Intragenomic and intraspecific heterogeneity in *rrs* may surpass interspecific variability in a natural population of *Veillonella*

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As well as intraspecific heterogeneity, intragenomic heterogeneity between 16S rRNA gene copies has been described for a range of bacteria. Due to the wide use of 16S rRNA gene sequence analysis for taxonomy, identification and metagenomics, evaluating the extent of these heterogeneities in natural populations is an essential prerequisite. We investigated inter- and intragenomic 16S rRNA gene heterogeneity of the variable region V3 in a population of 149 clinical isolates of *Veillonella* spp. of human origin and in 13 type or reference *Veillonella* strains using PCR-temporal temperature gel electrophoresis (TTGE). 16S rRNA gene diversity was high in the studied population, as 45 different banding patterns were observed. Intragenomic heterogeneity was demonstrated for 110 (74 %) isolates and 8 (61.5 %) type or reference strains displaying two or three different gene copies. Polymorphic nucleotide positions accounted for 0.5–2.5 % of the sequence and were scattered in helices H16 and H17 of the rRNA molecule. Some of them changed the secondary structure of H17. Phylotaxonomic structure of the population based on the single-copy housekeeping gene *rpoB* was compared with TTGE patterns. The intragenomic V3 heterogeneity, as well as recombination events between strains or isolates of different *rpoB* clades, impaired the 16S rRNA-based identification for some *Veillonella* species. Such approaches should be conducted in other bacterial populations to optimize the interpretation of 16S rRNA gene sequences in taxonomy and/or diversity studies.

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INTRODUCTION

The genus *Veillonella* currently groups 11 validated species of non-fermentative, strictly anaerobic, Gram-negative cocci. These bacteria belong to the oral, genito-urinary, respiratory and intestinal flora of humans and other animals. Six species, *Veillonella dispar*, *Veillonella parvula*, *Veillonella atypica*, *Veillonella montpellierensis*, *Veillonella*

rogosae and *Veillonella denticariosi* have so far been isolated from human flora whilst *Veillonella ratti*, *Veillonella criceti*, *Veillonella rodentium*, *Veillonella caviae* and *Veillonella magna* were only reported from non-human animal flora. Species of the genus *Veillonella* are usually regarded as early colonizers and highly represented members of the human microbiome, particularly in the mouth (Knnen *et al.*, 1999) and the gut (Favier *et al.*, 2002; Zilberstein *et al.*, 2007), and are frequently detected during culture-independent studies of the diversity of different microbiotae during health and disease (Paster *et al.*, 2001; Wang *et al.*, 2004; Aas *et al.*, 2005; Preza *et al.*, 2008). More frequently isolated from human clinical specimens in aerobic–anaerobic polymicrobial cultures, these bacteria were recovered in pure culture and implicated in severe opportunistic human infections (Bhatti & Frank, 2000; Marchandin *et al.*, 2001; Boo *et al.*, 2005; Marriott *et al.*, 2007). Molecular studies are required for species identification due to the lack of

Abbreviations: D2V3, 2D structure type of the V3 region; ML, maximum-likelihood; TTGE, temporal temperature gradient electrophoresis.

GenBank/EMBL/DDBJ accession numbers for RNA polymerase B subunit (*rpoB*) genes determined in this work are as follows: VA1–VA149, GU479077–GU479225, respectively; *V. parvula* CIP 60.1, GU479076; *V. denticariosi* CIP 109449, GU479074; and *V. magna* DSM 19857^T, GU479075.

Two supplementary figures, showing the secondary structure of the 16S rRNA gene and an ML tree based on partial *rpoB* sequences, are available with the online version of this paper.

discriminating phenotypic tests. Sequencing of 16S rRNA genes (16S rDNA or *rrs*) has proven to be a stable and specific marker for bacterial identification but has failed to discriminate between very related species (Marchandin *et al.*, 2003, 2005), such as *V. parvula* and *V. dispar*, or *V. ratti* and *V. criceti*, which shared 99% of their 16S rDNA nucleotide positions. Moreover, intragenomic heterogeneity has been previously demonstrated in a clinical isolate belonging to the genus *Veillonella*, consisting of 1.43% divergence between the four rRNA operons (*rrn*) (Marchandin *et al.*, 2003). An RFLP-PCR-based approach suggested that this phenomenon could be frequent in *Veillonella* spp. since 74.1% of the 27 clinical isolates studied produced unexpected restriction patterns compared with type strains (Marchandin *et al.*, 2003). More recently, Byun *et al.* (2007) reported ambiguous positions observed in the sequencing chromatograms of *V. denticariosi* consistent with intragenomic variations between 16S rRNA gene copies.

Although intragenomic heterogeneities between 16S rDNA copies are now well-known for several bacterial species (Ninet *et al.*, 1996; Nübel *et al.*, 1996; Conville & Witebsky, 2007), their general extent in the natural population remained poorly investigated. The aim of this study was to investigate intragenomic and intergenomic *rrs* heterogeneity levels in a population of 149 clinical isolates of *Veillonella* spp. of human origin. To address this point, we validated a PCR-temporal temperature gel electrophoresis (TTGE)-based approach suitable for rapid and convenient 16S rRNA gene heterogeneity analysis in such large bacterial populations.

METHODS

Bacterial strains and isolates, culture conditions and DNA extraction. The study included the type strains of the 11 characterized *Veillonella* species purchased from the corresponding culture collection (*rpoB* gene GenBank accession nos in parentheses): *V. atypica* ATCC 17744^T (EF185159), *V. parvula* ATCC 10790^T (EF185158), *V. dispar* ATCC 17748^T (EF185161), *V. ratti* ATCC 17746^T (EF185165), *V. rodentium* ATCC 17743^T (EF185166), *V. criceti* ATCC 17747^T (EF185164), *V. caviae* DSM 20738^T (EF185163), *V. rogosae* DSM 18960^T (EF211831), *V. magna* DSM 19857^T (GU479075), *V. montpellierensis* CIP 107992^T (EF411194) and *V. denticariosi* CIP 109448^T (EF185162). Reference strains were *V. parvula* CIP 60.1 and *V. denticariosi* CIP 109449 (gifts from Dr Carlier, Institut Pasteur, Paris). One hundred and forty-nine clinical isolates were obtained from the Bacteriology Laboratory of the University Hospital of Montpellier, France, and were designated VA1–VA149. These 149 clinical isolates were collected over an 8 year period (from 1999 to 2007) from diverse clinical samples collected from 140 patients attending various units of the institution. Clinical isolates were recovered in pure culture (seven isolates; VA1, 8, 81, 134 and 144–146, from bone and joint, blood, bronchoalveolar fluid and liver biopsy samples from four patients), in mixed aerobic–anaerobic cultures or in mixed anaerobic cultures (142 isolates from 137 different patients). The sources of these clinical isolates were as follows: respiratory tract including bronchoalveolar fluid (*n*=41), sinus (*n*=3), pleural fluid (*n*=2) and lung biopsy (*n*=1); skin and soft tissues (*n*=42); urogenital tract including semen (*n*=9), amniotic fluid (*n*=5) and urethral swab (*n*=1); intraabdominal samples

including peritoneal fluid (*n*=13) and liver biopsy (*n*=2); bone and joint (*n*=5); oral cavity (*n*=18); ear (*n*=4); blood (*n*=1); gastric fluid (*n*=1); and pacemaker (*n*=1).

Bacteria were cultured on Columbia sheep blood agar (bioMérieux) incubated for 2–5 days in anaerobic jars with the AnaeroGen System (Oxoid). Strictly anaerobic, Gram-negative cocci were identified as *Veillonella* sp. on the basis of their capacity to reduce nitrate and their susceptibility profile to special-potency disks as previously described (Marchandin *et al.*, 2005). Each strain and isolate was purified by one subculture of a single colony with particular care and frozen at –80 °C. For subsequent experiments, strains and isolates were cultured in the conditions described above and DNA was extracted using the MasterPure DNA purification kit (EPICENTRE Biotechnologies) according to the recommendations of the manufacturer. DNA yield was quantified by determining A₂₆₀/A₂₈₀. Extracts were then diluted to a final concentration of 50 µg ml^{–1}.

PCR-TTGE experiment. A 199 bp fragment (from position 338 to position 536, *Escherichia coli* numbering) overlapping the 16S rDNA V3 variable region (position 338 to position 534, *E. coli* numbering) (Neefs *et al.*, 1993; Sundquist *et al.*, 2007) was amplified using the primers HDA1-GC (primer HDA1 with a fragment rich in GC – the ‘GC clamp’ – added to the 5′ extremity) and HDA2 (Ogier *et al.*, 2002). The reaction mixture (50 µl) consisted of 200 nM each primer (Sigma Genosys), 200 µM each dNTP (Fermentas), 2.5 U FastStart *Taq* DNA polymerase (Roche) in the appropriate 1 × reaction buffer, with 1.8 mM MgCl₂. Template DNA extract (50 ng) was added to the reaction buffer and amplified by thermal cycling as follows: 95 °C for 2 min; 30 cycles of 95 °C for 1 min, 62 °C for 30 s, 72 °C for 1 min; and 72 °C for 7 min. PCR products were checked by electrophoresis in a 1.5% agarose gel before TTGE migration in the DCode Universal Mutation Detection System (Bio-Rad Laboratories). Gels (40 ml) were composed of 8% (w/v) bisacrylamide (37.5:1), 7 M urea, 40 µl N,N,N',N'-tetramethylethylenediamine, and 0.1% (w/v) ammonium persulfate, and were run in 1 × Tris/acetate/EDTA buffer at pH 8.3. DNA (6 µl) was loaded on the gel with 6 µl of in-house dye marker (50% sucrose, 0.1% bromophenol blue) using capillary tips. The electrophoresis conditions were 46 V for 16 h with an initial temperature of 63 °C and a final temperature of 70 °C corresponding to an increase of 0.4 °C h^{–1}. In order to obtain thin discrete bands, a pre-migration for 15 min at 63 °C and 20 V was done after loading. Pre-migration and migration were performed with additional magnetic shaking in the electrophoresis chamber. Gels were stained for 15 min with 0.5 µg ethidium bromide ml^{–1} in 1 × TAE buffer, washed for 45 min in 1 × TAE buffer, and photographed under UV illumination.

TTGE band analysis and sequencing. A specifically designed ladder was loaded on all TTGE gels corresponding to a mix of V3 region amplification products from the four isolates VA2, VA4, VA7 and VA69 (mixed TTGE pattern 1+2+5+6+7+8). TTGE bands were visually detected and analysed by migration distance measuring after standardization. Each TTGE band for further analysis was cut out of the gel with a disposable sterile scalpel to avoid contamination between bands. Gel slices were washed twice in molecular biology grade water and incubated overnight at 37 °C in 10 mM Tris buffer (pH 8.5) to allow DNA diffusion. Amplification of a single 16S rRNA gene V3 region copy was performed using 1 µl band eluate and the primers HDA1 without a GC-clamp and HDA2. The PCR was carried out in 50 µl reaction mixture containing 200 nM each primer, 200 µM each dNTP, 2.5 mM MgCl₂ and 2.5 U *Taq* DNA polymerase (Go *Taq*; Promega) in the appropriate 1 × buffer. PCR conditions were 94 °C for 2 min; 35 cycles of 45 s at 95 °C, 30 s at 62 °C, 1 min at 72 °C; and 10 min at 72 °C. PCR products were checked by electrophoresis in a 1.5% agarose gel and sequenced on an Applied Biosystems automatic sequencer (Cogenics) by using the forward

primer HDA1. Each band was labelled by numbering for bands identified by their distance migration and by adding a, b or c to the band number for co-migrating bands identified by sequencing only; the TTGE profile designation is indicated by a combination of band numbers with a + sign. The presence of different TTGE bands, i.e. 16S rRNA gene V3 region copies with distinct sequences, in a strain or an isolate will be referred to hereafter as having intragenomic heterogeneity, while the presence of different TTGE bands in different strains or isolates will be referred to as intergenomic heterogeneity.

DNA amplification and sequencing of *rpoB* genes. DNA (50 ng) was used as template in the PCR experiments. A 0.7 kb fragment of the β subunit of the ARN polymerase (*rpoB*) gene was amplified using primers Veill-rpoBF and Veill-rpoBR (Arif *et al.*, 2008) for all strains and isolates except for *V. montpellierensis* CIP 107992^T and VA50, VA100, VA110, for which primers Vmontp-rpoBF (5'-GTGTTG-TATCCCGCGTTATG-3') and Vmontp-rpoBR (5'-GCACCATCAA-ATACAGGAGTC-3') were used. The positions of the primers on the *rpoB* gene of *V. parvula* ATCC 10790^T (GenBank accession no. EF185158) were as follows: positions 2421–2442 for Veill-rpoBF, 3100–3120 for Veill-rpoBR and Vmontp-rpoBR, and 2430–2449 for Vmontp-rpoBF. The PCR was carried out by using the same amplification conditions as previously described for 16S rRNA gene V3 regions after TTGE. The amplification profile was as follows: 35 cycles of 1 min at 94 °C, 1 min at 65 °C, 2 min at 72 °C; and a final extension of 10 min at 72 °C. *rpoB* gene PCR products were sequenced on an Applied Biosystems automatic sequencer (Cogenics) by using forward or reverse primer.

Sequence analysis and tree construction. DNA sequences were visualized and analysed with BioEdit programme version 7.0.9 (Hall, 1999). RNA secondary structure by energy minimization was predicted using the Mfold programme version 3.4 (<http://mfold.bioinfo.rpi.edu/>) (Zuker, 2003). Before phylogenetic analysis, the *rpoB* sequences were aligned with CLUSTAL_X (Thompson *et al.*, 1997) and alignments were manually checked and corrected. *Clostridium perfringens* *rpoB* sequence (AB055810) was used as an outgroup. Distance-based phylogeny was computed using the PHYLIP package (Felsenstein, 1997) with F84 as a substitution model in the DNADIST program and neighbour-joining method (NEIGHBOR program). A total of 1000 bootstrapped trees were sampled to test the robustness of the nodes and a consensus tree was reconstructed with CONSENSE program. The maximum-likelihood (ML) tree was constructed using PhyML programs (Guindon & Gascuel, 2003) with GTR + invariant sites + gamma-distribution. For the ML tree, bootstraps were

determined by 100 reiterations. A distance matrix in nexus format was generated from *rpoB* clade types and from the presence or absence of each of the 43 TTGE bands in the TTGE profiles, and then used for decomposition analysis with SplitsTree 4.0 software (Huson & Bryant, 2006).

RESULTS

PCR-TTGE patterns reflect the diversity of the V3 region among the *Veillonella* population

PCR-TTGE analysis generated interpretable patterns for the 149 clinical isolates and the 13 type or reference strains (Fig. 1). A total of 307 TTGE bands were observed. Among them, 201 (65.5 %) bands were selected for sequencing as follows: (i) 36 bands with different migration distances; (ii) 1–12 bands for each of the 36 migration distances; and (iii) one of each band of the 54 TTGE pattern types. This sequencing strategy confirmed that the sequence of the 16S rDNA V3 region differed according to the migration distance. Conversely, in most cases, bands with identical migration distance displayed the same sequence except for 7 bands found in 11 isolates that showed very close migration distances and variable sequences (7.4 % of the bands) (Table 1).

Some extra fragments generated during PCR or TTGE should be noted and excluded from pattern analysis in order to avoid any overestimation of band number (Satokari *et al.*, 2001). As previously described, some artefacts may be generated during PCR by reassociation of DNA strands with slightly different sequences. Heteroduplex bands are recognized by their low intensity associated with reduced migration distances when compared with homoduplex bands, owing to their lower melting temperatures. Between one and five heteroduplex bands were observed within a TTGE pattern composed of multiple bands (Fig. 1); these bands were confirmed as heteroduplexes by a novel run of 16S rDNA PCR-TTGE showing an identical double band profile. Identical

Fig. 1. TTGE profiles of amplified V3 16S rDNA for 16 *Veillonella* spp. clinical isolates. Lanes: 1–16, isolates VA74, VA83–85 and VA87–98, respectively; ladder, mix of four isolates with profiles 1 + 2, 1 + 7, 5 + 6 and 8. Each band number is noted on the band. Dotted arrows indicate heteroduplexes. Solid arrows indicate bands with delayed migration, which had identical sequences to the homoduplex band below.

heteroduplex bands were observed for all the strains or isolates displaying a specific TTGE pattern. We also observed bands of low intensity migrating slightly above the homoduplex bands (Fig. 1). As previously observed by Janse *et al.* (2004), sequencing showed that these bands with delayed migration had identical sequences to the homoduplex band below and were not further considered during pattern analysis. Hereafter, 'TTGE band' will refer to non-artefactual bands excluding heteroduplex and delayed bands.

A very large diversity of TTGE patterns was noted among the 162 strains and isolates, as 54 distinct profiles were observed; 45 among the clinical isolates and 13 for the 13 type or reference strains. Only four TTGE patterns were common to clinical isolates and type strains: *V. rogosae* DSM 18960^T and 13 clinical isolates shared the same profile, and *V. dispar* ATCC 17748^T, *V. atypica* ATCC 17744^T and *V. parvula* ATCC 10790^T displayed identical profiles to those of seven, three and two clinical isolates, respectively. Thus, diversity observed from TTGE patterns in the studied population was higher than expected from the diversity of the known species in the genus. Forming the 54 patterns, 43 TTGE bands differing by their migration distance and/or sequence were identified (Table 1). Sequencing detected a total of 30 variable positions localized from position 415 to position 492. These variable positions were located in the helices H16 and H17 of the rRNA molecule (Case *et al.*, 2007) corresponding to helices H406 (position 406–436, *E. coli* numbering) and H441 (position 441–493, *E. coli* numbering) according to the nomenclature of the Gutell Lab database (<http://www.rna.cccb.utexas.edu/>). Six of the 30 variable positions accounted for intergenomic diversity only, six were variable at the intragenomic level only and 18 were variable at both intragenomic and intergenomic levels (Table 1). Sequencing and 2D reconstruction of the mostly complete H17 rRNA helix revealed 10 different secondary structure types for the 43 different sequences (Supplementary Fig. S1, available with the online version of this paper).

Intragenomic heterogeneity among *Veillonella* spp.

Intragenomic heterogeneity among *rrs* copies was revealed by TTGE profiles with multiple bands further called 'heterogeneous profiles'. Heterogeneous profiles were shown for 110 of the 149 isolates (74 % of the isolates, 37 profiles). They displayed two (84 isolates, 56 %) or three bands (26 isolates, 18 %). Five main heterogeneous profiles were observed, 1 + 2 ($n=19$), 2 + 4 ($n=19$), 8 + 10 ($n=13$), 5 + 6 ($n=11$) and 5 + 6 + 11 ($n=5$), including 60.9 % of the clinical isolates. Heterogeneous profiles were observed for 61.5 % of type and reference strains (8 of 13 strains), with each strain displaying a specific profile (Tables 2 and 3). Sequencing showed that within a genome, between 1 and 5 positions of the V3 region (0.5 % to 2.5 %) were divergent between 16S rDNA copies. However, in 75 % of the heterogeneous TTGE profiles, no more than two hetero-

geneous positions (1 %) were detected. Intragenomic heterogeneity was mainly associated with transitions CT or AG (23 cases) or more rarely with a GT transversion (two cases). The polymorphic position 458 was the only variable nucleotide position characterized either by transition CT (TTGE patterns 1 + 7, 1 + 10, 8 + 10 and 7b + 8 + 10) or by transversion (profiles 1a + 34 and 1a + 5a + 34). The majority of these positions are localized in the helix H17 between nt 444 and 492 (*E. coli* numbering) (Table 1), resulting in 10 different secondary structure types for the 43 TTGE bands (Supplementary Fig. S1). In 15 of the 44 heterogeneous profiles (34.1 %), intragenomic heterogeneity resulted in 16S rRNA gene copies with different secondary structure to the H17 helix. The impact of heterogeneous positions on the secondary structure was independent of their number. For example, two heterogeneous positions may change the secondary structure of the stem-loop (TTGE profile 5 + 13), while five heterogeneous positions did not change this structure due to compensating base co-variation in the stem-loop region (TTGE profile 7 + 1). Different secondary structure types were more frequently observed in strains and isolates with three different copies (9 of 15 heterogeneous profiles, 60 %) than in strains and isolates with two different copies (6 of 29 heterogeneous profiles, 20.7 %). The TTGE pattern 1 + 9a + 2, observed for the clinical isolate VA79, was the only one displaying three secondary structure types (structures 1, 2 and 3).

Structure of the bacterial population on the basis of *rpoB* gene phylogeny

Distance and ML *rpoB*-based phylogenetic trees were congruent (an ML *rpoB*-based tree is shown in Supplementary Fig. S2). The population of clinical isolates was distributed in eight clades. Six of the eight clades included type strains of species of the genus *Veillonella*; clade 1 included *V. parvula*, clade 2 *V. rogosae*, clade 3 *V. dispar*, clade 5 *V. atypica*, clade 6 *V. montpellierensis* and clade 7 *V. ratti* (Supplementary Fig. S2). The polyphyletic clade 1 was considered as a taxonomically unique clade, since both sub-branches supported a reference strain of *V. parvula*. Phylogenetic analysis indicated the presence of two other clades (clades 4 and 8) that did not contain a type strain of a known species. *V. denticariosi*, *V. rodentium*, *V. caviae*, *V. criceti* and *V. magna* type and reference strains did not group with clinical isolates. *V. denticariosi* and *V. rodentium* formed an additional clade. Distribution of clinical isolates in the eight *rpoB*-based clades is given in Table 2. It is noteworthy that 16S rRNA gene intragenomic heterogeneity was observed in all *rpoB*-based clades – ranging from 20 % of clinical isolates belonging to *V. dispar* clade 3 to 100 % of clinical isolates belonging to *V. ratti* clade 7 – and in *V. denticariosi*, *V. caviae* and *V. criceti* strains (Tables 2 and 3).

For further analysis, we considered that *rpoB* clades 1 to 8 were each representative of a known or unknown *Veillonella* species, and comparisons at both intra- and

Table 1. Sequence differences among the 43 TTGE bands observed in the *Veillonella* population, *rrs* V3 region secondary structures, TTGE patterns and *rpoB*-based clades

Nucleotide positions compared with those in band 1 are indicated by their variant nucleotide when divergent or by “–” when identical. For divergent positions, bold type indicates intergenomic heterogeneity, italic type indicates an intragenomic transition CT or AG, and Roman type (no bold or italic) indicates an intragenomic transversion GT.

TTGE band*	Variable nucleotide position (<i>E. coli</i> numbering)																																	TTGE pattern with band†	<i>rpoB</i> -based clade or species	Secondary structure type
	415	418	425	444	446	449	453	455	456	457	458	459	460	461	462	463	464	465	468	470	472	473	474	475	476	477	478	479	480	492						
1‡	C	C	G	C	G	C	A	G	C	C	T	T	C	T	T	G	C	G	T	G	T	A	G	A	A	G	G	A	T	G	1, <u>1+2</u> , 1+4, 1+7, 1+7a, 1+36, 1+8, 1+10, <u>1+2+4</u> , <u>1+9a+2</u> , <u>1+2+9</u> , <u>1+3a+9</u> , <u>1+4+9</u>	1, 2, 3, 4 <i>V. denticariosi</i>	S1			
8‡	–	–	–	–	–	–	–	–	<i>T</i>	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>A</i>	–	–	–	8, 1+8, 7b+8, 8+10, <u>2+4+8</u> , 7b+8+10	1, 2, 3	S1			
10a	–	<i>T</i>	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<u>10a+2</u>	1	S1			
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>C</i>	–	–	–	–	–	–	–	–	–	–	–	4, 1+4, <u>2+4</u> , <u>1+2+4</u> , <u>1+4+9</u> , <u>2+4+8</u> , <u>2+4+9</u>	1	S1			
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>G</i>	–	–	–	–	–	–	–	–	–	2, <u>1+2</u> , <u>10a+2</u> , <u>2+4</u> , 2+9, <u>1+2+4</u> , <u>1+9a+2</u> , <u>1+2+9</u> , <u>2+4+8</u> , <u>2+4+9</u>	1	S2			
9a	–	–	–	–	<i>A</i>	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>G</i>	–	–	–	–	–	–	–	–	–	<u>1+9a+2</u>	1	S3			
9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>A</i>	–	–	<i>G</i>	–	–	–	–	–	–	–	–	–	2+9, <u>1+2+9</u> , <u>1+3a+9</u> , <u>1+4+9</u> , <u>2+4+9</u>	1	S2			
3a	–	–	–	–	–	–	–	–	–	–	–	–	<i>T</i>	–	–	–	–	–	–	–	<i>G</i>	–	–	–	–	–	–	–	–	–	<u>1+3a+9</u>	1	S2			
7b	–	–	–	–	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>G</i>	–	<i>A</i>	–	–	–	7b+8, 7b+8+10	2	S1			
10	–	–	–	–	–	–	–	–	<i>T</i>	–	<i>C</i>	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>G</i>	–	<i>A</i>	–	–	–	10, 1+10, 8+10, 7b+8+10	2	S1			
7	–	–	–	–	–	–	–	–	<i>T</i>	–	<i>C</i>	–	–	–	–	–	–	<i>A</i>	–	–	–	–	–	–	<i>G</i>	–	<i>A</i>	–	–	–	1+7	4	S1			
36	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>A</i>	1+36	4	S1			
7a	–	–	–	<i>T</i>	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1+7a	3	S1			
33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>G</i>	–	–	–	–	–	–	1+33, 32+33	<i>V. denticariosi</i>	S1			
32	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	<i>A</i>	–	–	–	–	–	G	–	–	–	–	–	–	32+33	<i>V. denticariosi</i>	S1			
23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G	–	–	–	–	–	–	–	–	–	23	<i>V. rodentium</i>	S1			
21	–	–	–	–	–	–	–	–	T	–	C	–	–	–	–	–	T	–	–	A	–	–	–	–	G	–	<i>A</i>	–	–	–	<u>21+22</u>	<i>V. caviae</i>	S1			
22	–	–	–	–	–	–	–	–	T	–	C	–	–	–	–	–	T	–	–	A	–	–	–	–	G	–	–	–	–	–	<u>21+22</u>	<i>V. caviae</i>	S7			

Table 1. cont.

TTGE band*	Variable nucleotide position (<i>E. coli</i> numbering)																														TTGE pattern with band†	<i>rpoB</i> -based clade or species	Secondary structure type	
	415	418	425	444	446	449	453	455	456	457	458	459	460	461	462	463	464	465	468	470	472	473	474	475	476	477	478	479	480	492				
6	-	-	-	-	-	-	T	-	T	T	C	-	T	G	-	-	-	-	-	-	G	C	-	-	G	-	A	T	-	-	6, 5+6, 6+35, 6+14, 3+5+6, 5+6+11, 5+6+12, 5+6+14	5	S4	
35	-	-	-	-	-	-	T	-	T	T	C	-	T	G	-	-	-	-	-	-	G	C	-	-	G	-	A	C	-	-	6+35	5	S4	
5	-	-	-	-	-	-	T	-	T	T	C	-	T	G	-	-	-	A	-	-	G	C	-	-	G	-	A	T	-	-	3+5, 5+6, <u>5+13</u> , 3+5+6, 5+6+11, 5+6+12, 5+6+14	5	S4	
3	-	-	-	-	-	-	T	-	T	T	C	-	T	G	-	-	-	A	-	-	G	C	A	-	G	-	A	T	-	-	3+5, 3+5+6	5	S4	
11	-	-	-	-	-	-	T	-	T	T	C	-	T	G	-	-	-	-	-	-	G	C	A	-	G	-	A	T	-	-	5+6+11	5	S4	
12	-	-	-	-	-	-	T	-	T	T	C	-	T	G	-	-	-	-	-	-	G	C	-	-	G	A	A	T	-	-	5+6+12	5	S4	
13	-	-	-	-	-	-	T	-	T	T	C	-	T	G	-	A	-	A	-	-	G	C	-	-	G	A	A	T	-	-	<u>5+13</u>	5	S10	
14	-	-	-	-	-	-	T	-	T	T	C	-	T	G	-	-	T	-	-	-	G	C	-	-	G	-	A	T	-	-	6+14, 5+6+14	5	S4	
15	-	-	-	-	-	-	T	-	T	-	-	-	T	A	-	-	T	-	-	A	G	T	A	-	-	-	A	T	-	-	15+17, 15+16+17	7	S10	
16	-	-	-	-	-	-	T	-	T	-	-	-	T	G	-	-	T	A	-	A	G	C	A	-	-	-	A	T	-	-	16+17, 15+16+17	7	S10	
17	-	-	-	-	-	-	T	-	T	-	-	-	T	G	-	-	T	-	-	A	G	C	A	-	-	-	A	T	-	-	15+17, 16+17, 17+18, 15+16+17	7	S10	
18	-	-	-	-	-	-	T	-	T	-	-	-	T	G	-	-	T	-	-	A	G	C	-	-	-	-	A	T	-	-	17+18	7	S10	
19	-	-	-	-	-	-	T	-	T	-	-	-	T	G	-	-	-	-	-	-	G	C	A	-	G	-	A	T	-	-	19+20	7	S4	
20	-	-	-	-	-	-	T	-	T	-	-	-	T	G	-	-	-	-	-	-	G	C	A	G	-	-	A	T	-	-	19+20	7	S4	
34	-	T	A	-	-	-	T	-	T	-	G	G	T	G	-	-	-	A	-	-	G	C	A	T	G	-	A	C	A	-	-	34, <u>1a+34</u> , <u>1a+5a+34</u>	6	S6
5a	-	T	A	-	-	-	T	-	T	-	-	G	T	G	-	-	-	A	-	-	G	C	A	T	-	-	A	C	A	-	-	<u>1a+5a+34</u>	6	S5
1a	-	T	A	-	-	-	T	-	T	T	-	G	T	G	-	-	-	A	-	-	G	C	A	T	-	-	A	C	A	-	-	<u>1a+5a+34</u>	6	S5
31	-	-	-	-	-	-	-	A	G	-	C	-	T	G	-	-	T	-	-	A	G	C	-	-	G	-	A	-	G	-	-	31	<i>V. magna</i>	S8
24	A	T	A	-	-	-	-	A	G	T	-	C	T	G	-	-	T	A	-	A	G	C	A	G	-	A	A	-	G	-	-	24+25, 24+26, 24+28	8	S8
25	A	T	A	-	-	-	-	A	G	T	-	C	T	G	-	-	T	-	-	A	G	C	A	G	-	A	A	-	G	-	-	25, 24+25, <u>25+26+27</u>	8	S8
26	A	T	A	-	-	-	-	A	G	T	-	C	T	G	C	-	T	-	-	A	G	C	A	G	-	A	A	-	G	-	-	24+26, <u>25+26+27</u>	8	S8
27	A	T	A	-	A	-	-	A	G	T	-	C	T	G	-	-	T	A	-	A	G	C	A	G	-	A	A	-	G	-	-	<u>25+26+27</u>	8	S9
28	A	T	A	-	-	-	-	A	G	T	-	C	T	G	-	-	T	A	-	A	G	C	A	G	G	A	A	-	G	-	-	24+28	8	S8
29	A	T	A	-	-	T	-	A	G	T	-	C	T	G	-	-	T	-	-	A	G	C	A	G	-	A	A	-	G	-	-	29+30	8	S8
30	A	T	A	-	-	-	-	-	G	T	-	C	T	G	-	-	T	A	-	A	G	C	A	G	-	A	A	-	G	-	-	29+30	8	S8

*TTGE bands differing by their migration distance are numbered; bands identified by sequencing only are indicated by a and b.

†TTGE patterns, including bands with different secondary structures are underlined.

‡Bands were found in TTGE patterns from isolates belonging to several *rpoB*-based clades or several *Veillonella* species.

Table 2. V3 16S rDNA PCR-TTGE results for isolates and strains of *Veillonella* spp. of the eight *rpoB*-based clades

Bands not present in the TTGE patterns of strains and isolates belonging to different *rpoB*-based clades are in bold type; bands shared by the TTGE patterns of strains or isolates belonging to different *rpoB*-based clades are underlined.

<i>rpoB</i> -based clade (no. of isolates, %)	Clade's type and reference strain (TTGE pattern)	Isolates with TTGE heterogeneity profile no. (%)	Intragenomic variable position no. (% of isolates)	Main TTGE bands (%)*	Main TTGE patterns (%)*	Other TTGE bands (%)*
Clade 1 (72, 48.3)	<i>V. parvula</i> ATCC 10790 ^T (4); <i>V. parvula</i> CIP 60.1 (1 + 4)	50 (69.4)	0 (30.6); 1 (29.2); 2 (33.3); 3 (5.5); 4 (1.4)	2 (94.4); 4 (37.5); <u>1</u> (34.7)	2 (27.7); 1 + 2 (26.4); 2 + 4 (26.4)	9 (9.7); 10a, 9a, 3a , <u>8</u> (1.4 each)
Clade 2 (21, 14.1)	<i>V. rogosae</i> DSM 18960 ^T (8 + 10)	18 (85.7)	0 (14.3); 1 (4.75); 2 (76.2); 4 (4.75)	10 (90.5); <u>8</u> (85.7)	8 + 10 (61.9)	7b (19); <u>1</u> (5)
Clade 3 (10, 6.7)	<i>V. dispar</i> ATCC 17748 ^T (1)	2 (20)	0 (80); 1 (10); 2 (10)	<u>1</u> (90)	1 (70)	<u>8</u> (20); 7a (10)
Clade 4 (2, 1.4)	–	2 (100)	1 (50); 5 (50)	<u>1</u> (100)	7 + 1 (50); 36 + 1 (50)	7, 36 (50 each)
Clade 5 (30, 20.1)	<i>V. atypica</i> ATCC 17744 ^T (6)	27 (90)	0 (10); 1 (50); 2 (40)	6 (93.3); 5 (83.3)	5 + 6 (36.6)	11 (16.7); 3 (13.3); 14 (10); 12 (6.7); 35 (3.3); 13 (3.3)
Clade 6 (3, 2)	<i>V. montpellierensis</i> CIP 107992 ^T (1a + 5a + 34)	2 (66.7)	0 (33.3); 3 (66.7)	34 (100); 1a (66.7)	1a + 34 (66.7)	–
Clade 7 (4, 2.7)	<i>V. ratti</i> ATCC 17746 ^T (17 + 18)	4 (100)	1 (25); 2 (25); 3 (50)	17 (100); 15 (75); 16 (75)	15 + 16 + 17 (50)	–
Clade 8 (7, 4.7)	–	5 (71.4)	0 (28.6); 1 (28.6); 2 (14.2); 3 (28.6)	25 (57.1); 24 (42.9)	25 (28.6)	26 (28.6); 27, 28 , 29, 30 (14.3 each)

*TTGE bands and TTGE patterns are described in Methods.

interclade levels were referred to as comparisons at intra- and interspecific levels, respectively.

Comparative analysis of *rpoB*-based bacterial population structure and TTGE band patterns

Inter- and intraspecific V3 heterogeneities were analysed with respect to *rpoB* population structure. To this end, we used a decomposition analysis based on the *rpoB* clade and TTGE banding profiles. Congruence between *rpoB*-based clade/species type and *rrs* V3 region type would lead to a

radial structure of the graph. Dissociation between *rpoB*-based clade/species type and *rrs* V3 region type suggestive of recombination events would lead to a network-like graph. On the split tree (Fig. 2), two groups of strains and isolates were observed. Strains and isolates belonging to clades 5–8 together with *V. rodentium*, *V. caviae*, *V. criceti* and *V. magna* type strains formed the first group, known hereafter as group A. They were organized in radial branches without network-like links among branches supporting clades and species. Network-like links were observed inside each clade. The split tree suggested that

Table 3. V3 16S rDNA PCR-TTGE results of species for which type and reference strains did not group with clinical isolates in the *rpoB*-based phylogenetic tree

Species	Type or reference strain	TTGE profile*	No. of intragenomic variable positions
<i>V. denticariosi</i>	<i>V. denticariosi</i> CIP 109448 ^T	32 + 33	1
	<i>V. denticariosi</i> CIP 109449	<u>1</u> + 33	1
<i>V. rodentium</i>	<i>V. rodentium</i> ATCC 17743 ^T	23	0
<i>V. caviae</i>	<i>V. caviae</i> DSM 20738 ^T	21 + 22	1
<i>V. criceti</i>	<i>V. criceti</i> DSM 20734 ^T	19 + 20	2
<i>V. magna</i>	<i>V. magna</i> DSM 19857 ^T	31	0

*TTGE profiles are described in Methods; TTGE band shared by TTGE patterns of strains or isolates belonging to different *rpoB*-based clades in Table 2 are underlined.

Fig. 2. SplitsTree decomposition analyses of PCR-TTGE and *rpoB*-based clustering data for clinical isolates and type and reference strains of the genus *Veillonella*. PCR-TTGE patterns are indicated at the extremity of each tree branch with the number of clinical isolates and/or name of reference or type strain displaying the corresponding pattern indicated in parentheses. *rpoB*-based clades of clinical isolates are indicated by the clade's number and are delimited by dotted lines.

recombination events occurred inside clades but each clade and species appeared genetically isolated from each other. TTGE bands were clade-specific in this group (Tables 2 and 3). High V3 region polymorphism (6–17 different nucleotides) was noted between group A members; in particular interspecific V3 polymorphism was always greater than intraspecific and intragenomic V3 heterogeneity among each of clades 5, 6, 7 and 8.

Strains and isolates belonging to clades 1–4 and to *V. denticariosi* formed a second group named group B. A network-like structure was observed on the split tree among group B strains (Fig. 2). Genetic recombination was observed inside each clade and also between strains and isolates belonging to different clades/species. Some TTGE bands were shared among clades of group B, such as band 1 among clades 1–4 and band 8 among clades 1–3 (Table 2).

For example, a copy of the V3 region observed in 90 % of *V. dispar* group isolates was present in 34.7 % of the *V. parvula* group isolates, and was also observed in the reference strain *V. denticariosi* CIP 109449. Similarly, a V3 region copy found in 85 % of members of the *V. rogosae* clade was identical to a V3 region copy found in 20 % of *V. dispar* group isolates. These observations suggested that horizontal gene transfer of *rrn* might occur between these species. At the interspecific level, a low polymorphism (1–6 divergent positions) was observed among clades and species of group B (data not shown). In these cases, intragenomic microheterogeneity and intraspecific *rrs* polymorphism levels may be higher than interspecific polymorphism. Finally, high V3 region polymorphism was noted between groups A and B and recombination did not occur between these two groups. We compared the tree in Fig. 2 with a split tree constructed on the basis of *rpoB* clade/species type, *rrs* V3 region type and 2D structure type of the V3 region (D2V3) (data not shown). On the basis of this comparison and of data in Table 1, D2V3 appeared to be shared inside clades, particularly in clades 5 and 8, independently of the V3 region sequence type. D2V3 was also shared between clades/species that did not experience V3 exchanges or recombination. This was particularly notable between clade 5 and *V. criceti*, between clade 8 and *V. magna*, between *V. rodentium* and group B representatives, and finally between clade 1 and *V. caviae* (Table 1). This suggested that the same secondary structure based on different sequences could appear independent from genetic exchanges in different lineages.

DISCUSSION

Intragenomic heterogeneity between 16S rRNA gene copies has been described for a range of bacteria. Therefore, evaluating the extent of these heterogeneities in natural populations is essential for determining how they impair the results of 16S rDNA-based identification, taxonomy and metagenomics (Dahllöf *et al.*, 2000). Operon heterogeneity may be detected by sequencing chromatogram examination revealing double peaks or by unexpected RFLP patterns of amplified rDNA but it could not be further investigated using these approaches (Marchandin *et al.*, 2003). Visualization of heteroduplexes after 16S rDNA PCR amplification and PAGE was also reported to be indicative of intragenomic polymorphism; it is suitable for easy testing of large numbers of strains but a cloning step remains necessary for a precise study of microheterogeneity (Moreno *et al.*, 2002). Cloning experiments involving sequencing of numerous clones for each isolate, complete genome sequencing and PFGE (Marchandin *et al.*, 2003) allowed the study of a limited number of strains and are not appropriate for population studies (Marchandin *et al.*, 2003; Conville & Witebsky, 2007). PCR-TTGE is based on electrophoretic separation of amplicons with the same length but different sequences by using a temperature gradient to denature the DNA. It

was previously optimized to reveal differences in the 16S rDNA V3 regions of bacteria (Roudière *et al.*, 2009), each sequence variation producing a distinctive band migration distance. PCR-TTGE was previously demonstrated to be suitable for detection of divergence in 16S rRNA genes in four *Paenibacillus polymyxa* strains (Nübel *et al.*, 1996) but has remained very rarely applied to bacterial populations since the study by Nübel and colleagues.

We used PCR-TTGE to describe 16S rRNA gene intragenomic, intraspecific and interspecific diversity in a natural population of clinical isolates of *Veillonella* species. We showed that PCR-TTGE was an accurate, sensitive, powerful and rapid method for revealing *rrs* sequence heterogeneities in a large population. Exhaustive detection of the different *rrs* sequences represented in the population is easier than using cloning experiments, which require analysis of a statistically significant number of clones to determine the number of different *rrs* copies. The combined approach associating comparisons of band migration distances with a specifically designed ladder and sequencing from selected excised bands made this method inexpensive compared with other methods.

High occurrence (74 %) of intragenomic heterogeneity was demonstrated for the 149 studied clinical isolates, which is in accordance with previous PCR-RFLP-based results (Marchandin *et al.*, 2003). Although the H17 helix constituted one of the more variable *rrs* regions in the genus *Veillonella* (Marchandin *et al.*, 2003) and in 111 genomes studied by Case *et al.* (2007), intragenomic microheterogeneity may concern an even higher rate of isolates if the complete gene was studied (Coenye & Vandamme, 2003). Analysis of sequencing patterns for 475 representatives of the genus *Streptomyces* and RFLP-based analysis conducted on 82 *Aeromonas* strains revealed the presence of different *rrs* copies in 21 and 6.9 % of strains, respectively (Ueda *et al.*, 1999; Graf, 1999). Moreno *et al.* (2002) studied four type strains and 20 environmental isolates of *Vibrio* spp. by PCR-PAGE, which revealed 16S rDNA polymorphism for all strains. Thus, the genus *Veillonella* displayed one of the highest occurrences of 16S rRNA gene intragenomic heterogeneity described so far.

Among the two types of operon heterogeneity currently described in bacterial genomes, i.e. microheterogeneity and macroheterogeneity (Teyssier *et al.*, 2003), microheterogeneity mostly accounted for 16S rRNA molecule heterogeneity (Acinas *et al.*, 2004), as previously reported for *Veillonella* sp. strain ADV360.1 (Marchandin *et al.*, 2003) and several other taxa (Cilia *et al.*, 1996; Martínez-Murcia *et al.*, 1999; Ninet *et al.*, 1996; Nübel *et al.*, 1996; Yap *et al.*, 1999). Acinas *et al.* (2004) noted divergence between *rrs* copies in 62 % of the 76 bacterial genomes, which possessed at least two copies of 16S rDNA. Four *rrn* operons were present in strains of the genus *Veillonella* analysed so far (Marchandin *et al.*, 2003; data not shown). Heterogeneity occurred at between 0 and 5 positions (0–2.5 %) of the variable region V3 and the average percentage

of nucleotide divergence was 0.89 %. Although we did not review here the 87 sequenced genomes containing four *rrn* operons currently referenced in *rrnDB* (<http://ribosome.mmg.msu.edu/rrndb/>), this value seemed particularly high when compared with results previously published for most genomes with four *rrs* copies (Coenye & Vandamme, 2003; Acinas *et al.*, 2004). Except for *Thermobispora bispora* and *Thermoanaerobacter tengcongensis*, which display 6.4 and 6.5 % *rrs* microheterogeneity, respectively, the 11 other examined genomes showed a mean divergence of 0.025 % between copies (range, 0–0.07 %). Among variable positions, transitions were the dominant type of mutation, as previously reported (Coenye & Vandamme, 2003), suggesting that divergence of *rrs* copies may occur by mutation after gene duplication (Yap *et al.*, 1999). Transversions were observed for 48 isolates belonging to *V. parvula* clade 1 and for the three representatives of *V. montpellierensis* clade 6. In some cases, substitutions change the secondary structure of helix H17 and may thus impact ribosomal function, since H17 plays a major role in the first steps of the 3D folding (Adilakshmi *et al.*, 2005). At the interspecies level, the same secondary structures were observed among different clades. These common secondary structures derived from different sequences indicated they were not the result of recombination events but rather suggested convergent evolution of some *rrs*.

Among the housekeeping genes previously studied in the genus *Veillonella*, *dnaK*, *gyrB* and *rpoB* were demonstrated as more discriminatory between species than 16S rRNA gene, with *rpoB* being the most discriminatory. Indeed, several species, such as *V. parvula* and *V. dispar* (Marchandin *et al.*, 2003), *V. denticariosi* and *V. rodentium* (Byun *et al.*, 2007), or *V. ratti* and *V. criceti* (Marchandin *et al.*, 2005), differed by only 1 % of their 16S rRNA gene nucleotide positions. Several other species, such as *V. rogosae* and *V. parvula* (Arif *et al.*, 2008), as well as *V. atypica* and *V. dispar* or *V. parvula* (Marchandin *et al.*, 2003), displayed no more than 2 % of *rrs* sequence divergence. Housekeeping genes, considered as single-copy genes, are thought to overcome limitations due to 16S rRNA gene heterogeneity (Dahlöf *et al.*, 2000). Previously used for taxonomic purposes in the genus *Veillonella*, the *rpoB* gene was used to structure the population of *Veillonella* spp.; isolates were affiliated to taxonomically relevant clades according to the *rpoB*-based phylogeny. Intragenomic 16S rRNA gene V3 region heterogeneity was observed in all the *rpoB* clades, with heterogeneity levels of ≤ 1 % observed for *V. dispar* and *V. atypica* clades and heterogeneity levels ranging from >1 to 2.5 % reported for the six other clades. Thus, intragenomic (0.5–2.5 %) and intraspecific (0.5–3 %) V3 heterogeneity may be as high as interspecific divergence (1–7.5 %) of the whole 16S rRNA gene and may impair 16S rDNA-based identification of closely related species in the genus *Veillonella*. As previously described for *Veillonella* sp. strain ADV 360.1, which shows 1.43 % *rrs* heterogeneity, or for *Aeromonas veronii* strain LMG13695, which shows 1.52 % divergence

between copies, such high levels of intragenomic heterogeneity may also confuse phylogeny (Marchandin *et al.*, 2003; Morandi *et al.*, 2005). Finally, high levels of intragenomic heterogeneity raise the problem of the bacterial species delineation. Stackebrandt & Ebers (2006) have recently proposed to raise the threshold of 97 % of *rrs* identity previously retained for species delineation (Stackebrandt & Goebel, 1994) to 98.7 % (Stackebrandt & Ebers, 2006). For an *rrs* heterogeneity level >1.3 %, the existence of novel taxa could be erroneously suggested. Intragenomic 16S rRNA gene heterogeneities could also affect diversity studies, leading to an overestimation of the number of different clones in studied samples. PCR-TTGE used for studying the bacterial diversity in different communities (Muyzer, 1999; Roudière *et al.*, 2009) could also be used for preliminary testing of known important bacteria of the community in order to avoid diversity overestimation due to *rrs* heterogeneity or to artefactual bands, with the aim to propose appropriate correction factors for diversity studies.

Heterogeneity between *rrs* copies in the genus *Veillonella* may be attributed to mutations after gene duplication or to horizontal *rrn* transfer (Yap *et al.*, 1999; van Berkum *et al.*, 2003), followed by a lack of intragenomic concerted evolution (Santoyo & Romero, 2005). Rupture in concerted evolution could occur if heterogeneity conferred a selective advantage to the bacteria. These heterogeneities previously demonstrated for other bacteria belonging to human microflora (Satokari *et al.*, 2001; Vásquez *et al.*, 2001; Roudière *et al.*, 2007) could have a function in the adaptation to the environment of these bacteria and in maintaining their diversity in human or other mammal microflora (López-López *et al.*, 2007; Jensen *et al.*, 2009).

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