

RosE represses Std fimbrial expression in *Salmonella enterica* serotype Typhimurium

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Summary

The *Salmonella enterica* serotype Typhimurium (*S. typhimurium*) genome contains a large repertoire of putative fimbrial operons that remain poorly characterized because they are not expressed *in vitro*. In this study, insertions that induced expression of the putative *stdABCD* fimbrial operon were identified from a random bank of transposon mutants by screening with immuno-magnetic particles for ligand expression (SIMPLE). Transposon insertions upstream of *csgC* and *IrhA* or within *dam*, *setB* and *STM4463* (renamed *rosE*) resulted in expression of StdA and its assembly into fimbrial filaments on the cell surface. RosE is a novel negative regulator of Std fimbrial expression as indicated by its repression of a *std::lacZ* reporter construct and by binding of the purified protein to a DNA region upstream of the *stdA* start codon. Expression of Std fimbriae in the *rosE* mutant resulted in increased attachment of *S. typhimurium* to human colonic epithelial cell lines (T-84 and CaCo-2). A *rosE* mutant exhibited a reduced ability to compete with virulent *S. typhimurium* for colonization of murine organs, while no defect was observed when both competing strains carried a *stdAB* deletion. These data suggest that a tight control of Std fimbrial expression mediated by RosE is required during host pathogen interaction.

Introduction

Laboratory-grown cultures of *Salmonella enterica* serotype Typhimurium (*S. typhimurium*) commonly elaborate

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only type 1 fimbriae (Duguid *et al.*, 1966) encoded by the *fim* operon (Clegg *et al.*, 1987) and thin curled fimbriae (also known as thin aggregative fimbriae or curli) (Grund and Weber, 1988; Stolpe *et al.*, 1994) encoded by the *csg* (*agf*) gene cluster (Romling *et al.*, 1998). Two other *S. typhimurium* fimbrial gene clusters, termed *pef* (Friedrich *et al.*, 1993) and *lpf* (Bäumler and Heffron, 1995), induce expression of fimbriae when the cloned genes are introduced into *Escherichia coli*. However, nucleotide sequencing identified nine additional putative fimbrial operons in the *S. typhimurium* genome, termed *bcf* (Tsolis *et al.*, 1999), *stf* (Emmerth *et al.*, 1999; Morrow *et al.*, 1999), *saf* (Folkesson *et al.*, 1999), *stb*, *stc*, *std*, *sth*, *sti* and *stj* (McClelland *et al.*, 2001). The fact that nine putative fimbrial operons were only identified by sequence analysis in this genetically well-characterized organism reflects the tight control of their expression *in vitro*, which is detectable neither by Western blot (Humphries *et al.*, 2005) nor by flow cytometry (Humphries *et al.*, 2003) after growth of *S. typhimurium* under standard laboratory conditions. This lack of *in vitro* expression has prevented a thorough functional characterization of the encoded adhesins.

The *std* operon was initially identified during sequence analysis of the human-adapted *S. enterica* serotype Typhi strain CT18 (Townsend *et al.*, 2001). Many of the putative fimbrial operons identified in the genomes of human-adapted *Salmonella* serotypes, including *S. enterica* serotypes Paratyphi A and Typhi (strains CT18 and Ty2), contain pseudogenes (i.e. genes carrying frameshift mutations or stop codons) (Parkhill *et al.*, 2001; Deng *et al.*, 2003; McClelland *et al.*, 2004). Interestingly, only two fimbrial operons, *tcf* and *std*, share the following characteristics: they are present and apparently intact in all three available genomes of human-adapted *Salmonella* serotypes (Andrews-Polymenis and Bäumlér, 2006). DNA–DNA hybridization studies show that the majority of *Salmonella* serotypes investigated contain orthologues of the *std* operon (Townsend *et al.*, 2001; Porwollik *et al.*, 2002; 2004; Chan *et al.*, 2003; Anjum *et al.*, 2005). Sequence comparison of its usher protein identifies the *std* operon as a member of the π -fimbriae, a group including well-characterized virulence factors such as pyelonephritis-associated (P) fimbriae of *E. coli* and the Mannose-resistant/*Proteus*-like (MR/P) fimbriae of

Proteus mirabilis (Nuccio and Bäuml, 2007). Deletion of the *std* operon in *S. typhimurium* causes a competitive disadvantage during intestinal persistence in a mouse model (Weening *et al.*, 2005). Mice infected with *S. typhimurium* seroconvert to StdA, which provides indirect evidence for *in vivo* expression of the *std* operon (Humphries *et al.*, 2005). Gene expression profiling of an *S. typhimurium* *dam* mutant recently demonstrated that transcription of the *std* operon is repressed by Dam methylation (Balbontin *et al.*, 2006). However, as the elaboration of surface structures has not been demonstrated in *S. typhimurium*, *std* must still be considered a putative fimbrial operon.

In this study we used a genetic screen to identify novel genes silencing expression of the *std* operon *in vitro* and demonstrated for the first time expression of the encoded fimbriae in *S. typhimurium*. Our results represent an important first step in characterizing Std fimbriae and lead the way for future functional studies on the remaining putative fimbrial operons present in the *S. typhimurium* genome.

Results

Identification of *S. typhimurium* mutants expressing StdA using immuno-magnetic particles for ligand expression (SIMPLE)

To identify novel regulatory genes controlling expression of StdA *in vitro*, we generated mutant libraries in an *S. typhimurium* *fim* mutant (AJB4) using the transposons Mud-Cam (Elliott, 1993) and T-POP (Rapple and Roth, 1997). Approximately 18 500 Mud-Cam mutants were generated, approximately 40% of which resulted from homologous recombination of the transposon into the *hisD* gene (data not shown), because the transposon was transduced from a donor strain (TE3461) carrying a *hisD::Mud-Cam* insertion. Thus, the Mud-Cam mutant bank contained an estimated 11 000 random insertion mutants. In addition, a bank containing approximately 9000 random T-POP insertion mutants was generated in an *S. typhimurium* *fim* mutant (AJB4). To this end, a plasmid encoding a Tn10 transposase with broad target specificity (pNK2880) was introduced into the *S. typhimurium* *fim* mutant. The T-POP transposon was delivered into this strain by transduction from an *S. typhimurium* donor (TH3923) (Lee *et al.*, 2007) carrying the transposon on an *E. coli* F' plasmid. A derivative of *S. typhimurium* isolate SR11 was used for this procedure, because this strain can be transduced with P22 to chloramphenicol (Mud-Cam) or tetracycline (T-POP) resistance, but is resistant to P22-mediated lysis, thus avoiding a selection for phage-resistant mutants during culture of mutant pools.

The immuno-magnetic particles for ligand expression (SIMPLE) approach (Nuccio *et al.*, 2007) is based on the

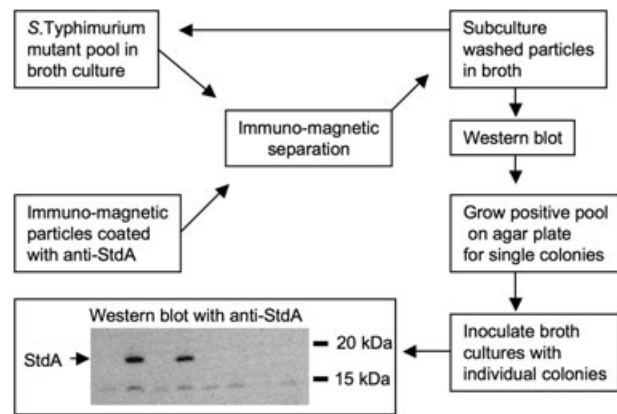


Fig. 1. Flow chart of the SIMPLE approach used to identify *S. typhimurium* mutants expressing StdA. The bottom panel shows a Western blot detected with rabbit anti-StdA serum. Each lane represents a culture inoculated with a single colony from a mutant pool after two rounds of enrichment with immuno-magnetic particles coated with anti-StdA serum. The molecular mass of standard proteins is indicated on the right.

idea that incubation of a mutant bank with magnetic particles coated with antiserum raised against a fimbrial protein can be used to enrich for fimbriated mutants by immuno-magnetic separation (Nuccio *et al.*, 2007). The Mud-Cam and T-POP mutant banks were divided into 16 and 7 pools, respectively, and each pool was used to inoculate a static Luria-Bertani (LB) broth (pH 7) culture with (for T-POP) or without (for Mud-Cam) tetracycline. Each pool was incubated with magnetic particles coated with anti-StdA serum; particle-bound bacteria were re-suspended in broth and used to inoculate a second static broth culture to repeat the enrichment procedure. After two enrichment cycles, a sample from each pool was grown statically in LB broth and expression of StdA by bacteria in the pool was assessed by Western blot (Fig. 1). Four T-POP pools and five Mud-Cam pools were positive for StdA expression, suggesting that enrichment for a mutant expressing this protein had occurred. Twenty bacterial colonies from each positive pool were grown statically in LB broth to identify individual transposon mutants expressing StdA. One StdA-expressing mutant from each pool was selected, a lysate was prepared using phage KB1 *int* (because SR11 derivatives are resistant to P22-mediated lysis) and the transposon insertion was transduced into an *S. typhimurium* *fim* mutant (AJB4). Transduction yielded four T-POP mutants (CD6R, CD7R, CD12R and CD15R) and five Mud-Cam mutants (CD2R, CD8R, CD10R, DB2R and DB3R) that expressed StdA in LB broth.

Identification of transposon insertion sites

DNA regions flanking the T-POP transposon insertion sites in strains CD2R, CD6R, CD7R, CD8R, CD12R,

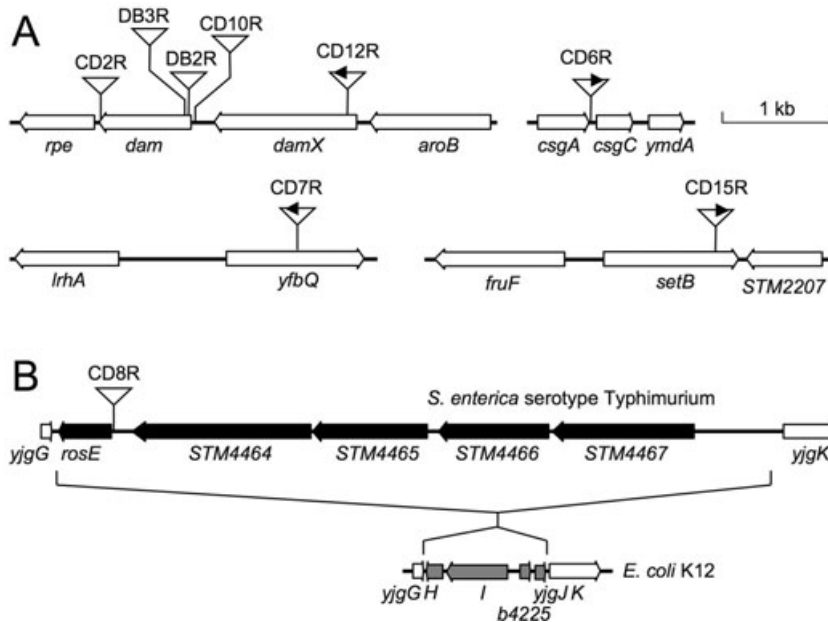


Fig. 2. Transposon insertion sites in *S. typhimurium* mutants expressing StdA. A. Arrows indicate the size and orientation of genes in the *S. typhimurium* genome. The location of transposon insertions is indicated above each genetic map. Arrowheads indicate the orientation of the *tet* promoter of T-POP (CD12R, CD6R, CD7R, CD15R). B. Comparison of the *yjgG*–*yjgK* intergenic regions of *S. typhimurium* strain LT2 (top) and *E. coli* strain K12 (bottom). Closed arrows indicate the size and orientation of genes present in the *S. typhimurium* genome but absent from *E. coli* K12. Open arrows indicate genes present in *S. typhimurium* and *E. coli*. Grey arrows indicate genes present in *E. coli* K12 but absent in *S. typhimurium*.

CD10R, CD15R, DB2R and DB3R were cloned by inverse polymerase chain reaction (PCR) and the respective nucleotide sequences were determined. Four Mud-Cam insertion sites were either within or immediately upstream of the *dam* open reading frame, at base pair positions +803 (CD2R), +36 (DB3R), +10 (DB2R) or –40 (CD10R) relative to the start codon. One T-POP insertion was located in *damX*, the open reading frame located directly upstream of *dam*, at base pair position +20 (CD12R) relative to the start codon (Fig. 2). The deoxyadenosine methylase (Dam) encoded by the *dam* gene has previously been implicated in controlling expression of fimbriae in *E. coli* (Blyn *et al.*, 1990; van der Woude and Low, 1994) and *S. typhimurium* (Nicholson and Low, 2000).

The T-POP insertion in CD6R was located in the *csgA* *csgC* intergenic region, at base pair position –60 relative to the *csgC* start codon. The *csg* gene cluster is involved in the biosynthesis of thin curled fimbriae in *S. typhimurium* (Romling *et al.*, 1998); however, no function in fimbrial biosynthesis has been assigned to the *csgC* open reading frame. One T-POP insertion (CD15R) was found in the *setB* gene, at base pair position +1145 relative to the start codon. The *setB* gene encodes a sugar transporter (Liu *et al.*, 1999) and mutations have been associated with pleiotrophic phenotypes, including a delay in chromosome segregation (Espeli *et al.*, 2003).

One T-POP mutant (CD7R) did not express StdA after growth in LB broth in the absence of tetracycline (Fig. 3), suggesting that the *tetA* promoter of T-POP may drive expression of a positive regulatory element in this mutant. The T-POP insertion in CD7R was located in the *yfbQ* open reading frame, at base pair position +600 relative to the start codon. Mutant CD7R carried the T-POP insertion

in an orientation in which the *lrhA* gene is located downstream of the *tetA* promoter (Fig. 2). LrhA is a negative regulator of flagellar, chemotactic, motility and type 1 fimbrial gene expression in *E. coli* (Lehnen *et al.*, 2002; Blumer *et al.*, 2005).

One Mud-Cam insertion was located upstream of open reading frame STM4463 (from hereon referred to as *rosE*), at base pair position –40 relative to the start codon. The protein encoded by *rosE* had homology to transcriptional regulators and showed the highest sequence identity to the ArgR (29%) (Lim *et al.*, 1987) and Fur (16%) (Schäffer *et al.*, 1985) proteins of *E. coli*. The *rosE* gene was located on an approximately 5 kb islet that was found to be present in all sequenced genomes of *Salmonella* serotypes and in genomes of uropathogenic *E. coli*, but absent from other sequenced *E. coli* genomes (Fig. 2). Open reading frames on this islet encode a putative arginine deiminase system composed of arginine deiminase (STM4467), carbamate kinase (STM4466), ornithine carbamyltransferase (STM4465) and an arginine-ornithine antiporter (STM4464). Similar arginine deiminase operons have been described in both Gram-positive and Gram-negative bacteria where they are involved in catabolizing arginine to ornithine, ammonia and carbon dioxide with the concomitant production of ATP (Luthi *et al.*, 1986; 1990; Maghnoij *et al.*, 1998).

StdA expression in *S. typhimurium* mutants is associated with enhanced transcription from the *std* promoter

Analysis of StdA expression divided mutants identified by SIMPLE into those exhibiting a strong signal by Western

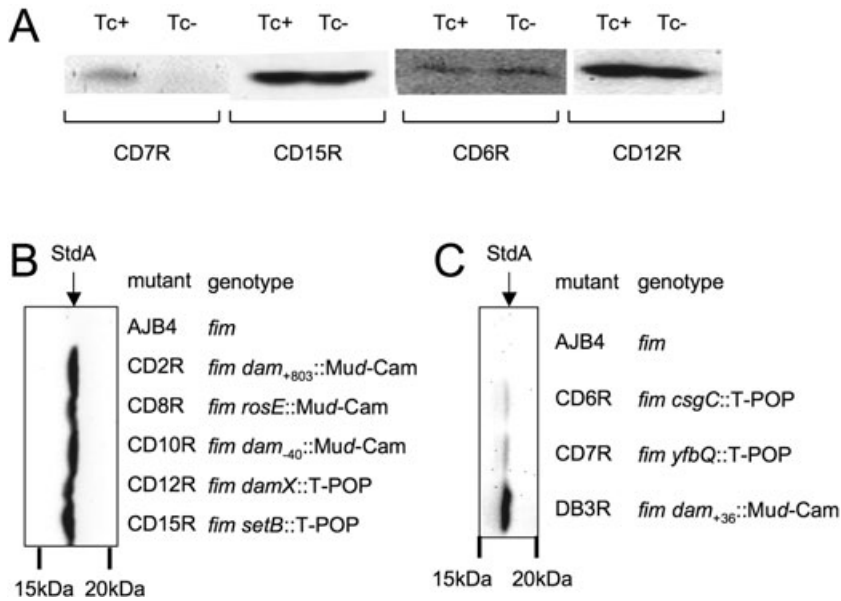


Fig. 3. Expression of StdA by *S. typhimurium* mutants.

A. Expression of StdA detected by Western blot in T-POP insertion mutants (CD7R, CD15R, CD6R and CD12R) grown in the presence (Tc+) or absence (Tc-) of tetracycline.

B. Expression of StdA (arrow) detected after short (20 min) exposure of a Western blot. Bacterial strains analysed are indicated on the right. The molecular mass of standard proteins is indicated at the bottom.

C. Expression of StdA (arrow) detected after overnight exposure of a Western blot. Bacterial strains analysed are indicated on the right. The molecular mass of standard proteins is indicated at the bottom.

blot minutes after exposure (Fig. 3B) and those in which a signal was only detectable after prolonged exposure of Western blots (Fig. 3C). Mutants with high levels of StdA expression included *rosE::Mud-Cam* (CD8R), *setB::Mud-Cam* (CD15R), *dam₊₈₀₃::Mud-Cam* (CD2R), *dam₋₄₀::Mud-Cam* (CD10R), *dam₊₁₀::Mud-Cam* (DB2R) and *damX::T-POP* (CD12R). The *damX::T-POP* mutant (CD12R) expressed StdA when cultures were inoculated from glycerol stocks, but not when cultures were inoculated from a colony grown on LB agar plates, which may result from selection for compensatory mutations (however, this was not further analysed). None of the other mutants exhibited this phenotype. Mutants with lower levels of StdA expression included *yfbQ::T-POP* (CD7R) and *csgC::T-POP* (CD6R), while the *dam₊₃₆::Mud-Cam* mutant (DB3R) exhibited an intermediate level of expression (Fig. 3C).

To determine whether expression of StdA in individual mutants was accompanied with increased expression of the *std* operon, the promoter region upstream of *stdA* (P_{std}) was introduced into vector pUJ10 oriented such that it controlled expression of a promoterless *lacZ* gene. The resulting plasmid (pDC57) was introduced into each of the mutants and their isogenic parent (AJB4). The parent strain AJB4(pDC57) expressed only low levels of β -galactosidase activity (approximately 2 Miller units on average) while all the T-POP or *Mud-Cam* insertions characterized in this study exhibited a significantly increased expression of the $P_{std}::lacZ$ reporter construct. The lowest levels of β -galactosidase expression were detected in the *yfbQ::T-POP* mutant (fivefold induction) and the *csgC::T-POP* mutant (sixfold induction) (Fig. 4), which also exhibited the weakest expression of StdA by Western blot

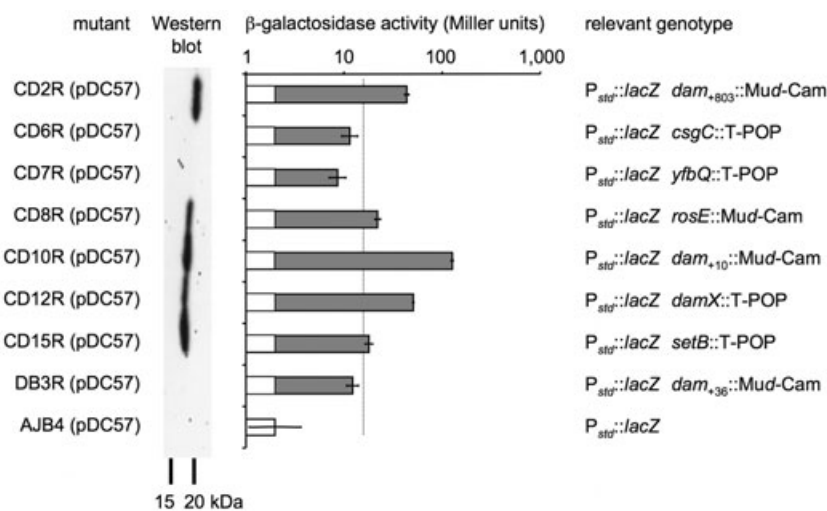


Fig. 4. Transcription of the *stdA* gene in *S. typhimurium* strains detected with a plasmid-based (pDC57) *lacZ* transcriptional fusion ($P_{std}::lacZ$). Bacterial strains analysed are indicated on the right. The second panel from the left shows a Western blot of each strain detected with anti-StdA serum. The molecular mass of standard proteins is indicated at the bottom. The β -galactosidase activities detected in each strain are shown at the centre. Each bar represents the average from three independent experiments \pm standard deviation. The genotype of each strain is given on the right.

(Fig. 3C). A stronger induction of the $P_{std}::lacZ$ reporter construct was detected in the $setB::Mud$ -Cam mutant (ninefold induction), the $rosE::Mud$ -Cam mutant (11-fold induction), the $damX::T$ -POP mutant (26-fold induction) and the dam mutants. Expression of StdA was detected after short exposure of Western blots in all mutants in which β -galactosidase expression from the $P_{std}::lacZ$ reporter construct was increased ninefold or higher compared with the parental strain (Fig. 4). These data suggest that expression of StdA in the T-POP or Mud-Cam mutants identified in this study were at least in part due to increased expression from the P_{std} promoter. However, expression of the $P_{std}::lacZ$ reporter fusion below the threshold level observed in the $setB::Mud$ -Cam mutant resulted in a dramatic reduction of StdA expression detected by Western blot (Fig. 4) to levels only detectable after prolonged exposure (Fig. 3C). Due to the relatively low level of StdA expression detected in the $yfbQ::T$ -POP mutant (CD7R) and the $csgC::T$ -POP mutant (CD6R) (Fig. 3C), we did not further analyse the respective mutations.

StdA assembles into fimbrial filaments on the surface of S. typhimurium that mediate attachment to human intestinal epithelial cells

Mutants that strongly expressed StdA were further analysed by electron microscopy to determine whether they elaborated fimbriae on their surface. When bacteria were cultured statically at 37°C in LB broth no fimbrial filaments were detected on the surface of the parental *S. typhimurium fim* mutant (AJB4) (data not shown), which is consistent with previous observations that type 1 fimbriae, but not thin-curved fimbriae, are expressed under this growth condition (Grund and Weber, 1988). As expected, introduction of the dam , $setB$ and $rosE$ mutations into a $\Delta stdAB$ derivative of AJB4 (CD21) did not result in expression of StdA (data not shown). However, surface appendages were visualized by negative staining on the surface of the dam (CD2R), $setB$ (CD15R) and $rosE$ (CD8R) mutants identified by SIMPLE (Fig. 5A). The identity of these surface structures was further investigated by immunogold labelling with anti-StdA serum. Gold particles labelled filamentous surface structures on the surfaces of the *S. typhimurium dam*₊₈₀₃::Mud-Cam (CD2R), $rosE::Mud$ -Cam (CD8R) and $setB::T$ -POP (CD15R) mutants, demonstrating that StdA was incorporated into fimbrial structures in these strains (Fig. 5B–D). In contrast, labelling with rabbit anti-StdA serum and goat anti-rabbit gold conjugate did not result in deposition of gold particles on the surface of the parental *S. typhimurium fim* mutant (AJB4) (data not shown). Immunoelectron microscopy thus provided the first evidence that the std operon encodes fimbriae in *S. typhimurium*.

Fimbriae were purified from an *S. typhimurium fim fliC fliB dam*₊₈₀₃::Mud-Cam mutant (to prevent contamination with flagella and type 1 fimbriae) after their removal from the surface by mechanical shearing. Separation of proteins by SDS-PAGE followed by Coomassie blue staining revealed that the fimbrial preparation contained a single protein band with an apparent molecular mass that was similar to that predicted for the mature StdA protein (16 kDa) (Fig. 5E). In addition to a fimbrial usher (StdB) and a chaperone (StdC), the std operon encodes another putative subunit (StdD) whose predicted molecular mass was considerably larger than that of StdA. Western blot analysis showed that the protein band present in the fimbrial extract reacted with anti-StdA antiserum (Fig. 5F). Collectively, these data suggest that StdA is the major subunit of fimbriae encoded by the std operon.

To investigate the function of fimbriae encoded by the std operon, we studied bacterial adhesion to two human colonic epithelial cell lines, T-84 cells and CaCo-2 cells. Compared with its parent (AJB4), the $rosE::Mud$ -Cam mutant (CD8R) was recovered in significantly higher numbers from both CaCo-2 cells and T84 cells (Fig. 6A and B). This increased recovery was due to the presence of std , because deletion of this fimbrial operon abolished adherence of the $rosE::Mud$ -Cam mutant (CD20). Western blot analysis of the bacterial inoculum confirmed that only adherent bacterial cultures expressed StdA (Fig. 6C). These data demonstrated that fimbriae encoded by the std operon mediate attachment to human colonic epithelial cells.

RosE is a negative regulator of StdA expression

Complementation of dam mutations was not attempted because both gene inactivation and introduction of the cloned gene can result in the same phenotype (e.g. increased spontaneous mutation rates in *S. typhimurium*) (Torreblanca and Casadesus, 1996). To complement the $rosE::Mud$ -Cam and $setB::T$ -POP mutations, both open reading frames were PCR amplified along with their ribosome binding sites, but without their promoter sequences, and cloned in vector pBAD30 behind the *E. coli* arabinose promoter (P_{BAD}). The resulting plasmids (pCD58 and pCD59 respectively) were introduced into the respective *S. typhimurium* mutants and strains were cultured in the presence of glucose (to repress expression from P_{BAD}) or in the presence of arabinose (to induce expression from P_{BAD}). Complementation of the $setB::T$ -POP mutant (CD15R) with the cloned $setB$ gene (pCD59) resulted in reduced StdA expression detected by Western blot. Similarly, introduction of the cloned $rosE$ gene (pCD58) into the $rosE::Mud$ -Cam (CD8R) mutant resulted in repression of StdA expression (Fig. 7). These data suggested that inactivation of $rosE$ and $setB$ were responsible for

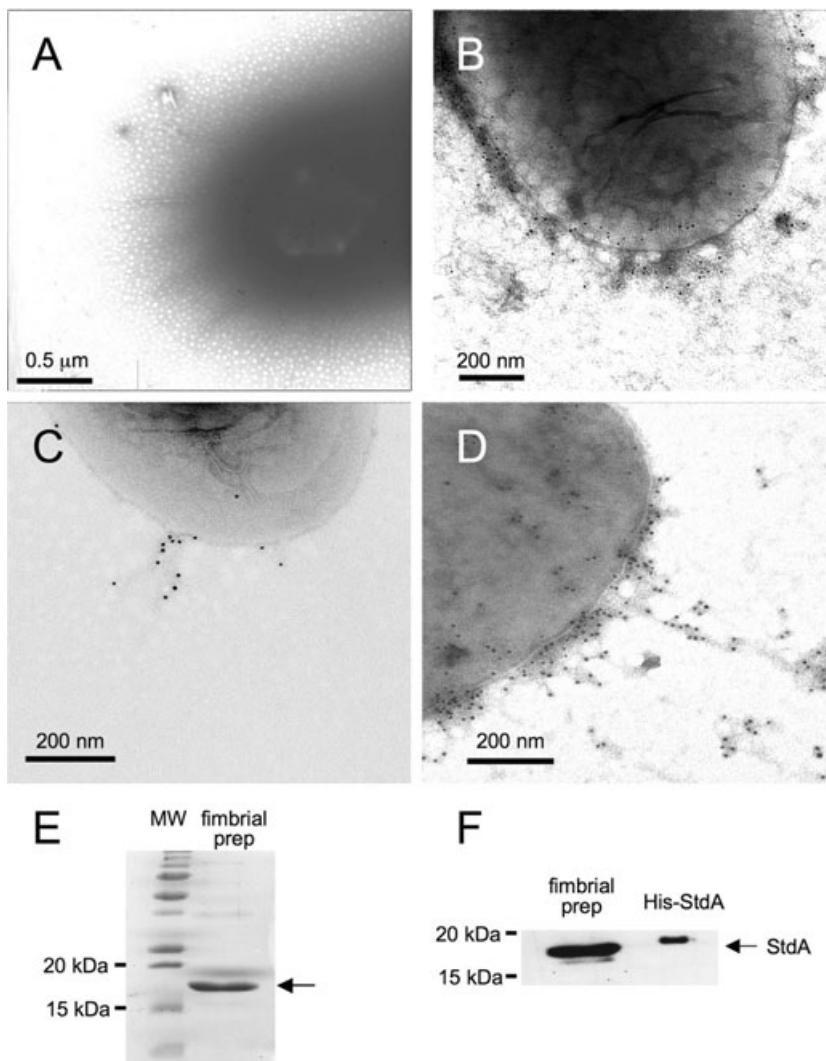


Fig. 5. Expression of fimbriae on the surface of *S. typhimurium* transposon mutants investigated by electron microscopy and purification of fimbriae.

A. Expression of fimbrial filaments on the surface of a *dam*₇₈₀₃::Mud-Cam mutant (CD2R) was visualized by negative staining. B–D. Immunoelectron microscopy detecting expression of StdA on the surface of a *dam*₇₈₀₃::Mud-Cam mutant (CD2R) (B), a *rosE*::Mud-Cam mutant (CD8R) (C) and a *setB*::T-POP mutant (CD15R) (D). E and F. Coomassie stain (E) and Western blot (F) of a fimbrial preparation (fimbrial prep) obtained from an *S. typhimurium* *fim fliC fliB dam*₇₈₀₃::Mud-Cam mutant. (E) The Coomassie-stained gel shows standard proteins (MW, left lane) and a fimbrial preparation (right lane). The molecular mass of relevant standard proteins is indicated on the left. The arrow indicates a single major protein band present in the fimbrial preparation. (F) The Western blot shows the fimbrial preparation (left lane) and purified His–StdA fusion protein (right lane). Note that due to its 6xHis tag, the His–StdA protein has a slightly greater molecular mass than native StdA (arrow). The molecular mass of standard proteins is indicated on the left.

increased expression of StdA strains CD8R and CD15R respectively.

As RosE showed homology to transcriptional regulators, we investigated whether this protein would bind to a DNA region upstream of the *stdA* open reading frame. To this end, a fusion protein between RosE and a histidine tag (His–RosE) was constructed and purified. Purified His–RosE protein was tested for its ability to bind the *stdA* upstream DNA region using an electrophoretic mobility shift assay (EMSA). Addition of increasing concentrations of His–RosE to a biotin-labelled PCR product containing nucleotides –225 to –375 relative to the *stdA* start codon (Fig. 8A, region 2) resulted in appearance of a band with higher molecular weight (Fig. 8B). In contrast, His–RosE did not result in an electrophoretic mobility shift of a biotin-labelled DNA region comprising nucleotides –275 to –126 relative to the *stdA* start codon (Fig. 8A, region 1). The electrophoretic mobility shift of biotin-labelled DNA region 2 could be inhibited by pre-incubation of His–RosE with

unlabelled DNA region 2, indicating specific binding of His–RosE to the *stdA* upstream DNA region (Fig. 8C).

Collectively, our data showed that RosE bound to a DNA region upstream of the *stdA* start codon (Fig. 8), repressed transcription from the *stdA* promoter (Fig. 4) and prevented expression (Fig. 7) and assembly of StdA into fimbrial filaments (Fig. 5). These results identified RosE as a transcriptional regulator of Std fimbrial expression in *S. typhimurium*.

RosE-mediated repression of the *std* operon is required for full infectivity of *S. typhimurium*

Next we wanted to determine whether constitutive expression of the *std* operon in a *rosE* mutant would alter the course of infection in mice. To this end, the *rosE*::Mud-Cam insertion was introduced into the *S. typhimurium* wild type (SR11) to give rise to strain CD30. The *S. typhimurium* wild type was marked with a mutation in

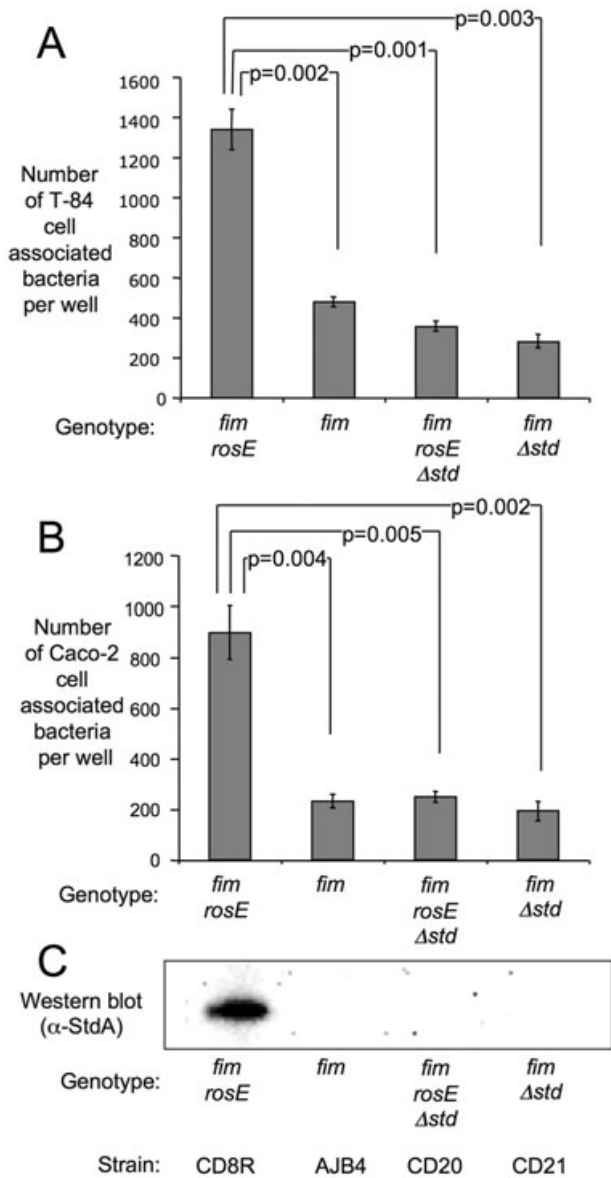


Fig. 6. Adherence of *S. typhimurium* strains to human colonic epithelial cells.

A and B. Adherence to T84-cells (A) or CaCo-2 cells (B) was determined at 4°C to prevent bacterial invasion. Data are shown as averages of cell-associated bacteria \pm standard deviation. Statistical significance of differences is indicated by brackets. C. Expression of StdA in the bacterial inoculum was investigated by Western blot with anti-StdA serum.

phoN (CD31), encoding alkaline phosphatase. Inactivation of *phoN* abolishes the ability to cleave 5-bromo-4-chloro-3-indolyl phosphate (XP). Growth on LB agar plates supplemented with XP thus provided an easy means to distinguish between mutants carrying a mutation in *rosE* (*PhoN*⁺ blue colonies) and CD31 (*PhoN*⁻ white colonies). Inactivation of *phoN* does not reduce the ability of *S. typhimurium* to colonize organs or faeces of mice

during competitive infections (Kingsley *et al.*, 2003; Weening *et al.*, 2005). A group of six genetically resistant mice (CBA/J) was inoculated with a 1:1 mixture of the *S. typhimurium phoN* mutant (CD31) and a *rosE* mutant (CD30). Recovery of bacteria from the faeces showed that the *rosE* mutant was recovered at significantly reduced numbers at 7 days after infection (Fig. 9A). Determination of the bacterial tissue load at 10 days after infection revealed a significant competitive disadvantage of the *rosE* mutant for colonizing caecal contents, caecal wall and the spleen (Fig. 9B).

We next determined whether reduced recovery of the *rosE* mutant from the faeces and organs of mice was due to uncontrolled expression of the *std* operon or due to altered expression of other genes that may be regulated by RosE. We reasoned that deletion of the *std* fimbrial biosynthesis genes from both competing strains would eliminate a competitive defect only if recovery of the *rosE* mutant was due to uncontrolled expression of the *std* operon. The *std* operon was deleted from the *S. typhimurium phoN* mutant (CD31) and the *rosE* mutant (CD30) to give rise to strains CD32 and CD33 respectively. A group of six genetically resistant mice (CBA/J) was inoculated with a 1:1 mixture of the *std phoN* mutant (CD32) and the *std rosE* mutant (CD33). Recovery of bacteria from the faeces and organs of mice showed that the *std rosE* mutant (CD33) was fully able to compete with the *std phoN* mutant (CD32) for colonization of mice (Fig. 9). These data provided evidence that RosE-mediated repression of the *std* operon is required for full virulence of *S. typhimurium* in mice.

Discussion

Sequence analysis has identified multiple putative fimbrial operons in the *S. typhimurium* genome that remain poorly characterized (Emmerth *et al.*, 1999; Folkesson *et al.*, 1999; Morrow *et al.*, 1999; Tsois *et al.*, 1999; McClelland *et al.*, 2001). We have recently developed a new method, termed SIMPLE, that can be used to isolate fimbriated *S. typhimurium* mutants from a random transposon library (Nuccio *et al.*, 2007). Here we have applied this method for the first time to a putative fimbrial operon, *stdABCD*, which was identified in the *S. typhimurium* genome by whole genome sequencing. The SIMPLE method proved to be a powerful approach for isolating mutants expressing fimbriae encoded by the *std* operon *in vitro*. The isolation of such mutants represents an important first step in the characterization of fimbriae encoded by the *std* operon and opened the way for functional studies on their binding specificity. Specifically, *in vitro* expression of the *std* operon enabled us to demonstrate that the encoded fimbriae mediate attachment of *S. typhimurium* to human colonic epithelial cell lines (i.e. T-84 and CaCo-2).

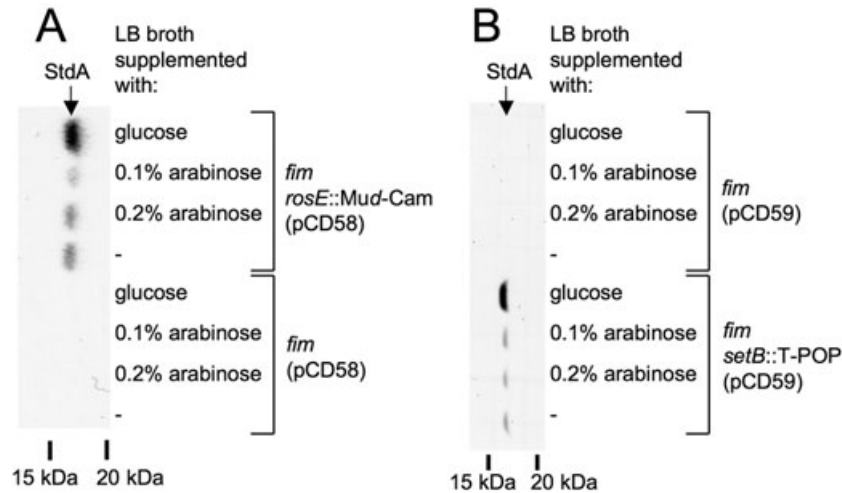


Fig. 7. Complementation of *S. typhimurium* mutants expressing StdA.

A. Complementation of the CD8R mutant (*fim rosE::Mud-Cam*) (top) and its parent AJB4 (*fim*) (bottom) with the *rosE* gene cloned under control of the arabinose promoter (pCD58). A Western blot detected with anti-StdA serum is shown on the left. The molecular mass of standard proteins is indicated at the bottom. The presence or absence of glucose or arabinose is indicated on the right.

B. Complementation of the CD15R mutant (*fim setB::T-POP*) (bottom) and its parent AJB4 (*fim*) (top) with the *setB* gene cloned under control of the arabinose promoter (pCD59). A Western blot detected with anti-StdA serum is shown on the left. The molecular mass of standard proteins is indicated at the bottom. The presence or absence of glucose or arabinose is indicated on the right.

While this work was in progress, Balbontin *et al.* (2006) demonstrated that transcription of the *std* operon is repressed by Dam methylation in *S. typhimurium*. Our data confirmed and extended this observation by showing that mutations in *dam* resulted in expression of StdA filaments on the bacterial cell surface. The Dam protein methylates adenine residues located within the palindromic sequence GATC (Razin and Friedman, 1981). Inspection of the *std* promoter region reveals three GATC sites arranged in the motif 5'-acGATCa-(N₆)-tcGATCgt-atcGATCta-3' located upstream of a putative *stdA* promoter (Balbontin *et al.*, 2006) (211 bp upstream of the *stdA* start codon). A similar arrangement of GATC sites and flanking regions is found in the promoter region of the *E. coli agn43* gene, which contains the sequence 5'-acGATCa-(N₁₂)-tgGATCgt-(N₄)-atcGATCga-3' located downstream of the transcriptional start site (32 bp upstream of the *agn43* start codon) (Waldron *et al.*, 2002; Wallecha *et al.*, 2002). Dam-mediated methylation of these GATC sites is part of a phase variation mechanism controlling expression of antigen 43, an adhesin of the autotransporter family (Henderson and Owen, 1999; Haagmans and van der Woude, 2000). However, while *E. coli dam* mutants do not express antigen 43, *S. typhimurium dam* mutants did express StdA, which illustrated that regulation of Std fimbriae differed from that of the *E. coli* autotransporter.

A novel transcriptional regulator of *std* expression identified in this study is RosE, a protein with homology to the arginine repressor ArgR of *E. coli* (Lim *et al.*, 1987). Analysis of a P_{rosE}::*lacZ* reporter construct and comple-

mentation of a *rosE-40::Mud-Cam* mutant (CD8R) with the cloned *rosE* gene suggested that RosE repressed transcription of the *std* operon. Furthermore, EMSAs showed that RosE bound a DNA region located between base pairs -375 and -225 relative to the *stdA* start codon. For comparison, the GATC sites implicated in Dam-mediated repression of *stdA* expression (Balbontin *et al.*, 2006) are located between base pairs -239 and -214 relative to the *stdA* start codon. Collectively, these data suggested that similar to its homologue ArgR, the RosE protein functioned as a transcriptional repressor. The *rosE* gene is located on a genetic islet that is well conserved among the genus *Salmonella*, thus making it likely that co-ordination of gene expression by RosE is a general feature of *Salmonella* serotypes.

While our data show that RosE prevented StdA expression *in vitro*, characterization of an *S. typhimurium* Δ *stdAB* mutant in the mouse model suggests that RosE-mediated suppression must be relieved under some conditions *in vivo*. The *S. typhimurium* Δ *stdAB* mutant has a competitive defect in colonizing the caecum of mice and in being shed with the faeces (Weening *et al.*, 2005), suggesting that fimbriae encoded by the *std* operon are expressed in the intestinal lumen. StdA seroconversion of mice infected with *S. typhimurium* provides further indirect evidence for *in vivo* expression of fimbriae encoded by the *std* operon (Humphries *et al.*, 2005). Interestingly, we found that a mutation in *rosE* resulted in a competitive defect of *S. typhimurium* during colonization of mice. This competitive colonization defect was no longer observed when *std* biosynthesis genes were deleted from both competing

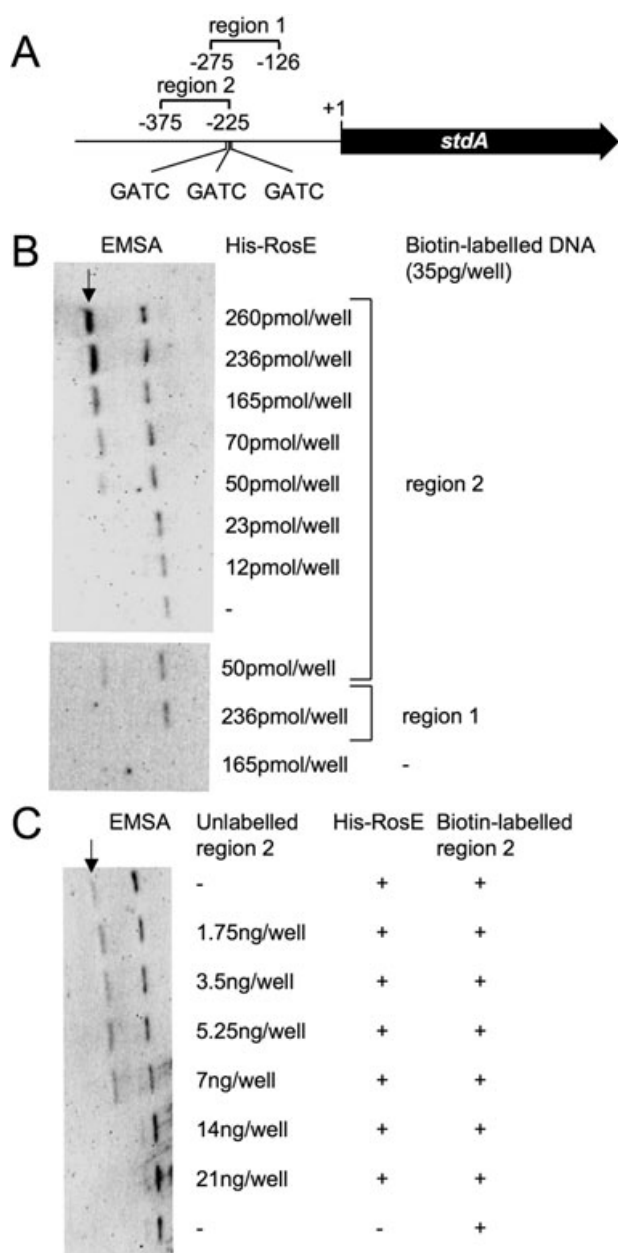


Fig. 8. Electrophoretic mobility shift assay (EMSA) of DNA regions located upstream of the *stdA* start codon using purified His-RosE protein. A. The locations of DNA fragments (brackets) used for the EMSA (region 1 and region 2) are shown relative to the *stdA* open reading frame (filled arrow). The positions of three Dam methylation sites (GATC) present in the putative *std* promoter region are indicated. B. EMSA of biotin-labelled DNA (region 1 or region 2) and different concentrations of His-RosE. Shifted DNA fragments are indicated by an arrow. C. Competitive EMSA of biotin-labelled DNA (region 2, 35 pg well⁻¹) in the presence (+) or absence (-) of purified His-RosE protein (70 pmol well⁻¹) and the indicated amounts of unlabelled competitor DNA (region 2). Shifted DNA fragments are indicated by an arrow.

strains. These data suggest that a mutation in *rosE* resulted in attenuated mouse virulence of *S. typhimurium* because fimbriae encoded by the *std* operon were expressed *in vivo* at an inappropriate time or location. Deletion of the *std* operon results in a competitive defect of *S. typhimurium* in colonizing the caecum of mice, while colonization of the spleen is not altered (Weening *et al.*, 2005). In contrast, the uncontrolled expression of the *std* operon in a *rosE* mutant reduced the ability of *S. typhimurium* to colonize both intestinal sites (i.e. the caecum) and extra-intestinal sites (i.e. the spleen). Collectively, these data illustrate that a tight control of *std* fimbrial expression is critical during host pathogen interaction *in vivo*.

Experimental procedures

Bacterial strains, media and growth conditions

Salmonella enterica serotype Typhimurium strains used in this study are shown in Table 1. An *S. typhimurium* *fim fliC fljB dam₊803::Mud-Cam* mutant was generated by transducing the *fliC* and *fljB* mutations of *S. typhimurium* strain EHW26 (Rafatellu *et al.*, 2005) into strain CD2R. *E. coli* strains DH5 α MCR (Gibco BRL) and TOP10 (Invitrogen) were used for cloning experiments. Insertions in the *phoN* gene were introduced into *S. typhimurium* strains by P22-mediated transduction from strain AJB715.

All strains were cultured aerobically or statically at 37°C in LB broth (10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 5 g l⁻¹ NaCl). For the SIMPLE approach, bacteria were cultured in LB buffered to pH 7 with 100 mM 2-(N-morpholino)ethanesulphonic acid (MES) (Nicholson and Low, 2000). When appropriate, antibiotics were added at the following concentrations (mg l⁻¹): kanamycin (Km), 100; nalidixic acid (Nal), 50; carbenicillin (Carb), 100; tetracycline (Tc), 20. To detect expression of beta-galactosidase on agar plates, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) was added at a concentration of 40 mg l⁻¹. For detection of alkaline phosphatase (PhoN) activity in *S. typhimurium* 30 mg l⁻¹ XP was added to LB agar plates.

Genetic techniques

Generalized transducing phages P22 HT *int-105* and KB1 *int* were used to generate lysates of *S. typhimurium* as previously described (Miller, 1972). Transductants were streaked for single colonies on Evans blue Uridine (EBU) agar (Bochner, 1984) and phage-free colonies were cross-streaked against P22 H5 (for P22 HT *int-105*), or KB1 *int* (for KB1) to confirm phage sensitivity.

Mud-Cam insertion mutants of *S. typhimurium* strain AJB4 were isolated using the method of Hughes and Roth (1988). In brief, a P22 HT *int-105* lysate of TE3461 (Elliott, 1993) was used to transduce *S. typhimurium* to chloramphenicol resistance. *Mud-Cam* insertions were selected on LB+Cm plates (approximately 1000–2000 mutants per plate) and pooled by flooding with 5 ml of LB broth and re-suspending colonies.

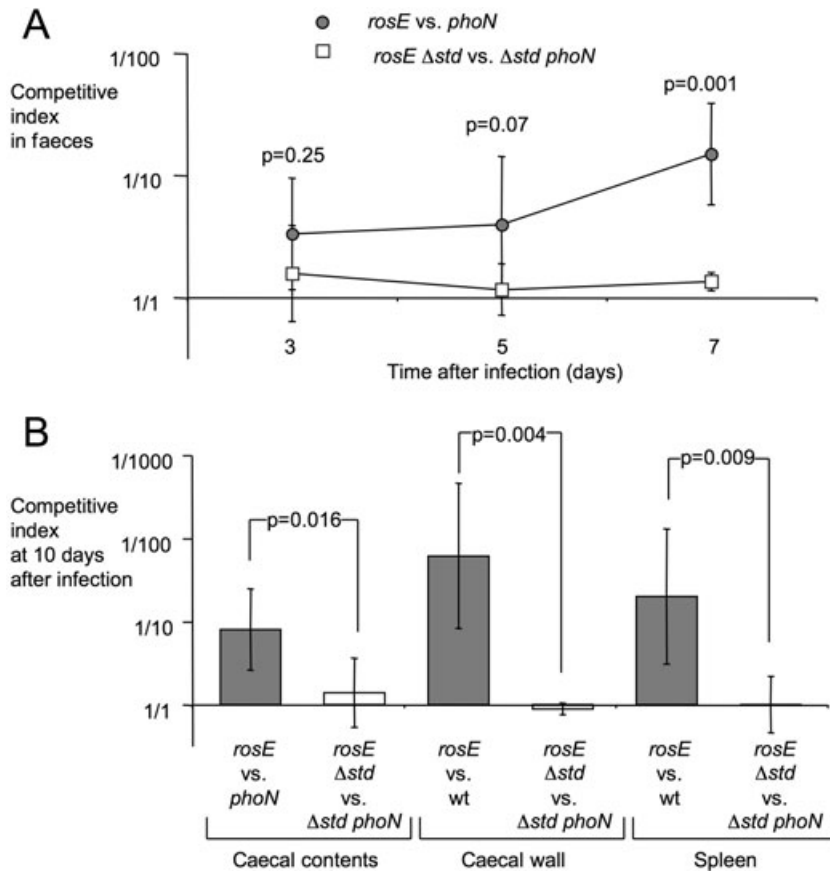


Fig. 9. Effect of a mutation in *rosE* on the ability of *S. typhimurium* to colonize genetically resistant (CBA/J) mice.

A. Mice were infected with a 1:1 mixture of the *S. typhimurium* *phoN* mutant and a *rosE* mutant (grey circles) or with a 1:1 mixture of *std phoN* mutant and a *std rosE* mutant (open squares) and bacteria were recovered from faeces over time. Data are shown as geometric means of competitive indices \pm standard deviation. Statistical significance of differences between both competitive infection experiments are indicated by *P*-values.

B. Mice were infected with a 1:1 mixture of the *S. typhimurium* *phoN* mutant and a *rosE* mutant (grey bars) or with a 1:1 mixture of *std phoN* mutant and a *std rosE* mutant (open bars) and bacteria were recovered from organs at 10 days after infection. Data are shown as geometric means of competitive indices \pm standard deviation. Statistical significance of differences between both competitive infection experiments are indicated by *P*-values.

Tn10dTc[del-25](T-POP) insertion mutants of *S. typhimurium* AJB4 were generated as described previously (Rappleye and Roth, 1997), using a P22 lysate grown on TH3923 to transduce into *S. typhimurium* AJB4 carrying plasmid pNK2880 (Rappleye and Roth, 1997; Lee *et al.*, 2007). Transductants were selected on LB+Tc agar (approximately 1000–2000 mutants per plate) and pooled by flooding with 5 ml of LB broth and re-suspending colonies.

SIMPLE protocol

SIMPLE was performed as described previously (Nuccio *et al.*, 2007). In brief, 100 μ l aliquots of BioMag Protein G particles (Qiagen) were washed three times in 750 μ l of TN buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5) and incubated for 1 h at room temperature in 100 μ l of pre-absorbed anti-StdA serum on an automatic roller. The particles were washed as described above and incubated at room temperature for half an hour with constant inversion on an automatic roller in 650 μ l of Particle-Blocking (PB) buffer (TN buffer + 1% casein, prepared fresh). Bacteria (500 μ l of a culture grown statically at 37°C for 2 days in LB-MES pH 7) were added and the tubes were incubated for 1 h at room temperature with constant inversion. The particles were washed three times with PB buffer by gentle inversion and re-suspended in 1 ml of PBS (to generate serial 10-fold dilutions that were spread on agar plates) or in the appropriate growth medium (to incubate bacteria statically at 37°C for

2 days and use the resulting culture to repeat the above protocol). For each mutant pool, 10 single colonies from a plate were analysed by Western blot using anti-StdA serum.

Western blotting

The polyclonal rabbit anti-StdA serum (Humphries *et al.*, 2003) was diluted 1:5 in PBS supplemented with 0.2% sodium azide and pre-absorbed with ADH17 as described previously (Gruber and Zingales, 1995). For Western blot analysis, 10 μ l containing approximately 2×10^8 colony-forming units (cfu) re-suspended in PBS was mixed with an equal volume of sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer and boiled for 10 min. These whole-cell lysates were separated by 15% SDS-PAGE, transferred to Immobilon-P (Millipore) membranes using a Trans-Blot semi-dry transfer cell (Bio-Rad) and incubated with anti-StdA serum diluted 1:500 (final). Binding of anti-StdA serum was detected with Goat anti-Rabbit-Alkaline Phosphatase conjugate and the ImmunoStar chemiluminescent substrate (Bio-Rad). Bands were visualized with a BioSpectrumAC Imaging System (UVP).

Cloning of transposon flanking DNA

DNA regions adjacent to the transposon insertion sites were amplified by an inverse PCR as described previously

Table 1. *S. typhimurium* strains used in this study.

Strain	Genotype	Source or reference
SR11	Xylose fermenting mutant of wild-type isolate BA2	Schneider and Zinder (1956)
ADH17	SR11, Δ stdAB::Km	Humphries <i>et al.</i> (2003)
CD30	SR11, <i>rosE</i> ::Mud-Cam	This study
CD31	SR11, <i>phoN</i> ::Km	This study
CD32	SR11, <i>phoN</i> ::Km Δ stdAB::Km	This study
CD33	SR11, <i>rosE</i> ::Mud-Cam Δ stdAB::Km	This study
AJB4	SR11, nalidixic acid resistant, <i>fim</i>	Bäumler <i>et al.</i> (1996)
CD6R	AJB4, <i>csgC</i> ::T-pop	This study
CD7R	AJB4, <i>yfbQ</i> ::T-pop	This study
CD8R	AJB4, <i>rosE</i> ::Mud-Cam	This study
CD10R	AJB4, <i>dam</i> ₋₄₀ ::Mud-Cam	This study
CD12R	AJB4, <i>damX</i> ::Mud-Cam	This study
CD15R	AJB4, <i>setB</i> ::T-pop	This study
DB3R	AJB4, <i>dam</i> ₊₃₆ ::Mud-Cam	This study
CD20	AJB4, <i>rosE</i> ::Mud-Cam Δ stdAB::Km	This study
CD21	AJB4, Δ stdAB::Km	This study
CD2R	AJB4, <i>dam</i> ₊₈₀₃ ::Mud-Cam	This study
CD3	CD2R, <i>fliC fljB</i>	This study
ATCC14028	Wild-type isolate from cattle	American Type Culture Collection
IR715	Nalidixic acid-resistant derivative of ATCC14028	Stojiljkovic <i>et al.</i> (1995)
EHW26	IR715, <i>fliC</i> ::Tn10 <i>fljB5001</i> ::MudJ	Raffatellu <i>et al.</i> (2005)
AJB715	IR715, <i>phoN</i> ::Km	Kingsley <i>et al.</i> (2003)
LT2	ATCC 700720	Lilleengen (1948)
TE3461	LT2, <i>hisD9953</i> ::Mud-Cam <i>hisA9944</i> ::Mud-1	Elliott (1993)
TH3923	LT2, pJS28(Carb ^R , P22-9+)/F'114ts Lac ⁺ <i>zzf-20</i> ::Tn10[<i>tetA</i> ::MudPJ](Tc ^R) <i>zzf-3823</i> ::Tn10dTc[Δ del-25](T-POP)/ <i>leuA414 hsdSB</i> Fels2-	Rappleye and Roth (1997)

(Bäumler *et al.*, 1994). Genomic DNA was digested with either Alul or TaqI and ligated with T4 DNA Ligase. DNA flanking Mud-Cam insertions was amplified using primer 5'-CCGAATAATCCAATGTCTCCCGGT-3' in combination with either 5'-AGTGCGCAATAACTTGCTCTCGTTC-3' (for TaqI-digested DNA) or 5'-CGAAAAACAAAACACTGCAAATCATTTCATAAC-3' (for Alul-digested DNA). DNA flanking T-POP insertions was amplified using primers 5'-CGCTTTTCCCGAGATCATATG-3' and TPOP-AluTaq, 5'-GCACTTGCTCCTGTTTACTCC-3' (for Alul-digested DNA) and PCR SuperMix HiFi (Invitrogen). PCR products were gel-purified (QIAEX II gel purification kit, Qiagen) and cloned into pCR2.1 with the TOPO TA cloning kit (Invitrogen). The inserts of the resulting plasmids were sequenced by SeqWright (Houston, TX).

Complementation

The *rosE* gene was PCR amplified with the primers 5'-GAGCTCTAAGGTGCATTTATGAAGGA-3' and 5'-AAGCTTACTCATCGCAAACGGTTCTTA-3', cloned into pCR2.1 and the insert excised with SacI and HindIII and cloned into vector pBAD30 (Guzman *et al.*, 1995) to give rise to plasmid pCD58. The *setB* gene was PCR amplified with the primers 5'-GAATCCGTAACCTCCGCCTCTTTCACAC-3' and 5'-AAGCTTGCTGAAATGTGTGCGAAGAGTAAA-3', cloned into pCR2.1 and the insert excised with EcoRI and HindIII and cloned into vector pBAD30 to give rise to plasmid pCD59. The plasmids were introduced into *S. typhimurium* strains by electroporation. The resulting strains were grown overnight at 37°C in LB+Carb supplemented with 10 mM glucose. This overnight culture was used to inoculate (1:100)

LB+Carb or LB+Carb supplemented with 0.2% of glucose, 0.1% arabinose or 0.2% arabinose. Cultures were grown at 37°C statically to an OD₆₀₀ of 0.4–0.6 and StdA expression assessed by Western blot with rabbit anti-StdA serum (Humphries *et al.*, 2003).

Electron microscopy

For microscopy, bacteria were grown for 2 days in a static culture, washed twice in PBS and re-suspended in EM grade water (EM Science) at a titre of approximately 1×10^9 cfu ml⁻¹. Bacteria were allowed to attach to a formvar/carbon-coated grid (EM Science) for 2 min. For negative staining of fimbriae, the grids were incubated for 1 min in 1% uranyl-acetate (UA). For immunogold labelling the grids were incubated for 20 min with rabbit anti-StdA serum diluted 1/250 in PBS containing 1% bovine serum albumin (BSA). Grids were washed five times for 1 min in PBS containing 1% BSA. Grids were then incubated for 20 min in goat anti-rabbit 10 nm gold conjugate (EM Science) diluted 1/20 in PBS containing 1% BSA. Grids were washed three times for 1 min in PBS containing 1% BSA and three times for 1 min in EM grade water. Grids were incubated for 1 min with 1% UA before they were analysed by electron microscopy.

Purification of fimbriae

An *S. typhimurium* *fim fliC fljB dam*₊₈₀₃::Mud-Cam mutant was grown statically in 2 l of LB broth at 37°C overnight, harvested by centrifugation and re-suspended in 10 ml of 0.5 mM Tris 75 mM NaCl. StdA fimbriae were separated from the cells by

mechanical shearing in a blender for three 1 min periods, after which cells and cellular debris were removed by centrifugation (3500 r.p.m. 30 min, 4°C). The supernatant was collected and passed through a 0.45 µm filter (Millipore), and (NH₄)₂SO₄ (60% final concentration) was added to precipitate the fimbriae. Precipitated fimbriae were recovered by centrifugation (14 000 r.p.m. 30 min, 4°C). The pellet was re-suspended in 50 µl of sterile water and was analysed by SDS-PAGE and Western blot.

Tissue culture experiments

The colorectal carcinoma cell lines T84 (ATCC CCL-248) and Caco-2 (HTB-37) were obtained from the American Type Culture Collection. T84 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Gibco), containing 1.2 g l⁻¹ sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate (Gibco), supplemented with 10% fetal calf serum (FCS). Caco-2 cells were maintained in Minimum essential medium (Eagle) (Gibco) containing 2.5 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, Earle's balanced salt solution (BSS) and 15% FCS. For assays, T84 cells were seeded in 24-well plates at a density of approximately 1 × 10⁵ cells per well or in six-well plates at a density of 5 × 10⁵ cells per well and incubated for 48 h. Caco-2 cells were grown to confluency in 24-well plates for 5 days. Prior to adherence assays, epithelial cells were washed with ice-cold PBS. Ice-cold medium was added, cells were infected with bacteria (10⁵ cfu well⁻¹) and allow to adhere for 1 h at 4°C (to prevent invasion). Non-adherent bacteria were removed by five washes with PBS and adherent bacteria re-suspended in PBS containing 1% (v/v) Triton X-100. Serial 10-fold dilutions were spread on LB agar plates to determine the number of cell-associated bacteria per well.

β-Galactosidase assay

The promoter region of *StdA* was PCR amplified with the primers 5'-TCTAGACCTGAACTTTCCATCGAA-3' and 5'-CCCGGGATATCCCCAGCCTGCTG-3'. The PCR product was cloned into pCR2.1 (Invitrogen), the insert excised with XbaI and EcoRI and cloned into vector pUJ10 (deLorenzo *et al.*, 1990) to give rise to plasmid pCD57. Plasmid pCD57 was introduced into *S. typhimurium* strains by electroporation. For β-galactosidase measurements, LB+Carb was inoculated 1:100 from an overnight culture and bacteria were grown to an optical density at 600 nm (OD₆₀₀) of 0.4–0.6. The enzymatic activity of β-galactosidase in each culture was determined using standard methodology (Miller, 1972).

Purification of His-RosE

An N-terminal 6xHis-RosE fusion protein was constructed using the cloning vector pQE30 (Qiagen). The *rosE* gene was PCR amplified using the primers 5'-GGATCCATGAAGG AATACGATGATTAC-3' and 5'-GAGCTCTTATGAATTTAAA TTCATTTAAG-3' and the product was cloned into pCR2.1 (Invitrogen). The insert was then cloned into the BamHI and SacI sites of pQE30 to give rise to plasmid pCD62. Plasmid

pCD62 was electroporated into *E. coli* TOP10 and the resulting strain was grown overnight at 37°C in LB media supplemented with 10 mM glucose and carbenicillin (100 mg l⁻¹) to maintain the plasmid. To induce the production of His-RosE, cells were grown to mid-log phase before IPTG was added to a final concentration of 2 mM. Cells were harvested by centrifugation after a 4 h incubation. His-RosE was purified by nickel-affinity chromatography (Ni-NTA, Invitrogen) according to the manufacturer's protocol, but after cell fragments had been removed from cell lysates by centrifugation, the supernatant was centrifuged at 15000 r.p.m. for 30 min to remove denatured protein before being applied to the affinity column. The fractions containing His-RosE were immediately aliquoted and frozen at -80°C in 5% sucrose. The His-RosE concentration was determined by measuring the absorbance at 280 nm.

Electrophoretic mobility shift assay

Fragments of the region upstream of the *stdA* open reading frame were generated by PCR amplification using the primer sets 5'-CCTGAACTTTCCATCGAAAAA-3', 5'-TAAAAAGTA TTTCTTTGAT-3' (region 1) and 5'-TCGATGATTATGAT CGTAAT-3', 5'-ATGGAAAAGCAAAACATA-3' (region 2) which were biotinylated at the 5' end (MWG Biosciences, High Point, NC). The PCR products were gel-purified in a vertical acrylamide gel. The vertical acrylamide gel was prepared by adding 0.1 ml of 50× Tris-acetate EDTA (242 g of Tris, 57.1 ml of glacial acetic acid, 37.2 g of Na₂EDTA·2H₂O in 1 l of distilled H₂O), 0.66 ml of acrylamide/bis-acrylamide (37.5:1, Roche), 6 µl of TEMED and 30 µl of ammonium persulphate to 5.33 ml of distilled H₂O. After electrophoresis, fragments were excised and eluted by electrophoresis in dialysis tubes for 15 min at 100 V. The DNA concentration was determined by spectrophotometry. The binding assays were performed in a total volume of 20 µl containing the following: 1 µg ml⁻¹ Poly(dI-dC), 50% glycerol, 1% non-ylphenyl-polyethylene glycol (NP-40), 1.05 M KCl, 10 mM TRIS (pH 7.5), 1 mM DTT, 100 mM MgCl₂, 200 mM EDTA, 35 pg of biotinylated DNA, various concentrations of His-RosE and unlabelled competitor DNA as required. Competitor DNA was generated by PCR amplification using the primers listed above without the biotin label. Binding reactions were allowed to incubate for 20 min at room temperature prior to adding loading buffer and separating fragments on a pre-run 5% acrylamide gel in Tris-borate EDTA buffer (Bio-Rad Precast Gels). Gels were run at 4°C at 100 V until the bromophenol blue dye front had migrated approximately three-fourths down the length of the gel. The DNA-protein complexes were transferred to a nylon membrane (Roche Applied Science) using a semi-dry blotter (Bio-Rad) at 15 V for 30 min. The DNA was UV cross-linked to the membrane, and biotinylated DNA on the membrane was detected using the LighShift™ Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol.

Animal experiments

Eight- to 12-week-old CBA/J (Jackson Laboratory) mice were used throughout this study. Bacteria were routinely cultured

as standing overnight cultures prior to infection. In all experiments the bacterial titre of the inoculum was determined by spreading serial 10-fold dilutions on agar plates containing appropriate antibiotics and determining cfu. For competitive infection experiments, groups of five mice were infected by oral gavage with a 1:1 mixture of mutant and isogenic parent at a dose of approximately 5×10^8 cfu per mouse. Faecal pellets were homogenized in 1 ml of PBS. The limit of detection was approximately 0.08 cfu per milligram of faeces. Caecum, caecal contents and spleen were harvested and homogenized in 5 ml of PBS pH 7.4. Dilutions of faecal pellets and homogenized organs were spread on LB agar plates containing the appropriate antibiotics. Agar plates were supplemented with XP to distinguished between colonies expressing PhoN and colonies that were PhoN negative. Data were normalized and competitive indices calculated by dividing the output ratio (cfu mutant/cfu wild type) by the input ratio (cfu mutant/cfu wild type). Competitive indices were converted logarithmically prior to the calculation of averages and statistical analysis. A Student's *t*-test was used to determine whether differences were statistically significant.

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References

- Andrews-Polymeris, H., and Bäuml, A.J. (2006) Pathogenomics of *Salmonella* species. In *Pathogenomics*. Hacker, J., and Dobrindt, U. (eds). Weinheim: Wiley-VCH, pp. 109–124.
- Anjum, M.F., Marooney, C., Fookes, M., Baker, S., Dougan, G., Ivens, A., and Woodward, M.J. (2005) Identification of core and variable components of the *Salmonella enterica* subspecies I genome by microarray. *Infect Immun* **73**: 7894–7905.
- Balbontin, R., Rowley, G., Pucciarelli, M.G., Lopez-Garrido, J., Wormstone, Y., Lucchini, S., *et al.* (2006) DNA adenine methylation regulates virulence gene expression in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **188**: 8160–8168.
- Bäuml, A.J., and Heffron, F. (1995) Identification and sequence analysis of *IpfABCDE*, a putative fimbrial operon of *Salmonella typhimurium*. *J Bacteriol* **177**: 2087–2097.
- Bäuml, A.J., Kusters, J.G., Stojiljkovic, I., and Heffron, F. (1994) *Salmonella typhimurium* loci involved in survival within macrophages. *Infect Immun* **62**: 1623–1630.
- Bäuml, A.J., Tsois, R.M., and Heffron, F. (1996) Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. *Infect Immun* **64**: 1862–1865.
- Blumer, C., Kleefeld, A., Lehnen, D., Heintz, M., Dobrindt, U., Nagy, G., *et al.* (2005) Regulation of type 1 fimbriae synthesis and biofilm formation by the transcriptional regulator LrhA of *Escherichia coli*. *Microbiology* **151**: 3287–3298.
- Blyn, L.B., Braaten, B.A., and Low, D.A. (1990) Regulation of pap pilin phase variation by a mechanism involving differential *dam* methylation states. *EMBO J* **9**: 4045–4054.
- Bochner, B.R. (1984) Curing bacterial cells of lysogenic viruses by using UCB indicator plates. *Biotechniques* **2**: 234–240.
- Chan, K., Baker, S., Kim, C.C., Detweiler, C.S., Dougan, G., and Falkow, S. (2003) Genomic comparison of *Salmonella enterica* serovars and *Salmonella bongori* by use of an *S. enterica* serovar typhimurium DNA microarray. *J Bacteriol* **185**: 553–563.
- Clegg, S., Purcell, B.K., and Pruckler, J. (1987) Characterization of genes encoding type 1 fimbriae of *Klebsiella pneumoniae*, *Salmonella typhimurium*, and *Serratia marcescens*. *Infect Immun* **55**: 281–287.
- Deng, W., Liou, S.R., Plunkett, G., 3rd, Mayhew, G.F., Rose, D.J., Burland, V., *et al.* (2003) Comparative genomics of *Salmonella enterica* serovar Typhi strains Ty2 and CT18. *J Bacteriol* **185**: 2330–2337.
- Duguid, J.P., Anderson, E.S., and Campbell, I. (1966) Fimbriae and adhesive properties in *Salmonellae*. *J Pathol Bacteriol* **92**: 107–138.
- Elliott, T. (1993) Transport of 5-aminolevulinic acid by the dipeptide permease in *Salmonella typhimurium*. *J Bacteriol* **175**: 325–331.
- Emmerth, M., Goebel, W., Miller, S.I., and Hueck, C.J. (1999) Genomic subtraction identifies *Salmonella typhimurium* prophages, F-related plasmid sequences, and a novel fimbrial operon, *stf*, which are absent in *Salmonella typhi*. *J Bacteriol* **181**: 5652–5661.
- Espeli, O., Nurse, P., Levine, C., Lee, C., and Mariani, K.J. (2003) SetB: an integral membrane protein that affects chromosome segregation in *Escherichia coli*. *Mol Microbiol* **50**: 495–509.
- Folkesson, A., Advani, A., Sukopolvi, S., Pfeifer, J.D., Normark, S., and Lofdahl, S. (1999) Multiple insertions of fimbrial operons correlate with the evolution of *Salmonella serovars* responsible for human disease. *Mol Microbiol* **33**: 612–622.
- Friedrich, M.J., Kinsey, N.E., Vila, J., and Kadner, R.J. (1993) Nucleotide sequence of a 13.9 kb segment of the 90 kb virulence plasmid of *Salmonella typhimurium*: the presence of fimbrial biosynthetic genes. *Mol Microbiol* **8**: 543–558.
- Gruber, A., and Zingales, B. (1995) Alternative method to remove antibacterial antibodies from antisera used for screening of expression libraries. *Biotechniques* **19**: 28–30.
- Grund, S., and Weber, A. (1988) A new type of fimbriae on *Salmonella typhimurium*. *Zentralbl Veterinarmed [B]* **35**: 779–782.
- Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* **177**: 4121–4130.
- Haagmans, W., and van der Woude, M. (2000) Phase variation of Ag43 in *Escherichia coli*: Dam-dependent methylation abrogates OxyR binding and OxyR-mediated repression of transcription. *Mol Microbiol* **35**: 877–887.

- Henderson, I.R., and Owen, P. (1999) The major phase-variable outer membrane protein of *Escherichia coli* structurally resembles the immunoglobulin A1 protease class of exported protein and is regulated by a novel mechanism involving Dam and oxyR. *J Bacteriol* **181**: 2132–2141.
- Hughes, K.T., and Roth, J.R. (1988) Transitory *cis* complementation: a method for providing transposition functions to defective transposons. *Genetics* **119**: 9–12.
- Humphries, A.D., Raffatellu, M., Winter, S., Weening, E.H., Kingsley, R.A., Droleskey, R., et al. (2003) The use of flow cytometry to detect expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. *Mol Microbiol* **48**: 1357–1376.
- Humphries, A.D., DeRidder, S., and Bäumler, A.J. (2005) *Salmonella enterica* serotype Typhimurium fimbrial proteins serve as antigens during infection of mice. *Infect Immun* **73**: 5329–5338.
- Kingsley, R.A., Humphries, A.D., Weening, E.H., De Zoete, M.R., Winter, S., Papaconstantinopoulou, A. et al. (2003) Molecular and phenotypic analysis of the CS54 island of *Salmonella enterica* serotype typhimurium: identification of intestinal colonization and persistence determinants. *Infect Immun* **71**: 629–640.
- Lee, C., Wozniak, C., Karlinsky, J.E., and Hughes, K.T. (2007) Genomic screening for regulatory genes using the T-POP transposon. *Methods Enzymol* **421**: 159–167.
- Lehnen, D., Blumer, C., Polen, T., Wackwitz, B., Wendisch, V.F., and Unden, G. (2002) LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in *Escherichia coli*. *Mol Microbiol* **45**: 521–532.
- Lilleengen, K. (1948) Typing of *Salmonella typhimurium* by means of bacteriophage. *Acta Pathol Microbiol Scand Suppl* **77**: 2–125.
- Lim, D.B., Oppenheim, J.D., Eckhardt, T., and Maas, W.K. (1987) Nucleotide sequence of the *argR* gene of *Escherichia coli* K-12 and isolation of its product, the arginine repressor. *Proc Natl Acad Sci USA* **84**: 6697–6701.
- Liu, J.Y., Miller, P.F., Willard, J., and Olson, E.R. (1999) Functional and biochemical characterization of *Escherichia coli* sugar efflux transporters. *J Biol Chem* **274**: 22977–22984.
- deLorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K.N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* **172**: 6568–6572.
- Luthi, E., Mercenier, A., and Haas, D. (1986) The *arcABC* operon required for fermentative growth of *Pseudomonas aeruginosa* on arginine: Tn5-751-assisted cloning and localization of structural genes. *J Gen Microbiol* **132**: 2667–2675.
- Luthi, E., Baur, H., Gamper, M., Brunner, F., Villeval, D., Mercenier, A., and Haas, D. (1990) The *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa* contains an additional gene, *arcD*, encoding a membrane protein. *Gene* **87**: 37–43.
- McClelland, M.K.E., Sanderson, J., Spieth, S.W., Clifton, P., Latreille, L., Courtney, S., et al. (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**: 852–856.
- McClelland, M.K.E., Sanderson, S.W., Clifton, P., Latreille, S., Porwollik, A., Sabo, R., et al. (2004) Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of *Salmonella enterica* that cause typhoid. *Nat Genet* **36**: 1268–1274.
- Maghnoouj, A., de Sousa Cabral, T.F., Stalon, V., and Vander Wauven, C. (1998) The *arcABDC* gene cluster, encoding the arginine deiminase pathway of *Bacillus licheniformis*, and its activation by the arginine repressor *argR*. *J Bacteriol* **180**: 6468–6475.
- Miller, J.H. (1972) *Experiments in Molecular Genetics*. New York: Cold Spring Harbor Laboratory Press.
- Morrow, B.J., Graham, J.E., and Curtiss, R., 3rd (1999) Genomic subtractive hybridization and selective capture of transcribed sequences identify a novel *Salmonella typhimurium* fimbrial operon and putative transcriptional regulator that are absent from the *Salmonella typhi* genome. *Infect Immun* **67**: 5106–5116.
- Nicholson, B., and Low, D. (2000) DNA methylation-dependent regulation of *pef* expression in *Salmonella typhimurium*. *Mol Microbiol* **35**: 728–742.
- Nuccio, S.P., and Bäumler, A.J. (2007) Evolution of the chaperone/usher assembly pathway: fimbrial classification goes greek. *Microbiol Mol Biol Rev* **71**: 551–575.
- Nuccio, S.P., Chessa, D., Weening, E.H., Raffatellu, M., Clegg, S., and Bäumler, A.J. (2007) SIMPLE approach for isolating mutants expressing fimbriae. *Appl Environ Microbiol* **73**: 4455–4462.
- Parkhill, J., Dougan, G., James, K.D., Thomson, N.R., Pickard, D., Wain, J., et al. (2001) Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* **413**: 848–852.
- Porwollik, S., Wong, R.M., and McClelland, M. (2002) Evolutionary genomics of *Salmonella*: gene acquisitions revealed by microarray analysis. *Proc Natl Acad Sci USA* **99**: 8956–8961.
- Porwollik, S., Boyd, E.F., Choy, C., Cheng, P., Florea, L., Proctor, E., and McClelland, M. (2004) Characterization of *Salmonella enterica* subspecies I genovars by use of microarrays. *J Bacteriol* **186**: 5883–5898.
- Raffatellu, M., Chessa, D., Wilson, R.P., Dusold, R., Rubino, S., and Bäumler, A.J. (2005) The Vi capsular antigen of *Salmonella enterica* serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. *Infect Immun* **73**: 3367–3374.
- Rappleye, C.A., and Roth, J.R. (1997) A Tn10 derivative (T-POP) for isolation of insertions with conditional (tetracycline-dependent) phenotypes. *J Bacteriol* **179**: 5827–5834.
- Razin, A., and Friedman, J. (1981) DNA methylation and its possible biological roles. *Prog Nucleic Acid Res Mol Biol* **25**: 33–52.
- Romling, U., Bian, Z., Hammar, M., Sierralta, W.D., and Normark, S. (1998) Curli fibers are highly conserved between *Salmonella typhimurium* and *Escherichia coli* with respect to operon structure and regulation. *J Bacteriol* **180**: 722–731.
- Schäffer, S., Hantke, K., and Braun, V. (1985) Nucleotide sequence of the iron regulatory gene. *Fur Mol Gen Genet* **200**: 110–113.
- Schneider, H.A., and Zinder, N.D. (1956) Nutrition of the host and natural resistance to infection. V. An improved assay

- employing genetic markers in the double strain inoculation test. *J Exp Med* **103**: 207–223.
- Stojiljkovic, I., Bäumler, A.J., and Heffron, F. (1995) Ethanolamine utilization in *Salmonella typhimurium*: nucleotide sequence, protein expression and mutational analysis of the *cchA cchB eutE eutJ eutG eutH* gene cluster. *J Bacteriol* **177**: 1357–1366.
- Stolpe, H., Grund, S., and Schroder, W. (1994) Purification and partial characterization of type 3 fimbriae from *Salmonella typhimurium* var. copenhagen. *Zentralbl Bakteriologie* **281**: 8–15.
- Torreblanca, J., and Casadesus, J. (1996) DNA adenine methylase mutants of *Salmonella typhimurium* and a novel *dam*-regulated locus. *Genetics* **144**: 15–26.
- Townsend, S.M., Kramer, N.E., Edwards, R., Baker, S., Hamlin, N., Simmonds, M., et al. (2001) *Salmonella enterica* serotype Typhi possesses a unique repertoire of fimbrial gene sequences. *Infect Immun* **69**: 2894–2901.
- Tsolis, R.M., Townsend, S.M., Miao, E.A., Miller, S.I., Ficht, T.A., Adams, L.G., and Bäumler, A.J. (1999) Identification of a putative *Salmonella enterica* serotype typhimurium host range factor with homology to IpaH and YopM by signature-tagged mutagenesis. *Infect Immun* **67**: 6385–6393.
- Waldron, D.E., Owen, P., and Dorman, C.J. (2002) Competitive interaction of the OxyR DNA-binding protein and the Dam methylase at the antigen 43 gene regulatory region in *Escherichia coli*. *Mol Microbiol* **44**: 509–520.
- Wallecha, A., Munster, V., Correnti, J., Chan, T., and van der Woude, M. (2002) Dam- and OxyR-dependent phase variation of *agn43*: essential elements and evidence for a new role of DNA methylation. *J Bacteriol* **184**: 3338–3347.
- Weening, E.H., Barker, J.D., Laarakker, M.C., Humphries, A.D., Tsolis, R.M., and Bäumler, A.J. (2005) The *Salmonella enterica* serotype Typhimurium *lpf*, *bcf*, *stb*, *stc*, *std*, and *sth* fimbrial operons are required for intestinal persistence in mice. *Infect Immun* **73**: 3358–3366.
- van der Woude, M.W., and Low, D.A. (1994) Leucine-responsive regulatory protein and deoxyadenosine methylase control the phase variation and expression of the *sfa* and *daa* pili operons in *Escherichia coli*. *Mol Microbiol* **11**: 605–618.