Putative horizontally acquired genes, highly transcribed during *Yersinia pestis* flea infection, are induced by hyperosmotic stress and function in aromatic amino acid metabolism.

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ABSTRACT

While alternating between insects and mammals during its lifecycle, *Yersinia pestis*, the flea transmitted bacterium that causes plague, regulates its gene expression appropriately to adapt to these two physiologically disparate host environments. In fleas competent to transmit *Y. pestis*, low GC content genes y3555, y3551 and y3550 are highly transcribed, suggesting that these genes have a highly prioritized role in flea infection. Here we demonstrate that y3555, y3551 and y3550 are transcribed as part of a single polycistronic mRNA comprising the y3555-y3554-y3553-y355x-y3551-y3550 genes. Additionally, y355x-y3551-y3550 compose another operon while y3550 can be also transcribed as a monocistronic mRNA. Expression of these genes is induced by hyperosmotic salinity stress, which serves as an explicit environmental stimulus that initiates transcriptional activity from the predicted y3550 promoter. Y3555 has homology to PLP-dependent aromatic aminotransferases, while Y3550 and Y3551 are homologous to the Rid protein superfamily (YjgF/YER057c/UK114) members that forestall damage caused by reactive intermediates formed during PLP-dependent enzymatic activity. We demonstrate that y3551 specifically encodes an archetypal RidA protein with 2-aminoacrylate deaminase activity, but Y3550 lacks Rid deaminase function. Heterologous expression of y3555 generates a critical aspartate requirement in a *Salmonella enterica* aspC mutant while its *in vitro* expression, and specifically heterologous co-expression with y3550, enhances the growth rate of an *Escherichia coli* ΔaspC ΔtyrB mutant in a defined minimal amino acid supplemented medium. Our data suggest that y3555-y3551-y3550 genes operate cooperatively to optimize aromatic amino acid metabolism and are induced under conditions of hyperosmotic salinity stress.
Distinct gene repertoires are expressed during *Y. pestis* infection of its flea and mammalian hosts. The function of many of these genes remain predicted or unknown; necessitating their characterization as this may provide better understanding of *Y. pestis* specialized biological adaptations to the discrete environments of its two hosts. This study provides functional context to adjacently clustered horizontally acquired genes predominantly expressed in the flea host, by deciphering their fundamental processes with regards to (i) transcriptional organization, (ii) transcription activation signals, and (iii) biochemical function. Our data support a role for these genes in osmoadaptation and aromatic amino acid metabolism, highlighting these as preferential processes to which *Y. pestis* gene expression is modulated during flea infection.
INTRODUCTION

Plague, caused by Yersinia pestis, is a severe zoonotic disease infamous for having caused the death of millions of people in three major pandemics. This disease primarily affects rodents which become infected mainly through blood feeding by rodent-associated infected fleas. Transmission by fleas is a recent adaptation that has likely evolved in the last 1,500 to 20,000 years during transformation of Y. pestis from its clonal ancestor, Yersinia pseudotuberculosis, which is transmitted by a fecal-oral route and causes a mild enteric self-resolving illness (1-3). Central to the biological mechanism of transmission via flea bite is Y. pestis biofilm blockage of the flea foregut proventriculus (4-6).

After acquisition from the blood of a highly bacteremic host, Y. pestis must adapt to the insect gut environment to multiply and form biofilm-mediated blockage by seven days post-infection (7). The various physicochemical stresses Y. pestis encounters in the flea gut such as low pH, reactive oxygen species (ROS), hyperosmolarity, nutrient-limitation and the immune response are slowly being uncovered along with how Y. pestis overcomes such stresses (8, 9). For example reactive oxygen species (ROS) have been demonstrated to limit Y. pestis survival during early flea infection (10) but this oxidative stress can be counteracted by modulation of antioxidant defenses by the Y. pestis OxyR transcriptional regulator (10). The flea gut was determined as a mildly acidic environment with a pH of ~6.5 to 6.8 (11). Implicated in adapting to this mild acidic condition is the PhoP transcriptional regulator (12) required to maintain optimal blockage and competitive fitness in fleas, and that modulates transcription of genes required for acid stress (12-14).
Moreover, hyperosmotic stress conditions occur in the flea gut as its content is measured to be ~500 mOsm, much greater than that of mouse blood at 320 mOsm (11). However, genes of *Y. pestis* belonging to pathways involved in alleviating osmotic stress are robustly expressed during flea infection (15-17). Examples of these are (i) genes belonging to the uptake and catabolic pathways of the L-glu amino acid group (his, glu, pro, and arg) which encodes proteins that generate the osmoprotectant glutamate (17) and, (ii) the *betT* and *proVWX* genes of the synthesis and transport system operons for the osmoprotectant glycine-betaine, respectively (15). In addition, the two component signal transduction regulatory system OmpR-EnvZ that is involved in sensing and mediating adaptation to osmolarity and pH stresses in many bacterial species (18-21) including *Y. pestis* (22-24) is shown to be required for efficient flea blockage (11).

To further understand the flea host specific response of *Y. pestis* we turned our attention to a cluster of genes (*y3555, y3551* and *y3550*) with lower %GC content (39.8%) than that typically noted for *Y. pestis* (48%) genes, and that ranked in the top 150 most highly expressed genes in the flea (17). Close inspection of the chromosomal context of *y3555, y3551* and *y3550* revealed that they are located adjacent to three other genes (*y3554, y3553, y355x*) having similarly low GC content. The *y3554, y3553, y355x* genes showed robust expression in the flea gut (9, 17). The *y355x* gene is not annotated as such in the *Y. pestis* KIM10+ genome but it is 100% identical to *ypo0625* of the *Y. pestis* CO92 genome to which the microarrays were developed for *Y. pestis* flea gut infection transcriptomics studies (12, 17). All six of these low GC content genes were either poorly expressed or not detected at mammalian conditions. Genes sharing 99-100% identity to *y3555, y3554, y3553, y355x, y3551* and *y3550* are present in *Y. pseudotuberculosis*. Because genetic loss is thought to drive clonal evolution enabling emergence of *Y. pestis* and its flea-borne transmissibility (25-28), we
reasoned that evolutionary conservation of encoded traits of these six genes are likely advantageous for growth and survival within new environmental niches like the flea gut. This study therefore aimed to understand the transcriptional organization and activation, as well as the role of the y3555, y3554, y3553, y335x, y3551 and y3550 genes in flea colonization, with a specific emphasis on the biochemical roles of the most highly expressed genes y3555, y3550 and y3551. Our data reveals that while these preferentially expressed genes that have been evolutionarily maintained are not essential for flea colonization in the context of our experimental laboratory flea model of Y. pestis infection, they are nonetheless coordinately induced during hyperosmotic stress, contribute to aromatic amino acid metabolism, and y3555 confers enhanced growth fitness traits to Escherichia coli and Y. pestis in vitro.

RESULTS

In silico analysis of the genetic context and predicted function of Y. pestis KIM10+ genes y3555, y3554, y3553, y335x, y3551, and y3550

The lower %GC content of the gene cluster comprising y3555 (3,935,132..3,936,364), y3554 (3,933,688..3,935,139) y3553 (3,933,238..3,933,684), y355x (3,932,478..3,933,068), y3551 (3,932,082..3,932,462) and y3550 (3,931,393..3,931,797) suggest their acquisition by horizontal gene transfer. In silico analysis using FGENESB predicts that the continuous region of this genomic island comprises three transcriptional units with y3555-y3554-y3553 and y355x-y3551 constituting two separate operons, and y3550 a monocistronic transcriptional unit. Additionally, BPROM identifies distinct promoter sequences upstream of genes y3555,
BLAST searches were used to identify homologous proteins and conserved functional domains of genes comprising the genomic island.

The y3555 gene product exhibits homology to uncharacterized pyridoxal 5'-phosphate (PLP)-dependent transaminases, including aromatic and aspartate aminotransferases encoded in some bacteria. These PLP-dependent aminotransferases typically catalyze the interconversion of an aromatic amino acid and α-ketoglutarate to oxaloacetate and glutamate. The Y. pestis KIM10+ genome encodes two PLP-dependent aromatic aminotransferases, y2760 (aspC), and y0579 (tyrB).

The y3554 gene encodes a putative Na+/H+ antiporter (nhaC-type) of which there are two others (nhaA and nhaB) in the reported genome sequence of Y. pestis. A primary role for Na+/H+ cation exchange antiporters (29-31) is to extrude Na ions to counter-balance Na+ toxicity thereby protecting against hyperosmotic saline stress. The NhaA and NhaB antiporters are essential for Y. pestis virulence in a mouse model of septicemic plague (31, 32) where they are thought to function in protecting Y. pestis against Na+ toxicity in blood.

The y3553 gene product is predicted to belong to the PYR/PYL/RCAR-like family of proteins, a part of the SRPBCC (START/RHO_alpha_C/PITP/Bet_v1/CoxG/CalC) domain protein superfamily, that are known to bind hydrophobic ligands and mediate signal transduction (33, 34). PYR/PYL/RCAR proteins are particularly well characterized in plants where they bind phytohormones enabling its regulation of growth, development and environmental stress responses. For example, osmotic stress responses and high salt tolerance is mediated by PYR/PYL/RCAR proteins binding interactions with plant phytohormones (34-36).

The gene product of the y355x gene is predicted to be a transcriptional regulator because it contains a helix-turn-helix motif and Per-ARNT-Sim (PAS) sensory domain. PAS
domains are ubiquitous in bacterial proteins involved in signal transduction and enable sensing of physical and chemical stimuli like oxygen and redox potential (37).

The y3551 and y3550 gene products belong to the reactive intermediate deaminase (Rid) protein superfamily, originally called the YjgF/YER057c/UK114 family of proteins. Rid enzymes eliminate reactive intermediates that are produced by PLP-dependent enzymatic activity, which can alter the function of cellular targets and prevent inhibition of transaminases (38-40). Interestingly, some Rid encoding genes cluster with genes encoding PLP-dependent enzymes, like aminotransferases, which are susceptible to damage by the enamine, 2-aminoacrylate (2AA) (41). The clustering of y3551 and y3550 with y3555, predicted to encode an aminotransferase, is consistent with this clustering pattern.

Genes y3555, y3554, y3553, y3551 and y3550 are transcribed as a polycistronic mRNA

Gene clustering in bacteria often implies a functional association of its member genes. Previous transcriptomic analyses have shown that the y3555, y3554, y3553, y3551 and y3550 genes exhibit coordinated gene expression in the flea gut and under similar in vitro conditions (12, 17, 24). To determine if the coordinated expression of this genomic island can occur from a single long polycistronic mRNA, a two-step RT-PCR using Y. pestis parental cDNA to amplify the entire locus from y3555 to y3550 (Figure 1B) was performed. A negative control to verify absence of genomic DNA contamination was included by omitting reverse transcriptase during cDNA synthesis (Figure 1B, lane 7) and a positive control using Y. pestis chromosomal DNA as template with DNA polymerase verified the expected amplicon size (Figure 1B, lane 6). As shown in Figure 1B, a single 5,014 bp predicted fragment spanning all six genes from y3555
to y3550 was amplified. Together, these results confirmed that genes y3555-y3554-y3553-
y355x-y3551-y3550 are transcribed as a long single polycistronic mRNA transcript.

Next to determine if the predicted promoters upstream of y3555, y355x and y3550 drive expression of their respective downstream genes, transcriptional fusions spanning different regions of the genomic island to the luxCDABE reporter genes (Figure 1C) were used. Luminescence directed by the plasmid carrying these transcriptional fusions was determined in the parental Y. pestis strain grown at 26°C to simulate conditions within the flea. Strains harboring fusions to the promoter of y3555 (pCS::P55) and y355x (pCS::P5x) showed ~10² to 10³ fold higher levels of luminescence expression in comparison with the strains carrying the empty vector (pCS26-Pac) or transcriptional fusions with potential regulatory regions of y3554 (pCS::P54), y3553 (pCS::P53) and y3550 (pCS::P50). This suggests that only the upstream regulatory regions of y3555 and y355x, and not y3550 are able to promote gene transcription using flea-matched temperature testing conditions. To confirm this observation, we generated two other transcriptional fusion constructs that allowed testing of transcription directed from the predicted upstream regulatory regions of y3555 or y355x. Transcriptional fusion construct pCS::P55-54 contains the region covered in pCS::P54 extended up to the regulatory region of y3555, and construct pCS::P5x-51-50 contains the region of fusion pCS::P50 extended up to the regulatory region of y355x (Figure 1C). The expression of fusions pCS::P55-54 and pCS::P5x-51-50 was drastically increased and similar to that shown in fusions P55 and P5x, respectively. (Figure 1C). This suggested that the promoter located upstream of y3555 can drive expression of y3555-y3554-y3553 -y355x-y3551-y3550 as a single operon and that y355x-y3551-y3550 can be transcribed as another operon from the promoter located upstream of the y355x gene.
Neither single gene nor whole genomic island mutant strains exhibit fitness attenuation in competitive coinfection with the parental strain.

To test if the most highly expressed genes y3555 and y3550 contributed to efficient colonization and formation of proventricular blockage of the flea, we constructed and tested mutants lacking: y3555 or y3550 genes alone, y3551 and y3550, or y3550 and y3555, and the entire locus comprising y3555-y3554-y3553-y355x-y3551-y3550 (referred to as 'All' mutant). A previously reported y3555-y3554-y3553 mutant was also used (42). The six mutants displayed parental abilities to infect and block fleas (data not shown). Observing no difference in single flea infection, a more sensitive competitive coinfection assay (43) designed to discern the fitness of a mutant strain relative to the parental strain was performed. Fleas were coinfect ed with a 1:1 ratio of mutant to parental strain, harboring a kanamycin resistance cassette in the non-deleterious glmS-pstS site, and called WT glmS-pstS::kanR which was previously shown to exhibit equal fitness during competition with an unmarked parental strain (43). All mutants tested in coinfections with the glmS-pstS::kanR WT strain were maintained at equal rates to the glmS-pstS::kanR WT as reflected by constant occurrence of the glmS-pstS::kanR strain population at a mean~50% of the total population of coinfecting Y. pestis immediately after infection (T0) and at seven days (T7) post-infection (Figure 2). These in vivo assays showed the deletion mutations did not have attenuated fitness in the flea.

Predicted promoters encoded upstream of y3555, y355x and y3550 are responsive to hyperosmotic salinity stress.

In a previous study the genome-wide transcriptional responses of the enzootic strain Y. pestis biovar Microtus to hyperosmotic stress mediated by high salt found that genes...
homologous to *Y. pestis* KIM6+ genes y3555, y3554, y3553, y355x, y3551 and y3550 genes, are up-regulated (24). This study suggested that the genomic island is responsive to hyperosmotic stress. To determine if the y3555-y3554-y3553-y355x-y3551-3550 genes were induced in response to hyperosmolarity stress in the KIM6+ epidemic strain we exposed this strain to 0.5 M NaCl (reflects high osmolarity of 1000 mOsm) and determined steady state mRNA expression levels of these genes. Expression levels for genes y3555-y3554-y3553 showed between 1.5-2.0-fold increases, while expression of the genes determined to compose the second operon i.e. y355x-y3551-y3550, showed between 4-6-fold increases in gene expression (Figure 3A).

Additionally, induction of lux transcriptional reporter fusion activity was assessed for the pCS::P55, pCS::P5x and pCS::P50 transcriptional fusion reporter constructs to determine if reporter activity increased after NaCl exposure. This time readings were taken 100 min post exposure to 0.5 M NaCl (Figure 3B). Interestingly while the lux reporter activity from pCS::P55 and pCS::P5x showed 2- and 4-fold increases respectively, a remarkable 45-fold increase in lux reporter activity from pCS::P50 was observed following exposure to 0.5M NaCl. This data indicated that while the promoters upstream of y3555 and y355x promote transcription at flea-matched temperatures, they exhibit increased transcriptional activity in hyperosmotic/saline conditions, whereas the promoter upstream of y3550 is only active under hyperosmotic/saline conditions. RT-PCR analysis (Figure 1B) indicates that the genomic island continues to be expressed as a single long transcript following exposure to 0.5M NaCl.

Y3555 generates a critical requirement for aspartate in a *Salmonella enterica aspC* mutant
The ridA and aspC genes, encoding aspartate aminotransferases, cluster in many bacterial chromosomes (41). Some online genome resources including KEGG, Phyre2 (44), and BLAST predicts that Y3555 is an aspartate aminotransferase and for this reason aspartate was considered as a possible substrate for the enzyme. However, based on structural alignments it appears Y3555 has a glutamate, instead of an aspartate residue in the active site to coordinate the pyridine nitrogen of PLP. These initial alignments suggest Y3555 may be a fold type IV aminotransferase (45), not a fold type I aminotransferase like AspC (45) (Figure S1).

Nonetheless, to experimentally query if indeed Y3555 had predicted aspartate aminotransferase activity, the ability of y3555 to restore growth of a S. enterica aspC789::km mutant strain was tested. Growth of S. enterica aspC789::km mutant containing either the empty pTRc99A plasmid or plasmid pTRc_55, encoding an IPTG-inducible y3555 was determined (Figure 4). The S. enterica aspC789::km mutant has a growth defect in glucose minimal medium, since it relies on low level promiscuous aspartate aminotransferase activity of the tyrosine aminotransferase, TyrB. The S. enterica aspC789::km mutant strains containing either the empty pTRc99A plasmid or plasmid pTRc_55 had similar growth on minimal glucose medium with aspartate. Expression of the aspC gene from S. enterica allows full growth of the mutant strain (Figure S2). Surprisingly, in the absence of aspartate, addition of 100μM IPTG, presumed to induce expression of y3555, inhibited growth of the S. enterica aspC789::km mutant (Figure 4). This growth defect was eliminated by the addition of aspartate suggesting a critical aspartate requirement was facilitated by Y3555 expression. There are a few possibilities for this effect that cannot be distinguished by the data here, including that Y3555 could affect TyrB flux or produce tyrosine and phenylalanine that shut down TyrB expression and/or activity (46-48).
Y3551, but not Y3550 is a functional RidA deaminase

Proteins of the Rid superfamily are defined as belonging to the archetypal RidA subfamily or Rid1-7 subfamilies based on phylogenetic analysis (41). RidA enzymes hydrolyze reactive imine/enamine metabolic intermediates (i.e., 2-aminoacrylate (2AA)) (40), which are generated as obligate catalytic intermediates by some PLP-dependent enzymes. An active site arginine (Arg105) is critical for RidA imine-hydrolysis activity (40). Rid4-7 subfamily members lack the active site arginine and an ascribed biochemical function. Amino acid alignment of Y3550 and Y3551 sequences with Rid family homologs (Figure 5A) revealed that Y3551 had the requisite arginine in the active site. Y3550 is missing an active site arginine, contains a phenylalanine at position 17 rather than the typical tyrosine, and has a variable asparagine at position 88. These features of Y3550 conform with those described for Rid5 or Rid6 proteins (41).

To test if Y3551 and/or Y3550 had the deaminase activity conserved for RidA enzymes the S. enterica ridA1::tn10 (Tc) mutant strain was transformed with plasmid constructs, pET28b::ridA, pET28a::y3551 and pET28a::y3550. Minimal defined medium supplemented with serine was used for growth analysis. In the S. enterica ridA1::tn10 (Tc) mutant, 2AA is generated from serine by the serine/threonine dehydratase, IlvA (EC 4.3.1.19). In the absence of RidA, 2AA accumulates and damages some PLP-dependent enzymes which can result in growth defects (49). Growth was monitored to determine if Y3551 and/or Y3550 could restore growth to the S. enterica ridA1::tn10 (Tc) mutant. The S. enterica ridA1::tn10 (Tc) mutant strain carrying the empty vector control failed to grow in this medium, and as expected was complemented by the pET28b::ridA plasmid. Significantly, the pET28a::y3551 plasmid restored
significant growth to the strain indicating that Y3551 had 2AA deaminase activity (Figure 5B). However, the pET28a::y3550 plasmid failed to restore growth to the S. enterica ridA1::tn10 (Tc) mutant strain (Figure 5B). Control experiments confirmed that the addition of isoleucine restored full growth to all strains, confirming the weak growth was due to 2AA accumulation (data not shown).

To confirm that Y3551 had 2AA hydrolysis activity as indicated by the complementation analyses, assays were done with pure proteins. Cysteine desulphydrase (CdsH) converts cysteine to 2AA which can be deaminated to pyruvate by solvent water or a RidA enzyme. Thus, RidA activity is detected as an accelerated rate of pyruvate formation in a CdsH assay. The assay was performed using purified Y3551 and S. enterica RidA enzymes, and the data are in Figure 5C. In the presence of either purified S. enterica RidA or Y3551, the initial rate of pyruvate formation was accelerated over the control lacking a RidA when cysteine concentration was 1mM or greater (Figure 5C). These data confirmed that Y3551 is a RidA family enzyme with 2AA hydrolysis activity.

One other disparate biochemical activity previously assigned to Rid enzymes is that of an endoribonuclease (41, 50). The Y3551 and Y3550 proteins were in fact previously designated as L-PSP family endonucleases by BLAST analyses (12, 17). However, our purified Y3551 and Y3550 did not show any ribonuclease activity (data not shown) supporting that these proteins do not encode such a function in Y. pestis.

Overexpression of y3555 enhances growth of E. coli and Y. pestis

The uncharacterized protein, Y3555 enhanced the growth of Y. pestis strains grown in rich media. The Y. pestis y3555 and y3550 mutants expressing Y3555 from a high copy
number plasmid (pCR4-TOPO) grew significantly faster than the wild-type strain grown under the same conditions (Figure 6). A similar observation was made in an E. coli aspartate (aspC) and tyrosine (tyrB) aromatic aminotransferase double mutant that requires either aspartate or tyrosine for growth (46, 51). This strain called DG44 was used in early trial experiments replicating those in the S. enterica aspC359::km mutant, but was abandoned due to lack of genetic complementation tools. It has been suggested that Rid enzymes may be encoded with genes for PLP-dependent enzymes that are targets of 2AA damage, including branched-chain amino acid transaminases and aspartate amino transferases (41). These reports along with the gene synteny of y3550, y3551, and y3555 suggest that either y3551 or y3550 may prevent accumulation of a toxic metabolic intermediate of y3555. The improvement in growth observed with over-expression of y3555 presented an opportunity to test if y3550 or y3551 expression combined with that of y3555 could further improve growth kinetics of the DG44 strain. The DG44 strain was transformed with inducible plasmids carrying a single y3555, y3550 or y3551 gene, and either y3555 and y3550, or y3555 and y3551 genes. Significantly higher growth rate and biomass yield was observed for the DG44 strain expressing y3555 (μ=0.0203±0.0009), but not y3550 (μ=0.0110±0.0012), and y3551 (μ=0.0105±0.0002) relative to the empty vector (μ=0.0120±0.0009) containing strain (Figure 7). The co-expression of y3555 with y3550 (μ=0.0256±0.0005) or y3551 (μ=0.0154±0.0007) increased growth rate and biomass yield similar to that of the y3555 expressing strain, indicating that the increased growth rate and yield was y3555 dependent (Figure 7). However, highest growth rates occurred in the strain co-expressing y3550 and y3555 followed by the strain expressing y3555 alone, and next the strain co-expressing y3555 and y3551. The y3550 and y3555 co-expressing strain achieved overall higher biomass yield than all other strains tested except the
y3555 expressing strain. Strains expressing y3550 or y3551 alone grew at similar growth rates and biomass yields to the empty vector containing strain.

DISCUSSION

In this work we have determined that the coordinated expression of an uncharacterized cluster of low GC content genes, y3555-y3554-y3553-y355x-y3551-y3550, that show exceptionally robust expression during flea infection, is accounted for by their co-transcription as a single polycistronic mRNA transcript. Adjacently clustered genes are often grouped transcriptionally as an operon and frequently encode functionally related proteins. This feature allows protein synthesis to be controlled coordinately in response to the needs of the bacterium, only when and where they are required. While the genomic island is expressed only at flea-matched temperatures (16, 17), the further induction of these genes following exposure to hyperosmotic saline stress implicates these genes as functionally concerned with osmoadaptation during the Y. pestis flea life stage. Indeed, protein homology for the y3553 and y3554 gene products clearly predict that these proteins have direct roles in hypersaline osmotic stress adaptation. In the case of Y3553 active exchange of a sodium ion for a proton is predicted whereas Y3554 is likely involved in transducing osmotic stress signals to mediate osmoadaptation. However, disruption of the genomic island in the ‘All’ mutant, did not result in any survival defects after exposure to 0.5M NaCl during in vitro LB broth culture as this mutant survived at comparable levels to the parental Y. pestis strain (data not shown). This suggested that absence of the genomic island is not critical for adaptation to osmotic stress in vitro. Nonetheless, y3555 which encodes a metabolic gene showing putative roles in amino acid
metabolism confers a striking ability to enhance growth fitness of *E. coli*, a trait that is also made apparent during overexpression of *y3555* in *Y. pestis* strains. These two findings allude to a role of the genomic island in optimizing amino acid metabolism in the context of hyperosmotic stress. This is fitting since: (i) *Y. pestis* primarily metabolizes amino acids during flea infection (15-17) and (ii) the flea gut has a demonstrated physicochemical status of high osmolarity (52), while several *Y. pestis* metabolic pathways involved in osmoprotection are transcriptionally induced during flea gut infection (15, 17).

To begin to understand the function of Y3555, it was expressed *in trans* in a well-characterized *S. enterica aspC* mutant. An *S. enterica aspC* mutant has a growth defect that is restored with the addition of aspartate or *aspC* expressed *in trans*. However, when Y3555 was expressed in the *S. enterica aspC359::km* mutant, growth was not restored but in fact further retarded. This surprising result may however hint at the function of Y3555. There is evidence to show that in an *aspC* mutant of *S. enterica* or *E. coli*, TyrB produces minimal aspartate that allows for some growth (48, 53), but the addition of tyrosine or phenylalanine inhibits *tyrB* expression and growth (51). As such one explanation for the growth defect of the *S. enterica aspC359::km* mutant expressing Y3555, is that Y3555 increases tyrosine and/or phenylalanine pools that leads to *tyrB* inhibition. Thus, our findings suggest that while Y3555 is not an aspartate aminotransferase it could instead be an aromatic aminotransferase. Alignments of Y3555 with AspC and TyrB from both *S. enterica* and *Y. pestis* suggest Y3555 are fairly dissimilar (Figure S1). Instead, Y3555 aligns with over 85% identity to uncharacterized PLP-dependent aminotransferases from foodborne isolates of *S. enterica* sequenced by the Centers for Disease Control (Figure S3). Crystallization of Y3555 may help to determine the substrates or products of the protein and characterization is on-going.
During active metabolism, cellular function can be compromised by anomalous accumulation of endogenously produced reactive intermediates of metabolism e.g. 2AA. Elimination of such metabolic intermediates is therefore needed to optimize cellular function, growth and fitness. The highly conserved Rid family of proteins prevalent in all domains of life (41, 49) are amongst the first enzymes characterized to support optimization of metabolic processes by neutralizing obligatory reactive metabolic intermediates. Currently RidA enzymes are well characterized as detoxifiers of 2AA in several bacterial species (38, 40, 41, 49, 54). Our data contributes to this list by establishing that in Y. pestis the y3551 gene encodes as an archetypal RidA protein that can deaminate the reactive enamine intermediate 2AA. Additionally, the Y3550 gene product likely belongs to the Rid 5-6 subclass of the Rid family proteins whose functions have not yet been characterized. Further to its distinctive status is that while the promoters predicted immediately upstream of y3555 and y355x can drive transcription of y3550, a dedicated promoter predicted immediately upstream of the Y3550 coding sequence is exclusively activated by hyperosmotic stress to inclusively drive y3550 transcription.

It remains elusive how Y3555, Y3550 and Y3551 operate collaboratively to achieve optimal metabolic activity in response to osmotic stress. The achievement of increased growth rate in the DG44 strain co-expressing y3555 and y3550 suggests a synergistic activity of these two proteins. Such a case can be explained by the specific Rid activity meted out by Y3550 being able to potentially eliminate a metabolic by-product that is toxic, and/or produced by Y3555. One complementary observation within a context of Y3555 serving in aromatic amino acid metabolic processes is that BPROM predicts a binding box of the transcriptional factor, TyrR (55-57), in the promoter sequence of y3550 (predicted promoter region spans positions -120 to -87 nts). In many enteric bacteria TyrR regulates genes involved in uptake and
biosynthesis of aromatic amino acids in response to presence or absence of each of the three aromatic amino acids (tyrosine, tryptophan, phenylalanine) which function as cofactors for TyrR (55, 56). For example in E. coli, TyrR is responsive to tyrosine and able to regulate expression of tyrosine biosynthesis genes tyrB, aroF-tyrA and aroLM, and genes encoding aromatic amino acid transporters, aroP and mtrR (56).

Conversely, the suppressed growth rate in the DG44 strain co-expressing y3555 and y3551 versus that of y3555 expression alone suggests antagonistic activity of these two encoded proteins. Such a scenario can be explained by Y3551 RidA deaminase activity protecting an unknown enzyme whose activity either competes with or generates reactive intermediates to which Y3555 is susceptible. Co-expression of Y3551 RidA and Y3550 in Y. pestis might therefore accomplish a balance in metabolic fitness as it pertains to aromatic amino acid metabolic activities.

Our experimental data demonstrates that mutations in the genomic island afford no compromise in growth and survival or competitive fitness of Y. pestis in fleas. This is confounding because highly elevated expression of unrequired genes would be energetically expensive if not required in the flea gut environment. It is possible that controlled lab-reared flea experiments cannot discern a crucial function of gene products that in wild fleas feeding on wild rodent blood sources (different from lab mouse species) is otherwise apparent. It has been demonstrated that infectious blood source influences Y. pestis infection in fleas (58) and that the gut microbiota of wild fleas is more diverse than that of lab-reared fleas (59). Perhaps in the wild, a competitive coinfection arises between Y. pestis and diverse gut microbes necessitating genomic island gene product function to heighten its competitive fitness.

Moreover, it is known that metabolic robustness is contributed by more than a single pathway and metabolic versatility is tolerated in the repertoire of metabolic gene pathways that
might be switched on to a common physiological purpose, e.g. sustaining *Y. pestis* growth and survival in the flea gut environment during hyperosmotic stress conditions. For example, the osmoprotectant glycine betaine, is produced by *proVW* and *betI* and these genes have been shown to be expressed by *Y. pestis* during flea infection (15). Additionally, the *Y. pestis* genome encodes homologs of some members of the genomic island. Two PLP-dependent aromatic aminotransferases, *y2760* (*aspC*), and *y0579* (*tyrB*), that can convert aspartate and tyrosine to glutamate are induced at robust levels during a *Y. pestis* flea infection (17). The *y3554* gene is one of three genes encoding a Na⁺/H⁺ antiporter (*nhaA*, *nhaB*, *nhaC*). Two *ridA* homologs *y2240* and *y1830*, a putative Rid1-7 superfamily encoding gene, *y0163* may be able to compensate for the function of their homolog in the genomic island (Figure 5A).

Overall our data reinforces a model wherein once *Y. pestis* enters the flea in the blood meal it transitions into an environment of elevated osmotic stress (11) likely exacerbated by water removal from the blood meal during flea digestive processes (60). Osmotic pressure in the blood meal is contributed by the salts it contains, the concentration of which is expected to increase after water removal. The requirement for NhaB and NhaC Na⁺/H⁺ antiporters for virulence of *Y. pestis* during septicemic infection supports that this bacterium faces hyperosmotic stress in mammalian blood (31, 32). Simultaneously *Y. pestis* must optimize its growth fitness to establish a transmissible infection in the hyperosmotic flea gut environment. The mechanisms by which *Y. pestis* counterbalances osmotic stress in the flea however remains unclear although our current data suggests that this is linked to amino acid metabolism shifts that support osmoadaptation within the flea gut. Interestingly *Y. pestis* encodes and expresses several biochemical and regulatory pathways that function in alleviating osmotic stress emphasizing (15, 52) that osmotic stress is a perilous stress that *Y. pestis* must adapt to in the flea gut. Preservation of compensatory/redundant osmotic stress
resistance pathways that allow simultaneous optimization of Y. pestis growth fitness to establish a transmissible infection in fleas is as such critical for maintenance of disease cycles.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. Bacterial strains used in this work are listed in Table S1. Bacterial cultures were grown at 28°C with shaking in BHI medium. When necessary, media were supplemented with carbenicillin (100 µg/ml) or kanamycin (50 µg/ml). For the osmolarity assays, overnight cultures of parental KIM6+ strains were used to inoculate fresh LB medium and were incubated at 28°C to reach mid-logarithmic phase, thereafter cultures were exposed to 0.5M NaCl. Bacteria were immediately mixed with RNA protect bacteria reagent (Qiagen) to minimize RNA degradation and then harvested at 4°C and stored at -80°C until RNA isolation.

Salmonella enterica serovar Typhimurium LT2 was the parental strain for all S. enterica mutants. Overnight cultures of S. enterica were grown in Difco nutrient broth (NB, 8 g/liter) with NaCl (5 g/liter) and the appropriate antibiotic (ampicillin 150 µg/mL or kanamycin 50 µg/mL). Minimal no-carbon E (NCE) was supplemented with MgSO₄ (1 mM), trace minerals, and glucose (11 mM) and where indicated serine (5 mM), isoleucine (0.3 mM), aspartate (1 mM) and/or Isopropyl β-d-1-thiogalactopyranoside (IPTG, 100 µM) (61, 62). All chemicals were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO.

Construction of plasmids. Plasmids and primers used in this study are listed in Tables S1 and S2, respectively.
Lux transcriptional fusion constructs. The lux transcriptional fusions were generated by cloning a PCR product containing the corresponding regulatory region into the low copy vector pCS26-Pac that carries the promoterless luxCDABE operon (63). PCR products were obtained with primer pairs, 50R-BHI/50F-XhoI, 51R-BHI/51F-XhoI, 50R-BHI/51F-XhoI, 53R-BHI/53F-XhoI, 54R-BHI/54F-XhoI, 55R-BHI/55F-XhoI and 54R-BHI/55F-XhoI, respectively (Table S2). They were then digested with BamHI and XhoI restriction enzymes (NEB) and cloned into the pCS26-Pac previously digested with the same restriction enzymes, generating plasmids pCS::P50, pCS::P5x, pCS::P5x_51_50, pCS::P53, pCS::P54, pCS::P55, pCS::P55_54 respectively (Table S1). Lux transcriptional fusion plasmids were transformed into the Y. pestis KIM6+ parental strain.

pTRc99A and pET28a constructs. The pTRc99A and pET28a inducible expression plasmids were generated by cloning PCR products obtained for gene y3550, y3551 and y3555 from primer pairs p270/p271, p272/p273 and p274/p275 respectively. The PCR products were digested with BamHI and XhoI and ligated into pTrc99A or pET28a previously digested with BamHI and SalI restriction enzymes. The plasmid constructs generated from cloning y3550, y3551 and y3555 into pTRc99A are referred to as pTRc55, pTRc50, pTRc51, and for pET28a cloning they are referred to as pET28a::y3550, pET28a::y3551 and pET28a::y3555. To clone y3555 together with y3551 or y3550 into pTrc99A, y3555 was amplified with primers p508 and p509, digested with HindIII, and ligated into pTRc50 or pTRc51 such that y3555 was inserted downstream of y3550 or y3551. These plasmids were referred to as pTRc50_55 and pTRc51_55 respectively. Plasmids were transformed into transformed into E. coli DG44 and selected for on LB Carbenicillin 100μg/mL.
Tn7-based chromosomal integration of Kanamycin resistance cassette into Y. pestis KIM6+ strain. The Kanamycin resistance cassette from plasmid pKD4 was amplified with primers p395 and p396 (Table S2). The PCR product was digested with BamHI and EcoRI and ligated into pUC18R6K-mini-Tn7T previously digested with the same restriction enzymes and transformed into DH5α lambda pir chemical competent E. coli and selected for on LB Kanamycin 50ug/mL. The plasmid, referred to as pUC18R6K-mini-Tn7T::kanR was isolated and verified for sequence. Electrocompetent Y. pestis KIM6+ was transformed with the pUC18R6K-mini-Tn7T::kanR and pTNS2 and selected for on HIB Kanamycin 50ug/mL plates. Colonies were screened with pstSup2 and pTn7R primers for insertion at the glmS-pstS site. This Y. pestis KIM6+ strain with the Kanamycin cassette integrated at the glmS-pstS site was referred to as Y. pestis KIM6+ glm-pstS::kanR

Lux transcriptional reporter assay. Y. pestis KIM6+ carrying plasmids containing the lux fusions were grown overnight in LB supplemented with appropriate antibiotics. An aliquot was used to inoculate fresh LB medium the next day. The cultures were grown on a shaker at 28°C to mid-logarithmic phase, then treated or not with 0.5M NaCl for 100 min. Aliquots of 1 mL were taken out to determine the optical density of the culture by measuring OD600. 0.1 mL of each culture was dispensed into a 96-well plate (Costar) and luminescence of each well was measured in the Infinite M1000 reader (Tecan), with an integration time of 1 s and using the Tecan i-control software version 1.7.1.12. Plates were first shaken with a 2 mm-linear amplitude 5-s pulse. Data were normalized by dividing relative light units (RLU) by OD600 to obtain the final RLU corrected for bacterial growth.
RT-PCR. *Y. pestis* KIM6+ strain was grown in Miller’s LB broth at 26°C to mid-logarithmic phase, culture was split in two and one half received 0.5M NaCl and the other distilled water vehicle control treatment. After 20 minutes, culture RNA was stabilized immediately with RNAprotect Bacteria reagent (Qiagen). Total RNA was purified using the Quick-RNA Miniprep kit (Zymo). Purified RNA was subject to TURBO DNase I (Ambion) treatment according to the manufacturer’s instructions. Then 800-1600ng of RNA was used in the first-strand cDNA synthesis reaction using Maxima H Minus Reverse Transcriptase (ThermoFisher) according to manufacturer’s instructions. Negative controls were performed by omitting the Reverse Transcriptase. The cDNA was then treated with RNase H (NEB) at 37°C for 30 min. The PCR amplification step was performed using Phusion polymerase (ThermoFisher), 100ng of cDNA, and the following pairs of primers, each at a final concentration of 0.5 µM (Table S2):

Y3555Set2Fw /5051RT-R, amplifies a 5,014 bp region upstream of y3555 to within y3550. RT-PCR cycles were as follows: one cycle of 1 min at 98°C for initial denaturation; 31 cycles of 10 sec at 98°C, 20 sec at 68°C, and 1 min 40 sec at 72°C for PCR amplification with primers pairs Y3555Set2Fw /5051RT-R. A final elongation step of 5 min at 72°C was included. Controls for the expected size of the PCR products were performed by using chromosomal DNA from *Y. pestis* KIM6+ as the template. The PCR products were analyzed by 1% agarose gel electrophoresis. The Generuler 1kb DNA ladder (ThermoScientific) was run alongside PCR products.

**Construction of Mutant and Complementation Strains.** Non-polar gene-deletion mutant strains were generated by the lambda (λ) Red recombinase system (64), (65) and gene-specific primer pairs shown in Table S2. The genes y3550, y3555, and entire continuous
region spanning genes y3555, y3554, y3553, y355x, y3551 and y3550 (referred to as ‘all’) and tandem encoded genes y3551-y3550 were replaced with a selectable kanamycin (Km) resistance cassette in the Y. pestis KIM6+ strain (Table S1). The Δy3550Δy3555 double mutant was constructed similarly but for this the Δy3550 mutant was used. Then the FRT-flanked Km cassette was excised from these strains after transformation with the helper plasmid pFLP3 (Table S1), expressing the FLP recombinase that acts on the directly repeated FRT (66). Both pKOBEG-sacB and pFLP3 were cured by sucrose counter-selection by growing the strains on LB without NaCl and supplemented with 10% sucrose. Mutant strains generated were Δy3550 (VV150), Δy3555 (VV151), Δy3550Δy3551 (VV278), Δy3550Δy3555 (VV279) and ‘all’ (VV280) (Table S1) and retained a FRT scar. To construct plasmids expressing y3550 or y3555 a DNA fragment containing the y3550 gene alone or y3555 with its native promoter sequence was amplified by PCR using primer pairs 3550-F/3555-R or 3550-F/3555-R (Table S2) and cloned into vector pCR4-TOPO, generating plasmids pCR4::3550 and pCR4::3555 respectively. All mutant strains were verified by PCR and sequencing.

**RT-qPCR.** Total RNA of Y. pestis KIM6+ was purified using the Quick-RNA Miniprep kit (Zymo). Chromosomal DNA was removed by incubating 5 µg of RNA from each strain with TURBO DNase I (Ambion) according to the manufacturer’s instructions. cDNA was synthesized in a reaction containing 2 µg of each DNase-treated-RNA, 100 pmol of random hexamers and 0.5 mM of dNTPs, using RiboLock RNase Inhibitor (ThermoFisher) and Maxima H Minus Reverse Transcriptase (ThermoFisher) according to the manufacturer’s protocol. The obtained cDNA was used as template for qPCR assays, with 0.5 µM of primer pairs for y3550, y3551, y3553, y3554, y3555 or gyrB (Table S2) and the SsoAdvanced Universal SYBR Green Supermix (BIO-RAD). Real-time PCR reactions were performed with the c1000 Touch Thermal
Cycler and the CFX96 Real Time System (BIO-RAD) and data collected using the CFX Manager Software 2.1 (BIO-RAD). Reaction conditions were 95°C for 3 min, 95°C for 10 s and 58°C for 30 s during 40 cycles. The level of gyrB mRNA was used as an internal control to normalize the results. The quantification technique used to analyze data from cultures was the 2–△△CT method (67). All qPCR reactions were performed in triplicate and were repeated using RNA purified from three independent bacterial cultures or flea infections.

**Flea infections.** For coinfections, *Xenopsylla cheopis* fleas were infected with blood containing a 1:1 mix of parental KIM6+ marked at the *attt7* site (66) called KIM6+ *glmS-pstS:kan^R* and a mutant strain as previously described (43). Infected fleas were fed twice weekly on a live neonatal mouse. To compare the bacterial load over the infection, 20 fleas were individually triturated and plated on BHI agar and BHI agar containing kanamycin to select for the KIM6+ *glmS-pstS:kan^R* at 2 h (time 0) and 7 days post infection. All experiments involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington State University, USA, and conducted in strict accordance with institutional guidelines based on the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Growth fitness of *E. coli* stains DH5α and DG44 expressing *Y. pestis* low GC content genes.** *E. coli* strains harboring IPTG-inducible constructs were grown overnight in LB containing carbenicillin 100μg/mL with shaking at 37°C. For the *E. coli* DH5α strains the overnight inoculum was diluted 1:200 into fresh M9 medium supplemented with 0.4% glucose and 0.1% casamino acids medium (Difco). For the *E. coli* D44 strains the overnight inoculum was diluted 1:200 into fresh M9 medium supplemented with 0.4% glucose and 0.1% casamino acids.
acids medium (Difco), succinate (2.5 mg/ml), malate (2.5 mg/ml), a-ketoglutarate (1 mg/ml) as well as 100ug/mL each of asparagine, glutamine and glutamic acid as previously described (46). Growth curves were generated at 37°C using a Bioscreen C (Growth Curves USA, Piscataway, NJ) shaking incubator.

Growth quantification of *S. enterica* strains. Growth of *S. enterica* in liquid culture was assessed using a BioTek EL808 microtiter plate reader by measuring the optical density at 650 nm at 37°C with slow shaking speed. Overnight cultures of *S. enterica* in biological triplicate were grown in rich medium with appropriate antibiotic at 37°C. Cultures were pelleted and resuspended in an equal volume of sterile NaCl (8.5 g/L). The cell suspension was used to inoculate growth medium (2% inoculum) and growth was monitored. The growth data were plotted in a log₁₀-format using GraphPad Prism 7.0c and the error bars represent the standard error of the mean for three biological replicates.

Protein purification. Proteins were purified as previously described (54, 68, 69). EcRidA and Y3551 were purified from *E. coli* strain BL21AI harboring either pET20-ridA (DM12740) or pET28b-y3551 (DM16279). Overnight cultures grown in superbroth with ampicillin were used to inoculate 3 liters of superbroth with ampicillin. Cultures were grown for ~3 h at 37°C shaking until the OD₅₅₀ of 0.7 was reached. Fresh arabinose was added to a final concentration of 0.2%, and cultures were incubated with shaking at 37°C overnight. A clarified supernatant was injected onto a Ni-NTA Superflow resin and proteins were purified according to the manufacturer’s protocol (Qiagen). Protein aliquots were frozen in liquid nitrogen and stored at -80°C. Purified CdsH was a gift from Dustin Ernst and was purified similarly from *E. coli* strain BL21AI containing pET14b-cdsH as previously described (68).
Cysteine desulfhydrase (CdsH) assay. 2AA deaminase activity was determined using a coupled assay with purified cysteine desulfhydrase (CsdH), RidA, and Y3551 as previously described (68, 70). Assay mixtures (100 µL) contained Tris-HCl (100 mM, pH 8), NADH (250 µM), pyridoxal 5′(PLP)-phosphate (30 µM), lactate dehydrogenase (5 U), and purified CdsH (0.27 µM). Reaction mixtures contained RidA or Y3551 (0.19 µM) or an equal volume buffer as a control. The reactions were initiated in triplicate with addition of freshly prepared L-cysteine (final concentration 0.5 – 2.5 mM). Reactions were monitored continuously in a 96-well quartz plate by measuring the absorbance at 340 nm for 2 min using a Spectramax M2 microplate reader. The initial rate of pyruvate formation was calculated from the rate of NADH oxidation in the first 30 s, along with the molar extinction coefficient (ε = 6,200 M⁻¹ cm⁻¹).

In silico analyses. In silico predictions of genomic island genetic organization and prediction of promoters was achieved using Softberry’s FGENESH and BPROM (Softberry Inc. Mt Kisco, NY, USA, http://www.softberry.com) freeware. Alignment of amino acid sequences of Rid proteins was accomplished using EMBL-MUSCLE tool (https://www.ebi.ac.uk/Tools/msa/muscle/). The protein secondary structure prediction and structural alignments of Y3555 was made using Phyre² (44).

Statistical analysis. Statistical analysis was accomplished using Graphpad Prism 7.0 details for which are provided in the specific methods section or the figure legend.

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**FIGURE LEGENDS**

**Figure 1.** The low GC content gene cluster is transcribed as a single polycistronic mRNA. Six genes compose the low GC content gene cluster. The three bioinformatically predicted transcriptional units are marked by the presence of predicted promoters upstream of genes y3555, y355x and y3550 (blue arrows) (A). RT-PCR analysis of regions (indicated by dashed lines) spanning y3555 to y3550. RT-PCR reactions were performed with reverse transcriptase and DNA polymerase mix (lanes 2, 3) or only with DNA polymerase (lanes 4-7). PCR reactions in the presence of Y. pestis KIM6+ chromosomal DNA (lane 6) or absence of template (lane 7) with the same set of primers were performed as controls (B). Activity of transcriptional fusions plasmids, pCS::P55, pCS::P54, pCS::P53, pCS::P55_54, pCS::P5x, pCS::P50 and pCS::P5x_51_50, carrying the transcriptional fusions of the luciferase (lux) genes and predicted promoter regions (yellow arrows) representing predicted regulatory regions of y3555, y3554, y3553, y3555-y3554, y355x, y3550 and y355x-y3551-y3550, respectively, as well as the low copy number empty vector pCS26-Pac. Error bars represent mean (±SD) of three independent experiments; Significance determined by One-Way ANOVA and a Dunnett’s post-test of lux/OD values of transcriptional fusion constructs compared to the empty vector are given as ****=P<0.0001 and **= P<0.01 (C).
Figure 2. *Y. pestis* low GC content gene mutants coinfected with the parental strain do not exhibit competitive fitness defects in fleas.

Fleas were infected using a 1:1 ratio of mutant and parental strain harboring a kanamycin resistance cassette in the chromosome, referred to as WT *glmS*-pstS::*kan*<sup>R</sup>. The percentage WT *glmS*-pstS::*kan*<sup>R</sup> from the total coinfecting *Y. pestis* population per flea (black dots) at days 0 and day 7 is reflected. Error bars represent the mean plus minimum and maximum percentage WT *glmS*-pstS::*kan*<sup>R</sup> per fleas. The data were analyzed using an unpaired student’s T-test. dpi=days post-infection

Figure 3. Gene expression of low GC content genes is induced during exposure to saline hyperosmotic stress. Steady state mRNA transcript levels of low GC content genes in *Y. pestis* KIM6+ parental strain treated or not with 0.5M NaCl was determined. The data is represented as fold change gene expression of treated versus untreated mRNA transcript levels. Error bars represent mean (±SD) of 3 independent experiments (A). Activity of transcriptional fusions plasmids, pCS::P55, pCS::P5x, pCS::P50, carrying the transcriptional fusions of the luciferase genes and predicted promoter regions of *y3555*, *y355x*, *y3550*, respectively, in pre-treatment with vehicle (white bars) or 0.5M NaCl (gray bars) and 100 mins post-treatment with vehicle (white dotted bars) or 0.5M NaCl (gray dotted bars). Error bars represent mean (±SD) of three independent experiments. Data was analyzed using Students t-test, **= P<0.01 and ****= P<0.0001 (B).

Figure 4. Induction of *y3555* generates an aspartate requirement for a *S. enterica* aspC789::km mutant. The *S. enterica* aspC789::km mutant expressing Y3555 DM16435
(circle) or the empty vector control DM16434 (square) were grown in minimal glucose (11 mM) medium plus IPTG (100 µM), with (solid) or without (open) aspartate (1 mM). Error bars represent mean(±SEM) from three biological replicates.

Figure 5. **Y3551 is a RidA enzyme.** Multiple sequence alignment of all *Y. pestis* KIM6+ Rid family homologs. Amino acids highlighted in yellow represent conserved residues found in archetypal RidA subfamily members (A). Y3551 complements a *S. enterica* ridA mutant. The *S. enterica* ridA1::tn10 (Tc) mutant carried the empty vector control DM16293 (squares), or plasmids pET28b expressing *E. coli* ridA DM16294 (triangles), Y3551 DM16284 (circles), or Y3550 DM16283 (diamonds). Strains were grown in a 96-well plate at 37 °C shaking in minimal NCE glucose (11 mM) with serine (5 mM). Error bars represent mean (±SEM) from three biological replicates (B). Y3551 deaminates 2-aminoacrylate *in vitro*. Purified *S. enterica* cysteine desulphhydrase (CdsH) was used to generate 2AA from cysteine. Reactions with or without the addition of Y3551 or *S. enterica* RidA measured coupled pyruvate formation and NADH oxidation, as previously described [Ernst, 2014; Kredich, 1974]. NADH oxidation was used as a measurement of pyruvate formation. RidA enzymes deaminate 2AA *in vitro*, increasing the reaction rate. The initial reaction rate of pyruvate formation is plotted vs concentration of cysteine. As controls CdsH alone (circles) or *S. enterica* RidA (square) were used. Y3551 (inverted triangle) deaminated 2AA derived from cysteine. Error bars represent the standard error of the mean from technical triplicates (C).

Figure 6. **Overexpression of y3555 increases growth fitness of *Y. pestis* strains.** Growth kinetics of *Y. pestis* KIM6+ parental strain, ∆y3550, ∆y3555, and ∆y3550 ∆y3555 alone or
overexpressing either y3555 (pCR4::3555) or y3550 (pCR4::3550) in LB medium. Data represents the mean of three independent experiments. Growth rate (μ) calculated from regression analysis is given next to strain name. Data was analyzed using One-Way ANOVA and a Tukey’s post-hoc test. * represents the growth rate (μ) of mutant strains versus parental strain that are significantly different.

Figure 7. Heterologous expression of y3555 increases growth rate of Escherichia coli ΔaspC ΔtyrB (DG44). Growth kinetics of E. coli DG44 in M9 minimal medium supplemented with 0.4% glucose, 0.1% casamino acids, succinate (2.5 mg/ml), malate (2.5 mg/ml), α-ketoglutarate (1 mg/ml) as well as 100µg/mL each of asparagine, glutamine and glutamic acid. Growth rate (μ) is given next to plasmid name. Error bars represent mean of seven independent replicates for E. coli DG44 studies. One Way ANOVA and Tukey’s multiple comparison test was used to determine significant differences in growth rate and final biomass yield. *= p<0.05, **= P<0.01, ***=P<0.001 and ****= P<0.000. ns=not significant.
REFERENCES


A

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Tyr17  Ser30

Asn88    Arg105

B

![Graph showing OD reading over time](image)

C

![Graph showing pyruvate formed](image)