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The *Salmonella typhimurium* Virulence Plasmid Increases the Growth Rate of Salmonellae in Mice

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The virulence plasmids of *Salmonella typhimurium* and other invasive *Salmonella* serovars have long been associated with the ability of these bacteria to cause systemic infection beyond the intestines in orally inoculated animals. Genetic analysis of virulence genes on the high-molecular-weight plasmids has revealed that no more than five genes spanning a 6.2-kb region are sufficient to replace the entire plasmid for conferring virulence. However, the exact virulence function(s) encoded by these genes has not been elucidated. In this report, we measured the possible effect of the virulence plasmid on the growth rate of *S. typhimurium* in mice by two complementary procedures. The first procedure used segregation of a temperature-sensitive plasmid in vivo to provide a measure of bacterial divisions and the number of recovered marker plasmid-containing salmonellae as a measure of killing. In the second procedure, *aroA* deletions were transduced into virulence plasmid-containing and plasmid-cured *S. typhimurium*. Since AroA$^-$ salmonellae are inhibited for growth in vivo, if the virulence plasmid affected only growth rate, no difference in the recoveries of the paired AroA$^-$ strains would be seen. Virulence plasmid-containing *S. typhimurium* segregated the marker plasmid more rapidly than did the virulence plasmid-cured strain, and AroA$^-$ derivatives of both strains were recovered equally from mice. Therefore, the *S. typhimurium* virulence plasmid increased growth rate but had no detectable effect on killing or bacterial movement into deep tissues. To examine whether the plasmid accomplished this function by affecting the intracellular/extracellular location of bacteria, orally infected mice were injected with gentamicin to kill the extracellular bacteria. Wild-type and plasmid-cured *S. typhimurium* strains were equally resistant to gentamicin in vivo and hence most likely located intracellularly to equal degrees. When wild-type and plasmid-cured *S. typhimurium* strains were sequestered within peritoneal chambers in mice, the resulting extracellular growth was equal. Therefore, the virulence plasmid increases the growth rate of *S. typhimurium* in mice, probably within mouse cells.

Salmonella typhimurium and other *Salmonella* serovars cause self-limiting gastroenteritis in the healthy human population. However, in immunocompromised patients such as those with AIDS (5, 12, 13, 33) or with certain cancers undergoing chemotherapy (3, 6, 23, 37), a more invasive infection, i.e., bacteremia, which can be life threatening, results. The *Salmonella* serovars with invasive potential possess genetically related high-molecular-weight virulence plasmids (14), and a higher percentage of invasive clinical isolates of *S. typhimurium* and *Salmonella enteritidis* carries the virulence plasmid than noninvasive isolates (29). In a mouse model of oral infection, the recovery of *S. typhimurium* from the mesenteric lymph nodes and spleens is dependent on the presence of the virulence plasmid (17). The plasmid is not necessary for infection of the intestines, resistance to complement-mediated bacteriolysis of serum, resistance to phagocytosis and killing by macrophages, or adherence to, invasion into, and growth within certain cell lines (17). The way that the plasmid affects the spread of the infection to deep tissues has not been determined.

The number of bacteria recovered from a tissue of an infected animal is affected by three components: (i) the rate of killing by the host, (ii) the rate of movement of the bacteria into and out of the tissue, and (iii) the growth rate of the bacteria. Because virulence plasmid-containing salmonellae are recovered in greater numbers from lymphoid tissues than are isogenic plasmid-cured strains, many have hypothesized that the plasmid affects resistance to macrophages or growth within these cells; however, no data supporting these hypotheses have been published.

In this study, we have examined the growth rates of wild-type and virulence plasmid-cured *S. typhimurium* strains in a mouse model of infection by using procedures developed by Benjamin et al. and Fallon et al. (1, 11). The use of a temperature-sensitive (ts) genetic marker to measure the relative number of bacterial cell divisions indicated that wild-type *S. typhimurium* grew more rapidly in vivo than virulence plasmid-cured *S. typhimurium* but was killed and moved through tissues at rates equal to those of the virulence plasmid-cured strain. This finding was confirmed by using wild-type and plasmid-cured salmonellae with an *aroA* deletion inhibiting growth in vivo. Essentially all bacteria of both strains were gentamicin resistant in mice by day 4 postinoculation, and extracellular growth of the two strains in mice was equal. Therefore, the virulence plasmid functions to increase the growth rate of *S. typhimurium*, most likely within the host cells of mice.

(A preliminary report of these results has been presented previously [20].)

MATERIALS AND METHODS

Bacterial strains and culture. Bacteria were grown in L broth or L agar (28) supplemented with antibiotics at the following concentrations as appropriate: chloramphenicol, 30 μg/ml; tetracycline, 12.5 μg/ml; kanamycin, 40 μg/ml; ampicillin, 100 μg/ml; and nalidixic acid, 25 μg/ml. Virulence

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plasmid-containing *S. typhimurium* SR-11 χ3456 and χ3181 and isogenic virulence plasmid-cured χ3337 have been described (17). The Δ*aroA* mutation of *S. typhimurium* WB266 was transduced into χ3181 and χ3337 by using the linked *zbj-465*::Tn*10* insertion (11) by P22HT*int*-mediated generalized transduction as described previously (32). Transductants were selected on tetracycline and screened for the $AroA^-$ phenotype (Tyr^- Trp^- Phe^-, para-amino benzoic acid requiring). The Δ*aroA* derivatives of χ3181 and χ3337 were named UF020 and UF021, respectively. To verify that any phenotype associated with UF020 and UF021 was due to the Δ*aroA*, the wild-type *aroA* gene was transduced back into UF020 and UF021 from *S. typhimurium* χ3000 (17), selecting for Tc^r and prototrophy. The $AroA^+$ wild-type transductants were named UF025 and UF026 for the virulence plasmid-containing and cured strains, respectively.

Infection of mice. Mice were orally inoculated with *S. typhimurium* as described previously (16). Approximately 2×10^8 CFU of *S. typhimurium* was fed to 7- to 11-week-old female BALB/c mice (Charles River, Wilmington, Mass.) after food and water deprivation and feeding of bicarbonate. Five days later, Peyer's patches, mesenteric lymph nodes, and spleens were removed, homogenized in glass tissue homogenizers with phosphate-buffered saline containing gelatin (BSG) (8), and plated to enumerate CFU. Additionally, the hind footpads of the mice were inoculated subcutaneously as described previously (31). Approximately 10^5 CFU of salmonellae suspended in 0.05 ml of BSG was injected into the hind footpads of the mice. Three days later, popliteal lymph nodes and spleens were removed, homogenized in BSG, and plated to enumerate CFU.

Use of plasmid pHSG422 to measure growth rate of salmonellae in mice. To examine the relative growth rates of wild-type and virulence plasmid-cured *S. typhimurium* strains in mice, we used a modification of the procedure of Benjamin et al. (1). Wild-type *S. typhimurium* χ3456 and virulence plasmid-cured χ3337 were transformed with the ts Cm^r plasmid pHSG422 (21). Maintenance of the plasmid during growth at 30°C and loss of the plasmid during growth at 37°C were confirmed by growth in vitro in L broth. The segregation kinetics of pHSG422 from χ3456 and χ3337 were essentially the same. For inoculation of mice, χ3456 (pHSG422) and χ3337(pHSG422) were grown statically overnight at 30°C in L broth containing chloramphenicol. The next day, these cultures were diluted 1:20 into fresh 37°C L broth without antibiotic and shaken at 37°C for approximately 2 h until the cultures reached an optical density at 600 nm of approximately 0.5. The percentage of each strain maintaining pHSG422 and the total number of pHSG422 plasmids in each culture were determined as described previously (1). The 2-h incubation period at 37°C resulted in approximately 75% of each strain maintaining the plasmid with a copy number of one or two plasmids per cell. The bacterial cultures were pelleted at 37°C by centrifugation and suspended in prewarmed BSG at 37°C for oral inoculation into mice at 2×10^8 CFU per mouse. Mice were housed at room temperature. Five days later, mice were killed, and tissues were homogenized and plated on media either containing or not containing chloramphenicol. The proportion of bacteria maintaining pHSG422 was determined by calculating the difference in the $\log_{10}$ total CFU and the $\log_{10}$ CFU carrying pHSG422. This proportion is expressed as the $\log_{10}$.

Use of gentamicin to kill extracellular bacteria. Examining the extracellular/intracellular nature of salmonellae was done by killing extracellular bacteria in mice by injection of gentamicin as described previously (9), with the following modifications. Mice were orally inoculated with mixtures of wild-type *S. typhimurium* χ3456 (Tc^r) or plasmid-cured χ3337 (Nal^r). Four days later, each mouse in one group was injected intraperitoneally twice with 0.1 mg of gentamicin sulfate (Elkins-Sinn, Cherry Hill, N.J.) at 24 and 18 h presacrifice. On day 5 postinoculation, 0.1 mg of gentamicin was injected intravenously into the lateral tail veins of the first set of mice, and the mice were killed 45 min later for quantitative analysis of CFU of salmonellae in the Peyer's patches, mesenteric lymph nodes, and spleens. A second set of mice received 0.1 mg of gentamicin intravenously only on day 5 and was examined 45 min later for CFU. A third set of infected mice receiving no gentamicin served as a control and was also examined for CFU of χ3456 and χ3337 in tissues on day 5 postinoculation.

Peritoneal chamber implants. Salmonellae were grown in the peritoneal cavities of mice by using peritoneal chambers as described previously (7). Pharmaceutical-grade in-line syringe filters (0.22-μm pore size, 8-mm wide; Filtertek Inc., Hebron, Ill.) were kindly provided by K. D. Coleman. The male and female ends of separate filters were removed, the male end of one filter was filled with 0.05 ml of salmonellae suspended in BSG, and the corresponding female end was glued into place with Krazy Glue (Krazy Glue, New York, N.Y.). Mice were anesthetized by intraperitoneal injection of 1 mg of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, Ill.). The abdomens were shaved and disinfected, a 1-cm incision was made in each abdominal wall, and then a 1-cm incision was made in each peritoneum. The sealed chambers were placed into the peritoneal cavities, the peritoneums were closed with two sutures, and the abdominal walls were each sealed with two surgical clips (9-mm length, Autoclips; Clay Adams, Parsippany, N.J.). The mice were observed for 2 days, at which time they were killed, the chambers were removed, and the contents were diluted and plated on agar media containing appropriate antibiotics. Peritoneal cavities were tested for leakage of bacteria from the chamber by swabbing and plating on L agar. No leakage was detected.

Statistical analysis. Analysis of CFU using $\log_{10}$ CFU was performed as described previously (4). For a comparison of mean CFU between groups of mice inoculated with single salmonella strains, the Student *t* test was used for analysis of the $\log_{10}$ CFU to determine whether there was a significant difference between the means found for plasmid-containing and plasmid-cured strains. In experiments in which individual mice were inoculated with two strains, the mean paired difference in the $\log_{10}$ CFU was determined by using a paired Student *t* test. In some cases, results from two or three experiments were pooled for analysis.

RESULTS

Rationale for determination of in vivo growth rate. Wild-type *S. typhimurium* is recovered in higher numbers than the isogenic plasmid-cured derivative from the mesenteric lymph nodes and spleens of orally inoculated mice (17). This difference in infectivity can be the product of the three independent factors, i.e., relative rates of growth, killing, and movement of the two strains in mouse tissues. To measure the growth rate in vivo in mice independently of the other two factors, we used an unstable genetic element which would be lost with each bacterial division in vivo. The genetic element, plasmid pHSG422, is extremely ts for replication at 37°C (21) and therefore is lost from a portion of the bacterial population with each division in mice (1, 11). As

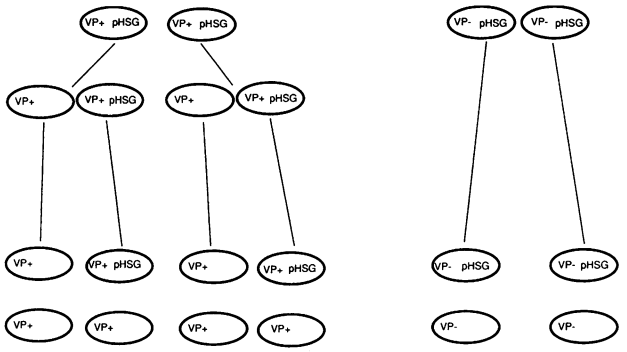

FIG. 1. Use of plasmid pHSG422 to measure relative growth rates of wild-type χ3456 and virulence plasmid-cured χ3337 in mice. The ovals represent salmonellae containing the virulence plasmid (VP+) (left) or cured of the virulence plasmid (VP−) (right), each beginning with 100% carriage of the ts marker plasmid pHSG422 (pHSG). In this hypothetical situation during growth at the nonpermissive temperature of 37°C in mice, the virulence plasmid-positive strain undergoes two generations, segregating pHSG422 with each division, resulting in a final carriage rate of 0.25. The virulence plasmid-negative strain undergoes one generation, resulting in a final carriage rate for pHSG422 of 0.50.

long as the presence of pHSG422 does not affect the rates of killing or movement of *S. typhimurium* in mice, the proportion of salmonellae still carrying pHSG422 recovered from mice after a given amount of time will be inversely proportional to the growth rate of the bacteria. This is illustrated in Fig. 1, and theoretical results are presented in Table 1. Mutant A represents the results obtained when the only difference between the mutant and the wild type was a slower growth rate (less segregation of pHSG422). Mutant B represents the results obtained if the growth rate was unchanged but if differences in killing or movement occurred (total pHSG422-containing bacteria decreased, but the proportion remained constant). Since there is no replication of the ts plasmid, differences in the total numbers of pHSG422-containing salmonellae in a tissue will not be compensated for by growth of salmonellae. Mutant C demonstrates that if multiple factors are affected in a given strain, a differential growth rate can still be detected independently from rates of killing and/or movement (the proportion and the total number of pHSG422-containing bacteria are different from those of the wild type).

TABLE 1. Recovery of wild-type χ3456 and virulence plasmid-cured χ3337 from mice after oral inoculation and segregation of the marker plasmid pHSG422; illustrative theoretical results[a]

Strain	Recovery of strain (log_{10}) from:					
	Peyer's patches			Spleens		
	Total CFU	CFU pHSG	Prop pHSG[b]	Total CFU	CFU pHSG	Prop pHSG
Wild type	5	3	−2	6	3	−3
Mutant A	5	3	−2	4	3	−1
Mutant B	5	3	−2	4	1	−3
Mutant C	5	3	−2	4	2	−2

[a] Theoretical results demonstrate different outcomes obtained with wild-type and mutant strains. All strains equally infected the Peyer's patches and segregated the marker plasmid to equal degrees. The splenic infections were different. In comparison with the wild type, mutant A exhibited slower growth and equal killing and movement (100-fold lower total CFU, equal CFU of pHSG422 [pHSG], yielding 100-fold less segregation). Mutant B exhibited an equal growth rate, increased killing and/or less efficient movement (100-fold lower total CFU, 100-fold lower pHSG422, equal segregation). Mutant C exhibited a combined decreased growth rate and increased killing and/or less efficient movement (100-fold lower total CFU, 10-fold lower pHSG422, 10-fold less segregation).

[b] Prop pHSG, proportion of each strain containing pHSG422 expressed as log_{10}.

The growth rates of wild-type *S. typhimurium* χ3456 and virulence plasmid-cured χ3337 are equal at 37°C in L broth (17) (data not shown). Similarly, the rates of segregation of pHSG422 from χ3456(pHSG422) and χ3337(pHSG422) were equal during growth of the bacteria at 37°C in the absence of antibiotic to select for pHSG422 (data not shown). As had been reported by Benjamin et al. (1) and Fallon et al. (11), pHSG422 could serve as a marker for the number of salmonella divisions in vivo. To confirm that the presence or absence of pHSG422 did not affect the recovery of *S. typhimurium* from mice, we inoculated mice with wild-type χ3456(pHSG422) and wild-type χ3306. χ3456 is Tc^r, while χ3306 is Nal^r. Five days after oral inoculation with 5×10^8 CFU, equal numbers of each strain were recovered from the Peyer's patches, mesenteric lymph nodes, and spleens of mice (data not shown). pHSG422, therefore, did not affect the infectivity of *S. typhimurium*; hence, no selection for or against salmonellae with the marker plasmid occurred in vivo.

Virulence plasmid-containing *S. typhimurium* replicates more rapidly in mice than does virulence plasmid-cured *S. typhimurium*. Mice were orally inoculated with either wild-type χ3456(pHSG422) or virulence-plasmid-cured χ3337 (pHSG422). Five days later, the total number of salmonellae, the total number of pHSG422-containing salmonellae, and the proportion of salmonellae still carrying pHSG422 in tissues were determined. The data in Table 2 are the combined results of three experiments. In the Peyer's patches, χ3337 was recovered in slightly higher yields than χ3456 ($10^{5.3}$ and $10^{4.8}$ CFU, respectively); however, this was not a consistent finding in other experiments of wild-type and plasmid-cured salmonellae (17, 18) (see below for UF025 and UF026). The proportion of each strain carrying pHSG422 was the same, $10^{-2.0}$. Therefore, in a tissue in which the presence or absence of the virulence plasmid generally has no effect on infectivity, the growth rates of the two strains were essentially the same. In contrast, in the mesenteric lymph nodes, approximately fivefold more χ3456 was recovered than χ3337 ($10^{5.1}$ and $10^{4.4}$ CFU, respectively), and the proportion of χ3456 maintaining pHSG422 was one-eighth that of χ3337 ($10^{-3.1}$ and $10^{-2.2}$, respectively; $P = 0.015$). This result indicated that χ3456 replicated more rapidly, contributing to the increased infectivity of the wild-type strain at this site. An even greater difference in the recovery of wild-type over that of virulence plasmid-cured salmonellae was observed in the spleens ($P < 0.0005$), and the carriage rate for pHSG422 for χ3456 was 1/16 that of χ3337 ($P < 0.0005$). Therefore, the greater recoveries of wild-type χ3456 in mesenteric lymph nodes and spleens could be explained at least in part by the increased growth rate relative to the virulence plasmid-cured strain, χ3337.

Further analysis of data for CFU recovered from Peyer's patches, mesenteric lymph nodes, and spleens revealed additional insight into the possible difference in the mechanism of virulence. In the mesenteric lymph nodes and spleens, although the carriage rate for pHSG422 was lower for χ3456 than for χ3337, the total recovery of CFU containing pHSG422 was not significantly different between χ3456 and χ3337. Had increased killing of χ3337 occurred, thereby

TABLE 2. Recovery of wild-type χ3456 and virulence plasmid-cured χ3337 from mice after oral inoculation and segregation of the marker plasmid pHSG422: experimental results[a]

Strain	Recovery of strain (mean $\log_{10}$ ± SD) from:								
	Peyer's patches			Mesenteric lymph nodes			Spleens		
	Total CFU	CFU pHSG	Prop pHSG	Total CFU	CFU pHSG	Prop pHSG	Total CFU	CFU pHSG	Prop pHSG
χ3456	4.8 ± 0.48	2.7 ± 1.0	−2.0 ± 0.86	5.1 ± 0.52	1.9 ± 0.75	−3.1 ± 0.79	5.6 ± 0.67	3.1 ± 0.78	−2.5 ± 0.67
χ3337	5.3 ± 0.43	3.4 ± 1.3	−2.0 ± 1.3	4.4 ± 0.42	2.1 ± 1.1	−2.2 ± 1.1	3.8 ± 0.95	2.5 ± 1.2	−1.3 ± 0.84
P value[b]	<0.005	>0.05	>0.25	<0.0005	>0.25	0.015	<0.0005	>0.1	<0.0025

[a] Mice were inoculated orally with 2×10^8 CFU of wild-type χ3456(pHSG422) or virulence plasmid-cured χ3337(pHSG422). Five days later, Peyer's patches, mesenteric lymph nodes, and spleens were examined for total CFU of each strain, CFU maintaining pHSG422 (pHSG), and the proportion of each strain containing pHSG422 (Prop pHSG). For each group for Peyer's patches and mesenteric lymph nodes, *n* equals 13; for each group for spleens, *n* equals 9. These are the results of three experiments.

[b] *P* value for χ3456 different from χ3337.

irreversibly lowering the number of pHSG422-containing CFU for χ3337, or if χ3337 had moved at a lower rate to the mesenteric lymph nodes and spleens, the total number of pHSG422-containing χ3337 would have been lower than that of χ3456 (Table 1, mutants B and C). The equal recoveries of pHSG422-containing CFU for wild-type χ3456 and virulence plasmid-cured χ3337 confirmed that the increased yield of virulence plasmid-containing *S. typhimurium* from mouse tissues correlated with an increased growth rate observed for salmonellae recovered from those tissues. These experimental data most closely fit the theoretical results for mutant A in Table 1, in which only growth rate is affected by the mutation.

Δ*aroA* renders virulence plasmid-containing and cured *S. typhimurium* strains equal in virulence. The data reported above indicate that the virulence plasmid increased the growth rate of *S. typhimurium* at the mesenteric lymph nodes and spleens and suggest that there was no difference in the rates of killing or movement through tissues by bacteria between wild-type and virulence plasmid-cured salmonellae. To examine these latter two points in another way, we used a method developed with *S. typhimurium* by Fallon et al. (11). The ability of salmonellae to grow in vivo was inhibited by transducing into the bacteria a deletion of the *aroA* gene. AroA$^-$ salmonellae require the aromatic amino acids, cannot make the siderophore enterobactin, and require para-amino benzoic acid for growth (25). The amino acids are probably supplied by the host, and it has been reported that enterobactin is not required for infectivity of *S. typhimurium* (2). The major defect of AroA$^-$ strains is probably the requirement for para-amino benzoic acid, which cannot be supplied by the host in sufficient amounts, resulting in a deficiency in replication (1) due to an inability to make thymidine for DNA synthesis. If virulence plasmid-cured salmonellae experienced an increase in susceptibility to killing by the host or a decrease in movement through tissues (and if the *aroA* mutation itself did not differentially affect killing, as shown by Benjamin et al. [1], or movement), then virulence plasmid-containing AroA$^-$ *S. typhimurium* would still be recovered in higher numbers than virulence plasmid-cured AroA$^-$ because the nongrowing plasmid-cured salmonellae would be differentially killed or still not move to the tissues. However, if the only difference between the wild-type and plasmid-cured salmonellae was the growth rate, then inhibition of growth with Δ*aroA* would equalize the virulence of the two strains.

One set of mice was orally inoculated with both strains of AroA$^+$ *S. typhimurium*: plasmid-positive UF025 and plasmid-negative UF026. A second set of mice was inoculated with both strains of AroA$^-$ *S. typhimurium*: plasmid-positive UF020 and plasmid-negative UF021. The paired differences in the log CFU of the two strains recovered from tissues were compared for mice infected with wild-type and plasmid-cured salmonellae (Table 3). Since all strains were Tc^r and only plasmid-cured UF021 and UF026 were Nal^r, the CFU of UF020 and UF025 were calculated as Tc^r CFU minus Nal^r CFU and by replica-plating Tc^r CFU to nalidixic acid plates. As is usually seen for AroA$^+$ strains, equal numbers of CFU were found in the Peyer's patches ($P > 0.25$), whereas 8- and 32-fold more virulence plasmid-containing UF025 than plasmid-cured UF026 was recovered from the mesenteric lymph nodes ($P < 0.0005$) and spleens ($P < 0.0005$), respectively.

A different result was obtained with the AroA$^-$ derivatives. Because of the inhibited replication of each strain, grossly lower numbers of CFU were recovered from all tissues, especially the mesenteric lymph nodes and spleens. The recovery of CFU from the spleen was too low for statistical analysis. However, the mean paired differences in

TABLE 3. Effects of Δ*aroA* on recovery of wild-type and virulence plasmid-cured *S. typhimurium* from tissues of orally inoculated mice

Strain	Virulence plasmid	*S. typhimurium* recovered (log CFU ± SD[a])		
		Peyer's patches	Mesenteric lymph nodes	Spleens
AroA$^+$:				
UF025	+	4.7 ± 0.68	4.8 ± 0.68	4.6 ± 0.83
UF026	−	4.8 ± 0.59	3.9 + 0.61	3.1 ± 1.1
Paired[b]		−0.18 ± 0.48	0.91 ± 0.35	1.5 ± 0.44
P value		>0.25	<0.0005	<0.0005
AroA$^-$:				
UF020	+	3.3 ± 0.87	1.6 ± 1.0	ND[c]
UF021	−	3.2 ± 0.72	1.9 ± 0.78	ND
Paired[b]		0.05 ± 0.77	−0.35 ± 0.93	
P value		>0.25	>0.10	

[a] Eight mice were inoculated orally with 2×10^8 CFU of AroA$^+$ *S. typhimurium* UF025 (virulence plasmid positive) and UF026 (virulence plasmid negative) or AroA$^-$ *S. typhimurium* UF020 (virulence plasmid positive) and UF021 (virulence plasmid negative). Five days layer, Peyer's patches, mesenteric lymph nodes, and spleens were examined for CFU of each strain. These are the results from two experiments.

[b] Mean paired difference in log CFU. To compare relative infectivities of each strain, the mean paired differences of $\log_{10}$ CFU UF025-UF026 or UF020-UF021 were examined for values different from 0.

[c] ND, not determined. Too few CFU were recovered from spleens from some mice for statistical analysis.

TABLE 4. Effects of ΔaroA on recovery of wild-type and virulence plasmid-cured *S. typhimurium* from tissues of subcutaneously inoculated mice[a]

Strain	Virulence plasmid	*S. typhimurium* recovered (log CFU ± SD)	
		Popliteal lymph nodes	Spleens
UF025	+	6.4 ± 0.68	7.2 ± 0.52
UF026	−	6.2 ± 0.68	5.8 ± 0.63
Paired		0.21 ± 0.28	1.4 ± 0.54
P value		<0.05	<0.0005
UF020	+	3.8 ± 1.3	3.0 ± 0.48
UF021	−	4.5 ± 0.32	2.9 ± 0.54
Paired		−0.74 ± 1.5	0.10 ± 0.21
P value		>0.10	>0.10

[a] The hind footpads of groups of eight mice were each injected subcutaneously with mixtures of AroA$^+$ *S. typhimurium* UF025 (virulence plasmid positive) and UF026 (virulence plasmid negative) or AroA$^-$ *S. typhimurium* UF020 (virulence plasmid positive) and UF021 (virulence plasmid negative). Three days later, popliteal lymph nodes and spleens were examined for CFU as described in Table 3, footnote *a*. These are the results of two experiments.

the log CFU of wild-type and virulence plasmid-cured salmonellae in all of the other tissues were not significantly different. Therefore, the inhibition of replication by ΔaroA removed all measurable differences in the infectivities of wild-type *S. typhimurium* relative to the isogenic virulence plasmid-cured derivative, indicating that the increased growth rate of wild-type salmonellae was the major reason for the increased recovery of wild-type over plasmid-cured salmonellae from mesenteric lymph nodes and spleens.

Because of problems in recovering enough AroA$^-$ salmonellae from deep tissues after oral inoculation, we studied the effect of the ΔaroA mutation by using a subcutaneous injection into the hind footpads (Table 4). It has been reported that wild-type and cured *S. typhimurium* strains equally invade the draining popliteal lymph nodes, while the virulence plasmid-containing strain is more efficient at invading spleens (31). AroA$^+$ virulence plasmid-containing UF025 infected the popliteal lymph nodes slightly more effectively (1.6 fold) than did AroA$^+$ plasmid-cured UF026 ($P < 0.05$), whereas UF025 achieved 25-fold higher splenic CFU relative to UF026 ($P < 0.0005$). However, the AroA$^-$ derivatives UF020 and UF021 equally infected both the popliteal lymph nodes and spleens, again demonstrating that the inhibition of the replicative potential of *S. typhimurium* by ΔaroA fully eliminated the virulence plasmid phenotype of invasive infection to deep tissues.

Virulence plasmid-containing and plasmid-cured *S. typhimurium* strains are most likely intracellular after oral inoculation. Since *S. typhimurium* is capable of intracellular as well as extracellular infection, it was possible that the virulence plasmid affected the growth rate by altering the intracellular/extracellular location of salmonellae. To compare the intracellular/extracellular environment of wild-type and plasmid-cured salmonellae in orally inoculated mice, we used the procedure of Dunlap et al. (9) in which gentamicin is administered to mice to kill extracellular, but not intracellular, salmonellae. Mice were orally inoculated with mixtures of wild-type χ3456 and plasmid-cured χ3337. At various times after inoculation, mice were injected with gentamicin, and the yields of gentamicin-resistant CFU of each strain were examined (Table 5).

At day 5 postinoculation in mice not receiving gentamicin, typical results were obtained for infections with wild-type and plasmid-cured *S. typhimurium*. Recoveries from Peyer's patches were similar, with 1.5-fold more χ3337 recovered than χ3456 (however, this was significant because of an unusually small standard deviation), and significantly more χ3456 than χ3337 was recovered from the mesenteric lymph nodes and spleens. Administration of gentamicin for as long as 24 h or as short as 45 min before sampling tissues did not significantly affect the yield of either strain or the paired difference in CFU between the strains. Similar results were obtained in a repetition of this experiment. Therefore, as had been reported by Dunlap et al. for intravenously inoculated *S. typhimurium* (9), essentially all *S. typhimurium* strains in all tissues examined after oral inoculation of mice were gentamicin resistant and probably intracellular. Since the presence or absence of the virulence plasmid did not affect gentamicin resistance, the plasmid probably did not alter the intracellular/extracellular location of the bacteria. The increased growth rate identified above for wild-type salmonellae most likely occurs within cells of the mice.

The virulence plasmid does not affect growth of extracellular salmonellae in mice. A converse hypothesis to the virulence plasmid affecting the intracellular growth rate of *S. typhimurium* is that extracellular growth rates in mice inoculated with wild-type and plasmid-cured salmonellae would be equal. To examine this possibility in vivo, wild-type χ3456 and plasmid-cured χ3337 were placed within chambers and implanted into the peritoneal cavities of separate mice. The chambers were sealed with 0.22-μm-pore-size filters which prevent the escape of bacteria and the entry of host cells; however, diffusion of solutes can occur, enabling bacterial growth in the extracellular environment of the peritoneal cavity (7). Two days after mice were surgically

TABLE 5. Effects of gentamicin on recovery of wild-type and virulence plasmid-cured *S. typhimurium* from mice[a]

Strain	*S. typhimurium* recovered (log CFU ± SD)								
	Peyer's patches			Mesenteric lymph nodes			Spleen		
	−Gent	Gent 45m	Gent 24h	−Gent	Gent 45m	Gent 24h	−Gent	Gent 45m	Gent 24h
χ3456	4.4 ± 0.06	4.0 ± 0.83	4.3 ± 0.50	4.4 ± 0.34	4.4 ± 0.46	4.3 ± 0.52	5.0 ± 0.39	5.9 ± 0.68	4.8 ± 0.47
χ3337	4.5 ± 0.14	4.1 ± 0.64	3.4 ± 1.8	2.6 ± 1.7	2.7 ± 1.5	1.7 ± 1.3	1.8 ± 1.3	2.6 ± 0.99	2.1 ± 1.1
Paired[b]	−0.18 ± 0.01	−0.02 ± 0.47	0.82 ± 1.5	1.8 ± 1.4	1.7 ± 1.2	2.6 ± 1.0	3.2 ± 1.0	2.4 ± 1.4	2.7 ± 1.3
P value	<0.0005	>0.25	>0.1	<0.05	<0.05	<0.01	<0.005	<0.025	<0.025

[a] Groups of four mice were inoculated orally with mixtures of 10^8 CFU each of wild-type χ3456 and plasmid-cured χ3337. On day 4 postinoculation, one set of mice (Gent 24h) received two intraperitoneal injections of 0.1 mg of gentamicin and then 0.1 mg intravenously on day 5 postinoculation. Forty-five minutes later, tissues were sampled for CFU of χ3456 and χ3337. On day 5, a second set of mice was given a single dose of 0.1 mg of gentamicin intravenously 45 min before the tissues were sampled (Gent 45m). A third set of mice received no gentamicin before the tissues were sampled on day 5 (−Gent).

[b] The mean paired differences in log CFU χ3456-χ3337 were examined for values different than 0.

TABLE 6. The subcloned *spv* virulence genes confer increased growth rate in mice[a]

Strain	Splenic CFU (mean ± SD)		
	Total	pGTR253	Prop pGTR253
χ3337(pGTR061)	5.2 ± 0.46	2.3 ± 0.90	−2.9 ± 0.64
χ3337(pYA2204)	3.2 ± 1.4	2.5 ± 1.7	−0.73 ± 0.50
P value	<0.0005	>0.1	<0.0005

[a] Five mice were orally inoculated with χ3337(pGTR061/pGTR253) or χ3337(pYA2204/pGTR253). Five days later, splenic bacteria were examined for total CFU, CFU containing the ts marker plasmid pGTR253 derived from pHSG422, and the proportion of CFU carrying pGTR253 (Prop p253). pGTR061 is a subclone of the virulence plasmid encoding *spvRABCD* and *orfE*, and pYA2204 is the vector.

implanted with peritoneal chambers containing 10^5 CFU of either χ3456 or χ3337 per ml, the strains grew to equal yields (8.8 ± 0.06 and 9.2 ± 0.47 log CFU, respectively; $P > 0.1$). In a pilot experiment, the yields of the two strains were equal from day 1 to 3 postimplantation (data not shown). Therefore, the virulence plasmid did not affect the growth of *S. typhimurium* in mice when bacteria were sequestered extracellularly.

The *spv* genes are sufficient to confer an increased growth rate. All of the experiments described above examined the effect of the presence or absence of the entire virulence plasmid on the growth rate in mice. We (15) and others (26, 36) have described an approximately 8-kb region of the virulence plasmid encoding the essential virulence genes. We constructed plasmid pGTR061 encoding the five *spv* virulence genes and an additional open reading frame, *orfE* (15). pGTR061 could replace the entire virulence plasmid of *S. typhimurium* to confer splenic infection and oral 50% lethal dose (LD_{50}). We therefore examined whether this virulence region subclone would similarly confer the increased growth rate observed with the entire virulence plasmid. pGTR061 and its vector, pYA2204, were transformed into virulence plasmid-cured *S. typhimurium* χ3337. Since pYA2204 and pGTR061 encode Ap^r, also encoded on pHSG422, we deleted the Ap^r gene from pHSG422, yielding pGTR253. The ts phenotype of pGTR253 was identical to that of pHSG422 (data not shown). Mice were orally inoculated with either χ3337(pGTR061/pGTR253) or χ3337(pYA2204/pGTR253). Five days later, 98-fold-more pGTR061-containing salmonellae than vector controls were recovered from spleens ($P < 0.0005$); however, the numbers of pGTR253-containing bacteria of each strain were not significantly different (Table 6). χ3337(pGTR061) segregated the ts marker to about 1/150th of the vector construct ($P < 0.0005$), indicating that, as was observed for the entire virulence plasmid, the *spv* genes alone were sufficient to confer an increased growth rate in vivo. Similar results were obtained in a repetition of this experiment.

DISCUSSION

It is well established that the virulence plasmid of *S. typhimurium* is involved in the ability of salmonellae to invade from the intestines into deeper tissues, such as the mesenteric lymph nodes and spleens, after oral inoculation of mice (14). Although an understanding of the genetic organization of the essential virulence genes and their regulation has emerged (19), the virulence functions resulting in infectivity in areas beyond the intestines have heretofore not been elucidated. Several groups have hypothesized that the plasmid affects intracellular killing by macrophages or growth within macrophages, although no definitive data have ever been published. By using a variety of techniques involving the mouse model of infection, we show that the virulence plasmid increases the growth rate of *S. typhimurium* most likely within as-of-yet-unidentified host cells of mice. The major experimental problem was to separate the roles of the rates of killing, movement, and growth in vivo. This was initially done by using an unstable genetic element, the plasmid pHSG422, to measure the bacterial cell divisions occurring in vivo. This procedure was pioneered by William Benjamin and David Briles to show that the major effect of the Ity^s genotype in mice is to permit an increased growth rate of *S. typhimurium* (1) and that *S. typhimurium* grows more slowly in mice infected with murine hepatitis virus (11).

In our studies, wild-type *S. typhimurium* χ3456 segregated the marker pHSG422 more rapidly in tissues of mice which yielded higher numbers of χ3456 than virulence plasmid-cured χ3337 (Table 2). The necessary conclusion from these data is that χ3456 underwent more divisions in the mice because of a more rapid growth rate. Analysis of the total CFU of each strain carrying the marker also allowed us to conclude that equal rates of killing and movement occurred for the strains. Equal numbers of CFU of wild-type and virulence plasmid-cured *S. typhimurium* possessed pHSG422. If χ3337 were killed more efficiently in mice than χ3456, fewer pHSG422-containing CFU would have been recovered because once a pHSG422-containing cell is killed, it cannot be replaced with another pHSG422-containing cell. Similarly, if χ3337 had moved less efficiently, fewer pHSG422-containing bacteria would have been recovered from deeper tissues.

A complementary procedure was used to more specifically examine the possibilities that rates of killing and movement of bacteria were responsible for the differences in yields of wild-type and plasmid-cured salmonellae from mesenteric lymph nodes and spleens. Both strains were endowed with deletions of the *aroA* gene, rendering the strains defective for replication in mice (1, 11, 25). We found that replication-defective plasmid-containing and plasmid-cured *S. typhimurium* strains were now equal in their infectivities for mesenteric lymph nodes and spleens. Had the plasmid-cured salmonellae been more susceptible to killing by the mice, they would have been recovered in numbers lower than those of the wild-type strain. Similarly, as long as the *aroA* deletion itself did not affect the movement of bacteria through tissues, increased movement caused by the virulence plasmid would have resulted in a lower recovery of the plasmid-cured strain from deeper tissues. However, by restricting the growth of plasmid-containing and plasmid-cured *S. typhimurium* strains to what is assumed to be equal rates of near zero, the virulences of the two strains were made equal. It therefore appeared that different rates of killing or movement were not responsible for different recoveries of plasmid-containing and plasmid-cured salmonellae from mesenteric lymph nodes and spleens.

To begin to understand where the virulence plasmid affected growth rate, we examined the intracellular/extracellular nature of the bacteria in mice by using a procedure developed by Dunlap et al. (9) involving the use of gentamicin to kill extracellular bacteria. Gentamicin is commonly used in tissue culture to kill extracellular bacteria since this antibiotic is not effective intracellularly (17). We found that gentamicin treatment of salmonella-infected mice for as long as 24 h before enumeration of CFU in tissues had no effect

on the recovery of either wild-type or virulence plasmid-cured *S. typhimurium* (Table 5). Dunlap et al. (9) similarly found that essentially all of the CFU of wild-type *S. typhimurium* were protected from gentamicin administered 30 min before CFU enumeration. Dunlap et al. used intravenous inoculation of mice, whereas we used oral inoculation. We also utilized a longer period of gentamicin treatment than Dunlap et al. since it could be argued that salmonellae growing slowly in mice might be resistant to short-term treatment with gentamicin. However, even 24 h of gentamicin treatment had no significant effect on the recovery of salmonellae. Our conclusions are the same as those of Dunlap et al., i.e., that most, if not all, salmonellae are present within a safe site in infected mice. However, no direct proof has been obtained that this safe site is necessarily intracellular.

A corollary hypothesis to one of different intracellular growth rates is that extracellular growth rates of wild-type and plasmid-cured salmonellae would be equal. We previously showed that the virulence plasmid did not affect the ability of *S. typhimurium* to grow in heat-treated serum or to be resistant to killing by complement (17). Therefore, different extracellular growth rates were not expected. Salmonellae were sequestered in an extracellular environment in the peritoneal cavities of mice by using growth chambers sealed with 0.22-μm-pore-size filters (7). When plasmid-containing and plasmid-cured *S. typhimurium* strains were inoculated at 10^5 CFU into the chambers in the peritoneal cavities of mice, increases in CFU higher than 1,000-fold occurred for both strains. The plasmid therefore did not affect the extracellular growth of *S. typhimurium* in mice, as modeled by the peritoneal chamber implants.

We have not identified the putative host cell in which the wild-type salmonellae replicate more rapidly than the plasmid-cured derivative. We hypothesize several mechanisms by which the plasmid may increase the intracellular growth rate in vivo. First, the plasmid may enable *S. typhimurium* to seek out an environment more permissive for growth. Examples of mechanisms include altering the specificity for infection of host cells, altering the intracellular location of salmonellae within host cells, or affecting the response of host cells to infection by *S. typhimurium*. Alternatively, the virulence plasmid may enable *S. typhimurium* to replicate more rapidly in a given environment of the appropriate host cell.

Our results that a subclone, pGTR061 (15), encoding the five virulence genes *spvRABCD*, conferred an increased growth rate to virulence plasmid-cured *S. typhimurium* χ3337 (Table 6) raise questions as to the role of other potential virulence loci of the virulence plasmid (22, 27, 30, 34, 35). Some loci have been identified solely through cloning (22, 35), while others have been identified solely through mutagenesis (27, 34). We previously showed that pGTR061 could replace the entire virulence plasmid to confer wild-type splenic infection and LD_{50} after oral inoculation of mice (15), raising the question of other virulence genes on the plasmid.

Hoertt et al. (24) reported that virulence plasmid-containing *S. typhimurium* caused immunosuppression in mice. Most recently, Emoto et al. (10) reported that wild-type *Salmonella choleraesuis* failed to elicit γ/δ T cells to the peritoneal cavity after intraperitoneal inoculation, in contrast to the isogenic virulence plasmid-cured strain which did elicit the T cells. This is an intriguing result; however, no direct linkage between the virulence plasmid and failure to elicit γ/δ T cells can be drawn since other types of attenuated salmonellae were not examined. Although the data presently reported do not address possible immunosuppression, should plasmid-mediated immunosuppression occur in orally inoculated mice, it would have to result in an altered growth rate, as opposed to killing, of salmonellae. It should be noted that the plasmid does not affect system-wide responses of mice to salmonella infection since wild-type bacteria are incapable of helping plasmid-cured salmonellae, and, conversely, infection with plasmid-cured salmonellae does not hinder the infection by wild-type salmonellae (17). Immunosuppression would therefore have to act at the level of salmonellae interacting with individual host cells. This situation seems to be inconsistent with the results of Emoto et al. (10). In any case, we can now focus attention on a limited set of virulence genes, *spvRABCD*, encoding a defined phenotype of increased growth rate in mice.

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