

Disruption of type III secretion in *Salmonella enterica* serovar Typhimurium by external guide sequences

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ABSTRACT

The type III secretion system involved in *Salmonella enterica* serovar Typhimurium invasion of host cells has been disrupted using inducibly expressed oligonucleotide external guide sequences (EGSs) complementary to *invB* or *invC* mRNA. These EGSs direct single site cleavage in these mRNAs by endogenous RNase P, and their expression in *Salmonella* results in *invC* mRNA and InvC protein depletion, decreased type III secretion and interference with host cell invasion. Comparison of these effects with those from studies of *Salmonella invB* and *invC* mutants suggests that *invB* EGSs have polar effects on *invC* mRNA.

INTRODUCTION

External guide sequence (EGS) oligonucleotides target complementary mRNA for specific cleavage catalyzed by RNase P (1). EGS oligonucleotides require an accessible single-stranded region on their target mRNA to base-pair with and create the stem structure recognized as a cleavage substrate by RNase P (1). Using EGSs complementary to essential genes, *Escherichia coli* viability can be decreased in a manner which is EGS oligonucleotide sequence specific, dose dependent and dependent on time elapsed after EGS expression (2). Here, EGS studies are extended to *Salmonella*, using EGSs complementary to two *Salmonella* pathogenicity island SPI-1 genes (3), *invB* and *invC*, neither of which are essential for bacterial viability (4). The *invB* and *invC* DNA sequences occur directly adjacent to each other in the multigene pathogenicity island SPI-1 of *Salmonella*, with the last nucleotide in the final codon of *invB* also serving as the first nucleotide in the first codon of *invC* (4). Prior studies of *Salmonella invB* and *invC* mutants have shown that *invC* is required for host cell invasion and that the gene encodes a protein with ATPase activity (4). The ATPase encoded by *invC* is postulated to provide energy to power the type III secretion system involved in host cell invasion (4) and

pathogenesis (5) by *Salmonella*. In contrast, *invB* does not appear necessary for invasion. InvB is a type III secretion chaperone specific for SipA, a translocated *Salmonella* protein which facilitates actin rearrangements within infected eukaryotic cells (6). Mutations in *invB* do not alter the secretion of other type III secreted proteins (6) and do not disrupt invasion (4). Using a tightly regulated inducible EGS expression system in *Salmonella* (7), we show that EGSs complementary to either *invB* or *invC* mRNA can disrupt type III secretion and *Salmonella* invasion assayed *in vitro*.

MATERIALS AND METHODS

Plasmids and bacterial strains

The EGSs listed below were cloned as previously described into high copy number EGS expression plasmids, derived from pUC19 (2) or into low copy number plasmids derived from pWKS30 (8). These plasmids were transformed into the *Salmonella enterica* serovar Typhimurium strain SB300A#1 (7). SB300A#1 has a T7 RNA polymerase gene integrated with an adjacent araC-P(BAD) control element into the bacterial chromosome of parent strain SB300. SB300A#1 allows tightly controlled arabinose-inducible T7 promoter-driven transcription of our EGSs in *Salmonella* (7). The *invA*-deficient *Salmonella* strain SB136 (4), which is disrupted for type III secretion, was used as a control. An *invC* deletion mutant *Salmonella* (J. E. Galán and Y. Akeda) was used as a negative control strain for studies of InvC intracellular protein level and of type III secretion. *Salmonella* was grown in 0.3 M NaCl Luria–Bertani (LB) medium. Liquid culture incubation conditions and EGS induction with arabinose at 0.2% final concentration are as previously described (7). Following addition of arabinose for EGS induction, *Salmonella* liquid cultures were grown to late log phase prior to northern blot analysis, assay of *Salmonella* type III secretion or quantification of bacterial entry, as detailed below.

Design of external guide sequences

EGS oligonucleotides were designed to be complementary to single-stranded regions of *invB* and *invC* mRNA, followed by

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an additional 3'-ACCA EGS terminal sequence. This strategy allows formation of a duplex EGS-mRNA molecule recognized as a substrate by endogenous RNase P with resultant cleavage of target mRNA (9). The individual EGS oligonucleotide sequences were named according to their predicted site of target mRNA cleavage. For example, *invB* 108 EGS (5'-AAUGCAAUAAAUCCacca-3') is complementary to *invB* mRNA nucleotides 108-122 (5'-GGAUUUUUUGCAUU-3') and will result in RNase P cleavage of *invB* mRNA at nucleotide number 108. The other *invB* or *invC* EGS sequences were: *invC* 98 EGS (5'-GGCGUGAUUUCACAAacca-3'), *invC* 269 EGS (5'-ACCGCGCCUAAUACCacca-3') and *invC* 293 EGS (5'-ACGAUUUCCCUGUCacca-3'). Two previously characterized EGSs which are not complementary to *invB* or *invC* were also used: synthC5 EGS 21 and synthC5 EGS 45 (2). The EGSs synthC5 EGS 21 and synthC5 EGS 45 are complementary to, and can guide the RNase P cleavage of, mRNA used for the recombinant synthesis of the C5 protein subunit of the RNase P holoenzyme of *E.coli*, but are not complementary (containing at least five unpaired nucleotides per EGS) to the mRNA encoding C5 in *Salmonella*. Herein, the EGSs synthC5 EGS 21 and synthC5 EGS 45 are referred to as synthC5 EGS 1 and 2, respectively.

Partial RNase T1 digest mapping of *invB* and *invC* mRNA

Single-stranded regions of *invB* and *invC* mRNA were identified using RNase T1 digestion (1). Two mRNAs were digested: (i) a joint *in vitro* transcript containing *invC* mRNA 3' to *invB* mRNA, transcribed from the plasmid pSB553 (4) DNA after digestion with BamHI; and (ii) an *invC* mRNA *in vitro* transcript alone, expressed from plasmid pIC001 (a pSB553 derivative, with *invB* coding sequence removed via KpnI and BspEI excision) DNA after digestion with EcoRI.

In vitro RNase P assays

Assays of mRNA cleavage *in vitro* by RNase P were performed as previously described (10), using the EGS sequences and the *invB* and *invC* mRNA targets detailed above. RNase P M1 RNA was folded in a buffer containing 10 mM magnesium, using a heat block to first heat the sample at 65°C for 5 min and then slowly cool the sample to room temperature. For conditions of substrate excess, reagent concentrations were: 11 fmol labeled substrate (1100 c.p.m.), 1, 5 and 10 pmol EGS, and 1 pmol of enzymatically active recombinant *E.coli* RNase P M1 RNA. For conditions of limited substrate, 10 fmol of labeled substrate RNA (1000 c.p.m.) and 50, 100 and 500 fmol of EGS were used. Samples were electrophoresed in 5% polyacrylamide-7 M urea gels.

Northern blots

Northern blots were performed on total RNA extracts of *Salmonella*, using previously published techniques (11). Each lane of a 2.5% agarose gel was loaded with 4 µg of total RNA. The UV transillumination pattern of rRNA bands after separation of each sample on an agarose gel revealed similarity in rRNA band patterns in terms of both gross quantity and quality. Northern blot probes were 5'-end-labeled DNA oligonucleotide probes. They included probes comple-

mentary to *invC* mRNA, 5S rRNA and each of the four *invB* and *invC* EGS oligonucleotides listed above. In each case, 8 pmol of oligonucleotide labeled with 4 pmol (30 µCi) of [γ -³²P]ATP was used per 40 ml of rapid hybridization buffer (Amersham). Signal was detected using a phosphoimager (Fuji) and quantitated using image analysis software (Fuji ImageGauge). Quantitative results are reported as the ratio, expressed as a percentage, of northern blot *invC* mRNA band signal intensity for the matched culture specimens of a given *Salmonella* strain with EGS induction versus without EGS induction.

Assay of *Salmonella* type III secretion

Salmonella culture supernatant proteins were prepared and analyzed as previously described (12). Western blots were probed with polyclonal antibodies against SipB and SipC and chemiluminescent signals produced using ECL Plus western blotting detection reagents from Amersham Biosciences. Signal was detected using autoradiograph film (Kodak) and quantitated using image analysis software (Fuji ImageGauge). Quantitative results are reported as the ratio, expressed as a percentage, of western blot band signal intensity for the matched culture specimens of a given *Salmonella* strain with EGS induction versus without EGS induction.

Matched culture specimens were employed as internal controls for western blots. Specifically, a given *Salmonella* transformant was grown to early log phase in a single liquid culture, then split into paired cultures which were grown simultaneously either with or without arabinose induction of EGS expression. Equal volumes of paired cultures were harvested for protein preparation at the same point of their late log phase growth, as assessed by optical density. Equal volumes of these protein preparations were loaded per well for western blot analysis.

Assay of intracellular InvC protein level

Salmonella cultures in liquid media were pelleted and resuspended in one-twentieth volume of phosphate-buffered saline (PBS)-Tris (77 mM Tris-HCl, pH 8.0). Samples were denatured by boiling with SDS-PAGE loading buffer and separated on a 9% polyacrylamide-SDS gel. Western blots were probed with a polyclonal antibody against InvC (J. E. Galán and Y. Akeda), and chemiluminescent signals produced using ECL Plus western blotting detection reagents from Amersham Biosciences. Signals were detected using autoradiograph film (Kodak). Quantitation of results, using matched culture specimens, was performed as described for type III secretion assays, above.

Quantification of bacterial entry

Entry of different *Salmonella* strains into Henle-407 cells in a gentamicin protection assay of bacterial entry into host tissue culture cell monolayers was performed and quantified as previously described (13).

RESULTS

Design of EGSs for *invB* and *invC* mRNA *in vitro*

To design the EGSs reported here, mRNA transcripts of *invB* and *invC* made *in vitro* were mapped using partial RNase T1

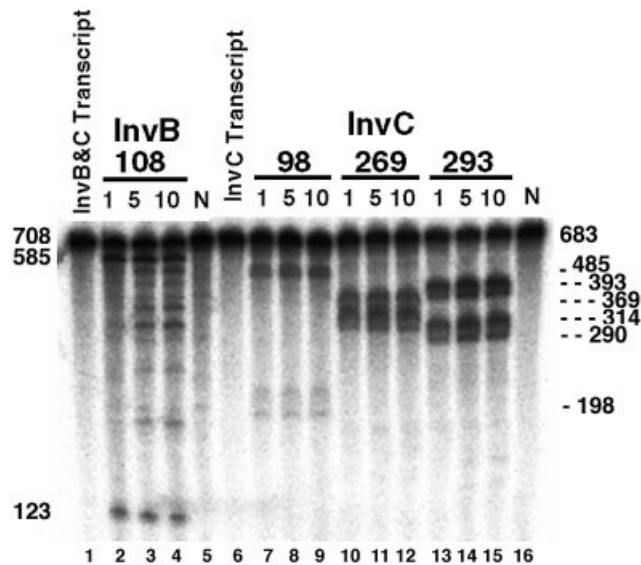


Figure 1. RNase P-EGS cleavage of mRNA *in vitro*. Target substrates for cleavage include a joint transcript including both *invB* and *invC* mRNA (lane 1), and a transcript of *invC* mRNA alone (lane 6). The former was incubated with increasing amounts of the *invB* EGS 108 (lanes 2–4); the latter as a target for *invC* EGSs *invC* 98 (lanes 7–9), *invC* 269 (lanes 10–12) and *invC* 293 (lanes 13–15). Lanes labeled N (lanes 5 and 16) lack any EGS, but do have active RNase P with the *invB* and *invC* joint transcript (lane 5) or the *invC* transcript (lane 16) and show no non-specific target cleavage in the absence of EGS. Cleavage products were separated by size using electrophoresis in a 5% polyacrylamide–7 M urea gel. Cleavage product sizes are consistent with RNase P cleavage occurring at the predicted site at the 5' end of the mRNA region to which each EGS hybridizes. Predicted sizes of reaction products following RNase P enzymatic cleavage of *in vitro* transcripts are listed on the left and right of the image (e.g. *invB* and *invC* joint transcript mRNA cleavage products of 585 and 123 nucleotides for EGS *invB* 108; *invC* transcript mRNA cleavage products of 485 and 198 nucleotides for EGS *invC* 98, etc.).

nuclease digestion to suggest EGS-accessible single-stranded mRNA regions. The first nucleotide of the start codon of each gene is labeled as nucleotide 1; single-stranded guanine residues of *invB* mRNA were identified via partial RNase T1 nuclease digestion at *invB* nucleotides 108 and 217. For *invC* mRNA, single-stranded guanines were identified at *invC* nucleotides 98, 237, 269 and 293. Given uncertainty about whether *invC* mRNA exists in cells independently from *invB* mRNA or as a joint transcript with *invB*, both possibilities were examined in RNase T1 digestion *in vitro*. RNase T1 digestions were performed on two *in vitro* transcripts: *invC* mRNA alone, as well as a tandem transcript of *invC* mRNA immediately 3' to *invB* mRNA. Single-stranded regions of *invC* identified in the joint *invB*–*invC* *in vitro* transcript were notably also found for the *invC* *in vitro* transcript alone (data not shown). EGS oligonucleotides were designed to be complementary to the RNase T1-accessible mRNA sequences *invB* 108–122, *invC* 98–112, *invC* 269–283 and *invC* 293–307, and were named for their predicted nucleotide cleavage sites by RNase P: *invB* 108, *invC* 98, *invC* 269 and *invC* 293, respectively.

RNase P-specific cleavage of mRNA *in vitro*

RNase P hydrolyzes the phosphodiester bond (in the target mRNAs) that precedes the first base pair in the 5' end of the

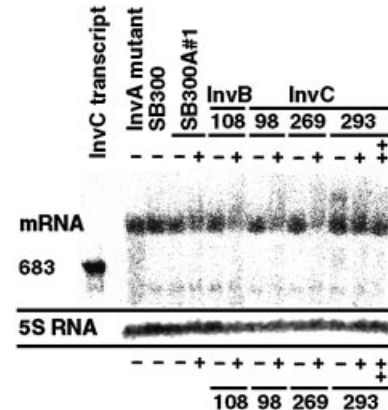


Figure 2. Northern blots in *Salmonella* with inducible expression of EGS molecules. RNA was isolated from *Salmonella*, electrophoresed in a 2.5% agarose gel, and probed for *invC* mRNA or for constitutively produced non-targeted 5S rRNA. The partial *invC* *in vitro* transcript expressed from plasmid pIC001 DNA after digestion with EcoRI (as described for T1 digest mapping) serves as a size marker (683 nt). The *invA* mutant is SB136 (also used for invasion assays). SB300 is the parent *Salmonella* strain, from which SB300A#1 was constructed. Other lanes are paired by SB300A#1 *Salmonella* transformant type, either with (+) or without (–) the addition of arabinose for the induced expression of the EGS molecules listed. Longer term induction (++) was suspected to be accompanied by the loss of EGS expression plasmid *invC* 293, and an accompanying lack of effect on target mRNA. Note the decrease in *invC* mRNA after the induction of EGS expression, whereas non-targeted constitutive 5S rRNA levels are independent of EGS expression.

target mRNA–EGS complex, akin to the site-specific cleavage reaction the enzyme catalyzes in the 5' processing of precursor tRNA (9). The reaction ingredients for RNase P assays *in vitro* were *E. coli* RNase P, internally radiolabeled *invB* and *invC* mRNA target transcribed *in vitro*, and EGSs complementary to portions of *invB* or *invC* mRNA. All four EGSs guide RNase P to cleave the mRNA at the predicted sites of EGS mRNA hybridization, yielding appropriately sized 5' and 3' cleavage products (Fig. 1). RNase P cleavage of mRNA increases with increasing EGS dose, with the EGSs *invB* 108 and *invC* 293 most efficient at guiding mRNA cleavage *in vitro* in conditions of limited substrate (data not shown).

Specific mRNA targeting in *Salmonella*

Induction of expression of *invB* or *invC* EGSs in *Salmonella* is followed by a decrease in *invC* mRNA compared with identical *Salmonella* transformants lacking EGS induction. Northern blots of equal microgram amounts of total RNA isolated from various *Salmonella* liquid cultures are shown in Figure 2, where northern blot signals for *invC* mRNA in matched *Salmonella* cultures decreased between 27 and 50% following relevant EGS induction. The *invB* or *invC* EGS expression effects appear specific, in that there is no similar change detected in the level of constitutive 5S rRNA after EGS induction (Fig. 2). Arabinose was used to induce *Salmonella* EGS expression. It did not have as pronounced an effect on *invC* mRNA in the absence of EGS expression plasmids, either in the case of *Salmonella* which was not transformed with an EGS expression plasmid, or in the case of *Salmonella* for which the EGS expression plasmid was presumably lost after prolonged culture (Fig. 2). Plasmid

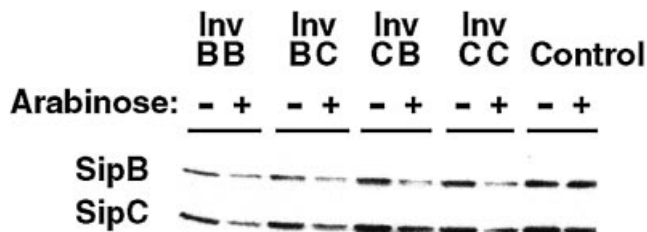


Figure 3. Western blots of cell culture supernatants from *Salmonella* with (+) or without (-) the induced expression of EGS molecules listed. Western blot detects the proteins SipB and SipC, secreted into cell culture supernatant by the type III secretion system and subsequently electrophoresed in a 9% polyacrylamide-SDS gel. The transformants shown contain a pUC19-derived high-copy number plasmid (2) from which two EGSs are concurrently expressed. InvBB denotes inducible expression of *invB* 108 and *invB* 108 EGSs in tandem, InvBC denotes *invB* 108 and *invC* 98 EGS expression, InvCB denotes *invC* 293 and *invB* 108 EGS expression, and InvCC denotes *invC* 293 and *invC* 98 EGS expression. The control concurrently expresses two EGSs targeting the mRNA used for synthetic C5 protein over-expression in *E.coli*. Migration patterns of SipB and SipC bands relative to protein molecular weight markers are as shown in Figure 4.

maintenance in transformants was assessed by parallel quantitative plating on LB or LB ampicillin plates as previously reported (2,11). Plasmid loss was considered to have occurred when colony counts on LB plates were greater than on LB ampicillin plates by at least an order of magnitude. As previously demonstrated for other EGS transcription in this *Salmonella* system (7), northern blot probes complementary to EGS oligonucleotides detected EGS expression only after arabinose induction (data not shown).

The effect of expression of EGSs on type III secretion by *Salmonella*

A standard functional assay of InvC-dependent type III secretion was employed, in which proteins secreted by the type III secretion system were measured in cell culture supernatants (12). These secreted proteins, SipB and SipC, are also encoded in the *Salmonella* pathogenicity island I gene complex and are reviewed elsewhere (14,15).

A panel of *Salmonella* transformants, containing EGSs in various inducible expression plasmid vectors, was used for InvC-dependent type III secretion assays. The concurrent expression of two EGSs using any one of four high copy number plasmids derived from pUC19 (2) consistently decreased SipB secretion by $\geq 65\%$ from that detected for the same *Salmonella* transformant grown in parallel under non-EGS-inducing conditions, based on the relative signal intensities of SipB western blot bands for a given *Salmonella* strain with versus without EGS induction (Fig. 3). Using the same method of quantitative analysis, induction of the same set of EGS pairs in *Salmonella* transformed with a low copy number plasmid derived from pWKS30 showed a 20–30% decrease in secretion (data not shown), as did any of the four EGSs when expressed alone from pUC19-derived plasmids (data not shown). These findings are consistent with prior studies of EGS dose-response features in *E.coli* comparing EGS expression plasmids encoding single versus multiple EGSs (2) and comparing EGS expression plasmids with strong versus weak promoters (10).

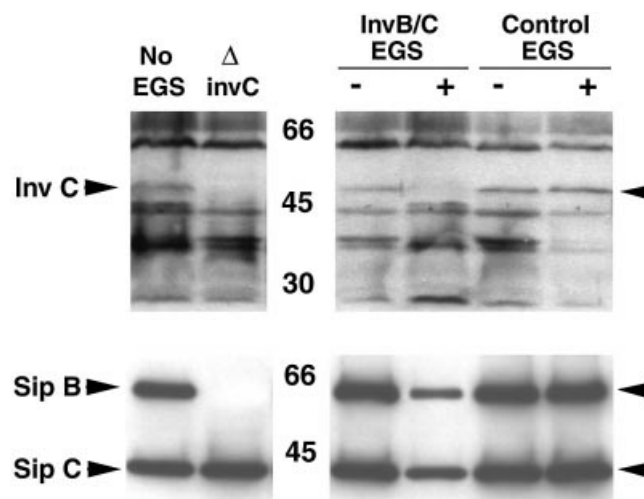


Figure 4. Western blots for *Salmonella* intracellular InvC (top) and for SipB and SipC secreted into *Salmonella* cell culture supernatants (bottom, as for Fig. 3). SB300A#1 without an EGS expression plasmid (no EGS) and a SB300 *invC* deletion mutant which does not express InvC (Δ invC) (produced by J. E. Galán and Y. Akeda) are shown to the left of protein molecular weight markers. At right: SB300A#1 transformed with the high copy number plasmids for arabinose-inducible expression of either *invB* 108 and *invC* 98 EGSs (InvB/C EGS) or the control EGSs (Control EGS) described for Figure 3. EGS induction status by arabinose addition is shown as (+) or (-). Arrowheads denote the predicted locations of InvC, SipB and SipC after electrophoresis in 9% polyacrylamide-SDS gels.

To assess sequence specificity, the negative control EGSs, synthC5 EGS 2 and 1, against the synthetic C5 component of *E.coli* RNase P, described above and in McKinney *et al.* (2), was also used. Induction of this negative control EGS was accompanied by a $<10\%$ decrease in SipB secretion as compared with non-induced parallel cultures (Fig. 3). In addition, the loss of an *invB* or *invC* EGS expression plasmid from a bacterial strain was accompanied by a loss of previously observed inhibitory effects on secretion (data not shown).

The effect of EGSs on intracellular InvC protein in *Salmonella*

A polyclonal antibody (J. E. Galán and Y. Akeda) raised against recombinant InvC protein was used for western blot analysis of cellular InvC protein level as an assessment of EGS effects (Fig. 4, top). A band consistent with the 47 kDa InvC protein is found in the *Salmonella* strain SB300A#1 with no EGS. An SB300 strain (constructed by Y. Akeda) in which InvC expression has been disrupted via an *invC* deletion mutation serves as a negative control for InvC expression. Intracellular expression of InvC was also assessed in SB300A#1 transformed with the EGS expression plasmid for the concurrent expression of either the EGSs *invB* 108/*invC* 98 or the negative control EGSs synthC5 EGS 2 and 1 (also shown in Fig. 3). After the induction of EGS expression, the relevant protein band is not detected for SB300A#1 with the *invB/invC* EGSs. No effect on the detection of this protein band is seen after the induction of the negative control EGS. Figure 4 is representative of repeated assays in that the decrease in putative ~47 kDa InvC signal cannot be explained by a general decrease in the intensity of other bands detected

Table 1. Invasion of Henle-407 cells by *Salmonella* strains

<i>Salmonella</i> strain	% Invasion No arabinose	% Invasion Plus arabinose
SB136 (<i>invA</i> -)	0.08 ± 0.04	0.1 ± 0.06
SB300A#1	27 ± 4.7	
SB300A#1 transformants		
pUC synthC5 EGS 1	26	20
pUC synthC5 EGS 2 and 1	29 ± 2.1	
pUC <i>InvB/B</i> EGS	10 ± 1.5	2.9 ± 0.2
pUC <i>InvB/C</i> EGS	9.6 ± 1.2	2.9 ± 0.5
pUC <i>InvC/B</i> EGS	8.8 ± 1.0	2.8 ± 0.3
pUC <i>InvC/C</i> EGS	12	3.5
pWKS <i>InvB/B</i> EGS	25	19
pWKS <i>InvB/C</i> EGS	23	23
pWKS <i>InvC/B</i> EGS	18	18
pWKS <i>InvC/C</i> EGS	13	13

Invasiveness of different *Salmonella* strains into Henle-407 cells, in a standard gentamicin protection assay. The numbers of internalized bacteria are shown as a percentage of bacteria input. Means ± SDs are shown for triplicate well assays, with no standard deviations shown for single-well assays. *Salmonella* strains include SB300A#1, SB300A#1 transformants expressing various EGSs described in the text, and the *invA*-deficient SB136.

by the polyclonal antibody. Indeed, for the two cases of EGS induction shown here, *invB/invC* EGS induction is accompanied by decreased ~47 kDa *InvC* signal in the context of strong signals for other bands, whereas negative control EGS induction is accompanied by strong ~47 kDa *InvC* signal in the context of relatively weak signals for other bands.

Cell supernatants from the same experiment were used for type III secretion assays (Fig. 4, bottom) as above. In each case, detection of the band representing intracellular *InvC* protein correlates with type III secretion. Type III secretion is decreased when intracellular *InvC* protein levels are decreased, either by the static *invC* deletion mutation, or by the dynamic disruption of *InvC* expression following the induction of the *invB/invC* EGSs. While the presumptive *InvC* protein band disappears in both situations, the decrease in type III secretion is more pronounced following the static deletion of the *invC* gene than following the induction of the *invB/invC* EGS. This functional assay of *InvC* cellular activity suggests that our *invB/invC* EGS system inhibits *InvC*-dependent type III secretion less completely than does the *invC* deletion-mediated ablation of *InvC* expression. Wild-type levels of secretion are observed for SB300A#1 with no EGS plasmid, without induction of *invB/invC* EGSs, or with negative control EGS expression.

EGS impact on *Salmonella* invasion into host cells

Salmonella strains containing EGSs in various inducible expression plasmid vectors were also tested for their ability to invade Henle-407 cells in tissue culture. In each case, a given *Salmonella* strain was grown in parallel liquid cultures, either with arabinose added to induce EGS induction or without the addition of arabinose. For invasion, these *Salmonella* strains were first incubated with Henle cells for 45 min in Hanks buffered salt solution. This was followed by a 2 h treatment with gentamicin to kill extracellular bacteria, and subsequent washes with tissue culture buffer. Invasion was quantified as

the percentage of bacteria inoculated into the tissue culture wells which were recovered from lysed Henle cells.

As shown in Table 1, a control *Salmonella* strain, SB136, with a null mutation in *invA* and a previously documented functional defect in type III secretion and host cell invasion (4), achieves <1% invasion into Henle cells, independent of arabinose addition. *Salmonella* SB300A#1 transformants which express an EGS complementary to mRNA encoding synthetic C5 (also shown as a negative control for type III secretion assays, above) have a ≥20% rate of invasion, with a small decrease in invasion observed after arabinose addition. *Salmonella* SB300A#1 transformants which inducibly express EGSs against *invB* or *invC* from a high copy number pUC19-derived plasmid exhibit an 8–12% rate of invasion without arabinose addition, which decrease to 2.8–3.5% after arabinose induction of EGS expression. The same EGS constructs, expressed from low copy number pWKS30-derived plasmids, did not affect invasion rates after EGS induction.

DISCUSSION

The pathogenicity island genes *invC* and *invB* of *Salmonella* provide intriguing targets for gene product disruption. In the case of *invC*, mutagenesis studies clearly show that the ATPase encoded by *invC* is required for type III secretion in assays *in vitro* and is important for pathogenicity in animal models (4,5). Following appropriate EGS expression, we observe a decrease in *invC* mRNA, *InvC* intracellular protein, *InvC*-powered type III secretion and type III secretion-dependent host cell invasion.

The inhibition of type III secretion and of *Salmonella* invasion using EGSs to disrupt *invC* mRNA is less complete than that resulting from *invC* deletion mutagenesis. This suggests that a certain critical level of mRNA disruption is able to partially inhibit type III secretion and host cell invasion. The level of mRNA disruption required for phenotypic changes probably varies for different target mRNAs, depending on factors such as the ratio of EGS to target mRNA (2,11), the relative efficiency of various EGSs and the functional reserve capacity a cell has for a given target mRNA and the protein that mRNA encodes. The EGSs reported here show greater phenotypic effects when expressed from high copy number, rather than low copy number, plasmids. This is consistent with prior EGS dose–response observations in bacteria. Phenotypic effects in *E.coli* are greater following EGS expression driven by a strong promoter as compared with a weak promoter (11), and the concomitant expression of different EGSs in *E.coli* results in phenotypic effects exhibiting additive synergy (2). The application of EGS technologies to regulate gene expression in bacteria in *in vivo* models of infection may benefit from EGS expression plasmids which can be stably maintained in bacteria within an animal. We produced the low copy number plasmids reported here in an initial effort toward this end. The fact that EGSs expressed from our low copy number plasmid system did partially inhibit type III secretion but showed no apparent effect on host cell invasion suggests a possible threshold effect, in which inhibition of type III secretion must reach a critical threshold to result in inhibition of cell invasion.

In contrast to the static effects on gene product disruption produced by mutagenesis techniques, our techniques of gene

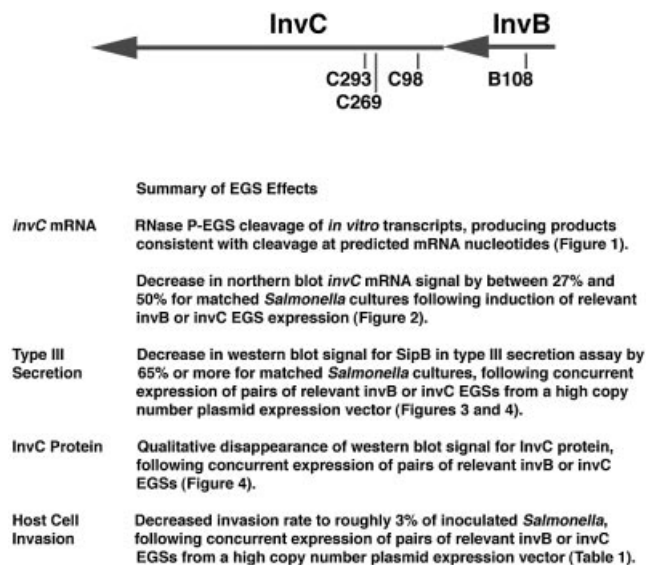


Figure 5. Summary of *invB* and *invC* EGSs and their effects. A schematic of the *invB* and *invC* mRNA transcripts is shown, with the predicted sites of RNase P-mediated cleavage identified for each *invB* or *invC* EGS (*invB* 108 EGS, *invC* 98 EGS, *invC* 269 EGS and *invC* 293 EGS). The last nucleotide in the final codon of *invB* also serves as the first nucleotide in the first codon of *invC*. Effects of these EGSs on *Salmonella invC* mRNA, type III secretion, *InvC* protein and host cell invasion are summarized, and relevant figures cited. Further details are provided in the text.

product disruption involve a more dynamic process of EGS-guided, RNase P-mediated, mRNA cleavage commencing after EGS induction. As observed in past studies of EGSs complementary in sequence to essential genes (2) or to antimicrobial resistance genes (11) in *E.coli*, a lag occurs between initiation of EGS expression and the observed molecular and phenotypic effects. This lag is consistent with the time required to accrue sufficient amounts of mRNA cleavage for observable phenotypic effects.

Reconciling the relative magnitude of effects we observe after EGS expression upon *invC* gene product expression and functions (Fig. 5) requires consideration of the kinetics of several steps of cellular physiology and our measurement methods. For example, the assays we employed showed a smaller relative effect of EGSs on type III secretion than on invasion. Our invasion assays detect host cell invasion over a short time period commencing at a time when differences between conditions of EGS or no EGS are relatively great. In contrast, our secretion assays detect the accumulated SipB or SipC secreted into supernatants throughout the period of EGS induction and include a lag time when EGS effects are minimal. Accordingly, the relative difference between conditions of EGS expression and no EGS expression which we detect for secretion might be predicted to be less than the relative difference we detect for invasion.

Effects following expression of *invB* EGSs were somewhat surprising given the results of prior *invB* studies. *InvB* is a type III secretion chaperone specific for SipA (6). Mutations of *invB* do not affect the type III secretion of SipB or SipC (6) or the invasion of host cells (4). Yet, an EGS complementary in nucleotide sequence to *invB* and which cleaves *invB* mRNA *in vitro*, does inhibit type III secretion of SipB and SipC and

host cell invasion. Furthermore, this EGS against *invB* decreases *invC* mRNA levels in *Salmonella*.

The coding regions of the *invB* and *invC* genes overlap, such that the 3' nucleotide of the final codon of *invB* serves as the 5' nucleotide of the first codon of *invC*. We postulate that the effects of *invB* EGSs are consistent with the existence of a joint *invB*-*invC* transcript in *Salmonella* (Fig. 5), which becomes destabilized or functionally disrupted after cleavage of its *invB* portion. If this is true, the fact that an EGS targeting *invB* mRNA also has effects on *invC* suggests that, in polycistronic mRNA transcripts, EGS targeting the 5' portions of mRNAs may also have effects on mRNAs encoded by genes 3' to the EGS target site.

Ultimately, pathogenicity island gene product disruption may lead to novel anti-pathogenicity agents and strategies. Since these genes are not essential to bacterial viability, interfering with their functions could conceivably decrease morbidity without the relatively broad-spectrum effects and selective pressure for resistance seen with many current antimicrobial agents.

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