

Type III secretion systems and the evolution of mutualistic endosymbiosis

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The view that parasites can develop cooperative symbiotic relationships with their hosts is both appealing and widely held; however, there is no molecular genetic evidence of such a transition. Here we demonstrate that a mutualistic bacterial endosymbiont of grain weevils maintains and expresses *inv/spa* genes encoding a type III secretion system homologous to that used for invasion by bacterial pathogens. Phylogenetic analyses indicate that *inv/spa* genes were present in presymbiotic ancestors of the weevil endosymbionts, occurring at least 50 million years ago. The function of *inv/spa* genes in maintaining symbiosis is demonstrated by the up-regulation of their expression under both *in vivo* and *in vitro* conditions that coincide with host cell invasion.

Many bacterial lineages have evolved close associations with eukaryotic hosts, with effects ranging from invasive parasitism to obligate mutualism. In many of these associations, bacteria must enter and replicate within host cells. The principal mechanism by which Gram-negative bacterial pathogens invade eukaryotic cells involves the deployment of a type III secretion system (TTSS) to deliver effector proteins that assist in host cell entry (1). Gene clusters encoding TTSSs, often situated within pathogenicity islands, have been acquired by a diverse array of horizontally transmitted plant and animal pathogens and symbionts, including species of *Shigella*, *Salmonella*, *Yersinia*, *Pseudomonas*, *Xanthomonas*, *Erwinia*, and *Rhizobium* (2). Recently, the insect endosymbiont, *Sodalis glossinidius*, was also shown to depend on a TTSS for invasion of host cells (3). These symbionts contain homologs of *inv/spa* genes encoding the *Salmonella/Shigella* TTSS, and mutants lacking a key component of type III secretion (*invC*) are known to be neither invasive *in vitro* nor symbiotic in their natural tsetse fly host.

As a vertically transmitted symbiont, *S. glossinidius* is not expected to greatly reduce the fitness of its host. Nonetheless, such “secondary” endosymbionts of insects resemble pathogens in that they confer no known benefits on hosts, are not essential for host growth or reproduction, invade a diverse range of host tissues, and undergo occasional horizontal transmission (4, 5). On the basis of 16S ribosomal RNA phylogenies, the closest known relatives of *S. glossinidius* are the primary endosymbionts of grain weevils (genus *Sitophilus*; Insecta: Coleoptera: Curculionidae; ref. 6). “Primary” endosymbionts differ from “secondary” symbionts in several respects: they are essential for host survival, reside only within specialized host organs (bacteriomes), and are strictly vertically transmitted. Primary symbionts show stereotyped patterns of replication and migration within specific host tissues; this movement results in their incorporation into bacteriocytes and developing eggs. On the basis of microscopic observations, these patterns have been interpreted as reflecting host-based mechanisms governing movement of the bacterial cells (5).

In the case of the primary symbionts of *Sitophilus* spp., eggs are infected before they are laid. The inoculating bacteria multiply in the developing embryo and are ultimately localized within a bacteriome at the foregut periphery that persists throughout the larval stages (5, 7). At metamorphosis, the larval bacteriome disappears, and the symbionts must migrate and infect a new

bacteriome associated with the hindgut outer lining in the adult weevil (5). Experimental reduction and removal of bacteria have confirmed that they are required for normal growth, development, and reproduction in *Sitophilus* (8). The endosymbionts of *Sitophilus* have not yet been cultivated *in vitro* and currently have no official nomenclature. In the present study, we have focused on the *Sitophilus zeamais* primary endosymbiont (SZPE).

It has been proposed that parasites can evolve into mutualists after the establishment of a vertical mode of transmission, concomitant with the attenuation of parasite virulence (9–12). To date, there is little empirical evidence supporting this evolutionary transition. In particular, if obligate “primary” symbionts evolve from pathogens, we might expect continuity in the molecular basis of the infection mechanism, despite changes in how the association affects the host, the loss of horizontal transmission, and the inability to infect many host cell types. Until now, there have been no reported examples of vertically transmitted mutualistic symbionts that use pathogenicity determinants to facilitate their associations with hosts. We searched for TTSS-encoding genes that might enable SZPE to invade the cells of the host bacteriome (bacteriocytes), as required during the insect life cycle.

SZPE and *S. glossinidius* provide an excellent model for studying the evolutionary transition to mutualism, because the divergence of these two lineages from a shared ancestor has produced different symbiotic outcomes. In this study, we show that SZPE, a weevil endosymbiont, harbors TTSS-encoding genes closely related to those found in *S. glossinidius*, and that expression of these genes coincides with the timing of bacteriome infection within the developing weevil. We then discuss the implications of these findings for the evolution of mutualistic associations involving bacteria and animals.

Materials and Methods

PCR Amplification and Sequence Determination. *S. glossinidius* was grown in liquid culture, as described previously (4). *Sitophilus zeamais* weevils, the host of SZPE, were maintained in maize at 27.5°C and 70% humidity. Bacteriomes (containing SZPE) were dissected from fifth-instar *S. zeamais* larvae, and DNA was prepared from cultured *S. glossinidius* and *S. zeamais* bacteriomes with the DNeasy tissue kit (Qiagen, Valencia, CA). To determine whether SZPE harbors *inv/spa* homologs, we designed degenerate oligonucleotide primers based on alignments of the *inv/spa* genes from *S. glossinidius*, *Salmonella enterica*, and *Shigella flexneri*. PCR primers used for the amplification of *invA* and *rplB* were synthesized with inosine (I) as follows (listed 5' to 3'): *invA* (1,020-bp product) ATG CCI GGI AAR CAR ATG and TIG TYT CYT GDT TIC CRA A; *rplB* (665 bp) AAC CCT GAR YTI CAY AAR GGI AA and TTA CCY TTI GTY TGI ACI CCC CA. An *invA-spaM* fragment, a *spaP-spaR* fragment,

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Abbreviations: TTSS, type III secretion system; SZPE, *S. zeamais* primary endosymbiont.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF426456–AF426460 and AF467290).

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Table 1. Pairwise distances between InvA amino acid sequences

	Sz	Se	Sf	Yp	Ea	Ps	Xc	Rs	Bb	Cp	Rh
Sg	7.1	45.8	48.6	43.8	63.1	63.5	62.5	64.0	59.5	62.3	63.1
Sz		53.4	53.9	51.8	68.8	69.4	68.0	71.3	66.2	67.5	66.9
Se			34.9	47.0	61.1	60.6	61.5	64.9	55.6	62.4	60.8
Sf				48.5	63.3	62.0	62.2	65.8	59.5	60.5	62.6
Yp					64.7	64.8	62.2	64.8	60.8	62.5	64.1
Ea						35.8	62.4	62.9	57.3	63.2	63.9
Ps							61.8	62.5	56.8	65.1	64.8
Xc								33.5	53.1	60.1	59.7
Rs									54.1	60.4	62.1
Bb										52.6	55.6
Cp											61.2

Sg, *S. glossinidius*; Sz, *SZPE*; Se, *S. enterica*; Sf, *S. flexneri*; Yp, *Yersinia pestis*; Ea, *E. amylovora*; Ps, *P. syringae*; Xc, *X. campestris*; Rs, *R. solanacearum*; Bb, *B. bronchiseptica*; Cp, *C. pneumoniae*; Rh, *Rhizobium* spp. NGR 234.

and a *fusA* fragment were amplified with the following primers (listed 5' to 3'): *invA-spaM* (3,323 bp) GTC AAC TGT ACG GTG CGC TTG and CCG CGT AAA ACC CTG CTG TTT CC; *spaPQR* (614 bp) ATG ATG ATG ATG AGC CCG and AGC CCA TGC ATA ACC CAA AA; *fusA* (760 bp) CAT CGG CAT CAT GGC NCA YAT HGA and CAG CAT CGG CTG CAY NCC YTT RTT. PCR reactions contained 50–100 ng of template DNA, 25 pmol of each primer, and 2.5 units of *Taq*DNA polymerase (Promega) in a final reaction volume of 50 μ l with 3.5 mM MgCl₂. Reactions proceeded with an initial denaturation step (5 min at 95°C) followed by 40 cycles of denaturation (1 min at 95°C), annealing (1 min at 50°C), and extension (1 min/kb at 72°C), followed by a final extension (5 min at 72°C) to promote A-tailing. PCR products were gel-purified, extracted with a Qiaquick PCR purification kit (Qiagen), and cloned into pGEM T-easy vector (Promega) or pTOPO-XL (Invitrogen), according to manufacturers' instructions. For each PCR product, at least six clones were sequenced in both directions to establish error-free consensus sequences.

Phylogenetic Analysis. DNA sequences of *invA* were translated and the inferred amino acid sequences aligned by using PILEUP (Genetics Computer Group package, Madison, WI). Phylogenetic trees were obtained by both maximum parsimony and distance methods, as implemented in PAUP*4.0 (13). Similarly, 16S rDNA sequences were aligned with PILEUP, and tree topologies were ascertained with PAUP. The uncorrected pairwise distances in Table 1 were computed by using the GCG package (Genetics Computer Group).

Estimation of Nucleotide Sequence Divergence. Sequence divergence at synonymous and nonsynonymous sites was computed by using the method of Li (14), implemented in the GCG package (Genetics Computer Group). Analyses were performed on a 1,020-bp fragment of *invA*, a 760-bp fragment of *fusA*, a 665-bp fragment of *rplB*, and the complete coding sequences of *invB* (408 bp), *invC* (1,311 bp), and *spaQ* (261 bp). Note that *Escherichia coli* does not harbor *inv/spa* homologs, thus precluding pairwise comparisons for these genes.

In Vivo Gene Expression Assays. To analyze *inv/spa* gene expression *in vivo*, *S. zeamais* development was synchronized by allowing 250 adults to mate and oviposit overnight in maize at 27.5°C and 70% relative humidity. Progeny were extracted from maize grains at 3-day intervals from day 6 after oviposition through day 33. After each collection, insects were dissected on ice in saline (0.85% wt/vol), and bacteriomes were removed and frozen in a dry ice/ethanol bath. RNA was prepared with the RNeasy minikit (Qiagen) with on-column DNase treatment. Relative

transcript levels were obtained by real-time quantitative RT-PCR in a LightCycler (Roche Molecular Biochemicals). Reactions consisted of 10 ng of RNA, 0.3 μ M of primers, and 2.75 mM (for *invA* only) or 3.25 mM Mn(OAc)₂ in 1 \times Hot Start LightCycler-RNA Master SYBR Green I (Roche Molecular Biochemicals). The following primers (listed 5' to 3') were designed to amplify 220 bp of *invA* (TCA AGA AAC GAC GTG AAG TAC and ACA AGT GGG TAT AAA CGG TAA G), 280 bp of *spaPQ* (AAT TTG TTG TTT GCC GGT AAC and AGA GGC GAG ATC ATC AGT CAG) and 220 bp of *fusA* (GTC CAT TTT GTT TAC GAA CGC and CAT CAA TAT CAT CGA CAC CCC). RT-PCR cycling parameters followed the manufacturer's recommendations, with a 10-sec annealing step at 57°C for all reactions. The integrity of reactions was confirmed by analyzing reaction product melting curves; no nonspecific products were observed. All RNA samples were free of DNA contamination, tested in standard LightCycler PCR reactions with no reverse transcription step. Relative expression levels of each gene were obtained through comparisons to external standard curves generated for each primer set with serial dilutions of template RNA.

In Vitro Gene Expression Assays. To analyze *inv/spa* gene expression *in vitro*, bacteriomes were removed from 14-day-old *S. zeamais* larvae under sterile conditions and homogenized in 0.85% (wt/vol) saline by using a Dounce tissue homogenizer (Kontes). The homogenate was filtered through a 5- μ m poly(vinylidene difluoride) membrane, and the filtrate was centrifuged (6,000 \times g, 10 min, 25°C) to pellet bacterial symbionts. Symbionts were purified away from cellular debris by three successive washes in sterile 0.85% (wt/vol) saline and equilibrated for assay by three additional washes in minimal medium (20 mM NaHPO₄/0.1% casamino acids/25 mM glucose). Bacteria were resuspended in minimal medium at a concentration of 10⁷ cells/ml. The bacterial suspension was combined with solutions of minimal medium containing either 2 mM CaCl₂, 2 mM MgCl₂, or 2 mM CuCl₂ to provide a range of samples of equal volume and cell densities having metal cation concentrations ranging from 20 μ M to 1 mM. Bacterial suspensions were then maintained under uniform conditions at 15 and 27.5°C for 15 h. After incubation, an aliquot of each suspension was plated on LB agar to ensure that samples were free of contamination. After pelleting bacteria from each culture, RNA was extracted by using the RNeasy minikit (Qiagen). Relative transcript levels of *invA*, *spaPQ*, and *fusA* were determined by LightCycler RT-PCR, as described above.

Results

Cloning and Sequencing of SZPE *inv/spa* Homologues. By using degenerate PCR primers, we amplified and sequenced fragments

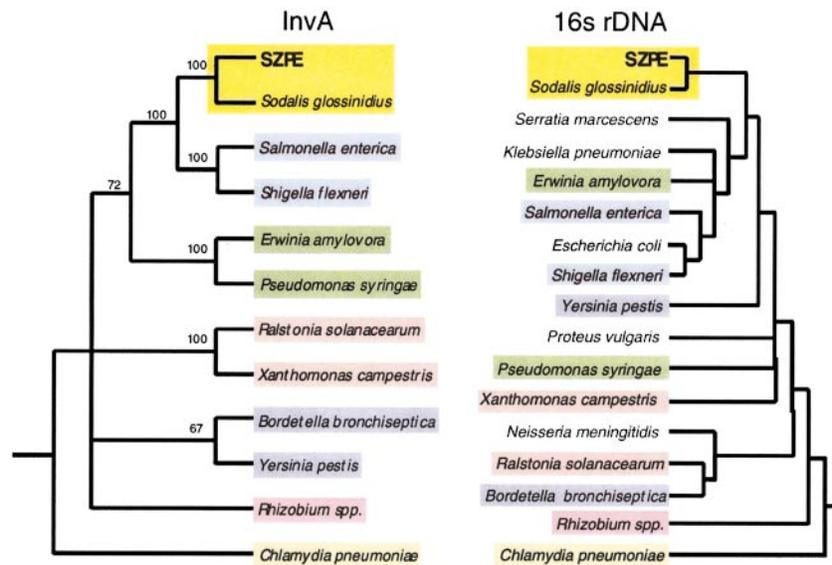


Fig. 1. Phylogenetic trees based on the *InvA* and 16S rDNA sequences from SZPE, *S. glossinidius*, and selected plant and animal pathogens. For the *InvA* tree, only clades supported by bootstrap values greater than 60% are shown as resolved. Accession numbers of *InvA* sequences are as follows: SZPE (AF467290), *S. glossinidius* (AF306649), *Yersinia pestis* (NC001972), *S. enterica* (U43239), *S. flexneri* (NC002698), *P. syringae* (AAG33877), *Ralstonia solanacearum* (P35656), *Chlamydia pneumoniae* (NC000922), *Erwinia amylovora* (X99768), *Rhizobium* sp. NGR234 (P55726), *Xanthomonas campestris* (P80150). The *invA* homolog from *Bordetella bronchiseptica* was obtained from a dataset produced by the *Bordetella bronchiseptica* Sequencing Group at the Sanger Center, and can be obtained from <ftp://ftp.sanger.ac.uk/pub/pathogens/bb/>.

of appropriate lengths from template DNA extracted from the bacteriomes of *S. zeamais*. The SZPE *inv/spa* sequences are 86.5, 81.9, 73.8, and 83.7% identical to *invA*, *invB*, *invC*, and *spaPQR* of *S. glossinidius*, respectively (3), and have intact ORFs with no mutations resulting in frameshifts or stop codons. The conceptual translations of *invA* and *invC* contain conserved functional protein domains corresponding to an inner cytoplasmic membrane component (*invA*) and a cytoplasmic ATP synthase nucleotide-binding domain (*invC*). The base compositions of the *inv/spa* genes (G+C: *invA*, 47.7%; *invB*, 45.1%; *invC*, 54.6%; *spaPQR*, 54.9%) are in agreement with the estimated genomic base composition of the primary endosymbiont of rice weevils (6) and are almost identical to those reported for the *inv/spa* genes of *S. glossinidius* (3).

Phylogenetic Analysis. We determined the ancestry of the SZPE *inv/spa* genes through phylogenetic reconstructions with homologs from *S. glossinidius* and several plant and animal pathogens. For comparison, we also constructed a tree based on the sequences of 16S rDNA from the same bacterial species. Results are shown for

the phylogeny generated by parsimony (Fig. 1, Table 1), with bootstrap support based on 1,000 replicates, and identical tree topologies were obtained by using maximum parsimony and distance methods. In both the 16S rDNA and *InvA* trees, the SZPE and *S. glossinidius* sequences form a single robust clade distinct from the enteric pathogens, indicative of a single acquisition of the *invA* homologs in the common ancestor of these symbionts.

Nucleotide Sequence Divergence Estimates. Because our phylogenetic analyses supported the presence of *invA* in the common ancestor to SZPE and *S. glossinidius*, the *inv/spa* gene cluster should follow the same evolutionary history as other genes ancestral to these species. To test this hypothesis, we compared the extent of sequence divergence of several *inv/spa* genes to that of two informational genes, *fusA* and *rplB*. Informational genes that are involved in transcription, translation, and related processes provide a robust standard for evolutionary comparisons, because these genes are rarely subject to horizontal transfer (15, 16).

For all genes, frequencies of both synonymous and nonsynonymous substitutions (K_s and K_a , respectively) are lower for *S.*

Table 2. Divergence among *E. coli*, *Salmonella*, *Sodalis*, and SZPE homologs

	Synonymous substitutions per site, K_s					
	<i>invA</i>	<i>invB</i>	<i>invC</i>	<i>spaQ</i>	<i>fusA</i>	<i>rplB</i>
SZPE– <i>S. glossinidius</i>	0.57	0.70	0.87	0.58	0.19	0.13
SZPE– <i>S. enterica</i>	>2	>2	>2	>2	0.66	0.57
<i>S. glossinidius</i> – <i>S. enterica</i>	>2	>2	>2	>2	0.70	0.61
<i>S. enterica</i> – <i>E. coli</i>	—	—	—	—	0.19	0.11
	Nonsynonymous substitutions per site, K_a					
	<i>invA</i>	<i>invB</i>	<i>invC</i>	<i>spaQ</i>	<i>fusA</i>	<i>rplB</i>
SZPE– <i>S. glossinidius</i>	0.04	0.09	0.21	0.04	0.002	0.002
SZPE– <i>S. enterica</i>	0.53	0.90	0.46	0.24	0.070	0.047
<i>S. glossinidius</i> – <i>S. enterica</i>	0.53	0.90	0.42	0.23	0.065	0.042
<i>S. enterica</i> – <i>E. coli</i>	—	—	—	—	0.017	0.003

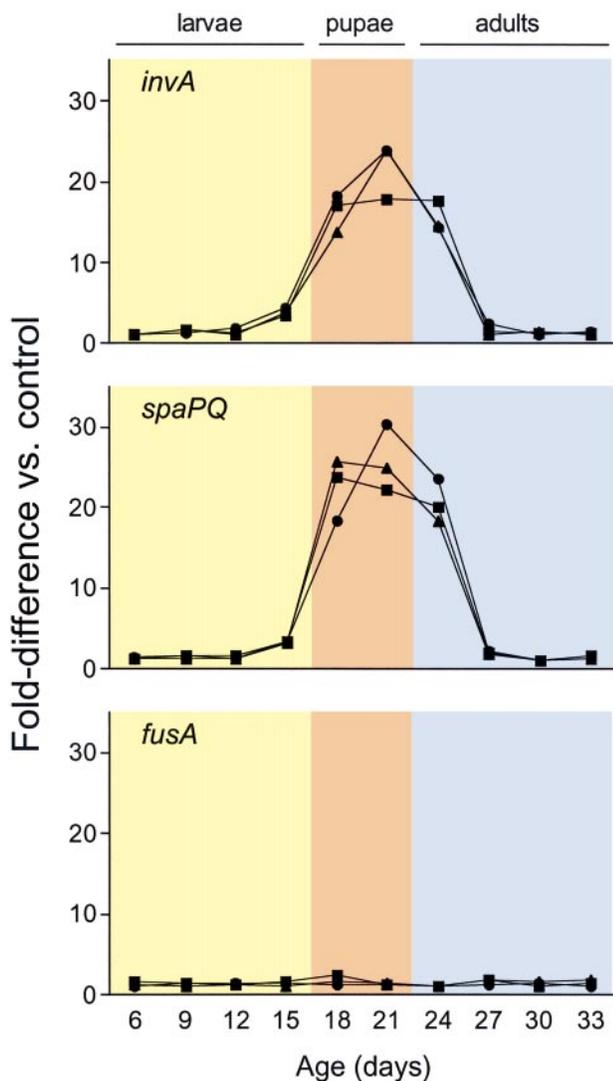


Fig. 2. Quantitative RT-PCR analysis of gene expression in weevil bacteriomes at different developmental stages. Expression of *invA*, *spaPQ*, and *fusA* was analyzed for three individual cohorts of reproductively synchronized animals at 3-day intervals through the course of their development into adulthood. To calculate relative transcript numbers, standard curves were generated for each gene by serially diluting RNA templates with the highest transcript numbers for each gene. “Fold-difference” values were determined by comparing transcript numbers in each sample to a control sample, defined within each cohort as the sample with the lowest number of transcripts for a given gene. Peak levels of the *invA* and *spaPQ* transcripts are detected in weevils undertaking pupation and metamorphosis, whereas levels of the housekeeping gene (*fusA*) transcripts remain constant throughout weevil development.

glossinidius—SZPE than for SZPE—*S. enterica* and *S. glossinidius*—*S. enterica*, supporting the occurrence of all six genes in the shared ancestor of *S. glossinidius* and SZPE (Table 2). In addition, pair-wise comparisons yielded similar K_a values for each gene between SZPE—*S. enterica* and *S. glossinidius*—*S. enterica*, as expected if inheritance of each gene has been strictly vertical since the divergence of *S. glossinidius* and SZPE. Estimates of K_s for *S. glossinidius*—SZPE are similar between the *inv/spa* genes but substantially higher than K_s values for both *fusA* and *rplB*, reflecting the strong codon bias in *fusA* and *rplB* within this group of bacteria. For example, *fusA* and *rplB* have high codon adaptation indices in *E. coli* (0.75 and 0.72, respectively), and thus these genes are expected to show atypically low K_s values for the *E. coli*—*S. enterica* comparison (17, 18).

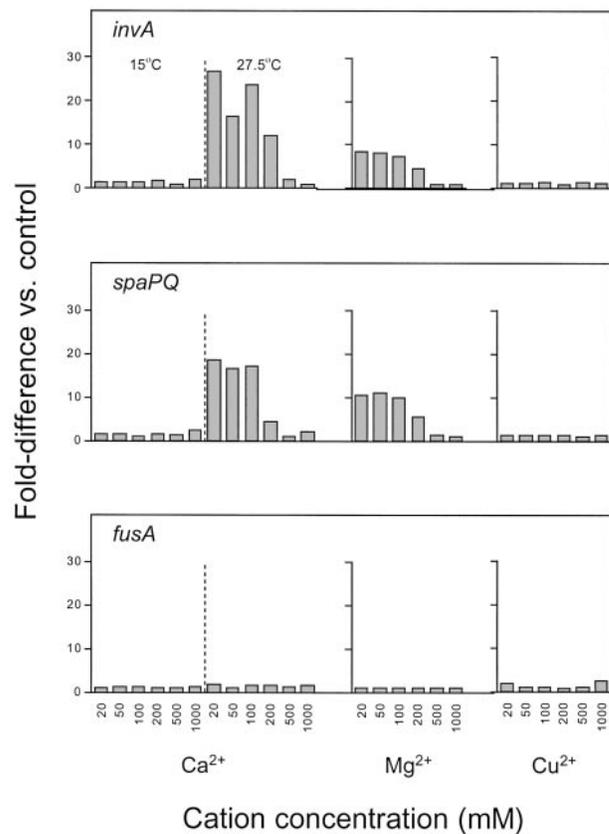


Fig. 3. Effect of divalent cations and temperature on *invA*, *spaPQ*, and *fusA* gene expression in SZPE. To calculate relative transcript numbers, standard curves for each gene were generated by serially diluting RNA templates with the highest transcript numbers for each gene. “Fold-difference” values were determined by comparing transcript numbers in each sample to a control sample, defined within each experiment as the sample with the lowest number of transcripts for a given gene. At high concentrations of Ca^{2+} or Mg^{2+} ($\geq 200 \mu\text{M}$), but not Cu^{2+} , *invA* and *spaPQ* transcription is repressed. Transcription of the housekeeping gene, *fusA*, is not affected by any of the metal cations tested. At a reduced assay temperature (15°C), *inv/spa* expression is completely repressed regardless of Ca^{2+} availability.

In Vivo Gene Expression Assays. Because SZPE has not been cultivated *in vitro*, we were unable to use gene knockouts to examine the function of the *inv/spa* genes in the SZPE—weevil symbiosis. Instead, we examined *inv/spa* gene expression at different points in the host life cycle to determine whether transcript levels change when SZPE invades bacteriomes during metamorphosis of the insect host (5). Expression patterns of *invA* and *spaPQ* were assayed by quantitative RT-PCR by using RNA extracted from the bacteriomes of weevils throughout the 33-day period of weevil development.

As anticipated from the polycistronic organization of the *inv/spa* genes (2, 3), the expression patterns of *invA* and *spaPQ* were very similar (Fig. 2). Both *invA* and *spaPQ* exhibited a 20-fold increase in expression coincident with the onset of metamorphosis, when symbionts must infect new bacteriomes. We observed no significant changes in the number of *fusA* transcripts in weevil bacteriomes throughout the entire period of weevil development. Because *fusA* is known to encode a ribosomal translocase (elongation factor G) expressed consistently during translation in bacteria, these results demonstrate that up-regulation of *inv/spa* expression occurs independently of any global changes in SZPE transcription and translation during insect metamorphosis. The integrity of the experimental approach was confirmed by testing RNA extracted from

three individual cohorts of animals, maintained under identical conditions.

In Vitro Gene Expression Assays. Because the regulation of the *inv/spa* genes in *S. enterica* is governed by the PhoP/PhoQ signal transduction system and is modulated by environmental Mg^{2+} and Ca^{2+} (19, 20), we tested whether *inv/spa* expression in SZPE is similarly controlled by external concentrations of divalent cations. For these assays, symbionts were purified directly from bacteriocytes of *S. zeamais* and resuspended in aliquots of minimal medium containing a series (20 μM to 1 mM) of three different divalent cations. After 15-h exposure, the relative levels of the symbiont *invA*, *spaPQ*, and *fusA* transcripts were measured by quantitative RT-PCR. In these *in vitro* assays, we observed at least a 10-fold increase in the relative expression of both *invA* and *spaPQ*—but not of *fusA*—as the extracellular concentrations of Ca^{2+} or Mg^{2+} decreased (Fig. 3), showing that the *inv/spa* genes in SPZE are regulated by the same cues as their *Salmonella* homologs.

To test whether the up-regulation of *inv/spa* was due simply to a change in the ionic strength of the medium, we measured transcript levels in symbionts exposed to varying concentrations of Cu^{2+} . The relative expression levels of the *inv/spa* genes, as well as the *fusA* controls, were unaffected by Cu^{2+} concentration in the medium (Fig. 3). To test the effect of temperature, which is known to affect *inv/spa* expression in *Shigella*, we set up a duplicate series of Ca^{2+} assays that were maintained at 15°C instead of 27.5°C. At the reduced incubation temperature, we detected only a 2-fold reduction in the numbers of *fusA* transcripts in these samples, indicating that global transcription and translation processes are still active in SZPE at 15°C. We were unable to detect any *invA* or *spaPQ* transcripts in RNA extracted from symbionts maintained at 15°C, suggesting that the processes of cell invasion are repressed in SZPE at reduced temperature.

Discussion

Many intracellular bacterial pathogens of animals and plants harbor a TTSS that mediates their uptake and entry into eukaryotic cells (21). In contrast, the transmission of primary endosymbionts of insects has traditionally been viewed as the result of host-directed mechanisms (5), a view that was recently corroborated by analysis of the full genome sequence of *Buchnera aphidicola*, the primary bacteriome-associated endosymbiont of aphids. Inspection of the highly reduced gene inventory of *Buchnera* suggested that this symbiont is a passive captive of its host (22); in particular, *Buchnera* was found to lack a TTSS. For these reasons, symbionts have not been expected to exploit protein translocation systems to actively invade host cells. In the present study, we have established that the infection of new host bacteriomes by certain endosymbionts is facilitated by a TTSS homologous to those identified in enteric pathogens.

Our findings indicate that the weevil endosymbiont, SZPE, contains a tandem array of genes (*inv/spa*) that share high levels of sequence identity with genes encoding components of the TTSS molecular syringe in other bacteria. The SZPE genes have intact ORFs and share an identical spatial and cistronic organization with their homologs in *S. glossinidius*, *S. enterica*, and *S. flexneri*. The lineage comprising SZPE and *S. glossinidius* is of interest, because these endosymbionts diverged from a common ancestor and have evolved in symbiotic relationships with different insect hosts. On the basis of sequence analyses of *inv/spa* and other genes, it appears that the TTSS was acquired by the common ancestor of SZPE and *S. glossinidius*. Subsequently, the TTSS was retained in both lineages as an essential element in the establishment and maintenance of infection.

Comparisons of the extent of nucleotide sequence divergence in the *inv/spa* genes with that of the housekeeping genes provide additional evidence for the origin of the *inv/spa* genes in the common ancestor of SZPE and *S. glossinidius*. Furthermore,

nonsynonymous nucleotide sequence divergence values are nearly identical for the *S. glossinidius*—SZPE comparisons and for the corresponding *E. coli*—*S. enterica* comparisons. If these genes evolve at similar rates in all four lineages, an assumption supported by sequence comparisons of *E. coli* and endosymbionts (23), then *S. glossinidius* and SZPE diverged at the same time as *E. coli* and *S. enterica*, approximately 100 million years ago (24). This date is consistent with estimates derived from independent analyses indicating that *Sitophilus* and its symbionts have coevolved through strict vertical transmission for only 50–100 million years (6). The recent establishment of the weevil–bacterial endosymbiosis may account for the observation that the genomes of the weevil endosymbionts do not display the typical A+T bias and extreme reduction in genome size, as observed in more ancient obligate bacterial endosymbionts such as *B. aphidicola* and *Wigglesworthia glossinidia* (8, 22, 25).

Although SZPE resides exclusively in bacteriomes and provides its weevil host with vitamins and amino acids essential for growth and reproduction (8), this endosymbiont also displays certain characteristics that are typical of facultative “secondary” endosymbionts. This includes a requirement for SZPE to invade new host cells during weevil metamorphosis, as indicated by the 20-fold increase in *inv/spa* transcript numbers in SZPE during metamorphosis relative to the entire period of larval development. Because there is no global change in SZPE transcription over the host life cycle, it appears that SZPE is responding to host metamorphosis by specifically up-regulating transcription of the *inv/spa* genes, promoting cell invasion.

Many bacteria have evolved mechanisms enabling them to modulate gene expression in response to environment cues (26, 27). For example, *Salmonella* species are able to sense the nature of their immediate environment by measuring Ca^{2+} and Mg^{2+} availability through the PhoP/PhoQ signal transduction system (20). In conditions of low Ca^{2+} and Mg^{2+} availability, as encountered intracellularly, PhoP/PhoQ modulates expression of *Salmonella* invasion genes, including *inv/spa* (28). By using a transient *in vitro* culture system and RT-PCR assays, we determined that SZPE is also able to modulate expression of *inv/spa* genes in response to Ca^{2+} and Mg^{2+} availability and temperature. Under conditions of low Ca^{2+} and Mg^{2+} availabilities (<100 μM), we observed an order of magnitude increase in the expression of the *inv/spa* genes, whereas expression of housekeeping genes was largely unchanged. We postulate that SZPE is responding to differences in the concentrations of these cations to coordinate the timing of cell invasion, in the same way as *Salmonella* spp. An alternative possibility is that SZPE retains, but no longer utilizes, a regulatory system that served to coordinate opportunistic host cell invasion by a free-living ancestor. However, the divergence at neutral nucleotide sites between *S. glossinidius* and SZPE is sufficiently high (Table 2) that we would expect functionless genes to have been inactivated by mutation.

We have demonstrated that two distinct members of an endosymbiotic clade have evolved in different hosts with dependence on a specialized cell invasion apparatus that is also commonly used by pathogens. It has been suggested that parasites, through a coevolutionary process, can develop commensal or mutualistic relationships, and that the molecular mechanisms of symbiosis and pathogenesis might be similar (29, 30). Our findings support these notions, indicating that a TTSS has been adapted in the context of mutualism to maintain a permanent intracellular association between symbiotic bacteria and their hosts.

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