Regulation, Integrase-Dependent Excision, and Horizontal Transfer of Genomic Islands in *Legionella pneumophila*

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Legionella pneumophila is a Gram-negative freshwater agent which multiplies in specialized nutrient-rich vacuoles of amoebae. When replicating in human alveolar macrophages, *Legionella* can cause Legionnaires' disease. Recently, we identified a new type of conjugation/type IVA secretion system (T4ASS) in *L. pneumophila* Corby (named *trb-tra*). Analogous versions of *trb-tra* are localized on the genomic islands Trb-1 and Trb-2. Both can exist as an episomal circular form, and Trb-1 can be transferred horizontally to other *Legionella* strains by conjugation. In our current work, we discovered the importance of a site-specific integrase (Int-1, *lpc2818*) for the excision and conjugation process of Trb-1. Furthermore, we identified the genes *lvrRABC* (*lpc2813* to *lpc2816*) to be involved in the regulation of Trb-1 excision. In addition, we demonstrated for the first time that a *Legionella* genomic island (LGI) of *L. pneumophila* Corby (LpcGI-2) encodes a functional type IV secretion system. The island can be transferred horizontally by conjugation and is integrated site specifically into the genome of the transconjugants. LpcGI-2 generates three different episomal forms. The predominant episomal form, form A, is generated integrase dependently (Lpc1833) and transferred by conjugation in a *pilT*-dependent manner. Therefore, the genomic islands Trb-1 and LpcGI-2 should be classified as integrative and conjugative elements (ICEs). Coculture studies of *L. pneumophila* wild-type and mutant strains revealed that the *int-1* and *lvrRABC* genes (located on Trb-1) as well as *lpc1833* and *pilT* (located on LpcGI-2) do not influence the *in vivo* fitness of *L. pneumophila* in *Acanthamoeba castellanii*.

Legionella pneumophila is a Gram-negative bacterium found ubiquitously in freshwater habitats (1). It resides in biofilms or invades free-living protozoa such as *Acanthamoeba castellanii* (2, 3). Furthermore, *Legionella* is able to infect human lung alveolar macrophages. When inhaled with contaminated aerosols, *Legionella* can cause a severe, life-threatening pneumonia, Legionnaires' disease (4). *L. pneumophila* strain Corby (serogroup 1 [Sg1], monoclonal antibody type Knoxville) is a highly virulent human isolate (5). In aerosol-infected guinea pigs, the strain multiplies very rapidly within the lung, and the bacteria spread to the blood, liver, spleen, and kidney (5, 6). In host cells, *Legionella* avoids killing by the phagolysosomal pathway and establishes a specialized *Legionella*-containing vacuole (LCV) for replication (7, 8). When nutrition becomes limiting, the bacterium switches to the virulent phase, evades the vacuole, and destroys the host cell. *Legionella* manipulates the host cell by introducing virulence factors via specialized secretion systems. This is crucial for intracellular survival and the establishment of the replication-permissive LCV in amoebae and macrophages (9–12).

Type IV secretion systems (T4SSs) are needed for conjugation and for transport of proteins and nucleic acids into the host cell during infection (13–15). They are widespread and grouped into the IVA and IVB families (16). The *Legionella* type IVB secretion system (T4BSS) *dot* (defect in organelle trafficking)-*icm* (intracellular multiplication) is similar to the *tra-trb* system of IncI plasmids (16, 17). It enables intracellular multiplication by translocating effector proteins into the host cell (9, 11, 18–21). Type IVA secretion systems (T4ASSs) are similar to the *tra* system of IncN plasmids (17, 22). The T4ASS *lvh* is dispensable for intracellular growth of *Legionella* at 37°C but is involved in host cell infection at lower temperatures (23–25). In *L. pneumophila* Corby, further T4ASSs are encoded by the *trb-tra* genes on the genomic islands Trb-1 and Trb-2. Trb-1 and Trb-2 are integrated within the tRNA^{Pro} gene (*lpc2778*) and the tmRNA gene, respectively. Both

islands exhibit an origin of transfer (*oriT*) region and are excised from the chromosome, forming episomal circles (ci's). The episomal form of Trb-1 can be transferred horizontally to another *L. pneumophila* strain by conjugation and is then integrated site specifically into the genome of the transconjugants (26). This finding may explain the observed horizontal transfer of chromosomal DNA in *Legionella* (27, 28). Recently, two further genomic islands, Trb-3 (*L. pneumophila* strain Lorraine) and Trb-4 (*L. longbeachae* NSW150), were identified. So far, nothing is known about excision of these elements (27).

In another classification system, the T4SSs are grouped into three distinct clusters, due to their homology: F-like (IncF, plasmid F), P-like (IncP, plasmid RP4), and I-like (IncI, plasmid R64) (14, 29). Juhas and colleagues described a further class of T4SSs, named the genomic island T4SS (GI-like) (30, 31). For *L. pneumophila*, two new GI-like islands (*Legionella* genomic island 1 [LGI-1] and LGI-2) were identified by genome sequence analysis of strain 130b (32). The authors speculate that these islands may be new T4SSs belonging to the integrative and conjugative elements (ICEs) and could contribute to mobilization of genomic islands in *L. pneumophila*. However, no experimental data were given.

The intention of our present work was to screen the *L. pneumophila* Corby genome for further genomic islands and to gain

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TABLE 1 *Legionella* strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
<i>L. pneumophila</i> Corby	WT	Jepras et al. (5)
<i>L. pneumophila</i> Philadelphia I JR32	Restriction-deficient strain of <i>L. pneumophila</i> Philadelphia I (Sm ^r)	Marra and Shuman (34)
<i>L. oakridgensis</i>	ATCC 33761	C. Lück, Dresden, Germany
<i>L. pneumophila</i> Corby WT ^o	Km ^r cassette between <i>lpc2816</i> and <i>lpc2817</i>	This study
<i>L. pneumophila</i> Corby WT*	Km ^r cassette between <i>lpc1856</i> and <i>lpc1857</i>	This study
<i>L. pneumophila</i> Corby Δ int-1	<i>lpc2818::Km^r</i>	This study
<i>L. pneumophila</i> Corby Δ lvrR	<i>lpc2816::Km^r</i>	This study
<i>L. pneumophila</i> Corby Δ lvrRABC	<i>lpc2813</i> to <i>lpc2816::Km^r</i>	This study
<i>L. pneumophila</i> Corby Δ lpc1833	<i>lpc1833::Km^r</i>	This study
<i>L. pneumophila</i> Corby Δ lpc1884	<i>lpc1884::Km^r</i>	This study
<i>L. pneumophila</i> Corby Δ lpc2123	<i>lpc2123::Km^r</i>	This study
<i>L. pneumophila</i> Corby Δ pilT	<i>lpc1876::Km^r</i>	This study
Plasmids^a		
pML2	2,990-bp PCR fragment (primers Trb1-Km-U/Trb1-Km-R)	This study
pML3	pML2 after inverse PCR (primers Trb1-Km-MU/Trb1-Km-MR)	This study
pML4	pML3 with Km ^r cassette cloned into XbaI site between <i>lpc2816</i> and <i>lpc2817</i>	This study
pML9	3,208-bp PCR fragment (primers Int-U/Int-R) in pGEM-T Easy	This study
pML11	pML9 after inverse PCR (primers Int-MU/Int-MR)	This study
pML12	pML11 with Km ^r cassette cloned into XbaI site instead of <i>lpc2818</i>	This study
pML14	3,418-bp PCR fragment (primers pilT-1876-U/pilT-1876-R)	This study
pML15	pML14 after inverse PCR (primers pilT-1876-MU/pilT-1876-MR)	This study
pML16	pML15 with Km ^r cassette cloned into XbaI site instead of <i>lpc1876</i>	This study
pML17	2,816-bp PCR fragment (primers Int-1884-U/Int-1884-R)	This study
pML18	pML17 after inverse PCR (primers Int-1884-MU/Int-1884-MR)	This study
pML19	pML18 with Km ^r cassette cloned into XbaI site instead of <i>lpc1884</i>	This study
pML20	2,198-bp PCR fragment (primers LpcGI-2-Km-U/LpcGI-2-Km-R)	This study
pML21	pML20 after inverse PCR (primers LpcGI-2-Km-MU/LpcGI-2-Km-MR)	This study
pML22B	pML21 with Km ^r cassette cloned into XbaI site between <i>lpc1856</i> and <i>lpc2857</i>	This study
pML23	3,103-bp PCR fragment (primers Int-1833-U/Int-1833-R)	This study
pML24	pML23 after inverse PCR (primers Int-1833-MU/Int-1833-MR)	This study
pML25A	pML24 with Km ^r cassette cloned into XbaI site instead of <i>lpc1833</i>	This study
pML26	2,377-bp PCR fragment (primers Int-2123-U/Int-2123-R)	This study
pML27	pML26 after inverse PCR (primers Int-2123-MU/Int-2123-MR)	This study
pML28	pML27 with Km ^r cassette cloned into XbaI site instead of <i>lpc2123</i>	This study
pML54	2,530-bp PCR fragment (primers LvrR-2816-U/LvrR-2816-R)	This study
pML56	pML54 after inverse PCR (primers LvrR-2816-MU/LvrR-2816-MR)	This study
pML58	pML56 with Km ^r cassette cloned into XbaI site instead of <i>lpc2816</i>	This study
pVH7	1,130-bp PCR fragment (primers LvrRABC-1U/LvrRABC-1R)	This study
pVH8	1,090-bp PCR fragment (primers LvrRABC-2U/LvrRABC-2R)	This study
pVH9	Insert of pVH8 cloned into XbaI and XhoI site in pVH7	This study
pVH10	pVH9 with Km ^r cassette cloned into XbaI site instead of <i>lpc2816</i> to <i>lpc2813</i>	This study

^a For all plasmids, pGEM-T Easy (Promega) was used as the vector.

more insight into the horizontal transfer process. To further analyze the excision of genomic islands from the chromosome and to verify if LGI-2 is a functional ICE, we generated and analyzed integrase mutants of the genomic islands Trb-1 and LGI-2 of *L. pneumophila* Corby (LpcGI-2). We could verify our hypothesis (26) that a defined integrase located on the genomic island itself is indispensable for the site-specific excision of Trb-1 from the chromosome and expand this conclusion to the process of LpcGI-2 excision. Furthermore, we were able to demonstrate that the circularization of Trb-1 is regulated by the *lvrRABC* gene cluster, which we assumed because *lvrR* is predicted to be a transcriptional regulator and *lvrC* encodes a paralog of CsrA. CsrA is known to be involved in gene regulation of *L. pneumophila* (33).

MATERIALS AND METHODS

Bacterial strains, amoeba, and cell lines. Experiments were done with *L. pneumophila* Sg1 strain Corby (5), *L. pneumophila* Sg1 strain Philadelphia I (ATCC 33152) (34), and *L. oakridgensis* ATCC 33761. The *L. pneumophila* Corby wild-type (WT) strains WT^o and WT* were used as positive controls in mating experiments. *L. pneumophila* Corby WT^o contains a kanamycin resistance (Km^r) cassette between the genes *lpc2816* and *lpc2817*, and in *L. pneumophila* Corby WT*, a Km resistance cassette was introduced between the genes *lpc1856* and *lpc1857*. Mutant strains of *L. pneumophila* Corby used in this study contained the mutations Δ int-1, Δ lvrRABC, Δ lvrR, Δ pilT, Δ lpc1833, Δ lpc1884, and Δ lpc2123. All strains are listed in Table 1. *Escherichia coli* strain DH5 α was used as the host for recombinant plasmids (35). *Acanthamoeba castellanii* ATCC 30010 (36) and the U937 cell line (ATCC CRL-1593.2) were used for infection assays.

Media and growth conditions. *Legionella* was grown in AYE medium [1% yeast extract supplemented with 1% ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid], 0.025% ferric PP₃, 0.04% L-cysteine] or on buffered charcoal-yeast extract (BCYE) agar at 37°C. Antibiotics used for *L. pneumophila* were kanamycin (Km) at a concentration of 12.5 mg ml⁻¹ and streptomycin (Sm) at a concentration of 50 mg ml⁻¹. Bacterial growth in broth was monitored by determining the optical density at 600 nm (OD₆₀₀) with a Thermo Scientific GENESYS 10 Bio spectrophotometer (VWR, Darmstadt, Germany). Growth phases were defined as follows: an OD₆₀₀ of ~1.0 corresponded to the exponential growth phase (E), additional growth for 8 h and an OD₆₀₀ of ~1.7 corresponded to the late exponential growth phase (LE), additional growth for 4 h and an OD₆₀₀ of ~1.8 corresponded to the postexponential growth phase (PE), and additional growth for 8 h and an OD₆₀₀ of ~2.0 corresponded to the stationary growth phase (S). *E. coli* was cultivated in Luria-Bertani (LB) medium or on LB agar. The antibiotics used for *E. coli* were ampicillin (Ap) at a concentration of 100 mg ml⁻¹ and Km at concentrations of 40 mg ml⁻¹. *Acanthamoeba castellanii* ATCC 30010 was cultured in PYG 712 medium [2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO₄, 0.4 M CaCl₂, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH₄)₂(SO₄)₂ · 6 H₂O, 2.5 mM NaH₂PO₄, 2.5 mM K₂HPO₄] at 20°C. U937 cells were cultured in RPMI with 10% fetal calf serum (FCS) at 37°C in 5% CO₂.

DNA techniques and sequence analysis. Genomic DNA for PCR was prepared with a Generation capture column kit (Qiagen, Hilden, Germany), and genomic DNA for real-time PCR was prepared with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The preparation of plasmid DNA was done with an Invisorb spin plasmid mini-two kit (Strattec, Berlin, Germany). Plasmid DNA was introduced into *E. coli* by electroporation with a gene pulser (Bio-Rad, Munich, Germany) at 1.7 kV, 100 Ω, and 25 μF. Both strands of plasmid DNA or a PCR product were sequenced with infrared dye-labeled primers by using an automated DNA sequencer (LI-COR-DNA4000; MWG-Biotech, Ebersberg, Germany). Oligonucleotides were obtained from Eurofins MWG Operon (Ebersberg, Germany). Restriction enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany).

***Legionella* mutant construction.** The *int-1* (*lpc2818*) gene-encoding DNA region was amplified by PCR with the primers Int-U and Int-R and cloned into the pGEM-T Easy vector. The resulting plasmid (pML9) was used as a template in an inverse PCR, using KAPAHifi DNA polymerase (Peqlab, Erlangen, Germany) for reaction. The PCR product was amplified with the primer pair Int-MU/Int-MR, with one of the primers containing an XbaI restriction site and enabling religation. The resulting plasmid (pML11) and a Km^r cassette were restricted with XbaI and ligated. To generate the mutant, the insert of the plasmid (pML12) containing the Km^r cassette and the flanking DNA sequences was amplified with the primer pair Int-U/Int-R. To generate other mutants of this study, plasmids containing the Km^r cassette and the flanking sequences were used as follows: pML56 for the *ΔlvrR* mutant, pVH10 for the *ΔlvrRABC* mutant, pML25A for the *Δlpc1833* mutant, pML19 for the *Δlpc1884* mutant, pML27 for the *Δlpc2123* mutant, and pML16 for the *ΔpilT* mutant. The following plasmids were used to clone a Km^r cassette in genomic islands: pML4 for Trb-1 and pML22B for LpcGI-2. Natural transformation of *L. pneumophila* Corby was done as described before with modifications (37). In brief, 2 ml of a culture exponentially grown overnight at 30°C was transferred to a plastic tube and incubated with the PCR product for 3 days at 30°C without agitation. Subsequently, bacteria were grown on antibiotic selective medium for 4 more days at 37°C. All mutant strains of *L. pneumophila* Corby generated in this study were produced by a method analogous to the method used for the *Δint-1* mutant. Mutant strains and plasmids are given in Table 1; specific primers used for mutant construction are listed in Table S1 in the supplemental material.

PCR analysis. PCR was carried out using a TRIO-Thermoblock thermocycler (Biometra, Göttingen, Germany) and HotStar Taq DNA polymerase (Qiagen, Hilden, Germany). The characterization of Trb-1 was

done as described before (26). PCR analysis of the genomic islands LpcGI-2 and LpcGI-1 was done with specific primer pairs analogous to those used for Trb-1. The tRNA regions of LpcGI-2 (tRNA^{Met}) and LpcGI-1 (tRNA^{Thr}) were amplified with the primer pairs LpcGI-2-1U/LpcGI-2-4R (referred to as primers 1/4), LpcGI-2-2R/LpcGI-2-6U (primers 2/6), and LpcGI-2-4R/LpcGI-2-6U (primers 4/6). The amplification of the different circular forms of the genomic islands was done with the primer pairs LpcGI-2-2R/LpcGI-2-3U (primers 2/3), LpcGI-2-1U/LpcGI-2-5R (primers 1/5), and LpcGI-2-3U/LpcGI-2-5R (primers 3/5). Points of integration of LpcGI-2 and LpcGI-1 into the chromosome of *L. pneumophila* Corby were shown by the primer combinations 1/2, 3/4, and 5/6. The characterization of LpcGI-Asn and LpcGI-Phe was done by a method analogous to the method used for Trb-1. The specific primer pairs 2/3, 1/4, 1/2, and 3/4 were used to demonstrate the circular form of the genomic island, to amplify the equal tRNA-encoding region, and for the amplification of the 5' and 3' regions of the point of integration of LpcGI-Asn or LpcGI-Phe into the genome, respectively. In general, initial denaturation was performed at 95°C for 15 min and final extension was performed at 72°C for 10 min. The cycling conditions (35 cycles) were 94°C for 1 min, 60°C for 45 s, and 72°C for 2 min. All primers specific for the genomic islands are listed in Table S1 in the supplemental material and illustrated in Fig. 2, 3, and 5.

***L. pneumophila* mating experiments.** Recipient (*L. pneumophila* JR32 Sm^r or *L. oakridgensis* ATCC 33761) and donor (*L. pneumophila* Corby WT⁺, WT⁺, *Δint-1*, *ΔlvrRABC*, *ΔlvrR*, *ΔpilT*, *Δlpc1833*, *Δlpc1884*, or *Δlpc2123*) strains were grown in AYE medium at 37°C. One milliliter of the donor strain (exponential phase) was mixed with 2 ml of the recipient strain (stationary phase) strain. Matings were performed in triplicate by incubating the mixed bacterial cultures for 24 h at 30°C on BCYE agar plates with or without the presence of DNase (1 μg/μl). After mating, *L. pneumophila* transconjugants were selected on BCYE plates containing kanamycin and streptomycin. For the selection of *L. oakridgensis* transconjugants, BCYE plates with kanamycin but without additional L-cysteine were used. In contrast to *L. oakridgensis*, *L. pneumophila* is not able to grow on these agar plates (38). Dilutions of transconjugants were plated on agar plates, and the number of transconjugants was determined by determination of the numbers of CFU. Conjugation frequencies were calculated as the number of transconjugants divided by the number of donor cells.

RNA techniques and cDNA synthesis. For RNA preparation, an overnight culture was diluted in AYE medium to an OD₆₀₀ of ~0.3 and cultured at 37°C to the favored growth phase. Total RNA was extracted from exponential and postexponential growth phase using a High Pure RNA isolation kit (Roche, Mannheim, Germany). Purified RNA was incubated with 100 U DNase I per ml (Qiagen, Hilden, Germany) for 30 min at room temperature. After DNase treatment, RNA was repurified with an RNeasy minikit (Qiagen, Hilden, Germany). PCR with primers specific for *gyrA* was done to analyze the purified RNA for the absence of genomic DNA. Synthesis of cDNA was performed with a SuperScript VILO cDNA synthesis kit (Invitrogen, Darmstadt, Germany) and started with 50 ng μl⁻¹ of total RNA. Synthesis was done according to the instructions of the manufacturer.

Real-time PCR. Real-time quantitative PCR (qPCR) was performed using an Mx3000P thermal cycler (Stratagene) and an Express SYBR GreenER qPCR SuperMix universal kit (Invitrogen, Darmstadt, Germany) according to the instructions of the manufacturers. A standard curve was used to quantify the amount of target present in unknown samples. For a standard curve, 10 μl from each probe was mixed and diluted from 10⁷ to 10¹ in diethyl pyrocarbonate water. The primer pair RT-*gyrA*-U/RT-*gyrA*-R was used for the standard curve. Genomic DNA isolated from the exponential and stationary phases was used to determine the amount of the episomal forms of the genomic islands Trb-1 (DNA concentration, 35 ng μl⁻¹) and LpcGI-2 (DNA concentration, 50 ng μl⁻¹). Episomal forms of LpcGI-2 were amplified with the primer pairs 2/3, 1/5, and 3/5. The episomal form of Trb-1 was shown by the primer

FIG 1 Genetic organization of the genomic islands LpcGI-2, LppGI-2, LpwGI-2, and LpcGI-1 of *L. pneumophila* (Lp) strains Corby (Lpc), Paris (Lpp), and 130b (Lpw) encoding LGI-like T4SSs. LpcGI-2 and LppGI-2 are integrated within the tRNA^{Met} gene, whereas LpwGI-2 and LpcGI-1 are integrated within the tRNA^{Arg} and tRNA^{Thr} genes, respectively. The gene numbers are given above the genes, which are indicated by arrows. DNA regions encoding clustered homologous proteins are boxed in the same color. The colors of the genes indicate their degree of homology to the genes on LpcGI-2. Subregions (I to IV) of the islands are given below the genes.

pair RT-trb-2R/RT-trb-3U. To determine the relative amounts of amplicons of the episomal forms, the chromosomal gene *flaA* was used as an internal standard. All specific primers for qPCR are listed in Table S1 in the supplemental material. Data analysis and calculation of quantity (gene copies) were done with the Stratagene MxPro software.

Intracellular replication in *A. castellanii*. Infection assays of *L. pneumophila* Corby and the mutant strains in *A. castellanii* were performed as described previously (39). In brief, 3-day-old cultures of *A. castellanii* were washed in AC buffer (PYG 712 medium without proteose peptone, glucose, and yeast extract), adjusted to 1×10^5 cells per ml, and incubated in 24-well plates for 2 h at 37°C in 5% CO₂. Stationary-phase *Legionella* bacteria grown on BCYE agar were diluted in AC buffer and mixed with *A. castellanii* at a multiplicity of infection (MOI) of 0.01. After invasion for 2 h at 37°C, the *A. castellanii* layer was washed twice with AC buffer. To determine the numbers of CFU of *L. pneumophila*, different dilutions of the *Legionella*-amoeba mix were plated on BCYE agar. Each infection was carried out in duplicate wells and was done at least three times.

Infection/survival assay in *A. castellanii*. To study the intracellular multiplication and survival in *A. castellanii*, a protocol was used as described recently (40). After 3 days of infection, *A. castellanii* cells were resuspended, 100-μl aliquots were lysed, and several dilutions were plated on BCYE agar to determine the number of CFU. To study the replication rates, the infection was repeated weekly with fresh amoebae. Afterwards, the remaining solution was incubated at 37°C in 5% CO₂ for a further 4 days, diluted in AC buffer (1:1,000), and plated on BCYE agar. Of this dilution, 1 ml was used to infect fresh amoeba cultures as described above. Four rounds of infection were performed. Each infection was carried out in duplicate wells.

Infection/survival assay in competition. For intracellular multiplication in competition, the infection protocol was carried out as described recently (40). The procedure is similar to the assay described above, except that a 1:1 mixture of *L. pneumophila* wild type and one of its isogenic mutant strains was used for infection. The number of CFU was determined by plating serial dilutions on BCYE with and without kanamycin. The number of wild-type bacteria was calculated by subtracting the number of CFU on BCYE agar with kanamycin from the number of CFU on BCYE plates without kanamycin. Each infection was carried out in duplicate wells.

Intracellular multiplication in human macrophages. Transformation and infection of U937 cells were done as previously described with

modifications (41–43). U937 cells were adjusted to 3×10^5 cells/ml and transferred to 100 ml fresh RPMI medium containing 10% FCS. For differentiation into macrophage-like cells, phorbol-12-myristate-13-acetate (PMA; stock concentration, 1 mg/ml in double-distilled H₂O [P-8139; Sigma-Aldrich]) was added at a concentration of 1:20,000, and cells were incubated for 36 h at 37°C in 5% CO₂. Afterwards, the supernatant was discarded and cells were washed once with 10 ml 0.2% EDTA in phosphate-buffered saline (PBS). Cells were removed from the flask bottom with RPMI–10% FCS, transferred to 50-ml tubes, and centrifuged at $800 \times g$ for 10 min. To determine the cell number, 100 μl of cell solution was treated with 100 μl trypan blue. Viable cells were counted in a Neubauer counting chamber, and the concentration of the cell solution was adjusted to 1×10^6 cells/ml with RPMI–10% FCS. To each well of a 24-well plate, 1 ml of the cell suspension was added and the mixture was incubated for 2 h at 37°C in 5% CO₂ to allow adhesion. Stationary-phase *Legionella* grown on BCYE agar was diluted in PBS and added to the macrophage cells. Infection was done with an MOI of 0.01 (time zero) for 2 h at 37°C in 5% CO₂. Thereafter, infected cells were washed 3 times with RPMI and covered with 1 ml RPMI–10% FCS. To determine the number of CFU, coinfections of U937 cells and legionellae were lysed by addition of 10 μl 10% saponin (S4521; Sigma-Aldrich) for 5 min, and different dilutions were plated on BCYE agar. Each infection was carried out in duplicate wells and was done at least three times.

RESULTS

Genomic islands of *L. pneumophila* Corby. There are six genomic islands (LpcGI-1 and -2, LpcGI-Asn, LpcGI-Phe, Trb-1 and -2) present in the genome sequence of *L. pneumophila* Corby (Fig. 1 and 2) (26). The organization of the LpcGI-1 and -2 islands is shown in Fig. 1. The genomic island LpcGI-2 of *L. pneumophila* Corby exhibits a putative T4-like secretion system. It seems to belong to a class of T4SSs named genomic island-associated T4SS (GI-like), and this class of T4SSs was recently identified within the genome sequence of *L. pneumophila* 130b (30, 32). LpcGI-2 shares similarity with the genomic island LGI-2 of *L. pneumophila* strains 130b and Paris and LpcGI-1 (Fig. 1; see below for LpcGI-1). The island LpcGI-2 (64,401 bp, 39% G+C content, *lpc1833* to *lpc1888* and *lpc2136* to *lpc2121*) is integrated within the tRNA^{Met} gene

FIG 2 Genomic islands LpcGI-1, LpcGI-Asn, and LpcGI-Phe of *L. pneumophila* Corby. (A) Genomic island LpcGI-1 is integrated within the $tRNA^{Thr}$ gene. Episomal forms of rings A and B were detected, and thereby, chromosomal DNA regions were generated with primers 2/3 and 1/4 and primers 1/5 and 2/6, respectively. No amplification product was detected by using primers 3/5, specific for the episomal form of LpcGI-1-AB. The A-B fusion point (*attR*-1) was detected with primers 1/2, and the 5' and 3' sites of integrated LpcGI-1 (5'i and 3'i) were detected with primers 3/4 and 5/6, respectively. (B) LpcGI-Asn is integrated within the $tRNA^{Asn}$ gene (3'i, primers 1/2; 5'i, primers 3/4) and can also exist in an episomal form (ci, primers 2/3). (C) LpcGI-Phe is integrated within the $tRNA^{Phe}$ gene (3'i, primers 1/2; 5'i, primers 3/4), but no episomal form could be detected (ci, primers 2/3, and tRNA, primers 1/4). Numbers with arrows, specific primers and their orientations. PCR was done for 35 or 40 (*) amplification cycles. All primers are specific for the respective islands and are named LpcGI-1, LpcGI-Asn, and LpcGI-Phe plus the following suffixes: 1U (primer 1), 2R (primer 2), 3U (primer 3), 4R (primer 4), 5R (primer 5), and 6U (primer 6) (see Table S1 in the supplemental material). Abbreviations: ci, episomal circular form; tRNA, chromosomal tRNA without integrated island; *attR*, chromosomal LpcGI-1 without B; i, integrated form.

lpc1832 (Fig. 1). In contrast to Trb-1, the island exhibits two *attR* sites (*attR*-1 and -2; Fig. 1 and 3). The putative T4SS is encoded by the region *lpc1857* to *lpc1880* with a DNA identity of approximately 87% to the respective region of strain 130b (*lpw_21631* to *lpw_21861*) (Fig. 1, green box, region I). However, in strain 130b, LGI-2 (LpwGI-2) is integrated within the $tRNA^{Arg}$ gene, and therefore, it is not surprising that the site-specific integrase Lpc1833 of LpcGI-2 is only 59% identical to the respective putative integrase (*lpw_21181*) of LpwGI-2. In addition, LpcGI-2 and LpwGI-2 exhibit a divergent genomic organization, predominantly within regions II and III, whereas region IV is not present in LpwGI-2 (Fig. 1).

LpcGI-2 seems to contain all genes necessary for a functional T4ASS/conjugation system and additional genes encoding putative regulatory proteins (*lvrRABC*, *lpc1838*, *lpc2122*), putative persistence or fitness factors (*helABC*, *cadA*, proline/betaine transport protein; Fig. 1, region III), metabolic enzymes, transposases (*lpc2127*, *lpc2136*, *lpc1856*), and three putative integrases (*lpc1833*, *lpc1884*, *lpc2123*) (Table 2 and Fig. 1). Within region IV of LpcGI-2, we identified a gene encoding a homolog of *traK* and close to its 5' site a region containing a partial *oriT* (Fig. 1). This region exhibits a putative TraI- and TraK-binding site, including the putative nick site, but without the inverted repeat responsible for TraJ binding (data not shown). The presence of a partial *oriT* region indicates that this island may be transferable by conjugation (see below).

The genomic island LpcGI-1 (120,190 bp, 40.5% G+C content) exhibits a region (*lpc2190* to *lpc2314*) encoding another putative LGI T4-like secretion system (LpcGI-1 [30, 32]), two *attR*

sites, and three integrases and is inserted into the $tRNA^{Thr}$ gene (Fig. 1 and Fig. 2A, primers 3/4 and 5/6). Like LpcGI-2, LpcGI-1 exhibits an *lvrRABC* region, several genes encoding putative persistence and fitness factors, metabolic proteins, and resistance factors (Fig. 1). The genomic islands LpcGI-Asn (6,066 bp, 37.4% G+C content) and LpcGI-Phe (11,555 bp, 37.3% G+C content), depicted in Fig. 2B and C, do not encode T4SSs (data not shown). LpcGI-Asn (*lpc0085* to *lpc0092*) is integrated within the $tRNA^{Asn}$ gene and exhibits one *attR* site, only the *lvrA* paralog of the *lvrRABC* region, and a putative integrase gene, *lpc0085* (Fig. 2B, primers 3/4 and 1/2). LpcGI-Phe (*lpc1383* to *lpc1395*) is integrated within the $tRNA^{Phe}$ gene (Fig. 2C, primers 3/4 and 1/2) and exhibits one *attR* site, several putative transposases, and two putative integrase genes but no *lvr* paralog (data not shown).

We analyzed *L. pneumophila* Corby for the presence of episomal forms of LpcGI-1, LpcGI-2, LpcGI-Asn, and LpcGI-Phe. Therefore, we employed PCR analysis to investigate whether an episomal ring is generated. For LpcGI-1, two episomal forms (A and B) were detectable, indicating that this island is excised from the genome of *L. pneumophila* Corby (Fig. 2A, primers 2/3 and 1/5). In contrast to LpcGI-2 (see below), the AB form, exhibiting the complete genomic island, does not seem to be generated (primers 3/5). However, the PCR product obtained using primers 4/6 indicated that both islands could be in the episomal state at the same time (Fig. 2A). While we were able to identify an episomal form of LpcGI-Asn (Fig. 2B, primers 2/3), no episomal form could be detected for LpcGI-Phe (Fig. 2C, primers 2/3). PCR with primers 1/4 revealed no PCR product, corroborating the finding that LpcGI-Phe is not able to be excised from the genome. For

LpcGI-2, PCR analysis for the detection of episomal forms revealed the presence of three different episomal forms of LpcGI-2, the medium-sized A ring (primers 2/3), the small-sized B ring (primers 1/5), and the complete AB ring (primers 3/5) (Fig. 3A and B), thereby generating a chromosomal region without part A (primers 1/4), part B (primers 2/6), and parts A and B (primers 4/6), respectively. This indicates that the island is excised without leaving a copy within the genome. The generation of episomal forms of genomic islands was then analyzed for LpcGI-2 and Trb-1 in detail.

Analysis of mechanism of LpcGI-2 excision. We chose LpcGI-2 to further analyze the excision of the island and to investigate if the island-associated new T4SS encodes a functional con-

jugation system. First, we amplified the *attP* sites of the three different episomal forms of LpcGI-2 by PCR and determined the DNA sequences (Fig. 3C). Sequence analysis of the *attP* sites revealed a site-specific excision between *attL* and *attR*-1 or *attL* and *attR*-2, generating the episomal forms A and AB, respectively (Fig. 3D). It is obvious that the *attP* sites of the episomal forms exhibit the typical format of mobile elements using an integrase-dependent excision/integration mechanism (5, 10, 44). The *attP* site is >200 bp long and exhibits arm sites (short repeats; Fig. 3C, underlined), a core site (crossover segment *attP*), and two putative integration host factor (IHF)-binding sites (Fig. 3C, marked in gray). We numbered the different IHF-binding sites (sites 1 to 5) as they appear in the integrated form within LpcGI-2 (Fig. 3D).

TABLE 2 Structure of LpcGI-2 (65,401 bp) of *L. pneumophila* Corby

Gene	Name	Putative function or similar protein
	Repeat	<i>attL</i> site
<i>lpc1833</i>	<i>int</i>	Integrase, similar to <i>lpp2312</i>
<i>lpc1834</i>		Acetyltransferase, similar to <i>lpp2313</i>
<i>lpc1835</i>		Proline/betaine transport protein-like protein, similar to <i>lpp2314</i>
<i>lpc1836</i>		Acetyltransferase, similar to <i>lpw21221</i>
<i>lpc1837</i>		Lipolytic enzyme, similar to <i>lp12_2062</i>
<i>lpc1838</i>		Transcription regulator protein, response regulator containing CheY-like receiver domain and a helix-turn-helix DNA-binding domain, similar to <i>lp12_2063</i>
<i>lpc1839</i>		Similar to <i>lpp2318</i>
<i>lpc1840</i>		Similar to <i>lpg1012</i>
<i>lpc1841</i>		Similar to <i>lpg1011</i>
<i>lpc1842</i>		Putative cadmium efflux ATPase, similar to <i>lp12_2067</i>
<i>lpc1843</i>		Cadmium efflux ATPase, similar to <i>lpg1010</i>
<i>lpc1844</i>		Similar to <i>lpc2269</i>
<i>lpc1845</i>		Similar to <i>lpc2267</i>
<i>lpc1846</i>	<i>cadA</i>	Cadmium-translocating P-type ATPase CadA, similar to <i>lp12_2070</i>
<i>lpc1847</i>	<i>helA</i>	Cobalt/zinc/cadmium efflux RND ^a transporter permease HelA, similar to <i>lp12_2071</i>
<i>lpc1848</i>	<i>helB</i>	Cation efflux system HelB, similar to <i>lp12_2072</i>
<i>lpc1849</i>	<i>helC</i>	Cobalt/zinc/cadmium efflux RND transporter outer membrane protein, similar to <i>lp12_2073</i>
<i>lpc1850</i>		Reverse transcriptase, similar to MEALZ2163 of <i>Methylomicrobium alcaliphilum</i>
<i>lpc1851</i>		Similar to serine/threonine protein kinase/putative ATPase of <i>Moorea product</i> 3L
<i>lpc1852</i>		Similar to <i>llo0765</i>
<i>lpc1853</i>		Similar to <i>llo1727</i>
<i>lpc1854</i>		Similar to hypothetical protein NH8B_0948 of <i>Pseudogulbenkiania</i> sp. strain NH8B
<i>lpc1855</i>		Similar to retron-type reverse transcriptase of <i>Bacteroides</i> sp. strain 1_1_14
<i>lpc1856</i>		Transposase IS4, similar to <i>lpl0192</i>
<i>lpc1857</i>	<i>lvrR</i>	Phage repressor, similar to <i>lp12_2074</i>
<i>lpc1858</i>	<i>lvrA</i>	<i>Legionella vir</i> region protein LvrA, similar to <i>lp12_2075</i>
<i>lpc1859</i>	<i>lvrB</i>	<i>Legionella vir</i> region protein LvrB, similar to <i>lp12_2076</i>
<i>lpc1860</i>	<i>lvrC</i>	CsrA paralog, similar to <i>lp12_2077</i>
<i>lpc1861</i>	<i>pilL</i>	Putative exported protein, similar to PilL of <i>Vibrio tubiashii</i> ATCC 19109
<i>lpc1862</i>		Similar to <i>lp12_2079</i>
<i>lpc1863</i>		Similar to <i>lp12_2080</i> , TIGR03759, integrating conjugative element protein, PFL_4693 family
<i>lpc1864</i>		Similar to <i>lp12_2081</i>
<i>lpc1865</i>		Similar to <i>lp12_2082</i>
<i>lpc1866</i>		Similar to <i>lp12_2083</i>
<i>lpc1867</i>		Similar to <i>lpp2385</i>
<i>lpc1868</i>		Similar to <i>lp12_2085</i>
<i>lpc1869</i>		Similar to <i>lpp2387</i>
<i>lpc1870</i>		Similar to <i>lpp2388</i> , integrating conjugative element protein of <i>Gallibacterium anatis</i> UMN179
<i>lpc1871</i>		Similar to <i>lpp2389</i>
<i>lpc1872</i>		Exported membrane protein, similar to <i>lp12_2089</i>
<i>lpc1873</i>		Similar to <i>lpp2391</i>
<i>lpc1874</i>	<i>virB4</i>	Type IV secretory protein VirB4 component, similar to <i>lpp2392</i>
<i>lpc1875</i>		Similar to <i>lpp2393</i> , TraU superfamily protein
<i>lpc1876</i>	<i>pilT</i>	Membrane protein, Tfp pilus assembly, pilus retraction ATPase PilT, similar to <i>lp12_2093</i>
<i>lpc1877</i>	<i>traG</i>	Membrane protein, TraG-like protein, N-terminal region, similar to <i>lpp2395</i>
<i>lpc1878</i>		Similar to <i>lp12_2095</i>
<i>lpc1879</i>		Similar to <i>lp12_2096</i>
<i>lpc1880</i>	<i>traD</i>	Conjugative coupling factor TraD, similar to <i>lp12_2097</i>
<i>lpc1881</i>		Similar to <i>lp12_2098</i>
<i>lpc1882</i>		Similar to <i>lpp2400</i>
<i>lpc1883</i>		Similar to <i>lp12_2101</i>
<i>lpc1884</i>	<i>int</i>	Putative integrase, similar to <i>lp12_2102</i>
<i>lpc1885</i>		Similar to <i>lp12_2103</i>
<i>lpc1886</i>		Antirestriction protein, similar to <i>lp12_2104</i>
<i>lpc1887</i>		Similar to <i>lpp2408</i>
<i>lpc1888</i>		Similar to <i>lpp2409</i>
	Repeat	<i>attR</i> -1 site
<i>lpc2136</i>		TnpA transposase, similar to <i>ldg6041</i>
<i>lpc2135</i>		Hypothetical protein
<i>lpc2134</i>		Similar to <i>GM18_2913</i> of <i>Geobacter</i> sp. strain M18

(Continued on following page)

TABLE 2 (Continued)

Gene	Name	Putative function or similar protein
<i>lpc2133</i>	<i>traK</i>	Similar to <i>lpw25661</i>
<i>lpc2132</i>		TraK protein, similar to <i>lpp0067</i>
<i>lpc2131</i>		Similar to <i>lpp2428</i>
<i>lpc2130</i>		Similar to <i>lpw25801</i> , putative Dot/Icm T4SS effector
<i>lpc2129</i>		Similar to <i>lpp2419</i>
<i>lpc2128</i>	<i>int</i>	Similar to <i>lpc0225</i> , SidC (llo_p0059) homolog <i>Legionella longbeachae</i> NSW150
<i>lpc2127</i>		Transposase (IS652), similar to <i>lpp2402</i>
<i>lpc2126</i>		Similar to <i>llo1617</i>
<i>lpc2125</i>		Similar to <i>lpc2174</i> , <i>lpa03424</i>
<i>lpc2124</i>		Similar to <i>lpa03424</i>
<i>lpc2123</i>		Putative prophage CP4-6 integrase, similar to <i>lpa03425</i>
<i>lpc2122</i>		Transcriptional regulator, LysR family, similar to <i>lpa03426</i>
<i>lpc2121</i>		Similar to <i>lpa03427</i>
	Repeat	<i>attR</i> -2 site

^a RND, resistance-nodulation-cell division.

IHF-binding site 5 is located within the chromosomal DNA and therefore is not part of LpcGI-2. A similar structure, but without IHF-binding sites and only one *attR* site, was identified for the *attP* site of Trb-1 (26).

To investigate which of the three integrases present on LpcGI-2 is responsible for the excision of the island, we then replaced the integrase genes *lpc1833*, *lpc1884*, and *lpc2123* with a Km^r cassette. The Δ *lpc1833*, Δ *lpc1884*, and Δ *lpc2123* mutants obtained were verified by PCR analysis (data not shown). Next, we demonstrated that growth of the mutant strains was similar to that of the wild type in AYE medium (data not shown). We then performed qPCR analysis to quantify the number of episomal forms of LpcGI-2-A, LpcGI-2-B, and LpcGI-2-AB within the wild type and the three integrase mutant strains. Results are given in Fig. 4A and Table 3. The ratio of the episomal form of LpcGI-2-A to the chromosomal *flaA* gene was 2.43×10^{-3} :1, for LpcGI-2-B the ratio was 7.57×10^{-7} :1, and for LpcGI-2-AB the ratio was 4.65×10^{-7} :1 in E phase. Furthermore, the quantities of the circular forms were nearly equal in S and E phases, and differences showed no or only a low significance (Table 3). Therefore, the A ring was the predominant form (Fig. 4A, WT LpcGI-2-A) and the AB and B rings were 3,352-fold (ratio of LpcGI-2-AB to LpcGI-2-A) and 1,826-fold less present than the A ring, respectively, in E phase (Fig. 4A, WT LpcGI-2-AB and GI-3B; Table 3). However, the amount of the A ring in E phase was reduced 3,945-fold in the Δ *lpc1833* (integrase) mutant strain, whereas the amounts of the AB and B rings were not significantly influenced (Table 4, Δ *lpc1833* mutant). This indicated that the excision of LpcGI-2-A is an *lpc1833*-dependent process. The integrase genes *lpc1884* and *lpc2123* both slightly increased the presence of the episomal form of LpcGI-2-B in exponential growth phase (Fig. 4A and Table 4, Δ *lpc1884* and Δ *lpc2123* mutants).

To investigate a putative role of the episomal form of LpcGI-2 for intracellular replication of *L. pneumophila* Corby within *A. castellanii*, we performed infection assays. However, the intracellular replication rate was not influenced in any of the three integrase mutant strains investigated (data not shown).

LpcGI-2 encodes a functional conjugation system. Since we identified a partial *oriT* region within LpcGI-2 (see above), we performed conjugation assays with the *L. pneumophila* Corby wild type as the donor strain and *L. pneumophila* JR32 as the acceptor

strain. We could demonstrate for the first time that a genomic island without a complete classical *oriT* region can be transferred horizontally to another *Legionella* strain by conjugation (Fig. 4B, WT*). In addition, we analyzed 10 transconjugants by PCR using specific primers, and we could corroborate that LpcGI-2-A is the predominant episomal form transferred by conjugation (Table 5, circular form of LpcGI-2). This was surprising, since the partial *oriT* region is not present on LpcGI-2-A (Fig. 1 and 3A). Next, we did the same conjugation experiment using the Δ *lpc1833* mutant as the donor. The conjugation rate was reduced $\sim$ 148 times compared to that for the wild-type strain (Fig. 4B, Δ *lpc1833* mutant). In contrast to the experiment using the wild-type strain as the donor, all 10 transconjugants analyzed were positive for LpcGI-2-AB and LpcGI-2-B but not for LpcGI-2-A (Table 5, circular form of LpcGI-2).

To verify if the new T4SS, encoded by LpcGI-2, is functional and necessary for horizontal transfer, we generated a Δ *pilT* mutant strain. We performed conjugation assays with the Δ *pilT* mutant strain as the donor strain and *L. pneumophila* JR32 as the acceptor strain. The transconjugation rate was reduced and comparable to that for the Δ *lpc1833* mutant strain (Fig. 4B, Δ *pilT*). Surprisingly, all 10 transconjugants investigated were positive for all three episomal forms of LpcGI-2, indicating that LpcGI-2-A is, in contrast to the ring formation in the wild-type strain, not the predominant form transferred by the Δ *pilT* mutant. The same results were obtained for transconjugants using the Δ *lpc2123* mutant as the donor strain. However, in these experiments, the marker for the selection of transconjugants was present in region IV; thus, transconjugants which received only LpcGI-2-A could not be selected. Three transconjugants of each experiment were also analyzed for the integration of the island within the tRNA^{Met} gene (Table 5, integration in tRNA^{Met}). The experiments revealed that the received island was integrated into the tRNA^{Met} gene of each transconjugant. Altogether, the results prove that the new T4SS of LpcGI-2 encodes a functional conjugation system.

First evidence for genes involved in the regulatory process of genomic island excision. We assumed that the *lvrRABC* genes may be involved in the regulation of genomic island excision, since *lvrC* encodes a paralog of CsrA which is known to be involved in gene regulation in *L. pneumophila* (33, 45). In addition, LvrR is a putative transcriptional regulator and the *lvrRABC* gene

FIG 4 Relative quantification of the three episomal forms of LpcGI-2 and conjugation frequency. (A) SYBR green quantitative PCR was done with chromosomal DNA from *L. pneumophila* wild-type, $\Delta lpc1833$, $\Delta lpc1884$, and $\Delta lpc2123$ strain in exponential (E) and stationary (S) growth phases. Circularization of LpcGI-2 was detected with primers RT-LpcGI-2-2R/RT-LpcGI-2-3U for GI-2-A, RT-LpcGI-2-1U/RT-LpcGI-2-5R for GI-2-B, and RT-LpcGI-2-3U/RT-LpcGI-2-5R for GI-2-AB. The *flaA* gene served as a chromosomal control, and the relative amount of copies was calculated in relation to a standard curve. Results are means of three independent experiments. Statistical significance is characterized by symbols above the columns: ∞ , comparison of GI-2-B and GI-2-AB versus GI-2-A of the wild type; $\sim$, comparison of the episomal forms of the mutant strains versus the wild type. (B) For conjugation experiments, *L. pneumophila* Corby wild type (WT*) or the $\Delta lpc1833$ and $\Delta pilT$ mutant strains were used as the donor and the *L. pneumophila* Philadelphia I JR32 strain served as the acceptor. Conjugation was done at 30°C on BCYE agar plates in the presence of DNase I. The transconjugation rates (ratios of transconjugants/donor) were 7.3×10^{-3} for the wild-type strain, 5×10^{-5} for the $\Delta lpc1833$ mutant strain, and 4.9×10^{-5} for the $\Delta pilT$ mutant strain. Results of the conjugation experiments are means of two independent experiments.

region is often found in association with T4ASSs in *Legionella* (26, 27, 32). Since the genomic island Trb-1 also exhibits an *lvrRABC* gene locus, only one episomal form is generated, and because the island is relatively small and composed primarily of *trb* and *tra* genes (26), we decided to use this island to analyze the regulatory mechanisms of island excision. In addition, within the genomic island Trb-1, only one site-specific putative integrase (*int-1*, *lpc2818*) was identified, and it was hypothesized that this enzyme may be necessary for the excision of Trb-1 (26). To verify that this integrase is responsible for the excision of Trb-1 from the genome,

we constructed a specific $\Delta int-1$ (*lpc2818*) mutant strain of *L. pneumophila* Corby. To study the role of the putative regulatory elements, we constructed *L. pneumophila* Corby $\Delta lvrRABC$ (*lpc2816* to *lpc2813*) and $\Delta lvrR$ (*lpc2816*) deletion mutants by replacing the respective genes with a kanamycin resistance cassette (see Materials and Methods). The three mutants obtained were verified by PCR analysis (data not shown). Next, we investigated the mutant strains for the excision of Trb-1 from the genome by PCR and qPCR analyses (Fig. 5 and 6A). The PCR and qPCR analyses revealed that *int-1* is necessary for the excision of Trb-1 from the genome. In contrast to the wild type, the episomal circular form of Trb-1 (Trb-1_{ci}) and the intact chromosomal form of the tRNA^{Pro} gene (without the integrated island) thereby generated were not detectable in the $\Delta int-1$ strain (Fig. 5B, $\Delta int-1$ with primers 2/3 and 1/4). As expected, the integrated form of Trb-1 (Trb-1_i) was present in both strains (Fig. 5B, primers 1/2 and 3/4). In a control experiment, we showed that the episomal form of Trb-2 (Trb-2_{ci}) was still present in the $\Delta int-1$ mutant (Fig. 5B, primers 6/7). Our data revealed that Int-1 is specifically necessary for the excision of Trb-1 from the genome of *L. pneumophila* Corby but does not influence the excision of the second *trb-tra* gene containing genomic island Trb-2.

In addition, the results of PCR analyses suggested that the amount of Trb-1_{ci} is upregulated in the $\Delta lvrRABC$ and $\Delta lvrR$ mutant strains (Fig. 5C, *in vitro*, primers 2/3), and thus, the amount of the tRNA^{Pro} gene without integrated Trb-1 was elevated compared to that in the wild type (Fig. 5C, *in vitro*, primers 1/4). However, the integrated form of Trb-1 was still detectable (Fig. 5C, *in vitro*, primers 1/2 and 3/4). Subsequently, we performed a PCR analysis with intracellularly grown wild-type and $\Delta lvrRABC$ mutant strains and demonstrated that the upregulation of Trb-1_{ci} in the $\Delta lvrRABC$ mutant strain also occurs during replication within *A. castellanii* (Fig. 5C, *in vivo*, primers 2/3).

To further investigate (quantitatively) if the generation of Trb-1_{ci} is negatively regulated by the *lvrRABC* gene cluster and the *lvrR* gene, we performed qPCR analysis using chromosomal DNA as the template (Fig. 6A). The *flaA* gene was used as the chromosomal control, and the value for the *flaA* gene was used as the reference value for qPCR analysis. The qPCR results demonstrated that Trb-1_i is the predominant form in *L. pneumophila*, since the ratio of Trb-1_{ci} to the chromosomal *flaA* gene was approximately 1×10^{-4} :1 in E phase in the wild-type strain (Fig. 6A, WT Trb-1_{ci}-E, and Table 6). Compared to the wild-type strain, the amount of Trb-1_{ci} was reduced ~ 30 -fold in the $\Delta int-1$ strain (Fig. 6A, $\Delta int-1$ Trb-1_{ci}, and Table 6). In contrast, the amount of Trb-1_{ci} was ~ 147 -fold higher in the $\Delta lvrRABC$ strain than in the wild-type strain (Fig. 6A, $\Delta lvrRABC$ Trb-1_{ci}, and Table 6). Similar results were obtained with the $\Delta lvrR$ mutant strain (Fig. 6A, $\Delta lvrR$ Trb-1_{ci}, and Table 6). The results corroborate the finding that the excision of Trb-1 is an Int-1-dependent but rare event. In addition, the excision of Trb-1_i is negatively regulated by the *lvrRABC* gene cluster and the *lvrR* gene. The experiments revealed a minor but significant difference between $\Delta lvrRABC$ and $\Delta lvrR$ mutant strains in both growth phases, indicating a further regulatory influence of *lvrABC* on the excision of Trb-1 (Fig. 6A). The influence of the growth phase on the presence of Trb-1_{ci} is low, since Trb-1_{ci} was induced only ~ 2 -fold from E to S phase (Table 6).

We recently demonstrated that Trb-1 can be transferred horizontally between *Legionella* strains by conjugation (26). Therefore, we performed conjugation assays to investigate the frequency

TABLE 3 Quantitative analysis (qPCR) of episomal forms of LpcGI-2 of *L. pneumophila* Corby WT

Genes compared ^a	Growth phase	No. of copies		<i>P</i> value ^b
		Mean	SEM	
LpcGI-2-A vs <i>flaA</i>	E	2.436×10^{-3}	1.447×10^{-3}	<0.001
	S	1.283×10^{-3}	5.819×10^{-4}	<0.001
LpcGI-2-B vs <i>flaA</i>	E	7.578×10^{-7}	4.007×10^{-8}	<0.001
	S	8.174×10^{-7}	3.702×10^{-7}	<0.001
LpcGI-2-AB vs <i>flaA</i>	E	4.655×10^{-7}	1.043×10^{-7}	<0.001
	S	6.639×10^{-7}	2.383×10^{-7}	<0.001
LpcGI-2-B vs LpcGI-2-A	E	5.475×10^{-4}	2.036×10^{-4}	<0.01
	S	9.465×10^{-4}	5.518×10^{-4}	<0.01
LpcGI-2-AB vs LpcGI-2-A	E	2.983×10^{-4}	1.037×10^{-4}	<0.01
	S	7.756×10^{-4}	3.829×10^{-4}	<0.01

^a The ratios of the amount in S phase to that in E phase were 0.968 ± 0.304 (*P*, not significant), 1.278 ± 0.141 (*P*, not significant), and 2.064 ± 0.594 (*P* < 0.05) for LpcGI-2-A, LpcGI-2-B, and LpcGI-2-AB, respectively.

^b Statistical significance was determined by Student's *t* test. NS, not significant (*P* > 0.05).

of Trb-1 conjugation using the *L. pneumophila* Corby wild type (WT^o) or the Δ *int-1* mutant strain as the donor and the *L. pneumophila* JR32 strain as the acceptor. The conjugation rates were 2.6×10^{-4} for the wild-type strain and 3.8×10^{-6} for the Δ *int-1* mutant strain; thus, the conjugation rate of Trb-1 from the Δ *int-1* mutant was reduced ~68-fold in comparison to that from the wild-type strain (Fig. 6B).

Trb-1_{ci} is not necessary for intracellular replication of *L. pneumophila* within host cells. To round up Trb-1 analysis, we then investigated if Trb-1_{ci} is involved in the *in vivo* fitness of *L. pneumophila*, since Trb-1_{ci} was shown to be present during replication in *A. castellanii* cells (see above; Fig. 5C). The growth of the Δ *int-1* and the Δ *lvrRABC* mutant strains in AYE medium was similar to that of the wild-type strain (data not shown). We then performed infection assays and infection/survival assays with and without competition of the wild type and the respective mutant strain with *A. castellanii*. The infection experiments revealed no differences between the wild type and the Δ *int-1* or Δ *lvrRABC* mutant strain (data not shown). The results demonstrated that the Δ *int-1* and Δ *lvrRABC* genes do not influence the intracellular replication or fitness of *L. pneumophila* in *A. castellanii*.

To further analyze a putative role of Trb-1 for intracellular replication, we transferred Trb-1_{ci} to *L. oakridgensis* by conjugation. *L. oakridgensis* is a less virulent strain and is negative for Trb-1 and other genomic islands, with the exception of the *lvh*

system (unpublished results; see Fig. S1A in the supplemental material, Lvh_{ci}). In the transconjugants of *L. oakridgensis*, Trb-1_i and Trb-1_{ci} were present and also detectable after passage (10 times) (see Fig. S1A in the supplemental material, TC3 and TC3/10). Since *L. oakridgensis* was described not to replicate in amoebae (46), we investigated the *L. oakridgensis* wild-type strain and the Trb-1-positive transconjugants for their ability to replicate within the human macrophage-like cell line U937. The replication of the transconjugants was similar to the replication of *L. oakridgensis* wild type (see Fig. S1B in the supplemental material). These results demonstrate that Trb-1_{ci} has no supporting effect on the ability of *L. oakridgensis* to replicate within human macrophages.

DISCUSSION

Recently, we identified and described two genomic islands in *L. pneumophila* Corby (Trb-1 and Trb-2) which can be excised from the chromosome, forming episomal plasmid-like forms. Both genomic islands exhibit an *oriT* region, and the whole Trb-1 island can be transferred to other *Legionella* strains by conjugation. After conjugation, the island is integrated site specifically within the genome of the transconjugants (26). Among further genes, Trb-1 contains the putative integrase *Int-1* and the *lvrRABC* region, which shows homology to regulatory proteins. So, the questions were if genomic islands in *L. pneumophila* are excised integrase dependently and if the *lvrABC* region is involved in this process. In

TABLE 4 Quantitative analysis (qPCR) of episomal forms of LpcGI-2 of *L. pneumophila* Corby Δ *lpc1833*, Δ *lpc1884*, and Δ *lpc2123*

Circular form of LpcGI-2	Growth phase	Δ <i>lpc1833</i> mutant vs WT			Δ <i>lpc1884</i> mutant vs WT			Δ <i>lpc2123</i> mutant vs WT		
		No. of copies		<i>P</i> value ^a	No. of copies		<i>P</i> value	No. of copies		<i>P</i> value
		Mean	SEM		Mean	SEM		Mean	SEM	
A	E	2.535×10^{-4}	7.198×10^{-5}	<0.01	1.227	0.448	NS	0.831	0.503	NS
	S	6.318×10^{-4}	2.456×10^{-4}	<0.01	1.277	0.308	NS	0.674	0.340	NS
B	E	1.137	0.120	NS	2.190	0.495	<0.001	2.693	0.860	<0.001
	S	0.926	0.192	NS	1.234	0.262	NS	1.534	0.578	NS
AB	E	0.868	0.179	NS	1.375	0.313	NS	0.922	0.314	NS
	S	0.886	0.117	NS	1.121	0.265	NS	0.813	0.168	<0.05

^a Statistical significance was determined by Student's *t* test. NS, not significant (*P* > 0.05).

TABLE 5 PCR analysis of transconjugants, recipient *L. pneumophila* JR32, and donor strains *L. pneumophila* Corby WT*, $\Delta pilT$, $\Delta lpc1833$, and $\Delta lpc2123^h$

Region used in analysis	PCR result				<i>L. pneumophila</i> JR32 Sm ^r recipient	<i>L. pneumophila</i> Corby donor			
	Transconjugants					WT*	$\Delta pilT$	$\Delta lpc1833$	$\Delta lpc2123$
	WT ^{*a}	$\Delta pilT^a$	$\Delta lpc1833^a$	$\Delta lpc2123^b$					
Genes									
<i>lpg0402^c</i>	+	+	+	+	+	—	—	—	—
<i>lpc1850^c</i>	+	+	+	+	—	+	+	+	+
<i>lpc2123^d</i>	—	+	+	ni	—	+	+	+	ni
Circular forms of LpcGI-2 ^d (primers)									
A (2/3)	+	+	—	+	—	+	+	—	+
B (1/5)	—	+	+	+	—	+	+	+	+
AB (3/5)	—	+	+	+	—	+	+	+	+
Primers for analysis of integration in tRNA ^{Met}									
2/6	—	—	—	—	—	+	+	+	+
2/6 ^f	+	+	+	+	—	—	—	—	—
3/4	—	—	—	—	—	+	+	+	+
3/4 ^g	+	+	+	+	—	+	+	+	+
5/6	—	—	—	—	—	+	+	+	+
5/6 ^f	—	+	+	+	—	—	—	—	—
4 ^g /6 ^f	+	+	+	+	+	—	—	—	—

^a Transconjugant selection only for LpcGI-2-A and LpcGI-2-AB and not for LpcGI-2-B.^b Transconjugant selection only for LpcGI-2-B and LpcGI-2-AB and not for LpcGI-2-A.^c Thirty transconjugants were analyzed.^d Ten transconjugants were analyzed.^e Three transconjugants were analyzed. tRNA-Met gene *lpc1832* or *lpg2362*.^f Primer specific for strain JR32 (trnM-lpg2362-U).^g Primer specific for strain JR32 (trnM-lpg2362-R).^h All specific primers are listed in Table S1 in the supplemental material. +, detected by PCR; —, not detected by PCR; ni, not investigated; Sm^r streptomycin-resistant strain.

addition, *L. pneumophila* Corby exhibits four further genomic islands within its genome (LpcGI-1 and -2, LpcGI-Asn, and LpcGI-Phe). Genomic islands LpcGI-1 and LpcGI-2 exhibit GI-like secretion systems which are similar to those of LGI-2 of *L. pneumophila* strains 130b and Paris (27, 32). Both islands exhibit gene loci with genes involved in the metal ion resistance, persistence, and fitness of *L. pneumophila*. LpcGI-1 and -2 are integrated within the tRNA^{Thr} and the tRNA^{Met} genes, respectively. In contrast to Trb-1, both islands exhibit two *attR* sites. Surprisingly, only the two episomal forms LpcGI-1-A and LpcGI-1-B were detectable, whereas for LpcGI-2, the three episomal forms A, B, and AB were present. It remains to be elucidated why the episomal LpcGI-1-AB form is absent. Like Trb-1, both islands exhibit a region encoding paralogs of the *lvrRABC* genes. The genomic islands LpcGI-Asn and LpcGI-Phe do not encode a T4ASS but are also integrated within tRNA genes. In contrast to LpcGI-Phe, LpcGI-Asn also exists as an episomal form. While LpcGI-Asn exhibits at least an *lvrA* paralog, LpcGI-Phe lacks the complete *lvr* region. Nothing is yet known about the putative horizontal transfer of these islands.

In the present work, we could demonstrate for the first time that the site-specific integrases Int-1 and Lpc1833 are responsible for the generation of the episomal forms of Trb-1 and LpcGI-2-A, respectively. In the $\Delta int-1$ and $\Delta lpc1833$ mutant strains, the rate of Trb-1 and LpcGI-2-A conjugation was drastically reduced. The results indicate that the excision of the genomic islands Trb-1 and

LpcGI-2-A in *L. pneumophila* Corby depends on a functional site-specific integrase and that both islands are mobilizable via conjugation. The generation of the episomal Trb-1_{ci} and LpcGI-2-A is an even rarer event, since the ratios of Trb-1_{ci} and LpcGI-2-A to chromosomal *flaA* were shown to be 1×10^{-4} :1 and 2×10^{-3} :1, respectively. In contrast to our findings, the *lvh* island of *L. pneumophila* Paris, encoding another T4ASS, was described to be present as a multicopy plasmid (47). The authors also published findings that the episomal form of *lvh* is more frequently generated in the exponential growth phase. However, this is not the case for Trb-1 or LpcGI-2-A.

We then investigated the mechanism of genomic island excision. We demonstrated that the island LpcGI-2 can exist in three different episomal forms and that integrase *lpc1833* is necessary for the excision of the predominant episomal form LpcGI-2-A (see above). Furthermore, the excision of LpcGI-2-A was independent from the integrase genes *lpc1884* and *lpc2123*, but in the respective mutant strains, the episomal form of LpcGI-2-B was slightly increased. We do not yet know which (additional) proteins (e.g., an excisionase) are involved in the excision of LpcGI-2-AB and LpcGI-2-B. It was shown that an excisionase helps the site-specific recombinases in the direction of excision (48). On Trb-1 and Trb-2, putative excisionase-like proteins are present (*lpc2780* [65 amino acids] and *lpc0198* [68 amino acids], respectively). Nevertheless, we could not identify an excisionase-like protein on LpcGI-2. Low cross activity of other integrases is prob-

FIG 5 Genomic island *Trb-1* of *L. pneumophila* Corby. (A) The chromosomal form of *Trb-1* (*Trb-1_i*) is integrated in the *tRNA^{Pro}* gene and bordered by the *attL* and *attR* sites. The organization of the *int-1* and *lvrRABC* genes is given above. After excision, *Trb-1* is present as an episomal circular form (*Trb-1_{ci}*) and an intact *tRNA^{Pro}* gene remains in the genome. Numbers and arrows, the specific primers and their orientations, respectively (26). (B) Determination of the *int-1*-dependent excision mechanism via PCR. Integrase-1 is essential for ring formation, because the episomal island (primers 2/3) was detectable in the wild type but not in the $\Delta int-1$ mutant strain ($\Delta int-1$). An intact *tRNA^{Pro}* gene (primers 1/4) was detected in the wild type but not in the $\Delta int-1$ mutant. The chromosomal form of *Trb-1* was present in both strains (primers 1/2 and 3/4). The episomal form of the genomic island *Trb-2* (*Trb-2_{ci}*) was detected in the wild type and the $\Delta int-1$ mutant strains (primers 6/7). (C) Determination of the circular forms of *Trb-1* in $\Delta lvrRABC$ and $\Delta lvrR$ mutants. *Trb-1_{ci}* was analyzed *in vitro* in the wild type as well as in the $\Delta lvrRABC$ and $\Delta lvrR$ mutant strains and *in vivo* (after 20 h of intracellular growth) in the wild type and in the $\Delta lvrRABC$ mutant (primers 2/3). The amount of *Trb-1_{ci}* and intact *tRNA^{Pro}* gene (primers 2/3 and 1/4) was upregulated in both mutants, but the integrated form *Trb-1_i* was still detectable (primers 1/2 and 3/4). Primers: 1, *trb-1*; 2, *trb-2*; 3, *trb-3*; 4, *trb-4*; 6, *trb-6*; 7, *trb-7*. Abbreviations: *attP*, episomal integration sites; *attL* and *attR*, chromosome-genomic island junctions. Results of PCR analysis were confirmed by using two independently generated mutant strains. Lanes M, molecular markers.

ably involved, since the relative quantity of LpcGI-2-AB and LpcGI-2-B was similar to the amount of episomal LpcGI-2-A in the $\Delta lpc1833$ integrase mutant strain (Fig. 4). On the other hand, sequencing of the *attP'* and *attP''* sites of LpcGI-2-B and LpcGI-2-AB revealed site-specific recombination for the excision of both islands between *attR*-1 and *attR*-2 or *attL* and *attR*-2, respectively. In addition, conjugation experiments using $\Delta lpc1833$ as the donor revealed that 10 out of 10 analyzed transconjugants were negative for the episomal form of LpcGI-2-A (Table 5). However, three of these transconjugants were analyzed for LpcGI-2 integration into the *tRNA^{Met}* gene, revealing that they were positive for integrated LpcGI-2-A and the episomal form of LpcGI-2-B. These results confirmed that the generation of the episomal form of LpcGI-2-A

is an *lpc1833*-dependent process, whereas this is not the case for LpcGI-2-B. Remarkably, LpcGI-2-AB was integrated into the *tRNA^{Met}* gene within the transconjugants, although *Lpc1833* was not present. In this case, integration into the genome of the recipient may be due to the action of RecA, as shown recently for the high-pathogenicity island of *Yersinia pseudotuberculosis* (49). Furthermore, it is likely that an integration host factor (IHF) is involved in excision/integration of LpcGI-2, since we found four putative IHF sites on the genomic island directly associated with the *attP/attR* sites. The presence of putative IHF-binding sites and the role of the IHFs for the function of integrative and conjugative elements (ICEs) are known (50–54).

It was discussed that *oriT*-negative islands have been ac-

FIG 6 Relative quantification of episomal form of Trb-1 ($Trb-1_{ci}$) and conjugation frequency. (A) Quantitative PCR for circularization frequency of Trb-1. SYBR green real-time PCR was done with chromosomal DNA from *L. pneumophila* Corby wild type and the $\Delta int-1$, $\Delta lvrRABC$, and $\Delta lvrR$ mutant strains in exponential (E) and stationary (S) growth phases. $Trb-1_{ci}$ formation was a rare, *int-1*-dependent event and occurred more often in the $\Delta lvrRABC$ and $\Delta lvrR$ mutants. The circularization frequencies in E and S growth phases ($Trb-1_{ci}$ -E and -S) are given. The *flaA* gene served as a chromosomal control, and the relative amount of copies was calculated in relation to a standard curve. Results are means of three independent experiments. Statistical significance (Student's *t* test) is characterized by symbols above the columns: ∞ , comparison of $Trb-1_{ci}$ of mutant strain versus wild type; $\sim$, comparison of $Trb-1_{ci}$ of $\Delta lvrRABC$ mutant versus $\Delta lvrR$ mutant. (B) Conjugation frequency of Trb-1. *L. pneumophila* Corby wild type (WT^a) or the $\Delta int-1$ mutant strain was used as the donor, and *L. pneumophila* Philadelphia I JR32 served as the acceptor. Conjugation was done at 30°C on BCYE agar plates in the presence of DNase I. The transconjugation rates (ratios of transconjugants/donor) were 2.6×10^{-4} for the wild-type strain and 3.8×10^{-6} for the $\Delta int-1$ mutant strain. Results are means of two independent experiments.

quired horizontally by *Legionella*, but it has not been shown experimentally (27). In this work, we could demonstrate for the first time that LpcGI-2-A is transferred horizontally by conjugation using *L. pneumophila* Corby as the donor strain and *L. pneumophila* Philadelphia I as the acceptor strain. The island was integrated site specifically into the genome of the transconjugants (Table 5). Since we were not able to identify a classical *oriT* region on LpcGI-2-A, even classical *oriT*-negative islands of *L. pneumophila* can be transferred horizontally by conjugation. Further experiments are needed to identify the mechanism of this transfer.

In addition, we investigated if LpcGI-2 encodes a functional conjugation system by analyzing the transfer of LpcGI-2 using a $\Delta pilT$ mutant strain as the donor. *pilT* is located in region I and therefore present on the DNA, forming rings A and AB. In the $\Delta pilT$ mutant strain, the rate of conjugation of LpcGI-2 or LpcGI-2-A was reduced 149-fold, and the conjugation rate was comparable to the conjugation rate obtained using the $\Delta lpc1833$ mutant as the donor. Therefore, LpcGI-2 is transferred horizontally in a *pilT*-dependent process. Surprisingly, LpcGI-2-AB and not LpcGI-2-A was mainly transferred from the $\Delta lpc1833$ or $\Delta pilT$ donor strain. It seems as if LpcGI-2-AB could be transferred by using another conjugation system, probably the *oriT*-dependent system present on Trb-1 and Trb-2, since a partial *oriT* region is present on LpcGI-2-AB. However, we can demonstrate for the first time that LpcGI-2 encodes a functional conjugation system in *L. pneumophila* which is integrated site specifically into the genome of the transconjugants and that the excision of this new GI-like secretion system from the genome depends on a site-specific integrase.

We then analyzed *lvrRABC* and *lvrR* for their putative roles in the regulatory process of genomic island excision. Most of the genomic islands identified so far in *Legionella* exhibit an *lvr* region (26, 27, 55), encoding the putative phage-repressor LvrR; LvrA and LvrB, two proteins with unknown function; and LvrC, a CsrA paralog. In *Legionella*, CsrA is a regulatory protein acting on the mRNA level of its target genes (33, 45, 56). It negatively regulates the switch from the replicative to the transmissive phase in *L. pneumophila* (33). LvrR exhibits Pfam_HTH_XRE and S24-LexA-like peptidase motifs. These motifs are found in proteins involved in bacterial plasmid copy control, repressors of the SOS system, and other DNA-binding proteins (57, 58). To analyze the role of the Trb-1 *lvr* region, we first deleted *lvrRABC*. We found that the excision of Trb-1 is negatively regulated by the *lvr* region, since the episomal form was upregulated 147 times in the mutant strain. Results obtained using the generated $\Delta lvrR$ mutant strain confirmed that the phage repressor-like protein LvrR is involved

TABLE 6 Quantitative analysis (qPCR) of Trb-1_{ci} of *L. pneumophila* Corby WT and *L. pneumophila* Corby $\Delta int-1$, $\Delta lvrRABC$, and $\Delta lvrR$ mutant strains^a

Growth phase	WT ($Trb-1_{ci}$ vs <i>flaA</i>)			$\Delta int-1$ vs WT			$\Delta lvrRABC$ vs WT			$\Delta lvrR$ vs WT		
	No. of copies			No. of copies			No. of copies			No. of copies		
	Mean	SEM	P value	Mean	SEM	P value	Mean	SEM	P value	Mean	SEM	P value
E	1.065×10^{-4}	4.865×10^{-6}	<0.001	0.034	0.009	<0.001	146.80	11.50	<0.001	68.12	9.66	<0.001
S	2.122×10^{-4}	2.451×10^{-5}	<0.001	0.029	0.014	<0.001	74.24	27.99	<0.001	125.09	35.61	<0.001

^a Statistical significance was determined by Student's *t* test. NS, not significant ($P > 0.05$). The mean $\pm$ SEM ratios of the number of copies for S phase versus number of copies for E phase were 1.867 ± 0.653 ($P < 0.05$) for the wild type, 1.691 ± 0.868 (P was not significant) for $\Delta int-1$, 0.839 ± 0.241 (P was not significant) for $\Delta lvrRABC$, and 2.916 ± 0.627 ($P < 0.001$) for $\Delta lvrR$.

in this repression. In addition, the *lvr* gene (*lpc2273*) of LpcGI-1 encodes a putative LvrR protein of only 84 amino acids in length. The protein exhibits the HTH-Xre motif, but it lacks the S24_LexA-like motif found within LvrR of Trb-1, suggesting that this protein may be nonfunctional. This assumption is supported by the finding that the amount of LpcGI-1-A and LpcGI-1-B was similar to the quantity of Trb-1_{ci} in the $\Delta lvrR$ (*lpc2816*) mutant strain (Fig. 2A and Fig. 5C). Furthermore, qPCR analyses revealed that the expression of *lpc2819* and *int-1* is induced in the $\Delta lvrRABC$ mutant strains (data not shown). The observed induction of *int-1* expression is in line with the upregulation of Trb-1_{ci} in the $\Delta lvrRABC$ mutant, since we demonstrated that the generation of Trb-1_{ci} is Int-1 dependent. The influence of *lvrRABC* on genomic island gene expression and conjugation will be analyzed in detail in a further study.

We then asked if the islands may be involved in the *in vivo* fitness of *L. pneumophila*, since Trb-1_{ci} was also present during the replication within *A. castellanii* (26). Neither the mutant strains exhibiting an elevated level of Trb-1_{ci} nor the *int-1* mutant showed a modified rate of intracellular multiplication. In addition, the conjugation of Trb-1_{ci} into the less virulent strain *L. oakridgensis* did not support the ability of the transconjugants to replicate within U937 cells. Although we could confirm that Trb-1_{ci} is generated *in vivo*, its relevance for the life cycle of *L. pneumophila* remains unknown. As demonstrated for Trb-1, none of the three integrase mutant strains of LpcGI-2 showed an effect on the intracellular replication of *L. pneumophila* in *A. castellanii*, suggesting that the excision of LpcGI-2 does not influence the *in vivo* fitness of *L. pneumophila*. Accordingly, Kim and colleagues published findings that a metal efflux island of *L. pneumophila* (similar to LpcGI-1) is not required for survival in macrophages and amoeba (59).

In conclusion, we could demonstrate that the excision and conjugation of the genomic islands Trb-1 and LpcGI-2-A are site-specific integrase-dependent events in *L. pneumophila*. The elements are integrated into the genome of the transconjugants. The *attP* and *attL/attR* sites and probably the IHF-binding sites of LpcGI-2 are involved in these processes. Therefore, the genomic islands should be classified as ICEs. ICEs are defined to be elements that excise site specifically from the chromosomal DNA, leading to an episomal circular form that is generally transient. After conjugation, the element is integrated into the recipient chromosome. The target site for the integrase- or recombinase-dependent integration is often a tRNA gene (44, 60, 61). The recently defined new class of GI-associated T4SSs (GI-like) identified in *Haemophilus influenzae* (14, 30, 31) has also been identified in genome sequences of *L. pneumophila* (32). We can now demonstrate that the GI-like element of *L. pneumophila* (LpcGI-2) encodes a functional conjugation system localized on an ICE. Furthermore, we present first experimental data for the involvement of the *lvrRABC* gene cluster in the regulation of the process of excision of ICEs (Trb-1) in *L. pneumophila*.

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