Detection of three different types of '*Tropheryma whippelii*' directly from clinical specimens by sequencing, single-strand conformation polymorphism (SSCP) analysis and type-specific PCR of their 16S–23S ribosomal intergenic spacer region

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The 16S–23S rDNA intergenic spacer region of organisms identical with or closely related to '*Tropheryma whippelii*', the uncultivated causative agent of Whipple's disease, was analysed directly from 38 clinical specimens of 28 patients using a specific nested PCR followed by direct sequencing. As compared to the reference sequence in public databases, two novel '*T. whippelii*' spacer types were recognized. In the absence of DNA–DNA hybridization data it is uncertain whether the three types found represent subtypes of a single species or three different but closely related species. Methods were developed to detect all three variants by single-strand conformation polymorphism analysis and by type-specific PCR assays, thus allowing the screening of large numbers of specimens. Further studies may provide a clue to the possible associations between the type of infecting strain and the various clinical presentations of Whipple's disease.

Keywords: Whipple's disease, '*Tropheryma whippelii*' typing, 16S–23S ribosomal intergenic spacer region, sequence analysis, single-strand conformation polymorphism

INTRODUCTION

Comparative sequence analysis of the 16S rRNA gene (16S rDNA) allowed the hitherto uncultivated presumptive agent of Whipple's disease to be identified as an actinomycete bacterium constituting the novel but not yet validated taxon '*Tropheryma whippelii*' (Wilson *et al.*, 1991; Relman *et al.*, 1992). Further, the sequence of the intergenic spacer region between the 16S and the 23S rDNA as well as the 5' end of the 23S rDNA have also been determined (Maiwald *et al.*, 1996).

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Abbreviation: SSCP, single-strand conformation polymorphism.

The GenBank accession numbers for the sequences of the '*T. whippelii*' 16S–23S rDNA spacer types 2 and 3 are AF100950 and AF100951, respectively (type 1 represents the original '*T. whippelii*' spacer sequence as deposited previously in GenBank under accession no. X99636).

Several diagnostic '*T. whippelii*'-PCRs that target parts of the 16S rDNA have been established (Relman *et al.*, 1992; Rickman *et al.*, 1995; Altwegg *et al.*, 1996; Dauga *et al.*, 1997). However, such systems may not discriminate between closely related species as illustrated by identical or almost identical 16S rRNA gene sequences of *Mycobacterium kansasii* and *Mycobacterium gastri*, *Mycobacterium mageritense* and *Mycobacterium szulgai*, or *Aeromonas trota* and *Aeromonas caviae*, respectively (Martínez-Murcia *et al.*, 1992; Roth *et al.*, 1998). Since the intergenic spacer is more variable than the flanking structural genes it has been widely used for species identification and particularly for subtyping purposes in many other bacterial groups (Gürtler & Stanisich, 1996). We have designed a nested PCR for the detection of the '*T. whippelii*' 16S–23S rDNA spacer region directly in clinical specimens. Sequencing of the resulting PCR products from nine independent Swiss patients with Whipple's disease revealed no spacer polymorphism at all (Hinrikson *et*

Table 1. Source and molecular characterization of the clinical specimens used for genotyping of '*T. whippelii*'

Symbols and abbreviations: + and -, positive and negative result; 1 to 3, '*T. whippelii*' spacer types 1 to 3, respectively; NT, not tested.

Patient no.	Specimen		16S rDNA PCR*	16S-23S rDNA spacer							
	Origin	Code		PCR†	Sequencing‡	SSCP	Type-specific PCRs§				
							A	B	C	D	E
1	Duodenum	5713/96	+	+	1	1	+	—	+	—	—
2	Heart valve	724/98	+	+	1	NT	+	—	+	—	—
	Heart valve¶	838/98	+	+	1	NT	+	—	+	—	—
3	Gastric aspirate	1124/97	+ #	+	1	NT	+	—	+	—	—
	Duodenum	1125/97	+ #	+	1	NT	+	—	+	—	—
	Duodenum	1125II/97	+	+	NT	1	+	—	+	—	—
4	Gastric aspirate	616/97	+	+	1	NT	+	—	+	—	—
	Gastric aspirate	616II/97	+	+	1	1	+	—	+	—	—
5	Small intestine	368/97	+ #	+	1	NT	+	—	+	—	—
6	Cerebrospinal fluid	1613/97	+	+	1	1	+	—	+	—	—
	Cerebrospinal fluid	1615/97	+	+	1	1	+	—	+	—	—
7	Duodenum¶	459-98	+ **	+	1	1	+	—	+	—	—
8	Duodenum¶	836-96	+ **	+	1	1	+	—	+	—	—
9	Duodenum¶	4549-94	+ **	+	1	1	+	—	+	—	—
10	Duodenum	725/97	+ #	+	1	1	+	—	+	—	—
11	Ileum	168/97	+ #	+	1	1	+	—	+	—	—
12	Duodenum	1908/96	+	+	1	NT	+	—	+	—	—
13	Synovial fluid	5794/96	+	+	1	NT	+	—	+	—	—
	Synovial fluid	5831/96	+	+	NT	1	+	—	+	—	—
14	Cerebrospinal fluid	141/97	+ #	+	1	NT	+	—	+	—	—
15	Heart valve	214/96	+	+	1	1	+	—	+	—	—
16	Gastric aspirate	895/97	+	+	2	2	—	+	—	+	—
17	Synovial fluid	749/98	+	+	NT	2	—	+	—	+	—
	Intraoperative swab	875/98	+	+	NT	2	—	+	—	+	—
18	Ileum	2249/96	+	+	2	2	—	+	—	+	—
19	Small intestine	5996/96	+ #	+	2	2	—	+	—	+	—
20	Duodenum	604/97	+ #	+	NT	2	—	+	—	+	—
21	Gastric aspirate	1037/97	+ #	+	2	2	—	+	—	+	—
22	Intervertebral disc	3994/95	+	+	NT	2	—	+	—	+	—
23	Heart valve	271/98	+	+	2	2	—	+	—	+	—
24	Duodenum	771/97	+ #	+	2	2	—	+	—	+	—
	Ileum	906/97	+ #	+	2	2	—	+	—	+	—
25	Heart valve	5194/95	+	+	2	2	—	+	—	+	—
26	Duodenum	530/97	+ #	+	3	NT	—	+	—	—	+
	Gastric aspirate	532/97	+ #	+	3	3	—	+	—	—	+
	Gastric aspirate	952/97	+ #	+	3	NT	—	+	—	—	+
27	Duodenum	220/97	+ #	+	NT	3	—	+	—	—	+
28	Gastric aspirate	672/97	+ #	+	NT	3	—	+	—	—	+

* '*T. whippelii*'-specific amplification with primers TW-1 & TW-2 (Altwegg *et al.*, 1996).

† '*T. whippelii*'-specific nested PCR using primer pairs tws1 & tws2 followed by tws3 & tws4.

‡ Analysis of products derived from '*T. whippelii*'-specific nested PCR using primer pairs tws1 & tws2 followed by tws3 & tws4.

§ Primer combinations: A, twsA1 & twsB1; B, twsA2 & twsB2; C, twsA1 & twsC1; D, twsA2 & twsC1; E, twsA2 & twsC2.

|| Confirmed by sequencing of products derived from broad-range (universal eubacterial) amplification (Goldenberger *et al.*, 1997).

¶ Paraffin-embedded tissue.

Confirmed by sequencing of products derived from '*T. whippelii*'-specific semi-nested PCR with primer pairs TW-1 & TW-2 followed by TW-4 & TW-2 (Brändle *et al.*, 1999).

** Confirmed by sequencing of products derived from '*T. whippelii*'-specific amplification with primers analogous to TW-1 & TW-2.

al., 1999). Nonetheless, specimens from additional patients were investigated using the same approach.

In the present study, we describe two new 16S-23S rDNA spacer types of '*T. whippelii*' or closely related organisms. In addition, two new approaches, single-strand conformation polymorphism (SSCP) analysis and type-specific PCR assays, were developed that are much easier to perform than sequencing and thus allow the screening of large numbers of specimens.

METHODS

Specimens. A total of 38 clinical specimens from 28 patients shown to contain '*T. whippelii*' 16S rDNA by semi-nested PCR using primer pairs TW-1 & TW-2 followed by TW-4 & TW-2 (Brändle *et al.*, 1999) were investigated (Table 1). They included cerebrospinal fluids ($n = 3$), heart valves ($n = 5$), a biopsy of an intervertebral disc, synovial fluids ($n = 3$), intestinal biopsies ($n = 17$), gastric aspirates ($n = 8$), and an intraoperative swab from a joint. In 19 specimens representing 13 patients the identity of 16S rDNA amplicons (229 bp) had previously been confirmed by direct sequencing. For an additional eight specimens from eight patients comparable results had been obtained by broad-range (universal eubacterial) amplification and sequencing of a ± 530 bp fragment (Goldenberger *et al.*, 1997). No sequence data were available for the remaining 11 samples. Negative controls consisted of intestinal biopsies ($n = 23$), a bowel aspirate, and gastric aspirates ($n = 17$) that were negative for '*T. whippelii*' by semi-nested PCR with the above primer pairs.

Extraction of DNA. Cerebrospinal fluids, synovial fluids, gastric aspirates, the 0.85% NaCl suspension (2 ml) of the intraoperative swab, and the bowel aspirate were centrifuged for 10 min at 14000 *g*. These pellets as well as the biopsies were suspended in digestion buffer (50 mM Tris/HCl, pH 8.5, 1 mM EDTA, 0.5% SDS, 200 μ g proteinase K ml⁻¹) and incubated at 55 °C for 1 h 30 min with agitation. DNA was

extracted with QIAamp DNA-binding columns (QIAGEN) according to the manufacturer's protocol except for the final step using only 100 μ l (instead of 200 μ l) of elution buffer AE. Five microlitres of the eluate were used for PCR.

PCR assays. Primers used for amplification and/or nested reamplification are listed in Table 2. Amplifications were done in a final volume of 50 μ l containing 200 μ M each deoxynucleotide, 2.5 U AmpliTaq Gold polymerase with the appropriate amount of its optimized buffer (Perkin-Elmer), 25 pmol each primer, 2% (v/v) Tween 20, and 5 μ l DNA extract. Reamplification was identical, except that 1–5 μ l of amplicon was used as template and Tween 20 was omitted. Each PCR run included molecular-grade H₂O (LAL reagent water; BioWhittaker Europe, Belgium) as negative control. All PCRs were performed on a GeneAmp PCR System 9600 (Perkin-Elmer) and started at 95 °C for 12 min. Amplifications and reamplifications with primer pairs tws1 & tws2 and tws3 & tws4 were done for 40 cycles at 95 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 1 min. Reamplifications with primer combinations (i) twsA1 & twsB1 (system A, targeting spacer type 1), (ii) twsA2 & twsB2 (system B, types 2 and 3), (iii) twsA1 & twsC1 (system C, type 1), (iv) twsA2 & twsC1 (system D, type 2), and (v) twsA2 & twsC2 (system E, type 3) were performed using 20 cycles at 95 °C for 1 min, at 70 °C for 1 min, and at 72 °C for 1 min. All PCRs ended at 72 °C for 10 min. PCR products (10 μ l) were separated by electrophoresis on a 2% (w/v) agarose gel, stained with ethidium bromide, and detected under UV light.

Sequence analysis. Direct sequencing of products derived from nested PCR with primer pairs tws1 & tws2 followed by tws3 & tws4 was performed on an ALFexpress DNA Sequencer (Pharmacia Biotech). Amplicons were purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. The cycle sequencing reaction was performed in both directions with the 5'-fluorescence-labelled primers tws3 or tws4, respectively, and the Thermo Sequenase Kit with 7-deaza-dGTP (Amersham). The cycling conditions were the same as described for the PCR assays except for an initial denaturation at 95 °C for only 5 min. Data analysis included

Table 2. Oligonucleotides used to analyse the 16S-23S rDNA intergenic spacer region polymorphisms of '*T. whippelii*'

Primer* /sense†	Sequence (5' → 3')	Position‡	'T. whippelii' target	
			rDNA	Spacer type(s)
tws1/ f	ATCGCAAGGTGGAGCGAATCT	1213 → 1233	3' end of 16S	1, 2 and 3
tws2/r	CGCATTCTGGCGCCCCAC	1940 → 1923	5' end of 23S	1, 2 and 3
tws3/f	CCGGTGACTTAACCTTTTGGAGA	1387 → 1410	3' end of 16S	1, 2 and 3
tws4/r	TCCCGAGGCTTATCGCAGATTG	1875 → 1854	5' end of 23S	1, 2 and 3
twsA1/f	AAGTGATACCGCCATAGTGCCTGT	1527 → 1551	16S-23S spacer	1
twsA2/f	AAGTGATACCGCCATAGTGCCTGC	1527 → 1551	16S-23S spacer	2 and 3
twsB1/r	CTCCCGTGAGCTTGTGCCAAAAC	1600 → 1577	16S-23S spacer	1
twsB2/r	CTCCCGTGAGCTTGTGCCAAAAC	1600 → 1577	16S-23S spacer	2 and 3
twsC1/r	AATAGTGACACAAAGTGCATAAGCA	1667 → 1643	16S-23S spacer	1 and 2
twsC2/r	AATAGTGACACAAAGCGCATAAGCG	1667 → 1643	16S-23S spacer	3

* This study except tws1, tws2, tws3 and tws4 (Hinrikson *et al.*, 1999).

† f, forward; r, reverse.

‡ According to '*T. whippelii*' sequence, GenBank accession no. X99636 (Maiwald *et al.*, 1996).

the softwares ALFwin version 1.10 (Pharmacia Biotech) and the Genetic Computer Group (GCG) package (University of Wisconsin, Madison, USA) for comparison with entries in the GenBank/EMBL databases.

HaeIII-restriction and SSCP analysis. The same QIAGEN-purified amplicons (1–3 µl) as used for sequencing were digested with 1 unit of endonuclease *HaeIII* (restriction sites are indicated in Fig. 1) according to the provider's protocol (Boehringer Mannheim). Performance of digestion was checked by 2% agarose gel electrophoresis. Digests (1 µl) were mixed with denaturation buffer (4 µl) according to Widjoatmodjo *et al.* (1994), incubated at 95 °C for 10 min, and stored on ice before loading onto a non-denaturing 10% polyacrylamide gel (CleanGel 36S; Pharmacia Biotech). Samples (2 µl) were separated with DNA Disc buffer (Pharmacia Biotech) on a 2117 Multiphor II Electrophoresis Unit (Pharmacia Biotech) at 4 °C using 100 V for 30 min followed by 400 V for 180 min, and DNA was visualized by silver-staining. Profiles were interpreted visually.

RESULTS

Amplification and direct sequencing of the 16S–23S rDNA spacer region

All DNA extracts were investigated by nested PCR with primer pairs *tws1* & *tws2* followed by *tws3* & *tws4* targeting the '*T. whippelii*' 16S–23S rDNA spacer and flanking coding regions. Amplicons of the expected size (~490 bp) were detected in all specimens previously shown to harbour '*T. whippelii*' 16S rDNA but none of the negative controls (results not shown). All amplicons that were sequenced ($n = 30$) perfectly matched the revised '*T. whippelii*' reference sequence (GenBank accession no. AF074933; Hinrikson *et al.*,

Type 1	AAGGAGC	TTTTGCGGT	ACTTTATAAC	1522
Type 2	.....	.....	.....	
Type 3	.....	.....	.....	
Primer pair <i>twsA1/twsA2</i>				
Type 1	TTTAAGTGA	TACCGCCATA	GTGCACCTGG	1572
Type 2	.....	.....	.....	
Type 3	.....	.....	.....	
Primer pair <i>twsB1/twsB2</i>				
Type 1	GTCTGTTTG	GGCACAAGCT	CACGGGAGTG	1622
Type 2	.....	.....	.....	
Type 3	.....	.....	.....	
Primer pair <i>twsC1/twsC2</i>				
Type 1	GGTTTGTTTA	CCGGTCTGGG	TGCTTATGCA	1672
Type 2	.....	.....	.....	
Type 3	.....	.....	.....	
Primer pair <i>twsA2/twsA1</i>				
Type 1	TTGAGAGGCC	AGGCTTTTAC	GGGTGGGCAG	1722
Type 2	.....	.....	.....	
Type 3	.....	.....	.....	
Primer pair <i>twsA2/twsC1</i>				
Type 1	ACTGCACAGT	GGACGCGAGC	ATCTTTCTAG	1772
Type 2	.....	.....	.....	
Type 3	.....	.....	.....	
Primer pair <i>twsA2/twsC2</i>				
Type 1	TTATGCGCAG	TATTTGT		1789
Type 2	.....	.....		
Type 3	.....	.....		

Fig. 1. 16S–23S rDNA intergenic spacer region variants of '*T. whippelii*'. The base numbering is given according to Maiwald *et al.* (1996); dots and hyphens symbolize identity and alignment gaps, respectively. Target regions of primers used for type-specific reamplification as well as restriction sites of endonuclease *HaeIII* are indicated by bold type.

Fig. 2. Representative results of nested PCR assays for direct detection of '*T. whippelii*' 16S–23S rDNA spacer types in clinical specimens on ethidium bromide-stained agarose gels. PCR products derived from amplification using primer pair *tws3* & *tws4* were reamplified with various type-specific primer combinations: *twsA1* & *twsB1* (A, targeting spacer type 1), *twsA2* & *twsB2* (B, types 2 and 3), *twsA1* & *twsC1* (C, type 1), *twsA2* & *twsC1* (D, type 2), and *twsA2* & *twsC2* (E, type 3). The expected products are indicated with arrows. Lanes 1–10: Clinical specimens positive for '*T. whippelii*' spacer type 1 (lanes 1, 2, 5 and 8), spacer type 2 (lanes 4 and 9), and spacer type 3 (lane 7), and negative controls (lanes 3, 6 and 10). Lane 11, molecular mass marker (50 bp ladder; Boehringer Mannheim).

1999) in the 3' and 5' terminal regions of the 16S and 23S rRNA genes, respectively. However, sequence variability was repeatedly observed within the 16S–23S rDNA spacer sequence with the following five dimorphic sites: (i) T or C [position 1551 of original spacer reference sequence (Maiwald *et al.*, 1996)]; (ii) TTTT or (TTT (positions 1578–1581); (iii) T or C (position 1619); (iv) T or C (position 1643); and (v) A or G (position 1652). According to these single-base alterations three spacer types were recognized (Fig. 1).

SSCP analysis

All 27 amplicons digested with *HaeIII* yielded two fragments of the expected sizes (295/294 and 166 bp, respectively) by agarose gel electrophoresis while the remaining 29 bp fragments were not clearly visible

(results not shown). SSCP analysis using electrophoretic conditions optimized for the single strands of the 295/294 bp fragments (spanning the dimorphic sites) resulted overall in three distinct patterns (not shown). They all consisted of two upper (very closely migrating) bands (near the double-stranded 622 bp marker) and one lower band (between the double-stranded 622 and 527 bp marker). As compared to the profiles corresponding to spacer type 1, a reduced mobility of the lower band was characteristic for the patterns derived from fragments of spacer type 2; a decreased migration of both the lower as well as the upper bands has been detected for all the samples representing spacer type 3.

Type-specific PCR assays

All DNA extracts were amplified with primer pair *tws3* & *tws4* and directly reamplified in five separate reactions with primer combinations targeting dimorphic spacer sites (Fig. 2). All specimens known to contain '*T. whippelii*' 16S rDNA were classified according to their type-specific amplicons (74/73 bp and 141/140 bp, respectively), with type-specific reamplification systems A and C being positive for type 1, systems B and D for type 2, and systems B and E for type 3. None of these samples was positive for more than one spacer type (Table 1). The appearance of *tws3* & *tws4* amplicons and other minor products (>200 bp; representing amplicons derived from combinations of 1st and 2nd round primers) correlated with the amount of target DNA present in the clinical samples. All 41 controls tested remained negative for spacer products and did not show non-specific bands.

Frequency of the three spacer types in clinical specimens

Spacer type 1 was found in 15 patients, types 2 and 3 in 10 and 3, respectively (Table 1). Spacer types did not correlate with the type of specimen analysed.

DISCUSSION

The various clinical manifestations of Whipple's disease ranging from chronic low grade fever to life-threatening endocarditis (Dobbins, 1987) have often been assumed to represent differences between the infecting strains of '*T. whippelii*' or to be due to closely related, '*T. whippelii*'-like species. This, however, remained pure speculation as nobody has ever looked at such differences, mainly because of the absence of reliable cultures (Schoedon *et al.*, 1997). In this study, we have analysed the 16S–23S rDNA spacer sequences directly from 38 clinical specimens of 28 patients. These specimens had previously been shown to contain 16S rDNA of '*T. whippelii*' by a specific assay (Table 1). The identity of the respective amplicons from 19 samples of 13 patients had been confirmed by sequence analysis (Brändle *et al.*, 1999; Ehrbar *et al.*, 1999). For an additional eight specimens from eight patients

comparable results had been obtained by broad-range (universal eubacterial) amplification and sequencing of a $\pm$ 530 bp fragment (Goldenberger *et al.*, 1997), thus excluding also the presence of significant amounts of DNA from unrelated bacteria.

We considered the analysis of the 16S–23S rDNA spacer region a promising approach to possibly detect strain differences as it has been shown for a variety of organisms that the spacer region is more variable than the flanking regions encoding 16S and 23S rRNA, respectively (Gürtler & Stanisich, 1996). In a first series of nine patients no variability had been found (Hinrikson *et al.*, 1999) and we, therefore, decided to analyse as many different samples as possible to verify these findings using the same approach, including sequence analysis of PCR products. This resulted in the detection of five dimorphic sites constituting three different spacer types (Fig. 1). In each of the eight patients with more than one positive specimen the same type was found in all independently processed samples (Table 1). The most frequent type detected, i.e. spacer type 1, perfectly matched the original '*T. whippelii*' spacer sequence (294 bp) previously deposited in GenBank (Maiwald *et al.*, 1996). As compared to this reference sequence, spacer types 2 and 3 differed solely at two and five nucleotide positions, respectively (Fig. 1). Nonetheless, these slight DNA alterations could be confirmed by SSCP analysis of *Hae*III-generated spacer fragments spanning all variable nucleotide positions found (not shown). As anticipated from the sequencing data, a total of three distinct SSCP profiles were obtained, each corresponding to one of the spacer types. Because both sequencing and, to some extent, SSCP analysis are tedious and time-consuming, we decided to establish type-specific PCR assays based on the polymorphisms found (Fig. 2). For each single specimen, the results of the five different assays were as expected (Table 1), thus confirming the reliability of each of the three methods used. While type-specific PCR is straightforward for detecting the presently known three types, SSCP and sequence analysis have the potential of recognizing additional types not related to the currently known five dimorphic sites.

In all specimens analysed only one single spacer type was detectable. Assuming that each patient is infected with only one single strain as suggested by the eight patients with multiple specimens, this may be explained by the presence of one single rRNA operon, i.e. spacer region, per strain as described for other actinobacteria (Gürtler & Stanisich, 1996). If so, any fixed single-base mutation in the rDNA spacer leading to an inappropriately folded molecule would have to be compensated by further sequence variation. Interestingly, the spacer types now found seem to have evolved mainly by pairwise DNA alterations (position 1551 $\leftrightarrow$ position 1578, and position 1643 $\leftrightarrow$ position 1652, Fig. 1) as indicated by the fact that five dimorphic sites (with 32 possible combinations) but only three different spacer types have been found.

The variations found raise the question whether they represent three different, closely related species or three subtypes of the single species '*T. whippelii*'. Partial 16S sequences determined for most specimens included in this study did not reveal any differences as compared to the published sequence. However, as has been shown for other organisms, even complete identity of 16S rDNA sequences does not prove species identity (Fox *et al.*, 1992) but suggests a very close relationship (Stackebrandt & Goebel, 1994). To solve the problem DNA-DNA hybridization studies are definitely needed but not feasible due to the fact that '*T. whippelii*' has not yet been cultured on artificial media. As long as hybridization data are not available, we suggest that the three types found be regarded as subtypes of the single species '*T. whippelii*' for practical reasons and because of the small number of variable nucleotides which is similar or even less than the variations found within other species (Gürtler & Stanisich, 1996).

We conclude that at least three different '*T. whippelii*' 16S-23S rDNA spacer types can be detected with different frequencies directly from human clinical specimens. Further studies including samples from other geographic areas as well as from environmental sources (Maiwald *et al.*, 1998) may show whether additional spacer types can be found. Such investigations are much easier to perform when using either type-specific PCR assays and/or SSCP analysis as compared to sequencing. Although an obvious association of spacer types and types of specimens analysed was not found, it remains to be shown whether the '*T. whippelii*' spacer types may be correlated with different clinical presentations of Whipple's disease or even with the possible carrier state for 'Whipple bacilli' as postulated previously (Ehrbar *et al.*, 1999).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Swiss National Science Foundation (32-50790.97) to M.A. We thank D. Harmsen (Würzburg, Germany) for providing specimens of patients 7, 8 and 9. A. von Graevenitz is acknowledged for careful reading of the manuscript.

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