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ORIGINAL ARTICLE

# Full characterization of plasmids from *Achromobacter ruhlandii* isolates recovered from a single patient with cystic fibrosis (CF)

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**KEYWORDS**
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**Abstract** In the last decade *Achromobacter* spp. has been associated with chronic colonization in patients with cystic fibrosis (CF). Although *Achromobacter xylosoxidans* is the most frequent species recovered within this genus, other species such as *A. ruhlandii* have also been reported in these patients. Descriptions of mobile elements are scarce in *Achromobacter* and none of them have been originated in *A. ruhlandii*. The aim of this study was to report the full characterization of a plasmid which was maintained in four clonally related *A. ruhlandii* isolates. Between 2013 and 2015, nine *A. ruhlandii* isolates were recovered from a pediatric patient with CF at a hospital in Buenos Aires. Four selected clonally related isolates were sequenced by Illumina MiSeq, annotated using RAST and manually curated. The presence of a unique plasmid of 34096-bp and 50 CDS was observed in the four isolates, displaying only 1 nucleotide substitution translated into one amino acid change among them. These plasmids have a class 1 integron containing the *aac-(6′)-Ib* gene, a mercury resistance operon region and the *relE/stbE* toxin/antitoxin system. Plasmids showed 79% similarity and 99% identity with pmatvim-7 from *Pseudomonas aeruginosa*. This is the first full description and characterization of a plasmid from *A. ruhlandii* which was maintained over time.


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**PALABRAS CLAVE**
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**Caracterización completa de plásmidos provenientes de aislamientos de *Achromobacter ruhlandii* recuperados de un único paciente con fibrosis quística**

**Resumen** Durante la última década, *Achromobacter* spp. han sido asociadas con la colonización crónica en pacientes con fibrosis quística. Si bien *Achromobacter xylosoxidans* es la especie más frecuentemente recuperada, otras especies como *Achromobacter ruhlandii* también fueron reportadas en nuestra región. Sin embargo, pocos reportes se han centrado en la descripción de elementos móviles, y ninguno de ellos los documenta en *A. ruhlandii*. El objetivo de este estudio fue reportar la caracterización completa de un plásmido conservado en 4 aislamientos clonalmente relacionados de *A. ruhlandii*. Se recuperaron 9 aislamientos de *A. ruhlandii* entre 2013 y 2015 de un único paciente con fibrosis quística proveniente de un hospital pediátrico de Buenos Aires, Argentina. Se realizó la secuenciación completa del genoma de los 4 aislamientos seleccionados según el perfil de resistencia antibiótica en un equipo Illumina MiSeq. Estos fueron anotados mediante RAST y curados manualmente. Se detectó la presencia de un solo plásmido de 34.096 pb y 50 CDS en los 4 aislamientos, observándose únicamente un cambio nucleotídico traducido en un cambio aminoacídico en un aislamiento. Los plásmidos ensamblados se caracterizaron por presentar un integrón de clase 1 que contenía el gen *aac-(6′)-Ib*, un operón de resistencia a mercurio y el sistema de toxina-antitoxina *relE/stbE*. Cabe destacar que estos plásmidos poseen un 79% de similitud y un 99% de identidad con el plásmido pmatvim-7 de *Pseudomonas aeruginosa*. Esta es la primera descripción y caracterización completa de un plásmido proveniente de *A. ruhlandii*.


## Introduction

*Achromobacter* spp. are gram negative rods capable of causing infections, including bacteremia, pneumonia and meningitis, mainly in immunocompromised patients. However, infections in immunocompetent patients have also been reported[11,13]. In addition, *Achromobacter* spp. are increasingly recognized as pathogens in patients with cystic fibrosis (CF). Different *Achromobacter* species, such as *Achromobacter xylosoxidans*, *Achromobacter ruhlandii*, *Achromobacter dolens* and *Achromobacter insuavis*, have been recovered from these patients, displaying variable frequencies among studies reported in different countries[16]. Members of this genus are naturally resistant to many antibiotics including ampicillin, cephalotin, aztreonam, cefotaxime, cefoxitin and aminoglycosides. The expression of multiple efflux pumps and the production of chromosomally encoded ß-lactamases[1] may be responsible for this resistance profile. Moreover, isolates displaying resistance to clinically relevant antibiotics such as colistin, carbapenem and fluoroquinolones have been described[16]. Little is known about the resistance markers involved in this acquired resistance profile. Thus, effective antimicrobial therapy can be challenging due to its inherent and acquired multidrug resistance patterns. Only a few plasmid-encoded ß-lactamases have been identified in *A. xylosoxidans*, as is the case of VEB-1[15] and class B carbapenemases, such as $bla_{IMP-1}$, $bla_{IMP-10}$, $bla_{VIM-11}$ and $bla_{VIM-2}$[1]. Moreover, plasmid descriptions are scarce in this genus, and no characterization of plasmids from *A. ruhlandii* have been reported, possibly due to misidentification of the species belonging to this genus.

Here we report the full characterization of a plasmid which was maintained in four clonally related *A. ruhlandii* isolates recovered from a single pediatric patient with CF, between 2013 and 2014.

## Materials and methods

Nine *Achromobacter* spp. isolates were recovered from a pediatric patient with CF at a hospital in Buenos Aires between 2013 and 2015 (isolation dates are shown in Table 1). The isolates were cultured under aerobic conditions at 37 °C on TSA (Tryptic Soy Agar) and accurate identification was achieved by amplification and sequencing of the *nrdA* gene according to Spilker et al.[18] *Xba*I-PFGE was performed according to Mireille-Cheron et al.[5] to evaluate the clonal relationship between successive isolates. Minimum inhibitory concentration (MIC) values were determined for a representative set of antibiotics: ampicillin, piperacillin, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, kanamycin, gentamicin and colistin. The antimicrobial susceptibility test was assayed by the agar dilution method according to CLSI recommendations, using the breakpoints established for the category ''other non-*Enterobacteriaceae*''[7]. The Master pure DNA purification kit (Epicenter, Madison, WI, USA) was used for DNA extraction in four selected isolates (Table 1). These 4 isolates were chosen because, in addition to being clonally related, they displayed differences in their antibiotic susceptibility profiles regarding antimicrobial therapy in patients with CF.

**Table 1** Antimicrobial resistance profiles of the tested isolates.

| Isolate–isolation date | CIM AMP (μg/ml) | CIM FEP (μg/ml) | CIM CAZ (μg/ml) | CIM CIP (μg/ml) | CIM COL (μg/ml) | CIM GEN (μg/ml) | CIM IMI (μg/ml) | CIM KAN (μg/ml) | CIM LEV (μg/ml) | CIM MER (μg/ml) | CIM PIP (μg/ml) | CIM PTZ (μg/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *1–10/13* | *32* | *128* | *16* | *4* | *64* | *>1024* | *4* | *>1024* | *4* | *32* | *≤0.5* | *0.5* |
| *2–12/13* | *>1024* | *>256* | *>256* | *4* | *256* | *>1024* | *4* | *>1024* | *4* | *32* | *512* | *512* |
| *3–12/13* | *32* | *>256* | *>256* | *4* | *64* | *1024* | *4* | *>1024* | *2* | *32* | *1* | *1* |
| 4–01/14 | 512 | 64 | 16 | 8 | 64 | >1024 | 8 | >1024 | 2 | 32 | 1 | 1 |
| 5–03/14 | 128 | 64 | 16 | 2 | 16 | >1024 | 8 | >1024 | 2 | 32 | ≤0.5 | 0.5 |
| *6–08/14* | *256* | *64* | *32* | *4* | *64* | *>1024* | *64* | *>1024* | *2* | *64* | *8* | *8* |
| 7–04/15 | 32 | 32 | 8 | 4 | 64 | >1024 | 1 | >1024 | 16 | 4 | 1 | 1 |
| 8–04/15 | 32 | 32 | 8 | 4 | 64 | >1024 | 1 | >1024 | 8 | 4 | ≤0.5 | 1 |
| 9–05/15 | 64 | 32 | 8 | 2 | 128 | >1024 | 64 | >1024 | 2 | 32 | 4 | 16 |

The isolates marked in italic (1, 2, 3 and 6) were selected for WGS.

Libraries were prepared for sequencing using the Illumina NexteraXT kit (Illumina Inc., San Diego, CA), and sequenced using paired-end reads of 150 base pairs in an Illumina NextSeq500 system at the Microbial Genome Sequencing Center. *De novo* assembly was performed with SPAdes v3.10[4], using a pre-assembly approach with Velvet v1.2.10[21]. All possible *k-mer* lengths were explored. The assembly was annotated using the RAST online server followed by manual curation. The presence of resistance determinants was assessed by Resfinder, MARA and CARD databases[10,20].

Plasmids from each isolate were assembled by contig overlapping. The comparison between plasmids was carried out using RAST, BLAST and the Artemis Comparison Tool. One of these plasmids was deposited in GenBank under AN: MK423762.1.

Plasmids from the *A. ruhlandii* isolates were extracted according to Kado and Liu[12]. Digestion with *KpnI* was carried out to compare the restriction profiles. Moreover, PCR amplification of class 1 integron carrying the *aac-(6′)-Ib* gene was performed[6].

To assess their mobilization potential, plasmids were first introduced by electroporation into *E. coli* DH5α. Transformants were selected in LBA medium containing 32 μg/ml kanamycin. Afterwards, conjugation assays were carried out on LBA agar plates using the selected transformants as donor strains and *E. coli* CAG12177 as recipient cells. Different proportions of donor and recipient cells (10:1, 2:1, 1:1) were spread on agar plates and incubated overnight at 37 °C. Transconjugants were selected on agar plates containing 32 μg/ml kanamycin and 32 μg/ml tetracycline.

## Results

Nine clonally related *A. ruhlandii* isolates were identified from a single patient, based on the results of PCR amplification and sequencing of the *nrdA* gene, and *XbaI*-PFGE (data not shown). Different resistance profiles were observed among successive isolates. All isolates were resistant to ampicillin, cefepime, and aminoglycosides, which correlates with the intrinsic resistance profile observed in *A. xylosoxidans*, and to colistin. Almost all of them were intermediate or resistant to fluoroquinolones. Piperacillin, piperacillin/tazobactam and imipenem were the most active antibiotics. Nevertheless, one *A. ruhlandii* isolate was resistant to piperacillin and piperacillin/tazobactam and four out of nine *A. ruhlandii* isolates were categorized as intermediate or resistant to imipenem (Table 1). According to the different susceptibility patterns observed, isolates 1, 2, 3 and 6 were selected for WGS.

All genomes showed a size of approximately 6 000 000 bp with 6000 coding sequences and a GC content of 67.7%. Different antibiotic resistance determinants were found in all 4 genomes corresponding to: 6 putative β-lactamases, 33 putative efflux pump coding genes, 1 aminoglycoside modifying enzyme (*aac-(6′)-Ib*), the dihydropteroate synthase and chloramphenicol O-acetyltransferase and mutations in *gyrA*, *gyrB*, *parC*, and *parD*. Among the detected β-lactamase ORFs no differences were observed among the compared isolates. These ORFs corresponded to: (i) $bla_{OXA\text{-}258a}$ previously reported as species-specific marker in *A. ruhlandii*[15]; (ii) $bla_{axc}$, recently described by Fleurbaaij et al.[9] in *A. xylosoxidans* as a novel β-lactamase with carbapenemase activity; and (iii) one molecular class A and three class C non identified β-lactamases. Complete sequences for 2 RND efflux pumps (AxyABM and AxyXY-OprZ) were detected in the 4 genomes and no differences were observed among them. AxyABM displayed 91% identity with that reported in *A. xylosoxidans* and 97% with that reported in *A. ruhlandii*[17]. The complete sequence for *axyXY-oprZ* coding genes displayed 96% identity with the previously described in *A. xylosoxidans*[2,3]. Twelve putative outer membrane coding genes were detected, being identical in all isolates. No *Pseudomonas aeruginosa* OprD porin homologues were found.

With regard to plasmid characterization, the four identified plasmids had a size of 34 096 bp with 50 coding sequences and an average G + C content of 63.3% (Supplementary Table 1).

These plasmids contained a mercury resistance operon region of 3351 bp containing *merT*, *merR*, *merP*, *merA*, *merD*, and *merE*; which were flanked by a Tn*3* family transposase and the *tra* operon. In addition, it had a partition and replication region of 2217 bp comprising genes *tnpR*, *repA*, *parA* and an ATPase gene, which showed 99% coverage and 100% identity with an IncP6 plasmid found in *P. aeruginosa* (CP033834.1). A transference region of 4923 bp containing the *trb* (*trbL*, *trbK*, *trbJ*) and *tra* (*traI*, *traJ*, *traK*) *loci* was observed. These plasmids also contained a class 1 integron of 2835 bp carrying the *aac-(6′)-Ib* gene, the *relE/stbE* toxin/antitoxin system, two copies of the dihydropteroate synthase gene, -one of them being truncated-, and one copy of the chloramphenicol-O-acetyltransferase (Fig. 1).

The four completely sequenced plasmids were almost identical. Only one of them showed one amino acid difference in both dihydropteroate synthase coding genes. The presence of this plasmid was assumed in the other clonally related isolates, based on the identical *KpnI* restriction profiles and the presence of the *aac-(6′)-Ib* gene located in the variable region of a class 1 integron displayed among them.

The overall backbone of the plasmids showed high homology (79% query cover and 99% identity) with *P. aeruginosa* plasmid pmatVim-7 (AM:778842.1), the *tra* and replication regions being almost identical as mentioned above (Supplementary Fig. 1).

The conjugation assays rendered negative results under the performed conditions.

## Discussion

In this study we report nine clonally related *A. ruhlandii* isolates, recovered from the same patient. These isolates displayed different antibiotic resistance profiles, in concordance with previous reports in this species[8]. Variations observed among these isolates suggest adaptive strategies that could lead to microevolution of resistance patterns in these patients. *In silico* analysis of *A. ruhlandii* genomes showed a great diversity of antimicrobial resistance determinants, which reveals the genomic plasticity of *Achromobacter* spp. and suggests that this genus could be a reservoir of antibiotic resistance markers. However, in the *in silico* comparative analysis no significant differences

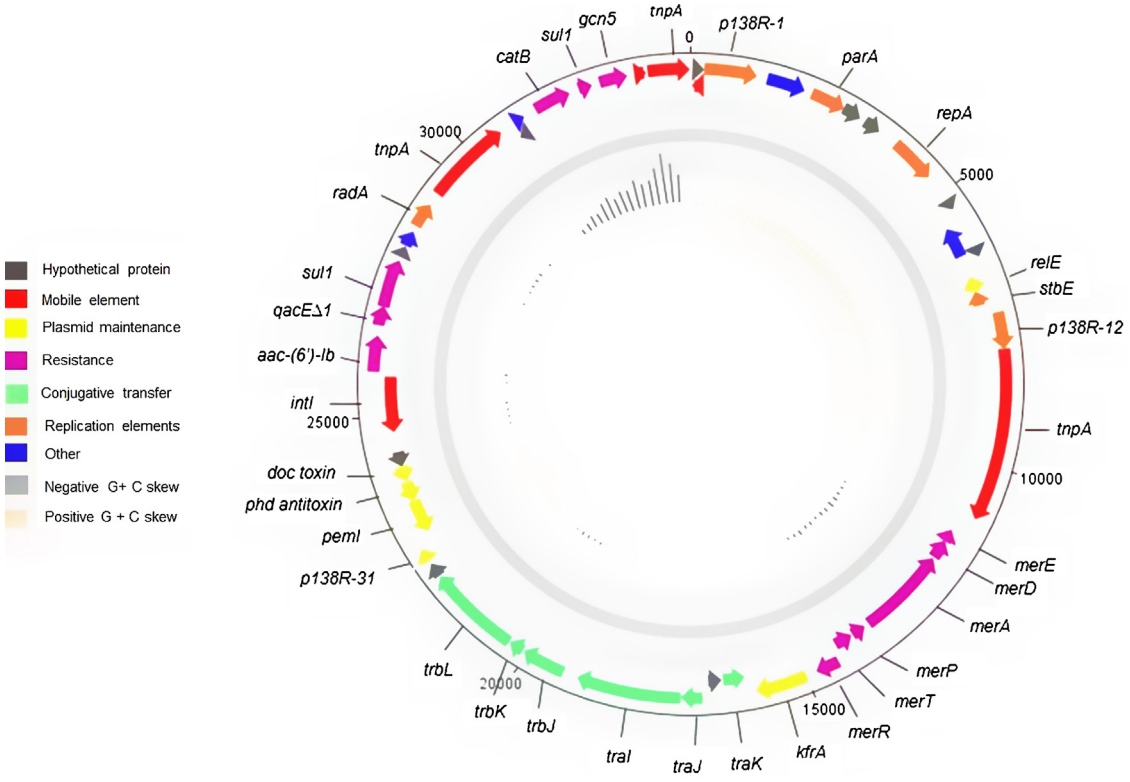


**Figure 1** Plasmid recovered from *A.ruhlandii* AN: MK423762. Overview and description.

were observed among the 4 analyzed genomes, despite their different resistance profiles. Further studies on putative resistance markers identified in the whole genome analysis should be conducted to understand the resistance patterns observed in *A. ruhlandii* isolates.

This is the first full description and characterization of a plasmid from *A. ruhlandii*. This plasmid was maintained in nine isolates recovered from the same patient. No significant mutations were found *in silico* among the four sequenced plasmids, indicating that this plasmid was preserved over time. Only an aminoglycoside resistance marker was found on this plasmid, indicating that mutations and/or variations in chromosomal gene expression should be involved in the resistant patterns observed in the isolates.

A comparative analysis of this plasmid with other 26 *Achromobacter* spp. available plasmids (*A. xylosoxidans*, *Achromobacter insolitus*, *Achromobacter denitrificans*, *Achromobacter pestifer*) indicated that the present plasmid from *A. ruhlandii* was not related with the others. The class 1 integron containing the *aac-6′-Ib* gene and the mercury resistance operon were detected in 6/26 studied plasmids. Only 2 plasmids corresponding to *A. insolitus* shared both the replicon and the addition system (*relE*/*stbE*), while 2 *A. xylosoxidans* plasmids harbored only the same replicon. The *doc*/*phD* toxin/antitoxin system was not observed in any of the studied plasmids.

Despite its high homology with pmatVim-7 from *P. aeruginosa*, $bla_{VIM-7}$ was not present in this plasmid. The metallo-carbapenemase coding gene was carried in a mobile element containing the *tnpA15* gene, which may indicate that this element could have been either acquired within *P. aeruginosa* or lost once the plasmid was incorporated into *A. ruhlandii*[14,19]. This description provides new insights into horizontally acquired elements in *A. ruhlandii*, highlighting its capability of acquiring or transferring genes horizontally from/to other non-fermenters.

The conjugation experiments performed by Li et al. on *P. aeruginosa* were not successful under laboratory conditions, probably due to the lack of certain genes on the *tra* and *trb* regions, such as *traF*, *traG*, *trbA* and *trbI*[14]. However, a putative *oriT* was detected, which could make this plasmid mobilizable. These outcomes are also in accordance with our results, which showed the failure to render transconjugants under the assayed conditions, even if some genes of the *tra* region such as *traR* and *traG* that were missing on the plasmid were encoded within the genome. However, the *traG* gene was truncated and genes of the *trb* region such as *trbP*, *trbN*, and *trbM* were missing.

This study provides new insights into this emerging pathogen, which may contribute to develop the necessary knowledge to better understand the persistence in patients colonized/infected by this pathogen and likely contribute to a better management of its evolution.

## Data availability

The plasmid sequence reported here has been deposited in GenBank under the accession number: MK423762.1.

## Conflict of interest

The authors declare that they have no conflicts of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2021.01.005.

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