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Deciphering regulatory mechanism influencing *qepA* efflux pump expression in *Escherichia coli*

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Abstract

QepA is a plasmid-mediated efflux pump found in some strains of *Escherichia coli*, in which it significantly elevates the resistance against quinolones. The protein has similarities with 14-TMS major facilitator superfamily transporters and is situated in the inner membrane of the bacteria. It was acquired by horizontal gene transfer and integrated into a now inactivated class 1 integron, also harbouring several other antibiotic resistance genes such as *rmtB* and *blaTEM-1*. QepA alone is not sufficient to raise the resistance level over the clinical breakpoint and is in clinical isolates therefore associated with other quinolone antibiotic resistance genes or quinolone target point mutations. The mechanisms regulating *qepA* expression are not yet understood. Therefore, in this study the *qepA* gene was amplified from an *E. coli* clinical isolate and, together with its upstream promotor sequence, was inserted into the *E. coli* chromosome. It was shown that *qepA* gene expression can be induced by exposure to 0.5-fold MIC concentrations of ciprofloxacin, trimethoprim and other DNA damaging antimicrobials. The deletion of a LexA binding site situated after a PcW promotor, which was predicted to drive *qepA* expression, did not alter this induction behaviour. Nested deletions of up to 200 nts downstream sequence of the PcW promotor, led to the identification of a sequence region required for expression induction. This study showed that *qepA* expression is induced by environmental factors leading to DNA damage and further identified a previously unknown DNA sequence required for expression regulation.

Keywords: *qepA*, gene regulation, quinolone resistance, class 1 integron, *lexA*

Popular Scientific Summary

Shiny *E. coli*: How glowing bacteria can help us to understand how antibiotic resistance is regulated

Antibiotics are the medication that revolutionised our health care. They are the most powerful tool humanity has at its disposal to fight the various bacterial infections lingering in the environment. However, this advance could be reversed. Resistance against those crucial medications is occurring rapidly in bacteria and is becoming an emerging problem in modern health care. While researches take years to develop safe and effective medications, bacteria can evolve at a much quicker pace and acquire protection mechanisms swiftly. But how can glowing bacteria help us win this race?

Fluorescent proteins are emitting coloured light and are used by researchers since ages to visualise different processes. In this study, a yellow fluorescent protein (YFP) was used to quantify the presence of an antibiotic resistance protein. A fusion of both proteins was created, and the amount of the resistance protein expressed correlated with the measured fluorescence of YFP. Different DNA damaging antibiotics in different concentrations were then added to the bacteria, which resulted in an increase in YFP fluorescence. This effect was dependent on the effect of those antibiotics, which were damaging the genetic material of the bacteria, and thereby inducing an SOS response. This SOS response, dependent on DNA damage, was shown to lead to the expression of the protein in question.

The protein that was analysed is QepA, a pump located in the membrane of *Escherichia coli* that removes antibiotics (more specifically quinolones) from the intracellular compartment. Ciprofloxacin, one of the most commonly prescribed antibiotics, is one of the targets of QepA. The QepA protein itself is apparently toxic to the cells that express it, and therefore it is important for the bacteria to only produce QepA when they need it for their survival. Due to this aspect, the regulation of this protein needs to be very finely tuned. In this case, a special binding site for the LexA protein was identified in the region of the DNA coding for QepA. This LexA protein is a repressor that silences transcription. However, when DNA damage is occurring inside of the cell LexA is cleaved, no longer acts as a repressor, and neighbouring genes are transcribed. To test this hypothesis, the relevant LexA binding site was deleted, but no change of expression behaviour of QepA could be shown. However, after deleting a longer sequence in this region, the induction of expression was abolished. Thereby, in this study, a newly found regulatory sequence was discovered. This segment could together with the LexA regulatory system, be responsible for the necessary fine tuning of the resistance mediated by the QepA protein.

It is imperative to understand how antibiotic resistance is orchestrated in order for researchers to find effective ways to circumvent those processes or develop new antimicrobials that are less prone to lead to resistance development. Further research in this area can be expected, and especially the inhibition of those antibiotic resistance activating pathways will be an interesting alternative target to prevent resistance.

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1 Introduction

The discovery of antibiotics revolutionised modern medicine, as doctors were handed a tool to effectively diminish bacterial populations. This enabled a new generation of treatments, where previously lethal infections could be cured in a matter of days without significant side effects. However, evolved resistance against those drugs is becoming an emerging problem in modern healthcare, as more and more antibiotics are becoming ineffective. Therefore, often unfavourable antibiotics with higher toxicity need to be used [1]. Transfer of genetic material containing gene cassettes coding for the respective antibiotic resistance can lead to a rapid spread of resistance between bacteria. This can be observed with extended-spectrum beta-lactamases (ESBL), which are becoming more and more common and are therefore threatening to render the entire class of beta-lactam antibiotics ineffective [23].

Furthermore, usage of antibiotics is creating a selective pressure favouring acquisition of resistance genes and mutations lowering the effectiveness of antibiotics. Due to the massive overuse of antibiotics, concentrations of several different molecules can be found in low concentrations in a variety of environments, where they significantly favour the evolution of highly resistant, low fitness cost bacterial strains [4]. One of those environments is the hospital, where a multitude of different antimicrobials are used, and they can be found for as contaminants for example the wastewater. Improper disposal and treatment of those residues favours the selection and evolution of resistance significantly [5].

To sustain the high standards in modern healthcare currently available, it is imperative for researchers to find ways to combat antibiotic resistance. This could be approached by preventive techniques but also by finding new antibiotic classes and compounds. As the development of new molecules is very time intensive, a detailed understanding of antibiotic resistance development and its regulation is necessary to maximise the effectiveness of current and future antibiotics.

1.1 Quinolones and Fluoroquinolones

Quinolones belong among the most commonly prescribed antibiotics worldwide [6] and are based on a central 4-quinolone structure. Their pharmacological activities are favourable, especially broad-spectrum activity, good bioavailability and low side effects. This class of antibiotics is especially used to treat urinary tract infections (UTI), bone- and joint infections, and other severe community- and hospital acquired infections [7].

The first discovered quinolone antibiotic was nalidixic acid in 1962 [8]. It showed effectiveness against Gram-negative enteric bacteria and was mainly used to treat uncomplicated UTIs [9]. In the 1970s- 1980s, the second generation of quinolones lead to a major breakthrough in coverage, as those molecules showed activity against a significantly broader spectrum of bacteria. This was achieved by the addition of a fluorine to the 4-quinolone structure at the C_6-position combined with a piperazinyl side chain at the C_7-position (Figure 1) [10]. Due to the addition of the fluorine, those compounds were then referred to as fluoroquinolones.

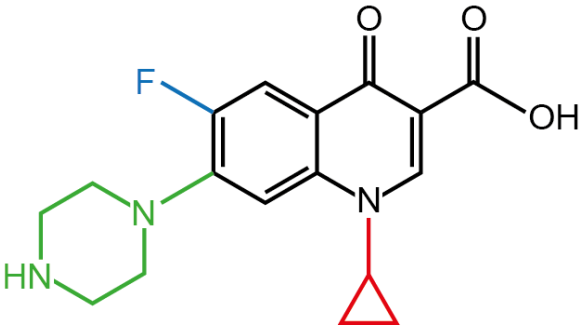

Figure 1: Structure of ciprofloxacin
Black: central 4-qionolone structure; Blue: fluorine at C_6-position; Green: piperazinyl at C_7-position; Red: cyclopropyl (affects potency)

The first used fluoroquinolones were ciprofloxacin (1983), ofloxacin (1982) and norfloxacin (1983), which showed activity against all gram-negatives as well as some gram-positive bacteria. More importantly, they were also able to inhibit *Mycobacterium tuberculosis* [11,12]. Ciprofloxacin is one of the most commonly prescribed and consumed antibiotics worldwide [6], as well as listed on the WHO "list of essential medicines", where it is recommended as treatment against acute invasive bacterial diarrhoea/dysentery, low-risk febrile neutropenia and pyelonephritis or prostatitis [13].

1.1.1 Mechanism of action

Quinolones target the type II topoisomerases of the bacterial cell, DNA gyrase and topoisomerase IV. Those enzymes are heterotetramers, DNA gyrase is formed by the $GyrA_2GyrB_2$ complex [14] and topoisomerase IV by the $ParC_2ParE_2$ complex [15]. In the bacterial cell, type II topoisomerases are responsible for altering DNA topology by introducing double strand breaks in the DNA, passing another DNA duplex through the break, and religating the cut DNA [16]. Quinolones are able to bind to those type II topoisomerases, where they disturb the balance between the cleavage and ligation processes. Hence, DNA breaks leading to chromosome fragmentation accumulate, showing that the drugs effectively convert DNA gyrase and topoisomerase IV to cellular toxins [17]. Thus, drugs inducing this effect are referred to as "topoisomerase poisons". The SOS response system will trigger DNA repair genes, which will be overwhelmed at high drug or chromosome fragmentation levels, ultimately leading to bacterial cell death. Additionally, quinolones can further inhibit bacterial growth due to binding and stabilising DNA-topoisomerase complexes, arresting progress of the replication fork [18] and sterically hindering transcription by RNA polymerase [19].

1.1.2 Resistance against quinolones

Resistance against quinolones is becoming a more and more relevant clinical issue, threatening the effective usage of this class of antimicrobials. There are a variety of pathways evolved in order to mediate resistance against quinolone antibiotics, and especially the accumulation of multiple mechanisms can lead to highly resistant strains. If the clinical breakpoint for resistance is exceeded, the antibiotic becomes ineffective for treatment. Ciprofloxacin has its clinical breakpoint to treat *Enterobacterales* at 0.5 mg/L [20].

1.1.2.1 Target-mediated resistance against quinolones

In many cases, quinolone resistance is associated with specific point mutations in the drug targets DNA gyrase and topoisomerase IV. These mutations lead to a diminished binding activity of the drug, and if several accumulate they can confer high levels of resistance [21]. Accumulation of those mutations happens stepwise, with MIC constantly increasing. The mutations occur in the *gyrA* and *parC* subunits of the enzyme complexes at the "quinolone-resistance determining regions" (QRDR) [22], have little to no fitness costs [23] and are very prevalent in clinical settings [24–26].

1.1.2.2 Plasmid-mediated resistance against quinolones

Several genes carried on plasmids were shown to mediate resistance against quinolones. *qnrA*, the first reported case of transferable quinolone resistance, was discovered 1998 in *Klebsiella pneumoniae* [27]. A multitude of *qnr* genes were subsequently discovered [28–30] and it was shown that their products elevate resistance against quinolones by binding to the type II topoisomerases, protecting them from the drug [31].

Another plasmid-borne protein increasing resistance against quinolones is aac(6′)-Ib-cr [32]. This fluoroquinolone-modifying enzyme is a variant of an aminoglycoside acetyltransferase and mediates resistance by acetylating the unsubstituted nitrogen in the piperazine ring at the C_7-position found in norfloxacin and ciprofloxacin. This modification decreases the drug activity, leading to an approximately fourfold increase in MIC [32]. Also, recently the ciprofloxacin-modifying enzyme CrpP was discovered, which was reported to decrease activity of the antibiotic by phosphorylation [33]. Lastly, efflux pumps mediating quinolone resistance were also found on plasmids [34,35].

1.1.2.3 Chromosomal encoded fluoroquinolone resistance

In the bacterial cell, the concentration of quinolone antibiotics is regulated by the balance between influx due to diffusion, as expulsion primarily due to the activity of efflux pumps. Especially in Gram-negatives, where the drug needs to pass the inner and outer membrane, influx is dependent on the presence of protein channels (porins). Therefore, mutations leading to a downregulation of those porins can confer a low level of resistance [36]. Also, upregulation of chromosomal encoded efflux systems increases resistance levels.

1.2 QepA as a plasmid-borne quinolone efflux pump

QepA is a plasmid-borne quinolone efflux pump first described in 2007 in Japan, where it was found on the plasmid pHPA in a clinical UTI *E. coli* isolate [34]. When this plasmid was then conjugated into a susceptible strain, ciprofloxacin MIC was increased 16-fold. Furthermore, isolating the gene and cloning it on another vector lead to a reported MIC increase of 32-fold [34]. Similar results were observed with other fluoroquinolones, such as norfloxacin and enrofloxacin.

1.2.1 *qepA* was acquired by horizontal gene transfer

Nucleotide analysis of the *qepA* gene revealed a high GC-content of 72% as well as a codon usage pattern unusual for *E. coli*. Therefore, the gene was most likely acquired on the plasmid by horizontal gene transfer. Initial analytics concerning the close relatives of this efflux pump claimed high similarities to the MFS-type efflux pumps in members of the order *Actinomycetales*, which led to the assumption, that the *qepA* gene originated in Gram-positive bacteria [34].

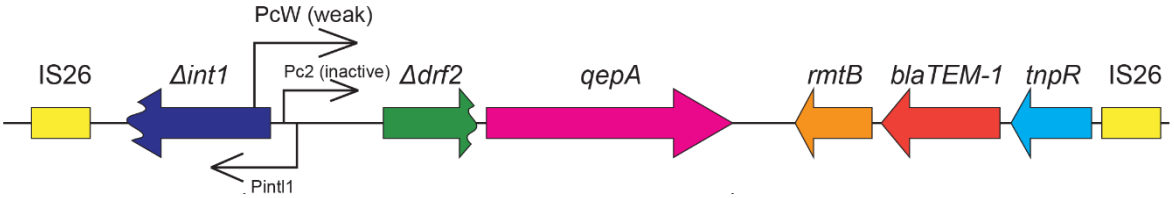

Figure 2: Scheme of the *qepA* pHPA plasmid region
The region of the pHPA plasmid harbouring the *qepA* gene is flanked by IS26 sequences. Upstream of the *qepA* gene is a fragment of *int1* and *drf2* gene, downstream are associated antibiotic resistance cassettes *rmtB* and *blaTEM-1*, as well as the resolvase *tnpR*. Promoters are annotated at the beginning of the integrase gene, corresponding to class 1 integron structures.

1.2.2 *qepA* associated features on the plasmid pHPA

Addressing the genetic context of the *qepA* gene, the region is flanked by IS26 sequences (Figure 2). These motifs are often associated with antibiotic resistance genes and have been shown to play an important role in the dissemination of resistance genes in Gram-negative bacteria [37]. Furthermore, an *int1* fragment from a class 1 integron is also present. This suggests that the *qepA* gene was inserted into a Class 1 integron, inactivating the integron in the process [34,35]. The promotor sequence from this integron is constructed in a Pc-P2 combination, with the P2 promotor being annotated as inactive due to insufficient spacer sequence length between the -10 and -35 box [38]. The PcW promotor is predicted to be responsible for QepA expression. Additionally, a LexA binding site is annotated between PcW and Pint1 promotors. This site is disrupted when the P2 promotor is in its active state (Supplementary Figure 2) [38].

Another gene present in the region which is associated with mobile genetic elements is the *tnpR* gene, which codes for a resolvase, able to catalyse site-specific recombination [39]. The *qepA* gene is further associated with multiple other resistance cassettes: *rmtB*, which is a 16S rRNA methylase, conferring high levels of aminoglycoside resistance [40]; *blaTEM-1*, a beta-lactamase, leading to resistance against β-lactam antibiotics [41]; as well as a fragment of *drf2*, which in its full length mediates resistance against trimethoprim [42].

1.3 SOS response

The so-called SOS response in *E. coli* can be induced by DNA damage as well as other environmental stresses [43]. In total, the SOS response upregulates approximately 50 unlinked genes. The function of those genes are numerous, ranging from cell division inhibitors [44] to DNA repair enzymes and low fidelity DNA polymerases [45]. The expression of those genes is either LexA-dependent or independent. The main purpose of the SOS response is to increase cell survival by inducing DNA repair, often at the

cost of acquiring mutations, whose potency is increased in different stages of the response. The first set of genes upregulated by SOS signals contains mostly enzymes for high fidelity repair [43]. Cell division inhibitor genes, such as *sulA*, cause an additional DNA-damage checkpoint during the cell cycle that gives the cell more time to repair their DNA before chromosomes are segregated [46]. Later in the SOS response, genes with high mutagenic potential, including DNA-damage-bypass polymerases are expressed in order to replicate the chromosome and mediate cell survival.

1.3.1 LexA-dependent SOS response

LexA is a major regulator in the SOS response. The protein forms dimer structures at their binding sites in DNA strands, one of which can be found in the upstream region of the *qepA* gene (Figure 3A). Thereby, transcription is inhibited in the "off-state" of the SOS response due to steric hindrance, and LexA is a negative regulator of SOS response. For LexA-dependent SOS response, inactive RecA protein interacts with DNA breaks (single stranded DNA) and is activated. This protein in turn activates LexA dimers bound to the LexA-binding site, acting as a co-protease by inducing self-cleavage of LexA (Figure 3B) [47].

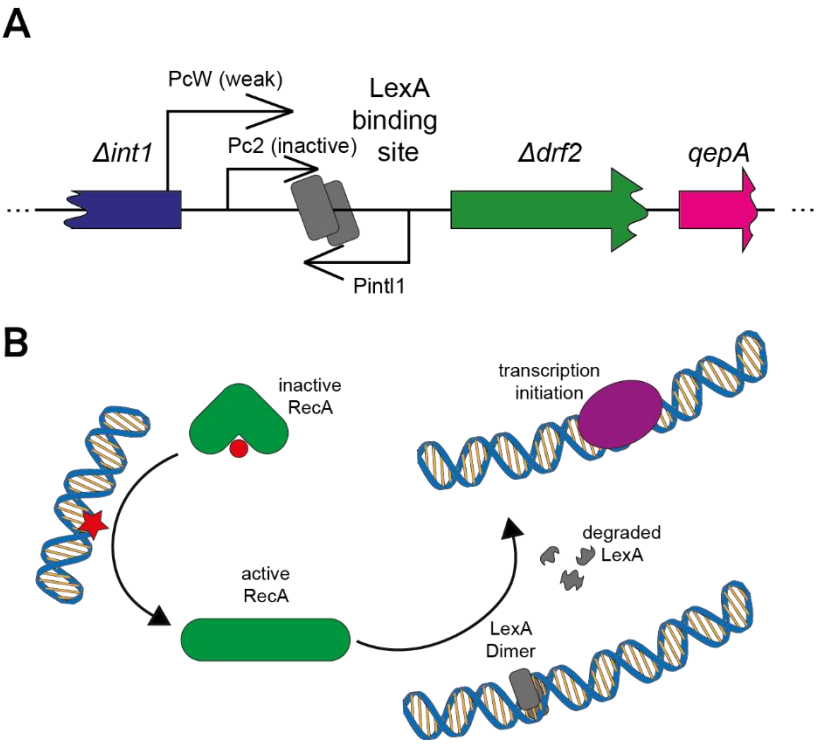

Figure 3: LexA dependent SOS response
(A) LexA binding site is characterised after the PcW promotor, which was predicted to drive *qepA* expression
(B) Induction of LexA SOS response is dependent on DNA damage, which activates RecA. RecA in turn stimulates self-cleavage of the DNA-bound LexA dimers, enabling transcription by RNA polymerase.

1.4 Aim of this study

QepA was shown to significantly increase the MIC against quinolones in susceptible bacteria [34]. However, the exact regulation this efflux pump is not deciphered yet. This study is aimed to unravel the gene regulation mechanism(s) influencing *qepA* expression, further addressing the potential for associated cytotoxic effects, environmental signals able to effect expression, as well as deciphering regulatory sequences in the upstream promotor region. To achieve this, the *qepA* gene was amplified from a clinical *E. coli* isolate. Translational YFP-fusions to truncated versions of the gene under the control of the native promotor sequence were constructed and their expression in response to different environmental stimuli were measured as a function of nested deletions of the upstream region.

2 Results

2.1 QepA has a high similarity towards efflux pumps in different Gram-negative species

In order to perform a comprehensive analysis of *qepA* and its regulation, the origin of the gene and its encoded protein, QepA, was assessed using extensive databank research as well as phylogenetic approaches. The predicted amino acid sequence of QepA shows similarities towards membrane transporter proteins of the major facilitator superfamily (MFS). The highest similarities are with the MFS from the proteobacteria *Pseudorhodoferax soli* (82%, WP_114470992.1), *Variovorax paradoxus* (82%, PZQ01053.1), *Variovorax boronicumulans* (80%, WP_145547738.1) and *Pseudomonas asiatica* (80%, WP_137187765.1). A more distant cluster of MFS transporters with around 65% identity is found in *Pseudoxanthomonas sp.* (WP_125111614.1), *Methyloversatilis discipulorum* (WP_088178717.1) and *Pigmentiphaga kullae* (WP_130357002.1). Phylogenetic analysis of these and other widespread efflux systems, chosen to represent different species, indicates that the *qepA* gene has most probably originated from Gram-negative bacteria (Figure 4).

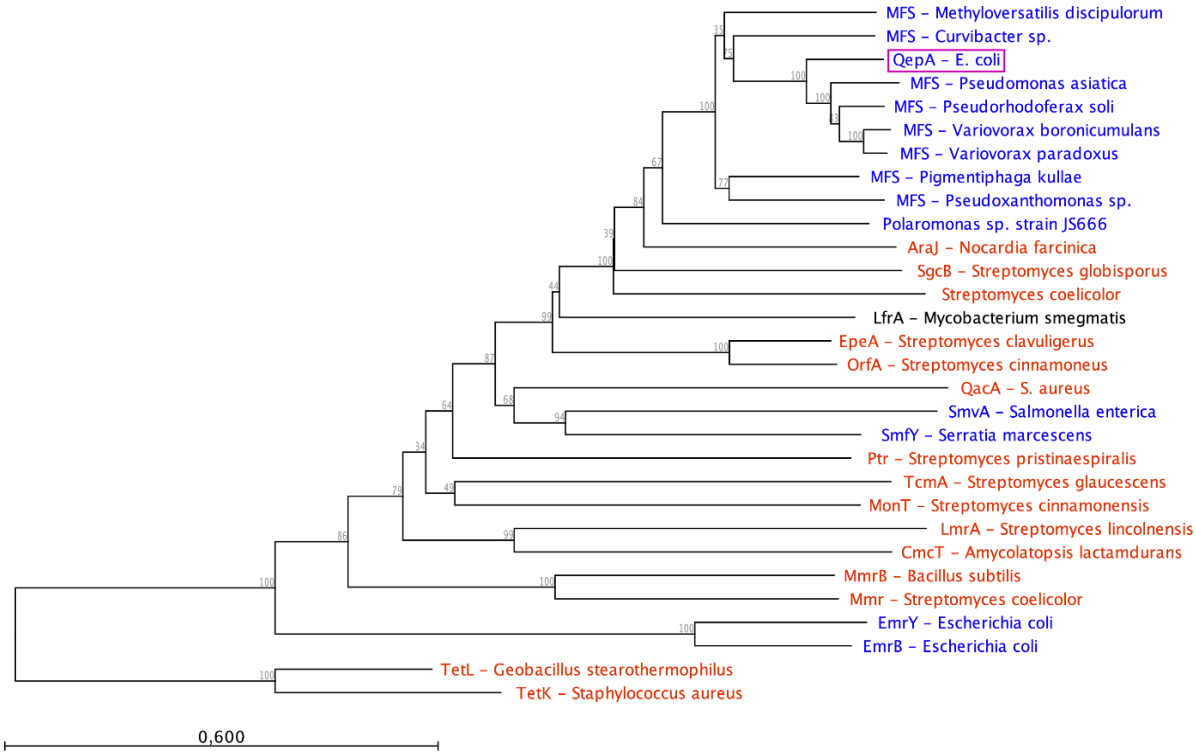

Figure 4: Phylogenetic tree of different bacterial efflux pump proteins
Amino acid sequences of different bacterial efflux pump proteins were aligned, and the phylogenetic tree was constructed using the neighbour joining method and Jukes-Cantor as protein distance measure. Gram-negative bacteria are labelled blue, Gram-positives red. QepA (boxed) is closely related to other, undescribed MFS proteins from Gram-negatives. The amino acid sequences used to construct this phylogenetic tree are referred to as: MFS from *Methyloversatilis discipulorum* (WP_088178717.1); MFS from *Curvibacter sp.* (HBH38318.1), QepA from *Escherichia coli* (AB263754); MFS from *Pseudomonas asiatica* (WP_137187765.1); MFS from *Pseudorhodoferax soli* (WP_114470992.1); MFS from *Variovorax boronicumulans* (WP_145547738.1); MFS from *Variovorax paradoxus* (PZQ01053.1); MFS from *Pigmentiphaga kullae* (WP_130357002.1); MFS from *Pseudoxanthomonas sp.* (WP_125111614.1); MFS from *Polaromonas sp.* (ABE43318); AraJ from *Nocardia farcinica* (BAD57397); SgcB from *Streptomyces globisporus* (AY048670); MFS from *Streptomyces coelicolor* (M18263); LfrA from *Mycobacterium smegmatis* (U40487); EpeA from *Streptomyces clavuligerus* (AJ302083); OrfA from *Streptomyces cinnamoneus* (U80063); QacA from *Staphylococcus aureus* (X56628); SmvA from *Salmonella enterica* (P37594); SmfY from *Serratia marcescens* (AB251607); Ptr from *Streptomyces pristinaespiralis* (X84072); TcmA from *Streptomyces glaucescens* (M80674); MonT from *Streptomyces cinnamonensis* (AAO65793); LmrA from *Streptomyces lincolnensis* (X59926); CmcT from *Amycolatopsis lactamdurans* (Q04733); MmrB from *Bacillus subtilis* (X66121); Mmr from *Streptomyces coelicolor* (M18263); EmrY from *Escherichia coli* (D78168); EmrB from *Escherichia coli* (AAC75733); TetL from *Geobacillus stearothermophilus* (P07561); and TetK from *Staphylococcus aureus* (M16217).

2.2 QepA protein has numerous variants with small amino acid substitutions

While analysing the *qepA* DNA sequence, it became apparent that there are a multitude of variants of this gene present in the NCBI databases. Taking *qepA1* as the standard, the resulting variant proteins are mostly only divergent in a few amino acids. The most distinct variants are encoded by *qepA3* (5 amino acid substitutions) and *qepA10* (deletion of 5 amino acids) (Figure 5). A common amino acid substitution is valine to isoleucine at position 134 (V134I), which is present in 5 out of the 10 variants. Most of the predicted variations of the amino acid sequence map inside the transmembrane segments (TMS), which

account for approximately 63% of the complete protein sequence. In the *qepA8* gene, the linker between the 11th and 12th TMS is extended by a lysine-glycine addition, while in *qepA5* the alanine at position 507 is substituted by threonine.

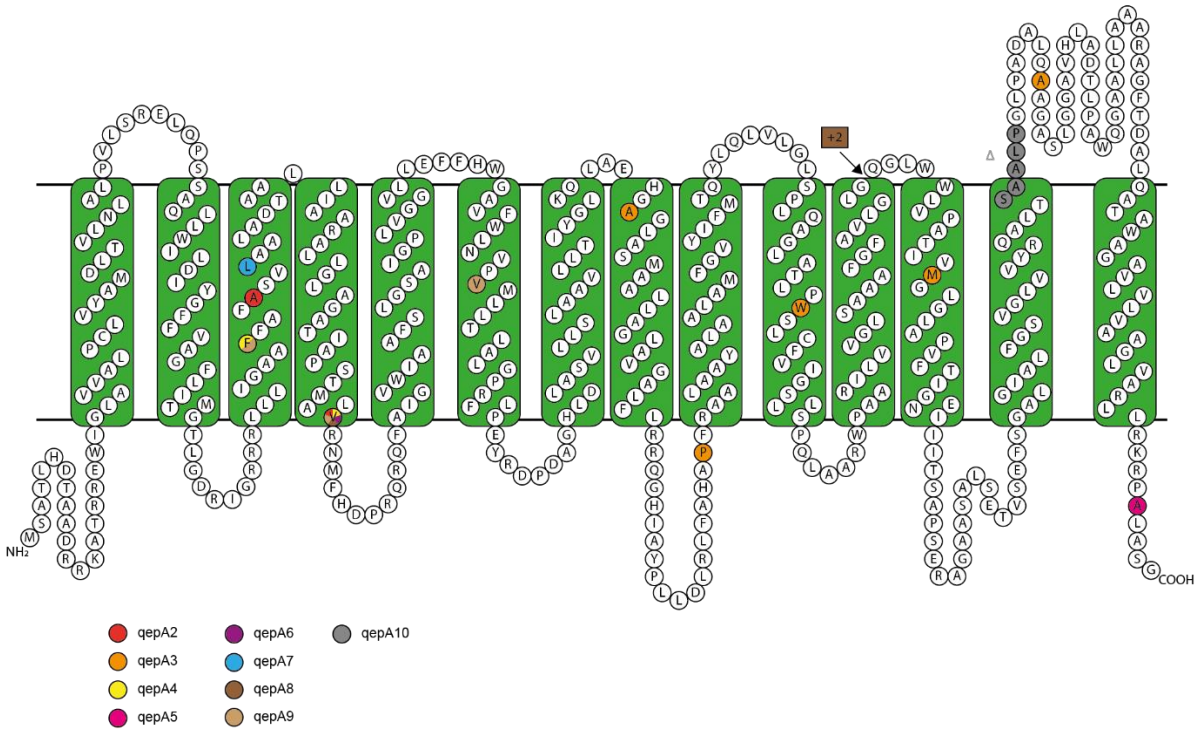

Figure 5: QepA protein variants
Differences in the amino acid sequence of QepA variants relative to the protein encoded by the *qepA1* gene are indicated in different colours marked on the predicted structure. The QepA efflux pump protein is build up from 14 transmembrane segments (TMS). In half of the QepA variants valine at position 134 is substituted to isoleucine, separating the variants into two different "lineages". QepA3 is the most diverse with 5 amino acid substitutions. QepA8 has an insertion of 2 amino acid at a TMS-linker, while QepA10 lacks 5 amino acids at the end of the 13th TMS. (Figure was adapted from Yamane *et al.*)

2.3 Integration of the *qepA* gene into *E. coli* chromosome leads to toxic effects

To analyse the expression and regulation of *qepA*, the coding sequence and the native promotor (enclosed within a ~550nt of upstream sequence) was prepared to be inserted into the *galK* region of an *E. coli* MG1655 strain by λ-red-recombineering. To achieve this goal, a *cat-sacB* gene cassette was first inserted into the region. This construct allowed for the selection of *qepA* recombinants by counterselection with sucrose, as the *sacB* gene mediates sucrose sensitivity. After performing a multitude of recombineering experiments aimed at integrating the *qepA* construct without finding positive clones, it became apparent that this construct might be failing because it introduces a significant fitness cost or toxicity into the recombineered bacteria.

2.3.1 *qepA* truncations are able to integrate into the *E. coli* chromosome

To circumvent the putative toxicity issue, truncation variants of *qepA* were constructed, which consist of the upstream regulatory region plus the first 60, 600 or 1200 bp of the coding sequence. Integration of sequences containing the 60 and 600 bp *qepA* fragments was successful and was confirmed by local DNA sequencing. In contrast, bacterial clones that harboured the 1200 bp fragment were obtained but, in every case, they had acquired point mutations leading to amino acid changes in the TMS region of the QepA protein sequence.

In order to analyse *qepA* gene expression and regulation, a YFP-*kan* cassette was introduced as a translational YFP-fusion following the 60 and 600 bp truncated *qepA* constructs. All further experiments were conducted using the *qepA* 600 bp truncation strain, as this construct resembled the natural regulatory circuit in the closest available manner.

2.4 Expression of *qepA* can be induced by ciprofloxacin

Due to the previously described toxicity effect or fitness costs of *qepA*, it was hypothesised that the efflux pump might only be beneficial when the corresponding substrate, ciprofloxacin, is present. Therefore, it is likely that ciprofloxacin has a direct effect on *qepA* expression. To test this hypothesis, bacteria harbouring the *qepA*(600 bp) YFP-fusion were grown in the presence of different concentrations of ciprofloxacin and the expression of YFP was measured in a cytometer. A wildtype bacteria strain was also measured under the different conditions, and its values afterwards subtracted from the experimental strains. After induction with approximately 6 µg/L ciprofloxacin, the YFP-signal was found to increase, reaching its peak at 8 µg/L (Figure 6A, blue dots). After this point, measurements became unreliable and highly variable (data not shown) likely due to the effects of increasing cell death from the effects of the high concentrations of antibiotic. To negate this effect a double *gyrA* mutation (S83L, D87N) was introduced in the experimental strain by phage-mediated transduction to further analyse whether induction is dependent on ciprofloxacin as a molecule or on the effects it has on the cell. This led to an abolishment of the induction (Figure 6A, red dots, Figure 6B), indicating that it is most likely secondary effects of the drug that are responsible for the increase in expression. This was confirmed by relativising ciprofloxacin concentration from µg/L to “fold of MIC”. Both strains show a similar induction behaviour when similar fractions of the corresponding MICs are present in the medium (Figure 6C).

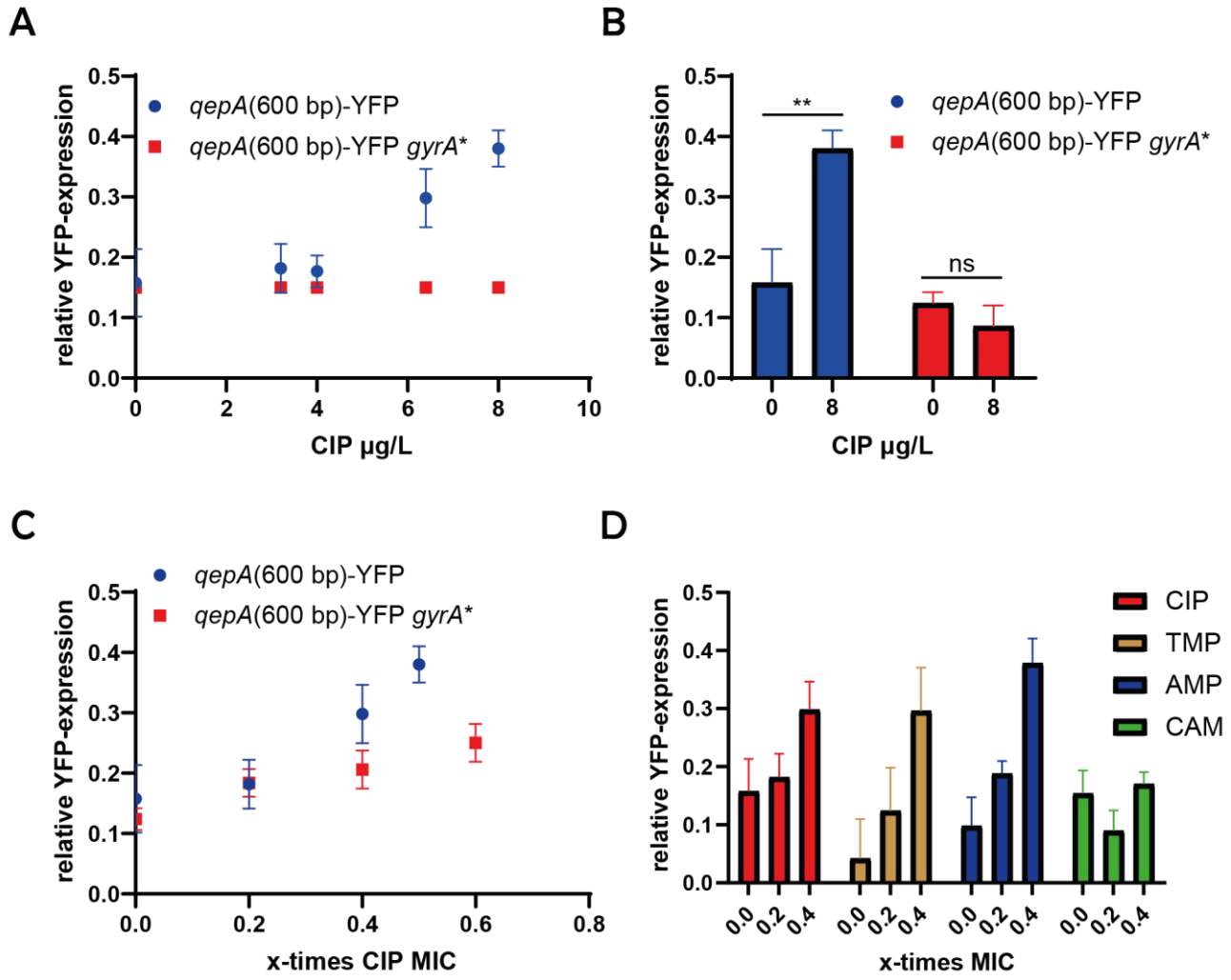

Figure 6: Induction of *qepA* expression
qepA expression was induced by different antimicrobials to evaluate the regulatory principles of the gene, analysed by YFP signal in a cytometer. Measurements were performed in quintuplicates. (A) Expression of QepA-YFP is induced by ciprofloxacin, starting to show a response at around 6 µg/L, while the same strain with a double gyrase mutation (S83L, D87N) is not responsive. (B) Induction of expression at 8 µg/L ciprofloxacin is successful for the *qepA*(600 bp)-YFP construct but is abolished when introducing gyrase mutations. Significance calculated with an unpaired t-test. ** = $p<0.01$; ns = not significant. (C) Induction results with ciprofloxacin plotted against x-times MIC, showing similar expression pattern in both strains. (D) Different antibiotics were tested for their potency to induce *qepA* expression. Ciprofloxacin (CIP), trimethoprim (TMP) and ampicillin (AMP) were able to induce expression, while there was no induction behaviour observed with chloramphenicol (CAM).

2.4.1 *qepA* expression can be induced by a multitude of different antimicrobials

After demonstrating that ciprofloxacin induces *qepA* expression, a variety of antimicrobial agents were assessed towards their induction potential for the *qepA*(600 bp) YFP-fusion, utilising the same assay. Due to ciprofloxacin inducing DNA damage it was hypothesised that induction is dependent on a LexA mediated SOS-response, as mentioned earlier.

To cover a wide variety of potential induction mechanics, antibiotics with different mechanisms and targets were assayed. Ciprofloxacin and trimethoprim are both DNA damaging antibiotics and were both shown to induce *qepA* expression, while chloramphenicol, inhibiting the 50S ribosomal subunit, was not able to elevate expression levels of YFP in induced cultures (Figure 6D). Ampicillin was also able to induce expression of *qepA*. Moving away from antibiotics, hydrogen peroxide treatment was also shown to lead to an upregulation of *qepA* (data not shown). Therefore, a more general induction behaviour towards different antimicrobials and environmental stimuli was observed.

2.5 Upstream deletions reveal regulatory region approximately 200 nt downstream of native promotor

Following the shown induction behaviour, deletions in the upstream region that includes several annotated promoter sequences (Figure 7A, B) were constructed in order to more accurately determine the regulatory region involved in antimicrobial-induced expression of *qepA*.

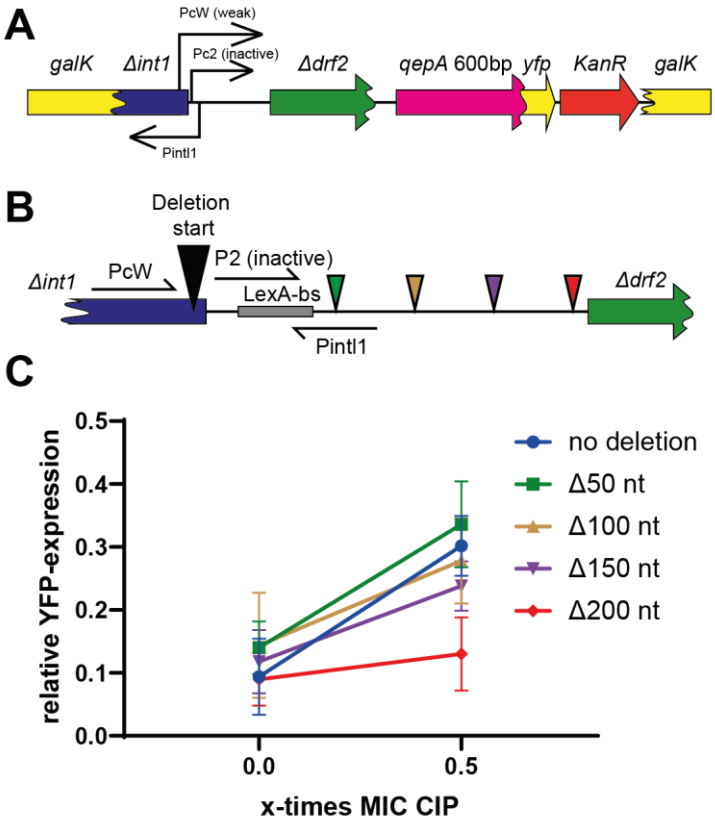

Figure 7: *qepA* upstream deletions
Deletions were introduced into the upstream region of *qepA* downstream of the PcW promotor sequence. (A) Circuit of the *qepA*(600)-YFP strain. (B) Experimental construct showing the different deletion steps by coloured boxes corresponding to colours used in Figure 7C. (LexA-bs: LexA binding site) (C) YFP-signal of deletions after induction with ciprofloxacin. Up to 150 nt deleted, a induction behaviour can still be observed, which is abolished after deleting 200 nt downstream of the PcW promotor.

Deletions were introduced into the *qepA*(600 bp)-YFP strain by λ-red-recombineering downstream to the annotated PcW promoter using synthetic DNA oligonucleotides to remove successive 50 nt sequences (Figure 4A).

The first deletion removes the previously described LexA binding site (Figure 7B, Supplementary Figure 2), which is responsible for regulating PcW transcription due to steric hindrance of LexA protein binding

to the DNA strand and is removed during DNA damage. This deletion showed no significant effect on the induction behaviour (Figure 7C, green line). The next two deletions, 100 and 150 nt, also did not alter the induction. However, when 200 nt were deleted, the antibiotic-dependent induction was abolished (Figure 7C, red line). Those results demonstrate that the induction of expression of *qepA* upon addition of ciprofloxacin is independent from the LexA binding site but is regulated by an element located at around 200 nt downstream from the PcW promoter.

3 Discussion

Transport of molecules across membranes is critical for cells to maintain a clear boundary towards the extracellular room. Passive transport will always occur from the higher concentrated area towards the lower concentrated area. To maintain cell viability, this concentration gradient needs to be maintained, which can be achieved by active efflux pump systems. Those efflux pumps are also vitally important for bacterial species to develop resistance against antibiotics. Recently, the efflux pump QepA was shown to facilitate resistance against quinolones [34], while the regulatory mechanisms influencing QepA expression remain mostly unknown. This study was aimed to decipher those mechanisms.

3.1 Origin of the QepA protein

When the first *qepA* gene was discovered in 2007, initial analysis suggested that *qepA* originated from Gram-positive *Actinomycetales* species. This result was most likely due to the limited dataset available, which explains why the here presented study found more close variants in other Gram-negatives (Figure 4). In the analysis, all genes used by Yamane *et al.* were included. The closest variants were found in bacteria from the soil [48,49]. It was previously reported that the soil bacteria harbour many different antibiotic resistance cassettes [50], and those results suggest the transfer of the *qepA* gene from a soil-dwelling Gram-negative bacterium into *E. coli*. It is possible that the *qepA* gene further spreads into other bacteria, leading to an elevation of quinolone resistance in multiple clinically relevant species. If the *qepA* gene will be associated with other transferable quinolone resistance cassettes, there is a risk of rapid and widespread development of highly resistant strains capable of exceeding the clinical breakpoint for quinolones.

3.2 Toxicity of QepA efflux pump and its variants

At the start of the study multiple recombineering attempts to integrate the *qepA* gene into the *E. coli* chromosome were unsuccessful. Due to the proven effectiveness of the method used, it was concluded that *qepA* has a significant fitness cost, and therefore recombination was not possible. Another factor influencing the recombineering effectivity is the usage of a *cat-sacB* gene cassette to select for integration. The *sacB* gene is mediating sucrose sensitivity, and deletion of this cassette is thereby selected. However, single mutations can inactivate the gene and thereby the background in the recombineering approaches is very high. This can be circumvented by additionally integrating a resistance marker.

After truncated variants of the *qepA* sequence were constructed, recombineering was successful in the 60 bp and 600 bp truncation. Multiple recombineering reactions of the 1200 bp fragment showed colonies where an amino acid substitution occurred in the TMS. The 3D structure of the QepA protein is predicted to form a kind of barrel (Supplementary Figure 1), which would be mostly intact in the 1200 bp truncation. Therefore, it is plausible that the 1200 bp truncation is still able to integrate into the bacterial cell membrane and form this barrel structure, leading to toxicity issues. Therefore, in the recombineering reactions mutations were selected that lead to a prevention of this structure. Taking this theory into account, addressing the different *qepA* variants is intriguing. If singe amino acid changes in the TMS regions can significantly lower the toxicity, potentially by inactivating the protein due to the inability to integrate properly into the membrane, many of the described variants could actually be inactive versions of the protein. This theory goes hand in hand with the observation that many of the variants have amino

acid substitutions inside the TMS sequences. Evolutionary, this could be beneficial in the context of inactivating a potential toxic gene, but still harbouring its associated, highly effective resistance cassettes. Contradicting this point, the *qepA2* gene variant was shown to confer a similar phenotype like QepA [51]. Therefore, a clear analysis of the different *qepA* variants is needed to assess their activity.

3.3 *qepA* gene expression is induced by DNA damage

As *qepA* gene expression is associated with a fitness cost / toxic effect, a tight regulation is necessary, where the gene should only be expressed when needed. Ciprofloxacin, trimethoprim and ampicillin were able to induce *qepA* gene expression, chloramphenicol was not (Figure 6D). The three antibiotics inducing expression were also shown to induce bacterial SOS stress response in *E. coli* [52–54], which does not apply to chloramphenicol [55]. Furthermore, ciprofloxacin, trimethoprim and ampicillin are all DNA damaging molecules, while chloramphenicol is suppressing protein biosynthesis. Those results show that *qepA* gene expression is most likely induced by DNA damage, as a part of the bacterial SOS stress response. Furthermore, hydrogen peroxide, which is also a potent inducer of DNA damage, was able to induce expression of *qepA*. Those results also shed new light on the observed toxicity issues to recombine the entire *qepA* gene into the *E. coli* chromosome. Utilising the λ-red-recombineering system, DNA breaks occur during the recombination and therefore the expression of *qepA* gene could be activated to a lethal amount.

As mentioned previously, behind the PcW promotor predicted to drive *qepA* gene expression, a LexA binding site was identified. It was shown, that LexA dependent SOS response is involved in the integron recombination processes by activating the Pint1 promotor [56]. Furthermore, derepression of Pint1 induced by mutating the LexA binding site led to a 5-fold increase of the gene regulated by PcW [57]. It is thereby likely, that *qepA* expression is also regulated by a LexA dependent SOS response. Contradicting this hypothesis, instead of leading to a constitutive high expression, a deletion of the LexA binding site did not alter the expression pattern of the *qepA* gene (Figure 7B). Therefore, eventually the PcW promotor is not responsible for *qepA* expression, but it is another unidentified promotor further downstream. In a study conducted by Cattoir *et. al.*, researchers claimed to have found a promotor further downstream regulating *qepA* expression. This cattoir promotor was not present in any of the *qepA* gene sequences accessible in different databases as well as our *qepA* isolate sequence and could only be found with an alteration in the -10 box. Additionally, this promotor (or promotor derivative) was not affected by the 200 nt deletion shown to diminish induction behaviour of *qepA* expression.

Further taking the mechanism of LexA dependent gene repression into context, namely that transcription is sterically hindered, another explanation of those results is that a different inhibitor binds at the downstream sequence inhibiting gene expression. However, this would most likely result in a higher expression even without antibiotic induction, and therefore an additional activator is more plausible. A potential region, possibly interacting with another repressor / activator protein or harbouring an unidentified promotor, was identified by successive deletions of the PcW downstream region at approximately -151-200 nt.

3.4 Outlook

While this study shed new light on the regulatory segments involved in *qepA* gene expression, various experiments can be conducted to further test the suggested hypothesises.

First, analysing the entire *qepA* gene integrated into the chromosome will be beneficial to prevent any artificial effects from using a truncated variant. This study used a truncation to prevent arising toxicity issues and mainly focus on the upstream regulatory region. Whole *qepA* gene integration could be achieved by using direct selection methods with coupled antibiotic resistance cassettes. To prevent the induction of the gene cassette in a LexA dependent SOS response during recombination, those constructs could further be inserted into a $LexA^{ind}$ strain. In this strain, SOS response cannot be induced by LexA due to the protein being unable to detach from its binding site. After integration, this mutation can be reversed by phage transduction.

Regarding the mechanism of induction, several, more targeted deletions of the upstream promotor sequence could be introduced. Firstly, a deletion of the PcW promotor would indicate, if this promotor is the driving force for *qepA* expression. Deletions removing only the LexA binding site and/or the 151-200 nt region would further show if one or both regions are essential for induction behaviour of *qepA*. Also, a $LexA^{ind}$ strain will be used to further analyse the effect of LexA dependent SOS response towards *qepA* gene expression.

In order to get an understanding of the different QepA variants, a detailed analysis of their associated fitness cost and benefit towards quinolone resistance would be compelling. Those results would be especially interesting in the light of QepA toxicity and how important QepA is towards the development of clinical resistant bacterial strains.

4 Conclusion

This study was dedicated to deciphering regulatory mechanisms involved in regulating the level of expression of the *qepA* gene. Truncated variants of the QepA protein were translationally fused to a YFP cassette and the expression was determined in a fluorescent readout utilising cell cytometry. SOS response towards DNA damage was shown to induce the expression of the *qepA* gene. Furthermore, by deleting a LexA binding site, it was demonstrated that the induction of *qepA* was not solely dependent on a LexA mediated SOS stress response. By successive deletions a regulatory element was discovered approximately 150-200 nt downstream of the PcW promotor. Thereby this study discovered a previously unknown regulatory element, influencing the expression of *qepA*, showing that the regulation of this gene is dependent on a novel, previously uncharacterised mechanism.

5 Material and Methods

5.1 Bacterial strains and growth conditions

The *E. coli* strain MG1655 was employed in this study and subsequently genetically modified for the specific experiments (Supplementary Table 1). Strains were cultured at 37°C 200 rpm shaking in Luria Broth (LB: 10g/L yeast extract, 5g/L tryptone, 10g/L NaCl). If harbouring the temperature sensitive plasmid pSIM5-tet, those strains were grown at 30°C 200 rpm shaking.

Luria agar (LA: LB + 15g/L agar) was used as a solid growth medium. When appropriate, colonies where selected by supplementing plates with antibiotics (chloramphenicol (Cam): 25 µg/mL; tetracycline (Tet): 15 µg/mL; kanamycin (Kan): 50 µg/mL). Sucrose counter-selection (to select loss of *sacB*) was made on LA plates containing sucrose (5%, in absence of NaCl).

5.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify linear DNA fragments for λ-red recombineering, as well as for verification of genetic modifications of the strains. Reactions, where the amplicon was further used in recombineering, were performed using Phusion High Fidelity Master Mix w/HF Buffer (New England Biolabs, Massachusetts, USA). Other reactions were performed using Taq-Polymerase PCR Master Mix (Thermo Scientific, Massachusetts, USA). Polymerase Master Mix was combined with sterile water, 100% DMSO, forward and reverse primers, and template DNA in a PCR tube (Table 1).

DNA template was prepared by picking the desired colony from an agar plate and dispersing it into 100 µL of sterile water. This was followed by 5 min microwaving at 1000 W and vortexing.

All used primers were ordered from Sigma-Aldrich and their sequences can be found in Supplementary Table 2.

PCR was performed using a S1000™ Thermal Cycler (Bio-Rad Laboratories, California, USA) with standard programmes respective to the used polymerase (Table 2, Table 3). Annealing temperature was adjusted per primer pair to be approximately 5°C lower than the melting temperature (Tm) or was evaluated using a temperature gradient. Elongation times were adjusted according to the amplicon size (1000 bp/min).

After the reaction, PCR products were evaluated on a 1% agarose gel.

Table 1: PCR Master Mix

Polymerase Master Mix (2x)	12.5 µL
Primer forward (10 mM)	1 µL
Primer reverse (10 mM)	1 µL
DNA template	1 µL
DMSO (100%)	2.5 µL
H_2O	7 µL
Total volume	25µL

Table 2: Phusion PCR Program

Temperature (°C)	Time (s)	
98	30	
98	30	X 35
X	30	
72	Y	
72	300	
4	∞	

X dependent on Primer Pair (Tm-5°C)
Y dependent on amplicon length (60 s per kb)

Table 3: Taq PCR Program

Temperature (°C)	Time (s)	
95	30	
95	30	X 35
X	30	
72	Y	
72	300	
4	∞	

X dependent on Primer Pair (Tm-5°C)
Y dependent on amplicon length (60 s per kb)

5.3 Gel electrophoresis

Agarose gel electrophoresis was performed to validate PCR products. The gel was prepared using a 1% agarose in TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA), supplemented with 1:10000 GelRed.

3 µL of PCR product were mixed with 3 µL of 2x TriTrack Loading dye (Thermo Scientific, Massachusetts, USA) and loaded on the Gel in parallel with the GeneRuler 1 kb DNA ladder (Thermo Scientific, Massachusetts, USA) serving as a marker. Electrophoresis was performed at 90 V for 30 min utilising the PowerPac™ (Bio-Rad Laboratories, California, USA). Gels were imaged using a UV Transilluminator (VWR GenoSmart).

5.4 PCR purification

PCR products were purified using two different commercially available kits: SureClean Plus (Bioline, UK), used for λ-red-recombineering amplicons, and QIAquick PCR Purification Kit (Qiagen, Germany), to clean amplicons for sequencing. Centrifuge 5415D (Eppendorf, Germany) was used for all centrifugation steps.

Using the SureClean Plus kit, 6 µL pink co-precipitant was added to the PCR sample and then vortexed. An equal volume of SureClean solution was added, the sample vortexed thoroughly and incubated at room temperature for 10 min. DNA was pelleted (10 min, room temperature, 14000 g), Supernatant was aspirated and 2x original sample volume of 70% ethanol was added followed by 10 s of vortexing. The sample was pelleted again (10 min, room temperature, 14000 g) and the supernatant removed. After approximately 5 min of air-drying the pellet was resuspended using 10 µL of sterile water. Purified DNA was stored at -20°C until used.

Following the manufactures instructions for the QIAquick PCR Purification Kit, 5x sample volume of Buffer PB was added and mixed. 10 µL of 3M sodium acetate (pH 5.0) was added to adjust the pH. Samples were applied to the QIAquick column and centrifuged (1 min, room temperature, 13000 g). Column was washed using 750 µL Buffer PE and centrifuged again (1 min, room temperature, 13000 g). An additional centrifugation step (1 min, room temperature, 13000 g) was conducted to ensure sufficient removal of washing buffers. Columns were placed into a clean 1.5 mL Eppendorf tube and DNA was eluted using 30 µL of sterile H_2O. DNA concentration was then measured using the NanoDrop1000 (Saveen Werner, Sweden).

5.5 λ-red recombineering

To create desired chromosomal mutant *E. coli* strains, λ-red recombineering was utilised. Linear DNA was transformed into recipient *E. coli* which harbour the pSIM5-tet plasmid. This plasmid encodes the recombination system derived from a defective λ prophage, compromised out of three proteins essential for the recombineering process: *gam*, *exo* and *beta*. Furthermore, the plasmid is temperature sensitive and expression of the target proteins can be induced at 43°C.

Recipient strains were grown overnight in 2 mL LB + Tet at 30°C 200 RPM shaking. 50 mL LB containing 15 µg/mL Tet and 2% Glucose (w/v) was inoculated with 500 µL overnight culture and grown in a baffled Erlenmeyer flask at 30°C 200 rpm shaking till an OD_{600nm} of 0.25-0.4, corresponding to exponential growth, was reached. The culture was then transferred to a 43°C 200 rpm shaking water bath for 15 min to induce expression of *gam*, *exo* and *beta*, followed by 15 min incubation on ice.

The culture was then harvested by centrifugation (4500 rpm, 4°C, 8 min) and the supernatant discarded. The cell pellet was washed 3 times with 20 mL ice-cold sterile H_2O (4500 rpm, 4°C, 8 min) to remove salt residues and then resuspended in 1 mL of ice-cold sterile H_2O for the final washing step (13000 rpm, 4°C, 3 min). The cell pellet was then resuspended in an appropriate volume of ice-cold sterile H_2O, depending on the number of samples (50-200 µL). 50 µL of cell suspension was mixed with linear DNA fragments and incubated for 5 min on ice. Transformation was achieved by electroporation using the BioRad Gene Pulser Xcell at 1800 V and 200 Ω. Transformed cells were immediately transferred into 1 mL prewarmed LB (when *cat-sacB* counterselection was performed in 10 mL salt-free LB) and recovered overnight at 37°C. Recovered cells were than plated on the appropriate selective medium and further validated by PCR.

When *cat-sacB* counterselection was performed, clones were additionally patched on sucrose and chloramphenicol plates to select for colonies with complete deletion of the *cat-sacB* cassette, indicated by chloramphenicol sensitivity.

5.6 Sequencing

All constructed strains were validated by local sequencing performed by Macrogen Europe Laboratory (Amsterdam. Netherlands). Purified PCR product as well as custom sequencing primers were sent to the facility, and resulting sequences were analysed using CLC Main Workbench 8.

Whole Genome Sequencing was performed in an Illumina MiSeq™ (Illumina Inc., Madison, Wisconsin)

5.7 Plasmid Isolation

To acquire plasmid DNA for transformation, the QIAprep Spin Miniprep Kit (Qiagen, Germany) was used. Centrifuge 5415D (Eppendorf, Germany) was used for all centrifugation steps.

1.5 mL of bacterial culture was pelleted by centrifugation (13000 rpm, RT, 3 min) and the supernatant removed. The bacterial pellet was resuspended in 250 µL of Buffer P1. To start the alkaline lysis, 250 µL of Buffer P2 were added and mixed by inverting the tube 6 times. Lysis was stopped by neutralisation after max 5 min by addition of 350 µL Buffer N3. Debris was pelleted by centrifugation (13000 rpm, RT, 10 min) and the clear supernatant was transferred to a QIAprep 2.0 spin column and centrifuged (13000 rpm, RT, 1 min). The column was washed using 500 µL Buffer PB followed by centrifugation (13000 rpm, RT, 1 min) and an additional washing step using 750 µL Buffer PE. After centrifugation (13000 rpm, RT, 1min), an additional centrifugation step (13000 rpm, RT, 1 min) was performed to remove wash buffer residues. DNA was then eluted into a 1.5 mL Eppendorf tube by pipetting 25 µL of water onto the column, incubating at RT for 1 min, and final centrifugation (13000 rpm, RT, 1min). Plasmid DNA was then quantified by spectroscopy using the NanoDrop1000 (Saveen Werner, Sweden) and qualitatively analysed on a 1% agarose gel.

5.8 Minimal inhibitory concentration (MIC) assays

To determine the susceptibility of bacterial strains to different antibiotics, broth microdilution MIC assays were performed. Minimal inhibitory concentration is defined as the lowest concentration of a compound in which bacterial growth is inhibited.

Relevant strains were plated, and colonies resuspended in 500 µL PBS until the 0.5 McFarland standard was reached (measured using a Sensititre Nephelometer). The assay was performed in 96-well plates (round bottom). 200 µL LB with corresponding antibiotic concentrations was transferred in the wells and inoculated with 1 µL of the adjusted PBS-bacteria mixture. Antibiotic concentrations were chosen according to literature expected values and then serial diluted. Plates were incubated overnight at 37°C. Bacterial growth was evaluated visually.

5.9 Induction assays

To evaluate ciprofloxacin-induced expression of *qepA*, translational fusions with YFP were created. Overnight cultures of different colonies were inoculated in LB and used to inoculate the experimental cultures containing different Sub-MIC concentrations of ciprofloxacin in LB (1 µL inoculum). Cultures were then grown overnight at 37°C 200 rpm shaking and diluted (1:200) in 200 µL PBS on a 96-well plate. YFP expression was assessed by exciting the cultures with a blue laser at 488 nm and measuring emission after a 525/50 nm filter in a MACSQuant VYB flow cytometer (Miltenyi Biotec, Germany).

5.10 Phage transduction

To transduce DNA into *E. coli*, P1 bacteriophage were used. 1 mL of overnight cultures of the recipient were supplemented with 5 mM $CaCl_2$ and shaken for 5 min at 37°C. 100 µL of the cells supplemented with $CaCl_2$ were mixed with 100 µL of corresponding P1 phage lysate. Samples were incubated for 20 min at 37°C without shaking and plated on corresponding selective medium.

5.11 Plasmid transformation

Plasmids were transformed into *E. coli* by electroporation. 1 mL of cells from recipient overnight cultures were harvested and washed five times with sterile water. Cells were resuspended in 50 µL of water and 3 µL plasmid DNA was added. Cells were electroporated using the BioRad Gene Pulser Xcell at 1800 V and 200 Ω; and recovered at 37°C for <1 h and afterwards plated on selective medium.

5.12 Preparation of genomic DNA

Genomic DNA was prepared for whole-genome-sequencing using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre, Illumina Inc., Madison, Wisconsin).

1 µL of 50 mg/mL Proteinase K was diluted into 300 µL of Tissue and Cell Lysis Solution per sample. Cells were pelleted by centrifugation (13000 rpm, RT, 2 min) from either an overnight culture or from colonies resuspended in 0.9% NaCl. The supernatant was discarded leaving approximately 25 µL of liquid in which the cells were suspended by vortexing. The prepared Tissue and Cell Lysis Solution containing Proteinase K is added to the cells and mixed thoroughly. Samples are incubated at 65°C for 15 min and vortexed every 5 min. Afterwards, the samples are cooled down to 37°C and 1 µL of 5 mg/mL RNase A is added to the sample and mixed thoroughly. Samples are finally incubated at 37°C for 30 min.

To precipitate the total DNA, 175 µL of MPC Protein Precipitation Reagent is added to 300 µL of lysed sample and vortexed thoroughly. Debris is pelleted by centrifugation (15000 rpm, 4°C, 20 min) and supernatant transferred into a clean 1.5 mL Eppendorf tube. 500 µL of isopropanol is added to the recovered supernatant and mixed by inverting 35-40 times, resulting in the precipitation of DNA. To pellet DNA, samples are centrifuged (10 min, 4°C, 15000 rpm). Isopropanol supernatant is removed, and the pellet washed twice with 200 µL 75% ethanol. DNA pellet is air-dried to ensure evaporation of ethanol and resuspended into 50 µL of Elution Buffer.

5.13 Bioinformatics

Databank sequences of *qepA* were extracted from NCBI Genome Database and analysed in CLC Main Workbench 8. Alignments were created and different QepA protein variants annotated and compared. Phylogenic trees were also generated using CLC Main Workbench 8.

Structural prediction of QepA protein was performed by Phyre[2] [58]and visualised using the USCF Chimera tool [59].

Graphs and statistical analysis were done using GraphPad Prism 8. All Figures were designed in Adobe Illustrator.

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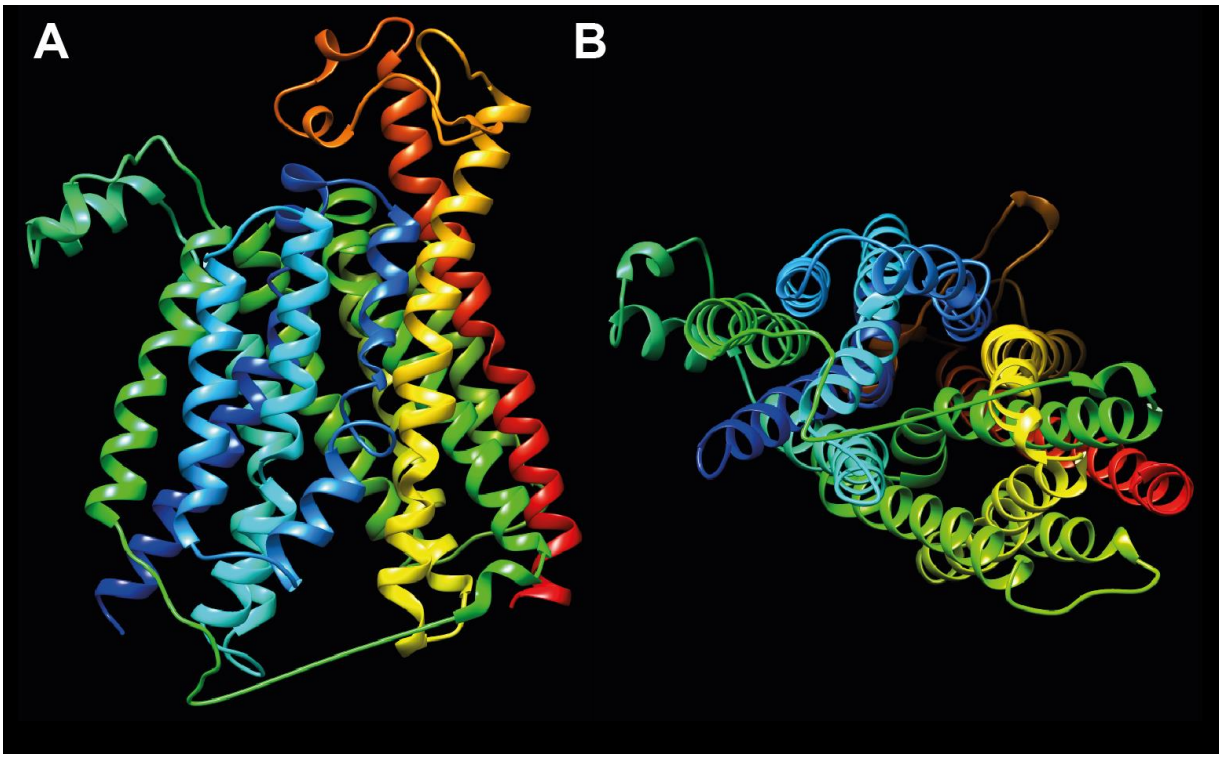
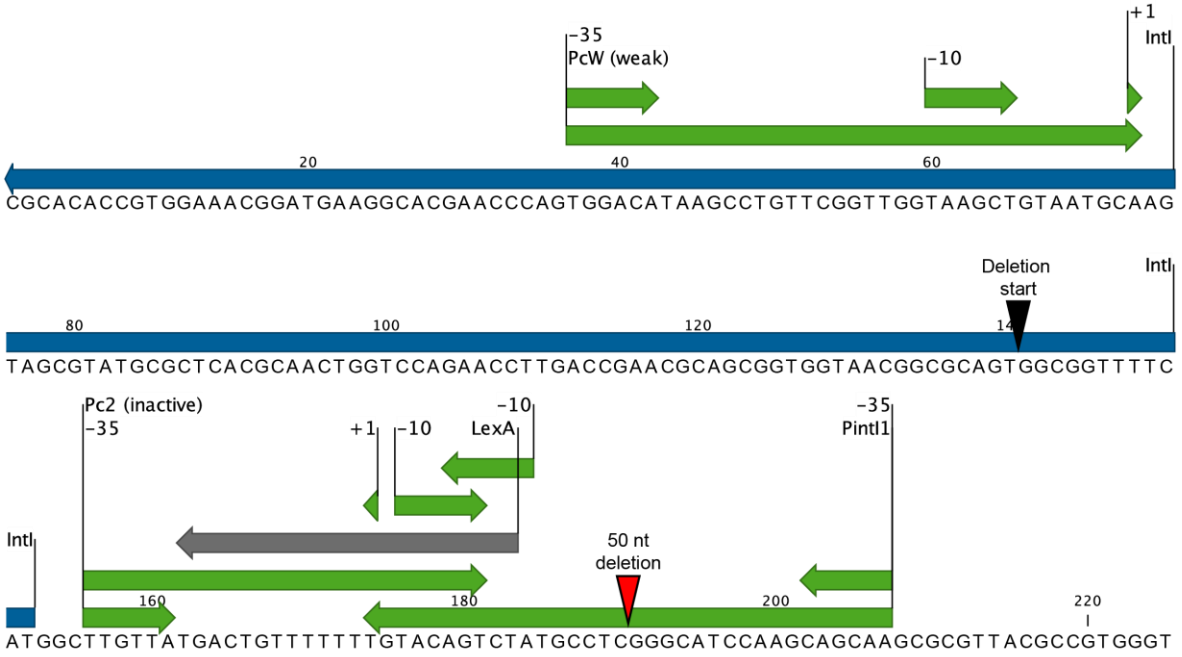
7 Appendix



Supplementary Figure 1: Predicted 3D structure of QepA
3D structure of QepA was predicted by the Phyre[2] Online Tool [58], and visualised using the UCSF Chimera Molecular Modelling System [59]
(A) QepA protein structure forming a barrel is shown from the side. (B) Upper view of QepA protein indicates pore-like structure.



Supplementary Figure 2: *qepA* promotor region
The predicted promotor driving qepA expression is PcW, which is shown in green on top of the figure. Deletions to assess downstream regulatory segments were introduced approximately 60 nt away from PcW (Black arrowhead). The first deletion, removing 50 nt, knocks out both the LexA binding site and the inactive P2 promotor (red arrowhead). Also, the -10 box of the Pint1 promotor is removed.

Supplementary Table 1: Strain list

Strain ID	Genotype	Parental strain
MG1655	Wildtype	-
CH9279	*galK*:*cat-sacB*	MG1655
JG01	*galK*:*qepA*(600 bp)	CH9279
JG03	*galK*:*qepA*(600 bp) / pSIM5-*tet*	JG01
JG05	*galK*:*qepA*(1200 bp)	CH9279
JG06	*galK*:*qepA*(1200 bp)	CH9279
JG08	*galK*:*qepA*(60 bp)	CH9279
JG09	*galK*:*qepA*(1200 bp) / pSIM5-*tet*	JG06
JG10	*galK*:*qepA*(600 bp) -YFP-*kan*	JG03
JG11	*galK*:*qepA*(600 bp) -YFP-*kan*	JG04

JG13	*galK*:*qepA*(1200 bp) / pSIM5-*tet*	JG05
JG15	*galK*:*qepA*(60 bp) / pSIM5-*tet*	JG08
JG16	*galK*:*qepA*(60 bp) –YPF-*kan*	JG14
JG17	*galK*:*qepA*(1200 bp)	CH9279
JG18	*galK*:*qepA*(60 bp) –YFP-*kan*	JG15
JG19	*galK*:*qepA*(60 bp) –YFP-*kan*	JG15
JG20	*galK*:*qepA*(1200 bp)	CH9279
JG21	*galK*:*qepA*(600 bp) -YFP-*kan* / psIM5tet	JG11
JG22	*galK*:*cat-sacB_qepA*(600 bp) -YFP-*kan*	JG21
JG25	*galK*:*cat-sacB_qepA*(600 bp) -YFP-kan / pSIM5-*tet*	JG22
JG26	*galK*:*qepA*(600 bp) -YFP-*kan* // *gyrA* S83L, D87N	JG11
JG28	*galK*:Δ50 nt_*qepA*(600 bp) -YFP-*kan*	JG25
JG30	*galK*:Δ100 nt_*qepA*(600 bp) -YFP-*kan*	JG25
JG31	*galK*:Δ150 nt_*qepA*(600 bp) -YFP-*kan*	JG25
JG33	*galK*:Δ200 nt_*qepA*(600 bp) -YFP-*kan*	JG25

Supplementary Table 2: DNA oligo list

ID#	Sequence	Purpose
LC67	GGTTTCGCACTGTTCACG	Sequencing *galK*
LC71	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATG	Deletes part of the sequence between promoter and qepA 50nt
LC72	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTTGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTA	Deletes part of the sequence between promoter and qepA 100 nt

LC73	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGGG CTTCACCAGAGTATCAAGTTGCCGAGCTGGCGATAAC	Deletes part of the sequence between promoter and qepA 150nt
LC74	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTAAA AAGAGGTTTGAAATGAATGAAGGAAAAAATGAGGTCA	Deletes part of the sequence between promoter and qepA 200nt
LC75	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTTGCT GGCCGGCTACGACCGTGCCCCCTGATGTGCGACGGG	Deletes part of the sequence between promoter and qepA 250nt
LC76	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTGCG TGTCACGCGCGCCTGAATGCGGATGGGTGTTGCCATC	Deletes part of the sequence between promoter and qepA 300 nt
LC77	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTCGA AATGCGCGCGTTGCCTTCGTCGTTAAAGCATTCTTGT	Deletes part of the sequence between promoter and qepA 350 nt
LC78	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTTTG AGTATGTCCGCCACGCTCCACGACACCGCAGCGGATC	Deletes part of the sequence between promoter and qepA 400 nt
LC79	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTCAC CCGCCGCGAATGGATCGGCCTGGCCGTGGTCGCCCTG	Deletes part of the sequence between promoter and qepA 450 nt
LC89	AACGGTCAGGAAGCAGAAAA	Sequencing *galK*
JG01	CATGGAAACTGCTTTGGTCCCCAGTGAGCGGCAATCGATCCGCA CACCGTGGAAACGGAT	clone qepA into galK gene
JG02	CAGCTAATTTCCGCGCTCGGCAAGAAAGATCATGCCTTGCTTTC CGACGCAGACCGTCTA	clone qepA into galK gene
JG03	CCAGAACCTTGACCGAACGCAGCGGTGGTAACGGCGCAGTATC AAAGGGAAAACTGTCCA	insert catsacB into galK:qepA
JG04	CTGTACAAAAAAACAGTCATAACAAGCCATGAAAACCGCCAAA ATGAGACGTTGATCGGCACG	insert catsacB into galK:qepA
JG09	ACACCTTTGGGCATGGAA	sequencing

		galK:*qepA*
JG10	ATTCAGGCGCGCGTGACA	sequencing *galK*:*qepA*
JG11	GGAGACGGTGTCCGAATT	sequencing *galK*:*qepA*
JG12	TCAGCTAATTTCCGCGCT	sequencing *galK*:*qepA*
JG13	CAGCTAATTTCCGCGCTCGGCAAGAAAGATCATGCCTTGCTTACCATTCGCGGCGGGTGGCCT	insert 60 bp *qepA* in *galK*
JG14	CAGCTAATTTCCGCGCTCGGCAAGAAAGATCATGCCTTGCTTAATACTCGGGCAGGAAGCGAG	insert 600 bp *qepA* in *galK*
JG15	CAGCTAATTTCCGCGCTCGGCAAGAAAGATCATGCCTTGCTTACGAGGCCGCGCCCGCGCGCT	insert 1200 bp *qepA* in *galK*
JG16	CACCGCAGCGGATCGTCGGAAGGCCACCCGCCGCGAATGGGTTAGCAAGGGCGAAGAACT	60 bp *qepA* translational YFP fusion
JG17	CAGCTAATTTCCGCGCTCGGCAAGAAAGATCATGCCTTGCCATATGAATATCCTCCTTAG	*qepA* truncations translational YFP fusion
JG18	GCTGACGCTGGCGCTCGGCCCTCGCTTCCTGCCCGAGTATGTTAGCAAGGGCGAAGAACT	600 bp *qepA* translational YFP fusion
JG19	CATCACCAGCGCGCCGTCCGAGCGCGCGGGCGCGGCCTCGGTTAGCAAGGGCGAAGAACT	1200 bp *qepA* translational YFP fusion
JG20	GCCTGTAAAAAGTTCTTCGC	*qepA* sequencing primer in YFP
JG21	TCTTGTCCCGGAAGTTTT	YFP scar primer
JG22	CGCTCACGCTAAACTTAT	YFP scar primer
JG23	AGCGCGCGGGCGCGGCCTCGAAAATGAGACGTTGATCGGCACGTGTAGGCTGGAGCTGCT	insert *cat-sacB* into *qepA*(1200 bp) -YFP-*kan*
JG24	TCGGTACTACGCCTGTAAAAAGTTCTTCGCCCTTGCTAACATCAAAGGGAAAACTGTCCA	insert *cat-sacB* into *qepA*(1200 bp) -YFP-*kan*

JG25	CTCGCTTCCTGCCCGAGTATAAAATGAGACGTTGATCGGCACGT GTAGGCTGGAGCTGCT	insert *cat-sacB* into *qepA*(600 bp) -YFP-*kan*
JG26	CTAAGGAGGATATTCATATGTGGACAGTTTTCCCTTTGATGTTAG CAAGGGCGAAGAACT	insert *cat-sacB* into *qepA*(600 bp) -YFP-*kan*
JG29	GCTGACGCTGGCGCTCGGCCCTCGCTTCCTGCCCGAGTATCGTG ATCCGGACGCGGGGCA	reconstruct *qepA* from 600 bp
JG30	TCGGTACTACGCCTGTAAAAAGTTCTTCGCCCTTGCTAACACCA GATGCGAGCGCTGGGC	reconstruct *qepA* from 600 bp
JG31	GCTGACGCTGGCGCTCGGCCCTCGCTTCCTGCCCGAGTATAAAA TGAGACGTTGATCGGC	insert *cat-sacB* into *qepA*(600 bp) -YFP-*kan*
JG32	TCGGTACTACGCCTGTAAAAAGTTCTTCGCCCTTGCTAACATCA AAGGGAAAACTGTCCA	insert *cat-sacB* into *qepA*(600 bp) -YFP-*kan*

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