Partial Disruption of Translational and Posttranslational Machinery Reshapes Growth Rates of Bartonella birtlesii

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ABSTRACT Specialization of bacteria in a new niche is associated with genome repertoire changes, and speciation in bacterial specialists is associated with genome reduction. Here, we tested a signature-tagged mutant library of 3,456 Bartonella birtlesii clones to detect mutants that could grow rapidly in vitro. Overall, we found 124 mutants that grew faster than the parental wild-type strain in vitro. We sequenced the genomes of the four mutants with the most rapid growth (formedvisible colonies in only 1 to 2 days compared with 5 days for the wild type) and compared them to the parental isolate genome. We found that the number of disrupted genes associated with translation in the 124 rapid-growth clones was significantly higher than the number of genes involved in translation in the full genome (P < 10^{-6}). Analysis of transposon integration in the genome of the four most rapidly growing clones revealed that one clone lacked one of the two wild-type RNA ribosomal operons. Finally, one of the four clones did not induce bacteremia in our mouse model, whereas infection with the other three resulted in a significantly lower bacterial count in blood than that with the wild-type strain.

RESULTS Rapid-growing clones of B. birtlesii. Using a 96-well puncture machine, small volumes (3 to 5 μl) of B. birtlesii mutant clones...
were plated on 5% sheep’s blood agar. Of the 3,456 clones tested, 124 were able to grow more rapidly (1 to 4 days) than the wild-type strain of *B. birtlesii* (5 days). A list of these clones is provided in Table S1 in the supplemental material. Among these 124 clones, four (E4, E7, E11, and H12) grew to full size in only one or two days (see Table S1).

**Gene sequence analysis.** Using the Genome Walker universal kit and the restriction enzyme DraI, the 124 mutant clones of *B. birtlesii* were PCR amplified and sequenced using both forward and reverse primers, and the sequences were compared with the *B. birtlesii* genome sequence (9) via BLAST analysis. The results are shown in Table S1 in the supplemental material. Among the 124 rapid-growth clones, 43 of the disrupted genes could be identified confidently with a known COG (cluster of orthologous group of proteins) function (see Table S1). We found that 16/43 of these genes disrupted in the rapid-growth clones belong to the translation COG (see Fig. S1), including three clones with disruptions of the 16S rRNA and 23S rRNA genes (H12, 43C4, and 43B10, respectively; see Table S1) and one clone with disruption of the 305 ribosomal protein S18/S6 (43A1; see Table S1). Moreover, the number of disrupted genes associated with translation in the rapid-growth clones was significantly higher than that expected by chance based on the number of translation genes in the genome ($P < 10^{-6}$). Finally, we compare the COG functions of these 43 disrupted genes to the 100 COGs previously found to be conserved in all bacteria (10), and we found that 24 disrupted genes belong to this set of genes, including 16 genes involved in translation. Conversely, none of the remaining 19 disrupted genes belonging to the set of 100 orthologous genes lost in specialists was associated with translation system ($P < 10^{-6}$) (see Fig. S1).

**Transposon integration in the genome.** Analysis of transposon integration in the genomes of the four most rapidly growing clones compared to the genome of the wild-type strain revealed that the transposon was integrated one or several times in each clone. While the H12 mutant contained one transposon integration, the E11 and E4 mutants contained two integrations each, and the E7 mutant contained three integrations. Figure 1 shows the sites of transposon integration in the wild-type genome and in the disrupted genes of the four clones. The integration sites were similar to those produced by Genome Walker analysis (see Table S1 in the supplemental material) and included the following: integration of the transposon in the 16S rRNA gene of mutant H12; integrations in a noncoding region flanked by a hypothetical protein and a tRNA-methyltransferase, an adenine-specific DNA methyltransferase, and a hypothetical protein for E7; integrations in an outer membrane efflux protein of the NodT family and likely part of a type I secretion system. This gene belongs to an operon in the resistance-nodulation-division (RND) efflux system that is well conserved in the *Bartonella* genus and *Rhizobiales* (see Fig. S2 in the supplemental material).

**Mouse infection model.** The wild-type strain of *B. birtlesii* induced bacteremia in our mouse model, but similar to the negative control, clone E11 did not induce bacteremia. Clones E4, E7, and H12 produced a significantly lower bacterial count in the blood (UFC/ml) than the wild-type strain (Fig. 3).

**DISCUSSION**

In this report, using a signature-tagged mutant library of more than 3,000 *Bartonella birtlesii* clones, we found that 124 clones were associated with significantly faster growth in axenic medium, i.e., an increased “agar fitness” in this artificial environment that mimicked rapid specialization. These mutants became specialists for *in vitro* culture on agar. Whole-genome sequencing was done for the four most rapidly growing clones to decipher the specific sites of integration of the transposon in the chromosome. Interestingly we found that the transposon could integrate the chromosome several times for the same clone. This has been also recently reported for a signature-tagged mutant library in *Borrelia burgdorferi* with an average of 2.68 unique insertions per kilobyte of DNA (11). One of the rapid-growth strains was unable to colonize its former niche—mouse erythrocytes (8)—and the three others had slower growth in mice (lower fitness). This phenomenon resembles the reduced virulence of *Pasteurella multocida*, the etiologic agent of fowl cholera, after specialization for *in vitro* cul-

![FIG 1 Transposon integration sites in the Bartonella birtlesii genome. The transposon integration events in the H12, E11, E7, and E4 strains are coded in blue, green, violet, and light orange, respectively.](image-url)

Rolain et al.
The group containing rapid growth.

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fitness and multiplication (10). We speculated that the better rRNA locus was not (11). Nevertheless, growth rates for these of two 5S rRNA genes were disrupted, whereas the single 16S revealed that each of the two 23S rRNA-encoding loci and one feri signature-tagged mutant library recently reported in suggests that if the bacteria do not use as many ribosomal operons apparatus does not need to be used extensively. This hypothesis eruption in 4 rapidly growing clones. Our first hypothesis was that belong to the translation COG, including ribosomal operon dis-

FIG 2 Sites of transposon integration and identification of the disrupted genes in the four clones with rapid growth.

ture. This model of evolution has been well described and starts with the principle that gene loss or acquisition required for fitness in one niche (here, the agar fitness) could inhibit the fitness of the microbe in another environment (here, in the animal model). Bacterial specialization involves potentially dramatic gene loss in both extra- and intracellular bacteria. The evolution of specialized bacteria is associated with various types of genome reduction, including decreases in gene number, GC percentage, and the numbers of both complete and intact ribosomal operons (10, 12, 13). Previously, we were surprised by the number of abnormal or split ribosomal operons in specialized bacteria, particularly intracellular bacteria (10). It appeared that the presence of an abnormal ribosomal operon was a triggering event in several groups of specialized bacteria and that this event occurred independently at least eight times. The presence of an abnormal ribosomal operon appeared to be especially common during the emergence of the Rickettsiales but also in Helicobacter pylori, Leptospira species, and the group containing Mycoplasma and Buchnera. This phenomenon is unlikely to be explained by chance. Similarly, in this work, we found that 16 of the genes disrupted in the rapid-growth clones belong to the translation COG, including ribosomal operon disruption in 4 rapidly growing clones. Our first hypothesis was that the number of active genes in a very restrictive niche must be lower than in a widely changing environment, because the translation apparatus does not need to be used extensively. This hypothesis suggests that if the bacteria do not use as many ribosomal operons in the new niche, they are lost (14). Interestingly, analysis of the signature-tagged mutant library recently reported in B. burgdorferi revealed that each of the two 235 rRNA-encoding loci and one of two 5S rRNA genes were disrupted, whereas the single 16S rRNA locus was not (11). Nevertheless, growth rates for these clones were not evaluated in this study (11).

The disruption of the rRNA operon is followed by increased fitness and multiplication (10). We speculated that the better the number of RNA operons is linked to bacterial ecological strategies (1). To the best of our knowledge, this hypothesis suggested that the duplication of the ribosomal operon was the key component in sympatric bacteria. Here, we demonstrated that rRNA operon disruption is a key factor for specialization in a specific niche.

The adenine-specific DNA methylase is an enzyme that methylates specific DNA targets (GANTC for alphaproteobacteria), resulting in a reduction of the thermodynamic stability of DNA (1) and changes in transcription regulation. In Escherichia coli, mutants lacking this gene exhibit increased spontaneous mutations (mutator phenotype), which may explain the rapid gene loss in specialized bacteria that lack this gene. Furthermore, this gene is critical in host-pathogen interactions, and it is missing from several specialized bacteria. Among the disrupted genes, we found efflux proteins associated with increased growth rate. Although the role of these proteins with growth rate is unknown, there are some experimental evidences that expression of the type III secretion system was associated with a growth penalty in a nonhost environment in Salmonella (18). Finally, the number of deleted genes in rapid growers was significantly higher than the number of conserved genes in specialized bacteria (Fig. 3). Genes associated with transcription and those encoding outer membrane proteins are frequently inactivated; interestingly, the same families of genes are specifically inactivated in E. coli that has been cured for thousands of passages (19).

Based on the mechanistic approach to pathogenicity, bacterial pathogens were thought to have repertoires for accumulating virulence genes (besides toxins), and Shigella was the paradigm of the Red Queen theory, which postulates an arms race for pathogenicity (6, 10).

Comparative genomics has contradicted the previous dogma and has shown that specialists (including pathogens) and specifically Shigella (6, 20) have smaller genome repertoires. Rickettsia
and mycobacterial (10) “killer bug” genomes contain a subset of the genes from their neighbors’ “nonkiller” genomes. Finally, it has been clarified that the most obvious changes from nonpathogenic E. coli to pathogenic and specialized Shigella were gene losses (22, 23).

Here, we show that specialization in a specific niche could be caused by the disruption of critical genes. Most of these genes were involved in translation, and we show that evolution of obligate parasitism bacteria was specifically associated with inactivation of translation system-encoding genes and that unique gene inactivation is a major source of specialization.

**MATERIALS AND METHODS**

**Culture of B. birtlesii wild-type and mutant clones.** Wild-type B. birtlesii (IBS 135, CIP 106691T) (24) and 3,456 signature-tagged mutant B. birtlesii clones (8) were cultured on Columbia agar containing 5% sheep’s blood in a humidified atmosphere containing 5% CO₂ at 37°C. These clones were screened for rapid growth using a 96-well puncture machine and compared to the B. birtlesii parental isolate. Bacterial growth was monitored every day until growth appeared, and the number of days required for bacterial growth was recorded for each clone.

**Identification of genes disrupted by transposon insertion.** The Genome Walker universal kit (Clontech Laboratories, United States) was used to identify genes disrupted by transposon insertion. Rapid-growth clones of B. birtlesii mutants were enzymatically digested with the DraI restriction enzyme. The regions upstream and downstream of Tn were sequenced, and the obtained sequences were analyzed and identified using BLAST analysis at the NCBI website (http://www.ncbi.nlm.nih.gov/Blast.cgi). Primers specific to the identified genes were used for PCR amplification and sequencing to confirm the identification of the disrupted genes associated with the rapid-growth phenotypes.

**Phylogeny of the disrupted genes in rapid-growth clones.** The protein sequences of disrupted genes were analyzed by BLASTP against NR. The first 20 best BLAST hits were retrieved and subjected to multiple alignment using MUSCLE. Multiple alignments were curated using triM, and a maximum likelihood phylogeny reconstruction was generated by phyML. Finally, the protein sequences of disrupted genes from rapid-growth clones with known functions were compared to the set of 100 orthologous genes lost in all obligate intracellular bacteria (specialists) (10).

**Genome sequencing, assembly, and annotation.** The genomes of four rapid-growth clones of B. birtlesii (clones E4, E11, H12, and E7) were fully sequenced with the 454 pyrosequencing platform (454 Life Sciences, Branford, CT) (25). A library of paired-end fragments was created following the manufacturer’s instructions. This library was sequenced using the GS titanium sequencer. Reads from each strain were assembled into contigs and scaffolds using Newbler 2.53 (454 Life Sciences, Branford, CT). The assembly was verified using the CLC Genomics software (CLC Bio, Massachusetts).

**Identification of transposon insertion sites.** Mutant reads were mapped to the wild-type B. birtlesii genome (9) with CLC Genomics software (CLC Bio, Massachusetts) to identify the genes disrupted by the 2,281-bp transposon (8). The sequences upstream and downstream of the transposon insertion were identified by alignment with the wild-type reference genome (9) and verified at the NCBI website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

**Bartonella birtlesii mouse infection model.** Thirty-week-old female BALB/c mice from Charles River Laboratories were housed in an animal facility (5 animals/cage) and allowed to acclimate to the facility and the

![Graph](image-url)
diet for at least 5 days prior to infection. Food and water were provided ad libitum (8).

* B. birtlesii* wild-type or signature-tagged mutants were grown on agar plates for 5 days. Each strain was suspended in phosphate-buffered saline (PBS) immediately before infection. On days 7, 10, 14, and 21 postinfection, 50 μl blood was taken from the tail vein of each infected mouse. Bacteria were released from erythrocytes by a freeze-thaw cycle and plated on CBA-km medium (mutants) or CBA medium (wild type). After 10 days, bacterial colonies were counted, and the degree of bacteremia was calculated.

**Nucleotide sequence accession numbers.** The complete genome sequences of the four *Bartonella birtlesii* mutants have been submitted to GenBank under the bioproject numbers PRJNA168998 (strain H12), PRJNA168999 (strain E7), PRJNA169001 (strain E11), and PRJNA169101 (strain E4).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00115-13/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Figure S2, PDF file, 0.1 MB.
Table S1, DOC file, 0.2 MB.

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