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Presence of OXA-Type Enzymes in *Achromobacter insuavis* and *A. dolens*

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Abstract The accurate species identification of *Achromobacter* isolates is difficult and the clinical isolates of this genus are mostly referred as *A. xylosoxidans*. Here, we report new OXA variants in 2 isolates identified as *A. insuavis* (A114, A79) and 1 isolate identified as *A. dolens* (A336). These results suggest that different bla_{OXA} genes are ubiquitous in the different species of *Achromobacter* spp. The role of the other species of *Achromobacter* in clinical samples needs to be reevaluated, and the proper identification is absolutely necessary to understand the epidemiology of this genus.

Introduction

Achromobacter xylosoxidans is recognized as capable of causing persistent respiratory tract infections in cystic fibrosis (CF) patients. Prevalence rates of *Achromobacter* spp. recovered from respiratory secretions have increased in recent years, probably due to the extended CF patients' life span, the selective pressure imposed by the multiple antimicrobial therapy, and the improved microbiologic and molecular techniques [1].

In Argentina, Vay et al. reported that the relative frequency of *Achromobacter* spp. isolates among non-fermentative gram-negative bacilli (NFGNB) infections, excluding *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* corresponded to 6,6 % of the total number of isolates (35/529), in a hospital setting (Personal communication). Accurate identification of *Achromobacter* species is difficult, and clinical isolates of *Achromobacter* are mostly referred as *A. xylosoxidans* leading to serious difficulties in determining the pathogenic role of the other species that may be also associated to persistent respiratory tract infections in CF patients [1–4].

Recently, Spilker et al. [1] have shown that only the 42 % of *Achromobacter* infections in CF patients in the U.S.A corresponded to *A. xylosoxidans,* whereas other species such as *A. ruhlandii* (23.5 %), *A. dolens* (17 %), *A. insuavis* (4.4 %), and *A. aegrifaciens* (3.8 %) were also recovered.

In 2011, Turton et al. [5] proposed the amplification of an inner fragment of the $bla_{OXA-114}$ gene as a PCR-based method for specific identification of *A. xylosoxidans*. Thirteen different $bla_{OXA-114}$ and even four different $bla_{OXA-243}$ variants were reported in isolates identified as *A. xylosoxidans* [2, 5–7]. Moreover, $bla_{OXA-258}$ has been identified in *A. ruhlandii* clinical isolates [8].

The aim of the present study was to determine the presence of bla_{OXA} genes in clinical isolates of *Achromobacter* different from *A. xylosoxidans,* in order to investigate if different $bla_{OXA-like}$ genes are ubiquitous in other *Achromobacter* species.

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Table 1 Biochemical and molecular identification of *Achromobacter* spp. isolates

Results[a] for							
Biochemical test	Clinical isolates			Control strains[b]			
	A79	A114	A336	*A. xylosoxidans* ATCC 27061	*A. denitrificans* ATCC 15173	*A. ruhlandii* ATCC 15749	*A. piechaudii* ATCC 43552
Oxidation of							
Glucose	+	+	−	+	−	(+)	−
Lactose	−	−	−	−	−	−	−
Galactosa	+	+	−	+	−	−	−
Maltose	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−
Fructose	−	−	−	−	−	−	−
Manitol	−	−	−	−	−	−	−
Xilose	+	+	+	+	−	(+)	−
Glycerol	(+)	(+)	−	(+)	(+)	−	−
L-Arabinose	−	−	+	−	−	(+)	−
Growth on acetamida	+	+	+	ne[d]	ne	ne	ne
Urea—Christensen	−	−	−	−	−	−	−
Hydrolysis of esculin	−	−	−	−	−	−	−
Nitrate reduction	+	+	+	+	+	+	+
Nitrite reduction	+	+	−	+	+	−	−
Arginine dehydrolase	−	−	−	−	−	−	−
Pyr[c] hydrolysis	+	+	+	ne	ne	ne	ne
API 20 NE	1042477 99 % *A. xylosoxidans*	1042477 99 % *A. xylosoxidans*	1042467 99 % *A. xylosoxidans*				
MALDI-TOF	A. *xylosoxidans* 2.048	*A. xylosoxidans* 2.048	*A. xylosoxidans* 2.01	ne	ne	ne	ne
16S rRNA gene sequence	99 % *A. xylosoxidans*	99 % *A. xylosoxidans*	99 % *A. xylosoxidans*	ne	ne	ne	ne
MLST	*A. insuavis* ST64	*A. insuavis* ST165	*A. dolens* ST 164	ne	ne	ne	ne

[a] + positive; (+) delayed reaction; − negative

[b] Data from reference [9]

[c] *p*-naphthylamide pyrrolidonyl

[d] *ne* not evaluated in reference [9]

Materials and Methods

Bacterial Strains

From a total of 27 *Achromobacter* spp. clinical isolates, recovered from CF patients during 2002–2007, 19 were identified as *A. xylosoxidans* [7], 5 corresponded to *A. ruhlandii* [8], while 3 isolates (A79, A114 and A336) remained to be accurately identified.

Bacterial Identification and DNA Techniques

Conventional phenotypic tests, API 20NE (bioMérieux), matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry (MS) (Bruker Daltonik), PCR amplification and sequencing of the 16S rRNA gene, and a MLST approach were performed in order to arrive to a correct identification of the species [9, 10] (Table 1).

Table 2 Antibiotic susceptibility (MIC) of the isolates (A114, A79 and A336) included in this study

Isolate numbers	MIC (μg/ml)		
	A114	A79	A336
Ampicillin	≥32	≥32	≥32
Ampicillin/sulbactam	≥32	≥32	≥32
Piperacillin/Tazobactam	≤4	≤4	≤4
Cefalotin	≥64	≥64	≥64
Cefotaxime	≥64	≥64	≥64
Ceftazidime	≥64	8	8
Cefepime	≥64	≥64	≥64
Imipenem	1	4	1
Meropenem	≤0.25	≤0.25	≤0.25
Amikacin	≥64	≥64	≥64
Gentamicin	≥16	≥16	≥16
Ciprofloxacin	≥4	≥4	4
SXT	≥4	≤2	≤2
Colistin	≤0.5	2	≤0.5

Total DNA was extracted using the Master Pure DNA purification kit by following manufacturer's instructions (Epicentre, Madison, WI, USA). Amplification of the 16S rRNA gene was carried out with the primers fD2 5′-AGAGTTTGATCATGGCTCAG and Rp2 5′-ACGGCTACCTTGTTACGACTT described by Weisburg et al. [11]. MALDI-TOF spectrometer and MALDI Biotyper 2.0 software (Bruker Daltonics, Bremen, Germany) were used according to manufacturers' recommendations.

For the Multilocus Sequence Typing (MLST) approach, the scheme suggested by Spilker et al. [12] was conducted. Allele profiles and sequence types (ST) were assigned according to http://pubmlst.org/achromobacter/.

The antibiotic susceptibility test was performed using the VITEK 2 System employing the panel AST-279 (GNS susceptibility card). The MIC results were interpreted using CLSI categories [13].

The presence of bla_{OXA} genes was investigated by the PCR amplification proposed by Turton et al. [5]. To further characterize the OXA-type coding genes present in these isolates, the complete sequence of bla_{OXA} genes was obtained by PCR reactions using the primers EntF (5′-CTGGGACATCCGCCTGTC-3′) and AXXAR (5′-ACCAGCAGGATCGACAGTC-3′) [2, 7].

All PCR reactions were carried out in 50 μl volumes containing 10 ng of DNA, 10 μl of 5X PCR buffer, 0.5 μl of 10X deoxynucleoside triphosphate mix (2 mM each dATP, dCTP, dGTP, and dTTP), 2 μl of each primer stock solution (2.5 pmol of each primer per μl), and sterile distilled water. Taq DNA polymerase was added according to manufacturer's instructions (Promega, Madison, USA). The thermocycler used was from Perkin-Elmer Cetus, Emeryville, CA, and a three-step profile was used.

Sequence Analysis

Amplicons sequencing were carried on both DNA strands using an ABIPrism 3100 BioAnalyzer and Taq FS Terminator Chemistry (Taq FS, Perkin-Elmer). Sequences were analyzed and assembled with Sequencher 4.7 software (Gene Codes Corp.) and BLAST (version 2.0) software (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence comparison and phylogenetic analysis were performed using Clustal X (version 2.0) and Mega5 (version 5.05) softwares.

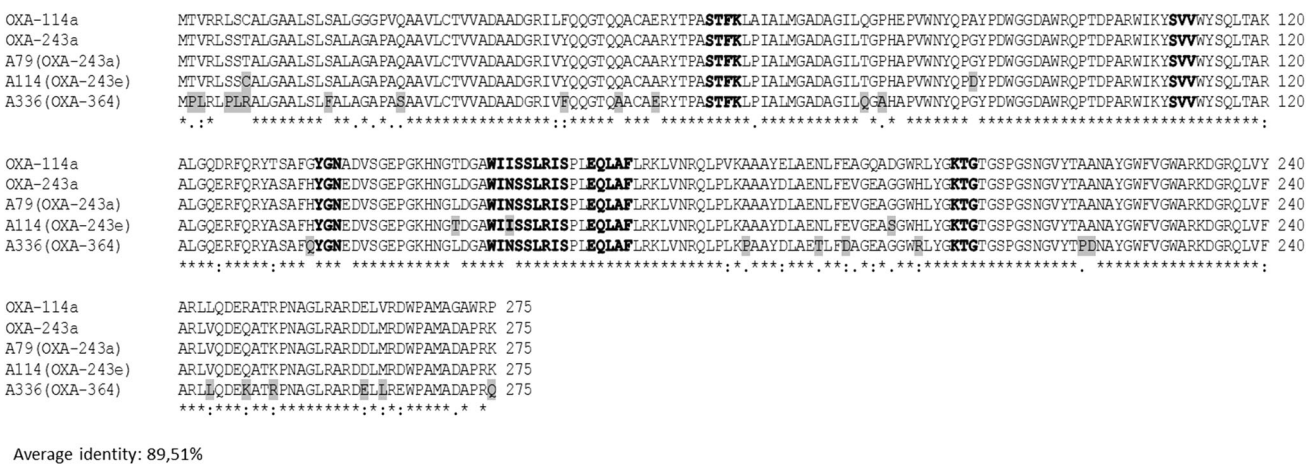

Fig. 1 **a** Amino acid sequence comparison of the OXA enzymes described in this study. In *bold* it was represented the oxacillinase-conserved amino acid motifs (S–T–F–K, Y–G–N, S–V–V, E–Q–L–A–F, W–I–I–S–S–L–R–I–S and K–T–G). The comparison was performed using the OXA-114a as reference. The *highlight letters* showed changes in the amino acid sequence of the new variant (OXA-243e) and the novel OXA (OXA-364) compared to OXA-243a. In *white letters* it was represented the amino acid substitutions inside the conserved motifs

Results and Discussion

Neither biochemical methods performed (conventional phenotypic tests, API 20NE and MALDI-TOF) nor 16S rRNA gene sequencing was conclusive to identify the isolates A79, A114, and A336 at the species level. Instead, the MLST scheme suggested by Spilker et al. [12] allowed the correct identification of the 3 isolates. The isolates A79 and A114 were identified as *A. insuavis* (ex genogroup 2b), ST 64 and ST165, respectively, and isolate A336 corresponded to *A. dolens* (ex genogroup 14) ST 164 [14]. ST 164, and ST 165 were not previously described. The 3 isolates were resistant to aminoglycosides, fluorquinolones, and third and fourth-generation cephalosporins, with the exception of ceftazidime in the isolates A114 and A336. Table 2 showed the obtained MIC values results for the 3 analyzed isolates.

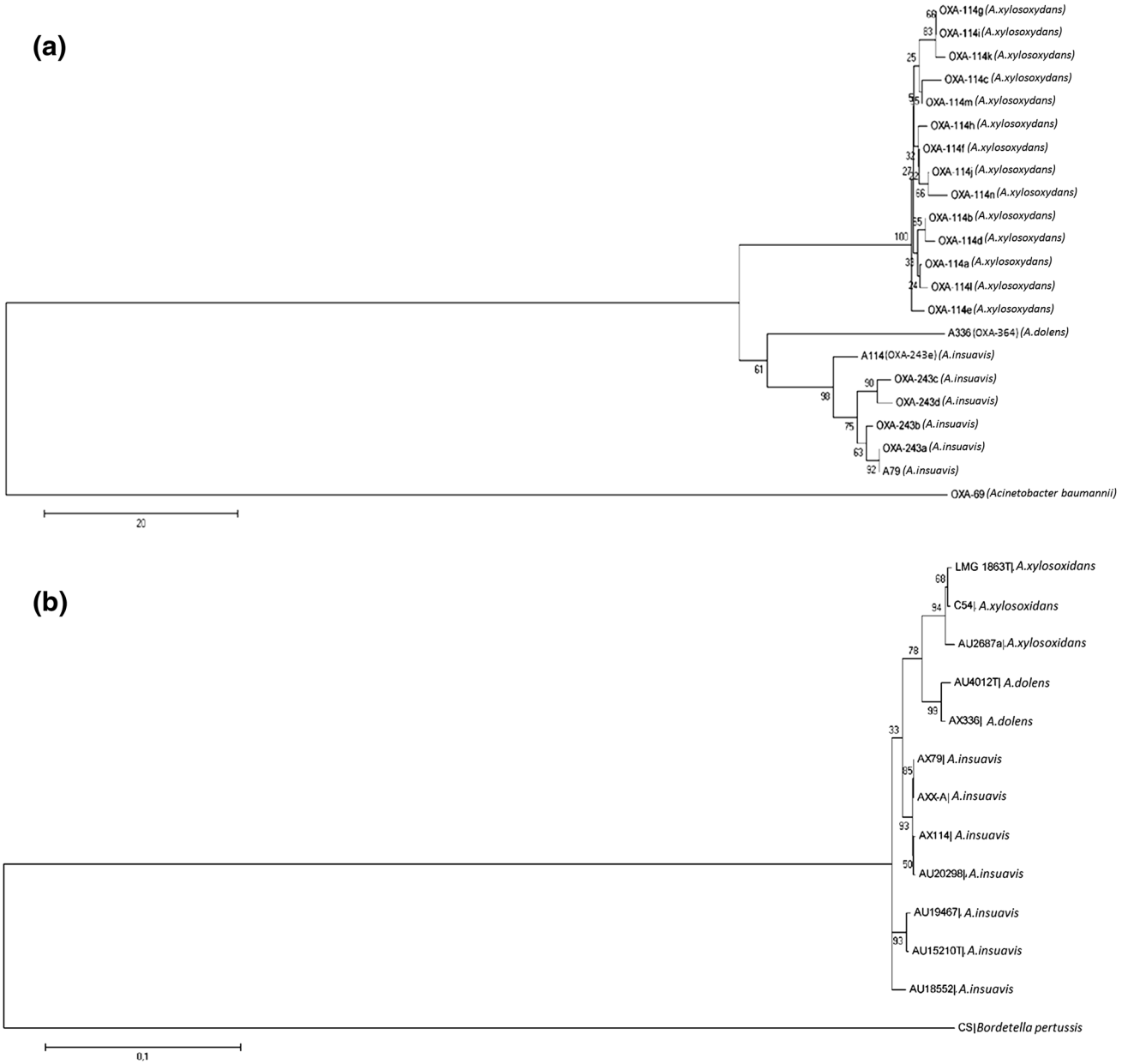

Fig. 2 **a** Tree representing the OXA enzymes. The dendrogram was inferred using the neighbor-joining method for OXA-114, OXA-243, and the OXAs from this study. *Branch lengths* are drawn to scale and are proportional to the number of amino acid changes. The distance along the *vertical axis* has no significance. **b** Tree of the species harboring OXA-114, OXA-243, the new variant (OXA-243e) and the novel OXA (OXA-364) enzymes. The dendrogram was inferred using the neighbor-joining method of the concatenated housekeeping genes of representatives *Achromobacter* species

In addition, the 3 isolates gave positive amplification for the presence of bla_{OXA} genes using the primers proposed by Turton et al. [5]. Sequence analysis of these amplified fragments showed 87–89 % of nucleotide identity (translated in 15–28 amino acid changes) in comparison with the $bla_{OXA-114a}$. The complete sequence for these genes was obtained using the primers EntF and AXXAR [7]. The $bla_{OXA-type}$ harbored by isolate A114 displayed 97 % of identity to the previously described $bla_{OXA-243a}$, resulting in 5 amino acids substitutions (Fig. 1a). This novel OXA-243 (JX306688) was named as OXA-243e. The $bla_{OXA-type}$ gene harbored by isolate A79, corresponded to the previously described $bla_{OXA-243a}$ [2]. Isolate A336 harbored a new OXA-type enzyme which displayed 26 amino acids substitutions respect OXA-243a (90 % of identity) (Fig. 1a), named as OXA-364 (JX306689).

The dendrogram obtained applying the Distances Method (Fig. 1) clearly showed that the variants found in *A. insuavis* grouped with the OXA-243 cluster and variant found in *A. dolens* grouped in a different branch (Fig. 2a). Figure 2b, constructed with the concatenated MLST genes proposed by Spilker et al. [12], represents the distances among the species harboring the mention OXA-type enzymes (Fig. 2b).

Here, we described the presence of the new OXA-364 in *A. dolens* and the presence of OXA-243 variants (OXA-243a and OXA-243e) in *A. insuavis*. The OXA-243 variants have been previously described in *A. xylosoxidans* clinical isolates, however, since MLST technique was not employed to define species in that report [2], we consider that probably a more accurate identification would be useful to define if that OXA-type enzyme is present in both *Achromobacter* species or if it is naturally harbored by *A. insuavis*.

Different OXA-type coding genes have been reported as species-specific markers in several species, including *A. xylosoxidans*.

The results obtained from the comparison analysis of both trees showed that the OXA genes can clearly discriminate among the *Achromobacter* species including in this study, letting to the correct identification of the different species.

Although a mayor number of isolates should be analyzed, our results suggest that different OXA-type coding genes seem to be specie-specific markers within *Achromobacter*. A robust identification of *Achromobacter* species should be achieved by sequencing the amplified fragment obtained in the PCR reaction proposed by Turton et al. as well as with the more laborious MLST scheme. Nowadays, it is clear that not only *A. xylosoxidans* represents the genus in CF secretions, but also suggesting that the role of the other species needs to be reevaluated and that proper identification is absolutely necessary to understand the epidemiology.

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