Novel Toxin-Antitoxin System Composed of Serine Protease and AAA-ATPase Homologues Determines the High Level of Stability and Incompatibility of the Tumor-Inducing Plasmid pTiC58^{∇†}

Shinji Yamamoto, Kazuya Kiyokawa, Katsuyuki Tanaka, Kazuki Moriguchi, and Katsunori Suzuki*

Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8526, Japan

Received 30 January 2009/Accepted 9 May 2009

Stability of plant tumor-inducing (Ti) plasmids differs among strains. A high level of stability prevents basic and applied studies including the development of useful strains. The nopaline type Ti plasmid pTiC58 significantly reduces the transconjugant efficiency for incoming incompatible plasmids relative to the other type, such as octopine-type plasmids. In this study we identified a region that increases the incompatibility and stability of the plasmid. This region was located on a 4.3-kbp segment about 38 kbp downstream of the replication locus, *repABC*. We named two open reading frames in the segment, *ietA* and *ietS*, both of which were essential for the high level of incompatibility and stability. Plasmid stabilization by *ietAS* was accomplished by a toxin-antitoxin (TA) mechanism, where *IetS* is the toxin and *IetA* is the antitoxin. A database search revealed that putative *IetA* and *IetS* proteins are highly similar to AAA-ATPases and subtilisin-like serine proteases, respectively. Amino acid substitution experiments in each of the highly conserved characteristic residues, in both putative enzymes, suggested that the protease activity is essential and that ATP binding activity is important for the operation of the TA system. The *ietAS*-containing *repABC* plasmids expelled Ti plasmids even in strains which were tolerant to conventional Ti-curing treatments.

Agrobacterium tumefaciens strains bearing a tumor-inducing (Ti) plasmid are the etiological agents of crown gall disease. Most genes required for pathogenicity are located on the plasmids (17, 33). Ti plasmids are kept stable at a low copy number equivalent to that of the chromosomal DNA in the bacterial cells (32) due to the *repABC* locus (16, 30, 34). The stability of Ti plasmids differs among strains (11).

Many genes for keeping plasmids stable have been reported in eubacteria, and these are divided into three categories based on their mechanism: multimer resolution systems, active partitioning systems, and toxin-antitoxin (TA) systems (15). Multimer resolution systems increase the number of plasmid molecules by resolving a multimer plasmid into monomers, resulting in a higher probability of plasmid distribution to daughter cells during cell division even when plasmid distribution occurs randomly (29). Active partitioning systems deliver plasmid copies to each progeny cell at cell division (21). In the *repABC* locus, the RepA and RepB proteins and *parS* site(s) ensure stable plasmid inheritance by the active partitioning system (2). TA systems contribute to plasmid maintenance in cell populations by initiating growth inhibition or death of plasmid-free cells and are widely distributed among eubacterial and archaeal plasmids as well as their chromosomes (9). Generally, the TA module consists of two genes which encode toxin and antitoxin. The antitoxin neutralizes the action of a

cognate toxin by interaction with the toxin or its target molecules. When a plasmid harboring the TA module is lost from a host cell, the antitoxin molecules decrease to an ineffective level because the antitoxin is degraded quickly or diluted by cell division (15). Thereafter, the toxin exerts its toxicity and inhibits the host cell growth. RNA antitoxins can suppress toxin expression by binding to the toxin mRNA as an antisense RNA or repress toxicity effects by an unknown mechanism (6, 4). In pTi-SAKURA, the Ti plasmid in the *A. tumefaciens* strain MAFF301001, it was shown that the *tiorf24* and *tiorf25* module increased plasmid stability by the TA mechanism (40; also S. Yamamoto, unpublished data).

Differences in Ti plasmid stability are critical for plasmid engineering and evolution (33). However, little is known about the stability factors of Ti plasmids other than the *repABC* locus. In our previous study (40), *tiorf24* and *tiorf25* were shown to increase the segregational stability and incompatibility of Ti plasmids and reduce the efficiency of transconjugants by the introduction of incompatible plasmids into host cells. The two genes are located 2.5-kbp downstream of *repABC* (8). Generally, incompatibility has been defined as a situation where two plasmids contain a related replication and/or partitioning system and are unable to exist in a cell simultaneously without external selection (1). *A. tumefaciens* strain C58, which contains a Ti plasmid pTiC58, allows entry of an incompatible *repABC* plasmid into the cell 60-fold less efficiently than a derivative of C58. The derivative of C58 harbors a small *repABC* vector instead of pTiC58 (40). This suggests the presence of incompatibility-enhancing genes on pTiC58.

In this study, we located the responsible genes in pTiC58 and found that the novel genes *ietA* and *ietS* enhance the incompatibility and stability of the plasmid by the TA mechanism.

* Corresponding author. Mailing address: Department of Biological Science, Graduate School of Science, Hiroshima University, Higashi Hiroshima 739-8526, Japan. Phone: 81 82 424 7455. Fax: 81 82 424 0734. E-mail: ksuzuki@hiroshima-u.ac.jp.

† Supplemental material for this article may be found at <http://jbb.asm.org/>.

[∇] Published ahead of print on 15 May 2009.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *A. tumefaciens* (biovar 1) and *Escherichia coli* strains were cultured at 28°C and 37°C in LB medium (22), respectively. The *Agrobacterium rhizogenes* (biovar 2) strains were cultured at 28°C in IFO medium 702 (35) (0.5% [wt/vol] polypeptone, 0.2% yeast extract, 0.01% MgSO₄, pH 7.0). MG/L (mannitol glutamate-Luria salts) medium (38) was used for protein induction experiments in *A. tumefaciens* cells. When necessary, antibiotics were added to the medium at the following final concentrations: gentamicin (45 µg/ml), kanamycin (50 µg/ml), rifampin (40 µg/ml), nalidixic acid (30 µg/ml), and ampicillin (50 µg/ml). *A. tumefaciens* strains were also grown on AB minimal salts medium (3) supplemented with glucose (ABG) or sucrose (ABS) at a final concentration of 0.5% as the sole source of carbon.

Plasmid construction and DNA preparation. *E. coli* S17-1 λ pir (20, 27) was used as a host strain for plasmid construction and as a donor strain for conjugal transfer of mobilizable plasmids.

DNA manipulations for plasmid construction were performed using standard procedures (22). Plasmids were prepared as described in the following paragraphs. All structural configurations of plasmids were confirmed by sequencing or polymorphism of appropriate restriction enzyme digestion.

Deletion derivatives of pTiC58. A series of deletion derivatives of pTiC58 labeled as pTiABC, pTiACD, pTiADE, pTiAEA, pTiABC1, pTiABC2, pTiABC3, pTiABC4, pTiABC5, and pTiABC6 were produced by a double crossover with each deletion-inducing plasmid. The deletion-inducing plasmids contain a Gm^r cassette sandwiched between two short fragments. One of the two fragments is located immediately to the left, and the other is located immediately to the right of the target region for deletion of pTiC58. Therefore, each Ti plasmid variant has the Gm^r gene instead of the deleted region. The two fragments, left and right, were amplified by PCR using the primers listed in Table 2. The average distance between a pair of short fragments in pTiC58 was about 48 kbp (in a large deletion) and 7 kbp (in a small deletion). In the deletion-inducing plasmids used for the construction of pTiABC, pTiACD, pTiADE, and pTiAEA, left and right fragments were digested by XbaI before being inserted into the XbaI and NheI sites of pK18mobsacB, respectively. After the two fragments were cloned, each recombinant plasmid was cleaved with PstI at a unique restriction site located between the two fragments, and the 2.9-kbp PstI fragment containing the Gm^r gene from pUC19Gm was inserted into the site. In the deletion-inducing plasmids for the production of pTiABC1, pTiABC2, pTiABC3, pTiABC4, pTiABC5, and pTiABC6, left and right fragments were digested by XbaI or SpeI before being inserted into the XbaI and NheI sites of pK18msg. pK18msg was made by inserting the 2.6-kbp NheI-PstI fragment containing the Gm^r gene from pUC19Gm into pK18mobsacB between the NheI and PstI sites.

Cloning of *iet* genes and introduction of a mutation in *iet* genes. pK18fr5 was made by insertion of a 9.3-kbp XbaI fragment of pTiC58 into an XbaI site of pK18mobsacB. pK18fr5rep (Fig. 1C) was assembled by insertion of a 5.1-kbp XbaI-NheI *repABC*-bearing fragment of pK18msr into a unique NheI site of pK18fr5. pK18fr5LFrep was produced by insertion of a 6.0-kbp NheI-XbaI fragment containing three complete open reading frames (ORFs) (atu6082 to atu6084) of pK18fr5rep into a unique NheI site of pK18msr. pK18SalDrep and pK18SphDrep were constructed by digesting pK18fr5LFrep with SalI and SphI, respectively, followed by self-ligation and insertion of a 5.1-kbp XbaI-NheI *repABC*-containing fragment into the NheI site. pK18fr5LFrep was digested with SalI and then self-ligated, resulting in the plasmid pK18SalD. pK18SDI was prepared by insertion of a Gm^r-containing PstI fragment of pUC19Gm into a unique PstI site of pK18SalD. pTi6082::Gm^r was made by a double crossover between pTiC58 and the disruption-inducing plasmid pK18SDI. pK186083 was created by insertion of a 2.8-kbp fragment containing *ietS* into pK18msr. pJNLf and pJNSph were prepared by insertion of an EcoRI-SalI fragment containing *ietAS* of pK18SalDrep and an EcoRI-SalI fragment containing *ietA* of pK18SphDrep into pJN105 (19), respectively. A frameshift mutant *ietA* was made by digestion of a unique PstI site situated in the *ietA* gene on pK18SalD, followed by trimming of the four-nucleotide protruding ends and self-ligation. pSPDR was constructed by replacing a 4.3-kbp *ietAS*-containing fragment of pK18SalDrep with a fragment containing the frameshift mutant *ietA*. pK18SCat was assembled by insertion of a 2.6-kbp NheI fragment containing *tiorf24* and *tiorf25* of pTi-SAKURA into a unique NheI site of pK18SalDrep.

Plasmids for controlled expression of *ietS*. pSRKietS was constructed by insertion of a 2.5-kbp NdeI-XbaI *ietS*-bearing fragment into pSRKKm between unique NdeI and XbaI sites. The 2.5-kbp fragment was prepared by NdeI and XbaI digestion of the *ietS* fragment amplified by PCR from pK18SalD. The oligonucleotide primers used were IetS-NdeATG and IetS-Hisx6 (Table 3). The forward and reverse primers contained an NdeI and an XbaI site, respectively.

The start codon of *ietS* in the pSRKietS was located at the same position as that of *lacZα* in the pSRKKm vector. pMWR was created by insertion of a 4.2-kbp BamHI *repABC* fragment of pRi1724 into the unique BamHI site of pMW109. The *repABC*-containing fragment was made by BamHI digestion of a *repABC*-harboring PCR fragment amplified from pRi1724 using oligonucleotide primers pRi-rep-L+Bam and pRi-rep-R+Bam, which contained BamHI sites at their 5' ends (Table 3). pMWRGietA was assembled by a combination of a 3.2-kbp fragment containing the *ietA* and the Gm^r genes with the XbaI-ScaI large fragment of pMWR. The 3.2-kbp fragment was prepared by XbaI digestion of a PCR fragment amplified from pJNSph using oligonucleotide primers pJNGmfw and pietAfwRv (Table 3).

A series of amino acid substitution mutant plasmids. p99R, p101R, p103R, p105R, p107R, and p109R were constructed by replacing a 4.3-kbp *ietAS*-harboring fragment of pK18SalDrep with the same fragment containing each of the mutations. The mutations were the following: K153N (AAG to AAC) in p99R, E206Q (GAG to CAG) in p101R, R260L (CGG to CTG) in p103R, D297A (GAT to GCT) in p105R, H336A (CAT to GCT) in p107R, and S566A (AGT to GCT) in p109R. Site-directed mutagenesis for creation of the amino acid substitutions in IetA and IetS was performed by the PCR method (10) using the mutagenic oligonucleotides shown in Table 3. The 4.3-kbp EcoRI-SalI fragment containing *ietAS* was excised from pK18SalD and then inserted into the corresponding site of pUC19. The resulting plasmid pUCSalD was used as a template for PCR, which was carried out using KOD-plus DNA polymerase as described in the manufacturer's instructions (Toyobo, Osaka, Japan). A mutagenized *ietAS* segment in the in vitro synthesized DNA was cut out by digestion with EcoRI and SalI. After the wild-type *ietAS* fragment was replaced in pK18SalD with each of the mutagenized fragments, the 5.1-kbp XbaI-NheI *repABC* fragment from pK18msr was inserted into a unique NheI site of the mutagenized recombinant plasmids. The correctness of the sequences of the *ietAS* genes, except the targeted point mutations, was confirmed by sequencing experiments.

Conjugation assay. Plasmids were delivered from *E. coli* to *Agrobacterium* strains by conjugal transfer as described elsewhere (36). Conjugation efficiency was calculated by dividing the number of transconjugant colonies by the number of input donors. An ejection ratio of Ti plasmids was calculated as the percentage of colonies that lost the resident Ti plasmid after conjugation (40). In order to obtain an ejection ratio, 100 transconjugant colonies were checked for the presence or absence of the Ti plasmid; our method utilized colony hybridization using the *virB* gene fragment as a probe, as previously described (40).

Segregational stability assay using counter-selection by the *sacB* gene. *A. tumefaciens* C58C1 cells harboring a *sacB*-containing plasmid were grown to $\sim 1 \times 10^8$ cells ml⁻¹ in LB medium. Approximately 1×10^8 cells were collected and washed with 0.9% (wt/vol) NaCl. The washed cells were suspended and diluted in 0.9% NaCl. Subsequently, the suspension was spread onto ABG and ABS agar plates. After incubation at 28°C for 2 days, colony numbers were counted. A CFU ratio was calculated by dividing the number of colonies on ABS plates by the number of colonies on ABG plates.

Homology search and multiple sequence alignment. Searches for protein homologs in databases were carried out using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the nonredundant sequence databases. All protein alignments were performed by the CLUSTALW program (<http://clustalw.ddbj.nig.ac.jp/top-j.html>).

RESULTS

Localization of potential incompatibility-enhancing locus on pTiC58. Our previous study (40) implied that pTiC58 contained a locus which enhanced the incompatibility of the plasmid.

To localize the incompatibility-enhancing region, we performed extensive deletion and subcloning analyses of pTiC58. Four large regions, which cover the entire pTiC58 plasmid, were deleted (Fig. 1A), except for the replicator locus *repABC*. The incompatible *repABC* plasmid pK18msr was introduced via conjugation into the wild-type C58 strain (C58rif) and the C58C1 cells that contained any one of the four mutant pTiC58 derivatives. Cells having pTiABC were transformed by pK18msr at about a 20-fold-higher frequency than cells having wild-type pTiC58 or other deletion derivatives (Fig. 1A). This

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
<i>A. tumefaciens</i> strains		
C58	Wild-type pathogenic strain carrying pTiC58; biovar 1	7
C58rif	Wild-type pathogenic strain carrying pTiC58; Rif ^r	Our collection
C58C1	pTiC58-less and Rif ^r derivative of the wild-type strain C58; Rif ^r	Our collection
<i>A. rhizogenes</i> strains		
Ch-Ag-2	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
Ch-Ag-6	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
Ch-Ag-10	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
Ch-Ag-14	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
Pch-Ag-2	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
Pch-Ag-4	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
Pl-Ag-1	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
Ro-Ag-10	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
Ro-Ag-13	Biovar 2 strain carrying Ti plasmid; Nal ^r	23
<i>E. coli</i> strain		
S17-1 λ pir	Host strain for recombinant plasmid construction and conjugal transfer; <i>recA</i> Sm ^r λ pir	27
Plasmids		
pTiC58	Wild-type Ti plasmid (214,233 bp) of <i>A. tumefaciens</i> C58; IncRh1	7
pTiABC	pTiC58 with a deletion of 52.1-kbp (nt 59428 to 111574); Gm ^r	This study
pTiACD	pTiC58 with a deletion of 40.4-kbp (nt 114891 to 155335); Gm ^r	This study
pTiADE	pTiC58 with a deletion of 50.4-kbp (nt 158464 to 208859); Gm ^r	This study
pTiAEA	pTiC58 with a deletion of 50.0-kbp (nt 212167 to 47919); Gm ^r	This study
pTiABC1	pTiC58 with a deletion of 7.4-kbp (nt 59428 to 66787); Gm ^r	This study
pTiABC2	pTiC58 with a deletion of 7.4-kbp (nt 68829 to 76271); Gm ^r	This study
pTiABC3	pTiC58 with a deletion of 5.7-kbp (nt 78298 to 84010); Gm ^r	This study
pTiABC4	pTiC58 with a deletion of 8.1-kbp (nt 86156 to 94269); Gm ^r	This study
pTiABC5	pTiC58 with a deletion of 5.2-kbp (nt 96382 to 101612); Gm ^r	This study
pTiABC6	pTiC58 with a deletion of 8.0-kbp (nt 103559 to 111574); Gm ^r	This study
pTi6082::Gm	pTiC58 with <i>ietA</i> ::Gm ^r mutation; Gm ^r	This study
pTi-SAKURA	Ti plasmid of <i>A. tumefaciens</i> MAFF301001	31
pK18mobsacB	Mobilizable cloning vector; ColE1-ori ^V <i>mob sacB</i> (sucrose ^s) Km ^r	24
pK18msg	pK18mobsacB with Gm ^r gene of pHRP311; Gm ^r Km ^r	This study
pK18msr	pK18mobsacB with <i>repABC</i> of pTi-SAKURA; Km ^r	40
pTG18msr	pK18msr with Tc ^r (<i>tetC</i>) and Gm ^r genes instead of Km ^r gene; Gm ^r Tc ^r	Our collection
pK18fr5rep	pK18msr with 9.3-kbp segment of pTiC58 (nt 94270 to 103558); Km ^r	This study
pK18fr5LFrep	pK18msr with 6.0-kbp segment of pTiC58 (nt 97533 to 103558); Km ^r	This study
pK18SalD	pK18mobsacB with 4.3-kbp segment of pTiC58 (nt 97533 to 101803); Km ^r	This study
pK18SDI	pK18SalD contains the Gm ^r cassette inserted in a unique PstI site situated within <i>ietA</i> open reading frame, Km ^r Gm ^r	This study
pK18SalDrep	pK18msr with 4.3-kbp segment of pTiC58 (nt 97533 to 101803); Km ^r	This study
pSPDR	pK18SalDrep with frameshift mutation of <i>ietA</i> by 4-bp deletion at nt 138 to 141; Km ^r	This study
pK18SphDrep	pK18msr with 1.5-kbp <i>ietA</i> segment of pTiC58 (nt 97533 to 98989); Km ^r	This study
pK186083	pK18msr with 2.8-kbp <i>ietS</i> segment of pTiC58 (nt 98691 to 101464); Km ^r	This study
pMWR	pMW109 with 4.2-kbp <i>repABC</i> segment of pRi1724; Ap ^r	This study
pMWRGietA	pMWR with 3.2-kbp <i>ietA</i> and Gm ^r segment of pJNSph instead of <i>P_{lac}</i> and Ap ^r gene; Gm ^r	This study
p99R	pK18SalDrep with an amino acid substitution in Walker motif A of IetA (K153N); Km ^r	This study
p101R	pK18SalDrep with an amino acid substitution in Walker motif B of IetA (E206Q); Km ^r	This study
p103R	pK18SalDrep with an amino acid substitution in SRH of IetA (R260L); Km ^r	This study
p105R	pK18SalDrep with an amino acid substitution at catalytic triad D of IetS (D297A); Km ^r	This study
p107R	pK18SalDrep with an amino acid substitution at catalytic triad H of IetS (H336A); Km ^r	This study
p109R	pK18SalDrep with an amino acid substitution at catalytic triad S of IetS (S566A); Km ^r	This study
pK18SCat	pK18SalDrep with 2.6-kbp fragment containing <i>tiorf24</i> and <i>tiorf25</i> of pTi-SAKURA; Km ^r	This study
pUC19Gm	pUC19 with 2.9-kbp PstI fragment containing Gm ^r gene cassette; Gm ^r Ap ^r	Our collection
pUCSalD	pUC19 with 4.3-kbp <i>ietAS</i> fragment; Ap ^r	This study
pJN105	Mobilizable cloning vector; pBBR1-ori <i>araC mob</i> Gm ^r	19
pJNLF	pJN105 with 4.3-kbp segment of pTiC58 (nt 97533 to 101803); Gm ^r	This study
pJNSph	pJN105 with 1.5-kbp segment of pTiC58 (nt 97533 to 98989); Gm ^r	This study
pSRKKm	pBBR expression vector; pBBR1-ori Km ^r	14
pSRKietS	pSRKKm with 2.5-kbp <i>ietS</i> segment under <i>lac</i> promoter; pBBR1-ori Km ^r	This study
pBHR1	Mobilizable pBBR plasmid; pBBR1-ori Km ^r	Purchased from Funakoshi Co., Ltd.

^a For the plasmids, nucleotide (nt) coordinate numbers indicate the position of the fragment in pTiC58 (accession no. NC_003065).

TABLE 2. Oligonucleotide primers used for constructing deletion-inducing plasmids

Constructed pTiC58 variant ^a	Fragment and direction ^b	Sequence ^c
pTiΔBC (pDI-BC)	L, forward L, reverse R, forward R, reverse	GCTCTAGATCGATCAGGTTCTTGACGGAC GCTCTAGAAAGGGTCAGCGACTTCAGACG GCTCTAGATGGTGGATCGCAATTCAGTGG GCTCTAGATTCCACTGGCCTCTCCTGTAT
pTiΔCD (pDI-CD)	L, forward L, reverse R, forward R, reverse	GCTCTAGATGGTGGATCGCAATTCAGTGG GCTCTAGATTCCACTGGCCTCTCCTGTAT GCTCTAGAGTGAGCAACCGAGACGATATG GCTCTAGACGACATTGCTGATTGCATTGAC
pTiΔDE (pDI-DE)	L, forward L, reverse R, forward R, reverse	GCTCTAGAGTGAGCAACCGAGACGATATG GCTCTAGACGACATTGCTGATTGCATTGAC GCTCTAGAACGAGCACCAAGCATAACGTTTC GCTCTAGAGGTCCATCTCTCGATGTCCA
pTiΔEA (pDI-EA)	L, forward L, reverse R, forward R, reverse	GCTCTAGAACGAGCACCAAGCATAACGTTTC GCTCTAGAGGTCCATCTCTCGATGTCCA GCTCTAGAGACAAGTGTCTCGATCCACTC GCTCTAGACAGAAGTTATCATTGGCAGCG
pTiΔBC1 (pDI-BC1)	L, forward L, reverse R, forward R, reverse	GGACTAGTTTTTCATCGATGTCACGTCCATCG GGACTAGTCATTCAAGAACTCGAACACCTG GCGTGGCTTTGGCTAGGAGAAC GGACTAGTTTGGCATCCAGGAAATGAGAGC
pTiΔBC2 (pDI-BC2)	L, forward L, reverse R, forward R, reverse	GCTCTAGAATCAAGGTGGGACAGACCGAG GGACTAGTTTGGCATCCAGGAAATGAGAGC GCTCTAGACTGACGTCCTACCATCCCATCG GCTCTAGACGAATGACATGGCGATCTTTGC
pTiΔBC3 (pDI-BC3)	L, forward L, reverse R, forward R, reverse	GCTCTAGACTGACGTCCTACCATCCCATCG GCTCTAGACGAATGACATGGCGATCTTTGC GCTCTAGAGCATCGTTGATGATGGTGTTCC GCTCTAGAACGAACCATCCAACGCCTGGAC
pTiΔBC4 (pDI-BC4)	L, forward L, reverse R, forward R, reverse	GCTCTAGAGCATCGTTGATGATGGTGTTCC GCTCTAGAACGAACCATCCAACGCCTGGAC GCTCTAGAGTCCAAAGACGATGTCTGAAGC GCTCTAGACACATCTCCAATCGTGAGATCGA
pTiΔBC5 (pDI-BC5)	L, forward L, reverse R, forward R, reverse	GCTCTAGAGTCCAAAGACGATGTCTGAAGC GCTCTAGACACATCTCCAATCGTGAGATCGA GCTCTAGAAGTCCCTTGAGGCTTCTTTTCG GCTCTAGACGTGATGGCGTCTTCCTTGAG
pTiΔBC6 (pDI-BC6)	L, forward L, reverse R, forward R, reverse	GCTCTAGAAGTCCCTTGAGGCTTCTTTTCG GCTCTAGACGTGATGGCGTCTTCCTTGAG GCTCTAGATGGTGGATCGCAATTCAGTGG GCTCTAGATTCCACTGGCCTCTCCTGTAT

^a The deletion in the pTiC58 variant was induced by the deletion-inducing plasmid.^b L, left fragment; R, right fragment. See Materials and Methods for details.^c Italic letters represent the artificially added restriction sites for XbaI (TCTAGA) and SpeI (ACTAGT).

result indicates that the 52-kbp region, which is lacking in pTiΔBC, includes the incompatibility-enhancing region.

The 52-kbp fragment was divided into six segments, all of which were deleted from pTiC58 (Fig. 1B). Cells possessing the mutant plasmid pTiΔBC5 showed an apparently high level of transconjugant efficiency that was comparable to that of cells with pTiΔBC (Fig. 1B). This result indicates that the incompatibility-enhancing region is located on a 5.2-kbp segment that is absent in pTiΔBC5 or its neighboring loci. The 5.2-kbp segment contained three complete ORFs (atu6081 to atu6083) and one incomplete ORF (atu6080) of pTiC58, according to the accessed NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov>; accession no. NC_003065).

In order to determine which ORFs on the 5.2-kbp segment

and its overlapping regions are responsible for the incompatibility-enhancing effect, we examined the introduction efficiency of the incompatible plasmid (pTG18msr) into the C58C1 cells possessing subclones, as shown in Fig. 1C. pK18SalDrep reduced the transconjugant efficiency by introduction of pTG18msr into host cells as efficiently as pTiC58. However, pK18SphDrep and pK186083 did not (Fig. 1C). The data suggest that the two ORFs, atu6082 and atu6083, are essential for the incompatibility-enhancing effect. We named the two ORFs *ietA* and *ietS*, respectively, after the incompatibility enhancer of the *Ti* plasmid. In fact, cells containing *ietA*-disrupted pTiC58 showed an efficiency level as high as that of cells having an *repABC* vector for introduction of an incompatible plasmid pK18msr (Fig. 1D). This result indicates that

FIG. 1. Transconjugant efficiency of cells harboring pTiC58 deletion clones or subclone plasmids. Schematic illustration of resident pTiC58 and its derivatives (left) and efficiency of transconjugant colony formation (right) following the introduction of an incompatible *repABC* plasmid, pK18msr (A, B, and D) or pTG18msr (C), into C58C1 cells containing the resident plasmid. Each transconjugant efficiency is the average of at least three independent experiments. (A) pTiC58 with a large deletion. (B) pTiC58 with a smaller deletion within the 52.1-kbp segment deleted in pTiΔBC. (C) The *repABC* plasmid pK18fr5rep, which contains a 9.3-kbp fragment including the 5.2-kbp segment deleted in pTiΔBC5, and its mutant derivatives. (D) pTiC58 with an insertion of Gm^r within the *ietA* gene (pTi6082::Gm). Filled boxes and open boxes indicate the *repABC* region and other gene clusters in pTiC58, respectively. Thin dashed lines indicate the deleted region. Black arrows represent ORFs and their transcriptional orientations. Gray arrows and thick gray lines indicate incomplete ORFs. The hatched box indicates the Gm^r gene.

ietA and *ietS* in the 4.3-kbp fragment ensure a high level of incompatibility of pTiC58.

Mode of action of *ietAS*. We examined whether *ietAS* affects transconjugant efficiency when *ietAS* is present on an incoming plasmid or when it is present on a resident plasmid of a different type of replicon. pK18SalDrep, a *repABC* replicon pK18msr containing *ietAS*, was introduced into wild-type C58rif cells which harbored the incompatible Ti plasmid pTiC58 80-fold more efficiently than pK18msr (Fig. 2A). pJNLF, a pBBR replicon pJN105 harboring *ietAS*, reduced the transconjugant efficiency by introduction of another pBBR replicon, pBHR1, into host C58C1 cells sevenfold more efficiently than pJN105 (Fig. 2B). However, a similar suppression was not observed in *E. coli* (data not shown). These data indicate that *ietAS* can improve the efficiency of plasmid introduction when it is present on the incoming plasmid and also enhance incompatibility of the plasmid regardless of its replication type in *Agrobacterium* cells. However, *E. coli* cells are not affected by the presence of *ietAS*.

When the incompatible plasmid pTG18msr was introduced by electroporation into *Agrobacterium* cells which contained a plas-

mid with or without *ietAS*, the *ietAS*-containing cells exhibited a 36-fold-lower transformation efficiency than cells lacking *ietAS*. This result suggests that the suppressive effect of *ietAS* is not due to an inefficient conjugation process but to the enhancement of plasmid stability. Therefore, we examined the stability of the *ietAS*-bearing plasmids. C58C1 cells that contained the *ietAS*-bearing plasmid pK18SalDrep or the vector plasmid pK18msr were spread on the sucrose medium ABS agar. Because the two plasmids contained the *sacB* gene, which confers hypersensitivity to sucrose, the cells with the plasmids should be unable to grow on ABS agar, whereas cells that had lost the plasmid could grow on sucrose. Fewer colonies growing on ABS agar exhibit higher plasmid maintenance. Cells with pK18SalDrep exhibited a 15-fold decrease in colony formation on ABS agar relative to cells with the control plasmid (Fig. 2C). This finding demonstrates that *ietAS* increases plasmid stability.

Toxin and antitoxin properties of IetA and IetS. Our BLAST search revealed that putative IetA and IetS proteins have significant similarity to AAA-ATPases and a subtilisin-like serine protease, respectively. For each of the IetA homologues, we found an IetS homologue belonging to the same

TABLE 3. Primers used for constructing plasmids other than pTiC58 variants

Name	Oligonucleotide sequence ^a	Constructed plasmid
p99R-fw	CACAATGACCGCGGCTGCGCTCGCCG	p99R
p99R-rv	TTTCCGGATCCGGGGGACCGATGA	p99R
p101R-fw	CAGTTCGATGCGATCGGCGCGAAGCG	p101R
p101R-rv	GTCGAAGAAGTAGACGCCTTTGGTT	p101R
p103R-fw	TGTTTCGATGACGTCATCGAGTATGCA	p103R
p103R-rv	GCCGAAAGAGCGCTGGATCGAGCAA	p103R
p105R-fw	CTTCAGGCTCTACCCGACGTCACCCT	p105R
p105R-rv	CGAGAAGGCAGACGGCGGGACGGTCA	p105R
p107R-fw	GCTGGCACCCAGCTTCCGGCGTC	p107R
p107R-rv	TCCGCCGTGCCCTGATTACTGGTG	p107R
p109R-fw	GCTGCAGCAACTGCGCTCGCGGCAAG	p109R
p109R-rv	GGTCTCGCCGGTCTCGTGAAC	p109R
IetS-NdeATG	GGAATTCCATATGGCAGAAGAACCCGCGTCC	pSRKietS
IetScHisx6	GCTCTAGACTAATGATGATGATGATGATGAGTCTCAATCA GAATTTCCG	pSRKietS
pRi-rep-L+Bam	CGCGGATCCGCGCGGTGTTGAGATCGCGAGA	pMWR
pRi-rep-R+Bam	CGCGGATCCGCGAGCTTGTGGTCAGCTGACAACA	pMWR
pJNGmfw	AGCGCCTGAAGCCCGTTCTG	pMWRGietA
pietAfwRv	GCTCTAGACATCCAATGCGGTCCGGGCTC	pMWRGietA

^a Underlining indicates mutated nucleotides.

organism (Table 4). Genes coding the IetA and IetS homologue proteins were located close together and in the same transcriptional direction as a pair on a plasmid or a chromosome (see Fig. S1 in the supplemental material). It is likely that these homologues are functionally and transcriptionally cooperative. Among the amino acid sequences of the putative IetA and its homologues, 17 residues were found only at the N terminus of IetA (see Fig. S2 in the supplemental material).

We disrupted an *ietA* gene by a frameshift, which might not affect the *ietS* expression. The new *ietA* mutant, *ietS*⁺ plasmid pSPDR contained a 4.3-kbp fragment, which had a 4-nucleotide deletion (138th to 141st) in the *ietA* gene and eventually might express a C-terminal truncated mutant protein consisting of 107 amino acids. The plasmid was introduced into C58C1 cells by conjugation (Fig. 3). The transconjugant efficiency of the *ietA* mutant, *ietS*⁺ plasmid pSPDR was significantly lower than that of the *repABC* vector pK18msr when recipient cells had a compati-

ble vector plasmid, pJN105. Conversely, the efficiency of pSPDR was restored to an efficiency similar to that of pK18msr when recipient cells contained a compatible plasmid with wild-type *ietA*, pJNLF, or pJNSph instead of pJN105. This suggests that the expression of IetS is toxic to recipient C58C1 cells in the absence of IetA. An IetA solo expression (*ietA*⁺ *ietS* mutant) construct, pK18SphDrep, was introduced into *Agrobacterium* cells as efficiently as pK18msr. These findings also suggest that the two genes exert a plasmid-stabilizing effect by the TA mechanism, with IetS as the toxin and IetA the antitoxin.

We confirmed that a very low introduction efficiency of pSPDR (*ietA* mutant, *ietS*⁺) into *Agrobacterium* cells without the *ietA* gene is caused by IetS toxicity. The *ietS* gene was placed under an inducible promoter in pSRKKm to check the effect of expression of IetS on cell proliferation. A resultant plasmid, pSRKietS, which tightly regulates the expression of *ietS* by *P*_{lac} and *lacI*^q, was introduced into C58C1 cells. How-

FIG. 2. Incompatibility and stability tests of the plasmids containing *ietAS*. (A) Effect of *ietAS* when present in the incoming plasmid. *E. coli* cells harboring an incoming *repABC* replicon with (+) or without a 4.3-kbp *ietAS* fragment were subjected to conjugation with recipient *Agrobacterium* cells containing the resident pTiC58. (B) Effect of *ietAS* when present in the pBBR replicon. *Agrobacterium* cells containing a resident pBBR replicon with or without the 4.3-kbp *ietAS* fragment were subjected to conjugation with donor *E. coli* cells containing an incoming incompatible plasmid pBHR1. (C) Effect of *ietAS* on segregational plasmid stability. CFU ratio is the number of CFU on ABS agar divided by the number of CFU on ABG agar. See Materials and Methods for details. Each CFU value is the average of at least three independent experiments. Thin vertical bars represent standard deviations.

TABLE 4. Properties of putative IetA and IetS protein and their homologues

Accession no.	Locus tag (name)	Source organism and locus of gene	Amino acid identity (%)	No. of intervening nucleotides ^a	Product
AAK91042	Atu6082 (<i>ietA</i>)	<i>Agrobacterium tumefaciens</i> C58,	100	3	AAA-family ATPase
AAK91043	Atu6083 (<i>ietS</i>)	pTiC58	100		Conserved hypothetical protein
ABS70019	Xaut_4803	<i>Xanthobacter autotrophicus</i> Py2,	82	0	AAA ATPase central domain protein
ABS70020	Xaut_4804	pXAUT01	79		Conserved hypothetical protein
ACB96249	Bind_2677	<i>Beijerinckia indica</i> subsp. <i>indica</i>	65	15	AAA ATPase central domain protein
ACB96248	Bind_2676	ATCC 9039, chromosome	51		Conserved hypothetical protein
AAM48293	RHE_PD00006	<i>Rhizobium etli</i> CFN 42, p42d	62	−1	Probable ATPase (ATP-binding) protein
AAM55046	RHE_PD00007		49		Conserved hypothetical protein
CAK10261	pRL100036	<i>Rhizobium leguminosarum</i> bv.	59	15	Putative AAA ATPase protein
CAK10260	pRL100035	viciae 3841, pRL10	50		Conserved hypothetical protein
ABK18534	Sfum_2857	<i>Syntrophobacter fumaroxidans</i>	54	21	AAA ATPase central domain protein
ABK18535	Sfum_2858	MPOB, chromosome	43		Conserved hypothetical protein
ABK43802	Mmc1_1291	<i>Magnetococcus</i> sp. strain MC-1,	52	20	AAA ATPase central domain protein
ABK43803	Mmc1_1292	chromosome	40		Conserved hypothetical protein
AAZ97645	Tbd_1692	<i>Thiobacillus denitrificans</i> ATCC	52	20	Putative ATPase
AAZ97646	Tbd_1693	25259, chromosome	41		Conserved hypothetical protein
ABS28407	Anae109_4229	<i>Anaeromyxobacter</i> sp. strain	55	90	AAA ATPase central domain protein
ABS28408	Anae109_4230	Fw109-5, chromosome	42		Conserved hypothetical protein
ABH00724	RHA1_ro11077	<i>Rhodococcus jostii</i> RHA1,	56	17	Probable AAA ATPase
ABH00723	RHA1_ro11076	pRHL3	41		Conserved hypothetical protein
ABC30163	HCH_03415	<i>Hahella chejuensis</i> KCTC 2396,	48	17	ATPase of the AAA ⁺ class
ABC30162	HCH_03413	chromosome	39		Subtilisin-like serine protease

^a The numbers of nucleotides present between *ietA* and *ietS* homologues; −1 means that two homologues are overlapped by single nucleotide.

ever, the introduction efficiency of pSRKietS was unexpectedly low (data not shown), suggesting the leakage of *ietS* expression. To neutralize the effect by basal expression of *ietS* on the plasmid, an *ietA*-containing low-copy-number plasmid pMWRGietA was introduced into C58C1 cells. Then, pSRKietS was efficiently introduced into C58C1 cells that contained pMWRGietA (data not shown). The growth of the cells that contained the two plasmids was monitored as shown in Fig. 4A. Growth of cells with pMWRGietA and pSRKietS was depressed by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), whereas a similar depression was not observed in cells bearing the vector plasmid pSRKKm instead of pSRKietS (Fig. 4A). In addition to the effect on cell growth, the IetS induction decreased the number of CFU of the culture severely (Fig. 4B). These results demonstrate that IetS is highly toxic.

FIG. 3. Conjugation efficiency of the plasmids with or without *ietA* and *ietS* genes. Recipient C58C1 cells contain a resident compatible pBBR replicon, pJN105 (vector; black bars), or its derivatives pJNLf (*ietA*⁺ *ietS*⁺; gray bars) and pJNSph (*ietA*⁺ *ietS* mutant; open bars). The incoming plasmids are the *repABC* replicon pK18msr and its derivatives harboring the *iet* genes. Each value is the average of at least three independent experiments. Thin vertical bars represent standard deviations.

Location of survivor mutations induced by a suicide genotype. Despite the high toxicity of IetS in the absence of IetA, a small number of transconjugant colonies grew in the presence of pSPDR (*ietA* mutant, *ietS*⁺) in C58C1 cells (Fig. 3). We wanted to determine how transconjugant cells could survive in a suicide genotype. Four transconjugants (TC1, TC2, TC3, and TC4) were isolated, and the pSPDR plasmids were extracted. At the same time, the plasmid was cured by the *sacB* counter-selection from the four strains. Fresh pSPDR plasmids were introduced again via conjugation into the four plasmid-cured strains (Table 5). The four strains received pSPDR at a low efficiency, at a level similar to that of their parental wild-type C58C1 strain. The toxicity of the pSPDR derivatives recovered from the four transconjugants was also examined. The plasmids pSPDR-E1, pSPDR-E2, pSPDR-E3, and pSPDR-E4 obtained from each of the four transconjugants (TC1, TC2, TC3, and TC4, respectively) were introduced into plasmid-free C58C1 cells by conjugation (Table 6). The introduction efficiency of any of the four pSPDR derivatives was about 1,000 times higher than that of the original pSPDR. These results show that the pSPDR-induced transconjugants maintained the toxicity-attenuated mutant pSPDR but had not acquired resistance to IetS toxicity.

Analyses of amino acid substitution mutants of IetA and IetS. As previously described, IetA and IetS are homologues of AAA-ATPase and subtilisin-like serine protease, respectively. Multiple sequence alignment data suggested that IetA and IetS have conserved catalytic residues required for ATPase and protease activity, respectively (Fig. 5). The three ATP domains (Walker A, Walker B, and second region of homology [SRH]) containing catalytic residues, such as Lys-201 and Asp-307 of *E. coli* FtsH (12), were highly conserved among the IetA homologues. In IetS homologues, a peptidase S8 (subtilisin-like serine protease) domain was detected in all the sequences using the

FIG. 4. Effect of induction of IetS expression on cell proliferation. C58C1 cells harboring pMWRGietA together with the IetS-inducible plasmid pSRKietS (open and filled squares) or with vector plasmid pSRKKm (cross) were inoculated into kanamycin-supplemented MG/L medium at 6×10^6 cells ml⁻¹. IPTG was added to the two cultures (filled square and cross) after 3 h from the start of the culture (indicated by arrows) to a final concentration of 1 mM. The turbidity and the numbers of viable cells at various time points were checked. (A) Growth curve of the cultures. (B) Viable cell numbers in the cultures at each point in time. Colony numbers on LB agar containing kanamycin were counted. OD₆₀₀, optical density at 600 nm.

BLAST program (data not shown). Furthermore, three putative catalytic residues (Asp-297, His-336, and Ser-566 in IetS) of protease were identical among the homologues and the three functional subtilisin-like serine proteases (Fig. 5, PepC, EpiP, and subtilisin).

To determine whether the plasmid-stabilizing effect of the two genes required the putative enzyme activities, we prepared amino acid substitution mutants at catalytic residue sites of the ORFs. In IetA, the three amino acids residues required for ATPase activity, Lys of Walker A, Glu of Walker B, and Arg of SRH (12, 39), were replaced with Asn, Gln, and Leu, respectively. The resulting IetA mutant plasmids, p99R (K153N), p101R (E206Q), and p103R (R260L) were introduced into C58C1 via conjugation. A significant growth defect was observed in the transconjugants by p99R (K153N), which has the mutation within the Walker A domain in IetA (see Fig. S3 in the supplemental material). The p99R (K153N) transconjugants required six days of incubation after conjugation for all the transconjugant colonies to appear (Fig. 6A). A slight delay in colony number (Fig. 6A) and a serious reduction in colony size (see Fig. S3 in the supplemental material) were observed in the transconjugants by the Walker B mutant p101R (E206Q). However, amino acid substitution at the invariant Arg within the SRH domain (p103R) did not affect growth. When the cultures were incubated for 6 days and the efficien-

cies were evaluated, the three IetA mutants were comparable with the wild-type IetA (Fig. 6A).

In IetS, each of the three essential catalytic triads for protease activity of subtilisin-like serine protease, i.e., Asp, His, and Ser, was replaced with Ala. The resident plasmids that contained any of the three mutations apparently failed to reduce the transconjugant efficiency for the incoming incompatible plasmid. The mutant's efficiency was as high as the vector plasmid's efficiency (Fig. 6B). This suggests that the protease activity is essential for the IetS toxicity and indispensable for the plasmid-stabilizing effects by IetS and IetA.

Effect on Ti plasmid ejection. Curing of Ti plasmid(s) from *Agrobacterium* cells is essential to determine the contribution of Ti plasmid genes and chromosomal genes on pathogenicity and to develop useful strains for biotechnology. Uraji and coauthors (36) proposed a simple curing method that induced a resident Ti plasmid to compete with an incoming counter-selectable, incompatible plasmid. This method could expel many Ti plasmids efficiently. We expected that the addition of *ietAS* to the expelling plasmid could improve the curing method. As shown in Table 7, 10 tumorigenic strains were subjected to curing experiments. We introduced, conjugationally, pK18msr and pK18msr with *ietAS* (pK18SalDrep) to the strains and checked for ejection of their resident Ti plasmids in the resulting transconjugants. *ietAS* increased the Ti plasmid ejection ratio in 9 strains of the 10 (Table 7). As an exception, the strain Ro-Ag-10 was still resistant to the

TABLE 5. Reintroduction of fresh pSPDR (*ietA* mutant, *ietS*⁺) into the pSPDR-induced transconjugants

Recipient strain ^a	Transconjugant efficiency with the indicated incoming plasmid (10 ⁻³) ^b	
	pK18msr	pSPDR
C58C1	172	0.03
TC1	166	0.03
TC2	NT	0.07
TC3	NT	0.04
TC4	NT	0.02

^a Four transconjugant cells (TC1 to TC4) were chosen randomly from pSPDR (*ietA* mutant, *ietS*⁺)-induced transconjugants, and then their plasmids were cured for reintroduction of fresh pSPDR.

^b Transconjugant efficiency is calculated as the number of transconjugant colonies/number of input colonies. NT, not tested.

TABLE 6. Toxicity of the pSPDR plasmids isolated from the pSPDR-induced transconjugants

Incoming plasmid ^a	Transconjugant efficiency (10 ⁻³) ^b
pK18msr (vector)	172
pSPDR	0.03
pSPDR-E1	68
pSPDR-E2	43
pSPDR-E3	35
pSPDR-E4	43

^a Derivatives of the pSPDR plasmid (pSPDR-E1 to pSPDR-E4) were isolated from the four transconjugants (TC1 to TC4), respectively.

^b Transconjugant efficiency is calculated as the number of transconjugant colonies/number of input colonies.

FIG. 5. Multiple alignments of amino acid sequences at catalytic regions of IetA, IetS, and their homologues. (A) Multiple alignments of amino acid sequences of conserved ATPase domains referred to as AAA modules among IetA and its homologues together with the members of AAA-ATPase, FtsH, and FIGL-1. (B) Multiple alignments of amino acid sequences of catalytic regions containing three essential residues required for protease activity among IetS and its homologues together with the three subtilisin-like serine proteases, PepC, EpiP, and subtilisin. Bold letters indicate the residues perfectly conserved among each group of homologues. Arrows indicate the residues replaced in the amino acid substitution experiments. The database accession numbers of the sequences are the following: *E. coli* FtsH, AAA23813; *C. elegans* FIGL-1, NP_504197; *Aspergillus niger* PepC, P33295; *S. epidermidis* EpiP, P30199; *B. subtilis* subsp. *natto* subtilisin, P35835. The accession numbers of homologues of IetA and IetS are listed in Table 4. The number of amino acid residues in FtsH is according to the revised sequence (37).

reinforced expelling plasmid. The addition of the pTi-SAKURA's plasmid-stabilizing genes *tiorf24* and *tiorf25* to pK18SalDrep (pK18SCat) further enhanced the expelling effect, as shown in Table 7. Notably, Ti-cured colonies were successfully obtained in every strain.

DISCUSSION

This study revealed that *ietAS* on pTiC58 depresses the entry of incoming incompatible plasmids into *Agrobacterium* cells by stabilizing the Ti plasmid (Fig. 1 and 2). The high level of incompatibility of pTiC58 was due to the postsegregational

killing effect produced by the IetS toxin and the IetA antitoxin. When the *ietAS*-containing Ti plasmid pTiC58 is replaced by an incompatible plasmid in C58 strains, the growth of the cells is inhibited by IetS toxin. Eventually, pTiC58 remained in the majority of the population. The TA system composed of *ietAS* is a novel type. The IetA antitoxin is a homologue of AAA-ATPase, and the IetS toxin is a homologue of subtilisin-like serine protease. The genes coding for IetA and IetS homologue proteins were observed in various groups of gram-negative and gram-positive bacteria. Both of the homologue genes were located very close to each other on the genome in each

FIG. 6. Effect of amino acid substitution mutations on IetA and IetS functions. (A) Transconjugant efficiency of incoming plasmids with a frameshift mutation or amino acid substitution mutation in *ietA*. Transconjugant colonies were counted 2 days (open bars) and 6 days (black bars) after the conjugation. C58C1 cells were used in the conjugation assay as recipients. Incoming plasmids are indicated as follows: pK18SalDrep, wild-type (wt) *ietA*; pSPDR, frameshift; p99R, K153N; p101R, E206Q; and p103R, R260L. (B) Transconjugant efficiency of C58C1 cells harboring the resident plasmid with or without amino acid substitution mutant *ietS*. The incoming incompatible plasmid is the pTG18msr. Transconjugant efficiency was calculated based on colony counts 3 days after the conjugation. Resident plasmids are indicated as follows: pK18msr, vector; pK18SalDrep, wt *ietS*; p105R, D297A; p107R, H336A; and p109R, S566A. Each value is the average of at least three independent experiments. Thin vertical bars represent standard deviations.

TABLE 7. Induction of Ti plasmid-cured strains among pathogenic strains using *repABC* plasmids reinforced by the stability genes

Recipient strain	Ejection ratio (%) by the incoming plasmid ^a		
	pK18msr	pK18SalDrep (<i>ietAS</i>)	pK18SCat (<i>tiorf24</i> , <i>tiorf25</i> , <i>ietAS</i>)
C58	70	91	99
Ch-Ag-6	96	100	100
Ch-Ag-14	84	98	100
Ch-Ag-2	46	98	100
Pch-Ag-4	21	97	99
Pch-Ag-2	0	88	100
Ch-Ag-10	0	76	79
Pl-Ag-1	10	14	54
Ro-Ag-10	0	0	1
Ro-Ag-13	0	1	2

^a The ejection ratio is the percentage of colonies that lost the Ti plasmid after conjugation. Stability genes carried by the plasmids are indicated in parentheses.

bacterium (Table 4; see also Fig. S1 in the supplemental material). This implies that each pair of homologues constitutes the stability module and that both genes disseminated as a unit by horizontal transfer.

A small number of colonies emerged from the suicide genotype construct induced by pSPDR (*ietA* mutant, *ietS*⁺) (Fig. 3), despite the absence of the *ietA* antitoxin gene. However, the “survivor” phenotype was due to some alteration in pSPDR (Table 6). A small fraction of survivors was also observed in our forced IetS expression experiment (Fig. 4B). The difference in transconjugant efficiency was approximately 10⁴ between the wild-type and the frameshift *ietA* mutation in *ietAS* plasmids. The ratio was much smaller than expected based on the mutation frequency. One explanation for the ratio is an activation of some unknown indigenous defense mechanism of cells to avoid death by the toxin.

Members of the AAA family ATPases are involved in a wide range of processes, such as protein degradation, membrane fusion, and the regulation of gene expression (5). Multiple alignment data of putative IetA proteins and their homologues indicated their potential ATPase activity (Table 4 and Fig. 5). IetA contained three conservative ATP domains, referred to as the AAA modules in AAA family ATPase. In a *Caenorhabditis elegans* fidgetin homologue, FIGL-1, the three catalytic residues, namely Lys in Walker A, Glu in Walker B, and Arg in the SRH domains, are essential for its ATPase activity (39). In addition, in *E. coli* FtsH, amino acid mutations of corresponding Lys-201 to Asp in Walker A or Arg-315 to Leu in the SRH domain eliminated its ATPase activity (12). Among the three catalytic residues, the Lys residue was essential for ATP binding, and the other two residues were essential for ATP hydrolysis but not for ATP binding (13, 39). In our amino acid substitution mutations in the IetA gene, the K153N mutant *ietA* severely decreased antitoxin activity (Fig. 6A; see also Fig. S3 in the supplemental material). The E206Q (p101R) mutant exerted a slight effect, and the R260L (p103R) mutant had no effect on growth. These data suggested that ATP binding is more important for IetA antitoxin activity than ATP hydrolysis, but the underlying mechanism is not clear. This is the first indication that suggested the involvement of an AAA family ATPase member functioning as an antitoxin.

The putative IetS protein is significantly similar to the subtilisin-like serine protease homologues (Table 4 and Fig. 5). Subtilisin-like serine proteases are widely distributed from prokaryotes to higher eukaryotes (25). The proteases contain three invariant residues, His, Asn, and Ser, as an essential catalytic triad. The replacement of any of the three residues with Ala (D297A, H336A, and S566A) eliminated the incompatibility-enhancing effect of *ietAS* (Fig. 6B). These results suggest that the protease activity of IetS is essential for any toxic effect. To the best of our knowledge, there has been no report that indicates involvement of a subtilisin-like serine protease in the TA mechanism.

Siezen et al. (26) reported that some subtilisin-like serine proteases play a role as lantibiotic leader peptidases. Lantibiotics are antimicrobial peptides that are secreted extracellularly and exert toxic effects on other cells. NisP of *Lactococcus lactis* and EpiP of *Staphylococcus epidermidis* are examples. Lantibiotic leader peptidases are essential enzymes for maturation of lantibiotics. However, because no growth inhibition effect was observed in cell cultures having the pK18SalDrep (data not shown), the *ietS*-derived toxicity is not due to secretion of toxic compounds. Generally, lantibiotic gene clusters contain one or two immunity genes that provide protection from lantibiotic toxicity (28). In the *ietAS* genes, IetA was immune to IetS toxicity, but it had no similarity to known lantibiotic immunity proteins, such as NisI and NisEFG of *L. lactis*, at the secondary-structure level (data not shown). To our knowledge, there has been no report that lantibiotics or their processing enzymes function as toxins in a TA system. The *Bacillus subtilis* plasmid pTA1050 contains a *parABC* locus, which confers segregational stability to the plasmid. The putative ParA encoded in the *parABC* locus is very similar to the subtilisin-like serine protease. However, its function is still unknown (18). It is possible that the IetS protein degrades various proteins randomly or targets specific proteins essential for cell proliferation. To address the mechanism of IetS toxic action, we are investigating the protease activity of IetS in vitro.

Curing of Ti plasmids is an important methodology for pure and applied research in *Agrobacterium*. For example, curing is an unavoidable step in order to determine whether the Ti plasmid or the chromosomal background of a strain contains some genes that elevate gene transfer efficiency. Uraji and coauthors (36) proposed a Ti plasmid-curing method that uses the incompatibility between Ti and small *repABC* plasmids. However, strains resistant to curing exist among laboratory strains and natural isolates, as shown in Table 7. Addition of *ietAS* to the incoming incompatible plasmid increased the number of Ti-free cells in the transconjugants (Table 7). The 2.6-kbp fragment containing *tiorf24* and *tiorf25* of pTi-SAKURA also enhanced the incompatibility of incoming plasmids. These stability genes enable us to utilize larger numbers of pathogenic strains for research.

ACKNOWLEDGMENTS

This work was supported in part by the Japanese Ministry of Education, Science, Sports, and Culture, Grant-in-Aid for Scientific Research (grant 20570221).

We thank T. L. Lauver for critical comments and discussion to improve the manuscript; B. Goodner, E. W. Nester, and H. Sawada for providing bacterial strains; and S. K. Farrand for the pSRK plasmids.

REFERENCES

1. Austin, S. J., and K. Nordström. 1990. Partition-mediated incompatibility of bacterial plasmids. *Cell* **60**:351–354.
2. Cevallos, M. A., R. Cervantes-Rivera, and R. M. Gutiérrez-Ríos. 2008. The *repABC* plasmid family. *Plasmid* **60**:19–37.
3. Chilton, M. D., T. C. Currier, S. K. Farrand, A. J. Bendich, M. P. Gordon, and E. W. Nester. 1974. *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc. Natl. Acad. Sci. USA* **71**:3672–3676.
4. Fineran, P. C., T. R. Blower, I. J. Foulds, D. P. Humphreys, K. S. Lilley, and G. P. C. Salmund. 2009. The phage abortive infection system, ToxIN, functions as a protein-RNA toxin-antitoxin pair. *Proc. Natl. Acad. Sci. USA* **106**:894–899.
5. Frickey, T., and A. N. Lupas. 2004. Phylogenetic analysis of AAA proteins. *J. Struct. Biol.* **146**:2–10.
6. Gerdes, K., and E. G. Wagner. 2007. RNA antitoxins. *Curr. Opin. Microbiol.* **10**:117–124.
7. Goodner, B., G. Hinkle, S. Gattung, N. Miller, M. Blanchard, B. Quorllo, B. S. Goldman, Y. Cao, M. Askenazi, C. Halling, L. Mullin, K. Houmiel, J. Gordon, M. Vaudin, O. Iartchouk, A. Epp, F. Liu, C. Wollam, M. Allinger, D. Doughty, C. Scott, C. Lappas, B. Markelz, C. Flanagan, C. Crowell, J. Gurson, C. Lomo, C. Sear, G. Strub, C. Cielo, and S. Slater. 2001. Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* **294**:2323–2328.
8. Hattori, Y., M. Uraji, K. Suzuki, N. Ohta, K. Iwata, A. Kato, and K. Yoshida. 2000. Gene list on a plant tumor-inducing plasmid, pTi-SAKURA in *Agrobacterium tumefaciens* MAFF301001. *DNA Res.* **7**:371–380.
9. Hayes, F. 2003. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* **301**:1496–1499.
10. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51–59.
11. Holsters, M., J. P. Hernalsteens, M. Van Montagu, and J. Schell. 1982. Ti plasmids of *Agrobacterium tumefaciens*: the nature of the tip, p. 269–298. In G. Kahl, and J. S. Schell (ed.), *Molecular biology of plant tumors*. Academic Press, New York, NY.
12. Karata, K., T. Inagawa, A. J. Wilkinson, T. Tatsuta, and T. Ogura. 1999. Dissecting the role of a conserved motif (the second region of homology) in the AAA family of ATPases. Site-directed mutagenesis of the ATP-dependent protease FtsH. *J. Biol. Chem.* **274**:26225–26232.
13. Karata, K., C. S. Verma, A. J. Wilkinson, and T. Ogura. 2001. Probing the mechanism of ATP hydrolysis and substrate translocation in the AAA protease FtsH by modelling and mutagenesis. *Mol. Microbiol.* **39**:890–903.
14. Khan, S. R., J. Gaines, R. M. Roop II, and S. K. Farrand. 2008. Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. *Appl. Environ. Microbiol.* **74**:5053–5062.
15. Kobayashi, I. 2004. Genetic addiction: a principle of gene symbiosis in a genome, p. 105–144. In B. E. Funnell and G. J. Phillips (ed.), *Plasmid biology*. ASM Press, Washington, DC.
16. Li, P., and S. K. Farrand. 2000. The replicator of the nopaline-type Ti plasmid pTiC58 is a member of *repABC* family and is influenced by the TraR-dependent quorum-sensing regulatory system. *J. Bacteriol.* **182**:179–188.
17. McCullen, C. A., and A. N. Binns. 2006. *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. *Annu. Rev. Cell Dev. Biol.* **22**:101–127.
18. Meijer, W. J., G. B. Wisman, P. Terpstra, P. B. Thorsted, C. M. Thomas, S. Holsappel, G. Venema, and S. Bron. 1998. Rolling-circle plasmids from *Bacillus subtilis*: complete nucleotide sequences and analyses of genes of pTA1015, pTA1040, pTA1050 and pTA1060, and comparisons with related plasmids from gram-positive bacteria. *FEMS Microbiol. Rev.* **21**:337–368.
19. Newman, J. R., and C. Fuqua. 1999. Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli* *araBAD* promoter and the *araC* regulator. *Gene* **18**:197–203.
20. Penfold, R. J., and J. M. Pemberton. 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. *Gene* **118**:145–146.
21. Pogliano, J. 2002. Dynamic cellular location of bacterial plasmids. *Curr. Opin. Microbiol.* **5**:586–590.
22. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. Sawada, H., and H. Ieki. 1992. Phenotypic characteristics of the genus *Agrobacterium*. *Ann. Phytopath. Soc. Jpn.* **58**:37–45.
24. Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**:69–73.
25. Siezen, R. J., B. Renckens, and J. Boekhorst. 2007. Evolution of prokaryotic subtilases: genome-wide analysis reveals novel subfamilies with different catalytic residues. *Proteins* **67**:681–694.
26. Siezen, R. J., O. P. Kuipers, and W. M. de Vos. 1996. Comparison of lantibiotic gene clusters and encoded proteins. *Antonie van Leeuwenhoek* **69**:171–184.
27. Simon, R., U. Priefer, and A. Pühler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Bio/Technology* **1**:784–794.
28. Stein, T., S. Heinzmann, I. Solovieva, and K. D. Entian. 2003. Function of *Lactococcus lactis* nisin immunity genes *nisI* and *nisFEG* after coordinated expression in the surrogate host *Bacillus subtilis*. *J. Biol. Chem.* **278**:89–94.
29. Summers, D. 1998. Timing, self-control and a sense of direction are the secrets of multicopy plasmid stability. *Mol. Microbiol.* **29**:1137–1145.
30. Suzuki, K., N. Ohta, Y. Hattori, M. Uraji, A. Kato, and K. Yoshida. 1998. Novel structural difference between nopaline- and octopine-type *trbI* genes: construction of genetic and physical map and sequencing of *trbI/traI* and *rep* gene clusters of a new Ti plasmid pTi-SAKURA. *Biochim. Biophys. Acta* **1396**:1–7.
31. Suzuki, K., Y. Hattori, M. Uraji, N. Ohta, K. Iwata, K. Murata, A. Kato, and K. Yoshida. 2000. Complete nucleotide sequence of a plant tumor-inducing Ti plasmid. *Gene* **242**:331–336.
32. Suzuki, K., K. Iwata, and K. Yoshida. 2001. Genome analysis of *Agrobacterium tumefaciens*: construction of physical maps for linear and circular chromosomal DNAs, determination of copy number ratio and mapping of chromosomal virulence genes. *DNA Res.* **8**:141–152.
33. Suzuki, K., K. Tanaka, S. Yamamoto, K. Kiyokawa, K. Moriguchi, and K. Yoshida. 2009. Ti and Ri plasmids, p. 133–147. In E. Schwartz (ed.), *Microbial megaplasmids*. Springer Verlag, Heidelberg, Germany.
34. Tabata, S., P. J. J. Hooykaas, and A. Oka. 1989. Sequence determination and characterization of the replicator region in the tumor-inducing plasmid pTiB6S3. *J. Bacteriol.* **171**:1665–1672.
35. Tanaka, K., H. H. Arafat, H. Urbanczyk, S. Yamamoto, K. Moriguchi, H. Sawada, and K. Suzuki. 2009. Ability of *Agrobacterium tumefaciens* and *A. rhizogenes* strains, inability of *A. vitis* and *A. rubi* strains to adapt to salt-insufficient environment, and taxonomic significance of a simple salt requirement test in the pathogenic *Agrobacterium* species. *J. Gen. Appl. Microbiol.* **55**:35–41.
36. Uraji, M., K. Suzuki, and K. Yoshida. 2002. A novel plasmid curing method using incompatibility of plant pathogenic Ti plasmids in *Agrobacterium tumefaciens*. *Genes Genet. Syst.* **77**:1–9.
37. Wang, R. F., E. B. O'Hara, M. Aldea, C. I. Bargmann, H. Gromley, and S. R. Kushner. 1998. *Escherichia coli* *mrsC* is an allele of *hflB*, encoding a membrane-associated ATPase and protease that is required for mRNA decay. *J. Bacteriol.* **180**:1929–1938.
38. Wise, A. A., Z. Liu, and A. N. Binns. 2006. Culture and maintenance of *Agrobacterium* strains. *Methods Mol. Biol.* **343**:3–13.
39. Yakushiji, Y., S. Nishikori, K. Yamanaka, and T. Ogura. 2006. Mutational analysis of the functional motifs in the ATPase domain of *Caenorhabditis elegans* fidgetin homologue FIGL-1: firm evidence for an intersubunit catalysis mechanism of ATP hydrolysis by AAA ATPases. *J. Struct. Biol.* **156**:93–100.
40. Yamamoto, S., M. Uraji, K. Tanaka, K. Moriguchi, and K. Suzuki. 2007. Identification of pTi-SAKURA DNA region conferring enhancement of plasmid incompatibility and stability. *Genes Genet. Syst.* **82**:197–206.