Genetic and Phenotypic Study of the *V*ersatile Beta-Lactamase, the Enzyme Most Similar to the Plasmid-Encoded TEM-1

Guilhem Royer, Zoya Dixit, Jacques Pédron, Gautier Pierrat, Vanessa Demontant, Béatrice Berçot, Christophe Rodriguez, Marie-Anne Barny, Hervé Jacquier, Paul-Louis Woerther

▶ **To cite this version:**

Guilhem Royer, Zoya Dixit, Jacques Pédron, Gautier Pierrat, Vanessa Demontant, et al.. Genetic and Phenotypic Study of the *V*ersatile Beta-Lactamase, the Enzyme Most Similar to the Plasmid-Encoded TEM-1. Applied and Environmental Microbiology, 2022, 88 (11), pp.e00220-22. 10.1128/aem.00220-22 . hal-04298127

HAL Id: hal-04298127

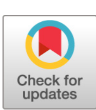
https://hal.u-pec.fr/hal-04298127

Submitted on 6 May 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

GENETICS AND MOLECULAR BIOLOGY



Genetic and Phenotypic Study of the *Pectobacterium versatile* Beta-Lactamase, the Enzyme Most Similar to the Plasmid-Encoded TEM-1

Guilhem Royer,[a,b] Zoya Dixit,[c] Jacques Pédron,[d] Gautier Pierrat,[c] Vanessa Demontant,[e] Béatrice Berçot,[f] Christophe Rodriguez,[e,g] Marie-Anne Barny,[d] Hervé Jacquier,[c,f] Paul-Louis Woerther[a,b,e]

[a]Département de Prévention, Diagnostic et Traitement des Infections, Hôpital Henri Mondor, APHP, Créteil, France

[b]EA 7380 Dynamyc, Université Paris-Est Créteil, Créteil, France

[c]Université de Paris, IAME UMR 1137 INSERM, Paris, France

[d]Sorbonne Université, INRAE, Institute of Ecology and Environmental Sciences-Paris, Paris, France

[e]Plateforme de Génomique, Hôpital Henri Mondor, APHP, Créteil, France

[f]Laboratory of Microbiology, Saint Louis-Lariboisiere-Fernand Widal Hospital Group, Assistance Publique-Hôpitaux de Paris, APHP, Paris, France

[g]INSERM U955, Institut Mondor de Recherche Biomédicale, Créteil, France

ABSTRACT Genus *Pectobacterium* bacteria include important agricultural pathogens. *Pectobacterium versatile* isolates contain a chromosome-borne beta-lactamase, PEC-1. This enzyme is the closest relative of TEM-1, a plasmid-borne beta-lactamase widespread in the *Enterobacterales*. We performed bioinformatics and phenotypic analyses to investigate the genetic and phenotypic features of PEC-1 and its frequency and ability to spread within genus *Pectobacterium*. We also compared the characteristics of PEC-1 and TEM-1 and evaluated the likelihood of transfer. We found that bla_{PEC-1} was present principally in a small number of genetic environments in *P. versatile*. Identical bla_{PEC-1} genetic environments were present in closely related species, consistent with the high frequency of genetic exchange within the genus *Pectobacterium*. Despite the similarities between PEC-1 and TEM-1, their genetic environments displayed no significant identity, suggesting an absence of recent transfer. Phenotypic analyses on clonal constructs revealed similar hydrolysis spectra. Our results suggest that *P. versatile* is the main reservoir of PEC-1, which seems to transfer to closely related species. The genetic distance between PEC-1 and TEM-1, and the lack of conserved elements in their genetic environments, suggest that any transfer that may have occurred must have taken place well before the antibiotic era.

IMPORTANCE This study aimed to compare the chromosomal beta-lactamase from *Pectobacterium versatile*, PEC-1, with the well-known and globally distributed TEM-1 in terms of genetic and functional properties. Despite the similarities between the enzymes, we obtained no definitive proof of gene transfer for the emergence of bla_{PEC-1} from bla_{TEM-1}. Indeed, given the limited degree of sequence identity and the absence of a common genetic environment, it seems unlikely that any transfer of this gene has occurred recently. However, although bla_{PEC-1} was found mostly in one specific clade of the *P. versatile* species, certain isolates from other closely related species, such as *Pectobacterium brasiliense* and *Pectobacterium polaris*, may also carry this gene inserted into common genetic environments. This observation suggests that genetic exchanges are frequent, accounting for the diffusion of bla_{PEC-1} between isolates from different *Pectobacterium* species and, potentially, to exogenous mobile genetic elements.

KEYWORDS antibiotic resistance, beta lactamase, emergence, progenitor, whole genome

Editor Christopher A. Elkins, Centers for Disease Control and Prevention

Copyright © 2022 American Society for Microbiology. All Rights Reserved.

Address correspondence to Paul-Louis Woerther, paul-louis.woerther@aphp.fr.

The authors declare no conflict of interest.

Received 4 February 2022
Accepted 14 April 2022
Published 16 May 2022

The soft rot pectobacteria (SRP) of the genera *Pectobacterium*, *Dickeya*, and *Musicola* of order *Enterobacterales* are ubiquitous pathogens that infect many different plant species worldwide, including various economically important crops, such as potato, tomato, carrot, and onion (1). These pathogens function as a species complex, with several species responsible for the same disease. Ten different SRP species have been recorded on potato, the principal crop affected by SRP, and mixed infections are common. *Pectobacterium carotovorum*, one of the first species described, is among the top 10 plant pathogenic bacteria (2). Recent genomic analysis led to the description of 20 species within genus *Pectobacterium*, including *Pectobacterium versatile*, which was described in 2019 (3). A taxonomic update of 265 *Pectobacterium* isolates from the CIRM-CFBP collection obtained worldwide since 1944 showed that *P. versatile* was pandemic, with isolates from diverse host plants and environments (4). *P. versatile* is not pathogenic to humans. However, it carries a putative beta-lactamase very closely related genetically to bla_{TEM-1} (5), one of the most widespread beta-lactamases in clinical isolates from the *Enterobacterales*.

Beta-lactams were discovered in the early 1940s. Their widespread use has led to the continual emergence of new enzymes conferring resistance to these antibiotics in Gram-negative bacilli of clinical interest (6). Most of the enzymes identified initially arose in environmental bacteria, suggesting that the resistance genes were transferred from the progenitor bacterial chromosome on mobile genetic elements (MGE), such as plasmids. These MGEs have the potential to spread to other pathogenic species, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. The recent pandemic of CTX-M-type extended-spectrum beta-lactamase (ESBL)-carrying enterobacteria provides a striking example of the mobilization of chromosomal genes from environmental *Enterobacterales* in this way, leading to worldwide spread in species of medical interest within a few decades (7). CTX-M-type ESBL were first described in the mid-1990s (8). They initially spread silently, but their spread through the community and hospitals has been exponential since the turn of the century, posing a major threat in terms of the development of antibiotic resistance in clinical bacteria (9). Several species from genus *Kluyvera* have been identified as the most probable progenitors of the various alleles of this enzyme family based on the presence of residual sequences corresponding to the chromosome of this genus in the immediate genetic environment of bla_{CTX-M} on plasmids (10). It is widely thought that the current pandemic has been driven by a small number of transfer events, mostly involving insertion sequences (IS) in a few plasmids (10). The extraordinary success of these bacteria may result from the fine-tuning of certain plasmids to particular bacterial hosts, as demonstrated for CTX-M-15-producing sequence type 131 (ST131) *E. coli* (11).

The TEM-1 beta-lactamase was first described in 1966. It rapidly spread worldwide, and community carriage of aminopenicillin-resistant *E. coli* is now reported worldwide. According to the annual reports of the European Antimicrobial Resistance Surveillance Network (EARS-Net) for 30 European Union/European Economic Area (EEA) countries (https://www.ecdc.europa.eu/en/antimicrobial-resistance), the percentage of aminopenicillin-resistant invasive *E. coli* isolates has remained stable since the turn of the century, at values exceeding 50% in most countries. The TEM-encoding genes are carried by genetically related transposons—Tn*1*, Tn*2*, and Tn*3* (12)—carrying allelic variants of TEM (TEM-2, TEM-1B, and TEM-1A, respectively). These transposons consist of a *tnpA* gene encoding a transposase and a *tnpR* gene encoding a resolvase and a *res* resolving site, and they are flanked by two 38-bp inverted repeat (IR) sequences. Tn*3* seems to be the most frequent form (12), but variants of these transposons generated by homologous recombination (13) or the integration of insertion sequences, such as IS*26* (14), have been described. These transposons are present on diverse plasmids from different incompatibility groups, underlying the rapid increase in the prevalence of TEM-1-producing isolates (15), but they have also been detected on the chromosome (14). No particular species has yet been unambiguously identified as the progenitor of bla_{TEM-1}, but the chromosomal beta-lactamase-encoding gene of *Pectobacterium versatile*, bla_{PEC-1}, seems to be the gene most closely related to the chromosomal beta-lactamase gene (5).

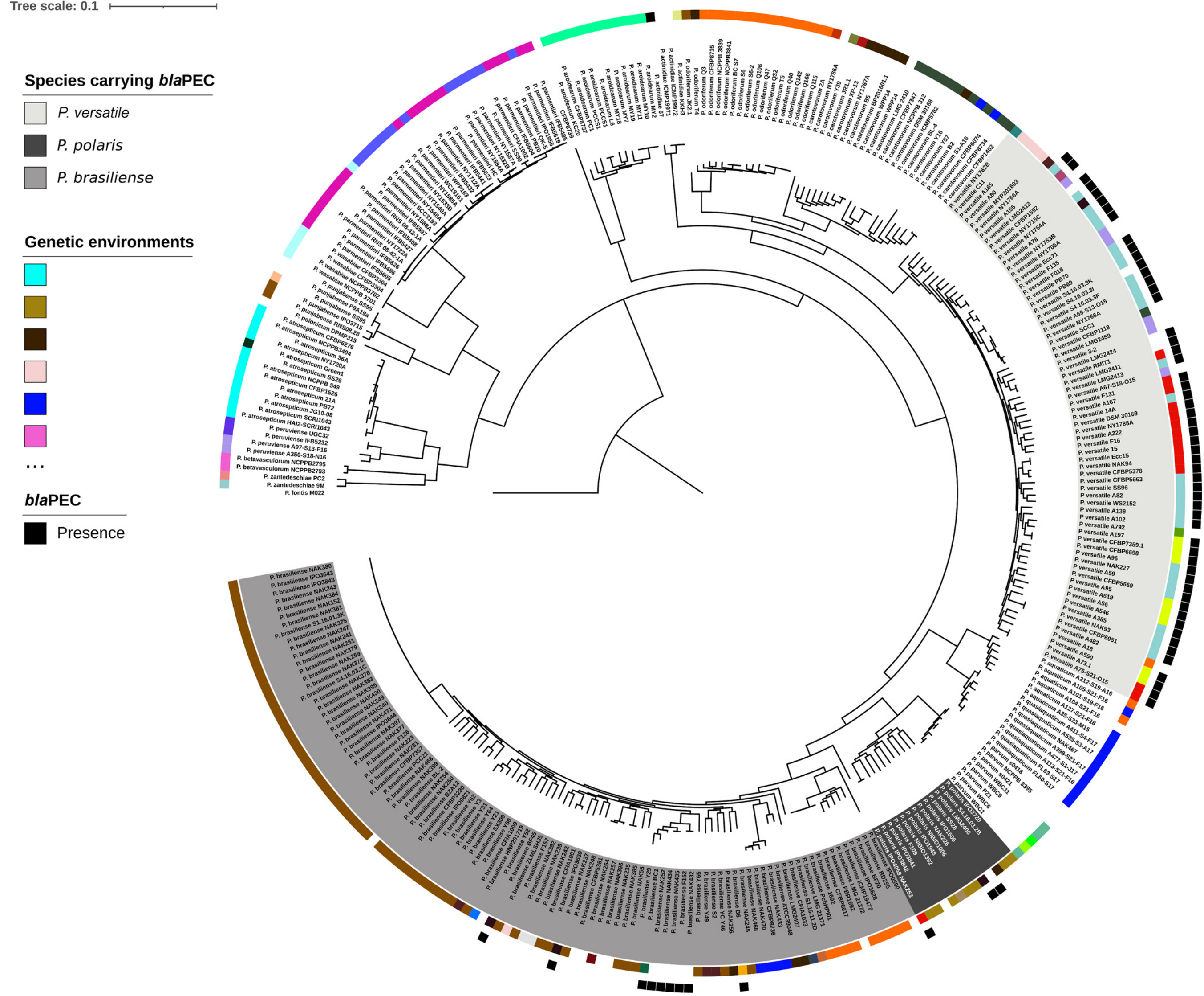


FIG 1 Core gene-based phylogenetic tree of genus *Pectobacterium*. The different genetic environments identified in the bla_{PEC-1} region are represented with colored squares in the inner circle around the tree. The presence of bla_{PEC-1} is highlighted by black squares in the outer circle around the tree. The three *Pectobacterium* species containing bla_{PEC-1} genes are shaded in gray. The tree is rooted on *Pectobacterium fontis* M022.

We investigated the genetic environment of bla_{PEC-1} and its diversity within the genus *Pectobacterium* in a large collection of isolates. We investigated the differences between TEM-1 and PEC-1 sequences to decipher putative functional and structural effects, and we performed phenotypic assays to compare the resistance spectra of the two enzymes. Finally, a comparison between the genetic environments of bla_{PEC-1} and bla_{TEM-1} suggested that if transfer actually occurred, it probably took place before the discovery of penicillin at the start of the 20th century.

RESULTS

Phylogenetic analysis of genus *Pectobacterium*. We retrieved 295 *Pectobacterium* spp. meeting our quality criteria from the GenBank database. We completed the collection with 31 newly sequenced genomes. We generated a pangenome from this collection that included 32,252 gene families, with a core genome (genes present in at least 99% of the genomes) of 1,710 genes. We then computed a phylogenetic tree from the core gene alignment (Fig. 1). As misclassification has been reported in this genus for classifications based on limited numbers of determinants (16), we identified species

with this core genome-based phylogenetic tree and the average nucleotide identity (ANI) values against the representative genomes of the different species (see Table S1 in the supplemental material). We were able to identify 19 of the 20 described species of genus *Pectobacterium* with this approach.

Putative beta-lactamase-encoding genes are found in a limited subset of *Pectobacterium* genomes. We scanned the whole collection of genomes for putative beta-lactamases. Nucleotide sequence matches to bla_{TEM-94} (GenBank accession number CAC85661) (identity = [80.72 to 81.31%]; coverage = 100%) were identified in 67 genomes. No additional beta-lactamase-encoding genes were identified. BLASTP analysis against the NCBI Bacterial Antimicrobial Resistance Reference Gene Database revealed that the closest protein sequences were either TEM-57 (GenBank accession number ACJ43254) (*n* = 51/67; identity = [82.04 to 82.39%]; coverage = 100%) or TEM-166 (GenBank accession number ACI25375) (*n* = 16/67; identity = [82.04 to 82.75%]; coverage = 100%), both considered to be TEM-1 variant beta-lactamases. Two genomes—*P. versatile* RMIT1 and LMG2424—had protein sequences truncated due to a nonsense mutation (774C > A/Y258X). Only minor variations were found between the 67 sequences obtained (see "Analysis of the primary and tertiary structures of PEC-1 and TEM-1") (see Fig. S1 in the supplemental material). We assessed the reciprocity of the similarity of these beta-lactamases by comparing TEM-1 (considered representative of TEM beta-lactamases), bla_{PEC-1}, and the other previously described beta-lactamases. At both nucleotide and protein sequence levels, bla_{PEC-1} was by far the most similar enzyme to TEM-1, with 80.2% nucleic acid sequence identity and 82.0% amino-acid identity (see Fig. S2 in the supplemental material). Screening of UniRef90, a more general database, confirmed that the TEM beta-lactamase (UniProt accession number P62593) was the protein most similar to PEC-1.

The putative beta-lactamase-encoding gene bla_{PEC-1} was found in only a restricted number of species. The prevalence of bla_{PEC-1} differed between *Pectobacterium* species (*P. versatile*, *n* = 55/70; *Pectobacterium brasiliense*, *n* = 9/99; and *Pectobacterium polaris*, *n* = 3/13). On the phylogenetic tree, all of the *P. versatile* genomes carrying bla_{PEC-1} were grouped into a monophyletic clade (Fig. 1). These data suggest acquisition by the common ancestor of this clade, with rare deletion events. Conversely, *P. brasiliense* and *P. polaris* strains carrying bla_{PEC-1} were infrequent and more dispersed in the phylogenetic tree, suggesting multiple independent acquisition events. Finally, we tried to determine the location of the bla_{PEC-1} gene (i.e., on a plasmid or the chromosome). In completely sequenced genomes (i.e., assembly level = "Complete" or "Chromosome"), the gene was systematically found on the chromosome as follows: *P. versatile* SCC1, F131, and DSM30169; and *P. brasiliense* NAK237 and BC1. In *P. versatile* genomes without bla_{PEC-1}, we found either no gene (*n* = 3/15) or an alginate lyase-encoding gene (*n* = 12/15) (see Fig. S3 in the supplemental material). This alginate lyase gene is present in many strains from other species as follows: *Pectobacterium zantedeschiae* (*n* = 2/2), *Pectobacterium peruviense* (*n* = 4/4), *Pectobacterium polonicum* (*n* = 1/1), *Pectobacterium punjabense* (*n* = 2/5), *Pectobacterium aroidearum* (*n* = 12/12), *Pectobacterium actinidiae* (*n* = 4/4), *Pectobacterium carotovorum* (*n* = 2/23), and *P. brasiliense* (*n* = 69/99), suggesting the presence of a hot spot for gene mobility.

In summary, we identified bla_{PEC-1}, a gene displaying marked sequence identity to bla_{TEM-1}, in almost all *P. versatile* genomes. This gene appeared to be located on the chromosome and was identified only rarely in the following two other *Pectobacterium* species: *P. brasiliense* and *P. polaris*. TEM was the known beta-lactamase with which PEC-1 displayed the highest level of sequence identity.

The restricted number of genetic environments for bla_{PEC-1} indicates a high level of conservation and probable interspecies exchange. We first characterized the genetic environments by aligning and comparing sequences in the region previously identified as containing bla_{PEC-1}. Seven distinct genetic contexts were identified, mostly linked to bacterial species, although identical environments were sometimes identified in different species (Fig. 1). Indeed, the genetic environments in some bla_{PEC-1}-containing *P. brasiliense* or *P. polaris* genomes were similar to that in certain *P. versatile* genomes (Fig. 2A and B). For example, *P. brasiliense* BF45, *P. brasiliense* NAK248, and *P. polaris* IPO1606 contained the same environment as *P. versatile* A78. We then compared the

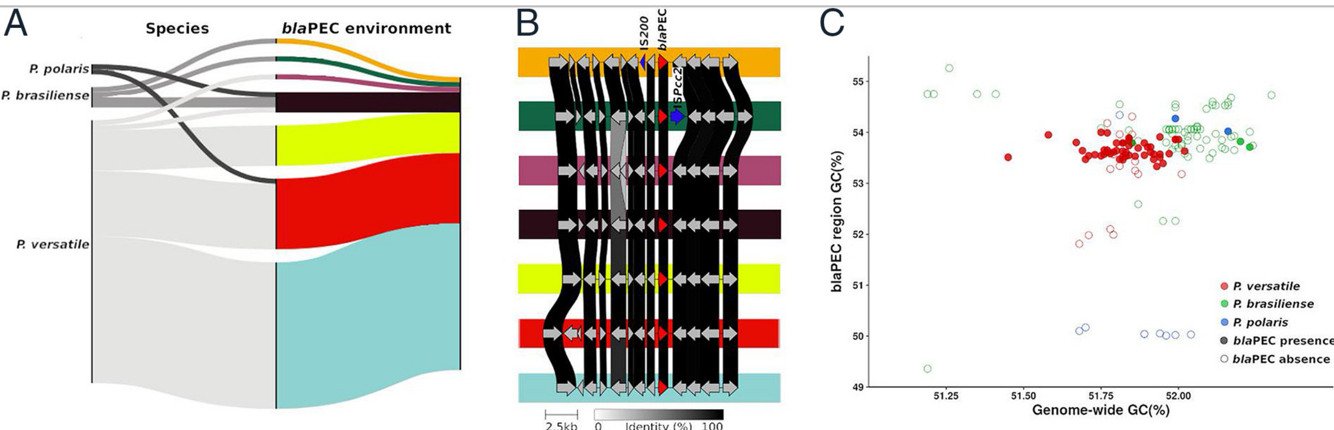


FIG 2 Detailed analysis of the bla_{PEC-1} region in *P. versatile*, *P. brasiliense*, and *P. polaris*. (A) Sankey diagram representing the three species in which bla_{PEC-1} was found (gray shading) and the seven different environments, each highlighted in the same color as in Fig. 1. (B) Gene cluster comparison of the different environments identified around bla_{PEC-1} (each environment is colored as in panel A). The presence of bla_{PEC-1} is shown by a red arrowhead, whereas insertion sequences are indicated in by a blue arrow. The alignment was generated with Clinker (44). A version of this figure including amino-acid sequence identity values is provided as a supplemental figure (see Fig. S5 in the supplemental material). (C) Distribution of %GC across the whole genome and the bla_{PEC-1} region of the three species, *P. versatile* (red), *P. brasiliense* (green), and *P. polaris* (blue). Solid circles represent genomes carrying bla_{PEC-1}, whereas empty circles represent genomes without this beta-lactamase gene.

%GC of the whole genomes of the three species carrying bla_{PEC-1}; all had similar %GC values, with median values of 51.83, 51.94, and 52.14% for *P. versatile*, *P. polaris*, and *P. brasiliense*, respectively (Fig. 2C). The observations focusing on the region of interest were similar, except for a few genomes not containing bla_{PEC-1}, mostly from *P. polaris*, which had a low %GC in this region. Finally, we analyzed a wider region ($\pm$100,000 bp) in genomes with bla_{PEC-1} and without bla_{PEC-1} of each of the three species (see Fig. S4 in the supplemental material). We found no major differences in %GC between bla_{PEC-1}-containing strains. No sequence identity was observed between these environments and the three known TEM-carrying transposons.

We then focused on the intergenic regions directly upstream and downstream from bla_{PEC-1}. The blastN alignment obtained against the nucleotide sequence collection of the NCBI database led to the identification of a partial match (length = 98 bp; nucleotide sequence identity = 89.90%) downstream from bla_{PEC-1}, with an IS*256* from *Salmonella enterica* strain 2011K-1440 (GenBank accession number CP053585). We found this remnant in all bla_{PEC-1}-positive genomes, whatever the *Pectobacterium* species. Moreover, we found an IS*Pcc2* sequence downstream from bla_{PEC-1} in *P. brasiliense* BC1 (GenBank accession number CP009769, 4502644 to 4503687) (Fig. 2B) and in five of the seven different environments. In this case, the contig was truncated just downstream from bla_{PEC-1} (*P. versatile* LMG2424 and RMIT1; *P. brasiliense* F152, NAK432, NAK434, and NAK435). Finally, the genome of *P. brasiliense* B6 carried another IS*200* upstream from bla_{PEC-1} (GenBank accession number JUJT01000005, 178075 to 178563).

In short, the genetic environment of bla_{PEC-1} was very similar, and sometimes identical, between the different species of *Pectobacterium*. The very similar whole-genome %GC values may facilitate genetic exchanges between species, and the similar values for the region surrounding bla_{PEC-1} provide evidence of a common origin. A larger-scale analysis of the region suggested that the presence of bla_{PEC-1} was probably either not linked to an acquisition event or that acquisition from a different bacterial species might have happened outside the time frame of antibiotic use in medicine. Fine-scale analysis revealed the presence of part of an IS*256* element in all bla_{PEC-1}-positive strains and the presence of other IS*Pcc2* elements in a few strains of *P. versatile* and *P. brasiliense*.

The dates of *P. versatile* isolation confirm the ancient nature of bla_{PEC-1} acquisition. We obtained isolation dates for 36 *P. versatile* isolates from GenBank reports and for 29 of the genomes that we sequenced (see Table S1). These dates ranged from 1918 to

B.

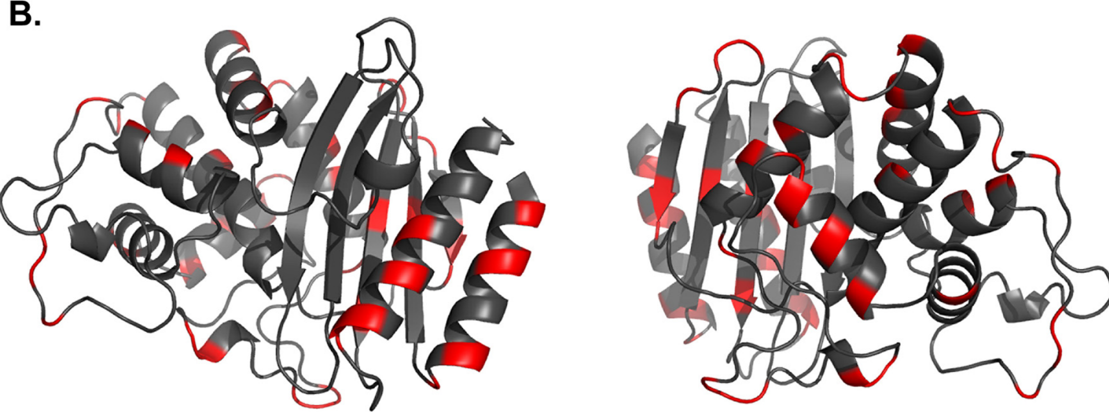


FIG 3 (A) Alignment of the amino-acid sequences of bla_{PEC-1} and bla_{TEM-1}. Numbering according to the numbering scheme described by Ambler et al. (47). Identical residues are indicated by dots. Signal peptides are shaded in gray. Conserved elements of class A beta-lactamases (S70XXK, S130DN, and K234S/TG) are boxed in red. The omega-loop is boxed in blue. (B) Three-dimensional structure of bla_{PEC-1} and bla_{TEM-1} beta-lactamases (PDB accession number 1BTL). Differences in amino acids between bla_{PEC-1} and bla_{PEC-1} are indicated in red.

2017. The oldest strain (*P. versatile* LMG2424) was isolated in 1918 from *Solanum tuberosum* in Canada, and it carried bla_{PEC-1}.

In parallel, we searched for the oldest strains of *E. coli* harboring bla_{TEM-1} present in the Enterobase database (17), one of the most comprehensive databases currently available. We identified only four genomes carrying bla_{TEM-1} among the 267 isolates obtained before 1970. All carried the classic transposons Tn*2* ($n = 3$) or Tn*3* ($n = 1$).

Together with the observation that the two genes encode proteins that are only 81.4% identical and the absence of a common genetic environment, the known isolation times support the hypothesis that any gene transfer must have happened long before the 1960s. The gene probably emerged through multiple intermediate steps, subsequently spreading under the effect of antibiotic pressure.

Analysis of the primary and tertiary structures of PEC-1 and TEM-1. We compared the sequence of PEC-1 from isolate A69-S13-O15 to that of TEM-1 (Fig. 3A). Conserved elements of class A beta-lactamases (S_{70}XXK, S_{130}DN, and K_{234}S/TG) were identified in PEC-1. Mutations were identified along the length of the beta-lactamase sequence, including the signal peptide. In total, two deletions and two point mutations were identified in the signal peptide, and 48 point mutations were identified in the core enzyme. In the three-dimensional (3D) structure (Fig. 3B), 24 of the 48 (50.0%) point mutations were located in the alpha helix, 5/48 (10.4%) were located in beta-sheets, and 19/48 (39.6%) were located in loops. In TEM-1, 117 of the 263 (44.5%) amino acids were present in alpha helices, 36/263 (13.7%) were in beta-sheets, and 110/263 (41.8%) were present in loops.

TABLE 1 MICs of beta-lactams for *E. coli* TOP10 recombinant strains producing TEM-1 or PEC-1, the *E. coli* TOP10 recipient strain, and *P. versatile* strain A69-S13-O15

	MIC for strain:			
Drug	***E. coli* TOP10 pBRHJ-TEM-1**	***E. coli* TOP10 pBRHJ-PEC-1**	***E. coli* TOP10**	***P. versatile strain* A69-S13-O15**
Amoxicillin	>128	>128	8	32
Amoxicillin/clavulanic acid[a]	32	32	8	≤2
Ticarcillin	>128	>128	4	32
Ticarcillin/clavulanic acid[a]	32	32	4	≤2
Piperacillin	>128	>128	2	16
Piperacillin/tazobactam[a]	≤2	≤2	≤2	≤2
Cefalexin	≤8	≤8	≤8	≤8
Cefuroxime	8	8	≤8	≤8
Cefixime	1	1	≤0.5	≤0.5
Cefotaxime	≤0.5	≤0.5	≤0.5	≤0.5
Ceftazidime	≤0.5	0.5	≤0.5	≤0.5
Cefepime	≤0.5	≤0.5	≤0.5	≤0.5
Cefoxitin	8	8	8	≤2
Aztreonam	≤0.12	≤0.12	≤0.12	≤0.12
Meropenem	≤0.12	≤0.12	≤0.12	≤0.12
Ertapenem	≤0.06	≤0.06	≤0.06	≤0.06

[a]Clavulanic acid was added at a fixed concentration of 2 mg/L and tazobactam at 4 mg/L.

The proportions of mutations in the different secondary structures and the proportions of amino acids in the different secondary structures were, therefore, quite similar, suggesting that the differences in amino-acid sequences were equally distributed between secondary structures in PEC-1 and TEM-1. However, the mutations within secondary structures did not appear to be distributed by chance and probably occurred through pairwise associations to maintain interactions within the secondary structure (Fig. 3B) to maintain the global structure and stability of the enzyme. The omega loop, which is rigidified by a salt bridge and contributes to delimitation of the active site, contained at least five mutations.

Ten of the 48 point mutations identified in PEC-1 were also found in TEM-producing clinical isolates, and four were associated with functional effects on TEM-1. The Q39K mutation has been associated with TEM-2 and TEM-2-derived alleles. The role of this mutation remains a matter of debate, as Q39K may be associated with a slight increase in the MIC of ceftazidime (18) or may enhance secondary mutations conferring higher levels of resistance to ceftazidime (19). The E104T mutation has never been described in clinical isolates but was shown to be associated with a slight increase in the MIC of ceftazidime in a directed mutagenesis experiment (20). The R120G, H153R, and M182T mutations have all been reported to stabilize TEM-1 (21–23). This stabilizing effect was particularly strong for M182T, which has been found in more than 30 TEM alleles from clinical isolates and in many strains from laboratory experiments (19).

However, residue interactions, or epistasis, play a crucial role in enzyme activity and/or stability (24). The effect of each individual mutation must therefore be interpreted with caution. Finally, the identification of these key residues involved in resistance to third-generation cephalosporins could explain the slight increase in the MIC of ceftazidime observed in *E. coli* TOP10 pBRHJ-PEC-1 (see "Resistance phenotypes of PEC-1-producing *Enterobacterales*" below; see also Table 1).

PEC-1-encoding genes were identified in 67 genomes from *Pectobacterium* spp. We identified only minor sequence variations relative to the sequence of PEC-1 identified in A69-S13-O15, and these sequences were described as "PEC-1-like" (see Fig. S1 in the supplemental material). In total, eight different variants combining seven mutations were found. S268G was the only one of these mutations known to enhance resistance to third-generation cephalosporins slightly in combination with other TEM-1 mutations (25).

Resistance phenotypes of PEC-1-producing *Enterobacterales*. *P. versatile* had a weakly expressed penicillinase (Table 1), and *E. coli* pBRHJ-PEC-1 had a resistance

spectrum similar to that of *E. coli* pBRHJ-TEM-1. Briefly, *E. coli* pBRHJ-PEC-1 was resistant to penicillins, with significant beta-lactamase inhibitor activity and a MIC of ceftazidime slightly higher than that of TEM-1. Due to this low resistance of PEC-1-producing *E. coli* to ceftazidime, the ESBL screen was negative (data not shown).

DISCUSSION

We described the chromosomal beta-lactamase of *P. versatile*, PEC-1, and its genetic environment in a large collection of genomes. We used nucleotide and amino-acid sequence identity data to compare the genetic and functional properties of PEC-1 with those of the well-known globally distributed TEM-1 (the enzyme to which it displays the highest degree of sequence identity). The two enzymes displayed 81.4% amino-acid sequence identity, but the genetic environment of bla_{PEC-1} was not similar to that surrounding bla_{TEM-1}, regardless of the transposon considered. Our data therefore provide no significant genetic evidence in favor of gene transfer as the mechanism of emergence of bla_{PEC-1} from bla_{TEM-1}.

The bla_{PEC-1} gene was found mostly in one specific clade of *P. versatile*, but certain isolates from other closely related species, such as *P. brasiliense* and *P. polaris*, also carried this gene inserted into similar genetic environments. These three species occupy the same ecological niche and can be isolated from the same plants, potentially accounting for this observation. Conversely, we found an alginate lyase gene in place of bla_{PEC-1} in a few *P. versatile* isolates. All genetic environments containing bla_{PEC-1} were very similar in terms of gene content and synteny, not only in *P. versatile*, but also in *P. brasiliense* and *P. polaris*. This observation is consistent with current knowledge for *Pectobacterium* spp., as previous genetic studies have demonstrated the existence of a large accessory genome common to species within this genus (16). This high degree of variability between isolates suggests that genetic exchanges are frequent, accounting for the diffusion of bla_{PEC-1} between isolates of different *Pectobacterium* species and, potentially, to exogenous mobile genetic elements, which remains to be demonstrated. This observation is also supported by the presence of various insertion sequences in isolates upstream (e.g., IS*200*) or downstream (e.g., IS*Pcc2*) from the gene, consistent with the occurrence of multiple events in the past. We also detected a 190-bp remnant including a 98-bp sequence displaying similarity to an IS*256* element, again suggestive of ancient gene transfer. These observations suggest that the region surrounding bla_{PEC-1} in *P. versatile* may be subject to frequent insertions, but the limited degree of sequence identity and the absence of a common genetic environment make it impossible to confirm that *P. versatile* was the original progenitor of bla_{TEM-1}.

Phenotypic characterization of *P. versatile* revealed that beta-lactamase expression levels were low. However, despite the antibiotic resistance conferred by bla_{PEC-1}, the widespread use of antibiotics in human and veterinary medicine is probably too recent to account for the selection and maintenance of this chromosomal gene in *P. versatile*. It, therefore, appears more likely that the basic function of the encoded enzyme may be different in *P. versatile* and may contribute to the ecological success of this species on diverse plants (4) in different environments (3). Similar situations have frequently been reported for beta-lactamase progenitors (26, 27).

Pectobacterium is an environmental bacterium from order *Enterobacterales*. This order includes most of the species recognized as progenitors of plasmid-encoded beta-lactamases, including *Kluyvera* spp. for CTX-M group extended-spectrum beta-lactamases, *Shewanella* spp. for OXA-48-derived carbapenemases, and *Morganella morganii* for AmpC cephalosporinases (27–29). However, despite the surprising similarities in the genetic and functional properties of the two enzymes considered here, not all of the conditions generally required to validate gene transfer are satisfied (30), possibly due to the long period of time since the hypothetical event, erasing the genetic trace of the initial event through evolution, but such traces may persist in unidentified intermediate hosts. Finally, only studies of more ancient, preantibiotic era TEM-producing isolates, which, to our knowledge, are not available, or the discovery of isolates at intermediate steps in the emergence process could shed additional light on the progenitor of this enzyme.

MATERIALS AND METHODS

Genome collection. We retrieved all of the available genus *Pectobacterium* genomes from the GenBank database on 12 August 2021. We retrieved 299 genomes of various species of *Pectobacterium* from the database (see Table S1 in the supplemental material) and sequenced 24 strains of *P. versatile* isolated from various diseased host plants and a few from other environments, such as water, insects, or soil (see Table S1 in the supplemental material). The study was extended to older isolates by the inclusion of 14 strains of *Pectobacterium carotovorum* isolated between 1918 and 1960 obtained from the Belgian Coordinated Collections of Microorganisms (BCCM). *P. versatile* and *P. carotovorum* were only recently distinguished (4). We therefore amplified and sequenced the *gapA* gene to identify *Pectobacterium* spp. to species level (31). Five strains identified as *P. versatile*, one as *P. polaris* and one as *P. brasiliense* (isolated in 1918, 1946, and two in 1957) were then sequenced. We also sequenced 31 new strains, including 29 *P. versatile*, one *P. brasiliense*, and one *P. polaris* (Table S1) strain. The cultured strains were diluted to a McFarland standard of 1 in sterile water. Bead beating was performed as a preextraction step, and DNA was then extracted with the DSP DNA midi kit on a QIAsymphony machine (Qiagen) as previously described (32). The extracts were prepared with the Nextera XT kit (Illumina) and pair-end sequenced (2×150 bp) with the NextSeq 500/550 high output kit v2.5 (300 cycles) on a NextSeq500 (Illumina). The newly sequenced genomes are available from BioProject PRJEB49284.

Assembly, quality control, and screening for beta-lactamase-encoding genes. The newly sequenced genomes were assembled with shovill 1.0.4 (https://github.com/tseemann/shovill) using standard parameters. Quality control was performed for all genomes, with CheckM 1.0.11 (33) and a lineage-specific workflow. Genomes with >5% contamination and/or <95% completeness were discarded. We calculated the genome-level %GC with Quast 5.0.2 (34). We then searched the entire collection of genome sequences for beta-lactamase-encoding genes, with Resfinder (35) and abricate (https://github.com/tseemann/abricate), with a minimum nucleotide sequence identity and target coverage of 20%. We then aligned putative beta-lactamase sequences with sequences from the NCBI Bacterial Antimicrobial Resistance Reference Gene Database (BioProject PRJNA313047), using both nucleotide (blastN) and protein (blastP) sequences to check for matches with known beta-lactamases. We also searched for amino-acid sequence identity against the whole UniRef90 database (36) (accessed on 13 October 2021), including protein sequences unrelated to resistance.

Phylogenetic analysis of genus *Pectobacterium*. We first computed the pangenome of the whole genus with Roary 3.12.0 (37) using a protein identity cutoff of 65% as suggested by Jonkheer et al. (16). This analysis yielded a core-gene alignment, which was used for phylogenetic analysis with IQ-tree 1.6.12 (38) and a general time reversible model, allowing for a proportion of invariant sites and using a discrete Gamma model with four rate categories. For species delineation, we computed average nucleotide identity (ANI) with fastANI (39) using a value of at least 95% relative to the representative genome according to the RefSeq database (https://www.ncbi.nlm.nih.gov/genome/). A higher cutoff value of 96% was used in three cases as follows: (i) *P. carotovorum*, *P. versatile*, and *Pectobacterium odoriferum* (4); (ii) *P. polaris* and *Pectobacterium parvum* (40); and (iii) *Pectobacterium aquaticum* and *Pectobacterium quasiaquaticum* (41). Furthermore, as previously reported (16), a few *P. brasiliense* genomes were found to have lower ANI values (*P. brasiliense* NAK468, *P. brasiliense* NAK470, and *P. brasiliense* NAK433) but should nevertheless be assigned to this species.

Phylogenetic analyses of *P. versatile*. We performed a more detailed analysis of *P. versatile*. All of the genomes from this species were aligned against the *P. versatile* SCC1 genome (GenBank accession number CP021894) with Snippy (https://github.com/tseemann/snippy). We then filtered on recombination using Gubbins with standard parameters (42). Finally, we constructed a phylogenetic tree with FastTree 2.1.8 (43) and the general time reversible model.

Coding sequence content and %GC of the genomic region surrounding bla_{PEC-1}. We performed a comprehensive analysis of the genomic region containing the beta-lactamase gene in all of the available isolates, including those lacking this gene. The comparison was facilitated by extracting the sequences surrounded by core genes on both sides as follows: *glpT* encoding a glycerol-3-phosphate transporter and *btsS* encoding a sensor histidine kinase. The annotations of these genomic regions are available from Zenodo in GenBank format (see https://doi.org/10.5281/zenodo.5550484 and Table S1). Excluding truncated contigs, which were not considered in the subsequent analysis, the median sequence length was 16,177 bp (interquartile range [IQR] = 14,972 to 17,247 bp). We aligned the sequences and plotted a cluster alignment with Clinker (44). The genomic region was considered different for each variation of coding sequence composition. We also computed the %GC of these regions, as described above, with Quast 5.0.2.

Alignment of the amino-acid sequences of PEC-1 and TEM-1. MAFFT was used to align the amino-acid sequences of PEC-1 and TEM-1 (45). The TEM variants described in clinical isolates were those referenced in the Beta-Lactamase Data Resources (https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/).

3D structure analysis. The TEM-1 3D structure (PDB accession number 1BTL) was used to map amino-acid differences between TEM-1 and PEC-1 with PyMOL (PyMOL Molecular Graphics System, version 1.2r3pre; Schrödinger, LLC).

Beta-lactamase cloning. The bla_{TEM-1} gene was first inserted into the pBRHJ plasmid (21). The bla_{PEC-1} gene was amplified from the DNA of *P. versatile* strain A69-S13-O15 with the primers blaPEC_F (5′-ATAAATGCTTCAATAATATTGAAAAAGGAAGACCATGCAACATTTCCGTATAGCG-3′) and blaPEC_R (5′-TATGAGTAAACTTGGTCTGACAGGCGGCCGCTTACCAGTGCTTAATCAGTG-3′). The pBRHJ plasmid backbone was amplified with the primers BB_PBRHJ_F (5′-CACTGATTAAGCACTGGTAAGCGGCCGCCTGTCAGACCAAGTTTACTCATA-3′) and BB_PBRHJ_R (5′-GGTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGG-3′). The bla_{PEC-1}

gene was then integrated into the pBRHJ backbone in place of the bla_{TEM-1} gene by a circular polymerase extension cloning (CPEC) approach (46). Briefly, the primers for amplifying the bla_{PEC-1} gene and those for the pBRHJ plasmid backbone were designed such that their tails overlapped. The bla_{PEC-1} gene and pBRHJ backbone amplicons were then mixed for the second step of amplification; overlapping regions were then extended between the insert and vector backbone to form a complete circular plasmid. The recombinant plasmid was used to transform *E. coli* strain TOP10 with selection on Luria-Bertani agar supplemented with gentamicin (6 mg/L). The recombinant plasmid was subjected to Sanger sequencing. Plasmids carrying bla_{TEM-1} and bla_{PEC-1} are referred to as pBRHJ-TEM-1 and pBRHJ-PEC-1, respectively.

MIC determinations. MIC determinations were performed in triplicate by the broth microdilution method in Mueller-Hinton broth (Bio-Rad, Marnes-La-Coquette, France) at optimal growth temperatures for *E. coli* and *P. versatile*. For *E. coli* transformed with pBRHJ-TEM-1 and pBRHJ-PEC-1, MICs were determined at 37°C. For *P. versatile* strain A69-S13-O15, MICs were determined at 30°C.

Data availability. Assemblies of genomes sequenced in this study are available under BioProject accession number PRJEB49284. Annotation files for the region between *glpT* and *btsS*, which sometimes includes bla_{PEC}, are available from Zenodo at https://doi.org/10.5281/zenodo.5550484.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 5.4 MB.

ACKNOWLEDGMENTS

We warmly thank Guillaume Arlet and Olivier Tenaillon for their help and useful suggestions.

We have no competing interests to declare.

REFERENCES

1. Czajkowski R, Perombelon M, Jafra S, Lojkowska E, Potrykus M, van der Wolf J, Sledz W. 2015. Detection, identification and differentiation of Pectobacterium and Dickeya species causing potato blackleg and tuber soft rot: a review. Ann Appl Biol 166:18–38. https://doi.org/10.1111/aab.12166.
2. Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer SV, Machado MA, Toth I, Salmond G, Foster GD. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. Mol Plant Pathol 13:614–629. https://doi.org/10.1111/j.1364-3703.2012.00804.x.
3. Portier P, Pedron J, Taghouti G, Fischer-Le Saux M, Caullireau E, Bertrand C, Laurent A, Chawki K, Oulgazi S, Moumni M, Andrivon D, Dutrieux C, Faure D, Helias V, Barny MA. 2019. Elevation of Pectobacterium carotovorum subsp. odoriferum to species level as Pectobacterium odoriferum sp. nov., proposal of Pectobacterium brasiliense sp. nov. and Pectobacterium actinidiae sp. nov., emended description of Pectobacterium carotovorum and description of Pectobacterium versatile sp. nov., isolated from streams and symptoms on diverse plants. Int J Syst Evol Microbiol 69: 3207–3216. https://doi.org/10.1099/ijsem.0.003611.
4. Portier P, Pedron J, Taghouti G, Dutrieux C, Barny MA. 2020. Updated taxonomy of Pectobacterium genus in the CIRM-CFBP bacterial collection: when newly described species reveal "old" endemic population. Microorganisms 8:1441. https://doi.org/10.3390/microorganisms8091441.
5. Philippon A, Jacquier H, Ruppe E, Labia R. 2019. Structure-based classification of class A beta-lactamases, an update. Curr Res Transl Med 67: 115–122. https://doi.org/10.1016/j.retram.2019.05.003.
6. De Oliveira DMP, Forde BM, Kidd TJ, Harris PNA, Schembri MA, Beatson SA, Paterson DL, Walker MJ. 2020. Antimicrobial resistance in ESKAPE pathogens. Clin Microbiol Rev 33:e00181-19. https://doi.org/10.1128/CMR.00181-19.
7. D'Andrea MM, Arena F, Pallecchi L, Rossolini GM. 2013. CTX-M-type beta-lactamases: a successful story of antibiotic resistance. Int J Med Microbiol 303:305–317. https://doi.org/10.1016/j.ijmm.2013.02.008.
8. Bauernfeind A, Stemplinger I, Jungwirth R, Ernst S, Casellas JM. 1996. Sequences of beta-lactamase genes encoding CTX-M-1 (MEN-1) and CTX-M-2 and relationship of their amino acid sequences with those of other beta-lactamases. Antimicrob Agents Chemother 40:509–513. https://doi.org/10.1128/AAC.40.2.509.
9. Woerther PL, Burdet C, Chachaty E, Andremont A. 2013. Trends in human fecal carriage of extended-spectrum beta-lactamases in the community: toward the globalization of CTX-M. Clin Microbiol Rev 26:744–758. https://doi.org/10.1128/CMR.00023-13.
10. Canton R, Gonzalez-Alba JM, Galan JC. 2012. CTX-M enzymes: origin and diffusion. Front Microbiol 3:110. https://doi.org/10.3389/fmicb.2012.00110.
11. Nicolas-Chanoine MH, Bertrand X, Madec JY. 2014. Escherichia coli ST131, an intriguing clonal group. Clin Microbiol Rev 27:543–574. https://doi.org/10.1128/CMR.00125-13.
12. Bailey JK, Pinyon JL, Anantham S, Hall RM. 2011. Distribution of the bla-TEM gene and blaTEM-containing transposons in commensal Escherichia coli. J Antimicrob Chemother 66:745–751. https://doi.org/10.1093/jac/dkq529.
13. Partridge SR, Hall RM. 2005. Evolution of transposons containing bla_{TEM} genes. Antimicrob Agents Chemother 49:1267–1268. https://doi.org/10.1128/AAC.49.3.1267-1268.2005.
14. Oliva M, Calia C, Ferrara M, D'Addabbo P, Scrascia M, Mule G, Monno R, Pazzani C. 2020. Antimicrobial resistance gene shuffling and a three-element mobilisation system in the monophasic Salmonella typhimurium strain ST1030. Plasmid 111:102532. https://doi.org/10.1016/j.plasmid.2020.102532.
15. Cantón R, Morosini MI, Martín O, de la Maza OMS, de la Pedrosa EGG. 2008. IRT and CMT beta-lactamases and inhibitor resistance. Clin Microbiol Infect 14(Suppl 1):53–62. https://doi.org/10.1111/j.1469-0691.2007.01849.x.
16. Jonkheer EM, Brankovics B, Houwers IM, van der Wolf JM, Bonants PJM, Vreeburg RAM, Bollema R, de Haan JR, Berke L, Smit S, de Ridder D, van der Lee TAJ. 2021. The Pectobacterium pangenome, with a focus on Pectobacterium brasiliense, shows a robust core and extensive exchange of genes from a shared gene pool. BMC Genomics 22:265. https://doi.org/10.1186/s12864-021-07583-5.
17. Zhou Z, Alikhan NF, Mohamed K, Fan Y, Achtman M, Agama Study Group. 2020. The EnteroBase user's guide, with case studies on Salmonella transmissions, Yersinia pestis phylogeny, and Escherichia core genomic diversity. Genome Res 30:138–152. https://doi.org/10.1101/gr.251678.119.
18. Blazquez J, Morosini MI, Negri MC, Gonzalez-Leiza M, Baquero F. 1995. Single amino acid replacements at positions altered in naturally occurring extended-spectrum TEM beta-lactamases. Antimicrob Agents Chemother 39:145–149. https://doi.org/10.1128/aac.39.1.145.
19. Salverda ML, De Visser JA, Barlow M. 2010. Natural evolution of TEM-1 beta-lactamase: experimental reconstruction and clinical relevance. FEMS Microbiol Rev 34:1015–1036. https://doi.org/10.1111/j.1574-6976.2010.00222.x.
20. Lenfant F, Petit A, Labia R, Maveyraud L, Samama JP, Masson JM. 1993. Site-directed mutagenesis of beta-lactamase TEM-1. Investigating the potential role of specific residues on the activity of Pseudomonas-specific

enzymes. Eur J Biochem 217:939–946. https://doi.org/10.1111/j.1432-1033.1993.tb18324.x.
21. Jacquier H, Birgy A, Le Nagard H, Mechulam Y, Schmitt E, Glodt J, Bercot B, Petit E, Poulain J, Barnaud G, Gros PA, Tenaillon O. 2013. Capturing the mutational landscape of the beta-lactamase TEM-1. Proc Natl Acad Sci U S A 110:13067–13072. https://doi.org/10.1073/pnas.1215206110.
22. Bershtein S, Goldin K, Tawfik DS. 2008. Intense neutral drifts yield robust and evolvable consensus proteins. J Mol Biol 379:1029–1044. https://doi.org/10.1016/j.jmb.2008.04.024.
23. Hecky J, Muller KM. 2005. Structural perturbation and compensation by directed evolution at physiological temperature leads to thermostabilization of beta-lactamase. Biochemistry 44:12640–12654. https://doi.org/10.1021/bi0501885.
24. Wang X, Minasov G, Shoichet BK. 2002. Evolution of an antibiotic resistance enzyme constrained by stability and activity trade-offs. J Mol Biol 320:85–95. https://doi.org/10.1016/S0022-2836(02)00400-X.
25. Vakulenko S, Golemi D. 2002. Mutant TEM beta-lactamase producing resistance to ceftazidime, ampicillins, and beta-lactamase inhibitors. Antimicrob Agents Chemother 46:646–653. https://doi.org/10.1128/AAC.46.3.646-653.2002.
26. Chaves J, Ladona MG, Segura C, Coira A, Reig R, Ampurdanes C. 2001. SHV-1 beta-lactamase is mainly a chromosomally encoded species-specific enzyme in Klebsiella pneumoniae. Antimicrob Agents Chemother 45:2856–2861. https://doi.org/10.1128/AAC.45.10.2856-2861.2001.
27. Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, Philippon A. 2002. Beta-lactamases of Kluyvera ascorbata, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob Agents Chemother 46:3045–3049. https://doi.org/10.1128/AAC.46.9.3045-3049.2002.
28. Barnaud G, Arlet G, Verdet C, Gaillot O, Lagrange PH, Philippon A. 1998. Salmonella enteritidis: AmpC plasmid-mediated inducible beta-lactamase (DHA-1) with an ampR gene from Morganella morganii. Antimicrob Agents Chemother 42:2352–2358. https://doi.org/10.1128/AAC.42.9.2352.
29. Poirel L, Heritier C, Nordmann P. 2004. Chromosome-encoded ambler class D beta-lactamase of Shewanella oneidensis as a progenitor of carbapenem-hydrolyzing oxacillinase. Antimicrob Agents Chemother 48:348–351. https://doi.org/10.1128/AAC.48.1.348-351.2004.
30. Ebmeyer S, Kristiansson E, Larsson DGJ. 2021. A framework for identifying the recent origins of mobile antibiotic resistance genes. Commun Biol 4:8. https://doi.org/10.1038/s42003-020-01545-5.
31. Cigna J, Dewaegeneire P, Beury A, Gobert V, Faure D. 2017. A gapA PCR-sequencing assay for identifying the Dickeya and Pectobacterium potato pathogens. Plant Dis 101:1278–1282. https://doi.org/10.1094/PDIS-12-16-1810-RE.
32. Rodriguez C, Jary A, Hua C, Woerther PL, Bosc R, Desroches M, Sitterle E, Gricourt G, De Prost N, Pawlotsky JM, Chosidow O, Sbidian E, Decousser JW, the Multidisciplinary Necrotizing Fasciitis Study Group. 2020. Pathogen identification by shotgun metagenomics of patients with necrotizing soft-tissue infections. Br J Dermatol 183:105–113. https://doi.org/10.1111/bjd.18611.
33. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res 25:1043–1055. https://doi.org/10.1101/gr.186072.114.
34. Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29:1072–1075. https://doi.org/10.1093/bioinformatics/btt086.
35. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother 67:2640–2644. https://doi.org/10.1093/jac/dks261.
36. The UniProt Consortium. 2021. UniProt: the universal protein knowledgebase in 2021. Nucleic Acids Res 49:D480–D489. https://doi.org/10.1093/nar/gkaa1100.
37. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, Fookes M, Falush D, Keane JA, Parkhill J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31:3691–3693. https://doi.org/10.1093/bioinformatics/btv421.
38. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 32:268–274. https://doi.org/10.1093/molbev/msu300.
39. Jain C, Rodriguez RL, Phillippy AM, Konstantinidis KT, Aluru S. 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nat Commun 9:5114. https://doi.org/10.1038/s41467-018-07641-9.
40. Pasanen M, Waleron M, Schott T, Cleenwerck I, Misztak A, Waleron K, Pritchard L, Bakr R, Degefu Y, van der Wolf J, Vandamme P, Pirhonen M. 2020. Pectobacterium parvum sp. nov., having a Salmonella SPI-1-like type III secretion system and low virulence. Int J Syst Evol Microbiol 70:2440–2448. https://doi.org/10.1099/ijsem.0.004057.
41. Ben Moussa H, Pedron J, Bertrand C, Hecquet A, Barny MA. 2021. Pectobacterium quasiaquaticum sp. nov., isolated from waterways. Int J Syst Evol Microbiol 71:005042. https://doi.org/10.1099/ijsem.0.005042.
42. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, Parkhill J, Harris SR. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res 43:e15. https://doi.org/10.1093/nar/gku1196.
43. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490. https://doi.org/10.1371/journal.pone.0009490.
44. Gilchrist CLM, Chooi YH. 2021. Clinker & clustermap.js: automatic generation of gene cluster comparison figures. Bioinformatics 18:btab007. https://doi.org/10.1093/bioinformatics/btab007.
45. Katoh K, Rozewicki J, Yamada KD. 2019. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform 20:1160–1166. https://doi.org/10.1093/bib/bbx108.
46. Quan J, Tian J. 2009. Circular polymerase extension cloning of complex gene libraries and pathways. PLoS One 4:e6441. https://doi.org/10.1371/journal.pone.0006441.
47. Ambler RP, Coulson AF, Frere JM, Ghuysen JM, Joris B, Forsman M, Levesque RC, Tiraby G, Waley SG. 1991. A standard numbering scheme for the class A beta-lactamases. Biochem J 276:269–270. https://doi.org/10.1042/bj2760269.