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MECHANISMS OF RESISTANCE

Detection of *mcr-1* Gene among *Escherichia coli* Isolates from Farmed Fish and Characterization of *mcr-1*-Bearing IncP Plasmids

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ABSTRACT The presence of the *mcr-1* gene in *Escherichia coli* isolated from retail freshwater fish was investigated. Seven (3.65%) clonally unrelated original *E. coli* isolates from grass carp were positive for *mcr-1*. The *mcr-1* genes were encoded by either chromosomes ($n = 2$) or conjugative plasmids (2 IncI2, 2 IncP, and 1 IncX4). The IncP plasmids were similar to other *mcr-1*-harboring IncP plasmids from China, though the insertion sites varied. Our report warrants further surveillance of resistance genes in aquaculture.

KEYWORDS colistin resistance, *mcr-1*, *Escherichia coli*, fish, IncP, *Enterobacteriaceae*, plasmid mediated

Since the first identification of the plasmid-mediated colistin resistance gene *mcr-1*, it has been detected worldwide, mainly in *Escherichia coli* from livestock, food, humans, and the environment (1–3). Recently, the presence of *mcr-1* has been reported in scampi, rivers, well water, and sewage from farms and hospitals (4–8), suggesting its possible spread to aquaculture. It has been proposed that aquaculture may promote the origination, mobilization, and selection of *mcr* genes (9). However, a systematic study on the prevalence of *mcr-1* genes in aquaculture is lacking. Therefore, we carried out an investigation of the *mcr-1* gene in *E. coli* from retail grass carp, one of the leading freshwater fishes produced in China.

Between March 2016 and December 2016, a total of 192 nonduplicated *E. coli* strains were recovered from 700 grass carp collected from 11 fish markets (A to K) located throughout Guangzhou, the largest trading center of aquatic products in southern China. The susceptibilities of these isolates to 19 antimicrobial agents were determined by the agar dilution method or the broth microdilution method (colistin) (10). Eight isolates showed reduced susceptibility to colistin, with MICs of 4 to 8 mg/liter, and exhibited a multidrug resistance phenotype (Table 1).

The presence of *mcr-1* was confirmed by PCR screening with specific primers described by Liu et al. (1) and by sequencing. Colistin-resistant isolates were also screened for *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* genes as detailed previously (11–14). Seven *E. coli* isolates (3.65%) were positive for *mcr-1*. One *E. coli* isolate exhibited reduced susceptibility to colistin, but was negative for all *mcr* genes. Although the origin of *mcr-1* in retail freshwater fish is unclear, improper disposal of human or animal sewage to the aquatic environment might be one source, as Cabello and Godfrey reported that aquacultural water is likely to be contaminated by human and animal pathogens (9). Additionally, *mcr-1* may occur in integrated agriculture where aquacultured fish was fed animal manure containing colistin. Furthermore, the possibility of *mcr-1* originating from aquatic bacteria could not be ruled out (15). It has been reported that enzymes encoded by *mcr-1*, such as ethanolamine phosphotransferase (EptA and PmrC), were detected in *Shewanella algae*, a member of a genus that contains opportunistic fish and

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TABLE 1 Characterization of *mcr-1*-carrying *E. coli* isolates and plasmids

Isolate	Isolation date	Market	MLST[a]	Resistance pattern[b]	Colistin (mg/liter)	Other resistance genes	Location and plasmid size (kb)	Conjugation frequency (cells per recipient)	Genetic context
GDP6F1	Sep 2016	A	ST48 (ST10 Cplx)	AMP, STR, NEO, CHL, FLR, SXT, TET, DOX	4	*aadA2*, *aadA1*, *aph(3′)-Ic*, $bla_{TEM-176}$, bla_{OXA-10}, bla_{CARB-2}, *mcr-1*, *qnrS1*, *oqxB*, *oqxA*, *cmlA1*, *floR*, *arr-2*, *sul2*, *sul3*, *tet*(A), *dfrA14*, *dfrA16*	IncP, 50.4	5.0×10^{-4}	IncP-*mcr-1*-hp-IncP
GDT6F13	Apr 2016	B	ST4014	AMP, CTX, FOS, STR, GEN, NEO, CHL, FLR, SXT, TET, DOX	8	*strA*, *strB*, *aph(3′)-Ic*, *aac(3)-IId*, $bla_{CTX-M-55}$, *mcr-1*, *oqxA*, *oqxB*, *fosA3*, *erm*(B), *mph*(A), *floR*, *sul2*, *tet*(A), *dfrA17*	chromosome		IS*Apl1*-*mcr-1*-*hp*-IS*Apl1*
GDT6F36	May 2016	C	ST7508	STR, CHL, FLR, TET	8	*strA*, *strB*, *mcr-1*, *qnrS1*, *floR*, *sul2*, *tet(A)*	IncP, 52.7	1.4×10^{-4}	IS*Apl1*-*mcr-1*-*hp*-IS*Apl1*
GDT6F38	May 2016	C	ST101 (ST101Clpx)	AMP, NEO, CHL, FLR, SXT, TET, DOX	8	*aadA1*, *aph(3′)-Ia*, *aadA2*, *mcr-1*, *floR*, *cmlA1*, *sul2*, *sul3*, *tet*(M), *tet*(A), *dfrA12*	chromosome		IS*Apl1*-*mcr-1*-*hp*-IS*1294*- IS*Apl1*
GDT6F49	May 2016	C	ST2040	AMP, CTX, STR, CHL, FLR, SXT, TET, DOX	8	*aadA1*, bla_{OXA-10}, $bla_{CTX-M-65}$, *mcr-1*, *qnrS1*, *cmlA1*, *floR*, *arr-2*, *tet*(A), *tet*(A), *dfrA14*	IncX4, 33.3	5.5×10^{-4}	IncX4-*mcr-1*-IncX4
GDT6F93	July 2016	D	ST7013	AMP, CL, STR, CHL, FLR, CIP, SXT, TET, DOX	4	*strA*, *strB*, *mcr-1*, *aadA5*, *aadA1*, bla_{OXA-10}, bla_{TEM-1A}, *qnrS1*, *cmlA1*, *floR*, *arr-2*, *sul2*, *tet*(A), *dfrA17*, *dfrA14*	IncI2, 60.8	1.7×10^{-3}	IncI2-*mcr-1*-IncI2
GDT6F97	July 2017	D	ST156	AMP, CTX, FOS, STR, GEN, NEO, CHL, FLR, CIP, SXT, TET, DOX	4	*tet*(A), *aac(3)-IId*, *fosA3*, *dfrA12*, *floR*, *strB*, *tet*(M), *aadA22*, *strA*, *aph(3′)-Ia*, *oqxA*, *oqxB*, *erm*(B), *sul2*, *blaCTX-M-14*	IncI2, 60	4.8×10^{-4}	IncI2-*mcr-1*-IncI2

[a]MLST, multilocus sequence type.

[b]Antimicrobial susceptibility was determined and evaluated according to CLSI document no. M100-S27 (https://clsi.org/standards/products/microbiology/documents/m100/). Resistance to florfenicol (>16 mg/L) and neomycin (>8 mg/L) was interpreted according to EUCAST clinical breakpoints and epidemiological cutoff values (http://mic.eucast.org/Eucast2/). AMP, ampicillin; CTX, cefotaxime; FOS, fosfomycin; STR, streptomycin; GEN, gentamicin; NEO, neomycin; CHL, chloramphenicol; FFC, florfenicol; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; DOX, doxycycline.

human pathogens (16). Furthermore, the amino acid sequence of the *mcr-1*-encoding protein is significantly similar to that of the phosphoethanolamine transferase of *Enhydrobacter aerosaccus*, an aquatic bacterium (1). The emergence of *mcr-1* in aquatic products should raise concerns, as *mcr-1* may spread globally via international trade, as evidenced by reports in Norway (4). In addition, considering high consumption of aquatic products and the habit of eating sashimi (raw fish meat), it is highly possible that *mcr-1* could be transferred directly to humans through the food chain.

Molecular typing results demonstrated that *mcr-1*-positive isolates belonged to different sequence types (STs), including ST48 (ST10 Cplx), ST4014, ST101 (ST101 Cplx), ST2040, ST7013, ST156, and a novel ST, ST7508 (Table 1). Interestingly, ST48 and ST156 *E. coli* isolates were also found as carriers of *mcr-1* in scampi and Muscovy duck, respectively (4, 17).

S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and Southern blotting indicated that the *mcr-1* genes were located on chromosomes in two isolates (GDT6F13 and GDT6F38) and on plasmids in the remaining five isolates (GDP6F1, GDT6F36, GDT6F49, GDT6F93, and GDT6F97). Streptomycin-resistant *E. coli* strain C600 was used as a recipient in the conjugation experiment to study the transferability of the *mcr-1* genes. All five *mcr-1*-bearing plasmids were successfully transferred to the recipient strain at frequencies varied from 1.4×10^{-4} to 5.5×10^{-4} cells per donor cell (Table 1).

To investigate the genetic backgrounds of the *mcr-1*-positive isolates, whole-genome sequencing was conducted on an Illumina HiSeq 2500-PE125 platform (Beijing Novogene Bioinformatics Cp., Ltd.). The sequences were assembled using SOAPdenovo (http://soap.genomics.org.cn/soapdenovo.html) and analyzed using the online tools MLST (MultiLocus Sequence Typing), PlasmidFinder, and ResFinder (http://cge.cbs.dtu.dk/services). Based on reference plasmids, *mcr-1*-bearing contigs were comparatively analyzed, and gaps between contigs were filled by PCR and Sanger sequencing. Four complete sequences of *mcr-1*-bearing plasmids were obtained, namely, IncP plasmids pHNGDF1-1 and pHNGDF36-1, IncX4 plasmid pHNGDF49, and IncI2 plasmid pHNGDF93. Previous studies revealed that the IncX4 and IncI2 plasmids were dominant carriers of *mcr-1* (18, 19). In this study, pHNGDF49 and pHNGDF93 were highly similar to those reported *mcr-1*-bearing IncX4 and IncI2 plasmids from various origins, including from animals, humans, and the environment (see supplemental material), suggesting the dissemination of these epidemic *mcr-1*-carrying plasmids to aquaculture.

Additionally, broad-host-range IncP plasmids were also the vectors for *mcr-1* in this study. *mcr-1*-positive IncP plasmids have been described in various species of bacteria in China, including *Klebsiella pneumoniae* (pMCR_1511, GenBank accession number KX377410), *E. coli* (pMCR_WCHEC1622, KY463452; pMCR3_WCHEC-LL123, MF489760; and pHKSHmcr_P2_p1, MF136778), *Citrobacter braakii* (pSCC4, NZ_CP021078), and *Salmonella enterica* serovar Typhimurium (pMCR16_P053, KY352406), originating from diverse sources such as hospital sewage, pig feces, chickens, and humans (Fig. 1) (20, 21), highlighting the significance of IncP in the transmission of *mcr-1*. The backbones of pHNGDF1-1 and pHNGDF36-1 were almost identical to that of pHNFP671 (KP324830), which belonged to a new IncP-1 plasmid clade and did not harbor *mcr-1*. pHNFP671 was found in an *E. coli* isolate obtained from swine feces in China. Comparative analysis of pHNGDF1-1, pHNGDF36-1, other IncP plasmids encoding *mcr-1*, and pHNFP671 revealed a high level of conservation across the plasmid backbones, except for pSCC4, which has an ~8,400 bp deletion of the transfer region (Fig. 1).

Although *mcr-1*-carrying IncP plasmids shared conserved backbones with pHNFP671, the variable regions were different (Fig. 1). In pHNFP671, IS*Apl1* was present ~630 bp upstream of *trbP*. Interestingly, *mcr-1-hp* and IS*Apl1-mcr-1-hp*-IS*Apl1* were also inserted within the same locus in pHNGDF1-1 and pSCC4, respectively (Fig. 1). This suggests that this locus is a hot spot for the insertion of IS*Apl1*. As for pHNGDF36-1, it represented a common mechanism mediating the spread of *mcr-1*. A composite transposon, IS*Apl1-mcr-1-hp*-IS*Apl1*, flanked by 2-bp (GT) direct repeats (DRs), was inserted downstream of *higA*, which was in agreement with the report that IS*Apl1* could generate 2-bp DRs (22).

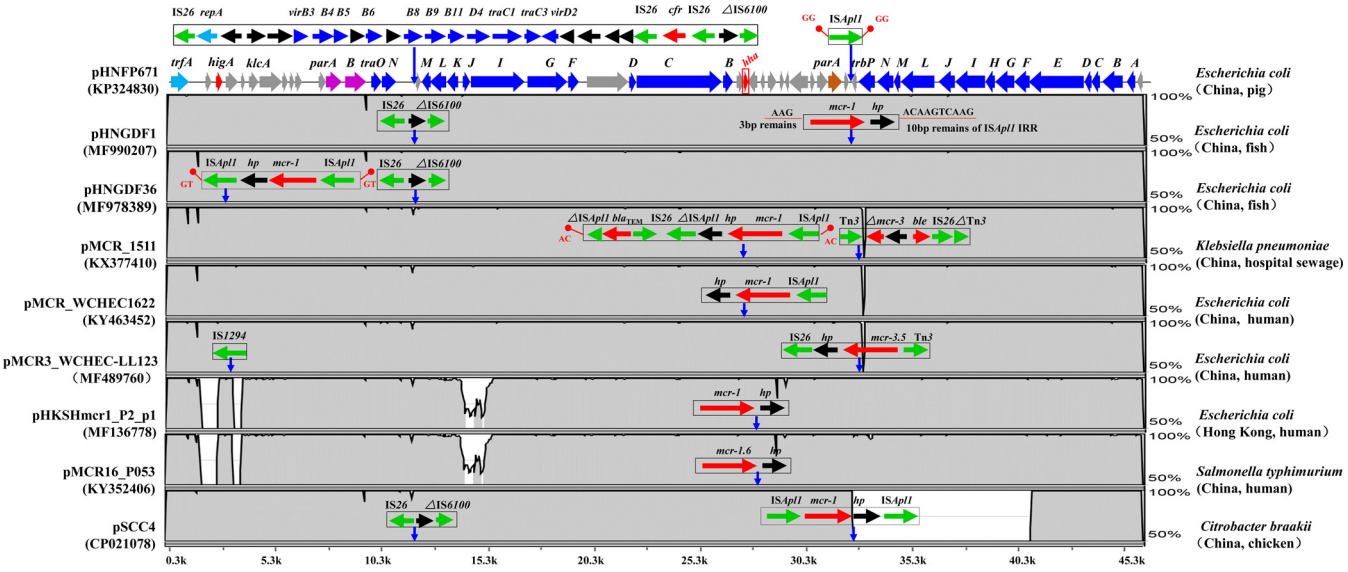

FIG 1 Levels of identity between the pHNGDF1-1, pHNGDF36-1, pMCR_1511, pMCR_WCHEC1622, pMCR3_WCHEC-LL123, pHKSHmcr_P2_p1, pMCR16_P053, and pSCC4 backbones and the pHNFP671 backbone. The scale of identity is displayed on the right. With *tra* genes shown by appropriate capital letters, the extents and directions of specific genes are shown by labeled arrows in different color. Labeled vertical arrows are used to annotate the insertion loci of mobile elements that were removed before alignment of the backbones. Sequences of other plasmids (accession numbers shown in the figure) were obtained from GenBank.

A BLAST search against the GenBank WGS database showed that the pMCR_1511-like IncP plasmid carrying *mcr-1* was also present in the *E. coli* strain CT37C A1 isolated from *Gallus gallus* feces in Netherlands (GenBank accession number FLZF01000032; 99% coverage and 99% nucleotide identity), a *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* ST34 strain GMR-S-1257 isolated from human feces in Colombia (MVPR01000083; 99% coverage and 99% nucleotide identity) (23), and three *E. coli* strains isolated from human vaginal secretions in Colombia (MVPN01000072; 99% coverage and 99% nucleotide identity). It is likely that the pMCR_1511-like IncP plasmid is widely distributed.

pHNGDF1-1 and pHNGDF36-1 were stably maintained after passage (data not shown). Additionally, pairwise competition assays were carried out using transformants, which were obtained by chemical transformation with *E. coli* DH5α as the recipient and competed with *E. coli* DH5α. It was found that that carriage of pHNGDF1-1 and pHNGDF36-1 enhanced biological fitness in the host (Fig. 2). Recently, IncP plasmids

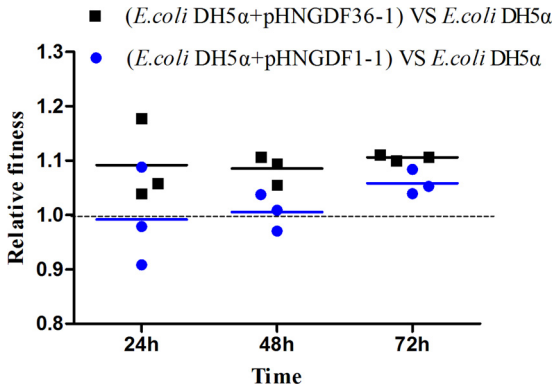

FIG 2 Fitness cost of pHNGDF1-1 and pHNGDF36-1 in *E. coli* DH5α *in vitro*. Growth competition between recipient *E. coli* DH5α and transformants containing pHNGDF1-1 and pHNGDF36-1. Each transformant was cultured in the presence of DH5α but without any antibiotics. The results were calculated and expressed as relative fitness against DH5α. The initial ratio was 1:1.

were described as mediating the dissemination of the *mcr-3* gene between *Enterobacteriaceae* and *Aeromonas* spp., which are pathogens of aquacultured fish (24). Moreover, Zhao et al. proposed that the IncP plasmid could mediate the transmission of *mcr-1* from *Enterobacteriaceae* to other Gram-negative bacteria, such as *Pseudomonas aeruginosa* (20). Thus, considering its high conjugation frequency and broad host range, the IncP plasmid may facilitate the dissemination of *mcr-1* across various hosts, and it might potentially become as dominant a carrier of *mcr-1* as the IncI2 and IncX4 plasmids.

In summary, to the best of our knowledge, ours is the first report of the *mcr-1* gene in fish products. The presence of *mcr-1* in retail freshwater fish is of great concern, given that this gene has the possibility to spread globally via international trade of aquatic products and to threaten human health through the food chain. This study warrants further investigation in aquaculture to prevent the spread of antimicrobial resistance.

Accession number(s). Plasmids pHNGDF1-1, pHNGDF36-1, pHNGDF49, and pHNGDF93 have been deposited in GenBank under the accession numbers MF990207, MF978389, MF978387, and MF978388, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02378-17.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We have no conflicts of interest to declare.

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