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The *Yersinia pseudotuberculosis* Degradosome is Required for Oxidative Stress, While its PNPase Subunit Plays a Degradosome-Independent Role in Cold Growth

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Abstract

Yersinia polynucleotide phosphorylase (PNPase), a 3'–5' exoribonuclease, has been shown to affect growth during several stress responses. In *E. coli*, PNPase is one of the subunits of a multi-protein complex known as the degradosome, but also has degradosome-independent functions. The carboxy–terminus of *E. coli* ribonuclease E (RNase E) serves as the scaffold upon which PNPase, enolase (a glycolytic enzyme), and RhlB helicase all have been shown to bind. In the yersiniae, only PNPase has thus far been shown to physically interact with RNase E. We show by bacterial two-hybrid and co-immunoprecipitation assays that RhlB and enolase also interact with RNase E. Interestingly, although PNPase is required for normal growth at cold temperatures, assembly of the yersiniae degradosome was not required. However, degradosome assembly was required for growth in the presence of reactive oxygen species. These data suggest that while the *Y. pseudotuberculosis* PNPase plays a role in the oxidative stress response through a degradosome-dependent mechanism, PNPase's role during cold stress is degradosome-independent.

Keywords

bacterial stress response; RNase E; cold shock

Introduction

Like other closely related Gram-negative enteric pathogens, *Y. pseudotuberculosis* employs a type III secretion system (T3SS) to infect host cells, and polynucleotide phosphorylase (PNPase), a phosphorolytic 3'–5' exo-ribonuclease involved in RNA decay, is required for its optimal functioning (Rosenzweig et al 2005; Rosenzweig et al. 2007). Furthermore, we (and others) have observed that PNPase is required for the cold-shock response and/or acclimation for a number of organisms including: *Yersinia pestis* and *Y. pseudotuberculosis*

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(Rosenzweig et al. 2005; Rosenzweig et al. 2007), *Escherichia coli* (Jones et al. 1987; Polissi et al. 203; Mathy et al. 2001), and *Yersinia enterocolitica* (Goverde et al. 1998). Intriguingly, PNPase has been shown to physically interact with an essential endoribonuclease, RNase E, in both *E. coli* (Carpousis et al. 1994; Vanzo et al. 1998; Khemici and Carpousis 2004) and *Y. pseudotuberculosis* (Yang et al. 2008) forming a large multi-protein RNA surveillance/quality control complex termed the degradosome. However, the role of the degradosome in various yersiniae stress responses has not been well studied.

RNase E, PNPase, RhlB RNA helicase and enolase have all been identified as components of the *E. coli* degradosome (Khemici and Carpousis 2004; Rosenzweig et al. 2010). PNPase, enolase, and RhlB each bind to the carboxy terminal domain (CTD) of RNase E that serves as the scaffold. Interestingly, the CTD of RNase E is not well conserved and varies widely in various bacterial species (Erce *et al*. 2010). Typically a degradosome consists of both an exo- and endoribonuclease (e.g. PNPase and RNase E), and they are thought to work together in concert producing a synergistic effect that optimizes RNA decay of unwanted transcripts (Rosenzweig et al. 2010). However, a degradosome consisting of both RNase R, a cold-inducible exoribonuclease in *E. coli* (Cairrao et al. 2003; Chen and Deutscher 2010) required for the maturation of SsrA/tmRNA (Cairrao et al. 2003;), and RNase E has also been identified in the psychrotrophic *Pseudomonas syringae*, possibly suggesting the existence of a specialized cold adapted degradosome (Purusharth et al. 2005). What remains uncertain within the field of RNA biology is the exact contribution the degradosome plays in RNA decay/maturation relative to its individual components. In fact, an inability of *E. coli* to assemble a degradosome resulted in affecting only some RNA decay outcomes and had only a modest impact on growth kinetics (Kido et al. 1996; Jiang et al. 2000). Perhaps the degradosome specifically degrades subsets of transcripts following periods of induced gene expression (e.g. stress response) while other stress-induced transcripts are degraded by degradosome-independent mechanisms. In support of this, an *E. coli* PNPase-deficient mutant was found to be more sensitive to oxidative stress in the form of H_2O_2, and this PNPase requirement for tolerating oxidative stress was independent of degradosome association (Wu et al. 2009). However, a dominant-negative, carboxy-truncated RNase E variant in *E. coli* (unable to form a degradosome) resulted in poor autoregulation of the *rne* transcript, suggesting that the degradosome might be required for degradation of specific transcripts in *E. coli* (Briegel et al. 2006). When a similar carboxy-truncated RNase E variant was expressed in *Y. pseudotuberculosis*, increased sensitivity to host cell induced stress (HCIS), prompted by macrophage challenge ensued (Yang et al. 2008).

In addition to degradosome constituents' physical interactions being demonstrated by co-immunoprecipitation (Co-IP) (Coburn et al. 1999; Yang et al. 2008), several bacterial 2 hybrid, B2H, (Karimova et al. 1998) assay studies have supported earlier Co-IP findings. More specifically, the B2H demonstrated an interaction between *E. coli*-derived PNPase and RhlB helicase (Liou et al. 2002). Additionally, the B2H assay demonstrated interactions between full-length PNPase, enolase, RhlB, and RNase E carboxy- terminal domains (CTD) as well as interactions between micro-domains of RNase E's CTD and the aforementioned full-length binding partners derived from *Vibrio angustum* S14 (Erce et al. 2009; Erce et al. 2010). Therefore, we sought to characterize the *Y. pseudotuberculosis* degradosome further

since only PNPase has been shown to physically interact with RNase E (Yang et al. 2008) and to determine whether it is required for various abiotic stress responses.

Results

The RhlB RNA helicase and enolase are subunits of the Yersinia degradosome

In an attempt to further identify *Y. pseudotuberculosis* degradosome constituents, we employed the B2H assay (Karimova et al. 1998) to determine whether RhlB and enolase also associate with the RNase E CTD. In this B2H assay, interaction between two proteins results in transcription of the Lac operon, and thus blue color on plates containing X-gal. Our data indicated that the RNase E CTD interacted very strongly with full-length RhlB helicase as evidenced by intensely blue colonies (Fig. 1 panel C). In fact, the intensity of blue mirrored that of the positive control Zip-Zip (compare panel C to B). Blue colonies also appeared when PNPase interacted with RNase E CTD (panel D); however, the overall intensity of blue was less than that of an RhlB-RNase E CTD interaction (compare panel D to B). Little interaction occurred between enolase and the RNase E CTD, as evidenced by weekly blue colonies (panel E). All experimental colonies observed appeared bluer than the empty vector negative control, $pKT25_{RNE\text{-}CTD}$ vs. $pUT18C_{empty\ vector}$ (compare all panels to panel A).

In addition to evaluating degradosome interaction of *Y. pseudotuberculosis* proteins, we also evaluated degradosome interaction of closely related *Y. enterocolitica* proteins and tested whether the *Y. enterocolitica* RNase E CTD interacted with both the *Y. pseudotuberculosis* and *Y. enterocolitica* RhlB degradosome-associated proteins. We chose looking at RhlB since it was the strongest binding partner for the *Y. pseudotuberculosis* RNase E CTD tested earlier (Fig. 1). Interestingly, the *Y. enterocolitica* RNase E CTD appeared to bind as well to the *Y. enterocolitica* RhlB protein as it did to the *Y. pseudotuberculosis* RhlB protein (Fig. 2). As was observed earlier with the *Y. pseudotuberculosis* RNase E CTD vs. *Y. pseudotuberculosis* enolase (Fig. 1), the *Y. enterocolitica*-derived RNase E CTD also interacted poorly with the *Y. pseudotuberculosis* derived enolase (Fig. 2). The positive control Zip-Zip appeared blue (as expected) while the two empty vector negative controls were white (as expected), $pKT25_{RNE}$ vs. $pUT18C_{empty}$ and $pKT25_{empty}$ vs. $pUT18C_{RhlB}$ (Fig. 2).

To validate our B2H findings (Fig. 1 and 2), co-immunoprecipitation (Co-IP) assays, utilizing polyclonal anti-RNase E antibodies fused to Protein G agarose beads, were employed. Immunoprecipitated complexes were resolved by SDS-PAGE and probed with polyclonal anti-RhlB or anti-PNPase antibodies. In agreement with our B2H results, RhlB clearly co-immunoprecipitated with RNase E (Fig. 3). PNPase also appeared to co-immunoprecipitate with RNase E (Fig. 3) as was demonstrated in earlier work (Yang et al. 2008). These B2H and co-IP experiments indicate that the RhlB and enolase are conserved subunits of the degradosome in *Yersiniae*.

Degradosome involvement in Y. pseudotuberculosis stress responses

The degradosome and PNPase have previously been implicated in various stress responses, including macrophage induced stress, and cold stress (see discussion). To more completely

understand their role during stress, we exposed a Δ*pnp* mutant and a strain over-expressing an *rne* truncation to a variety of stresses. This *rne* truncation removed the CTD responsible for interaction with the other degradosome subunits, and its overexpression has previously been shown to interfere with degradosome assembly (Briegel et al. 2006; Yang et al. 2008).

Since the ability of *Y. pseudotuberculosis* to respond to HCIS was previously shown to be dependent upon PNPase (Rosenzweig et al. 2005; Rosenzweig et al. 2007) as well as upon degradosome assembly (Yang et al. 2008), we were curious as to whether degradosome assembly was required for growth under oxidative stress which would be experienced during macrophage encounters. To test this directly, H_2O_2 liquid- and plate-based experiments were carried out. For plate-based assays, 0, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 4, 5, 10, 20, 50, and 100mM H_2O_2 plate concentrations were all evaluated. The Δ*pnp* mutant formed smaller colonies on plates, which was exacerbated by 0.1–0.4mM H2O2 (Fig 4). In a manner similar to how *E. coli* did not require degradosome assembly during oxidative stress (Wu et al. 2009), interfering with degradosome assembly did not affect growth on H_2O_2-containing plates (Fig. 4B).

To better quantify H_2O_2 sensitivity, a liquid-based H_2O_2 stress test was carried out in which subcultures were optically monitored for growth following the addition of either 0mM, 20mM, 50mM, or 100mM H_2O_2 (Fig. 5). Remarkably, the more sensitive liquid-based assay revealed two significant effects. First, as indicated by the change in the slope of the graphs in figure 6, the Δ*pnp* mutant had a longer doubling time in H_2O_2 containing media, but not in control media. In addition, interfering with degradosome assembly caused a reduced culture density as cultures entered stationary phase. Both of these differences were statistically significant. For reasons not well understood, interfering with degradosome assembly in the Δ*pnp* mutant mirrored the phenotype of the Δ*pnp* mutant strain and suppressed the early stationary phenotype when only degradosome assembly was disrupted (Fig. 5).

We also tested growth of these same strains at 4°C (Fig 6). Not surprisingly, and in agreement with previously published data (Rosenzweig et al. 2005; Rosenzweig et al. 2007), the Δ*pnp* mutant was unable to grow at 4°C (Fig. 6B) despite relatively normal growth at 28°C (Fig.6A). When $RNE_{1–465}$ was expressed, there was no effect on the cold-sensitive phenotype (Fig. 6). These data strongly suggest that the psychrotropic yersiniae's ability to grow in the cold depends on PNPase in a degradosome-independent manner. To further evaluate the role that degradosome assembly might be playing in yersiniae stress responses, we challenged the strains with several antibiotics that target protein translation, membrane integrity, and cell wall integrity and found that neither the presence of PNPase nor the ability of the yersiniae degradosome to assemble altered antibiotic susceptibility profiles (data not shown).

Since we observed that over-expression of $RNE_{1–465}$ led to a significant reduction in biomass during oxidative stress, but that there was no similar reduction in biomass when expressed in the Δ*pnp* background (Fig. 7), we hypothesized that perhaps PNPase affected expression of the plasmid-encoded $RNE_{1–465}$. Following a 1.5 hour induction of $RNE_{1–465}$ in both strains and Western blot analysis, we concluded that the truncated $RNE_{1–465}$ was expressed similarly in both strains, and that PNPase was not modulating $RNE_{1–465}$

expression levels. More specifically, the *Y. pseudotuberculosis* + empty vector pBAD24 (WT) and Y. *pseudotuberculosis* Δ*pnp* + empty vector pBAD24 (pnp) controls did not express $RNE_{1–465}$ when either induced with 0.02% arabinose or not (lanes 1, 2, 5, and 6). However, the *Y. pseudotuberculosis* + pBAD-$RNE_{1–465}$ (RNE) and the *Y. pseudotuberculosis* Δ*pnp* + pBAD-$RNE_{1–465}$ (pnp/RNE) both expressed the ~ 52KDa $RNE_{1–465}$ when induced with 0.02% arabinose (lanes 3 and 7).

Discussion

Y. pseudotuberculosis is a very close relative of the etiological agent of plague, *Y. pestis*, which diverged from *Y. pseudotuberculosis* between 15,000–20,000 years ago (Achtman et al. 1999). In fact, their RNase E, PNPase, RhlB and enolase proteins are 97–100% identical. Unlike *Y. pestis* (which has caused three major human pandemics), *Y. pseudotuberculosis* (like the more distantly related *Y. enterocolitica*) causes a relatively benign self-limiting gastro-intestinal disease in humans (Galindo et al. 2011). Being psychrotropic and a human pathogen, a better understanding of *Y. pseudotuberculosis* stress responses could result in the discovery of novel targets for chemotherapeutic design. Both temperature (i.e. cold) and oxidative stress responses have been characterized in this manuscript, the former potentially experienced by *Y. pseudotuberculosis* or *Y. enterocolitica* during food processing and shipping and the latter experienced when attacked by host innate immune cells during an infection. Knowing that the exoribonuclease, PNPase, is required for cold growth of several organisms (Jones et al. 1987; Goverde et al. 1998) including *Y. pseudotuberculosis* (Rosenzweig et al. 2005), we strove to evaluate whether the PNPase requirement for cold growth of *Y. pseudotuberculosis* was degradosome-dependent. Similarly, we chose to characterize the *Y. pseudotuberculosis* oxidative stress response since PNPase had already been implicated in the *E. coli* H_2O_2 stress response in a degradosome-independent manner (Wu et al. 2009). In fact, PNPase has already been shown to promote yersiniae virulence and is required for optimal T3SS function (Rosenzweig et al. 2005 & 2007), so identifying the exact constituents of the *Y. pseudotuberculosis* degradosome improves our understanding of how RNA metabolism impacts bacterial virulence as well.

Our data have identified RhlB, PNPase, and RNase E as components of the *Y. pseudotuberculosis* degradosome, which previously had been shown to only inlcude PNPase and RNase E (Yang et al. 2008). Furthermore, using the B2H assay, we demonstrated how the carboxy-terminus of a *Y. enterocolitica* derived RNase E protein can also interact with *Y. pseudotuberculosis* RhlB helicase strongly supporting the notion that all pathogenic yersiniae can assemble a degradosome. We further characterized the role the *Y. pseudotuberculosis* degradosome plays in various stress responses and surprisingly found that the *Y. pseudotuberculosis* degradosome is not implicated in all stress responses that require PNPase involvement. More specifically, we determined that the *Y. pseudotuberculosis* cold-growth requirement for PNPase (Rosenzweig et al. 2005; Rosenzweig et al. 2007) is degradosome independent. However, *Y. pseudotuberculosis* degradosome assembly was required for the oxidative stress response. Degradosome involvement with oxidative stress is in agreement with a previously published report of its requirement for macrophage-induced stress (Yang et al. 2008) and in contrast to its dispensability in the *E. coli* oxidative stress response (Wu et al. 2009). This is a shining

example of how even closely related Gram-negative, enteric bacteria, e.g. *E. coli* and *Y. pseudotuberculosis*, might behave differently to various stressors and employ different coping mechanisms to overcome the stress itself.

Unexpectedly, PNPase and the degradosome affect growth during H_2O_2 stress in different phases of growth. PNPase appeared important during log-phase growth of *Y. pseudotuberculosis*, while degradosome assembly affected biomass accumulation resulting in an early stationary phase. Even more unexpected was that the absence of PNPase suppressed the H_2O_2 sensitive phenotype of $RNE_{1–465}$. Furthermore, the deletion of the PNPase-encoding gene did not diminish expression levels of $RNE_{1–465}$, so the observation remains both intriguing and unexplained. In one scenario, PNPase responds to oxidative stress in *Y. pseudotuberculosis* independently during early growth; however, during later growth PNPase associates with the degradosome to overcome the stress and enter into an acclimation phase. Of course, such a scenario fails to explain the surprising and unexplained phenomenon in which the absence of PNPase suppressed the H_2O_2-sensitive phenotype of $RNE_{1–465}$. Perhaps a global evaluation of transcript abundance in each strain during oxidative stress is warranted and could reveal clues to help explain why PNPase and the degradosome affect growth during H_2O_2 stress differently despite PNPase not diminishing expression levels of $RNE_{1–465}$.

Taken together, these data have expanded our understanding of the *Y. pseudotuberculosis* degradosome by clearly identifying RhlB helicase as a member of the multi-protein complex. Additionally, these data have delineated the role of the *Y. pseudotuberculosis* degradosome in various stress responses. Whereas PNPase seemingly affects growth at 4°C in a degradosome-independent manner, the *Y. pseudotuberculosis* oxidative stress response clearly requires degradosome assembly to achieve optimal biomass during late log-phase growth. Realizing the unique contributions made by the degradosome during various stress responses could enable us to uncover novel chemotherapeutic targets more specifically aimed at disarming pathogens and making them more vulnerable/susceptible to those agents.

Materials and Methods

Strains and plasmids

DHM1 is a *cya*-deleted *E. coli,* slow growing, temperature sensitive mutant strain that is used for the B2H screening.

For the immunoprecipitations, *Yersinia pseudotuberculosis* ATCC® 6902™ was used.

YPT YPIII pIB102 (Bölin and Wolf-Watz 1984) (WT) and YPIII pIB100Δ*pnp* (Rosenzweig et al. 2005) were used for the cold growth and H_2O_2 plate-based assays.

The arabinose-inducible promoter containing pBAD24 (Guzman et al. 1995) plasmid was used as a cloning vector into which a carboxy-truncated RNase E (encoding only the first 465 amino acid residues in the amino terminus) was cloned (Yang et al. 2008). For all inductions 0.02% arbinose was used unless otherwise noted. Ampicillin working concentrations were 100μg/mL.

Y. pseudotuberculosis Primers for B2H cloning

RNaseE CTD:

Forward: tcaggattcctccagcattggctacc

Reverse: tcagaattcttactcaacagattgc

PNPase:

Forward: tcaggatcctttgctgactccgattattcg

Reverse: tcagaattcttactctgctgctgcttc

RhlB helicase:

Forward: tcaggatcctatgagcaaaacacacttg

Reverse: tcagaattctcagcctggtcgcttacgg

Enolase:

Forward: tcaggatcctatgtccaaaattgttaaag

Reverse: tcagaattcttactggcctttaacttc

Y. enterocolitica Primers for B2H cloning

RNE CTD:

Forward: tcaggatcctctggcgacgttctctctg

Reverse: tcagaattcctattcaaccgattgtg

RhlB helicase:

Forward: tcaggatcctttgaccgaacagaag

Reverse: tcagaattctcagctcggtcgcttac

Cloning of Proteins for B2H

RNase E CTD was cloned into the plasmid pKT25 while full-length enolase, PNPase, and RhlB were cloned into plasmid pUT18C. PCR products were generated using a 2× PCR master mix (New England Biolabs), and all cloning was done using BamH1 and EcoR1 high fidelity enzymes (New England Biolabs). All constructs were sequenced to confirm that they were correct.

DHM1 *E. coli* transformations and B2H assay

After DHM1 were harvested at OD_{600} of 0.5–0.6, pellets were washed twice in 1.0mL of ice cold water, washed once in ice cold 10% polyethalene glycol (PEG), and resuspended in ~750μl 10% PEG. To introduce plasmids, the cells were electroporated at 1,700 V (Biorad Inc.). Following a 1 hour recovery at 30°C with agitation, transformations were plated on LB agar plates containing 40μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid), IPTG (0.5mM), 100μg/ml of ampicillin, and 50μg/ml of kanamycin. Plates were placed at 30°C, and colonies were observed between 48–72 hours later (Euromedex Inc.).

Co-immunoprecipitation

YPT was grown in 100mL of LB medium to OD 620_{nm} of 0.7. Cells were harvested by centrifugation at 5000g for 15 minutes at 4°C. Pellets were then resuspended in 5mL 1× IP Buffer as part of a commercially available Protein G immunoprecipiation Kit (Sigam Aldrich IP50). Complete EDTA-free protease inhibitor cocktail (Roche) (one tablet per 5mL of solution) was added to the resuspended cells. Cells were lysed via sonication and 600μl of sonicate/lysate was used for downstream IP reaction (Sigma IP Kit protcol).

Immunoblotting

Polyacrylamide gel electrophoresis (PAGE) was carried out using pre-cast, 10 well, 4–12% gradient BIS-TRIS gels (Invitrogen Life Technologies), and proteins were transferred unto nitrocellulose membranes (Biorad) and blocked with non-fat milk. Polyclonal rabbit anti-PNPase, anti-RNase E, and/or anti-RhlB helicase antibodies were used to probe RNase E complexes or whole cell extracts (at a dilution of 1:3000) for 1 hour at room temperature.

Cold growth assay

A previously published protocol (Rosenzweig et al. 2005) was used with several modifications. In short, 10-fold serial dilutions of saturated bacterial cultures were spotted in duplicate in ~ 2μl volumes (using a pronger) on 2 LB agar (Difco) plates containing 100μg/mL ampicillin (Sigma) and 0.02% arabinose (Sigma). One plate was placed at 30°C while the other was placed at 4°C and monitored for an 11 day period. Alternatively, cultures were streaked out on the aforementioned plates and monitored for their growth over an 11 day period.

H_2O_2 plate and liquid-based assays

Previously published protocols (Wu et al. 2009) were employed. In short, saturated cultures were diluted, and subcultures of OD 600_{nm} ~0.2 were established in triplicate 100μl volumes of LB medium (Difco) in 96 well plates. Following static growth @ 30°C for 1.0 hours (with the appropriate antibiotic added and arabinose @ 0.02%), a stock 0.88 M H_2O_2 was added to the various cultures yielding H_2O_2 concentrations of either 0mM, 20mM, 50mM, or 100mM, respectively. Growth in the liquid cultures was monitored every 30 minutes over a 12 hour period with continuous agitation. Growth curves were plotted, and the Student's T-test was used to determine statistical significance with p values < 0.05 considered significant.

For plate-based H_2O_2 assays, 10-fold serial dilutions of saturated bacterial cultures were spotted in duplicate (using a pronger) in ~2μl volumes on 2 LB agar (Difco) plates containing 100μg/mL Ampicillin (Sigma) and 0.02% Arabinose (Sigma). Plate H_2O_2 concentrations were 0mM, 0.4mM, 1mM, 2mM, 4mM, and 100mM.

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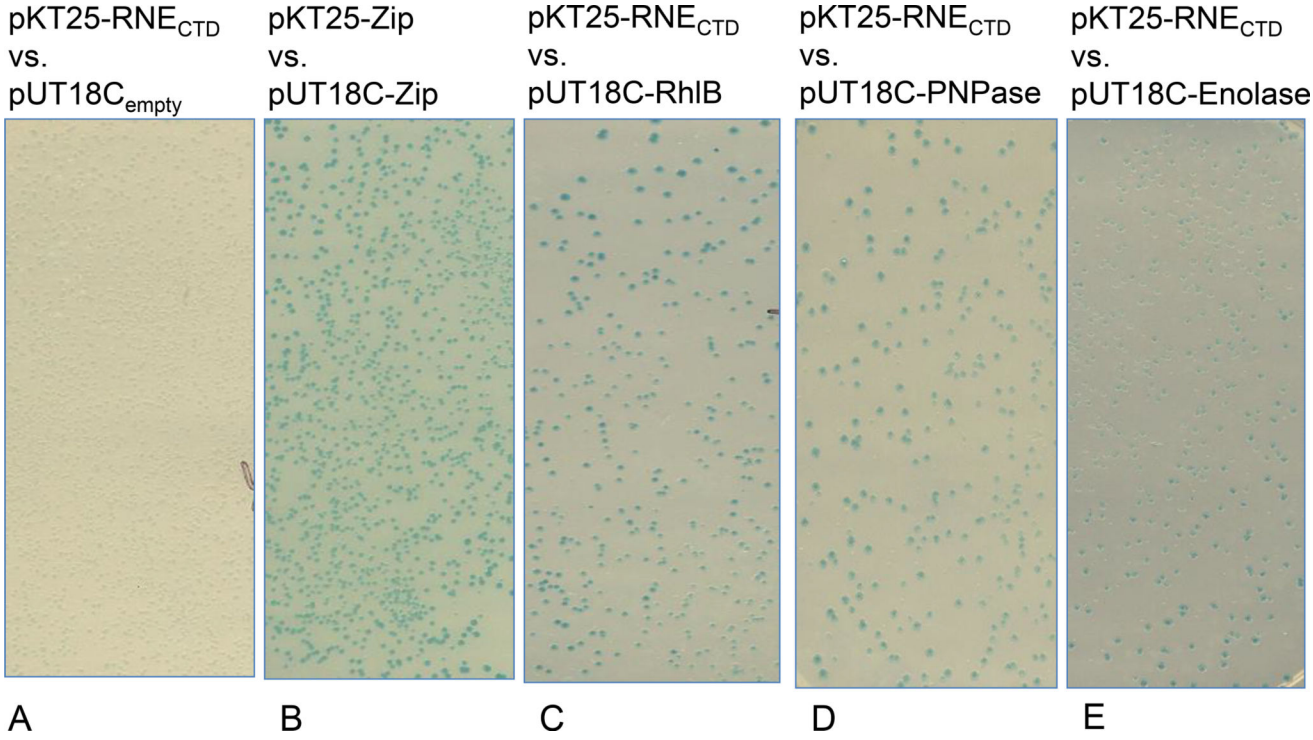

Figure 1.

Representative B2H experiment testing f or interactions between the *Y. pseudotuberculosis* RNase E CTD scaffolding region and full length RhlB, enolase, or PNPase. Individual bacterial colony forming units were evaluated, and the positive control was pKT25-Zip vs. pUT18C-Zip. The negative control was pKT25-RNE$_{1–465}$ vs. pUT18C$_{empty}$.

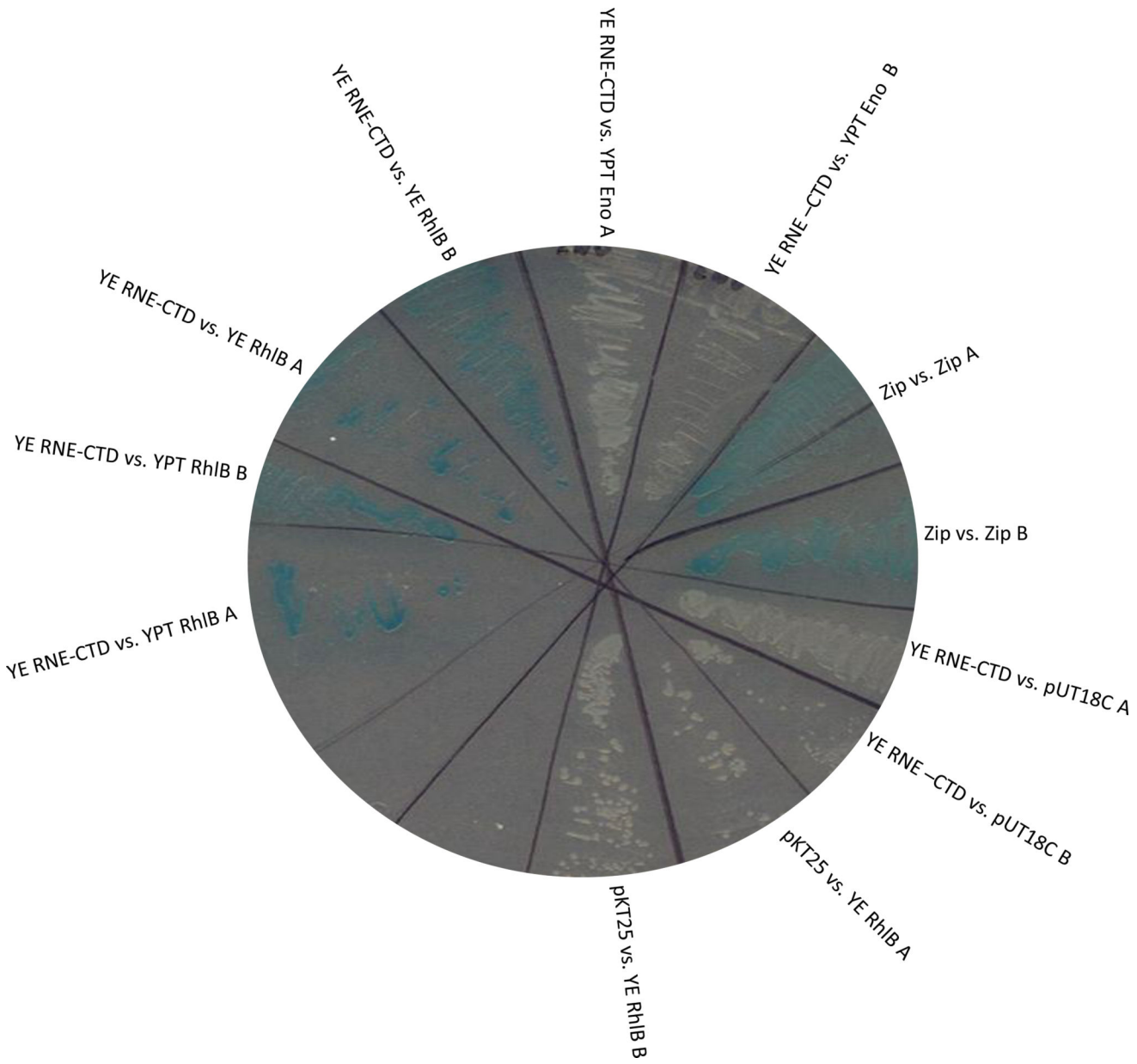

Figure 2.

Representative B2H experiment testing for interactions between the *Y. enterocolitica* RNase E CTD scaffolding region and full length *Y. pseudotuberculosis* and *Y. enterocolitica* RhlB. Single colony bacterial isolates were evaluated, and the positive control was pKT25-Zip vs. pUT18C-Zip. The negative controls were pKT25 vs. pUT18C- *Y. enterocolitica* RhlB and pKT25- *Y. enterocolitica* $RNE_{1–465}$ vs. pUT18C.

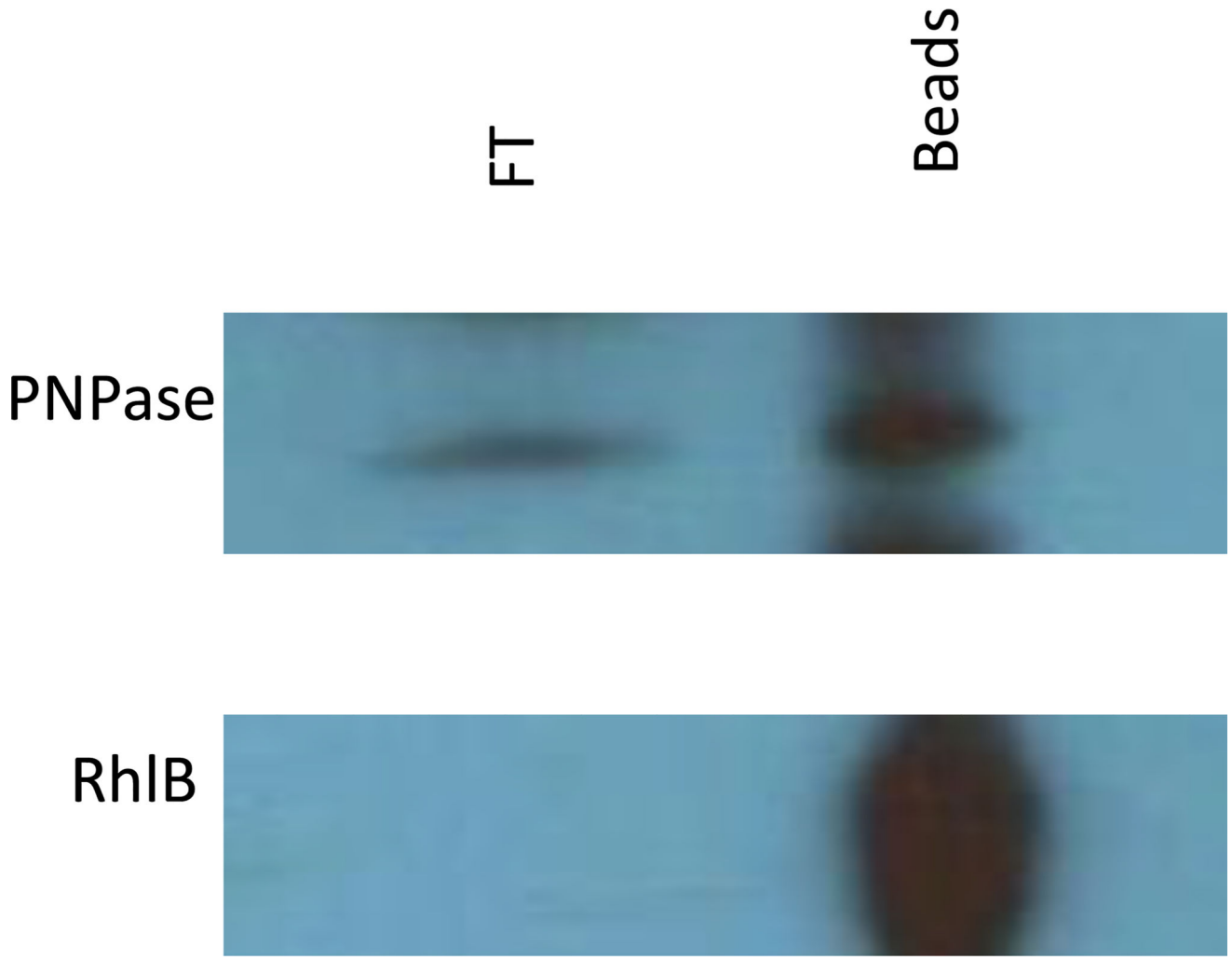

Figure 3.

Co-immunoprecipitations using anti-RNase E antibody fused Protein G sepharose beads. Depicted is an immunoblot probing the co-precipitated protein complex (bead) and probing the flow through (FT) using either rabbit polyclonal anti-PNPase or -RhlB antibodies.

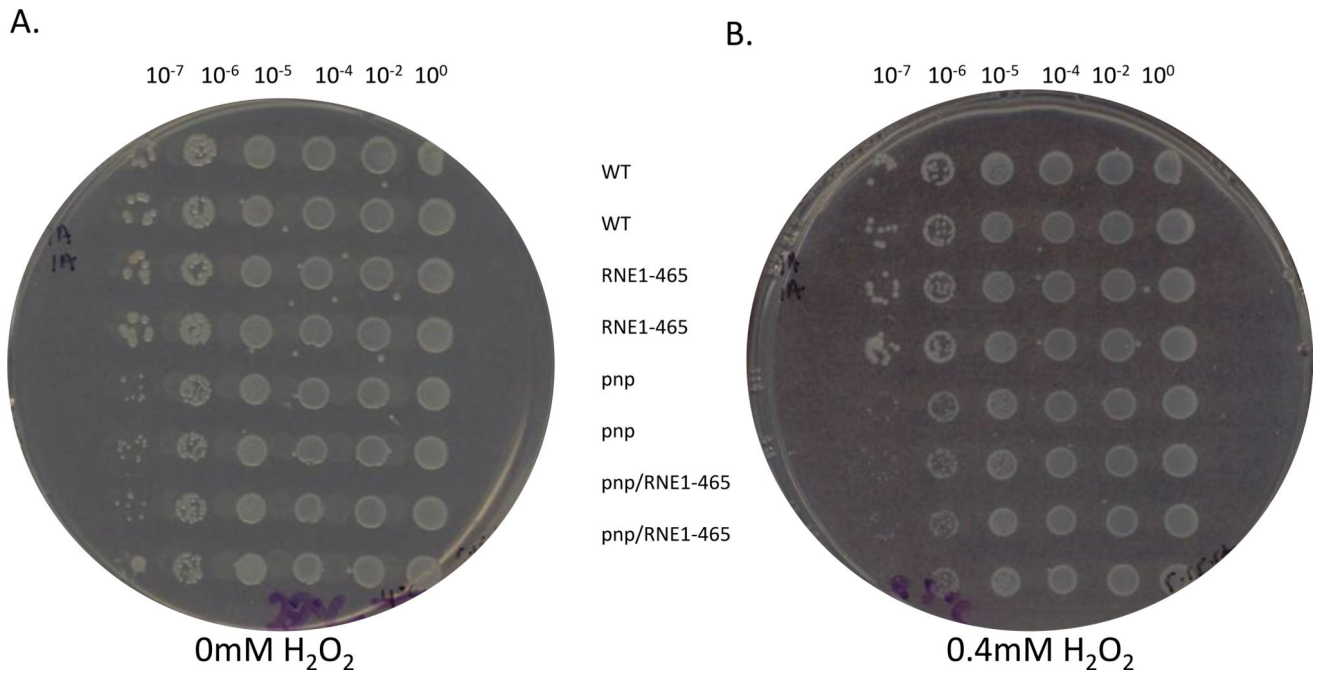

Figure 4.

Representative H_2O_2 plate experiment. *Y. pseudotuberculosis* + empty vector pBAD24 (WT), *Y. pseudotuberculosis* Δ*pnp* + empty vector pBAD24 (*pnp*), *Y. pseudotuberculosis* Δ*pnp* + pBAD-$RNE_{1–465}$ (*pnp*/RNE), and *Y. pseudotuberculosis* + pBAD-$RNE_{1–465}$ (RNE) strains were grown on 0mM H_2O_2 (A) or 0.4mM H_2O_2 (B) at 30°C for 16 hours at which point the plates were scanned. All strains were spotted in duplicate for internal dilution and spotting controls.

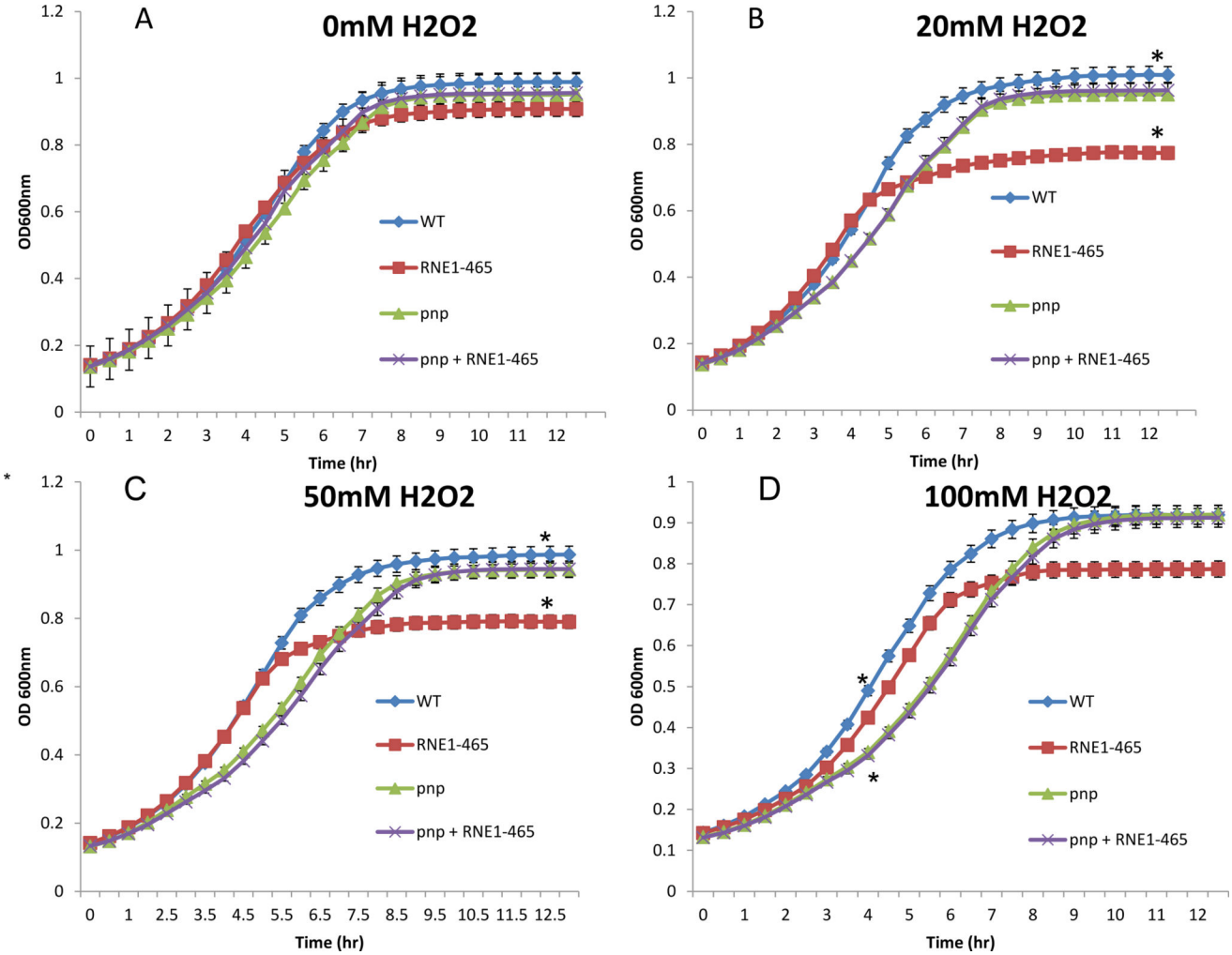

Figure 5.

Representative H_2O_2 liquid growth experiment. Subcultures of *Y. pseudotuberculosis* + empty vector pBAD24 (WT), *Y. pseudotuberculosis* Δ*pnp* + empty vector pBAD24 (*pnp*), *Y. pseudotuberculosis* Δ*pnp* + pBAD-$RNE_{1–465}$ (*pnp*/RNE), and *Y. pseudotuberculosis* + pBAD-$RNE_{1–465}$ (RNE) were grown (in triplicate) in 100μl volumes using a 96 well plate. Following 1 hour of static growth, 0mM (A), 20mM (B), 50mM (C), or 100mM (D) H_2O_2 was a added to the appropriate wells, and plates were constantly agitated while grown at 30°C for 12 hours. Samples were read at optical density 600_{nm} every 30 minutes. Asterisk between the WT and pnp samples in panel D at 4 hours and between WT and RNE in panel B and C at 12 hours denote statistical significance ($p < 0.5$). All statistical tests employed the Student's T-test.

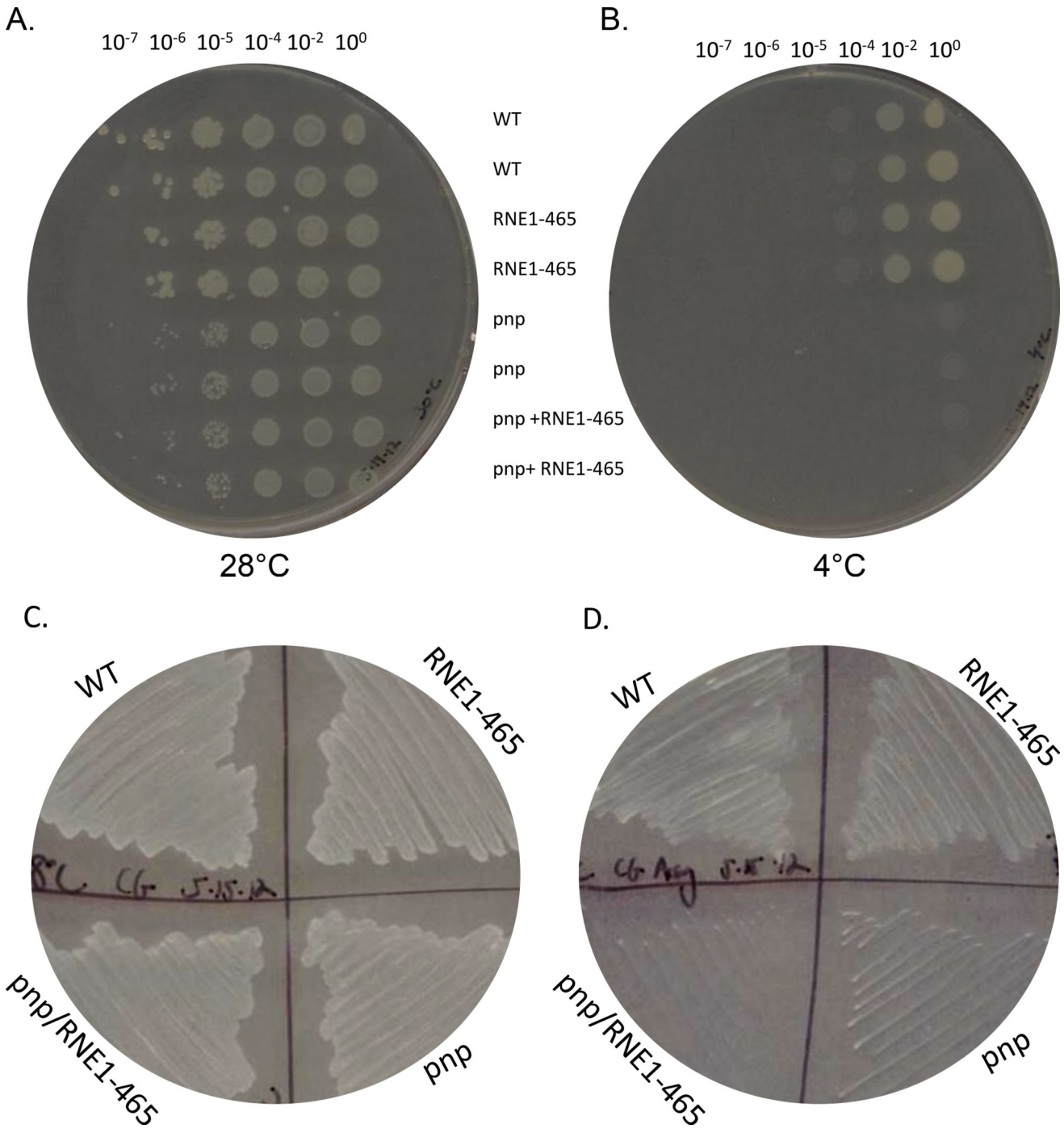

Figure 6.

Representative cold-growth experiments. A. Various dilutions of saturated *Y. pseudotuberculosis* + empty vector pBAD24 (WT), *Y. pseudotuberculosis* Δ*pnp* + empty vector pBAD24 (pnp), *Y. pseudotuberculosis* Δ*pnp* + pBAD-$RNE_{1–465}$ (pnp/RNE), and *Y. pseudotuberculosis* + pBAD-$RNE_{1–465}$ (RNE) cultures were spotted (using a pronger) on LB agar plates and grown at 30°C for 16 hours (A) or at 4°C for 11 days (B) at which point the plates were scanned. The same aforementioned strains were streaked on plates and

images were acquired following 16 hours of growth at 30°C (C) or following 11 days of growth at 4°C (D).

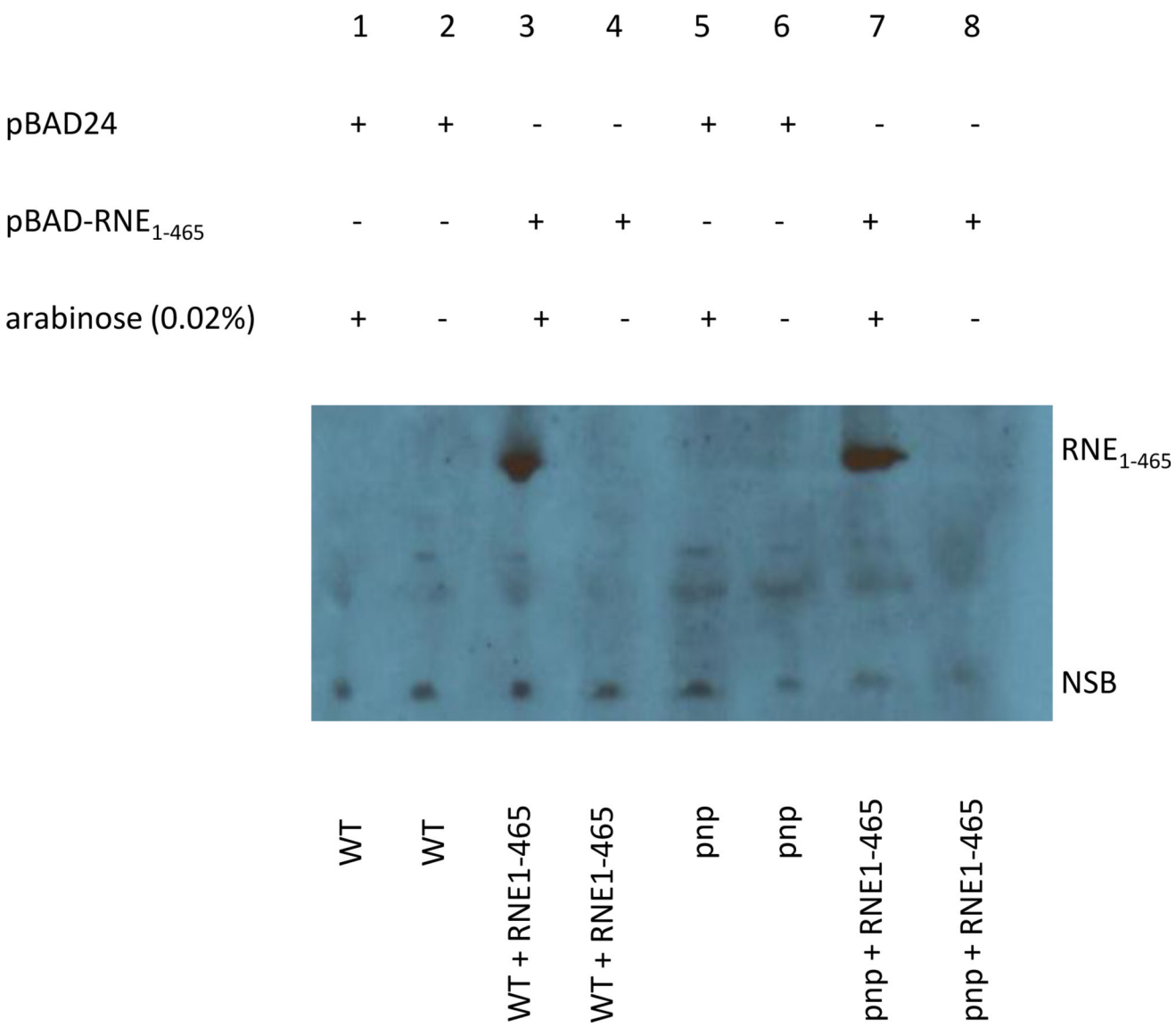

Figure 7.

Arabinose induction of the truncated $RNE_{1–465}$. *Y. pseudotuberculosis* + empty vector pBAD24 (WT), *Y. pseudotuberculosis* Δ*pnp* + empty vector pBAD24 (*pnp*), *Y. pseudotuberculosis* Δ*pnp* + pBAD-$RNE_{1–465}$ (*pnp* + RNE), and *Y. pseudotuberculosis* + pBAD-$RNE_{1–465}$ (WT + RNE) cultures were grown and induced for 1.5 hours with 0.02% arabinose. An immunoblot was performed employing polyclonal anti-RNase E antibody. CTD-deficient $RNE_{1–465}$ was expressed in strains that contained the appropriate plasmid and that were induced with 0.02% arabinose. NSB= non-specific band.