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Antibiotic detection using bioreporters based on SOS and cold shock responses

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Antibiotic detection using bioreporters based on SOS and cold shock responses

Abstract:

With emerging antibiotic resistance, development of methods for screening new antibiotics in environmental and metabolite samples is necessary. Whole-cell bacterial bioreporters are a unique tool in that they only detect the bioavailable fraction of antibiotic concentration and can also aid with investigating the mechanism of action. In this study, two reporters were constructed based on SOS and cold shock stress promoters to detect genotoxic compounds and C group translation inhibitors. Both reporters displayed specific and dose-dependent response to sub-inhibitory antibiotic concentrations.

Keywords:

Bioreporter, antibiotic, stress response

CERCS: B230 Microbiology, bacterioloy, virology, mycology; T490 Biotechnology

Antibiootikumite detekteerimine SOS ja külmašoki stressivastustel põhinevate bioreporteritega

Lühikokkuvõte:

Antibiootikumresistentsuse kasvuga kasvab ka vajadus arendada välja meetodeid uute antibiootikumide tuvastamiseks keskkonnast ja mikroobide metaboliitide seast. Bioreporterid kui elusorganisme teeb eriliseks nende võime tuvastada just elusorganismile mõjuvat antibiootikumi kontsentratsiooni ning uurida selle toimemehhanismi. Käesolevas töös konstrueeriti kaks bioreporterit kasutades SOS ja külmašoki stressipromootoreid, et tuvastada genotoksilisi ühendeid ja C-grupi translatsiooni inhibiitoreid. Mõlema bioreporteri puhul nähti spetsiifilist ja annusest sõltuvat vastust subinhibitoorsetele antibiootikumide kontsentratsioonidele.

Võtmesõnad:

Bioreporter, antibiootikum, stressivastus

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia; T490 Biotehnoloogia

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Abbreviations

5' UTR - 5' untranslated region

CIP - ciprofloxacin

CHL - chloramphenicol

CPEC - circular polymerase chain reaction

GFP - green fluorescent protein

IF3 - initiation factor 3

KAN - kanamycin

MBC - minimum bactericidal concentration

MIC - minimum inhibitory concentration

NAL - nalidixic acid

NOR - norfloxacin

OD - optical density

PCR - polymerase chain reaction

SD - Shine-Dalgarno ribosomal binding sequence

TET - tetracycline

Introduction

Antibiotic resistance is a growing problem that, if not combated, could cause millions of deaths per year due to small injuries and currently well-treatable infections becoming unmanageable. The solution is likely to be multi-faceted, combining reduced and optimized use of antibiotics, abolished agricultural use, resistance tracking, monitoring of environmental residues and development of new drugs.

The aim of this study was to develop two bacterial bioreporters responding to genotoxic compounds, and to C group translation inhibitors. Aside from detecting sub-inhibitory concentrations of existing drugs or potential candidates from environmental samples and identifying their mechanisms of action, such reporters can measure antibiotic concentration in *in vitro* and *in vivo* infection models and intracellular infections. For the latter there is currently a lack of good single cell level tools.

Two reporters were constructed with red fluorescent protein expressed from *cda* or *cspA* promoters and green fluorescent protein expressed constitutively to control for general inhibition of gene expression by antibiotics. The ratio of red to green fluorescence was calculated as the reporters were tested with a range of antibiotics, sub-inhibitory concentrations and incubation times. The specificity and concentration-dependence of signal and optimal duration was assessed for both reporters.

The developed bioreporters displayed a clear dose-dependent response to their expected antibiotics. As a surprising finding, *cspA* promoter was also induced by fluoroquinolones ciprofloxacin and norfloxacin.

1 Literature review

1.1 Antibiotics

Antibiotics are substances that can kill bacteria or inhibit their growth. Antibiotics are used as medicine to treat bacterial infections. They are among the most important discoveries of the 20th century and have saved countless lives. (Davies and Davies, 2010) Among the first antibiotics is arsphenamine discovered by Paul Ehrlich in 1909. It was used to treat syphilis. (Ehrlich and Hata, 1910) Another early discovery was sulfanilamide by Bayer chemists (Domagk, 1935). Also famously, in 1928 Sir Alexander Fleming accidentally discovered that a fungus from the *Penicillium* genus was lethal to neighboring *Staphylococcus* colonies. He figured out that a substance excreted by the fungus was responsible for the effect and named the substance penicillin. (Fleming, 1929) In his 1945 Nobel lecture, Fleming was also one of the first to warn about the possibility of emerging antibiotic resistance (Fleming, 1945).

There are various ways to classify antibiotics. They can be bactericidal or bacteriostatic based on whether they kill bacteria or merely inhibit growth respectively. The distinction is not that clear because a bacteriostatic antibiotic may be bactericidal at a higher concentration. Two measures are important here - minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). For a given antibiotic and bacterial strain, MIC is the minimum antibiotic concentration required to inhibit growth. It is commonly determined using a standardized protocol where the strain is incubated with twofold dilutions of the antibiotic at 35-37 °C for 18-24 hours to determine the lowest concentration that inhibits visible growth. For MBC, the samples with no growth (≥MIC concentrations) are plated on antibiotic free solid medium and incubated for additional 18-24 hours. The lowest concentration able to decrease the initial inoculum at least 1000-fold corresponds to MBC. The MBC / MIC ratio above 4 is used as an indication of bacteriostatic activity. (Pankey and Sabath, 2004).

MIC alone isn't sufficient to determine antibiotic concentration necessary for infection treatment. The drug concentration must reach the inhibitory concentration at the site of infection for successful treatment. The *in vivo* concentration depends on dosage and varies over time and between tissues. Other varying factors include the number and growth speed of bacteria, pH, oxygen concentration and the immune response of the host which contributes to

clearing infections. (Estes, 1998) Clinical breakpoints are a tool for translating MIC to likely treatment outcome. Such breakpoints are determined per pathogen and per drug and classify MIC ranges as susceptible (S) or resistant (R) which correlate respectively with treatment success and failure. (Humphries et al., 2019)

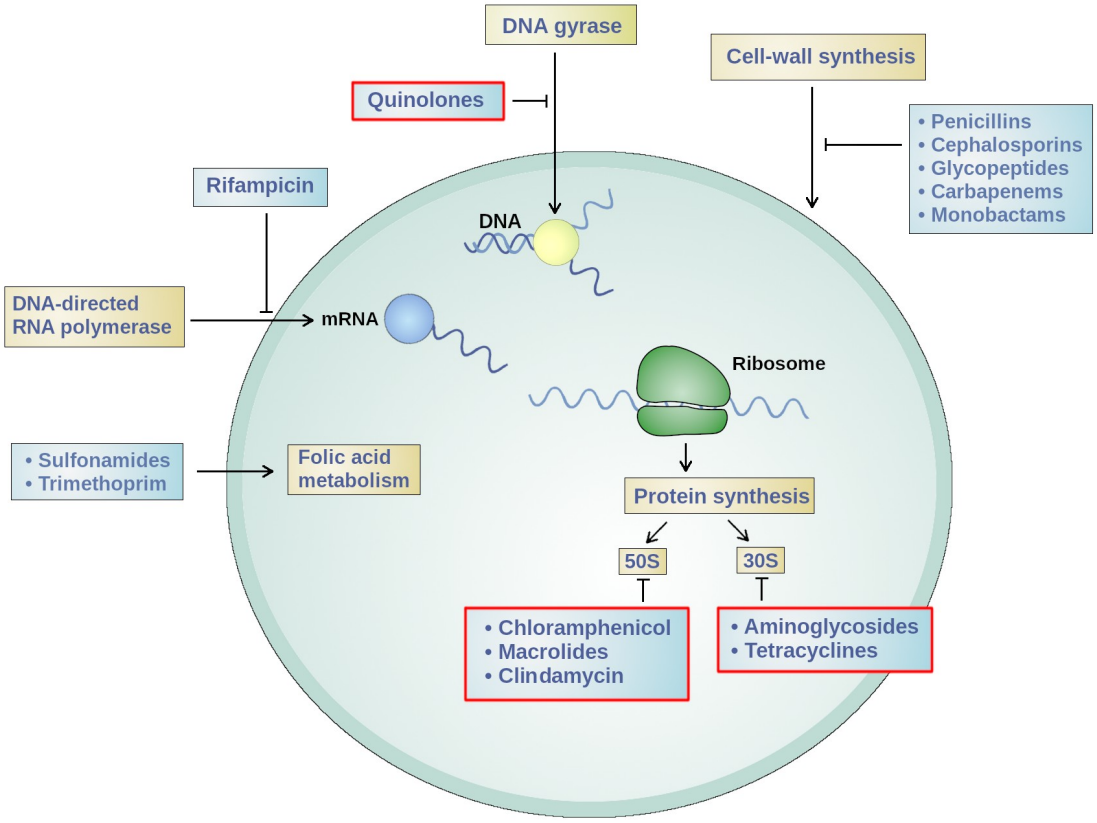

Figure 1: Common cellular targets of antibiotics. Relevant for this study (in red boxes) are quinolones acting on DNA gyrase, aminoglycosides and tetracyclines acting on the 30S ribosomal subunit and chloramphenicol acting on the 50S ribosomal subunit. Figure adapted from Lewis, 2013.

Antibiotics have various mechanisms of action (Figure 1). Antibiotics are classified as having a broad spectrum, meaning they target many types of bacteria, or narrow spectrum, which target only specific types. Penicillin G discovered by Fleming mainly targets Gram-positive bacteria and is an example of a narrow spectrum antibiotic. Tetracyclines and chloramphenicol have a broad spectrum and target both Gram-positive and Gram-negative bacteria. (Ory and Yow, 1963) Whether a particular strain is Gram-positive or Gram-negative can be assessed using membrane structure dependent Gram stain. It is an important method dividing bacteria into two large groups. (Coico, 2006) It has been argued that antibiotic

classification based on spectrum isn't clearly defined and that overuse of ambiguous terms is unhelpful. (Acar, 1997) The nonspecific activity of broad spectrum antibiotics is useful for complex surgeries and for fast treatment of an infection with an unidentified microbe but it also has more off-target effects. Beneficial symbionts are targeted along with the infection. More targets also means greater possibility for the emergence of resistance. (Casadevall, 2009) The mechanisms of action relevant for this study are discussed in the following sections.

1.1.1 Protein synthesis inhibitors

Protein synthesis is an important cellular process inhibited by many antibiotics. It is carried out by ribosomes, which are complicated molecular machines that translate the nucleotide sequence carried by mRNA to amino acid sequence of a protein. Ribosomes consist of small and large subunits, denoted 30S and 50S respectively. Protein synthesis initiates with the formation of a complex of the two subunits and mRNA. Aminoacyl-tRNAs are transferred through the ribosome, each bringing a single amino acid to the growing polypeptide chain. Three sites, the A- P- and E- site (aminoacyl-, peptidyl- and exit-sites, respectively) are occupied by the tRNAs. An incoming aminoacyl-tRNA enters the A-site while P-site tRNA holds the growing polypeptide chain. A peptidyl transferase reaction in 50S catalyses peptide bond formation by transferring the polypeptide chain from the P site tRNA to the amino acid of the A-site tRNA. This is followed by translocation – stepping the ribosome to the next codon while moving the A- and P-site tRNAs to P- and E-sites respectively. The dischcarged tRNA can leave through the E-site and next tRNA is ready to be accepted. The elongation cycle repeats, extruding the nascent polypeptide through an exit tunnel until a stop codon is encountered. The synthesis is terminated by releasing the newly formed protein. (Moore, 2009)

Chloramphenicol inhibits protein synthesis by binding to ribosomal 50S subunit (Pongs et al., 1973) and blocking the peptidyl transferase reaction (Moazed and Noller, 1987). It was isolated in 1947 from *Streptomyces* (Ehrlich et al., 1947). Chloramphenicol isn’t widely used because of toxic side effects to the patient (Nitzan et al., 2010). Tetracyclines also inhibit protein synthesis, but they bind to the 30S subunit and block the binding of aminoacyl-tRNA (Brodersen et al., 2000). Tetracyclines are characterized by a four-ring core and include tetracycline, chlortetracycline and doxycycline (Griffin et al., 2010). Chlortetracycline was isolated from *Streptomyces* in 1945 (Duggar, 1948; Walker, 1982). A third protein synthesis

inhibitor used in this study is kanamycin, isolated in 1957 from *Streptomyces kanamyceticus* (Umezawa et al., 1957). Kanamycin belongs to the class of aminoglycosides, which bind to the A-site of the 30S subunit and modify its conformation. This causes mistranslation and generates faulty proteins (Noller, 1991). Chloramphenicol, tetracycline and kanamycin are broad-spectrum antibiotics targeting many Gram-positive and Gram-negative bacteria (Griffin et al., 2010; HN and R, 1970; Krause et al., 2016).

1.1.2 Genotoxins

This study uses quinolone genotoxins, which target type II topoisomerases gyrase (Gellert et al., 1977) and topoisomerase IV (Heisig, 1996). Both enzymes can relax positive DNA supercoils (Brown and Cozzarelli, 1979; Hiasa and Marians, 1996). Gyrase is also necessary for negative supercoiling of DNA (Gellert et al., 1976). Topoisomerase IV is needed for separation of daughter chromosomes during DNA synthesis (Peng and Marians, 1993). Both function by creating a temporary double-stranded break in DNA and transferring an intact double helix through the break. The break is then fixed by ligation. (Liu et al., 1980) Quinolones bind to the topoisomerases and prevent ligation, forcing the double-strand break to persist (Drlica et al., 2009). The DNA damage induces DNA repair pathways and SOS response (Lewin et al., 1989).

Quinolones are a class of antibiotics originating from nalidixic acid, introduced by George Lesher in 1962 (Lesher et al., 1962). In early 1980s, a number of new members containing fluorine were synthesized. The new compounds were called fluoroquinolones and they include norfloxacin (Koga et al., 1980) and ciprofloxacin (Wise et al., 1983) used in this study. Fluoroquinolones have a significantly higher potency than nalidixic acid (Domagala et al., 1986). Ciprofloxacin is a clinical success as one of the most frequently prescribed antibiotics. It is mainly used against Gram-negative but also against Gram-positive bacteria. (Emmerson and Jones, 2003)

1.1.3 Resistance

Emerging antibiotic resistance is a growing problem. World Health Organization (WHO) 2014 report warns that return to post-antibiotic era is possible within this century. This means that small injuries and currently well-treatable infections could once again become deadly. (World Health Organization, 2014) Another WHO report from 2019 estimates that the number of

deaths from drug-resistant diseases could increase from the current 700,000 per year to 10 million per year by 2050, if no action is taken to combat resistance (World Health Organization, 2019).

With the presence of an antibiotic, bacteria with genetic changes that provide resistance against that antibiotic are selected as part of natural evolution. Resistant population can grow quickly because of vertical gene transfer during clonal expansion, and mutations can then spread within and between species through horizontal gene transfer. (Aslam et al., 2018; Li et al., 2019) A concept related to resistance is persistence, where a small fraction of a clonal population can survive upon antibiotic exposure. As opposed to resistance though, persistence is a transient phenotypic trait and is not genetically inheritable. (Gefen and Balaban, 2009) Percistence phenomenon has been a matter of scientific debate in the recent years (Kaldalu et al., 2016). It is the overuse of antibiotics that drives the emergence of resistance. On the one hand, there is over-prescription by doctors (Ventola, 2015). Patients contribute by pressuring doctors for prescription, buying without prescription and misusing antibiotics by skipping doses or saving part of the course for later use (Pechère, 2001). On the other hand, antibiotics are used extensively in agriculture to prevent and treat infections but also for increased yield (Marshall and Levy, 2011).

Main mechanisms to antibiotic resistance are illustrated on Figure 2.

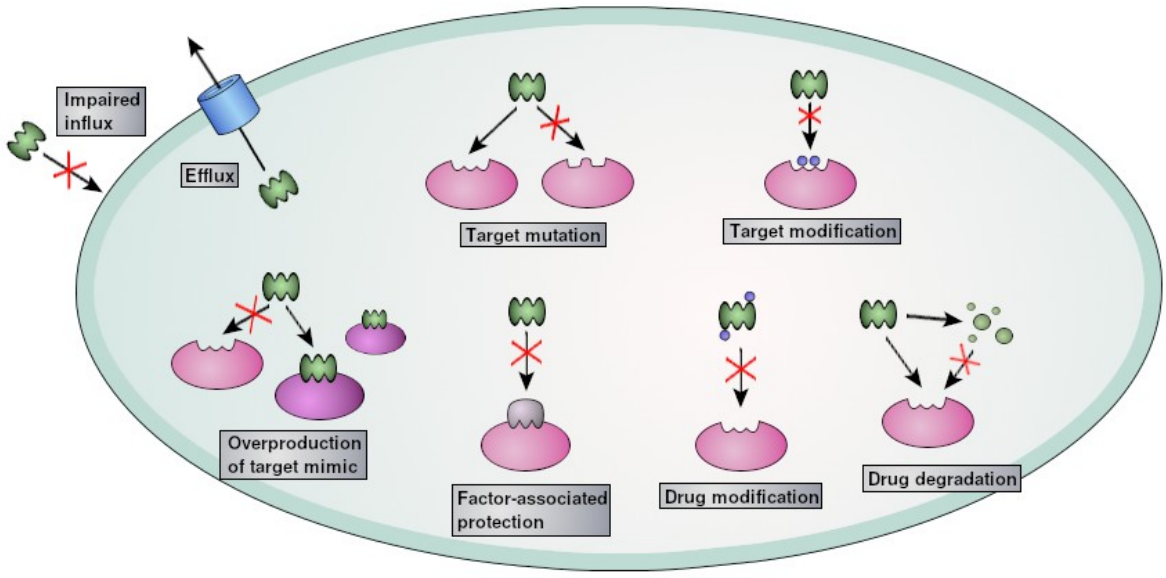

Figure 2: Antibiotic resistance mechanisms. Figure adapted from Wilson 2014.

Blocking influx is the first line of defense for bacteria. Hydrophilic molecules often use porin channels to cross cell membrane. (Delcour, 2003) As a response, bacteria could either decrease the expression or change the structure of porins (Nikaido, 2003).

Decrease of influx can be accompanied by efflux pumps which selectively remove the antibiotic from the cell. One of the first such pumps was found in *E. coli* against tetracycline (McMurry et al., 1980). Many classes of efflux pumps have been described (Piddock, 2006).

Another common mechanism employed is the blocking of the interaction between antibiotic and its target by various means. One possibility is the production of a protective protein that binds to the target and blocks the association of the drug. As an example, the Qnr protein binds to type II topoisomerases and protects them from quinolones (Rodríguez-Martínez et al., 2011). Another possibility is the modification of the target. This could occur as an underlying genetic change as exemplified again by quinolone resistance - point mutations between amino acids 67 - 106 of the GyrA protein are associated with resistance (Yoshida et al., 1990). But target modification can also be enzymatic as seen by enzymes encoded by *erm* genes. They methylate a specific adenine base of 30S rRNA to block the binding of the macrolide, lincosamide and streptogramin B families of antibiotics (Weisblum, 1995).

The antibiotic molecule can be chemically modified upon entry into cell to inhibit its interaction with the target. As a common example, over 100 aminoglycoside-modifying enzymes have been found. These enzymes can acetylate, phosphorylate or attach the adenosine monophosphate (AMP) nucleotide to aminoglycoside rendering it inactive. (Ramirez and Tolmasky, 2010)

The antibiotic can also be degraded. This was already seen in 1940, not long after penicillin was discovered, with the identification of the penicillinase enzyme which could degrade penicillin (Abraham and Chain, 1940). Interestingly, evidence for resistance against penicillin has been found in ancient DNA, indicating that antibiotic resistance occurred naturally long before modern clinical selective pressure (D'Costa et al., 2011).

1.2 Stress responses

Bacteria have developed a number of stress responses as a survival strategy for coping with unfavorable environmental conditions. Among such stressors are heat, cold, acidity and lack of nutrients. Stress responses involve coordinated expression of genes necessary for defense

mechanisms. (Chung et al., 2006) Such gene regulation comes in handy for biotechnology – responses can be measured by placing a reporter protein under the expression of a stress-induced promoter (Figure 5) (Xu et al., 2013). Two types of stress responses relevant for this study are discussed in detail.

1.2.1 SOS response

SOS response was first proposed by Miroslav Radman in a 1975 article as a mechanism that responds to DNA damage by activating normally repressed DNA repair mechanisms. (Radman, 1975) Mainly two proteins are important for SOS response. The transcriptional repressor LexA blocks the expression of DNA repair mechanisms in normal conditions. In case of DNA damage, the RecA protease blocks the activity of LexA by cleaving it to induce SOS response (Figure 3). (Gudas and Pardee, 1975; Little et al., 1980) The target genes of LexA share a homologous 20 bp LexA binding site in the promoter region called the SOS box (Little and Mount, 1982). Among targets repressed by LexA are *uvrA* and *uvrB* necessary for excision repair (Kenyon and Walker, 1981; Sancar et al., 1982) and *sulA* necessary to block cell division (Mizusawa et al., 1983). Interestingly for this study, SOS response also induces the *cda* gene encoding for the Colicin D antibacterial protein. The *cda* gene is found on the ColD-CA23 plasmid. (Frey et al., 1986)

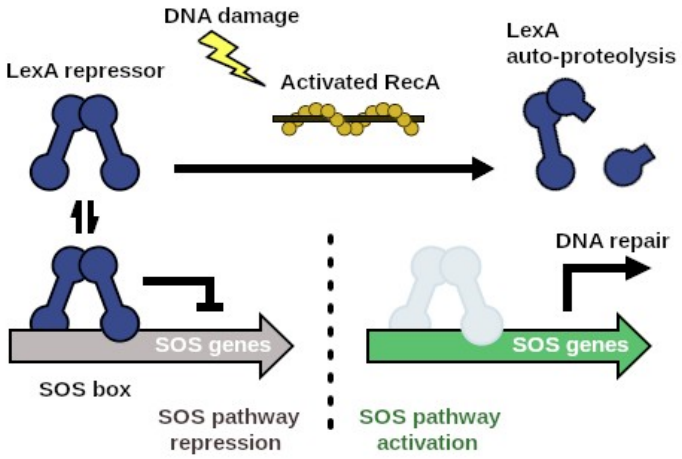

Figure 3: LexA repressor blocks the expression of SOS genes. RecA induces LexA cleavage upon DNA damage to initiate SOS response. Figure adapted from Selwood et al., 2018.

Since quinolones damage DNA, as discussed above, they would be expected to induce SOS response. Indeed, nalidixic acid was first found to induce SOS response (Gudas and Pardee,

1976). Later the same was found to be true for newer quinolones, in particular the ciprofloxacin and norfloxacin used in this study (Phillips et al., 1987).

1.2.2 Cold shock response

Escherichia coli cells transferred from 37 °C to 10 °C undergo what's called a cold shock response. A 4-hour acclimation lag phase is followed by slow growth with a 24-hour generation time. During lag phase, most of protein synthesis is turned off. (Jones et al., 1987) Yet many proteins are induced during lag phase, most notably the CspA designated as the major cold shock protein (Goldstein et al., 1990; Jones et al., 1987). Low temperature is accompanied by increased secondary structure for almost all mRNA, which correlates with lower translation efficiency. CspA binds to single-stranded RNA with low specificity to reduce secondary structure and resume translation (Figure 4). (Jiang et al., 1997; Zhang et al., 2018) In spite of the name, CspA has other roles unrelated to cold shock and is also heavily induced during early exponential growth (Brandi et al., 1999).

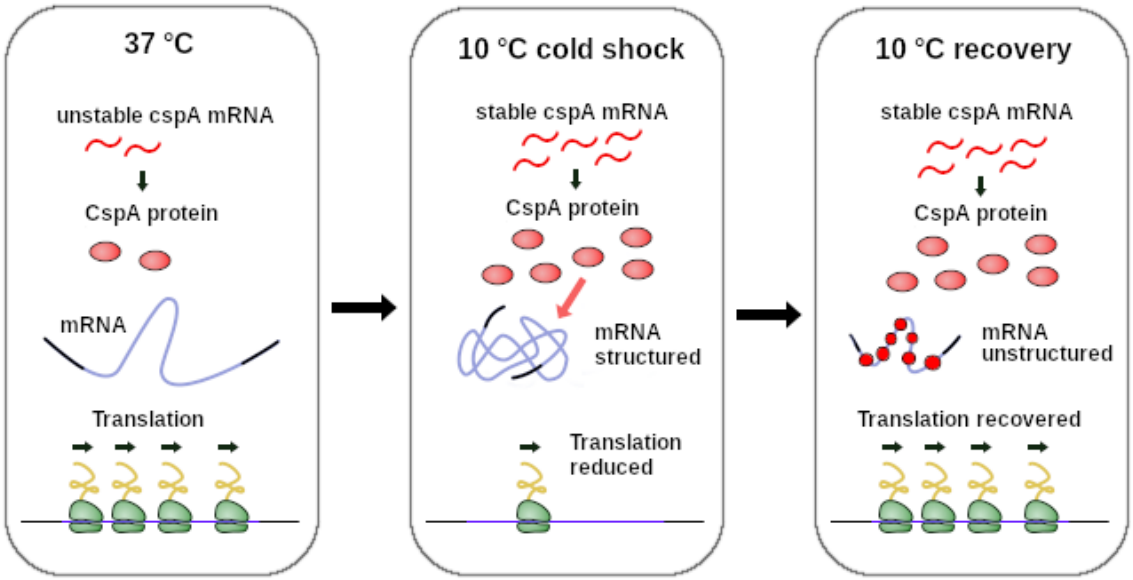

Figure 4: Cold shock response. At 37 °C the cspA mRNA is unstable and not much CspA is produced. With cold shock, cellular mRNA becomes more structured which inhibits translation. Also, cspA mRNA changes structure and becomes more stable with cold shock, increasing CspA synthesis. CspA binds to cellular mRNA to reduce secondary structure and recover translation. Figure adapted from Zhang et al., 2018.

The regulation of CspA expression both at 37 °C and during cold shock has been extensively studied and occurs at the level of transcription (Brandi et al., 1999), mRNA stability and translation (Brandi et al., 1996). The transcription of CspA is antagonistically regulated by Fis and H-NS proteins at 37 °C. These account for the fluctuation of CspA with different growth phases but not with cold shock. (Brandi et al., 1999) The mRNA of CspA was found to be very unstable at 37 °C with a significantly longer half-life at 10 °C (Figure 4) (Brandi et al., 1996). An unusually long 5' UTR of *cspA* gene was found to fold into different structures at 37 °C and 10 °C. At 37 °C the ribosomal binding site and start codon are buried within the mRNA secondary structure while at low temperature they remain accessible, facilitating translation. (Giuliodori et al., 2010) Also, *trans*-acting factors like the translation initiation factor IF3 and CspA itself increase with cold shock and influence the selectivity of translation towards cold shock proteins (Gualerzi et al., 2003).

Interestingly, it has been shown that some ribosome inhibiting antibiotics induce cold shock response (C group) while others induce heat-shock response (H group) (VanBogelen and Neidhardt, 1990). This study uses chloramphenicol and tetracycline from the C group and kanamycin from the H group. While cold shock has been studied thoroughly, less is known about induction of CspA through antibiotics and an interesting question is whether there is a common underlying mechanism. As a hint, half-life of CspA mRNA was found to increase with the addition of chloramphenicol, suggesting that C group antibiotics may have an effect through mRNA stability. (Jiang et al., 1993)

1.3 Bioreporters

Through genetic engineering, a promoter which responds to a particular external substance or type of stress can be fused with a reporter gene to produce a measurable signal (Figure 5) (Leveau and Lindow, 2002). Such a construct yields a whole-cell bioreporter useful for various applications.

Bioreporters have been used for the detection of substances like antibiotics (Hansen et al., 2001), nutrients (Leveau and Lindow, 2001) and metals (Joyner and Lindow, 2000). Also, physical signals like UV radiation (Kim and Sundin, 2000) and temperature shift (Ullrich et al., 2000) have been detected. In addition, bioreporters can respond to specific types of cell stress. An early example from 1982 fused the *sulA* promoter to *lacZ* reporter to detect SOS stress response induced by DNA damage (Quillardet et al., 1982).

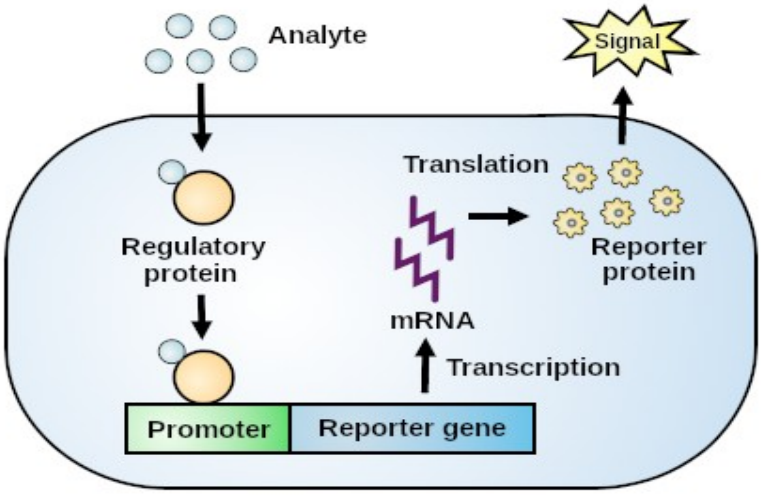

Figure 5: General schematic of a whole-cell bioreporter. A promoter under the control of a regulatory protein is fused with the reporter gene. Binding of analyte by the regulatory protein induces production of reporter protein which generates a measurable signal. Figure adapted from Shemer et al., 2015.

Common reporter genes used induce a colorimetric, luminescent or fluorescent response. For colorimetric signal, *lacZ* gene is widely used, which encodes for β-galactosidase, an enzyme that breaks down lactose. Lactose analogs such as o-nitrophenyl-β-D-galactoside (ONPG) produce a colored substance when cleaved by β-galactosidase. (Lederberg, 1950; Xu et al., 2013).

Luminescence is the emission of light due to anything other than heat. Bioluminescence is a form of luminescence where light is emitted by a living organism using energy from a chemical reaction. Bioluminescent signals are commonly produced using the bacterial l*ux* and firefly l*uc* luciferase systems (Xu et al., 2013). Fluorescence is another form of luminescence where emission energy is obtained by absorption of light. Absorption and emission occur at separate wavelengths with the emission wavelength normally being longer. (Valeur and Berberan-Santos, 2011) Fluorescent signal in bioreporters is achieved through fluorescent proteins. The use of such proteins began with the isolation of green fluorescent protein (GFP; gene *gfp*) from *Aequorea* jellyfish in 1962 (Shimomura et al., 1962). Since then, the range of available fluorescent proteins has extended widely (Rodriguez et al., 2017). Several fluorescent proteins may be incorporated in a single reporter strain and can be measured simultaneously (Osterman et al., 2016). A comparison of *gfp* and *lux* reporters reveals a higher sensitivity with *lux* but a more stable signal with *gfp (Justus and Thomas, 1999)*. Contrary to *lacZ* and *lux* systems, *gfp* doesn't need any substrate to function. The signal from *gfp* can be

measured nearly instantly without cell lysis, allowing for continuous measurements. (Norman et al., 2005) In addition, fluorescence measurement doesn't require bulk culture. Fluorescence-based sorting of individual cells with flow cytometry is a common procedure and allows to discover possible heterogeneity within a population. (Adan et al., 2017)

Whole-cell bioreporters are useful for screening new antibiotics and detecting antibiotic mechanisms of action. Samples with sub-inhibitory antibiotic concentrations can be screened for, as well as combinatory effects of different substances. (Eltzov et al., 2008; Zhang et al., 2012) Only bioavailable fraction is measured (Harms et al., 2006). Reporters may be combined in a single strain (Osterman et al., 2016) or a panel of strains (Melamed et al., 2012) to cover a range of mechanisms of action. Previous reporters have been constructed that distinguish SOS response and ribosome stalling (Osterman et al., 2016) and cell envelope damage, heat and cold shock responses (Bianchi and Baneyx, 1999). Various SOS promoters have been tested for detection of DNA damaging agents. The response from *cda* promoter based reporter was found to be stronger and more sensitive than those from *recA*, *sulA* and *umuDC* reporters. (Norman et al., 2005)

2 Aim of the thesis

1. Construct concentration-dependent double-fluorescent reporter, using GFPmut2 as a control protein for the general level of gene expression, and red fluorescent protein mScarlet-I as a reporter protein induced by certain antibiotics. Previous works with reporters often lack the control protein.
2. Test the reporter specificity to antibiotics of interest.
3. Determine the useful concentration range and the dose-dependence of reporter signal.
4. Initial verification of the reporter system in a laboratory strain. In the future these reporters could be used in *in vitro* and *in vivo* infection models for measurement of antibiotic effects on bacteria.

3 Experimental part

Experimental part was carried out in following steps:

1. Construction of reporter plasmids and strains.
2. Antibiotic MIC determination for planned experimental conditions.
3. Incubation of reporter strains with four subinhibitory concentrations of six antibiotics and fluorescence measurements in timepoints between 12h - 60h.
4. Data analysis.

3.1 Materials and methods

3.1.1 Strains

Bioreporter assay was carried out with MG1655 strain of *Escherichia coli.* MG1655 is similar to the wild-type K-12 strain but lacks λ phage and F-plasmid (Guyer et al., 1981; Lederberg and Lederberg, 1953).

Another strain of *E. coli*, DH5α was used for the initial cloning. It is a common laboratory strain also based on K-12 and contains many mutations for easier cloning. Notably, DH5α lacks endonuclease I (*endA1*), disrupts EcoKI endonuclease (*hsdR17*) and has reduced

homologous recombination (*recA1*). This results in better quality and stability of plasmid DNA. (Anton and Raleigh, 2016)

3.1.2 Media

BD Difco™ Lennox LB broth was used for the preparation of DH5α and MG1655 competent cells and during transformation procedures. The LB broth was prepared according to manufacturer's instructions and filter-sterilized using 0.22 µm pore filter. Filter-sterilization was preferred over autoclaving in order to preserve uniform amino acid composition of the media.

M9 minimal medium was prepared (Table 1) from 5x M9 salts stock solution (Table 2), 100 mM $CaCl_2$, 1 M $MgSO_4$ and 20% glucose, all provided by Mariliis Hinnu, filter-sterilized with 0.22 µm pore filter and stored at 4 °C. M9 medium was used for MIC measurement and for testing the response of constructed bioreporters to various antibiotics. Minimal medium was used for increased sensitivity during fluorescence analysis because of its low fluorescence background compared to rich media, such as LB. As a defined medium it is also less prone to content variability.

Table 1: M9 minimal medium

Component	**Final concentration**
5x M9 salts stock solution (Table 2)	1x
$MgSO_4$	2.0 mM
$CaCl_2$	0.1 mM
glucose	0.2 % w/V

Table 2: 5x M9 salts stock solution

Component	**Final concentration**
$Na_2HPO_4 \cdot 7H_2O$	238 mM
KH_2PO_4	110 mM
NH_4Cl	93 mM
NaCl	43 mM

3.1.3 Antibiotics

A number of antibiotics were used in this study (Table 3). Ampicillin (100 µg/ml) was used for selection of plasmid-carrying cells. Ampicillin solution was stored at -20 °C. Chloramphenicol, tetracycline, kanamycin, nalidixic acid, ciprofloxacin and norfloxacin were used to test the response of bioreporters and the solutions were stored at 4 °C.

Table 3: Antibiotic stock solutions used in this study

Antibiotic	**Concentration**	**Solvent**	**Manufacturer, product number**
chloramphenicol (CHL)	30 mg/ml	96% EtOH	AppliChem, A6435
tetracycline (TET)	10 mg/ml	70% EtOH	Sigma, T7660-5G
kanamycin (KAN)	25 mg/ml	H_2O	AppliChem, A1493
nalidixid acid (NAL)	25 mg/ml	H_2O	Sigma, 70162
ciprofloxacin (CIP)	10 mg/ml	H_2O	Sigma, 17850
norfloxacin (NOR)	10 mg/ml	H_2O	Sigma, N-9890
ampicillin (AMP) (as sodium salt)	100 mg/ml	H_2O	Carl Roth, K029 / Sandoz, Standacillin

The solutions were prepared as follows. Chloramphenicol was dissolved in 96% ethanol by vortexing. Tetracycline hydrochloride was dissolved in 70% ethanol by vortexing. Nalidixic acid was added to sterile Milli-Q water (75% of total volume). 1 M NaOH was added drop-by-drop until all nalidixic acid was dissolved. The solution was vortexed after each drop. Milli-Q water was then used to fill to volume. Ciprofloxacin and norfloxacin stocks were prepared as follows – antibiotic was added to Milli-Q water (50% of total volume). 0.1 M HCl was added (30% of total volume). Additional acid was added drop-by-drop until all the antibiotic was dissolved. The solution was vortexed after each drop. Milli-Q water was used to fill to volume. Kanamycin and ampicillin solutions were dissolved in Milli-Q water by Mariliis Hinnu.

3.1.4 Plasmids and primers

A number of plasmids were used in this study (Table 4). The primers for plasmid construction are shown in Table 5. Primers were designed using SnapGene v4.1.5 software and supplied by Metabion (Germany). Plasmid maps are presented in appendix 1.

Table 4: Plasmids used in this study

Name	**Characterization**	**Source**
pSC101-GFPmut2-mScarlet-I	pSC101-based template plasmid with ampicillin resistance (*ampR*). GFPmut2 and mScarlet-I are expressed together by constitutive tet-promoter and stress-inducible dnaK1 promoter. Positive control.	Preem et al., 2019.
pRFPCER-TrpL2A	Source for *trpT* terminator (TtrpT).	Osterman et al., 2012
pANO1::*cda'*	Source for *cda* promoter originating from pColD-CA23.	Norman et al., 2005
pSC101-GFPmut2-TrpT-ΔP-mScarlet-I	TtrpT is inserted between GFPmut2 and mScarlet-I of pSC101-GFPmut2-mScarlet-I. No promoter for mScarlet-I. Red fluorescence negative control.	This study
pSC101-GFPmut2-Pcda-mScarlet-I	SOS response reporter. cda promoter from pANO1::*cda`* inserted in front of mScarlet-I of pSC101-GFPmut2-TrpT-ΔP-mScarlet-I plasmid	This study
pSC101-GFPmut2-PcspA-mScarlet-I	Cold shock reporter. cspA promoter from *E. coli* MG1655 inserted in front of mScarlet-I of pSC101-GFPmut2-TrpT-ΔP-mScarlet-I plasmid.	This study

Table 5: *Primers used for plasmid construction.*

Name	**5'→3' sequence**
term_vector_fwd	CAATATGGTGAGCAAGGGCGAGG
promoter_vector_rev	CTGTCAGGTCATTTCCAAGCTTGTCGA
TestprimerAmp.rev	GACACGGAAATGTTGAATAC
AmpCPEC.for	GGAAGAGTATGAGTATTCAACATTTCCGTGTC
pcda_insert_fwd	TCGACAAGCTTGGAAATGACCTGACAGCGCTCTTCGGCTTCGGTCA
pcda_insert_rev	CCTCGCCCTTGCTCACCATATTGCACCTCCTTGACTTTTAAAACAATGCGTTAAAAACAACAAAC

Name	5'→3' sequence
pcspA_insert_fwd	TCGACAAGCTTGGAAATGACCTGACAGGGAATATAAAGATCCAATGCATGAGCTGTTGA
pcspA_insert_rev	CCTCGCCCTTGCTCACCATATTGCACCTCCTTTAATAATTAAGTGTGCCTTTCGGCG

PCR with vec1 primers was problematic possibly due to difficult DNA secondary structure for Phusion polymerase in the *ampR* region. The TestprimerAmp.rev and AmpCPEC.for primers provided by Arvi Jõers were used to separate the vector into two segments from the *ampR* region.

3.1.5 Plasmid construction

All plasmids were constructed using the Circular Polymerase Extension Cloning (CPEC) method (Quan and Tian, 2009). Shortly, fragments of DNA are PCR-amplified such that neighboring fragments have overlapping complementary ends through primer design. Overlaps with about 60 °C melting temperature (Tm) are aimed for. Fragments of correct size are purified from agarose gel. The purified fragments are mixed for a second round of PCR, where double strands are denatured and during annealing overlapping ends of vector and insert hybridize. DNA polymerase fills the gaps to generate a double-stranded plasmid. CPEC product is then directly transformed into chemically competent cells and plasmid is purified from transformants.

The PCR reaction mix is shown in Table 6 and program steps are shown in Table 7.

Table 6: PCR reaction mix, total volume 50 µl

Component	Amount	Final concentration
Phusion HF buffer (5x)	10 µl	1x
dNTP mix (2 mM each)	5 µl	0.2 mM each
Phusion DNA polymerase (2 U/µl)	0.5 µl	1 U
Forward primer (10 µM)	2.5 µl	0.5 µM
Reverse primer (10 µM)	2.5 µl	0.5 µM
Template plasmid DNA	5 ng	
Milli-Q water	Up to 50 µl	

Table 7: PCR program

Cycle number	**Denaturation**	**Annealing**	**Extension**
1	98 °C, 30 s		
2-34	98 °C, 10 s	63 °C, 30 s	72 °C, 90 s (15 s/kb)
35			72 °C, 5 min

The *cspA* promoter was amplified from MG1655 genomic DNA. The template DNA for PCR was obtained by transferring bacteria from a glycerol stock to 100 µl Milli-Q water with inoculation loop and using 1 µl of this cell suspension for PCR. The denaturation time of the first cycle was extended to 2 minutes in order to lyse the cells.

The PCR result was confirmed by gel electrophoresis. 1% agarose gel was prepared by dissolving 1 g of agarose in 100 ml 1x TAE (Tris-acetate-EDTA) buffer via boiling in the microwave. 3 µl of ethidium bromide was added for DNA visualization. Agarose gel was poured and allowed to solidify. 50 µl of PCR product was mixed with 10 µl of 6x loading dye and loaded into two wells on the agarose gel, 30 µl in each well. 2 µl of GeneRuler 1 kb DNA ladder (Thermo Scientific, United States) was added in a parallel lane for reference. Electrophoresis was performed at 100 V. The band corresponding to correct fragment size was cut from gel on a UV-transilluminator and purified using FavorPrep™ Gel/PCR Purification Kit (Favorgen, Taiwan). Concentrations of gel-purified PCR products were measured with NanoDrop spectrophotometer (Thermo Scientific, United States).

The fragments were combined by CPEC (Quan and Tian, 2009). The reaction mix is shown in Table 8 and program steps in Table 9.

Table 8: CPEC reaction mix, total volume 20 µl

Component	**Amount**	**Final concentration**
Phusion HF buffer (5x)	4 µl	1x
dNTP mix (2 mM each)	2 µl	0.2 mM each
Phusion DNA polymerase (2 U/µl)	0.2 µl	0.4 U
Insert and vector DNA	7-13 ng for insert 30-60 ng for vector fragments	insert/vector molar ratio of 1.5

Table 9: CPEC program

Cycle number	**Denaturation**	**Slow ramp annealing**	**Annealing**	**Extension**
1	98 °C, 30 s			
2-26	98 °C, 10 s	70 to 55 °C, 3 min	55 °C, 30 s	72 °C, 90 s
27				72 °C, 5 min

CPEC product was transformed directly into DH5α chemical competent cells. All reporter construct sequences were verified by sequencing.

3.1.6 Preparing competent cells

Competent cells were prepared according to the OpenWetWare RbCl protocol (RbCl competent cell - OpenWetWare). *E. coli* cells were streaked out from a glycerol stock onto LB-agar plate and incubated overnight at 37 °C. 3 ml of LB was inoculated with a single *E. coli* colony in a test tube and aerobically incubated overnight at 37 °C, 220 rpm with Orbi-Safe incubator (Sanyo, Japan). 1 ml of the overnight culture was inoculated into 100 ml of LB (100x dilution) in a 250 ml flask and incubated at above-mentioned conditions until optical density at 600 nm (OD_{600}) reached about 0.4 (mid-exponential growth phase). The exponential culture was aliquoted into two centrifuge tubes and chilled on ice for 10 minutes followed by centrifugation at 2700 g, 4 °C for 10 minutes. Supernatant was discarded and cells in each resuspended in RF1 buffer (Table 10) and kept on ice for another 15 minutes. Another centrifugation was performed at 580 g for 15 minutes. Supernatant was once again discarded and cells were resuspended in RF2 (Table 11) buffer and kept on ice for 15 minutes. Final competent cells were aliquoted on ice into 200 µl tubes, flash frozen in liquid nitrogen and stored at -80 °C.

Table 10: 100 ml RF1 (RbCl competent cell - OpenWetWare)

Component	**Weight**	**Final concentration**
RbCl	1.21 g	100 mM
$MnCl_2 \cdot 4H_2O$	0.99 g	50 mM
CH_3COOK	0.294 g	30 mM
$CaCl_2 \cdot 2H_2O$	0.148 g	10 mM

Component	Weight	Final concentration
glycerol	15 g	15% w/V

pH is adjusted to 5.8 with 0.2 M acetic acid.

Table 11: 50 ml RF2 (RbCl competent cell - OpenWetWare)

Component	Weight	Final concentration
MOPS	0.105 g	10 mM
RbCl	0.06 g	10 mM
$CaCl_2 \cdot 2H_2O$	0.55 g	75 mM
glycerol	7.5 g	15% wt/vol

pH is adjusted to 6.8 using 1M NaOH.

3.1.7 Transformation, plasmid purification and strain construction

Competent cells were thawed on ice. 10 µl of CPEC product was added to 100 µl of competent DH5α cells for transformation. The cells were incubated on ice for 30 minutes followed by heat shock at 42 °C for 45 seconds on a thermoblock and returned to ice for 5 minutes. 900 µl of LB was added and the culture was incubated on a 37 °C thermoblock with shaking for 45 minutes. Culture was pelleted by centrifugation at maximum speed on a tabletop centrifuge for 30 seconds. Supernatant was discarded, cells were resuspended in 100 µl of LB, plated on LB plates containing 100 µg/ml ampicillin and incubated overnight at 37 °C.

Next day three single colonies were inoculated into 3 ml of LB (with 100 µg/ml ampicillin). The culture was incubated at 37 °C overnight and 100 µl was transferred to a transparent flat-bottomed 96-well microtiter plate. Green (excitation 485 nm, emission 510 nm) and red (excitation 569 nm, emission 600 nm) fluorescence was measured using microplate reader Synergy™ Mx (BioTek, United States). The presence of green fluorescence was confirmed and suitable colonies were selected for future work. Plasmids were purified from the overnight culture using FavorPrep Plasmid Extraction MiniKit (Favorgen, Taiwan). 100 µl of competent *E. coli* MG1655 cells were transformed with 1 ng of purified plasmid as was described above with transformation of DH5α cells.

DMSO (dimythyl sulfoxide) stocks were prepared from MG1655 strains carrying reporter plasmids. Four strains were used for the following work, Scda, Scspa, Sneg and Spos. They harbor the plasmids pSC101-GFPmut2-Pcda-mScarlet-I, pSC101-GFPmut2-PcspA-mScarlet-I, pSC101-GFPmut2-TrpT-ΔP-mScarlet-I and pSC101-GFPmut2-mScarlet-I respectively. Scda and Scspa are the reporters while Sneg and Spos are negative and positive control. DMSO stocks of all four strains were prepared. A 3 ml LB culture started from a single colony was incubated at 37 °C overnight for 16 hours. It was diluted 100x into 10 ml LB and incubated aerobically until optical density at 600 nm (OD_{600}) of 0.5-0.6 was reached. Cultures were chilled on ice. 8% DMSO stocks were prepared on ice, aliquoted into PCR tubes and stored at -80 °C.

3.1.8 MIC measurement

The reporters were tested against six antibiotics, CHL, TET, KAN, NAL, CIP and NOR. In order to determine the range of interesting concentrations, MIC was first measured for all antibiotics with Spos. Spos is the only strain to produce mScarlet-I constitutively and therefore is expected to be most sensitive to antibiotics. MIC was determined according to bioreporter assay experimental procedures. A pre-culture was grown by adding 100 µl of DMSO stock thawed on ice to 3 ml of M9 minimal medium (with 100 µg/ml ampicillin) and incubated at 37 °C until OD_{600} of 0.5. The culture was diluted to OD_{600} 0.1 with M9 medium. Twofold dilutions were prepared on a microtiter plate to achieve a range of final antibiotic concentrations (in µg/ml): 64-0.25 for CHL, TET and KAN, 256-1 for NAL, 1-0.0039 for CIP and 4-0.016 for NOR. 50 µl of diluted culture was added to 50 µl of antibiotic serial dilutions (containing 2x of final concentration of antibiotic). No ampicillin was added to growth medium during MIC measurement. The plate was sealed with Parafilm M, placed in a ziplock bag along with wet towels preheated at 37 °C to reduce evaporation and condensation, and incubated at 37 °C, 380 rpm on a Unimax 1010 (Heidolph Instruments, Germany) shaker-incubator for 20 hours. OD_{600} of each well was measured using Tecan Sunrise™ microplate reader. The minimal antibiotic concentration with no visible growth was determined from the OD values. Growth was defined as OD_{600} 0.07 or higher. Experiment was repeated 3 times for each antibiotic.

3.1.9 Reporter evaluation

The four strains were tested against the six antibiotics in a 60-hour growth experiment. A pre-culture of all strains was grown as described for the MIC measurement and diluted to OD_{600} 0.1. Three 96-well microtiter plates were prepared with two antibiotics per plate. Each antibiotic was tested in combination with all four strains at final concentrations of MIC/2 to MIC/16 using twofold dilutions. 50 µl of diluted culture was added to the same volume of diluted antibiotic (containing 2x of final concentration of antibiotic). A no-antibiotic control was present with 50 µl of culture added to 50 µl of medium without antibiotics. Edge wells of plates were filled with 200 µl plain medium as an attempt to reduce the effect of evaporation. Aside from the pre-culture, no ampicillin was added to growth media.

The plates were incubated for 60 hours on Stuart Microtitre Plate Shaker Incubator SI505 (Cole-Parmer, UK) at 37 °C and 750 rpm in a humidified environment. Fluorescence and OD600 measurements were taken with Synergy Mx every 12 hours with 5 measurements in total, first one taken at 12 hours. Green fluorescence was measured at 485/9 nm excitation (485 nm with 9 nm bandwidth), 510/9 nm emission (gain 80) and red at 569/13.5 nm excitation, 600/17.0 nm emission (gain 100).

The experiment was performed in three repeats. Data was processed with NumPy python library and graphs were generated using Matplotlib. The average OD_{600} of media was calculated for edge well readings and subtracted from all OD_{600} readings. Same was done for green and red fluorescence. The following was then performed for each repetition, timepoint, antibiotic and antibiotic concentration. The ratio of red to green fluorescence was calculated for each strain. The percentage of the fluorescence ratio for a reporter strain relative to Spos strain yielded the Spos-relative reporter signal. Induction factor (F_i) was calculated by dividing the antibiotic-induced Spos-relative signal to that of the no-antibiotic control. Thus, F_i is a unitless quantity describing the fold-induction of reporter signal above the no-antibiotic control. The average and standard deviation for the three repetitions was found.

3.2 Results

3.2.1 Constructed reporters

Three plasmids were constructed with CPEC. First, pSC101-GFPmut2-TrpT-ΔP-mScarlet-I was derived from pSC101-GFPmut2-mScarlet-I by inserting TtrpT terminator between GFPmut2 and mScarlet-I genes. pSC101-GFPmut2-Pcda-mScarlet-I and pSC101-GFPmut2-PcspA-mScarlet-I were constructed by inserting the corresponding promoter into pSC101-GFPmut2-TrpT-ΔP-mScarlet-I in front of mScarlet-I gene (Figure 6). *cda* and *cspA* promoters were amplified from the pANO1::*cda'* plasmid and *E. coli* MG1655 genomic DNA respectively. The constructs were verified by sequencing. Detailed plasmid construction history is available upon request from the author / supervisor.

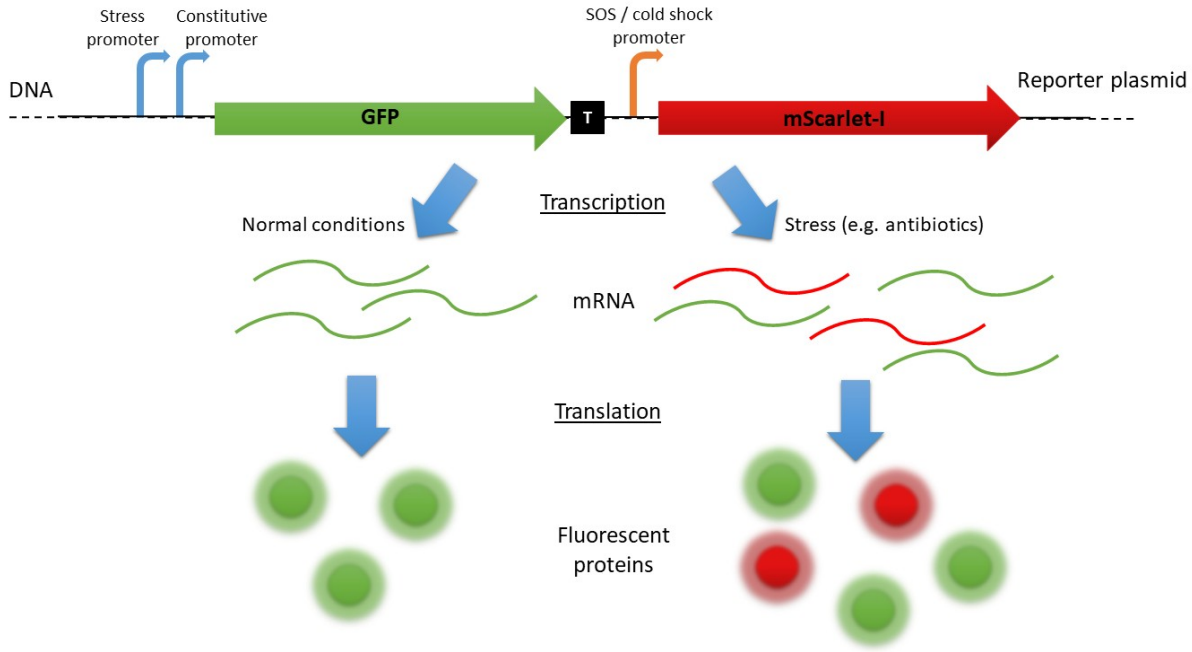

Figure 6: Working principle of the bioreporter. GFP is expressed under a constitutive promoter and controls for antibiotic growth inhibition. A terminator (T) follows GFP and mScarlet-I is expressed from a separate transcript under SOS / cold shock promoter. Genotoxic and C group antibiotics induce SOS and cold shock respectively, increasing the expression of mScarlet-I mRNA and protein. The ratio of mScarlet-I / GFP fluorescence is measured as the reporter signal.

The working principle of the constructed reporters is shown in Figure 6. The red fluorescent reporter protein mScarlet-I is expressed from *cda* and *cspA* promoters while green fluorescent

control protein (GFPmut2) is produced constitutively. Genotoxins and C group translation inhibitors induce SOS and cold shock respectively, which increases mScarlet-I production. Green fluorescence on the other hand is expected to decrease along with general inhibition of growth by antibiotics. Both effects increase the ratio of red to green fluorescence, which is calculated as reporter signal. With near-MIC antibiotic concentrations, GFPmut2 production can drop close to zero, driving up signal uncertainty. This is alleviated by an additional stress promoter for GFPmut2.

3.2.2 MIC measurement

The MICs observed for the Spos strain are shown in Table 12. The presented values were observed at least twice out of 3 experiment repetitions.

Table 12: MIC values for the Spos strain.

Antibiotic	CPH	TET	KAN	NAL	CIP	NOR
MIC (µg/ml)	32	16	8	128	0.25	1

3.2.3 *cda* reporter

Figure 7A shows the response of the Scda reporter to all tested antibiotics after 24 hours of incubation. As expected, SOS response is induced by the quinolones NOR, CIP and NAL. The strongest response is due to NOR. At the highest concentration, NOR induces reporter signal 325x stronger than the no-antibiotic control. The slight increase of NOR signal from MIC/2 to MIC/4 indicates that the reporter is saturated at such high concentrations. A significant signal 114-fold above the no-antibiotic control is still produced at the lowest concentration, suggesting that even much lower concentrations can be detected. Panel 7B models reporter signal as a function of log2(concentration) with linear regression. Panel 7C shows OD_{600} of Scda after 24 hours of incubation with different concentrations of NOR and NAL. As expected, there is stronger growth inhibition with higher concentrations and the inhibition is similar with other antibiotics.

3.2.4 *cspA* reporter

Figure 8A shows dose-dependent response of the Scspa reporter to all tested antibiotics after 48 hours of incubation. As expected, the cold shock response is induced by the C group

antibiotics CPH and TET. But interestingly, there also appears to be a response to fluoroquinolones NOR and CIP.

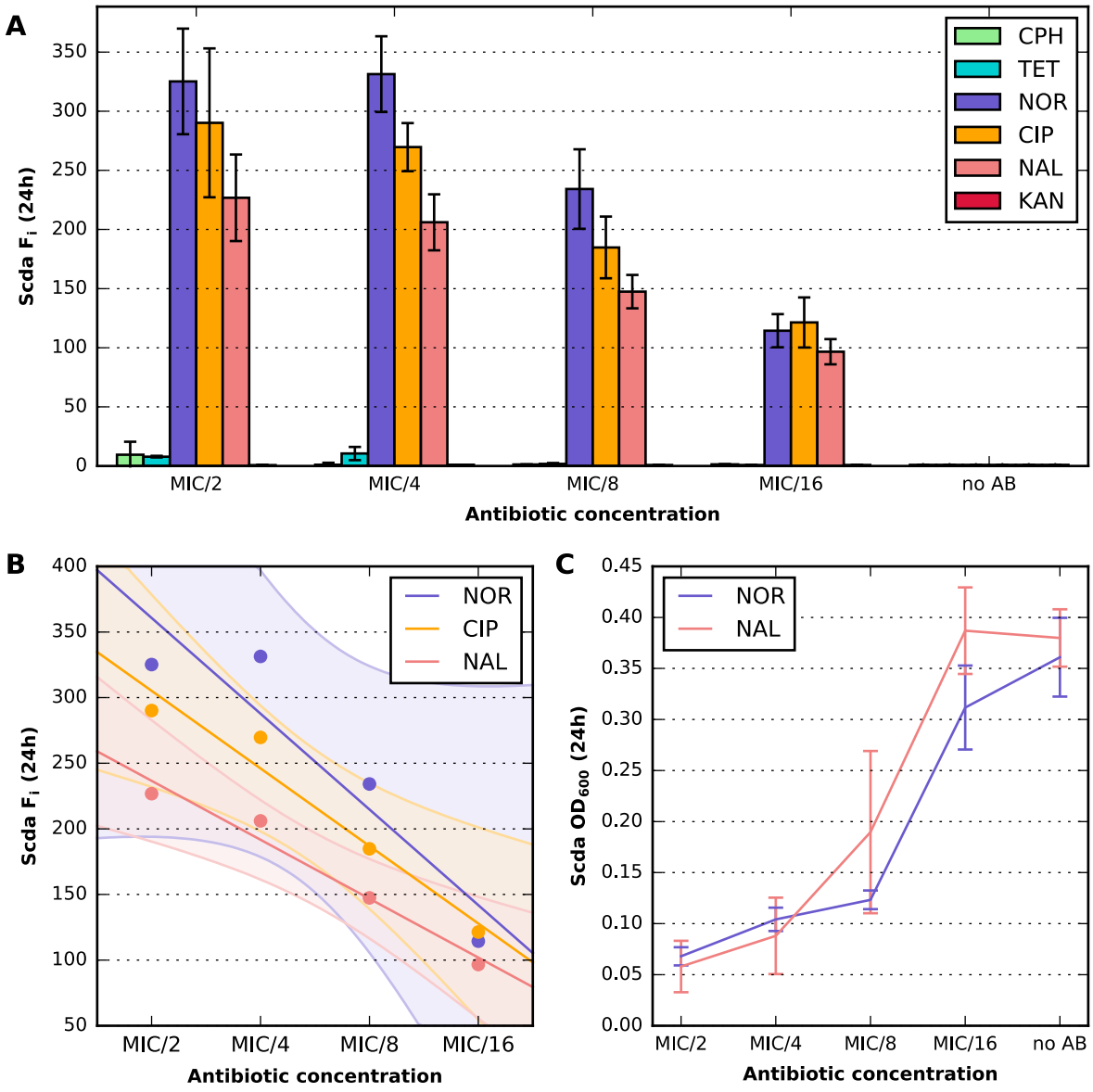

Figure 7: Scda reporter response to six antibiotics. MIC of each antibiotic was measured with the Spos strain and the reporter was incubated with antibiotic concentrations from MIC/2 to MIC/16 at 37 °C for 48 hours. F_i was calculated as the ratio of red to green fluorescence relative to the no-antibiotic control. ***(A)*** *Response of Scda to four concentrations of six antibiotics.* ***(B)*** *Linear regression displays concentration-dependent response to the quinolones NOR, CIP and NAL. The shaded areas display 95% confidence bands.* ***(C)*** *Growth inhibition due to antibiotics.*

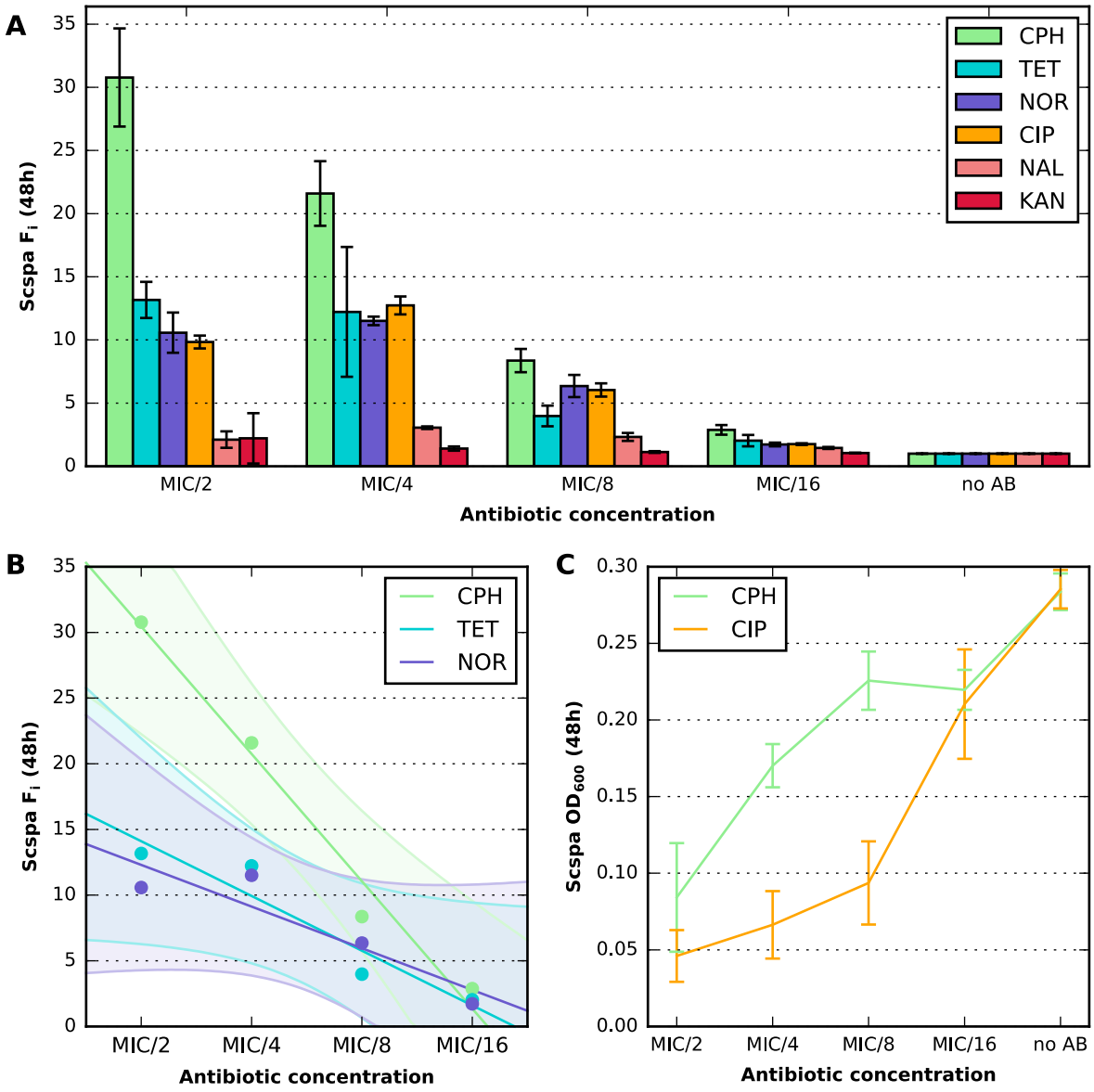

Figure 8: Scspa response to six antibiotics. MIC of each antibiotic was measured with the Spos strain and the reporter was incubated with antibiotic concentrations from MIC/2 to MIC/16 at 37 °C for 48 hours. F_i was calculated as the ratio of red to green fluorescence relative to the no-antibiotic control. ***(A)*** *Response of Scspa to four concentrations of six antibiotics.* ***(B)*** *Linear regression displays concentration-dependent response to CPH, TET and NOR. The regression of CIP (not shown) is similar to that of NOR. The shaded areas display 95% confidence bands.* ***(C)*** *Culture OD_{600} after 24 hour incubation with a range of CPH and CIP concentrations.*

Scspa responses are an order of magnitude weaker than Scda responses, yet still much higher than the no-antibiotic control. CPH shows the strongest concentration dependent response. Other responders, TET, NOR and CIP display saturation close to MIC similar to Scda, but

otherwise show a decrease in F_i in along with the concentration. As with Figure 7, panels 8B and 8C show linear regression and OD_{600} for selected antibiotics.

3.2.5 Time-dependent response

In figures 7 and 8 the 24h and 48h timepoints chosen for Scda and Scspa respectively display the clearest concentration-dependent response. For Scda, the five measured timepoints from 12h to 60h are rather similar. In contrast, Scspa exhibits more variation. There is a general upward trend going from 12h to 24h, indicating that full signal strength has not yet developed by 12 hours. From 24 hours onward the responses remain fairly stable. As an exception, CPH signal with MIC/2 concentration peaks at 48 hours (Figure 9). Complete data with all antibiotics and all timepoints is shown in Appendix 2.

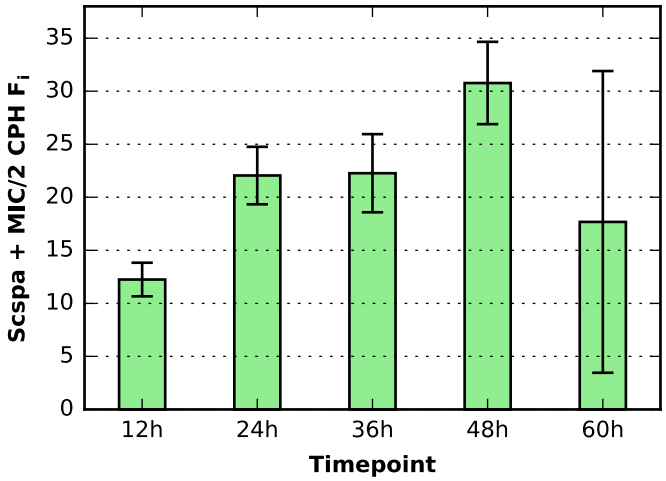

Figure 9: Scspa shows time-dependent response with CPH at half-MIC concentration, while other combinations of reporter, antibiotic and antibiotic concentration are less variable in time. MIC/2 corresponds to 16 µg/ml of CPH and F_i is calculated as the ratio of red to greed fluorescence relative to no-antibiotic control.

3.2.6 Spos-relative signal

During antibiotic-induced stress, the reporters produced signals many times higher than the Spos positive control in response to some antibiotics (Figure 10). The strongest response displayed by Scda was ~7x above Spos after 60 hours of incubation with NOR. For Scspa, the highest was ~1.9x above Spos signal for 48 hours of incubation with CPH. Yet, with the no-antibiotic control, Scda and Scspa were induced only at 2.3% and 6.2% of Spos respectively. This corresponds to the leakiness of the promoters under stress-free conditions.

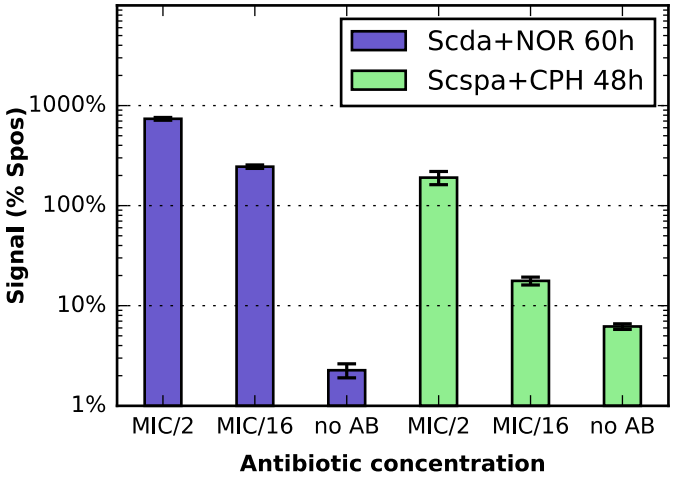

Figure 10: Response of Scda and Scspa reporters relative to the Spos positive control strain. The ratio of red to green fluorescence of Scda reporter is shown as percentage of that of Spos strain in response to NOR concentrations MIC/2 and MIC/16 and the no-antibiotic control after 60 hours of incubation (green). Same is shown for the Scspa reporter in response to CPH with 48 hours of incubation (turquoise). The 60h NOR and 48h CPH combinations shown induced the strongest response relative to Spos in Scda and Scspa respectively.

The Sneg negative control strain was used to assess the red autofluorescence of cells. Red autofluorescence remained within limits of measurement noise under experimental conditions and was not accounted for during data analysis.

3.3 Discussion

Both constructed reporters respond well to predicted antibiotics, Scda to quinolones and Scspa to C group translation inhibitors. As expected, neither of them reacts to KAN. Interestingly, Scspa also responds to the fluoroquinolones CIP and NOR (Figure 8). The implication that fluoroquinolones induce *cspA* expression doesn't appear to be confirmed by literature and is currently dubious. If confirmed, this serendipitous finding might shine new light on the already well-studied control of CspA expression. Induction may be through known mechanisms like *cspA* mRNA stability, translation efficiency or due to something entirely new.

As a comparison of *cda*-based reporters, Scda and another reporter developed by Norman et al., 2005 were tested with a similar concentration of NAL. Scda produced 6x stronger signal relative to no-antibiotic control for 1.7x higher NAL concentration. An exact cause for this can't be pinpointed due to many design differences between the two reporters. While Scda controls for general inhibition of gene expression with the green fluorescent control protein, the reporter by Norman et al. controls for growth inhibition with OD_{600}. Scda is based on the low-copy pSC101 plasmid, which might result in a lower basal expression from the *cda* promoter (Norman et al., 2005).

The constructed Scspa reporter is comparable to another one developed by Bianchi and Baneyx, 1999, which was tested with CPH, TET and NAL. Scspa produces comparable results with similar concentrations (relative to no-antibiotic control), but this is likely to be coincidental as Scspa relies heavily on the differential expression of two fluorescent proteins while the reporter by Bianchi and Baneyx is based on *lacZ* and doesn't account for general inhibition of gene expression. For example, with TET comparison, lacZ signal is entirely due to increased expression from *cspA* promoter relative to no-antibiotic control while Scspa signal is entirely due to drop in green fluorescence.

Scda signal is already fully developed in the first timepoint at 12 hours (Figure 15), while for Scspa development takes between 12 and 24 hours (Figure 16). Experiments with finer time granularity are needed to determine the minimum incubation time required for Scda. Short incubation time is beneficial for infection experiments and may yield a stronger signal. Indeed, the reporter by Norman et al. reached maximum values at around 2 hours and showed decline after 3 hours. The currently used low granularity of 12 hours is partly due to

fluorescence measurements requiring a certain culture density with a microtiter plate. Flow cytometry or fluorescence microscopy can be used for improved granularity, sensitivity and to examine sub-populations. But the current method is simple, cheap and good enough for preliminary screening.

The lowest concentration of quinolones tested with Scda (MIC/16) produced responses two orders of magnitude above the no-antibiotic control (Figure 7), indicating that much smaller amounts are likely detectable. Both reporters display saturation at concentrations close to MIC. This may be due to induction of the dnaK1 promoter of GFPmut2 at such high concentrations, lowering the ratio of red to green fluorescence. Additional antibiotic concentrations should be tested to find the limits of detection and a precise calibration curve. Unfortunately, the 96-well microtiter plates used provide limited space for testing many strains, antibiotics, concentrations and media. Also, evaporation is an issue to a varying degree with different incubators.

The ability of constructed reporters to produce fluorescent signal in complex infection environments should be assessed, starting from simpler *in vitro* models and moving to mouse models. The reporters could then be used for measuring antibiotic concentration reaching a particular tissue, investigating causes of failed antibiotic therapy and devising more effective treatments.

Summary

Whole-cell bioreporters are a promising tool for detecting bioavailable fraction of antibiotics in environmental samples and classifying them according to mechanism of action. They can be used to measure antibiotic concentrations with *in vitro* and *in vivo* infection models, for which there is currently a lack of accurate single cell level tools.

In this study, two reporters were constructed using the *cda* and *cspA* stress promoters. The *cda* promoter is induced by genotoxins and *cspA* promoter by C group translation inhibitors. Red fluorescent protein mScarlet-I was placed under the control of stress promoters and green fluorescent protein GFPmut2 under constitutive promoter to control for general inhibition of gene expression by antibiotics. The ratio of red to green fluorescence relative to no-antibiotic control was calculated as the signal of the reporters.

Each reporter was tested with six antibiotics – kanamycin, C group translation inhibitors chloramphenicol and tetracycline and genotoxins nalidixic acid, ciprofloxacin and norfloxacin. MIC was identified for each antibiotic and the response of reporters to concentrations from MIC/2 to MIC/16 was assessed. Five timepoints from 12 hours to 60 hours were examined to narrow down the optimal incubation time.

Both reporters work well. Of all antibiotics tested, *cda*-based reporter responds only to genotoxins as expected. It yields high signals 100 to 340 times stronger than the no-antibiotic control. Full signal is measurable with a microtiter plate reader within 12 hours of incubation. *cspA*-based reporter responds to chloramphenicol and tetracycline as expected, but unexpectedly also to fluoroquinolones ciprofloxacin and norfloxacin. It takes 24 hours of incubation to measure full signal. Basal expression with no antibiotic remains low for both reporters with red to green fluorescence ratio below 3% of positive control for Scda and below 7% for Scspa.

In conclusion, the two reporters constructed are suitable for further experiments with infection models, in search for new antibiotics and for classification of the two mechanisms of action.

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Appendix 1 – plasmid maps

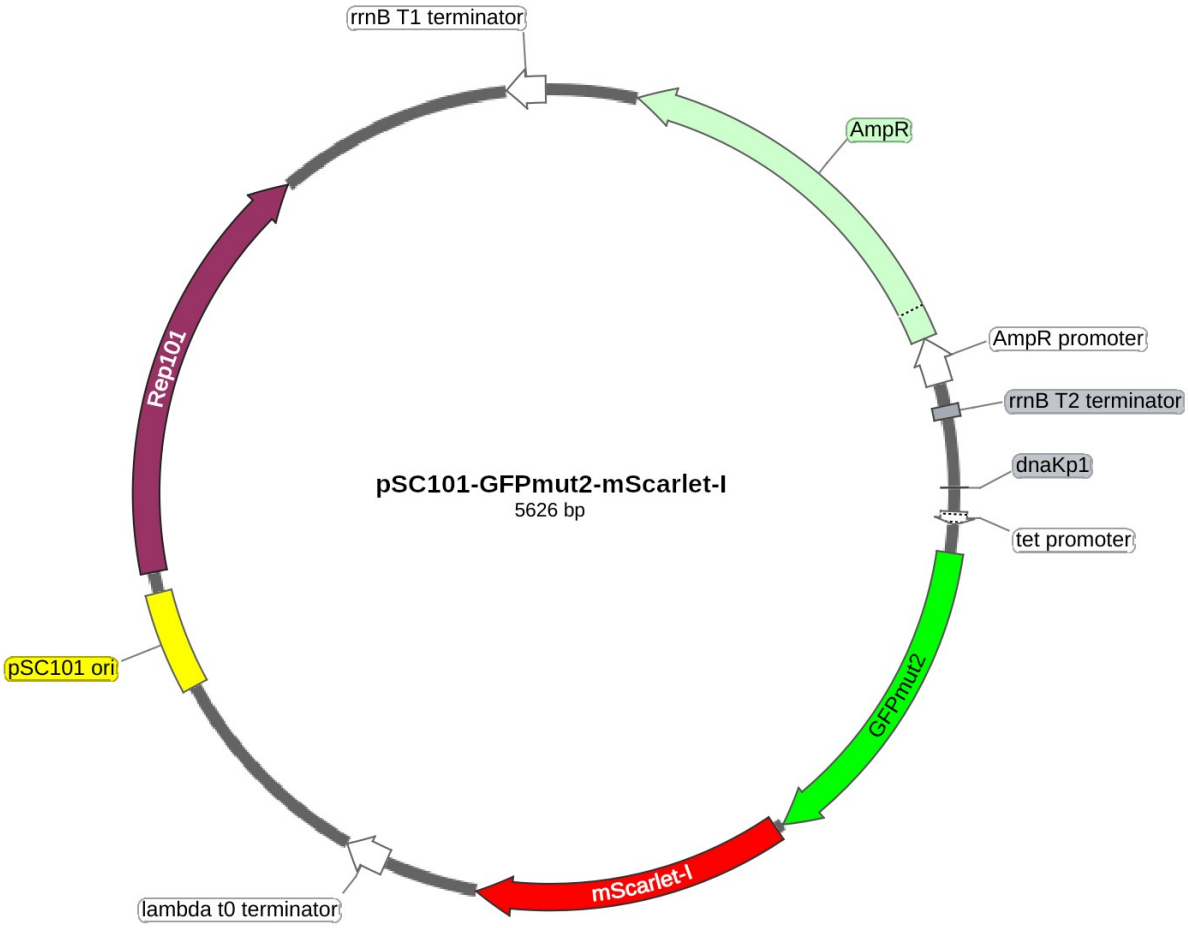

Figure 11: The initial pSC101-GFPmut2-mScarlet-I plasmid (positive control) contains GFPmut2 and mScarlet-I under the expression of constitutive tet-promoter and stress-induced dnaK1 promoter.

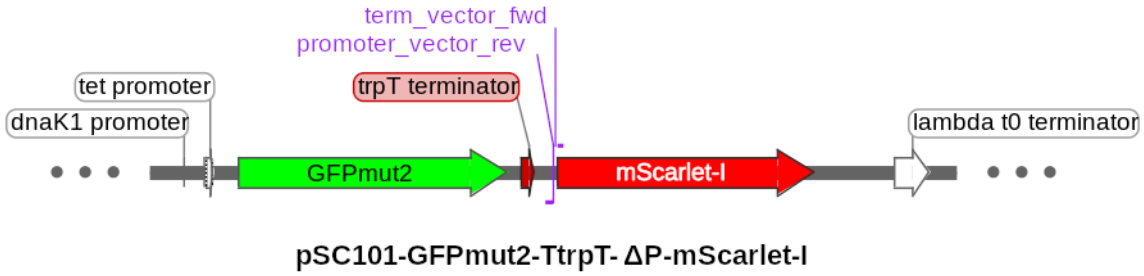

Figure 12: Negative control plasmid with insertion of trpT terminator (TtrpT) between GFPmut2 and mScarlet-I. The TtrpT insert originates from pRFPCER-TrpL2A of *Osterman et al. 2012. Also show are term_vector_fwd and promoter_vector_rev primers used for insertion of mScarlet-I promoters.*

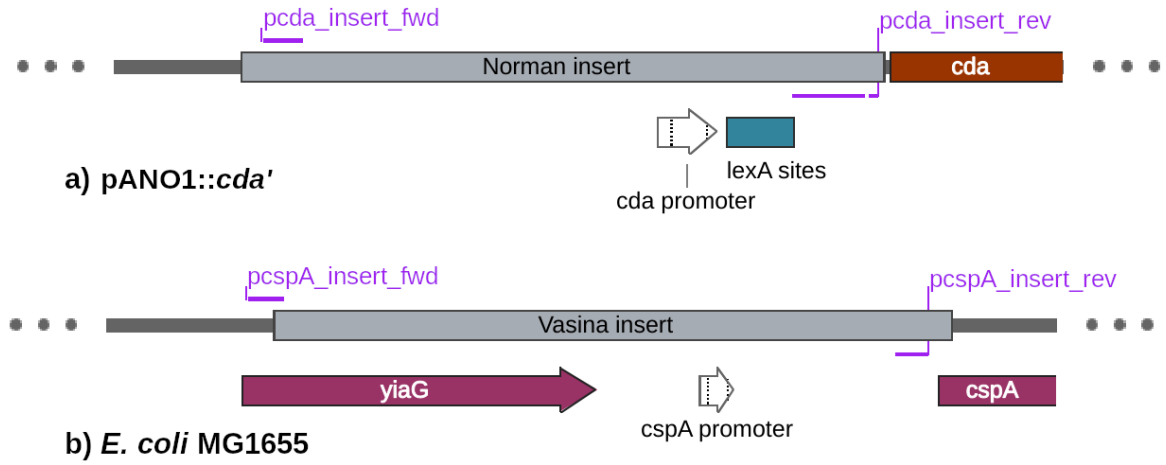

Figure 13: Amplification of a) cda promoter insert from pANO1::cda` plasmid and b) cspA promoter insert from E. coli MG1655 genomic DNA. Binding sites of primers are shown. For comparison, the region used for cda promoter by Norman et al. 2005 and the one for cspA promoter by Vasina and Baneyx 1996 are shown.

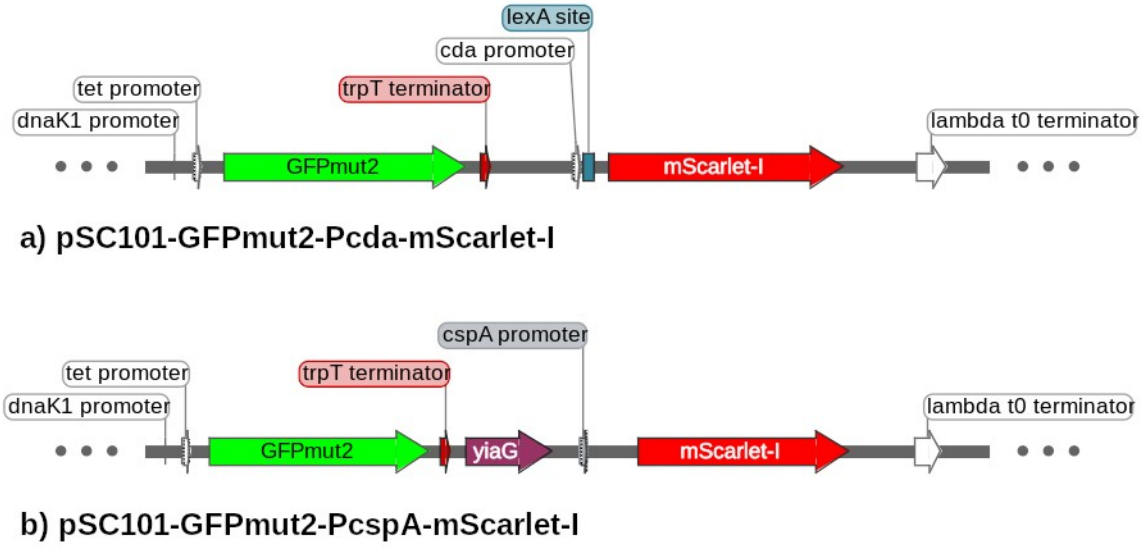

Figure 14: The bioreporter constructs. GFPmut2 is under the control of constitutive tet-promoter and stress-induced dnaK1 promoter. mScarlet-I is under the control of a) cda and b) cspA promoter.

Appendix 2 – reporter antibiotic response charts

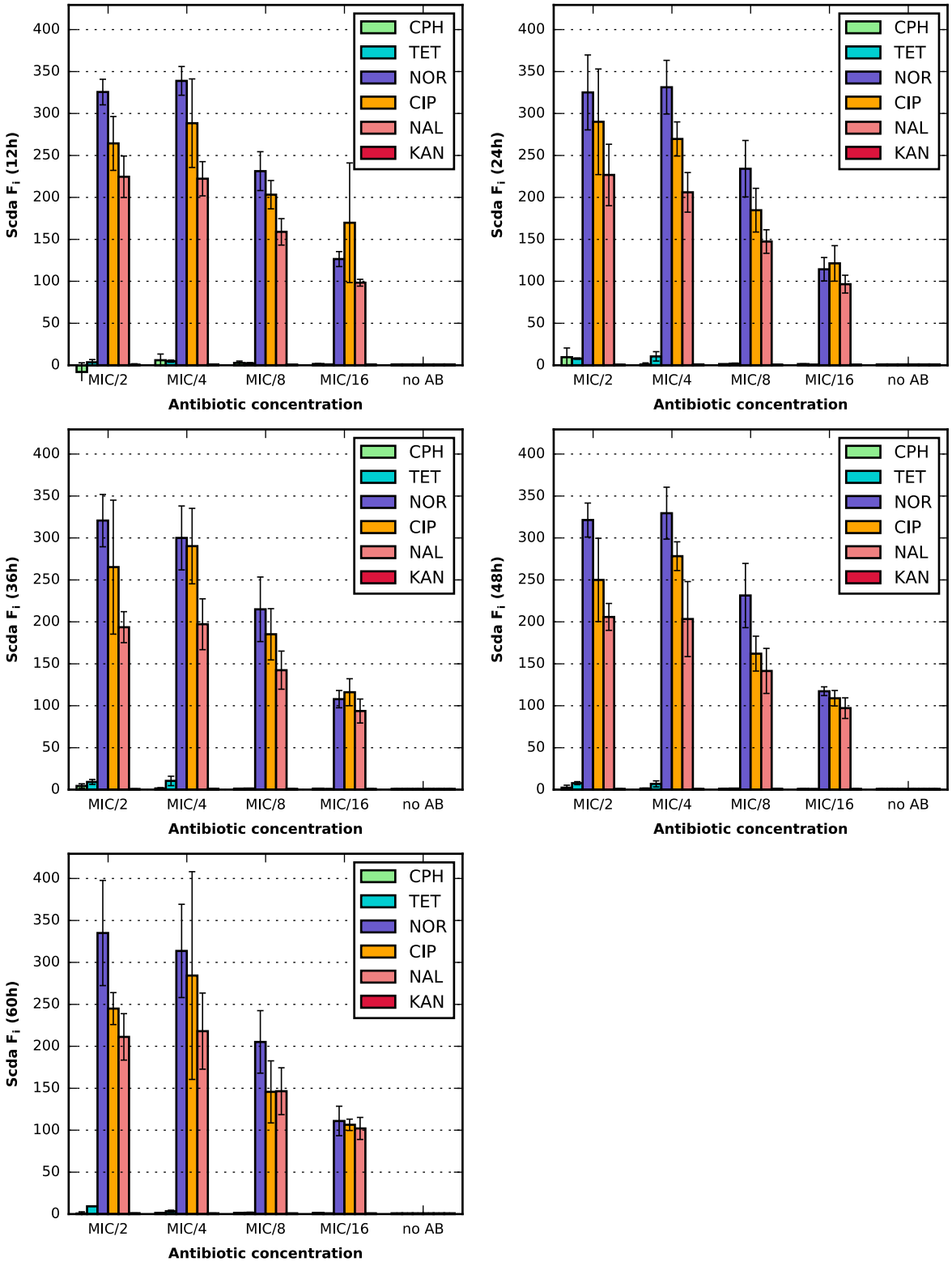

Figure 15: Response of cda reporter to 4 concentrations of 6 antibiotics at 5 timepoints. F_i is calculated as red to green fluorescence relative to the no-antibiotic control.

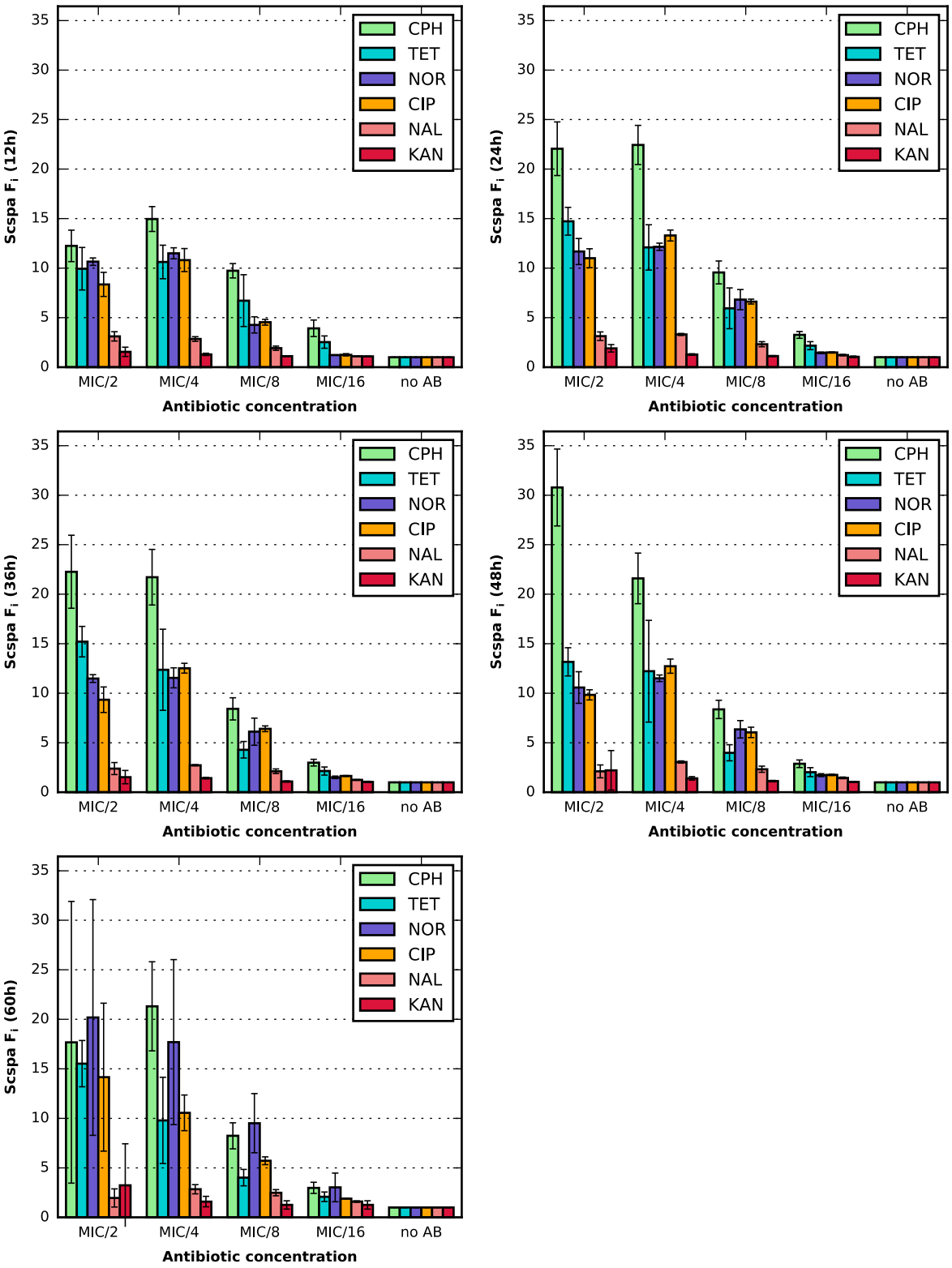

Figure 16: Response of cspA reporter to 4 concentrations of 6 antibiotics at 5 timepoints. F_i is calculated as red to green fluorescence relative to the no-antibiotic control.

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