Both low temperatures and encounters with host phagocytes are two stresses that have been relatively well studied in many species of bacteria. Previous work has shown that the exoribonuclease polynucleotide phosphorylase (PNPase) is required for Yersinia to grow at low temperatures. Here, we show that PNPase also enhances the ability of Yersinia pseudotuberculosis and Yersinia pestis to withstand the killing activities of murine macrophages. PNPase is required for the optimal functioning of the Yersinia type three secretion system (TTSS), an organelle that injects effector proteins directly into host cells. Unexpectedly, the effect of PNPase on the TTSS is independent of its ribonuclease activity and instead requires its S1 RNA binding domain. In contrast, catalytically inactive enzyme does not enhance the low temperature growth effect of PNPase. Surprisingly, wild-type-like TTSS functioning was restored to the pnp mutant strain by expressing just the ~70 amino acid S1 domains from either PNPase, RNase R, RNase II, or RpsA. Our findings suggest that PNPase plays multifaceted roles in enhancing Yersinia survival in response to stressful conditions.

Bacteria can rapidly modulate their metabolism to enhance their survival during periods of environmental change. For example, the cold shock response of Escherichia coli involves the induction of several genes including cold shock proteins (CSPs) as well as exoribonucleases PNPase and RNaseR involved in RNA metabolism (1, 2). In addition to E. coli, Bacillus subtilis and Yersinia enterocolitica have been shown to require PNPase for growth in the cold (3–5). It is believed that PNPase is required for the restart of growth following cold shock by degrading the remaining CSP mRNA molecules (5, 6).

Similar to cold-induced responses, interactions with immune cells also triggers bacterial responses that serve to enhance bacterial survival. Viewed in this light, at least some virulence gene products can be thought of as host cell-induced stress response proteins. Several bacterial pathogens secrete virulence factors that mollify anti-microbial killing systems of host cells. Many plant- and animal-interacting Gram-negative bacteria, including the pathogenic Yersiniae, utilize a type three secretion system (TTSS) to inject virulence factors directly from the bacterium into the host cell (7). Yersinia outer membrane protein virulence factors (Yops) are either effector or facilitator proteins, the latter being necessary for the direct injection of the effector Yops into the host cell. YopE, an effector Yop, has GTPase-activating protein (GAP) activity, and disrupts the host cell cytoskeleton (8, 9). YopB and YopD have been shown to be required for the directed translocation of effector Yops into the host cell (10, 11). Therefore, it appears as though TTSS has evolved to directly address host cell-induced stresses.

Through microarray analysis, a Salmonella enterica pnp mutant strain was recently shown to have altered patterns of gene expression in 3.44% of all genes analyzed including TTSS and fimbriae genes (12). Differential expression of TTSS and fimbriae-encoding genes may account for the differences observed between Yersinia and pnp strains in cell culture and animal infection assays (12). Here, we examined whether Yersinia ribonucleases are involved in host cell-induced stress responses and specifically tested whether PNPase affects the functioning of Yersinia TTSS.

**Experimental Procedures**

**Bacterial Strains and Plasmids—Oligonucleotide primer sequences used for constructing strains and plasmids are provided under Supplemental Materials.** The Y. pseudotuberculosis (YPT) pnp strain was derived from the parental YPT/pB100 yopE::gfp (13) using a recombinase-based method (14). In brief, a kanamycin or a chloramphenicol resistant cassette was PCR-amplified by using forward and reverse oligonucleotide primers flanked by 30 nucleotides of complementary target gene sequence. These PCR products were transformed into YPT strains previously transformed with a plasmid encoding the lambda red recombinase. Following transformation of the PCR product and subsequent recombination, the inserted resistance cassette was then excised by virtue of a flanking FRT site. The recombinase-encoded plasmids were then cured, and deletion of the entire pnp coding sequence was confirmed by PCR and immunoblotting. The YPIIIB/pIB604 strain was used as the TTSS-deficient (yopB) control (10). All Y. pestis (YP) strains originate from the KIM5–3001 strain (15). A KIM5–3001 yopB mutant strain was generated by an allelic replacement strategy as described by Day et al. (16). The YP ribonuclease mutant strains were generated using the same method as described above, and the YP pnp strain was deleted from residues 79–578. For the Elk-tagged translocation assay (Fig. 7), the KIM5–3001.P39 and P.41 strains (PNPase+/yopB) respectively were used (16). The PNPase−/YopB strain was generated by transferring the pCD1 virulence plasmid from KIM-3001.P39 into the pnp strain described above.

 Constructs encoding either the full-length E. coli PNPase or PNPase variants were supplied by Claude Portier (17). Various S1 domains from YP proteins (Fig. 10) were cloned into Bluescript (pSK, Stratagene), which was previously converted into a Gateway-compatible destination vector (Invitrogen).
Cell Infection Assays—Viability assays (Fig. 1) were performed essentially as described by Bartra et al. (13). YPT wild-type and yopB strains were transformed with the empty control vector pCL-1920, and the YPT pnp strain was transformed with either the control vector pCL-1920 or pCL-1920 encoding E. coli PNPase ([pnp]/PNP+). Transformants were added to tissue culture wells containing RAW 264 mouse macrophages at MOIs of 0.2–0.5. Following a 30-min attachment period, excess bacteria were removed, and the number of viable cell-associated bacteria was determined by plating either immediately (0 h) or 6 h later. Three independent wells per strain were analyzed, and the average fold-increases over the 6-h infection period for each strain are shown graphically. Using the Student’s t test and calculating for unequal variance, p < 0.01 for the differences between the wild type and yopB, wild type and pnp, and pnp and pnp/PNP+. For an independent YPT viability assay showing actual numbers of cfu per well see Supplemental Materials (Fig. S1A). B, YP wild-type, yopB, and pnp strains were assayed in the same manner as the YPT strains shown in A with the exception that a 5-h infection period was used. p values <0.01 were calculated when comparing differences between wild type and yopB, and wild type and pnp. For an independent YP viability assay showing actual numbers of cfu per well see Supplemental Materials (Fig. S1B).

RESULTS

Modulating Effect of PNPase on Yersinia TTSS

The method of Plano and Straley (18) was employed for the growth restriction assay (Fig. 4). In brief, cultures were grown to saturation in defined TMH medium (19), and strains were diluted in 3 ml of TMH (either without or with 2.5 mM of Ca2+) to optical densities (620 nm) of 0.2. Diluted cultures were then grown for 1 h at 26 °C and then shifted to 37 °C where optical densities at 620 nm were read every hour for a 5-h period.

For flow cytometric analysis of yopE promoter activity (Fig. 5), cultures were grown to saturation in 2 ml of TMH containing 2.5 mM Ca2+ and 5.0 mM MgCl2 at 37 °C. Cultures were diluted to an A620 of 0.2 in 5 ml of TMH that was prewarmed to 37 °C and contained 2.5 mM Ca2+ and 5.0 mM MgCl2. After 1 h of shaking at 37 °C, the TTSS was induced by adding 5.0 mM EGTA, a calcium chelator. At various times, 300-μl samples were removed and placed in prechilled tubes and kept on ice. Bacterial cells were then washed once in Hank's Buffered Saline Solution (HBSS, Invitrogen Life Technologies, Inc.) and resuspended in 300 μl of HBSS. Flow analysis was then carried out using a Facscan 488 with an argon laser (BD Biosciences), and mean fluorescent intensities (MFIs) were analyzed using the Cellquest Pro software (BD Biosciences).

In the secretion assay shown in Figs. 6 and 11, cultures were grown exactly the same way as for the above flow cytometric analysis except that some cultures did not receive EGTA. At the indicated time points, 1-ml samples were removed and placed in prechilled tubes and were kept on ice. Cells and supernatants were fractionated by centrifugation at 12,000 × g for 5 min. 500 μl of the resulting supernatant were removed and precipitated with 55 μl of trichloroacetic acid overnight on ice after which samples were centrifuged 12,000 × g for 10 min at 4 °C. The resulting pellets were resolved by SDS-PAGE and analyzed by immunoblotting with a rabbit polyclonal mix of anti-YopE and anti-YopD antibodies (Fig. 6) or anti-YopE alone (Fig. 11). Cell pellets were resuspended directly in sample buffer and were analyzed together with the secreted proteins.

Cold Growth Assays—For the experiment shown in Fig. 9, strains were grown to saturation at 26 °C in HIB with the appropriate antibiotics, diluted 10−1, and grown for 1 h at 26 °C. Cultures were then diluted 10−3, and 50 μl were plated on LB plates containing antibiotics, if necessary, and were incubated at 4–6 °C for 10 days.

Viability of PNPase- and Various Ribonuclease-deficient Strains in an Infection Assay—The viability of a YPT PNPase-deficient mutant strain was tested in a cell infection assay.
Using a mouse macrophage-like cell line. Previously, it had been demonstrated that this assay is sensitive to well characterized virulence determinants of YPT (13). There was a 14.8-fold increase in cfu of the wild-type YPT strain between the start of the infection and the 6-h end point (Fig. 1A). In contrast, there was a decrease in the number of cfu of the YPT TTSS-deficient yopB strain during the 6-h infection period. The cfu fold-increase of the YPT and YP strains in the above assays, we further analyzed TTSS functioning in the YP and YPT strains. Normally, Yersinia possessing an intact TTSS arrest their growth when shifted from their optimal growing temperature of 26–37 °C in medium lacking calcium; this is referred to as “growth restriction” (21). Shown in Fig. 4, similar to the wild-type strain, the YP pnp strain ceased growing when shifted to 37 °C in medium lacking calcium in contrast to YP strains with disregulated TTSSs that either constitutively secrete Yops in both the absence and presence of calcium termed calcium blind (22) or constitutively grow in either the presence or absence of calcium, calcium-independent. These data indicate that PNPase is not required for TTSS-dependent growth restriction.

We then tested whether PNPase plays a role in Yop virulence factor expression and export by the TTSS. We measured Yop secretion in YPT since YP possesses a unique extracellular protease, Pla that rapidly degrades secreted Yops (23). An additional feature of the YPT strains used in Fig. 1 is that they contain a yopE:gfp transcriptional fusion gene that allows for yopE promoter activity measurements without affecting the expression or function of YopE (13). Strains were grown at 37 °C in the presence of calcium, and the expression and secretion of Yops were induced by adding a calcium chelator to the medium. For evaluating yopE promoter activity in the wild-type and pnp YPT strains, samples were removed from the
protein, and no detectable YopD protein were observed in the cultural supernatant from the \textit{pnp} strain prior to induction (lanes 3 and 4). In fact, YopE and YopD protein were not detectable in the \textit{pnp} culture supernatants until either 5 or 20 min, respectively (lanes 7 and 11). Although YopE protein was detected 5 min after induction, the levels were severalfold lower than that present in the culture supernatant from the wild-type strain (compare lanes 5 with 7 and 9 with 11). However, by 60 and 120 min following induction, there were little appreciable differences in YopE and YopD protein levels between the wild-type and \textit{pnp} culture supernatants (data not shown). These data suggest that PNPase is involved in configuring \textit{Yersinia} TTSS in such a way that maximal Yop effector proteins are exported from the bacterium upon induction.

A translocation assay was employed to test whether the reduced initial Yop secretion rates in the \textit{pnp} strain translated into reduced Yop injection into host cells. A plasmid encoding a hybrid protein consisting of YopE (residues 1–130) and a 40-residue Elk tag was transformed into the YP wild-type, \textit{ypB}, and \textit{pnp} strains. The relative level of YopE-Elk translocation into HeLa cells can be determined by probing for Elk phosphorylation that only occurs within the host cell (16). Phosphorylated YopE-Elk was readily detected in lysates prepared from cells infected with the wild-type strain after a 3-h infection period (Fig. 7A, lanes 1 and 2). As described previously by Day et al. (16), phosphorylated YopE-Elk was not detected in lysates prepared from cells infected with the TTSS-deficient \textit{ypB} mutant strain (lanes 3 and 4). In lysates prepared from cells infected with the \textit{pnp} strain, phosphorylated YopE-Elk was detected after a three hour infection period but at levels greatly reduced compared with levels in lysates from wild-type-infected cells (lanes 5 and 6; signal quantification shown in Fig. 7B). These data are consistent with what was observed in the secretion and infection assays and further supports a model in which PNPase is required for the optimal functioning of \textit{Yersinia} TTSS following host cell contact.

**PNPase Determinants Required for Affecting TTSS Functioning**—The exoribonuclease PNPase is comprised of several recognizable domains including 2 distinct RNA binding domains as well as 2 catalytic centers (17, 24). To determine whether any of these specific domains are required for PNPase effect on TTSS functioning, a panel of plasmids encoding \textit{E. coli} PNPase variants (17) were transformed into the \textit{Yersinia} \textit{pnp} strains. PNPase variants tested included a PNPase\text{R100D} variant that is deficient in all of the enzymatic activities associated with PNPase, as well as a PNPase\text{deltaKH} and PNPase\text{deltaS1} variants that have internal deletions in the KH and S1 RNA binding domains, respectively. By immunoblotting, it was found that all of the PNPase variants were stably expressed in YP (Fig. 8, \textit{panel A, inset}). In fact, PNPase\text{R100D}, PNPase\text{deltaKH}, and PNPase\text{deltaS1} were expressed at notably higher levels than the wild-type PNPase; this observation was consistent with the pre-

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Fig. 3. Quantification of \textit{Yersinia}-induced HeLa cell cytotoxicity. A, cytotoxicity of the YPT-infected HeLa cells shown in Fig. 2A were quantified using a scoring system in which all cells from two fields were evaluated (200 cell minimum) based upon the severity of the morphological alteration of the infected HeLa cell. Definition of the scoring system is provided in Supplemental Materials (Fig. S2). B, cytotoxicity of the YP-infected HeLa cells shown in Fig. 2B were quantified as described above and in Supplemental Materials.

Fig. 4. YP calcium-dependent growth restriction. Cultures were grown at 37 °C either in the presence or absence of calcium represented by solid and dashed lines, respectively, and optical density readings of the wild-type (A), \textit{pnp} (B), \textit{ypB} (C), and \textit{ypB} \textit{ypN} \textit{ypP} \textit{P}_{\text{F245A}} (D) strains were recorded every hour for 5 h. The latter two strains are referred to as either calcium blind or calcium independent, respectively. Absolute \textit{A}_{620} values are provided in Supplemental Materials.

medium at various times shortly after induction and analyzed by flow cytometry. As previously reported (13), the wild-type strain had a detectable increase in yop\textit{E} promoter-driven GFP signal within 5 min following induction that steadily increased to 5.5-fold compared with initial levels by 60 min (Fig. 5). In the \textit{pnp} strain, initial GFP levels were comparable to those observed in the wild-type strain, and following TTSS induction, there was a similar increase in GFP signal (Fig. 5). As measured by immunoblotting, it was found that all of the PNPase variants were stably expressed in YP (Fig. 8, \textit{panel A, inset}). In fact, PNPase\text{R100D}, PNPasedeltaKH, and PNPasedeltaS1 variants that have internal deletions in the KH and S1 RNA binding domains, respectively. By immunoblotting, it was found that all of the PNPase variants were stably expressed in YP (Fig. 8, \textit{panel A, inset}). In fact, PNPase\text{R100D}, PNPase\text{deltaKH}, and PNPase\text{deltaS1} were expressed at notably higher levels than the wild-type PNPase; this observation was consistent with the pre-
was added. In these strains, containing calcium, at which time EGTA mid-log phase at 37 °C in defined medium dashed lines, respectively) were grown to infected with the YP and YPT cytotoxicity-based infection assays. Surprisingly, HeLa cells analytically inactive PNPaseR100D displayed levels of cytotoxicity shown in mean fluorescent intensities of data points following the addition of EGTA. By flow cytometry at the indicated time moter, and GFP levels were determined shown in A.

levels of cytotoxicity were observed in cells infected with the pnp strain expressing the PNPasedeltaS1 variant. In fact, the level of cytotoxicity observed in cells infected with PNPasedel- taS1-expressing YP was essentially equivalent to the levels observed in cells infected with the YP pnp strain (Fig. 8). Collectively, these data indicate that the enzymatic activity and the KH RNA binding domain are not required for PNPase affect on the TTSS. However, our results suggest that PNPase S1 domain is required for TTSS functioning.

PNPase Determinants Required for Low Temperature Growth—We were surprised by our finding that the PNPase effect on Yersinia TTSS was independent of PNPase ribonuclease activity. This observation prompted us to test whether the phenomenon of PNPase enhancing growth at low temperatures was also independent of its ribonuclease activity, an issue that to the best of our knowledge has not been addressed. Similar to what has been previously reported for E. coli, B. subtilis, and Y. enterocolitica (3–5), PNPase is clearly required for YPT and YP to grow at low temperatures (Fig. 9a; data for YPT not shown). The YPT strains expressing the various PNPases described above were also tested for their ability to grow at low temperature. Plasmid-encoded PNPase of E. coli was able to enhance the ability of the YPT pnp strain to grow at low temperatures (Fig. 9b). In stark contrast, the pnp strain expressing the catalytically inactive PNPasedeltaR110D remained clearly defective for growth at low temperature. The PNPasedeltaS1- and PNPasedeltaKH-expressing strains were also compromised in their ability to grow at low temperature although not to the same degree as the PNPasedeltaR110D-expressing strain (Fig. 9b). These data indicate that a catalytically active PNPase is required for Yersinia to grow at low temperature.

The S1 Domain and TTSS Functioning—To address whether the S1 domain alone was sufficient to complement the pnp strain in regard to TTSS functioning, the sequence encoding the YP 69-amino acid S1 domain from PNPase was cloned into an expression vector and transformed into the YP strain expressing the catalytically inactive PNPasedeltaR110D-expressing strain. Additionally, in the viability assay, the PNPasedeltaR110D-expressing strain was as viable as the pnp strain expressing wild-type PNPase (data not shown). Like the PNPasedeltaR110D-expressing strain, the YP pnp strain expressing the PNPasedeltaKH variant caused similar levels of cytotoxicity in HeLa cells as the levels observed in cells infected with YP-expressing wild-type PNPase (Fig. 8).

In contrast to the catalytically inactive and KH-deleted PNPases being fully active in our infection assay, reduced

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FIG. 5. yopE promoter activity in the wild-type and pnp YPT strains. A, the wild-type and pnp strains (solid and dashed lines, respectively) were grown to mid-log phase at 37 °C in defined medium containing calcium, at which time EGTA was added. In these strains, gfp expression is under the control of the yopE promoter, and GFP levels were determined by flow cytometry at the indicated time points following the addition of EGTA. B, mean fluorescent intensities of data shown in A.

FIG. 6. YopE and YopD expression in the wild-type and pnp YPT strains. The strains used in Fig. 5 were either grown and induced as described in Fig. 5 (by adding EGTA) or left uninduced. Samples were removed from the cultures at the indicated time points, and YopE and YopD levels from both whole cell and supernatant fractions were determined by immunoblotting. The 0 min time point reflects sample taken as quickly as possible after the addition of EGTA resulting in an induction period of ~20 s.
PNPase\textsubscript{R100D} variant (Supplemental Materials, Fig. S5). To similarly test S1 domains from other proteins, we cloned S1 domains from YP proteins ribosomal S1, RNase II, and RNase R. These various S1 domains had similar effects on YP TTSS as the S1 domain from PNPase (Fig. 10). As might be expected, expression of S1 domains did not restore low temperature growth to the YPT\textsubscript{pnp} strain in an assay similar to the one shown in Fig. 8 (data not shown). The data suggest that these various S1 domains share some structural attribute that is sufficient to enhance the activity of \textit{Yersinia} TTSS.

To determine whether S1 domains affected the yersinial TTSS, the YPT\textsubscript{pnp} strain was transformed with Bluescript vectors containing the cloned S1 domains from either PNPase or RNaseR (see Fig. 10). We used the YPT strains here for the same reason as in the experiment shown in Fig. 6. Samples were collected in the same manner as in Fig. 6 in that they were removed from the cultures as quickly as possible after the addition of the TTSS inducer EGTA. YopE levels in the whole cell pellets of all strains were similar (Fig. 11). As shown previously in Fig. 6, YopE levels in the supernatant fraction of
FIG. 10. S1-mediated cytotoxicity of YP-infected HeLa cells. A, cells were infected with either the wild-type or pnp YP strains at a MOI of 100 (panels A and B, respectively). Shown in C–F are cells similarly infected with the pnp strain transformed with plasmids encoding the ~70-amino acid S1 domains from the following proteins: C, PNPase; D, ribosomal protein S1; E, RNase II; and F, RNase R. The above photographs were taken after 2 h of infection at 37 °C. Cytotoxicity of the corresponding panels shown in A were quantified and described in Supplemental Materials (Fig. S4).

FIG. 11. S1-mediated effects on YPT YopE expression and secretion. The wild-type and pnp strains used in Fig. 5 were transformed with either an empty Bluescript vector (lanes 1–4) or a Bluescript vector expressing either the PNPase S1 domain or RNaseR S1 domain (S1PNPase or S1RNaseR, respectively). All strains were grown and induced as described in the legend to Fig. 4 or left uninduced. Samples were removed as quickly as possible after the addition of the TTSS inducer EGTA (~20-s time point), and YopE levels from both whole cell and supernatant fractions were determined by immunoblotting.

The wild-type strain was severalfold greater than the YopE levels observed in the supernatant fraction from the pnp strain (compare lanes 1 and 3). Strikingly, the expression of just PNPase S1 domain restored wild-type-like YopE secretion to the pnp strain (lanes 5 and 6). An even more pronounced effect on YopE secretion was observed in the pnp strain expressing the RNaseR S1 domain (lanes 7 and 8). These data, together with the data shown in Fig. 8, indicate that overexpressed S1 domains are sufficient to enhance Yop secretion and translocation by the pathogenic Yersinia.

DISCUSSION

The yersinial TTSS is charged with rapidly delivering Yop effector proteins into the host cell where they modulate cellular processes that normally serve to limit bacterial viability. In order to outpace the microbial killing responses of the host cell, the Yersinia possess both a preformed pool of Yop effectors for immediate injection, as well as the ability to quickly synthesize Yops following host cell attachment (26). Yersinia survival vis-à-vis the host cell is dependent on the proper functioning of both of these levels of regulation.

We present evidence here that the exoribonuclease PNPase is required for the proper functioning of Yersinia TTSS. Functional studies revealed that pnp mutant strains had reduced viability in infection assays that was likely due to a malfunctioning Yop delivering system. Despite the pnp strain having similar Yop steady-state expression levels as the wild-type strain, we found that the pnp strain was defective in rapidly exporting Yop effector proteins upon both TTSS induction in culture as well as injecting Yops into host cells. Thus, it appears that PNPase is somehow required for the Yersiniae to rapidly deliver preformed Yops into the host cell. This defect could conceivably be caused by either fewer TTSS complexes present in the bacterial membrane or to qualitative alterations resulting in a suboptimally functioning TTSS. Whatever the defect, the TTSS in the pnp strain appears to eventually function normally following its induction. For an individual bacterium, however, by that time the host cell has had ample opportunities to initiate and complete its microbial killing program.

Ribonuclease have been shown to be involved in bacterial virulence. In a transposon mutagenesis screen, a Shigella flexneri was isolated that displayed reduced host cell invasion. The disruption was mapped to a locus that was later identified as the gene (vacB/rnr) encoding RNaseR (27, 28). More pertinent to this study and as mentioned in the introduction, a pnp mutant of Salmonella enterica was found to have a disregulated TTSS (12). In contrast to the findings presented here though, Clements et al. (12) found that a lack of a fully functional PNPase in Salmonella correlated to increased TTSS activity. In the Salmonellae, TTSS activity mediates host cell invasion and intracellular proliferation whereas in the Yersinia, TTSS activity results in the diametrically opposite effect of blocking the host cell from internalizing surface-bound bacteria (7). It is unclear why or how PNPase exerts opposite effects on the Yersinia and Salmonellae TTSSs. It is interesting to note though, that for both genera, disrupting the pnp locus results in an apparent increase in the levels of bacterial internalization. In the case of the Yersinia, however, this does not bode well for its viability (29).

How does PNPase affect the functioning of Yersinia TTSS? One possible mechanism could be that the pnp cells suffer from a general metabolic sickness. Previously, it has been shown that an E. coli pnp strain had slightly slower doubling times compared with the isogenic wild-type strain (30). Similarly, when grown in some media, we observed a slightly slower growth rate of the YP pnp strain when the strains were tested...
at *Yersinia* optimal growth temperature (data not shown). However, when the YP *pnp* strain was grown in defined TMH medium, no differences were observed between its growth rate and that of the wild-type strain (Fig. 4). A slightly slower growth rate could, in theory, contribute to the reduced viability of the *pnp* strains shown in Fig. 1. This explanation, though, we find unlikely since the PNPase<sub>R100D</sub>-expressing *yp* cells, like PNPase-deficient cells, displayed a slightly reduced growth rate when grown in the same conditions. Despite this defect, however, the PNPase<sub>R100D</sub>-expressing strain performed as well as, if not better than, the wild-type strain both in the viability assay (data not shown) as well as the cytotoxicity assay (Fig. 8 and Supplemental Materials, Fig. S5). Additionally, Clements et al. (12) found that the growth rates of the wild-type and *pnp* strains of *Salmonella* were identical when tested at 37°C. Therefore, although full-length, enzymatically active PNPase is clearly required for optimal growth of *Yersinia* at low temperature (see below), at higher temperatures it appears to be dispensable for maximal growth in most laboratory conditions.

At low temperatures, or in cells recovering from a cold shock, PNPase plays a central role in reprogramming cellular metabolism (4, 5, 31). Following a cold shock, restart of growth in both *E. coli* and *Y. enterocolitica* is preceded by PNPase-mediated degradation of transcripts encoding so-called CSPs (5, 31). It is believed that PNPase might act to free ribosomes that are inactivated by bound CSP-encoding transcripts. In fact, our data support this model in that PNPase catalytic activity was found to be essential for YPT cold growth ability (Fig. 9B). CSPs, which consist of a 5-stranded anti-parallel β-barrel (32), share structural similarity to the S1 domains found in PNPase and the ribosomal protein S1 and have been shown to melt out mRNA secondary structures that form at lower temperatures (33). Xia and Inouye (34) reported that a quadruple deletion of 4 of the 9 CSPs rendered *E. coli* cold-sensitive, and that overexpressing any 8 of the 9 CSPs or the S1 domain from *E. coli* PNPase alone enabled the quadruple-deleted strain to grow in the cold. Even thus, several proteins with *bona fide* S1 domains as well as proteins that resemble S1 domains (e.g. CSPs) are involved in low temperature stress responses in Gram-negative bacteria.

In this report, we show that PNPase S1 domain is required for optimal growth of YPT at low temperature (Fig. 9). PNPase KH RNA-binding domain, and to a greater extent, its catalytic for optimal growth of YPT at low temperature (Fig. 9). PNPase affects least when expressed at relatively high levels, that positively suggests that there is a general feature of these peptides, at contrast, expression of the S1 domain by itself did not restore low growth rate could, in theory, contribute to the reduced viability of the *pnp* strain (data not shown) indicating further the necessity of PNPase catalytic activity during this condition. The fact that S1 domains from other proteins could likewise complement the *pnp* strain (Fig. 10) suggests that there is a general feature of these peptides, at least when expressed at relatively high levels, that positively affects *Yersinia* TTSS. This latter finding is puzzling given the fact that although the S1 domains from various proteins are structurally similar, they differ substantially in their surface residue composition that is thought be the basis for their specificity for their respective cognate RNAs (32). Another issue that is unclear is why other S1 domains, as they naturally occur in their native contexts in a variety of other proteins (e.g. RNase R, RNase II, etc.), do not functionally complement the *pnp* strain in our infection assays. Is there a unique feature of PNPase S1 domain in the context of the entire PNPase, or are these other S1 domains not present at sufficient levels? Our S1-related data may offer an as of yet undiscovered aspect of the process by which the *Yersinia* enhance their viability by quickly injecting virulence factors into the host cell.

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REFERENCES

1. Jones, P. G., VanBogelen, R. A., and Neidhardt, F. C. (1987) *J. Bacteriol.* 169, 2092–2095
2. Cairao, F., Cruz, A., Mori, H., and Arraiano, C. M. (2003) *Mol. Microbiol.* 50, 1349–1360
3. Luttinger, A., Hahn, J., and Dubnau, D. (1996) *Mol. Microbiol.* 19, 343–356
4. Gouvereau, R. L., Huis in't Veld, J. H., Kusters, J. G., and Moi, F. M. (1998) *Mol. Microbiol.* 28, 555–569
5. Yamanaka, K., and Inouye, M. (2001) *J. Bacteriol.* 183, 2808–2816
6. Neuhauer, K., Rapposch, S., Francis, K. P., and Scherer, S. (2000) *J. Bacteriol.* 182, 3285–3298
7. Galan, J. E., and Collmer, A. (1999) *Science* 284, 1322–1328
8. Black, D. S., and Bliska, J. B. (2000) *Mol. Microbiol.* 37, 515–527
9. Von Pawel-Rammingen, U., Telepnev, M. V., Schmidt, G., Aktories, K., Wolf-Watz, H., and Rosqvist, R. (2000) *Mol. Microbiol.* 36, 737–748
10. Hakansson, S., Schesser, K., Persson, C., Galyon, K. E., Rosqvist, R., Homble, H., and Wolf-Watz, H. (1996) *EMBO J.* 15, 5812–5823
11. Williams, A. W., and Straley, S. C. (1998) *J. Bacteriol.* 180, 350–358
12. Clements, M. O., Eriksson, S., Thompson, A., Lucchini, S., Hinton, J. C., Nornmark, S., and Bihm, M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 8784–8789
13. Bartra, S., Cherepanov, P., Forsberg, A., and Schesser, K. (2001) *BMC Microbiol.* 1, 22–33
14. Otsuka, K. A., and Wanner, B. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 6640–6645
15. Landler, L. E., Klempner, M. S., and Straley, S. C. (1990) *Infect. Immun.* 58, 2569–2577
16. Day, J. B., Ferracci, F., and Plano, G. V. (2003) *Mol. Microbiol.* 47, 807–823
17. Jarrige, A., Brechhemier-Bacy, D., Mathy, N., Duchê, O., and Portier, C. (2002) *J. Mol. Biol.* 321, 397–409
18. Plano, G. V., and Straley, S. C. (1993) *J. Bacteriol.* 175, 3536–3545
19. Straley, S. C., and Bowmer, W. S. (1986) *Infect. Immun.* 51, 445–454
20. Rosqvist, R., Forsberg, A., Rimplainen, M., Bergman, T., and Wolf-Watz, H. (1990) *Mol. Microbiol.* 4, 657–667
21. Hueck, C. J. (1998) *Microbiol. Mol. Biol. Rev.* 62, 379–433
22. Day, J. B., and Plano, G. V. (1998) *Mol. Microbiol.* 30, 777–788
23. Sedeinbe, O. A., Sample, A. K., Brushaker, B. R., and Gogeun, J. D. (1988) *Infect. Immun.* 56, 2749–2752
24. Symmons, M. F., Jones, G. H., and Luís, B. F. (2000) *Struct. Fold Des.* 5, 1215–1226
25. Garcia-Mena, J., Das, A., Sanchez-Trujillo, A., Portier, C., and Montanez, C. (1999) *Mol. Microbiol.* 33, 235–248
26. Pettersson, J., Nordfeldt, R., Dubina, E., Bergman, T., Gustafsson, M., Magnusson, K. R., and Wolf-Watz, H. (1996) *Science* 275, 1231–1233
27. Toke, T., Sasakawa, C., Okada, N., Honma, Y., and Yoshikawa, M. (1992) *J. Bacteriol.* 174, 6359–6367
28. Cheng, Z. F., Zuo, Y., Li, X., Rudel, K. E., and Deutscher, M. P. (1998) *J. Biol. Chem.* 273, 14077–14080
29. Mills, S. D., and Finlay, B. B. (1998) *Eur. J. Cell Biol.* 77, 35–47
30. Cheng, Z. F., and Deutscher, M. P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 6388–6393
31. Polissi, A., De Laurentis, W., Zangrossi, S., Briani, F., Longhi, V., Pesole, G., and Deho, G. (2003) *Res. Microbiol.* 154, 573–580
32. Brycich, M., Hubbard, T. J., Proctor, M., Freund, S. M., and Murzin, A. G. (1997) *Cell* 88, 235–242
33. Jiang, W., Hou, Y., and Inouye, M. (1997) *J. Biol. Chem.* 272, 196–202
34. Xia, B., Ke, H., and Inouye, M. (2004) *Mol. Microbiol.* 40, 179–188
Modulation of *Yersinia* Type Three Secretion System by the S1 Domain of Polynucleotide Phosphorylase

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