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

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ABSTRACT

Legionella pneumophila is a Gram-negative freshwater agent, which multiplies in specialized nutrient rich vacuoles of amoeba. 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, respectively. 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* (*lpc2813-16*) to be involved in the regulation of Trb-1 excision. In addition, we could demonstrate for the first time that the *Legionella* genomic island (LGI) LpcGI-2 encodes a functional T4SS. 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 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). Co-culture 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*.

INTRODUCTION

Legionella pneumophila is a Gram-negative bacterium found ubiquitously in fresh water 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- the Legionnaires` disease (4). *L. pneumophila* strain Corby (Sg1, Mab 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 phago-lysosomal 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 (T4SS) 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* T4BSS *dot* (defect in organelle trafficking)*/icm* (intracellular multiplication) is similar to the *tra/trb* system of IncI plasmids (16, 17). It enables the intracellular multiplication by translocating effector proteins into the host cell (9, 11, 18-21). T4ASS 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 T4ASS 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. 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 a Trb-4 *(L. longbeachae* NSW150) have been identified. So far, nothing is known about excision of these elements (27).

In another classification system, the T4SS 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 T4SS, named Genomic Island T4SS (GI-like) (30, 31). For *L. pneumophila* two new GI-like islands were identified by genome sequence analysis of strain 130b (LGI-1 and -2) (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 more insight into the horizontal transfer process. To further analyse the excision of genomic islands from the chromosome and to verify if LGI-2 is a functional ICE, we generated and analysed 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 excision process of LpcGI-2. 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 as 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 strains WT^{o} and WT^{*} were used as a positive control in mating experiments. *L. pneumophila* Corby WT^{o} contains a kanamycin cassette between the genes *lpc2816* and *lpc2817* and in *L. pneumophila* Corby WT* a kanamycin cassette was introduced between the genes *lpc1856* and *lpc1857*. Mutant strains of *L. pneumophila* Corby used in this study were Δ*int-1*, Δ*lvrRABC*, Δ*lvrR*, Δ*pilT*, Δ*lpc1833*, Δ*lpc1884* and Δ*lpc2123*. All strains are listed in Table 1. *E. coli* strain DH5α was used as 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 PP_i and 0.04% L-cysteine] or on buffered charcoal-yeast extract (BCYE) agar at 37°C. Antibiotic concentrations used for *L. pneumophila* were kanamycin (Km), 12.5 mg ml^{-1} and streptomycin (Sm), 50 mg ml^{-1}. Bacterial growth in broth was monitored by determining the optical density at 600 nm (OD_{600}) with a Thermo Scientific GENESYS 10 Bio spectrophotometer (VWR, Darmstadt, Germany). Growth phases were defined as follows: OD_{600}~1.0 corresponded to the exponential (E), additional growth for 8 hours and OD_{600}~1.7 to the late exponential (LE), additional growth for 4 hours and OD_{600}~1.8 to the post exponential (PE) and additional growth for 8 hours and OD_{600}~2.0 to the stationary (S) growth phase. *E. coli* was cultivated in Luria-Bertani (LB) medium or on LB agar. The antibiotic concentrations used for *E. coli* were ampicillin (Ap), 100 mg ml^{-1} and kanamycin

(Km), 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 $MgSO_4$, 0.4 M $CaCl_2$, 0.1% sodium citrate dihydrate, 0.05 mM $Fe(NH_4)_2(SO_4)_2$ x 6 H_2O, 2.5 mM NaH_2PO_4 and 2.5 mM K_2HPO_4] at 20 °C. U937 cells were cultured in RPMI with 10% fetal calf serum (FCS), at 37°C and 5% CO_2.

DNA techniques and sequence analysis

Genomic DNA for PCR was prepared with the Generation Capture Column Kit (Qiagen, Hilden, Germany) and for real-time PCR with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The preparation of plasmid DNA was done with the Invisorb Spin Plasmid Mini Two Kit (Stratec, 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 a.M., 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 the 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 *Xba*I restriction site and enabling religation. The resulting plasmid (pML11) and a kanamycin resistance (Km^r) cassette were restricted with *Xba*I 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 followed: pML56 for Δ*lvrR*, pVH10 for Δ*lvrRABC*, pML25A for Δ*lpc1833*, pML19 for Δ*lpc1884*, pML27 for Δ*lpc2123*, and pML16 for Δ*pilT*. 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 were 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 analogous to Δ*int-1*. Mutant strains and plasmids are given in Table 1, specific primers used for mutant construction are listed in Table S1.

PCR analysis

PCR was carried out using a Thermocycler TRIO-Thermoblock (Biometra, Göttingen, Germany) and the HotStar Taq DNA polymerase (Qiagen, Hilden, Germany). The characterisation 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 Trb-1. The tRNA regions of LpcGI-2 (tRNA^{Met}) and LpcGI-1 (tRNA^{Thr}) were amplified with the primer pairs 1U/4R, 2R/6U and 4R/6U respectively. The amplification of the different circular forms of the genomic islands was done with the primer pairs 2R/3U, 1U/5R and 3U/5R. Integration points of LpcGI-2 and LpcGI-1 into the chromosome of *L. pneumophila* Corby were shown by the primer combinations 1U/2R, 3U/4R and 5R/6U. The characterisation of LpcGI-Asn and LpcGI-Phe was done analogous to Trb-1. The specific primer pairs 2R/3U, 1U/4R, 1U/2R and 3U/4R 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` region of the integration

point 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 seconds and 72°C for 2 min. All specific primers for the genomic islands are listed in Table S1 and illustrated in Figures 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^o, *L. pneumophila* Corby WT*, Δ*int-1*, Δ*lvrRABC*, Δ*lvrR*, Δ*pilT*, Δ*lpc1833*, Δ*lpc1884* and Δ*lpc2123*) were grown in AYE medium at 37°C. 1 ml 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 bacteria cultures for 24 h at 30°C on BCYE agar plates with or without the presence of DNase (1µg/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 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 to OD_{600}~0.3 and cultured at 37°C to the favored growth phase. Total RNA was extracted from exponential and post-exponential growth phase using the High Pure RNA Isolation Kit (Roche, Mannheim, Germany). Purified RNA was incubated with 100 U DNaseI per ml (Qiagen, Hilden,

Germany) for 30 min at room temperature. After DNAse treatment, RNA was repurified with the Rneasy Mini Kit (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 the 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 PCR (qPCR) was performed using the Mx3000P thermal cycler (Stratagene) and the EXPRESS SYBR GreenER qPCR SuperMix Universal Kit (Invitrogen, Darmstadt, Germany) according to the instructions of the manufacturer. A standard curve was used to quantify the amount of target present in unknown samples. For a standard curve 10 µl from each probe were mixed and diluted from 10^7 to 10^1 in DEPC water. The primer pair RT-gyrA-U/RT-gyrA-R was used for the standard curve. Genomic DNA isolated from the exponential and stationary phase 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}) respectively. Episomal forms of LpcGI-2 were amplified with the primer pairs (RT-LpcGI-2-) 2R/3U, 1U/5R and 3U/5R. The episomal form of Trb-1 was shown by the primer pair (RT-trb-) 2R/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. 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 x 10^5 cells per ml and incubated in 24-well plates for 2 hours at 37°C and 5% CO_2. Stationary-phase *Legionella* bacteria grown on BCYE agar were diluted in AC buffer and mixed with *A. castellanii* at an 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 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 and 5% CO_2 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 CFU on BCYE agar with kanamycin from the 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 was done as previously described with modifications (41-43). U937 cells were adjusted to 3 x 10^5 cells/ml and transferred to 100 ml fresh RPMI medium containing 10% FCS. For differentiation into macrophage-like cells PMA (phorbol-12-myristate-13-acetate, Stock 1 mg/ml in H_2Odd [P-8139; Sigma-Aldrich]) was added in a concentration of 1:20,000 and cells were incubated for 36 h at 37°C and 5% CO_2. Afterwards the supernatant was discarded and cells were washed once with 10 ml 0.2% EDTA in PBS. Cells were removed from the flask bottom with RPMI+10% FCS, transferred to 50 ml tubes and centrifuged at 800 g for 10 min. To determine the cell number, 100 µl of cell solution were 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 x 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 incubated for 2 h at 37°C and 5% CO_2 to allow adhesion. Stationary phase *Legionella* grown on BCYE agar were diluted in PBS and added to the macrophage cells. Infection was done with an MOI of 0.01 (time point 0 h) for 2h at 37°C and 5% CO_2. Thereafter infected cells were washed 3 times with RPMI and covered with 1 ml RPMI+10% FCS. To determine number of CFU, co-incubations of U937 cells and legionellae were lysed by addition of 10 µl 10% Saponin (Sigma-Aldrich S4521) 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 at the genome sequence of *L. pneumophila* Corby (Fig. 1 and 2)(26). The organization of the islands LpcGI-1 and -2 is shown in Fig. 1. The genomic island LpcGI-2 of *L.*

pneumophila Corby exhibits an 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* strain 130b, strain Paris and LpcGI-1 (Fig. 1 and see below for LpcGI-1). The island LpcGI-2 (64,401 bp; 39 % G+C content, *lpc1833-1888* and *lpc2136-2121*) is integrated within the $tRNA^{Met}$ gene *lpc1832* (Fig. 1). In contrast to Trb-1, the island exhibits two attR sites (Fig. 1 and 3, *attR*-1 and -2). 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 region 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*) (Tab. 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) exhibits a region (*lpc2190-2314*) encoding another putative LGI T4-like secretion system (LpcGI-1; (30, 32)), two attR

sites, 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) and LpcGI-Phe (11,555 bp, 37.3 % G+C) do not encode T4SSs (Fig. 2B and C). LpcGI-Asn (*lpc0085-0092*) is integrated within the $tRNA^{Asn}$ gene, 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 (*lpc1383-1395*) is integrated within the $tRNA^{Phe}$ gene (Fig. 2C, primers 3/4 and 1/2), exhibits one attR site, several putative transposases and two putative integrase genes, but no *lvr* paralog (Fig. 2C).

We analysed *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, seems not to be generated (primers 3/5). However, the PCR product 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 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) or AB (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.

333

334 **Analysis of the excision mechanism of LpcGI-2**

335 We choosed LpcGI-2 to further analyse the excision of the island and to investigate if
336 the island-associated new T4SS encodes a functional conjugation system. First, we amplified
337 the attP-sites of the three different episomal forms of LpcGI-2 by PCR and determined the
338 DNA sequences (Fig. 3C). Sequence analysis of the attP sites revealed a site-specific excision
339 between attL and attR-1 or attL and attR-2 generating the episomal forms A and AB,
340 respectively (Fig. 3D). It is obvious that the attP sites of the episomal forms exhibit the typical
341 format of mobile elements using an integrase-dependent excision/integration mechanism (5,
342 10, 60). The attP site is >200 bp long and exhibits arm sites (short repeats, Fig. 3C,
343 underlined), a core site (cross-over segment attP) and two putative integration host factor
344 (IHF)-binding sites (Fig. 3C, marked in grey). We numbered the different IHF-binding sites
345 (1-5) as they appear in the integrated form within LpcGI-2 (Fig. 3D). IHF-binding site
346 number 5 is located within the chromosomal DNA and therefore not part of LpcGI-2. A
347 similar structure, but without IHF-binding sites and only one attR site, was identified for the
348 attP site of Trb-1 (26).

349 To investigate which of the three integrases present on LpcGI-2 is responsible for the
350 excision of the island, we then replaced the integrases *lpc1833*, *lpc1884* and *lpc2123* with a
351 Km^R cassette, respectively. The obtained mutants *Δlpc1833*, *Δlpc1884* and *Δlpc2123* were
352 verified by PCR analysis (data not shown). Next, we demonstrated that growth of the mutant
353 strains was similar to the wild-type in AYE medium (data not shown). We then performed
354 qPCR analysis to quantify the number of the episomal LpcGI-2-A, LpcGI-2-B and LpcGI-2-
355 AB within the wild-type and the three integrase mutant strains. Results are given in Figure 4A
356 and Table 2. The ratio of the episomal form of LpcGI-2-A to the chromosomal *flaA* gene was
357 2×10^{-3}:1, for LpcGI-2-B 8×10^{-7}:1 and for LpcGI-2-AB 5×10^{-7}:1 in E phase. Furthermore,
358 the quantities of the circular forms were nearly equal in S and E phase and differences showed

no or only low significance (Tab. 2A). 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 LpcGI-2-AB vs. LpcGI-2-A) and 1,826-fold less present in E phase than the A-ring, respectively (Fig. 4A, WT LpcGI-2-AB and GI-3B, Tab. 2A). However, the amount of the A-ring in E phase was reduced ~4,000-fold in the Δ*lpc1833* (integrase) mutant strain, whereas the amounts of the AB- and B-ring were not significantly influenced (Tab. 2B, Δ*lpc1833*). This indicated that the excision of LpcGI-2-A is an *lpc1833*-dependent process. The integrases *lpc1884* and *lpc2123* both did slightly increase the presence of the episomal form of LpcGI-2-B in exponential growth phase (Fig. 4A and Tab. 2B, Δ*lpc1884* and Δ*lpc2123*).

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, in none of the three integrase mutant strains investigated, the intracellular replication rate was influenced (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 donor and *L. pneumophila* JR32 as 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 analysed ten transconjugants by PCR using specific primers and we could corroborate that LpcGI-2-A is the predominant episomal form transferred by conjugation (Tab. 3, middle). This was surprising since the partial *oriT*-region is not present on LpcGI-2-A (Fig. 1). Next we did the same conjugation experiment using Δ*lpc1833* as donor. The conjugation rate was reduced ~148-times compared to the wild-type strain (Fig. 4B, Δ*lpc1833*). In contrast to the experiment using the wild-type as donor, all of

384 the ten transconjugants analysed were positive for LpcGI-2-AB and LpcGI-2-B, but not for
385 LpcGI-2-A (Tab. 3, middle).

386 To verify if the new T4SS, encoded by LpcGI-2, is functional and necessary for the
387 horizontal transfer, we generated a *ΔpilT* mutant strain. We performed conjugation assays
388 with the *ΔpilT* mutant strain as donor and *L. pneumophila* JR32 as acceptor strain. The
389 transconjugation rate was reduced and comparable to the *Δlpc1833* mutant strain (Fig. 4B,
390 *ΔpilT*). Surprisingly, all 10 transconjugants investigated were positive for all three episomal
391 forms of LpcGI-2, indicating that LpcGI-2-A is, in contrast to the ring formation in the wild-
392 type strain, not the predominant form transferred by the Δ*pilT* mutant. The same results were
393 obtained for transconjugants using Δ*lpc2123* as donor strain. However, in these experiments,
394 the marker for the selection of transconjugants was present in region IV, thus transconjugants
395 which received only LpcGI-2-A could not be selected. Three transconjugants of each
396 experiment were also analysed for the integration of the island within the $tRNA^{Met}$ gene (Tab.
397 3, bottom). The experiments revealed that the received island was integrated into the $tRNA^{Met}$
398 gene of each transconjugant. Altogether, the results prove that the new T4SS of LpcGI-2
399 encodes a functional conjugation system.

400

401 **First evidence for genes involved in the regulatory process of genomic island excision**

402 We assumed that the *lvrRABC* genes may be involved in the regulation of genomic
403 island excision, since *lvrC* encodes a paralog of CsrA which is known to be involved in gene
404 regulation in *L. pneumophila* (33, 44). In addition, LvrR is a putative transcriptional regulator
405 and the *lvrRABC* gene region is often found in association with T4ASSs in *Legionella* (26, 27,
406 32). Since the genomic island Trb-1 also exhibits a *lvrRABC* gene locus, only one episomal
407 form is generated and because the island is relatively small and composed primarily of *trb* and
408 *tra* genes (26), we decided this island for analysing the regulatory mechanisms of island
409 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 Δ*int-1* (*lpc2818*) mutant strain of *L. pneumophila* Corby. To study the role of the putative regulatory elements, we constructed *L. pneumophila* Corby, Δ*lvrRABC* (*lpc2816-2813*) and Δ*lvrR* (*lpc2816*) deletion mutants by replacing the respective genes with a kanamycin resistance cassette (Materials and Methods). The obtained three mutants 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 quantitative PCR (qPCR) analysis (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 thereby generated intact chromosomal form of the $tRNA^{Pro}$ gene (without integrated island) were not detectable in the Δ*int-1* strain (Fig. 5B, *Δint-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 Δ*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 Δ*lvrRABC* and Δ*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 the wild-type (Fig. 5C *in vitro*, primers 1/4). However, the integrated form of Trb-1 was still detectable (Fig. 5C *in vitro*, primers1/2 and 3/4). Subsequently, we performed a PCR analysis with intracellular grown wild-type and Δ*lvrRABC* mutant strains and demonstrated that the upregulation of Trb-1_{ci} in the Δ*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 template **(**Fig. 6A). The *flaA* gene was used as chromosomal control and 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 x 10^{-4}:1 in E phase in the wild-type strain (Fig. 6A, WT Trb-1_{ci}-E and Tab. 4). Compared to the wild-type strain, Trb-1_{ci} was reduced ~30-fold in the Δ*int-1* strain (Fig. 6A *Δint-1* Trb-1_{ci} and Tab. 4). In contrast, the amount of Trb-1_{ci} was ~147-fold higher in the Δ*lvrRABC* strain than in the wild-type strain (Fig. 6A, Δ*lvrRABC* Trb-1_{ci} and Tab. 4). Similar results were obtained with the Δ*lvrR* mutant strain (Fig. 6A, Δ*lvrR* Trb-1_{ci} and Tab. 4). The results corroborate 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 Δ*lvrRABC* and Δ*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 only induced ~2-fold from E to S phase (Tab. 4).

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 of Trb-1 conjugation using *L. pneumophila* Corby wild-type (WT^o) or the Δ*int-1* mutant strain as donors and the *L. pneumophila* JR32 strain as acceptor. The conjugation rate was 2.6 x 10^{-4} for the wild-type strain and 3.8 x 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 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). Then we 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 strains (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 analyse 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 and Fig. S1A, Lvh_{ci}). In the transconjugants of *L. oakridgensis*, Trb-1_i and Trb-1_{ci} were present and also detectable after passage (10 times) (Fig. S1A, TC3 and TC3/10). Since *L. oakridgensis* was described not to replicate in amoebae (45), we investigated the *L. oakridgensis* wild-type strain and the Trb-1 positive tranconjugants 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 (Fig. S1B). 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). Amongst further genes, Trb-1 contains the putative integrase Int-1 and the *lvrRABC* region, which shows homology to regulatory proteins. So the question was, if genomic islands in *L. pneumophila* are excised integrase-dependently and if the *lvrABC* region is involved in this process. In addition, *L. pneumophila* Corby exhibits further four genomic islands within its genome (LpcGI-1 and -2, LpcGI-Asn and LpcGI-Phe). The genomic island LpcGI-1 and LpcGI-2 exhibit GI-like secretion systems which are similar to LGI-2 of *L. pneumophila* strains 130b and Paris (27, 32). Both islands exhibit gene loci encoding genes involved in metal-ion resistance, persistence and fitness of *L. pneumophila*. LpcGI-1 and -2 are integrated within the $tRNA^{Thr}$ and the $tRNA^{Met}$ gene, 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 are 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 known yet 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 Δ*int-1* and Δ*lpc1833* mutant strains the conjugation rate of Trb-1 and LpcGI-2-A 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 $Trb\text{-}1_{ci}$ and LpcGI-2-A is an even rare event, since the ratio of $Trb\text{-}1_{ci}$ and LpcGI-2-A to chromosomal *flaA* was shown to be 1×10^{-4}:1 and 2×10^{-3}:1, respectively. In contrast to

513 our findings, the *lvh*-island of *L. pneumophila* Paris, encoding another T4ASS, was described
514 to be present as a multi-copy plasmid (46). The authors also published that the episomal form
515 of *lvh* is more frequently generated in the exponential growth phase. However, this is not the
516 case for Trb-1 or LpcGI-2-A.

517 We then investigated the mechanism of genomic island excision. We demonstrated
518 that the island LpcGI-2 can exist in three different episomal forms and that integrase *lpc1833*
519 is necessary for the excision of the predominant episomal form LpcGI-2-A (see above).
520 Furthermore, the excision of LpcGI-2-A was independent from the integrases *lpc1884* and
521 *lpc2123*, but in the respective mutant strains the episomal form of LpcGI-2-B was slightly
522 increased. We do not know yet which (additional) proteins (e. g. an excisionase) are involved
523 in the excision of LpcGI-2-AB and LpcGI-2-B. It was shown that an excisionase helps the
524 site-specific recombinases in the direction of excision (47). On Trb-1 and Trb-2, putative
525 excisionase-like proteins are present (*lpc2780*; 65 amino acids and *lpc0198*; 68 amino acids,
526 respectively). Nevertheless, we could not identify an excisionase-like protein on LpcGI-2.
527 Probably, low cross-activity of other integrases may be involved, since the relative quantity of
528 LpcGI-2-AB and LpcGI-2-B was similar to the amount of episomal LpcGI-2-A in the
529 Δ*lpc1833* integrase mutant strain (Fig. 4). On the other hand, sequencing of the attP' and attP"
530 sites of LpcGI-2-B and LpcGI-2-AB revealed site-specific recombination for the excision of
531 both islands between attR-1 and attR-2 or attL and attR-2, respectively. In addition,
532 conjugation experiments using Δ*lpc1833* as donor revealed that ten out of ten analysed
533 transconjugants were negative for the episomal form of LpcGI-2-A (Tab. 5). However, three
534 of these transconjugants were analysed for LpcGI-2 integration into the $tRNA^{Met}$ gene,
535 revealing them positive for integrated LpcGI-2-A and the episomal form of LpcGI-2-B. These
536 results confirmed that the generation of the episomal form of LpcGI-2-A is an *lpc1833*-
537 dependent process, whereas this is not the case for LpcGI-2-B. Remarkably, LpcGI-2-AB was
538 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* (48). 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 (49-53).

It was discussed that *oriT*-negative islands have been acquired 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 donor and *L. pneumophila* Philadelphia as acceptor strain. The island was integrated site-specifically into the genome of the transconjugants (Tab. 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 analysing the transfer of LpcGI-2 using a Δ*pilT* mutant strain as donor. *pilT* is located in the region I and therefore present on the DNA forming ring A and AB. In the Δ*pilT* mutant strain the conjugation of LpcGI-2-(A) was reduced 149-fold and comparable to the conjugation rate using Δ*lpc1833* as donor. Therefore, LpcGI-2 is transferred horizontally in a *pilT*-dependent process. Surprisingly, mainly LpcGI-2-AB was transferred from the donor strains Δ*lpc1833* or Δ*pilT* and not LpcGI-2-A. 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.

Then we analyzed *lvrRABC* and *lvrR* for its putative role in the regulatory process of genomic island excision. Most of the genomic islands identified so far in *Legionella* exhibit an *lvr* region (26, 27, 54), encoding the putative phage-repressor LvrR, LvrA and B, 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, 44, 55). It negatively regulates the switch from the replicative to the transmissive phase in *L. pneumophila* (33). LvrR exhibits a Pfam_HTH_XRE and a S24-LexA-like peptidase motif. These motifs are found in proteins involved in bacterial plasmid copy control, repressors of the SOS system and other DNA-binding proteins (56, 57). To analyse 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 using the generated Δ*lvrR* mutant strain confirmed that the phage repressor-like protein LvrR is involved 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 non-functional. 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 Δ*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 Δ*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 Δ*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 that a metal efflux island of *L. pneumophila* (similar to LpcGI-1) is not required for survival in macrophages and amoeba (58).

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/R sites and probably the IHF-binding sites of LpcGI-2 are involved in these processes. Therefore, the genomic islands should be classified as integrative and conjugative elements (ICEs). ICEs are defined as 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 (59-61). The recently defined new class of GI-associated T4SSs (GI-like) identified in *H. influenzae* (14, 30 ,31) has also been identified in genome sequences of *L. pneumophila* (32). We now could demonstrate that the GI-like element of *L. pneumophila* (Lpc-GI-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 excision process of ICEs (Trb-1) in *L. pneumophila*.

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796 **Figure Legends**

797 FIG. 1. Genetic organization of the genomic islands LpcGI-2, LppGI-2, LpwGI-2, and LpcGI-
798 1 of *L. pneumophila* Corby (Lpc), *L. pneumophila* strains Paris (Lpp) and 130b (Lpw)
799 encoding LGI-like T4SSs. LpcGI-2 and LppGI-2 are integrated within the $tRNA^{Met}$ gene,
800 whereas LpwGI-2 and LpcGI-1 are integrated within the $tRNA^{Arg}$ and $tRNA^{Thr}$ genes,
801 respectively. The gene numbers are given above the genes which are indicated by arrows.
802 DNA regions encoding clustered homologous proteins are boxed in the same color. The
803 colors of the genes indicate their degree of homology to the genes on LpcGI-2. Subregions (I-
804 IV) of the islands are given below the genes.

805 FIG. 2. The genomic islands LpcGI-1, LpcGI-Asn and LpcGI-Phe of *L. pneumophila* Corby.
806 (A) Genomic island LpcGI-1 is integrated within the $tRNA^{Thr}$ gene. Detection of episomal
807 forms of A and B and thereby generated chromosomal DNA regions with primers 2/3 and 1/4
808 and primers 1/5 and 2/6, respectively. No amplification product was detected by using primers
809 3/5, specific for the episomal form of LpcGI-1-AB. The A-B fusion point (attR-1) was
810 detected with primers 1/2 and the 5` and 3` sites of integrated LpcGI-1 (5´i and 3`i) with
811 primers 3/4 and 5/6, respectively. (B) LpcGI-Asn is integrated within the $tRNA^{Asn}$ gene (3´i,
812 primers 1/2; 5´i primers 3/4) and can exist also in an episomal form (ci, primers 2/3). (C)
813 LpcGI-Phe is integrated within the $tRNA^{Phe}$ gene (3´i, primers 1/2; 5´i primers 3/4), but no
814 episomal form could be detected (ci, primers 2/3 and tRNA, primers 1/4). Numbers with
815 arrows stand for the specific primer and its orientation. PCR was done at 35 or 40 (*)
816 amplification cycles. All primers are specific for the respective islands and are named LpcGI-
817 1, LpcGI-Asn and LpcGI-Phe plus the following suffix: 1, -1U; 2, -2R; 3, -3U; 4, -4R; 5, -5R;
818 6, -6U (see Tab. S1). Abbreviations: ci, episomal circular form; tRNA, chromosomal tRNA
819 without integrated island; attR, chromosomal LpcGI-1 without B; i, integrated form.

820 FIG. 3. Circularization of LpcGI-2 of *L. pneumophila* Corby. (A) Mechanism for the
821 formation of the episomal rings A, B and AB of LpcGI-2. The chromosomal form of LpcGI-2

(blue double headed arrows) is integrated within the $tRNA^{Met}$ gene (grey arrow) and bordered by the attL and attR-2 sites (black arrows). Numbers with arrows stand for the specific primer and its orientation. (B) Detection of episomal forms of A, B and AB and thereby generated chromosomal DNA region with primers 2/3 and 1/4, primers 1/5 and 2/6 and primers 3/5 and 4/6, respectively; as well as the A-B fusion point (attR-1) with primers 1/2 and the 5` and 3` sites of integrated LpcGI-2 (5´i and 3`i) with primers 3/4 and 5/6, respectively. PCR was done with 35 or 40 (*) amplification cycles. Primers: 1, LpcGI-2-1U; 2, LpcGI-2-2R; 3, LpcGI-2-3U; 4, LpcGI-2-4R; 5, LpcGI-2-5R; 6, LpcGI-2-6U. (C) Nucleotide sequence of the attP, attP` and attP``sites (black arrows) of episomal LpcGI-2-A, -B and -AB, respectively. Putative IHF-binding sites (grey boxes) and identified direct (NTTTN, underlined) and indirect repeats (green arrows) are indicated. (D) Nucleotide sequences of the attL, attR-1 and attR-2 sites (black arrows) of chromosomal LpcGI-2. The $tRNA^{Met}$ gene (grey arrow) and sequence variations between attL and attR sites (marked in red) are indicated. LpcGI-2 consists of region A (dark blue) and region B (light blue). Chromosomal DNA is shown in black. The numbers 1-5 indicate putative IHF-binding sites (WATCAANNNNTTR; W=dA or dT; R=dA or dG; N= any nucleotide) and are highlighted as grey boxes.

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, *Δlpc1833*, *Δlpc1884* and *Δlpc2123* 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 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; ~,

comparison of the episomal forms of the mutant strains versus wild-type. (B) For conjugation experiments, *L. pneumophila* Corby wild-type (WT^{*}) or the mutant strains *Δlpc1833* and *ΔpilT* were used as donor and the *L. pneumophila* Philadelphia I JR32 strain served as acceptor. Conjugation was done at 30°C on BCYE agar plates in the presence of DNAseI. The transconjugation rate (ratio transconjugants/donor) was 7.3×10^{-3} for the wild-type strain, 5×10^{-5} for *Δlpc1833* and 4.9×10^{-5} for the *ΔpilT* mutant strain. Results of the conjugation experiments are means of two independent experiments.

FIG. 5. The 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 genes *int-1* and *lvrRABC* are 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 stand for the specific primers and its orientation. (26), modified). (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 (WT) but not in the *Δint-1* mutant strain (*Δint-1*). An intact $tRNA^{Pro}$ gene (primers 1/4) was detected in the wild-type but not in the *Δint-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 *Δint-1* mutant strain (primers 6/7). (C) Determination of the circular forms of Trb-1 in *ΔlvrRABC* and *ΔlvrR* mutants. Trb-1_{ci} was analyzed *in vitro* in the wild-type as well as in *ΔlvrRABC* and ΔlvrR mutant strains and *in vivo* (after 20 h of intracellular growth) in the wild-type and in the *ΔlvrRABC* 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.

FIG. 6. Relative Quantification of the 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 mutant strains *Δint-1*, *ΔlvrRABC* and *ΔlvrR* 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 *ΔlvrRABC* and *ΔlvrR* mutants. The circularization frequencies in E and S growth phases (Trb-1_{ci}-E and -S) are given. The *flaA* gene served as 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 (Students T-test) is characterized by symbols above the columns: ∞, comparison of Trb-1_{ci} of mutant strain versus wild-type; ~, comparison of Trb-1_{ci} of Δ*lvrRABC* versus Δ*lvrR*. (B) Conjugation frequency of Trb-1. *L. pneumophila* Corby wild-type (WTº) or the *Δint-1* mutant strain was used as donor and *Lp* Phil-1 JR32 served as acceptor. Conjugation was done at 30°C on BCYE agar plates in the presence of DNAseI. The transconjugation rate (ratio transconjugants/donor) was 2.6×10^{-4} for the wild-type strain and 3.8×10^{-6} for the *Δint-1* mutant strain. Results are means of two independent experiments.

TABLE 1. *Legionella* strains and plasmids used in this study. For all plasmids pGEM-T Easy (Promega) was used as a vector.

Strain	Characteristics	Reference
L. pneumophila (*Lp*) Corby	Wild-type	Jepras et al. 1985
Lp Philadelphia I JR32	restriction deficient strain of *Lp* Philadelphia I (Sm^r)	Marra and Shuman, 1989
L. oakridgensis	ATCC 33761	C. Lück, Dresden
Lp Corby, WT^o	Km^r-cassette between *lpc2816* and *lpc2817*	This study
Lp Corby, WT*	Km^r-cassette between *lpc1856* and *lpc1857*	This study
Lp Corby, Δ*int-1*	*lpc2818*::Km^r	This study
Lp Corby, Δ*lvrR*	*lpc2816*::Km^r	This study
Lp Corby, Δ*lvrRABC*	*lpc2813-2816*::Km^r	This study
Lp Corby, Δ*lpc1833*	*lpc1833*::Km^r	This study
Lp Corby, Δ*lpc1884*	*lpc1884*::Km^r	This study
Lp Corby, Δ*lpc2123*	*lpc2123*::Km^r	This study
Lp Corby, Δ*pilT*	*lpc1876*::Km^r	This study
Plasmids		
pML2	2990 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 *Xba*I site between *lpc2816*/*lpc2817*	This study
pML9	3208 bp PCR fragment (primers Int-U/Int-R) in pGEMTEasy	This study
pML11	pML9 after inverse PCR (primers Int-MU/Int-MR)	This study
pML12	pML11 with Km^r-cassette cloned into *Xba*I site instead of *lpc2818*	This study
pML14	3418 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 *Xba*I site instead of *lpc1876*	This study
pML17	2816 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 *Xba*I site instead of *lpc1884*	This study
pML20	2198 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 *Xba*I site between *lpc1856/ lpc2857*	This study
pML23	3103 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 *Xba*I site instead of *lpc1833*	This study
pML26	2377 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 *Xba*I site instead of *lpc2123*	This study
pML54	2530 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 *Xba*I site instead of *lpc2816*	This study
pVH7	1130 bp PCR fragment (primers LvrRABC-1U/LvrRABC-1R)	This study
pVH8	1090 bp PCR fragment (primers LvrRABC-2U/LvrRABC-2R)	This study
pVH9	Insert of pVH8 cloned into *Xba*I and *Xho*I site in pVH7	This study
pVH10	pVH9 with Km^r-cassette cloned into *Xba*I site instead of *lpc2816-lpc2813*	This study

898 TABLE 2. Structure of the genomic island LpcGI-2 of *L. pneumophila* Corby.

899

LpcGI-2 (65.401 bp)		
Gene (*lpc*)	Name	Putative function or similar protein
-	Repeat	att-L site
1833	*int*	Integrase, similar to *lpp2312*
1834		Acetyltransferase, similar to *lpp2313*
1835		Proline/betaine transport protein like protein, similar to *lpp2314*
1836		Acetyltransferase, similar to *lpw21221*
1837		Lipolytic enzyme, similar to *lp12_2062*
1838		Transcription regulator protein, response regulator containing CheY-like receiver domain and HTH DNA-binding domain, similar to *lp12_2063*
1839		Similar to *lpp2318*
1840		Similar to *lpg1012*
1841		Similar to *lpg1011*
1842		Putative cadmium efflux ATPase, similar to *lp12_2067*
1843		Cadmium efflux ATPase, similar to *lpg1010*
1844		Similar to *lpc2269*
1845		Similar to *lpc2267*
1846	*cadA*	Cadmium translocating P-type ATPase CadA, similar to *lp12_2070*
1847	*helA*	Cobalt/zinc/cadmium efflux RND transporter permease HelA, similar to *lp12_2071*
1848	*helB*	Cation efflux system HelB, similar to *lp12_2072*
1849	*helC*	Cobalt/zinc/cadmium efflux RND transporter outer membrane protein, similar to *lp12_2073*
1850		Reverse Transcriptase, similar to MEALZ2163 of *Methylomicrobium alcaliphilum*
1851		Similar to serine/threonine protein kinase/putative ATPase of *Moorea product* 3L
1852		Similar to *llo0765*
1853		Similar to *llo1727*
1854		Similar to hypothetical protein NH8B_0948 of *Pseudogulbenkiania sp*. NH8B
1855		Similar to retron-type reverse transcriptase of *Bacteroides sp*. 1_1_14
1856		Transposase IS4, similar to *lpl0192*
1857	*lvrR*	Phage repressor, similar to *lp12_2074*
1858	*lvrA*	*Legionella vir* region protein LvrA, similar to *lp12_2075*
1859	*lvrB*	*Legionella vir* region protein LvrB, similar to *lp12_2076*
1860	*lvrC*	CsrA paralog, similar to *lp12_2077*
1861	*pilL*	Putative exported protein, similar to PilL of *Vibrio tubiashii* ATCC 19109
1862		Similar to *lp12_2079*
1863		Similar to *lp12_2080*, TIGR03759, integrating conjugative element protein, PFL_4693 family
1864		Similar to *lp12_2081*

1865		Similar to *lp12_2082*
1866		Similar to *lp12_2083*
1867		Similar to *lpp2385*
1868		Similar to *lp12_2085*
1869		Similar to *lpp2387*
1870		Similar to *lpp2388*, integrating conjugative element protein of *Gallibacterium anatis* UMN179
1871		Similar to *lpp2389*
1872		Exported membrane protein, similar to *lp12_2089*
1873		Similar to *lpp2391*
1874	*virB4*	Type IV secretory protein VirB4 component, similar to *lpp2392*
1875		Similar to *lpp2393*, TraU superfamily protein
1876	*pilT*	Membrane protein, Tfp pilus assembly, pilus retraction ATPase PilT, similar to *lp12_2093*
1877	*traG*	Membrane Protein, TraG-like protein, N-terminal region, similar to *lpp2395*
1878		Similar to *lp12_2095*
1879		Similar to *lp12_2096*
1880	*traD*	Conjugative coupling factor TraD, similar to *lp12_2097*
1881		Similar to *lp12_2098*
1882		Similar to *lpp2400*
1883		Similar to *lp12_2101*
1884	*int*	Putative integrase, similar to *lp12_2102*
1885		Similar to *lp12_2103*
1886		Antirestriction protein, similar to *lp12_2104*
1887		Similar to *lpp2408*
1888		Similar to *lpp2409*
-	Repeat	att-R1 site
2136		TnpA transposase, similar to *ldg6041*
2135		Hypothetical protein
2134		Similar to *GM18_2913* of *Geobacter sp*. M18
2133		Similar to *lpw25661*
2132	*traK*	TraK protein, similar to *lpp0067*
2131		Similar to *lpp2428*
2130		Similar to *lpw25801*, putative Dot/Icm T4SS effector
2129		Similar to *lpp2419*
2128		Similar to *lpc0225*, SidC (llo_p0059) homolog *Legionella longbeachae* NSW150
2127		Transposase (IS652), similar to *lpp2402*
2126		Similar to *llo1617*
2125		Similar to *lpc2174, lpa03424*
2124		Similar to *lpa03424*
2123	*int*	Putative prophage CP4-6 integrase, similar to *lpa03425*
2122		Transcriptional regulator, LysR family, similar to *lpa03426*
2121		Similar to *lpa03427*
-	Repeat	att-R2 site

900
901

TABLE 3. Quantitative analysis (qPCR) of the episomal forms of LpcGI-2 of (A) *L. pneumophila* Corby wild-type (WT) and (B) *Δlpc1833*, *Δlpc1884* and *Δlpc2123*. Statistical significance (student T-test; P<0.05) is shown by stars (*) and "ns" for not significant.

A.

		WT		
	Growth phase	Mean	SEM	P value
LpcGI-2-A vs. *flaA*	E	2.436×10^{-3}	1.447×10^{-3}	***
	S	1.283×10^{-3}	5.819×10^{-4}	***
LpcGI-2-B vs. *flaA*	E	7.578×10^{-7}	4.007×10^{-8}	***
	S	8.174×10^{-7}	3.702×10^{-7}	***
LpcGI-2-AB vs. *flaA*	E	4.655×10^{-7}	1.043×10^{-7}	***
	S	6.639×10^{-7}	2.383×10^{-7}	***
LpcGI-2-B vs. LpcGI-2-A	E	5.475×10^{-4}	2.036×10^{-4}	**
	S	9.465×10^{-4}	5.518×10^{-4}	**
LpcGI-2-AB vs. LpcGI-2-A	E	2.983×10^{-4}	1.037×10^{-4}	**
	S	7.756×10^{-4}	3.829×10^{-4}	**
LpcGI-2-A	S vs. E	0.968	0.304	ns
LpcGI-2-B	S vs. E	1.278	0.141	ns
LpcGI-2-AB	S vs. E	2.064	0.594	*

*** <0.001; ** <0.01; * <0.05; ns >0.05

B.

Circular form		**Δ*lpc1833* vs. WT**			**Δ*lpc1884* vs. WT**			**Δ*lpc2123* vs.WT**		
of LpcGI-2	Growth phase	Mean	SEM	P value	Mean	SEM	P value	Mean	SEM	P value
-A	E	2.535×10^{-4}	7.198×10^{-5}	**	1.227	0.448	ns	0.831	0.503	ns
	S	6.318×10^{-4}	2.456×10^{-4}	**	1.277	0.308	ns	0.674	0.340	ns
-B	E	1.137	0.120	ns	2.190	0.495	***	2.693	0.860	***
	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	*

*** <0.001; ** <0.01; * <0.05; ns >0.05

TABLE 4. PCR analysis of the transconjugants (TC), the recipient *L. pneumophila* JR32 (*Lp* JR32) and the donor strains *L. pneumophila* Corby (Lpc) WT*, Δ*pilT*, Δ*lpc1833*, Δ*lpc2123*. All specific primers are listed in Table S1.

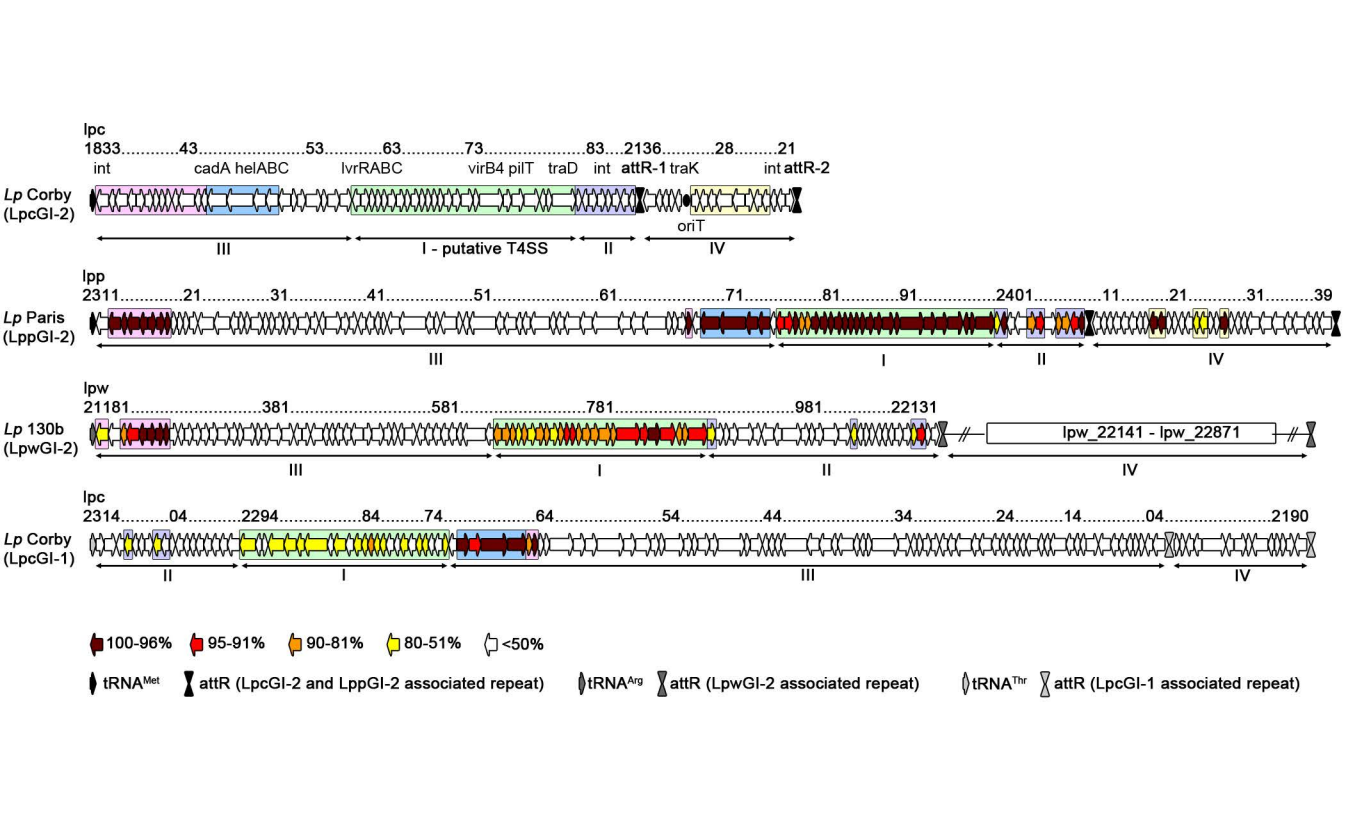
Gene	Transconjugants				Recipient	Donor			
	TC_{WT*}[a]	$TC_{\Delta pilT}$[a]	$TC_{\Delta lpc1833}$[a]	$TC_{\Delta lpc2123}$[b]	*Lp* JR32, Sm^R	Lpc WT*	Lpc Δ*pilT*	Lpc Δ*lpc1833*	Lpc Δ*lpc2123*
lpg0402[c]	+	+	+	+	+	-	-	-	-
lpc1850[c]	+	+	+	+	-	+	+	+	+
lpc2123[d]	-	+	+	(ni)	-	+	+	+	(ni)
Circular form GI-2[d]									
A (2/3)	+	+	-	+	-	+	+	-	+
B (1/5)	-	+	+	+	-	+	+	+	+
AB (3/5)	-	+	+	+	-	+	+	+	+
Integration in tRNA-Met[e, f]									
2/6	-	-	-	-	-	+	+	+	+
2/6[g]	+	+	+	+	-	-	-	-	-
3/4	-	-	-	-	-	+	+	+	+
3/4[h]	+	+	+	+	-	+	+	+	+
5/6	-	-	-	-	-	+	+	+	+
5/6[g]	-	+	+	+	-	-	-	-	-
4[h]/6[g]	+	+	+	+	+	-	-	-	-

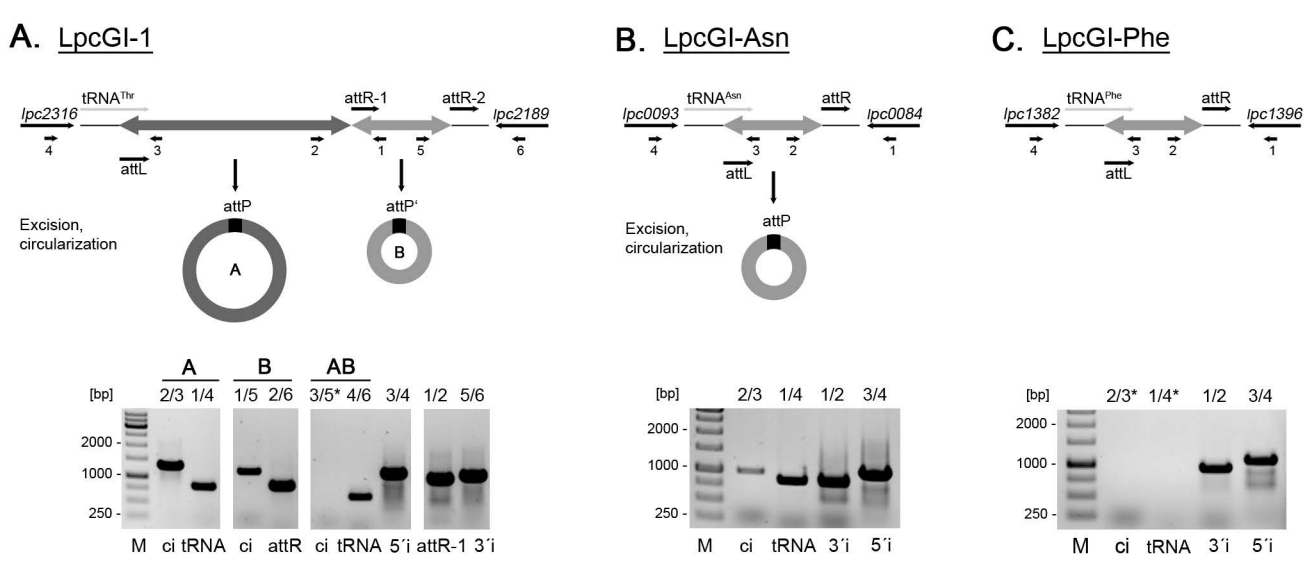
[a] TC selection only for LpcGI-2-A and LpcGI-2-AB, not for LpcGI-2-B
[b] TC selection only for LpcGI-2-B and LpcGI-2-AB, not for LpcGI-2-A
[c] 30 TCs analysed
[d] 10 TCs analysed
[e] 3 TCs analysed
[f] tRNA-Met gene *lpc1832* or *lpg2362*
[g] specific primer for strain JR32 (trnM-lpg2362-U)
[h] specific primer for strain JR32 (trnM-lpg2362-R)
(+) detected by PCR, (-) not detected by PCR, (ni) not investigated, (Sm^R) streptomycin resistant strain

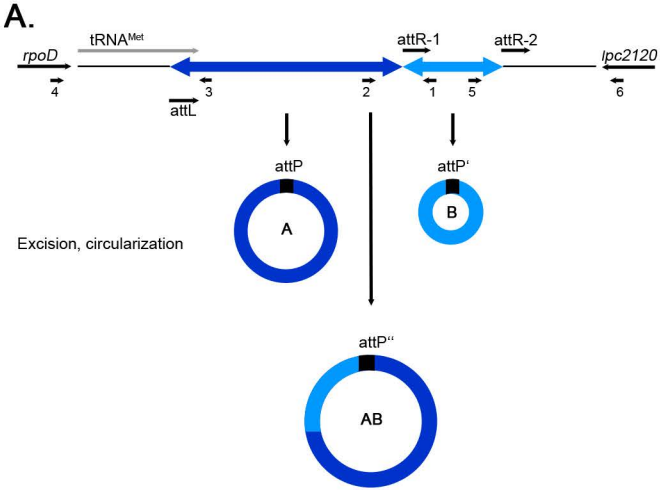
TABLE 5. Quantitative analysis (qPCR) of the episomal form of Trb-1 (Trb-1_{ci}) of *L.pneumophila* Corby wild-type (WT) and the mutant strains Δ*int-1*, Δ*lvrRABC* and Δ*lvrR*. Statistical significance (students T-test; P<0.05) is indicated by stars (*) and "ns" for not significant.

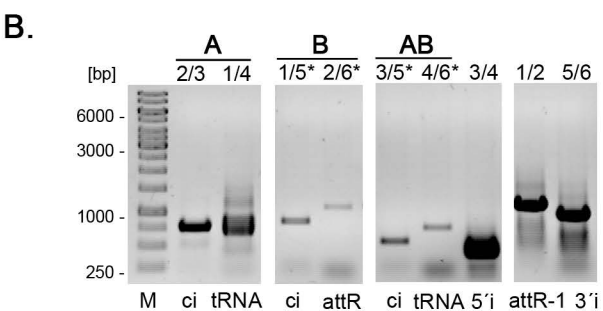
	WT (Trb-1_{ci} vs. *flaA*)			Δ***int-1*** vs. WT			Δ***lvrRABC*** vs. WT			Δ***lvrR*** vs. WT		
Growth phase	Mean	SEM	P value	Mean	SEM	P value	Mean	SEM	P value	Mean	SEM	P value
E phase	1.065×10^{-4}	4.865×10^{-06}	***	**0.034**	0.009	***	**146.80**	11.50	***	**68.12**	9.66	***
S phase	2.122×10^{-4}	2.451×10^{-05}	***	**0.029**	0.014	***	**74.24**	27.99	***	**125.09**	35.61	***
	WT			Δ***int-1***			Δ***lvrRABC***			Δ***lvrR***		
S vs. E phase	**1.867**	0.653	*	1.691	0.868	ns	0.839	0.241	ns	**2.916**	0.627	***

*** <0.001; ** <0.01; * <0.05; ns >0.05


lpc
1833
43
53
63
73
83
2136
28
21
int
cadA helABC
lvrRABC
virB4 pilT
traD
int
attR-1 traK
int attR-2
Lp Corby
(LpcGI-2)
oriT
III
I - putative T4SS
II
IV
lpp
2311
21
31
41
51
61
71
81
91
2401
11
21
31
39
Lp Paris
(LppGI-2)
III
I
II
IV
lpw
21181
381
581
781
981
22131
Lp 130b
(LpwGI-2)
lpw_22141 - lpw_22871
III
I
II
IV
lpc
2314
04
2294
84
74
64
54
44
34
24
14
04
2190
Lp Corby
(LpcGI-1)
II
I
III
IV
100-96%
95-91%
90-81%
80-51%
<50%
tRNAMet
attR (LpcGI-2 and LppGI-2 associated repeat)
tRNAArg
attR (LpwGI-2 associated repeat)
tRNAThr
attR (LpcGI-1 associated repeat)


A. LpcGI-1
lpc2316
tRNAThr
attR-1
attR-2
lpc2189
4
3
2
1
5
6
attL
Excision, circularization
attP
attP´
A
B
A
B
AB
[bp]
2/3 1/4
1/5 2/6
3/5* 4/6
3/4
1/2 5/6
2000
1000
250
M ci tRNA ci attR ci tRNA 5´i attR-1 3´i
B. LpcGI-Asn
lpc0093
tRNAAsn
attR
lpc0084
4
3
2
1
attL
Excision, circularization
attP
[bp]
2/3 1/4 1/2 3/4
2000
1000
250
M ci tRNA 3´i 5´i
C. LpcGI-Phe
lpc1382
tRNAPhe
attR
lpc1396
4
3
2
1
attL
[bp]
2/3* 1/4* 1/2 3/4
2000
1000
250
M ci tRNA 3´i 5´i





C.

LpcGI-2$_{ci}$-A

```
...ATTGATAATTTACCTCATAAGAAGCTAAAATAGCCTCGCGTGAGATT
TGCGTGTTTGAGTACGACGCAGTACGCCATAATACGCCATTTGTAGTGGT
                                              2
TGTTACGCAGCAAATTTCATAATAAAATCAAATGGTTAAAATAGAGATTT
                    attP
ATGGCTCATAATCCGTTGGTCCTAGGTTCAAGTCCTAGTGGGCCCACCAA
                                  1
TTAAAGCCAGTAAAATCAATAAGTTACACTTGAAACCTTAGTCACCTCAA
AAGTCCTTGCGTGACTTTTACGTGACCTAATCGAATTCCCGCGGCCGCCA
TGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGA...
```

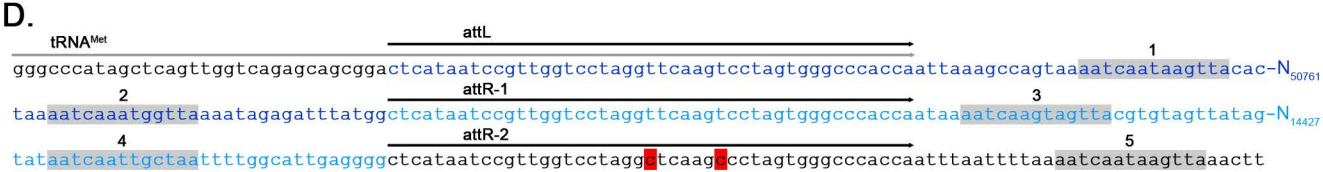
LpcGI-2$_{ci}$-B

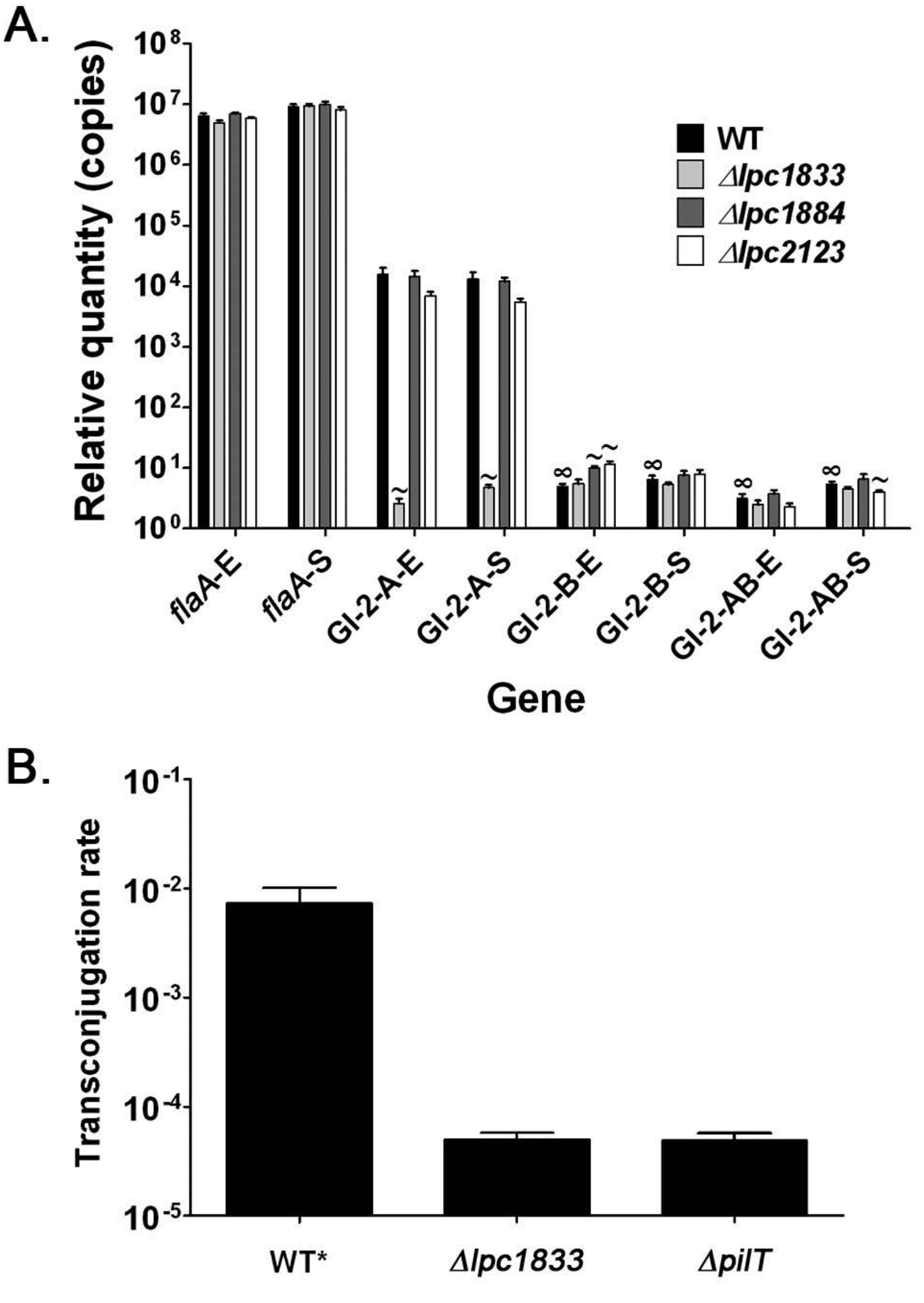
```
...TGCAAGCACAAGTATTTGAGCAATGGAATTTTATCAGAGAGAACTTA
AATAATGTCATTATTCAGTCAAATTTCTAAAACAACTAATATTTGTAGTT
                                          4
AATTCCTGCTATAAAAATTACAATATAATCAATTGCTAATTTTGGCATTG
                    attP'
AGGGGCTCATAATCCGTTGGTCCTAGGTTCAAGTCCTAGTGGGCCCACCA
                3
ATAAAATCAAGTAGTTACGTATAGTTATAGAGGGCGAATTTTAGGCTCTT
TCCCATTTAAGGGGGCAGTCTTTAATCACCCGCAGAGCACCCTAACACGC
ACTCTATAATTTTCAAAATTTCTAAAACCATAAGCCCTTCGCTGAAT...
```

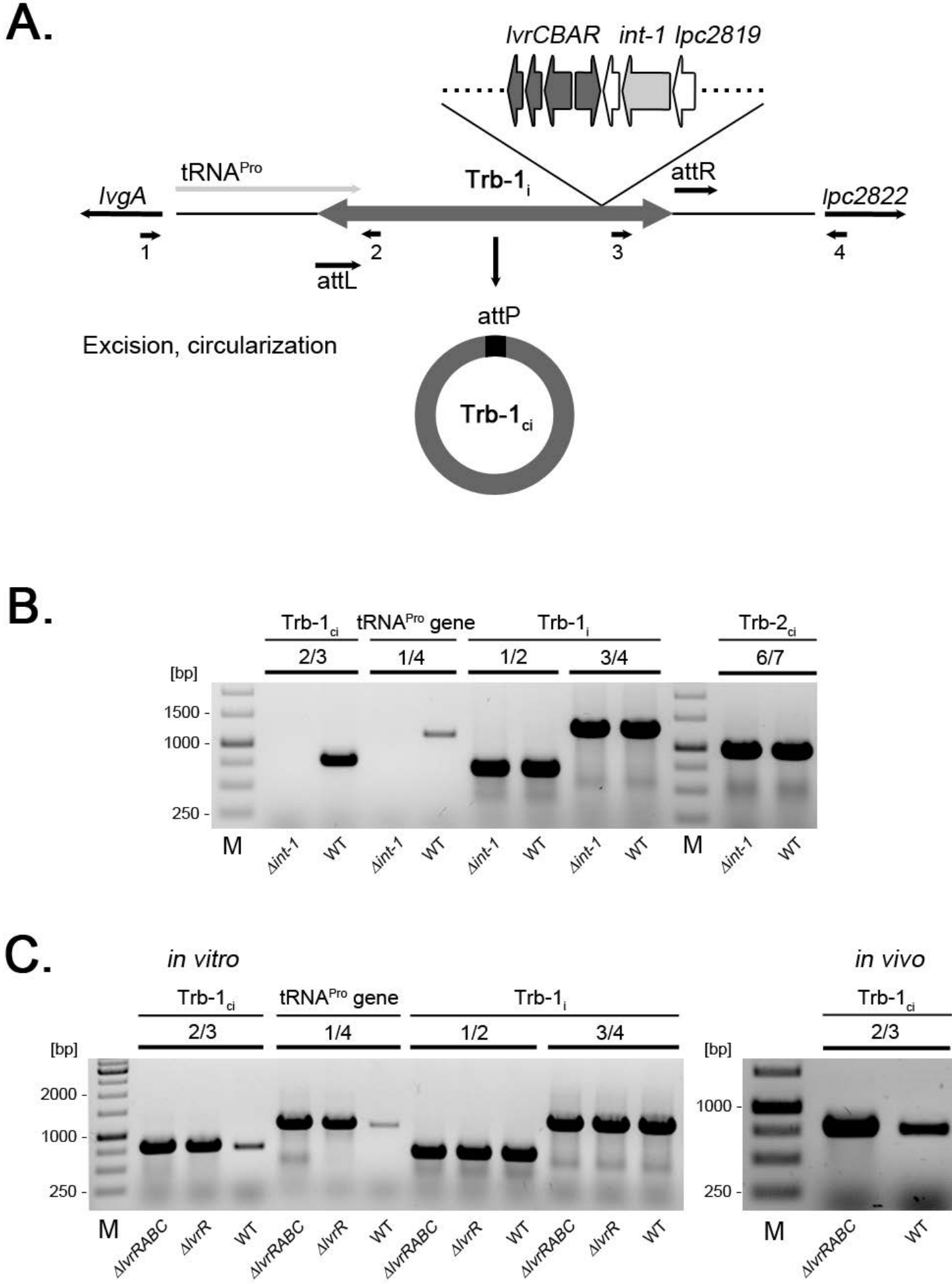
LpcGI-2$_{ci}$-AB

```
...CAAGCACAAGTATTTGAGCAATGGAATTTTATCAAAGAGAACTTAAA
TAATGTCATTATTCAGTCAAATTTCTAAAACAACTAATATTTGTAGTTAA
                                          4
TTCCTGCTATAAAAATTACAATATAATCAATTGCTAATTTTGGCATTGAG
                    attP“
GGGCTCATAATCCGTTGGTCCTAGGCTCAAGCCCTAGTGGGCCCACCAAT
                1
TAAAGCCAGTAAAATCAATAAGTTACACTTGAAACCTTAGTCACCTCAAA
AGTCCTTGCGTGACTTTTACGTGACCTAATCACTAGTGAATTCGCGGCCG
CCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATA...
```

D.




A.
Relative quantity (copies)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
WT
Δlpc1833
Δlpc1884
Δlpc2123
flaA-E
flaA-S
GI-2-A-E
GI-2-A-S
GI-2-B-E
GI-2-B-S
GI-2-AB-E
GI-2-AB-S
Gene
B.
Transconjugation rate
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
WT*
Δlpc1833
ΔpilT


A.
lvrCBAR int-1 lpc2819
tRNAPro
Trb-1i
attR
lvgA
lpc2822
1
2
3
4
attL
attP
Excision, circularization
Trb-1ci
B.
Trb-1ci
tRNAPro gene
Trb-1i
Trb-2ci
2/3
1/4
1/2
3/4
6/7
[bp]
1500
1000
250
M
Δint-1
WT
C.
in vitro
in vivo
Trb-1ci
tRNAPro gene
Trb-1i
2/3
1/4
1/2
3/4
[bp]
2000
1000
250
M
ΔlvrRABC
ΔlvrR
WT

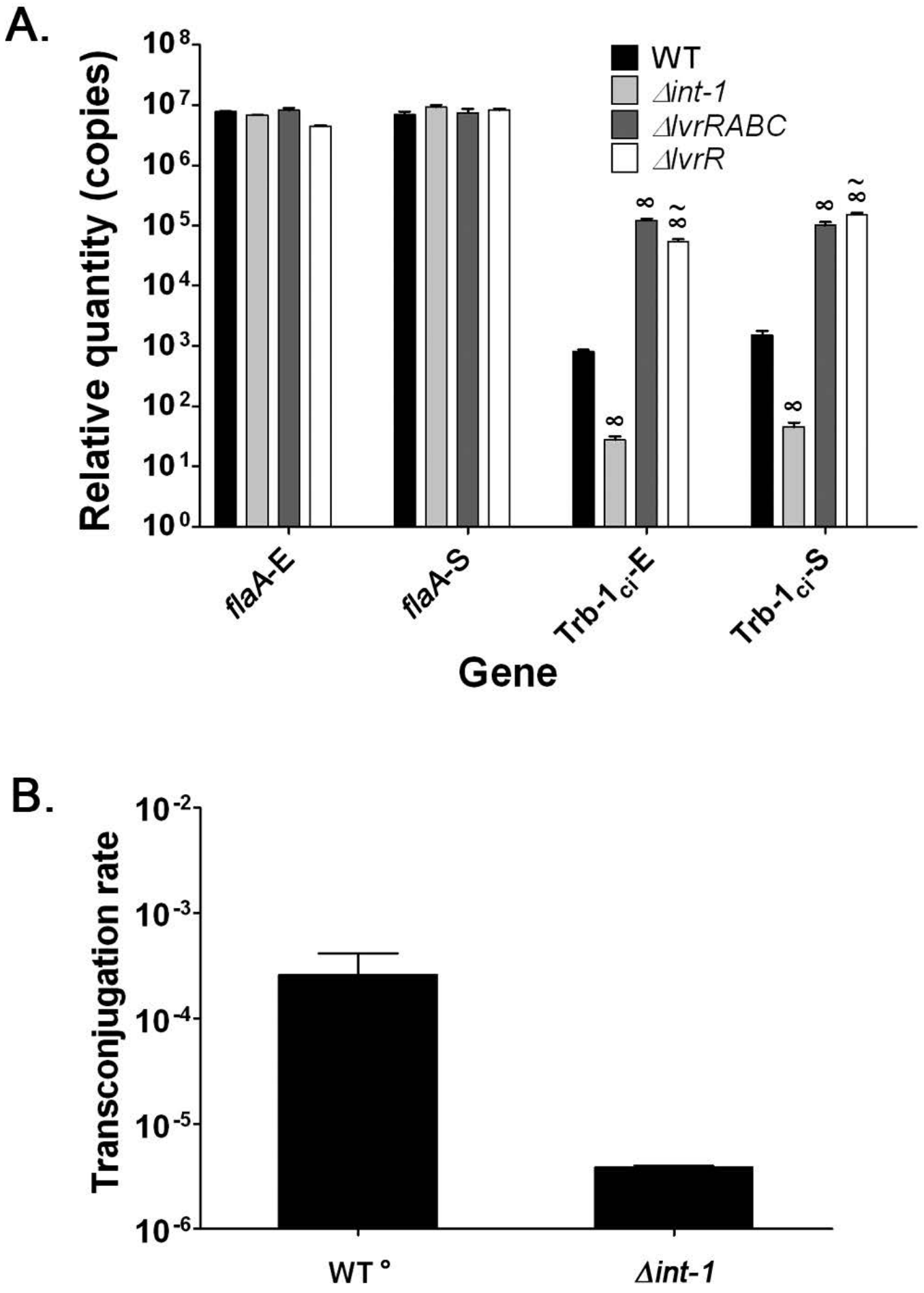

A.
Relative quantity (copies)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
WT
Δint-1
ΔlvrRABC
ΔlvrR
flaA-E
flaA-S
Trb-1ci-E
Trb-1ci-S
Gene
B.
Transconjugation rate
10^{-2}
10^{-3}
10^{-4}
10^{-5}
10^{-6}
WT °
Δint-1

TABLE S1. Oligonucleotides (primers) used in this study.

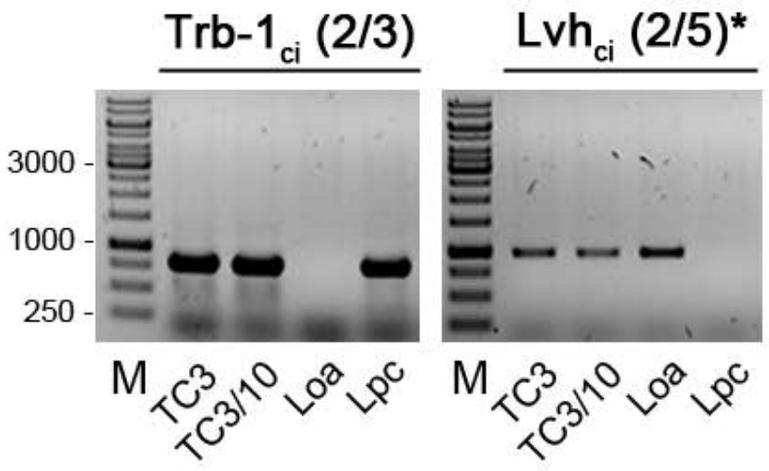
Name	Sequence (5'-3')	Reference or source
PCR analysis of genomic islands		
trb-1	TTCGATATCGCCGTCTGCCAT	Glöckner et al. (2008)
trb-2	AGATTGCTCATCAATCAAACTTCC	Glöckner et al. (2008)
trb-3	GTAAGCCTATCTATTGATAATGCAC	Glöckner et al. (2008)
trb-4	GAGAAGGTCGATGGTGATC	Glöckner et al. (2008)
trb-6	GCATGGTTCATAGCTCAACGTTGGAG	Glöckner et al. (2008)
trb-7	GTTGTAAAGTAGTGTTGGCCTTGG	Glöckner et al. (2008)
LpcGI-2-1U	GCCATCTATCAATTTCAGCAAGAGC	This study
LpcGI-2-2R	CTGCATGAATAGAACCACGATATCGAC	This study
LpcGI-2-3U	AGGTCACGTAAAAGTCACGCAAG	This study
LpcGI-2-4R	GGAAGAAGTTGGTAAACAATTTGACG	This study
LpcGI-2-5R	CATTGCCCACAATTTAGTCGAGAAGT	This study
LpcGI-2-6U	GAATATCTCAAAGAGCTTTCTGCACTG	This study
LpcGI-1-1U	CATCTGCACCAATGTTCAAGCTG	This study
LpcGI-1-2R	CGCTTTAGCAAGATATTCACGAGC	This study
LpcGI-1-3U	GGATGGCAGTAATATACATAGGCTCC	This study
LpcGI-1-4R	GTGTTCAACTACTGCATGCTACC	This study
LpcGI-1-5R	GCGATCTCTGGTTATTGATAACATTG	This study
LpcGI-1-6U	GAGCAAACATTCACGAGGAGTGAT	This study
LpcGI-Asn-1U	GTCGCATAAGTTTCAATGCTATGG	This study
LpcGI-Asn-2R	GCTAATATCTTTGGTTTGCACGTC	This study
LpcGI-Asn-3U	GTATCCCGATTAAAGAGTTCTGC	This study
LpcGI-Asn-4R	CTTTACGAACATCCTCAATGCCT	This study
LpcGI-Phe-1U	GTCATAGGTTATGCCACAGACA	This study
LpcGI-Phe-2R	GCATGAGTGTCGTAAAGTAATACC	This study
LpcGI-Phe-3U	CATCTTCAAGCTGATATTCATGTGC	This study
LpcGI-Phe-4R	GACAAGCCTATTACTCAACTCATC	This study
Loa-lvh-2R	GAAATCCACACTATCTACTGAAG	This study
Loa-lvh-5U	ACTGAACTGATGAGGAGCAAAC	This study
Characterization of transconjugants		
lpg0402-U	GATGATCGAATTAGGATTACCATCC	This study
lpg0402-R	GACATTGATGCAATCAACACCTTC	This study
TraG-F	TGGAGACGGTCAATGAGCTTGA	This study
TraG-R	TAGTCTGATTTCCTCATCTTCACG	This study
TraM-F	GTTATCACTGGTGGTCAAGC	This study
TraM-R	TGGATGCATGAGTTATCTCGC	This study
Loa-lvhB4-U	GAAGGGCAATAGTGAGTTGTCTGA	This study
Loa-lvhB4-R	GATATAGGAGCTGTTACCGGAAG	This study
Loa-orf0933-U	AGTCACCATAGGTCATAACGTCAA	This study
Loa-orf0933-R	TGCCTACACACTGTTTCAGCTATT	This study
lpc1833-U	AAGTCCTTGCGTGACTTTTACGTG	This study
lpc1833-R	AGCAACCAATCAAAGGAGTATGG	This study
lpc1850-U	CGCCAGCAGTGAAAGAAGTG	This study
lpc1850-R	CAAGCCATCCCCTAAGCATC	This study
lpc1884-U	CGCTTTTGGAGTTATCTGCATG	This study
lpc1884-R	CACGTTTGATTGATTTCATCCAC	This study
lpc2123-U	AGAGAAGTCCTAGGGGATGTAG	This study
lpc2123-R	AAGAAATACATCTTGCCTATGTCTG	This study
trnM-lpg2362-R	CAGTCTGAAAGAAAGCCCTTTTGA	This study
trnM-lpg2362-U	GACGACTCTCATCTGGGTGATTTT	This study
qPCR – episomal forms of genomic islands		
RT-LpcGI-2-1U	GGAAAGAGCCTAAAATTCGCCC	This study
RT-LpcGI-2-2R	GTGTTTGAGTACGACGCAGTAC	This study
RT-LpcGI-2-3U	AGGTCACGTAAAAGTCACGCAAG	This study

RT-LpcGI-2-5R	CCCACATCTGCAAGCACAAGTA	This study
RT-trb-2R	CACATTCAATTTGCACCCTGTG	This study
RT-trb-3U	CAAGGAGTTATTGTTCATGGAG	This study
RT-GyrA-F	GCCAAAGAAGTCTTACCAGTCAAC	This study
RT-GyrA-R	CGCAATACCGGAAGAGCCATTAA	This study

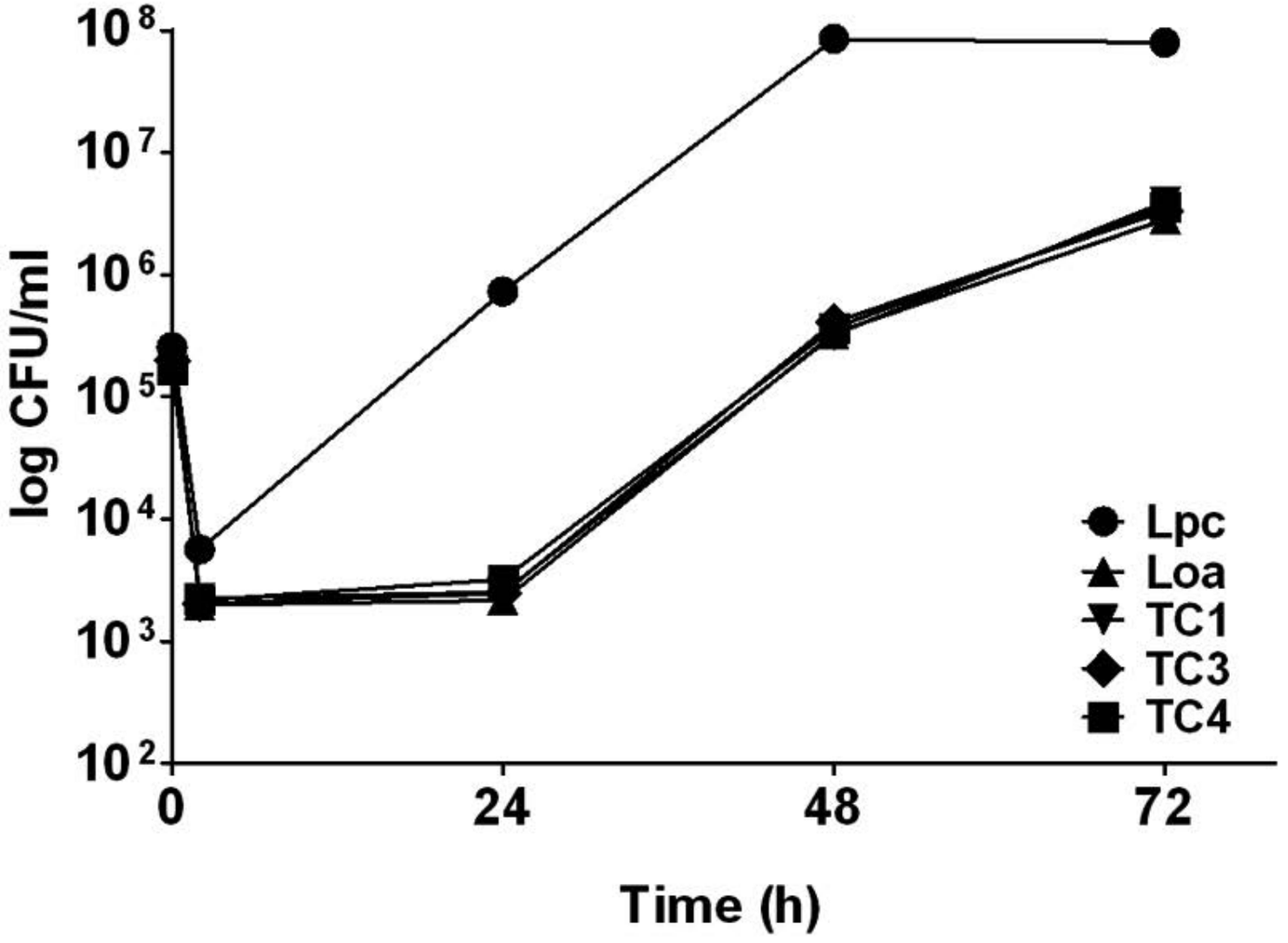
Legionella mutant construction

Int-U	CTAAAAGCTTACGTTGTAATTATG	This study
Int-R	AAGAGCTCGTATGCTTGCTGGCTTAT	This study
Int-MU	GCATAAAGTGAATGCCGTTAGAA	This study
Int-MR	GATCTAGATCATGAACTTCGTATT	This study
Int-Uin	GTTATGACAGCAGCTCAAGCTC	This study
Int-Rin	CTCAGCGTACTGGCAATATCAG	This study
Int-Rout	AGAGGCATCCTTACACTCATAACG	This study
LvrR-2816-U	GGATATGGTGAATTTGTCAGAAGG	This study
LvrR-2816-R	CTCCTGCGTTACTTCGATTTTGAC	This study
LvrR-2816-MU	GCGTTGTCCCATTCTAGAACTATC	This study
LvrR-2816-MR	CAAACTAGGGCATACGATCAACC	This study
LvrR-2816-Uin	GAATCAGTATCCATACGCTCC	This study
LvrR-2816-Rin	CTGCTGACATAACGGGATCA	This study
LvrRABC-1U	GAGTCGAATGGAAGTCCTCCA	This study
LvrRABC-1R	GCTTCTAGAACAAATTCCTGAGAATT	This study
LvrRABC-2U	TTACTCGAGCTTTCAGAAGACAATG	This study
LvrRABC-2R	CAATCTAGAGCATACGATCAACC	This study
LvrRABC-Uout	CATGGCTGAGACTCGATTGCTGTCAT	This study
LvrRABC-Rout	TCACTGATGACCAAGGCAGTTGAATGG	This study
LpcGI-2-Km-U	CTAATACCACGACGAATACCGATC	This study
LpcGI-2-Km-R	CAACCGTTCAAGCCAATATAG	This study
LpcGI-2-Km-MU	CGCATTAACACCTGGCTAAAATC	This study
LpcGI-2-Km-MR	CAATACCCATCTAGAGCAACTAACA	This study
LpcGI-2-Km-Rout	ACAGATGTTTAACCTCACTGATCG	This study
Int-1833-U	GCTAACTTGCGTCTTGTGATTTC	This study
Int-1833-R	GAAGATTGATACGTTTGGAATCTA	This study
Int-1833-MU	CCATTTACCCATACTCCTTTGATTG	This study
Int-1833-MR	GCGTAATCTAGATTTGAGAATGC	This study
Int-1833-Uin	CTGCATCATCATGAAGACTACG	This study
Int-1833-Rout	CAACTCCTATAGTGATGATGTGG	This study
Int-1884-U	GAGGCAAGCTTCGCAACATCATTG	This study
Int-1884-R	CAACACTAGTTCGTAGAGACTTTG	This study
Int-1884-MU	GATTGACTGGCATAGGAGTGGTTA	This study
Int-1884-MR	CGCATATCTAGAACAAGGATCTATTG	This study
Int-1884-Uout	CAAATCCTCCGGTATTGTACTGCTGT	This study
Int-1884-Rin	AGACGATATAGAGAGCTAACCC	This study
Int-2123-U	TGCTTCAAGCGCCTCTTTAGTG	This study
Int-2123-R	AAGCGAAGCTTTACTCAAGCCG	This study
Int-2123-MU	CTTCCGCGGTCCATTAACACATAGA	This study
Int-2123-MR	GCTAAAGCATCTAGAAGGGGATAATTC	This study
Int-2123-Uout	AAGGTTGGTCATCACACACTACC	This study
Int-2123-Rin	TAGCCTTGATTTGCCTGCTTATGC	This study
pilT-1876-U	GTCTTCTGAGCTCAACTGCGAAC	This study
pilT-1876-R	GCTGAATTCCTTGCAGAACAACTC	This study
pilT-1876-MU	GTACATCTAGATTGGATGAGCCAC	This study
pilT-1876-Uin	GACTATCCGACACAGAAAGCATGC	This study
pilT-1876-Rout	GCTATTACCCAAAACGTCACCGATATG	This study
Trb1-Km-U	GCTAAGTAATGCCAATAAGGG	This study
Trb1-Km-R	GCAACATCATTCGCAATAGATG	This study
Trb1-Km-MU	TATCTAGATGATTTTTAGGGGTAGATTGGG	This study
Trb1-Km-MR	CAAGTGGATTACTAATTTCGATTGA	This study
Trb1-Km-Rout	CTCTCTGTATATTCTGACA	This study

A.


Trb-1ci (2/3)
Lvhci (2/5)*
3000
1000
250
M
TC3
TC3/10
Loa
Lpc
M
TC3
TC3/10
Loa
Lpc

B.


log CFU/ml
10 8
10 7
10 6
10 5
10 4
10 3
10 2
0
24
48
72
Time (h)
Lpc
Loa
TC1
TC3
TC4