Roles of Long and Short Replication Initiation Proteins in the Fate of IncP-1 Plasmids

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Broad-host-range IncP-1 plasmids generally encode two replication initiation proteins, TrfA1 and TrfA2. TrfA2 is produced from an internal translational start site within *trfA1*. While TrfA1 was previously shown to be essential for replication in *Pseudomonas aeruginosa*, its role in other bacteria within its broad host range has not been established. To address the role of TrfA1 and TrfA2 in other hosts, efficiency of transformation, plasmid copy number (PCN), and plasmid stability were first compared between a mini-IncP-1β plasmid and its *trfA1* frameshift variant in four phylogenetically distant hosts: *Escherichia coli*, *Pseudomonas putida*, *Sphingobium japonicum*, and *Cupriavidus necator*. TrfA2 was sufficient for replication in these hosts, but the presence of TrfA1 enhanced transformation efficiency and PCN. However, TrfA1 did not contribute to, and even negatively affected, long-term plasmid persistence. When *trfA* genes were cloned under a constitutive promoter in the chromosomes of the four hosts, strains expressing either both TrfA1 and TrfA2 or TrfA1 alone, again, generally elicited a higher PCN of an IncP1-β replicon than strains expressing TrfA2 alone. When a single species of TrfA was produced at different concentrations in *E. coli* cells, TrfA1 maintained a 3- to 4-fold higher PCN than TrfA2 at the same TrfA concentrations, indicating that replication mediated by TrfA1 is more efficient than that by TrfA2. These results suggest that the broad-host-range properties of IncP-1 plasmids are essentially conferred by TrfA2 and the intact replication origin alone but that TrfA1 is nonetheless important to efficiently establish plasmid replication upon transfer into a broad range of hosts.

Plasmids belonging to incompatibility group P-1 (IncP-1 group) are generally considered to have a broad host range owing to their ability to replicate in at least two classes within the phylum *Proteobacteria* (e.g., *Alphaproteobacteria* and *Gammaproteobacteria*). The majority of IncP-1 plasmids have been identified as agents that mediate transfer of beneficial traits between these phylogenetically distinct bacteria, such as antibiotic resistance, resistance to heavy metals, or catabolic activity of xenobiotic compounds (2, 51, 60). Since the worldwide spread of antibiotic resistance is a growing concern in human health care (40), it is important that we improve our understanding of the mechanisms by which IncP-1 plasmids are maintained in a broad range of hosts.

Plasmid RK2, identified in *Pseudomonas aeruginosa*, has been studied as a model IncP-1 plasmid to gain understanding of basic plasmid functions (i.e., replication, stable inheritance, and conjugative transfer) and of the promiscuous nature of the IncP-1 group (32). It is known that the replication initiation protein (*trans*-acting factor A, TrfA) and the replication origin (*oriV*) are responsible for RK2 replication in a broad range of hosts (20, 52). RK2 encodes two replication initiation proteins (Rep proteins), TrfA-44 and TrfA-33 (54, 56). TrfA-33 is the truncated version of TrfA-44 and is produced from a second translational start site in the *trfA-44* reading frame. According to homology modeling, TrfA-33 shows structural similarity to Rep proteins from narrow-host-range plasmids such as the F plasmid and pPS10 (49). The N terminus of TrfA-44 is absent in the Rep proteins of those narrow-host-range plasmids (Fig. 1).

It has been shown that TrfA-44 is essential for replication of plasmid RK2 in *P. aeruginosa* (16, 55). In this host TrfA-33 functions poorly in positioning the host's DnaB helicase at the plasmid *oriV* with or without the presence of the host's DnaA, while TrfA-44 is able to position DnaB at the *oriV* of plasmid RK2 ($oriV_{RK2}$) without the assistance of DnaA (27). In fact, all DnaA

boxes in $oriV_{RK2}$ are dispensable for RK2 replication in *P. aeruginosa* (15). For RK2 replication in *Pseudomonas putida*, one of the four DnaA-boxes in $oriV_{RK2}$ is indispensable, but *in vitro* TrfA-44 is able to position DnaB at $oriV_{RK2}$ without DnaA of *P. putida*, just as observed for *P. aeruginosa* (15, 27). These observations suggested that TrfA-44 is able to modulate the plasmid's replication to be less dependent on the host's DnaA. Therefore, TrfA-44 has been postulated to play a role in extending the host range of IncP-1 plasmids (27, 63). However, *P. aeruginosa* is the only host reported so far in which RK2 required the longer TrfA-44 for replication. An experimentally generated mini-RK2 encoding only TrfA-33 was shown to replicate in several hosts currently classified as *Alphaproteobacteria* or *Gammaproteobacteria* (16). Furthermore, two naturally occurring plasmids of the IncP-1δ subgroup that encode only the TrfA-33 homolog have been found in natural hosts belonging to *Betaproteobacteria* (see below) (53, 68).These findings imply that the shorter protein alone might confer broad-host-range replication to the plasmid. Thus, the role of TrfA-44 and its homologs in broad-host-range replication of IncP-1 plasmids in hosts other than *P. aeruginosa* still needs to be elucidated.

IncP-1 plasmids have been classified into six subgroups (α, β, γ, δ, ε, and ζ) according to the phylogeny of the *trfA* gene or other plasmid backbone gene sequences (6, 25, 28, 46, 68). Plasmid sequence analyses have suggested that IncP-1 plasmids belonging to the α, β, ε, and ζ subgroups encode a long and shorter Rep pro-
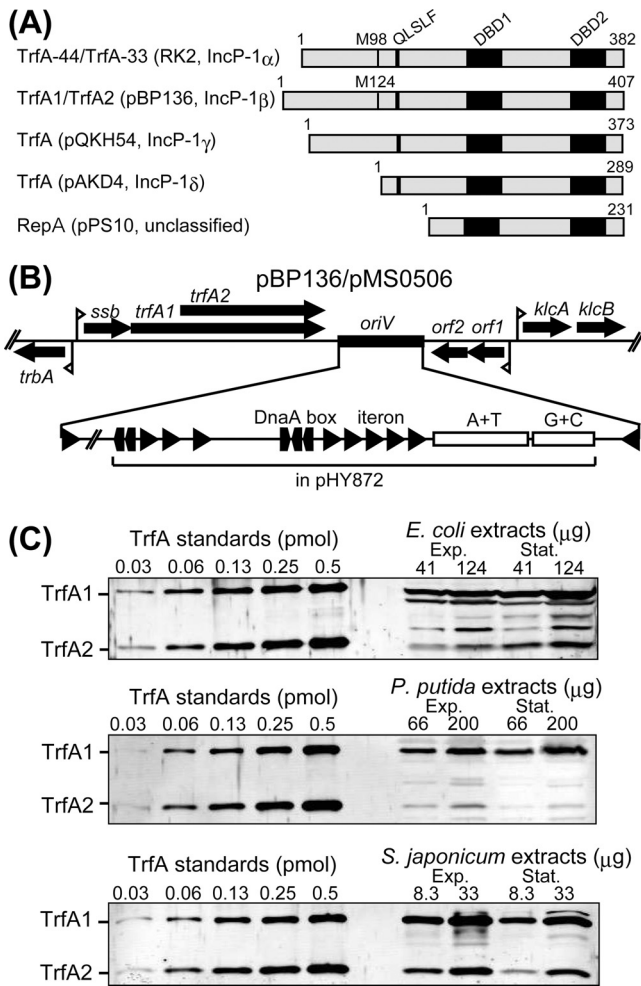
FIG 1 (A) Schematic representation of the diversity in TrfA structure. M98 and M124 indicate the start methionines of TrfA-33 in RK2 and TrfA2 in pBP136, respectively. QLSLF indicates the conserved amino acid sequence motif responsible for interaction with DNA polymerase III (33). DBD1 and DBD2 indicate DNA-binding (winged-helix) domains deduced from secondary structure comparisons (22, 49). (B) The *trfA* gene locus of pBP136 and its mini-pBP136 derivative pMS0506. A+T and G+C indicate the A/T- and G/C-rich regions, respectively. Triangles and pentagons indicate iterons (TrfA-binding motif) and DnaA-boxes (DnaA-binding motif), respectively. Flags indicate promoters. The region cloned in pHY872 is indicated. (C) Western blots showing TrfA production from pMS0506 in three hosts: *E. coli*, *P. putida*, and *S. japonicum*. The quantities of cell extracts were normalized to total protein content using a Bradford assay (11). Exp, exponential phase; Stat, stationary phase.

teins, just like the IncP-1α plasmid RK2. In contrast, plasmids from γ and δ subgroups encode only a single species of TrfA (Fig. 1A) (6, 25, 53, 68). The TrfA-44 and TrfA-33 homologs encoded by IncP-1 plasmids other than RK2 have been referred to as TrfA1 and TrfA2, respectively (61). From here on we will refer to TrfA1 and TrfA2 for all non-RK2 IncP-1 Rep protein homologs. The TrfA protein of IncP-1γ plasmid pQKH54 is equivalent to TrfA1, whereas the TrfA proteins of IncP-1δ plasmids pEST1044 and pAKD4 are essentially TrfA2 (25, 53, 68). In contrast to rapidly increasing information about the genetic diversity of IncP-1 plasmids, the significance of encoding TrfA1 alone, TrfA2 alone, or both proteins together is poorly understood. It was previously

shown that the copy number of RK2 in *Escherichia coli* decreased when translation of TrfA-33 was eliminated by point mutation at the translational start site (17). On the other hand, elimination of TrfA-44 translation decreased the copy number of RK2 in *Agrobacterium tumefaciens* but not in *E. coli* or *Azotobacter vinelandii* (17). While these observations seemed to suggest that the presence of the shorter TrfA is important for a high plasmid copy number (PCN) in *E. coli*, it is unclear if the longer TrfA participates in the plasmid's replication in hosts other than *P. aeruginosa* or *A. tumefaciens*.

To further examine the role of TrfA1 and TrfA2 in the host range of IncP-1 plasmids, here we have characterized the replication activity *in vivo* of a mini-IncP-1β replicon derived from plasmid pBP136 (29) in four phylogenetically distinct hosts. TrfA1 and TrfA2 of pBP136 (TrfA1$_{pBP136}$ and TrfA2$_{pBP136}$, respectively) show 65% and 83% amino acid identity to TrfA-44 and TrfA-33 of RK2, respectively, and 94% and 95% identity to TrfA1 and TrfA2 of the archetype IncP-1β plasmid R751. We chose this minirepli-con as our model system for two reasons: (i) all previous work on the role of long and short TrfA proteins in IncP-1 plasmid host range was done with the IncP-1α plasmid RK2 and not with any of the very widespread IncP-1β plasmids, and (ii) our previous experimental evolution study showed that *trfA1* frameshift variants of the mini-IncP-1β replicon had a different host range and stability than the wild-type replicon (57). We have compared efficiency of transformation, plasmid copy number, and plasmid stability between the mini-pBP136 replicon and its *trfA1* frameshift variant. In addition, plasmid copy number in the presence of TrfA1 alone, TrfA2 alone, or both proteins was determined. The results suggest the following: (i) that TrfA2 is sufficient for plasmid replication in a broad range of hosts; (ii) that TrfA1, but not TrfA2, plays a role in maintaining high plasmid copy number and increasing transformation efficiency; and (iii) that TrfA1 does not contribute to long-term plasmid persistence in hosts wherein TrfA2 is sufficient for plasmid replication.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. Bacterial strains and plasmids used in this study are listed in Table 1. pMS0506, a deletion derivative of the cryptic IncP-1β plasmid pBP136 isolated from *Bordetella pertussis* (29, 57), was used as a model IncP-1β plasmid. This minireplicon is no longer self-transmissible due to the removal of transfer genes. Transcription of the *trfA* gene in pMS0506 is controlled by cognate promoter and transcriptional regulators encoded by pMS0506 itself (KorB and KorA). The *trfA1* variant of pMS0506, pEvo-Sh15, has a frameshift mutation in the 5′ end of *trfA1* caused by a 13-bp duplication and was obtained previously after experimentally evolving pMS0506 to higher stability in *Shewanella oneidensis* MR-1. This plasmid no longer replicates in *Pseudomonas aeruginosa* (57). Western analysis has confirmed the production of TrfA2 and the lack of production of TrfA1 from pEvo-Sh15 (see Fig. S1 in the supplemental material).

To address the role of the two Rep proteins in the ability of IncP-1 plasmids to establish themselves and be stably maintained in a wide range of bacteria, we used four model hosts. The two hosts, *E. coli* and *P. putida* (both belonging to the *Gammaproteobacteria*) were selected because the behavior of RK2 in these hosts has been relatively well studied. In addition, since IncP-1 plasmids have been found in strains within the *Betaproteobacteria* (for example, in the genera *Achromobacter*, *Bordetella*, *Comamonas*, *Cupriavidus*, *Delftia*, and *Neisseria*) and *Alphaproteobacteria* (genus *Sphingomonas*) (10, 26, 29, 37, 47, 67, 68), we also included representative hosts from those two classes: the betaproteobacterium *Cupria-*

TABLE 1 Strains and plasmids used in this study

Strain (class) or plasmid	Genotypes and relevant characteristic(s)[a]	Reference or source
E. coli strains (*Gammaproteobacteria*)		
BW25113	F⁻ *rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	4
BW29427	*thrB1004 pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)*567* Δ*dapA1341::(erm-pir)* Erm^r	B. L. Warner, Purdue University
DH5α	λ⁻ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r$_K^-$ m$_K^-$) *supE44 thi-1 gyrA relA1*	50
DY380	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara-leu*)*7697 gal490 pgl*Δ8 *rpsL nupG* [λ *cI857indl* Δ(*cro-bioA*):: *tet*] Tc^r	39
EC100D*pir*⁺	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL nupG pir* (DHFR)	Epicentre (Madison, WI)
HY390	BW25113 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA1*(*M124L*)	This study
HY407	BW25113 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA2*	This study
HY414	BW25113 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA1/trfA2*[b]	This study
P. putida strains (*Gammaproteobacteria*)		
KT2440	Type strain, ATCC 47054	5
HY566	KT2440 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA1/trfA2*; Gm^r	This study
HY569	KT2440 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA1*(*M124L*); Gm^r	This study
HY570	KT2440 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA2*; Gm^r	This study
S. japonicum strains (*Alphaproteobacteria*)		
UT26S	Type strain, JCM17232	45
HY848	UT26S *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA1/trfA2*; Gm^r	This study
HY849	UT26S *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA1*(*M124L*); Gm^r	This study
HY850	UT26S *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA2*; Gm^r	This study
C. necator strains (*Betaproteobacteria*)		
JMP228	JMP134 derivative cured of plasmid pJP4; Rif^r	3
HY876	JMP228 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA1/trfA2*; Rif^r Gm^r	This study
HY877	JMP228 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA1*(*M124L*); Rif^r Gm^r	This study
HY878	JMP228 *att*Tn*7::*mini-Tn*7::aacC1-lacI*^{q}-P*tac-trfA2*; Rif^r Gm^r	This study
Plasmids		
pBAD24	pMB1 replicon, protein expression vector; Ap^r	24
pBAD24C	pBAD24 derivative in which *bla* was replaced by *cat*; Cm^r	This study
pEvo-Sh15	pMS0506 derivative with a frameshift mutation in 5′ end of *trfA1*, unable to replicate in *P. aeruginosa*; Km^r	57
pHSG399	pMB1 replicon, cloning vector; Cm^r	59
pHY819	pBAD24C carrying *trfA1*(*M124L*); Cm^r	This study
pHY820	pBAD24C carrying *trfA2*; Cm^r	This study
pHY835	pUCR6KT-mini-Tn*7*T derivative in which the HindIII site in the R6K origin was disrupted; Ap^r Gm^r	This study
pHY835LAC	R6K replicon, mini-Tn*7::aacC1-lacI*^{q}-P*tac*; Ap^r Gm^r	This study
pHY837	pHSG399 carrying *trfA1*(*M124L*); Cm^r	This study
pHY838	pHSG399 carrying *trfA2*; Cm^r	This study
pHY853	pHSG399 carrying *trfA1/trfA2*; Cm^r	This study
pHY872	pJP5608 ΔP*lac* carrying the 0.5-kb fragment of *oriV*$_{pBP136}$; Tc^r	This study
pHY884	pHY835LAC carrying *trfA1/trfA2*; Ap^r Gm^r	This study
pHY885	pHY835LAC carrying *trfA1*(*M124L*); Ap^r Gm^r	This study
pHY886	pHY835LAC carrying *trfA2*; Ap^r Gm^r	This study
pHY922	pSTV28 carrying the 1.5-kb PstI-EcoRI fragment containing *kleE* of pBP136; Cm^r	This study
pHY924	pHY872 carrying the 2.0-kb EcoRI fragment containing *atpB* of the BW25113 chromosome; Tc^r	This study
pHY925	pTXB1 carrying *trfA2*; Ap^r	This study
pHY926	pTXB1 carrying *trfA1*(*M124L*); Ap^r	This study
pHY934	pHY922 carrying the 2.0-kb EcoRI fragment containing *atpB* of the *E. coli* BW25113 chromosome; Cm^r	This study
pHY950	pHY922 carrying the 1.8-kb EcoRI fragment containing *parB* of the KT2440 chromosome; Cm^r	This study
pHY951	pHY872 carrying the 1.8-kb EcoRI fragment containing *parB* of the KT2440 chromosome; Tc^r	This study
pHY974	pHY872 carrying the 2-kb EcoRI fragment of SJA-00140 of the UT26S chromosome I; Tc^r	This study
pHY976	pHY922 carrying the 2-kb EcoRI fragment of SJA-00140 in the UT26S chromosome I; Cm^r	This study
pHY982	pHY872 carrying the 0.8-kb EcoRI fragment containing *parB* of the JMP228 chromosome I; Tc^r	This study
pHY983	pHY922 carrying the 0.8-kb EcoRI fragment containing *parB* of the JMP228 chromosome I; Cm^r	This study
pJP5608	R6K replicon, suicide vector; Tc^r	48
pMS0506	IncP-1β replicon, pBP136 Δ(*trbB-traM*)::(*oriT*$_{RP4}$-*kan*); Km^r	57
pTXB1	pMB1 replicon, protein expression vector; Ap^r	New England Biolabs (Ipswich, MA)
pUCR6KT-mini-Tn*7*T	R6K replicon, *oriT*$_{RP4}$, mini-Tn*7* vector; Ap^r	14
pUC18-mini-Tn*7*T-LAC	pUC18 replicon, mini-Tn*7::aacC1-lacI*^{q}-P*tac*; Ap^r Gm^r	14
pUX-BF13	R6K replicon, Tn*7* transposase expression vector, *tnsABCDE oriT*$_{RP4}$; Ap^r	8

[a] Abbreviations for are as follows: Tc, tetracycline; Ap, ampicillin; Gm, gentamicin; Cm, chloramphenicol; Km, kanamycin; Erm, erythromycin; Rif, rifampin. *kan* is the kanamycin resistance gene.
[b] *trfA1/trfA2*, wild-type *trfA1* encoding both TrfA1 and TrfA2.

vidus necator JMP228, a derivative of JMP134 (41), and the alphaproteobacterium *Sphingobium japonicum* UT26S (45).

Luria-Bertani (LB) medium (50) was used for cultivation of *E. coli* and *P. putida*, and 1/3-LB medium (3.3 g of Bacto tryptone, 1.7 g of yeast extract, and 5 g of NaCl/liter of H_2O) was used for *S. japonicum*; 1/10-TSB (1/10 dilution of Bacto tryptic soy broth; BD Bioscience, Sparks, MD) was used for *C. necator*. Solid medium was made by addition of 1.5% agar to LB (referred to as LBA), 1/3-LB (1/3-LBA), or 1/10-TSB (1/10-TSBA). *E. coli* strains were incubated at 37°C for DNA cloning, while *P. putida*, *S. japonicum*, and *C. necator* strains were incubated at 30°C. Once the IncP-1 replicon was introduced, all strains including *E. coli* were incubated at 30°C. If necessary, the medium was supplemented with antibiotics at the following concentrations: for *E. coli*, 100 μg/ml ampicillin (Ap), 50 μg/ml kanamycin (Km), 10 μg/ml gentamicin (Gm), 25 μg/ml chloramphenicol (Cm), and 10 μg/ml tetracycline (Tc); for *P. putida*, 20 μg/ml Gm, 50 μg/ml Km, and 10 μg/ml Tc; for *C. necator*, 70 μg/ml Gm, 50 μg/ml Km, and 10 μg/ml Tc; for *S. japonicum*, 2.5 μg/ml Gm, 50 μg/ml Km, and 10 μg/ml Tc.

General molecular techniques. For DNA cloning, heat shock transformation of *E. coli* DH5α ultracompetent cells (50) and electroporation of *E. coli* EC100D*pir*$^+$ (Epicentre, Madison, WI) were routinely used. Plasmid DNA was isolated from *E. coli* using a GeneJET plasmid miniprep kit (Fermentas, Inc., Ontario, Canada). DNA fragment purifications from PCR mixtures and agarose gels were performed using a GeneJET PCR purification kit and a GeneJET gel extraction kit (Fermentas, Inc., Ontario, Canada), respectively. Phusion High-Fidelity DNA polymerase (New England BioLabs, Ipswich, MA) was used for conventional PCR. Total DNA extraction was performed using a GenElute bacterial genomic kit (Sigma, St. Louis, MO). Fast SYBR premix *Taq* (Applied Biosystems, Foster City, CA) was used for quantitative PCR (qPCR). Southern blotting experiments were performed using a DIG-High Prime DNA Labeling and Detection Kit (Roche Applied Science, Mannheim, Germany) (where DIG is a digoxigenin). Oligonucleotides used in this study were synthesized by Sigma (Sigma-Aldrich, St. Louis, MO) and are listed in Table S1 in the supplemental material.

For preparation of electrocompetent cells of *E. coli*, *P. putida*, and *C. necator*, 1 ml of overnight bacterial cultures was diluted in 100 ml of fresh medium, grown up to an optical density at 600 nm (OD_{600}) of 0.8, washed with an electroporation solution (300 mM sucrose for *E. coli* and *P. putida*; 10% glycerol for *C. necator*), and resuspended in 2 ml of the solution. Cell suspensions (100 μl) containing 10^8 to 10^9 cells were used for electroporation. Electrocompetent cells of *S. japonicum* were prepared from bacterial colonies grown on 1/3-LBA for 48 h; the bacterial colonies were gently scraped from the agar surface, washed, and resuspended in distilled water. Electroporation was conducted using a 1-mm gap cuvette and a Gene Pulser Xcell (Bio-Rad, Hercules, CA) at 1.8 kV. After electroporation, cells were incubated in their appropriate liquid medium for 2 h and subsequently plated on plain agar medium for total cell counts and on agar medium containing Km for transformant counts.

Plasmid stability assay. Plasmid stability assays were done essentially as described previously (57). Briefly, for *E. coli* and *P. putida*, each strain was first grown with Km overnight, and 4.9 μl of overnight culture was transferred to 5 ml of LB medium, while an aliquot of the overnight culture was serially diluted and plated on LBA to obtain single colonies. The following day, 52 single colonies were replicated on LBA and on LBA containing Km. The ratio of Km-resistant colonies to total colonies was considered the plasmid-containing fraction. This procedure was repeated every 24 h for 10 days. For *C. necator*, the strains were grown in 1/10-TSB. For *S. japonicum*, the strains were grown in 20 ml of 1/3-LB medium to prevent aggregation of bacterial cells, and the culture was transferred and plated every 2 days. Single colonies of *S. japonicum* were replicated on 1/3-LBA and 1/3-LBA containing Km.

Construction of strains carrying *trfA* in the chromosome. The mini-Tn7 vector (14) was used to insert *trfA* genes into a specific site (*att*Tn7) in the chromosomes. The mini-Tn7-*aac*C1-*lacI*^{q}-P*tac* region of pUC18T-

mini-Tn7T-LAC (14) was cloned into the NdeI-HindIII region of pHY835, a derivative of pUCR6KT-mini-Tn7T carrying a mutation in the HindIII site in the R6K origin. The transcriptional terminator region in pColdIV (Takara, Shiga, Japan) was cloned into the HindIII-KpnI region of the pHY835 derivative. The resulting R6K replicon carrying mini-Tn7-*aac*C1-*lacI*^{q}-P*tac* was designated pHY835LAC. The *trfA1* gene of pBP136 was first PCR amplified using primers that included a ribosome-binding binding site. The PCR products were cloned into the SacI-HindIII region of pHSG399 (59) to confirm the *trfA* sequences. To eliminate translation of TrfA2 from the *trfA1* gene, the *trfA2* start codon, a methionine (ATG) at 124, was replaced with the codon for leucine (CTG). This mutation (the M124L mutation) was introduced into *trfA1* [*trfA1(M124L)*] in the pHSG399 derivatives by PCR, followed by phosphorylation and ligation. pHSG399 derivatives carrying either the wild-type *trfA1* encoding both TrfA1 and TrfA2 (*trfA1/trfA2*), *trfA1(M124L)*, or *trfA2* were designated pHY853, pHY837, and pHY838, respectively. The SacI-HindIII region carrying *trfA1/trfA2*, *trfA1(M124L)*, or *trfA2* was cloned from pHY853, pHY837, or pHY838 into pHY835LAC, yielding pHY884, pHY885, and pHY886. To introduce the mini-Tn7::*aac*C1-*lacI*^{q}-P*tac*-*trfA* fragments to the recipient chromosomes, *E. coli* BW29427 harboring pHY884, pHY885, or pHY886, *E. coli* BW29427(pUX-BF13), and a recipient strain were mated on LBA or 1/3-LBA containing 200 μM diaminopimelic acid overnight. The recipient strains tagged by mini-Tn7 were selected on LBA or 1/3-LBA containing the appropriate concentration of Gm depending on the host. Mini-Tn7 insertion to *att*Tn7 was confirmed by PCR amplification of the mini-Tn7-*glmS* junction. The presence of a single copy of *trfA* in the genomes of the constructed strains was confirmed by Southern hybridization analysis using the *trfA2* region as a probe. PCR primers used to clone *trfA* genes or to confirm mini-Tn7 insertion are listed in Table S1 in the supplemental material. Location of the mini-Tn7-*trfA* insertion site in *S. japonicum* UT26S and *C. necator* JMP228 was determined by sequencing and was 26 bp downstream from the stop codon of the *glmS* gene in chromosome I for both strains.

Construction of TrfA expression plasmids. Protein expression plasmid pBAD24C was constructed by replacing the ampicillin resistance gene on pBAD24 (24) with the pHSG399-derived chloramphenicol resistance gene in *E. coli* DY380 using λ-Red recombination according to a previously published protocol (39). The PCR-amplified *trfA1(M124L)* and *trfA2* gene fragments were first cloned into pHSG399 to confirm the sequences and then moved into the EcoRI-HindIII region of pBAD24C. The resulting pBAD24C derivatives carrying either *trfA1(M124L)* or *trfA2* were designated pHY820 or pHY819, respectively. For affinity protein purification, *trfA1(M124L)* and *trfA2* were PCR amplified and cloned into the NdeI-XhoI region of pTXB1 (New England BioLabs, Ipswich, MA), yielding pHY926 and pHY927, respectively.

Determination of plasmid copy number. To measure copy numbers of pMS0506 and pEvo-Sh15 by qPCR, a series of copy number reference plasmids was constructed carrying a pBP136 fragment and a chromosomal fragment (the *oriC* region) at a 1:1 ratio. The 1.7-kb PstI-HindIII fragment (the *kleE* region) of pBP136 was cloned from pMS0506 into pSTV28 (Takara, Shiga, Japan). The resulting plasmid was designated pHY922. The 0.8- to 2.0-kb EcoRI fragments near *oriC* in the chromosomes of *E. coli*, *P. putida*, *C. necator*, and *S. japonicum* were PCR amplified using primers shown in Table S1 in the supplemental material, and the PCR products were cloned into the EcoRI site of pHY922, yielding pHY934 (*E. coli*), pHY950 (*P. putida*), pHY982 (*C. necator*), and pHY974 (*S. japonicum*). The chromosomal fragment contained *atpB* for *E. coli*, *parB* for *P. putida*, *parB* of chromosome I for *C. necator*, or a partial fragment of the TonB-dependent receptor-like protein gene (SJA-0140) of chromosome I for *S. japonicum*. A copy number reporter plasmid, pHY872 (Fig. 1B), was constructed by PCR amplification of the pBP136 *oriV* (*oriV*$_{pBP136}$) containing eight iteron copies, cloning of the PCR product into pJP5608 (48), and subsequent removal of the *lac* promoter in the pJP5608 backbone using PCR. To measure copy number of pHY872, copy number reference plasmids were constructed by cloning the chromo-

somal fragments described above into the EcoRI site of pHY872. The resulting copy number reference plasmids were designated pHY924 (for *E. coli*), pHY951 (*P. putida*), pHY983 (*C. necator*), and pHY976 (*S. japonicum*).

For the copy number analysis of pMS0506 and pEvo-Sh15, total DNA was purified from 1 ml of exponential-phase culture. For the copy number analysis of pHY872 in the strains carrying *trfA* genes in the chromosome, total DNA was purified from 0.5 ml of stationary-phase cultures grown with 10 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Relative copy numbers of IncP-1β replicons compared to a gene in the *oriC* region in the chromosome in total DNA were determined using qPCR. Prior to qPCR, purified total DNA was linearized by restriction digestion and recovered by ethanol precipitation. Digested total DNA (1.5 μl) at a concentration of 6.0 to 10.0 ng/μl was added to the 20-μl-scale qPCR mixtures. The EcoRI and PstI digests of pHY934, pHY950, pHY974, and pHY982 were used to generate standard curves in the copy number analysis for pMS0506 and pEvo-Sh15, while the EcoRI digests of pHY924, pHY951, pHY976, and pHY983 were used to generate standard curves in the copy number analysis of pHY872. Primers used in qPCR are listed in Table S1 in the supplemental material. Data analysis was performed using the SDS2 software supplied with the ABI 7900HT thermal cycler (Applied Biosystems, Foster City, CA). Quantities of the target sequence in the total DNA were determined on the standard curves with R^2 of >0.994.

Proteins. TrfA1 and TrfA2 were purified using an Impact kit (New England BioLabs, Ipswich, MA) and buffers used for purification of TrfA-33 and TrfA-44, with modifications (65). Protein expression was induced in *E. coli* Rosetta 2 (DE3) carrying pHY926 or pHY927 in 50 ml of LB medium. Cells were resuspended in 3 ml of a buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 0.05% Triton-X) and sheared by French press at 900 lb/in^2 (repeated three times), followed by ultrasonic treatment for 2 min. After the cell extract was clarified by centrifugation at 20,000 × *g* for 25 min, phenylmethylsulfonyl fluoride (PMSF) was added to 1 mM. The supernatant was incubated with 1 ml of chitin beads for 1 h at 4°C to bind the expressed protein. The bead-TrfA complex was washed in a 2-ml column with 40 ml of wash buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 10% glycerol) and then incubated in 1 ml of wash buffer containing 50 mM dithiothreitol (DTT) and 1 mM PMSF at 30°C overnight to induce intein cleavage. The eluate containing tag-free TrfA was dialyzed overnight against 1 liter of stock buffer (50 mM sodium phosphate [pH 6.0], 100 mM NaCl, 10% glycerol). This protocol yielded >95% pure TrfA as assessed by SDS-PAGE gels. Protein concentration of purified TrfA was normalized to a bovine serum albumin (BSA) standard in SDS-PAGE gels stained with Coomassie brilliant blue (CBB).

To prepare whole-cell extracts, bacterial cells were suspended in an appropriate volume of buffer containing 10 mM sodium phosphate (pH 7.6) and 145 mM NaCl and then disrupted using a French press, followed by ultrasonic treatment as above. The disrupted cells were clarified by centrifugation at 20,000 × *g* for 25 min, and then PMSF was added to 1 mM. The supernatant containing 1 mM PMSF was used as whole-cell extract throughout this study. Protein concentrations of whole-cell extracts were determined by Bradford assay (11) using BSA as the standard.

Quantitative Western blotting. Whole-cell extracts and purified TrfA2 (quantity standards) diluted in phosphate buffer containing 1 mg/ml BSA were denatured in sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol) and electrophoresed in an 11% SDS-polyacrylamide gel for 90 min at 120V to separate TrfA1 and TrfA2. Proteins were electrotransferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-FL; Millipore, Billerica, MA) overnight (~15 h) at 10V in Towbin buffer (66) using a TE 62 transfer unit (GE Healthcare, Piscataway, NJ). The membrane was hybridized with anti-TrfA2-serum diluted 1:2,000 in blocking buffer (1× phosphate-buffered saline [PBS], 5% dry milk). Polyclonal anti-TrfA2 antibodies were raised in rabbits immunized three times with denatured TrfA2$_{pBP136}$ contained in an SDS-polyacrylamide gel slice (Harlan Laboratories, Inc., Indianapolis, IN). After being washed with blocking buffer, the membrane was

incubated with IRDye 800CW goat anti-rabbit IgG (Rockland, Gilbertsville, PA), according to the manufacturer's protocols. Densitometry analysis was performed with an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). TrfA proteins were quantified on standard curves with an R^2 of >0.99. Each experiment was performed at least three times.

RESULTS

Production of TrfA proteins encoded on IncP-1β plasmid pBP136. Although plasmid sequence analysis suggests that pBP136 encodes both TrfA1 and TrfA2, no direct evidence had been obtained so far. Therefore, we first confirmed production of both proteins from the mini-pBP136 replicon, pMS0506, in our model hosts, *E. coli*, *P. putida*, and *S. japonicum*, using Western blotting and determined the ratio of TrfA1 to TrfA2. Production of both TrfA1 and TrfA2 was observed for the three hosts analyzed, but TrfA levels and the TrfA2/TrfA1 ratios differed among hosts (Fig. 1C). In exponential phase, TrfA1 and TrfA2 levels (fmol/μg of total protein) were, respectively, 2.9 ± 0.3 and <0.2 for *E. coli*, 2.1 ± 0.5 and 0.2 ± 0.1 for *P. putida*, and 40 ± 2 and 13 ± 1 for *S. japonicum*. In stationary phase, TrfA1 and TrfA2 levels were, respectively, 7.0 ± 0.9 and 0.7 ± 0.1 for *E. coli*, 1.3 ± 0.2 and <0.2 for *P. putida*, and 18 ± 1 and 7.0 ± 1.1 for *S. japonicum*. The TrfA2/TrfA1 ratios in exponential phase were <0.1:1 for *E. coli*, 0.1:1 for *P. putida*, and 0.3:1 for *S. japonicum*. The TrfA2/TrfA1 ratios in stationary phase were 0.1:1 for *E. coli*, <0.1:1 for *P. putida*, and 0.4:1 for *S. japonicum*. These results indicate that pBP136, indeed, encodes both TrfA1 and TrfA2 and that TrfA1 was consistently more abundant than TrfA2 regardless of growth phase and host. However, in previous studies using plasmid RK2 in *P. putida*, TrfA-44 was not produced at detectable levels (7). Thus, even within the IncP-1 group, there are differences in the levels of production of the larger TrfA protein.

TrfA2 is sufficient for broad host range but not for high transformation efficiency. Previous studies have shown that elimination of TrfA1 expression abolished replication activity of IncP-1 plasmids in *P. aeruginosa* (16, 54, 57). This suggests that TrfA1 could be responsible for broad-host-range replication of IncP-1 plasmids. However, these studies used limited numbers of model hosts, and the requirement for TrfA1 in plasmid replication was not demonstrated outside *P. aeruginosa*. To address the role of TrfA1 in determining host range, we first determined whether the *trfA1* frameshift variant of pMS0506, pEvo-Sh15 (57), could replicate in *S. japonicum* and *C. necator* when introduced into these hosts by electroporation. These two strains are phylogenetically distant from the two model hosts, *E. coli* and *P. putida*, in which the TrfA2 homolog TrfA-33 was previously shown to be sufficient for RK2 replication (16, 55). Efficiency of transformation was then compared between pMS0506 and pEvo-Sh15 for four hosts: *E. coli*, *P. putida*, *S. japonicum*, and *C. necator* (Table 2). All four hosts were successfully transformed with pEvo-Sh15, indicating that the presence of TrfA2 and the intact *oriV* was sufficient for the IncP-1β plasmid to replicate in a broad range of hosts. However, the transformation frequency of pEvo-Sh15 was decreased more than 10-fold in *E. coli*, 5-fold in *P. putida* and *S. japonicum*, and 3-fold in *C. necator* (Table 2). This suggests that the presence of TrfA1 increases the efficiency of establishment in a broad range of hosts even though TrfA2 is sufficient for broad-host-range replication.

Requirement of TrfA1 for a high plasmid copy number. Sev-

TABLE 2 Effect of the presence of TrfA1 on the transformation efficiency of mini-IncP-1β plasmids

Plasmid (description)	No. of transformants/10^9 cells/μg of DNA (relative efficiency)^a			
	E. coli	P. putida	S. japonicum	C. necator
pMS0506 (wt)	$6.7 \times 10^5 \pm 0.8 \times 10^5$ (1.0)	$1.4 \times 10^4 \pm 0.7 \times 10^4$ (1.0)	$4.6 \times 10^4 \pm 0.2 \times 10^4$ (1.0)	$1.4 \times 10^2 \pm 0.3 \times 10^2$ (1.0)
pEvo-Sh15 (trfA1)	$4.4 \times 10^4 \pm 0.2 \times 10^4$ (0.1)**	$3.4 \times 10^3 \pm 0.7 \times 10^3$ (0.2)	$9.8 \times 10^3 \pm 1.1 \times 10^3$ (0.2)**	$4.4 \times 10^1 \pm 1.1 \times 10^1$ (0.3)*

^{a} The value indicates the mean and standard error of the mean obtained from four electroporation experiments. The concentration of plasmid DNA used per electroporation was normalized on a standard curve generated by densitometric analysis of linearized and serially diluted plasmid DNA in an agarose gel stained with ethidium bromide. Relative efficiency indicates transformation efficiency relative to that for pMS0506. Significance was determined in comparison to pMS0506 (t test): *, $P < 0.05$; **, $P < 0.01$.

eral studies have shown a correlation between high transformation efficiency and high plasmid copy number (PCN) (19, 42, 69). To investigate if the observed change in transformation efficiency was associated with PCN, copy numbers of pMS0506 and pEvo-Sh15 were determined in the four hosts using qPCR, as described in Materials and Methods. The copy number of pMS0506 ranged from 3.0 to 5.4 copies per chromosomal *oriC* region in the four hosts, while that of pEvo-Sh15 was lower, 0.9 to 2.5 copies per *oriC* region (Table 3). Elimination of TrfA1 thus decreased the PCN in *P. putida* by a factor of 2 and by a factor of 3 in *E. coli*, *S. japonicum*, and *C. necator*. This suggests that TrfA1 plays a role in maintaining a high PCN in a broad range of hosts.

TrfA1 does not promote plasmid stability. A decrease in plasmid copy number might negatively affect plasmid stability in a growing bacterial population by increasing the plasmid segregational loss rate. To assess this possibility, plasmid stability was analyzed for pMS0506 and pEvo-Sh15 in four hosts: *E. coli*, *P. putida*, *S. japonicum*, and *C. necator*. It should be noted that both plasmids used here carried intact partitioning genes. In *E. coli*, *P. putida*, and *C. necator*, the fraction of plasmid-bearing cells decreased at similar rates for both pMS0506 and pEvo-Sh15 (Fig. 2) even though there was a significant difference in PCN between the two plasmids in each host. Surprisingly, in *S. japonicum* plasmid pEvo-Sh15 carrying the *trfA1* frameshift variant was drastically more stable than the wild-type pMS0506 (Fig. 2). These results indicate that when the plasmid carries the genes involved in plasmid inheritance, the presence of TrfA1 and the resulting high PCN do not contribute to, or even negatively affect, plasmid persistence.

Plasmid copy number in the presence of a single species of TrfA in the four hosts. The significant decrease in both transformation frequency and PCN resulting from eliminating TrfA1 production in the cell prompted us to hypothesize that TrfA1 and TrfA2 are distinct in their abilities to generate plasmid copies. Alternatively, these decreases in transformation frequency and PCN may be simply due to decreased concentrations of total intracellular TrfA. In fact, total TrfA levels in pEvo-Sh15 were less

than one-fourth of the wild-type level (see Fig. S1 and Table S2 in the supplemental material). To address this caveat, we inserted different variants of *trfA* in the chromosomes of the four hosts and measured the copy number of a synthetic plasmid, pHY872, which carried the pBP136 *oriV* but not *trfA* (Fig. 1B). This was done as follows. First, three *trfA* gene alleles were cloned under a *tac* promoter and a common ribosome-binding sequence: wild-type *trfA* (*trfA1/trfA2*), the *trfA1* variant that does not produce TrfA2 [*trfA1*(M124L)], and *trfA2* (Fig. 3A). These were inserted into the *att*Tn7 site of the four host chromosomes, thus resulting in expression of the various TrfA proteins in the presence of IPTG. Subsequently, pHY872 was introduced into these 12 hosts, and its copy number was determined. It should be noted that, except in the presence of TrfA1(M124L) alone in *P. putida*, the total TrfA levels were higher than those produced by pMS0506 in *E. coli*, *P. putida*, and *S. japonicum*. The TrfA levels in *C. necator* could not be measured because the signal-to-noise ratio was too low in Western analysis. Below, we describe a pairwise comparison of the effect of the three TrfA variants on the PCN in each of the four hosts.

In three of the four hosts, the clones with wild-type *trfA* (producing both TrfA1 and TrfA2) showed higher PCNs than the clones with *trfA2* alone. This was true even though similar or even higher levels of TrfA2 were found in the clones producing only TrfA2 than were found for TrfA1 produced from wild-type *trfA* (Fig. 3B, compare the third and first lanes of Western blots). This positive effect of the presence of TrfA1 on PCN was generally consistent with the result obtained for wild-type minireplicon pMS0506 and its derivative pEvo-Sh15 carrying the *trfA1* frameshift variant (Table 2). Therefore, it is likely that the wild-type *trfA* gene of IncP-1β plasmids, which produces TrfA1 and TrfA2 at a certain host-specific ratio, typically generates equal or higher PCNs than the *trfA2* gene, which produces only TrfA2.

When clones producing TrfA1(M124L) alone and TrfA2 alone were compared, a higher PCN was observed for TrfA1(M124L) in three of the four hosts (*E. coli*, *S. japonicum*, and *C. necator*) although the TrfA2 concentration was again higher than that of

TABLE 3 Effect of the presence of TrfA1 on the copy number of mini-IncP-1β plasmids

Plasmid (description)	PCN (relative PCN)^a			
	E. coli	P. putida	S. japonicum	C. necator
pMS0506 (wt)	5.2 ± 0.2 (1.0)	4.6 ± 0.3 (1.0)	3.0 ± 0.3 (1.0)	5.4 ± 0.4 (1.0)
pEvo-Sh15 (trfA1)	1.7 ± 0.1 (0.3)**	2.5 ± 0.2 (0.5)**	0.9 ± 0.1 (0.3)*	1.6 ± 0.4 (0.3)**

^{a} Plasmid copy number (PCN) is represented by the ratio of *kleE* in the plasmids and a gene in the *oriC* region of the chromosomes (*atpB* for *E. coli*, *parB* for *P. putida*, SJA-00140 of chromosome I for *S. japonicum*, and *parB* of chromosome I for *C. necator*) at exponential phase. The values indicate the mean and standard error of the mean obtained from the analyses of four total DNA samples. Relative PCN is the ratio of PCN relative to that for pMS0506. Significance was determined by comparison to pMS0506 (t test): *, $P < 0.01$; **, $P < 0.001$. Total TrfA levels in three of the four hosts under this experimental condition were as follows (in fmol/μg of total protein for pMS0506 and pEvo-Sh15, respectively): 2.2 and <0.4 for *E. coli*; 2.3 and 0.5 for *P. putida*; 53 and 13 for *S. japonicum*.
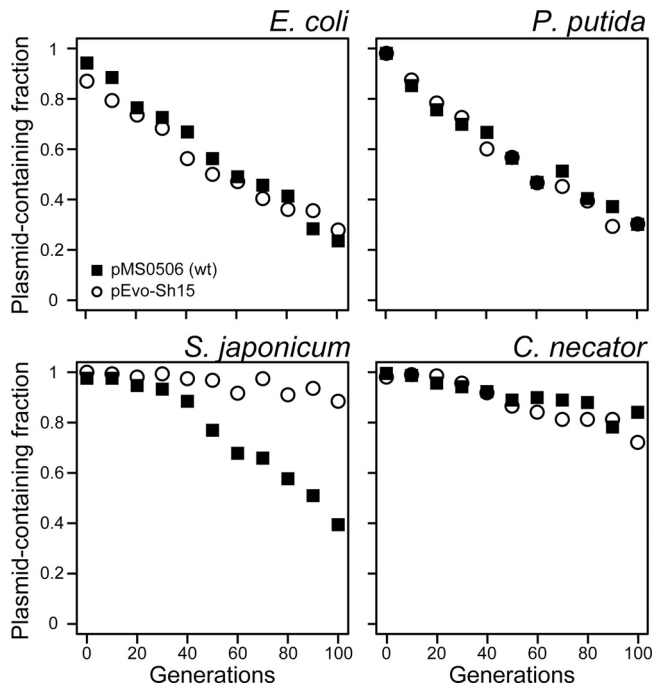
FIG 2 Persistence of pMS0506 and pEvo-Sh15 (the *trfA1* frameshift variant) in four different bacterial hosts. Each data point represents the mean value obtained from four replicate assays for *E. coli*, *S. japonicum*, and *C. necator*, and three assays for *P. putida*.

FIG 3 Plasmid copy numbers in the presence of TrfA1 alone, TrfA2 alone, or both proteins. (A) Experimental design. TrfA proteins were expressed in the presence of 10 μM IPTG from the *tac* promoter inserted at the *att*Tn*7* site in the chromosomes. Plasmid copy number was determined as the ratio of the copy number of *tetA* in the introduced IncP-1β replicon pHY872 to the copy number of a gene in the chromosomal *oriC* region at stationary phase. (B) Plasmid copy numbers and TrfA levels in the four hosts. In the graphs, the value and the error bars indicate the mean and standard error of the mean obtained from at least three replicate experiments. Significance was determined by multiple comparisons (Tukey's test): *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The lower panels show the Western blots of the corresponding TrfA production under each condition. For each strain, the same amount of total protein was loaded in each lane of the SDS-PAGE gel: 6 μg for *E. coli* and *P. putida*, 3 μg for *S. japonicum*, and 25 μg for *C. necator*. Total TrfA levels in three hosts under this experimental condition were as follows (in fmol/μg of total protein, blots from left to right, respectively): 27, 12, and 68 for *E. coli*; 47, <10, and 30 for *P. putida*; 160, 80, and 290 for *S. japonicum*.

TrfA1(M124L) alone in *E. coli* and *S. japonicum*; in *C. necator* the TrfA2 level was lower than the TrfA1(M124L) level (Fig. 3B, compare third to second lanes in Western blots). In *P. putida*, the M124L mutation caused a decrease in the amount of TrfA1(M124L) to levels indistinguishable from the background, and the PCN was concurrently lowest in the presence of TrfA1(M124L) alone (Fig. 3B). In a second *P. putida* clone constructed in an independent experiment, TrfA1(M124L) was also produced at a similarly low level (data not shown), suggesting that poor production of TrfA1(M124L) was not due to a clone-specific DNA rearrangement or mutation in *P. putida*. The reasons for decreased TrfA1 production due to the M124L mutation are currently unclear. Together, these results suggest that when both TrfA1 and TrfA2 are synthesized at detectable levels in four phylogenetically distinct hosts, TrfA1 helps generate a higher PCN than TrfA2 can generate by itself.

Except for *P. putida*, clones carrying wild-type *trfA* produced equal or lower PCNs than clones carrying *trfA1(M124L)* even though the TrfA2 concentrations were much lower than those of TrfA1 (Fig. 3B, left lanes in Western blots). This suggests that a low level of expression of TrfA2 in the presence of a relatively high level of TrfA1 is sufficient to suppress the high PCN generated by TrfA1.

TrfA1 maintains higher plasmid copy number than TrfA2. To clarify the differences in replication control between TrfA1 and TrfA2, the copy number of pHY872 was measured at different concentrations of TrfA1 or TrfA2 in *E. coli*. TrfA proteins were expressed from pBAD24C in the presence of different concentrations of arabinose (Fig. 4A). The TrfA concentration and the PCN (plasmid-to-*oriC* ratio) were determined by quantitative Western blotting and qPCR, respectively. By increasing the concentration
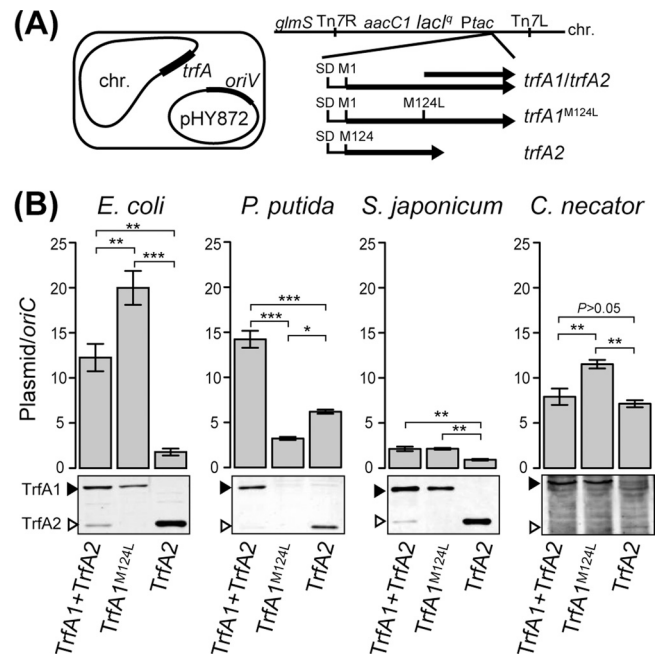
of arabinose used for induction of protein expression, the TrfA1(M124L) concentration increased from 7.4 fmol/μg of total protein, which was approximately equivalent to the TrfA1 level in *E. coli* carrying pMS0506 (7.0 fmol/μg of total protein), to a level more than 400-fold higher. The TrfA2 concentration increased from twice the TrfA1 level in *E. coli* carrying pMS0506 to more than 600-fold the TrfA1 level. With increasing TrfA expression, the PCN decreased from 5.5 to 3.5 copies/*oriC* for TrfA1(M124L) and from 1.5 to 0.8 copies/*oriC* for TrfA2, indicating that overproduction of TrfA negatively affected replication initiation (Fig. 4B). Under these experimental conditions, TrfA1(M124L) maintained a 3- to 4-fold higher PCN than TrfA2 at any concentration of TrfA analyzed. This suggests that plasmid replication mediated by TrfA1 is more efficient than that by TrfA2.

DISCUSSION

Recent isolation and genome sequence analysis of a large number of IncP-1 plasmids have revealed a great diversity in replication initiation proteins encoded by these plasmids. Although the majority of sequenced plasmids encode both TrfA1 and TrfA2 homologs, the IncP-1β plasmid pADP-1 and IncP-1δ plasmids
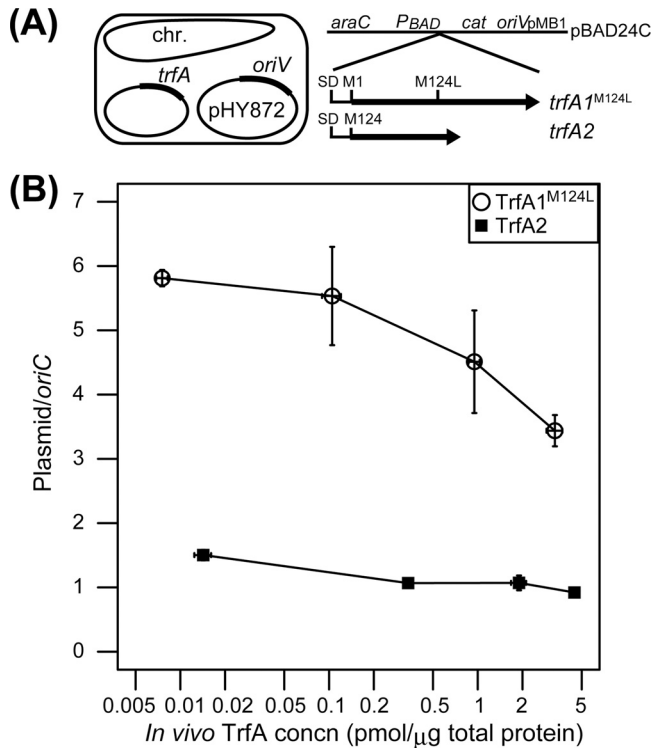
FIG 4 Plasmid copy numbers at different TrfA concentrations in *E. coli*. (A) Experimental design. Expression of TrfA1(M124L) and TrfA2 was induced from the pBAD24C constructs, pHY819 and pHY820, respectively, by addition of arabinose. Plasmid copy number was determined as the ratio of the copy number of *tetA* in the introduced IncP-1β replicon pHY872 to the copy number of a gene in the chromosomal *oriC* region at stationary phase. (B) A plot showing relationship between PCN and TrfA concentrations. Arabinose was added at the following concentrations (%, wt/vol): 0.2×10^{-5}, 2×10^{-4}, and 2×10^{-3} (data points from left to right). Each data point with error bars represents the mean and standard error of the mean obtained from three total DNA samples and more than three quantitative Western blot experiments. Note that the TrfA1(M124L) level without arabinose was equivalent to the TrfA1 level in *E. coli* harboring pMS0506.

pEST1044 and pAKD4 encode TrfA2 alone (44, 53, 68), while the IncP-1γ plasmids encode TrfA1 alone (25; also H. Yano, L. M. Rogers, D. Sen, and E. M. Top, unpublished data) (Fig. 1A). Our study was aimed at investigating the significance of this diversity in the replication initiation protein gene, *trfA*, of IncP-1 plasmids. To address the role of the two Rep proteins in the ability of IncP-1 plasmids to establish themselves and be stably maintained in a wide range of bacteria, we used four model hosts belonging to three different proteobacterial subgroups, the IncP-1β minireplicon pMS0506 and its *trfA1* frameshift variant pEvo-Sh15, as well as systems wherein the TrfA proteins were provided in *trans* to *oriV*.

Several reports have indicated the requirement of the TrfA1 N terminus for delivery and activation of DnaB helicase at *oriV* during replication initiation in *P. aeruginosa* (16, 55, 57). Discovery of this function of the TrfA1 N terminus led to the hypothesis that TrfA1 may be responsible for the wide host range of IncP-1 plasmids (27, 63, 70). However, there are some caveats to the assumption that the long TrfA1 protein is needed to ensure a broad host range for IncP-1 plasmids. As shown in this study, the *trfA1* frameshift variant of mini-pBP136, pEvo-Sh15, was also able to replicate

in bacteria from three proteobacterial subgroups (in not only the four model hosts but also *A. tumefaciens* [*Alphaproteobacteria*], *Burkholderia multivorans* [*Betaproteobacteria*], and *P. fluorescens* [*Gammaproteobacteria*]) (data not shown). Moreover, the stability of pEvo-Sh15 was equivalent to or higher than that of the wild-type plasmid in spite of decreased plasmid copy number (Fig. 2). Similarly, a *trfA-44* null mutant of RK2 was previously shown to replicate in hosts currently classified as *Alphaproteobacteria* and *Gammaproteobacteria* (16). Thus, TrfA1 seems to be required for IncP-1 plasmid replication in only a limited number of bacterial species. The TrfA2 domain and the intact replication origin contain all the elements necessary for an IncP-1 plasmid to have a broad host range. Consistent with this idea is the similarity in secondary structure of the Rep protein of the broad-host-range IncW plasmid R388 (18) to TrfA2 rather than TrfA1 (data not shown).

To address the role of TrfA1, we analyzed two of the most fundamental plasmid traits, infection rate (transformation efficiency) and plasmid persistence, using pMS0506 (wild type) and pEvo-Sh15 (*trfA1*). First, differences in transformation efficiency were observed for most hosts analyzed (Table 2). This comparative analysis is analogous to an earlier study that used mini-RK2 and its *trfA-44* frameshift variant (55). In that study, however, no differences in infection rates (transformation and conjugative transfer) between the two plasmids were observed in either *E. coli* or *P. putida* (55). Therefore, the role of the long TrfA protein in these and other hosts may be different for IncP-1α and IncP-1β plasmids. Here, we showed that TrfA1 seems to improve the plasmid's initial establishment efficiency even though TrfA2 is sufficient for broad-host-range replication.

Generally, high transformation efficiency correlates with high PCN (19, 42, 69) although this does not seem to be true for all iteron-containing plasmids (1). Plasmid pMS0506, indeed, showed a higher copy number than pEvo-Sh15 in all hosts tested (Table 3). This suggested that the coexpression of TrfA1 and TrfA2 ensures more efficient replication than the expression of TrfA2 alone. This result was confirmed in experiments using strains carrying *trfA* genes under a common promoter in the chromosome. Except for *C. necator*, hosts expressing both TrfA1 and TrfA2 produced a higher PCN than hosts expressing TrfA2 alone even though the TrfA2 level under the TrfA2-alone condition was as high as the TrfA1 level under the TrfA1-plus-TrfA2 condition. These findings suggest that the naturally occurring IncP-1 plasmids pADP-1 (44), pEST1044 (68), and pAKD4 (53), which do not encode the longer TrfA1, may exhibit lower PCNs than other IncP-1 plasmids encoding both Rep proteins. Under some environmental conditions, a low plasmid copy number may be advantageous for large plasmids encoding catabolic pathways like pADP-1 and pEST4011 because of a possibly high cost of maintaining more plasmid copies. Future studies should determine if the loss of TrfA1 production in these natural plasmids resulted in lower copy numbers than for other IncP-1 plasmids and, if so, if that in turn decreased the plasmid cost.

To eliminate TrfA2 production, we introduced the M124L mutation in the *trfA1* gene, which was equivalent to the M98L mutation in *trfA-44* of RK2 (17). It was previously demonstrated that the M98L mutation did not affect replication efficiency *in vitro* (17, 31). In our study, the equivalent M124L mutation reduced TrfA1 levels in most hosts analyzed (Fig. 3B). This was most striking in *P. putida* even though the introduced codon (CTG) is fre-

quently used in *P. putida.* It has been reported that even a neutral mutation in the *trfA-33* region of RK2 reduced both TrfA levels and replication efficiency in a temperature-dependent and host-specific manner, possibly by affecting local mRNA structure and translational efficiency (30). It is currently unclear how introduction of CTG to create the M124L mutation in *trfA1* affected the mRNA structure. Another possible explanation for reduced TrfA1 levels is the potential requirement for TrfA2 in the cell in mediating stability or synthesis of TrfA1. However, contrary to our expectations, supplying TrfA2 using an additional expression plasmid vector in the *P. putida* strain carrying *trfA1*(M124L) in the chromosome did not significantly improve the TrfA1(M124L) level (data not shown). Interestingly, Fang and Helinski (17) observed a decrease in copy number of RK2 in *E. coli* upon introduction of the M98L mutation, and their results were also consistent with a reduction in the total TrfA levels. At this point we can assume that either elimination of TrfA2 expression from the same molecule or potential changes in the mRNA or protein structure of TrfA1 negatively affected the TrfA1 level, thus resulting in a lower PCN in *P. putida.* Recently, TrfA stability *in vivo* was shown to increase in a Lon protease-deficient host (38). The TrfA2-TrfA1 interaction may increase the stability of TrfA1 by protecting it from such proteases.

Even though the TrfA1(M124L) level was lower than the TrfA2 level, a higher PCN was observed in three of the four hosts when TrfA1(M124L) alone was expressed (Fig. 3). This suggests that replication control by TrfA1 is generally more relaxed than that by TrfA2. We confirmed this for *E. coli* by comparing PCNs at a range of concentrations that were similar for TrfA1 and TrfA2 (Fig. 4B). A 30% (TrfA2) or 40% [TrfA1(M124L)] decrease in the PCN of the IncP-1β replicon was observed when the TrfA level was increased to more than 45-fold (TrfA2) or 440-fold [TrfA1(M124L)] of the normal TrfA1 level of pMS0506. Replication inhibition by overproduction of the Rep protein (above the normal level) could be common throughout iteron-containing plasmids since similar conclusions were previously obtained for P1 replication and R6K γ-origin replication (13, 21).

It was unexpected that the PCNs were higher in strains wherein TrfA, especially TrfA1, was expressed from the chromosome (Fig. 3) than when it was expressed from the plasmid (Fig. 4). We note that the levels of expressed TrfA1 and TrfA2 shown in Fig. 3 (12 to 68 fmol/μg of total protein in *E. coli*) were in the range shown in Fig. 4, and the same copy number reference plasmid was used to generate a standard curve in qPCR in both experiments. This suggests that an additional PCN-limiting factor other than the TrfA level influenced the results in the two different experimental systems. The PCN-limiting factor involved in these experiments is currently unresolved.

Replication initiation proteins of iteron-containing plasmids mediate both activation and inhibition of replication initiation to keep the PCN constant (12). Several mechanisms could be involved in the observed differences in PCN generated by TrfA1 and TrfA2; we describe the three major ones below. The first mechanism is related to the process that generates the active form of TrfA. Both *in vivo* and *in vitro*, TrfA is present mostly as a dimer that cannot bind to iterons efficiently (64, 65). The active monomer form of TrfA, which binds to an iteron with high affinity and mediates *oriV* melting, can be generated by the DnaK/DnaJ/GrpE chaperone during protein synthesis or by conversion from inactive dimers by the protein chaperone ClpX or the ClpB/DnaK/

DnaJ/GrpE bichaperone system (34, 36). These protein chaperones may act on inactive molecules of TrfA1 and TrfA2 with different efficiencies. Alternatively, the active fraction of TrfA1 may be higher than that of TrfA2 even without protein chaperones.

A second possible mechanism is the DnaB helicase loading and activation after *oriV* melting. TrfA-44 interacts with DnaB more efficiently than TrfA-33 at *oriV*, depending on the source of the DnaB (27, 70). In the case of pPS10, the increased interaction of RepA mutants with DnaA resulted in an increased PCN in *E. coli* (42, 43). In *E. coli*, DnaA is required in the process of DnaB loading to *oriV* during RK2 replication (35). Thus, it is possible that the difference in the interactions of TrfA1 versus TrfA2 with host proteins such as DnaA and DnaB affected efficiency in the DnaB loading and activation and resulted in the difference in PCN.

A third mechanism that may underlie the difference in PCNs between TrfA1 and TrfA2 is the inhibition of *oriV* melting by blocking the replication origin by TrfA dimers that bridge iteron clusters on separate molecules (so-called handcuffing) (31) or iteron clusters on the same molecule (71). Although the polypeptides involved in the TrfA dimer formation seem to be localized in the C-terminal region of TrfA2 (9, 64), it is unclear how TrfA monomers and dimers interact in the replication inhibition process. The presence of the N-terminal extension in TrfA1 may affect the efficiency of inhibition through monomer-monomer or monomer-dimer interactions. Future work will address these possibilities.

In addition to efficient plasmid establishment and replication in new hosts, plasmid stability is an important factor that affects the fate of a plasmid in natural bacterial communities. In three of the four hosts, no difference was observed in levels of plasmid stability between pMS0506 and pEvo-Sh15, yet in *S. japonicum*, pEvo-Sh15 was more stable than pMS0506. Thus, elimination of TrfA1 was beneficial for plasmid persistence in an *S. japonicum* population, in spite of the reduced PCN. It is likely that as long as the plasmids carry functional partitioning genes, an increase in PCN greater than 1 to 2 copies/*oriC* does not necessarily improve plasmid persistence. Greater plasmid stability of the pEvo-Sh15 than pMS0506 was also previously observed by us in the gamma-proteobacterium *Shewanella oneidensis* MR-1, and in that host the copy numbers of both plasmids were indistinguishable (57). Factors other than segregational plasmid loss may limit plasmid persistence, such as fitness cost of the plasmid and low transferability (58). Since our model plasmid is conjugation deficient, we hypothesize that the low stability of pMS0506 compared to pEvo-Sh15 is due to a high fitness cost of wild-type TrfA1 in some hosts. One possible molecular mechanism is the titration of DnaB and DnaA by the TrfA1 N terminus. A second is related to the unstructured (disordered) nature of the N terminus that has been suggested by bioinformatic analysis (57, 70). Proteins carrying intrinsically unstructured regions are generally sensitive to proteolysis (23, 62), which seems to be consistent with our observation of the low TrfA1 level in the absence of TrfA2 in *P. putida* (Fig. 3). Expression of unstructured proteins is tightly controlled in eukaryotic systems, which implies that it imposes a high cost to the cells (23). It is therefore possible that a high rate of degradation of TrfA1 or the nature of structural disorder itself negatively affects host fitness, thereby resulting in occasional poor plasmid stability of plasmids encoding TrfA1. These hypotheses obviously need to be tested in future studies. In sum, while the molecular mecha-

nism has not yet been elucidated, the negative effect of TrfA1 on long-term persistence of an IncP-1β plasmid has now been demonstrated in members of two different proteobacterial subgroups (*Alphaproteobacteria* and *Gammaproteobacteria*). These findings suggest that IncP-1 plasmid persistence does not always benefit from encoding the long TrfA1 protein.

In conclusion, we presented evidence that the IncP-1β plasmid-encoded TrfA1 protein participates in plasmid replication in a broad range of hosts but is not essential for replication in these hosts. However, in natural, highly diverse bacterial communities, the relaxed replication control mediated by TrfA1 could play an important role in the efficient establishment of theta replication in a broad range of hosts to which the plasmids transfer. Thus, in spite of the poor stability of plasmids encoding TrfA1 in some hosts, the promiscuous nature of IncP-1 plasmids may present strong enough selective pressure for plasmids to maintain the rather unique genetic system of a *trfA* locus that produces two replication initiation proteins.

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