A trimeric autotransporter enhances biofilm cohesiveness in *Yersinia pseudotuberculosis* but not in *Yersinia pestis*

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ABSTRACT

Cohesion of biofilms made by *Yersinia pestis* and *Yersinia pseudotuberculosis* (*Yptb*) has been attributed solely to an extracellular polysaccharide matrix encoded by the *hms* genes (Hms-ECM). However, mutations in the *Yptb* BarA/UvrY/CsrB regulatory cascade enhance biofilm stability without dramatically increasing Hms-ECM production. We found that treatment with proteinase K enzyme effectively destabilized *Yptb csrB* mutant biofilms, suggesting that cell-cell interactions might be mediated by protein adhesins or extracellular matrix proteins. We identified an uncharacterized trimeric autotransporter lipoprotein (YPTB2394), repressed by *csrB*, which has been referred to as YadE. Biofilms made by a Δ*yadE* mutant strain were extremely sensitive to mechanical disruption. Overexpression of *yadE* in wild-type *Yptb* increased biofilm cohesion, similar to biofilms made by *csrB* or *uvrY* mutants. We found that the Rcs signaling cascade, which represses Hms-ECM production, activated expression of *yadE*. The *yadE* gene appears to be functional in *Yptb* but is a pseudogene in modern *Y. pes*tis strains. Expression of functional *yadE* in *Y. pestis* KIM6+ altered the production of Hms-ECM and weakened biofilms made by these bacteria. This suggests that although the YadE autotransporter protein increases *Yptb* biofilm stability, it may be incompatible with Hms-ECM production that is essential for *Y. pestis* biofilm production in fleas. Inactivation of *yadE* in *Y. pestis* may be another instance of selective gene loss in the evolution of flea-borne transmission by this species.

IMPORTANCE

The evolution of *Yersinia pestis* from its *Y. pseudotuberculosis* (*Yptb*) ancestor involved gene acquisition and gene losses, leading to differences in biofilm production. Characterizing the unique biofilm features of both species may provide better understanding of how each adapts to its specific niches. This study identifies a trimeric autotransporter YadE that promotes biofilm stability of *Yptb* but which has been inactivated in *Y. pestis*, likely because it is not compatible with Hms polysaccharide that is crucial for biofilms inside fleas. We also reveal that the Rcs signaling cascade, which represses Hms expression in *Y. pestis*, activates YadE in *Yptb*. The ability of *Yptb* to use polysaccharide or YadE protein for cell-cell adhesion may help it produce biofilms in different environments.

INTRODUCTION

Environmental persistence, host interaction, and transmission of *Yersinia* depend on biofilms, which are tightly regulated by both transcriptional and post-transcriptional control mechanisms [1, 2]. Arguably, the best studied *Yersinia* biofilms are those made by *Y. pestis* while in the flea digestive tract that block the proventriculus and increase transmission to new hosts during flea feeding. These biofilms require the HmsHFRS proteins to produce and export a polysaccharide extracellular matrix of poly-ß-1,6-N-acetylglucosamine that is crucial in forming and maintaining bacterial cell-cell attachments [3, 4]. Without high levels of Hms-dependent extracellular matrix (Hms-ECM), the biofilms formed by *Y. pestis* while in fleas are not sufficiently cohesive to cause proventricular blockage. Adaptation to flea-borne transmission was precipitated in part by mutations that led to high levels of Hms-ECM compared to its *Y. pseudotuberculosis* (*Yptb*) ancestral lineage [5-7].

Among these mutations, modification of the Rcs regulatory system was especially important in enhancing Hms-ECM production. The Rcs signaling system includes the inner membrane kinase RcsC and the phosphorelay protein RcsD. RcsC phosphorylates itself when an inducing signal is present, and that phosphate is passed to RcsD and then to RcsB. Phosphorylated RcsB is a transcriptional regulator, binding to target promoters either as homodimers or as heterodimers with the auxiliary protein RcsA or other proteins [8]. Normally, the system is kept in the 'off' state by an inner membrane protein IgaA that blocks signaling through RcsD. When an appropriate activating signal is present, the RcsF lipoprotein sensor interacts with IgaA and relieves the inhibition [9]. RcsAB heterodimers negatively regulate *Yersinia* biofilms by binding to the promoters of the diguanylate cyclase genes *hmsT* and *hmsD*, as well as the *hmsHFRS* operon itself [10-12]. The *rcsA* gene is inactive in *Y. pestis* due to an

insertion in the open reading frame, and restoring the function of this gene prevents biofilm formation and flea blockage [13].

Like many bacteria, exopolysaccharide production is positively regulated in *Yersinia* by cyclic di-GMP made by diguanylate cyclases [14, 15]. When RcsAB repression of *hmsT* and *hmsD* transcription is eliminated, the resultant high levels of the second messenger enhance Hms-ECM production, likely by activating the HmsRS inner membrane proteins [11, 16, 17]. Two phosphodiesterases that degrade cyclic-di-GMP are functional in *Yptb* but not in *Y. pestis*. A *Yptb* mutant strain wherein these genes (*rcsA* and both phosphodiesterases) are replaced with *Y. pestis* non-functional alleles can block fleas, but not to the same extent as *Y. pestis* [5]. This indicates that additional biofilm-related differences exist between the two species.

Several other regulatory influences on biofilm production in both *Yptb* and *Y. pestis* have been identified. We recently reported that the BarA/UvrY two-component system represses biofilms in *Yptb* by activating the CsrB small RNA [18]. Although *Yptb* mutants lacking *csrB* or *uvrY* make more cohesive biofilms than the wild type strain, their production of Hms-ECM does not approach that of *Y. pestis*. This suggested that *Yptb* biofilms may contain additional extracellular matrix components that are responsible for their cohesion. In this study, we investigated the basis for the increased cohesiveness of *csrB* mutant biofilms in an effort to identify novel *Yptb*-specific biofilm features. We found that *Yptb* biofilms have a significant protein component not present in *Y. pestis*. We focused on the uncharacterized YPTB2394 protein (YadE) which is repressed by *csrB*. This predicted lipoprotein is a part of the trimeric autotransporter family of proteins that function in bacterial adhesion to host surfaces or bacterial cell-cell attachments in biofilms [19-21]. Here, we show that production of YadE leads to *Yptb* biofilms that strongly resist disruption. Expression of *yadE* is activated by the Rcs system, in

contrast to repression of Hms-ECM by RcsAB. Conversely, *yadE* is a pseudogene in *Y. pestis* and we demonstrate that *yadE* expression alters the production of Hms-ECM and weakens *Y. pestis* biofilms.

RESULTS

Yptb biofilm cohesion requires proteins

We previously demonstrated that mutations in the BarA/UvrY two-component regulatory system, or in the CsrB small RNA activated by UvrY, increase cohesion of *Yptb* strain IP32953 biofilms [18]. In that study, lectin staining of the *csrB* mutant strain indicated only moderately increased Hms-ECM on the surface of these bacteria. This suggested the possibility of additional structural components that are increased in the *csrB* mutant strain which assist in holding *Yptb* biofilms together. Protein adhesins and extracellular DNA are present in the biofilm extracellular matrices of numerous other bacteria. We therefore treated wild type and *csrB* mutant *Yptb* biofilms with DNase or proteinase K enzymes prior to testing their dispersal in mechanical disruption assays (Fig. 1). As expected, the *csrB* mutant formed more cohesive biofilms than the wild type strain. DNase did not significantly increase disruption of either wild type or *csrB* mutant biofilms compared with the control saline treatment. In contrast, proteinase K markedly increased the proportion of the *csrB* mutant strain biofilms that were dispersed. *Y. pestis* biofilms were very stable in these assays and were not affected by proteinase K or DNase treatment.

YPTB2394 putative autotransporter expression confers biofilm cohesion

Since we had demonstrated that *Yptb csrB* mutant biofilms are likely more cohesive in part due to a protein component of the extracellular matrix, we searched for proteins that were expressed more abundantly in the *csrB* mutant strain. We had previously conducted a

comparison of the wild type and *csrB* mutant proteomes and found a large number of proteins that were expressed at lower levels in the *csrB* mutant, which is consistent with the role of CsrA as a translational repressor [15]. YPTB2394 was among the few proteins that were expressed more abundantly (approximately 30-fold higher) by the *csrB* mutant. YPTB2394 is annotated as a predicted lipoprotein with homology to type Vc autotransporter proteins. Trimeric autotransporter adhesins are membrane-anchored proteins known to mediate cell-cell attachments in other Gram-negative bacteria and promote greatly enhanced biofilm production in diverse species [22-24]. YPTB2394 is predicted to have a C-terminal YadA-like anchor, and several YadA-like stalk domains. The orthologous gene in *Y. pseudotuberculosis* strain YPIII (YPK_0761) has been referred to as *yadE* [25], although its function has not been investigated.

To examine the role of *yadE* in biofilm cohesion, we first deleted the gene from the wild type *Yptb* IP32953 strain. Biofilms formed by the mutant strain were extremely fragile compared to the wild type strain (Fig. 2). We then complemented the mutant strain by inserting *yadE* on a low-copy plasmid with a constitutive promoter. This resulted in biofilms that were highly resistant to disruption, even more so than *csrB*::Tn5 mutant biofilms. This strongly suggests that YadE helps maintain intracellular contacts and promotes *Yptb* biofilm cohesion.

yadE gene expression is regulated by CsrB and by the Rcs regulatory cascade

YadE (YPTB2394) protein levels are approximately 30-fold greater in *csrB* mutant bacteria compared to wild-type cells [18]. CsrB is a regulatory RNA that sequesters CsrA protein [26, 27]. Typically, CsrA binds to target sequences found near the Shine-Delgarno region of target transcripts and represses their translation. CsrB accumulation frees mRNA targets of CsrA to be translated more efficiently. However, translation of some CsrA-regulated proteins is enhanced by CsrA binding, and it is possible that CsrA is a direct translational activator of YadE.

Alternatively, CsrA could repress translation of another transcriptional regulator, making its effect on *yadE* expression indirect. To further investigate regulation of *yadE*, we created a reporter plasmid containing the 356 bp region upstream of the start codon fused to a promoterless green fluorescent protein (*gfp*) gene. Transformation of wild type cells with this plasmid resulted in low but detectable levels of fluorescence. Expression was significantly enhanced in *csrB*::Tn5 mutant bacteria (Fig. 3), confirming the negative effect on YadE expression by CsrB.

In order to identify possible transcriptional repressors of *yadE*, we created a transposon mutant library in a wild type IP32953 strain carrying the *yadE*::*gfp* reporter plasmid. We then used fluorescence activated cell sorting to enrich for mutants with higher fluorescence than the wild type strain. After sorting, we verified enhanced *yadE::gfp* activity in isolated single colonies of 48 mutants by flow cytometry and determined their transposon insertion sites. We determined 23 distinct insertion sites among these mutants (Supplementary Table 1). We obtained 9 separate transposon mutants with insertions in the *igaA* gene, encoding the periplasmic IgaA protein that prevents overactivation of the Rcs signaling cascade [8]. We also obtained multiple mutants with insertions in genes encoding adenylate cyclase (*cya*) and the stringent starvation protein (*sspA*).. We tested biofilms made by *cya*, *sspA*, and *igaA* transposon mutants and found that they were also more cohesive than the wild type strain (Fig. 4), correlating with increased expression of *yadE*.

IgaA limits the phosphorylation of RcsC and thereby prevents activation of RcsB. In some Enterobacteriaceae, *igaA* is an essential gene as overactivation of the Rcs cascade is lethal [28, 29]. In contrast, *Y. pestis* mutants with transposon insertions in *igaA* have been reported [30, 31] and our results confirm that this gene is also not essential in *Yptb* despite its fully functioning

Rcs cascade. We verified that plasmid complementation of the *igaA* mutation could restore lower *yadE* expression similar to the wild type strain (Fig. 5). To confirm that overactive Rcs signaling was responsible for the enhanced *yadE* expression in *igaA* mutants, we compared *yadE* promoter activity in an *rcsB*::Tn5 mutant background, and in bacteria overexpressing *rcsB* via a multicopy plasmid. Lack of *rcsB* did not measurably decrease *yadE* expression compared to the wild type strain, but overexpression resulted in much higher fluorescence, similar to the *igaA* mutant (Fig. 5). These results indicate that the Rcs signaling cascade positively regulates *yadE* expression, opposite to Rcs repression of Hms-ECM production [11].

yadE is a pseudogene in _Y. pestis_, and expression of functional _yadE_ prevents Hms-ECM production

Selective gene loss in *Y. pestis* during its divergence from *Yptb* has contributed to the emergence of flea-borne transmission [5, 6, 13, 32]. All publicly available genome sequences contain a *yadE* gene fragment that is identical among *Y. pestis* strains. Pairwise alignment shows four small deletions in the N-terminal region of the *Y. pestis* sequences relative to the *Yptb* IP32953 *yadE*. These are predicted to maintain the reading frame with 98.5% amino acid identity across the first 706 residues. However, a single insertion in a poly-G tract starting at nucleotide 2118 alters the reading frame of the *Y. pestis* sequence and results in five premature stop codons (Fig. 6). These are predicted to result in a non-functional protein missing the C-terminal YadA-like membrane anchoring domain. To investigate the possible consequences of *yadE* loss on *Y. pestis* biofilm stability, we transformed *Y. pestis* KIM6+ with the same plasmid conferring constitutive expression of the functional *yadE* gene. In contrast to its effect on *Yptb*, expression of *yadE* in *Y. pestis* resulted in biofilms that were more easily disrupted (Fig. 7a). This strain also produced colonies that were less pigmented on Congo-red agar plates, which is suggestive of

reduced Hms-ECM production (Fig. 7b). We also tested the production of Hms-ECM using lectin staining and flow cytometry. Wild type *Y. pestis* binds strongly to wheat-germ agglutinin specific for N-acetylglucosamine polysaccharides. Conversely, *Y. pestis* expressing *yadE* exhibited a bipolar WGA-lectin staining pattern, where a significant portion of the cells did not bind to the lectin while another population bound at higher levels than the wild type strain (Fig. 7c). Thus, expression of functional *yadE* from *Yptb* in *Y. pestis* weakens biofilm cohesiveness, perhaps by altering production of the Hms-ECM.

DISCUSSION

Biofilms may be held together by extracellular polysaccharides, DNA, or proteins. Among *Yptb* strains, carriage of the *hms* genes encoding for polysaccharide extracellular matrix is ubiquitous, although they vary greatly in their expression of these genes [33]. The Hms-ECM is required for *Yptb* biofilm production in some conditions, including on the mouthparts of predatory nematodes [34, 35]. Extracellular DNA has also been detected in at least some *Yptb* nematode-associated biofilms, although the source and functional significance of the DNA remain unclear [36]. The extracellular matrix of biofilms formed by *Y. pestis* while they are in the flea digestive tract incorporates material derived from blood digestion [4], but the bacteria are not known to produce any other biofilm extracellular matrix material other than Hms-ECM. Our study is the first to demonstrate a role for a protein in mediating cohesion of *Yersinia* biofilms. The YPTB2394 (*yadE*) gene encodes a trimeric autotransporter protein vital to the stability of *Yptb* biofilms, as its inactivation results in a fragile biofilm and its overexpression significantly strengthens cohesiveness.

Multiple autotransporter proteins belonging to either type Va, Vc, or Ve families are present in *Y. pestis* and/or *Yptb* [37]. Type Va *Yersinia* autotransporters have been mainly investigated in the context of *Y. pestis* infection. YapE, YapJ, YapK, and YapV promote development of bubonic and/or pneumonic plague in mice [38-40], likely by enhancing adherence to epithelial cells. YapC can induce aggregation when expressed in *E. coli* but no effects on *Y. pestis* biofilms following deletion of this gene were observed [41]. *Yptb* strains also possess an additional type Va autotransporter gene *yapX*, whose function is unknown, which is also a pseudogene in all *Y. pestis* strains [38].

YadE is predicted to be a member of the type Vc trimeric autotransporter family. Numerous species use trimeric autotransporters to adhere to other bacteria within biofilms, host extracellular matrix proteins, or inanimate surfaces [21]. The prototype of the Vc family is YadA, made by *Yptb* and *Y. enterocolitica* but which has been inactivated in *Y. pestis*. YadA promotes autoagglutination and tight adherence to eukaryotic cells necessary for proper injection of effector proteins via type III secretion [42]. Restoring *yadA* gene function in *Y. pestis* has been reported to decrease its virulence in mouse infections [43]. It is not known whether YadA expression also interferes with Hms-ECM production by *Y. pestis*. However, it is co-expressed along with the type III secretion effectors encoded on the virulence plasmid at 37°C rather than at lower temperatures when Hms-ECM is produced. *Yptb* and *Y. pestis* both express two additional type Vc proteins, YadB and YadC, also at 37°C, which promote invasion of host cells and bacterial survival in skin [44].

Versatile biofilm production strategies are probably most helpful to bacteria such as *Yptb* that are found in many different environments (free-living or within amoeba in soil or water, in plants, or in the digestive tracts of multiple animals) [45]. Conversely, niche specialization might

be promoted by the selection of one biofilm pathway at the expense of others. Many *Staphylococcus* strains produce biofilms that are dependent on polysaccharide intracellular adhesin, which is identical to Hms-ECM [46]. However, expression of this polymer tends to be suppressed in methicillin-resistant *S. aureus* strains in favor of fibronectin binding proteins [47]. Staphylococci that predominantly inhabit environments with high shear stress or grow on medical devices produce primarily polysaccharide-dependent biofilms [48, 49], whereas those that interact more directly with host tissues may benefit from protein-based biofilms that allow them to incorporate fibrin or other host proteins into a protective shield [50]. It is tempting to speculate that *Yptb* strains retain multiple biofilm strategies that provide flexibility according to changing environments, whereas *Y. pestis* may have jettisoned alternative biofilm strategies to Hms-ECM as it adapted to its restricted lifestyle of flea-rodent transmission.

Although *yadE* (y1486 in the *Y. pestis* KIM sequence) is a pseudogene in *Y. pestis*, it is one of the 100 most highly transcribed genes by this strain during infection of fleas [51]. Furthermore, even greater expression was measured in a *Yptb* mutant strain that is able to infect and block fleas [52]. Thus, the regulatory influences necessary for strong induction of this gene exist during flea infections. We found that Tn5 insertions in the adenylate cyclase responsible for producing cyclic AMP greatly enhance *yadE* expression. This is consistent with previously reported transcriptome comparisons of a cAMP receptor protein (CRP) mutant to the wild type YPIII strain [25]. Our results also demonstrate that induction of Rcs signaling, either due to inactivation of *igaA* or overexpression of *rcsB*, dramatically increases expression of *yadE*. The Rcs cascade is very complex and can respond to many different signals, including lipopolysaccharide and peptidoglycan perturbations [53, 54]. Innate immune defenses, osmotic changes, or other factors present in the flea digestive tract may inflict similar stresses to *Yersinia*.

At the same time, high levels of *yadE* expression are disruptive to production, accumulation, or stability of the Hms-ECM. Hms-ECM is essential for proper biofilm formation and proventricular blockage, the major transmission mode of *Y. pestis* by rat fleas. Therefore, loss of *yadE* gene function may have been an additional key step in the divergence of *Y. pestis* from *Yptb*.

EXPERIMENTAL PROCEDURES

Bacterial strains, media and growth conditions

Y. pestis KIM6+ and *Y. pseudotuberculosis* IP32953 were grown at 21°C in Luria-Bertani (LB) or heart infusion broth containing 0.2% galactose (HIG) [18]. *E. coli* strains were grown in LB agar or broth at 37°C. Where required, kanamycin (30 µg/ml), chloramphenicol (10 µg/ml), or ampicillin (100 µg/ml) were added to media.

Deletion of *yadE* from *Yptb* IP32953 was accomplished through allelic exchange. The PCR primers used for creating the strains and plasmids for these experiments are listed in Supplementary Table S2. Upstream and downstream regions adjacent to YPTB2394 were cloned by overlapping extension PCR [55] and inserted into plasmid pRE112 [56] to create pREΔ*yadE.* The ampicillin resistance (*bla*) gene from pHSG415 [57] was then inserted into this plasmid replacing the *yadE* coding region to create pREΔ*yadE::bla*. After biparental mating with *E. coli* strain MFDλpir (pREΔ*yadE::bla*) [58] as donor and *Yptb* IP32953 as recipient, double-crossover mutations were selected on media containing 10% sucrose and ampicillin. The mutation was verified using PCR and Sanger sequencing (Eton Biosciences Inc.).

To overexpress *yadE*, the YPTB2394 gene as well as the upstream 200 bp region were placed under the control of the tetracycline resistance promoter on plasmid pACYC184. PCR

products for the *yadE* gene and the pACYC184 backbone were combined in an overlapping-extension PCR reaction and transformed into *E. coli* DH5α to create plasmid pACYC-*yadE*. In a similar way, the pACYC-*yadE::gfp* reporter plasmid was created by amplifying the *gfp* coding region from pUC18R6K-miniTn7T sig70c35_GFP [59], the YPTB2394 promoter region, and the pACYC184 backbone. These PCR products were purified and combined in an overlapping-extension PCR reaction and transformed into *E. coli* DH5α. Plasmids containing *igaA* and *rcsB* (pJET-*igaA* and pJET-*rcsB*) were created by PCR amplification of the respective genes and cloning into the pJET1.2 plasmid (ThermoFisher). All plasmids were sequence verified and transformed into *Yersinia* strains by electroporation.

Biofilm disruption assay

Biofilm stability was measured as previously described [18]. Overnight broth cultures of strains to be tested were transferred to polycarbonate track etched (PCTE) membrane filters (n=4-5) on HIG agar plates. After 72h of growth at 21°C, individual filters containing the biofilm samples were placed in 10 mL of phosphate buffered saline (PBS). Tubes were shaken vertically at 200 rpm and the optical density of dislodged cells was measured (A_{600nm}) at specific time points. The filters were then vortexed at high speed until biofilms were completely disrupted and the A_{600nm} measured. Enzymatic treatment of biofilms prior to disruption tests were performed with proteinase K (Sigma) or DNase (Ambion) at 5 mg/ml. Solutions of enzyme in PBS (100 µl) were placed directly on top of the biofilms and incubated at 21°C for 60 minutes.

Transposon mutagenesis and fluorescence activated cell sorting

A Tn5 transposon mutant library in strain IP32953 (pACYC-*yadE::gfp*) was created using the pRL27 donor plasmid as previously described [60]. After plating the mating mixture on HIB agar containing kanamycin and chloramphenicol, ~100,000 individual mutant colonies were

suspended and washed in PBS and diluted to 10^6 cfu/ml for FACS sorting. A total of ~$4x10^6$ individual *Yptb* mutant bacteria were sorted using FACS Aria Fusion Cell Sorter (BD Biosciences) at the BYU Cell Sorting/Bio-Mass Spectrometry core facility. Cells exhibiting one standard deviation greater than the wild-type IP32953 strain in the GFP channel were collected, diluted and plated on HIB agar containing kanamycin and chloramphenicol to grow single colonies. The plates were then incubated for 24 h. Approximately 1000 colonies from these plates were examined individually using an LED mini-blue transilluminator (IO Rodeo). From this pool, 48 colonies were confirmed visually to express high levels of GFP after re-growth and were selected for further verification using flow cytometry. The Tn5 insertion sites in these mutants were determined by arbitrary PCR and sequencing as previously described [58]. Of the 48 colonies, 23 were found to have unique insertion sites as reported in Supplementary Table 1.

Flow cytometry

To measure *yadE::gfp* expression, bacteria containing the reporter plasmid were grown on HIG agar plates at 21°C for 24 h. For each strain, bacteria from individual colonies were suspended in PBS and the GFP fluorescence measured by flow cytometry. For Hms-ECM measurement on the surface of bacteria, fixed bacterial cells were incubated with FITC-labeled wheat-germ agglutinin (Sigma) as previously described [18]. GFP expression and lectin binding of individual cells was measured using a BD Accuri C6 Flow Cytometer and analysed using FACSDiva software (BD Biosciences).

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6.0. The details for each test are provided in the relevant figure legend.

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FIGURE LEGENDS

Figure 1. Increased stability of *Yptb csrB*::Tn5 mutant biofilms may be mediated by proteins. Biofilms were grown on polycarbonate filters for 72 hours. Filter biofilms were then treated with proteinase K or DNase for 30 minutes prior to agitation for 15 minutes. The proportion of the biofilms that were dislodged was measured by spectrophotometry. Pre-treatment of *Yptb csrB*::Tn5 mutant biofilms with proteinase K enhances their dispersal relative to the PBS control (****$p<0.001$ by Student's T test) while DNase had no effect. *Y. pestis* biofilm stability was not affected by pretreatment with either enzyme.

Figure 2. YPTB2394 (*yadE*) affects biofilm cohesion. Biofilms formed by the wild type IP32953, YPTB2394 (Δ*yadE*) mutant, and strain overexpressing *yadE* were each agitated and their dispersal measured at 5-minute intervals. Results were analyzed using repeated measures ANOVA with Tukey's multiple comparison test (****significantly different from wild type, $p<0.0001$).

Figure 3. Expression of *yadE* is repressed by CsrB. The *Yptb* IP32953 wild type and *csrB*::Tn5 mutant strains were transformed with a *yadE*::*gfp* reporter plasmid. After growth for 48h on solid media the fluorescence of the population was measured by flow cytometry. *** indicates a significant difference from the wild-type strain ($p=0.0002$) by unpaired T-test.

342 **Figure 4. Mutants that overexpress *yadE* produce more cohesive biofilms.** Transposon
343 mutants derived from IP32953 with high *yadE*::*gfp* reporter activity were selected by FACS.
344 Individual mutants with insertions in *igaA*, *sspA*, and *cya* genes were tested in biofilm disruption
345 assays in comparison with the wild type IP32953 strain and the wild type strain overexpressing
346 *yadE* (pACYC-*yadE*). One-way ANOVA analysis was performed with Tukey's correction for
347 multiple comparisons. Columns with the same letter (a, b, c) are not significantly different from
348 each other (95% confidence interval).
349
350 **Figure 5. Expression of *yadE* is controlled by the Rcs phosphorelay.** Inactivation of *igaA* or
351 overexpression of *rcsB* increased *yadE*::*gfp* reporter activity. Fluorescence was measured by
352 flow cytometry and compared with the wild type strain (** indicates a significant difference by
353 unpaired T test, $p<0.01$).
354
355 **Figure 6. The YPTB2394 and y1486 orthologues of *Yptb* and *Y. pestis*.** Domains predicted in
356 the YPTB2394 protein sequence typical of trimeric autotransporters [61] include a
357 transmembrane helix containing a secretion signal peptide (30-51), a HANS domain that
358 typically connects α-to-β regions of proximal to head regions, COG5295 comprising the β-strand
359 head domains with repetitive motifs, and the YadA-like anchor consisting of a 12-stranded outer
360 membrane β-barrel. The asterisk notes the region containing the premature stop codons found in
361 the *Y. pestis* sequences. Pairwise alignment of the *Yptb* IP32953 *yadE* (YPTB2394) and *Y. pestis*
362 KIM pseudogene (y1486) nucleotide sequences shows that the sequences are highly similar
363 across the 2643 bp *Yptb* sequence. All *Y. pestis* sequences have 14, 7, 4, and 5 bp deletions
364 between nucleotides 185-209 and 326-340, which maintain the reading frame (not shown). A

guanine insertion at nucleotide 2118 changes the reading frame and results in 5 predicted stop codons in the C-terminal region beginning at nucleotide 2230 that are predicted to eliminate the C-terminal YadA-like anchor.

Figure 7. Expression of functional *yadE* in *Y. pestis* reduces biofilm stability and alters Hms-ECM production. A. Disruption of biofilms formed by *Y. pestis* KIM6+ wild type or strains transformed with pACYC-*yadE* or the pACYC184 empty vector. Results were analyzed using repeated measures ANOVA with Tukey's multiple comparison test (**p<0.01, ***p<0.001, ****p<0.0001) B. Pigmentation of *Y. pestis* (*yadE*+ or empty vector) grown on Congo-red agar plates. C. Flow cytometry analysis of *Y. pestis* stained with GFP-labeled WGA lectin that binds to HMS-ECM. The wild type strain (orange) is uniformly labeled compared to the unstained control (red) while the strain expressing functional *yadE* (blue) exhibits a bipolar staining pattern with either no detectable lectin bound to the surface or with very high staining.

REFERENCES

1. Hinnebusch BJ, Erickson DL. Yersinia pestis biofilm in the flea vector and its role in the transmission of plague. Curr Top Microbiol Immunol. 2008;322:229-48. Epub 2008/05/06. doi: 10.1007/978-3-540-75418-3_11. PubMed PMID: 18453279; PubMed Central PMCID: PMCPMC3727414.

2. Zhou D, Yang R. Formation and regulation of Yersinia biofilms. Protein Cell. 2011;2(3):173-9. Epub 2011/03/08. doi: 10.1007/s13238-011-1024-3. PubMed PMID: 21380640; PubMed Central PMCID: PMCPMC4875308.

387 3. Hinnebusch BJ, Perry RD, Schwan TG. Role of the Yersinia pestis hemin storage (hms)
388 locus in the transmission of plague by fleas. Science. 1996;273(5273):367-70. PubMed PMID:
389 8662526.
390 4. Jarrett CO, Deak E, Isherwood KE, Oyston PC, Fischer ER, Whitney AR, et al.
391 Transmission of Yersinia pestis from an infectious biofilm in the flea vector. J Infect Dis.
392 2004;190(4):783-92. PubMed PMID: 15272407.
393 5. Sun YC, Jarrett CO, Bosio CF, Hinnebusch BJ. Retracing the evolutionary path that led
394 to flea-borne transmission of Yersinia pestis. Cell Host Microbe. 2014;15(5):578-86. Epub
395 2014/05/17. doi: 10.1016/j.chom.2014.04.003. PubMed PMID: 24832452; PubMed Central
396 PMCID: PMCPMC4084870.
397 6. Erickson DL, Jarrett CO, Callison JA, Fischer ER, Hinnebusch BJ. Loss of a biofilm-
398 inhibiting glycosyl hydrolase during the emergence of Yersinia pestis. J Bacteriol.
399 2008;190(24):8163-70. Epub 2008/10/22. doi: 10.1128/JB.01181-08. PubMed PMID: 18931111;
400 PubMed Central PMCID: PMCPMC2593235.
401 7. Hinnebusch BJ. The evolution of flea-borne transmission in Yersinia pestis. Curr Issues
402 Mol Biol. 2005;7(2):197-212. Epub 2005/08/02. PubMed PMID: 16053250.
403 8. Wall E, Majdalani N, Gottesman S. The Complex Rcs Regulatory Cascade. Annual
404 review of microbiology. 2018;72:111-39. Epub 2018/06/14. doi: 10.1146/annurev-micro-
405 090817-062640. PubMed PMID: 29897834.
406 9. Hussein NA, Cho SH, Laloux G, Siam R, Collet JF. Distinct domains of Escherichia coli
407 IgaA connect envelope stress sensing and down-regulation of the Rcs phosphorelay across
408 subcellular compartments. PLoS genetics. 2018;14(5):e1007398. Epub 2018/06/01. doi:

409 10.1371/journal.pgen.1007398. PubMed PMID: 29852010; PubMed Central PMCID:
410 PMCPMC5978795.
411 10. Guo XP, Ren GX, Zhu H, Mao XJ, Sun YC. Differential regulation of the hmsCDE
412 operon in Yersinia pestis and Yersinia pseudotuberculosis by the Rcs phosphorelay system.
413 Scientific reports. 2015;5:8412. Epub 2015/02/13. doi: 10.1038/srep08412. PubMed PMID:
414 25672461; PubMed Central PMCID: PMCPMC4325325.
415 11. Sun YC, Guo XP, Hinnebusch BJ, Darby C. The Yersinia pestis Rcs phosphorelay
416 inhibits biofilm formation by repressing transcription of the diguanylate cyclase gene hmsT. J
417 Bacteriol. 2012;194(8):2020-6. Epub 2012/02/14. doi: 10.1128/JB.06243-11. PubMed PMID:
418 22328676; PubMed Central PMCID: PMCPMC3318482.
419 12. Fang N, Yang H, Fang H, Liu L, Zhang Y, Wang L, et al. RcsAB is a major repressor of
420 Yersinia biofilm development through directly acting on hmsCDE, hmsT, and hmsHFRS.
421 Scientific reports. 2015;5:9566. Epub 2015/04/02. doi: 10.1038/srep09566. PubMed PMID:
422 25828910; PubMed Central PMCID: PMCPMC4381331.
423 13. Sun YC, Hinnebusch BJ, Darby C. Experimental evidence for negative selection in the
424 evolution of a Yersinia pestis pseudogene. Proc Natl Acad Sci U S A. 2008;105(23):8097-101.
425 Epub 2008/06/05. doi: 10.1073/pnas.0803525105. PubMed PMID: 18523005; PubMed Central
426 PMCID: PMCPMC2430365.
427 14. Kirillina O, Fetherston JD, Bobrov AG, Abney J, Perry RD. HmsP, a putative
428 phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm
429 formation in Yersinia pestis. Mol Microbiol. 2004;54(1):75-88. Epub 2004/10/02. doi:
430 10.1111/j.1365-2958.2004.04253.x. PubMed PMID: 15458406.

15. Ren GX, Yan HQ, Zhu H, Guo XP, Sun YC. HmsC, a periplasmic protein, controls biofilm formation via repression of HmsD, a diguanylate cyclase in Yersinia pestis. Environ Microbiol. 2014;16(4):1202-16. Epub 2013/11/07. doi: 10.1111/1462-2920.12323. PubMed PMID: 24192006.

16. Bobrov AG, Kirillina O, Forman S, Mack D, Perry RD. Insights into Yersinia pestis biofilm development: topology and co-interaction of Hms inner membrane proteins involved in exopolysaccharide production. Environ Microbiol. 2008;10(6):1419-32. Epub 2008/02/19. doi: 10.1111/j.1462-2920.2007.01554.x. PubMed PMID: 18279344.

17. Bobrov AG, Kirillina O, Perry RD. Regulation of biofilm formation in Yersinia pestis. Adv Exp Med Biol. 2007;603:201-10. Epub 2007/10/31. doi: 10.1007/978-0-387-72124-8_17. PubMed PMID: 17966416.

18. Schachterle JK, Stewart RM, Schachterle MB, Calder JT, Kang H, Prince JT, et al. Yersinia pseudotuberculosis BarA-UvrY Two-Component Regulatory System Represses Biofilms via CsrB. Front Cell Infect Microbiol. 2018;8:323. Epub 2018/10/04. doi: 10.3389/fcimb.2018.00323. PubMed PMID: 30280093; PubMed Central PMCID: PMCPMC6153318.

19. Wells TJ, Tree JJ, Ulett GC, Schembri MA. Autotransporter proteins: novel targets at the bacterial cell surface. Fems Microbiology Letters. 2007;274(2):163-72. doi: 10.1111/j.1574-6968.2007.00833.x. PubMed PMID: WOS:000248960500001.

20. Linke D, Riess T, Autenrieth IB, Lupas A, Kempf VA. Trimeric autotransporter adhesins: variable structure, common function. Trends Microbiol. 2006;14(6):264-70. Epub 2006/05/09. doi: 10.1016/j.tim.2006.04.005. PubMed PMID: 16678419.

453 21. Meuskens I, Saragliadis A, Leo JC, Linke D. Type V Secretion Systems: An Overview of
454 Passenger Domain Functions. Front Microbiol. 2019;10:1163. Epub 2019/06/20. doi:
455 10.3389/fmicb.2019.01163. PubMed PMID: 31214135; PubMed Central PMCID:
456 PMCPMC6555100.
457 22. Klemm P, Hjerrild L, Gjermansen M, Schembri MA. Structure-function analysis of the
458 self-recognizing Antigen 43 autotransporter protein from Escherichia coli. Mol Microbiol.
459 2004;51(1):283-96. Epub 2003/12/04. doi: 10.1046/j.1365-2958.2003.03833.x. PubMed PMID:
460 14651628.
461 23. Okaro U, Green R, Mohapatra S, Anderson B. The trimeric autotransporter adhesin BadA
462 is required for in vitro biofilm formation by Bartonella henselae. NPJ Biofilms Microbiomes.
463 2019;5:10. Epub 2019/03/20. doi: 10.1038/s41522-019-0083-8. PubMed PMID: 30886729;
464 PubMed Central PMCID: PMCPMC6418236.
465 24. Lazar Adler NR, Dean RE, Saint RJ, Stevens MP, Prior JL, Atkins TP, et al.
466 Identification of a predicted trimeric autotransporter adhesin required for biofilm formation of
467 Burkholderia pseudomallei. PLoS ONE. 2013;8(11):e79461. Epub 2013/11/14. doi:
468 10.1371/journal.pone.0079461. PubMed PMID: 24223950; PubMed Central PMCID:
469 PMCPMC3818227.
470 25. Nuss AM, Heroven AK, Waldmann B, Reinkensmeier J, Jarek M, Beckstette M, et al.
471 Transcriptomic profiling of Yersinia pseudotuberculosis reveals reprogramming of the Crp
472 regulon by temperature and uncovers Crp as a master regulator of small RNAs. PLoS genetics.
473 2015;11(3):e1005087. Epub 2015/03/31. doi: 10.1371/journal.pgen.1005087. PubMed PMID:
474 25816203; PubMed Central PMCID: PMCPMC4376681.

475 26. Romeo T, Vakulskas CA, Babitzke P. Post-transcriptional regulation on a global scale:
476 form and function of Csr/Rsm systems. Environ Microbiol. 2013;15(2):313-24. Epub
477 2012/06/08. doi: 10.1111/j.1462-2920.2012.02794.x. PubMed PMID: 22672726; PubMed
478 Central PMCID: PMCPMC3443267.
479 27. Heroven AK, Bohme K, Rohde M, Dersch P. A Csr-type regulatory system, including
480 small non-coding RNAs, regulates the global virulence regulator RovA of Yersinia
481 pseudotuberculosis through RovM. Mol Microbiol. 2008;68(5):1179-95. Epub 2008/04/24. doi:
482 10.1111/j.1365-2958.2008.06218.x. PubMed PMID: 18430141.
483 28. Cano DA, Dominguez-Bernal G, Tierrez A, Garcia-Del Portillo F, Casadesus J.
484 Regulation of capsule synthesis and cell motility in Salmonella enterica by the essential gene
485 igaA. Genetics. 2002;162(4):1513-23. Epub 2003/01/14. PubMed PMID: 12524328; PubMed
486 Central PMCID: PMCPMC1462382.
487 29. Cho SH, Szewczyk J, Pesavento C, Zietek M, Banzhaf M, Roszczenko P, et al. Detecting
488 envelope stress by monitoring beta-barrel assembly. Cell. 2014;159(7):1652-64. Epub
489 2014/12/20. doi: 10.1016/j.cell.2014.11.045. PubMed PMID: 25525882.
490 30. Palace SG, Proulx MK, Lu S, Baker RE, Goguen JD. Genome-wide mutant fitness
491 profiling identifies nutritional requirements for optimal growth of Yersinia pestis in deep tissue.
492 mBio. 2014;5(4). Epub 2014/08/21. doi: 10.1128/mBio.01385-14. PubMed PMID: 25139902;
493 PubMed Central PMCID: PMCPMC4147864.
494 31. Senior NJ, Sasidharan K, Saint RJ, Scott AE, Sarkar-Tyson M, Ireland PM, et al. An
495 integrated computational-experimental approach reveals Yersinia pestis genes essential across a
496 narrow or a broad range of environmental conditions. BMC Microbiol. 2017;17(1):163. Epub

2017/07/25. doi: 10.1186/s12866-017-1073-8. PubMed PMID: 28732479; PubMed Central PMCID: PMCPMC5521123.

32. Erickson DL, Waterfield NR, Vadyvaloo V, Long D, Fischer ER, Ffrench-Constant R, et al. Acute oral toxicity of Yersinia pseudotuberculosis to fleas: implications for the evolution of vector-borne transmission of plague. Cell Microbiol. 2007;9(11):2658-66. Epub 2007/06/26. doi: 10.1111/j.1462-5822.2007.00986.x. PubMed PMID: 17587333.

33. Erickson DL, Jarrett CO, Wren BW, Hinnebusch BJ. Serotype differences and lack of biofilm formation characterize Yersinia pseudotuberculosis infection of the Xenopsylla cheopis flea vector of Yersinia pestis. J Bacteriol. 2006;188(3):1113-9. Epub 2006/01/24. doi: 10.1128/JB.188.3.1113-1119.2006. PubMed PMID: 16428415; PubMed Central PMCID: PMCPMC1347331.

34. Darby C, Hsu JW, Ghori N, Falkow S. Caenorhabditis elegans: plague bacteria biofilm blocks food intake. Nature. 2002;417(6886):243-4. PubMed PMID: 12015591.

35. Joshua GW, Karlyshev AV, Smith MP, Isherwood KE, Titball RW, Wren BW. A Caenorhabditis elegans model of Yersinia infection: biofilm formation on a biotic surface. Microbiology. 2003;149(Pt 11):3221-9. PubMed PMID: 14600234.

36. Atkinson S, Goldstone RJ, Joshua GW, Chang CY, Patrick HL, Camara M, et al. Biofilm development on Caenorhabditis elegans by Yersinia is facilitated by quorum sensing-dependent repression of type III secretion. PLoS Pathog. 2011;7(1):e1001250. Epub 2011/01/22. doi: 10.1371/journal.ppat.1001250. PubMed PMID: 21253572; PubMed Central PMCID: PMCPMC3017118.

518 37. Chauhan N, Wrobel A, Skurnik M, Leo JC. Yersinia adhesins: An arsenal for infection.
519 Proteomics Clin Appl. 2016;10(9-10):949-63. Epub 2016/04/14. doi: 10.1002/prca.201600012.
520 PubMed PMID: 27068449.
521 38. Nair MK, De Masi L, Yue M, Galvan EM, Chen H, Wang F, et al. Adhesive properties of
522 YapV and paralogous autotransporter proteins of Yersinia pestis. Infect Immun.
523 2015;83(5):1809-19. Epub 2015/02/19. doi: 10.1128/IAI.00094-15. PubMed PMID: 25690102;
524 PubMed Central PMCID: PMCPMC4399037.
525 39. Lawrenz MB, Pennington J, Miller VL. Acquisition of omptin reveals cryptic virulence
526 function of autotransporter YapE in Yersinia pestis. Mol Microbiol. 2013;89(2):276-87. Epub
527 2013/05/25. doi: 10.1111/mmi.12273. PubMed PMID: 23701256; PubMed Central PMCID:
528 PMCPMC3708302.
529 40. Lenz JD, Lawrenz MB, Cotter DG, Lane MC, Gonzalez RJ, Palacios M, et al. Expression
530 during host infection and localization of Yersinia pestis autotransporter proteins. J Bacteriol.
531 2011;193(21):5936-49. Epub 2011/08/30. doi: 10.1128/JB.05877-11. PubMed PMID: 21873491;
532 PubMed Central PMCID: PMCPMC3194903.
533 41. Felek S, Lawrenz MB, Krukonis ES. The Yersinia pestis autotransporter YapC mediates
534 host cell binding, autoaggregation and biofilm formation. Microbiology. 2008;154(Pt 6):1802-
535 12. Epub 2008/06/06. doi: 154/6/1802 [pii]
536 10.1099/mic.0.2007/010918-0. PubMed PMID: 18524935.
537 42. El Tahir Y, Skurnik M. YadA, the multifaceted Yersinia adhesin. Int J Med Microbiol.
538 2001;291(3):209-18. Epub 2001/09/14. doi: 10.1078/1438-4221-00119. PubMed PMID:
539 11554561.

43. Rosqvist R, Skurnik M, Wolf-Watz H. Increased virulence of Yersinia pseudotuberculosis by two independent mutations. Nature. 1988;334(6182):522-4. Epub 1988/08/11. doi: 10.1038/334522a0. PubMed PMID: 3043229.

44. Uittenbogaard AM, Myers-Morales T, Gorman AA, Welsh E, Wulff C, Hinnebusch BJ, et al. Temperature-dependence of yadBC phenotypes in Yersinia pestis. Microbiology. 2014;160(Pt 2):396-405. Epub 2013/11/14. doi: 10.1099/mic.0.073205-0. PubMed PMID: 24222617; PubMed Central PMCID: PMCPMC3919538.

45. Martinez-Chavarria LC, Vadyvaloo V. Yersinia pestis and Yersinia pseudotuberculosis infection: a regulatory RNA perspective. Front Microbiol. 2015;6:956. Epub 2015/10/07. doi: 10.3389/fmicb.2015.00956. PubMed PMID: 26441890; PubMed Central PMCID: PMCPMC4585118.

46. O'Gara JP. ica and beyond: biofilm mechanisms and regulation in Staphylococcus epidermidis and Staphylococcus aureus. FEMS Microbiol Lett. 2007;270(2):179-88. Epub 2007/04/11. doi: 10.1111/j.1574-6968.2007.00688.x. PubMed PMID: 17419768.

47. O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman A, et al. A novel Staphylococcus aureus biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J Bacteriol. 2008;190(11):3835-50. Epub 2008/04/01. doi: 10.1128/JB.00167-08. PubMed PMID: 18375547; PubMed Central PMCID: PMCPMC2395027.

48. Schaeffer CR, Hoang TN, Sudbeck CM, Alawi M, Tolo IE, Robinson DA, et al. Versatility of Biofilm Matrix Molecules in Staphylococcus epidermidis Clinical Isolates and Importance of Polysaccharide Intercellular Adhesin Expression during High Shear Stress. mSphere. 2016;1(5). Epub 2016/10/18. doi: 10.1128/mSphere.00165-16. PubMed PMID: 27747298; PubMed Central PMCID: PMCPMC5064449.

563 49. Yu L, Hisatsune J, Hayashi I, Tatsukawa N, Sato'o Y, Mizumachi E, et al. A Novel
564 Repressor of the ica Locus Discovered in Clinically Isolated Super-Biofilm-Elaborating
565 Staphylococcus aureus. mBio. 2017;8(1). Epub 2017/02/02. doi: 10.1128/mBio.02282-16.
566 PubMed PMID: 28143981; PubMed Central PMCID: PMCPMC5285506.
567 50. Zapotoczna M, O'Neill E, O'Gara JP. Untangling the Diverse and Redundant
568 Mechanisms of Staphylococcus aureus Biofilm Formation. PLoS Pathog. 2016;12(7):e1005671.
569 Epub 2016/07/22. doi: 10.1371/journal.ppat.1005671. PubMed PMID: 27442433; PubMed
570 Central PMCID: PMCPMC4956047.
571 51. Vadyvaloo V, Jarrett C, Sturdevant DE, Sebbane F, Hinnebusch BJ. Transit through the
572 flea vector induces a pretransmission innate immunity resistance phenotype in Yersinia pestis.
573 PLoS Pathog. 2010;6(2):e1000783. Epub 2010/03/03. doi: 10.1371/journal.ppat.1000783.
574 PubMed PMID: 20195507; PubMed Central PMCID: PMC2829055.
575 52. Chouikha I, Sturdevant DE, Jarrett C, Sun YC, Hinnebusch BJ. Differential Gene
576 Expression Patterns of Yersinia pestis and Yersinia pseudotuberculosis during Infection and
577 Biofilm Formation in the Flea Digestive Tract. mSystems. 2019;4(1). Epub 2019/02/26. doi:
578 10.1128/mSystems.00217-18. PubMed PMID: 30801031; PubMed Central PMCID:
579 PMCPMC6381227.
580 53. Szczesny M, Beloin C, Ghigo JM. Increased Osmolarity in Biofilm Triggers RcsB-
581 Dependent Lipid A Palmitoylation in Escherichia coli. mBio. 2018;9(4). Epub 2018/08/23. doi:
582 10.1128/mBio.01415-18. PubMed PMID: 30131361; PubMed Central PMCID:
583 PMCPMC6106083.
584 54. Klein G, Stupak A, Biernacka D, Wojtkiewicz P, Lindner B, Raina S. Multiple
585 Transcriptional Factors Regulate Transcription of the rpoE Gene in Escherichia coli under

586 Different Growth Conditions and When the Lipopolysaccharide Biosynthesis Is Defective. J Biol
587 Chem. 2016;291(44):22999-3019. Epub 2016/10/30. doi: 10.1074/jbc.M116.748954. PubMed
588 PMID: 27629414; PubMed Central PMCID: PMCPMC5087721.
589 55. You C, Zhang YH. Simple cloning and DNA assembly in Escherichia coli by prolonged
590 overlap extension PCR. Methods Mol Biol. 2014;1116:183-92. Epub 2014/01/08. doi:
591 10.1007/978-1-62703-764-8_13. PubMed PMID: 24395365.
592 56. Edwards RA, Keller LH, Schifferli DM. Improved allelic exchange vectors and their use
593 to analyze 987P fimbria gene expression. Gene. 1998;207(2):149-57. doi: 10.1016/s0378-
594 1119(97)00619-7. PubMed PMID: WOS:000072270400007.
595 57. Hashimoto-Gotoh T, Franklin FC, Nordheim A, Timmis KN. Specific-purpose plasmid
596 cloning vectors. I. Low copy number, temperature-sensitive, mobilization-defective pSC101-
597 derived containment vectors. Gene. 1981;16(1-3):227-35. Epub 1981/12/01. doi: 10.1016/0378-
598 1119(81)90079-2. PubMed PMID: 6282694.
599 58. Ferrieres L, Hemery G, Nham T, Guerout AM, Mazel D, Beloin C, et al. Silent mischief:
600 bacteriophage Mu insertions contaminate products of Escherichia coli random mutagenesis
601 performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4
602 conjugative machinery. J Bacteriol. 2010;192(24):6418-27. doi: 10.1128/JB.00621-10. PubMed
603 PMID: 20935093; PubMed Central PMCID: PMCPMC3008518.
604 59. Shivak DJ, MacKenzie KD, Watson NL, Pasternak JA, Jones BD, Wang YJ, et al. A
605 Modular, Tn7-Based System for Making Bioluminescent or Fluorescent Salmonella and
606 Escherichia coli Strains. Appl Environ Microbiol. 2016;82(16):4931-43. doi:
607 10.1128/Aem.01346-16. PubMed PMID: WOS:000380550900008.

608 60. Erickson DL, Lew CS, Kartchner B, Porter NT, McDaniel SW, Jones NM, et al.
609 Lipopolysaccharide Biosynthesis Genes of Yersinia pseudotuberculosis Promote Resistance to
610 Antimicrobial Chemokines. PLoS ONE. 2016;11(6):e0157092. doi:
611 10.1371/journal.pone.0157092. PubMed PMID: 27275606; PubMed Central PMCID:
612 PMCPMC4898787.
613 61. Bassler J, Hernandez Alvarez B, Hartmann MD, Lupas AN. A domain dictionary of
614 trimeric autotransporter adhesins. Int J Med Microbiol. 2015;305(2):265-75. Epub 2015/01/15.
615 doi: 10.1016/j.ijmm.2014.12.010. PubMed PMID: 25583454.

616

617 **Supplementary Table 1. Tn5 insertion mutants with increased *yadE::gfp* expression**

Tn5 Insertion point (orientation)	Mean fluorescence	YPTB gene number	Annotation
Wild type IP32953	1352		
70558 (-)	1744	YPTB0058	*yibQ* hypothetical protein
224414 (+)	3395	YPTB0185	*cyaA* adenylate cyclase
223538 (+)	3104	YPTB0185	*cyaA*
757367 (-)	5851	intergenic	Near Ysr105
757452 (-)	3965	intergenic	Near Ysr105
757520 (+)	3791	Intergenic	Near Ysr105
757978 (-)	4206	Intergenic	Near Ysr105
1732316 (-)	7024	YPTB1440	Hypothetical protein
2242048 (-)	12513	pseudogene	transposase (non-functional)
2475581 (-)	4932	intergenic	Upstream of *hns*
3138894 (+)	1658	intergenic	T6SS island
4176313 (-)	13980	YPTB3506	*sspA* stringent starvation protein
4176973 (-)	11465	YPTB3506	*sspA* stringent starvation protein
4176992 (+)	9682	YPTB3506	*sspA* stringent starvation protein
4459942 (+)	9948	YPTB3758	*igaA* prevents activation of RcsC
4459955 (+)	8919	YPTB3758	*igaA* prevents activation of RcsC
4459959 (-)	11687	YPTB3758	*igaA* prevents activation of RcsC
4459976 (-)	9543	YPTB3758	*igaA* prevents activation of RcsC
4460097 (+)	9138	YPTB3758	*igaA* prevents activation of RcsC
4460363 (-)	10221	YPTB3758	*igaA* prevents activation of RcsC
4460771 (+)	10589	YPTB3758	*igaA* prevents activation of RcsC
4460832 (+)	8849	YPTB3758	*igaA* prevents activation of RcsC
4461288 (+)	9740	YPTB3758	*igaA* prevents activation of RcsC

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619

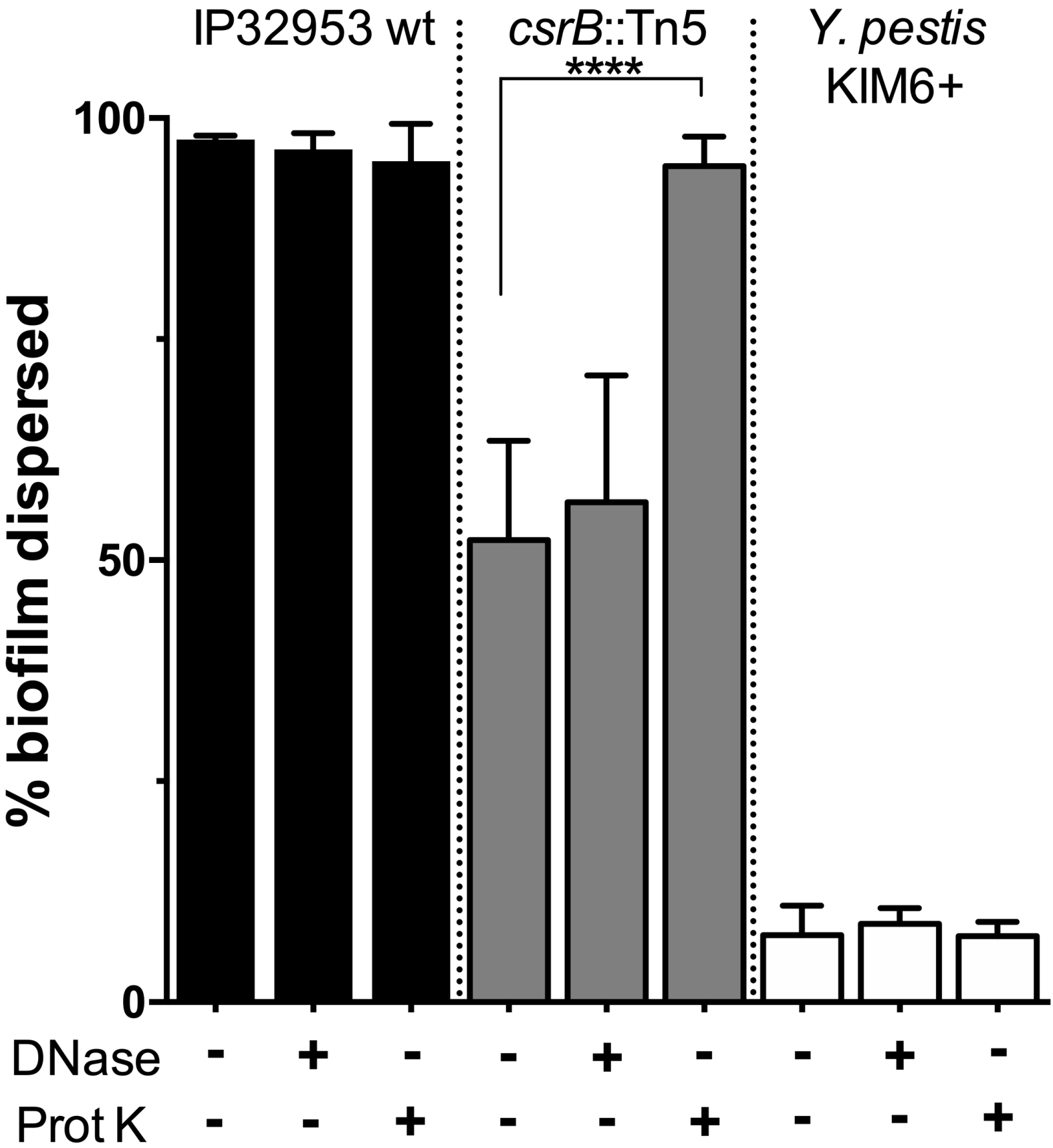
620 **Supplementary Table 2. PCR Primers**

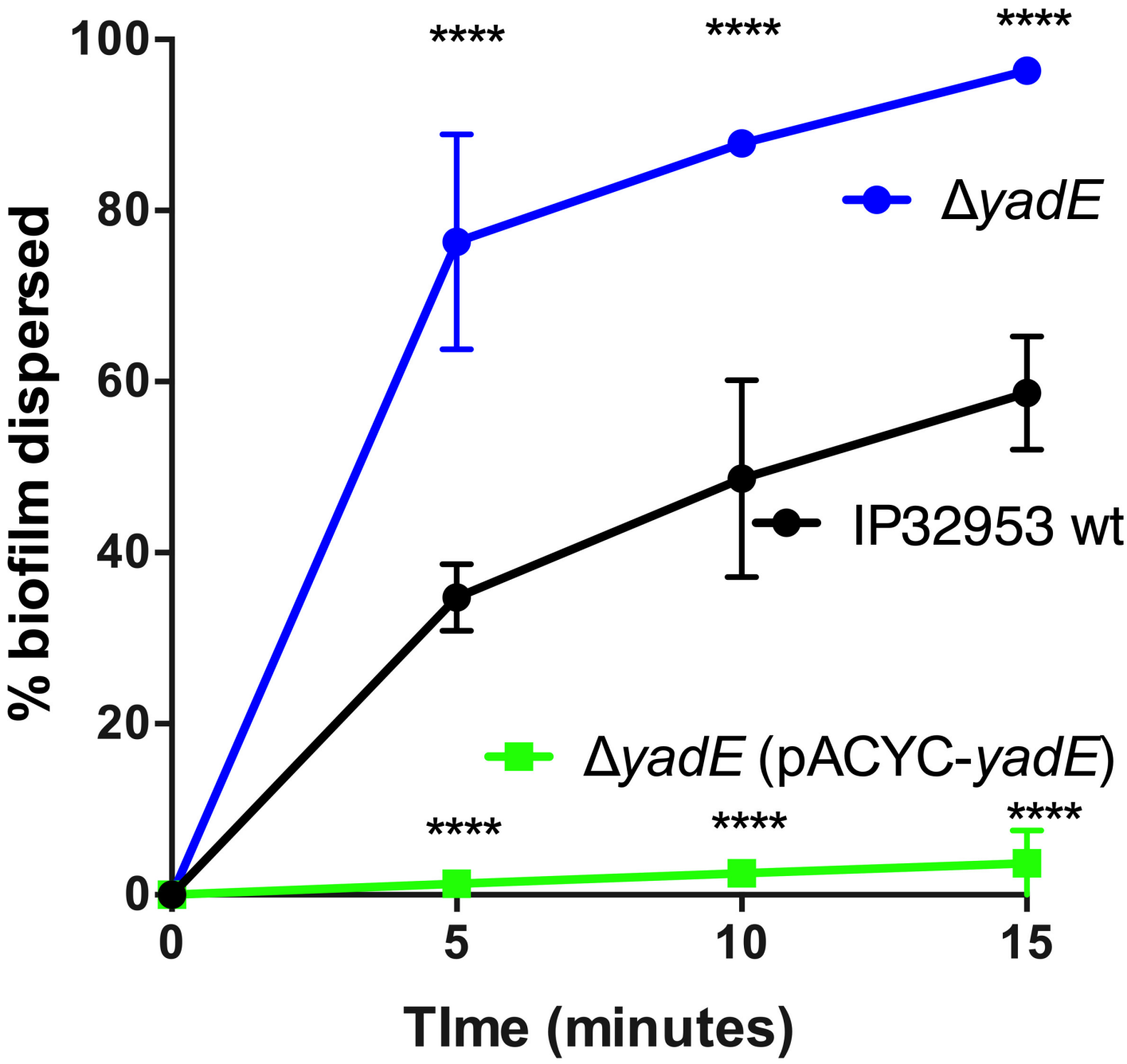
Primer	Sequence (5' – 3')*	Purpose
2394-F	GCAGTCAGGCACCGTGTATG*GGGGATGGTATGAAAAGTATCC*	Amplify *yadE* to create pACYC-*yadE*
2394-R	CCATTCAGGTCGAGGTGGCC*CAATATTCAGTGTTGGCCTTTC*	
pACYCa	GGCCACCTCGACCTGAATGG	pACYC184 vector primers
pACYCb	CATACACGGTGCCTGACTGC	
2394A	GGTTTCATCGACCTCCACCG*GGTGGGGTCAGTGGGTAATG*	5' portion of *yadE* for plasmid pREΔ*yadE*
2394B	GGCAAATGGCTAACCGTATT*CCCAATATTCAGTGTTGGCCTTTC*	
2394C	CATTACCCACTGACCCCACC*CGGTGGAGGTCGATGAAACC*	3' portion of *yadE* for plasmid pREΔ*yadE*
2394D	CCTTAAACGCCTGGTTGCTAC*GGGGATGGTATGAAAAGTATCC*	
pRE112a	GAAAGGCCAACACTGAATATTG*GGAATACGGTTAGCCATTTGCC*	pRE112 vector primers
pRE112b	GGATACTTTTCATACCATCCCC*GTAGCAACCAGGCGTTTAAGG*	
ampR F	CATTACCCACTGACCCCACC*CTTAGACGTCAGGTGGCACTT*	Amplify *bla* from pHSG415
ampR R	GGTTTCATCGACCTCCACCG*GTAAACTTGGTCTGACAGTTACCAATG*	
pRE112c	AAGTGCCACCTGACGTCTAAG*GGTGGGGTCAGTGGGTAATG*	Inserting *bla* to create pREΔ*yadE::bla*
pRE112d	CATTGGTAACTGTCAGACCAAGTTTAC*CGGTGGAGGTCGATGAAAC C*	
2394 int F	GACCGCTGTTGTTGTTCACC	*yadE* internal primers to verify deletion
2394 int R	GGAGCACAGTCTCTGGCATT	
2394 proF	CTGAAGTCAGCCCCATACGA*AATATATTTTTTCTACCGCT*	*yadE* promoter for pACYC*yadE::gfp*
2394 proR	TCTTCTCCTTTACG*CATACCATCCCCATTT*	
GFP F	GGTGAACAAGAATAAAATGGGGATGGT*ATGCGTAAAGGAGAAGAAC*	*gfp* from

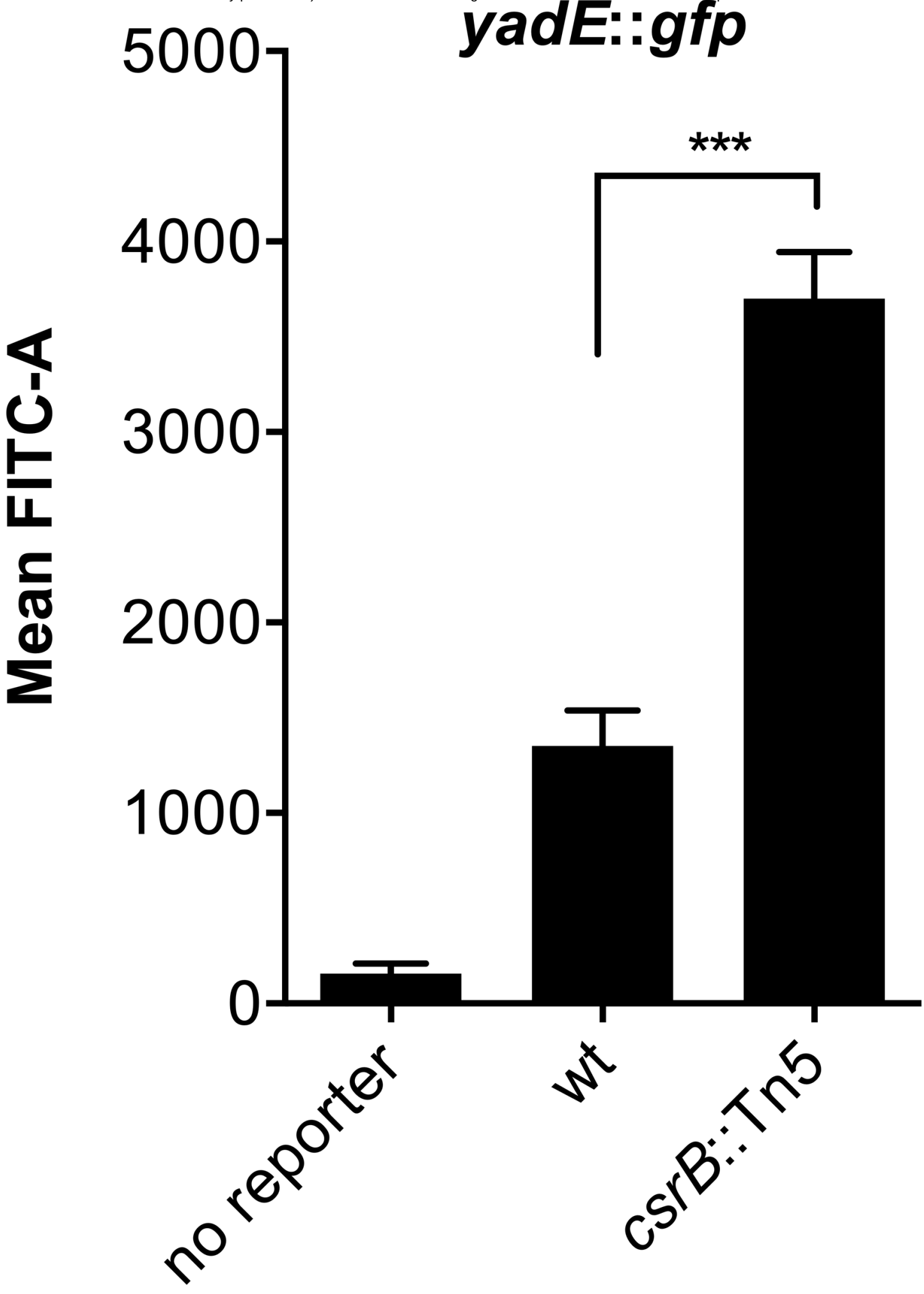
GFP R	GTTCAGGGCAGGGTCGTTAA*TTATTTGTATAGTTCATCCATG*	pUC18R6K-miniTn7T
pACYCc	TCTTCTCCTTTACGCAT*ACCATCCCCATTTTATTCTTGTTC*	pACYC*yadE::gfp*
pACYCd	CATGGATGAACTATACAAATA*ATTAACGACCCTGCCCTGAAC*	
igaA F	GCAACGTCAAGGACAAAGGG	pJET-*igaA*
igaA R	TGAGCGATGATCAACCGGAG	
rcsB F	TCGGCAAGCAGCTATGTGAA	pJET-*rcsB*
rcsB R	GATACTCTGCGCCAGATGCT	
Arb1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	Arbitrary PCR and sequencing of Tn5 insertion sites
Arb2	GGCCACGCGTCGACTAGTAC	
extsx	GACAACAAGCCAGGGATG	
intsx	CGCACTGAGAAGCCCTTAGAGC	
kanarb3	TGACACAGGAACACTTAACGGCTGAC	
tpnRL13-2	CAGCAACACCTTCTTCACGA	
intdx	GAGTCGACCTGCAGGCATGC	
kanarb2	GCATGCAAGCTTCAGGGTTGAG	

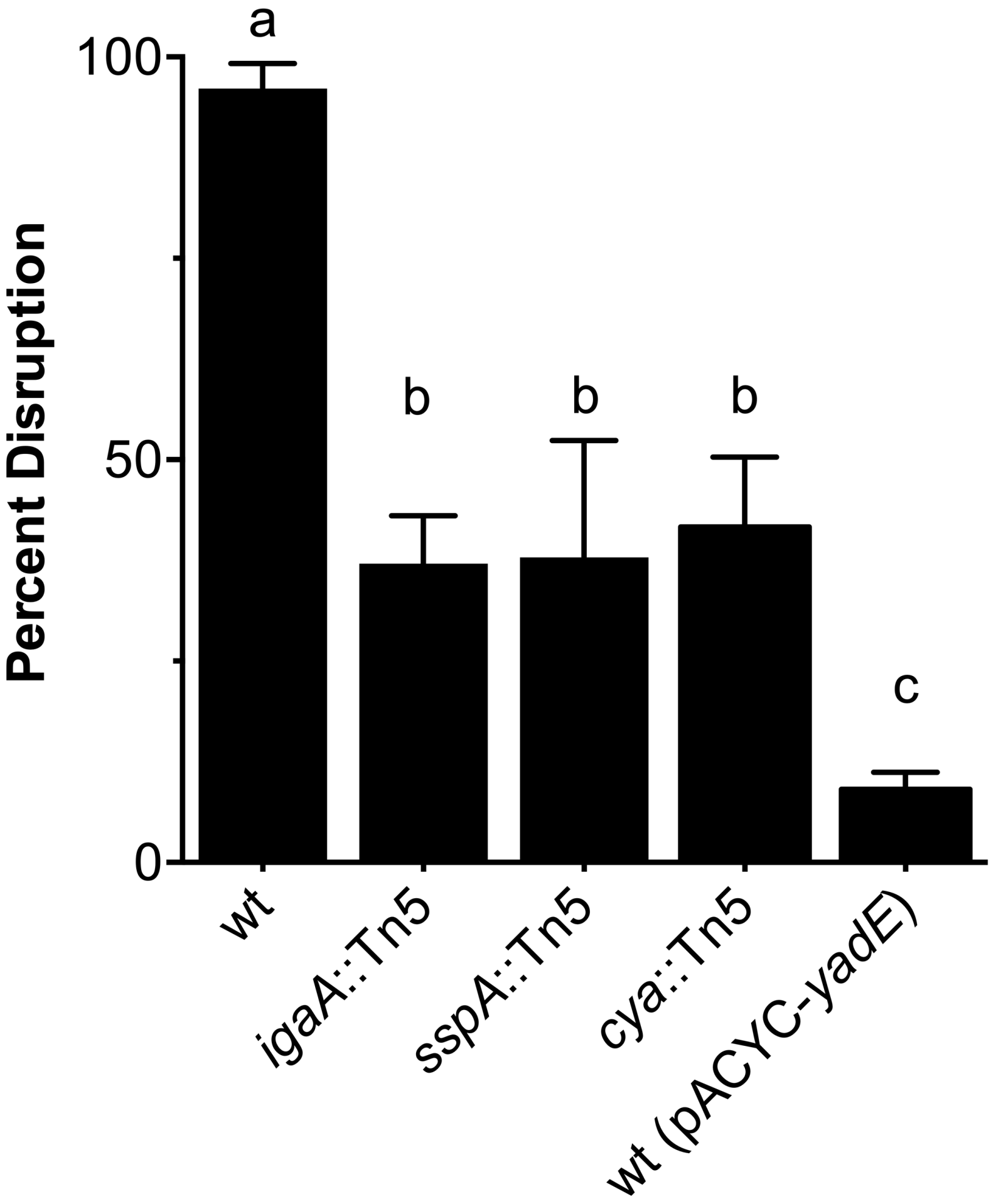
621 * Italicized portion of primers denote target binding region where applicable, and non-italicized portion is

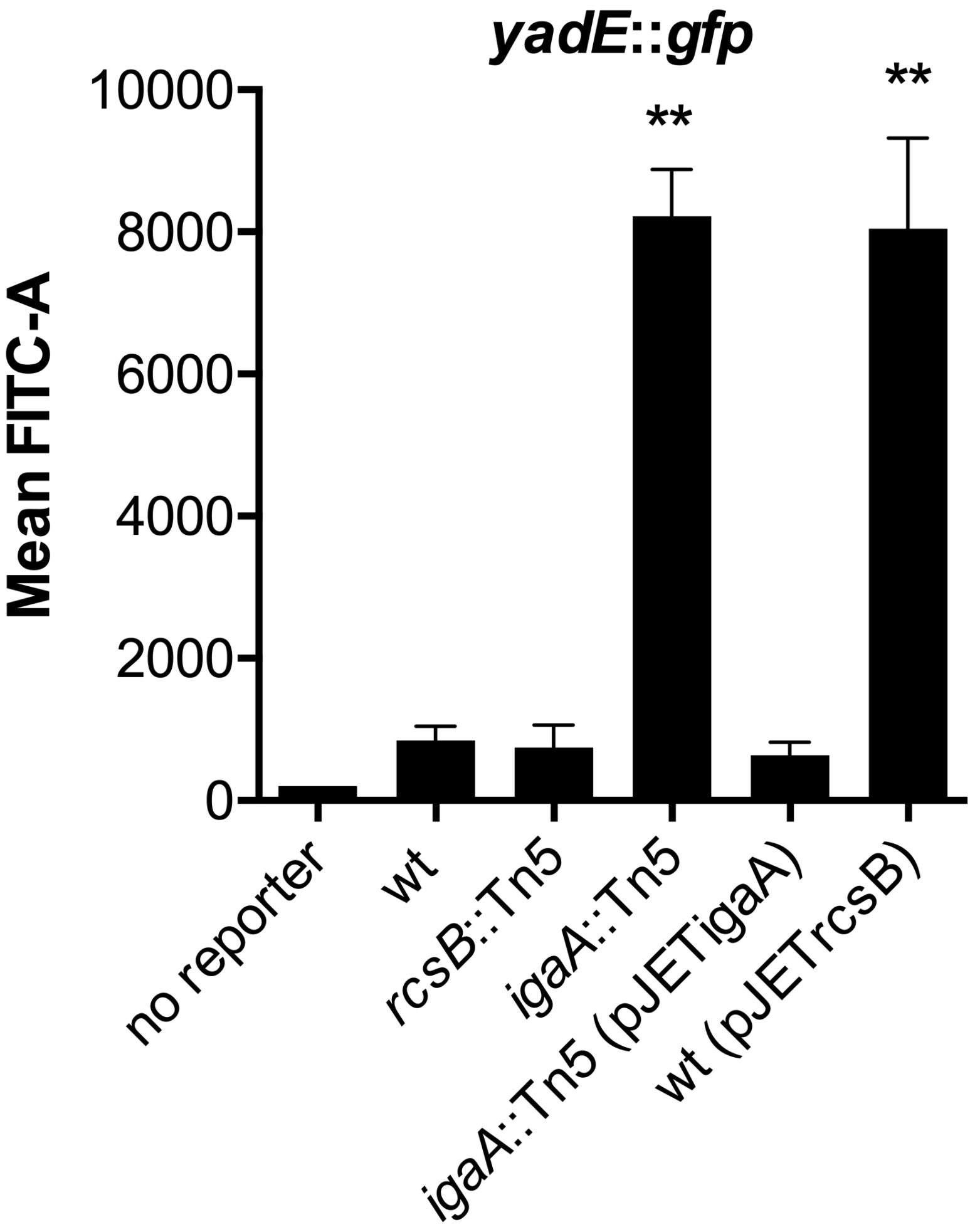
622 complementary to vector sequence for overlapping PCR.

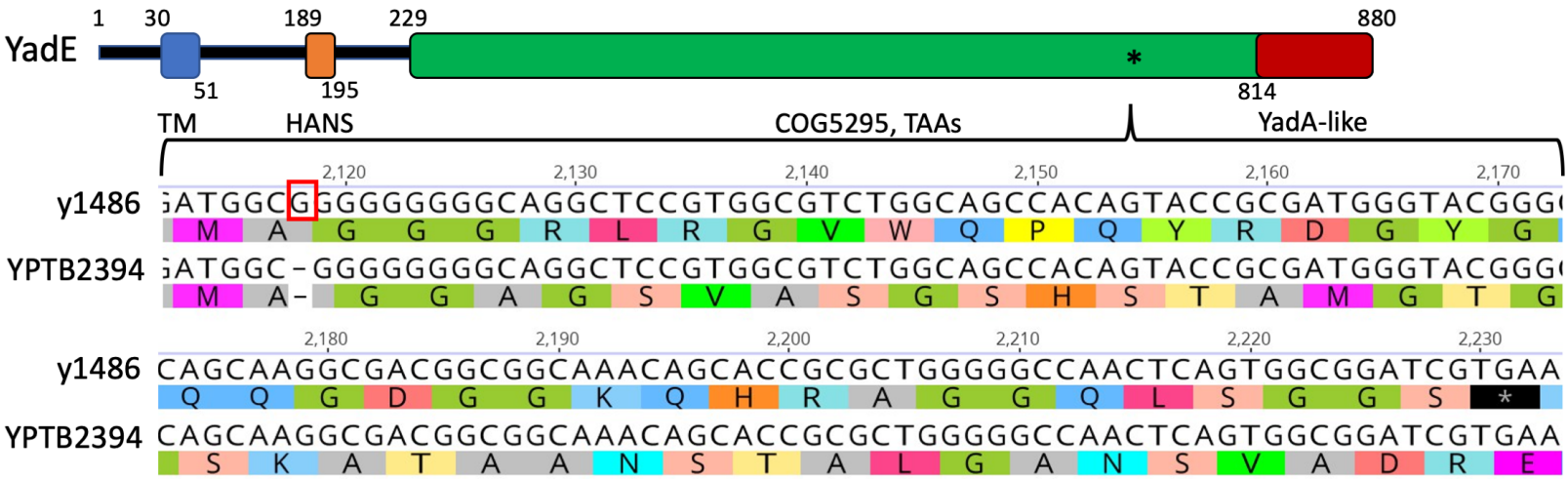












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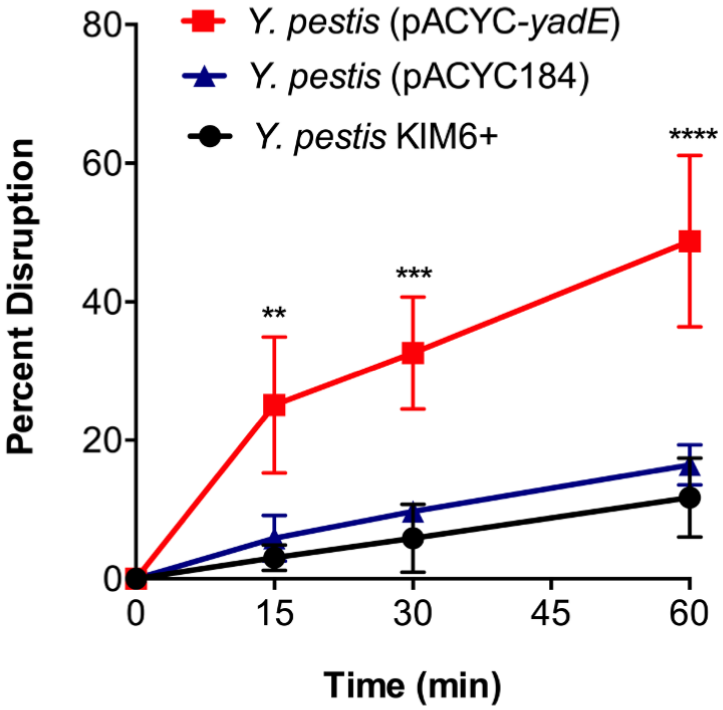

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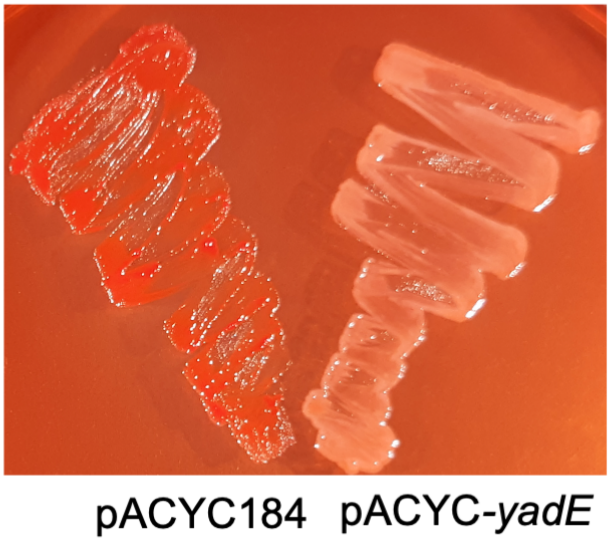

C