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Original article

IpgB1 and IpgB2, two homologous effectors secreted via the Mxi-Spa type III secretion apparatus, cooperate to mediate polarized cell invasion and inflammatory potential of *Shigella flexenri*

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Abstract

Type III secretion systems (T3SS) are present in many pathogenic gram-negative bacteria and mediate the translocation of bacterial effector proteins into host cells. Here, we report the phenotypic characterization of *S. flexneri ipgB1* and *ipgB2* mutants, in which the genes encoding the IpgB1 and IpgB2 effectors have been inactivated, either independently or simultaneously. Like IpgB1, we found that IpgB2 is secreted by the T3SS and its secretion requires the Spa15 chaperone. Upon infection of semi-confluent HeLa cells, the *ipgB2* mutant exhibited the same invasive capacity as the wild-type strain and the *ipgB1* mutant was 50% less invasive. Upon infection of polarised Caco2-cells, the *ipgB2* mutant did not show a significant defect in invasion and the *ipgB1* mutant was slightly more invasive than the wild-type strain. Entry of the *ipgB1 ipgB2* mutant in polarized cells was reduced by 70% compared to the wild-type strain. Upon infection of the cornea in Guinea pigs, the *ipgB2* mutant exhibited a wild-type phenotype, the *ipgB1* mutant was hypervirulent and elicited a more pronounced proinflammatory response, while the *ipgB1 ipgB2* mutant was highly attenuated. The attenuated phenotype of the *ipgB1 ipgB2* mutant was confirmed using a murine pulmonary model of infection and histopathology and immunochemistry studies.

Keywords: *Shigella*; Type III secretion; Invasion; Tight junction

1. Introduction

Shigella flexneri causes bacillary dysentery and is the major aetiological agent of the endemic form of this disease. *Shigella* and many other gram-negative bacteria use a type III secretion apparatus (T3SA) to interact with host cells. The major function of the T3SA is to deliver effector proteins into the cytoplasm of the host cell, where they interfere with host components to subvert cellular process [1,2]. In *Shigella*, the T3SA is responsible of the entry of bacteria into epithelial cells and for cell-to-cell spread [3]. The genes required for

Abbreviations: T3SA, type 3 secretion apparatus; BALT, bronchus-associated lymphoid tissue; MHC, major histocompatibility complex; CFU, colony forming unit; MCDK, Madin-Darby canine kidney; PMN, polymorphonuclear; LPS, lipopolysaccharides; TJ, tight junction.

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entry are located on a 30-kb entry region of the virulence plasmid pWR100 which carries 31 genes clustered in two divergently transcribed loci [3]. The *mxi-spa* locus encodes mostly components of the Mxi-Spa T3SA [3–6]. The *ipa-ipg* locus encodes the secreted IpaA, IpaB, IpaC and IpaD proteins that are the major factors mediating entry into epithelial cells [3]. Contact of bacteria with the host cell induces secretion through the T3SA, which leads to IpaB and IpaC insertion into the host cell membrane to form a pore used for the translocation of bacterial effectors into cells [7]. After internalization, *Shigella* lyses the membrane surrounding the phagocyte vacuole and then migrates from cell to cell through an actin-based motility mechanism [3].

Upon bacterial entry, the NF-κB pathway is activated [8], which in turn induces production and secretion of interleukin 8 (IL-8). In animal models of Shigellosis, IL-8 contributes to the massive inflammatory response characteristic of natural infections [9]. Inflammation is sustained mainly by a polymorphonuclear leukocyte (PMN) influx that destroys intercellular junctions and allows bacteria to access the basolateral pole of epithelial cells [9]. Bacteria are proposed to cross the epithelial barrier through M cells [10], before becoming internalized by macrophages, or invading epithelial cells via their basolateral side. Wells et al. [11] showed that preferential adherence of *S. flexneri* on the exposed lateral surface of the intestinal epithelial cells is associated with enhanced *S. flexneri* internalization.

Here, we investigated the function of the *ipgB1* and *ipgB2* genes encoding two proposed T3S effectors. The biological role of IpgB1 and IpgB2 has been extensively studied in vitro. IpgB2 was reported to act as a molecular mimic of Rho [12,13], and to induce stress fibers [13]. In contrast, IpgB1 was shown to be a functional Rac mimic capable of inducing cell surface lamelliopodia. More recently, IpgB1 was demonstrated to produce membrane ruffles by exploiting the RhoG-ELMO-Dock 180 pathway [14]. Although there are recent in vitro studies addressing the question about the role of these proteins, there is an absolute need to understand the impact of these genes on *Shigella* virulence. Previous reports have shown that IpgB1, encoded by a gene located within the *ipa* operon, is secreted via the T3SA [15,16], and requires the Spa15 chaperone for its stability [16]. Despite the relevant information about the biological activity of these proteins, the role of IpgB1 and IpgB2 in *Shigella* virulence has been not fully unveiled. To acquire more information about the role the *ipgB1* and *ipgB2* genes in the virulence of *Shigella*, in this study we inactivated the two genes either individually or together. The virulence of the *ipgB* mutants was analyzed in vitro using cell culture assays, and in vivo with both the Sereny test and the murine pulmonary model of shigellosis.

2. Materials and methods

2.1. Bacterial strains and growth media

S. flexneri strains were derivatives of the wild-type M90T-Sm (Sm^R) [4]. *Escherichia coli* DH5αλpir and Sm10λpir were used for cloning of the suicide vector pGP704 derivatives, and for their transfer to *S. flexneri*. Bacteria were grown at 37 °C in Luria-Bertani (LB) medium or tryptic casein soy broth (TSB). Antibiotics were used at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; gentamicin, 50 μg ml^{-1}; and zeocin, 25 μg ml^{-1}.

2.2. Construction of plasmids and strains

Strains and plasmids used in this study are listed in Table 1. The *ipgB1* mutant was constructed using the allelic exchange method. Firstly, plasmid pRM34 was constructed by cloning a 1442-bp MluI-EcoRV DNA fragment of pWR100 [15], encompassing the 3′end of *ipgA*, the entire *ipgB1* gene, and the 5′ end of *ipgC* into SmaI digested pUC19. Plasmid pRM35 was constructed by replacing the 248-bp NruI-MscI fragment of pRM34, internal to *ipgB1* by the 850-bp SmaI DNA fragment carrying the *aphA-3* gene. Then, a 2342-bp PvuII fragment, encompassing the inactivated *ipgB1* gene, was inserted into EcoRV digested pGP704 suicide vector. The resulting pRM36 plasmid was then transferred to *S. flexneri* M90T-Sm by conjugation, and transconjugants were selected on plates containing streptomycin and kanamycin. Clones in which a double recombination event had exchanged the wild-type *ipgB1* gene for the mutated copy were screened for their sensitivity to ampicillin. The structure of the *ipgB1* mutation (designated SBF1) was further confirmed by PCR. For the inactivation of the *ipgB2* gene, firstly, a DNA fragment of pWR100 encompassing a region from position 428 upstream from the 5′ end of the *ipgB2* gene through to nucleotide 60 downstream from the 5′ end was amplified by PCR. The 1052-bp DNA fragment was digested by EcoRI and NheI and cloned into pTZ18R to generate plasmid pHA18. A SpeI-XbaI fragment containing the non-polar *ble* gene conferring resistance to zeomycin, amplified from pZeoSv, was inserted into SpeI-XbaI sites internal to *ipgB2* giving rise to pHA23. A 1310-bp PvuII-SalI fragment encompassing the inactivated *ipgB2* gene was then inserted into EcoRV-SalI digested pGP704. The resulting plasmid, pHA24, was then transferred to M90T-Sm and to the *ipgB1* by conjugal mating. Transconjugants were selected for their resistance to zeocin

Table 1
Strains used in this study

Strain	Genotype	Reference
M90T-Sm	Sm^R derivative of wild-type strain M90T	[4]
SF401	M90T-Sm *mxiD* (*mxiD*::*aphA-3*)	[3]
SBF1	M90T-Sm *ipgB1* (*ipgB1*::*aphA-3*)	This study
SBH55	M90T-Sm *ipgB2* (*ipgB2*::*ble*)	This study
SBH57	M90T-Sm *ipgB1 ipgB2* (*ipgB1*::*aphA-3, ipgB2*::*ble*)	This study
SBH63	SBH55/pHA28 (native IpgB2)	This study
SBH58	SBH57/pHA28 (native IpgB2)	This study
SBH 148	SBH57/pHA61 (native IpgB1)	This study
SBH147	SBF1/pHA61 (native IpgB1)	This study
SF1601	M90T-Sm *spa15* (*spa15*::*aphA-3*)	[16]
SBH137	SF1601/pHA57 (native Spa15)	This study