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Activation of Phenotypic Subpopulations in Response to Ciprofloxacin Treatment in *Acinetobacter baumannii*

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Summary

The multidrug-resistant, opportunistic pathogen, *Acinetobacter baumannii*, has spread swiftly through hospitals worldwide. Previously, we demonstrated that *A. baumannii* regulates the expression of various genes in response to DNA damage. Some of these regulated genes, especially those encoding the multiple error-prone DNA polymerases, can be implicated in induced mutagenesis, leading to antibiotic resistance. Here, we further explore the DNA damage-inducible system at the single cell level using chromosomal transcriptional reporters for selected DNA damage response genes. We found the genes examined respond in a bimodal fashion to ciprofloxacin treatment, forming two phenotypic subpopulations: induced and uninduced. This bimodal response to ciprofloxacin treatment in *A. baumannii* is unique and quite different than the *Escherichia coli* paradigm. The subpopulations are not genetically different, with each subpopulation returning to a starting state and differentiating with repeated treatment. We then identified a palindromic motif upstream of certain DNA damage response genes, and have shown alterations to this sequence to diminish the bimodal induction in response to DNA damaging treatment. Lastly, we are able to show a biological advantage for a bimodal response, finding that one subpopulation survives ciprofloxacin treatment better than the other.

Introduction

Acinetobacter baumannii has gained notoriety in recent years as a nosocomial pathogen (Munoz-Price & Weinstein, 2008). Causing pneumonia, meningitis and severe wound infections in immunocompromised patients (Dijkshoorn *et al.*, 2007), *A. baumannii* is able to persist in the hospital environment by resisting disinfection and desiccation (Jawad *et al.*, 1998, Musa *et al.*, 1990). Compounding the problem, antibiotic resistance is on the rise among *A. baumannii* strains, with some emerging strains showing resistance to all clinically relevant antibiotics (Doi *et al.*, 2009, Park *et al.*, 2009).

It has been shown that the genome of *A. baumannii* is readily changeable through the incorporation of insertion sequences, transposons and genetic material horizontally

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transferred from other organisms (Peleg *et al.*, 2008, Mugnier *et al.*, 2010). This ease of acquisition and incorporation of foreign genetic material facilitates the rapid adjustment of this bacterium to new antibiotic treatments and ultimately determines its success as a pathogen (Hornsey *et al.*, 2011, Hua *et al.*, 2012, Adams *et al.*, 2010). While other organisms utilize numerous systems to preserve genomic stability, *A. baumannii* appears to favor genomic plasticity. With this knowledge, it was intriguing that *A. baumannii* appears to lack a DNA damage response system, known to contribute, in other systems, to mutagenesis and genetic flexibility under certain conditions (Ubeda *et al.*, 2005, Friedberg *et al.*, 2006). Such systems have been researched extensively, *Escherichia coli* being the paradigm, in which over 40 genes, coding for proteins predominantly involved in DNA repair, mutagenesis and growth cycle arrest are simultaneously regulated by the global regulator LexA (Courcelle *et al.*, 2001, Little *et al.*, 1980, Little *et al.*, 1981). In *E. coli*, DNA damage response induction begins with the DNA damage-dependent accumulation of single stranded DNA (ssDNA). RecA coats this ssDNA, forming the RecA nucleoprotein filament. Finally, interaction of LexA with the RecA nucleoprotein filament promotes cleavage of the DNA damage response repressor, LexA, resulting in the concerted induction of the DNA damage response genes in both whole populations and individual cells (Walker *et al.*, 1985, Friedman *et al.*, 2005).

Initially, it was thought that there was no DNA damage response system in *Acinetobacter* spp. due to the lack of genes normally associated with this type of response, such as the global repressor, *lexA* and the cell division factor, *sulA* (Robinson *et al.*, 2010). However, several groups have linked the induction of certain genes to DNA damage within the *Acinetobacter* genus (Rauch *et al.*, 1996, Hare *et al.*, 2006, Aranda *et al.*, 2013) and have shown the capacity for DNA damage-induced mutagenesis within the *A. baumannii* species (Hare *et al.*, 2012). We have recently shown the RecA-dependent regulation of multiple copies of genes encoding error-prone, Y-family DNA polymerases in response to DNA damage in *A. baumannii*. Additionally, these proteins seem to be involved in DNA damage- or desiccation-induced mutagenesis, leading to antibiotic resistance (Norton *et al.*, 2013). Because the *A. baumannii* *recA* strain we used is not a complete deletion, we also observed elevated *recA* transcript in the absence of a functional RecA protein, contradicting conventional DNA damage system induction. Though the full elucidation of the DNA damage system in *A. baumannii* is unfinished, the lack of a LexA-like repressor, the existence of multiple copies of error-prone polymerases and the RecA-independent induction of *recA*, indicate that *A. baumannii* possesses a DNA damage response that is different from the defined *E. coli* paradigm (Norton *et al.*, 2013). A recent work by Aranda *et al.*, suggested that the A1S_1389 gene might be the missing LexA. However, the authors indicate it is likely not as the *E. coli* paradigm LexA global repressor since it functions as an activator of other DNA damage response genes (Aranda *et al.*, 2013).

In the present study, we used single-cell analyses to investigate the potential for a unique mode of regulation of several DNA damage-inducible genes in *A. baumannii* in response to DNA damaging treatment. Using green fluorescent protein (GFP) fused to DNA damage-inducible gene promoters, we studied the induction of DNA damage response genes in individual cells in response to treatment with the antibiotic ciprofloxacin, which inhibits

DNA gyrase and causes DNA double strand breaks (Chen *et al.*, 1996), as well as the alkylating agent, methyl methanesulfonate (MMS). Using this technique, we were able to detect a bimodal pattern of induction in response to DNA damage that could contribute to the rapid adaptability of *A. baumannii*. Additionally, we identified a palindromic regulatory motif upstream of the DNA damage response genes *umuDC* and *ddrR*, representing a potential binding site for regulator(s) involved in this response. We found that changes to the sequence of this conserved motif greatly reduced the bimodal induction of a selected *umuDC* operon, indicating its importance *in vivo*.

Results

***A. baumannii* cells differentiate into phenotypic subpopulations in response to ciprofloxacin treatment**

Our previous work indicates that the DNA damage response in *A. baumannii* is likely different than the *E. coli* paradigm (Norton *et al.*, 2013). Here, we sought to explore the response further by analyzing the induction of several DNA damage response genes using sensitive single cell analyses with flow cytometry. We constructed reporter strains in *A. baumannii* containing the putative promoter region of the gene of interest fused to green fluorescent protein (GFP) at the natural chromosomal locus (Fig. S1) for *recA* (A1S_1962, Recombinase A), *dinB* (A1S_0186, DNA Polymerase IV), *umuDC* (A1S_0636-0637, DNA Polymerase V), *uvrA* (A1S_3295, UvrABC endonuclease), and a non-DNA damage inducible gene control, *trpB* (A1S_1692, Tryptophan synthase subunit B). Downstream of the GFP gene, the putative promoter region is duplicated leading into the wild-type gene on the chromosome, thus avoiding any disruption of the natural promoter and gene.

The reporter strains were untreated or treated with varying concentrations of ciprofloxacin (cip) at the beginning of exponential growth for 3 hours. Treatment levels were calculated based on the minimum inhibitory concentration (MIC) of cip in *A. baumannii* ($0.6 \mu\text{g mL}^{-1}$), with each strain experiencing treatment ranging from sub-inhibitory to inhibitory ($0.3\times$, $1\times$, $3\times$ and $10\times$ the MIC). Strains were analyzed for gene induction (i.e. fluorescent levels) directly before treatment (T=0), at 90 minutes (T=90), and after 180 minutes (T=180) of treatment. The end-point was selected based on whole population survival curves in response to $10\times$ the MIC cip in *A. baumannii* and *E. coli* (Fig. S2).

At T=0, reporter strains for *recA*, *dinB*, *umuDC*, *uvrA* and *trpB* were largely unimodal and non-fluorescent for all treatment conditions, indicating no induction of the genes (Fig. 1). Over the 180-minute time-course, and in response to varying levels of cip, the *recA*, *dinB*, *umuDC* and *uvrA* reporter strains followed a similar trend of differentiation into two subpopulations, both dim and bright. Generally, increasing treatment time and increasing cip concentration led to a higher proportion of bright cells and a decreasing proportion of dim cells, though some genes were more highly induced under lower concentrations of cip, such as *umuDC* and *uvrA*. The reporter strain for *trpB* was not induced by any concentration of cip and remained largely dim throughout the time course (Fig. 1). Uniform, whole population fluorescence was observed for this strain however, after nutrient starvation on Nematode Growth Medium (NGM) plates using microscopy, indicating that the *trpB* fusion is indeed functional (Fig. S3).

We next compared this mode of induction in response to DNA damaging treatment to that of a comparable *E. coli* gene. Though the induction of DNA damage response genes has been explored extensively in *E. coli* (Anderson & Kowalczykowski, 1998, Fijalkowska *et al.*, 1997, Sutton *et al.*, 2000, Pennington & Rosenberg, 2007), we performed the same single-cell analysis described above on an *E. coli* *dinB* (b0231) reporter strain containing a plasmid-borne promoter-GFP fusion to compare it to *A. baumannii*. The *E. coli* *dinB* reporter strain was treated with the same range of cip concentrations, relative to its own MIC ($0.01\mu\text{g mL}^{-1}$). When analyzed using flow cytometry at T=0, the *Ec* *dinB* reporter showed one largely dim population under all treatment conditions (Fig. 2A), much as in the *A. baumannii* reporters. However, with increasing time and increasing cip concentration, the *Ec* *dinB* reporter strain shifted into the fluorescent range, showing a unimodal distribution, compared to the bimodal shift seen in *A. baumannii*. This unimodal shift in *E. coli* in response to DNA damaging agents agrees with data shown by Pennington, et al. in 2007, where the addition of a DNA damaging agent creates a unimodal induction of DNA damage response genes (Pennington & Rosenberg, 2007).

To ensure that the *E. coli* *dinB* fusion was not affected by the use of a plasmid carrier, we also constructed an *E. coli* *dinB* protein fusion to GFP, expressed from the chromosome. We used the chromosomal protein fusion rather than the chromosomal promoter fusion because the promoter fusion was not stable in *E. coli* for this gene. This chromosomal protein fusion strain was untreated or treated with 10× the MIC cip for 240 minutes and analyzed by flow cytometry. As with the plasmid-borne reporter, the *dinB* protein-GFP strain showed a unimodal distribution. Without treatment, a single, wide peak formed in the dim range (Fig. 2B). With treatment, again a single peak formed. This peak was more tightly composed and shifted into the fluorescent range (Fig. 2B).

Finally, to further ensure that the bimodal induction seen in *A. baumannii* was different than the induction of the DNA damage response in *E. coli* regardless of the location of the fusion, we analyzed induction of a different *E. coli* DNA damage induced gene, *sulA*, with a promoter fusion expressed both from the chromosome and from a plasmid. As before, these strains were untreated or treated with 10× the MIC cip for 180 minutes before analysis. When the fusion was expressed both from the chromosome and from a plasmid, we saw a unimodal distribution of cells. The single peak was in the dim range for the untreated cells and shifted into the bright range for the treated cells (Fig. 2C). Taken together, these data suggest that whether expressed from a plasmid, or the chromosome, *E. coli* reporters for DNA damage genes show one main population of cells in response to damage, while *A. baumannii* forms two subpopulations.

We next wanted to determine if subpopulation formation in *A. baumannii* was resulting from genetic changes that could be passed to future generations.

Bimodal response to ciprofloxacin treatment is not heritable

To attribute subpopulation formation in *A. baumannii* to a genotypic or phenotypic change, we determined if the characteristics of each subpopulation were passed to future generations. We hypothesized that a genetic change or mutation would persist in future generations, causing dim-sorted cells to all remain dim and bright-sorted cells to all become bright upon

recurrent challenge. Conversely, should the differentiation be caused by a phenotypic change, both the dim and bright subpopulations should again be able to generate two subpopulations upon treatment. We treated the *A. baumannii* *dinB*, *umuDC* and *uvrA* reporter strains with 10× MIC cip for three hours, as before. These strains were then sorted by fluorescence into dim and bright subpopulations (Fig. 3A). Each subpopulation was then inoculated into fresh medium and recovered for 20 hours. The recovered subpopulations for each reporter were then treated with a second round of 10× MIC cip for 3 hours, and analyzed by flow cytometry directly before and after treatment. After the initial overnight recovery, both subpopulations had returned to a unimodal, dim state (Fig. S4). After 3 hours of cip treatment, both bright and dim subpopulations again differentiated into two subpopulations, bright and dim (Fig. 3B). This result thus established the subpopulation formation was not heritable to future generations and was reversible upon removal of cip, allowing each subpopulation to differentiate to bright and dim cells.

We then sought to determine if the bimodal response to DNA damage was caused only by treatment with cip or if it resulted from other types of damaging treatment as well.

***A. baumannii* response to methyl methanesulfonate treatment varies depending on gene analyzed**

To determine if the differentiation into subpopulations was caused exclusively by cip treatment, or if it was a more general response to DNA damage, we treated several of the *Ab* promoter fusion strains (*Ab recA*, *Ab dinB*, *Ab umuDC*, *Ab uvrA* and *Ab trpB*) with the alkylating agent, methyl methanesulfonate (MMS) and analyzed fluorescence.

Reporter strains were treated with 1× the MIC MMS at the beginning of exponential growth for 60 minutes and then analyzed by flow cytometry. Interestingly, the population dynamics in response to MMS varied based on the gene analyzed. The *Ab dinB* reporter strain formed two main subpopulations, bright and dim (Fig. 4). The *Ab umuDC* reporter strain also formed two populations, though the induction levels were higher than those for *dinB*. *umuDC* was more highly induced than any other gene analyzed, suggesting that *umuDC* is highly responsive to MMS treatment (Fig. 4). The *Ab recA* and *uvrA* reporter strains formed one main population, however, these populations were found closer to the dim range, suggesting *recA* and *uvrA* may not be highly induced by MMS (Fig. 4). Lastly, the *Ab trpB* reporter strain was not fluorescent in response to MMS treatment. This strain formed one main population of dim cells (Fig. 4).

As little is known about the factors causing this unique type of regulation of the DNA damage response genes in *A. baumannii*, we next took a bioinformatics approach to identify possible regulatory motifs within the putative promoter regions of the genes analyzed in this study.

Strong palindromic, regulatory motif found upstream of *umuD*, *umuC* and *ddrR* genes

Initially, to search for a putative regulatory motif, the intergenic regions between *ddrR* (A1S_1388) and *umuD* (A1S_1389) were collected from six complete and nine partial genomic sequences of *Acinetobacter* spp. from NCBI Genbank. A conserved palindromic

motif was identified between the *ddrR* gene and *umuD* gene in eleven genomes (six complete, five partially sequenced) (Fig. 5A). A whole-genome search with this motif identified additional motifs, upstream of the *umuDC* operons, the single *umuD* and single *umuC* genes and *ddrR* in all six complete genomes (Table 1). In the strain used predominantly in this study, *A. baumannii* ATCC 17978, the palindromic motif was found upstream of *umuDC* (A1S_1174-1173), *umuDC* (A1S_0636-0637), *umuC* (A1S_2008), *umuD* (A1S_1389), *umuC* (A1S_2015) and *ddrR*.

To ascertain the *in vivo* importance of this motif, we created a reporter GFP strain for the *umuDC* (A1S_0636-0637) gene at the chromosomal locus as before, but altered the regulatory motif in the promoter region by changing 7 of the most highly conserved nucleotides (Fig. 5B). This reporter strain is isogenic to the *umuDC* reporter strain (*umuDC* pr-GFP) used in Fig. 1, with the exception of the altered motif.

The altered motif strain (*umuDC* pr*-GFP) was then either left untreated or treated with 10× MIC cip for 180 minutes as before, and analyzed by FACS at 0, 90 and 180 minutes. The untreated strain remained largely unimodal and dim throughout the time course, showing no induction of the gene (Fig. 5C, left), much as in *umuDC* pr-GFP with the wild-type motif. However, the treated *umuDC* pr*-GFP strain shifted into the fluorescent range by 90 minutes and remained fluorescent at the 180-minute time point. The *umuDC* pr*-GFP reporter strain was almost completely lacking a dim subpopulation (Fig. 5C, right), dramatically contrasting what was seen under the same conditions in the isogenic *umuDC* pr-GFP strain (Fig. 1), indicating a role for the conserved motif in subpopulation formation.

To explore the response of this mutant reporter to other treatments, the *umuDC* pr*-GFP and *umuDC* pr-GFP strains were next untreated or treated at exponential phase with 0.3× and 1× the MIC MMS for 60 minutes followed by flow cytometry analysis. The untreated *umuDC* pr*-GFP and *umuDC* pr-GFP strains each formed one peak. Both strains showed some background fluorescence, though the reporter for the wild-type promoter was shifted farther into the fluorescent range (Fig. 5D). Upon treatment with 0.3× and 1× the MIC MMS, both strains remained largely unimodal and shifted to the fluorescent range above their background fluorescence. Interestingly, the *umuDC* pr*-GFP strain remained less induced than the strain with the wild-type reporter at both 0.3× and 1× MIC treatment (Fig. 5D).

Lastly, to identify possible survival advantages, we then explored the killing effect of cip on the bright and dim subpopulations individually.

Dim subpopulation has survival advantage over bright subpopulation in ciprofloxacin

To determine survival of each subpopulation in cip, we sorted *A. baumannii* cells into bright and dim subpopulations after 3 hours of 10× MIC cip treatment, using the *umuDC* reporter. We then plated these cells and determined the percent survival for each subpopulation compared to the total number of cells sorted (250,000 cells per subpopulation). We also analyzed the survival of bright-sorted cells containing the *umuDC* pr*-GFP reporter. Through six independent trials, we found the dim cells, on average, to survive 10 times better than the sorted bright cells from both the wild-type and promoter mutant reporters in 10× MIC cip (Fig. 6).

Discussion

We have examined the induction of several DNA damage response genes by fusing the putative promoter region of each gene to GFP at the natural chromosomal locus and observing fluorescence in response to DNA damaging treatment using flow cytometry. In this way, we have found *A. baumannii* to foster two subpopulations in response to DNA damage caused by ciprofloxacin (Fig. 1). Upon treatment, *dinB*, *umuDC*, *uvrA* and *recA* are induced in one subpopulation, while another shows no induction. We have found that the differentiation into bright and dim subpopulations is not heritable to future generations beyond 20 hours (Fig. 3). We also found subpopulation formation in response to MMS treatment, though the extent of this varied based on the gene analyzed (Fig. 4). To our knowledge, bimodal induction in response to a DNA damaging agent has not been shown before. The wealth of knowledge pertaining to DNA damage response systems centers mainly on *E. coli*, where upon DNA damaging treatment, genes are expressed unimodally, with genes with lower promoter affinity for the repressor being transcribed first (Friedberg *et al.*, 2006, Courcelle *et al.*, 2001). Previously, in the absence of DNA damaging treatment, population heterogeneity has been observed in expression of DNA damage response genes in *E. coli*, in strains mutated for genes such as *priA*, which is involved in replication fork restart, or *polA*, which codes for DNA Polymerase I (McCool *et al.*, 2004). Spontaneous, heterogeneous DNA damage response has also been seen in *E. coli*, again in the absence of treatment, by Pennington, *et al.* (Pennington & Rosenberg, 2007), however, when treated with UV light, *E. coli* cells were induced unimodally.

Research has previously indicated that the DNA damage response in *Acinetobacter* spp., including *A. baumannii*, differs from the well-described paradigm found in *E. coli*. Rauch *et al.* describe the induction of RecA in *A. baylyi* to occur independently of the functional protein itself (Rauch *et al.*, 1996). This contradicts the system in *E. coli* where *recA* induction is reliant on functional RecA, to cleave the LexA repressor (Little *et al.*, 1981). Recently, we have shown similar findings in *A. baumannii*, demonstrating a DNA damage-dependent increase in transcription of *recA* in the absence of the functional protein itself (Norton *et al.*, 2013). These observations indicate that the DNA damage response system in *Acinetobacter* spp. may operate independently of a repressor. Indeed, Aranda *et al.* recently described a regulatory role for the A1S_1389 gene product (Aranda *et al.*, 2013). Insertional inactivation of this gene on the *A. baumannii* 17978 strain chromosome resulted in decreased expression of other genes known to be part of the DNA damage response, suggesting it functions as an activator. Taking all of these result together, it is clear that a different regulatory mechanism is likely controlling the DNA damage response in *A. baumannii* and leading to the subpopulation formation we have observed.

Because bacteria divide symmetrically and grow as clonal populations, whole population analyses often assume all cells will respond identically; yet the differentiation into subpopulations, through phenotypic variation, has been shown to be prevalent in bacterial species, rendering these types of studies unable to uncover the intricacies of some regulatory systems (Dubnau & Losick, 2006, Smits *et al.*, 2006). We have shown that the subpopulations are not due to genetic changes (Fig 3). However, because the recovery time after cip treatment and sorting is approximately 20 hours, and most of the bright cells are

killed by the treatment, the recovered subpopulation could be the offspring of a small number of survivors, and perhaps not fully representative of the starting population. Alternatively, the changes might be explained by gene amplification. Gene amplification is a proven mechanism for evolution and acquisition of new gene product functions that includes the adaptation of genes acquired by horizontal gene transfer (Elliott *et al.*, 2013), and antibiotic resistance (Elliott *et al.*, 2013, Seaton *et al.*, 2012, Reams & Neidle, 2004, Reams & Neidle, 2003). Such changes are usually unstable and difficult to detect (Andersson & Hughes, 2009), however they allow bacteria to increase the chance of acquiring mutations (Bergthorsson *et al.*, 2007, Nasvall *et al.*, 2012, Andersson & Hughes, 2009).

A possible explanation for the regulation of the DNA damage response genes leading to subpopulation formation in *A. baumannii* is bistability. An example of bistability is the transcriptional regulation of the *lac* operon, in which cells within a clonal population show varying induction of this operon in response to the same stimuli (Novick & Weiner, 1957, Monod & Jacob, 1961, Ozbudak *et al.*, 2004). In the case of *A. baumannii* however, further research is needed to fully understand the mechanisms underlying the formation of subpopulations and to determine if this system is bistable.

A. baumannii is a highly adaptable pathogen, able to survive desiccation and colonize human hosts (Gaddy & Actis, 2009, Giannouli *et al.*, 2013, Stacy *et al.*, 2012). In response to DNA damage, *A. baumannii* could be using bimodal induction of DNA damage response genes within the population as one option to increase odds of survival in ever-changing environments. Similarly, in *Saccharomyces cerevisiae*, increased phenotypic switching capabilities have been shown to give growth advantages (Acar *et al.*, 2008, Bishop *et al.*, 2007). Additionally, entry into sporulation in *Bacillus subtilis* has been identified as a bet-hedging strategy, in which a subpopulation of cells enter sporulation to give a reproductive advantage to the growing subpopulation (Veening *et al.*, 2008). We were able to detect an approximate 10-fold difference in survival of dim cells over bright cells in ciprofloxacin (Fig. 6), showing an advantage for differentiation into subpopulations. In addition to their survival advantage, the dim cells would also likely be better suited to conjugate with other species, as DNA damage genes are often suppressed by conjugative elements (Bagdasarian *et al.*, 1986, Golub *et al.*, 1988). Dim cells could therefore adapt through DNA exchange within the species and with other species. It is possible that the dim cells possess a survival advantage over the bright cells because the multiple copies of *umuD* and *umuC* create higher intercellular concentrations of the error-prone DNA polymerase V upon induction, creating perhaps unusually high levels of mutagenesis. It is known that high intracellular levels of the *umuDC* or *dinB* genes products are lethal to *E. coli*. It is also known that translesion synthesis proficient *Salmonella* strains are prone to spontaneous deletions (Koskiniemi & Andersson, 2009), which again can prove lethal. All of these reasons might cause us to observe fewer surviving bright cells. However, cells belonging to the bright subpopulation, with such heightened expression of DNA Pol V would be more likely to accumulate base pair substitutions (Napolitano *et al.*, 2000, Watanabe-Akanuma *et al.*, 1997) that might occasionally prove useful for acquiring resistance to other antibiotics, such as rifampicin (Giannouli *et al.*, 2012), or give an evolutionary advantage. In those cases, the one mutated cell could then propagate and replenish the population. By fostering both subpopulations in

response to DNA damage, *A. baumannii* creates two subsets of cells, each employing very different strategies to increase chances of survival in a changing environment.

In efforts to shed light on the regulatory circuitry behind the DNA damage response system in *A. baumannii*, we identified a palindromic regulatory motif, upstream of the DNA damage response *ddrR* and *umuDC* genes (Fig. 5A). This motif is conserved throughout other *A. baumannii* strains, as well as different *Acinetobacter* species. Upon altering the nucleotide sequence of the motif in the *umuDC* reporter strain, we saw a change in the induction profile of *umuDC* from bimodal induction with the motif intact, to nearly unimodal induction in its absence (Fig. 5C). We also saw diminished bright fluorescence (i.e. gene induction levels) compared to the wild-type motif in response to MMS treatment (Fig. 5D). These changes show the importance of the motif in the regulation of the *umuDC* genes *in vivo* and indicate that the motif likely represents a binding site for the regulator responsible for the bimodal phenotype.

To our knowledge, the bimodal expression of DNA damage response genes upon treatment with a DNA damaging agent has not been observed in other well-described systems, such as in *E. coli* (Friedberg *et al.*, 2006, Radman, 1975). This unusual type of regulation gives a survival advantage to the uninduced cells and could be responsible for quick adaptability in *A. baumannii*, ultimately promoting survival in changing environments. Further investigation into the network and possible regulator(s) involved in this system will lead to a more complete picture, giving insights into its effects on pathogenicity and adaptability. Heterogeneous populations have been seen in many types of bacteria and it is likely that bimodal regulation happens much more readily than is known, since many assays operate at the population level. With more focused analyses of single cells, bacterial survival strategies can be dissected more finely, giving forth results that are more pertinent to natural systems.

Experimental Procedures

Bacterial strains and culturing conditions

Strains and plasmid used are listed in Table 2. Luria-Bertani (LB) medium (Becton Dickinson, Sparks, MD) was used for the growth of all strains. Cultures were incubated at 37 °C and 225 rpm. Kanamycin (kan; 35 µg mL⁻¹; EMD Biosciences, Inc., La Jolla, CA), chloramphenicol (cam; *E. coli* MIC = 20 µg mL⁻¹; Sigma-Aldrich, St. Louis, MO) and ciprofloxacin (cip; *A. baumannii* MIC= 0.6 µg mL⁻¹, *E. coli* MIC= 0.01 µg mL⁻¹; Sigma-Aldrich) and methyl methanesulfonate (MMS; *A. baumannii* MIC= 25 mM; Sigma-Aldrich) were added to the medium as indicated in the text.

A. baumannii reporter strain construction

The *A. baumannii* *dinB*, *umuD*, *uvrA*, *recA* and *trpB* putative promoter regions (Supplementary Table 1) were amplified using polymerase chain reaction (PCR) with the oligonucleotides listed in Table 3. The forward and reverse oligonucleotides included the restriction sites XhoI and BamHI, respectively, for use in the downstream construction of recombinant plasmids. The PCR amplicons were cleaned up using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and digested with the appropriate enzymes in a

double digest as suggested by the manufacturer (New England Biolabs, Ipswich, MA). The plasmid, pUA66 (Zaslaver *et al.*, 2006), was also digested in the same manner. Both digests were separated using a 1% agarose gel and extracted using a QIAquick Gel Purification Kit (Qiagen). The insert and vector were then ligated using T4 ligase (Promega, Madison, WI) at 4 °C overnight. Electrocompetent *A. baumannii* cells were prepared as follows. Cultures were grown in 30 mL LB at 37 °C to exponential phase and made electrocompetent by washing three times with ice-cold 10% glycerol and then concentrating 200-fold. 150 ng DNA was introduced into 50 µL cells by electroporation. Transformants were plated on LB agar supplemented with kan. Colonies that resulted were suspected to contain the full plasmid on the chromosome as a result of a single crossover event at the homologous promoter region, as the pUA66 plasmid does not replicate in *A. baumannii* (Fig. S1). Expected chromosomal insertions were confirmed by sequencing using the internal and flanking oligonucleotides listed in Table 3.

***E. coli* DinB protein-GFP reporter construction**

The *E. coli* *dinB*-GFP fusion gene was originally generated in pCA24N-*dinB* (Kitagawa *et al.*, 2005). Site-directed mutagenesis using the GeneTailor™ kit was performed in accordance with manufacturer's instructions using oligonucleotides listed in Table 3 to generate the A206K mutation in the *GFP* gene. This mutation assures the monomeric state of GFP (von Stetten *et al.*, 2012). The *dinB*-GFP (A206K) and *cam*^R from pKD3 (Datsenko & Wanner, 2000) amplicons were generated and fused by overlap extension PCR using oligonucleotides listed in Table 3. PCR products were separated by 1% agarose gel and extracted using a QIAquick Gel Purification Kit (Qiagen). The resulting DNA fragment was recombined onto the chromosome by SOE-LRED (Benson *et al.*, 2011). Transformants were selected for on LB agar supplemented with cam. The successful integration of the *dinB*-GFP allele onto the chromosome was confirmed by PCR and DNA sequencing.

Flow cytometry analyses

All *A. baumannii* strains were grown overnight with kan. Overnight cultures were subcultured 1:1000 into fresh medium supplemented with kan. Cultures were grown to exponential phase (approximately 2.5 hours), and then cip or MMS were added to the media, or cells were left untreated. Cultures were monitored for 180 minutes with FACS analysis at 0, 90 and 180 minutes (cip) or 60 minutes with FACS analysis at endpoint (MMS). For FACS analysis, cultures were diluted 1:100 into 1% sodium chloride solution (Thermo Fisher Scientific, Waltham, MA) and 1,000,000 cells were analyzed for fluorescence per sample using BD FACS Aria II sorter (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Version 9.6.3).

For *E. coli* reporter strains, an overnight culture was subcultured 1:100 and grown for 3 hours twice a day, and subcultured 1:1000 overnight. This set of outgrowths was repeated twice. Cam was added to the *E. coli* *dinB*-GFP cultures grown to exponential phase at 30°C, and either cip was added to the media or cells were left untreated. At 240 minutes after adding cip, FACS analysis was performed on 1×10⁶ cells for fluorescence as above. The *E. coli* chromosomal and plasmid borne *sulA*-GFP (Benson *et al.*, 2011) were grown with no

antibiotic or kan, respectively. After cultures reached exponential phase, these were treated with cip and analyzed by fluorescence at 180 minutes as above.

Fluorescence automated cell sorting and re-treatment

Strains were prepared for flow cytometry analyses as described above. After cip treatment, 10,000 cells from each strain were analyzed for fluorescence. Each strain was then sorted into a dim (10^1 - 10^3 FITC-H units) and bright (10^3 - 10^5 FITC-H units) subpopulation, each consisting of 200,000 cells. Duplicate sorted subpopulations were re-analyzed using FACS to ensure proper sorting of dim and bright cells. The sorted cells were inoculated into 30 ml LB supplemented with kan and grown 20 hours. After 20 hours, each sorted subpopulation was treated in exponential phase with $10\times$ MIC cip and flow cytometry analyses again as described above. Cultures were analyzed using FACS at 0, 60, 120 and 180 minutes treatment.

Bioinformatics analyses

Complete genome sequences of six *Acinetobacter* species uploaded from NCBI GenBank database (Benson *et al.*, 2013) were analyzed in this study: *Acinetobacter baumannii* AB0057 (NC_011586), *Acinetobacter baumannii* ATCC 17978 (NC_009085), *Acinetobacter baumannii* MDR-ZJ06 (NC_017171), *Acinetobacter baylyi* ADP1 (NC_005966), *Acinetobacter calcoaceticus* PHEA-2 (NC_016603) and *Acinetobacter oleivorans* DR1 (NC_014259).

Motif detection was performed using Discover Profile tool implemented in RegPredict web server (Novichkov *et al.*, 2010). For discovery of a putative regulatory motif upstream of *umuDC* operons, intergenic regions between *ddrR* and *umuD* genes were collected from all studied genomes. Nine additional *ddrR-umuD* intergenic regions from other *Acinetobacter* species were added to the training set to achieve higher specificity of motif discovery. The search for the palindromic motif was conducted with default parameters except motif size range, which was 16-28 basepairs (bp).

The motif identified was initially validated by whole-genome search in *A. baumannii* AB0057 and *A. baylyi* ADP1 available in RegPredict using Run Profile tool and then detailed regulon reconstruction was done in all six genomes using GenomeExplorer package (Mironov *et al.*, 2000). The search was performed in a range -250 through +50 bp with respect to the gene start, with a threshold for the search defined as a lowest score observed in the training set.

Construction and FACS analysis of *umuDC* altered motif pr-GFP strain

A double-stranded DNA fragment consisting of 548 basepairs upstream of the *A. baumannii umuDC*(A1S_0636-0637) start codon flanked by an XhoI restriction site at the 5' end and the first 30 basepairs of the GFP gene at the 3' end was manufactured by Life Technologies (GeneArt® Strings™ DNA fragments, Grand Island, NY). The predicted regulatory motif in this fragment was altered as shown in Fig. 5B. This fragment was joined to *gfp* using splicing overlap extension (SOE) PCR with the *umuDC* upstream and GFP gene R primers listed in Table 3. Next, the kanamycin gene from the pUA66 plasmid and the *umuDC* gene

were amplified using Kan F and Kan R and *umuDC* gene F and *umuDC* gene R, respectively (Table 3). The *umuDC* gene F primer contained homology to the kan F primer (underlined) to aid in SOE PCR. These two fragments were then joined using SOE PCR with the primers Kan R and *umuDC* gene R (Table 3). The *umuDC* upstream – GFP fragment and kan – *umuDC* gene fragment were then ligated separately into the pGEM Easy T-vector according to the manufacturer's instructions (Promega). The *umuDC* upstream – GFP T-vector was digested with XhoI to remove the insert and the kan – *umuDC* gene T-vector was linearized with SalI according to the manufacturer's instructions (NEB). The digests were separated using 1% agarose gel electrophoresis and extracted using a QIAquick Gel Purification Kit (Qiagen). The *umuDC* – GFP insert was then ligated into the linearized T-Vector containing kan – *umuDC* gene fragment. Recombinant plasmids were introduced into *E. coli* cells by transformation and checked for correct orientation (upstream *umuDC*– GFP – kan – *umuDC* gene). Plasmids containing the desired inserts were then introduced into *A. baumannii* cells by transformation where a double cross-over event allowed the upstream *umuDC*– GFP – kan – *umuDC* gene construct to move onto the chromosome as with the other promoter – GFP reporters. Chromosomal insertions were checked by PCR. The *umuDC* pr*-GFP strain was then analyzed by flow cytometry after 10× MIC cip treatment at T=0 and T=180 minutes and 0.3× and 1× MIC MMS treatment at T=60 minutes, as detailed in Flow cytometry analyses section, above.

Ciprofloxacin killing in sorted populations

The *umuDC* reporter strain and *umuDC* pr*-GFP reporter strain were treated with 10× MIC cip during exponential phase as above and sorted into dim and bright subpopulations after 180 minutes, as described in fluorescence automated cell sorting and re-treatment section. 250,000 cells were sorted for each subpopulation. Liquid from cell sorting was deposited on LB agar plates supplemented with kan and plates were incubated at 37 °C overnight. Recovery was calculated as the percent of 250,000 cells that formed colonies on plates. Percent survival was calculated by first standardizing the fraction of surviving sorted dim or bright (or bright promoter mutant) with respect to sorted untreated cells. From such fractions the highest recovery was considered as 100%. Data are comprised of 6 independent experiments and 5 independent experiments for the promoter mutant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *A. baumannii* DNA damage response genes show bimodal induction after treatment with ciprofloxacin

Reporter strains were created in *A. baumannii* by fusing GFP to the upstream, putative promoter region of the gene of interest at the natural chromosomal locus. The promoter region was then duplicated leading into the wild-type gene. Green fluorescence is thus the readout of gene transcription. To analyze gene induction, each reporter was treated with a range of cip relative to the MIC ($0.61 \mu\text{g mL}^{-1}$) during exponential phase and 1,000,000 cells were analyzed by FACS at T=0, 90 and 180 minutes.

Histograms show cell number vs. fluorescent intensity (FITC-A) over the time course at each concentration. (A-D) All reporters show differentiation into dim and bright subpopulations. Generally, increasing cip concentration and time leads to a higher proportion of bright cells and a decreasing proportion of dim cells. Though some reporters (e.g. *umuDC*) show higher induction levels at subinhibitory drug concentrations. (E) The *trpB* reporter remains largely unimodal and dim throughout treatment.

Figure 2. *E. coli dinB* is induced unimodally by DNA damage

(A) A plasmid-borne reporter containing GFP fused to the upstream, putative promoter region of *dinB* was used in *E. coli*. To analyze gene induction, cells were treated with a range of cip relative to the MIC ($0.01 \mu\text{g mL}^{-1}$) during exponential phase and 1,000,000 cells were analyzed by FACS at T=0, 90 and 180 minutes. The *E. coli dinB* pr-GFP strain shows increasing fluorescence with increasing cip concentration and time, while remaining a unimodal population. (B-C) Cells were untreated or treated with 10 $\times$ MIC cip and analyzed by FACS at 240 and 180 minutes, respectively. (B) A chromosomal reporter containing GFP fused to the *dinB* protein was used in *E. coli*. Populations again remain unimodal with an increase in fluorescence in response to treatment. (C) Both a chromosomal and plasmid-borne reporter containing GFP fused to the upstream, putative promoter region of *sulA* were used in *E. coli*. Both reporters show unimodal populations, with an increase in fluorescence in response to treatment. Histograms show cell number vs. fluorescent intensity (FITC-A) over the time course at each concentration.

Figure 3. High or low induction levels are not heritable to future generations

(A) GFP transcriptional reporters for *A. baumannii* *dinB*, *umuDC* and *uvrA* were treated with 10× MIC (6.1 $\mu\text{g mL}^{-1}$) cip during exponential phase for 180 minutes. Each reporter was then sorted using FACS into a dim subpopulation and bright subpopulation, consisting of 100,000 cells each. Histograms show *Ab umuDC* reporter and are representative of all reporters tested. Top panel shows analysis of 10,000 cells after 3 hours cip treatment, immediately prior to sorting. Bottom panels show analysis 1,000 cells from sorted subpopulations, dim (Subpopulation A, left) and bright (Subpopulation B, right), to show sorting accuracy. (B) Sorted subpopulations were inoculated into fresh LB medium and grown for 20 hours. Each subpopulation was then subjected to cip treatment matching that of the previous day. FACS was used to analyze cultures after 3 hours of treatment. Again, representative histograms from *Ab umuDC* are shown. Both Subpopulation A (top) and Subpopulation B (bottom) differentiate into the two subpopulations upon repeated treatment.

Figure 4. *A. baumannii* genes show variable induction after treatment with MMS

To analyze gene induction, each *A. baumannii* reporter was treated with $1\times$ the MIC MMS (25 mM) during exponential phase and 1,000,000 cells were analyzed by FACS at T=60 minutes. Histograms show cell number vs. fluorescent intensity (FITC-A). The reporter strain for *dinB* is most reminiscent of the *cip* treated strains, with two sharply defined subpopulations, one dim and one bright. The reporter strain for *umuD* again forms two subpopulations, though both are shifted further into the fluorescent range. The *uvrA* and *recA* reporters are unimodal and induced to a lower extent than *dinB* or *umuD*. The *trpB* reporter is also unimodal and found largely in the dim range. Basal level is found to the left of the broken line.

Figure 5. Palindromic regulatory motifs found upstream of *umuD*, *umuC* and *ddrR* genes in *Acinetobacter* strains contribute to subpopulation formation

(A) Palindromic repeat motif identified upstream of *umuD*, *umuC* and *ddrR* genes in *Acinetobacter* species. Letter height indicates conservation level. (B) *In vivo* activity of the motif was determined by introducing changes to the nucleotide sequence of the motif (as indicated by black arrows) in the *A. baumannii umuDC*(0636-0637) reporter strain and analyzing gene induction in response to treatment. Most highly conserved motif residues are underlined. The distance between the motif and the starting ATG is also indicated. bp; base pair (C) To analyze gene induction, the *umuDC* pr*-GFP strain was left untreated or treated with 10× the MIC cip (6.1 $\mu\text{g ml}^{-1}$) during exponential phase and 1,000,000 cells were analyzed by FACS at T=0, 90 and 180 minutes. Alterations to the motif in the *umuDC* pr*-GFP reporter strain cause a great reduction of the dim subpopulation, as compared to the isogenic *umuDC* pr-GFP strain under the same conditions. (D) To compare induction in response to MMS, both the *umuDC* pr*-GFP strain and the *umuDC* pr-GFP strains were untreated or treated during exponential phase with 0.3× and 1× the MIC MMC (7.5 mM and 25 mM, respectively) and 1,000,000 cells were analyzed by FACS. Populations are largely unimodal with a decrease in bright fluorescence in the mutant strain. Histograms show cell number vs. fluorescent intensity (FITC-A) over the time course.

Figure 6. Dim subpopulation has 10-fold survival advantage over bright subpopulation after ciprofloxacin treatment
 A GFP transcriptional reporter for *A. baumannii umuDC* and *umuDC* (mutated promoter) were treated with 10× MIC ($6.1 \mu\text{g mL}^{-1}$) cip during exponential phase for 180 minutes. Using FACS, cells were sorted into a dim and bright subpopulation, consisting of 250,000 cells each. These cells were then washed twice with SMO (NaCl 0.85%, Tris pH 7.5) and plated without treatment. Untreated cells were also sorted and plated to standardize the recovery of either the bright and dim sorted cells. The bright cells coming from the promoter motif mutant was treated and sorted as indicated above and plated on LB Kan. These were also standardized to untreated cells. Error bars indicate standard deviation of the mean for 5 (wild type cells) and 6 independent (mutant cells) replicates. On average, dim cells survive 10-fold more than bright cells during cip treatment.

Table 1

Gene preceded by motif (orthologous genes sorted by row)	Strain						
	<i>Acinetobacter baumannii</i> ATCC 17978	<i>Acinetobacter baumannii</i> AB0057	<i>Acinetobacter baumannii</i> MDR-Z106	<i>Acinetobacter calcoaceticus</i> PHEA-2	<i>Acinetobacter oleivorans</i> DR1	<i>Acinetobacter baylyi</i> ADP1	
<i>ddlR</i>	AIS_1388	AB57_1622	ABZI_01590	BDGL_000771	AOLE_11750	ACIAD2730	
<i>umuD</i>	AIS_1389	AB57_1623	ABZI_01591	BDGL_000772	AOLE_11745	ACIAD2729	
<i>umuD</i>	AIS_0636	--	--	--	--	--	
<i>umuC</i>	AIS_0637 ^a	--	--	--	--	--	
<i>umuD</i>	AIS_1174	AB57_1306	--	--	AOLE_07970	--	
<i>umuC</i>	AIS_1173 ^a	AB57_1305 ^a	--	--	AOLE_07965 ^a	--	
<i>umuC</i>	AIS_2008	AB57_2356-2357 ^b	ABZI_02316	BDGL_001504	AOLE_07085	ACIAD2257	
<i>umuD</i>	--	--	--	--	AOLE_14875	--	
<i>umuC</i>	--	--	--	--	AOLE_14880 ^a	--	
<i>umuC</i>	AIS_2015	--	--	--	--	--	

^a second gene in regulated operon

^b single gene annotated as two genes

--- no orthologous gene in strain

Table 2

Strains and plasmid used in this study.

Strain/plasmid	Description	Reference
<i>Acinetobacter baumannii</i>	ATCC17978, amp ^R	American Type Culture Collection
<i>Acinetobacter baumannii</i> <i>dinB</i>	As ATCC17978 with <i>dinB</i> (A1S_0186) promoter-GFP fusion on chromosome, kan ^R	This work
<i>Acinetobacter baumannii</i> <i>umuDC</i>	As ATCC17978 with <i>umuDC</i> (A1S_0636-0637) promoter-GFP fusion on chromosome, kan ^R	This work
<i>Acinetobacter baumannii</i> <i>uvrA</i>	As ATCC17978 with <i>uvrA</i> (A1S_3295) promoter-GFP fusion on chromosome, kan ^R	This work
<i>Acinetobacter baumannii</i> <i>recA</i>	As ATCC17978 with <i>recA</i> (A1S_1962) promoter-GFP fusion, kan ^R	This work
<i>Acinetobacter baumannii</i> <i>trpB</i>	As ATCC17978 with <i>trpB</i> (A1S_1692) promoter-GFP fusion on chromosome, kan ^R	This work
<i>Escherichia coli</i> <i>dinB</i>	<i>Escherichia coli</i> P90C, F- <i>ara</i> (<i>lac-proB</i>) _{XIII} <i>thi</i> , with pUA66- <i>dinB</i> -GFP, kan ^R	This work; plasmid from (Zaslaver <i>et al.</i> , 2006)
<i>Escherichia coli</i> DinB-GFP	<i>Escherichia coli</i> BW25513, <i>dinB</i> -GFP	This work. Strain from (Datsenko and Wanner, 2000). GFP gene from Kitagawa <i>et al.</i> , 2005.
<i>Escherichia coli</i> <i>sulA</i> (plasmid)	<i>Escherichia coli</i> P90C, F- <i>ara</i> (<i>lac-proB</i>) _{XIII} <i>thi</i> , with pUA66- <i>sulA</i> -GFP, kan ^R	Benson <i>et al.</i> , 2011B; plasmid from (Zaslaver <i>et al.</i> , 2006)
<i>Escherichia coli</i> <i>sulA</i> (chromosomal)	<i>Escherichia coli</i> P90C, F- <i>ara</i> (<i>lac-proB</i>) _{XIII} <i>thi</i> , with <i>sulA</i> -GFP on chromosome	McCool <i>et al.</i> , 2004
<i>Acinetobacter baumannii</i> <i>umuDC</i> (altered motif)	As ATCC17978 with <i>umuDC</i> (A1S_0636-0637) promoter (altered motif)-GFP fusion on chromosome, kan ^R	This work
pUA66	SC101 ori, GFP reporter plasmid carrying <i>gfpmut2</i> , kan ^R	(Zaslaver <i>et al.</i> , 2006)

Table 3

Oligonucleotides used in this study.

Oligonucleotide	Sequence
<i>Abau dinB</i> pr	5'-GCAGCTCGAGGATCTGGAATATGACAGCAAC
<i>Abau dinB</i> pr	5'-GCAGGGATCCATTATTGTGCTTTCACGATGCC
<i>Abau umuDC</i>	5'-GCGCCTCGAGCTGGCACACCAAAGTTAATACA
<i>Abau umuDC</i>	5'-GCCGGATCCGCTCCAATTCTGAATTAATTACTC
<i>Abau uvrA</i> pr	5'-GAGCCTCGAGTCTAAAATGTGTTGTCGTGTGGT
<i>Abau uvrA</i> pr	5'-CAGCGGATCCTCGAATACGGATATGACTTTGG
<i>Abau recA</i> pr	5'-GCGGCCTCGAGGATCACTGGTCTTATAATGATG
<i>Abau recA</i> pr	5'-GCATAGGATCCGCTTGAACAGTGTTCACCA
<i>Abau trpB</i> pr	5'-GCCACTCGAGGTCGTAGGTGATGCCAGTAT
<i>Abau trpB</i> pr	5'-GGATGGATCCCTGGTGTATGGCGAATTTCT
GFP R	5'-CCATGGAACAGGTAGTTTTTC
pUA66 F	5'-GCGTGCAATCCATCTTGTTTC
<i>dinB</i> upstream	5'-GCGAACATGGCTTTCATGTC
<i>dinB</i> gene R	5'-GAGGGCATAGTTTTCTTGCT
<i>umuDC</i>	5'-GCTAGAGTCTTTGAAGTGTG
<i>umuDC</i> gene R	5'-GCATGGAAAGGACATCCGTA
<i>uvrA</i> upstream	5'-GTTGCCAAAGCTGTTCATA
<i>uvrA</i> gene R	5'-GCCGAAAGTGATTCGACATA
<i>recA</i> upstream	5'-GAAATTCGTGTCGGTATGGA
<i>recA</i> gene R	5'-GCTCAGCATCAATGAAGGCA
<i>trpB</i> upstream	5'-GCATGAACACATGGGACTTG
<i>trpB</i> gene R	5'-TATCAACTTGACCATGTGG
GFP gene R	5'-TTTACTCGAGCCTCTAGAGCTTGCATGCCT
<i>umuDC</i> gene F	5'- <u>AGCTGGCAATTCCGACGCT</u> CTGATTACGTTACGT
<i>umuDC</i> gene R	5'-GTGACGACTCCCCATACGAT
Kan F	5'-AGACGTCGGAATTGCCAGCT
Kan R	5'-ATCGCTGCAGATCAACAGGAGTCCAAGCGAG
EC <i>dinB</i> -GFP 1	5'-CTCGCCAGGGGTGAAATTAAGTTTCGACGATTTTCAGCAAACCAACCCAGGAGCACG
EC <i>dinB</i> -GFP 2	5'-CTCCAGCCTACACAATCGCTCTTATTTGTATAGTTCATCCATGCC ATGTGTAATCCCAGCAGC
EC <i>dinB</i> -GFP 3	5'-GCATGGATGAACATATAAAATAAGAGCGATTGTGTAGGCTGGAG CTGCTTCGAAGTTCCTATACTTTCTAGAG
EC <i>dinB</i> -GFP 4	5'-GCGAGAATTTCGATGCATACAGTGATACCCTCATAATAATGCACA CCAGGCCATGGTCCATATGAATATCCTCC
GFP A206K F	5'-ATTACCTGTGACACAACTTAACTTTCGAAAG
GFP A206K R	5'-AGATTGTGTGACAGGTAATGGTTGTCTGGTA

^{1,2} Primers contain XhoI¹ restriction site or BamHI² restriction site (underlined) for cloning.