The *Salmonella* Genomic Island 1 Is Specifically Mobilized In Trans by the IncA/C Multidrug Resistance Plasmid Family

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Abstract

**Background:** The *Salmonella* genomic island 1 (SGI1) is a *Salmonella enterica*-derived integrative mobilizable element (IME) containing various complex multiple resistance integrons identified in several *S. enterica* serovars and in *Proteus mirabilis*. Previous studies have shown that SGI1 transfers horizontally by *in trans* mobilization in the presence of the IncA/C conjugative helper plasmid pR55.

**Methodology/Principal Findings:** Here, we report the ability of different prevalent multidrug resistance (MDR) plasmids including extended-spectrum β-lactamase (ESBL) gene-carrying plasmids to mobilize the multidrug resistance genomic island SGI1. Through conjugation experiments, none of the 24 conjugative plasmids tested of the IncFI, FII, HII, I1, L/M, N, P incompatibility groups were able to mobilize SGI1 at a detectable level (transfer frequency \(<10^{-9}\)). In our collection, ESBL gene-carrying plasmids were mainly from the IncHII and I1 groups and thus were unable to mobilize SGI1. However, the horizontal transfer of SGI1 was shown to be specifically mediated by conjugative helper plasmids of the broad-host-range IncA/C incompatibility group. Several conjugative IncA/C MDR plasmids as well as the sequenced IncA/C reference plasmid pRA1 of 143,963 bp were shown to mobilize *in trans* SGI1 from a *S. enterica* donor to the *Escherichia coli* recipient strain. Depending on the IncA/C plasmid used, the conjugative transfer of SGI1 occurred at frequencies ranging from \(10^{-3}\) to \(10^{-6}\) transconjugants per donor. Of particular concern, some large IncA/C MDR plasmids carrying the extended-spectrum cephalosporinase *bla*CMY-2 gene were shown to mobilize *in trans* SGI1.

**Conclusions/Significance:** The ability of the IncA/C MDR plasmid family to mobilize SGI1 could contribute to its spread by horizontal transfer among enteric pathogens. Moreover, the increasing prevalence of IncA/C plasmids in MDR *S. enterica* isolates worldwide has potential implications for the epidemic success of the antibiotic resistance genomic island SGI1 and its close derivatives.

Introduction

Acquisition of foreign DNA by horizontal gene transfer (HGT) is a crucial mechanism that allows bacteria to acquire new traits, and it represents a key driving force in bacterial evolution. HGT is widely recognized as the mechanism responsible for the widespread distribution of antibiotic resistance genes. Among HGT mechanisms, those specified by conjugative plasmids seem to be the most sophisticated and implicated in the diffusion of antibiotic resistance genes [1]. Bacterial conjugation appears to be a conserved molecular process by which transfer of DNA from a donor to a recipient cell occurs during a close cell-cell contact. In Gram-negative bacteria, conjugation requires the elaboration of a type IV secretion system, called the transferosome and the formation of a nucleoprotein complex involved in processing the DNA and delivering it to the transferosome, called the relaxosome [2]. Although conjugation is a conserved mechanism, conjugative systems (transferosome and relaxosome) have shown some differences depending on the incompatibility (Inc) group of plasmids [2]. Incompatibility is a manifestation of the relatedness of plasmids that share common replication controls. Incompatibility was defined as the inability of two related plasmids to be propagated stably in the same bacterial strain [3,4].

Localization of antimicrobial resistance genes on transferable genetic elements such as broad-host range plasmids, integrative conjugative elements facilitates the HGT of these genes among bacteria and provides a rapid mean of spread [5]. Certain replicon types (correlated with Inc groups) are associated with multidrug resistance (MDR) from bacteria implicated in disease outbreaks or found in food-producing animals [6]. In this context, IncA/C MDR plasmids are widely distributed among foodborne pathogens such as *Salmonella* [7]. IncA/C replicon types are associated with plasmids carrying and disseminating extended-spectrum β-lactamase (ESBL) genes in animals and humans [6]. During the
past decade, specific attention has been focused on IncA/C MDR plasmids that encode the AmpC β-lactamase CMY-2 (blaCMY-2 gene) especially among Salmonella enterica and Escherichia coli [5,6,8,9].

On the other hand, the 43-kb Salmonella genomic island 1 (SGI1) is the first genomic island conferring a MDR phenotype identified in S. enterica [10]. SGI1 is an integrative mobilizable element (IME) which contains a complex class 1 integron, named Ini104, located at the 3' end of the island [11]. The Ini104 complex integron confers the common penta-resistance profile to ampicillin (Amp), chloramphenicol (Chl), streptomycin (Str), sulfonamides (Sul) and tetracycline (Tet) of the epidemic MDR S. enterica serovar Typhimurium defined phage type DT104 (S. Typhimurium DT104) strains [10,11]. Since the identification of SGI1 in S. Typhimurium DT104, variant SGI1 MDR complex integrons have been described in a wide variety of S. enterica serovars and more recently also in Proteus mirabilis [11–14]. The identification of SGI1 in P. mirabilis clinical isolates is of great concern as the spread of the SGI1 (or variants) MDR phenotype could have significant implications in other pathogenic bacteria.

In all cases and until now, in field or clinical S. enterica and P. mirabilis strains, SGI1 is found integrated into the bacterial chromosome within the last 18 bp of the trmE gene (also named thld) [10,11]. In 2005, we reported that SGI1 could be conjugally transferred from S. enterica donor strains to non-SGI1 S. enterica and E. coli recipient strains where it integrated into the recipient chromosome in a site-specific manner [15]. Briefly, after excision of SGI1 from the Salmonella chromosome, the conjugative mobilization in trans by the conjugative helper IncA/C plasmid pR55 occurs between donor and recipient strains. In the recipient cell, the circular form of SGI1 integrates in a site-specific manner at the 3' end of the chromosomal trmE gene [15]. SGI1 appeared to be a non-self-transmissible but mobilizable element and was thus classified within the group of site-specific integrative mobilizable elements (IMEs) that are related to integrative conjugative elements (ICEs) [15].

In the present study, we report the conjugative in trans mobilization assays of SGI1 by several plasmids of different incompatibility groups. We have tested epidemic successful plasmids carrying Extended Spectrum Cephalosporins (ESC) resistance genes encoding CTX-Ms, TEM-52 ESBLs, and CMY-2 AmpC β-lactamase [16]. According to the results of our plasmid collection tested, we found that only the broad-host-range conjugative IncA/C plasmids were able to mobilize in trans SGI1. Conjugative IncH2 and IncI1 plasmids carrying ESBL genes (blaCTX-M or blaTEM) were unable to mobilize SGI1. Although certain IncA/C MDR blaCMY-2 plasmids seemed to have lost their self-transferable capacity, some conjugative IncA/C plasmids blaCMY-2 plasmids permitted the conjugative mobilization of SGI1 [5,7,8]. Our findings suggest that a close relationship, probably at a molecular conjugative process level, may exist between the IncA/C MDR plasmid family and the MDR genomic island SGI1.

**Results and Discussion**

The identification of SGI1 in the chromosome of several S. enterica serovars, i.e. Agona, Cerro, Derby, Dusseldorf, Emek, Haifa, Infantis, Kedougou, Kentucky, Kiambu, Kingston, Melaga-grids, Newport, Paratyphi B, Tallahassee, Typhimurium, Virchow, and also in P. mirabilis suggested that SGI1 might be horizontally mobilized by conjugative elements concomitantly borne by field strains [11–15]. Mobilization experiments were undertaken to determine whether SGI1 was mobilizable in trans by different conjugative plasmids previously described as spreading among MDR Salmonella strains and/or other pathogenic Enterobacteriaceae [16]. We firstly introduced by conjugation a set of 17 conjugative ESBL plasmids (CTX-M-1, -2, -9 and TEM-52) of IncH2 and IncI1 groups in the SGI1-carrying S. Agona strain 9598A97 previously described to conjugally transfer SGI1 in the presence of the conjugative IncA/C helper plasmid pR55 (Table 1 and data not shown) [15]. These ESBL IncH2 and IncI1 plasmids are representative of the ESBL plasmid families that spread among several S. enterica serovars in animal and human isolates representing a major concern [17,18]. In all mobilization assays between ESBL plasmids/SGI1-carrying S. Agona strain 9598A97 and the recipient E. coli K-12 strain BM14, no SGI1 transconjugants could be obtained in 3 repeated attempts (Table S1 in the supplemental material). In all mating experiments, control of conjugative transfer of the introduced helper plasmid was positive (data not shown). Two TEM-52 IncI1 conjugative plasmids p7778A01 and p04-3486 were initially isolated from S. Agona and S. Typhimurium field strains that also harboured SGI1 or SGI1-A variant, respectively (Table 1) [17]. Mobilization of SGI1 was also tested from these field strains and resulted also negatively (Table S1). These results indicated that SGI1 is not mobilized by our set of ESBL plasmids of IncH2 and IncI1 groups from S. enterica to E. coli. These data suggested that IncH12 and IncI1 plasmids are not able to mobilize SGI1. Moreover, these results indicated that the presence of any conjugative plasmid, which would provide all conjugal transfer functions for successful conjugation, is not the only sufficient prerequisite for mobilization of SGI1. However, our ESBL plasmid collection is mainly from S. enterica sources and does not contain plasmids of other Inc groups than H2 and I1 that are also described to harbour various ESBL genes (blaCTX-M, blaTEM, blaCMY-2) in bacterial isolates from other sources such as E. coli from humans [19].

In spite of these negative results, we decided to test reference conjugative plasmids of main different incompatibility groups previously described for plasmid typing [20–22]. Conjugative plasmids pRA1 (IncA/C), pOXY38 (IncFII), R1-16 (IncFII), R112 (IncI1), R69 (IncL/M), RPC3 (IncN), RP4 (IncP), and Rsa (IncW) were introduced by conjugation into SGI1-carrying S. Agona strain 9598A97 or SGI1-C-carrying S. Agona strain 478A97 depending on antibiotic selection used to introduce the plasmid and thereafter for the transfer of SGI1 (Table 1). The presence of each reference plasmid in S. Agona SGI1 donor strains was confirmed by PCR based-replicon typing [3]. Among all the reference plasmids tested, only the IncA/C reference plasmid pRA1 initially isolated from Aeromonas hydrophila in 1971 was able to mobilize variant SGI1-C from S. Agona donor strain 478A97 in mating experiments with the E. coli recipient strain BM14 (Table 2). E. coli SGI1-C transconjugants showed the antibiotic resistance profile conferred by SGI1-C (StrSul) and the additional resistance to sodium azide. The presence of SGI1 or variants of it was confirmed by several PCRs indicating that the entire SGI1 was presented in E. coli transconjugants and integrated into the chromosome (data not shown). The kanamycin resistance conferred by plasmid pRA1 was not transferred to the E. coli recipient. The absence of plasmid pRA1 in E. coli transconjugants was also confirmed by the specific IncA/C replicin PCR (data not shown) [3]. These results indicated that the IncA/C reference plasmid pRA1 was not transferred into the transconjugants tested and confirmed that SGI1 was mobilized in trans by pRA1. Using the IncA/C reference plasmid pRA1 as helper plasmid, E. coli SGI1-C transconjugants were found at a frequency of around 10⁻⁻ (Table 2). All other conjugative reference plasmids of IncFII, IncFII, IncI1, IncL/M, IncN, IncP, and IncW were unable to mobilize SGI1 or variant SGI1-C in repeated mating experiments.
shown). These results suggested that the in trans mobilization of SGI1, we introduced by in trans IncA/C plasmids may mobilize SGI1, and whether different S. Agona strain 959SA97 or SGI1-C-carrying S. enterica conjugative IncA/C plasmids may mobilize SGI1, we introduced by in trans IncA/C plasmids to the SGI1 variant horizontally transferred and susceptibility to specific antibiotic markers of the different helper IncA/C plasmids used in the donor strains. All these results were confirmed by PCRs on E. coli transconjugants (data not shown). Depending on the helper conjugative IncA/C plasmid used, and also on the conjugative IncA/C plasmid used, and also on the donor strain, the SGI1 transfer frequencies were found to range from $10^{-4}$ to $10^{-5}$. These results indicated that several conjugative IncA/C plasmids were able to mobilize in trans SGI1 or variants of it. Thus, these results strengthen the hypothesis that there is a specific relation between the IncA/C plasmid family and the ability to mobilize SGI1.

To further confirm this hypothesis, other current MDR IncA/C plasmids encoding the extended spectrum cephalosporinase CMY-2 were also tested in SGI1 mobilization assays (Table 1). Since 2000, the emergence of extended-spectrum cephalosporin-resistant S. enterica conjugative IncA/C plasmids into SGI1-carrying S. Agona strain 959SA97 or SGI1-C-carrying S. Agona strain 475SA97 or SGI1-F-carrying S. Albany strain 7205.00 (Table 1). A first set of historical MDR IncA/C plasmids isolated at the end of the 1960's like the reference IncA/C plasmid pRA1, i.e. pIP40a (from Pseudomonas aeruginosa in 1969), pR16a (from Providencia Stauriti in 1966), and pR55 (from Klebsiella pneumoniae in 1969) that was firstly used to experimentally mobilize SGI1, was tested in SGI1 mobilization assays (Table 1) [15,21,23,24]. E. coli SGI1 transconjugants were obtained in all mobilization assays (Table 2). Transconjugants showed the antibiotic resistance profile of the SGI1 variant horizontally transferred and susceptibility to specific antibiotic markers of the different helper IncA/C plasmids used in the donor strains. All these results were confirmed by PCRs on E. coli transconjugants (data not shown). Depending on the helper conjugative IncA/C plasmid used, and also on the S. enterica SGI1 donor strain, the SGI1 transfer frequencies were found to range from $10^{-4}$ to $10^{-5}$. These results indicated that several conjugative IncA/C plasmids were able to mobilize in trans SGI1 or variants of it. Thus, these results strengthen the hypothesis that there is a specific relation between the IncA/C plasmid family and the ability to mobilize SGI1.

*Table 1. Bacterial strains, and plasmids used in this study.*

| Strains and plasmid | Relevant genotype, resistance profile, original host or characteristics | Reference or source |
|---------------------|---------------------------------------------------------------|---------------------|
| **S. enterica SGI1 donors** | | |
| Agona 959SA97       | SGI1; AmpChlStrSulTet                                           | [10] |
| Agona 959SA97:5009:kan | SGI1:5009:kan; AmpChlKanStrSulTet                                 | [10] |
| Agona 475SA97       | IncA-C; StrSul                                                  | [30] |
| Agona 475SA97:xis:kan | SGI1-C:xis:kan; KanStrSul                                          | [30] |
| Agona 777SA01       | SGI1-A; Inc11’ (TEM-52); AmpChlStrSulTetTmcp-3GC                | [17] |
| Albany 7205.00      | SGI1-F; AmpChlStrSulTmcp                                         | [31] |
| Typhimurium 04-3486 | SGI1; Inc11’ (TEM-52); AmpChlStrSulTet-3GC                     | [17] |
| **E. coli recipient** | | |
| BM14                | K-12 JS3 derivative; F- pro met azi; Az²                        | Institut Pasteur, France |

**Plasmids**

| Plasmids                  | Genotype, resistance profile, original host or characteristics | Reference or source |
|---------------------------|-----------------------------------------------------------------|---------------------|
| IncA/C pRA1               | Tra; SulTet; A. hydrophila                                       | [32] |
| IncA/C pIp40A             | Tra; AmpKanSul; P. aeruginosa                                    | [33] |
| IncA/C pR55               | Tra; AmpChlGenKanSul; K. pneumoniae                              | [24] |
| IncA/C pR16a              | Tra; AmpKanSul; P. stuartii                                      | [21] |
| IncA/C p13688             | Tra; AmpChlGenSulStrSulTet-3GC; CMY-2; E. coli                  | [9] |
| IncA/C p13956             | Tra; AmpChlStrSulStrSulTet-3GC; CMY-2; E. coli                  | [9] |
| IncA/C pM40528            | Tra; AmpChlStrSulSulStrSulTet-3GC; CMY-2; S. Newport            | [8] |
| IncA/C pN418              | Tra; AmpChlGenKanStrSulTetStrSulTet-3GC; CMY-2; S. Heidelberg    | [7] |
| IncF1 pOX38               | Tra; Kan; F factor derivative; E. coli                         | Institut Pasteur, France |
| IncFII R1-16              | Tra; Kan; R1 derivative; E. coli                               | [20] |
| IncH2 pCEB6542            | Tra; SulTet-3GC; CTX-M-1; S. Landaoff                           | [34] |
| IncH2 p1639-5A-00         | Tra; AmpSulTeStetStr-3GC; CTX-M-2; S. Virchow                   | [18] |
| IncH2 p142-5A-01          | Tra; AmpSulTeStetStr-3GC; CTX-M-2; S. Virchow                   | [fernandez2007] |
| IncH2 p3464b              | Tra; AmpStrSulTeStetStr-3GC; CTX-M-9; S. Virchow                | [35] |
| Inc1 p777-5A-01           | Tra; Amp-3CG; TEM-52; S. Agona                                  | [17] |
| Inc1 p04-3486             | Tra; Amp-3CG; TEM-52; S. Typhimurium                             | [17] |
| Inc1 R112                 | Tra; Kan; S. Panama                                             | [21] |
| IncL/R M R69              | Tra; AmpKanTet; S. Paratyphi B                                   | [21] |
| IncN RPC3                 | Tra; KanStr; E. coli                                           | [21] |
| IncP RP4                  | Tra; AmpKanTet; P. aeruginosa                                   | [33] |
| IncW RSa                  | Tra; ChlKanSulSul; Shigella flexner                            | [22] |

*Abbreviations: Amp, ampicillin; Az, sodium azide; Chl, chloramphenicol; Gen, gentamicin; Kan, kanamycin; Str, streptomycin; Sul, sulphonamides; Tet, tetracyclines; Tmp, trimethoprim; 3GC, third generation cephalosporin.*

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Table 2. SGI1 mobilization by IncA/C plasmids.

| S. enterica donor strain | SGI1 variant | Conjugative plasmid | SGI1 transfer frequency* |
|-------------------------|--------------|----------------------|--------------------------|
| **Field strain**        |              |                      |                          |
| Agona 959SA97           | SGI1         | –                    | <10^{-9}                 |
| Agona 47SA97            | SGI1-C       | –                    | <10^{-9}                 |
| Albany 7205.00          | SGI1-F       | –                    | <10^{-9}                 |
| **Derivative of field strain** |           |                      |                          |
| Agona 959SA97:AS009:kan | SGI1:AS009:kan | –                  | <10^{-9}                 |
| Agona 47SA97:Δxis:kan    | SGI1-Δxis:kan | –                  | <10^{-9}                 |
| **Transconjugant strain** |          |                      |                          |
| Agona 47SA97            | SGI1-C       | IncA/C pRA1          | 9.8 × 10^{-3}            |
| Agona 47SA97            | SGI1-C       | IncA/C pIP40a        | 1.9 × 10^{-2}            |
| Agona 959SA97           | SGI1         | IncA/C pRS55         | 1.9 × 10^{-3}            |
| Agona 47SA97            | SGI1-C       | IncA/C pRS55         | 3.4 × 10^{-3}            |
| Albany 7205.00          | SGI1-F       | IncA/C pRS55         | 7.2 × 10^{-5}            |
| Agona 959SA97           | SGI1         | IncA/C pR16a         | 4.4 × 10^{-3}            |
| Agona 47SA97            | SGI1-C       | IncA/C pR16a         | 8.9 × 10^{-3}            |
| Agona 959SA97:AS009:kan | SGI1:AS009:kan | IncA/C pRS55       | 3.1 × 10^{-7}            |
| Agona 47SA97:Δxis:kan    | SGI1-Δxis:kan | IncA/C pRS55       | 6.7 × 10^{-5}            |
| Agona 959SA97:AS009:kan | SGI1:AS009:kan | IncA/C p13668 (CMY-2) | 2.2 × 10^{-5}            |
| Agona 47SA97:Δxis:kan    | SGI1-Δxis:kan | IncA/C p13668 (CMY-2) | 5.2 × 10^{-6}            |
| Albany 7205.00          | SGI1-F       | IncA/C pAM04528      | <10^{-9}                 |
| Albany 7205.00          | SGI1-F       | IncA/C pN418 (CMY-2) | <10^{-9}                 |

*The frequency of transfer was calculated by dividing the number of SGI1 transconjugants by the number of SGI1 donor cells. Transfer frequencies correspond to the result of one experiment which has been repeated two times and showing the same results.

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entica carrying IncA/C plasmid-mediated CMY-2 AmpC β-lactamase has been reported in numerous countries around the world. Such CMY-2 IncA/C plasmids have also been identified in E. coli isolates [8,9]. Two CMY-2 IncA/C plasmids p13688 and p13956 isolated from E. coli animal strains were introduced by conjugation from an E. coli transconjugant to S. Agona derivative strain 959SA97ΔS009::kan or S. Agona derivative strain 47SA97Δxis::kan [9]. These mutant strains contain a kanamycin marker in SGI1 that was necessary for the selection of SGI1 in mating experiments according to the large antibiotic resistance profile of these plasmids (Table 1). The ORF S009 initially annotated in the SGI1 sequence revealed to be non-functional, thus the insertion of the kanamycin marker gene would not affect the transfer of SGI1. However surprisingly, SGI1 mobilization (Table 2). Control of conjugative transfer of helper plasmid was positive for pN418 and as suspected, negative for pAM04528. As expected, these results indicated that a non-self-transferable IncA/C plasmid, i.e. pAM04528 from S. Newport, was not able to mobilize in trans SGI1. However surprisingly, the CMY-2 MDR IncA/C plasmid pN418 from S. Heidelberg, which is self-transmissible, was also not able to mobilize SGI1. These two plasmids have been previously described in different studies and seemed to be very similar [7,8]. Thus, these data suggested that there might exist genetic differences between these two CMY-2 MDR IncA/C plasmids and also between the 2 others CMY-2 MDR IncA/C plasmids isolated from E. coli strains of this study that could explain the efficiency or not to mobilize SGI1. Moreover, the difference in SGI1 transfer rates ranging from 10^{-3} to 10^{-6} could be another result of transfer function differences between IncA/C plasmids. These results are in accordance with other studies which showed that transfer rates for different IncA/C plasmids can vary by as much as 10^4 fold [7,25].

A comparative genetic analysis of the large IncA/C MDR plasmids used in this study was set up based on restriction profile and comparison of two sequenced plasmids pRA1 and pAM04528 to assess their genetic relationship and to determine if their transfer efficiency could be explained by particular traits. According to the full-length sequences of plasmids pRA1 and pAM04528, enzymatic digestion of the historical and current IncA/C MDR plasmids was

Plasmid pAM04528 was transferred by electroporation into the same SGI1 donor strain. For both plasmids no E. coli SGI1 transconjugants were obtained in 3 independent assays of SGI1 mobilization (Table 2). Control of conjugative transfer of helper plasmid was positive for pN418 and as suspected, negative for pAM04528. As expected, these results indicated that a non-self-transferable IncA/C plasmid, i.e. pAM04528 from S. Newport, was not able to mobilize in trans SGI1. However surprisingly, the CMY-2 MDR IncA/C plasmid pN418 from S. Heidelberg, which is self-transmissible, was also not able to mobilize SGI1. These two plasmids have been previously described in different studies and seemed to be very similar [7,8]. Thus, these data suggested that there might exist genetic differences between these two CMY-2 MDR IncA/C plasmids and also between the 2 others CMY-2 MDR IncA/C plasmids isolated from E. coli strains of this study that could explain the efficiency or not to mobilize SGI1. Moreover, the difference in SGI1 transfer rates ranging from 10^{-3} to 10^{-6} could be another result of transfer function differences between IncA/C plasmids. These results are in accordance with other studies which showed that transfer rates for different IncA/C plasmids can vary by as much as 10^4 fold [7,25].
performed using restriction enzyme DraI. Among the IncA/C MDR plasmids studied, the historical plasmids pR55, pR16, and pIP40a showed related DraI patterns being only different by few fragments of different sizes (Figure 1). The same result was also observed for the 4 current epidemic CMY-2 IncA/C MDR plasmids, i.e. p13956, p13688, pN418, and pAM04528 (Figure 1). However, this second set of plasmids presented really distinct profiles compared to the historical ones. Interestingly, the IncA/C reference plasmid pRA1 revealed a distinct DraI profile from all other IncA/C plasmids (Figure 1).

Sequence comparison of the two sequenced IncA/C plasmids pRA1 and pAM04528 used in this study allowed to define a common IncA/C plasmid backbone between these 2 plasmids which represents around 100 kb in size indicated as an inner red circle in Figure 2. This common IncA/C plasmid backbone showed an overall genomic synteny and a nucleotide identity ranging from 88 to 94%. Moreover, the functional predictions inferred from the plasmid annotations showed that this IncA/C backbone corresponds to essential functions of a plasmid lifestyle such as replication, maintenance, and conjugative transfer (Figure 2). The main regions of difference between these plasmids correspond to different antibiotic resistance gene clusters and insertion sequences as shown by the deviating nucleotide composition (Figure 2), which seem to be acquired by insertion/transposition events. It is worth to note that although a large common IncA/C backbone is shared, the DraI restriction profiles of these plasmids are very different from each other (Figure 1).

A recent study from Lindsey et al. showed that the combination of Salmonella, IncA/C plasmids, and MDR is very ancient. In their work, they demonstrated an interesting relation between the presence/absence of IncA/C MDR plasmids, the presence/absence of SGI1 and epidemic successful serovars of S. enterica like Typhimurium DT104 and Newport [6]. MDR plasmids belonging to the IncA/C family are widely distributed among...
Salmonella and other Enterobacteriaceae from animal sources and have caused considerable concern in public health community [6,7]. Moreover, a stronger association of MDR with IncA/C replicon was observed in Salmonella than with other replicon types [6]. This association is probably related to a highly conserved IncA/C plasmid backbone into which horizontally acquired antibiotic resistance fragments were integrated in few sites [25].

An important trait for the epidemic spread of IncA/C plasmids lies in their conjugative self-transmissibility. Welch et al. demonstrated that a large majority of IncA/C plasmids found in Salmonella strains were able to transfer horizontally [25]. However, several studies showed that the conjugal transfer could not be demonstrated for all CMY-2 MDR IncA/C plasmids of epidemic S. Newport isolates in the U.S. [25]. Poole et al. also observed that CMY-2 MDR IncA/C plasmids from Salmonella strains rarely transferred when it was the only replicon detected in the donor strain [5]. Regarding the majority of large MDR IncA/C plasmids sequenced and their self-transferability when it is known, there are few differences, which could explain the conjugative efficiency or not. The only difference identified involving tra genes was a partial duplication of traC genes associated with the duplication of the blaCMY-2 locus in non-self-transferable plasmid identified in S. Newport isolates [7,8,25]. Only one larger fragment upstream of the parAB cluster is absent from pAM04528 but present on the IncA/C reference plasmid pRA1 [7]. However, this region contains several ORFs of unknown function and only one putative ATPase which could be implicated in transfer function. When transferability of sequenced plasmids were known, no correlation was observed between the presence of this region and the self-transferability [5,7,8]. The loss of transfer efficiency of current epidemic CMY-2 IncA/C plasmids of S. Newport isolates may be due to numerous insertion sequences present on these plasmids compared to historical ones. However, several questions remain to be answered: (i) Why the self-transferable IncA/C plasmid pN418 is unable to mobilize SGI1; and (ii) What and/or where is the specific link between IncA/C transferability and SGI1 mobilization. To answer these questions, further studies need to be undertaken on specific transfer elements of SGI1 to connect them to IncA/C transfer functions.

In summary, we have shown that the MDR genomic island SGI1 is specifically mobilized in trans by the conjugative IncA/C plasmid family. Depending on the IncA/C plasmid used, the conjugative transfer of SGI1 occurred at frequencies ranging from $10^{-3}$ to $10^{-6}$ transconjugants per donor. Of particular concern, some large IncA/C MDR plasmids carrying the extended-spectrum cephalosporinase blaCMY-2 gene were shown to mobilize in trans SGI1. To the best of our knowledge, this study represents the first description of a specific relation between an incompatibility plasmid group and the specific mobilization of another mobile genetic element. Related IncA/C MDR plasmids have been detected in several fish pathogens such as Aeromonas, Yersinia, Photobacterium, Edwardsiella indicating that IncA/C plasmids mediates environmental MDR dissemination between bacteria from mammalian enteric flora and from aquatic ecosystem [7,8,23–27]. Thus, aquatic environment may be a favourable ecologic niche where horizontal transfer takes place between different bacterial genera. More than just their own dissemination, conjugative IncA/C plasmids may also contribute to the spread of the antibiotic resistance genomic island SGI1 and its close-derivatives among enteric pathogens and potentially more widely.

**Materials and Methods**

**Bacterial strains, media, plasmids and antibiotic susceptibility testing**

The Salmonella strains harbouring SGI1 or variants of it used in conjugation experiments are described in Table 1. S. enterica strains 959SA97 (serovar Agona harbouring SGI1), 47SA97 (serovar....
third generation cephalosporins, ceftriaxone (30 μg) and/or Tet (10 μg). All antibiotic disks were purchased from BioRad (Marnes la Coquette, France).

Bacterial conjugations

Bacterial conjugation was performed to introduce the different helper plasmids into the SGI1-containing S. enterica strains. Briefly, end-log exponential phase liquid cultures of an E. coli donor containing helper plasmid and a recipient SGI1-carrying S. enterica strain were mixed together in a approximately 1:1 ratio. After overnight incubation without shaking at 37°C, the mating was streaked on appropriate selective SS agar plates. Antibiotics for which resistances were conferred by SGI1, were used to select Salmonella SGI1 recipient strains and antibiotic resistances displayed by helper plasmids were chosen to select transconjugants.

SGI1 mobilization assays were performed by mixing S. enterica SGI1 donor strain harbouring different helper plasmid and the sodium azide-resistant E. coli recipient strain BM14 together with a donor-to-recipient ratio of 4:1. This broth was incubated overnight at 37°C without shaking. The next day, the cells were streaked on appropriate selective SS agar plates. Sodium azide (500 μg/ml) was used to select against Salmonella donor cells, and Str (50 μg/ml) and/or Tet (10 μg/ml) and/or Tmp (40 μg/ml) to select against unmutated recipient cells. Salmonella donors in the mating were numbered on SS agar plates supplemented with antibiotics selecting for SGI1 (Str, Tet, Tmp) and the helper plasmid (antibiotic resistance only conferred by the plasmid and not by SGI1). Appropriate control plates were performed for the conjutive transfer of the helper plasmid in each mating experiment. The SGI1 frequency of transfer was determined by dividing the number of E. coli SGI1 transconjugants by the number of Salmonella donor cells.

SGI1 PCR detection, PCR-based replicon typing, IncA/C plasmid restriction profile, and sequence analysis

Detection of SGI1 and its location in Salmonella strains and in E. coli transconjugants were performed using primers corresponding to the left and right junctions in the chromosome as described previously [15,23]. Primers U7-L12 (located in the traM gene) and 104D (located in the yidY gene) corresponding to the Salmonella chromosome and primers EcU7-L12 and Ec104D (located in the traU gene) corresponding to the E. coli chromosome were used to assess SGI1 junctions in its specific attachment site in Salmonella and E. coli, respectively (Table S1).

Conjutive helper plasmids in E. coli donor strains were typed by the PCR-based replicon typing as previously described [3]. The same method was applied to confirm the presence of the different helper plasmids in the Salmonella SGI1 donor strains. Additional PCRs were performed to amplify known resistance genes carried by the helper plasmids especially for ESC resistance genes blaTEM, blablaC, blamSET, and for phenicol resistance genes, cat and floR (Table S1).

To assess the genetic relationship between IncA/C helper plasmids used to mobilize SGI1, plasmid DNA was extracted and purified from E. coli host strains with the QIAGEN plasmid midi kit (Courtaboeuf, France) used according to the manufacturer's recommendations. According to the sequence of the IncA/C reference plasmid pRA1, plasmid DNA was digested with the restriction enzyme DdeI (Promega, Charbonnières-les-Bains, France). Fragments of DNA were separated by electrophoresis in 0.6% ultra pure DNA grade Agarose gel (BioRad, Marnes la Coquette, France).

Sequence comparisons at the nucleotide level were carried out with the BLAST algorithm using the Artemis Comparison Tool [29]. Plasmid maps of pRA1 and pAM04528 were designed using DNA plotter according the annotations in GenBank database under accession numbers FJ705807 and FJ621387, respectively. Their common IncA/C backbone was assigned for region of high nucleotide identity and of genomic synteny.

Supporting Information

Table S1 SGI1 mobilization assays by different incompatibility group plasmids.

(DOC)

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Author Contributions

Conceived and designed the experiments: AC BD. Performed the experiments: GD KP BD. Analyzed the data: GD KP BD. Contributed reagents/materials/analysis tools: GD KP AC BD. Wrote the paper: GD AC BD.

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