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# Transfer of the cloned *Salmonella* SPI-1 type III secretion system and characterization of its expression mechanisms in Gram negative bacteria in comparison with cloned SPI-2

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ABSTRACT

Cloned type III secretion systems have much potential to be used for bacterial engineering purposes involving protein secretion and substrate translocation directly into eukaryotic cells. We have previously cloned the SPI-1 and SPI-2 type III systems from the *Salmonella enterica* serovar Typhimurium genome using plasmid R995 which can conveniently capture large genomic segments for transfer between bacterial strains. However, though expressed and functional in *Salmonella* strains, cloned SPI-1 was previously observed to have a serious expression defect in other Gram negative bacteria including *Escherichia coli*. Here we show that cloned SPI-1 expression and secretion can be detected in the secretion preps from *E. coli* and *Citrobacter* indicating the first observation of non-*Salmonella* SPI-1 expression. We describe a compatible plasmid system to introduce engineered SPI-1 substrates into cloned SPI-1 strains. However, a SPI-1 translocation defect is still observed in *E. coli*, and we show that this is likely due to a defect in SipB expression/secretion in this species. In addition, we also examined the requirement for the *hilA* and *ssrAB* regulators in the expression of cloned SPI-1 and SPI-2, respectively. We found a strict requirement for *hilA* for full cloned SPI-1 expression and secretion. However, though we found that *ssrAB* is required for full cloned SPI-2 expression in a range of media across different bacteria, it is not required for cloned SPI-2 expression in MgM8 inducing media in *S.* Typhimurium. This suggests that under SPI-2 inducing conditions in *S.* Typhimurium, other factors can substitute for loss of *ssrAB* in cloned SPI-2 expression. The results provide key foundational information for the future use of these cloned systems in bacteria.
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## 1. Introduction

Bacterial protein secretion systems can be cloned on large contiguous DNA segments, transferred to other bacterial strains, and used for the study and application of the particular system for bacterial engineering purposes (Karaolis et al. 1997; McDaniel and Kaper 1997; Hansen-Wester et al. 2004; Wilson and Nickerson 2006a; Wilson et al. 2007; Blondel et al. 2010; Akeda et al. 2012; Bao et al. 2012). In addition, important information about the evolution of the cloned system can be obtained when its expression and functional activity is studied outside of its original genomic context in bacteria other than the species of origin (Hansen-Wester et al. 2004; Wilson et al. 2004; Wilson and Nickerson 2006a,b; Wilson et al. 2007; Akeda et al. 2012; Bao et al. 2012). Cloned type III secretion systems (T3SS) have much potential for bacterial engineering applications involving vaccine delivery, anti-cancer strategies, and other specifically-designed bacterial-eukaryotic cell interactions (Panthel et al. 2008; Xiong et al. 2010; Carleton et al. 2013; Xu et al. 2014). The effective use of different cloned T3SS in different bacteria involves establishing parameters for the optimal expression and function of the cloned systems across genera.

We have previously reported the separate cloning of the *Salmonella enterica* serovar Typhimuirum SPI-1 and SPI-2 T3SS using a plasmid vector that can transfer to a range of other bacterial genera (Wilson and Nickerson 2006a; Wilson et al. 2007). These constructs allow greater ease of transfer and better flexibility in the range of bacteria available for future applications compared to other T3SS constructs (McDaniel and Kaper 1997; Ham et al. 1998; Hansen-Wester et al. 2004). In contrast to the cloned SPI-2 system, which was readily expressed in other genera, the cloned SPI-1 system displayed a severe expression defect (where expression was undetectable in cell lysates) in bacterial genera other than

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*Salmonella* (Wilson and Nickerson 2006a; Wilson et al. 2007). In addition, the expression pattern of both cloned SPI-1 and SPI-2 was different from that observed for the corresponding systems located in their original genomic contexts with the cloned systems being expressed under “non-inducing” conditions which normally repress the genomic systems (Wilson and Nickerson 2006a; Wilson et al. 2007). This suggests that different regulatory mechanisms may be at play for these systems in the cloned context where the systems are maintained at about 10 copies per cell (Wilson et al. 1997; Wilson and Nickerson 2006a). Identifying which gene regulation mechanisms are utilized/required for the cloned systems allows engineering and optimization of the systems for future applications.

In this report, we establish expression and secretion activity of the SPI-1 system in bacteria other than *Salmonella*, but we observe a translocation defect that is in part due to a defect in SipB expression/secretion. In addition, we examine the role of the *hilA* and *ssrAB* genes in cloned SPI-1 and SPI-2 system expression, respectively. The findings in this report fill important gaps in our knowledge of how these cloned systems express and function in other bacterial genera. Moreover, this work further highlights potential hurdles to the use of certain cloned T3SS (including SPI-1) when transferred to other bacteria and studied outside the chromosomal context of the original strain.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and media

Please refer to Table 1 for a list of strains and plasmids used in this study. All experiments were performed in accordance with established biosafety guidelines and have been approved by the investigators’ institutional biosafety committee. Strains were grown in Lennox broth (LB), LB 0.3 M NaCl, LB pH5.5, MgM10, and MgM8 media as previously described (Lennox 1955; Wilson and Nickerson 2006a; Wilson et al. 2007; Cangelosi et al. 2013). Deletion of *invJ*, *prgI*, and *hilA* from R995 + SPI-1 and deletion of *ssrAB* from R995 + SPI-2 were performed using standard recombineering techniques as described previously (Datsenko and Wanner 2000). The pBAD18 + *sopE*-HA, pBAD18 + *sopA*-Flag, pBAD18 + *sopE-cyaA*, and pBAD18 + *sopA-cyaA* plasmids were made by subcloning the indicated commercially synthesized gene constructs (Genewiz, South Plainfield, NJ) onto pBAD18 downstream of the pBAD promoter using standard cloning procedures as described previously (Guzman et al. 1995). The *sopE* and *sopA S.* Typhimurium coordinates used in these constructs were: *sopE* 1952422–1952734 and *sopA* 2141570–2141855. The HA, Flag, and CyaA sequences used are standard and commonly available as previously described (Sory et al. 1995; Wilson and Nickerson 2006a). The R995 + SPI-1 *sipB*-Flag plasmid was constructed using recombineering via the FRUIT technique as previously described (Stringer et al. 2012). DNA primers (with PCR template plasmids indicated) used for all recombineering constructs are provided in Table 2.

### 2.2. Protein techniques and Western blot analysis

Analysis of SPI-1 and SPI-2 protein expression via Western blot for SipC, SseB, p25, p15, HA, and Flag was performed as described previously (Wilson and Nickerson 2006a; Wilson et al. 2007; Jennings et al. 2012; Cangelosi et al. 2013). Preparation of secreted SPI-1 and SPI-2 proteins from bacterial cultures was performed as described previously (Wilson and Nickerson 2006a; Wilson et al. 2007; Jennings et al. 2012; Cangelosi et al. 2013). Briefly, bacterial cultures grown for 4 h (late exponential phase) were centrifuged and supernatants were saved for the analysis of secreted proteins.

**Table 1**
Strains and plasmids used in this study.

| Organism | Strain designation | Reference |
|---|---|---|
| *Salmonella* Typhimurium | χ3339 ΔSPI-1 | Wilson and Nickerson (2006a) |
| *Salmonella* Typhimurium | χ3339 ΔSPI-2 | Wilson et al. (2007) |
| *Salmonella* Typhimurium | 14028 ΔSPI-2 | This study |
| *Salmonella* Typhimurium | UK-1 ΔSPI-2 | This study |
| *Salmonella* Typhimurium | NCIMB ΔSPI-2 | This study |
| *Escherichia coli* | TOP10 | Invitrogen |
| *Escherichia coli* | MG1655 | ATCC 700926 |
| Escherichia coli | DH5α | Invitrogen |
| *Escherichia coli* | JA221 | ATCC 33875 |
| *Citrobacter freundii* | ATCC 8090 | American Type Culture Collection |
| *Enterobacter cloacae* | ATCC 23355 | American Type Culture Collection |
| *Salmonella bongori* | ATCC 43975 | American Type Culture Collection |
| Plasmid | Reference | |
| R995 | Pansegrau et al. (1994) | |
| R995 + SPI-1 | Wilson and Nickerson (2006a) | |
| R995 + SPI-1 Δ*invJ* | This study | |
| R995 + SPI-1 Δ*prgI* | This study | |
| R995 + SPI-1 Δ*invA* | Wilson and Nickerson (2006a) | |
| R995 + SPI-1 Δ*hilA* | This study | |
| R995 + SPI-1 *sipA*-HA | Wilson and Nickerson (2006a) | |
| R995 + SPI-1 *sipA*-Flag | Wilson and Nickerson (2006a) | |
| R995 + SPI-1 *sipB*-Flag | This study | |
| R995 + SPI-2 | Wilson et al. (2007) | |
| R995 + SPI-2 | Δ*ssrAB* this study | |
| pBAD18 | Guzman et al. (1995) | |
| pBAD18 + *sopE*-HA | This study | |
| pBAD18 + *sopE*-Flag | This study | |
| pBAD18 + *sopA*-HA | This study | |
| pBAD18 + *sopA*-Flag | This study | |
| pBAD18 + *sopE-cyaA* | This study | |
| pBAD18 + *sopA-cyaA* | This study | |
| pKD3 | (Datsenko and Wanner 2000) | |
| pAMD135 | (Stringer et al. 2012) | |

Supernatants were filtered through a 0.22 μm membrane filtration unit and proteins were precipitated in the presence of 10% trichloroacetic acid overnight. Precipitated proteins were then separated by centrifugation at 12,000 × *g* for 30 min, washed with cold acetone, and re-suspended in a small volume of PBS Tris pH 8 buffer.

### 2.3. CyaA translocation assay

Translocation of SopE-CyaA and SopA-CyaA into Int407 cells was assayed as described previously using the Amersham cAMP Biotrak Enzyme-immuno-assay System kit (GE Healthcare Life Science) per manufacturer’s instructions (Barker et al. 2009).

## 3. Results

### 3.1. The cloned SPI-1 T3SS is functional for expression and secretion activity in other Gram negative bacteria

We previously reported the cloning of the SPI-1 T3SS from the *S.* Typhimurium genome onto the plasmid vector R995 and its use to functionally complement *S.* Typhimurium ΔSPI-1 strains (Wilson and Nickerson 2006a). Moreover, R995 + SPI-1 causes biofilm formation in *S.* Typhimurium strains that is mediated by a SPI-1 mechanism that is independent of known biofilm

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