1   Properties and distribution of a Metallo-β-Lactamase (ALI-1) from the fish

2   pathogen *Aliivibrio salmonicida* LFI1238

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11   Running title: ALI-1, a class B β-lactamase from *A. salmonicida*

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13   ***Objectives:*** To characterize the chromosome-encoded Metallo-β-Lactamase (MBL) from the

14   psychrophilic, marine fish-pathogenic bacterium *Aliivibrio salmonicida* LFI1238 and check

15   for the presence of the gene in other *Aliivibrio* isolates both connected to the fish farming

16   industry and from the environment.

17

18   ***Methods:*** The MBL gene was cloned and intracellularly expressed in *Escherichia coli*.

19   Kinetic parameters, NaCl dependence, pH optimum and temperature optimum were

20   determined using purified enzyme. The VIM-2 enzyme from a *Pseudomonas aeruginosa*

21   hospital isolate was used as a counterpart in comparative analysis. PCR with degenerate MBL

22   primers were used to screen different *A. salmonicida* isolates for the presence of the gene.

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24   ***Results:*** *A. salmonicida* MBL (ALI-1) is an Ambler class B β-lactamase sharing 39% and

25   29% amino acid identity with IMP-1 and VIM-2, respectively. ALI-1 hydrolyzed all β-lactam

26 antibiotics, except from the monobactam aztreonam and the penicillin piperacillin. A

27 profound increase in activity was observed when adding NaCl to the assay mix (60% active

28 without addition of NaCl, increasing to 100% at 0.5M NaCl). The increase was less noticeable

29 for VIM-2 (100% active at 0.2M NaCl). The ALI-1 appears to be ubiquitous in nature as it is

30 found in *Aliivibrio* isolates not affected by human activity.

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32 *Conclusions:* This work provides more data in the ever-expanding MBL group of enzymes.

33 These periplasmic enzymes are activated by addition of sodium chloride, and the marine

34 enzyme is highly salt tolerant and cold active. The observed enzyme properties very likely

35 reflect the conditions the enzymes face in-situ.

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Introduction

Metallo-β-lactamases (MBLs) belong to the protein family of β-lactamases, a group of enzymes which deactivates β-lactam antibiotics by cleaving the amide bond in the β-lactam ring. MBLs are considered a major cause of bacterial resistance towards β-lactam antibiotics.[1] According to the Ambler molecular classification, MBL from *Aliivibrio salmonicida* (ALI-1) belongs into the molecular class B, subclass B1.[2] Enzymes from the subclasses B1 and B3 need two zincs bound for maximum activity, while B2-enzymes are inhibited if two zincs are bound to them.[3] Active-site residues that are coordinating zinc ions are conserved among all three subclasses.[4]

β-lactamase-induced antibiotic resistance, which is developing into a serious threat in recent years, is thought to be mostly induced by man's thoughtless use of antibiotics. The most common way of transferring antibiotic resistance in bacteria is through horizontal transfer of a plasmid between different bacterial species,[5] but the origin of β-lactamases is still not well known. First and foremost, it is reasonable to suppose that the β-lactamases were originally important in fighting for the natural habitat amongst bacteria. β-lactamase production can be seen as a response to the different organism's capability of producing antibiotics. For example in an Alaskan environment, where no human antibiotic pressure has been applied, a diverse set of lactamases have been found and also other substrates than the β-lactam ring were suggested as substrates for these β-lactamases.[6] In addition, there have also been found many different freshwater species of enterobacteria with a broad spectrum of resistance with no apparent connection to human activities.[7] This indicates that human antibiotic overuse is perhaps not the only reason for antibiotic multi-resistance found in bacteria. A possible hypothesis for finding resistance to antibiotics in natural bacterial colonies could be that the resistance functions as a regulator of quorum sensing. It has been

67   suggested that antibiotics are used to communicate between bacteria, which could mean that,

68   for instance, the role of β-lactamases is to adjust quorum sensing signals.[8]

69   The MBL studied in this article originates from *Aliivibrio salmonicida*, a Gram

70   negative, motile and rod-shaped bacteria, previously known as *Vibrio salmonicida*.[9] It locates

71   to the marine environment and is often associated with fish; some species have been regarded

72   as pathogens of marine animals as for example the causative agent of the so-called Hitra-

73   disease or cold-water vibrosis.[10]

74   Organisms are generally adapted to their surroundings, and there are numerous

75   organisms that have adapted to life under cold conditions. On the molecular level, these

76   adaptations can result in increased specific activity of enzymes at low temperatures, which is

77   usually associated with higher flexibility and lower stability.[11-14] The marine environment,

78   where *A. salmonicida* resides, is quite hostile in terms of low temperature and high salt

79   concentrations. This will potentially also effect ALI-1 as it locates to the periplasm. In this

80   study, we have investigated whether ALI-1 is affected by its surroundings and further have

81   attempted to rationalize this adaptation.

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Materials and methods

Bacterial strains

The bacterial strains used in the study were *Aliivibrio salmonicida* LFI1238[15] from which the gene encoding ALI-1 was amplified, *A. salmonicida* isolates originating from different disease outbreaks in the fish farming industry and environmental *Aliivibrio* isolates from the Barents Sea. *Escherichia coli* Rosetta2 pLysS (Novagen) was used for recombinant protein expression.

Cloning, expression and purification of ALI-1

The gene encoding ALI-1 was amplified from *A. salmonicida* LFI1238 and cloned into the expression vector pET26b(+) containing a T7 promoter using standard molecular biology techniques. The gene was cloned without its N-terminal periplasmic signal peptide and with six histidines added in the N-terminus.

The cells were grown in 250 mL cultures of Lysogeny broth (LB) and protein expression was induced with IPTG (Takara Bio, Otsu Japan) at a final concentration of 0.5 mM. Following overnight expression of protein at 20°C, the cells were harvested by centrifugation at 6000 rpm for 25 min and at 4°C. The supernatant was discarded and each pellet was resuspended in 30 mL of lysis buffer (50 mM Tris, 250 mM NaCl, 5 mM β-mercaptoethanol, 10 mM Imidazole and 0.1 mM $ZnCl_2$ at pH 7.5) also containing 1 tablet of protease inhibitor cocktail (Roche, Germany) and DNaseI (Sigma Aldrich). The cells were disrupted by sonication, and centrifuged at 9000 g for 30 min at 4°C after which the supernatant was used further in purification. The purification was done on an ÄKTA purifier (GE healthcare) using a 5 mL HisTrap crude FF column. The protein was eluted with buffer containing 50 mM Tris, 0.1 mM $ZnCl_2$, 250 mM NaCl and 500 mM Imidazole at a pH 7.5.

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117   Biochemical characterization

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119   pH, NaCl and temperature optimum

120   The appropriate wavelengths for quantifying the substrate, nitrocefin, as well as the product

121   were found by comparing wavelengths as previously described,[16,17] and by making

122   experimental measurements of the absorbance spectra of the compounds. All measurements

123   were done using a Spectramax M2^e (Molecular devices, Sunnyvale California USA).

124   The optimal pH for MBL was identified using HEPES buffer in the pH range 6.5-8.5. The

125   experiments were done in a reaction mixture containing 500 mM NaCl, 0.2 mM nitrocefin, 50

126   mM HEPES with varying pH and 100 µM $ZnCl_2$ at room temperature (22°C).

127   The search for the optimal concentration of NaCl for MBL activity was performed in a

128   reaction mixture with 25 µM $ZnCl_2$, 10 mM Tris, 0.2 mM nitrocefin at pH 7.5 and at 37°C.

129   The enzyme was incubated in reaction mixture at 37°C for 5 min prior to adding substrate.

130   For investigation of temperature optimum, the reaction mixture was made of these

131   components: 0.2 mM nitrocefin, 25 mM HEPES at pH 7.5, 200 µM $ZnCl_2$ with concentrations

132   of NaCl at 0, 200 and 500 mM. The experiments were carried out having the reaction mixture

133   preincubated at the intended temperature before adding enzyme. After 10 min, the reaction

134   was stopped by adding a surplus of EDTA and incubating the mixture on ice before

135   measuring its endpoint activity by the spectrophotometer.

136   The blanks had the same components as the reaction mixture but without enzyme. From these

137   measurements an assumption of the stability of the substrate was found.

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140   Zinc-influence

141   The reaction mixture used when testing zinc-influence was 500 mM NaCl, 10 mM Tris, 0.2

142   mM nitrocefin at pH 7.5 and at a temperature of 37°C, where the enzyme was incubated for 5

143   min in the reaction mixture at 37°C. The zinc concentrations tested were 100 μM and 5 mM.

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145   Reducing agents

146   To observe the influence of reducing agents on the enzymatic activity, 5 mM of β-

147   mercaptoethanol, DTT (Dithiothreitol) or TCEP (*Tris*(2-carboxyethyl)phosphine HCl) was

148   added to a reaction mixture containing 500 mM NaCl, 10 mM Tris, 25 μM $ZnCl_2$ and 10 μL

149   20 μG/mL ALI-1, pH 7.5 at 37°C. The reaction mixture was equilibrated for 5 min at 37°C

150   before starting the reaction by adding 0.2 mM nitrocefin.

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152   Kinetics

153   The different substrates were dissolved in a solution of 50 mM HEPES, pH 7.2 and 100 μM

154   $ZnCl_2$. The substrate concentration of this stock solution was decided using the

155   spectrophotometer and extinction coefficient of each substrate. Based on the concentration of

156   the stock solution, the solutions for the kinetic analysis were made in the range of 2-1,000

157   μM. The kinetic analysis was performed in a reaction mixture containing 50 mM HEPES, pH

158   7.2 and 100 μM $ZnCl_2$ and measured on a SpectraMax using 96-well falcon UV microplates

159   (BD Biosciences, USA) on all substrates, except for nitrocefin, where the 96-well flat-bottom,

160   non-binding-surface plates (Corning, Edison, New Jersey USA) were used. The reaction

161   mixture was pre-incubated for 5 min at 30°C. The extinction coefficients, wavelengths and

162   also plate specific extinction coefficients were calculated.

163   The results of the kinetic analysis were processed in Microsoft Excel using solver, an

164   algorithm that can be used for non-linear regression.[18]

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166   Screening of *Aliivibrio* strains by PCR

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168   The degenerate primers MBL-F1 (CAYTTTCATGAAGAYCAAAC) and MBL-R1

169   (GCAYCACCWGTCCASCCAAT) were constructed based on a selection of MBL nucleotide

170   sequences from Vibrionaceae and used in order to screen *Aliivibrio* isolates from the Barents

171   Sea. If positive, a PCR product of about 300 nucleotide length should be formed. The

172   chemicals used were; dNTP mix F-560 (Thermo Scientific, Rockford USA), Taq polymerase

173   (VWR, Dublin Ireland) and Thermo Pol buffer (New England Biolabs, Ipswich USA). The

174   PCR products were verified by electrophoresis in 1% agarose gel and by sequencing using

175   BigDye 3.1 Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

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177   Results

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179   Cloning, expression and purification of ALI-1

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181   The gene encoding ALI-1 was successfully cloned into the expression vector and the protein

182   was overexpressed. After optimization of the purification protocol, pure protein was obtained

183   (figure 1.). The presence of ALI-1 in the collected fractions was indicated using nitrocefin and

184   verified using other substrates (table 1).

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186   Zinc influence

187   Zinc is crucial to ALI-1 function, as the enzyme is inactive without Zn^{2+} bound to it. This is

188   verified in the temperature experiments when a surplus of EDTA is added to the reaction

189   mixture and stops the hydrolysis reaction, as have been shown also for other MBLs.[19] The

190   zinc concentration appears to have no further impact on the activity as long as the minimum

191   concentration of zinc is present.[20]

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193   Reducing agents

194   In the presence of the reducing agents DTT and β-mercaptoethanol, the activity of ALI-1 is

195   somewhat lowered, whereas when TCEP is added, the activity of the enzyme is completely

196   abolished (data not shown).

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198   pH optimum

199   ALI-1 is active over a broad pH range (data not shown). Compared to the pH in coastal water

200   (pH 8.0-8.3), ALI-1 is expected to have its optimal activity in this range. Although the

201   original intention of the pH screening was to test the activity at even higher pH than presented

202   in this study, nitrocefin was noticed not to be stable at high pH and some buffers destabilized

203   nitrocefin more than others. At higher pH the autohydrolysis of nitrocefin (*i.e.* nitrocefin is

204   broken down without enzyme present) is increased, but only when Tris was used as buffer.

205   This effect has also been observed in other studies.[21] Both the concentration of Tris and pH of

206   the solution were found to influence the autohydrolysing effect of nitrocefin. At 20 mM Tris

207   at pH 7.5 it was observed that after 5, 30 and 60 minutes respectively 3%, 6% and 11% of the

208   substrate was autohydrolysed. When having very high concentration of Tris, *i.e.* 1 M at pH

209   7.5, it was observed that after 5, 30, and 60 minutes respectively 6%, 15% and 29% of the

210   substrate was autohydrolysed. After 15 minutes in a solution with 20 mM Tris at pH 9.0 it

211   was observed that 33% of the substrate was autohydrolysed. Because of this observed effect

212   the autohydrolysing effect was corrected for when Tris buffer was used. When nitrocefin is

213   present in HEPES buffer no autohydrolysis is observed except to a slight degree after

214   prolonged incubation (two hours) at pH>8.

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NaCl and temperature optimum

As ALI-1 originates from a bacterium which thrives in the sea, it is interesting to investigate how the protein is adapted to salt and low temperatures. As can be seen from figure 2, the salt optimum for ALI-1 is 500 mM NaCl. This is expected since the enzyme is excreted into the periplasm of a marine bacterium and knowing that this is approximately the same concentration as in seawater, but the enzyme is also highly active in the range of 0.5-2 M NaCl. Even at salt concentrations of around 2.5 M NaCl, the activity is still about 90% of its activity at the optimal concentration of NaCl.

The optimal salt conditions for ALI-1 were further compared to the optimal salt conditions for VIM-2 from *Pseudomonas aeruginosa,* which is a bacterium shown to be pathogenic in humans.[22] It can be observed from figure 2 that VIM-2 has an optimal activity at around 200 mM NaCl, which is substantially lower than ALI-1. Furthermore, VIM-2 does not display the same extreme degree of salt tolerance as ALI-1, with the activity dropping faster and in a nearly linear fashion at salt concentrations above its optimum.

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In order to find the optimal temperature for each enzyme, reaction mixture were prepared for both ALI-1 and VIM-2 at 0.5 M and 0.2 M NaCl, respectively (optimum NaCl concentration for each enzyme). At both of these salt concentrations ALI-1 has a lower temperature optimum than VIM-2 (figure 3). These findings are not unexpected since ALI-1 originates from an environment that is characterized with low temperatures and high salt concentrations, while VIM-2, first identified in a human pathogen, is expected to be adapted to lower salt concentration and higher temperature. The amount of salt present has marked effects on the optimal temperature for enzymatic activity. This is seen both for VIM-2 and ALI-1 (figure 4A

240 and B), where the temperature optimum is lowered by approximately 5-10 degrees when the

241 salt concentration is reduced from 0.5 M to 0.2 M. VIM-2 has its highest measured relative

242 activity at 40°C and 200 mM NaCl (figure 4A), which correlates well to the NaCl optimum of

243 VIM-2 (figure 2). From figure 4B a reduction of overall activity of ALI-1 can be observed

244 when lowering the salt concentration, which correlates well with the NaCl optimum of ALI-1

245 shown in figure 2.

246 Another point of interest is the stability of the substrate nitrocefin at higher temperatures. As

247 seen in figure 3, nitrocefin decays at higher temperatures as it is being turned into product.

248 The instability of nitrocefin is not a problem at temperatures ranging from 10°C to 60°C, while

249 at higher temperatures it could have impact on the results.

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251 Kinetics

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253 Results from the kinetic study were compared to similar MBLs from functional group B1. The

254 comparison focused on the acquired MBLs IMP-1[19] and VIM-2,[22] because of their sequence

255 similarity (figure 5) to ALI-1. ALI-1 hydrolyzed more or less all β-lactam antibiotics, except

256 from the monobactam aztreonam and the penicillin piperacillin (see table 1).

257 The catalytic efficiency of ALI-1 is generally poorer than the other enzymes, ranging from

258 10^6 to 10^3 M^{-1}*s^{-1}, while for instance IMP-1 has a catalytic efficiency ranging from about 10^6

259 to 10^5 M^{-1}*s^{-1} for the same substrates.

260 IMP-1 is the MBL with determined crystal structure which has the most similar amino acid

261 sequence as ALI-1, but their kinetic constants are very different. Generally, ALI-1 has both

262 higher K_M and lower k_{cat} than IMP-1.

263 When comparing the catalytic efficiency for the different substrates, ALI-1 seem to have a

264 better affinity for carbapenems than cephalosporins and cephamycins, in which cefuroxime is

265 an exception. Nitrocefin is secluded from this comparison as it is made to be readily

266 hydrolyzed. Piperacillin is a penicillin which is used as an extended spectrum antibiotic,

267 which could explain ALI-1s apparent inability to hydrolyze penicillins in this study. As long

268 as this is the only penicillin substrate in this study there is no definite conclusion to be made

269 about ALI-1's capability of hydrolyzing penicillins in general.

270 The presence of 0.5M NaCl in the reaction mixture raised ALI-1's catalytic efficiency for the

271 hydrolysis of ertapenem. The improvement in the catalytic efficiency, when having salt

272 present, is not caused by a rise in k_{cat}, but rather a lowering (*i.e.* strengthening) of the enzyme

273 affinity, K_M (table 1).

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275 Screening of *Aliivibrio* strains by PCR

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277 Two of the PCR products obtained in the PCR screening of environmental *Aliivibrio* isolates

278 (A) and those obtained from screening *Aliivibrio salmonicida* strains associated with the fish

279 farming industry (B) show a size of about 300 bp, which coincides with the expected product

280 size the primers should generate (298 bp). We also observed some unspecific bands of higher

281 mw from some of the environmental *Aliivibrio* isolates. The two positive PCR products from

282 the environmental strains were verified by sequencing (not shown).

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284 Discussion

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286 We have performed a comparative analysis of the properties belonging to a chromosome-

287 encoded marine MBL and compared those with the plasmid borne MBL (VIM-2) encoded by

288 a human pathogen. The two enzymes originate from two profoundly differently adapted

289 bacteria. *A. salmonicida* will secrete its MBL into a cold and salty environment, while VIM-2

290 is meant to function under warmer and less salty conditions in body fluids. In previous work

291 we have investigated how NaCl affected the properties of a secreted marine endonuclease

292 from *A. salmonicida*.[11] Here we show that also the ALI-1 is activated by NaCl up to 0.5M,

293 approximately the same concentration as in seawater. The VIM-2 has a less salt- dependent

294 activity, with NaCl optimum at 200 mM. The Michaelis constant (K_M) and the turnover

295 number (k_{cat}) are affected by the NaCl concentration. The K_M goes dramatically down when

296 adding NaCl, and the k_{cat} increases slightly. This is measured with ertapenem as a substrate,

297 and the salt probably favours the substrate binding by increasing the hydrophobic interactions

298 between substrate and enzyme, thereby lowering the K_M. The detailed mechanism for an

299 increased catalytic efficiency when adding NaCl remains to be explored. The effect of NaCl

300 on the kinetic values is measured using only one substrate. It is possible that the ranking of

301 which substrate is best (*i.e.* has the highest catalytic efficiency) might be different when

302 assaying with NaCl in the buffer.

303

304 The two enzymes also showed significant differences in optimal temperature for activity. The

305 marine enzyme is more cold-active compared to VIM-2, showing increased activity at lower

306 temperatures and a lower temperature optimum. This can be explained by a lower temperature

307 stability which leads to a more rapid temperature-induced denaturation. NaCl is increasing the

308 stability of both proteins probably by reducing repulsive interactions between charges on the

309 surface and by strengthening the hydrophobic effect. This was clearly visible in our

310 experiments as the optimum temperature for activity shifted several degrees when assaying

311 with NaCl in the buffer (see *e.g.* figure 4).

312 Orthologues of ALI-1 can be found in several other *Aliivibrio*, *Vibrio* and *Photobacterium*

313 species as judged from online BLAST searches against the nucleotide databases at The

314 National Center for Biotechnology Information, USA (NCBI). The gene resides on

The chromogenic substrate nitrocefin displayed low stability in buffers with Tris present. The auto-degradation of nitrocefin was proportional to the Tris concentration and more severe at higher pH. Also, the nitrocefin stays intact only up to about 65°C.

The chosen expression construct with the N-terminal periplasmic signal replaced with six histidines proved to be a highly efficient method to produce fair quantities of pure protein. In ordinary LB-medium the yield is about 16 mG/L. Based on visualization of X-ray structures of related MBLs (*e.g.* pdb entry 1ddk) we believe that the histidine-tag will not interfere with active-site residues.

When testing the effect of different reducing agents we observed that activity was lost using 5 mM TCEP, but both β-mercaptoethanol and DTT at the same concentrations had nearly no effect. The ALI-1 contain only one cysteine which is thought to coordinate the second Zinc ion in the active site. It is reasonable to believe that only TCEP has a redox potential strong enough to reduce the thiolate (S-) to the sulfhydryl form (SH). The Zn^{2+} concentrations in these experiments were kept at 0.1 mM and we do not believe that the TCEP is capable of reducing Zn^{2+} to Zn or chelating Zn^{2+} and thereby abolishing the activity. TCEP is proposed as a treatment after botulinum toxin exposure as it reduces key disulphide bonds.[24] At 1 mM, TCEP is not toxic to neuronal cells and therefore we suggest that TCEP in some cases could

340 be used as a combinational treatment together with β-lactam antibiotics to evade the MBL

341 activity of the pathogen.

342

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345 enzyme and the antibiotics.

346

350 **Transparency declarations**

351 None to declare.

352 **Author contributions**

353 Study conception and design, BA and IL; Conducting research, AK, MG and BA; Analysis

354 and interpretation of data, AK, MG, BA and IL; Drafting and writing the manuscript AK,

355 MG, BA and IL. All authors read and approved the final manuscript version to be published.

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357 **References**
358
359 1. Bebrone C. Metallo-beta-lactamases (classification, activity, genetic organization, structure, zinc
360   coordination) and their superfamily. *Biochem Pharmacol* 2007; **74**: 1686-701.
361 2. Ambler RP. The structure of beta-lactamases. *Philos Trans R Soc Lond B Biol Sci* 1980; **289**: 321-31.
362 3. Garau G, Garcia-Saez I, Bebrone C *et al.* Update of the standard numbering scheme for class B beta-
363   lactamases. *Antimicrob Agents Chemother* 2004; **48**: 2347-9.
364 4. Galleni M, Lamotte-Brasseur J, Rossolini GM *et al.* Standard numbering scheme for class B beta-
365   lactamases. *Antimicrob Agents Chemother* 2001; **45**: 660-3.
366 5. Hawkey PM, Jones AM. The changing epidemiology of resistance. *J Antimicrob Chemother* 2009; **64**
367   **Suppl 1**: i3-10.
368 6. Allen HK, Moe LA, Rodbumrer J *et al.* Functional metagenomics reveals diverse beta-lactamases in a
369   remote Alaskan soil. *ISME J* 2009; **3**: 243-51.
370 7. Lima-Bittencourt CI, Cursino L, Goncalves-Dornelas H *et al.* Multiple antimicrobial resistance in
371   Enterobacteriaceae isolates from pristine freshwater. *Genet Mol Res* 2007; **6**: 510-21.
372 8. Aminov RI. The role of antibiotics and antibiotic resistance in nature. *Environ Microbiol* 2009; **11**: 2970-
373   88.

9. Urbanczyk H, Ast JC, Higgins MJ *et al.* Reclassification of *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. *Int J Syst Evol Microbiol* 2007; **57**: 2823-9.

10. Egidius E, Wiik R, Andersen K *et al. Vibrio salmonicida* sp. nov., a new fish pathogen. *Int J Syst Bacteriol* 1986; **36**: 518-20.

11. Altermark B, Niiranen L, Willassen NP *et al.* Comparative studies of endonuclease I from cold-adapted *Vibrio salmonicida* and mesophilic *Vibrio cholerae*. *FEBS J* 2007; **274**: 252-63.

12. Fields PA, Somero GN. Hot spots in cold adaptation: localized increases in conformational flexibility in lactate dehydrogenase A4 orthologs of Antarctic notothenioid fishes. *Proc Natl Acad Sci U S A* 1998; **95**: 11476-81.

13. Leiros I, Moe E, Lanes O *et al.* The structure of uracil-DNA glycosylase from Atlantic cod (*Gadus morhua*) reveals cold-adaptation features. *Acta Cryst D* 2003; **59**: 1357-65.

14. Siddiqui KS, Cavicchioli R. Cold-adapted enzymes. *Annu Rev Biochem* 2006; **75**: 403-33.

15. Hjerde E, Lorentzen MS, Holden MT *et al.* The genome sequence of the fish pathogen *Aliivibrio salmonicida* strain LFI1238 shows extensive evidence of gene decay. *BMC Genomics* 2008; **9**: 616.

16. Hu Z, Periyannan G, Bennett B *et al.* Role of the Zn1 and Zn2 sites in metallo-beta-lactamase L1. *J Am Chem Soc* 2008; **130**: 14207-16.

17. Lisa MN, Hemmingsen L, Vila AJ. Catalytic role of the metal ion in the metallo-beta-lactamase GOB. *J Biol Chem* 2010; **285**: 4570-7.

18. Brown AM. A step-by-step guide to non-linear regression analysis of experimental data using a Microsoft Excel spreadsheet. *Comput Methods Programs Biomed* 2001; **65**: 191-200.

19. Laraki N, Franceschini N, Rossolini GM *et al.* Biochemical characterization of the *Pseudomonas aeruginosa* 101/1477 metallo-beta-lactamase IMP-1 produced by *Escherichia coli*. *Antimicrob Agents Chemother* 1999; **43**: 902-6.

20. Badarau A, Page MI. Loss of enzyme activity during turnover of the *Bacillus cereus* beta-lactamase catalysed hydrolysis of beta-lactams due to loss of zinc ion. *J Biol Inorg Chem* 2008; **13**: 919-28.

21. Wang Z, Benkovic SJ. Purification, characterization, and kinetic studies of a soluble *Bacteroides fragilis* metallo-beta-lactamase that provides multiple antibiotic resistance. *J Biol Chem* 1998; **273**: 22402-8.

22. Poirel L, Naas T, Nicolas D *et al.* Characterization of VIM-2, a carbapenem-hydrolyzing metallo-beta-lactamase and its plasmid- and integron-borne gene from a *Pseudomonas aeruginosa* clinical isolate in France. *Antimicrob Agents Chemother* 2000; **44**: 891-7.

23. Docquier JD, Lamotte-Brasseur J, Galleni M *et al.* On functional and structural heterogeneity of VIM-type metallo-beta-lactamases. *J Antimicrob Chemother* 2003; **51**: 257-66.

24. Shi X, Garcia GE, Neill RJ *et al.* TCEP treatment reduces proteolytic activity of BoNT/B in human neuronal SHSY-5Y cells. *J Cell Biochem* 2009; **107**: 1021-30.
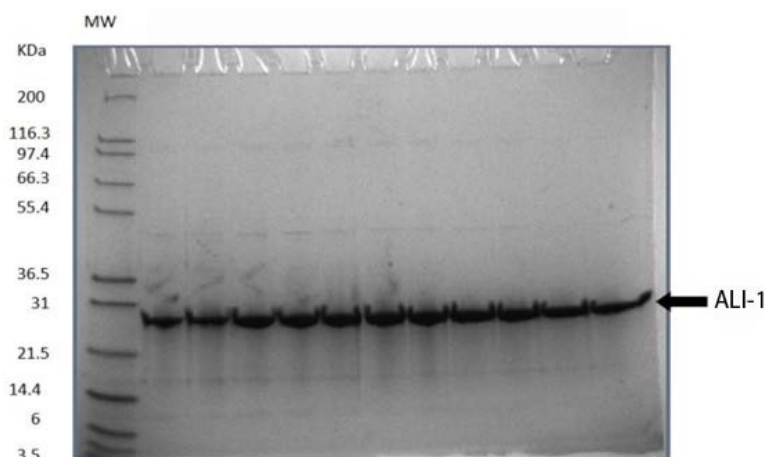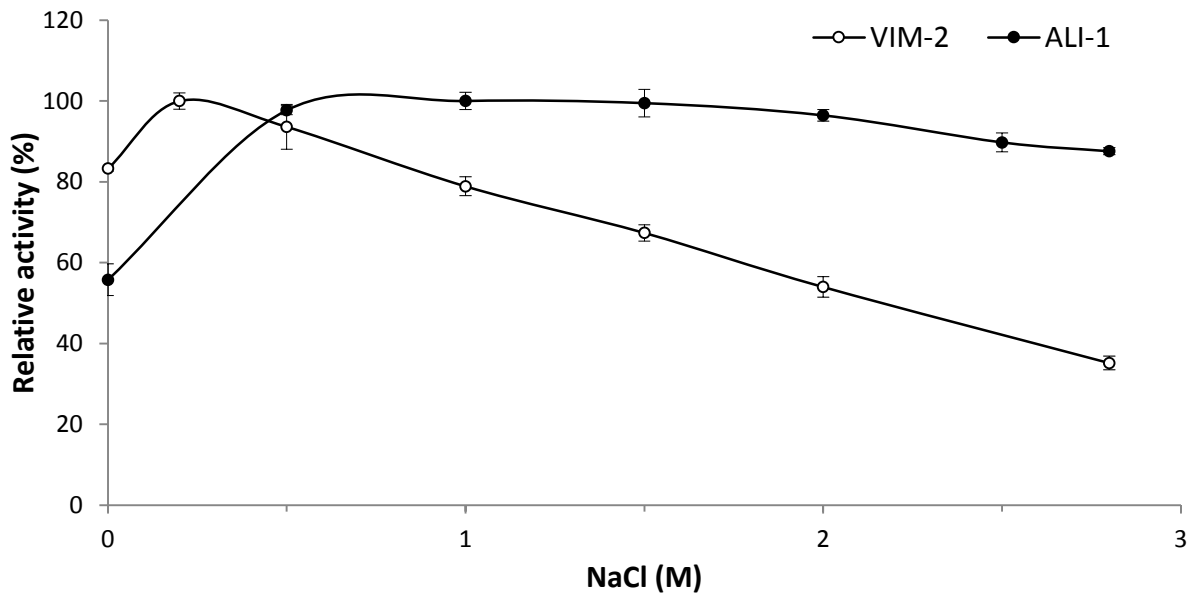
Figure 1. SDS-PAGE gel picture from the His Trap purification of ALI-1. MW: molecular weight marker, (Mark12, Invitrogen); others: fractions from the elution peak that were positive for breakdown of nitrocefin.

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Figure 2. The salt optimum for ALI-1 and VIM-2. All residual activities are relative to the highest average activity for the respective enzyme. For ALI-1 the highest activity is at 0.5 M NaCl, while for VIM-2 it is at 0.2 M NaCl. The other measured NaCl concentrations are 0, 1, 1.5, 2, 2.5, and 2.8 M. Other salt optimum trials for ALI-1 showed a good salt tolerance (approximately 80%) up to 3.5 M NaCl (not shown). It is a clear difference between the psychrophilic, marine MBL and mesophilic, terrestrial MBL. At each salt concentration, the uncertainty is indicated as the range of activity measured.

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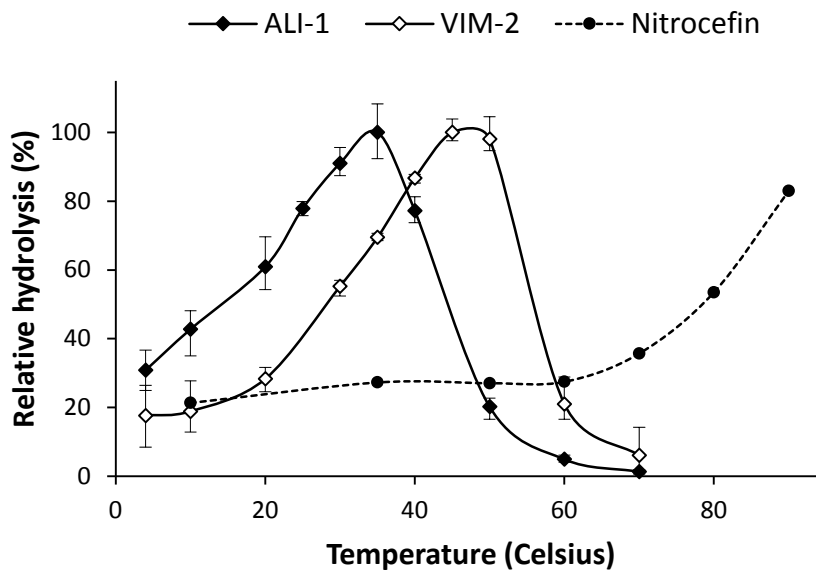
Figure 3. Comparison of temperature optimum for activity at 500 mM NaCl for ALI-1 and VIM-2 and also the temperature-dependent breakdown of nitrocefin. The activity was measured at the temperatures; 4, 10, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 and 90^{0}C. All the measurements in a series were set relative to its series maximum value. For ALI-1 it was at 35^{0}C and for VIM-2 it was at 45^{0}C. The values of

428   substrate stability measurements were relative to the maximum value of the <mark>ALI-1</mark> series. The range of measured enzyme activities is

429   displayed by the antennas.

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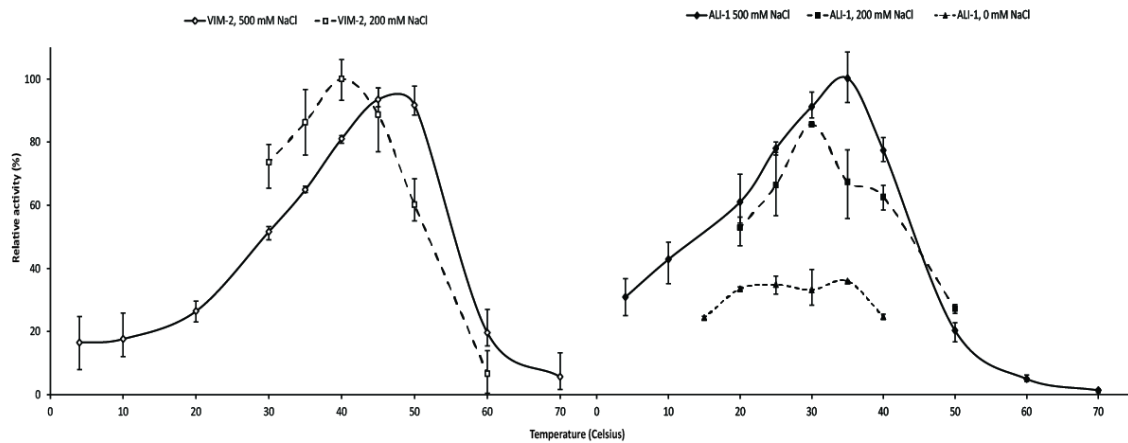
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439   A.   B.

440

441   Figure 4. A. Comparing VIM-2 optimal temperature for activity under different NaCl concentrations. Activity was measured at the

442   temperatures; 4, 10, 15, 20, 25, 30, 35, 40, 50, 60 and 70^{0}C. All measurements were made relative according to optimal temperature at 500

443   mM NaCl. The range of measured enzyme activities is displayed by the antennas. B. The optimal temperature for <mark>ALI-1</mark> activity at different

444   concentrations of NaCl. The activity was measured at the temperatures; 4, 10, 20, 30, 35, 40, 45, 50, 60 and 70^{0}C. All measurements were

445   made relative according to optimal temperature at 200 mM NaCl. The antenna at each measured temperature displays the range of enzyme

446   activity measured.

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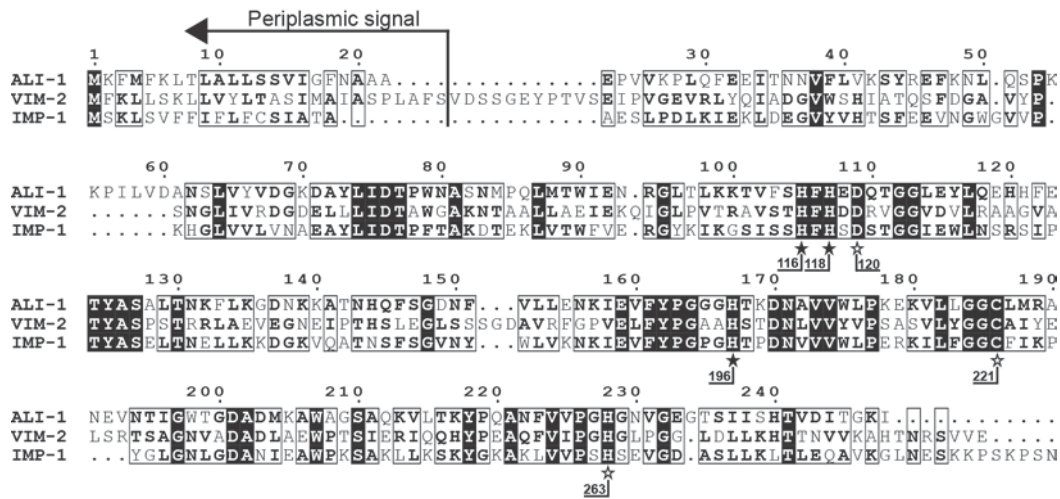
Figure 5. Protein alignment showing ALI-1 (acc. number YP_002262687), the VIM-2 MBL (acc. number ACT32123) and IMP-1 (acc. number GI:560552). The Zn-coordinating residues are indicated with filled (Zn1) and open (Zn2) star symbols, and the amino acid numbers, according to the standard class B β-lactamase (BBL) numbering system, is shown.
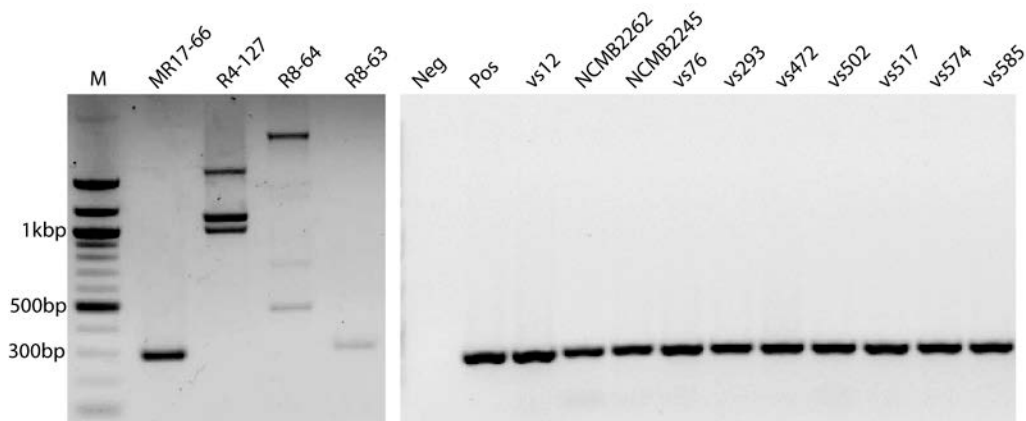
A.   B.

Figure 6. Results from PCR screening using MBL-specific primers. Agarose gel showing PCR products from A. Environmentally derived *Aliivibrio* isolates originating from the Barents Sea, and B. *A. salmonicida* strains isolated from disease outbreaks in marine aquaculture. M= 100 bp molecular weight marker, Neg = Negative control, Pos = Positive control.

466   Table 1. Comparison of k_{cat}, K_M and catalytic efficiency for ALI-1 (values from current work), IMP-1[19] and VIM-2.[23] The kinetic parameters

467   for VIM-2 had standard deviations that always were <10%. NA=Results not available, NM=No activity measured, ND=Not determined

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	k_{cat} (s-1)			K_M (µM)			k_{cat}/K_M (1/µM*s)		
Substrate	ALI-1	IMP-1	VIM-2	ALI-1	IMP-1	VIM-2	ALI-1	IMP-1	VIM-2
Meropenem	13 ± 5	50 ± 5	5	142 ± 100	10 ± 2	2	0.094	0.12	2.5
Imipenem	8.0 ± 0.6	46 ± 3	34	933 ± 70	39 ± 4	9	0.0085	1.2	3.8
Ertapenem	4 ± 1	NA	NA	73 ± 30	NA	NA	0.054	NA	NA
Ertapenem (NaCl)	4.3 ± 0.4	NA	NA	17 ± 7	NA	NA	0.25	NA	NA
Nitrocefin	6.3 ± 0.6	63 ± 10	770	6 ± 3	27 ± 3	18	1.0	2.3	43
Cefuroxime	11 ± 3	8 ± 1	8	68 ± 30	37 ± 3	20	0.16	0.22	0.40
Cefepime	0.581 ± 0.005	7.0 ± 0.5	>40	177 ± 14	11 ± 1	>400	0.0033	0.66	0.10
Ceftazidime	0.09 ± 0.03	8 ± 1	3.6	37 ± 12	44 ± 3	72	0.0024	0.18	0.050
Cefoxitin	0.27 ± 0.09	16 ± 1	15	79 ± 30	8 ± 1	13	0.0034	2	1.2
Piperacillin	NM	ND	300	NM	ND	125	-	0.72	2.4
Aztreonam	NM	>0.01	<0.01	NM	>1,000	>1000	-	<0.0001	<0.00010

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