A single *Proteus mirabilis* lineage from human and animal sources: a hidden reservoir of OXA-23 or OXA-58 carbapenemases in Enterobacterales

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In Enterobacterales, the most common carbapenemases are Ambler’s class A (KPC-like), class B (NDM-, VIM- or IMP-like) or class D (OXA-48-like) enzymes. This study describes the characterization of twenty-four OXA-23 or OXA-58 producing *Proteus mirabilis* isolates recovered from human and veterinary samples from France and Belgium. Twenty-two *P. mirabilis* isolates producing either OXA-23 (*n* = 21) or OXA-58 (*n* = 1), collected between 2013 and 2018, as well as 2 reference strains isolated in 1996 and 2015 were fully sequenced. Phylogenetic analysis revealed that 22 of the 24 isolates, including the isolate from 1996, belonged to a single lineage that has disseminated in humans and animals over a long period of time. The *bla*<sub>OXA-23</sub> gene was located on the chromosome and was part of a composite transposon, Tn6703, bracketed by two copies of IS15ΔII. Sequencing using Pacbio long read technology of OXA-23-producing *P. mirabilis* VAC allowed the assembly of a 55.5-kb structure encompassing the *bla*<sub>OXA-23</sub> gene in that isolate. By contrast to the *bla*<sub>OXA-23</sub> genes, the *bla*<sub>OXA-58</sub> gene of *P. mirabilis* CNR20130297 was identified on a 6-kb plasmid. The acquisition of the *bla*<sub>OXA-58</sub> gene on this plasmid involved XerC-XerD recombinases. Our results suggest that a major clone of OXA-23-producing *P. mirabilis* is circulating in France and Belgium since 1996.

*Proteus* spp. are Gram-negative rods and belong to the order of Enterobacterales and to the family of Morganellaceae. This genus is part of the natural gut microbiota in humans and animals. Six species compose this genus being *Proteus mirabilis, Proteus vulgaris, Proteus penneri, Proteus cibarius, Proteus terrae* and *Proteus hauseri*, and three genomospecies 4, 5, and 6¹². Among these species, *P. mirabilis* is the most commonly identified from clinical samples, mainly in context of urinary tract infections (UTIs) but also from a wide range of clinical samples related to healthcare associated infections². *P. mirabilis* does not produce any intrinsic β-lactamase. Accordingly, the wild-type resistance pattern is fully susceptible to all β-lactams active on Enterobacterales. Resistance to cephalosporins in *P. mirabilis* is caused by the acquisition of extended-spectrum β-lactamases (ESBLs) of CTX-M-, VEB- and PER-types or of plasmid-mediated cephalosporinas such as CMY-type³⁴⁶.

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observed on gentamicin and tobramycin, with few isolates being susceptible to these compounds whereas the broad-spectrum cephalosporins, fluoroquinolones, tigecycline, fosfomycin and amikacin. Few differences were summarized in Table S1. A similar pattern was observed for all isolates with antibiotic susceptibility pattern of clavulanate-amoxicillin resistance and decreased susceptibility to carbapenem. They were all susceptible to

\[ \text{ml or diameter inhibition zone size} < \text{0.125} \mu g/\text{ml or diameter inhibition zone size} < 25 \text{ nm}. \]

Resistance phenotypes of all OXA-23-producing \( P. \) mirabilis are summarized in Table S1. A similar pattern was observed for all isolates with a antibiotic susceptibility pattern of clavulanate-amoxicillin resistance and decreased susceptibility to carbapenem. They were all susceptible to broad-spectrum cephalosporins, fluoroquinolones, tigecycline, fosfomycin and amikacin. Few differences were observed on gentamicin and tobramycin, with few isolates being susceptible to these compounds whereas the others were resistant to both of them.

Resistome of OXA-23-/OXA-58-producing \( P. \) mirabilis isolates. WGS of all the OXA-23- and OXA-58-producing \( P. \) mirabilis isolates (\( n = 22 \)) of this study along with OXA-58-producing \( P. \) mirabilis 1091, and OXA-23-producing \( P. \) mirabilis S4 \cite{14,16} were performed using Illumina technology. Resistomes were determined using the Resfinder 3.1 and the CARD database \cite{17,18}. They are summarized in Table S2. The \( P. \) mirabilis VAC possessed the highest number of acquired resistance determinants. It carried multiple aminoglycoside resistance genes (two copies of aph(6′)-Id, three copies of aph(3′)-Ib, aac(3)-IV, aph(4)-Ia, aadA1, aadA14-like, two copies

| Isolates | Source, sample | Carbapenemase | City, Country (F/B) | Year of isolation | Reference | GenBank accession numbers |
|----------|----------------|----------------|---------------------|-------------------|-----------|--------------------------|
| 1091     | Human, blood   | OXA-58         | Yvoire, B           | 2015              | 10        | MCO90000000               |
| CNR20130297 | Human, N/A     | OXA-58         | Kortrijk, B         | 2013              | This study | SPTE00000000               |
| S4       | Human, N/A     | OXA-23         | Clermont-Ferrand, F | 1996              | 16        | SPTF00000000               |
| Cow-15-39117 | Cow, blood     | OXA-23         | Mauriac, F          | 2015              | This study | SPDD00000000               |
| Dog-06-37860 | Dog, otitis    | OXA-23         | Grasse, F           | 2014              | This study | SPTC00000000               |
| Dog-35-37761 | Dog, otitis    | OXA-23         | Lyon, F             | 2015              | This study | SPTB00000000               |
| L100     | Human, skin biopsy | OXA-23     | Limoges, F           | 2016              | This study | SPTA00000000               |
| L92      | Human, stool   | OXA-23         | Limoges, F           | 2016              | This study | SPSZ00000000               |
| CNR20160679 | Human, Respiratory | OXA-23     | Brussels, B         | 2016              | This study | SPSY00000000               |
| CNR20160877 | Human, Urine    | OXA-23         | Brussels, B         | 2016              | This study | SPSX00000000               |
| CNR20160617 | Human, Urine    | OXA-23         | Eeklo, B            | 2016              | This study | SPSW00000000               |
| GUI      | Human, Urine   | OXA-23         | Les Mureaux, F      | 2016              | This study | SPSV00000000               |
| VAC      | Human, Rectal swab | OXA-23     | Chartres, F         | 2016              | This study | CP042907                  |
| MOR      | Human, Urine   | OXA-23         | Montevrain, F       | 2016              | This study | SPST00000000               |
| BCT11    | Human, Urine   | OXA-23         | Le Kremlin-Bicetre, F | 2017            | This study | SPSS00000000               |
| BCT17    | Human, Urine   | OXA-23         | Le Kremlin-Bicetre, F | 2017            | This study | SPRS00000000               |
| 130B9    | Human, Urine   | OXA-23         | Sens, F             | 2017              | This study | SPTN00000000               |
| 160A10   | Human, Urine   | OXA-23         | Limoges, F           | 2018              | This study | SPTM00000000               |
| 168F7    | Human, Urine   | OXA-23         | Villenur sur Tarn, F | 2018            | This study | SPTL00000000               |
| 172C2    | Human, Blood   | OXA-23         | Tarbes, F           | 2018              | This study | SPTK00000000               |
| 172J1    | Human, Urine   | OXA-23         | Abbeville, F        | 2018              | This study | SPTJ00000000               |
| 175H8    | Human, Urine   | OXA-23         | Saint-Étienne, F   | 2018              | This study | SPTO00000000               |
| 188H6    | Human, Urine   | OXA-23         | Rouen, F            | 2018              | This study | SPTH00000000               |
| 189B4    | Human, N/A     | OXA-23         | Sanary/Mer, F       | 2018              | This study | SPTG00000000               |

Table 1. Clinical features of OXA-23 or OXA-58-producing \( P. \) mirabilis isolates. *F*: France, and *B*: Belgium. *N/A*: not available.
of *aph(3′)-Ia* and *aac(3′)-II*, the phenicol resistance gene *floR*, the lincosamide nucleotidyltransferase gene *lnuG*, the sulfonamide resistance gene *sul2*, a streptothricin acetyltransferase gene *sat2*, and the carbapenem resistance *bla*OXA-23 gene (Table S2). Accordingly, this strain was selected as reference for further analyses and sequenced using the PacBio technology. Sequencing gave 27,741 reads representing a total of 166,521,740 nucleotides. The genome of *P. mirabilis* VAC was reconstructed and was 4.08 Mb in size with a GC content of 39% (Fig. 2A).

**Phylogenetic analysis of *bla*OXA-containing *P. mirabilis* isolates.** To dive deeper into the understanding of the dissemination of the *bla*OXA-23 or *bla*OXA-58 genes, the genome sequences of all sequenced *P. mirabilis* isolates were compared. In addition, 122 available reference genomes of *P. mirabilis* from GenBank were also included in the analysis (Table S2). Surprisingly, 22 of the 24 CHDL-producing isolates, including the OXA-23-producing *P. mirabilis* S4 and the OXA-58-producing *P. mirabilis* 1091, belonged to the same lineage (Fig. 3). Single nucleotide polymorphisms (SNPs) count revealed that 22 isolates possessed the same background (less than 50 SNPs vs >2000 SNPs for unrelated clones) confirming that all these isolates belonged to the same lineage. Moreover, despite the fact that three isolates (NEYX, NJFA and LDIU) were branched to OXA-producing lineage, they are not related with an average of 4,200, 4,900 and 5,000 SNPs respectively with the OXA-23/OXA-58-producing isolates (Fig. 3 and Table S3). Two OXA-producing isolates (*P. mirabilis* 160A10 and CNR20130297) were not related to the main lineage (>2000 SNPs). Isolate 160A10 and SDUJ01 are close with 185 SNPs (Table S3) whereas isolate CNR20130297 was a singleton.

In addition to the carbapenemase-encoding gene (*bla*OXA-23 or *bla*OXA-58), all isolates from the main cluster carried acquired aminoglycoside and sulfonamide resistance genes (Table S2). The unrelated OXA-58-producing *P. mirabilis* CNR20130297 and OXA-23-producing *P. mirabilis* 160A10 displayed different resistance features. As opposed to the isolates of the main cluster, *P. mirabilis* CNR20130297 remained susceptible to all tested aminoglycosides (gentamicin, tobramycin, kanamycin, amikacin and netilmicin), and both isolates (CNR20130297 and 160A10) produced an additional β-lactamase TEM-1 (Table S2).

Of note, a chloramphenicol acetyltransferase gene (*cat*) and a tetracycline efflux pump encoding gene (*tet(J)*), both related to the intrinsic resistance to tetracyclines and chloramphenicol of *P. mirabilis* species were present in all genomes.

**The *bla*OXA-23 gene is carried by a transposon on the chromosome.** Attempts to transfer the *bla*OXA-23 carbapenemase gene from *P. mirabilis* VAC by conjugation and transformation failed. Genome analysis using *P. mirabilis* VAC as reference for the dominant OXA-23-producing clone (see above) confirmed that the *bla*OXA-23 gene was located on the chromosome. Comparative genomics between the *P. mirabilis* VAC isolate and the fully
Figure 2. (A) Comparative genomic of *P. mirabilis* VAC. Genome analysis of *P. mirabilis* VAC and its comparison with *P. mirabilis* S4 (1996), *P. mirabilis* BCT17 (2017) and *P. mirabilis* BB2000 reference genome (CP004022). Circular representation was obtained using CGViewer. Inner circles represent CG content (black circle) and CG Skew (green & purple circle). GI = Genomic Island (B) Schematic representation of IS15AIH-based composite transposon (Tn6703) and its insertion site. Red boxes represent resistance genes and orange boxes represent mobile elements. (C) Schematic representation of Tn7. Genes are indicated by arrows. Red arrows represent resistance genes and orange arrows represent mobile elements. (D) Analysis of the genetic context of *blaOXA-58* in *P. mirabilis* 1091 and 20130297 isolates. XerC-XerD binding sites are indicated by triangles. Dashed lines represent DNA insertions.
susceptible *P. mirabilis* BB2000 reference strain revealed the presence of genomic islands (GIs) only in the *P. mirabilis* VAC isolate (Fig. 2A). Here, GIs refer to large DNA sequences coming from an horizontal transfer and integrated in the chromosome. Among these GIs, GI1 corresponds to the Tn6703 transposon that carries the *bla*OXA-23 gene. GI3 shares 97% of nucleotide identity with an integrative and conjugative elements (ICE) ICEPmJpn1 identified in *P. mirabilis* (KY437729). GI4 another ICE identified in different *P. mirabilis* isolates as well as in *Klebsiella quasipneumoniae* strain KPC142 (CP023478), *Providencia stuartii* strain BE2467 (CP017054) and *Morganella morganii* strain AR_0133 (CP028956). GI5 is a copy of the class 2 transposon Tn7. Finally, GI6 contains a putative type VI secretion system encoding operon.

In *P. mirabilis* VAC, the *bla*OXA-23 gene is carried on GI1 of 55-kb in size. It is bracketed by two copies of IS15∆II, an IS26 point mutant variant belonging to the IS6 family. IS15∆II themselves are bracketed by a target site duplication (TSD) TAATTTCC (Fig. 2B), typical of IS15∆II (as well as IS26) transposition events. This composite transposon was named Tn6703 according to the transposon registry database (https://transposon.lstmed.ac.uk/). It has been previously demonstrated that at least 6 copies of *bla*OXA-58 gene were duplicated in tandem in *P. mirabilis* isolate, only one copy of the *bla*OXA-23 gene was present in all isolates of the main clone (ratio *bla*OXA-23/housekeeping genes at 1). Analysis of the close genetic structure of *bla*OXA-23 gene revealed that it was carried by a Tn2008-like transposon named Tn6704 (Fig. S1). Tn6704 was inserted in a fragment of Tn5393 within a non-coding region between the resolvase and strA genes. Noticeably, a plasmid replicase from *Acinetobacter* was identified within this Tn6704. However, this replicase encoding gene is interrupted by ISAba125 (bracketed by TSD of 3 bp, TAG). This Tn6704 is, itself, bracketed by TSD of 9-bp (GATGAAGCG) consistent in all transposons carrying *bla*OXA-23 genes. Downstream of the *bla*OXA-23 gene, an ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23. Following this ATPase-encoding gene was identified, as described in all transposons carrying *bla*OXA-23.
two aminoglycoside resistance genes (aph(6′)-Id and aph(3′)-Ib) and two genes involved in plasmid transfer (traA/traD). This structure was identified in all OXA-23-producing *P. mirabilis* of this study. Intriguingly, close to this region and only in *P. mirabilis* VAC, a fragment of Tn6260 carrying *lnuG* resistance gene originating from *Enterococcus faecalis* was identified. The *lnuG* gene was bracketed by two copies of ISCR2, an IS91-like mobile element. Ultimately, two copies of IS15AI bracketed this MDR GI with a TSD of 8-bp (TAATTTCGC) leading to a putative composite transposon. Of note, all isolates of this clone do not share the same resistome (Fig. 2A). The alignment of whole genome sequences revealed some differences in this region. This can be explained, for instance, by the presence of the transposon carrying *lnuG* only in *P. mirabilis* VAC. Accordingly, this *lnuG*-carrying transposon was most likely acquired recently. Some aminoglycoside resistance genes are also present in few isolates. The genetic diversity of Tn6703 is not surprising since studied isolates were recovered from different countries, over a long period and from animal or human. They were likely submitted to different selective pressures that might explain this diversity.

In *P. mirabilis* VAC and other isolates of the same clone, GI1 was inserted within the remnant (15 kb in size) of a prophage sharing 75% nucleotide identity with a prophage identified in *Provetia retgerri* RB151 (CP017671). *P. mirabilis* BB2000 reference strain (CP004022) also harboured this prophage, but neither Tn6703 nor any resistance genes were inserted in it (Fig. 2B). In the unrelated *P. mirabilis* 160A10, the *bla*<sub>OXA-23</sub> gene was also part of a Tn6703-like element. However, since 160A10 possessed an intact homolog of the phage Burkho_BcepB1A tail protein-encoding gene (GenBank NC005886). To decipher the genetic context of the carbapenemase gene in this isolate, *P. mirabilis* 160A10 was sequenced using MinIon technology. In this isolate, the *bla*<sub>OXA-23</sub> gene is carried by a conjugal plasmid of 67 kb in size (Fig. S2). This plasmid carried a full transfer operon and was not typeable using PlasmidFinder v2.1 for replicon typing of Enterobacteriales. The *bla*<sub>OXA-23</sub> gene was present within a fragment of Tn6703 carried by the plasmid (Fig. S2).

The other resistance genes (aadA1, sat2 and dfrA1) were identified within a class 2 integron carried by a Tn7 transposon (G1S) (Fig. 2C). This transposon has been identified in many isolates of *P. mirabilis*. As previously reported, the class 2 integrase gene contains a premature stop codon leading to a pseudo-gene (Fig. 2C).

### The *bla*<sub>OXA-58</sub> Gene Might Be Mobilized by XerC/XerD Recombination Events

Within *P. mirabilis* CNR20130297, the *bla*<sub>OXA-58</sub> gene is carried on a plasmid of 6,219 bp that shared 99% nucleotide identity (only one SNP), with plasmid p10797-OXA-58 (KU871396). This plasmid has been previously identified in an OXA-58-producing *P. mirabilis* from Germany.<sup>44</sup> The plasmid replicase showed 51% amino acid identity with a replicase of *Stenotrophomonas maltophilia* (GenBank accession number WP_029214130.1) and to a lesser extent with another replicase of *Acinetobacter baumannii* (50% amino acid identity) (GenBank accession number WP_005102557.1). Analysis of the closed genetic environment of the *bla*<sub>OXA-58</sub> gene revealed that XerC-XerD recombination was likely involved in its acquisition (Fig. 2D). The process of site-specific recombination can be performed by two chromosomally-encoded tyrosine recombinases (XerC and XerD). These recombinases recognize a 28-bp recombination site named *dly* and may allow resolution of the recombination event.<sup>29</sup> XerC and XerD recombination sites are composed of two sequences of 11 nucleotides separated by a spacer of 6 nucleotides<sup>29</sup>. In *P. mirabilis* 1091, the *bla*<sub>OXA-58</sub> gene was bracketed by two fragments of ISaba3, and a gene coding for a cephalosporinase as previously described<sup>31,43</sup>. Bracketing ISaba3-*bla*<sub>OXA-58</sub>-ISaba3, two XerC-XerD sites were identified named XerC3/XerD3 and XerC4/XerD4. Downstream of the *bla*<sub>OXA-58</sub>-emp gene, another site was identified called XerC5/XerD5. In *P. mirabilis* VAC, only XerC5/XerD5 is present and might be considered as an empty XerC-XerD binding site within a prophage (Fig. 2D). In *P. mirabilis* CNR20130297, harbouring the p20130297-OXA-58 plasmid, XerC1/XerD1 binding site is found at the 5′ end extremity of the structure whereas a XerC2-XerD2 binding site is present at the 3′ end extremity. Analysis of XerC-XerD sites suggests a mobilisation of this structure via XerC-XerD recombinases.

### Discussion

OXA-23 is the main carbapenemase identified in *Acinetobacter* species. The *bla*<sub>OXA-23</sub> gene is now widespread and even endemic in some areas.<sup>32</sup> However, this carbapenemase is very rarely identified in Enterobacteriales. Only a few CHDL, other than OXA-48-like carbapenemases, have been reported in Enterobacteriales and especially in *Proteus* spp.<sup>14–15</sup>

Here, we report the first genomic characterization of twenty-one OXA-23- and one OXA-58-producing *P. mirabilis* isolates from 2013 to 2018. Two reference OXA-producing *P. mirabilis* isolates were also sequenced: an OXA-23-producer isolated in France in 1996<sup>16</sup> and the OXA-58-producing *P. mirabilis* 1091 isolated in Yvoir, Belgium, in 2015<sup>11</sup>. This analysis revealed that one clone carrying *bla*<sub>OXA-23</sub> gene is circulating since 1996 and had spread over the last twenty years among humans and animals. Interestingly, the recently described OXA-58-producing *P. mirabilis* 1091 isolate<sup>11</sup> also belonged to this lineage (Fig. 3). The comparison with genomes recovered from GenBank revealed that this lineage is distantly related to others lineages except a branch represented by three isolates (NEYX02.1, LDIU01.1 and NJFA02.1). Nevertheless, despite being of the same lineage, these three isolates that do not carry any carbapenemase-encoding gene, are not part of this OXA-23/OXA-58-producing “successful” clone (4000 to 5000 SNPs) (Fig. 3 and Table S3).

The *bla*<sub>OXA-23</sub> gene is part of a Tn6703, which is embedded in a 55-kb DNA sequence bracketed by two copies of IS15AI, thus forming a composite transposon, named Tn6703. This transposon is bracketed by an 8-bp target site duplication compatible with an IS15AI-mediated transposition event<sup>21–23</sup>. It is unlikely that this structure was acquired in one step since the mapping of reads on G11 revealed variability of its content among different isolates. Of note, three resistance genes (*aadA1, sat2 and dfrA1*) were not present in Tn6703 transposon but carried by Tn7 (Fig. 2C). The class 2 integron, carrying these genes, does not seem to be functional anymore. Indeed, the int2 gene carried a premature stop codon leading to an incomplete integrase. Regarding the *bla*<sub>OXA-58</sub> gene, its acquisition involved a XerC-XerD tyrosine recombinases and it has been identified either on the chromosome or on a
plasmid. XerC-XerD tyrosine recombinases have been involved in the resolution of plasmid co-integrates carrying the bla\textsubscript{OXA-23} gene in A. baumannii\textsuperscript{33}. Interestingly, this plasmid was reported to replicate in Enterobacterales and in A. baumannii ATCC17978\textsuperscript{32}. Accordingly, we might hypothesize that this plasmid might be the shuttle vector between the Acinetobacter genus and P. mirabilis.

Comparative genomics also revealed the presence of other GIs in P. mirabilis VAC as compared to the P. mirabilis BB2000 reference strain. Among the identified GIs, an ICE sharing high homology with ICEPmJpn1 (KY437729) has been identified (GI3)\textsuperscript{34}. Interestingly thisICE was identified in only two isolates of the main lineage (Fig. 3A.). Several other GIs carrying potential virulence genes were identified in P. mirabilis VAC including Gf6 carrying a putative type VI secretion system encoding operon. The content of all genomic islands is indicated in Tables S4 and S5. Accordingly, we can speculate that these GIs might be involved in the spread of this clone. Investigations of these elements will be further conducted to decipher their potential role in the spread of this clone.

Here, we described the clonal relationship of OXA-producing P. mirabilis over a twenty-one-year period (1996-2017). The spread of the bla\textsubscript{OXA-23} gene is due to a single clone possessing a complex IS\textsubscript{2017}. The spread of this clone.

Investigations of these elements will be further conducted to decipher their potential role in the spread of this clone.

Material and methods
Strain collection and reference strains. P. mirabilis resistant to amoxicillin and amoxicillin-clavulanate sent to the French and Belgium National Reference Centres (NRC) for antibiotic resistance as well as isolates collected through the National Monitoring Network for Antimicrobial Resistance in Diseased Animals (Resapath; https://resapath.anses.fr) were screened for the presence of the bla\textsubscript{OXA-23} or bla\textsubscript{OXA-58} gene. Thus, a total of 61 P. mirabilis isolates (4 from the Belgium NRC; 54 from the French NRC and 3 from the Resapath) were collected with a phenotype compatible with the production of a CHDL (Table 1). A collection of 22 OXA-23- and 2 OXA-58-producing P. mirabilis were included in this study (Table 1 & Fig. 1). Three isolates were recovered from veterinary samples whereas the others were from human origin. All available reference genomes of P. mirabilis from GenBank at the date of November 1\textsuperscript{4} 2019 (n = 122) were used for phylogenetic or comparative genomic analyses.

Susceptibility testing and carbapenemase detection. Antimicrobial susceptibility testing was performed by the disc diffusion method on Mueller-Hinton (MH) agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to EUCAST guidelines (http://www.eucast.org). MICs were determined as recommended using Etest\textsuperscript{8} (bioMérieux, Marcy l’Etoile, France). Carbapenemase detection was performed using the Carba NP and CarbaR+ MCR (Mobidiag, Paris, France) PCR-based assays are the only commercially-available molecular tests targeting the big 5 carbapenemases (KPC, NDM, VIM, IMP, OXA-48-like), and the main CHDLs from A. baumannii (OXA-23, OXA-24/40, OXA-58, and the over-expressed chromosomally-encoded OXA-51-like β-lactamase associated with an upstream inserted ISA\textsubscript{ba1}). These kits are thus able to detect these carbapenemase producers\textsuperscript{35}. Recently, a novel assay either immunochromatographic test targeting OXA-23 in Acinetobacter spp., OXA-23 K-Set\textsuperscript{36} test (Coris BioConcept, Gembloux, Belgium), or molecular assays such as Amplidiag\textsuperscript{8} Carba-R + MCR’s that detects the major carbapenemases: KPC, NDM, VIM, IMP, and OXA-48, as well as the main OXA-type carbapenemases from Acinetobacter spp. have been demonstrated to accurately identify OXA-23-producing P. mirabilis isolates\textsuperscript{35,36}. The use of these assays might help to decipher the underestimated carriage of these OXA-23/58-producing P. mirabilis. However, the clinical impact and the need to set-up hygiene measures around these OXA-23/58-producing P. mirabilis need to be evaluated since these isolates remain multi-susceptible to most antimicrobials including carbapenems.

DNA extraction, PCR, and sequencing. Total DNA for Illumina’s sequencing and conventional PCR was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Ozyme, Saint-Quentin, France) following manufacturer’s instructions. DNA concentration and purity assessments were determined using a Qubit\textsuperscript{8} 2.0 Fluorometer using the dsDNA HS and/or BR assay kit and Nanodrop 2000 (Thermofisher, Saint-Herblain, France). Conventional PCRs were performed as previously described\textsuperscript{39}. Main acquired-carbapenemase encoding genes (bla\textsubscript{NDM}, bla\textsubscript{OXA-24}, bla\textsubscript{OXA-58}, bla\textsubscript{KPC}, bla\textsubscript{OXA-23}, bla\textsubscript{OXA-24/40}, bla\textsubscript{OXA-58}) in Enterobacterales and Acinetobacter spp. were sought by PCR using primers as previously described\textsuperscript{10,31}. The DNA library was prepared using the Nextera XT-v2 kit (Illumina, Paris, France) and then run on NextSeq. 500 automated system (Illumina), using a 2 × 100-bp paired-end approach. P. mirabilis VAC DNA was sequenced using PacBio’s technology (www.macrogen.com) and used as reference genome. P. mirabilis 160A10 was sequenced using MinIon technology as previously described\textsuperscript{40}.

Bioinformatic analysis. De novo assembly and read mappings were performed using CLC Genomics Workbench v10.1 (Qiagen, Les Ulis, France). The acquired antimicrobial resistance genes were identified using Resfinder server v3.1 (https://cge.cbs.dtu.dk/services/ResFinder/) and CARD database (https://card.mcmaster.ca)\textsuperscript{17,44}. The genome was annotated using the RAST server\textsuperscript{41}. Detection of phage was performed using the PHASTER server (www.phaster.ca)\textsuperscript{42}. Genomic Island were detected using Island Viewer 4 (http://www.pathogenomics.sfu.ca/islandviewer/). Phylogenetic analysis was performed using CIPhylogeny v1.4\textsuperscript{43}. The parameters used were as follows: minimum distance between SNPs at 10 bp, minimum Z-score at 1.96, and minimum depth at 10X with a relative depth at 10% per position.
The copy number of blaOXA-23 was assessed to identify a potential gene duplication event as observed for blaOXA-58. The gene copy number was calculated using the ratio of the coverage of the blaOXA-23 gene and that of distantly located single copy chromosomal genes (rpoB, dnaA and mdh). Insertion sequences were identified using the ISfinder database44.

**Transfer of β-lactam resistance determinants.** Plasmids were extracted using Kieser’s method as previously described45. Plasmids were extracted using Kieser’s method and subsequently analysed by electrophoresis on a 0.7% agarose gel as previously described45, and attempted to be introduced by electroporation into E. coli TOP10. Recombinant E. coli were selected on TSA supplemented with 50 µg/ml of amoxicillin as previously described49. Conjugation assays using P mirabilis isolates as donors and E. coli J53 as recipient strains were performed as previously described46.

**Ethic statements.** No animal or human experiments were performed in this study. All the human isolates were sent anonymously to the NRCs, and none of the authors had access to any identifying information along with the isolates, and that thus ethical approvals and informed consents were not needed.

**Nucleotide sequence accession number.** The whole genome sequences generated in the study have been submitted to the GenBank nucleotide sequence database under the accession numbers detailed in Table 1. The nucleotide sequence of the 6-kb plasmid carrying blaOXA-58 in P mirabilis CNR20130297 was submitted to the GenBank nucleotide sequence database under the accession number MK533136. The genomes of OXA-23- or OXA-58-producing P mirabilis were submitted to GenBank (bioproject number PRJNA521327).

**Transparency declarations:** L.D. is co-inventor of the Carba NP Test, which patent has been licensed to bioMérieux (La Balme les Grottes, France).

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**References**

1. O’Hara, C. M., Brenner, F. W. & Miller, J. M. Classification, identification, and clinical significance of Proteus. Providencia, and Morganella. *Clin. Microbiol. Rev.* 13, 534–546 (2000).

2. Frontiers | Genetics of Acquired Antibiotic Resistance Genes in *Proteus* spp. | Microbiology, https://www.frontiersin.org/articles/10.3389/fmicb.2020.00256/full.

3. Schaffer, J. N. & Pearson, M. M. Proteus mirabilis and Urinary Tract Infections. *Microbiol. Spectr.* 3 (2015).

4. Decré, D. *et al.* Characterization of CMY-type β-lactamases in clinical strains of Proteus mirabilis and Klebsiella pneumoniae isolated in four hospitals in the Paris area. *J. Antimicrob. Chemother.* 50, 681–688 (2002).

5. Schultz, E. *et al.* Survey of multidrug resistance integrative mobilizable elements SGI1 and PGI1 in Proteus mirabilis in humans and dogs in France, 2010-13. *J. Antimicrob. Chemother.* 70, 2543–2546 (2015).

6. Nakama, R. *et al.* Current status of extended spectrum β-lactamase-producing *Escherichia coli*, Klebsiella pneumoniae and Proteus mirabilis in Okinawa prefecture, Japan. *J. Infect. Chemother.* 22, 281–286 (2016).

7. Valentín, T. *et al.* Proteus mirabilis harboring carbapenemase NDM-5 and ESBL VEB-6 detected in Austria. *Diagn. Microbiol. Infect. Dis.* 91, 284–288 (2018).

8. Tibbetts, R., Frye, J. G., Marschall, J., Warren, D. & Dunne, W. Detection of KPC-2 in a clinical isolate of *Enterobacter cloacae*. *Antimicrob. Agents Chemother.* 50, 3080–3083 (2006).

9. Markovska, R. *et al.* Dissemination of a Multidrug-Resistant VIM-1- and CMY-99-Producing Proteus mirabilis Clone in Bulgaria. *Microb. Drug Resist. Larchmt.* N 23, 345–350 (2017).

10. Bonnin, R. A., Nordmann, P. & Poirel, L. Screening and deciphering antibiotic resistance in *Acinetobacter baumannii*: a state of the art. *Expert Rev. Anti Infect. Ther.* 11, 571–583 (2013).

11. Girlich, D. *et al.* Chromosomal amplification of the blaOXA-58 carbapenemase gene in a Proteus mirabilis clinical isolate. *Antimicrob. Agents Chemother.* https://doi.org/10.1128/AAC.01697-16 (2016).

12. Lange, F. *et al.* Dissemination of blaOXA-58 in Proteus mirabilis isolates from Germany. *J. Antimicrob. Chemother.* 72, 1334–1339 (2017).

13. Leulmi, Z. *et al.* First report of blaOXA-23 Carbapenemase-encoding gene, *armA* Methyltransferase and *aac(6’)-Ib-cr* producing multidrug-resistant clinical isolates of *Proteus mirabilis* in Algeria. *J. Glob. Antimicrob. Resist.* https://doi.org/10.1016/j.jgar.2018.08.019 (2018).

14. Bonnet, R. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. *Antimicrob. Agents Chemother.* 48, 1–14 (2004).

15. Osterblad, M. *et al.* Rare Detection of the *Acinetobacter* Class D Carbapenemase *bla*OXA-23 Gene in Proteus mirabilis. *Antimicrob. Agents Chemother.* 60, 3243–3245 (2016).

16. Bonnet, R. *et al.* Chromosome-encoded class D β-lactamase OXA-23 in *Proteus mirabilis*. *Antimicrob. Agents Chemother.* 46, 2004–2006 (2002).

17. Jia, B. *et al.* CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res.* 45, D566–D573 (2017).

18. Zankari, E. *et al.* Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644 (2012).

19. Juhás, M. *et al.* Genomic islands: tools of bacterial horizontal gene transfer and evolution. *Fems Microbiol. Rev.* 33, 376–393 (2009).

20. Trieu-Cuot, P. & Courvalin, P. Nucleotide sequence of the transposable element IS15. *Gene* 30, 113–120 (1984).

21. Mollet, B., Ida, S. & Arber, W. Gene organization and target specificity of the prokaryotic mobile genetic element IS26. *Mol. Gen. Genet.* 201, 198–203 (1985).

22. Trieu-Cuot, P., Labigne-Roussel, A. & Courvalin, P. An IS15 insertion generates an eight-base-pair duplication of the target DNA. *Gene* 24, 125–129 (1983).

23. Hammer, C. J., Moran, R. A. & Hall, R. M. Movement of IS26-associated antibiotic resistance genes occurs via a translocatable unit that includes a single IS26 and preferentially inserts adjacent to another IS26. *mBio* 5, e01801–01814 (2014).

24. Wang, X., Zong, Z. & Lu, X. In 2008 is a major vehicle carrying *bla*OXA-23 in *Acinetobacter baumannii* from China. *Diagn. Microbiol. Infect. Dis.* 69, 218–222 (2011).

25. Muguinier, P. D., Poirel, L. & Nordmann, P. Functional analysis of insertion sequence ISAbA1, responsible for genomic plasticity of *Acinetobacter baumannii*. *J. Bacteriol.* 191, 2414–2418 (2009).
26. Zhu, X.-Q. et al. Novel lau(G) gene conferring resistance to lincomycin by nucletidyl tylation, located on Tn6260 from Enterococcus faecalis E531. J. Antimicrob. Chemother. 74, 1560–1562 (2019).
27. Mendes Moreira, A. et al. Proteae: a reservoir of class 2 integrons? J. Antimicrob. Chemother. 72, 993–997 (2017).
28. Márquez, C. et al. Recovery of a Functional Class 2 Integron from an Escherichia coli Strain Mediating a Urinary Tract Infection. Antimicrob. Agents Chemother. 52, 4133–4134 (2008).
29. Castillo, F., BennMohamed, A. & Statmari, G. Xer Site Specific Recombination: Double and Single Recombinease Systems. Front. Microbiol 8, 453 (2017).
30. Carnoy, C. & Roten, C.-A. The dif/Xer recombination systems in proteobacteria. PLoS One 4, e6531 (2009).
31. Poirel, L. et al. OXA-58, a novel class D β-lactamase involved in resistance to carbapenems in Acinetobacter baumannii. Antimicrob. Agents Chemother. 49, 202–208 (2005).
32. Peleg, A. Y., Seifert, H. & Paterson, D. L. Acinetobacter baumannii: emergence of a successful pathogen. Clin. Microbiol. Rev. 21, 538–582 (2008).
33. Cameranesi, M. M., Morán-Barrio, I., Limansky, A. S., Repizo, G. D. & Viale, A. M. Site-Specific Recombination at XerC/D Sites Mediates the Formation and Resolution of Plasmid Co-integrates Carrying a blα-carB and TaqPha6-Resistance Module in Acinetobacter baumannii. Front. Microbiol 9, 66 (2018).
34. Lei, C.-W. et al. Characterization of SXT/K91 Integrative and Conjugative Elements in Proteus mirabilis Isolates from Food-Producing Animals in China. Antimicrob. Agents Chemother. 60, 1935–1938 (2016).
35. Girlich, D. et al. Evaluation of the Amplidiag CarbaR+MCR Kit for Accurate Detection of Carbapenemase-Producing and Colistin-Resistant Bacteria. J. Clin. Microbiol. 57 (2019).
36. Riccobono, E. et al. Evaluation of the OXA-23 K-Set® immunochromatographic assay for the rapid detection of OXA-23-like carbapenemase-producing Acinetobacter spp. J. Antimicrob. Chemother., https://doi.org/10.1093/jac/dka4801 (2019).
37. Dortet, L., Brechard, L., Poirel, L. & Nordmann, P. Impact of the isolation medium for detection of carbapenemase-producing Enterobacteriaceae using an updated version of the Carba NP test. J. Med. Microbiol 63, 772–776 (2014).
38. Boutal, H. et al. A multiplex lateral flow immunoassay for the rapid identification of NDM-, KPC-, IMP- and VIM-type and OXA-48-like carbapenemase-producing Enterobacteriaceae. J. Antimicrob. Chemother 73, 909–915 (2018).
39. Bonnin, R. A. et al. Carbapenem-hydrolyzing GES-type extended-spectrum beta-lactamase in Acinetobacter baumannii. Antimicrob. Agents Chemother. 55, 349–354 (2011).
40. Bonnin, R. A. et al. First occurrence of the OXA-198 carbapenemase in Enterobacteriales. Antimicrob. Agents Chemother., https://doi.org/10.1128/AAC.01471-19 (2020).
41. Aziz, R. K. et al. SEED servers: high-performance access to the SEED genomes, annotations, and metabolic models. PloS One 7, e48053 (2012).
42. Arnott, D. et al. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res 44, W16–21 (2016).
43. Kaas, R. S., Leekitcharoensophon, P., Aarestrup, F. M. & Lund, O. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. PloS One 9, e104984 (2014).
44. Siguer, P., Porechaon, J., Lestrede, L., Mahillon, J. & Chandler, M. ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res 34, D52–36 (2006).
45. Kieser, T. Factors affecting the isolation of CCC DNA from Streptomyces livids and Escherichia coli. Plasmid 12, 19–36 (1984).
46. Pottou, A., Poirel, L. & Nordmann, P. Plasmid-mediated transfer of the blα-carB-1 gene in Gram-negative rods. FEMS Microbiol. Lett. 324, 111–116 (2011).

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Author contributions
R.A.B., P.G., L.D. and T.N. conceived and designed the study; R.A.B., D.G., L.G. performed the experiments; R.A.B., D.G., A.B.J., L.G., G.C., P.G., L.D. analyzed the data. P.B., M.H., J.Y.M., E.C.D., O.B., N.F. provided strains. R.A.B., P.G., L.D. and T.N. conceived and designed the study; R.A.B., D.G., L.G. performed the experiments; R.A.B., P.G., L.D. and T.N. wrote the paper; all authors revised and approved the manuscript.

Competing interests
The authors declare no competing interests.

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