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To cite this version:
Charbel Al Bayssari, S. Kumar Gupta, Fouad Dabboussi, M Hamze, Jean-Marc Rolain. MUS-2, a novel variant of the chromosome-encoded $\beta$-lactamase MUS-1, from Myroides odoratimimus. New Microbes and New Infections, Wiley Online Library 2015, 7, pp.67-71. 10.1016/j.nmni.2015.06.007. hal-01772724

HAL Id: hal-01772724
https://hal-amu.archives-ouvertes.fr/hal-01772724
Submitted on 20 Apr 2018

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MUS-2, a novel variant of the chromosome-encoded β-lactamase MUS-1, from Myroides odoratimimus

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Abstract

The aim of the present study was to investigate the molecular mechanism of carbapenem resistance of three imipenem-resistant isolates of Myroides odoratimimus recovered from two livestock farms of cows and pigeons by rectal swab in Lebanon in January 2014. Investigation of imipenem resistance of these isolates using the modified Hodge test, the EDTA test, the modified CarbaNP test and the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry Ultraflex assay showed a carbapenemase activity due to the presence of a chromosome-encoded β-lactamase MUS, verified by PCR. However amplification and sequencing of this chromosomal gene showed a novel variant of it designated MUS-2 by the curators of the Lahey database of β-lactamas (http://www.lahey.org/Studies/webt.asp). Cloning of the \( \text{bla} \)MUS-2 was performed, followed by protein expression in Escherichia coli TOP 10. Pulsed-field gel electrophoresis clearly showed that the three isolates belonged to the same clone. This study reports a novel variant of the chromosome-encoded \( \text{bla} \)MUS-1 associated with carbapenem resistance in Myroides odoratimimus and shows that animals may represent a reservoir of bacteria harbouring several variants of resistance genes.

New Microbes and New Infections © 2015 The Authors. Published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases.

Keywords: Animals, \( \text{bla} \)MUS-2, carbapenem-resistance, metallo-β-lactamase, Myroides odoratimimus

Original Submission: 8 April 2015; Accepted: 22 June 2015
Available online 27 June 2015

Introduction

Myroides species, previously named as Flavobacterium odoratum, are aerobic Gram-negative bacteria that can be found in environmental sources such as soil and water, but are not residents of the normal human microflora [1]. The genus Myroides comprises two species, Myroides odoratus and Myroides odoratimimus [2]. Taking into consideration their role as causative agents in human disease, both M. odoratimimus and M. odoratus are seen rarely as opportunistic pathogens, causing infections in severely immunocompromised patients and rarely, in immunocompetent hosts [1,3]. Myroides odoratimimus strains have been found to be responsible for urinary tract infections [4,5], cellulitis [6,7] in immunocompromised patients, and also for septic shock, pneumonia and soft-tissue infections [1,8] in immunocompetent hosts. In addition, three outbreaks of M. odoratimimus, two of urinary tract infections and the third of central venous catheter-associated bloodstream infections due to contaminated water, have been reported [2,4].

Antibiotic resistance patterns of Myroides strains show variable susceptibility to β-lactams [5], with a conserved decreased susceptibility to cephalosporins and carbapenems [9]. This decreased susceptibility is the result of the production of a chromosome-encoded β-lactamase MUS-1. This enzyme is a member of the subclass B1 of metallo-β-lactamases and is distantly related to other metalloenzymes, being most closely
related to IND-1 from *Chryseobacterium indologenes* (42% amino acid identity). However, phylogenetic analysis showed that MUS-1 belongs to the same phylogenetic lineage of subclass B1 enzymes that groups the subclass B1 β-lactamases of *Flavobacterium* species [9]. Here we report a novel variant, MUS-2, of the chromosome-encoded β-lactamase MUS-1 from *M. odoratimimus* isolated from livestock animals in Lebanon.

**Materials and methods**

**Bacterial isolates**

Three imipenem-resistant *M. odoratimimus* strains (32, 35a and 104b) were isolated from two livestock farms of cows and pigeons by rectal swabs (32: farm 1, cow; 35a: farm 1, pigeon; 104b: farm 2, cow) in January 2014. These strains were collected during a study that aimed to detect carbapenemase-encoding genes in animals in Lebanon [10]. They were isolated on MacConkey agar supplemented with ertapenem (1 mg/L), from alive and non-sick animals without contact with any other animals. Strains were sub-cultured on trypticase soy agar plates at 37°C for 18–24 h and identification was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Microflex™; Bruker Daltonics, Bremen, Germany) with FLEXCONTROL software (Bruker Daltonics).

**Antibiotic susceptibility testing**

Antibiotic susceptibility testing was determined on Müller–Hinton agar by standard disc diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org). Seventeen antibiotics were tested, including ticarcillin, ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam, cefazidime, cefotaxime, cefepime, aztreonam, amikacin, tobramycin, gentamicin, ciprofloxacin, rifampicin, ertapenem, meropenem, imipenem and colistin (Bio-Rad, Marnes-la-Coquette, France). The MIC for imipenem was determined using the Etest method (bioMérieux, Saint-Arnold, France) with running conditions were 6 V/cm at 14°C for 20 h.

**Phenotypic and molecular detection of carbapenemases**

Isolates were screened for carbapenemase production using the modified Hodge test, the EDTA, the Modified CarbaNP test as previously described [11–13] and also by MALDI-TOF MS Ultraflex assay [14]. Screening for class A, B and D carbapenemases was carried out using PCR according to previous protocols and previously described primers including for *bla*<sub>VIM</sub>, *bla*<sub>PER</sub>, *bla*<sub>NDM-1</sub>, *bla*<sub>KPC</sub> and all the *bla*<sub>OXA</sub> variants [15–20]. In addition, amplification of the chromosomal MUS gene was performed using primers designed in this study (MUS-F/R 5′-CGTATGGCAGCAGGAAAGA-3′/ gagctttggaatttactctg-3′). These primers were designed outside the region containing the *bla*<sub>MUS</sub>-1, located on the chromosome of a random *M. odoratimimus* genome deposited in GenBank. PCR products were purified and sequenced using the Big dye terminator chemistry on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were analysed using BLASTN and BLASTP against the NCBI database (www.ncbi.nlm.nih.gov).

**Cloning of the MUS-2 gene**

The full length of the *bla*<sub>MUS</sub>-2 from *M. odoratimimus* was amplified using *Herculase II Fusion Enzyme* with dNTPs Combo (Agilent Technologies, Santa Clara, CA, USA) with primer MUS_F/MUS_R. The gene was then cloned into the high-copy-number plasmid pCR-BluntII-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA). The resulting plasmid pTOPO-MUS-2 was transformed into TOP10 Electrocomp™ *Escherichia coli*. Transformants were selected on Luria–Bertani agar supplemented with 100 mg/L of kanamycin; the presence of the insert was confirmed by PCR. The antibiotic resistance profile of the TOP10 *E. coli* containing pTOPO-MUS vector was then determined.

**Molecular strain typing**

The three isolates were typed by the pulsed-field gel electrophoresis (PFGE) method as previously described [4]. The DNA was digested with Smal restriction enzyme and the fragments of DNA were separated using a GenePath system (Bio-Rad). The running conditions were 6 V/cm at 14°C for 20 h.

**Results**

During a previous study that was aiming to detect carbapenemases encoding genes in animals in livestock farms in Lebanon [10], three isolates of *M. odoratimimus* were isolated and were studied separately. These isolates were obtained after culture of animal faeces in MacConkey agar supplemented with ertapenem (1 mg/L) and were identified by MALDI-TOF MS as *M. odoratimimus* (score: 2.439). The results of antibiotic susceptibility testing revealed that the isolates were resistant to almost all antibiotics, including β-lactams, aminoglycosides and colistin but remain susceptible to sulfamethoxazole-trimetoprim, rifampicin and fluoroquinolones (Table 1). In addition, all the isolates showed resistance to carbapenem with MIC for imipenem at 8 mg/L. The isolates were then analysed via the modified Hodge test, the MALDI-TOF MS Ultraflex
In this study, we report the identification of a novel β-lactamase from Myroides odoratimimus, designated MUS-2. The enzyme variant was designated MUS-2 by the curators of the Lahey database of β-lactamases and deposited in GenBank under accession number KP658209.

**TABLE 1. Antimicrobial susceptibility testing of the three Myroides isolates**

| Substrate | MO_32   | MO_35a  | MO_104b |
|-----------|---------|---------|---------|
| Ticarcillin   | R       | R       | R       |
| Piperacillin  | R       | R       | R       |
| Ticarcillin-clavulanic acid (CL) | R       | R       | R       |
| Piperacillin-Tazobactam (TZB) | R       | R       | R       |
| Cephalosporins | R       | R       | R       |
| Carbapenems | R       | R       | R       |
| Tobramycin    | R       | R       | R       |
| Amikacin      | R       | R       | R       |
| Gentamicin    | R       | R       | R       |
| Ciprofloxacin  | R       | R       | R       |
| Amoxicillin   | R       | R       | R       |
| Azetronem     | R       | R       | R       |
| Rifampicin    | R       | R       | R       |
| Sulfamethoxazole-trimethoprim | R       | R       | R       |

**TABLE 2. MICs of β-lactams for Myroides odoratimimus 35a, TOP10 E. coli and TOP10 E. coli pTOPO-MUS-2**

| Substrate | M. odoratimimus 35a | TOP10 E. coli pTOPO-MUS-2 | TOP10 E. coli |
|-----------|---------------------|--------------------------|--------------|
| Amoxicillin | 32                  | 128                      | 2            |
| Amoxicillin-CLA | 32                  | 128                      | 2            |
| Ticarcillin | 256                 | 256                      | 2            |
| Piperacillin | 128                 | 8                        | 2            |
| Piperacillin-TZB | 24                 | 8                        | 1            |
| Cefoxitin    | 32                  | 4                        | 2            |
| Mosalactam   | 0.064               | 0.19                     |              |
| Cefotaxime   | 32                  | 0.094                    | 0.047        |
| Cefuroxime   | 128                 | 6                        | 1            |
| Cefazidime   | 128                 | 0.38                     | 0.064        |
| Ceftipime    | 32                  | 0.064                    | 0.064        |
| Ceftriaxone  | 32                  | 0.064                    | 0.064        |
| Cefuroxime   | 128                 | 6                        | 1            |
| Aztreonem    | 64                  | 0.064                    | 0.064        |
| Imipenem     | 8                   | 0.38                     | 0.064        |
| Meropenem    | 4                   | 0.38                     | 0.064        |

*CLA, clavulanic acid at 2 μg/mL; TZB, tazobactam at 4 μg/mL.*

**FIG. 1.** Amino acid sequence alignment between the MUS-1 and MUS-2 proteins.

Discussion

To date, infections caused by *M. odoratimimus* in immunocompromised and immunocompetent hosts had been rarely reported. The novelty of the enzyme and its potential impact on the treatment of infections caused by this species highlights the importance of further studies to better understand its epidemiology and antibiotic susceptibility profile.
reported [1,2,4–8]. The fact that this bacterium might cause infection in immunocompetent hosts is a real health problem because it is resistant to β-lactams including carbapenems, aminoglycoside but also to colistin. Biochemical detection of β-lactamases in clinical strains of Myroides spp. (formerly F. odoratum) was performed in 1985 by Sato et al. [21], but the molecular characteristics of β-lactamase were not reported. We would have to wait until 2002 when Mammeri et al. biochemically and genetically characterized β-lactamases expressed in M. odoratimimus and M. odoratus [9]. In fact, M. odoratimimus species produce chromosomal MUS-1 β-lactamase, which belongs to the subclass B1 of Ambler classification, and their antibiotic resistance patterns show a constant decreased susceptibility to imipenem [9]. The β-lactamase produced by the three M. odoratimimus isolates in our study shared 98.78% amino acid identity with the known MUS-1 protein. At the present time, each bacterial species of the Flavobacteriaceae family that has been investigated for β-lactamase characterization produces a subclass B1 metalloenzyme [9]. Our study confirms a previous study performed on M. odoratimimus species; however, here we report a new variant of this enzyme in Lebanon. PFGE analysis showed that these isolates belong to the same clone because they had the same PFGE profile. This confirms that contamination between farms has occurred and precautions should be established to limit the emergence of this clone in livestock farms because that could be a source of human infection.

Taking into consideration the biochemical criteria established by Rasmussen and Bush to classify metallo-β-lactamases [22], MUS-1 and most probably MUS-2 belong to functional subgroup 3a, as their catalytic efficiencies for penicillins are at least 60% of that for imipenem [9]. However, these data are discrepant with those from Rasmussen and Bush [22], who mentioned that a metallo-β-lactamase produced by F. odoratum strain belongs to subgroup 3b, which groups true carbapenem-hydrolysing β-lactamases. Regarding this conflict, it is very difficult to estimate the exact role of either MUS-1 or MUS-2 in the intrinsic β-lactam resistance of M. odoratimimus because the metalloenzymes expressed in E. coli give much lower levels of resistance to β-lactams than those seen in the original producers as seen in the TOP10 E. coli after cloning.

Finally, it remains unknown if these bacteria could be a source of such a variety of metalloenzymes. The most serious finding is that animals could be a reservoir for such genes and are a source of new and/or emerging multidrug-resistant bacteria and due to possible links with animals, humans may have high risk factors for colonization/infection with such bacteria.

In conclusion, we report a novel variant of the chromosome-encoded β-lactamase MUS-1 that has been designated MUS-2 from M. odoratimimus isolates from livestock animals in Lebanon.

**GenBank accession number**

The full sequence of the BlaMUS-2 described in this study is deposited in the GenBank database with accession no. KP658209.

**Funding**

This work was supported by AZM research centre for biotechnology and its application, Lebanese University, the National Council for Scientific Research, Lebanon, the IHU Méditerranée Infection and the French CNRS.
None to declare; all authors have read and approved the manuscript.

None declared.

The authors thank Linda Hadjadj, Said Azza, Taha Abdo and Maryam Yehya for technical assistance.

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