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## Atypical 16S rRNA Gene Copies in *Ochrobactrum intermedium* Strains Reveal a Large Genomic Rearrangement by Recombination between *rrn* Copies

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*Ochrobactrum intermedium* is an opportunistic human pathogen belonging to the alpha 2 subgroup of proteobacteria. The 16S rDNA sequences of nine *O. intermedium* isolates from a collection of clinical and environmental isolates exhibited a 46-bp insertion at position 187, which was present in only one sequence among the 82 complete or partial 16S rDNA sequences of *Ochrobactrum* spp. available in data banks. Reverse transcription-PCR experiments showed that the 46-bp insertion remained in the 16S rRNA. The inserted sequence folded into a stem-loop structure, which took place in and prolonged helix H184 of the 16S rRNA molecule. Helix H184 has been described as conserved in length among eubacteria, suggesting the idiosyncratic character of the 46-bp insertion. Pulsed-field gel electrophoresis experiments showed that seven of the clinical isolates carrying the 46-bp insertion belonged to the same clone. Insertion and *rrn* copy numbers were determined by hybridization and I-CeuI digestion. In the set of clonal isolates, the loss of two insertion copies revealed the deletion of a large genomic fragment of 150 kb, which included one *rrn* copy; deletion occurred during the in vivo evolution of the clone. Determination of the *rrn* skeleton suggested that the large genomic rearrangement occurred during events involving homologous recombination between *rrn* copies. The loss of insertion copies suggested a phenomenon of concerted evolution among heterogeneous *rrn* copies.

The genus *Ochrobactrum* belongs to the alpha 2 subgroup of Proteobacteria and is the closest relative to the genus *Brucella*. *Ochrobactrum anthropi* was considered the sole and type species of the genus *Ochrobactrum* (15) until 1998, when *Ochrobactrum intermedium* was proposed as a new species of this genus (50). Factors discriminating between *O. anthropi* and *O. intermedium* were their low DNA-DNA hybridization, their different 16S ribosomal DNA (rDNA) sequences, the different Western blot profiles of sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated whole-cell proteins, and the resistance of *O. intermedium* to colistin and polymyxin B (50). In a recent study, Lebuhn et al. (21) performed a polyphasic analysis of a large collection of *Ochrobactrum* sp. strains isolated from the wheat rhizosphere. They described two new species, *Ochrobactrum grignonense* and *Ochrobactrum tritici* and proposed the separation of the *Brucella-Ochrobactrum* group into five clades with *O. intermedium* belonging to clade 1 (21).

*Ochrobactrum* spp. are members of the soil microbiota (21), and an increasing number of studies have reported the isolation of *O. anthropi* and *O. intermedium* from clinical specimens, especially from immunocompromised patients or from patients with nosocomial infections (28, 32, 47). To date, 16S rDNA sequencing is the most reliable procedure used for identification of *Ochrobactrum* strains at the species level, especially in clinical specimens (32, 47).

The genomes of *O. anthropi* and *O. intermedium* have been

previously described as complex with two independent circular chromosomes. Each chromosome hybridized with a 16S rDNA probe, but the *rrn* copy number has not been determined (17). The organization of rRNA genes as a multigene family is widespread in eubacteria. The members of an rRNA multigene family are subject to a homogenization process, allowing several gene copies to evolve in concert. In a concerted-evolution mode, a mutation occurring in one copy will be either fixed for all of them or lost for all. The recombination events involved in concerted evolution probably occur by gene conversion, a non-reciprocal recombination event in which the sequence of one copy of a gene is converted to the sequence present in another one (13, 22). Thus, rRNA sequences show low variability within species, subspecies, or genomes. However, the general extent of intraspecific variation in rDNA sequences has been observed among sequences deposited in the GenBank database (8). Furthermore, intragenomic heterogeneity in the form of nucleotide differences between 16S rDNA copies, so-called microheterogeneity, has been described in few cases. For example, microheterogeneity has been identified in *Escherichia coli* (7, 31), *Mycobacterium terrae* (34), *Paenibacillus polymyxa* (36), members of the class Mollicutes (14, 23, 39), and the Actinomycetales *Thermomonospora chromogena* (53), *Thermobispora bispora* (38), and *Streptomyces* spp. (49). Expression of two *rrn* operons differing in 5% of the nucleotide positions has also been described for the archeon *Haloarcula marismortui* (10). Microheterogeneity appeared to be more common than macroheterogeneity, involving large insertions ranging from 50 to several hundred nucleotides. Macroheterogeneity of the 16S rDNA, involving an intervening sequence (IVS) present in the gene but absent in the 16S rRNA molecule, has been observed

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TABLE 1. Data for *O. intermedium* strains used in this work

Strain(s)	Origin	Copy no. of:		16S rDNA sequence accession no.	Source and reference(s):
		<i>rrn</i>	Insertion		
ADV1	Human (clinical)	4	3	AF526509	This study
ADV2	Human (clinical)	4	3		This study
ADV3	Human (clinical)	3	1	AF526516	This study
ADV4-ADV7	Human (clinical)	3	1		This study
ADV9	Human (clinical)	4	2	AF526510 and AF526517	This study
ADV10	Human (clinical)	4	0	AF526511	This study
ADV11	Human (clinical)	4	0	AF526512	This study
ADV14	Human (clinical)	4	0	AF526513	This study
ADV21	Human (clinical)	4	0	AF526514	This study
ADV24	Human (clinical)	4	0	AF526515	This study
PR17/sat	Nematode	4	4	AJ245941	3 and this study
LMG 3301 <sup>T</sup>	Human (clinical)	4	0	U70978	15, 49, and this study

in the archaeons *Pyrobaculum aerophilum* (5), *Aeropyron pernix* (35), and *Thermoproteus* spp. (16). IVSs have also been found in the 16S rDNA of some eubacteria, for instance, in the genera *Campylobacter* (11, 12, 25), *Helicobacter* (24, 45), and *Clostridium* (41), and in two endosymbiotic proteobacteria (42, 44). In the two eubacterial species *Desulfotomaculum australicum* (37) and *Bacillus sporothermodurans* (40), the insertion in the 16S rDNA persisted in the 16S rRNA.

Direct sequencing after PCR amplification of 16S rDNA of *O. intermedium* isolates gave ambiguous results with double sequencing signals; this is due to an atypical insertion of 46 bp in certain 16S rDNA copies. We studied the persistence and location of the insertion in the 16S rRNA molecule. In a set of clonal isolates, the 46-bp insertion was unstable and variation in the insertion copy number revealed a large genomic deletion including one *rrn* operon.

#### MATERIALS AND METHODS

**Bacterial strains, cultivation, identification, and growth curves.** The reference strain of *O. intermedium*, LMG 3301<sup>T</sup> (deposited as *O. anthropi* and now transferred to *O. intermedium* as the type strain [50]), was obtained from the Collection Française des Bactéries Phytopathogènes. Thirteen *O. intermedium* isolates were obtained from patients hospitalized in the Academic Hospital of Montpellier (Montpellier, France). Among these clinical isolates, strains ADV1 to -7 were isolated from respiratory samples of the same patient with chronic carriage over a 1-year period. *O. intermedium* strain PR17/sat had already been isolated from the nematode *Heterorhabditis indica* in Puerto Rico (3) (Personal gift from Noel Boemare, Laboratoire de Pathologie Comparée, Université de Montpellier, Montpellier, France). *O. intermedium* strains used in this study are listed in Table 1. Bacteria were grown on tryptic soy (TS) agar for 24 h at 37°C. The identification of the isolates as members of the species *O. intermedium* was based on phenotypic criteria such as Gram stain, presence of oxidase, analytical profile index (API 20E and API 20NE systems; bioMérieux, Marcy l'Etoile, France), and antibiotic susceptibility profiles (47). 16S rDNA sequencing was used to confirm the species affiliation.

Growth curves of *O. intermedium* strains were performed as follows. Bacterial strains grown overnight in TS medium were diluted 50-fold in fresh TS medium and incubated at 37°C with gentle shaking. A bacterial count was performed every 15 min for 8 h by measuring the turbidity at 600 nm. Each strain was tested for growth curves in five independent experiments. For statistical analysis, the turbidity versus time was plotted. The plotted points were subjected to a non-linear regression analysis. The comparison of the curves among strains was performed by using the one-sided Wilcoxon rank sum test. The level of the statistical significance was set at 5%.

**rDNA amplification, sequencing, and analysis.** One isolated colony was suspended in 50 µl of sterile distilled water, and the DNA was rapidly extracted by a method involving boiling and freezing (43). 16S rDNAs were selectively amplified by PCR using 27f (5'-GTGCTGCAGAGATTTGATCCTGGCTCAG-

3'; positions 8 to 36 [*E. coli* numbering]) as the forward primer and 1492r (5'-CACGGATCTACGGGTACCTTGTACGACTT-3'; positions 1478 to 1508 [*E. coli* numbering]) as the reverse primer. An internal primer in the 46-bp insertion, ins1 (5'-GCCCCCTTTAAATTTTCAG-3'), was used in association with the primer 1492r for specific amplification of 16S rDNA copies carrying the insertion. Primers for 23S rDNA amplification were LS24f (5'-ATTTGGTGGATGCCTTGG-3'; positions 24 to 41 [*E. coli* numbering]) and LS2744r (5'-CCCGCTTAGATGCCTTCAGC-3'; positions 2744 to 2763 [*E. coli* numbering]). The PCRs were carried out in 50 µl of reaction mixture containing 200 nM (each) primer, 200 µM (each) deoxynucleoside triphosphate (dNTP), 1 U of *Taq* polymerase (Roche, Meylan, France) in the appropriate reaction buffer, and 2 µl of crude DNA extract as the template. PCR conditions were 30 cycles of 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C. Amplification of a single 16S rDNA copy was performed by cutting a restriction fragment from agarose gel and using 2 µl of the melted agarose as the PCR template. Determination of fragments harboring a 16S rDNA copy was done by Southern blotting (see below). Restriction analysis of PCR products was done with 10 U of *DraI* (New England Biolabs, Hertfordshire, United Kingdom) by following the supplier's recommendations.

PCR products were directly sequenced on an Applied Biosystems automatic sequencer (Genome Express, Meylan, France) in both directions by using forward and reverse primers. The 16S rDNA sequences were compared with sequences deposited in the GenBank, EMBL, and sequencing-genome databases by using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast>) and with sequences deposited in the Ribosomal Database Project, version 7.0, by using SIMILARITY RANK and SUGGEST TREE (29). Prediction of RNA secondary structure by energy minimization was performed online by the MFOLD program (51).

**PFGE and DNA electrophoresis.** Genomic DNAs were prepared in agarose plugs for enzymatic digestion and pulsed-field gel electrophoresis (PFGE) of intact chromosomes as previously described (17, 18). DNAs were digested with 40 U of *SpeI* or *HindIII* (New England Biolabs) or with 1 U of the intronic endonuclease *I-CeuI* (New England Biolabs) (18). *SpeI* and small *I-CeuI* fragments were separated by PFGE using a contour-clamped homogeneous electric field apparatus (CHEF-DRII; Bio-Rad, Hercules, Calif.) in a 1% agarose gel in Tris-borate-EDTA buffer (TBE; 0.5×). Pulse ramps were 5 to 35 s for 40 h at 150 V for *SpeI* fragments and 90 to 150 s for 24 h at 170 V for small *I-CeuI* fragments. Separation of undigested DNA and large *I-CeuI* fragments was obtained in the same PFGE apparatus in a 0.8% agarose gel in 0.5× TBE by using a pulse ramp of 90 to 300 s for 45 h at 150 V. DNAs digested with *HindIII* were subjected to electrophoresis for 3 h at 80 V in a 0.8% agarose gel in 0.5× TBE by using a SubCell apparatus (Bio-Rad).

**Southern blotting, probes, and hybridization.** Electrophoresis gels were transferred onto a Nytran N (Schleicher & Schuell, Dassel, Germany) nylon membrane by vacuum blotting (vacuum blotter; Bio-Rad) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). 16S rDNA and 23S rDNA digoxigenin-labeled probes were obtained by PCR as described before using primer pairs 27f and 1492r and LS24f and LS2744r, respectively, with a dNTP mixture containing 0.1 mM digoxigenin-dUTP (Roche). The digoxigenin-labeled oligonucleotide corresponding to the 46-bp insertion was purchased from Sigma-Genosys. The hybridization of the probes was detected by a CSPD chemiluminescence system (Roche).

**RNA preparation and RT-PCR experiment.** *O. intermedium* total RNA was isolated and purified by using the SV total-RNA isolation system (Promega, Madison, Wis.) in accordance with the supplier's protocol. Reverse transcription-PCR (RT-PCR) was performed by using the Access RT-PCR kit (Promega). The reaction volume of 50  $\mu$ l contained 50 ng of the DNase-treated RNA extract, 200 nM concentrations of primers 27f or ins1 and 590r (5'-TGACTTATTGCCCG CCTACG-3'; positions 580 to 599 by *E. coli* numbering), 200  $\mu$ M (each) dNTP, 5 U of *Tfl* DNA polymerase, and 5 U of avian myeloblastosis virus reverse transcriptase in the buffer recommended by the supplier. The incubation conditions were 48°C for 45 min for reverse transcription followed by 94°C for 5 min and 30 cycles of 95°C for 1 min, 60°C for 1 min, and 68°C for 2 min for PCR. Non-reverse-transcribed RNA was used as the PCR template for the negative control. *Dra*I digestion of the RT-PCR products was performed as previously described for the digestion of the PCR products.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the 16S rDNA sequences of *O. intermedium* strains are listed in Table 1. The accession number of the 23S rDNA sequence of *O. intermedium* strain ADV1 was AY223505.

## RESULTS

**Evidence for different copies of 16S rDNA *O. intermedium* strains.** The 16S rDNA sequence of *O. intermedium* PR17/sat isolated from a nematode included a sequence of 46 bp inserted at position 187 (*E. coli* numbering). This inserted sequence was absent from the 82 complete or partial 16S rDNA sequences of *Ochrobactrum* spp. available in data banks in February 2003, except for one sequence obtained from an *Ochrobactrum* sp. isolated from the rhizosphere of rice (48). Moreover, BLAST analysis of the inserted sequence did not show significant similarity with sequences of genomes currently being sequenced. Ambiguous sequencing results consisting of double signals were obtained for strains ADV1 to -7 and strain ADV9. After several attempts an exploitable sequence for the strain ADV1 was obtained. The sequence showed the same 46-bp insertion at position 187 as that observed in the isolate PR17/sat. When the 46-bp insertion was excluded from analysis, sequences of 16S rRNA genes of strains ADV1 and PR17/sat were 99.9% identical to those of *O. intermedium* soil isolates OiC8a and OiC8-6 and clinical isolates 609360I and Relman 99, previously deposited in the databases. To rapidly screen *Ochrobactrum* isolates that carried the 46-bp insertion, a PCR anchored in the insertion was performed. Amplification products were obtained for *O. intermedium* strains ADV1 to -7, ADV9, and PR17/sat (data not shown). Since direct sequencing of 16S rDNA amplification products remained unsuccessful for strain ADV9, we sequenced the product obtained by a PCR anchored in the insertion. The partial sequence obtained showed 100% identity to that of strain ADV1. The 46-bp insertion was present in 9 of the 15 *O. intermedium* strains tested. Therefore, the insertion appeared to be widely represented though it was not a characteristic of the species. In particular, LMG 3301<sup>T</sup> did not harbor the insertion. The 46-bp insertion was absent from a collection of 10 *O. anthropi* isolates tested by a PCR anchored in the insertion (data not shown).

**16S rDNA copy number and 46-bp insertion copy number.** The copy number of 16S rRNA gene was determined by a Southern blotting experiment, using a 16S rDNA probe, on genomic DNAs digested by *Hind*III (restriction site absent from the 16S rDNA in the genus *Ochrobactrum*). Four 16S rDNA hybridizing fragments were found in 10 (including LMG 3301<sup>T</sup>) of the 15 strains of *O. intermedium* (Fig. 1A and Table 1). The four fragments, named A, B, C, and D, comprised 20,

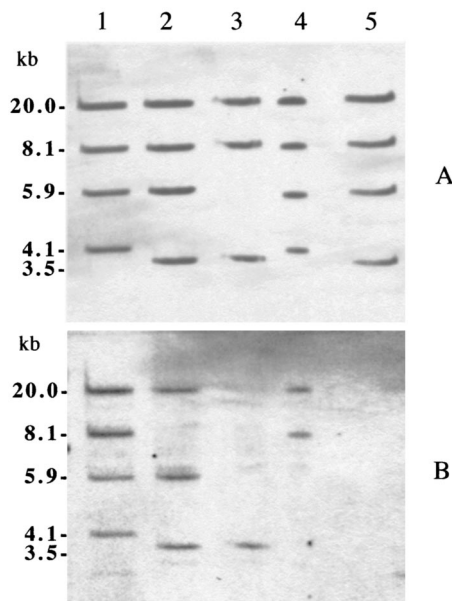


FIG. 1. Copy numbers of 16S rDNA (A) and 46-bp insertion (B) in the genome of *O. intermedium*. Shown are Southern blots of *Hind*III-digested genomic DNA from strains PR17/sat (lane 1), ADV1 (lane 2), ADV3 (lane 3), and ADV9 (lane 4) and reference strain LMG 3301<sup>T</sup> (lane 5) hybridized with the 16S rDNA probe (A) and the 46-bp insertion probe (B). Strain ADV2 showed a hybridization profile identical to that of strain ADV1; strains ADV4 to -7 showed hybridization profiles identical to that of strain ADV3; strains ADV10, ADV11, ADV14, and ADV24 showed hybridization profiles identical to the strain LMG 3301<sup>T</sup> profile (data not shown). Sizes of hybridizing fragments were calculated by using  $\lambda$  digested by *Hind*III as a molecular marker.

8.1, 5.9, and 4.1 kb, respectively. Surprisingly, the five remaining clinical isolates (strains ADV3 to -7) showed only three restriction fragments hybridizing with the 16S rDNA probe (Fig. 1A and Table 1). The restriction fragment C was absent from the hybridization profile. Genomic digestion with *I-Ceu*I, an intronic endonuclease that cleaved specifically a 26-bp site in eubacterial 23S rDNA (position 1909 in *E. coli* numbering), gave four restriction fragments (2,130, 1,850, 250, and 150 kb) for 10 (including the strain LMG 3301<sup>T</sup>) of the 15 strains but only three fragments (2,130, 1,850, and 250 kb) for *O. intermedium* strains ADV3 to -7 (Fig. 2; see below). These results suggested the presence of four *rrn* operons in a majority of *O. intermedium* strains including the reference strain. Operons were named *rrnA*, *rrnB*, *rrnC*, and *rrnD*, corresponding to the nomenclature for the *Hind*III fragments. However, *O. intermedium* strains ADV3 to -7 carried only three copies of the *rrn* operon. Operon *rrnC* was absent from these strains.

The 46-bp insertion introduced a unique *Dra*I restriction site in 16S rDNA PCR products. *Dra*I digestion did not cut amplicons obtained for *O. intermedium* strains LMG 3301<sup>T</sup>, ADV10, ADV11, ADV14, ADV21, and ADV24 and totally digested amplification products from *O. intermedium* strain PR17/sat. In contrast, *Dra*I partially digested the 16S rDNA PCR products obtained from strains ADV1 to -7 and ADV9, suggesting that not all their 16S rDNA copies harbored the 46-bp insertion (data not shown). The copy number of the insertion was determined by a Southern blotting experiment using the 46-bp

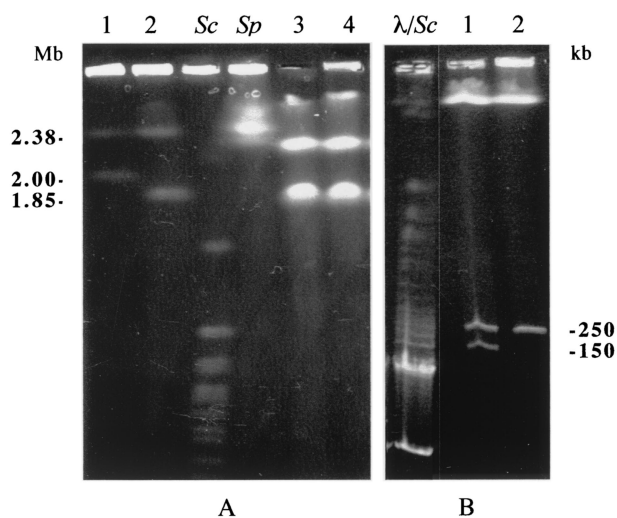


FIG. 2. PFGE migration of undigested and I-CeuI-digested genomic DNAs of *O. intermedium* strains ADV1 and ADV3. (A) Migration of high-molecular-weight fragments. Lanes 1 and 2, undigested DNA from strain ADV1 (lane 1) and strain ADV3 (lane 2); lanes 3 and 4, I-CeuI-digested DNA from strain ADV1 (lane 3) and strain ADV3 (lane 4); Lanes *Sc* and *Sp*, *Saccharomyces cerevisiae* (*Sc*) and *Schizosaccharomyces pombe* (*Sp*) chromosomes (Bio-Rad) as molecular size markers. (B) Migration of low-molecular-weight I-CeuI fragments. Lane 1, strain ADV1; lane 2, strain ADV3. Chromosomes and I-CeuI digestion patterns of *O. intermedium* strains ADV2 and ADV4 to -7 are identical to patterns for strains ADV1 and ADV3, respectively (data not shown). A mixture of  $\lambda$  digested by *Hind*III, the  $\lambda$  concatemer, and *Saccharomyces cerevisiae* chromosomes was used as the molecular size marker (lane  $\lambda$ /Sc); the bands useful for the measure were, from the bottom, 27, 50, 100, 150, 200, 225, 250, and 285 kb.

probe on the products of *Hind*III genomic digestion (Fig. 1B and Table 1). Among the fragments not carrying 16S rDNA, no hybridizing fragments were observed. The four 16S rDNAs of strain PR17/sat possessed the insertion. There were three copies of the insertion for strains ADV1 and -2 in *rmA*, *rmC*, and *rmD*. Operons *rmA* and *rmB* of strain ADV9 harbored the 46-bp insertion, whereas it was observed only in *rmD* for strains ADV3 to -7.

**Instability of the 46-bp insertion and of a large genomic deletion in clonal isolates.** *O. intermedium* strains ADV1 to -7 were chronologically isolated from the same patient over a 1-year period of chronic carriage. The clonality of the seven strains was studied by PFGE after *Spe*I macrorestriction. Identical PFGE patterns were obtained on one hand for strains ADV1 and -2 and on the other hand for strains ADV3 to -7 (Fig. 3). These two patterns differed slightly, with one additional band of about 150 kb present only in the former pattern. On the basis of the criteria of Tenover et al. (46) and the high degree of PFGE pattern polymorphism among unrelated *O. intermedium* strains (47), the isolates ADV1 to -7 should be considered a clone. Seven isolates of a single clone provided the opportunity to study the stability of the 46-bp insertion in "field" conditions. In these clonal isolates, the copy number of the insertion decreased from three (strains ADV1 and -2) to one (strains ADV3 to -7) whereas the *rm* copy number decreased from four to three for the same strains (Fig. 1). Hybridization of the 16S rDNA probe on *Spe*I patterns confirmed the loss of one *rm* copy in strains ADV3 to -7. This copy was

carried on the 150-kb fragment present in the genomes of strains ADV1 and -2 but was absent from strains ADV3 to -7 (data not shown).

PFGE migration of undigested DNAs of strains ADV1 to -7 allowed the visualization of two chromosomes (Fig. 2A, lanes 1 and 2) as previously observed for the type strain (17) and for all the isolates belonging to the species *O. intermedium* tested in this work (data not shown). The two chromosomes migrated in PFGE as faint bands, suggesting their circular topology. Strains ADV1 and -2 harbored two chromosomes of 2.38 and 2 Mb, whereas in strains ADV3 to -7 the size of the large chromosome was 2.38 Mb and that of the small one was 1.85 Mb. Owing to the sizes of I-CeuI fragments (Fig. 2A, lanes 3 and 4, and B, lanes 1 and 2) and the sizes of undigested chromosomes, we deduced that each chromosome of strains ADV1 and -2 carried two *rm* copies separated by 250 kb on the large chromosome and by 150 kb on the small one. Moreover, I-CeuI digestion indicated the loss of the 150-kb fragment in strains ADV3 to -7 (Fig. 2B). These results suggested that a large deletion of 150 kb occurred in the small chromosome and that it was related to the loss of one *rm* copy in strains ADV3 to -7. The sequencing of the 23S rRNA gene of strain ADV1 showed the presence of a *Spe*I site in position 932 (*E. coli* numbering), about 1 kb before the I-CeuI site. The location of this *Spe*I site explained why DNA deleted by genomic rearrangement corresponded to *Spe*I and I-CeuI restriction fragments of nearly the same size in PFGE experiments.

To determine the orientation of *rm* operons on the two chromosomes, we performed hybridization experiments using 16S rDNA and 23S rDNA probes on I-CeuI digestion products (data not shown). Then, insertion copies were located using

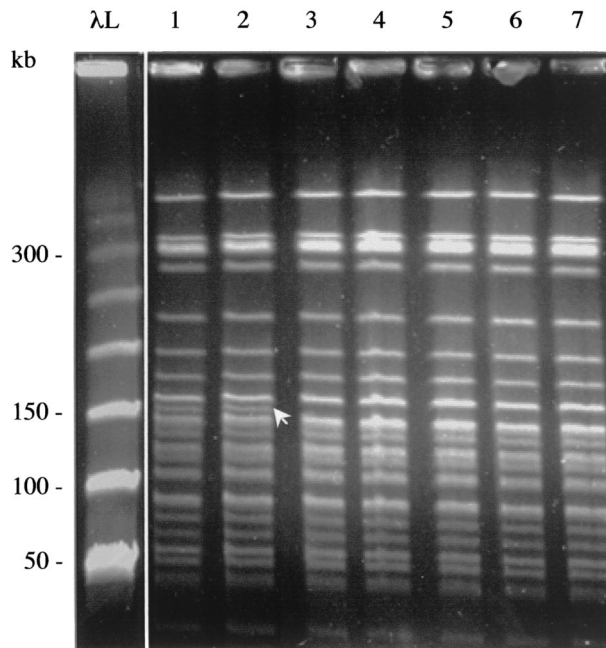


FIG. 3. PFGE of *Spe*I-digested genomic DNA from *O. intermedium* strains. Lanes 1 to 7, strains ADV1 to -7, respectively; lane  $\lambda$ L,  $\lambda$  ladder, PFGE Marker I (Roche), as a molecular size marker. Arrowhead, 150-kb band present in lanes 1 and 2.

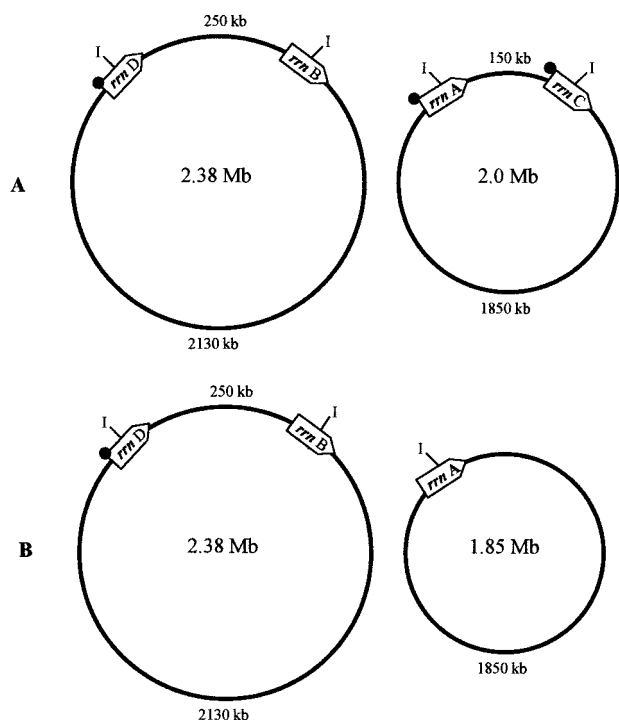


FIG. 4. Schematic representation of I-CeuI map and *rrm* skeleton of the two chromosomes of strains ADV1 and -2 (A) and ADV3 to -7 (B). I, I-CeuI restriction site. Sizes of chromosomes and I-CeuI restriction fragments are indicated in megabases and kilobases, respectively. Arrows indicate the orientation of *rrm* operons. Black circles, 46-bp insertion.

the 46-bp insertion probes on I-CeuI digestion products. An I-CeuI map and *rrm* skeleton of the two chromosomes of strains ADV1 and ADV3 are schematically represented in Fig. 4. We observed in the small chromosomes of strains ADV3 to -7 a large deletion of 150 kb, which included *rrmC*. Moreover, *rrmA* remained on the small chromosome but lost the 46-bp insertion. Direct sequencing of 16S rDNA amplification product obtained with *Hind*III fragment A of strain ADV3 as the template gave a partial sequence of *rrsA* (accession no. AF526516), which was strictly identical to those of insertion-free *rrs* copies of *O. intermedium*.

The loss of one 16S rDNA copy and/or the genomic deletion that occurred seemed to affect the phenotypes of the strains. Indeed, the colonies of strains ADV1 and -2 were mucoid and quickly became confluent during incubation at 37°C, as described for the type strain (50) and for clinical isolates (47) of *O. intermedium*, whereas the colonies of strains ADV3 to -7 were smooth and nonmucoid. Moreover, strains ADV3 to ADV7 showed a growth rate about 30% lower than that for strains ADV1 and ADV 2 (data not shown). Statistical analysis showed that the difference in growth rates was significant ( $P = 0.0143$ ).

**Expression and two-dimensional structure of the 46-bp insertion.** We selectively amplified 16S rRNA molecules carrying the 46-bp insertion by RT-PCR using the insertion-specific primer *ins1* and the consensual primer 590r as forward and reverse primers on total-RNA extracts. RT-PCR products of about 380 bp were obtained from *O. intermedium* strains

ADV1, ADV3, ADV9, and PR17/sat (Fig. 5A) and strains ADV2 and ADV4 to -7 (data not shown). The size of a fragment was in accordance with the position of the 46-bp insertion in the 16S rRNA gene. No amplification was obtained for strain LMG 3301<sup>T</sup> (Fig. 5A) and the other strains (data not shown). The RT-PCR performed using the consensual primers 27f and 590r on RNA extracts of strains ADV1, ADV3, ADV9, PR17/sat, and LMG 3301<sup>T</sup> gave positive controls. PCR amplification of RNA samples without reverse transcription was negative in all experiments. We concluded that the 46-bp insertion was expressed in the 16S rRNA.

RT-PCR products obtained by using the consensual primers 27f and 590r on RNA extracts of strains LMG 3301<sup>T</sup>, ADV1, ADV3, ADV9, and PR17/sat were then digested by the endonuclease *Dra*I. As seen before, the 46-bp insertion introduced a unique *Dra*I restriction site in the 16S rDNA of *O. intermedium*, and then *Dra*I digestion could be used to visualize the amplification of both 16S rRNA types. *Dra*I partially digested the RT-PCR products obtained from strains ADV1, ADV3, and ADV9, confirming the expression of both types of 16S rRNA in the strains harboring heterogeneous 16S rDNA copies (Fig. 5B). The sizes of the digested fragments (380 and 170 bp) were in accordance with the position of the 46-bp insertion in the 16S rDNA. The intensity of the undigested band of 550 bp, which corresponded to the 16S rRNA without insertion, seemed to decrease slightly as the number of insertion copies in 16S rDNA increased (Fig. 5B). This result was obtained in three independent experiments. The insertion-free strain LMG 3301<sup>T</sup> and strain PR17/sat, which carried the 46-bp insertion on all the 16S rDNA copies, were, respectively, used as

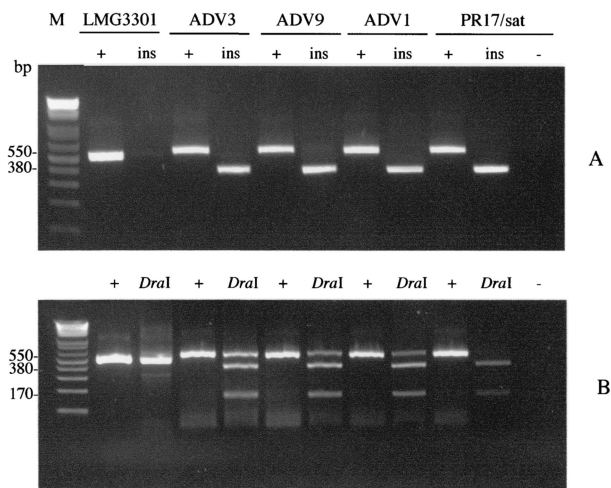


FIG. 5. Expression of the 46-bp insertion and detection of the two types of 16S rRNA by RT-PCR. The strains analyzed are indicated at the top. (A) Lanes +, use of consensual primer pair 27f and 590r; lanes ins, use of an insertion-specific pair of primers, *ins1* and 590r. (B) Lanes +, RT-PCR products obtained with the primers 27f and 590r; lanes *Dra*I, RT-PCR products obtained in + lanes digested by *Dra*I. Lanes - (A and B), negative controls performed on the RNA of strain PR17/sat without reverse transcription and with the primers 27f and 590r (negative controls were also obtained for other strains and for the primers *ins1* and 590r but are not shown); lane M, 100-bp DNA ladder (Promega) as a molecular weight marker.

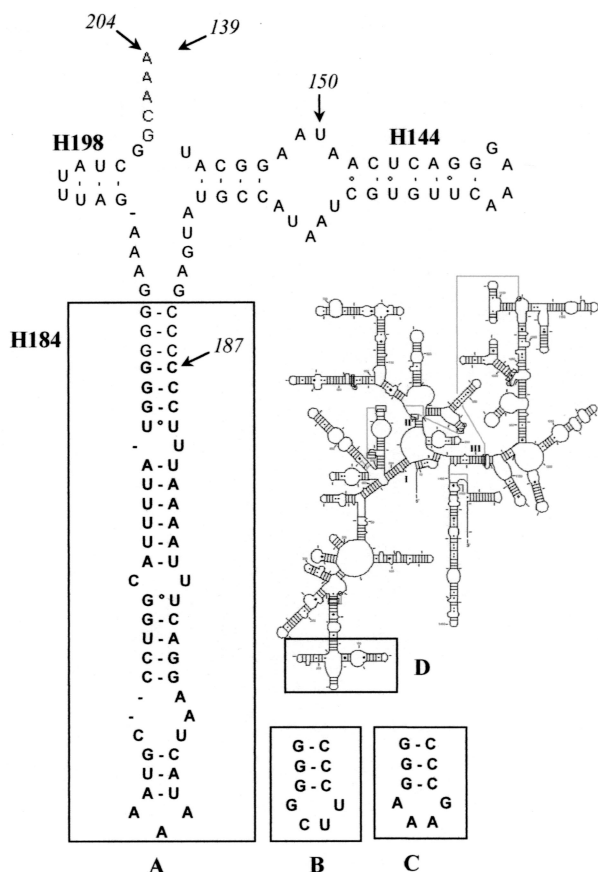


FIG. 6. Secondary structure of the 46-bp insertion. (A) Secondary structure of the insertion (boldface) replaced in *O. intermedium* 16S rRNA partial two-dimensional structure (positions 139 to 204; *E. coli* numbering). (B and C) Structures of helix H184 in 16S rRNA of *O. anthropi* and *O. intermedium*, respectively. (D) Location of the region carrying helix H184 on the *E. coli* 16S rRNA secondary-structure model.

negative and positive controls for the *DraI* digestion of RT-PCR products (Fig. 5B).

When analyzed with the MFOLD program, the sequence folded into a stem-loop structure with a predicted free energy  $-36.4$  kcal/mol at  $37^{\circ}\text{C}$ . This secondary structure was placed on the secondary structure model of the 16S rRNA molecule of *Brucella suis* described by Gutell et al. (<http://www.rna.icmb.utexas.edu/>). The 46-bp insertion took place in, and prolonged helix H184 of, the 16S rRNA (Fig. 6A and D). Helix H184 was variable in sequence among alpha proteobacteria and carried a tetranucleotide, allowing distinction between *O. anthropi* (TTTCG) and *O. intermedium* (GAAA) (Fig. 6B and C). However, in 2,184 proteobacteria analyzed in the Gutell laboratory database (<http://www.rna.icmb.utexas.edu/>), this stem-loop is formed by 10 bp and neither less nor more nucleotides are known to exist in this region, indicating the very unconventional character of the 46-bp insertion.

We tested a few phenotypical traits of the strains LMG 3301<sup>T</sup>, ADV9, and ADV1. These strains displayed the same overall genomic organization, as judged by *I-CeuI* digestion profiles (data not shown), but differed in the copy number of the insertion (0, 2, and 3, respectively). We saw no difference

in colony aspect, morphology after Gram stain, biochemical traits on API 20E and API 20NE systems, and antibiotic susceptibility profiles. Moreover, strains LMG 3301<sup>T</sup>, ADV9, and ADV1 showed similar growth curve profiles. Thus, the few characters we tested did not allow us to relate the expression of the insertion to a particular phenotype.

## DISCUSSION

We found in the *O. intermedium* genome four *rrm* copies. Two copies were located on the large chromosome, and the two others were located on the small one. The two copies were in the same orientation on each chromosome. While there are limited examples where multiple copies have been characterized for the same alpha proteobacterium, 16S rDNA copies appeared to be homogeneous in this subphylum. Thus, alignment of *rrs* sequences obtained from totally sequenced alpha proteobacterial genomes by using the BLAST program showed that the *rrs* copies in *Brucella melitensis*, *Brucella suis*, *Agrobacterium tumefaciens*, *Sinorhizobium meliloti*, *Mesorhizobium loti*, and *Caulobacter vibrioides* genomes were strictly identical. In contrast, we observed different copies of 16S rDNA in the genome of *O. intermedium*, a species closely related to the genus *Brucella*. This is the first described case of *rrs* heterogeneity in the alpha subgroup of proteobacteria. We found atypical copies of 16S rRNA genes with an additional sequence of 46 bp at position 187 (*E. coli* numbering) in several isolates of *O. intermedium*. Since this insertion was also present in 16S rRNA, it could not be related to IVSs that have been described for a few eubacterial species (11, 12, 24, 25, 41, 42, 44, 45). Up to this date, such a large insertion remaining in the 16S rRNA had only been described for *D. australicum* (37) and *Bacillus sporothermodurans* (40), two species belonging to the phylum comprising *Bacillus* and *Clostridium*. We present here the first description of such an insertion in the *Proteobacteria* phylum. The 46-bp insertion was found in seven clinical isolates belonging to the same clone and in another clinical strain epidemiologically unrelated. Moreover, we found the insertion in an *O. intermedium* strain isolated from the nematode *Heterorhabditis indica* in association with the bacterial symbiont *Photorhabdus luminescens* (3). Clinical and nematode isolates were geographically unrelated since the former were isolated in France and the latter were isolated in Puerto Rico. Thus, the atypical insertion in 16S rRNA of *O. intermedium* is not an idiosyncrasy of a particular strain but is on the contrary widely represented among natural isolates. However, the 46-bp insertion was absent from *O. intermedium* 16S rDNA sequences previously deposited in the databases. 16S rRNA gene sequences obtained by direct sequencing of PCR products leading to preferential amplification of one copy or by cloning one isolated copy could lead to underestimation of the occurrence of the insertion.

The question about the origin of the 46-bp insertion in the 16S rRNA genes can be discussed only hypothetically. The GC content of the insertion (47%) was compared to the GC content of the entire 16S rDNA of *O. intermedium* (55%) and to that of the region surrounding the atypical insertion, from position 139 to 204 (45%). The similarity in GC content neither suggested nor ruled out the hypothesis of acquisition by lateral transfer. Moreover, the sequence of the 46-bp insertion

did not match any other sequences of other species. Thus, arguments for lateral transfer were quite inconclusive.

When it was included in the secondary-structure model of the 16S rRNA molecule of *Brucella suis* and *E. coli*, the 46-bp insertion prolonged the helix H184. This stem-loop has been described as conserved in length among the proteobacteria (<http://www.rna.icmb.utexas.edu/>) suggesting the atypical character of the 46-bp insertion. The Database of Ribosomal Cross-Links (4) indicated that helix H184, precisely from position 189 to 191, was involved in the cross-link between 16S rRNA and ribosomal protein S13. Ribosomal protein S13 has been described as important for both translation initiation and elongation because it is cross-linked to the three translation initiation factors and to tRNA in the P site (6). RT-PCR experiments showed that copies with and without the insertion were expressed. Although these experiments did not quantify the expression of each type of 16S rRNA gene, the amounts of the two types of 16S rRNA appeared to correlate with the proportion of both types of 16S rDNA, as judged by the intensity of the RT-PCR-amplified fragments. However, the problem of a potential phenotypic impact of the 46-bp insertion could not be solved in this work.

We analyzed clonal strains chronologically isolated from respiratory samples of a patient with chronic *O. intermedium* carriage over a 1-year period. The loss of two insertion copies and a genomic rearrangement occurred between the second (ADV2) and the third (ADV3) isolates obtained. In vitro, a variation in colony aspect and growth rate between strains ADV2 and ADV3 was observed. The elongation of doubling time could be related to the loss of one *rrn* in strain ADV3, as previously described for *E. coli* after inactivation of a variable number of *rrn* copies (9).

The new genomic organization and the new phenotype were maintained in four subsequent isolates obtained over a 4-month period. We considered that genomic modifications appeared naturally but not in vitro, because isolates were analyzed with no more than one subculture event. This suggests that the new genomic organization gave a selective advantage to the strain in vivo. We never observed a mixture of mucoid and nonmucoid phenotypes in the seven clinical samples obtained from the patient. Moreover, the PFGE patterns did not suggest a mixture of two different genomic structures in the same DNA preparation. As a consequence, both genotypes and both phenotypes did not coexist in the samples tested. This did not rule out the possibility of coexistence of the two types of strain in the patient at an unexplored stage of the carriage.

The relation between host-restricted life style and a small genome size is patent in bacteria, particularly in alpha *Proteobacteria* (33). *O. intermedium* is a free-living bacterium and has a larger genome than its phylogenetic neighbors with an intracellular life style, such as *Brucella* and *Bartonella* (17, 33). It might be suggested that the 1-year restriction of isolates ADV1 to -7 to a very narrow ecological niche, i.e., the human respiratory system, led to reductive evolution (2, 33).

The 16S rDNA copy that lost the insertion (*rrsA* in strains ADV3 to -7) exhibited exactly the same sequence as the insertion-free copy (*rrsB*). When present, the 46-bp insertion replaced the tetranucleotide GAAA classically observed in *O. intermedium* 16S rDNA. Excision of an inserted element by a site-specific recombination event did not lead to the reforma-

tion of the GAAA tetranucleotide (Fig. 6). Gene conversion has been previously proposed as the mechanism of homogenization among *rrn* copies in bacteria (13, 22). Thus, a homogenization process due to gene conversion among *rrn* copies could explain the loss of the insertion. Physical mapping of the two chromosomes showed that *rrnA* and *rrnB* were on different chromosomes. Thus, we describe here a case of genetic recombination between two independent bacterial chromosomes.

A second insertion copy was lost due to the deletion of one *rrn* copy and 150 kb of the small chromosome. The two *rrn* copies flanking the 150-kb fragment on the small chromosome of strain ADV1 were in the same orientation, suggesting that a deletion event occurred by homologous recombination between the two *rrn* copies of the small chromosome (2, 18). Variations in genome structure by rearrangements at *rrn* loci have previously been described in host-specialized *Salmonella* serovars (*S. enterica* serovars Typhi, Paratyphi C, Gallinarum, and Pullorum) (26), in *Vibrio cholerae* (20), and in the genus *Pasteurella* (27). In the alpha proteobacterium *Brucella suis*, the differences in chromosome size and number have been explained by rearrangements at chromosomal regions containing the three *rrn* genes. The location and orientation of these genes suggested that these rearrangements were due to homologous recombination at the *rrn* loci (18). In the case described here, homologous recombination between *rrn* copies in the same orientation led to a deletion of a 150-kb genomic region flanked by the two copies. One of the two *rrn* copies was also deleted in this rearrangement process. Although deletion or duplication of *rrn* was observed a long time ago in *Salmonella enterica* serovar Typhimurium (1) and *Bacillus subtilis* (52) during laboratory maintenance, the first descriptions of natural isolates of *V. cholerae* were done recently (19, 20). The presence of tandemly repeated operons and the occasional deletion of one of the pairs are similar to the situation for a *Bacillus subtilis* laboratory strain (52). Deletion of an *rrn* operon is observed to occur quite often, presumably by intrachromosomal recombination within the tandemly repeated sets. In contrast, a recombination event in the small chromosome of *O. intermedium* strain ADV1 occurred between two distant *rrn* copies separated by 150 kb.

The alpha proteobacterial phyletic group is characterized by a high occurrence of complex genomes and often-important genome structure variations among bacteria belonging to the same species or the same genus. This is the case in the species *Brucella suis* (18) and in the genera *Azospirillum* (30), *Rhizobium*, *Agrobacterium*, and *Rhodobacter* (17). A high frequency of rearrangement processes involving homologous recombination between *rrn* copies could explain some of the variations observed in the genome structure (18). Moreover, as we showed before, a 100% sequence identity among 16S rDNA copies has been observed in all alpha proteobacterial genomes sequenced so far. In contrast, a low level of heterogeneity among copies was observed in the majority of totally sequenced genomes of bacteria belonging to other phyla. The homogeneity in *rrn* copy sequences observed in alpha proteobacteria might reflect very efficient concerted evolution among members of the *rrn* multigenic family in this bacterial phylum. The atypical insertion we observed in some strains of *O. intermedium* allowed us to monitor the genetic exchange among *rrn* copies in a set of clonal isolates. The results gave a

new illustration of genomic rearrangement among *rrn* copies in alpha proteobacteria and an additional argument in favor of a particularly high frequency of genetic exchanges among *rrn* copies in this bacterial subphylum.

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