Polynucleobacter necessarius, a model for genome reduction in both free-living and symbiotic bacteria

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Edited by Nancy A. Moran, University of Texas at Austin, Austin, TX, and approved October 1, 2013 (received for review September 6, 2013)

We present the complete genomic sequence of the essential symbiont *Polynucleobacter necessarius* (*Betaproteobacteria*), which is a valuable case study for several reasons. First, it is hosted by a ciliated protist, *Euplotes*; bacterial symbionts of ciliates are still poorly known because of a lack of extensive molecular data. Second, the single species *P. necessarius* contains both symbiotic and free-living strains, allowing for a comparison between closely related organisms with different ecologies. Third, free-living *P. necessarius* strains are exceptional by themselves because of their small genome size, reduced metabolic flexibility, and high worldwide abundance in freshwater systems. We provide a comparative analysis of *P. necessarius* metabolism and explore the peculiar features of a genome reduction that occurred on an already streamlined genome. We compare this unusual system with current hypotheses for genome erosion in symbionts and free-living bacteria, propose modifications to the presently accepted model, and discuss the potential consequences of translesion DNA polymerase loss.

symbiosis | nonsynonymous mutation rates | *Burkholderiales* | protozoa | genome streamlining

Symbiosis, defined as a close relationship between organisms belonging to different species (1), is a ubiquitous, diverse, and important mechanism in ecology and evolution (e.g., refs. 2–4). In extreme cases, through the establishment of symbiotic relationships, quite unrelated lineages can functionally combine their genomes and generate advantageous emergent features or initiate parasite/host arms races. Ciliates, common unicellular protists of the phylum Ciliophora, are extraordinary receptacles for prokaryotic ecto- and endosymbionts (5, 6) that provide varied examples of biodiversity and ecological roles (6). Nevertheless, most of these symbionts are understudied, partially owing to the scarcity of available molecular data and the absence of sequenced genomes. Yet, thanks to their various biologies and the ease of sampling and cultivating their protist hosts, they are excellent potential models for symbioses between bacteria and heterotrophic eukaryotes. Until recently this field was dominated by studies on endosymbionts of invertebrates, especially insects (e.g., ref. 7), although unicellular systems like amoebas (e.g., refs. 8 and 9) have been shown to be suitable models.

Polynucleobacter necessarius was first described as a cytoplasmic endosymbiont of the ciliate *Euplotes aediculatus* (10, 11). Further surveys detected its presence in a monophyletic group of fresh and brackish water *Euplotes* species (12, 13). All of the investigated strains of these species die soon after being cured of the endosymbiont (10, 12, 13). In the few cases in which *P. necessarius* is not present, a different and rarer bacterium apparently supplies the same function (12, 14). No attempt to grow symbiotic *P. necessarius* outside their hosts has yet been successful (15), strongly suggesting that the relationship is obligate for both partners, in contrast to most other known prokaryote/ciliate symbioses (6).

Thus, the findings of many environmental 16S rRNA gene sequences similar to that of the symbiotic *P. necessarius* (16) but belonging to free-living freshwater bacteria came as a surprise. These free-living strains, which have been isolated and cultivated (17), are ubiquitous and abundant in the plankton of lentic environments (17, 18). They are smaller and do not show the most prominent morphological feature of the symbiotic form: the presence of multiple nucleoids, each containing one copy of the genome (10, 11). It is clear that free-living and endosymbiotic *P. necessarius* are not different life stages of the same organism (15). Nevertheless, these strikingly different bacteria, occupying separate ecological niches, exhibit >99% 16S rRNA gene sequence identity, and phylogenetic analyses fail to separate them into two distinct groups (15). Rather, several lines of evidence point to multiple, recent origins of symbiotic strains from the free-living bacterial pool (14, 15).

Thus, the *Euplotes–Polynucleobacter* symbiosis provides a promising system for the study of changes promoting or caused by the shift to an intracellular lifestyle. The remarkably small (2.16 Mbp) genome of the free-living strain QLW-P1DMWA-1 has been sequenced and studied, especially for features that would explain the success of this lineage in freshwater systems worldwide (19, 20). Phylogenies based on the 16S rRNA gene (13, 14) and multiple-gene analyses (19, 21, 22) consistently cluster *Polynucleobacter* with bacteria of the family *Burkholderiaceae* (*Betaproteobacteria*), either in a basal position or as the sister group of *Ralstonia* and *Cupriavidus*.

Significance

We have investigated multiple aspects of the *Euplotes-Polynucleobacter* system, which provides a unique opportunity for the study of an obligate symbiont with a closely related free-living organism that itself possesses a peculiarly reduced genome and metabolism. We confirmed the robustness and generality of patterns in the evolution of bacterial symbionts' genome, adding at the same time new elements and hypotheses concerning genome reduction in both symbiotic and free-living bacteria. We argue that this system will provide an exceptionally useful model for investigations on symbiosis, because of its peculiarities and the commonness and ease of handling of the ciliate hosts. Genome sequences for independently derived *Polynucleobacter* symbionts will be particularly telling.

Author contributions: C.V., M.L., and G.P. designed research; C.V., P.S.G.C., S.M., L.M.V., and M.S. performed research; V.B., M.F., M.S.A., P.S.G.C., S.M., L.M.V., M.S., and T.G.D. analyzed data; V.B. and M.F. wrote the paper; and G.P. coordinated research.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. NC_010531.1).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1316687110/-/DCSupplemental.

Here we provide the complete genomic sequence of a symbiotic *P. necessarius* harbored in the cytoplasm of *E. aediculatus* and present a comparative analysis of the two sequenced *Polynucleobacter* genomes, addressing the possible biological basis of the *Euplotes–Polynucleobacter* symbiosis. We also provide insights into the evolution of the unique two-step genome reduction in this bacterial species: the first step involving streamlining in a free-living ancestor and the second a more recent period of genome erosion confined to the symbiotic lineage.

Results and Discussion

General Features of the Genome. The circular chromosome (Fig. S1) of the symbiotic *P. necessarius* strain is 1.56 Mbp long and contains approximately 1,279 protein-coding genes (*SI Results and Discussion*, *Genome Composition Analysis* and Table S1). The reduction in genome size that has occurred since the establishment of symbiosis can be estimated by comparing this genome with that of the *P. necessarius* free-living strain QLW-P1DMWA-1 (see also Fig. S2), an acceptable procedure given that the symbiont's gene set is largely a subset of that of the free-living strain (*SI Results and Discussion*, *Genome Composition Analysis* and Fig. S3). This reduction is more apparent as a decrease in the amount of coding DNA (42.3%) than in genome size (27.7%) because of the massive number of pseudogenes in the symbiont (Fig. 1). There are virtually no genes related to mobile elements and extremely few recently duplicated genes in the symbiotic isolate. Horizontally transmitted genes, mostly originated before the split between the two strains, are present (*SI Results and Discussion*, *Genome Composition Analysis*), but there is no sign of DNA exchange between the symbiont and its host *Euplotes*, nor any other eukaryote. A total of 277 genes in the symbiont genome (105 of which are not shared with the free-living relative) have unknown functions (*SI Results and Discussion*, *Genome Composition Analysis*).

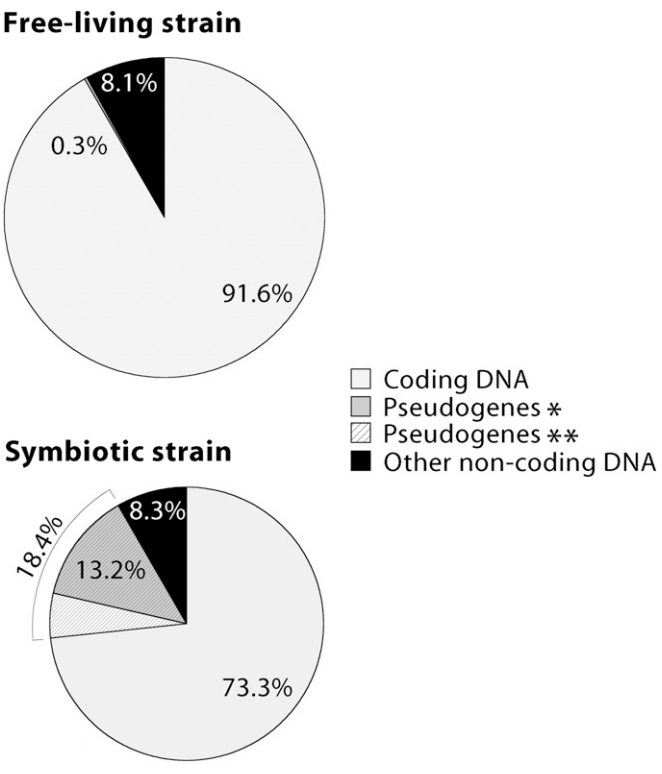

Fig. 1. Percentage allocation of coding and noncoding DNA in the free-living and symbiotic *Polynucleobacter* genomes. The graph areas are proportional to genome sizes (2.16 and 1.56 Mbp, respectively). Pseudogenes were identified with a more conservative (*) and a more permissive (**) approach, as detailed in *SI Results and Discussion*, *Genome Composition Analysis*.

Metabolism. ***Central metabolism and carbon sources.*** Both the symbiont and the free-living *P. necessarius* lack a glycolytic pathway. They do not possess the central regulatory enzyme of the Embden-Meyerhof pathway (6-phosphofructokinase) nor enzymes specific to the Entner-Doudoroff variant, which is used by most *Burkholderiaceae* bacteria. They also lack any enzyme that could phosphorylate glucose to glucose-6-phosphate or be involved in the assimilation of other monosaccharides. The inability to exploit sugars as carbon or energy sources reflects a general poverty in catabolic pathways.

Genomic analysis (Fig. 2 and *SI Results and Discussion*, *Details on Metabolic Analysis*; see also ref. 19) suggests that the principal carbon sources for the free-living strain are pyruvate, acetate, carboxylic acids, and probably compounds convertible to them. These can be metabolized to acetyl-CoA, which is the key intermediate of both energy production and anabolism, thanks to a complete glyoxylate cycle, tricarboxylic acids cycle (TCA), and gluconeogenesis pathways. Acetyl-CoA can additionally be directed to the synthesis of polyhydroxybutyrate (PHB), a storage polymer whose production has been investigated in the bacterium *Ralstonia eutropha*, also a member of *Burkholderiaceae* (23). A total of eight amino acids and all TCA intermediates can also be converted to glucose.

The symbiont possesses all of the aforementioned enzymatic paths with the exception of the glyoxylate cycle, the metabolic link between acetyl-CoA and biosynthetic pathways. Notably, genes involved in the polymerization, depolymerization, and metabolic regulation of PHB are still present. Thus, non-TCA carboxylic acids are not exploitable as sole carbon sources, and most enzymes acting on related compounds are missing. Only three amino acids can potentially be converted to glucose in the symbiont (*SI Results and Discussion*, *Details on Metabolic Analysis*). Thus, it either relies on a very small range of compounds as sole carbon sources, or directly imports various metabolic precursors from its host.

Nitrogen and sulfur metabolism. The free-living *P. necessarius* can perform the assimilatory reduction of nitrate imported from the environment, but the symbiont has lost this ability. An operon including all necessary enzymes for import and catabolism of urea is present only in the free-living strain. Both the free-living and symbiotic *Polynucleobacter* do not possess enzymes involved in nitrification, denitrification, or nitrogen fixation.

The free-living strain can assimilate elemental sulfur and sulfate, whereas the pathway is absent in the symbiont. Moreover, as discussed by Hahn and colleagues (19), the free-living strain possesses an entire set of *sox* genes and hence can probably obtain electrons from hydrogen sulfide (chemolithoheterotrophy). Many of these genes remain in the symbiont, but it is unclear whether the pathway is still functional.

Electron transport chain. Both genomes encode the entire electron transport chain complex and an F-type ATPase. Electrons must come mostly from carboxylic acids and, in the free-living strain, also from hydrogen sulfide. In addition to the most widespread cytochrome *c* oxidase complex, there is a variant in both—the cbb_3 complex—and additionally a *bd* complex in the free-living strain. The cbb_3 complex is present in the genera *Ralstonia* and *Cupriavidus*, and the *bd* complex is present also in *Burkholderia*; both are used in microaerophilic respiration. The symbiont apparently does not posses enzymes able to exploit terminal electron acceptors other than oxygen, whereas the free-living strain possesses a set of alcohol dehydrogenases, suggesting the possibility of energy production through fermentation under anaerobic conditions.

Amino acid and cofactor metabolism. Experimental evidence finds that the free-living isolate can grow on single carbon sources (like acetate) with a few cofactors (19), so we must assume that all amino acid biosynthetic pathways are somehow present in the genome, although it seems that in certain cases the bacterium

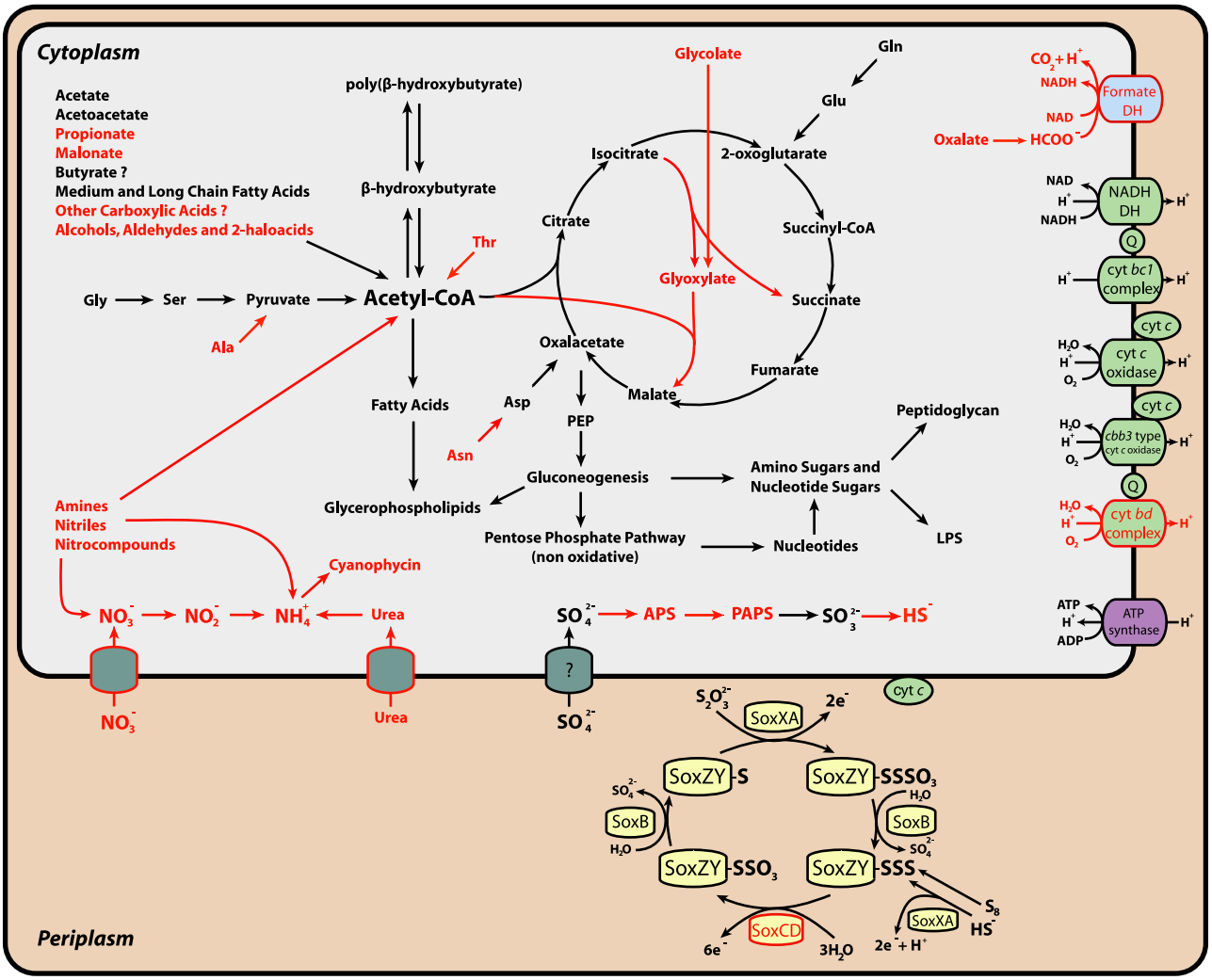

Fig. 2. Schematic drawing of selected pathways of *Polynucleobacter necessarius* metabolism as inferred by genomic analysis. Elements in red are exclusive to the free-living strain's genome. DH, dehydrogenase; cyt, cytochrome.

uses unconventional synthetic strategies (*SI Results and Discussion*, *Details on Metabolic Analysis*). The biosynthesis of four amino acids (alanine, aspartate, serine, and cysteine) has been lost in the symbiont. In a similar way, the symbiont cannot produce many cofactors, although the free-living strain lacks the ability to synthesize some of them as well (*SI Results and Discussion*, *Details on Metabolic Analysis*).

Other biosynthetic pathways. The major biosynthetic pathways stemming from TCA and gluconeogenesis (fatty acids biosynthesis, de novo and salvage nucleotide biosynthesis, and all those providing the necessary intermediates, e.g., the nonoxidative part of the pentose phosphate pathway) are present in both bacteria. As suggested by encoded genes, *P. necessarius* can synthesize phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, but not phosphatidylinositol or lecithin, as membrane phospholipids. Typical bacterial structures of the cell wall (peptidoglycan and lipopolysaccharide) can be synthesized as well, although the symbiont apparently has a reduced set of genes involved in the recycling and modification of both.

Other Genomic Features. ***Gene classification.*** We classified the genes of both genomes in broad functional categories (*SI Results and Discussion*, *Genome Composition Analysis*), highlighting the differential amounts of gene loss in the symbiont compared with the free-living strain. Genes involved in core metabolism, protein metabolism, and growth are relatively more retained, whereas those related to membranes and transport, sensing and regulation, and other/unknown functions are much more reduced (Fig. S3).

DNA repair systems. DNA repair systems are considerably underrepresented in both *Polynucleobacter* strains. Although base and nucleotide excision repair pathways are intact, the enzymes for mismatch repair (MMR) are missing. Of the DNA polymerases able to perform translesion synthesis and rescue replication from arrest at points of chromosomal lesions (Pol II, Pol IV, and Pol V), only the error-prone Pol V is present in the free-living strain, and none in the symbiont.

No intact homologous recombination pathway is present, either: the *recBCD* system is completely missing, whereas the *recFOR* system lacks the essential gene *recF*. The Holliday junction resolution system (*ruvABC* and *recG*), however, is present. This suggests that in *Polynucleobacter* illegitimate recombination mechanisms are present. Indeed, the genes *recJ*, *recO*, *recR*, and *recA*, which are up-regulators of illegitimate recombination (24), are maintained despite the absence of *recF*.

Membranes and transport. There are fewer than 100 putatively functional genes for transport-related proteins in the symbiotic *P. necessarius* [mostly belonging to large families like ATP-binding cassette (ABC) transporters, tripartite ATP-independent periplasmic transporters (TRAP-T), and major facilitators]. In both strains the phosphotransferase system is reduced to nonspecific

components and a few putative cytoplasmic enzymes. The specificity of most transporters is difficult to assess (see also ref. 19).

Fewer differences between the strains were found in genes related to protein export. Both a Sec and Sec-independent pathways were found, as well as enzymes involved in the recognition of signal peptides, but no complete bacterial secretion system complex is encoded in either strain. Other membrane-related proteins are limited to lipoproteins and very conserved complexes involved in outer membrane integrity (Tol/Par operon), or outer membrane proteins' correct folding and targeting (YaeT complex). A huge putative membrane protein (10,429 aa) of unknown function is encoded by the free-living strain's genome (19) but is absent in the symbiont.

Cell-cycle, sensing, and stress resistance. Whereas genes involved in DNA metabolism (including replication) and cell cycle are relatively conserved between the *P. necessarius* strains, those involved in sensing and resistance were largely lost in the symbiont and are already few in number in the free-living isolate. Many genes of the two-component and TolB systems are likely nonfunctional in the symbiont. This suggests a strongly reduced ability to react to changes in the environment—the ciliate cytoplasm, which is probably more stable than the water column. Among environmental defenses, oxidative stress response systems are particularly reduced; the symbiont lacks, for example, glutathione synthetase, notwithstanding the presence of enzymes requiring glutathione (e.g., glutathione S-transferases).

Physiological Bases of the *Polynucleobacter–Euplotes* Symbiosis. The metabolic profile provides a clear explanation for the inability to grow symbiotic *P. necessarius* strains outside their hosts (15). The symbiont relies on the ciliate at least for carbon sources, organic nitrogen and sulfur, and other essential molecules, including many cofactors. In contrast to ancient or extremely specialized symbionts (e.g., ref. 21), though, it can still perform its own basic anabolic processes and energy production. This condition is similar to that of the other symbiotic member of the family *Burkholderiaceae* with a severely reduced genome: "*Candidatus* Glomeribacter gigasporarum" (22), a beneficial endosymbiont of arbuscular mychorrhizal fungi. *P. necessarius* and "*Ca.* G. gigasporarum" underwent independent events of genome reduction [see also the phylogenetic tree of Ghignone et al. (22)] that produced the loss of different metabolic pathways. For example, "*Ca.* G. gigasporarum" has conserved more amino acid degradation pathways than *P. necessarius* (*SI Results and Discussion*, *Details on Metabolic Analysis*) but lacks instead the β-oxidation pathway (22). Both share the loss of glycolytic pathways.

The symbiotic *P. necessarius* is probably specialized for the intracellular environment in other aspects too, as suggested by its reduced set of genes for sensing and stress resistance. Defensive and regulation mechanisms, as well as membrane and cell wall plasticity, seem to be weakened, providing other plausible reasons for the inability of the bacterium to grow outside its host.

It is more difficult to understand why the symbiont is essential for *Euplotes* survival (12, 15). Many obligate symbionts of eukaryotes described as mutualists serve as a source of essential molecules (7, 25). The possibility that *P. necessarius* provides at least some metabolites to its host cannot be completely ruled out, but we consider it unlikely. *Euplotes* are heterotrophic algal and bacterial feeders and can probably obtain all required amino acids and cofactors from their diet, unlike specialized feeders like sap-feeding insects. More likely, the ciliate host requires *Polynucleobacter* to fix a catabolic deficiency (e.g., in compound degradation) in a pathway usually conserved in both bacteria and eukaryotes but lost in the clade of *Euplotes* species harboring *Polynucleobacter*. Vannini et al. (26) worked on a similar premise and provided evidence for a possible role of these bacteria in glycogen depolymerization; nevertheless, we found nothing in the genome supporting this hypothesis, so the real catabolic pathway involved remains uncertain. The genome of a *Polynucleobacter*-harboring *Euplotes* and further experimental investigation will be able to better address the matter, now that the genomic bases of *Polynucleobacter* biology are established.

It has been pointed out (27) that obligate bacterial symbionts generally do not have "symbiotic genes" coding for exotic functions. Their genomes are fundamentally a subset of those of free-living relatives, and the functional role of symbiosis is better explained by a metabolic cooperation between partners (28, 29). An interesting exception are bacterial secretion systems (BSSs), which are probably involved in the ancestral invasion process and are often found in an active or degraded form in symbiont genomes (25). The symbiotic *P. necessarius* strain does not encode a complete BSS, contrary to some more parasitic-like bacteria that infect amoebas (8). Nevertheless, we found a region similar to a pathogenicity island (positions 625,503–639,686) including 16 ORFs, 10 of which show similarities with type II and type IV secretion system assembly protein genes (the other 6 ORFs do not share significant similarities with any available sequence). This region is of horizontal origin (*SI Results and Discussion*, *Genome Composition Analysis*) and is absent in the free-living strain genome. Most of the genes are considerably shorter (range, 13–78%) than their closest homologs and are possibly nonfunctional. An ancestral free-living strain may have acquired these "invasion genes," which allowed it to survive ingestion and digestion in a predatory *Euplotes*, and was then trapped in the cytoplasm. The ubiquity of free-living *P. necessarius* and the high frequency of BSS genes horizontal transmission, together with no apparent requirement for the evolution of novel functions (at least at the genomic level), may have facilitated the multiple origins and complex pattern of replacement inferred for these essential *Euplotes* symbionts (14).

Genomic Reduction in the Symbiont. The symbiotic *P. necessarius* isolate possesses one of the smallest genomes observed so far in *Betaproteobacteria*, being surpassed only by two exceptional cases that blur the distinction between organism and organelle: "*Candidatus* Tremblaya princeps" (0.14 Mbp, 148 genes) (30) and "*Candidatus* Zinderia insecticola" (0.21 Mbp, 232 genes) (29).

Progressive genome reduction is the rule for obligate symbionts (30, 31). This process has been explained by nonselective mechanisms in insects' symbionts: either relaxed selection and enhanced genetic drift (32, 33) or an increase in mutation rates (34). The first hypothesis stems from the decreased number of essential functions in symbionts and their small population sizes, reduced gene exchange, and frequent occurrence of bottlenecks. The prediction is for a higher synonymous/nonsynonymous (dN/dS) ratio in symbiotic lineages, which has often been reported (32, 33), although a potential bias resulting from synonymous-site saturation has been suggested (34, 35). In *P. necessarius*, dN is slightly higher in the symbiotic than in the free-living strain (Fig. 3), in accordance with expectations. Nevertheless, it was impossible to obtain confident estimates of dS in the two lineages despite their unparalleled level of sequence similarity, because synonymous sites were still saturated on the branch leading to the closest outgroup. When more *P. necessarius* genomes of both free-living and symbiotic strains become available, this problem will almost certainly be circumvented with the help of an intraspecific outgroup.

Moran et al. (31) and McCutcheon and Moran (36) described the steps of genome erosion: the crucial turning point is the loss of DNA repair mechanisms, which brings increased mutation rates, an A+T bias, and massive gene inactivation driven by the spread of mobile elements (MEs). Under these conditions, elimination of DNA in noncoding regions is expected to be due to an intrinsic deletion bias (30, 37). Most of our findings are compatible with this scenario, notwithstanding the differences between the systems studied: *P. necessarius* does not experience a bottleneck during the host asexual division, and ciliate effective population sizes are arguably larger than those of insects. Where our system stands in striking contrast to the prior model is the virtual absence of MEs, paired with the abundance of pseudogenes. MEs and pseudogenes should be a signature of the first

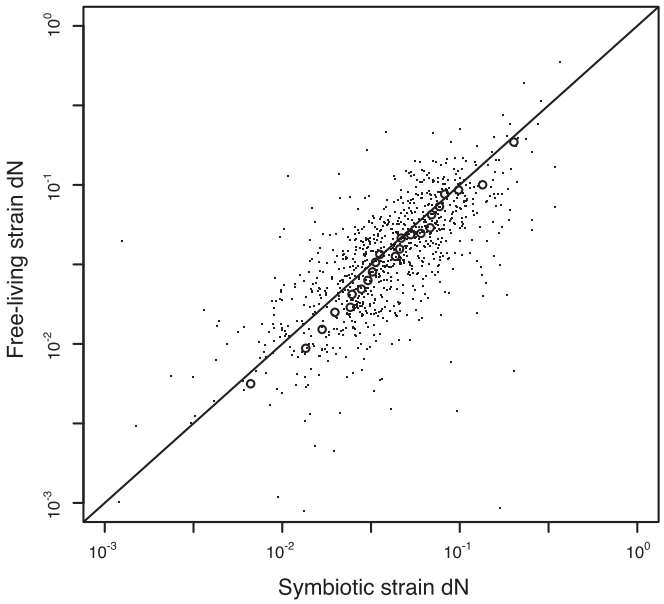

Fig. 3. Nonsynonymous divergence between 904 homolog functional genes in the free-living and symbiotic *Polynucleobacter necessarius*. The rate of divergence from the outgroup is higher in the symbiont (averages: 0.0556, SE: 0.0049 vs. 0.0479, SE: 0.0035; $P = 0.006$, two-tailed t test). Axes are in logarithmic scale, and each circle represents the average position of 40 gene-windows projected onto the diagonal.

stages of genome erosion and until now have always been found together and in large numbers in recently evolved symbionts (30, 35, 36), but in very low quantity, or entirely missing, in the extremely reduced genomes of more ancient symbionts (36). In this regard, the *P. necessarius* case is an exception in recent symbionts and may be explained by the preexisting small size of the ancestral genome or by a relatively large effective population size. It demonstrates, however, that the multiplication of MEs is not a required step of genome erosion.

An aspect that has been less investigated is the role of translesion DNA polymerases (TLPs), and we propose here a scenario that may deserve attention in future studies. The symbiotic *P. necessarius* lacks all TLPs. Although the loss of MMR and homologous recombination may increase the rate of mildly deleterious mutations, the loss of TLPs introduces the risk that a single damaged nucleotide, even in noncoding regions, can entirely block replication. This expectation is supported by the severe reduction in survival of bacteria with artificially impaired translesion synthesis under DNA-damaging conditions (38, 39) and reduced fitness under nonstress conditions (40). A small genome, however, provides less target sites for damage. If the last TLP was lost passively during the early stages of gene inactivation, this would have exerted an additional pressure toward deletions in sequences whose function is not selected for maintenance. The *umuC* and *umuD* genes (encoding the two subunits of Pol V, the only TLP present in the free-living strain) are not even recognizable as pseudogenes in the genome of the symbiotic *Polynucleobacter*, suggesting that the loss of translesion synthesis happened relatively early during the genome erosion process.

Not all proximate causes of genome reduction are known, but among them there is illegitimate recombination, apparently more so when coupled with MMR loss (24). Although the molecular pathways involved are not entirely characterized, TLPs actually participate in deletion accumulation during illegitimate recombination in *Salmonella* (41). However, other mechanisms have been proposed in different systems (e.g., ref. 42). Illegitimate recombination and absence of MMR likely constitute the main source of deletions in *Polynucleobacter* through TLP-independent mechanisms.

The probability that an aborted chromosome replication leads to cell death could be alleviated by multiple copies of the genome. It is thus intriguing to also link the loss of TLPs with the presence of multiple nucleoids in *Polynucleobacter*. Polyploidy has been observed in other intracellular symbionts with reduced genomes, such as *Buchnera* (27), and this explanation might apply to them too (and is not in contradiction with the possibility that a multiple-copy genome is advantageous because of increased gene expression). An alternative hypothesis is that multiple nucleoids arose in *P. necessarius* before the loss of the last remaining TLP, perhaps as byproduct of an unbalanced cell cycle, and paved the way for reduced selection on translesion synthesis maintenance. However, the proximal causes of the polyploidy in the symbiont are not immediately apparent from genomic sequences. The symbiont and the free-living *P. necessarius* share a very similar set of genes involved in chromosome segregation and cell division [including the ParAB-*parS*, structural maintenance of chromosome (SMC), and filamentous temperature-sensitive proteins (Fts) systems (43)].

Genomic Reduction in the Free-Living Progenitor. As stressed above, the genome of the symbiotic *P. necessarius* strain is largely a subset of that of its conspecific relative. However, the free-living *P. necessarius* strain QLW-P1DMWA-1 already possesses a remarkably small genome (19), comparable in size to that of beta-proteobacterial obligate pathogens like *Neisseria* (2.09–2.28 Mbp) and "*Ca.* G. gigasporarum" (approximately 1.72 Mbp) and much smaller than those of other free-living or facultative symbiotic *Burkholderiaceae* (range, 3.75–9.73; Table S2). Thus, a first event of gene loss in the *Polynucleobacter* lineage occurred before the establishment of the symbiosis, presumably in a free-living ancestor.

Genome streamlining in free-living bacteria is less understood than genome erosion in symbionts but has drawn attention in *Prochlorococcus* (44) and "*Candidatus* Pelagibacter ubiquae" (45), two marine taxa with huge global populations. Most authors have argued for adaptive explanations of genome streamlining in free-living bacteria (44, 46), and selection-driven gene loss has been reported for experimental populations (47, 48). In particular, mechanisms requiring relaxed selection and increased drift are generally considered unrealistic because of the huge population sizes of these bacteria.

Nevertheless, the reduced genomes of symbionts and the streamlined genomes of free-living bacteria share many analogies, like a higher AT content and reduced DNA repair mechanisms, in particular MMR loss [reported here for *P. necessarius* and also observed in strains of *Prochlorococcus* (44) and "*Candidatus* Pelagibacter" (49)]. This suggests that nonadaptive mechanisms may have shaped the genomes of these free-living lineages too, perhaps acting in the past when the population sizes were smaller. Although likely detrimental during the first stages, a genome erosion driven by the same mechanisms invoked for symbionts may produce a compact, specialized, and less costly metabolism that could be very successful in the right environment.

Materials and Methods

Purification of the Endosymbiont DNA. Cultures of the ciliate host *E. aediculatus* strain STIR1 were starved, filtered, and treated with chloramphenicol to minimize the amount of contaminating bacteria in the culture medium. The cells were mechanically ruptured to release the symbionts, which were then separated from the eukaryote's cellular fragments through centrifugations at increasing accelerations. Total genomic DNA was extracted from the supernatant after the final centrifugation (protocols and quality controls are detailed in *SI Materials and Methods*).

Sequencing, Assembling, and Annotation. The genome of this symbiotic strain of *P. necessarius* was selected for sequencing on the basis of the US Department of Energy Joint Joint Genome Institute Community Sequencing Program 2006 and is publicly available (accession number NC_010531.1). Sequencing, assembling, and annotation were performed as described elsewhere for the conspecific free-living strain QLW-P1DMWA-1 genome (20). Pseudogenes were identified on the base of reading frame interruptions and/or substantially shorter sequence length with respect to

orthologs, using one more conservative and one more permissive threshold (details in *SI Results and Discussion, Genome Composition Analysis* and Table S1).

Functional Analysis. Predicted ORFs from symbiotic and free-living *P. necessarius* were compared against the nonredundant protein sequences database using BlastP (50). A list of best results was produced for each ORF, and the putative protein product inferred. The KAAS–KEGG Automatic Annotation Server (51) was used as an aid in the interpretation of metabolic pathways in *Polynucleobacter* and to comparatively screen a set of complete genomes from other *Burkholderiaceae* bacteria (Table S2). Data on available genomes were obtained from the National Center for Biotechnology Information microbial genomes webpage (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html).

Substitution Rates. Homologous sequences were defined by closed groups of reciprocal best hits determined from BlastP (50) among the pooled genes belonging to the genomes listed in Table S2. Amino acid sequences were aligned with MUSCLE (52), and nonsynonymous substitution rates were inferred from using PAML (53).

ACKNOWLEDGMENTS. We thank Martin W. Hahn for his fundamental contribution in supporting, as main applicant, the *Polynucleobacter* genome project, performed by the US Department of Energy Joint Genome Institute with support by the Office of Science of the US Department of Energy under contract no. DE-AC02-05CH11231; Matteo Meliani and Clizia Gennai for help in DNA isolation; and Simone Gabrielli for the technical assistance in graphic artwork. This work was supported by European Commission FP7-PEOPLE-2009-IRSES project CINAR PATHOBACTER (Project 247658) and by European Cooperation in Science and Technology (COST) action BM1102.

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