IncC helper dependent plasmid-like replication of Salmonella Genomic Island 1

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ABSTRACT

The Salmonella genomic island 1 (SGI1) and its variants are mobilized by IncA and IncC conjugative plasmids. SGI1-family elements and their helper plasmids are effective transporters of multidrug resistance determinants. SGI1 exploits the transfer apparatus of the helper plasmid and hijacks its activator complex, AcaCD, to trigger the expression of several SGI1 genes. In this way, SGI1 times its excision from the chromosome to the helper entry and expresses mating pore components that enhance SGI1 transfer. The SGI1-encoded T4SS components and the FlhDC-family activator proved to be interchangeable with their IncC-encoded homologs, indicating multiple interactions between SGI1 and its helpers. As a new aspect of this crosstalk, we report here the helper-induced replication of SGI1, which requires both activators, AcaCD and FlhDCSGI1, and significantly increases the stability of SGI1 when coexists with the helper plasmid. We have identified the oriVSGI1 and shown that S004-repA operon encodes for a translationally coupled leader protein and an IncN2/N3-related RepA that are expressed under the control of the AcaCD-responsive promoter P S004. This replicon transiently maintains SGI1 as a 4–8-copy plasmid, not only stabilizing the island but also contributing to the fast displacement of the helper plasmid.

INTRODUCTION

Integrative mobilizable elements (IMEs) are prevalent vehicles of the antibiotic resistance and virulence factors in bacteria (1). More than 250 IMEs have been identified to date, occurring in a wide range of Gram− and Gram+ species (1,2). Unlike integrative conjugative elements (ICEs, formerly known as conjugative transposons (3)), IMEs devoid of the complete conjugation gene set. To ensure their horizontal spread, they have to hijack the conjugative apparatus of other elements, such as conjuga-

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SGI1 is a founder element of a large IME family that was identified in a multidrug resistant (MDR) clone of Salmonella enterica serovar Typhimurium DT104 (6) in the mid-1980s (7). This pandemic clone spread worldwide, possibly due to the resistance to many antibiotics (ACSSuT phenotype) conferred by the 42.4-kb island. SGI1 and most of its variants identified in numerous S. enterica serovars and Proteus mirabilis strains (8–12) share a conserved backbone consisting of an integration/excision (int and xis) module, a rep gene encoding a putative replication initiation protein, genes related to plasmid-borne transposases. The elements with Tyr recombinases often target the 3′ end of tRNA genes or other conserved genes, while members of the other two groups show lower target specificity (1).

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mobilized exclusively by the broad host range conjugative IncA and IncC helper plasmids \( (19,20) \), whose conjugative system is classified into the MOBH12 group \( (21) \). The conjugation apparatus and several other genes of these plasmids are controlled by the FlhDC-family activator complex, AcaCD \( (22) \), which is essential for the plasmid transfer and the mobilization of SGI1 \( (14) \). In addition to the conjugation genes of the plasmid, AcaCD also activates Xis expression on SGI1 leading to the excision of the island \( (14,22) \). In absence of the helper plasmid, SGI1 is very stable \( (23) \) although spontaneous excision is still detectable by nested PCR \( (19) \). This stability is probably based on the low excision activity of the constitutively expressed Int \( (14) \) and the very low level of Xis \( (24) \). In contrast, a high rate of SGI1 loss has been detected in the presence of the helper plasmid due to the AcaCD-dependent triggering of excision \( (14) \). This effect is reduced by the TA system of the island \( (16) \), however, it cannot completely stabilize the co-habitation of SGI1 and the helper plasmid \( (14) \). Furthermore, SGI1 destabilizes the IncA and IncC plasmids \( (16,25) \) by a yet unknown manner, which might also contribute to the long-term stability of the island. The transient replication can also increase the stability of the excised element. Relaxase-dependent rolling circle replication in donor cells appears to be general among SXT/R391-family \( (26) \) and some other ICEs, like ICEBs1, ICESt3 and Tn916 \( (27) \). This type of replication begins at the nick site of oriT used as an origin of replication \( (oriV) \) and relies on the relaxase acting as the replication initiator \( (28) \). In the case of ICEBs1, this conjugation-associated transient replication is required for the stability the ICE \( (29) \). This sigma-type or rolling-circle replication may be an inherent feature of the conjugation module of ICEs, which can contribute to their stable maintenance. Another way of replication is a circle-to-circle or theta-replication, which is common in many plasmid families and has been shown to occur also in several ICEs. For example, theta-replication ensures the stability of the extrachromosomal forms of TnGBS1 and TnGBS2 from \( S. agalactiae \) \( (30) \). Regarding IMEs, similar replication mechanisms have not yet been reported, even though many IMEs encode for relaxases and proteins related to replication initiation proteins of RepA, RepC or Rep3 families \( (5) \). SGI1 also encodes for a Rep3 family Rep protein \( (S003) \), but the cognate \( oriV \) has not been identified previously \( (31) \), and the island has been thought to be a non-replicable element \( (19) \).

SGI1 not only stabilizes itself in various ways when the helper plasmid enters, but enhances the self-transfer rate by remodelling its conjugation apparatus by the AcaCD-induced expression of TraNc, TraHc and TraGc. These SGI1 proteins are homologous to and can replace the TraNc, TraHc and TraGc of the IncC plasmids and ensure some advantages for SGI1 transfer at the expense of the helper \( (32) \). Similar compatibility has been shown between AcaCD and the closely related SGI1-encoded regulator, FlhDC\_SGI1 (also known as SgaCD). The latter is able to rescue the transfer-deficiency of the \( acaCD \)-deleted IncC plasmid and activate the AcaCD-controlled promoters of SGI1 \( (33) \), however, the exact function of FlhDC\_SGI1 is not yet clear. In addition to \( xis, traN \) and the \( traGH \) operon, the ORFs \( S004 \) and \( S018 \) are also controlled by AcaCD-responsive promoters, but the functions of the putative proteins encoded are unknown. However, S004 protein, which proved to be translated from an inner START codon of ORF \( S004 \) (and named as \( S004S \)), has some negative effects on the bacterial growth \( (33) \).

As a new aspect of the complex crosstalk between SGI1 and IncC plasmids, we report here the plasmid-like replication of SGI1, which requires both activators, AcaCD and FlhDC\_SGI1. This is the first example of a helper-induced replication of an IME. We have identified the \( oriV \) and show that the Rep protein encoded by ORF \( S003 \) is related to RepA of IncN2 plasmids. The ORF \( S004 \) overlapping with \( repA \) encodes for a leader peptide and its translation is essential for RepA expression. The AcaCD-induced replication results in \( 4–8 \) circular plasmid-like SGI1 copies per cell, which is indispensable for stable maintenance when the helper plasmid is present and may also contribute to the fast elimination of the helper and the high frequency of SGI1 transfer.
MATERIALS AND METHODS

DNA and microbial techniques

Standard molecular biology procedures were carried out according to (34). For comparison of the amount of excised SGI1 with or without excess RepA, plasmid DNA was isolated from 1.5 ml overnight cultures of TG1Nal::SGI1-C/R55ΔTn5KmR strain /- pGMY9 (RepA producer plasmid) using the alkaline lysis method. Total DNA was prepared as described in (35). Enzymes were purchased from Thermo Fisher Scientific, New England Biolabs and Roche, chemicals are from Sigma, Roth and Reanal. Test/colony PCRs were performed using Dream Taq polymerase (Thermo Fisher Scientific) as described in (23). For cloning purposes, high-fidelity DNA polymerases Pwo (Roche) or Phusion (Thermo Scientific) were applied and the cloned PCR products were sequenced on ABI Prism 3100 Genetic Analyzer (Perkin Elmer). Oligonucleotides used in this work are listed in Supplementary Table S1. Primers were designed using the published sequence of R55 (GenBank: JQ010984) and the ‘excised’ SGI1 generated by deleting the first 145bp of the cloned PCR products were sequenced on ABI Prism 3100 Genetic Analyzer (Perkin Elmer). Oligonucleotides used in this work are listed in Supplementary Table S1. Primers were designed using the published sequence of R55 (GenBank: JQ010984) and the ‘excised’ SGI1 generated by deleting the first 145bp of the published SGI1 sequence (GenBank: AF261825).

Gene KO experiments were carried out by the one-step recombination method (36) using the λ Red recombinase producer plasmid pKD46 and pKD3 or pKD4 (where it is indicated) template plasmids for amplification of the KO fragments. For deletion of oriV with or without P\_cis in TG1Nal::SGI1-C, sgiPxisdelfor1-sgiPxisdelrev or sgiPxisdelfor2-sgiPxisdelrev primers were used to amplify the KO fragments, respectively. The full rep region along with P\_cis was deleted using sgiPxisdelfor1-SGI\_S004-005delrev primer pairs resulting in SGI1-C\_Δrep\_region. ORF S003 (rep\_ASGI1) was deleted using primers SGI\_S004-005delfor-SGI\_S003delrev resulting in SGI1-C\_Δrep\_A. For the construction of SGI1-C\_Δrep\_As004, SGI\_S004-005delfor-SGI\_S004-005delrev primers were used. The flhDC of SGI1-C\_Δrep\_region and the accCD of R55ΔTn5KmR were deleted using the pKD4 template plasmid and sgi006delfor-sgi007delrev and delflhDfor-delflhDCrev primer pairs, respectively. Electroporation of KO fragments was carried out using BTX Electro Cell Manipulator 600 with 2-mm gap electroporation cuvettes as described (35).

Bacterial strains listed in Supplementary Table S2 were routinely grown at 37°C in LB broth/agar supplemented with the appropriate antibiotics used at a final concentration as follows: ampicillin (Ap) 150 µg/ml, chloramphenicol (Cm) 20 µg/ml, kanamycin (Km) 30 µg/ml, spectinomycin (Sp) 50 µg/ml, streptomycin (Sm) 50 µg/ml, nalidixic acid (Nal) 20 µg/ml, gentamicin (Gm) 25 µg/ml, tetracycline (Tc) 10µg/ml. For maintaining and curing the strains were maintained at 80°C in LB broth containing 30% glycerol. In conjugation-, stability-, and RT-qPCR-assays, the interchangeable IncC plasmid R16a or R55 was applied as helper depending on the required resistance markers in the particular experimental setup. Their equivalence in excision induction and mobilization of SGI1, as well as in their self-transfer rate has previously been shown (20,15). Matings were carried out as described previously (23).

The β-galactosidase assays (37) were performed in 4 replicates as described by (14). The activity of P\_S004 promoter was measured using the β-galactosidase test plasmid pMSZ965 harbouring the upstream region of ORF S004S. To test the translation activity of RepA depending on the translation of S004 protein, the 3’ truncated rep\_A gene was fused in-frame to the eighth codon of lacZ (38) in the pMSZ1030 derivative plasmids pMSZ1032, pMSZ1034, pMSZ1037, pMSZ1039 and the β-galactosidase activity of the resulting fusion proteins was measured.

For compatibility tests, pMSZ1012 ( oriV\_SGI1 + P\_lac::rep\_ASGI1) was transformed into TG1 cells containing R16a or pCU999. Four transformant colonies were grown overnight (ON) in 2 ml LB+Cm+Km (selection for both plasmids) and 40 µl of the 10^5 x diluted cultures (ca. 500–1000 cells) were transferred into 2 ml LB+Cm (selection only for pMSZ1012) and grown ON. This passage step representing ca. 20 generations was repeated five times. Cultures from each passage were titered on LB+Cm and LB+Km plates. The frequency of Cm^R and Km^R colonies in the fifth passage was tested also by replica plating and the rate of plasmid loss was calculated accordingly.

Plasmid constructions

Relevant features of plasmids are listed in Supplementary Table S3. The methodology of plasmid constructions is described in detail in Text S1.

Alignments and phylogenetic tree construction

For construction of the phylogenetic tree of IncN RepA proteins, the selected RepA sequences were aligned by ClustalW algorithm and the tree was generated by MEGA X software package (39) using the Maximum Likelihood method with the default settings. The tree was drawn to scale with branch lengths measured by the number of substitutions per site. Bootstrapping was repeated 2000 times. For the alignment of IncN2/N3 oriV sequences, the Multalin software (40) was applied.

Construction of strains expressing RepASGI1 or harbouring attP site deriving from circularized SGI1

For chromosomal integration of rep\_ASGI1 and attP\_SGI1, the pLOFKm (41) derivative plasmids pMSZ1014 and pMSZ1041 were introduced into E. coli strain S17–1 Δpir, respectively, which allows the replication and transfer of R6K-based plasmids carrying the oriT of RK2. One colony of each transformation was used as donor strain in a standard mating (23) with TG1Nal recipient. The miniTn10 chromosomal integrants containing the rep\_A or attP site of SGI1 were selected on LB+Nal+Km plates at 37°C ON. Km^R/Nal^R transconjugants were streaked onto LB+Nal+Km plates and tested for Ap^R phenotype indicating the loss of plasmid backbone of pMSZ1014 or pMSZ1041 (conservative transposition of the miniTn10
unit). Single-copy integrations of miniTn10::repASGI1-KmR or miniTn10::attPSGI1-KmR were confirmed by Southern hybridization. Total DNA was extracted from three parallel colonies of both transconjugants and ~400 ng DNA was digested with multiple restriction enzyme combinations. DNA blots using Hybond-N nylon membrane (Amersham) and labelled DNA probes were made using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim) according to the manufacturer’s instructions. DIG-labelled probes were amplified with primers S003_BXhrev-pxissequrev for miniTn10::repASGI1-KmR insertion and LJ3-RJ5 for miniTn10::attPSGI1-KmR insertion.

### Mobility shift assay

**Purification of RepASGI1.** Overnight culture of *E. coli* strain Tuner (DE3) (Novagen) transformed with pMSZ1066 (RepA-producer plasmid containing PrT::repASGI1) was diluted 100 × in 25 ml fresh LB+Ap medium and grown to OD600 of 0.5 at 37°C. The culture was induced with 0.2 mM IPTG at 30°C for 5 h under vigorous shaking. Bacteria were harvested by centrifugation and resuspended in 1 ml buffer (50 mM Tris pH 8.1, 300 mM NaCl, 0.01% Triton X-100) supplemented with 60 μg/ml lysozyme and 30 μl of Complete protease inhibitor cocktail (Roche). Cells were frozen at −70°C, then thawed on ice and sonicated at 50% activity for 4 × 10 s. The lysate was centrifuged at 16 000 × g for 30 min at 4°C and RepASGI1 was purified from the supernatant (cleared lysate) using the Dynabeads® Histag Isolation & Pulldown Kit (Novex life Technologies) designed for the isolation of histidine-tagged protein by magnetic separation. The purified protein (final concentration of ca. 600 ng/μl) was kept on ice until use.

**Labelling of oriV fragments.** The high copy pBluescript-derivative plasmids, pMSZ1095 and pMSZ1098 were digested with EcoRI-BamHI, while pMSZ1120 was digested with XhoI–SacI to isolate the 209 bp, the 124 bp and the 83 bp DNA fragments containing the complete or partial oriV sequences, respectively. 100 ng DNA from each fragment was DIG-labelled by terminal transferase (TdT) using the second Generation DIG Gel Shift Kit (Roche) according to the manufacturer’s protocol. The labelling reactions were verified by dot-blotting to ensure that they produced the expected amount of labeled DNAs.

**EMSA assays.** Binding reactions were performed in binding buffer (20 mM Tris pH 8.1, 60 mM NaCl, 40 mM KCl, 0.1 mM EDTA, 1 mM Mg-acetate, 2.5 mM ATP, 1 mM DTT, 5% glycerol), containing 1 μg poly [d(I-C)], 0.1 μg poly-l-lysine, 0.1 ng labelled DNA and different amounts of purified RepA protein (0, 0.3, 0.6, 1.2, 2.4, 3.6, 6.0 μg) in a final volume of 20 μl. For the binding specificity test, 0.1 ng labeled and 250-fold unlabeled competitor DNA fragments were mixed and 1.2 μg protein was added to the binding reaction. Binding reactions were kept on ice for 15 min and 8 μl samples were loaded onto a 5% non-denaturing polyacrylamide gel. Gel electrophoresis was performed in TBE buffer at 8 V/cm and 4°C. Protein–DNA complexes were electro-transferred and crosslinked to a Hybond-N+ membrane (Amersham) using LKB 2117 electrophoresis unit and Amersham ultraviolet crosslinker, respectively. The DIG-label was detected by chemiluminescence and film exposure using alkaline phosphatase-conjugated anti-DIG antibody and CDP-Star according to the protocol of DIG Gel Shift second generation Kit (Roche).

### RT-qPCR assays for relative quantification of attB, attP and repA

Real-time quantitative PCR assays were used to measure the amount of excised circular SGII (attP), and the unoccupied integration site (attB) per cell in TG1Nal::SGII-C strain with or without R55^Atb^87. The amount of attB and attP were normalized to the amount of single-copy chromosomal gene (trmE) in each sample. An *E. coli* strain, TG1Nal::attPSGI1, containing single chromosomal copies of attB and attP, was constructed to calibrate the RT-qPCR assay. The LJ3-RJ5, attsigf2-attsgirev2 and attsigf2-attgirev3 primer pairs were used to amplify the 251 bp attP, the 257 bp attB and the 207 bp trmE specific fragments, respectively. The RT-qPCR reactions were performed in a final volume of 10 μl using a LightCycler®480 detection system (Roche). Each reaction mixture contained 1 × qPCR-BIO SyGreen Lo-ROX master mix (PCR Biosystems), 400 nM of forward and reverse primers and 1 μl *E. coli* cultures (OD600 = 0.5) as templates. The PCR conditions were as: initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 15 s and 60°C for 30 s. Specificity was confirmed by determining the melting curves and agarose electrophoresis of the final PCR products. At least three reactions were performed for each sample. The relative amount of the amplified attP and attB sequences was calculated based on the Ct deviation of an experimental sample compared to the control sample (TG1Nal::attPSGI1) and was expressed in comparison to the calibrator (trmE) sequence. Consequently, the ratio of attP and trmE measured the copy number of excised SGII and the ratio of attB and trmE measured the excision of the island.

The copy number of pMSZ1016 was measured as the ratio of repA and trmE. The primers SGII_repfor-SGII_reprev were used to amplify the 239 bp repA fragment. The qPCR reactions were performed as described above. The relative amount of the amplified repA sequences was calculated based on the Ct deviation of an experimental sample compared with the control sample (TG1Nal containing pMSZ1016 and pGMY6 grown without IPTG induction) and was expressed in comparison to the calibrator (trmE) sequence.

### SGII stability tests

For monitoring the stability of SGII in the ‘rep’ mutant strains harbouring an IncC plasmid, R55^Atb^87 was conjugated from *E. coli* TG90 into the TG1Nal recipient strains carrying SGII-C KO mutants for oriV, repA, P^SGII^ or FlhDCSGII. Conjugation assays were carried out in 4–6 replicates as described (23). Transconjugants were selected on LB+Nal+Cm plates and then replica plated onto LB+S+m+Splates to count Sm^3^/Sp^3^ (SGII-free) segregants.
RESULTS

SGI1 encodes for a functional RepA protein related to the replication initiators of IncN plasmids

ORF S003 of SGI1 has been annotated as a repA gene (31), but the role of its putative protein product has never been demonstrated. The repA transcript was previously reported to be one of the less abundant mRNAs synthesized from a chromosomally integrated island (24) and it was not clear whether this gene is functional at all. Pfam search indicated that the putative RepA protein of SGI1 belongs to the RepA_C-domain-containing proteins and further comparative analysis suggested that it is related to the replication initiator proteins of the broad host range plasmids of IncN family (42) (Supplementary Figure S1). BLASTp search using the amino acid sequence of RepASGI1 showed 10–60% similarities to IncN RepA proteins, depending on the subgroup of IncN family. RepASGI1 significantly differed (<10% identity) from the RepA proteins of IncN1 plasmids (43), and appeared much more similar (50–60% identity) to those of IncN2 group (42,41) and pN-Cit classified as IncN3 (44). The phylogenetic reconstruction based on the RepA proteins did not support the separation of IncN2 and IncN3 groups, while IncN1 was clearly distinct from them and possibly forms a different family. Although RepASGI1 was located on a separate branch of the tree along with several RepA proteins of unclassified plasmids, this cluster appeared more closely related to the IncN2 and IncN3 than to the IncN1 group.

To examine the functionality of the putative IncN-related replicon, the repA gene of SGI1 was cloned into the p15A-based expression vector, pJKI391, and placed under the control of P lac promoter. The resulting RepASGI1-producer plasmid pGMY9 was transformed into the E. coli strain TG1Nal::SGI1-C and the transformants were selected for SGI1 (Sm R/Sp R) and pGMY9 (Km R) (lane 3) and digested with PstI. Black arrowheads point to the characteristic 2.26, 1.16 and 0.73 kb fragments deriving from the circular SGI1-C. The bracket shows the additional SGI1 fragments (7.75, 5.27, 4.49, 4.25 kb) with or without the 6.3 kb linearized RepA producer plasmid, pGMY9. For comparison, lanes 1* and 2* shows the original lanes 1 and 2 with high contrast to make the fragments of the very low copy plasmid-like SGI1 visible. (B) Schematic representation of the cells in the three populations of panel A. SGI1 is shown as a bar integrated into the chromosome or as free circular plasmids. RepA gene is indicated as an orange box. The helper is shown as a single plasmid with the acaCD activator gene (blue bars) required for triggering RepA expression from SGI1, while pGMY9 expressing RepASGI1 from the P lac promoter (open arrowhead) is drawn as an ellipse.

The transformants should be viable when the same oriV is located extrachromosomally. SGI1 was known to be excised in the presence of IncC plasmids (14), i.e. they ensure the extrachromosomal location of SGI1 including its oriV. Therefore, the transformation of pGMY9 was repeated into the same host but containing the IncC plasmid R55 ΔTn6187, and the transformants were selected for SGI1 (Sm R/Sp R), R55 (Cm R) and pGMY9 (Km R). As expected, triple-resistant transformants were obtained and their rate was ca. 5 × 10−3 CFU/0.1 μg DNA.

To prove the replication of SGI1-C, plasmid DNA was isolated from transformant colonies and analyzed on agarose gel. SGI1-specific bands were not observed without R55 ΔTn6187 and pGMY9, but in the presence of both plasmids, strong SGI1-specific fragments appeared along with the band characteristic for pGMY9 (Figure 2A, lanes 1, 3). This confirmed that the repA gene of SGI1 encodes for an active RepA protein, which promotes the replication of the excised circular SGI1 and also proved that SGI1 has a functional oriV. On the other hand, in the presence of R55 ΔTn6187 without pGMY9 (lack of excess RepA), the excised SGI1 could not reach such a high copy number observed with pGMY9, however, the weak SGI1-derived bands, especially the characteristic 1.16 and 2.26 kb fragments, were also visible in the plasmid preparation from TG1Nal::SGI1-C/R55 ΔTn6187 strain (Figure 2, lanes 2 and
can be detected only by nested PCR (19). In contrast, when the $R_{55}^{A\text{tn}6187}$ helper plasmid was present in the cells, SGI1 was excised in more than 90% of the population. Moreover, the amount of $attP$ per cell was 4–8 times higher in this population than in the control strain TG1NaI::$attP_{SGI1}$, referring to a significantly increased copy number of the free circular SGI1 form (Figure 3B). These findings clearly indicated a helper-dependent transient replication of the excised island, maintaining an average of 6.6±1.9 extrachromosomal SGI1 copies per cell.

**Identification of oriV sequence of SGI1**

During the previous analysis of the noncoding region between $xis$ and $repA$, imperfect direct repeat (DR) motifs were found, which proved not to be involved in the regulation of $xis$ gene (14), but resembled the iteron-like repeats of oriV regions of many plasmids (45,46). Based on the homologies with IncN2/N3 plasmids, the putative oriV was predicted downstream of $repA_{SGI1}$, BLASTn alignment of downstream sequences of $repA$ in the related plasmids and SGI1 revealed a ca. 70% conserved 185-bp noncoding region beginning 50 bp downstream of the STOP codon of $repA_{SGI1}$ (Supplementary Figure S2). This region includes six putative DnaA binding sites, a 15-bp inverted repeat (IR) motif and the previously recognized iteron-like segment with four 16-bp DR motifs (Figure 4A). These DRs differ at several positions and can be characterized by the GGGGHRATTTAATCGY consensus.

To examine the functionality of the predicted oriV, the 1901–2161 bp region located downstream of $repA_{SGI1}$, including the conserved segment and additional two IR motifs and two putative DnaA-boxes, was cloned into a conditionally replicating R6K-based CmR vector pSG76-CS. The resulting plasmid, pMSZ1003, and the parental pSG76-CS vector were introduced into the $E.\ coli$ strain TG1NaI::$repA_{SGI1}$, which expresses the $RepA_{SGI1}$ protein from a chromosomally integrated $P_{lac::repA}$ cassette but does not support the maintenance of R6K-based replicons. While no CmR transformants were obtained with pSG76-CS, transformation with pMSZ1003 resulted in 1.2±0.4×10^6 CFU/0.1 μg DNA. Restriction mapping of plasmid DNA from several transformant colonies confirmed the presence of pMSZ1003 (Supplementary Figure S3A), indicating that the cloned fragment contains the functional $oriV_{SGI1}$.

As a next step, the $P_{lac::repA}$ cassette was inserted into pMSZ1003 and the resulting pMSZ1012 was introduced into the $polA$ mutant $E.\ coli$ strain and its isogenic wild type counterpart (ME6266 and ME7772, respectively). The transformation resulted in ca. 10^9 CmR CFU/0.1 μg DNA in both strains. The plasmid DNA isolated from several colonies also indicated that pMSZ1012 replicates in a PolA-independent manner (Supplementary Figure S3B). The SGI1-derived minimal replicon was fully replicable in $E.\ coli$ strain TG1 containing the KmR pCU999, a derivative of the IncN1 plasmid pCU1, supported by the analysis of plasmid DNA isolated from TG1/pCU999+pMSZ1012 cells (Supplementary Figure S3C). In addition, a compatibility test was performed with these transformants. No pCU999 loss (<0.5±0.3%) was observed during 100 generations in the
presence of pMSZ1012 without a selection for pCU999, confirming their compatibility. A similar assay was carried out with the IncC plasmid R16a, which also proved to be stable together with the minimal SGI1 replicon (loss of pCU999 was not detected, <0.7±0.2%).

In order to further specify the minimal oriVSG1, a deletion analysis was performed. Maintenance of pMSZ1003-like plasmids carrying shortened oriV regions was tested in the RepA expressing strain TG1Nal::repASG1 as described above. Transformation of plasmids pMSZ1073, pMSZ1074, pMSZ1075 carrying the four DRs with different lengths of the 3′ flanking region but lacking the IR1 motif resulted in no CmR transformants, indicating that these constructs are not able to replicate even in the presence of RepASG1 (Figure 4A). Similarly, the plasmids pMSZ1165 and pMSZ1164, lacking one or two copies of the DR motifs, could not replicate. In contrast, all the other clones resulted in viable transformants. The shortest replicable plasmid was pMSZ1071 possessing a 155-bp sequence extending from 1929 to 2083 bp of SGI1. Nevertheless, this construct resulted in less and smaller colonies than the others. Moreover, the pMSZ1071 transformants contained less plasmid DNA, referring to its lower copy number (Figure 4B). pMSZ1070, containing the oriV segment from IR1 to IR2R and pMSZ1113, lacking IR2R but containing an additional 28 bp flanking the IR1L at the 5′ end, replicated similarly to any other constructs harbouring longer oriV segment. Interestingly, pMSZ1071 containing the common region of the fully replicable pMSZ1070 and pMSZ1113 was probably less stable (gave smaller colonies) and maintained at a lower copy number. Altogether, we showed that the 1929–1973 bp AT-rich region with the 15 bp imperfect inverted repeat (IR1R has 3 bp insertion compared to IR1L) and the well-conserved 1974–2083 bp core region containing four DRs and the five potential DnaA binding sites are essential for SGI1 replication. The 2084–2123 bp region, which is highly conserved in IncN2/N3 plasmids (Supplementary Figure S2), and the 1901–1928 bp sequence may contain auxiliary sequences (possibly the further three DnaA sites) that are not essential but can facilitate the replication.

Detection of RepA-binding to oriV

The sequence-specific interaction of RepASG1 with the oriV region was investigated by EMSA using the purified Histagged RepASG1 protein (Supplementary Figure S4) and different fragments of oriVSG1. First, the fully replicable fragment of SGI1 containing the most conserved part of oriVSG1 (1929–2123 bp cloned in pMSZ1070, Figure 4) was end-labelled and used to optimize the binding conditions. When using a higher amount (≥2.4 μg) of RepA protein, four slower migrating complexes were detected (not shown), indicating that four binding sites occur on this fragment. We assumed that the four binding sites correspond to the four 16-bp DRs located in the central region of oriV, thus in the second binding assay, the labelled DNA probe was shortened to the four DRs of oriV (1974–2083 bp). As expected, increased amount (0.3–6 μg) of RepA protein also resulted in four retarded bands indicating the consecutive filling of
the four binding sites (Figure 4C). To test whether the RepA binds to a single DR sequence, the 2035–2055 bp SGI region containing the DR3 was used in EMSA. The shifted band already appeared when using 0.3 μg RepA and no further complexes were detected with higher amounts of the protein (Figure 4D).

The role of FlhDC-family activators in SGI1 replication

The results described above clearly suggest that SGI1 is capable for plasmid-like replication, which occurs only in the presence of the helper plasmid. Thus, we assumed that IncC plasmids can induce SGI1 replication. The last four codons of ORF S004 overlap with repA and this region is located inside of a long IR motif that can form a potential stem-loop structure on the mRNA transcript (Figure 5A). Similar genetic constitution often occurs in different plasmid families, where a leader protein contributes to the control of repA expression (47). The translation of leader peptides is required for or enhances the translation of the downstream reading frames (48). This analogy and our previous observation that expression of ORF S004 is activated by AcaCD and FlhDCSGI1 (33) suggest that repA is expressed as a part of the S004-repA operon under the control of the AcaCD-responsive promoter P_{S004}. Since the expression from a test plasmid carrying the lacZ gene fused to the START codon of repA was not detectable even in the presence of excess AcaCD (33), the possibility of AcaCD/FlhDCSGI1-controlled repA expression was re-examined here using newly designed plasmid constructs (Figure 5A). For this, two methods were applied: (i) the translation of repA gene and S004 was monitored by β-galactosidase assays in hosts producing one or both activators and (ii) the replication ability of the SGI1-derived minimal replicon was tested under the same conditions.

For the first approach, two β-gal tester plasmids were constructed where the lacZ expression was controlled by the AcaCD-responsive promoter P_{S004}, the putative promoter of the S004-repA operon. Plasmid pMSZ1032 contained a translational repA-lacZ fusion (the codon 14 of repA_SGI1 was fused in frame to the codon 8 of lacZ), retaining the long IR motif. Therefore, the β-galactosidase activity measured with pMSZ1032 referred to the translation level of RepA_SGI1. In the other tester plasmid, pMSZ965, the START codon of lacZ was placed at the ATG of ORF S004_SGI1, thus the amount of RepA and no further complexes were detectable in the absence of both activators, while it was measurable either in the presence of any p15A-based producer plasmids providing the activators in excess or if both activators were supplied from their original sources. In contrast, transcription from P_{S004} was undetectable in hosts with ΔacaCD or ΔflhDC_SGI1 background, indicating that none of the activators could induce P_{S004} alone at a concentration measured with pMSZ965 referred to the translation level of RepA.
centration supplied by the helper plasmid or SGI1 (Figure 5B). Translation measurements using pMSZ1032 correlated well with the transcriptional activities of pMSZ965 in most cases, indicating that RepA production is under the control of P\text{S004S} and its activators. One exception was the non-induced FlhDC\text{SGI1}-producer host, in which the promoter activity was well detectable (24.9±2.8 units of β-gal) while the translation activity was lacking (≤0.8±0.6 units of β-gal). Interestingly, pMSZ1032 could not be introduced into the TG1Nal/pJK1888 strain expressing AcaCD from \text{P}_{\text{ac}} even without IPTG-induction, which could be due to the cumulative negative effect of the overexpressed AcaCD and the high level of AcaCD-induced S004S protein (33) on the bacterial growth. Thus, in order to detect the translational activity, a lower level of AcaCD expression was achieved using pJK1828. This plasmid contains the entire operon encoding AcaCD under the feedback control of the repressor Acr1 (22) and previously has been shown to cause much weaker expression than the uninduced pJK1888 producing AcaCD by leaking of \text{P}_{\text{ac}} (14). Our data supported the previous observations that the efficiency of the two activators is different (33) (compare hosts 3 and 4 in Figure 5B) and the expression level of RepA depends on the amount of activators. Furthermore, both activators were necessary for a detectable RepA expression if they were supplied from the helper plasmid and SGI1 under their original expression control.

As a second approach, the maintenance and copy number of the SGI1-derived minimal replicon were investigated in the activator-producing hosts applied in β-gal assays. The suspected minimal functional rep region of SGI1 (1901–3494 bp including the oriV sequence and the S004-repA operon with the −10 box and the AcaCD-binding site of P\text{S004}) was joined to a CmR gene (Supplementary Figure S5). Therefore, maintenance of the resulting construct pMSZ1016 was possible only if the expression of RepA was induced by AcaCD and/or FlhDC\text{SGI1} through the promoter P\text{S004}. In case of the p15A-based producer plasmids, the impact of different amounts of the activators was examined. In hosts carrying a helper plasmid, R5\text{S}^{\Delta\text{Tn}687} was replaced with R16a due to its more convenient resistance. Replication of pMSZ1016 in these hosts was monitored by determining transcriptant frequencies, RT-qPCR-based copy number measurement and analysis of plasmid DNA prepared from viable transformants (Table 1, Supplementary Figure S5). The results correlated well with the outcome of β-gal assays: transformants were obtained only if either activator was expressed from a p15A-based producer plasmid (AcaCD from pJK1828 or pJK1888 and FlhDC\text{SGI1} from IPTG-induced pGMY6) or both were expressed from R16a and the chromosomally integrated SGI1-C\text{rep-region} (Table 1). As expected, pMSZ1016 proved not to be replicable (i.e. no transformant colonies were obtained) in hosts with ΔacaCD or ΔflhDC\text{SGI1} background. This supported again the conclusion that both activators are required for SGI1 replication under the quasi-natural situation when they are supplied by the coexisting helper plasmid and SGI1. In this case, pMSZ1016 was maintained in a similar copy number (4–5/cell) measured previously for the wt SGI1 in the presence of R5\text{S}^{\Delta\text{Tn}687}, while higher copy numbers were observed if either AcaCD or FlhDC\text{SGI1} alone was present in excess. Unlike FlhDC\text{SGI1}, overexpression of AcaCD proved to be lethal for pMSZ1016 transformants likely due to the same reason as for pMSZ1032 in β-gal assays. Therefore, pJK1828 producing a lower level of AcaCD was used for comparison with the uninduced pJK1888. The frequency of the transformants and their pMSZ1016-content were proportional to the amount of the activators. FlhDC\text{SGI1} alone induced the replication less efficiently than AcaCD, as pMSZ1016 could only be maintained by IPTG-induced pGMY6 (Table 1, Supplementary Figure S5). In conclusion, the copy number of SGI1, at least in a certain range, is proportional to the amount of RepA, which depends on the amount of the activators. Furthermore, unlike the excision induction (14), both AcaCD and FlhDC\text{SGI1} are required for proper replication of SGI1 under natural conditions.

**Translation of S004S is required for RepA synthesis**

If S004S protein acts as a classical leader peptide, its translation is required to normal expression of RepA. Consequently, nonsense mutations in ORF S004S should decrease or prevent RepA synthesis. To test this hypothesis, the 24th Leu codon of ORF S004S was replaced with a UAA (ochre) stop codon in both test plasmids, pMSZ1016 and pMSZ1032 (resulting in plasmids pMSZ1028 and pMSZ1034, respectively). pMSZ1028 was used to detect the replication ability of the SGI1 rep region without S004S protein as described for pMSZ1016, while pMSZ1034 was applied to monitor the expression level of RepA by β-galactosidase assay. Although pMSZ1028 could be maintained in strain TG1Nal::repA\text{SGI1}, in contrast to its parental plasmid pMSZ1016, no pMSZ1028 transformants were obtained within the strain TG1Nal::SGI1-C\text{rep-region}/R16a (producing both activators) even with expressing the S004S protein from plasmid pMSZ1040 containing the \text{P}_{\text{lac}}::S004S cassette (33). Thus, the nonsense mutation in S004S prevented the replication and this deficiency was not rescued by S004S protein overexpressed in trans. Similarly, pMSZ1028 did not replicate in any activator-producing strains independently of the expression levels.

The β-galactosidase assay showed very low lacZ expression from pMSZ1034, which was near the detection limit (0.8±0.5 units) and it was not increased (1.1±0.4 units) by S004S protein overexpressed from pMSZ1040, indicating that RepA synthesis is very limited in the absence of S004S translation in cis. Since β-galactosidase expression from the wt parental plasmid pMSZ1032 was relatively low (44.2±7.1 units) in the host harbouring R55 and SGI1, a more sensitive test was also applied. For this, the AcaCD-inducible S004S promoter of pMSZ1032 and pMSZ1034 was replaced with the \text{P}_{\text{lac}} promoter to ensure elevated expression of the repA-lacZ fusion. The resulting plasmid pMSZ1037 bearing wt S004S ORF produced 118.8±4.5 units of β-galactosidase without IPTG induction, and 1612.7±81.8 units after induction with 0.05 mM IPTG. Contrarily, pMSZ1039 containing a stop codon in S004S produced only 0.7±0.3 and 2.2±0.6 units under the same conditions, and the mutation was not complemented by S004S protein overexpressed from pMSZ1040. These re-
Replication has a key role in the stability of SGI1 in the presence of IncC helpers

Transient replication of the extrachromosomal form of ICEs has been shown to contribute to the stable maintenance of the islands (27). In most of these cases, relaxase-dependent RC-replication occurs, while the SGI1-derived replicon proved to belong to the theta-type IncN2/N3-family. Nevertheless, we supposed that the helper-induced replication of SGI1 has a similar role in SGI1 maintenance when it coexists with the mobilization helper. To test this hypothesis, deletions affecting the replication functions of the island were generated in TG1Nal::::SGI1-C strain and these mutants were used to monitor their co-habitation with the helper plasmid. In addition to knocking out the oriVSGI1, repAsgI1 and the AcaCD binding site of P\text{S004}, a ΔflhDCsgI1 mutant was also generated, as FlhDCsgI1 appeared to be an important factor in proper replication of pMSZ1016, maintained by the SGI1-derived basic replicon (Table 1).

For the first attempt, TG1Nal::::SGI1-C strain and its KO mutant derivatives were used as recipients in mating with an IncC plasmid-bearing donor strain TG90/R55\text{Ty6618/2} and the frequency of transconjugants was determined under selecting only for the incoming plasmid (LB+Nal+Cm) and for both the plasmid and SGI1 (LB+Nal+Cm+Sm+Sp), respectively. As in any similar assays, the frequency of transconjugants depends not only on the primary rate of transfer but also on the stability of SGI1 and helper plasmid co-habiting in the transconjugant cells. Thus, the transconjugants obtained without selection for SGI1 were also tested for the presence of SGI1 by replica plating. The transconjugant frequencies with the recipient bearing wt SGI1-C were similar independently of the selection method (Figure 6A) and the phenotype test showed 11.5±3.7% loss of SGI1 from the transconjugants not selected for the Sm\text{R}Sp\text{R} markers of the island. Similar segregation rate was observed previously in Salmonella (14).

In contrast, ca. 3 orders of magnitude lower transconjugant frequencies were observed when the recipients carried one of the three replication-deficient SGI1-C mutants (ΔoriV, ΔrepA and ΔP\text{S004}) and the transconjugants were also selected for SGI1 than without selecting for it. The phenotype test of individual R55\text{Ty6618/2} transconjugants selected only for the plasmid marker indicated a high rate of SGI1 loss (ΔoriV: >99.7±0.1%, ΔrepA: >99.6±0.1 and ΔP\text{S004}: 96.8±2.0% SGI1-free transconjugants). A characteristic feature of these replica-plated colonies was that only a few small secondary colonies appeared in the footprint of the original transconjugant colonies on the plates selective for both SGI1 and R55 (Supplementary Figure S6). These data indicate that the replication-deficient SGI1 had been lost almost completely from the colonies of the transconjugant cells without a selection for SGI1. These observations confirmed a drastic decrease in the stability of all rep mutant islands in the presence of the helper plasmid.

Table 1. Transformability and copy number of the SGI1-derived replicon pMSZ1016 in strains expressing different amounts of the transcriptional activators AcaCD and FlhDC<SGI1

| Strain                      | Activator | Transformants | Copy number of pMSZ1016 |
|----------------------------|-----------|---------------|-------------------------|
| TG1Nal::::SGI1-C            | None      | <1            | -                       |
| TG1Nal::::SGI1-C            | AcaCD     | 9±2.1×10^2    | 2.4±10.5               |
| TG1Nal::::SGI1-C            | FlhDC     | 2.1±1.4×10^6  | 3.1±2.5                |
| TG1Nal::::SGI1-C            | AcaCD+FlhDC | 2.3±1.5×10^6 | 4.4±1.4                |

4 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

5 Transformants obtained with the host containing pJKI828 grew considerably slower compared to that carrying pJKI888.

6 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

7 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

8 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

9 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

10 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

11 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

12 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

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15 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

16 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

17 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

18 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

19 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

20 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

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25 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

26 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

27 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

28 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

29 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.

30 The copy number measurement was carried out under non-inductive conditions using transformant colonies obtained from plates supplemented with 50 μM IPTG.
FlhDCSG11 is an important factor in SG11 replication

The ΔflhDCSG11 mutant appeared rather different from the rep mutants in similar tests. The frequency of R55 transconjugants obtained with the recipient containing this mutant was ca. 50 × higher compared to the wt SG11-containing recipient, independently of the selection (ΔflhDCSG11: 7.3±4.1 × 10^{-2} versus 7.5±4.3 × 10^{-2} and wt: 1.5±0.7 × 10^{-3} versus 1.4±0.9 × 10^{-3}, Figure 6A). The finding that the SG11-derived replicon (pMSZ1016) was not maintained in an flhDCSG11-acaCD+ background (Table 1) suggested an insufficient replication in the absence of FlhDCSG11. Thus, to determine the copy number of the wt SG11-C and the ΔflhDCSG11 mutant in cells containing the IncC plasmid R16a, RT-qPCR assay for attP and attB was performed, including the three rep mutants. The results confirmed that the wt island is present in 7–8 copies/cell when a wt (acaCD+) helper is present, while neither excision nor replication was detectable in an acaCD- background (Figure 6B). The excision of SG11 KO-mutants, including ΔflhDCSG11, was almost complete in an acaCD+ background similar to that of wt SG11-C (>80%, except ΔP5004 mutant showing a somewhat lower, 32±6%, excision rate). The copy number of the excised rep-mutant islands was <0.2 per cell (Figure 6B), indicating their proper excision but the lack of replication. This can explain the frequent SG11 loss and the low rate of transconjugants retaining SG11 after the intake of the helper plasmid (Figure 6A). The only exception was the ΔflhDCSG11 mutant, whose copy number proved to be around 1/cell. Although the FlhDCSG11 depletion caused a significant reduction in the copy number (from 7–8 to 1/cell), the intact SG11-C, unlike the SG11-derived minimal replicon (pMSZ1016), was maintained as a single-copy plasmid.

Copy-number of SG11 influences its incompatibility with the helper plasmid

At a first glance, the lower copy number of ΔflhDCSG11 mutant cannot explain the 50× higher frequency of R55 transconjugants (Figure 6A) compared to that obtained with the recipient carrying wt SG11-C. However, the data suggested a more stable co-habitation of the helper plasmid and the island if SG11 is present in single copy due to the lack of its own activator.

To further analyse this phenomenon, isogenic strains harbouring R55ΔTn6187 and wt or ΔflhDC mutant SG11-C were grown for 20 generations without antibiotic selection and the proportion of cells still containing one or both elements was determined. In case of wt SG11-C, >84% of the cell population carried only SG11 and <1% retained both partners. In contrast, SG11-only cells almost disappeared (1.6%) in the case of the ΔflhDC mutant and the population was dominated by cells retaining both the plasmid and the island (Figure 6C). These findings suggest that the lack of FlhDCSG11 can contribute to the stability of the co-habitation, which can also explain the higher frequency of R55 transconjugants obtained with SG11-ΔflhDC-bearing recipient. Nevertheless, the background of this phenomenon is not yet clarified.

DISCUSSION

SG11-family IMEs are known as passengers hijacking the transfer apparatus of IncA and IncC plasmids. Several important characteristics of the relations between the IME and its mobilization helper have recently been uncovered (14,16,22,32,33,49). The present study reports a new aspect of the SG11-helper crosstalk, which highly contributes to...
the stability of SGI1 when coexisting with the helper plasmid. We provide evidence for the helper-induced plasmid-like replication of the excised SGI1, which seems indispensable for the maintenance of the island in helper-bearing cells and may also contribute to the efficient displacement of the helper.

The basic replicon identified on SGI1 includes the S004-repA (S003) operon and the downstream oriV. ORF S003 encodes for a functional replication initiator, which is related to the IncN2/N3 family RepA proteins (Supplementary Figure S1). The cognate oriV region identified near the 3' end of repA-SGI1 includes four iteron-like DRs with a GC-rich GGGGGHRATTATGCGY consensus preceded by an AT-rich region containing the imperfect IR1 motif. This arrangement of oriV is characteristic for the iteron-containing replicons (50). Similar conclusions have been reported on the SGI1-derived replicon in a very recent study published after the submission of this work (51). Both repA and the cis elements of oriV are well conserved in the IncN2/N3 group of plasmids having closely related replicon to that of SGI1 (Supplementary Figure S2). The specific protein-DNA interaction between RepA-SGI1 and the DRs of oriV has been confirmed by EMSA, showing that the four DRs serve as binding sites for RepA-SGI1. The oriV region contains putative DnaA-boxes neighbouring or overlapping the DR1–4 motifs. DnaA binding sites are usually found in the region containing putative DnaA-boxes near oriV in the same constitution as found in SGI1. The only exception is the chromosome of S. enterica serovar Kentucky strain VNSEC0 (CP039439), where an SGI1-like element showing less than 40% coverage to the SGI1 backbone also contains the homolog of SGI1 rep region. A search for more divergent SGI1-related elements revealed two SGI1-like GIs sharing a similar structure to SGI1, but having a different rep gene (51). These elements, GIvchO27–1 and GIvSen–26, identified in V. cholerae O27 and S. enterica serovar Muenster 26 strains, respectively, are also located at the 3' end of trmE and show 94% identity to each other but have much lower sequence similarity to SGI1. Their repA is unrelated to that of the IncN2/N3 group and encodes a Rep-Primase family protein, whose homologs occur also in several SGI1-related elements found in P. mirabilis, S. enterica serovars and some other species (51). The repA of GIvchO27–1 and GIvSen–26, like in SGI1, is preceded by an S004 homolog whose putative C-terminal portion shares only 33% identity to S004 of SGI1. Although these S004-rep operons also appear to be driven by an AcaCD-responsive promoter, the different genetic context of their two genes (i.e., there is no overlap, instead, a ca. 200 bp space separates the STOP of S004 and START of repA) suggests that S004 and repA are not translationally coupled. Therefore the amount of RepA, and consequently, the copy number of the excised GIs are probably controlled in a different way than in SGI1.

The copy number of SGI1, similarly to the SGI1-derived replicon pMSZ1016, is determined by the amount of RepA within a certain range (Figures 2, 5, Supplementary Figure S5, and Table 1). The β-galactosidase assays showed that both the promoter activity of S004 and the translation of RepA are proportional to the amount of that is regulated through the AcaCD-responsive promoter P004 by the AcaCD and FlhDCSGI1 activators encoded by IncC plasmids and SGI1, respectively. The plasmid pMSZ1016, composed of a resistance gene and the whole rep region of SGI1 including oriV and the S004-repA operon along with P004, proved to be replicable in the presence of any FlhDC-like activators if they are in excess. However, the maintenance of pMSZ1016 required both activators when expressed under their native control systems of the IncC plasmid and SGI1, respectively. Therefore, SGI1 synchronizes its plasmid-like replication with the presence of the helper plasmid similarly to the timing of excision, i.e. the replication machinery is also triggered by the helper-encoded activator AcaCD. Thus, SGI1 is the first example for a helper-induced plasmid-like replication of an IME. This seems to be a good strategy, as a constitutive replication would be harmful to the host when the IME is integrated into the chromosome.

The comparison of search results in GenBank database with the rep region and the 27.4 kb backbone sequence of SGI1 showed that all the 122 retrieved SGI homologs having >60% coverage to the backbone sequence carry the homolog of SGI1-derived replicon including P004, S004, repA and oriV in the same constitution as found in SGI1. This indicates that the SGI1 family elements. The only exception is the chromosome of the S. enterica serovar Kentucky strain VNSEC0 (CP039439), where an SGI1-like element showing less than 40% coverage to the SGI1 backbone also contains the homolog of SGI1 rep region. A search for more divergent SGI1-related elements revealed two SGI1-like GIs sharing a similar structure to SGI1, but having a different rep gene (51). These elements, GIvchO27–1 and GIvSen–26, identified in V. cholerae O27 and S. enterica serovar Muenster 26 strains, respectively, are also located at the 3' end of trmE and show 94% identity to each other but have much lower sequence similarity to SGI1. Their repA is unrelated to that of the IncN2/N3 group and encodes a Rep-Primase family protein, whose homologs occur also in several SGI1-related elements found in P. mirabilis, S. enterica serovars and some other species (51). The repA of GIvchO27–1 and GIvSen–26, like in SGI1, is preceded by an S004 homolog whose putative protein product shares only 33% identity to S004 of SGI1. Although these S004-rep operons also appear to be driven by an AcaCD-responsive promoter, the different genetic context of their two genes (i.e., there is no overlap, instead, a ca. 200 bp space separates the STOP of S004 and START of repA) suggests that S004 and repA are not translationally coupled. Therefore the amount of RepA, and consequently, the copy number of the excised GIs are probably controlled in a different way than in SGI1.

The copy number of SGI1, similarly to the SGI1-derived replicon pMSZ1016, is determined by the amount of RepA within a certain range (Figures 2, 5, Supplementary Figure S5, and Table 1). The β-galactosidase assays showed that both the promoter activity of S004 and the translation of RepA are proportional to the amount of AcaCD or FlhDCSGI1 when present in excess and both activa-
tors are necessary if they are expressed from their original source (Figure 5). These results support that expression of RepA<sub>SGI1</sub> and consequently, the copy number of SGI1 are under the double control of AcaCD and FlhDC<sub>SGI1</sub>. AcaCD proved to be a stronger activator on the AcaCD-responsive promoters of SGI1 than FlhDC<sub>SGI1</sub> (33) and seems to be expressed from the helper plasmid in sufficient amount for triggering SGI1 excision, but not for inducing proper replication alone. To maintain the 4–8 SGI1 copies/cell, both AcaCD and FlhDC<sub>SGI1</sub> are required in the amount provided by their own expression mechanisms (14,22), while in the absence of FlhDC<sub>SGI1</sub>, the island seems to be maintained only as a single copy plasmid (Figure 6B). Thus, SGI1 controls, at least partially, its copy number by expressing its own activator FlhDC<sub>SGI1</sub>.

Our results indicate that the copy number of SGI1 is controlled through the expression level of RepA<sub>SGI1</sub>. We have shown that in addition to the transcriptional activation of P<sub>S004</sub> by the two FlhDC-family activators, the other key element in this control mechanism is the translational coupling of repA and ORF S004. It has been reported that the expression of the repA gene cannot be measured with a β-galactosidase test plasmid where the lacZ is fused to the START codon of repA even in the presence of excess AcaCD (33). However, the analysis of the sequence near the S004-repA overlap uncovered an IR-motif, which can form a stem-loop structure on the S004-repA transcript. Such long-range RNA structures have a central role in controlling repA expression and regulation of copy number in numerous plasmid families (56). Using β-galactosidase test plasmids that were designed by taking into consideration the potential requirement for the intactness of this stem-loop structure, we have confirmed that the AcaCD- and FlhDC<sub>SGI1</sub>-controlled P<sub>S004</sub> drives the expression of RepA<sub>SGI1</sub> (Figure 5). We have also shown that translation of S004 and RepA is tightly coupled as an early STOP mutation in ORF S004S prevents RepA expression and SGI1 replication and this mutation cannot be rescued by providing S004S protein in trans. This result supports that the S004 protein acts as a leader peptide, of which translation in cis is indispensable for the translation of RepA.

Proper expression of RepA was observed only when the IR motif remained intact (Figure 5), indicating the importance of the potential stem-loop structure in the S004-repA transcript (33). The IR overlaps the translation START site of repA along with the downstream STOP codon of S004, which are normally positioned close to each other. The putative stem-loop structure efficiently masks the Shine-Dalgaro sequence (SD) and the START codon of repA on the mRNA (note that RepA expression was not observed in the absence of S004 translation even using a strong promoter like the IPTG-induced P<sub>lac</sub>). However, when translating the C-terminus of S004 leader protein, the stalled ribosomes possibly resolve the stem-loop, which uncovers the translation START site of repA. This constitution ensures the reinitiation of the ribosome at the SD site of RepA. The early STOP codon in ORF S004 prevents the ribosomes from reaching the stem-loop and thus the initiation of RepA translation fails. Similar translational coupling mechanisms are prevalent among plasmid replication systems and appear as a general evolutionary strategy in copy number control (56,57). The genetic constitution seen in the SGI1 rep region suggests that the IncN2/N3-related SGI1-derived replicon has also adopted such a control mechanism.

The stable vertical transfer of an IME is mostly based on the chromosomal integration, however, when the mobilization helper element appears, the IME has to give this safe position up for the benefits of horizontal transfer. The chance for the lateral transfer accompanies the transient destabilization of SGI1 when coexisting with the IncC helper plasmid (14). This negative effect of the helper is considerably reduced by the SGI1-encoded TA-system (16), however, it cannot entirely prevent SGI1 loss (14). Our results clearly indicate that plasmid-like replication of the excised circular SGI1 significantly contributes to its stability. It was obvious for all replication-deficient SGI1 mutants, which showed extremely high rate of segregation in the presence of the helper. Moreover, maintenance of SGI1 at ca. 6 copies per cell in the presence of the helper plasmid may have further important advantages. SGI1 has been suspected to encode for destabilizing functions against its helper plasmids (16,25). These might be more efficient when expressed from elevated number of SGI1 copies. The copy number of the ΔflhDC<sub>SGI1</sub> mutant decreased to ca. 1 per cell when the helper was present and showed not only lower stability, but it was much less efficient in the displacement of the helper (Figure 6C). The SGI1-K variant lacking flhDC<sub>SGI1</sub> was shown not to destabilize pRMH760 helper (25). These observations might be explained by the direct destabilizing effect of FlhDC<sub>SGI1</sub> protein on the IncC plasmids. On the other hand, the fact that R55 appeared stable together with the FlhDC<sub>SGI1</sub>-producer plasmid pGMY6 in a 100-generation growth without selection (unpublished data) makes this explanation unlikely. Instead, we propose that the lower copy-number of the ΔflhDC<sub>SGI1</sub> mutant better explains this phenomenon. Although our results also support that the SGI1 replication is linked to the incompatibility with IncC plasmids as suggested by (51), this effect is unlikely to be based on the direct effect of FlhDC<sub>SGI1</sub> or the conflict of the replication machineries, as indicated by the compatibility of the SGI1-derived basic replicon with the IncC helper. We suggest instead that the increased dosage of a yet unidentified SGI1-encoded incompatibility factor (due to the elevated copy number of SGI1) serves as a better explanation for the efficient destabilization of the helper.

Outperforming the transfer of helper plasmid by SGI1 has been reported several times (22,25), and the role of SGI1-encoded TA-components TraN<sub>S</sub>, TraG<sub>S</sub> and TraH<sub>S</sub> in remodelling the helper-encoded mating pore has been demonstrated. It seems that the ‘hybrid’ TA-components modified by the SGI1-encoded components favours SGI1 transfer over that of the helper plasmid and helps SGI1 to evade the helper-encoded entry exclusion (32). However, the fact that SGI1 transiently overreplicates the single-copy helper plasmid raises the possibility that merely the higher copy-number of SGI1 contributes to the more efficient transfer, which is further supported by the elevated expression of SGI1-genes involved in the transfer.

Interesting analogies can be seen in the interrelations of SGI1-IncC helpers and the viral satellites, the phage-inducible chromosomal island-like elements (PLE), and their target phages. PLE1–5 are chromosomal islands of <i>V. cholerae</i> whose excision is induced by ICP1 phage. While in SGI1, the RDF (Xis) expression is induced by the helper-
encoded activator, the ICP1 helper phage itself provides the RDF (PexA) for PLE excision (58). Further similarities are that the excised PLE overreplicates its helper phage, out-performs the phage genome in packaging and consequently, highly reduces the spread of the helper phage (59). Thereby PLEs provide efficient defence for their host against its lytic phage invaders and ensure their own horizontal transfer at the expense of the helper phage (59,60). PLE also has its own replication origin and encodes a RepA resembling Gram+ plasmid replication initiation factors. Similarly to SGI1, the replication is helper-dependent as it is triggered by an unknown helper-encoded factor (59). Although the molecular mechanisms are different, PLEs also exploit their helper phages in multiple ways, which is performed by hijacking the helper-encoded proteins as in case of SGI1. Despite that the genetic background of these interventions seems completely different, the analogies suggest that there are common selection forces acting in the co-evolution of SGI1-like IMEs and PLEs with their respective helper elements.

SGI1 appears exceptional among IMEs as it is not simply an opportunistic hitch-hiker of its mobilization helpers, the IncA and IncC plasmids, but has been evolved to efficiently hijack the helper-encoded machinery to its own benefit, ensuring its most efficient vertical and horizontal transfer. Advantages over the helper plasmid are gained by multiple fine-tuned interventions into the crosstalk between SGI1 and its helper. This occurs mostly by transient expression of several SGI1 genes triggered by the helper-encoded AcaCD activator (22,14,32,31). Furthermore, SGI1 destabilizes its helper plasmid (25), reducing the time of co-habitation. Its evolutionary benefits possibly root in the fact that the helper also destabilizes SGI1 by triggering its excision (14). The helper-induced transient replication of SGI1 seems to be a refined adaptive trait, ensuring the more stable vertical transfer and more efficient horizontal transfer of SGI1 when the helper element appears. The diverse ways by which SGI1 exploits the IncA and IncC plasmids certainly played an important role in the outstanding success of SGI1-family elements in their distribution among pathogenic enteric bacteria.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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