Removal of the phage-shock protein PspB causes reduction of virulence in *Salmonella enterica* serovar Typhimurium independently of NRAMP1

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The phage-shock protein (Psp) system is believed to manage membrane stress in all *Enterobacteriaceae* and has recently emerged as being important for virulence in several pathogenic species of this phylum. The core of the Psp system consists of the *pspA–D* operon and the distantly located *pspG* gene. In *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), it has recently been reported that PspA is essential for systemic infection of mice, but only in NRAMP1$^+$ mice, signifying that attenuation is related to coping with divalent cation starvation in the intracellular environment. In the present study, we investigated the contribution of individual *psp* genes to virulence of *S.* Typhimurium. Interestingly, deletion of the whole *pspA–D* set of genes caused attenuation in both NRAMP1$^+$ and NRAMP1$^-$ mice, indicating that one or more of the *psp* genes contribute to virulence independently of NRAMP1 expression in the host. Investigations of single gene mutants showed that knock out of *pspB* reduced virulence in both types of mice, while deletion of *pspA* only caused attenuation in NRAMP1$^+$ mice, and deletion of *pspD* had a minor effect in NRAMP1$^-$ mice, while deletions of either *pspC* or *pspG* did not affect virulence. Experiments addressed at elucidating the role of PspB in virulence revealed that PspB is dispensable for uptake to and intracellular replication in cultured macrophages and resistance to complement-induced killing. Furthermore, the Psp system of *S.* Typhimurium was dispensable during pIV-induced secretin stress. In conclusion, our results demonstrate that removal of PspB reduces virulence in *S.* Typhimurium independently of host NRAMP1 expression, demonstrating that PspB has roles in intra-host survival distinct from the reported contributions of PspA.

INTRODUCTION

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is an important intracellular pathogen that causes diseases ranging from self-limiting gastroenteritis to life-threatening systemic infections. In susceptible mice it causes a typhoid-like disease, which is widely used as a model of human typhoid fever (Haraga *et al.*, 2008; Wallis & Galyov, 2000). A central feature of *Salmonella* infections is the ability of *Salmonella* to survive and replicate in host cells inside a specialized compartment termed the *Salmonella*-containing vacuole (Mastroeni & Grant, 2011). Pathogenicity of *S.* Typhimurium is dependent on many tightly regulated factors including large gene clusters, so-called *Salmonella* pathogenicity islands (SPIs). The two major SPIs, SPI1 and SPI2, encode type three secretion systems (T3SSs), which release effector proteins in the cytosol of the host cell. Effectors of the SPI1 T3SS are mainly associated with invasion of epithelial cells, inducing an inflammatory response and fluid secretion in the intestine (Galán, 1996; Watson *et al.*, 1998), while those of the SPI2 T3SS are associated with survival and replication within eukaryotic cells and thus establishment of

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Abbreviations: CI, competition index; PMF, proton motive force; *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; SPI, *Salmonella* pathogenicity island; T3SS, type three secretion system.

A supplementary table is available with the online version of this paper.

systemic disease (Hensel *et al.*, 1998). Other features important for *S.* Typhimurium virulence include the ability to kill macrophages by pyroptosis (Fink & Cookson, 2007) and the ability to survive killing by the complement system (Heffernan *et al.*, 1992).

Mouse strains vary considerably in natural resistance to *Salmonella* infection. This is mainly due to the gene SLC11A1 (NRAMP1), which encodes a divalent cation transporter. The wt allele of this gene confers resistance to *Salmonella* infection (in this study termed NRAMP1$^+$) primarily due to divalent cation starvation of *S.* Typhimurium in the *Salmonella*-containing vacuole, while a mutated allele confers infection susceptibility (in this study termed NRAMP1$^-$) (Blackwell *et al.*, 2000). It is therefore important to relate the results of studies of host–pathogen interaction to the type of mice used, both for *Salmonella* as investigated in this study and in general, when using mouse models to screen for virulence factors of intracellular pathogens.

The Psp system, which is present in all *Enterobacteriaceae*, belongs to the extra-cytoplasmic stress response systems and is believed to help bacteria to resist membrane stress by maintaining the membrane integrity and by controlling the energy status in the cell (Darwin, 2005; Joly *et al.*, 2010; Model *et al.*, 1997). The system is encoded by two loci in *S.* Typhimurium and *E. coli*, the *pspA–E* operon, the divergently transcribed *pspF* gene and the separate single *pspG* gene (Huvet *et al.*, 2011). Expression of these genes, described in *E. coli*, is driven by a σ^{54} RNA polymerase and activated by the enhancer-binding protein PspF (Joly *et al.*, 2010; Jovanovic *et al.*, 1996; Weiner *et al.*, 1991; Yamaguchi & Darwin, 2012), while the peripheral membrane protein, PspA, negatively regulates expression by binding to PspF and blocking its ATPase activity (Dworkin *et al.*, 2000; Elderkin *et al.*, 2005). The fifth gene of the *pspA–E* operon, *pspE*, is transcribed independently from a *pspE*-specific σ^{70}-dependent promoter, which has been demonstrated in *E. coli* (Huvet *et al.*, 2011). This gene encodes a putative thiosulfate sulfurtransferase (TST) and we have recently shown that *pspE* contributes to virulence only in combination with a deletion of *glpE*, the other major TST of *S.* Typhimurium (Wallrodt *et al.*, 2013). Expression of *pspE* independently of *pspABCD* in *S.* Typhimurium has recently been confirmed, supporting the possibility that PspE has functions unrelated to the Psp system (Kröger *et al.*, 2013).

PspA functions as the major effector of the system, by promoting maintenance of the proton motive force (PMF) in both *S.* Typhimurium and *E. coli* (Becker *et al.*, 2005; Hankamer *et al.*, 2004; Joly *et al.*, 2009; Standar *et al.*, 2008). It is considered important for many cell functions, as a drop in PMF has negative effects on PMF-driven processes, for example aerobic respiration, metabolite transport, motility and translocation of flagellin, and T3SS secreted effector molecules (Galán, 2008; Minamino & Namba, 2008).

In *Salmonella*, the roles of individual Psp proteins are mostly unknown; however, the *psp* genes are highly expressed during *S.* Typhimurium infection of cultured epithelial cells and macrophages (Eriksson *et al.*, 2003; Hautefort *et al.*, 2008), and also in non-proliferating *Salmonella* bacteria in a fibroblast cell culture model of resting infection (Núñez-Hernández *et al.*, 2013). This suggests a role for the Psp system in host–pathogen interactions. In support of this, it was recently shown that PspA is required for virulence of *S.* Typhimurium in mice; however, only in mice expressing NRAMP1 (Karlinsey *et al.*, 2010). This was elegantly shown to be related to PspA-dependent maintenance of the PMF, a prerequisite for the function of multiple bacterial transporters facilitating divalent metal uptake during infection. Interestingly, in this study it was also revealed that deletion of *pspA* did not affect motility, type three secretion and survival of oxidative and nitrosative stresses, despite the strong induction of *pspA* expression during infection of cell cultures (Karlinsey *et al.*, 2010).

In the related enteric pathogen *Yersinia enterocolitica*, a *pspA* deletion only has a small effect on virulence, whereas *pspC* is essential for virulence in a mouse model of infection (Darwin & Miller, 2001). Further studies revealed that PspC and PspB, but not PspA, of the *Y. enterocolitica* Psp system are essential for secretin stress tolerance induced by expression of the T3SS during infection (Horstman & Darwin, 2012). The contrasting roles of the Psp system in *S.* Typhimurium and *Y. enterocolitica* may reflect their different host niches, as *S.* Typhimurium is an intracellular pathogen, while *Y. enterocolitica* is an extracellular pathogen. These differences have been discussed in a recent review (Darwin, 2013).

Based on the findings on the roles of individual Psp proteins in virulence of *Y. enterocolitica* and the fact that only the role of PspA in *S.* Typhimurium has been investigated, the aim of the present study was to investigate if the PspB, PspC, PspD and PspG proteins of the Psp system contribute to virulence of *S.* Typhimurium. Interestingly, we find that removal of PspB independently of the other Psp proteins reduces virulence in both NRAMP1$^+$ and NRAMP1$^-$ mice, suggesting that PspB has a role in virulence distinct from the role of PspA, which only contributes to virulence in Nramp1-expressing mice.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this work are listed in Table 1. Gene knockouts in *S.* Typhimurium 4/74 were obtained by cloning of PCR-amplified resistance genes encoded on pKD3 or pKD4 into the target gene using the λ-Red recombinase system (Datsenko & Wanner, 2000) with primers listed in Table S1 (available in the online Supplementary Material). Insertions were confirmed by PCR and sequencing with gene-flanking primers (Table S1) following standard procedures. The mutations were then re-introduced into a wt *S.* Typhimurium ST4/74 background via transduction with phage P22 HT105/1 *int201* using previously described protocols (Thomsen *et al.*, 2003). Bacterial

Table 1. Strains and plasmids used in this study

Strains	Relevant genotype	Reference or source
JEO3774	*S.* Typhimurium 4/74 wt	Watson *et al.* (1998)
JEO4533	*S.* Typhimurium 4/74 Δ*pspA*::Kan (kan^r)	This work
JEO4534	*S.* Typhimurium 4/74 Δ*pspB*::Kan (kan^r)	This work
JEO4909	*S.* Typhimurium 4/74 Δ*pspB*::Kan+pINS06 (kan^r, amp^r)	This work
JEO4535	*S.* Typhimurium 4/74 Δ*pspC*::Kan (kan^r)	This work
JEO4536	*S.* Typhimurium 4/74 Δ*pspD*::Kan (kan^r)	This work
JEO4542	*S. Typhimurium* 4/74 Δ*pspG*::Kan (kan^r)	This work
JEO4837	*S.* Typhimurium 4/74 Δ*pspABCD*::Cm (cm^r)	This work
JEO3775	*S.* Typhimurium 4/74 *invH201*::Tn*phoA*	Watson *et al.* (1998)
LJ607	*S.* Typhimurium 4/74 Δ*ssaV*::Kan (kan^r)	This work
KP1274	*S.* Typhimurium LT2. Restriction-deficient	Enomoto & Stocker (1974)
Top10	*E. coli* cloning host	Invitrogen
Plasmids		
pGJ4	P*lacUV5*-*gIV* (pIV). Plasmid that expresses the F1 pIV protein constitutively (tet^r)	Jovanovic *et al.* (2009)
pKD3	Template for amplification of Cm resistance cassette (amp^r, cm^r)	Datsenko & Wanner (2000)
pKD4	Template for amplification of Kan resistance cassette (amp^r, kan^r)	Datsenko & Wanner (2000)
pKD46	Vector for λ-Red-mediated mutagenesis; λ-Red expression from arabinose-inducible promoter; temperature-sensitive (amp^r)	Datsenko & Wanner (2000)
pACYC177	Cloning vector (amp^r, kan^r)	Chang & Cohen (1978)
pINS06	*pspB* in pACYC177 (amp^r)	This work

strains were maintained in LB–Lennox broth (LB) with 30 % (v/v) glycerol at −80 °C. LB agar plates (LB+1.5 % agar) were used for growth on solid medium. If not stated otherwise, growth in liquid medium was performed in M9 medium containing 12.8 g Na$_2$HPO$_4$.12H$_2$0 l^{-1}, 3.0 g KH$_2$PO$_4$ l^{-1}, 0.5 g NaCl l^{-1}, 1.0 g NH$_4$Cl l^{-1}, 0.1 mM CaCl$_2$, 2 mM MgSO$_4$ and 0.4 % (w/v) glucose. When required, media were supplemented with antibiotics in the following concentrations: 50 μg ampicillin ml^{-1}, 50 μg kanamycin ml^{-1}, 10 μg chloramphenicol ml^{-1} and 12.5 μg tetracycline ml^{-1}. Investigation of growth kinetics was performed in 96-well plates and followed by OD$_{600}$ measurements in a microplate spectrophotometer (PowerWave XS; Biotek) with shaking of the plate.

Plasmid construction and complementation. Plasmids for complementation purposes were constructed by cloning gene-specific PCR products into pACYC177 (Chang & Cohen, 1978) following standard procedures. Oligonucleotides used for amplification are listed in Table S1. For construction of pINS06 (*pspB* into pACYC177), the *pspB*-specific region plus a shorter, approximately 60 bp, large flanking upstream sequence was cloned into the *Xho*I/*Hin*dIII restriction sites of pACYC177, enabling complementation from the pACYC177-deriving kanamycin promoter. Cloning of *pspB* in pINS06 was verified by PCR and sequencing with pACYC177-specific oligonucleotides (Table S1). The plasmid was transformed into One Shot *E. coli* TOP10 chemically competent cells (Invitrogen), after which it was transformed into the restriction-deficient *S.* Typhimurium strain KP1274 (Enomoto & Stocker, 1974) and finally into the *pspB* mutant to test for genetic complementation. It has been shown that pACYC177 does not affect virulence of *S.* Typhimurium (Jelsbak *et al.*, 2012; Knodler *et al.*, 2005), and hence no control for the role of the plasmid on wt virulence was included in the present study.

Serum resistance. To test for the ability to resist complement killing, the cultures were mixed with calf serum (Invitrogen) in a ratio of 10 : 1. Samples were taken at the beginning of the experiment and after 1 h of incubation at 37 °C without shaking. Tenfold dilutions were spotted on LB agar plates for enumeration.

Induction of secretin stress. To induce secretin stress, *psp* mutant bacteria as well as wt were transformed with the pGJ4 plasmid, which expresses the F1 pIV protein constitutively (Jovanovic *et al.*, 2009). Expression was confirmed by Western blot, as detailed below. The backbone plasmid pBR325D (ColD origin; Jovanovic *et al.*, 2006) has been shown not to affect a large range of phenotypes, including *psp* induction, motility and drop in PMF in the Δ*pspF* strain (Jovanovic *et al.* 2006, 2009, 2014). Hence, no control for the plasmid was included in the present study.

Western blot analysis. Bacterial cultures grown to stationary phase in M9 were pelleted and adjusted to the same bacterial number (OD$_{600}$ 10) in saline buffer and cells were then lysed by boiling (99 °C for 10 min). The samples were separated in NuPage Novex 4–12 % BisTris Gels (Invitrogen) using MOPS buffer. Proteins were blotted on Invitrolon PVDF membranes with the help of the XCell II blotting module (Invitrogen) as described in Jelsbak *et al.* (2010). Primary antibodies to pIV were used at a 1 : 5000 dilution. Protein bands were visualized with the WesternBreeze Chemiluminescent kit-Anti-Rabbit according to the supplier's recommendations (Invitrogen). The experiment was performed two independent times to ensure reproducibility.

Mouse virulence studies. Infection of 6-week-old C3H/HeN (NRAMP1$^+$) and C57BL/6 (NRAMP1$^-$) mice was done as described previously (Jelsbak *et al.*, 2012). Briefly, four or five mice were infected intraperitoneally with a 50 : 50 mixture of 5×10^3 c.f.u. of each of the wt and the mutant strain. After 4–6 days (within a 24 h interval) bacteria were recovered from spleen samples. Samples below 10^2 c.f.u. ml^{-1} were regarded as failed and excluded from further analysis. One hundred randomly picked colonies of both the inoculum and the spleen samples from each mouse were streaked on LB agar plates containing the appropriate antibiotic to determine the proportion of the mutant to the wt strain. Competition indices (CIs) were calculated as the mutant/wt ratio of the output versus the mutant/wt ratio of the inoculum (Jelsbak *et al.*, 2012). Mice infection studies were performed with permission from the Danish Animal Experiments Inspectorate, licence number 2009/561-1675.

Infection of macrophage-like cells. Intracellular survival/replication and cytotoxicity/pyroptosis towards macrophages were tested using J774 macrophage-like cells as previously described (Wallrodt *et al.*, 2013). Essentially, J774 cells were infected with complement-opsonized bacteria at an m.o.i. of 10:1. After 25 min of infection cells were washed and treated with 100 µg ml^{-1} gentamicin for 1 h to kill extracellular bacteria. Cells were either lysed in the presence of 0.1 % (v/v) Triton-X to release intracellular bacteria (time point 1 h p.i.) or further incubated in the presence of 25 µg gentamicin ml^{-1} for 3 h (time point 4 h p.i.) and 23 h (time point 24 h p.i.) and then lysed. The number of intracellular bacteria was determined by c.f.u. ml^{-1} calculations. Survival/replication was expressed as the number of intracellular bacteria at 24 h p.i. relative to the number at 4 h p.i. An SPI2 mutant (Δ*ssaV*) unable to propagate intracellularly (Shea *et al.*, 1996) was used as control.

Cytotoxicity towards macrophages was determined on cell culture supernatants that were collected 24 h p.i., using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) following the supplier instructions. Cytotoxicity was calculated as the percentage of lactate dehydrogenase (LDH) released in infected cells in relation to LDH released in non-infected, enzymically lysed cells (maximum release). The values were normalized against the wt data. All experiments were repeated at least three times.

Statistical analyses. Outliers with a significance of 0.05 were identified by Grubb's outlier test and removed for further analysis. Statistical significance of differences between wt and mutated strains in mouse experiments was determined by Students *t*-test using GraphPad Prism software version 6.0 (GraphPad), while statistical comparison of uptake in macrophages, fold changes of intracellular bacteria, cytotoxicity and serum resistance was performed using GraphPad Prism software version 6.0 (GraphPad) using ANOVA with Dunnett's correction for multiple comparison.

RESULTS AND DISCUSSION

The Psp system contributes to virulence of *S.* Typhimurium independently of Nramp1

It has previously been reported that PspA is essential for virulence in NRAMP1-expressing mice (Karlinsey *et al.*, 2010) and that the *psp* operon is highly induced in intracellular *S.* Typhimurium bacteria in cultured cells

(Eriksson *et al.*, 2003; Hautefort *et al.*, 2008; Núñez-Hernández *et al.*, 2013). This prompted us to investigate if the Psp system had additional roles in virulence independent of Nramp1. To this end, we constructed a Δ*pspABCD* mutant. As an initial control we confirmed that this mutant grew equally as well as the isogenic wt (4/74) strain in both LB and M9 media at 37 °C (data not shown). The infection potential of the *pspABCD* mutant was investigated in both NRAMP1$^+$ mice (C3H/HeN) and NRAMP1$^-$ mice (C57BL/6) by competitive infection using intraperitoneal infections with equal amounts of wt and mutant bacteria as described in Beuzón & Holden (2001) and Jelsbak *et al.* (2012).

Interestingly, the Δ*pspABCD* strain was slightly, but significantly ($P<0.01$), affected in virulence in both NRAMP1$^+$ and NRAMP1$^-$ mice, suggesting a role for one or more of the genes in the *pspA–D* gene cluster in systemic infection independent of NRAMP1 (Table 2).

PspB is required for virulence independently of Nramp1

To obtain more detailed information on the reason for the attenuation observed when the *pspABCD* fragment was removed, we constructed mutants deleting individual genes in the *pspABCD* operon and investigated their role in virulence as above. As the *pspG* gene, located distantly from the *pspABCD* genes, has been associated with the Psp system in other bacteria, we included a *pspG* deletion mutant in our studies. The Δ*pspA* mutation caused a slightly reduced virulence in NRAMP1$^+$ mice (CI, 0.40 ± 0.08; $P<0.001$) and had a slight increase in CI in NRAMP1$^-$ mice (CI, 2.16 ± 1.00) (Table 2). These results adhere to previous reports on the role of PspA in *S.* Typhimurium virulence (Karlinsey *et al.*, 2010), where it has been shown that PspA contributes to virulence in mice expressing the NRAMP1 divalent metal transporter and that this is associated with altered membrane transport of divalent ions (Karlinsey *et al.*, 2010).

The phenotype of the Δ*pspA* mutant in our study is milder than previously reported. This could be due to differences

Table 2. Virulence of Δ*psp* mutants in *Nramp1*$^-$ and *Nramp1*$^+$ mice as shown by mixed infection with *S.* Typhimurium 4/74 wt and *psp* mutant bacteria

Mice were infected intraperitoneally with equal numbers of mutant and wt bacteria (each 5×10^3 c.f.u.). Competition indices (CI) were calculated based on input (inoculum) and output (spleen sample) proportions of wt versus mutant bacteria. The results show mean values ± SD based on the number of mice tested as indicated in parentheses. Asterisks indicate significantly different from 1.0 (with *$P<0.05$, **$P<0.01$, ***$P<0.001$) as determined by one sample *t*-test analysis. ND, Not determined.

Indicated mutant versus 4/74 wt	CI ± SD in NRAMP1$^-$ mice (C57/BL6)	CI ± SD in NRAMP1$^+$ mice (C3H/HeN)
Δ*pspABCD*	0.46 ± 0.09** (4)	0.58 ± 0.09** (4)
Δ*pspA*	2.16 ± 1.00* (8)	0.40 ± 0.08*** (4)
Δ*pspB*	0.14 ± 0.04*** (3)	0.29 ± 0.08*** (4)
Δ*pspB*/pACYC177-*pspB*	0.66 ± 0.44 (5)	ND
Δ*pspC*	1.00 ± 0.18 (5)	1.39 ± 0.59 (4)
Δ*pspD*	0.46 ± 0.09** (4)	1.25 ± 0.14 (2)
Δ*pspG*	0.85 ± 0.28 (4)	1.17 ± 0.30 (4)

in experimental set-up; Karlinsey *et al.* (2010) performed survival experiments lasting more than 20 days whereas we performed a CI experiment lasting 6 days, and our results are based on competition between wt and mutant strain, while Karlinsey *et al.* (2010) tested strains individually. Additionally, the genetic backgrounds of *S.* Typhimurium differ. In our study we use ST4/74, a close relative to SL1344, whereas Karlinsey *et al.* (2010) investigated the role of PspA in *S.* Typhimurium ATCC 14028s. It has recently been reported how these two genetic lineages of *S.* Typhimurium differ in their virulence potential (Clark *et al.*, 2011).

Interestingly, the Δ*pspB* strain was reduced in CI in both types of mice (CI, 0.29; *P*<0.001 and 0.14, *P*<0.001, respectively), indicating that removal of PspB affects virulence of *S.* Typhimurium by a mechanism distinct from PspA. Virulence could be partially restored by re-introducing the *pspB* gene on a plasmid (CI, 0.66±0.44). C3H/HeN mice, as used here, provide the standard model to study the influence of NRAMP1 on infectivity, and this type of mouse was used in the original studies of the role of host genes in mice susceptibility to *Salmonella* (Plant & Glynn, 1974). However, there are other differences between the two lines of mice that we have employed, and since we have not performed functional assays, we can only assume that the phenotype seen with PspB in *Nramp1*^{+} mice is related to metal transport, as demonstrated for PspA by Karlinsey *et al.* (2010), but we cannot rule out that other differences between the mouse strains may be important.

Deletion of *pspC* and *pspG* did not affect virulence in any of the mouse types, whereas lack of *pspD* reduced virulence slightly in NRAMP1^{−} mice only (CI, 0.46±0.09; *P*<0.05). In summary, PspA contributes to virulence in NRAMP1^{+} mice only, removal of PspB affects virulence independently of NRAMP1, the *pspC* and *pspG* mutants do not exhibit any virulence attenuation, while PspD only contributes to virulence in NRAMP1^{−} mice. Together, these results indicate that the Psp system of *S.* Typhimurium is highly complex, with distinct roles in intra-host survival for individual proteins of the system. Furthermore, in contrast to reports of the role the PspC protein plays in virulence of *Y. enterocolitica* (Darwin & Miller, 2001), this protein is dispensable for *S.* Typhimurium virulence.

The Psp system does not contribute to uptake, intracellular survival and replication in cultured J774 macrophages

To investigate if the reduced virulence of the *psp* mutants was linked to defects in survival inside host cells, a hallmark of *S.* Typhimurium pathogenicity, uptake and intracellular survival and replication of the mutants were assessed by enumerating intracellular bacteria 1, 4 and 24 h post-infection of J774 macrophage-like cells. The number of bacteria that were taken up by the macrophages, as estimated by c.f.u. counts at 1 h post-infection, did not differ between strains, nor did the c.f.u. at 4 h (data not shown). Further, they had a 10–15-fold net replication between 4 and 24 h which did not differ

significantly between strains (Fig. 1). As a control, an isogenic SPI2 mutant (Δ*ssaV*) exhibited a reduced replication of approximately twofold, as expected (Hensel *et al.*, 1998). These data indicate that the Psp system does not contribute significantly to uptake by macrophages and to intracellular survival or growth; however, further studies employing activated macrophages are indicated, as these may require a higher degree of resistance from the bacteria to survive.

Furthermore, cytotoxicity 24 h post-infection did not differ between the strains (data not shown), ruling out that some strains in particular were subject to gentamicin killing, which could mask an increased ability to multiply in the intracellular compartment.

The Psp system does not contribute to resistance to complement-induced killing

Human and animal pathogens are able to resist complement-induced killing (serum resistance) by the innate immune system. This process can consequently be a decisive factor for the success of pathogens during systemic infections. In *S.* Typhimurium, serum resistance is mediated by several virulence factors that inhibit or destroy complement factors (Ho *et al.*, 2011). To investigate if the virulence phenotypes of the *psp* mutants were related to resistance to complement-induced killing, we tested resistance of the *psp* mutants to serum by subjecting them to fresh calf serum. Also in this assay, survival of single and multiple deletion mutants did not differ from that of the wt strain (data not shown).

The Psp system is dispensable for coping with secretin stress

In *Y. enterocolitica* both PspB and PspC are important to maintain normal growth in the presence of secretin-induced
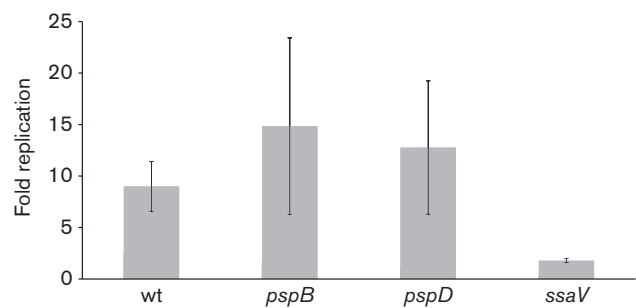

Fig. 1. Replication of *S.* Typhimurium 4/74 wt, Δ*pspB* and Δ*pspD* mutants inside J774 macrophages. The bars show the fold changes ± SD of c.f.u. between 4 and 24 h post-challenge. No significant differences between wt and mutated strains were observed. To demonstrate that the assay was able to show differences between strains, an isogenic SPI-2 Δ*ssaV* mutant was included, and as expected this strain was clearly attenuated for intracellular growth.
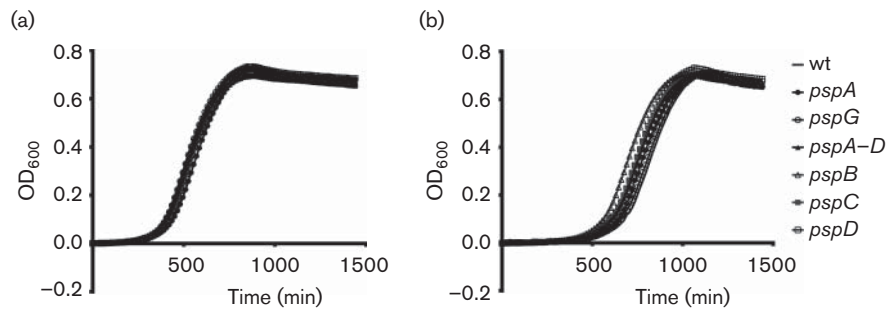
Fig. 2. Growth of *S.* Typhimurium 4/74 wt and mutated strains without (a) and with (b) secretin-induced stress in M9 medium. The mutated strains did not differ from the wt strains in growth rate and length of lag phase in either condition. Growth in the presence of secretin stress was found to prolong the lag phase of all strains.

stress (Darwin & Miller, 2001). As virulence in a mouse model of infection correlated with the secretin-induced growth defects, attenuation of *Y. enterocolitica* was concluded to be linked to impaired growth during secretin production (Darwin & Miller, 2001). To investigate if the reduced virulence observed for the *pspB* and *pspD* mutants in this study was related to diminished secretin stress resistance, we tested whether the Psp proteins of *S.* Typhimurium played a role in growth and virulence during secretin-induced stress by overproducing the pIV secretin in our Δ*psp* strains. Overexpression of pIV has previously been shown to strongly induce expression of the *psp* operon, including *pspA* in *S.* Typhimurium (Lloyd *et al.*, 2004). To investigate this possibility, the wt and *psp* single and multiple mutant strains of *S.* Typhimurium were transformed with a plasmid expressing pIV (pGJ4) (Jovanovic *et al.*, 2006). Expression of pIV was confirmed by Western blot analysis (data not shown). Presence of pGJ4 extended the lag phase of *S.* Typhimurium during growth in M9; however, this effect was the same in all strains, wt as well as Δ*psp* mutants (Fig. 2). Additionally, the secretin stress caused similar attenuation of the Δ*pspA–D* strain in *Nramp1⁻* mice to that observed without secretin stress (Table 3). Interestingly, expression of pIV eliminated the attenuation previously observed with the Δ*pspA* and Δ*pspA–D* strains in *Nramp1⁺* mice. Together these results show that the Psp system is dispensable for coping with secretin stress in *S.* Typhimurium and indicate that other genes and associated stress response mechanisms are the major players in secretin stress

survival in *S.* Typhimurium. This is in contrast to the roles of PspB and PspC in *Y. enterocolitica*, and indicates that the Psp system has a different role in *S.* Typhimurium, or that additional systems coping with secretin stress are present in *S.* Typhimurium.

Concluding remarks

In the present study we demonstrated a clear role of the Psp system during infection independent of Nramp1. Analysis of phenotypes of single genes indicated that this could be attributed mainly to the lack of *pspB*, and also to *pspA* in the NRAMP1⁺ mice, confirming previous reports (Karlinsey *et al.*, 2010). Genetic complementation of the Δ*pspB* phenotype supports this conclusion. The mechanism behind attenuation caused by deletion of *pspB* is unknown, but since we see an effect in both NRAMP1⁺ and NRAMP1⁻, it is independent of NRAMP1 and as a consequence different from PspA. The PspB protein is located in the inner membrane (Huvet *et al.*, 2011) and the virulent phenotype could theoretically be associated with a general morphological change of the membrane in the absence of PspB. Further studies are needed to detail this.

From our studies it was clear that, although the Psp system is very similar in organization in different *Enterobacteriaceae* (Joly *et al.*, 2010; Model *et al.*, 1997), it has evolved to have either different functions or most likely different degrees of redundancy in different bacteria. In *Y.*

Table 3. Virulence of Δ*psp* mutants in *Nramp1⁻* and *Nramp1⁺* mice in the presence of secretin stress (pGJ4) as shown by mixed infection with *S.* Typhimurium 4/74 wt and *psp* mutant bacteria

Indicated mutant versus 4/74/pGJ4	CI ± SD in NRAMP1⁻ mice (C57/BL6)	CI ± SD in NRAMP1⁺ mice (C3H/HeN)
Δ*pspABCD*/pGJ4	0.62 ± 0.15** (6)	1.58 ± 1.25 (4)
Δ*pspA*/pGJ4	1.79 ± 0.97 (4)	1.43 ± 0.73 (4)

Mice were infected intraperitoneally with equal numbers of mutant and wt bacteria (each 5×10^3 c.f.u.). Competition indices (CI) were calculated based on input (inoculum) and output (spleen sample) proportions of wt versus mutant bacteria. The results show mean values ± SD. **, Significantly different from the wt ($P<0.01$) as determined by one sample *t*-test analysis.

enterocolitica PspB and PspC are virulence factors (Darwin & Miller, 2001; Horstman & Darwin, 2012). In contrast, in *S.* Typhimurium PspA and lack of PspB contribute to virulence, while *pspC* is dispensable. The most remarkable difference, perhaps, is that secretin stress did not affect the *psp* mutants differently from the wt strain in our study, which is in contrast to observations in *Y. enterocolitica* (Darwin & Miller, 2001). The discrepancy between the functions of Psp proteins in related species has been reported by others, and recently it has been shown that PspBC and not PspA (as in *E. coli*) was important for coping with leaks in the membrane in *Y. enterocolitica* (Horstman & Darwin, 2012).

During our attempts to elucidate the underlying mechanism behind the virulence phenotype of a Δ*pspB* mutant, we were able to eliminate several potential mechanisms. Most important, secretin stress resistance and survival and replication inside cultured macrophages were not affected. Finally, our results have revealed important differences in the essentiality of the Psp system for a number of phenotypes in *S.* Typhimurium compared with what has been reported in *E. coli* and *Y. enterocolitica*. Further studies should focus on clarification of these differences, specifically whether they are caused by a higher degree of redundancy between stress-supporting systems in *Salmonella* compared with the other species.

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