Components of the peptidoglycan-recycling pathway modulate invasion and intracellular survival of *Salmonella enterica* serovar Typhimurium

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Summary

β-Lactam resistance in enteric bacteria is frequently caused by mutations in ampD encoding a cytosolic N-acetylmuramyl-L-alanine amidase. Such mutants are blocked in murein (peptidoglycan) recycling and accumulate cytoplasmic muropeptides that interact with the transcriptional activator ampR, which de-represses β-lactamase expression. *Salmonella enterica* serovar Typhimurium, an extensively studied enteric pathogen, was used to show that mutations in ampD decreased the ability of *S. typhimurium* to enter a macrophage derived cell line and made the bacteria more potent as inducers of inducible nitric oxide synthase (iNOS), as compared with the wild-type. More potent as inducers of inducible nitric oxide synthase (iNOS), as compared with the wild-type. As compared with the wild-type.

Introduction

Bacterial development of resistance to antibiotics is often associated with a fitness cost for the resistant microorganism by reducing its ability to compete with its sensitive parental strain in infection model systems. In vitro generated *Salmonella enterica* serovar Typhimurium mutants resistant to streptomycin, nalidixic acid, or rifampicin are all outcompeted by the sensitive parental strain in mixed oral infections in mice (Björkman et al., 1998). These findings concern antibiotics rarely used to treat Gram-negative infections and the resistance mechanisms in all three examples involve point mutations affecting essential housekeeping enzymes (Björkman et al., 1998; 1999; 2000). Much less is known about fitness cost in bacteria with multifactorial resistance such as resistance to β-lactam antibiotics, the clinically most important group of antibiotics (Normark and Normark, 2002). Many Gram-negative organisms like *Enterobacter cloacae*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica* and *Serratia marcescens* express a chromosomal AmpC β-lactamase that is inducible by β-lactams and under the control of the transcriptional regulator AmpR (Lindberg and Normark, 1987; Bartowsky and Normark, 1993). During growth in the absence of β-lactams, the regulator acts as a repressor preventing ampC transcription (Jacobs et al., 1997). AmpR is interacting with the cytosolic murein precursor, UDP-MurNAc-pentapeptide and the interaction prevents AmpR from activating the ampC gene during normal growth (Jacobs et al., 1997). In the presence of β-lactams, there is an increased degradation of cell wall derived muropeptides (Vollmer and Holtje, 2001). The degradation products N-acetylgalactosaminyl-1,6-anhydro-N-acetylmuramyl-tri (tetra) peptides are taken up into the bacterial cytosol via the AmpG transporter (Lindquist, 1993; Cheng and Park, 2002). After enzymatic removal of the N-acetylgalactosamine, the anhydro-MurNAc tripeptide displaces UDP-MurNAc-pentapeptide from AmpR, converting AmpR into an activator inducing ampC transcription (Jacobs et al., 1997).

In organisms with inducible AmpC β-lactamases, mutants arise spontaneously at a very high frequency (10^5–10^7) producing high constitutive levels of enzyme (Normark and Normark, 2002). The high levels of β-lactamase confer resistance to many β-lactam antibiotics including the more potent third generation cephalosporins (Lindberg and Normark, 1987). The mutants frequently contain loss of function mutations in the ampD gene encoding a cytoplasmic N-acetylmuramyl-L-alanine amidase (Holtje et al., 1994; Jacobs et al., 1995). *Escherichia coli* carries a chromosomal ampC locus but it normally...
lacks the ampR gene. Inactivation of ampD has therefore no consequence for ampC-transcription in this organism (Park, 1996).

In many Gram-negative bacteria, recycled muropeptides are reused for peptidoglycan synthesis, a process involving the AmpD and AmpG proteins (Park, 1995; 1996). A key step in the reutilization process is the cleavage of the stem peptide from the recycled muropeptide by the cytoplasmic AmpD amidase (Holtje et al., 1994; Jacobs et al., 1995). The stem peptide is then reutilized in the murein biosynthesis (Park, 1996). The recycling of muropeptides has always been presumed to be an energy conserving system (Vollmer and Holtje, 1999). However, interference with the pathway does not affect the cell growth, and ampD and ampG mutants are as fit as the wild-type in vitro (Park, 1996).

The bacterial peptidoglycan not only plays a central role for the maintenance and growth of the organism but also represents an important recognition target for the innate immune system (Yoshimura et al., 1999). Recently, the Nod/CARD family of cytosolic receptors was found to recognize diaminopimelate-containing muropeptide motifs found in Gram-negative bacterial peptidoglycan, to induce an innate immune response via activation of NF-kB (Girardin et al., 2003b). Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection (Girardin et al., 2003a). Human variants in Nod2/CARD15 are predisposed for Crohn’s disease and it was shown that these Crohn’s disease-associated variants of Nod2/CARD15 did not respond to peptidoglycan fragments (Chamaillard et al., 2003).

Bordetella pertussis, produces the tracheal cytotoxin (TCT), which in essence is the peptidoglycan turnover fragment N-acetylglucosaminyl-1,6-anhydro-N-acetylmuramyl-tetrapeptide (Luker et al., 1995). The biochemically active part of this molecule is identical to the muropeptide accumulated in the cytoplasm of ampD mutants of other Gram-negative bacteria (Luker et al., 1995). The TCT is together with endotoxin responsible for the respiratory cytopathology observed during whooping cough that is mainly mediated via nitric oxide (NO) after induction of inducible nitric oxide synthase (iNOS) within nonciliated cells (Luker et al., 1995; Flak and Goldman, 1999; Flak et al., 2000). The accumulation of muropeptides in AmpC hyper producing ampD mutants suggested that such mutants might be compromised during infection by inducing higher levels of reactive nitrogen intermediates (RNI) that are important in microbial host defence.

RNI synthesized by iNOS are involved in growth control of the intracellular pathogen S. typhimurium (Fang, 1997; Eriksson et al., 2000). Although, Salmonella lacks an ampC gene, the genome sequence shows that it has a complete cell wall recycling pathway with intact ampG and ampD genes (McClelland et al., 2001). Here we constructed ampD and ampG mutants of Salmonella. We show that the ampD mutant is less able to enter mouse macrophages and it induced NO to a higher degree than both the wild-type and the ampG mutant in these cells. The Salmonella ampD mutant is also outcompeted in a competition experiment against its wild-type in BALB/c mice. Taken together our data suggest that it is the cytoplasmic accumulation of muropeptide rather than the block in peptidoglycan recycling that causes a decreased fitness of ampD mutants.

**Results**

Isolation of spontaneous S. typhimurium ampD mutants

ampD mutants of E. coli were previously obtained by selecting for cefotaxime resistant mutants of E. coli harbouring plasmid pNU305 carrying the ampR and ampC genes of C. freundii (Lindberg and Normark, 1987). Plasmid pNU305 was introduced into S. typhimurium LT2 that has an ampD gene encoding a protein with a 90% overall identity to that of E. coli (McClelland et al., 2001). Mutants of S. typhimurium LT2/pNU305 resistant to 50 mg l^{-1} of cefotaxime were obtained at a high frequency, 1 × 10^{-7}. A total of 20 independent mutants were isolated and the mutations backtransduced into S. typhimurium LT2/pNU305. Introduction of plasmid pNU404, encoding the wild-type AmpD protein of C. freundii, made all mutants sensitive to 20 mg l^{-1} of cefotaxime implying that all mutants as expected were located in the S. typhimurium ampD gene.

One cefotaxime resistant mutant of S. typhimurium LT2/pNU305 denoted AF19/pNU305 was chosen for further study. The ampD gene of this strain was sequenced and a point deletion of a cytosine was found at position +118 from the start codon resulting in a frame-shift and loss of function. The growth rate of AF19/pNU305 measured as increase in optical density at 600 nm or as viable count in Luria broth, E-minimal medium with glucose or glycerol and in brain–heart infusion media at either 37°C or 30°C was not significantly different from that of the isogenic wild-type strain LT2/pNU305 (data not shown). Hence, like in E. coli a block in the peptidoglycan-recycling pathway does not affect the in vitro growth of S. typhimurium and inactivation of ampD leads to hyper-induction of ampC in the presence of ampR.

Muropeptides accumulate in the cytoplasm of Salmonella ampD mutants but not in an ampG or an ampD ampG mutant

There is a remote possibility that ampD affects the regulation of ampR and ampC carried on pNU305 in a differential way compared with the E. coli system.
We therefore investigated if muropeptides accumulate in the cytoplasm of Salmonella ampD mutants. Hot water extracts of 3H-DAP (diaminopimelic acid) labelled LT2/pNU305 and AF19/pNU305 were analysed by high-performance liquid chromatography (HPLC) using a reverse phase column (Jacobs et al., 1994). As expected, in the Salmonella ampD mutant an additional peak was detected (Fig. 1A). The identity of the accumulate muropeptide was confirmed with mass analysis as anhydro-N-acetylmuramyl-tripeptide (Fig. 2).

It is well known that S. typhimurium LT2 is attenuated because of a change in the regulation of the sigma factor gene rpoS whereas S. typhimurium ATCC 14028s is considerably more virulent in mice, and also more invasive in cell culture experiments (Swords et al., 1997). We therefore constructed defined ampD and ampG mutations by one-step allelic replacement in the ATCC 14028s derivate AF63 resulting in the isogenic ampD strain AF218 and ampG strain AF212 (Datsenko and Wanner, 2000). To be able to differentiate between recycling and accumulation of muropeptides in the cytosol as the cause of the invasion defect, an ampD/ampG double mutant was constructed designated AF253. The ampD strain AF218 accumulates large amounts of anhMurNAc-tripeptide while the wild-type strain AF63, the ampG strain AF212, and the ampD/ampG strain AF253 does not as confirmed by HPLC and mass analysis (Figs 1 and 2). Cefotaxime resistance in the equivalent E. coli/pNU305 system is attributed to the cytosolic accumulation of anhMurNAc-tripeptide as a consequence of abolished cytosolic AmpD amidase activity.

We show here that mutation of ampD in S. typhimurium blocks the cell wall recycling pathway resulting in cytoplasmic muropeptide accumulation.

Salmonella invasion into J774-A.1 cells is decreased in an ampD but not an ampG mutant

Entry and survival in host macrophages is an essential step in the pathogenicity of the Salmonellae (Hensel et al., 1998). We therefore investigated if the ampD mutant was impaired in its interaction with the murine macrophage cell line J774-A.1. Although strain LT2/pNU305 was relatively poor in its ability to invade, as monitored by a gentamicin survival assay, invasion was even lower for its ampD mutant derivative AF19/pNU305 (Fig. 3). We also investigated the ability of the ATCC 14028 derived strains to enter and survive within J774-A.1 cells. Interestingly, invasion was also considerably lower for the ampD mutant AF218 as compared with its parental wild-type AF63 while the ampG mutant AF212 did not significantly differ (Fig. 4). The double mutant AF253 is not significantly affected in its ability to invade cultured murine macrophages (Fig. 4). It appears that cytoplasmic accumulation of muropeptides affects invasion rather than a defect in the recycling pathway that is a common feature for both the ampD and ampG mutants.
Salmonella ampD mutants are also affected in intracellular growth

To investigate the cell wall recycling mutants’ ability for intracellular growth in J774-A.1 cells the bacterial inoculums were opsonized with 10% normal mouse serum as described in Experimental procedures to ensure equal levels of uptake. The ampD mutants had a decreased ability to grow inside this murine cell line and exhibited significantly lower cfu after 14 h coincubation compared with the wild-type, ampG and ampD/ampG double mutant (Fig. 3). The accumulation of muropeptides exhibited in the ampD mutants not only affects the organisms’ ability to invade but also proliferates inside murine macrophages.

The ampD mutant induced NO to a higher degree than both the wild-type and the ampG mutant

The induction of RNI synthesized by iNOS is involved in the control of the growth intracellular pathogen S. typhimurium (Fang, 1997). In J774-A.1 cells infected with S. typhimurium, NO is produced as measured by nitrite accumulation in the cell culture medium (Fig. 5). Interestingly, the ampD mutant AF218 causes a doubling of accumulated nitrite, as compared with the wild-type, the ampG and the ampD/ampG double mutant (Fig. 5).

The Salmonella ampD mutant is less fit than wild-type in competition experiments in mice

Competition experiments suggest that Salmonella ampD mutants are affected in their in vivo fitness. When BALB/c mice are orally infected with either LT2/pNU305 or AF19/pNU305, no significant differences could be detected in cfu in the liver and spleen after 7 days of infection, arguing that Salmonella ampD mutants remain virulent (data not shown). However, when wild-type Salmonella AF19/pNU305 and the ampD mutant Salmonella LT2/pNU305 are mixed in equal numbers and of a total of 10^7 cfu are administrated orally to BALB/c mice, after 7 days of infection more than 90% of the recovered organisms from liver
and spleen were LT2/pNU305, suggesting that the in vivo fitness of the \textit{ampD} mutant is decreased relative to the wild-type (Fig. 6).

**Discussion**

In this study we have examined the role of cell wall recycling in the biology of \textit{S. typhimurium}. It has been shown previously that cell wall recycling is not only an unspecified energy saving device but is also important for the bacterial cell to sense and react to environmental insults such as those evoked by \beta-lactam antibiotics. Here we show that an intact cell wall recycling pathway is important for full virulence of \textit{S. typhimurium}. Spontaneous mutations or insertion of an antibiotic resistance cassette in \textit{ampD}, encoding a cytosolic muropeptide amidase, lead to a decrease in the ability to invade and grow within cultured murine macrophages. An \textit{ampD} mutant remains able to cause disease in mice but is outcompeted by the wild-type in the BALB/c animal model in a mixed infection. Moreover, \textit{ampD} mutants of \textit{Salmonella} induced higher levels of NO when infecting murine macrophages as compared with the wild-type.

In \textit{E. coli} and other enterics, \textit{ampD} mutants block cell wall recycling at a cytosolic step. As a consequence cytosolic muropeptides, mainly anhydromuramyl-tripeptide, accumulate in the cytosol at very high levels (Jacobs et al., 1994). Cell wall recycling can also be completely blocked by mutating the \textit{ampG} gene encoding a transporter for muropeptide turnover products. Such mutants do not accumulate cytoplasmic muropeptides. Instead muropeptide turnover products are released into the medium. The \textit{ampD/ampG} double mutants have the same phenotype as \textit{ampG} single mutants, in that they do not accumulate cytoplasmic muropeptides. \textit{Salmonella} contain an \textit{ampG} gene highly homologous to that of \textit{E. coli} and other enteric bacteria. \textit{Salmonella} \textit{ampG} mutants or \textit{ampD/ampG} double mutants behaved essentially as the wild-type with respect to invasive ability and ability to activate NO production. Based on these findings we suggest that the effects of \textit{ampD} on \textit{in vivo} fitness in mice, invasion ability and ability to induce NO production do not primarily depend on the blocked cell wall recycling and the release of muropeptides, but rather on the accumulation of cytoplasmic muropeptides in the bacterium affecting for example the regulation of virulence factors. Abnormal accumulation of cytoplasmic muropeptides may interfere with the normal regulation of important virulence determinants such as the SPI-1 and SPI-2 type three secretion systems leading to differential delivery of bacterial effector proteins, such as AvrA, to the eukaryotic cell reducing the ability of the bacteria to limit the inflammatory response (Collier-Hyams et al., 2002).

Accumulation of cytosolic muropeptides may have signalling effects in the pathogen. We know that these molecules can displace negatively acting cell wall precursor ligands from the transcriptional regulator AmpR present in many enterics. AmpR is thereby converted into a transcriptional activator for AmpC-\beta-lactamase. Although \textit{S. typhimurium} lacks an \textit{ampR} gene, it may well be that cytosolic muropeptide turnover products act as ligands for other regulators. AmpR belongs to the LysR family of transcriptional regulators of which there are several representatives in \textit{Salmonella}. SinR is one such protein encoded by a horizontally acquired genomic island also containing genes involved in invasion (Folkesson et al., 1999; 2002). SpvR is another LysR-family regulator involved in the regulation of the spv operon on the virulence plasmid (Gulig et al., 1993). The spv genes are important for the intracellular replication of \textit{Salmonella} in host cells (Gulig et al., 1993).

The tracheal cytotoxin (TCT) of \textit{Bordetella pertussis} has been shown to be the muropeptide turnover product anhydromuramyl-tetrapeptide (Luker et al., 1993). TCT acts in concert with endotoxin to activate iNOS in ciliated epithelial cells (Flak and Goldman, 1999). In the present study \textit{Salmonella} \textit{ampG} mutants, likely secreting muropeptides, behaved as the wild-type with respect to NO induction in the murine macrophage cell line J774-A.1. It is therefore possible that muropeptides do not evoke a signal response from the surface of macrophages. Nothing is currently known about internalization of muropeptides. Bacteria with mutations in \textit{ampD} accumulate muropeptides in the cytoplasm. It is possible that the muropeptides are released from an intracellular compartment as a consequence of bacterial uptake. Interestingly, it has recently been shown that proteins in the Nod/CARD family recognize muropeptides derived from Gram-negative bacteria.
and future work will show if the hyper-induction of iNOS and hence hyperproduction of antibacterial reactive nitrogen intermediates by ampD mutants, are mediated via this pathway (Inohara et al., 2001; Chamaillard et al., 2003; Girardin et al., 2003a,b). It was recently demonstrated that growth of Salmonella within Caco2 cells was reduced by expression of wild-type Nod2, but not by a Crohn’s disease-associated Nod2 variant. The growth-inhibiting factor was however, not demonstrated (Hisamatsu et al., 2003).

Resistance to β-lactam antibiotics, including the more potent cephalosporins, is frequently caused by ampD mutations in opportunistic Gram-negative pathogens, encoding a chromosomal ampC-β-lactamase that is under the control of an AmpR regulator (Normark and Normark, 2002). The mutation frequency is very high, as any null-mutation in ampD will cause resistance. It is not known to what extent these β-lactam resistant ampD-mutants have a decreased fitness in vivo as shown here for S. typhimurium. However, preliminary data suggest that also ampD mutants of En. cloacae are hyperinducers of iNOS (S. Normark, unpubl. results).

Antibiotic resistance is probably associated with a fitness cost in most instances, which will have an effect on spread of resistant clones in the absence of a selection pressure. However, fitness cost can be ameliorated by additional mutations, usually in the same gene (Björkman et al., 1998; 2000). Fitness cost associated with β-lactam resistance attributed to ampD mutations is likely difficult to compensate for, as the fitness cost and resistance is attributed to the same process, namely accumulation of cytoplasmic muropeptide turnover products.

**Experimental procedures**

**Bacterial strains, plasmids, bacterial genetic techniques and growth conditions**

Strains and plasmids used in this study are listed in Table 1. All Salmonella strains are derivatives of Salmonella enterica serovar Typhimurium ATCC 14028s except LT2/pNU305 and AF19/pNU305, which are derived from serovar Typhimurium LT2. The bacteria were routinely grown in Luria–Bertani media or in M9 minimal medium supplemented with glucose (0.2%), casamino-acids (0.1%), thiamine (1.0 μg ml⁻¹), uracil (50 μg ml⁻¹), MgCl₂ (1 mM), lysine (100 μg ml⁻¹), threonine (100 μg ml⁻¹) and methionine (100 μg ml⁻¹). The antibiotics ampicillin, kanamycin, chloramphenicol and tetracycline were used at the concentrations of 50 μg ml⁻¹, 50 μg ml⁻¹, 10 μl ml⁻¹ and 10 μl ml⁻¹ respectively. Unless otherwise stated all genetic methods followed standard procedures as described by Ausubel, F.M., and others (Ausubel et al., 1996). Phage P22 HT105/1-int-201-mediated transductions and MuD transposon mutagenesis were performed as described (Schmeiger, 1972; Maloy et al., 1996). Spontaneous Salmonella ampD mutants were obtained as described previously (Lindberg and Normark, 1987). The ampD gene of strain AF19 was polymerase chain reaction (PCR)-amplified using Expand High Fidelity System (Roche) using the primers ampDfwstm 5’-AGAACCAGGCAGGCAGGATAT-3’ and ampDrevstm 5’-CGTCGACATACGACCCAGCAG-3’. Sequencing of the resulting PCR products was performed by the dyeoxy chain termination method (Sanger et al., 1977) by the genomic service provider GATC (GATC Biotech AG, Constance, Germany). The sequence data have been submitted to the EMBL/GenBank databases under accession number AJ748846.

The defined insertion mutations in ampD and ampG were produced by using one-step allelic replacement utilizing the λ Red recombinase system described by Datsenko and Wanner (Datsenko and Wanner, 2000). In short, transformants of AF20 carrying the Red recombinase plasmid pKD46 were grown in 5 ml SOB cultures with ampicillin and L-arabinose at 30°C. pKD46 shows temperature-sensitive replication and the Red recombinase genes are under the control of a arabinose-sensitive promoter. The culture was grown to an OD₆₀₀ of approximatively 0.6 and then made electrocompetent by washing four times in ice cold 15% glycerol. PCR products were amplified using the plasmids pKD4 and pKD3 as templates for kanamycin resistant and chloramphenicol resistant insertions respectively. The amplified products were gel-purified, digested with DpnI, repurified and suspended in water. Electroporation was performed by using a Gene pulser (Bio-Rad) as recommended by the manufacturer using 50 μl bacterial cells and 10–100 ng PCR product. The cells were then added to 1 ml of SOC medium and incubated for 1 h at 37°C and then one-half was spread onto selective agar plates. After primary selection putative mutants were restreaked on selective media at 37°C and then tested for

**Table 1. Bacterial strains and plasmid used in this study.**

| Strains | Description | Reference |
|---------|-------------|-----------|
| Salmonella | | |
| LT2/pNU305 | WT | Lilleangen (1948) |
| AF19/pNU305 | LT2 ampDpNU305 | This work |
| AF83 | ATCC 14028s Nal' | This work |
| AF218 | ampD:kan | This work |
| AF212 | ampG:cm | This work |
| AF253 | ampD:kan ampG:cm | This work |
| Plasmids | | |
| pNU305 | pBR322 with ampRampC from Citrobacter freundii | Lindberg and Normark (1987) |
| pNU404 | pBR322 with ampD from Citrobacter freundii | Lindberg et al. (1987) |
| pKD3 | Chloramphenicol insertion template plasmid | Datsenko and Wanner (2000) |
| pKD4 | Kanamycin insertion template plasmid | Datsenko and Wanner (2000) |
| pKD46 | Red recombinase encoding plasmid | Datsenko and Wanner (2000) |
ampicillin sensitivity. Putative insertion mutations were transduced with phage P22 HT105/1nt-201-mediated transduction into fresh AF20 background for further phenotypic characterization. The following primers were used for allelic replacement. AmpD4: 5'-GCATTGTCGGGAGTATTGCTCCTC-3'; AmpD4r: 5'-GCGATTCTGTCATGATGATACATATCCAAG-3'.

**Preparation of hot water extracts and HPLC analysis**

Extracts were prepared as previously described (Jacobs et al., 1994). The samples represent the hot water extract from 8 ml of culture of exponentially growing bacteria in M9 minimal medium. The cultures were labelled with 5 μCi of [14C]-DAP (Morovek Biochemicals, Brea, CA, USA). When the culture reached a turbidity of OD600 of 0.6 the cultures were quickly chilled to 4°C, centrifuged and washed once with 2 ml water. The collected cells were suspended in 2 ml water, heated at 100°C for 5 min, centrifuged (12 000 g, 10 min, 4°C). The supernatants (hot water extracts) were collected and lyophilized. HPLC analysis was performed using a reverse phase column and 0.05% trifluoro acetic acid (TFA). The material was eluted with 0.05% TFA in water followed by a shallow gradient of 0.035% TFA in acetonitrile. The material of interest eluted after about 77 ml when the concentration of acetonitrile was almost 10%.

**Mass analysis of accumulated muropeptides**

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis (MALDI TOF MS) was carried out using a Reflex III (Bruker Daltronics, Germany). The samples were dissolved in 60% acetonitrile/0.1% TFA and spotted onto a target plate. The crystals were washed once with 0.1% TFA and data were collected with positive selection in the linear mode.

**Tissue culture conditions and invasion assays**

Cultivation of J774-A1 cells (ATCC TIB 67) was performed in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamine (10 mM final concentration; Gibco) and HEPEs (10 mM final concentration; Gibco). The batches of RPMI and FBS were screened before use to assure that they did not trigger host NO responses. Invasion assays were performed as described (Elsingorhorst, 1994). In short, a 24-well dish with 106 cells seeded the previous day was inoculated with 2 x 106 bacteria to each well. Plates were centrifuged 5 min at 1000 g. Bacteria were allowed to invade for 1 h before washing each well four times with PBS. One milliliter of fresh RPMI 1640 media with 50 μg ml⁻¹ gentamicin was added to each well and incubated 1 h at 37°C. Wells were washed four times with PBS and the cells were then lysed using 0.1% Triton X-100 in PBS. Serial dilutions from each well were plated to determine the number of intracellular bacteria. Studies of intracellular replication were performed as described for the invasion assay with the difference that bacteria were opsonized for 30 min at 37°C with normal mouse serum before infection. Equal uptake in the replication experiments was monitored by lysis of the appropriate number of wells of each strain after 1 h invasion and 1 h incubation in killing medium as described for the invasion assay. For continued incubations, killing medium was replaced by maintenance medium containing gentamicin (10 μg ml⁻¹).

**Measurement of NO production**

Host NO response was analysed by following the accumulation of nitrite in the cell culture medium using Griess reagent (Colantoni et al., 1995). A standard curve, using sodium nitrate (Merck) was used to convert values from absorbance to μM.

**Animal experiments**

Female BALB/c mice aged 6–8 weeks were used for all animal infections. For per-oral infection, mice were deprived of food overnight and then deprived of water for 3 h prior to inoculation. The animals were then fed 50 μl of 1% sodium bicarbonate followed by 10–20 μl of bacteria suspended in phosphate-buffered saline (PBS). Food and water were withheld until 30 min post inoculation. On day 1, 3, 7 and 15 after inoculation, mice were euthanized, and their organs were aseptically removed, weighed and homogenized in sterile PBS at room temperature. Serial dilutions of organ homogenates were plated on Luria agar plates.

**Statistical analysis**

Statistical significance was determined using unpaired Student’s t-test, values of P > 0.05 were considered significant.

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