Genome Evolution and Phylogenomic Analysis of Candidatus Kinetoplastibacterium, the Betaproteobacterial Endosymbionts of Strigomonas and Angomonas

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Abstract

It has been long known that insect-infecting trypanosomatid flagellates from the genera Angomonas and Strigomonas harbor bacterial endosymbionts (Candidatus Kinetoplastibacterium or TPE [trypanosomatid proteobacterial endosymbiont]) that supplement the host metabolism. Based on previous analyses of other bacterial endosymbiont genomes from other lineages, a stereotypical path of genome evolution in such bacteria over the duration of their association with the eukaryotic host has been characterized. In this work, we sequence and analyze the genomes of five TPEs, perform their metabolic reconstruction, do an extensive phylogenomic analyses with all available Betaproteobacteria, and compare the TPEs with their nearest betaproteobacterial relatives. We also identify a number of housekeeping and central metabolism genes that seem to have undergone positive selection. Our genome structure analyses show total synteny among the five TPEs despite millions of years of divergence, and that this lineage follows the common path of genome evolution observed in other endosymbionts of diverse ancestries. As previously suggested by cell biology and biochemistry experiments, Ca. Kinetoplastibacterium spp. preferentially maintain those genes necessary for the biosynthesis of compounds needed by their hosts. We have also shown that metabolic and informational genes related to the cooperation with the host are overrepresented amongst genes shown to be under positive selection. Finally, our phylogenomic analysis shows that, while being in the Alcaligenaceae family of Betaproteobacteria, the closest relatives of these endosymbionts are not in the genus Bordetella as previously reported, but more likely in the Taylorella genus.

Key words: endosymbiont biology, phylogenomics, comparative genomics, Trypanosomatidae, selective pressure.

Introduction

The phenomenon of bacterial endosymbiosis has been increasingly shown to occur in a widespread range of eukaryotes ranging from protozoa to Metazoa. In Metazoa, insects (Kikuchi 2009) are most commonly implicated, but plants (Markmann and Parniske 2009), nematodes, Amoebozoa (Schmitz-Esser et al. 2010), and leeches (Perkins et al. 2005) have also been shown to harbor prokaryotic symbionts, which perform a variety of important roles for their hosts. Endosymbiotic associations characterized to date have exhibited a common pattern of genome evolution, the extent of which is directly proportional to the age of the association (Moya et al. 2008). Such common alterations in the endosymbiont genomes include marked genome size reduction, lowering of GC content, loss of transcriptional regulation genes (possibly due to the stability of the intracellular milieu), loss of DNA repair genes (possibly due to bottleneck effects given the small number of bacteria, frequently just one, in each host cell), extensive loss of biosynthetic metabolic capabilities (except those required by the host), reduction or loss of codon-usage bias, loss of mobile elements, reduction of the length of intergenic regions (frequently leading to a significant
number of overlapping genes), and endosymbiotic gene transfer to the host nucleus (Moya et al. 2008; Nowack and Melkonian 2010). The Kinetoplastida, one of the three classes of the phylum Euglenozoa, is comprised of a number of protozoan taxa of great ecological, medical, veterinary, and economical importance. The class is divided into the Trypanosomatidae, comprised only of highly successful parasitic species, and several bodonid clades, containing parasitic, commensal, or free-living species (Vickerman 1976; Campbell 1992; Cavalier-Smith 1993; Podlipaev 2001). Trypanosomatids are a highly successful taxon of parasitic organisms that are known to infect all classes of vertebrates, several classes of invertebrates, and many plants. Monoxenic parasites including Angomonas, Crithidia, Blastocrithidia, Strigomonas, Herpetomonas, and Leptomonas infect a wide range of insects (Vickerman 1976; Campbell 1992; Cavalier-Smith 1993; Podlipaev 2001). Newton and Horne (1957) first reported the presence of self-reproducing structures in the cytoplasm of Strigomonas oncocoli. These so-called bipolar bodies were the first endosymbionts described in trypanosomatids and were later shown to be of betaproteobacterial nature (Du et al. 1994; Umaki et al. 2009; Teixeira et al. 2011). Typically, there is only a single bacterium per trypanosomatid cell, and the host and parasite display a sophisticated interrelationship. Thus, the endosymbiont affects the morphology and ultrastructure of the host cells (Motta et al. 1997; de Souza and Motta 1999) and supplies the host with essential compounds including heme, nucleotides, and essential amino acids (de Souza and Motta 1999; Alves et al. 2011). Conversely, the endosymbionts are supplied with a stable environment and nutrients. Finally, the host nucleus and the bacterium have developed a form of communication that controls their cell cycles such that they replicate in synchrony (Motta et al. 2010).

Chang and coworkers (Chang and Trager 1974; Chang 1975a, 1975b; Chang et al. 1975) characterized the presence of endosymbionts in S. culicis and demonstrated the ability of this flagellate to synthesize heme. The latter ability was absent in flagellates artificially cured of their symbionts by chloramphenicol treatment, showing that the bacterium was responsible for this biosynthetic capability. The details of this biochemical collaboration have been recently published, showing that heme biosynthesis is performed partly by the endosymbiont and partly by host enzymes (which were laterally transferred from a Gammaproteobacteria), with neither organism capable of completing synthesis on its own (Alves et al. 2011). It is interesting to note that, while the flagellate can live in culture without the endosymbiont if the medium is supplemented with compounds the bacterium supplies, the opposite has not been possible, that is, the bacterial endosymbiont has not been successfully cultured outside its host to date (de Souza and Motta 1999).

Presently, six trypanosomatid species are known to harbor symbionts (Teixeira et al. 2011). They are referred to as SHTs (symbiont-harboring trypanosomatids) in contrast to regular trypanosomatids lacking symbionts. In this work, we sequenced and analyzed the entire genomes of the betaproteobacterial endosymbionts Candidatus Kinetoplastibacterium oncopolii, Ca. K. blastocrithidii, Ca. K. galati, Ca. K. desouzaei, and Ca. K. crithidii, which are referred to as TPEs (trypanosomatid proteobacterial endosymbionts) (Teixeira et al. 2011), and found in association with five SHTs: S. oncocoli, S. culicis, S. galati, Angomonas desouzaei, and A. deanei, respectively. We have extensively annotated and analyzed the completed genome sequences of the five TPEs above, performing phylogenomic analyses with all sequenced betaproteobacterial genomes available to date (including two highly reduced and fast evolving genomes of endosymbionts from insects (McCutcheon and Moran 2010; Lopez-Madrigal et al. 2011), and three organisms each from the Alpha- and Gammaproteobacteria as outgroups. We also report the comparison of the TPE genomes with those of two of their relatives from the Alcaligenaceae family, that is, Taylorella equigenitalis, which is a pathogen of the equine genital tract (Hebert et al. 2011), and Achromobacter xylosoxidans, a free-living bacterium that is commonly found on human skin and gut, but can also be an opportunistic pathogen (Holmes et al. 1977; Strnad et al. 2011). These latter bacteria were chosen for being, respectively, the closest known relative of the TPEs and a free-living representative in the family.

Materials and Methods
Organisms and Growth Conditions
As the endosymbionts cannot be grown outside of their trypanosomatid host, we proceeded to shotgun sequence the mixture of host and bacterial DNA directly from the cultures, without any enrichment or separation of cells or DNA. The genomes of the following organisms and their endosymbionts were sequenced (numbers are culture identifiers from the Trypanosomatid Culture Collection of the University of São Paulo): A. deanei (TCC036E), A. desouzae (TCC079E), S. culicis (TCC012E), S. galati (TCC219), S. oncocoli (TCC290E). Organisms were grown in LIT media (Camargo 1964) supplemented with 2% fetal bovine serum.

DNA Extraction and kDNA Depletion
Genomic DNA was extracted with the phenol/chloroform method (Ozaki and Czeko 1984). Steps were taken to minimize the amount of kinetoplast DNA present in the sequence, as previously described (Alves et al. 2011). Our results indicate that this enrichment protocol reduces the proportion of kDNA in the sample to less than approximately 5%, yielding good coverage of nuclear DNA, kDNA, and endosymbiont DNA (data not shown).
Genome Sequencing and Analysis, and Gene Calling and Annotation

Five micrograms of total kDNA-depleted genomic DNA was sequenced using standard pyrosequencing shotgun methodology according to Roche 454 protocols. Reads were assembled using Newbler software (Roche, version 2.3). Resulting contigs were initially segregated into endosymbiont- or host-derived sets by similarity of their sequences to currently available betaproteobacterial genomes. Bacterial chromosome sequence closing was performed by multiplex long-range polymerase chain reaction using primers designed based on contig ends, followed by capillary sequencing of the resulting products and manual gap closing. Genome atlases were drawn using DNAplotter (Carver et al. 2009). Dot plot comparisons were performed using the mummerplot program from MUMmer3 (Kurtz et al. 2004). Genes from TPEs were called using Glimmer (Delcher et al. 1999), and manually checked for completeness and adequate translation start and stop sites in comparison with previously characterized bacterial genes. Genes missed by Glimmer but identified by BLAST similarity searches were also added when shown to be complete. Ribosomal clusters were identified by similarity compared with their homologs from other Betaproteobacteria. Transfer RNAs were predicted using tRNAscan (Lowe and Eddy 1997) and predictions were refined using TFAM (Ardell and Andersson 2006) and its bacterial tRNA models and two of three previously described methodologies (Silva et al. 2006), namely the phenogram analysis and the comparison with the three precompiled fMet (X), Met (M), and Ile (I) tRNA profiles. For the phenogram method, the PHYLIP (Felsenstein 2005) package was used to calculate Jukes–Cantor distances and build the neighbor-joining tree. Prediction of cellular localization for proteins was done by SecretomeP (Bendtsen et al. 2005). Metabolic reconstruction and functional classification were performed by running ASGARD (Alves and Buck 2007), using as general databases both KEGG (Ogata et al. 1999) and UniRef100 (Suzek et al. 2007). Further Clusters of Orthologous Genes (COG) comparisons with other Alcaligenaceae bacteria were done using RPSBLAST (Altschul et al. 1997) against the COG database (Tatusov et al. 1997), and significant P values were calculated using Ca. K. blastocrithidii as the representative TPE. All searches were performed using an E value cutoff of 1E–6.

Orthologous Group Classification

Orthologous groups of Betaproteobacteria were determined using OrthoMCL (Li et al. 2003) version 2, using as input the predicted proteins from 119 organisms: the 5 TPEs, 108 other Betaproteobacteria, three Alphaproteobacteria, and three Gammaproteobacteria (supplementary table S1, Supplementary Material online). After the OrthoMCL run, we filtered the resulting groups to keep only those presenting at least 95% of the total number of organisms. For comparison of the five TPE genomes with each other, the same analysis was performed using only the five relevant protein sets.

Analysis of Natural Selection Pressure in Ca. Kinetoplastibacterium

Investigation of natural selection pressure was performed on all Ca. Kinetoplastibacterium orthologous groups where all five TPEs where represented, except EF-Tu. Amount and direction of selective pressure were calculated using the codeml program from PAML (Yang 2007), testing different models of codon evolution. We have investigated models allowing different dN/dS ratios (o, nonsynonymous to synonymous substitution) for different codon positions along the gene. Site models used were M0, M1a, M2a, M7, and M8 (Yang et al. 2000; Wong et al. 2004), using the Bayes Empirical Bayes test with a probability of greater than 0.95 to identify positively selected codons (Yang et al. 2005).

Phylogenomic Inference

Phylogenetic analyses were performed in a two-step manner: First, a fast maximum likelihood phylogenetic inference of each orthologous group was performed to identify and remove inparalogs (sequences that appeared duplicated only inside a taxon) and misclassified orthologs—genes that were grouped in an orthologous group erroneously, due to partial similarity of sequence. Such sequences usually presented very long branch lengths or divided the phylogenetic tree in two parts that partially mirrored each other, and in such cases the smaller of the two subtrees was removed. Sequence alignments were redone after any removal of taxa. After filtering and cleanup, only groups containing at least 95% of the original number of taxa (i.e., 113) and no more than one gene per taxon were used in subsequent analyses. The second and final step of phylogenetic analysis was a maximum likelihood run using the supermatrix generated by concatenating the 233 protein alignments from the filtered orthologous groups described earlier. Protein sequences were aligned by ClustalW2 (Larkin et al. 2007) using default parameters. Alignments were concatenated using FASconCAT v.1.0 (Kück and Meusemann 2010) after removal of ambiguously aligned columns by Gblocks (Castresana 2000) with default parameters except for the minimum length of a block (5 instead of 10 amino acids) and allowing gaps if present in less than half of the sequences in each column. Maximum likelihood phylogenetic inferences were performed by RAxML v.7.2.8 (Stamatakis 2006) on a Linux computer using 40 processor cores, under the WAG substitution model (Whelan and Goldman 2001), with four gamma-distributed heterogeneity rate categories and estimated empirical residue frequencies (model PROTGAMMAWAGF). One hundred different best tree searches were performed, and the tree with best likelihood found was kept. RAxML’s rapid bootstrap was performed with 100 pseudoreplicates and support is only shown for
Results and Discussion

Overall Genomic Content

In this work, we sequence and characterize the genomes of five endosymbionts from trypanosomatid hosts. As previously suggested, the symbiotic association between ancestral trypanosomatids and Betaproteobacteria was probably the result of a single event that occurred in the late Cretaceous Period (Du et al. 1994; Teixeira et al. 2011), leading to tens of millions of years of symbiont and host co-evolution. Figure 1 presents the genome maps of Ca. K. crithidii and Ca. K. blastocrithidii, showing the typical bacterial features of GC skew and differential distribution of the protein-coding genes in the two chromosomal strands. As determined by comparing the 652 genes used for natural selection pressure analysis (discussed later), the genomes of the TPEs from Strigomonas hosts are quite similar and exhibit overall approximately 83% base sequence identity (with the highest similarity being ~85%, between Ca. K. oncopeltii and Ca. K. galatii). The genomes of TPEs from Angomonas species exhibit lower, approximately 73% base sequence identity. However, the base sequences of the Strigomonas TPEs exhibit only approximately 65% identity to those of the Angomonas TPEs. All these findings are concordant with these organisms’ positioning and branch lengths in the phylogeny (fig. 2).

The genomes of the TPEs are markedly reduced in size compared with their closest nonendosymbiotic relatives, being between 810 and 833 kbp in length and presenting 693–742 protein-coding genes, 43 (or 44, in Ca. K. crithidii) tRNA genes, 9 ribosomal RNA genes and 4, 7, and 20 pseudogenes in Ca. K. galatii, Ca. K. blastocrithidii, and Ca. K. oncopeltii, respectively; only one pseudogene was identified in each of the endosymbionts from Angomonas (table 1). In comparison, the genome of T. equigenitalis, the closest known relative of Ca. Kinetoplastibacterium (fig. 2), is over twice the size and bears over twice as many protein-coding genes as the TPE genomes. However, the number of structural RNA genes in the endosymbionts and T. equigenitalis is similar (~40 tRNA genes and 3 rRNA clusters). The three CAT anti-codon tRNAs identified by tRNAscan could be confidently annotated as the initiator (fMet or X) tRNA, the elongator (Met or M), and a modified tRNA that is charged with isoleucine (Ile) instead of methionine, by using previously described methods (Silva et al. 2006). The presence of this modified tRNA is consistent with the presence of the gene for TiLS (tRNA(Ile)-lysidine synthase), the enzyme responsible for the modification of a cytidine to lysidine. This modification is important because it allows the decoding of the AUA codon, for which no other tRNA was found in our predictions. These results were achieved unambiguously by the phenogram method, where the three different CAT tRNAs from the
FIG. 2.—Maximum likelihood supermatrix phylogeny (233 concatenated orthologs) of the Betaproteobacteria class, with Alpha- and Gammaproteobacteria as outgroups. For the Betaproteobacteria, families are indicated on the right of each clade. Numbers on branches represent bootstrap support (only support of 50 or greater shown). Black circles with white numbers mark the different betaproteobacterial orders: 1, Neisseriales; 2, Nitrosomonadales; 3, Gallionellales; 4, Methylophilales; 5, Hydrogenophilales; 6, Burkholderiales; 7, Rhodocyclales. The five endosymbionts sequenced in this work are represented in bold.

Alphaproteobacteria

Gammaproteobacteria

Neisseriaceae

Nitrosomonadaeae

Gallionellaceae

Methylophilaceae

Hydrogenophilaceae

Burkholderiaceae

Oxalobacteraceae

Comamonadaceae

Burkholderiales incertae sedis

Sutterellaceae

Burkholderiaceae

Alcaligenaceae

Rhodocyclaceae

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TPEs cluster deep within each of the three sequence types (data not shown); comparison of the TPE sequences with the three profiles available from Silva et al. were not as definitive when it came to the distinction of the Ile and Met CAT tRNAs, although the distinction of fMet was very clear, as already observed by those authors for other organisms. Besides these three cases, all other tRNAscan predictions have been confirmed by matches to the corresponding bacterial TFAM profiles, which could also more easily distinguish the Ile and Met CAT tRNAs, although the distinction of fMet was very clear, as already observed by those authors for other organisms. It is not clear why the endosymbionts, in all other genomic aspects so reduced, would maintain two identical copies of this gene—although it would be tempting to speculate that these bacteria, which have also maintained the three ribosomal cluster copies customary in Betaproteobacteria and a high proportion of translation genes, still need to maintain high protein synthesis efficiency during at least part of their life cycle. The fact that one of them, the pseudogene in Ca. K. galatii, seems to be in the process of being lost suggests that this redundancy might not be essential.

**Genome Organization and Synteny**

Our genome structure analyses show that the genomes of these five Ca. Kinetoplastibacterium bacteria follow the path of genome evolution previously observed in other endosymbionts of diverse ancestries, and also exhibit total synteny. Synteny with *T. equigenitalis* is also relatively highly maintained (supplementary fig. S1, Supplementary Material online), with most blocks of matching sequence along the main diagonal lines. Interestingly, the dot-plot presents an overall X-shape, suggesting that bacteria in the Alcaligenaceae family follow the same pattern of inversions centered around the axis of replication—that is, the line connecting the origin and termination of replication in the genome map—as other bacteria previously studied (Tillier and Collins 2000; Silva et al. 2001). Comparison of Ca. Kinetoplastibacterium with *Pusillimonas* sp., a more distantly related Alcaligenaceae bacterium, by the same method yielded the same X-shaped dot-plot (not shown).

As in other endosymbiotic associations (Moya et al. 2008; Nowack and Melkonian 2010), the genomes of the TPEs

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**Table 1**

| Comparative Genome Statistics | CKbla | CKcri | CKdes | CKgal | CKonc | Tequi | Axylo |
|------------------------------|-------|-------|-------|-------|-------|-------|-------|
| Genome length                | 820,029 | 821,932 | 833,125 | 822,140 | 810,172 | 1,695,860 | 7,359,146 |
| Overall GC %                  | 32.55 | 30.96 | 30.17 | 32.36 | 31.23 | 37.42 | 65.78 |
| Protein-coding genes          | 723   | 730   | 742   | 726   | 693   | 1,556 | 6,815 |
| % of genome in genes<sup>a</sup> | 92.03 | 91.90 | 91.68 | 91.85 | 90.92 | 93.45 | 91.57 |
| rRNA genes<sup>b</sup>        | 9     | 9     | 9     | 9     | 9     | 9     | 10    |
| tRNA genes                    | 43    | 44    | 43    | 43    | 43    | 38    | 60    |
| Other noncoding RNAs          | 7     | 7     | 6     | 7     | 7     | ND    | ND    |
| Pseudogenes                   | 1,004 | 1,010 | 1,007 | 1,012 | 1,017 | 1,008 | 986   |
| Average gene length<sup>d</sup> | 84    | 85    | 87    | 86    | 96    | 82    | 94    |

<sup>a</sup>Includes protein-coding and RNA genes, and pseudogenes.

<sup>b</sup>Includes protein-coding and RNA genes, and pseudogenes.

<sup>c</sup>Arranged in three identical clusters of the 16S, 23S, and 5S rRNA genes (except for *A. xylosoxidans*, where one of the clusters has a duplicated 5S gene).

<sup>d</sup>Only protein-coding genes.
apparently underwent a downward shift of their GC compositions to approximately 30% (table 1). In contrast, the genome of \textit{Achromobacter} exhibits approximately 65.78% GC, whereas \textit{T. equigenitalis}, the closest available relative of the TPEs, presents 37.42% GC. These values could suggest that the low GC content of the TPE genomes might not be completely associated with the process of becoming endosymbiotic, because their closest nonendosymbiotic relative has a relatively low GC value in comparison with other bacteria of the Alcaligenaceae family. All other Alcaligenaceae genomes currently available show GC contents ranging between 56.92% and 68.10%. Interestingly, like the TPEs, \textit{T. equigenitalis} exhibits a markedly reduced genome size compared with the other Alcaligenaceae. Moreover, \textit{T. equigenitalis} is an obligate intracellular pathogen and could be considered to be evolving toward an “endosymbiotic” life style. A more definitive conclusion on the significance of the variation in genome size and GC content among these bacteria will only be possible with more widespread sampling of taxa in the family.

Differences of Gene Content and Function among the TPEs

Although the endosymbionts present complete synteny and very similar overall characteristics, some differences in gene content, apparently due to differential gene loss, were observed. Figure 3 displays the presence or absence of protein-coding genes from the five TPE genomes aligned and in order from first (\textit{dnaA}) to last gene. Interestingly, differences in gene content seem to appear in “hot-spots” of gene loss, interspersed with more stable blocks of genes. There also seems to be a trend of pseudogenes being located preferentially close to these “hot spots,” suggesting that the presence of these pseudogenes is an early intermediate stage in the genome reduction process, as previously observed (Silva et al. 2001). Together, these observations suggest that functionally related genes from specific operons are being preferentially lost, as we have noticed in at least a couple of cases, for example, an operon related to ubiquinone biosynthesis, defective in the TPEs from \textit{Angomonas}, and another related to thiamine biosynthesis, this time in process of loss from the genomes of TPEs from \textit{Strigomonas} instead (not shown). This hypothesis is supported by the analysis of COG classifications for these “lost” genes. Although most COG categories are represented among the gene losses and pseudogenes, the distribution of these genes is not random ($P < 0.05$). Underrepresented (i.e., presenting less loss than expected by chance) categories include genes required for basic informational tasks and those related to the host-endosymbiont relationship; that is, “translation, ribosomal structure, and biogenesis” ($P = 2.7 \times 10^{-5}$ using the two-tailed Fisher’s exact test), “posttranslational modification, protein turnover,
chaperones” \( (P=4.7E-06) \), “DNA replication, recombination, and repair” \( (P=4.8E-05) \), “energy production and conversion” \( (P=2.0E-04) \), “amino acid transport and metabolism” \( (P=0.001) \), and “nucleotide transport and metabolism” \( (P=0.02) \). The finding that loss of genes from these categories is lower than expected suggests that most of the genes that could be lost from these categories have already been lost, and that many of the remaining ones might be needed to fulfill the needs of the endosymbionts or hosts. Overrepresented categories among gene losses and pseudogenes include, for the most part, genes of unknown or uncertain function, and genes related to the interface between the bacterium and its host; for example, “hypothetical protein” \( (P=9.5E-11) \), “function unknown” \( (P=0.02) \), “cell wall, membrane, and envelope biogenesis” \( (P=2.1E-08) \), “coenzyme metabolism” \( (P=7.2E-06) \), and “lipid metabolism” \( (P=0.02) \) (note: although “hypothetical protein” is not a COG category, we have included such proteins in this analysis due to their abundance among genes lost in the TPEs).

The COG category “general function prediction only,” which was not statistically overrepresented \( (P=0.40) \), and the “hypothetical protein” class include a few genes predicted to encode membrane associated or transporter proteins, reinforcing the trend mentioned earlier of gene losses related to host–bacterium interface proteins. These observations argue that the genes of these functional categories are more dispensable by the TPEs. Cell wall- and lipid-related proteins, for example, might be dispensable given the protected environment inside the trypanosomatid host cell. Hypothetical proteins, on the other hand, could represent genes that were specific to the TPEs’ former independent life, but which are uncharacterized given their obscure roles or limited taxonomic distribution, and that are now superfluous for their symbiotic intracellular life style.

Differences of Gene Content between the TPEs and Other Alcaligenaceae

Comparison of the presence of different functional categories as defined by COG has also shown interesting differences between the TPEs and their betaproteobacterial relatives (supplementary fig. S2, Supplementary Material online), demonstrating potential ancient losses that could have already occurred in all five TPEs, probably in their common ancestor—as a shorthand, we will be referring to the differences found as losses in the reduced TPE genomes, but it should be noted that, given the currently unsolvable lack of the relevant ancestral genomes, only a more thorough parsimony analysis of the gene presence patterns, and taking into account the underlying phylogeny, would be able to indicate the most likely reason for the differences with higher certainty. As previously observed in other endosymbionts (Moran et al. 2008), there is clear retention, compared with *Taylorella* and *Achromobacter*, of genes involved in replication \( (P=0.009 \text{ and } 1.2E-10, \text{ respectively}) \) and translation \( (P=1.6E-09 \text{ and } P=1.2E-10) \), as a proportion of whole genome gene content—although absolute numbers would seem to indicate otherwise given the large difference in genome size, specially between *Achromobacter* and the TPEs. In contrast, transcription-related genes are less represented in the TPE genomes than in *Achromobacter* \( (P=4.0E-10) \). This finding might reflect the stability of the endosymbionts’ intracellular milieu, which may decrease the requirement for regulatory molecules in relation to free-living organisms, which are much more likely to encounter differing environmental challenges. There was no statistically significant difference in the proportions of transcription-related genes between the TPEs and *T. equigenitalis*, which might reflect either shared ancestry of the reduction, or, more counter intuitively, that the pathogenic parasite *Taylorella* does not need extensive transcriptional regulatory capabilities in its intracelular niche in the equine genital tract. Other functional categories overrepresented in the TPEs in relation to both *Taylorella* and *Achromobacter* are “coenzyme transport and metabolism” \( (P=0.002 \text{ and } P=5.8E-11) \) and “nucleotide transport and metabolism” \( (P=0.01 \text{ and } P=1.2E-10) \), suggesting collaboration between the endosymbionts and their hosts in the synthesis of these compounds and, therefore, maintenance of necessary genes. Heme synthesis genes, for example, which were previously shown to be provided by the TPEs (Alves et al. 2011), are included in the “coenzyme” category. A more detailed analysis of the host genomes will be required to clarify whether a collaboration on the synthesis of nucleotides would benefit either, or both, of the two organisms. However, the “inorganic ion transport and metabolism” category is underrepresented in the TPEs as compared with the other two Alcaligenaceae examined \( (P=0.007 \text{ and } P=2.9E-06) \), suggesting that the host could be supplying some of these metabolites.

Another interesting observation is the lower representation of “poorly characterized” genes (e.g., not matching any COG category) in the TPE genomes compared with the other Alcaligenaceae. This observation likely reflects the loss of nonessential genes, or of genes specific to independent life that have not yet been studied. The proportions of genes not classified in any COG are strikingly lower in the TPEs than in either *Taylorella* \( (P=1.8E-10) \) or *Achromobacter* \( (P=3E-10) \). The category “lipid transport and metabolism” is overrepresented in the endosymbionts compared with *Taylorella* but not *Achromobacter* \( (P=0.03 \text{ and } P=0.16, \text{ respectively}) \). The significance of this lower amount of *Taylorella* lipid-related genes, compared with the other two organisms, is unclear. Compared with *Achromobacter*, the number of other underrepresented categories in the TPEs is higher, and includes “secondary metabolites biosynthesis, transport and catabolism” \( (3.7E-05) \), “cell motility” \( (P=1.3E-04) \), “function unknown” \( (P=0.002) \), “amino acid transport and metabolism” \( (P=0.05) \), and “signal transduction mechanisms” \( P=2.0E-04 \) (note: although “hypothetical protein” is not a COG category, we have included such proteins in this analysis due to their abundance among genes lost in the TPEs).
translational modification, protein turnover, chaperone. Taylorella and endosymbionts. to synthesize more of these compounds than parasites or endosymbionts.

Several categories of genes are overrepresented in the TPEs and Taylorella in relation to Achromobacter, including "post-translational modification, protein turnover, chaperone" ($P = 5.8 \times 10^{-5}$), "cell wall/membrane biogenesis" ($P = 4.0 \times 10^{-4}$), and "cell cycle control, cell division, chromosome partitioning" ($P = 6.4 \times 10^{-4}$). Neither of these categories is statistically different between TPEs and Taylorella ($P = 0.23, 0.93, \text{and} 0.59$, respectively), suggesting that the TPEs are close to having the minimal complement of genes from these categories, although some may still be lost (discussed earlier in the comparative analyses of the gene losses and pseudogenes in the five TPE genomes).

Recombination and Repair Genes

Recombination and repair-related genes are also of interest in endosymbionts, given that the extreme organizational stability of their genomes is commonly thought to be due to the loss of recombination genes and mobile elements (Silva et al. 2003). However, the Ca. Kinetoplastibacterium genomes contain all genes for the RecOR pathway of homologous recombination involving single-strand DNA breaks: recAGJOR, ssb, and ruvABC. The exact same arrangement is present in the genomes of Taylorella and Achromobacter, both of which also lack recF and thus also apparently use the RecOR version of the homologous recombination pathway (Sakai and Cox 2009). As recBCD are missing in all of these genomes, homologous recombination involving double-strand breaks does not seem possible in any of these organisms. On the other hand, two bacterial genes for nonhomologous end-joining (Ku protein and DNA ligase D1) are absent from both the TPEs and Taylorella but present in Achromobacter and many other betaproteobacterial genera currently present in KEGG. Given the isolation of the TPEs inside of the host precluding contact with other bacterial DNA, lack of recBCD is probably dispensable, and the presence of the RecOR pathway in the endosymbionts is maintained to perform essential functions of DNA damage repair. We speculate that the RecOR homologous recombination system could potentially mediate chromosomal inversions, and therefore explain the diversity in cluster arrangements in the Alcaligenaceae, described later.

Ribosomal Gene Cluster Organization

The ribosomal gene clusters in the endosymbiont genomes exhibit the customary betaproteobacterial organization (supplementary fig. S3, Supplementary Material online). There are three ribosomal clusters in all of the endosymbionts, as in other known Alcaligenaceae—although Pullulomonas has only two. With the exception of A. xylosoxidans, which bears a duplicated 5S rRNA gene in one of the clusters, the genes in the rDNA clusters from all bacteria included in table 1 are arranged in exactly the same in order: 16S-Ile-Ala-23S-5S, where Ile and Ala represent isoleucine and alanine tRNA genes, respectively. However, the orientations of the individual clusters in relation to each other vary. In T. equigenitalis and Bordetella avium, all of the clusters are in the plus strand (defined here as being the strand of the dnaA4 gene), as are all genes in each cluster. In contrast, in T. asinigenitalis, A. xylosoxidans, and other Bordetella species, two clusters are encoded in one strand (sometimes the plus strand, other times the minus strand), and the third is encoded from the other strand.

The organization of the rDNA gene clusters of these members of the Alcaligenaceae varies surprisingly, and often is not conserved across closely related taxa; compare the Bordetella species shown in supplementary figure S3, Supplementary Material online. These observations argue that the ribosomal region of these organisms is structurally pliable and subject to rearrangement, potentially by the RecOR system.

Phylogenomic Analysis of the Betaproteobacteria

We performed maximum likelihood analysis of 233 concatenated proteins from the 5 endosymbionts, 109 Betaproteobacteria from 7 different orders and 11 different families (fig. 2), and 3 Alpha- and 3 Gammaproteobacteria as outgroups. Organisms were selected from GenBank based on the availability of both genome sequences and predicted protein sequences. Genes from the 119 organisms were selected utilizing a “relaxed single-copy” approach, where inparalogs were initially allowed (all but one were removed from the final analyses) and genes were required to be present in only 95% of the organisms. This approach permitted us to use many more genes and organisms than a strict “single-copy in all organisms” approach would permit (not shown).

After removal of ambiguous positions from the alignment, 71,747 columns were analyzed, with only approximately 0.68% and 1.94% of the matrix composed of gaps or missing data, respectively. The resulting phylogenetic tree (fig. 2) shows, as previously suggested by more restricted phylogenetic analyses of a few genes (Alves et al. 2011), that the five TPEs are divided in two clades reflecting the taxonomy and phylogeny of their host species, trypanosomatids from the Angomonas and Strigomonas genera. Also as previously shown (Du et al. 1994; Umaki et al. 2009; Teixeira et al. 2011), the TPEs are within the family Alcaligenaceae but, in
contrast to previous analyses, they group with the *Taylorella* instead of the *Bordetella*. This discrepancy is due to the previous lack of sequences intermediary between *Bordetella* and the TPEs; that is, *Taylorella* and *Pusillimonas*, which have had their genomes sequenced only very recently (Cao et al. 2011; Hebert et al. 2011). In general, bootstrap support values are very high (more than 95, and most often 100) across the whole tree, showing strong support of the phylogeny by the data. Bootstrap values for the branches among the TPEs are all 100, as are the ones for the branch-linking *Taylorella* and the TPEs, and the branch grouping all the Alcaligenaceae.

Only one low support value (of 47) is present, for the branch-positioning *Limnobacter* (classified as a Burkholderiaceae) as sister group of the Alcaligenaceae. The only other disagreement between our phylogeny and the previous taxonomic classification of the Betaproteobacteria here analyzed is the positioning of *Launtonia*, classified as a Burkholderiaceae, as a sister group of the Sutterellaceae with reasonably high bootstrap support (value of 81). Bootstrap support for other orders and families of the Betaproteobacteria is very high and groups are nearly all monophyletic, with few exceptions. The basal family, amongst those with sequenced genomes, seems to be the Neisseriaceae, as shown by rooting the tree with the Alpha- and Gammaproteobacteria.

A few previously unclassified (according to NCBI’s Taxonomy database) bacterial species present in our tree can now be confidently placed in families. Thus, the Burkholderiales *incertae sedis Methylibium, Leptothrix*, and *Rubribivax* cluster together, and as sister group of the Family Comamonadaeae with bootstrap support of 100 and divergence level consistent with its potential placement in this family. The next sister group of these bacteria, *Thianomas*, is also currently unclassified at the family level; its divergence level from any other bacterial group included in our phylogeny is similar to that observed among the known families included, and might warrant the creation of a new family. *Ca. Accumulibacter* and the extremely reduced endosymbiont *Ca. Tremblaya*, both also previously unclassified at the family level, have been placed by our phylogenomic analysis in the Rhodocyclaceae and Burkholderiaceae families, respectively, with very high bootstrap support values of 100 and 95.

Two branches in the phylogeny were very long, that is, the branches for the highly reduced endosymbionts *Ca. Tremblaya* and the *Ca. Zinderia*. It is well known that long branches can cause problems for phylogenetic inference, by leading to the positively misleading long-branch attraction phenomenon (Felsenstein 1978), which can be a problem for any phylogenetic method if it is extremely enough. This would lead to unnatural groups being highly supported by bootstrap, whereas sometimes lowering bootstrap support values in other parts of the tree. With that in mind, we have also run the betaproteobacterial phylogeny described without including these two fast-evolving lineages to check for any changes in tree topology and bootstrap support. Except for the lack of the two species, the rest of the resulting tree (not shown) has identical topology to the one presented in figure 2, and bootstrap values are very similar, therefore indicating that the presence of the two long branches is not biasing the rest of the phylogeny in any appreciable way.

**Analysis of Natural Selection Pressure**

We have analyzed all 652 single-copy genes that were present in all 5 TPEs to scan for potential instances of positive selection in these bacteria. The nature of selective pressure is determined by calculating the dN/dS (nonsynonymous to synonymous substitution) ratio (\(\omega\)), where \(\omega < 1\), \(\omega = 1\), and \(\omega > 1\) mean negative, neutral, or positive evolution, respectively (Yang and Bielawski 2000). We tested a number of different models accounting for some modes of evolution (see Materials and Methods). As expected (Yang et al. 2000), tests of model M0 (allowing for only one \(\omega\) category over time and across codon sites in genes) identified no genes with any signal of positive selection, with \(\omega\) ranging from 0.00168 to 0.17109, thus indicating strong purifying evolution overall in TPE genes. However, it is common that a few codon sites in a gene could be subjected to positive selection (e.g., epitopes and extra-cellular residues) among a mostly conserved sequence, leading to a very low average \(\omega\) for the gene as a whole. Thus, it is necessary to test sequence evolution with models (M2a and M8, here) that allow the occurrence of \(\omega\) values above 1 on different codon sites along the genes, and compare those with their corresponding null models, which do not allow for \(\omega > 1\) (M1a and M7, respectively). Using such models, we have identified several genes that contain one or more codons that seem to have undergone positive selection among the orthologs tested (table 2). As expected (Anisimova et al. 2001), the M1a-M2a test is more conservative and identified only two genes containing one codon each undergoing positive selection, whereas the M7-M8 test identified 20 genes containing codons with at least 0.95 probability of being under positive selection (one gene had one codon with better than 0.99 probability). All of these genes presented just one codon undergoing positive selection, except for one gene (ATP-dependent RNA helicase RhIE) with two such codons.

Categories of genes undergoing positive selection often include those related to membrane or extracellular proteins, proteins related to defense and environmental adaptation, or proteins involved in host–pathogen interaction (Aris-Brosou 2005; Anisimova et al. 2007). Less common, however, are reports of less obvious housekeeping genes undergoing positive evolution, although the significance of these findings is usually unexplored (Xu et al. 2011). In this work, we have seen many such genes amongst the candidates for positive selection (table 2), including many genes belonging to COG categories dealing with “amino acid transport and metabolism” (four genes—or five, if the methyltransferase is included),
Table 2
Genes Under Positive Selection in Ca. Kinetoplastibacterium

| Annotation                              | COG Category Class*                  |
|-----------------------------------------|--------------------------------------|
| Shikimate kinase                        | Amino acid transport and metabolism  |
| Glutamine amidotransferase<sup>b,c</sup> | Amino acid transport and metabolism  |
| 3-Isopropylmalate dehydrogenase small subunit | Amino acid transport and metabolism  |
| Leucyl aminopeptidase                    | Amino acid transport and metabolism  |
| Small-conductance mechanosensitive ion channel protein (MscS) | DNA replication, recombination, and repair |
| Lipoprotein NlpD                         | Cell envelope biogenesis, outer membrane |
| Uroporphyrin-III C-methyltransferase (hemX) | Cell envelope biogenesis, outer membrane |
| Chromosomal replication initiator protein (dnaA) | Coenzyme metabolism |
| DNA polymerase III subunit delta         | DNA replication, recombination, and repair |
| TatD DNAse family protein               | DNA replication, recombination, and repair |
| ATP-dependent RNA helicase (rhlE)<sup>d</sup> | DNA replication, recombination, and repair |
| F-type H<sup>+</sup>-transporting ATPase subunit gamma | Energy production and conversion |
| F-type H<sup>+</sup>-transporting ATPase subunit epsilon | Energy production and conversion |
| Methyltransferase                       | General function prediction only     |
| FeS cluster insertion protein ErpA<sup>c</sup> | Posttranslational modification, protein turnover, and chaperones |
| Predicted ATPase of the DUF815 family and AAA + superfamily | Posttranslational modification, protein turnover, and chaperones |
| Small subunit ribosomal protein S14     | Translation, ribosomal structure, and biogenesis |
| Large subunit ribosomal protein L2      | Translation, ribosomal structure, and biogenesis |
| Large subunit ribosomal protein L9      | Translation, ribosomal structure, and biogenesis |
| Glutaminyl-tRNA synthetase               | Translation, ribosomal structure, and biogenesis |

*COG category classes in bold typeface are overrepresented (see text) in relation to the genome; those in normal typeface have representation not significantly different from the genome.

<sup>b</sup>Confidence of the positive selection inference, as calculated by PAML, is greater than 0.99 (all others are between 0.95 and 0.99).

<sup>c</sup>Genes identified both by M1a-M2a and M7-M8 tests.

<sup>d</sup>Two amino acids were detected as being under positive selection (all other had one).

“translation, ribosomal structure and biogenesis” (four genes), and “DNA replication, recombination, and repair” (four genes). Of these categories, only the amino acid- and DNA replication-related ones are overrepresented or nearly so, at the 0.05 level, in relation to the whole genome (Fisher’s exact test P values of 0.0249 and 0.0867, respectively). Out of the 20 genes identified as possibly having undergone positive selection, only two (lipoprotein NlpD and small-conductance mechanosensitive ion channel protein MscS) belong to a category more obviously associated with this mode of evolution, namely “cell envelope biogenesis, outer membrane,” because such proteins face the exterior of the cell and might participate more directly in interactions with hosts or, in case of free-living organisms, the environment. Although the delta and epsilon subunits of the F-type H<sup>+</sup>-transporting ATPase (ATP synthase) participate in a complex of proteins that is membrane-associated, these two specific subunits are part of the F1-unit, located on the cytoplasmic side. A previous report (Xu et al. 2011) on the selective pressure in genes from the parasitic bacterium Actinobacillus pneumonieae also found, in addition to the regularly found membrane-associated and virulence-related genes, several housekeeping genes presenting evidence for positive selection. Interestingly, some of the genes were in common with our observations (e.g., isopropylmalate dehydrogenase small subunit and mechanosensitive ion channel) or from the same pathways or processes (e.g., genes related to amino acid biosynthesis, and translation). In the endosymbionts of trypanosomatids, we hypothesize that the categories of genes found to be under positive selection probably reflect their interactions with their hosts, which might have led to accelerated evolution in processes related to compounds made by the TPEs for the host (amino acids, heme) and to DNA replication (helicase, polymerase, replication initiator)—synchronization of replication between the two organisms could be responsible for positive evolution in this category of genes.

Conclusions

As observed in other endosymbiont genomes previously analyzed, despite significant sequence divergence, genome rearrangement has not occurred in millions of years of separation between the TPEs. This is probably associated with the loss of some, but not all, of the DNA recombination genes. More specifically, nonhomologous end-joining genes are absent, whereas homologous recombination genes for the RecOR pathway are present.

The observed genome size difference between the TPEs and their nearest nonendosymbiont relatives is clearly due to loss of genes instead of gene or intergenic region size reduction, which are both essentially identical among all Alcaligenaceae analyzed here. Gene loss may occur by accumulation of substitutions that turn the gene into a pseudogene, followed by eventual sequence loss during replication. The categories of
Supplementary Material

Supplementary table S1 and figures S1–S3 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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