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# A high-throughput screen for the engineered production of β-lactam antibiotics

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## Abstract

High-throughput screens and selections have had profound impact on our ability to engineer proteins possessing new, desired properties. These methods are especially useful when applied to the modification of existing enzymes to create natural and unnatural products. In an advance upon existing methods we developed a high-throughput, genetically-regulated screen for the *in vivo* production of β-lactam antibiotics using a green fluorescent protein (gfp) reporter. This assay proved reliable, sensitive and presents a dynamic range under which a wide array of β-lactam architectural sub-classes can be detected. Moreover, the graded response elicited in this assay can be used to rank mutant activity. The utility of this development was demonstrated *in vivo* and then applied to the first experimental investigation of a putative catalytic residue in carbapenem synthase (CarC). Information gained about the mutability of this residue defines one parameter for enzymatic activity and sets boundaries for future mechanistic and engineering efforts.

β-Lactam antibiotics are a mainstay in the treatment of bacterial infections. More than 50% of clinically-used antibiotics contain a β-lactam core.[1] This class of antibiotics is structurally diverse owing to their differing biological origins. In addition to naturally-occurring β-lactams, many of clinical relevance are produced synthetically or semi-synthetically giving rise to an expanded arsenal of antibiotics.[2] Differentiation of the core β-lactam-containing ring system, variation of appendages to the bicyclic core and their oxidation state[3] modulate antibacterial effectiveness and stability to commonly encountered β-lactamase (βL) resistance enzymes. Inactivation of β-lactam antibiotics by βL is a problem of great concern for health care giving rise to a need for new or improved antibiotics to overcome resistance.[4] Recent advances in biosynthetic chemistry and metabolic engineering have proved useful in both lowering the cost and increasing the availability of complex pharmaceuticals.[5–8] Moreover, modification of known enzymes to create mutants with new substrate specificities has proved to be a viable tactic for producing modified natural products.[9–12] Generation of catalysts that can aid in synthesizing β-lactams with rationally altered structures would be desirable for the production of next-generation β-lactam antibiotics.[2] To this end, a reliable and high-throughput screen possessing these properties, as well as potentially others such as heightened thermostability or increased activity in organic solvents, would be particularly useful when applied to the interrogation of mutant enzyme libraries to achieve engineered β-lactam biosynthesis. In contrast to *in vitro*[13,14] or traditional growth-inhibition assays,[15,16] creation of a screen that is run on a single agar plate with hundreds to thousands of enzyme variants present would allow conservation of substrate while greatly increasing capacity. With this goal in mind, we aimed to design a

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high-throughput, highly sensitive and reproducible screen that would function on agar plates to report the *in vivo* production of β-lactam antibiotics in single bacterial colonies.

In this paper we describe the development of such an assay and demonstrate its utility in a mutational investigation of a putative catalytic residue in carbapenem synthase (CarC) from *Pectobacterium carotovorum*, the terminal enzyme in the biosynthesis of the simple carbapenem **3**.[15,16] This iron-dependent oxygenase catalyzes both coupled and uncoupled epimerization and desaturation of carbapenam **1** as shown in Figure 1. We have unambiguously established that the C-5 bridgehead hydrogen (H°) in **1** is lost during a presumed radical abstraction catalyzed by the CarC iron-oxo center and replaced by another hydrogen from the opposite face of the substrate to achieve epimerization.[15,16] The identity of this hydrogen donor is thought to be Tyr67 based on a crystal structure of the enzyme[17] and molecular modeling. This proposal is backed by substrate specificity studies[15,16,18] and computation.[19,20] In the absence of experimental support for this unusual mechanism, we elected to carry out saturation mutagenesis[21–23] of this residue to give insight into the requirements at position 67. We, therefore, charged these mutants with the task of catalyzing a reaction with both its native **1** and epimerized substrate **2**. Information gained about the mutability of this residue will give insight into the mechanism of CarC and aid future endeavors to expand its substrate specificity.

To create the desired *in vivo* screen, we turned to the well-studied, inducible AmpC β-lactamase from *Citrobacter freundii*.[24,25] The *C. freundii* AmpC βL allows for selective induction of transcription from the *ampC* promoter (*PampC*) in the presence of a β-lactam antibiotic. An additional benefit of the *ampC* βL is that it can be heterologously expressed and behaves in *E. coli* in a fashion analogous to that of *C. freundii.* Of specific importance, the *C. freundii ampC* βL has been shown to be hyperinducible when used in the *ampD* mutant *E. coli* strain SN0301.[26,27] AmpD is involved in recycling the cell wall fragment anhydro-muramic acid *N*-acetyl pentapeptide (anhMurNAc) that is accumulated from β-lactam inhibition of cell wall biosynthesis.[26] As a consequence, this hypersensitive strain can detect β-lactam antibiotics in the low nM to high pM range, concentrations well below those that are cytotoxic to the bacterium.

*PampC* is regulated by the product of the *ampR* gene.[28] In the presence of the cell wall degradation product, anhMurNAc, AmpR converts from a repressor to a transcriptional activator of *ampC* (Scheme 1).[24] To achieve induction of fluorescence in the presence of a β-lactam antibiotic, we placed GFPMut2[29] (*gfp*) under the control of *PampC*, which was cloned with its regulatory gene, *ampR*, from pNU305.[30] The *gfp-PampC-ampR* gene segment was placed in the EcoRI-PstI fragment of pBR322 containing the tetracycline resistance marker. The sequence of the resultant plasmid, known as pRP5.199, was verified and further tests were initiated to validate its use in the envisioned screen.

To substantiate the assay and ascertain its reliability and robustness, it was necessary to demonstrate that the fluorescent phenotype was induced in the presence of β-lactam antibiotics. This fluorescence response should ideally show a large induction coefficient (β-lactam induced colonies *vs.* uninduced colonies) allowing for the easy identification of β-lactam-producing colonies. Additionally, the elicited response should be reproducible, reflected in a low coefficient of variance (CV). Use of the *PampC* system would optimally also produce a graded fluorescence response permitting differentiation among mutants of differing catalytic activities. It would be further desirable if the system were robust enough to detect a wide array of β-lactam structural types ranging from simple monobactams to the more highly elaborated bicyclic classes. A series of *in vitro* assays was conducted to establish the extent to which this assay met all of these criteria. The assay was then applied

*in vivo* to assay for production of the simple carbapenem 3 to verify its utility and reliability *in vivo*.

To first demonstrate the range and reproducibility of the pRP5.199 assay, a series of experiments was designed in which external supplementation of β-lactam antibiotic was used to induce a fluorescence response. *E. coli* SN0301 containing pRP5.199 was grown overnight and replica plated on agar plates containing either 50% the measured minimum inhibitory concentration (MIC) of a given β-lactam antibiotic or a control plate lacking the compound. Clear results were obtained after incubation for 16 h revealing a marked increase in fluorescence for the β-lactam-containing plates (Table 1). An 11 to 43-fold increase in fluorescence was observed when induced colonies were compared to their controls. The results also demonstrated that the responses caused by these inducers were well behaved and reproducible, reflected by a CV of less than 10% for each trial. Finally, these experiments established that this assay was amenable to a wide variety of β-lactam architectures, exemplifying its applicability to all known classes of the β-lactam antibiotics.

The induction of *gfp* is triggered by cell-wall fragments (anhMurNAc) generated during β-lactam inhibition of cell wall biosynthesis. Since this system indirectly detects β-lactam antibiotics, it is important to show that it is not triggered by other cell wall synthesis inhibitors. To demonstrate specificity for β-lactam-induced cell wall degradation products, we incubated SN0301 containing pRP5.199 with phosphomycin or vancomycin at 50% the MIC determined for this strain. These two well-characterized antibiotics inhibit bacterial cell wall biosynthesis, as do β-lactam antibiotics, but act on different stages of biosynthesis than do β-lactams.[31,32] The results of these control experiments show that neither of these bacterial cell wall inhibitors induces fluorescence above the uninduced state, inspiring confidence that this assay is specific for β-lactams. This result is in accord with the literature.[33]

Another desirable feature of any successful screen is a graded response to the input stimulus. To determine whether *PampC* exhibits such a response to β-lactam antibiotics, colonies of SNO301 containing pRP5.199 were grown on agar plates containing varying concentrations of Amoxicillin. The results clearly demonstrated that as the concentration of β-lactam antibiotic increased, so did the fluorescence signal (Figure 2). The response is non-linear showing greater sensitivity at low concentrations when compared to changes observed as β-lactam concentrations approach ~50% of the minimum inhibitory concentration (MIC = 1 μg/mL), then falling at higher concentrations as increased cellular damage occurs. One salient feature of this screening system was that Amoxicillin down to the low nanomolar range (*ca.* 30 nM) elicited approximately a 500% increase in fluorescence when compared to the uninduced control. This acute sensitivity enables detection of even weakly producing mutants, especially when considering more intrinsically potent β-lactam antibiotics such as Imipenem and related naturally-occurring carbapenem antibiotics.

To validate this assay for *in vivo* production of antibiotic, we turned to a well- characterized biosynthetic pathway, that of the simple carbapenem **3**, to use as a model system. In particular, we focused on the enzyme CarC, which catalyzes the unusual bridgehead epimerization and desaturation of the antibiotically inactive (3*S*,5*S*)-carbapenam **1** to the active (5*R*)-carbapenem **3**, with (3*S*,5*R*)-carbapenam **2** as a freely-diffusible intermediate.[15,16] Initial evaluation focused on the direct comparison of two *E. coli* strains which either possessed or lacked the ability to catalyze the reaction of **2** to **3**. Expression of CarC in *E. coli* SN0301 was achieved by placing a codon-optimized variant of CarC, CarCopt, under control of the *tac* promoter contained in the expression vector pDIMC8K (see Supporting Information). *E. coli* SN0301 was transformed with pDIMC8K-CarCopt or the control vector, pDIMC8K-MalE (*malE* codes for the maltose-binding protein, which has

no effect on the assay and was used in control reactions) in conjunction with pRP5.199. These strains were grown overnight and replica plated on bioassay plates containing the appropriate antibiotics, $Fe^{2+}$, IPTG, and **2**. Bioassay plates were incubated at 37 °C for 16 h and then scanned for fluorescence using a phosphorimager. A dramatic response was observed in which the cell line possessing the ability to catalyze the formation of the active antibiotic **3** showed a 45-fold increase in fluorescence as compared to the control cell line (Supporting Information, Table S1). This result was encouraging in several ways. Not only did *in vivo* production of the antibiotic occur on the agar plate, but it elicited a fluorescence response that rivaled the best of the *in vitro* experiments. To solidify this assay as a reliable tool for the identification and ranking of β-lactam producing colonies, *in vivo* comparison of three *E. coli* strains that either possessed to varying degrees or lacked entirely the ability to catalyze the reaction of **1** to **3** was executed. Two cell lines harbored either CarCopt (*E. coli* RP1) or wild-type (wt) CarC (*E. coli* RP2), in addition to pRP5.199. Owing to the greater *in vivo* production of CarC by the optimized gene *vs*. wt, the reaction is catalyzed in a more efficient manner, which translates into a greater fluorescence signal for the CarCopt-bearing cell line. Additionally, a control cell line absent the ability to catalyze the reaction, pDIMC8K-MalE (*E. coli* RP3) containing pRP5.199, was generated (*E. coli* RP3). It was expected that all three cell lines would show differing fluorescence intensities (RP1>RP2>RP3) and that these cells could be differentiated and separated using this assay. To test this hypothesis, these strains were combined in an approximately 1:1:8 (RP1:2:3) ratio, grown overnight and replica plated on bioassay plates as previously described. Evaluation of the plates showed three cell lines exhibiting different fluorescence intensities (Figure 3; Supporting Information, Table S2). Members of groups exhibiting high, medium and low fluorescence intensities were grouped and their sequences were verified. The results showed, as expected, that cell lines could be distinguished using this assay with no crossover of the RP1, 2, or 3 cell lines into an incorrect fluorescence intensity group. With this mock screen verifying the capabilities of this assay to not only distinguish β-lactam producers from non-producers but also allow ranking of catalytically superior to inferior cell lines, we sought to use this assay to address a central question about the catalytic mechanism of CarC.

CarC is a unique bifunctional member of the non-heme iron a-ketogluarate dependent oxygenase family that has been proposed to catalyze the C5 epimerization of **1** producing **2** by way of iron-oxygen catalyzed radical abstraction and hydrogen donation from the opposite face.[15–20] A model depicting the likely positioning of (3*S*,5*S*)-carbapenam **1** in the CarC crystal structure was generated *in silico* and revealed that a small movement of less than 2.5 Á by the Tyr67 β-carbon, followed by rotation around the α,β-carbon bond would position Tyr67 for hydrogen donation to C5, resulting in bridgehead epimerization (Figure 4). Since efforts to redesign the substrate specificity of CarC require biochemical understanding of its mechanism, that is whether or not Tyr67 is essential for catalysis, we chose to address the proposed role of Tyr67 in both the full-reaction (1 → 3) and the second half-reaction (2 → 3). To investigate the residue requirements for position 67, we performed saturation mutagenesis[21–23] at this locus in wild-type CarC and screened the point-mutants against both **1** and **2**. Screening of all amino acid variants with **1** found that Tyr was the only amino acid tolerated at this site that could carry out the full reaction. Although this observation does not strictly prove the involvement of Tyr67 in the reaction as a radical shuttle, it is the first experimental evidence to support the participation of this residue in catalysis. Results for the second-half reaction were expected to differ, as C5 epimerization had already occurred, and, therefore, a hydrogen donor (tyrosine) would no longer be necessary for catalysis. Interestingly, a limited set of highly similar amino acids, Tyr67, Y67F and Y67W, was required to sustain the reaction with the latter two mutants possessing reduced catalytic efficiency (*ca.* 30% of wt; Supporting Information, Tables S3 and S4). Combined, these results reveal the necessity of Tyr67 for substrate epimerization, but not

desaturation, and demonstrate the requirement for an aromatic residue at position 67, even for the simple desaturation reaction.

Reported here is the first high-throughput assay for the *in vivo* production of β-lactam antibiotics. This assay was shown to be reliable and reproducible as demonstrated by the low CV exhibited in both the *in vivo* and *in vitro* experiments. Owing to the fact that the assay indirectly detects β-lactam antibiotics by the production of unique β-lactam-induced cell wall degradation products (anhMurNAc), the assay is specific to β-lactam antibiotics, but general to all known structural classes. This assay shows a good dynamic range across these diverse structural types represented by an 11 to 45-fold increase in detected fluorescence, with the *in vivo* synthesis of the simple carbapenem **3** displaying the largest fluorescence increase. The graded response offers a means to discern highly active from less active mutants, improving the screening process to rapidly identify the most active clone(s). Of further importance, the ability of this assay to sensitively operate in the nanomolar range enables identification of even weakly producing mutants that can be carried on to additional rounds of mutagenesis to potentially enhance activity. In a test of the assay, it was applied to probe the amino acid requirement at position 67 in CarC, proposed to serve as a key radical shuttle in the overall catalytic cycle of the enzyme. This experiment, the first biochemical investigation of residue requirements in the active site of CarC, supports hypotheses advanced to account for the unusual bifunctional reaction catalyzed by this enzyme. The strict tolerance observed for aromatic residues at this site defines one important parameter for future engineering of this enzyme and taken together these experiments demonstrate an assay superior to current methods for identification of β-lactam production with greater reliability, conservation of substrate and high-throughput.

## Methods

### *In vitro* assay for detection of β-lactam antibiotics

*E. coli* SN0301 containing pRP5.199 was grown overnight at 37 °C on 2xYT (1.5% agar) containing 1.0% glycerol, tetracycline (5 μg/mL) and kanamycin (25 μg/mL). The cells were grown until colonies were visible and then replica plated on LB agar (1.5% agar) containing 1% glycerol, tetracycline (5 μg/mL) and kanamycin (25 μg/mL) in addition to the appropriate β-lactam antibiotic at 50% of the determined MIC or no β-lactam, which served as the control plate. The replica plates were grown 16 h at 37 °C. Plate analysis was performed on a Typhoon 9410 phosphorimager (GE Healthcare, Piscataway, NJ) using 300 V excitation with a 488 nm laser and a 526 short-pass cutoff filter with 100 μm resolution and +3 mm top focusing. All images were quantitated using Image Quant TL (GE Healthcare).

### *In vivo* assay for detection of β-lactam production

*E. coli* SN0301 containing pRP5.199 the desired pDIMC8K vector (pDIMC8K-CarCopt, pDIMC8K-CarAC or pDIMC8K-MalE at a 1:1:8 ratio (RP1:2:3) for separation trials or pDIMC8K-CarCY67X for saturation mutagenesis experiments) was grown overnight at 37 °C on 2xYT (1.5% agar) containing 1.0% glycerol, tetracycline (5 μg/mL) and kanamycin (25 μg/mL). The cells were grown 18 h and replica plated on LB agar (1.5% agar) containing ferrous ammonium sulfate (80 μM), IPTG (1 mM), tetracycline (5 μg/mL), kanamycin (25 μg/mL) and (3*S*,5*S*)-carbapenam (1, 500 μM) or (3*S*,5*R*)-carbapenam (2, 250 μM for initial *in vivo* trials or 200 μM for separation trials). The replica plates were grown 16 h at 37 °C. Plate analysis was performed on a Typhoon 9410 phosphorimager (GE Healthcare, Piscataway, NJ) using 300 V excitation with a 488 nm laser and a 526 short-pass cutoff filter with 100 μm resolution and +3 mm top focusing. All images were quantitated

using Image Quant TL (GE Healthcare). The Y67X point mutants were screened against 1 and 2 with >95% confidence that all codons were present.

### Quantitative analysis of pRP5.199 trial assay

After fluorescence was read, the data were analyzed using ImageQuant TL (GE Lifesciences). Each colony was interrogated separately using a correction for background fluorescence. Arbitrary fluorescence units were recorded for each colony. The average of the control cell line (*E. coli* SN0301-pDIMC8K-MalE/pRP5.199) was used to determine fluorescence intensity attributed to the basal transcription of *gfp* from pRP5.199. Normalized data are the raw fluorescence less the background fluorescence.

### Computational modeling of (3*S*,5*S*)-carbapenam in the CarC active site

All computational work was performed using Discovery Studio 2.1. The carbapenem was created using the Discovery Studio drawing function and energy minimized using the Smart Minimizer program. The substrate was manually docked in the CarC (pdb ID: 1NX8) B-subunit active site the and constraints were set so that the iron-oxo center would be within 2.75–3.25 Å of the carbapenam C5 and a further constraint was to set the tyrosyl hydroxyl at 2.75–3.25 Å. The system was typed with the 3 CHARMm[33] algorithm and minimized using an adopted basis NR algorithm with a generalized Born implicit solvent model with molecular volume. The resultant minimized model was visualized using PyMol.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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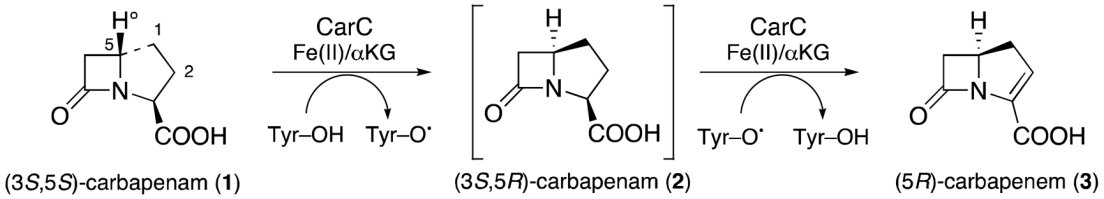



(3*S*,5*S*)-carbapenam (**1**) (3*S*,5*R*)-carbapenam (**2**) (5*R*)-carbapenem (**3**)

**Figure 1.**
Epimerization and desaturation of the simple carbapenam **1** catalyzed by CarC.

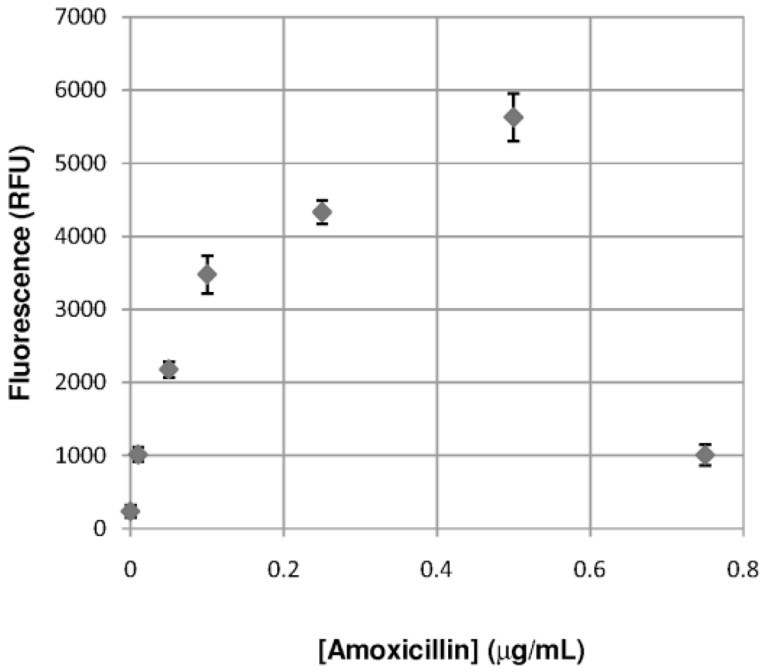



**Figure 2.**
Fluorescence response to increasing concentrations of Amoxicillin.

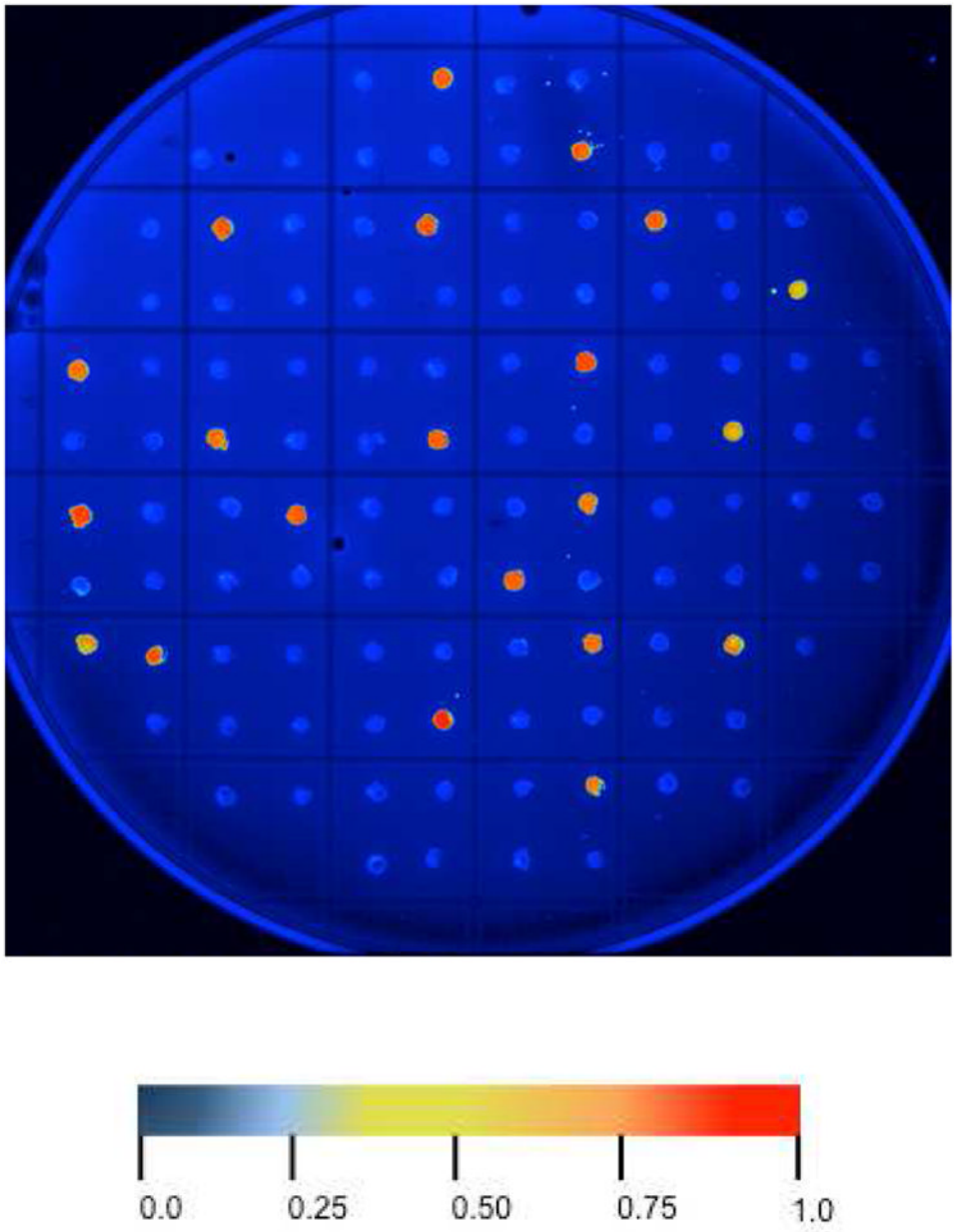



**Figure 3.**
Image of fluorescence intensities of *E. coli* RP1, 2 and 3. Intensity ranges from weak fluorescence (blue), medium (orange) and strong (red). Dark blue indicates no florescence continuing to bright red, which indicates wild-type activity.

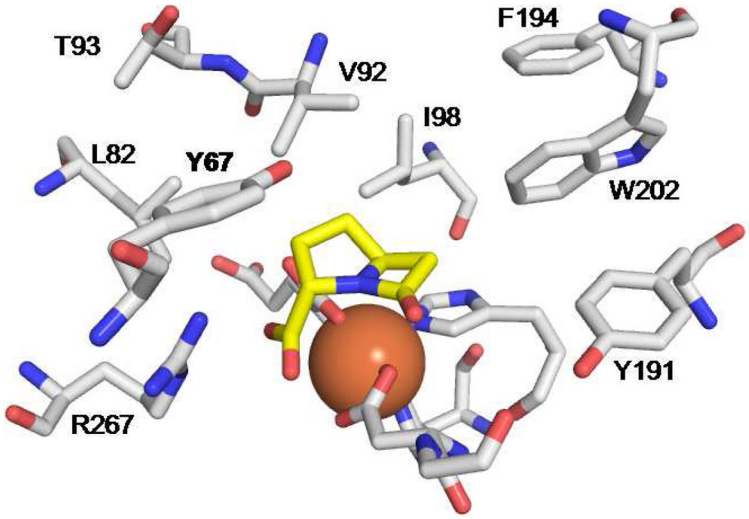
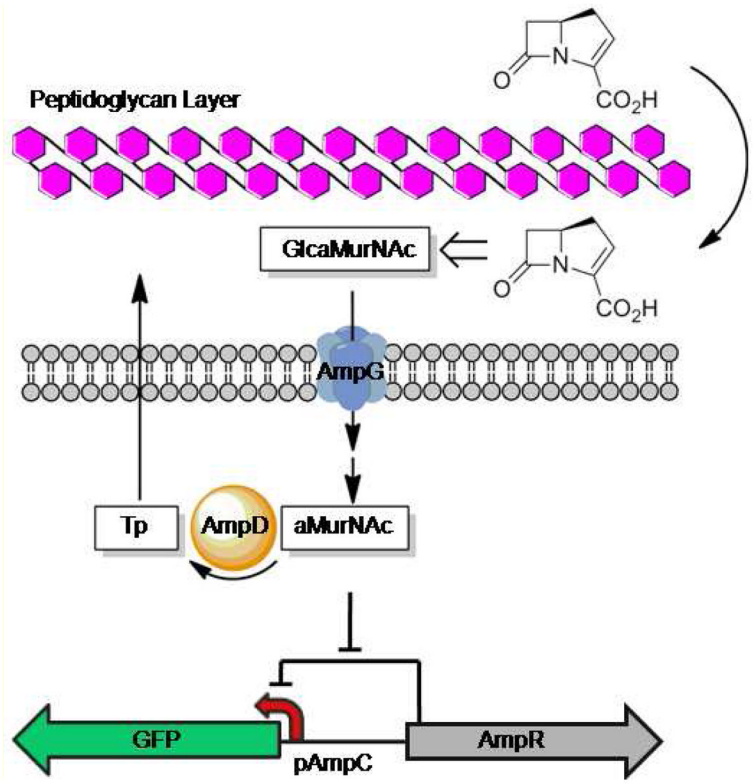



**Figure 4.**

Depiction of how (3*S*,5*S*)-carbapenam may sit in the active site with the C5 hydrogen poised for abstraction by the iron-oxo (orange) center followed by replacement of the hydrogen by Y67 leading to C5 inversion.




**Scheme 1.**
Induction of *gfp* by cell-wall breakdown byproducts induced by β-lactam antibiotics

**Table 1**

pRP5.199 Response to β-Lactam Stimuli. IC: Induction Coefficient, CV: Coefficient of Variance.

| β-lactam | IC | CV |
|---|---|---|
| Amoxicillin | 32 | 2.1 |
| Ampicillin | 21 | 8.5 |
| Carbenicillin | 19 | 8.2 |
| Cefmetazole | 37 | 9.1 |
| Clavulanic Acid | 11 | 8.5 |
| Imipenem | 16 | 9.4 |
| Nocardicin A | 15 | 3.7 |
| Penicillin G | 43 | 7.3 |
| Vancomycin | 1.1 | 9.7 |
| Phosphomycin | 1.3 | 15.3 |