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mcr-9, an Inducible Gene Encoding an Acquired Phosphoethanolamine Transferase in *Escherichia coli*, and Its Origin

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ABSTRACT The plasmid-located *mcr-9* gene, encoding a putative phosphoethanolamine transferase, was identified in a colistin-resistant human fecal *Escherichia coli* strain belonging to a very rare phylogroup, the D-ST69-O15:H6 clone. This MCR-9 protein shares 33% to 65% identity with the other plasmid-encoded MCR-type enzymes identified (MCR-1 to -8) that have been found as sources of acquired resistance to polymyxins in *Enterobacteriaceae*. Analysis of the lipopolysaccharide of the MCR-9-producing isolate revealed a function similar to that of MCR-1 by adding a phosphoethanolamine group to lipid A and subsequently modifying the structure of the lipopolysaccharide. However, a minor impact on susceptibility to polymyxins was noticed once the *mcr-9* gene was cloned and produced in an *E. coli* K-12-derived strain. Nevertheless, we showed here that subinhibitory concentrations of colistin induced the expression of the *mcr-9* gene, leading to increased MIC levels. This inducible expression was mediated by a two-component regulatory system encoded by the *qseC* and *qseB* genes located downstream of *mcr-9*. Genetic analysis showed that the *mcr-9* gene was carried by an IncHI2 plasmid. *In silico* analysis revealed that the plasmid-encoded MCR-9 shared significant amino acid identity (ca. 80%) with the chromosomally encoded MCR-like proteins from *Buttiauxella* spp. In particular, *Buttiauxella gaviniae* was found to harbor a gene encoding MCR-BG, sharing 84% identity with MCR-9. That gene was neither expressed nor inducible in its original host, which was fully susceptible to polymyxins. This work showed that *mcr* genes may circulate silently and remain undetected unless induced by colistin.

KEYWORDS *Escherichia coli*, MCR, MCR-9, colistin, plasmid, polymyxin

Acquired resistance to polymyxins in *Enterobacteriaceae* may arise from two main pathways, namely, chromosomally encoded resistance mechanisms corresponding to mutations or deletions in genes involved in the biosynthesis of the lipopolysaccharide (LPS) or the acquisition of plasmid-mediated *mcr* genes. The latter genes encode phosphoethanolamine transferases that modify the lipid A moiety of the LPS, subsequently leading to resistance to polymyxins (1, 2). There have been eight different

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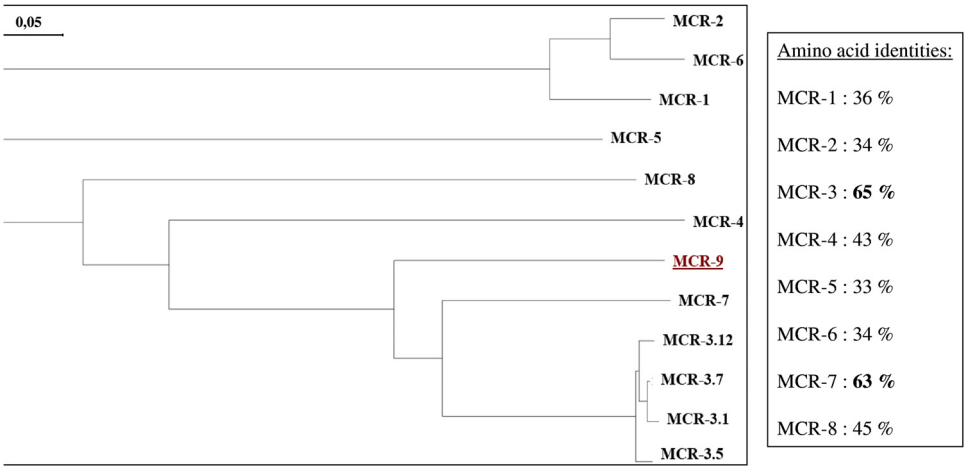

FIG 1 Phylogenetic tree obtained for the MCR-type phosphoethanolamine transferases identified by the distance method using the Neighbor-Joining algorithm (SeaView software, version 4 [44]). Branch lengths are drawn to scale and are proportional to the number of amino acid substitutions with 500 bootstrap replications. The distance along the vertical axis has no significance. The percentage of amino acid identity compared to the MCR-9 enzyme is indicated. The tree was rooted with the MCR-3.1 enzyme. The scale along the horizontal axis is indicated.

groups of *mcr* genes identified, with some variants described within some given groups (3–11). *mcr-1* likely originates from a *Moraxella* species (12), while *Moraxella pluranimalium* is the exact progenitor of *mcr-2* (13), *Aeromonas* spp. that of *mcr-3*-like genes (14, 15), and *Shewanella* spp. that of *mcr-4*-like genes (7). The origins of the more recently discovered *mcr* genes (*mcr-5* to *-8*) remain unknown. In addition, a very recent study identified the *mcr-9* gene from a colistin-susceptible *Salmonella enterica* serotype Typhimurium isolate recovered from a U.S. patient in 2010 (16).

The high prevalence of MCR (particularly MCR-1)-producing *Escherichia coli* isolates in food-producing animals, and therefore the high rate of colistin-resistant isolates, may be explained by the significant use of colistin in veterinary medicine, in particular in livestock for the treatment of poultry, swine, and cattle (17–20). In humans, the prevalence of MCR producers seems to be more limited, with many studies reporting a low prevalence of the *mcr-1* gene in *Enterobacteriaceae* (1, 21). On the other hand, the prevalence of the *mcr-2* to *-9* genes remains unknown overall, even though the few reports are mainly on animal isolates.

In a recent prospective study performed in six different French university hospitals, despite the fact that the prevalence of fecal colistin-resistant *E. coli* carried by inpatients was found to be unexpectedly high (12.7%), the rate of MCR-1 producers was low (4.6% of the colistin-resistant isolates), and no other known MCR-like enzyme was identified (22). Two-thirds of the resistant isolates had mutations in the PmrA/PmrB chromosomally encoded two-component systems that are the likely sources of the resistance phenotype (22). Among those colistin-resistant *E. coli* isolates that remained negative for all known *mcr*-like genes (*mcr-1* to *mcr-8*) and that did not harbor obvious mutations in chromosomal genes that might be responsible for acquired resistance, *E. coli* 68A, showing a colistin MIC of 8 μg/ml, was retained for further characterization.

RESULTS

Identification of the *mcr-9* gene. Whole-genome sequencing (WGS) of the *E. coli* isolate 68A (GenBank assembly accession no. GCA_900500325.1) was used in an attempt to identify the mechanism leading to this reduced susceptibility to colistin, and a gene showing significant identity to *mcr*-like genes was identified. It corresponded to the newly reported *mcr-9* gene (16) encoding the MCR-9 enzyme, sharing 65% and 63% amino acid identity with the most closely related MCR-3 and MCR-7 enzymes, respectively, and between 33% and 45% with the other MCR-like enzymes (Fig. 1) (GenBank accession number MK070339). Phylogenetic analysis performed with MCR-9 and all

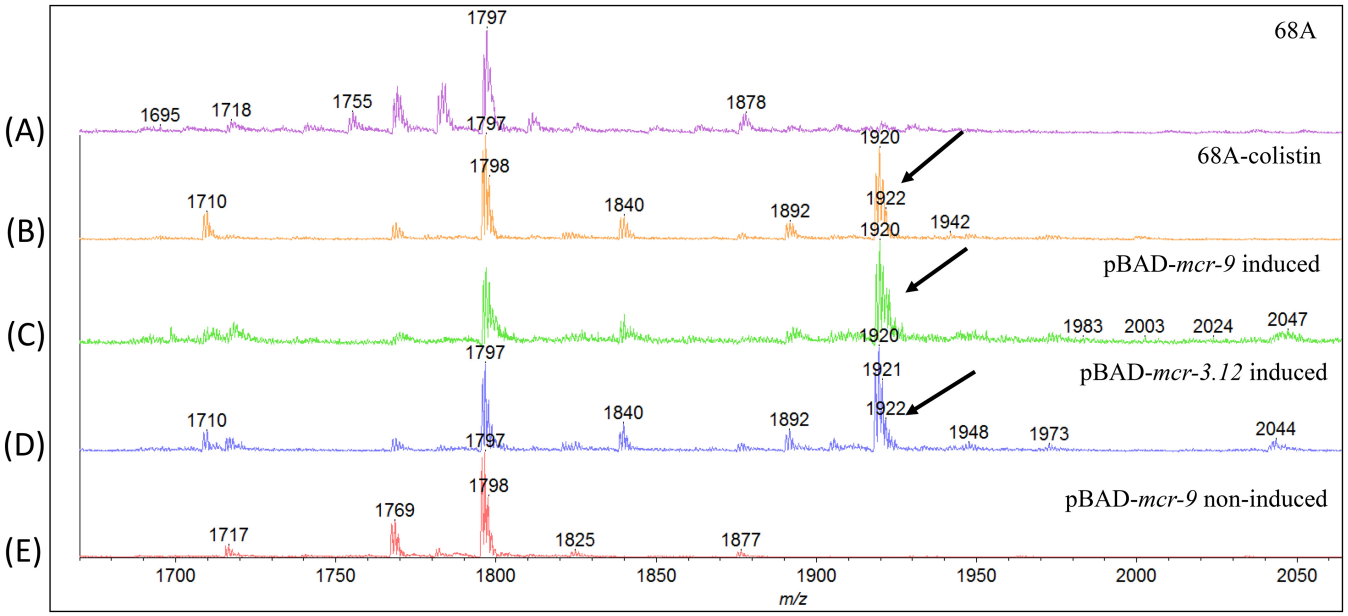

FIG 2 Mass spectrometry analysis of lipid A from isolate *E. coli* 68A after growth on a colistin-free LB plate (A), *E. coli* 68A after growth on a colistin-supplemented (2 μg/ml) LB plate (B), *E. coli* TOP10(pBAD-*mcr-9*) recombinant strain induced by L-arabinose (C), *E. coli* TOP10(pBAD-*mcr-3.12*) recombinant strain induced by L-arabinose (D), and *E. coli* TOP10(pBAD-*mcr-9*) (not induced by L-arabinose) (E). The addition of phosphoethanolamine is indicated by an arrow.

other MCR-type enzymes revealed that MCR-9 was distantly related to all those enzymes and constituted a distinct cluster.

Isolate 68A showed resistance only to penicillins (with susceptibility to penicillin-β-lactamase inhibitors) and tetracycline due to the presence of bla_{TEM-1} and *tet*(A) genes. Multilocus sequence typing analysis showed that isolate 68A belonged to the phylogroup D-ST69 and was of the O15:H6 serotype. It possessed numerous extraintestinal virulence genes either on the chromosome (*fyuA*, *irp2*, and *ompT*) or on a pS88-like IncFIB virulence plasmid (*iro* operon, *iss*, *ompT*, *hlyF*, and *iuc* operon) (23). Thus, this strain corresponds to an extraintestinal pathogenic *E. coli* (ExPEC) isolate.

Localization of the *mcr-9* gene. Analysis of the whole-genome sequence of *E. coli* 68A using PlaScope (24) showed that the *mcr-9* gene was located on an IncHI2 (pMLST2) incompatibility group plasmid (p68) of 225 kb. This plasmid did not carry any other putative resistance gene. PCR-based replicon typing (PBRT) analysis (25) and Kieser extraction (26) performed from isolate 68A, followed by gel electrophoresis analysis, confirmed the whole-genome sequencing (WGS) data.

MCR-9 is a phosphoethanolamine transferase conferring reduced susceptibility to colistin. Mass spectrometry analysis of the lipid A moiety of the LPS of the MCR-9-producing *E. coli* isolate 68A recovered from a colistin-supplemented agar plate showed an additional peak at *m/z* 1,920 (Fig. 2). This modification corresponded to the addition of a phosphoethanolamine (PEtN) group to lipid A, as previously reported for MCR-1 to MCR-3 producers (27–29) (Fig. 2). Surprisingly, no peak at *m/z* 1,920 was observed when we performed the same experiment with isolate 68A recovered from a colistin-free medium (Fig. 2). These results strongly suggested that the isolate 68A did not produce MCR-9 at a level sufficient to significantly modify its LPS under the colistin-free condition and therefore that the expression of *mcr-9* was induced in the colistin-containing plate.

The *mcr-9* gene alone then was cloned into the arabinose-inducible pBAD vector, leading to *E. coli* TOP10(pBAD-*mcr-9*), in which the expression of *mcr-9* is high in the presence of L-arabinose. Mass spectrometry analysis of the lipid A moiety of the LPS of both MCR-9- and MCR-3.12-producing recombinant *E. coli* TOP10 pBAD-*mcr-9* and pBAD-*mcr-3.12* recombinant strains, both recovered from an L-arabinose-supplemented

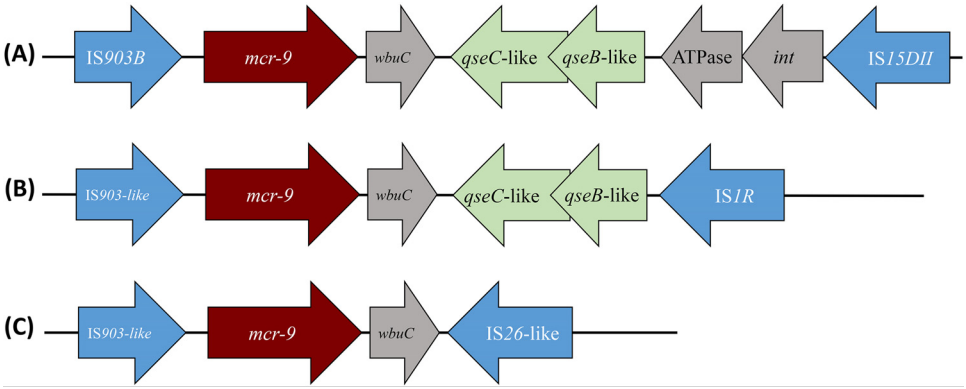

FIG 3 Different genetic structure surrounding the *mcr-9* gene. The *wbuC*, *qseC*, *qseB*, and ATPase-encoding genes located downstream of *mcr-9* are represented, as well as the different insertion sequences (IS) bracketing that DNA fragment, namely, IS*903B*, IS*1R*, IS*26*-like, and IS*15DII*. Arrows indicate the sense of transcription of those different genes. (A) Structure surrounding the *mcr-9* gene in *E. coli* isolate 68A (present study) and those identified *in silico* in *Leclercia* species (GenBank accession no. CP031102.1), *Enterobacter hormaechei* (CP031575.1), *Salmonella enterica* (CP029037.1 and CP006057.1), and *Enterobacter kobei* (CP032893.1). (B) Structure surrounding the *mcr-9* gene identified *in silico* in *Leclercia adecarboxylata* (MH909331.1) and *Salmonella enterica* (CP026661.1). (C) Structure surrounding the *mcr-9* gene identified *in silico* in *Enterobacter cloacae* (CP020529.1), *Salmonella enterica* (MK191844.1), *Cronobacter sakazakii* (CP028975.1), and *Enterobacter hormaechei* (CP031575.1).

agar plate, showed additional peaks at *m/z* 1,920 not seen for the *E. coli* TOP10(pBAD-*mcr-9*) strain recovered from an L-arabinose-free plate (Fig. 2).

Apart from colistin induction, and since the *mcr-9* gene seemed to be poorly transcribed in its natural host, we evaluated the level of resistance (or reduced susceptibility) it might confer by comparing it to that of the *mcr-1* gene. After induction with arabinose, *E. coli* TOP10(pBAD-*mcr-9*) showed a colistin MIC of 0.15 μg/ml (susceptibility range), whereas the same noninduced strain presented a MIC of 0.03 μg/ml, corresponding to a 5-fold increase of the colistin MIC. Conversely, the MIC of colistin for the *E. coli*(pBAD-*mcr-1*) recombinant strain was 4 μg/ml. These data confirmed the impact of the MCR-9 enzyme on colistin susceptibility but to a lower extent than that of MCR-1.

Genetic context of the *mcr-9* gene. Analysis of the genomic data of *E. coli* 68A showed that the *mcr-9* gene was bracketed by two insertion sequences (IS). Upstream of *mcr-9*, IS*15DII* (IS*5* family) was identified, in which no obvious promoter sequences that might be responsible for *mcr-9* expression could be identified (Fig. 3). Downstream of *mcr-9*, another IS, namely, IS*903B* (IS*6* family), was identified (Fig. 3). No target site duplication was identified at the extremities of each IS or when looking at the left-hand extremity of IS*903B* and the right-hand extremity of IS*903B*. The latter observation and the fact that those two IS are distantly related (with totally different inverted repeat sequences) likely rule out the hypothesis that *mcr-9* was acquired through a composite transposon bracketed by those two IS.

Analysis of the DNA fragment bracketed by the two above-mentioned IS showed that four open reading frames were present downstream of *mcr-9*, including *wbuC*, *qseC*, *qseB*, and an ATPase gene (Fig. 3). Interestingly, although *wbuC* encodes a putative enzyme of the cupin superfamily of unknown function, the *qseC* and *qseB* genes encode a putative two-component system corresponding to a histidine kinase sensor (QseC) and its cognate partner (QseB). Similar proteins have been shown to play a role in the signaling network inducing resistance to colistin (30). In order to evaluate whether those two genes located downstream of *mcr-9* play a role in colistin resistance, the whole structure encompassing the *mcr-9*, *qseC*, and *qseB* genes along with the two IS*903B* and IS*15DII* elements was cloned, leading to the *E. coli* TOP10(pBAD-Tot) strain. The MIC of colistin for this recombinant strain was compared with that of the isogenic recombinant *E. coli* TOP10(pBAD-*mcr-9*), and only a slight increase was observed for *E. coli* TOP10(pBAD-Tot) (0.3 versus 0.15 μg/ml).

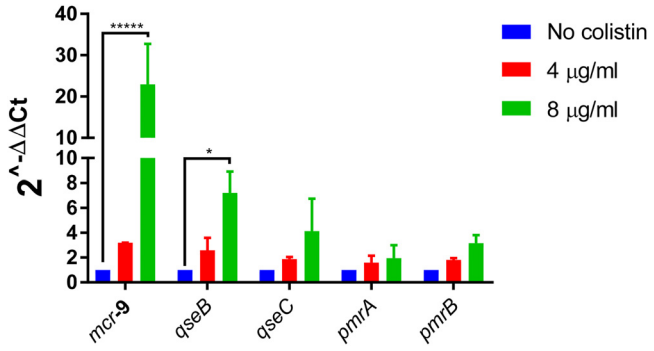

FIG 4 Expression of five selected genes in clinical isolate 68A with and without colistin exposure. The genes are the plasmid-located *mcr-9*, *qseB*, and *qseC* as well as the chromosome-located *pmrA* (DNA-binding response regulator) and *pmrB* (sensor protein) genes. Fold changes in mRNA levels were determined by RT-qPCR. The data have been normalized to values for reference 16S rRNA genes. Three independent replicates were performed; the data shown represent the means, and the error bars represent standard deviations. The asterisks indicate statistical significance at different levels by analysis of variance: *, $P \leq 0.1$; **, $P \leq 0.05$; ***, $P \leq 0.01$; ****, $P \leq 0.001$; *****, $P \leq 0.0001$. The different colistin concentrations used in our assays are shown by color, with blue indicating no colistin, red indicating 4 μg/ml colistin, and green indicating 8 μg/ml colistin.

Inducibility of *mcr-9* gene expression. In order to evaluate whether the expression of *mcr-9* was constitutive or inducible in its original host, induction assays were performed using isolate 68A as the template for mRNA extraction. It is noteworthy that a 3-fold increase of the *mcr-9* mRNA amount was detected once the culture was induced with 4 μg/ml colistin, and a 24-fold increase was detected when using a concentration of 8 μg/ml (Fig. 4). In parallel, the transcription levels of the chromosomal *pmrA* and *pmrB* genes of *E. coli* 68A, encoding a response regulator and a sensor kinase, respectively, which may be involved in acquired resistance to colistin, remained almost unchanged upon induction (Fig. 4). Also, the amount of transcripts for the IncHI2 plasmid replicase gene remained unchanged, ruling out the possibility that induction assays have led to an increased plasmid copy number, thereby biasing the measurement of the *mcr-9* transcription level (data not shown). Of note, no induction of *mcr-9* expression was observed when performing the induction assay with ampicillin (50 μg/ml), suggesting that the colistin inducer effect was quite specific. In parallel, the same experiment with colistin (4 μg/ml) as the inducer was conducted with *E. coli* isolate Af23 harboring the *mcr-1* gene (31) and exhibiting a colistin MIC of 4 μg/ml, but no induction of *mcr-1* was noticed, highlighting the specific effect observed in *E. coli* 68A.

Induction experiments then were performed using recombinant strains *E. coli* TOP10(pNK1-*mcr-9*) (*mcr-9* gene alone) and *E. coli* TOP10(pNK1-Tot) (*mcr-9* together with downstream-located genes *wbuC*, *qseC*, and *qseB*), corresponding to low-copy-number plasmids not depending on arabinose inducibility. Although no induction of the *mcr-9* expression was observed for *E. coli* TOP10(pNK1-*mcr-9*) (data not shown), a significant increase was observed for *E. coli* TOP10(pNK1-Tot) in the presence of subinhibitory concentrations of colistin (Fig. 5). Moreover, higher expression levels of the *qseC* and *qseB* genes were concomitantly observed in the presence of colistin (Fig. 5). These data strongly suggest that overexpression of *mcr-9* observed in the presence of colistin is related to that of the QseC-QseB two-component system. Interestingly, higher expression levels were obtained in the presence of 0.5 μg/ml colistin than with 0.06 μg/ml colistin, highlighting a concentration-based modulating effect.

Origin of the *mcr-9* gene. *In silico* analysis using the GenBank databases showed that *mcr-9*-like genes were present in the chromosomes of *Buttiauxella* species strains, including *Buttiauxella ferragutiae*, *Buttiauxella izardii*, *Buttiauxella noackiae*, *Buttiauxella warmboldiae*, *Buttiauxella brennerae*, *Buttiauxella agrestis*, and *Buttiauxella gaviniae*. The most closely related gene, called *mcr-BG*, was identified in *Buttiauxella gaviniae* strain

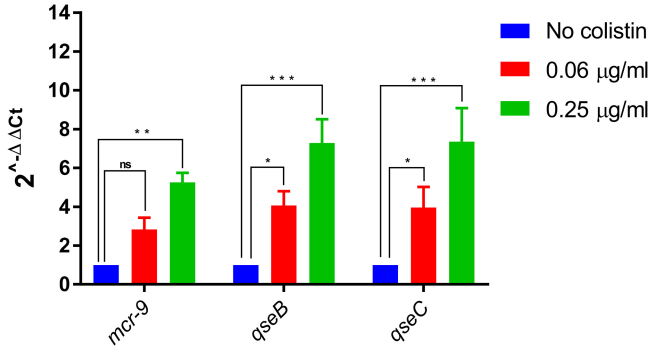

FIG 5 Expression of three selected genes cloned in *E. coli* TOP10(pNK1-Tot) with and without colistin exposure. The genes are *mcr-9*, *qseB*, and *qseC*. Fold changes in mRNA levels were determined by RT-qPCR. The data have been normalized to values for reference 16S rRNA genes. Three independent replicates were performed; the data shown represent the means, and the error bars represent standard deviations. The asterisks indicate statistical significance at different levels by ANOVA: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. The different colistin concentrations used in our assays are shown by color, with blue indicating no colistin, red indicating 0.06 μg/ml colistin, and green indicating 0.25 μg/ml colistin.

CIP106356T (GenBank accession no. NZ_LXEP00000000.1). The MCR-BG protein shared 84% identity with MCR-9 and 30 to 60% with the other plasmid-encoded MCR-type enzymes (MCR-1 to -8) (GenBank accession no. WP_064511805.1). Looking at the sequences located downstream of *mcr-BG*, a gene homologous to *wbuC* was found, but neither *qseC* nor *qseB* homologues were identified. This suggested that acquisition of the *mcr-9* gene from *Buttiauxella* spp. occurred together with *wbuC* as a whole fragment but that the *qseC-qseB* tandem originated from another source.

In fact, by performing an *in silico* BLAST analysis over the GenBank databases with those two genes, it appeared that they both shared 87% nucleotide identity with similar genes from *Klebsiella aerogenes* and *Salmonella enterica*, respectively. Induction experiments were performed using *B. gaviniae* strain CIP106356T with subinhibitory concentrations of colistin, but no overexpression of *mcr-BG* was observed (data not shown).

The *mcr-BG* gene was cloned in plasmid pBAD and expressed in *E. coli* TOP10. A minor impact of the *mcr-BG* gene on susceptibility to polymyxins was observed once cloned and produced in *E. coli*, similar to what was observed with *mcr-9* expressed in *E. coli* TOP10. After induction with arabinose, an 8-fold increase in the MIC of colistin was noticed for *E. coli* TOP10 producing MCR-BG (0.125 μg/ml) or producing MCR-9 (0.25 μg/ml) compared to that of *E. coli* TOP10 (0.015 μg/ml), while the MIC of colistin for the MCR-1-producing *E. coli* TOP10 recombinant strain was 4 μg/ml. Analysis of the LPS pattern of the MCR-BG-producing *E. coli* confirmed that a PEtN group was added to the lipid A component of the LPS of *E. coli*, while no specific peak was observed when analyzing the *B. gaviniae* strain (data not shown).

Distribution of the *mcr-9* gene. We performed an *in silico* analysis using the GenBank databases to see whether the *mcr-9* gene was already present in the available bacterial genome sequences. Surprisingly, a 100% match at the nucleotide level was found in ca. 50 genomes, all corresponding to enterobacterial isolates, recovered from worldwide sources, either from humans or animals (swine). Among the different enterobacterial species in which *mcr-9* was present were *Klebsiella* spp., *Enterobacter* spp., *Salmonella* spp., *Leclercia* spp., *Citrobacter* spp., *Raoultella* spp., *Phytobacter ursingii*, and *Cronobacter sakazakii*. Information about the colistin susceptibility status of all these strains unfortunately is not available. Diversity was observed by examining the genetic environment of the *mcr-9* gene in all these genomes, with three main structures identified (Fig. 3A to C). The IS*903B* element was always identified upstream of *mcr-9*, and the *wbuC* gene also was systematically present downstream of it. However, different IS could be identified on the right-hand extremity, being either IS*15DII*, IS*1R*, or IS*26*-like. It is noteworthy that some structures did not encompass the *qseC-qseB* regulatory genes.

DISCUSSION

We report here the identification of an MCR determinant, detected in a colistin-resistant *E. coli* isolate recovered from a patient in France. Interestingly, in contrast to other MCR determinants identified so far, MCR-9 confers only reduced susceptibility, not resistance, to colistin under normal conditions in an *E. coli* wild-type background. However, this gene showed increased expression once induced with subinhibitory colistin concentrations, leading to resistance in *E. coli* according to EUCAST breakpoints (www.eucast.org).

The fact that acquisition of *mcr-9* does not confer clinical resistance *per se* suggests a silent spread of that gene, with elevated MICs of colistin observed for the corresponding producers still categorized as susceptible. It is worth noting that we showed that the expression of *mcr-9* was inducible by subinhibitory concentrations of colistin, leading to resistance in *E. coli*. This inducible expression was shown to be related to the presence of the *qseC* and *qseB* genes downstream of *mcr-9*. Those two genes encode a putative sensor kinase (QseC) and its cognate partner (QseB), with the former likely mediating dephosphorylation of the latter (30). This tandem likely has been acquired through an independent mobilization event with respect to *mcr-9* acquisition. This is suggested by the lack of those genes downstream of the *mcr-9*-like gene in its original progenitor, *B. gaviniae*.

A so-called inducibility of *mcr-1* gene expression was recently reported; however, the phenomenon observed was quite different. In one case, it was shown that the *mcr-1* gene was truncated by IS*1294b*, leading to colistin susceptibility in *E. coli* (32). Upon colistin-based selective pressure, excision of the IS was observed, leading to restoration of the intact *mcr-1* gene and subsequently to acquired resistance. In the other case, two copies of a 22-bp DNA insertion were identified in *mcr-1* in *Shigella sonnei*, leading to colistin susceptibility (33). Upon loss of one of those two copies, restoration of the *mcr-1* open reading frame was observed, leading to colistin resistance. Here, we verified that the overexpression of *mcr-9* was not related to modifications in the sequences located in its upstream vicinity (data not shown).

Hence, we speculate that MCR-9 production is a source of colistin resistance upon selective pressure with that antibiotic, playing a significant clinical role in strain selection. The inducibility property observed with *mcr-9* was shown to be related to the two-component system present downstream of *mcr-9*. Further work will be conducted to decipher the exact interactions between those plasmid-borne two-component regulatory systems and the chromosomally encoded proteins involved in lipopolysaccharide biosynthesis, particularly those modifying lipid A.

Interestingly, *in silico* analysis identified the *mcr-9* gene in isolates recovered in different parts of the world and in a variety of enterobacterial species. It would be interesting to learn about the colistin susceptibility of those *mcr-9*-positive isolates, since that information is not available. Broad epidemiological studies will be necessary to evaluate the extent of dissemination of that gene worldwide among human and animal isolates. Detection of the *mcr-9* gene can be performed by using primers MCR-9F (5′-GGT AGT TAT TCC GCT GG-3′) and MCR-9R (5′-TCG CGG TCA GGA TTA AC-3′), which were actually validated during our study (data not shown). Looking at those genomes available *in silico*, it appeared that different genetic structures surrounding the *mcr-9* gene could be identified, with some of them lacking the *qseC-qseB* regulatory genes. We speculate that the expression of *mcr-9* is not inducible in strains possessing the latter structure.

Isolate 68A belongs to the phylogroup D-ST69 and is of serotype O15:H6. The phylogroup D-ST69 lineage includes the clonal group A strains first described by Johnson et al. (34) as ExPEC strains responsible for human urinary tract infections and resistant to trimethoprim-sulfamethoxazole. These strains have a typical virulence factor profile and exhibit specific O antigens (O11, O17, O73, and O77) always associated with the H18 type. However, isolate 68A was susceptible to trimethoprim-sulfamethoxazole, and the presence of the pS88-like virulence plasmid (35) and of the

O15:H6 serotype has not been reported in ST69. A search of ca. 12,000 *E. coli* genomes in the NCBI database identified only 3 genomes corresponding to phylogroup D-ST69-O15:H6, all being of human origin (one recovered from blood in the United States, one from an intra-abdominal abscess in China, and one from feces in France), but none of these strains possessed the *mcr-9* gene.

On the other hand, by searching the PLSD plasmid database (36), a total of 26 similar IncHI2 plasmids were identified from *E. coli*, *K. pneumoniae,* and *Salmonella enterica*, but none of them possessed the *mcr-9* gene. However, 16 out of those 29 plasmid sequences contained the *mcr-1.1* gene, further suggesting that such a plasmid backbone originates from strains present in animal reservoirs subjected to colistin selective pressure.

Isolate 68A was found to be resistant to colistin in susceptibility testing by broth microdilution (BMD), as recommended (EUCAST). However, it is noteworthy that the expression of *mcr-9* was actually induced in the presence of colistin when performing BMD. Therefore, the MIC value actually reflects the impact of MCR-9 once the corresponding gene is induced.

Analysis of the LPS profile of isolate 68A as well as MCR-9-producing recombinant strains showed that the MCR-9 enzyme confers colistin resistance the same way as MCR-1 and MCR-3 enzymes, by adding a phosphoethanolamine group to the lipid A, although the different enzymes shared significant sequence diversities. The fact that MCR-1, -2, and -3 share similar functions was previously hypothesized through an *in silico* protein structure analysis (5). Here, we showed that MCR-9, along with MCR-BG and MCR-3, exhibited a very similar three-dimensional structure.

Analysis of the genetic context of the *mcr-9* gene did not allow us to explain its acquisition by the IncHI2 plasmid. The putative involvement of IS*903B* or IS*15DII* in the original mobilization of the *mcr-9* gene from *Buttiauxella* spp. remains elusive. It is noteworthy that this plasmid did not possess any other antibiotic resistance determinant; therefore, its acquisition through a coselection pressure is unlikely.

MATERIALS AND METHODS

Bacterial isolates and susceptibility testing. The isolates were initially tested for colistin resistance using agar dilution methods. All colonies growing on plates supplemented with >2 μg/ml colistin were confirmed by the commercialized rapid polymyxin NP test (ELITech Microbiology, France) (37, 38), and MICs were determined by the BMD method using cation-adjusted Mueller-Hinton (MH) broth (38). Antimicrobial susceptibility testing for other antibiotic families was performed according to the standard disk diffusion method on MH agar plates by following CLSI recommendations (39).

WGS and bioinformatic analysis. Whole genomic DNA of isolate 68A was sequenced as previously described (22) using Illumina NextSeq 2× 150-bp technology. The sequences were analyzed with an in-house bioinformatic pipeline named PETANC, for plasmid exploration typing assembly N′ contig ordering (22), including the PlaScope tool to assess the plasmid origin of the sequence (24).

Plasmid analysis. Plasmid analysis was performed using the Kieser extraction method (26), followed by gel electrophoresis in order to further confirm the size of the plasmid containing the *mcr-9* gene using the *E. coli* strain 50192 harboring four plasmids of 154, 66, 48, and 7 kb as plasmid size markers. The determination of the incompatibility group was confirmed by PBRT (27).

Analysis of the LPS modification. The LPS of *E. coli* TOP10 (unmodified lipid A), of isolate *E. coli* 68A (*mcr-9* positive) grown in the presence or absence of colistin, of the *E. coli*(pBAD-*mcr-9*) clone carrying the *mcr-9* gene in the pBAD vector, grown in the presence or absence of L-arabinose, of *B. gaviniae*, and of the *E. coli*(pBAD-*mcr-3.12*) clone carrying the *mcr-3.12* gene as a control were analyzed by mass spectrometry (29). Lipid A was extracted using an acetic acid-based procedure as previously described (40, 41). Once extracted, 0.7 μl of the concentrate was spotted on a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) plate, followed by addition of 0.7 μl of norharmane matrix (Sigma-Aldrich, St. Louis, Missouri), and then air dried. The samples were analyzed on a Vitek MS instrument (bioMérieux, Marcy l'Étoile, France) in the negative-ion mode.

Cloning and overexpression of the *mcr-9* gene. The *mcr-9* gene was cloned into the arabinose-inducible pBAD vector in order to determine the impact of the expression of the MCR-9 putative phosphoethanolamine transferase on colistin susceptibility. Primers used for cloning are indicated in Table 1. Induction of the pBAD vector was performed using MH broth supplemented with 1% L-arabinose as previously described (42). In addition, to avoid using an arabinose-inducible vector, for the purpose of colistin inducibility assays, the *mcr-9* gene and the full *mcr-9* cassette were cloned in low-copy-number plasmid pNK1 (42) and transformed in *E. coli* TOP10, giving recombinant plasmids pNK-*mcr-9* and pNK-Tot.

Colistin induction assays. For induction assays with colistin, growth conditions were as follows. *Buttiauxella gaviniae* was grown in an LB agar plate, clinical isolate 68A was grown in an LB agar plate

TABLE 1 Oligonucleotide sequences used in this work

Primer	Sequence	Application
16S	Forward, 5′-GTGCAATATTCCCCACTGCT-3′ Reverse, 5′-CGATCCCTAGCTGGTCTGAG-3′	RT-qPCR
mcr-BG	Forward, 5′-ACTACCAGGATTACGCCTCG-3′ Reverse, 5′-GCGACTGGAAGGGTTCTTTG-3′	RT-qPCR
mcr-9	Forward, 5′-CGGTACCGCTACCGCAATAT-3′ Reverse, 5′-ATAACAGCGAGACACCGGTT-3′	RT-qPCR
mcr-1	Forward, 5′-ACACTTATGGCACGGTCTATG-3′ Reverse, 5′-GCACACCCAAACCAATGATAC-3′	RT-qPCR
repA IncHI2	Forward, 5′-GTAACCACTAAATACCCGGG-3′ Reverse, 5′-TTCCTGGTTTCGGTTTAGCC-3′	RT-qPCR
qseB	Forward, 5′-TGACATTGTGCTCCTGGATC-3′ Reverse, 5′-AGTTCGCTGAACTCGAACGG-3′	RT-qPCR
qseC	Forward, 5′-ATATGACAACCCTGACTCGC-3′ Reverse, 5′-AATTGGGTGGGTGAAAGTCG-3′	RT-qPCR
mcr-9 (for cloning in pBAD)	Forward, 5′-ATGCCTGTACTTTTCAGGG-3′ Reverse, 5′-AACAATCGATTAGCCACGGC-3′	Cloning
mcr-9 (for cloning in pNK1)	Forward, 5′-TTCTCTGATGAACGTTCCGC-3′ Reverse, 5′-TGTCACGTCAACTGGATGAC-3′	Cloning

supplemented with colistin (1 μg/ml) or left unsupplemented, and *E. coli* TOP10 pNK1-*mcr-9* and pNK1-Tot recombinant strains were plated in LB agar supplemented with kanamycin (30 μg/ml). All these strains then were grown overnight in liquid medium before the mRNA extraction assay, with kanamycin (30 μg/ml) being the selective agent of the pNK1 plasmid. A 1/10 dilution culture then was performed with or without colistin. Induction assays were performed using subinhibitory concentrations of colistin, namely, 0.12 μg/ml for *B. gaviniae*, *E. coli* TOP10(pNK1-*mcr-9*), and *E. coli* TOP10(pNK1-Tot), 2 μg/ml for clinical isolate 68A, originally recovered onto an LB agar plate without colistin, and 12 μg/ml for clinical isolate 68A, originally recovered from an LB agar plate with colistin. Primers used for cloning are indicated in Table 1.

mRNA extraction. Cultures of isolate 68A and the recombinant *E. coli* TOP10 strains harboring plasmids pNK1-mcr-9 and pNK1-Tot were used for mRNA extraction. They were grown for 3 h. Total RNA was extracted by using the TRIzol Max bacterial RNA isolation kit (Thermo Fisher Scientific, Waltham, MA) as specified by the manufacturer. The RNA samples were treated with DNase to remove contaminating DNA traces by a DNA-free kit (Thermo Fisher Scientific). Finally, RNA samples without traces of DNA were cleaned with the RNeasy PowerClean Pro cleanup kit (Qiagen, Hilden, Germany) and measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Littau, Switzerland).

RT-qPCR. A total of 200 ng of each RNA sample was reverse transcribed using the PrimeScript RT reagent kit (TaKaRa, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. Quantitative real-time PCR (RT-qPCR) was performed using a RotorGene Q (Qiagen, Hilden, Germany). Sequences of primers used are listed in Table 1. Reactions were set up in a total volume of 25 μl with a Rotor-Gene SYBR green PCR kit (Qiagen, Hilden, Germany). The selected genes in this assay were 16S rRNA (*E. coli* and *B. gaviniae* reference gene), *mcr-1*, and *mcr-9*, together with a fragment corresponding to the iteron of the IncHI2 plasmid. Three independent replicates were performed with and without induction. The obtained threshold cycle (C_T) values were analyzed by the $2^{-\Delta\Delta CT}$ method (43). Relative expression levels were calculated and compared with those of samples that were not induced. All values were corrected with values for the appropriate reference gene.

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