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Whole-Genome Analysis of an Extensively Drug-Resistance *Empedobacter falsenii* Strain Reveals Distinct Features and the Presence of a Novel Metallo-ß-Lactamase (EBR-2)

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Abstract

The spread of antibiotic resistance is rapidly threatening the effectiveness of antibiotics in the clinical setting. Many infections are being caused by known and unknown pathogenic bacteria that are resistant to many or all antibiotics currently available. *Empedobacter falsenii* is a nosocomial pathogen that can cause human infections. *E. falsenii* Wf282 strain was found to be resistant to many antibiotics, including carbapenems and colistin. Whole-genome shotgun sequencing of the strain was performed, and distinct features were identified. A novel metallo-β-lactamase, named EBR-2, was found, suggesting a potential role of *E. falsenii* as a reservoir of β-lactamases and other resistance determinants also found in its genome. The EBR-2 protein showed the highest catalytic efficiency for penicillin G as compared to meropenem and ampicillin and was unable to hydrolyze cefepime. The results described in this work broaden the current understanding of the role of β-lactamases in the *Flavobacteriaceae* family and suggest that *E. falsenii* Wf282 may be a reservoir of these novel resistance determinants.

Introduction

Antimicrobial resistance is a worldwide growing problem [1–3]. In February 2017, the World Health Organization published a list of twelve bacterial pathogens that are extensively or multidrug resistant and are common pathogens in the clinical setting [4]. The emergence of antibiotic resistance is affected by numerous factors, some of which are lack of completion of the prescribed treatment, or inappropriate human use, use of antibiotics as nutritional supplement for livestock [2]. As a consequence, many infections that used to require relatively short and cheap treatments are now difficult or even impossible to treat [5–7]. Among pathogens that can cause human infections and can harbor resistance genes in their genomes, several species of Non-Fermenting Gram-Negative Bacilli (NFGNB) such as *Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Empedobacter falsenii* have been identified as pathogens with increasingly higher levels of antibiotic resistance [8–10]. NFGNB are widely distributed opportunistic pathogens that can survive in hospital environments.

In 2013, an isolate of *E. falsenii* (WF282) was isolated from a cervical neck abscess from an 18-year-old female patient admitted to the otolaryngology department with acute otitis media. *E. falsenii* Wf282 was resistant to several

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antibiotics, including carbapenems. Whole-genome shotgun sequencing analysis identified a novel metallo-β-lactamase named bla_{EBR-2} [11]. In this work, we characterize EBR-2 and show that its presence is associated with a decrease in susceptibility to imipenem and meropenem but not cefepime.

Materials and Methods

Bacterial Strains and Antimicrobial Agents

E. falsenii strain Wf282 was obtained from an 18-year-old patient with acute otitis media admitted to the otolaryngology department at a public hospital in Buenos Aires, Argentina [11]. pZero-Blunt vector, pGEX-4T-1 expression vector and BL21 (DE3) pLysS cells were used to performed the production and purification of EBR-2 protein.

Meropenem, imipenem, cefepime, ampicillin, and penicillin G were purchased from Sigma Aldrich (St. Louis, MO USA) and were stored as specified. Aliquots were thawed only once for use during kinetics assays and then discarded.

Whole-Genome Sequence Analysis and Genomic Comparison

Genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit from Promega Corporation (Madison, WI USA). Whole-genome shotgun sequencing was performed using Illumina MiSeq-I and assembled by means of SPAdes assembler version 3.6.0 [12] with a preassembly done using Velvet version 1.2 [13]. RAST server version 2.0 [14] was used to predict open reading frames and BLAST version 2.0 [15] software was utilized to confirm the predictions. ARG-ANNOT and ISfinder programs were used to identify antibiotic resistance genes and insertion sequences within the genome of Wf282, respectively [16, 17]. Phage and prophage sequences were identified using PHAST [18]. SignalIP, Uniprot, and InterProScan5 softwares were used to identify the signal peptide, the conserved domain and conserved amino acid of the active site [19–21].

For phylogenetic analysis, 822 assemblies from the *Flavobacteriaceae* family were downloaded via FTP from the NCBI assembly database. Twenty-one highly conserved ribosomal protein-coding genes were identified in all the assemblies annotation using BLASTP [15] (Supplementary Table 1). 363 assemblies were subsequently sampled as representatives of the genetic diversity of the family (Supplementary Table 2). In brief, the 21 conserved proteins were independently aligned using MUSCLE version 3.8.31 [22]. The resulting amino acid alignments were concatenated, then CD-HIT version 4.6 [23] was used to cluster assemblies based on a 99% identity threshold. An assembly from each cluster was randomly selected. High quality regions were selected using GBLOCK version 0.91b [24] and subsequently the phylogram was built using RAXML version 8.2.11 [25].

Based on the phylogenetic results, 12 closely related genomes to Wf282 strain were selected and used for further comparative analyses (Supplementary Table 2 and Fig. 1). The OrthoMCL [26] method, as implemented in the get_homologues software package [27], was used to cluster homologous genes with 1e-5 blast *e*-value, 30% identity and 75% coverage as threshold for significance blastp hits.

Production and Purification of EBR-2

For purification of the EBR-2 protein, bla_{EBR-2} was amplified by PCR using the expression primers (forward primer 5′-GGATCCATAAAACCAATTCAAATTGAT-3′ and reverse primer 5′-CTCGAGTTATTTCTTTTCTAAAAG TTT-3′) to remove the signal peptide and was introduced into the pZero-Blunt vector from ThermoFisher Scientific (Waltham, MA USA). The β-lactamase bla_{EBR-2} was excised from the pZero-Blunt vector using restriction sites BamHI and XhoI. Following digestion, bla_{EBR-2} was ligated into the pGEX-4T-1 expression vector purchased from GE Healthcare Life Sciences (Pittsburgh, PA USA). The positive recombinant plasmid (pGEX EBR-2) was confirmed by sequencing.

Chemically competent BL21 (DE3) pLysS cells were transformed with pGEX EBR-2. Cells were cultured until they reached $OD_{600} = 0.6$ and expression of the gene encoding EBR-2 was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were incubated for 24 h at 30 °C with agitation. The bacterial suspension was centrifuged at 5000×*g* for 10 min at 4 °C. The pellet was resuspended in 1X phosphate buffer saline (PBS) containing 0.1 mM phenylmethane sulfonyl fluoride (PMSF) and cells were disrupted by sonication. The suspension was centrifuged and the supernatant was applied to a pre-equilibrated 1 ml GSTrap HP prepacked column from GE Healthcare Life Sciences (Pittsburgh, PA USA) as specified by the manufacturer. Thrombin (80 U) was applied to the column to cleave the protein of interest from GST. The cleaved protein was collected in approximately 4–5 ml 1X PBS. Efficiency of the purification was analyzed using SDS PAGE. The concentration of the final elution was determined by Bradford assay. Final eluates from the column were concentrated using Pierce™ Protein Concentrators PES, 20K MWCO, 0.5 ml from ThermoFisher Scientific (Rockford, IL) according to the manufacturer's instructions.

Biochemical Characterization of EBR-2

A Jasco V-550 spectrometer was used for the determination of the kinetic parameters. All kinetics assays were performed

in 100 mM HEPES Buffer (pH 7.5) containing 50 μM $ZnCl_2$. Assays were performed at 30 °C in 0.1-cm light path cuvettes. Non-linear fitting of the data to the Michaelis–Menten equation was used for estimation of the K_m and K_{cat}. Kinetics assays were performed for meropenem (wavelength = 300 nm), ampicillin (wavelength = 240 nm), and penicillin G (wavelength = 235 nm). Antibiotic agents used in the kinetics assays were purchased from Sigma Aldrich (St. Louis, MO USA) and were stored as specified by the manufacturer. Aliquots of EBR-2 were thawed only once for use during kinetics assays and then discarded. Assays were performed twice independently for each substrate.

Nucleotide Accession Number

The sequence of bla_{EBR-2} can be found in the GenBank under accession number KR131616.

Results and Discussion

E. falsenii Strain Wf282 Phenotypic Characteristics

E. falsenii strain Wf282 is a non-motile, Gram-negative rod that grows aerobically at 20, 30, and 37 °C on tryptic soy agar and other standard growth media. *E. falsenii* Wf282 was resistant to ampicillin, ampicillin/sulbactam, cefalotin, meropenem, and colistin. Wf282 exhibited intermediate resistance to imipenem, piperacillin/tazobactam, cefotaxime, ceftazidime, and ciprofloxacin). Also, it was only susceptible to amikacin, cefepime, gentamicin, and trimethoprim-sulfamethoxazole [11].

Wf282 Genome Sequence, Features, and Comparative Genomic Analysis

The complete genome assembly of *E. falsenii* Wf282 comprises 3,738,626 base pairs with an N50 contig size of 113,944 bp [11]. In the assembly database, the predicted gene complement of Wf282 strain is 3542 protein-coding genes, 1351 of which were annotated as hypothetical proteins. Using the available genomic information, we studied the genetic bases of resistance of Wf282 strain and compared its gene complement, focusing on those genes linked to antibiotic resistance, in a robust phylogenetic framework.

Twenty-one highly conserved genes were aligned and subsequently used for phylogenetic analysis (see "Whole-genome sequence analysis and genomic comparison" section). After quality trimming the alignment, 3175 sites were used for building the phylogenetic tree of the family, which was highly congruent with the taxonomy of the family *Flavobacteriaceae*. It is noteworthy that species from the same genus cluster together, each as a monophyletic group, with only a very few exceptions (Supplementary Fig. 1). *Empedobacter* genomes, *E. brevis* and *E. falsenii*, cluster together and are phylogenetically close to *Chishuiella changwenlii* and *Algoriella xinjiangensis*. These four genomes also form a monophyletic group with *Weeksella* species, *Vaginella massiliensis, Moheibacter sediminis*, and *Ornithobacterium rhinotracheale* ("Blue lineage" in Supplementary Fig. 1). The comparative genome analysis was done using these closely related genomes. A group of 953 gene families were identified as conserved. Among these genes, 913 were considered as putative orthologs, with only one copy in each genome. This result further supports the idea that these genomes are closely related as shown in the phylogeny.

Empedobacter genomes share 2424 homologous gene families. A preliminary analysis suggests that 656 genes are unique of *E. falsenii* Wf282; among these, 419 are annotated as hypothetical proteins (Supplementary Table 3). Within unique genes, 95 clusters were identified. Genes are considered clustered if they are in the same contig closer than 1000 bp. from a contiguous gene in the same cluster. Fifteen of these clusters comprised 5 or more genes. These are good candidate genes for being acquired by horizontal gene transfer.

Antibiotic Resistance Determinants and Mobile Elements Found in Wf282

A group of 29 antibiotic resistance genes were identified by RAST, ARDB, and ARG-ANNOT database. These genes were not linked to any mobile elements; they were identified as putative β-lactamases, efflux pumps, and tripartite multidrug resistance systems. The predicted β-lactamase coding genes in the genome of Wf282 included one class A β-lactamase, one metallo-β-lactamase (EBR-2), and three class C β-lactamases. The class A and class C β-lactamases had between 84 and 91% identity at the amino acid level when compared to β-lactamases that were present in *E. brevis* ATCC 43,319 (NZ_ARNT00000000.1), suggesting some conservation of these gene types between these two closely related species according to the phylogeny described above. Furthermore, eight genes coding for efflux pump systems, of which seven belonged to the Resistance-Nodulation-Division (RND) superfamily were identified in the Wf282 genome. Other systems that were identified were the tripartite multidrug resistance systems (non-RND and non-multi-antimicrobial extrusion protein, MATE, efflux pump systems). One of them contains four genes, one of them duplicated, that code for the membrane fusion protein, the outer membrane component and the inner membrane component of the tripartite multidrug system. Another tripartite system consisting of three genes coding for each of the components was identified. Additionally, we identified two

putative genes associated with streptogramin resistance; one putative gene linked with trimethoprim resistance; one putative gene related with bacitracin resistance, and two putative genes related with macrolide resistances.

Comparative analysis revealed that all but two of the genes linked to antibiotic resistance were also found in the closely related genomes of the genera *Ornithobacterium, Moheibacter, Vaginella, Weeksella, Chishuiella, Algoriella*, and *Empedobacter* (see Supplementary Fig. 1). While six were completely conserved, 23 were restricted to the monophyletic group of *Chishuiella changwenlii* DSM_27989, *Algoriella xinjiangensis* XJ109, *Empedobacter brevis* NBRC_14943, and *E. falsenii* Wf282. These results are consistent with previous findings that species containing a metallo-β-lactamase also harbor other β-lactamases and non-β-lactamase resistance genes [28, 29].

Using PHAST tool to predict phage sequences, two incomplete prophages in the Wf282 genome were identified. The first incomplete predicted prophage, composed of 8502 bp, while the second incomplete prophage was composed of 8055 bp. Both prophages possessed ten predicted open reading frames. Similarly, analyses of nonphage genes into the predicted prophage showed the presence of genes with unknown functions. In both putative prophages, integrase, transposases, and specific site recombination were not found. However, insertion sequences, transposases, and plasmids were not identified in the *E. falsenii* Wf282 genome.

Sequence Analysis and Characterization of the Novel Metallo-ß-Lactamase EBR-2

A novel carbapenemase was identified in the genome of Wf282 with a 97% identity at the amino acid level to a previously described carbapenemase from *E. brevis*, EBR-1 [30]. The novel carbapenemase has been named EBR-2. A sequence alignment showed a 59% identity at the amino acid level to IND-1 from *Chryseobacterium indologenes* (Fig. 1). Moreover, we identified that the conserved domain of the β-lactamase class B1 was present in EBR-1, EBR-2, and also in IND-1 but with four amino acid of difference in these last. In addition, ten conserved residues within the active site of these three β-lactamase were also identified. When we compared the signal peptide, we observed that EBR-1 and EBR-2 have a signal peptide compound of 15 residues; 5 of them are different from the ones present in IND-1.

Purified EBR-2 showed the highest affinity for penicillin G (K_m = 160 µM) as compared to meropenem and ampicillin, with K_m values of 557 and 263 µM, respectively. EBR-2 had the highest catalytic efficiency for penicillin G with a K_{cat}/K_m (s^{-1} M^{-1}) of 8×10^6, compared to a K_{cat}/K_m (s^{-1} M^{-1}) of 4. $\times10^6$ for ampicillin and 2×10^6 for meropenem. However, EBR-2 had a higher catalytic efficiency for meropenem than

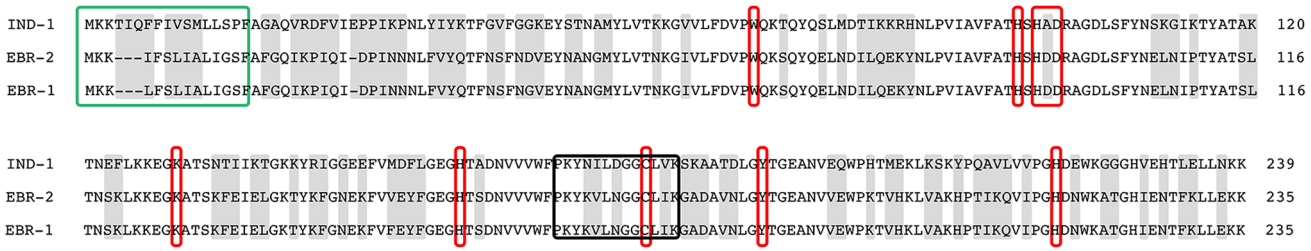

Fig. 1 Protein alignment among to IND-1, EBR-2, and EBR-1 protein. The amino acid changes are represented with gray labels. The conserved domain of β-lactamase class B1 was represented with a black square. The signal peptide of β-lactamase class B1 was represented with a green square and the conserved histidine and other conserved amino acids within the active sites were represented with a red square

Table 1 Kinetic parameters of EBR-2 purified from BL21(DE3) pLysS cells.

Substrate	EBR-2 parameters			K_{cat}/K_m (s^{-1} M^{-1})		
	K_m (µM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} M^{-1})	EBR-1	IND-2	NDM-1
Meropenem	557	923.5	2×10^6	10×10^4	2×10^5	3×10^6
Ampicillin	263	1056.9	4×10^6	ND	7×10^5	2×10^6
Penicillin G	160	1247	8×10^6	2×10^6	ND	3×10^6
Cefepime	NH	NH	NH	NH	5×10^2	ND

Catalytic efficiencies of EBR-1 [35], IND-2 [36], and NDM-1 are also shown for comparison. EBR-2 assays were performed twice independently for each substrate

NH non-hydrolyzed, *ND* not determined

EBR-1 or IND-2 (Table 1). Surprisingly, EBR-2 was also observed to have a higher catalytic efficiency than NDM-1 for penicillin G and ampicillin, suggesting that EBR-2 is more effective at hydrolyzing these antibiotics than the clinically relevant β-lactamase NDM-1 (Table 1). EBR-2, as well as EBR-1 [30], NDM-1 [31], and IND-2 [32], were unable to hydrolyze cefepime (Table 1).

Although imipenem was not also tested, the ability of EBR-2 to hydrolyze meropenem suggests that this protein may also efficiently hydrolyze imipenem. Identifying the hydrolytic spectrum of EBR-2 revealed the importance of using caution in the clinical setting when β-lactams are administered for an *E. falsenii* or other *Flavobacteriaceae* infection. The results obtained during the kinetics assays suggest that EBR-2 has catalytic efficiencies similar to or higher that an emergent and well know carbapenamase, NDM-1. However, to accurately compare the catalytic efficiencies of these proteins, they should be isolated and tested in a singular lab to ensure the same methods used on each protein. Following purification of the protein, crystallization of the proteins could provide valuable information regarding the effect of the structure of the protein on its hydrolytic ability.

Conclusion

Research has shown that antibiotic resistance has been growing around the world in recent years [2, 3, 32, 33]. The spread of this resistance threatens to render clinically relevant antibiotics no longer affective against highly resistant pathogens. The emergence of novel carbapenemase-producing bacteria which threaten to spread resistance to other pathogens has been extensively described in the literature [28, 29, 34–37].

In summary, a novel enzyme has been identified and appears to have catalytic efficiencies higher than or similar to those of a clinically relevant metallo-β-lactamase, NDM-1. However, further experimentation needs to be performed in order to determine the potential relevance of EBR-2 in comparison to NDM-1.

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