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TolC, but not AcrB, is involved in the invasiveness of multidrug-resistant *Salmonella enterica* serovar Typhimurium by increasing type III secretion system-1 expression

Isabelle Virlogeux-Payant*, Sylvie Baucheron, Julien Pelet, Jérôme Trotereau, Elisabeth Bottreau, Philippe Velge, Axel Cloeckaert

INRA, UR1282 Infectiologie Animale et Santé Publique, IASP, F-37380 Nouzilly, France

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Abstract

The AcrAB-TolC efflux system is involved in multidrug and bile salt resistances. In addition, this pump has recently been suggested to increase the invasion of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) into host cells in vitro and could therefore have an important clinical relevance for multidrug-resistant strains. The aim of this study was to investigate the role of the TolC outer membrane channel and the AcrB transporter in the interaction of multidrug-resistant *S.* Typhimurium strains with eukaryotic cells, especially in relation to the expression of the type III secretion system-1 (TTSS-1) required for *Salmonella* invasion. Deletion of *tolC* led to a reduced transcription of the *Salmonella* pathogenicity island-1 genes *sipA*, *invF* and *hilA*, demonstrating that all genes required for TTSS-1 biosynthesis are down-regulated in this mutant. Consequently, *tolC* mutants secreted smaller amounts of the TTSS-1 effector proteins SipA and SipC, and invasion tests performed with one mutant showed that it was significantly less able to invade HT-29 epithelial cells than its parental strain. This control seems specific to the TTSS-1 among the three TTSS of *Salmonella* as no down-regulation of expression of TTSS-2 or flagella was observed in this mutant. By contrast, *acrB* mutants behaved as their parents except that they secrete a slightly greater amount of SipA and SipC proteins. These data indicate that TolC but not AcrB mediates the uptake of multidrug-resistant *S.* Typhimurium into target host cells. Therefore, this role of TolC in the invasion of the intestine in addition to its role in bile salt resistance reinforces the interest of targeting TolC for fighting multidrug-resistant *Salmonella*.

Keywords: Multidrug resistance; Efflux systems; *Salmonella*; Invasion; Type III secretion; Adhesion

Introduction

Salmonellosis is an important worldwide health problem. *Salmonella enterica* causes diseases ranging from gastroenteritis to life-threatening systemic infections. Usually, systemic infections are caused by serovars with restricted host range, such as Typhi in humans, or Pullorum and Gallinarum in poultry, while non-typhoidal serovars are mainly responsible for gastroenteritis. The most frequent serovars isolated from human foodborne infections in the EU and in the USA are Typhimurium (*S.* Typhimurium) and Enteritidis (*S.* Enteritidis) (Velge et al., 2005). In recent

*Corresponding author. Tel.: +33 2 4742 7660; fax: +33 2 4742 7779.
E-mail address: Isabelle.Virlogeux@tours.inra.fr (I. Virlogeux-Payant).

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years, one of the most important clinical problems with salmonellosis is the emergence of multidrug-resistant strains such as the epidemic *S.* Typhimurium DT104 clone (Mulvey et al., 2006). This clone carries a genomic island called SGI1 containing an antibiotic resistance cluster conferring resistance mostly to five antibiotic classes. Active efflux has also recently been shown to play an important role in multidrug resistance, particularly in strains that are also resistant to quinolones (Baucheron et al., 2004). In addition to this role in antibiotic resistance, these efflux pumps seem to have very important clinical relevance since they have been shown to be involved in the virulence of different bacterial pathogens (Baucheron et al., 2005; Buckley et al., 2006; Hirakata et al., 2002; Lacroix et al., 1996; Lin et al., 2003; Nishino et al., 2006; Piddock, 2006; Posadas et al., 2007; Stone and Miller, 1995).

The AcrAB-TolC efflux system is one of the pumps for which a role in antibiotic resistance and in virulence has been demonstrated in *S.* Typhimurium (Baucheron et al., 2005; Buckley et al., 2006; Lacroix et al., 1996; Nishino et al., 2006). Overexpression of this efflux system has clearly been associated with multidrug resistance in human and animal isolates in *S.* Typhimurium (Baucheron et al., 2004; Giraud et al., 2000; Piddock et al., 2000). This efflux system allows the export of a broad spectrum of antibiotics, including quinolones/fluoroquinolones which are therapeutics of choice to treat life-threatening salmonellosis (Baucheron et al., 2004). The AcrAB-TolC efflux system is also involved in resistance to detergents and dyes and it has been shown to play an important role in bile salt resistance (Baucheron et al., 2005; Bina and Mekalanos, 2001; Lacroix et al., 1996; Thanassi et al., 1997). In addition, several laboratories have reported the role of the AcrAB-TolC efflux pump, or its homologues, in the virulence of Gram-negative bacteria (for a review, see (Piddock, 2006)). In *Salmonella*, *acrB* and *tolC* mutants have been shown to be attenuated in the chick and mouse models of infection (Baucheron et al., 2005; Buckley et al., 2006; Lacroix et al., 1996; Nishino et al., 2006; Stone and Miller, 1995) and notably in the colonization of the intestine (Baucheron et al., 2005; Buckley et al., 2006; Lacroix et al., 1996). The first explanation that could be proposed for this attenuation is the bile salt resistance conferred by the AcrAB-TolC efflux system. However, *tolC* and *acrB* mutants of the commonly used antibiotic-sensitive *S.* Typhimurium SL1344 strain have recently been shown to be less able to invade intestinal epithelial cells in vitro than their respective wild-type strains (Buckley et al., 2006), suggesting that the AcrAB-TolC efflux pump could also play a role in the interaction of *Salmonella* with the intestine.

Invasion of the intestinal epithelium is an essential feature of the *Salmonella* pathogenic life cycle. The entry process mainly involves the type-three secretion system (TTSS)-1 encoded on the *Salmonella* pathogenicity island (SPI)-1. This system allows the translocation of a large set of effector proteins directly from the bacterial cytoplasm into the cytosol of the host cell. These effector proteins induce local cytoskeleton rearrangements, leading to membrane ruffling, macropinocytosis and finally to *Salmonella* internalization (Patel et al., 2005). Expression of the TTSS-1 is mostly dependent on the central transcriptional regulator HilA encoded on SPI-1. HilA activates the SPI-1 *inv/spa* and *prg* operons, encoding components of the TTSS-1 secretion apparatus and indirectly regulates the expression of TTSS-1-secreted proteins by activating the transcription of the SPI-1-encoded transcriptional regulator InvF (Bajaj et al., 1995; Darwin and Miller, 1999). Two other TTSS are important for the virulence of *Salmonella.* The flagella, which – according to their structure – are considered to be a TTSS, confer motility to the bacteria and therefore favour their interaction with the intestinal epithelium (La Ragione et al., 2003; Schmitt et al., 2001). The TTSS-2 is required for the intracellular survival of *Salmonella* (Waterman and Holden, 2003).

The hypothesis of a role of the AcrAB-TolC efflux pump in the interaction of *Salmonella* with the intestine is of particular interest in the case of multidrug-resistant strains, such as the epidemic clone of *S.* Typhimurium DT104, with the aim of combating the further spread of these strains of clinical importance. Indeed, such demonstration would reinforce the interest of using this efflux system as a target against multidrug-resistant *Salmonella*. The aim of this study was therefore to investigate the role of TolC and AcrB in the uptake of representative multidrug-resistant epidemic strains of *S.* Typhimurium into host cells. As the TTSS-1 is the most important factor involved in invasion, we focused our intention on the study of the expression of the TTSS-1 and of the secretion of TTSS-1 effector proteins in *tolC* and *acrB* mutants of these multidrug-resistant strains of *S.* Typhimurium. The adhesion ability of these mutants was also studied.

Materials and methods

Bacterial strains and culture conditions

The *S.* Typhimurium DT104 strains used in this study were isolated from cattle. *S.* Typhimurium 1948SA96 is a multidrug-resistant and quinolone-susceptible strain and *S.* Typhimurium BN10055 is a multidrug- and quinolone-resistant strain. *S.* Typhimurium DT204 strain 102SA00 is a multidrug- and high-level fluoroquinolone-resistant strain isolated in Belgium from animal feed imported from China. *acrB* and *tolC* mutants have previously been described (Baucheron et al., 2004, 2005). Bacteria were