Paradoxical β-lactamase activity of archaeal encoding enzymes

Seydina M. Diene[1], Lucile Pinault[2], Nicholas Armstrong[2], Vivek Keshri[1], Saber Khelaifia[3], Eric Chabrière[1], Gustavo Caetano-Anolles[4], Jean-Marc Rolain[1,2], Pierre Pontarotti[1,5], Didier Raoult[1,2,3*]

1. Aix-Marseille Univ., MEPHI, IHU-Mediterranee Infection, Marseille, France.

2. Assistance Publique-Hôpitaux de Marseille (AP-HM), IHU-Méditerranée Infection, Marseille, France.

3. IHU-Mediterranee Infection, Marseille, France.

4. Evolutionary Bioinformatics Laboratory, Department of Crop Sciences, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.

5. CNRS, Marseille, France.

*** Corresponding author :** Prof. Didier Raoult

Address : MEPHI, IHU-Mediterranee Infection, 19-21 Bd Jean Moulin, 13005 Marseille, France. Phone: (+33) 4 13 73 24 01. Fax: (+33) 4 13 73 24 02.

Email: didier.raoult@gmail.com

Abstract: 176

Text words: 1337

Figures: 3

Suppl. Figures: 4; suppl. Tables: 2

Keywords: common ancestor sequence, metallo-β-lactamases, archaea, core genes,

Abstract

β-lactams targeting the bacterial cell wall are not efficient on archaea. Using phylogenetic analysis and common ancestor sequences for bacterial β-lactamases, we found serendipitously class B and class C-like β-lactamase genes in most archaea genomes. The class B β-lactamase appears to be highly conserved in archaea and to has been transferred in the bacterial genus *Elizabethkingia*. The experimentaly expressed class B enzyme from *Methanosarcina barkeri* was able to digest penicillin G and was inhibited by a β-lactamase inhibitor (i.e. sulbactam). The class C-like β-lactamase was more closely related to DD-peptidase enzymes than know bacterial class C β-lactamases. The use of these very conserved genes in this domain cannot be explored as a defense system against β-lactams but may be used to feed β-lactams as a source of carbon as shown in bacteria.

Introduction

Antibiotics are part of the microorganism's arsenal in their struggle to master microbial ecosystems (1). Most antibiotics are non-ribosomal peptides assembled by megaenzymes, the non-ribosomal peptide synthetases (NRPS) that have structural motifs which appear to be among the oldest of the living world (2, 3). As part of the Red Queen theory of evolutionary law (4), in the fight against β-lactam antibiotics that act on the cell wall, bacteria have developed enzymes hydrolyzing these molecules, the β-lactamases. These enzymes, acting as hydrolases, also have extremely archaic motifs (3). Four molecular classes (labelled A, B, C and D) are described todays (5). The three classes A, C, and D are characterized by a serine residue in their catalytic active site whereas the class B, metallo-β-lactamase enzymes, is characterized by zinc as an essential metal cofactor in their catalytic active site (5). The struggle between β-lactams and β-lactamases appears to be essentially limited to bacteria. In archaea microorganisms, it may be useless in this context as the antibiotic target in their cell wall is lacking (6, 7). In the current study, following a phylogeny analysis, we have investigated the presence of β-lactamase enzymes in archaeal species. The reconstruction of a common ancestor for β-lactamases easily identify β-lactamases in genomic databases and in most archaeal genomes. Here, we demonstrate that the gene annotated as a β-lactamase in an encoding enzyme which when expressed, exhibits a typical β-lactamase activity.

Results

Blast analysis of known bacterial β-lactamase genes such as class A (TEM-24, SHV-12), class B (VIM-2, NDM-1), class C (CMY-12, AAC-1), and class D (OXA-23, OXA-58) show no or insignificant results (% identity ≤ 24) against the NCBI archaeal database. However, as described, ancestral sequences are capable of detecting remote homologous sequences from published biological databases (8). Consequently, using constructed phylogenetic trees (cf. suppl. figures) of the four bacterial β-lactamase classes, an ancestral sequence for each class was inferred. From the four inferred ancestral sequences, homologous sequences in the archaeal database were identified for the class B and C β-lactamases (**fig. S1 and S2**). No significant hits were obtained for the class A and D.

Archaeal Class B metallo-β-lactamase. An archaeal β-lactamase appeared highly conserved in several classes of archaea including *Archaeoglobi*, *Methanomicrobia*, *Methanobacteria*, *Thermococci*, *Methanococci*, *Thermoplasmata* and *Thermoprotei* (**fig. 1; Suppl. Table S1**) (9). To evaluate these archaeal enzymes activity, the protein from *Methanosarcina barkeri* (gi|851225341; 213 aa; 25.5 kDa)(**fig. 1** and **Suppl. Table S1**) was experimentally tested. Protein alignment of this latter with known bacterial metallo-β-lactamase proteins reveals conserved motifs/amino acids including Histidine118 (His118), Aspartic acid 120 (Asp120), His196, and His263, markers of this metallo-β-lactamase class B as previously described (10)(**fig. S3**). Three-dimensional (3D) structure comparison of this enzyme with known and well characterized proteins in the Phyre2 investigator database reveals 100% of confidence and 94% of coverage with the crystal structure of the New Delhi metallo-β-lactamase 1 (NDM-1; Phyre2 ID: c3rkjA) (**Table S2**). To evaluate these archaeal enzymes activity, the MetbaB protein from *Methanosarcina barkeri* was experimentally tested. As expected, this enzyme exhibits a significant hydrolysis activity on nitrocefin (**fig. 2A, 2B**) (with determined kinetic parameters $k_{cat}=18.2\times10^{-3}$ s^{-1}, $K_M=820$ µM and resulting

79 k_{cat}/ K_M=22.19 $s^{-1}.M^{-1}$) and on penicillin G, when measuring its complete degradation toward
80 a single metabolite i.e. benzyl penilloic acid within three hours (**fig. 2C**). As shown on **Suppl.**
81 **Figure S4**, the MetbaB activity was also evaluated in different pH and was optimal on
82 nitrocefin at pH 7. Furthermore, to confirm the β-lactamase activity of this enzyme, the
83 combination of nitrocefin with β-lactamase inhibitor sulbactam (at 1 µg/mL) was tested. As
84 shown in **Figure 2A** (column 4), in the presence of sulbactam, no degradation of the
85 nitrocefin β-lactam could be detected, suggesting a complete inhibition of the archaeal β-
86 lactamase enzyme. This neutralizing activity was confirmed microbiologically on a
87 *Pneumococcus* strain highly susceptible to penicillin (MIC =0.012 µg/ml) and highly resistant
88 to sulbactam (MIC =32 µg/ml). Indeed, bacteria could grow in the presence of 0.1 µg/ml of
89 penicillin incubated with the archaeal β-lactamase, but not when sulbactam was added,
90 suggesting an inhibition of penicillin G enzymatic digestion (**fig. 2D**).

91 The antibiotic susceptibility testing of a recombinant *E. coli* mutant containing this
92 archaeal β-lactamase also revealed a reduced susceptibility to penicillin (from 1 µg/ml to 4
93 µg/ml) (data not shown). Interestingly, it appears that these archaeal β-lactamases are closely
94 related to bacterial enzymes known as "GOB" (AF090141), which are fully functional in vivo
95 and present in a single bacterial genus, namely *Elizabethkingia* (11, 12) (**fig. 1**). However, the
96 MBL protein sequences of this bacterial genus compared to those of archaea reveal low
97 similarities (less than 36%) and this therefore suggests an ancient HGT from an archaic
98 phylum to this bacterial group, which furthermore exhibited β-lactam hydrolysis activity,
99 previously considered to be fairly atypical for a bacterium (**Table S3**). Indeed, because
100 archaea are naturally resistant to ß-lactams, the role of these β-lactamases in these
101 microorganisms remains to be clarified, but the digestion of β-lactams by β-lactamases in
102 Archae to use it as a carbon source, as in bacteria, should be investigated (13).

Archaeal class C-like β-lactamases: Four significant sequences homologous to bacterial class C β-lactamase sequences were identified in archaea database using the inferred bacterial class C ancestor sequence (**fig. 3; Suppl. Table S1**). The phylogeny analysis shows that this third-class C-like of β-lactamases appears to be a very old class, a putative new clade, which cannot be identified without the reconstruction of the common ancestor (**fig. 3**). As shown in this figure, this class C-like enzyme appears more closely related to DD-peptidase enzymes than the known bacterial class C β-lactamases. Protein alignment reveals the same conserved motifs ($S^{64}XXK$ and $Y^{150}XN$) identified in bacteria, the signature motifs of this class C β-lactamase (**fig. S5**). The three-dimensional (3D) structure comparison of this archaeal class C-like enzyme with known and well characterized proteins in the Phyre2 investigator database reveals 100% of confidence and 66% of coverage with the crystal structure of the octameric penicillin-binding protein (PBP) homologue from *pyrococcus abyssi* (Phyre2 ID: c2qmiH) (**Table S2**). Similarly, the identified archaeal enzyme of this class C (gi|919167542) was also cloned in *E. coli* and found to be active in enzymatic level by hydrolyzing the nitrocefin (data not shown). This enzymatic activity was also confirmed by the kinetic assays showing the catalytic parameters kcat=9.67×10-3 s-1, Km=583.6 µM and kcat/ Km=16.57 s-1.M-1, according to Michaelis-Menten equation fitting (R^2=0.984). However, the β-lactams susceptibility testing of the recombinant *E. coli* strains harboring this sequence reveals no reduced susceptibility as compared to the control *E. coli* strains.

Discussion

The archaea microorganisms, in which these β-lactamases were identified, are fully resistant to β-lactam antibiotics. So far, β-lactamases have been described and considered as one of the elements in the fight against β-lactams acting on the cell wall (14). Nevertheless, given the well-known and documented natural resistance of archaea to β-lactam antibiotics, it did not make sense to discover the existence of archaic β-lactamases in this microorganism group. In this current study, we show that two classes of β-lactamases can be found in archaea, especially in *Methanosarcina* species. These latter have the largest genomes in the archaea kingdom because of a massive horizontal gene transfer (HGT) from bacteria (15). The identified class B appears highly conserved in archaea, with a unique transfer event in *Elizabethkingia* species whereas, the class C enzyme appears as a new clade and more closely related to the DD-peptidase enzymes i.e. the penicillin binding proteins. So far, metallo-β-lactamase enzymes in Archaea are essentially described with respect to their role in the DNA and RNA metabolism (16, 17). Here, we show that these archaeal enzymes can hydrolyze also β-lactam antibiotics, as known for bacteria, and are inhibited by β-lactamase inhibitors. So, the role of β-lactamases in Archaea is not totally understood. Our findings suggest that archaeal β-lactamases are as ancestral as those of bacteria, and HGT events have occurred from archaea to bacteria. Moreover, we highlight here that the use of consensual ancestor sequences from phylogenetic analyses, is an interesting approach to fish out remote homologous sequences to known ones in any sequences database.

Finally, the existence of β-lactamases in the world of archaea is showing that β-lactamases are not only a defense system against β-lactams. The use of antibiotics as a nutriment sources for archaea as key to degrade β-lactam molecules and use them as carbon sources as described in bacteria, is a plausible hypothesis (13, 18–20).

Materials and Methods

Sequence analysis:

A total of 1,155 amino acid sequences were retrieved (Class A: 620; B: 174; C: 151, and D: 210) from the ARG-ANNOT database (21). The phylogenetic trees were inferred using the approximate maximum-likelihood method in FastTree (22). For a detailed and comprehensive diversity analysis, a few sequences from each clade of the trees were selected as representatives of the corresponding clades (labeled in red in **fig. S1 and S2**).

The ancestral sequence was inferred using the maximum-likelihood method conducted by MEGA6 (23) software. Then, these ancestral sequences were used as queries in a BlastP (24) search (≥ 30% sequence identity and ≥ 50% query coverage) against the NCBI-nr archaeal database. For Class C β-lactamase analysis, DD-peptidase sequences (penicillin binding proteins) were downloaded from the NCBI database. 2515 sequences were selected for local Blast analysis with the archaeal Class C-like β-lactamase used as query sequence (**GI: 919167542**). From this analysis, 24 DD-peptidase sequences were identified as homologous to the query and thus used for further phylogenetic tree analysis. The selected archaeal sequences were aligned with known bacterial β-lactamase sequences (representative sequences of a known clade from the guide tree) using the multiple sequence alignment algorithm MUSCLE (25) and the phylogenetic tree was inferred using FastTree (22).

Antibiotic susceptibility testing

The antibiotic susceptibility testing was performed on 15 antibiotics including ampicillin, ampicillin/sulbactam, penicillin, piperacillin, piperacillin/tazobactam, cefoxitin, ceftriaxone, ceftazidime imipenem, meropenem, aztreonam, gentamicin, ciprofloxacin, amikacin, and trimethoprim-sulfamethoxazole (I2a, SirScan Discs, France). A filtred aqueous solution of each antibiotic was prepared anaerobically in a sterilized Hungate tubes at concentration of 5 mg/ml. Then, 0.1 ml of each one of these solutions was added to a freshly

inoculated culture tube containing 4.9 ml of the tested stain to obtain a final concentration of 100µg/ml for each antibiotic herein tested. The mixture of antibiotic and archaeal culture was then incubated at 37°C and the growth of archaea was observed after 5 to 10 days incubation depending on the tested strain. Control cultures without antibiotic were also incubated in the same conditions to assess the strain growth and non-inoculated culture tubes were used as negative control.

In vitro activity test:

Protein expression and purification: The selected beta-lactamases were optimized for protein expression in *Escherichia coli* and synthesized by GenScript (Piscataway, NJ, USA) and then cloned into the pET24a(+) expression vector. Recombinant β-lactamases were expressed in *E. coli* BL21(DE3)-pGro7/GroEL (TaKaRa) using ZYP-5052 media. Each culture was grown at 37°C until reaching an OD600 nm = 0.8, followed by addition of L-arabinose (0.2% m/v) and induction with a temperature transition to 16°C over 20 hours. Cells were harvested by centrifugation (5000 g, 30 min, 4°C) and the resulting pellets were resuspended in Wash buffer (50 mM Tris pH 8, 300 mM NaCl) and stored at -80°C overnight. Frozen cells were thawed and incubated on ice for 1 hour after adding lysozyme, DNAse I and PMSF (Phenylmethylsulfonyl fluoride) to final concentrations of, respectively, 0.25 mg/mL, 10µg/mL and 0.1 mM. Partially lysed cells were then disrupted by three consecutive cycles of sonication (30 seconds, amplitude 45) performed on a Q700 sonicator system (QSonica). Cell debris was discarded following a centrifugation step (10,000 g, 20 min, 4°C). Recombinant β-lactamases were purified using Strep-tag affinity chromatography (Wash buffer: 50 mM Tris pH 8, 300 mM NaCl and Elution buffer: 50 mM Tris pH 8, 300 mM NaCl, 2.5 mM desthiobiotin) on a 5 mL StrepTrap HP column (GE Healthcare). Fractions containing each protein of interest were pooled. Protein expression and purity were assessed

using a 10% SDS-PAGE analysis (Coomassie stain). Protein concentrations were measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific).

β-Lactamase detection: Purified recombinant β-lactamases were submitted for a BBL™ Cefinase™ paper disc test(26) (Becton Dickinson). All protein samples were adjusted to a final concentration of 2 mg/ml. 15 µl of each recombinant β-lactamase were deposited onto a paper disc impregnated with nitrocefin and incubated at room temperature. 15 µl of extracted proteins from induced BL21(DE3)-pGro7/GroEL strain that did not contain any β-lactamase genes, was used as negative control. When a change of color from yellow to red was visible within 30 minutes of incubation, corresponding to the hydrolysis of the amide bond in the beta-lactam ring of nitrocefin, it was considered that the tested fraction contained an active β-lactamase enzyme. The hydrolysis of the nitrocefin and penicillin G in presence of sulbactam, was also monitored using a Synergy HT microplate reader (BioTek, USA). Reactions were performed at 25°C in a 96-well plate in PBS buffer and 5 % DMSO with a final volume of 100 µl for each well. Time course hydrolysis of nitrocefin (0.5 mM) was monitored for 10 minutes after adding 50 µL of previously prepared protein sample, with absorbance at 486 nm. For the inhibition assay, active β-lactamases at a final concentration of 0.5 mg/ml were briefly incubated with 0.1 mM sulbactam. Negative controls with only sulbactam in buffer and positive controls containing enzymes without any inhibitor were also prepared. After adding 0.5 mM nitrocefin, its hydrolysis was monitored over time with absorbance at 486 nm. Furthermore, the activity of MetbaB enzyme was evaluated at different pH (between pH7 and pH10) using the same nitrocefin assay conditions.

β-lactamase kinetic characterization: Kinetic assays were monitored with a Synergy HT microplate reader (BioTek, USA). Reactions were performed at 25°C in a 96-well plate (6.2 mm path length cell) in buffer 50 mM Tris pH 8, 300 mM NaCl, 5% DMSO with a final volume of 100 µl for each well. The time course hydrolysis of nitrocefin (ε486 nm = 20 500

M-1 .cm-1) with final concentrations varying between 0.05 and 1.5 mM was monitored for 10 minutes following absorbance variations at 486 nm, corresponding to the appearance of a red product. Both enzymes were kept at a final concentration of 0.3 mg/ml for kinetic studies. For each substrate concentration, the initial velocity was evaluated by Gen5.1 software. Mean values obtained were fitted using the Michaelis-Menten equation on GraphPad Prism 5 software in order to determine catalytic parameters.

β-lactam hydrolysis monitored by Liquid Chromatography-Mass Spectrometry (LC-MS). Water and acetonitrile solvents were ULC-MS grade (Biosolve). Penicillin G and sulbactam stock solutions at 10 mg/ml were freshly prepared in water from the corresponding high purity salts (Sigma Aldrich). A 1X phosphate-buffered saline (PBS) solution at pH 7.4 was prepared in water from a commercial salt mixture (bioMerieux). Pure solutions of the archaeal class B (MetbaB) β-lactamase enzyme was buffer-exchanged in PBS, and the concentration was adjusted to 1 mg/ml. 30 µl was then spiked with penicillin G and sulbactam at a final concentration of 10 µg/ml. Negative controls consisted of PBS spiked with penicillin G and sulbactam Several solutions were prepared to measure metabolites at different incubation times at room temperature. Each time point corresponded to triplicate sample preparations. Then, 70 µl of acetonitrile was added to each sample, and tubes were vortexed 10 minutes at 16000 g to precipitate proteins. The clear supernatant was collected for analysis using an Acquity I-Class UPLC chromatography system connected to a Vion IMS Qtof ion mobility-quadrupole-time of flight mass spectrometer. For each sample, 5 µl stored at 4°C was injected into a reverse phase column (Acquity BEH C18 1.7 µm 2.1x50 mm, Waters) maintained at 50°C. Compounds were eluted at 0.5 ml/min using water and acetonitrile solvents containing 0.1% formic acid. The following composition gradient was used: 10-70% acetonitrile within 3 minutes, 95 % acetonitrile for a 1-minute wash step, and back to the initial composition for 1-minute. Compounds were ionized in the positive mode using a

Zspray electrospray ion source with the following parameters: capillary/cone voltages 3 kV/80 V, and source/desolvation temperatures 120/450°C. Ions were then monitored using a High Definition MS(E) data independent acquisition method with the following settings: travelling wave ion mobility survey, 50-1000 m/z, 0.1 s scan time, 6 eV low energy ion transfer, and 20-40 eV high energy for collision-induced dissociation of all ions (low/high energy alternate scans). Mass calibration was adjusted within each run using a lockmass correction (Leucin Enkephalin 556.2766 m/z). The Vion instrument ion mobility cell and time-of-flight tube were calibrated beforehand using a Major Mix solution (Waters) to calculate collision cross section (CCS) values from ion mobility drift times and mass-to-charge ratios. 4D peaks, corresponding to a chromatographic retention time, ion mobility drift time and parents/fragments masses, were then collected from raw data using UNIFI software (version 1.9.3, Waters). As reported, penicillin G can be degraded in alkaline or acidic pH and in the presence of β-lactamase into different metabolites, including benzyl penilloic acid or benzylpenillic acid. A list of known chemical structures, including penicillin G and its metabolites (27, 28), were targeted with the following parameters: 0.1 minutes retention time window, 5 % CCS tolerance, 5 ppm m/z tolerance on parent adducts (H+ and Na+) and 10 mDa m/z tolerance on predicted fragments. Retention times and CCS values were previously measured from penicillin G degradation experiments at pH 2 and pH 10 in order to perform subsequent accurate structures screening. Detector counts of the targeted structures were then collected for data interpretation.

Acknowledgements

Financial support from the IHU Mediterranee Infection, Marseille France and American Journal Experts (AJE) for English corrections of the manuscript are gratefully acknowledged.

Author contributions:

D.R. conceived and designed the study. S.M.D., L.P., V.K., N.A, P.C, S.K., G.C-A., J.-M.R., B.L, P.P., and D.R. analysed and interpreted data. S.M.D., L.P., V.K., N.A, P.C, S.K., G.C-A., J.-M.R., B.L, P.P., and D.R. drafted the manuscript and/or made critical revisions. All of the authors read and approved the final manuscript.

Funding:

This work was supported by the French Government under the "Investments for the Future" program managed by the National Agency for Research (ANR), Méditerranée-Infection 10-IAHU-03 and was also supported by Région Provence Alpes Côte d'Azur and European funding FEDER PRIMMI (Fonds Européen de Développement Régional - Plateformes de Recherche et d'Innovation Mutualisées Méditerranée Infection).

Competing interests:

We declare that we have no conflicts of interest.

Figure legends

Figure 1: Phylogenetic Tree of Class B β-lactamases from archaea and bacteria. Archaeal sequence colored in green is which expressed and experimentaly tested.

Figure 2: Characterization of the archaeal class B MBL (MetbaB) identified in *Methanosarcina barkerii*. **(A and B)**: β-lactamase activity of the *M. barkeri* Class B MBL enzyme (MetbaB) on a chromogenic cephalosporin substrate (Nitrocefin). **A1 and A2** refer to the nitrocefin degradation test using the BBL™ Cefinase™ paper disc respectively at t=0 and t=30 min. **A3** refers to this same test performed in liquid medium in the absence of sulbactam while **A4**, with the addition of 1 µg/ml sulbactam, both after 30 minutes of incubation; (**B**) monitored nitrocefin degradation by following the absorbance at 486 nm over time in the presence and absence of the β-lactamase inhibitor. **(C)**: LC/MS average relative response of screened metabolite compounds of penicillin G in the presence the *M. barkeri* Class B MBL enzyme monitored for three hours. Penicillin G (in orange) refers to the intact form of the antibiotic while penilloic acid (in purple) and penillic acid (in light blue) refer to the penicillin G metabolites. Penicillin G control in PBS did not show any degradation towards any metabolite (data not shown). **(D),** Microbiological test of the mixture of penicillin G (0.1 µg/ml) with the MetbaB enzyme in the presence and absence of sulbactam (15 µg/ml) on a *Pneumococcus* strain highly susceptible to penicillin G (MIC= 0.012 µg/ml) and highly resistant to sulbactam (MIC= 32 µg/ml). The halo around holes 1 and 5 reveals growth inhibition of the *Pneumococcus* strain. The absence of this halo around holes 2, 3, and 4 means no effect of the mixture on the *Pneumococcus* growth could be observed.

Figure 3: Phylogenetic Tree of Class C β-lactamases and DD-peptidases proteins (penicillin binding proteins). The class A β-lactamases is used as root.

References

1. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. 2010. Bacterial competition: surviving and thriving in the microbial Jungle. Natl Rev Microbiol 8:15–25.

2. Caetano-Anollés G, Kim KM, Caetano-Anollés D. 2012. The phylogenomic roots of modern biochemistry: Origins of proteins, cofactors and protein biosynthesis. J Mol Evol 74:1–34.

3. Caetano-Anollés D, Kim KM, Mittenthal JE, Caetano-Anollés G. 2011. Proteome evolution and the metabolic origins of translation and cellular life. J Mol Evol 72:14–33.

4. Van V L. 1973. A new evolutionary law. Evol Theory 30:1–30.

5. Walther-Rasmussen J, Høiby N. 2007. Class A carbapenemases. J Antimicrob Chemother 60:470–82.

6. Kok J. 2011. Murein and pseudomurein cell wall binding domains of bacteria and archaea — a comparative view 921–928.

7. Dridi B, Fardeau ML, Ollivier B, Raoult D, Drancourt M. 2011. The antimicrobial resistance pattern of cultured human methanogens reflects the unique phylogenetic position of archaea. J Antimicrob Chemother 66:2038–2044.

8. Sharma V, Colson P, Giorgi R, Pontarotti P, Raoult D. 2014. DNA-Dependent RNA Polymerase Detects Hidden Giant Viruses in Published Databanks. Genome Biol Evol 6:1603–10.

9. Vivek Keshri, Arup Panda, Anthony Levasseur, Jean-Marc Rolain, Pierre Pontarotti DR. 2018. Phylogenomic analysis of betalactamase in archae and bacteria enables the identification of putative new members. Genome Biol Evol 10:1106–1114.

10. Bush K. 2013. The ABCD's of β-lactamase nomenclature. J Infect Chemother 19:549–559.

11. Opota O, Diene SM, Bertelli C, Prod'hom G, Eckert P, Greub G. 2016. Genome of the carbapenemase-producing clinical isolate Elizabethkingia miricola EM_CHUV and comparative genomics with Elizabethkingia meningoseptica and Elizabethkingia anophelis: evidence for intrinsic multidrug resistance trait of emerging pathogens. Int J Antimicrob Agents 1:93–97.

12. Horsfall LE, Izougarhane Y, Lassaux P, Selevsek N, Li??nard BMR, Poirel L, Kupper MB, Hoffmann KM, Fr????re JM, Galleni M, Bebrone C. 2011. Broad antibiotic resistance profile of the subclass B3 metallo-β- lactamase GOB-1, a di-zinc enzyme. FEBS J 278:1252–1263.

13. Crofts TS, Wang B, Spivak A, Gianoulis TA, Forsberg KJ, Gibson MK, Johnsky LA, Broomall SM, Rosenzweig CN, Skowronski EW, Gibbons HS, Sommer MOA, Dantas G. 2018. Shared strategies for β-lactam catabolism in the soil microbiome. Nat Chem Biol 14:556–564.

14. Bush K, Bradford PA. 2016. β-lactams and β-lactamase inhibitors: An overview. Cold Spring Harb Perspect Med.

15. Deppenmeier U, Johann A, Hartsch T, Merkl R, Schmitz RA, Martinez-Arias R, Henne A, Wiezer A, Bäumer S, Jacobi C, Brüggemann H, Lienard T, Christmann A, Bömeke

M, Steckel S, Bhattacharyya A, Lykidis A, Overbeek R, Klenk H-P, Gunsalus RP, Fritz H-J, Gottschalk G. 2002. The Genome of Methanosarcina mazei: Evidence for Lateral Gene Transfer Between Bacteria and Archaea JMMB Research Article. J Mol Microbiol Biotechnol 4:453–461.

16. Dominski Z. 2007. Nucleases of the Metallo- β -lactamase Family and Their Role in DNA and RNA Metabolism 67–93.

17. Fischer S, John von Freyend S, Sabag-Daigle A, Daniels CJ, Allers T, Marchfelder A. 2012. Assigning a function to a conserved archaeal metallo-β-lactamase from Haloferax volcanii. Extremophiles.

18. Woappi Y, Gabani P, Singh A, Singh O V. 2016. Antibiotrophs: The complexity of antibiotic-subsisting and antibiotic-resistant microorganisms. Crit Rev Microbiol 42:17–30.

19. González T de JB, Zuidema T, Bor G, Smidt H, van Passel MWJ. 2016. Study of the aminoglycoside subsistence phenotype of bacteria residing in the gut of humans and zoo animals. Front Microbiol 6:1–7.

20. Xin Z, Fengwei T, Gang W, Xiaoming L, Qiuxiang Z, Hao Z, Wei C. 2012. Isolation, identification and characterization of human intestinal bacteria with the ability to utilize chloramphenicol as the sole source of carbon and energy. FEMS Microbiol Ecol 82:703–712.

21. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, Rolain J-M. 2014. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother 58:212–20.

22. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490.

23. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729.

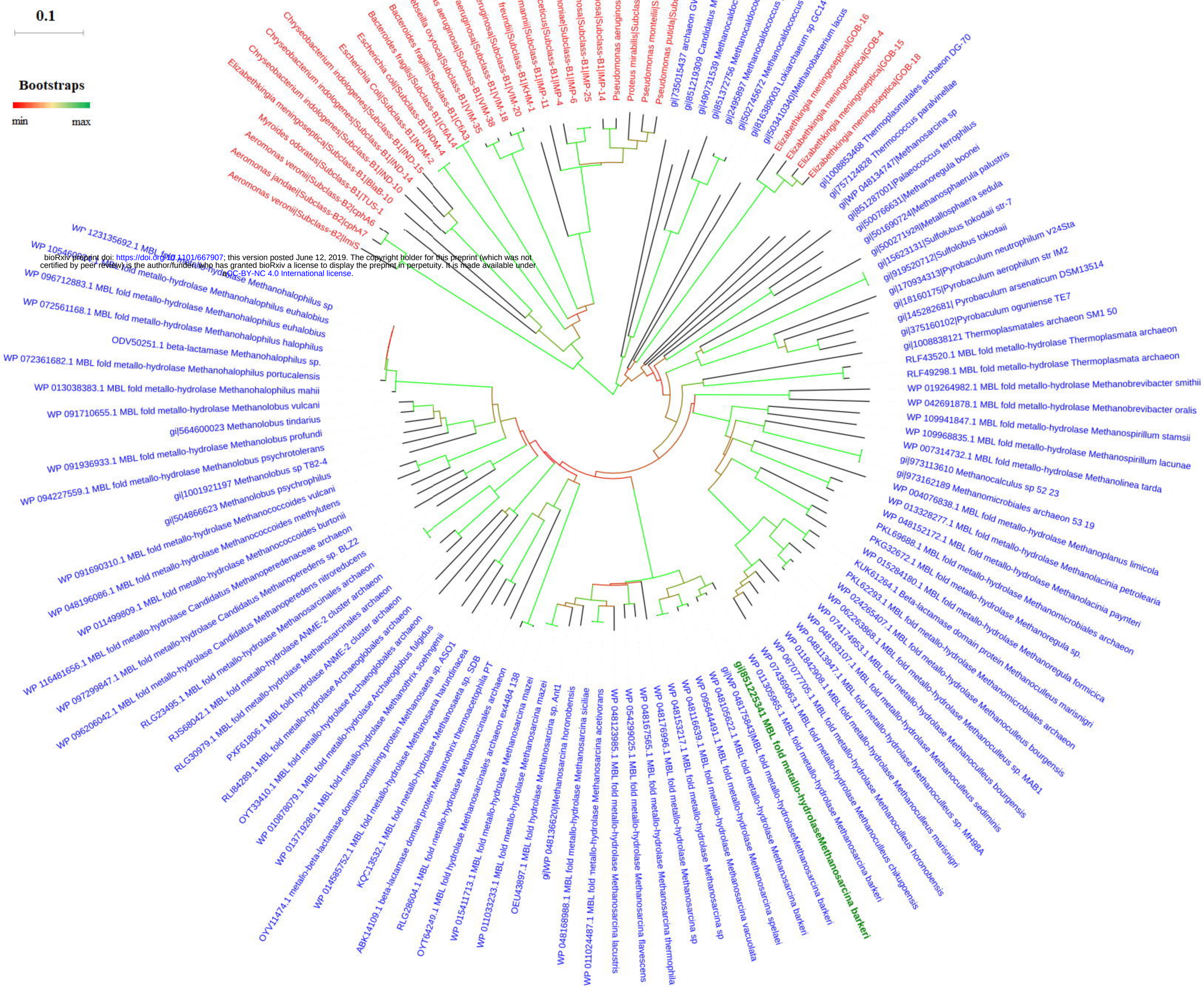
24. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic Local Alignment Search Tool. J Mol Biol 215:403–410.

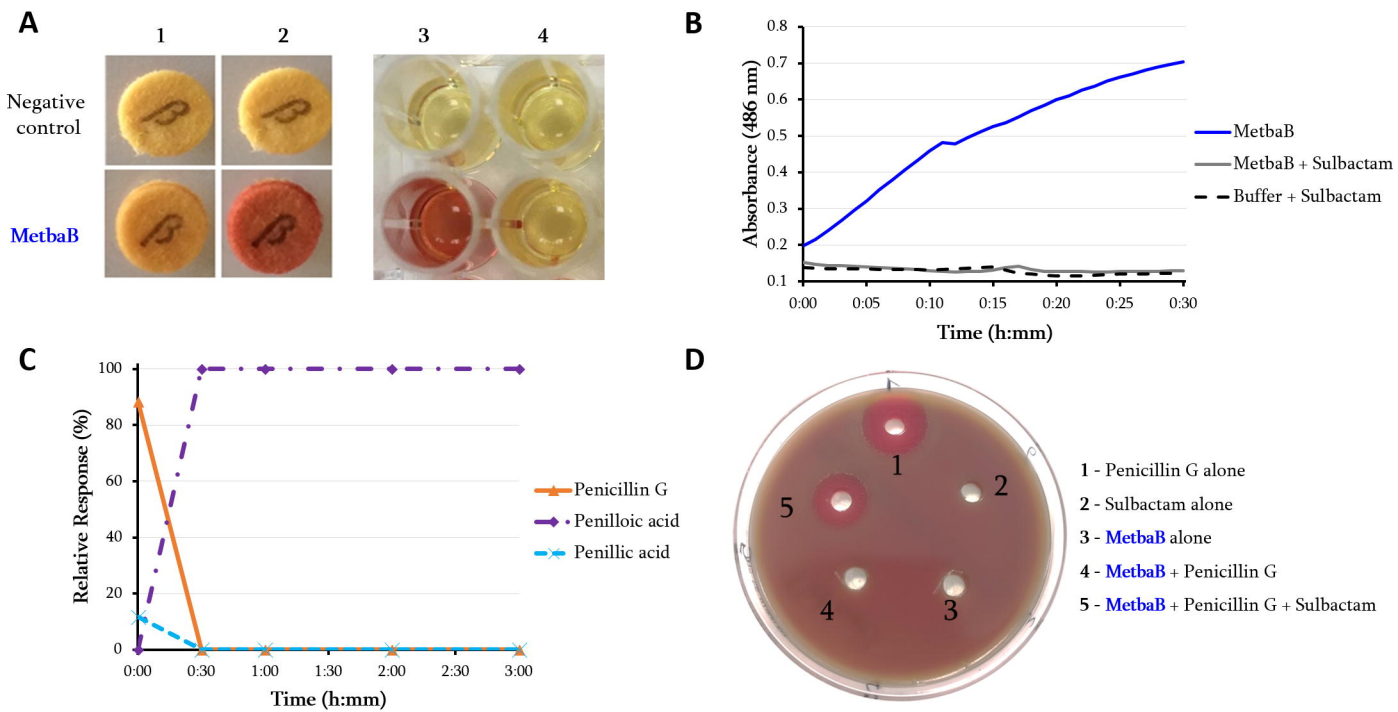
25. Edgar RC. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797.

26. Qadri SMH, Perryman F, Flournoy D. 1983. Detection of Beta Lactamase Producing Bacteria by Cefinase□: Nachweis von Beta-Laktamase produzierenden Bakterien mit Cefinase□: Zentralblatt fr Bakteriol Mikrobiol und Hyg / A Medizinische Mikrobiol Infekt und Parasitol 255:489–493.

27. Deshpande AD, Baheti KG, Chatterjee NR. 2004. Degradation of β-lactam antibiotics. Curr Sci 87:1684–1695.

28. Aldeek F, Canzani D, Standland M, Crosswhite MR, Hammack W, Gerard G, Cook J-M. 2016. Identification of Penicillin G Metabolites under Various Environmental Conditions Using UHPLC-MS/MS. J Agric Food Chem 64:6100–6107.




A
1
2
3
4
Negative control
MetbaB
B
Absorbance (486 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0:00
0:05
0:10
0:15
0:20
0:25
0:30
Time (h:mm)
MetbaB
MetbaB + Sulbactam
Buffer + Sulbactam
C
Relative Response (%)
100
80
60
40
20
0
0:00
0:30
1:00
1:30
2:00
2:30
3:00
Time (h:mm)
Penicillin G
Penilloic acid
Penillic acid
D
1
2
3
4
5
1 - Penicillin G alone
2 - Sulbactam alone
3 - MetbaB alone
4 - MetbaB + Penicillin G
5 - MetbaB + Penicillin G + Sulbactam

CMY-12 Enterobacter cloacae
CMY-27 Escherichia coli
CMY-17 Escherichia coli
CMY-22 Escherichia coli
CMY-28 Escherichia coli
CMY-20 Escherichia coli
CMY-13 Escherichia coli
CMY-80 Citrobacter freundii
CMY-39 Citrobacter freundii
CMY-64 Escherichia coli
CMY-79 Citrobacter freundii
CMY-48 Citrobacter freundii
CMY-67 Citrobacter freundii
CMY-78 Citrobacter freundii
CMY-81 Citrobacter freundii
CMY-83 Citrobacter braakii
ACT-3 Enterobacter asburiae
ACT-12 Enterobacter cloacae
DHA-7 Enterobacter cloacae
ACC-1 Klebsiella pneumoniae
ACC-2 Hafnia alvei
OCH-6 Ochrobactrum anthropi
OCH-8 Ochrobactrum anthropi
MOX-4 Aeromonas caviae
CMY-19 Klebsiella pneumonaie
FOX-5 Klebsiella pneumonaie
FOX-7 Enterobacter cloacae

Bacterial Class C Beta-lactamases

gi|919167542|ref|WP 052723173.1|Methanosarcina sp. WH1
gi|919167113|ref|WP 052722744.1|Methanosarcina sp. WWM596
gi|1011504614|ref|WP 062399151.1|Methanogenium cariaci
gi|445600523|gb|ELY54529.1|Natronococcus amylolyticus DSM10524

Archaeal class C-like-β-lactamases

AEY86313.1|Putative DD-peptidase | Streptomyces hygroscopicus jinggangensis
ALO99834.1|DD-peptidase |Streptomyces hygroscopicus subsp. limoneus
CAJ89414.1|putative DD-peptidase| Streptomyces ambofaciens ATCC23877
EMF29789.1|DD-peptidase |Streptomyces gancidicus BKS 13-15
EFL13097.1| DD-peptidase Streptomyces sp. C
pdb|1PWD|DD-peptidase With Cephalosporin C|Streptomyces R61
pdb|1PW1|DD-Peptidase With A Highly Specific Penicillin Streptomyces R61
sp|P15555.2|D-alanyl-D-alanine carboxypeptidase|Streptomyces sp.
CAA28756.1|DD-peptidase precursor Streptomyces lividans

DD-Peptidase proteins

KPC-2
KPC-5
IMI-2
GES-2
GES-4
HERA-1
HERA-4
PSE-4
OKP-A-5
OKP-A-9
OKP-A-13
TEM-177
TEM-167
TEM-169
TEM-182

Bacterial Class A Beta-lactamases

0.1