Comparative Genomic Analysis of Holospora spp., Intranuclear Symbionts of Paramecia

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While most endosymbiotic bacteria are transmitted only vertically, Holospora spp., an alphaproteobacterium from the Rickettsiales order, can desert its host and invade a new one. All bacteria from the genus Holospora are intranuclear symbionts of ciliates Paramecium spp. with strict species and nuclear specificity. Comparative metabolic reconstruction based on the newly sequenced genome of Holospora curviuscula, a macronuclear symbiont of Paramecium bursaria, and known genomes of other Holospora species shows that even though all Holospora spp. can persist outside the host, they cannot synthesize most of the essential small molecules, such as amino acids, and lack some central energy metabolic pathways, including glycolysis and the citric acid cycle. As the main energy source, Holospora spp. likely rely on nucleotides pirated from the host. Holospora-specific genes absent from other Rickettsiales are possibly involved in the lifestyle switch from the infectious to the reproductive form and in cell invasion.

Keywords: Holospora, Rickettsiales, endosymbionts, paramecia, ciliates, nuclear symbionts, genome

INTRODUCTION

Symbiotic associations with bacteria are common to many, if not all, eukaryotes. Endosymbiotic bacteria are well studied in plants, insects, and some other organisms (Moran et al., 2005; Kawaguchi and Minamisawa, 2010; McCutcheon and Moran, 2010; Bennett and Moran, 2013; Becker et al., 2015; Wu et al., 2015; Wexler et al., 2016; Zipfel and Oldroyd, 2017). By the original definition (Oulhen et al., 2016), a symbiotic relationship does not imply benefits for the host. Nevertheless, in symbiotic relationships, symbiont explores its host as a habitat. In turn, host can acquire some necessary products and/or properties which it unable to synthesize, e.g., ammonium produced for plants by nitrogen fixing bacteria, essential amino acids produced by endosymbionts of sap-sucking insects (Moran et al., 2005; Archibald, 2015; Sabater-Muñoz et al., 2017), or vitamins for blood-feeding tsetse fly (Dale and Welburn, 2001). Some symbionts, like some strains of Wolbachia spp., protect the host from pathogenic viruses (Hedges et al., 2008); or are necessary for the maturation of the immune system, as e.g., Wigglesworthia for tsetse flies (Weiss et al., 2011). Caedibacter creates growth advantages for infected paramecia and helps them to out-compete uninfected ones (Kusch et al., 2000). An endosymbiont of ciliates from the genus Euplotes is...
essential for the host cell proliferation (Vannini et al., 2012, 2017). However, most relations are not well understood, and an advantage for the host is not always demonstrated, especially for facultative endosymbionts (Lu et al., 2016; Yañez et al., 2016).

Endosymbiotic bacteria and endonuclear parasites typically have several characteristic features, such as small genome size, low GC content, and short intergenic spacers (reviewed in McCutcheon and Moran, 2012; Batut et al., 2014; Martínez-Cano et al., 2015). Another important trait of these bacteria is an accelerated rate of genome evolution, caused by the minimal gene flow and substantial genetic drift due to the small effective population size of endosymbionts (Marais et al., 2008; Boscaro et al., 2017; Sabater-Muñoz et al., 2017). This leads to formation of pseudogenes and gene loss, and an overall decrease of the metabolic capacity (McCutcheon and Moran, 2012; Boscaro et al., 2017). The loss of genes involved in the replication drives an even faster evolution of symbiotic species (McCutcheon and Moran, 2012). It also can lead to the decrease in the GC content (Horst et al., 1999; Moran et al., 2005; Long et al., 2018). A stable environment in the host cell does not require complicated regulatory systems, yielding reduction of intergenic regions (McCutcheon and Moran, 2012).

Holospora spp. are endonuclear symbiotic Alphaproteobacteria from the order Rickettsiales that inhabit either macro- or micronucleus of Paramecium spp. It has been suggested that Holospora spp. are parasites, as other Rickettsiales. A recent analysis of 16S rRNA lead some researchers to separate the basal rickettsial lineage of Holospora-like bacteria in a separate order Holosporales (Ferla et al., 2013; Szokoli et al., 2016), but for convenience we discuss both orders here together as Rickettsiales, because they have similar lifestyle and metabolic capacities. The genus Holospora currently is comprised of nine species, each showing clear nuclear and host specificity (Görtz and Schmidt, 2015). Holospora are not able to reproduce outside the host cell but can leave the host in order to invade a new one. Unlike most of the studied symbiotic species, Holospora have a complex life cycle involving two morphologically different forms. The short reproductive form exists only in the host nucleus and multiplies by binary fission, while the long infectious form does not multiply and is capable of infecting new host cells (Gromov and Ossipov, 1981; Fokin and Görtz, 2009). This form shows a unique cytological organization with a pronounced polarity (a large perinuclear space and a special tip), which seems to be of functional significance for the infection process. The infectious form enters new host via Paramecium food vacuole together with food bacteria, but escapes digestion, exits the vacuole and enters the cytoplasm followed by nuclear invasion. Two main mechanisms of this process have been proposed: Holospora either disrupt the digestive vacuole or enter the transport vacuoles (Schweikert et al., 2013). Then Holospora reach the nucleus by utilizing the host’s actin cytoskeleton (Fokin and Görtz, 2009; Sabaneyeva et al., 2009). In Holospora obtusa, the 89 kDa periplasmic protein was shown to be associated with cell invasion (Iwatani et al., 2005). The invasion of macronucleus by H. obtusa is associated with release of the 63 kDa periplasmic protein into the macronucleus of the host (Abamo et al., 2008). Additionally, 15 and 39 kDa periplasmic proteins are released from the cell tip during the macronucleus infection (Fujishima et al., 1997). Mechanisms underlying the transition from the reproductive to the infectious form and back are not well understood, but the 5.4 kDa protein with a signal peptide is only detected in the intermediate and infectious forms (Dohra et al., 1997).

In the presence of Holospora, Paramecium is able to grow, divide and mate (Schweikert et al., 2013). Holospora contribute to the heat-shock resistance in Paramecium caudatum, as cells infected with H. obtusa express high levels of hsp70 mRNA (Fujishima et al., 2005). They may also assist in acquiring high-salt and osmotic-shock resistance to the host (Fujishima, 2009). The presence of H. caryophila in the Paramecium biaurelia nucleus is advantageous in several cell lines during the exponential growth (Bell et al., 2016). Despite these observations of benefits for the host, Holospora have been shown to negatively affect host cells. For example, the presence of Holospora elegans in Paramecium leads to the formation of dysfunctional macronucleus during conjugation (Görtz and Fujishima, 1983). Holospora undulata increases mortality of the host, especially at low-food treatment (Restif and Kaltz, 2006), and high concentrations of the infectious form in the macronucleus inhibit the host cell growth (Fujishima, 2009). The relationship between Holospora spp. and their paramecia hosts seems to be a complex system in which benefits or damages for the host can be highly context-dependent.

The metabolism of Rickettsiales, and in particular of Rickettsia spp., has been studied in detail (Andersson et al., 1998; Hotopp et al., 2006; Fuxelius et al., 2007; Georgiades et al., 2011). Rickettsia export at least 51 metabolites from the host (Driscoll et al., 2017). They are not able to synthesize amino acids, nucleotides, lack glycolysis, and have to import such compounds as coenzyme A, pyruvate, FAD, biotin, etc.

So far, three Holospora genomes have been sequenced, all of which are endosymbionts of Paramecium caudatum: macronucleus-specific H. obtusa and micronucleus-specific H. elegans and H. undulata (Dohra et al., 2013, 2014). Analysis of common orthologous genes has yielded 572 single-copy core genes shared by the three genomes, and Holospora have been shown to rely on the host for energy production (Dohra et al., 2014). However, no detailed metabolic pathway reconstruction has been performed.

Here, we report a comparative analysis of four Holospora species, including Holospora curviuscula, a newly sequenced macronuclear endosymbiont of P. bursaria. We propose that Holospora use host-produced nucleotides as its energy source. We also identify the essential compounds that Holospora are likely to synthesize, and compare their metabolism to that of other Rickettsiales.

**MATERIALS AND METHODS**

**Growth Conditions and Genomic DNA Preparation**

Holospora curviuscula has an obligate association with its host, Paramecium bursaria, and is therefore uncultivable, so the bacteria have been grown inside host cells. *Holospora*
curviscula strain NRB217 from Paramecium bursaria isolated in the Novosibirsk Akademgorodok were obtained from the infected clones maintained in the CCCS (Culture Collection of Ciliates and their Symbionts), Research Park, Saint-Petersburg State University. The host cells were cultivated at the room temperature on the lettuce medium inoculated with Enterobacter aerogenes as a food resource for paramecia. The culture of P. bursaria bearing H. curviscula was concentrated by centrifugation (10 min at 4500 g) and then homogenized using 10% solution of detergent Nonidet P-40 (Sigma-Aldrich Cat No. 21-3277 SAJ). The infectious forms of H. curviscula were isolated from the homogenate by centrifugation in Percoll density gradient (Sigma-Aldrich Cat No. P1644) as described previously (Rautian and Wackerow-Kouzova, 2013). Genomic DNA was isolated with the DNeasy Blood and Tissue kit (QIAGEN Cat No. 69504) using a modified protocol — the time of incubation of cell homogenate with ATL buffer and proteinase K was increased to 16 h. All the subsequent steps were performed according to the standard Quick-Start Protocol.

**Genome Assembly and Annotation**

Two libraries were generated, paired-end MiSeq Illumina library (2 × 250) (PE), and mate-pair library with insertion size 4 kbp (MP). The initial MP library size was 2.5 million reads, and the PE library size, 4.5 million. MP reads were filtered with NextClip v1.3 (Leggett et al., 2014) and only category A pairs of reads (both reads in a pair contain adapters) were selected for further analysis. Both MP and PE were filtered by quality with trimmomatic version 0.33 (Leading 15, sliding −15, slidingwindow −4:25), after filtering 1.3 million MP reads and 1.7 million PE reads were retained for assembly. Processed reads were assembled with platanus version 1.2.1. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession PHHC00000000. The version described in this paper is version PHHC01000000.

To estimate the completeness of the Holospora assemblies, we used HMMer to search for essential PFAM domains (Rinke et al., 2013). A domain was considered to be present, if it was found by hmmsearch (with default parameters) (Mistry et al., 2013) and the bias was lower than the hit score. For all missing genes we checked hmmsearch (with default parameters) (Mistry et al., 2013). A domain was considered to be present, if it was found by HMMer package. To find phage-like regions in the genome assemblies, we used PHAST (Zhou et al., 2011). Additionally, we searched for all PFAM-domains with the keywords “transposase” and “phage” (328 PFAM domains).

**16S rRNA-Based Phylogeny**

To reconstruct the phylogenetic tree of Holospora spp., the PhyML software (Guindon et al., 2010) was used. 16S rRNAs from H. curviscula NRB217, H. elegans E1, H. obtusa, and H. undulata HU1 were extracted from the full genome sequences, while 16S rRNAs from H. acuminata and Candidatus Gortzia infectiva were obtained from the SILVA database (accession numbers: KC164379 and HE797907, respectively). To root the tree, we used 16S rRNA of Rickettsiales (SILVA accession number: CP009217). The phylogenetic tree was constructed with PhyML v. 3.1 with 100 bootstrap replicates, GTR substitution model, and -o tlr parameter.

**Analysis of Repeats**

To estimate the fraction of repetitive DNA in the Holospora genome assemblies, reads were realigned with Bowtie 2 (end-to-end mode) (Langmead and Salzberg, 2012) back to the respective assemblies, and the coverage for each nucleotide was calculated. In the case of H. curviscula, we used PE reads generated in this study, reads for H. obtusa and H. undulata were downloaded from DDBJ (accession numbers DRP001203 and DRA001008, respectively). A fragment was presumed to be duplicated if it was longer than 20 bp, and its coverage was at least 1.6-fold higher than the median contig coverage.

**Orthologous Groups and Genome Comparisons**

To investigate the phylogenetic relations between H. undulata and H. elegans, pairwise genome alignments of all Holospora genomes were constructed with MAUVE (snapshot 2015-02-13) (Darling et al., 2010). Alignments of orthologous genes from the essential list (see above) were extracted from the MAUVE output, and all gaps were removed. The number of substitutions was calculated for each gene for each pair of genomes.

To investigate the gene repertoire of all Holospora and other Rickettsiales, we constructed groups of orthologous genes using OrthoMCL v. 2.0.9 (Li et al., 2003) with the MCL software v. 14-137. The output gene clusters were grouped in five subgroups using an ad hoc perl script which considered orthologs, co-orthologs and inparalogs. All Rickettsiales and other Holospora genomes (for the complete list see Supplementary Table 2) were downloaded from NCBI Genbank (Benson et al., 2013).
RESULTS

Holospora curviuscula Genome

The H. curviuscula draft genome sequenced here is comprised of 152 scaffolds (210 contigs) with N50 of ~39 kbp, total length of 1.7 Mb, and GC content of 37.6% (Table 1). The longest scaffold is 153367 bp and contains 126 CDSs. We predicted 1594 genes, including 1555 protein-coding genes with the average encoded protein length of 218 aa (Supplementary Figure 1). We assigned protein function to 683 genes. The genome assembly contains all rRNA genes and a set of 36 tRNAs necessary to recognize all codons. The 16S rRNA analysis revealed that the sequenced genome indeed belongs to the genus Holospora, and specifically to H. curviuscula, and is closer to Holospora acuminata, another endosymbiont of P. bursaria, than to Holospora from P. caudatum (Figure 1). The H. curviuscula genome is the largest Holospora genome sequenced so far (Table 1). Its GC content is slightly higher than that of most other non-Rickettsiales obligate endosymbionts (McCutcheon and Moran, 2012), but is typical for Rickettsiales (Supplementary Figure 2).

We estimated the quality of the assembly by searching for nearly universal bacterial genes (see Materials and Methods). Among the 139 universal PFAM domains, 121 are present in the H. curviuscula genome. Nine of the 18 missing domains have been found with a lower similarity threshold, which makes their presence uncertain. The remaining nine of the 18 missing domains are absent in all other Holospora as well. Moreover, these domains are also absent either in some endosymbiotic bacteria with small genomes or in some Rickettsiales genomes (Figure 2), meaning that their absence can be tolerated. We further searched for these 18 missing domains in unassembled sequencing reads of H. curviuscula (14% of all reads), and found no indication of their presence.

TABLE 1 | Results of genome assembly and annotation of H. curviuscula, and comparison with other Holospora species.

|            | H. curviuscula | H. obtusa | H. undulata | H. elegans |
|------------|----------------|-----------|-------------|------------|
| Genome (bp)| 1715500        | 1334837   | 1402636     | 1268333    |
| No. of contigs | 210           | 91        | 203         | 152        |
| GC content (%) | 37.6%         | 35.2%     | 36.1%       | 36.0%      |
| CDS        | 1594           | 1117      | 1224        | 1212       |

We manually checked 615 ORFs for frameshifts, and found only nine ORFs with frameshifts. This number may be an underestimate, because H. curviuscula genome contains short ORFs with unknown functions that have no homologs outside Holospora and can be remnants of some ancestral genes. Still, it implies that the H. curviuscula genome contains few pseudogenes.

Rickettsiales have been reported to have an atypical rRNA operon (Andersson et al., 1998). However, all Holospora have a standard rRNA operon, with 23S and 16S genes located close to each other and separated by several tRNA genes.

Reanalysis H. undulata and H. elegans Genomes

Holospora undulata HU1 and H. elegans E1 extracted from the micronucleus of P. caudatum have been recognized as separate species based on differences in the cell morphology (Görtz and Schmidt, 2015). However, our comparison of 16S rRNA genes from the available genome assemblies (Genbank IDs NZ_ARPM000000000 and NZ_BAUP000000000, respectively) of these species revealed just a single-nucleotide difference (Figure 1), yielding > 99.9% sequence identity, which is above the common thresholds of 97 or 98.7% used to define species (Janda and Abbott, 2007). That led us to inquire whether the genomes of H. undulata and H. elegans indeed represented different species or just strains of the same species. We constructed whole-genome pairwise alignments of the Holospora genomes and counted mismatches in the essential genes (Figure 3). The H. elegans–H. undulata pair carried ~100-fold fewer mismatches than all other genome pairs.

Holospora spp. Have Multiple Repeat Sequences and Contain Prophages

All available Holospora assemblies are comprised of multiple contigs, and reassembly has not led to better assemblies even with added genome coverage. Moreover, the genome length of H. curviuscula has been estimated at ca. 2 Mb (unpublished data), while the total assembly length is around 1.7 Mb. This suggests that Holospora genomes may contain a considerable fraction of repetitive DNA. To test this, we searched for fragments with high read coverage (see Materials and Methods). In H. curviuscula, we found ~300 repeats with the total length of ~300 kb (Figure 4A) and the estimated copy number varying from 2 to 21 (Figure 4B). In H. obtusa and H. undulata, the similarly estimated lengths of repetitive DNA were 60 and 161 kb, respectively. We were not able to estimate the fraction of repetitive DNA for H. elegans, as no raw sequencing data were available. The calculated repeat lengths could be underestimates, as we have applied strict coverage cutoffs yielding conservative repeat boundaries. For the same reason, we could overestimate the number of repeats by splitting long repeats into individual repeats in regions of low coverage. Still, the match between the difference in the assembly length and the genome length and the total coverage of repetitive DNA suggests that unassembled repeats comprise ~15% of the genome in H. curviuscula. The high copy-number repetitive regions in H. curviuscula include short ORFs of
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FIGURE 2 | The presence/absence pattern of 18 essential PFAM domains in Holospora, endosymbiotic bacteria with tiny genomes, and Rickettsiales (Supplementary Table 1). Color code: dark blue, the domain is present; gray, the domain is absent; cyan represents domains found with decreased similarity threshold (see Materials and Methods). The number in each cell is the number of genomes where the domain has not been found.

possible transposases. In addition, the identified repeats include two copies of rRNA operons with the similar structure, and also genes encoding prophage-like proteins.

To characterize prophages further, we searched for complete prophages and, additionally, for phage-related PFAM domains in all Holospora genomes. We found two possible prophage regions in H. elegans, one in H. obtusa, and two in H. undulata. One of the H. undulata prophages was intact, contained all proteins necessary for the phage replication and was surrounded by integration sequences (Supplementary Figure 3); the remaining copies were severely disrupted and lacked insertion sequences. Although no intact prophages were found in H. curviuscula, scaffold565 had a locus with two DDE superfamily endonucleases and two putative transposases, and other scaffolds carried genes encoding phage capsid and phage portal proteins similar to the ones found in other Holospora as well as some full-length and fragmentary transposases (data not shown).

**Holospora spp. Cannot Synthesize Amino Acids and Some Other Essential Small Molecules**

Holospora have a reduced metabolism even by the standards of the already gene-poor order Rickettsiales (Georgiades et al., 2011). In particular, Holospora lack genes encoding enzymes of the citric acid cycle (CAC) (Supplementary Figure 4). By contrast, Rickettsia and other Rickettsiales have at least some of these enzymes and use them to convert small molecules (Driscoll et al., 2017).

All sequenced Holospora lack amino acid synthesis pathways. For most amino acids, all enzymes of the pathway are missing. The exceptions are the partial pathways for the biosynthesis of L-tryptophane, L-lysine, and L-glutamate and for the conversion of L-alanine to D-alanine, an essential compound for formation of the cell wall (Figure 5A). This means that all amino acids have to be imported from the host. While Rickettsia also cannot produce amino acids and probably import them from the host (Driscoll et al., 2017), other Rickettsiales are capable of synthesizing some amino acids such as L-lysine and L-glutamine (Fuxelius et al., 2007). Similar to Rickettsia, Holospora cannot produce chorismate or use it as a substrate for downstream reactions, and need to import it or its derivates from the host.

Like Rickettsia, Holospora are not capable of purine and pyrimidine synthesis (Figure 5B), although, also like Rickettsia, they can convert UTP to NTP. Therefore, they need to obtain all nucleoside triphosphates from the host.
On the other hand, *Holospora* carry genes for ribonucleotide reductases and therefore can convert ribonucleotides into deoxyribonucleotides.

The ubiquinone biosynthesis pathway is also partial, as it is comprised of only dimethylallyl diphosphate (DMAPP) and the enzymes downstream of it (Supplementary Figure 6), similarly to what has been reported for *Rickettsia* (Driscoll et al., 2017). Unlike *Rickettsia*, *Holospora* are unable to convert DMAPP to isopentenyl diphosphate, although they carry geranyl-diphosphate synthase which is missing in *Rickettsia*. Therefore, DMAPP seems to be obligatorily imported from the host.

The only major metabolic pathway that is almost intact is the fatty acids synthesis (Supplementary Figure 5).

In order to determine how the missing compounds are imported from the host, we analyzed predicted transporters. In *H. curviuscula*, thirty transport-associated genes have been identified. As expected, *Holospora* genomes encode oligopeptide and amino acid transporters, as well as proteases, some of which are periplasmic. In particular, we have found arginine/glutamine, proline, acetyl-serine/cysteine, choline/glycine/betaine, and putative branched amino acids candidate transporters. In addition, we found putative transport systems for magnesium, ferric ions, ribose, purines, sulfoacetate, L-galactonate or other sugars, and putrescine. However, these transport systems are insufficient to deliver all missing compounds to *Holospora*; in particular, it is not clear, how the remaining amino acids are delivered, unless as components of oligopeptides.

**Holospora** spp. Use Nucleotides as the Main Energy Source

All *Holospora* lack most genes involved in energy production. In particular, all enzymes necessary for the glycolysis except phosphoglycerate mutase (Figure 5C) and all enzymes of the citric acid cycle except malate dehydrogenase (Supplementary Figure 4) are missing. The F$_{1}$F$_{0}$-ATPase is also missing. In addition, *Holospora* cannot produce coenzyme A from scratch, although as *Rickettsia*, they seem to have CoaE (PF01121, Figure 2) and thus are able to synthesize coenzyme A from dephospho-CoA (Driscoll et al., 2017). Of the pentose phosphate pathway, only the non-oxidative branch is present, as well as the downstream enzyme ribose-phosphate mutase that converts phosphoribosyl pyrophosphate (PRPP) to sugars (Figure 5C); the energy-producing oxidative pathway leading to the ribulose-5-phosphate is missing. By contrast, all *Holospora* have the pyruvate dehydrogenase complex and are able to convert pyruvate to acetyl-CoA, and further to acetoacetyl-CoA and acetoacetate, with the production of ATP (Figure 5D). All *Holospora* have a set of ribonucleotide reductases, which would allow them to use either nucleotides or ribonucleotides as an energy source. No obvious source of energy other than nucleotides was found.

**Secretory Systems and Putative Invasins**

Although most *Rickettsiales* are parasites and their genomes are highly reduced, they retain secretory systems such as the Tat and Sec pathways, a type IV system, and the TolC protein from a type I secretion system (Gillespie et al., 2015). Moreover, the VirB protein from a type IV secretory system is thought to be essential for the host invasion in most *Rickettsiales* (Rennoll-Bankert et al., 2015; Gillespie et al., 2016). By contrast, all studied *Holospora* demonstrate a significant decay of secretion pathways. They still possess the complete Sec system, additional systems helping to translocate proteins to the outer membrane (LolA, LolD, and possibly LolE) or transport them outside the cell (BamA, BamB, BamD, and chaperone DegP), and a TolC-like protein. However,
we found no proteins similar to components of the Tat-system (TatA, TatB, or TatC). Since Tat-system proteins may be hard to identify (Gillespie et al., 2015), we performed a genome-wide search for proteins with the twin-arginine signal required for the recognition by the Tat-system and found no significant hits. Together, these observations show that the Tat transport system is indeed missing, which means that Holospora are unable to export folded substrates.

In Rickettsia, invasion is associated with proteins RalF, RickA, and Sca (Gillespie et al., 2015) which are missing in Holospora, implying that Holospora use other mechanisms to invade the host cell. There are also no proteins with ankyrin domains assumed to play a role in the pathogenesis in Rickettsia (Gillespie et al., 2015). Although Sec proteins are present in all Holospora, only one protein with a putative autotransporter domain (PF03797) was found in H. curviuscula (HCUR_00103). Even though this gene has homologs in other Holospora, this domain was not predicted in them, implying that it can be a false positive. However, we found multiple (3–9 copies per genome) genes similar to ompA in all Holospora. OmpA has been previously shown to be involved...
Holospora genomes encode numerous short (<100 aa) peptides with unknown functions. Short proteins have been shown to be involved in spore formation, regulation of transport, regulation of transcription, and signal transduction, or to possess antimicrobial or other toxic activities (Wang et al., 2008; Storz et al., 2014). Of the 102 HOGs, seventeen contained short proteins, including five HOGs that contained proteins with secretory signal peptide sequences. One of these HOGs has been described earlier as the 5.4 kDa protein involved in the switch between the reproductive and infectious forms (Dohra et al., 1997).

To determine whether there are proteins that could determine the nuclear specificity of Holospora, we searched for HOGs specific to the two macronuclear species, H. curviuscula and H. obtusa. We found eight HOGs present in both H. curviuscula and H. obtusa and absent in H. undulata, H. elegans and in other studied Rickettsiales. Of these, two HOGs encoded alpha/beta hydrolases; one, a possible aspartate/glutamate racemase or a malate isomerase; one, nucleotide sugar epimerase; one HOG had an unknown function; and three remaining HOGs encoded short proteins, one of which was similar to DDE superendonuclease, and another one had a predicted signal peptide.

DISCUSSION

Here we report a comparative analysis of the newly sequenced genome of H. curviuscula NRB217 and other available Holospora genomes. The phylogenetic analysis of all available Holospora has confirmed (Rautian and Wackerow-Kouzova, 2013) that they are clustered by the host species rather than by micro- vs. macronuclear specificity, so that H. curviuscula is the outgroup to the previously analyzed genomes. Furthermore, the fact that we found only one substitution in the 16S rRNA gene and few substitutions genome-wide between the sequenced samples of H. elegans and H. undulata suggests that these are in fact strains of the same species.

The previous genomic analysis of Holospora spp. (Dohra et al., 2014) included only a general description of the available COG categories and some reconstruction of the metabolism. It has been observed that many pathways are missing in Holospora. The pyruvate dehydrogenase complex that is present in Holospora has been proposed to be a possible relic of an ancestral pathway. Additionally, it has been suggested that Holospora strongly relies on the host for energy production.

Addition of H. curviuscula allows for a detailed comparative genomic analysis of the metabolism of Holospora spp. We found that all Holospora have reduced metabolic capacities and have to import many metabolites from the host.

Even though the genomes of Holospora are relatively large (Table 1) in comparison with other symbiotic species such as Buchnera, Candidatus Baumannia, or Candidatus Carsonella, Holospora are unable to produce most of the essential compounds. Furthermore, all available Holospora genomes seem to contain a large fraction of repetitive sequences, which complicates the genome assembly. Arguably, as all available...
Holospora genomes consist of multiple contigs, some of the enzymes could have been missed. However, quantitative analysis suggests that all universal genes are indeed present and that nearly all non-repetitive regions are contained in contigs; any potential missing part of the genome would have to be small, and cannot account for a large number of missing genes. Furthermore, our analysis is based on independent assemblies of multiple moderately related species, and it is unlikely that the same gene would be missing in several assemblies.

While smaller endosymbionts can produce at least some amino acids or retain some parts of the central metabolism, Holospora are unable to synthesize any amino acid, and have to acquire them from the host, despite the fact that some relics of pathways are seen, such as a partial pathway for the tryptophan synthesis. Holospora lack glycolysis, the Entner-Doudoroff pathway, and the pentose phosphate pathway. Surprisingly, Holospora have no enzymes of the citric acid cycle, which is unusual for Rickettsiales, as even the most reduced Rickettsia retain it.

Although we have not performed a complete metabolic reconstruction, it is evident that a broad range of compounds, including amino acids, DMAPP, and chorismate derivatives have to be imported from the host for Holospora to survive. It is unlikely that these compounds are degraded in the host's nuclear proteins, and these compounds have to be acquired from the host. Amino acids can be obtained by degrading host nuclear proteins, and indeed the Holospora infection is associated with increased proteolytic activity in the macronucleus (Freiburg, 1985). The situation with other compounds is more intriguing. All these small molecules can passively diffuse through the nuclear pore complex (Knockenhauer and Schwartz, 2016). Further, Holospora alter the host's gene expression and increase RNA synthesis (Freiburg, 1985; Fujishima, 2009). These alterations may help bacteria to acquire the necessary nutrients. To understand how the missing nutrients are delivered to Holospora, we searched for transport proteins and attempted to predict their specificity. This has explained some, but not all of the missing nutrients, and this topic has to be investigated further.

We propose that Holospora not only relies on the host for the energy production, but specifically use nucleotides or ribonucleotides as the energy source. Indeed, they are able to interconvert them and to convert UTP to CTP, and putative ribose transport proteins, which can also transport nucleotides, are present. We suggest that ribonucleotides are the preferred energy source, as the intracellular abundance of ribonucleotides can be 10- to 100-fold higher than that of dNTPs (Traut, 1994). This seems natural, given the nuclear habitat of Holospora.

The interactions between Holospora and their hosts have been studied in some depth. Holospora infection can either decrease or increase the Paramecium viability under a variety of conditions (Fujishima et al., 2005; Hori et al., 2008; Fujishima, 2009; Bella et al., 2016). Addition of Holospora curviuscula to the analysis allowed us not only to investigate proteins specific to Holospora in a particular host, but also to find proteins that may play a role in the macronuclear infection. While the details of the mechanism remain unclear, it is likely that the interaction and stable infection rely on Holospora-specific proteins, in particular, secreted short conserved peptides. The 89 kDa periplasmic protein shown to be involved in the H. obtusa infection (Iwatani et al., 2005) is conserved in all studied Holospora spp., which indicates its importance. Finally, we propose that OmpA-like proteins may be involved in the Holospora invasion.

Overall, the analysis of the Holospora genomes demonstrates that their metabolic capabilities are unusually restricted, especially given the genome size, and provides a list of candidate genes essential for their unique lifestyle.

AUTHOR CONTRIBUTIONS

SG, MR, and MG designed the research. AB and MR isolated and cultivated bacteria. ML prepared the sequencing libraries and sequenced the genome. ML and SG assembled the genome. SG, AB, DM, and MG annotated the genome and performed the comparative analysis. SG, AB, MR, and MG wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00738/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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