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Cloning and Transposon Insertion Mutagenesis of Virulence Genes of the 100-Kilobase Plasmid of *Salmonella typhimurium*

PAUL A. GULIG† AND ROY CURTISS III*

Department of Biology, Washington University, St. Louis, Missouri 63130

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We have cloned regions of the 100-kilobase (kb) plasmid, pStSR100, of *Salmonella typhimurium* SR-11 that confer virulence to plasmid-cured *S. typhimurium*. Cells carrying recombinant plasmids that conferred virulence were selected by inoculating mice orally with recombinant libraries in virulence plasmid-cured *S. typhimurium* and harvesting isolates that infected spleens. Three plasmids, pYA401, pYA402, and pYA403, constructed with the cosmid vector pCVD305 conferred wild-type levels of virulence to plasmid-cured *S. typhimurium* and had a common 14-kb DNA insert sequence. Another recombinant plasmid, pYA422, constructed with the vector pACYC184, conferred to plasmid-cured *S. typhimurium* a wild-type 50% lethal dose (LD_{50}) level, but mice died more slowly than when infected with wild-type *S. typhimurium*. Furthermore, pYA422 conferred the ability to cause a higher, but not a wild-type, level of splenic infection on plasmid-cured *S. typhimurium*. pYA422 had a 3.2-kb insert sequence which mapped to the center of the 14-kb common sequence of the cosmid clones. Transposon Tn*5* insertion mutations in pYA403 inhibited virulence to various degrees, and when transduced into the native virulence plasmid of *S. typhimurium*, these Tn*5* insertions decreased virulence to degrees similar to those observed when the Tn*5* insertions were present in pYA403. *vir-22*::Tn*5* in pStSR100 greatly lowered infection of spleens relative to unmutagenized virulence plasmid, while *vir-26*::Tn*5* and *vir-27*::Tn*5* lowered splenic infection to lesser degrees. At least three proteins were encoded by pYA403 containing 23 kb of insert sequence and subclone pYA420, containing the 14-kb common insert sequence present in all of the cosmid clones. One of these proteins, with an apparent molecular weight of 28,000, was also encoded by pYA422. The Tn*5* insertion that most attenuated virulence, *vir-22*::Tn*5*, inhibited synthesis of the 28,000-molecular-weight protein. The *vir-22*::Tn*5* insertion was complemented by recombinant plasmids encoding only the 28,000-molecular-weight protein, suggesting a role of this protein in virulence. However, recombinant plasmids, exemplified by pYA422, that encoded only the 28,000-molecular-weight protein did not confer full virulence.

The importance of large plasmids for the virulence of *Salmonella typhimurium* and other serotypes of *Salmonella* has been the subject of much research in recent years (1, 4, 14, 16, 18, 20, 24, 27–29, 32). Although the exact contribution of the virulence plasmids to pathogenesis is still a matter of debate (14, 16, 18, 20, 24), progress has been made in identifying regions of the plasmid necessary for conferring virulence. Popoff et al. (29) and Helmuth et al. (20) identified, by Southern blot hybridization, restriction digest bands that were shared among various serotypes. Baird et al. (1) identified regions of the *S. typhimurium* and *Salmonella dublin* virulence plasmids that had very similar restriction enzyme maps. They also constructed transposon insertion mutations that inhibited virulence to various degrees. Hackett and Wyk (17) cloned a gene encoding a 23,000-molecular-weight membrane protein (23K protein), but they did not report any role for this gene in virulence. Hackett et al. (18) also cloned a gene from the *S. typhimurium* virulence plasmid that encoded an 11K protein which conferred serum resistance both to their virulence plasmid-cured *S. typhimurium* LT2 and to *Escherichia coli* K-12. Michiels et al. (27) identified a virulence-associated region of the *S. typhimurium* plasmid by deletion analysis. Most recently, Beninger et al. (4) further defined the virulence region of the *S. dublin* plasmid by transposon and deletion mutation analyses.

We (14) and others (16, 28) have found that the 100-kilobase (kb) virulence plasmid of *S. typhimurium* is primarily responsible for conferring the potential for systemic infection from Peyer's patches to spleens of mice inoculated orally (p.o.) with *S. typhimurium*. We therefore constructed recombinant libraries of virulence plasmid DNA in a plasmid-cured *S. typhimurium* strain, and we report here the isolation of virulence-conferring recombinant plasmids by inoculating mice p.o. and selecting bacteria that infected spleens. Transposon Tn*5* insertion mutagenesis of these plasmids further identified plasmid sequences that were involved in virulence, and three proteins were identified that were encoded by the cloned virulence region.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and their genotypes and descriptions are listed in Table 1. Unless noted otherwise, cells were grown in L broth (25) or on L broth containing 1.5% (wt/vol) agar at 37°C. For infection with phage λ, bacteria were grown in lambda broth (10). The following antibiotics were used at the indicated concentrations (micrograms per milliliter): tetracycline (Tc), 12.5 to 25; chloramphenicol (Cm), 25; kanamycin (Km), 50; ampicillin (Ap), 100 to 200; and streptomycin (Str), 50.

DNA manipulations. Cesium chloride density gradient centrifugation for purification of plasmid DNA was done as described before (26). Restriction enzyme digestion and ligation were done with enzymes from Bethesda Research Laboratories, Gaithersburg, Md.; ProMega Biotec, Madison, Wis.; and International Biotechnologies, Inc., New

* Corresponding author.
† Present address: Dept. of Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, FL 32610.

TABLE 1. Bacterial strains

Strain	Relevant genotype	Virulence plasmid	Comments[a]
S. typhimurium			
χ3306 (SR-11)	*gyrA1816*	+	Virulent (14)
χ3337 (SR-11)	*gyrA1816*	−	100-kb plasmid-cured χ3306 (14)
χ3385 (LT2-Z)	*hsdL6 galE496 trpB2 flaA66 his-6165 rpsL120 xyl-404 metE551 metA22 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29 val*	−	Rough LPS, restriction$^-$, modification$^+$, λ^s, derived from AS68 of T. Palva by S. A. Tinge and R. Curtiss (unpublished)
χ3456 (SR-11)	pStSR100::Tn *mini-tet*	+	Virulent (14)
χ3477 (LT2-Z)	*hsdL6* Δ(*galE-uvrB*)*1005 flaA66 rpsL20 xyl-404 lamB*$^+$ (*E. coli*) Δ(*zja*::Tn*10*) *hsdSA29*	−	From χ3385 by Tinge and Curtiss (unpublished); Δ(*galE-uvrB*)*1005* from SL5400 of B. A. D. Stocker
χ3589 (SR-11)	pStSR100 *vir-22*::Tn*5*	+	χ3306 with *vir-22*::Tn*5* transduced from χ3477(pYA403 *vir-22*::Tn*5*)
E. coli			
χ925 (K-12)	*thr-1 ara-13 leu-6 azi-8 tonA2 lacY1 minA1 glnV44 gal-6 minB2 rpsL135 xyl-7 mtl-2 thi-1*		Minicell producer (12)
χ2819 (K-12)	*lacY1 glnV44 galK2 galT22* λ(*c*I857 *b2 red3 S7*) *recA56* Δ*thyA57 metB1 hsdR2*		In vivo packaging of cosmid DNA (23)
LE392 (K-12)	*lacY1 glnV44 galK2 galT22 tyrT58 metB1 hsdR514 trpR55*		Efficient transformation, amber suppressor (26)
MG1655 (K-12)			Suppressor free (15)

[a] LPS, Lipopolysaccharide.

Haven, Conn., according to the suppliers' instructions. Nick translation was done with a kit from Bethesda Research Laboratories according to the supplier's instructions with [α-^{32}P]dATP (specific activity, 500 mCi/mmol; Amersham Corp., Arlington Heights, Ill.). Transformation was done by the procedure of Humphreys et al. (21), and plasmid DNA extraction was done by the method of Birnboim (6).

Construction of pStSR100 library in pCVD305. The cosmid cloning vector pCVD305 was a gift of James Kaper, University of Maryland School of Medicine. pCVD305 is a 23-kb IncP low-copy-number plasmid which carries the Tc^r gene from pRK290, the *lacZ* multiple cloning site of M13mp8, and the λ *cos* site from pREG152. Thus, approximately 25 kb of insert DNA can be cloned into pCVD305 for efficient packaging into λ.

The virulence plasmid of χ3306, pStSR100, was partially cleaved with *Sau*3A, and fragments of approximately 25 kb were isolated by centrifugation through a 5 to 30% (wt/vol) sucrose gradient formed by freezing and thawing a 20% (wt/vol) solution of sucrose (2). Equimolar amounts of *Sau*3A-cut pStSR100 and *Bam*HI-cut pCVD305 were ligated at room temperature for 48 h. Ligated DNA was packaged in vitro into phage λ with Packagene (Promega Biotec). The packaged cosmid library was transduced into *E. coli* χ2819 (23) with selection for Tc^r. Because dimerization of pCVD305 could result in a packageable product, Tc^r transductants were screened for the presence of virulence plasmid DNA inserts by colony blot hybridization with ^{32}P-labeled, nick-translated pStSR100 (26).

For movement of the cosmid library into *S. typhimurium*, cells in colonies of strain χ2819 carrying the individual recombinant plasmids were thermally induced to package cosmids into λ in vivo by incubation at 42°C as described before (23). After thermal induction and packaging, cells in colonies were lysed by incubation over chloroform. The packaged library was transduced into *S. typhimurium* χ3385 by replica-plating lysed χ2819 cells to cells of χ3385 spread on an L-agar plate containing tetracycline and streptomycin. Successful transduction of individual plasmids into χ3385 was confirmed by colony blot hybridization with ^{32}P-labeled pStSR100.

The library was moved into virulence plasmid-cured *S. typhimurium* SR-11 (strain χ3337) by extraction of plasmid DNA of individual isolates of χ3385 and transformation.

Construction of pStSR100 library in pACYC184. pStSR100 DNA was partially digested with *Sau*3A, and fragments of less than 5 kb were isolated by centrifugation through a 5 to 30% (wt/vol) sucrose gradient. *Sau*3A-cut pStSR100 and *Bam*HI-cut, calf alkaline phosphatase-treated (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) pACYC184 (7) were ligated at 12°C for 16 h. Ligated DNA was transformed into *S. typhimurium* χ3477, extracted from χ3477, purified by cesium chloride density gradient centrifugation, and transformed into strain χ3337.

Infection of mice. The procedures for infection of mice were based on results of our previous work on pathogenesis mediated by the virulence plasmid (14). BALB/c mice (6 to 11 weeks old; Harlan Sprague-Dawley, Indianapolis, Ind.; Sasco Inc., St. Louis, Mo.) were inoculated p.o. after food and water deprivation and feeding of sodium bicarbonate as described before (14). Spleens of infected mice were sampled at various times postinoculation and homogenized in 2.5 ml of buffered saline containing 0.1% (wt/vol) gelatin (BSG) (10) in glass tissue homogenizers. Alternatively, spleens were homogenized in 3 ml of BSG with a Brinkman tissue homogenizer (Brinkman Instruments, Inc., Westbury, N.Y.; model PT10/35). Samples were diluted and plated on L-agar plates containing appropriate antibiotics, and values are reported as total CFU per spleen. Most splenic infections were determined at 1 week postinoculation because this time point yielded the greatest difference in plasmid-mediated infection of spleens and preceded the death of mice infected with fully virulent *Salmonella* strains (14). For 50% lethal dose (LD_{50}) determinations, mice were inoculated p.o. and monitored for death up to 30 days postinoculation. Previous studies indicated that essentially all deaths occurred by 30 days postinoculation in our system. LD_{50}s were determined by the method of Reed and Muench (30) with four to six mice

per inoculation dose. Infected mice were housed in cages with raised wire floors and filter lids to prevent cross-contamination between cages.

Tn*5* insertion mutagenesis. Phage λ::Tn*5* (λ *O*am *P*am *rex*::Tn*5* *c*I857 *b*221), a gift of Douglas Berg (5), was used as a suicide Tn*5* donor in suppressor-free *E. coli* MG1655. Recombinant plasmids were transformed into MG1655, and cells were grown in lambda broth with appropriate antibiotics. Bacteria were pelleted by centrifugation, suspended in 10 mM Tris (pH 7.4)–10 mM $MgSO_4$–0.01% (wt/vol) gelatin–100 mM NaCl, and infected with λ::Tn*5* at a multiplicity of 0.1 to 0.5. Phages were allowed to adsorb for 30 min at room temperature, followed by addition of 1 ml of L broth at 37°C. After incubation at 37°C for at least 1 h to allow expression of Km^r, the cultures were added to 35 ml of L broth containing 100 μg of kanamycin per ml and incubated overnight at 37°C to enrich for Tn*5* insertions in plasmids. The next day, plasmid DNA was extracted and transformed into *E. coli* LE392, with selection for Km^r. Restriction maps of plasmids in Km^r transformants were analyzed to verify insertion of Tn*5* into cloned virulence plasmid DNA sequences. Recombinant plasmids with Tn*5* insertions of choice were transformed into *S. typhimurium* χ3337 by using χ3477 as an intermediate *S. typhimurium* host. Recombination of Tn*5* insertions from cosmid clones into the wild-type virulence plasmid of χ3306 was achieved by generalized transduction with phage P22 HT*int* (31). *S. typhimurium* χ3477 possessing a Tn*5*-labeled recombinant plasmid was grown in L broth containing 0.01% (wt/vol) galactose instead of glucose to allow production of complete lipopolysaccharide and productive infection with P22 HT*int*. P22 HT*int* lysates from χ3477 were used to transduce Tn*5* insertions into χ3306, with selection for Km^r. Recombination of Tn*5* insertions into pStSR100 was confirmed by restriction enzyme mapping.

***E. coli* minicells.** The *E. coli* minicell-producing strain χ925 was used to identify plasmid-encoded proteins as described before (8, 11, 12). χ925 carrying each plasmid was grown to stationary phase in minicell labeling medium (minimal salts-glucose medium [10] containing all amino acids except methionine and containing thiamine hydrochloride), diluted 10^{-4} in minicell labeling medium, and grown overnight with vigorous aeration. The following day, whole cells were removed by centrifugation at 2,000 × *g* for 10 min at room temperature, and minicells were harvested from the resulting supernatant fluid by centrifugation at 12,000 × *g* for 10 min. Minicells were suspended in BSG and centrifuged through a 5 to 30% (wt/vol) sucrose gradient in BSG at 3,000 × *g* for 15 min at room temperature in an SW-27 rotor (Beckman Instruments, Inc., Fullerton, Calif.). The upper band containing minicells was removed and slowly diluted with BSG, and minicells were pelleted by centrifugation at 12,000 × *g* for 10 min. The minicell pellet was suspended in 1 ml of minicell labeling medium and incubated at 37°C for 10 min, and plasmid-encoded proteins were intrinsically radiolabeled by addition of 10 to 25 μCi of [^{35}S]methionine (Amersham; specific activity, 1,500 μCi/mmol). After incubation at 37°C for 10 min, labeling was stopped by incubation on ice, followed by pelleting of minicells and solubilization of proteins in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (19). Radiolabeled proteins were resolved by SDS-PAGE in 10% (wt/vol) polyacrylamide gels (19) or 5 to 20% (wt/vol) linear gradient polyacrylamide gels (as described in Hoefer Scientific Instruments, San Francisco, catalog 3), and identified by fluorography (En^{3}Hance; Dupont, Wilmington, Del.).

In vitro transcription-translation of plasmid-encoded proteins. A DNA-directed transcription-translation kit from Amersham was used according to the supplier's instructions with [^{35}S]methionine (Amersham) to radiolabel proteins. Radiolabeled proteins were resolved in 10% (wt/vol) polyacrylamide gels and identified by fluorography.

Statistical analysis. For comparison of CFU in spleens of mice inoculated p.o. with a single *S. typhimurium* strain, geometric means (log_{10}) of total splenic CFU of different strains were compared by using the one-tailed Student *t* test. For analysis of results from mixed infections of mice with two different *S. typhimurium* strains, the geometric mean (log_{10}) of the ratios of CFU of the first strain to CFU of the second strain was examined for the difference from the ratio being 1 by using the one-tailed Student *t* test.

RESULTS

Construction of pStSR100 library in pCVD305 and selection of virulence-conferring clones in mice. The virulence plasmid of *S. typhimurium* SR-11, pStSR100, was cloned into the cosmid vector pCVD305 by ligating 25-kb *Sau*3A fragments of pStSR100 into *Bam*HI-cut pCVD305. A library consisting of 44 pStSR100-hybridization-positive recombinant clones in χ2819 was successfully transferred into χ3337. This number of clones possessing 25 kb of insert DNA was greater than the 31 clones necessary for a 99% probability of constructing a representative library of the 100-kb virulence plasmid (9). Furthermore, digestion of the combined recombinant plasmid DNA present in the χ3337 library with *Pvu*II, *Eco*RI, and *Hin*dIII revealed that all bands of 10 kb or less from pStSR100 were present in the library (data not shown).

We (14) and others (16, 28) have determined that the virulence plasmid of *S. typhimurium* is essential for systemic infection from Peyer's patches to spleens. Recombinant plasmids that conferred the ability to infect spleens on pStSR100-cured *S. typhimurium* χ3337 were selected by infecting mice p.o. and isolating bacteria from spleens. Six or eight BALB/c mice were inoculated p.o. with 10^7 or 10^9 CFU, respectively, of χ3337 carrying the pStSR100 library. To ensure that approximately equal numbers of CFU of each construct were inoculated, the 44 isolates were grown together on a single L-agar plate and harvested by suspension in BSG for infection of mice. In addition to examining the plasmid DNA content of cultures of infected spleen homogenates, individual colonies were obtained from each spleen homogenate culture and the plasmid content of these individual isolates was examined by restriction enzyme digestion. The DNA from three plasmids, pYA401, pYA402, and pYA403, obtained from the spleen of a single mouse harvested at 5 days after inoculation with 10^9 CFU, had all of the restriction enzyme digestion fragments of plasmid DNA from the spleen homogenate cultures from all of the mice infected with the pStSR100 library (data not shown). This was examined by using *Eco*RI, *Hin*dIII, *Pst*I, and *Pvu*II, which each yielded three to eight bands after restriction of the plasmid DNA of cultures of infected spleen homogenates.

Restriction endonuclease maps of pYA401, pYA402, and pYA403. Plasmids pYA401, pYA402, and pYA403 were extracted from χ3337 and restricted with *Eco*RI and *Hin*dIII, and the resulting fragments were compared with those of pStSR100 generated by *Hin*dIII and *Eco*RI digestion. The three recombinant plasmids were further mapped with the restriction enzymes *Bam*HI, *Bgl*II, *Xho*I, and *Sal*I (Fig. 1). The recombinant plasmids had inserts of 22 to 26 kb and had

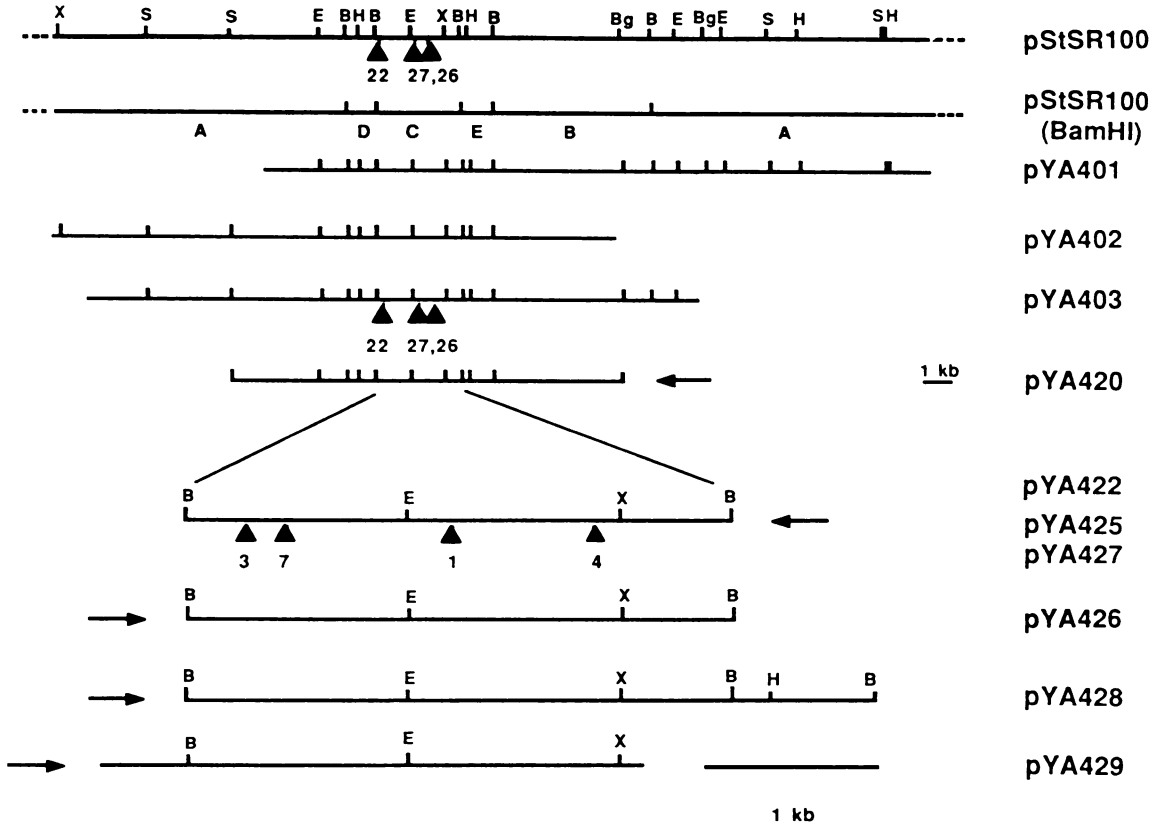

FIG. 1. Restriction enzyme maps of the *S. typhimurium* virulence plasmid, recombinant plasmids, and Tn*5* insertions. Numbers and solid triangles below lines indicate allele numbers and locations of *vir*::Tn*5* insertions. Letters below pStSR100(*Bam*HI) indicate *Bam*HI fragment designations. Letters above lines indicate restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I. Arrows indicate direction of transcription of tetracycline resistance gene in pACYC184 clones.

a common 14-kb insert sequence representing 14% of the virulence plasmid pStSR100 (Fig. 1). The 14-kb common insert sequence present in pYA403 was subcloned into pACYC184 by cutting pYA403 with *Sal*I and *Bgl*II and ligating into pACYC184 cut with *Sal*I and *Bam*HI. The subclone was designated pYA420 (Fig. 1).

Virulence conferred by cosmid clones. The ability to infect spleens after p.o. inoculation conferred on χ3337 by pYA401, pYA402, and pYA403 was compared with that of χ3306, χ3337, and χ3337(pCVD305) (Table 2, part A). To ensure that virulence-conferring recombinant plasmids had been selected in χ3337 as opposed to chromosomal mutations which conferred increased virulence, the recombinant plasmids were extracted and retransformed into χ3337. All three recombinant cosmids conferred on χ3337 a significantly higher spleen infection ability than did either pCVD305 or no plasmid ($P < 0.0005$). All mice infected with χ3337 carrying the recombinant plasmids and χ3306 were diseased (i.e., scruffy with enlarged, granulomatous spleens). pCVD305 conferred no splenic infection ability on χ3337. Furthermore, χ3337 containing the recombinant plasmids yielded splenic CFU values at least as high as did χ3306. Because pYA403 represented the central region of the pStSR100 sequence of the three cosmid clones, it was chosen for further virulence and genetic analyses. The p.o. LD_{50} of χ3337(pYA403) was $<10^5$ CFU, which is similar to that of χ3306, 3×10^5 CFU (Table 2, part A). Furthermore, the mean times to death for both strains were very similar, 12 and 10 days postinoculation, respectively.

Construction of pStSR100 library in pACYC184 and selection of virulence-conferring clones. Because the insert DNA in each plasmid in the pCVD305 library constituted a large portion (22 to 26 kb) of pStSR100 (100 kb), we constructed a library consisting of smaller insert sizes (<5 kb) in a different vector, pACYC184. pStSR100 was restricted with *Sau*3A and ligated to pACYC184 restricted with *Bam*HI. A library consisting of 1.5×10^3 independent clones in χ3337 was constructed in this manner. Ten mice were inoculated p.o. with χ3337 carrying the pACYC184 library, and cultures of homogenates of infected spleens had what appeared to be 18 different plasmid species. Individual colonies from homogenate cultures which collectively carried plasmids which comigrated with each of the 18 plasmids were then pooled and inoculated into a second set of six mice. pYA422 was the only plasmid recovered from cultures of spleens of the secondarily infected mice that consistently conferred on χ3337 the ability for increased infection of spleens of mice inoculated p.o.

Virulence of χ3337(pYA422). p.o. LD_{50} and splenic infection were determined for χ3337(pYA422). The p.o. LD_{50} for χ3337(pYA422) was 3.0×10^5 CFU (Table 2, part A). This value was very close to that of wild-type *S. typhimurium* SR-11, strain χ3306; however, the mean time to death was significantly greater for χ3337(pYA422) than for wild-type SR-11 χ3306 ($P < 0.001$, Student's one-tailed *t* test). At 6 days postinoculation with 4×10^8 CFU of χ3337(pYA422), spleens contained 2.6×10^4 CFU, compared with 10^6 to 10^7 CFU obtained with χ3306 in other experiments (14). The relative contribution to virulence by pYA422 in χ3337 was examined in more detail in mixed-infection experiments

TABLE 2. p.o. LD_{50}s and infection of spleens

Part[a]	Strain	LD_{50} (CFU)	CFU in spleen[b]	
			Mean ± SD (antilog mean)	*P* value
A	χ3306	3×10^5[c]	5.3 ± 0.9 (2.0×10^5)	
	χ3337	$>10^8$[c]	2.3 ± 1.0 (200)	<0.0025[d]
	χ3337(pCVD305)	ND[e]	1.65 ± 0.35 (45)	<0.0005[d]
	χ3337(pYA401)	ND	6.68 ± 0.96 (4.8×10^6)	<0.0005[f]
	χ3337(pYA402)	ND	5.94 ± 0.36 (8.7×10^5)	<0.0005[f]
	χ3337(pYA403)	$<1 \times 10^5$	5.5 ± 1.2 (3.2×10^5)	<0.0005[f]
	χ3337(pYA422)	3×10^5[g]		
B	χ3306	ND	5.46 ± 0.49 (2.9×10^5)	
	χ3589	ND	2.66 ± 0.29 (460)	<0.0005[d]
	χ3589(pYA427)	ND	2.52 ± 0.68 (330)	<0.001[d]
	χ3589(pYA428)	ND	5.01 ± 0.75 (1.0×10^5)	<0.005[h]
	χ3589(pYA429)	ND	4.27 ± 0.77 (1.9×10^4)	<0.05[d],<0.025[h]
C	χ3306	ND	5.41 ± 0.90 (2.6×10^5)	
	χ3337(pYA427)	ND	3.49 ± 0.71 (3.1×10^3)	<0.001[d]
	χ3337(pYA428)	ND	3.42 ± 0.98 (2.6×10^3)	<0.001[d]
	χ3337(pYA429)	ND	3.59 ± 0.68 (3.9×10^3)	<0.001[d]
D	χ3589(pYA425)	ND	2.55 ± 0.73 (355)	
	χ3589(pYA426)	ND	6.00 ± 0.93 (1.0×10^6)	<0.001[i]

[a] Each part represents a separate experiment.
[b] Mean $\log_{10}$ CFU in spleens 6 to 7 days postinfection with p.o. inocula of 4×10^8 to 1×10^9 CFU, four or five mice per group; unless stated otherwise, $P > 0.05$ compared with controls.
[c] Data from reference 14.
[d] *P* value vs. χ3306 within experiment.
[e] ND, Not determined.
[f] *P* value vs. χ3337 and χ3337(pCVD305) within experiment.
[g] Mean time to death of 20 days for χ3337(pYA422) compared with 10 days for χ3306 with inocula of 10^5 to 10^6 CFU ($P < 0.001$). Infection of spleens for χ3337(pYA422) not run in parallel with other strains for part A.
[h] *P* value vs. χ3589 within experiment.
[i] *P* value for χ3589(pYA426) > χ3589(pYA425).

(Table 3, part A). Twenty-two-fold more χ3337(pYA422) than χ3337(pACYC184) cells were recovered from spleens of infected mice ($P < 0.025$). However, 260-fold more wild-type χ3456 than χ3337(pYA422) cells were recovered from mice infected with both of these strains ($P < 0.0025$). Thus, pYA422 conferred virulence plasmid-cured *S. typhimurium* with increased but not wild-type levels of virulence, as measured by infection of spleens after p.o. inoculation.

Physical mapping of pYA403 and pYA422. The plasmids pACYC184 and pYA422 were digested with *Bam*HI or the combination of *Bam*HI and *Eco*RI and examined by agarose gel electrophoresis. After restriction with *Bam*HI, pYA422 generated a 4.0-kb fragment which comigrated with pACYC184 and a 3.2-kb fragment which comigrated with *Bam*HI fragment C of pStSR100, indicating that the *Sau*3A sites defining the insert sequence of pYA422 were present in the *Bam*HI sites which define *Bam*HI fragment C of pStSR100 (Fig. 1). The cloned insert sequence of pYA422 also yielded two *Bam*HI-*Eco*RI fragments that comigrated with *Eco*RI-generated fragments from *Bam*HI fragment C. That the insert of pYA422 consisted of *Bam*HI fragment C was confirmed by Southern blot analysis with ^{32}P-labeled pYA422 with pStSR100, pYA401, pYA402, and pYA403 (data not shown). Thus, pYA422 carried the 3.2-kb *Bam*HI fragment C of pStSR100 DNA, which was also present on the three cosmid clones which conferred the ability for splenic infection on plasmid-cured *S. typhimurium*.

Transposon insertion mutagenesis of pYA403, pYA422, and pStSR100. The cloned DNA sequences of pStSR100 present in pYA403 and pYA422 were subjected to transposon insertion mutagenesis. Three different Tn*5* insertions in *Bam*HI fragment C of pYA403 were analyzed for virulence (Fig. 1). The loci in the recombinant plasmids were designated *vir* (for virulence) for purposes of identifying Tn*5* insertion alleles. Mice were inoculated p.o. with mixtures of 1×10^8 to 2×10^8 CFU each of χ3337(pYA403) and χ3337(pYA403 *vir*::Tn*5*), and the CFU of each strain in infected spleens were enumerated at 5 days postinoculation to determine the

TABLE 3. Results of mixed p.o. infections[a]

Part	Strain A	Strain B	Antilog[b]		Ratio A/B[c]	*P* value
			CFU A	CFU B		
A	χ3337(pYA422)	χ3337(pACYC184)	2.8×10^5	1.2×10^4	1.35 ± 0.23 (22)	$P < 0.025$
	χ3456	χ3337(pYA422)	8.2×10^6	3.2×10^4	2.41 ± 0.59 (260)	$P < 0.0025$
B	χ3306	χ3306(pStSR100 *vir-22*::Tn*5*)	9.2×10^6	3.7×10^3	3.39 ± 0.34 (2.5×10^3)	$P < 0.0025$
	χ3306	χ3306(pStSR100 *vir-26*::Tn*5*)	1.9×10^6	6.5×10^4	1.5 ± 1.3 (32)	$P > 0.05$
	χ3306	χ3306(pStSR100 *vir-27*::Tn*5*)	1.5×10^5	1.8×10^4	0.92 ± 0.32 (8.3)	$P > 0.05$

[a] Three to four mice were inoculated p.o. with equal CFU (10^8 to 10^9 CFU) of strain A and strain B. At 1 week (experiment B) and 2 weeks (experiment A) postinfection, spleens were harvested, and CFU of each strain were determined. Parts A and B represent results of different experiments.
[b] Antilog of mean $\log_{10}$ CFU at 1 to 2 weeks postinoculation.
[c] Mean ± standard deviation of $\log_{10}$ ratio for individual mice (antilog mean); *P* value shown for ratio > 1.

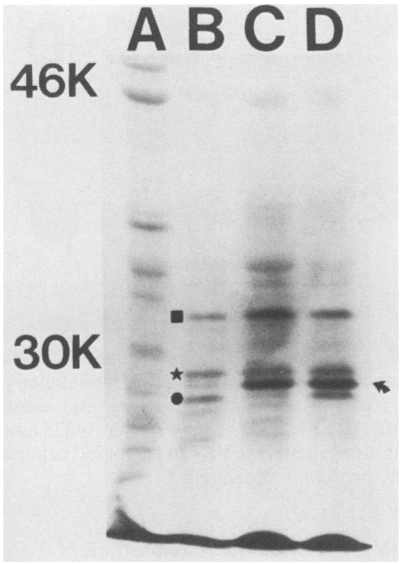

FIG. 2. Proteins synthesized in *E. coli* minicells containing recombinant plasmids constructed with pCVD305. Proteins were synthesized and radiolabeled with [^{35}S]methionine as described in Materials and Methods, resolved in a 10% polyacrylamide–SDS gel and identified by fluorography. The relevant portion of the 24-cm-long gel is shown. Lane A, pCVD305; lane B, pYA403; lane C, pYA403 *vir-22*::Tn*5*; lane D, pYA403 *vir-26*::Tn*5*. Symbols between lanes A and B indicate locations of pYA403-encoded proteins: ●, 28K protein; ★, 29K protein; ■, 32K protein. ◀, Tn*5*-encoded Km^r protein. Molecular weight markers were ^{14}C-proteins (Amersham). The vector control, pCVD305, in lane A was overloaded relative to the other lanes to prevent misinterpretation of proteins in lanes B to D as being insert-encoded.

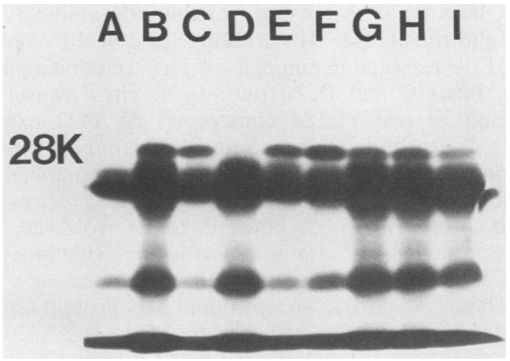

FIG. 3. Proteins synthesized in an in vitro transcription-translation system by recombinant plasmids constructed with pACYC184. Proteins were resolved in a 10% polyacrylamide–SDS gel and identified by fluorography. The relevant portion of the 24-cm-long gel is shown. Lane A, pACYC184; lane B, pYA422; lane C, pYA422 *vir-1*::Tn*5*; lane D, pYA422 *vir-3*::Tn*5*; lane E, pYA422 *vir-4*::Tn*5*; lane F, pYA422 *vir-7*::Tn*5*; lane G, pYA427; lane H, pYA428; lane I, pYA429. Migration of the 28K protein is indicated to the left.

ratio of χ3337(pYA403) to χ3337(pYA403 *vir*::Tn*5*). *vir-22*::Tn*5* had the greatest effect on virulence (mean ratio, 2.9 × 10^3) relative to *vir-26*::Tn*5* and *vir-27*::Tn*5* (ratios of 23 and 60, respectively). It is interesting that the latter two Tn*5* insertions mapped together near the center of *Bam*HI fragment C, while *vir-22*::Tn*5* mapped to the left end of the fragment.

vir-22::Tn*5*, *vir-26*::Tn*5*, and *vir-27*::Tn*5* in pYA403 were introduced into the wild-type virulence plasmid, pStSR100, of χ3306 by P22-mediated transduction. Restriction enzyme mapping of Km^r transductants confirmed that the Tn*5* insertions had recombined into the wild-type plasmid (data not shown). Mice were inoculated p.o. with mixtures of 10^8 CFU each of χ3306 and χ3306 with one of the *vir*::Tn*5* insertions in pStSR100. Six days postinoculation, results similar to those obtained with the Tn*5* insertions in pYA403 were found with the same Tn*5* insertions in the native virulence plasmid (Table 3, part B). χ3306(pStSR100 *vir-22*::Tn*5*) yielded the highest ratio of wild-type to Tn*5* mutant spleen isolates (2.5 × 10^3; $P < 0.0025$), while χ3306(pStSR100 *vir-26*::Tn*5*) and χ3306(pStSR100 *vir-27*::Tn*5*) yielded lower ratios (32 and 8.3, respectively; $P > 0.05$). The different levels of attenuation mediated by the three Tn*5* insertions were not due to plasmid stability or instability, as all three Tn*5*-mutated virulence plasmids were 100% stable after growth of bacteria in vitro for 20 generations and in vivo in mice for 4 days.

Identification of recombinant plasmid-encoded proteins. We used *E. coli* minicells to identify proteins expressed by virulence plasmid genes cloned into pCVD305 (Fig. 2). The pCVD305 constructs did not efficiently segregate into minicells, and hence longer exposures of autoradiographs resulting in increased background were necessary to identify plasmid-encoded proteins. Three proteins of 32K, 29K, and 28K were encoded by pYA403 (Fig. 2, lane B). pYA403 containing *vir-22*::Tn*5*, which mapped to the left end of *Bam*HI fragment C, no longer expressed the 28K protein in minicells (Fig. 2, lane C), while pYA403 containing *vir-26*::Tn*5* expressed all three proteins expressed by pYA403

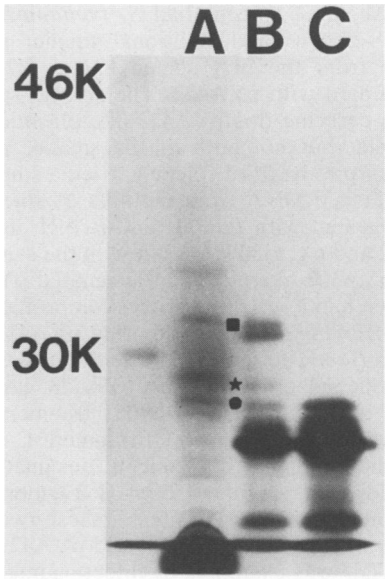

FIG. 4. Comparison of proteins synthesized from recombinant plasmids pYA403, pYA420, and pYA422. Samples depicted in Fig. 2 and 3 were resolved in a 10% polyacrylamide–SDS gel to allow direct comparison of proteins. pYA420 was examined by in vitro transcription-translation run concomitantly with the samples depicted in Fig. 3. The relevant portion of the gel is shown. Locations of ^{14}C-molecular weight standards and sizes are shown to the left. Lane A, pYA403 in minicells; lane B, pYA420 in in vitro transcription-translation; lane C, pYA422 in in vitro transcription-translation. Symbols between lanes A and B indicate the 28K (●), 29K (★), and 32K (■) proteins.

(Fig. 2, lane D). The presence of the 29K aminoglycoside 3′-phosphotransferase II protein (calculated molecular weight) (3) was noted in minicells of Tn*5*-containing plasmids (Fig. 2, lanes C and D, arrow). In in vitro transcription-translation of pACYC184 constructs, pYA422 expressed only the 28K protein (Fig. 3, lane B). Of four Tn*5* insertion mutations in pYA422, only *vir-3*::Tn*5*, which mapped to the extreme left of *Bam*HI fragment C, inhibited expression of the 28K protein (Fig. 3, lanes C to F). pYA420, which contains the 14-kb common sequence subcloned into pACYC184, expressed the same three proteins as pYA403 (Fig. 4, lanes A and B). An additional 31K protein (apparent molecular weight) was also produced by pYA420. This protein may be a truncated product of a higher-molecular-weight protein encoded in this region of the virulence plasmid but not identified by using pYA403 due to the inefficiency of pCVD305 constructs in minicells. The combined results of analysis of pYA422 and Tn*5* insertions indicated that the 28K protein was encoded at the extreme left region of *Bam*HI fragment C.

Complementation of *vir-22*::Tn*5*. The *vir-22*::Tn*5* insertion mutation was originally examined for effects on virulence because it was located within the 3.2-kb *Bam*HI fragment C of pStSR100 which had been cloned in virulence-conferring pYA422. We therefore investigated the ability of pYA422 to complement in *trans* the *vir-22*::Tn*5* insertion in pStSR100 of χ3589. pYA422 was unable to restore the ability to infect spleens inhibited by the *vir-22*::Tn*5* mutation (data not shown).

To isolate recombinant plasmids capable of complementing the *vir-22*::Tn*5* insertion, χ3589 was transformed with the same recombinant library used to isolate pYA422 (see above). Three mice were infected p.o. with 3×10^8 CFU of χ3589 containing this library, and spleens were harvested 6 days later. All three spleens had *S. typhimurium* carrying pStSR100 *vir-22*::Tn*5* and additional smaller plasmids. *S. typhimurium* from the first spleen had a 7.2-kb plasmid which comigrated with pYA422. The second spleen had *S. typhimurium* carrying this pYA422-like plasmid or another 8.0-kb plasmid, but not both of the smaller plasmids. *S. typhimurium* from the third spleen carried a smaller plasmid of 7.0 kb. Plasmid DNA from cultures of spleen homogenates was digested with *Bam*HI and *Eco*RI and compared with pYA422 and pYA420 DNA cut with the same enzymes. The 7.2-kb pYA422-like plasmid, designated pYA427, from the first spleen had a restriction digestion profile identical to that of pYA422 (Fig. 1), indicating that pYA427 must have also carried a *Bam*HI fragment C insert. The 8.0-kb plasmid from the second spleen, designated pYA428, carried *Bam*HI fragment C, with the 0.9-kb *Bam*HI fragment E located immediately to the right of *Bam*HI fragment C (Fig. 1). The 7.0-kb plasmid from the third spleen, designated pYA429, carried the adjacent regions of *Bam*HI fragments C and D, but not the outermost *Bam*HI sites of these two fragments.

The ability of pYA427, pYA428, and pYA429 to complement *vir-22*::Tn*5* was examined by infecting mice with χ3589 which had been retransformed with each of the recombinant plasmids (Table 2, part B). Retransformation ensured that complementation by recombinant clones had been originally selected, as opposed to suppressor mutations in pStSR100 *vir-22*::Tn*5*. One week after p.o. inoculation with 4×10^8 to 7×10^8 CFU, the total number of splenic CFU in mice infected with χ3306 was much greater than that in mice infected with χ3589 (2.9×10^5 versus 460 CFU, respectively; $P < 0.0005$). pYA427, which was identical to pYA422, failed to significantly complement *vir-22*::Tn*5* of χ3589 (330 CFU; $P > 0.1$). pYA428 was the most efficient complementing plasmid for *vir-22*::Tn*5* (1.0×10^5 CFU; $P > 0.1$ compared with χ3306, $P < 0.005$ compared with χ3589). pYA429 increased infection of spleens by χ3589 (1.9×10^4 CFU; $P < 0.025$ compared with χ3589) but did not restore wild-type virulence ($P < 0.05$ compared with χ3306).

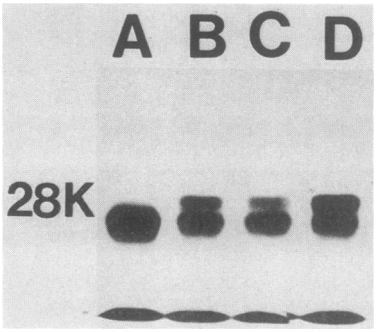

FIG. 5. Effects of reversing insert sequence of pYA422 on proteins synthesized in *E. coli* χ925 minicells. ^{35}S-labeled proteins were resolved in a 10% polyacrylamide–SDS gel and identified by fluorography. Lane A, pACYC184; lane B, pYA422; lane C, pYA425 (original orientation); lane D, pYA426 (reversed orientation). Migration of the 28K protein is indicated.

The degree of virulence conferred by each of these plasmids alone was examined by transforming them into χ3337 and inoculating mice p.o. with 5×10^8 to 1.1×10^9 CFU. Six days postinoculation, mice infected with wild-type χ3306 had 2.6×10^5 CFU in their spleens, while mice inoculated with χ3337 carrying pYA427, pYA428, or pYA429 had 2.6×10^3 to 3.1×10^3 CFU in their spleens (Table 2, part C). Thus, the plasmids that complemented *vir-22*::Tn*5* did not confer virulence by themselves.

Because *vir-22*::Tn*5* inactivated the gene encoding the 28K protein, we examined the proteins encoded by pYA427, pYA428, and pYA429 by in vitro transcription-translation (Fig. 3, lanes G to I). As expected, each of these plasmids encoded a 28K protein.

Reversal of insert DNA of pYA422. After analysis of more detailed restriction maps of pYA422 and the *vir-22*::Tn*5*-complementing clones, we determined that the insert sequences of the most efficient complementing clones, pYA428 and pYA429, were in the opposite orientation relative to that of the lesser-complementing clones pYA422 and pYA427. Specifically, the insert sequences of pYA428 and pYA429 were oriented so that transcription from the tetracycline resistance gene of pACYC184 (into which sequences were cloned) was from the left (Fig. 1, arrow), and transcription of the tetracycline resistance gene in pYA422 and pYA427 was from the right (Fig. 1). The *vir-22*::Tn*5* insertion and the gene encoding the 28K protein were located at the extreme left side of the *Bam*HI fragment C shared by all of these plasmids. To determine whether the difference in orientation of insert sequences could have been responsible for the different complementing abilities of the constructs, pYA422 was cleaved with *Bam*HI, which precisely removes the insert sequence, and religated. pYA425 and pYA426, which have the *Bam*HI fragment C insert of pYA422 in the original and opposite orientations, respectively, were isolated (Fig. 1) and tested for their ability to complement *vir-22*::Tn*5*. One week after p.o. inoculation of mice with 5×10^8 CFU of χ3589(pYA425) or χ3589(pYA426), splenic infections of 355 CFU and 1.0×10^6 CFU, respectively, were obtained (Table 2, part D; $P < 0.001$). Thus, the inability of pYA422 to

complement the *vir-22*::Tn*5* mutation was due to the orientation of the insert DNA.

Both pYA425 and pYA426 expressed only the 28K protein in *E. coli* minicells (Fig. 5). Examination of proteins expressed from minicells by gradient SDS-PAGE did not reveal any additional smaller proteins that might have accounted for the different abilities of these clones to complement *vir-22*::Tn*5* (data not shown). No differences in plasmid stability were detected between pYA425 and pYA426 in χ3589 after infection of mice for 4 days (both plasmids being greater than 95% stable). Because χ3589 is *recA*$^+$, we also examined the possibility that differential recombination of pYA425 or pYA426 into the Tn*5*-mutated virulence plasmid could have resulted in different levels of complementation of *vir-22*::Tn*5*. Mice were inoculated p.o. with χ3589(pYA425) or χ3589(pYA426), and 4 days later spleens were harvested. Plasmid DNA was extracted from spleen cultures and resolved on a 0.6% agarose gel. The plasmid DNA was then examined by Southern blot analysis with pACYC184 (to test for a single crossover causing cointegration of the plasmids) and the central *Hin*dIII fragment of Tn*5* (to test for retention of Tn*5* in the virulence plasmid and recombination of Tn*5* into the complementing plasmids) used as probes. None of the virulence plasmids hybridized with pACYC184, indicating that no single crossover events had been selected during infection of mice (data not shown). All of the cultures of χ3589 recovered from mouse spleens retained the Tn*5* insertion in the virulence plasmid. Furthermore, no Tn*5* sequences were detected in plasmids of lower molecular weight than pStSR100 of χ3589 from infected mice. These latter two results indicated that no double-crossover events causing replacement of *vir-22*::Tn*5* in pStSR100 with wild-type plasmid sequences from either pYA425 or pYA426 were selected during infection of mice. Therefore, the differences in complementation by pYA425 and pYA426 were not due to recombinational events with pStSR100 *vir-22*::Tn*5* that might have been selected in vivo. Thus, the exact reason for the lack of complementation by pYA422, pYA427, and pYA425 and complementation by pYA426, pYA428, and pYA429 has yet to be determined.

Serum resistance. Although we found no relationship between possession of the 100-kb virulence plasmid and serum resistance of *S. typhimurium* (14), others have reported such a relationship (16, 18, 20, 32). Hackett et al. (18) found that cloned serum resistance genes from *S. typhimurium* LT2 conferred serum resistance on *E. coli* K-12 E138. We therefore examined the serum resistance of *E. coli* K-12 LE392 and MG1655 carrying pYA403 or pYA422. Neither plasmid, which conferred different levels of virulence on pStSR100-cured *S. typhimurium*, conferred increased serum resistance on either *E. coli* K-12 strain (<0.01% survival after 1 h at 37°C in 90% [vol/vol] normal rabbit serum).

DISCUSSION

We have isolated, by genetic cloning, regions of the 100-kb virulence plasmid of *S. typhimurium* that confer virulence to plasmid-cured isogenic derivatives. To our knowledge, this constitutes the first report of in vivo selection of recombinant virulence-conferring clones in an animal model system. Perhaps the first in vivo selection for virulence by genetic recombination was the classic experiment of Griffith in 1928 (13), in which avirulent rough *Streptococcus pneumoniae* was rendered virulent by transformation of capsular genes from killed virulent encapsulated *S. pneumoniae*. Most recombinant bacteria that have cloned virulence genes have been screened for expression of gene products in either immunological (e.g., colony blot) or functional (e.g., hemolysis) assays. Isberg and Falkow used in vitro cell culture to select clones carrying the cellular adherence and invasion genes of *Yersinia pseudotuberculosis* in *E. coli* (22). Perhaps one limitation to in vivo selection of chromosomal virulence genes is that a properly avirulent strain must be available for use as a recipient, and such a strain would probably have to be deficient in only the particular gene(s) being cloned.

Three cosmid clones present in isolates of *S. typhimurium* which were able to infect spleens of mice inoculated p.o. with cured *S. typhimurium* χ3337 carrying a recombinant library each possessed 22 to 26 kb of insert DNA and a 14-kb common sequence of insert DNA. All of these cosmid clones conferred wild-type levels of virulence on the 100-kb-plasmid-cured *S. typhimurium*, as determined by infection of spleens of p.o. inoculated mice. Furthermore, pYA403 conferred a wild-type p.o. LD_{50} (Table 2). The map of our 14-kb common virulence region is very similar to those published by Michiels et al. (27), who used deletion mutagenesis of the *S. typhimurium* plasmid; Baird et al. (1), who used transposon insertion mutagenesis of the *S. dublin* virulence plasmid; and Beninger et al. (4), who used both types of mutagenesis.

To identify smaller sequences of the 100-kb plasmid that conferred virulence, a library with DNA inserts of less than 5 kb in pACYC184 was introduced into plasmid-cured *S. typhimurium* and fed to mice. A single recombinant clone, pYA422, was isolated from spleens of infected mice that conferred increased but not wild-type levels of splenic infection on virulence plasmid-cured *S. typhimurium* (Tables 2 and 3). The insert DNA of pYA422 corresponded to the 3.2-kb *Bam*HI fragment C of the virulence plasmid and mapped to the central region of the insert DNA of pYA403. Although a wild-type p.o. LD_{50} of approximately 10^5 CFU was found for χ3337(pYA422), the mean time to death was twice as long as for the wild-type χ3306 (20 versus 10 days, respectively, with inocula of 10^5 to 10^6 CFU). It appears that infection of mice with χ3337(pYA422) proceeds at a slower rate than does infection with χ3306. Because the p.o. LD_{50} of virulence plasmid-cured *S. typhimurium* χ3337 is $>10^8$ CFU, it appears that pYA422 encodes a product that determines the minimum number of CFU that ultimately leads to death; however, pYA422 lacks some gene(s) needed for wild-type plasmid-mediated virulence in terms of kinetics of spleen infection.

Transposon Tn*5* insertion mutagenesis of *Bam*HI fragment C of pYA403 was used to further identify cloned sequences important for virulence. pYA403 *vir-22*::Tn*5* resulted in 10^3-fold-decreased infection of spleens compared with pYA403. Recombination of the *vir-22*::Tn*5* insertion into the wild-type 100-kb plasmid of χ3306 resulted in a similar decrease in virulence (Table 3, part B). The *vir-26*::Tn*5* and *vir-27*::Tn*5* insertions resulted in some attenuation in both pYA403 and pStSR100, but not at a significant level. The latter two insertions mapped together at least 1 kb from *vir-22*::Tn*5*. Baird et al. (1) constructed transposon insertions in the virulence plasmids of *S. typhimurium* and *S. dublin* and identified common DNA sequences important for virulence. None of the insertions investigated by Baird et al. (1) fell within the *Bam*HI fragment C studied here; however, the restriction map of the common sequence in the *S. typhimurium* and *S. dublin* virulence plasmids was very similar, if not identical, to our map. Two Tn*A* insertions made by Baird et al. (1) resulting in decreased virulence of *S. dublin* were located immediately to the right of our *Bam*HI fragment C and to the left of our *Bam*HI fragment D. A Tn*10*

insertion in the *S. typhimurium* plasmid partially decreased virulence (1) and mapped in a location corresponding to the left end of pYA401. Beninger et al. (4) recently identified a 4-kb *Eco*RI fragment of the *S. dublin* plasmid as being important for plasmid-mediated virulence of *S. dublin*, with homologous sequences detected in the virulence plasmids of *S. enteritidis* and *S. choleraesuis*. By analogy with our plasmid map, this *Eco*RI fragment could encode the 28K protein.

Expression of proteins from the recombinant clones was examined by using *E. coli* minicell and in vitro transcription-translation analyses. Proteins of 32K, 29K, and 28K (apparent molecular weights) were encoded by pYA403 and pYA420 (Fig. 2 and 4), and the 28K protein was encoded by pYA422 (Fig. 3 and 4). *vir-22*::Tn*5* and *vir-3*::Tn*5* inhibited production of the 28K protein (Fig. 2 and 3, respectively). The fact that *vir-22*::Tn*5* inhibited virulence conferred by pYA403 and the native virulence plasmid, pStSR100 (Tables 2 and 3), suggested that the 28K protein was necessary for virulence. This was indicated further by complementation of *vir-22*::Tn*5* by pYA428 and pYA429, which encode the 28K protein. pYA422 and pYA427, which are identical and also encode the 28K protein, did not complement *vir-22*::Tn*5* as well as did pYA428 and pYA429. pYA422 did not complement *vir-22*::Tn*5* because its insert sequence was in the opposite orientation from the tetracycline resistance gene of pACYC184, into which the insert was cloned. This was shown by pYA426, in which the insert sequence of pYA422 was in the reverse orientation and which complemented *vir-22*::Tn*5*, and pYA425, which had the insert in the original orientation of pYA422 and did not complement *vir-22*::Tn*5*. All three plasmids, pYA422, pYA425, and pYA426, expressed the 28K protein in *E. coli* minicells (Fig. 5), and pYA426 may have expressed slightly higher amounts of the protein. However, the slightly higher expression does not apparently explain the difference in complementing abilities between these plasmids. Reversal of the insert did not allow expression of new or different proteins between pYA422, pYA425, and pYA426. The reversal of the insert of pYA422 demonstrated that the promoter of the 28K protein gene is encoded within *Bam*HI fragment C. Placement of the 28K protein gene downstream from the tetracycline resistance gene may affect expression of the gene encoding the 28K protein in the genetic background of the virulence plasmid present in χ3589, used for complementation experiments. It is also possible that different proteins are expressed in vivo in infected mice but not in minicells or in in vitro transcription-translation. We are currently examining these possibilities. We did eliminate the possibilities that the different complementing abilities were due to plasmid stability differences or recombinational events with the Tn*5*-mutated virulence plasmid. However, until the anomaly of the lack of complementation of *vir-22*::Tn*5* by certain 28K protein-expressing plasmids is resolved, a definitive role for the 28K protein in plasmid-mediated virulence cannot be made. The 28K protein by itself is not sufficient to confer a wild-type level of virulence, as evidenced by the lack of wild-type infection of spleens by χ3337 carrying pYA422, pYA427, pYA428, or pYA429 (Tables 2 and 3).

A major difference in our results and those of others concerning pathogenesis associated with the *S. typhimurium* virulence plasmid is its role in serum resistance. We have found that curing of the virulence plasmid had no effect on resistance of *S. typhimurium* to normal human serum, rabbit serum, or guinea pig serum (14). Hackett et al. (18) recently identified a clone from the *S. typhimurium* virulence plasmid that conferred serum resistance on both their plasmid-cured *S. typhimurium* LT2 and *E. coli* K-12. Similarly, VandenBosch and Jones (J. L. VandenBosch and G. W. Jones, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, abstr. B169, p. 53) reported cloning a gene from the virulence plasmid that conferred resistance to normal human serum on plasmid-cured *S. typhimurium*. The restriction map of the region of the serum resistance-conferring clone of VandenBosch and Jones appeared very similar to the map of the extreme right-hand region of pYA403. We could not accurately compare the restriction map of the clone of Hackett et al. (18) encoding an 11K serum resistance protein with that of our presently described virulence-encoding region. Furthermore, we did not detect an 11K protein in in vitro transcription-translation and minicell analyses of any of our recombinant plasmids in gradient SDS-PAGE. Similarly, the restriction map of the gene encoding the 23K protein of Hackett and Wyk (17) did not appear to be similar to the region near *vir-22*; however, no definite conclusions can be made about any possible relationships between these two genes without further mapping and comparison.

To examine whether serum resistance was conferred by pYA403 and pYA422, *E. coli* K-12 LE392 and MG1655 carrying each of these plasmids were examined for resistance to normal rabbit serum. Both constructs were as sensitive to serum bactericidal activity as the parental strains (<0.01% survival). It should be emphasized that we could not examine the contributions of these recombinant plasmids to serum resistance in virulence plasmid-cured *S. typhimurium* because all cured strains in our possession are serum resistant (14). As we discussed previously (14), it is possible that our *S. typhimurium* SR-11 isolate does not depend on any plasmid-encoded factors for serum resistance, as opposed to strains tested by others (16, 18, 32). The use of the presently described recombinant clones and Tn*5* mutations will aid in our ongoing studies of the role of the 100-kb plasmid in *S. typhimurium* virulence. For example, we are attempting to identify other plasmid-encoded virulence genes by selecting clones that act synergistically with *Bam*HI fragment C to mediate virulence (i.e., infection of spleens after p.o. inoculation).

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