor and fumarate (40 mM) or PCE as electron acceptor. PCE was added to the medium (10 mM nominal concentration) from a hexadecane stock solution (0.5 M). Pre-cultures were grown in rubber-stoppered 200 ml glass serum bottles and the main cultures in rubber-stoppered 2 l glass bottles. The ratio of aqueous to gas phase was always 1:1. In order to generate *S. multivorans* cells with down-

regulated *pceA* gene expression (12), the organism was cultivated for 60 transfers on pyruvate (40 mM) and fumarate (40 mM). The resulting culture was used as inoculum for all pre-cultures. The bacterial growth was photometrically monitored by measuring the optical density at 578 nm.

Genome sequencing, assembly and annotation, *in silico* sequence analysis

The genomes of *Sulfurospirillum multivorans* strain N and *Sulfurospirillum* sp. JPD-1 were sequenced by Macrogen, Seoul, Korea using PacBio RSII 4.0 chemistry. The genome sequencing depth of the genomes was 180-fold for strain N and 169-fold for JPD-1. HGAP v3.0 was used as assembler (with default parameters). Genomes were annotated with *S. multivorans* as reference annotation (CP72001) using Prokka (44) and the annotation was manually refined. Topology prediction of membrane proteins was performed using TOPCONS (45). Protein motif prediction was performed with CD search (46) and INTERproScan (47). The secondary structures were predicted using the Predict a Secondary Structure server (v. 6.0.0) (48).

Isolation of RNA

RNA was isolated from *S. multivorans* cells harvested after four transfers on pyruvate/PCE-containing medium at an $OD_{578} \approx 0.26$ and after 64 transfers on pyruvate and fumarate (40 mM each) at an $OD_{578} \approx 0.43$. The cell suspension (PCE-cultivated cells: 30 ml, fumarate-cultivated cells: 18 ml) was mixed by inversion with 1/6 volume of 95% v/v ethanol / 5% v/v Roti-Aqua-phenol followed by 10 min centrifugation at 1,700 x g at 4 °C. The cells were snap-frozen in liquid nitrogen and stored at -80 °C.
Frozen cell pellets were thawed on ice and resuspended in 600 µl lysis solution containing 0.5 mg/ml lysozyme in TE buffer, pH 8.0, and 60 µl 10% SDS. The cells were lysed by incubating the samples for 1-2 min at 64 °C. After the incubation, 1 M NaOAc, pH 5.2 (66 µl) was added and the sample was mixed by inversion. Total RNA was extracted by adding 750 µl phenol and using the hot-phenol method. The solution was mixed by inversion and incubated for 6 min at 64 °C. Afterwards, the samples were mixed 6-10 times by inversion and cooled on ice. After centrifugation for 15 min at 14,000 x g at 4 °C the aqueous layer was transferred and the chloroform extraction was performed in a 2 ml Phase Lock Gel tube (Eppendorf, Hamburg, Germany). 750 µl chloroform was added and mixed by inversion. After centrifugation for 12 min at 14,000 x g and 15 °C, the aqueous layer was used for the ethanol precipitation. To the RNA containing sample, 0.1 volume 3 M NaOAc, pH 5.2 and two volumes of 96% ethanol (-20 °C) were added. The sample was incubated for 2 h at -20 °C. Ethanol was discarded after centrifugation for 20 min at 14,000 x g and 4 °C. The RNA was washed once with 200 µl 70% ethanol (-20 °C). Ethanol was removed and the RNA was used for sequencing.
In order to prepare RNA samples for RT-qPCR, $\approx 1 \times 10^9$ *S. multivorans* cells were harvested during the exponential growth phase ($OD_{578} \approx 0.15$). Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The DNA was digested with recombinant DNase I (RNase free, Roche,

Mannheim, Germany) in the presence of RNase inhibitor (RiboLock, Thermo Scientific, Schwerte, Germany).

Reverse transcription-PCR (RT-PCR)

The OneStep RT-PCR kit (Qiagen, Hilden, Germany) was used. The reaction mixture contained 5 µl 5x reaction buffer, 25 pmol reverse primer, 25 pmol forward primer, 1 µl 10 mM dNTP mix, 1 µg total RNA or 70 ng *S. halorespirans* genomic DNA as positive control, 1 µl enzyme mix and nuclease free water up to a final volume of 25 µl. As negative control nuclease free water was added instead of nucleic acid. The reaction mixture was incubated for 1 h at 50 °C followed by a PCR with an initial denaturation of 95 °C for 15 min, followed by an indicated number of cycles of denaturation for 1 min at 94 °C, annealing for 30 s at 50 °C and elongation for 1 min at 72 °C. The final elongation lasted 10 min. The amplified DNA was separated on a 2% agarose gel and stained with ethidium bromide. The primers used for RT-PCR analysis are listed in Tab. 2. The 16S cDNA was diluted 1:10.000 before PCR.

Tab. 2: List of oligonucleotides used in this study.

Oligo	Sequence (5' -> 3')	Gene	Name
T563	CCGGCCTGCAACACATGAGCCTAA	SMUL_1574	
T564	CCGGAAGGAAGGCTTTTCCCTTCA	SMUL_1574	
T598	GAGACACGGTCCAGACTCCTAC	SMUL_2335/SMUL_3269 SHALO_2082/SHALO_2989	*rrsA1/rrsA2*
T599	CTCGACTTGATTTCCAGCCTAC	SMUL_2335/SMUL_3269 SHALO_2082/SHALO_2989	*rrsA1/rrsA2*
T732	CGAATTAATTATACAAACCATACTA	SMUL_1573	
T736	CATTGAAACAATGAATAAAACAGC	SMUL_1542	
T737	AAGACATTGTATTAATGCAACGTG	SMUL_1542	
T738	CCGTTAGAACAATATTTTTTTCTG	SMUL_1543	*cbiB*
T739	GCAAAAAAAGCTATTAATGCGG	SMUL_1543	*cbiB*
T776	TTAATGTATTTGTAAAACAGCAC	SMUL_1568	*sirC*
T777	TTCCCAAAAAAGTTCCATTGC	SMUL_1569	
T779	GGTGAGCCGGATACTCC	SMUL_1573	
T886	TATGCAGAAGATGATGCAGG	SMUL_1539	*pceP*
T887	GTAGCCATGACAATCGGG	SMUL_1539	*pceP*
T888	TATATGTTGAAAAATCCTAATAGAG	SMUL_1539	*pceP*
T889	TGAAGAACCAGCTTGTATCC	SMUL_1539	*pceP*

Reverse transcription-quantitative real-time PCR (RT-qPCR)

The RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Schwerte, Germany) was used for the reverse transcription. The reaction mixture contained 3.5 µl 5x reaction buffer, 25 pmol reverse primer, 2 µl 10 mM dNTP mix, 1 µg total RNA and nuclease free water up to a final volume of 17.5 µl. 10 µl were transferred into a new PCR tube and 0.5 µl reverse transcriptase was added. The remaining mixture was used as a reverse transcriptase minus (RT-) negative control to assess for genomic DNA contamination in the RNA sample. The reaction mixtures were incubated for 1 h at 42 °C plus 5 min at 70 °C in order to stop the RT reaction. The qPCR was performed in technical triplicates using a CFX96

qPCR machine (Bio-Rad, Munich, Germany). Each reaction mixture contained 6 µl 2x Maxima SYBR green qPCR master mix (Fermentas, St. Leon Rot, Germany), 5 pmol of both, forward and reverse primer, and finally 2.5 µl cDNA sample, 175 ng *S. halorespirans* genomic DNA as positive control or nuclease free water as negative control, respectively. The mixtures were filled up to a final volume of 12 µl with nuclease free water. The initial denaturation was performed for 3 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 50 °C and extension for 30 s at 72 °C. Directly after qPCR melting curves were measured and the transitions were checked for primer dimer formation and wrong PCR products. The primers used for RT-qPCR analysis are listed in Tab. 3.

Tab. 3: List of oligonucleotides used in this study.

Oligo	Sequence (5' -> 3')	Gene	Name
T598	GAGACACGGTCCAGACTCCTAC	SMUL_2335/SMUL_3269 SHALO_2082/SHALO_2989	*rrsA1/rrsA2*
T599	CTCGACTTGATTTCCAGCCTAC	SMUL_2335/SMUL_3269 SHALO_2082/SHALO_2989	*rrsA1/rrsA2*
T774	AATATTGCAAAAACATTAGAAGAG	SMUL_1540	
T775	ATTAAAAATTTTCTTCGATTAAAATTC	SMUL_1541	
T776	TTAATGTATTTGTAAAACAGCAC	SMUL_1568	*sirC*
T777	TTCCCAAAAAAGTTCCATTGC	SMUL_1569	
T778	AACCATACTAGCACTGCTC	SMUL_1572	
T779	GGTGAGCCGGATACTCC	SMUL_1573	

cDNA library preparation and sequencing

Terminator exonuclease (TEX) treatment of RNA samples was performed as previously described (31). The cDNA libraries for Illumina sequencing were constructed by Vertis Biotechnology AG, Germany (http://www.vertis-biotech.com/) in a strand-specific manner as previously described for eukaryotic microRNA (49) but omitting the RNA size-fractionation step prior to cDNA synthesis.

In brief, the RNA samples were poly(A)-tailed using poly(A) polymerase. Terminator exonuclease treatment (+TEX) and mock treatment without the enzyme (-TEX) were carried out after poly(A)-tailing. In this way, corresponding cDNA pairs were generated. Then, the 5'PPP structures were removed using tobacco acid pyrophosphatase (TAP). Afterwards, an RNA adapter was ligated to the 5'-monophosphate of the RNA. First-strand cDNA synthesis was performed using an oligo(dT)-adapter primer and the M-MLV reverse transcriptase. The resulting cDNAs were PCR-amplified to about 10-20 ng/µl using a high-fidelity DNA polymerase. The cDNAs were purified using the Agencourt AMPure XP kit (Beckman Coulter Genomics) and were analyzed by capillary electrophoresis.

For Illumina sequencing, samples were pooled in approximately equimolar amounts. The cDNA pool was size fractionated in the size range of 150-600 bp (replicate A) or 200-600 bp (replicate B) using a differential clean-up with the Agencourt AMPure kit. An aliquot of the cDNA pool was analyzed by capillary electrophoresis.

The primers used for PCR amplification were designed for TruSeq sequencing according to the instructions of Illumina. The following adapter sequences flank the cDNA inserts:
TruSeq_Sense_primer:
5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'
TruSeq_Antisense_NNNNNN_primer
Barcode:
5'-CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC(dT25)-3'
The combined length of the flanking sequences is 146 bases. All libraries were sequenced on an Illumina HiSeq 2500 machine with 100 cycles in single-end mode.

Computational analysis of dRNA-seq data

To assure high sequence quality, Illumina reads in FASTQ format were quality trimmed with a cutoff Phred score of 20 using fastq_quality_trimmer (FASTX toolkit version 0.0.13, http://hannonlab.cshl.edu/fastx_toolkit/) discarding reads without any remaining bases. High-quality reads were converted to FASTA format via fastq_to_fasta (FASTX toolkit).

Afterwards, we applied the pipeline READemption (50) version 0.3.5 for trimming of poly(A)-tail sequences and to align all reads longer than 11 nt to the *Sulfurospirillum multivorans* DSM 12446^T (GenBank Acc.-No.: CP007201.1) genome using segemehl version 0.2.0 (51) with an accuracy cut-off of 95%.

To facilitate visualization in a genome browser, coverage plots representing the numbers of mapped reads per nucleotide were generated. Reads that mapped to multiple locations contributed a fraction to the coverage value. For example, reads mapping to three positions contributed only 1/3 to the coverage values. Each graph was normalized to the number of reads that could be mapped from the respective library. To restore the original data range, each graph was then multiplied by the minimum number of mapped reads calculated over all libraries.

We applied READemption to assess the overlap of read alignments for each library to GenBank annotations for CDS, tRNA, rRNA and ncRNA features (CP007201.1, download on 2015-09-22) on the sense strand. Each read with a minimum overlap of 10 nt was counted with a value based on the number of locations where the read was mapped. If the read overlapped more than one annotation, the value was divided by the number of regions and counted separately for each region (e.g., 1/3 for a read mapped to 3 locations). The resulting read counts were subjected to differential expression analysis of Py_PCE vs. Py_Fu total RNA samples (-TEX) using DESeq2 (52) version 1.8.1 via READemption. All features with log2FoldChange ≤ -1 or ≥ 1 and Benjamini Hochberg-corrected p-values (padj) < 0.05 were con-

sidered significantly differentially expressed. The Integrated Genome Browser (33) was used for the dRNA-seq data evaluation.

Plasmid construction, strains and medium

The gene encoding PceP (SHALO_1503) was amplified from *S. halorespirans* genomic DNA using the cloning primers listed in Tab. 4, digested with the restriction enzyme Esp3I and ligated to the multiple cloning site of pASG-IBA105 (IBA, Göttingen, Germany). The construct was verified by sequencing across the inserts using the sequencing primers listed in Tab. 4. *E. coli* DH5α was used for plasmid proliferation. *E. coli* BL21(DE3) was used for PceP-twin-Strep overproduction. Both strains were cultivated in lysogeny broth (LB) containing 100 µg/ml ampicillin. For gene expression the cultivation temperature was set to 28 °C and decreases to 18 °C after induction with 100 µg/l anhydrotetracycline. The cells were harvested after 1 h.

Tab. 4: List of oligonucleotides used in this study.

Oligo	Sequence (5' -> 3')	Gene	Name
	Cloning primer pair		
T906	AGCGCGTCTCCAATGTTTAAAAACTATAAAGTAC TTTATGCAGAAG	SHALO_1503	*pceP*
T907	AGCGCGTCTCCTCCCTCATTTTTGAAGAACCAGC	SHALO_1503	*pceP*
	DNA sequencing primers		
T954	GAGTTATTTTACCACTCCCT	pASG-IBA105 (SHALO_1503)	*pceP*
T955	CGCAGTAGCGGTAAACG	pASG-IBA105 (SHALO_1503)	*pceP*

Electrophoretic mobility shift assay (EMSA)

Twin-Strep tagged PceP protein was purified on Strep-Tactin XT Superflow columns (IBA, Göttingen, Germany) using the standard protocol provided by the manufacturer. The purity was proven by Coomassie- or silver-stained polyacrylamide gels. Silver staining comprised fixation for 20 min (50% (v/v) methanol, 10% (v/v) acetic acid, 10 mM ammonium acetate), followed by two washing steps à 10 min with ultra-pure water, sensitization for 20 min (20 µM sodium thiosulfate), staining for another 20 min (0.6 mM silver nitrate), rinsing with ultra-pure water, approx. 1 min developing (0.1% (v/v) formaldehyde, 20 mM sodium carbonate), and finally stopping for 10 min (50 mM EDTA).

The 6FAM-labeled double-stranded DNA probes contain the entire promoters or truncated sequences and were amplified from *S. halorespirans* genomic DNA using the indicated primers (Tab. 5). EMSA reactions (15 µl) contained 2.5 pmol DNA probe, 250 ng (≈ 0.1 pmol) poly (dI/dC) and the indicated amounts of PceP-twin-Strep in 1x reaction buffer (10 mM HEPES, pH 7.6, 2 mM $MgCl_2 \cdot 6\ H_2O$, 200 mM KCl, 0.1 mM EDTA, 5 mM DTT, 10% (v/v) glycerol, 50 µg/µl Ficoll 70). The reaction was performed at room temperature for 20 min. The reaction mixtures were separated using a protocol

adapted from Sidorova *et al.* (53). The native 10% polyacrylamide gels (acrylamide:bis-acrylamide = 29:1) contained 30% (v/v) triethylene glycol in 1x TAE buffer (40 mM Tris, pH 8.3, 20 mM acetic acid, 2 mM EDTA). 20 µl of 30% (v/v) triethylene glycol in 1x TAE was added to the sample wells as soon as the gel was immersed in the electrophoresis buffer in order to minimize loss of the osmolyte. The gels were run at 200 V for 2 h and were scanned with a Typhoon FLA 7000 device (GE Healthcare) with a 473 nm LD laser and an Y520 filter.

Tab. 5: List of oligonucleotides used in this study.

Oligo	Sequence (5' -> 3')	Gene	Name
T599	CTCGACTTGATTTCCAGCCTAC	SHALO_2082/ SHALO_2989	*rrsA1/rrsA2*
T898	6-FAM-GAGACACGGTCCAGACTCCTAC	SHALO_2082/ SHALO_2989	*rrsA1/rrsA2*
T982	6-FAM-GCCTTTAGTTCCAGATAAGCCTATCGAC	SHALO_1495	*pceA*
T607	GGCCCGCCACAATATCCACCAGAT	SHALO_1495	*pceA*
T983	6-FAM-TTGTATATTTTATTAATATTAGCTTATAAATG	pSHALO_1552	
T984	CATTAAATTCTTAATTACATCATTATATC	pSHALO_1552	
T890	6-FAM-TTAAGAAAAAGTTATAGATGTAGAT	pSHALO_1494	
T719	CATATTCTATCCTTATTTTTAAAAT	pSHALO_1494	
T787	6-FAM-ATTGTCTGATATATTGACAAAATCT	pSHALO_1495	p*pceA* (x4)
T721	CATACTTCCTCCTTAAAAAATACC	pSHALO_1495	p*pceA* (x1)
T891	6-FAM-CAAAATTTATCATTGATCTTAGCC	pSHALO_1497	
T723	CATAGTGTTTATTCCTTTAGTTATA	pSHALO_1497	
T892	6-FAM-CCTTCTTGTAACCTTTAATTAAATG	pSHALO_1499	pTCS1
T725	CATGTTAAATCCCTGAAATTTTATG	pSHALO_1499	pTCS1
T893	6-FAM-AGTACATAAAACTTGTAGTAACTTT	pSHALO_1503	pTCS2 (x1-5)
T727	CATGTGTGAACCTATCCTATAG	pSHALO_1503	pTCS2
T894	6-FAM-TCTATCAGATATTTTGTTAAAAATT	pSHALO_1504	(x6-10)
T729	CATGTCGTTCCTTTATTTTTG	pSHALO_1504	
T895	6-FAM-CACATAATGAAGGACTTTATGATAA	pSHALO_1505	p*pceMN*/B12 (x1-2)
T731	CATCTTTTTCTCTCTTAAAGATTGT	pSHALO_1505	p*pceMN*/B12
T896	6-FAM-CGAATTAATTATACAAACCATACTA	pSHALO_1534	
T733	CATTTGTTCTTCCTTCTTTGTTATA	pSHALO_1534	(x1-2)
T897	6-FAM-CTGCATAGAGTATTGTATAAGAAG	pSHALO_1500	p*rdhAB*
T735	CATATTTACTCCTTAAAAAATCATC	pSHALO_1500	p*rdhAB*
T986	TAAAAGAGTAAAAATTATCTATTTAATAG	pSHALO_1503/1504	pTCS2/SHALO_1504x5
T987	AATAGTTACCCTATATTTCACCT	pSHALO_1503/1504	pTCS2/SHALO_1504x4
T991	CCCTATATTTCACCTATAAGTTA	pSHALO_1503/1504	pTCS2/SHALO_1504x3
T992	TTCACCTATAAGTTACCTATAAATA	pSHALO_1503/1504	pTCS2/SHALO_1504x2
T993	TAAGTTACCTATAAATATTCATAAA	pSHALO_1503/1504	pTCS2/SHALO_1504x1
T989	GCAATAAAGAGAGAACCTTG	pSHALO_1503/1504	pTCS2/SHALO_1504x6
T990	TTATGAATATTTATAGGTAACTTAT	pSHALO_1503/1504	pTCS2/SHALO_1504x7
T994	ATTTATAGGTAACTTATAGGTG	pSHALO_1503/1504	pTCS2/SHALO_1504x8

Oligo	Sequence (5' -> 3')	Gene	Name
T995	GTAACTTATAGGTGAAATATAG	pSHALO_1503/1504	pTCS2/SHALO_1504x9
T996	TAGGTGAAATATAGGGTAACT	pSHALO_1503/1504	pTCS2/SHALO_1504x10
T1006	6-FAM-CTATAACTTTTGTATATCATTC TTAAAAT	pSHALO_1495	p*pceAB*x1
T1005	ATATTATTAACTTTCATTATTTTAAAA TTAAA	pSHALO_1495	p*pceAB*x2
T1007	6-FAM-ATTTTAAGAATGATATACAAA AGTTATAG	pSHALO_1495	p*pceAB*x2
T1002	AAAGAAAAGTATTTACTTATTACTTC	pSHALO_1495	p*pceAB*x3
T997	6-FAM-CTTTGTTATAGGAAAGATATA GATT	pSHALO_1495	p*pceAB*x3
T998	GAAAGTTAATAATATTTACGAAGTA	pSHALO_1495	p*pceAB*x4
T1009	TTTTATTCATCTATTTTCTCCGCA	pSHALO_1505	p*pceMN*/B12x3
T1010	CCGCACAACTTATCTATTTTTTTTT	pSHALO_1505	p*pceMN*/B12x4
T1008	6-FAM-CATCTTTTTCTCTCTTAAAGAT TGT	pSHALO_1505	p*pceMN*/B12 (x3-4)
T1011	AGAAAAAAAAATAGATAAGTTGTGCG	pSHALO_1505	p*pceMN*/B12x2
T1012	TTGTGCGGAGAAAATAGATGAATA	pSHALO_1505	p*pceMN*/B12x1
T1014	TGGAACAAATATTCCAGAATACGT	pSHALO_1534	pSHALO_1534x3
T1015	CTCCACAAGTTACCTATATATTTT	pSHALO_1534	pSHALO_1534x4
T1013	6-FAM-CATTTGTTCTTCCTTCTTTGTTA TA	pSHALO_1534	(x3-4)
T1016	AATGAAAATATATAGGTAACTTGTGG	pSHALO_1534	pSHALO_1534x2
T1017	TGTGGAGTAAAAGTAGATAATCAC	pSHALO_1534	pSHALO_1534x1

Data availability

RNA-seq data are available at NBCI Gene Expression Omnibus (GEO) (54) under accession number GSE139083. The genomes of *S. multivorans* strain N and *S.* sp. JPD-1 are available at NCBI under the GenBank accession numbers CP042966 and CP023275, respectively.

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2.2 A Retentive Memory of Tetrachloroethene Respiration in *Sulfurospirillum halorespirans* – involved Proteins and a possible link to Acetylation of a Two-Component Regulatory System

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This publication provides proteomes and acetylomes of *S. halorespirans* cultivated under dehalogenating and non-dehalogenating conditions. Similar to *S. multivorans*, this organism is subject to a retentive memory effect in terms of a long-term downregulation of the OHR gene region once PCE is depleted. Protein lysine acetylations especially during adaptation to the non-halogenated substrate indicate a regulatory role of this modification.

My own contribution to this manuscript covers about 40%.

This publication resulted from a fruitful cooperation with Dominique Türkowsky at the Helmholtz Centre for Environmental Research – UFZ in Leipzig. Together we planned the study conception, analyzed the data, and wrote the manuscript. While I was conducting the cultivations, sampling, activity assays, Western blots, and the structural modeling of TCS2, Dominique Türkowsky performed the sample preparations and the LC-MS analyses. All co-authors reviewed the manuscript before submission.

For supplementary information see appendix, pp. xxiii - xxvii

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A Retentive Memory of Tetrachloroethene Respiration in *Sulfurospirillum halorespirans* - involved Proteins and a possible link to Acetylation of a Two-Component Regulatory System

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ABSTRACT

Organohalide respiration (OHR), comprising the reductive dehalogenation of halogenated organic compounds, is subject to a unique memory effect and long-term transcriptional downregulation of the involved genes in *Sulfurospirillum multivorans*. Gene expression ceases slowly over approximately 100 generations in the absence of tetrachloroethene (PCE). However, the molecular mechanisms of this regulation process are not understood. We show here that *Sulfurospirillum halorespirans* undergoes the same type of regulation when cultivated without chlorinated ethenes for a long period of time. In addition, we compared the proteomes of *S. halorespirans* cells cultivated in the presence of PCE with those of cells long- and short-term cultivated with nitrate as the sole electron acceptor. Important OHR-related proteins previously unidentified in *S. multivorans* include a histidine kinase, a putative quinol dehydrogenase membrane protein, and a PCE-induced porin. Since for some regulatory proteins a posttranslational regulation of activity by lysine acetylations is known, we also analyzed the acetylome of *S. halorespirans*, revealing that 32% of the proteome was acetylated in at least one condition. The data indicate that the response regulator and the histidine kinase of a two-component system most probably involved in induction of PCE respiration are highly acetylated during short-term cultivation with nitrate in the absence of PCE.

Significance: The so far unique long-term downregulation of organohalide respiration is now identified in a second species suggesting a broader distribution of this regulatory phenomenon. An improved protein extraction method allowed the identification of proteins most probably involved in transcriptional regulation of OHR in *Sulfurospirillum* spp. Our data indicate that acetylations of regulatory proteins are involved in this extreme, sustained standby-mode of metabolic enzymes in the absence of a substrate. This first published acetylome of Epsilonproteobacteria might help to study other ecologically or medically important species of this clade.

1. Introduction

The anaerobic respiration with halogenated compounds, called organohalide respiration (OHR), is of importance for bioremediation and the global halogen cycle. OHR relies on a reductive dehalogenase as terminal reductase [1]. The molecular mechanisms underlying OHR, including its regulation, are unresolved to a great extent. The Epsilonproteobacterium *Sulfurospirillum multivorans* is able to reductively dehalogenate the environmentally harmful but widely distributed degreasing agent tetrachloroethene (PCE) to *cis*-dichloroethene. This is catalyzed by the PCE reductive dehalogenase (PceA). When *S. multivorans* is cultivated in the absence of PCE, PceA is subject to a memory effect: PceA is still produced, but during a long-term downregulation gene expression ceases slowly within approximately 100 generations [2]. A similar long-term loss of PCE respiration was observed in *Desulfitobacterium hafniense* strains Y51, PCE-S, and TCE1 [3–5]. However, in these *D. hafniense* strains, the loss of PCE respiration was not due to a regulatory effect but a transposon-mediated loss of the *pce* gene cluster in the majority of the bacterial population after cultivation in the absence of PCE [3,5]. In *S. multivorans*, the *pceA* gene is still present after

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long-term cultivation without PCE but is not transcribed anymore [2]. The *pceA* transcription can be induced again by PCE. The *pceA* gene of *S. multivorans* is embedded in a large gene region encoding putative electron-transfer proteins for the PCE respiratory chain and proteins necessary for the production of its cobamide cofactor (norpseudo-B_{12}) [6]. Evidence was obtained that this gene region undergoes concerted, PCE-induced transcriptional regulation [6,7]. Until recently, *S. multivorans* was the only organohalide-respiring Epsilonproteobacterium with a sequenced genome, hindering global comparisons of PCE respiration and its regulation on a genetic basis so far. Therefore, the genome of a second PCE-respiring Epsilonproteobacterium, *Sulfurospirillum halorespirans*, was sequenced and described [8]. Overall, both organisms show similar genome features, although unlike *S. multivorans* and unusual for an organohalide-respiratory bacterium, *S. halorespirans* harbors *nos* and *sox* proteins, involved in nitrous oxide respiration and thiosulfate oxidation, respectively. The OHR gene region of *S. halorespirans* displays nearly 100% nucleotide sequence identity compared to *S. multivorans*, with three exceptions: (1) The *pceA* gene is only 95% identical to that of *S. multivorans*, (2) 106 nucleotides that might contain a small ORF are present upstream of the norpseudo-B_{12} (*cbi*) biosynthesis gene cluster in *S. halorespirans* and (3) an intact *tetR*-like repressor gene downstream of this *cbi* cluster is interrupted by a transposase in *S. multivorans*. The latter two features and a PceA with an even lower degree of sequence identity distinguish also the OHR regions of the recently sequenced *Sulfurospirillum* sp. JPD-1 (accession number CP023275) and Candidatus *Sulfurospirillum diekertiae* [9] from *S. multivorans*. Further regulators encoded in the OHR region of both organisms are two two-component regulatory systems (TCSs). The first, TCS I, is located downstream of *pceAB*. A second, similar TCS, TCS II, is encoded downstream of a second reductive dehalogenase gene set (*rdhAB*). Both TCSs are formed by two multidomain proteins, a histidine protein kinase (HK) and a response regulator (RR). The HKs consist of a periplasmic N-terminal domain putatively involved in ligand binding, a transmembrane domain with seven transmembrane helices, a dimerization/histidine phosphotransfer domain and a catalytic and ATP-binding domain [6]. The RRs harbor a receiver domain as well as a winged helix turn helix (HTH) motif and might be involved in transcriptional activation of the OHR regulon. The role of the second reductive dehalogenase is up to now unknown and its expression was never observed regardless of the substrate tested [7]. However, the TCS II was described as a candidate for PCE-sensing, because the RR (SMUL_1539) was detected in the proteome of *S. multivorans* cells cultivated with and without PCE [7], which is opposed to the TCS I, which has never been detected in proteome studies. Besides OHR region genes, the expression of which was highly upregulated by PCE, only a few genes in *S. multivorans* were found to undergo regulation in the presence of PCE. Among them, stress-induced proteins such as an Hsp20 family protein might play a role in a specific stress response in *S. multivorans* [7].

The trigger which retains expression of the OHR-related genes in the absence of PCE is still unknown. Post-translational modifications (PTMs) of proteins are important regulators, but their role has been underestimated in bacteria for a long time. One important PTM in bacteria with diverse functions is the acetylation of proteins. Proteins get reversibly modified by an interplay of acetyltransferases and deacetylases or non-enzymatically by acetylphosphate [10]. By neutralizing the positive charge of lysine, acetylations may alter local or global protein structure, which can have an impact on the protein's stability, activity, localization or interaction with other proteins and other biomolecules. In bacteria, these processes were observed to play a role e.g. in the regulation of cell motility and shape, RNA degradation and gene expression [10].

The aim of our study was to show that the long-term downregulation of OHR is present in another *Sulfurospirillum* sp. and to get insight into its regulation by analyzing the proteome during different stages of the long-term downregulation. We hypothesized that lysine acetylations could play a role in the delay of downregulation in *Sulfurospirillum* species.

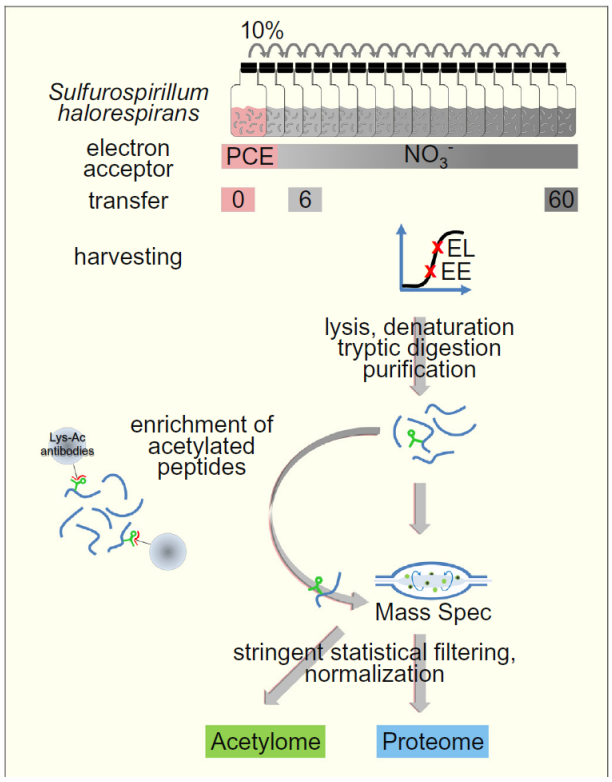

Fig. 1. Monitoring the proteomic and acetylation profile of *S. halorespirans* during cultivation on different electron acceptors. Three biological replicates of *S. halorespirans* were cultivated with PCE/pyruvate (t0, transfer 0) and subsequently on nitrate/pyruvate (t1-t60). Each culture was inoculated with 10% of a culture in exponential phase. Protein was extracted and digested from t0, t6 and t60 during early (EE) or late exponential (EL) growth phase. A subfraction of the peptide digest was subjected to acetylpeptide enrichment. Peptide and acetylpeptide digest were analyzed by LC-MS/MS, followed by bioinformatic analysis.

2. Methods

2.1. Cultivation of S. multivorans

S. halorespirans DSM 13726 (PCE-M2) was cultivated anaerobically at 28 °C in a defined mineral medium [11] in the absence of exogenous cobamide and yeast extract. Pyruvate (40 mM) was used as the electron donor and nitrate (40 mM) or PCE as the electron acceptor. PCE was added to the medium (10 mM nominal concentration) from a hexadecane stock solution (0.5 M). In order to generate *S. halorespirans* cells with downregulated *pceA* gene expression [2], the organism was cultivated for 60 transfers (10% inoculum each) with nitrate as the sole electron acceptor (Fig. 1). The inoculum corresponded to about 6 µg protein per mL medium. Each cultivation was performed in 100 mL glass serum bottles. For proteomic analyses, cells were cultivated in 1 L or 500 mL medium in rubber-stoppered 2 L or 1 L glass bottles, respectively. Cells were harvested during early ($OD_{578} \approx 0.11$) and late ($OD_{578} \approx 0.20$) exponential phase. The ratio of aqueous to gas phase was always 1:1. The bacterial growth was monitored photometrically by measuring the optical density at 578 nm. All cultivations were performed in triplicates.

2.2. Cell harvest, disruption and PceA activity assay

S. halorespirans cells were harvested from a 100 mL culture in the late exponential growth phase by centrifugation (12,000 × *g*, 10 min at 10 °C). Cell pellets were washed three times with 50 mM Tris-HCl (pH 7.5) to ensure the removal of PCE and nitrate. The cell pellets were transferred into an anoxic glove box and resuspended (1:2) in anoxic buffer (50 mM Tris-HCl, pH 7.5). An equal volume of glass beads (0.25–0.5 mm diameter, Carl Roth GmbH, Karlsruhe, Germany) was added and the cells were disrupted using a bead mill (5 min at 25 Hz; MixerMill MM400, Retsch GmbH, Haan, Germany). The crude extracts were separated from the glass beads by centrifugation (14,000 x g, 2 min) under anoxic conditions. The measurements of PceA activity were performed as described using a photometric assay with reduced methyl viologen as artificial electron donor [12].

2.3. Immunoblot analysis

Cells were harvested as described above. Protein concentration was determined using the Bio-Rad Bradford reagent (Bio-Rad, Munich, Germany) and bovine serum albumin as a protein standard. Soluble fractions (10 µg protein per lane) were subjected to denaturing SDS-PAGE (12.5%) and afterwards blotted onto a polyvinylidene difluoride (PVDF) membrane (Roche, Mannheim, Germany) using a semi-dry transfer cell (Bio-Rad, Munich, Germany) according to the protocol described by John et al. [2]. The PceA antiserum (primary antibody) was diluted 500,000-fold. The primary antibody was detected via a secondary antibody (diluted 1:30,000) coupled to alkaline phosphatase (Sigma-Aldrich, Munich, Germany).

2.4. Structural modeling of the two-component system

The structural models of the cytoplasmic domain of the HK and the RR were generated using the I-TASSER server for protein structure and function prediction [13,14]. The best threading templates used by the platform were the HK KinB of *Geobacillus stearothermophilus* with the inhibitor Sda (PDB ID 3D36, [15]) and the RR MtrA of *Mycobacterium tuberculosis* (PDB ID 2GWR, [16]). The acetylations were added to the structural model using the PyTMs plugin [17] of the PyMOL Molecular Graphics System [18].

2.5. Peptide preparation from lysed cells

S. halorespirans cells were harvested in the early and late exponential growth phases by centrifugation (12,000 × *g*, 10 min at 10 °C). The cell pellets were washed once in PBS buffer (140 mM NaCl, 10 mM KCl, 6.4 mM Na_2PO_4, and 2 mM KH_2PO_4). Protein extraction, digestion, and peptide purification were performed as described before [19]. Briefly, cells were dissolved in 8 M urea lysis buffer (20 mM HEPES, 8 M urea, 1 mM sodium vanadate, 1 mM β-glycerolphosphate, 2.5 mM sodium pyrophosphate) and lysed by four cycles of freeze/thaw/ultrasonic bath treatment. Cell debris was removed by centrifugation (15 min, 4 °C, 20,000 g), proteins were quantified by BCA-assay (Thermo Fisher Scientific, USA), reduced (4.5 mM dithiothreitol, 30 min, 55 °C) and alkylated (10 mM iodoacetamide, 15 min, room temperature, in the dark). Samples were diluted four-fold with 20 mM HEPES, pH 8, and 5.3 mg of each sample was digested overnight with 20 µg trypsin (Promega) and subsequently with 3.5 µg lysyl endopeptidase (Wako, Japan) for six hours. Digested peptides were acidified with 1% trifluoroacetic acid (TFA), desalted over SEP PAK Classic C18 columns (Waters, USA) and eluted using 40% acetonitrile in 0.1% TFA. 5% of the eluate was aliquoted for the proteome analysis; all samples were lyophilized and stored at −80 °C.

2.6. Immunoaffinity enrichment of lysine-acetylated peptides

Enrichment of acetylated peptides was conducted using the PTMScan Acetyl-Lysine Motif Kit (Cell Signaling Technology, USA) as described in [19]. Peptides were dissolved in IAP buffer and incubated with the antibody beads for 2 h at 4 °C. The beads were washed with IAP buffer and water and the peptides were eluted in two steps with 0.15% TFA. Non-modified and acetylated peptides were desalted by using C18 Zip Tip columns (Millipore, Germany), dissolved in 0.1% formic acid and injected into a liquid chromatography tandem mass spectrometer (LC-MS/MS).

2.7. Mass spectrometry

Separation of tryptic peptides was performed using a 120 min non-linear gradient from 3.2% to 40% acetonitrile, 0.1% formic acid on a C18 analytical column (Acclaim PepMap100, 75 µm inner diameter, 25 cm, C18, Thermo Scientific) in a UHPLC system (Ultimate 3000, Dionex/Thermo Fisher Scientific, Idstein, Germany). Mass spectrometry was performed on a Q Exactive HF MS (Thermo Fisher Scientific, Waltham, MA, USA) with a TriVersa NanoMate (Advion, Ltd., Harlow, UK) source in LC chip coupling mode. Mass spectrometer full scans were measured in the Orbitrap mass analyzer within the mass range of 400–1600 m/z, at 60,000 resolution using an automatic gain control target of 3×10^6 and maximum fill time of 50 ms. An MS/MS isolation window for ions in the quadrupole was set to 1.4 m/z. MS/MS scans were acquired using the higher energy dissociation mode at a normalized collision-induced energy of 28%, within a scan range of 200–2000 m/z and a resolution of 15,000. The exclusion time to reject masses from repetitive MS/MS fragmentation was set to 30 s.

2.8. Data analysis

The acquired MS spectra were processed in Proteome Discoverer (v1.4 and v2.1, Thermo Scientific). MS/MS spectra were searched against an *S. halorespirans* database containing 2965 non-redundant protein-coding sequence entries (downloaded December 2016 from NCBI Genbank accession number CP017111.1) using the SEQUEST HT algorithm. Enzyme specificity was selected as trypsin with up to two missed cleavages allowed for the proteome-analysis and four missed cleavages allowed for the acetylome-analysis. The latter yielded more acetylated peptide hits than applying two or three missed cleavages in the searches. Peptide ion tolerance was set to 10 ppm and MS/MS tolerance to 0.02 Da. Oxidation (methionine) was selected as a dynamic and carbamidomethylation (cysteine) as a static modification. A maximum of three equal and four dynamic modifications per peptide were allowed. Only peptides with a false discovery rate (FDR) < 0.01, calculated by Percolator, and XCorr > 2.1 were considered as identified. Quantification of proteins was performed using the average of top three peptide MS1-areas. Protein quantification was considered successful for proteins quantified in $> 50\%$ of biological replicates, otherwise, they were classified as identified proteins. After log10 transformation, the protein values were median-normalized and scaled, so that the global minimum is zero. Throughout the text protein abundances are given in relation to the median of all proteins of a condition, i.e. $> M + 2\sigma$ relates to proteins with a higher abundance value than the median plus two standard deviations. MS1-areas of acetylated peptides which only differ in their modification status (oxidation, carbamidomethylation) were summed and counted as one acetylation site. To correct acetylation differences for different protein amounts, acetylation abundance ratios were obtained by subtracting the lg-area of the most abundant acetylated peptide of a protein which was detected in at least two replicates from the logarithmized protein abundance values. For statistical analysis, data of proteins quantifiable in $\geq 50\%$ of replicates were imputed using Prostar (imp4p, 10 iterations, no Lapala, http://www.prostar-proteomics.org). Differential proteome analysis was performed

using a Limma moderated *t*-test, corrected with the Benjamini – Hochberg method at false discovery rate (FDR) < 0.05. *P*-values in the non-parametrical multiple dimensional scaling (nMDS)-plots were calculated with the anosim-function of the vegan package in R v. 3.4.1 [20,21]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (https://www.ebi.ac.uk/pride) partner repository with the dataset identifier PXD008953.

Orthologs with *S. multivorans* and *E. coli* were obtained by using BLAST reciprocal best hits on the Galaxy-platform (Minimum percentage identity 60% for *S. m.* and 35% for *E. c.*, Minimum percentage query coverage 50%) [22,23]. Data from Goris et al. [24] were re-analyzed using the same stringent criteria to ensure comparability. Areas of the membrane and cytoplasm fraction were median-transformed and summed. Protein localizations were calculated based on the amino acid sequence using psortb version 3.0.2 [25]. Protein functions were determined with Prophane [26]. Enrichment analyses were conducted using R packages clusterProfiler, dose and splitstackshape [27–29]. Figures were created using R packages ggplot2, vegan and pheatmap [30,31].

3. Results and discussion

3.1. Long-term downregulation of PCE respiration in Sulfurospirillum halorespirans

In order to assess whether *S. halorespirans* shows the same kind of long-term downregulation of *pceA* gene expression as *S. multivorans* [2,7], *S. halorespirans* was cultivated for 60 transfers with nitrate as the sole electron acceptor (Fig. 1). This corresponds to approximately 200 generations. The amount and specific enzyme activity of PceA in crude extract decreased to about 1% of the initial activity detected in the presence of PCE (Fig. 2A, B), similar to the values observed for *S. multivorans* with fumarate or nitrate as the electron acceptor [2,7].

We extracted the proteome of *S. halorespirans* cultivated with tetrachloroethene (PCE) as the electron acceptor (t0), and from cells that were cultivated for six (t6, short-term) or 60 transfers (t60 long-term cultivated) with nitrate as the electron acceptor. Cells were harvested at the early and late exponential phase (Fig. S1). Of the in total 18 samples (six conditions, three replicates), 2029 proteins could be identified (68% of the predicted proteome) and 1799 proteins were quantifiable (i.e., had an abundance value in > 50% of all replicates). This was a substantial improvement compared to a previous study on *S. multivorans* cultivated with different electron donors and acceptors [7], where 1716 proteins (53% coverage) were identified in 36 samples with less stringent filter criteria applied during proteome analysis. Of all identified proteins, 19% were predicted to be membrane-integral, according to classification by the localization prediction tool PSORTb [25] (Fig. S2A). This is closer to the theoretical value of 26% membrane-integral proteins than the 10% membrane-integral proteins identified in the study on *S. multivorans*. The distribution of protein sequence lengths of the measured proteome was almost identical to that of the predicted proteome, except for the underrepresented extremely small proteins of below 50 amino acids length (Fig. S2B). The bias against small proteins, however, was less than in the study on *S. multivorans*. This higher yield in protein identifications can be attributed to an optimized extraction protocol using harsher conditions with concentrated urea buffer and the combined use of trypsin and lysyl endopeptidase for a more efficient proteolytic cleavage [32] as opposed to *S. multivorans*, where a mild detergent (digitonin) was used. Consequently, our proteome dataset most likely represents the actual protein content of the cell closer than the previous study with *S. multivorans*.

3.2. Proteome dynamics during long-term cultivation without PCE

The number of identified proteins in samples gained from the three different subcultivation steps was similar (Fig. 3A). An nMDS was performed to assess the dissimilarity between protein abundances of the different time points. The results show a clear separation between the proteomes of t0, t6 and t60 cells, indicated by the reasonable variance and good reproducibility (Fig. 3C).

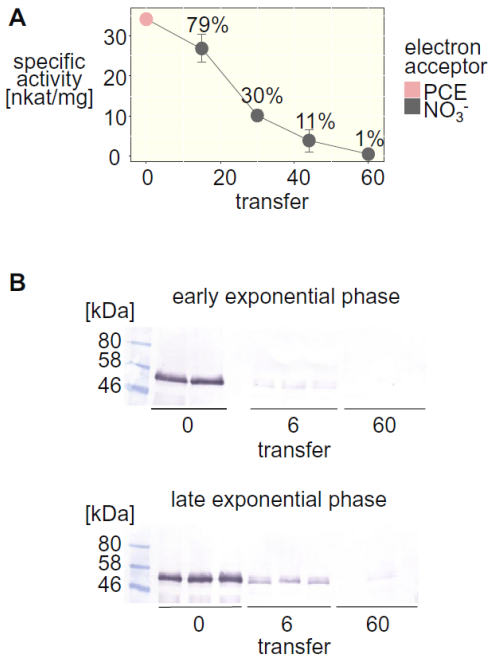

Fig. 2. (A) PCE dechlorination activity of PceA in crude cell extracts from *S. halorespirans*, measured photometrically with reduced methyl viologen as the electron donor. Activity presented from cells cultivated with PCE (t0, 100% activity) and from cells cultivated for 15, 30, 44 and 60 successive transfers to medium with nitrate as the sole electron acceptor. (B) Immunostaining of PceA in crude extracts from *S. halorespirans* cultivated with PCE (t0) or for six or 60 transfers with nitrate (t6, t60). 10 µg protein per lane applied to SDS-PAGE, subsequent blotting to a PVDF membrane. Biological replicates of the samples harvested after the transfers t0, t6 and t60 during the early (OD_{578} ≈ 0.11) and late exponential (OD_{578} ≈ 0.20) growth phase are shown.

Further, we compared the protein functions under the different growth conditions. In PCE-grown cells, proteins of coenzyme metabolism, cell motility, and amino acid metabolism were significantly more abundant than in nitrate-grown cells (Fig. 4A). The significantly upregulated functional classes in t6 and t60 were similar to each other. These proteins were particularly related to translation and energy conservation. In t60, also proteins of the categories cell motility and amino acid metabolism were enriched compared to t6 cells.

3.3. OHR gene region

Analogous to the situation in the *S. multivorans* proteome [7], the most striking difference between cells adapted to PCE and to nitrate was the expression of the genes of the OHR core region. Among the overall five highest expressed proteins on PCE was the reductive dehalogenase PceA (> M + 2σ, Table S1, S2, Fig. 5). It was among the 20 most differentially expressed proteins in PCE-grown cells compared to nitrate-grown cells (Table S3A), with downregulation in t6 to 11% of its original abundance and no quantifiable enzyme in t60. This is comparable to *S. multivorans*, where PceA had 0.1% of its original abundance in t60. Accordingly, its putative membrane anchor PceB was among the 20 most differentially produced proteins in t0 cells (Table S3B). Also, many other proteins encoded in the OHR gene region were among the most differentially produced proteins (Table S2, S3). Of all analyzed

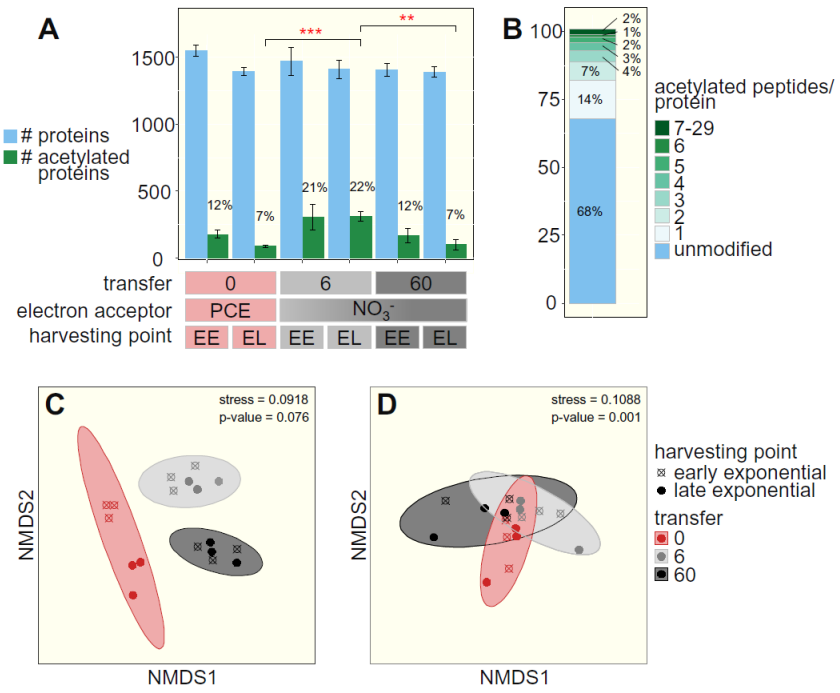

Fig. 3. (A) Number of quantifiable proteins and proteins with at least one acetylation site and *p*-values of differences between transfers if significant: **, $p < .01$; ***, $p < .001$. (B) Number of acetylation sites per protein. (C) nMDS-analysis of proteome profiles. (D) nMDS-analysis of acetylome profiles (logarithmized average of top three acetylated peptide areas normalized to the intensity of the corresponding unmodified protein). EE, early exponential phase; EL, late exponential phase.

transfers, almost all quantifiable proteins encoded in the OHR region were highest abundant in t0 cells and were only slightly lower abundant in t6 (on average 57% of the abundance in t0). In t60, they were not detectable or only identifiable (Fig. 6B). Thus, most proteins showed a similar downregulation as the reductive dehalogenase PceA, including the putative quinol dehydrogenase (Qdh) and the norpseudo-B_{12} biosynthesis genes, suggesting the genes of the OHR region to be included within a single regulon.

Almost all proteins encoded in the OHR gene region were quantifiable after cultivation on PCE (Fig. 6B). This includes also several membrane-integral proteins, of which only a few were detected in the *S. multivorans* dataset [5]. For example, the membrane subunit of the Qdh (SHALO_1506) was detected in *S. halorespirans* t0 and t6 samples, underpinning its suggested role in the electron transport chain (Fig. 5). Not quantifiable in both organisms were TCS I (SHALO_1498–1499) and the second reductive dehalogenase RdhAB (SHALO_1500–1501), even though they are part of the 100% conserved region in organohalide-respiring *Sulfurospirillum* spp. In both species, of

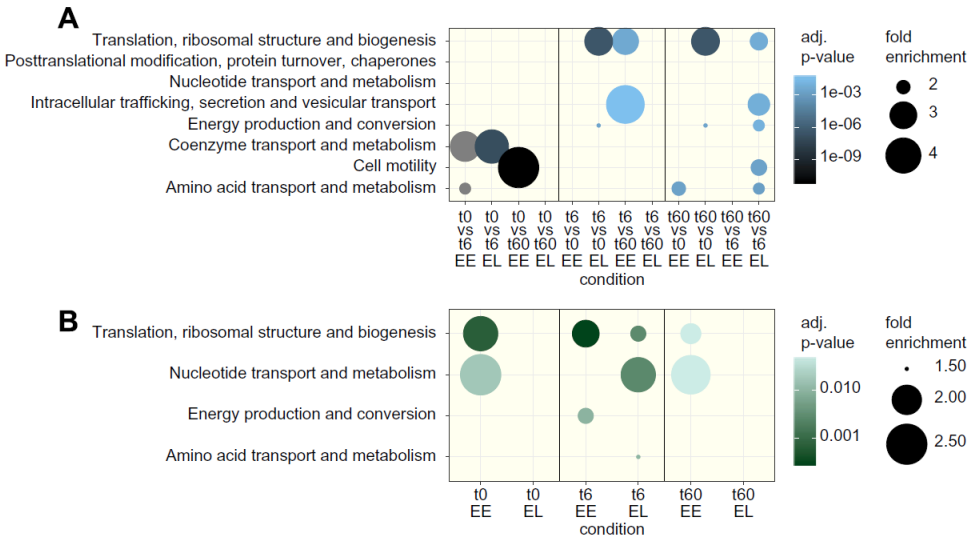

Fig. 4. Enrichment analysis of functional protein classes. (A) Functional protein classes overrepresented within the significantly upregulated proteins ($p < .05$) of cells cultivated on PCE (t0) compared to cells cultivated for six or 60 transfers on nitrate (t6, t60). (B) Functional protein classes overrepresented within the acetylated proteins compared to all proteins of each condition. Y-axis lists the functions according to the database of Clusters of Orthologous Groups of proteins (COGs), x-axis the compared conditions, bubble size corresponds to fold enrichment of the respective pathway among the significantly regulated proteins in the condition mentioned first, color scale of bubbles corresponds to the adjusted p-values. EE, early exponential phase; EL, late exponential phase.

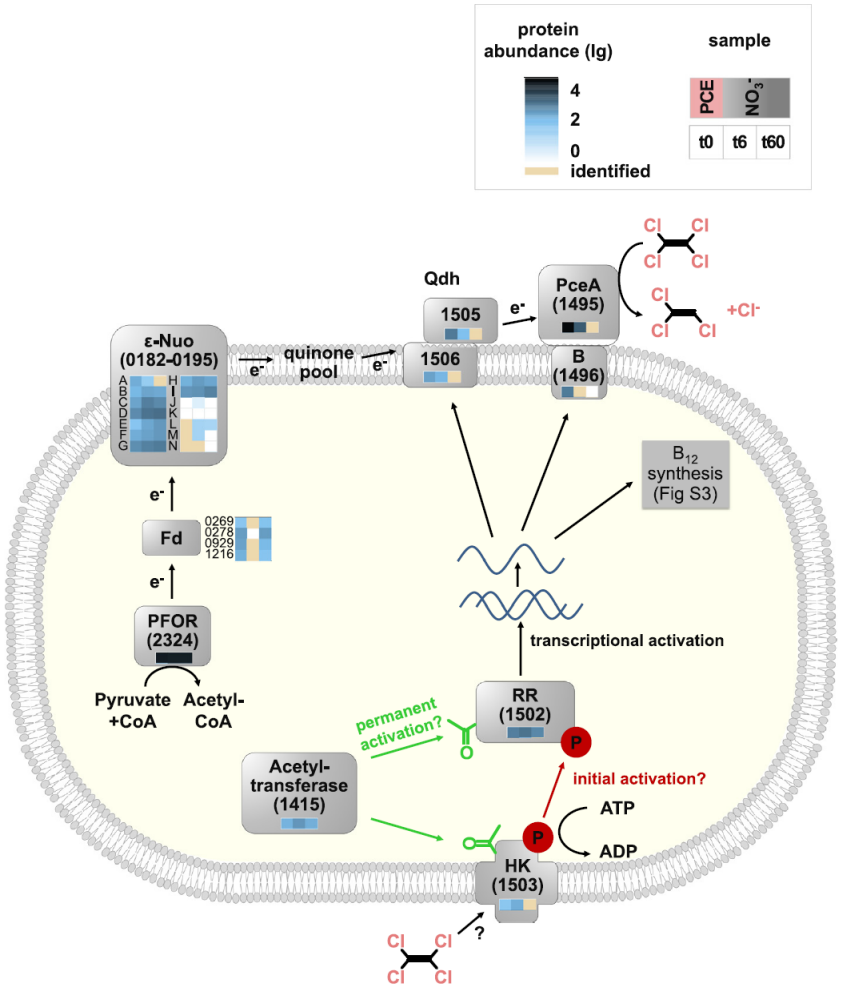

Fig. 5. Model of the organohalide respiratory chain and its putative two-component regulatory system in *S. halorespirans* with pyruvate as the electron donor. The locus tag suffix (prefix SHALO) and the average protein abundances with PCE (t0), after six (t6) and 60 transfers (t60) with nitrate are given as heatmaps inside the protein sketches (early and late exponential phase pooled). PCE is reduced with electrons derived from pyruvate oxidation, the route of electrons is indicated by arrows. PCE is probably sensed by the histidine kinase, which is autophosphorylated and transfers the signal by phosphorylating the response regulator. The phosphorylated response regulator binds the DNA, which leads to expression of the OHR gene region. The acetyltransferase SHALO_1415 is a candidate for acetylating both components of the two-component system, which might lead to the prolonged activation of the response regulator when PCE is not present anymore. PFOR, pyruvate:ferredoxin oxidoreductase; Fd, ferredoxin; ε-Nuo, epsilonproteobacterial complex I; Qdh, quinol dehydrogenase; RR, response regulator; HK, histidine kinase.

RdhA, only one peptide was identified in a single replicate and no *rdhA*-transcription could be detected in *S. multivorans* [6,7]. Other non- or only partly detected proteins may have escaped from extraction due to their tight membrane interaction and/or small size, and include a small, putative membrane protein possibly involved in electron transport (SHALO_1504), adenosylcobinamide-phosphate synthase CbiB (SHALO_1507), the permease component BtuC of the corrinoid ABC transporter (SHALO_1516) and the cysteine-rich SHALO_1512 and SHALO_1531 gene products.

Some proteins of the OHR core region were found in the proteomes of all conditions. Among them was the TCS II response regulator (RR, SHALO_1503), with abundances between the median and M + 2δ of all proteins, and only found in 1.2–1.9-fold higher abundances in t0 cells (Fig. 5, 6B). The corresponding histidine protein kinase (HK, SHALO_1502) was only quantifiable in the t0 and t6 proteomes. However, low amounts of this protein could have been undetected due to its seven predicted transmembrane helices. Comparably to *S. multivorans*, only the TCS proteins encoded adjacent to *rdhAB* rather than the one adjacent to *pceAB* were produced and therefore are most likely involved in the regulation of OHR (Fig. 6B).

Nearly all proteins encoded by genes of the *cbi* cluster were detected in the proteome of *S. halorespirans* (Fig. 6B, S3). These included several proteins which have not been assigned a function and which were not quantifiable in *S. multivorans* [7], namely two cysteine-rich proteins (SHALO_1512, SHALO_1531) and the MsbA-like protein (SHALO_1529). The cysteine-rich proteins could play a role in metal supply or unknown redox processes in corrinoid biosynthesis. The MsbA-like putative lipid A export protein is probably not involved in the transport of corrinoids or cobalt, as these roles are already assigned to BtuCDF (SHALO_1516–1518) and the ECF cobalt transporter (SHALO_1589–1593), respectively. Both of these transporters were quantified in the proteomes of both species (Fig. S3, Table S1) [7]. Aside from similarities of the SHALO_1529 gene product to MsbA (30% amino acid sequence similarity), which might suggest a role in lipid export, nothing is currently known about its function.

One of the observed differences between the *S. multivorans* and *S. halorespirans* OHR gene region was the presence of 106 inserted nucleotides upstream of the *cbi* gene cluster in the latter [6]. A putative ORF is located in this region, but no peptides were detected when the proteome data were searched against a six-frame-translation-database. It either escaped proteomic detection due to its small size or the region does not encode an ORF but serves regulatory functions. Downstream of the *cbi* gene region, a regulatory gene encodes a TetR-like transcriptional repressor, which could not be assigned a function up to now. The TetR-like transcriptional regulator (SHALO_1533) was produced to low abundances (< M-1σ) on PCE. The abundance of the proteins encoded downstream of the corresponding gene, however, showed a similar pattern as in *S. multivorans* (Fig. 6B): Two flavin-containing proteins (SHALO_1534, SHALO_1536) were among the most abundant proteins in the proteome of PCE-cultivated cells (Table S2) and have an

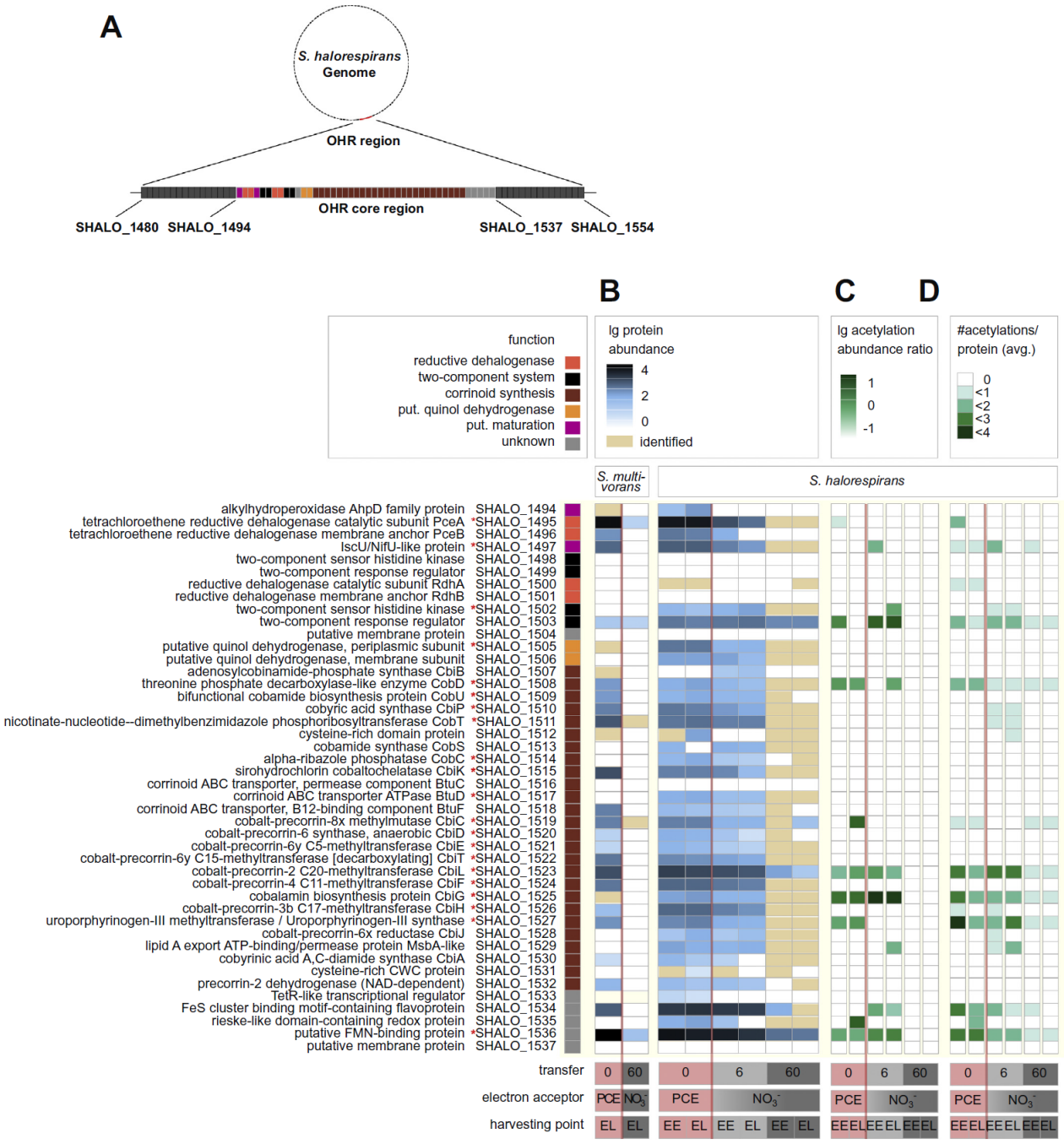

Fig. 6. The organohalide respiratory (OHR) core gene region (A), protein intensity pattern of *S. halorespirans* and *S. multivorans* (B) and acetylome pattern (C + D) of *S. halorespirans*. Locus tags and protein descriptions are given on the left of the protein intensity pattern. Colored squares adjacent to the protein locus tags indicate their function in OHR. Each square of the protein intensity pattern (B) corresponds to a given protein quantified (shown in blue, color code on top, normalized and logarithmized average of top three peptide MS1-area as described in the methods section) or identified (beige). Cultivation conditions at the bottom. Proteins with significant abundance differences between any of the cultivation conditions are marked with a red asterisk (for details see Table S1). Data for *S. multivorans* calculated from the dataset in [24]. (C) Acetylation of a given protein quantifiable in > 50% of all replicates is marked in green. The relative acetylation intensity is provided (color code on top, logarithmized top acetylated peptide area normalized to the intensity of the corresponding unmodified protein). (D) Number of acetylation sites identified per protein, average of three replicates. EE, early exponential phase; EL, late exponential phase; put., putative. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

abundance level similar to PceA. However, both were also detectable in the proteome in t60. Based on its sequence, SHALO_1534 is predicted to be an electron-transfer flavoprotein, whereas SHALO_1536 is classified to a group of redox proteins, which also includes pyridoxamine 5′-phosphate oxidases involved in the vitamin B_6 metabolism. They were suggested to play a role in corrinoid biosynthesis or modification [7], but their presence even after 60 transfers without PCE remains enigmatic.

The proteins encoded in the regions flanking the OHR core region (SHALO_1480–1493, SHALO_1538–1554, Fig. 6A) are hardly detectable in any of the proteomes of *S. halorespirans* or *S. multivorans* (Table S1) [7] and their role remains obscure. The contained genes are conserved in the two organohalide-respiring species *S. multivorans* and *S. halorespirans* and do not have orthologs in the *S. deleyianum* and *S. barnesii*, which are not capable of OHR [6,8]. Only a hypothetical protein (SHALO_1552) and a putative membrane-bound protein (SHALO_1553) were detected but showed low abundance levels (< M). To conclude, production of almost all proteins encoded in the OHR core region continues during the absence of PCE and only ceases after prolonged cultivation without their specific substrate in *S. halorespirans*, comparable to *S. multivorans*, suggesting that the three differences in the OHR region have no detectable influence on the long-term downregulation.

3.4. PCE-induced proteins outside the OHR gene region

The outer membrane porin SHALO_0946 was among the 20 most abundant proteins in t0 (Table S2), whereas on nitrate, it only had a rank ranging from 187 to 310 and was significantly more abundant in t0 than in t6 (Table S1), indicating a role in PCE-import. The only stress-related protein among the 20 most differentially produced proteins was the heat shock protein Hsp20 (SHALO_0540), which was up to 12-fold higher on PCE (Table S3). The biosynthesis of this protein was also found to be PCE-dependent in *S. multivorans* [7]. The higher abundance of Hsp20 in both proteomes strengthens our suggestion for its role in a PCE-dependent stress response.

3.5. Proteins involved in nitrate respiration

Proteins related to nitrate respiration were among the most abundant proteins in *S. halorespirans* cells cultivated with nitrate (Table S2). In general, coverage of proteins involved in nitrate respiration was higher than in *S. multivorans*: All subunits of the nitrate reductase NapAGHBLD (SHALO_0949–0955) and the nitrite reductase NrfHAIJ (SHALO_0905–0908) were quantified with abundances of up to > M + 2σ (Fig. S4, Table S1). Most of the Nos proteins, responsible for the reduction of nitrous oxide to nitrogen (encoded by SHALO_0349–0357), were produced in *S. halorespirans* to abundances below median under all conditions but higher with nitrate than with PCE. For unknown reasons, NosZ (SHALO_0357) was only detected in PCE-grown cells. In *S. multivorans,* this cluster is not encoded [8]. As in *S. multivorans*, on nitrate, the hydroxylamine reductase (SHALO_0596) had similar abundances as Nap and Nrf, but it was also quantifiable on PCE. It was suggested to scavenge intermediates formed during denitrification, such as nitrite or nitric oxide [7]. An outer membrane porin (SHALO_2097) was up to 44-fold higher abundant on nitrate (Table S3A), indicating a specific function in nitrate import.

3.6. Other redox proteins involved in the energy metabolism

In the proteome of *S. halorespirans*, two proteins were quantified serving the oxidation of the electron donor pyruvate, the pyruvate:ferredoxin/flavodoxin oxidoreductase (PFOR, SHALO_2324) and the quinone-dependent pyruvate dehydrogenase (PoxB, SHALO_1660, exclusively identified in organohalide-respiring *Sulfurospirillum* spp. up to now [7]). Opposed to *S. multivorans*, PoxB might have lower importance in *S. halorespirans*, since the abundance of this enzyme was up to 800-fold less than that of PFOR (Table S1).

Ferredoxin most probably serves in accepting the electrons from pyruvate oxidation via PFOR (Fig. 5). Similar to *S. multivorans*, ferredoxin SHALO_0269 (ortholog SMUL_0303) was produced, even though lower abundant in *S. halorespirans* (around median vs. > M + σ). Additionally and opposed to *S. multivorans*, two other ferredoxins were quantifiable, SHALO_0929 (SMUL_0908) and, preferentially in the late exponential phase, SHALO_0278 (SMUL_0312). These ferredoxins are conserved in most Epsilonproteobacteria [6]. Furthermore, ferredoxin SHALO_1216 (SMUL_1235), which is encoded in the nitrogenase gene region, was quantified, whereas the nitrogenase itself was not found. As in *S. multivorans*, flavodoxins (SHALO_1212, SHALO_2554) were not detected, stressing the role of ferredoxin as a central electron carrier in *Sulfurospirillum* spp.

As in *S. multivorans*, of the four encoded hydrogenases, the periplasmic membrane-bound NiFe hydrogenase (MBH, SHALO_1400–1402) was constitutively produced to high abundances under all conditions (up to > M + 2σ), even though the cultures were not supplied with hydrogen. Interestingly, the putative hydrogen-evolving Ech-like hydrogenase Coo (SHALO_1293–1297) did show a PCE-dependent production. This hydrogenase has never been detected in *S. multivorans* [7]. The hydrogenase-4 Hyf (SHALO_2129–2141) bears a frameshift mutation caused by a transposon insertion, which is the reason why only few of its subunits were produced, in contrast to *S. multivorans*. This presumably also explains the observation that *S. halorespirans* cannot be cultivated on pyruvate alone, in contrast to *S. multivorans*, which can grow fermentatively on pyruvate without any electron acceptor [33]. Hyf might also function as an electron sink using the electrons from ferredoxin to generate H_2 when excess electron donor is supplied. Coo might partly substitute the defective Hyf in *S. halorespirans*.

Apart from the small differences in the genome, some physiological differences to *S. multivorans* became apparent, among them the more important role of chemotaxis in *S. halorespirans*. Among the proteins encoded in both species but produced to a much higher level in *S. halorespirans*, were 20 chemotaxis proteins. Most of the chemotaxis-related proteins had abundances of at least M + σ (Table S1) and were up to 400-times higher in *S. halorespirans* or not even produced in *S. multivorans* (Table S4). Similar to *S. multivorans* [7], cell motility-related proteins were enriched within the significantly more abundant proteins of t0- compared to t60-cells (Fig. 4A).

3.7. Acetylome

Protein acetylations are more and more recognized as an important modifier of protein activity and function. This comprehensive lysine-acetylome study was designed in order to prove the hypothesis that acetylations of key regulators are involved in controlling long-term regulation in *S. halorespirans*. This first acetylome of an Epsilonproteobacterium revealed that one-third of all identified proteins possess acetylation sites (Fig. 3B). This corresponds to 22% of the predicted proteome in *S. halorespirans* (Table S5) and is a relatively large number compared to on average 9% acetylated proteins in comparable studies of other bacteria [34], implying that acetylations are involved in many physiological processes in *S. halorespirans*. Of the 640 identified acetylated proteins, 368 were acetylated at various Lys sites (57%, Fig. 3B). Proteins with the most acetylation sites were PFOR (SHALO_2324, 29 sites, highest in t6), heat shock protein 60 family chaperone GroEL (SHALO_1004), chaperone protein DnaK (SHALO_1713) and ferredoxin-sulfite reductase (SHALO_2857, 22 sites each and highest in t6, Table S1). Apart from the latter, these highly acetylated proteins had also acetylated orthologs in *E. coli* and are in general known to be targets for lysine acetylations (e.g [35–37].), even though their exact role is unknown, as in general, to date, only little has been explored about specific effects of protein acetylations.

60% of the acetylated proteins in *S. halorespirans* had orthologs in *E. coli* and 58% of these orthologs were also acetylated in *E. coli*. This and

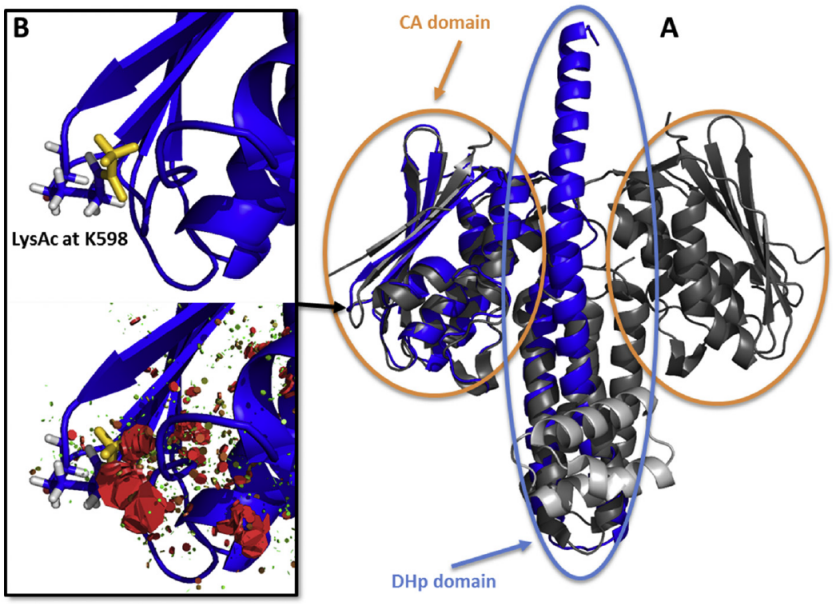

Fig. 7. (A) Predicted structural model of the catalytic core of the histidine kinase (SHALO_1501) of *S. halorespirans* (blue). Alignment with the crystal structure of the Sda/KinB catalytic core complex of *Geobacillus stearothermophilus* (PDB ID 3D36, gray). Orange circle: catalytic and ATP-binding domains (CA); blue circle: dimerization and histidine phosphotransfer (DHp) domains. (B) Part of the SHALO_1501 CA domain harboring the acetylated lysine K598. The acetylation is colored gold. In the lower panel, steric clashes based on van der Waals overlap are represented by the colored discs. The increasing size and redness of the discs correlate with stronger van der Waals strains [17]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the constitutive production of most of these proteins under all conditions points at a higher incidence of acetylations on housekeeping proteins (Table S1). Furthermore, the more acetylation sites on a protein, the higher was the conversation grade of the protein in *E. coli* and its probability to be acetylated as well, i.e. to be found in the CPLM database of lysine-modified proteins (Fig. S5). The enrichment of acetylated proteins in energy conservation, translation, and nucleotide metabolism (Fig. 4B) is consistent with previous studies of other bacteria [34,38], indicating the conserved role of acetylations in the regulation of physiological processes.

The nMDS analysis of the acetylome resulted in a significant segregation of t0, t6 and t60 samples ($p = 0.001$), indicating that the acetylation pattern differed between transfers (Fig. 3D). Most acetylated proteins were detected in t6 (EL t0 vs. t6 $p = 0.0005$, EL t6 vs. t60 $p = 0.002$, Fig. 3A), which might reflect the metabolic transition of *S. halorespirans* adapting to a different electron acceptor. Only one acetyltransferase was higher abundant in t6 than in other phases, namely a putative *N*-acetyltransferase (SHALO_1415).

Two flavoproteins (SHALO_1534 and SHALO_1536) encoded in the OHR gene region were acetylated (Fig. 6C, D). There are several studies about flavoproteins which are inhibited by acetylations, e.g. the flavoprotein subunit of the succinate dehydrogenase [35,39] and the pyridoxine 5′-phosphate oxidase [40–42].

3.8. Acetylation of the two-component system

In TCS II (SHALO_1502–1503), which is most probably involved in the regulation of PCE respiration [6,7], several acetylated sites were identified and their spectra manually curated (Fig. S6). The number of acetylations in the TCS II was at a maximum in the transition phase (t6), when the OHR genes were expressed in the absence of PCE (Table S6). The HK (SHALO_1502) was acetylated in two out of three replicates of the sample harvested in the late exponential phase and in one of three replicates in the early exponential phase of t6. In t0, no HK acetylations were detected and in t60, the HK was not identified in the proteome. The acetylated K598 is part of the catalytic domain, which belongs to the HATPase_c domain family, and is located adjacent to the ATP-binding site. The acetylated lysine clashes with the amino acids D552 and S553 in the structural model of the HK (Fig. 7A, B). However, the mode of influencing the protein functionality might be indirect, since the K598 side chain is most likely oriented outwards the protein with some steric freedom.

The cognate response regulator shows two acetylations in t6, K114 and K218, of which only K114 was detected in t0 but with lower abundance (Table S6). Acetylation of K114 during t60 was only present in one replicate (of EE and EL), interestingly the one, in which PceA was also identified (Table S1). The acetylation of K114 was highly abundant ($> M + \sigma$) and found in three replicates of the early and in two replicates of the late exponential phase of t6 (Fig. 6D). In the predicted structural model of the RR, an acetylation of K114, which is part of the receiver domain (REC), would interfere with D111 of the REC domain (Fig. 8A, B). The acetylation of K218 was detected in only one replicate of the early exponential phase of t6. K218 is located at the C-terminus of the DNA binding domain and part of the smaller β-sheet. In the structural model, the acetylation is exposed to the protein surface and interferes most likely with N171 (Fig. 8C), which is likewise part of the DNA-binding domain. Both amino acids face away from the DNA and both acetylation sites are most probably not directly involved in intramolecular interactions. Although no direct effect of the acetylations can be concluded, the specificity and stability of protein-protein interactions or protein-DNA binding as well as the efficiency of the initiation of the transcription could be influenced by conformational changes or neutralization of the positive charge of a lysine.

Overall, the acetylations specific for t6 during the transition phase could have both, an activating or an inhibiting effect on the gene regulation of the OHR gene cluster. Assuming that the phosphorylation of the RR by the HK has stopped, acetylation might mimic the phosphorylation and keep the RR in the active, DNA-binding state but with lower affinity (Fig. 5). An activating effect was described for the transcription factor HilD in *Salmonella enterica* serovar Typhimurium, which is stabilized by an acetylation leading to a continuous gene expression [43]. However, of the few TCSs analyzed for their functional modulation by acetylation, mainly inhibiting effects have been reported. An acetylation within the winged HTH motif was described to decrease the DNA-binding activity of the RR RcsB in *E. coli* [44,45] and PhoP in *S. enterica* serovar Typhimurium [46] but only slightly of the global RR

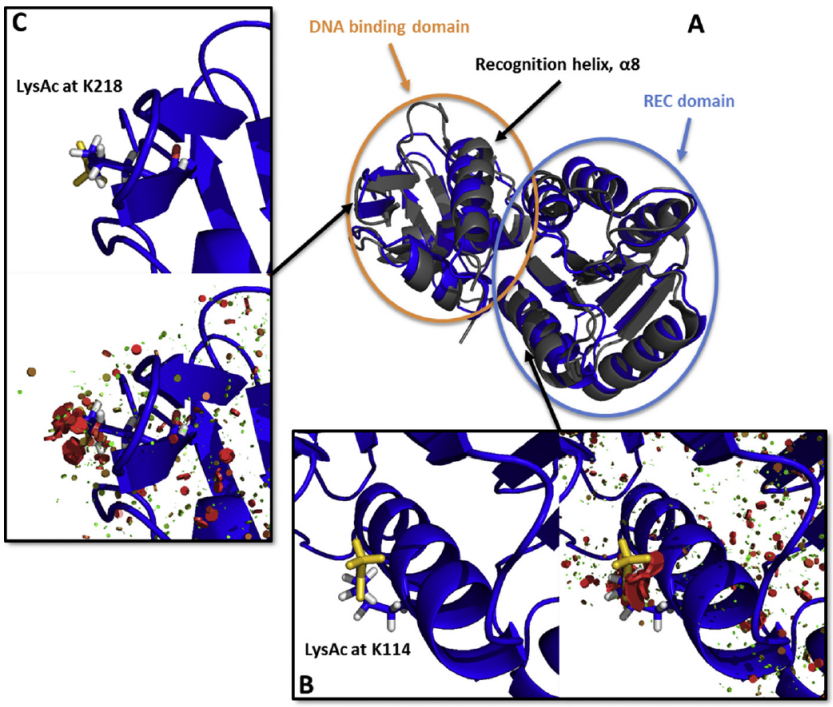

Fig. 8. (A) Predicted structural model of the response regulator (SHALO_1502) of *S. halorespirans* (blue). Alignment with the crystal structure of MtrA of *Mycobacterium tuberculosis* (PDB ID 2GWR, gray). Blue circle: receiver (REC) domains; orange circle: DNA-binding domains. (B) Part of the SHALO_1502 DNA-binding domain harboring the acetylated lysine K218 in the winged helix-turn-helix (HTH) motif. (C) Part of the SHALO_1502 REC domain with the acetylated lysine K114. The acetylations are colored gold. Steric clashes based on van der Waals overlap are represented by the colored discs in the right or lower panel, respectively. The increasing size and redness of the discs correlate with stronger van der Waals strains [17]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

GlnR in *Streptomyces coelicolor* [47]. Another RR, CheY, regulating chemotaxis in *E. coli*, was described to have a repressed binding affinity to all target proteins due to lysine acetylation [48,49]. In vivo, two acetylation sites of CheY are located within the receiver domain and one within the active site, which is part of the dimerization interface [49]. In general, the acetylation of the TCS II in *S. halorespirans* could lead to a reduction in the protein stability, the dimerization of the RR or its promoter binding affinity. Assuming that the phosphorylation of the RR by the HK is not immediately ceasing in the absence of PCE, this loss in functionality of the RR might cause the delay in OHR gene expression.

Other RRs were suggested to be specifically acetylated via an *N*-acetyltransferase [44–49] and deacetylated via a Sir2 family deacetylase [44,46,47,49]. In *Sulfurospirillum* spp. the putative *N*-acetyltransferase (SHALO_1415), which was higher abundant in t6 than at other time points (Fig. 5), and the NAD^+-dependent protein deacetylase of the Sir2 family (SHALO_1877), which is the only annotated Sir2 family deacetylase in the genome of *S. halorespirans*, might be responsible for the acetylation and deacetylation of the TCS II. The characterization of the molecular mechanism of the signal transduction via phosphorylation and further fine-tuning via acetylation as well as its impact on the maintenance of the gene expression of the OHR gene cluster in the absence of PCE should be investigated in future studies.

4. Conclusion

In this study, we proved that tetrachloroethene respiration of *Sulfurospirillum halorespirans* is long-term regulated. The physiology of PCE respiration seems to be similar to *S. multivorans*, although some differences were observed in the pyruvate-oxidizing enzymes and the ferredoxin abundance. The regulation of OHR in *Sulfurospirillum* spp. seems to depend mainly on a two-component regulatory system. The acetylome provided evidence that acetylations might play a major role in the OHR gene expression regulation via the TCS II. However, our findings need to be confirmed by in vitro studies with the regulatory gene products.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.03.030.

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2.3 Cobalt - an essential micronutrient for organohalide respiration in dehalogenating *Sulfurospirillum* spp.

Esken J, Schubert T, Diekert G, *FEMS Microbiology Ecology* (in preparation for submission)

* Authors contributed equally to this work.

In this study, *S. halorespirans* and *S. multivorans* were cultivated under ecological relevant cobalt concentrations and a limitation in the norcobalamin biosynthesis, PceA availability, and OHR was observed. When PCE was the only electron acceptor, growth was affected and a transcriptional downregulation of *pceA* and *cbiB* was observed. However, when an alternative electron acceptor was present, the culture continued growing and the long-term downregulation of the OHR gene expression was detected, although slightly accelerated.

My own contribution to this manuscript covers about 80%.

I conducted study conception, cultivation, activity assays, Western blots, norcobalamin extraction and quantification, RNA isolation, RT-PCR, RT-qPCR, data analysis, and manuscript writing. The trace element analysis was done at the Institute of Geoscience, Friedrich Schiller University in cooperation with Dirk Merten. Torsten Schubert and Gabriele Diekert helped with the revision of the manuscript.

For supplementary information see appendix, pp. xxviii - xxxi

Cobalt - an essential micronutrient for organohalide respiration in dehalogenating *Sulfurospirillum* spp.

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Abstract

Except for land plants and fungi, cobalt is an essential trace element in all living organisms, especially due to its role as central atom in cobamides (Cba, complete corrinoids) such as vitamin B_{12}. Cba-containing enzymes catalyze important reactions including methylations, structural rearrangements, and reductive dehalogenations. Only selected prokaryotes can synthesize Cba cofactors *de novo* and bioavailable Co^{2+} is limited in natural habitats. This study focusses on the impact of Co^{2+} limitation on the Cba production in the Campylobacterota (formerly Epsilonproteobacteria) *Sulfurospirillum halorespirans* and *Sulfurospirillum multivorans*, the biosynthesis of the Cba-containing reductive dehalogenase, and the consequences for the organohalide respiration in both organisms. Due to the lack of a *btuB* gene, both organisms lack a functional uptake system for exogenous Cbas. Hence, both organisms rely on *de novo* Cba biosynthesis and Co^{2+} supply while growing on halogenated organic compounds. Although it was shown that Co^{2+} limitation affects the amount of functional reductive dehalogenase, the biosynthesis of the corrinoid cofactor was not Co^{2+}-dependent transcriptionally regulated.

Introduction

Cobalt is a first-row transition metal located in the periodic table of chemical elements between iron and nickel. In nature cobalt occurs in the oxidation states Co^{2+} and Co^{3+}, but it can exhibit oxidation states from -1 to +4 (Kobayashi and Shimizu 1999) (henceforth, metal ions are only named with their metabolically most relevant oxidation state). It is an essential trace element for the metabolism in both prokaryotes and eukaryotes. Except for land plants and fungi, the redox-active cobalt ion is a unique biological cofactor as it is the central ion in the corrin ring of cobamides (Cbas) (Cracan and Banerjee 2013, Helliwell *et al.* 2011, Kräutler 2005, Okamoto and Eltis 2011). It is utilized in metalloproteins, but less frequently than other first-row metals such as manganese, iron, copper or zinc (Okamoto and Eltis 2011).

In the vast majority of cobalt-containing proteins, the ion is part of Cbas. Only eight noncorrin cobalt-containing metalloenzymes have been characterized (Kobayashi and Shimizu 1999, Okamoto and Eltis 2011). One of which is the methionine aminopeptidase ubiquitously found in both prokaryotes and eukaryotes (Bazan *et al.* 1994, Roderick and Matthews 1993). This enzyme is able to catalyze the cleavage of the N-terminal methionine from polypeptide chains after translation. In addition, cobalt can compete with other biologically essential metal ions for metal binding sites. Hence, Co^{2+} can be bound by macromolecules instead of the physiologically correct ion. For example, several of the typical Mg^{2+}-activated enzymes such as esterases can function with Co^{2+}, though usually with a lower activity (Vallee and Coleman 1964). Co^{2+}-incorporation is particularly acute in Fe^{2+}-binding metalloproteins, since Co^{2+} and Fe^{2+} have similar radii and the same biologically relevant oxidation states (Andreini *et al.* 2008).

Prokaryotes are the only natural sources of Cbas. A unique Cba is adeninyl-norcobamide (AdeNCba, *synonym*: norpseudo-B_{12}). AdeNCba displays an exceptional nucleotide loop structure (Kräutler *et al.* 2003). Instead of 5,6-dimethylbenzimidazole (DMB), adenine serves as lower base and a methyl group is lacking at carbon 176 in the aminopropanol phosphate linker. It is solely produced by members of the genus *Sulfurospirillum*, which belong to the class of Campylobacterota (formerly Epsilonproteobacteria). Recently, two members, namely *Sulfurospirillum halorespirans* and its close relative *Sulfurospirillum multivorans*, have been studied and genomic (Goris *et al.* 2017, Goris *et al.* 2014) and proteomic (Goris *et al.* 2015, Türkowsky *et al.* 2018) data became available. The microaerophilic *S. halorespirans* was isolated from flow through soil columns inoculated with a sample polluted with chlorinated aliphatics (Middeldorp *et al.* 1998) and *S. multivorans* from activated sludge of a wastewater treatment plant (Scholz-Muramatsu *et al.* 1995). *Sulfurospirillum* spp. seem to be globally distributed and mostly found in sediments, groundwater or soil and often in environments polluted with organohalides occurring together with *Dehalococcoides*, *Desulfitobacterium* or *Dehalobacter* species (Goris and Diekert 2016). It is assumed that *Sulfurospirillum* is involved in a syntrophic relationship with other dehalogenating bacteria providing Cba derivatives, acetate, and hydrogen produced via fermentation (Kruse *et al.* 2018). *S. multivorans* effectively produces up to 0.5 mg/l AdeNCba *de novo* (final OD_{578} 0.25 – 0.3) (Schubert 2017). The norcobamide biosynthesis pursues the “anaerobic” or “early cobalt insertion” pathway in which cobalt is incorporated into the precorrin-2, which is formed out of uroporphyrinogen III in the first step of the biosynthesis pathway (Warren *et al.* 2002).

AdeNCba serves as redox-active cofactor of the tetrachloroethene (PCE) reductive dehalogenase (PceA). This enzyme is an iron-sulfur protein that serves as a terminal reductase of a membrane-associated electron transfer chain (Hug *et al.* 2013, Kräutler *et al.* 2003). Electrons are transferred to the Co^{2+}, which finally dechlorinates the substrates (Fig. 1). This process is coupled to the energy metabolism via menaquinone and described as organohalide respiration (Adrian and Löffler 2016, Schubert *et al.* 2018). Except for the genes needed for the biosynthesis of iron-sulfur clusters, all genes required for the reductive dehalogenation are organized in a 40 kb gene region. *S. halorespirans* and *S. multivorans* share this OHR gene region with nearly 100% nucleotide sequence identity (Fig. 1) (Goris *et al.* 2017, Goris *et al.* 2014). The gene region comprises reductive dehalogenase genes *pceA* and *rdhA*, their putative membrane anchors *pceB* and *rdhB*, all genes needed for the AdeNCba biosynthesis and regulators including a two-component system (TCS) that induces OHR gene expression in the presence of the substrate PCE (Chapter 2.1) (Goris *et al.* 2015, Türkowsky *et al.* 2018). In the absence of PCE, the transcription ceases over more than 100 generations. This retentive memory effect has been reported for both *S. halorespirans* (Türkowsky *et al.* 2018) and *S. multivorans* (John *et al.* 2009). Both species have a similar versatile energy metabolism and are suitable candidates for the analysis of Co^{2+} limitation.

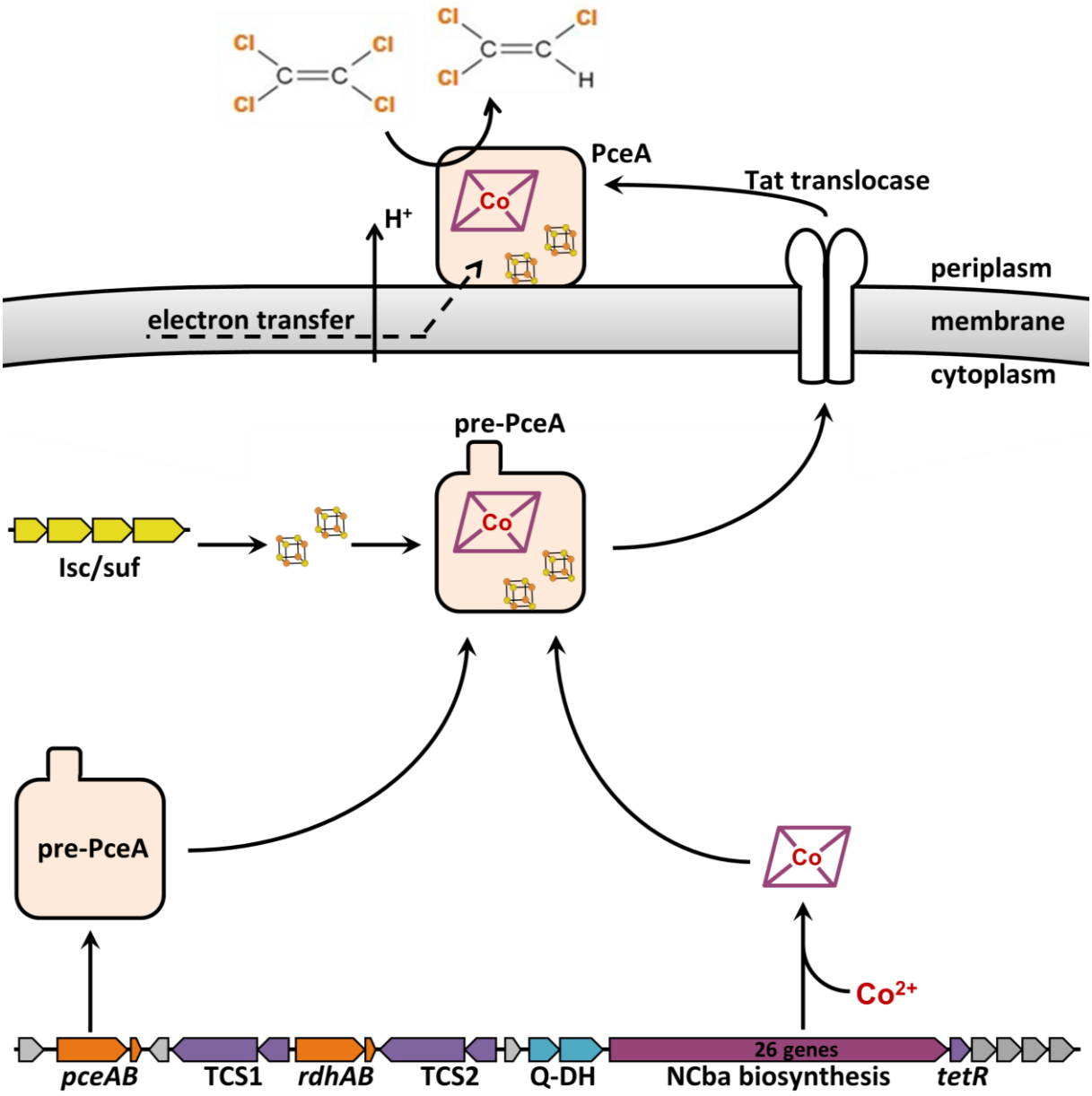

Figure 1: Illustration of the biosynthesis of the reductive dehalogenase PceA orchestrated by the organohalide respiration (OHR) gene region. The gene region encodes for two reductive dehalogenases and their putative membrane anchors (orange), two two-component systems as well as a TetR-like repressor (purple), two genes, which encode for components of a putative quinol dehydrogenase (cyan), and genes for the biosynthesis of the norcobamide cofactor (pink) with three genes for the incomplete cobamide transport system (pale pink). Some of the genes have a yet unknown function (gray). The genes for the synthesis of the [4Fe-4S] clusters (yellow) are not encoded within the OHR gene cluster.

Under laboratory conditions standardly an amount of 47 µg/l Co^{2+} is used for *Sulfurospirillum* cultivations. This amount is by two orders of magnitude higher than the amount of Co^{2+} detected in the Saale river (Proft and Schrön 2008), in which PceA similar to that in *Sulfurospirillum* species has been reported (von Wintzingerode *et al.* 2001). In this study, the impact of Co^{2+} limitation on dehalogenating bacteria as it occurs in natural habitats was analyzed. For that purpose, the norcobamide biosynthesis as

well as the amount and enzymatic activity of PceA and the bacterial viability was examined. The effect of Co^{2+} limitation on the transcriptional level and its impact on the retentive memory effect reported in *S. halorespirans* (Türkowsky *et al.* 2018) and *S. multivorans* (John *et al.* 2009) was now investigated.

Experimental Procedures

Cultivation of *Sulfurospirillum multivorans* and *Sulfurospirillum halorespirans*

S. multivorans (DSM 12446) and *S. halorespirans* (DSM 13726) were cultivated anaerobically at 28°C in a defined mineral medium in the absence of exogenous cobamide and yeast extract. The basal medium contained per liter: 70 mg Na_2SO_4, 200 mg KH_2PO_4, 250 mg NH_4Cl, 1 g NaCl, 400 mg $MgCl_2 \cdot H_2O$, 500 mg KCl, and 150 mg $CaCl_2 \cdot 2\ H_2O$. The basal medium was supplemented with a trace element solution SL10, a vitamin, a selenite, and a tungsten solution. The 1000x fold concentrated SL10 solution was composed of 1 l H_2O, 10 ml HCl [25% (w/v)], 1 g $FeSO_4 \cdot 7\ H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2 \cdot 4\ H_2O$, 6 mg H_3BO_3, 190 mg $CoCl_2 \cdot 6\ H_2O$, 2 mg $CuCl_2 \cdot 2\ H_2O$, 24 mg $NiCl_2 \cdot 6\ H_2O$, and mg $Na_2MoO_4 \cdot 2\ H_2O$. The 2000x fold concentrated vitamin solution contained per liter: 80 mg *p*-amino-benzoic acid, 20 mg D(+)biotin, 200 mg nicotinic acid, 100 mg Ca-D(+)pantothenate, 300 mg pyridox-amine · 2 HCl, and 200 mg thiamine · HCl. The selenite solution was 5000x fold (26 mg/l $Na_2SeO_3 \cdot 5\ H_2O$) and the tungsten solution was 10000x fold (33 mg/l $Na_2WO_4 \cdot 2\ H_2O$) concentrated. The medium was further complemented with 84 g/l $NaHCO_3$ (25x fold), 50 g/l cysteine · HCl (1000x fold), and 1 g $FeSO_4$ in 100 ml 50 mM H_2SO_4 (400x fold).

40 mM pyruvate was used as electron donor and 40 mM nitrate, 40 mM fumarate or 10 mM nominal concentrated PCE as electron acceptor. PCE was added to the medium from a 0.5 M hexadecane stock solution. Co^{2+} limiting conditions were accomplished by omitting $CoCl_2$ from the mineral medium. The cultivations were performed in 100 ml or 200 ml glass serum bottles or in 2 l glass bottles. The ratio of aqueous to gas phase was always 1:1. The microorganisms were always transferred with a 10% inoculum and harvested during the exponential phase ($OD_{578} \approx 0.20$) by centrifugation (12,000 x g, 10 min at 10 °C). The bacterial growth was monitored photometrically by measuring the optical density at 578 nm and by determining the protein concentration in triplicates with the method of Bradford (Bradford 1976) using the Roti-Nanoquant reagent (Carl Roth GmbH) after an alkaline lysis of the cells with 0.1 N NaOH incubated 5 min at 95 °C. In order to generate *S. halorespirans* cells with completely downregulated *pceA* gene expression (John *et al.* 2009), the organism was cultivated for 60 transfers with nitrate as sole electron acceptor. All cultivations were at least performed in duplicates.

PceA activity assay

The cell pellets of a 100 ml cultivation were washed three times with 50 mM Tris-HCl (pH 7.5) and resuspended (1:2) in the same buffer under anaerobic conditions. An equal volume of glass beads

(0.25-0.5 mm diameter, Carl Roth GmbH, Karlsruhe, Germany) was added and the cells were disrupted using a bead mill (5 min at 25 Hz; MixerMill MM400, Retsch GmbH, Haan, Germany). The crude extracts were separated from the glass beads by centrifugation (14,000 x g, 2 min) under anoxic conditions. The measurements of PceA activity were performed as described using a photometric assay with reduced methyl viologen as artificial electron donor (Neumann, Wohlfarth and Diekert 1996).

Immunoblot analysis

Soluble extracts (10 µg protein per lane) were subjected to denaturing SDS-PAGE (12.5%) and afterwards blotted onto a polyvinylidene difluorid (PVDF) membrane (Roche, Mannheim, Germany) using a semi-dry transfer cell (Bio-Rad, Munich, Germany) according to the protocol described by John *et al.* (John *et al.* 2009). The PceA antiserum (primary antibody) was diluted 500,000-fold. The primary antibody was detected via a secondary antibody (diluted 1:30,000) coupled to alkaline phosphatase (Sigma-Aldrich, Munich, Germany).

Cobamide extraction and quantification

The cell pellets of a 1 l cultivation were resuspended in three volumes 50 mM Tris-HCl (pH 7.5). The cobamide extraction and purification was performed accordingly to the protocol described by Stupperich *et al.* (Stupperich, Steiner and Rühlemann 1986). Acetic acid was added to the crude extract to adjust a pH < 5 and 100 mM potassium cyanide was added. The crude extract was boiled for 10 min, cooled down to room temperature and centrifuged (12,000 x g, 10 min at 10 °C). The supernatant was stored on ice. The extraction was repeated twice. The addition of 250 mg Amberlite XAD4 (Sigma-Aldrich, Steinheim, Germany) per ml supernatant allowed the adsorption of the cobamide overnight, gently shaken at room temperature. The resin was washed ten times with an equal volume of UPW. The elution was performed three times by incubating the resin for one hour in one volume of 100% methanol. The eluate was dried in a vacuum concentrator, resuspended in 2 ml UPW and transferred onto a 3 g Al_2O_3-containing column in order to get rid of hydrophilic compounds. The cobamide was recovered with 40 ml UPW, dried and resuspended in 100 µl UPW.

The purified cobamide was quantified by means of reversed phase high performance liquid chromatography (RP-HPLC, Smartline System, Knauer GmbH, Berlin, Germany) with a C18 column (Chromolith Performance, RP-18e, 100-4.6 mm, Merck, Darmstadt, Germany). The RP-HPLC operated at a temperature of 30 °C and a flow rate of 1.0 ml/min. Mobile phases used were 18% methanol / 0.2% acetic acid (solvent A) and 99.8% methanol / 0.2% acetic acid (solvent B). The cobamides were separated 10 min in solvent A, followed by a gradient to 100% solvent B within 4 min, and finally in 100% solvent B for 3 min. The sample was detected photometrically at 360 nm.

Isolation of RNA

The RNA was isolated from a cell pellet of 2/OD_{578} ml ($1x10^9$ cells) of a cultivation harvested in the exponential growth phase using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The DNA was digested with recombinant DNase I (RNase free, Roche, Mannheim, Germany) in the presence of RNase inhibitor (RiboLock, Thermo Scientific, Schwerte, Germany).

Reverse transcription-PCR (RT-PCR)

The OneStep RT-PCR kit (Qiagen, Hilden, Germany) was used. The reaction mixture contained 5 µl 5x reaction buffer, 25 pmol reverse primer, 25 pmol forward primer, 1 µl 10 mM dNTP mix, 1 µg total RNA or 70 ng *S. halorespirans* genomic DNA as positive control, 1µl enzyme mix and nuclease free water up to a final volume of 25 µl. As negative control nuclease free water was added instead of nucleic acid. The reaction mixture was incubated for 1 h at 50 °C followed by a PCR with an initial denaturation of 95 °C for 15 min, followed by 40 cycles of denaturation for 1 min at 94 °C, annealing for 30 s at 50 °C and elongation for 1 min at 72 °C. The final elongation lasts 10 min. The amplified DNA was separated on a 2% agarose gel and labeled with ethidium bromide. The primers used for RT-PCR analysis are listed in Tab. S1.

Reverse transcription-quantitative real-time PCR (RT-qPCR)

The RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Schwerte, Germany) was used for the RT. The reaction mixture contained 3.5 µl 5x reaction buffer, 1.4 µM reverse primer, 230 µM dNTPs, 1 µg RNA and nuclease free water up to a final volume of 17.5 µl. 10 µl were transferred into a new PCR tube and 0.5 µl reverse transcriptase was added. The remaining mixture was used as an NRT control. The reaction mixture was incubated for 1 h at 42 °C plus 5 min at 70 °C in order to stop the RT. The reaction mixture contained 6.0 µl 2x Maxima SYBR green qPCR master mix (Fermentas, St. Leon Rot, Germany), 0.42 µM forward and reverse primer, each, 2.5 µl cDNA sample, 175 ng *S. halorespirans* genomic DNA as positive control or nuclease free water as negative control, respectively. The mixtures were filled up to a final volume of 12 µl with nuclease free water. The qPCR was performed in triplicates using a CFX96 qPCR machine (Bio-Rad, Munich, Germany). 16S rRNA was used for normalization. Its cDNA was diluted 1:10,000. The change in gene expression was calculated with the $\Delta\Delta C_q$ method (formerly $\Delta\Delta C_t$ method (Livak and Schmittgen 2001)). The results were evaluated with the Welch's unequal variances *t*-test (Welch 1947). The primers used for RT-qPCR analysis are listed in Tab. S1.

***In silico* verification of RNA riboswitches in the PCE regulon**

The Denison Riboswitch Detector was used to identify putative riboswitches in DNA sequences on the scale of the OHR regulatory region (Havill *et al.* 2014). RiboSW was additionally used to identify

riboswitches within messenger RNA sequences (Chang *et al.* 2009). Similarly, RibEx (Abreu-Goodger and Merino 2005) and Riboswitch finder (Bengert and Dandekar 2004) were used. In order to manually check for cobamide and Ni^{2+}/Co^{2+}-binding riboswitches, secondary structures of the 5'-untranslated regions were predicted using RNAstructure 6.0.1 (02/12/2018) (Bellaousov *et al.* 2013).

Results and Discussion

The role of Co^{2+} in the metabolism of dehalogenating *Sulfurospirillum* spp.

When *S. multivorans* was cultivated under nitrate reducing conditions, the cultures grew to a final protein concentration of about 400 - 500 µg/ml, which correlates to an OD_{578nm} of 0.25 - 0.30 (Fig. 2A). In order to evaluate the impact of Co^{2+} starvation, *S. multivorans* was cultivated on a defined medium, lacking the standardly added amount of 0.8 µM $CoCl_2$, which corresponds to a theoretical amount of 47.1 µg/l Co^{2+}. When the culture was transferred on $CoCl_2$-free medium growth remained unaffected. The viability of the cells was not altered by Co^{2+} limitation. Using nitrate as alternative electron acceptor the energy conservation is coupled to the nitrate respiration, which is independent of the reductive dehalogenation and does not require AdeNCba. In *Sulfurospirillum* Co^{2+} is only used by one other non-corrinoid metalloenzyme, namely the methionine aminopeptidase (SMUL_2684; SHALO_2416), which was quantified in the proteomes of *S. halorespirans* (Türkowsky *et al.* 2018) and *S. multivorans* (Goris *et al.* 2015) under all conditions tested. Since growth remains unaffected by Co^{2+} limitation while cultivating with an alternative electron acceptor rather than PCE, this enzyme does not seem to play an essential role in growing cells. Under the conditions tested, AdeNCba is solely essential for the enzyme activity of the reductive dehalogenase. Other genes encoding putative Cba-containing proteins (Goris and Diekert 2016), namely the glutamate-1-semialdehyde 2,1-aminomutase and the ethanolamine ammonia-lyase (existing in *S. multivorans*, but not in *S. halorespirans*), are not expressed (Türkowsky *et al.* 2018).

The bioavailability of Co^{2+} is essential in any microbial community that performs organohalide degradation either because bacteria like *Sulfurospirillum* synthesize the cobamide cofactor *de novo* or because the bacteria acquire Cba through a symbiotic relationship with Cba-producing bacteria (Gruber, Puffer and Krautler 2011). Although the worldwide background concentration of cobalt in soil ranges from 1-40 mg/kg and the average concentration of cobalt in soil amounts to 8 mg/kg (Suthersan 2001), the bioavailability of Co^{2+} in the environment is low due to the sorption characteristics and the low solubility of cobalt. In 31 Dutch groundwater measurement sites the concentration of cobalt was determined to range between 0.01-19.3 µg/l (Arends, Van der Sloot and Van Duijvenbooden 1987). Investigations of ten North American rivers as well as at the Amazon and the Rhone in the years 1965 and 1966 result in an average cobalt concentration of 0.19 µg/l (Wedepohl 1969). The Saale River, in which organohalide-respiring bacteria like *Desulfitobacterium hafniense* TCP-A (Breitenstein *et al.* 2001) or *Dehalococcoides* and *Sulfurospirillum* (Ballerstedt *et al.* 2004) were reported, contained an average

cobalt concentration of 0.6 µg/l in the years 1948-1959 and 0.44 µg/l in the years 1995-1996 (Proft and Schrön 2008). The standard cobalt concentration of 47 µg/l in the defined liquid medium used for the cultivation of *S. multivorans* and *S. halorespirans* is by two orders of magnitude higher than the natural concentration in the Saale River. Therefore, Co^{2+} limitation is of ecological relevance and might be a limiting factor in a dehalogenating community that includes cobalamin synthesizing bacteria. Especially since it was proven that *S. multivorans* (Kruse *et al.* 2018) and *Geobacter* (Yan *et al.* 2012) are able to share their Cbas in a community with *Dehalococcoides mccartyi*, it is comprehensible that a Co^{2+} limitation can lead to a general Cba limitation. As shown here, cobalamin is a limiting factor in dehalogenating bacteria affecting growth and organohalide conversion.

Both organisms used in this study *S. halorespirans* and *S. multivorans* lack a complete vitamin B_{12} ABC transporter system and therefore rely on environmental Co^{2+} supply. The Co^{2+} homeostasis can be mediated by six transporter proteins. Based on the respective gene expression levels published together with the proteome of *S. halorespirans* (Türkowsky *et al.* 2018) three of which are mainly involved. *S. halorespirans* and *S. multivorans* express the Mg^{2+} and Co^{2+} transport protein CorA (SHALO_1642; SHALO_1970; SMUL_2226), which is described to mediate influx of Mg^{2+}, Co^{2+} and Mn^{2+} in *Escherichia coli* (Park, Wong and Lusk 1976) and Mg^{2+}, Co^{2+} and Ni^{2+} in *Helicobacter pylori* (Pfeiffer *et al.* 2002). Moreover, they express the Mg^{2+} and Co^{2+} efflux protein CorC (SHALO_1845; SMUL_2091). There is also the $Co^{2+}/Zn^{2+}/Cd^{2+}$ efflux system protein CzcD (SHALO_1864; SMUL_2110) highly expressed, which was described to be involved in the regulation of heavy metal resistance in *Ralstonia* sp. strain CH34 (Anton *et al.* 1999) and characterized as a Cd^{2+}, Co^{2+} and Zn^{2+}/H^{+}-K^{+} antiporter involved in divalent cation and potassium homeostasis in *Bacillus subtilis* (Guffanti *et al.* 2002). In addition to these three transporter proteins, the cation efflux system proteins CusA (SHALO_2839; SMUL_3074) and CusB (SHALO_2840; SMUL_3075) were slightly expressed. These proteins mediate resistance to copper and silver in *E. coli* (Franke *et al.* 2003, Outten *et al.* 2001). CusA is also annotated as the cation efflux system protein CzcA, which is essential for cobalt, zinc and cadmium resistance, but with a low cation transport activity for Co^{2+} (UniProtKB: P13511; P94177). Lastly, the genomes of *S. halorespirans* and *S. multivorans* encode for the *nikKMNQO* energy-coupling factor (ECF) Ni^{2+} transport system (SHALO_1589-1593; SMUL_1631-1635). The genes *nikK* and *nikO* encode for the periplasmic component and the ATP-binding protein, respectively, and are highly expressed. In addition, the membrane-bound component NikN could be identified in the proteome. The substrate specific component NikM and the permease NikQ could not be detected in the proteome, possibly due to difficulties in the enrichment of membrane-bound proteins. The complex can also transport Co^{2+} with a low affinity in *E. coli* (Rodionov *et al.* 2006). All these expressed proteins are able to transport Co^{2+} and mediating the Co^{2+} homeostasis.

Effects of Co^{2+} limitation on organohalide respiration

The ability of the organism to perform reductive dehalogenation was tested on a liquid growth medium containing 10 mM PCE as terminal electron acceptor and 40 mM lactate as electron donor. *S. multivorans* cannot grow on lactate as sole energy and carbon source due to the high redox potential of the pyruvate/lactate pair (E_0' = -190 mV) (Thauer, Jungermann and Decker 1977). Lactate is presumably oxidized to pyruvate gaining NADH, which has to be further oxidized to balance the reducing equivalents. Since the reduction of protons to hydrogen gas with electrons from NADH oxidation is thermodynamically not feasible, *S. multivorans* is not capable of lactate fermentation. However, in syntrophic interactions with hydrogen consuming bacteria, such as *Dehalococcoides*, *S. multivorans* was shown to grow fermentatively on lactate (Kruse *et al.* 2019). Under these conditions *S. multivorans* grew to a final protein concentration above 200 µg/ml corresponding to an OD_{578nm} > 0.15 (Fig. 2B). When the culture was transferred on Co^{2+}-depleted medium, growth was strongly affected, and the cells grew slower after each transfer finally reaching a final protein concentration of almost 60 µg/ml (OD < 0.06). Growth is strictly dependent on the amount of remaining Co^{2+} after each transfer to a new batch culture and the impact of Co^{2+} limitation can be monitored as a limitation of growth.

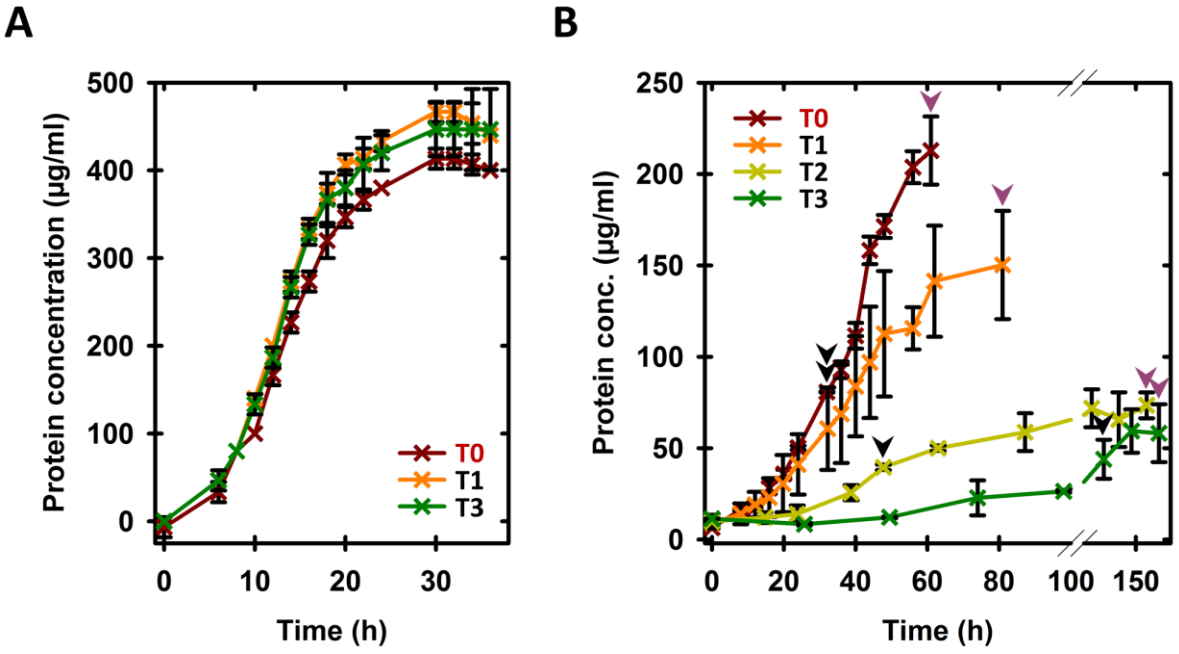

Figure 2: Growth curves of *S. multivorans* cells under cobalt limitation. (A) The cells were grown on 40 mM lactate and 40 mM nitrate-containing medium for initially 7 transfers. Three growth curves are plotted: T0 – growth in the presence of $CoCl_2$; T1 – first and T3 – third transfer lacking $CoCl_2$. The growth of three biological replicates is shown. (B) The medium contains 40 mM lactate and 10 mM PCE. Four growth curves are plotted: T0 – growth in the presence of $CoCl_2$; T1 – first, T2 – second and T3 – third transfer on $CoCl_2$ lacking medium. Two biological replicates are shown. For RNA isolation, cells were harvested during the exponential growth phase (⮟). For AdeNCba extraction and PceA analyses, cells were harvested at the end of the cultivation (⮟).

Each batch cultivation was inoculated with 10% of the previous culture. After inoculation of the liquid cultures the concentration of metal ions was quantified (Tab. S2). Fig. 3 displays a selection of biological relevant trace elements. From 33.1 µg/l, the initial Co^{2+} concentration decreased stepwise to 3.2 µg/l after the first transfer, to subsequently 0.4 µg/l and 0.2 µg/l. The difference compared to the theoretical amount might result from sorption effects of Co^{2+} and the glass serum bottle used for cultivation. Other trace elements did not change in their amount. Zn^{2+} was measured remarkably four times higher than the theoretical amount, potentially due to chemical impurities mainly in $FeSO_4$ and $NaHCO_3$.

The Co^{2+} dilution resulted in lower levels of the norcobamide cofactor produced by the cells (Fig. 4A). The enzyme activity of PceA measured in crude extract decreased in a correlating manner. The immunoblot demonstrates the decline of PceA in the cells (Fig. 4B). Without Co^{2+}, the biosynthesis of the cobamide cofactor cannot be completed resulting in a reduced total amount of PceA. This leads finally to a deficiency in OHR although the inductor of the OHR gene region, PCE, is still present. This implies that the presence of the cobamide cofactor is essential either for the *pceA* gene expression or for the stability of the cytoplasmic precursor of the enzyme.

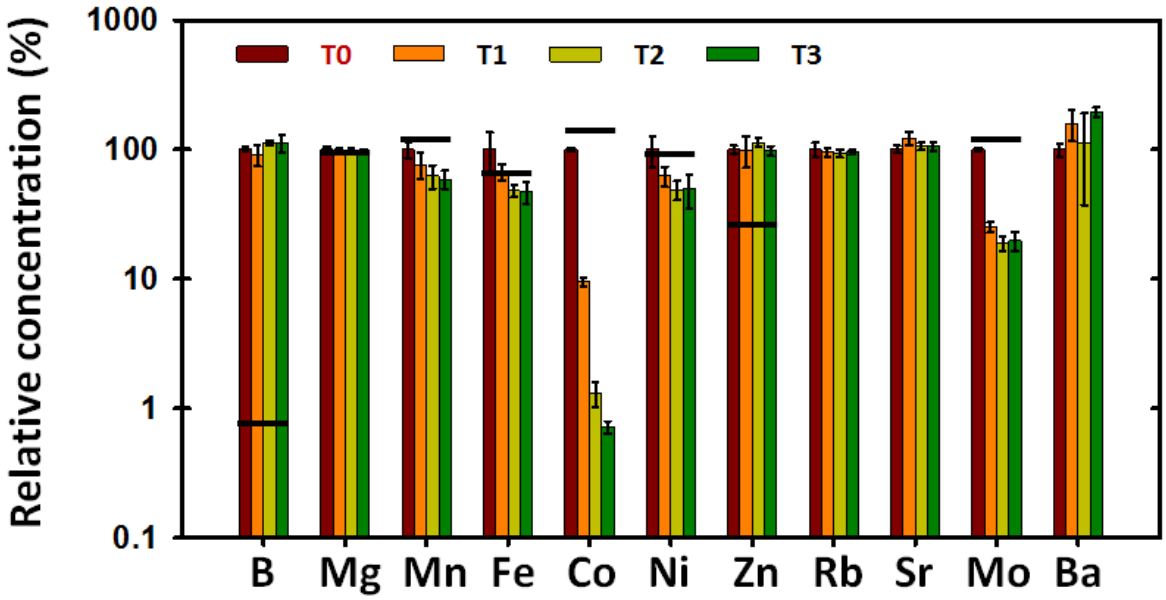

Figure 3: Relative concentrations of trace elements in the cultivation media of *S. multivorans* measured after inoculation. The values were normalized to the measured values of the Co^{2+}-containing T0 medium (≙ 100%). Theoretical values added as supplement solution (SL10) were marked as black line. Two biological replicates were measured in technical triplicates.

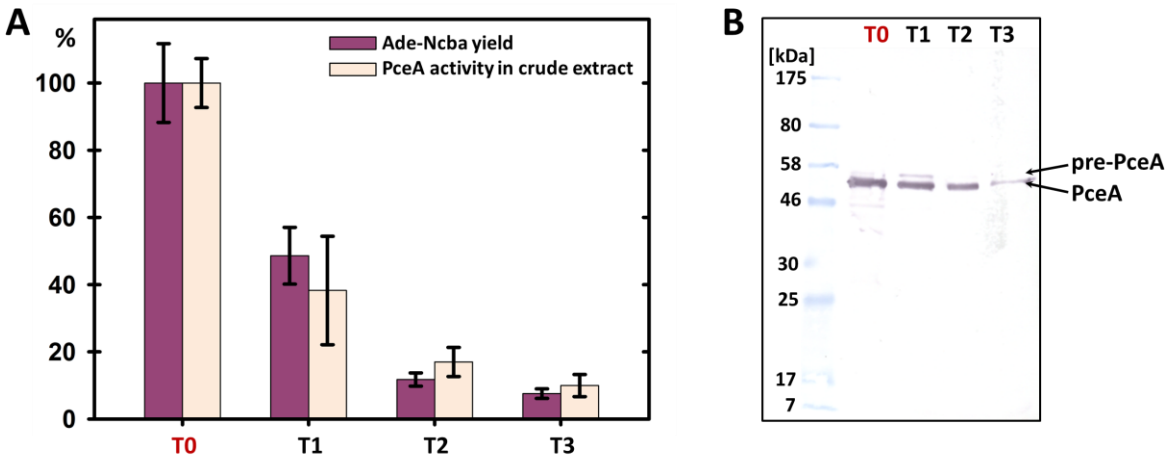

Figure 4: Decline of norpseudo-B_{12} (AdeNCba) and PceA biosynthesis under Co^{2+} limitation. *S. multivorans* was cultivated on a medium containing 10 mM PCE and 40 mM lactate. Four samples are plotted: T0 – growth in the presence of $CoCl_2$; T1 – first, T2 – second and T3 – third transfer on $CoCl_2$ lacking medium. (A) AdeNCba yield (100% = 0.58 µmol/g protein) and the PceA specific enzyme activity in crude extract (100% = 76.5 nkat/mg). The plot represents results from two biological and two to four technical replicates. (B) PceA immunoblot of one replicate.

Effects on OHR gene regulation

The RT-PCR analyses of the two biological replicates sampled at the time points noted in Fig. 2B demonstrated that the transcript levels of *pceA* and *cbiB* (the first gene of the norcobamide biosynthesis), although still present, decreased under Co^{2+} limitation (Fig. 5). Whether the amounts of Co^{2+} and AdeNCba also affect OHR gene expression in the presence of an alternative electron acceptor was focused in a second approach. For these analyses *S. halorespirans* was chosen as preferred organism, since it represents the archetype along the dehalogenating *Sulfurospirillum* spp. with an unaltered version of the OHR gene region (Goris *et al.* 2017).

For studying the PCE-dependent induction of OHR under Co^{2+} limitation *S. halorespirans* was cultivated on pyruvate and nitrate rather than PCE for 66 transfers (approximately 200 bacterial generations) followed by three transfers in the absence of Co^{2+}. When the culture was transferred to a PCE-containing medium lacking Co^{2+} the cultures grow slowly but surely to a final OD_{578} of 0.4 within five days, whereby the culture is not fermenting pyruvate as one gene encoding a hydrogenase subunit involved in producing hydrogen from pyruvate oxidation is disrupted by a transposase gene (Goris *et al.* 2017). When the amount of Co^{2+} is limited, OHR must still proceed, whereby growth was slowed down due to the limited availability of functional PceA. Consequently, PCE-dependent induction of OHR seems to be unaffected of low Co^{2+} concentrations.

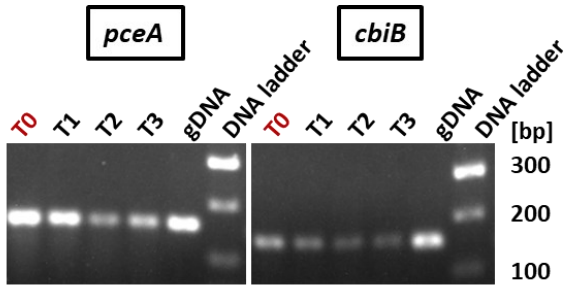

Figure 5: RT-PCRs of *pceA* and *cbiB* from samples plotted in Fig. 2B. The PCR was stopped after 21 cycles. Genomic DNA (gDNA) was used as control.

Catalytically active PceA (Goris *et al.* 2014, John *et al.* 2009) as well as other proteins encoded in the OHR gene region (Türkowsky *et al.* 2018) were previously observed to be downregulated over a long term of at least 100 generations. This retentive memory effect seems to exclusively exist for the PCE regulon, since other enzymes like the nitrate dehydrogenase NapA were confirmed to undergo a short downregulation within two transfers on fresh medium in the absence of nitrate, which corresponds to less than ten generations (Fig. 6).

The retentive memory effect cannot be explained by the function of the TCS itself. The signal keeping the gene expression ongoing is not part of the culture medium and must be produced by the cells. There might be an internal effector molecule encoded keeping the transcription ongoing in *S. halorespirans* and *S. multivorans*. Co^{2+} and AdeNCba are potential candidates, which could affect OHR gene regulation. Cobalamin (Cbl) riboswitches have been identified in the genomes of the organohalide-respiring bacteria *Dehalococcoides mccartii* (Johnson *et al.* 2009, Men *et al.* 2012), *Desulfitobacterium hafniense* (Choudhary *et al.* 2013) and *Dehalobacter restrictus* (Rupakula *et al.* 2015). However, within the OHR gene region no *cis*-regulatory Cbl, adenosylcobalamin (AdoCbl), and AdoCbl-variant riboswitches could be identified. In addition, The 5'-untranslated regions harbor neither Ni^{2+}- nor Co^{2+}-binding riboswitches, which were described to bind metal cooperatively with affinities in the low micromolar range and regulate the expression of Co^{2+} transporters in *Clostridium scindens* (Furukawa *et al.* 2015).

In order to further evaluate a Co^{2+}-dependence in the downregulation of the OHR, *S. halorespirans* was transferred from a 10 mM PCE- to a 40 mM fumarate-containing medium. With fumarate as the terminal electron acceptor, the cells do not show any Co^{2+} sensitivity and grow as usual to a maximum OD_{578} of 0.7. After ten transfers, the amount of AdeNCba per entire protein mass averages 3% compared to the initial cultivation on PCE (Fig. 7A). In addition, the amount of PceA is not detectable on the immunoblot (Fig. 7B). Via RT-qPCR, the change in gene expression was measured again for *pceA* and *cbiB* (Fig. 8). The genes seem to be transcriptionally downregulated in a shorter period of time, when Co^{2+} and AdeNCba were absent. Under standard conditions ($+Co^{2+}$) the transcript level of *pceA* decreased 1.6-fold after ten transfers on fumarate-containing medium to an amount of 62% compared to the PCE-containing initial cultivation. Under Co^{2+} limitation ($-Co^{2+}$) the transcript level decreased even further (5.2-fold) to an amount of 19%. The *cbiB* transcript was measured to be slightly (1.6-fold) increased under Co^{2+}-containing conditions, whereas it decreased 2.9-fold to an amount of 34% when Co^{2+} is limited. Comparing the $+Co^{2+}$ and the $-Co^{2+}$ conditions, the p values were calculated to be 0.09 and 0.03 for the

pceA and the *cbiB* transcript, respectively. The change in *cbiB* gene expression is significant considering a significance level of 0.05. In conclusion, the amount of AdeNCba has a small impact on the long-term downregulation in *S. halorespirans*. The availability of Co^{2+} and the biosynthesis of AdeNCba delay the downregulation by a factor of three, but the retentive memory effect is still persistent.

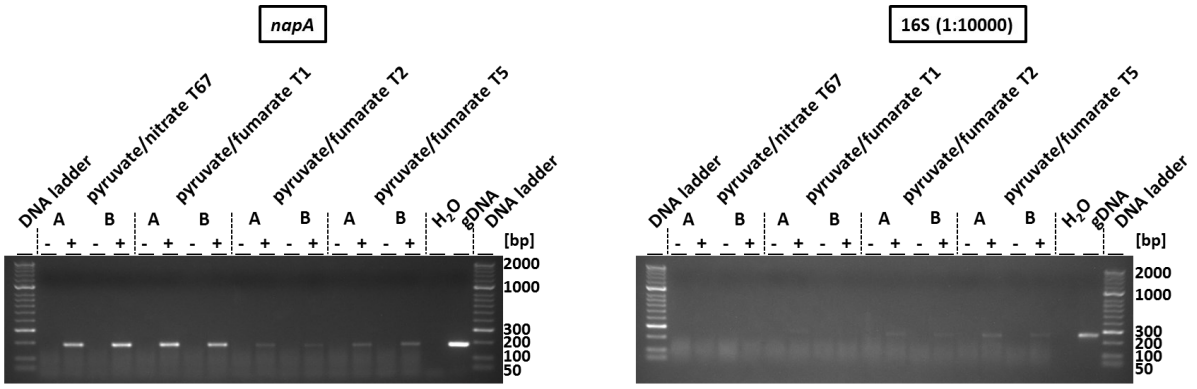

Figure 6: RT-PCR of the nitrate reductase gene *napA*. *S. halorespirans* was transferred 67 times on 40 mM pyruvate and 40 mM nitrate-containing medium. The culture was further transferred on a medium containing 40 mM fumarate rather than nitrate. Samples were harvested after the first, second and fifth transfer. Two biological replicates, A and B, are presented with an RT minus (-) control as well as an RNA minus (H_2O) and a genomic DNA positive control (gDNA). 16S rRNA served as reference transcript. The PCR was stopped for *napA* and 16S after 21 and 16 cycles, respectively.

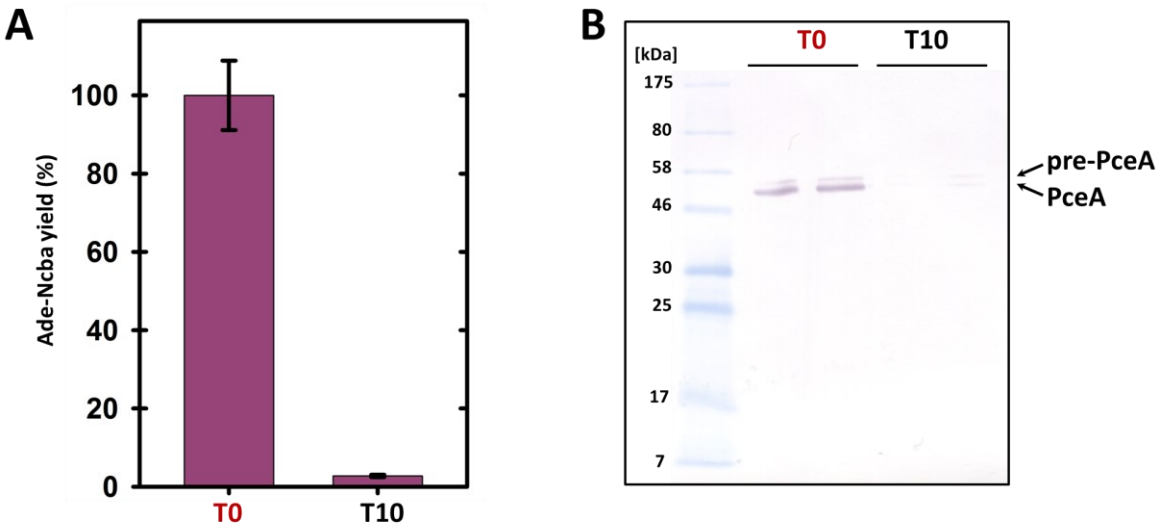

Figure 7: Decline of norpseudocobalamin (AdeNCba) and PceA biosynthesis in *S. halorespirans* cells cultivated under Co^{2+} limitation. The results of biological and technical duplicates are plotted. T0 – growth in the presence of 40 mM pyruvate, 10 mM PCE and $CoCl_2$; T10 – tenth transfer on 40 mM pyruvate and 40 mM fumarate-containing medium lacking $CoCl_2$. (A) The relative amounts of AdeNCba per entire protein concentration (100% = 2.2 µmol/g). (B) Immunoblot of PceA.

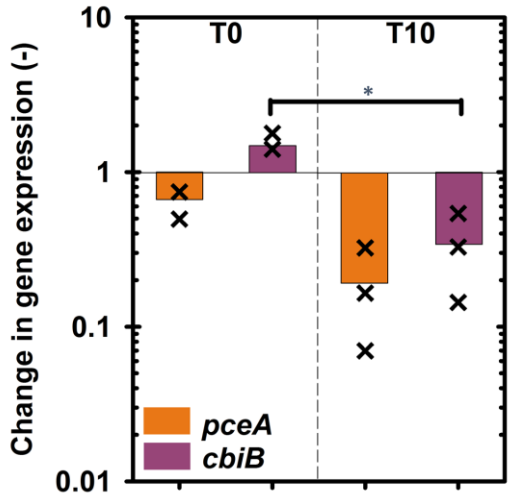

Figure 8: Co^{2+}-dependent changes in gene expression during the long-term downregulation in *S. halorespirans*. The changes in gene expression of *pceA* and *cbiB*, first gene of the norcobamide biosynthesis genes, after 10 transfers on 40 mM pyruvate and 40 mM fumarate with and without $CoCl_2$ are plotted. The original cultivation T0 was performed on a 40 mM pyruvate and 10 mM PCE-containing medium with $CoCl_2$. Two to three biological replicates are shown.

The retentive memory effect is also detectable in *S. multivorans* cells. An RT-PCR of *pceA* was performed with *S. multivorans* cultures cultivated seven passages on nitrate-containing medium and seven further passages on nitrate-containing medium lacking Co^{2+} (Fig. 9). While the *pceA* transcript is detectible in this sample, another sample analyzed after 67 transfers with nitrate as electron acceptor, confirms the entire downregulation of *pceA*. Therefore, the availability of Co^{2+} and the intracellular amount of AdeNCba has only a minor role in the transcriptional regulation of OHR in *Sulfurospirillum* species.

In another approach, it was verified whether the Cba structure affect the long-term downregulation in *S. multivorans*. The lower ligand was changed by supplementing the medium with 25 µM 5-hydroxybenzimidazole (5-OH-Bza), which does not inhibit the PceA enzyme activity as it was described for DMB (Keller *et al.* 2014). This stopped the biosynthesis of AdeNCba and 5-hydroxybenzimidazolyl norcobalamin was synthesized instead (Keller *et al.* 2018). The long-term downregulation was monitored by use of RT-qPCR of samples taken from the initial cultivation with PCE as terminal electron acceptor and after 10, 20, and 30 transfers on medium containing nitrate rather than PCE (Fig. 10). The retentive memory effect was recorded and significant decreases in the RNA levels of *pceA* and *cbiB* were not detectable until transfer 30. The Cba structure does not seem to affect OHR gene regulation. There is no change in gene expression of the response regulator of TCS2. This is consistent with the assumption that this TCS is responsible for the PCE-dependent induction of the OHR gene cluster, which is likely due to comparative genomic data of the non-dechlorinating *S. multivorans* strain N, which harbors an insertion sequence in the TCS2 response regulator (Chapter 2.1).

In the end, OHR gene expression is transcriptionally downregulated in particular when Co^{2+} limitation affects the energy metabolism of the cells, i.e. when the cells cannot ferment or use an alternative electron acceptor. When fumarate served as electron acceptor, growth was not affected and the impact on gene transcription was less distinct. Since the PceA protein level is far more reduced than the respective transcript level, its downregulation occurs posttranscriptionally and most likely due to PceA protein

instability when AdeNCba is missing. Since the retentive memory effect was still proven, it must have another origin and Co^{2+} limitation seems to play a subordinate role in OHR gene regulation. The role of the memory effect remains doubtful. It might be beneficial when PCE appears only as trace element in the environment. In addition, the organism cannot take up entire cobalamin, but is restricted to synthesize its norcobamide cofactor *de novo*. The biosynthesis is however limited to the availability of cobalt. Hence, it is beneficial for the organism to store cobalt in the form of stable and atoxic cobalamin.

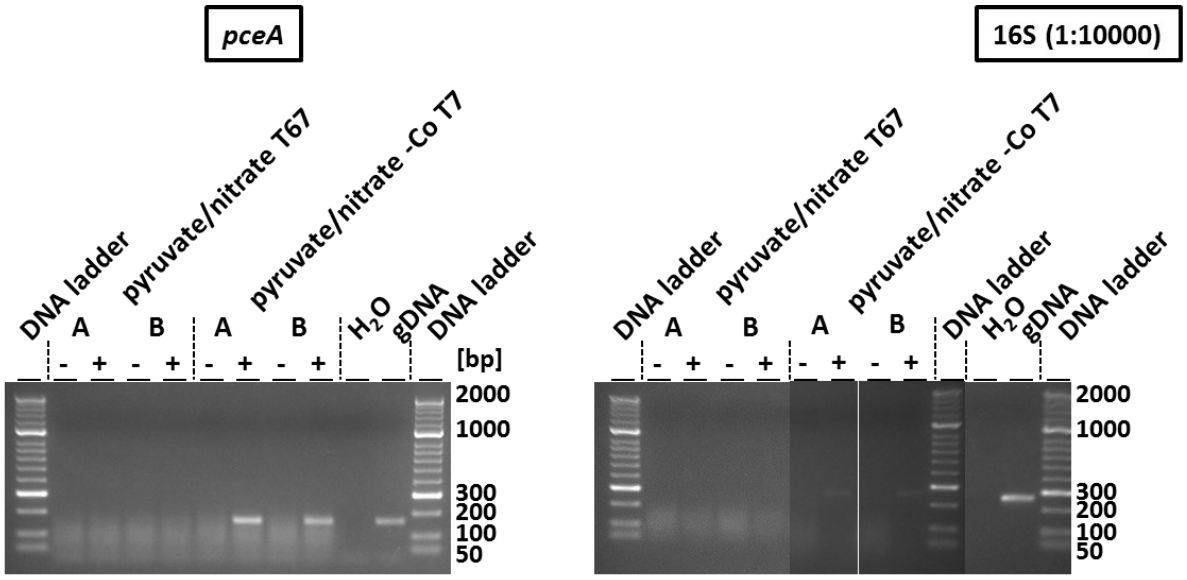

Figure 9: Comparative RT-PCR of *pceA* from two *S. multivorans* cultures. The first culture was transferred 67 times on 40 mM pyruvate and 40 mM nitrate-containing medium. The second culture originate from a PCE-growing stock and was transferred for seven passages on nitrate rather than PCE and subsequently for another seven passages on the same nitrate-containing medium lacking Co^{2+}. Two biological replicates, A and B, are presented with an RT minus (-) control as well as an RNA minus (H_2O) and a genomic DNA positive control (gDNA).

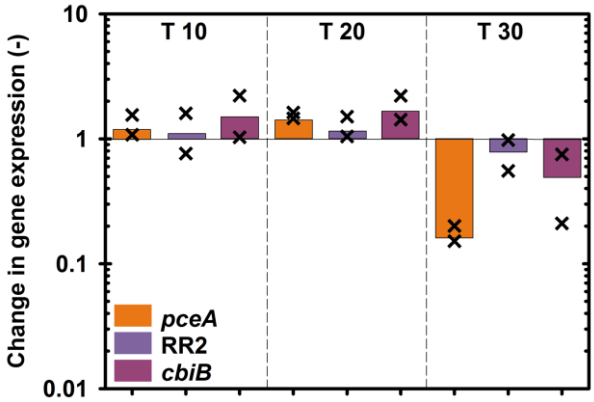

Figure 10: Changes in gene expression of *pceA*, RR2 and *cbiB* of *S. multivorans* samples harvested after 10, 20 and 30 transfers on a medium containing 40 mM pyruvate, 40 mM nitrate and 25 µM 5-OH-Bza are plotted. The data were normalized to the original cultivation T0, which was performed on a 40 mM pyruvate and 10 mM PCE-containing medium without 5-OH-Bza. Two biological replicates are shown.

Conclusion

Co^{2+} is an essential trace element not only for dehalogenating *Sulfurospirillum* species but also for the microbial community that relies on the availability of Cba, including other organohalide-respiring bacteria such as *Dehalococcoides* and *Desulfitobacterium*. However, Co^{2+} is next to Cd^{2+} and W^{6+} the rarest of all known essential trace elements. Therefore, Co^{2+} limitation is the rule rather than an exception for microorganisms that need bioavailable Co^{2+} for their metabolism, e.g. for Cba biosynthesis. Under laboratory conditions *S. multivorans* produces up to 0.5 mg/l AdeNCba (Schubert 2017). At Co^{2+} concentrations corresponding to the amounts measured in natural habitats the AdeNCba production attenuates accordingly by two orders of magnitude. Under these ecologically relevant conditions, reductive dehalogenation is less effective than described in former literature, since the amount of reductive dehalogenase is reduced to a minimum. Although Co^{2+} limitation causes a marginal transcriptional downregulation, most of the gene product is removed posttranscriptionally. Furthermore, the growth rate on PCE is getting more comparable to that of other dehalogenating bacteria, indicating that the exceptional high growth rates of *Sulfurospirillum* are the result of artificial growth conditions.

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3 Discussion

Bacteria use TCSs to sense various environmental stimuli and to respond to changing conditions by adjusting gene expression. In contrast to all other organohalide-respiring bacteria, *Sulfurospirillum* species mediate signal transduction of environmental halogenated organic compounds via membrane bound classical TCSs. The following chapters address the characterization of this TCS and its important role in the transcriptional regulation of the PCE regulon. For this purpose, the methods and results gained from the manuscripts will be discussed. The omics results assembled and compared, complemented by the conclusions of the electrophoretic mobility shift assays (EMSAs), will illuminate the mechanism of PCE-mediated induction of OHR gene expression in *Sulfurospirillum* species harboring a functional OHR gene cluster. The retentive memory effect observed during the downregulation of the OHR gene cluster indicates an interplay of the TCS with an unknown effector molecule. Based on the cobalt limitation analysis and the acetylome, norcobamides, acetyltransferases, and deacetylases will be evaluated and discussed as potential candidates together with conceivable alternatives.

3.1 Comparative omics analyses of *Sulfurospirillum* species

The simplest way standardly used to determine the role of a regulator would be the creation of deletion mutants with a transcription factor that is knocked out. Afterwards, changes in the bacteria's phenotype and the expression profiles of relevant genes could be gathered. Accessory proteins that are involved in gene regulation could be identified in the same way. Unfortunately, no gene editing method was established for *Sulfurospirillum* species and until now there is only one mutant of *S. multivorans* available (Kunze *et al.*, 2017b). Therefore, other methods were utilized. The basis was the complete genome sequence of two dehalogenating species, *S. multivorans* (T. Goris *et al.*, 2014) and *S. halorespirans* (T. Goris *et al.*, 2017), which allowed the localization of the two reductive dehalogenase genes, *pceA* and *rdhA*, as well as the AdeNCba biosynthesis genes. All genes are located in close vicinity to each other together with two TCSs and a *tetR*-like repressor gene. The repressor gene is disrupted by an insertion element in *S. multivorans*. Since both organisms share the same PCE dechlorinating phenotype and exhibit the same retentive memory effect (Chapter 2.2), it can be concluded that the repressor has no essential effect on the transcriptional regulation of OHR. Although it was not possible to create deletion mutants in order to investigate the role of the TCSs, fortunately, two natural mutants could be identified (Chapter 2.1). The complete genome sequence of *S. multivorans*

strain N, which was coisolated together with *S. multivorans* and described as a non-dechlorinating strain (Siebert *et al.*, 2002), revealed that the RR gene of TCS2 is disrupted by a transposase gene. In addition, the complete genome of *Sulfurospirillum* sp. JPD-1 was sequenced. This species was originally described as dechlorinating, but the strain that was ordered from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and sequenced was not capable of dechlorinating PCE. Indeed, similar to *S. multivorans* strain N, the RR of TCS2 is disrupted by another transposable element. Consequently, TCS2 could be identified as essential transcriptional regulator of OHR.

The most powerful method used within this work was the dRNA-seq performed in cooperation with Prof. Dr. Cynthia Sharma at the Institute for Molecular Infection Biology at the University of Würzburg, who developed the method in 2010 (Sharma *et al.*, 2010). The method enabled the identification of eight transcriptional units in *S. multivorans* that are regulated PCE-dependently and their respective TSSs (Chapter 2.1). Neither internal nor orphan TSSs are located within the OHR gene region. With the knowledge of the TSS locations, the promoter regions could be characterized including the identification and classification of the -35 and -10 boxes. Interestingly, *rdhAB* transcription was not induced by PCE and no TSS was measurable. Nevertheless, the promoter region could be predicted by aligning the sequence upstream of *rdhA* and the untranslated sequences of the other eight transcripts. All promoters share -35 and -10 boxes with the typical consensus sequence of the housekeeping σ^{70} factor (Pribnow, 1975; Siebenlist *et al.*, 1980), which is in accordance to the transcription of the OHR genes during the exponential growth phase. No transcript of TCS1 was detected in the absence of PCE and only small amounts in the PCE-containing sample. If this TCS is functional, it will require a first step PCE induction before enough gene products are synthesized to guarantee signal transduction of a yet unknown ligand. Whether TCS1 induces *rdhAB* transcription and whether the ligand is also the substrate of RdhA remains unknown. However, TCS2 showed a basal transcription in the absence of PCE and an autoregulation as it is commonly described for TCSs (Groisman, 2016). Regulatory sRNAs do not seem to be involved in the regulation of OHR, since the only sRNA detected within the gene cluster was an anti-sense RNA (asRNA) of the transposable element disrupting the *tetR*-like repressor gene in *S. multivorans*. The expression profile on the RNA level confirmed the expression profile measured in the proteomes of *S. multivorans* (T. Goris *et al.*, 2015a) and *S. halorespirans* (Chapter 2.2).

An overview of all omics data of the OHR gene region is summarized in Fig. 3.1. In the presence of the inductor *pceAB* was the most upregulated operon, had the most RNA reads, and PceA

was the most abundant protein of the PCE regulon, followed by the expression of the IscU/NifU-like protein (SHALO_1497 and SMUL_1533), which might help in the maturation of FeS clusters in PceA, and the last four genes of the gene cluster encoding putative redox proteins (SHAL_1534-1537 and SMUL_1573-1576), particularly an FeS cluster binding motif-containing flavoprotein, a Rieske-like domain-containing redox protein, a putative flavin mononucleotide (FMN)-binding protein, and a putative membrane protein. TCS1 and the first gene of the OHR gene cluster encoding an alkylhydroperoxidase AhpD family protein (SHALO_1494 and SMUL_1530) were the weakest upregulated genes. The HK and the RR of TCS1 were even not detectable in the proteomes. Differences in the abundances of proteins encoded on the same operon result from posttranscriptional regulations, such as RNA degradation, different translation rates, codon usage, amino acid composition, and protein stability (Abreu *et al.*, 2009; van Assche *et al.*, 2015), or from lower capturing rates of small and membrane-associated proteins (T. Goris *et al.*, 2015a). The determination of TSSs also allowed the characterization of the 5′ UTRs, which were extensively large for *pceAB* (134 bp) and *rdhAB* (115 bp estimated) enabling several and more complex secondary structures, which might affect translation efficiency and RNA stability. The simplest secondary structure is a single stem loop structure, which could be predicted for the 5′ UTRs of the first transcript as well as for the transcripts of TCS1, TCS2, the putative membrane protein (SHALO_1504 and SMUL_1540), the *pceMN*/AdeNCba transcript and the last transcriptional unit of the PCE regulon. The determination of the 3′ UTRs was however difficult, since no transcriptional terminators stop the transcription suddenly. On the contrary, the amount of RNA reads decreased gradually, i.e. some transcripts have a longer 3′ UTR with the capacity of more putatively regulatory secondary structures than others. After 60 transfers and more than 200 cell divisions most of the proteins were still detectable, but almost completely downregulated, except for TCS2. The RR of TCS2 was the most abundant protein of the PCE regulon in this sample.

The acetylomic analysis based on the method, which had been used successfully for *E. coli* and *B. subtilis* (J. Zhang *et al.*, 2009; Schilling *et al.*, 2015). Acetylations could be classified by the characteristic MS^2-shift of the y-ions upstream and the b-ions downstream of the acetylated lysine. The MS^2-shift of +42.011 Da could be distinguished from the shift of the isobaric trimethylation, which had a shift of +42.047 Da, due to the high resolution of the Orbitrap Fusion mass spectrometer and by manual inspection of the MS^2-spectra for the occurrence of a specific immonium-ion at m/z = 126 (Downey and Baetz, 2016; Ouidir *et al.*, 2016). The lysine acetylation pattern changed during the cultivation. After six transfers on PCE-free medium most of the proteins were still quantifiable and present in a large amount. At this time point, the

acetylation varied compared to the PCE-containing sample. Most interestingly, TCS2, which is essential for PCE signal transduction, was highly acetylated when the inductor was absent. After 60 transfers no lysine acetylations were detectable, especially due to the reduced protein amounts.

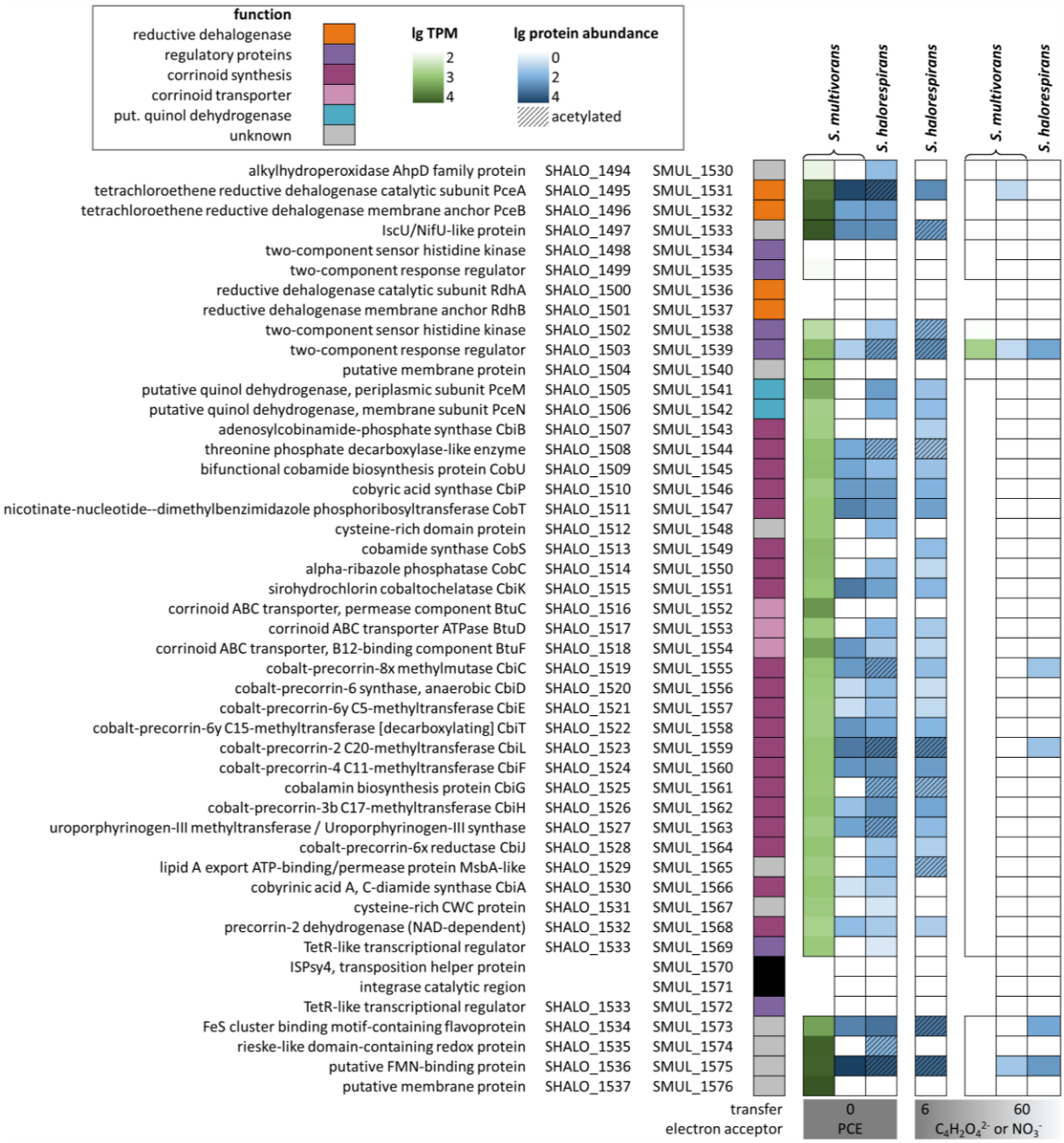

Fig. 3.1: Summary of the omics data of the OHR gene region. The transcripts in *S. multivorans*, the proteins in both *S. multivorans* and *S. halorespirans*, and the acetylome in *S. halorespirans* in the presence (transfer 0) and absence of the inductor PCE (transfers 6 and 60) are compared. Alterative electron acceptors used in the cultivations for the transcriptomic or proteomic analyses were fumarate and nitrate, respectively. Protein descriptions and locus

tags are given on the left side. Colored squares adjacent to the protein locus tags indicate their function in OHR. The transcriptome was quantified in duplicates (green, color code on top, logarithmized average of normalized transcripts per kilobase million, TPM). Both the proteome and the acetylome were quantified in triplicates (T. Goris *et al.*, 2015a; Türkowsky *et al.*, 2018b) and visualized together (blue, color code on top, normalized and logarithmized average of top three peptide MS^1-area, hatched when acetylation was quantifiable in > 50% of all replicates).

All omic approaches have both advantages and disadvantages. Genomic data conceal information about the physiological state of a bacterium, but serve as a starting point for further experiments and are a prerequisite for transcriptomic and proteomic mapping (Kube *et al.*, 2005; Seshadri *et al.*, 2005; T. Goris *et al.*, 2014). Although genomes of organohalide-respiring bacteria from all corresponding phyla are available, global transcriptome studies have been conducted only with *D. mccartyi* and *De. hafniense* (Türkowsky *et al.*, 2018a). Most published transcriptomes were gained from microarray data. RNA-seq data are only available for *D. mccartyi* CG (S. Wang *et al.*, 2014c) and for the consortium *D. mccartyi* HiTCE (Men *et al.*, 2017). Studies that compare transcriptomic and proteomic datasets elucidated that the expression profiles often do not correlate (P. K. H. Lee *et al.*, 2012; Men *et al.*, 2012), which might be influenced by the methodological deficits of microarrays. In the present study, RNA-seq data were compared to recent proteomic datasets. It was shown that the discrepancy between transcriptomic and proteomic data was less severe and, on the contrary, it predominantly correlated within the OHR gene region. The transcriptome provided a more complete picture of gene expression and information about promoter and untranslated regions as well as non-translated regulatory elements, such as riboswitches or sRNAs (Sharma and Vogel, 2014; Creecy and Conway, 2015; Thomason *et al.*, 2015). However, the biochemical machinery of an organism is in general more accurately reflected by the proteome. While the first proteomic approaches of organohalide-respiring bacteria covered less than 9% of the proteome (Morris *et al.*, 2006; Morris *et al.*, 2007), the yield increased in recent approaches up to 72%, but low abundant, small, and membrane-integral proteins were still difficult to identify (Rowe *et al.*, 2012; Schiffmann *et al.*, 2014; Depke *et al.*, 2015; Schiffmann *et al.*, 2016). Thus, the small, membrane-integral RdhB was previously detected in only one proteome study of *D. mccartyi* (Kublik *et al.*, 2016) and its homologue PceB only in the proteome of *S. multivorans* (T. Goris *et al.*, 2015a). There are a few proteomic studies published for organohalide-respiring bacteria

(Türkowsky *et al.*, 2018a). The present work provides the second proteome of a *Sulfurospirillum* species with 68% protein coverage, 29% higher than in the previous study on *S. multivorans*. The combination of transcriptomics and proteomics offsets the disadvantages given above and is crucial to understand the whole picture of regulation and induction processes in bacterial cells.

3.2 Regulation of the organohalide respiration in *Sulfurospirillum* species

Comparative genomics revealed that the TetR-like repressor is not essentially involved in OHR gene regulation. The respective gene is disrupted in *S. multivorans*, which exhibits the same dehalogenating phenotype as *S. halorespirans*, which harbors a functional *tetR* gene. Both organisms are indistinguishable in the up- and downregulation of the PCE regulon. Furthermore, the transcriptomic and proteomic studies identified TCS1 as not transcribed when nitrate or fumarate is the sole electron acceptor rather than PCE. In addition, the RR of TCS1 is lacking the REC domain in '*Candidatus* S. diekertiae' sp. SL2-2 and is therefore most certainly not functional. Nevertheless, this species has a PCE dechlorinating phenotype (Buttet *et al.*, 2018). Therefore, TCS1 is certainly not essential and even not available to sense an environmental signal and induce gene expression as long as TCS2 is not inducing TCS1 gene expression. *S. multivorans* encodes more than 50 TCSs. In the transcriptome almost all of them had a basal transcription enabling their functionality. Thus, TCS2 remains the only regulatory system responsible for the induction of OHR, which was confirmed by both the basal transcription of the TCS2 genes and the fact that transposable elements disrupting the RR gene of TCS2 result in a non-dehalogenating phenotype as it was demonstrated for *S. multivorans* strain N and *S.* sp. JPD-1 introduced in this work (Chapter 2.1). Interestingly, both non-dehalogenating species feature a basal transcription of the 5′ fragment of TCS2 until the gene is disrupted by the respective insertion sequences. This observation substantiates the basal transcription and its theoretical functionality as TCS. In addition, the transcriptome of *S. multivorans* displayed the PCE-dependent upregulation of the transcription of the TCS2 operon indicating an autoregulation of this regulatory system. Indeed, this autoregulation could be confirmed by the proteome study of *S. halorespirans*. Such an autoregulation is typical for TCSs and provides opportunities for fine-tuning expression of a group of target genes controlled by a given regulator (Groisman, 2016). When an organism faces inducing conditions, the number of phosphorylated RR is

initially low, but increasing as the RR promotes its own transcription and thereby creating additional substrates that are phosphorylated by the cognate HK. In the case of the PhoP/PhoQ system in *Sa. enterica* serovar Typhimurium, the regulator PhoP binds initially only those promoters that have a high affinity binding site as well as those that are not a subject of silencing proteins (Zwir *et al.*, 2012; Zwir *et al.*, 2014). As the amount of phosphorylated RR increases, low-affinity promoters will be occupied and those that are subject of gene silencing. This results in an expression hierarchy that may reflect physiological responses as it was described for the PhoB/PhoR system in *E. coli*, which is activated under phosphate limiting conditions (Gao and Stock, 2015). PhoB is autoregulated and induces first the expression of genes directly involved in the phosphate uptake and afterwards genes that encode phosphate scavenging proteins (Gao and Stock, 2015). With this work (Chapter 2.1), the autoregulation was confirmed by means of electrophoretic mobility shift assay (EMSA) and a predicted hierarchy might reflect the dispersion of the RNA-seq intensities.

EMSA was first introduced in 1981 (Fried and Crothers, 1981; Garner and Revzin, 1981) and became one of the most popular techniques to detect interactions between proteins and nucleic acids (Lane *et al.*, 1992; Kerr, 1995; Fried and Garner, 1998; Molloy, 2000; Buratowski and Chodosh, 2001; Rippe *et al.*, 2001; Vigneault and Guérin, 2005; Hégarat *et al.*, 2008). The method basically uses a polyacrylamide gel electrophoresis (PAGE) for the separation of labeled pure nucleic acids and complexes of a protein and the labeled nucleic acid, which were created during a previous binding reaction. In this study, a new EMSA method was established for the functional characterization of RR2. Instead of a standard method with radioisotopes (Hellman and Fried, 2007), it based a fluorescence labeling method (Rye *et al.*, 1993) and the addition of the neutral osmolyte triethylene glycol to the gel before polymerization. While complexes with association binding constants $K_a > 10^9$ M^{-1} can be detected at physiological conditions of salt and pH, it was shown that triethylene glycol stabilizes complexes that have association binding constants in the range of 10^5-10^6 M^{-1} (Sidorova *et al.*, 2010). The stabilizing effect on protein-DNA complexes bases on the exclusion of osmolytes from exposed protein and DNA surfaces, while water is included. The extension of this effect depends on the affinities between osmolyte and DNA as well as between osmolyte and protein. Triethylene glycol is strongly excluded from the DNA and protein surfaces and likewise not particularly viscous (Sidorova *et al.*, 2010). Thus, it does not affect electrophoresis properties, but it stabilizes less specific binding reactions. The new method stabilized the RR2-DNA complexes and decrease the rate of complex dissociation significantly.

The RR of TCS2 was heterologously expressed and purified. Native PAGE results indicated that the overexpressed, unphosphorylated RR occurs in both an inactive monomer and an active dimer formation. An additional phosphorylation step with either acetyl phosphate or phosphorylated HK cytoplasmic domain was not necessary. The gel shift indicated that the RR binds DNA only as a dimer pair as it is typically described for RRs and other activating transcription factors (Browning and Busby, 2016). The assay confirmed that the RR of TCS2 specifically binds to the promoter sequences of all eight transcriptional units (Chapter 2.1). Other DNA sequences, such as the promoter sequence of a gene encoding a phage CI repressor superfamily protein that is located in the downstream flanking region of the OHR gene cluster and PCE-independently transcribed, were not bound by the regulator. Due to promoter DNA truncations the location of the *cis*-regulatory element (CRE) recognized by the RR could be further determined. Activators typically bind DNA sequences containing two nearly identical half-sites organized as direct, inverted or palindromic repeats. These sequences are usually located upstream of the core promoter site, i.e. upstream of the -35 box, in order to avoid steric clashes with the RNA polymerase (RNAP) holoenzyme, particularly with the σ-factor, which binds the -35 and -10 boxes for the initiation of gene transcription (Ruff *et al.*, 2015). These activators can be classified as class I transcription activators and usually make directly contact with the RNAP, e.g. with the C-terminal domain of the α-subunit (αCTD). More exceptional are class II transcriptional activators, which function by binding DNA sequences overlapping the core promoter region or even downstream of the TSS as it has been reported for *Caulobacter crescentus* GcrA (Wu *et al.*, 2018) or *Thermus thermophilus* transcription activator protein TTHB099 (Y. Feng *et al.*, 2016). Class II activators stimulate isomerization of an initially closed into an open complex of RNAP and promoter DNA (Y. Feng *et al.*, 2016). The CRE of the *S. halorespirans* TCS2 regulator has a consensus sequence identified as CTATW-N_{17}-CTATW overlapping the -35 box, except for the intergenic region between TCS2 and the upstream located SHALO_1504 encoding a putative membrane protein. In that case the CRE is located in the center of the intergenic region 85 bp upstream of each TSS and presumably bound by two RR dimer pairs. Therefore, the regulator functions as both class I and class II transcriptional activator. Such an independent transcriptional activation has been proven for the cyclic adenosine 3′, 5′-cyclic monophosphate (cAMP) receptor protein (CAP) in *E. coli* as well (B. Liu *et al.*, 2017a). On the one hand, CAP binds the *lac* promoter as a class I activator and facilitates RNAP binding (Busby and Ebright, 1999; Lawson *et al.*, 2004; Decker and Hinton, 2013). On the other hand, CAP binds the *gal* promoter as a class II activator and assists the RNAP in unwinding the DNA in the core promoter sequence (Busby and Ebright, 1999;

Lawson *et al.*, 2004; Decker and Hinton, 2013). The fact that the RR regulates its own transcription as a class I activator and therefore mechanistically different than the other OHR genes allows for a distinctly different core sequence overlapping the -35 box, which would be an explanation for the promoter providing both basal and autoregulated expression. According to the RNA quantities of the eight transcriptional units measured in the presence of PCE, the CRE sequence varies. The more imperfect the direct repeat of the CRE sequence, the lower the binding affinities indicated *in vitro* by EMSA and the lower the RNA level measured in the transcriptomic study. The promoter region of the *pceAB* operon and the overlapping TCS2/SHALO_1504 promoter share a direct repeat sequence of CTATA-N_6-CTATA-N_6-CTATA. Since TCS2 and SHALO_1504 were not transcribed in the amounts as *pceAB*, the class I transcriptional activation seems to be less efficient than the class II transcriptional activation. Whether the central repetition, which contains the -35 box, is involved in complex formation is yet unknown. The sequence TAT seems to be essential, since only the putative promoter of *rdhAB* misses this repetition and CTATA seems to be more attractive than CTATT.

In order to further understand the molecular mechanism of sensing, signal integration, and gene regulation, the protein sequences of TCS2 were blasted, conserved regions were identified, and the structure was predicted *in silico* and compared to other protein family members. HKs have a highly variable N-terminal input domain and a conserved C-terminal HK domain. The sensor kinase of TCS2 harbors a 170 amino acids long putative periplasmic sensing domain, which might have a *β*-galactosidase-, *β*-glucosidase- or glycoside hydrolase-like structure followed by seven transmembrane helices. The putative ligand PCE is available in the periplasm but accumulates also within the membrane due to its hydrophobicity. Therefore, PCE might be sensed directly as a ligand binding to either the periplasmic or the transmembrane domain. Another putative scenario could be that PCE is sensed in an indirect and more unspecific manner. Upon PCE accumulation in the cytoplasmic membrane, the membrane integrity and membrane lipid fluidity changes. These changes could potentially result in a conformational change of the transmembrane helices. The membrane-associated HK DesK from *B. subtilis* is a paradigm for such a molecular mechanism. The TCS DesR/DesK responds to changes in the membrane lipid fluidity upon a temperature shift and induces the synthesis of unsaturated fatty acids (Martín and de Mendoza, 2013). Another example is the PhoQ/PhoP system in Entero-bacteria including *E. coli* and *Salmonella*. This system senses different stimuli in the periplasm, such as Mg^{2+} limitation, low pH, and the presence of cationic antimicrobial peptides as well as an osmotic upshift by a conformational change within the transmembrane domain of PhoQ induced by a perturbation in cell membrane thickness and lateral pressure under hyperosmotic

conditions (Yuan *et al.*, 2017). It is also conceivable that the periplasmic and the transmembrane domain receive different input signals. While the periplasmic domain putatively binds PCE, the transmembrane domain might bind an effector molecule, such as a small membrane protein. The C-terminal domain of the HK is conserved, consists of a DHp and a CA domain, and has the highest conformity to the BaeS superfamily. The BaeR/BaeS TCS in *Salmonella* and *E. coli* responds to envelope stress (Raffa and Raivio, 2002) and plays a role in multidrug resistance (Baranova and Nikaido, 2002; Sivakumar *et al.*, 2013).

The RR belongs to the OmpR/PhoB superfamily. The active site of the highly conserved N-terminal REC domain contains the aspartate residue, which receives the phosphoryl group from the HK as well as the HK recognition site and a dimerization interface. Native PAGE and EMSA results in this work provided further evidence that most of the OmpR/PhoB superfamily members share a common activation mechanism via dimerization. The diverse C-terminal DNA-binding domain consists of a wHTH-motif. Most bacterial RRs have a DNA-binding output domain and the OmpR/PhoB-like domain architecture is the predominant type of RRs. The domains are well studied and have known three-dimensional structures (Kondo *et al.*, 1997; Martínez-Hackert and Stock, 1997; Okamura *et al.*, 2000; Blanco *et al.*, 2002; Robinson *et al.*, 2003). The predicted structure of RR2 revealed α8 as the recognition helix, which extend into the major groove of the DNA double helix. The local amino acids P191, R194, T195, K198, R201 and K202 were predicted to be exposed to the protein's surface and face the DNA. Thus, these amino acids were suspected to be involved in the consensus sequence specification and probably affect the specific binding affinity to the consensus sequence. The consensus sequence itself revealed that the RR homodimer binds the DNA from one site. The α8 helix of one protein reaches into the first major groove (CTATW) and the α8 helix of the other protein reaches into the third major groove. The gap of 17 bp contains two minor and one major groove. This major groove is in the case of class II promoters the -35 box, which is typically bound by the HTH-motif of the $\sigma^{70}{}_4$ domain of the RNAP (B. Liu *et al.*, 2017a). Both RR and RNAP bind the DNA from the same direction probably similar to the mechanism described for the *E. coli* CAP protein (B. Liu *et al.*, 2017a). This allows a strong interaction between transcription factor and RNAP. It is possible that the RR dimer sterically clashes with the α helix of the $\sigma^{70}{}_4$ domain, but since the -35 major groove seems to be unoccupied by the RR, the α helix could still interact with the DNA. Other OmpR/PhoB regulators bind consensus sequences that are similarly arranged, typically direct repeats with a gap spanning one minor groove (Martínez-Hackert and Stock, 1997). One example is the TCS SaeR/SaeS in *Staphylococcus aureus*, which is hypothesized to respond to membrane disturbances and regulates the expression of many virulence

factors (Mascher, 2006). After dimerization the regulator SaeR binds two neighboring major grooves with the direct repeat sequence GTTAA-N_6-GTTAA (Sun *et al.*, 2010). Similar consensus sequences have been identified for many OmpR/PhoB-type regulators including PmrA, which regulates virulence in *Legionella pneumophila* and *Coxiella burnetii* (Zusman *et al.*, 2007), the cell wall metabolism and/or cell division regulator MtrA of *Corynebacterium glutamicum* (Brocker *et al.*, 2011), and the virulence regulator PhoP of *Mycobacterium tuberculosis* (X. He and Wang, 2014). In fact, the consensus sequence of PmrA overlaps the -35 box, but, in contrast to RR2 in *Sulfurospirillum*, PmrA binds two neighboring major grooves (Altman and Segal, 2008). This work provides an example for a more exceptional consensus sequence for OmpR/PhoB-type regulators with a separation by 17 bp.

The PCE-dependent induction of OHR in *S. halorespirans* and *S. multivorans* has been proven to be mediated only by TCS2 and its canonical structure is pointing towards a straightforward signaling pathway with no other regulatory active elements required, such as other transcription factors or regulatory sRNAs. Only the putative N-terminal sensing domain of the HK is extended by seven transmembrane helices suggesting a more complex sensing mechanism. However, the delay in downregulating OHR gene expression cannot be explained without additional components interacting with TCS2.

3.3 The retentive memory effect in organohalide-respiring *Sulfurospirillum* species

TCS proteins control their output by altering the amount of active regulator by positively regulating their own transcription, by changing the biochemical activity of the TCS or by interactions with extrinsic factors. Positive autoregulation is common in TCSs and was now proven in TCS2. This autoregulation helps the organism to respond immediately and fast upon environmental PCE. Many HKs exert both an autokinase and a phosphatase activity. When the amount of phosphorylated RR increases, the activity shifts more towards the phosphatase activity until a steady-state of active RR is reached (Yeo *et al.*, 2012). Nevertheless, this intrinsic control cannot be a reason for the retentive memory effect. The ongoing transcription of the OHR gene region in the absence of PCE requires an extrinsic effector. In this work, the retentive memory effect previously observed in *S. multivorans* (John *et al.*, 2009) was confirmed in *S. halorespirans* (Chapter 2.2). This retentive memory effect is unique and only described for the OHR in both *Sulfurospirillum* species. Indeed, the downregulation of nitrate

respiration was shown to occur instantly when nitrate was consumed (Chapter 2.3). Since the retentive memory effect is limited to OHR and both *Sulfurospirillum* species share the almost 100% conserved OHR gene region, the reason for this effect was hypothesized to rest upon this gene region, but this does not necessarily have to be the case. Both *Sulfurospirillum* species are closely related and share a lot of genes encoding for regulatory proteins or sRNAs that are not directly connected to OHR, but that could interact with TCS2. Such enzymes could be the putative *N*-acetyltransferase (SHALO_1415), which was found to be upregulated during the long-term downregulation process in *S. halorespirans* (Chapter 2.2), and the NAD^+-dependent Sir2 family deacetylase (SHALO_1877). Homologous proteins are available in *S. multivorans* with sequence identities of 100% and 94% for the putative *N*-acetyltransferase (SMUL_1438) and the deacetylase (SMUL_2127), respectively. These enzymes mediate the specific acetylation and deacetylation of lysine residues and might be responsible for the PTM of TCS2 observed in the acetylomic study of *S. halorespirans*. Both HK and RR were highly acetylated during the transition phase when the TCS mediated transcription of the PCE regulon was ongoing, although the inductor PCE was absent. Protein acetylations in general can alter local or global protein structure by neutralizing the positive charge of the lysine and thereby affect the protein's properties including its enzymatic activity and its intramolecular interactions (Cain *et al.*, 2014; Hentchel and Escalante-Semerena, 2015). One acetylation site was identified in the CA domain of the HK. More precisely, it was localized adjacent to the ATP-binding site. If this acetylation causes the ongoing transcription of the PCE regulon, it will keep the ATPase domain presumably active, while any phosphatase activity of the HK will be eliminated, resulting in a continuous autophosphorylation of the HK. The RR in turn was detected to be acetylated at two sites. One site is located in the REC domain adjacent to the dimerization interface and possibly promotes dimer formation without the necessity of receiving a phosphoryl group from the HK. *In vivo*, dimerization occurs normally upon phosphorylation in order to avoid any unspecific response, but, as it has been shown in the EMSA analyses, unphosphorylated dimerization is possible under designated conditions. The second acetylation site is located in the DNA-binding domain. Although the site was not predicted to be exposed towards the DNA, it could conceivably promote binding affinities. Only a few TCSs have already been investigated in respect of lysine acetylations and all of them result in an inhibiting effect. The regulator RcsB for instance regulates multiple processes in *E. coli* including cell division, capsule and flagellum synthesis (Majdalani and Gottesman, 2005; Huang *et al.*, 2006). It can function in an RcsA-dependent or RcsA-independent manner and its function is repressed due to an acetylation within the wHTH motif (Thao *et al.*, 2011; Hu *et al.*, 2013). A similar

repression was attested for the virulence regulator PhoP in *Sa. enterica* serovar Typhimurium (Ren *et al.*, 2016), whereas the acetylation of the chemotaxis regulator CheY of *E. coli* leads to a repression of its protein-binding output domain (R. Li *et al.*, 2010a; Liarzi *et al.*, 2010). However, there are other transcription factors that exhibit an increased DNA-binding affinity upon lysine acetylation, such as the virulence factor HilD of *Sa. enterica* serovar Typhimurium (Sang *et al.*, 2016). Although an activating effect of TCS acetylation has not been reported yet, it is not unexpected that such a mechanism exists (Wolfe and Stock, 2018).

The prevalent role of TCS signaling underscores their tremendous versatility and their utility in bacteria. These systems have been implicated in mediating the response to a wide range of signals. Therefore, cross-talk, in terms of cross-phosphorylation between different TCSs, must be kept to a minimum in order to ensure the high-fidelity transmission of signals and the desired, beneficial response. Most cross-talks that have been documented in the literature were observed only in the absence of the cognate HK (Fisher *et al.*, 1995; Haldimann *et al.*, 1997; Silva *et al.*, 1998). RR competition as well as the bifunctionality of HKs acting as phosphatases for the cognate RR can effectively prevent cross-talk. Therefore, it is rather unlikely that another HK, which is not related to OHR, phosphorylates not only its cognate RR but also the regulator of TCS2 in amounts, which explain the ongoing transcription when PCE and TCE are consumed. TCSs can also be cross-phosphorylated by small-molecular-weight phosphoryl donors, such as acetyl phosphate (J. Feng *et al.*, 1992; Lukat *et al.*, 1992; Wanner and Wilmes-Riesenberg, 1992; McCleary *et al.*, 1993; Wolfe, 2005; Trajtenberg *et al.*, 2014). Acetyl phosphate is also a critical determinant of lysine acetylation as it was shown for *E. coli* (Brian T. Weinert *et al.*, 2013). It is an intermediate of the acetate activation pathways. It comes from either acetate dissimilation catalyzed by the phosphotransacetylase-acetate kinase pathway or from acetate assimilation catalyzed by the acetyl-coenzyme A synthetase. Its concentration varies depending on the metabolic state of the organism (Wolfe, 2005), but it is independent from PCE-induced OHR gene regulation. In fact, acetyl phosphate is a nonspecific phosphodonor to many regulators and could function as a global signal that feeds into various TSC signaling pathways (Wanner, 1992; McCleary *et al.*, 1993; Wolfe, 2005). But as there is no indication that any other pathway features a similar retentive memory mechanism or that other systems are connected to the PCE-dependent OHR gene regulation, it is rather unlikely that acetyl phosphate is responsible for the ongoing transcription in the absence of the putative stimulus PCE. Indeed, similar to cross-talk between different TCSs, acetyl phosphate mediated cross-phosphorylation of the regulators has been observed in most cases in the absence of the cognate HK (Laub and Goulian,

2007). Although phosphorylation of the RR with acetyl phosphate is very useful *in vitro*, its impact seems to be very limited *in vivo*.

The transgenerational downregulation of the PCE regulon could putatively be implemented by changes in the DNA methylation pattern as well. While epigenetics is of fundamental importance in eukaryotic gene regulation, it plays a minor role in bacteria. Most DNA methyltransferases allow for DNA mismatch repair (Iyer *et al.*, 2006) or belong to the restriction-modification system, which provides a defense against foreign DNA invasion, e.g. from virulent phages (Arber and Linn, 1969). Only a few transcription factors were characterized as methylated DNA-binding regulators. Recently, the transcriptional activator GcrA in *Caulobacter crescentus* was described to recognize a N^6-adenine methylated CRE and initiate transcription by interacting with the primary sigma factor (Wu *et al.*, 2018). However, no TCS was identified so far, which responds to a signal by binding preferentially to a methylated CRE. In the omics analyses of *S. halorespirans* and *S. multivorans*, no DNA methyltransferase was PCE-dependently up- or downregulated. In addition, a comparative DNA analysis unveiled no significant change in the methylation pattern between cells grown in the presence and absence of PCE (Rubick, 2010). Thus, the retentive memory effect does not seem to rely on an epigenetic-like mechanism.

The retentive memory effect could also originate from the conserved OHR gene region itself. In that case, the effector causing the ongoing transcription in the absence of the signal PCE would be a gene product under regulatory control of the TCS (Fig. 3.2). There are manifold examples of feedback control mechanisms by gene products regulated by TCSs. These gene products can target either the sensor or the regulator. The well-studied PhoP/PhoQ system in *E. coli* and *Sa. enterica* serovar Typhimurium for example responds to low Mg^{2+} in the periplasm and initiates the transcription of the Mg^{2+} transporter gene *mgtA* (Soncini *et al.*, 1996; Minagawa *et al.*, 2003). MgtA imports Mg^{2+} and removes a repressing signal of PhoQ resulting in an amplification of the signal intensity (Snavely *et al.*, 1989; S.-Y. Park and Groisman, 2014). The cytosolic Mg^{2+} concentration is sensed by a Mg^{2+}-responding riboswitch, which allows *mgtA* transcriptional elongation only when Mg^{2+} drops beyond a certain concentration (Cromie *et al.*, 2006). PhoP induces also the transcription of different connector proteins in *Sa. enterica* serovar Typhimurium. These accessory proteins interact with other HKs and RRs and therefore connect different signaling pathways. The connector protein PmrD binds to the phosphorylated RR PmrA and protects it from dephosphorylation by the cognate HK PmrB (Kato and Groisman, 2004). As a consequence, PmrA regulated antibiotic resistance genes are not only

expressed in the PmrB-mediated response to Fe^{3+} (Wösten *et al.*, 2000) but also in response to low Mg^{2+} (García Véscovi *et al.*, 1996). Another connector protein IraP was shown to bind the RR MviA resulting in an accumulation of the alternative sigma factor RpoS (Bougdour *et al.*, 2008). RpoS in turn suppresses the expression of the connector protein IgaA, which is an inner membrane protein that represses the RcsC/RcsD/RcsB TCS, which is needed for virulence (Cano *et al.*, 2002; Domínguez-Bernal *et al.*, 2004). This example points out that effectors are structurally and functionally very divers. They can modify signal access to the sensor and/or the activity of either the sensor or the regulator and allow for more complex regulatory networks. This helps the organism to adjust the particular levels of gene expression and since many effectors are subject to regulation by signals other than those recognized by the given TCS, the integration of additional inputs to the TCS is possible. The effector does not necessarily have to be an accessory protein. Certain TCSs are feedback regulated by small regulatory RNAs (Göpel and Görke, 2012; Mandin and Guillier, 2013). Especially in Proteobacteria and Firmicutes, sRNAs are regular members of many TCS regulons (Göpel and Görke, 2012). In *E. coli* 6 of 30 TCSs are connected to sRNAs (Göpel and Görke, 2012). One example is the OmpR regulator of *E. coli*, which initiates the transcription of two sRNAs OmrA and OmrB. When these sRNAs are overexpressed, the *ompR* mRNA levels are downregulated and cause a negative feedback loop (Guillier and Gottesman, 2008). Feedback regulation via sRNAs controlling the translation or stability of corresponding transcripts operates on the output of a TCS not as fast as accessory proteins directly affecting the HK or the RR, but they react faster on preexisting mRNA targets. This allows sRNAs to extend the timing, flexibility, and the dynamic range of TCS signaling and to provide robustness against signal fluctuations (Göpel and Görke, 2012).

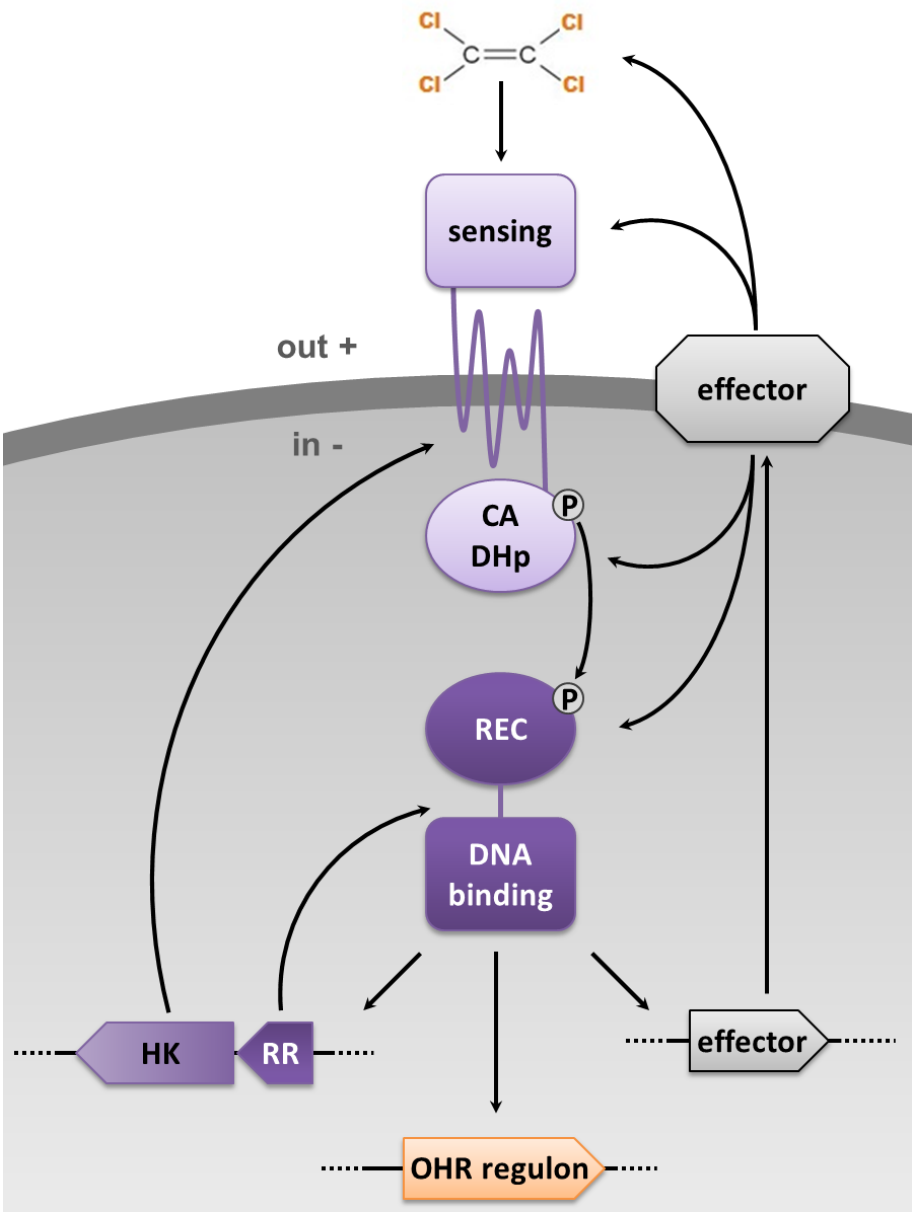

Fig. 3.2: Feedback control of TCS2. TCS2 is positively autoregulated by the regulator governing transcription of the genes specifying the sensor and regulator (purple) and by a putative effector encoded by a gene under transcriptional control of the regulator (gray). Effectors may act at other levels and are not necessarily proteins. The figure is modified from (Groisman, 2016).

The dRNA-seq of *S. multivorans* confirmed that no *trans*-encoded sRNAs are located within the OHR gene region, which could activate or repress target genes. Only one *cis*-encoded asRNA is located at the insertion element disrupting the *tetR* gene. This asRNA is perfectly complementary to its target transposase mRNA. Although a lot of asRNAs have been shown to

enter TCS signaling pathways (E.-J. Lee and Groisman, 2010; Wen *et al.*, 2011), no asRNA was validated at either TCS ORF. Cobalamin riboswitches have been identified in the genomes of *D. mccartyi* (Johnson *et al.*, 2009; Men *et al.*, 2012), *De. hafniense* (Choudhary *et al.*, 2013), and *Dehalobacter restrictus* (Rupakula *et al.*, 2015). These riboswitches are located at the 5′ UTRs of transcripts encoding cobalamin biosynthesis proteins, cobalamin transporters or reductive dehalogenases. In contrast to these organohalide-respiring bacteria, *Sulfurospirillum* species harbor no cobalamin riboswitches in the eight OHR transcripts (Chapters 2.1 and 2.3). Whether the norcobamide cofactor is nevertheless able to act as a regulatory effector molecule was matter of the third part of this study (Chapter 2.3). Since cobamides can be regulatory active in terms of binding to *cis*-regulatory riboswitches and since AdeNCba is unique for dehalogenating *Sulfurospirillum* species and produced in amounts up to 0.5 mg/l in a cell culture (Schubert, 2017), it was conceivable that the presence of AdeNCba in the absence of PCE might establish a positive feedback loop causing the retentive memory effect observed in *S. halorespirans* and *S. multivorans*. The impact of structural changes of the norcobamide cofactor has been analyzed in a first approach. By feeding a growing culture with alternative derivatives of the norcobamide cofactor's lower ligand the biosynthesis can be guided towards structurally changed products. One of the products tested was 5-hydroxybenzimidazolyl (5-OH-Bza)-norcobalamin, which has no impact on the enzyme activity of PceA and therefore should not affect protein misfolding or cellular stress. RNA quantities of *pceAB*, TCS2, and the *pceMN*/AdeNCba operon proved a change in gene expression not until more than 50 *S. multivorans* generations have been passed. The alteration of the lower ligand did not end the retentive memory effect. However, whether the presence of high amounts of AdeNCba cause the retentive memory effect was analyzed in the second approach. For this purpose, an organohalide-respiring bacterium was cultivated on a Co^{2+}-limited medium the first time. After three transfers into fresh Co^{2+}-limited medium, the amount of Co^{2+} decreased to a minimum of 0.2 µg/l, which indeed amounts to the concentration of bioavailable Co^{2+} in the natural environment of organohalide-respiring bacteria. The concentration of Co^{2+} measured in the Saale River, a natural habitat in which sediments *S. multivorans* (von Wintzingerode *et al.*, 2001), *D. mccartyi* (Adrian *et al.*, 1998; Adrian *et al.*, 2000), and *De. hafniense* (Breitenstein *et al.*, 2001) were identified, accounted for an average of 0.44 µg/l in the years 1995-1996 (Proft and Schrön, 2008). Under Co^{2+} limiting conditions, the amounts of AdeNCba and PceA decreased by one order of magnitude (3-10%) compared to standard cultivation conditions, which contain a concentration of 47 µg/l Co^{2+}. However, the RNA levels of both transcripts remained stable. The loss of PceA protein was not the consequence of a transcriptional

downregulation but a posttranscriptional effect, possibly the quick degradation of misfolded PceA. The retentive memory effect in the absence of PCE was also valid for a Co^{2+}-limited culture that lacks a sufficient AdeNCba concentration for PceA maturation. Both RNA of *pceAB* and *pceMN*/AdeNCba was detected after more than 15 generations. Although the retentive memory effect was still present under these conditions, the downregulation occurred to be accelerated. The effect was weak but reproducible. Since growth was not affected by Co^{2+} limitation when the cells grow in the presence of an alternative electron acceptor rather than PCE, a stress-induced effect seems to be not responsible for this observation. The question whether AdeNCba limitation directly affects the downregulation or other effects connected to Co^{2+} limitation are involved awaits further investigation.

Instead of AdeNCba, other gene products could be responsible for the retentive memory effect. Considering the OHR gene region of dehalogenating *Sulfurospirillum* species, all genes encode proteins that are either functionally characterized or annotated with a function justifying their organization within the OHR gene region. The sole exception is the putative membrane protein encoded upstream of the *pceMN*/AdeNCba operon (SMUL_1540 and SHALO_1504). The protein is predicted to consist of three transmembrane helices and a domain of unknown function (DUF4405). This domain contains two conserved histidine residues, H37 and H40, which were predicted to face the periplasm close to the second transmembrane helix and may be functionally important. There are a lot of arguments that underline the hypothesis that this gene product is a candidate that serves as the effector causing the retentive memory effect. Homologous genes of the DUF4405 family can be found mainly in Proteobacteria and the Flavobacterium *Lutibacter*. They share only a small protein sequence identity compared to the gene product in *Sulfurospirillum* species and are moreover identified in other genomic contexts. The role of this protein is likely different to those homologues and unique for *Sulfurospirillum* species. As most of the other OHR genes, it is transcribed in the presence of PCE and undergoes the long-term downregulation, i.e. its transcript is available during the downregulation phase. Although its gene product was not detectable in the proteomic approach, it is certainly available like the other PCE-dependently regulated proteins and was not captured due to its biochemical properties as a small membrane integral protein. It is organized in an own operon and not associated to proteins of a known function. In addition, it is located directly upstream of the TCS2 ORF and both share the promoter region-containing intergenic region. Both operons distinguish themselves by a class I transcriptional activation and express comparably high transcript amounts. The only conspicuous feature of the canonical TCS2 is the presence of both a periplasmatic domain and an extended transmembrane domain with seven transmembrane

helices. Both could be separate sensing domains for different signals and stimuli. While PCE is putatively sensed in the periplasm, a second input could be sensed within the membrane. The transmembrane protein could be sensed by the HK of TCS2, which activates the RR and keeps the PCE regulon upregulated. The previous mentioned connector protein IgaA of *Sa. enterica* serovar Typhimurium is for example a membrane integral protein with five transmembrane helices, which interacts with the transmembrane helices of the HK RcsC and inhibits its activity (Cano *et al.*, 2002; Domínguez-Bernal *et al.*, 2004). Another example is the small membrane protein SafA, which connects the signal transduction between the TCSs EvgS/EvgA and PhoQ/PhoP in *E. coli* (Eguchi *et al.*, 2007). It interacts directly with the membrane associated HK PhoQ and thus activates the PhoQ/PhoP system in response to acidic stress conditions. The membrane protein SHALO_1504/SMUL_1540 might activate TCS2 in a similar manner.

3.4 Conclusions and future prospects

TCSs are one of the most prevalent signal transduction systems by which bacteria sense, respond, and adapt to changes in their environment. In this study, the TCS that regulates OHR in the dehalogenating Campylobacterota *S. halorespirans*, *S. multivorans*, and the '*Candidatus* S. diekertiae' strains have been identified and characterized. The PCE regulon has been defined. It is induced by the substrate of the PCE reductive dehalogenase PceA and the regulator of the TCS was proven to bind specifically to all promoter sites of the PCE regulon. The comprehensive methods used within this work were state-of-the-art. The recently established dRNA-seq was applied to organohalide-respiring bacteria the first time and provided important information about the transcriptional regulation of OHR. An improved protocol for the isolation of membrane proteins substantially enlarged the coverage of the proteomic analysis and the enrichment of acetylated peptides allowed for the first quantified acetylome of an organohalide-respiring bacterium. The RR was functionally characterized using an adapted EMSA that allowed the analysis of the relatively unstable complex of the heterologously expressed regulator and its CRE-containing promoter DNA. For the analysis of the retentive memory effect unique in *Sulfurospirillum* species, the impact of Co^{2+} limitation on a dehalogenating organism was initially analyzed. The methods used herein extend the toolbox for the analysis of bacterial physiology and gene regulation of versatile organisms for which tools for the systematic genetic manipulation have not been established. The results of this work broaden the knowledge about signaling of halogenated organic compounds, the regulatory organization of OHR, and the binding mechanism of an OmpR-like transcriptional regulator that is highly acetylated and

binds as a dimer a direct repeat not separated by 6 bp (one minor groove) but 17 bp (two minor and on major groove). It will furthermore help to understand the ecophysiology of organo-halide-respiring bacteria in their natural habitats. Future investigations will display by which molecular mechanism the HK senses PCE. For this purpose and for the quantification of binding affinities of the RR to its target DNA a surface plasmon resonance system can be used. Mutagenesis studies can help to further understand the binding mechanism of the RR. In addition, it is worth proving promoter binding with the heterologously expressed regulator of TCS1 in order to define its regulon and deduce its potential role. Crystal structures of the RR2-DNA complex could provide insights in the atypical binding mechanism that target a direct repeat separated by 17 bp. Other candidates for the effector causing the retentive memory effect should be examined. It is highly recommended to functionally characterize the membrane protein of unknown function upstream of TCS2. With the construction of reporter gene fusions, the effect of putative effector genes on the gene expression could be monitored. Finally, the experimental detection of effects caused by Co^{2+} limitation could be transferred to other microbial systems that have for instance other cobalamin-dependent enzymes. This would allow for the determination of the ecological relevance of Co^{2+} and cobalamin limitation in other bacteria and bacterial communities.

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Appendix / Supplementary information of the manuscripts

Supplementary Tables and Figures

Tetrachloroethene respiration in *Sulfurospirillum* species is regulated by a two-component system as unraveled by comparative genomics, transcriptomics, and regulator binding studies

Jens Esken, Tobias Goris, Jennifer Gadkari, Thorsten Bischler, Konrad Förstner, Cynthia Sharma, Gabriele Diekert, Torsten Schubert

Supplementary Tables:

Supplementary Figures:

Tab. S1: Percent identity matrix of *Sulfurospirillum* PceA amino acid sequences, created by Clustal Omega 2.1 with standard settings. PceA of *S. multivorans* strain N was not included, since it shows 100% identity to that of *S. multivorans*.

	S.m.	***S.h.***	**JPD-1**	**SL2-1**	**SL2-2**	**NCBI Acc.No.**
S. multivorans	100.00	92.76	94.01	96.21	92.96	AHJ12791.1
S. halorespirans	92.76	100.00	89.74	92.15	92.80	AAG46194.1
***S.* JPD-1**	94.01	89.74	100.00	93.21	89.94	ATB69613.1
***Cand.* S.d. SL2-1**	92.96	92.80	89.94	100.00	94.57	ARU48750.1
***Cand.* S.d. SL2-2**	96.21	92.15	93.21	94.57	100.00	ASC93572.1

Tab. S2: Locus tags of the OHR gene region. Functions: gray = unknown; orange = reductive dehalogenase; violet = regulatory proteins; turquoise = putative quinol dehydrogenase; purple = norcobamide biosynthesis; pink = cobamide transporter.

locus tags				product
SHALO_1494	SMUL_1530	SMN_1515	SJPD1_1503	alkylhydroperoxidase AhpD family protein
SHALO_1495	SMUL_1531	SMN_1516	SJPD1_1504	tetrachloroethene reductive dehalogenase catalytic subunit PceA
SHALO_1496	SMUL_1532	SMN_1517	SJPD1_1505	tetrachloroethene reductive dehalogenase membrane anchor PceB
SHALO_1497	SMUL_1533	SMN_1518	SJPD1_1506	IscU/NifU-like protein
SHALO_1498	SMUL_1534	SMN_1519	SJPD1_1507	two-component sensor histidine kinase
SHALO_1499	SMUL_1535	SMN_1520	SJPD1_1508	two-component response regulator
SHALO_1500	SMUL_1536	SMN_1521	SJPD1_1509	reductive dehalogenase catalytic subunit RdhA
SHALO_1501	SMUL_1537	SMN_1522	SJPD1_1510	reductive dehalogenase membrane anchor RdhB
SHALO_1502	SMUL_1538	SMN_1523	SJPD1_1511	two-component sensor histidine kinase
SHALO_1503	SMUL_1539			two-component response regulator
		SMN_1524	SJPD1_1512	two-component response regulator, disrupted by transposase
		SMN_1525		transposase
			SJPD1_1513	putative transposase
			SJPD1_1514	putative transposase
			SJPD1_1515	two-component response regulator, disrupted by transposase
SHALO_1504	SMUL_1540	SMN_1526	SJPD1_1516	putative membrane protein
SHALO_1505	SMUL_1541	SMN_1527	SJPD1_1517	putative quinol dehydrogenase, periplasmic subunit
SHALO_1506	SMUL_1542	SMN_1528	SJPD1_1518	putative quinol dehydrogenase, membrane subunit
SHALO_1507	SMUL_1543	SMN_1529	SJPD1_1519	adenosylcobinamide-phosphate synthase CbiB
SHALO_1508	SMUL_1544	SMN_1530	SJPD1_1520	threonine phosphate decarboxylase-like enzyme
SHALO_1509	SMUL_1545	SMN_1531	SJPD1_1521	bifunctional cobamide biosynthesis protein CobU
SHALO_1510	SMUL_1546	SMN_1532	SJPD1_1522	cobyric acid synthase CbiP
SHALO_1511	SMUL_1547	SMN_1533	SJPD1_1523	nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase CobT
SHALO_1512	SMUL_1548	SMN_1534	SJPD1_1524	cysteine-rich domain protein
SHALO_1513	SMUL_1549	SMN_1535	SJPD1_1525	cobamide synthase CobS
SHALO_1514	SMUL_1550	SMN_1536	SJPD1_1526	alpha-ribazole phosphatase CobC

locus tags				product
SHALO_1515	SMUL_1551	SMN_1537	SJPD1_1527	sirohydrochlorin cobaltochelatase CbiK
SHALO_1516	SMUL_1552	SMN_1538	SJPD1_1528	corrinoid ABC transporter, permease component BtuC
SHALO_1517	SMUL_1553	SMN_1539	SJPD1_1529	corrinoid ABC transporter ATPase BtuD
SHALO_1518	SMUL_1554	SMN_1540	SJPD1_1530	corrinoid ABC transporter, B12-binding component BtuF
SHALO_1519	SMUL_1555	SMN_1541	SJPD1_1531	cobalt-precorrin-8x methylmutase CbiC
SHALO_1520	SMUL_1556	SMN_1542	SJPD1_1532	cobalt-precorrin-6 synthase, anaerobic CbiD
SHALO_1521	SMUL_1557	SMN_1543	SJPD1_1533	cobalt-precorrin-6y C5-methyltransferase CbiE
SHALO_1522	SMUL_1558	SMN_1544	SJPD1_1534	cobalt-precorrin-6y C15-methyltransferase [decarboxylating] CbiT
SHALO_1523	SMUL_1559	SMN_1545	SJPD1_1535	cobalt-precorrin-2 C20-methyltransferase CbiL
SHALO_1524	SMUL_1560	SMN_1546	SJPD1_1536	cobalt-precorrin-4 C11-methyltransferase CbiF
SHALO_1525	SMUL_1561	SMN_1547	SJPD1_1537	cobalamin biosynthesis protein CbiG
SHALO_1526	SMUL_1562	SMN_1548	SJPD1_1538	cobalt-precorrin-3b C17-methyltransferase CbiH
SHALO_1527	SMUL_1563	SMN_1549	SJPD1_1539	uroporphyrinogen-III methyltransferase / Uroporphyrinogen-III synthase
SHALO_1528	SMUL_1564	SMN_1550	SJPD1_1540	cobalt-precorrin-6x reductase CbiJ
SHALO_1529	SMUL_1565	SMN_1551	SJPD1_1541	lipid A export ATP-binding/permease protein MsbA-like
SHALO_1530	SMUL_1566	SMN_1552	SJPD1_1542	cobyrinic acid A,C-diamide synthase CbiA
SHALO_1531	SMUL_1567	SMN_3265	SJPD1_1543	cysteine-rich CWC protein
SHALO_1532	SMUL_1568	SMN_1553	SJPD1_1544	precorrin-2 dehydrogenase (NAD-dependent)
SHALO_1533			SJPD1_1545	TetR-like transcriptional regulator
	SMUL_1569	SMN_1554		TetR-like transcriptional regulator, disrupted by transposase
	SMUL_1570	SMN_1555		ISPsy4, transposition helper protein
	SMUL_1571	SMN_1556		integrase catalytic region
	SMUL_1572	SMN_1557		TetR-like transcriptional regulator, disrupted by transposase
SHALO_1534	SMUL_1573	SMN_1558	SJPD1_1546	FeS cluster binding motif-containing flavoprotein
SHALO_1535	SMUL_1574	SMN_1559	SJPD1_1547	Homologue to Rieske proteins, CxH(x)CxxH motif replaced with CxH(x)CxxN
SHALO_1536	SMUL_1575	SMN_1560	SJPD1_1548	putative FMN-binding protein
SHALO_1537	SMUL_1576	SMN_1561	SJPD1_1549	putative membrane protein

. S2: Blastp results of transposase genes detected in the OHR gene region.

Organism	SJPD1_1513: putative transposase			SJPD1_1514: putative transposase		
	Name	aa seq id	coverage	Name	aa seq id	coverage
furospirillum sp. JPD-1	SJPD1_0244	100%	100%	SJPD1_0245	100%	100%
furospirillum sp. JPD-1	SJPD1_0977			SJPD1_0976		
furospirillum sp. JPD-1	SJPD1_1067			SJPD1_1066		
furospirillum sp. JPD-1	SJPD1_1488			SJPD1_1489		
furospirillum sp. JPD-1	SJPD1_1646			SJPD1_1645		
furospirillum multivorans strain N	SMN_0981			SMN_0980		
furospirillum multivorans strain N	SMN_0280	99%	100%	SMN_0281	100%	100%
furospirillum multivorans	SMUL_0991			SMUL_0990	99%	100%
ndidatus S. diekertiae' SL2-1	Sdiek1_0868	94%	100%	Sdiek1_0867	95%	100%
ndidatus S. diekertiae' SL2-2	Sdiek2_0859			Sdiek2_0858	93%	77%
furospirillum multivorans	SMUL_0210	100%	93%	SMUL_0211	100%	100%
furospirillum multivorans	SMUL_0287			SMUL_0288		
furospirillum multivorans	SMUL_1589			SMUL_1588		
furospirillum multivorans	SMUL_2876			SMUL_2877		

ab. S3: Blastp results of transposase genes detected in the OHR gene region, continued.

Organism	SMUL_1570: ISPsy4, transposition helper protein			SMUL_1571: integrase catalytic region		
	Name	aa seq id	coverage	Name	aa seq id	coverag
Candidatus S. diekertiae' SL2-2	Sdiek2_1043	100%	100%	Sdiek2_1044	100%	100%
ulfurospirillum multivorans strain N	SMN_1555			SMN_1556		
ulfurospirillum multivorans	SMUL_0039	91%	100%	SMUL_0040	91%	100%
ulfurospirillum multivorans	SMUL_0261			SMUL_0260		
ulfurospirillum multivorans	SMUL_0276			SMUL_0275		
ulfurospirillum multivorans	SMUL_1000			SMUL_999		
ulfurospirillum multivorans	SMUL_2742			SMUL_2741		
ulfurospirillum halorespirans	SHALO_2283			SHALO_2284		
Candidatus S. diekertiae' SL2-1	Sdiek1_1055	99%	83%	Sdiek1_1056	100%	97%
ulfurospirillum multivorans	SMUL_3012	frameshift: not functional		SMUL_3011	98%	97%
ulfurospirillum multivorans strain N	not annotated			SMN_2977		
ulfurospirillum multivorans	SMUL_3014	frameshift: not functional		SMUL_3015	99%	100%
ulfurospirillum multivorans strain N	not annotated			SMN_2979		
ulfurospirillum multivorans strain N	SMN_0037	91%	100%	SMN_0038	90%	97%
ulfurospirillum multivorans strain N	SMN_0992			SMN_0991		
ulfurospirillum multivorans strain N	SMN_0254			SMN_0253		
ulfurospirillum multivorans strain N	SMN_0270			SMN_0269	91%	99%
ulfurospirillum multivorans strain N	SMN_2714			SMN_2713		

Tab. S3: Blastp results of transposase genes detected in the OHR gene region, continued.

Organism	SMN_1525: transposase		
	Name	aa seq id	coverage
Sulfurospirillum multivorans	SMUL_0896		
Sulfurospirillum multivorans	SMUL_1226		
Sulfurospirillum multivorans	SMUL_2120	100%	100%
Sulfurospirillum multivorans strain N	SMN_1982		
Sulfurospirillum multivorans strain N	SMN_2100		
Sulfurospirillum multivorans	SMUL_2437	99%	100%
Sulfurospirillum multivorans	SMUL_1516		
Sulfurospirillum halorespirans	SHALO_2308	94%	100%
Sulfurospirillum halorespirans	SHALO_2374		
Sulfurospirillum multivorans strain N	SMN_1501		

Read alignment statistics.

Libraries	Py_Fu_A_ minus_TEX	Py_Fu_A_ plus_TEX	Py_Fu_B_ minus_TEX	Py_Fu_B_ plus_TEX	Py_PCE_A_ minus_TEX	Py_PCE_A_ plus_TEX	Py_PCE_B_ minus_TEX	Py
No. of input reads	5469794	6112735	5304494	5014615	5614126	7164779	4375347	
f reads - PolyA detected and removed	2341132	3371730	1423646	1914606	2339894	3482963	2050260	
No. of reads - Single 3' A removed	515530	477573	327513	501826	590518	675424	421229	
No. of reads - Unmodified	2613132	2263432	3553335	2598183	2683714	3006392	1903858	
No. of reads - Removed as too short	30904	59388	35863	307333	24179	56722	50310	
ds - Long enough, used for alignment	5438890	6053347	5268631	4707282	5589947	7108057	4325037	
Total no. of aligned reads	5376025	5997438	5239759	4620634	5538334	7051727	4287282	
Total no. of unaligned reads	62865	55909	28872	86648	51613	56330	37755	
Total no. of uniquely aligned reads	3972686	4604113	2281527	3697868	4752217	5418877	3498178	
Total no. of alignments	6815238	7454212	8227512	5580782	6380790	8757868	5136737	
Percentage of aligned reads (compared to no. of input reads)	98.29	98.11	98.78	92.14	98.65	98.42	97.99	
Percentage of aligned reads ompared to no. of long enough reads)	98.84	99.08	99.45	98.16	99.08	99.21	99.13	
Percentage of uniquely aligned reads (in relation to all aligned reads)	73.9	76.77	43.54	80.03	85.81	76.84	81.59	

Tab. S4: Mostly regulated genes (Py_PCE vs. Py_Fu), padj: Benjamini-Hochberg adjusted p-value.

Locus_tag	Product	log2FoldChange	padj
SMUL_0188	manganese/zinc/iron chelate uptake transporter (MZT) family, periplasmic-binding protein	3.706141763	1.29009E-13
SMUL_0547	heat shock protein Hsp20	3.928639806	4.69236E-32
SMUL_0914	oleate hydratase	-5.328708478	6.67844E-66
SMUL_1376	indolepyruvate oxidoreductase subunit IorB	-3.357859244	4.61594E-13
SMUL_1531	PceA	7.957848413	1.90956E-93
SMUL_1532	PceB	5.529636956	1.37944E-10
SMUL_1533	IscU/NifU-like protein	6.203163354	3.00897E-36
SMUL_1540	putative membrane protein	4.091494694	9.32673E-08
SMUL_1541	PceM	6.185575879	6.59202E-76
SMUL_1542	PceN	7.617250082	9.76877E-62
SMUL_1543	CbiB	6.784071929	2.80479E-61
SMUL_1544	threonine phosphate decarboxylase-like enzyme	7.653246092	4.77859E-59
SMUL_1545	CobU	7.301835871	2.26884E-56
SMUL_1546	CbiP	7.229554336	8.21767E-94
SMUL_1547	CobT	7.450000181	2.15013E-81
SMUL_1548	cysteine-rich domain protein	7.15783996	1.56005E-35
SMUL_1549	CobS	7.110218148	1.42499E-50
SMUL_1550	CobC	7.570170467	1.10639E-46
SMUL_1551	CbiK	6.442502207	1.15417E-79
SMUL_1552	BtuC	6.975106327	8.8242E-101
SMUL_1553	BtuD	7.322787761	3.64873E-59
SMUL_1554	BtuF	6.870970537	2.23065E-63
SMUL_1555	CbiC	6.390476154	2.03371E-34
SMUL_1556	CbiD	6.293549376	4.05651E-85
SMUL_1557	CbiE	5.940440235	2.94744E-41
SMUL_1558	CbiT	6.377525645	1.48587E-40
SMUL_1559	CbiL	6.826321327	3.45861E-65
SMUL_1560	CbiF	7.12898108	2.37995E-69
SMUL_1561	CbiG	6.327298396	5.61016E-85
SMUL_1562	CbiH	6.783888426	2.94934E-59
SMUL_1563	uroporphyrinogen-III methyltransferase / Uroporphyrinogen-III synthase	5.675055838	1.15417E-79
SMUL_1564	CbiJ	4.857598806	6.31887E-71
SMUL_1565	lipid A export ATP-binding/permease protein MsbA-like	3.373444956	2.04003E-15
SMUL_1566	CbiA	4.123923514	6.67467E-31
SMUL_1567	cysteine-rich CWC protein	3.537696231	1.02731E-22
SMUL_1568	SirC	3.517441259	4.30141E-25
SMUL_1569	TetR-like transcriptional regulator	3.752933837	2.61372E-21
SMUL_1573	FeS cluster binding motif-containing flavoprotein	9.019230255	4.96274E-44
SMUL_1574	rieske-like domain-containing redox protein	8.715025983	3.63129E-59
SMUL_1575	putative FMN-binding protein	7.25225219	2.6604E-107
SMUL_1576	putative membrane protein	7.266423648	1.69691E-42
SMUL_1679	fumarate hydratase / tartrate dehydratase class I, beta subunit	-3.784971996	1.45393E-06
SMUL_1680	fumarate hydratase class I / tartrate dehydratase, alpha subunit	-3.631948264	1.66852E-13
SMUL_1681	anaerobic C4-dicarboxylate transporter	-3.995272359	1.82261E-44
SMUL_2666	methyl-accepting chemotaxis sensory transducer	-4.518658635	5.14879E-26
SMUL_2667	methyl-accepting chemotaxis sensory transducer	-4.340873976	2.68256E-40
SMUL_2817	aspartate ammonia-lyase	-9.11183091	1.3706E-150

Locus_tag	Product	log2FoldChange	padj
SMUL_2818	C4-dicarboxylate transporter DcuA	-8.750538177	4.2635E-102
SMUL_2819	L-asparaginase	-8.921077706	5.17731E-93

Tab. S5: OHR genes, padj: Benjamini-Hochberg adjusted p-value.

Locus_tag	Product	log2FoldChange	padj
SMUL_1530	alkylhydroperoxidase AhpD family protein	2.288230864	7.52726E-07
SMUL_1531	PceA	7.957848413	1.90956E-93
SMUL_1532	PceB	5.529636956	1.37944E-10
SMUL_1533	IscU/NifU-like protein	6.203163354	3.00897E-36
SMUL_1534	two-component sensor histidine kinase	1.64903125	0.156683843
SMUL_1535	two-component response regulator	2.183682739	0.001374947
SMUL_1536	RdhA	1.594020272	0.00172506
SMUL_1537	RdhB	0.342863587	0.963904841
SMUL_1538	PceS	1.692624888	1.51154E-05
SMUL_1539	PceP	0.892719153	0.092756887
SMUL_1540	putative membrane protein	4.091494694	9.32673E-08
SMUL_1541	PceM	6.185575879	6.59202E-76
SMUL_1542	PceN	7.617250082	9.76877E-62
SMUL_1543	CbiB	6.784071929	2.80479E-61
SMUL_1544	threonine phosphate decarboxylase-like enzyme	7.653246092	4.77859E-59
SMUL_1545	CobU	7.301835871	2.26884E-56
SMUL_1546	CbiP	7.229554336	8.21767E-94
SMUL_1547	CobT	7.450000181	2.15013E-81
SMUL_1548	cysteine-rich domain protein	7.15783996	1.56005E-35
SMUL_1549	CobS	7.110218148	1.42499E-50
SMUL_1550	CobC	7.570170467	1.10639E-46
SMUL_1551	CbiK	6.442502207	1.15417E-79
SMUL_1552	BtuC	6.975106327	8.8242E-101
SMUL_1553	BtuD	7.322787761	3.64873E-59
SMUL_1554	BtuF	6.870970537	2.23065E-63
SMUL_1555	CbiC	6.390476154	2.03371E-34
SMUL_1556	CbiD	6.293549376	4.05651E-85
SMUL_1557	CbiE	5.940440235	2.94744E-41
SMUL_1558	CbiT	6.377525645	1.48587E-40
SMUL_1559	CbiL	6.826321327	3.45861E-65
SMUL_1560	CbiF	7.12898108	2.37995E-69
SMUL_1561	CbiG	6.327298396	5.61016E-85
SMUL_1562	CbiH	6.783888426	2.94934E-59
SMUL_1563	CobA/HemD	5.675055838	1.15417E-79
SMUL_1564	CbiJ	4.857598806	6.31887E-71
SMUL_1565	lipid A export ATP-binding/permease protein MsbA-like	3.373444956	2.04003E-15
SMUL_1566	CbiA	4.123923514	6.67467E-31
SMUL_1567	cysteine-rich CWC protein	3.537696231	1.02731E-22
SMUL_1568	SirC	3.517441259	4.30141E-25
SMUL_1569	TetR-like transcriptional regulator	3.752933837	2.61372E-21
SMUL_1570	ISPsy4, transposition helper protein	-0.255044852	0.963904841
SMUL_1571	integrase catalytic region	-0.08886508	0.986159508
SMUL_1572	TetR-like transcriptional regulator	0.766545409	0.854250437
SMUL_1573	FeS cluster binding motif-containing flavoprotein	9.019230255	4.96274E-44
SMUL_1574	rieske-like domain-containing redox protein	8.715025983	3.63129E-59
SMUL_1575	putative FMN-binding protein	7.25225219	2.6604E-107
SMUL_1576	putative membrane protein	7.266423648	1.69691E-42

Tab. S6: Identified transcriptional units in the OHR gene region, locations of the transcriptional start sides (TSSs) in the genome of *S. multivorans*, and the genes organized in these operons.

Locus_tag	TSS_location	genes
pSMUL_1530	1490384	SMUL_1530
p*pceAB*	1491183	SMUL_1531-1532
pSMUL_1533	1493874	SMUL_1533
pTCS1	1496519	SMUL_1534-1535
pTCS2	1501090	SMUL_1538-1539
pSMUL_1540	1501260	SMUL_1540
p*pceMN*/B12	1531093	SMUL_1541-1569
pSMUL_1573	1528995	SMUL_1573-1576

Tab. S7: qPCR C(t) values of three sequences of intergenic regions. The first intergenic region is located between SHALO_1504/1505, the second is located between SHALO_1532/1533, the third is located between SHALO_1533/1534. Genomic DNA (gDNA) of *S. halorespirans* was used for positive control. Water served as a no template control. Complementary (cDNA) was used for the RT- control. The green hook highlights detected RNA.

Intergenic region content	Primer	Template	C(t) Mean	C(t) Std. Dev
Sample: SHALO_1504 -> SHALO_1505	T774/T775	Sh_cDNA	N/A ✗	N/A
RT- Ctrl: SHALO_1504 -> SHALO_1505	T774/T775	Sh_cDNA	N/A	N/A
Pos Ctrl: SHALO_1504 -> SHALO_1505	T774/T775	Sh_gDNA	23.83	1.627
Neg Ctrl: SHALO_1504 -> SHALO_1505	T774/T775	H_2O	N/A	N/A
Sample: SHALO_1532 -> SHALO_1533	T776/T777	Sh_cDNA	22.48 ✓	0.657
RT- Ctrl: SHALO_1532 -> SHALO_1533	T776/T777	Sh_cDNA	N/A	N/A
Pos Ctrl: SHALO_1532 -> SHALO_1533	T776/T777	Sh_gDNA	16.65	0.248
Neg Ctrl: SHALO_1532 -> SHALO_1533	T776/T777	H_2O	N/A	N/A
Sample: SHALO_1533 -> SHALO_1534	T778/T779	Sh_cDNA	29.94 ✓	0.445
RT- Ctrl: SHALO_1533 -> SHALO_1534	T778/T779	Sh_cDNA	N/A	N/A
Pos Ctrl: SHALO_1533 -> SHALO_1534	T778/T779	Sh_gDNA	15.77	0.351
Neg Ctrl: SHALO_1533 -> SHALO_1534	T778/T779	H_2O	N/A	N/A

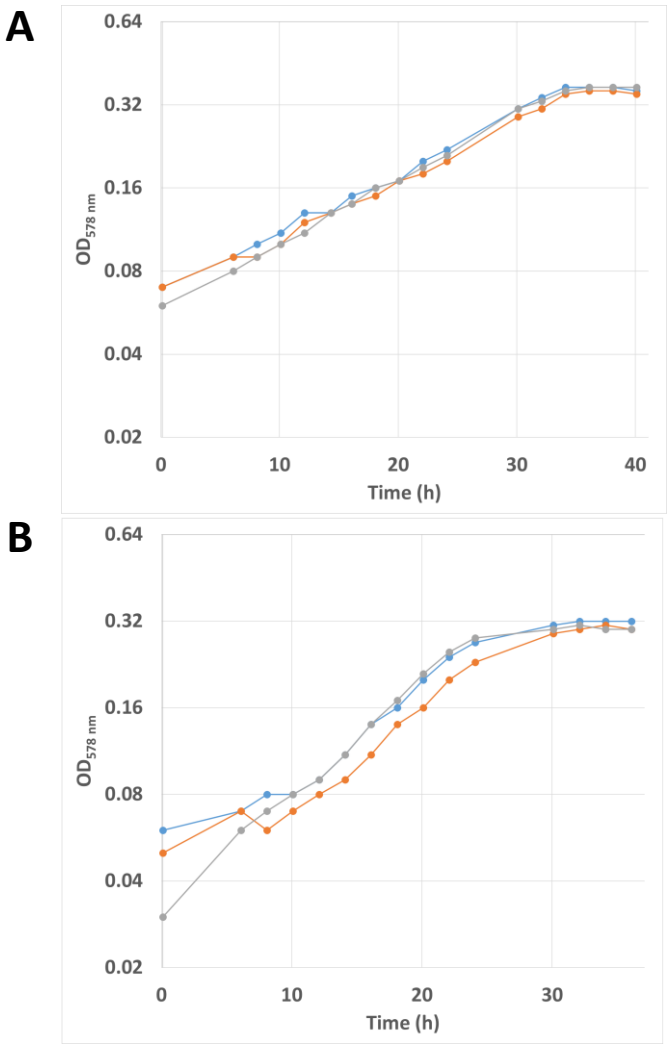
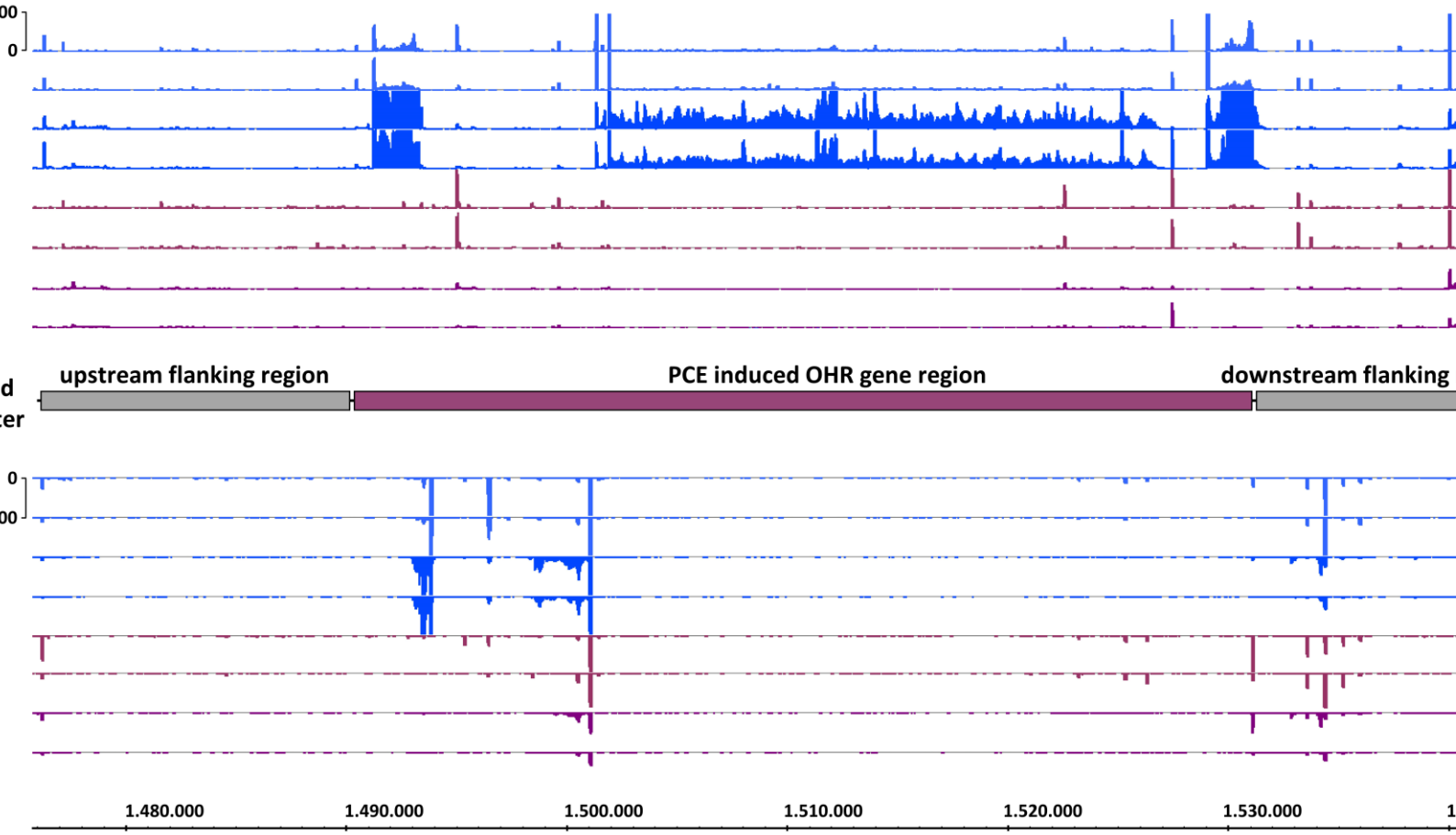


Fig. S1: Growth curves of A) *S.* sp. JPD-1 and B) *S. multivorans* strain N on pyruvate and fumarate (40 mM each). Three biological replicates were measured.



fferential RNA sequencing (dRNA-Seq) results of the gene region that is conserved among *Sulfurospirillum* species compared in this udes the OHR gene region visualized in Fig. 3. The "Integrated Genome Browser" (v. 9.0.1) was used for data evaluation.

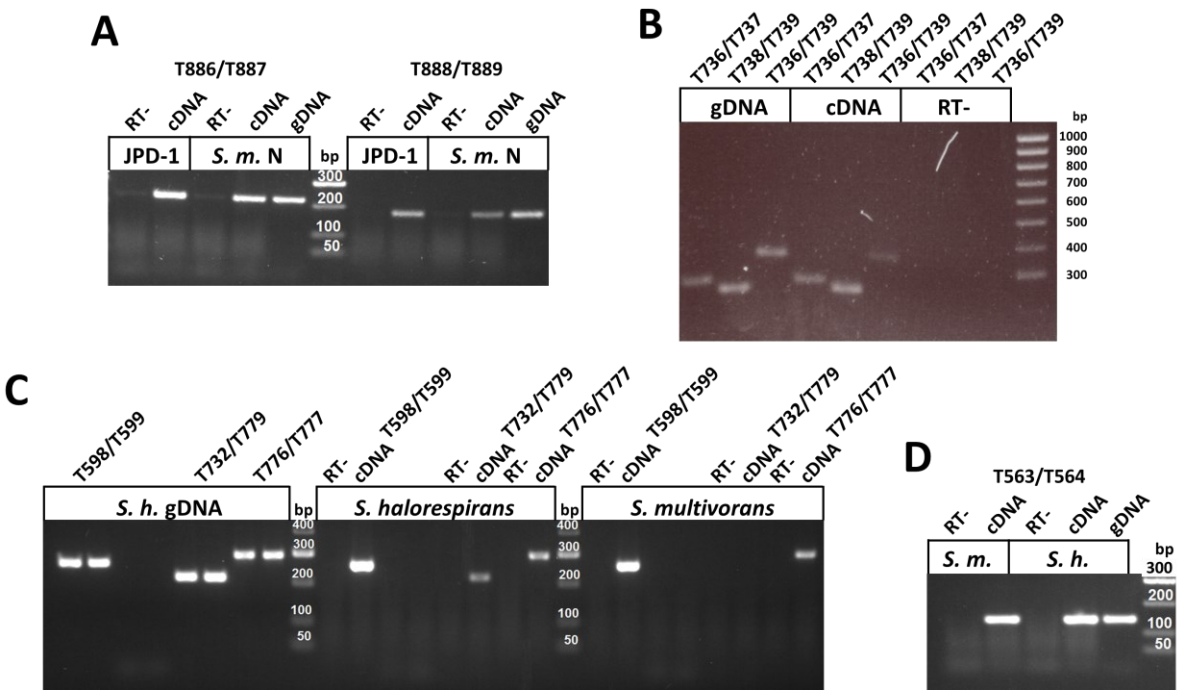


Fig. S3: Agarose gels of amplified cDNA. All separations were performed on 2% agarose gels after RT-PCR. cDNA: sample cDNA; gDNA: genomic DNA positive control; H_2O: no template negative control; RT-: no reverse transcriptase negative control; Txyz: primer ID (cf. Tab. 2). A) RT-PCR of RR2 sequences up- (T886/T887) and downstream (T888/T889) of the transposases in *S.* sp. JPD-1 and *S. multivorans* strain N. The PCR was stopped after 26 cycles. B) RT-PCR of the 106 nt sequence using different primers in *S. halorespirans*. The PCR was stopped after 21 cycles. C) RT-PCR of two sequences covering *tetR* and one of the neighboring gene, respectively. The first sequence (T776/T777) covers the gene encoding the precorrin-2 dehydrogenase and *tetR* in *S. multivorans* (SMUL_1568 and SMUL_1569) and *S. halorespirans* (SHALO_1532 and SHALO_1533). The second sequence (T732/T779) covers the Fe-S cluster binding-motif containing flavoprotein gene (SMUL_1573 or SHALO_1534) and its promoter region. The PCR was stopped after 21 cycles. Only the first sequence was detected in both species. The second sequence was only found in *S. halorespirans*. The 16S rRNA (T598/T599) served as positive control and was stopped after 16 cycles. D) RT-PCR of the Fe-S cluster binding motif-containing flavoprotein in *S. multivorans* (SMUL_1573) and *S. halorespirans* (SHALO_1543). PCR was stopped after 21 cycles.

```
             1                                                  41
Seq.         MFRQVVSLTL LVSLLAVGSS GILMIILNSF EFQFQMHPVH KIFGVLMVLS
TOPCONS      iiiiiiMMMM MMMMMMMMMM MMMMMMMooo oooooooooMM MMMMMMMMMM
OCTOPUS      iiiiiiMMMM MMMMMMMMMM MMMMMMMooo oooooooooMM MMMMMMMMMM
Philius      iiiiiiiMMM MMMMMMMMMM MMMMMMMooo oooooooooMM MMMMMMMMMM
PolyPhobius  SSSSSSSSSS SSSSSSSSSS SSSooooooo ooooooooooM MMMMMMMMMM
SCAMPI       iiiiiiMMMM MMMMMMMMMM MMMMMMMooo oooooooooMM MMMMMMMMMM
SPOCTOPUS    iiiiiiMMMM MMMMMMMMMM MMMMMMMooo oooooooooMM MMMMMMMMMM

             51                                                 91
Seq.         GSLHLYLNFG SVKKYLNIKK MALFTGVLSI IMVLLYGVGI NKPLNIEKIK
TOPCONS      MMMMMMMMMi iiiiiiiiiM MMMMMMMMMM MMMMMMMMMM oooooooooo
OCTOPUS      MMMMMMMMMi iiiiiiiiiM MMMMMMMMMM MMMMMMMMMM oooooooooo
Philius      MMMMMMMMMi iiiiiiiiii MMMMMMMMMM MMMMMMMMMM oooooooooo
PolyPhobius  MMMMMMMMMi iiiiiiiiii MMMMMMMMMM MMMMMMMMMM Mooooooooo
SCAMPI       MMMMMMMMMi iiiiiiiiii MMMMMMMMMM MMMMMMMMMM Mooooooooo
SPOCTOPUS    MMMMMMMMMi iiiiiiiiiM MMMMMMMMMM MMMMMMMMMM oooooooooo

             101        111
Seq.         QMENIAKTLE E
TOPCONS      oooooooooo o
OCTOPUS      oooooooooo o
Philius      oooooooooo o
PolyPhobius  oooooooooo o
SCAMPI       oooooooooo o
SPOCTOPUS    oooooooooo o
```

Fig. S4: Topological analysis of SMUL_1540 with TOPCONS. Abbreviations and symbols: Seq.: SMUL_1540 amino acid sequence, i - inside (cytoplasmic), M - membrane, S - signal peptide, o - outside

Fig. S5: Alignment of the OHR promoter regions and randomly chosen promoter sequences in *S. multivorans*. The sequences are up to 50 bp long and the 3'-end is the defined TSS (+1). The promoter region of *rdhAB* was predicted from sequence alignments.

Fig. S6: WebLogo of the promoter regions of the eight PCE-dependent regulated transcripts. The -35 and -10 boxes are highlighted. The TSSs vary between four bp as it is shown in Fig. S5.

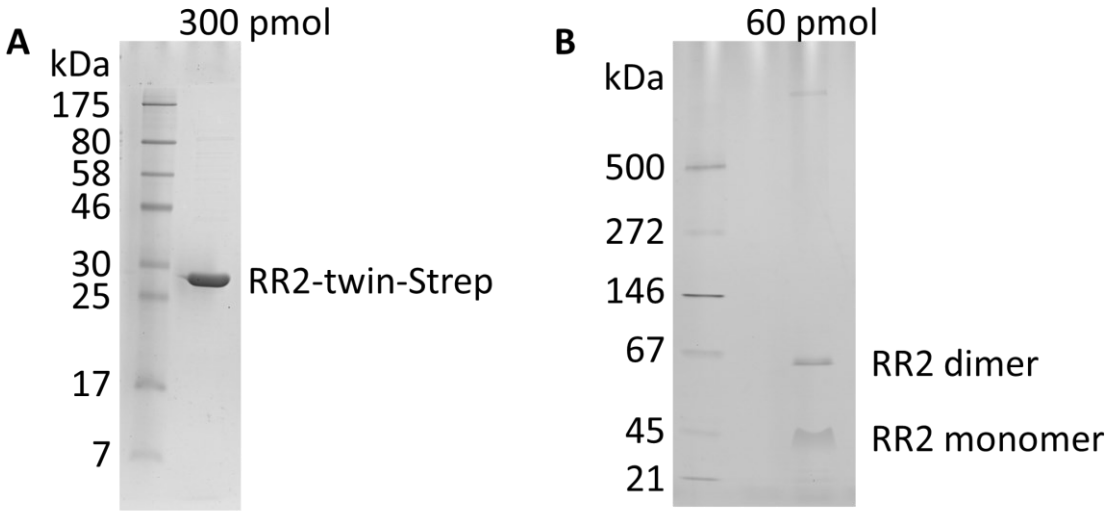


Fig. S7: Purified RR2-twin-Strep on A) Coomassie-stained denaturating and B) silver-stained native polyacrylamide gels.

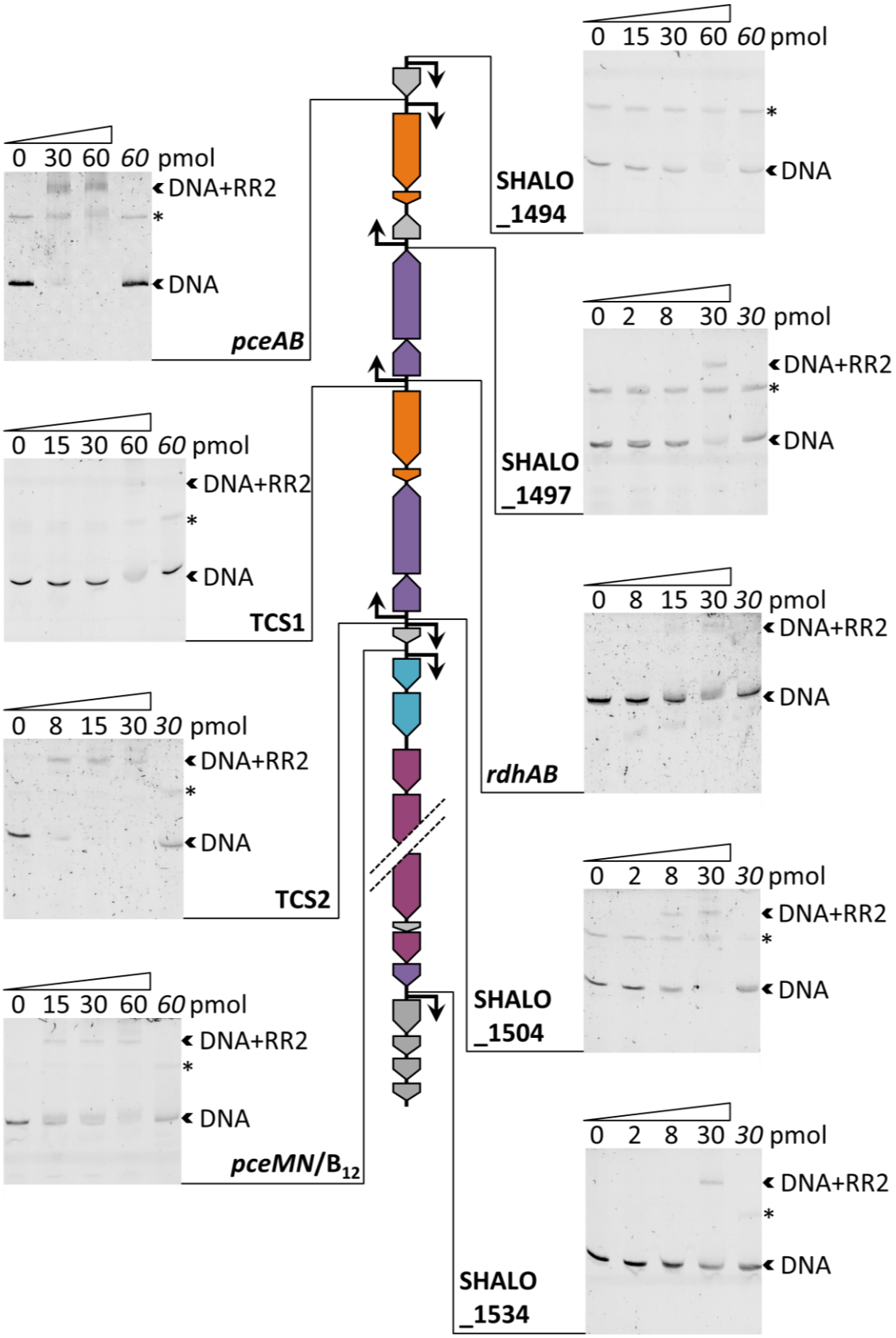


Fig. S8: Gel shift assays of *S. halorespirans* RR2-twin-Strep binding to the promoters of all transcriptional units of the OHR gene region. The amount of fluorescently labeled promoter DNA was 2.5 pmol, whereas the protein amount increased in each lane as indicated. As a negative control (protein amount written in italics) the sample mixture was boiled for 5 min after the binding reaction. A DNA artifact not targeted by RR2 is marked by an asterisk.

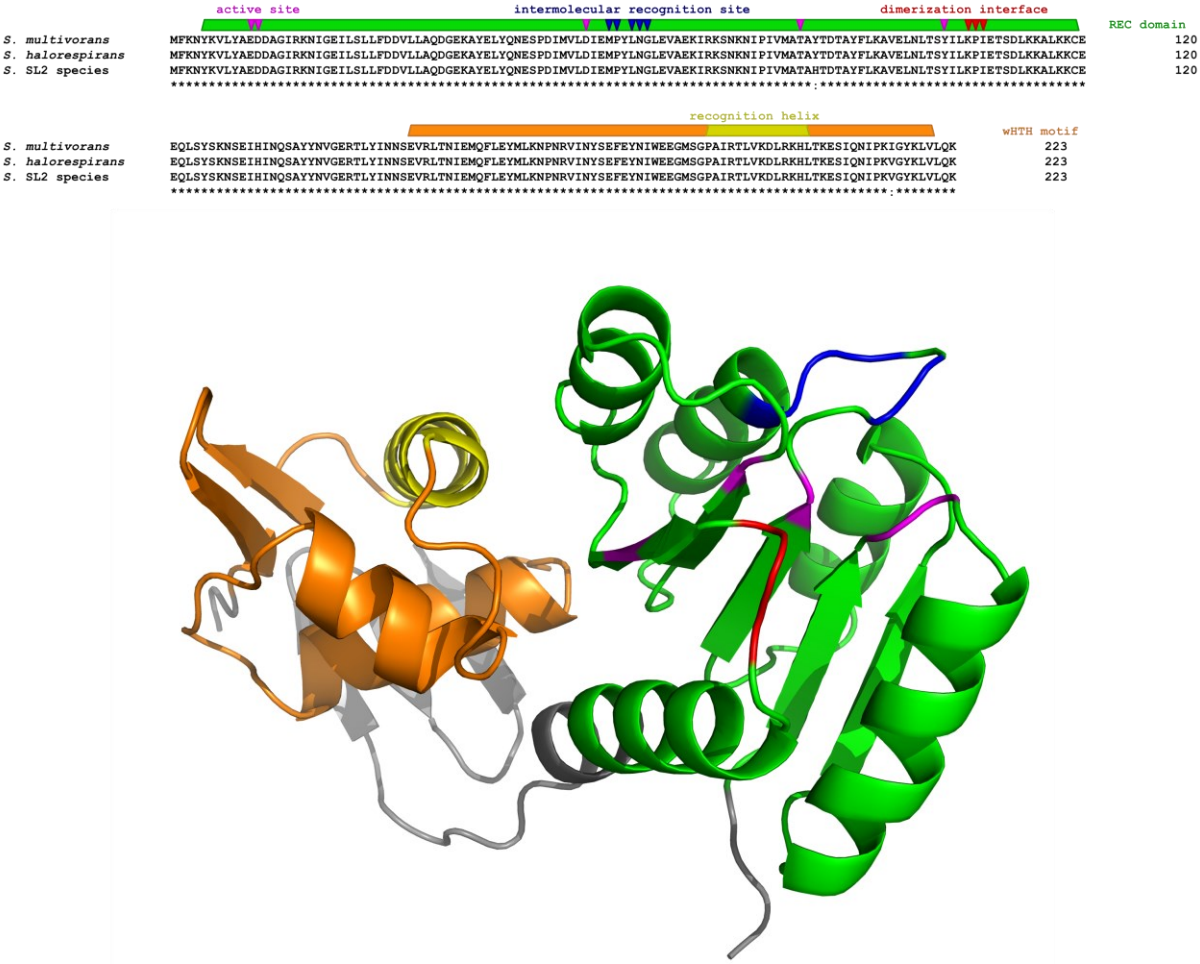
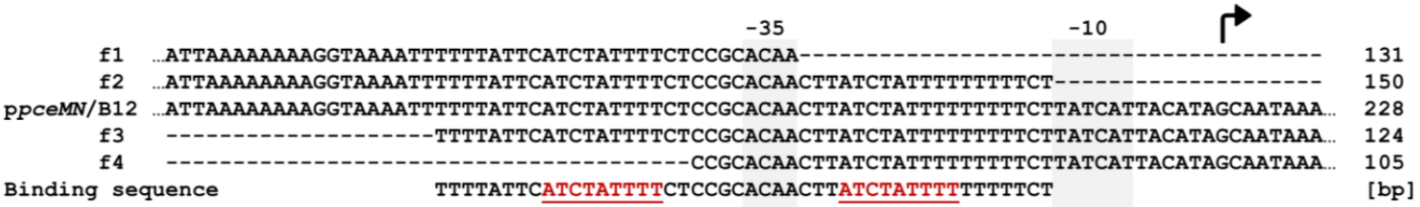
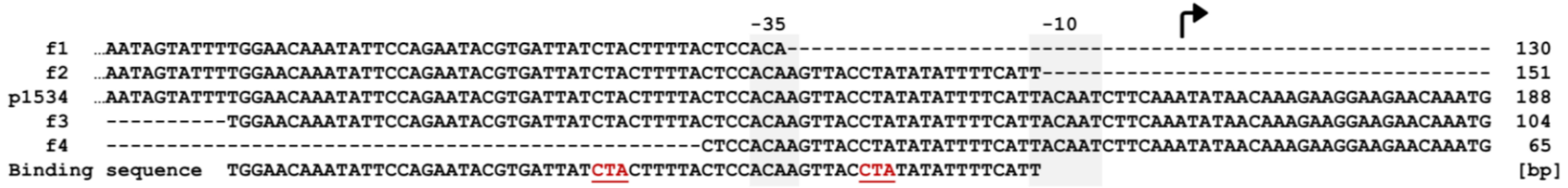


Fig. S9: Protein sequence alignment of RR2 in the dehalogenating *Sulfurospirillum* species. '*Candidatus* S. diekertiae' SL2-1 and SL2-2 share an identical sequence. The conserved domains are labeled in different colors that are recovered in the predicted protein structure below (green: REC domain; purple: active site; blue: intermolecular recognition site; red: dimerization interface; orange: wHTH motif; yellow: recognition helix).

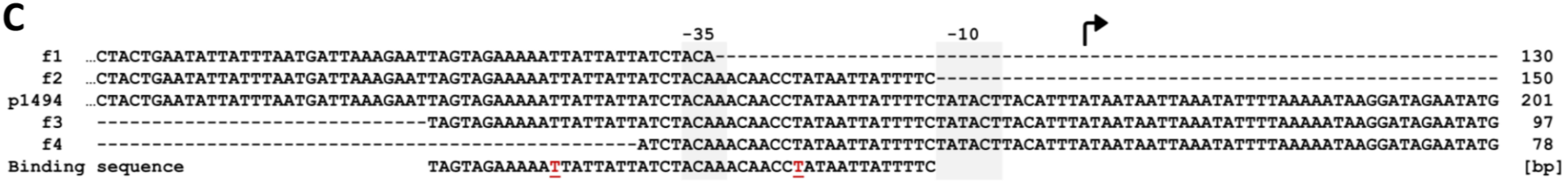
A


-35
-10
f1 …ATTAAAAAAAAGGTAAAATTTTTTATTCATCTATTTTCTCCGCACAA---------- 131
f2 …ATTAAAAAAAAGGTAAAATTTTTTATTCATCTATTTTCTCCGCACAACTTATCTATTTTTTTTTTCT---------- 150
ppceMN/B12 …ATTAAAAAAAAGGTAAAATTTTTTATTCATCTATTTTCTCCGCACAACTTATCTATTTTTTTTTTCTTATCATTACATAGCAATAAA… 228
f3 ----------TTTTATTCATCTATTTTCTCCGCACAACTTATCTATTTTTTTTTTCTTATCATTACATAGCAATAAA… 124
f4 ----------CCGCACAACTTATCTATTTTTTTTTTCTTATCATTACATAGCAATAAA… 105
Binding sequence TTTTATTCATCTATTTTCTCCGCACAACTTATCTATTTTTTTTCT [bp]

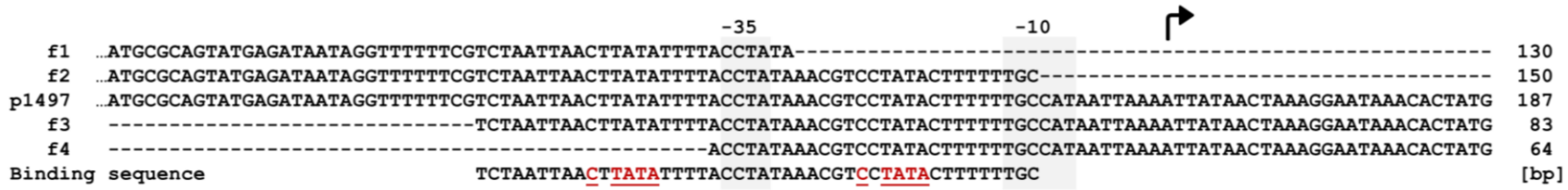
B


-35
-10
f1 …AATAGTATTTTGGAACAAATATTCCAGAATACGTGATTATCTACTTTTACTCCACA---------- 130
f2 …AATAGTATTTTGGAACAAATATTCCAGAATACGTGATTATCTACTTTTACTCCACAAGTTACCTATATATTTTCATT---------- 151
p1534 …AATAGTATTTTGGAACAAATATTCCAGAATACGTGATTATCTACTTTTACTCCACAAGTTACCTATATATTTTCATTACAATCTTCAAATATAACAAAGAAGGAAGAACAAATG 188
f3 ----------TGGAACAAATATTCCAGAATACGTGATTATCTACTTTTACTCCACAAGTTACCTATATATTTTCATTACAATCTTCAAATATAACAAAGAAGGAAGAACAAATG 104
f4 ----------CTCCACAAGTTACCTATATATTTTCATTACAATCTTCAAATATAACAAAGAAGGAAGAACAAATG 65
Binding sequence TGGAACAAATATTCCAGAATACGTGATTATCTACTTTTACTCCACAAGTTACCTATATTTTCATT [bp]

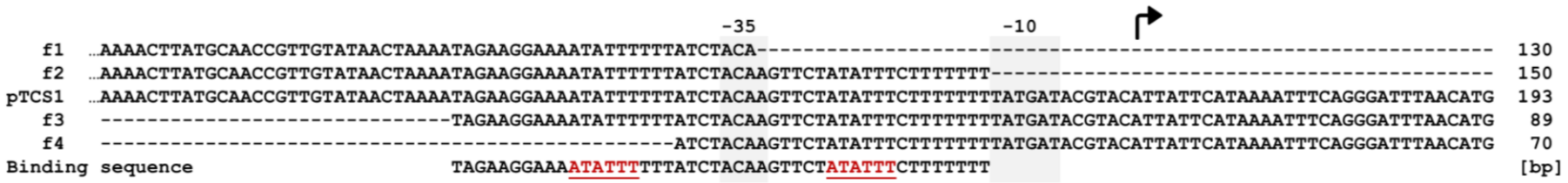
C


-35
-10
f1 …CTACTGAATATTATTTAATGATTAAAGAATTAGTAGAAAAATTATTATTATCTACA---------- 130
f2 …CTACTGAATATTATTTAATGATTAAAGAATTAGTAGAAAAATTATTATTATCTACAAACAACCTATAATTATTTTC---------- 150
p1494 …CTACTGAATATTATTTAATGATTAAAGAATTAGTAGAAAAATTATTATTATCTACAAACAACCTATAATTATTTTCTATACTTACATTTATAATAATTAAATATTTTAAAAATAAGGATAGAATATG 201
f3 ----------TAGTAGAAAAATTATTATTATCTACAAACAACCTATAATTATTTTCTATACTTACATTTATAATAATTAAATATTTTAAAAATAAGGATAGAATATG 97
f4 ----------ATCTACAAACAACCTATAATTATTTTCTATACTTACATTTATAATAATTAAATATTTTAAAAATAAGGATAGAATATG 78
Binding sequence TAGTAGAAAAATTATTATTATCTACAAACAACCTATAATTATTTTC [bp]

D


-35
-10
f1 …ATGCGCAGTATGAGATAATAGGTTTTTTCGTCTAATTAACTTATATTTTACCTATA---------- 130
f2 …ATGCGCAGTATGAGATAATAGGTTTTTTCGTCTAATTAACTTATATTTTACCTATAAACGTCCTATACTTTTTTGC---------- 150
p1497 …ATGCGCAGTATGAGATAATAGGTTTTTTCGTCTAATTAACTTATATTTTACCTATAAACGTCCTATACTTTTTTGCCATAATTAAAATTATAACTAAAGGAATAAACACTATG 187
f3 ----------TCTAATTAACTTATATTTTACCTATAAACGTCCTATACTTTTTTGCCATAATTAAAATTATAACTAAAGGAATAAACACTATG 83
f4 ----------ACCTATAAACGTCCTATACTTTTTTGCCATAATTAAAATTATAACTAAAGGAATAAACACTATG 64
Binding sequence TCTAATTAACTTATATTTTACCTATAAACGTCCTATACTTTTTTGC [bp]

E


-35
-10
f1 …AAAACTTATGCAACCGTTGTATAACTAAAATAGAAGGAAAATATTTTTTATCTACA---------- 130
f2 …AAAACTTATGCAACCGTTGTATAACTAAAATAGAAGGAAAATATTTTTTATCTACAAGTTCTATATTTCTTTTTTT---------- 150
pTCS1 …AAAACTTATGCAACCGTTGTATAACTAAAATAGAAGGAAAATATTTTTTATCTACAAGTTCTATATTTCTTTTTTTTTATGATACGTACATTATTCATAAAATTTCAGGGATTTAACATG 193
f3 ----------TAGAAGGAAAATATTTTTTATCTACAAGTTCTATATTTCTTTTTTTTTATGATACGTACATTATTCATAAAATTTCAGGGATTTAACATG 89
f4 ----------ATCTACAAGTTCTATATTTCTTTTTTTTTATGATACGTACATTATTCATAAAATTTCAGGGATTTAACATG 70
Binding sequence TAGAAGGAAAATATTTTTTATCTACAAGTTCTATATTTCTTTTTTT [bp]

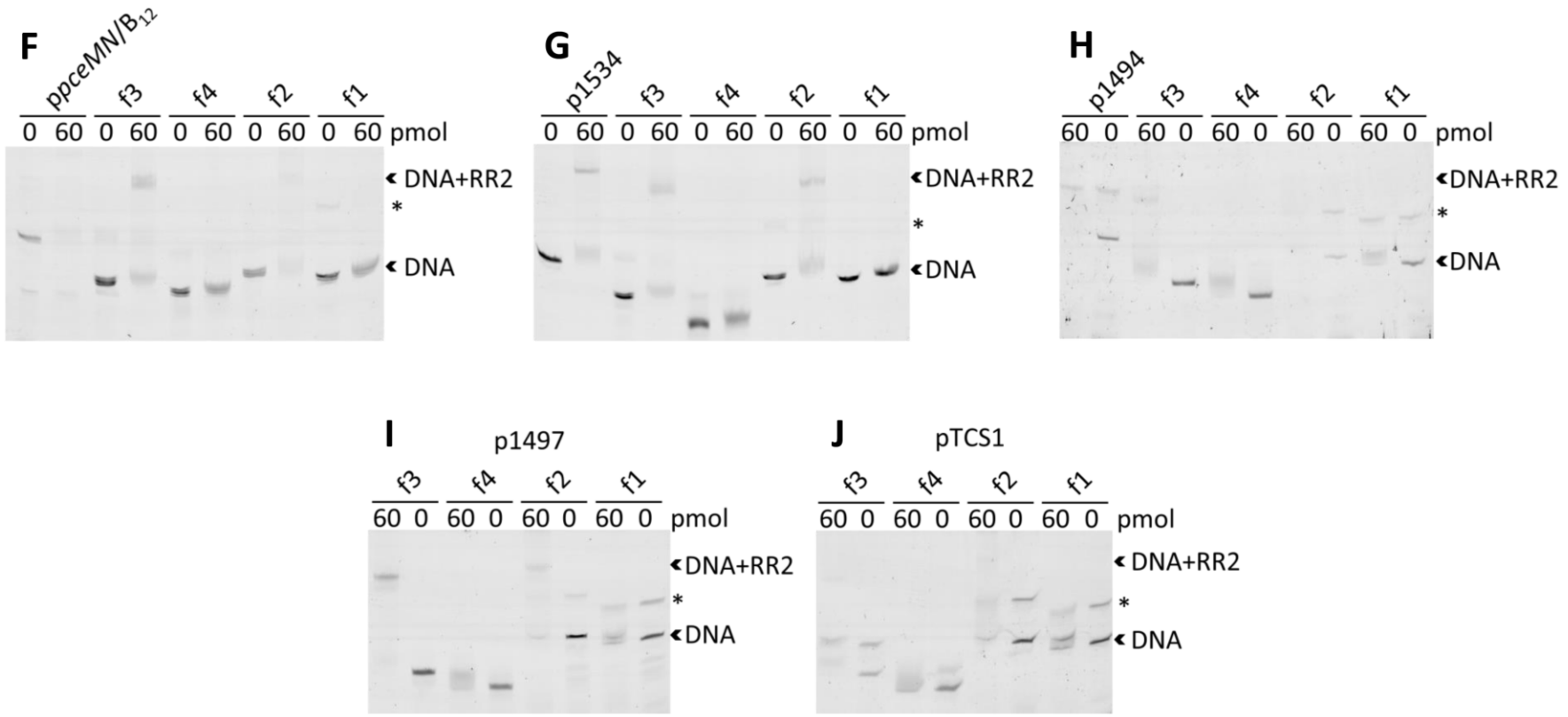


Fig. S10: Truncated promoter DNA sequences (f1-4) of A) p*pceMN*/B_{12}, B) p1534, C) p1494, D) p1497, and E) pTCS1 are shown together with the respective gel shift assays performed with RR2 and the DNA fragments of F) p*pceMN*/B_{12}, G) p1534, H) p1494, I) p1497, and J) pTCS1. The direct repeat in the suggested binding sequence is underlined. The amount of 6Fam-labeled promoter DNA was 2.5 pmol, whereas the protein amount to 0 or 60 pmol as indicated. A DNA artifact not targeted by RR2 is marked by an asterisk. See also Fig. 5 for further EMSA analyses of the promoter sequences of p*pceAB* and pTCS2/1504.

Supplementary RNA data set with raw data of the dRNA-seq, gene annotations and countings is appended as excel file: DESeq2_SMUL_Py_PCE_vs_Py_Fum.xlsx

References:

1. Alikhan N-F, Petty NK, Ben Zakour NL, Beatson SA. 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics 12:402.

The calculated TMP values visualized in Figure 3.1 in the discussion are appended in an excel file: TPM_SMUL_Py_PCE_vs_Py_Fum.xlsx

Supplementary Figures and Tables

A Retentive Memory of Tetrachloroethene Respiration in *Sulfurospirillum halorespirans* - involved Proteins and a possible link to Acetylation of a Two-Component Regulatory System

Dominique Türkowsky, Jens Esken, Tobias Goris, Torsten Schubert, Gabriele Diekert, Nico Jehmlich, Martin von Bergen

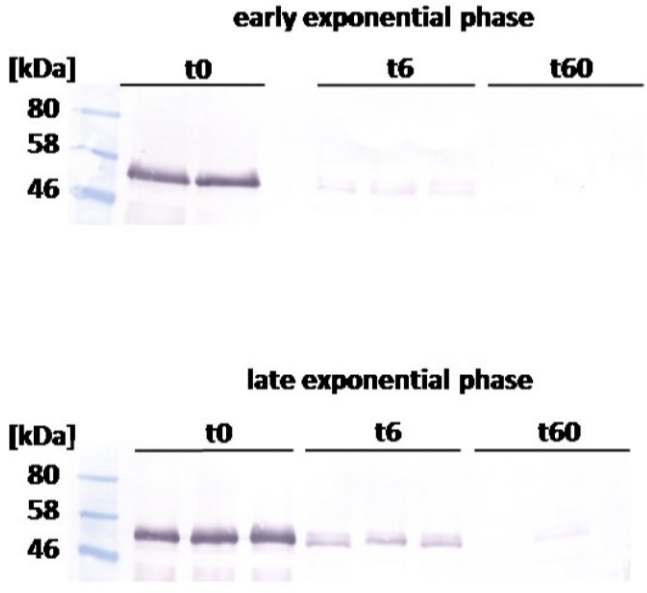
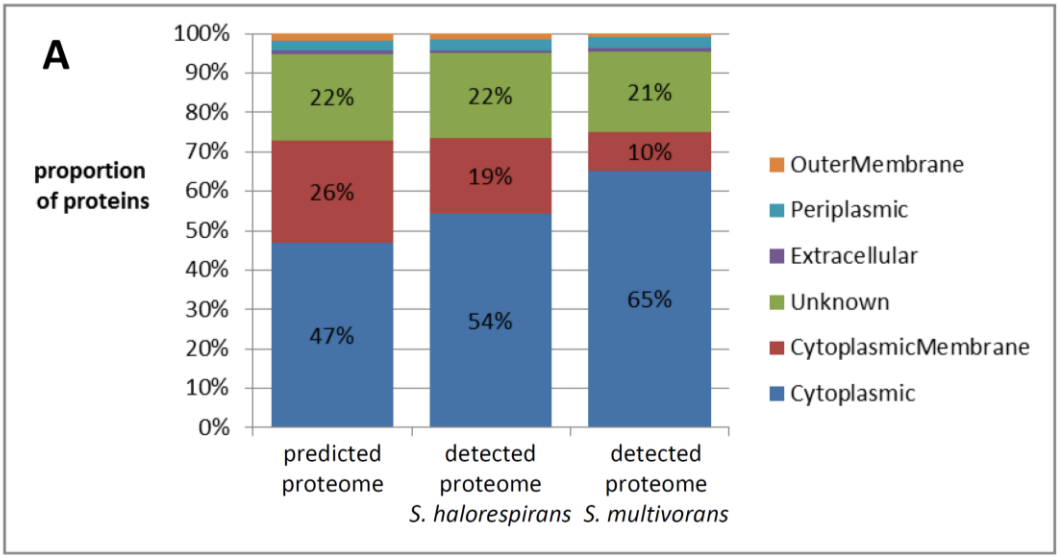


Fig. S1. Immunostaining of of PceA in crude extracts from *S. halorespirans* cultivated on PCE (t0) or for six or 60 transfers on nitrate (t6, t60). 10 µg protein per lane applied to SDS-PAGE, subsequent blotting to a PVDF membrane. Biological replicates of the samples harvested after the transfers t0, t6 and t60 during the early (OD_{578} ≈0.11) and late (OD_{578} ≈0.20) exponential growth phase are shown.



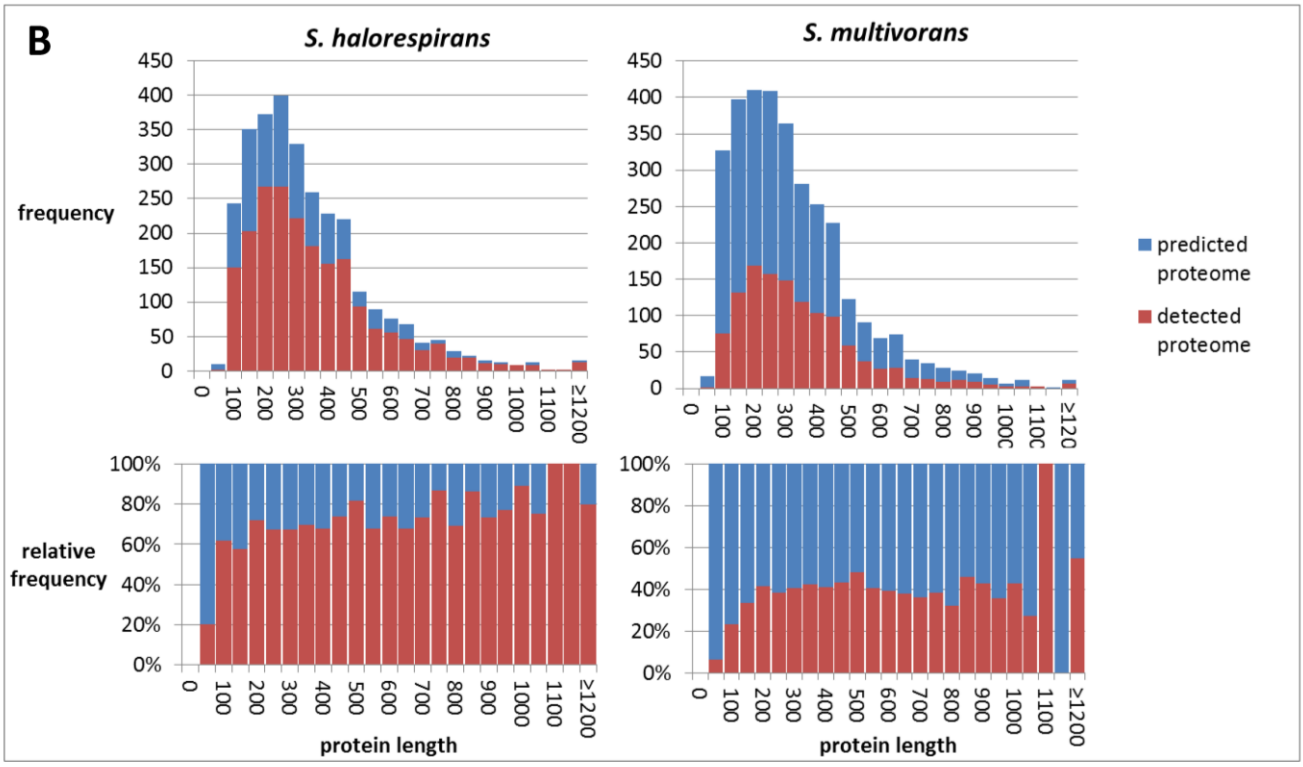


Fig. S2. Comparison of the measured and predicted proteome of *S. halorespirans* (this study) and *S. multivorans* [1]. A Distribution of protein localization of proteins compared for the predicted proteome of *S. halorespirans* and the detected proteome of *S. halorespirans* and *S. multivorans*. The distribution of the predicted proteome of *S. multivorans* is comparable to *S. halorespirans*. B Distribution of length of proteins, absolute and percentage.

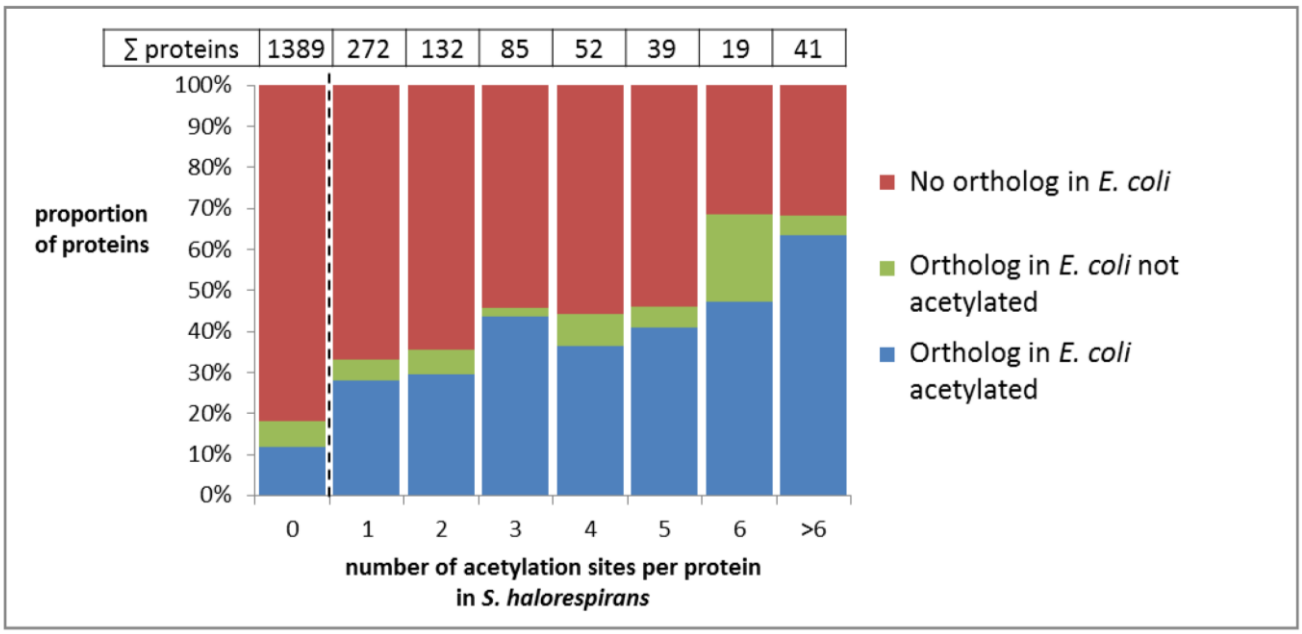


Fig. S3. Proportion of proteins of *S. halorespirans* with an ortholog in *E. coli* acetylated (blue), not acetylated (green) or without any ortholog in *E. coli* (red).
The probability of having an ortholog in *E. coli* increases with the number of acetylation sites of proteins from *S. halorespirans*. Orthologs were defined as having >35% amino acid sequence identity, >50% query coverage and reciprocal best blast hits, as calculated with BLAST Reciprocal Best Hits (RBH) from two FASTA files (Galaxy Version 0.1.11, [2,3]). In total, 21% of *S. halorespirans*-proteins had orthologs in *E. coli* respectively.

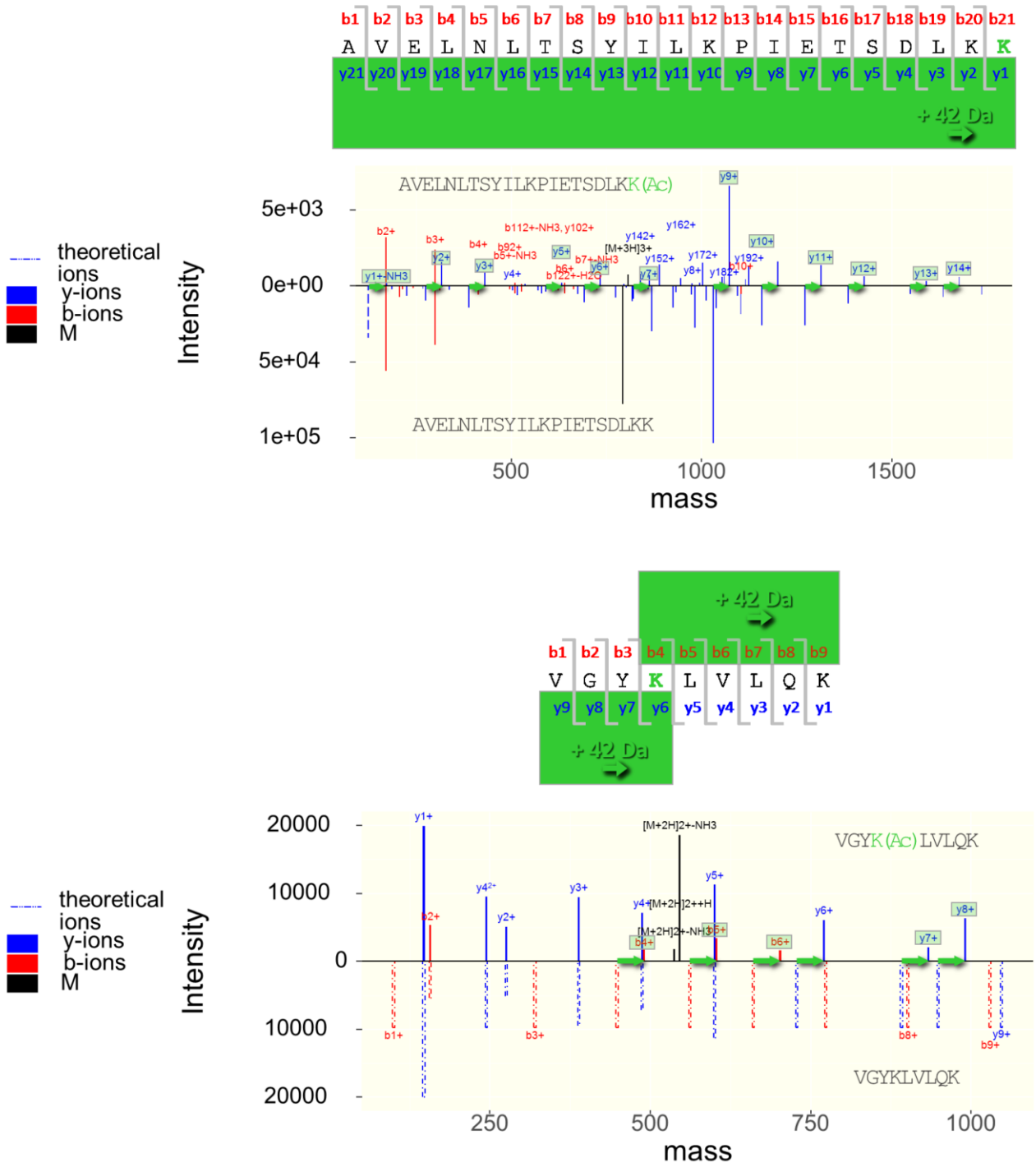
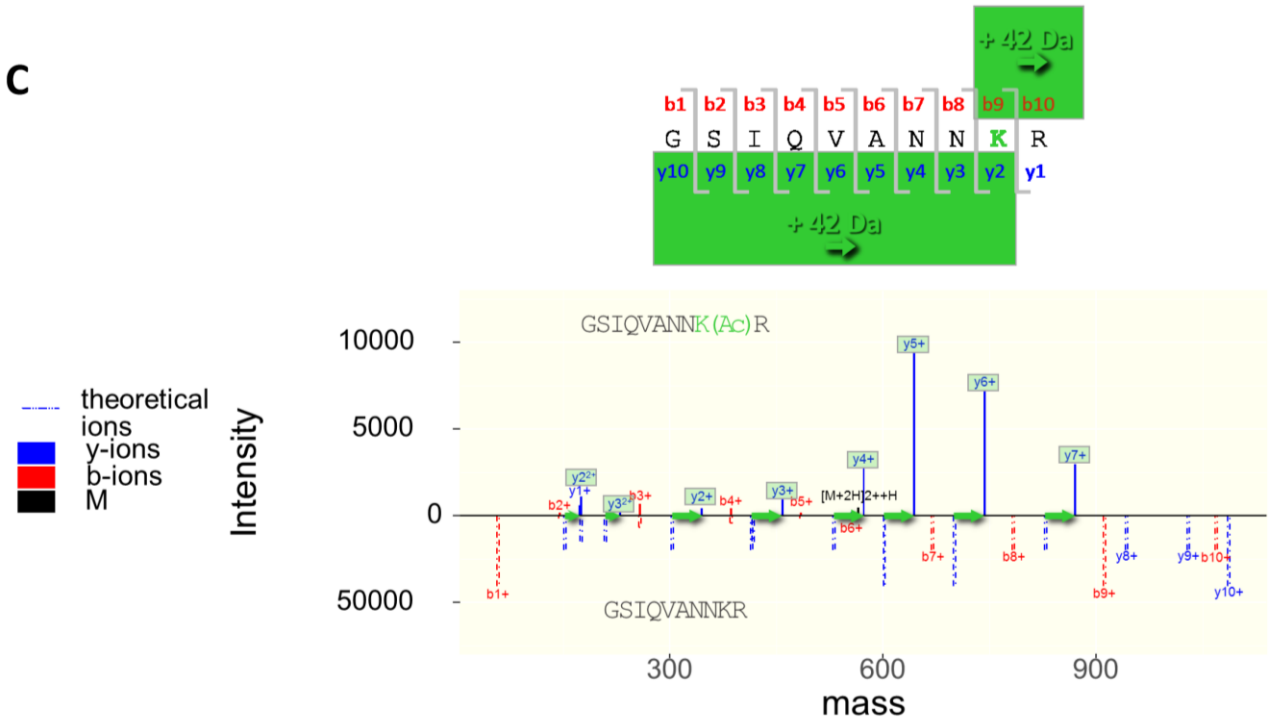


Fig. S4. Measured fragmentation spectra of acetylated peptides of the two-component-system compared with the measured or theoretical spectra of the unmodified peptide. (A) In the example of AVELNLTSYILKPIETSDLKK of the response regulator (SHALO_1503) the acetylation is on K21 (K114 on protein), which shifts the mass (m/z) of the peptide fragments y1-y21 by 42 Da. (B) In the example of VGYKLVLQK of the response regulator the acetylation is on K4 (K218 on protein), which shifts the mass (m/z) of the peptide fragments y6-y9 and b4-b10 by 42 Da. (C) In the example of GSIQVANNKR of the histidine kinase (SHALO_1502) the acetylation is on K9 (K598 on protein), which shifts the mass (m/z) of the single charged peptide fragments $y2^{+}$-$y10^{+}$ and $b9^{+}$-$b10^{+}$ by 42 Da and of the double charged peptide fragments $y2^{2+}$-$y10^{2+}$ and $b9^{2+}$-$b10^{2+}$ by 21 Da.



Tab. S1-5 with raw data of the proteomic analyses are appended as excel files.

The supplementary data can also be found online at https://doi.org/10.1016/j.jprot.2018.03.030

Tab. S6. Abundances of acetylated peptides of the two-component system II. Abundance values were logarithmized and normalized to protein abundances (see Method section for details). The three columns of each sample correspond to the three replicates.

Locus tag (gene name)	Description	Peptide Sequence	Acetylation site	t0 EE			t0 EL			t6 EE			t6 EL			t60 EE			t60 EL		
				1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
SHALO_1502	histidine kinase	GSIQVANNKR	K598								2.9			2.2	1.9						
SHALO_1503	response regulator	AVELNLTSYILKP IETSDLKK	K114	2.0	2.7	2.6	2.3				3.1	2.9	3.1	3.1	3.2		2.3			2.6	
SHALO_1503	response regulator	VGYKLVLQK	K218								1.8										

References

[1] T. Goris, C.L. Schiffmann, J. Gadkari, T. Schubert, J. Seifert, N. Jehmlich, M. von Bergen, G. Diekert, Proteomics of the organohalide-respiring Epsilonproteobacterium *Sulfurospirillum multivorans* adapted to tetrachloroethene and other energy substrates, Sci Rep 5 (2015) 13794.
[2] C. Camacho, G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos, K. Bealer, T.L. Madden, BLAST+: architecture and applications, BMC Bioinformatics 10(1) (2009) 421.
[3] P.J.A. Cock, J.M. Chilton, B. Grüning, J.E. Johnson, N. Soranzo, NCBI BLAST+ integrated into Galaxy, GigaScience 4(1) (2015) 1-7.

Supplementary Tables

Cobalt - an essential micronutrient for organohalide respiration in dehalogenating *Sulfurospirillum* spp.

Jens Esken, Torsten Schubert, Gabriele Diekert

Table S1: List of oligonucleotides used in this study.

Gene	Name	Sequence (5' -> 3')
SMUL_1538/SHALO_1502	HK2	CCGGTCAAAGGATGTGCGCATAAA
SMUL_1538/SHALO_1502	HK2	CCGGTGCATTTGCAGATTCGTCAT
SMUL_2335/SMUL_3269 SHALO_2082/SHALO_2989	*rrsA1/rrsA2*	GAGACACGGTCCAGACTCCTAC
SMUL_2335/SMUL_3269 SHALO_2082/SHALO_2989	*rrsA1/rrsA2*	CTCGACTTGATTTCCAGCCTAC
SMUL_1531/SHALO_1495	*pceA*	GCCCGCACGTGAGTGTCCTTCAAA
SMUL_1531/SHALO_1495	*pceA*	GGCCCGCCACAATATCCACCAGAT
SMUL_1543/SHALO_1507	*cbiB*	GGCCGCTGGATTGGCATTTCATTT
SMUL_1543/SHALO_1507	*cbiB*	GGCCTCTTTGAATGCAGCGTGAGT
SMUL_0934/SHALO_0949	*napA*	TGGGACAAATCCGTTTGCCGTTTCTGTGG
SMUL_0934/SHALO_0949	*napA*	AAAAGTGGCTCTTTAAGACGATCCGCGCC

Table S2: Trace elements in µg per l culture medium; blanked when blank exceeded the detection limit.

Sample	T0 (Rep.1)	T0 (Rep.2)	T0 (mean)	T1 (Rep.1)	T1 (Rep.2)	T1 (mean)	T2 (Rep.1)	T2 (Rep.2)	T2 (mean)	T3 (Rep.1)	T3 (Rep.2)	T3 (mean)	Detection limit	Theoretical value
Li	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	0.5	0
Li (SD)														
Be	< 1.4	< 1.4	< 1.4	< 1.4	< 1.4	< 1.4	< 1.4	< 1.4	< 1.4	< 1.4	< 1.4	< 1.4	1.4	0
Be (SD)														
B (blanked)	129.5	129.5	129.5	102.3	132.5	117.4	142.75	148	145	132.5	160	146	2.1	1
B (SD)	2.0	3.0	4.8	0.3	0.3	21.9	0.07	2	6	0.2	3	23		
Mg (blanked)	50720	48983	49851.5	50083	49170	49626.5	50013	48084	49048.5	49447	49105	49276	1	47821
Mg (SD)	619	594	2441.2	244	14	903.6	251	364	1979.0	386	7	635		
Al (blanked)	6.8	5.7	6.3	18.3	19.8	19.0	8.0	7.8	7.9	11.9	7.6	9.8	0.6	0
Al (SD)	0.5	0.2	1.5	0.3	0.7	2.1	0.2	0.0	0.3	0.8	0.1	3.9		
Sc	0.31	0.31	0.31	0.33	0.37	0.35	0.37	0.37	0.37	0.48	0.40	0.44	0.3	0
Sc (SD)	0.05	0.02	0.07	0.04	0.03	0.10	0.05	0.05	0.10	0.01	0.02	0.09		
V	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	0.8	0
V (SD)														
Cr	< 1	< 1	< 1	1.0	< 1	< 1	1.83	< 1	< 1.42	< 1	1.2	< 1.1	1	0
Cr (SD)				0.1			0.02				0.4			
Mn (blanked)	21.5	25.3	23.4	15.5	20.3	17.9	12.58	16.7	14.7	12.5	15.1	13.8	0.5	27.8
Mn (SD)	0.3	0.3	3.3	0.2	0.4	4.0	0.01	0.1	3.0	0.2	0.2	2.2		
Fe (blanked)	1299	2137.5	1718	1041	1262	1152	778	874	826	707	903	805	0.4	1120
Fe (SD)	6	0.7	600	0	0	156	7	13	88	7	8	154		
Co (blanked)	33.4	32.8	33.1	3.1	3.2	3.2	0.42	0.44	0.43	0.223	0.251	0.237	0.15	47.1
Co (SD)	0.2	0.1	0.8	0.1	0.1	0.3	0.04	0.04	0.09	0.000	0.009	0.026		
Ni (blanked)	6.9	6	7	4.2	3.92	4.1	3.03	3.3	3.2	3.0	3.48	3.2	0.6	5.9
Ni (SD)	0.5	1	2	0.5	0.02	0.7	0.02	0.3	0.5	0.5	0.02	0.9		
Cu	< 1	< 1	< 1	1.1	< 1	< 1.1	< 1	< 1	< 1	< 1	< 1	< 1	1	0
Cu (SD)				0.3										
Zn (blanked)	131	125	128	147	106	127	144	147	145	126	124	125	3.2	33.6
Zn (SD)	4	2	10	4	2	35	6	4	12	8	1	10		
As	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	1.5	0
As (SD)														
Rb (blanked)	15.0	13.2	14.1	13.8	13.0	13.4	13.6	12.67	13.1	13.32	13.6	13.5	0.2	0
Rb (SD)	0.4	0.1	1.8	0.2	0.3	1.1	0.2	0.02	0.9	0.07	0.2	0.5		
Sr (blanked)	21.3	21.7	21.5	24.4	28.1	26.2	21.8	23.6	22.7	23.9	21.8	22.9	0.2	0
Sr (SD)	0.6	0.5	1.4	0.0	0.3	3.0	0.1	0.0	1.4	0.2	0.2	1.9		
Y	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.1	0
Y (SD)														
Zr	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	0.8	0
Zr (SD)														
Nb	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	0.5	0
Nb (SD)														

Mo	12.19	12.1	12.2	3.176	2.91	3.04	2.3	2.3	2.3	2.4	2.4	2.4	1	14.3
Mo (SD)	0.01	0.2	0.3	0.005	0.09	0.28	0.2	0.1	0.3	0.1	0.3	0.4		
Ag	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	0.37	< 0.2	< 0.29	< 0.2	< 0.2	< 0.2	0.2	0
Ag (SD)							0.04							
Cd	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	0.3	0
Cd (SD)														
Sn	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	0.2	0
Sn (SD)														
Sb	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	0.3	0
Sb (SD)														
Cs	0.068	< 0.05	< 0.059	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Cs (SD)	0.004													
Ba (blanked)	9.9	8.8	9.4	12.4	17.5	14.9	6.1	15.2	10.7	17.7	28.7	18.2	0.5	0
Ba (SD)	0.1	0.2	1.1	0.2	0.3	4.0	0.3	0.2	7.2	0.5	0.4	1.6		
La	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.058	< 0.05	< 0.054	0.05	0
La (SD)										0.008				
Ce	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.1	0
Ce (SD)														
Pr	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Pr (SD)														
Nd	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.1	0
Nd (SD)														
Sm	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.1	0
Sm (SD)														
Eu	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Eu (SD)														
Tb	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Tb (SD)														
Gd	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Gd (SD)														
Dy	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Dy (SD)														
Ho	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Ho (SD)														
Er	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	0.2	0
Er (SD)														
Tm	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Tm (SD)														
Yb	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.1	0
Yb (SD)														
Lu	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Lu (SD)														
Hf	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	0.2	0
Hf (SD)														
Ta	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.1	0

Ta (SD)														
W	1.21	1.17	1.19	1.42	1.3155	1.37	1.39	1.26	1.33	1.36	1.35	1.36	0.2	0
W (SD)	0.03	0.02	0.08	0.06	0.0007	0.14	0.06	0.06	0.21	0.01	0.09	0.11		
Hg	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	2	0
Hg (SD)														
Pb	< 0.5	< 0.5	< 0.5	< 0.5	0.54	< 0.52	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	0.5	0
Pb (SD)					0.01									
Th	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.05	0
Th (SD)														
U	0.059	0.069	0.064	0.063	0.066	0.065	0.074	0.065	0.070	0.08	0.07	0.075	0.05	0
U (SD)	0.004	0.007	0.018	0.009	0.006	0.017	0.002	0	0.008	0.005	0.007	0.019		

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Author's declaration of originality

I, Jens Esken, born on 16th February 1988 in Aachen (Germany), hereby declare that I am familiar with the valid doctoral examination regulations of the Faculty of Biological Sciences of the Friedrich Schiller University Jena. Furthermore, I confirm that I produced the doctoral thesis project myself, that I neither did use any text passages from third parties nor my own previous final theses without citing those. In addition, I also confirm that I cited the tools, personal communication, and sources having been used. Support in the preparation of the manuscripts was only received from named coauthors as well as anonymous reviewers within the peer-review processes during manuscript submission. All persons who assisted in cooperation were acknowledged in this thesis. I confirm that I did not receive any assistance from specialized consultants and that any third party did not receive either direct or indirect financial benefits for work connected to the doctoral thesis submitted. I declare that I have not already submitted the doctoral thesis project as final thesis for a state examination or other scientific examination. I did not submit the same, a substantially similar, or another scientific paper to any other institution of higher education or to any other faculty as a doctoral thesis.

Jena, 02.04.2020

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Jens Esken