

# The Active Partition Gene *incC* of IncP Plasmids Is Required for Stable Maintenance in a Broad Range of Hosts

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**Plasmids of incompatibility group P (IncP) are capable of replication and stable inheritance in a wide variety of gram-negative bacteria. Three determinants of IncP plasmids are components of an active partition locus that is predicted to function in the segregation of plasmid copies to daughter cells. These determinants are *incC*, which codes for a member of the ParA family of partition ATPases; *korB*, which specifies a DNA-binding protein that also functions as a global transcriptional repressor; and  $O_B$ , the DNA target for KorB, which occurs at multiple locations on IncP plasmids. To determine the importance and host range of the IncC/KorB partition system in the maintenance of IncP plasmids, we constructed an in-frame deletion of *incC* in the otherwise intact 60-kb IncP $\alpha$  plasmid R995. R995 $\Delta$ *incC* was found to be highly unstable in *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Agrobacterium tumefaciens*, and *Acinetobacter calcoaceticus*, whereas wild-type R995 is stable in all these hosts. In addition, R995 $\Delta$ *incC* could not be established in *Actinobacillus actinomycetemcomitans*. *trans*-Complementation analysis showed that the coding region for IncC2 polypeptide, which is expressed from an internal translational start within the *incC* gene, was sufficient to restore stable maintenance to wild-type levels. The results show that the IncC/KorB active partition system of IncP plasmids is remarkably proficient for stable maintenance in diverse bacteria.**

Active partition of bacterial plasmids and chromosomes into newly forming daughter cells is the functional equivalent of mitosis in eukaryotes (10, 14, 16, 19, 29). Early studies on plasmid maintenance and plasmid-plasmid incompatibility led to the identification of genetic loci for active partition (2, 27, 31–33). From sequence analysis, such loci are now known to be widespread among plasmids and bacterial chromosomes (12, 18, 51). The prototypical active partition locus consists of an autoregulated operon of two genes and a *cis*-acting DNA element functionally analogous to a chromosomal centromere. The genes specify an ATPase and a DNA-binding protein specific for the *cis*-acting element (3, 12). The partition locus constitutes a functional cassette sufficient to stabilize heterologous, unstable plasmids (1, 11, 27, 33). In a widely accepted model for active partition, replicated DNA molecules are paired in the form of nucleoprotein complexes containing the partition ATPase and the site-specific DNA-binding protein. The paired complexes are proposed to interact with a host DNA segregation apparatus at midcell, which facilitates the separation of the molecules and their translocation towards opposite poles of the cell (4, 21). The nucleoprotein partition complexes of plasmids P1, F, and R1 are understood in considerable molecular detail (8, 17, 21, 25, 37, 44). Additional critical support for the model has emerged from remarkable fluorescence microscopy studies of bacteria showing specific localization and migration of both plasmid and chromosomal DNA (14, 16, 19, 20, 36, 48). However, virtually nothing is known about the identity of the bacterial segregation appara-

tus or the specific events following the formation of nucleoprotein complexes.

The mechanism for ensuring the segregation of DNA in dividing bacterial cells promises to be especially interesting for the remarkable broad-host-range plasmids of incompatibility group P (IncP). These plasmids are stably maintained as autonomously replicating elements in a wide spectrum of gram-negative bacteria (9, 34). To understand how IncP plasmids are able to persist in markedly different bacterial hosts, it will be necessary to identify their strategies for stable inheritance and to learn how these systems interact productively with different hosts. From studies on the 60,099-bp IncP $\alpha$  plasmid RK2 (35), it is clear that stable plasmid maintenance in diverse hosts is not simply a function of replication control and that other plasmid determinants are required (42). Several stabilization loci have been identified thus far, including the *parCBA* operon, which specifies a multimer resolution system that maintains the normal plasmid copy number by reducing plasmid multimers to monomers (13, 38); the *parDE* operon, which encodes a plasmid addiction system that is toxic to plasmidless segregants (22, 39); and the *kilE* locus, which specifically stabilizes RK2 in *Pseudomonas aeruginosa* by an as yet unknown mechanism (50). In addition, RK2 encodes an active partition system with strong similarities to, but also notable differences from, partition systems common among other plasmids (30, 41). However, the significance of this partition system to the maintenance and host range of IncP plasmids has not been addressed.

Active partition in IncP plasmids was first indicated by Meyer and Hinds (28), who found an incompatibility determinant indicative of a maintenance function in the RK2 *korA* operon, which encodes the transcriptional repressors KorA and KorB. The gene responsible for incompatibility (*incC*) overlaps *korA* in a different reading frame and extends to the

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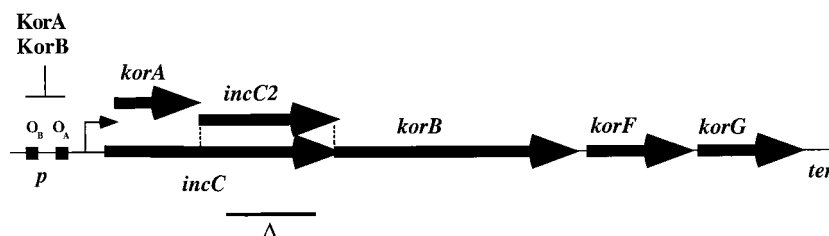


FIG. 1. *korA* operon of IncP $\alpha$  plasmids. Genes are shown as bold arrows. The *korA* gene is within the *incC* coding sequence but in a different reading frame. *incC2*, the coding region for the IncC2 polypeptide translated from an internal initiation site in *incC*; line  $\Delta$ , the 465-bp region deleted in R995 $\Delta$ *incC* (pR9401); *korF* and *korG*, genes for basic proteins of unknown function (35); p, promoter; O<sub>A</sub> and O<sub>B</sub>, binding sites for the KorA and KorB repressors, respectively; angled arrow, transcriptional start site; *ter*, a putative transcriptional terminator.

beginning of *korB* (Fig. 1) (45). *incC* expresses two polypeptides: full-length IncC1 (38.1 kDa) and the shorter IncC2 (27.5 kDa), which is initiated from an internal translation start site (24, 45). Based on their amino acid sequences, both polypeptides are members of the large family of predicted ATPases related to the ParA partition ATPase of plasmid P1 (12, 30). Fluorescence microscopy studies have shown that RK2 displays localization and migration behavior in *Escherichia coli* consistent with an active partition system (36). Recently, we reported that IncC protein interacts with KorB in vivo and in vitro and that IncC2, KorB, and one KorB-binding site (O<sub>B</sub>) are sufficient to confer the replication-independent incompatibility properties characteristic of an active partition system (41). These three elements are the RK2 counterparts to the partition ATPase, DNA-binding protein, and *cis*-acting DNA element, respectively, of the prototypical partition system. However, unlike in other systems, these RK2 partition elements are not sufficient for stabilization of a heterologous replicon in *E. coli*. A larger region of RK2 containing additional genes and O<sub>B</sub> sites is partially able to stabilize plasmids in *E. coli* in an *incC*-dependent manner (7, 41). Thus, while *incC*, *korB*, and an O<sub>B</sub> site are components of an active partition system, the genetic structure of the minimal IncP active partition system has not yet been determined.

In this study we sought to address a fundamental question concerning the IncP active partition system: is the system important for stable maintenance in a wide variety of bacteria or is it functional only in a subset of specific hosts? The answer has considerable significance. If the IncP system is host specific, then it will be critical to identify the appropriate host(s) before further analysis of the partition system can be undertaken. If the IncP active partition system is found to function in

a broad range of hosts, then it will be important to understand how such a system can be expressed appropriately and function efficiently in very different hosts.

**IncC is required for stable maintenance of R995 in *E. coli*.** We studied the function of the IncC/KorB partition system in the context of the natural ampicillin-sensitive IncP $\alpha$  plasmid R995, which is essentially equivalent to RK2 except for the absence of TnI in the *kilC* operon (Table 1) (47). The nucleotide sequence of the R995 *incC* gene is identical to that of RK2 (unpublished results). A derivative of R995 with a deletion of the *incC* gene (pR9401) was constructed by allelic exchange with a mutated *incC* subclone (Table 1). The deletion mutant is missing 465 bp of the 780-bp coding region of *incC2*, which lies between *korA* and *korB* (Fig. 1). The mutation is not expected to affect the regulation of the *korA* operon, whose transcription is controlled by the KorA and KorB repressors (Fig. 1). The in-frame deletion of *incC2* leaves *korA* intact, and it is predicted to be nonpolar on expression of the downstream *korB* gene. This property was confirmed in *E. coli* by Western blot analysis using polyclonal antiserum specific for the KorB protein (data not shown).

Wild-type R995 is completely stable in *E. coli*, with no detectable loss of the plasmid during 65 generations of unselected growth (Fig. 2A). In contrast, the R995 $\Delta$ *incC* mutant was found to be highly unstable in *E. coli*, with 50% of the population having lost the plasmid in 35 to 40 generations of unselected growth (Fig. 2A). To confirm that the high loss rate of R995 $\Delta$ *incC* resulted from the absence of the *incC* gene product, we tested the ability of *incC2* to complement the  $\Delta$ *incC* mutation in *trans*. The RK2 *incC2* coding region was inserted downstream of the inducible *tac* promoter in a compatible IncQ plasmid vector, pJAK16, to generate pRK21985

TABLE 1. Plasmids

Plasmid	Marker(s) <sup>a</sup>	Relevant genotype	Description	Reference or source
pJAK16	Cm <sup>r</sup>	<i>lacI<sup>q</sup> tacp</i>	IncQ replicon; expression vector	J. Kornacki
pRK21985	Cm <sup>r</sup>	<i>lacI<sup>q</sup> <math>\Phi</math>(tacp-<i>incC2</i>)</i>	pJAK16 with <i>incC2</i> coding region expressed from <i>tacp</i>	41
pRK22324	Ap <sup>r</sup>	$\Delta$ <i>incC</i>	P15A replicon carrying the <i>korA incC korB</i> region of RK2 with 465-bp, in-frame deletion within <i>incC</i>	41
pR9401 <sup>b</sup>	Km <sup>r</sup> Tc <sup>r</sup>	$\Delta$ <i>incC</i>	R995 with 465-bp, in-frame deletion within <i>incC</i>	This work
R995	Km <sup>r</sup> Tc <sup>r</sup>	<i>incC</i> <sup>+</sup>	Natural IncP $\alpha$ plasmid	47

<sup>a</sup> Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; r, resistance.

<sup>b</sup> A pRK22324/R995 cointegrate was selected by conjugation of *E. coli* donor cells containing both plasmids with a Nal<sup>r</sup> recipient. The cointegrate was transferred to *penB* mutant strain LS1443 (26), in which the P15A replicon of pRK22324 is unstable. Resolved cointegrates were screened for Ap<sup>r</sup>, and the  $\Delta$ *incC* mutation was detected by PCR. pR9401 was confirmed by restriction analysis.

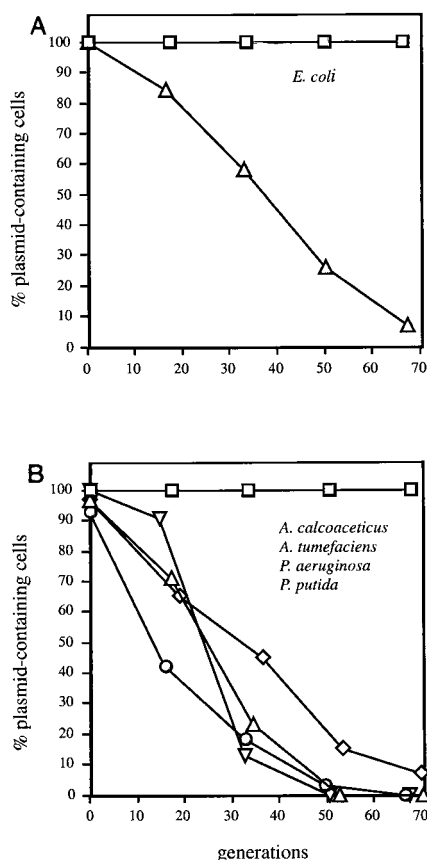


FIG. 2. Stability of R995 and R995 $\Delta$ incC in different hosts. (A) *E. coli* host. R995 ( $\square$ ) and R995 $\Delta$ incC ( $\Delta$ ) in *E. coli* DF4063 (43). (B) Other gram-negative hosts. R995 $\Delta$ incC in *A. calcoaceticus* BD413 (42) ( $\diamond$ ), *A. tumefaciens* A136 (42) ( $\nabla$ ), *P. aeruginosa* PAC452 (43) ( $\Delta$ ), and *P. putida* ATCC 12633 (6) ( $\circ$ ). The results for all hosts with wild-type R995 were indistinguishable from the results for *P. aeruginosa*, which are displayed here ( $\square$ ). Strains were grown to saturation in the absence of selection and then diluted  $10^5$ -fold in fresh broth. This procedure was repeated until the strains had grown for at least 65 generations. Growth conditions for all bacteria have been described previously (6, 42, 43). Colonies were screened for the presence of plasmids on medium containing kanamycin or tetracycline.

(Table 1). The maintenance of R995 $\Delta$ incC was then assayed in *E. coli* cells containing either the pJAK16 vector or the *tacp*-*incC2* derivative (Fig. 3A). The pJAK16 vector showed no ability to stabilize R995 $\Delta$ incC. In contrast, uninduced expression of *incC2* from pRK21985 (without isopropyl- $\beta$ -D-thiogalactopyranoside [IPTG]) allowed complete stabilization of R995 $\Delta$ incC to wild-type levels. No plasmid loss was detected in 65 generations. With the pJAK16 vector in *trans*, 95% of the cells emerging over the same period lacked R995 $\Delta$ incC. At 1 mM IPTG, R995 $\Delta$ incC was destabilized, as expected from our previous results showing that elevated expression of *incC2* causes rapid loss of IncP plasmid RK2 (41). Thus, low-level expression of *incC2* from the leaky *tacp* promoter is sufficient to fully complement the  $\Delta$ incC mutation. We conclude that *incC* is required for the stable maintenance of R995 in *E. coli*.

Agarose gel electrophoresis revealed no differences in the amounts of R995 and R995 $\Delta$ incC relative to that of a coresident IncQ plasmid (data not shown). Thus, the R995 copy

number was not affected by the  $\Delta$ incC mutation. Conjugative plasmids can promote their maintenance in a cell line by self-transfer to plasmidless segregants (43), raising the possibility that the  $\Delta$ incC mutation affects conjugative transfer. However, the R995 $\Delta$ incC mutant was found to transfer at the same frequency as that of the wild type, R995, in matings of *E. coli* with *E. coli* (data not shown). Thus, the properties of R995 $\Delta$ incC are consistent with a defect in DNA segregation.

**IncC is required for stable maintenance of R995 in diverse gram-negative hosts.** We compared the maintenance properties of R995 and R995 $\Delta$ incC in broth cultures of *P. aeruginosa*, *Pseudomonas putida*, *Acinetobacter calcoaceticus*, and *Agrobacterium tumefaciens* (Fig. 2B). As expected for an IncP plasmid, R995 was stably maintained in all these hosts. In striking contrast, R995 $\Delta$ incC was rapidly lost from growing populations of each of these hosts. To confirm *trans* complementation in a non-*E. coli* host, we placed the broad-host-range IncQ plasmid with *tacp*-*incC2* (pRK21985) and the *tacp* vector control (pJAK16) in *trans* to R995 $\Delta$ incC in *P. aeruginosa*. Similar to the results observed for *E. coli*, low-level expression of *incC* was sufficient to fully stabilize the R995 $\Delta$ incC mutant in *P.*

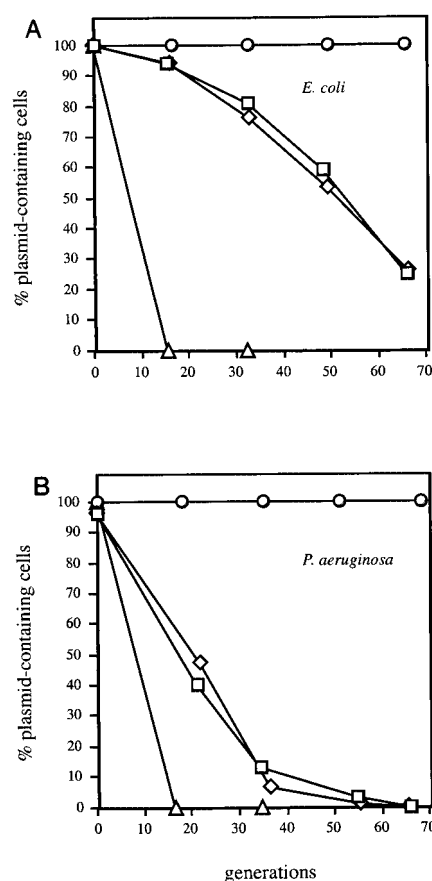


FIG. 3. Complementation of R995 $\Delta$ incC by *incC2* in *trans*. (A) *E. coli* DF4063 host; (B) *P. aeruginosa* PAC452 host. Results for strains containing R995 $\Delta$ incC and pJAK16 (*tacp* vector) without IPTG ( $\square$ ) and with 1 mM IPTG ( $\diamond$ ), and containing R995 $\Delta$ incC and pRK21985 (*tacp*-*incC2*) without IPTG ( $\circ$ ) and with 1 mM IPTG ( $\Delta$ ) are shown. Assays were done as described in the legend to Fig. 2, except that selection for pJAK16 and pRK21985 was maintained.

TABLE 2. Phenotype of R995 $\Delta$ incC in *A. actinomycetemcomitans*

Plasmid(s) in donor <sup>a</sup>	Plasmid in recipient <sup>b</sup>	Relative no. of transconjugants <sup>c</sup>
R995	NA	1.0
R995 $\Delta$ incC	NA	<10 <sup>-4</sup>
R995, pJAK16	NA	1.0, 0.9 <sup>d</sup>
R995 $\Delta$ incC, pJAK16	NA	<10 <sup>-4</sup> , 1.0 <sup>d</sup>
R995	<i>tacp</i> vector	1.0
R995 $\Delta$ incC	<i>tacp</i> vector	<10 <sup>-4</sup>
R995 $\Delta$ incC	<i>tacp-incC2</i>	1.0

<sup>a</sup> *E. coli* LS1443 (26).

<sup>b</sup> *A. actinomycetemcomitans* Y4Nal (46). NA, not applicable.

<sup>c</sup> Four-hour matings at 37°C were done as described previously (46). Transconjugants were selected on media containing a combination of the following antibiotics: kanamycin (20 µg/ml), chloramphenicol (2 µg/ml), and nalidixic acid (20 µg/ml). The relative number of transconjugants was obtained by dividing the number of transconjugants per donor for the test plasmid by the number of transconjugants per donor for R995 for each experiment. Results are the average of two experiments.

<sup>d</sup> Relative numbers of R995 and pJAK16 transconjugants, respectively.

*aeruginosa* and high-level expression destabilized the plasmid (Fig. 3B). The results show that *incC* is required for stable maintenance of R995 in the four gram-negative hosts.

We encountered a severe phenotype expressed by R995 $\Delta$ incC when we attempted to transfer the plasmid into *Actinobacillus actinomycetemcomitans* for stability assays. Wild-type R995 readily formed healthy transconjugants, but R995 $\Delta$ incC produced transconjugant colonies at a frequency lower than 10<sup>-4</sup> that of R995 (Table 2). The R995 $\Delta$ incC transconjugant colonies that did appear were pinpoint and grew poorly, if at all, when restreaked on selective medium. Conjugative transfer of R995 $\Delta$ incC was shown above to be normal with an *E. coli* recipient. To confirm that conjugative transfer to *A. actinomycetemcomitans* was also normal, we examined the ability of R995 $\Delta$ incC to mobilize the IncQ plasmid pJAK16 to this host. IncQ plasmids are not able to self-transfer, but they are efficiently mobilized by the IncP plasmid transfer apparatus and they replicate in *A. actinomycetemcomitans* (15). We found that R995 and R995 $\Delta$ incC mobilized pJAK16 from *E. coli* to *A. actinomycetemcomitans* at the same frequencies (Table 2). Thus, the failure to obtain R995 $\Delta$ incC transconjugants of *A. actinomycetemcomitans* indicates a defect in plasmid establishment or maintenance in this host and leads to the prediction that *incC* in the recipient should complement the defect. This prediction was confirmed in matings with an *A. actinomycetemcomitans* recipient carrying the *tacp-incC2* plasmid pRK21985. Healthy transconjugants of R995 $\Delta$ incC appeared at the same frequency as those of wild-type R995 (Table 2). These results show that *incC* is essential for stable inheritance of R995 in *A. actinomycetemcomitans*.

**Significance.** This study was undertaken to determine the importance and host range of the IncC/KorB active partition system of promiscuous IncP plasmids. The results show unequivocally that *incC* is required for stable maintenance of IncP plasmid R995 in *E. coli* and five other gram-negative hosts. In each case, the nonpolar  $\Delta$ incC mutation of an otherwise wild-type IncP plasmid caused the plasmid to be lost rapidly from growing cultures. Furthermore, the shorter *incC2* gene in *trans* was sufficient to restore a wild-type level of

stability. The function of the full-length *incC* gene remains to be determined.

The R995 $\Delta$ incC loss rates appear to be greater than those expected for a randomly diffusing plasmid with a copy number of 10 to 20 at cell division (5, 50). Recent fluorescence microscopy studies by Pogliano et al. (36) and Weitao et al. (49) may suggest an explanation. Their studies showed that multicopy plasmids are not randomly distributed in cells and that they may organize in clusters. Alternatively, abortive partition complexes may be formed in the absence of IncC, such that plasmids are localized but fail to segregate (7).

The significant rates of R995 $\Delta$ incC loss observable for all hosts tested also underline the importance of the active partition system to the broad host range of IncP plasmids. R995 $\Delta$ incC carries the determinants for multimer resolution (*parCBA*) and plasmid addiction (*parDE*), both of which function to stabilize IncP plasmids to varying degrees in these hosts (43). In addition, the *kilE* locus needed for maintenance in *P. aeruginosa* is intact. There is no reason to expect that multimer resolution and plasmid addiction are dependent upon the IncC protein, and indeed studies with a derivative of R995 with deletions of both the *incC* gene and the *par* region, which contains *parCBA* and *parDE*, show that the phenotypes are additive, as expected for independent pathways. At 33 generations, R995 $\Delta$ par was present in 98% of cells, R995 $\Delta$ incC was present in 75% of cells, and R995 $\Delta$ par $\Delta$ incC was present in 6% of cells. It is not yet known whether the *kilE* locus acts independently of *incC*.

Active partition systems are generally considered to be non-essential accessory functions that enable replicons to be stably maintained by their bacterial hosts. However, *A. actinomycetemcomitans* cells that have received R995 $\Delta$ incC do not form colonies under selection. The *parDE* postsegregational toxicity system is not responsible for this phenotype, since the removal of *parDE* from the R995 $\Delta$ incC mutant did not cause a change in phenotype (unpublished results). Clearly the absence of *incC* in *A. actinomycetemcomitans* has serious consequences, and there are several possible explanations for the phenotype. While the level of KorB is unchanged by the absence of IncC in *E. coli*, it remains possible that it is affected in *A. actinomycetemcomitans*. Increased levels of KorB could inhibit replication, whereas decreased levels of KorB may allow derepression of toxic plasmid genes (35). It has also been observed for plasmids F and P1 that, under certain circumstances, the DNA-binding protein causes the silencing of several kilobases of DNA surrounding the *cis*-acting DNA-binding site (17, 40). Perhaps a similar silencing of R995 $\Delta$ incC is induced in *A. actinomycetemcomitans* by the absence of IncC. Another possibility is that the absence of IncC causes IncP plasmids to aggregate, thereby blocking their segregation to daughter cells. Such a phenotype has been suggested for partition mutants of P1 (5). The failure to form colonies might also be caused by inhibition of cell growth, which might, for example, be due to interference with the host DNA segregation apparatus caused by its interaction with an incomplete IncP partition system. The strong phenotype in *A. actinomycetemcomitans* may prove useful for selecting plasmid or host mutants that reveal other components or properties of the IncP partition system.

How is the IncP partition system able to function efficiently in a broad range of hosts? Recent experiments have shown that

the Soj/Spo0J chromosomal partition system of *Bacillus subtilis* can function in *E. coli*, suggesting that DNA segregation machinery is highly conserved in bacteria. It is therefore possible that the broad-host-range IncP partition system has evolved to fully exploit the conserved features of bacterial systems for efficient function in multiple hosts. Alternatively, functioning of the basic IncC/KorB partition system of IncP plasmids in various bacteria may be facilitated by additional plasmid-encoded, host-specific adapters. For example, the *kilE* locus, which is required for stable maintenance in *P. aeruginosa*, may express partition adapters specific for this host. Such a collaboration might serve to explain why *incC/korB* and *kilE* are coregulated as part of the unique *kor* regulon of IncP plasmids (23). The answer awaits further investigation of the IncP plasmid partition system.

In summary, we have determined that the IncC partition protein of IncP plasmids is required for stable maintenance in diverse gram-negative bacterial hosts of the  $\alpha$  and  $\gamma$  subdivisions of the *Proteobacteria*. The clear instability phenotype of the partition defect, as revealed by the deletion of *incC*, will allow the identification of other components of the IncP active partition system. In addition, the ability to fully complement the  $\Delta incC$  mutation in *trans* has opened the door to a detailed molecular genetic analysis of the functions of the IncC protein. We anticipate that future studies on the IncP partition system will lead to a better understanding of plasmid host range and DNA segregation in bacteria.

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