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Isolation of the Replication and Partitioning Regions of the *Salmonella typhimurium* Virulence Plasmid and Stabilization of Heterologous Replicons

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Although the virulence plasmid of *Salmonella typhimurium* has a copy number of one to two per chromosome, plasmid-free segregants are produced at a rate less than 10^{-7} per cell per generation. Three regions appear to be involved in the maintenance of this virulence plasmid. The first two, *repB* and *repC*, are functional replicons hybridizing with IncFII and IncFI plasmids, respectively, neither exhibiting the segregational stability of the parent virulence plasmid. The third region, *par*, cloned as a 3.9-kilobase *Sau*3A fragment, is not a functional replicon but exhibits incompatibility with the virulence plasmid. Subsequent tests revealed the ability of this 3.9-kilobase *par* insert to increase the stability of pACYC184 in *S. typhimurium* from less than 34% to 99% plasmid-containing cells after 50 generations. In addition, the *par* region increased the stability of *oriC*, R388, and *repC* replicons in both *S. typhimurium* and *Escherichia coli* hosts. The *par* region encodes 44,000- and 40,000-molecular-weight proteins essential for the Par$^+$ phenotype but not for the Inc$^+$ phenotype. Although actual sequestering of plasmids within the cell was not demonstrated, all results indicate that the *par* region described is an actual partitioning locus, similar in organization to those described for plasmids F, P1, and NR1.

The reliable maintenance of plasmids within a population requires both accurate replication and consistent distribution of plasmids to viable progeny. Although mechanisms ensuring plasmid maintenance have been described for other replicon systems, the mechanisms employed by the virulence plasmid of *Salmonella typhimurium* remain obscure. Previous studies of *S. typhimurium* have revealed a correlation between the presence of a large (90- to 100-kilobase [kb]), low-copy-number plasmid (49), called pSLT by Jones et al. (27), and invasive virulence (21, 27, 46). The ubiquitous distribution of large plasmids among *S. typhimurium* strains isolated from various sources (8, 25, 47) indicates that natural systems for reliable maintenance of these plasmids have evolved in *S. typhimurium*.

The complex nature of many large stable plasmids involves various mechanisms to ensure their maintenance, including multiple replicons (4), partitioning regions (1), host killing (18), or control-of-division functions (44). The localization and function of the two replication regions (36) or other possible maintenance regions of the *S. typhimurium* virulence plasmid, however, remain unresolved. The segregational stability ($<10^{-7}$ segregants per cell per generation) of the virulence plasmid (21) is similar to that observed for other low-copy-number plasmids, such as F (29), P1 (2), and NR1 (37). Plasmids with copy numbers this low require some form of active partitioning if they are to avoid the production of 25% plasmid-free cells per generation inherent with the random distribution of such plasmids (1).

Various approaches to increase the proportion of plasmid-containing cells within a population have been investigated, including those selecting against plasmid-free cells, such as the balanced-lethal *asd* vector system (38) and the *valS* system (39), or the implementation of maintenance functions isolated from naturally occurring plasmids (7). This study examines the maintenance functions of the virulence plasmid and the potential use of the *par* region to stabilize heterologous replicons. Two self-replicating regions on the 91-kb virulence plasmid are described, one from within the *repB* region described by Michiels et al. (36) and the other occurring outside either previously described *rep* region. The correlation of incompatibility with partition system function (43) was used as the basis for the selection of potential virulence plasmid partitioning region clones. A 3.9-kb *Sau*3A fragment cloned from the virulence plasmid was found to exhibit incompatibility with the parent virulence plasmid while increasing the segregational stability of the cloning vector, pACYC184. This region, called *par*, is analyzed for similarities in function (including the stabilization of other replicons) and organization with those partitioning regions described for other low-copy-number plasmids such as F (45), P1 (2), and NR1 (51).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Tables 1 and 2, respectively.

Culture media and growth conditions. The complex media used were L broth and L agar (32). The defined media used contained minimal salts (12) and supplements as previously described (14). Media were supplemented with antibiotics at the following concentrations (micrograms per milliliter): ampicillin, 50 or 100; chloramphenicol, 25; kanamycin, 50; and tetracycline, 15. Fusaric acid-containing medium was made as described by Bochner et al. (5).

Genetic exchange. Transformation was performed by using the protocol described by Dagert and Ehrlich (15). Conjugations were performed by using plate matings (13).

DNA manipulations. Mini-lysate and large-scale plasmid extractions were performed as described by Birnboim and Doly (6). Agarose gel electrophoresis, Southern blot hybridization, and cesium chloride density gradient centrifugation were performed as described by Maniatis et al. (33). Restric-

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TABLE 1. Bacterial strains used

Strain	Genotype	Source and description
E. coli K-12		
χ1891	F⁻ *thr-16 tsx-63 purE41 glnV42* λ⁻ Δ*trpE63 his-53 gyrA23 srl-2* Δ*thyA57* T3ʳ *mtlA9 polA12*(Ts) *cycA1 cycB2*	Met⁺ *PolA*(Ts) transductant of χ1806 obtained by using P1L4 grown on χ1693
C118	F⁻ *araD139* Δ(*ara-leu*)*7697* Δ*lacX74* Δ*phoA20 galE galK recA1 rpsE argE*(Am) *rpoB thi*	Obtained from C. Manoil
SE5000	F⁻ *araD139* Δ(*argF-lac*)*U169 flbB5301 ptsF25 recA56 relA1 rpsL150 rbsR deoC1*	Obtained from G. Weinstock
LE392	*lacY1 glnV44 galK2 galT22 tyrT58 metB1 hsdR514 trpR55*	Efficient transformer received from P. Leder (32)
χ6106	F⁻ pStSR101⁺ *thr-1 ara-14 leuB6 proA2 lacY1 tsx-33 glnV44 galK2 sbcB15 his-4 recB21 recC22 rpsL31 xyl-5 mtl-1 argE3 thi-1*	JC7623; Kushner et al. (31); obtained from A. J. Clark; transformed with pStSR101
S. typhimurium SR-11		
χ3337	pStSR100⁻ *gyrA1816*	χ3306 cured of the 91-kb virulence plasmid; Gulig and Curtiss (21)
S. typhimurium LT-2		
χ3364	pStLT100⁺ *hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*⁺ (*E. coli*) Δ*zja*::Tn*10 hsdSA29 val*	Tetˢ derivative of AS68 received from T. Palva
χ3385	pStLT100⁻ *hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*⁺ (*E. coli*) Δ*zja*::Tn*10 hsdSA29 val*	Tcˢ plasmid-cured derivative of AS68 obtained from T. Palva
χ3387	pStLT100⁻ *hsdL6 galE496 trpB2 flaA66 his-6165 recA1 srl-202*::ΔTn*10 rpsL120 xyl-404 metE551 metA22 lamB*⁺ (*E. coli*) Δ*zja*::Tn*10 hsdSA29 val*	RecA⁻ derivative of χ3385 obtained by P22HT *int* transduction of *recA1* from TT521 (28)
χ3477	pStSL100⁻ *hsdL6* Δ(*gal-uvrB*)*-1005 flaA66 rpsL120 xyl-404 lamB*⁺ (*E. coli*) Δ*zja*::Tn*10 hsdSA29 val*	Derived from χ3385 after transduction with P22HT *int* lysate from SL5400, obtained from B. A. D. Stocker, to introduce Δ(*gal-uvrB*)*-1005*
χ3934	pStSR101⁺ *hsdL6 galE496 trpB2 flaA66 his-6165 recA1 srl-202*::ΔTn*10 rpsL120 xyl-404 metE551 metA22 lamB*⁺ (*E. coli*) Δ*zja*::Tn*10 hsdSA29 val*	Tcʳ derivative of χ3387 transformed with pStSR101

tion enzyme digests and ligations were carried out with enzymes from International Biotechnologies, Inc. (New Haven, Conn.), and Promega Biotec (Madison, Wis.) as instructed by the manufacturer. DNA fragments were isolated from Tris-acetate agarose gels, using an Elutrap from Schleicher & Schuell, Inc. (Keene, N.H.) as instructed by the manufacturer. In vitro packaging of cosmids was accomplished using the Packagene system from Promega Biotec as instructed by the manufacturer. Production of [α-^{32}P]ATP (specific activity, 800 Ci/mmol; Du Pont Co., Wilmington, Del.)-labeled DNA was performed by using either the nick translation kit from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), or the random primer labeling kit from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) as instructed by the manufacturer. In vitro transcription-translation was performed by using [^{35}S]methionine from Amersham Corp. (Arlington Heights, Ill.) with the DNA-directed transcription-translation kit from Amersham, as instructed by the supplier. Radiolabeled proteins were resolved in 10% (wt/vol) polyacrylamide gels and identified by fluorography with En^{3}Hance from Du Pont.

Stability and incompatibility tests. Stability tests for each plasmid-containing strain were carried out in duplicate. Serial dilutions of 1:1,000 were performed by using standing overnight cultures in L broth or aerated overnight cultures in minimal media without antibiotics as indicated. Dilutions were plated on nonselective L agar, and colonies were replicated to selective media to monitor loss of plasmids. Incompatibility tests were performed in a similar manner. After the transfer of both plasmids to be tested into the same host, strains were grown overnight selecting for both plasmids, diluted 1:1,000 into nonselective L broth or L broth containing antibiotics selecting for one of the plasmids, and grown without aeration at 37°C. Loss of plasmids at the end of 10 generations was again determined by plating on nonselective media and replicating to selective media.

Construction of the pStSR100 *Sau*3A library in pACYC184. pStSR100 DNA was partially digested with *Sau*3A and fractionated on a 5 to 30% (wt/vol) neutral sucrose gradient (3) to isolate fragments predominately in the 4- to 6-kb range. pACYC184 was cut with *Bam*HI and then treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals) as instructed by the manufacturer before ligation to the *Sau*3A-generated pStSR100 fragments. Ligated DNA was then transformed into χ3477, the library colonies were pooled, and plasmid DNA was extracted. The library DNA was then purified by cesium chloride density gradient centrifugation and transformed into χ3934 (Table 1).

Functional replicons from the virulence plasmid were obtained by ligating random fragments generated by either *Sau*3A or *Bst*EII digests of pStSR100 DNA to nonreplicating antibiotic resistance markers.

Estimation of plasmid copy number. Plasmid copy number was estimated relative to an internal plasmid standard (53) by isolating plasmid DNA as described by Birnboim and Doly (6). Plasmid DNA was then run on a 0.6% agarose gel before staining with ethidium bromide. Photographic negatives of the gel were then subjected to densitometer quantification using a Joyce Loebl densitometer (Gateshead 11, England) to estimate relative amounts of each plasmid.

RESULTS

Isolation of the *repB* replicon. Three clones, each containing one independent replicon, *repB*, were isolated from the

TABLE 2. Plasmids used

Plasmid	Phenotype	Description
F		IncFI from χ15
F-amp	Ap^r	IncFI from χ2948
F-kan	Km^r	IncFI from χ2949
R100	Cm^r Sm^r Sp^r Su^r Tc^r	IncFII from χ1781
R64*drd-11*	Sm^r Tc^r	IncI from χ1780
R702	Km^r Sm^r Su^r Tc^r	IncP from χ1909 received from R. Olsen
R726-1	Cm^r Sm^r Su^r	IncH from χ2086
pACYC184	Cm^r Tc^r	4.2-kb medium-copy-number, DNA polymerase I-dependent plasmid; Chang and Cohen (9)
pHSG422	Ap^r Cm^r Km^r	8.76-kb temperature-sensitive pSC101 cosmid vector; Hashimoto-Gotoh et al. (24)
pStSR100		91-kb virulence plasmid of *S. typhimurium*; Gulig and Curtiss (21)
pStSR101	Tc^r	Tn*mini-tet*-labeled virulence plasmid; Gulig and Curtiss (21)
pStLT200	Km^r Tc^r	pStLT100Ω*parB*[90.1 kb *Eco*RV::Km]
pStLT201	Km^r Tc^r	pStLT100Ω[88.2 kb *Eco*RV::Km]
pStLT202	Km^r Tc^r	pStLT100Ω[88.0 kb *Sma*I::Km]
pStLT203	Km^r Tc^r	pStLT100Ω*parA*[89.1 kb *Sma*I::Km]; unstable
pUC-4K	Ap^r Km^r	High-copy-number plasmid used as source of Km^r cartridge; Vieira and Messing (55)
pUC18	Ap^r αLacZ$^+$	2.7-kb high-copy-number cloning vector; Yanisch-Perron et al. (58)
pXX199	Ap^r	*oriC*-based plasmid containing the F plasmid *sop* genes; obtained from S. Hiraga (45)
pYA2012	Ap^r Cm^r	pUC18 containing a *Bam*HI Cm^r cartridge from pACYC184
pYA2013	Cm^r	Self-replicating 4.9-kb *repB* clone obtained by ligating a *Sau*3A partial digest of pStSR100 to the Cm^r cartridge of pYA2012
pYA2014	Cm^r	Self-replicating 6.6-kb *repB* clone obtained as for pYA2013
pYA2015	Cm^r	Self-replicating 7.1-kb *repB* clone obtained as for pYA2013
pYA2018	Km^r	Self-replicating 4.4-kb *repB* subclone of pYA2015 containing the Km^r cartridge of pUC-4K
pYA2027	Cm^r Par^+	*par* region of pStSR100 in a 3.9-kb *Sau*3A fragment cloned into the *Bam*HI site of pACYC184
pYA2028	Ap^r	pUC18 containing *par* from pYA2027
pYA2029	Cm^r	*Hin*dIII subclone of pYA2027 containing *parB* in pACYC184
pYA2030	Cm^r	*Hin*dIII subclone of pYA2027 in pACYC184
pYA2034	Tc^r	*Sma*I subclone of pYA2027 in pACYC184 *Pvu*II site
pYA2039	Cm^r	*Eco*RI subclone of pYA2027 in pACYC184
pYA2041	Cm^r	*Kpn*I subclone of pYA2027 containing *parA* in pACYC184
pYA2044	Ap^r	*Bst*EII cosmid clone of pStSR100 containing *repC* and *par* obtained by ligation of the Ap^r *cos Bst*EII fragment to pStSR100 *Bst*EII fragments
pYA2045	Ap^r	*oriC* obtained by deleting the *Eco*RI-*Pst*I fragment containing the *sop* region of pXX199
pYA2046	Ap^r	*oriC* (pYA2045) containing *par* from pYA2028
pYA2047	Ap^r Km^r	*oriC* (pYA2045) containing *par* 88.0-kb *Sma*::Km insert from pYA2057
pYA2050	Km^r	3.2-kb *Bgl*II fragment of pStSR100 containing *repC* ligated to the Km^r fragment of pUC-4K
pYA2051	Ap^r Km^r	pUC18 containing *par* with a Km^r insert at *Eco*RV 90.1 kb
pYA2052	Cm^r Km^r	pACYC184 containing *par* with a Km^r insert at *Eco*RV 90.1 kb; inactivates *parB*
pYA2054	Ap^r Km^r	pUC18 containing *par* with a Km^r insert at *Eco*RV 88.2 kb
pYA2055	Cm^r Km^r	pACYC184 containing *par* with a Km^r insert at *Eco*RV 88.2 kb; does not inactivate either *par* gene
pYA2057	Ap^r Km^r	pUC18 containing the *par* region with a Km^r insert at *Sma*I 88.0 kb
pYA2058	Cm^r Km^r	pACYC184 containing *par* with a Km^r insert at *Sma*I 88.0 kb; does not inactivate either *par* gene
pYA2060	Ap^r Km^r	pUC18 containing *par* with a Km^r insert at *Sma*I 89.1 kb
pYA2061	Cm^r Km^r	pACYC184 containing *par* with a Km^r insert at *Sma*I 89.1 kb
pYA2063	Ap^r Km^r	*par* containing the Km^r insert at *Sma*I 88.0 kb, in the *Bam*HI site of pYA2204
pYA2064	Ap^r Km^r	pYA2204 containing a *Sau*3A fragment of the *par* region with the Km^r insert at *Sma*I 88.0 kb, obtained from pYA2057
pYA2065	Km^r	3.2-kb *Bgl*II *repC* fragment from pStSR100 ligated to the *par* region containing the Km^r insert at *Sma*I 88.0 kb, obtained from pYA2064
pYA2067	Cm^r Km^r	pACYC184 containing *par* with the Km^r insert at *Sma*I 88.0 kb, obtained from pYA2064
pYA2068	Cm^r	*Pst*I subclone of pYA2027 containing *parA* in pACYC184
pYA2070	Km^r	46-kb *Xho*I fragment of pStSR100 containing *repB* and *repC* ligated to the Km^r cartridge of pUC-4K
pYA2071	Ap^r Km^r	pYA2045 containing *parA parB*[90.1 kb *Eco*RV::Km] from pYA2051
pYA2072	Ap^r Km^r	pYA2045 containing *parA*[89.1 kb *Sma*I::Km] *parB* from pYA2060
pYA2204	Ap^r αLacZ$^+$	8.9-kb low-copy-number derivative of pREG153; Galan et al. (17)

virulence plasmid (Fig. 1) by ligating 2- to 6-kb *Sau*3A fragments of pStSR100 to the nonreplicating 1.4-kb *Bam*HI Cm^r cartridge of pYA2012. The restriction patterns of these clones, pYA2013 (4.9 kb), pYA2014 (6.6 kb), and pYA2015 (7.1 kb), revealed a common 3.4-kb region, with the *Hin*dIII and *Bgl*II sites of pYA2015 (Fig. 2) corresponding to those within the *repB* region of pStSR100 (Fig. 1) described by Michiels et al. (36). Blunt-end ligation of the T4 DNA polymerase-treated 3.0-kb *Hin*dIII-to-*Kpn*I fragment of pYA2015 to the 1.4-kb *Hin*cII Km^r cartridge from pUC-4K produced a functional replicon, pYA2018 (Fig. 2), whereas insertion of the Cm^r marker of pYA2012 into either the *Bgl*II

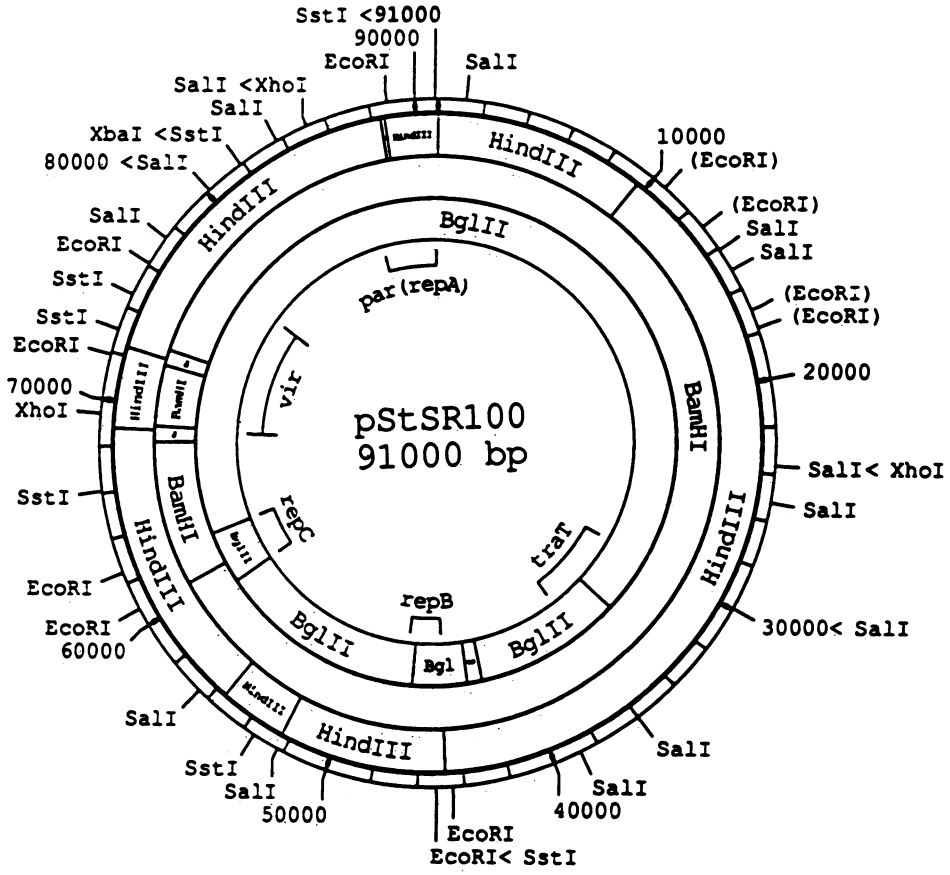

FIG. 1. Map of the virulence plasmid, pStSR100, of *S. typhimurium*, based on the map of Michiels et al. (36). The designations of *repA* and *repB* are from Michiels et al. (36), the position of *vir* is from references 20 and 22, the position of *traT* was determined by Rhen et al. (48), and the positions of *par*, *repB*, and *repC* were deduced from this work. Sites in parentheses have not been confirmed.

or the *Hin*cII site of pYA2018 failed to produce any functional replicons, suggesting that the midsection of this 3.0-kb region is required for replication.

Confirmation that these replicon clones were derived from the *repB* region of pStSR100 was accomplished by probing a Southern blot of pStSR100 fragments with one of the *repB* clones, pYA2013. The Southern blot (data not shown) revealed that pYA2013 hybridized with the 7.6- and 35-kb *Hin*dIII bands and the 60-kb *Bam*HI fragment of pStSR100 (Fig. 1). Hybridization data, along with the presence of a single *Bgl*II site within pYA2015 (Fig. 2), indicated that the *Hin*dIII site of pYA2015 corresponds to the 44.8-kb *Hin*dIII site of pStSR100, while the *Bgl*II site corresponds to the 46.6-kb *Bgl*II site of pStSR100, as shown for pYA2015 in Fig. 2.

Isolation of the *repC* replicon. A second replicon of the virulence plasmid is purported to lie within the *repA* region (Fig. 1) defined by Michiels et al. (36). Cosmids containing virulence plasmid DNA were constructed by ligating the nonreplicating 2.2-kb *Bst*EII fragment of pHSG422 (24), containing the λ *cos* site and Ap^r, to >40-kb fragments isolated from a *Bst*EII partial digest of pStSR100. This cosmid ligation mix was packaged in vitro and transduced into LE392, followed by selection for Ap^r. Eight isolated clones were screened for hybridization with the *repB* replicon, pYA2015, and the presence of the 3.6-kb *Bgl*II and 7.6-kb *Hin*dIII fragments associated with the *repB* region. Only one of the four $RepB^-$ cosmid replicons, pYA2044, contained the 2.4-kb *Hin*dIII fragment from the *repA* region. Subcloning of pYA2044, however, revealed that the region providing the autonomous replication functions was the 3.2-kb *Bgl*II fragment located between coordinates 59.4 and 62.6 kb on pStSR100 (Fig. 1), not *repA*. Replicon function was confirmed by ligating the 3.2-kb *Bgl*II fragment isolated from pStSR100 to the 1.4-kb *Bam*HI Km^r cartridge of pUC-4K to produce the self-replicating *repC* plasmid, pYA2050, shown in Fig. 2. Probing a Southern blot of virulence plasmid fragments with pYA2050 (Fig. 3) confirmed the location of *repC* within the 13.3-kb *Hin*dIII fragment of pStSR100 (Fig. 1) and lack of DNA homology with *repB*, pYA2015, or the *repA* region.

Characteristics of the *repB* and *repC* replicons. These two discrete replicons were then characterized. The copy number of both plasmids in χ3387 was estimated relative to the pYA2045 *oriC* plasmid internal standard (two to four copies per chromosome) (45). The *repB* replicon, represented by pYA2018, and the *repC* replicon, represented by pYA2050), have a copy number of 2 to 3 per chromosome, whereas the pYA2013 subclone with Cm^r in place of the Km^r marker has a higher copy number of approximately 10 per chromosome. Hybridization studies using the 1.8-kb internal *Bgl*II-*Hin*dIII fragment from pYA2015, *repB*, or the 1.7-kb internal *Eco*RI fragment from pYA2050, *repC*, revealed that *repB* hybridized weakly with the IncFII plasmid R100, whereas *repC* hybridized weakly with the IncFI plasmid F (data not shown), but neither replicon hybridized with the IncH plasmid R726-1. Neither pYA2018 (*repB*) nor pYA2050 (*repC*) exhibited incompatibility with F-amp or R100 during

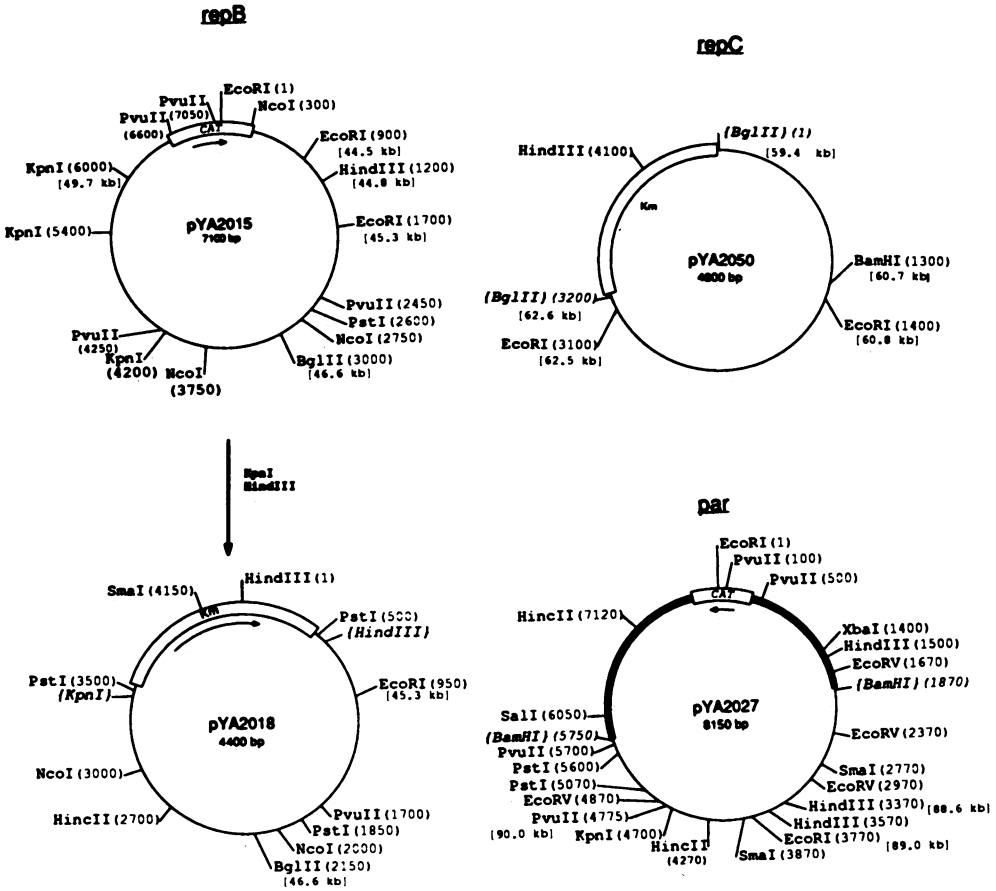

FIG. 2. Virulence plasmid maintenance region clones and subclones. The numbers in brackets represent coordinates on the virulence plasmid. Sites in braces are those destroyed during cloning.

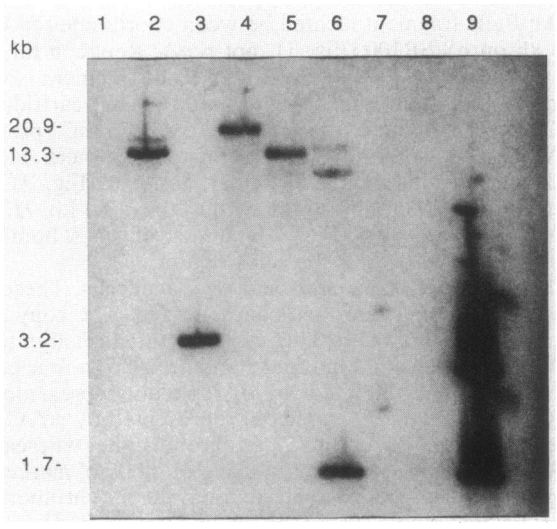

FIG. 3. Southern blot of virulence plasmid fragments probed with the 1.7-kb *Eco*RI fragment obtained from *repC*(pYA2050). Lanes: 1, high-molecular-weight marker; 2, pStSR100 cut with *Hin*dIII; 3, pStSR100 cut with *Bgl*II; 4, pStSR100 cut with *Sal*I; 5, pStSR100 cut with *Sst*I; 6, pStSR100 cut with *Eco*RI; 7, pYA2027(pACYC184 + *par*) cut with *Pvu*II; 8, pYA2015(*repB*) cut with *Pvu*II; 9, pYA2050(*repC*) cut with *Eco*RI. Sizes given on the left are for virulence plasmid bands hybridizing with *repC*.

10 generations of growth selecting for the larger plasmids, although hybridization indicated >65% homology. Both *repB* and *repC* replicons, however, exhibited incompatibility with pStSR101 (although to different degrees), as revealed by the inability to maintain pYA2050 in χ3934 and the decreased stability of pYA2015 in χ3934 (37.7% pYA2015$^-$ after 10 generations) relative to the segregation rate in χ3387 without pStSR101 (<1% pYA2015$^-$ after 10 generations).

The dependence of the *repB* replicon pYA2018 and the *repC* replicon pYA2050 on DNA polymerase I was tested by transforming each plasmid into a temperature-sensitive *polA* mutant, χ1891. When these transformants were grown at 30, 37, or 42°C in nonselective media, both the *repB* plasmid pYA2018 and the *repC* plasmid pYA2050 replicated, whereas pACYC184 was lost within 10 generations (Table 3).

TABLE 3. DNA polymerase I dependence of virulence plasmid clones in χ1891

Plasmid	% Plasmid-containing CFU after 10 generations at:		
	30°C	37°C	42°C
pACYC184	19.0	<0.8	<0.8
pYA2027 (*par*)	76.0	23.0	<0.9
pYA2018 (*repB*)	85.0	83.0	78.3
pYA2050 (*repC*)	85.0	88.0	47.4

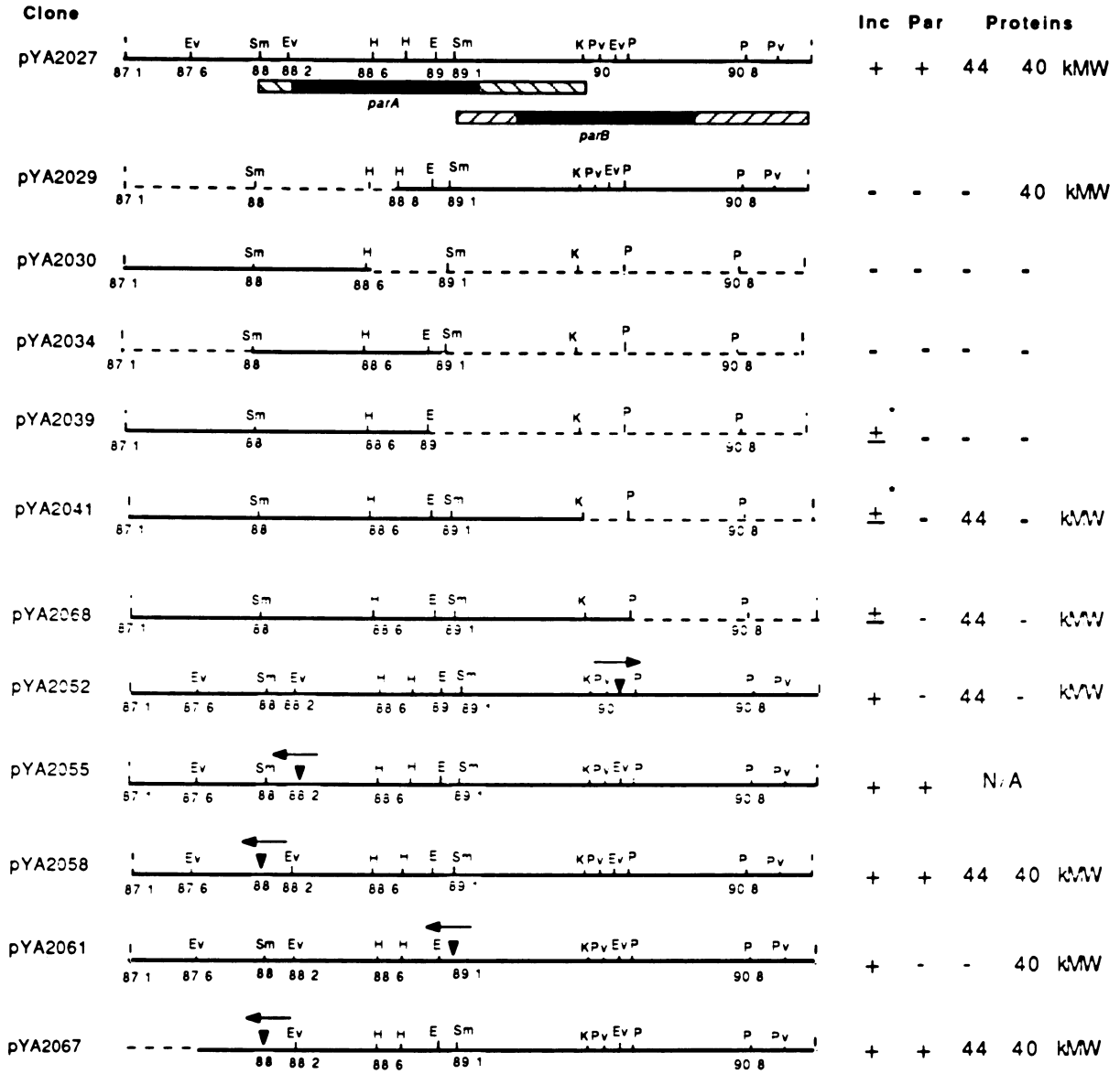

FIG. 4. Internal regions of the *par* subclones and Km^r inserts, indicating the *parA* and *parB* genes encoding 44,000- and 40,000-MW proteins, respectively. Inc$^+$ phenotype is indicated as follows: + if the pACYC184-based clone excluded pStSR101, ± if <5% virulence plasmid segregants were detected, and − if no (<1%) segregants were detected. Subclones marked with an asterisk exclude the virulence plasmid as pUC18 clones. The Par$^+$ phenotype is based on the ability of the subclone to increase the stability of pACYC184. Arrows over the Km^r inserts (▼) represent the orientation of the Km^r promoter; dotted lines represent DNA segments removed. Restriction sites: E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; Sm, *Sma*I.

The ability of these plasmids to replicate in the absence of protein synthesis was tested by placing cells containing the *repB* derivative, pYA2018, and the *repC* derivative, pYA2050, in L broth containing chloramphenicol. Neither replicon was amplifiable, as determined from the amount of DNA visualized after agarose gel electrophoresis of DNA obtained from these strains before and after incubation with chloramphenicol (170 μg/ml) for 4 to 6 h.

Segregational stability of *repB* relative to that of *repC*. Batch cultures of χ3477 containing the *repB* replicon pYA2018 revealed a segregational stability (95.8% pYA2018$^+$ after 60 generations) greater than that expected for a replicon with a copy number of two to three (7) if random segregation occurs. While the stability of the *repB* replicon pYA2018 was greater than that of the *repC* replicon pYA2050 (66.9% pYA2050$^+$ after 60 generations), the stability of either replicon was substantially less than that of the parent plasmid, pStSR100, indicating that neither replicon alone specifies all of the maintenance and partitioning functions required for the consistent inheritance of the virulence plasmid.

Isolation of the virulence plasmid partitioning region on the basis of incompatibility functions. Assuming that all partitioning regions exhibit incompatibility with related plasmids (43), a search was initiated to isolate clones capable of excluding the virulence plasmid. The pStSR100 *Sau*3A library in pACYC184, described in Materials and Methods, was transformed into the *recA S. typhimurium* strain χ3934, which contains the Tn*mini-tet*-labeled virulence plasmid. Pooled Cm^r transformants were then plated on medium containing chloramphenicol and fusaric acid to select for loss

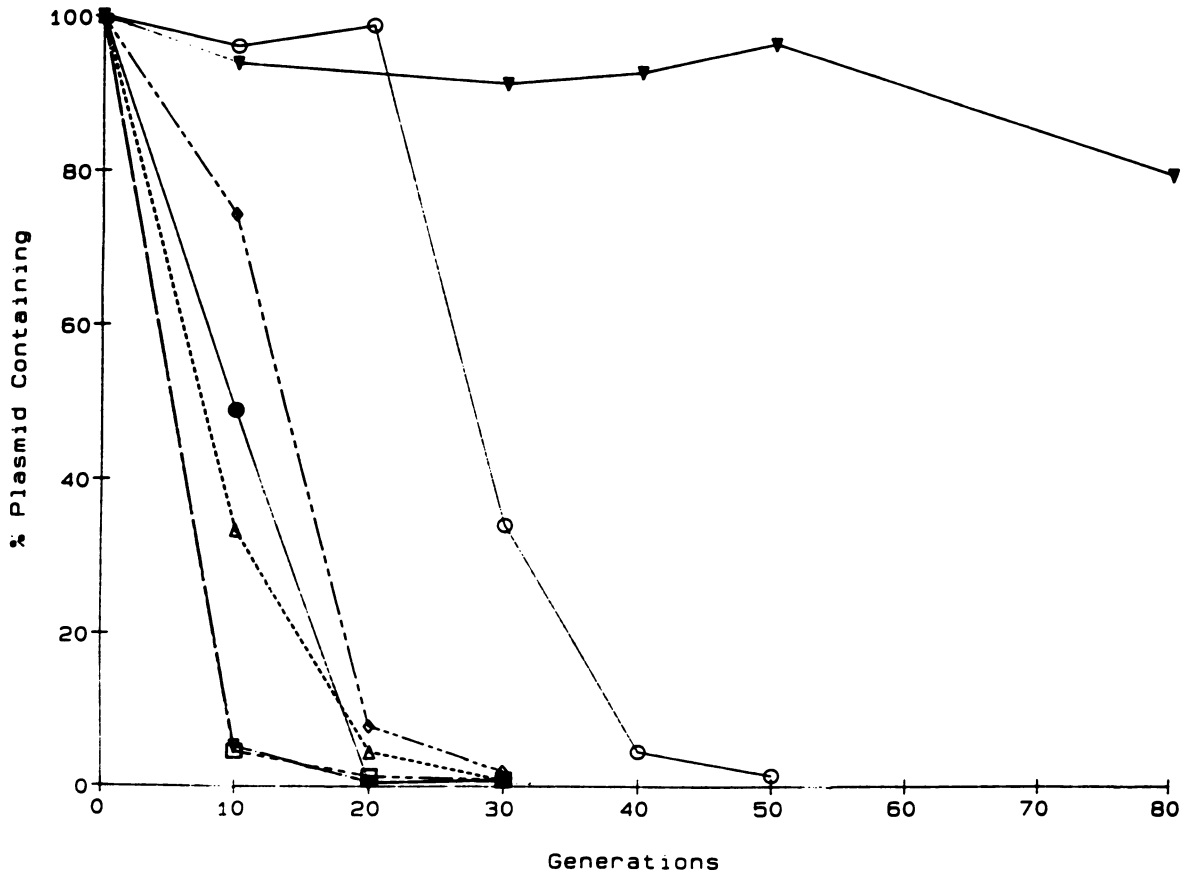

FIG. 5. Stability of pACYC184-based *par* subclones in the host *S. typhimurium* χ3337 during batch culture in minimal media at 37°C. Symbols: ●, pACYC184; △, pYA2041; ▽, pYA2039; ◇, pYA2029; □, pYA2030; ○, pYA2034; ▼, pYA2027 (*par* clone parent).

of pStSR101. Ten fusaric acid-resistant Tc^s colonies were found to carry an 8-kb Cm^r plasmid. Transformation of these Cm^r plasmids back into χ3934 and the subsequent production of Tc^s colonies confirmed their ability to exclude the virulence plasmid within 10 generations. When one of these Inc^+ clones, pYA2027, was tested for segregational stability in χ3477 during 50 generations of growth in L broth, less than 1% plasmid-free segregants were detected, whereas the cloning vector, pACYC184, produced greater than 66% plasmid-free segregants by this time. Thus, the DNA sequence in pYA2027 imparted both virulence plasmid incompatibility and stabilizing functions to pACYC184.

Determining the location of the *par* region on the virulence plasmid. The source of the 3.9-kb *Sau*3A insert in pYA2027 (Fig. 2) encoding the Inc^+ Par^+ phenotype was not readily apparent when its restriction map was compared with the map of the virulence plasmid (Fig. 1). Probing a Southern blot of pStSR100 with pYA2027 revealed that the insert hybridized with the 16.2- and 2.4-kb *Hin*dIII, 60-kb *Bgl*II, 7.3-kb *Sal*I, 8.8-kb *Sst*I, and 12.7- and 12.2-kb *Eco*RI fragments of the virulence plasmid (data not shown). This placed the Inc^+ Par^+ clone, pYA2027, within the region identified by Michiels et al. (36) as *repA* (Fig. 1), the *Hin*dIII sites corresponding to coordinates 88.6 and 88.8 kb of the virulence plasmid (Fig. 1). The inability of pYA2028 (*par*) to exclude F-kan (IncFI), R100 (IncFII), R726-1 (IncH), R64*drd-11* (IncI), or R702 (IncP) and the lack of hybridization between the 1.5-kb internal *Pst*I-to-*Hin*dIII fragment of pYA2027 (*par*) and F, R100, or R726-1 (data not shown) indicated that the incompatibility function of *par* was not related to these plasmids.

Obtaining the minimal region exhibiting the Inc^+ Par^+ phenotype. To delimit the Inc^+ Par^+ region, the pYA2027 subclones illustrated in Fig. 4 were analyzed for Inc^+ and Par^+ phenotypes. The Inc^+ phenotype of each subclone was determined by transforming either the pACYC184- or the pUC18-based clone into χ3934 and growing the transformants for 10 generations while selecting for the subclone. The Par^+ phenotype was determined by transforming each pACYC184-based subclone into χ3337, growing these strains in minimal medium batch cultures for 80 generations, and determining the segregation rate of each clone. The proportion of the population retaining each pYA2027 subclone is presented in Fig. 5. Unlike pYA2027, all subclones failed to exhibit stability greater than that of pACYC184 except for pYA2034, which appeared to possess a slightly increased stability.

Use of site-directed Km^r inserts to inactivate *par* functions. Further definition of the region required for the stabilizing phenotype of *par* involved inserting the pUC-4K Km^r cartridge into the *Eco*RV site at pStSR100 coordinate 90.1 kb of pYA2027, yielding pYA2052, or into the *Sma*I site at 89.1 kb, producing pYA2061, both eliminating the Par^+ phenotype of pYA2027. On the other hand, Km^r insertions at the *Eco*RV site at 88.2 kb, yielding pYA2055, or the *Sma*I site at 88.0 kb, producing pYA2058, failed to alter the Par^+ phenotype (Fig. 4). None of these Km^r inserts eliminated the Inc^+

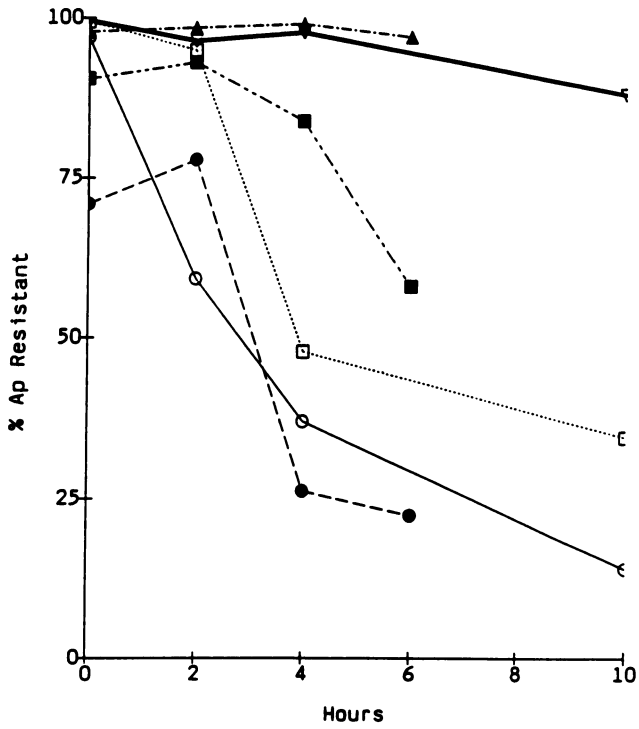

FIG. 6. Stability of *oriC* plasmids containing the *par* region of the virulence plasmid compared with that of *oriC* containing the *sop* locus from F. Growth was in L broth at 37°C, using both *E. coli* SE5000 and *S. typhimurium* χ3477 as hosts. Symbols: ○, χ3477(pYA2045 [*oriC*]); ▽, χ3477(pYA2046 [*oriC* + *par*]); □, χ3477(pXX199 [*oriC* + *sop*]); ●, SE5000(pYA2045 [*oriC*]); ▲, SE5000(pYA2046 [*oriC* + *par*]); ■, SE5000(pXX199 [*oriC* + *sop*]).

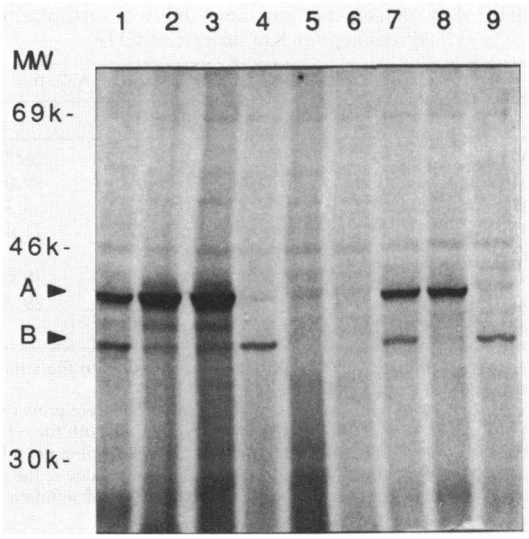

FIG. 7. Proteins synthesized by using an in vitro transcription-translation system and the pACYC184-based subclones of *par*. Proteins were resolved on a 10% (wt/vol) polyacrylamide-sodium dodecyl sulfate gel and identified by fluorography. The portion of the gel revealing the ParA 44,000-MW (A) and the ParB 40,000-MW (B) proteins is shown; the 25,000-MW chloramphenicol acetyltransferase protein and the 27,000-MW aminoglycoside 3′-phosphotransferase protein specified by pACYC184 and the Km^r cartridge are not shown. Lanes: 1, pYA2067; 2, pYA2068; 3, pYA2041; 4, pYA2029; 5, pYA2039; 6, pYA2030; 7, pYA2058; 8, pYA2052; 9, pYA2061.

phenotype of pYA2027, even when the Par$^+$ phenotype was reduced.

Since the region required for the Par$^+$ phenotype could not be reduced by using defined restriction sites, *Sau*3A partial digests of pYA2057, containing the 6.1-kb *par* region with a Km^r insert at *Sma*I coordinate 88.0 kb, were ligated into the *Bam*HI site of pYA2204, which is unstable in both *Escherichia coli* and *S. typhimurium*. The smallest stable clone obtained, pYA2064, still contained 3.4 kb of the *par* region shown for the pACYC184 derivative, pYA2067, in Fig. 4. This requirement for approximately 3.4 kb of DNA for the Par$^+$ phenotype suggested that more than just a small *cis*-acting partitioning site, as in the *par* region of pSC101 (35), was involved in the stabilizing phenotype observed.

Use of *par* to alter the stability of heterologous replicons. The demonstration that the *par* region was capable of increasing the segregational stability of the p15A vector, pACYC184, shown by the comparison of the *par* subclone stabilities in Fig. 5, suggested that *par* could be developed into a stabilizing cartridge for other replicons. To test this possibility, the 4.6-kb *Xba*I-*Sal*I fragment of pYA2027 was removed and inserted into the *Xba*I-*Sal*I site of pUC18, producing the high-copy-number clone pYA2028. Although ColE1-derived plasmids are thought to undergo random segregation (50), pUC18 is relatively stable in the *E. coli* and *S. typhimurium* backgrounds used. No decrease in the stability of pUC18 was expected upon the introduction of this *par* region. However, the same *par* insert that had increased the stability of the moderate copy number vector pACYC184 now appeared deleterious to host cells containing *par* as a high-copy-number pUC18 clone. To determine whether the poor growth observed was due to a lethal effect of the high copy number of *par* or extremely erratic segregation, χ3385 containing pYA2028 was grown with and without ampicillin selection and compared to χ3385 with the pUC18 cloning vector. The generation times determined for χ3385(pUC18) and χ3385(pYA2028) in L broth with ampicillin were 0.42 and 0.51 h, respectively. In nonselective media, however, the growth rates were similar, 0.36 and 0.38 h, respectively, suggesting that the production of plasmid-free cells is responsible for the poor growth of χ3385(pYA2028) in selective media.

The *par* cartridge isolated from pYA2028 was subsequently inserted into other lower-copy-number replicons which exhibited segregational instability to determine whether *par* could function effectively on other replicons even though it decreased the stability of pUC18. Installing *par* in the *oriC* plasmid obtained from the *E. coli* chromosome increased segregational stability in both *E. coli* and *S. typhimurium* backgrounds (Fig. 6) to a greater extent than provided by the *sop* region of F in pXX199 (Fig. 6). Introducing *par* into the IncW replicon pYA2204 increased the percentage of plasmid-containing cells from 60.6 (pYA2204) to 99.3 (pYA2063), whereas the percentage of *repC par*-containing cells increased from 33.6 (pYA2050) to 99.2 (pYA2065) after 60 generations in the *E. coli* host C118. This result demonstrated that the *par* cartridge, taken from the unstable pUC18 derivative pYA2028, could be used to increase the segregational stability of various lower-copy-number replicons unrelated to the virulence plasmid.

Identifying the proteins encoded by the *par* region. The proteins encoded by the *par* region were determined by using in vitro transcription-translation of the pACYC184 subclones. For all clones exhibiting the Par$^+$ phenotype, there were two proteins produced, one of approximately

TABLE 4. Complementation and stability of *oriC* plasmids containing *par* Km^r inserts in C118

Plasmid (proteins)[a]	% CFU[b] Ap^r after:	
	0 h	4 h
pYA2045(ParA$^-$ ParB$^-$)	91.9 ± 2.3	26.7 ± 4.3
pYA2046(ParA$^+$ ParB$^+$)	>99.6 ± 0.1	99.0 ± 0.4
pYA2071(ParA$^+$ ParB$^-$)	68.0 ± 7.9	5.3 ± 2.5
pYA2071(ParA$^+$ ParB$^-$) + pYA2029*(ParA$^-$ ParB$^+$)	80.6 ± 5.5	11.4 ± 1.1
pYA2072(ParA$^-$ ParB$^+$)	52.3 ± 3.6	8.0 ± 7.3
pYA2072(ParA$^-$ ParB$^+$) + pYA2041*(ParA$^+$ ParB$^-$)	93.1 ± 1.2	29.8 ± 5.2

[a] The complementing pACYC184 clones transformed into the strains containing the *oriC* clones are indicated by asterisks.

[b] Three isolated Ap^r transformants for each construct were grown without aeration in L broth with ampicillin, chloramphenicol, or both for ~12 h and then for 1 h with aeration with an additional 50 μg of ampicillin per ml before dilution (1:1,000) into L broth without antibiotics. Each value is the average for three isolates ± the standard deviation after growth with aeration at 37°C for 4 h.

44,000 molecular weight (MW) and another of 40,000 MW (Fig. 7). The association of these proteins with either the Inc$^+$ or Par$^+$ phenotype (Fig. 4) revealed that although both proteins were required for the Par$^+$ phenotype, the Inc$^+$ phenotype could be obtained with neither protein present if the copy number of the subclone was sufficiently high. This high-copy-number-induced exclusion was demonstrated by the frequent generation of virulence plasmid-free cells when a pUC18-based *par* subclone, pYA2039 or pYA2041, was introduced into χ3934. On the basis of these results, it is proposed that the 44,000-MW protein, requiring 1.19 kb of DNA, is produced by the region depicted as *parA* in Fig. 4, whereas the 40,000-MW protein is encoded by the 1.08-kb *parB* region.

Complementing the Par$^-$ phenotype of *parA* and *parB* mutants. To determine whether the *par* proteins function in *trans*, the *par* Km^r inserts eliminating production of either the 44,000-MW protein, in pYA2052, or the 40,000-MW protein, in pYA2061, were isolated on 5.3-kb *Sal*I fragments and ligated into the *Xho*I site of the unstable *oriC* plasmid pYA2045. The unstable Km^r clones obtained were then transformed into C118 along with the pACYC184-based subclone encoding only one *par* protein. These combinations were then tested for segregational stability relative to isogenic strains lacking the pACYC184 *par* clone. Although all strains lacking the intact *par* region contained plasmid-free segregants at T_0 even in the presence of ampicillin, there was a consistent trend of increasing the proportion of *oriC* plasmid-containing cells when the complementing clone was present with the mutant (Table 4). These results revealed that the *parA* and *parB* loci encode *trans*-acting gene products capable of partially complementing the Par$^-$ phenotypes of pYA2071 and pYA2072.

TABLE 5. Stability of virulence plasmids containing Km^r inserts within *par* in χ3364

Plasmid (pACYC184 clone) genotype	% CFU Km^r after:	
	60 generations	100 generations
pStLT200 (pYA2052) pStLT100Ω*parB*[90.1 kb *Eco*RV::Km]	99.3	99.5
pStLT201 (pYA2055) pStLT100Ω[88.2 kb *Eco*RV::Km]	99.4	99.4
pStLT202 (pYA2058) pStLT100Ω[88.0 kb *Sma*I::Km]	99.6	99.4
pStLT203 (pYA2061) pStLT100Ω*parA*[89.1 kb *Sma*I::Km]	79.0	1.4

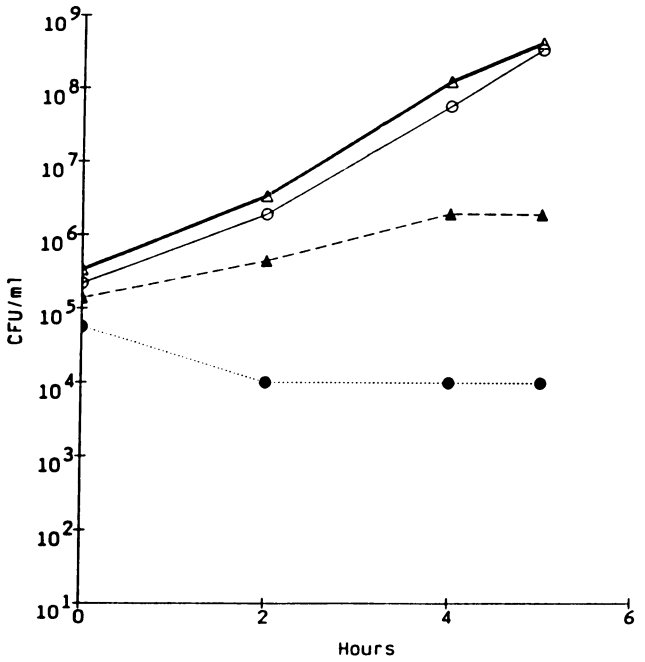

FIG. 8. Stability of pYA2027 and viability of plasmid-free segregants, tested in a *pol*A(Ts) mutant, χ1891, during growth in L broth at 30°C. Symbols: ○, total CFU with pACYC184; △, total CFU with pYA2027 (pACYC184 + *par*); ●, Cm^r CFU with pACYC184; ▲, Cm^r CFU with pYA2027.

Effect of allele replacement on stability of the virulence plasmid. Using *par* genes inactivated by a Km^r insert, mutants were introduced into the parent virulence plasmid, using linear transformation as described by Winans et al. (56), in the *recB recC sbcB E. coli* strain χ6106 containing pStSR101. The integration of the Km^r cartridge containing the *par* region of pYA2051, pYA2054, pYA2057, and pYA2060 into pStSR101 was confirmed by observing a 1.4-kb increase in the size of the 7.3-kb *Sal*I fragment of pStSR101. These inserts within the *par* region were then transduced into *S. typhimurium* χ3364, using P1L4 grown on each plasmid-containing strain. The transductants used received both Km^r from the insert and Tc^r from pStSR101. The insert inactivating the *parA* gene encoding the 44,000-MW protein in pStLT203 was the only insert greatly destabilizing the virulence plasmid (Table 5); still, plasmid-free segregants appeared at a much lower frequency than would be expected if random segregation were the only means of ensuring distribution of plasmids to progeny cells without the *par* region.

Testing for mechanisms other than partitioning associated with the *par* region. The presence of a stabilizing function does not necessarily ensure that a region is exhibiting a partitioning mechanism. Other mechanisms that could provide increased plasmid stability include the existence of a second replicon (4), increases in the plasmid copy number (50), expression of a control-of-cell-division function (44), or a plasmid-free host-killing system process (18). The possibility of each of these mechanisms being specified by the *par* region was investigated. Other mechanisms for plasmid

distribution within the cell related to partition function such as association with the chromosome (30) or segregation into minicells (26) were not investigated.

The *par* region does not contain a functional DNA polymerase I-independent replicon, as revealed by the inability of *par* to rescue the pACYC184 cloning vector in the *polA*(Ts) host χ1891 (Table 3). The *par* region also does not appear to be associated with a functional replicon in the *repA* region, as determined from our inability to isolate a functional replicon from the *par* region by either self-ligation of the 7.3-kb *Sal*I fragment containing the 88.0-kb *Sma*I::Km insert within the *par* region, ligation of partial *Xho*I digests of pStSR100 to the Km^r cartridge from pUC-4K (even though the 46-kb *Xho*I fragment containing the *repB* and *repC* replicons was recovered as pYA2070), or the isolation of only *repC* from the *Bst*EII-generated cosmids encompassing the *repA* region.

The increased stability imparted by the *par* region did not appear to be due to increased relative copy number of *oriC*, pACYC184, R388, or *repC* replicons containing *par*. The relative copy numbers per chromosome DNA equivalent determined for the *oriC* clones in SE5000(pACYC184) were 3.5 for *oriC*(pYA2045), 2.2 for *oriC* + *par*(pYA2046), and 3.7 for *oriC* + *sop*(pXX199), based on a copy number of 20 per chromosome for pACYC184 (34).

No variations in cell growth rate were associated with pYA2027 to indicate the presence of a control-of-cell-division (44) or host-killing function (19) acting to reduce the proportion of plasmid-free cells within the population. Comparison of growth rates at 37°C for χ3337 with or without pYA2027 in L broth failed to show any variation in generation time, both being 0.31 h. Likewise, no deviations in growth rate at 30°C or reduction of viability could be detected when the *polA*(Ts) *E. coli* strain χ1891 was grown with or without pYA2027, even though viable Cm^s plasmid-free cells were being produced (Fig. 8). Also, no clear difference in morphology could be observed with χ1891 containing either pACYC184 or pYA2027, as might be expected if a *hok* region were acting (19).

DISCUSSION

Although our isolation of two separate replicons from the virulence plasmid of *S. typhimurium* agrees with the conclusions of Michiels et al. (36), our results differ in regard to the precise location and incompatibility group assignments of these replicons. The first replicon, pYA2018, was localized in the *repB* region, but it hybridized with the IncFII plasmid R100, not the IncFI plasmid F, as stated by Michiels et al. (36). The second replicon, *repC*, was isolated from a region distinct from either the *repA* or *repB* region described by Michiels et al. (36) within the 3.2-kb *Bgl*II fragment of pStSR100. The location of *repC* suggested that it may have been responsible for the replication functions assigned to *repA* by Michiels et al. (36), since no autonomous replication functions were found associated with *par* during our study. Although *repB* and *repC* hybridized to IncFII and IncFI plasmids, respectively, our incompatibility group assignments were inverted relative to those proposed by Michiels et al. (36). Their IncFIB classification of *repB* is based on the demonstration that a naturally occurring 12-kb deletion mutant of the virulence plasmid failed to hybridize with the *rep*FI probe (36). The present classification of *repB* as IncFII is reinforced by the observation that the *Salmonella dublin* virulence plasmid is of the IncFII incompatibility group (52), in agreement with our observation that *S. dublin* hybridizes with only the *repB* and *par* regions (54). Although neither the *repB* replicon, pYA2018, nor the *repC* replicon, pYA2050, was excluded by the IncFI or IncFII plasmids tested, the hybridization data would place these replicons in the IncFI and IncFII incompatibility groups on the basis of the classification scheme proposed by Couturier et al. (11). This classification based on homology does not necessarily contradict the separate IncTV functional incompatibility classification proposed by Ou (J. T. Ou, L. Baron, X. Dai, and C. Life, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, H-281, p. 216), even though the presence of multiple replicons complicates any functional incompatibility grouping as discussed by Couturier et al. (11). The failure of *par* to hybridize with IncFI, IncFII, or IncH plasmids strengthens the unique incompatibility assignment of the virulence plasmid, even though other Inc groups have not been tested.

The search for a region of the virulence plasmid involved in the partitioning of plasmids to progeny cells revealed that a region responsible for incompatibility was associated with increasing segregational stability, a function also observed for F (45), P1 (2), and R1 (40) partitioning regions. This region, expressing both incompatibility and partitioning functions, has been reduced to a 3.4-kb fragment within the 20-kb area designated *repA* by Michiels et al. (36). Our results, however, failed to demonstrate the presence of a functional replicon within the *repA* region associated with *par*. Although the presence of an independent replicon near the *repA* region is not ruled out, we have no evidence of its existence on pStSR100, which appears the same as the plasmid described by Michiels et al. (36) on the basis of restriction digest patterns. Unlike the F partitioning region, which is closely associated with the FIA replicon (45), the partitioning region of the virulence plasmid is distant from either origin of replication, resembling the partitioning region arrangement of NR1, in which *par* and *rep* functions are separated by a 28-kb region containing antibiotic resistance genes (57).

The *par* region has been shown to increase the segregational stability of the *oriC* plasmid, the virulence plasmid *repC* replicon, the IncW plasmid pYA2204, and the multicopy plasmid pACYC184. The virulence plasmid *par* region has the potential to be used as a partitioning cartridge to stabilize other cloning vectors, as described for the *sop* locus of F or the *parA* locus of R1 (7). The size of the region required to provide the Par^+ phenotype, similar in size to the 3.0-kb *sop* region of F (45), is due to the requirement for the 44,000- and 40,000-MW proteins encoded by the region, loss of either protein resulting in reduced segregational stability of the clone. Recently, Cerin and Hackett (10) have cloned what appears to be the same *par* region, referred to as *parVP*. The 40- and 37-kilodalton (kDa) proteins that they observe are similar to those observed in this study and by Norel et al. (41); however, only the larger 40-kilodalton protein is required for the partitioning phenotype of the clone of Cerin and Hackett. Although the clones cover slightly different areas (our largest is 3.9 kb, whereas theirs is 4.4 kb), the restriction maps are very similar. They also infer that their *par* clone covers the same 2.4-kb *Hin*dIII fragment within *repA* coinciding with the *virB* locus described by Norel et al. (41) and the *par* region reported here. The orientation of both promoters presented by Cerin and Hackett (10) does not conflict with our observations; however, the absence of any truncated proteins in our study has restricted exact positioning of the *parA* and *parB* regions or confirmation of promoter location.

Functional and organizational similarities are found be-

tween the virulence plasmid *par* region and those of F (45), P1 (16), and NR1 (51), all of which have 1.7- to 3.0-kb regions encoding two proteins that act on a *cis* region essential for partitioning. The Inc$^+$ phenotype, however, is not strictly related to the production of the two proteins but varies depending on the copy number of the cloned region. Novick (42) has placed incompatibility functions into three groups according to the maintenance functions shared: copy number control, origin of replication, or partitioning regions. The *par* region discussed here exhibits incompatibility functions yet shows no incompatibility with or homology to either *repB* or *repC* replicon even when present as the multicopy-number clone, pYA2027. This finding indicates that *par* is not a remote copy number control region for *repB* or *repC* as suggested by Cerin and Hackett (10). The inability of *par* to replicate either as a pACYC184 clone in a *polA* mutant background or alone when ligated to a Km^r marker indicates that this *par* region is not a replicon. Further unsuccessful attempts to clone *repA* as a replicon strongly suggest that the 20-kb *repA* region does not contain a functional replicon. The assignment of *repA* as the major replicon of the virulence plasmid by Michiels et al. (36) was based on two observations: (i) very few deletions were obtained in the *repA* region, and (ii) when a deletion did occur within that region, the apparent copy number decreased. These characteristics attributed to replicon function (36) could also relate to the reduction of plasmid-containing cells in any population suffering disruption of the *par* functions. This same production of plasmid-free cells and potential reduction of growth rate due to partitioning malfunction could also relate to the assignment of virulence functions to this region (41). Norel et al. (41) describe a region including a 2.4-kb *Hin*dIII fragment of the virulence plasmid as *virB*; their 43-kilodalton protein is encoded by a region encompassing the *Eco*RI site at 89 kb within *parA* on the virulence plasmid. The other region, encoding a 38-kilodalton protein, appears to start just before the *Kpn*I site at 89.8 kb, corresponding to our *parB* encoding a 40,000-MW protein. Although we have not conducted any virulence tests with any of the *par* mutants or clones, the reduction of virulence attributed to Tn*5* insertions within this *virB* region may be the by-product of partitioning problems producing plasmid-free cells in vivo which would, in turn, reduce virulence (21, 23, 46).

Although the actual mechanism involved in the partitioning of plasmids containing the *par* region is not identified, there is no evidence indicating that another stabilizing mechanism is involved. The *par* region is not an autonomous replicon, does not increase the copy number of plasmids containing it, is not involved in the resolution of plasmid multimers, and does not appear to alter host growth or kill plasmid-free segregants. The similarities between the *par* region of the virulence plasmid and those of F, P1, and NR1, combined with the report of virulence plasmid hybridization with *rep*FIB and *rep*FIIA minireplicons (36), suggested that *par* may share sequence homology with either F or R100. Hybridization studies, however, revealed that the *repC* and *repB* replicons, not *par*, are the ones sharing sequence homology with F and R100. The organizational similarities between the virulence plasmid and the IncFI and IncFII plasmids suggest that the maintenance functions identified may be supplemented by an additional mechanism debilitating the plasmid-free cells that do occur. The combination of these mechanisms results in the virulence plasmid being maintained with great fidelity although large in size and low in copy number.

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