

# Antibiotic resistance and heavy metal tolerance plasmids: the antimicrobial bulletproof properties of *Escherichia fergusonii* isolated from poultry

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**Abstract:** We describe the mobilome of *Escherichia fergusonii* 40A isolated from poultry, consisting of four different plasmids, p46\_40A (IncX1, 45,869 bp), p80\_40A (non-typable, 79,635 bp), p150\_40A (IncI1-ST1, 148,340 bp) and p280\_40A (IncHI2A-ST2, 279,537 bp). The mobilome-40A carries a blend of several different resistance and virulence genes, heavy metal tolerance operons and conjugation system. This mobilome 40A is a perfect tool to preserve and disseminate antimicrobial resistance and makes the bacterial isolate incredibly adapted to survive under constant antimicrobial pressure.

**Keywords:** plasmids, antibiotic resistance, heavy metal tolerance, poultry

## Introduction

Antimicrobials are widely used for disease prevention and growth promotion in food animals.<sup>1</sup> Global consumption of antimicrobials in food animal production was estimated at approximately 63 tons in 2010 and is projected to rise approximately to 105,596 tons, by 2030.<sup>2</sup> The globalization of the food trade makes the fight against antimicrobial resistance an international issue.<sup>2</sup> According to the Ministry of Agriculture, Livestock and Supply, Brazil is the main exporter of chicken meat in the world, making poultry industry one of the main economic activities in the country. Besides, in Brazil, a great effort has been done in order to prevent antibiotic resistance in livestock activities through the rational use of antibiotics. Poultry gut microbiome is very diversified and has been characterized as a potential reservoir of resistance genes.<sup>3</sup> Furthermore, a great effort has been done in order to track foodborne infections by antibiotic-resistant *Salmonella Enterococcus* spp., *Clostridium perfringens* and *Escherichia coli*; however, there are few data available regarding completely sequenced resistance plasmids from animal sources and even less considering plasmids isolated from other bacterial species than above-cited. The presence of resistance genes carried by mobile genetic elements, such as plasmids, increases the spread of antibiotic resistance to other bacteria of potential food safety concern. *E. fergusonii* is considered a safe bacterium, and no preventive measures to control *E. fergusonii* have been recommended. However, the use of antibiotics in the poultry industry may contribute to the emergence of multidrug-resistant (MDR) *E. fergusonii* strains.<sup>4</sup> The aim of this study was to characterize the mobilome involved the MDR phenotype of *Escherichia fergusonii* previously isolated from commercial broiler during a surveillance study in Brazil and evaluate the genetic potential of persistence and dissemination in an environment with antimicrobials usage.

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## Methods

During a surveillance study, between 2011 and 2012, 200 cloacal swabs were harvested from 40-day-old commercial broilers in two different poultry farms from Brazil and streaked on MacConkey (MC) agar containing cefotaxime (1 µg/mL) and on MC agar with ceftazidime (1 µg/mL), in order to select third-generation cephalosporin-resistant *Enterobacteria*.<sup>3</sup> Among all isolates, only two MDR *E. fergusonii* were recovered (same clone by PFGE). *E. fergusonii* 40A was phenotypically (by disc diffusion test) resistant to beta-lactams (cefotaxime, cefepime, aztreonam), quinolones (ciprofloxacin, levofloxacin), tetracycline, aminoglycosides (amikacin, gentamicin) and trimethoprim/sulfamethoxazole. Besides, S1-PFGE was performed and four different plasmids were identified in the gel, a mega-plasmid being one of them.

Therefore, in order to know the genome content and better characterize the plasmids involved in the MDR phenotype, we performed the whole genome sequencing (WGS) of *E. fergusonii* 40A using the PacBio (Pacific Bioscience) platform. *De novo* assembly was carried out with SMRT Analysis Software. The assembled genome and mobilome achieved the consensus concordance of ~99.7% and ~80X coverage. Gene prediction was performed for each plasmid sequence using Prokka pipeline<sup>5</sup> and RAST Server. The annotation was manually curated using BLASTN and BLASTP searches against GenBank NR database. Web tools provided by the Center for Genomic Epidemiology were used to predict the antimicrobial resistance genes,<sup>6</sup> plasmids incompatibility groups, multilocus sequence type (MLST), virulence-related genes and plasmid multilocus sequence typing (pMLST).<sup>7</sup> The Whole Genome Sequence project has been deposited at DDBJ/ENA/GenBank under the accession CP031282.

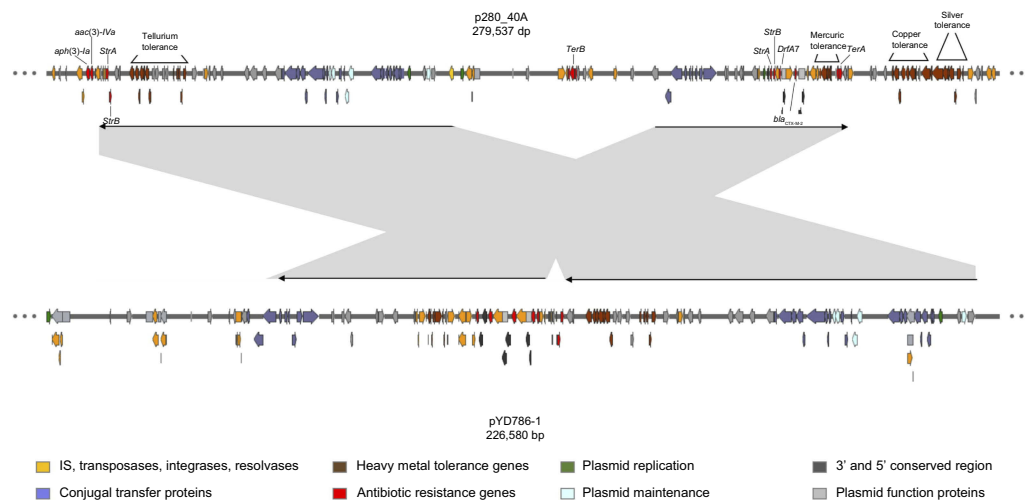
## Results

The WGS of *E. fergusonii* 40A revealed a circular chromosome spanning 4,515,966 bp and the four different plasmids p46\_40A (45,869 bp), p80\_40A (79,635 bp), p150\_40A (148,340 bp) and p280\_40A (279,537 bp) (Figure S1). In silico analysis of the genome showed that no acquired antibiotic resistance gene was present in the chromosome of 40A isolate. Meanwhile, only one conserved mutation in *gyrA* (S83L) related to quinolone resistance was detected. Besides, interestingly, two plasmids (p46\_40A and p80\_40A) also did not carry acquired resistance genes. However, both presented different families of transposases, including the Tn3 family, suggesting that they are able to capture and propagate resistance genes. Plasmid p46\_40A belongs to the IncX1

incompatibility group and shows 99% of coverage and 99% of identity with pECD227\_46 isolated from *E. fergusonii* ECD227 (CM001145) from healthy broiler chicken from British Columbia, Canada, and 100% coverage and 99% of identity with pCVM29188\_46 from *Salmonella enterica* subsp. *enterica* serovar Kentucky CVM29188 (CP001123) isolated from chicken breast meat from Georgia, USA. Plasmid p80\_40A does not belong to any incompatibility group and was considered a non-typable plasmid. Besides, p80\_40A shows low similarity with any other sequenced plasmids available at NCBI databases, being pSal8934a from *S. Typhimurium* 8934 (NC\_019111) and pBJ114-78 from *E. coli* (MF679144) the closest matches with 26% of coverage and 97% of identity, for both of them. In contrast, plasmid p150\_40A was classified into incompatibility group Inc11-ST 1. This plasmid carries antibiotic resistance genes (*tetA*, *aadA1* and *dfrA1*) and three operons related to the heavy metals manganese, mercury and nickel tolerance. Moreover, two conjugation regions, Tra (A, B, C, E–Y) and Pil (I–S, V), are present as well as restriction/anti-restriction system *ard*. Furthermore, p150\_40A shows 56% coverage and 99% of identity with pV404 from *E. coli* (LM651376) isolated from a healthy child and 66% coverage and 99% of identity p9134dAT from *S. Typhimurium* 13B5 isolated in a surveillance program taken in the Czech Republic during 1984–2002 (KF705207).

In addition to the previously mentioned plasmids, a mega-plasmid named p280\_40A was identified. p280\_40A belongs to the IncHI2-ST2 incompatibility group and carries virulence and several antibiotic resistance genes (*aph(4)-Ia*, *aac(3)-IVa*, two copies of *strA* and *strB*, *bla<sub>CTX-M-2</sub>*, *sul2*, two copies of *sul1*, *tetA*, *tetB* and *dfrA7Δ6*). Moreover, *dfrA7Δ6* is a cassette gene in a new class 1 integron, In1554. Furthermore, p280\_40A presents the two conjugation regions Tra1 and Tra2 and four heavy metal tolerance operons (tellurium, copper, mercuric and silver) (Figure 1). Three operons were carried by transposons (Tn7 carrying copper and silver operons and Tn21 carrying mercury tolerance operon). Moreover, p280\_40A shows 73% of coverage and 99% of identity with pYD786-1 from *E. coli* (KU254578) isolated from human urine samples from the USA and 75% of coverage and 99% of identity pN13-01290\_23 from *S. Heidelberg* N13-01290 isolated from turkey meat (CP012931) from Quebec, Canada.

The set of the four plasmids was here called mobilome-40A. The mobilome-40A can be considered a versatile weapon against environmental stress and antimicrobial pressure for *E. fergusonii*, because in addition to antibiotics, the non-antibiotic compounds with antimicrobial activity,



**Figure 1** Comparison between p280\_40A and pYD786-1 plasmid. Gray region shows the two main regions shared by the two plasmids. Hypothetical proteins are not represented to simplify graphing.

such as disinfectants, are currently used in the animal environment to reduce challenge by pathogenic virus or bacteria, including foodborne zoonotic bacteria such as *Salmonella*, *Campylobacter* and *Escherichia*.<sup>8</sup> It was estimated that globally each kilogram of meat harvested from cattle, chickens and pigs would lead to the consumption of 45 mg, 148 mg and 172 mg of antimicrobial compounds, respectively.<sup>2</sup> The mobilome-40A carries a resistome conferring resistance to aminoglycosides, tetracycline, sulfonamide, trimethoprim and beta-lactams and the chromosomal mutation on *gyrA* lead resistance to quinolone. Furthermore, operons related to tolerance to the heavy metals manganese, mercury, nickel, tellurium, silver, copper and cobalt-zinc-cadmium (cation tolerance operon) were found. It is known that some heavy metals are used as trace minerals required for animal health and growth (copper, zinc and nickel) in animal feed, as topical treatments of superficial wounds to prevent microbial contamination and proliferation (silver and copper), and there are also those considered feed contaminants (mercury and arsenic).<sup>1</sup> Metals can accumulate in the animal production environment and reach toxic concentrations, representing a long-term selective pressure potentially driving co-selection of antibiotic-resistant bacteria, including *Salmonella*,<sup>8</sup> when mobilomes carrying heavy metal tolerance operons are present. Besides, p280\_40A was conjugative in vitro using filter-mating protocol, suggesting that even a mega-plasmid could be spread to other bacterial species and *E. fergusonii* may work as an antibiotic resistance genes reservoir, such as *bla*<sub>CTX-M-2</sub>, harbored in this.

## Conclusion

Thus, the present study provided the WGS of a MDR *E. fergusonii* isolated from poultry showing a mobilome-40A consisting of four large plasmids, including one mega-plasmid. The characterized mobilome-40A is a “bulletproof” feature, protecting the bacteria against different antimicrobial classes and providing it a great advantage to survive under extremely unfavorable conditions.

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## Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work

## Disclosure

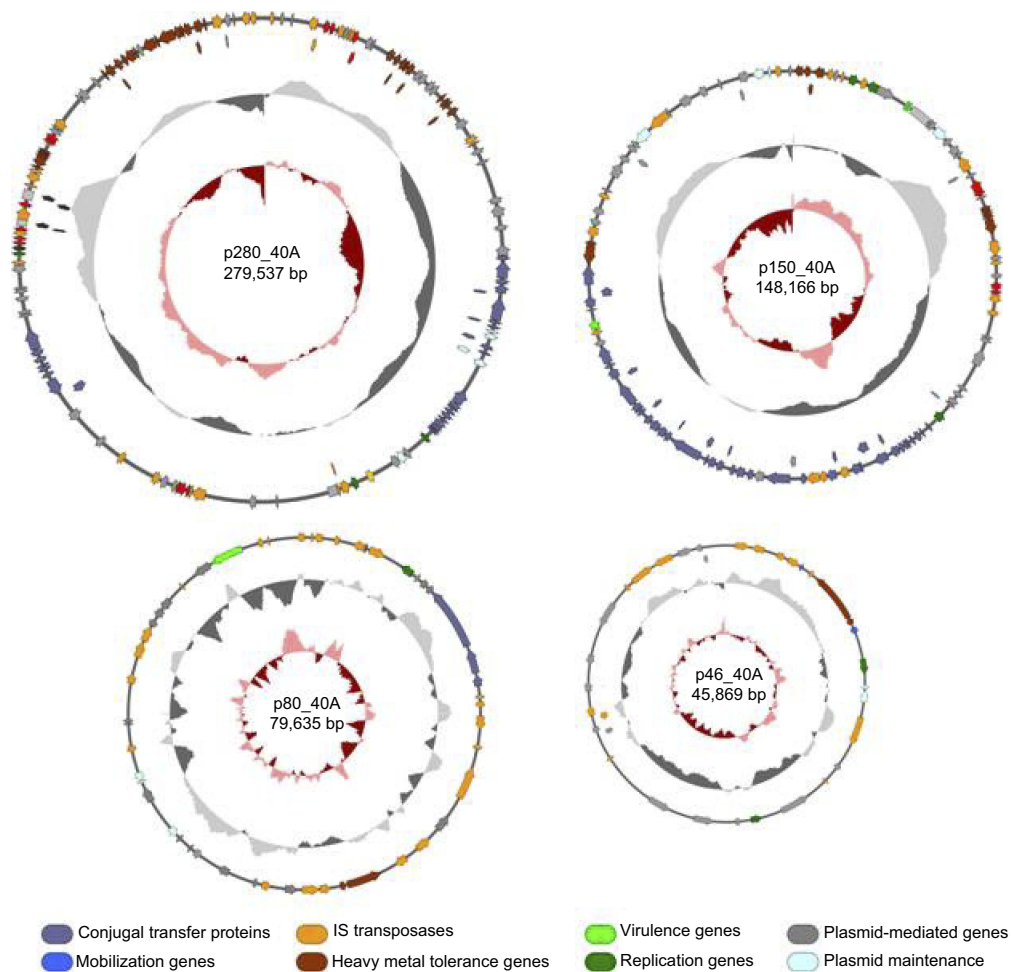
The authors report no conflicts of interest in this work.

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## Supplementary material



**Figure S1** Mobilome 40A: p280\_40A, p46\_40A, p80\_40A and p150\_40A. Big external circle: encoded genes-features (hypothetical proteins were not represented in order to simplify the graphics). Middle circle: CG content. Small internal circle: CG Skew (light red-CG skew positive; dark red-CG skew negative).

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