

## Polynucleotide Phosphorylase Negatively Controls *spv* Virulence Gene Expression in *Salmonella enterica*†

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**Mutational inactivation of the cold-shock-associated exoribonuclease polynucleotide phosphorylase (PNPase; encoded by the *pnp* gene) in *Salmonella enterica* serovar Typhimurium was previously shown to enable the bacteria to cause chronic infection and to affect the bacterial replication in BALB/c mice (M. O. Clements et al., Proc. Natl. Acad. Sci. USA 99:8784–8789, 2002). Here, we report that PNPase deficiency results in increased expression of *Salmonella* plasmid virulence (*spv*) genes under in vitro growth conditions that allow induction of *spv* expression. Furthermore, whole-genome microarray-based transcriptome analyses of bacteria growing inside murine macrophage-like J774.A.1 cells revealed six genes as being significantly up-regulated in the PNPase-deficient background, which included *spvABC*, *rtcB*, *entC*, and STM2236. Mutational inactivation of the *spvR* regulator diminished the increased expression of *spv* observed in the *pnp* mutant background, implying that PNPase acts upstream of or at the level of SpvR. Finally, competition experiments revealed that the growth advantage of the *pnp* mutant in BALB/c mice was dependent on *spvR* as well. Combined, our results support the idea that in *S. enterica* PNPase, apart from being a regulator of the cold shock response, also functions in tuning the expression of virulence genes and bacterial fitness during infection.**

Members of the genus *Salmonella* have evolved through the acquisition of several genetic elements that enhance bacterial virulence and enable these bacteria to act as facultative intracellular pathogens (31, 34). These acquired genetic elements include the *Salmonella* pathogenicity islands (SPIs) (31, 34, 40), the *spv* virulence gene cluster (33), and selected prophages (25). In addition, *Salmonella* pathogenicity is dependent on a strict transcriptional regulation of horizontally acquired genetic elements (27, 30, 40, 60, 70, 71). This regulation is coordinated through the bacterial responses to environmental cues experienced during infection (30, 71) and includes the expression of SPI1 genes needed for bacterial invasion (12, 26, 34) and for the induction of proinflammatory responses (37) and the expression of *spv* and SPI2 genes needed for intracellular survival and replication (10, 30, 34, 56, 57, 71, 77). It has, however, remained enigmatic as to how the imported genetic elements became adapted to the repertoire of preexisting gene regulators. In part, this regulatory compatibility can be explained by the fact that the imported genetic elements rely to a degree on evolutionary conserved regulatory factors for their expression, such as two-component sensor regulatory systems, the RNA-polymerase  $\sigma$  factors, and nucleoid-associated proteins. These factors then act in concert with specific virulence-

associated gene regulatory proteins to achieve the proper induction of virulence genes (71).

Polynucleotide phosphorylase (PNPase; encoded by the *pnp* gene) belongs to an expanding family of exoribonucleases (7) with homologues identified in eubacteria (22, 29, 79), *Drosophila melanogaster* (47), plants (44, 87), and even mice and humans (48, 75, 87). The *Escherichia coli* PNPase participates in RNA degradation (2) and plays a central role in adaptation to growth at low temperature (86). In this context, PNPase assists adaptation to the new environmental situation by specifically degrading mRNAs that code for cold shock proteins (CSPs), a process which is needed for the resumption of bacterial replication after shifting to decreased temperature (86). PNPase is also a crucial component of the RNA degradosome, the multiprotein complex that is responsible in part for mRNA degradation in *E. coli* (2, 69).

The facultative intracellular pathogen *Salmonella enterica* serovar Typhimurium causes a systemic infection in mice (8, 53). The murine infection resembles human typhoid fever and involves invasion of the intestinal epithelial cell barrier, subsequent visceral colonization, and replication in phagocytic cells (8, 26, 34, 53). *S. enterica* serovars Typhimurium and Typhi both contain a gene which is very similar to the *E. coli pnp* gene (11, 55), and an *S. enterica* serovar Typhimurium mutant deficient for PNPase exoribonuclease activity shows the expected defect in cold adaptation (11). When grown at 37°C in rich complex medium, the *S. enterica* serovar Typhimurium *pnp* mutation does not show alterations in mRNA levels of *csp* genes but instead expresses increased levels of other mRNA species, in particular those coded for by SPI1 and, to a lesser

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degree, SPI2 (11). Mutational inactivation of the *S. enterica* serovar Typhimurium PNPase also results in an altered infection pathogenesis; in contrast to acute systemic infections caused by wild-type (wt) *S. enterica* serovar Typhimurium, the *pnp* mutation gains the ability to establish a persistent infection in BALB/c mice (11).

The complexity by which environmental signals regulate the expression of virulence genes in *Salmonella* is well illustrated by the fact that the bulk of the essential virulence genes are not properly expressed during growth in ordinary complex laboratory medium (71). Therefore, to find out whether virulence genes apart from SPI1 and SPI2 would depend on PNPase for their expression, we have in this report studied the effect of PNPase deficiency on bacterial gene expression under in vitro conditions that simulate selected aspects of the intracellular environment and during actual bacterial replication inside murine macrophage-like J774-A.1 cells. The concomitant results reported here show that the expression of the *spv* operon is strongly increased in the absence of PNPase under conditions that allow the induction of *spv* gene expression. In the absence of the transcriptional activator gene *spvR*, however, no accumulation in *spv* gene expression was observed with PNPase deficiency, implying that the relaxed expression of *spv* genes resulting from PNPase deficiency did not relieve *spv* expression from the gene regulator SpvR. Significantly, competition experiments with BALB/c mice showed that the apparent growth advantage of the *pnp* mutant depended on SpvR as well.

#### MATERIALS AND METHODS

**Bacterial strains, growth media, and plasmids.** *Salmonella enterica* serovar Typhimurium MC1 (SR-11 variant  $\chi$ 3181) and the isogenic *pnp* mutant MC71 are described in Clements et al. (11). Strains were grown in Luria broth (LB) or on Luria agar with antibiotics as appropriate (ampicillin, 100  $\mu$ g/ml; chloramphenicol, 10  $\mu$ g/ml; kanamycin, 30  $\mu$ g/ml; tetracycline, 10  $\mu$ g/ml). Low-pH minimal medium (MM5.8), as adapted from Kox et al. (42), included 100 mM Bis/Tris (Sigma) buffer (pH 5.8), 0.1% (wt/vol) Casamino Acids, 0.16% (wt/vol) glycerol, and 10  $\mu$ M MgCl<sub>2</sub>. Complete cell tissue culture medium (CC) consisted of RPMI 1640 medium (Gibco) supplemented with 10% (wt/vol) fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma), and 20 mM HEPES (Sigma) as previously described (17). To generate *phoP*, *rpoS*, or *spvR* mutants of wt MC1 and the *pnp* mutant MC71 bacteria, the strains were transduced with P22 *int* phage lysates (76) containing *phoP::Tn10* (23), *spvR::Tn5* (72), or *rpoS* inactivated through insertion with the suicide vector pRR10 (21). A Tn10 tetracycline resistance marker, carried on the virulence plasmid pSLT and shown to be neutral in virulence assays applying BALB/c mice (4), was used to tag bacteria in competition experiments. Plasmid pFF-1 was obtained by cloning the PCR-amplified *pnp* gene into the T7 RNA-polymerase-dependent expression vector pET21(c) using the primers 5'CGGGATCCCGATGCGAGAAGATCGGGTA TT3' and 5'CCGCTCGAGCGGCTCGGCCTGTTCGCTCGC3' with the BamHI and XhoI cloning sites, respectively, in boldface type (11). The fragment thus amplified included 17 bp upstream of the *pnp* initiation codon, as well as the last codon before the stop signal. In this construct, expression is driven from the plasmid-encoded start codon and ribosomal-binding site. The pET-based vector pHAM-1 encoding the catalytic carboxy-terminal portion of SpvB was available from previous work (67, 83).

**Cell culture and infection models.** The murine macrophage-like J774-A.1 cells (ATCC TIB67) were grown in RPMI 1640 medium (Gibco) supplemented with 10% (wt/vol) fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma), and 20 mM HEPES (Sigma). To avoid the rapid cytotoxicity mediated by expression of invasion-associated SPI1 genes (9, 50, 63), the bacterial inocula were taken from bacteria grown on agar plates, a condition that does not induce SPI1 expression (16, 71), and opsonized with fresh mouse serum to facilitate bacterial uptake into J774-A.1 cells. To increase the uptake of salmonella, plates were centrifuged at 1,000  $\times$  g for 5 min, and this was defined as time zero hour. After 1 h of phagocytosis, extracellular bacteria were killed with 30- $\mu$ g/ml gentamicin. This medium was replaced after 1 h with medium containing 5- $\mu$ g/ml gentamicin.

Incubations were continued for the time required for each time point tested. The relatively low levels of gentamicin were chosen to avoid any potential effects on intracellular bacterial gene expression by gentamicin (17). Bacteria were released from host cells by hypotonic lysis and enumerated by viable counting on Luria agar plates.

**RNA extraction for microarrays.** Total bacterial RNA for the microarray experiments was isolated from bacteria propagated in either of three separate growth media. First, for experiments referred to as MM5.8, bacteria were grown aerobically in MM5.8 until they reached optical density at 600 nm (OD<sub>600</sub>) of 0.5. Second, for CC samples, the bacteria were grown as described in Eriksson et al. (17). The bacterial RNA was in each experiment stabilized in 1% (wt/vol) acidic phenol and 19% (wt/vol) ethanol in water (17, 36, 82). Third, for extraction of bacterial RNA from intracellular bacteria, we refer to a recent description (17). Briefly, for each extraction of *Salmonella* RNA, a total of 10<sup>8</sup> J774-A.1 murine macrophage-like cells were seeded in 20 six-well cell culture plates and infected with mouse complement-opsonized *S. enterica* serovar Typhimurium MC1 or MC71 prepared as described in Eriksson et al. (17), at a multiplicity of infection (MOI) of 100:1 or 10:1 bacteria to cells (17). Eight hours postinfection, the macrophages were lysed for 30 min on ice in 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 1% (wt/vol) acidic phenol, and 19% (wt/vol) ethanol in water, causing lysis of eukaryotic cells but not lysing the intracellular bacteria. The phenol-ethanol mixture acted to stabilize all bacterial RNA (36, 82). Bacteria were isolated by centrifugation (10,000  $\times$  g; 30 min) from the host cell lysate obtained from 120 wells of infected J774-A.1 murine macrophage-like cells and pooled. For all experiments, total RNA was prepared using the Promega SV total RNA purification kit. The quality of bacterial RNA samples was analyzed by size fractionation on a microfluid-based system where the fractionated RNA is detected by being stained with an intercalating fluorescent dye (2100 Bioanalyzer; Agilent). This allowed us to simultaneously define the RNA samples with respect to quantity and quality.

**Microarray procedures.** DNA microarray analysis of gene expression was performed essentially as previously described (17). Briefly, RNA was first reverse transcribed into cDNA and subsequently labeled by random priming with Cy5-dCTP (for labeling protocols, see [www.ifr.bbsrc.ac.uk/Safety/Microarrays/#Protocols](http://www.ifr.bbsrc.ac.uk/Safety/Microarrays/#Protocols)). Genomic DNA (gDNA) was fluorescently labeled with Cy3-dCTP and used as a reference channel in each experiment. The labeled cDNA and gDNA were mixed together, denatured, and hybridized to the microarray under standard glass coverslips (22 by 22 mm). Hybridization was performed at 65°C overnight, after which the slides were washed twice in 2 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 sodium citrate) at 65°C for 5 min, followed by washing twice, once with 1 $\times$  SSC and once with 0.2 $\times$  SSC for 5 min, each at room temperature. The washed slides were scanned on a GenePix 4000A scanner (Axon Instruments, Inc.). Fluorescent spots and local background intensities were quantified using Genepix Pro software (Axon Instruments, Inc.). All hybridizations were repeated three or four times. Spots showing a reference signal that was lower than the background plus 2 standard deviations or obvious blemishes were excluded from subsequent analyses. The local background was subtracted from spot signals, and fluorescence ratios were calculated. To compensate for unequal dye incorporation or any effect of the amount of template, data centering was performed by bringing the median natural logarithm of the ratios for each group of spots printed by the same pin to zero. The averages from the four data sets from each strain were calculated and compared to each other, and genes that had a >2-fold difference in expression were further analyzed. This new subset of data was analyzed by analysis of variance, and genes for which the difference in expression that had a *P* value of <0.005 were considered significant.

**RNA blot analyses.** For Northern blotting, bacteria were grown in MM5.8 to an OD<sub>600</sub> of 0.3. Rifampin (200  $\mu$ g/ml; Sigma) was added, and samples were taken immediately and at defined intervals (0, 2.5, 5, 10, and 15 min). Total RNA was prepared and analyzed for purity as above. Ten micrograms of RNA per lane was separated on denaturing agarose gels and finally transferred onto nylon sheets (Amersham) by capillary blotting. Hybridization conditions were applied as specified by the manufacturer of the membrane (Amersham). Briefly, the membrane was baked for 2 h at 80°C and then prehybridized in 50% (wt/vol) formamide, 4 $\times$  SSC, 0.2% (wt/vol) SDS, 2.5 $\times$  Denhardt solution, and 0.05-mg/ml sonicated herring sperm DNA at 42°C for 3 h. Internal fragments of the genes encoding *spvR* (positions 31630 to 32131, as annotated in the sequenced LT2 genome), *spvA* (30302 to 30810, as annotated in the sequenced LT2 genome), and 16S rRNA (positions 289339 to 289840, as annotated in the sequenced LT2 genome) were amplified by PCR, labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham) using a random priming labeling kit (Roche), and used as probes. The synthesized probes were added to the prehybridization solution and incubated at constant agitation at 42°C overnight. The membrane was washed twice with 2 $\times$  SSC and 0.1% (wt/vol) SDS and then twice for 20 min each in 0.1 $\times$  SSC-0.1%

(wt/vol) SDS. A radioisotope imaging system (Phosphorimager 445SI; Molecular Dynamics) was used to detect radioactivity. Half-lives were calculated using the rate equation for a one-substrate enzyme-catalyzed reaction. For the dot blot experiment, bacteria were grown to OD<sub>600</sub> of 0.6 in LB and then transferred to MM5.8 to mimic transition to an intracellular environment. Samples were taken at 1, 1.5, and 2 h and finally overnight, posttransition. Total RNA was prepared and analyzed as above. Twelve micrograms of total RNA per dot was applied onto nylon sheets (Amersham) and UV cross-linked. After this, the same procedure as for Northern blots was used.

**Determination of  $\beta$ -galactosidase activities.** Enzyme activities expressed by *lacZ* transcriptional constructs were measured according to Miller (59) using 2-nitrophenyl- $\beta$ -galactoside (Sigma) as a substrate. The *spvR-lacZ* (pHUB70), *spvA-lacZ* (pHUB61), and *tlpA-lacZ* (pOF1) constructs have been described previously (38, 72, 80). For bacteria grown in MM5.8, the samples used for  $\beta$ -galactosidase activity measurements were neutralized with an equal volume of 1 M Tris-HCl buffer, pH 8.0, before addition to the reaction mixture. For comparing  $\beta$ -galactosidase activities, we used an unpaired *t* test with Welch correction.

**Immunostaining and immunofluorescence.** Murine macrophage-like J774-A.1 cells were infected as described for the microarray experiment (using MOI as indicated) and subsequently fixed in a freshly prepared 4% (wt/vol) formaldehyde solution, prepared as described by Eriksson et al. (18). The fixed cells were subsequently permeabilized in 0.1% (wt/vol) saponin and stained with antibodies as indicated in a solution with 10% (wt/vol) horse serum and 0.1% (wt/vol) saponin (3). Texas red-conjugated phalloidin was from Molecular Probes. For primary antibodies, we used a 1:200 dilution of the rabbit 156 anti-lysosome-associated membrane glycoprotein 1 (LAMP-1) (3) and a 1:200 dilution of goat anti-*Salmonella* (CSA-1) (3), while for secondary antibodies we used rhodamine red X-conjugated donkey anti-rabbit and Cy2-conjugated donkey anti-goat antibodies (both diluted 1:200). The samples were mounted using Aqua polymount (Polysciences) and analyzed by confocal microscopy (3).

**Assessment of cytotoxicity.** Three separate methods were used to score the viability of the macrophage-like J774.A.1 cells during infection. (i) The proportion of attached to detached cells was estimated by phase-contrast microscopy. To document these observations, the cells were grown on glass coverslips, fixed, and stained for bacteria and actin after infection as specified above. Thereafter, the density of infected murine macrophage-like J774 cells was monitored by microscopical counting. (ii) As release of lactate dehydrogenase (LDH) is used as a defined measure of SPI1- and SPI2-induced cell death (14, 62, 63), we used a cytotoxicity detection kit (Roche) measuring LDH activity in the cell culture supernatants after given time points postinfection. The activities are given as the percentage of LDH activity released in relation to the LDH activity obtained by lysing a comparable amount of uninfected cells with Triton X-100. (iii) Apoptosis was monitored by measurement of nuclear fragmentation (28, 51). For these experiments, infected murine macrophage-like J774-A.1 cells were collected by centrifugation (800  $\times$  g; 1 min). This included both the attached cells and the potentially detached cells (in total, approximately  $2 \times 10^5$  cells). The pelleted cells were washed in cold phosphate-buffered saline. The pellet was then rapidly dissolved in 200  $\mu$ l of 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% (wt/vol) Triton X-100 buffer. Thereafter, the suspension was separated into "Triton-insoluble" and "Triton-soluble" fractions by centrifugation (14,000  $\times$  g; 5 min). A small proportion of the supernatant was then immediately run on a 1% (wt/vol) agarose gel. Meanwhile, the pellet was solubilized into 100  $\mu$ l of a 10 mM Tris-HCl-2 mM EDTA-1% (vol/vol) buffer (pH 7.5) and analyzed on a 1% agarose gel. Finally, the rest of the Triton-soluble material was extracted with phenol-chloroform and precipitated with ethanol by using glycogen (Roche) as a carrier. The material was dissolved into 10  $\mu$ l distilled water, treated with RNase, and analyzed on 1% (wt/vol) agarose gels. Agarose gels included ethidium bromide (0.5  $\mu$ g/ml; Sigma) to reveal nucleic acids.

**Infection experiments.** For *in vivo* infection experiments, we used *S. enterica* serovar Typhimurium strains MC1 and MC1 *spvR* mutant (11, 72) tagged with a Tn10 element on pSLT and previously shown to be neutral in mice infection experiments (4) and MC71 and MC71 *spvR* mutant strains. Infection experiments were carried out as previously described (11, 78). Briefly, female BALB/c mice, 6 to 8 weeks of age, were infected intraperitoneally with a 50:50 mixture of MC1-Tn10 and MC71 bacteria in a total dose of approximately  $5 \times 10^3$  bacteria per mouse or with a 50:50 mixture of MC1-Tn10-*spvR* mutant strain and MC71 *spvR* mutant bacteria, also in a total dose of  $5 \times 10^3$  bacteria per mouse. Groups of five mice were used for each of two series of experiments (10 mice per competition in total). Three days after infection, mice were sacrificed, and the livers and spleens were removed for enumeration of the total amount of viable bacteria, as well as the percentage of tetracycline-resistant bacteria. To compare the significance in ratios, we applied the two-sided Mann-Whitney U test.

## RESULTS

**Whole-genome scale probing for bacterial gene expression in vitro reveals a strong association between PNPase and *spv* gene expression.** Our previous transcriptome analyses of *Salmonella enterica* serovar Typhimurium gene expression in vitro were carried out with bacterial cultures replicating in complex LB medium (11). While these analyses defined a strong association between SPI1 gene expression and PNPase, the growth conditions applied were not optimal for the expression of several virulence genes, such as SPI2 (39) or *spv* genes (17, 71). To further define the repertoire of virulence genes affected by PNPase, we extended the microarray-based transcriptome analysis to include bacteria grown in deprived medium with a low Mg<sup>2+</sup> concentration and a pH of 5.8 (MM5.8). The composition of the medium was primarily designed to mimic the intravacuolar environment in which *Salmonella* replicates (17, 42) and is known to induce expression of SPI2 (78).

We first defined the transcriptome for the wild-type, PNPase-proficient *S. enterica* serovar Typhimurium strain MC1 grown in MM5.8 medium. Samples for RNA preparation were taken when the culture entered the transition into the stationary phase (OD<sub>600</sub>, 0.5). To probe for alterations in gene expression, we used cultures grown in CC as a comparator and applied a false discovery rate of 0.005. Under these conditions, we defined 447 genes as up-regulated and 479 genes as down-regulated during replication in MM5.8 (see Table S1 in the supplemental material). Among the genes strongly induced for expression, we identified representatives of the SPI2 and the *spv* gene clusters, whereas among the genes strongly suppressed we identified SPI1 and genes for flagellation and motility (see Table S1 in the supplemental material).

Having shown that MM5.8 did induce expression of genes that are normally induced when bacteria are residing in an intracellular compartment (17), we next tested whether PNPase affected the mRNA levels of any of these genes. To probe for the effect of PNPase deficiency, we applied *S. enterica* serovar Typhimurium strain MC71, which was constructed from MC1 by introducing a STOP codon in *pnp* by site-directed mutagenesis (11). When MC71 was grown in MM5.8 to the transition to the stationary phase of growth (OD<sub>600</sub>, 0.5), MC71 showed a relative increase in expression of 37 genes and a relative decrease in the expression of 9 genes compared to wt MC1 (Fig. 1). More specifically, the comparison revealed a strong (7- to 8-fold) relative increase in *spv* expression in MC71, in addition to an increase (2- to 10-fold) in mRNA levels of genes located in prophages (STM0906, STM0908, STM0914, STM2233 to STM2237, STM2587, STM2616, and STM2620) and of genes encoding proteins involved in stress responses (STM1251 and *ibpAB*) and DNA repair (*mutM* and *recF*). In parallel, we found a PNPase-dependent decrease (two- to threefold) in the expression of genes associated with purine synthesis (*carA*, *purK*, *trpCD*, and *purF*) and of genes involved in flagellar synthesis (*flgBC*) (Fig. 1).

**mRNA analyses reveal increased levels of *spv* gene mRNA at PNPase deficiency in vitro.** The *spv* gene cluster is transcribed from two main promoters, one in front of the transcriptional activator protein gene *spvR* (*PspvR*) and another in front of *spvA* (*PspvA*) directing the expression of Spv effector proteins (13, 43, 52, 81). To verify the association between PNPase and

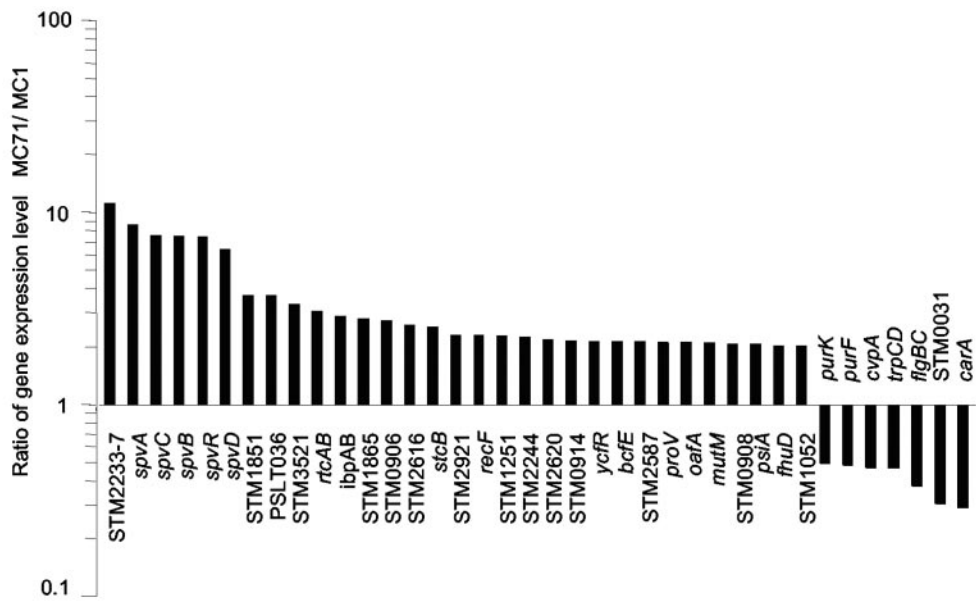


FIG. 1. Differences in the transcriptomes of PNPase-proficient MC1 and PNPase-deficient MC71 *S. enterica* serovar Typhimurium during growth in low-pH minimal medium (MM5.8). Alterations are defined as the ratio in mRNA signals between MC71 and MC1, when the bacteria were grown in MM5.8 to OD<sub>600</sub> values of 0.5. Alterations in gene mRNA contents were measured by whole-genome microarray-based analyses. The data include quadruplicate measurements and show all genes that show alterations of >2-fold and with a *P* value of  $\leq 0.005$ .

*spv* expression, we next determined *spvA* expression by comparing *spvA* mRNA levels in the wt MC1 PNPase-proficient and the MC71 PNPase-deficient mutant background by carrying out Northern blotting to probe for *spvA* mRNA during logarithmic growth (OD<sub>600</sub> 0.3) in MM5.8 after blocking RNA synthesis with rifampin. This analysis clearly showed expression of *spvA* mRNA in both MC1 and MC71. The analysis also revealed an overall higher *spvA* mRNA content and an alternative *spvA* mRNA pattern in MC71 (Fig. 2A); whereas both MC1 and MC71 showed *spvA* mRNA of apparently higher molecular weight named A1, MC71 revealed clear accumula-

tion of a second *spvA* mRNA named A2 that was only weakly detected from MC1 (Fig. 2A and B). When using rifampin to block the emergence of nascent mRNA species, we could not demonstrate any stabilization of the *spvA1* mRNA in MC71 in either of two separate sets of experiments (Fig. 2A and data not shown). In contrast, the *spvA1* mRNA appeared more stable in MC1 (Fig. 2A and B), suggesting that this transcript was differentially degraded by PNPase.

Parallel Northern analyses using a <sup>32</sup>P-labeled PCR-generated *spvR* fragment revealed a clear signal for *spvR* in MC71 but no signal in MC1 (data not shown). This is consistent with

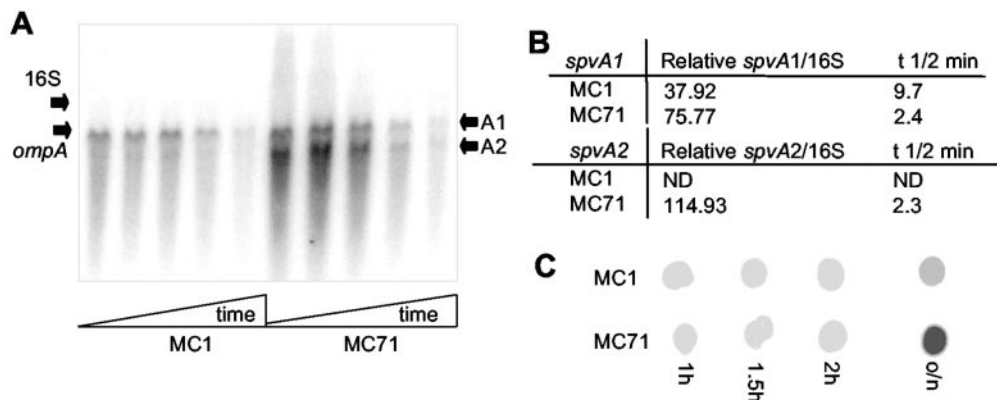


FIG. 2. Analysis of *spvA* mRNA expression in bacteria grown in low-pH minimal medium (MM5.8). (A) One representative Northern blot probing for *spvA* mRNA after the addition of rifampin to cultures growing in MM5.8 to OD<sub>600</sub> values of 0.3. RNA was isolated at 0, 2.5, 5, 10, and 15 min after RNA synthesis was blocked. A1 and A2 indicate the positions of the two *spvA*-reactive mRNA species, whereas arrows labeled 16S and *ompA* show the positions of the signals obtained with a 16S rRNA and *ompA* probes on the same blot after the membrane was stripped and the images were overlaid. (B) Phosphorimager-assisted quantification of the A1 and A2 *spvA* mRNA signals shown in panel A at the time of addition of rifampin and the estimated mRNA half-life measurements of *spvA* mRNAs. (C) One representative dot blot probing for *spvA* mRNA obtained from PNPase-proficient MC1 and PNPase-deficient MC71 at various time points after transfer of growth in MM5.8, detected with a <sup>32</sup>P-labeled *spvA* gene fragment.

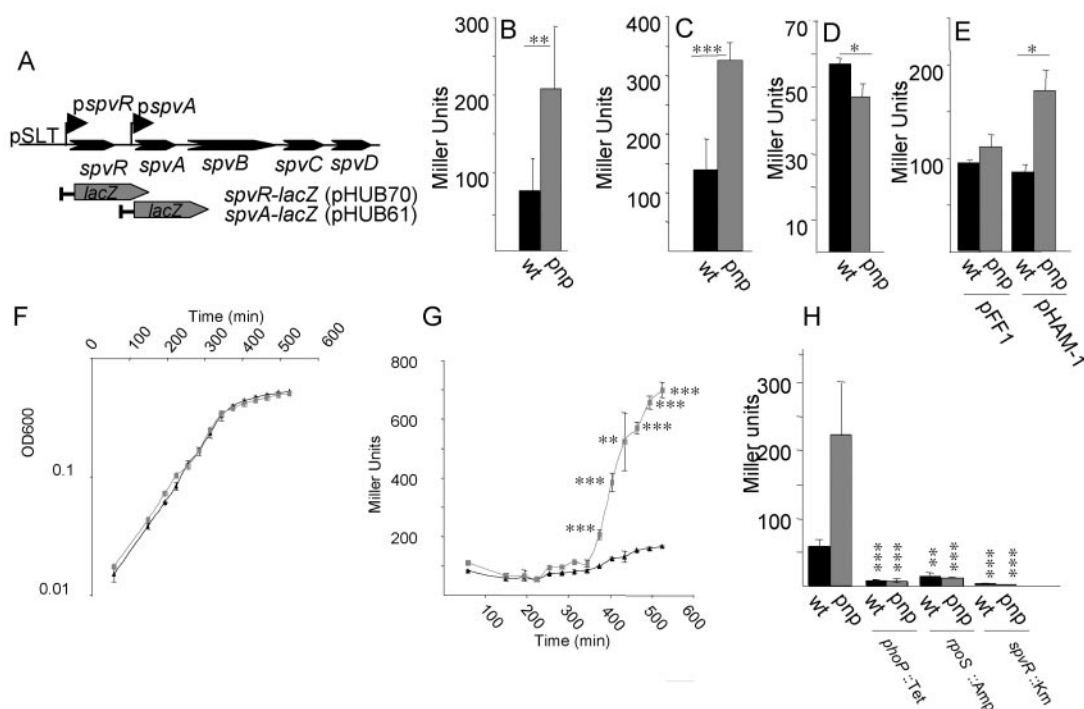


FIG. 3. Expression of  $\beta$ -galactosidase by *spv-lacZ* fusions. (A) Summary of the genetic organization of the *spv* gene cluster, indicating the position of the *lacZ* cartridges in relation to the *spvR* and *spvA* promoters. The bars upstream of the shaded *lacZ* arrows indicate the *spv* region included in the constructs (72, 81). (B and C) Stacked bar presentations of  $\beta$ -galactosidase activities expressed by *spvA-lacZ* (B) or *spvR-lacZ* (C) in PNPase-proficient MC1 (black columns) or PNPase-deficient MC71 (gray columns) grown in MM5.8. (D)  $\beta$ -Galactosidase activities of the control *tlpA-lacZ* transcriptional fusion construct in MC1 (black columns) or MC71 (gray columns) grown in MM5.8. (E) Complementation in *trans* when MC1/*spvA-lacZ* (black columns) or MC71/*spvA-lacZ* (gray columns) is provided with pFF-1 containing *pnp* or with pHAM-1 containing parts of *spvB* as a control. (F and G) Growth slopes (F) and concomitant  $\beta$ -galactosidase activities (G) expressed by MC1/*spvA-lacZ* (black line) and MC71/*spvA-lacZ* (gray line) bacteria growing in MM5.8. Combined data from three separate cultures grown in parallel generated the data. (H) Effect of *phoP*, *rpoS*, or *spvR* mutations on  $\beta$ -galactosidase activities expressed by *spvA-lacZ* in MC1 (black columns) or MC71 (gray columns) when grown in MM5.8. For panels B, C, D, E, G, and H, the measurements were carried out on stationary-phase cultures, and each value represents the mean of at least three separate experiments. Asterisks (C to H) indicate the *P* values obtained when an unpaired *t* test was performed with Welch correction, as follows: \*,  $P < 0.02$ ; \*\*,  $P < 0.005$ ; and \*\*\*,  $P < 0.001$ .

the previously reported difficulty in detecting the *spvR* transcript in *S. enterica* (1). Nevertheless, these analyses confirmed the increased *spv* mRNA levels in MC71 implied by microarray analyses.

**Transcriptional *spv-lacZ* fusions reveal PNPase-dependent expression of *spv* genes.** The fact that we could not conclusively demonstrate any increase in the half-life of the *spvA* mRNA in the PNPase-deficient strain MC71 (Fig. 2A and B) prompted us to test whether an increased transcriptional strength of *spv* promoters could account for the increased *spvA* mRNA levels observed with the PNPase-deficient background. The constructs used included the *spvA* (pHUB61) or *spvR* (pHUB70) promoter positioned to direct *lacZ* expression in a pACYC184-based construct (Fig. 3A) (72). As the expression of *spvA-lacZ* fusions tends to be weak in rich growth medium (84) and indeed confirmed for pHUB61 grown in CC (data not shown), we initiated a series of experiments to compare the expression of  $\beta$ -galactosidase from *spv-lacZ* fusions when the bacteria were grown overnight in MM5.8 in batch cultures (15 to 17 h). Regarding the *spvA-lacZ* construct pHUB61, the  $\beta$ -galactosidase levels obtained when the bacteria were grown overnight in MM5.8 were significantly higher for MC71 than for PNPase-proficient MC1 (Fig. 3B). Likewise, in comparison to MC1/

pHUB70, we noted a significant increase in the  $\beta$ -galactosidase levels expressed by MC71/pHUB70 grown in MM5.8 (Fig. 3C).

To ensure that the alterations in  $\beta$ -galactosidase activities in the PNPase-deficient MC71 background were not due to stabilization of *lacZ* mRNA or to alterations in the copy number of the operon fusion constructs in MC71, we applied the *tlpA-lacZ* fusion pOF1 as a control (38). Like the *spv* genes, *tlpA* is contained on the virulence plasmid pSLT but was not induced during bacterial replication in MM5.8 (see Table S1 in the supplemental material). Furthermore, pOF1 relied on the same *lacZ* cartridge as pHUB61 and pHUB70 and cloned with *tlpA* in the same position in the same cloning vector. The *tlpA-lacZ* fusion did not show increased but rather a slightly decreased level of activity in the *pnp* mutant MC71 background when grown in MM5.8 (Fig. 3D), which indeed suggests that the increased  $\beta$ -galactosidase activities obtained with *spvA-lacZ* and *spvR-lacZ* in MC71 originated from increased transcriptional activity.

The increased expression of *spvA* in overnight cultures of PNPase-deficient MC71 was also verified by blotting for *spvA* mRNA. For this experiment, PNPase-proficient MC1 and MC71 were grown in MM5.8, and the bacterial RNA was extracted at early (1 h, 1.5 h, and 2 h) and late (overnight) time

points of growth. Dot blot assays applying the PCR-amplified and  $^{32}\text{P}$ -labeled *spvA* gene as a probe revealed no significant signal for *spvA* mRNA during the very early time points in either MC1 or MC71 (Fig. 2C). Also, we could not detect any significant accumulation of *spvA* mRNA in the overnight cultures of MC1. This would be consistent with the observation that *spvA* mRNA becomes induced during logarithmic growth in minimal medium low in magnesium, to become only weakly detectable after prolonged incubation (84). In contrast and in accordance with the LacZ measurements from MC71/pHUB61, PNPase-deficient strain MC71 showed a substantial accumulation of *spvA* mRNA in MC71 after overnight incubation (Fig. 2C).

We next tested if complementation of the PNPase deficiency in MC71 could restore the *spvA-lacZ* activity to wild-type levels. Initial attempts using cloned *pnp* to complement for the *pnp* mutation in *trans* were not successful, due to our inability to maintain *pnp*-containing plasmid constructs in *S. enterica* serovar Typhimurium. However, when the *pnp* open reading frame was contained in the pET21(c) vector under the T7 promoter (pFF-1), the constructs could be maintained. The pFF-1 construct caused a reduction in the *spvA-lacZ* activities in the MC71 *pnp* mutant background but did not affect *lacZ* fusion expression in the MC1 wt background (Fig. 3E). A control construct containing the 3'-terminal portion of *spvB* in the pET32 vector (pHAM-1) (67, 83) did not mediate complementation (Fig. 3E). These observations implied that the T7 promoter shows weak expression in *S. enterica* serovar Typhimurium, even though the bacterium formally lacks the T7 RNA polymerase and the PNPase deficiency of MC71 can be complemented in *trans*.

**PNPase deficiency does not relieve *spvA* gene expression from dependency on growth phase, PhoP, RpoS, or SpvR.** Expression of *spv-lacZ* fusions in bacteria grown in low-magnesium minimal medium is reported to be dependent on the bacterial growth phase, the transcription factor SpvR, and the alternative  $\sigma$ -factor RpoS (84, 85). Furthermore, the conditions prevailing in MM5.8 are likely to activate the PhoP/PhoQ response regulatory system (42), also implicated in *spv* gene expression (30). Therefore, we set out to test whether the  $\beta$ -galactosidase activity expressed by the *spvA-lacZ* constructs in the PNPase-deficient background MC71 grown in MM5.8 still depended on the bacterial growth phase and the transcription factors PhoP, RpoS, and SpvR.

The growth slopes in MM5.8 appeared similar for both PNPase-proficient MC1 and PNPase-deficient MC71 (Fig. 3F). However, the concomitant expression of  $\beta$ -galactosidase became much stronger in MC71/*spvA-lacZ* when the cultures entered late logarithmic phase of growth (Fig. 3G). In parallel, the *spvR-lacZ* fusion construct pHUB70 was observed to express a similar growth-phase-dependent induction of  $\beta$ -galactosidase activity when the bacteria were grown in MM5.8, albeit the difference in  $\beta$ -galactosidase activity between MC1 and MC71 remained lower (data not shown).

To test to what extent the increase in *spvA* expression that associated with PNPase deficiency during propagation in MM5.8 still depended on the transcription factors PhoP, RpoS, or SpvR implicated in *spv* gene activation (5, 20, 21, 30, 35, 43, 68, 71, 84, 85), we measured  $\beta$ -galactosidase activities encoded by pHUB61 (*spvA-lacZ*) in *phoP*, *rpoS*, and *spvR*

mutants of MC1 and MC71. Inactivation either of *phoP*, *rpoS*, or *spvR* in PNPase-proficient MC1 or PNPase-deficient MC71 resulted in a significant reduction of *spvA-lacZ* expression in overnight cultures grown in MM5.8 (Fig. 3H). Thus, PNPase deficiency did affect the transcriptional strengths from both the *spvA* and *spvR* promoters, yet PNPase deficiency apparently did not evoke or apply any alternative bypass routes for *spvA* gene activation.

**PNPase-proficient and PNPase-deficient bacteria do not differ in their ability to induce cytotoxicity in J774-A.1 cells 8 h postinfection.** We have recently developed a method that enables transcriptometric analysis of bacteria growing within host cells (17, 36). However, to generate enough bacterial RNA for microarray analyses, we needed to apply an MOI of 100:1 (17), potentially generating infection-associated cytotoxicity and apoptosis (14, 45, 46, 49, 62, 63). Therefore, before defining and comparing the transcriptomes of PNPase-proficient MC1 and PNPase-deficient MC71 while growth occurred in host cells, we analyzed the murine macrophage-like J774-A.1 cells infected with *S. enterica* serovar Typhimurium MC1 and MC71 for possible alterations in toxicity or bacterial growth.

Upon infection of J774-A.1 macrophage-like cells with PNPase-proficient MC1 or PNPase-deficient MC71 at an MOI of 100:1, no signs of cytotoxicity or detachment of murine macrophage-like J774-A.1 cells as judged by phase-contrast microscopy were observed at 8 h postinfection (data not shown). Likewise, as followed by immunofluorescence microscopy labeling for *Salmonella* bacteria and filamentous actin at 10 h postinfection, the density and appearance of the host cells on glass slides were very similar whether the cells were infected with MC1 or MC71 bacteria (Fig. 4A and B). When the infected murine macrophage-like J774-A.1 cells were monitored for the release of lactate dehydrogenase (LDH) as a general indicator of cytotoxicity, the levels of released LDH observed at 8 h postchallenge by application of an MOI of 100:1 or 18 h postchallenge by application of an MOI of 10:1 remained low (Fig. 4C). The LDH levels released by cells infected with MC71 at an MOI of 100:1 were slightly but statistically significantly higher than those released upon infection with MC1. Yet, the LDH levels remained very low compared to the levels reported with invasive SPI1-expressing bacteria (14, 63) and to the levels contained in a comparable number of uninfected cells (Fig. 4C) (63). Similarly, when analyzed on ethidium bromide-stained agarose gels, no detectable DNA fragmentation was apparent at 8 h postinfection when an MOI of 100:1 of noninvasive complement-opsonized bacteria was applied (Fig. 4D). However, if the uninfected cells were allowed to overgrow the cell culture, leading to acidified and crowded cell conditions, DNA fragmentation was indeed detected (Fig. 4D).

In separate infection experiments, immunofluorescence microscopy labeling for intracellular bacteria revealed PNPase-proficient MC1 and PNPase-deficient MC71 bacteria residing in an LAMP-1-positive compartment, with no differences observed in the labeling pattern for the two strains (Fig. 4E to J). Viable counts, performed in parallel from nonfixed and unstained cells 8 h postinfection, revealed a 5.4-fold and 5.0-fold growth index, respectively, for the MC1 wt and MC71 *pnp* mutation, when the growth yields were compared to intracellular bacterial counts at 2 h postinfection. A repetition of the

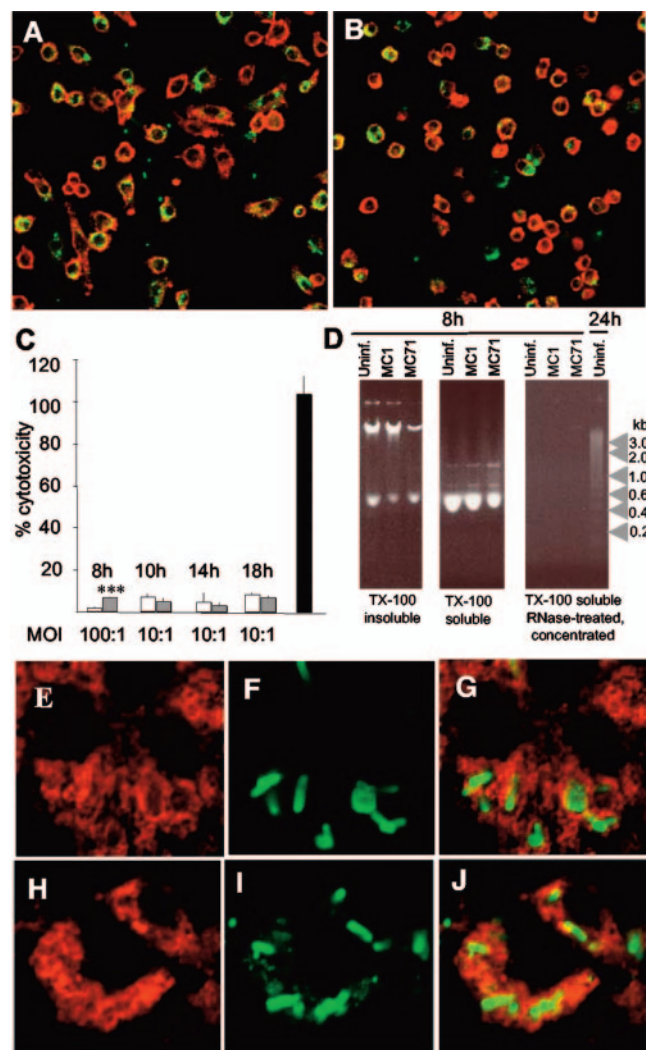


FIG. 4. Assessment of cytotoxicity in murine macrophage-like J774-A.1 cells infected with *S. enterica* serovar Typhimurium. Murine macrophage-like J774-A.1 cells grown on glass coverslips were allowed to phagocytose opsonized PNPase-proficient MC1 (A) or PNPase-deficient MC71 (B) bacteria. Ten hours postinfection, the coverslips were washed, fixed, permeabilized, stained for bacteria (green) and filamentous actin (red), and examined by confocal fluorescence microscopy at a magnification of  $\times 40$ . (C) Levels of lactate dehydrogenase in the supernatants of murine macrophage-like J774-A.1 cell cultures infected with either MC1 (white columns) or MC71 (gray columns) with the MOIs and at the time points postinfection as indicated. The black column represents the total LDH activity released from uninfected murine macrophage-like J774-A.1 cells by detergent treatment. (D) Nucleic acid contents in Triton X-100-insoluble and Triton X-100-soluble fractions of macrophage-like J774-A.1 cells infected with either MC1 or MC71. Uninfected cultures are included as controls. Samples were taken at the time points given. (E to J) Confocal microscopic images (magnification,  $\times 92$ ) of macrophages 10 h postinfection, using an MOI of 100:1 of either MC1 (E to G) or MC71 (H to J) and immunolabeled with the lysosomal marker protein LAMP-1 (red) (E and H) and *S. enterica* serovar Typhimurium (green) (F and I). Panels G and J show the merged images of panels E and H and I, respectively.

growth experiment did not provide any apparent alterations in the growth yields (data not shown).

In summary, the host cells appeared intact 8 h postinfection, and no significant differences in cytotoxicity, intracellular lo-

calization, or bacterial growth yields were found between cells infected with either PNPase-proficient or PNPase-deficient *S. enterica* serovar Typhimurium strains.

**Intracellular transcriptome of *S. enterica* serovar Typhimurium SR-11.** Having established the infection procedures, we next determined the transcriptome of the PNPase-proficient MC1 strain when replicating in the macrophage-like cells. For these experiments, bacterial RNA was extracted at 8 h postinfection was compared to RNA extracted from CC-grown bacteria. This time point was previously defined to reveal the majority of the gene expression profile alterations upon infection of murine macrophage-like J774-A.1 cells (17). Since we previously determined the transcriptome of *S. enterica* serovar Typhimurium SL1344 when replication occurs within host cells, we first compared the transcriptomes of intracellular *S. enterica* serovar Typhimurium of lines SR-11 and SL1344 to define to what extent the pattern of gene expression was dependent on the bacterial strain background. The gene expression profiles of the two lines were consistent: 94.4% of the genes revealed similar expression patterns in both strains after application of a twofold cutoff (false-discovery rate = 0.005) (see Table S1 in the supplemental material) (17). A proportion of the expressional difference reflected the histidine auxotrophy of strain SL1344. Nevertheless, these observations show that the transcriptomes of the two different strains of *S. enterica* serovar Typhimurium were comparable when the bacteria were replicating within murine macrophage-like J774-A.1 cells.

We then evaluated to what extent the intracellular transcriptome reflected the one obtained when bacteria were replicating in MM5.8. The comparison showed that 42% of the MC1 genes that were up-regulated in murine macrophage-like J774-A.1 cells were also more highly expressed in MM5.8 (see Table S1 in the supplemental material). It is noteworthy that virulence genes within SPI2 (*ssaG*), SPI3 (*mgtB*), and the *spv* locus (*spvA*) and a gene within SPI5 (*pipD*) that functionally associates with SPI2 were clearly represented within this category of genes (see Table S1 in the supplemental material). Almost one-third (30.5%) of MC1 genes that were down-regulated in murine macrophage-like J774-A.1 cells were also expressed at lower levels in MM5.8 (see Table S1 in the supplemental material), including genes within SPI1 (*hilC*) and SPI4 (STM4260) and motility-related (*fliC*) genes, as well as a gene within SPI5 (*pipC*) that associates with SPI1 gene expression (see Table S1 in the supplemental material) (40). Thus, while growth in vitro in MM5.8 obviously did not reproduce all aspects of the SCV, the large proportion of genes that showed parallel trends of expression in MM5.8 and during infection of murine macrophage-like J774-A.1 cells confirms that MM5.8 indeed does represent certain crucial aspects of the intravacuolar compartment.

**PNPase deficiency affects *spv* expression not only in vitro but also in murine macrophage-like J774-A.1 cells.** Having defined the intracellular transcriptomes for PNPase-proficient *S. enterica* serovar Typhimurium MC1, we compared the transcriptomes of intracellular MC1 and PNPase-deficient MC71. The gene expression profiles obtained at 8 h postuptake were found to reveal an extremely high degree of similarity. Only six genes (*spvABC*, *entC*, *rtcB*, and STM2236) were up-regulated, whereas the motility-associated *cheAW* and *motB* genes were

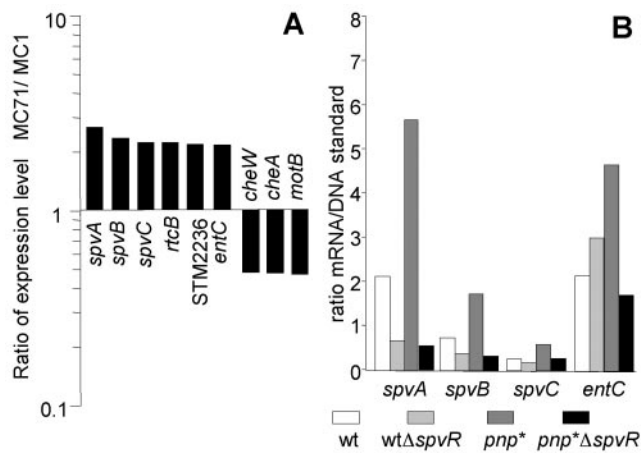


FIG. 5. The effect of PNPase on *spv* expression during replication within murine macrophage-like J774-A.1 cells. (A) PNPase-proficient MC1 and PNPase-deficient MC71 bacteria were grown in J774-A.1 cells for 8 h, and the levels of bacterial mRNA were measured with microarray technology to reveal the ratio in signals between MC71 and MC1. The averages from quadruplicate hybridizations for each strain were compared, and it was found that nine genes had >2-fold difference and a *P* value of <0.005. These significant alterations are expressed as the ratio of mRNA levels between MC71 and MC1, and displayed on a log base 10 scale. (B) MC1 and MC71 with and without *spvR* were grown within murine macrophage-like J774-A.1 cells for 8 h, and the levels of bacterial mRNA were measured with microarray technology. The averages from quadruplicate hybridizations for each strain were compared. Fluorescence hybridization signals compared to an internal gDNA standard are shown for genes that were altered between MC1 and MC71 (A).

down-regulated in intracellular MC71 compared to intracellular MC1 bacteria (Fig. 5A). These data showed that PNPase specifically affected the expression of a small set of genes during macrophage infection.

We next assessed to what extent inactivation of *spvR*, the gene encoding a key activator of *spvABCD* transcription (Fig. 3H) (71, 81), influenced the intracellular transcriptome. As measured by microarray analysis, expression of *spvAB* and to some extent *spvC* became significantly reduced in both PNPase-proficient MC1 and PNPase-deficient MC71 upon mutational inactivation of *spvR* (Fig. 5B). More significantly, the differences observed for *spv* gene expression between intracellular MC1 and MC71 were not observed in the *spvR* mutant backgrounds. Surprisingly, inactivation of *spvR* also affected the increased mRNA levels observed for *entC* in the PNPase-deficient MC71 (Fig. 5B). This shows that much of the differences observed in the intracellular transcriptomes for PNPase-proficient MC1 and PNPase-deficient MC71 depended on SpvR.

**PNPase deficiency associates with a *spvR*-dependent alteration in virulence in BALB/c mice.** Apart from affecting virulence gene expression, PNPase deficiency was previously reported to affect infection pathogenesis and replication of the bacteria in BALB/c mice (11). Here, we followed up this phenomenon by performing in vivo competition experiments between PNPase-proficient MC1 and PNPase-deficient MC71 bacteria in BALB/c mice after intraperitoneal infection. Upon recovery of bacteria from the livers and spleens 3 days postin-

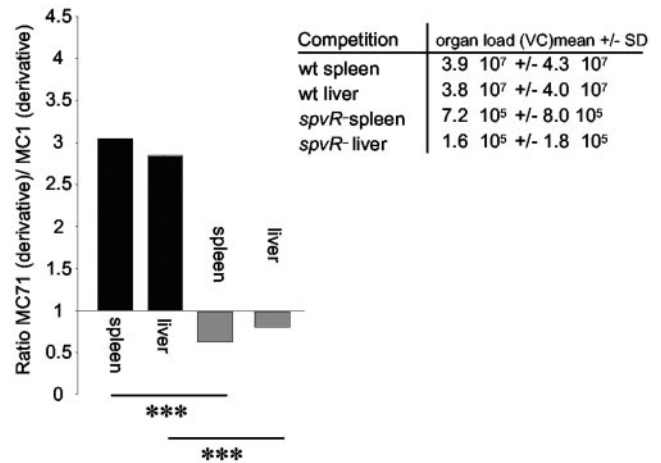


FIG. 6. Infection experiments probing the competitive indexes of PNPase-proficient MC1 and PNPase-deficient MC71 strains in BALB/c mice. Mice were challenged intraperitoneally with either a mixture of MC71 and MC1 (Tet<sup>r</sup>) or MC71 *spvR* mutant and MC1 *spvR* mutant (Tet<sup>r</sup>). The bacteria were given at a ratio of 1:1 and in a total dose of approximately  $5 \times 10^3$  bacteria per mouse. Three days after infection, the mice were sacrificed for enumeration of the bacteria in the livers and spleens. The figure shows the ratios between MC1 (Tet<sup>r</sup>) and MC71 (black columns), or MC1 *spvR* mutant (Tet<sup>r</sup>) MC71 *spvR* mutant (gray columns) and total bacterial loads. The competitions were carried out twice using five mice per competition each time, and the results are shown after the data from the two separate series of infections were combined. Asterisks show the level of significance (\*\*\*, *P* < 0.001; Mann-Witney double-sided U test) when the ratios between the viable counts from competitions between MC71 and MC1 versus the MC71 *spvR* mutant to the MC1 *spvR* mutant in either the spleen or liver were compared.

fection, the MC71 bacteria showed a higher competitive index in both the livers and spleens in two separate sets of experiments (the combined data are shown Fig. 6). As PNPase deficiency was found associated with increased *spv* gene expression in murine macrophage-like J774-A.1 cells (Fig. 5A) and as the *spv* genes participate in bacterial replication during the systemic phase of infection (32, 33, 73), we next tested the effect of inactivating *spvR* on the competitive indexes in mice. Thus, *spvR* mutants of MC1 and MC71 were competed against each other in BALB/c mice after intraperitoneal challenge. Astonishingly, under these premises MC71 lost its competitive advantage in mice in each of two separate sets of experiments, and in fact the competitive index for MC71 decreased (the combined data are shown in Fig. 6). Therefore, a noticeable proportion of the in vivo alteration in competitive index conferred by PNPase deficiency was dependent on alterations in *spvR* and SpvR-regulated gene expression.

## DISCUSSION

For many serovariants of *Salmonella enterica*, virulence in the mouse model for systemic salmonellosis is dependent on the bacterial *spv* gene cluster that promotes bacterial replication in the liver and spleen, as the bacteria reside in macrophages (32, 33). While the exact details by which Spv proteins participate in the disease process are not known, the molecular function has been determined for two of the five Spv proteins: SpvB is an actin-specific mono(ADP-ribosyl)transferase (46,

49, 67, 83), whereas SpvR acts as transcriptional activator for *spv* expression (5, 43, 54, 68, 80, 81, 84, 85). However, the genetic control of *spv* gene expression is complex and requires selected environmental cues, as well as the interplay between several transcriptional regulators (71). Expression of *spv* genes is clearly induced when the bacteria reside within mammalian cells (17, 24, 72) and in vitro when the bacteria are grown in minimal medium mimicking the intravacuolar environment (84, 85). Expression of *spv* genes can also be achieved, at least to some extent, as the bacteria enter the stationary phase of growth in rich medium (13, 15, 21, 80, 84, 85). Apart from SpvR, the regulatory factors that participate in *spv* gene induction include the response regulator PhoP (30) the alternative  $\sigma$ -factor RpoS (15, 21, 41, 71), and integration host factor (52). In addition, the global gene regulator H-NS (65, 71), leucine-responsive regulatory protein (52), and the catabolite repression system (66) have been identified as repressors of *spv* gene expression.

We have previously demonstrated that mutational inactivation of the *pnp* gene encoding the exoribonuclease PNPase in *Salmonella enterica* serovar Typhimurium resulted in apparent increased bacterial replication in the host and in the establishment of chronic carrier states among convalescent mice (11). In parallel, when the bacteria were grown in vitro in rich growth medium, the PNPase-deficient mutant revealed stronger expression of invasion-associated SPI1 genes and to some extent increased expression of SPI2 genes (11), implying that PNPase-activity somehow connected with virulence gene expression. A recent investigation demonstrated that persistent salmonellosis in the mouse involves prolonged bacterial colonization of macrophages (61). Therefore, in the current study we probed the effect of PNPase on *S. enterica* serovar Typhimurium gene expression while the bacteria were replicating in an in vitro growth medium that partially mimicked the intravacuolar environment (MM5.8) or within murine macrophage-like J774-A.1 cells.

When whole-genome-based microarray technology was applied to define different bacterial transcriptomes, growth in MM5.8 was found to result in decreased expression of SPI1 genes and in a strong increase in the SPI2 and *spv* gene expression in both the PNPase-proficient MC1 and PNPase-deficient MC71 backgrounds. Somewhat surprisingly, PNPase deficiency associated only with a fairly moderate alteration in the transcriptome when the bacteria were grown in MM5.8. The alteration recorded in the PNPase-deficient MC71 background included genes associated with stress responses, DNA repair, and, significantly, the *spv* genes (Fig. 1; see Table S1 in the supplemental material).

Yet while Northern blot analyses clearly confirmed an accumulation of *spvA* mRNA in the PNPase-deficient MC71 background grown in MM5.8, we could not demonstrate any increase in the half-life of the *spvA* mRNA in MC71 (Fig. 2A and B). In parallel, *lacZ* fusions probing the transcription of the *spvA* and *spvR* promoters also revealed higher  $\beta$ -galactosidase levels in the PNPase-deficient MC71 background (Fig. 3B and C). These observations suggest that the increased levels of *spvA* expression observed at PNPase deficiency, at least partially, could originate from an increased transcription from the *spvA* promoter. Still, the expression of *spvA-lacZ* remained dependent on growth phase and on the transcriptional regula-

tors PhoP, RpoS, and SpvR, even in the PNPase-deficient MC71 strain (Fig. 3F to H). This would be in accordance with the previously defined strict dependency of the *spvA* promoter on RpoS and SpvR (35, 84, 85). Thus, one could envision a model in which *spvA* mRNA is not directly affected by PNPase. Instead PNPase could, by affecting the amount or activity of accessory transcription factors or regulatory RNAs, control *spv* expression indirectly. However, the array data did not reveal any significant increase in the mRNAs in MC71 for the activator genes *phoP* or *rpoS* or any significant decrease in the mRNAs for H-NS or the leucine-responsive regulatory protein (see Table S1 in the supplemental material). Yet, when we compared the genes altered for their expression at shift to MM5.8, we did find parallels to the PhoPQ-regulon recently revealed for *S. enterica* serovar Typhimurium grown in magnesium-deprived M9 minimal medium (64). The transcriptomes of MC1 and MC71 grown in MM5.8 showed induction in the expression of the PhoPQ-connected *bioA*, *cysCDJN*, *mig-3*, *pagOC*, *oat*, *udg*, *ybaY*, and *ygaY* genes and a repression in the expression of the PhoPQ-connected *nrdFH*, *slyB*, and *yfbE* genes, compared to the transcriptomes expressed in CC (see Table S1 in the supplemental material). Furthermore, it is interesting to note that the promoter region of *spvA* in particular does share sequences reminiscent of the PhoP-binding site (38). It thus remains quite possible that MM5.8 provokes activation of the PhoPQ regulon, while it may not activate *phoPQ* expression in itself. Nevertheless, the growth medium applied by Monsieurs et al. (64) to provoke the PhoPQ regulon differs in many respects from MM5.8, which might explain differences in the transcriptome profiles observed.

The transition from CC into murine macrophage-like J774-A.1 cells is known to have a drastic effect on the bacterial transcriptome, and this alteration also includes induction of *spv* gene expression (17). Therefore, we used the microarray analysis to define differences in the intracellular transcriptomes displayed by the PNPase-proficient MC1 and PNPase-deficient MC71 strains while the bacteria were grown in murine macrophage-like J774-A.1 cells. The comparison of the intracellular transcriptomes of MC1 and MC71 revealed a significantly different expression of only nine genes and included *spvA*, *spvB*, and *spvC* as being up-regulated in MC71 (Fig. 5A). The expression of *spvA*, *spvB*, and *spvC* genes was reduced to equal levels in *spvR* mutant derivatives of MC1 and MC71 grown in murine macrophage-like J774-A.1 cells (Fig. 5B). Somewhat unexpectedly, introducing the *spvR* mutation into MC71 also reduced the increase in *entC* expression conferred by PNPase deficiency. Thus, it remains possible that expression of the isochorismate synthetase EntC is affected by the expression of *spv* genes. As *entC* expression was grossly unaffected by *spvR* in the PNPase-proficient MC1 strain, it is unlikely that *entC* would directly rely on SpvR for its expression. However, we cannot exclude the possibility that *entC* expression would respond to an alternative PNPase-suppressed and SpvR-dependent transcriptional activation.

Mutational inactivation of PNPase was originally reported to affect not only SPI1 and SPI2 expression, but also infection pathogenesis in BALB/c mice (11). The *pnp* mutant MC71 displayed higher bacteria doses in the spleens of the infected mice at early time points after infection; the mutant bacteria showed increased rates of replication in mice and finally were

capable of establishing persistent infections (11). As PNPase deficiency clearly caused an increase in *spv* gene expression when the bacteria were growing in MM5.8 or in murine macrophage-like J774-A.1 cells, we probed the effect of inactivating the *spvR* gene on bacterial replication *in vivo*. This was done by comparing the competitive indexes of the *pnp* mutant when competed against a PNPase-proficient strain in BALB/c mice and by competing corresponding *spvR* mutants. The results obtained (Fig. 6) clearly showed that the growth advantage conferred in mice by PNPase deficiency depended on *spv* gene expression. Thus, PNPase seems not only to suppress *spv* gene expression under conditions that induce *spv* gene expression, but also to suppress bacterial replication in BALB/c mice.

In many respects, the pattern of regulation of PNPase-dependent expression of virulence in *S. enterica* genes resembles that of the PNPase-directed cold shock response in *E. coli*; *spv* genes, as well as *csp* genes, are induced by environmental changes. Both classes of genes are needed to adapt the bacteria to new environmental demands, and the mRNA levels for all of these genes are modulated by PNPase. In *E. coli*, PNPase is not required for the induction of the cold shock response itself but for degradation of the *csp* mRNAs during adaptation to decreased growth temperature (86). A parallel scenario could be envisaged for the interplay between PNPase and virulence gene expression in *Salmonella*. According to the gene expression profile data presented here, PNPase is not necessary for the induction of *spv* virulence gene expression but may function to restrict gene expression once the genes have been induced. Nevertheless, the effect of PNPase on the expression of a given virulence gene may not necessarily reflect a PNPase-mediated degradation of the given mRNA. Instead, PNPase could affect gene expression indirectly through affecting the stability of the mRNA encoding a gene regulatory factor, the stability of regulatory RNA molecules, or the regulation of a factor otherwise needed for proper SPI expression (58). Any such scenario could well explain the increased *spvA* expression observed here for the PNPase-deficient strain MC71.

Recent studies of *Yersinia* virulence suggest yet another mechanism for PNPase-mediated regulation of virulence expression. As for *Salmonella*, the virulence of *Yersinia* relies on the expression of type III secretion (6). The effector proteins secreted by the *Yersinia* type III secretion system prevent phagocytosis and expression of the oxidative burst (6, 74). Inactivation of *Yersinia* PNPase caused a delay in the secretion of such virulence proteins to the extent that the mutants expressed decreased survival in murine macrophage-like RAW cells (74). The defect in the expression did not rely on the level of gene expression but rather occurred at the posttranslational level. In parallel, another component of the RNA degradosome, the endoribonuclease RNaseE, has also been implicated in the expression of *hilA*, a main transcriptional regulatory gene of SPI1 (19).

We propose that PNPase can fine-tune the expression of environmentally induced genes, such as cold-shock or virulence genes, and allow bacteria to successfully adapt to alternating biological niches. Such observations suggest that imported genetic virulence elements also could be subjected to posttranscriptional regulation and that their regulation has become integrated not only into the network of existing gene

regulatory proteins, but also to the activity of preexisting ribonucleases.

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