Genotyping of *Ochrobactrum* spp. by AFLP Analysis

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AFLP was used to analyze the genetic diversity among *Ochrobactrum* strains. AFLP patterns showed a great genomic variability that separated the samples into three distinct clusters. *Ochrobactrum intermedium* was found to be closely related to *Brucella abortus* S99.

*Ochrobactrum* spp. are potential human pathogens. *Ochrobactrum anthropi* bacteremia is usually associated with contaminated intravenous lines in immunocompromised human patients, water sources, and environmental conditions in hospitals (1, 5, 8, 10, 11). *O. anthropi* isolates have also recently been obtained from other sources such as water, concrete, soils, termites, feces, activated sludges, oil spills, etc. Many of these isolates present interesting degradative properties not only towards multiple antibiotics but also towards herbicides, hemicellulose, anthracene, and other complex organic molecules including crude oil (2–5, 8, 12, 15, 17, 19, 25), displaying an opportunism that allows them to succeed in a wide range of environments.

*Ochrobactrum* (formerly called *Achromobacter* CDC group Vd) was originally described as a monospecific genus (9). The first species described was named *O. anthropi* since all original isolates were obtained from human clinical specimens. Three biotypes (A, C, and D) were suggested for this genus based on phenotype, G+C content, and DNA-DNA hybridization (9). Based on protein profiling, Western blotting, immunoelectrophoresis, and 16S rRNA gene analyses, Velasco et al. (22) proposed the recognition of a new species, *Ochrobactrum intermedium*, after its observed relationship to the genus *Brucella*.

*Ochrobactrum* is phylogenetically related to members of the genus *Brucella*, belonging to the alpha-2 subdivision of *Proteobacteria*, and catalogued on the *Brucella* rRNA branch of rRNA superfamily IV.

AFLP is a useful tool for establishing relationships between and within different species and genera. This method was used to measure the genetic diversity among *Ochrobactrum* strains. A *Brucella abortus* isolate was also included in the analysis to compare the relationship between *Brucella* and the *Ochrobactrum* strains.

*O. intermedium*ENC was obtained from the Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional (ENCB-IPN). All other *Ochrobactrum* strains analyzed were obtained from the University of Göteborg Culture Collection, Göteborg, Sweden. *B. abortus* S99 was obtained from the Central Veterinary Laboratory, New Haw, Weybridge, United Kingdom.

Bacterial DNA was extracted as described previously (14). The AFLP procedure was based on the protocol of Vos et al. (24), but using one selective nucleotide (rather than three) due to the small genome size of the bacteria, 4.8 Mb (21). The selective oligonucleotides used were the following: EcoRI+A, 5′-AGACTGCGTACCAATTC/A-3′, and MseI+A, 5′-GACGATGAGTCCTGAGTAA/A-3′.

Our modifications of the AFLP method provided significant numbers of well-defined bands (Fig. 1A). Data analysis of short, medium, and long gel runs (2,000 V for 2.5, 5, and 7.5 h) allowed us to read from 149 to 237 bands of different molecular weights, compared with running at a single length of time (<80 bands). Details on the methods are available upon request.

Bands were numbered consecutively from the highest to the lowest molecular weights and a binary code, where 0 is the absence of a band and 1 is the presence of a band, was used to capture the data. Genetic distances between individuals were determined by estimating the simple matching coefficient (20) and the distance matrix generated was used to produce a dendrogram by the unweighted-pair group method with arithmetic averages by using S-plus 4.0 for Windows software (Insightful Corp.).

Bootstrap analysis (7) involving 1,000 repetitions was carried out to determine the robustness of the dendrogram; the most robust topology is shown in Fig. 1B.

Analysis of the banding patterns showed great genomic variability, distinctive for each strain. We were able to identify three distinct clusters of strains, I to III (Fig. 1B). Group I contains *B. abortus* S99, *O. intermedium* ENCB, and *O. intermedium* 24694; group II includes *O. anthropi* 772, 25934, 24566, 20050, 24695, 28303, 30656, 32009, and 33786; group III contains only *O. anthropi* 29689. Each of these clusters contains members with a distance index of around 0.3, which is also the approximate relationship found between *O. intermedium* and *B. abortus*.

Most *Ochrobactrum* strains group within a single cluster (cluster II) (9), sharing multiple AFLP bands. A few strains were more distant (Fig. 1, clusters I and III). One strain in particular (29689) separates from the rest, with an average similarity of only 0.52. More strains should be analyzed to

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2537
determine the importance of cluster III within the genus. Analysis of a larger number of strains isolated from more diverse environments may reveal an even more diverse picture and other strains more closely related to 29689.

We found *O. intermedium* to be closely related to *B. abortus* S99, even closer than any other strain belonging to the *Ochrobactrum* genus, coinciding with previous reports (18, 22). The close relationship between *O. intermedium* and *B. abortus* S99 is shared by all the other members of the genus *Brucella* in view of the fact that, unlike *Ochrobactrum* strains, the *Brucella* strains showed a marked similarity by AFLP analysis that included reference, vaccine, and field strains of *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* (data not shown). The low diversity of *Brucella* has been shown in previous reports (6, 16, 23).

*O. intermedium* ENCB and *O. intermedium* 24694 have a high degree of similarity, observable even with the naked eye (Fig. 1A). Both of these strains gave positive results with the PCR designed for *O. intermedium* (22). These strains were donated from two different laboratories but showed a remarkable similarity, suggesting either that they share a recent clonal origin or that the DNA sequences of the genome of the *O. intermedium* species are far more conserved than the rest of the genus. AFLP is presented here as a reliable alternative to classical morphological and physiological identification of *O. intermedium*, since there is no useful phenotypic pattern to discriminate the two *Ochrobactrum* species.

The results presented here suggest that *Ochrobactrum* is a genetically diverse genus, with a similarity of 0.52 or higher. This genus is so diverse that it includes bacteria isolated from diverse environments and its members have previously been classified within the genera *Pseudomonas*, *Micrococcus*, and...
Achromobacter. Ochrobactrum has been described as a physiologically diverse genus and shown to have only 163 out of 284 phenotypic characteristics (57%) in common among its members (9, 22). These bacteria are being isolated from a wide variety of environments and described as presenting a wide variety of metabolic features. An adaptable physiology suggests an equally adaptable genome. It will be interesting to investigate whether genetically unstable genomes underlie the adaptability of this genus and whether inherently unstable regions provide the molecular basis of the observed natural variation.

The close relationship between the genera Ochrobactrum and Brucella has been repeatedly documented in recent years and both old and new data sustain the proposal of revising the family Brucellaceae (6). Our results suggest that this family should include only Brucella and Ochrobactrum. This conclusion is based on the work presented here, the bibliography cited throughout this report, and DNA hybridization studies performed using Brucella probes on species of Ochrobactrum, Phyllobacterium, Rhizobium, Agrobacterium, Bradyrhizobium, Pseudomonas, Vibrio, Yersinia, and Escherichia (13), where only Ochrobactrum presented the greatest number of hybridizing strains at the highest stringencies.

It seems that the putative Brucellaceae family includes members of an opportunistic nature and, accordingly, highly adaptable genomes (mostly within cluster II). In agreement with previous reports, our results may also suggest that other members of the family are bacteria of highly specific habitats and therefore they may have highly conserved DNA sequences, such as Brucella spp. and O. intermedium (cluster I) (6, 16, 23).

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