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PipB2 is a substrate of the *Salmonella* pathogenicity island 1-encoded type III secretion system

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ABSTRACT

Salmonella harbors two type III secretion systems, T3SS1 and T3SS2, encoded on the pathogenicity islands SPI1 and SPI2, respectively. Several effector proteins are secreted through these systems into the eukaryotic host cells. PipB2 is a T3SS2 effector that contributes to the modulation of kinesin-1 motor complex activity. Here, we show that PipB2 is also a substrate of T3SS1. This result was obtained infecting human epithelial HeLa cells for 2 h and was confirmed in murine RAW264.7 macrophages, and rat NRK fibroblasts. Analysis at different time points after infection revealed that translocation of PipB2 is T3SS1-dependent in epithelial cells throughout the infection. In contrast, translocation into macrophages is T3SS1-dependent during invasion but T3SS2-dependent at later time points. The N-terminal 10 amino acid residues contain the signal necessary for translocation through both systems. These results confirm the functional overlap between these virulence-related secretion systems and suggest a new role for the effector PipB2.

1. Introduction

Salmonella enterica is a facultative intracellular bacterium responsible for gastroenteritis and systemic infections in many animals including humans [1]. *S. enterica* virulence depends on two distinct type three secretion systems (T3SS). T3SS1 translocates proteins, called effectors, through the plasma membrane of the host cell and is necessary for invasion. T3SS2 is induced intracellularly, injects effectors through the membrane of the *Salmonella* containing vacuole (SCV), and is essential for survival and proliferation inside host cells. Both systems, however, depend on each other for efficient functioning [2], and, although most effectors are specific substrates of one T3SS, some effectors can be secreted by both. The genes encoding the structural components, many effectors and some regulators of T3SS1 and T3SS2 are located in two *Salmonella* pathogenicity islands, SPI1 and SPI2, respectively [2].

PipB2 was described as a *Salmonella* T3SS2 effector [3] with sequence similarity to a previously identified effector of the same system, PipB [4]. PipB2 is synthesized under SPI2-inducing growth conditions and upon infection of macrophages [5], where it localizes to the SCV and to *Salmonella*-induced filaments (SIFs) 12 h post-infection. PipB2 reorganizes late endosome/lysosome compartments in mammalian cells resulting in the centrifugal extension of SIFs away from the SCV along microtubules. This activity is a consequence of its kinesin-1 binding activity [6]. Since SifA, another T3SS2 effector, down-regulates kinesin-1 recruitment, PipB2 and SifA demonstrate antagonistic activities [6]. PipB2 promotes outward movement of the SCV when myosin II activity is inhibited [7]. The characteristic positioning of SCVs to juxtanuclear regions suggests that the kinesin-inhibitory action of SifA may be dominant over the effects of PipB2 at 8–14 h post infection. However, at later stages of epithelial cell infection there is an outward displacement of a significant proportion of SCVs that is dependent upon host microtubules, kinesin and PipB2, and that is involved in cell-to-cell spread of *Salmonella* during infection [8].

Here, we describe the screens that we carried out to detect T3SS effectors based on the generation of fusions with a fragment of the gene *cyaA* from *Bordetella pertussis*, called *cyaA′*, encoding the catalytic domain of a calmodulin-dependent adenylate cyclase. This enzyme catalyzes the conversion of ATP to cyclic AMP (cAMP) in the presence of calmodulin. Because calmodulin is present in eukaryotic host cells, but not in bacteria, translocation of a CyaA′ fusion can be detected as an increase in the level of cAMP in cell

Abbreviations: T3SS, type III secretion system; SPI, *Salmonella* pathogenicity island; SCV, *Salmonella* containing vacuole; SIF, *Salmonella*-induced filament; cAMP, cyclic AMP; LB, Luria–Bertani medium; Km, kanamycin; Cm, chloramphenicol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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cultures infected with *Salmonella*. As a result of these screens, we found that PipB2 can be translocated into the host cell under SPI1-inducing conditions. We show here that this effector is secreted into several mammalian cell types in a T3SS1-dependent manner at different time points after infection, and that the N-terminal 10 amino acid residues contain the signal necessary for secretion through T3SS1 and T3SS2.

2. Materials and methods

2.1. Bacterial strains, bacteriophages, strain construction, and bacterial culture

Escherichia coli and *S. enterica* serovar Typhimurium strains used in this study are described in Table 1. Transductional crosses using phage P22 HT 105/1 *int201* [9] were used for strain construction [10]. The standard culture medium for *S. enterica* and *E. coli* was Luria–Bertani (LB) broth. Solid LB contained agar 1.5% final concentration. Antibiotics were used at the following concentrations: kanamycin (Km), 50 μg ml^{-1}; chloramphenicol (Cm), 20 μg ml^{-1}; ampicillin, 100 μg ml^{-1}. For SPI1-inducing conditions, *Salmonella* strains were grown overnight at 37 °C in LB-0.3 M NaCl medium in static conditions. For SPI2-inducing conditions, bacteria were inoculated in minimal medium at pH 5.8 (LPM) containing 80 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 5.8), 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 0.1% casamino acids, 38 mM glycerol, 337.5 μM K_2HPO_4–KH_2PO_4 (pH 7.4) and 8 μM $MgCl_2$, and incubated 16–24 h at 37 °C with shaking.

2.2. DNA amplification with the polymerase chain reaction (PCR)

Amplification reactions were carried out as previously described [11]. PCR constructs were sequenced with an automated DNA sequencer (Stab Vida, Oeiras, Portugal) to confirm that the sequence was correct.

2.3. Construction of mutants

Disruption and replacement of 34.8 kb of SPI1, from *avrA* to *invH* genes, with a Km resistance gene (mutant ΔSPI1) and of 21.4 kb of SPI2, from *ssaU* to *ssaB*, with a Cm resistance gene (mutant ΔSPI2) were performed as previously described [12], using specific primers (Table 2). The antibiotic resistance cassette introduced by the gene-targeting procedure was eliminated by recombination using the FLP helper plasmid pCP20 [12].

2.4. Transposon mutagenesis

Mutagenesis with mini-Tn*5cyaA′* was achieved by conjugation between the donor strain *E. coli* S17-1 λpir carrying pUT/mini-Tn*5cyaA′* [13] and the recipient strains *S. enterica* serovar Typhimurium 14028, SV5030 (14028 *slrP*::Cm^r), or SV6151 (14028 ΔSPI1 ΔSPI2 *slrP*::Cm^r). Recipient strains were incubated for 30 min at 50 °C prior to mating in order to temporarily inactivate host restriction and increase the frequency of conjugation [14]. Aliquots of the donor and the recipient (500 μl) were harvested by centrifugation, mixed on a 0.45-μm-pore-size membrane filter placed on LB plates, and incubated at 37 °C for 4 h. After mating, the mixtures were suspended in 10 mM $MgSO_4$ and spread on LB agar with Km.

2.5. Plasmids

Plasmids used in this study are listed in Table 1. Plasmids expressing CyaA′ fusions were derivatives of pIZ1673 [11], a modification of pSIF003-R1 [15]. This plasmids were constructed as previously described [11,15] using primers listed in Table 2.

2.6. Mammalian cell culture

HeLa cells (human epithelial; ECAC No. 93021013), RAW264.7 cells (murine macrophages; ECACC No. 91062702), and NRK-49F (normal rat kidney fibroblasts; ATCC CRL1570) were cultured in DMEM supplemented with 10% fetal calf serum. About 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin were

Table 1
Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics	Source/reference
E. coli		
DH5α	*supE44* Δ *lacU169* (Ø80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[23]
S17-1 λpir	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn*7* λpir	[24]
TP610	F-, *thi-1 thr-1 leuB6 lacY1 tonA21 supE44 hsdR hsdM recBC lop-11 lig*$^+$*cya-610*	[25]
S. enterica serovar Typhimurium[a]		
14028	Wild type	ATCC
SV5030	14028 Δ*slrP*::Cm^r	Laboratory stock
SV6017	14028 ΔSPI2::Cm^r	This study
SV6055	14028 ΔSPI1::Km^r	This study
SV6151	14028 ΔSPI1 ΔSPI2 ΔslrP::Cm	This study
SV6619	14028 Δ*slrP*::Cm^r *pipB2*::mini-Tn*5cyaA′*	This study
Plasmids		
pKD3	*bla* FRT *cat* FRT PS1 PS2 oriR6 K	[12]
pKD4	*bla* FRT *aph* FRT PS1 PS2 oriR6 K	[12]
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS	[12]
pCP20	*bla cat* cI857 λP_R *flp* pSC101 oriTS	[26]
pIZ1673	pSIF003-R1 ΔlacI	[11]
pIZ1907	pIZ1673-PipB2(1–350)	This study
pIZ1908	pIZ1673-PipB2(11–350)	This study
pIZ1911	pIZ1673-PipB2(1–10)	This study
pIZ1913	pIZ1673-PipB2(1–48)	This study
pIZ1947	pIZ1673-PipB2(1–100)	This study
pIZ1948	pIZ1673-PipB2(1–225)	This study
pUTmini-Tn*5cyaA′*	Suicide delivery plasmid for mini-Tn*5cyaA′*	[13]

[a] Derivatives of these strains were used as indicated in the text.