**Introduction**

*Pseudomonas aeruginosa* is an opportunistic, Gram-negative pathogen (Mace et al. 2008), and it is the primary cause of important chronic infections including those associated with cystic fibrosis (CF) (Moker et al. 2010), burn wound infections, bacterial keratitis, and urinary and peritoneal dialysis catheter infections (Mace et al. 2008). It is difficult to treat infections from *P. aeruginosa* because this species is highly tolerant to antibiotics (Lewis 2010). For example, late isolates of *P. aeruginosa* from CF patients can be high-persister mutants with 100-fold greater antibiotic tolerance (Mulcahy et al. 2010). The cause of antibiotic tolerance in many strains is thought to be due to activation of toxins of toxin/antitoxin (TA) systems (Jayaraman 2008; Lewis 2008) which causes dormancy by inactivating key metabolic functions like protein and ATP production (Kwan et al. 2013; Wood et al. 2013).

The first TA operons were discovered over 30 years ago (Ogura and Hiraga 1983) for stabilizing low copy number plasmids via postsegregational killing (Gerdes et al. 1986) and some additional roles of TA systems in cell physiology have become clear. For example, they are antiphage measures (Pecota and Wood 1996; Hazan and Engelberg-Kulka 2004; Fineran et al. 2009), and TA systems slow metabolism to allow cells to withstand stress such as that from antibiotics (Cheng et al. 2014; Islam et al. 2015) and bile acid (Kwan et al. 2015). Furthermore, the expression of the toxin genes are induced under stress conditions (Aizenman et al. 1996; Sat et al. 2001; Hazan et al. 2004), and antitoxins like MqsA and DinJ directly control the stress response by regulating the stress response sigma factor RpoS (Wang et al. 2011; Hu et al. 2012).

---

**The HigB/HigA toxin/antitoxin system of *Pseudomonas aeruginosa* influences the virulence factors pyochelin, pyocyanin, and biofilm formation**

Thammajun L. Wood¹,² & Thomas K. Wood¹,³

¹Department of Chemical Engineering, Pennsylvania State University, University Park, Pennsylvania 16802
²Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, Pennsylvania 16802
³Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, Pennsylvania 16802

**Keywords**

biofilm, HigA, HigB, pyochelin, pyocyanin, toxin/antitoxin system, virulence factors

**Abstract**

Toxin/antitoxin (TA) systems are prevalent in most bacterial and archaeal genomes, and one of the emerging physiological roles of TA systems is to help regulate pathogenicity. Although TA systems have been studied in several model organisms, few studies have investigated the role of TA systems in pseudomonads. Here, we demonstrate that the previously uncharacterized proteins HigB (unannotated) and HigA (PA4674) of *Pseudomonas aeruginosa* PA14 form a type II TA system in which antitoxin HigA masks the RNase activity of toxin HigB through direct binding. Furthermore, toxin HigB reduces production of the virulence factors pyochelin, pyocyanin, swarming, and biofilm formation; hence, this system affects the pathogenicity of this strain in a manner that has not been demonstrated previously for TA systems.
TA systems also have a role in biofilm formation (Ren et al. 2004; González Barrios et al. 2006; Kim et al. 2009).

Beyond phage inhibition, stress response, and biofilm formation, the role of TA systems in pathogenicity is also becoming more clear. Production of almost any active toxin to the point of making the cell dormant increases antibiotic tolerance (i.e., persistence) (Wang and Wood 2011), and deletion of some single TA systems decreases persistence (Dörr et al. 2010; Kim and Wood 2010). Although Mycobacterium tuberculosis has about 88 putative TA systems (Ramage et al. 2009), the nonpathogenic Mycobacterium smegmatis has only two putative TA systems, so pathogenicity might be related to the number of TA systems (Yamaguchi and Inouye 2011).

As anticipated, inactivation of three of M. tuberculosis MazF/MazE TA systems reduced its persistence in vitro, its survival in macrophages, and its cell numbers in the spleen and lungs of guinea pigs (Tiwari et al. 2015). Similarly, TA systems play a role in the persistence of Salmonella typhimurium in macrophages in a mouse model for typhoid fever (Helaine et al. 2014). There are at least 11 type II TA systems in S. typhimurium, and several of them are conserved in other pathogenic bacteria but absent from other nonpathogenic strains (De la Cruz et al. 2013). Also, inactivation of three Vap-type TA systems reduced virulence for nontypeable Haemophilus influenzae (NTHi) in a chinchilla model for otitis media (Ren et al. 2012), and inactivation of YoeB/YeM, Hha/TomB (García Contreras et al. 2008), and PasT/Pasl TA systems are important for uropathogenic Escherichia coli infections in the bladder and spleen in murine models (Norton and Mulvey 2012). Hence, determining the function of TA systems and the target of these toxins is very important because of their roles in bacterial physiology and pathogenicity (Yamaguchi and Inouye 2011).

One prominent TA system found in many pathogens is HigB/HigA in which the antitoxin HigA masks the toxicity of the toxin HigB. Genes for the HigB/HigA TA system are found on the Rts1 plasmid originally isolated from Proteus vulgaris and are found in the chromosomes of the pathogens Vibrio cholera (Christensen-Dalsgaard and Gerdes 2006; Budde et al. 2007), Streptococcus pneumonia (Chan et al. 2012), Acinetobacter baumannii (Jurenaite et al. 2013), S. typhimurium (De la Cruz et al. 2013), Yersinia pestis (Gould et al. 2010), M. tuberculosis (Schuessler et al. 2013), E. coli CFT073 (Pandey and Gerdes 2005), and E. coli O157:H7 (Pandey and Gerdes 2005) and is also present in E. coli K12 (Christensen-Dalsgaard et al. 2010). Additionally, higBA is widespread in P. aeruginosa clinical isolates (Williams et al. 2011). The HigB/HigA system has the uncommon gene arrangement with the toxin gene (higB) upstream of the antitoxin gene (higA), which is the reverse arrangement of most other TA systems (Tian et al. 1996).

HigB functions as an endoribonuclease in Proteus spp. (Hurley and Woychik 2009), V. cholera (Christensen-Dalsgaard and Gerdes 2006), A. baumannii (Jurenaite et al. 2013), and E. coli K12 (Christensen-Dalsgaard et al. 2010). Although the mechanism of how the SehA (Salmonella enterica Hig-like) toxin works is unknown, it shares 40% identity with HigB from E. coli K12 (De la Cruz et al. 2013); hence, SehA is likely to be an endoribonuclease and have the same target as HigB (De la Cruz et al. 2013). SehAB was also found to play an important role in virulence in mice (De la Cruz et al. 2013).

Here, we identified and characterized the HigB/HigA system in the chromosome of P. aeruginosa PA14. The HigB/HigA system is shown to be a bona fide TA system. Moreover, the HigB/HigA system affects the virulence factors of the strain in a fashion that has not been demonstrated previously for TA systems, since activation of toxin HigB reduces pyocyanin, a toxin produced and secreted by P. aeruginosa, reduces the siderophore pyochelin, reduces swarming, and reduces biofilm formation.

The closest HigB homolog to that we identify here in P. aeruginosa has only 34.3% identity (from V. cholera), so our findings are for a novel HigB/HigA TA system.

**Experimental Procedures**

**Bacterial strains and culture conditions**

The strains and plasmids used in this study are shown in Table 1. All strains were grown in lysogeny broth (LB) (Sambrook and Russell 2001) at 37°C except for the biofilm assay where M9 medium with 0.4% glucose and 0.4% casamino acids (Rodriguez and Tait 1983) was used. Chloramphenicol (30 μg/mL) was used to maintain pCA24N-based plasmids (Kitagawa et al. 2005) in E. coli. To obtain the specific growth rates, the P. aeruginosa PA14 wild-type and higA-mutant strains were inoculated into LB medium at an initial turbidity of 0.05 at 600 nm, and the turbidity at 600 nm was measured every an hour. At least two replicates were performed.

**Bioinformatics search**

The web-based search tool RASTA (Sevin and Barloy-Hubler 2007) was used to search for type II TA systems. The search was performed against the complete genome sequence of P. aeruginosa PA14 (Lee et al. 2006) with standard parameters.

**Plasmid construction**

Plasmid pCA24N(His-higB) was constructed by amplifying the higB gene from the chromosome of P. aeruginosa.
PA14 (Lee et al. 2006) and cloning into pCA24N at the NotI and PstI restriction sites placed higB under the control of T5-lac promoter. The higB gene was fused with His tag in the pCA24N plasmid at the N terminus. Plasmids pCA24N(higA-FLAG) and pCA24N(His-higB-higA-FLAG), Figure 3B, were constructed by amplifying the higA gene from the chromosome of P. aeruginosa PA14 with the addition of a FLAG tag at the C terminus and cloning into pCA24N and pCA24N(His-higB), respectively, at the PstI and HindIII restriction sites. All plasmids were verified by DNA sequencing. All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA), and the primer sequences are listed in Table 2.

### Site-directed mutagenesis of higA

Plasmid pCA24N(His-higB-higA-FLAG) was used as a PCR template to replace the start codon of HigA with Thr; since methionine residues exist at amino acid positions 1 and 6, both methionines were converted to threonine (i.e., M1T and M6T) in case either one is the start codon. The primers higA- PstI- start- QC- f and higA- FLAG- HindIII- r (Table 2) were used in the PCR reaction to generate the mutations. The PCR product was cloned into pCA24N(His-higB) using the PstI and HindIII sites. The resulting plasmid pCA24N(His-higB-higAX-FLAG) was sequenced to confirm the mutations.

### Random mutagenesis of higB toxin

The plasmid pCA24N(His-higB) was used for the error-prone PCR template. Using the pCA24N- f and pCA24N- r primers (Table 2), higB was randomly mutated under error-prone conditions (0.5 mmol/L Mn²⁺ and 5 mmol/L Mg²⁺) (Cadwell and Joyce 1992). Mutated higB inserts were digested and cloned into pCA24N at the NotI and PstI restriction sites. Ligated product was electroporated into E. coli TG1 (supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM5, rK−mK−) F′ [traD36 proAB+ lacIq lacZΔM15] Sambrook et al. (1989).
into *E. coli* TG1. The electroporated population was plated on LB–chloramphenicol agars. To select for growth, recovered colonies were restreaked on LB–chloramphenicol agar with 1 mmol/L IPTG. Variants were chosen for DNA sequencing analysis and retransformation tests.

**Toxicity assay**

Overnight cultures of strains *E. coli* TG1/pCA24N, *E. coli* TG1/pCA24N(lacZ), *E. coli* TG1/pCA24N(His-higB), *E. coli* TG1/pCA24N(higA-FLAG), *E. coli* TG1/pCA24N(His-higB-higA-FLAG), and *E. coli* TG1/pCA24N(His-higB-higAX-FLAG) were inoculated into 25 mL of LB medium at an initial of turbidity of 0.05 at 600 nm. IPTG (0.01 mmol/L) was added after 1 h, and the turbidity was recorded to determine growth.

**Western blot analysis and pull-down assay**

*Escherichia coli* TG1/pCA24N(His-higB) and *E. coli* TG1/pCA24N(His-higB-HigA-FLAG) were inoculated into LB medium from the overnight culture at an initial turbidity of 0.05 at 600 nm, then 0.1 mmol/L of IPTG was added to produce HigB and HigA for 5 h. The cell pellets were resuspended in buffer (50 mmol/L NaCl and 20 mmol/L Tris, pH 7.4) with protease inhibitor, and the cells were lysed using a French Press (Thermo Electron, Waltham, MA); centrifugation (15,000 g for 1 h at 4°C) was used to remove cell debris. The lysate was filtered through a 0.22-μm membrane and loaded on a HisTrap column (GE Healthcare, Pittsburgh, PA, USA). HigB and associated proteins were eluted with a 5–500 mmol/L imidazole gradient. The fractions containing HigB and HigA were identified using western blot analysis using anti-FLAG antibody (Thermo Scientific, Waltham, MA, USA). HigB were detected using a chemiluminescence kit (Thermo Scientific). DNA probes were labeled using Biotin 3′ End DNA Labeling Kit (Thermo Scientific). Northern blot analysis method was described previously (Sambrook et al. 1989). RNA levels were detected using Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific).

**Pyocyanin assay**

The *P. aeruginosa* PA14 wild-type, higA-mutant, and negative control (phzM and phzS mutants) strains were inoculated at a 1/1000 dilution from the overnight culture in LB medium and incubated for 24 h. As described previously (Essar et al. 1990), a 1-mL sample was centrifuged. The 800 μL of supernatant was extracted with 480 μL of chloroform. The sample was vortexed and centrifuged, then re-extracted with 0.2 N HCl. The absorbance of this sample was measured at 520 nm. The OD values at 520 nm were normalized with bacteria growth to eliminate any possible growth affects. At least three replicates were performed.

**Pyochelin assays**

The *P. aeruginosa* PA14 wild-type, higA-mutant, and negative control (pchB and pchF mutants) strains were grown overnight in LB medium. As described previously (Takase et al. 2000), acetic acid (0.3 mL) and dichloromethane (1.5 mL) were added into LB medium and incubated for 24 h. The samples were vortexed briefly and centrifuged for 5 min. One milliliter of dichloromethane fractions (bottom layer) were collected and evaporated. The samples were resuspended in 10 μL of dichloromethane and applied onto silica thin-layer plates for chromatography in
chloroform–acetic acid–ethanol (90:5:2.5). The pyochelin spots were scraped from the plates, eluted with 1 mL of methanol, and measured using a spectrophotometer at 313 nm and using a spectrofluorimeter (excitation at 355 nm and emission at 430 nm). At least three replicates were performed.

**Pyoverdine assays**

The *P. aeruginosa* PA14 wild-type, higA-mutant, and negative control (pvdF mutant) strains were grown overnight in LB medium. For the chrome azurol S (CAS) agar plate assay (Owen and Ackerley 2011), 1 μL of the overnight culture was placed in the middle of CAS agar plate. After incubation at 37°C for 1 and 2 days, the plate was observed under UV light by the formation of a fluorescent zone around the cells (Yu et al. 2014). For CAS liquid assay (Yu et al. 2014), an overnight culture in LB was diluted to an initial turbidity of 0.1 at 600 nm in King’s B medium (Yu et al. 2014). After 1 or 2 days incubation at 37°C, the samples were centrifuged, and the supernatant was used to measure the pyoverdine production using a fluorescence spectrometer (excitation at 405 nm and emission at 460 nm).

**Biofilm assay using crystal violet**

Biofilm formation was assayed in 96-well polystyrene plates using 0.1% crystal violet staining as described previously (Fletcher 1977) with some modifications. Diluted overnight cultures at an initial turbidity of 0.05 at 600 nm were inoculated into 96-well plates with M9 medium with 0.4% glucose and 0.4% casamino acids (Rodriguez and Tait 1983) and the bacteria were cultured for 48 h without shaking. After the crystal violet was added to each well, the wells were rinsed and dried, and ethanol was added to dissolve the crystal violet. The total biofilm formation samples were measured at 540 nm, whereas cell growth was measured at 620 nm. Biofilm formation was normalized by the bacterial growth to reduce any growth effect. At least two independent cultures were used for each strain.

**Swarming assay**

The *P. aeruginosa* PA14 wild-type, higA-mutant, and negative control (rhlR mutant) strains were grown overnight in LB medium. The culture (1 μL) was inoculated in the middle of fresh BM2 plates (Overhage et al. 2008) that were dry for 3 h before inoculation, and the plates were incubated for 18 h. The agar plate coverage was measured using ImageJ software (www.imagej.nih.gov/ij/). At least three replicates were performed.

**Results**

**Identification of the putative HigB/HigA TA system**

We identified a putative HigB/HigA system in the genome of *P. aeruginosa* PA14 by using the RASTA Bacteria program (Sevin and Barloy-Hubler 2007). This program searches for type II TA systems, and the HigB/HigA system got the highest score (90%) which indicates it is likely to be a TA system (scores above 70% are indicated to likely be TA systems). HigB was not annotated previously. HigB is a small protein (92 amino acids), and HigA consists of 106 amino acids (Fig. 1A). Their genes overlap by one nucleotide, which is the same as the HigB/HigA system in *P. vulgaris* (Schureck et al. 2014), and gene overlap is a common characteristic of TA systems (Yamaguchi et al. 2011).

**HigB and HigA form a TA system**

To assess whether HigB functions as a toxin and whether HigA masks HigB toxicity, the activity of the two proteins were evaluated in *E. coli*. HigB toxin produced from

![Figure 1. higBA loci and results showing HigB toxicity is masked by HigA.](image-url)

(A) The higBA operon of *Pseudomonas aeruginosa* PA14. The toxin gene *higB* (not annotated, 5514513–5514791) and the antitoxin gene *higA* (PA4674, PA14_61840, 5514196–5514516) were unveiled using RASTA software. (B) Overnight cultures of strains of *Escherichia coli* colT1/pCA24N (pCA24N, control), *E. coli* colT1/pCA24N (lacZ) (pCA24N(lacZ), control), *E. coli* colT1/pCA24N(His-higB) (pCA24N(higB)), *E. coli* colT1/pCA24N(higA) (pCA24N(higA)), and *E. coli* colT1/pCA24N(higBA) (pCA24N(higBA)), where “X” indicates the translation start signal was changed to a threonine codon so antitoxin HigA is not produced (pCA24N(higBAX)), were inoculated into 25 mL of LB medium at an initial of turbidity of 0.05 at 600 nm at 37°C. IPTG (0.01 mmol/L) was added after 1 h. The error bars shown are standard deviation from three independent cultures.
plasmid pCA24N inhibited the growth in *E. coli* TG1 (Fig. 1B); hence, HigB showed strong toxin activity in this non-native host. When both HigB and HigA were produced simultaneously, the toxicity of HigB was counteracted (Fig. 1B). Therefore, HigB/HigA is a bona fide TA system.

**Antitoxin HigA binds to toxin HigB and functions as a protein**

Antitoxin HigA could mask HigB toxicity as either RNA or as a protein; hence, the start codon of *higA* was converted to Thr and its effect on HigB toxicity was evaluated. Growth in *E. coli* showed that the translation mutation in *higA* abolished its antitoxin activity. Therefore, HigA functions as a protein antitoxin (Fig. 1B).

We then hypothesized that the HigA antitoxin directly binds toxin HigB to inactivate it. To verify the direct interaction between antitoxin HigA and toxin HigB, a pull-down experiment was performed. The protein HigB from pCA24N(His-higB) and from pCA24N(His-higB-higA-FLAG) was tagged with six histidines at the N terminus, and the protein HigA from pCA24N(His-higB-higA-FLAG) was tagged using the FLAG octapeptide at the C terminus.

Using the His-tagged HigB protein to pull down the Flag-tagged HigA antitoxin, a western blot was performed. Both proteins HigB and HigA were detected using His and FLAG tag antibodies, respectively; hence, antitoxin HigA interacts directly with toxin HigB (Fig. 2A). Therefore, HigB and HigA interact at the protein level and are a type II TA system (i.e., protein–protein TA system).

**Toxin HigB functions as an RNase**

To determine the enzyme activity of HigB in *E. coli*, a Northern blot analysis for *ompA* and *ompF* was performed (Fig. 2B); these two loci encode large genes so they are used frequently in TA studies (Hurley and Woychik 2009). The degradation of the mRNAs was detectable immediately after induction (1 min) of *higB*. The level of the control mRNAs at the 60-min time point without *higB* induction was also decreased due to low-level activity of the plasmid promoter, but it was not as strong as in the samples with *higB* induction.

**Toxin HigB catalytic sites**

Error-prone PCR of *higB* was performed to generate a library of HigB variants in *E. coli* to determine the residues required for its RNase activity. The HigB variants were selected on LB plates with 0.1 mmol/L of IPTG to produce the toxin; under these conditions, native HigB prevented the formation of colonies. Variants were selected based on their enhanced growth, and the substitutions were identified as G17D/A61E, V28D, R31H, Q57P, Q63H, H64P, R67W, and W72C substitutions were found multiple times. These results indicate that the amino acids at these positions are crucial for the toxicity of HigB. We also aligned HigB sequences of *P. aeruginosa* PA14, *P. vulgaris*, and *V. cholerae* and found that most of the crucial amino acids we identified in the variants (G17, I66, W72, and R73, Fig. 3A) are conserved or nearby the conserved amino acids which are likely to be the active site of HigB (Schureck et al. 2014).

**Toxin HigB reduces pyochelin production**

To determine the physiological role of HigB/HigA in *P. aeruginosa*, a whole-transcriptome experiment was performed for the *higA* antitoxin deletion mutant compared with
to the *P. aeruginosa* PA14 wild-type strain. The rationale was that for the strain that lacks the antitoxin, the effect of the toxin could be discerned due to enhanced activity of the toxin. Notably, the specific growth rates of *P. aeruginosa* PA14 and the *higA* antitoxin deletion mutant strain were 1.2 and 0.8 h⁻¹, respectively, so there is a modest decrease in growth upon activation of toxin HigB (Fig. 4A).

The microarray results (Table 3) indicate that deletion of the antitoxin induces toxin transcription by 28-fold, as expected since antitoxins normally repress the TA operon (Brown et al. 2013). Furthermore, the PA2405-2410 operon, which is part of the pyoverdine locus (Ravel and Cornelis 2003), has the most induced genes upon activating toxin HigB (i.e., deletion of *higA*); these genes probably encode proteins related to membrane transport. However, there was not much difference in pyoverdine production between the wild-type strain and the *higA* mutant (data not shown) which collaborated previous work which reported that the mutations in this PvdS-regulated PA2403-PA2410 cluster did not affect pyoverdine production (Ravel and Cornelis 2003).

Critically, the microarray data show that pyochelin-related genes (*pchA*, *pchB*, *pchC*, *pchD*, *pchE*, *pchF*, *pchG*, and *fptA*), which encode proteins related to iron transfer, are repressed upon activating the toxin HigB. Pyochelin is a siderophore produced by *P. aeruginosa* which increases the growth and lethality of pathogenic bacteria (Cox 1982). FptA is the receptor for ferric pyochelin, and its expression is pyochelin dependent (Heinrichs and Poole 1996). A number of studies have shown that there is a correlation between pyochelin and virulence (Cox 1982; Sokol 1987; Wang et al. 1996). Pyochelin levels were assayed in the *higA* mutant and found to be reduced by 2.0 ± 0.1-fold (Fig. 4B); hence, the microarray data were corroborated by the reduced pyochelin levels. The
T. L. Wood & T. K. Wood

HigB of \textit{P. aeruginosa} Reduces Virulence

OD$_{313}$ values for the pyochelin assay for \textit{P. aeruginosa} PA14 and the \textit{higA} mutant were 0.104 and 0.05, respectively. Therefore, the HigB toxin reduces virulence via a reduction in pyochelin, and HigA antitoxin represses its own transcription. These results were not affected by growth since the samples for pyochelin assay were collected in the stationary phase at the same turbidity.

Toxin HigB reduces pyocyanin production

Pyocyanin is a blue redox-active secondary metabolite that interferes with multiple cellular functions and has crucial roles in \textit{P. aeruginosa} infections (Lau et al. 2004). The \textit{higA} antitoxin deletion mutant was less blue compared to the wild type; hence, chloroform extraction was performed to more accurately measure the amount of the pyocyanin in an overnight culture. The \textit{higA} mutant strain produced 5 ± 3-fold less pyocyanin than the \textit{P. aeruginosa} PA14 wild-type strain (Fig. 4B), which showed that the toxin HigB affected the pyocyanin level in \textit{P. aeruginosa}. The OD$_{520}$/OD$_{600}$ values for the pyocyanin assay for \textit{P. aeruginosa} PA14 and the \textit{higA} mutant were 0.0086 ± 0.0012 and 0.0018 ± 0.00007, respectively. These results were not affected by growth because the samples were collected after 24 h at the same turbidity.

Toxin HigB reduces biofilm formation and swarming motility

Since TA systems are related to biofilm formation (Ren et al. 2004; González Barrios et al. 2006; Kim et al. 2009) as well as quorum sensing and motility (González Barrios et al. 2006), biofilm formation and swarming were investigated for the HigB/HigA TA system. After 48 h in M9 medium with 0.4% glucose and 0.4% casamino acids (Rodriguez and Tait 1983), the \textit{higA} mutant had 11.0 ± 0.5-fold less normalized biofilm than the \textit{P. aeruginosa} PA14 wild-type strain (Fig. 4B). The OD$_{540}$/OD$_{600}$ values for the biofilm assay for \textit{P. aeruginosa} PA14 and the \textit{higA} mutant were 1.3 ± 0.4 and 0.12 ± 0.04, respectively. These results were not affected by growth because the samples were collected after 48 h, and the planktonic cell densities were nearly identical. Furthermore, the \textit{higA} mutant had 5 ± 2-fold lower swarming motility than the \textit{P. aeruginosa} PA14 wild-type strain (Figs. 4B and 5). Hence, toxin HigB reduces both biofilm formation and swarming.

Discussion

We present additional evidence in this report that TA systems are involved in pathogenicity. Furthermore, we find that the HigB/HigA TA system of \textit{P. aeruginosa} affects its virulence in a manner that is distinct from the way any other TA system has been linked to virulence, since here we found HigB/HigA affects virulence through pyochelin, pyocyanin, swarming (as well as through biofilm formation). We also demonstrate clearly that the \textit{P. aeruginosa} HigB/HigA system is a type II bona fide TA system.

\textit{Pseudomonas aeruginosa} produces two siderophores, pyoverdine and pyochelin (Ankenbauer and Quan 1994), and gene expression related to both siderophores was affected by the HigB/HigA TA system. Based on our microarray results, \textit{fpvA}, which encodes the receptor of the siderophore ferripyoverdine, was induced in the \textit{higA} mutant compared to the \textit{P. aeruginosa} PA14 wild-type strain; this would make cells with the HigB toxin activated more susceptible to pyocins S2 and S3 since these pyocins use the ferripyoverdine receptor (Denayer et al. 2007).
In contrast, fptA, which encodes the receptor of the siderophore pyochelin, was reduced in the higA mutant. pyoS5, which encodes a toxin that uses the FptA ferrirypochelin receptor to enter the cell (Elfarash et al. 2014), was also repressed.

The HigB toxin is prevalent in pseudomonads; for example, P. aeruginosa DK2, P. aeruginosa B136-33, Pseudomonas stutzeri DSM 10701, Pseudomonas putida F1, Pseudomonas putida KT2440, Pseudomonas fluorescens F113, and Pseudomonas denitrificans all contain genes for the toxin. Although the genes for putative HigB/HigA TA systems have been found in many organisms, most of them have not been characterized. An exception is the HigB toxin from Proteus vulgaris which has a highly conserved residue N71 for controlling mRNA specificity by interacting with the 16S rRNA residue C1054 (Schureck et al. 2015). However, the HigB from P. aeruginosa has glutamine at this position. The HigB toxin protein we found in P. aeruginosa PA14 was aligned with the HigB from V. cholera, Proteus vulgaris, E. coli K12, and SehA from S. typhimurium to reveal that there is only 34.3%, 28%, 10.6%, and 8.7% identity, respectively (Fig. 3C). Hence, the P. aeruginosa system that we describe here is distinct since proteins that share about 20% identity are not related, in that only those with identities of 50% or greater are usually considered related in databases (Seffernick et al. 2001). Therefore, we have characterized a novel HigB/HigA TA family member and shown how the TA system affects virulence factors of an important opportunistic pathogen. This study also represents the first TA system that has been characterized in P. aeruginosa.
Acknowledgments

T. K. W. is the Biotechnology Endowed Professor at the Pennsylvania State University. We appreciate the help of Olga Vinogradova and Kenneth Hubbell in generating, screening, and sequencing some HigB variants.

Conflict of Interest

None declared.

References

Aizenman, E., H. Engelberg-Kulka, and G. Glaser. 1996. An Escherichia coli chromosomal “addiction module” regulated by 3',5'-bispyrophosphate: a model for programmed bacterial cell death. Proc. Natl. Acad. Sci. USA 93:6059–6063.

Ankenbauer, R. G., and H. N. Quan. 1994. Fpta, the Fe(III)-pyochelin receptor of Pseudomonas aeruginosa - a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. J. Bacteriol. 176:307–319.

Brown, B. L., D. M. Lord, S. Grigoriu, W. Peti, and R. Page. 2013. The Escherichia coli toxin MqsR destabilizes the transcriptional repression complex formed between the antitoxin MqsA and the mqsRA operon promoter. J. Biol. Chem. 288:1286–1294.

Budde, P. P., B. M. Davis, J. Yuan, and M. K. Waldor. 2007. Characterization of a higBA toxin-antitoxin locus in Vibrio cholerae. J. Bacteriol. 189:491–500.

Cadwell, R. C., and G. F. Joyce. 1992. Randomization of genes by PCR mutagenesis. PCR Methods Appl. 2:28–33.

Chan, W. T., I. Moreno-Cordoba, C. C. Yeo, and M. Espinosa. 2012. Toxin-antitoxin genes of the gram-positive pathogen Streptococcus pneumoniae: so few and yet so many. Microbiol. Mol. Biol. Rev. 76:773–791.

Cheng, H. Y., V. W. C. Soo, S. Islam, M. J. McAnulty, M. J. Benedik, and T. K. Wood. 2014. Toxin GhoT of the GhoT/GhoS toxin/antitoxin system damages the cell.
membrane to reduce adenosine triphosphate and to reduce growth under stress. Environ. Microbiol. 16:1741–1754.

Christensen-Dalsgaard, M., and K. Gerdes. 2006. Two higBA loci in the Vibrio cholerae superintegron encode mRNA cleaving enzymes and can stabilize plasmids. Mol. Microbiol. 62:397–411.

Christensen-Dalsgaard, M., M. G. Jorgensen, and K. Gerdes. 2010. Three new RelE-homologous mRNA interferases of Escherichia coli differentially induced by environmental stresses. Mol. Microbiol. 75:333–348.

Cox, C. D. 1982. Effect of pyochelin on the virulence of Pseudomonas aeruginosa. Infect. Immun. 36:17–23.

De la Cruz, M. A., W. D. Zhao, C. Farenc, G. Gimenez, D. Cox, C. D. 1982. Effect of pyochelin on the virulence of Escherichia coli. Biochem. Biophys. Res. Commun. 106:894–899.

Elfarash, A., J. Dingemans, L. M. Ye, A. A. Hassan, M. Craggs, C. Reimmann, et al. 2014. Pore-forming pyocin S5 kills Pseudomonas aeruginosa strains via the FpvA type I ferripyoverdine receptor. J. Bacteriol. 189:7663–7668.

Dörr, T., M. Vulic, and K. Lewis. 2010. Ciprofloxacin causes persister formation by inducing the TisB toxin in Escherichia coli. PLoS Biol. 8:e1000317.

Elfarash, A., J. Dingemans, L. M. Ye, A. A. Hassan, M. Craggs, C. Reimmann, et al. 2014. Pore-forming pyocin S5 utilizes the FptA ferripyochelin receptor to kill Pseudomonas aeruginosa. Microbiology 160:261–269.

Essar, D. W., L. Eberly, A. Hadero, and I. P. Crawford. 1990. Identification and characterization of genes for a 2nd anthranilate synthase in Pseudomonas aeruginosa - interchangeability of the 2 anthranilate synthases and evolutionary implications. J. Bacteriol. 172:884–900.

Fineran, P. C., T. R. Blower, I. J. Foulds, D. P. Humphreys, K. S. Lilley, and G. P. C. Salmond. 2009. The phage abortive infection system, ToxIN, functions as a protein–RNA toxin–antitoxin pair. Proc. Natl. Acad. Sci. USA 106:894–899.

Fletcher, M. 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23:1–6.

Garcia Contreras, R., X.-S. Zhang, Y. Kim, and T. K. Wood. 2008. Protein translation and cell death: the role of rare tRNAs in biofilm formation and in activating dormant phage kinase genes. PLoS ONE 3:e2394.

Gerdes, K., F. W. Bech, S. T. Jorgensen, A. Lønberg-Olesen, P. B. Rasmussen, T. Atlung, et al. 1986. Mechanism of postsegregational killing by the hok gene product of the parB system of plasmid R1 and its homology with the relF gene product of the E. coli relB operon. EMBO J. 5:2023–2029.

González Barrios, A. F., R. Zuo, Y. Hashimoto, L. Yang, W. E. Bentley, and T. K. Wood. 2006. Autoinducer 2 controls biofilm formation in Escherichia coli through a novel motil quorum-sensing regulator (MqsR, B3022). J. Bacteriol. 188:305–316.

Goulard, C., S. Langrand, E. Carniel, and S. Chauvaux. 2010. The Yersinia pestis chromosome encodes active addiction toxins. J. Bacteriol. 192:3669–3677.

Hazan, R., and H. Engelberg-Kulka. 2004. Escherichia coli mazEF-mediated cell death as a defense mechanism that inhibits the spread of phage P1. Mol. Genet. Genomics 272:227–234.

Hazen, R., B. Sat, and H. Engelberg-Kulka. 2004. Escherichia coli mazEF-mediated cell death is triggered by various stressful conditions. J. Bacteriol. 186:3663–3669.

Heinrichs, D. E., and K. Poole. 1996. PchR, a regulator of ferripyochelin receptor gene (fptA) expression in Pseudomonas aeruginosa, functions both as an activator and as a repressor. J. Bacteriol. 178:2586–2592.

Helaine, S., A. M. Cheverton, K. G. Watson, L. M. Faure, S. A. Matthews, and D. W. Holden. 2014. Internalization of Salmonella by macrophages induces formation of nonreplicating persisters. Science 343:204–208.

Hu, Y., M. J. Benedik, and T. K. Wood. 2012. Antitoxin DinJ influences the general stress response through transcript stabilizer CspE. Environ. Microbiol. 14:669–679.

Hurley, J. M., and N. A. Woychik. 2009. Bacterial toxin HigB associates with ribosomes and mediates translation-dependent mRNA cleavage at A-rich sites. J. Biol. Chem. 284:18605–18613.

Islam, S., M. J. Benedik, and T. K. Wood. 2015. Orphan toxin OrfT (YdcX) of Escherichia coli reduces growth during the stringent response. Toxins 7:299–321.

Jayaraman, R. 2008. Bacterial persistence: some new insights into an old phenomenon. J. Biosci. 33:795–805.

Jurenaite, M., A. Markuckas, and E. Suziedeliene. 2013. Identification and characterization of type II toxin-antitoxin systems in the opportunistic pathogen Acinetobacter baumannii. J. Bacteriol. 195:3165–3172.

Kim, Y., and T. K. Wood. 2010. Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in Escherichia coli. Biochem. Biophys. Res. Commun. 391:209–213.

Kim, Y., X. Wang, Q. Ma, X.-S. Zhang, and T. K. Wood. 2009. Toxin-antitoxin systems in Escherichia coli influence biofilm formation through YjgK (TabA) and fimbriae. J. Bacteriol. 191:1258–1267.

Kitagawa, M., T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, et al. 2005. Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. DNA Res. 12:291–299.

Kwan, B. W., J. A. Valenta, M. J. Benedik, and T. K. Wood. 2013. Arrested protein synthesis increases persister-like cell formation. Antimicrob. Agents Chemother. 57:1468–1473.
HigB of *P. aeruginosa* Reduces Virulence

Kwan, B. W., D. M. Lord, W. Peti, R. Page, M. J. Benedik, and T. K. Wood. 2015. The MqsR/MqsA toxin-antitoxin system protects *Escherichia coli* during bile acid stress. Environ. Microbiol. 17:3168–3181.

Lau, G. W., D. J. Hassett, H. M. Ran, and F. S. Kong. 2004. The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol. Med. 10:599–606.

Lee, D. G., J. M. Urbach, G. Wu, N. T. Liberati, R. L. Feinbaum, S. Miyata, et al. 2006. Genomic analysis reveals that *Pseudomonas aeruginosa* virulence is combinatorial. Genome Biol. 7:R90.

Lewis, K. 2008. Multidrug tolerance of biofilms and persister cells. *in T. Romeo*, ed. Bacterial Biofilms. Springer, Berlin.

Lewis, K. 2010. Persister cells. *Annu. Rev. Microbiol.* 64:357–372.

Liberati, N. T., J. M. Urbach, S. Miyata, D. G. Lee, E. Drenkard, G. Wu, et al. 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc. Natl. Acad. Sci. USA* 103:2833–2838.

Mace, C., D. Seyer, C. Chemani, P. Cosette, P. Di-Martino, B. Guery, et al. 2008. Identification of biofilm-associated cluster (bac) in *Pseudomonas aeruginosa* involved in biofilm formation and virulence. *PLoS ONE* 3:e3897.

Moker, N., C. R. Dean, and J. Tao. 2010. *Pseudomonas aeruginosa* increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. *J. Bacteriol.* 192:1946–1955.

Mulcahy, L. R., J. L. Burns, S. Lory, and K. Lewis. 2010. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J. Bacteriol.* 192:6191–6199.

Norton, J. P., and M. A. Mulvey. 2012. *Toxin-antitoxin* systems are important for niche-specific colonization and stress resistance of uropathogenic *Escherichia coli*. *PLoS Pathog.* 8:e1002954.

Ogura, T., and S. Hiraga. 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc. Natl. Acad. Sci. USA* 80:4784–4788.

Overhage, J., M. Bains, M. D. Brazas, and R. E. W. Hancock. 2008. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J. Bacteriol.* 190:2671–2679.

Owen, J. G., and D. F. Ackerly. 2011. Characterization of pyoverdine and achromobactin in *Pseudomonas syringae* pv. phaseolicola 1448a. *BMCMicrobiol.* 11:218.

Pandey, D. P., and K. Gerdes. 2005. *Toxin-antitoxin* loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res.* 33:966–976.

Pecota, D. C., and T. K. Wood. 1996. Exclusion of T4 phage by the hok/sok killer locus from plasmid R1. *J. Bacteriol.* 178:2044–2050.

Ramage, H. R., L. E. Connolly, and J. S. Cox. 2009. Comprehensive functional analysis of *Mycobacterium tuberculosis* toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. *PLoS Genet.* 5:e1000767.

Ravel, J., and P. Cornelis. 2003. Genomics of pyoverdine-mediated iron uptake in pseudomonads. *Trends Microbiol.* 11:195–200.

Ren, D., L. A. Bedzyk, S. M. Thomas, R. W. Ye, and T. K. Wood. 2004. Gene expression in *Escherichia coli* biofilms. *Appl. Microbiol. Biotechnol.* 64:515–524.

Renn, D., A. N. Walker, and D. A. Daines. 2012. *Toxin-antitoxin* loci vapBC-1 and vapXD contribute to survival and virulence in nontypeable *Haemophilus influenzae*. *BMC Microbiol.* 12:263.

Rodríguez, R. L., and R. C. Tait. 1983. Recombinant DNA techniques: an introduction. Benjamin/Cummings Publishing, Menlo Park, CA.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning, a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sat, B., R. Hazan, T. Fisher, H. Khaner, G. Glaser, and H. Engelberg-Kulka. 2001. Programmed cell death in *Escherichia coli*: some antibiotics can trigger mazEF lethality. *J. Bacteriol.* 183:2041–2045.

Schuessler, D. P., and K. Gerdes. 2005. *Toxin-antitoxin* loci vapBC involved in multidrug tolerance of biofilms. *Appl. Microbiol. Biotechnol.* 64:515–524.

Schuette-Kesseler, M. A., T. Maehigashi, S. J. Miles, J. Marquez, S. Cho, R. Erdman, et al. 2014. Structure of the *Proteus vulgaris* HigB-(HigA)(2)-HigB toxin-antitoxin complex. *J. Biol. Chem.* 289:1060–1070.

Schuette-Kesseler, M. A., J. A. Dunkle, T. Maehigashi, S. J. Miles, and C. M. Dunham. 2015. Defining the mRNA recognition signature of a bacterial toxin protein. *Proc. Natl. Acad. Sci. USA* 112:13862–13867.

Sefernick, J. L., M. L. de Souza, M. J. Sadowsky, and L. P. Wackett. 2001. Melamine deaminase and atrazine chlorohydrolase: 98 percent identical but functionally different. *J. Bacteriol.* 183:2405–2410.

Sevin, E. W., and F. Barloy-Hubler. 2007. RASTA-Bacteria: a web-based tool for identifying toxin-antitoxin loci in prokaryotes. *Genome Biol.* 8:R155.

Sokol, P. A. 1987. Surface expression of ferripyochelin-binding protein is required for virulence of *Pseudomonas aeruginosa*. *Infect. Immun.* 55:2021–2025.

Takase, H., N. Nitanai, K. Hoshino, and T. Otani. 2000. Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. *Infect. Immun.* 68:1834–1839.
Tian, Q. B., T. Hayashi, T. Murata, and Y. Terawaki. 1996. Gene product identification and promoter analysis of hig locus of plasmid Rts1. Biochem. Biophys. Res. Commun. 225:679–684.

Tiwari, P., G. Arora, M. Singh, S. Kidwai, O. P. Narayan, and R. Singh. 2015. MazF ribonucleases promote Mycobacterium tuberculosis drug tolerance and virulence in guinea pigs. Nat. Commun. 6:6059.

Wang, X., and T. K. Wood. 2011. Toxin/Antitoxin systems influence biofilm and persister cell formation and the general stress response. Appl. Environ. Microbiol. 77:5577–5583.

Wang, J. Y., A. Mushegian, S. Lory, and S. G. Jin. 1996. Large-scale isolation of candidate virulence genes of Pseudomonas aeruginosa by in vivo selection. Proc. Natl. Acad. Sci. USA 93:10434–10439.

Wang, X. X., Y. Kim, S. H. Hong, Q. Ma, B. L. Brown, M. M. Pu, et al. 2011. Antitoxin MqsA helps mediate the bacterial general stress response. Nat. Chem. Biol. 7:359–366.

Williams, J. J., E. M. Halvorsen, E. M. Dwyer, R. M. DiFazio, and P. J. Hergenrother. 2011. Toxin-antitoxin (TA) systems are prevalent and transcribed in clinical isolates of Pseudomonas aeruginosa and methicillin-resistant Staphylococcus aureus. FEMS Microbiol. Lett. 322:41–50.

Wood, T. K., S. J. Knabel, and B. W. Kwan. 2013. Bacterial persister cell formation and dormancy. Appl. Environ. Microbiol. 79:7116–7121.

Yamaguchi, Y., and M. Inouye. 2011. Regulation of growth and death in Escherichia coli by toxin-antitoxin systems. Nat. Rev. Microbiol. 9:779–790.

Yamaguchi, Y., J. H. Park, and M. Inouye. 2011. Toxin-antitoxin systems in bacteria and archaea. Annu. Rev. Genet. 45:61–79.

Yu, X., M. Chen, Z. Jiang, Y. Hu, and Z. Xie. 2014. The two-component regulators GacS and GacA positively regulate a nonfluorescent siderophore through the Gac/ Rsm signaling cascade in high-siderophore-yielding Pseudomonas sp. strain HYS. J. Bacteriol. 196:3259–3270.