INFECTION AND IMMUNITY, Sept. 1990, p. 3084–3092
0019-9567/90/093084-09$02.00/0

Vol. 58, No. 9

Conservation of *Salmonella typhimurium* Virulence Plasmid Maintenance Regions among *Salmonella* Serovars as a Basis for Plasmid Curing

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Received 18 December 1989/Accepted 20 June 1990

The association of large plasmids with virulence in invasive *Salmonella* serovars has led to a number of studies designed to uncover the role of these plasmids in virulence. This study addresses two aspects of virulence-associated plasmids. The first is the distribution of the replication and maintenance regions among the plasmids of different *Salmonella* serovars, and the second is the use of the conserved virulence plasmid *par* region to provide a rapid method for eliminating the virulence plasmids specifically. Colony blots revealed that the *par* and *repB* regions of the *S. typhimurium* virulence plasmid hybridized with 80% of the isolates of *S. choleraesuis*, *S. dublin*, *S. enteritidis*, *S. gallinarum*, *S. pullorum*, and *S. typhimurium*, while the *repC* region was not detected in any of the isolates of *S. dublin*, *S. gallinarum*, or *S. pullorum*. None of these maintenance regions was found in any of the 30 additional serovars tested. The large plasmids of those serovars that hybridized with *par* were labeled with a Km^r insert within *parA* via P22HT*int* or P1L4 transduction, which destabilized the plasmids and allowed the rapid isolation of plasmid-free derivatives for all of the serovars, except for *S. dublin*, which exhibited weak homology with *par*.

The association of virulence with plasmid content in different *Salmonella* serovars, specifically *S. choleraesuis* (22), *S. dublin* (10, 28, 41), *S. enteritidis* (30), *S. gallinarum* (4), *S. pullorum* (3), and *S. typhimurium* (15, 17, 18, 20, 31), has expanded the knowledge of plasmid function in the genus *Salmonella*. Although these studies report a correlation between large (50 to 100 kilobases [kb]) plasmids and the virulence of these serovars, the complete function and organization of these plasmids is still unclear.

Several studies have demonstrated the conservation of sequences homologous to portions of the *S. typhimurium* virulence plasmid *vir* region by hybridization among *Salmonella* serovars (2, 33, 34, 44), supporting the relationships indicated by the use of restriction fragment analysis of these plasmids (8, 32, 33). Korpela et al. (24) have also demonstrated the conservation of major regions of the *S. typhimurium* virulence plasmid among *Salmonella* serovars by DNA hybridization with various virulence plasmid subclones representing 85% of the plasmid. Beninger et al. (5) demonstrated hybridization between *S. dublin*, *S. enteritidis*, and *S. choleraesuis* virulence and replication regions. The general conclusion of these studies is that the virulence plasmids within a serovar are tightly conserved while the *vir* region is conserved among serovars. Although the study by Korpela et al. (24) utilizing large subclones of the *S. typhimurium* virulence plasmid indicated that most regions are conserved among the different serovars, the probes used, because of their size, could not confirm that the replication and partitioning regions identified previously (42) are present in all serovars. We present the results of hybridization studies indicating that although the partitioning region and the *repB* region of the *S. typhimurium* virulence plasmid are strongly conserved among the invasive *Salmonella* serovars, the second replicon, *repC*, is not.

This information was applied to the development of a specialized curing scheme allowing the elimination of the large virulence plasmid from invasive *Salmonella* serovars. The persistence of the large plasmids within *Salmonella* serovars is suggested by their wide distribution among different strains (18) and confirmed by the low frequency of plasmid-free isolates obtained during curing attempts (19, 35, 36, 38, 45). Although general curing strategies employing acridine orange, novobiocin, ethidium bromide, and other chemical agents or growth at high temperature have successfully eliminated plasmids from *Salmonella* serovars (3, 15, 28, 35), these treatments have potential problems. One problem involves the general curing effect produced by these chemicals or growth conditions, resulting in some or all of the other plasmids being eliminated during the process of curing the virulence plasmid. The next concern is the possibility of mutational changes induced in the host during the curing treatment, resulting in rough strains (19), reductions in virulence due to unknown mutations other than loss of the virulence plasmid (35), or integration of the virulence plasmid or other plasmids into the chromosome (45). A final problem complicating the elimination of these virulence-associated plasmids is their lack of any detectable marker. This cryptic nature has been remedied by introducing Tn*3* or Tn*10* transposons encoding antibiotic resistance, thus allowing screening for antibiotic-sensitive plasmid-free segregants. However, this tagging procedure has the drawback that transposons can hop into the chromosome or another plasmid (even if transposition to plasmids is favored [25]), and this is complicated by possible deletions within the transposon (45), leading to false-cured isolates. The use of transposase-defective transposons such as Tn*mini-tet* (43) to label the virulence plasmid (15) circumvents the last obstacle.

This study analyzed the distribution among *Salmonella* serovars of maintenance regions homologous to those of the *S. typhimurium* virulence plasmid and demonstrated that the *par* region has a similar function in the other serovars. This information was subsequently utilized to develop a specific

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TABLE 1. Bacterial strains

Strain	Description, genotype, or phenotype	Reference, derivation, or source
χ3000	*S. typhimurium* LT-2, contains 91-kb pStLT100, prototroph	15
χ3364	*S. typhimurium* LT-2 pStLT100$^+$ *hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*$^+$ (*Escherichia coli*) Δ(*zja*::Tn*10*) *hsdA29 val*	Tc^s, fusaric acid-resistant derivative of AS68; AS68 obtained from T. Palva
χ3385	*S. typhimurium* LT-2 pStLT100$^-$ *hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	Tc^s plasmid-cured derivative of χ3364
χ3387	*S. typhimurium* LT-2 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 (23) followed by fusaric acid selection (7) for loss of Tn*10*
χ3477	*S. typhimurium* LT-2 pStSL100$^-$ *hsdL6* Δ(*gal-uvrB*)*1005 flaA66 rpsL120 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	Derived from χ3385 following transduction with P22HT*int* lysate from SL5400, obtained from B. A. D. Stocker, to introduce Δ(*gal-uvrB*)*1005*
χ3540	*S. gallinarum*, contains 85-kb pSgV1 and 2.5-kb plasmids	Received from National Veterinary Science Lab, Ames, Iowa
χ3759	*S. enteritidis*, chicken passaged 4973, contains 55-kb pSeV1 and 4.5-kb plasmids	Received from J. G. Morris, Baltimore, Md., as 4973
χ3760	*S. typhimurium*, chicken-passaged 6850C, contains 91-kb pStV1 and 7-kb plasmids	Received from R. Clarke, Animal Health Labs, Guelph, Ontario, Canada, as calf isolate 6850C
χ3761	*S. typhimurium*, chicken-passaged 30875, contains 91-kb pStV1 plasmid	Received from P. McDonough as equine isolate 30875
χ3762	*S. pullorum*, chicken-passaged 3045, contains 85-kb pSpV1, 2.7-, and 2.5-kb plasmids	Received from National Veterinary Science Lab as chicken isolate 3045
χ3790	*S. dublin*, contains 80-kb pSDL2 plasmid	Received from D. Guiney, University of California, San Diego, as *S. dublin* Lane (10)
χ3866	*S. enteritidis*, χ3759 cured of 55-kb pSeV1 plasmid	This study
χ3868	*S. typhimurium*, χ3760 cured of 91-kb pStV1 plasmid	This study
χ3872	*S. pullorum*, χ3762 cured of 85-kb pSpV1 plasmid	This study
χ3903	*S. choleraesuis*, 5451-84 cured of 50-kb pScKunV1 plasmid	This study
χ3905	*S. gallinarum*, χ3540 cured of 85-kb pSgV1 plasmid	This study
χ3918	*S. typhimurium* LT-2 pStLT203$^+$ *hsdL6* Δ(*gal-uvrB*)*1005 flaA66 rpsL120 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	χ3477 transformed with pStLT203, Km^r Tc^r unstable virulence plasmid (42)
χ3934	*S. typhimurium* 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*	χ3387 transformed with pStSR101, Tc^r virulence plasmid
TT521	*S. typhimurium* LT-2 *recA1 rpsL srl-202*::Tn*10*	Received from J. Roth as TT521
88/8	*S. dublin*, contains 70-kb pSdV1 plasmid	Received from C. Poppe, Animal Health Labs, as 88/8
1092	*S. dublin*, contains 70-kb pSdV2 plasmid	Received from National Veterinary Science Lab as 1092
5451-84	*S. choleraesuis*, contains 50-kb pScKunV1 and 8-kb plasmids	Received from W. Fales, University of Missouri, Columbia, as 5451-84

Salmonella virulence plasmid-curing protocol devoid of harsh chemical or physical treatment.

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 (LB) and L agar (LA) (26). Media were supplemented with antibiotics at the following concentrations; ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 15 μg/ml.

Genetic exchange. Transformation was performed by the method of Dagert and Ehrlich (13). Transductions mediated by bacteriophages P1L4 (12) and P22HT*int* (14, 37) were performed using *galE hsd* mutants of *S. typhimurium* χ3387 or χ3477, which are sensitive to P1L4 when grown in LB containing 5 mM $CaCl_2$ without galactose and sensitive to P22HT*int* when grown in LB containing 0.05% galactose in place of 0.1% glucose.

DNA manipulations. Plasmid extractions were performed by the methods of Birnboim and Doly (6) or Kado and Liu (21). Agarose gel electrophoresis was performed by the procedures described by Maniatis et al. (27). Restriction enzyme digestions were carried out with enzymes from International Biotechnologies, Inc. (New Haven, Conn.) and Promega Corp. (Madison, Wis.) according to instructions of the manufacturers. DNA fragments were excised from Tris acetate-agarose gels and isolated by using a Gene Clean Kit from BIO 101, Inc. (LaJolla, Calif.). DNA probes labeled with [α-^{32}P]ATP (specific activity, 3,000 Ci/mmol) from Dupont, NEN Research Products (Boston, Mass.) were obtained by using the Random Primer Labeling Kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the instructions of the manufacturer. Southern blots, colony blots, and hybridizations were carried out using GeneScreen Plus membranes from Dupont according to the instructions of the manufacturer. Hybridization was carried out at 37°C in hybridization solutions (27) containing 50% formamide and 1% sodium dodecyl sulfate for 12 to 16 h, followed by washing at 55°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% sodium dodecyl sulfate, providing a stringency requiring ~65% or greater homology for binding (1).

Stability tests. Stability tests for each plasmid-containing

TABLE 2. Plasmids

Plasmid	Description, genotype, or phenotype	Reference, derivation, or source
F	IncFI plasmid from *E. coli* χ15	11
R100	IncFII plasmid from *E. coli* χ1781 Cm^r Sm^r Sp^r Su^r Tc^r	Curtiss collection
R726-1	IncHI plasmid from *E. coli* χ2086 Cm^r Sm^r Su^r	Curtiss collection
pGTR001	*virA* region of pStSR100 within a 1.2-kb *Bam*HI-*Eco*RI fragment from pYA422 (16) cloned into the *Bam*HI-*Eco*RI site of pUC19, Ap^r	P. Gulig
pStLT100	91-kb virulence plasmid of *S. typhimurium* LT-2	15
pStSR100	91-kb virulence plasmid of *S. typhimurium* SR-11	15
pStSR101	Tn*mini-tet*-labeled virulence plasmid, Tc^r	15
pStLT203	pStLT101 destabilized by Ω *parA*[89.1 kb *Sma*I::Km], Km^r	42
pYA2015	Self-replicating *repB* region of pStSR100 in a 5.7-kb *Sau*3A fragment ligated to a 1.4-kb *Bam*HI Cm^r cartridge, Cm^r	42
pYA2027	*par* region of pStSR100 in a 3.9-kb *Sau*3A fragment cloned into the *Bam*HI site of pACYC184, Cm^r	42
pYA2028	pUC18 containing the *Xba*I-*Sal*I *par* fragment of pYA2027, Ap^r	42
pYA2050	Self-replicating *repC* region of pStSR100 in a 3.2-kb *Bgl*II fragment ligated to the 1.4-kb *Bam*HI Km^r cartridge from pUC-4K, Km^r	42

strain were performed using serial dilutions of 1:1,000 from standing overnight cultures in LB. Dilutions were plated on LA without antibiotics, and colonies were replicated to selective media to determine plasmid loss.

RESULTS

Preparation of *par*, *repB*, and *repC* probes. The maintenance regions of the *S. typhimurium* virulence plasmid identified previously (42) are each separated by greater than 5 kb of intervening DNA. The *par* region is located at 88.6 to 91 kb, *repB* is located at 44.8 to 52 kb, and *repC* is located at 59.4 to 62.6 kb (Fig. 1). To determine the conservation of these maintenance regions among *Salmonella* serovars, internal regions were utilized as probes against Southern blots and colony blots of various serovars. The 1.5-kb *par*, 1.8-kb *repB*, and 1.5-kb *repC* fragments used as probes from each

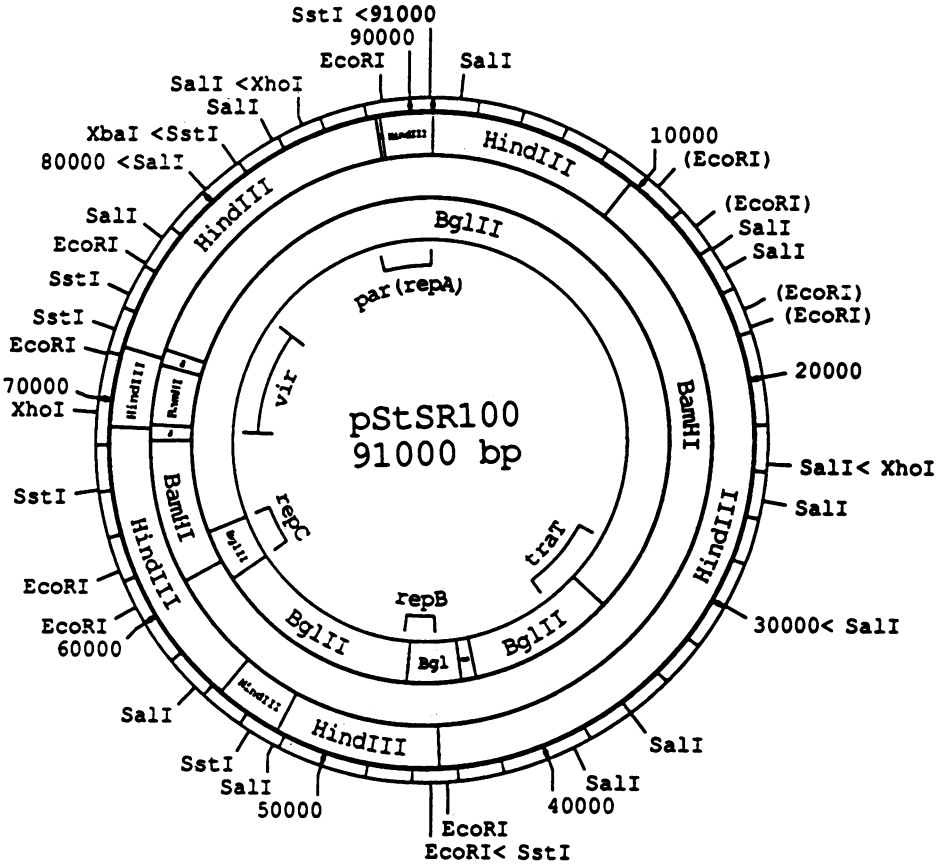

FIG. 1. Map of the virulence plasmid of *S. typhimurium*, indicating the location of the maintenance regions. bp, Base pairs. (Reprinted from the *Journal of Bacteriology* [42] with permission of the publisher.)

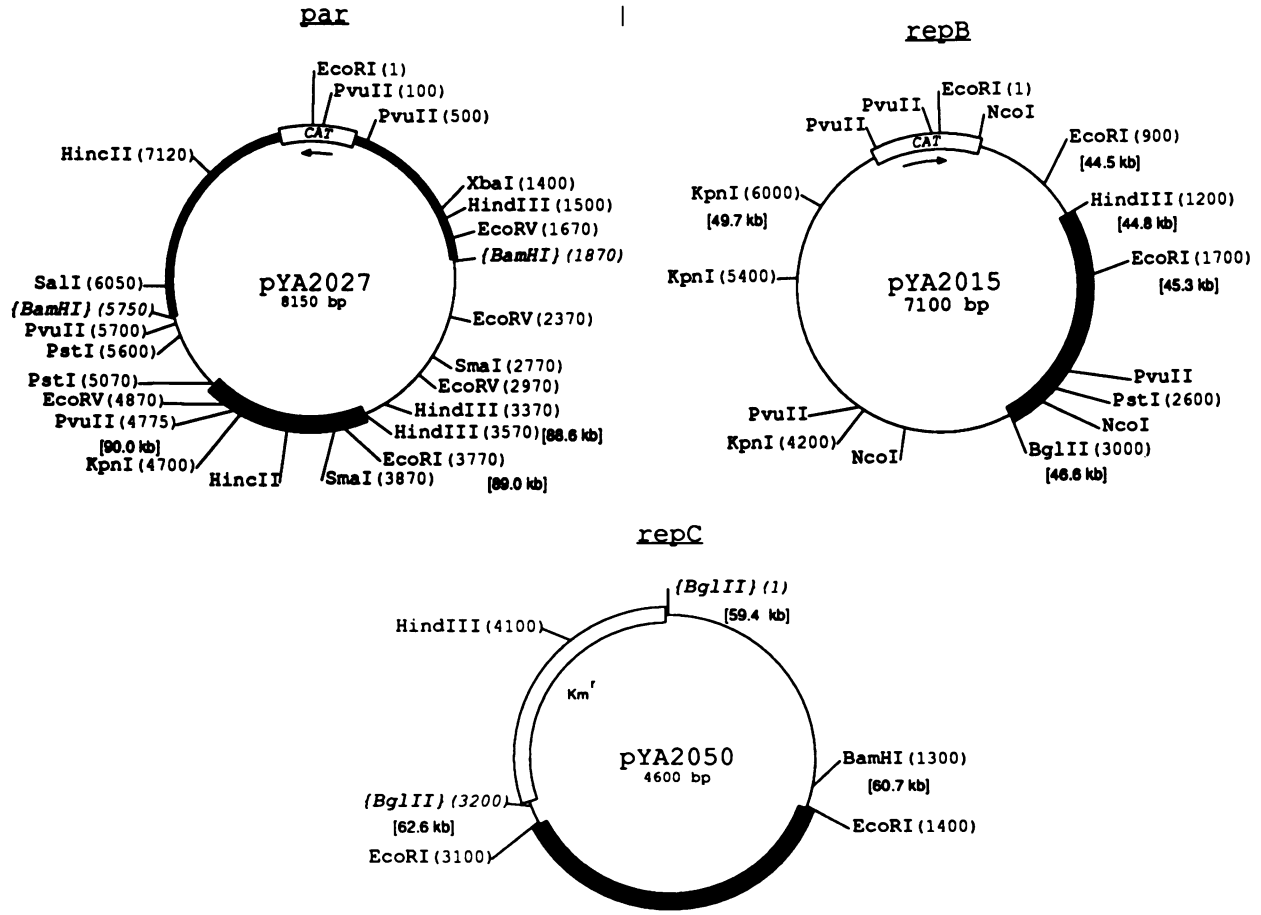

FIG. 2. Virulence plasmid maintenance region subclones, with the regions utilized as probes in hybridization studies shown as hatched bars. The thin lines represent virulence plasmid regions, while the closed bars represent pACYC184 vector regions. The numbers in brackets represent kilobase coordinates on the virulence plasmid. The sites in braces are those destroyed during cloning. bp, Base pairs.

region are highlighted in Fig. 2. The fragments were isolated from agarose gels by using the Gene Clean system and subsequently labeled with ^{32}P, using a random primer labeling system as described in Materials and Methods.

Hybridization of *par*, *repB*, and *repC* to plasmids isolated from invasive serovars. Although other studies have demonstrated the conservation of the *vir* region among invasive *Salmonella* serovars (34, 44), the conservation of the three maintenance regions has not been addressed. One strain from each invasive *Salmonella* serovar possessing a large virulence plasmid was selected for the hybridization experiments. Plasmid DNA was isolated from each strain and run on an agarose gel, followed by Southern transfer to a GeneScreen membrane. This membrane was then hybridized sequentially with each of the probes described above. The results of these hybridizations are illustrated by the autoradiographs shown in Fig. 3. The weak blurry band present for the pStSR100 positive control in lane 2 in all panels was due to the lower quantity of DNA loaded, not a lack of homology, compounded by the presence of nicked open circular DNA failing to enter the gel readily. *par* from *S. typhimurium* hybridized with the large plasmids of each serovar (Fig. 3A), as revealed by a strong supercoiled band, with a lower faint band indicating the presence of topoisomers in most samples, except that hybridization of *par* with the 70-kb plasmid of *S. dublin* was less than for other plasmids. In addition, some of the other small plasmids (barely visible at the bottom of Fig. 3A), including the 4.5-kb plasmid of *S. enteritidis* χ3759, the 2.5-kb plasmid of *S. gallinarum* χ3540, the 2.5- and 2.7-kb (dimer or open circular) plasmids of *S. pullorum* χ3762, and the 7-kb plasmid of *S. typhimurium* χ3760, hybridized weakly, indicating slight homology with *par*. The *par* probe also failed to hybridize with the IncFI plasmid F, IncFII plasmid R100, or IncH plasmid R726-1 at a stringency requiring ~65% homology for detection. The *repB* probe also hybridized to the large plasmids in all serovars in addition to the IncFII plasmid R100, while it failed to hybridize with F, R726-1, or the smaller plasmids present in any of the serovars tested (Fig. 3). *repC* hybridized with the large plasmids of *S. choleraesuis*, *S. enteritidis*, and *S. typhimurium* along with F but not with R100 or R726-1 and only very weakly with the large plasmids of *S. dublin*, *S. gallinarum*, and *S. pullorum* (Fig. 3C).

The distribution of *par*, *repB*, and *repC* among 91 isolated strains representing 36 *Salmonella* serovars was tested by using a colony blot probed with the same maintenance regions described above, using the same conditions as for the Southern blot. The presence of each region and the number of isolates hybridizing among the total tested for each serovar are listed in Table 3. The colony blot revealed sequences homologous to both the *par* and *repB* probes in *S. choleraesuis*, *S. dublin*, *S. enteritidis*, *S. gallinarum*, *S. pullorum*, and *S. typhimurium*, while *S. dublin*, *S. galli-*

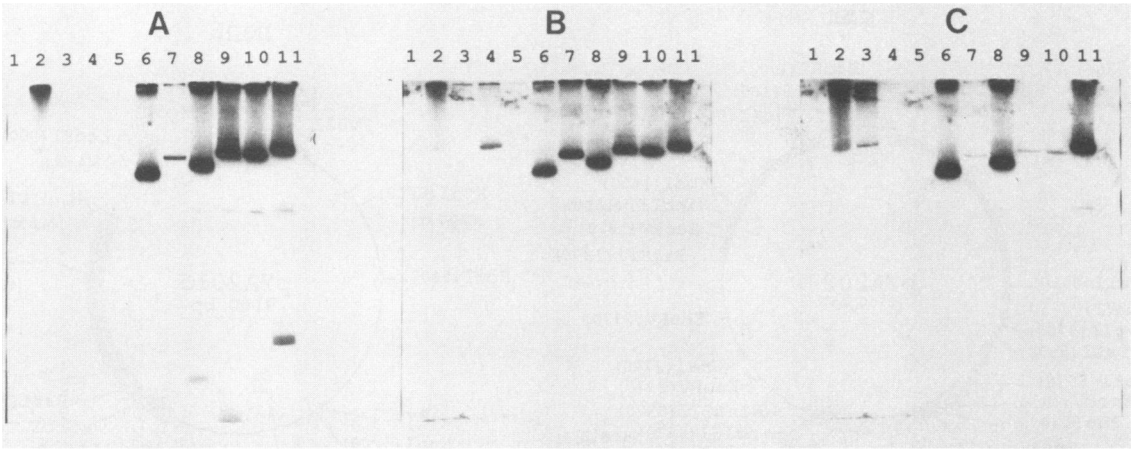

FIG. 3. Southern blot of plasmid DNA from *Salmonella* serovars hybridized with the following fragments: (A) *par* 1.5-kb *Hin*dIII-*Pst*I; (B) *repB* 1.8-kb *Hin*dIII-*Bgl*II; (C) *repC* 1.7-kb *Eco*RI. Each panel shows the results of hybridizing the same Southern blot with the probes indicated. Lanes 1, λ cut with *Hin*dIII; lanes 2, pStSR100 (χ3461); lanes 3, F (χ15); lanes 4, R100 (χ1781); lanes 5, R726-1 (χ2086); lanes 6, *S. choleraesuis* (5451-84); lanes 7, *S. dublin* (88/8); lanes 8, *S. enteritidis* (χ3759); lanes 9, *S. gallinarum* (χ3540); lanes 10, *S. pullorum* (χ3762); lanes 11, *S. typhimurium* (χ3760).

narum, and *S. pullorum* failed to hybridize with the *repC* probe. The colony blot also indicated that *par* and *repB* either both hybridized or failed to hybridize to each isolate in serovars that hybridized with either probe (except *S. dublin*). Although all three strains of *S. dublin* tested hybridized with the *repB* replicon probe, only χ3790 and 88/8 appeared to hybridize with the *par* probe (although weakly), while hybridization of *par* with 1092 could not be detected. The limited number of isolates tested for each serovar revealed that for *S. enteritidis* and *S. typhimurium*, respectively, 88 and 69% of the same isolates of these serovars hybridized with all three probes, although no additional tests were conducted to determine whether any virulence plasmid sequences or large plasmids were present in those isolates that failed to hybridize with these maintenance gene clones. This conservation of the *par* region among the invasive *Salmonella* serovars suggested that the incompatibility exhibited by the cloned *par* region could be used to exclude the virulence plasmids of these serovars.

Exclusion of the virulence plasmid of *S. typhimurium* by using high-copy-number *par* clones or destabilization with specific Km^r inserts in the *par* region. Having observed that the *par* region clones or Km^r insert *parA*[89.1 kb *Sma*I::Km] could produce virulence plasmid-free cells of *S. typhimurium* χ3934, we investigated the possibility of curing other strains of *S. typhimurium* or other serovars. When the high-copy-number *par* clone pYA2028 was transformed into χ3934, followed by growth of isolated Ap^r Tc^r transformants in LB containing only ampicillin, a rapid decline of Tc^r virulence plasmid-containing cells to 18% was observed after 10 generations of growth (42). Further growth of χ3934(pYA2028) for 10 generations in LB without selection for the pUC18 *par* clone resulted in 18.5% of the cells losing pYA2028 and becoming Ap^s. The instability of pYA2028 observed in χ3934, however, was not reliably exhibited in other *S. typhimurium* backgrounds, including χ3761 and χ3762. Thus, introduction of pYA2028 eliminates the virulence plasmid, but the pYA2028 *par* clone may remain.

Introduction of the *parA*[89.1 kb *Sma*I::Km] insert by P22HT*int*-mediated transduction into the virulence plasmid of *S. typhimurium* χ3000, χ3760, and χ3761 resulted in 4 to 15% Km^s plasmid-free cells after 30 generations of growth.

TABLE 3. Hybridization of virulence plasmid maintenance regions with *Salmonella* serovars

Serovar	No. of isolates hybridizing/no. tested		
	par	*repB*	*repC*
S. agona	0/1	0/1	0/1
S. albany	0/1	0/1	0/1
S. anatum	0/2	0/2	0/2
S. arizonae	0/2	0/2	0/2
S. bovismorbificans	0/2	0/2	0/2
S. brandenberg	0/1	0/1	0/1
S. bredeny	0/1	0/1	0/1
S. branderup	0/1	0/1	0/1
S. choleraesuis[a]	2/2	2/2	2/2
S. derby	0/1	0/1	0/1
S. dublin[a]	2/3	3/3	0/3
S. duisburg	0/1	0/1	0/1
S. enteritidis[a]	15/17	15/17	15/17
S. gallinarum[a]	2/2	2/2	0/2
S. give	0/1	0/1	0/1
S. glostrup	0/1	0/1	0/1
S. hadar	0/2	0/2	0/2
S. heidelberg	0/2	0/2	0/2
S. infantis	0/3	0/3	0/3
S. java	0/1	0/1	0/1
S. london	0/2	0/2	0/2
S. manhattan	0/2	0/2	0/2
S. montevideo	0/1	0/1	0/1
S. newport	0/1	0/1	0/1
S. nienstaden	0/1	0/1	0/1
S. ohio	0/1	0/1	0/1
S. othmarschen	0/1	0/1	0/1
S. panama	0/2	0/2	0/2
S. pullorum[a]	3/3	3/3	0/3
S. schwarzengrund	0/1	0/1	0/1
S. sieburg	0/1	0/1	0/1
S. tennessee	0/1	0/1	0/1
S. thompson	0/1	0/1	0/1
S. typhimurium[a]	16/23	16/23	16/23
S. vejle	0/1	0/1	0/1
S. virchow	0/2	0/2	0/2

[a] Serovars that often possess a large virulence plasmid that hybridizes to the *S. typhimurium* virulence plasmid *virA* gene (34).

TABLE 4. Stability of *Salmonella* virulence plasmids containing either Tn*mini-tet* or *parA*[89.1 kb *Sma*I::Km][a]

Serovar (parent strain)	+Tn*mini-tet* (~transduction frequency) % Tc^r	+*parA*[89.1 kb *Sma*I::Km] (~transduction frequency) % Km^r
S. choleraesuis (5451-84)	(1×10^{-6}) >99.4	(5×10^{-8}) 30.9
S. dublin (88/8)	($<2 \times 10^{-8}$) ND[b]	($<2 \times 10^{-8}$) ND
S. enteritidis (χ3759)	(5×10^{-7}) >99.5	(1×10^{-7}) 80.9
S. gallinarum (χ3540)	(2×10^{-7}) >99.3	(1×10^{-6}) 20.5
S. pullorum (χ3762)	(3×10^{-7}) >99.4	(2×10^{-6}) 96.6
S. typhimurium (χ3364)	ND	ND 79.0

[a] Each strain indicated was transduced to Tc^r or Km^r by using P22HT*int* or P1L4 grown on χ3918. Transductants were then grown for 60 generations without selection as nonaerated cultures at 37°C in LB.
[b] ND, Not determined.

This destabilization of the *S. typhimurium* virulence plasmid, due to a Km^r insert within *parA* (42), was the basis for the following tests conducted to determine whether the virulence-associated plasmids of other serovars that hybridized with the *par* region could be destabilized by *par* inactivation.

Curing other serovars of virulence plasmids by using the *parA* Km^r insert. Although the *par* region is conserved among the different invasive *Salmonella* serovars, with the possible exception of *S. dublin*, the function or effect of *par* on the stability of virulence plasmids in other serovars is unknown. The use of either P22HT*int*- or P1L4-mediated transduction to replace the functional *parA* gene with that containing the Km^r insert *parA*[89.1 kb *Sma*I::Km] obtained from χ3918 allowed analysis of *par* region function in other serovars while providing a curing regimen that does not involve additional treatment. The destabilized virulence plasmid pStLT203 was obtained by replacing the functional *parA* of pStLT100 with *parA* containing a Km^r cartridge that inactivates *parA* (42). Transforming pStLT203 into χ3477 produced χ3918, a *galE* deletion mutant of *S. typhimurium* used for the production of P22HT*int* and P1L4 lysates. The unstable pStLT203 was maintained by continued selection for Km^r. One strain of each serovar containing a large virulence plasmid shown to hybridize with *par* (Fig. 3A) was transduced to Km^r for the destabilization test, while controls were obtained by transducing the same strains to Tc^r, using the same bacteriophage lysates but selecting for transductants containing Tn*mini-tet* insertions on LA containing tetracycline. Although the *parA* Km^r insert of pStLT203 destabilizes the virulence plasmid in *S. typhimurium* (42), the Tn*mini-tet* insertion has no detectable effect on virulence plasmid stability (15). Single transductants of each serovar were streaked to obtain isolated colonies for use in stability tests. The stability of each Km^r- or Tc^r-labeled plasmid was determined by picking one isolated colony of each strain and growing it in a series of standing overnight LB cultures without selection for 60 generations, while monitoring the percentage of the population retaining either antibiotic resistance marker by replica plating. Km^s plasmid-free segregants were detected within 30 generations, while <1% Tc^s plasmid-free segregants were detected after 60 generations (Table 4). The smaller colony size observed for strains retaining the *parA*[89.1 kb *Sma*I::Km] insert aided in the isolation of Km^s plasmid-free segregants, although the extent of this apparent difference in growth rate was not quantified. Although strains of all other serovars that hybridized with the *par* region allowed allele replacement with *parA*[89.1 kb *Sma*I::Km], we have been unable to introduce this *parA* Km^r insert or the Tn*mini-tet* insert from *S. typhimurium* into the virulence plasmid of any *S. dublin* strain.

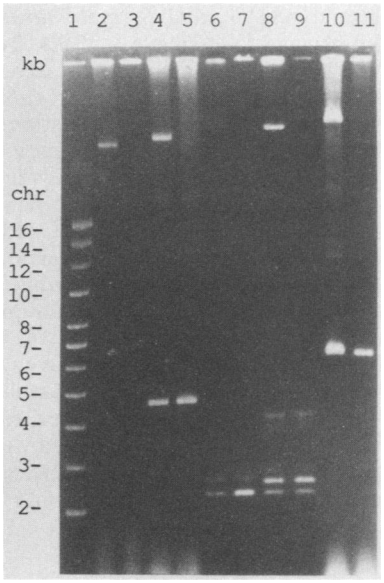

FIG. 4. Parental and cured *Salmonella* serovars produced by *parA* allele replacement. Lane 1, Supercoiled ladder; lane 2, *S. choleraesuis* (5451-84); lane 3, cured *S. choleraesuis* (χ3903); lane 4, *S. enteritidis* (χ3759); lane 5, cured *S. enteritidis* (χ3866); lane 6, *S. gallinarum* (χ3540); lane 7, cured *S. gallinarum* (χ3905); lane 8, *S. pullorum* (χ3762); lane 9, cured *S. pullorum* (χ3872); lane 10, *S. typhimurium* (χ3760); lane 11, cured *S. typhimurium* (χ3868). chr, Chromosomal DNA.

Additional attempts to cure the virulence plasmid of *S. dublin* from strains 1092 and 88/8 by using the pUC18 *par* clone pYA2028 were also unsuccessful. Ten isolated Ap^r colonies obtained after growing isolated pYA2028 transformants for >10 generations in LB containing ampicillin were screened for the presence of the virulence plasmid by agarose gel electrophoresis as described in Materials and Methods. Although all 10 isolates of *S. typhimurium* χ3000 subjected to the same curing treatment as the *S. dublin* strains lost their virulence plasmid, 10 of 10 isolates of both *S. dublin* strains still contained virulence plasmids.

Confirmation of plasmid loss in *Salmonella* serovars. Although the homology of the *par* region of the *S. typhimurium* virulence plasmid with the large plasmids of other invasive *Salmonella* serovars suggested that only the large plasmid should be labeled with the Km^r insert and subsequently lost, confirmation of this was still required since *par* weakly hybridized to several of the small plasmids in *S. enteritidis*, *S. gallinarum*, *S. pullorum*, and *S. typhimurium* (Fig. 3A).

Demonstration that the large virulence plasmids were actually the only plasmids eliminated was shown by agarose gel electrophoresis of plasmid DNA isolated from both the parent strains and their cured derivatives (Fig. 4). Analysis of each cured strain confirmed that only the large virulence plasmid was eliminated, while the remainder of the plasmid profile remained unchanged: *S. choleraesuis* 5451-84 still contained the 8-kb plasmid following elimination of the 50-kb plasmid pScKunV1, *S. enteritidis* χ3759 retained the 4.5-kb plasmid after elimination of the 55-kb plasmid pSeV1, *S. gallinarum* χ3540 retained the 2.5-kb plasmid following elimination of the 85-kb plasmid pSgV1, *S. pullorum* χ3762 retained the 2.5- and 2.7-kb plasmids following elimination of

the 85-kb plasmid pSpV1, and *S. typhimurium* χ3760 retained the 7-kb plasmid following loss of the 91-kb virulence plasmid pStV1. The slight homology indicated between *par* and the smaller plasmids or with the *S. dublin* virulence plasmid was insufficient to allow recombination and labeling.

The possibility that the large plasmid had integrated into the chromosome while losing Km^r was analyzed by probing a colony blot of the cured strains and their parents with the 1.2-kb *Bam*HI-*Eco*RI *virA* fragment of pGTR001 obtained from the *S. typhimurium* virulence plasmid. All parent strains hybridized with the *virA* probe, while none of the cured derivatives showed any hybridization (data not shown). Failure to hybridize with *virA* was taken as an indication of virulence plasmid absence, even though Korpela et al. (24) have reported instances of plasmid integration with loss of the *vir* region. Nevertheless, our tests confirmed that loss of Km^r was consistently associated with the loss of only the large virulence plasmid and that the plasmid was eliminated and not frequently integrated into the chromosome.

DISCUSSION

The invasive *Salmonella* serovars, *S. choleraesuis*, *S. dublin*, *S. enteritidis*, *S. gallinarum*, *S. pullorum*, and *S. typhimurium*, have previously been shown to contain plasmids that hybridize with the *vir* region of the *S. typhimurium* virulence plasmid (34). The hybridization experiments described here revealed that both the *repB* (~20 kb counterclockwise from *vir*) and *par* (~14 kb clockwise from *vir*) regions also hybridized with the same group of serovar-specific plasmids, although the *par* region hybridized weakly and inconsistently with the virulence plasmids in *S. dublin*. Although the second replicon of the *S. typhimurium* virulence plasmid, *repC*, is closer to the *vir* region (~6 kb counterclockwise from *vir*) than the other maintenance regions analyzed, it failed to hybridize with the plasmids of *S. dublin*, *S. gallinarum*, or *S. pullorum*. The absence of all three maintenance regions from all of the 30 additional serovars tested is similar to the findings of Poppe et al. (34) for the *vir* region of the *S. typhimurium* virulence plasmid, even though large plasmids are present in a number of these serovars. The conservation of virulence plasmid regions among *Salmonella* serovars determined by Korpela et al. (24) also supports the distribution of maintenance regions determined in this study. The probes utilized by Korpela et al. (24), however, were substantially larger than those employed in this study. The 1.5-kb *par* probe is within their 8-kb probe 5, the 1.8-kb *repB* probe is within their 15-kb probe 7, and the 1.7-kb *repC* probe is adjacent to their 8-kb probe 1. Although the *vir* region is well conserved among *Salmonella* serovars, it is interesting that probe 7, containing the *repB* region, was found in all isolates that hybridized with any virulence plasmid sequences, including some "cured" isolates, *S. give*, and three serovars we have not tested (24). The *repB* region thus appears highly conserved among *Salmonella* serovars on the basis of this study and the results of Korpela et al. (24). This suggests that *repB* may actually be the primary replicon instead of the *repA* region containing *par*, as proposed by Michiels et al. (29). The structural and functional differences between the *par* region of the *S. typhimurium* virulence plasmid and that of the *S. dublin* virulence plasmid indicated by reduced hybridization of *par* with the virulence plasmid of *S. dublin* 88/8, the inability to introduce the Km^r insert of *parA* via P22HT*int*-mediated transduction, and the absence of detectable incompatibility between the *S. dublin* virulence plasmid and the *S. typhimurium* virulence plasmid *par* clone pYA2028 are in contrast to the observation of *par* clone incompatibility with *S. dublin* plasmids made by Cerin and Hackett (9). Their *par* clone pADE78 used to exclude two large plasmids from an *S. dublin* strain, SL2972, contains an additional region of DNA, designated *incR*, which is not present in the *par* clones used in the present study and may be involved in the different incompatibility observed.

The distribution of the *repC* replicon among the *Salmonella* serovars tested appears to divide them into two categories, those that are $RepC^+$, including *S. choleraesuis*, *S. enteritidis*, and *S. typhimurium*, and those that are $RepC^-$, consisting of *S. dublin*, *S. gallinarum*, and *S. pullorum*. The distribution of *repC* was not checked by Korpela et al. (24) since their probes excluded this region. The three serovars sharing common plasmid maintenance functions are also from different O-antigen groups: *S. choleraesuis* is from group C, *S. enteritidis* is from group D, and *S. typhimurium* is from group B. Although there have been mixed reports associating virulence plasmids with differences in lipopolysaccharide core and side chain composition (17, 40, 41), the presence of plasmids containing related maintenance and virulence regions in different O-antigen groups raises questions as to the coevolution of plasmids within different serovars. The restricted distribution of these maintenance regions within all the serovars screened suggests that a common precursor containing all regions may have given rise to these plasmid-containing serovars. The inability of these virulence plasmids to mobilize themselves (39) restricts their exchange among strains, although mobilization with the help of IncFI (4) or IncFII (19) plasmids is possible. The weak hybridization of *repB* with R100 (IncFII) and *repC* with F (IncFI) shown in this study indicates that the plasmids of these invasive *Salmonella* serovars have replication mechanisms related to the IncFI and IncFII plasmids, as previously reported for *S. typhimurium* (29, 42) and *S. dublin* (40).

The conservation of the *par* region among these invasive *Salmonella* serovars allowed for allele replacement and demonstrated that absence of a functional ParA protein as determined previously for the virulence plasmid of *S. typhimurium* (42), also affects the stability of the plasmids containing regions homologous to *par* in other *Salmonella* serovars.

Although plasmid-cured strains can be produced by chemical or physical treatments, the problems encountered encumber expeditious strain construction. The demonstration that the destabilized *par* region obtained from *S. typhimurium* can be used to replace the *par* regions in all invasive serovars tested, except *S. dublin*, simplifies the production of plasmid-cured derivatives. Once a P22HT*int* or P1L4 lysate has been made on a strain containing the *parA* Km^r insert, the mutation can readily be transferred to other strains, labeling and destabilizing only the virulence plasmid, in one step. Although this principle does not allow the curing of nonrelated plasmids within these serovars, it does provide a consistent means of eliminating virulence plasmids without the additional mutational risk of other curing methods. This generation of isogenic plasmid-cured and plasmid-containing strains allows elucidation of the role of virulence plasmids in invasive disease and the elicitation of protective immune responses against *Salmonella* infections.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DE06669 and AI24533 from the National Institutes of Health. We thank Jorge Galan, Paul Gulig, David Howe, Sandra Kelly, and Hank Lockman for critical review and assistance.

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