More than the "Killer Trait": Infection with the Bacterial Endosymbiont *Caedibacter taeniospiralis* Causes Transcriptomic Modulation in *Paramecium* Host

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Abstract

Endosymbiosis is a widespread phenomenon and hosts of bacterial endosymbionts can be found all-over the eukaryotic tree of life. Likely, this evolutionary success is connected to the altered phenotype arising from a symbiotic association. The potential variety of symbiont's contributions to new characteristics or abilities of host organisms are largely unstudied. Addressing this aspect, we focused on an obligate bacterial endosymbiont that confers an intraspecific killer phenotype to its host. The symbiosis between *Paramecium tetraurelia* and *Caedibacter taeniospiralis*, living in the host's cytoplasm, enables the infected paramecia to release *Caedibacter* symbionts, which can simultaneously produce a peculiar protein structure and a toxin. The ingestion of bacteria that harbor both components leads to the death of symbiont-free congeners. Thus, the symbiosis provides *Caedibacter*-infected cells a competitive advantage, the "killer trait." We characterized the adaptive gene expression patterns in symbiont-harboring *Paramecium* as a second symbiosis-derived aspect next to the killer phenotype. Comparative transcriptomics of infected *P. tetraurelia* and genetically identical symbiont-free cells confirmed altered gene expression in the symbiont-bearing line. Our results show up-regulation of specific metabolic and heat shock genes whereas down-regulated genes were involved in signaling pathways and cell cycle regulation. Functional analyses to validate the transcriptomics results demonstrated that the symbiont increases host density hence providing a fitness advantage. Comparative transcriptomics shows gene expression modulation of a ciliate caused by its bacterial endosymbiont thus revealing new adaptive advantages of the symbiosis. *Caedibacter taeniospiralis* apparently increases its host fitness via manipulation of metabolic pathways and cell cycle control.

Key words: ciliate, fitness advantage, mutualist, parasite, R-body, RNA-Seq.

Introduction

New phenotypes and abilities arise from symbioses. Some of those have been studied at the functional and transcriptome level. Examples comprise nutritional symbioses where the symbionts substitute the diet of their host by provision of nutrients or by cooperation in the digestion of complex compounds, for example, in cellulose-digesting termites (Tartar et al. 2009; He et al. 2013), phloem-feeding insects (Sloan et al. 2014), and mice (Sonnenburg et al. 2006). Defensive symbioses provide another kind of altered phenotype, for example, immune system activation by murine and human microbiomes (Anahtar et al. 2015; Zelante et al. 2013) or resistance to chytridiomycosis in amphibians (Rebollar et al. 2016). More "exotic" cases report developmental and

behavioral modifications (Stilling et al. 2015; Heijtz et al. 2011; Shin et al. 2011).

The association between the eukaryote *Paramecium tetraurelia* (Ciliophora, Alveolata) and the cytoplasmic endosymbiont *Caedibacter taeniospiralis* (Gammaproteobacteria) might be considered a less complex symbiosis as it includes only two participants. However, complexity is not necessarily restricted to the number and variety of involved partners. The *Paramecium-Caedibacter* symbiosis exhibits a rather peculiar phenotypic change: Paramecia carrying these obligate intracellular bacteria are termed killer paramecia because they become toxic for uninfected *Paramecium* cells, a phenomenon called killer trait (Schrallhammer and Schweikert 2009; Pond et al. 1989; Preer et al. 1974). The underlying mechanism is not yet completely understood. *Caedibacter taeniospiralis* residing in the cytoplasm of *P. tetraurelia* occur in two morphologically different forms: Normal bacterial rods ("nonbright") and slightly bigger "bright" cells. The first multiply by standard cell division whereas the latter are incapable of division and contain a microscopically visible (up to 20 µm in length, 0.4 µm in width), proteinaceous structure termed Refractile body (R-body) due to its bright appearance in phase contrast microscopy. The R-body is build-up from a large, tightly coiled protein ribbon. As a prerequisite for the killer trait, bright bacteria need to be released and ingested by *Caedibacter*-free and thus sensitive paramecia (Preer et al. 1974; Schrallhammer et al. 2012). Phagocytosis represents the normal feeding behavior of bacterivorous paramecia. Incorporation in a phagosome followed by acidification triggers the R-body to unroll and thereby to destroy bacterial and vacuolar membranes. This action is probably connected with the release of an unidentified toxin, which ultimately leads to the death of the *Caedibacter*-free *Paramecium* cell. Infected paramecia are protected from the killer trait by an unknown mechanism provided by the symbiotic bacteria. Thus, they gain a selective advantage compared with symbiont-free competitors (Schrallhammer and Schweikert 2009).

While environmental paramecia can be found either free of symbiotic bacteria or infected by one or more bacterial endosymbionts from diverse phylogenetic origins (Görtz and Fokin 2009), *C. taeniospiralis* reproduces exclusively in the cytoplasm of *P. tetraurelia* and is vertically transmitted. Hence, its survival, reproduction, and evolution are coupled to its host. At the first glance, all benefits of expressing the killer trait are gained by *Paramecium* while *Caedibacter* pays the costs. It devotes a part of its population to R-body production, an evolutionary dead-end because those bacteria lose the ability to divide. Finally, those cells die when the R-body unrolls, albeit once inside a phagosome *Caedibacter* would be digested in any case. However, by sacrificing a proportion of individual members, the host and therewith the rest of the symbiont population can thrive.

Clearly, the killer trait and the corresponding resistance are beneficial phenotype modifications provided by *Caedibacter*

(Görtz et al. 2009). Interestingly, the alphaproteobacterial symbiont *Caedimonas varicaedens* (basonym *Caedibacter varicaedens*, Schrallhammer et al. 2018) also confers its *Paramecium* host the killer trait. The question regarding the nature of additional effects of these symbionts is controversial. Preer et al. (1974) state that under laboratory conditions *Caedibacter* generally do not harm their hosts. Experimental studies performing growth assays with genetically identical lines either infected by *Caedibacter* or symbiont-free highlight the complexity of this question (Kusch et al. 2002; Dusi et al. 2014). While both studies arrive at the conclusion that *C. taeniospiralis* is a parasite, they present contradicting results: One observes a significant difference in population growth rates (Kusch et al. 2002), the other exclusively during at carrying capacity but not during exponential growth (Dusi et al. 2014). Thus, cultivation conditions (e.g., food abundance, food quality, medium composition, temperature) could have a strong influence on the outcome of the interaction between *Caedibacter* and *Paramecium*.

Among ciliate-bacteria symbioses, the *Paramecium-Caedibacter* system is exceptional because it is relatively well-studied (reviewed by Schrallhammer and Schweikert 2009; Preer et al. 1974; Pond et al. 1989; Schweikert et al. 2013). We introduce a new perspective on this interaction by applying mRNA-Seq to reveal differential gene expression in *Caedibacter*-carrying paramecia compared with symbiont-free *Paramecium* cells. Fitness assays are used as a methodological independent confirmation of transcriptome results. This approach aims to elucidate so far hidden aspects of host's phenotype modification caused by its bacterial symbionts.

Materials and Methods

Organisms and Cultivation

Paramecium tetraurelia 51K infected by the bacterial symbiont *C. taeniospiralis* (Preer et al. 1974) was obtained from the Culture Collection of Algae and Protozoa (CCAP Scotland, UK; CCAP 1660/3F). "K" stands for "Killer" indicating that this strain exhibits the killer trait and thus harbors the causative symbiont. The line *P. tetraurelia* 51 (genetically identical *P. tetraurelia* background as 51K) was generated from 51K cells by curing them from *C. taeniospiralis* infection via antibiotic treatment. From now on, the two lines are referred to as 51K and 51.

Paramecium cells were maintained at 24 °C in Wheat Grass Powder (WGP, Pines International Co., Lawrence, KS, USA) infusion medium supplemented with 0.8 µg ml^{-1} stigmasterol (Sigma–Aldrich, Seelze, Germany). As food source *Klebsiella pneumoniae* (Cheaib et al. 2015) inoculated in WGP was provided twice a week.

For the elimination of the bacterial symbionts, 51K cells were incubated two times with 100 µg ml^{-1} streptomycin (Carl Roth GmbH, Karlsruhe, Germany). Briefly, 30 cells of

51K were placed in antibiotic solution (100 µg ml^{-1} streptomycin in sterile Volvic) at 24 °C for 24 h. Thereafter, cells were washed individually four times and individual cells were incubated in antibiotic solution as described. Then the cells were washed as before and transferred to 0.5 ml of bacterized CM diluted in a ratio 1:2 with sterile Volvic. Cultivation was continued as described above until >100 cells per batch were available to control for the successful elimination of *C. taeniospiralis* by Fluorescence In Situ Hybridization (FISH, see below). The line 51 was established circa seven months prior to the preparation for RNA extraction and fitness experiments by pooling two batches of *Caedibacter*-free paramecia.

For analysis of the potential killer effect of 51K and 51 an additional line with reported susceptibility to this phenomenon (*P. tetraurelia* 51S, "S" stands for "Sensitive") was used (Schrallhammer et al. 2012). Furthermore, to exclude potential undesired side effects of streptomycin on paramecia, 51S was also subjected to the above-described antibiotic treatment as control.

FISH

Presence or absence of endosymbionts was monitored regularly (before and after antibiotic treatment and fitness assays, before killer tests, cell age synchronization, and harvesting for RNA extraction). Therefore, bacteria were visualized and identified inside the *Paramecium* cells by FISH applying the universal probe EUB338 (Amann 1990) and the species-specific probe Ctaenio-998 (Beier et al. 2002). For each FISH experiment, at least 20 *Paramecium* cells were collected and fixed in 2% paraformaldehyde (w/v) on an adhesion slide (Superfrost Ultra Plus, Thermo Scientific, Braunschweig, Germany). Hybridizations were carried out in hybridization buffer (900 mM NaCl, 20 mM Tris–HCl, 0.01% SDS, 35% formamide) at 46 °C for 18 h, followed by washing (70 mM NaCl, 20 mM Tris–HCl, 0.01% SDS, 5 mM EDTA) at 48 °C for 20 min. The slides were examined with an epifluorescence microscope (Axio Imager M2, Carl Zeiss Microscopy GmbH, Jena, Germany). Pictures were taken (AxioCam MRM, Zeiss Microscopy GmbH, Jena, Germany) and processed using the implemented ZEN software package (blue edition).

Killer Tests

Caedibacter-bearing paramecia confer the killer trait to their host and hence kill sensitive cells while infected cells are resistant. Without the symbionts, the *Paramecium* cells become susceptible. The occurrence of this characteristic behavior was assessed for 51K and 51 using the symbiont-free and reportedly sensitive line *P. tetraurelia* 51S (Schrallhammer et al. 2012) as susceptibility control. Ten cells of 51S were exposed to a lysate corresponding to ~25 cells of 51K or 51, respectively. The lysate was prepared by vortexing the *Paramecium* suspension with glass beads until no intact paramecia were observed. Thus, potentially present R-body containing

Caedibacter, the lethal agents of the killer trait, were released at least in case of 51K. The number of living 51S was determined at several time points. Six replicates were included for each experiment. A one-way ANOVA with repeated measures followed by a multiple comparison test was performed using the standard settings of PRISM 6 (version 6, GraphPad Software, Inc., La Jolla, USA).

RNA Extraction, cDNA Library Generation, and Illumina Sequencing

For the identification of Differentially Expressed Genes (DEGs) in *P. tetraurelia* as response to *C. taeniospiralis*, the transcriptomes of the genetically identical lines 51K (fig. 1A) and 51 (fig. 1B) were analyzed. The symbiont-free 51 was used as reference.

In order to avoid differences in gene expression resulting from sexual recombination (autogamy), 51K and 51 were subjected to cell age synchronization (fig. 1). Therefore, the paramecia were triggered to undergo autogamy, a self-fertilization process that results in homozygote cells which remain immature (unable to undergo a new round of autogamy) for about seven cell divisions (Ishikawa et al. 1998). The F1 paramecia were then cultivated to obtain the desired cell number before reaching maturity again.

Starting from single cells, both 51K and 51 were grown in bacterized 0.2× WGP until the cells completed ~25 divisions and thus should be fully sexually mature. The cells were harvested by centrifugation and resuspended in exhausted 0.2× WGP to obtain a final cell density of 2,000 cells ml^{-1}. Exhausted medium is the cell-free supernatant of bacterized 0.2× WGP obtained after centrifugation (6,000 × g for 20 min). High cell density and starvation triggered the cells to undergo autogamy as microscopically confirmed by visualization of macronuclear fragmentation after DAPI staining (fig. 1C). Mass cultivation of F1 paramecia were performed with *Paramecium* lines that contained >95% autogamous cells. The respective cells were transferred to 1× WGP and incubated for three days (approximately max. seven divisions) till the cell number exceeded 200,000. After harvesting, the collected paramecia were resuspended in sterile Volvic water and incubated for 20 min at 24 °C to reduce bacteria in *Paramecium* food vacuoles. Total RNA of ~200,000–400,000 cells per line was isolated (modified from Chomczynski and Sacchi 1987) using TriReagent (Sigma–Aldrich, Seelze, Germany). After DNAse I treatment and subsequent purification by acid phenol, DNA-free total RNA integrity was checked by denaturing gel electrophoresis. For the generation of cDNA libraries, poly-A RNA was enriched using the NEBNext Poly(A) mRNA Magnetic Isolation Module followed by library preparation with NEBNext Ultra Directional RNA Library Prep Kit for Illumina. Both steps were carried out according to manufacturer's (NEB, Frankfurt a. M., Germany) recommendations using 11 PCR cycles of library enrichment.
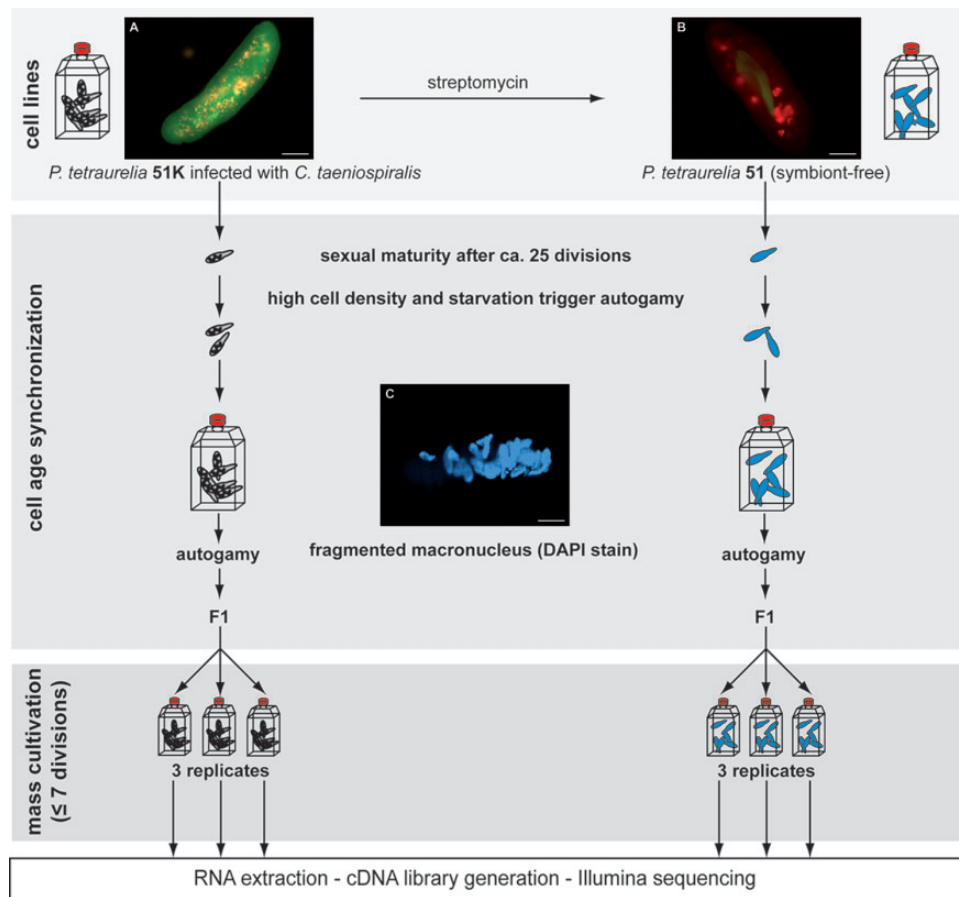
Fig. 1.—Experimental set-up. Fluorescence In Situ hybridizations of used cell lines *Paramecium tetraurelia* 51K infected by *Caedibacter taeniospiralis* (*A*) and symbiont-free *P. tetraurelia* 51 (*B*) generated by treatment with streptomycin. Applied probes are the universal EUB338 (labeled with Cy3) and the symbiont-specific Ctaenio-998 (fluorescein). Merged images show the presence of numerous cytoplasmic bacteria (*A*) respectively bacteria in digestive vacuoles (*B*). For visualization of the macronuclear fragmentation during autogamy, DAPI staining was performed (*C*). Bars represent 20 μm. To obtain cell age synchronized cells, paramecia were cultivated for circa 25 divisions and then triggered to undergo autogamy, which was confirmed by the observation of fragmented macronuclei after DAPI staining (*C*). F1 of lines with > 95% autogamous cells were split into three replicates, cultivated for max. seven divisions and harvested for subsequent steps.

DNA quantity of the prepared cDNA libraries was measured using the Qubit 1.0 Fluorometer (Life Technologies, United States). Illumina sequencing was carried out on a HiSeq2500 platform generating 2×100 nt paired end reads. Read numbers were: 51-1, 115.094.792; 51-2, 19.897.636; 51-3, 24.146.344; 51K-1, 16.888.465; 51K-2, 15.131.427; 51K-3, 13.962.278 (study accession PRJEB21163). Reads were demultiplexed with bcl2fastq (v1.8.4) and trimmed for adaptor contamination and low quality bases with the cutadapt (v1.4.1) wrapper trim_galore (v0.3.3).

Bioinformatics

Gene expression levels for each individual library were quantified using Sailfish (version 0.9.2), an alignment-free quantification algorithm (Patro et al. 2014). The expression values were normalized using Transcripts Per Million (TPM) values reported by Sailfish. However, differential expression of genes was determined by DESeq2 (version 3.3; Love et al. 2014) using read counts per gene not TPM. DESeq2 uses negative binomial linear models for dispersion estimates. After performing multiple testing correction, genes with a False Discovery Rate (FDR) of ≤0.01 were considered as DEGs.

Gene Ontology (GO) enrichment was performed via Ontologizer (Bauer et al. 2008), using the Java webstart application (version 2.1). Thereby, the DEGs were categorized into different GO terms. Those are concepts to describe gene molecular functions or biological processes. The GO annotation file was obtained from the Gene Ontology Consortium webpage (http://geneontology.org, accessed December 15, 2017). The GO term association file has been created according to Cheaib et al. (2015) using their gene association file as template. Gene enrichment was determined for up- and down-regulated genes using the Parent–Child-Union method

with Benjamini–Hochberg multiple testing correction (Bauer et al. 2008). Multidimensional scaling scatter plots of GO terms exhibiting a P-value of ≤0.001 were generated using the REViGO tool (Supek et al. 2011). This visualization summarizes biological processes according to their similarity and GO enrichment P-values.

Fitness Assays

The impact of *Caedibacter* on *P. tetraurelia* was functionally analyzed. Thus, the exponential growth rate r (division rate during the exponential growth phase) and the carrying capacity k (maximal cell density in the stationary phase) were used as indicative fitness parameters for 51K and symbiont-free 51 (modified from Bella et al. 2016). In preparation for the fitness assay, cells were washed and adjusted to exponential growth. Therefore, ten single cells per line were individually washed three times and cultivated until a density of $\sim$50 cells ml^{-1} was reached. In order to adapt the cells for the assays, nine cultures (for 51K as well as 51) were pooled and each day provided with $^{1}/_{4}$ volume of bacterized WGP to ensure exponential growth for three days. For the actual assay, five replicates per line were included. Each replicate started with identical volumes of bacterized medium and $\sim$80 *Paramecium* cells ml^{-1}. The total volume of each replicate was set to 30 ml. In order to obtain growth curves, samples for density counts (Castelli et al. 2015) were collected at regular time points and fixed with 2.9% Bouin's solution (Sigma–Aldrich, Seelze, Germany). The exponential growth rate r and carrying capacity k were calculated by fitting a nonlinear parametric regression based on a logistic growth model as described elsewhere (Bella et al. 2016). Differences of those two fitness parameters between symbiont-bearing 51K and symbiont-free 51 were determined by unpaired *t*-test with Welch's correction (PRISM 6, version 6, GraphPad Software, Inc., La Jolla, USA).

Results

Phenotypes of Infected and Symbiont-free *P. tetraurelia*

Caedibacter taeniospiralis was removed from infected cells via antibiotic treatment enabling subsequent comparisons between killer and symbiont-free paramecia. Ideally, cured cells should be re-infected and used in parallel for transcriptome or fitness analyses. However, *C. taeniospiralis* is strictly vertically transmitted and experimental infections are not feasible. By streptomycin treatment of *C. taeniospiralis*-bearing 51K, the genetically identical endosymbiont-free line *P. tetraurelia* 51 was successfully generated (fig. 1B). In analogously treated 51S cells no physiological effects (abnormal cell morphology or division rate, increased mortality) caused by the antibiotics were observed.

The prominent feature of *Caedibacter* symbionts is the alteration of their host's phenotype into so-called killer
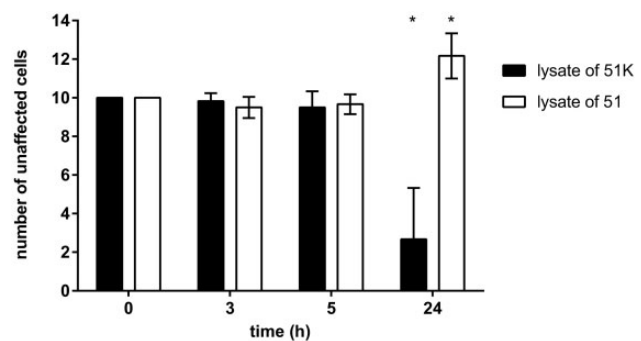

FIG. 2.—Survival rates of *Paramecium tetraurelia* 51S in killer tests. Ten cells of 51S were exposed to lysate corresponding to $\sim$25 paramecia up to 24 h. Lysates were prepared from *P. tetraurelia* 51K infected with *Caedibacter taeniospiralis* (black) and *Caedibacter*-free 51 (white). After exposure, the number of unaffected 51S cells was reported at different time points. Statistically significant differences to initial cell number were observed (* P-value ≤0.001; repeated measurements one-way ANOVA followed by a multiple comparison test). Bars represent the mean of six replicates ± SD.

paramecia. We performed killer tests to verify the presence of the symbiont and hence if 51K can cause the death of sensitive 51S. Vice versa, in case of 51 we tested for the loss of this ability together with the elimination of *C. taeniospiralis*. Therefore, 51K and 51 were mechanically lysed to maximize the number of potentially released bacteria. Exposing 51S cells to these lysates confirmed toxic effects of 51K (fig. 2): After 24 h, the cell number of 51S mixed with this lysate had decreased significantly (P-value < 0.001). Those exposed to 51 lysate did not show reduced cell numbers or any prelethal symptoms. On the contrary, 51S multiplied in these replicates (fig. 2).

Analysis of Differential Gene Expression in Infected and Symbiont-Free Cells

RNA sequencing with three biological replicates was carried out to determine the genome-wide gene expression patterns for *C. taeniospiralis*-infected 51K and symbiont-free 51. A hierarchical clustering analysis of these transcriptomes revealed major reproducible differences between both lines as the replicates of infected and symbiont-free cells cluster clearly together (fig. 3A). Using the DESeq2 approach (Love et al. 2014) with an adjusted P-value of ≤0.01 as cut-off, we identified 1,037 genes to be differentially expressed between *C. taeniospiralis*-carrying and symbiont-free paramecia (fig. 3B). Considering symbiont-free 51 as reference, out of 39,495 annotated genes, slightly more were significantly up- (548) than down-regulated (489) in *Caedibacter*-infected paramecia (fig. 3C). Up-regulated genes show more variation than down-regulated genes concerning both their mean expression values and absolute fold changes (fig. 3B).
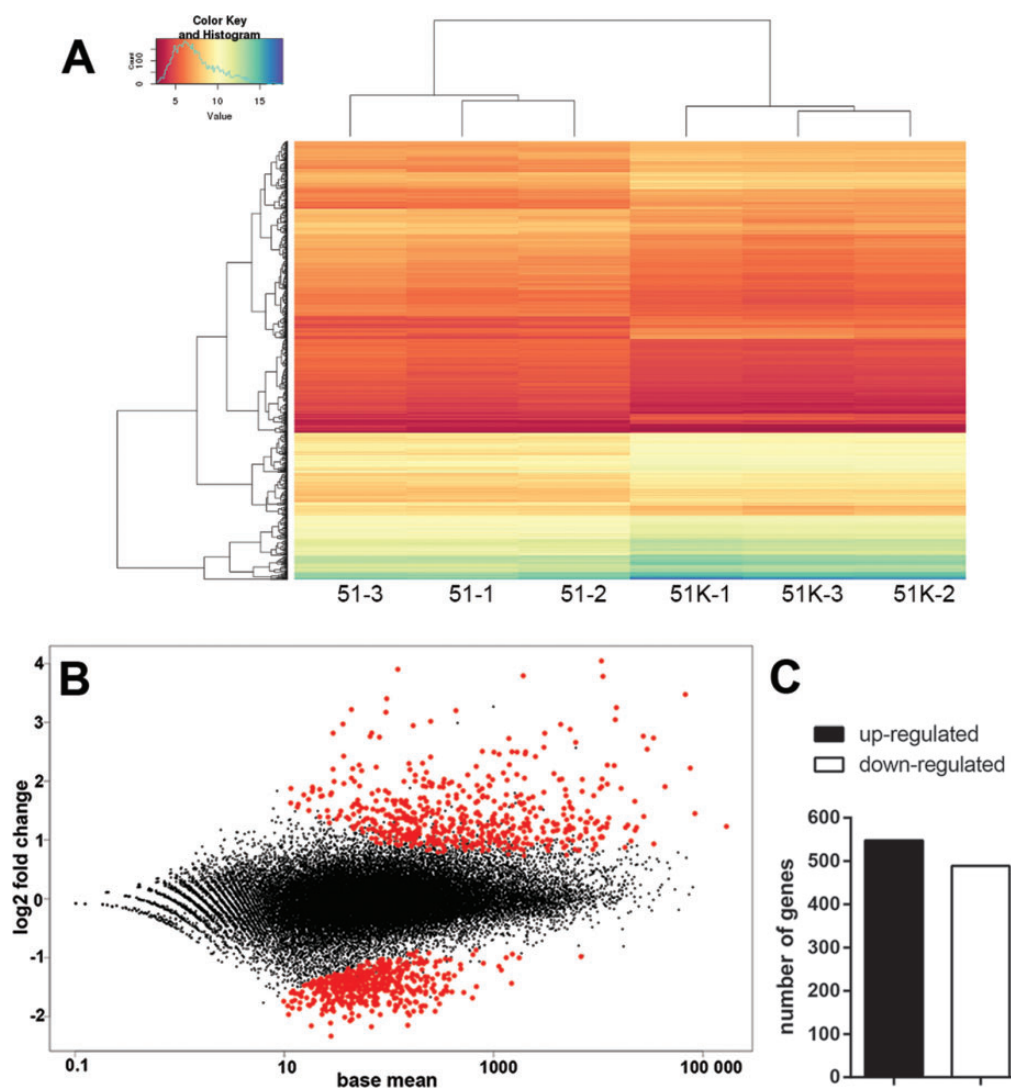
Fig. 3.—Gene expression in *Paramecium tetraurelia* 51K infected with *Caedibacter taeniospiralis* compared with symbiont-free line 51. (*A*) Hierarchical clustering of gene expression values (logTPM) in rows and columns for all six samples. (*B*) MA plot of log2 fold change of gene expression level (*y*-axis) against mean gene expression level (*x*-axis). Each point represents a gene; those with significant differential expression (FDR ≤0.01) are indicated in red. (*C*) Bar plot of DEGs in 51K with the number of up- and down-regulated genes, in black and white respectively.

GO Term Enrichment in Infected *P. tetraurelia*

DEGs with a *P*-value of ≤0.001 were subjected to GO enrichment analysis with the Ontologizer (Bauer et al. 2008) using the Biological Process subontology. Enriched GO terms are specified (supplementary tables S1 and S2, Supplementary Material online) and depicted by REViGO (fig. 4; Supek et al. 2011). The most significantly up-regulated GO term in infected paramecia (fig. 4*A*) is *lipid oxidation* (including *cellular lipid metabolism*, *cellular lipid catabolism*, and *lipid catabolism*). It comprises six DEGs (table 1) with sequence homologies to peroxisomal acyl-CoA oxidases, important enzymes for kinetics and substrate specificity in the β-oxidation (Poirier et al. 2006). Further up-regulated processes (fig. 4*A*) are *organic acid catabolism* (included DEGs almost identical with those in *lipid oxidation*, table 1) and *carbohydrate metabolism*. The latter comprises mostly transcripts involved in citrate cycle, glycolysis and glycogenesis and therefore indicates an increased energy metabolism (table 1).

The most significantly down-regulated process (fig. 4*B*) is *phosphorus metabolism* (82 DEGs), directly followed by *macromolecule modification* (79 DEGs, all also included in *phosphorus metabolism*). These DEGs show homologies either to phosphatases or protein kinases (supplementary table S2, Supplementary Material online). The four transcripts comprised by the GO term *nitrogen compound* belong to ammonium transporters (supplementary table S2, Supplementary Material online), which are generally involved in nitrogen uptake for nutritional purposes and ammonia excretion for
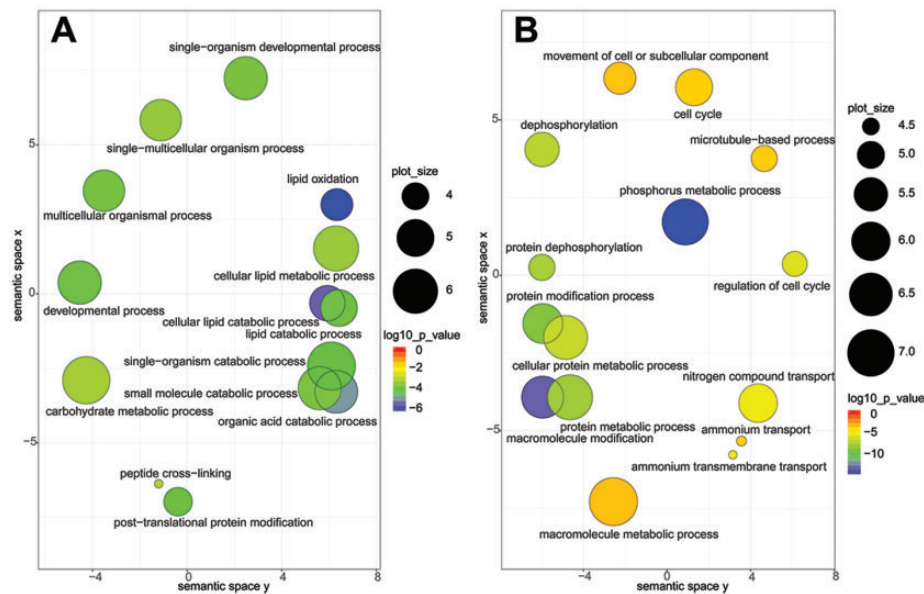
Fig. 4.—Visualization of Gene Ontology (GO) terms representing biological processes. Up- (A) and down-regulated (B) GO terms (FDR ≤0.01) are depicted as circles; the distance between those indicates the relationship between terms: closer distance means higher similarity. Color indicates significance of differential expression of an individual GO term (red low and blue high); size (in log10 P-value) indicates the percentage of genes annotated with a term in the reference database (UniProt) and thus indicates more general terms (large) and more specific ones (small).

waste removal (Kirsten et al. 2008). Additionally, the GO term *cell cycle* contains four genes annotated as cyclin-dependent kinases (CDKs).

Caedibacter taeniospiralis Infection Affects Host Fitness

Comparative transcriptomics of infected and symbiont-free *P. tetraurelia* indicated that 51K carrying *C. taeniospiralis* has an increased energy metabolism. In parallel growth assays with both lines the possible consequences of *Caedibacter* infection on *Paramecium* fitness were tested. FISH was applied at the beginning and end of the fitness assay to determine the infection level of *C. taeniospiralis* in 51K and confirmed a constant infection rate (100% infection prevalence). After application of a logistic growth model to fit a nonlinear regression (fig. 5A), exponential growth rate and carrying capacity (cell density that is reached and remains constant during stationary phase) were used for comparisons. Symbiont-bearing 51K reached a significantly higher carrying capacity (P-value < 0.001) than genetically identical symbiont-free 51, whereas no relevant difference was observed during exponential growth (fig. 5B). Thus, in the stationary phase infected cells had a fitness advantage (fig. 5C).

Infection Causes Permanent Activation of Individual HSP70 Isoforms

Expression of Heat Shock Protein (HSP70) genes is not only related to classical heat shock situations, it furthermore involves constitutively expressed isoforms and additionally those up-regulated in response to different stressors. In *P. tetraurelia*, five cytosolic HSP70 isoforms have been identified (HSP70Pt01-05; Cheaib et al. 2015). HSP70Pt01 is the major constitutively expressed in *P. tetraurelia* and shows the highest total abundance of transcripts in response to heat shock. This gene is significantly up-regulated in *Caedibacter*-harboring 51K (fig. 6).

Discussion

Comparative Transcriptomics Reveal Modulation of the Host Transcriptome Caused by Caedibacter

Differential gene expression analysis comparing the transcriptome of *Caedibacter*-harboring to symbiont-free cells as reference reveals that the presence of the bacterial endosymbiont causes a rather discrete modification of the host transcriptome with 2.6% of the sequenced genes classified as DEGs. In comparison, a study analyzing *P. tetraurelia* gene expression alterations in response to environmental changes revealed a much higher number of DEGs (starvation: ca. 11.4%, cold adaptation: ca. 8.2%) using the same significance criteria (Cheaib et al. 2015). Apparently, a discrete number of genes are involved in the host's response to the presence of *C. taeniospiralis* rather than a massive modulation of the transcriptome taking place.

Up- and down-regulated DEGs were analyzed by GO enrichment analysis, revealing that up-regulated GO terms relate to several metabolic and catabolic pathways

Table 1

Metabolic Pathways and Cellular Functions of DEGs Included in Most Significantly Enriched GO Terms

GO Term	Transcript Stable ID	Protein	Involved In
Up-regulated DEGs			
Lipid oxidation	CAK94090, CAK59990, CAK90106, CAK92996, CAK77450, CAK79917	Acyl-coenzyme A oxidase	Peroxisomal β-oxidation
Organic acid catabolic process	CAK94090, CAK59990, CAK90106, CAK92996, CAK77450, CAK79917	Acyl-coenzyme A oxidase	Peroxisomal β-oxidation
	CAK76270	Homogentisate 1, 2-dioxygenase	Catabolization of aromatic amion acids
Carbohydrate metabolic process	CAK89181, CAK72962, CAK86438, CAK94271	Fructose-1, 6-bisphosphatase	Glycolysis and glycogenesis
	CAK61489, CAK90992	Citrate synthase	Citrate cycle
	CAK60798	Glycosyl hydrolase family 31 protein	Hydrolysis of complex sugars
	CAK89297	Malate dehydrogenase	Citrate cycle
	CAK93624	Amylo-alpha-1, 6-glucosidase	Glycogen degradation
	CAK74015	Poly [ADP-ribose] polymerase	DNA repair and programmed cell death
	CAK69096	Lipid A-disaccharide synthase	Lipid A biosynthesis
	CAK88889	Proteobacterial glycerol kinase-like protein	Triglyceride and glycero-phospholipid synthesis or degradation
	CAK94394	Hypothetical protein	Unknown
Down-regulated DEGs			
Phosphorus metabolic process [1]	CAK74095, CAK82985, CAK63555, CAK77180, CAK77847, CAK89645, CAK93119, CAK59145, CAK65257, CAK69124	Dual specificity phosphatase domain protein	Protein phosphorylation
	CAK92571, CAK72540, CAK70776, CAK75609, CAK77917	Protein kinase domain protein	Protein phosphorylation
	CAK81054, CAK71834, CAK84440	Hypothetical protein	Unknown
	CAK71484	Serine/threonine kinase domain protein	Protein phosphorylation
	CAK92511	Cyclin-dependent kinase-like serine/threonine kinase family protein	Cell cycle regulation

[1]Only the 20 DEGs with the lowest *P*-values comprised by this GO term are listed (in total: 82).

(supplementary table S1, Supplementary Material online). These observations suggest that *Caedibacter*-bearing 51K probably possess an increased fatty acid catabolism by peroxisomal β-oxidation as well as increased glycolysis and citrate cycle activity. Enzymes involved in each of these pathways were detected in up-regulated processes by GO enrichment analysis (table 1). Those pathways are connected by substrates and cofactors and are used, also via the electron transport chain, to generate energy. Thus, our findings suggest that *Caedibacter*-harboring paramecia have an increased energy metabolism by catabolizing the available resources faster and/or activating storage compounds such as fatty acids. This additional (or faster) available energy could be used by infected paramecia to sustain their cytoplasmic symbionts and their

higher cell density compared with symbiont-free cells (fig. 5).

Prominent among the down-regulated DEGs (supplementary table S2, Supplementary Material online) are kinases and phosphatases comprised in the significantly enriched GO terms *phosphorus metabolism* (table 1) and *macromolecule modification*. Both enzyme types are expected to play major roles in various signaling pathways (Patterson et al. 2009). The observed down-regulation of kinases and phosphatases in infected cells compared with symbiont-free ones amounts to ~1/6 of all DEGs with reduced expression levels. Down-regulation of kinases and phosphatases can result in a modified activity in phosphorylation-dependent signaling cascades. This suggests that symbiont-bearing 51K have altered
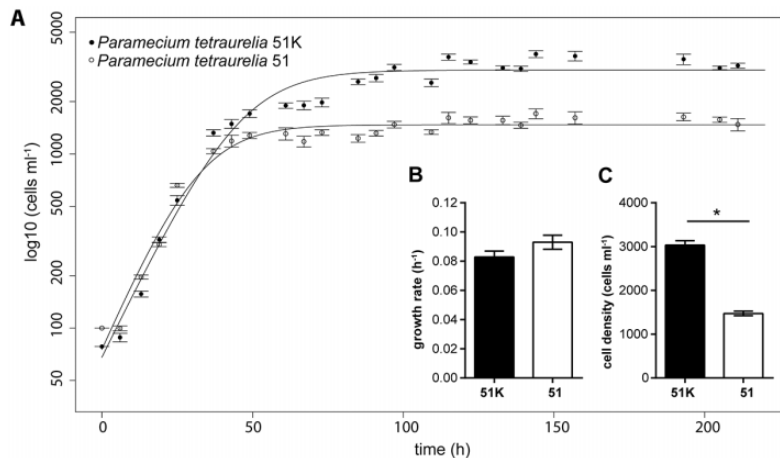
Fig. 5.—Fitness parameters of *Paramecium tetraurelia* infected with *Caedibacter* and symbiont-free cells. (*A*) Data points represent the mean cell density of five replicates ± SD at different time points (in hours). A nonlinear parametric regression model fits the regressions (lines). Filled circles indicate *P. tetraurelia* infected by *Caedibacter taeniospiralis* 51K, empty circles indicate symbiont-free 51 cells. Bar plots depict exponential growth rate (*B*) and carrying capacity (*C*) of infected 51K (black) and symbiont-free 51 (white). Statistically significant differences were only observed at carrying capacity (* *P*-value ≤0.001; unpaired *t*-test with Welch's correction). Bars represent the mean of five replicates ± SD.
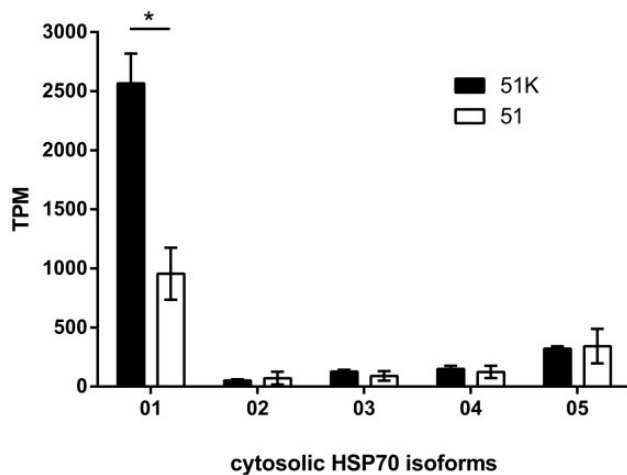

Fig. 6.—Cytosolic HSP70 expression of *Paramecium tetraurelia*. Transcripts Per Million (TPM) normalized expression values for the five cytosolic heat shock (HSP70) genes in *Caedibacter*-infected *P. tetraurelia* 51K (black) and symbiont-free 51 (white). Statistically significant differences were observed (* *P*-value ≤0.001; unpaired *t*-test with Welch's correction).

signaling and regulatory functions compared with symbiont-free 51.

A specific group of kinases, CDKs, are comprised in the GO term *cell cycle* which is also enriched in down-regulated DEGs. Like in other eukaryotes, *P. tetraurelia* CDKs are major regulators of the cell cycle (Zhang et al. 1999; Zhang and Berger 1999; Berger 2001). Thus, down-regulated cell cycle signaling pathways could result in altered density-dependent regulation of the cell cycle via CDKs and cause modified cell division inhibition, which might partially explain the higher cell

number of *Caedibacter*-bearing 51K (compared with symbiont-free 51) at carrying capacity as observed in the fitness assays.

Caedibacter Induces Multiple Modifications of Its Host Phenotype

The killer tests demonstrate that the analyzed lines represent killer paramecia (51K) and cells that have lost this ability (51) together with the bacterial symbionts. *Paramecium* may serve as a model for comparative studies addressing the function of various symbionts, as it can harbor a large variety of bacteria (Görtz and Fokin 2009) or algae (Kodama et al. 2014). Symbiont-free genetically identical lines are easily established as well as – in case of infective symbionts – infected *Paramecium* lines (Bella et al. 2016). Additional to the killer trait, fitness assays reveal the significant impact of *Caedibacter* on the cell density of its host. The obtained results differ from those of previous studies and hence stress the influence of biotic and abiotic factors such as cultivation medium and food organisms on the outcome of the *Paramecium-Caedibacter* symbiosis. Presumably, these factors not only alter the magnitude of the fitness impact but also shift a mutualistic interaction (fig. 5) into a parasitic one (Kusch et al. 2002; Dusi et al. 2014). Accordingly, *C. taeniospiralis* can cause a broad spectrum of effects ranging from harmful to beneficial.

The obligate cytoplasmic symbiont *C. taeniospiralis* induces a permanent up-regulation of the HSP70Pt01 isoform in its host. Similarly, HSP70Pt01 was found to be permanently up-regulated in *P. tetraurelia* expressing a specific surface antigen (51D, Cheaib et al. 2015). Both cases do not represent classic short-term stress situations. Thus, elevated HSP70Pt01

expression might be indicative for adaptations of the *P. tetraurelia* proteome in situations such as specific serotypes or infections considering the chaperone nature of HSPs. Interestingly, a similar up-regulation of HSP70 genes was observed in *Paramecium bursaria* and *Paramecium caudatum* carrying endosymbionts: The transcriptome alteration analysis of *P. bursaria* carrying the microalgae *Chlorella* sp. compared with symbiont-free *P. bursaria* revealed an up-regulation of individual HSP70 genes in the symbiosis (Kodama et al. 2014). Hori and Fujishima (2003) observed by RT-PCR that *P. caudatum* infected with the alphaproteobacterial symbiont *Holospora obtusa* express higher levels of HSP70 mRNA already at physiological cultivation temperature (25 °C) which was further enhanced when transferred to 35 °C. This may indicate that HSP70Pt01 is involved in the host–symbiont interaction in *P. tetraurelia* or it could represent a general adaptation of the host to exogenous intracellular particles.

Symbiont-Induced Modulation of Host Energy Metabolism and Evolutionary Implications

The combination of comparative transcriptomics and comparative functional analyses provides new insights into the interactions of bacteria with their unicellular host. Killer paramecia represent an evolutionary outstanding symbiosis as the symbiont provides its host with a phenotype to kill other paramecia. Although the host's killer phenotype is well-studied in terms of the bacterial protein structure involved in intraspecific competition, the complexity of the host's adaptation processes to infection remains hardly understood.

Here, comparative transcriptomics reveals discrete but significant gene expression differences in *C. taeniospiralis*-harboring 51K cells relative to symbiont-free 51. Most strikingly, enzymes of several connected metabolic pathways (*β*-oxidation, glycolysis, gluconeogenesis, and citrate cycle) are up-regulated in infected paramecia. The energy gained from 51K up-regulated metabolism is apparently used to increase the host cell number at carrying capacity. *Caedibacter* should profit from an increased amount of infected paramecia as it is vertically transmitted. Thus, the increased host cell number implies an increase also in *Caedibacter* population size. Modified expression of kinases and cell cycle regulatory genes could assist in increasing the host population density. As the genome of *C. taeniospiralis* (Zaburannyi et al. 2018) encodes various transporters, the cytoplasmic symbiont might profit even more directly from the up-regulated metabolism of its host by importing additionally available metabolites. Accordingly, 51K maintains *C. taeniospiralis* in exchange for the killer advantage and the symbionts maintain their intracellular niche.

Possibly, these obligate endosymbionts have evolved an indirect response to react to environmental changes by manipulating their host's metabolism. In our fitness assays they act as mutualists and cause an increased host cell density.

Further investigations will show which factors can trigger a switch from mutualistic to parasitic behavior and how those are sensed by the symbiont. Currently the true nature of this symbiosis in the environment is still undisclosed.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Authors' Contributions

Acknowledgments

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