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PER extended-spectrum β-lactamases originate from *Pararheinheimera* spp

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ABSTRACT

To investigate the origin of PER extended-spectrum β-lactamases, publicly available sequence databases were searched for $bla_{\text{PER-like}}$ genes. Three genomes from *Pararheinheimera*, a genus associated with water and soil environments, were found to carry $bla_{\text{PER-like}}$ genes but lacked the IS*CR1*/IS*Pa12*/IS*Pa13* insertion sequences commonly associated with bla_{PER} in clinical isolates. Sequence analysis revealed 78–96% nucleotide identity and conserved synteny between the clinical mobile genetic elements (MGEs) encoding $bla_{\text{PER-1}}$ and the bla_{PER} locus in the *Pararheinheimera* genomes. Notably, bla_{PER} genes were only identified in 3 of 21 *Pararheinheimera* and *Rheinheimera* genomes, whereas the genetic environment of bla_{PER} genes as found in clinical MGEs was conserved in all *Pararheinheimera* and *Rheinheimera* genomes. These findings indicate that bla_{PER} genes were likely acquired by a branch of the *Pararheinheimera* genus long before the antibiotic era. Later, bla_{PER} genes were mobilised, likely through the involvement of insertion sequences, from one or several *Pararheinheimera* species, allowing their dissemination into human pathogens.

1. Introduction

Enzymes belonging to the PER family of extended-spectrum β-lactamases (ESBLs) confer resistance to cephalosporins, penicillins and aztreonam but are inhibited by clavulanic acid, tazobactam and sulbactam [1]. Since the discovery of PER-1 in 1993, seven new variants (PER-2 to -8) have been described in the literature. They can be divided into two distinct groups based on their similarity to PER-1. Whereas PER-3, -4, -5, -7 and -8 share 98–99% amino acid identity with PER-1, the amino acid sequences of PER-2 and PER-6 are only 86% similar to that of PER-1. Initially described in *Pseudomonas aeruginosa*, PER ESBLs have been reported in several Gram-negative human pathogens including *Salmonella enterica, Klebsiella pneumoniae, Acinetobacter baumannii* and *Aeromonas caviae* [2–5] from different geographical locations and have thus become clinically relevant resistance determinants. The effective dissemination of bla_{PER} genes is likely due to their association with multiple different mobile genetic elements (MGEs) located either on the host chromosome or on plasmids [6,7]. The most commonly encountered bla_{PER}-associated MGEs are the composite transposon Tn*1213* [6], in which the bla_{PER} gene is enclosed by the insertion sequences IS*Pa12* and IS*Pa13*, and an IS*CR1* element often associated with *sul1*-type integrons. As IS*CR* elements have been shown to mobilise adjacent genes [8], it has been hypothesised that this element played a role in the mobilisation of $bla_{\text{PER-1}}$ genes [9] and its variants, and Tn*1213* has recently been shown to be actively involved in the gene's dissemination [10]. A glutathione S-transferase (*gst*) gene followed by an ABC-type transporter (*abct*) are encoded downstream of $bla_{\text{PER-1}}$ on most IS*CR1*/integron-associated MGEs [7,9,11], whereas Tn*1213* contains $bla_{\text{PER-1}}$ followed by a truncated *gst* gene [6], suggesting a common ancestry of both elements [5]. Despite the significant amount of research on bla_{PER} genes, their source remains unknown. So far, some antimicrobial resistance genes have been reported to have been mobilised from pathogens or human commensals such as $bla_{\text{OXA-23}}$ from *Acinetobacter radioresistens* [12] and *qnrB* from *Citrobacter freundii* [13], whereas others appear to have been mobilised from species more closely associated with the environment such as *Shewanella* spp. [14,15]. Identifying the origins of antimicrobial resistance genes is crucial to shed light on how human practices, especially the use of antibiotics, may have influenced their mobilisation [16] and, ultimately, their appearance in clinically relevant pathogens. Understanding these processes might help us to develop strategies on how to prevent

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the spread of new, more effective resistance determinants into the clinic.

2. Materials and methods

2.1. Sequence identification, annotation and comparison

To identify sequences harbouring $bla_{\text{PER-like}}$ genes, all available genomes and plasmids were downloaded from the National Center for Biotechnology Information (NCBI) assembly/nucleotide database (February/March 2018) and were searched for all PER protein variants (PER-1 to -8) available in the Comprehensive Antibiotic Resistance Database (CARD) [17] using DIAMOND v.0.8.36 [18] with an 85% identity cut-off. All contigs harbouring the bla_{PER} gene were annotated using PROKKA 1.12 [19]. If an open reading frame (ORF) was annotated as a hypothetical protein, DIAMOND v.0.8.36.98 was used to compare the respective sequence with the UniProt Knowledgebase (—id 50, —subject_cover 50, —more-sensitive) to investigate the function of the gene. As the aim was to compare the context of bla_{PER} genes, contigs that were annotated with less than six ORFs were excluded from the analysis.

Only sequences from two genera (*Rheinheimera* and *Pararheinheimera*) were found not to carry transposases close to the bla_{PER} gene. These sequences were further subjected to a manual search against the ISfinder database [20]. Furthermore, the contigs of the *Pararheinheimera*/*Rheinheimera* genomes containing bla_{PER} genes were also compared with the NCBI nucleotide database using discontinuous megablast and megablast to investigate whether they matched any MGEs, other than the PER-1-associated transposon/integron structures (see Results).

To investigate whether the bla_{PER} gene is acquired in certain *Pararheinheimera* and *Rheinheimera* lineages or whether bla_{PER} homologs are native to all members of these genera, all available *Pararheinheimera* and *Rheinheimera* genomes were downloaded. As some species of the genus *Rheinheimera* have recently been reclassified as the novel genus *Pararheinheimera*, we found it necessary to reinvestigate the classification of all *Rheinheimera* genomes containing sufficiently long 16S rRNA sequences using the genus-specific 16S rRNA signature nucleotides reported by Sisinthy et al. [21]. The barrnap software v0.9 [22] was used to identify 16S rRNA sequences in the genomes.

The nucleotide identity and synteny was then compared between the *Rheinheimera*/*Pararheinheimera* isolates and the bla_{PER}-positive sequences from other isolates.

DIAMOND was used to identify orthologs of proteins (blastx, —id 40, —subject_cover 60, —more-sensitive) encoded close to the bla_{PER} gene in all *Pararheinheimera*/*Rheinheimera* spp. using the respective protein sequences from sp. KL1 as reference (as the *Rheinheimera* sp. KL1 bla_{PER} gene was most similar to mobile bla_{PER} genes; see Results). Genes encoded a maximum of 10 kb upstream or downstream of bla_{PER} were analysed, including a glutathione S-transferase (*gst*) gene, an ABC-type transporter (*abct*) gene, a methionine–tRNA ligase (*metG*) gene, an iron-sulfur cluster carrier protein (*apbC*) gene, a hypothetical putative membrane protein gene and the $bla_{\text{PER-like}}$ class A β-lactamase gene.

2.2. Phylogenetic analysis

Phylogenetic analysis based on the PER amino acid sequences was conducted using MUSCLE [23] for multiple sequence alignment and RAxML [24] with the PROTCAT model and 1000 times rapid bootstrapping for creation of maximum likelihood trees. The same method (using the GTRCAT model instead of PROTCAT) was used for phylogenetic analysis of *Pararheinheimera* and *Rheinheimera* genomes based on *rpoB, gyrB, dnaK, metG* and *gst* nucleotide sequences. Calculation of the average nucleotide identity (ANI) over the whole genome for all *Pararheinheimera* and *Rheinheimera* isolates was conducted using dRep [25].

2.3. GenBank accession numbers

The following *Pararheinheimera* and *Rheinheimera* genome assemblies were included (GenBank assembly accession nos.): GCA_000986865.1; GCA_000711985.1; GCA_000382165.1; GCA_000425345.1; GCA_000296695.1; GCA_001752395.1; GCA_000217935.2; GCA_001275035.1; GCA_001513785.1; GCA_001669775.1; GCA_900215315.1; GCA_900108485.1; GCA_001518915.1; GCA_002683675.1; GCA_002685395.1; GCA_002721625.1; GCA_002862515.1; GCA_002381885.1; GCA_003504155.1; GCA_003482455.1; and GCA_003543135.1.

The MGEs containing bla_{PER} genes included the following GenBank accession nos.: *Morganella morganii* (KU133347.1); *P. aeruginosa* (AY779042.1); *Proteus vulgaris* (CVRZ01000031.1); and *Shewanella* sp. (GCA_002196695.1). The MGEs associated with bla_{PER} discussed here were chosen to reflect a broad host range as well as the variety of elements the gene is associated with.

The following PER variants were used in the phylogenetic analysis (GenBank accession nos.): WP_001100753.1; WP_032491907.1; WP_049637349.1; WP_063864593.1; WP_063864594.1; WP_063864595.1; WP_032495440.1; WP_063864596.1; KOO59254.1; KKL00294.1; and EGM79323.1.

3. Results

3.1. The Pararheinheimera bla_{PER} locus is highly similar to clinical mobile genetic elements, but lacks insertion sequences

The similarity search of the bla_{PER} genes against the NCBI assembly/nucleotide database yielded 95 high amino acid identity (85–100%) hits. Of these, 92 were from genomes or plasmids isolated from the genera *Vibrio, Acinetobacter, Pseudomonas, Proteus* and *Citrobacter*. Annotation showed that in all of these, the bla_{PER} genes were associated with ISs or IS*CRs*. Three hits, ranging from 85–92% sequence identity to PER-1 and PER-6, were associated with isolates from the genera *Rheinheimera* and *Pararheinheimera*, namely *Pararheinheimera mesophila* (87% amino acid identity to PER-1), *Rheinheimera* sp. A13L (85% amino acid identity to PER-6) and *Rheinheimera* sp. KL1 (92% amino acid identity to PER-1). Interestingly, the ISs commonly associated with $bla_{\text{PER-1}}$ in the other PER-positive isolates, namely IS*CR1*/IS*Pa12*/IS*Pa13*, were not identified on the bla_{PER}-carrying *Pararheinheimera* and *Rheinheimera* contigs, and no other ISs or transposases were found. Searches of the whole contigs against the ISfinder database supported the absence of intact ISs and associated transposons. No plasmids or MGEs except those previously reported in clinical isolates could be identified by searching the nucleotide database using the PER-containing *Pararheinheimera* contigs as query. However, other bla_{PER}-carrying MGEs (*M. morganii* KU133347.1, *Shewanella* sp. GCA_002196695.1) not contained in the previous results were identified this way.

Comparison with genus-specific 16S rRNA signature nucleotides of the recently established genera *Rheinheimera* and *Pararheinheimera* identified all three genomes as *Pararheinheimera*, with 11–12 of 14 signature nucleotides matching [21]. Two other *Rheinheimera* genomes, namely *Rheinheimera* sp. F8 (GCA_001518915.1) and *Rheinheimera* sp. SA1 (GCA_001669775.1), were also reclassified as *Pararheinheimera* based on their 16S rRNA signature nucleotides (Supplementary Table S1). The similarity in ANI between the three isolates was 86–89%, indicating that the three isolates belong to different *Pararheinheimera* species. Annotation of the respective genomes identified a glutathione S-synthase (*gst*) and an

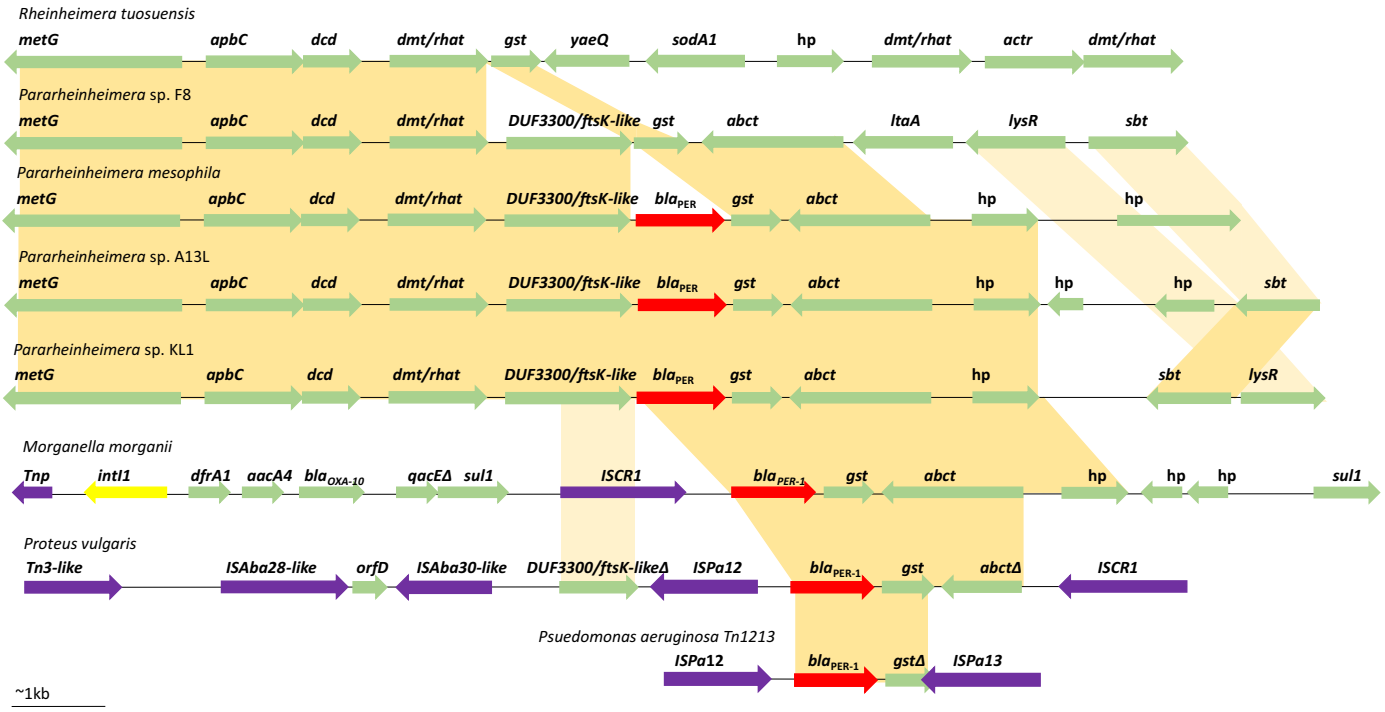

Fig. 1. Synteny comparison of the bla_{PER} locus from *Rheinheimera, Pararheinheimera* spp. and clinical bla_{PER-1}-carrying mobile genetic elements. Shared regions between two sequences are shown with a yellow background. Average nucleotide identities over shared regions: *Rheinheimera tuosuensis–Pararheinheimera* sp. F8, 74%; *R. tuosuensis–Pararheinheimera* sp. A13L, 73%; *R. tuosuensis–Pararheinheimera* sp. KL1, 73%; *Pararheinheimera* sp. F8–*Pararheinheimera mesophila*, 74%; *R. tuosuensis–P. mesophila*, 74%; *Pararheinheimera* sp. F8–*Pararheinheimera* sp. A13L, 75%; *Pararheinheimera* sp. F8–*Pararheinheimera* sp. KL1, 75%; *P. mesophila–Pararheinheimera* sp. A13L, 88%; *Pararheinheimera* sp. A13L–*Pararheinheimera* sp. KL1, 88%; *Pararheinheimera* sp. KL1–*Morganella morganii* class 1 integron, 96%; *M. morganii* class 1 integron–*Proteus vulgaris* mosaic element, 99%; *P. vulgaris* mosaic element–*Pseudomonas aeruginosa* Tn*1213*, 99%. Transposases, integrase genes, bla_{PER} and other genes are coloured in purple, yellow and red and green, respectively. Hypothetical proteins are denoted as 'hp'.

ABC-type transporter (*abct*) downstream of the $bla_{PER-like}$ gene, and an ORF containing an *ftsK*-like domain and a long hydrophobic chain upstream in each of the genomes (Fig. 1). Other genes annotated on the respective contigs are mainly involved in efflux and metabolism. Nucleotide sequence comparison between the isolates and the selected clinical MGEs revealed distinct similarity of each isolate to the mobilised DNA fragments. *Pararheinheimera* sp. KL1 shared 96% nucleotide identity over 4511 bp with the bla_{PER}-containing class 1 integron from *M. morganii*, 94–96% nucleotide identity with the MGE carrying bla_{PER} in *P. vulgaris* over 945 bp and 2450 bp, and 96% identity over 1413 bp with Tn*1213* from *P. aeruginosa. Pararheinheimera mesophila* and *Pararheinheimera* sp. A13L were 82% and 81% similar to the *M. morganii* integron over 4515–4544 bp, 83% and 78% similar to the *P. vulgaris* MGE over 3400 bp and 3162 bp, and 83% and 80% similar to Tn*1213* over 1406 bp and 1332 bp, respectively. The ORFs contained in each alignment were bla_{PER}–*gst*–*abct*–hp for the *M. morganii* integron (with 'hp' denoting a hypothetical protein), Δ*ftsK*-like and bla_{PER}–*gst*–Δ*abct* for the *P. vulgaris* MGE, and bla_{PER}–Δ*gst* for Tn*1213*.

3.2. The genetic environment of bla_{PER} is conserved in Rheinheimera and Pararheinheimera

Phylogenetic analysis of the *Pararheinheimera* PER amino acid sequences and previously described PER variants placed PER-1, -3, -4, -5, -7 and -8 on a clade with the PER proteins from *Pararheinheimera* sp. KL1 and *P. mesophila*, whereas the PER-like protein from *Pararheinheimera* sp. A13L formed a clade with PER-2 and PER-6. These branches were supported by bootstrap values of ≥87%. Orthologs to five proteins (*gst, abct, metG, apbC* and DUF3300/FtsK-like) were encoded close to the $bla_{PER-1-like}$ gene in all three bla_{PER}-carrying *Pararheinheimera* isolates. The genetic context in which the bla_{PER} genes from *Pararheinheimera* were identified was present with different degrees of conservation in all *Pararheinheimera* and *Rheinheimera* species (Fig. 1; Supplementary Table S2). The maximum likelihood trees created based on the chromosomal genes *gyrB, dnaK, rpoB, gst* and *metG* (the latter two being associated with the *Pararheinheimera* bla_{PER} region) displayed highly similar patterns, grouping the three bla_{PER}-carrying *Pararheinheimera* isolates together on a clade distinct from other *Pararheinheimera* with high bootstrap support. bla_{PER}-like genes were not identified in any *Pararheinheimera* or *Rheinheimera* other than the three previously described isolates.

4. Discussion

In this study, the genus *Pararheinheimera* was identified as the origin of the PER β-lactamases based on conserved synteny and nucleotide similarities up to 96% between chromosomal *Pararheinheimera* sequences and clinical MGEs encoding PER. The absence of intact known ISs or transposases confirmed that bla_{PER} was not part of a clinical MGE in *Pararheinheimera*, and no similarities to known plasmids or conjugative elements were found except for the bla_{PER}-associated genes contained in clinical MGEs.

The glutathione S-synthase (*gst*) and ABC-type transporter (*abct*) found downstream of the $bla_{PER-like}$ gene in all three *Pararheinheimera* species have been reported previously in association with MGEs encoding PER-1 in Europe and Asia, such as Tn*1213* [6,26] and *sul1*-type integrons [7,11], in which bla_{PER} is usually linked to the IS*CR1* transposase. Furthermore, bla_{PER-6} was reported on a mobile element carrying a truncated *gst* downstream and a truncated protein containing an *ftsK*-like domain upstream of bla_{PER-6} [5], respectively. In all three PER-encoding *Pararhein-*

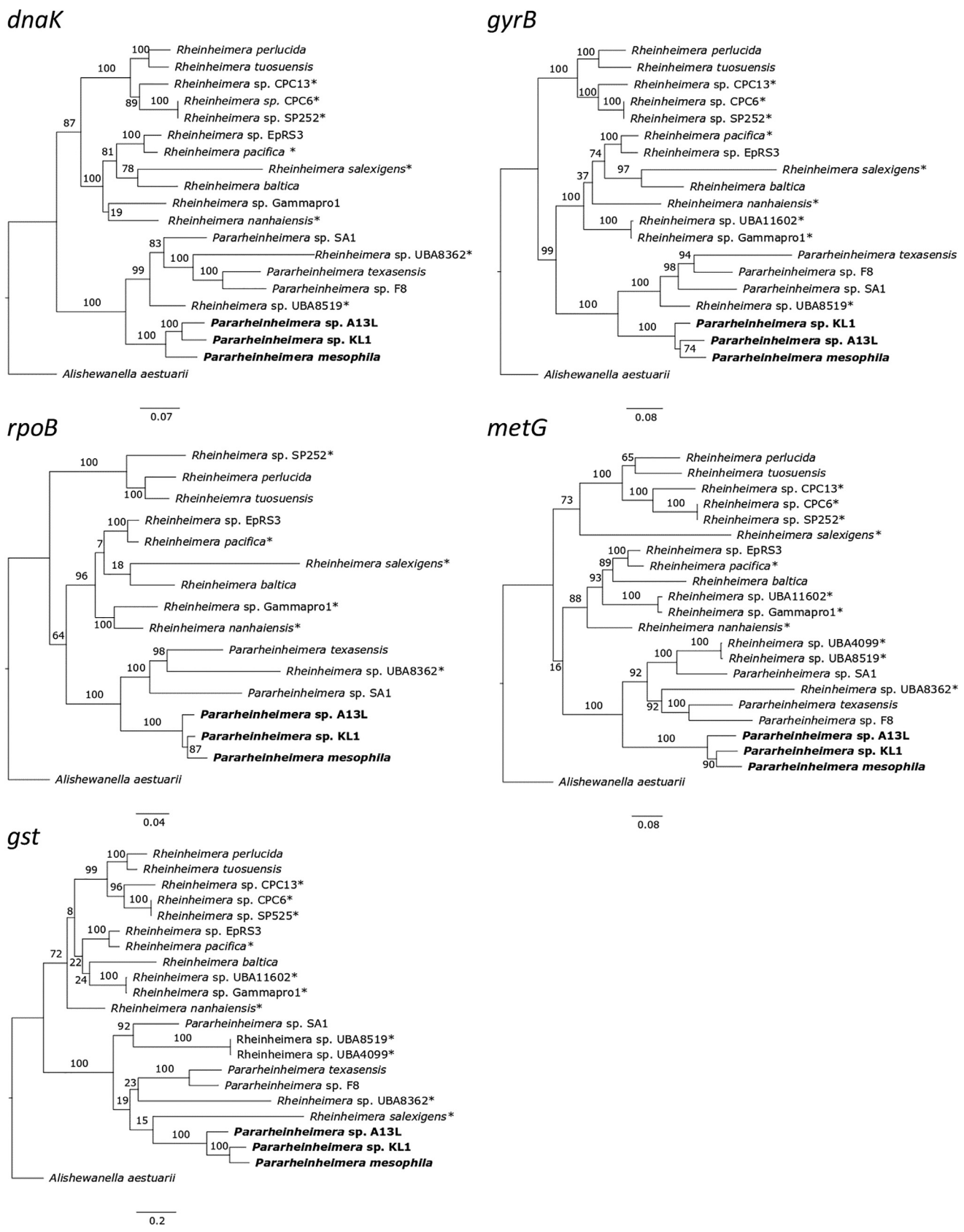

Fig. 2. Phylogenetic analyses based known chromosomal genes and genes closely located to bla_{PER} in *Pararheinheimera* spp. Maximum likelihood phylogenies inferred from nucleotide sequences extracted from the respective genome. Whereas *gyrB*, *dnaK* and *rpoB* are known chromosomal genes, *metG* and *gst* are associated with the bla_{PER} region in the *Pararheinheimera* genomes. bla_{PER}-positive isolates are shown in bold letters. Values on the branches represent bootstrap likelihood for the respective branch, and branch lengths indicate sequence change over time. Only isolates in which the respective gene was identified based on the specified thresholds were included in the phylogeny. The genus identity of the isolates marked with * could not be reassessed due to the lack of 16S rRNA in the respective assemblies. Corresponding *Alishewanella aestuarii* (GCF_000280055.1) genes were used as an outgroup.

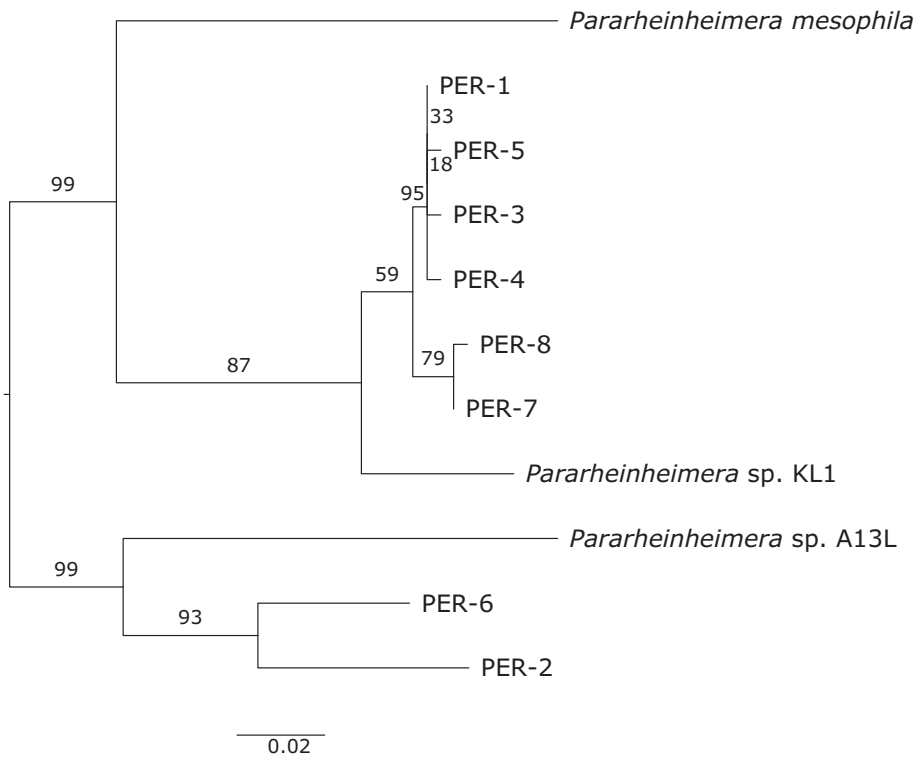

Fig. 3. Phylogeny of PER variants and *Pararheinheimera* PER. Phylogenetic analysis of the PER amino acid sequences from *Pararheinheimera* genomes and PER variants described in clinical isolates. Numbers on the branches reflect bootstrap support in percent, and branch lengths indicate sequence change over time.

heimera species, a protein of unknown function was identified upstream of bla_{PER}, harbouring a long hydrophobic chain and a conserved *ftsK*-like domain, which implies that bla_{PER-6}, despite greater sequence dissimilarity, has been mobilised from a very similar context as bla_{PER-1}, possibly from another *Pararheinheimera* species. A *Shewanella* isolate (GCA_002196695.1) carrying the bla_{PER-2}–*gst*–*abct* sequence suggests that bla_{PER-2} has also been mobilised from a *Pararheinheimera*-like context. This implies that some bla_{PER} variants might have been mobilised in separate mobilisation events.

The absence of ISs or transposons in the neighbourhood, in combination with the dissimilarity between the bla_{PER} gene sequences in different *Pararheinheimera* species, indicates that the bla_{PER} genes are permanently associated with the respective *Pararheinheimera* genomes. This notion is in line with the different sampling location of the different isolates. Whilst *Pararheinheimera* sp. KL1 was isolated from a freshwater lake in Canada [27] and *Pararheinheimera* sp. A13L was isolated from Pangong Lake [28], a saline lake in the Tibetan highlands, *P. mesophila* was isolated from soil from an industrial waste site in India [29].

The genetic environment of bla_{PER} is conserved in all *Rheinheimera* and *Pararheinheimera* genomes, and the different degrees of similarity of the locus from one genome to another show that this region has been associated with these genera throughout their evolution, underlining that this region is non-mobile in *Rheinheimera* and *Pararheinheimera* species (Fig. 1; Supplementary Table S2). This is further supported by comparison of the phylogenies based on *gyrB, dnaK, rpoB, gst* and *metG* (Fig. 2), which show that the three bla_{PER}-carrying isolates are closely related. That the bla_{PER} gene itself was only found in three *Pararheinheimera* isolates implies that the bla_{PER} progenitor was acquired by a common ancestor of the three bla_{PER}-positive isolates analysed in this study, before their speciation. This hypothesis is supported by the absence of bla_{PER} genes in the other *Pararheinheimera* isolates as well as in the other *Rheinheimera* genomes, whereas the genetic environment from clinical bla_{PER}-containing MGEs is present in all available *Rheinheimera* and *Pararheinheimera* genomes.

The MGEs carrying bla_{PER} and the *Pararheinheimera* sp. KL1 bla_{PER}-region shared 96% nucleotide sequence similarity. This high degree of similarity and the conserved synteny (Fig. 1) imply that the sequences are closely related to one another, as supported by the phylogenetic analysis of the PER variants in Fig. 3. Combined with the absence of transposons or ISs on the contigs containing bla_{PER} in the *Pararheinheimera* species, these findings suggest that bla_{PER} genes have been mobilised from *Pararheinheimera* species. Although bla_{PER-1} and its variants have likely been mobilised from a *Pararheinheimera* species closely related to *Pararheinheimera* sp. KL1, bla_{PER-2} and bla_{PER-6}-carrying MGEs appear too dissimilar in sequence identity to have been recently mobilised from the same species. Still, the genes mobilised with those bla_{PER} genes appear to be the same as in bla_{PER-1}-associated MGEs. bla_{PER-1} and bla_{PER-2}/bla_{PER-6} share ca. 86% sequence identity, a deviation in similarity that is also observed between other genes in different *Pararheinheimera* species (Supplementary Table S2). These dissimilarities in combination with the locally restricted appearance of some PER variants, such as PER-2 being mainly observed in South America [30,31], lead to the assumption that bla_{PER} genes have been mobilised more than once, presumably from different species of the *Pararheinheimera* genus. This is further supported by the different lengths of spacer sequences between bla_{PER} and the respective upstream transposase in different MGEs [11]. In this study, however, the resolution of the analysis is limited by the number of available *Pararheinheimera* genomes.

A transposase likely involved in the mobilisation of bla_{PER-1} and its neighbouring genes is, as previously hypothesised [11], IS*CR1*. IS*CR1* is, of all described MGEs containing bla_{PER}, associated with the longest *Pararheinheimera* sequence and has been shown to mobilise adjacent DNA segments [8]. However, it is uncertain whether

the IS*CR1*-associated sequences are indeed the longest fragments of DNA mobilised from *Pararheinheimera* and the presence of undetected larger elements is possible.

It could be hypothesised that the mobilisation of bla_{PER} involved the transfer of a plasmid or conjugative transposon carrying ISs or transposons into *Pararheinheimera*, which then lead to the capture of bla_{PER} and its surrounding sequences. Once transposed to a MGE encoding different ISs, the mobilised DNA fragment may have been gradually transformed by insertion of ISs. In this scenario, the transposition of bla_{PER} and associated sequences from the *Pararheinheimera* genome to a MGE carrying different transposases, such as a plasmid, would represent a key event, enabling the evolution of the variety of MGEs associated with bla_{PER} genes that are observed in the clinics. This hypothesis is supported by the fact that the transposed sequences in many bla_{PER-1}-containing MGEs are 99% similar in nucleotide sequence to one another, suggesting a common origin of these elements. Another explanation for the association of bla_{PER} with different ISs in different clinical isolates could be that $bla_{PER-like}$ genes have been mobilised repeatedly from *Pararheinheimera* sp. by different ISs.

5. Conclusion

To the best of our knowledge, *Pararheinheimera* has not yet been reported to be involved in human disease or associated with the human microbiome [32]. In contrast, *Pararheinheimera* have been isolated from a wide range of environmental habitats such as freshwater, seawater and soil [29,33,34]. This suggests that the mobilisation and dissemination of bla_{PER} genes has likely taken place in the external environment, several times and possibly as a consequence of antibiotic selection pressure [16]. Transfer of bla_{PER} genes from the environmental genus *Pararheinheimera* therefore highlights the importance of the environment as a reservoir for novel resistance determinants [35].

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Competing interests

None declared.

Ethical approval

Not required.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.10.019.

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