The *higBA* Toxin-Antitoxin Module From the Opportunistic Pathogen *Acinetobacter baumannii* – Regulation, Activity, and Evolution

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**INTRODUCTION**

*Acinetobacter baumannii* is an emerging Gram-negative opportunistic pathogen, causing serious hospital-acquired infections (Antunes et al., 2014). These bacteria are well adapted to survive in hospital environment such as intensive care units, burn wards, and field hospitals (Rosa et al., 2014). During the last decade, several highly successful multidrug-resistant *A. baumannii* clonal...
lineages have spread in clinical settings worldwide causing
difficult to treat hospital outbreaks (Howard et al., 2012).
A. baumannii is known for its ability to withstand harsh
environmental conditions such as prolonged periods of dryness,
disinfectants, and antibiotic treatment (Jawad et al., 1998;
Cardoso et al., 2010; Nwugo et al., 2012).

Bacterial type II toxin-antitoxin (TA) systems are the most
ubiquitous among six types of prokaryotic toxin-antitoxin
systems (TAs), known to date (Chan et al., 2016; Page and Peti,
2016; Rocker and Meinhardt, 2016). They are encoded on the
low copy plasmids or chromosomes and code for two proteins,
one of which (toxin) is toxic to the cell, whereas the other
(antitoxin) neutralizes its toxicity by forming strong protein–
protein complex, which is non-harmful. Upon release from the
complex, the toxin acts within the cell by interfering with essential
processes, such as protein (Diaz-Orejas et al., 2010; Goeders and
Van Melderen, 2014) or DNA synthesis (Harms et al., 2015),
cell wall synthesis (Mutschler et al., 2011), and cell division
(Masuda et al., 2012). The toxin action results in a rapid cell
growth arrest or even leads to cell death (Page and Peti, 2016).

The majority of toxins from type II systems, characterized to
date, are endoribonucleases (Cook et al., 2013), which, if not
neutralized by its cognate antitoxin protein, cleave mRNAs at
specific sequences either within or outside the ribosome and
inhibit translation (Chan et al., 2016). The plasmid-borne type II
TAs often function as plasmid stabilization elements by allowing
growth of the cells that inherit plasmid with the TA system upon
segregation, whereas cells that have lost plasmid are killed by
more stable toxin after the more labile antitoxin is degraded
by proteases (Engelberg-Kulka and Glaser, 1999; Hernández-
Arriaga et al., 2015). The biological role of chromosomally
encoded type II systems is not clearly elucidated yet. The proposed functions of type II TAs range from viewing them as
selfish DNA, anti-addiction elements to stress-responsive genes,
which can regulate bacterial growth and survival adapting to
various environmental changes (Magnuson, 2007; Van Melder
and Saavedra De Bast, 2009; Ramisetty and Santhosh, 2017).
The TA systems can adjust the metabolic processes at a large
scale, such as shutting down protein synthesis and switching to
a dormant cellular state (Kędzierska and Hayes, 2016; Lee and
Lee, 2016).

Genome analysis has shown a wide variety of TA modules
in pathogenic species (Makarova et al., 2009; Leplae et al.,
2011). The role of TAs in the life of bacterial pathogens is
now beginning to be explored (Fernández-García et al., 2016;
Kędzierska and Hayes, 2016; Lee and Lee, 2016; Lobato-Márquez
et al., 2016a). Recent reports have demonstrated the significance
of TAs in the stabilization of virulence plasmids in Shigella
(McVicker and Tang, 2016) and Salmonella enterica (Lobato-
Márquez et al., 2016b), superintegron in Vibrio cholerae (Jqbal
et al., 2015), also in the promoting of S. enterica persist
formation (Cheverton et al., 2016; Jaiswal et al., 2016) and in
mediating the transcriptional response to environmental cues in
Helicobacter pylori and Brucella abortus (Heaton et al., 2012;
Cárdenas-Mondragón et al., 2016).

We have recently shown that type II HigBA TA system is one of
the most prevalent plasmid-borne TA systems in A. baumannii
isolates of clinical origin. It is encoded by the higBA operon,
where higBAb toxin gene (357 bp) precedes higAAb antitoxin
(303 bp) (Jurénaitė et al., 2013; Sužiedėlienė et al., 2016). higBAAb
locus was also found to be encoded by the newly observed
ubiquitous A. baumannii 11 kb plasmid pAB120. Plasmid
carries two copies of blaOXA−72 genes, conferring resistance to
carbenepens, A broad spectrum β-lactam antibiotics class, which
is used to treat A. baumannii infections (Povilonis et al., 2013;
Supplementary Figure S1). Here we report characterization of
A. baumannii type II TA system, by demonstrating that higBA
locus is represented in A. baumannii by two functional variants,
named higBAAb1 and higBAAb2. The higBAAb2 is encoded on
pAB120 plasmid and was further thoroughly characterized. We
demonstrate that HigB2Ab toxin acts as a ribonuclease and forms
an unusually large complex with the antitoxin. Both HigA2Ab and
the HigBA2Ab protein complex transcriptionally autoregulate
their own operon. We show that higBA2Ab represents a stress
responsive TA locus, which also possesses plasmid stabilization
ability.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Escherichia coli and Acinetobacter strains were grown in
LB at 37°C with appropriate antibiotics added (ampicillin
100 μg/mL, meropenem 8 μg/mL, gentamicin 10 μg/mL,
kanamycin 50 μg/mL, and chloramphenicol 30 μg/mL), unless
otherwise indicated. The strains and plasmids used in the study
are described in Supplementary Table S1. A. baumannii was
transformed with plasmid pAB120 variants by electroporation,
and selected on LB containing meropenem. Minimal inhibitory
concentration (MIC) values were detected as described (Wie
gand et al., 2008).

Plasmid Construction

Gene cloning procedures were performed using high-fidelity
Phusion polymerase (Thermo Fisher Scientific), cleavage with
restriction enzymes and ligation was performed according
manufacturers recommendations (New England Biolabs,
Thermo Fisher Scientific). All final constructs were verified by
sequencing. pAB120ΔhigBA: to introduce higBA2Ab deletion
to pAB120, primers described in Supplementary Table S2 were
used for inverse PCR (Imai et al., 1991). The resulting PCR
product was cleaved with NcoI and ligated. The deletion of
higBA2Ab was confirmed by PCR. pAcORI*higBA: a derivative
of the plasmid pWH1266 (Hunger et al., 1990; Supplementary
Table S1), exhibiting faulty inheritance in Acinetobacter sp. was
constructed. pAcORI plasmid contained pUC19 backbone
with introduced gentamicin resistance gene and a defective
Acinetobacter calcoaceticus ORI from pWH1266, amplified
using primers listed in Supplementary Table S2. higBA2Ab TA
system from pAB120, together with predicted promoter region,
was then cloned to pAcORI Ab using primers in Supplementary
Table S2, higBA2Ab operon, meropenem 8 µg/mL, and chloramphenicol 30 µg/mL, unless
otherwise indicated. The strains and plasmids used in the study
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using primer pairs described in Supplementary Table S2. pAB120 plasmid was used as a template for higBA2Ab gene amplification, while for relBEAb, A.baumannii clinical (35, ECII) strain was used (Jurenaite et al., 2013). Both toxins and antitoxins were N-terminally fused to appropriate Cya domains. Protein expression plasmids: for protein purification, higBA2Ab operon was cloned to pET28b protein expression vector, fusing the antitoxin (C-terminally) or the toxin (N-terminally) with his-tag (Supplementary Table S2). To construct plasmids containing TEV cleavage sites, the thrombin recognition site in the plasmid was replaced to TEV site by inverse PCR using primers listed in Supplementary Table S2 to generate pET-His6-TEV-HigB-HigA plasmid. For pET-HigB-His6-TEV-HigA, TEV site and His-tag was first removed from previous plasmid and then added to the N-terminus of higA2Ab. Constructs for promoter repression assay: pPROBE’-gfp vectors were constructed by inserting PCR amplified predicted promoter DNA sequences (200 bp in length upstream of higB2Ab or higA2Ab genes; primers indicated in Supplementary Table S2) upstream of gfp gene. higBA2Ab and higA2Ab genes were cloned to pBAD24 under arabinose inducible promoter (primers indicated in Supplementary Table S2). pBAD-higBpAB120 and pUEHcat-higApAB120 for kill-rescue assay: the plasmids were constructed as described elsewhere (Jurenaite et al., 2013).

Bacterial Adenylate Cyclase Two Hybrid System (BACTH) Assay
Five independent clones, with compatible toxin and antitoxin containing pKNT25 and pUT18 vectors were grown in LB for 16 h, then the cultures of the five clones were mixed and 5 µL of the mix was spotted on LB agar plates containing appropriate antibiotics, 100 µg/mL IPTG and 100 µg/mL X-Gal. The plates were incubated for 24 h at 30°C.

Purification of HigBA2Ab Protein Complex
For purification of His-HigBA2 and HigBA2-His protein complexes, plasmids pET-His-HigBA or pET-HigBA-His, were introduced into E. coli strain BL21 (DE3) (Supplementary Table S1) and the expression of protein complex induced by 1 mM IPTG for 4 h during mid-logarithmic phase. Cells were collected by centrifugation for 10 min at 4°C 5500 g, bacterial pellets were resuspended in lysis buffer (20 mM NaH2PO4 pH 7.4, 500 mM NaCl, 20 mM imidazole) and lysed by sonication. Lysate was centrifuged for 10 min at 4°C 13000 g to remove cell debris. The protein complexes were purified from soluble fraction by affinity chromatography, using 1 mL HisTrapHP™ nickel-Sepharose column (GE Healthcare), equilibrated with 20 mM NaH2PO4 pH 7.4, 500 mM NaCl, 20 mM imidazole buffer. After loading the protein lysate, the column was washed with the same buffer for column volumes followed by 10 volumes of wash buffer (20 mM NaH2PO4 pH 7.4, 500 mM NaCl, 50 mM imidazole) to remove impurities. Proteins were eluted by linear gradient using buffer 20 mM NaH2PO4 pH 7.4, 500 mM NaCl, 50 mM imidazole. The eluted fractions were desalted using Sephadex G–25 (GE Healthcare) column, exchanging the buffer to 20 mM NaH2PO4 pH 7.2, 300 mM NaCl. Eluted proteins were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), stained with Coomassie Brilliant Blue. To generate tag-less complex, His-TEV-HigBA2 proteins were incubated with TEV protease at a molar ratio of 1 to 100 overnight at room temperature.

Toxin Purification From Protein Complex
To separate HigB2 toxin from His-HigBA2 complex, denaturant-induced dissociation of the toxin–antitoxin complex on-column method was used (Sterckx et al., 2015). Briefly, the protein complex was denatured on the affinity column using guanidine HCl buffer (5 volumes of 50 mM Tris, pH 8.0, 500 mM NaCl, 5 M guanidine HCl), followed by renaturation on the resin (5 volumes of 25 mM Tris pH 8.0, 250 mM NaCl, 5% glycerol, followed by 5 volumes of 25 mM Tris pH 8.0, 250 mM NaCl, 1% glycerol). The toxin was eluted with 50 mM Tris pH 8.0, 500 mM NaCl, 200 mM imidazole, then immediately dialyzed to 50 mM Tris–HCl pH 8.0.

RNA Cleavage Analysis in Vitro
Total A.baumannii RNA, 5S rRNA and E. coli total tRNA were used as substrates for in vitro RNA cleavage analysis by HigB2 toxin. The mixtures (20 µL) contained 1.5 µg total RNA or 3 µg of 5S or tRNA in 25 mM Tris–HCl pH 7.8, and 0–1 µM final concentration of His-HigB2 protein. As a control, 1 µM of His-HigBA2 protein complex was added. The mixes were incubated for 30 min at 37°C and fractionated using 2% agarose electrophoresis.

Promoter Activity and Repression Measurements in Vivo
For promoter activity assays D1624Δara cells were transformed with pPROBE’-gfp vectors with or without inserted predicted promoter sequences upstream from gfp. Together, pBAD24, pBAD24-HigBA or pBAD24-HigA vectors were co-transformed. Overnight cultures of resulting strains were diluted to optical density at 600 nm (OD600) = 0.02 in minimal M9 media with casamino acids, 50 µg/mL kanamycin, 100 µg/mL ampicillin and 0.2% glucose. 1 mL of cultures was grown in glass bottom
black 24 well plates (Greiner) in Spectramax microplate reader at 37°C with constant shaking. OD600 and fluorescence (excitation 485 nm emission 520 nm) were registered every 15 min. After 3 h of growth 0.2% arabinose was added to induce the protein expression from pBAD24 vectors and measurements were continued for 10 more hours.

Analysis of Gene Expression by qPCR

Acinetobacter baumannii clinical strain K60 (Povilonis et al., 2013), containing pAB120 plasmid, was grown in LB, to exponential phase (OD600 = 1) and to stationary phase (48 h, OD600 = 4.5). For stress conditions, the cells were grown in LB with 200 mM 2,2′-bipyridine (Eijkelkamp et al., 2011) or in LB with 1 or 2% ethanol to OD600 = 1. For antibiotic stress, 1/10 of MIC values for gentamicin and rifampicin (2 and 0.3 µg/mL, respectively) and 1/2 MIC for meropenem (16 µg/mL) was added to exponentially growing cells and incubated for 1 h to a final OD600 = 1. Total RNA was isolated, DNA removed and cDNA synthesized as recommended by the kit supplier (Thermo Fisher Scientific). qPCR was performed using primer pairs listed in Supplementary Table S2 (all primers exhibited 100–103% amplification efficiency at selected concentrations). The changes in gene expression were calculated as ΔΔCt, using rpoB as house-keeping gene. At least three biological replicas were performed.

Plasmid Stability Assay

Bacteria containing plasmid of interest were grown in LB containing appropriate antibiotic as an overnight culture (16 h), and then diluted 500-fold to a fresh media without antibiotic. The culture was restarted to a new batch of LB without antibiotic every 24 h. After each inoculation the colony forming units (CFU) of antibiotic resistant (retaining the plasmid encoding resistance) and sensitive bacteria (plasmid lost) was calculated by serial dilutions and plating on LB agar with or without appropriate antibiotic, and plasmid retention was calculated as a percentage of resistant bacteria.

Plasmid Copy Number (PCN) Calculation

Plasmid copy number (PCN) was calculated as described elsewhere (Providenti et al., 2006). Briefly, the bacteria were grown for 24 h without antibiotic pressure, the cells were collected and their lysates used for qPCR, with primers detecting rep gene of the pAB120 plasmid and rpOB as genome encoded control. Copy number was calculated as PCN = EACt (E = 2, if the product amplifies at 100% efficiency, which was the case for the primers used). The experiment was independently repeated 10 times.

Kill-Rescue Assay

The assay was performed as previously described (Jurenaite et al., 2013). Briefly, E. coli BW25113 F’ pUHEcat-“antitoxin” pBAD-“toxin” strains were grown in LB medium until the early logarithmic phase (optical density at 600 nm (OD600) = 0.1). The culture was then induced with arabinose (0 or 0.02%) and/or IPTG (0, 0.1, and 1 mM). The growth was observed in Tecan Infinite M200 Pro plate reader at 37°C with shaking.

In Silico Analysis

Cluster of GP49-domain toxin family was prepared as neighbor joining tree (Saitou and Nei, 1987) using BLOSUM62 matrix, average distance tree of two major lineages of HigB toxins based on sequence identity as well as alignment of HigBAb toxins obtained using ClustalW and Jalview 2.10.0b1.

RESULTS

higBAAb System in A. baumannii Is Represented by Two Variants

Acinetobacter baumannii higBA module has been classified on the basis of low but significant homology of its predicted GP49-like domain toxin HigB to the RelE/ParE superfamily toxins (Jurenaite et al., 2013). Further homology search has shown that the closest GP49-domain homologues of HigBAb, mentioned in the literature are Mycobacterium tuberculosis Rv2022c-Rv2021c (also called higBA2) and Rv3182-Rv3183 (higBA3) TA systems, whose toxins share more than 40% protein sequence identity with HigBAb (Figure 1A). Based on the BLAST search, close homologues of HigBAb (more than 50% sequence identity) are present in other gammaproteobacteria closely related to Acinetobacter, such as Psychrobacter, as well as various members of Enterobacteriaceae family, such as Klebsiella (Supplementary Figure S2).

Strikingly, BLAST search revealed a variety of amino acid sequences for HigBAb toxin in A. baumannii species. Therefore, we have aligned all A. baumannii HigBAb sequences existing to date and found that they are, in fact, represented by two distinct versions of HigBAb modules showing around 60% sequence identity (Figures 1B,C). The most prevalent variant is largely conserved (called higB1Ab further), while the other version of HigBAb is represented by many sequence variations ranging from 85 to 99% identity (HigB2Ab) (Figure 1B). The higBAAb module found in plasmid pAB120 (Povilonis et al., 2013), which we characterize in this study, represents the less conservative version higB2Ab, while the conserved higB1Ab has been characterized previously as a representative higBAAb module carried by strain 35 in a pilot study of A. baumannii TA systems (Jurenaite et al., 2013). We were interested whether the two versions of TA systems constitute stand-alone TA modules. Expression of both versions of HigBAb toxin resulted in an inhibition of E. coli growth, and interestingly, co-expression of HigAAb antitoxin counteracted toxin-mediated growth inhibition regardless the HigAAb variant used for growth rescue (Figures 1D–G). This experiment has shown that the components of two TA variants are still able to interact despite 42 and 45% amino acid sequence difference between the toxins and antitoxins of the two groups, respectively. Structure models of both versions of HigB and HigA (Supplementary Figure S3) also predicted high similarity of folds, despite some differences at the termini of the proteins. Notably, the pAB120-borne HigB2Ab pair exhibited more pronounced kill-rescue effect compared to the more conserved HigB1Ab version (Figures 1D vs. G). These results are in line with the proposed anti-addiction hypothesis (Saavedra De Bast...
et al., 2008), where the conserved but less active HigBA1Ab module could provide evolutionary pressure for the HigBA2Ab lineage.

We then searched for Acinetobacter sp. strains having both higBAAb versions in the GenBank sequence database. We were not able to find two distinct versions of higBAAb on the same plasmid or in the same strain. This phenomenon has been reported previously by analysis of identity of bacterial and chromosomal TAs (Ramisetty and Santhosh, 2016). However, we were able to find two sequenced Acinetobacter sp. isolates with copies of higBAAb on several plasmids [isolates DUT-2 (GenBank accessions CP014652, CP014654, CP014655) and VE-C3 (GenBank accessions NC_010310, NZ_ALIG01000010)], and five plasmids containing two copies of same version of higBAAb (GenBank accessions CM008888, CP010369, CM001803, NC_010404, NC_025173) (Supplementary Table...
HigB2\textsubscript{Ab} and HigA2\textsubscript{Ab} Proteins Form Complex

pAB120 plasmid-borne HigBA2\textsubscript{Ab} and its toxin HigB2\textsubscript{Ab} shares only low similarity to RelE/ParE type TA toxins. Therefore we asked whether this TA system is and acts as a canonical one. Type II TA systems are known to employ different mechanisms of action and their proteins may form different oligomeric complexes (Chan et al., 2016; Rocker and Meinhart, 2016). We asked whether HigB2\textsubscript{Ab} and HigA2\textsubscript{Ab} form a physical complex which neutralizes toxicity in a typical way to type II TA systems. For this purpose we employed bacterial adenylate cyclase two hybrid system (BACTH) (Karimova et al., 1998) and verified that HigB2\textsubscript{Ab} toxin and HigA2\textsubscript{Ab} antitoxin directly interact in vivo by restoring adenylate cyclase activity (Figure 2A).

Moreover, the A. baumannii RelE\textsubscript{Ab} and RelB\textsubscript{Ab} proteins [homologues of well-described RelBE TA system of E. coli (Jurenátk et al., 2013)], which we used as a control, showed even weaker interaction in comparison to that of HigB2\textsubscript{Ab} and HigA2\textsubscript{Ab}.

To further assess the complex forming ability between A. baumannii HigB2\textsubscript{Ab} and HigA2\textsubscript{Ab} proteins, we have constructed two versions of the expression vectors encoding higBA2\textsubscript{Ab} operon with HigB2\textsubscript{Ab} toxin N-terminal His-tag fusion (pET-His-HigBA) and with antitoxin HigA2\textsubscript{Ab} C-terminal His-tag fusion (pET-HigBA-His). Proteins were expressed in E. coli and purified by affinity chromatography as described in Section “Materials and Methods.” In both cases HigB2\textsubscript{Ab} and HigA2\textsubscript{Ab} proteins co-purified, indicating that they form a strong complex (Figures 2C,D). To assess the size of complexes we have analyzed them by size-exclusion chromatography. The elution profiles showed single peaks (not shown) corresponding to the entities with estimated molecular masses of approximately 99.3 kDa and 53.7 kDa for His-HigB2\textsubscript{Ab} and HigB2\textsubscript{Ab}-His complexes, respectively (Figure 2B). Protein analysis of pooled fractions that corresponded eluted peaks by 15% SDS–PAGE and subsequent gel densitometric analysis showed two bands present in ratio (toxin:antitoxin) of approximately 1:1 and 1:1 (Figure 2B). As the differences in observed complex sizes could have been due to the interference of His-tag, we aimed to confirm the complex size with the tag-less protein complex. To eliminate the probable His-tag effect, we constructed plasmids with cleavable His-tags situated at the N-terminus of HigB2\textsubscript{Ab} (plasmid pET-His6-TEV-HigB-HigA) or HigA2\textsubscript{Ab} (plasmid pET-HigB-His6-TEV-HigA). However, the cleavage of His-tag was only efficient for the protein complex with N-terminal His-tag fusion of HigA2\textsubscript{Ab}. Upon removal of His-tag from HigBA2\textsubscript{Ab} complex, size exclusion chromatography resulted in an entity of approximately 109 kDa (Supplementary Figure S4), thus confirming that HigBA2\textsubscript{Ab} is able to form unusually large complex.

HigB2\textsubscript{Ab} Toxin Is a Ribonuclease

We have demonstrated previously, that HigB1\textsubscript{Ab} toxin from higBA1\textsubscript{Ab} system (named as higBA\textsubscript{Ab} in the previous research) when expressed in E. coli caused degradation of cellular RNAs and inhibited translation by impairing the incorporation of protein synthesis precursor, therefore suggesting that it might act as a ribonuclease (Jurenátk et al., 2013). To investigate whether HigB2\textsubscript{Ab} possesses a ribonuclease activity in vitro, we have purified recombinant His-HigB2 toxin from His-HigBA2\textsubscript{Ab} complex and tested whether it is able to degrade A. baumannii total RNA as well as RNA with known secondary structure such as E. coli 5S rRNA and tRNA. HigB2\textsubscript{Ab} toxin efficiently degraded total A. baumannii RNA in a dose dependent manner, whereas in a complex with HigA2\textsubscript{Ab} protein its ribonuclease activity was blocked (Figure 3A). The degrading activity against E. coli 5S rRNA was only detected using high concentrations of HigB2\textsubscript{Ab} toxin (Figure 3B), whereas effect against tRNA at the same conditions was negligible, indicating that HigB2\textsubscript{Ab} toxin more likely targets unstructured RNAs.

HigA2\textsubscript{Ab} Antitoxin Represses Transcription From higBA2\textsubscript{Ab} Promoter Whereas HigB2\textsubscript{Ab} Toxin Acts as a Corepressor

Type II TA complexes or single antitoxin proteins have been shown to bind to the operator DNA of their own promoter and negatively autoregulate operon transcription (Kedzierska et al., 2007). HigA2\textsubscript{Ab} has a HTH domain and could bind to DNA alone or in complex with HigB2\textsubscript{Ab}. It is not known how reverse TA systems, where the toxin is the first gene in the operon, regulate their toxin:antitoxin ratio. We looked if an additional promoter could be found for higA2\textsubscript{Ab}, indicating additional regulation for antitoxin expression. BPROM tool (Solovyev and Salamov, 2011) predicted promoter elements (−10 and −35) upstream the higBA2\textsubscript{Ab} operon and higA2\textsubscript{Ab} coding sequence (Figure 4A), indicating that besides promoter for the operon, higA2\textsubscript{Ab} might also have an additional promoter. We have tested the activity of these promoters and their regulation by HigB2\textsubscript{Ab} complex and HigA2\textsubscript{Ab} protein. 200 bp DNA fragments containing higBA2\textsubscript{Ab} and higA2\textsubscript{Ab} promoters were cloned upstream of the gfp gene (plasmids pPROBE-PhigB-gfp and pPROBE-PhigA-gfp). Promoter activities were measured by following GFP fluorescence upon induction of HigA2\textsubscript{Ab} antitoxin and HigB2\textsubscript{Ab} complex (plasmids pBAD24-HigA, pBAD24-HigBA) in E. coli. Promoterless gfp (plasmid pPROBE-gfp) was used to assess autofluorescence level of the cells. Induction of HigA2\textsubscript{Ab} expression strongly repressed the activity of higBA2\textsubscript{Ab} promoter, and the induction of HigB2\textsubscript{Ab} complex showed even more pronounced inhibitory effect (Figure 4C). Putative higA2\textsubscript{Ab}
FIGURE 2 | Analysis of HigB2Ab–HigA2Ab protein interaction. (A) Two-hybrid analysis of interaction of toxin-antitoxin (TA) components. E. coli BTH101 with two plasmids, one encoding Cya-T18 fused to A. baumannii antitoxins HigA2Ab or RelB2Ab, and another encoding Cya-T25 fused to toxins HigB2Ab or RelE2Ab. The ability of the proteins to interact and reconstitute functional Cya from T25 and T18 was observed as blue colony formation when grown on LB agar with IPTG and X-gal for 24 h; (B) Size-exclusion chromatography and SDS–PAGE analysis of His-HigBA2 and HigBA2-His protein complexes. The proteins used for molecular mass standard curve are indicated as black diamonds, empty diamonds indicate the positions of His-HigBA2 and HigBA2-His protein complexes and single His-HigB2 protein and their calculated size; inside box – 15% SDS–PAGE gel analysis lane 1 – His-HigB2, lane 2 – His-HigBA2, lane 3 – HigBA2-His proteins and complexes after gel filtration; (C,D) E. coli BL21(DE3) strain, containing pET-HigBA-His (C) and pET-His-HigBA (D) plasmids were grown to mid-exponential phase and protein expression was induced with 1 mM IPTG for 4 h. Cells were disrupted by sonication and the His-tag containing proteins were purified by affinity chromatography from the soluble fraction as described in Section “Materials and Methods”. Proteins were visualized by 15% SDS–PAGE stained with Coomassie Brilliant Blue. M – protein molecular mass markers (band sizes in kDa are shown on the Left), –/+ cell lysate before and after induction with IPTG, S – soluble protein fraction after cell disruption, F – protein purification flow-through fraction, W – protein purification wash fraction, Elution – protein purification elution fractions.

FIGURE 3 | HigB2Ab toxin acts as a ribonuclease. His-HigBA2 protein complex at the concentration of 1 µM and His-HigB2 toxin at the concentrations of 0, 0.25, 0.5, and 1 µM, were incubated with 1.5 µg of total A. baumannii RNA (A) and with 3 µg of E. coli 5S rRNA (B) in 10 µl reactions for 30 min at 37°C. The samples were visualized in 1% agarose gel.
promoter located within the coding sequence of higB2<sub>Ab</sub> gene did not display any activity (Figures 4B,D). Our results clearly show that higBA2<sub>Ab</sub> operon is transcriptionally autorepressed by its cognate antitoxin HigA2<sub>Ab</sub>, and HigB2<sub>Ab</sub> toxin acts as a co-repressor. The predicted higA2<sub>Ab</sub> promoter was not functional.

Expression of A. baumannii higB2<sub>Ab</sub> and higA2<sub>Ab</sub> Genes in Stress Conditions

We have demonstrated that HigB2<sub>Ab</sub> and HigA2<sub>Ab</sub> form a strong complex which causes repression of its own operon. We next asked how higBA2<sub>Ab</sub> locus is expressed in A. baumannii K60 (Povilonis et al., 2013) under various conditions. We have chosen to investigate growth and stress conditions relevant to those found in clinical environment and within the host: the stationary phase, conditions mimicking the iron deficiency, growth with ethanol and sub-lethal amounts of antibiotics.

The expression changes in both higB2<sub>Ab</sub> and higA2<sub>Ab</sub> were evaluated separately by qPCR to see if there are any differences in expression levels which would indicate the presence of separate transcripts. We have found that transcripts of higB2<sub>Ab</sub> and higA2<sub>Ab</sub> genes were more abundant in stationary phase (more than fivefold increase) comparing to the exponential growth conditions (Figure 5). Interestingly, the iron deficiency caused by the addition of iron chelator 2,2′-bipyridine (Eijkelkamp et al., 2011) to the LB medium resulted in the up to threefold induction of the higB2<sub>Ab</sub> and higA2<sub>Ab</sub> genes (Figure 5). The presence of ethanol in the media did not have pronounced effect on the gene expression (Figure 5). We further tested sub-lethal concentrations of antibiotics. As A. baumannii strain K60 was highly resistant to several classes of antibiotics [aminoglycosides, β-lactams, fluoroquinolones, (Supplementary Table S4)], we chose antibiotics which exhibited moderate to low MIC (gentamicin MIC 20 µg/mL, rifampicin MIC 3.125 µg/mL). The antibiotics were added at 1/10 of the MIC, as higher concentrations resulted in severe growth impairment. Effect of meropenem was also tested (1/2 of MIC), due to the presence bla<sub>OXA</sub>-72 gene in the proximity to higBA2<sub>Ab</sub> in pAB120 plasmid. No significant changes in higBA2<sub>Ab</sub> expression were observed for gentamicin or meropenem, but the addition of rifampicin caused a decrease of expression of both genes (Figure 5). We therefore conclude that higBA2<sub>Ab</sub> module could be expressed during stress conditions linked to stationary phase and iron deficiency stress, as well as it could play a role during RNA synthesis inhibition or yet in other unknown conditions. Additionally, since all expression changes were similar for both higB2<sub>Ab</sub> and higA2<sub>Ab</sub>, we can expect both genes to be expressed from a single transcript of the whole operon.
Resistant to clinically important antibiotics may contribute to the persistence and spread of antibiotic resistant strains. We have previously shown that higBA2<sub>Ab</sub> module is widely spread among A. baumannii plasmids (Povilonis et al., 2013; Sužiedelienė et al., 2016), therefore we were interested whether it provides stabilization function for cognate plasmid carrying resistance genes, found in clinical A. baumannii isolates. For this purpose plasmid pAB120, which originally carries two copies of bla<sub>OXA-72</sub> gene conferring resistance to carbapenems (meropenem and imipenem) was purified from clinical A. baumannii strain K60 and higBA2<sub>Ab</sub> locus was deleted (Supplementary Figure S1). Given that pAB120 plasmid is equipped with another type II TA module, splTA (Jurenaite et al., 2013; Povilonis et al., 2013), we sought if the latter could be sufficient to supply plasmid stabilization even when higBA2<sub>Ab</sub> was deleted. Therefore, plasmid with deletion of both TA modules was also constructed (Supplementary Figure S1). The pAB120ΔhigBA<sub>Ab</sub> and pAB120ΔhigBA<sub>Ab</sub>,ΔsplTA<sub>Ab</sub> plasmids were transformed into non-pathogenic Acinetobacter baylyi strain ADP1, which is known not to contain any plasmids or higBA homologs (Supplementary Table S1). Unexpectedly, neither the deletion of higBA2<sub>Ab</sub> locus, nor the elimination of both higBA2<sub>Ab</sub> and splTA<sub>Ab</sub> modules caused any loss of pAB120 variants in A. baylyi (not shown). To eliminate the possibility of different plasmid maintenance effects in different Acinetobacter species, A. baumannii clinical strain K53 was then used as a host (the strain also does not contain any plasmids of known A. baumannii replication groups and higBA modules (Jurenaite et al., 2013; Povilonis et al., 2013; Supplementary Table S1). pAB120 variants did not show decrease in plasmid retention in A. baumannii as well (Figure 6A). We then analyzed whether higBA2<sub>Ab</sub> locus has an impact on the copy number of pAB120 plasmid. A. baumannii strains K53 bearing pAB120 with or without higBA2<sub>Ab</sub> were grown without antibiotic pressure for 24 h and PCN was calculated by qPCR. A slight reduction in PCN was observed, which was statistically significant (Figure 6A). These results indicate

![Image](https://example.com/image.png)

**The Role of higBA2<sub>Ab</sub> TA System in Stabilization of Acinetobacter Plasmids**

Toxin-antitoxin modules are known plasmid stabilization factors in bacteria (Engelberg-Kulka and Glaser, 1999; Hernández-Arriaga et al., 2015) and their presence on plasmids conferring resistance to clinically important antibiotics may contribute...
that while \textit{higBA2} and \textit{splTA} are not the main players in stabilization of pAB120 under tested conditions, the deletion of \textit{higBA2} has an impact on its copy number, and could influence plasmid retention in the long run or in different conditions.

To be sure if \textit{higBA2} is able to confer stability when introduced into unstable plasmid lacking any stabilizing determinants, we used \textit{higBA2} from pAB120 in additional plasmid stabilization experiments. The \textit{higBA2} locus with its own promoter region was cloned into unstable \textit{Acinetobacter} plasmid pAcORI\star, conferring resistance to gentamicin (Supplementary Table S1). \textit{A. baylyi} ADP1 strain containing plasmid pAcORI\star with \textit{higBA2} locus and plasmid without the TA system were grown without antibiotic pressure and plasmid retention was calculated. Strikingly, in this background the presence of \textit{higBA2} locus on the pAcORI\star plasmid ensured its stability when bacteria were grown for over 80 h (Figure 6B). This result together with the previous observations of the effect on PCN confirms that \textit{higBA2} module can play a role in plasmid maintenance.

**DISCUSSION**

The TA modules which are grouped into \textit{higBA} family (\textit{host inhibition of growth}) encode a RelE-like toxin and antitoxin that contains a HTH Xre-domain (Gerdes et al., 2005; Makarova et al., 2009). Toxins of \textit{HigB} family belong to a large RelE/ParE superfamily consisting of mRNAses such as \textit{RelE} as well as gyrase poisons such as \textit{ParE} toxin (Anantharaman and Aravind, 2003). Within this superfamily, GP49-domain toxins show low but significant homology to RelE (Makarova et al., 2009). TA modules containing GP49-domain toxin in all studied cases constitute the reverse type of TA operons where toxin is encoded first and is followed by an antitoxin (Dziejewit et al., 2007; Ramage et al., 2009; Jurenaitė et al., 2013; Sala et al., 2014). Despite being classified as \textit{HigB} family toxins, the GP49-domain toxins show little similarity to well-studied \textit{HigB} toxins from \textit{E. coli}, \textit{Vibrio cholerae}, \textit{Proteus vulgaris} (Tian et al., 1996; Christensen-Dalsgaard and Gerdes, 2006; Christensen-Dalsgaard et al., 2010) as well as to toxins of other TA families of RelE superfamily. \textit{HigBA2} shows only ~20% sequence identity to other validated TA modules that have GP49-domain toxins and were named as Tad toxins (Dziejewit et al., 2007).

pAB120 plasmid-borne \textit{HigBA2} proteins form strong complex with molecular mass of approximately 100 kDa suggesting that up to eight protein molecules might be present within the oligomer (the predicted molecular masses of \textit{HigB2} and \textit{HigA2} proteins are 13.5 and 11.2 kDa, respectively). According to the currently available structural data, the RelE toxin-based \textit{TA} superfamily TA complexes are known to form heterotrimers (\textit{E. coli} YefM-YeoB, PDB accession: 2A6Q), and most commonly, heterotetramers (\textit{E. coli} DinJ-YaqQ, PDB accession: 4Q2U; MqsR-MqsA, PDB accession: 3HI2, \textit{E. coli} RelB-RelE, PDB accession: 4FXE; \textit{Brucella abortus} BrnT-BrnA (Heaton et al., 2012), \textit{Proteus vulgaris} \textit{HigBA} (Schureck et al., 2014]). In some cases complexes crystallize as more complex oligomeric structures: a so far unique hetero-hexameric complex has been observed for ParE2-PaaA2 TA system (Sterckx et al., 2016). ParE-family toxins inhibit DNA replication and despite sharing common fold they are functionally different from RelE-family toxins. Therefore \textit{A. baumannii} \textit{HigBA2} complex of 100 kDa, the size strongly exceeding that of a heterotetramer, observed in this study might represent one of the most complex oligomeric structures known for RelE family TAs.

Taking into account the organization of \textit{A. baumannii} \textit{higBA2} module, where toxin gene is located upstream the antitoxin gene, a feature known for a limited number of TAs (Christensen-Dalsgaard and Gerdes, 2006), the expression of equal amounts of both proteins must be ensured in the cell to avoid a harmful effect of the toxin. According to our observations, equal levels of \textit{higB2} and \textit{higA2} transcripts are produced at the standard growth conditions in \textit{A. baumannii} indicating that TA balance at least at the transcription level is properly preserved. The transcript of the whole operon (spanning both \textit{higB2} and \textit{higA2} genes) could also be detected by qPCR (Armalytė, Unpublished data), whereas an additional promoter for the \textit{higA2} gene was not, indicating that most likely \textit{higBA2} is transcribed as a single transcript. \textit{HigA2} antitoxin and \textit{HigBA2} complex autorepressed their own promoter, indicating the transcriptional regulatory mode common to type II TA operons in other bacteria (Chan et al., 2016). \textit{HigA2} antitoxin harbors HTH Xre-like domain, which is present in some antitoxins of type II TAs such as \textit{HigA} from \textit{Proteus vulgaris}, and has been shown to bind operator sequences which overlap with the promoter region (Schureck et al., 2014). The more pronounced inhibition by \textit{HigBA2} complex than by single antitoxin indicates \textit{HigBA2} toxin also takes part in transcriptional autoregulation of the system.

Despite well studied transcriptional regulation for TA systems, the data on their expression \textit{in vivo} in conditions relevant to bacterial lifestyle, in particular to that of pathogenic species, is only beginning to emerge. \textit{A. baumannii} represents interesting example to study as it is well adapted to colonize the abiotic (glass, plastic, and metal) and biotic (human skin and mucous membranes) surfaces, withstand prolonged periods of dryness, treatment by disinfectants, antibiotics and nutrient restriction (Giannouli et al., 2013). In the host, \textit{A. baumannii} encounters responses mediated by complement and professional and non-professional phagocytic cells. Among stress factors playing essential role in the host defense is the nutritional immunity exerted by the iron limitation (McConnell et al., 2013). Our data suggest that stationary growth and iron depletion causes the induction of both toxin and antitoxin genes of \textit{higBA2} module at a similar level, therefore, expression of both components could play a role for \textit{A. baumannii} stress response. This is supported by recent observation that closest homologues of \textit{A. baumannii} \textit{higBA} in \textit{M. tuberculosis}, Rv2022c-Rv2021c (\textit{higBA2}) and Rv3182-Rv3183 (\textit{higBA3}) are among the strongest stress responsive modules of this pathogen, activated in response to multiple stressors including antibiotics, starvation and low pH (Gupta et al., 2017). In our study, we found that only rifampicin influenced the expression of \textit{higBA2} by reducing it, which was
the opposite effect from that observed for *M. tuberculosis* higBAs. Sub-inhibitory concentrations of antibiotics are known to induce variable gene expression outcomes in bacteria. SOS response is commonly induced, which includes upregulation of stress proteases such as Lon and Clp. Generally they target antitoxins and relieve transcriptional repression of TAs (Muthuramalingam et al., 2016). However, the observations that particular TAs are downregulated after antibiotic exposure indicate that specific inhibition of the activities of the TA promoters could be involved. Type II loci of other human pathogens have been recently shown to transcriptionally respond under stress conditions. *H. pylori* hp0893-hp0892 module, which is the most prevalent TA system among *H. pylori* clinical isolates was induced during stationary growth, in the low concentration of iron and nickel as well as in a high concentration of urea, conditions mimicking the host environment, suggesting that this TA gene pair might represent a novel *H. pylori* stress responsive virulence factor (Cárdenas-Mondragón et al., 2016). Intracellular pathogen *B. abortus* increased the expression of type II brnTA system in the presence of chloramphenicol, H₂O₂ and low pH stress (Heaton et al., 2012). In *S. enterica* type II sehAB module responded to minimal medium and within macrophages (De la Cruz et al., 2013), whereas different expression of some toxins of type II systems has been observed inside fibroblasts and epithelial cells (Lobato-Márquez et al., 2015).

The induction of bacterial TAs in stress conditions has been attributed to the enhanced degradation of antitoxin by stress peptidases or decreased translation under stress conditions (Ramisetty et al., 2016). This, in turn, might result in the derepression of transcription of TA operon to a different level depending on the stress influence on antitoxin proteolysis and translation rates. Therefore, the decrease in antitoxin concentration might result in various different physiological outcomes: the toxin, freed from the complex, could quickly come into action leading to growth reduction, or an increased transcriptional response of TA genes could be induced, led by derepression of the operon. Such a range in TA-mediated outcomes would be beneficial to *A. baumannii* in contributing to its persistence in clinical environment and in sensing the host.

The *higBA*₁₂₅₅ locus, with a few exceptions, is found so far on the *A. baumannii* plasmids, which frequently harbor antibiotic resistance genes coding for carbapenemases OXA-72, OXA-23, and OXA-58 (Sužiedelienė et al., 2016). We found that 67 out of 307 (21.8%) *Acinetobacter* sp. plasmids sequenced to date (GenBank accessed 2018/01/11) had *higBA*₁₂₅₅ TA systems. The location of *higBA*₁₂₅₅ could not be attributed to a certain plasmid replication group or plasmid size (plasmids sizes varied from 7.4 to 121 kb). The high prevalence of these TA systems indicates their easy spread and stable maintenance on the plasmids. The mechanisms responsible for plasmid stabilization in *A. baumannii* are largely unknown (Lean and Yeo, 2017) and this study is the first attempt to assess the role of TA systems in *A. baumannii*. Surprisingly, we have shown that despite the ability of *higBA*₂₅₅₅ module to function as a *bona fide* addiction system by supporting maintenance of unstable plasmid in *Acinetobacter*, it did not play that role for pAB120 plasmid from clinical *A. baumannii* strain, as did not another type II module, carried by the same plasmid. This data suggest that other elements than plasmid-borne post-segregational killing pathway components are responsible for pAB120 stabilization. Among the possible candidates are XerC/XerD sites, which are known to participate in recombinase-based proper resolution of plasmid copies (Sengupta and Austin, 2011) and are present in pAB120 plasmid in multiple copies as well as in series of *A. baumannii* plasmids of the same replicon type (Lean and Yeo, 2017).

Another unexpected observation was the detection of several *higBA*₁₂₅₅ copies in plasmids and *A. baumannii* isolates in the sequence databases. Interestingly, only the less conserved *higBA*₂₅₅₅ group was detected in that setting, and we were unable to find both groups *higBA*₁₂₅₅ in one isolate or plasmid in the pool of *Acinetobacter* sp. sequences, available to this day. In agreement with this observation we have demonstrated that both groups’ antitoxins are able to counteract the toxins. Such counter-activity has been reported for other TA systems (Zhu et al., 2010). In case of *HigBA*₁₂₅₅, the two versions might work as counter-addiction modules for each other. The observed duplication of *higBA*₂₅₅₅ could indicate the ongoing evolution and divergence of the novel TA species.

**AUTHOR CONTRIBUTIONS**

JA, DJ, RK, and ES designed the experiments. JA, DJ, RK, and AČ performed the experiments. JA, DJ, and ES analyzed the data and wrote the paper.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00732/full#supplementary-material

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