Simultaneous presence of two different copies of the 16S rRNA gene in *Bartonella henselae*

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Bartonella henselae is an emerging pathogen of increasing medical significance. Previous investigations have revealed two different 16S rRNA gene variants among *B. henselae* isolates, resulting in delineation of the *B. henselae* population into 16S RNA type I and type II isolates. While studying 191 *B. henselae* isolates by multi-locus sequence typing (MLST) we detected three isolates that could not be assigned to a distinct 16S RNA type upon direct sequencing because of ambiguous nucleotides in a distinct region of the 16S rRNA gene. Cloning and sequencing of the target region of the 16S rRNA gene suggested that these atypical isolates contained different 16S rRNA gene copies. Southern blot and hybridization experiments confirmed the presence of two different 16S RNA gene copies in each isolate. The isolates were further analysed by 16S RNA type-specific PCR, which assigned them to both 16S RNA types I and II. These results suggest that a small percentage of *B. henselae* isolates may harbour two different 16S rRNA gene copies. These isolates, which accounted for 1.6 % of the isolates in our study, have probably emerged by horizontal gene transfer. The implications of these findings for identification and genotyping studies on *B. henselae* are discussed.

INTRODUCTION

Bartonella henselae is the causative agent of a wide variety of infectious diseases including cat scratch disease, bacillary angiomatosis, prolonged fever ('fever of unknown origin'), and endocarditis (Spach & Koehler, 1998). Domestic cats represent the main host and reservoir for *B. henselae*. Infected cats develop a relapsing bacteraemia of several months' duration during which *B. henselae* may be transmitted to other cats or humans by *Ctenocephalides felis* (cat flea) or cat scratch or bite injuries.

Identification of *Bartonella* species is usually based on molecular methods rather than biochemical reactions because of the fastidious and relatively inert nature of bartonellae. Analysis of the nucleotide sequence of the 16S rRNA gene was the first molecular method used for the identification of *B. henselae* (Relman *et al.*, 1990). Bergmans *et al.* (1996) detected a 3 bp difference between the partial 16S rRNA sequences of clinical *B. henselae* isolates obtained from Dutch patients with cat scratch disease and divided the isolates into two distinct genotypes, 16S RNA type I and type II. Subsequently, isolates belonging to the 16S RNA types I or II were detected by different investigators in other countries (Birtles *et al.*, 2002; Chang *et al.*, 2002; Drancourt *et al.*, 1996; Gupta *et al.*, 2004; Melter *et al.*, 2003; Sander *et al.*, 1997). The 16S RNA types were designated 16S RNA

alleles 1 and 2 and included in the MLST scheme for *B. henselae* (Iredell *et al.*, 2003).

We have recently studied the molecular epidemiology of *B. henselae* isolates from different hosts and various geographical regions by MLST (Arvand *et al.*, 2007). During that study and in subsequent investigations, a total of 191 *B. henselae* isolates were subjected to direct 16S rRNA gene sequencing. Three of these isolates could not be assigned to a distinct 16S RNA type by direct sequencing because of ambiguous nucleotides within a circumscribed region of the 16S rRNA gene. We hypothesized that these isolates may harbour two different copies of the 16S rRNA gene within their genome, leading to ambiguous base sequences within and downstream of the variable region. The atypical isolates were characterized by cloning and sequencing of the 16S rRNA gene, Southern blot analysis and hybridization, and type-specific 16S RNA-PCR, which has frequently been used in previous studies for discrimination of the 16S RNA variants. Our results demonstrate that the atypical *B. henselae* isolates harbour two different copies of the 16S rRNA gene in their genomes.

METHODS

Bartonella isolates. A total of 191 feline and human *B. henselae* isolates from Europe, Australia and the USA were analysed. The epidemiological data of 184 isolates have been described previously (Arvand *et al.*, 2007). The *B. henselae* isolates Ber-K186, Is-479 and

Abbreviation: ST, sequence type.

Simba were isolated from blood cultures of naturally infected cats in Germany, Israel and the USA, respectively. The isolates were stored at -20°C or -80°C until use. Bacteria were grown on Columbia blood agar with 5% sheep blood at 37°C in 5% CO_2 for 7–14 days. A single colony of each isolate was passaged once on agar prior to isolation of bacterial DNA.

Direct 16S rRNA sequencing and MLST analysis. Partial sequences of eight genetic loci (16S rRNA, *batR*, *gltA*, *groEL*, *ftsZ*, *nlpD*, *ribC* and *rpoB*) were determined by direct sequencing on both strands as described before (Arvand & Viezens, 2007; Iredell *et al.*, 2003). The nucleotide sequences were analysed with the DNASTAR Lasergene software package 7. The isolates were assigned to alleles and sequence types (STs) as described previously (Arvand *et al.*, 2007).

Cloning and sequencing of the 16S rRNA gene. A 512 bp region of the 16S rRNA gene was amplified by PCR using the primers 16S-F (5'-AGAGTTTGATCCTGGYTCAG-3') and 16S-R (5'-CTTACGCCARTAAWTCGG-3'), which were originally described by Bergmans *et al.* (1996). Cloning was performed using the TOPO TA Cloning kit with the pCR2.1-TOPO vector (Invitrogen) in *Escherichia coli*; 5–9 clones of each isolate were sequenced (GATC Biotech).

Southern blotting and hybridization. Bacteria were embedded in SeaPlaque GTG agarose and DNA was prepared following treatment with proteinase K and lysozyme as described for PFGE analysis (Arvand *et al.*, 1998). The bacterial DNA was digested with *HincII*, or with *HindIII* and *Eco8II* simultaneously, at 37°C according to the manufacturer's instructions. All restriction enzymes were purchased from Fermentas. Restriction digests were separated by electrophoresis in a 0.8% agarose gel and transferred to a Biotodyne B nylon membrane (Pall Gelman) by vacuum blotting. A DNA probe which was complementary to a 183 bp region of the *B. henselae* 16S rRNA gene corresponding to bases 1414428–1414610 of the *B. henselae* strain Houston-1 sequence (accession no. BX897699) was designed using the primers 16S-F and BH1 (5'-CCGATAAATCTTCTCCCTAA-3'). The probe was labelled with dUTP by using the DIG Oligonucleotide Tailing kit (Roche Diagnostics). Hybridization was performed at 70°C overnight and detection was performed with the CSPD chemiluminescence system (Roche Diagnostics). The *B. henselae* isolates Houston-1 (type I), Berlin-1 (type I), Urly8 Marseille (type

II), Berlin-2 (type II) and Audrey (type II) were included in different experiments as controls.

16S RNA type-specific PCR assay. Type-specific PCR was performed using the primers 16S-F and BH1 or BH2 (5'-CCGATAAATCTTCTCCAAAT-3') as described previously (Bergmans *et al.*, 1996; Sander *et al.*, 1998). Briefly, amplification was carried out in a 50 μl reaction volume with 3 min at 95°C , 30 cycles of 20 s at 95°C , 30 s at 57°C (primers 16S-F and BH1) or 56°C (primers 16S-F and BH2), 60 s at 72°C , and a final extension of 5 min at 72°C . The products were separated by electrophoresis in 2% agarose, stained with ethidium bromide, and photographed.

RESULTS

Evidence for different 16S rRNA gene copies in a distinct *B. henselae* isolate by direct 16S rRNA gene sequencing

During the analysis of 191 *B. henselae* isolates by MLST, we could successfully determine the partial 16S rRNA gene sequence of 188 isolates on both strands by direct sequencing. However, we could not obtain 16S rRNA gene sequences of the appropriate length for the *B. henselae* isolates Ber-K186, IS-479 and Simba. Although the sequences were clear and of high quality at the beginning of both strands, they became undecipherable after approximately 150 bp in the forward or 320 bp in the reverse strand and remained unclear thereafter (Fig. 1). Therefore, we could not obtain overlapping sequences for those isolates.

Cloning and sequencing of the 16S rRNA gene reveals two different 16S rRNA gene copies in the atypical isolates

To determine the 16S rRNA gene sequences of the isolates Ber-K186, IS-479 and Simba, the 16S rRNA gene was

Fig. 1. Representative results of direct sequencing of the 16S rRNA gene of the *B. henselae* isolate Is-479. The sequences, obtained using the primers 16S-F (forward) and 16S-R (reverse), show an abrupt onset of ambiguous bases that is suggestive of overlapping sequences.

amplified by PCR, cloned in *E. coli*, and 5–9 clones from each isolate were sequenced. Two or more clones obtained from each isolate contained the 16S rRNA allele 1, corresponding to the 16S rRNA gene sequence of *B. henselae* Houston-1 (accession no. BX897699), whereas other clones obtained from the same isolate contained the 16S rRNA allele 2, corresponding to the 16S rRNA gene sequence of *B. henselae* Urly8 (accession no. AF214556). The sequences of alleles 1 and 2 differed in three nucleotides including a gap. In detail, the nucleotides CTA- in allele 1 corresponding to the position 1414445–1414447 and 1584454–1584456 of the *B. henselae* Houston-1 sequence (accession no. BX897699) were replaced by AAAT in allele 2. These results suggest that the atypical isolates possess two different copies of the 16S rRNA gene.

Determination of the copy number of the 16S rRNA gene by Southern blotting and hybridization

To determine the copy number of the 16S rRNA gene in the atypical isolates, chromosomal DNA of these isolates and reference strains was digested with *HincII*, which does not cleave within the 16S rRNA gene. The estimated size of the restriction digests that would contain the 16S rRNA genes was 4110 and 4723 bp, as deduced from the sequence of the *B. henselae* Houston-1 strain. Hybridization was performed with a 183 bp 16S rRNA gene-specific probe. The isolates Ber-K186, IS-479 and Simba, and the reference strains Houston-1 and Urly8, revealed two fragments, of approximately 4.1 and 4.7 kb (data not shown), indicating that all isolates harboured two copies of the 16S rRNA gene.

Evidence for two different 16S rRNA copies in a distinct *B. henselae* isolate by double digestion and Southern blotting

To confirm the presence of different 16S rRNA gene copies in the isolates Ber-K186, Is-479 and Simba, genomic DNA

was subjected to double digestion with *HindIII* and *Eco81I*. The former enzyme has no restriction site within the 16S rRNA gene. Restriction of *B. henselae* DNA with *HindIII* should generate two fragments of approximately 3.7 and 2.7 kb that harbour the 16S rRNA gene copies, as deduced from the genome sequence of the Houston-1 isolate. *Eco81I* possesses a recognition site within the 16S rRNA allele 1, but not in allele 2. Digestion of chromosomal DNA of a type I isolate with *HindIII* and *Eco81I* would yield four fragments, three of approximately 1.8 kb and one of approximately 0.8 kb, which contain the restriction fragments of the 16S rRNA gene. In contrast, digestion of chromosomal DNA of type II isolates with *HindIII* and *Eco81I* would yield two fragments of approximately 3.7 and 2.7 kb that harbour the two 16S rRNA gene copies. We hypothesized that the isolates with two different 16S rRNA gene copies would reveal a combination of type I and II patterns, presented schematically in Fig. 2(a) as option 1 and option 2. Hybridization was performed with the 183 bp 16S rRNA-specific probe as described above. As shown in Fig. 2(b), the isolates Ber-K186, Is-479 and Simba displayed a combined 'type I and II' pattern corresponding to option 2. In contrast, the reference strains Houston-1, which has 16S rRNA allele 1, and Urly8 and Audrey, which have 16S rRNA allele 2, showed representative restriction patterns for type I or type II isolates, respectively.

Isolates with different 16S rRNA gene copies are assigned to both genotypes by 16S RNA type-specific PCR

16S rRNA type-specific PCR has frequently been used to determine the 16S rRNA genotype of *B. henselae* isolates (Guptill *et al.*, 2004; Gurfield *et al.*, 1997; Melter *et al.*, 2003; Maruyama *et al.*, 2001; Matar *et al.*, 1999; Sander *et al.*, 1997). We therefore tested whether the atypical *B. henselae* isolates could be assigned to a distinct 16S rRNA type by using this assay. The isolates Ber-K186, Is-479 and

Fig. 2. Southern blot analysis of *B. henselae* DNA after double digestion with *HindIII* and *Eco81I*. (a) Expected size of restriction fragments containing the 16S rRNA genes of isolates with two copies of the 16S rRNA allele 1 (red lines) or allele 2 (blue lines), or one copy of each allele. (b) Restriction fragments of *B. henselae* isolates: lane 1, Houston-1 (type I); lane 2, Urly8 (type II); lane 3, Ber-K186; lane 4, Is-479; lane 5, Audrey (type II); lane 6, Simba.

Simba were subjected to 16S rRNA type-specific PCR along with reference strains. The isolates with different 16S rRNA gene copies revealed positive results in both type I- and type II-specific PCR (Fig. 3). In contrast, the reference strains revealed a positive result in either type I- or type II-specific PCR. Thus, *B. henselae* isolates with two different 16S rRNA alleles are recognized as double-positive isolates by the type-specific PCR assay.

Allelic profiles of isolates with different 16S rRNA gene copies by MLST

The allelic profiles of the isolates Ber-K186, Is-479 and Simba are presented in Table 1. They differed in one or more positions from each other, indicating that the isolates did not belong to the same ST. Since 16S rRNA is one of the genes included in the MLST scheme of *B. henselae* (Iredell *et al.*, 2003), the isolates with different 16S rRNA gene copies could not be assigned to any ST.

DISCUSSION

Presence of multiple copies of the 16S rRNA gene has been documented for several bacterial species (Acinas *et al.*, 2004). Generally, multiple copies of rRNA genes in an organism are either identical or nearly identical in nucleotide sequence. Recent reports have shown that within some species, the multiple rRNA gene copies may show distinct sequence differences (Conville & Witebsky, 2005, 2007; Ninet *et al.*, 1996; Reischl *et al.*, 1998; Wang *et al.*, 1997). Sequence diversity of the 16S rRNA gene in *B. henselae* was first described in 1996 (Bergmans *et al.*, 1996). Two 16S rRNA variants were detected among *B. henselae* isolates: type I represented by the Houston-1 strain, and type II represented by the Urlyl8 and the Berlin-2 strain (Arvand *et al.*, 2001; Drancourt *et al.*, 1996). The delineation of *B. henselae* isolates into two 16S rRNA types

Table 1. Allelic profiles of the *B. henselae* isolates with two different 16S rRNA gene copies

The sequences of all alleles have been previously described (Arvand *et al.*, 2007).

Isolate	16S rRNA	<i>batR</i>	<i>gltA</i>	<i>groEL</i>	<i>ftsZ</i>	<i>nlpD</i>	<i>ribC</i>	<i>rpoB</i>
Ber-K186	1 and 2	3	2	2	2	1	1	2
Is-479	1 and 2	1	1	2	1	1	1	1
Simba	1 and 2	1	1	1	1	1	1	1

has been frequently used in previous studies and is also part of the MLST scheme for *B. henselae* (Chang *et al.*, 2002; Guptill *et al.*, 2004; Heller *et al.*, 1997; Iredell *et al.*, 2003; Maruyama *et al.*, 2001; Melter *et al.*, 2003). Recently, the complete genome sequence of the *B. henselae* Houston-1 strain became available and was shown to contain two identical copies of the 16S rRNA gene (Alsmark *et al.*, 2004). In the present study, we demonstrate, we believe for the first time, the presence of two different 16S rRNA gene copies in the genomes of individual *B. henselae* isolates.

The *B. henselae* isolates with different 16S rRNA gene copies were detected during a study on molecular epidemiology of *B. henselae* isolates from different hosts and geographical regions by MLST. The atypical isolates were originally isolated from distinct hosts in different countries and were therefore not epidemiologically related. The MLST allelic profiles of these isolates displayed one or more allelic differences, indicating that they were also not very closely related genetically. We conclude that our isolates with different 16S rRNA gene copies are not clonally related but rather have emerged independently from each other. A possible mechanism for the emergence of isolates with two different 16S rRNA alleles is horizontal gene transfer. This could possibly occur when an arthropod vector or a feline host is simultaneously infected with different *B. henselae* strains harbouring distinct alleles. Other possible mechanisms such as mutation are less likely because the two alleles of the 16S rRNA gene differ from each other in three nucleotides, which would require three simultaneous mutations at distinct positions within the 16S rRNA gene copy.

Theoretically, a hybrid-type isolate with two different 16S rRNA copies may display two different genomic arrangements with allele 1 at position 1413128–1414619 and allele 2 at position 1583137–1584628 of the *B. henselae* Houston-1 sequence (accession no. BX897699), and vice versa. Restriction of these variants with *Hind*III and *Eco*81I would result in patterns presented as options 1 and 2 in Fig. 2(a). It is interesting that all three hybrid isolates detected in this study corresponded to option 2. Because of the limited number of the hybrid isolates in this study, we can only speculate whether this finding is a simple coincidence. Future studies on more hybrid isolates would help to elucidate this question.

Fig. 3. 16S rRNA type-specific PCR. M, 100 kb molecular mass marker; lane 1, *B. henselae* Houston-1 (type I); lane 2, Urlyl8 (type II); lane 3, Ber-K186; lane 4, Is-479; lane 5, Simba; lane 6, Berlin-1 (type I); lane 7, Berlin 2 (type II).

The presence of two different 16S rRNA gene copies in a distinct *B. henselae* isolate has implications for future studies on *B. henselae* and probably other *Bartonella* species. When 16S rRNA gene sequencing is used for identification of *Bartonella*-like organisms to the species level, the investigators should be aware of the possibility that some *B. henselae* isolates may harbour different 16S rRNA gene copies, which might lead to equivocal nucleotide sequences by direct sequencing. Furthermore, it is possible that some previously described cases of double infection of a distinct host by two *B. henselae* isolates of 16S rRNA types I and II, which were diagnosed by 16S rRNA type-specific PCR, were in reality caused by a single isolate that harboured both 16S rRNA alleles. It would be useful to subject those isolates to additional tests, e.g. direct sequencing of the 16S rRNA gene, MLST analysis or other genotyping methods, in order to differentiate between simultaneous infection by two different isolates and infection by one isolate that harbours two different copies of the 16S rRNA gene.

In summary, our data indicate that a small percentage of *B. henselae* isolates harbour two different copies of the 16S rRNA gene. These isolates have probably emerged by horizontal gene transfer. Investigators involved in sequence-based identification or typing of *B. henselae* should be aware of the existence of such atypical isolates.

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