Salmonella Genomic Island 3 Is an Integrative and Conjugative Element and Contributes to Copper and Arsenic Tolerance of *Salmonella enterica*

Nobuo Arai,[a,b] Tsuyoshi Sekizuka,[c] Yukino Tamamura,[b] Masahiro Kusumoto,[d] Atsushi Hinenoya,[a] Shinji Yamasaki,[a] Taketoshi Iwata,[b] Ayako Watanabe-Yanai,[b] Makoto Kuroda,[c] Masato Akiba[a,b]

[a]Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisano, Osaka, Japan
[b]Division of Bacterial and Parasitic Disease, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan
[c]Pathogen Genomics Center, National Institute of Infectious Diseases, Tokyo, Japan
[d]Kyushu Research Station, National Institute of Animal Health, Kagoshima, Japan

ABSTRACT *Salmonella* genomic island 3 (SGI3) was first described as a chromosomal island in *Salmonella* 4,[5],12:i:–, a monophasic variant of *Salmonella enterica* subsp. *enterica* serovar Typhimurium. The SGI3 DNA sequence detected from *Salmonella* 4,[5],12:i:– isolated in Japan was identical to that of a previously reported one across entire length of 81 kb. SGI3 consists of 86 open reading frames, including a copper homeostasis and silver resistance island (CHASRI) and an arsenic tolerance operon, in addition to genes related to conjugative transfer and DNA replication or partitioning, suggesting that the island is a mobile genetic element. We successfully selected transconjugants that acquired SGI3 after filter-mating experiments using the *S. enterica* serovars Typhimurium, Heidelberg, Hadar, Newport, Cerro, and Thompson as recipients. Southern blot analysis using I-CeuI-digested genomic DNA demonstrated that SGI3 was integrated into a chromosomal fragment of the transconjugants. PCR and sequencing analysis demonstrated that SGI3 was inserted into the 3′ end of the tRNA genes *pheV* or *pheR*. The length of the target site was 52 or 55 bp, and a 55-bp *attI* sequence indicating generation of the circular form of SGI3 was also detected. The transconjugants had a higher MIC against $CuSO_4$ compared to the recipient strains under anaerobic conditions. Tolerance was defined by the *cus* gene cluster in the CHASRI. The transconjugants also had distinctly higher MICs against Na_2HAsO_4 compared to recipient strains under aerobic conditions. These findings clearly demonstrate that SGI3 is an integrative and conjugative element and contributes to the copper and arsenic tolerance of *S. enterica*.

KEYWORDS *Salmonella enterica* serovar Typhimurium, *Salmonella* genomic island 3, arsenic, copper, integrative and conjugative element, metal resistance

Address correspondence to Masato Akiba, akiba@affrc.go.jp.

*S*almonella enterica subsp. *enterica* serovar Typhimurium (*Salmonella* Typhimurium) and its monophasic variant (*Salmonella* 4,[5],12:i:–) are major causes of gastroenteritis in humans and animals worldwide (1–4). In particular, *Salmonella* 4,[5],12:i:– infection of humans and animals has increased considerably in European countries since the mid-1990s. The epidemic *Salmonella* 4,[5],12:i:– strain is called the "European clone" and is characterized by sequence type 34 (ST34) and possession of a composite transposon containing antimicrobial resistance genes and a chromosomal island, *Salmonella* genomic island 3 (SGI3) (5). This clone has been spread from Europe to other areas, including North America and Asia (1, 2, 6–10).

In Japan, bovine salmonellosis caused by *Salmonella* 4,[5],12:i:– has been increasing in the last decade (7, 11, 12). We performed a phylogenetic analysis using whole-

genome sequencing data of 119 *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:– isolates obtained in Japan and Italy and identified nine distinct clades as genotypes for epidemiological analysis. Then, we established an allele-specific PCR-based genotyping system detecting a clade-specific single nucleotide polymorphism (SNP) to rapidly identify the clade of each isolate. Clade 9 was the most prevalent in recent years among 955 *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:– isolates obtained from food animals in Japan between 1976 and 2017. This clade mainly consists of *Salmonella* 4,[5],12:i:– and shares the same characteristics as the European clone. We speculate that the increased prevalence of clade 9 isolates in Japan is a part of the pandemic of the European clone (13).

All the clade 9 isolates harbored a composite transposon containing antimicrobial resistance genes defining resistance to ampicillin, streptomycin, sulfonamide, and tetracycline. However, these antimicrobials do not appear to be a main selection pressure because some former epidemic clones of *Salmonella* Typhimurium showed similar resistance patterns (14, 15). Clade 9 isolates also harbored SGI3, which contains genes for tolerance to copper, zinc, and arsenic, and the European clone exhibits an increased MIC for copper sulfate in broth culture (5). Since copper and zinc are generally added to feed of livestock to achieve growth promotion and increased feed efficiency (16), these feed additives might be an important selection pressure for this clone.

Integrative and conjugative elements (ICEs) are self-transmissible between chromosomes of different cells (17, 18) and are widely distributed among both Gram-negative and Gram-positive bacteria (19). ICEs include genes defining a type IV secretion system, which contributes to conjugative transfer, in addition to an integrase and an excisionase (18, 19). ICEs can excise themselves from the donor chromosome, form a circular intermediate, transfer by conjugation, and reintegrate into the recipient chromosome. Each ICE has variety of cargo genes that contribute to antimicrobial resistance (20–24), biofilm formation (25), metabolism of alternative carbon sources (26), and degradation of the persistent chemical substances (27, 28). Hence, horizontal gene transfer by ICEs plays an important role for bacterial evolution.

SGI3 includes genes encoding the type IV secretion system and a site-specific recombinase, suggesting that SGI3 might be an ICE and provide heavy metal tolerance to the host. However, functions of SGI3 as an ICEs are unproved to date. In this study, we determined the transfer frequencies of SGI3 using clade 9 strains as donors and other *Salmonella* strains as recipients, and the susceptibility of the transconjugants to heavy metals was compared to that of their parental strains to prove that SGI3 was a functional ICE. Moreover, we constructed deletion mutants of the copper homeostasis gene clusters among SGI3 and compared the susceptibility to copper to that of the parental strains to identify the functional gene clusters that contributed to copper tolerance.

RESULTS

Structure of SGI3 in wild-type strains. The clade 9 isolates L-3838 and L-3841 harbored a genomic island that was identical to SGI3 in the Australian *Salmonella* 4,[5],12:i:– strain TW-Stm6 (29). The open reading frames (ORFs) of SGI3 in L-3841 are listed in Table S2 in the supplemental material. The SGI3 length of L-3838 and L-3841 is 80,686 bp, except for a direct repeat in both ends, and the nucleotide sequence identity of both isolates was 100%. SGI3 of these isolates had a nucleotide sequence (80,685/80,686 bp) almost identical to that of TW-Stm6. SGI3 of L-3838 was integrated into the 3′ end of the chromosomal tRNA gene *pheV* and formed a 52-bp direct repeat at both ends. SGI3 of L-3841 was integrated into the 3′ end of the chromosomal tRNA gene *pheR* and formed a 55-bp direct repeat at both ends (Fig. S1). Among 86 ORFs identified in L-3841, 24 were predicted as heavy metal-resistant genes (Fig. 1). Of the 24 ORFs, 17 formed a heavy metal homeostasis/resistance island called the copper homeostasis and silver resistance island (CHASRI) (30). The CHASRI consists of a plasmid-borne copper homeostasis system (*pco*) cluster (*pcoABCDRSE*) (31), a *sil* heavy metal
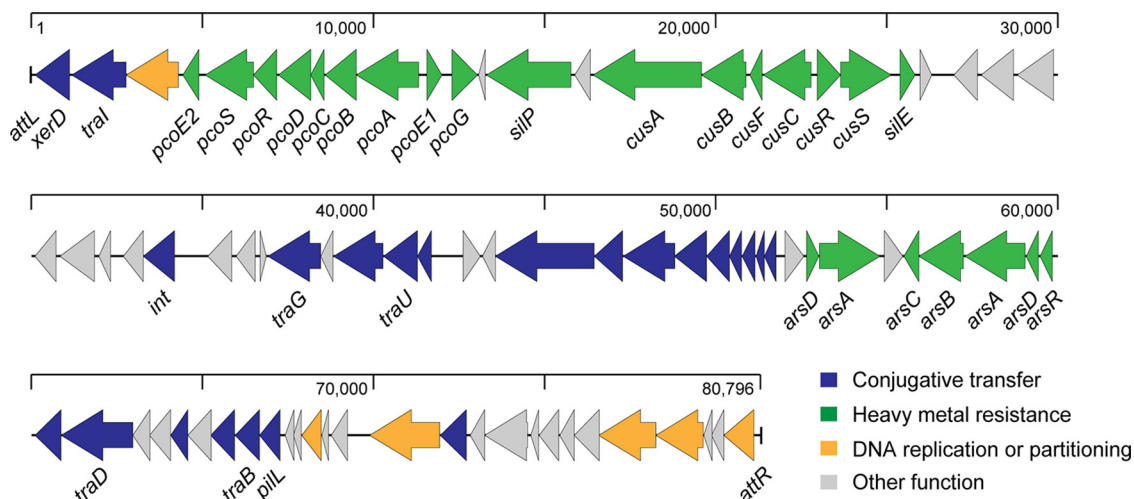
FIG 1 Schematic view of full-length SGI3 of *Salmonella* 4,[5],12:i:– L-3841. The diagram shows the predicted classification of each gene, which is represented by arrows according to the following scheme: violet, genes for conjugative transfer; green, genes for heavy metal resistance; orange, genes for DNA replication or partitioning; and gray, genes with other functions.

export system cluster (*silE* and *silP*) (32), and a copper-sensing copper efflux system (*cus*) cluster (*cusABFCRS*) (33). The nucleotide sequence identity of CHASRI located in SGI3 and *E. coli* strain APEC-O1 plasmid pAPEC-O1-R (GenBank accession number NC_009838.1) was 95% (Fig. 2). The remaining seven heavy metal-resistant genes formed an arsenic tolerance operon (*arsRDABC*) (34). The nucleotide sequence identity of the arsenic operon located in SGI3 and *Enterobacter cloacae* ATCC 13047 plasmid pECL_A (GenBank accession number CP001919.1) was 92% (Fig. 2). The structure of the remaining part of SGI3, including genes relating to conjugative transfer and DNA replication or partitioning, showed high similarity with the ICE-like chromosomal island of *Edwardsiella icutaluri* MS-17-156 (GenBank accession number CP028813.1). The nucleotide sequence identities of genes *traD*, *traG*, and *traI* located in the SGI3 and ICE-like genomic island of *E. icutaluri* MS-17-156 were 91, 92, and 86%, respectively (Fig. 2).

Transfer of SGI3. The results of the conjugation experiment are shown in Table 1. SGI3 of donor strain *Salmonella* 4,[5],12:i:– L-3841 (SNP genotype 9) was successfully transferred to recipient strains of *Salmonella* Typhimurium or *Salmonella* 4,[5],12:i:– with SNP genotypes 1 to 8. The transfer frequencies ranged from 1.2×10^{-7} to 1.3×10^{-4} per donor. The SGI3 was also transferred to other serovars, including Heidelberg, Thompson, Hadar, Newport, and Cerro, with the transfer frequencies ranging from 4.1×10^{-9} to 1.7×10^{-5} per donor.
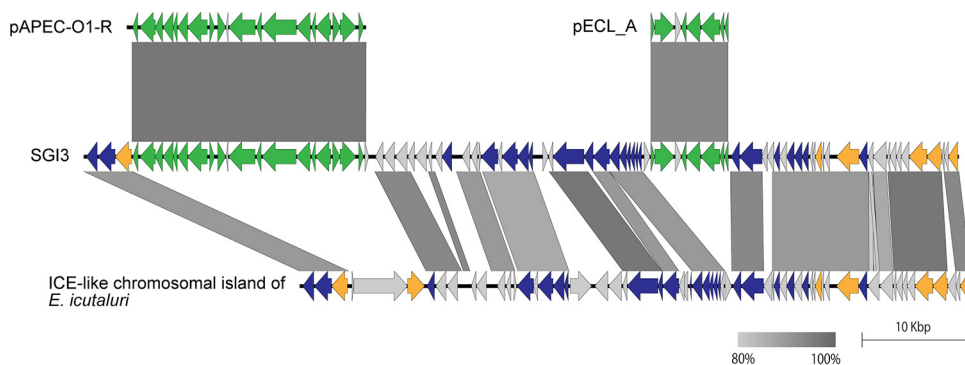

FIG 2 Pairwise alignment of SGI3 of *Salmonella* 4,[5],12:i:– L-3841 with the relevant mobile genetic elements, pAPEC-01-R of *Escherichia coli* APEC-O1, pECL_A of *Enterobacter cloacae* ATCC 13047, and ICE-like chromosomal island of *Edwardsiella icutaluri* MS-17-156.

TABLE 1 Transfer frequency of SGI3 to *S. enterica* strains[a]

Recipient				Transfer frequency
Strain	Serovar	O group	SNP genotype	(per donor)
L-3900	Typhimurium	4	1	1.4×10^{-6}
L-4131	Typhimurium	4	2	7.0×10^{-5}
LT2	Typhimurium	4	3	1.2×10^{-7}
L-3785	4,[5],12:i:–	4	4	4.5×10^{-5}
L-4153	Typhimurium	4	5	1.3×10^{-4}
L-4125	Typhimurium	4	6	2.3×10^{-5}
L-4239	Typhimurium	4	7	7.3×10^{-5}
L-3569	Typhimurium	4	8	2.4×10^{-5}
L-2707	Heidelberg	4	NA	1.7×10^{-5}
L-3038	Infantis	7	NA	ND
L-3202	Choleraesuis	7	NA	ND
L-4000	Thompson	7	NA	6.1×10^{-8}
L-2819	Hadar	8	NA	4.1×10^{-9}
L-3037	Newport	8	NA	7.4×10^{-6}
L-3046	Dublin	9	NA	ND
L-3241	Enteritidis	9	NA	ND
L-3289	Cerro	18	NA	6.4×10^{-8}

[a]NA, not applicable; ND, not detected.

Localization of SGI3 in the recipients. To confirm the chromosomal location of SGI3 among the donor and transconjugants, PCR, direct sequencing of the products, and PFGE (pulsed-field gel electrophoresis)-Southern blot analysis were performed. As shown in Fig. 3a, strains LT2, LT2TC*pheV*, and LT2TC*pheR* were identified as SNP genotype 3, whereas L-3838 and L-3841 were identified as SNP genotype 9 by the SNP
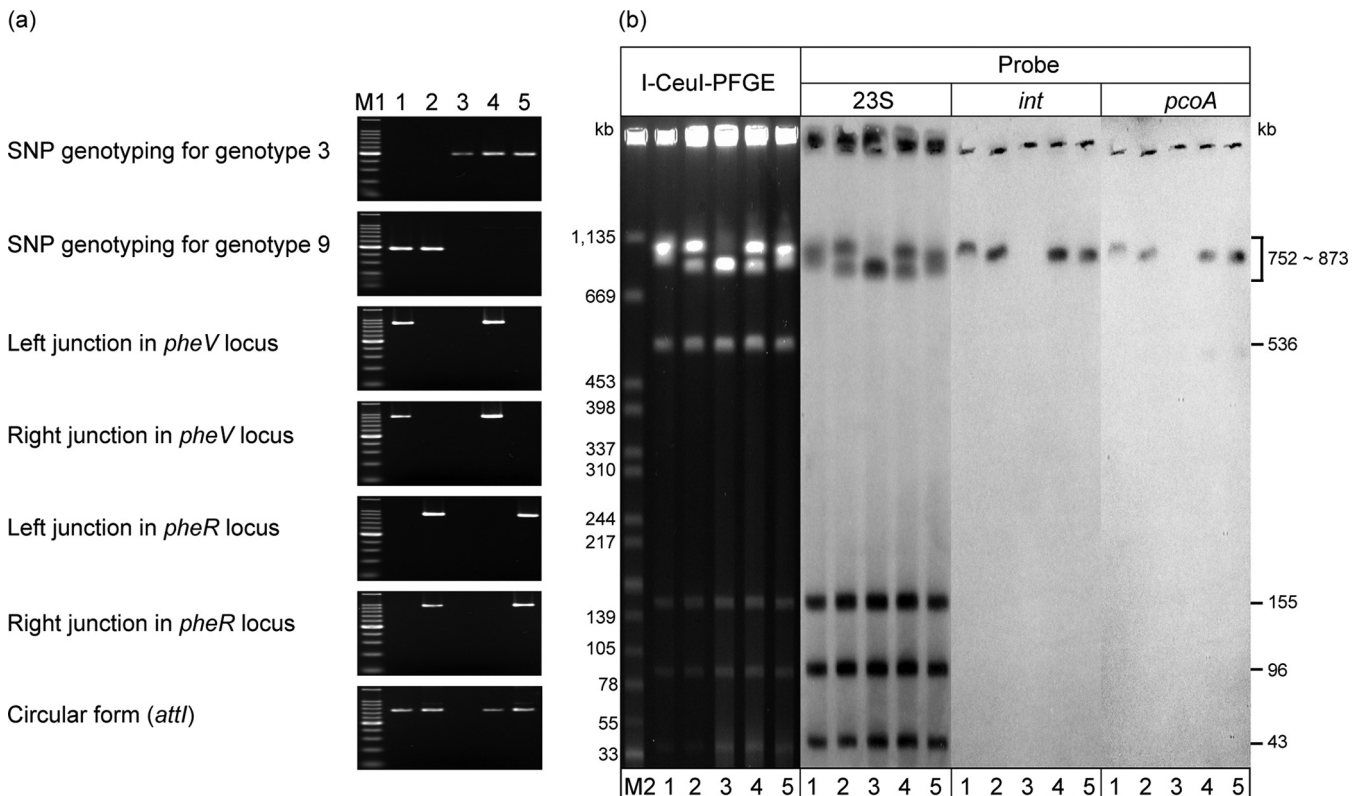

FIG 3 PCR and PFGE-Southern blot hybridization images demonstrating the chromosomal location and/or generation of the circular form of SGI3 in *Salmonella* 4,[5],12:i:– strains and *Salmonella* Typhimurium LT2 transconjugants. Lane 1, L-3838; lane 2, L-3841; lane 3, LT2; lane 4, LT2TC*pheV*; lane 5, LT2TC*pheR*; lane M1, 100-bp DNA ladder; lane M2, XbaI-digested *Salmonella* Braenderup. (a) SNP genotyping and PCR to detect junction regions of integrated or circular form of SGI3. (b) PFGE separation of I-CeuI-digested genomic DNA from *S. enterica* strains, followed by Southern blot hybridization with 23S rRNA, integrase, and *pcoA* gene probes.

genotyping system. PCR and the direct sequencing of the products was confirmed that the SGI3 insertion site of strains L-3838 and LT2TC*pheV* was the 3′ end of *pheV*, whereas that of L-3841 and LT2TC*pheR* was the 3′ end of *pheR*. The *attI* sequence indicating the generation of circular form of SGI3 was also detected from wild types and recipients by PCR and direct sequencing of the products.

LT2 contains seven I-CeuI restriction sites located on the 23S rRNA genes on the chromosome (GenBank accession number NC_003197.1). I-CeuI digestion of the LT2 chromosome generated fragments of 2,504, 770, 752, 536, 155, 96, and 43 kb. As shown in Fig. 3b, the 2,504-kb fragment did not migrate from the well, and the 770- and 752-kb fragments were indistinguishable under the PFGE conditions used in this study. To detect the chromosomal fragments by Southern blot analysis, the probe 23S, which targeted the 23S rRNA gene sequence, was used. This probe hybridized with six fragments of 2504, 770, 752, 155, 96, and 43 kb, as expected. The expected SGI3 insertion sites *pheV* and *pheR* are located on the 770- and 752-kb fragments, respectively. Thus, when SGI3 was inserted into the *pheV* and *pheR* locus in LT2, 851- and 833-kb fragments should be generated in the transconjugants instead of 770- and 752-kb fragments, respectively. The expected fragments were identified in strains LT2TC*pheV* and LT2TC*pheR*, respectively. The sizes of SGI3 containing I-CeuI-digested fragment of strains L-3838 and L-3841 were expected to be 861 and 873 kb, respectively. Both *int* and *pcoA* gene probes hybridized with an 861-kb fragment of L-3838, an 873-kb fragment of L-3841, an 852-kb fragment in LT2TC*pheV*, and an 833-kb fragment in LT2TC*pheR*, which were generated from the chromosome. Direct sequencing of the amplified fragments of the junction regions identified direct repeats of 52 and 55 bp in strains LT2TC*pheV* and LT2TC*pheR*, respectively, as shown in Fig. S1 in the supplemental material. The size of the *attI* sequence of the circular form of SGI3 was 55 bp in the both strains.

Susceptibility of wild types, transconjugants, and deletion mutants to heavy metals. The MICs of the SNP genotype 9 strains to $CuSO_4$ under anaerobic condition were four to six times higher than those of other SNP genotypes of *Salmonella* Typhimurium, i.e., serovars Heidelberg, Hadar, Newport, Cerro, Thompson, Infantis, and Enteritidis. Serovars Dublin and Choleraesuis were slightly more susceptible to $CuSO_4$ under anaerobic conditions. The MICs of the wild-type strains and their transconjugants to $CuSO_4$ were the same under aerobic conditions with the exception of strain L-4153. The MICs of transconjugants to $CuSO_4$ under anaerobic conditions were four to six times higher than those of their wild-type strains. The MICs were the same as that of the donor strain L-3841. The MICs of wild-type strains and their transconjugants to $ZnCl_2$ were the same or slightly different (1 mM difference) under both aerobic and anaerobic conditions. The MIC of genotype 9 strains to Na_2HAsO_4 under aerobic conditions was >64 μg/ml, whereas that of other genotypes of *Salmonella* Typhimurium and other serovars were ranged from <0.25 to 4 μg/ml. The MICs of the transconjugants to Na_2HAsO_4 were at least 32-fold increased compared to those for the wild-type strains. The MICs of LT2TC*pheR*ΔSGI3 and LT2TC*pheR*Δcus to $CuSO_4$ under anaerobic conditions were six times lower than those of their parental strains, LT2TC*pheR* (Table 2).

DISCUSSION

In this study, we characterized the genomic island SGI3, which was first described in the European *Salmonella* 4,[5],12:i:– clone (5). The corresponding clone has been isolated in Japan since 2000 and was designated clade 9 (SNP genotype 9) in a previous study (13). The SGI3 structure of Japanese strains was almost identical to that of the Australian strain TW-Stm6 (29) and the European strain SO4698-09 (5). The SGI3 nucleotide sequence identity of these isolates was almost 100% across the entire length of 80,685 bp. These data support the notion that the worldwide dissemination of the *Salmonella* 4,[5],12:i:– clone originated from Europe (13).

The nucleotide sequences of the core region of SGI3 showed low similarity to representative ICEs that were reported previously. However, SGI3 contains genes

TABLE 2 Susceptibility of strains to heavy metals

Strain	MIC (mM)				
	$CuSO_4$		$ZnCl_2$		Na_2HAsO_4
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic
L-3838	8	6	2	3	>64
L-3841	8	6	3	2	>64
L-3841ΔSGI3	6	1	3	3	≤0.25
L-3841Δpco	8	6	3	3	>64
L-3841Δcus	8	1	3	3	>64
L-3900	8	1	3	6	0.5
L-3900TC	8	6	3	6	64
L-4131	8	1	3	3	≤0.25
L-4131TC	8	6	3	4	>64
LT2	8	1	4	6	0.5
LT2TCpheV	8	6	3	6	64
LT2TCpheR	8	6	3	6	>64
LT2TCpheRΔSGI3	8	1	4	6	0.5
LT2TCpheRΔpco	8	6	4	6	>64
LT2TCpheRΔcus	8	1	4	6	>64
L-3785	8	1	3	3	0.5
L-3785TC	8	6	3	4	>64
L-4153	8	1	4	3	0.5
L-4153TC	6	6	3	3	>64
L-4125	8	1.5	2	2	0.5
L-4125TC	8	6	2	2	>64
L-4239	8	1	3	4	0.5
L-4239TC	8	6	3	4	64
L-3569	8	1	4	6	0.5
L-3569TC	8	6	3	6	64
L-2707	8	1	3	3	≤0.25
L-2707TC	8	6	3	3	64
L-2819	6	1.5	3	3	≤0.25
L-2819TC	6	6	3	3	>64
L-3037	6	1	3	4	≤0.25
L-3037TC	6	6	3	4	>64
L-3289	8	1	2	6	≤0.25
L-3289TC	8	6	2	6	>64
L-4000	8	1	4	3	4
L-4000TC	8	6	4	3	>64
L-3038	6	1	3	3	≤0.25
L-3046	8	0.75	3	4	0.5
L-3202	3	≤0.5	3	4	≤0.25
L-3241	8	1	3	3	0.5

encoding a site-specific recombinase XerD (35), an ICE relaxase TraI (36), and type VI secretion system (Table S2), suggesting that this island is a mobile genetic element. Transconjugants that acquired the SGI3 in their chromosome were successfully selected after the filter-mating experiment in this study (Table 1). The 3′ end of tRNA genes is a representative attachment site of ICEs reported previously (37), and SGI3 was inserted into the 3′ end of tRNA genes *pheV* or *pheR* (Fig. 3a). Southern blot analysis results supported the chromosomal location of SGI3 in the transconjugants (Fig. 3b). The *attI* sequence indicating the generation of circular form of SGI3 was detected by PCR in donor and recipient strains (Fig. 3a). These data suggested that SGI3 was excised from the donor chromosome, transferred by conjugation, and integrated into the host chromosome by site-specific recombination.

A direct repeat (*attL* and *attR*) was formed by the integration of SGI3 in the chromosome of the transconjugants. Although the size of the direct repeat generated from *pheV* (52 bp) was 3 bp shorter than that from *pheR* (55 bp), the nucleotide sequence was identical (Fig. S1). The 52- and 55-bp sequences seemed to be recognized as attachment sites of SGI3.

We could not detect any transconjugants from four strains of *S. enterica*, including serovars Infantis, Choleraesuis, Dublin, and Enteritidis, after three independent conju-

gation experiments, while transconjugants were successfully obtained from all genotypes of *Salmonella* Typhimurium, including *Salmonella* 4,[5],12:i:–. We detected the 55-bp attachment sequences among these strains by PCR and sequencing (data not shown). In this study, we performed the conjugation experiment without any chemicals inducing the SOS response. The addition of mitomycin C or antimicrobials might leads to successful conjugative transfer of SGI3 to these strains (19).

SGI3 contains CHASRI and the *ars* gene cluster, which contribute to heavy metal homeostasis (Fig. 1). CHASRI emerged and evolved in response to copper deposition across aerobic and anaerobic environments (30). CHASRI confers copper homeostasis under aerobic and anaerobic conditions and during the shift between aerobic and anaerobic environments, which could explain its wider distribution among facultative anaerobes.

CHASRI consists of two copper homeostasis gene clusters: *pco* and *cus*. The *pco* gene cluster includes *pcoABCDE1E2G-RS* and is potentially involved in periplasmic copper detoxification (38). The *cus* gene cluster consists of six genes: *cusCFBA-RS* (39). On the other hand, CusABC is a tripartite copper efflux pump consisting of an inner membrane pump (CusA), a periplasmic protein (CusB), and an outer membrane protein (CusC) and forms a channel spanning the entire cell envelope (38). PcoRS and CusRS are copper response two-component regulatory systems (38), and the latter has an important role for copper homeostasis under anaerobic conditions in *Escherichia coli* (40).

In our study, all of the SGI3 transconjugants had higher MICs to $CuSO_4$ compared to the recipient strains under anaerobic conditions. Furthermore, the MICs of the *cus* gene cluster deletion mutants L-3841Δ*cus* and LT2TC*pheR*Δ*cus* to $CuSO_4$ were equivalent to those of L-3841ΔSGI3 and LT2TC*pheR*ΔSGI3, respectively (Table 2). These data indicate that the products of the *cus* gene cluster contribute to copper tolerance of *S. enterica* under anaerobic conditions and are concordant with results of earlier studies (40–42). On the other hand, we did not detect MIC differences between *pco* gene cluster deletion mutants and parental strains. Given that the contribution of the *pco* gene cluster products to copper tolerance is subtle (43), intrinsic copper tolerance under aerobic conditions might mask this activity.

The *ars* gene cluster includes *arsABCDR* (44). ArsA is a soluble ATPase, which interacts with the integral membrane protein ArsB. Prior to efflux, arsenate is reduced to arsenite by small cytoplasmic arsenate reductase ArsC to activate ATPase activity of ArsA. Then, arsenite is pumped out through ArsB. This system involves the detoxification of As(III) and As(V). All of the transconjugants obtained in this study had distinctly higher MICs to Na_2HAsO_4 compared to recipient strains under aerobic conditions. Deletion of SGI3 in the strains L-3841 and LT2TC*pheR* reduced remarkably their MICs, while deletion of the gene clusters *pco* and *cus* did not affect their MICs. These data suggest that the *ars* gene cluster contributes to arsenic tolerance of *S. enterica* under aerobic conditions.

Heavy metals, such as copper and zinc, are added to livestock feed as micronutrients for growth promotion and suppressing gut pathogens (16). Aromatic organoarsenic compounds are also used as feed additives in the poultry industry for the same purposes (45). Medardus et al. reported in 2014 (16) that copper levels in swine feed samples ranged between 3.2 and 365.2 mg/kg. The copper levels in fecal samples were higher, ranging from 71.2 to 2,397 mg/kg. Zinc levels in swine feed samples varied between 77 and 2,000 mg/kg. The level of zinc in fecal samples was significantly higher, ranging between 536.5 and 12,557.2 mg/kg (16). The use of high concentrations of heavy metals as feed additives and biocides and the subsequent deposition across the environment might pose strong selection pressures to bacteria. Notably, SGI3 conferred copper tolerance under anaerobic conditions, suggesting that the acquisition of SGI3 might support *S. enterica* growth in the intestinal environment containing higher concentrations of heavy metals.

In conclusion, the findings of this study clearly demonstrate that SGI3 is an ICE and elevates host tolerance to copper and arsenic. The European clone has been one of the major clones of *Salmonella* Typhimurium worldwide for more than 20 years. Elevated

TABLE 3 Bacterial strains used in this study

Strain	Serovar	Description	SNP genotype	Accession no.	Reference or source
LT2	Typhimurium	ATCC 700726	3	AE006468.2	53
LT2TC*pheV*	Typhimurium	LT2 transconjugant which acquired SGI3 at *pheV* locus	3	NA[a]	This study
LT2TC*pheR*	Typhimurium	LT2 transconjugant which acquired SGI3 at *pheR* locus	3	NA	This study
LT2TC*pheR*ΔSGI3	Typhimurium	SGI3 deletion mutant derived from LT2TC*pheR*	3	NA	This study
LT2TC*pheR*Δ*pco*	Typhimurium	*pco* gene cluster deletion mutant derived from LT2TC*pheR*	3	NA	This study
LT2TC*pheR*Δ*cus*	Typhimurium	*cus* gene cluster deletion mutant derived from LT2TC*pheR*	3	NA	This study
L-3838	4,[5],12:i:–	Wild type	9	AP019374	This study
L-3841	4,[5],12:i:–	Wild type	9	AP019375	This study
L-3841ΔSGI3	4,[5],12:i:–	SGI3 deletion mutant derived from L-3841	9	NA	This study
L-3841Δ*pco*	4,[5],12:i:–	*pco* gene cluster deletion mutant derived from L-3841	9	NA	This study
L-3841Δ*cus*	4,[5],12:i:–	*cus* gene cluster deletion mutant derived from L-3841	9	NA	This study
L-3900	Typhimurium	Wild type	1	NA	11
L-3900TC	Typhimurium	L-3900 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	1	NA	This study
L-4131	Typhimurium	Wild type	2	SAMD00097416	13
L-4131TC	Typhimurium	L-4131 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	2	NA	This study
L-3785	4,[5],12:i:–	Wild type	4	SAMD00097434	13
L-3785TC	4,[5],12:i:–	L-3785 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	4	NA	This study
L-4153	Typhimurium	Wild type	5	SAMD00097444	13
L-4153TC	Typhimurium	L-4153 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	5	NA	This study
L-4125	Typhimurium	Wild type	6	SAMD00097448	13
L-4125TC	Typhimurium	L-4125 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	6	NA	This study
L-4239	Typhimurium	Wild type	7	SAMD00097461	13
L-4239TC	Typhimurium	L-4239 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	7	NA	This study
L-3569	Typhimurium	Wild type	8	NA	This study
L-3569TC	Typhimurium	L-3569 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	8	NA	This study
L-2707	Heidelberg	Wild type	NA	NA	This study
L-2707TC	Heidelberg	L-2707 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	NA	NA	This study
L-2819	Hadar	Wild type	NA	NA	This study
L-2819TC	Hadar	L-2819 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	NA	NA	This study
L-3037	Newport	Wild type	NA	NA	This study
L-3037TC	Newport	L-3037 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	NA	NA	This study
L-3038	Infantis	Wild type	NA	NA	This study
L-3046	Dublin	Wild type	NA	NA	This study
L-3202	Choleraesuis	Wild type	NA	NA	This study
L-3289	Cerro	Wild type	NA	NA	This study
L-3289TC	Cerro	L-3289 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	NA	NA	This study
L-3241	Enteritidis	Wild type	NA	NA	This study
L-4000	Thompson	Wild type	NA	NA	This study
L-4000TC	Thompson	L-4000 transconjugant which acquired SGI3 at *pheV* or *pheR* locus	NA	NA	This study

[a]NA, not applicable.

usage of heavy metals as feed additives might represent a selection pressure for this clone. Continuous monitoring of heavy metals as feed additives and the tolerance of bacteria detected in the livestock industry is required.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 3. *Salmonella* Typhimurium LT2 was purchased from the American Type Culture Collection (ATCC; Manassas, VA). Wild-type strains of *S. enterica* were isolated from organs or feces of diseased animals, including cattle, swine, or birds in Japan between 1997 and 2011. Whole-genome raw sequence reads of *S. enterica* strains L-3785, L-3838, L-3841, L-4125, L-4131, L-4153, and L-4239 were determined and deposited in the DDBJ Sequence Read Archive in the previous study (13). *Salmonella* 4,[5],12:i:– L-3841, which harbors SGI3 in its chromosome, was used as a donor in conjugation experiment. All strains but L-3838 and L-3841 were used as the recipients in conjugation experiments. Rifampin-resistant mutants of all recipients were selected on DHL agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 100 μg/ml of rifampin (Fujifilm Wako Pure Chemical Corp., Osaka, Japan).

Identification of SGI3 structure in wild-type strains. To determine the complete genome sequences of *Salmonella* 4,[5],12:i:– strains L-3838 and L-3841, chromosomal DNA was extracted, followed by long-read sequencing using a PacBio Sequel sequencer (Sequel SMRT Cell 1 M v2 [four/tray]; Sequel sequencing kit v2.1; insert size, approximately 10 kb). The long-read libraries were prepared with a SMRTbell library using a SMRTbell template prep kit 1.0 (PacBio, Menlo Park, CA) with barcoding adapters according to the manufacturer's instructions. These sequencing data were produced with >50-fold coverage, and preassembled reads were generated using SMRT Link software v5. *De novo* assembly with preassembled reads was performed using Canu version 1.4 (PMID: 28298431), Minimap version 0.2-r124

(PMID: 27153593), racon version 1.1.0 (PMID: 28100585), and Circlator version 1.5.3 (PMID: 26714481). Error correction of assembled sequences was performed using Pilon version 1.18 (PMID: 25409509) with Illumina short reads previously deposited in the SRA database (13). ORFs were predicted and automatically annotated by using the DDBJ Fast Annotation and Submission Tool (DFAST) (46). Then, each ORF in SGI3 structure was confirmed using the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) in reference to SGI3 in *Salmonella* 4,[5],12:i:– strain TW-Stm6 (accession number CP019649) (29).

Pairwise alignment of SGI3 with relevant DNA sequences. Pairwise alignment of SGI3 and other mobile genetic elements was performed using the BLAST search tool, followed by a homology search using the NUCmer alignment program from MUMmer in GENETYX version 13 (Genetyx Co., Ltd., Tokyo, Japan). The alignment image was visualized using Easyfig version 2.2.2 (47).

Conjugation experiment. Conjugation experiments were performed using the filter-mating method (48) with slight modifications. A detailed description can be found in the supplemental material. Transconjugants were selected on DHL agar plates containing rifampin (100 μg/ml) and Na$_2$HAsO$_4$ (8 mM) (Fujifilm Wako Pure Chemical). Donor was selected on DHL agar plates with Na$_2$HAsO$_4$ (8 mM). The transfer frequency was calculated as the number of transconjugants divided by the number of donor cells. We repeated the conjugation experiments up to three times for each recipient to obtain transconjugants.

PCR. The primers used in this study are listed in Table S1 in the supplemental material. SNP genotyping was performed to identify the genetic background of *Salmonella* Typhimurium and *Salmonella* 4,[5],12:i:– using the primer pairs SNP3_F/SNP3_R and SNP9_F/SNP9_R (13). IS*200*, located between the genes *fliA* and *fliB* of *Salmonella* Typhimurium, can be used as a specific marker of this serovar (49). The primer pair FFLIB/RFLIA was used for amplification of the intergenic region by PCR to determine whether the tested isolate is *Salmonella* Typhimurium. The amplicon sizes from *Salmonella* Typhimurium, *Salmonella* 4,[5],12:i:–, and other serovars were expected to be 1,000, 1,000, and 250 bp, respectively (50). To localize SGI3, we amplified the junction region between SGI3 and the recipient chromosome. The primer pairs SGI3_LJ_F/SGI3_LJ_R and SGI3_RJ_F/SGI3_RJ_R were used to amplify the left and right junctions of SGI3 at the *pheR* locus, respectively. The primer pairs SGI3_LJ_F2/SGI3_LJ_R and SGI3_RJ_F/SGI3_RJ_R2 were used to amplify the left and right junctions of SGI3 at *pheV* locus, respectively. To confirm the occurrence of the circular form of SGI3, the primer pair SGI3_RJ_F/SGI3_LJ_R was used. All of the oligonucleotides used in this study were purchased from Hokkaido System Science (Hokkaido, Japan). Detailed methods for PCRs are described in the supplemental material.

Localization of SGI3 by Southern blot analysis. To confirm the chromosomal location of SGI3, PFGE, followed by Southern blot analysis, was performed as described previously (51), with slight modifications. Briefly, the cell culture for plug preparation had an optical density at 600 nm of 2. The final concentration of proteinase K (Fujifilm Wako Pure Chemical) in the plugs was 1 mg/ml, and samples were washed with 10 mM Tris-HCl containing 1 mM EDTA buffer four times. The plugs were digested with 20 U/plug of I-CeuI (New England BioLabs, Inc., Ipswich, MA) or 40 U/plug of XbaI (TaKaRa Bio, Inc.) at 37°C for 6 h. XbaI-digested DNA from *Salmonella* Braenderup strain HB9812 was used as a molecular standard marker. The digested fragments were separated by PFGE (6.0 V/cm for 21 h, with a pulsing time linearly ramped from 2.2 to 63.8 s) using the CHEF DR-II apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) on a 1% agarose gel. The separated I-CeuI fingerprint was transferred to positively charged nylon membranes (Roche Diagnostics Co., Indianapolis, IN) and hybridized with PCR-generated digoxigenin-labeled probes (Roche Diagnostics) using DIG Easy Hyb hybridization solution (Roche Diagnostics) according to the manufacturer's instructions. The primer pairs 23S-For/23S-Rev, pcoA-F/pcoA-R, and int-F/int-R were used to generate the probes for the detection of 23S rRNA, *pcoA*, and integrase genes, respectively (Table S1).

Construction of gene replacement vectors and generation of deletion mutants. To generate deletion mutants of SGI3, the *pco* gene cluster, and the *cus* gene cluster, gene replacement was performed as described previously (52) with slight modifications. Detailed methods for the construction of gene replacement vectors can be found in the supplemental material.

The strains L-3841 and LT2TC*pheR* were transformed with one of each vector by electroporation. The cells were spread on LB agar (Becton Dickinson) supplemented with kanamycin (30 μg/ml) and incubated at 30°C overnight. The colonies were then streaked on the same agar plates prewarmed at 42°C and incubated at 42°C overnight. The single-crossover strains were purified under the same conditions and then passaged at 30°C several times. The double-crossover strains were screened for the loss of kanamycin resistance, and the mutation was checked by PCR and sequencing of the amplicon using appropriate primers.

Heavy metal susceptibility testing. MICs of strains to CuSO$_4$, ZnCl$_2$, and Na$_2$HAsO$_4$ (Fujifilm Wako Pure Chemical) were determined by the agar dilution method. Overnight cultures of *S. enterica* grown in LB broth were diluted to approximately 10^6 CFU/ml, and 5-μl portions were inoculated as spots onto Mueller-Hinton agar plates (Becton Dickinson) supplemented with CuSO$_4$ (0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 12 mM), ZnCl$_2$ (0.5, 0.75, 1, 1.5, 2, 3, 4, and 6 mM), or Na$_2$HAsO$_4$ (0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 mM). The spotted plates were incubated at 37°C under aerobic conditions for 20 h or anaerobic conditions for 48 h. An anaerobic atmosphere (not less than 16% carbon dioxide and under 0.1% oxygen) was generated by using AnaeroPack-Anaero (Mitsubishi GAS Chemical Company, Inc., Tokyo, Japan). *Salmonella* Typhimurium LT2 (53) was used as a quality control strain in each test.

Accession number(s). Complete genome sequences of *S. enterica* strains L-3841 and L-3838 were deposited in the DDBJ Sequence Read Archive under accession numbers AP019374 and AP019375, respectively.

SUPPLEMENTAL MATERIAL

ACKNOWLEDGMENTS

REFERENCES

1. Dionisi AM, Graziani C, Lucarelli C, Filetici E, Villa L, Owczarek S, Caprioli A, Luzzi I. 2009. Molecular characterization of multidrug-resistant strains of *Salmonella enterica* serotype Typhimurium and monophasic variant (*S.* 4,[5],12:i:–) isolated from human infections in Italy. Foodborne Pathog Dis 6:711–717. https://doi.org/10.1089/fpd.2008.0240.

2. Elnekave E, Hong S, Mather AE, Boxrud D, Taylor AJ, Lappi V, Johnson TJ, Vannucci F, Davies P, Hedberg C, Perez A, Alvarez J. 2018. *Salmonella enterica* serotype 4,[5],12:i:– in swine in the United States Midwest: an emerging multidrug-resistant clade. Clin Infect Dis 66:877–885. https://doi.org/10.1093/cid/cix909.

3. Foley SL, Lynne AM. 2008. Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. J Anim Sci 86:E173–E187. https://doi.org/10.2527/jas.2007-0447.

4. Herikstad H, Motarjemi Y, Tauxe RV. 2002. *Salmonella* surveillance: a global survey of public health serotyping. Epidemiol Infect 129:1–8. https://doi.org/10.1017/s0950268802006842.

5. Petrovska L, Mather AE, AbuOun M, Branchu P, Harris SR, Connor T, Hopkins KL, Underwood A, Lettini AA, Page A, Bagnall M, Wain J, Parkhill J, Dougan G, Davies R, Kingsley RA. 2016. Microevolution of monophasic *Salmonella* Typhimurium during epidemic, United Kingdom, 2005-2010. Emerg Infect Dis 22:617–624. https://doi.org/10.3201/eid2204.150531.

6. Gallati C, Stephan R, Hachler H, Malorny B, Schroeter A, Nuesch-Inderbinen M. 2013. Characterization of *Salmonella enterica* subsp. *enterica* serovar 4,[5],12:i:– clones isolated from human and other sources in Switzerland between 2007 and 2011. Foodborne Pathog Dis 10:549–554. https://doi.org/10.1089/fpd.2012.1407.

7. Ido N, Lee K, Iwabuchi K, Izumiya H, Uchida I, Kusumoto M, Iwata T, Ohnishi M, Akiba M. 2014. Characteristics of *Salmonella enterica* serovar 4,[5],12:i:– as a monophasic variant of serovar Typhimurium. PLoS One 9:e104380. https://doi.org/10.1371/journal.pone.0104380.

8. Majtan V, Majtanova L, Majtan J. 2011. Phenotypic and molecular characterization of human *Salmonella enterica* serovar 4,[5],12:i:– isolates in Slovakia. Curr Microbiol 63:491. https://doi.org/10.1007/s00284-011-0010-6.

9. Switt AI, Soyer Y, Warnick LD, Wiedmann M. 2009. Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,5,12:i. Foodborne Pathog Dis 6:407–415. https://doi.org/10.1089/fpd.2008.0213.

10. Yang X, Wu Q, Zhang J, Huang J, Guo W, Cai S. 2015. Prevalence and characterization of monophasic *Salmonella* serovar 1,4,[5],12:i:– of food origin in China. PLoS One 10:e0137967. https://doi.org/10.1371/journal.pone.0137967.

11. Ido N, Kudo T, Sasaki K, Motokawa M, Iwabuchi K, Matsudate H, Seimiya YM, Akiba M. 2011. Molecular and phenotypic characteristics of *Salmonella enterica* serovar 4,5,12:i:– isolated from cattle and humans in Iwate Prefecture, Japan. J Vet Med Sci 73:241–244. https://doi.org/10.1292/jvms.10-0140.

12. Kurosawa A, Imamura T, Tanaka K, Tamamura Y, Uchida I, Kobayashi A, Hata E, Kanno T, Akiba M, Yukawa S, Tamura Y. 2012. Molecular typing of *Salmonella enterica* serotype Typhimurium and serotype 4,5,12:i:– isolates from cattle by multiple-locus variable-number tandem-repeats analysis. Vet Microbiol 160:264–268. https://doi.org/10.1016/j.vetmic.2012.05.023.

13. Arai N, Sekizuka T, Tamamura Y, Tanaka K, Barco L, Izumiya H, Kusumoto M, Hinenoya A, Yamasaki S, Iwata T, Watanabe A, Kuroda M, Uchida I, Akiba M. 2018. Phylogenetic characterization of *Salmonella enterica* serovar Typhimurium and its monophasic variant isolated from food animals in Japan revealed replacement of major epidemic clones in the last 4 decades. J Clin Microbiol 56:e01758-17. https://doi.org/10.1128/JCM.01758-17.

14. Tamada Y, Nakaoka Y, Nishimori K, Doi A, Kumaki T, Uemura N, Tanaka K, Makino SI, Sameshima T, Akiba M, Nakazawa M, Uchida I. 2001. Molecular typing and epidemiological study of *Salmonella enterica* serotype Typhimurium isolates from cattle by fluorescent amplified-fragment length polymorphism fingerprinting and pulsed-field gel electrophoresis. J Clin Microbiol 39:1057–1066. https://doi.org/10.1128/JCM.39.3.1057-1066.2001.

15. Tamamura Y, Uchida I, Tanaka K, Okazaki H, Tezuka S, Hanyu H, Kataoka N, Makino S, Kishima M, Kubota T, Kanno T, Hatama S, Ishihara R, Hata E, Yamada H, Nakaoka Y, Akiba M. 2011. Molecular epidemiology of *Salmonella enterica* serovar Typhimurium isolates from cattle in Hokkaido, Japan: evidence of clonal replacement and characterization of the disseminated clone. Appl Environ Microbiol 77:1739–1750. https://doi.org/10.1128/AEM.01910-10.

16. Medardus JJ, Molla BZ, Nicol M, Morrow WM, Rajala-Schultz PJ, Kazwala R, Gebreyes WA. 2014. In-feed use of heavy metal micronutrients in U.S. swine production systems and its role in persistence of multidrug-resistant salmonellae. Appl Environ Microbiol 80:2317–2325. https://doi.org/10.1128/AEM.04283-13.

17. Bellanger X, Payot S, Leblond-Bourget N, Guédon G. 2014. Conjugative and mobilizable genomic islands in bacteria: evolution and diversity. FEMS Microbiol Rev 38:720–760. https://doi.org/10.1111/1574-6976.12058.

18. Johnson CM, Grossman AD. 2015. Integrative and conjugative elements (ICEs): what they do and how they work. Annu Rev Genet 49:577–601. https://doi.org/10.1146/annurev-genet-112414-055018.

19. Delavat F, Miyazaki R, Carraro N, Pradervand N, van der Meer JR. 2017. The hidden life of integrative and conjugative elements. FEMS Microbiol Rev 41:512–537. https://doi.org/10.1093/femsre/fux008.

20. Christie PJ, Korman RZ, Zahler SA, Adsit JC, Dunny GM. 1987. Two conjugation systems associated with *Streptococcus faecalis* plasmid pCF10: identification of a conjugative transposon that transfers between *S. faecalis* and *Bacillus subtilis*. J Bacteriol 169:2529–2536. https://doi.org/10.1128/jb.169.6.2529-2536.1987.

21. Harada S, Ishii Y, Saga T, Tateda K, Yamaguchi K. 2010. Chromosomally encoded bla_{CMY-2} located on a novel SXT/R391-related integrating conjugative element in a *Proteus mirabilis* clinical isolate. Antimicrob Agents Chemother 54:3545–3550. https://doi.org/10.1128/AAC.00111-10.

22. Shoemaker NB, Barber RD, Salyers AA. 1989. Cloning and characterization of a *Bacteroides* conjugal tetracycline-erythromycin resistance element by using a shuttle cosmid vector. J Bacteriol 171:1294–1302. https://doi.org/10.1128/jb.171.3.1294-1302.1989.

23. Whittle G, Shoemaker NB, Salyers AA. 2002. The role of *Bacteroides* conjugative transposons in the dissemination of antibiotic resistance genes. Cell Mol Life Sci 59:2044–2054. https://doi.org/10.1007/s000180200004.

24. Wozniak RA, Fouts DE, Spagnoletti M, Colombo MM, Ceccarelli D, Garriss G, Dery C, Burrus V, Waldor MK. 2009. Comparative ICE genomics: insights into the evolution of the SXT/R391 family of ICEs. PLoS Genet 5:e1000786. https://doi.org/10.1371/journal.pgen.1000786.

25. Carter MQ, Chen J, Lory S. 2010. The *Pseudomonas aeruginosa* pathogenicity island PAPI-1 is transferred via a novel type IV pilus. J Bacteriol 192:3249–3258. https://doi.org/10.1128/JB.00041-10.

26. Ravatn R, Studer S, Springael D, Zehnder AJ, van der Meer JR. 1998. Chromosomal integration, tandem amplification, and deamplification in *Pseudomonas putida* F1 of a 105-kilobase genetic element containing the chlorocatechol degradative genes from *Pseudomonas* sp. Strain B13. J Bacteriol 180:4360–4369.

27. Fukuda K, Hosoyama A, Tsuchikane K, Ohji S, Yamazoe A, Fujita N, Shintani M, Kimbara K. 2014. Complete genome sequence of polychlorinated biphenyl degrader *Comamonas testosteroni* TK102 (NBRC 109938). Genome Announc 2:e00865-14. https://doi.org/10.1128/genomeA.00865-14.

28. Ohtsubo Y, Ishibashi Y, Naganawa H, Hirokawa S, Atobe S, Nagata Y, Tsuda M. 2012. Conjugal transfer of polychlorinated biphenyl/biphenyl degradation genes in *Acidovorax* sp. strain KKS102, which are located on an integrative and conjugative element. J Bacteriol 194:4237–4248. https://doi.org/10.1128/JB.00352-12.

29. Dyall-Smith ML, Liu Y, Billman-Jacobe H. 2017. Genome sequence of an Australian monophasic *Salmonella enterica* subsp. *enterica* Typhimurium isolate (TW-Stm6) carrying a large plasmid with multiple antimicrobial resistance genes. Genome Announc 5:e00793-17. https://doi.org/10.1128/genomeA.00793-17.

30. Staehlin BM, Gibbons JG, Rokas A, O'Halloran TV, Slot JC. 2016. Evolution of a heavy metal homeostasis/resistance island reflects increasing copper stress in enterobacteria. Genome Biol Evol 8:811–826. https://doi.org/10.1093/gbe/evw031.

31. Brown NL, Barrett SR, Camakaris J, Lee BT, Rouch DA. 1995. Molecular genetics and transport analysis of the copper-resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ1004. Mol Microbiol 17:1153–1166. https://doi.org/10.1111/j.1365-2958.1995.mmi_17061153.x.

32. Gupta A, Matsui K, Lo JF, Silver S. 1999. Molecular basis for resistance to silver cations in *Salmonella*. Nat Med 5:183–188. https://doi.org/10.1038/5545.

33. Munson GP, Lam DL, Outten FW, O'Halloran TV. 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. J Bacteriol 182:5864–5871. https://doi.org/10.1128/jb.182.20.5864-5871.2000.

34. San Francisco MJ, Hope CL, Owolabi JB, Tisa LS, Rosen BP. 1990. Identification of the metalloregulatory element of the plasmid-encoded arsenical resistance operon. Nucleic Acids Res 18:619–624. https://doi.org/10.1093/nar/18.3.619.

35. Subramanya HS, Arciszewska LK, Baker RA, Bird LE, Sherratt DJ, Wigley DB. 1997. Crystal structure of the site-specific recombinase, XerD. EMBO J 16:5178–5187. https://doi.org/10.1093/emboj/16.17.5178.

36. Lang S, Kirchberger PC, Gruber CJ, Redzej A, Raffl S, Zellnig G, Zangger K, Zechner EL. 2011. An activation domain of plasmid R1 TraI protein delineates stages of gene transfer initiation. Mol Microbiol 82:1071–1085. https://doi.org/10.1111/j.1365-2958.2011.07872.x.

37. Wozniak RA, Waldor MK. 2010. Integrative and conjugative elements: mosaic mobile genetic elements enabling dynamic lateral gene flow. Nat Rev Microbiol 8:552–563. https://doi.org/10.1038/nrmicro2382.

38. Rensing C, Grass G. 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol Rev 27:197–213. https://doi.org/10.1016/S0168-6445(03)00049-4.

39. Rademacher C, Masepohl B. 2012. Copper-responsive gene regulation in bacteria. Microbiology 158:2451–2464. https://doi.org/10.1099/mic.0.058487-0.

40. Outten FW, Huffman DL, Hale JA, O'Halloran TV. 2001. The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. J Biol Chem 276:30670–30677. https://doi.org/10.1074/jbc.M104122200.

41. Espariz M, Checa SK, Audero ME, Pontel LB, Soncini FC. 2007. Dissecting the *Salmonella* response to copper. Microbiology 153:2989–2997. https://doi.org/10.1099/mic.0.2007/006536-0.

42. Pontel LB, Soncini FC. 2009. Alternative periplasmic copper-resistance mechanisms in Gram negative bacteria. Mol Microbiol 73:212–225. https://doi.org/10.1111/j.1365-2958.2009.06763.x.

43. Lee SM, Grass G, Rensing C, Barrett SR, Yates CJ, Stoyanov JV, Brown NL. 2002. The Pco proteins are involved in periplasmic copper handling in *Escherichia coli*. Biochem Biophys Res Commun 295:616–620. https://doi.org/10.1016/S0006-291X(02)00726-X.

44. Mukhopadhyay R, Rosen BP, Phung LT, Silver S. 2002. Microbial arsenic: from geocycles to genes and enzymes. FEMS Microbiol Rev 26:311–325. https://doi.org/10.1111/j.1574-6976.2002.tb00617.x.

45. Jackson BP, Bertsch PM. 2001. Determination of arsenic speciation in poultry wastes by IC-ICP-MS. Environ Sci Technol 35:4868–4873. https://doi.org/10.1021/es0107172.

46. Tanizawa Y, Fujisawa T, Nakamura Y. 2018. DFAST: a flexible prokaryotic genome annotation pipeline for faster genome publication. Bioinformatics 34:1037–1039. https://doi.org/10.1093/bioinformatics/btx713.

47. Sullivan MJ, Petty NK, Beatson SA. 2011. Easyfig: a genome comparison visualizer. Bioinformatics 27:1009–1010. https://doi.org/10.1093/bioinformatics/btr039.

48. Sasaki Y, Taketomo N, Sasaki T. 1988. Factors affecting transfer frequency of pAMβ1 from *Streptococcus faecalis* to *Lactobacillus plantarum*. J Bacteriol 170:5939–5942. https://doi.org/10.1128/jb.170.12.5939-5942.1988.

49. Burnens AP, Stanley J, Sack R, Hunziker P, Brodard I, Nicolet J. 1997. The flagellin N-methylase gene *fliB* and an adjacent serovar-specific IS*200* element in *Salmonella* Typhimurium. Microbiology 143:1539–1547. https://doi.org/10.1099/00221287-143-5-1539.

50. Echeita MA, Herrera S, Usera MA. 2001. Atypical, *fljB*-negative *Salmonella enterica* subsp. *enterica* strain of serovar 4,5,12:i:– appears to be a monophasic variant of serovar Typhimurium. J Clin Microbiol 39:2981–2983. https://doi.org/10.1128/JCM.39.8.2981-2983.2001.

51. Shahada F, Sekizuka T, Kuroda M, Kusumoto M, Ohishi D, Matsumoto A, Okazaki H, Tanaka K, Uchida I, Izumiya H, Watanabe H, Tamamura Y, Iwata T, Akiba M. 2011. Characterization of *Salmonella enterica* serovar Typhimurium isolates harboring a chromosomally encoded CMY-2 beta-lactamase gene located on a multidrug resistance genomic island. Antimicrob Agents Chemother 55:4114–4121. https://doi.org/10.1128/AAC.00560-11.

52. Akiba M, Nakaoka Y, Kida M, Ishioka Y, Sameshima T, Yoshii N, Nakazawa M, Uchida I, Terakado N. 2007. Changes in antimicrobial susceptibility in a population of *Salmonella enterica* serovar Dublin isolated from cattle in Japan from 1976 to 2005. J. Antimicrob Chemother 60:1235–1242. https://doi.org/10.1093/jac/dkm402.

53. McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, Courtney L, Porwollik S, Ali J, Dante M, Du F, Hou S, Layman D, Leonard S, Nguyen C, Scott K, Holmes A, Grewal N, Mulvaney E, Ryan E, Sun H, Florea L, Miller W, Stoneking T, Nhan M, Waterston R, Wilson RK. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413:852–856. https://doi.org/10.1038/35101614.