The *yefABEF* operon of *Salmonella* confers resistance to antimicrobial peptides and contributes to its virulence

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Pathogenic micro-organisms have evolved many strategies to counteract the antimicrobial peptides (AMPs) that they encounter upon entry into host systems. These strategies play vital roles in the virulence of pathogenic micro-organisms. The *Salmonella enterica* serovar Typhimurium genome has a gene cluster consisting of *yefA*, *yefB*, *yefE* and *yefF* genes, which encode a putative ATP-binding cassette (ABC) transporter. Our study shows that these genes constitute an operon. We deleted the *yefF* gene, which encodes the ATPase component of the putative ABC transporter. The $\Delta yefF$ strain showed increased sensitivity to protamine, melittin, polymyxin B, human defensin (HBD)-1 and HBD-2, and was compromised in its capacity to proliferate inside activated macrophages and epithelial cells. Inside Intestine 407 cells, *Salmonella* was found to co-localize with human defensins HD-5 and HBD-1; this suggests that the ability to counteract AMPs in the intracellular milieu is important for *Salmonella*. In a murine typhoid model, the $\Delta yefF$ strain displayed decreased virulence when infected intragastrically. These findings suggest that the putative transporter encoded by the *yefABEF* operon is involved in counteracting AMPs, and that it contributes to the virulence of *Salmonella*.

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INTRODUCTION

Salmonella enterica serovar Typhimurium is a Gram-negative facultative intracellular pathogen that causes a systemic disease in mice that is similar to typhoid caused by serovar Typhi in humans. Serovar Typhimurium is also an important causative agent of food poisoning in humans.

Multicellular organisms use various defence strategies to protect themselves from microbial infections. Production of antimicrobial peptides (AMPs) is one of these strategies. AMPs are widely distributed in phylogenetically diverse animal, plant and bacterial species (Hancock & Chapple, 1999; Lehrer & Ganz, 1999). Most of these peptides are cationic in nature, and they interact with the bacterial cytoplasmic membrane, which usually comprises negatively charged phospholipids. AMPs exhibit broad antimicrobial activity against Gram-negative and Gram-positive bacteria,

fungi and enveloped viruses (Lehrer, 2007). They also function as chemotactic agents, cytotoxins and opsonins (Ganz *et al.*, 1990).

A continuous monolayer of gastrointestinal epithelial cells functions as the primary physical barrier against microbes, such as *Salmonella*, that enter the host through the gastrointestinal tract. Intestinal epithelial cells, especially Paneth cells, produce several AMPs that are lethal to invading bacteria (Ayabe *et al.*, 2000; Ouellette *et al.*, 1994). Murine macrophages produce cathelin-related antimicrobial peptides (CRAMPs), which serve as antimicrobial agents against intracellular *Salmonella* (Rosenberger *et al.*, 2004). To cause disease, pathogenic bacteria, such as *Salmonella*, must endure the battery of these AMPs. Mutants of *Salmonella* that are sensitive to AMPs are compromised in their virulence (Groisman *et al.*, 1992). Bacteria have developed many strategies to protect themselves from AMPs. For example, they reduce the net negative charge on the cell envelope by modifying lipid A; this helps to reduce the attraction of cationic AMPs towards bacteria. They also pump AMPs out of the bacteria using energy-dependent transporters, and cleave AMPs using surface-associated proteases. A putative ATP-binding cassette (ABC) transporter encoded by the *sapABCDF*

Abbreviations: ABC, ATP-binding cassette; AMP, antimicrobial peptide; CRAMP, cathelin-related antimicrobial peptide; FRT, flippase recombinase target; HBD, human β -defensin; HD, human defensin; HPRT, hypoxanthine phosphoribosyl transferase; MHC, major histocompatibility complex; WT, wild-type.

Three supplementary tables and four supplementary figures are available with the online version of this paper.

operon is required to counteract AMPs, and it contributes to the virulence of *Salmonella* (Groisman *et al.*, 1992). The presence of such a variety of strategies to counteract AMPs suggests the significant role of AMPs in host defence against microbes (Guina *et al.*, 2000; Guo *et al.*, 1998; Peschel, 2002; Shafer *et al.*, 1998; Stumpe *et al.*, 1998).

The *S. Typhimurium* genome contains a gene cluster located at minute 49 that encodes the components of a putative ABC-type dipeptide/oligopeptide/nickel transport system. This putative operon consists of four genes: *yejA* (STM 2216), which encodes a putative periplasmic binding protein; *yejB* (STM 2217) and *yejE* (STM 2218), which encode putative permease components; and *yejF* (STM 2219), which encodes the ATPase component of this transporter. Qimron *et al.* have screened for genes that interfere with major histocompatibility complex (MHC) class I presentation in *S. Typhimurium*. In their screen, they identified that the *yej* operon interferes with MHC I presentation. They showed that specific CD8 T cells were elicited at a higher level in mice in response to immunization with *yej* mutants, compared with immunization with the parental strain (Qimron *et al.*, 2004). Antal *et al.* have observed that a small non-coding bacterial RNA of 62–64 nt, RydC, regulates the expression of the *yejABEF* gene cluster, and overexpression of RydC abolishes the expression of these genes (Antal *et al.*, 2005). It has also been reported that the *yejE* and *yejF* genes of *S. enterica* serovars Typhimurium and Typhi are upregulated inside host macrophages (Eriksson *et al.*, 2003; Faucher *et al.*, 2006), indicating the importance of these genes inside host cells. Apart from these reports, not much is known about the function of the *yejA*, *yejB*, *yejE* and *yejF* genes.

The objective of the present study was to probe into the other possible functions of *yejA*, *yejB*, *yejE* and *yejF* genes, and to investigate their role in the virulence of *Salmonella*. In this study, we show that mutants of the *yejABEF* operon are more sensitive to AMPs, are compromised to

proliferate inside activated macrophages and epithelial cells, and have decreased virulence in a murine typhoid model.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. Bacteria were grown at 37 °C in Luria broth (LB), unless mentioned otherwise. Carbenicillin, kanamycin, nalidixic acid (all at 50 µg ml⁻¹) and chloramphenicol (10 µg ml⁻¹) were added when required for selection of strains. F medium composition was as follows: 1 × N-salts (10 × N-salts: 50 mM potassium chloride, 75 mM ammonium sulphate, 5 mM potassium sulphate, 10 mM potassium dihydrogen phosphate and 1 M Bistris), 8 µM magnesium chloride, 38 mM glycerol and 0.1 % Casamino acids, pH 5.2. The growth study in a lower K⁺ concentration was done as described (Parra-Lopez *et al.*, 1994). The composition of the medium was: 50 mM MES, 5 mM Na₂HPO₄, 10 mM (NH₄)₂SO₄, 10 mM KCl, 1 mM MgSO₄, 4 mM Tricine and 10 µM FeSO₄. Glucose was added to a final concentration of 5 mM, and glycerol and DL-lactate to a final concentration of 10 mM each.

Eukaryotic cell lines and growth conditions. Eukaryotic cell lines used in this study are listed in Table S3 (available with the online version of this paper). RAW 264.7 cells were the kind gift of Professor Anjali Karande (Department of Biochemistry, Indian Institute of Science). J774A.1 cells were the kind gift of Dr K. N. Balaji (Department of Microbiology and Cell Biology, Indian Institute of Science). U-937, Intestine 407, COLO 205 and Caco-2 cells were obtained from the National Center for Cell Science, Pune, India. The cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma), except COLO 205, which was grown in RPMI 1640 medium (Sigma); both DMEM and RPMI 1640 were supplemented with 10 % fetal calf serum (Sigma). All cells were maintained at 37 °C in 5 % carbon dioxide.

Bacterial RNA extraction from infected cells. Bacterial RNA from infected cells was isolated as described previously (Eriksson *et al.*, 2003). Twelve hours after infection, cells were lysed on ice by incubating for 30 min in 0.1 % SDS, 1 % acidic phenol and 19 % ethanol in water. *Salmonella* were isolated from the lysate by

Table 1. Strains and plasmids used in this study

Strain	Description	Reference/source
WT	WT 12023, parental strain for all mutants, resistant to nalidixic acid	Chakravorty <i>et al.</i> (2005)
	WT 12023 with pFPV25.1, resistant to carbenicillin (used in competition experiment only)	Chakravorty <i>et al.</i> (2005)
<i>ΔyejF</i>	<i>ΔyejF</i> ::CAT	This study
<i>ΔyejA</i>	<i>ΔyejA</i> :: <i>kan</i>	This study
<i>ΔyejB</i>	<i>ΔyejB</i> :: <i>kan</i>	This study
<i>ΔyejE</i>	<i>ΔyejE</i> :: <i>kan</i>	This study
<i>Δsap</i>	<i>Δsap</i> :: <i>kan</i> (the entire <i>sap</i> operon, consisting of <i>sapABCDF</i> genes, was deleted)	This study
<i>ΔsapΔyejF</i>	<i>Δsap</i> :: <i>kan ΔyejF</i> ::CAT <i>S. typhimurium</i>	This study
Plasmid		
pTrc99A <i>yejF</i>	<i>S. typhimurium yejF</i> complementing vector (Amp ^R)	This study
pQE60 <i>yejF</i>	<i>S. typhimurium yejF</i> complementing vector (Amp ^R)	This study
pKD46	Plasmid expressing λ Red recombinase system (Amp ^R)	Datsenko & Wanner (2000)
pKD3	Plasmid with FRT-flanked chloramphenicol-resistance gene	Datsenko & Wanner (2000)
pKD4	Plasmid with FRT-flanked kanamycin-resistance gene	Datsenko & Wanner (2000)

centrifugation, and RNA was isolated using TRI reagent (Sigma), according to the manufacturer's protocol. In each case, bacteria were recovered from a six-well plate of infected cells and pooled to isolate RNA. RNA from *in vitro*-grown bacteria was obtained by growing bacteria statically at 37 °C to mid-exponential phase in DMEM medium, under 5% CO₂. These conditions mimicked those used for the cell-infection experiments.

RT-PCR. Bacterial RNA (extracted from infected cells, bacteria grown in LB medium or bacteria grown in DMEM medium) was reverse transcribed using a reverse transcription system (Promega) and a gene-specific primer (*yejF* or 16S rRNA). It was then amplified (35 cycles) using primers generated to amplify the intergenic regions of the *yejABEF* genes, to check co-transcription (Mason *et al.*, 2005). To check the expression level inside eukaryotic cells, cDNA was amplified (30 cycles) using primers (Table S1, available with the online version of this paper) generated to amplify the intergenic region of the *yejE* and *yejF* genes. Control reactions were done without reverse transcription to verify the absence of DNA in RNA preparations.

To check the expression of genes encoding human defensin (HD)-5 and human β defensin (HBD)-1, total RNA was isolated from 500 000 Intestine 407 cells, seeded in a 35 mm Petri dish, using TRI reagent (Sigma). RNA was reverse transcribed (Promega), and the cDNA was used to amplify the cDNA of the genes encoding HD-5, HBD-1 and hypoxanthine phosphoribosyl transferase (HPRT) (see Table S1).

Construction of gene deletions in *S. Typhimurium*. Knockout constructions were done as described (Datsenko & Wanner, 2000). This method has been used successfully to knock out single genes, as well as an operon. *Salmonella* transformants carrying a Red helper plasmid (pKD46) were grown in LB with carbenicillin and 10 mM L-arabinose (Sigma), at 30 °C, to an OD₆₀₀ of 0.35–0.4, and then made electrocompetent by washing three times with ice-cold 10% (v/v) glycerol and MilliQ water. The PCR product containing the chloramphenicol-resistance gene (from pKD3) or the kanamycin-resistance gene (from pKD4), flanked by sequences upstream and downstream of the target genes, was obtained using specific primers (Table S1). PCR products were digested with *DpnI* to digest the methylated template. Electroporation was done according to the manufacturer's instructions (Bio-Rad), using 500 ng PCR product. Transformants were selected on LB agar containing chloramphenicol or kanamycin. The knockouts were confirmed using specific primers (Table S1). The *yejF* knockout was also confirmed with a reverse primer (Table S1) specific for the *CAT* gene of pKD3.

Construction of the $\Delta yejF\Delta sap$ strain. pKD46 was transformed into the Δsap strain. To this, the PCR product containing the chloramphenicol-resistance gene (from pKD3), flanked by sequences upstream and downstream of the *yejF* gene, was electroporated. There was a possibility that the chloramphenicol cassette could replace the kanamycin cassette that was present in the Δsap strain, because of the presence of the flippase recombinase target (FRT) sequence. To avoid such recombinants, transformants were selected on LB agar containing kanamycin and chloramphenicol. The double-knockout strain was confirmed using specific primers designed for both *yejF* and the *sap* operon (see Table S1).

Complementation of $\Delta yejF$. First, the *yejF* gene was cloned in pET-21a(+) using *Bam*HI and *Xho*I. The *yejF* fragment was subcloned, after digesting with *Bam*HI and *Xho*I, into the *Bam*HI and *Sal*I sites of pTrc99A. The plasmid was electroporated into the $\Delta yejF$ strain to obtain the pTrc99A*yejF* strain. This strain showed a significant decrease in virulence in a murine typhoid model. This non-specific effect of some cloning vectors on virulence has been described recently (Knodler *et al.*, 2005). Hence, the *yejF* fragment from

pTrc99A was moved to pQE60, after digesting with *Bam*HI and *Hind*III, to obtain the pQE60*yejF* strain.

AMP sensitivity assay. This was done as described by Fields *et al.* (1989), with some modifications. Bacteria in exponential phase were seeded in a 96-well plate at a concentration of 2×10^4 to 5×10^4 bacteria per well, in 50 μ l of a solution containing 0.5% tryptone and 0.5% sodium chloride. A 100 μ l volume of specific AMP was added at a specified concentration (polymyxin B, 0.15 μ g ml⁻¹; protamine, 40 μ g ml⁻¹; melittin, 5 μ g ml⁻¹; HBD-1 and HBD-2, 2 μ g ml⁻¹ each) and incubated at 37 °C in shaking conditions for 1 h. The samples were then plated at different dilutions on LB plates. For the HBD-1 sensitivity assay, 10 mM potassium phosphate buffer was used, as the presence of sodium chloride is known to inhibit the action of HBD-1 (Singh *et al.*, 1998), and the incubation period was increased to 4 h. Data are presented as percentage survival relative to the untreated sample. Protamine, melittin and polymyxin B were purchased from Sigma. HBD-1 and HBD-2 were purchased from CytoLab/PeptoTech Asia.

Intracellular proliferation assay. Cells at a concentration of 1×10^5 to 2×10^5 per well were plated in 24-well plates 24 h prior to infection. These cells were infected with a specific strain at an m.o.i. of 1. To check transcription level of the *yej* genes by RT-PCR, an m.o.i. of 1:100 was used. Macrophages were infected with bacteria from an overnight culture. Overnight cultures were diluted to a ratio of 1:33 and grown for 3 h to late-exponential phase prior to the infection of epithelial cells. The plate was centrifuged at 120 g for 5 min and incubated at 37 °C for 20 min. The cells were then washed with PBS to remove excess bacteria and fresh medium containing 100 μ g gentamicin ml⁻¹ was added. After 1 h, the medium was discarded and the cells were washed with PBS, and then added to medium containing 25 μ g gentamicin ml⁻¹ and incubated at 37 °C for further time periods. After specified incubation periods, cells were lysed with 0.1% Triton X-100 (Sigma), the lysate was plated on LB agar containing the appropriate antibiotic at different dilutions and the colonies were counted to calculate the c.f.u. Fold intracellular replication was calculated by dividing the intracellular bacterial load at 16 h by the intracellular bacterial load at 2 h.

Confocal microscopy. To study the morphology of bacteria after treating with HBD-2, 2×10^4 to 5×10^4 exponential-phase bacteria were seeded on coverslips in 24-well plates and treated with HBD-2 at a concentration of 2 μ g ml⁻¹ for 30 min at 37 °C. Bacteria were then fixed using 3.5% paraformaldehyde for 20 min. They were stained for LPS using monoclonal anti-*S. Typhimurium* LPS antibody (HyTest), raised in mouse, as the primary antibody, and Cy3-conjugated anti-mouse IgG antibody (Dianova), raised in goat, as the secondary antibody. Both antibodies were diluted in PBS containing 2% BSA and 2% goat serum for staining. Images were taken using a confocal laser-scanning microscope (Zeiss LSM Meta). Bacteria showing blebbings were counted. The percentage of bacteria showing blebbings relative to total number of bacteria counted was calculated.

To study the localization of HD-5 and HBD-1, Intestine 407 cells were seeded on coverslips in a 24-well plate and infected with either the wild-type (WT) strain or the $\Delta yejF$ strain, as described above. At specified time points, cells were fixed with 3.5% paraformaldehyde and stained for HD-5, or HBD-1 and LPS. Affinity-purified anti-HD-5 antibody or anti-HBD-1 antibody (Alpha Diagnostic International), raised in rabbit, was used as the primary antibody, and Cy2-conjugated anti-rabbit antibody, raised in goat (Jackson ImmunoResearch Laboratories), was used as the secondary antibody. LPS staining was done using the antibodies described above. All antibodies were diluted in PBS containing 0.1% saponin, 2% BSA and 2% goat serum, for staining. After staining, images were taken using a confocal laser-scanning microscope (Zeiss LSM Meta).

Scanning electron microscopy. Bacteria (10^8) were treated with 1 mg polymyxin B ml^{-1} for 1 h and fixed using Karnovsky's fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer). After fixing, samples were treated with 1% osmium tetroxide for 1 h. Samples were subsequently washed and dehydrated in a series of ethanol washes (70% for 10 min, 90% for 10 min and 100% for 30 min) and air-dried prior to sputter coating with gold. Samples were then analysed using a scanning electron microscope (FEI Quanta 200).

Mouse experiments. Six- to eight-week-old BALB/c mice (Central Animal Facility, Indian Institute of Science, Bangalore, India) were maintained under specific-pathogen-free conditions. All procedures with animals were carried out in accordance with institutionally approved protocols. Mice were infected intragastrically or intraperitoneally under aseptic conditions with the indicated doses. For the survival assay, 10 mice were used for each strain of bacteria and observed twice daily for survival. In organ infiltration studies, liver, spleen and Peyer's patches were taken aseptically, 4 days after infection. The organs were weighed and homogenized in a tissue homogenizer, in 1 ml PBS. The homogenate was centrifuged and plated at different dilutions. For liver and spleen, LB agar was used, and for Peyer's patches, *Salmonella Shigella* agar (HiMedia) was used. The number of c.f.u. was calculated per gram weight of organ for liver and Peyer's patches, and per organ for spleen.

Determination of mouse competitive indices. This was done as described (Beuzon & Holden, 2001). The WT and $\Delta yefF$ strains were diluted and mixed at a ratio of 1:1 to a final bacterial concentration of 10^7 bacteria ml^{-1} . A 1 ml volume of this bacterial suspension was inoculated intragastrically into 6- to 8-week-old BALB/c mice. Five mice were used for each pair of strains. Four days after infection, the mice were sacrificed, and spleens and Peyer's patches were removed and homogenized in sterile PBS. The numbers of WT and $\Delta yefF$ strains in each infected organ were then quantified by serial dilution and plating on selective media. The $\Delta yefF$ strain was marked with chloramphenicol resistance, whereas the WT strain harboured carbenicillin resistance. The competitive index was calculated by dividing the number of mutant bacteria recovered from infected animals by the number of WT bacteria recovered. This value was then corrected by the initial ratio of mutant to WT bacteria used to infect the mice. This experiment was performed twice.

Statistical methods. For AMP sensitivity assays and the intracellular survival assay, Student's *t* test was employed. For mouse experiments, the Mann-Whitney *U* test was employed.

Bioinformatics analysis. Amino acid sequence identity and similarity were estimated with the help of the 'Align two sequences' option of BLAST.

RESULTS AND DISCUSSION

Products of *yejA*, *B*, *E* and *F* genes share amino acid sequence similarity with the components of other peptide transporters of *Salmonella*

yejA, *B*, *E* and *F* genes are annotated as genes encoding components of a putative ABC transporter system. In order to obtain an indication of their functions, we searched for other transporters that share amino acid sequence similarity with the products of the *yejA*, *B*, *E* and *F* genes. Bioinformatic analysis using BLAST revealed that the proteins encoded by the *yejABEF* operon share amino acid

sequence similarity with other peptide transporters, such as the transporter encoded by the *sapABCD* operon (henceforth referred to as the *sap* operon), the Opp transporter, which is involved in oligopeptide transport (Hiles *et al.*, 1987), and the Dpp transporter, which is involved in dipeptide transport (Abouhamad *et al.*, 1991) (Table S2, available with the online version of this paper). The *sap* operon is known to contribute to virulence in *Salmonella* spp., *Haemophilus* spp. and *Erwinia* spp. (Groisman *et al.*, 1992; Lopez-Solanilla *et al.*, 1998; Mason *et al.*, 2005) by counteracting AMPs. The Opp transporter has also been implicated in the virulence of *Staphylococcus aureus* (Mei *et al.*, 1997). Based on these facts, we hypothesized that the transporter system encoded by the *yejABEF* operon (henceforth referred to as the *yej* operon) might be involved in conferring virulence to *Salmonella*, and may be involved in counteracting AMPs, similar to the transporter system encoded by the *sap* operon.

The *yejA*, *B*, *E* and *F* genes are co-transcribed as a single message in *S. Typhimurium*

Before investigating the biological importance of the *yejA*, *B*, *E* and *F* genes, we demonstrated that these genes are co-transcribed as a single message using specific primers (Table S1) designed to amplify the nucleotide sequence spacing the junction region between each pair of adjacent genes (Fig. 1a). RNA was isolated from a stationary-phase culture, and reverse transcribed using a *yejF*-specific primer. The cDNA was used to amplify the intergenic regions. PCR products were detected for intergenic regions of the *yejA*, *B*, *E* and *F* genes. No product was detected for the *rtn-yej* region, or control samples lacking reverse transcriptase (Fig. 1a). This result demonstrated that *yejA*, *B*, *E* and *F* genes constitute an operon.

yej genes are upregulated inside host cells

A previous microarray study by Eriksson *et al.* showed that *yejE* and *yejF* genes of *S. Typhimurium* are upregulated (approx. two- and tenfold, respectively) upon infection in J774A.1 cells (Eriksson *et al.*, 2003). A similar finding was recently observed in *Salmonella* Typhi in THP-1 cells (Faucher *et al.*, 2006). To follow up this observation, RT-PCR was done to check the expression of *yej* genes inside J774A.1 and Intestine 407 cells. Twelve hours after infection, bacteria were recovered, RNA was isolated, and reverse transcription was done as described in Methods. Intergenic primers between the *yejE* and *yejF* genes were used for PCR (Fig. 1b). The results were analysed using Multi Gauge densitometry software and, after normalizing with an internal control (16S rRNA), it was observed that the *yej* genes are upregulated about sevenfold in J774A.1, which is a macrophage cell line, and in Intestine 407, which is an epithelial cell line. This result confirmed the previous result, and also indicated the importance of the *yej* operon in the epithelial cells that *Salmonella* encounters first during its course of infection.

Fig. 1. (a) *yejABEF* genes are co-transcribed as a single operon. RNA was prepared from stationary-phase cultures and the mRNA was reverse transcribed. Primers (indicated by black arrows), spanning the four ORFs, were used to amplify the intergenic regions. (b) Transcription of the *yejABEF* operon was enhanced in J774A.1 and Intestine 407 cells. RT-PCR was done to determine the transcription of the *yejABEF* operon inside J774A.1 and Intestine 407 cells. Primers designed for the intergenic region of *yejE* and *yejF* were used for this analysis and 16S rRNA was used as an endogenous control. RNA isolated from the bacteria grown in DMEM at 37 °C under 5% CO₂ was used for control RT-PCR to determine expression outside host cells. Numbers in the parentheses indicate the values of densitometric image analysis using Multi Gauge software. The densitometric values were normalized with that of 16srRNA. Images are representatives of two independent experiments.

Deletion of the *yejF* gene confers susceptibility to AMPs

yejF codes for a protein that has two putative ATP-binding cassettes, and could be the ATPase components for the transporter system encoded by the *yej* operon. We thus reasoned that YejF is energizing this transport system, and that deletion of the *yejF* gene would abolish the function of this transporter. We adopted the Lambda Red recombinase system to delete *yejF*, as described in Methods (Datsenko & Wanner, 2000). The $\Delta yejF$ strain grew normally in LB medium when compared with the WT strain (data not shown).

Based on our hypothesis that the *yej* operon may confer resistance to AMPs, we investigated the sensitivity of the $\Delta yejF$ strain to different AMPs. *Salmonella* spp. have been isolated from insects, amphibians, birds and a variety of mammals. *Salmonella* must have strategies to counteract AMPs found in all of these organisms. Protamine, melittin and polymyxin B represent AMPs from different organisms. Protamine is derived from salmon sperm. The role of the *sap* operon in counteracting AMPs was identified by Groisman *et al.* (1992) by screening for mutants that were sensitive to protamine. Melittin and polymyxin B are derived from honey bee and *Paenibacillus polymyxa*, respectively. It was observed that the $\Delta yejF$ was more sensitive to protamine, melittin and polymyxin B (Fig. 2a and Fig. S1, available with the online version of this paper) when compared with the WT. Sensitivity to polymyxin B, a product of another bacterium, reflects the importance of the *yej* transporter in competing with other bacteria in the

environment. *S. Typhimurium sap* mutant strains have been reported to be sensitive to protamine (Groisman *et al.*, 1992). We compared the protamine sensitivity of the $\Delta yejF$ and Δsap strains with that of the $\Delta sap\Delta yejF$ strain, in which transporters encoded by both the *yej* and the *sap* operons were inactivated. It was observed that $\Delta yejF$, Δsap and $\Delta sap\Delta yejF$ strains were equally sensitive to 40 μ g protamine ml⁻¹ (Fig. 2c). This may be due to other genes that have roles in counteracting AMPs in *Salmonella* taking over the function, at least to some extent, of counteracting AMPs when *yej* and *sap* systems are non-functional. The $\Delta yejB$ and $\Delta yejE$ strains were also sensitive to protamine and polymyxin B, confirming the role of the *yej* operon in conferring resistance to AMPs in *Salmonella* (Fig. 2b). Surprisingly the $\Delta yejF$ strain did not show any increased sensitivity to polymyxin, protamine (Fig. 2b) or melittin (data not shown), suggesting that YejA is dispensable in this aspect.

In order to confirm the hypersensitivity of the $\Delta yejF$ strain to AMPs, we looked at the morphology of bacteria treated with polymyxin B using a scanning electron microscope. In the case of the $\Delta yejF$ strain, we could see many damaged bacteria with membrane irregularities (inset, Fig. 3a), and many bacteria with their extruded cytoplasm formed an irregular mass of debris. When we scored for disrupted bacteria, we found 16% (20 out of 122) of the WT strain, and 54% (63 out of 117) of the $\Delta yejF$ strain, showed either membrane irregularities and/or other features of bacterial lysis (Fig. 3a).

Salmonella has been reported to induce the expression of HBD-2 in Caco-2 cells (Ogushi *et al.*, 2001, 2004). To

Fig. 2. (a) Susceptibility of the $\Delta yejF$ strain to protamine (40 $\mu\text{g ml}^{-1}$), melittin (5 $\mu\text{g ml}^{-1}$) and polymyxin B (0.15 $\mu\text{g ml}^{-1}$). Black bars, WT; light grey bars, $\Delta yejF$; dark grey bars, pTrc99AyejF. (b) Susceptibility of $\Delta yejA$ (light grey bars), $\Delta yejB$ (dark grey bars) and $\Delta yejE$ (white bars) strains to protamine (40 $\mu\text{g ml}^{-1}$) and polymyxin B (0.15 $\mu\text{g ml}^{-1}$). Black bars, WT. (c) Susceptibility of $\Delta yejF$, Δsap and $\Delta sap\Delta yejF$ strains to protamine (40 $\mu\text{g ml}^{-1}$). (d) and (e) Susceptibility of the $\Delta yejF$ strain to HBD-1 (2 $\mu\text{g ml}^{-1}$) and HBD-2 (2 $\mu\text{g ml}^{-1}$). Assays were done as described in Methods, with triplicate samples. Values represent means of two independent experiments, each with triplicate samples. Standard error bars are shown. Statistical significance was defined as follows (Student's *t* test): ** $P < 0.005$; * $P < 0.01$; # $P < 0.05$. (f) Growth of $\Delta yejF$ (○) and Δsap (▼) strains in minimal medium with 10 mM K⁺. ●, WT.

further characterize the role of the *yej* operon in counteracting mammalian AMPs, we investigated the sensitivity of the $\Delta yejF$ strain to HBD-2 and HBD-1. As expected the $\Delta yejF$ strain was more sensitive to both HBD-2 and HBD-1 at 2 $\mu\text{g ml}^{-1}$ concentration when compared with the WT strain (Fig. 2d and e). To further confirm this, we looked at the morphology of the bacteria after HBD-2 treatment (2 $\mu\text{g ml}^{-1}$) using a confocal laser-scanning microscope. A total of 84 % of cells of the $\Delta yejF$ strain showed blebbings on the membrane; this is a characteristic feature of bacteria exposed to AMPs, as observed in *Escherichia coli*, *Pseudomonas aeruginosa*, *Staph. aureus* and *Mycobacterium tuberculosis* (Lehrer *et al.*, 1989; Sawyer *et al.*, 1988; Shai, 2002; Shimoda *et al.*, 1995). However, only 11 % of cells of the WT strain showed such membrane defects at this concentration of HBD-2 (Fig. 3b).

In *E. coli*, the SapD protein, which is a product of the *sap* operon, confers ATP dependence to the Trk potassium-uptake system that mediates potassium homeostasis, contributing to extracytoplasmic protease activity by OmpT, and the subsequent degradation of toxic peptides (Harms *et al.*, 2001; Stumpe & Bakker, 1997; Stumpe *et al.*, 1998). However, the $\Delta yejF$ strain, which lacks the *sapD* homologue *yejF*, did not show any growth defect at a lower potassium concentration, unlike Δsap (Fig. 2f). This

showed that the mechanism of counteraction of AMPs by the transporter encoded by the *yej* operon is not connected to potassium transport, unlike that of the *sap* operon.

$\Delta yejF$ strain is attenuated for replication inside activated macrophages and epithelial cells

Since *yejF* is upregulated inside the macrophage-like cell line J774A.1, we looked for the intracellular replication capacity of the $\Delta yejF$ strain in RAW 264.7, J774A.1 and elicited mouse peritoneal macrophages (data not shown). We did not find any defect in the capacity of the $\Delta yejF$ strain to replicate inside any of these macrophage cell lines (Fig. 4a). This result was in accordance with the finding of Qimron *et al.* (2004). Brodsky *et al.* showed that *mig-14* confers resistance to AMPs in *Salmonella* (Brodsky *et al.*, 2002). It has also been reported that a *mig-14* mutant was compromised in its replication inside activated macrophages, but not inside naïve macrophages (Brodsky *et al.*, 2005). Increased expression of CRAMP in activated macrophages has been implicated for this phenotype of *mig-14* mutant of *Salmonella* (Brodsky *et al.*, 2005). We looked at the replication of the $\Delta yejF$ strain in RAW 264.7 cells, which were activated by pre-treating with interferon- γ and LPS. In agreement with the previous observation that

Fig. 3. (a) Scanning electron microscopic images of the WT and $\Delta yejF$ strains treated with polymyxin B. Insets show enlarged images of representative bacteria. Eighty-four per cent (102 out of 122 cells) of the WT strain, and 46 % (54 out of 117 cells) of the $\Delta yejF$ strain, appeared healthy and intact. Arrows indicate disrupted bacteria. Scale bar, 5 μm . (b) Confocal microscopy images of the WT and $\Delta yejF$ strains treated with HBD-2 (2 $\mu\text{g ml}^{-1}$) for 30 min and stained for LPS. Eighty-four per cent (43 out of 51 cells) of the $\Delta yejF$ strain, and 11 % (4 out of 36 cells) of the WT strain, showed membrane blebblings. Bars, 2 μm .

activated macrophages limit intracellular replication of *Salmonella* (Rosenberger & Finlay, 2002), we did not observe any significant replication of the WT strain in activated RAW 264.7 cells, and the $\Delta yejF$ strain showed decreased survival (Fig. 4b). The $\Delta yejF$ strain has also been shown to be compromised in its replication within phorbol-12-myristate-13-acetate-activated U937 cells, which are derived from human monocytes, and are known to produce LL-37 (Agerberth *et al.*, 2000) (Fig. S2, available with the online version of this paper).

Macrophages activated by IFN- γ upregulate a variety of effector functions, including production of reactive oxygen and reactive nitrogen species (Ding *et al.*, 1988), increased expression of AMPs (Brodsky *et al.*, 2005; Hiemstra *et al.*, 1993), and increased trafficking of phagocytosed material to the lysosome (Ishibashi & Arai, 1990). Though the $\Delta yejF$ strain can be sensitive to any of the above antimicrobial mechanisms of activated macrophages, the compromised phenotype of the $\Delta yejF$ strain inside activated macrophages is most likely to be due to its sensitivity to AMPs such as CRAMP, as suggested by the results of our *in vitro* studies (Figs 2 and 3). Moreover, CRAMP is known to colocalize

with *Salmonella* inside murine macrophages and impair its replication (Rosenberger *et al.*, 2004).

Salmonella encounters epithelial cells in the intestine before entering macrophages. Epithelial cells are known to express genes that encode for AMPs like defensins (Fahlgren *et al.*, 2004; Mineshiba *et al.*, 2005; O'Neil *et al.*, 1999; Ogushi *et al.*, 2001). We checked for the intracellular replication capacity of the $\Delta yejF$ strain in Intestine 407 cells, COLO 205 cells, HeLa cells and Caco-2 cells; these are epithelial cell lines derived from different tissues. It has been reported that Intestine 407 cells express genes coding for HBD-3 and HBD-4 (Fahlgren *et al.*, 2004), HeLa cells and Caco-2 cells express HBD-2, to which $\Delta yejF$ was more sensitive (Mineshiba *et al.*, 2005; Ogushi *et al.*, 2001, 2004), and Caco-2 cells also express HBD-1 constitutively (O'Neil *et al.*, 1999). The $\Delta yejF$ strain was attenuated in intracellular replication in these epithelial cells (Fig. 4c, d and e). However, the invasion was not defective, as the number of colonies of the $\Delta yejF$ strain after 30 min of infection was comparable to that of the WT strain (Fig. 4d). The $\Delta yejB$ and $\Delta yejE$ strains were also defective in intracellular replication within Intestine 407 cells (Fig. 4f).

Fig. 4. Intracellular replication of strains in (a) RAW 264.7 (black bars) and J774A.1 (grey bars) cells; (b) activated RAW 264.7 cells (+IFN- γ and LPS); (c), (d) and (f) Intestine 407 cells; (e) HeLa, COLO 205 and Caco-2 cells. Values represent means of two independent experiments, each with triplicate samples. Standard error bars are shown. Statistical significance was defined as follows: ** $P < 0.005$; # $P < 0.05$. (Student's t test). (d) ●, WT; ○, $\Delta yejF$ strain. (e) Black bars, WT; grey bars, $\Delta yejF$ strain.

However, the $\Delta yejA$ strain did not show any such defect, in agreement with the results of AMP-sensitivity experiments (Fig. 4f). These findings suggest that the *yej* operon confers the ability to proliferate inside activated macrophages and epithelial cells.

The minimal medium F, which is low in magnesium and phosphorous, has a low pH of 5.0, and is poor in aromatic amino acids. This medium mimics the environment of a *Salmonella*-containing vacuole inside host cells (Chakravorty *et al.*, 2005). The $\Delta yejF$ strain grew normally in F medium (data not shown), indicating that this strain is not compromised in its capacity to utilize the limited nutrition available inside the phagosomes of host cells, but that it may be compromised in its resistance to host intracellular defences, such as AMPs.

Surprisingly, unlike $\Delta yejF$, $\Delta yejE$ and $\Delta yejB$ strains, the $\Delta yejA$ strain did not show any difference when compared with the WT strain in its survival upon treatment with protamine, polymyxin B (Fig. 2b) and melittin (data not shown), and the $\Delta yejA$ strain proliferated normally in Intestine 407 cells (Fig. 4f). *yejA* encodes a putative periplasmic-binding protein of the Yej transporter. Periplasmic-binding proteins bind to the substrates and deliver them to the import complex in the inner membrane

of Gram-negative bacteria. The different phenotype of the $\Delta yejA$ strain, when compared with the $\Delta yejB$, $\Delta yejE$ and $\Delta yejF$ strains, was unexpected, as periplasmic-binding proteins are essential for the function of bacterial importers. However, it has been reported that some of the mutants of histidine and maltose transporters can function independent of their periplasmic-binding protein (Petronilli & Ames, 1991; Treptow & Shuman, 1988). So it is possible that YejA is dispensable for the function of Yej transporter, at least in counteracting AMPs. The transporter activity of the Yej transporter has to be investigated to validate this.

HD-5 and HBD-1 co-localize with intracellular *Salmonella* in Intestine 407 cells

It has been reported that HD-5 is secreted by Paneth cells into the crypts of the small intestine, and that it can clear *Salmonella* both *in vitro* and *in vivo* (Ghosh *et al.*, 2002; Salzman *et al.*, 2003). Transgenic mice expressing HD-5 are markedly resistant to oral challenge with *S. Typhimurium* (Salzman *et al.*, 2003). However, the intracellular role of HD-5 in clearing intracellular pathogens has not been established. We observed uniform distribution of HD-5 inside the cytoplasm of Intestine 407 cells, and the WT and

the $\Delta yefF$ strains were co-localized with HD-5 at 2 and 12 h (Fig. 5 and Fig. S3, available with the online version of this paper) post-infection. Similarly, HBD-1 was also found to co-localize with the WT and the $\Delta yefF$ strain (Fig. 6). We confirmed the expression of HD-5 and HBD-1 in Intestine 407 cells by RT-PCR (Fig. S4, available with the online version of this paper). These findings indicate that *S.*

Typhimurium is exposed to lethal AMPs, such as HD-5, in the intracellular environment. Because of the non-functional Yej transporter, the $\Delta yefF$ strain is more sensitive to intracellular AMPs such as HD-5, and is compromised in its proliferation within Intestine 407 cells. However, the mechanism of entry of AMPs into the *Salmonella*-containing vacuole remains to be investigated. These results

Fig. 5. Co-localization of *Salmonella* with HD-5 at 2 h (a) and 12 h (b) after infection of Intestine 407 cells. Arrows indicate bacterium/bacteria whose enlarged images are shown in adjoining panels. Bars, 5 μ m (a) and 10 μ m (b). Red, LPS; green, HD-5.

Fig. 6. Co-localization of *Salmonella* with HBD-1 2 h after infection of Intestine 407 cells. Arrows indicate bacterium/bacteria whose enlarged images are shown in adjoining panels. Bars, 5 μ m. Red, LPS; green, HBD-1

provide a strong indication of the intracellular role of defensins in clearing intracellular pathogens, such as *Salmonella*, from epithelial cells; this role remains largely unexplored.

The $\Delta yejF$ strain is attenuated for survival *in vivo*

Having demonstrated that the *yejF* is necessary for the replication of *Salmonella* in epithelial cells, we next

investigated the virulence of the $\Delta yejF$ strain in a murine model of typhoid fever. The intragastric route was chosen for infection, as bacteria are exposed to the intestinal epithelium and its secreted AMPs, such as cryptidins. Mice were intragastrically inoculated with the WT or the $\Delta yejF$ strain, with a dose of 2×10^8 bacteria per mouse, and survival was monitored. Mice infected with the $\Delta yejF$ strain had significantly higher survival rates (Fig. 7a). Then we analysed the bacterial load in Peyer's patches, liver and

Fig. 7. $\Delta yejF$ *S. Typhimurium* is attenuated for survival *in vivo*. (a) Survival of the WT (●) and $\Delta yejF$ (○) strains in BALB/c mice ($n=10$ for each group) infected intragastrically at a dose of 2×10^8 bacteria per mouse. Bacterial load after 4 days in Peyer's patches (b), spleen (c) and liver (d), of BALB/c mice ($n=6$) infected intragastrically with a dose of 10^7 bacteria per mouse. (e) Proliferation of WT and $\Delta yejF$ strains after 4 days in the liver and spleen of BALB/c mice ($n=4$) infected intraperitoneally with a dose of 10^3 bacteria per mouse. Plots are representative of two independent experiments. Statistical significance was defined as follows: ** $P < 0.005$. (Mann-Whitney *U* test).

spleen after infecting mice intragastrically at a dose of 10^7 bacteria per mouse. After 4 days of infection, there was a significant decrease in the bacterial burden in all these organs of mice infected with the $\Delta yefF$ strain when compared with mice infected with the WT strain (Fig. 7b–d). However, this difference was not observed after intraperitoneal infection (Fig. 7e). This observation clearly indicates that the transporter encoded by the *yef* operon is important in intragastric infections where *Salmonella* might encounter AMPs in both extracellular (intestinal epithelium) and intracellular environments (neutrophils, macrophages and epithelial cells).

In order to further evaluate the loss in relative virulence displayed by the $\Delta yefF$ strain, we tested for the ability of the $\Delta yefF$ strain to compete with the WT strain after intragastric infection. Table 2 shows the competitive indices resulting from co-infection using the strains indicated. The competitive index represents the ratio (normalized to the ratio of inocula) of the number of mutant to WT bacteria recovered from infected spleens and Peyer’s patches 4 days after the intragastric inoculation of 10^7 bacteria. The $\Delta yefF$ strain showed a reduced competitive index in both Peyer’s patches and spleen, indicating that attenuation of $\Delta yefF$ diminishes its ability to compete for survival in these organs.

Peyer’s patches are known to be the site where *Salmonella* crosses the intestinal barrier (Schauser *et al.*, 2004). The decrease in bacterial load in the liver and spleen of the $\Delta yefF$ -infected mice was most likely to be due to the decrease in the entry of bacteria to the systemic circulation, as most of the bacteria are cleared at the Peyer’s patches, and also due to attenuated replication of the $\Delta yefF$ strain inside activated macrophages. A similar observation was made in mutants of the PmrA–PmrB regulated locus *pmrHFIJKLM*, which is involved in resistance to polymyxin B. It has been shown that this locus is essential for the

Table 2. Competitive indices of mixed infections using WT, $\Delta yefF$ and $\Delta yefF$ (*pQE60yefF*) strains

The table is a representative of two experiments with five mice in each group.

Strain 1	Strain 2	Median competitive index of strain 2 with respect to strain 1	
		Spleen	Peyer’s patches
WT*	WT†	1	1.37
WT*	$\Delta yefF$	0.262‡	0.47‡
WT*	$\Delta yefF$ (<i>pQE60yefF</i>)	1.26	1.3

*Resistant to carbenicillin.
†Resistant to nalidixic acid, and the parent strain for all knockout strains. Both WT strains were isogenic.
‡ $P < 0.05$ (Mann–Whitney *U* test).

virulence of *Salmonella* when infected orally, but not intraperitoneally, in a murine model of typhoid fever (Gunn *et al.*, 2000). In accordance with this report, our findings show the importance for *Salmonella* to counteract AMPs at the intestinal level, in order to cause systemic disease.

To best of our knowledge, this is the first report to show that the *yef* operon is important for virulence in *S. Typhimurium*. Our results, along with the findings of Qimron *et al.* (2004), show that the *yef* operon is important for both innate and adaptive immunity. The exact mechanisms of action, and the link between the two entirely different kinds of function, need to be elucidated. One interesting possibility, based on the model proposed by Parra-Lopez *et al.* (1993) for the Sap transporter, is as follows: *Salmonella*, using the Yef transporter, can transport immunogenic peptides, which are otherwise targeted for loading on to MHC I molecules, by the same mechanism by which it imports AMPs for degradation. This makes these peptides unavailable for MHC I presentation. In fact, it has been showed that phagosomes are competent organelles for antigen cross-presentation (Houde *et al.*, 2003). Moreover, the MHC I molecule is known to co-localize with the *Salmonella*-containing vacuole, and peptides expressed in *Salmonella* have been reported to undergo MHC I presentation (Smith *et al.*, 2005; Yrlid & Wick, 2000). This hypothesis could explain the superior CD8 response observed in mice infected with *yef* mutants (Qimron *et al.*, 2004), and also their sensitivity to AMPs (Figs 2 and 3). These peptides, which are imported, can be a source of amino acids after proteolysis. Thus, the *yef* operon seems to have three functions: (i) interfering with MHC I presentation, which is a part of adaptive immunity; (ii) counteracting AMPs that are part of innate immunity; and (iii) providing a nutritive source in the form of peptides. All these functions are very crucial for *Salmonella* to survive and proliferate inside the hostile environment of the host.

The fact that importers are not found in mammalian hosts make them ideal targets for novel antimicrobial compounds. Drugs linked to the natural substrate of these transporters can easily cross the bacterial cell wall, and act more effectively. Further studies to understand the functional and structural details, and substrates, of transporters such as the one encoded by the *yef* operon, may help in the design of novel antimicrobial compounds. This is relevant in the present scenario, where pathogenic bacteria, such as *Salmonella*, are developing resistance to existing antibiotics (Glynn *et al.*, 1998; Molbak *et al.*, 1999).

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