Rare Spontaneous Loss of Multiresistance Gene Carrying IncI/ST12 Plasmid in *Escherichia coli* in Pig Microbiota

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Resistance to extended-spectrum cephalosporins (ESCs) is a matter of considerable concern for public health. Here, we studied the spontaneous loss of an extended-spectrum beta-lactamase (ESBL)-encoding plasmid from a rifampin-resistant *Escherichia coli* isolate orally inoculated into pigs under controlled conditions. Fecal samples were collected and cultured on rifampin-supplemented medium, and the resistance of the *E. coli* isolates to ESCs was studied by phenotypic tests, PCR detection of plasmid genes, and complete sequencing. The results showed that only 3 out of 353 rifampin-resistant *E. coli* isolates were ESC susceptible, and PCR and bioinformatics analysis confirmed the loss of the plasmid. These *in vivo* experiments indicate that the loss of an ESBL-encoding plasmid seems a rare event in gut microbiota.

Resistance to extended-spectrum cephalosporins (ESCs) is a considerable public health concern. Food and companion animals may also harbor ESC-resistant *Enterobacteriaceae* in their intestinal microbiota. ESC resistance in animals in Europe is most commonly due to conjugative plasmids carrying extended-spectrum beta-lactamase (ESBL)-encoding genes. It is sometimes hypothesized that, for bacteria, the presence of a conjugative plasmid may have a biological cost so that plasmid-free bacteria would outcompete their plasmid-harboring homologs in an antimicrobial-free environment. This assumption relies on the hypothesis that either both plasmid-bearing and plasmid-free strains are present in this environment or bacteria can lose their plasmid. However, plasmids have developed sophisticated mechanisms to ensure their transmission to daughter cells during cell division (1). Although several studies have addressed the *in vitro* stability of plasmids (2–4), the *in vivo* maintenance of a resistance plasmid has never been reported, to our knowledge. Thus, we took advantage of a series of experiments including pigs inoculated with ESC-resistant *Escherichia coli* to evaluate the maintenance of an ESBL-encoding plasmid in its *E. coli* host in pig gut.

MATERIALS AND METHODS

Four different trials were conducted in 2012 to 2013, using the previously described ESBL-producing *E. coli* M63 strain (5). The experiments were performed in accordance with French animal welfare regulations, and the protocols were approved by the ANSES/ENVA/UPEC ethics committee (ComEth approval no. 12/032 and authorization no. 12-003). Briefly, *E. coli* M63 was obtained by conjugation between a randomly chosen pan-susceptible *E. coli* isolate, UB12/059-3, obtained from a specific-pathogen-free pig of our herd, made resistant to 250 mg/liter rifampin, and an ESC-resistant *E. coli* isolate, 05-M63-1, from our slaughter pig strain collection. Thus, *E. coli* M63 is resistant to rifampin because of chromosomal mutations, and sequencing of the ESC resistance plasmid showed that it was an IncI1/ST12 plasmid harboring the *bla*_{CTX-M-15}, *bla*_{CMY-2}, *sul2*, *dfrA17*, and *aadA5* genes (5). The *E. coli* M63 cultures used for pig inoculation were prepared in cefotaxime (CTX)-supplemented medium. For each trial, 6- or 7-week-old specific-pathogen-free pigs were placed in air-filtered animal facilities. Each trial included one group of 6 to 10 noninoculated pigs in one room and one or more groups of 5 to 8 pigs in separate rooms that were orally inoculated with *E. coli* M63 on day 0 (trials 04-12, 10-12, 01-13, and 06-13) and on day 1 (trial 04-12), as described

previously (5) (Table 1). Additionally, for the first trial (trial 04-12), four noninoculated contact pigs were placed into the same pen as the six inoculated ones, whereas for the other trials, all pigs in the inoculated groups were inoculated with *E. coli* M63. For the second trial (trial 10-12), from day 8 to day 12, pigs orally received either *E. coli* ED1a (6) or *E. coli* Nissle 1917 (7). For the third and fourth trials (trials 01-13 and 06-13, respectively), pigs were given or not given *E. coli* ED1a daily, from day 8 to day 13. Details of the protocols are indicated in Table 1. Individual fecal samples were collected at regular intervals after inoculation. The samples were inoculated onto MacConkey agar supplemented with rifampin (250 mg/liter). For each sample, one typical colony was transferred onto Mueller-Hinton agar for identification by PCR (8) and future storage and onto MacConkey agar supplemented with 2 mg/liter CTX to evaluate its susceptibility to ESCs. The *bla*_{CTX-M}, *sul2*, *aadA5*, and IncI1 genes were looked for by PCR (9–12) in all CTX-susceptible isolates as well as *E. coli* M63 and *E. coli* UB12/059-3, which had been used as a recipient for the preparation of *E. coli* M63. The pulsed-field gel electrophoresis (PFGE) profiles of these isolates and strains were compared after restriction with the XbaI enzyme (13). Finally, the total DNA contents of the CTX-susceptible isolates, *E. coli* M63, and *E. coli* UB12/059-3 were sequenced. Total DNA was prepared by using the QIAamp DNA minikit (Qiagen), and the Ion Shear Plus Reagents kit (Thermo Fisher) was then used for enzymatic shearing of the DNA. The size, quantitation, and quality of the fragments were checked by using a BioAnalyzer (Agilent). Bar code adapters were ligated with the Ion XPress bar code adapter kit (Thermo Fisher), and DNA fragments of 250 to 290 bp were recovered on E-Gel SizeSelect (Invitrogen). The High Sensitivity DNA kit (Agilent) was then used to allow analysis of DNA libraries before sequencing using the Ion Proton system (Thermo Fisher).

For bioinformatics analysis, sample reads were aligned with *E. coli*

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TABLE 1 Design of animal experiments and culture results^c

Trial	Group receiving M63 inoculation on day 0 (dose per pig)	Other inoculation(s) (dose per pig)	Fecal sample origin(s)	No. of samples	No. of rifampin-resistant <i>E. coli</i> isolates	No. of CTX-susceptible <i>E. coli</i> isolates/total no. of isolates
04-12	Inoculated pigs (6.8×10^{10} CFU ^a)	Reinoculation with M63 on day 1	6 inoculated pigs sampled on days 1 to 11 and days 14, 16, 18, 20, 22, 24, and 27 ^b	97	97	0/97
	Contact pigs (no inoculation)		4 contact pigs sampled on days 1 to 11 and days 14, 18, 16, 20, 22, 24, and 27 ^b	66	55	2/55
10-12	All pigs (78×10^9 CFU)	ED1a (days 8–12) (11.2×10^9 CFU)	5 pigs sampled on days 2, 3, 9, and 13 ^b	16	15	0/15
		EcN (days 8–12) (17×10^9 CFU)	5 pigs sampled on days 2, 3, 9, 13, and 16 ^b	14	13	0/13
01-13	All pigs (78×10^8 CFU)	ED1a (days 8–13) (4.4×10^9 CFU)	8 pigs sampled on days 1, 3, 7, 10, 13, 17, and 22	55	45	0/45
06-13	All pigs (73×10^6 CFU)	No	8 pigs sampled on days 3, 10 ^b , 13, 16, 20, 23, 27, and 31 ^b	55	51	1/51
		ED1a (days 8–13) (74×10^6 CFU)	7 pigs sampled on days 3, 10, 16, 20, 23, 27, and 31 ^b	42	42	0/42
		ED1a (days 8–13) (65×10^7 CFU)	8 pigs sampled on days 3, 16, 20, 23, 27, and 31 ^b	36	36	0/36

^a Pigs were inoculated on day 0 and day 1.^b Not all pigs were sampled.^c There were 48 pigs inoculated with M63 that were not yet or not inoculated with ED1a or EcN with 200 samples, with 1 being a CTX-susceptible *E. coli* isolate out of 194 Rif^r *E. coli* isolates; there were 4 contact pigs with 66 samples, with 2 being CTX-susceptible *E. coli* out of 55 Rif^r *E. coli* isolates; there were 28 pigs inoculated with M63 and then with ED1a with 149 samples, with 0 being CTX-susceptible *E. coli* out of 99 Rif^r *E. coli* isolates; and there were 4 pigs inoculated with M63 and then with EcN with 6 samples, with 0 being CTX-susceptible *E. coli* out of 5 Rif^r *E. coli* isolates. In total, there were 3 CTX-susceptible *E. coli* isolates out of 353 Rif^r *E. coli* isolates.

MG1655 (GenBank accession no. [NC_000913.3](#)) by using BWA-MEM (14) to evaluate chromosome coverage depths. When needed, we downsampled read files to fit a coverage depth of 80. We ran KmerGenie (15) and MIRA assembler (16) on raw reads for each sample with the aim of reconstituting the chromosome. Due to the large difference in the coverage depths of the chromosome and plasmid (~300-fold greater for the plasmid), we did not try to assemble the plasmid but tried to align cleaned reads with the original pESCR plasmid already available for *E. coli* M63 (5). Sequences were cleaned with Trimmomatic (17).

RESULTS AND DISCUSSION

No rifampin-resistant (Rif^r) *E. coli* and no CTX-resistant *E. coli* strains were isolated from noninoculated pigs or before inoculation with *E. coli* M63. Thus, it was hypothesized that all Rif^r CTX-resistant *E. coli* isolates were the inoculated *E. coli* M63 strain. The results of cultures from inoculated pigs showed that most samples (298/355; 84%) collected from 1 to 31 days after inoculation contained the Rif^r *E. coli* strain M63. Rif^r *E. coli* could also be obtained from 55 out of 66 fecal samples from contact pigs in the first trial. All 353 Rif^r isolates were directly inoculated onto CTX-supplemented agar medium, and 350 of them were found to be CTX resistant. They were also shown to contain the *bla*_{CTX-M}, *sul2*, and *IncI1* genes. Conversely, three Rif^r isolates could not grow on CTX-supplemented medium and did not contain the *bla*_{CTX-M}, *aadA5*, *sul2*, and *IncI1* genes. All these isolates shared the PFGE profile of *E. coli* M63. Bioinformatics analysis showed that pESCR plasmid sequences were covered 72 times on average for the M63 sample, while we observed only spurious matches (average coverage depth of <1) for other samples (*E. coli* UB12/059-3 and the three Rif^r CTX-susceptible isolates), showing the absence of the

plasmid in these samples. Chromosome assemblies of the five samples were reordered according to M63 with Mauve (18) and aligned by using progressiveMauve (19) (Fig. 1), confirming that M63, *E. coli* UB12/059-3, and the three Rif^r CTX-susceptible isolates had the same chromosomal sequences.

Two of the three Rif^r CTX-susceptible isolates were obtained from contact pig 3716 (trial 04-12), first from the sample collected 7 days after the first inoculation (day 7) and then from the sample collected on day 18. This pig had also been sampled on seven occasions before day 7, on six occasions between day 7 and day 18, and once on day 20, and for all these samples, randomly chosen Rif^r *E. coli* isolates were found to be resistant to CTX. Thus, only 2 of the 16 consecutive isolates from this pig, isolates 8 and 15, were plasmid free. Contact pig 3716 was in the same pen as four other pigs (three *E. coli* M63-inoculated pigs and one other contact pig), and the 39 isolates obtained from these four pigs were all CTX resistant.

The third Rif^r CTX-susceptible isolate was obtained from a fecal sample of pig 4613 on day 20 after inoculation (trial 06-13). The samples of this pig collected on days 13, 16, 23, 27, and 31 gave 5 Rif^r CTX-resistant isolates, and the 18 other *E. coli* isolates obtained from the three pigs in the same pen were found to be Rif^r CTX-resistant isolates.

Thus, the results of these different trials showed that the *bla*_{CTX-M} plasmid was rarely lost by the inoculated *E. coli* M63 strain. Among the 47 pigs that had been inoculated and the 4 contact pigs, only 1 inoculated pig and 1 contact pig were found to harbor plasmid-free *E. coli* M63. Because this phenomenon was detected for only 3 out of 353 isolates, it is not possible to deter-

FIG 1 Chromosome assemblies of *E. coli* M63, UB12/059-3Rif, and the three rifampin-resistant cefotaxime-susceptible *E. coli* isolates reordered according to M63 with Mauve, aligned by using progressiveMauve. M63 is the inoculated strain; UB12/059-3Rif is the recipient strain used to prepare *E. coli* M63; and 3716D7, 3716D18, and 4613D20 are the rifampin-resistant cefotaxime-susceptible *E. coli* isolates obtained from pig 3716 on days 7 and 18 and pig 4613 on day 20, respectively.

mine whether the loss of the plasmid could have been favored in contact pigs compared to inoculated ones or in mono-inoculated pigs compared to the ones that also received *E. coli* ED1a or EcN. It should also be underlined that plasmid-free M63 could be found in animals that also harbored plasmid-bearing *E. coli* M63. Plasmid-free M63 seemed to persist for at least 11 days in the microbiota of contact pig 3716, but it did not seem to spread to the other animals in the pen.

Although mathematical models showed that plasmid loss could have a major impact on the persistence of resistance in flocks of animals (20), to our knowledge, this is the first evidence of *in vivo* spontaneous plasmid loss in a commensal *E. coli* isolate. Inversely, several studies have been conducted *in vitro*, and the authors have highlighted the difficulty in precisely evaluating the plasmid loss rate in serial-transfer experiments, particularly when the loss rate is low and the presence of the plasmid results in retarded growth (21). Godwin and Slater (22) showed that in a chemostat under carbon- or phosphorus-limited conditions, culture of an *E. coli* strain harboring a multiresistant plasmid led to the loss of one or more resistances, with mutants being more competitive than the plasmid-bearing parental strain. Oscillations of the proportions of plasmid-free and plasmid-positive *E. coli* isolates were described previously by Helling et al. (2). More recently, using batch culture, Dahlberg and Chao (23) showed that after 1,100 generations, no plasmid loss occurred, but the fitness cost associated with plasmid carriage was decreased through genetic changes in both the plasmid and the host. Fischer et al. (24) observed no loss of an IncI1-carrying *bla*_{CTX-M} gene during a 24-h culture, which they explained by the presence of two addition factors in the plasmid under study, whereas Mnif et al. (25) showed that ESBL CTX-M-producing *E. coli* strains had more addition systems than did TEM ESBL producers. Thus, the pres-

ence of several plasmid maintenance systems, toxin CcdB and antitoxin CcdA proteins, and toxin RelE and antitoxin RelB proteins in the M63 plasmid (5) is probably involved in the persistence of the *bla*_{CTX-M} gene in *E. coli* M63. Finally, the *in vitro* stability of resistance plasmids was also demonstrated in pathogenic bacteria such as *Haemophilus parasuis* or *Pasteurella multocida* (26).

In conclusion, whereas the *in vivo* transfer of the *bla*_{CTX-M} plasmid of *E. coli* M63 between *E. coli* isolates was frequently observed (5), the loss of this plasmid seems a rare event in gut microbiota, reinforcing the need for better monitoring of such resistances.

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