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A Small RNA is Functional in *Escherichia fergusonii* Despite Containing a Large Insertion.

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A small RNA is functional in *Escherichia fergusonii* despite containing a large insertion

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ABSTRACT

Bacterial small RNAs (sRNAs) are important regulators of gene expression; however, the impact of natural mutations on sRNA functions has not been studied extensively. Here we show that the sRNA MgrR contains a unique 53 bp insertion in *Escherichia fergusonii*, a close relative of *Escherichia coli* and *Salmonella enterica*. The insertion is a repetitive extragenic palindromic (REP) sequence that could block transcription, but full-length MgrR is produced in *E. fergusonii*, showing that the insertion has not affected sRNA production. Additionally, despite containing the large insertion, the sRNA appears to be functional because deletion of *mgrR* made *E. fergusonii* more susceptible to H_2O_2. The molecular details of MgrR's roles in H_2O_2defence are yet to be defined, but our results suggest that having an alternative function allowed the sRNA to be retained in *E. fergusonii* despite it sustaining a large, potentially disruptive mutation.

INTRODUCTION

Small RNAs (sRNAs) regulate gene expression in bacteria by modulating translation and mRNA stability [1]. In *Escherichia coli* and *Salmonella enterica*, expression of the sRNA MgrR is upregulated by the PhoP/PhoQ two-component system in response to low availability of Mg^{2+} [2, 3]. The sRNA in turn downregulates the production of the lipopolysaccharide-modifying enzyme EptB, and in concert with the small protein MgtS, boosts intracellular Mg^{2+} levels [2, 4, 5]. MgrR is conserved across enteric bacteria and is ~100 nt long [5], but in *E. fergusonii*, an understudied opportunistic pathogen [6, 7], the *mgrR* gene is interrupted by a 53 bp sequence. Large insertions typically disrupt the functions of protein-coding genes, but their impact on sRNA genes are unknown. In this study, we show that despite containing the insertion, full-length MgrR is produced in response to low Mg^{2+} availability, and the sRNA enhances *E. fergusonii*'s peroxide resistance. This function seems to be unique to *E. fergusonii*'s MgrR, indicating that it contributed to the retention of the sRNA despite it seemingly losing the ancestral function of *eptB* regulation.

METHODS

Bacterial strains and plasmids

Bacterial strains used in this study are listed in Table S2. All bacteria were either grown in Lysogeny Broth (LB) or in N-minimal medium containing 10 µM or 10 mM $MgCl_2$ [8]. Kanamycin (KAN) was used at a final concentration of 50 µg ml^{-1}, ampicillin (AMP) at 100 µg ml^{-1} and chloramphenicol (CAM) at 25 µg ml^{-1}. The wild-type (WT) strain used in this study was *E. fergusonii* ATCC 35469, which was obtained from American Type Culture Collection (ATCC) [6]. The RL7 (ΔmgrR::KANr) strain was constructed using primers listed in Table S3 and the λ Red recombination system [9]. Briefly, a PCR fragment obtained by amplifying the kanamycin resistance gene (KANr) from the pKD4 vector was recombined into the chromosome of wild-type *E. fergusonii* carrying the pKD78 vector that encodes the λ Red function. The transformed cells were selected on LB-KAN plates and deletion of *mgrR* gene was verified by PCR and DNA sequencing. KANr inserted in place of *mgrR* in the chromosome of RL7 was eliminated using thermal induction (43 °C) of FLP synthase present on pCP20 to generate the DEL strain.

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Abbreviations: COMP, *mgrR*-complementation strain; DEL, *mgrR*-deletion strain; OE, *mgrR*-overexpression strain; REP, repetitive extragenic palindromic sequence; sRNA, small RNA; WT, wild-type strain.

Three supplementary tables are available with the online version of this article.

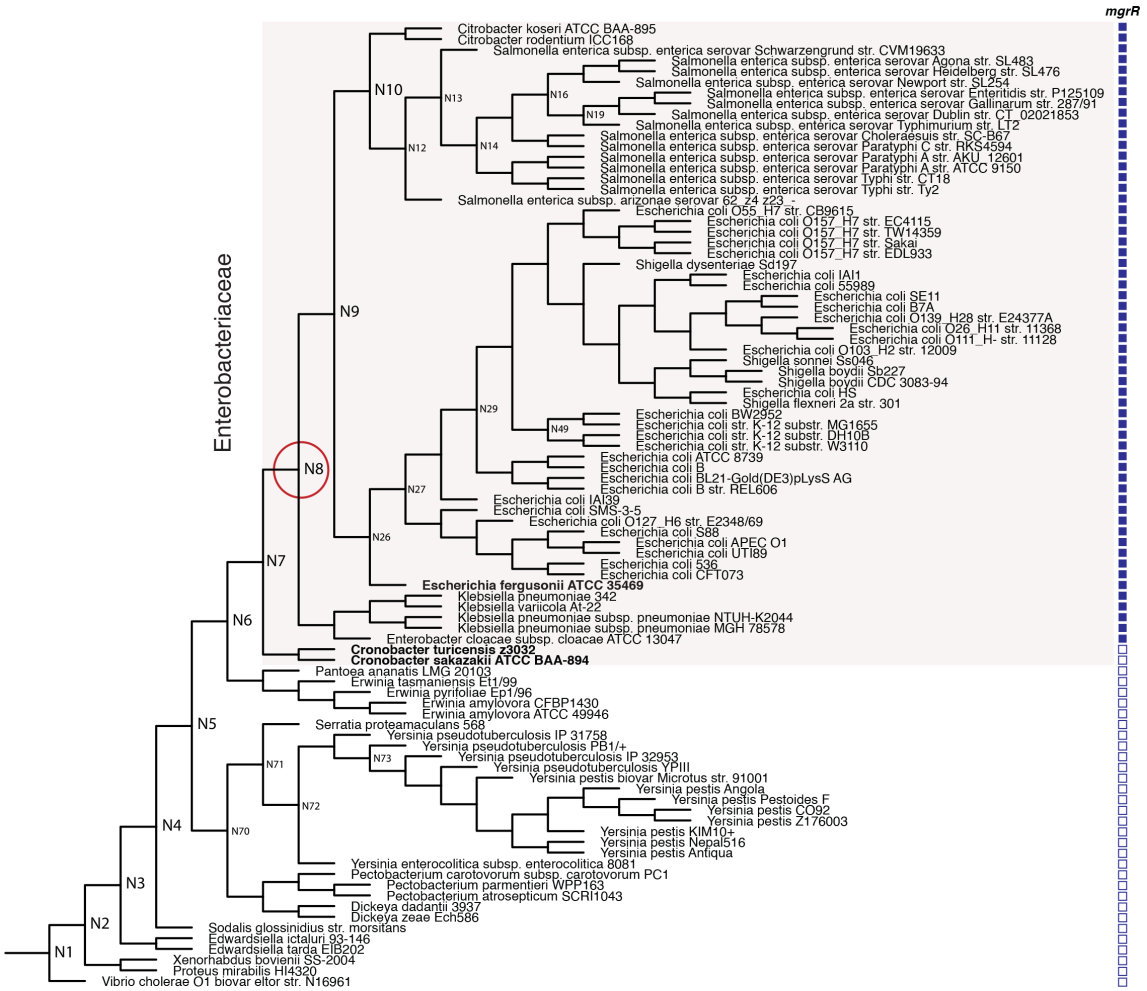

Fig. 1. MgrR arose in Enterobacteriaceae. An Enterobacterales phylogenetic tree downloaded from MicrobesOnLine is shown. The presence or absence of MgrR in each bacterium is depicted with filled or blank squares on the right. The predicted node of origin (N8) of MgrR is marked with a red circle.

A chromosomal-complementation strain (COMP) was constructed by introducing the *mgrR* gene along with its natural promoter within a defunct partial IS621 in the intergenic region between rhamnulose-1-phosphate aldolase (EFER_RS19330) and lactaldehyde reductase (EFER_RS19335) genes in DEL. *E. fergusonii*'s *mgrR* gene along with its promoter, 1000 bp of sequences from each side of the insertion site, and KANr (from pKD4) were amplified using PCR (see Table S3 for primer sequences). The PCR fragments along with linearized pKD78 (using BstXI) were assembled into a circular plasmid using an In-Fusion cloning kit (Clontech) and transformed into *E. coli* Stellar cells (Clontech) following the manufacturer's protocols. Transformed cells were plated on LB-CAM plates and incubated at 30 °C. Colonies that grew overnight were plated on LB-KAN at 30 °C overnight, and plasmids were isolated using GeneJet miniprep kit (Thermo Fisher Scientific). DEL cells were transformed with the newly assembled plasmid via electroporation (BioRad MicroPulser, 2.5 kV), allowed to recovered at 30 °C and were plated on LB-KAN and incubated overnight at 43 °C. Integration of *mgrR* gene along with its natural promoter was confirmed by DNA sequencing.

Measuring MgrR expression

N-minimal medium with 10 mM $MgCl_2$ was inoculated with WT *E. fergusonii* and incubated overnight at 37 °C. The overnight culture was diluted in N-minimal medium with either low (10 µM) or high (10 mM) Mg^{2+} concentrations and were grown to an OD_{600} of ~0.5 and RNA Stop Solution (5% phenol, 95% ethanol) was added. Total RNA was extracted using TRI reagent (Thermo Fisher Scientific), treated with DNase, and 500 µg of DNA-free RNA was used to generate cDNA using random hexamer primers. A SYBR Green master mix (Thermo Fisher Scientific) along with 2 µl of cDNA was used in qPCR reactions to measure *mgrR* expression under low and high Mg^{2+} conditions. As a control, expression of *pmrD*, a known PhoP-regulated gene, was also measured.

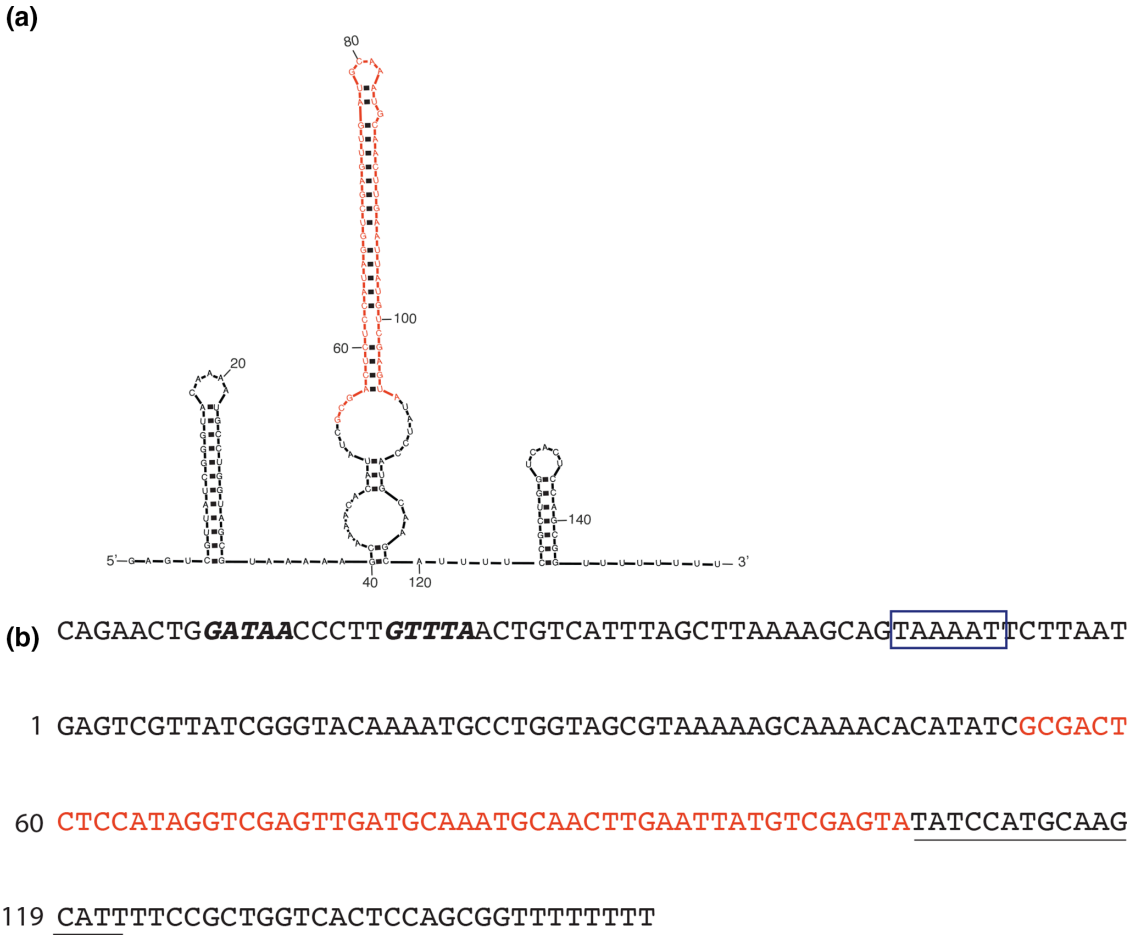

Fig. 2. MgrR contains a large insertion. (a) A predicted secondary structure of MgrR in *E. fergusonii*. The hairpin-like REP insertion is in red. (b) Genomic sequence around the *mgrR* gene in *E. fergusonii*. The sequences in bold italics are the predicted PhoP-binding sites, and a putative −10 sequence is boxed. The underlined nucleotides correspond to the *eptB*-binding site predicted in *E. coli*, and the REP sequence is in red [2].

Expression values were normalized using 16S rRNA gene expression.

Overexpression of MgrR was accomplished using the arabinose inducible promotor on the pBAD33 plasmid [10]. Overnight cultures of WT_{pBAD33}, $RL7_{pBAD33}$ and $RL7_{pBAD33\text{-}mgrR}$ (Table S2) were diluted 1:1000 in fresh LB-CAM containing 0.2% L-arabinose and grown to an OD_{600} of ~0.5. RNA stop solution (5% phenol, 95% ethanol) was added, cells were pelleted by centrifugation, total RNA was isolated and *mgrR* and *eptB* expression was measured using qRT-PCR, as described above.

Rapid amplification of 3′ cDNA ends (3′ RACE)

N-minimal medium with 10 mM Mg^{2+} was inoculated with WT *E. fergusonii* and grown overnight at 37 °C. The overnight culture was diluted in fresh N-minimal medium with 10 µM Mg^{+2} and grown to an OD_{600} of ~0.5, at which point rifampicin (300 µg ml^{-1}) was added and RNA was isolated using TRI reagent (Thermo Fisher Scientific). After DNase treatment, a poly-A tail was added to the 3′ ends of RNA using *E. coli* poly(A) polymerase, and cDNA was generated using the adapter primer provided in the kit (Table S3). MgrR cDNA was amplified using an *mgrR*-specific primer and the abridged universal amplification primer (Table S3). The PCR product was cloned into pGEM-T Easy Vector (Promega) and colonies were sequenced using M13 primers to determine the 3′ end of MgrR.

Hydrogen peroxide sensitivity assays

To perform a disc-based H_2O_2 sensitivity assay, 100 µl of overnight cultures of WT, DEL and COMP strains of *E. fergusonii*, and NM 22540 (wild-type) and KM129 (ΔmgrR::KANr) strains of *E. coli* were spread on LB plates that contained 25 ml of LB agarose. Then, 10 mm paper discs were placed in the centre of the LB plates, loaded with 10 µl of 10 M H_2O_2 and incubated at 37 °C overnight. The next day photographs were taken of each plate and the area of clearing was measured with ImageJ [11]. Statistical significance between the three *E. fergusonii* strains was measured using one-way ANOVA followed by Tukey's HSD test, and between the two *E. coli* strains using two-tailed, unpaired Student's *t*-test.

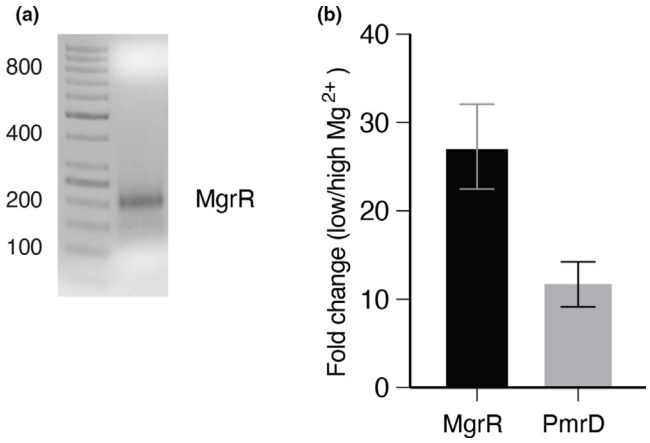

Fig. 3. Full-length MgrR is expressed in response to low magnesium availability. (a) A PCR product from the MgrR 3′ RACE assay is shown. The amplicon, which was sequenced to determine the 3′ end of MgrR, is larger (~200 bp) than MgrR (151 nt) due to the addition of a poly-A tail and an adapter sequence as part of the 3′ RACE assay. (b) Increase in expression of MgrR in *E. fergusonii* grown in low Mg^{2+} (10 µM) compared to high Mg^{2+} (10 mM). As a control, expression of *pmrD*, a known PhoP-regulated gene, was also measured under the same conditions. Fold-change values (mean+/-SD) were calculated from two independent growth experiments.

Evolutionary reconstruction

The Enterobacterales phylogenetic tree was downloaded from MicrobesOnLine. Prevalence of MgrR across Enterobacterales was determined using a covariance-modelling-based approach [12]. The presence/absence pattern for MgrR was input into Gain and Loss Mapping Engine (GLOOME) [13] to determine the sRNA's node of origin. The gain node was the most ancestral node with a posterior probability of ≥0.6, where all nodes leading from this ancestor to *E. coli* MG1655 had a posterior probability ≥0.6, as described previously [12].

RESULTS AND DISCUSSION

MgrR arose in Enterobacteriaceae

Using a covariance-modelling-based approach, we recently determined the prevalence of several sRNAs, including that of MgrR, across the order Enterobacterales [12]. An evolutionary reconstruction of MgrR origination based on this data indicated that the sRNA emerged in the family Enterobacteriaceae (Node 8 in Fig. 1). The only genus in this family without MgrR is *Cronobacter*, but it contains *mgtS*, a gene typically found next to *mgrR* [5]. Interestingly, the genome location of *mgtS* in *Cronobacter* is different from that in other Enterobacteriaceae members [5]. This divergent genetic locus of *mgtS* in *Cronobacter* was likely a result of genome rearrangement, a process that can disrupt sRNA genes [14]. Hence, it is possible that MgrR was present in the common ancestor of all Enterobacteriaceae members (Node 7 in Fig. 1) and was later lost in *Cronobacter* through genome rearrangements. Irrespective of the exact node-of-origin, this sRNA is present exclusively in the family Enterobacteriaceae.

MgrR in *E. fergusonii* contains an insertion

An analysis of *mgrR* genes across Enterobacteriaceae showed that the sRNA is ~100 nt in length in all taxa except in *E. fergusonii* (Table S1). On closer examination, we discovered that the 53 bp insertion within the *mgrR* gene in *E. fergusonii* is a repetitive extragenic palindromic (REP) sequence (Fig. 2). Typically, these hairpin-like elements are found in intergenic regions [15], and *E. fergusonii* contains several other copies of this element, suggesting that the REP sequence invaded the *mgrR* gene from another location in *E. fergusonii*'s genome.

E. fergusonii expresses full-length MgrR in response to low magnesium availability

In bacteria, REP-containing regions are transcribed [16, 17], but they form stem-loop-like structures that could prematurely terminate transcription [18]. To rule out this possibility, we performed a 3′ RACE assay, which confirmed that full-length MgrR was produced when *E. fergusonii* was grown in medium containing low (10 µM) Mg^{2+} concentration (Fig. 3). In addition, expression of MgrR was significantly higher in the low-magnesium condition in comparison to growth in medium with high (10 mM) Mg^{2+} concentration (Fig. 3). These data, along with the presence of a putative PhoP-binding site upstream of the *mgrR* gene (Fig. 2), indicate that expression of MgrR in *E. fergusonii* is regulated by the PhoP/PhoQ two-component system, as observed in *E. coli* [2].

MgrR may not regulate *eptB* in *E. fergusonii*

In *E. coli*, MgrR downregulates the expression of the *eptB* gene, which encodes a lipopolysaccharide-modifying enzyme [2]. To check whether MgrR has the same function in *E. fergusonii*, we measured *eptB* expression in wild-type (WT), *mgrR*-deletion (DEL) and *mgrR*-overexpression (OE) strains of *E. fergusonii* during exponential growth. Although OE produced ~12-fold more MgrR than WT, and MgrR was not detectable in DEL, the expression levels of *eptB* in the three *E. fergusonii* strains did not differ significantly from each other (P=0.235, One-way ANOVA) (Fig. 4). This result is different from what was observed in *E. coli*, where MgrR caused 2–3-fold downregulation of *eptB* expression in wild-type compared to an *mgrR*-deletion strain during exponential growth [2]. Although REP is inserted outside of the predicted *eptB*-binding site (Fig. 2), the presence of the hairpin-like element in close proximity might be interfering with MgrR-*eptB* interaction, thereby diminishing the sRNA's ability to control *eptB* expression in *E. fergusonii*.

MgrR improves *E. fergusonii*'s H_2O_2 resistance

Despite the apparent loss of *eptB* regulation, REP insertion does not seem to have rendered MgrR nonfunctional. An *mgrR*-deletion strain of *E. fergusonii* had significantly reduced resistance to H_2O_2 in comparison to wild-type and *mgrR*-complementation strains (Fig. 5), indicating that the sRNA has a role in *E. fergusonii*'s ability to withstand H_2O_2 stress. We could not test the contribution, if any, of REP towards this function because we were unable to create a version

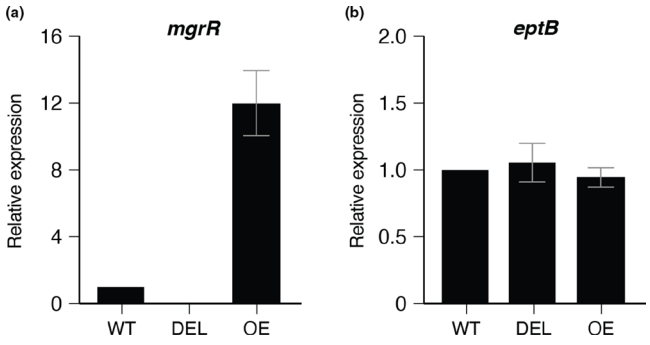

Fig. 4. Expression of *eptB* is not impacted by MgrR in *E. fergusonii*. Expression of (a) MgrR and (b) *eptB* was measured in wild-type (WT), *mgrR*-deletion (DEL) and *mgrR*-overexpression (OE) strains of *E. fergusonii*. Expression fold change values relative to WT (taken as 1) are shown. Fold-change (mean+/-SD) were calculated from two independent growth experiments. Expression of *eptB* did not differ significantly between the three strains (P=0.235, one-way ANOVA).

of MgrR without this hairpin-like element in *E. fergusonii*. However, it is unlikely that the double-stranded REP region has a direct role in MgrR function because binding of sRNAs to target mRNAs typically occur via single-stranded regions [19]. Alternatively, the highly structured nature of REP could improve MgrR's stability and prolong its survival into a different growth stage, thereby indirectly allowing the sRNA to acquire new targets [20].

MgrR's ability to enhance H_2O_2 resistance appears to be specific to *E. fergusonii* because deletion of *mgrR* did not alter *E. coli*'s H_2O_2 sensitivity (Fig. 5). Interestingly, wild-type *E. fergusonii* appear to be much more resistant to H_2O_2 than wild-type *E. coli*; however, the respective contributions of MgrR and peroxidases present in the two bacteria to this phenotype are currently unknown. Further studies are required to understand the molecular details of how MgrR improves *E. fergusonii's* H_2O_2 resistance, but if this function was gained prior to REP insertion, it could have contributed to the retention of the sRNA despite the apparent loss of *eptB* regulation. Alternatively, even after sustaining an insertion that affected *eptB* regulation, the sRNA gene's small size (151 bp out of the 4.6 million bp genome) could have buffered it against deletion, thereby providing MgrR enough time to gain new functions. Future studies should clarify whether other sRNAs have survived large perturbations, and whether having multiple functions and/or small size make sRNAs resilient to potentially disruptive mutations.

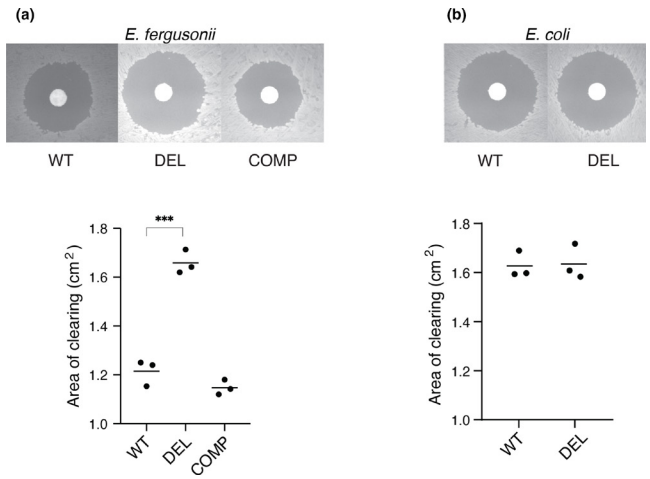

Fig. 5. MgrR improves *E. fergusonii's* H_2O_2 resistance. Bacterial growth in response to hydrogen peroxide exposure was measured using a disc-based assay. Representative assay images are shown in the top panels and horizontal bars in the bottom panel correspond to the mean of three biological replicates. Strains of *E. fergusonii* (a) and *E. coli* (b) used: WT, wild-type; DEL, *mgrR*-deletion; COMP, complementation. Statistical significance in (a) was calculated using one-way ANOVA followed by Tukey's HSD test (***P≤0.001), and in (b) using two-tailed, unpaired Student's *t*-test (P=0.865).

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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