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PII: S2213-7165(18)30067-5
DOI: https://doi.org/10.1016/j.jgar.2018.03.015
Reference: JGAR 633

To appear in:

Received date: 20-12-2017
Revised date: 8-3-2018
Accepted date: 31-3-2018

Please cite this article as: Mariana Papalia, Germán Traglia, Melina Ruggiero, Marisa Almuzara, Carlos Vay, Gabriel Gutkind, Soledad María Ramírez, Marcela Radice, Characterization of OXA-258 enzymes and AxyABM efflux pump from Achromobacter ruhlandii (2010), https://doi.org/10.1016/j.jgar.2018.03.015

Characterization of OXA-258 enzymes and AxyABM efflux pump from *Achromobacter ruhlandii*

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Highlights

- Biochemical and genetic characterization of OXA-258 from *A. ruhlandii*.
- First description of AxyABM and OprZ-AxyXY in *Achromobacter ruhlandii.*
- Characterization of efflux pump AxyABM in antimicrobial resistance

Abstract:

Objectives

The aim of this study was to characterize OXA-258 variants and other features that may contribute to the carbapenem resistant in *Achromobacter ruhlandii.*

Methods

Kinetic parameters for purified OXA-258a and OXA-258b were determined measuring the rate of hydrolysis of a representative group of antibiotics. Whole-genome shotgun sequencing was performed on *A. ruhlandii* 38 (producing OXA-258a) and *A. ruhlandii* 319 (producing OXA-258b) and i*n silico* analysis of antimicrobial resistant determinants was conducted. Substrates of AxyABM efflux pump were investigated by inhibition assays using phenylalanine (Phe)-arginine (Arg)-β-naphthylamide. Outer membrane proteins profiles were resolved by 12% SDS-PAGE.

Results

Kinetic measurements of purified OXA-258 variants displayed an overall weak catalytic efficiency toward β-lactams. A detectable hydrolysis of imipenem was observed.

In *silico* genomic analysis confirmed the presence of 32 and 35 putative efflux pump coding genes in *A. ruhlandii* 38 and 319, respectively. Complete sequences for AxyABM and AxyXY efflux pumps, previously described in *Achromobacter xylosoxidans,* were detected. Decrease in the MIC values for chloramphenicol, nalidixic acid, trimethoprim-sulfamethoxazole was observed in the presence of inhibitor suggesting that these antibiotics are substrates of AxyABM. AxyXY coding genes of *A. ruhlandii* 38 and *A. ruhlandii* 319 displayed 99% identity between them. No differences were observed in the outer membrane proteins profiles.

Conclusions

The contribution of OXA-258a enzymes to the final β-lactam resistance profile may be secondary. Further studies on other putative resistance markers identified in the whole genome analysis should be conducted to understand the carbapenem resistance observed in *A. ruhlandii.*

Keywords: *Achromobacter* spp., *A. ruhlandii*, OXA-258, antibiotic resistance, efflux pump.

1. Introduction

Achromobacter spp. are non-frequent nosocomial and community pathogens able to cause infections in patients with underlying diseases and immunocompromised (1, 2). Although *A. xylosoxidans* is the most frequent species recovered within this genus, other species have been also associated with human infections (3, 4). *A. ruhlandii* is considered an opportunistic pathogen known to be resistant to a wide range of antibiotics, however, the knowledge about the resistance mechanisms is limited. In a previous study we described two allelic variants of a new class D β-lactamase named OXA-258, which constitutes a naturally occurring β-lactamase in *A. ruhlandii* (5). OXA-258a differs from OXA-114a of *A. xylosoxidans* by 40 amino acids, displaying 85% identity (6). OXA-258a was detected in a carbapenem resistant strain, while OXA-258b was identified in a carbapenem susceptible one. This latter differs from OXA-258a in one amino acid, R170H, according to the class D β-lactamase numbering scheme (7). The aim of this study was to characterize these OXA-258 variants and other features that may contribute to the carbapenem resistant profile observed in *A. ruhlandii*.

2. Methods

2.1 Bacterial isolates

Carbapenem resistant *A. ruhlandii* 38, producing the allelic variant OXA-258a, and carbapenem susceptible *A. ruhlandii* 319, producing OXA-258b, were included (5).

As indicated below, different recipient strains were used for cloning and expression assays.

2.2 β-Lactamase production and purification

Full length $bla_{\text{OXA-258}}$ variants were amplified by PCR using custom designed primers OXA-258F (5' AGCCATATGGACGTCCTGTGCACCCT 3'), containing the NdeI restriction site, and OXA-258R (5' TGCTCGAGTTTGCGGGGCGCCGCGACCAT 3'), containing the XhoI restriction site. The NdeI-XhoI fragments of $bla_{\text{OXA-258a}}$ and $bla_{\text{OXA-258b}}$ were cloned into the pK19 vector and into the pET-28a(+) expression vector (Novagen, Inc., Madison, WI). The

authenticity of the cloned fragments was confirmed by sequencing. *Escherichia coli* TOP 10F' (F'[lacI^q Tn10(tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ$^-$) was transformed with the pK19-OXA-258a and pK19-OXA-258b recombinant plasmids, and the MICs of a representative panel of β-lactam antibiotics were determined using the broth microdilution test according to the CLSI (8).

pET-OXA-258a and pET-OXA-258b recombinant plasmids were introduced into *E. coli* BL21(DE3) (Novagen) to overproduce OXA-258a and OXA-258b, respectively. Overnight cultures of *E. coli* BL21(DE3)-pET-OXA-258a and *E. coli* BL21(DE3)-pET-OXA-258b were diluted (1/20) in 1 liter of LB medium containing 30 µg/ml of kanamycin and grown at 37°C until 0.8 OD units (λ = 600 nm). IPTG was added at a final concentration of 1mM and cultures were grown at 18°C overnight. After centrifugation at 8,000 *g* at 4ºC, cells were suspended in 25 ml of 25 mM phosphate buffer (pH 7.5) and disrupted by sonication. Cellular debris were separated by centrifugation. The supernatant fractions displayed the highest specific activities when measured spectrophotometrically using 100 µM cephalothin as substrate. Each supernatant fraction was clarified by centrifugation at 18.000 *g* for 30 min and filtering through a 0.22 µm pore filter membrane. The samples were loaded onto a 5ml HisTrap FF affinity column (flow rate, 0.5 ml/min) (GE Healthcare Life Sciences, USA) equilibrated with binding buffer (25 mM phosphate buffer +10 mM imidazole, pH 7.5). The column was extensively washed to remove unbound proteins. β-lactamases were eluted with a step gradient of 25 mM phosphate buffer supplemented with imidazole 100, 200 and 300 mM, pH 7.5. β-lactamase containing fractions were pooled and dialyzed overnight against 25 mM phosphate buffer (pH 7.5) containing 50 mM $NaHCO_3$. Purified OXA-258a and OXA-258b were stored at -20°C until use. Enzymes purity was over 99% as estimated by SDS-PAGE. The HisTag could not be removed as thrombin cleavage sites are present within the sequence of both enzymes.

2.3 Kinetic studies

Kinetic parameters (k_{cat} and K_m) were determined measuring the rate of hydrolysis of different substrates using a UV/Vis spectrophotometer. The reactions were performed at

25°C in 25 mM phosphate buffer (pH 7.5) containing 50 mM $NaHCO_3$ in order to avoid biphasic kinetics (6, 9). The k_{cat} and K_m values were determined for a representative set of β-lactam antibiotics: oxacillin, cephalothin, cephalexin, cephazolin, ceftazidime, cefotaxime, cefepime and imipenem.

2.4 In silico analysis of antimicrobial resistant determinants

Whole-genome shotgun sequencing was performed in OXA-258a and OXA-258b *A. ruhlandii* producing strains (*A. ruhlandii* 38 and *A. ruhlandii* 319, respectively) with Illumina MiSeq-I, using Nextera XT libraries for sample preparation. De novo assembly was performed with the SPAdes assembler version 3.1.0 (10), using a preassembly approach with Velvet (11). RAST server was used to predict all open reading frames and search for the $bla_{OXA-258a}$ and $bla_{OXA-258b}$ and their genetic context (12). BLAST (version 2.0) software was used to confirm the predictions. The presence of other resistance determinants was assessed by ARDB (13) and InterProScan (EMBL-EBI), and the existence of insertion sequences (IS), inverted repeat sequences (IR) or strong promoters were performed using ISfinder (14) and b-prom (Softberry) programs.

2.5 Phenotypic characterization of efflux pump AxyABM in *A. ruhlandii*

Inhibition assays were conducted in order to evaluate the contribution of the efflux pump AxyABM, previously described in *A. xylosoxydans* (15), in the imipenem resistance profile. Efflux pump coding genes were cloned into pK19 and transformed in *E. coli* TOP 10F'. Minimal inhibitory concentrations of chloramphenicol (CHL), nalidixic acid (NAL), trimethoprim-sulfamethoxazole (SXT), ceftazidime (CAZ), imipenem (IPM), ciprofloxacin (CIP) and norfloxacin (NOR) were determined in the presence and absence of 25 µg/ml of the inhibitor phenylalanine (Phe)-arginine (Arg)-β-naphthylamide (PAβN), in *A. ruhlandii* 38 and in the transformant *E. coli* TOP 10F' + pK19-AxyABM (16). A reduction of ≥ 2 dilutions in the MICs values of the antibiotic combined with PAβN respect the MIC of the antibiotic, was considered as positive inhibition (17).

2.6 Analysis of outer membrane porins

Porins extraction was performed according to Hancock and Poxton method and were resolved by 12% SDS-PAGE (18).

OprD homolog genes were investigated by whole-genome sequencing using RASTk server, and pfam and genbank databases.

3. Results

No differences were observed in the imipenem MIC values between transformants carrying OXA-258a and OXA-258b coding genes (Table 1a). β-lactam hydrolytic parameters are shown in Table 1b. Similar values were observed for both enzymes, displaying an overall weak catalytic efficiency toward the β-lactams included. No hydrolysis was detected for oxacillin and expanded-spectrum cephalosporins, neither for OXA-258a nor OXA-258b, as previously observed for other "oxacillinases", such as the naturally occurring OXA-114a from *A. xylosoxidans* (6). A detectable hydrolysis of imipenem was observed, however k_{cat} values could not be obtained because of high K_m values, indicating a very poor affinity for this substrate.

In *silico* genomic analysis confirmed the presence of 3 putative β-lactamase coding genes in each isolate. One of these open reading frames corresponded to $bla_{OXA-258}$ allelic variants ($bla_{OXA-258a}$ in *A. ruhlandii* 38 and $bla_{OXA-258b}$ in *A. ruhlandii* 319). Both markers had the same genetic context in the closest surrounding regions, however some differences could be observed in downstream remote regions. No insertion sequences (IS), inverted repeat sequences (IR) or strong promoters were identified upstream $bla_{OXA-258}$ alleles (Figure 1). About the other 2 β-lactamase putative genes they codified for molecular class A and class C enzymes β-lactamases, which will be subject of further studies.

Thirty two putative efflux pump coding genes were found in *A. ruhlandii* 38 genome, while 35 in *A. ruhlandii* 319 (GenBank accession nos. **PHIH00000000** and **PHIK00000000**). Among them, complete sequences for two RND efflux pumps, previously characterized in *A. xylosoxidans* (15, 19) were detected in both genomes. One of them presented 98% identity with AxyABM of *A. xylosoxidans* and presented 99% identity between isolate 38 and 319. MIC values for PaβN inhibition assays are shown in Table 2. A decrease in the MIC values for CHL, NAL and SXT was observed in the presence of PaβN in the transformant TOP 10-pK19–AxyABM, suggesting that these antibiotics are substrates of

AxyABM efflux pump. No changes were observed in the MIC values of CAZ, IPM, CIP and NOR in the presence of the inhibitor.

The complete sequence for AxyXY-OprZ coding genes displays 99% identity with the previously described in *A. xylosoxidans* (19). It has been reported that carbapenems, fluoroquinolones and aminoglycosides are substrate of this efflux pump. Using the BLAST program, *axy*Z, *axy*X, and *axy*Y genes could be detected in *A. ruhlandii* 38 and 319 genomes, sharing 99% nucleotide similarity with the corresponding genes in *A. xylosoxidans*. Downstream *axy*Y, it was located *opr*Z which encodes for an outer membrane protein that differs from OprZ of *A. xylosoxidans* by 2 amino acid, displaying 99% identity. No major differences were observed in AxyXY coding genes between *A. ruhlandii* 38 and *A. ruhlandii* 319.

No differences in the outer membrane proteins banding profile were observed among these isolates (data not shown). However, in *silico* genomic analysis showed the presence of 13 putative outer membrane coding genes in *A. ruhlandii* 38, while in *A. ruhlandii* 319 there were detected 15. No *Pseudomonas aeruginosa* OprD porin homologues were found in *A. ruhlandii*.

4. Discussion

Here we describe the kinetic parameters for both allelic variants of the *A. ruhlandii* naturally occurring class D OXA-258. Despite the different resistance profiles observed in *A. ruhlandii* clinical isolates, no significant differences were observed between OXA-258a and OXA-258b respect β-lactam hydrolysis. It has been hypothesized for the naturally occurring OXA-114 in *A. xylosoxidans* that its expression could be influenced by the presence of IS, leading to a higher level of OXA-114-mediated β-lactam resistance (6). However, in the analysis of $bla_{\text{OXA-258}}$ environment up to 5 Kb upstream $bla_{\text{OXA-258}}$, not a single genetic element able to contribute to an increased expression level of the enzymes or recombination sites, was detected. It is likely that the contribution of OXA-258 enzymes to the final β-lactam resistance profile may be secondary, as previously demonstrated for the naturally occurring oxacillinase of *A. xylosoxidans.*

Here we also describe in *A. ruhlandii* the presence of two efflux pumps homologous to those previously reported in *A. xylosoxidans*. Accordingly, AxyABM in *A. ruhlandii* is able to extrude CHL and SXT but not imipenem. As suggested by *Bador et al*. AxyXY-oprZ in *A. xylosoxidans*, may be involved in acquired resistance to carbapenem and fluoroquinolone, which are antibiotics widely used to treat pulmonary infections in cystic fibrosis patients. However its presence was observed in both susceptible and resistant *A. ruhlandii* isolates. Further studies on other putative resistance markers identified in the whole genome analysis should be conducted to understand the carbapenem resistance observed in *A. ruhlandii* as *t*he multiple approaches evaluated in this study are still not conclusive.

Declarations

Funding: The work was supported by grants from UBACyT to M. Radice and G. Gutkind; ANPCyT to M. Radice, and PIP to G. Gutkind.

Competing Interests: Radice and Gutkind are members of Carrera del Investigador Científico (CONICET). M. Papalia is recipient of a posdoctoral fellowship from CONICET.

Ethical Approval: Not required

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Figure 1: Genetic context of *bla*$_{OXA-258}$ genes. Arrows represent the direction of transcription. The grey box indicates the region coding for the same protein.

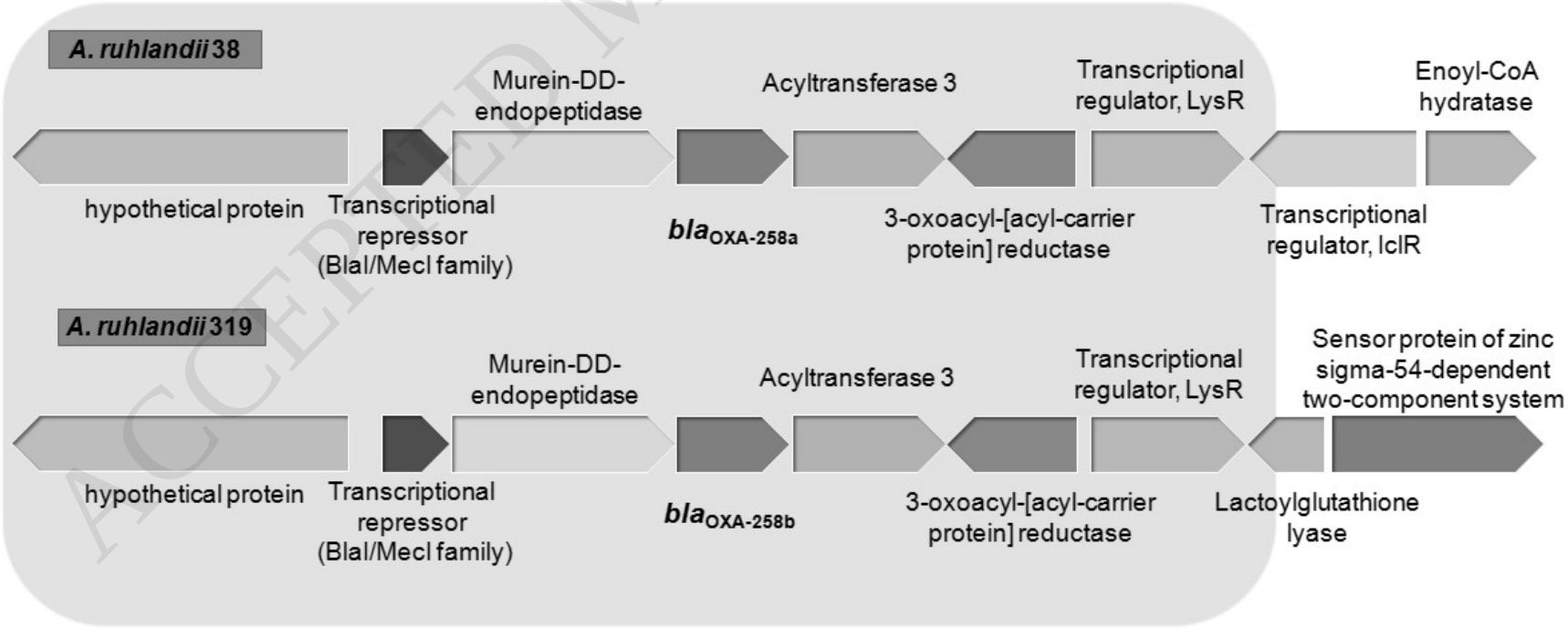

Table 1: a) β-lactams MICs for *A. ruhlandii* strains (38 and 319), *E. coli* TOP10F' harboring pK19 plasmid, *E. coli* TOP10F' harboring pK19 plasmid expressing OXA-258a and OXA-258b, and *E. coli* TOP10F' (reference strain). b) Kinetic parameters of purified β-lactamase OXA-258a and OXA-258b.

a)

	MIC (µg/ml)						
	CEF	**CFZ**	**LEX**	**FOX**	**CAZ**	**FEP**	**IPM**
***E. coli* TOP 10F'**	4	2	8	2	0.25	≤ 0.125	0.25
***E. coli* TOP 10F' + pK19**	4	2	8	2	0.25	≤ 0.125	0.125
***E. coli* TOP 10F' + pK19-OXA-258a**	16	2	8	2	0.5	≤ 0.125	0.25
***E. coli* TOP 10F' + pK19-OXA-258b**	8	2	8	2	0.25	≤ 0.125	0.25
***A. ruhlandii* 38**	512	1024	256	256	8	64	32
***A. ruhlandii* 319**	512	1024	32	32	4	32	2

CEF: cephalothin; CFZ: cefazolin; LEX: cephalexin, FOX: cefoxitin; CAZ: ceftazidime; FEP: cefepime; IPM: imipenem

b)

Antibiotic	**Enzyme**	K_m **(µM)**	k_{cat} **(s^{-1})**	k_{cat}/K_m **($mM^{-1}.s^{-1}$)**
Oxacillin	**OXA-258a**	ND		
	OXA-258b			
Cephalothin	**OXA-258a**	Biphasic	Biphasic	-
	OXA-258b			
Cephalexin	**OXA-258a**	425 ± 55	0.034 ± 0.003	0.08± 0.02
	OXA-258b	352 ± 69	0.023 ± 0.002	0.06 ± 0.02
Cephazolin	**OXA-258a**	49 ± 4	0.0099 ± 0.0003	0.20 ± 0.02
	OXA-258b	33 ± 3	0.0080 ± 0.0002	0.24 ± 0.03
Ceftazidime	**OXA-258a**	ND		
	OXA-258b			
Cefotaxime	**OXA-258a**	ND		
	OXA-258b			
Cefepime	**OXA-258a**	ND		
	OXA-258b			
Imipenem	**OXA-258a**	Detectable hydrolysis. Poor affinity for this substrate.		
	OXA-258b			
Nitrocefin	**OXA-258a**	6.6 ± 0.9	0.51 ± 0.02	80 ± 20
	OXA-258b	7.4 ± 0.6	0.77 ± 0.02	104 ± 11

ND: No detectable hydrolysis

Table 2: Representative antibiotic MICs for *A. ruhlandii* 38 strain, *E. coli* TOP10F' harboring pK19 plasmid, *E. coli* TOP10F' harboring pK19 plasmid expressing AxyABM efflux pump, and *E. coli* TOP10F' (reference strain).

Isolates	CHL (µg/ml)		Inhibition	NAL (µg/ml)		Inhibition	SXT (µg/ml)		Inhibition
	- PAβN	+ PAβN		- PAβN	+ PAβN		- PAβN	+ PAβN	
TOP 10	≤1	≤1	-	≤0.5	≤0.5	-	0.5	≤0.25	-
TOP 10 - pK19	≤1	≤1	-	≤0.5	≤0.5	-	≤0.25	≤0.25	-
TOP 10 - pK19 - AxyABM	16	≤1	+	8	≤0.5	+	1	≤0.25	+
A. ruhlandii 38	32	8	+	256	128	-	32	16	-

Isolates	CIP (µg/ml)		Inhibition	NOR (µg/ml)		Inhibition	CAZ (µg/ml)		Inhibition	IPM (µg/ml)		Inhibition
	- PAβN	+ PAβN		- PAβN	+ PAβN		- PAβN	+ PAβN		- PAβN	+ PAβN	
TOP 10	≤0.125	≤0.125	-	≤0.03	≤0.03	-	0.125	0.125	-	0.25	0.25	-
TOP 10 - pK19	≤0.125	≤0.125	-	≤0.03	≤0.03	-	0.125	0.125	-	0.25	0.25	-
TOP 10 - pK19 - AxyABM	≤0.125	≤0.125	-	0.06	0.06	-	0.25	0.25	-	0.25	0.25	-
A. ruhlandii 38	4	4	-	64	32	-	8	8	-	32	16	-