Plasmid Stability in *Pseudomonas fluorescens* in the Rhizosphere

ARJAN J. VAN DER BIJ,[1] LETTY A. DE WEGER,[1] WILLIAM T. TUCKER,[2]† AND BEN J. J. LUGTENBERG[1]*

*Leiden University, Institute of Molecular Plant Sciences, Clusius Laboratory, 2333 AL Leiden,
The Netherlands,[1] and DNA Plant Technology Corporation, Oakland, California 94608[2]*

Plasmids belonging to various incompatibility (Inc) groups were introduced into the efficiently root-colonizing strain *Pseudomonas fluorescens* WCS365, and their stabilities in complex and minimal media and in the rhizospheres of tomato, wheat, and potato plants grown under gnotobiotic conditions without selection pressure were tested. The IncP plasmid was found to be highly unstable under all conditions tested, whereas the IncQ and IncW plasmids showed intermediate stabilities and the plasmids pVSP41 and pWTT2081, for which the Inc group is unknown, both containing the origin of replication (*rep*) and stability (*sta*) regions of the *Pseudomonas aeruginosa* pVS1 replicon, were stably maintained under all conditions tested. Growth experiments in which cells of strain WCS365 carrying the plasmid pWTT2081 were grown in the presence of WCS365 without the plasmid showed that the presence of pWTT2081 acts as a burden. We conclude that pVSP41 and pWTT2081 are valuable as stable vectors for the functional analysis of genes involved in root colonization, provided that control cells carry the empty vector.

Many plant growth-promoting rhizobacteria belong to the genus *Pseudomonas* (18, 21, 28). Efficient colonization of the rhizosphere is thought to be one of the most important factors for bacterial plant growth promotion, since in unsuccessful biocontrol field experiments low numbers of plant growth-promoting rhizobacterium cells usually are found in the rhizospheres of plants (5, 27, 33). *Pseudomonas fluorescens* WCS365 is a highly efficient potato rhizosphere-colonizing strain which was isolated from potato roots grown under field conditions (4) and was also found to be a good colonizer of the rhizospheres of tomato and wheat plants (29). In our previous studies on traits involved in rhizosphere colonization, we showed that flagella (10) and the O-antigenic side chain of lipopolysaccharides (8) are important factors for efficient rhizosphere colonization. Furthermore, the availability of nutrients present in the exudate secreted by the roots of plants is also considered to play an important role in colonization (29).

Currently, we are following a genetic approach to find new traits and genes that are involved in rhizosphere colonization. Random *P. fluorescens* WCS365::Tn*5lacZ* mutants were screened for their abilities to colonize tomato roots. Mutants with impaired colonization abilities are complemented by wild-type genes in order to clone genes involved in colonization. For this purpose, plasmids that are stably maintained in the mutant bacteria in the rhizosphere are required. While naturally occurring plasmids are often stably maintained within the population in the absence of selection pressure, many known cloning vectors disappear from the bacterial population without the appropriate selection (23, 25, 26).

For the present study, a selection from existing cloning vectors belonging to different incompatibility groups was made and two new cloning vectors which contain the origin of replication (*rep*) and the stability (*sta*) regions of the native *Pseudomonas aeruginosa* plasmid pVS1 (16) were made. These plasmids were introduced into the efficiently colonizing strain

P. fluorescens WCS365, and the stabilities of resulting strains in various laboratory media and in the rhizospheres of various plant species were subsequently compared.

Introduction of plasmids into *P. fluorescens* WCS365. A constitutive Tn*5lacZ* construct (19) was introduced into *P. fluorescens* WCS365. One derivative, designated PCL1500, which was shown not to be affected in growth, motility, O-antigenic side chain of lipopolysaccharide, and colonization behavior (results not shown), was chosen for further studies. Cloning vectors that belong to the incompatibility groups P, Q, and W (IncP, IncQ, and IncW, respectively) were chosen (Table 1). Furthermore, the plasmids pVSP41 and pWTT2081 were tested. These new plasmids have a bireplicon, comprising the replication origin of the plasmid pACYC184 (6) and the *rep* and *sta* regions of the native *P. aeruginosa* plasmid pVS1 (16). The construction of these plasmids is outlined in Fig. 1. All plasmids except pVSP41 were introduced into strain PCL1500 (Table 1) by electroporation (11). Since the presence of pVSP41 has to be determined on medium supplemented with kanamycin, it is not possible to use strain PCL1500, which is kanamycin resistant (Km^r), as a host for pVSP41. Therefore, this plasmid was introduced into the wild-type strain *P. fluorescens* WCS365 (Table 1). Selection was performed by plating 100 μl of the electroporation samples on King's B (KB) medium (17) supplemented with the appropriate antibiotics (streptomycin, 500 μg · ml^{-1}; tetracycline, 40 μg · ml^{-1}; or kanamycin, 50 μg · ml^{-1}). After 2 days of growth at 28°C, transformed cells were purified and analyzed by Southern hybridization to verify the presence of the various plasmids. For this purpose, total DNA was isolated from the transformants and from the parental strains *P. fluorescens* WCS365 and PCL1500. Isolation, digestion, and blotting of total and plasmid DNA was performed as described by Maniatis et al. (22). Preparations of plasmid DNA, which were labelled in vitro with [α-^{32}P]dCTP, were used as probes. Detection was performed with the Phospho-Imager system (Molecular Dynamics, Sunnyvale, Calif.). A comparison of the lanes containing the total DNA from the transformants with those containing the appropriate plasmid DNA showed that fragments of indistinguishable sizes hybridized. Total DNA of the parental strains *P. fluorescens* WCS365 and PCL1500 did not hybridize with the various probes (data not shown). These results con-

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* Corresponding author. Mailing address: Leiden University, Institute of Molecular Plant Sciences, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands. Phone: (31) 71-5275063. Fax: (31) 71-5275088. Electronic mail address: lugtenb@rulsfb.leidenuniv.nl.

† Present address: Perkin-Elmer, Foster City, CA 94404.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)[a]	Reference or source
P. fluorescens strains		
WCS365	Efficient potato root-colonizing strain; Nal[r]	4
PCL1500	Tn*5lacZ*-marked derivative of WCS365; Nal[r] Km[r]	This study
PCL1501	Wild-type WCS365 containing pWTT2081	This study
PCL1502	PCL1500 containing pWTT2081	This study
PCL1503	PCL1500 containing pMP92	This study
PCL1504	PCL1500 containing pMP190	This study
PCL1505	PCL1500 containing pMP2740	This study
PCL1506	Wild-type WCS365 containing pVSP41	This study
Plasmids		
pCIB100	Carries Tn*5lacZ* which is constitutively expressed; mob site; Km[r]	19
pMP92	IncP; 7 kb; Tc[r]	31
pMP190	IncQ; 15 kb; Sm[r] Cm[r]	31
pMP2740	IncW; 13 kb; Sm[r] Spec[r]; derivative of pMP2733 (31) with a pIC20H polylinker in the *Hin*dIII site	This study
pVSP41	15.3 kb; ori pACYC184 and pVS1; Km[r]; cos site	This study
pWTT2081	Tc[r] derivative of pVSP41; 12 kb; no cos site	This study

[a] Abbreviations for resistance phenotypes: Nal, nalidixic acid; Km, kanamycin; Tc, tetracycline; Sm, streptomycin; Cm, chloramphenicol; Spec, spectinomycin.

firm that the expected plasmids were present in all the transformants. These transformants were used to study the stabilities of the various plasmids in laboratory media and in the rhizospheres of tomato, potato, and wheat plants.

Stabilities of various plasmids in laboratory media. To analyze the stabilities of the plasmids in laboratory media, single colonies of the *Pseudomonas* strains harboring the plasmids were used to inoculate the complex KB medium and the minimal standard succinate medium (SSM) (24) supplemented with the appropriate antibiotics. After 16 h of growth, bacterial suspensions were centrifuged and washed twice with sterile water to remove the antibiotics. Nonselective KB medium and SSM were inoculated with approximately 10^8 CFU of bacteria per ml, and the cultures were allowed to grow for 24 h at 28°C on a rotary shaker. Suspensions were diluted 1,000-fold in 10-ml aliquots of fresh nonselective KB medium and SSM. This procedure was repeated at least three times, resulting in cultures that had grown for at least 25 generations without antibiotic pressure after the initial inoculation. During the course of the experiment, samples were taken, diluted, and plated with the Spiral Plater (model C; Spiral System Instruments, Bethesda, Md.) on KB medium without or supplemented with the required antibiotic. Colonies were counted after 2 days of growth at 28°C. The stability of each plasmid was determined by comparing the numbers of colonies present on selective and nonselective plates. The experiment was performed once in SSM, whereas experiments were performed three times in KB medium. Means and standard deviations for these experiments were calculated and are presented in Table 2. Results revealed that the IncP plasmid pMP92 was very unstable in PCL1500 (Table 2). After 9 generations, more than 70% of the cells no longer carried the plasmid (data not shown), whereas after more than 25 generations, only 3 to 5% of the cells retained the plasmid (Table 2). The IncQ plasmid

pMP190 showed a stability of approximately 80 to 88% after more than 25 generations of growth, whereas the IncW plasmid pMP2740 was still present in 93 to 95% of the cells. A 100% stability was found exclusively for the plasmids pVSP41 and pWTT2081 in *P. fluorescens* WCS365 and PCL1500, respectively. Stability was hardly affected by the type of medium used (Table 2).

Since the plasmid may confer a metabolic burden to the cells (3, 24, 26), we investigated whether the presence of the stable plasmid pWTT2081 affects the competitiveness of the cells. Therefore, nonselective KB medium was inoculated with a 1:1 mixture of strain PCL1501 (*P. fluorescens* WCS365 harboring plasmid pWTT2081) and PCL1500 (a Tn*5lacZ* derivative of *P. fluorescens* WCS365 without the plasmid) and cultured for more than 25 generations in KB medium. During the experiment, samples were plated on selective and nonselective KB plates supplemented with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopgranoside; 40 μg · ml^{-1}). After more than 25 generations the majority of the colonies (95%) were blue, whereas the remainder of the colonies were tetracycline resistant (Tc[r]) and white. This result shows that the plasmid-carrying WCS365 derivative PCL1501 was outcompeted by the plasmid-free derivative PCL1500. When a similar experiment was performed, but with both strains carrying the pWTT2081 plasmid (strains PCL1501 and PCL1502) instead, the ratio of white and blue colonies remained 1:1, indicating that these two plasmid-carrying strains had indistinguishable growth rates. After streaking 100 colonies from these plates on KB medium supplemented with tetracycline, we determined that all colonies were Tc[r]. This result indicates that the decrease in cell numbers of PCL1501, the plasmid pWTT2081-carrying strain, in the first experiment was due to the presence of the plasmid, which behaves as a genetic and/or metabolic load (1, 3, 24, 26).

Stabilities of plasmids in rhizospheres of various plants. The stabilities of the various plasmids in *P. fluorescens* WCS365 and PCL1500 in the rhizospheres of potato, wheat, and tomato plants were tested. For potatoes, sterile plantlets (cultivar Bintje) were used as described by de Weger et al. (9). Surface-sterilized wheat seeds (cultivar Obelisk) and tomato seeds (cultivar Carmello; S&G Seeds B.V., Enkhuizen, The Netherlands) were allowed to germinate on agar plates containing plant nutrient solution (PNS) (13). Sterile seedlings with roots about 2 to 5 mm in length were dipped in a bacterial suspension (10^8 CFU/ml) for 1 min. Subsequently the inoculated seedlings were planted in a gnotobiotic system as described by Simons et al. (30). After 7 days of growth in the growth chamber (19°C; 16-h light period), bacteria were isolated from the tip of the root (1 to 2 cm) by vigorous shaking with glass beads (1- to 3-mm diameter) in 1 ml of PNS for 15 min. Five different plants per treatment were sampled. The experiment was performed twice. A twofold dilution was made for the suspensions from tomato roots, whereas the suspensions from potato and wheat roots were diluted 10 to 100 times before plating on KB medium that was supplemented (if required) with the appropriate antibiotic. Colonies were counted after 2 days of growth at 28°C. Maintenance of the various plasmids was determined by comparing the numbers of colonies present on selective and nonselective plates. The percentages of cells containing each plasmid, determined for each individual plant, were used to calculate means and standard deviations. Table 2 shows the results of two independent experiments.

The number of bacteria isolated from the root tip varied among plant species. Root tips of potato and wheat plants gave the largest numbers of bacteria (10^4 to 10^5 CFU · cm of
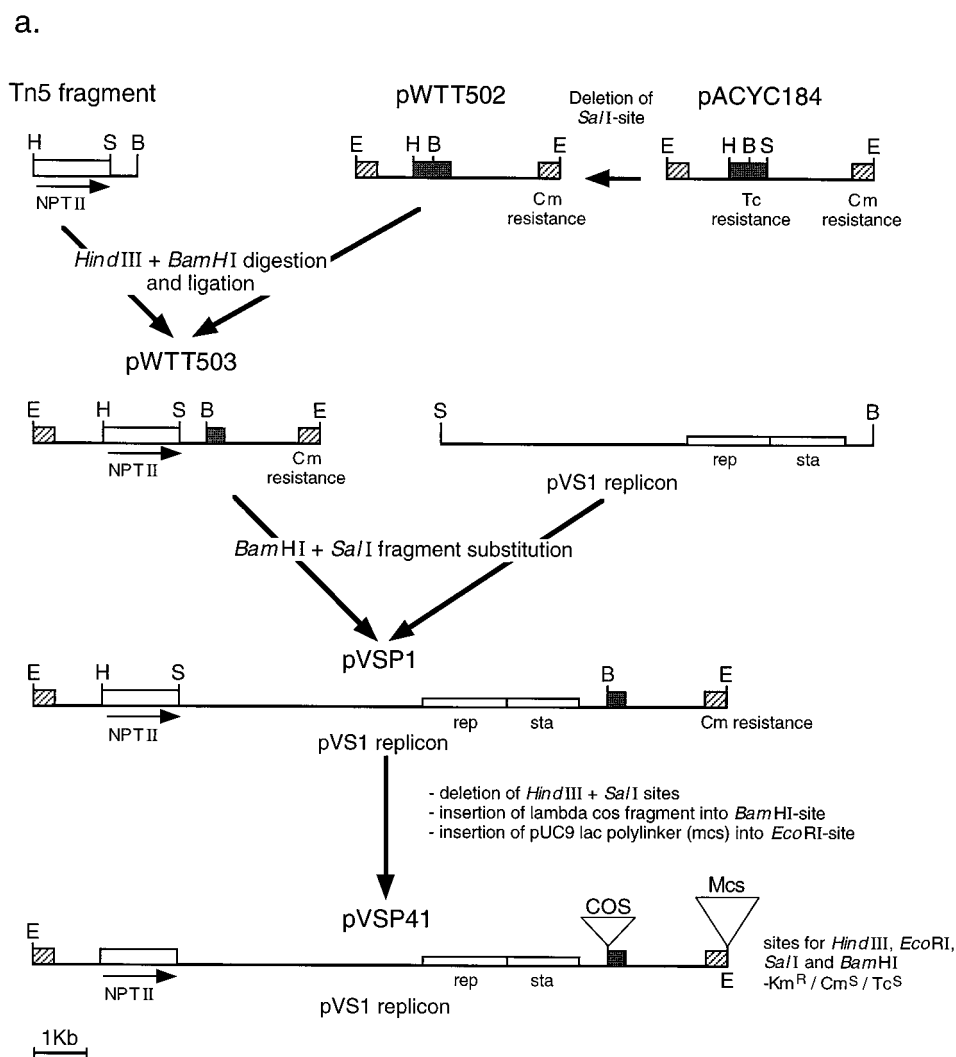
a.

FIG. 1. Construction of cloning vectors pVSP41 (a) and pWTT2081 (b). (a) For the construction of pVSP41, the *Sal*I site in pACYC184 (6) was deleted by restriction digestion, filling in, and ligation, thereby creating a new plasmid, pWTT502, in which the Tc[r] is destroyed. Subsequently, a *Hin*dIII-*Bam*HI fragment encoding the NPTII gene from the transposon Tn*5* (2) was substituted for the *Hin*dIII-*Bam*HI fragment in pACYC184 to create pWTT503, which encodes kanamycin and chloramphenicol resistance. The *Bam*HI-*Sal*I fragment encoding the replication (rep) and stability (sta) functions of pVS1 (16) was then substituted for the *Bam*HI-*Sal*I fragment (originally derived from Tn*5*) of pWTT503 to construct the precursor plasmid pVSP1. pVSP41 was constructed from pVS1 by sequential deletion of the unique *Hin*dIII and *Sal*I sites, insertion of a *Bgl*II fragment carrying the cohesive ends (cos) site of bacteriophage lambda derived from the plasmid pHC79 (14) into the *Bam*HI site, and finally insertion of the 452-bp *Hae*II fragment of plasmid pUC9 carrying the *lacZ*α region (32) into the filled-in *Eco*RI site. The final plasmid is approximately 15.3 kb in size and encodes Km[r]. (b) For the construction of the cloning vector pWTT2081, the *Bam*HI-*Sal*I fragment carrying the replication (*rep*) and stability (*sta*) regions of pVS1 (16) was ligated into the polylinker of the gentamicin-resistant (Gm[r]) plasmid pWTT595 (31a), which also contains the *Eco*RI-*Hin*dIII origin-containing fragment of pACYC184, a gentamicin resistance-encoding fragment, and a polylinker region. To construct pWTT2081, a Tc resistance gene derived from plasmid pLAFR3 (12) was modified by in vitro mutagenesis to remove internal *Sal*I and *Sma*I sites and substituted for the gentamicin resistance element. The resulting cloning vector, pWTT2081, is approximately 12 kb in size and encodes Tc[r]. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; Sp, *Sph*I. mcs, multi-cloning site.

root^{-1}), whereas tomato root tips contained approximately 10^3 to 10^4 CFU · cm of root^{-1}.

The results obtained on stability of the plasmids after growth in the rhizosphere were similar to those obtained after growth in laboratory media. Cells carrying the IncP plasmid (pMP92) were hardly detected on the root tips of the two plant species tested (Table 2). The IncQ (pMP190) and IncW (pMP2740) plasmids showed intermediate levels of stability, whereas the plasmids pVSP41 and pWTT2081 were present in all the colonies recovered from the root tips of the three plant species tested (Table 2). This result indicates that the latter plasmids are very stably maintained in the rhizosphere, independent of the plant species used.

As was shown already in laboratory media, concomitant growth of a plasmid-free and a plasmid-containing strain causes a dramatic loss in cell numbers for the latter strain. The same type of experiment was set up to examine whether this phenomenon also occurs in the rhizosphere. Therefore, seedlings or plantlets were inoculated with a 1:1 mixture of the wild-type strain WCS365 harboring the pWTT2081 plasmid (strain PCL1501) and the marked wild-type strain PCL1500 without the plasmid. After 7 days of growth, analysis of the root tip showed that hardly any bacteria (<10%) which were Tc[r] and white could be isolated on X-Gal-containing KB plates. When a 1:1 mixture of *P. fluorescens* WCS365 and PCL1500, both containing the plasmid pWTT2081 (strains PCL1501 and
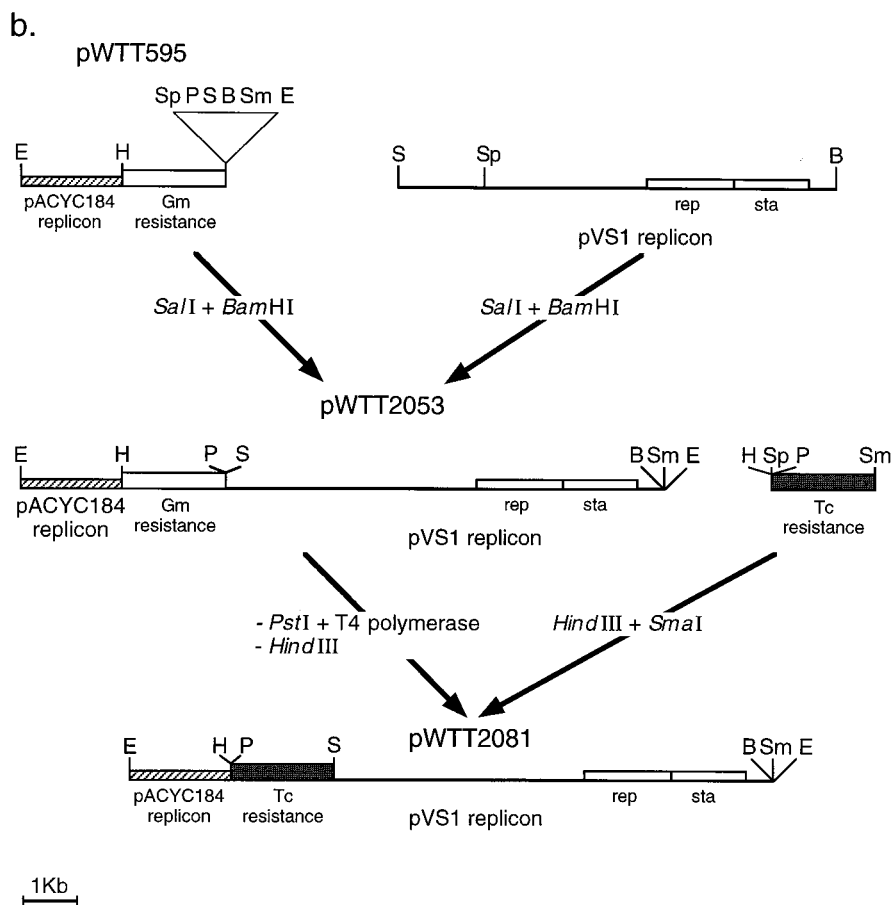
b.

pWTT595

FIG. 1—*Continued.*

PCL1502, respectively), was used for inoculating tomato, potato, and wheat plants, both cell types could be recovered from the root tip in a 1:1 ratio and all colonies were Tc[r]. The results from testing these strains together in the rhizosphere indicate that the presence of pWTT2081 negatively affects the colonization ability of the strain. However, when two strains harboring the same plasmid are used to inoculate the seedlings, the equilibrium is restored and both strains colonize the roots equally well. This knowledge is of crucial importance for colonization studies.

The results obtained in laboratory media and in the rhizospheres of the three plant species tested are all very similar. The IncP plasmid is very unstable and the IncQ and IncW plasmids show intermediate stabilities (44 to 95%), whereas the plasmids pVSP41 and pWTT2081 are completely stably maintained. The latter two plasmids carry the *rep* and *sta* regions of pVS1, which was originally isolated from *P. aeruginosa*. This region of pVS1 has previously been described as responsible for stable maintenance of *Rhizobium leguminosarum*, *Agrobacterium tumefaciens*, and several *Pseudomonas* species on the basis of tests performed in laboratory media (16). Recent results from our laboratory showed that pWTT2081 is also stable in *P. fluorescens* WCS307 in the rhizosphere of potato plants (10). A very similar plasmid, pVSP61, carrying the same pVS1 region, was also shown to be stably maintained in *Pseudomonas syringae* and *P. fluorescens* strains in the phyllosphere and rhizosphere of bean plants (20). The Inc group of the plasmid pVS1 has not been determined (15). These plas-

TABLE 2. Stabilities of various plasmids in *P. fluorescens* WCS365[a]

Plasmid	% Plasmid-containing cells in:			
	Laboratory medium[b]		Plant[c]	
	SSM	KB	Potato	Wheat
IncP pMP92	3	5 ± 3	0.2 ± 0.2	0.1 ± 0.1
			0.1 ± 0.1	0.1 ± 0.1
IncQ pMP190	80	88 ± 8	44 ± 18	84 ± 23
			44 ± 13	36 ± 8
IncW pMP2740	95	93 ± 7	87 ± 8	85 ± 18
			95 ± 13	71 ± 13
pVSP41	100	100 ± 0	100 ± 0	100 ± 0
			100 ± 0	100 ± 0
pWTT2081	100	100 ± 0	100 ± 0	100 ± 0
			100 ± 0	100 ± 0

[a] Stabilities of the various plasmids were tested in KB medium and SSM after more than 25 generations of growth without antibiotic pressure. Stabilities of the various plasmids in the rhizospheres of potato and wheat plants were tested after 7 days of growth at 19°C under gnotobiotic conditions.

[b] For SSM, the experiment was performed once and the values correspond to the percentages of cells containing the plasmid found in one experiment. For the complex medium KB, the percentages of cells containing the plasmid from three independent experiments were statistically analyzed and the means and standard deviations are presented.

[c] Five individual plants per treatment were sampled, and the percentages of cells containing the plasmid were determined for these plants. These values were statistically analyzed, and the means and standard deviations for two independent experiments are given.

mids are estimated to be present in six to eight copies per chromosome equivalent (16). The IncP plasmid with approximately 5 to 10 copies per cell appeared to be highly unstable, indicating failure of the equal distribution of the plasmids among the progeny cells. The IncQ plasmids, with an estimated copy number of approximately 20 per cell, showed intermediate stability. Plasmids pVSP41 and pWTT2081, with estimated copy numbers similar to those of the unstable IncP plasmid, appeared to be highly stable under the various conditions tested. This can most likely be attributed to the stability function encoded by the pVS1 region present in these plasmids. The very stable cloning vectors pWTT2081 and pVSP41 have been constructed (i) with a useful antibiotic marker, (ii) with unique restriction sites, and (iii) by combining the *rep* and *sta* regions of pVS1 with the replicon of pACYC184, which is functional in *Escherichia coli.*

The presence of an insert can influence the stability of the plasmid (25). Recently we have used plasmid pWTT2081 to study the complementation of a colonization-negative mutant which shows a 10- to 100-fold reduction in its colonization ability compared with that of the wild-type strain WCS365. In this experiment, plasmid pWTT2081 contained the wild-type DNA region which was affected in the mutant. When the mutant with this plasmid was inoculated onto potato plants in a 1:1 ratio with the wild-type strain WCS365 carrying the empty vector, similar numbers of plasmids were found for both strains after reisolation (7). This result indicates that the presence of an extra DNA fragment in plasmid pWTT2081 does not seriously act as a metabolic burden to the cells compared with cells carrying the empty vector. The effect of an insert will of course depend on the cloned genes as well as on the expression level of the genes, and therefore each insert should be tested separately for its effect. The results described in this paper show (i) that the plasmids pVSP41 and pWTT2081 are highly stable plasmids under rhizosphere conditions and (ii) that they are very valuable as cloning vectors for future research in which the influence of the presence of certain genes on rhizosphere colonization is tested.

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