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Cloning and Transfer of the *Salmonella* Pathogenicity Island 2 Type III Secretion System for Studies of a Range of Gram-Negative Genera▽†

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The engineering of bacterial strains with specific phenotypes frequently requires the use of blocks or "cassettes" of genes that act together to perform a desired function. The potential benefits of utilizing type III secretion systems in this regard are becoming increasingly realized since these systems can be used to direct interactions with host cells for beneficial purposes such as vaccine development, anticancer therapies, and targeted protein delivery. However, convenient methods to clone and transfer type III secretion systems for studies of a range of different types of bacteria are lacking. In addition to functional applications, such methods would also reveal important information about the evolution of a given type III secretion system, such as its ability to be expressed and functional outside of the strain of origin. We describe here the cloning of the *Salmonella enterica* serovar Typhimurium pathogenicity island 2 (SPI-2) type III secretion system onto a vector that can be easily transferred to a range of gram-negative bacterial genera. We found that expression of the cloned SPI-2 system in different *Gammaproteobacteria* and *Alphaproteobacteria* (as monitored by SseB protein levels) is dependent on the bacterial strain and growth medium. We also demonstrate that the cloned system is functional for secretion, can direct interactions with macrophages, and can be used as a novel tool to analyze the predicted interaction of SseB with host cells. This work provides a foundation for future applications where the cloned SPI-2 region (or other cloned type III systems) can provide a desired function to an engineered gram-negative strain.

The organized compilation and applied use of blocks or "cassettes" of genes that can be used to conveniently engineer microbes for specific purposes are being increasingly developed (3, 12, 14). The potential to use bacterial type III secretion systems in this regard is significant and can be illustrated by recent uses of these systems to direct interactions with host cells for beneficial purposes such as vaccine development, anticancer therapies, and targeted protein delivery (13, 28, 32, 33, 35, 36). Type III secretion systems allow secretion of protein substrates to the extracellular milieu and facilitate translocation of effector proteins from bacteria to eukaryotic host cells (7, 15). Originally, these systems were discovered and characterized by their ability to facilitate interactions of pathogens with their host cells, and the list of different gram-negative species that have evolved to use the type III pathway includes *Salmonella enterica* serovars, *Yersinia* spp., *Shigella* spp., *Escherichia coli*, and *Pseudomonas aeruginosa* (8). Recently, however, type III systems have been harnessed as tools for targeted beneficial applications. A growing body of work has demonstrated that type III systems can be used to deliver foreign protein antigens, resulting in epitope presentation to the immune system, a vigorous immune response, and subsequent protection against challenge by the organism from which the foreign epitope was derived (6, 21, 35, 36). Other studies have shown that protein delivery via type III systems can be used to elicit cytotoxic effector and memory $CD8^+$ T-cell responses, resulting in the prevention of new tumor growth and regression of established tumors in mice (13, 28, 31). In addition, type III systems have been used in several applications where targeted protein delivery to eukaryotic cells reveals key information about how translocated bacterial effectors serve to alter host cell signaling pathways via a variety of effector/host protein interactions (1, 9, 32, 33, 38).

An improved ability to genetically engineer different kinds of bacteria with type III secretion systems will lead to advances that could have profound effects on our strategies to utilize these systems as beneficial molecular tools. In this study, we report the cloning of the entire *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system onto a plasmid vector that can be conveniently self-transferred to a range of gram-negative bacterial genera. A previous study has demonstrated the cloning of SPI-2, but this construct was on a narrow-host-range vector and was not self-transmissible, thus limiting its application for studies in an extended range of bacterial genera (17). We transferred SPI-2 to several different nonpathogenic or attenuated gamma- and alphaproteobacterial genera and assayed for SPI-2 expression (by monitoring SseB protein levels) after culture in different growth media. Altered expression of the *ssrAB* two-component regulatory genes on the cloned SPI-2 was used to obtain SseB expression under a nonpermissive condition, but this result was found to be bacterium specific. The results demonstrate that the cloned SPI-2 system is functional for secretion and can direct interactions with macrophage cells. We also demonstrate that the cloned SPI-2 sys-

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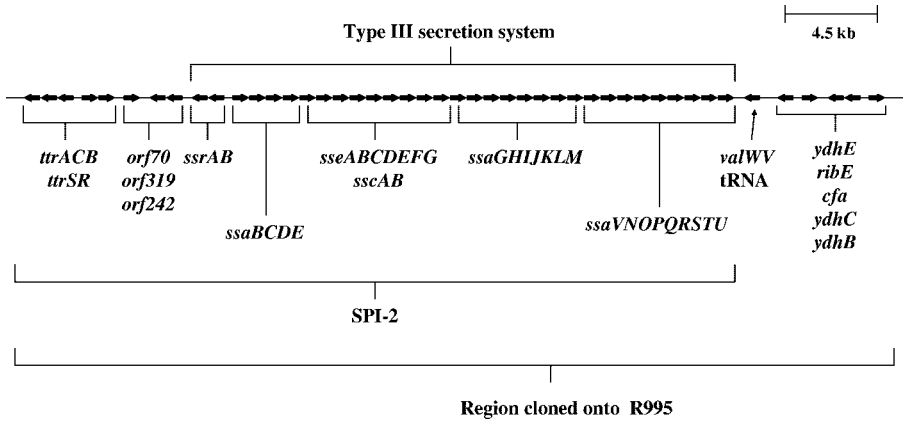

FIG. 1. *S. enterica* serovar Typhimurium SPI-2 region. A map of the *S. enterica* serovar Typhimurium SPI-2 region is depicted. The arrows indicate the genes of the region that have been cloned onto R995 using VEX-capture. The genes that comprise the SPI-2 genomic island and the type III secretion system within that island are also indicated.

tem can be used as a novel tool to analyze the predicted interaction of the SseB protein with eukaryotic cells. The results provide a foundation for future applications involving the transfer, expression, and function of SPI-2 and other secretion systems in an extended range of bacterial genera.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains used in this study are as follows: *Salmonella enterica* serovar Typhimurium χ3339 (16), χ3339 ΔSPI-2 (this study; contains a chloramphenicol marker at the site of the SPI-2 deletion), LT2 (25), and 14028 (ATCC 14028) (generously provided by Yakhya Dieye); *Salmonella enterica* serovar Typhi TY2 and ISP1820 (generously provided by Roy Curtiss III) (11, 20); *E. coli* TOP10 (Invitrogen, Carlsbad, CA) and MG1655 (5); *Salmonella bongori* SARC11 (generously provided by Michael McClelland) (34); *Pseudomonas putida* ATCC 12633 (40); *P. aeruginosa* PAK *pilA* and PAO1 *pilA* (generously provided by Michael Schurr); *Agrobacterium tumefaciens* A136 (40); and *Rhodobacter sphaeroides* 2.4.1 (42). Rifampin derivatives of the above strains were isolated and used as plasmid recipients in conjugative transfers. Bacterial cells were grown in magnesium minimal medium (MgM) (4) containing either 10 mM $MgCl_2$, pH 7.5 (MgM 10), or 8 μM $MgCl_2$, pH 5.0 (MgM 8), and Lennox broth (LB) (2, 24). The growth of strains in MgM 8 was generally not as robust as that in the other media used in this study. However, we found that initial growth in MgM 10 followed by a wash in MgM 8 and subsequent inoculation of the MgM 8 allowed improved growth under this condition in most cases. Antibiotics were used at the indicated concentrations (in μg per milliliter): rifampin, 75; streptomycin, 100; and spectinomycin, 125 or 250.

DNA methods. DNA manipulations and PCR were performed using standard protocols as described previously (2, 37). All plasmid DNA was isolated using QIAGEN columns as described by the manufacturer (QIAGEN, Valencia, CA).

Cloning and transfer of *S. enterica* serovar Typhimurium SPI-2 region. For cloning and transfer of the *S. enterica* serovar Typhimurium SPI-2 region, please refer to File S1 in the supplemental material.

Protein methods used for analysis of SseB protein expression and secretion. Analysis of SseB protein expression in strains containing cloned SPI-2 was performed as described previously via Western blotting and probing with rabbit polyclonal anti-SseB antisera (generously provided by Michael Hensel) (4, 41). Protein expression was tested with lysates from cultures inoculated from freshly streaked plates and grown for 16 to 18 h or inoculated by diluting an overnight culture and growing it for an additional 4 to 16 h. All the samples shown in the figures included here are from cultures grown under the former condition; however, similar results were obtained with either culture condition. Culture optical density measurement, Ponceau S staining for total protein, and control antibody probes were used in combination to obtain and verify equivalent sample loading for each strain as described previously (41). Samples from cultures that differed more than twofold in optical density (such cultures were usually slightly lower in density) were normalized for equal loading. In addition, a standard amount of SseB protein from the same batch of total cell lysate was routinely run on each gel as a standardization control for each Western blot assay. Preparation of secreted bacterial proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie blue staining, and Western blot transfers were performed using standard protocols as described previously (2, 27).

Construction of R995 + SPI-2::*plac-ssrAB* and R995 + SPI-2 *ssaV*. The plasmids R995 + SPI-2::*plac-ssrAB* and R995 + SPI-2 *ssaV* were constructed using the suicide plasmid pMAK705 via methods described previously (41). In R995 + SPI-2::*plac-ssrAB*, the suicide plasmid is integrated upstream of *ssrAB* on the cloned SPI-2 region (genome coordinates 1479612 to 1479987) so that the *lac* promoter on this plasmid is driving expression of the *ssrAB* genes. In R995 + SPI-2 *ssaV*, the suicide plasmid is integrated internally in the reading frame of the *ssaV* gene on the cloned SPI-2 region (genome coordinates 1494132 to 1494679) such that this gene is disrupted. The structures of each plasmid construct were verified by PCR analysis (data not shown).

Macrophage assays. The survival assay of bacterial strains in the murine macrophage cell line RAW264.7 (ATCC TIB-71) was performed as described previously using 2-h and 18-h time points and a multiplicity of infection of approximately 20 (26). The survival assays were replicated in four independent experiments using triplicate tissue culture sample wells in each experiment. Detection of SseB protein associated with J774 murine macrophages infected with bacterial strains containing SPI-2 was performed using methods described previously (39) except for the following modifications. To separate intracellular bacteria from the host cell fraction, infected J774 cells were lysed with 0.1% Triton X-100 and the lysate was centrifuged at 27,000 × *g* to remove intracellular bacteria (which remain intact during this treatment). The supernatant from this centrifugation was filtered with a 0.45-μm filter, precipitated with 10% trichloroacetic acid, and centrifuged at 12,000 × *g*, and the pellet was resuspended with phosphate-buffered saline and SDS-PAGE sample loading buffer. The J774 macrophages were infected in T-75 flasks with the indicated bacterial strains for 1.5 h at a multiplicity of infection of approximately 50. For this length of infection, all strains infected the macrophages to equivalent levels (data not shown and description of Fig. 7 in text). The results of this assay were replicated in three independent experiments.

Biosafety. All studies were performed in accordance with established biosafety guidelines and have been approved by the investigators' institutional biosafety committee. The cloned SPI-2 region was transferred to bacterial strains that are nonpathogenic or significantly attenuated for virulence.

RESULTS

Cloning of the *S. enterica* serovar Typhimurium SPI-2 type III secretion system. To obtain the type III secretion system encoded by *S. enterica* serovar Typhimurium SPI-2 as a single cloned fragment, we used the VEX-capture technique for the targeted excision and cloning of large bacterial chromosomal sections (40). The VEX-capture technique allows precise Cre/*lox*-mediated excision of the desired chromosomal fragment as a nonreplicating, covalently closed circular molecule and si-

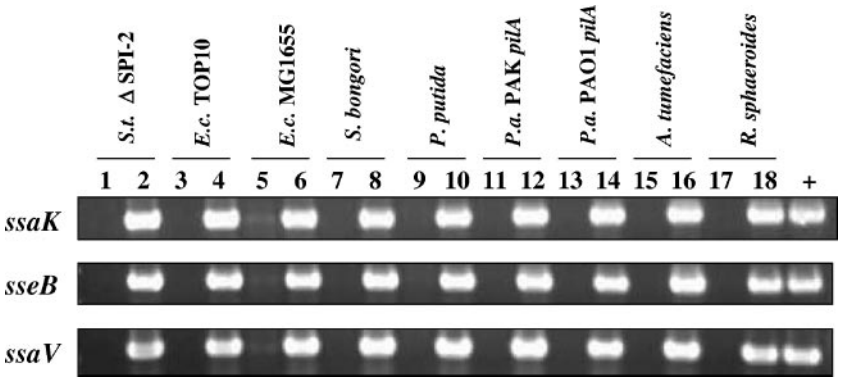

FIG. 2. Transfer of R995 + SPI-2 to different bacterial hosts as shown via PCR analysis. Plasmid DNA isolated from the indicated transconjugant strains containing either R995 or R995 + SPI-2 was used as a template in PCR analysis with primers hybridizing to portions of the indicated SPI-2 genes. The PCR products were run on DNA agarose gels and stained with ethidium bromide. Odd-numbered and even-numbered lanes indicate samples from strains containing R995 or R995 + SPI-2, respectively. The lane marked "+" indicates PCRs where chromosomal DNA from wild-type *S. enterica* serovar Typhimurium strain χ3339 was used as a template. Abbreviations: *S.t.*, *S. enterica* serovar Typhimurium; *E.c.*, *E. coli*; *P.a.*, *P. aeruginosa*.

multaneous cloning (or "capture") of the excised circular molecule by homologous recombination. The vector used to capture the excised chromosomal region was the self-transmissible, broad-host-range plasmid R995 (30) containing a DNA fragment from the *orf70-319-242* region of SPI-2 (18, 25). A map of *S. enterica* serovar Typhimurium SPI-2 and adjacent genes that are present in the R995 + SPI-2 clone is presented in Fig. 1. We transferred this plasmid to an *S. enterica* serovar Typhimurium strain containing a deletion of SPI-2 as well as to a range of other gram-negative proteobacterial genera, and we confirmed the presence of the cloned SPI-2 region in these strains via PCR analysis as shown in Fig. 2.

Analysis of SseB expression from R995 + SPI-2 in different bacterial genera. As a measure of SPI-2 gene expression from the R995 + SPI-2 construct, we probed Western blots of different bacterial cell lysates with antisera to the SseB protein (Fig. 3). SseB is a substrate of the SPI-2 type III secretion system that is essential for the translocation of other effector proteins (19, 27). We grew strains in two types of MgM that have previously been shown to be either noninducing (MgM 10) or inducing (MgM 8) for SPI-2 gene expression and secretion (4, 10). While the wild-type *S. enterica* serovar Typhimurium strain χ3339 exhibited the expected medium-specific regulation of SseB expression (i.e., expression only in MgM 8), the cloned SPI-2 island in the *S. enterica* serovar Typhimurium ΔSPI-2 background was expressed in both MgM 10 and MgM 8. The observed expression of SseB from cloned SPI-2 under the "noninducing" condition is likely due to the fact that multiple copies of the R995 + SPI-2 plasmid are present (about 10 copies per chromosome) (43). In the *E. coli* strains, the medium-specific expression of SseB from R995 + SPI-2 was found to be strain specific: SseB expression in TOP10 (R995 + SPI-2) was MgM specific while SseB in MG1655 (R995 + SPI-2) was expressed in both MgMs. In the other genera, SseB was expressed from R995 + SPI-2 at different levels depending on the strain, but the expression in each strain was observed to be similar in either MgM. Expression of SseB was undetectable under these conditions in the *P. putida* strain background.

The large majority of experiments involving in vitro expres-

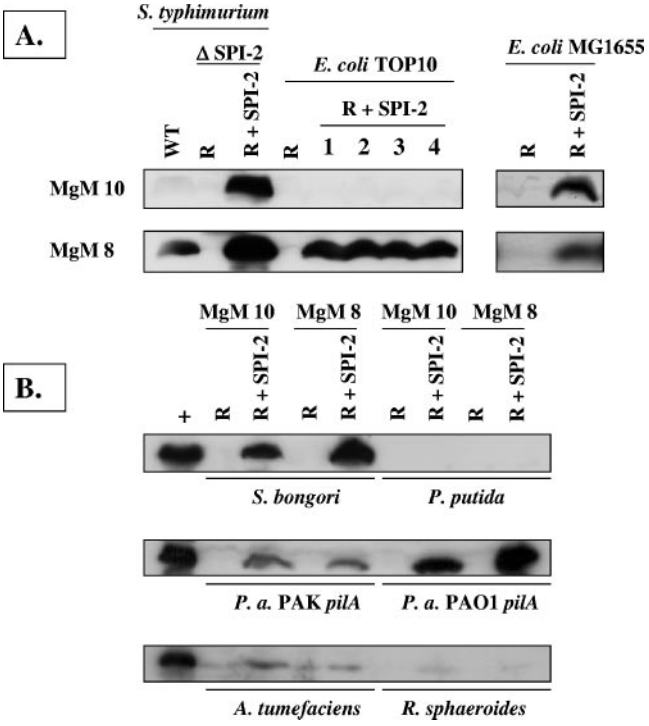

FIG. 3. Western blot analysis of SseB expression in different bacterial strains containing R995 + SPI-2 grown in MgM 10 and MgM 8. (A) Total cell lysates were obtained from *S. enterica* serovar Typhimurium and *E. coli* strains containing either R995 (R) or R995 + SPI-2 (R + SPI-2) grown in the indicated medium, and equivalent amounts of these lysates were analyzed via SDS-PAGE, Western blotting, and probing with anti-SseB antisera. Lanes numbered 1 to 4 indicate four separate isolates of the strain TOP10 (R + SPI-2). MgM 10 and MgM 8 indicate media used for SPI-2 noninducing and inducing conditions, respectively. WT, wild type. (B) An analysis identical to that described for panel A was performed for other gram-negative strains containing R995 (R) or R995 + SPI-2 (R + SPI-2). The lane marked "+" contains a sample from the same batch of lysate obtained from the strain *S. enterica* serovar Typhimurium ΔSPI-2 (R + SPI-2) as a positive control. *P.a.*, *P. aeruginosa*.

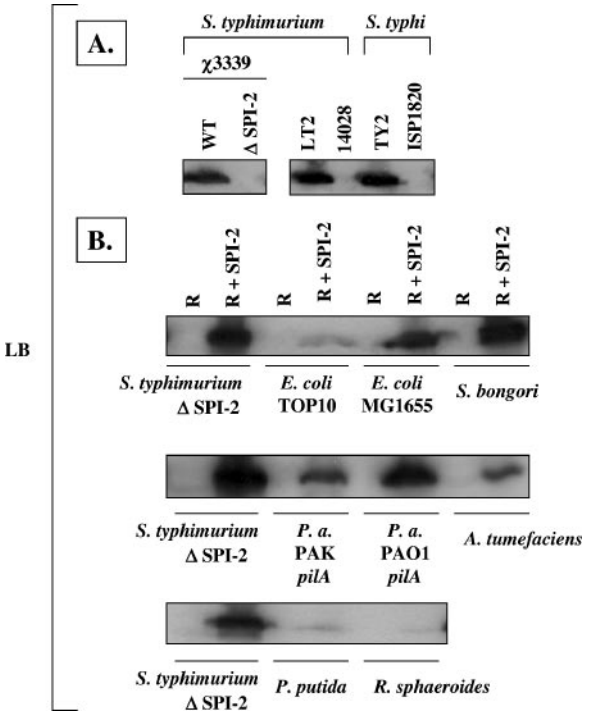

FIG. 4. Western blot analysis of SseB expression in different bacterial strains containing R995 + SPI-2 grown in LB medium. (A) Equivalent amounts of total cell lysates from the indicated strains grown in LB medium were analyzed via Western blotting and probing with anti-SseB antisera. WT, wild type. (B) Equivalent amounts of total cell lysates from the indicated strains containing R995 (R) or R995 + SPI-2 (R + SPI-2) grown in LB medium were analyzed as described for panel A. *P.a.*, *P. aeruginosa*.

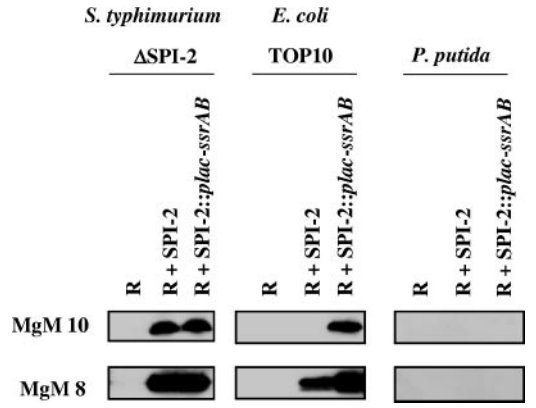

FIG. 5. Expression of SseB from the R995 + SPI-2::*plac-ssrAB* construct. Strains containing the plasmids R995 (R), R995 + SPI-2 (R + SPI-2), and R995 + SPI-2::*plac-ssrAB* (R + SPI-2::*plac-ssrAB*) were grown in the indicated media and assayed for SseB protein expression via Western blotting of equivalent amounts of cell lysate. Please refer to the text for additional details.

sion of SPI-2 genes have involved the use of media containing specific nutritional and environmental signals to induce SPI-2 gene expression, including a shift to low pH, low magnesium levels, and phosphate limitation (10, 23). We examined whether the SPI-2 protein SseB is expressed in a rich medium (LB) since bacterial growth in this medium is more robust and it can be used to grow a broader range of bacterial species than the other specialized minimal media frequently used in previous studies. We found that expression of SseB in the *S. enterica* serovar Typhimurium strains χ3339 and LT2 was robust and easily detectable (Fig. 4). However, this observation was found to be strain specific, as the *S. enterica* serovar Typhimurium strain 14028 did not express SseB under the same conditions. We also examined SseB expression in LB medium for two *S. enterica* serovar Typhi strains, TY2 and ISP1820. We found a strain-specific expression pattern in this species as well, as TY2 expressed SseB but ISP1820 did not under these conditions. These results indicate that LB medium can be used to obtain significant expression of chromosomally encoded SPI-2 SseB in strains of *S. enterica* serovar Typhimurium and *S. enterica* serovar Typhi. The expression of SseB from cloned SPI-2 in LB medium was readily observed in most of the bacterial host genera and generally followed a strain-specific pattern similar to that found in the MgM (Fig. 4). The low expression of SseB in LB in the bacterial hosts *P. putida* and *R. sphaeroides* was consistent with the low SseB expression in MgM in these species. Taken together, the results indicate that the cloned SPI-2 can be successfully established in a range of bacterial genera and SseB protein expression can be detected in most of these strains in a range of growth media.

The *ssrAB* genes located in SPI-2 encode a two-component regulatory system that is essential for expression of several SPI-2 genes including *sseB* (4). In strains containing cloned SPI-2 where SseB expression is low or undetectable, manipulation of *ssrAB* expression could allow increased SseB (and likely other SPI-2 gene) expression levels to be obtained. To test this hypothesis, we inserted a heterologous promoter upstream of the *ssrAB* genes on the cloned SPI-2 (R995 + SPI-2::*plac-ssrAB*) and tested SseB expression from this construct in three different bacterial genera (Fig. 5). In these strains, this promoter (*plac*) is active due to low or absent LacI repressor activity. Since SseB expression from cloned SPI-2 in *S. enterica* serovar Typhimurium ΔSPI-2 was already at significant levels in all media tested, the *plac-ssrAB* construct had little effect on SseB expression in this strain. However, in the *E. coli* strain TOP10, the R995 + SPI-2::*plac-ssrAB* plasmid produced detectable SseB expression under conditions where the R995 + SPI-2 plasmid did not. Under the same conditions in *P. putida*, R995 + SPI-2::*plac-ssrAB* did not allow detectable SseB expression to be observed. These results indicate that the R995 + SPI-2::*plac-ssrAB* construct can allow SseB expression under certain "nonpermissive" conditions but that this result is strain specific.

Protein secretion via the cloned SPI-2 type III system. To demonstrate that the cloned SPI-2 type III secretion system is functional for protein secretion, we made protein preparations from culture supernatants harvested from strains containing cloned SPI-2. The R995 + SPI-2 plasmid complemented the *S. enterica* serovar Typhimurium ΔSPI-2 strain for secretion as shown using Coomassie blue staining and Western blot analysis (Fig. 6). We constructed a mutation in the *ssaV* gene in the cloned SPI-2 region (resulting in plasmid R995 + SPI-2 *ssaV*) to demonstrate that the observed secretion was dependent on the activity of the type III secretion system. The *ssaV* gene encodes an essential component of the SPI-2 secretion apparatus, and the secretion of SseB has been previously shown to be dependent on a

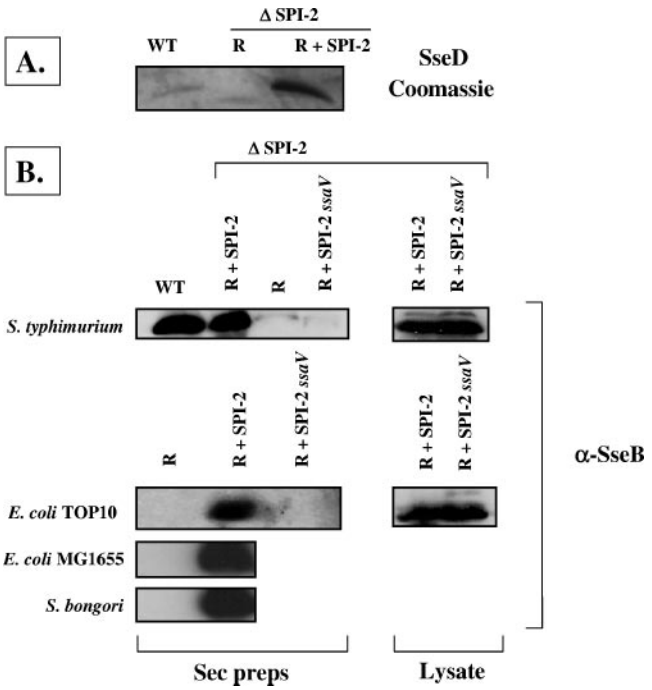

FIG. 6. Protein secretion via the cloned SPI-2 type III secretion system. Preparations of secreted proteins were obtained from culture supernatants of the indicated strains containing either R995 (R), R995 + SPI-2 (R + SPI-2), or R995 + SPI-2 *ssaV* (R + SPI-2 *ssaV*) grown in MgM 8. (A) Coomassie blue staining of secretion preparations obtained from the indicated *S. enterica* serovar Typhimurium strains and run on an SDS-polyacrylamide gel. In this assay, the SPI-2-encoded SseD protein is detected as a prominent band running at approximately 20 kDa. (B) Samples of secreted proteins from the indicated strains were Western blotted and probed with anti-SseB antisera. Samples from secretion preparations ("Sec preps") and cell lysates are indicated. WT, wild type.

functional *ssaV* gene (4). Accordingly, secretion of SseB from strains containing the R995 + SPI-2 *ssaV* construct was not observed. We tested SseB secretion from *E. coli* TOP10, *E. coli* MG1655, and *S. bongori* SARC11 containing cloned SPI-2 and observed secretion of SseB from these strains. This indicates that R995 + SPI-2 is functional for secretion in bacterial backgrounds other than *S. enterica* serovar Typhimurium.

Use of R995 + SPI-2 to mediate interactions with macrophages. One of the phenotypes associated with the SPI-2 secretion system is its role in the survival of *S. enterica* serovar Typhimurium in macrophages after entry into these cells (22, 29, 39). Since mutants in SPI-2 genes have been shown to be defective in macrophage intracellular survival, we tested whether the R995 + SPI-2 construct could complement this phenotype in the *S. enterica* serovar Typhimurium ΔSPI-2 background (Fig. 7). However, we found that the presence of cloned SPI-2 in this strain had the opposite effect: the survival of the *S. enterica* serovar Typhimurium ΔSPI-2 (R995 + SPI-2) strain was much less than that of both the wild-type and ΔSPI-2 strains. The *S. enterica* serovar Typhimurium ΔSPI-2 (R995 + SPI-2 *ssaV*) strain survived at a level equal to that of the ΔSPI-2 (R995) strain, thus indicating that it is likely that overactivity of the cloned SPI-2 secretion system is causing the enhanced survival defect of the *S. enterica* serovar Typhimurium ΔSPI-2 (R995 + SPI-2) strain. These strains all infected the macrophages at almost equal levels at a 2-h time point (the survival defect of the R995 + SPI-2 strain was at 18 h, per Fig. 7), indicating that decreased initial entry into the host cells was not the cause of the enhanced survival defect (data not shown). When *E. coli* strains TOP10 and MG1655 containing cloned SPI-2 were tested in the same assay, only very slight differences were observed between SPI-2-containing strains and the R995-only controls. Also, these strains were much less able to survive in macrophages than was wild-type *S. enterica* serovar Typhimurium. Both of these observations with the *E. coli* strains are most likely due to the fact that other substrates which are secreted by the SPI-2 system and which are encoded elsewhere on the *S. enterica* serovar Typhimurium chromosome are absent in these backgrounds. The severe survival defect observed in the *S. enterica* serovar Typhimurium ΔSPI-2 (R995 + SPI-2) strain could be potentially useful in cases where clearance of a SPI-2-containing strain is desirable after it has performed its desired function. Consequently, this phenotype is being further characterized in our laboratory for future applications.

To further characterize the ability of cloned SPI-2 to direct bacterial cell/host cell interactions, we tested for an association between the SseB protein and host cell macrophages. The SseB protein has been shown to be localized to the surface of *S. enterica* serovar Typhimurium cells and to function as a translocator protein that acts to allow movement of SPI-2 substrates from the bacterial cell to eukaryotic host cells (4, 27). This activity of the SseB protein predicts that SseB would interact with eukaryotic cells and possibly be found in the eukaryotic host cell fraction of an infection assay. After infection of J774 macrophage cells with strains containing cloned SPI-2 (and appropriate control strains), bacterium-free J774 total host cell fractions were obtained, Western blotted, and probed for the presence of the SseB protein (Fig. 8). Although detection of SseB association with the host cell fraction from the wild-type strain was somewhat variable and weak (Fig. 8 and data not shown), we readily detected SseB in the host cell fraction from infections using cloned SPI-2 in strains *S. enterica* serovar Typhimurium ΔSPI-2 and *E. coli* MG1655. This result was found to be dependent on the normal function of the cloned SPI-2 type III system since SseB was not detected in the host cell fraction from infections with *S. enterica* serovar Typhimurium ΔSPI-2 (R995 + SPI-2 *ssaV*). As an additional control, we incubated SseB protein from an amount of bacterial cells equivalent to the inoculum used in the infection asssays (approximately 5×10^8 cells) with the J774 cells. We did not observe SseB to be present in the host cell fractions in this control assay, indicating that the extensive washing of cells postinfection was sufficient to remove any extracellular SseB that may be present during the assays. Since our assay does not discriminate between host cell membrane and cytosolic fractions, the host cell-associated SseB could be targeted to either compartment in the host cells.

Taken together, the above results indicate that the cloned SPI-2 type III secretion system can function to direct interactions with host cells.

DISCUSSION

The ability to obtain entire type III secretion systems as single cloned DNA fragments that could be moved between

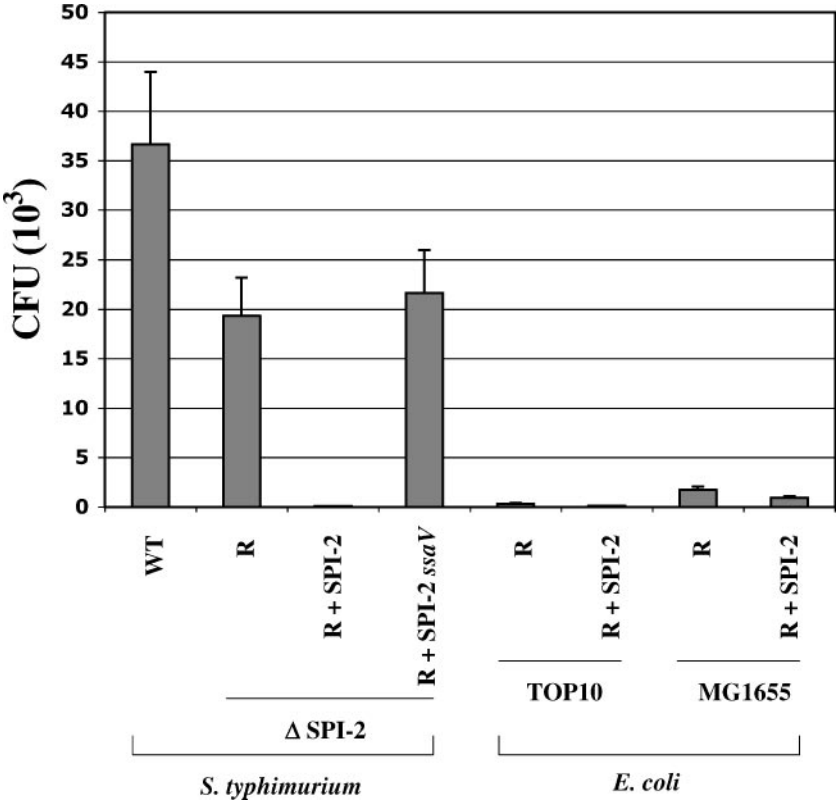

FIG. 7. Survival of strains containing the cloned SPI-2 region in RAW264.7 macrophages. Equivalent numbers of cells from cultures of the indicated bacterial strains containing either R995 (R), R995 + SPI-2 (R + SPI-2), or R995 + SPI-2 *ssaV* (R + SPI-2 *ssaV*) were incubated with RAW264.7 macrophages for 1 h. At this point, the infection medium was changed to contain gentamicin, and the infected-cell cultures were incubated for an additional 17 h (18-h total infection). Intracellular bacteria were obtained at this time and enumerated via plating for CFU. The results were replicated in four independent trials with each trial being performed in triplicate sample wells. WT, wild type.

bacterial cells would greatly enhance our ability to genetically engineer different bacteria for certain beneficial purposes. In this study, we report the cloning of the entire SPI-2 type III secretion system as a single DNA fragment contained on a plasmid vector that can be conveniently transferred to and established in a range of gram-negative proteobacteria. We demonstrate that the SPI-2 protein SseB is detectably expressed from the R995 + SPI-2 construct in strains of *S. enterica* serovar Typhimurium, *S. bongori*, *E. coli*, *P. aeruginosa*, *P. putida*, *A. tumefaciens*, and *R. sphaeroides*. However, SseB expression in the *P. putida* and *R. sphaeroides* backgrounds ranges from very low to undetectable. We attempted to obtain elevated SseB expression in the *P. putida* background by manipulating the expression of the SPI-2 *ssrAB* regulator genes,

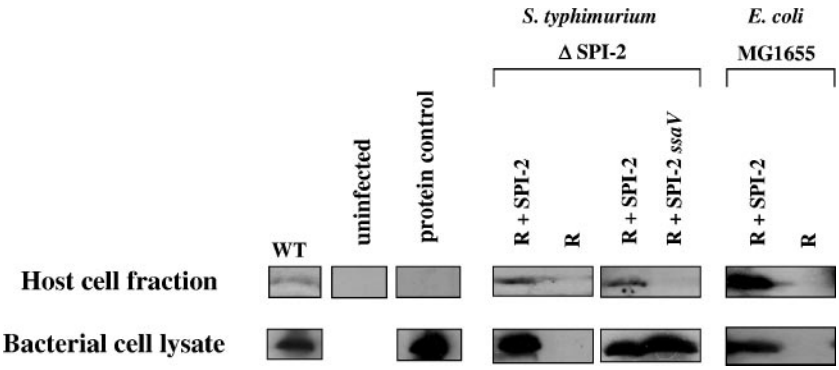

FIG. 8. Association of SseB with host macrophage cells. The eukaryotic host cell protein fraction from infections of J774 macrophages with the indicated bacterial strains was obtained as described in Materials and Methods and analyzed for the presence of SseB via Western blotting and probing with anti-SseB antisera. Samples of cell lysate from cultures of the bacterial strains used in the infections are also indicated. The protein control was performed by adding SseB protein from a cell lysate (equivalent to the number of bacterial cells used in the infection) to demonstrate that any extracellular SseB is washed away before macrophage lysis. WT, wild type.

but this did not result in increased SseB expression in this strain. However, this approach did result in detectable SseB expression under a "noninducing" condition in the strain *E. coli* TOP10. Further study will be required to determine if low levels of SseB expression in a particular strain are due to defects at the transcriptional, posttranscriptional, translational, or posttranslational levels. Interestingly, our laboratory has previously cloned the *S. enterica* serovar Typhimurium SPI-1 type III secretion system in a similar fashion onto the R995 vector, but we could not detect SPI-1 protein expression in strains other than those of *S. enterica* serovar Typhimurium upon its transfer to and establishment in other gram-negative genera (41). This difference in results between the cloned SPI-1 and SPI-2 systems highlights two important points: (i) the different evolution of the two type III systems such that SPI-2 appears to be able to be expressed and to function outside of the host bacterial species of origin while SPI-1 appears to have barriers that prevent this under the conditions that we have examined, and (ii) the potential advantage in obtaining and studying clones of different type III systems, since the activities and specificities of these different systems may vary, and this variability may provide flexibility such that a given type III system could be targeted for a specific application or environmental condition.

Our studies demonstrate that the cloned SPI-2 region is functional to secrete cognate substrates and direct interactions between bacterial strains containing SPI-2 and eukaryotic host cells. However, we observed an enhanced survival defect in macrophages with the strain *S. enterica* serovar Typhimurium ΔSPI-2 (R995 + SPI-2) that is dependent on the function of the cloned type III system. The most likely reason for this result is that the cloned SPI-2 system in the *S. enterica* serovar Typhimurium background is secreting and/or translocating substrates in an unregulated manner that is detrimental to the survival of this strain inside the macrophages. This observation may indicate a potentially beneficial phenomenon that can be used in particular applications where clearance of a strain containing SPI-2 can be obtained after the strain has performed its desired function. Regulating SPI-2 (or its substrate proteins) to function at different levels such that a desired result can be obtained at one level of activity and strain clearance can be obtained at another activity level could be very useful in vaccine delivery and other such applications. Further study will be needed to determine the specific SPI-2 substrates or specific aspects of SPI-2 type III secretion activity that are involved in causing the enhanced survival defect. We also demonstrate that the R995 + SPI-2 construct can be used to study interactions between SseB (and potentially other SPI-2 translocation proteins) and eukaryotic host cells. To our knowledge, this study provides the first results indicating that SseB does indeed associate with host cells as predicted by previous studies that document its activity as a translocation protein (4, 27).

The use of type III secretion systems to deliver antigens for vaccines is currently being developed, and recent results indicate much promise in utilizing type III systems for this purpose (6, 21, 35, 36). The potential to use cloned type III secretion systems to design particular bacterial vaccine delivery strains in this regard is significant. First, epitopes can be specifically designed and delivered by the type III system to induce a desired immune response and obtain potential protection against the source organism of the epitope. Second, since the design of polyvalent vaccines is frequently advantageous, the ability to use cloned type III systems and cognate substrates provides the potential for a range of different epitopes to be expressed from a single bacterial strain carrying the type III system. For example, a bacterial background strain that expresses endogenous antigens from its chromosome (either against the strain itself or another organism) could carry a cloned type III system that expresses a different set of antigen epitopes. The different set of epitopes could be directed toward the background organism itself (to provide a more potent polyvalent response against this strain) or toward a heterologous pathogenic organism. In the latter case, protection could be obtained against multiple organisms with a single vaccine. However, since the plasmid constructs described here are self-transmissible, the likelihood of their use in approved vaccines is low, though their convenient use for research studies in this area has much potential. Future studies aimed at permanently knocking out the R995 transfer system upon establishment of the cloned type III system in a given bacterial strain would help to solve this problem. Alternatively, a method to irreversibly integrate a cloned type III system into a given host chromosome can be developed to circumvent this issue as well.

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