

1 RESEARCH LETTER

2 Are new beta-lactamases posing a potential future threat?

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11 ABSTRACT

12 Antimicrobial resistance, a significant and escalating public health threat, has extended its
13 reach beyond healthcare settings to diverse environments such as urban beaches, wastewater
14 plants, and household pets. A recent study even found bacteria in permafrost with genes
15 encoding beta-lactamases, enzymes that confer resistance to beta-lactam antibiotics. Our
16 previous study identified 2340 potential beta-lactamases across 673 bacterial genera, with
17 only 139 of these enzymes sharing more than 70% amino acid identity with known beta-
18 lactamases. This study underscores the critical and widespread distribution of potential beta-
19 lactamases across different bacterial genera, regions, and hosts. The role of horizontal gene
20 transfer and mobile genetic elements in spreading antibiotic resistance is of utmost
21 importance, highlighting the gravity of the situation and the urgent need for monitoring and
22 research.

23
24 Keywords: antimicrobial resistance, beta-lactamases, horizontal gene transfer, public health

26 INTRODUCTION

27 Antimicrobial resistance, a looming and urgent global public health threat, is expected
28 to escalate in severity. Once confined to healthcare settings, antibiotic-resistant bacteria have
29 now proliferated in diverse environments, from washing machines (Schmithausen, Sib et al.
30 2019), urban beaches (Carney, Labbate et al. 2019), wastewater treatment plants (Parnanen,
31 Narciso-da-Rocha et al. 2019), post-cleaning hospital environments (Gouliouris, Coll et al.
32 2021), heavy metal environments (Gaeta, Bean et al. 2020), city bird droppings (Zhao, Sun et

al. 2020), incinerators and landfills (Li, Wang et al. 2020), dust (Ben Maamar, Glawe et al. 2020), wild dolphins (Schaefer, Bossart et al. 2019), household pets (Hamame, Davoust et al. 2022, Menezes, Moreira da Silva et al. 2022), bedbugs (Herrera, Chaussee et al. 2024), insects and spiders (Hassan, Ijaz et al. 2021), and microplastics (Liu, Liu et al. 2021). This widespread emergence of antibiotic-resistant microbes poses a significant risk to a larger population. A recent study in permafrost (Rigou, Christo-Foroux et al. 2022) even found bacteria with a high proportion of genes encoding beta-lactamases in their genomes, further emphasizing the immediate need for global monitoring and research.

Currently, 346 beta-lactamase types have been described worldwide according to the Beta-Lactamase DataBase (BLDB), a comprehensive and widely recognized repository of beta-lactamase information (<http://bldb.eu>; last accessed on 10 October 2024). In this context, more than 7280 beta-lactamase genes have been described in cultured bacteria, and new variants are emerging worldwide (Bao, Huang et al. 2022). However, new beta-lactamases are constantly being discovered.

In our previous study (Zdarska, Kolar et al. 2024), silico analysis of over 850 bacterial genomes from clinical and environmental isolates revealed more than 2340 potential beta-lactamases in over 673 bacterial genera. In the present study, we focused more on candidate beta-lactamases with 70% or greater amino acid sequence identity with known beta-lactamases.

METHODS

For the analysis, 139 bacterial genomes and gene sequences encoding putative beta-lactamases were downloaded from the National Center for Biotechnology Information (NCBI) and examined. These sequences, annotated using the NCBI prokaryotic genome annotation pipeline (Tatusova, DiCuccio et al. 2016), were filtered using the “find annotations” feature in Geneious Prime 2024.0.7 (Kearse, Moir et al. 2012).

This study only considered sequences with 70.0-92.3% amino acid identity with known beta-lactamases. The amino acid identity threshold of 92.3% was used to identify potential beta-lactamases as it indicates significant similarity to known beta-lactamases, suggesting a high likelihood of functional similarity. Amino acid matches were determined using the BLDB BLAST function (Naas, Oueslati et al. 2017).

The flanking regions of beta-lactamases were analyzed using Geneious Prime. The NCBI Protein database and Geneious data were also utilized to obtain information on the country of origin, host, and source of isolation of the bacterial isolates.

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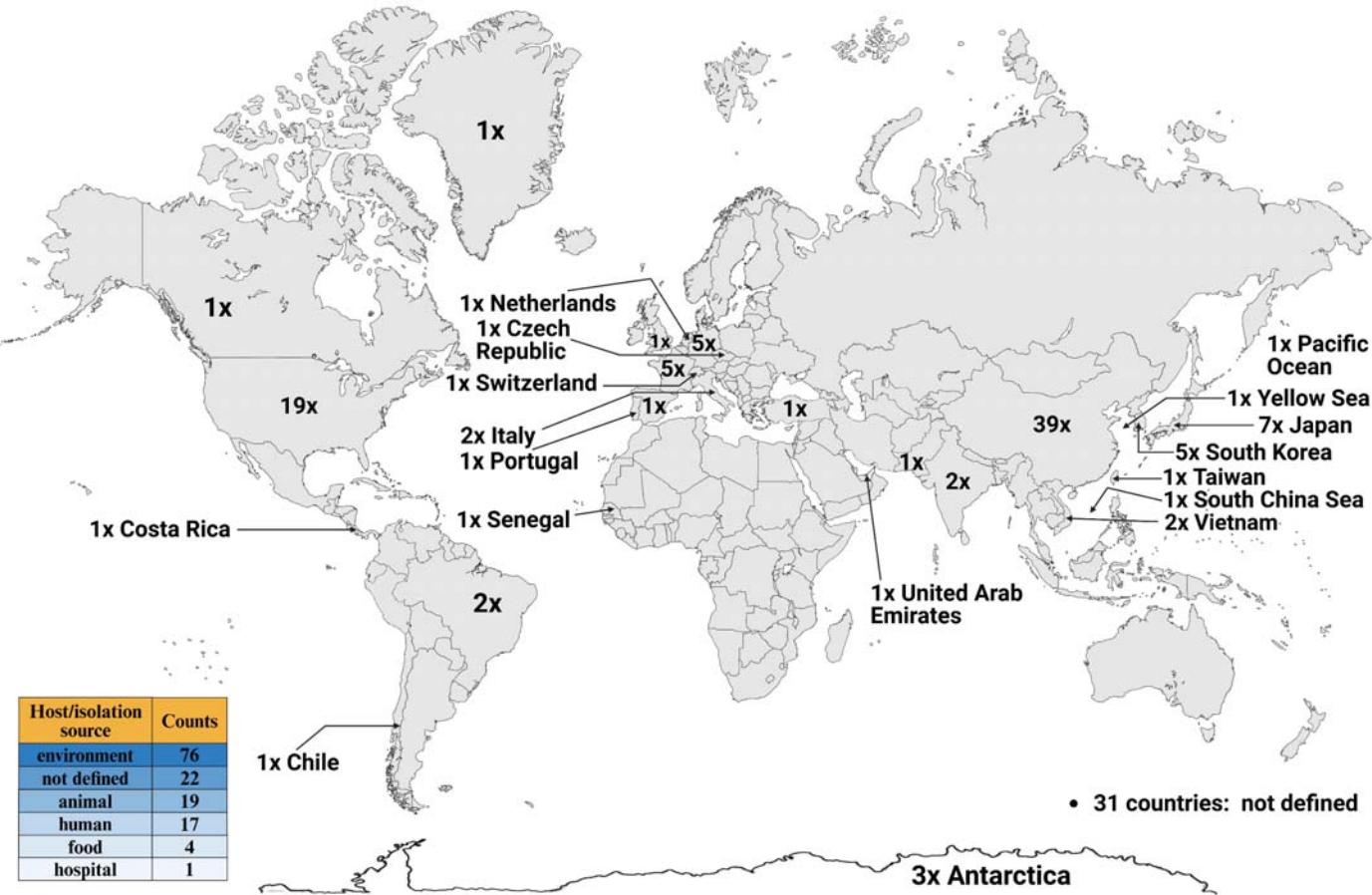
69 RESULTS AND DISCUSSION

70 One hundred thirty-nine beta-lactamases in this study exhibited $\geq 70\%$ amino acid
71 identity with known beta-lactamases. The distribution of potential beta-lactamases across
72 various regions and hosts is shown in Figure 1. In total, we identified 109 bacterial genera
73 and one *Candidatus Ornithobacterium* species (see Table S1), comprising the following
74 bacterial classes (*Actinomycetes*, *Alphaproteobacteria*, *Bacilli*, *Bacteroidia*,
75 *Betaproteobacteria*, *Cyanophyceae*, *Cytophagia*, *Deltaproteobacteria*,
76 *Epsilonproteobacteria*, *Flavobacteriia*, *Gammaproteobacteria*, *Chitinophagia*, *Chlorobiia*,
77 and *Sphingobacteriia*), in which the following novel potential beta-lactamases of group A (53
78 times), group B (27 times), group C (12 times) and group D (47 times) were detected.

79 The highest numbers of potential beta-lactamases were found in the genera
80 *Chryseobacterium*, *Enterobacter*, *Aeromonas* and *Chitinophaga*, in which six, four and three
81 enzymes (twice), respectively, were detected. For example, two potential class A beta-
82 lactamases (83.2% and 80.5% amino acid identity with CGA-1), three class B beta-
83 lactamases (87.9% identity with ESP-1; 71.6% and 70.7% identity with GOB-like enzymes),
84 and one class D beta-lactamase (89% identity with OXA-209) were detected in the genus
85 *Chryseobacterium*. Further, in the genus *Enterobacter*, there were two potential class A beta-
86 lactamases (89.1% and 82.5% amino acid identity with LAP-1 and PLA-6, respectively) and
87 two class C beta-lactamases (87.1% and 85.6% identity with ACT-96 and CDA-1,
88 respectively). In the case of the genus *Aeromonas*, there was one potential beta-lactamase of
89 class A (70.6% amino acid identity with BlaP-2), class C (86.4% identity with PAC-1), and
90 class D (86.2% identity with OXA-12).

91 Identification of mobile genetic elements revealed the presence of genes for
92 transposases, integrases/recombinases, and mobilization proteins in 14 cases (Table 1), with a
93 higher abundance of transposases, which may lead to overproduction of beta-lactamases and
94 facilitate gene transfer, spreading beta-lactamase genes. In addition, two potential beta-
95 lactamases were detected nearby in some cases (Figure 2A/B).

96 **Figure 1.** Global distribution of potential beta-lactamases in different regions and hosts. Created in BioRender.com.



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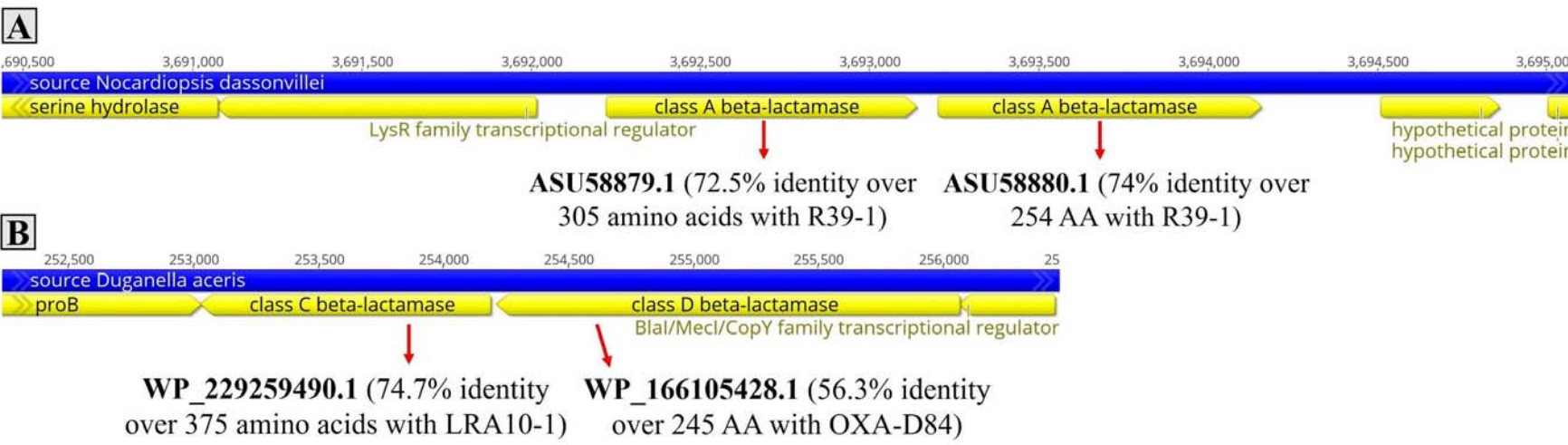
98

99 **Table 1.** Genetic neighborhood of selected putative beta-lactamases with mobile genetic elements.

Bacterial genera	Putative B-L class	Flanking proteins	Orientations	Size (AA)	Country	Host/isolation source	GenPeptID/Identity
<i>Cedecea</i>	A	IS3 family transposase=hypothetical protein	< < <	285	ND	mosquito	WP_008460137.1 (89.1% identity over 285 amino acids with LAP-1)
<i>Enterobacter</i>	A	transposase IS3/IS911 family protein=beta-lactam binding protein AmpH	< < <	285	USA	mosquito	EJF29815.1 (89.1% identity over 285 amino acids with LAP-1)
<i>Ornithobacterium</i> *	A	Tyrosine recombinase XerC=DUF4468 domain-containing protein	< > >	294	ND	nasopharynx	CAI9429279.1 (84.7% identity over 294 amino acids with RASA-1)
<i>Alistipes</i>	A	mobilization protein=alkaline phosphatase family protein	> < <	322	India	human	MBE5686174.1 (83.7% identity over 318 amino acids with CfxA-2)
<i>Bacteroides</i>	A	tRNA-Ser=mobilization protein	> > <	322	USA	human	UVS32091.1 (83.7% identity over 318 amino acids with CfxA-3)
<i>Parabacteroides</i>	A	mobilization protein=tRNA-Ser	> < <	322	USA	human	UVS67385.1 (83.7% identity over 318 amino acids with CfxA-2)
<i>Psychrobacter</i>	A	recombinase family protein=S-formylglutathione hydrolase mobilization	> < <	299	ND	seafood	WP_102076509.1 (78% identity over 296 amino acids with CARB-5)
<i>Capnocytophaga</i>	A	protein=mechanosensitive ion channel protein Msc	> > >	315	Japan	animal	GIJ97441.1 (71.5% identity over 316 amino acids with CfxA-6)
<i>Vibrio</i>	B	IS30 family transposase=pyridoxamine 5'-phosphate oxidase family protein	> > >	250	China	food	WP_318122257.1 (85.8% identity over 240 amino acids with TMB-1)
<i>Pseudomonas</i>	C	biotin/lipoyl-binding protein=tyrosine-type recombinase/integrase	> > >	396	China	cave sediment	UVJ43626.1 (92.2% identity over 396 amino acids with RSC1-1)
<i>Aeromonas</i>	C	IS91 family transposase=hypothetical protein	> > >	380	Japan	environment	BBT97416.1 (86.4% identity over 381 amino acids with PAC-1)
<i>Elizabethkingia</i>	D	IS110 family transposase=GNAT family N-acetyltransferase	< > >	274	Taiwan	human	UKY84509.1 (89% identity over 255 amino acids with OXA-209)
<i>Comamonas</i>	D	IS3 family transposase (frameshifted)=tRNA-Arg	< < <	256	France	rotting apple	WP_182328161.1 (83.9% identity over 255 amino acids with OXA-926)
<i>Aromatoleum</i>	D	IS3 family transposase=electron transfer flavoprotein-ubiquinone oxidoreductase	< < <	261	China	environment	WRL48780.1 (70.9% identity over 258 amino acids with LCR-1)

* - *Candidatus Ornithobacterium*. The orientation of beta-lactamases relative to their neighboring genes is indicated in bold. B-L: beta-lactamase. AA: amino acids. ND: not defined. Additional information on putative beta-lactamases with mobile genetic elements is provided in Table S1.

100 **Figure 2.** Detection of potential beta-lactamases in *Nocardiopsis dassonvillei* (A) and *Duganella aceris* (B). Arrows indicate the direction of
101 transcription. The length of the arrows is proportional to the size of the genes.



102
103 The study analyzed the presence of potential beta-lactamases in bacterial isolates from various countries and demonstrated a notable
104 distribution of these enzymes across different regions. China had the highest number of potential beta-lactamases, with 39 cases detected. In
105 addition, there were 31 cases where the source country was not specified. The USA reported 19 cases, Japan had seven cases, and France,
106 Germany, and South Korea each had five cases. Additionally, we explored the presence of potential beta-lactamases across different isolation
107 environments. The majority were detected in environmental samples, accounting for 76 cases, followed by 22 cases where the origin was not
108 determined. Furthermore, 19 cases were identified in animal and 17 in human hosts.

Our detailed study of potential beta-lactamases binding to mobile genetic elements resulted in a significant discovery. Specifically, we identified two potential class A beta-lactamases with identical amino acid sequences in *Enterobacter* sp. (AKXM01000000) and *Cedecea* sp. (NZ_JBBCPU010000048), both of which were found in mosquitoes and had an insertion sequence located nearby. This finding is crucial as it highlights potential sources of antibiotic resistance, with one case originating from the USA and the other from an unspecified location.

An insertion sequence, a short DNA segment that acts as a simple transposable element, was also found around potential class C beta-lactamase described in *Aeromonas veronii* (AP022282) isolated from a wastewater treatment plant effluent in Japan. This insertion sequence has also been detected in other bacterial species of this genus, such as *Aeromonas caviae* (AP022242, AP019196), all isolated from the same source and country.

The most worrying situation seems to be related to potential class A beta-lactamases, which show 99.9% nucleotide and 99.7-100% amino acid identity (change in one amino acid) with each other and have been detected in *Bacteroides faecis* (CP103274), *Parabacteroides distasonis* (CP103286) and *Alistipes* sp. (RKBL01000025), and these enzymes were located near the mobilization protein. More detailed *in silico* analysis indicated their occurrence in other species of the above genera, such as *Bacteroides ovatus* (CP103071), *Bacteroides fragilis* (CP036539, CP119600), *Bacteroides salyersiae* (CP072243), *Bacteroides* sp. (CP040630), *Parabacteroides distasonis* (CP072231), and *Parabacteroides goldsteinii* (CP081906). In addition, these enzymes have also been detected in other bacterial species, such as *Phocaeicola vulgatus* (CP096965) and *Prevotella* sp. (AP035786). These enzymes originated from humans and were found in India, the USA, Canada, Denmark, and the Netherlands, and from horses (*Equus caballus*) in Japan. This fact illustrates the seriousness of the situation, as the presence of beta-lactamases with a mobilization protein has been detected in many genera and species, indicating their widespread distribution and potential for the spread of antibiotic resistance.

Our study highlights the significant distribution of potential beta-lactamases across different bacterial genera, regions, and hosts. The presence of these enzymes, with exact amino acid identities with known beta-lactamases, in genera such as *Cedecea*, *Enterobacter*, *Alistipes*, *Bacteroides*, and *Parabacteroides*, suggests that horizontal gene transfer may play a role in spreading antibiotic resistance. This is supported by the identification of mobile genetic elements surrounding these genes, which may facilitate their transfer. China had the highest number of potential beta-lactamases, with the majority detected in environmental

samples. The detection of these enzymes in various environmental and clinical settings underscores the need for comprehensive monitoring and international surveillance. To combat antibiotic resistance, global cooperation is needed. Mobile genetic elements, such as transposases, highlight the potential for these resistance genes to spread. This is crucial for predicting treatment failures and understanding the spread of resistance within and across bacterial species. The presence of beta-lactamases in diverse environments suggests that natural habitats may serve as reservoirs for these resistance genes, potentially allowing them to be transferred to clinical settings. Environmental strains provide a significant reservoir of new resistance genes, including carbapenemases, which can be disseminated through the food chain, accelerating global antibiotic resistance. Human activities facilitate the spread of resistance from various reservoirs such as washing machines and urban beaches, contributing to the complexity of combating antibiotic resistance. This broad distribution underscores the severity of the issue and the need for further research to understand and tackle antimicrobial resistance. This is also why we are convinced that we will soon witness the discovery of numerous new types of beta-lactamases. Many of these previously undescribed beta-lactamases may also play a role in the resulting resistance to various beta-lactam antibiotics. This emphasizes the urgent need for continued surveillance and research to combat antibiotic resistance and protect public health. Addressing this challenge requires the integration of clinical and environmental perspectives to effectively monitor and mitigate the impact of beta-lactamases on global health.

SUPPLEMENTARY DATA

Table S1: Summary of putative beta-lactamases in various bacterial classes.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used ChatGPT to improve readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the publication's content.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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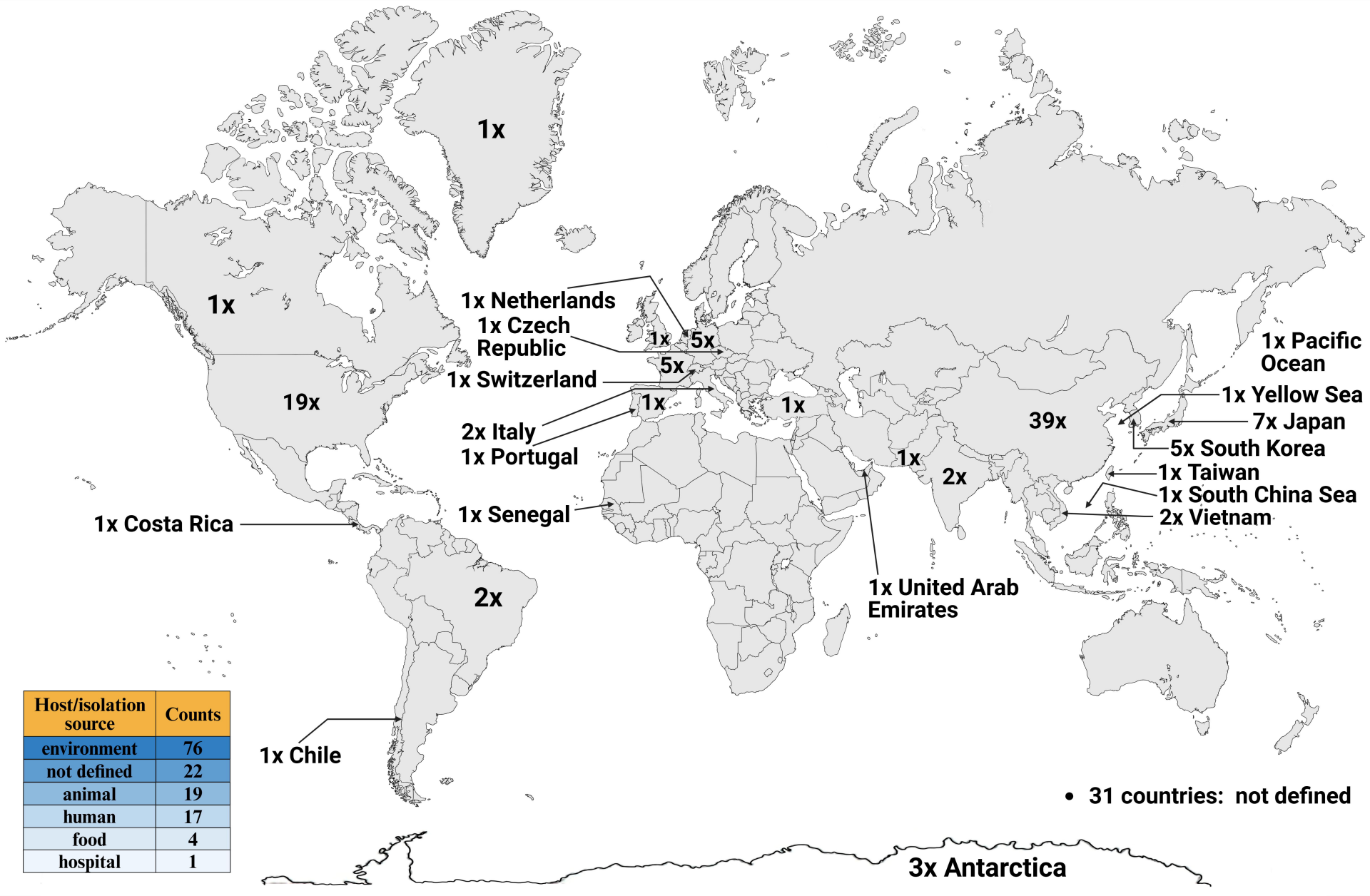
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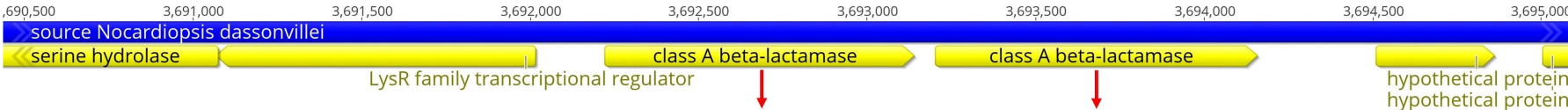
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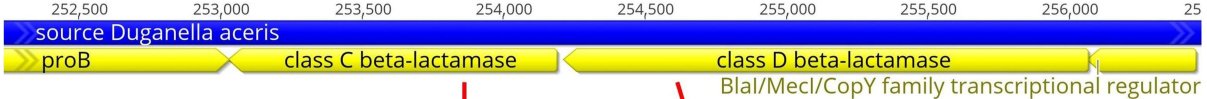
Host/isolation source	Counts
environment	76
not defined	22
animal	19
human	17
food	4
hospital	1

• 31 countries: not defined

A

ASU58879.1 (72.5% identity over 305 amino acids with R39-1)

ASU58880.1 (74% identity over 254 AA with R39-1)

B

WP_229259490.1 (74.7% identity over 375 amino acids with LRA10-1)

WP_166105428.1 (56.3% identity over 245 AA with OXA-D84)