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Virulence Plasmid-Borne *spvB* and *spvC* Genes Can Replace the 90-Kilobase Plasmid in Conferring Virulence to *Salmonella enterica* Serovar Typhimurium in Subcutaneously Inoculated Mice

HIDENORI MATSUI,[1,2] CHRISTOPHER M. BACOT,[2]† WENDY A. GARLINGTON,[2]‡ THOMAS J. DOYLE,[2] STEVE ROBERTS,[2] AND PAUL A. GULIG[2]*

Laboratory of Infectious Diseases and Immunology, Center for Basic Research, The Kitasato Institute, Tokyo, Japan,[1] *and Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, Florida*[2]

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In a mouse model of systemic infection, the *spv* genes carried on the *Salmonella enterica* serovar Typhimurium virulence plasmid increase the replication rate of salmonellae in host cells of the reticuloendothelial system, most likely within macrophages. A nonpolar deletion in the *spvB* gene greatly decreased virulence but could not be complemented by *spvB* alone. However, a low-copy-number plasmid expressing *spvBC* from a constitutive *lacUV5* promoter did complement the *spvB* deletion. By examining a series of *spv* mutations and cloned *spv* sequences, we deduced that *spvB* and *spvC* could be sufficient to confer plasmid-mediated virulence to *S. enterica* serovar Typhimurium. The *spvBC*-bearing plasmid was capable of replacing all of the *spv* genes, as well as the entire virulence plasmid, of serovar Typhimurium for causing systemic infection in BALB/c mice after subcutaneous, but not oral, inoculation. A point mutation in the *spvBC* plasmid preventing translation but not transcription of *spvC* eliminated the ability of the plasmid to confer virulence. Therefore, it appears that both *spvB* and *spvC* encode the principal effector factors for Spv- and plasmid-mediated virulence of serovar Typhimurium.

Salmonella enterica is best known as the cause of diarrhea produced by numerous serovars, including *Salmonella enterica* serovar Typhimurium. In fact, serovar Typhimurium is one of the leading causes of diarrhea in the United States, with more than 1 million cases estimated to occur each year (30). However, in compromised individuals, most notably those with immune deficiencies, the infection can spread beyond the intestines to cause enteric fever, resembling typhoid fever caused by *S. enterica* serovar Typhi (45). Of the thousands of diarrheagenic serovars of *S. enterica*, only a subset can cause systemic infection, and this subset has in common the presence of genetically related, high-molecular-weight virulence plasmids (14). Serovar Typhi does not possess a virulence plasmid (34).

The exact mechanism by which the virulence plasmid enables systemic infection is not known; however, the serovar Typhimurium virulence plasmid contributes to systemic disease in a mouse model of enteric fever by increasing the replication rate of the bacteria within host cells beyond the intestines (15). The primary plasmid-borne virulence attributes, including intracellular replication, are encoded by five *spv* genes (14). It is believed that the most relevant host cells for Spv-mediated intracellular replication are macrophages, based on cellular depletion experiments with mice infected with wild-type or Spv$^-$ serovar Typhimurium (17) and immunofluorescence analysis of tissues from mice infected with wild-type serovar Typhimurium (37; P. A. Gulig, S. A. Roberts, and T. J. Doyle, Abstr. 98th Gen. Meet. Am. Soc. Microbiol. abstr. B-212, 1998). Libby et al. (24) reproduced the Spv phenotype of increased growth rate within macrophages in cell culture. They subsequently determined that the *spv* genes were associated with the release of macrophages from cell culture substrates and rapid replication of salmonellae within these released macrophages (25). Most recently, SpvB was shown to be an ADP-ribosylating enzyme of actin, inhibiting its polymerization in host cells (23, 33, 42), and therefore could be involved with inhibition of phagosome-lysosome fusion. Others have shown that plasmid-cured *S. enterica* serovar Dublin was also less effective at lysing macrophages in culture than was the wild-type parent (9).

The relationship between the *spv* genes and virulence has been confirmed by both mutation of *spv* genes and placing the recombinant *spvABCD* genes into virulence plasmid-cured serovar Typhimurium or serovar Dublin (14). Virulence plasmid-cured salmonellae containing the cloned *spv* genes have virulence indistinguishable from that of the wild-type parent (10, 20, 47). The five *spv* genes, *spvRABCD*, have been sequenced; however, the only significant homology based on primary sequence offering a clue to their function was the identification of the *spvR* gene product as a positive regulatory protein in the LysR family (2, 4, 7, 27, 41). SpvR is essential for expression of the other *spv* genes, which form an operon (7, 19, 28). The

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Box 100266, Gainesville, FL 32610-0266. Phone: (352) 392-0050. Fax: (352) 392-3133. E-mail: gulig@ufl.edu.

† Present address: Florida Department of Law Enforcement, Tallahassee, FL 32308.

‡ Present address: 139 Main Rd., Glenalta, South Australia 5052, Australia.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype	Comment(s) (reference[s])
Serovar Typhimurium strains		
χ3181	pStSR100$^+$	Virulence plasmid positive (12)
χ3306	*gyrA1816* pStSR100$^+$	Virulence plasmid positive, Nal^r (12)
χ3337	*gyrA1816* pStSR100$^-$	Virulence plasmid-cured derivative of χ3306, Nal^r (12)
χ3456	pStSR100::Tn*minitet*	Tn*minitet* insertion in the virulence plasmid; does not affect virulence, Tet^r (12)
UF009	pStSR100::*vir-44*::*aph*	*aph* insertion in the virulence plasmid that does not affect virulence; used as Kan^r wild-type strain (10)
UF012	pStSR100*spvB5*::*aph*	*aph* insertion in the *spvB* gene of virulence plasmid that attenuates virulence (10)
UF051	pStSR100Δ*spvB30*	Δ*spvB30*, deleting amino acids 21 to 555 of *spvB* (49)
UF110	pStSR100Δ*spvRABCD′*::*tet*	*spvRABCD′* replaced with *tet* in χ3181; Tet^r (16, 17)
Plasmids		
pACYC184		Cloning vector (3)
pGTR040		*spvRABCD′* cloned into pYA2204 (10)
pGTR061		*spvRABCD* cloned into pYA2204 (10)
pGTR100		4-kb *Eco*RI fragment of the virulence plasmid carrying *spvABCD* cloned into *Eco*RI site of pACYC184 (10)
pGTR127		*spvC* cloned into pACYC184 on a *Bam*HI-*Eco*RI fragment so that *spvC* is expressed from the *tet* promoter (11)
pGTR147		pGTR061 *spvC22*::Tn*5* (10)
pGTR153		*spvD* cloned into pACYC184 so that *spvD* is expressed from the *tet* promoter (11)
pGTR175		*Pst*I deletion derivative of pGTR100, removing open reading frame of *spvA*; encodes *spvBC* downstream of *spvA* promoter (this work)
pGTR309		*Pst*I-*Eco*RI fragment of pGTR061 encoding *spvB* and *spvC* cloned into the *Pst*I-*Eco*RI site of pUC118 (this work)
pGTR333		2.4-kb *Eco*RI-*Stu*I fragment of pGTR175 cloned into the *Eco*RI site of pACYC184; carries *spvB* downstream of the *spvA* promoter and is codirectional with *cat* promoter of pACYC184 (this work)
pGTR337		2.4-kb *Eco*RI fragment from pGTR333 cloned into the *Eco*RI site of pYA2204; carries *spvB* downstream of the *spvA* promoter; *spvB* is in the opposite orientation from *lacZ′* (this work)
pGTR338		2.4-kb *Eco*RI fragment from pGTR333 cloned into the *Eco*RI site of pYA2204; carries *spvB* downstream of the *spvA* promoter; *spvB* is in the same orientation as *lacZ′* (this work)
pGTR339		2.2-kb *Msc*I-*Eco*RI fragment of pGTR333 cloned into pYA2204 digested with *Sma*I and *Eco*RI; carries the ribosome-binding site, start codon, and open reading frame of *spvB*, with *spvB* in the same orientation as *lacZ′* (this work)
pGTR356		*Sph*I-*Eco*RI fragment of pGTR309 cloned into the *Sph*I-*Eco*RI site of pMW119 *spvBC* is codirectional with *lacZ′* of pMW119 (this work)
pGTR357		pGTR356 with point mutation abolishing start codon of *spvC* (this work)
pMW119		Cloning vector derived from pSC101 (Nippon Gene Co., Tokyo, Japan)
pUC118		Cloning vector, Amp^r (44)
pYA426		*spvCD* cloned into pACYC184 as a *Bam*HI fragment so that *spvCD* are expressed by the *tet* promoter (11)
pYA2204		Low-copy-number cloning vector, Amp^r (8)

ADP-ribosylating activity of SpvB, initially examined because of secondary amino acid structure (33), is discussed above.

There are reports that genes carried on the virulence plasmid other than the *spv* genes are involved in virulence. The virulence plasmid has been associated with resistance of salmonellae to complement, adherence to or invasion of host cells, and suppression of elicitation of γδ T cells; however, these results have not been repeated or have been contradicted (reviewed in reference 14). The virulence plasmid can affect growth of salmonellae in vitro under certain conditions (18, 21, 35). In addition to the *spv* genes, insertion mutations in other genetic loci have been shown to decrease virulence in animal models (5, 26, 31, 32, 36, 40, 46).

We wanted to determine the minimum complement of *spv* genes necessary to confer the plasmid-mediated virulence phenotype to serovar Typhimurium to enable a more focused genetic and functional analysis. It should be noted that the demonstration that genes are necessary for virulence by analysis of specific mutations and the demonstration that genes are sufficient to confer virulence by using cloned genes are different matters. It had been shown that the *spvA* gene was dispensable for virulence in orally inoculated mice (38, 49), and we reasoned that the *spvR* gene could be replaced by a suitable promoter for *spvABCD* provided on a recombinant plasmid. Through a combination of mutagenesis and cloning, we determined that the *spvB* and *spvC* genes could replace all of the *spv* genes and the entire virulence plasmid of serovar Typhimurium after subcutaneous (s.c.) inoculation of mice, in which *spv* genes are essential for full virulence.

Bacterial strains and plasmids. Bacterial strains and plasmids are listed and described in Table 1, and plasmid maps are depicted in Fig. 1. Unless noted otherwise, bacterial culture was at 37°C in L broth (LB) (22) or on LB containing 1.5% (wt/vol) agar. Cultures were stored frozen at −80°F in LB

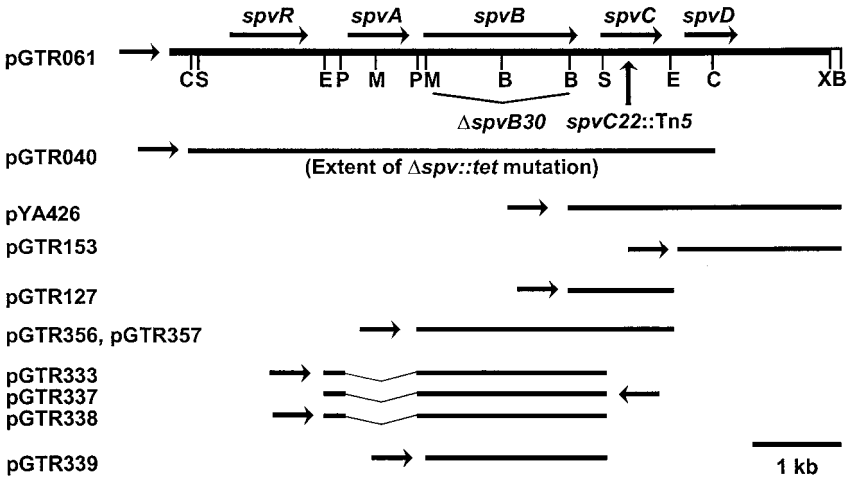

FIG. 1. Physical and genetic maps of virulence plasmid sequences and recombinant plasmids used in this study. The top line depicts the insert of pGTR061, which carries *spvRABCD*, whose open reading frames are indicated by arrows with the direction of transcription shown. Restriction sites indicated below pGTR061 are as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; M, *Msc*I; P, *Pst*I; and S, *Stu*I. Note that pGTR061 extends to the *Xho*I site on the right. The rightmost *Bam*HI site is indicated for reference to plasmids depicted below. The extent of the Δ*spvB30* deletion and the location of the *spvC22*::Tn*5* insertion are shown. The *Cla*I insert of pGTR040 corresponds to the Δ*spv*::*tet* deletion of UF110. The arrows next to the insertion sequences of each clone indicate the direction of transcription of the promoters of the respective vectors. For pGTR333, pGTR337, and pGTR338, the broken line indicates that the *Eco*RI-*Pst*I fragment carrying the *spvA* promoter has been juxtaposed to the *spvB* open reading frame by the *Pst*I deletion. Details of plasmid construction are presented in Table 1.

containing 35% (vol/vol) glycerol. For most applications, cultures were grown overnight as static cultures in LB. On the day of use, the cultures were diluted 1:20 into fresh, prewarmed LB and incubated with shaking at 37°C until the optical density at 600 nm reached approximately 0.4.

Construction of an *spvC* start codon mutation in pGTR356. To address the possibility that *spvC* is required for virulence conferred by pGTR356, we constructed a site-directed mutation in the start codon of *spvC* using the Transformer system (Clontech, Palo Alto, Calif.). The sequence of the start codon and the preceding three nucleotides, CCCATG, was changed to CCCGGG, which not only destroyed the start codon but also created a new and unique *Sma*I site as a simple screen for the mutation. The mutagenic oligonucleotide sequence was CGCAAAGGAGATTTCCCGGGCCCATAAATAGGC (*Sma*I site underlined), and the presence of the mutation was confirmed by *Sma*I digestion coupled with the lack of *Bsa*I digestion (as part of the mutagenesis procedure, a *Bsa*I site within the *bla* gene of the vector pMW119 was destroyed while conserving the amino acid sequence of Bla). The SpvC$^-$ derivative plasmid was named pGTR357.

Infection of mice. All mouse studies used BALB/c mice (Charles River, Wilmington, Mass.; University of Florida Department of Pathology, Immunology, and Laboratory Medicine Mouse Facility), which are sensitive to infection by serovar Typhimurium because of the *Ity*^{s} mutation (39). Mice were orally inoculated as described previously with approximately 10^8 CFU of serovar Typhimurium (11). Inoculation with 10^5 CFU of salmonellae was done s.c. either into both hind footpads, as described previously (15), or into the upper back near the shoulder blades. All experiments were repeated at least once, with results similar to those of the experiment shown.

Lack of the *spvD* gene can be complemented by increased expression of *spvC*. It has been shown through mutational and complementation analysis that the *spvD* gene is essential for full virulence of serovar Typhimurium (10). However, the effects of mutating *spvD* were small compared with the effects of mutations in *spvR* or *spvC*. As part of our attempts to construct a recombinant plasmid which contained the minimal *spv* sequence needed to confer full virulence in plasmid-cured serovar Typhimurium, we constructed plasmid pGTR040, which contains the 6.3-kb *Cla*I fragment bearing *spvRABCD′* subcloned into the low-copy-number vector pYA2204 (similarly to pGTR061; see Fig. 1) (10). Unlike pGTR061, pGTR040 could not restore virulence to plasmid-cured χ3337 (10). The lack of *spvD* in pGTR040 was complemented by placing into χ3337 (pGTR040) the plasmid pGTR153, which carries only the *spvD* gene (10) (Table 2). We also examined plasmid pYA426, which carries *spvCD* expressed from the *tet* promoter of pACYC184 (11, 13), for its ability to complement the lack of *spvD* in pGTR040. As expected, χ3337 (pGTR040, pYA426) was fully

TABLE 2. Expression of recombinant *spvC* can complement lack of *spvD* to confer virulence to serovar Typhimurium in mice

Plasmid used to transform χ3337(pGTR040)	CFU of[a,b]:		Paired difference[a]	*P* value[c]
	χ3337(pGTR040)	χ3456		
pGTR153	5.5 ± 1.2	5.1 ± 1.2	−0.4 ± 1.1	>0.35
pGTR127	4.8 ± 0.8	5.0 ± 1.0	0.2 ± 0.6	>0.35
pACYC184	4.1 ± 1.4	5.2 ± 1.6	1.1 ± 0.5	<0.005

[a] Means ± standard deviations of log-transformed data are shown. $n = 6$ (for all groups).

[b] Virulence plasmid-cured serovar Typhimurium χ3337(pGTR040), which carries *spvRABCD′*, was transformed with either pGTR153 (carrying only *spvD*), pGTR127 (carrying only *spvC*), or the vector pACYC184. These strains were mixed with wild-type serovar Typhimurium χ3456 and inoculated orally to mice at a dose of 10^8 CFU. Five days later, spleens were harvested and plated to enumerate CFU of each strain.

[c] *P* value for the paired difference being different from 0.

virulent in terms of splenic infection (data not shown). We then examined deletion derivatives of pYA426 produced from the 3′ end of *spvD* moving toward *spvC*. We expected that as soon as *spvD* experienced deletions, the resulting plasmids would not work with pGTR040 to confer full virulence. However, deletions of pYA426 extending completely through *spvD* still produced virulence in combination with pGTR040 (data not shown). The smallest deletion derivative conferring virulence was plasmid pGTR127, which carries only *spvC* (Table 2). Therefore, by providing *spvC* expressed from a recombinant plasmid, the necessity of providing *spvD* to *spvRABC*-carrying pGTR040 was abolished. It appeared that overexpressed *spvC* compensated for the lack of *spvD*.

If the mechanism of this *spvC*-mediated complementation of a lack of *spvD* was due to an effect of the *spvD* deletion on the *cis*-encoded *spvC*, then the *spvD* deletion of pGTR040 should not have been complemented by *spvD* alone. The data are consistent with SpvD either affecting expression of *spvC* in a *trans*-active manner or physically or functionally interacting with SpvC to facilitate its virulence function in a manner that is mimicked by overexpressing *spvC* in the absence of *spvD*.

A nonpolar deletion mutation of *spvB* attenuates virulence but is noncomplementable. It has been reported that an *aph* insertion in *spvB* greatly attenuates splenic infection for serovar Typhimurium after oral inoculation (10). However, we were unable to complement the mutation with cloned sequences expressing *spvB*. In fact, no one has been able to complement an *spvB* mutation with only *spvB* to date. We considered the possibility that the *aph* insertion was polar on downstream genes or otherwise exerted pleiotropic effects. We therefore constructed strain UF051, in which amino acids 21 to 555 of the *spvB* gene present on the virulence plasmid were deleted using inverse PCR mutagenesis, resulting in the Δ*spvB30* mutation (49). As expected, UF051 was greatly attenuated for splenic infection after oral inoculation of mice compared with wild-type χ3456 (paired difference in log splenic CFU, 3.8 ± 0.7 [mean ± standard deviation]; $P < 0.002$), similar to *spvB5*::*aph* strain UF012 compared with wild-type χ3456 (paired difference in log splenic CFU, 3.8 ± 1.2; $P < 0.01$).

To complete the molecular version of Koch's postulates and confirm that the Δ*spvB30* mutation was responsible for the observed attenuation, we attempted to complement the mutation with a variety of recombinant plasmids expressing *spvB*. We constructed a series of plasmids, each carrying only *spvB* transcribed by either a vector-borne promoter and/or the *spvABCD* promoter (Table 1; Fig. 1). None of the plasmids were capable of complementing the Δ*spvB30* mutation after oral inoculation of mice (data not shown). Whenever *spvB* was expressed from a vector-borne promoter, either *cat* or *lacUV5*, salmonellae containing these plasmids (pGTR333, pGTR338, and pGTR339) were poorly recovered in complementation experiments and were even attenuated for virulence when the plasmids were placed in wild-type serovar Typhimurium χ3181 (data not shown). pGTR337, which had *spvB* expressed from the *spvABCD* promoter alone, was not detrimental but did not complement. We verified that the *spvB*-containing plasmids expressed SpvB using in vitro transcription-translation and Western blot analyses (data not shown). This lack of complementation was not due to the Δ*spvB30* mutation being *trans* dominant (e.g., by producing an SpvB product that interfered with other Spv or cellular virulence functions), because UF051 containing pGTR061 (bearing the entire *spv* region) was fully virulent for splenic infection after oral inoculation of mice (paired difference for log CFU per spleen in a mixed infection with wild-type χ3456 was -0.06 ± 1.3; $P = 0.5$). Similarly, the Δ*spvB30* mutation was not polar on expression of *spvC* or *spvD*, because pGTR147, which is pGTR061 with an *spvC22*::Tn*5* insertion (10), was able to complement the Δ*spvB30* mutation (paired difference for log CFU per spleen in a mixed infection with wild-type χ3456 was -0.02 ± 1.3; $P = 0.5$).

A recombinant plasmid carrying *spvBC* complements Δ*spvB30* and confers virulence to Spv$^-$ serovar Typhimurium by the s.c. route of inoculation. Since previous results (38, 49) indicated that *spvA* was not essential for virulence and we demonstrated above that lack of *spvD* could be compensated for by providing excess *spvC*, we considered the possibility that the Δ*spvB30* mutation could be complemented by a plasmid carrying *spvB* and *spvC* together. If the *spvBC* genes were expressed from an exogenous promoter, then *spvR* would be obviated. We therefore constructed plasmid pGTR356, which contains the *spvBC* expressed from the *lacUV5* promoter genes in the low-copy-number vector pMW119 (Table 1; Fig. 1). In Lac$^-$ serovar Typhimurium, this promoter would be constitutive. UF051(pGTR356) was fully virulent when administered to mice by the oral route compared with wild-type χ3306 (mean log splenic CFU were 5.6 ± 0.76 and 6.0 ± 1.5, respectively; $P > 0.6$). UF051 containing the vector pMW119 yielded a log splenic CFU of 2.2 ± 1.0 [$P < 0.005$ compared with χ3306 or UF051(pGTR356)]. Therefore, the combined *spvBC* genes were able to complement the Δ*spvB30* mutation. We still do not know why the *spvB* gene alone could not complement the Δ*spvB30* mutation. However, with the breadth of *spv* clones attempted by us and others, it is unlikely that the reason was inadequate or insufficient expression.

Since pGTR356 complemented the Δ*spvB30* mutation, and in light of the ancillary roles of *spvA* and *spvD* and the ability to compensate for SpvR by an exogenous promoter, we asked if pGTR356 could restore virulence to either Δ*spv*::*tet* serovar Typhimurium UF110 (16, 17) or plasmid-cured χ3337 (12). In oral inoculations with mixed strains, UF110(pGTR356) sometimes approached wild-type UF009 for levels of splenic infection but most often failed to achieve wild-type levels (data not shown). UF110(pGTR356) was sometimes significantly higher than virulence plasmid-cured χ3337 for splenic infection but at other times was not significantly higher. Interestingly, UF110(pGTR356) was often recovered from Peyer's patches or feces in lower numbers, compared with UF009 or χ3337. Similarly, χ3337(pGTR356) failed to achieve wild-type levels of splenic infection after oral inoculation of mice (data not shown).

Since it appeared that pGTR356 could be detrimental to salmonellae in the gut, we injected pGTR356-containing strains s.c. into the hind footpads or backs of mice and examined splenic infection (Fig. 2 and 3). We had previously shown that the virulence plasmid and specifically the *spv* genes were essential for systemic infection of serovar Typhimurium inoculated s.c. into BALB/c mice (15, 17). pGTR356 was able to fully complement the Δ*spv*::*tet* mutation of UF110 when examined in mixed infection with wild-type χ3306 (paired difference

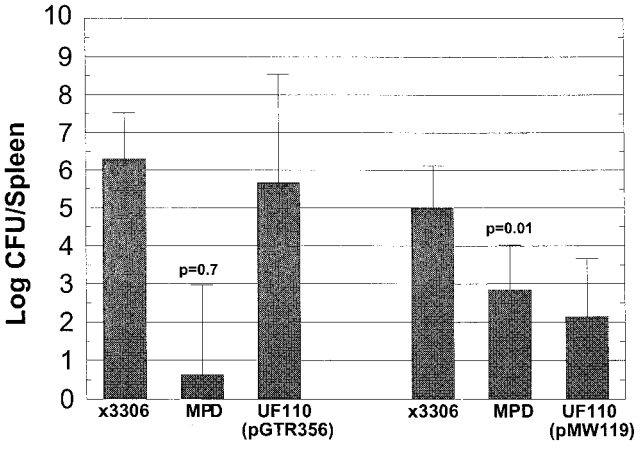

FIG. 2. Recombinant *spvBC* complements Δ*spv*::*tet* after s.c. inoculation of mice. Mice were inoculated s.c. with wild-type strain χ3306 and Δ*spv*::*tet* strain UF110 containing either *spvBC*-bearing pGTR356 or the vector pMW119. Four days later, spleens were removed, homogenized, and plated to enumerate CFU. *P* values are for the mean paired difference (MPD) being greater than 0. Error bars represent standard deviations. $n = 5$ (for all groups).

in log splenic CFU of 0.62 was not significantly different from 0; $P > 0.6$) (Fig. 2). Similarly, in the virulence plasmid-cured background of χ3337, pGTR356 fully restored splenic infection after s.c. inoculation, compared with wild-type χ3306 and χ3337 (pGTR061) ($P > 0.2$) (Fig. 3). Therefore, when the intestines, which are not involved with Spv-mediated pathogenesis in mice, are bypassed via s.c. inoculation, the *spvBC* genes are sufficient to replace the entire virulence plasmid for enabling splenic infection.

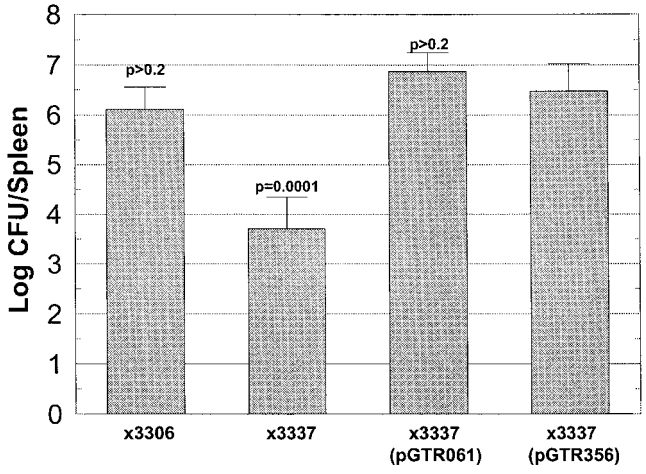

FIG. 3. Recombinant *spvBC* complements virulence plasmid-cured serovar Typhimurium after s.c. inoculation of mice. Mice were inoculated s.c. with wild-type strain χ3306 or virulence plasmid-cured strain χ3337 containing *spvBC*-bearing pGTR356, *spvRABCD*-bearing pGTR061, or no plasmid. Four days later, spleens were removed, homogenized, and plated to enumerate CFU. *P* values are for χ3337(pGTR356) being different from the other strains. Additionally, χ3456 and χ3337(pGTR061) were each significantly greater than χ3337 ($P < 0.0001$), and χ3337(pGTR061) was significantly greater than χ3456 ($P < 0.02$). Error bars represent standard deviations. $n = 5$ (for all groups).

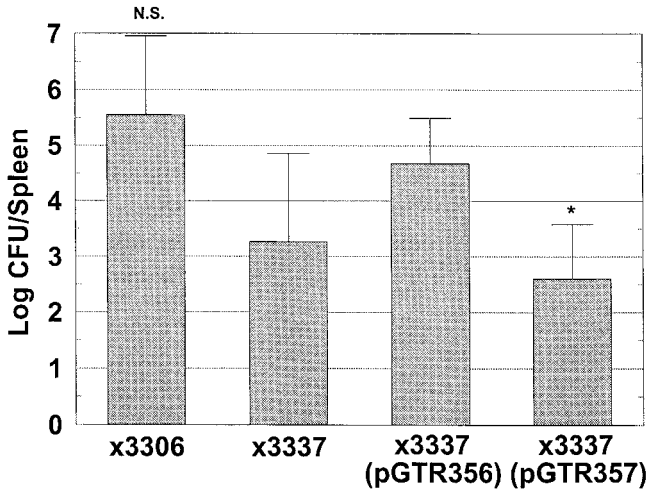

FIG. 4. *spvC* is required for virulence conferred by *spvBC*-bearing pGTR356. Mice were inoculated s.c. with 10^5 CFU of either wild-type χ3306, virulence plasmid-cured χ3337, χ3337(pGTR356), or χ3337(pGTR357). Six days later, spleens were removed, homogenized, and plated to enumerate CFU. The *spvC* mutation of pGTR357(*) significantly decreased infection, compared with *spvBC*-bearing pGTR356 ($P < 0.01$). N.S., χ3306 was not significantly different from χ3337(pGTR356) ($P > 0.3$). Error bars represent standard deviations. $n = 5$ (for all groups).

We considered the possibility that the presence of *spvC* on pGTR356 enabled virulence by affecting the stability of *spvB* mRNA in a *cis*-active manner by the presence of the *spvC* mRNA immediately downstream. Alternatively, the SpvC protein could interact with the SpvB protein to either aid in its function or prevent toxic effects to the salmonella cells. To confirm that the SpvC protein was essential for the virulence conferred by pGTR356 to plasmid-cured χ3337, we constructed a site-directed mutation in the start codon of *spvC* in pGTR356, yielding pGTR357 (Table 1). The mRNA structure of pGTR357 should have been intact, while translation of *spvC* should have been inhibited. When inoculated s.c. into the backs of BALB/c mice, pGTR357 was unable to confer virulence to χ3337 in terms of splenic CFU (Fig. 4), and splenic CFU were significantly lower than those attained by χ3306. Therefore, translation of SpvC protein is required for the virulence function encoded by pGTR356, and both *spvB* and *spvC* are necessary and sufficient to confer plasmid-mediated virulence to serovar Typhimurium after s.c. inoculation of mice.

Why are constitutively expressed *spvBC* genes unable to confer plasmid-mediated virulence after oral inoculation of mice? The answer could lie in the detrimental nature of inappropriate expression of these genes. For example, it appears that salmonella genes that are involved with infection of macrophages (*spv* and salmonella pathogenicity island 2 [SPI2]) are regulated in a manner opposite that of those involved with infection of the intestines (salmonella pathogenicity island 1 [SPI1]), especially with regard to regulation by PhoP and PhoQ (*spv* and SPI2 are PhoP activated [6]; SPI1 is PhoP repressed [1]). In fact, we recently showed that the attenuating effects of constitutively expressed *phoP* in serovar Typhimurium are only

apparent in an Spv$^+$ background (29). Most recently it was shown that SpvB is an ADP-ribosylating toxin of actin in macrophages and inhibits polymerization of actin (23, 33, 42). If this activity contributes to inhibition of phagosome-lysosome fusion mediated by SPI2 (43), then it is possible that expression of these macrophage-specific genes might inhibit SPI1-mediated invasion of or transcytosis through the intestinal epithelium after oral inoculation. However, after s.c. inoculation, in which *spv* genes are essential for efficient systemic infection but SPI1 is dispensable (17), constitutive expression could be beneficial. We did not perform intraperitoneal infections because we have found that the *spv* genes are not nearly as important for virulence by this route as by oral and s.c. inoculation (12). This could be due to the fact that after intraperitoneal inoculation the salmonellae replicate extensively in extracellular fluid, where the *spv* genes are not required for virulence (12) and are not even induced for expression (48).

The mechanism by which the SpvB protein is secreted out of the salmonella cells and into the cytoplasm of macrophages to interact with actin is not known. A type III secretion-mediated process would be plausible, but there are no published data to support this hypothesis. It is possible that the SpvC and perhaps SpvD proteins participate in this process. The detrimental nature of *spvB* expressed by itself, either when cloned alone or when *spvC* is mutated in pGTR357, suggests that SpvB requires SpvC for appropriate, functional activity. This phenomenon is not restricted to virulence in mice, since some *spvB*-bearing recombinant plasmids are detrimental to serovar Typhimurium growing in vitro. Furthermore, our data suggest that SpvD interacts with SpvC for its function, since lack of SpvD could be complemented with overexpressed SpvC. In any case, it is clear that *spvBC* are sufficient to replace the entire virulence plasmid to enable systemic infection of mice under some circumstances. Coupled with the recent discovery of the molecular function of SpvB, our data should focus future investigations of Spv function on these two genes and their products.

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