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MECHANISMS OF RESISTANCE

Multiple Copies of *qnrA1* on an IncA/C_2 Plasmid Explain Enhanced Quinolone Resistance in an *Escherichia coli* Mutant

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ABSTRACT In a previous study, mutants with enhanced ciprofloxacin resistance (Cip^r) were selected from *Escherichia coli* J53/pMG252 carrying *qnrA1*. Strain J53 Cip^r 8-2 showed an increase in the copy number and transcription level of *qnrA1*. We sequenced the plasmids on Illumina and MinION platforms. Parental plasmid pMG252 and plasmid pMG252A from strain J53 Cip^r 8-2 were almost identical, except for the region containing *qnrA1* that in pMG252A contained 4 additional copies of the *qnrA1-qacEΔ1-sul1*-IS*CR1* region.

KEYWORDS A/C_2 plasmid, *Escherichia coli*, IS*CR1*, ciprofloxacin, *qnrA1*

The plasmid-mediated quinolone resistance gene *qnrA1* causes a modest decrease in quinolone susceptibility but facilitates the selection of mutants with higher levels of quinolone resistance through additional chromosomally encoded mechanisms (1, 2). Since the report of the first plasmid-mediated quinolone resistance (PMQR) determinant, *qnrA1*, in 1998, PMQR genes have been identified worldwide (3, 4).

In our previous study, in which mutants with decreased ciprofloxacin susceptibility were selected from J53/pMG252 with *qnrA1* (1), we found that *qnrA* expression was 2.2-fold higher in mutant J53 Cip^r (ciprofloxaxin resistance) 8-2 carrying what we term plasmid pMG252A than in the parental strain, with no changes detected in the *qnrA* promoter region. This mutant also had a 3.1-fold increase in *qnrA* copy number without a change in copy number for bla_{FOX-5}, another marker on the plasmid, indicating that the *qnrA* copy number increase was gene specific and potentially responsible for increasing the ciprofloxacin MIC from 0.25 μg/ml in the parental strain to 2 μg/ml in the mutant. Furthermore, no mutations enhancing ciprofloxacin resistance were found in chromosomal genes of mutant J53 Cip^r 8-2, and the enhanced resistance could be transferred by conjugation and eliminated along with pMG252A, indicating that plasmid pMG252A was directly involved.

Therefore, to confirm an increase in the number of copies of *qnrA1* and the nature of the duplication, we sequenced both pMG252 and pMG252A from the J53 Cip^r 8-2 mutant.

In a first attempt, we extracted both plasmids using the Qiagen large construct kit and obtained some short-read sequences with the Illumina platform, performed by the Center for Computational and Integrative Biology (CCIB) DNA Core Facility at Massachusetts General Hospital (Cambridge, MA). Libraries were pooled in equimolar concentrations for multiplexed sequencing on the Illumina MiSeq platform with a 2 × 150-bp read run parameter. Purified plasmids were sequenced, producing 154×10^5 reads and 1.28×10^5 reads for plasmids pMG252 and pMG252A, respectively. Ninety-five percent of the reads had a mean sequence quality (Phred score) of >30. The total depth of coverage after deduplication for each plasmid was 108× for pMG252 and 98× for pMG252A. Because of the large size of pMG252 (~180 kb) (2) and

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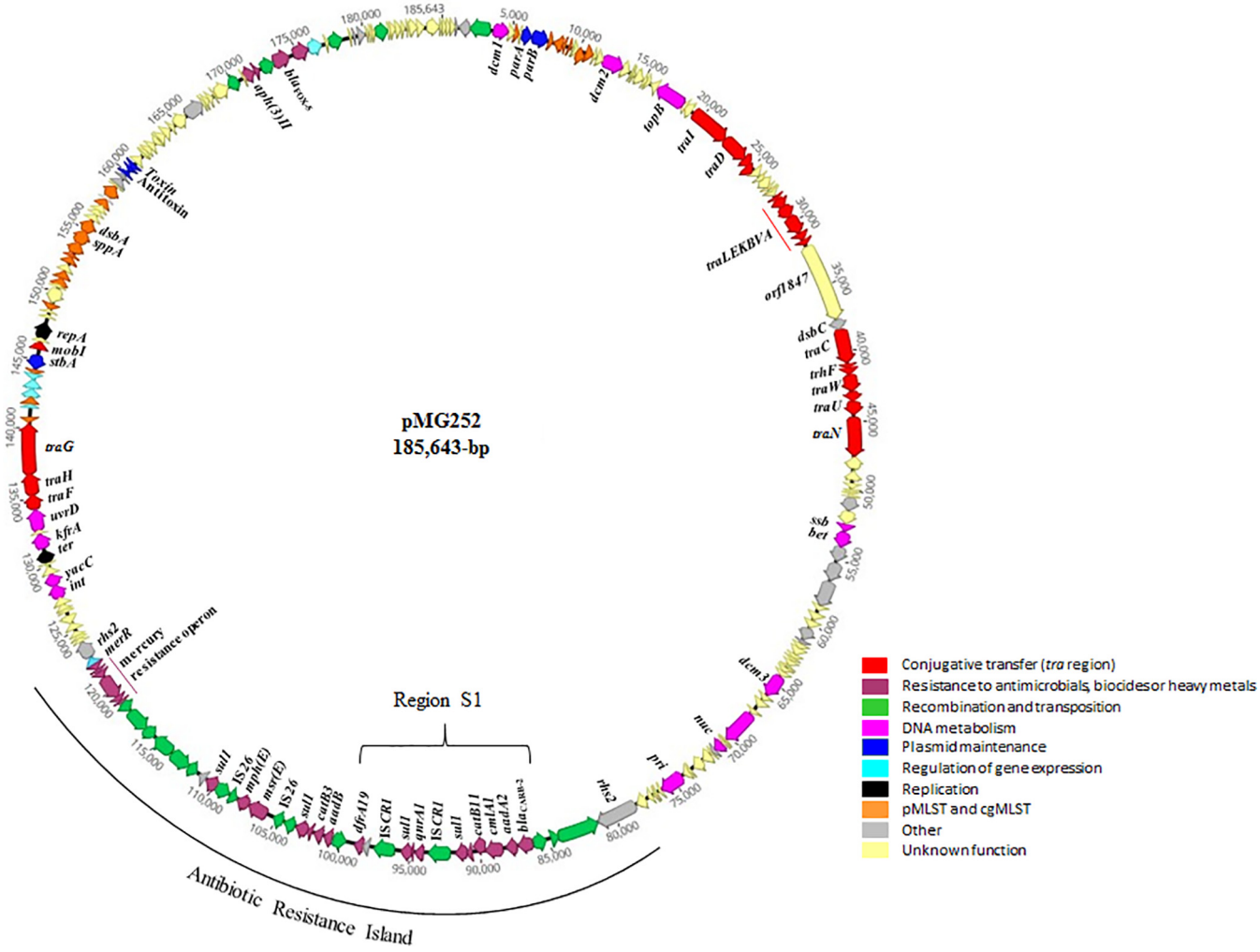

FIG 1 Genetic map of parental pMG252 plasmid. The figure was constructed with Geneious Prime 2019.0.4.

the challenges imposed by insertion elements, repetitive sequences, and duplications, *de novo* assembly from these data was unable to produce a circularized plasmid sequence or an unambiguous location and copy number for *qnrA1*. To overcome these challenges, we used the MinION platform (Oxford Nanopore Technologies) to produce long sequence reads via Day Zero Diagnostics (Boston, MA). Total DNA was extracted using the Genomic DNA kit (Genomic-tip 100/G; Qiagen), prepped with SQK-RBK004, run using an R9.4.1 flow cell on a MinION Mk1B sequencer controlled by MinKNOW 2.0, and base called using Albacore v2.3.1. A larger number of reads and higher read lengths were achieved, and circularized plasmids were obtained. The final assembly was polished using Illumina reads (Fig. 1).

The circularized sequence of plasmid pMG252 was 185,643 bp long, while that of pMG252A was 201,521 bp. The two plasmids had the common backbone of IncA/C plasmids (5, 6). They were identified as IncA/C_2 type 2 (5). Additionally, plasmid MLST (PMLST) typing assigned the pMG252 plasmid to sequence type 3 (ST3), which is the most common group of IncA/C plasmids (6). By the core gene PMLST (cgPMLST) scheme (7), pMG252 belongs to core-genome ST 3.3 (cgST3.3).

pMG252, which is the first plasmid identified with a *qnr* gene, was found in a *Klebsiella pneumoniae* clinical isolate (8). It had an average G+C content of 52.8%. It contained a large antibiotic resistance island (41,056 bp) located between the two ends of the *rhs2* gene. This module included a class 1 integron (termed region S1) with the following gene cassette array: *bla*$_{\text{CARB-2}}$*-aadA2-cmlA1-catB11-qacEΔ1-sul1*-IS*CR1-qnrA1-*

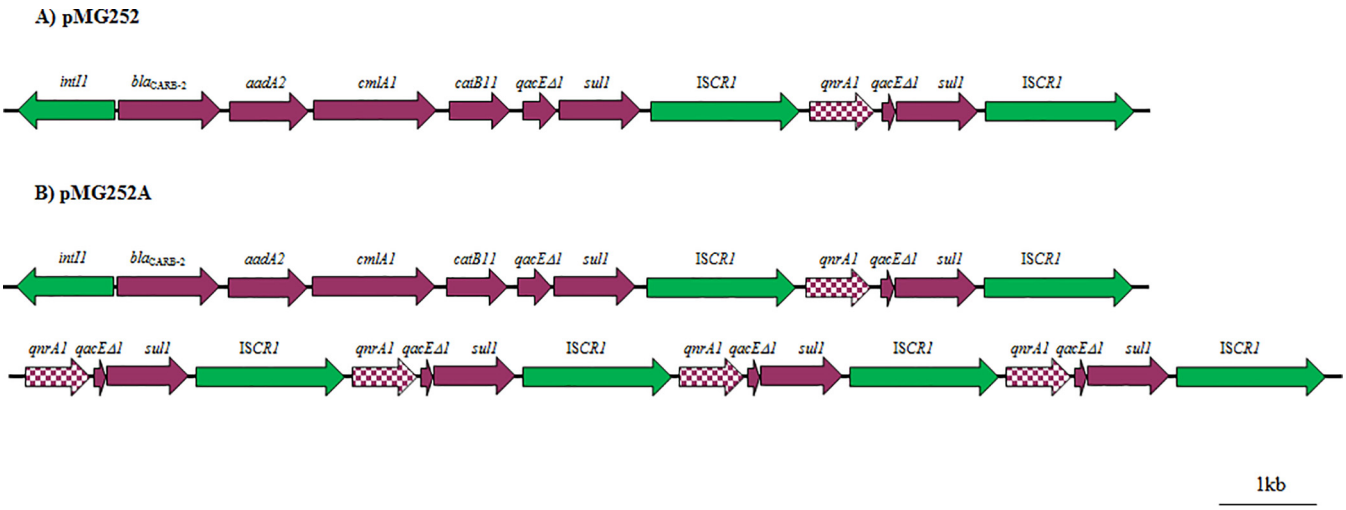

FIG 2 (A and B) Genetic environment of *qnrA1* in plasmids pMG252 (A) and pMG252A (B) in region S1. Purple arrows show genes conferring resistance to antimicrobials or biocides, dotted purple arrows indicate the multiple copies of *qnrA1,* and green arrows denote elements involved in recombination and/or transposition.

qacEΔ1-sul1-IS*CR1-dfrA19*, followed by another class 1 integron harboring an *aadB-catB3* array. Downstream from the integrons were *msr*(E), encoding a macrolide efflux pump, and *mph*(E), encoding a macrolide 2′-phosphotransferase, both associated with insertion sequence IS*26*. Different insertion sequences separated the *msr*(E)-*mph*(E) module from a mercury resistance operon that was bracketed by the end of the *rhs2* gene (Fig. 1).

Other plasmid-encoded functions that have been previously annotated in sequenced IncA/C_2 plasmids (5) have been also identified in pMG252 (Fig. 1). These functions included genes for methyltransferases (*dcm1, dcm2,* and *dcm3*), DNA metabolism (*nuc, topB, kfrA, uvrD, ter, int,* and *pri*), protein export and folding (*sppA, dsbA, dsbC,* and *yacC*), and plasmid stability (*parA-parB* and *stbA*) (Fig. 1).

In a comparison of the assemblies of pMG252 and pMG252A, they were found to be 88.9% identical, which was ascertained using the multiple-sequence alignment program MAFFT (Geneious). The major difference between them was in region S1 that indeed contained multiple copies of *qnrA1* (Fig. 2). In most *qnrA1*-containing plasmids, a single copy of IS*CR1* is found downstream from *qnrA1*, but in plasmid pMG252, *qnrA1* is surrounded by two copies of IS*CR1*. The *qnrA1*-IS*CR1* complex is inserted into *sul1*-type integron-containing cassettes that confer resistance to β-lactams (bla_{CARB-2}), chloramphenicol (*cmlA1* and *catB11*), streptomycin (*aadA2*), sulfonamide (*sul1*), and trimethoprim (*dfrA19*) (2). The assembled sequence of plasmid pMG252A revealed the presence of four additional copies of *qnrA1-qacEΔ1-sul1*-IS*CR1*, thus validating our findings of an increased *qnrA1* copy number.

There are numerous studies describing the prevalence of PMQR harboring *qnrA1* in *Enterobacteriaceae* (9, 10). *qnrA1* has been associated in the same plasmid with other PMQR genes, such as *qnrB, qnrS,* and *qepA* (11), but it is unusual to find more than one copy of the same PMQR gene within a plasmid. An exception is plasmid pSZ50 from Mexico, in which the entire integron containing IS*CR1*, *qnrA1,* and other resistance genes was duplicated in tandem, although quinolone resistance was not increased (12). In our study, plasmid pMG252A with 5 copies of *qnrA1* had an 8-fold higher MIC to ciprofloxacin than did its pMG252 parent, reaching an MIC of 2 μg/ml ciprofloxacin and thus exceeding the CLSI breakpoint for resistance (13). The elevated resistance was transmissible by pMG252A on conjugation. Unlike other *qnr* types, *qnrA1* is not inducible by quinolones (14). The duplication of IS*CR1* around *qnrA1* in pMG252 and related plasmids creates the structure of a composite transposon; this facilitates enhancement of the *qnrA1* copy number and, consequently, quinolone resistance under conditions of

quinolone stress, and it adds to the repertoire of mechanisms that can increase quinolone resistance to clinically important levels.

The genetic organization of bla_{FOX-5} in pMG252 was similar to that previously published (GenBank accession number CP007732). Upstream of the bla_{FOX-5} gene, there was a region with 98% DNA homology to the IS*As2* transposase gene from *Aeromonas salmonicida*, a member of the IS*30* family (15), followed by a bleomycin resistance gene. Immediately in the 3′ direction, there was a multidrug transporter gene (*mdrL*), followed by a transcriptional regulator gene (*lysR*) and another copy of IS*As2*.

In summary, we determined by plasmid sequencing the novel presence of 5 copies of *qnrA1* in plasmid pMG252A, as a result of transposition via insertion sequence IS*CR1* located downstream from *qnrA1* within a class 1 integron; we also characterized pMG252 as a sequence type 3.3 (ST3.3) member of the IncA/C_2 type 2 family.

Data availability. The sequences of the entire plasmids pMG252 and pMG252A have been deposited in the NCBI GenBank database under accession numbers MK638972 and MK733575, respectively.

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