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OXA-258 from *Achromobacter ruhlandii*: a Species-Specific Marker

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A new $bla_{OXA-258}$ gene is described as a species-specific taxonomic marker for *Achromobacter ruhlandii* isolates (all recovered from cystic fibrosis patients). Even though OXA-258 differs from OXA-114 variants, isolates could be misidentified as *A. xiloxosidans* by the amplification of an inner fragment from the OXA-coding gene. A robust identification of *A. ruhlandii* can be achieved by sequencing this single OXA gene, as well as by a more laborious recently proposed multilocus sequence-typing (MLST) scheme.

Although *Achromobacter xylosoxidans* was the only species when the genus *Achromobacter* was proposed (1), the genus currently comprises several species: *A. xylosoxidans* (the type species of the genus), *A. denitrificans*, *A. insolitus*, *A. marplatensis*, *A. piechaudii*, *A. ruhlandii*, and *A. spanius* (2). *A. ruhlandii* and *A. piechaudii* were reassigned from the genus *Alcaligenes*, while *A. xylosoxidans* subsp. *denitrificans* was reclassified as *A. denitrificans* (3). *A. spanius*, *A. insolitus*, and *A. marplatensis* were described later (4, 5). Moreover, the existence of several additional species, such as *A. animicus*, *A. mucicolens*, *A. pulmonis*, and *A. spiritinus*, has been recently reported (6, 7), so the taxonomic complexity of the genus is expected to increase.

A. xylosoxidans is recognized as being capable of causing persistent respiratory tract infections in cystic fibrosis (CF) patients, although its pathogenic role in disease progression remains to be elucidated (8). Prevalence rates of *Achromobacter* spp. recovered from respiratory secretions have increased in recent years, probably due to the extended life span of CF patients and the selective pressure imposed by multiple-antimicrobial therapy, but also to improved microbiological and molecular techniques (9).

Accurate species identification of *Achromobacter* isolates is difficult, and clinical isolates of *Achromobacter* are mostly referred as *A. xylosoxidans*. Also, *Achromobacter* species other than *A. xylosoxidans* have been considered environmental inhabitants and only recently associated with human infections (6, 9, 10). However, a clone of *A. ruhlandii*, designated the Danish epidemic strain (DES), spread among CF patients in Denmark in 2006, and its dissemination could not be stopped despite the control measures applied (10, 11).

In this study, different phenotype-based techniques and molecular tools were used to characterize *Achromobacter* isolates recovered from CF patients.

Twenty-eight clinical isolates of *Achromobacter* recovered from CF patients were included. Isolates were derived from respiratory secretions (19 isolates), blood (5 isolates), urine (2 isolates), skin and soft tissues (1 isolate), and bone (1 isolate). Phenotypic identification was performed by conventional biochemical tests and API 20 NE (bioMérieux) (3, 7). Twenty-three isolates could be identified as *A. xylosoxidans*. However, the results obtained for five isolates (namely, 38, 39, 67, 80, and 319) were not conclusive to identify them at the species level (Table 1). According to Vandamme et al. (7), these five isolates could correspond to *A. ruhlandii*, as galactose oxidation and nitrite reduction were negative; these reactions have been proposed as differentiating tests for the identification of *Achromobacter* species. Growth on acetamide has also been mentioned as a differential reaction, rendering positive results for *A. xylosoxidans* and negative results for *A. ruhlandii*; however, all the isolates included in this study except one (isolate 67) rendered a positive result. Bio codes and identification results obtained using the API 20 NE System (bioMérieux) are shown in Table 2.

Total protein extracts for these five isolates and a previously characterized *A. xylosoxidans* strain were obtained as previously described (12). Electrophoretical total protein profiles in 12% SDS-PAGE were identical among isolates 38, 39, 67, 80, and 319 but different from those of *A. xylosoxidans*.

The 16S rRNA gene was amplified by PCR (13). Purified amplicons were sequenced in both strands using an ABI Prism DNA 3700 sequencer and compared with databases using NCBI's BLAST. They displayed about 99% identity to the sequences of different species of *Achromobacter* and were unable to discriminate among them.

A multilocus sequence-typing (MLST) scheme was conducted to identify species of *Achromobacter* in those isolates that could not be unambiguously identified by biochemical reactions. For this, fragments of seven conserved housekeeping genes were amplified and sequenced, as recently described (6). Allele profiles and sequence types (ST) were assigned according to the *Achromobacter* MLST website (http://pubmlst.org/achromobacter/). Isolate 319 displayed the following alleles: *nusA*, 19; *rpoB*, 33; *eno*, 5; *gtlB*, 15; *lepA*, 47; *nuoL*, 19; and *nrdA*, 12, corresponding to *A. ruhlandii* ST 43. The other four isolates (38, 39, 67, and 80) displayed new alleles for three of these seven genes, showing the following allelic

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TABLE 1 Biochemical identification of *Achromobacter* sp. isolates

Biochemical test	Results[a] for: Clinical isolates: Ax isolates[c]	38	39	80	67	319	Control strains[b]: *A. xylosoxidans* ATCC 27061	*A. denitrificans* ATCC 15173	*A. ruhlandii* ATCC 15749	*A. piechaudii* ATCC 43552
Oxidation of:										
Glucose	+	+	+	+	+	+	+	−	(+)	−
Lactose	−	−	−	−	−	−	−	−	−	−
Galactose	+	−	−	−	−	−	+	−	−	−
Maltose	−	−	−	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−	−	−	−
Fructose	−	−	−	−	−	−	−	−	−	−
Manitol	−	−	−	−	−	−	−	−	−	−
Xilose	+	+	+	+	+	+	+	−	(+)	−
Glycerol	(+)	−	−	+	(+)	−	(+)	(+)	−	−
L-Arabinose	−	+	−	+	+	+	−	−	(+)	−
D-Arabinose	(+)	+	−	−	+	+	(+)	−	+	−
Growth on acetamide	+	+	+	+	−	+	NE[d]	NE	NE	NE
Urea-Christensen	−	−	−	−	−	−	−	−	−	−
Hydrolysis of esculin	−	−	−	−	−	−	−	−	−	−
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Nitrite reduction	+	−	−	−	−	−	+	+	−	−
Arginine dehydrolase	−	−	−	−	−	−	−	−	−	−
Pyr[e] hydrolysis	+	+	+	+	+	+	NE	NE	NE	NE

[a] +, positive; (+), delayed reaction; −, negative.
[b] Data from reference 3.
[c] Clinical isolates identified as *A. Xylosoxidans* ($n = 23$).
[d] NE, not evaluated in reference 3.
[e] Pyr, *p*-naphthylamide pyrrolidonyl.

profile: *nusA*, 17; *rpoB*, 32; *eno*, 74; *gtlB*, 7; *lepA*, 80; *nuoL*, 19; and *nrdA*, 73, belonging to a new ST assigned as ST 148. An alignment of the concatenated sequences (2,254 nucleotides) of these seven fragments with those corresponding to 142 *Achromobacter* spp. available in the database was conducted using the Clustal X 2.1 program. The statistical selection of the best-fit model of nucleotide substitution was assayed by JModelTest. Maximum-likelihood estimation was conducted using PhyML 3.0, while the distances method was performed using the MEGA5 program. A bootstrap with 1,000 resample matrices for both methods was executed to assess the statistical support for the identified groups (14). The dendrogram obtained with maximun likelihood is depicted in Fig. 1, showing that these five isolates grouped with *A. ruhlandii* strains. A similar tree was achieved when applying the distance method (data not shown).

TABLE 2 Biochemical identification performed with the API 20 NE system

Clinical isolate	Bio code	Identification (%)
Ax isolates[a] ($n = 23$)		
$n = 12$	1042477	*A. xylosoxidans* (99.9)
$n = 8$	1042467	*A. xylosoxidans* (99.9)
$n = 3$	1040477	*A. xylosoxidans* (94.5)
38	1042465	*A. xylosoxidans* (92.2)
39	1042465	*A. xylosoxidans* (92.2)
67	1040467	*A. xylosoxidans* (89.4)
80	1000465	*A. denitrificans* (46.7)
319	1042467	*A. xylosoxidans* (99.9)

[a] Clinical isolates identified as *A. xylosoxidans.*

The presence of the *A. xylosoxidans* species-specific marker $bla_{\text{OXA-114}}$ was investigated by PCR amplification as described by Turton et al. (15) with positive amplification in all isolates. Purified amplicons were sequenced in both strands as described above, and the nucleotide sequences were compared with those of all the different $bla_{\text{OXA-114}}$ allelic variants deposited in GenBank. Amplicons obtained from the isolates identified as *A. xylosoxidans* displayed 99 to 100% identity to the $bla_{\text{OXA-114}}$ variants. However, the nucleotide sequences of the amplicons corresponding to the five *A. ruhlandii* isolates differed significantly from those of all the $bla_{\text{OXA-114}}$ alleles. The complete sequence for the new bla_{OXA} gene was obtained using a thermal asymmetric interlaced (TAIL)-PCR approach (16). The enzyme was assigned the number OXA-258. This new class D β-lactamase differed from OXA-114a by 40 amino acids, displaying 85% identity (Fig. 2). Also, a variant of the enzyme, referred to here as OXA-258a, that differed in 1 amino acid was found in *A. ruhlandii* 319, corresponding to OXA-258b (Fig. 2).

Restriction sites of different endonucleases were inferred *in silico* for all $bla_{\text{OXA-114}}$ allelic variants deposited in GenBank in order to design an accurate restriction fragment length polymorphism (RFLP) PCR assay able to differentiate the $bla_{\text{OXA-258}}$ gene from all $bla_{\text{OXA-114}}$ variants. ApaI (Amersham Phamacia Biotech) was used to digest the amplicons obtained by the PCR proposed by Turton et al. (15). For this, amplicons were incubated with 15 U of this restriction enzyme for 2 h at 37°C. As expected, ApaI was able

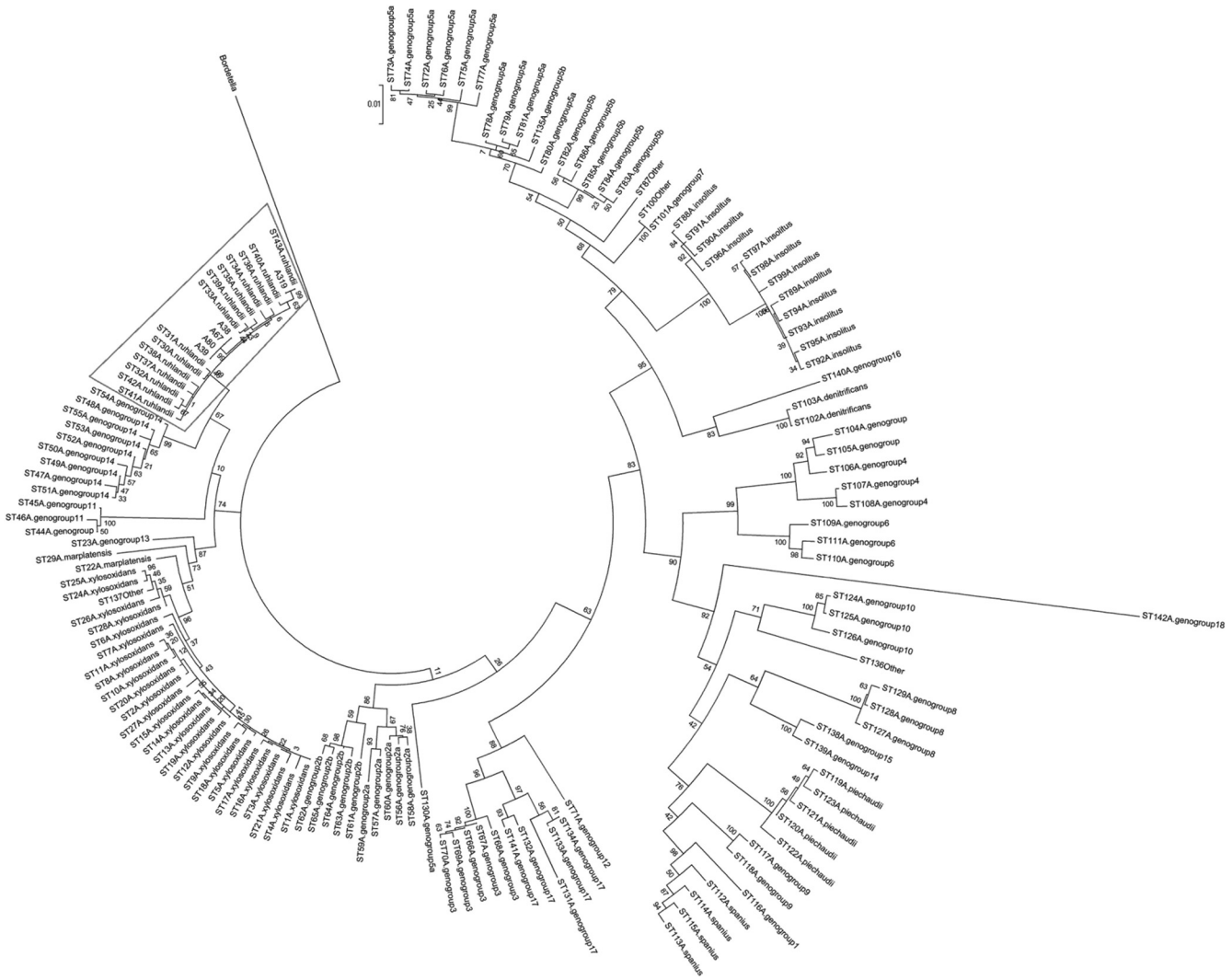

FIG 1 Dendrogram based on maximum-likelihood estimation conducted using PhyML 3.0 for 147 *Achromobacter* sp. concatenated housekeeping genes.

to digest $bla_{OXA-258}$ amplicons obtained from *A. ruhlandii* isolates but none of those for $bla_{OXA-114}$ variants obtained from *A. xylosoxidans* isolates.

Although at present *Achromobacter* is considered an emerging pathogen in CF patients, species of the genus are frequently misidentified (10, 11). Identification based on biochemical reactions was awkward and sometimes subjective and was conclusive only for true *A. xylosoxidans* isolates. In agreement with recent reports (6, 15), ARNr 16S gene sequencing was not able to discriminate among different species of *Achromobacter*. CLSI recommendations proposing this tool, when the phenotypic approach was not enough for species level identification (17), were made before the publication of the majority of today's species within the genus.

In 2008, the *A. xylosoxidans* species-specific class D β-lactamase OXA-114 was described (18), and later, in 2011, Turton et al. proposed the identification of the species based on the PCR amplification of an inner fragment of the coding gene (15). In our hands, not only *A. xylosoxidans* isolates, but also *A. ruhlandii* rendered positive amplifications, leading to the description of a new OXA-coding gene when *A. ruhlandii* amplicons were sequenced. This highlights the fact that amplification of the inner fragment without further sequencing, as initially described, may be misleading, resulting in the misidentification of some species of *Achromobacter*. A robust identification may be achieved by sequencing only this bla_{OXA} gene, as well as with the MLST scheme recently proposed (6), based on a comparison of either the allelic profiles or the concatenated sequences. Both display remarkable discriminatory power among *Achromobacter* species, allowing the identification of *A. ruhlandii* isolates. However, ApaI-based fragment restriction may constitute a convenient alternative to discriminate between $bla_{OXA-258}$, described in the present study and ubiquitous in *A. ruhlandii*, and the $bla_{OXA-114}$ variants present in *A. xylosoxidans*, leading to accurate, easy, and fast identification in laboratories that may not be able to achieve sequencing.

It is clear that not only *A. xylosoxidans* represents the genus in these patient secretions, suggesting that the roles of the other species need to be reevaluated and that proper identification is absolutely necessary to understand the epidemiology and clinical impact of the different *Achromobacter* species in this pathology and to guide proper antimicrobial therapy.

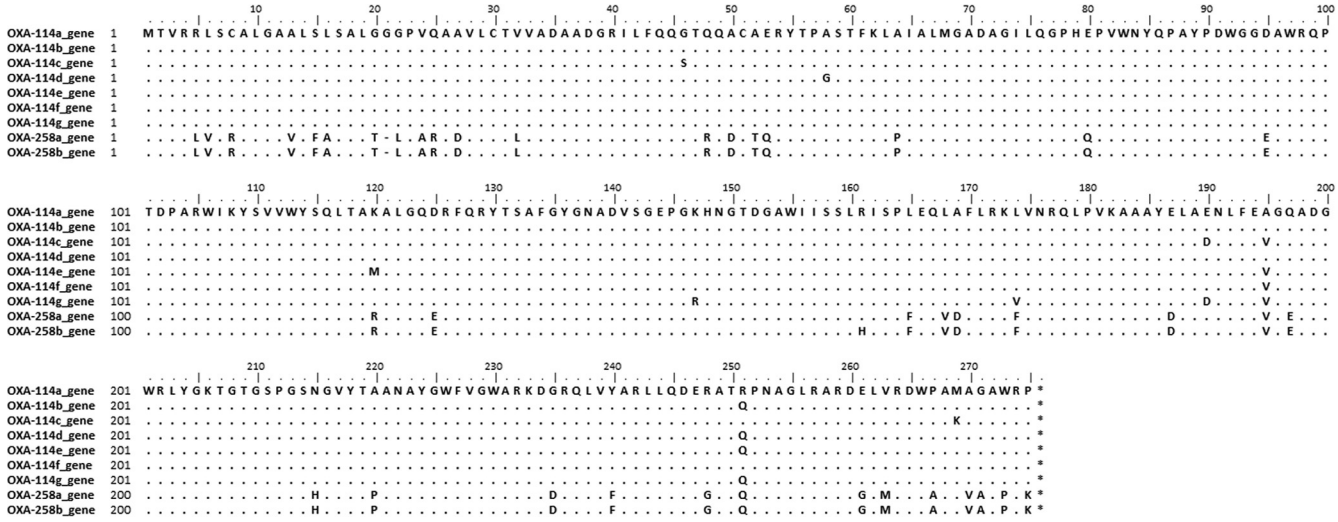

FIG 2 Clustal X 2.1 alignment of OXA-258 variants with respect to OXA-114 variants.

Nucleotide sequence accession numbers. The sequences for the enzymes OXA-258 and OXA-258a were assigned GenBank accession numbers EMBL HE614014.1 and EMBL HF562855, respectively.

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We declare no competing interests.

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