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Toxin Inactivation in Toxin/Antitoxin Systems

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Running title: Promoter mutations inactivate toxins.

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Key Contributions: In this manuscript we determined that (i) toxins may be inactivated in 6 to 12 hours due to mutations, (ii) these mutations occur primarily in the promoters of the toxins and not in the structural genes, and (iii) chromosomal proteins IraM and MhpR are necessary for toxin activity.

A BSTRACT

Toxin/antitoxin (TA) systems are used primarily to inhibit phage, reduce metabolic activity during stress, and maintain genetic elements. Given the extreme toxicity of some of the toxins of these TA systems, we were curious how the cell silences toxins, if the antitoxin is inactivated or when toxins are obtained without antitoxins via horizontal gene transfer. Here we find that the RalR (type I), MqsR (type II), GhoT (type V), and Hha (type VII) toxins are inactivated primarily by a mutation that inactivates the toxin promoter or via the chromosomal mutations *iraM* and *mhpR*.

INTRODUCTION

Toxin-antitoxin (TA) systems have been related to gene regulation during the stress response [1], persister cell generation [2,3], bacteriophage protection [4], and other functions [5]. TA systems were originally found on plasmids [6], 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;* hence, TA systems are related to pathogenicity [7]. 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 [8].

TA systems are primarily two component systems, composed of a toxin, which disrupts important cellular mechanisms, and an antitoxin, which blocks the toxin action [5]. 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 [5]. In type IV systems (e.g., CbtA/CbeA), the antitoxin competes with the toxin for the target [9], and in type V systems (e.g., GhoT/GhoS), the antitoxin is an enzyme that cleaves specifically the toxin mRNA [10]. In type VI systems (e.g., SocB/SocA), the antitoxin protein facilitates toxin degradation as an adaptor protein [7]. 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 [11,12].

The broad distribution and myriad types of TA systems implies there is an evolutionary advantage for utilizing them. Nevertheless, TA systems require tight regulation, to prevent undesired reductions in metabolism. For example, deletion of the gene encoding antitoxin MqsA is lethal [13]. 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 post-transcriptional cleavage of mRNA [14,15], and (iii) some antitoxins inhibit toxins. For example, toxin MqsR autoregulates itself by cleaving its own mRNA [16].

This study focuses on four different toxins, MqsR, GhoT, RalR, 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 [17]. MqsR also regulates another TA system, GhoT/GhoS [18]. MqsA antitoxin participates in the global stress response by regulating RpoS and activating biofilm formation under oxidative stress [1]. As an example of this oxidative stress response, the MqsR/MqsA system manages growth during stress due to bile acids in the gastrointestinal tract [19]. MqsR is also the first toxin that when inactivated, reduces persister cell formation [20]. In regard to its regulation, toxin MqsR disrupts the MqsA-DNA complex to activate transcription [16].

The first type V TA system, GhoT/GhoS [10], is named based on the ghost-cell phenotype seen when toxin GhoT is produced, which results from membrane damage and reduces ATP [21]. The RalR/RalA system is a type I TA system found in the cryptic prophage rac [22]. RalR is the only known, non-specific DNase TA system toxin (many toxins are RNases); it activates the SOS response to DNA damage and increases resistance to the antibiotic fosfomycin. Hence, the RalR/RalA systems improve bacterial fitness under stress conditions [22].

Three years ago, the first type VII TA system was described [11]. The haemolysin expression modulation protein (Hha) is the toxin, and TomB is the antitoxin. Hha is a global transcriptional regulator that modulates cell physiology [23] 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 (bacteriolytic effect) and fimbrial genes which reduces biofilm formation [24], (iii) having a pleiotropic

effect in catabolite repression [25], inducing protease ClpXP, which activates some prophage lytic genes [24], and inducing excision of prophages Cp4-57 and DLP-12 of *E. coli* [26]. Remarkably, instead of forming a complex between the toxin and the antitoxin, toxin Hha is inactivated by oxidation mediated by the antitoxin TomB [11].

The aim of this study was to provide insights into how bacteria cope with TA toxin activity by identifying what mutations take place in bacteria to 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 each toxin (an RNase, membrane-damaging peptide, DNase, and transcriptional regulator) from a common plasmid backbone and 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.

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 mM 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 6 to 12 h, growth inhibition ceased (**Fig. 1**) which indicated a mutation 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, eight plasmids (two encoding MqsR, four encoding GhoT, and two encoding RalR), showed a partial deletion of a core 32 nucleotides in the P*t5-lac* promotor rather than changes in the structural portion of the gene (**Fig. S1-S4**). Specifically, the core deletion included the -10 TATA box of the promotor. For 10 other strains with inactivated toxins (one MqsR, one GhoT, three RalR, and five Hha), no mutation in the promoter and toxin gene were found (**Fig.**

S1-S4); hence, a stable mutation occurred in the chromosome. Note that along with the five mutants with chromosomal changes that inactivate Hha shown in **Fig. 2**, another three mutants were obtained with chromosomal changes that inactivate Hha, so in all, Hha was inactivated eight times by changes 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 bacteriophage genes, RNA general metabolism genes and other metabolism genes. We focused on mutations in *lacI* (lac operon repressor), *iraM* (anti-RssB factor, involved in RpoS stabilization during Mg starvation) and *mhpR* (transcriptional regulator) genes. Modifications in these three genes were observed in the chromosome of the five strains analyzed. As shown in **Fig. 3**, the *lacI* and *iraM* mutations were unable to mask MqsR toxicity; however, GhoT was not toxic in both of the *iraM* and *mhpR* strains, and MqsR was inactive in the *mphR* strain. Hence, inactivating MhpR renders both GhoT and MqsR toxins inactive.

Bioinformatic analysis. To determine the presence and conservation of these toxins in *E. coli*, we made a bioinformatic analysis of 1,000 *E. coli* genomes. This analysis revealed that Hha is the most conserved toxin among the four, being present in 98% of the population analyzed (**Fig. 4**). Also, GhoT was conserved since it was present in the 91% of the genomes. This analysis also shows 50% of the changes in *hha* (49% with a 99% of homology and 1% with less) and 33% of the changes in *ghoT* (30% of the strains with a 99% of homology and 3% of strains with less than 99%), compared to 50% and 67% of the population with 100% of homology in their sequences. However, both genes were present with 100% of identity in the amino acid sequence in 99% and 98% of strains, respectively, with only 1% of the population has changes in the amino acid sequence in both cases and another 1% of the population in which the modification of the nucleotide sequence was translated with an early stop in the protein in the case of GhoT.

MqsR toxin was found in 29% of the 1,000 genomes analyzed, of which 58% have 100% nucleotide sequence homology and a 99% homology in the other 42% of the population. However, these percentages

of homology only implied 88% of the population has 100% of homology in its amino acid sequence, and 7% of the population has changes in the protein sequence and 5% had an early stop codon (**Fig. 4**).

RalR was the less conserved toxin since it was present in only 21% of the genomes analyzed. Only 41% of the strains had 100% homology in the nucleotide sequence compared to 36% that had 99% identity and 5% showed less than 99% homology. 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 widely distributed and conserved in *E. coli* genomes. Hence, these data indicate clearly the importance of these TAs are to bacteria.

DISCUSSION

In this study, we demonstrate that bacteria mutate rapidly (in the first 12 hours 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 [27-29].

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 [30]. 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 [30]. 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.

The whole-genome sequencing revealed the two chromosomal mutations that we verified 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 [31-33]. IraM has also been linked to the PhoP/PhoQ a two component system,

which is necessary to activate IraM, and to H-NS that inhibits IraM activation [34]. The second mutation, *mhpR*, encodes a DNA-binding transcriptional activator and is located upstream of the *lacI* reperessor [35]; MhpR is the regulator of the 3-hydroxyphenyl propionate catabolic pathway-10 [36] and is activated by the cAMP-CRP complex in the absence of glucose and in the presence of 3-hydroxyphenyl propionate [37]. How inactivating IraM and MhpR reduce toxin activity remains to be discerned.

Bacteria with high stress increase their mutation rate; for example, the mutation rate of *E.coli* is higher with prolonged growth arrest [38]. For antibiotic-induced resistance mutations, some studies suggest stress induces the mutations [39] while others suggest the mutations are due to selection [40]. Our data indicate that the appearance of toxin-inactivating mutations are due to selection rather than random mutation since the same promoter deletions were found repeatedly that inactivate the toxin and select faster-growing cells.

Overall, by using four toxins from four different type of TA systems, we found toxins are rapidly inactivated by changes in their promoters rather than changes in the structural genes. We also identified two proteins important for toxin activity, IraM and MhpR, and our results suggest selection is important for mutations.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* K12 BW25113 [41] with plasmids pCA24N [42], pCA24N-mqsR [18], pCA24N-ghoT [43], pCA24N-ralR [22] and pCA24N-hha [11] was used for producing toxins and BW25113 with plasmids pCA24N-mqsRA [18], pCA24N-ghoST [43], pCA24N-ralRA [22], and pCA24N-hha-tomB [11] was used for producing the toxin along with its antitoxin. Moreover, we used Keio mutants for *lacI*, *iraM*, and *mhpR* genes (*27*) with the pCA24N-based plasmids. All cultures were grown in lysogeny broth (LB) [44] supplemented with 30 μg/mL of chloramphenicol (to maintain the plasmids) at 37°C with shaking.

Toxin inactivation. The toxicity of each toxin was confirmed by growing each strain from an overnight culture on plates with and without 1mM isopropyl β-D-1-thiogalactopyranoside (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 1mM IPTG was added. The turbidity was measured every 15 minutes until it reached 0.7 to 0.8. After 12 hours, 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 1mM of IPTG.

Bioinformatic analysis. Sequences were analyzed by Clustal Omega. The analysis of 1,000 genomes of *E. coli* were made using "Integrated microbial genomes and microbiomes" (IMG/M).

Mutation analysis. Plasmids were sequenced (Quintara Biosciences) using reverse primer pCA24N-R (5'-GAACAAATCCAGATGGAGTTCTGAGGTCATT-3'). Strains that lost toxin activity but did not show any mutations in the plasmid-based toxin gene or its promoter were sequenced by Illumina HiSeq platform. Raw sequence data were trimmed by Sickle (https://github.com/najoshi/sickle) and quality was checked by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The raw data were mapped to reference the 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 1mM of IPTG after the mutation period, in order to assay the stability of the mutations, by measuring the turbidity at 600 nm.

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FIGURE CAPTIONS

Fig. 1. **Inactivation of plasmid-based toxins.** Plate results: BW25113 producing toxins from pCA24N-based plasmids (pCA24N-mqsR, pCA24N-ghoT, pCA24N-ralR, or pCA24N-hha) with (left) and without (right) 1mM IPTG to induce the toxin genes indicate the initial toxicity for MqsR, GhoT, RalR, and Hha. Five colonies of the original strain were streaked on each plate. Growth curves: Growth of cells harboring the empty plasmid pCA24N ("Empty", black circles and lines), the toxin and antitoxin (blue diamonds and lines), and toxin alone (triangles and red lines) with 1mM IPTG.

Fig. 2. **Stability of the toxin inactivation mutations.** Growth of BW25113 containing pCA24N-based plasmids with mutated promoters or chromosomal changes that inactivate the toxin in the presence of 1 mM IPTG. Blue indicates plasmid mutations whereas red indicates chromosomal changes. Sequences of the plasmid toxin genes and promoter are shown in Fig. S1 (for *mqsR*), Fig. S2 (for *ghoT*), Fig. S3 (for *ralR*), and Fig. S4 (for *hha*).

Fig. 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 mM IPTG to induce the toxins.

Fig. 4. **Conservation of the toxins in *E. coli.*** Presence (%, first column), nucleotide identity (%, 2nd column) and amino acid identity (%, 3rd column) of each toxin in 1,000 *E. coli* genomes.

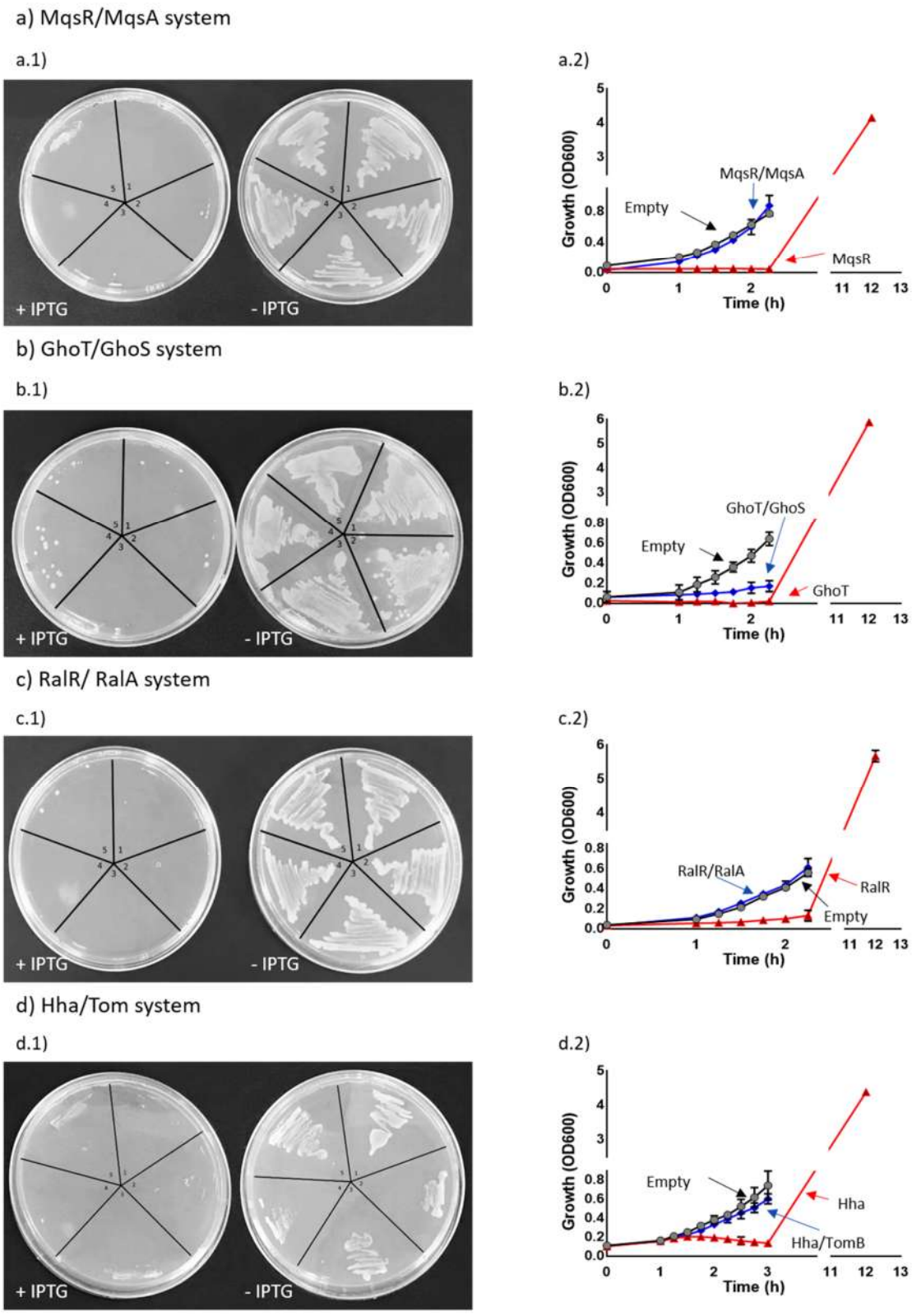

Figure 1

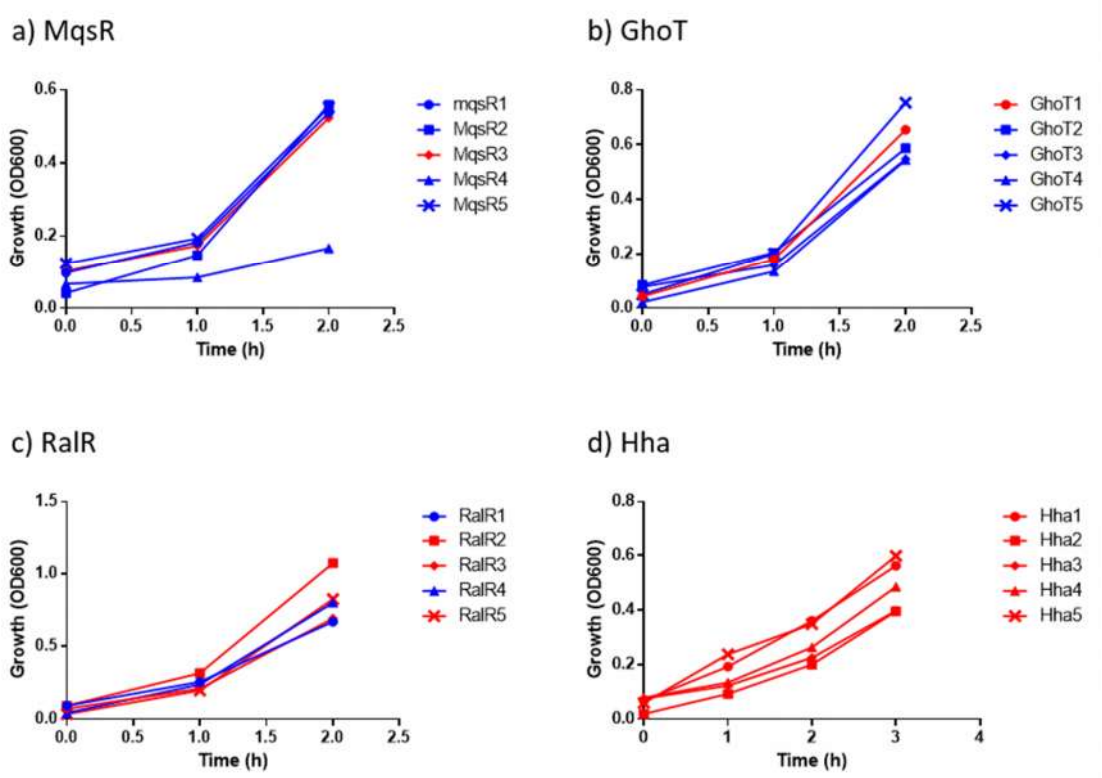

Figure 2

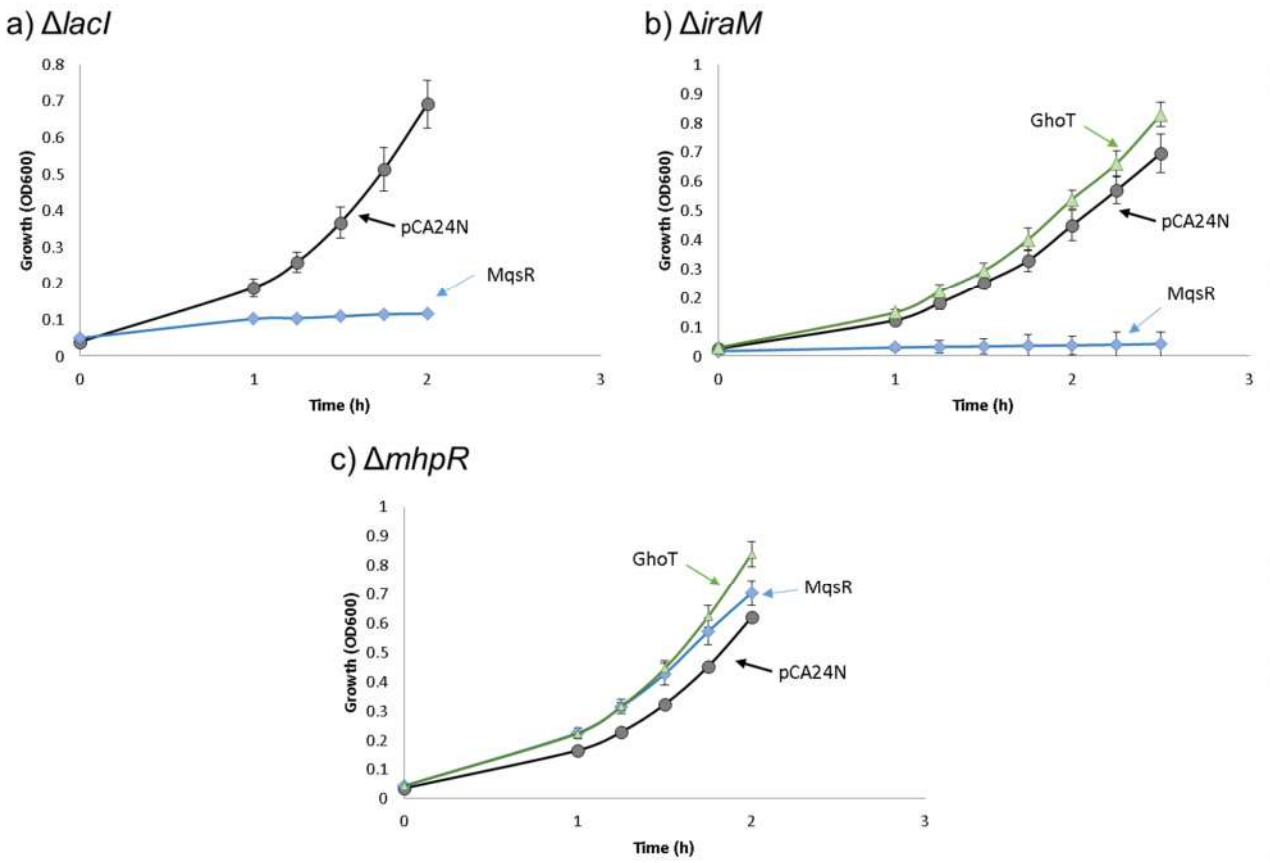

Figure 3

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Peer-reviewed version available at Journal of Applied Microbiology 2019; doi:10.1111/jam.14414

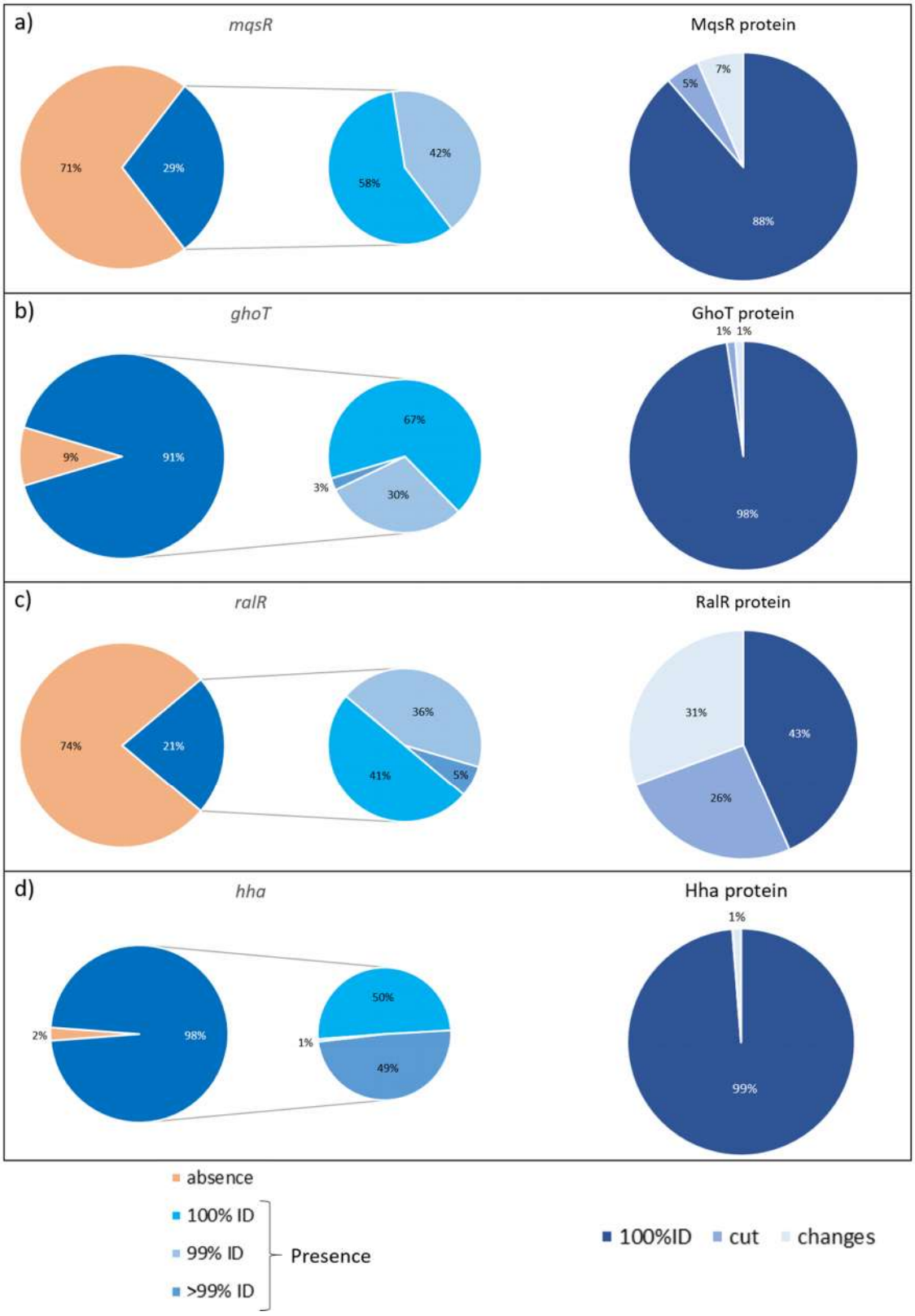

Figure 4

SUPPLEMENTARY INFORMATION

Toxin Inactivation in Toxin/Antitoxin Systems

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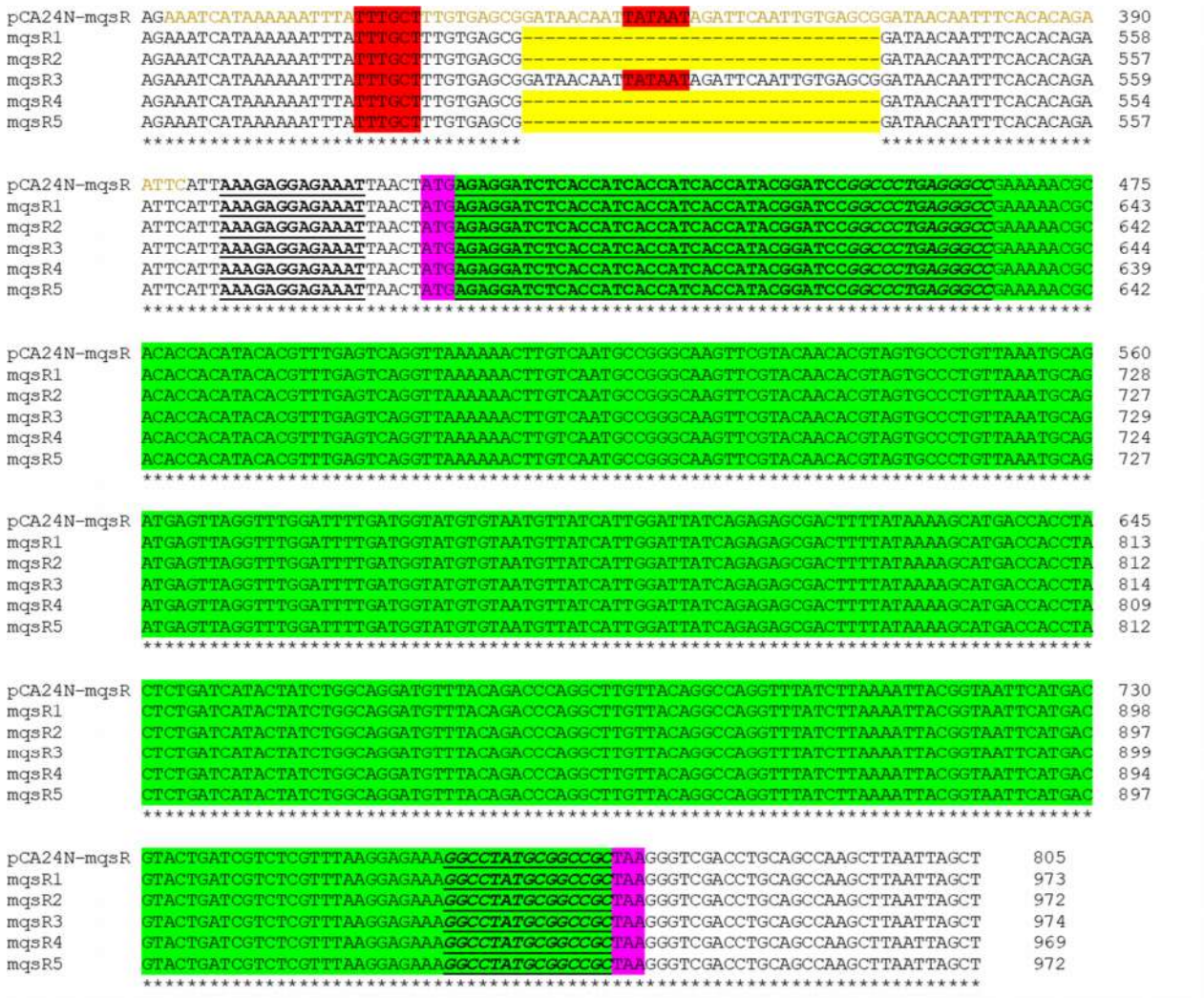

Fig. S1. Plasmid sequences for inactivated MqsR. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the p*t5-lac* promoter, bold and underlined letters indicate the RBS, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5' end contains the 6x His tag, italic bold green highlight at the 5' and 3'ends indicates *SfiI* restriction sites, and pink highlight indicates the start and stop codons.

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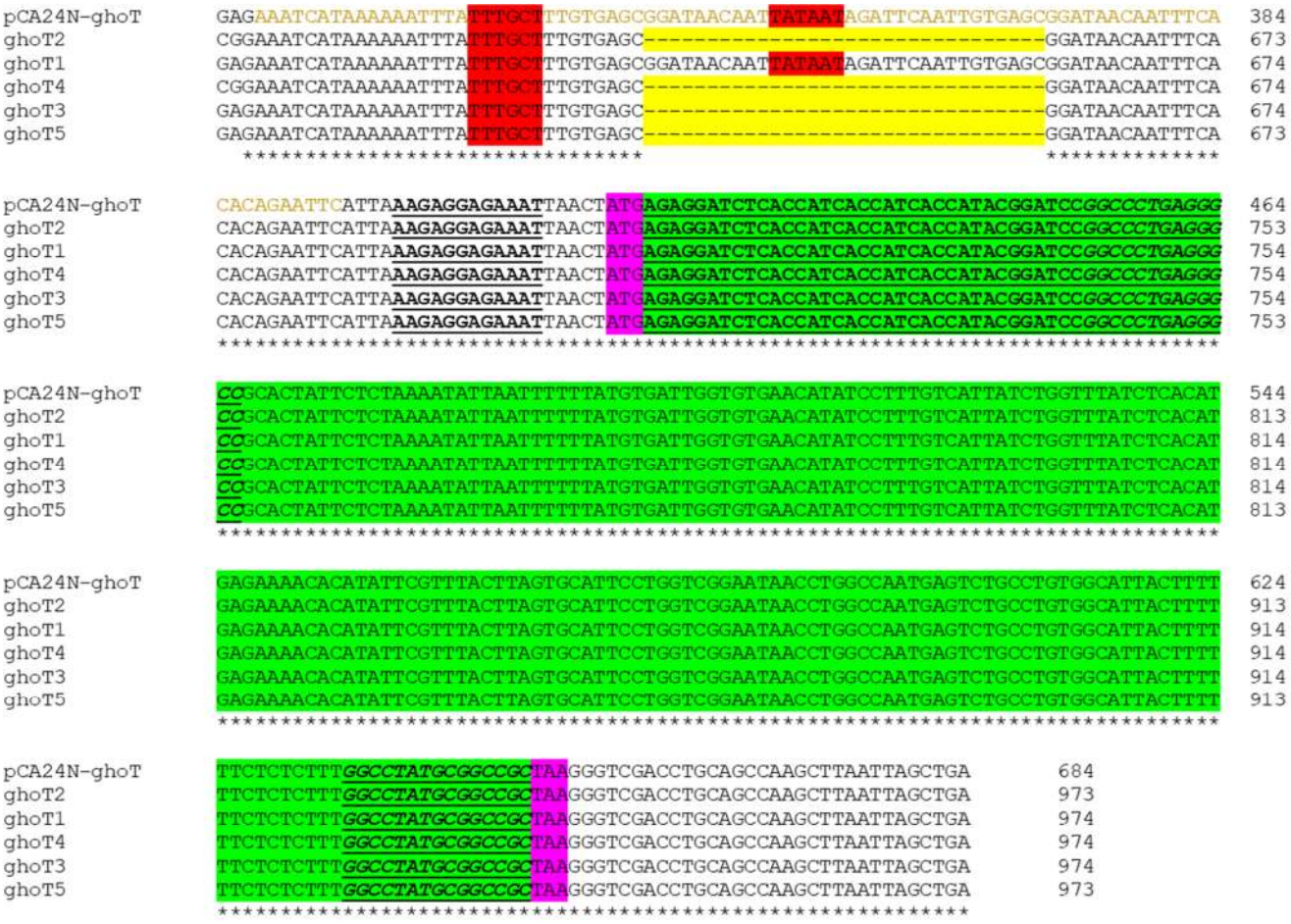

Fig. S2. Plasmid sequences for inactivated GhoT. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the p*t5-lac* promoter, bold and underlined letters indicate the RBS, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5' end contains the 6x His tag, italic bold green highlight at the 5' and 3'ends indicates *SfiI* restriction sites, and pink highlight indicates the start and stop codons.

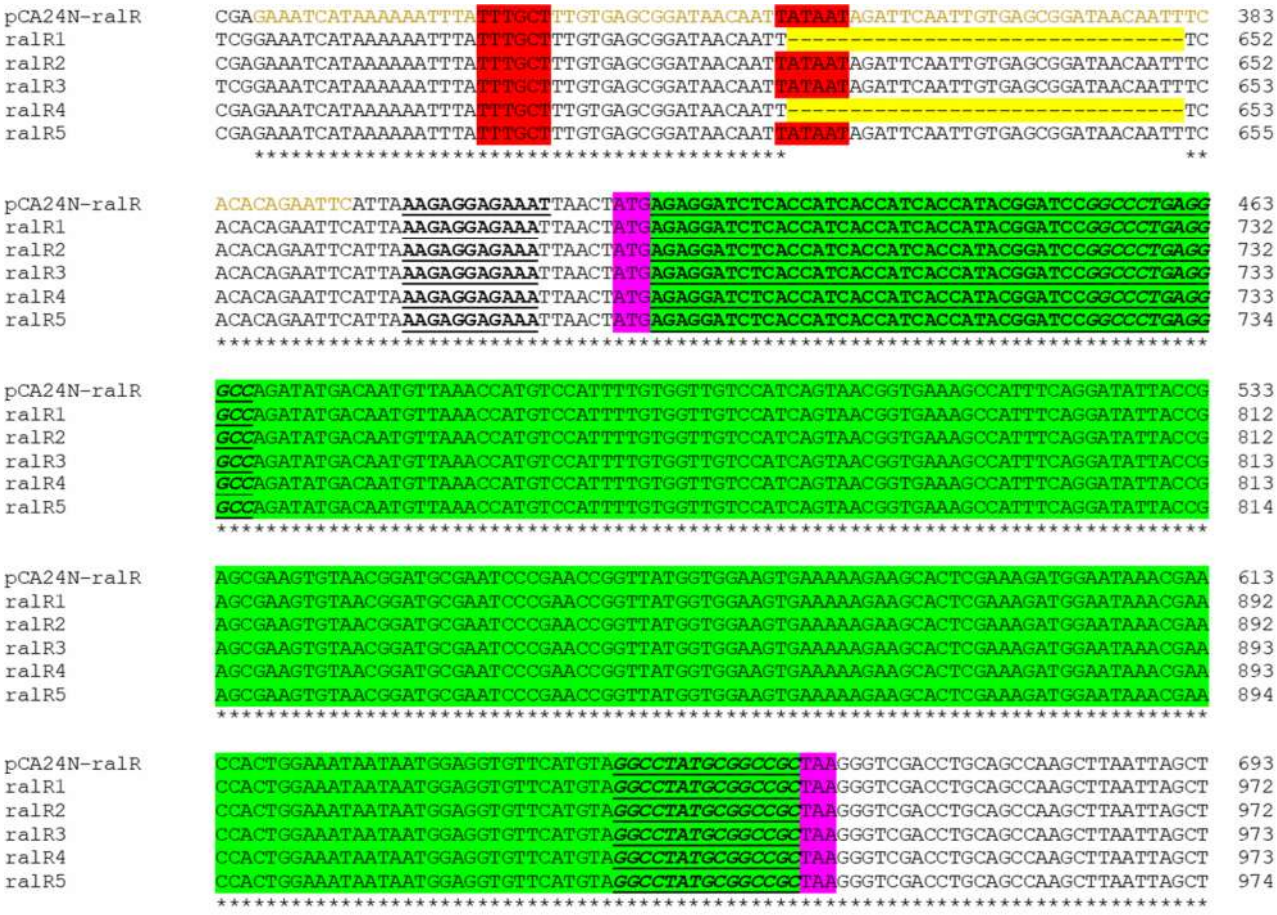

Fig. S3. Plasmid sequences for inactivated RalR. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the p*t5-lac* promoter, bold and underlined letters indicate the RBS, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5' end contains the 6x His tag, italic bold green highlight at the 5' and 3'ends indicates *SfiI* restriction sites, and pink highlight indicates the start and stop codons.

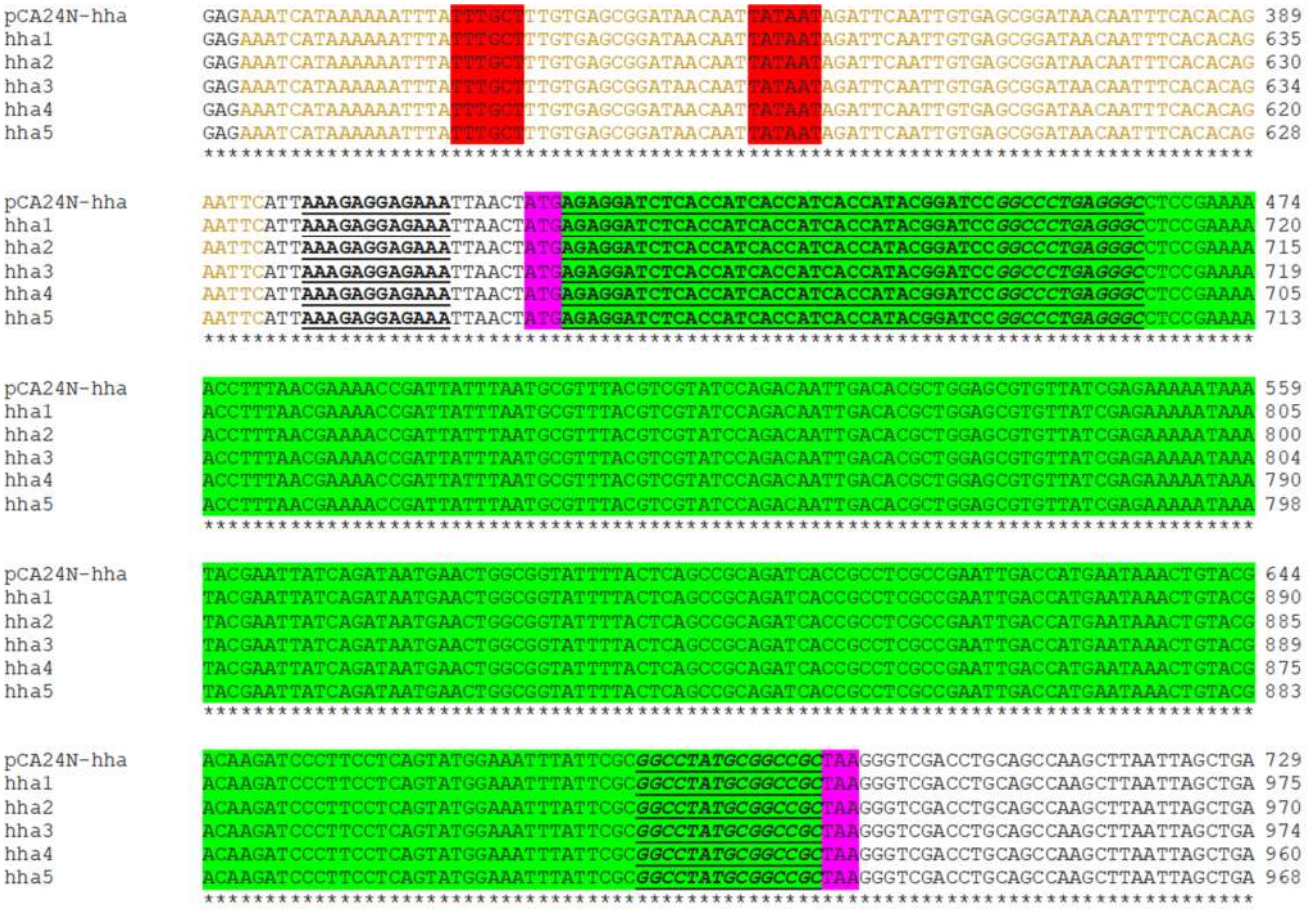

Fig. S4. Plasmid sequences for inactivated Hha. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the p*t5-lac* promoter, bold and underlined letters indicate the RBS, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5' end contains the 6x His tag, italic bold green highlight at the 5' and 3'ends indicates *SfiI* restriction sites, and pink highlight indicates the start and stop codons.