Approaching the Uncultured Endosymbiont of *Riftia pachyptila* by Physiological Proteomics

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The bacterial endosymbiont of the deep-sea tube worm *Riftia pachyptila* has never been successfully cultivated outside its host. In the absence of cultivation data we have taken a proteomic approach based on the metagenome sequence to study the metabolism of this peculiar microorganism in detail. As one result, we found that three major sulfide oxidation proteins constitute ~12% of the total cytosolic proteome, highlighting the essential role of these enzymes for the symbiont's energy metabolism. Unexpectedly, the symbiont uses the reductive tricarboxylic acid (TCA) cycle in addition to the previously identified Calvin cycle for CO_2 fixation.

Riftia pachyptila inhabits deep-sea hydrothermal vent areas along mid-ocean ridges in the East Pacific (*1*). Instead of containing a digestive system, the worm's coelomic cavity is densely populated by a single species of sulfide-oxidizing gamma-proteobacteria that provide for their host's carbon and energy supply by fixing CO_2 from the surrounding water (*2-4*). Microbial chemosynthesis is sustained by the presence of H_2S originating from reduced hydrothermal fluids and oxygen in the seawater (*5*). Compared with free-living sulfur-oxidizers, the symbionts benefit from high nutrient concentrations within the worm's body (*6*), which allows for a high metabolic activity. The microbially produced carbon compounds are transferred to the host, making *R. pachyptila* one of the fastest-growing marine invertebrates known (*7*).

We took a functional genomics approach to analyze the proteome and hence derive information on the physiology of the uncultured *Riftia* symbionts. The sequencing of the symbiont genome was conducted by a metagenome approach. Bacterial symbionts were isolated and separated from the host tissue, the so-called trophosome (*8*). Using one- and two-dimensional (2D) gel electrophoresis we established reference maps (master gels) of the soluble intracellular and the membrane-associated bacterial proteome, on which more than 220 identified proteins have been registered so far (Fig. 1 and Fig. S1). Our procedures were reproducible, showing the presence of a single endosymbiont species (*9*) with no indication of other bacterial or host tissue contaminants. The proteomic approach provided evidence regarding which of the predicted genes are expressed under natural growth conditions. Levels of protein synthesis reflect the relative proportion of translational capacity that is invested in the individual metabolic paths, and we can deduce the relevance of particular proteins for the symbionts' physiology. Based on our proteome data we were thus able to deduce major pathways of the symbionts' metabolism, e.g. the sulfide oxidation pathway (Fig. S2) and the

reverse tricarboxylic acid (TCA) cycle (Fig. S3), and to make inferences on the symbionts' response to oxidative stress (Fig. 3).

Although great advances have been made in our understanding of the sulfur oxidation pathways in a variety of sulfur-oxidizers in recent years, this pathway remains incompletely characterized in *Riftia* symbionts. In this proteome study the enzymes DsrA (dissimilatory sulfite reductase), AprA/AprB (adenosine phosphosulfate reductase) and SopT (ATP sulfurylase) were identified, suggesting the reactions displayed in Fig. S2: H_2S is oxidized to SO_3^{2-} in a six-electron step; this is subsequently converted to adenosine phosphosulfate (APS), with consumption of AMP. The final oxidation step yields ATP by substrate-level phosphorylation and simultaneously creates the end product, SO_4^{2-}. While these enzymes were originally described as part of the reverse pathway in sulfate-reducing bacteria (as reflected in their names), it has long been proposed that they can function just as well in the opposite direction (*10*). Quantitative analyses of the respective protein spots in our study revealed that the three major sulfide oxidation proteins DsrA, AprA/AprB and SopT constitute more than 12% of the total cytosolic symbiont proteome in a pH range of 4 – 7. This highlights the essential role of these enzymes for the symbiont's energy metabolism.

Using our intracellular protein master gel, we also examined the *Riftia* symbiont's carbon metabolism. We identified most of the enzymes involved in the energy-generating TCA cycle. Moreover, we found the enzyme 2-oxoglutarate:ferredoxin oxidoreductase (KorB), several copies of a pyruvate:ferredoxin oxidoreductase (Por1, Por2 and PorA/PorG), a putative fumarate reductase (SdhA/SdhC), and a protein highly similar to a citryl-CoA synthetase subunit (CcsB). These enzymes could run the TCA cycle in the reductive direction (Fig. S3). This cycle represents an alternative CO_2 fixation mechanism that requires less energy than the Calvin cycle per 3-carbon unit formed. Considering the high abundance of

these four key enzymes on the protein gels (see relative spot volumes in table S1) we suggest that the reductive TCA cycle is not only a possibility, but a very important feature of the *Riftia* symbiont's carbon metabolism.

To verify the symbiont's capability for using the reductive TCA cycle for CO_2 fixation, we tested cell extracts of isolated bacteria for activity of the above-mentioned key enzymes: The assays clearly revealed specific enzyme activities in all four cases that are higher than those previously reported for RubisCO (*11*) (table S2).

The results of our proteome analysis in combination with the measured enzyme activities provide strong evidence that the *Riftia* symbionts, which have been considered a prime example for chemolithoautotrophic carbon fixation via the Calvin cycle, utilize, at least partly, the reductive TCA cycle for autotrophic carbon fixation as well. This might explain the longstanding dilemma that the stable carbon isotopic composition of the *Riftia* symbiont is substantially heavier (Δ13C of –9‰ to -16‰) than would be expected by the use of the Calvin cycle alone (*12*). The observed isotopic variation most likely results from varying contributions by the reductive TCA cycle and the Calvin cycle. It is also interesting to note that, concordant with previous findings (*12*), RubisCO constitutes only ~1% of the *Riftia* symbiont's total protein on our gels. This is rather little compared to other bacteria solely using the Calvin cycle for CO_2 fixation, where RubisCO is usually the major protein and can account for 4 to 50% of the total soluble protein (*13*). The apparent discrepancy between the low concentration and the relatively high activity of RubisCO in the trophosome (*4*) and the possible occurrence of two carbon fixation pathways in one organism certainly warrant further investigations.

Riftia symbionts can store elemental sulfur in their periplasm if high concentrations of H_2S are available (*14*), resulting in a light green, almost yellow trophosome. The tissue appears dark green or black (*15*) when sulfur is limiting. To analyze the symbionts' potential

responses to changing environmental conditions we compared bacterial protein patterns from naturally occurring sulfur-rich and from sulfur-depleted trophosome tissues. Under high-sulfide conditions, the resulting 2D gels revealed a distinctly higher spot intensity for enzymes involved in sulfide oxidation and for RubisCO, compared to low-sulfur conditions (Tab. 1, Fig. 2). Spot intensities of the sulfide oxidation enzymes AprA and DsrA were about 8-fold and 4-fold greater, respectively. This indicates that *Riftia* symbionts are capable of adjusting the production of enzymes needed for energy metabolism to the prevailing environmental conditions. In bacteria from tissue with only little or no stored sulfur (dark trophosomes) the protein spot volume of the putative fumarate reductase subunit SdhA was about 24 times higher, and the spot volume of the putative pyruvate:ferredoxin oxidoreductase Por1 was about 9 times higher than in sulfur-rich, light trophosome (Fig. 2, Tab. 1). Several other enzymes involved in the reductive TCA cycle, including two other pyruvate:ferredoxin oxidoreductase subunits, PorG and PorA, and the 2-oxoglutarate synthase subunit KorB, could also be detected with elevated spot volumes in samples from sulfur-depleted trophosomes. This suggests that the *Riftia* symbiont might be capable of adapting to a temporary low-energy situation by adjusting its way of carbon fixation: The use of the Calvin cycle might be reduced in favor of the up-regulation of the energetically more favorable reductive TCA cycle. Since *Riftia* symbionts have also been shown to produce glycogen as a carbon storage compound (*16*) it might even be speculated that – for example under long-lasting or severe low-energy conditions – their metabolism switches from an autotrophic mode to a heterotrophic mode. In this case the symbiont might revert to burning carbon reserves through glycolysis and the oxidative TCA cycle to generate energy and precursors for biosynthesis. The symbiont might thus be able to use the TCA cycle in the oxidative and reductive direction, depending on the environmental conditions, which allows for a high metabolic flexibility. Apparently, the true nature of the *Riftia* symbiont's metabolic strategies

is much more complex than expected. Further detailed investigations are needed to explicitly evaluate and validate these hypotheses.

Physiological tests revealed that *Riftia* symbionts do not possess a catalase to protect the cells from H_2O_2 (*17*). This result is supported by the lack of the respective gene in the metagenome sequence. Since *Riftia* symbionts do not tolerate high oxygen concentrations (*18*), we tested their strategy of coping with stress situations caused by hydrogen peroxide. Our experiments revealed a strong induction of the alkyl hydroperoxide reductase AhpC (Fig. 3). This enzyme is present in high amounts in the cytoplasmic protein fraction (Fig. 1) and reduces organic hydroperoxides caused by H_2O_2. Our results indicate that AhpC plays a crucial role in the resistance of these microaerophilic bacteria against oxidative stress.

This study shows that a comparative proteomic view of the *Riftia* symbionts' cell physiology allows for a complex physiological description without their cultivation. It reaches beyond the mere prediction of putative metabolic functions as coded in the genome sequence.

References and notes

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19. We appreciate the great help of Monika Bright and thank James Childress and Craig Cary for inviting us on their cruises. Thanks also to Annette Dreisbach, Uwe Völker, Jörg Bernhardt, Manuel Kleiner and Martin Fraunholz. This work was supported by the DFG, grant Schw595/3-1. Other funding sources were: NSF (OCE 04-52333) and

NASA Astrobiology Institute (NNA04CC04A) for SMS, MH: postdoctoral scholarship from WHOI, HF: Academic Senate (RF811S and RE518S). The *Riftia* symbiont nucleotide sequence has been deposited as a Whole Genome Shotgun project at DDBJ/EMBL/GenBank under the project accession AASF00000000. For all proteins addressed in this study the annotation results were submitted to the NCBI GenBank (see table S1).

Table 1: Comparison of *Riftia* symbiont protein spot intensities under high- / low-sulfur conditions

Spot intensities of selected proteins were compared between 2D gel images from sulfur-rich and sulfur-depleted trophosome samples (see SOM for details). Positive ratios represent the factor by which the respective spot intensity was higher on the gels from sulfur-rich trophosomes. Negative ratios give the factor by which spot intensity was higher under low-sulfur conditions. The listed protein spots are highlighted in Fig. 2.

Protein	Function	Spot ratio
Proteins with higher spot intensity in sulfur-rich trophosome samples:		
AprA	adenylylsulfate reductase, alpha subunit	8.20
CbbM	ribulose-1,5-bisphosphate carboxylase / oxygenase form II, large subunit	4.29
DsrA	dissimilatory sulfite reductase, alpha subunit	3.55
HdrA2	heterodisulfide reductase, subunit A	2.26
DsrL	predicted NADPH:acceptor oxidoreductase	1.92
DsrC	dissimilatory sulfite reductase, gamma subunit	1.47
AprB	adenylylsulfate reductase, beta subunit	1.30
Proteins with higher spot intensity in sulfur-depleted trophosome samples:		
SdhA	putative fumarate reductase, flavoprotein subunit	- 23.83
OadA	oxaloacetate decarboxylase, alpha subunit	- 15.00
Por1	pyruvate:flavodoxin/ferredoxin oxidoreductase	- 8.58
SucC	succinyl CoA ligase, beta subunit	- 6.75
PorG	pyruvate:ferredoxin/flavodoxin oxidoreductase	- 6.13
KorB	2-oxoglutarate synthase	- 4.57
AcnA	aconitase A	- 4.50
Mdh1	malate dehydrogenase	- 2.36
SucD	succinyl-CoA synthetase, alpha subunit	- 2.12
PorA	pyruvate:flavodoxin/ferredoxin oxidoreductase	- 2.03

Legends

Figure 1: Reference map of the *Riftia pachyptila* endosymbionts' intracellular proteome

Identified symbiont proteins are indicated. Protein functions are listed in table S1.

Figure 2: Comparison of protein patterns under high- and low-sulfur conditions

Images of the 2D gels were colored (sulfur-rich: green, sulfur-depleted tissue: red spots) and overlaid. Selected proteins with distinct variations in their relative spot volumes from one gel to the other are indicated (see SOM for details).

Figure 3: Comparison of protein patterns in response to oxidative stress

The 2D gels show proteins from samples taken before and 60 minutes after the addition of 2 mM H_2O_2 to a suspension of isolated *Riftia* symbionts. Both images were colored (control: green spots, stress sample: red spots) and overlaid. The oxidative stress protein AhpC is indicated. Due to the delayed deformylation of the newly synthesized protein during the massive induction of AhpC production, the protein spot shifts to a slightly more acidic pH. The "new" AhpC, produced after the stress with H_2O_2 is thus visible as red spots right next to the "old" AhpC, visible as green spots.