Mobile Insertion Cassette Elements Found in Small Non-Transmissible Plasmids in Proteae May Explain qnrD Mobilization

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Abstract

qnrD is a plasmid mediated quinolone resistance gene from unknown origin, recently described in Enterobacteriaceae. It encodes a pentapeptide repeat protein 36–60% different from the other Qnr (A, B, C, S and V). Since most qnrD-positive strains were described as strains belonging to Proteus or Providencia genera, we hypothesized that qnrD originated in Proteae before disseminating to other enterobacterial species. We screened 317 strains of Proteae for qnrD and its genetic support by PCR. For all the seven qnrD-positive strains (4 Proteus mirabilis, 1 Proteus vulgaris and 2 Providencia rettgeri) the gene was carried onto a small non-transmissible plasmid, contrarily to other qnr genes that are usually carried onto large multi-resistant plasmids. Nucleotide sequences of the qnrD-bearing plasmids were 96% identical. Plasmids contained 3 ORFs apart from qnrD and belonged to an undescribed incompatibility group. Only one plasmid, in P. vulgaris, was slightly different with a 1,568 bp insertion between qnrD and its promoter, leading to absence of quinolone resistance. We sought for similar plasmids in 15 reference strains of Proteae, but which were tested negative for qnrD, and found a 48% identical plasmid (pVERM) in Providencia vermicola. In order to explain how qnrD could have been inserted into such native plasmid, we sought for gene mobilization structures. qnrD was found to be located within a mobile insertion cassette (mic) element which sequences are similar to one mic also found in pVERM. Our conclusions are that (i) the small non-transmissible qnrD-plasmids described here may result from the recombinant between an as-yet-unknown progenitor of qnrD and pVERM, (ii) these plasmids are maintained in Proteae being a qnrD reservoir (iii) the mic element may explain qnrD mobilization from non-transmissible plasmids to mobilizable or conjugative plasmids from other Enterobacteriaceae, (iv) they can recombined with larger multiresistant plasmids conjugated in Proteae.

Introduction

Quinolones inhibit replication and transcription by inhibiting the bacterial type II topoisomerases, DNA gyrase and topoiso-merase IV [1]. Quinolones attach to the DNA-topoisomerase complex, which becomes irreversible, leading to immobilization of the enzymes resulting in bacteriostasis and to the release of DNA double-strand breaks that activate the SOS system and produce the “poison” effect responsible for the intense bactericidal action of quinolones [2]. Fluoroquinolones (the main subgroup of quinolones) are currently among the most heavily prescribed antimicrobials in the world because of their pharmacodynamic and pharmacokinetic properties [1]. They are very potent, especially for treatment of urinary tract infections due to Enterobacteriaceae [1,2], and as a consequence of their intense use, quinolone resistance rate has increased much for the last years [1,3,4]. Quinolone resistance mechanisms are multiple such as those reducing permeability of the bacterial wall, increasing efflux, reducing target affinity, producing inactivating enzymes and target protection proteins. Clinical resistance mostly results from the combination of several mechanisms [1]. Most of these mechanisms are chromosome-mediated [1,2] but plasmid-mediated genes have been described for a decade [3]. qnr genes were the first plasmid-mediated quinolone resistance genes described in 1998 [6]. Qnr proteins are pentapeptide repeat proteins that protect DNA gyrase and topoisomerase IV from quinolone binding. Six families of plasmid-mediated qnr gene were described: qnrA, qnrB, qnrC, qnrD and qnrS [7–10]. Several alleles have been reported for each gene: 7 alleles of qnrA, 73 of qnrB, 1 of qnrC, 2 of qnrD, 9 of qnrS and 6 qnrVC. Alleles are numbered consecutively in a qnr library (http://lahey.org/qnrStudie, last update December 08, 2015). Most of qnr genes were detected in Enterobacteriaceae, where they located on large conjugative multi-resistance plasmids, such as pMG252 described in the original Klebsiella pneumoniae qnrA1-
positive isolate [6]. The qnrB gene is the most prevalent in clinical bacterial strains, whereas qnrA gene is prevalent in environmental strains. Since qnrA alleles were found also as chromosome-borne in Shewanella spp. [11], this species was proposed to be a qnrA progenitor. Similarly, a qnrB allele was found in the chromosome of Citrobacter spp. [12], a qnrS and qnrVC allele in Vibrionaceae [10,19]. qnr genetic environment usually showed mobile elements that can explain their spread from their progenitor, although they confer only a low level resistance to quinolones. Most of qnrA and qnrB genes were reported on large conjugative multi-drug resistant plasmids including aminoglycoside and beta-lactam resistance genes such as those encoding extended spectrum beta-lactamases, and in the vicinity of complex type 1 integrons [14,15]. On the contrary, qnrS and qnrD genes were described on small non-conjugative plasmids, that may harbor mob genes allowing their mobilization [16,17].

qnrD was first reported on a 4,270-bp (p2007057) plasmid in four Salmonella enterica isolates [7]. We subsequently described two Providencia retgeri clinical isolates carrying qnrD on a 2,683-bp plasmid [18]. We hypothesized that qnrD can be originated from Proteae or present in these bacteria as a reservoir. Our study determined how much qnrD is prevalent in recent clinical isolates of Proteae, and investigated how qnrD could have disseminated to other bacterial species. We found seven nearly identical small qnrD-bearing plasmids, where qnrD is surrounded by sequences of mobile insertion cassette (mic) that may explain qnrD dissemination. Since the half part of the plasmid sequence excluding qnrD, was found in a native plasmid of Providencia vermicola, we think that qnrD might be present on the chromosome of a close, as yet unknown, Proteae species and that its DNA recombination with P. vermicola native plasmid led to the small non-transmissible qnr-positive plasmid found in clinical isolates.

Materials and Methods

qnrD Detection in Proteae Clinical Isolates

A total of 317 clinical isolates including 190 Proteus mirabilis, 59 Morganella morgani, 33 Proteus vulgaris, 20 Providencia stuartia, 14 Providencia retgeri and 1 Proteus penner were screened. They were isolated between 2008 and 2011 at five University hospitals (CHU Dijon, CHU Lariboisière Paris, CHU Nancy, CHU Pitié-Salpêtrière Paris, CHU Reims) from clinical specimens regardless antibiotic susceptibility. Fifteen reference strains of the tribe of Proteae were purchased at the Collection de l’Institut Pasteur (CIP) (n = 13) and at the Leibniz-Institut DSMZ-German Collection of Microorganisms and Cells Cultures (Braunschweig, Germany) (n = 2) (Table 1).

Detection of qnr genes was performed by multiplex real time PCR as previously described [19]. Briefly, after DNA extraction using the NucliSens easyMAG (bioMérieux, Marcy l’Etoile, France), multiplex real-time PCR was carried out using LightCycler® 480 (LC480) with HRM Supermix (Roche Molecular Diagnostics, Germany). The melt-curve analysis showed characteristic curves for each qnr gene family. All strains detected were confirmed to be qnr-positives by conventional PCR-sequencing.

The qnrD-positive isolates were compared for genome fingerprinting by Random Amplification of Polymorphic DNA (RAPD) using the Ready to Go RAPD analysis kit according to the manufacturer’s instructions (Amersham Biosciences).

Characterization of qnrD-bearing Plasmids

Plasmid extraction was performed by the Kieser method from the qnrD-positive strains [20]. Plasmids harboring qnrD were sequenced on both strands using a Walk DS strategy [18]. Nucleotide sequences were aligned using the program BioEdit version 7.0.9.0 and BLAST searches using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) and the blastn algorithms. Putative promoters, ribosome binding site, and transcription start site were searched using BPROM (http://linux1.softberry.com) and Promoter Prediction by Neural Network [21].

Incompatibility groups of the plasmids were determined using the PCR-based replicon typing (PBRT) as described elsewhere [22]. Briefly, four multiplex PCR were used for the detection of A/C, T, FIIAs, W, N, FB, L/M, II-1γ, X, H2, FIA, and Y replicons. Replicons P, R, U, F, FIC, and K were detected by simple PCR, as previously described, and replicons FII1K, FII2K, NewXXX or also named ZK, LVPK and Amet (Table S1) as described by D. Decré and G. Arlet. The primer design was based on pGSH500 and pKNP4 (AJ009980 and CP000649 respectively), pLVPK (AY378100) and pK245 (DQ449578), and pMET-1 (EU383016) sequences.

Search for qnrD and Small Non-transmissible Plasmids in Reference Strains of Proteae

Plasmid extraction was performed by the Kieser method from the 15 reference strains and from 50 qnrD-negative strains randomly selected [20]. Based on the nucleotide sequences of the qnrD-plasmids characterized in the clinical isolates, six pairs of primers were designed corresponding to overlapping fragments (Table S1). Amplification products were visualized on 2% agar gel after electrophoresis and sequenced.

Investigation of qnrD Transfer

Conjugation experiments were carried out using liquid and filter mating methods. Liquid mating experiments were performed in brain–heart infusion (BHI) broth with E. coli J53 azide-resistant [15], P. mirabilis ATCC 29906 RifR® [23] and E. coli C600 RifR® [23] as recipient strains. Donor and recipient cells in the logarithmic phase of growth were mixed (1 ml each) and incubated at 37°C for 3 hours without shaking. For filter conjugation, cultures (logarithmic phase of growth) of the donor and the different recipient cells were collected on a MF-type filter (Millipore, Molsheim, France). The hydrophobic edge membrane was incubated at 37°C on the surface of a BHI agar plate overnight. Transconjugants were selected on BHI agar plates containing sodium azide (100 μg/ml), rifampin (250 μg/ml), with regard to the recipient strain, and nalidixic acid (50 μg/ml) or ciprofloxacin (0.015 μg/ml, 0.03 μg/ml, 0.06 μg/ml and 0.12 μg/ml) or ofloxacin (0.03 μg/ml, 0.06 μg/ml, 0.12 μg/ml and 0.25 μg/ml).

The main capture systems (intI1, ISCR1, IS26, IS10, and ISEcp1) that have been associated with plasmid-mediated quinolone resistance determinants were detected using specific PCR (Table S1). In silico analysis was conducted for searching insertion sequence (IS) using IS Finder (http://www-is.biotoul.fr/is.html) and inverted repeats using REPFIND [24].

Quinolone Resistance Testing

Since the qnrD-positive clinical isolates may have additional mechanisms conferring quinolone resistance, including chromosomal mutations, we determined the quinolone resistance of both the original Proteae strains and the transformants obtained after transfer of qnrD-bearing plasmids into competent E. coli DH10B cells (Invitrogen, Cergy-Pontoise, France). Plasmid DNA was extracted from the qnrD-positive strains using the QIAamp DNA mini Kit (Qiagen, Courtaboeuf, France) and introduced by electroporation (Gene Pulser, Biorad, Marne la Coquette, France).
Table 1. Reference strains of Proteaeae harbored native plasmids but no qnr genes.

| Organism                  | Strain collection designation | Plasmid content* |
|---------------------------|-------------------------------|------------------|
| Proteus mirabilis         | CIP 103181, ATCC 29906        | No plasmid       |
| Proteus hauseri           | CIP 106868, ATCC 700826       | No plasmid       |
| Proteus mputiens           | CIP 106872, ATCC 19692        | No plasmid       |
| Proteus penneri           | CIP 103030, ATCC 33519        | No plasmid       |
| Proteus vulgaris           | CIP 104989, ATCC 29905        | No plasmid       |
| Providencia alcalifaciens | CIP 82.90, ATCC 9886          | 14 kb (pPAC1, [35]) |
| Providencia rettgeri       | CIP 103182, ATCC 29944        | 5.5 kb (pPRET, [35]) |
| Providencia rustigianii    | CIP 103032                    | 150 Kb, 12 Kb    |
| Providencia stuartii       | CIP 104687, ATCC 29914        | No plasmid       |
| Providencia vermica       | CIP 108829, DSM 17385         | 3.6 kb (pVerm)   |
| Providencia heimbachae    | CIP 103031, ATCC 35613        | No plasmid       |
| Providencia burhodogranaria | DSM 19968, ATCC-BA1590      | –                |
| Providencia sneebia       | DSM 19967, ATCC-BA1589       | 10.8 Kb (pPSN1), 7.6 Kb (pPSN2), 4.3 Kb (pPSN3) [35] |
| Morganella morganii subsp. morganii | CIP A231, ATCC 25830 | No plasmid       |
| Morganella morganii subsp. siboni       | CIP 103648, ATCC 49948       | No plasmid       |

* Determined by agarose gel electrophoresis of plasmids extracted from the reference strains of Proteaeae using the Kieser method. Control plasmids for size were pIP55 (130 kb), pIP135 (70.4 kb) and pGH509-09 (2.6 kb); –, not done.

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France). Transformants were selected on BHI agar plates containing ciprofloxacin (0.015 g/ml, 0.03 g/ml, 0.06 g/ml and 0.12 g/ml) or ofloxacin (0.03 g/ml, 0.06 g/ml, 0.12 g/ml, and 0.25 g/ml). In order to distinguish transformants and quinolone resistant mutants growing on quinolone containing media, we sequenced the QRDRs in gyrA and parC, in addition to qnrD detection by PCR.

Minimal inhibitory concentrations (MICs) of nalidixic acid, ciprofloxacin, levofloxacin, moxifloxacin, norfloxacin, and ofloxacin were determined by the agar dilution method using Mueller–Hinton agar plates containing serially twofold-diluted antibiotics. Plates were inoculated with a Steers-type multiprong device with ca. 10^4 CFU per spot, and were read after incubation for 18 hours at 37°C.

Results

Small Non-transmissible Plasmids are the Genetic Support of qnrD in Proteaeae Isolates

qnr gene screening of the 317 clinical isolates resulted in eight (2.6%) qnr-positive strains with seven strains (27.5% of qnr-positive isolates) carrying qnrD: four P. mirabilis (DPROT11, DPROT104, DPROT189, and DPROT304), two P. rettgeri (DIJ09–518a and GHS09–09) and one P. vulgaris (DPROT78). The remaining qnrA-positive isolate was a P. mirabilis isolate harboring qnrA1 (DPROT289). The seven qnrD genes were 100% identical to the original gene described [7]. On the basis of epidemiological findings (Table 2) and RAPD profiles (data not shown), the isolates belonging to the same species (4 P. mirabilis, 2 P. rettgeri) were distinct strains.

Conjugation assays, performed extensively, failed in transferring qnrD from clinical isolates to any of the recipient strains. From the seven qnrD-positive isolates, DNA extraction by the Kieser method showed several plasmids but a similar small plasmid of ca. 2.6 Kb in six isolates and one ca. 4 Kb in the remaining isolate. qnrD-plasmids were negative for known incompatibility groups.

Purified qnrD-plasmids were successfully transferred by electroporation into E. coli DH10B. Plasmid sequencing showed that six of the seven plasmids were of ca. 2.6-Kb long: pDIJ09–518a, pGHS09–09, pRS12–11, pRS12–104, pRS12–189, and pLRB12–304 being 2,683-bp, 2,683-bp, 2,863-bp, 2,683-bp, 2,656-bp and 2,638-bp long, respectively. They were carried, respectively, by two P. rettgeri clinical isolates previously described [18] and 4 clinical strains of P. mirabilis. The remaining plasmid (pRS12–78) was 2,496-bp long and was carried by a P. vulgaris isolate.

Quinolone MICs are presented in Table 3. Quinolone resistance conferred by the qnrD-bearing plasmids was similar for all plasmids, with a 60-fold increase in ciprofloxacin MIC. For the plasmid pRS12–78, transformation experiments on ofloxacin or ciprofloxacin, even at low concentration, selected S83L gyrA chromosomal mutants harboring pRS12–78. Consequently we cannot measure the exact level of resistance conferred by the plasmid. However, since the ciprofloxacin MIC was similar to that on a gyrA mutant [25], we concluded that the plasmid did not confer quinolone resistance, which is explained by the insertion between the putative promoter and the qnrD start codon (see below). No mutation in the QRDR of gyrA and parC were found in the other transformants.
1,568 bp insert between qnrD and its promoter (Figure 1A). BLAST comparison highlighted a putative ORF5 exhibiting 76% identity with a hypothetical protein (GenBank accession number

| Strains      | Hospital             | Ward        | Date of isolation | Specimen |
|--------------|----------------------|-------------|-------------------|----------|
| P. mirabilis DPROT 11 | CHU Reims           | Nephrology  | 11/10/2010        | Urine    |
| P. mirabilis DPROT 104 | CHU Reims           | Diabetology | 04/09/2010        | Sputum   |
| P. mirabilis DPROT 189 | CHU Reims           | Nephrology  | 02/18/2011        | Urine    |
| P. mirabilis DPROT 304 | CHU Lariboisière-Paris | Long term care | 02/02/2010 | Urine    |
| P. vulgaris DPROT 78 | CHU Reims           | Long-term care | 12/12/2010      | Urine    |
| P. rettgeri DJU09–518a | CHU Dijon          | Hematology  | 12/08/2008        | Stool    |
| P. rettgeri GHS09-09 | CHU Pitié-Salpêtrière-Paris | Emergency room | 10/09/2009 | Urine    |

Table 2. Epidemiological findings of the 7 qnrD-positive strains detected among 317 clinical isolates of Proteae.

Table 3. Resistance conferred by qnrD-bearing plasmids as measured by determination of minimal inhibitory concentrations (MIC) of quinolones on transformants with regard to parental strains of Proteae.

Table 3. Resistance conferred by qnrD-bearing plasmids as measured by determination of minimal inhibitory concentrations (MIC) of quinolones on transformants with regard to parental strains of Proteae.

| Strains      | MIC (µg/ml) |
|--------------|-------------|
|              | NAL | NOR | OFX | CIP | MXF | LVX |
| P. mirabilis DPROT 11 | >256 | 12  | >32 | >32 | >32 | 16  |
| P. mirabilis DPROT 104 | 8   | 0.5 | 1   | 0.25 | 1   | 0.25 |
| P. mirabilis DPROT 189 | >256 | 12  | >32 | >32 | >32 | 16  |
| P. mirabilis DPROT 304 | >256 | 8   | >32 | >32 | >32 | 16  |
| P. rettgeri GHS09-09 | 4   | 0.25 | 0.5 | 0.125 | 0.5 | 0.25 |
| P. rettgeri DJU09–518 | >256 | 8   | 8   | 4   | 12  | 4   |
| P. vulgaris DPROT 78 | >256 | 8   | 8   | 4   | >32 | 4   |
| E. coli DH10B | 1 | 0.03 | 0.015 | 0.006 | 0.03 | 0.006 |
| E. coli DH10B/ pRS12–11 | 4 | 0.5 | 0.25 | 0.125 | 0.125 | 0.125 |
| E. coli DH10B/ pRS12–104 | 4 | 0.5 | 0.25 | 0.125 | 0.125 | 0.125 |
| E. coli DH10B/ pRS12–189 | 2 | 0.5 | 0.25 | 0.125 | 0.125 | 0.125 |
| E. coli DH10B/ pLRB12–304 | 4 | 0.5 | 0.25 | 0.125 | 0.125 | 0.125 |
| E. coli DH10B/ pGHS09-09 | 2 | 0.5 | 0.25 | 0.125 | 0.125 | 0.125 |
| E. coli DH10B/ pDJU09–518a | 2 | 0.5 | 0.25 | 0.125 | 0.125 | 0.125 |
| E. coli DH10B/ pRS78 | 16 | 0.125 | 0.125 | 0.03 | 0.06 | 0.006 |
| E. coli DH10B/ p2007057a | 2 | 0.125 | 0.25 | 0.125 | ND | ND |

**NAL**, Nalidixic acid; **NOR**, Norfloxacin; **OFX**, Ciprofloxacin; **CIP**, Ciprofloxacin; **MXF**, Levofloxacin; **LVX**, Metronidazole; **ND**, no data.

**qnrD** is included in a Mobile Insertion Cassette

In silico analysis did not show any gene involved in mobilization and specific PCRs for intI1, ISCR1, IS26 and ISEcp1 were negative. We also looked for mobile insertion cassette (mic) elements that are similar to transposons. A mic element is bracketed by two inverted repeats without any transposase-encoding gene found nearby and duplication of the target site is usually observed indicating the transposition. In the 2,683-bp long plasmids, **qnrD** was localized within a 1,519-bp “mic” element, bracketed by two 24-bp imperfect inverted repeats (IR) with six mismatches (Figure 1B). No transposase-encoding gene was found. Acquisition of the mic-**qnrD** element was likely the result of a transposition process because it is bracketed by a 2-bp duplication of the target site (GA). The mic-**qnrD** element described here carries the necessary elements for expression of the **qnrD** gene: (i) promoter sequences including a –35 box (TTTATA) and a –10 box (TCTTAAAAT) separated by 11-bps, (ii) and a Shine-Dalgarno sequence (AAAAGGG) upstream on the transposase-encoding gene (ATG).

In the 4,686-bp long plasmid pRS12–78, **qnrD** is a part of a 5,072-bp “mic” element given that the ORF5 described above was inserted between **qnrD** and a putative promoter (Figure 1A). Although this second mic-**qnrD** element is slightly different in length, it has the same characteristics.

Reference Strains of **Proteeae** Carried Native Plasmids but no **qnrD** Gene

**qnrD** was not detected by multiplex or simplex real-time PCR in the fifteen reference strains of **Proteeae** but plasmids were found in most of the reference strains (Table 1). These plasmids did not belong to any known incompatibility group detected using the PBRT method [22].

Overlapping PCRs designed on the sequence of pDIJ09–518a and performed using template DNA of the **Proteeae** reference strains showed that one small plasmid of **P. vermicola**, pVERM, carried a fragment exhibiting 89% identity with the sequence of pDIJ09–518a. **pVERM** was linearized using *AciI* restriction enzyme. After gel extraction, the sequence was determined using a walk DS strategy on the amplification product obtained with primers pDIJ-2558F and orf2–3’R. **pVERM** is a 3,682-bp long
plasmid containing a 1,794-bp region sharing more than 98% identity with the qnrD-plasmids. In this region, we identified three ORFs similar to ORF2, ORF3 and ORF4 described in pDIJ09–518a. BLAST of the 1,888-bp remaining region showed a 267-bp ORF1 (named pVERM_ORF1 in the following) covering 76% of a putative protein described in Vibrio brasiliensis (accession number ZP_080099857.1) with 31% similarities. Two 24-bp long imperfect IR, with eight mismatches, and CA duplications were found to bracket pVERM_ORF1 and pVERM_ORF2 leading to identify another mic element. The 2,664-bp long mic element harbored by pVERM is different from mic-qnrD for 1,145 bps containing pVERM_ORF1 and lacking qnrD (Figure 1B). Nonetheless, IRR and IRL are similar to those bracketing mic-qnrD on the qnrD plasmids such as pDIJ09–518a with 2 and 1 single nucleotide differences for IRLs and IRRs respectively (Figure 1B). No known mobilization structure such as IS were identified in this 2,664-bp long mic element.

Discussion

We investigated the emergence of the new plasmid-borne quinolone resistance gene, qnrD in Proteae clinical bacterial isolates. The qnr genes are encoding a group of pentapeptide repeat proteins recently discovered but certainly present on bacterial chromosomes long before quinolones were used clinically. They emerged 10 years ago, following the 1990–2010 worldwide increase in fluoroquinolone usage [3]. qnrD was initially described in Salmonella enterica Kentucky and Bovismorbificans isolates [7], our investigation showed that qnrD was the qnr gene mainly present in isolates belonging to the Proteae tribe (Proteus, Providencia and Morganella genera) since the screening of all qnr genes in 317 Proteae clinical isolates resulted in 8 qnr-positive strains but seven (87.5%) positive for qnrD. This unexpected result converges with data in GenBank where among 38 sequences of qnrD half were reported in Proteae (Table S2, last update October 30, 2013). We consequently hypothesized that Proteae are the reservoir of qnrD genes.
In Proteae clinical isolates, we found qnrD on small ca. 2.6-kb non-transmissible plasmids, in contrast with what was mainly described for other qnr genes carried by large conjugative plasmids [5]. Although qnrD was also reported to on such large conjugative plasmids, none of these plasmids was well characterized [26–29]. All the 15 qnrD-bearing plasmids found in Proteae (the 7 from our study and 8 plasmids from GenBank sequences, Table S2) have the same size (2,683-bp) and have nearly identical sequences, which is in favor of a common origin or ancestor [30–32].

Figure 2. qnrD may have emerged and disseminated from Proteae to Enterobacteriaceae using mic elements and a recombination event with the native PVERM plasmid. Comparison of the plasmid structures of pDU09-518a from P. rettgeri, pVERM from P. vermicola, p2007057 from S. enterica Bovismorbificans, pGC156 from E. coli and pSC101 from S. enterica Typhimurium. The open reading frames are shown as arrows with the arrowhead indicating the direction of transcription. Red color is used to shade arrows in the qnrD-mic element. Grey shading shows the areas of homology between the plasmids. doi:10.1371/journal.pone.0087801.g002

The only exception found during our study is the plasmid pRS12–78 that presented a plasmid scaffold identical to the other characterized plasmids but with a 1,368-bp insertion corresponding to a putative additional ORF. By contrast, the plasmids carrying qnrD found in strains of Enterobacteriaceae other than Proteae are 4,270 bp long with sequences nearly identical to p2007057 described in S. enterica. These latter plasmids usually harbor a mob gene that may explain their transfer. The two types of plasmids carrying qnrD show a highly conserved backbone, but the smallest are not transmissible. This was previously described for qnsS, as it was described on a 10,066-bp non-conjugative but mobilizable plasmid in S. enterica Typhimurium [16]. It was suggested that this plasmid results from the recombination of a qnsS1 conjugative plasmid from S. enterica Infantis (pINF5) and a small mobilizable plasmid from E. coli (pEC276). The qnsS-2,683-bp plasmids were stable in E. coli DH10B after electroporation, indicating a host range including other species of Enterobacteriaceae. However, the gene encoding the replicase is probably different to what is usually found since the incompatibility group cannot be assessed. Non classic replication systems were recently reported for some small plasmids in Proteae [18,33] and we share the view of Galac et al. that cryptic plasmids in Proteae, including the small qnsD-plasmids, may use a not yet characterized replication method [33]. Since reference type strains of Proteae did not harbor qnrD, we sought for similar plasmid backbone in their constitutive DNA. We found, in a reference strain of P. vermicola, a native plasmid that shares 1,794 bps with more than 98% identity with our qnrD-bearing plasmids.

We described here qnrD as located in a mobile insertion cassette (mic) element. Mic elements were first described in Bacillus cereus (MIC231A1) as a DNA cassette able to be mobilized in trans by the IS231A transposase [34]. MIC231A1 contains an active D-stereospecific endopeptidase (adp) gene instead of a transposase. Further studies conducted on B. cereus showed the presence of antibiotic resistance determinant such as a fosfomycin resistance gene in MIC231/D, described as a novel active composite cassette [35]. It was then proposed mic element could be also a vehicle for antibiotic resistance genes beside class 1 integrons where multiple antibiotic cassettes can be inserted. With regard to plasmid mediated quinolone resistance genes, qnsS2 was described in a mic element in a strain of Aeromonas fumigata isolated in water sampled from the River Seine in Paris [36]. Unfortunately, as reported by Cattoir et al., no transposases were found, on the basis of the IR nucleotide sequences, suitable for demonstrating trans-position in an in vitro assay [36]. qnsA genes were mainly associated with ISCR1 at the vicinity of complex class 1 integrons which include various other resistance determinants (e.g. bha genes or acr(6’)-Ib-cr). qnsB genes were also described beside class 1 integrons, but associated with ISBe1 and ORF1005, another type of inserted sequences [37,38]. Unlike qnsA and qnsB, qnsS genes were mainly associated with structure such as ISEs2, IS26 and mic structure [16,36,39], and were not reported as part of complex integrons. These gene-capture systems explain the mobilization of qns genes from their progenitors to self-transmissible plasmids leading to their widespread in Enterobacteriaceae.

We think that qnrD was imported from a Proteae species and inserted in pVERM through mic mobilization (Figure 2).
recombination, insertion or deletion events may have occurred after the mic-qnrD mobilization including qnrD deletion, resulting in the plasmids pVERM and pDIJ09-518 reported here. Indeed, the IRs found in the mic-qnrD plasmids from *Proteae* clinical strains are more similar to those found in pVERM than those found in plasmids in *S. enterica*. pVERM differed from *qnrD*-plasmids by 1,388 bps located inside the very same lacking *qnrD* mic. This *qnrD*-lacking-mic element found in pVERM is 1,145 bp longer than *mic-qnrD* and carried a *pVERM* _ORF1_ present neither on pDIJ09-518 nor on p2007057. While mic-*qnrD*-IRR and IRL showed seven unmatched nucleotides for pVERM vs six for mic-*qnrD*, more importantly a comparison of IRLs between mic-*qnrD* and pVERM found only 2 SNPs and only 1 SNPs was found comparing both IRRs. Finally, IRRs in both *qnrD*-plasmid and pVERM were 24-bp long. (Figure 1A). Moreover, none of the 50 *qnr*-negative clinical strains, randomly selected, harbored such small plasmids. Therefore, our current hypothesis is that pVERM could be the ancestor plasmid where *qnrD* was later deleted. Alternatively, pVERM could be a *qnrD*-plasmid resulting from another mic-*qnrD* harbored by an unknown *qnrD* progenitor, from which *qnrD* was subsequently lost. The *qnrD* progenitor is not yet identified. For the other *qnr* genes, progenitors are mainly bacteria living in the environment (*Shewanella* spp., *Citrobacter* spp., and *Vibrio* spp) where *Proteae* are also present [13,36,40,41]. The *Proteae* could have acquired *qnrD* from its progenitor by recombination between an as-yet-unknown bacterial source of *qnrD* and the cryptic plasmid pVERM harbored by *P. vermicola* leading to small non-mobilizable 2,683-bp long plasmids carrying *qnrD* embedded in a mic-*qnrD* element (Figure 2). Given that all plasmids of about 4,270 bps such as *p2007057* (e.g. *P. mirabilis* isolates CGP180 and CGH10 described by Zhang et al.) also harbored a mic-*qnrD* with a conserved 24-bp IRL and a modified 26-bp IRR showing 1 and 7 nucleotide differences, respectively, with regard to 2,683-bp *qnrD*-plasmids, we think that the mic-*qnrD* element could have been later inserted by transposition in a mobilizable plasmid such as the 4,270-bp *qnrD*-plasmid p2007057. It is also possible that the mic-*qnrD* was transferred from a common progenitor to both *Proteae* and *Salmonella* spp. in parallel onto two different types of plasmids in which the IRL sequences evolved and diverged especially for the IRR.

Finally, we investigate the quinolone resistance conferred by the *qnrD*-bearing plasmids. The MICs of fluoroquinolones were similar and comparable to MICs previously reported for p2007057, except for the plasmid *pRS12–78*. In this plasmid, the insertion of a 1,560-bp long sequence, can hamper *qnrD* expression from a putative promoter then located far from *qnrD*. Although the level of resistance is rather low for classical quinolones such as nalidixic acid, it can reach 20-fold increase in the MIC of ciprofloxacin. Such an increase can cause failure in experimental model of pneumonia and in pulmonary infections [42–44]. Further studies are needed to investigate the fitness cost of *qnrD* harbored by these small plasmids, but as described for *qnrA3* [45], *qnrD* could contribute to a fitness gain leading to the emergence of *qnrD*-bearing plasmids even in the absence of quinolones.

### Supporting Information

**Table S1** List of primers used in this study. (DOC)

**Table S2** List of *qnrD* deposited in GenBank. (DOC)

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### Author Contributions

Conceived and designed the experiments: TG CdC EC. Performed the experiments: TG AG CC JM. Analyzed the data: TG CaC EC.

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