

# Genome-scale mapping reveals complex regulatory activities of RpoN in *Yersinia pseudotuberculosis*

Running Title: RpoN regulation in *Yersinia pseudotuberculosis*

F.A.K.M. Mahmud<sup>1</sup>, K. Nilsson<sup>1</sup>, A. Fahlgren<sup>1</sup>, R.Navais<sup>1</sup>, R. Choudhry<sup>1</sup>, K. Avican<sup>1</sup>, and M. Fallman<sup>1</sup>

<sup>1</sup>Department of Molecular Biology, Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå Centre for Microbial Research (UCMR), Umeå University, Umeå, Sweden.

## ABSTRACT

RpoN, an alternative sigma factor commonly known as sigma 54, is implicated in persistent stages of *Yersinia pseudotuberculosis* infections in which genes associated with this regulator are upregulated. We here combined phenotypic and genomic assays to provide insight into its role and function in this pathogen. RpoN was found essential for *Y. pseudotuberculosis* virulence in mice, and *in vitro* functional assays showed that it controls biofilm formation and motility. Mapping genome-wide associations of *Y. pseudotuberculosis* RpoN using chromatin immunoprecipitation coupled with next-generation sequencing identified an RpoN-binding motif located at 103 inter- and intragenic sites on both sense and anti-sense strands. Deletion of *rpoN* had a large impact on gene expression, including down-regulation of genes encoding proteins involved in flagellar assembly, chemotaxis, and quorum sensing. There were also clear indications of cross talk with other sigma factors, together with indirect effects due to altered expression of other regulators. Matching differential gene expression with locations of the binding sites implicated around 130 genes or operons potentially activated or repressed by RpoN. Mutagenesis of selected intergenic binding sites confirmed both positive and negative regulatory effects of RpoN binding. Corresponding mutations of intragenic sense sites had less impact on associated gene expression. Surprisingly, mutating intragenic sites on the anti-sense strand commonly reduced expression of genes encoded by the corresponding sense strand.

**IMPORTANCE** The alternative sigma factor, RpoN ( $\sigma$  54), which is widely distributed in eubacteria have been implicated to control gene expression of importance for numerous functions including virulence. Proper responses to host environments are crucial for bacteria to establish infection and regulatory mechanisms involved are therefore of high interest for

development of future therapeutics. Little is known about the function of RpoN in the intestinal pathogen *Y. pseudotuberculosis* and we therefore investigated its regulatory role in this pathogen. This regulator was indeed found to be critical for establishment of infection in mice, likely involving its requirement for motility and biofilm formation. The RpoN regulon involved both activating and suppressive effects on gene expression which could be confirmed with mutagenesis of identified binding sites. This is the first of its kind study of RpoN in *Y. pseudotuberculosis* revealing complex regulation of gene expression involving both productive and silent effects of its binding to DNA providing important information about RpoN regulation in enterobacteria.

## INTRODUCTION

During infection of a host, bacteria are exposed to rapid changes in the environment, such as changes in temperature, pH, osmolarity, nutrient levels, and immune cell attacks. Bacteria usually cope with these types of changes through stress responses, alterations of their gene expression that are adaptive to the new environment (1). This type of infection-associated transcriptomic reprogramming was obvious in a previous *in vivo* transcriptomic study of *Y. pseudotuberculosis* isolated from cecal lymphoid compartments of infected mice (2). In that model, plasmid-encoded virulence genes known to be necessary for tissue invasion and resistance towards initial attacks from phagocytes were highly expressed during the early phase of the infection. After about one-and-a-half months of symptomless infection, the expression pattern had changed so that genes encoding proteins involved in adaption and resistance to different types of stresses dominated, while expression of the plasmid-encoded virulence genes was considerably reduced. This highlights the importance for bacteria of both adapting to new environments and the regulatory mechanisms involved. Hence, increasing our understanding of bacterial function during infection is of great interest. Mechanisms of bacterial adaptation inform choices of potential targets for new antibiotics, with gene products required to maintain infections considered more promising than those of classical virulence genes.

Transcriptional reprogramming is commonly controlled by various transcriptional regulators that are activated in response to external signals. A major class of transcriptional regulators are sigma factors, which upon activation associate with the core RNA polymerase (RNAP), promoting its binding to specific initiation sites and subsequent open complex formation for transcription of downstream genes (3). There are different types of sigma factors in bacteria,

where RpoD or sigma70 ( $\sigma^{70}$ ) is the primary and house-keeping sigma factor active during exponential growth (4-6). Other, alternative sigma factors such as RpoE, RpoS, RpoH, and RpoN, which recognize promoter sequences distinct from that of RpoD, regulate transcription during specific conditions, allowing expression of genes required for the bacteria to cope with and adapt to particular situations (5). One of the sigma factors that attracted interest during the analysis of data from our previous *in vivo* transcriptomic analysis of *Y. pseudotuberculosis* was RpoN or sigma 54 ( $\sigma^{54}$ ), which together with many of its associated proteins, including activators and modulating proteins, was upregulated during the persistent stages of infection, when the expression of genes important for adaptation to the tissue environment dominated (2). RpoN has been reported to control regulation of genes involved in nitrogen metabolism, flagella, and motility, but biofilm formation and quorum sensing can also be affected in *rpoN* mutant strains (7-10). In some species, RpoN also influences regulation of Type III and Type VI secretion (8, 11-13), and there are many reports implicating RpoN as a regulator of bacterial virulence (9, 14, 15).

RpoN is structurally and functionally distinct from other sigma factors in that transcription initiation commonly depends on its binding to activating proteins termed bacterial enhancer-binding proteins (EBPs) (6). These EBPs use ATP catalysis to remodel RNAP DNA binding to initiate transcription (16). The affinity of RpoN for the core RNAP is higher than for most other RpoD-related alternative sigma factors, allowing it to compete efficiently for RNAP binding. Regulation by RpoN can be either direct or indirect via activation of different positive or negative regulators, including other sigma factors (5, 17, 18). Compared with other sigma factors, direct cross talk whereby different sigma factors regulate the same gene is particularly high for RpoN and is also commonly seen for genes that encode proteins involved in complex processes with different levels of regulation, such as adaption, chemotaxis, adhesion, and protein secretion (19). Adding to the complexity is the variation between different bacteria in the specific signals regulating RpoN and the specific downstream outcomes. One example is biofilm formation, where a deletion of the *rpoN* gene results in severe effects on the capacity to form biofilms in many bacteria, but where the opposite is seen in some other bacteria (20, 21).

This study investigated the regulatory role of RpoN in *Y. pseudotuberculosis*, which had not previously been addressed in detail. Neither has the RpoN regulon been defined for this pathogen. The results revealed that RpoN is crucial in *Y. pseudotuberculosis* to establish infection and is required for biofilm formation and motility. Chromatin immunoprecipitation

coupled with next-generation sequencing (ChIP-seq) was used to determine genome-wide binding and revealed more than 100 RpoN binding sites with both inter- and intragenic locations. Transcriptomic data from bacteria lacking *rpoN* implied a complex regulatory network with direct or indirect effects. Matching the locations of ChIP peaks with transcriptomic data allowed retrieval of more than 130 genes potentially regulated by RpoN, some novel and some known from previous studies. Mutagenesis of selected RpoN binding sites confirmed both activating and suppressive roles of upstream intergenic RpoN-binding. This was not seen for sites of intragenic binding to the sense strand. In contrast, mutation of RpoN binding motifs on the anti-sense strand commonly resulted in suppressed expression of the gene on the sense strand, implicating a novel regulatory mechanism.

## RESULTS AND DISCUSSION

**RpoN participate in regulation of *Y. pseudotuberculosis* biofilm formation and motility and is required for virulence.** To reveal the importance of RpoN in *Y. pseudotuberculosis* virulence, mouse infection studies were conducted using the *Y. pseudotuberculosis* WT strain as well as an *rpoN* deletion mutant strain. In accordance with prior results in this virulence model for *Y. pseudotuberculosis* acute infection, the WT strain initially colonized all infected mice, and all showed clear signs of disease and succumbed at day 5–7 post infection (Fig. 1A and Fig. S1A). The  $\Delta rpoN$  strain on the other hand, had colonized all mice to a low extent one day after infection and showed increased colonization at day 3, but then the infection declined and the majority were cleared at day 14–21. At 29 days post infection, all mice had cleared the infection (Fig. 1A and Fig. S1A). None of the mice infected with the  $\Delta rpoN$  strain showed signs of disease during the infection period, indicating clear attenuation. We also employed the *Y. pseudotuberculosis* infection model for persistent infection of the cecal lymphoid compartment, where a fraction of mice infected with low doses of the WT strain carry bacteria in cecal tissue for prolonged times without showing symptoms of disease (22). Upon low-dose infection with the  $\Delta rpoN$  strain, only 75% of infected mice were initially colonized compared with mice infected with the corresponding WT strain, all of which were colonized (Fig S1B). This suggest a critical role for this regulator during the early stages of infection. We next tested the  $\Delta rpoN$  strain in different phenotypic assays and found that the mutant strain was deficient in biofilm formation and motility (Fig. 1C and D). The biofilm and motility phenotypes could be complemented by expressing RpoN in *trans* (Fig. 1C and D). Hence, it is obvious that the  $\Delta rpoN$  mutant strain has limited functional capacity that likely



contributes to the observed attenuation in virulence. Together, these data indicate a pivotal role for RpoN in *Y. pseudotuberculosis* virulence.

**RpoN binds to multiple sites on the *Y. pseudotuberculosis* chromosome.** Since previous studies of RpoN-mediated events have indicated variation in the regulatory network between different bacterial species, and since studies of RpoN in *Y. pseudotuberculosis* are limited, we set out to uncover the regulatory mechanisms involved. As a first step we employed ChIP-seq to determine the RpoN DNA-binding sites in *Y. pseudotuberculosis*. ChIP-seq strongly depends on an appropriate antibody, and we chose to fuse the C-terminus of RpoN to the 3×V5 epitope, which has been shown to function in similar approaches in other bacteria (23). We generated a vector construct to overexpress RpoN-V5 (*prpoN::V5*) and also a strain expressing RpoN-V5 from its native promoter, by inserting the sequence for the V5-tag in the 3' end of the *rpoN* gene (*rpoN::V5*). Repeating the assays for biofilm formation and motility and including the  $\Delta rpoN/prpoN::V5$  and the *rpoN::V5* strains showed that the V5 tag did not interfere with RpoN function (Fig. 1C and D). It is commonly assumed that RpoN levels and binding to RNAP and to DNA are relatively stable and that the EBPs play the regulatory role. However, our finding of differential expression of *rpoN* *in vivo* compared with its expression level *in vitro* (2), and the possibility of chromosomal structural changes influencing gene expression under certain conditions (24, 25), prompted us to map the binding of RpoN in bacteria subjected to more than one condition. The conditions used were exponential growth at 26°C, where RpoN-V5 either is over-expressed (*prpoN::V5*) in *trans* or in *cis* (*rpoN::V5*), the latter to ensure proper stoichiometry and thereby avoid side effects of competition with other sigma factors. We also included samples subjected to virulence-inducing conditions, which includes a shift to 37°C and depletion of extracellular  $Ca^{2+}$  for 75 minutes that results in expression of the virulence plasmid (26) in the *rpoN::V5* strain.

The resulting ChIP-seq data were subjected to a high-stringency bioinformatic analysis with a cut-off of a 2.5-fold difference over genomic noise. This identified 119 ChIP-seq peaks representing putative sites for RpoN binding. The number of peaks was in the range previously shown for *E. coli*, *Salmonella* Typhimurium, and *V. cholerae* (8, 27, 28). Some of the peaks were narrow and distinct, covering 200–300 nucleotides, whereas others were relatively broad and covered 300–800 nucleotides (Table S1). The predicted peaks were used to identify a common sequence motif in the  $\pm 50$  bp regions of the peak center. We identified a motif that resembled the RpoN -24/12 promoter element found in other bacteria (27, 29, 30) (Fig. 2A). To determine motif strength, NN-GG-N9-TGC-NN was used as base for position-

weight matrix calculations. The identified motifs had PSSM (position-specific scoring matrix) scores ranging from 3 to 12, and the motif sequences were positioned 20 bp upstream of the peak center (Fig. 2B).

We set a cut-off for the PSSM of scores  $>7$  for high-confidence peaks, which yielded 103 peaks encompassing 112 binding sites (Fig. 2C; Table 1 and 2). There were relatively few ChIP-seq peaks without motifs (16 out of 119; Table S2) compared with the findings in some other studies (23, 27, 28). This probably reflects the high-stringency analysis used, which limits the number of false positives commonly found in ChIP data from highly transcribed regions (31, 32). The majority of the peak-associated RpoN binding sites were found in bacteria expressing RpoN-V5 from its native promoter during logarithmic growth. The corresponding samples from bacteria overexpressing RpoN-V5 lacked six of those peaks, and even more peaks were missing from the samples of virulence inducing conditions. The reasons for these differences are not obvious, but in the case of peaks missing in bacteria induced for expression of the virulence plasmid, the availability of exposed sites might have been affected by structural changes in the chromosome *per se* that can be part of mechanisms suppressing chromosomal gene expression (24, 25). In bacteria overexpressing RpoN-V5 there might be a saturation effect at high RpoN-V5 concentrations, with the precipitation of RpoN molecules not associated with any binding site that dilute samples resulting in relatively less precipitated DNA where DNA fragments from low abundant binding sites are missed. Notably, unlike other sigma factors, RpoN can bind its DNA sequence without RNAP, although the binding is 10-fold less efficient than the RpoN:RNAP complex (33). ChIP-seq should therefore also detect RpoN-DNA interactions that are independent of active transcription.

The robustness of the analysis was further verified by the identification of RpoN binding sites at intergenic regions upstream of genes previously shown to be regulated by RpoN in other bacteria. Examples here are YPK\_1894 (*pspA*), YPK\_3220 (*glnK*), YPK\_3857 (*pspG*) and YPK\_4189 (*glnA*) (8, 27, 28). Comparing our high-stringency peaks with the peaks identified by ChIP-seq in *E. coli* and *S. Typhimurium*, it was obvious that the location of many in relation to coding DNA sequences (CDS) was conserved, and some were also shared with *V. cholerae* (Fig. 2D, Table 1, 2, and 3). The conserved locations of RpoN binding sites included locations upstream of *pspA* and *glnA* with orthologs in *E. coli*, and *S. Typhimurium* and *V. cholerae*, for example, YPK\_2229, YPK\_2908, and YPK\_1600, with orthologs in *E. coli*, and *S. Typhimurium* (Table 3). There were also many novel binding sites identified in *Y.*

*pseudotuberculosis*, where a majority were intragenic with some also on the non-coding strand (Fig. 2D, E and F). Presence of sense and antisense intragenic binding sites for RpoN is in accordance with what been previously found for *S. Typhimurium* and *E. coli* (27, 28). The function and mechanisms of RpoN intragenic binding are generally unknown, but there are examples where this binding can drive transcription of downstream genes with long 5' UTRs (27, 34). The putative RpoN binding sites identified were divided into groups (A–D) based on their position and orientation. Group A (26 sites) and B (3 sites) comprise binding sites in intergenic regions. Group A sites are oriented towards the 5' end of the nearest coding sequence and those in group B are oriented towards the 3' end of the neighboring coding sequence (Fig. 2E and F; Table 1). Groups C (49 sites) and D (25 sites) comprise intragenic binding sites; those in group C are oriented in the sense direction and those in group D in the anti-sense direction (Fig. 2E and F; Table 2). In general, binding sites in group A were associated with stronger peaks than the sites in groups C and D (Table 1 and 2, Fig. S2). Further, compared with the intergenic binding sites among which a relatively large fraction appears to be conserved among *E. coli*, *S. Typhimurium*, and *Y. pseudotuberculosis*, the fraction of conserved intragenic RpoN-binding sites was considerably lower (Table 1, 2, and 3).

To reveal possible sigma factor cross talk in *Y. pseudotuberculosis*, we also screened for RpoD, RpoE, RpoS, RpoH, and FliA binding sites close ( $\pm 200$  nt) to the identified RpoN binding sites. This screen showed many potential dual and sometimes triple sigma factor binding regions close to each other in 68% of all A sites. Even more multiple binding regions were found associated with intragenic sites, with clusters of 2–5 sigma factor sites in more than 90% of all C and D sites. Notably, all sigma factor binding regions associated with A sites had a binding site for RpoD ( $\sigma 70$ ), and nearly half of them contained sites for RpoE ( $\sigma 24$ ) and/or RpoS ( $\sigma 38$ ). In contrast, binding sites for FliA ( $\sigma 28$ ) were very rare and sites for RpoH ( $\sigma 32$ ) was absent. The multiple binding sites associated with C sites included sites for RpoE (60%), RpoH (54%), RpoS (40%), FliA (42%), and RpoD (30%). D sites were similar, with binding sites for RpoS (64%), RpoH (60%), FliA (52%), RpoE (48%) and RpoD (20%).

**Deletion of *rpoN* has a large impact on the *Y. pseudotuberculosis* transcriptome, with both direct and indirect effects.** Next, we aimed to determine whether the identified binding sites could indeed be coupled to gene expression in *Y. pseudotuberculosis*. For this we employed RNA-seq on WT and  $\Delta rpoN$  bacteria at 26°C in stationary phase and at 37°C with virulence induction. Analysis of differentially expressed genes revealed a markedly different

gene expression pattern in the  $\Delta rpoN$  strain compared with the isogenic WT strain. More than 500 genes were found to be differentially regulated at 26°C stationary phase: 294 were downregulated and 213 upregulated in  $\Delta rpoN$  (Fig. 3A and 3B). The effect was even more pronounced at 37°C in virulence-inducing conditions: almost 1700 genes were affected, with 766 genes downregulated and 929 upregulated. The reason for this discrepancy, with a much higher number of genes affected during virulence-inducing conditions compared with 26°C stationary phase, might be a higher degree of stress associated with the former. This is a condition known to involve activation of different alternative sigma factors as well as other global regulators. Functional annotation analysis of the differentially expressed genes showed down-regulation of genes involved in nitrogen metabolism, flagellar assembly, chemotaxis, and quorum sensing in both conditions (Fig. 3C and Fig. S3). There was also downregulation of fatty acid biosynthesis and metabolism, but this was not seen in samples of bacteria subjected to virulence-inducing conditions. For these samples, additional pathways were affected by the deletion of *rpoN*, including, for example, low expression of genes involved in T3SS that normally is highly upregulated at this condition, downregulation of DNA replication, and amino acid biosynthesis, and upregulation of genes involved in carbon metabolism, gluconeogenesis, and ribosomal organization, with the latter possibly reflecting the stress of translational reprogramming.

Although the expected effects of RpoN deletion, such as reduced expression of genes involved in nitrogen metabolism, flagella, and quorum sensing were obvious, the effect on the *Y. pseudotuberculosis* transcriptome was huge, clearly reflecting deletion of a global regulator. This accords with previous studies, which found that the absence of RpoN commonly results in a global effect involving both direct and indirect effects on gene expression (9, 23, 35). The differential gene expression analysis revealed changed expression levels of other sigma factors in the  $\Delta rpoN$  strain (Table S3). Expression of RpoD, for example, was significantly upregulated, whereas expression of RpoE was downregulated both in stationary phase and in virulence-inducing conditions. RpoS on the other hand was upregulated in stationary phase but downregulated during inducing conditions. In addition to other sigma factors, the mRNA levels of other transcriptional regulators were affected, such as CpxR, RovA, FlhCD, and others (Table S3). Hence, these effects, together with effects on the transcription of other sigma factors, are expected to contribute extensively to indirect effects on gene expression, adding further complexity to the data set.

**Identified RpoN-binding sites mediates both positive and negative regulation of gene expression.** Given the complexity, including indirect effects, in the RNA-seq data set, we next aimed to reveal direct effects of RpoN. The RpoN binding sites identified in the ChIP-seq analysis likely include both active promoters driving transcription and suppressive and silent binding of RpoN to the chromosome. The potential RpoN binding sites identified were therefore matched with detected changes in gene expression levels (Fig. 3D). Among the identified binding sites in group A, 88% were associated with differential gene expression, involving both upregulated and downregulated genes (Fig. S2). The intragenic C and D sites were also associated with differential gene expression, 79% for the C sites and 68% for the D sites. For C sites, the differential gene expression included both upregulation and downregulation of genes containing the motif as well as downstream genes. For the antisense D sites, there was a larger fraction of upregulated than downregulated genes, suggesting potential suppressive effects of the RpoN binding (Fig. S2). In general, a relatively large portion of the differentially expressed genes associated with RpoN binding showed increased expression in the  $\Delta rpoN$  strain. Binding by RpoN might suppress transcription by nearby sigma factors and possibly other transcription factors binding to the same region, where the absence of RpoN would then allow transcription to occur (8, 27). Also, collision as a consequence of RpoN intragenic binding has been suggested (36, 37).

To explore the potential importance of the RpoN binding sites identified in *Y. pseudotuberculosis*, we set out to mutate some of them to reveal their effects on gene expression. For this we chose binding sites with locations indicative of putative positive or negative regulation by RpoN, which was the case for many A and D sites. We could not identify any C-site ChIP-seq peak indicative of transcriptional activation of downstream genes in our data set. The sites were mutated by the exchange of 3–6 nucleotides in the conserved TGG and TGC sequences of the RpoN binding motif. In intragenic motifs, the exchanged nucleotides were selected in order to minimize changes in the encoded protein (see Table S5 for details). The selected sites included seven A and five D sites (Fig. 4 and Table S4) and the effects of the mutations on gene expression were verified by qPCR. For all putative activating A sites that is, those represented by down-stream genes downregulated in the absence of RpoN point mutations in the RpoN binding motifs in the WT strain resulted in reduced transcription. This class of binding sites also showed the highest degree of conservation. The most prominent effect of the disruptive nucleotide exchange was seen for *pspA* (YPK\_1894), a gene known to be activated by RpoN (38, 39). There were also indications of inhibitory

effects of RpoN binding to A sites, where the expression of down-stream genes was increased in both  $\Delta rpoN$  and the corresponding binding site mutant. Intriguingly, four of the five binding site mutations in D sites resulted in increased expression of the CDS on the opposite strand, suggesting a suppressive effect of RpoN binding. We also mutated some of the C sites, but here no effect on transcription compared with that in the WT strain could be seen. Notably, peaks associated with C sites were commonly flatter and broader than the peaks covering the mutated A and D binding sites (Fig. S4).

Taken together, we have by mutagenesis been able to verify effects of RpoN binding to the binding motifs identified in a ChIP-seq screen of *Y. pseudotuberculosis*. Activating as well as suppressing effects of intergenic RpoN-binding were verified by mutating intergenic A sites. Among these verified productive RpoN-binding sites, some were known, such as those in PspA, PspP, and GlnA, whereas RpoN regulation of TppB, UgpB, and DkgA is described for the first time. There were also indications of inhibitory effects of RpoN binding to intragenic D sites. As discussed earlier, how RpoN suppresses transcription is less clear but for binding to the sense strand it might occur by steric hindrance, either by the RpoN-RNA polymerase complex or RpoN alone binding to the DNA. How this can affect expression from the opposite strand, as would be the case for the observed inhibitory effect of mutating RpoN binding D sites is less clear, but it might involve disturbed strand separation. For intragenic RpoN binding, we saw effects on transcription only by mutating binding sites on the anti-sense strand. Thus, intragenic binding by RpoN to coding regions on the sense strand is likely silent, and may be used for storage of RpoN-RNAP. RpoN-mediated suppression by binding to internal anti-sense sequences of genes encoded on the opposite strand has not been shown previously and its mechanism remains to be elucidated.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Strains and plasmids are listed in Table S5. *Yersinia pseudotuberculosis* strain YPIII was used in this study. *Escherichia coli* S17-1  $\lambda$ pir was used for cloning and conjugation. Antibiotics were used at the following concentrations: ampicillin (100  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), and chloramphenicol (25  $\mu$ g/ml). Motility was tested on Luria Bertani (LB) medium with 0.6% agar. Biofilm assays were carried out as described previously, using LB medium in glass tubes (40). All strains were routinely grown at 26°C in LB medium containing kanamycin (50  $\mu$ g/ml). For ChIP- and RNA-Seq analyses, cultures were grown in LB medium to the desired OD<sub>600</sub>. Arabinose (0.005%) was used for



30-min induction at 26°C for overexpression of *rpoN*. To reach the virulence induction condition, overnight bacterial cultures were diluted to OD<sub>600</sub> 0.05 in LB and grown at 26°C. After 1 h, calcium was depleted by adding 5mM EGTA and 20 mM MgCl<sub>2</sub> and cultures were shifted to 37°C (26).

**Strain construction.** In-frame gene deletion and insertion of the V5 epitope and binding-site mutations in *Y. pseudotuberculosis* were performed using an In-fusionHD cloning kit (Clontech) according to the manufacturer's instructions. Briefly, the flanking regions of the respective gene were amplified by PCR and cloned into the suicide vector pDM4. This construct was used to transform S17-1, and then transferred into recipient strains through conjugation. Conjugants were purified and incubated on 5% sucrose to recombine out the vector together with wt sequence. Deletion or mutation was confirmed by PCR. For trans-complementation, the gene was PCR amplified and cloned into the pBAD24 plasmid. For gene induction, *rpoN* and a C-terminal 3×V5 epitope tag were PCR amplified and cloned into the pBAD18 plasmid. All constructs were verified by sequencing. Primers used in this study are listed in Table S6.

**Motility and biofilm assays.** Determination of swimming motility was performed as described previously (41). A 5 µl aliquot of a diluted overnight culture (OD<sub>600</sub> of 1.0) were spotted at the center of a 0.6% LA soft agar plate and incubated at 26°C for 24 h. Bacterial motility was determined by measuring the diameter of the bacterial growth area.

Biofilm formation was determined as previously described (40). Overnight cultures were diluted to an OD<sub>600</sub> of 0.05 and grown to an OD<sub>600</sub> of 0.5 in a 26°C shaking water bath. A 1 ml aliquot of the bacterial culture was pelleted and dissolved in 2 ml fresh LB. The suspension was transferred to glass tubes and incubated for 48 hours at 37°C without shaking. After incubation, the bacterial suspension was discarded and tubes were gently washed 3 times with PBS and stained with 0.1% crystal violet (Sigma-Aldrich) for 15 min, followed by 3 successive washes with PBS. The biofilms on the tube surface were thereafter dissolved with 33% acetic acid for 15 min and the absorbance at 590 nm was measured with an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Piscataway, NJ).

**RNA isolation and Illumina sequencing.** Total RNA was isolated from three independent biological replicates of WT *Y. pseudotuberculosis* and an isogenic *rpoN* deletion strain at stationary phase and under virulence-inducing conditions. For isolation and purification of RNA, the Trizol method (Ambion Life Technologies, Carlsbad, CA) and the Direct-zol RNA

kit (Zymo Research, USA) were used. To remove unwanted DNA, purified RNA was treated with DNase for 30 min at room temperature according to the manufacturer's instructions. RNA quantity was measured by Qubit (Nordic Biolabs AB, Sweden). RNA from stationary phase bacteria was collected after 8 hours of growth ( $OD_{600} \sim 2$ ) at 26°C in LB. For virulence-inducing conditions, RNA was isolated from cells grown exponentially at 26°C, shifted to 37°C, and incubated in  $Ca^{2+}$ -depleted medium for 3 h.

For sequencing, cDNA libraries were prepared using the ScriptSeq™ Complete Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. Ribosomal RNA was depleted from total RNA using a Ribo-Zero rRNA Removal Kit for Bacteria (Epicenter, Madison, WI, USA) following the manufacturer's protocol. The resulting cDNA libraries were purified using AMPure XP (Beckman Coulter) and quantified using an Agilent 2100 Bioanalyzer. Sequencing was done using an Illumina MiSeq.

Raw RNA-seq reads were trimmed from 5' and 3' ends by Trimmomatic (42) until all the adapter and low-quality bases (Phred quality score >30) were removed and the sequences passed Quality checking by FastQC (43). ProkSeq, a complete RNA-seq data analysis package for prokaryotic was used for further RNA-seq data processing, quality control, and visualization (44). It includes all the tools mentioned below as well as SAMtools (45) and BEDtools (46). Reads were aligned to *Y. pseudotuberculosis* YPIII chromosome (NC\_010465) and plasmid (NC\_006153) using bowtie2 (47) with the unique-mapping option. Post-mapped read quality checking was done using RseQC (48), and the numbers of reads for each gene were counted using featureCounts (49). Differential gene expression was determined using DESeq2, using the shrinkage estimation of dispersion option ( $lfcSrink = \text{True}$ ) to generate more accurate estimates of differential expression in fold changes (50). Differential expression of a gene was defined using absolute  $\log_2$  fold change values of  $\geq 1.5$  and false-discovery-rate values of  $< 0.05$ . Figures are plotted using the ggplot2 package in R (linux version 4.0.2) and GraphPad Prism (version 8.0)

### **Chromatin immunoprecipitation followed by next generation Sequencing (ChIP-Seq).**

*Y. pseudotuberculosis*  $\Delta rpoN$  expressing RpoN::V5 in trans under the inducible araBAD promoter (YPIII,  $\Delta rpoN$ /pIBX,  $prpoN::V5$ ) as well as *Y. pseudotuberculosis* expressing RpoN:V5 in *cis* under its native promoter (YPIII,  $rpoN::V5$ /pIBX) were grown overnight in LB, diluted to  $OD_{600}$  0.05, and grown for 4 h at 26°C, at which point the  $prpoN::V5$  plasmid was induced with 0.005% arabinose for 30 min. Virulence-induced cultures were shifted to 37°C after 1 h at 26°C and incubated for 75 min in calcium-depleted conditions.. Non-tagged

WT *Y. pseudotuberculosis* grown at 26°C for 4 h was used as a negative control. Cultures were cross-linked with 1% formaldehyde at room temperature for 10 min. For subsequent ChIP-seq, 10 OD<sub>600</sub> were used for each biological replicate. Chromatin immunoprecipitation was performed as described previously, with slight modifications (27). The 1 ml samples were sonicated in AFA milli-tubes using a Covaris E220 sonicator with the following settings: peak power, 140W; duty factor, –5%; cycles/burst, 200; time, 16 min. Immunoprecipitation was done using 90 µL Anti-V5 Agarose Affinity Gel (Sigma A 7345) overnight at 4°C. ChIP DNA was eluted in 100 µl elution buffer, treated with RNase A and proteinase K, and purified using a ChIP DNA clean & Concentrator Kit (Zymo Research, USA). AMPure XP magnetic beads (Beckman Coulter) 1.5× and 0.8× were used to remove adapters and clean and concentrate the DNA. Failsafe polymerase (Nordic Biolabs AB, Sweden) was used for efficient library amplification.

**ChIP-seq Data analysis.** A custom pipeline in Python was used for ChIP-seq data analysis, data visualization, and downstream bioinformatic and computational analyses (the pipeline is available on request). The quality of raw reads generated from Hiseq 2500 was checked using FastQC (43). Raw reads were trimmed by 5 nt at both the 5' and 3' ends and aligned with the reference *Y. pseudotuberculosis* YPIII chromosome (NC\_010465) and plasmid (NC\_006153) using BWA (51). Aligned reads were converted to BAM files using SAMtools (45) and duplicate reads were removed with picard (52) by deduplicate function. Before peak calling, two biological replicates for each sample and the input (control) were merged using SAMtools. Peak calling was done using MACS (2.1.2) (53) with the following custom settings: log2FC>1 over input, --broad –g, --broad-cutoff = 0.1. Identified peak co-ordinates in the genome were used to identify the probable regulated genes using the R packages ChIPpeakAnno and ChIPseeker (54, 55). De novo motif prediction was done using the bioconductor package BCRANK (56). A position- weight matrix (PWM) of the predicted motif was used to map the motif presence around the peak center using the FIMO-meme package (linux version) (57). A region of 50 bases upstream and downstream sequence of the peak center was used as input for searching for the PWM of the motif. The SIGffRid tool was used to predict the binding sites of other sigma factors within 200 bases upstream and downstream of each predicted RpoN-binding peak center. To check the conservation of RpoN-regulated genes, cross-species homology prediction was done by comparing *Y. pseudotuberculosis* YPIII, *V. cholerae* 037, *E. coli* 0157, and *S. Typhimurium* LT2 using OrthologueDB (58). *Yersinia* genes having orthologs in at least one of the species mentioned

above were checked for previous ChIP-seq studies (23, 27, 28). Figures are plotted using the ggplot2 package in R (linux version 4.0.2).

**Ethics statement.** Mice were housed and treated in accordance with the Swedish National Board for Laboratory Animals guidelines. All the animal procedures were approved by the Animal Ethics Committee of Umeå University (Dnr A108-10). Mice were allowed for one week to conform to the new environment before the experiments start.

**Mouse infections.** Female FVB/N mice (Taconic) 8 weeks old were deprived of food and water for 16 hours before infection. For infection of mice, overnight cultures of the *Y. pseudotuberculosis* strains were suspended in sterilized tap water supplemented with 150 mM NaCl, reaching an approximate colony forming unit (CFU) count of  $10^7$  CFU/ml for low-dose infection and  $10^8$  CFU/ml for acute infection. Mice were allowed to drink for 6 h. The infection dose was calculated based on viable count and the volume of drinking water supplemented with bacteria that was consumed. Frequent inspections of mice were carried out routinely to ensure no prominent clinical signs were overlooked. Infected mice showing notable clinical signs were euthanized promptly to prevent suffering.

The infections were monitored using IVIS Spectrum (Caliper Life Sciences) routinely every third day until 15 dpi (days post infection), and later every week up to 28 dpi. The mice were anesthetized using the XGI-8 gas anesthesia system (Caliper Life Sciences) and 2.5% IsoFloVet (Orion Pharma, Abbott Laboratories Ltd, Great Britain) in oxygen for initial anesthesia and 0.5% isoflurane in oxygen during IVIS imaging. After infection, some mice were euthanized and dissected to analyze bacterial localization and presence in various organs, including the intestine, mesenteric lymph nodes, liver, and spleen. The organs were imaged using bioluminescence imaging. Living Image software, version 3.1 (Caliper Life Sciences, Inc.) was used for image acquisition and data analysis.

**cDNA preparation and qPCR.** To validate the ChIP-Seq and RNA-Seq results, qPCR was performed using qPCRBIO SyGreen Mix (PCR Biosystems) and a BioRad CFX Maestro real-time PCR machine. For RpoN binding-site mutants, bacteria were grown for 2.5 hours at 37°C in inducing conditions in LB ( $OD_{600} \sim 0.6$ ) or at 26°C for 8 hours, and total RNA was extracted as described earlier. Isolated RNA was used as template for cDNA synthesis using a RevertAid First Strand cDNA Synthesis kit (ThermoScientific). Experiments were done in triplicate for each mutant. YPK\_0831 was selected as an internal control in order to calculate the relative expression levels of tested genes, using appropriate primers (Table S6).

**Data availability.** The RNA-seq and ChIP-seq data files have been deposited in Gene Expression Omnibus (GEO) under accession number GSE155608. All the computer code and pipeline used in this studies are available on request.

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Conception or design of the work: F.A.K.M.M, K.N,R.C and M.F; data collection: A.K.M.M, K.N,R.C.,R.N, K.A; data analysis and interpretation: F.A.K.M.M, K.N, A.F. and M.F.; drafting of the article: F.A.K.M.M, K.N and M.F; critical revision and contributions to the article: F.A.K.M.M, K.N, A.F., R.N, K.A. and M.F.; final approval of the version to be published: F.A.K.M.M, K.N, A.F., R.N., R.C, K.A. and M.F.

## FIGURE LEGENDS

**FIG 1 RpoN is required for *Y. pseudotuberculosis* virulence and essential for motility and biofilm formation.** (A) Female FVB/N mouse (n = 6) were infected with  $4.2 \times 10^8$  CFU/ml of *Y. pseudotuberculosis* WT (dashed line) or the isogenic  $\Delta rpoN$  mutant strain (solid line). Data are presented as percent live mice during the course of infection (0–28 days). (B) *In vivo* images showing anesthetized mice during the first 7 days of infection. Colors indicate levels of light emitted by bioluminescent bacteria. Mice infected with *Y. pseudotuberculosis* WT become sick at day 5–7 and were sacrificed. (C) Biofilm formation by *Y. pseudotuberculosis* WT, and the corresponding strains with *rpoN*::V5 inserted in the chromosome,  $\Delta rpoN$ ,  $\Delta rpoN$  complemented *in trans* with *prpoN*, and  $\Delta rpoN$  complemented *in trans* with *prpoN*::V5. Biofilm mass was determined by dissolving biofilm material and measuring the OD<sub>590</sub>. Data are presented as means  $\pm$  SEM for six individual experiments (D) Motility in agarose by the same strains as indicated in B. One representative experiment of three replicates is shown.

**FIG 2 ChIP-seq identifies RpoN binding sites in *Y. pseudotuberculosis* genome** (A) RpoN consensus binding motif in *Y. pseudotuberculosis* derived from 119 ChIP-seq peaks, determined using BCRANK (E-value =  $1.8e^{-110}$ ). (B) Position of motifs relative to peak

centers calculated using the Centrimo tool, MEME suite. The graph indicates the average density of motif position for all 119 motif-containing regions, using 10 bp bins from position -50 to +50 relative to the ChIP-seq peak. (C) Venn diagram showing the number of RpoN binding sites identified by ChIP-sequencing of *Y. pseudotuberculosis* in different growth conditions. (D) Venn diagram showing number of RpoN binding sites with homologous genomic positions in *Y. pseudotuberculosis*, *E. coli*, and *S. Typhimurium*. Those genes which were found to be conserved based on function and phylogenetic distance ratios among all three bacteria and whose corresponding gene was reported (by a ChIP-seq study) to be regulated by RpoN were included in the Venn diagram. (E) Schematic illustration of four classes of RpoN binding sites based on their positions in relation to gene coding sequences. (F) Distribution of each class of RpoN binding site.

**FIG 3 Deletion of *rpoN* has a substantial influence on the *Y. pseudotuberculosis* transcriptome.** (A) Volcano plot showing differentially expressed genes in the *Y. pseudotuberculosis*  $\Delta rpoN$  strain compared with the WT strain at stationary phase (OD = 2,0 at 26°C) and during virulence induction (Ca<sup>2+</sup> depletion, at 37°C). (B) Venn diagram showing the number of differentially expressed genes under the indicated conditions. The criteria for differential expression shown in A and B were fold changes >1.5 with a *P* value <0.05. (C) Pathway enrichment (KEGG) of genes differentially expressed both in stationary phase and after virulence induction. Pathways are ranked by the negative log 10 of the *P* value of the enrichment score. The *P* values were calculated using the Bonferroni correction. (D) Plots showing expression of genes associated with intergenic or intragenic high-confidence peaks. The y-axis indicates expression values from RNA-seq (log2 fold-change of  $\Delta rpoN$ /WT) and the x-axis indicates the strength of the associated ChIP peaks. For both A and D, significantly upregulated (red) and downregulated (green) genes, as well as non-significant changes (black circles) are indicated.

**FIG 4 Validation of productive RpoN-binding.** (Left) Relative expression of indicated genes in bacteria when the associated RpoN binding site is mutated (white bars) and in the  $\Delta rpoN$  strain (black bars). Expression of genes in the mutant strains was determined by qPCR and presented as log2 fold relative expression compared with expression in the WT strain, where positive values represent suppressed transcription and negative indicate activated transcription. Results are shown for expression during virulence-inducing conditions for



YPK\_0329, YPK\_1886, YPK\_1894, YPK\_2927, YPK\_2997, YPK\_3010, YPK\_3075, YPK\_3613, YPK\_3887, and YPK\_3962, and for expression during stationary phase at 26°C for YPK\_2908 and YPK\_2909. Values represent means  $\pm$  SEM of three independent experiments. (Right) Schematic illustration of the shapes and genomic contexts of ChIP-peaks together with the positions and orientations of RpoN-binding of associated motifs.

**FIG S1 High and low-dose infection of *Y. pseudotuberculosis* WT and  $\Delta$ rpoN in female FVB/N mice.** (S1A) Mice (one experiment, n = 6) were infected with a mean of  $4.2 \times 10^8$  CFU/ml. IVIS imaging was employed at each time point to determine if mice were infected. Severely sick WT mice were sacrificed before day 7. The color scale represents pseudocolors of the intensity of bioluminescence, where red is the most intense light emission and blue corresponds to the weakest signal. (S1B) Mice (one experiment :WT, n= 40 and  $\Delta$ rpoN, n= 20) were infected for 28 days with a mean of  $4.2 \times 10^6$  CFU/ml. IVIS imaging was used to determine if mice were infected. Mice showing acute clinical signs were sacrificed. Symptom-free infection was calculated at 28 days after infection.

**FIG S2 Different classes of ChIP peaks and their associated gene expression (WT/ $\Delta$ rpoN).** The y-axis represents the fold-change difference between WT and  $\Delta$ rpoN and the x-axis represents the average peak strength.

**FIG S3 Pathway enrichment in differentially expressed genes ( $\Delta$ rpoN/WT) at stationary phase and under virulence induction.** The pathway enrichment score was calculated as the probability of a gene's enrichment in a pathway other than by random chance. Pathways are ranked by the negative log 10 of the *P* value of the enrichment score. The *P* values were calculated using the Bonferroni correction and used to evaluate the significance of the pathway in a particular condition. Up- and downregulation of the pathway was predicted by calculating the Z score of differential expression of the genes in a certain pathway.

**FIG S4 Schematic representation of selected intragenic sense (C) RpoN binding sites for qPCR.** The arrow indicates the position and direction of the RpoN binding motif. ChIP peaks are shown in blue, representing their coverage in the genome in logarithmic phase (26°C).

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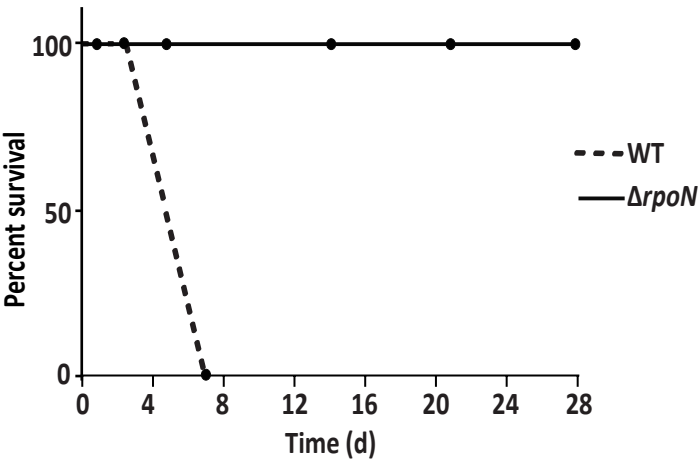
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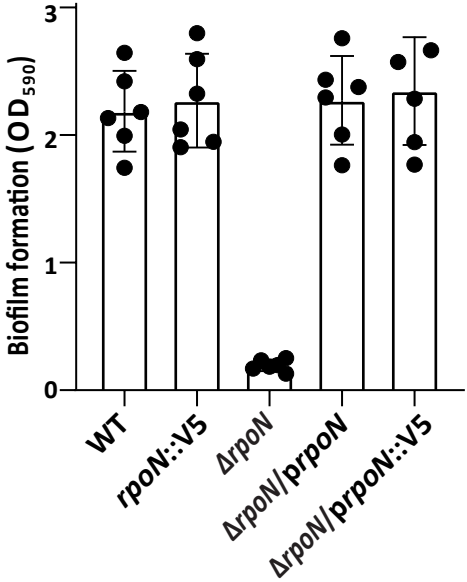
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Figure 1

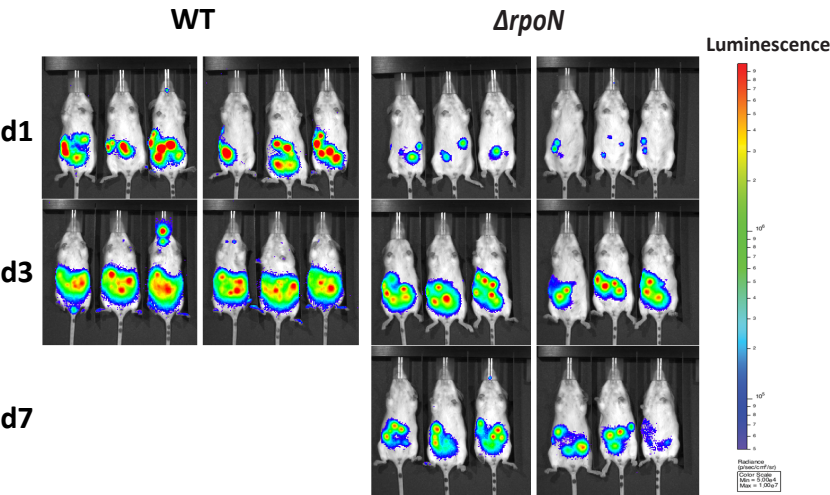
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(C)



(B)



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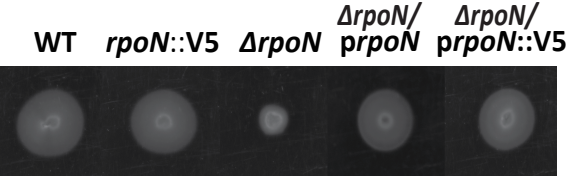


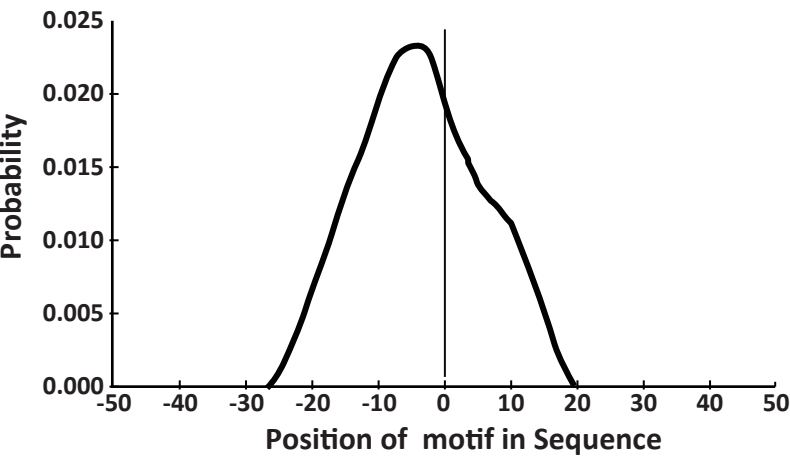


Figure 2

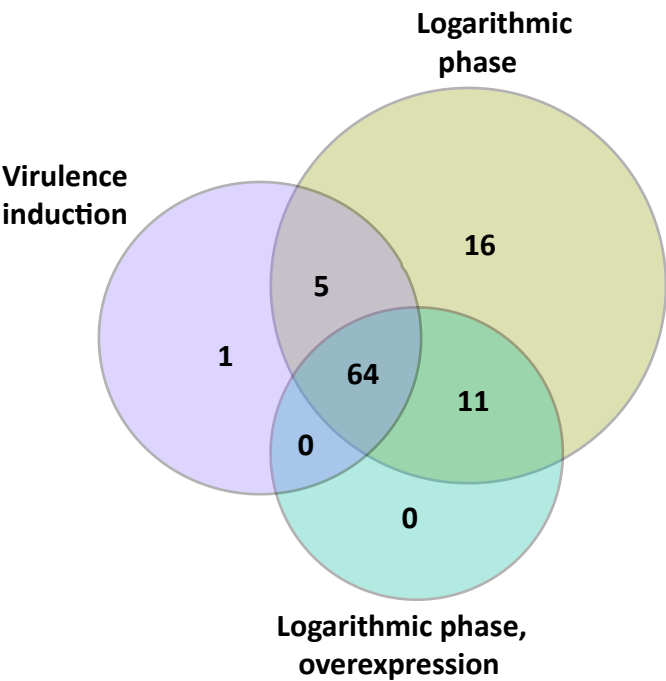
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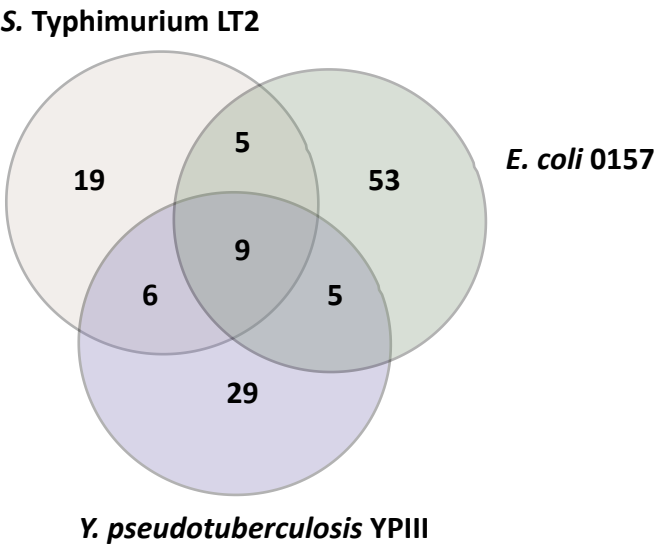
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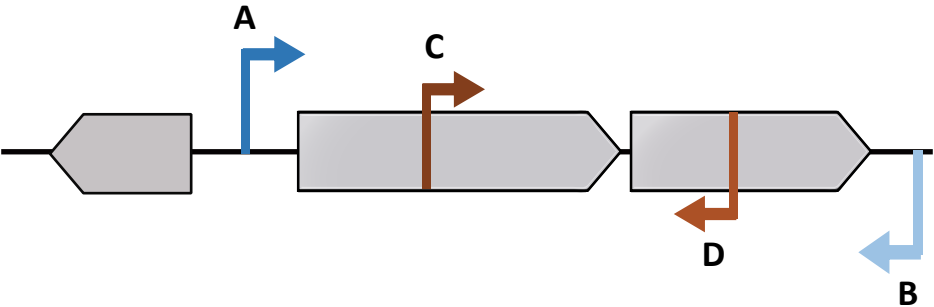
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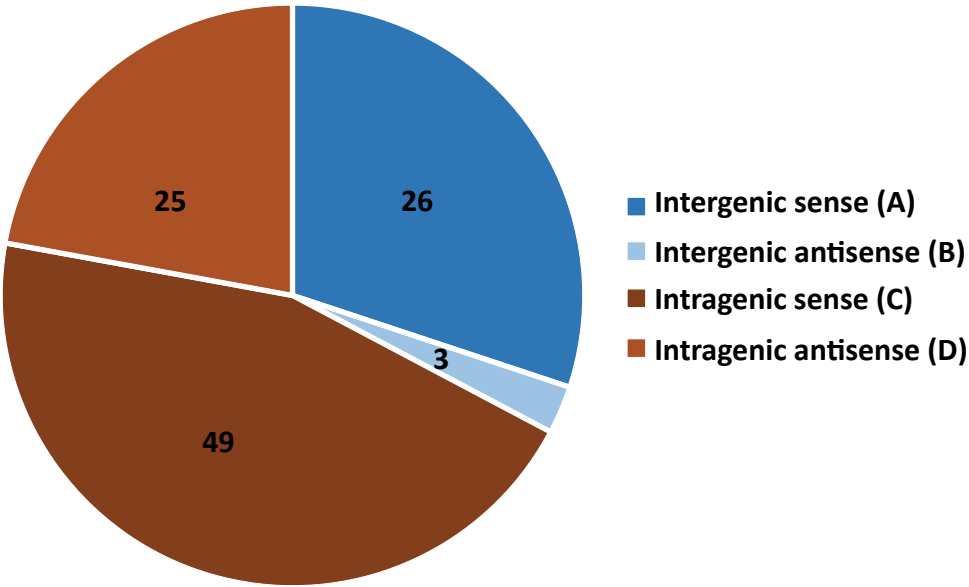


Figure 3

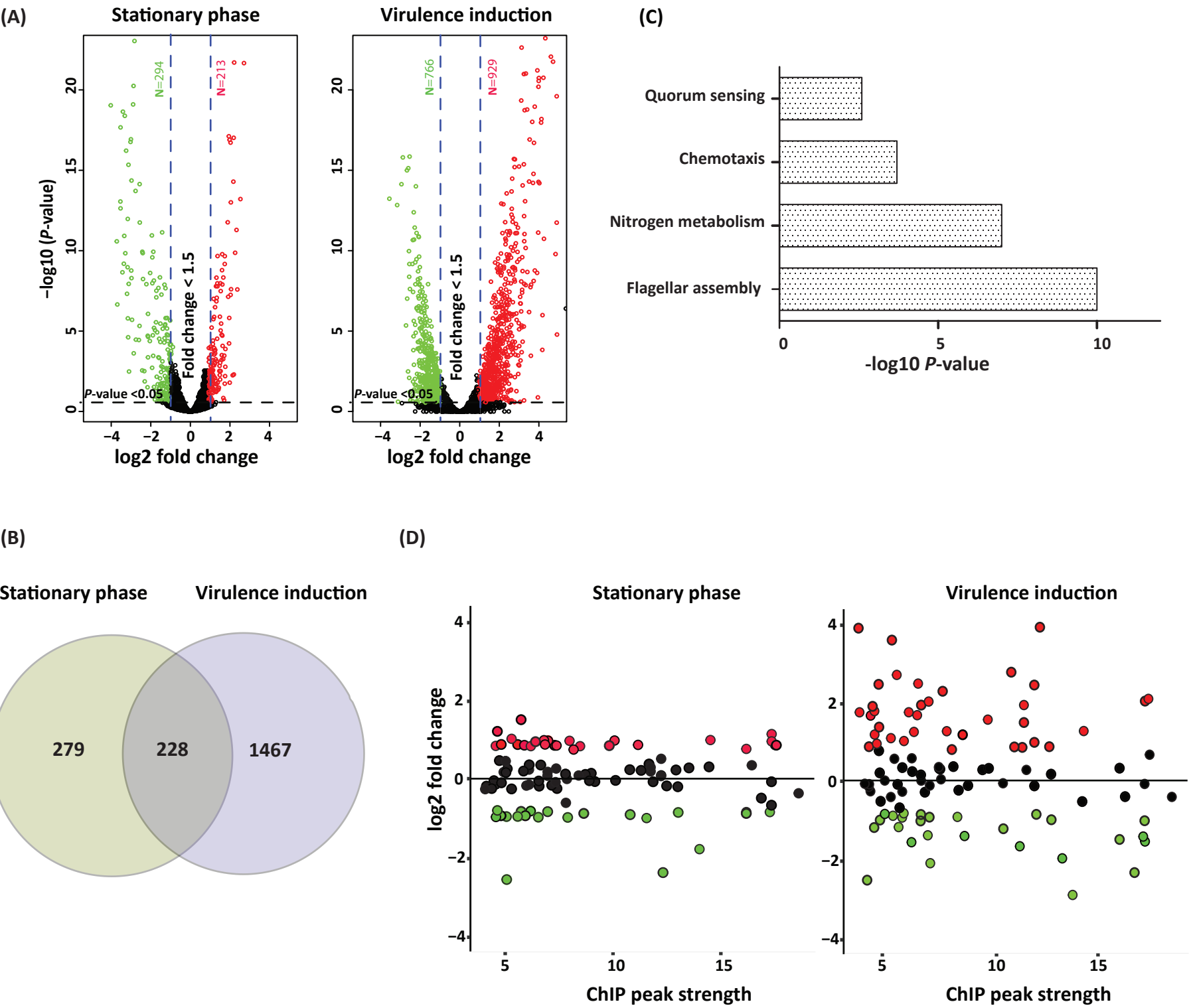
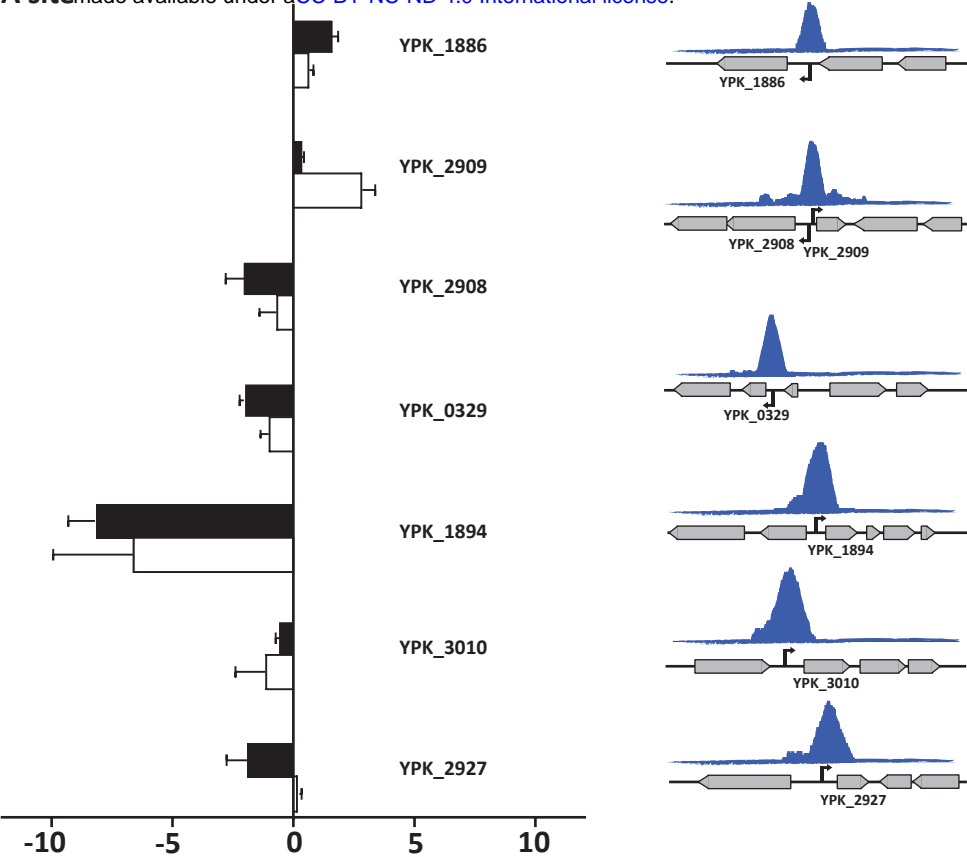
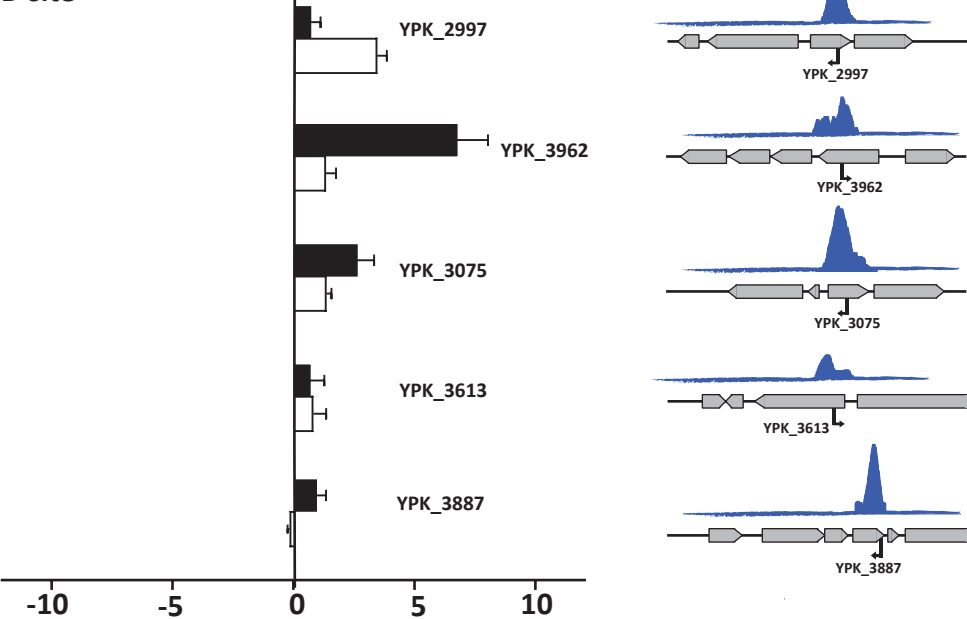


Figure 4

A site



D site



Relative expression compared to WT (log2 fold)

**Table 1: Intergenic sense (A) and antisense (B) RpoN binding sites**

**Intergenic sense (A sites)**

Peak	PC	PSL	PSI	PSO	Motif Sequence	Strand (+/-)	PSSM	Associated $\sigma$ factor	Downstream gene	Distance from PC
IGS1	117503	10.6	13.9	9.3	CTGGCTTGATTATGCAA	+	10.26	NA	YPK_0100	NA
IGS2	353423	9.6	11.0	8.7	TTGGCTTGTTATTGCTT	-	11.14	NA	YPK_0329	NA
IGS3	779812	10.5	11.5	8.6	CTGGCAGCTTAGTTGCAT	+	11.29	NA	YPK_0719	NA
IGS4	1127431	7.7	5.8	7.1	TAGGCTTGGATCTTGCTT	+	10.15	NA	YPK_1001	NA
IGS5	1384233	13.1	14.2	9.3	TTGGCAGCTAACTGCAT	+	10.82	NA	YPK_1257, YPK_1258 <sup>S</sup>	NA
IGS6	1469648	5.2	NA	3.4	CTGGCATGAGTCGTGCTC	-	10.08	$\sigma$ 24,54,70	YPK_1333	29,-70
IGS7	1700633	7.4	4.9	9.7	CTGGCAGCTATTATGCAA	+	10.75	$\sigma$ 54,70	YPK_1538	69
IGS8	1770585	5.9	4.4	7.3	CTGGCAGCATCTTTTCAT	-	10.32	$\sigma$ 70,54	YPK_1600 <sup>E,S</sup>	-23
IGS9	2092867	16.8	8.4	6.5	TGGGCACGCAATTTGCCT	-	10.54	$\sigma$ 70,28,54	YPK_1886	-28,-127
IGS10	2101765	16.5	15.0	13.5	TTGGCAGCACTCTTGATT	+	11.44	$\sigma$ 70,54	YPK_1894 <sup>E,S,V</sup>	-37
IGS11	2153679	4.7	5.4	NA	CTGGTGCAGATTTTGAG	-	9.6	$\sigma$ 70,24,54	YPK_1940	-4,-14
IGS12	2194598	16.6	16.8	13.8	TTGGCAGCACTAATGCTT	-	10.96	$\sigma$ 38,70,54	YPK_1974	-14,-43
IGS12	2194598	16.6	16.8	13.8	AGGGGTTAATATTTGCGT	+	9.34	$\sigma$ 54,70,38	YPK_1975	61,141
IGS13	2298808	7.2	3.5	5.2	TTGGGATGGTTGATGCAT	-	10.22	$\sigma$ 54,70	YPK_2070	39
IGS13	2298808	7.2	3.5	5.2	TTGGAATAGTTGATGCAT	-	9.94	$\sigma$ 54,70	YPK_2070	39
IGS14	2473234	4.3	NA	4.5	GTGGCAGCAAGCTGCTG	-	10	$\sigma$ 54,24,38,70	YPK_2229 <sup>E,S</sup>	12,38,52
IGS15	2990747	16.9	7.7	8.0	ATGGCATCTTATTGCTC	+	10.51	$\sigma$ 54,38,70,	YPK_2707	6,31
IGS16	3172389	16.6	14.6	11.7	TTGGCATGGCAATTGCGC	-	10.63	NA	YPK_2873	NA
IGS17	3205379	15.4	15.3	11.8	ATGGCATGATAATTGCTT	-	11.42	$\sigma$ 24,54,70	YPK_2908 <sup>E,S</sup>	44,-44
IGS17	3205379	15.4	15.3	11.8	TTGGCATAGGAATTGCCT	+	10.63	$\sigma$ 54,24,70	YPK_2909 <sup>E,S</sup>	66,104
IGS18	3225401	16.1	9.7	8.8	TTGGTCCAAGAGTTGCTT	+	10.11	NA	YPK_2927	NA
IGS19	3300593	8.9	7.7	6.2	TTGGCGTGTTTTTGAT	+	11.21	$\sigma$ 54,70	YPK_3001	39
IGS20	3311541	12.6	13.3	10.6	CTGGCACAAACCTGCAT	+	10.73	$\sigma$ 54,70,38,24	YPK_3010 <sup>S</sup>	15,17,23
IGS20	3311541	12.6	13.3	10.6	ATGGCTGCTTTACTGCTT	+	9.24	$\sigma$ 54,70,38,24	YPK_3010 <sup>S</sup>	15,17,23
IGS20	3311541	12.6	13.3	10.6	CAGGGCTATATCCGCTT	+	8.12	$\sigma$ 54,70,38,24	YPK_3010 <sup>S</sup>	15,17,23
IGS21	3520103	13.5	9.2	10.8	ATGGCATAGCCTTTGCTT	-	10.36	NA	YPK_3220 <sup>E,S</sup>	NA
IGS22	3650370	3.6	NA	2.7	TTGGCATGGTACTGCAA	-	11.11	$\sigma$ 24,70,54	YPK_3329	-29,-39
IGS23	3651607	11.2	6.3	5.4	TTGTCAAGTTTCGTGCTG	-	10.18	$\sigma$ 32,24,54,70	YPK_3330	-40,-9,54
IGS23	3651607	11.2	6.3	5.4	CTGGAACAGCTCTTGCTT	+	10.08	$\sigma$ 54,70	YPK_3331	60
IGS24	4250134	12.0	13.5	10.1	TTGGCACGTTTCTTGTA	-	10.43	NA	YPK_3857 <sup>S,E</sup>	NA
IGS25	4358596	5.6	3.3	4.0	TTGGCGCGATTCATGCTT	-	10.45	$\sigma$ 70,54,38	YPK_3950	-154,136
IGS25	4358596	5.6	3.3	4.0	GAGGCATGAATCGCGCCA	+	8.2	NA	YPK_3951	NA
IGS26	4621308	10.7	13.2	9.0	TTGGTGCCTCATTTGCGC	-	9.18	NA	YPK_4188	NA
IGS26	4621308	10.7	13.2	9.0	TTGGCATAGATTTGCGAA	+	9.96	$\sigma$ 70,28,54,24	YPK_4189 <sup>E,S,V</sup>	-112,-169,178

**Intergenic antisense (B sites)**

Peak	PC	PSL	PSI	PSO	Motif Sequence	Strand (+/-)	PSSM	Associated $\sigma$ factor	Downstream gene	Distance from PC
IGAs1	2892926	5.4	5.2	4.6	TTGGCGTGAATTTGCGC	-	10.68	$\sigma$ 32,54,28	YPK_2636	-17,14
IGAs2	3172389	16.6	14.6	11.7	AGGGCATAAACGGTGCAA	+	8.71	NA	YPK_2874	NA
IGAs3	4432739	9.6	11.8	8.2	CTGGCAGCTAAGTGCAA	-	10.25	NA	YPK_4019	NA

IGS=Intergenic Sense

IGAs=Intergenic Antisense

PC= Peak Center base pair

PSL=Peak Strength Logarithmic phase

PSI=Peak Strength Induction

PSO=Peak Strength Over express of RpoN

PSSM= Position Specific Score Matrix

X<sup>E/S/V</sup> - Prefix's indicates existence of RpoN binding motif in similar location in relation to orthologues genes in (E) *E.coli*, (V) *V.cholerae*, (S) *S.Typhimurium*

**Table 2: Intragenic sense (C) and antisense (D) RpoN binding sites**

**Intragenic sense (C sites)**

Peak	PC	PSL	PSI	PSO	Motif Sequence	Strand (+/-)	PSSM	Associated $\sigma$ factor	CDS	Downstream gene	Distance from PC
IrGS1	173082	7.9	3.8	4.3	ATGGCACGATTAATGCCA	+	10.32	$\sigma$ 54,32,28	YPK_0145 <sup>S</sup>	YPK_147,YPK_148,YPK_149	52,140
IrGS2	506362	6.0	5.9	4.9	ATGGTCCGTTTATTGCGT	+	10.2	$\sigma$ 54,32,24	YPK_0464	YPK_0465	85,123
IrGS3	535257	3.8	NA	NA	ATGGCACAAAATGTGCTG	-	10.09	$\sigma$ 54,24,32	YPK_0482	YPK_0481	57,187
IrGS4	613541	10.8	5.5	7.0	TTGGCTCGATTATGCGT	-	10.74	$\sigma$ 54,70,24	YPK_0560	YPK_0559	4,21
IrGS5	653552	6.5	5.3	3.3	ATGGCGCATTTATGCGT	+	9.99	$\sigma$ 54,32	YPK_0589	YPK_0591,YPK_0592	42
IrGS6	818023	4.2	NA	NA	GCGGCATGGTTGTGCAA	-	9.99	$\sigma$ 38,54,70,28,32	YPK_0744	NA	-16,35,36,39
IrGS7	821601	4.7	2.9	3.3	GCGGCATGGTTGTGCAA	-	9.99	$\sigma$ 54,28,32	YPK_0746	YPK_0745	175,132
IrGS8	940388	4.1	NA	3.4	TTGGCATTGTTGATGCTC	+	10.11	$\sigma$ 24,28,54,38	YPK_0824	YPK_0825	-26,-26,64,
IrGS9	1141660	6.5	4.2	3.4	TTGGTATGATTATGCCT	+	10.55	$\sigma$ 54,70,38,32,24	YPK_1016	YPK_1018	35,36,76,78
IrGS10	1225391	3.3	NA	NA	TTGGCTGAAACTGCGAG	+	9.88	$\sigma$ 54,24	YPK_1084	YPK_1085	28
IrGS11	1308478	3.5	NA	NA	CAGGCATGGATTATGCAA	+	9.59	$\sigma$ 70,24,32,38,54	YPK_1170	YPK_1171, YPK_1172	-4,-9,-19,-27
IrGS12	1475860	6.0	4.2	3.5	TTGCCCCACGCCTTGCTT	+	9.92	$\sigma$ 24,54,28	YPK_1338	YPK_1339	-13,16
IrGS13	1528613	6.6	4.6	3.7	CCGGCACGTTTTGTGCGAG	+	9.86	$\sigma$ 24,54,38	YPK_1386	YPK_1387	-19,14
IrGS14	1605153	18.0	9.3	9.0	TTGGTCTGACTTTTGCTT	-	10.42	$\sigma$ 54,32,38	YPK_1454	YPK_1452, YPK_1453	21,45
IrGS15	1930700	3.6	NA	2.8	TTGGCGGGAATATTGCTT	-	10.69	$\sigma$ 54,28	YPK_1742	NA	86
IrGS16	2058280	6.7	4.8	3.7	TTGGTATGTTATTGCAA	+	10.68	$\sigma$ 24,28,54,38,70	YPK_1853	NA	-44,-44,22,135
IrGS16	2058280	6.7	4.8	3.7	TAAGCATGAAACGTGCAT	+	9.04	$\sigma$ 32,70,54,38,28	YPK_1853	NA	-44,-44,22,135
IrGS17	2211354	7.4	3.4	4.5	TTGGTACATTATTGCGC	-	10.39	$\sigma$ 24,54,38	YPK_1988	NA	-49,123
IrGS18	2408285	4.5	NA	3.6	CTGGCACGCTGATGCAA	+	9.98	$\sigma$ 32,54	YPK_2169	YPK_2170,YPK_2171	-19
IrGS19	2536664	7.6	4.1	5.5	TTGGCATGGTAATTGAAT	-	10.65	$\sigma$ 24,54,28,32	YPK_2282	YPK_2281	-44,7,8
IrGS20	2598060	7.1	4.7	6.2	TTGGCACAAAGCTTGCTC	-	10.6	$\sigma$ 32,54,28,24	YPK_2364	YPK_2362	-16,4,15
IrGS21	2599974	6.9	7.5	6.3	CTGGCACGTAAATTGTAT	-	9.67	$\sigma$ 24,70,54,28,32	YPK_2365	YPK_2364	-30,-50,100,112
IrGS22	2663968	11.3	6.5	5.8	TTGGCCCGCTTCTTGCGC	-	10.55	$\sigma$ 70,24,54,28,32	YPK_2431 <sup>V</sup>	YPK_2429 <sup>S</sup> , YPK_2430	-29,-31,32,46
IrGS23	2760688	8.9	6.0	3.7	ACGGAGCACTTCTGCGAT	-	9.25	$\sigma$ 54,24,32	YPK_2519	YPK_2517, YPK_2518	69,120
IrGS24	2815385	3.0	NA	NA	ACGGTATAATTATTGCGT	-	10.04	$\sigma$ 28,54,70	YPK_2564	NA	-1,70
IrGS25	2870433	3.2	NA	NA	ATGGTATAAAAATTGCGC	-	9.85	NA	YPK_2615	NA	NA
IrGS26	2978162	15.7	8.2	7.7	TTGGCACGATTGATGCTC	-	10.88	$\sigma$ 70,28,38,54	YPK_2696	YPK_2695	-26,-53,-86,
IrGS27	3075166	4.1	3.3	2.2	CTGGCTTAATGTTTGCAT	+	10.49	NA	YPK_2785	YPK_2786,YPK_2787	NA
IrGS28	3195552	4.4	NA	NA	CTGGCAAAAATTATGCCC	+	9.63	$\sigma$ 32,38,28,54,24	YPK_2901	YPK_2902	-7,-24,-44,146,
IrGS29	3266581	3.4	NA	NA	CTGGAACGTTATTGCGAG	-	10.42	$\sigma$ 38,32,54,24,70	YPK_2968	YPK_2967	-1,-13,20,148,
IrGS30	3276693	3.3	NA	3.1	TTGGCATCAAAGTTGCCG	+	10.29	$\sigma$ 54,38,24	YPK_2976	YPK_2977	67,147
IrGS31	3386998	4.7	NA	2.9	TCGGCCCGTTTATTGCTC	-	10.04	$\sigma$ 54,28,32,38,28	YPK_3080	YPK_3079	44,86,91,
IrGS32	3448213	2.6	2.4	NA	GTGGCGCGTTATTGCGT	-	10.49	$\sigma$ 28,24	YPK_3152	NA	80,152
IrGS33	3491164	5.6	4.1	3.5	GTGGAACAGGTTTTGCAC	-	9.94	$\sigma$ 28,32,54,70	YPK_3192	YPK_3191	-5,-10,181,
IrGS34	3558532	6.5	5.8	4.2	TTGGTACGTACTTGCTC	+	10.66	$\sigma$ 28,54,32,38,24	YPK_3253	NA	-44,73,89,95
IrGS35	3621908	4.7	5.0	4.4	CTGGCAAAATTTCTGAAA	-	9.43	$\sigma$ 54,32,28	YPK_3308	YPK_3307	2,57
IrGS36	3658670	6.0	5.5	3.9	TTGGCACAAACGTGCGT	+	10.39	$\sigma$ 54,32,38	YPK_3338	YPK_3339	137,198
IrGS37	3674947	4.2	5.4	3.6	ATGGAACAGGACTTGCGAT	-	9.85	$\sigma$ 24,54	YPK_3349	NA	-42
IrGS38	3948038	5.0	3.0	2.3	CCGGCCCGCATGATGCCG	-	8.67	$\sigma$ 54,38,24	YPK_3583	NA	10,38
IrGS39	4007370	3.1	NA	NA	TTGGTGCAGATTATGCGAG	-	9.57	$\sigma$ 38,24,54	YPK_3630	YPK_3629	-30,-197
IrGS40	4106580	3.0	NA	2.5	CTGGCACCAGTTATGCGT	-	10.55	$\sigma$ 24,38,32,54,28	YPK_3718	YPK_3717	-47,-49,-50,107,
IrGS41	4186557	5.2	3.9	3.1	AAGGGGCAATCTTGCAA	-	8.94	$\sigma$ 38,54,24	YPK_3799	YPK_3798	-134,194
IrGS42	4216234	11.9	9.6	7.5	TTGGTATAGGTTTTCGAG	+	10.12	$\sigma$ 32,54	YPK_3826	YPK_3827,YPK_3828	-167
IrGS43	4341322	3.8	NA	NA	ATGGCACAGTTACTGCAG	+	7.68	NA	YPK_3934	NA	NA
IrGS44	4441905	10.2	14.2	9.2	TTGGCACGGAAAATGCTA	-	10.46	$\sigma$ 24,32,54	YPK_4027 <sup>S</sup>	YPK_4026	-104,-137
IrGS45	4448335	8.6	11.2	7.7	TTGGCATTGTCTTGCTT	-	11.07	$\sigma$ 24,54	YPK_4033	YPK_4032 <sup>E,S</sup>	-148
IrGS46	4561060	5.1	2.7	3.2	TTGGTATGGTTTTGCGCG	-	9.67	$\sigma$ 32,54,70	YPK_4127	YPK_4126	-179; 123
IrGS47	4584577	4.5	4.3	3.3	TTGGTACGGGTTTGCGT	+	10.07	$\sigma$ 54,70,24	YPK_4151	NA	133;191
IrGS48	4602274	5.6	4.2	4.0	TTGGCGCACAAATTGCTC	+	10.14	NA	YPK_4174	NA	NA
IrGS49	4612122	5.6	4.2	4.0	ATGGCACGCTAGTTGCGG	-	10.55	$\sigma$ 54,70,24	YPK_4181 <sup>V</sup>	NA	173;123

## Intragenic antisense (D sites)

Peak	PC	PSL	PSI	PSO	Motif Sequence	Strand (+/-)	PSSM	Associated $\sigma$ factor	CDS	Downstream gene	Distance from PC
IrGAs1	128696	3.0	NA	NA	CTGGCCCAATACATGCAT	-	10.06	$\sigma$ 54,28,24,32	YPK_0107	YPK_105,YPK_106	2,11,27
IrGAs2	318299	NA	2.9	NA	CTGGCTTGTCGGATGCAC	-	9.06	$\sigma$ 38,54,32	YPK_0280	YPK_0279	-44,132
IrGAs3	549836	4.7	3.3	NA	TTGGTCCAGTAATTGCTG	+	9.9	$\sigma$ 54,32,38	YPK_0496	YPK_0495	15,164
IrGAs4	630656	3.3	2.6	NA	ACGGCTCAATTGTGCAT	-	9.97	$\sigma$ 24,54,32,28	YPK_0712	YPK_0713,YPK_0714	-19,20,140
IrGAs5	1053922	3.6	NA	NA	CAGGCACGATACGTGCAT	+	10.12	$\sigma$ 54,24,38	YPK_0933	NA	168,172
IrGAs6	1099750	2.8	NA	NA	CCGGCATGCATCATGCAC	+	9.39	$\sigma$ 24,54,38	YPK_0978	YPK_0979	-8,159
IrGAs7	1204307	2.5	NA	NA	TTGGTACAGTTTTTCCA	-	9.45	$\sigma$ 54,32,28	YPK_1066	YPK_1065	109,112
IrGAs8	1265403	12.0	7.4	8.0	TTGGCATGCATATTGCGC	-	10.85	$\sigma$ 38,54,24,32,70	YPK_1125	YPK_1126	-27,4,8,21
IrGAs9	1357208	5.8	4.3	3.1	CTGGCATGTCAATTGCTG	-	10.58	$\sigma$ 54,70,38,28	YPK_1228	NA	63,152,182
IrGAs10	1894351	3.5	NA	NA	TGGGCACACTCGTTGCAT	-	10.02	$\sigma$ 54,28,24,38	YPK_1706	NA	42,44,167
IrGAs11	2058280	6.7	4.8	3.7	AATGCACGTTTCATGCTT	-	9.09	$\sigma$ 54,28,32,38	YPK_1853	NA	67,152,182
IrGAs12	2760688	8.9	6.0	3.7	TAGGGTTTCTGGCTGCGG	+	7.27	$\sigma$ 32,24,54,28	YPK_2519	YPK_2520	-39,-22,128
IrGAs13	3195552	4.2	NA	3.5	TGGGCATAATTTTTGCCA	-	10.6	$\sigma$ 54,28	YPK_2901	NA	19
IrGAs14	3296918	10.0	8.8	7.9	TAGGCACGAGATTTCAT	-	10.73	$\sigma$ 54,32,28,38	YPK_2997	YPK_2996 <sup>E</sup>	168,189,199
IrGAs15	3347078	3.5	NA	NA	TTGGCAAGAAATCTGCAC	+	10.74	$\sigma$ 38,70,54,28	YPK_3045	NA	-5,-7,3
IrGAs16	3383005	13.6	11.8	9.3	ATGGCACGGATTTTGCCC	-	10.84	$\sigma$ 38,24,54,70	YPK_3075	YPK_3074	-43,-50,72,
IrGAs17	3435112	5.4	3.7	NA	AAGGCACGATAGTTGCGT	-	10.48	$\sigma$ 54,28,32,38	YPK_3144	NA	52,104,119
IrGAs18	3603190	3.1	NA	NA	CTGGCACTGATATTGCCG	-	10.17	$\sigma$ 70,32,54,38	YPK_3293	NA	-26,-8,187
IrGAs19	3621908	4.7	5.0	4.4	ATGGCATCAATATTGCTA	+	10.46	$\sigma$ 54,32,28	YPK_3308	YPK_3309	106,113
IrGAs20	3984487	6.0	5.0	3.6	CTGGCTTAAATCTGCGT	+	10.39	$\sigma$ 54,24,38,32	YPK_3613	YPK_3614	75,110,125
IrGAs21	4135862	4.4	NA	3.5	CTGGCACGCTTTCTGCAA	+	10.55	$\sigma$ 32,54,24,38	YPK_3743 <sup>E</sup>	NA	-15,22,123
IrGAs22	4186557	5.2	3.9	3.1	CAGGAACGGATCTTGCAA	+	9.38	NA	YPK_3799	NA	
IrGAs23	4288435	11.2	13.7	10.2	CTGGCTCAATTAATGCAT	-	10.33	$\sigma$ 24,28,54	YPK_3887	NA	-102,-178
IrGAs24	4309656	5.1	5.0	3.4	CTGGTGTAATATTGCAC	+	9.89	NA	YPK_3909	NA	
IrGAs25	4371420	11.5	7.9	5.2	GCGGCGCATTTTTTGCAT	+	9.83	$\sigma$ 24,32,54	YPK_3962	NA	-143,-198

IrGAs=Intergenic Sense

IGAs=Intergenic antisense

PC= Peak Center base pair

PSL=Peak Strength Logarithmic phase

PSI=Peak Strength Induction condition

PSO=Peak Strength Over express of RpoN

PSSM= Position Specific Score Matrix

X<sup>E/S/V</sup>- Prefix's indicates existence of RpoN binding motif in similar location in relation to orthologues genes in (E) *E.coli*, (V) *V.cholerae*, (S) *S. Typhimurium*



**Table 3: RpoN binding motif with similar location in relation to orthologues genes**

Peaks	Locus Tag	Description	Type	<i>Y. pseudotuberculosis</i>	<i>E. coli.</i>	<i>V. cholerae</i>	<i>S. Typhimurium</i>
IGS8	YPK_1600	Glutamine ABC transporter periplasmic protein, GlnH	A	✓	✓		✓
IGS10	YPK_1894	Phage shock protein, PspA	A	✓	✓	✓	✓
IGS14	YPK_2229	Bifunctional succinylornithine aminotransferase, AstC	A	✓	✓		✓
IGS17	YPK_2908	Sensor kinase, ZraS	A	✓	✓		✓
IGS17	YPK_2909	Zinc resistance protein, ZraP	A	✓	✓		✓
IGS21	YPK_3220	Nitrogen regulatory protein P-II 2, GlnK	A	✓	✓		✓
IGS24	YPK_3857	Phage shock protein G, PspG	A	✓	✓		✓
IGS26	YPK_4189	Glutamine synthetase, GlnA (Nitrogen metabolism)	A	✓	✓	✓	✓
IrGS45	YPK_4032	Lipopolysaccharide biosynthesis protein, WzzE	C	✓	✓		✓

IGS=Intergenic Sense

IrGS=Intragenic Sense

Locus Tag = Locus tag of the gene in relation to predicted RpoN binding site in *Y. pseudotuberculosis* YPIII

✓ = Reported RpoN binding motif with similar location in relation to orthologues genes