Toxins of toxin/antitoxin systems are inactivated primarily through promoter mutations

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Abstract

Aims: Given the extreme toxicity of some of the toxins of toxin-antitoxin (TA) systems, we were curious how the cell silences toxins, if the antitoxin is inactivated or independent toxins are obtained via horizontal gene transfer.

Methods and Results: Growth curves of *Escherichia coli* K12 BW25113 harbouring plasmid pCA24N to produce RalR, MqsR, GhoT or Hha toxins, showed toxin inactivation after 3 h. Sequencing plasmids from these cultures revealed toxin inactivation occurred primarily due to consistent deletions in the promoter. The lack of mutation in the structural genes was corroborated by a bioinformatics analysis of 1000 *E. coli* genomes which showed both conservation and little variability in the four toxin genes. For those strains that lacked a mutation in the plasmid, single nucleotide polymorphism analysis was performed to identify that chromosomal mutations *iraM* and *mhpR* inactivate the toxins GhoT and MqsR/GhoT respectively.

Conclusion: We find that the RalR (type I), MqsR (type II), GhoT (type V) and Hha (type VII) toxins are inactivated primarily by mutation that inactivates the toxin promoter or via the chromosomal mutations *iraM* and *mhpR*.

Significance and Impact of the Study: This study demonstrates toxins of TA systems may be inactivated by mutations that primarily affect the toxin gene promoter instead of the toxin structural gene.

Introduction

Toxin-antitoxin (TA) systems have been associated with stress response (Wang *et al.*, 2011), persister cell generation (Shah *et al.*, 2006; Kim and Wood, 2010), bacteriophage inhibition (Pecota and Wood, 1996) and other functions (Schuster and Bertram, 2013). TA systems were originally found on plasmids (Ogura and Hiraga, 1983), but they have also been found in bacterial chromosomes and bacteriophages. Almost all bacteria have TA systems in their genomes, reaching 88 TA loci in case of *Mycobacterium tuberculosis* (Page and Peti, 2016). Moreover, TA systems have been related to pathogenicity in a wide range of nosocomial pathogens (Trastoy *et al.*, 2018). Furthermore, TA systems have also been described in 86 Archaea and even in some fungi, in which there have been identified fourteen Doc toxin homologs (Yamaguchi *et al.*, 2011).

TA systems are primarily two component systems, composed of a toxin, which disrupts important cellular mechanisms, and an antitoxin, which blocks the toxin action (Schuster and Bertram, 2013). Depending on how the antitoxin interacts with the toxin, TA systems are classified into seven types. In type I systems (e.g. Hok/Sok), the antitoxin is an antisense RNA of the toxin. In type II (e.g. CcdB/CcdA) and III (e.g. ToxN/ToxI) systems, the antitoxin protein or RNA, respectively, inhibits the toxin by direct binding (Schuster and Bertram, 2013). In type IV systems (e.g. CbtA/CbeA), the antitoxin competes with the toxin for the target (Masuda *et al.*, 2012),
and in type V systems (e.g. GhoT/GhoS), the antitoxin is an enzyme that cleaves specifically the toxin mRNA (Wang et al., 2012). In type VI systems (e.g. SocB/SocA), the antitoxin protein facilitates toxin degradation as an adaptor protein (Page and Peti, 2016). Recently, the type VII system has been described (Hha/TomB) in which the antitoxin is an enzyme that inactivates the toxin by oxidizing a cysteine residue (Marimon et al., 2016; Song and Wood, 2018).

The broad distribution and myriad types of TA systems implies there is an evolutionary advantage for utilizing them. Also, many of the TA systems require tight regulation, to prevent undesired reductions in metabolism. For example deletion of the gene encoding antitoxin MqsA is lethal (Brown et al., 2009). For this tight regulation, TA systems are often self-regulated, as (i) antitoxins of type II TAs repress expression via promoter binding, (ii) some toxins limit both antitoxin and toxin levels via posttranscriptional cleavage of mRNA (Wang et al., 2013; Hayes and Kędzierska, 2014) and (iii) some antitoxins inhibit toxins. For example toxin MqsR autoregulates itself by cleaving its own mRNA (Brown et al., 2012).

This study focuses on four different toxins, MqsR, GhoT, RaR and Hha, each one from a different TA system. The MqsR/MqsA system is a type II TA system in which the toxin, MqsR, was originally characterized as a biofilm formation regulator that is quorum-sensing related (Ren et al., 2004). MqsR also regulates another TA system, GhoT/GhoS (Wang et al., 2013). MqsA antitoxin participates in the global stress response by regulating RpoS and activating biofilm formation under oxidative stress (Wang et al., 2011). As an example of this oxidative stress response, the MqsR/MqsA system manages growth during stress due to bile acids in the gastrointestinal tract (Kwan et al., 2015). MqsR is also the first toxin that when inactivated, reduces persistor cell formation (Kim and Wood, 2010). In regard to its regulation, toxin MqsR disrupts the MqsA-DNA complex to activate transcription (Brown et al., 2012).

The first type V TA system, GhoT/GhoS (Wang et al., 2012), is named based on the ghost-cell phenotype seen when toxin GhoT is produced, which results from membrane damage and reduces ATP (Cheng et al., 2014). The RaR/RaA system is a type I TA system found in the cryptic prophage rac (Guo et al., 2014). RaR is the only known, nonspecific DNase TA system toxin (many toxins are RNases); it activates the SOS response via DNA damage and increases resistance to the antibiotic fosfomycin. Hence, the RaR/RaA system improves bacterial fitness under stress conditions (Guo et al., 2014).

Three years ago, the first type VII TA system was described (Marimon et al., 2016). The haemolysin expression modulation protein (Hha) is the toxin, and TomB is the antitoxin. Hha is a global transcriptional regulator that modulates cell physiology (Hong et al., 2010) by (i) forming the Hha-H-NS complex where it represses the pathogenicity locus of enterocyte effacement (LEE), (ii) repressing the transcription of rare codon tRNAs (bacterioletic effect) and fimbrial genes which reduces biofilm formation (García-Contreras et al., 2008), (iii) causing pleiotropic effects in catabolite repression (Balsalobre et al., 1999), inducing protease ClpXP, which activates some prophage lytic genes (García-Contreras et al., 2008), and (iv) inducing excision of prophages Cp4-57 and DLP-12 of E. coli (Wang et al., 2009). Remarkably, instead of forming a complex between the toxin and the antitoxin, toxin Hha is inactivated by oxidation mediated by the antitoxin TomB (Marimon et al., 2016).

The aim of this study was to provide insights into how bacteria inactivate toxins, for cases in which antitoxins do not function or are not present, such as after horizontal gene transfer. For this goal, we produced four different toxins of TA systems from E. coli (an RNase, a membrane-damaging peptidase, a DNase and a transcriptional regulator) from a common plasmid backbone with an isopropyl β-D-1-thiogalactopyranoside (IPTG) dependent promoter, and we determined that bacteria inactivate toxins primarily by mutating the promoter of the toxin or by mutating chromosomal copies of the iraM and mhpR genes.

Materials and methods

Bacterial strains and growth conditions

E. coli K12 BW25113 (Baba et al., 2006) with multicopy plasmids pCA24N (Kitagawa et al., 2005), pCA24N-mqsR (Wang et al., 2013), pCA24N-ghoT (Wang et al., 2012), pCA24N-raR (Guo et al., 2014) and pCA24N-hha (Marimon et al., 2016) was used for producing toxins, and BW25113 with plasmids pCA24N-mqsRA (Wang et al., 2013), pCA24N-ghoST (Wang et al., 2012), pCA24N-raRA (Guo et al., 2014) and pCA24N-hha-tomB (Marimon et al., 2016) was used for producing the toxin along with its antitoxin. Moreover, we used the Keio mutants lacI, iraM and mhpR (Baba et al., 2006) with the pCA24N-based plasmids to determine possible influence of these genes on the toxin activity; using these mutants allowed us to test the toxicity of the pCA24N-based plasmids in a strain with no other mutations. All cultures were grown in lysogeny broth (LB) (Sambrook et al. 1989) supplemented with 30 µg ml⁻¹ of chloramphenicol (to maintain the plasmids) at 37°C.

Toxin inactivation

The toxicity of each toxin was confirmed by growing each strain from an overnight culture on plates with and
without 1 mmol l⁻¹ IPTG. For inactivation, overnight cultures were used to inoculate fresh medium and grown until a turbidity at 600 nm of 0.05 to 0.1, then 1 mmol l⁻¹ IPTG was added. The turbidity was measured every 15 min until it reached 0.7 to 0.8. After 12 h, single colonies were isolated from each culture, regrown and the plasmid purified with an E.Z.N.A plasmid DNA Minikit Omega® for sequencing. To confirm that the toxins had been inactivated, the purified strains were grown in liquid cultures in the presence of 1 mmol l⁻¹ of IPTG.

Bioinformatic analysis

Sequences were analysed by Clustal Omega. The analysis of the 1000 genomes of E. coli was made using the Integrated Microbial Genomes and Microbiomes (IMG/M) web page and via a Blast search of each of the genes to determine their conservation and variability in the E. coli population.

Mutation analysis

Plasmids were sequenced (Quintara Biosciences) using reverse primer pCA24N-R (5'-GAACAATCCGAGTG-GAGTTCTGAGGTCATT-3'). Strains that lost toxin activity but did not show any mutations in the plasmid-based toxin gene or its promoter were sequenced by the Illumina HiSeq platform. Raw sequence data were trimmed by Sickle (https://github.com/najoshi/sickle), and the quality was checked by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The raw data were mapped to the reference genome by BWA (http://bio-bwa.sourceforge.net/). Mapping data were sorted, merged and deduplicated by Picard (https://broadinstitute.github.io/picard/). Realignment and unified-genotype data were performed by GATK (https://software.broadinstitute.org/gatk/). SnpEff (http://snpeff.sourceforge.net/) was used for genome annotation. REDTools (https://bedtools.readthedocs.io/en/latest/) and Samtools (http://samtools.sourceforge.net/) were used for calculating the depth of each base and for finding unique reads. All the strains were re-grown in the presence of 1 mmol l⁻¹ of IPTG after the mutation period, in order to confirm the stability of the mutations, by measuring the turbidity at 600 nm.

Results

Mutation analysis

To determine general insights into how toxins are inactivated, we studied four diverse toxins by choosing one from each of the type I (RalR), type II (MqsR), type V (GhoT) and type VII (Hha) systems. Initially, each toxin inhibited cell growth completely when induced with 1 mmol l⁻¹ IPTG, and each antitoxin was able to mask each toxin, restoring growth to that seen with the empty plasmid for antitoxins MqsA, RalA and TomB, whereas GhoS did not completely restore growth (Fig. 1).

For all four toxins, after 3 to 12 h, growth inhibition ceased (Fig. 1) which indicated a mutation occurred in the plasmid or in the chromosome that allowed the cells to resume growth. Also, purified colonies, obtained after the toxicity of each of the four toxins was inactivated, grew in the presence of IPTG (Fig. 2), indicating the mutations were stable.

Upon sequencing the plasmids of the strains with inactivated toxins, 10 plasmids (four encoding MqsR, four encoding GhoT and two encoding RalR), showed a partial deletion of the core 32 nucleotides in the P₅₆-lac promoter rather than changes in the structural portion of the gene (Figs S1–S4). Specifically, the core deletion included the −10 TATA box of the promoter. For 13 other strains with inactivated toxins (one for MqsR, one for GhoT, three for RalR and eight for Hha), no mutation in the promoter or toxin gene was found (Figs S1–S4); hence, a stable mutation occurred in the chromosome.

To investigate changes in the chromosome that led to growth in the presence of the four toxins in the absence of changes in the promoter and toxin genes, we selected five strains (one for MqsR, one for GhoT, two for RalR and one for Hha) and sequenced their chromosomes. We found several single nucleotide changes, including those in genes related to bacteriophage, RNA metabolism and other general metabolism. We focused on mutations related to regulators, specifically, those in lacI (lac operon repressor), iraM (anti-RssB factor, involved in RpoS stabilization during Mg starvation) and mphR (transcriptional regulator). Modifications in these three genes were observed in the chromosome of all five strains analysed. Using clean strains with single gene knockouts in either lacI, iraM or mphR, we electroporated plasmids with active GhoT and MqsR toxins and checked for the impact of the deletions on toxin activity. As shown in Fig. 3, the lacI and iraM mutations were unable to mask MqsR toxicity; however, GhoT was not toxic in both the iraM and mphR strains, and MqsR was inactive in the mphR strain. Hence, inactivating MhpR renders both GhoT and MqsR toxins inactive.

Bioinformatics analysis

Given the lack of mutation in the structural part of the toxin genes, we investigated the conservation of these toxins in E. coli by analysing 1000 E. coli genomes using the IMG/M internal database. This analysis revealed that
Figure 1  Inactivation of plasmid-based toxins. Plate results (left column): BW25113 producing toxins from pCA24N-based plasmids (pCA24N-mqsR, pCA24N-ghoT, pCA24N-ralR or pCA24N-hha) with (left) and without (right) 1 mmol l\(^{-1}\) IPTG to induce the toxin genes shows the initial toxicity of MqsR, GhoT, RalR and Hha. Five colonies of the original strain were streaked on each plate. Growth curves (right column): Growth of cells harbouring the empty plasmid pCA24N ("Empty", black circles and lines), producing both the toxin and antitoxin (blue diamonds and lines), and producing the toxin alone (red triangles and lines) with 1 mmol l\(^{-1}\) IPTG. [Colour figure can be viewed at wileyonlinelibrary.com]
Hha is the most conserved toxin among the four, as it is present in 98% of the population analysed (Fig. 4). Also, GhoT was conserved since it was present in the 91% of the genomes. This analysis also shows small variability in hha (1% with <99% identity) and small variability in ghoT (3% with <99% identity). Consistently, both genes were present with 100% of identity in the amino acid sequence in 98 to 99% of the population.

MqsR toxin was found in 29% of the 1000 genomes and was largely invariant when present (100% with 99 to 100% sequence identity). At the protein level, 88% of the population had 100% identity in the amino acid sequence, 7% of the population had changes in the protein sequence and 5% had an early stop codon (Fig. 4). RalR was the least conserved toxin since it was present in only 26% of the genomes; however, the sequence varied little when present (23% with <99% identity) (Fig. 4). For the RalR sequence, only 43% of the population had 100% amino acid identity, and 31% of strains had changes in the protein sequence (some with more than one amino acid change). Furthermore, we found that 26% of the genomes had an early stop codon in their sequence. Overall, our bioinformatic results show these four toxin genes are conserved in E. coli genomes with little changes in the gene sequence.

Discussion

In this study, we demonstrate that bacteria mutate rapidly (in the first 3 h of toxin production) to inactivate toxins of TAs and allow the cells to resume growth, when toxin production is not regulated. Hence our data indicate the importance of tight regulation for TA systems. The mutations that inactivate the toxins were found primarily in the −10 promoter region of the plasmids that carry the toxins or in lacI, iraM and mhpR. No mutations were found in the structural part of the toxin genes. These results are novel since previous work has shown that toxins are inactivated by mutations in the toxin gene itself (Silvaggi et al., 2005; Jahn et al., 2012; Masachis et al., 2018).

The lack of changes in the toxin structural genes for all four toxins is surprising since we have shown TA systems can evolve rapidly, transforming their genes into new toxins and antitoxins via a few mutations in their genes (Soo et al., 2014). For example a novel toxin was created from the ghoS antitoxin gene (with only two amino acid changes), and two novel antitoxins were created from the mqsA and from toxI antitoxin genes (Soo et al., 2014). Therefore, since small changes in the sequence of either a toxin or an antitoxin gene can radically alter the TA
system, perhaps this prevents mutations in the toxin structural gene. This result is corroborated by our bioinformatics analysis which shows the four toxin genes are both highly conserved and largely unvarying in the E. coli population. Moreover, since these TAs are widespread; they likely provide the evolutionary advantages of contributing to the formation of persister cells as well as protecting during stress (Trastoy et al., 2018).

The whole-genome sequencing revealed two chromosomal mutations that inactivate the toxins (Fig. 3). The first, iraM, encodes an anti-adaptor protein that has been related to the stabilization of RpoS during Mg starvation (Hemmi et al., 1998; Yang et al., 2004; Bougdour et al., 2008). IraM has also been linked to the PhoP/PhoQ two component system, which is necessary to activate IraM, and to H-NS that inhibits IraM expression (Battesti et al., 2012). The second mutation, mhpR, encodes a DNA-binding transcriptional activator and is located upstream of the lacI repressor (Zhang et al., 2006); MhpR is the regulator of the 3-hydroxyphenyl propionate catabolic pathway (Manso et al., 2011) and is activated by the cAMP-CRP complex in the absence of glucose and in the presence of 3-hydroxyphenyl propionate (Torres et al., 2003). How inactivating IraM and MhpR reduce toxin activity remains to be discerned.

We also observed some differences in toxin inactivation depending on the type of toxin. For example Hha has multiple functions in bacteria apart from toxicity (García-Contreras et al., 2008), and this might be the cause of the absence of promoter changes. Also, there were a higher proportion of changes in the chromosome during RalR inactivation compared to MqsR and GhoT. Bacteria with high stress increase their mutation rate; for example, the mutation rate of E. coli is higher with prolonged growth arrest (Loewe et al., 2003). For antibiotic-induced resistance mutations, some studies suggest stress induces the mutations (Bjedov et al., 2003), whereas others suggest the mutations are due to selection (Wrande et al., 2008). Our data indicate that the appearance of toxin-inactivating mutations are due to selection rather than random mutation since the same promoter deletions that inactivate the toxin were found repeatedly.

Figure 3 Inactivation of plasmid-based toxins by chromosomal mutations. Growth of the (a) lacI, (b) iraM and (c) mhpR strains containing the empty plasmid pCA24N (black), pCA24N-mqsR (blue) or pCA24N-ghoT (green) in the presence of 1 mmol l⁻¹ IPTG to induce the toxins. [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 4 Conservation of the toxins (a) MqsR, (b) GhoT, (c) RalR, and (d) Hha in *Escherichia coli*. Presence (%, first column), nucleotide identity (%, 2nd column) and amino acid identity (%, 3rd column) of each toxin in 1000 *E. coli* genomes. [Colour figure can be viewed at wileyonlinelibrary.com]
Overall, using four toxins from four different type of TA systems, we found toxins are rapidly inactivated primarily by changes in their promoters rather than changes in the structural genes. We also identified two proteins important for toxin activity, IraM for GhoT and MhpR for MqsR and GhoT; these two proteins may have potential as targets in the development of treatments to eliminate persister cells. Furthermore, for these results demonstrating rapid toxin inactivation, we utilized only one strain, E. coli, so it remains to be demonstrated that our results are general for other bacteria.

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Conflict of Interest
The authors have no conflict of interest to declare.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Plasmid sequences for inactivated MqsR.
Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the pr5-lac promoter, bold and underlined letters indicate the ribosome binding site, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5’ end contains the 6x His tag, itallic bold green highlight at the 5’ and 3’ ends indicates SfiI restriction sites and pink highlight indicates the start and stop codons.

**Figure S2.** Plasmid sequences for inactivated GhoT.
Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow
letters indicate the \textit{pt5-lac} promoter, bold and underlined letters indicate the ribosome binding site, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5’ end contains the 6x His tag, italic bold green highlight at the 5’ and 3’ ends indicates \textit{SfiI} restriction sites and pink highlight indicates the start and stop codons.

\textbf{Figure S3}. Plasmid sequences for inactivated RalR. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the \textit{pt5-lac} promoter, bold and underlined letters indicate the ribosome binding site, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5’ end contains the 6x His tag, italic bold green highlight at the 5’ and 3’ ends indicates \textit{SfiI} restriction sites and pink highlight indicates the start and stop codons.

\textbf{Figure S4}. Plasmid sequences for inactivated Hha. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the \textit{pt5-lac} promoter, bold and underlined letters indicate the ribosome-binding site, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5’ end contains the 6x His tag, italic bold green highlight at the 5’ and 3’ ends indicates \textit{SfiI} restriction sites and pink highlight indicates the start and stop codons.