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RESEARCH ARTICLE

A whole-cell hypersensitive biosensor for beta-lactams based on the AmpR-AmpC regulatory circuit from the Antarctic *Pseudomonas* sp. IB20

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Abstract

Detecting antibiotic residues is vital to minimize their impact. Yet, existing methods are complex and costly. Biosensors offer an alternative. While many biosensors detect various antibiotics, specific ones for beta-lactams are lacking. To address this gap, a biosensor based on the AmpC beta-lactamase regulation system (*ampR–ampC*) from *Pseudomonas* sp. IB20, an Antarctic isolate, was developed in this study. The AmpR–AmpC system is well-conserved in the genus *Pseudomonas* and has been extensively studied for its involvement in peptidoglycan recycling and beta-lactam resistance. To create the biosensor, the *ampC* coding sequence was replaced with the mCherry fluorescent protein as a reporter, resulting in a transcriptional fusion. This construct was then inserted into *Escherichia coli* SN0301, a beta-lactam hypersensitive strain, generating a whole-cell biosensor. The biosensor demonstrated dose-dependent detection of penicillins, cephalosporins and carbapenems. However, the most interesting aspect of this work is the high sensitivity presented by the biosensor in the detection of carbapenems, as it was able to detect 8 pg/mL of meropenem and 40 pg/mL of imipenem and reach levels of 1–10 ng/mL for penicillins and cephalosporins. This makes the biosensor a powerful tool for the detection of beta-lactam antibiotics, specifically carbapenems, in different matrices.

INTRODUCTION

The phenomenon of antibiotic resistance is a global public health issue (Coque et al., 2023; Morehead & Scarbrough, 2018) since a large quantity of these compounds is extensively used in human health, animal production and agriculture (Bengtsson-Palme et al., 2018; Bengtsson-Palme & Larsson, 2015). Antibiotics are now considered emerging pollutants in various matrices, such as water sources and food items (meat or vegetables; Feng et al., 2021; Rodríguez-González et al., 2022). This is why many countries have implemented maximum residual limits (MRLs) for food imported products (Griboff et al., 2020; Liu et al., 2018). Therefore, implementing reliable and efficient procedures to detect and measure antibiotic concentrations in different matrices is necessary.

Various methods exist for detecting antibiotic residues, such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS) assays (Kim et al., 2018). These methods are accurate but have drawbacks like high costs, time consumption and specialized equipment (Gaudin, 2017). Biosensors have emerged as a promising alternative for quick and cost-effective antibiotic measurement. While biosensors for aminoglycosides, macrolides, and tetracyclines are developed (Mehlhorn et al., 2018; Naseri et al., 2023; Yang et al., 2023), progress in detecting beta-lactam antibiotics is limited. Detecting penicillins and cephalosporins is until now addressed through enzymatic methods, aptamers and whole-cell biosensors (Au et al., 2020; Chinnappan et al., 2020; Lautenschläger et al., 2020; Valtonen et al., 2002; Xiao et al., 2016), but detecting carbapenems is challenging due to their complex structure, limited stability and low abundance (Codjoe & Donkor, 2017), presenting an ongoing challenge for accurate and affordable measurement.

Whole-cell biosensors are emerging as an alternative solution for detecting beta-lactam compounds. These biosensors involve modifying model organisms through genetic alterations to introduce complex beta-lactamase regulation systems, where the beta-lactamase is replaced by a reporter gene. These systems are triggered by the presence of beta-lactams, leading to the activation of the reporter gene that can be quantified for concentration measurement. Currently, only two studies describe biosensors with such characteristics. The first study modified *Escherichia coli* (*E. coli*) with the *ampR–ampC* beta-lactamase system from *Citrobacter freundii*, detecting various beta-lactams within specific concentration ranges (Valtonen et al., 2002). The second study involved modifying *Bacillus subtilis* with the *blaR1I* system from *Staphylococcus aureus*, enabling the detection of a wide range of beta-lactam antibiotics in specific concentration ranges as well (Lautenschläger et al., 2020).

The AmpR–AmpC system has been described as the most conserved and widespread resistance mechanism to beta-lactams in the *Pseudomonas* genus (Livermore & Woodford, 2006). This system's regulation has been extensively studied in *Pseudomonas aeruginosa*, where AmpC beta-lactamase expression is controlled by the AmpR transcriptional regulator, activated by the presence of 1,6-anhydromuropeptides linked to peptidoglycan recycling and degradation processes (Juan et al., 2017; Torrens et al., 2019).

In our laboratory, we have characterized and studied multiple *Pseudomonas* isolates from Antarctica (Higuera-Llantén et al., 2018; Vásquez-Ponce et al., 2017, 2018). Specifically, we have discovered that a particular isolate called *Pseudomonas* sp. IB20 exhibits significantly higher levels of induction of the *ampR–ampC* system compared to *P. aeruginosa* when exposed to various types of beta-lactam antibiotics. Based on this background, we have developed a whole-cell biosensor capable of detecting extremely low concentrations of beta-lactams, particularly those belonging to the carbapenem family.

EXPERIMENTAL PROCEDURES

Bacterial strains, culture conditions and antibiotic susceptibility tests

All *E. coli* strains used in this study are listed in Table 1. *E. coli* Top10® was employed as the host strain for routine DNA manipulation. *E. coli SN0301* strain is a deletion mutant in the following genes: *ampD1*, *ampA*, *ampC8*, *pyrB*, *recA* and *rpsL* (Lindquist et al., 1989); and it was used as a final host for all the genetic constructs. *Pseudomonas* sp. IB20 is an environmental strain isolated from Antarctic seawater previously described by our group (Vásquez-Ponce et al., 2018). *P. aeruginosa* PAO1-V was also used as a model strain for comparative purposes. All *E. coli* and *P. aeruginosa* strains were routinely grown in 20 mL of LB medium (BD™ DIFCO™) in 100 mL Erlenmayer flasks, incubated at 37°C with constant shaking at 250 rpm. *Pseudomonas* sp. strain IB20 was grown under the same incubation conditions, but at 25°C. Minimum inhibitory concentrations (MIC) for all bacterial strains used in this study were determined using the broth microdilution method in Müller-Hinton medium, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2018).

Quantification of *ampC* gene expression

To assess *ampC* gene induction in *Pseudomonas* sp. IB20, we measured gene expression levels using RT–qPCR after exposing the bacteria to its respective

TABLE 1 List of strains and plasmids used in this study.

Plasmids	Description	Reference/origin
pJET1.2	Commercial vector used for the cloning of PCR products (selection in ampicillin)	Thermofisher
pUC18	Cloning vector used for cloning the genetic constructs of biosensor 2 and of the construct that expresses mcherry constitutively (control mcherry) (selection in ampicillin)	Commercial
pSEVA541	Expression plasmid used as a vector for the genetic constructs of biosensors 1, 2 and mcherry control (selection in tetracycline)	Standard European Vector Architecture 4.0 (CNB)
pSEVA541-mcherry	Vector expressing mcherry constitutively (mcherry control) (selection on tetracycline)	This study
pSEV-B1	Vector expressing "Biosensor 1" (selection in tetracycline)	This study
pSEV-B2	Vector expressing "Biosensor 2" (selection in tetracycline)	This study
Pseudomonas sp. IB20	Environmental isolate obtained from antarctic seawater	This study
P. aeruginosa PAO1-V	Wild-type strain of clinical origin conserved in Madrid (CNB)	CNB
E. coli One Shot™ TOP10	Host strain used for transformation of cloned vectors with PCR products	Commercial
E. coli SN0301	*E. coli* SN03-derived strain mutant in ampD1 gene	Schmidt et al. (1995)
E. coli SN0301-pSEV-B1	Vector-carrying strain of pSEV-B1 (tetracycline selection)	This study
E. coli SN0301-pSEV-B2	Vector-carrying strain of pSEV-B2 (tetracycline selection)	This study
E. coli SN0301-mcherry	Vector-carrying strain of pSEVA541-mCherry (tetracycline selection)	This study

subinhibitory concentrations of imipenem, carbenicillin, ampicillin and ceftazidime as inducers. Overnight cultures of IB20 were diluted in 20 mL of fresh LB medium to an OD_{600} of 0.01 in 100 mL Erlenmeyer flasks. The cultures were incubated at 25°C with constant agitation of 250 rpm until they reached the exponential growth phase (OD_{600} = 0.6). To provide a comparison, we also evaluated *ampC* expression levels in *P. aeruginosa* PAO1-V. This strain was grown under the same above-mentioned conditions, excepting a temperature that was set at 37°C. Once both strains reached an OD_{600} of 0.6, one-fourth (1/4) of their respective MIC value of each antibiotic was individually added to the culture media, and the cultures were grown for an additional 90 min. The MIC values against antibiotics for all strains used in this study are listed in Table S2. Subsequently, 5 mL of each induced culture was harvested and centrifuged at 8000 *g* for 15 min at 4°C. The resulting cell pellets were resuspended in 270 μL of TE buffer with 20 μL of lysozyme (20 mg/mL) and 10 μL of proteinase K (20 mg/mL), followed by a 10-min incubation at room temperature. Next, 1 mL of TRIzol® reagent was added to extract RNA, following the manufacturer's guidelines (Invitrogen). The extracted RNA (5 μg) was treated with DNase TURBO (Ambion) in a 50 μL reaction. Approximately 1 μg of RNA was then reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). To demonstrate the absence of DNA contamination in the isolated RNA, two tests were conducted. The first test involved PCR analysis of samples that were not subjected to reverse transcriptase treatment, with the *rpsL* gene as the target. In the second test, all samples were subjected to PCR analysis targeting the *rpsL* gene. If a sample successfully amplified this gene, it underwent DNase treatment, and the process was repeated. Only samples that failed to yield a positive PCR result were then quantified by real-time PCR.

RT–PCR was performed in triplicate using the CFX96 Touch™ RT–PCR Detection System (Bio-Rad) and the KAPA SYBR® FAST Universal qPCR kit (Kapa Biosystems). Each reaction contained 150 mM of each primer, 25 ng of RNA and a total reaction volume of 25 μL. The relative expression in both strains was calculated using the $2^{-\Delta\Delta C_t}$ method (Pfaffl, 2001), with *rpsL* as the gene normalizer. The oligonucleotides used in the RT–PCR experiments are found in Table S1.

Genetic engineering for the construction of biosensors

Two strategies were used to construct the expression vector containing the transcriptional fusion of the *ampR–ampC* circuit and mCherry protein. For the first strategy, the *ampR–ampC* locus was amplified from the IB20 genome using overlap extension PCR (OE–PCR), with the ORF of *ampC* replaced by the mCherry gene (Figure S1). The resulting genetic construction was cloned into the pSEVA541 vector, creating the pSEV-B1 vector. The second strategy involved commercial synthesis by Genescript resulted in a transcriptional fusion where AmpR is constitutively expressed under the P3 promoter (Arce-Rodríguez et al., 2023). This construction was also cloned into the pSEVA541 vector, generating the pSEV-B2 vector. Additionally, a vector with the constitutive mCherry under the P3 promoter

was constructed based on pSEVA541, resulting in the pSEVA-mCherry vector. Finally, the pSEV-B1, pSEV-B2 and pSEV-mCherry plasmids were transformed into the *E. coli* SN301 strain, producing biosensor 1, biosensor 2 and the constitutive-mCherry-, respectively. The plasmid empty-pSEVA541 was transformed in the *E. coli* SN0301 strain as a control.

Demonstration of the correct functioning of biosensors: Visualization by microscopy

To demonstrate the proper functioning of the biosensors, overnight cultures of each biosensor were diluted at a ratio of 1:100 in 20 mL of LB medium. These diluted cultures were incubated at 37°C with 240 rpm shaking until they reached an optical density at OD_{600} of 0.6. Next, the culture's concentration was adjusted to an OD_{600} of 0.01 in 150 μL of medium, and the antibiotics ampicillin and imipenem were added to achieve final concentrations of 125 and 1000 ng/mL, respectively. These concentrations corresponded to a quarter (1/4) of the MIC of the antibiotics for each of the strains used. The resulting mixture was placed in a clear 96-well flat-bottom microplate. After 24 h of incubation at 37°C, the samples were observed using an inverted epifluorescence microscope (Nikon eclipse Ti-S) with a green light (540 nm) excitation filter. Pictures were captured using 10× magnification. The captured images are representative fields of visualization.

Biosensor induction assays

Once microscopy confirmed that all the biosensors could successfully detect imipenem, their response to increasing concentrations of this antibiotic was evaluated. The biosensors were cultured overnight and then diluted 100-fold in 20 mL of LB medium. They were subsequently incubated at 37°C with 250 rpm shaking until reaching an OD_{600} of 0.2 (~1.6×10^8 cells/mL) (corresponding to the exponential phase in a microplate) and 0.4 (~3×10^8 cells/mL) (corresponding to the stationary phase in a microplate) (Figure S2). Increasing concentrations of imipenem were added to the medium in a 2-fold dilution series, ranging from 0.04 to 125 ng/mL. Afterwards, 150 μL of the mixture was transferred to a 96-well Black/Clear Bottom microplate (Nunc, Thermo Scientific) and incubated at 37°C for 20 h in the VICTOR® Nivo™ plate reader (PerkinElmer). Measurements of OD_{600} and fluorescence production (using 580 nm excitation and 625 nm emission filters) were taken every 20 min. The fluorescence units produced at each antibiotic concentration were normalized to the OD_{600}, and the induction levels at each antibiotic concentration were calculated as the fold change relative to the absence of antibiotics.

Additionally, we evaluated the response of the biosensors to other types of beta-lactam antibiotics, such as meropenem (0.004–40 ng/mL) (Carbapenem), ampicillin (2–2000 ng/mL), carbenicillin (0.5–1000 ng/mL) (penicillins), amoxicillin-clavulanate (1–1000 ng/mL) (Penicillin + beta-lactamase inhibitor), cefuroxime (0.5–1000 ng/mL) and ceftazidime (0.5–1000 ng/mL) (Cephalosporins). Furthermore, we included polymyxin B (1–250 ng/mL) and gentamicin (1–1000 ng/mL) (aminoglycoside) to test the specificity of the biosensors in the presence of non-beta-lactam drugs. In these experiments, all inductions were performed in microplates using cultures with an initial OD_{600} of 0.2 (~1.6×10^8 cells/mL). All MIC values refer to the respective strains.

RESULTS

Pseudomonas sp. IB20 induce higher expression levels of the *ampC* gene compared to *P. aeruginosa* PAO1 in response to a broader spectrum of beta-lactam antibiotics

In previous studies conducted by our research group, we demonstrated that *Pseudomonas* sp. IB20 exhibits high resistance to beta-lactams, surpassing even *P. aeruginosa* (Vásquez-Ponce et al., 2018). Considering that AmpC beta-lactamase is a primary mechanism of resistance to beta-lactams in the *Pseudomonas* genus and is also inducible, we compared the expression of this system using RT–qPCR with subinhibitory concentrations of the antibiotics imipenem (carbapenem), carbenicillin, ampicillin (penicillins) and ceftazidime (a third-generation antipseudomonal cephalosporin) between this isolate and *P. aeruginosa* PAO1. The results revealed that *Pseudomonas* sp. IB20 strongly induces *ampC* expression in the presence of imipenem (540-fold), carbenicillin (490-fold) and ampicillin (510-fold). In contrast, ceftazidime showed only a three-fold induction compared to the uninduced state (Figure 1). In contrast, PAO1 only exhibited a strong *ampC* induction in the presence of imipenem (270-fold), while carbenicillin and ampicillin produced a two-fold induction, and no induction was observed with ceftazidime. These findings demonstrate the high inducibility of the AmpC beta-lactamase in IB20, surpassing even that of PAO1, and even responding to ceftazidime. Based on these results, we propose that IB20 possesses an AmpR–AmpC-dependent regulation system that is more sensitive to beta-lactams than that of PAO1.

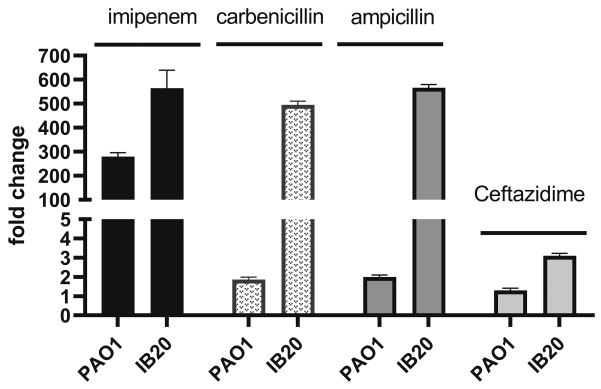

FIGURE 1 Relative expression of the ampC gene in *Pseudomonas* sp. IB20 after exposure to beta-lactam antibiotics. The expression levels of the *ampC* gene from IB20 were determined using RT–qPCR after exposure to subinhibitory concentrations (1/4 of MIC values) of several beta-lactams. For comparison, the expression levels of *ampC* in *P. aeruginosa* PAO1-V were also evaluated. The data obtained indicated that the *ampC* gene is highly induced in IB20, with the highest values observed in the presence of imipenem, ampicillin, and carbenicillin. In comparative terms, the expression levels of the IB20 *ampC* gene were 2.4-fold higher than those observed in PAO1 in the presence of imipenem, 2-fold higher in the presence of ceftazidime, and 270-fold higher in the presence of carbenicillin. Three biological and technical replicates were used for this assay, and *rpsL* was used as the normalizing gene (MICs PAO1-V: Imipenem 1 μg/mL; ceftazidime 1 μg/mL; carbenicillin 16 μg/mL; ampicillin 1024 μg/mL. MICs *Pseudomonas* sp IB20: Imipenem 1 μg/mL; ceftazidime 2 μg/mL; carbenicillin 4096 μg/mL; ampicillin 16,384 μg/mL).

The *E. coli* whole-cell biosensors, based on the AmpR–AmpC regulatory system of *Pseudomonas* sp. IB20, can detect beta-lactams

As mentioned in methods, two AmpR-based reporter constructs were created using different methods and inserted into a pSEVA541 plasmid. The first method involved replacing the *ampC* gene with the mCherry reporter gene, preserving the *ampR* gene and intergenic regions to form the pSEV-B1 plasmid. The second approach, proposed by Arce-Rodriguez et al. (2023), utilized a P3 constitutive promoter and a strong ribosome binding site (RBS) to regulate AmpR expression in the pSEV-B2 plasmid. A non-regulatory buffer sequence was placed between the P3 promoter and the DNA sequences upstream of the *ampC* gene. The mCherry reporter gene was also cloned alone into pSEVA541 for constitutive expression as a control (Figure 2). These constructs, along with an empty pSEVA541 vector, were transformed into *E. coli* SN0301 to generate specific strains: Biosensor 1 (pSEV-B1), Biosensor 2 (pSEV-B2), mCherry-constitutive control (pSEV-mCherry) and Biosensor control (empty pSEVA541). The strains were tested by incubation with imipenem and ampicillin, revealing significant fluorescence in Biosensors 1 and 2, while the Biosensor control showed no fluorescence, confirming strain functionality (Figure 3). The control biosensor was subsequently excluded due to consistent results, and the figure did not display the outcome of pSEV-mCherry due to saturation.

Biosensors 1 and 2 exhibit optimal activity when the initial OD_{600} is 0.2 and after 10 h of incubation

To determine the optimal response time, each biosensor was exposed to subinhibitory concentrations of imipenem and fluorescence was quantified for 20 h. The results demonstrated that the optimal induction time for both Biosensor 1 and Biosensor 2 was 10 h (Figure 4A,B, respectively). No induction was observed in the mCherry control biosensor (Figure 4C). Prior to 10 h, the measurements were unstable, as were the measurements obtained after this induction time. Once the optimal induction time was determined for the biosensors, we proceeded to estimate the optimal initial inoculum for the induction assays. Two different cell densities were employed: OD_{600} 0.2 (~1.6×10^8 cells/mL) (corresponding to the exponential phase in a microplate) and 0.4 (~3×10^8 cells/mL) (corresponding to the stationary phase in a microplate). For this purpose, a range of imipenem concentrations (0.04–20 ng/mL) was used. Figure 5 illustrates the dose-dependency trend observed with both biosensors 1 and 2 when an initial OD_{600} of 0.2 was used (Figure 5A,B, respectively). Additionally, it can be observed that biosensor 2 displayed a better response at an initial OD_{600} of 0.2 (Figure 5B). In contrast, when initiating with an OD_{600} of 0.4, the dose-dependent phenomenon is not observed in either biosensor 1 or biosensor 2 (Figure 5D,E, respectively). No induction was observed in the mCherry control biosensor (Figure 5C,F). Based on these findings, it can be concluded that the optimal cell density is an OD_{600} of 0.2, and the optimal incubation time is 10 h.

The biosensor 1 responds in a dose-dependent manner and exhibits high specificity for beta-lactams

To evaluate the responsiveness of biosensors 1 and 2 to beta-lactams in a dose-dependent manner, we monitored the relative fluorescence emitted in the presence of different types of beta-lactams: imipenem, meropenem (carbapenems), ampicillin, carbenicillin (penicillins), amoxicillin-clavulanate (Penicillin + beta-lactamase inhibitor), cefuroxime, ceftazidime (cephalosporins), using a wide range of subinhibitory concentrations where no growth defects were observed for any antibiotic. Biosensor 1 exhibited a dose-dependent response. Specifically, in the case of imipenem, the biosensor showed a dose-dependent response from 0.04 to 15.6 ng/mL, with the detection peak at 15.6 ng/mL (Figure 6A,B). On the other hand,

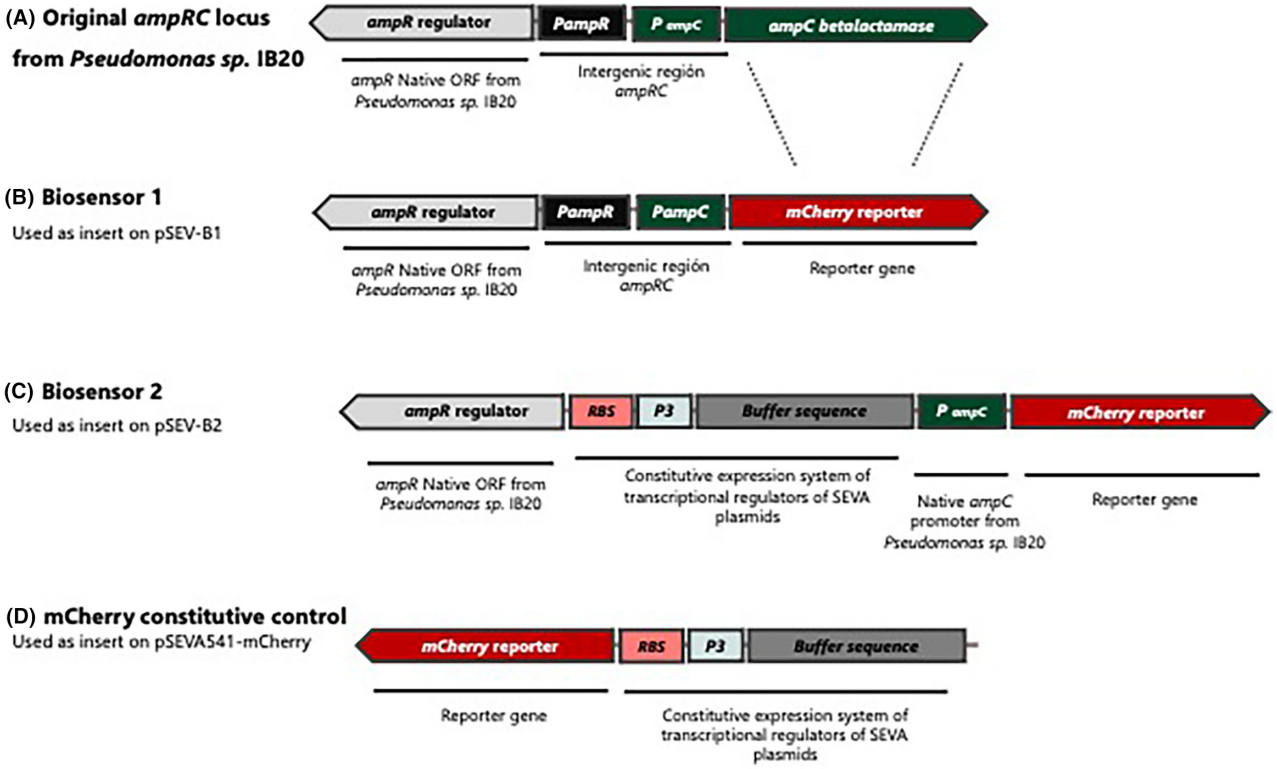

FIGURE 2 Scheme illustrating the construction of transcriptional fusions for biosensor generation. (A) Original *ampRC* gene-locus from *Pseudomonas* sp. IB20; (B) Biosensor 1; (C) Biosensor 2; (D) mCherry biosensor control. Key components: ampC betalactamase, Coding sequence for the AmpC betalactamase; ampR regulator, Coding sequence for the AmpR protein; mCherry reporter, coding sequence for the mCherry fluorescent protein; P3, Strong promoter for constitutive expression. Buffer sequence; pampC, promotor sequence from ampC-coding sequence; PampR, promotor sequence from ampR-coding sequence; RBS, improved ribosomal binding site.

the maximum detection capacity (MDC) was 250 ng/mL, corresponding to half the MIC. MDC is defined as the maximum concentration detected by the biosensor at which no growth problems are observed. For meropenem, the range of concentrations in which biosensor 1 responded in a dose-dependent manner was much more limited (0.016–0.5 ng/mL), with the detection peak at 0.5 ng/mL and an MDC of 1 ng/mL, which is six times less than the MIC (Figure 6C).

Regarding penicillins, for ampicillin, the range of concentrations for dose-dependent behaviour was between 3.9 and 2000 ng/mL, with the peak and MDC at 2000 ng/mL, which corresponds to half the MIC (Figure 6E). For carbenicillin, the concentration range goes from 3.9 to 1000 ng/mL, with a peak and an MDC of 1000 ng/mL, equivalent to 1/4 of the MIC (Figure 6F). In the case of cephalosporins (cefuroxime and ceftazidime), both showed similar behaviour with a range of concentrations for the dose-dependent response between 7.8 and 500 ng/mL, with a peak and an MDC of 500 ng/mL, equivalent to 1/8 MIC of cefuroxime and 1/2 MIC ceftazidime (Figure 6G,H). When evaluating amoxicillin-clavulanate (penicillin + beta-lactamase inhibitor), it is observed that the dose-dependent behaviour occurs at concentrations between 2 and 250 ng/mL, with a detection peak at 250 ng/mL and an MDC of 1000 ng/mL, corresponding to half the MIC (Figure 6D).

On the other hand, when evaluating biosensor 2, a notable difference is observed compared to biosensor 1 since it is much less sensitive. Its dose-dependent response can only be demonstrated with the antibiotic's imipenem (Figure 6A,B), amoxicillin-clavulanate (Figure 6D), ampicillin (Figure 6E) and carbenicillin (Figure 6F). Furthermore, it is important to mention that biosensor 2 could not detect any of the evaluated cephalosporins (cefuroxime and ceftazidime), or meropenem.

The specificity of the biosensors was assessed by exposing them to non-beta-lactam antibiotics: polymyxin B, targeting the outer membrane of Gram-negative bacteria and gentamicin (an aminoglycoside), affecting ribosomes and disrupting protein synthesis. None of these antibiotics activated the biosensors under evaluation, confirming their specificity towards beta-lactams (Figure S3).

Biosensor 1 exhibits very high sensitivity to carbapenems

The last aspect evaluated for both biosensors was the limit of detection (LOD), which represents the

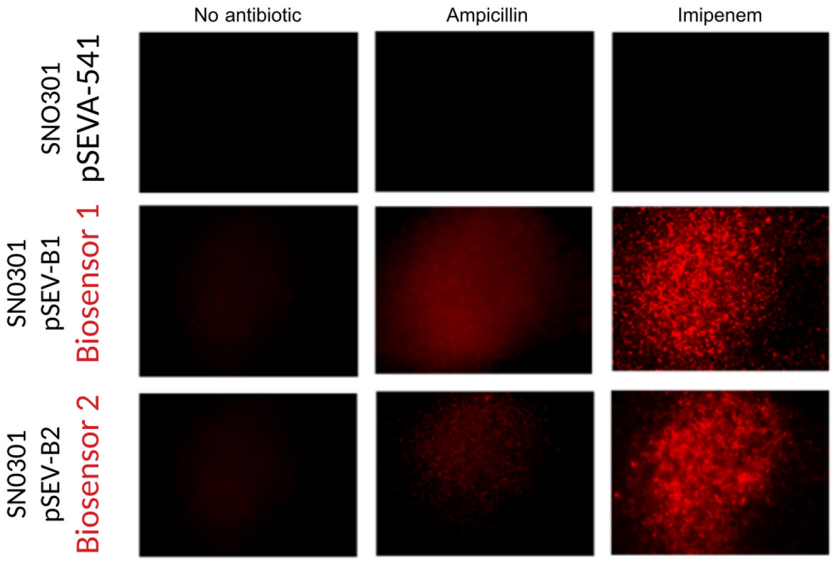

FIGURE 3 Microscopic visualization of biosensors exposed to sub-inhibitory concentrations of ampicillin and imipenem. Raw fluorescence production was evaluated by microscopic visualization of biosensors 1 and 2 in cultures grown on black 96-well plates with a transparent bottom. The cultures were grown from an initial OD_{600} of 0.05 using LB medium supplemented with ampicillin (1000 ng/mL) or imipenem (125 ng/mL) corresponding to 1/4 of their respective MIC values. For comparative purposes, strain SN0301 carrying the empty vector pSEVA541 was also included in the testing. Microscopic visualization was performed after 24 h of culture using a green light excitation filter (540 nm) and a 10× magnification on a fluorescence microscope.

lowest concentration at which the biosensor can detect a specific antibiotic (Table 2). When evaluating this characteristic, biosensor 1 showed extremely high sensitivity to carbapenems, being able to detect 0.04 ng/mL (40 pg/mL) of imipenem and 0.008 ng/mL (8 pg/mL) of meropenem. Likewise, when evaluating penicillins, it was found that the lower detection limit for ampicillin and carbenicillin was 3.9 ng/mL, which indicates a great sensitivity towards this type of beta-lactam antibiotics as well. When amoxicillin-clavulanate (penicillin + beta-lactamase inhibitor) was evaluated, we saw an LOD of 2, presenting a slightly higher sensitivity than penicillins alone. Undoubtedly, biosensor 1 exhibited a poorer performance with cephalosporins compared to the other families of beta-lactams evaluated. Both the dose-dependent behaviour and the LOD were significantly worse in this case.

Regarding biosensor 2 and its ability to detect antibiotics, amoxicillin-clavulanate and ampicillin showed a detection limit identical to that of biosensor 1 (2 ng and 3.9 ng, respectively). However, for imipenem, it presented a detection limit double that of biosensor 1, and for carbenicillin, showed a detection limit four times higher than biosensor 1.

Considering sensitivity, antibiotic detection capacity, and dose-dependent response, biosensor 1 showed a better performance than biosensor 2. Therefore, it is proposed to rule out biosensor 2 for any improvement and its possible implementation at a commercial biotechnological level.

DISCUSSION

In this study, we present the development of a whole-cell-based biosensor by genetically modifying the *E. coli* SN0301 strain, which is highly susceptible to beta-lactams, incorporating a high-copy-number plasmid containing the *ampR–ampC* system from the Antarctic strain *Pseudomonas* sp. IB20. This regulatory system controls the expression of the beta-lactamase AmpC, participating in the process of repair and recycling of peptidoglycan (PG). During this process, degradation products such as 1,6-anhydro-MurNAc peptides have been identified as *ampC* inducers. In the absence of antibiotics, the cytosolic 1,6-anhydro-MurNAc peptides are metabolized by the cytosolic protein AmpD, resulting in low levels of 1,6-anhydro-MurNAc peptides, which keeps the system repressed (Bartowsky & Normark, 1993; Jacobs et al., 1994; Lindquist et al., 1989). The presence of beta-lactams increases the influx of peptidoglycan degradation products into the cytoplasm, saturating AmpD and inducing the *ampC* expression (Dietz et al., 1996; Jacobs et al., 1994; Vollmer & Höltje, 2001). Hence, the role played by AmpD is essential in the regulation of the expression of this system.

Considering the above information, the simultaneous deletion of the non-inducible beta-lactamase *ampC8* and *ampD1* in the *E. coli* SN0301 strain makes this bacterium an ideal host for the development of beta-lactam biosensors. Eliminating *ampC8* enhances

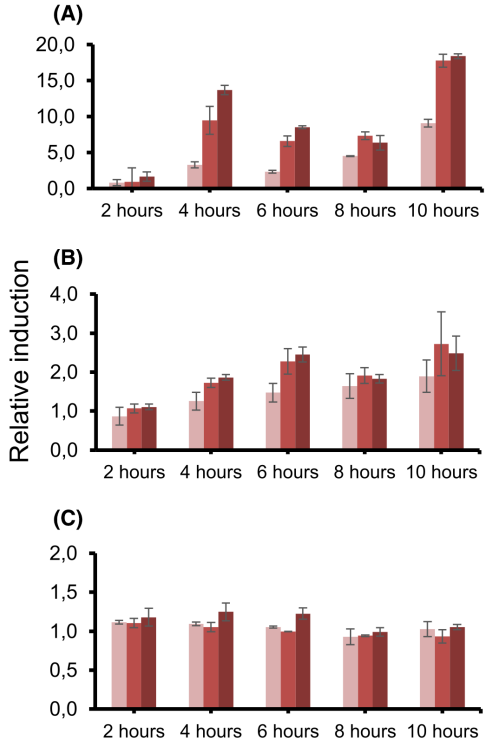

FIGURE 4 Determination of the optimal response time of the constructed biosensors. Cultures of each biosensor were supplemented with subinhibitory concentrations of imipenem to evaluate its effect on fluorescence production over time. The raw fluorescence values were normalized to OD_{600}, and the biosensor induction capacity was expressed as the fold change of the relative fluorescence compared to the absence of the drug. (A) Biosensor 1; (B) Biosensor 2; (C) mCherry **control biose**nsor. The red bars represent concentrations of 2 ng/mL (1/256 of the MIC), 16 ng/mL (1/32 of the MIC) and 125 ng/mL (1/4 of the MIC), from the lightest to the darkest.

susceptibility to beta-lactams and lowers the antibiotic concentration threshold at which this bacterium responds to damage in the PG structure. Similarly, the loss of AmpD leads to a more substantial accumulation of the inducing peptide, 1,6-anhydro-MurNAc, in the presence of beta-lactam antibiotics. This, in turn, significantly enhances the sensitivity of AmpR activation, consequently increasing the expression of AmpC (Dik et al., 2020), even in heterologously expressed *ampR–ampC* systems (Schmidt et al., 1995; Valtonen et al., 2002). In our experiments, we observed that in *Pseudomonas* sp. IB20 high levels of *ampC* gene expression are induced in the presence of imipenem, ampicillin and carbenicillin but not ceftazidime. On the other hand, biosensor 1 was able to produce a dose-dependent response in all tested antibiotics, including ceftazidime and cefuroxime. In that sense, it is likely that the genetic background of SN0301, and especially the mutation in *ampD*, could play a key role in the ability to induce the *ampR–ampC* system in this strain.

Until now, the heterologous expression of *ampR–ampC* systems in *ampD* mutants of *E. coli* has been addressed in two studies. One of them is the beta-lactam biosensor made by Valtonen et al. (2002), which expresses the *ampR–ampC* system from *C. freundii*, also in SN0301. Although this biosensor was able to respond to the presence of several cephalosporins, neither ceftazidime nor cefuroxime was included in this study. In another study, Schmidtke and Hanson (2006) reported that an *E. coli ampD* mutant susceptible to ceftazidime acquires resistance to this antibiotic when heterologously expressing a *blaACT-1-ampR* system. Although this study demonstrated an increase in *blaACT-1* expression, it remains unknown whether it occurred through a derepression or hyperinduction phenotype, since expression levels of the *blaACT-1* gene in the presence or absence of antibiotics were not evaluated.

While it is highly likely that the ability of biosensor 1 to respond to ceftazidime is significantly influenced by the lack of *ampD* in *E. coli* SN0301, other elements could be involved, such as penicillin-binding proteins (PBPs). In contrast, the malfunction of biosensor 2 would have its origin in this same regulation circuit. This biosensor overexpresses the *ampR* regulator, which allows AmpR to always be available for binding to PG residues. However, it seems that the constant presence of this regulator protein means that the system does not "turn on" or does not have precise regulation. Furthermore, AmpR has been designated as a global regulator in *P. aeruginosa* (Balasubramanian et al., 2015; Caille et al., 2014; Vadlamani et al., 2015). Therefore, its constitutive expression could impact gene expression in *E. coli*, potentially leading to failures in the biosensor. Taking these results into account, the constant presence of AmpR and its role as a global regulator does not allow the development of an optimal biosensor, which led us to discard biosensor 2.

Another interesting finding is that antibiotics belonging to the same beta-lactam subclass induce similar dose–response profiles in biosensor 1 (Figure 7). This similarity is supported by the affinity of different types of beta-lactams for penicillin-binding proteins (PBPs). Although this phenomenon has been extensively studied in both Gram-positive and Gram-negative bacteria, the output response of the cell-based biosensors developed by Valtonen et al. (2002) and Lautenschläger et al. (2020) does not seem to share similar dose–response profiles for related drugs. Nevertheless, the affinity of beta-lactams for these enzymes in *E. coli* has been described, and it may be crucial for the generation of 1,6-anhydro-MurNAc and the efficiency of the biosensor's activation. For instance, studies in *E. coli* have demonstrated that cephalosporins bind preferentially to PBP3 and PBP1, inhibiting them in a dose-dependent manner (Davies et al., 2007; Gutmann et al., 1986; Preston et al., 1990). Additionally, it has been observed that the inhibition of both PBPs by ceftazidime and

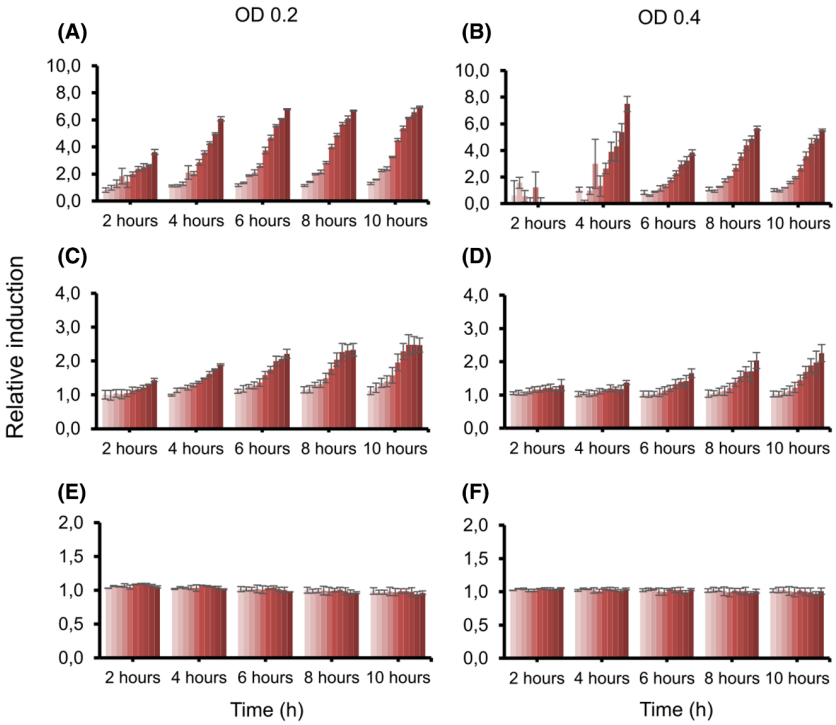

FIGURE 5 Effect of initial cell density on the fluorescent response of biosensors exposed to subinhibitory concentrations of imipenem. Fluorescence production of biosensors was evaluated in microplates at two different initial cell densities: OD_{600} 0.2 (exponential phase) and 0.4 (stationary phase). (A and D) Biosensor 1; (B and E) Biosensor 2; (C and F) mCherry control biosensor. The red bars represent the relative inductions of fluorescence production at 0.04, 0.08, 0.16, 0.3, 0.6, 1.25, 2.5, 5.0, 10 and 20 ng/mL, from the lightest to the darkest bar.

cefuroxime is significantly stronger in *E. coli* than in *P. aeruginosa* (Fontana et al., 2000), which supports the results obtained in this study. Alternatively, penicillins, especially carbenicillin and ampicillin, showed a linear dose–response profile (Figure 7). However, amoxicillin-clavulanate reached saturation points near the minimum inhibitory concentration (MIC). In general, penicillins bind to a wide and varied range of PBPs (Kocaoglu & Carlson, 2015), which leads to effective induction levels but could cause a kind of "stoichiometric dilution". Therefore, penicillins are effective inducers of AmpC, although they may not be as potent as carbapenems. In contrast, carbapenems exhibit superior performance by activating the circuit at very low concentrations, thanks to their exceptionally high affinity for PBP2 and PBP4 (Kocaoglu & Carlson, 2015; Preston et al., 1990). At this point, it is crucial to emphasize the role of PBP4 in the activation of AmpC in both *P. aeruginosa* and *E. coli*. It has been documented that mutations in this enzyme lead to significant alterations in the quality and quantity of modifications in the muropeptides, the AmpR inducers. Consequently, it has been reported that potent PBP4 inhibitors, such as imipenem and meropenem, act as robust inducers of AmpC (Moya et al., 2009; Sanders et al., 1997; Torrens et al., 2019). Finally, clavulanic acid, commonly used in combination with amoxicillin, is a potent inducer of AmpC expression (Ruppé et al., 2015) and binds strongly to PBP2 (Chambers & Miick, 1992). This could explain why biosensor 1 produces an output response that more closely resembles the asymptotic profile of carbapenems than the linear profile of penicillins (Figure 7).

Undoubtedly, the most outstanding characteristic of biosensor 1 is its remarkably high sensitivity for detecting carbapenems. It can detect as low as 40 pg/mL of imipenem and 8 pg/mL of meropenem, which greatly surpasses the sensitivity of whole-cell biosensors developed for this type of antibiotic. In fact, it is 60 times more sensitive than the biosensor developed by Valtonen et al. (2002), which can detect up to 2500 pg of imipenem, and 125 times more sensitive than the biosensor developed by Lautenschläger et al. (2020), capable of detecting 1000 pg of meropenem. Additionally, it exhibits detection limits comparable to biosensors based on beta-lactamases coupled to circuits (Andresen et al., 2018; Meng et al., 2021) and conventional methods such as HPLC or MS (Andresen et al., 2018; Parker et al., 2019). It is important to highlight that this biosensor is highly efficient in detecting low concentrations of evaluated antibiotics, particularly carbapenems. However, its performance has been observed to decrease at high concentrations. Nonetheless, it is crucial to consider that the concentrations of antibiotics to be detected in different matrices generally fall within

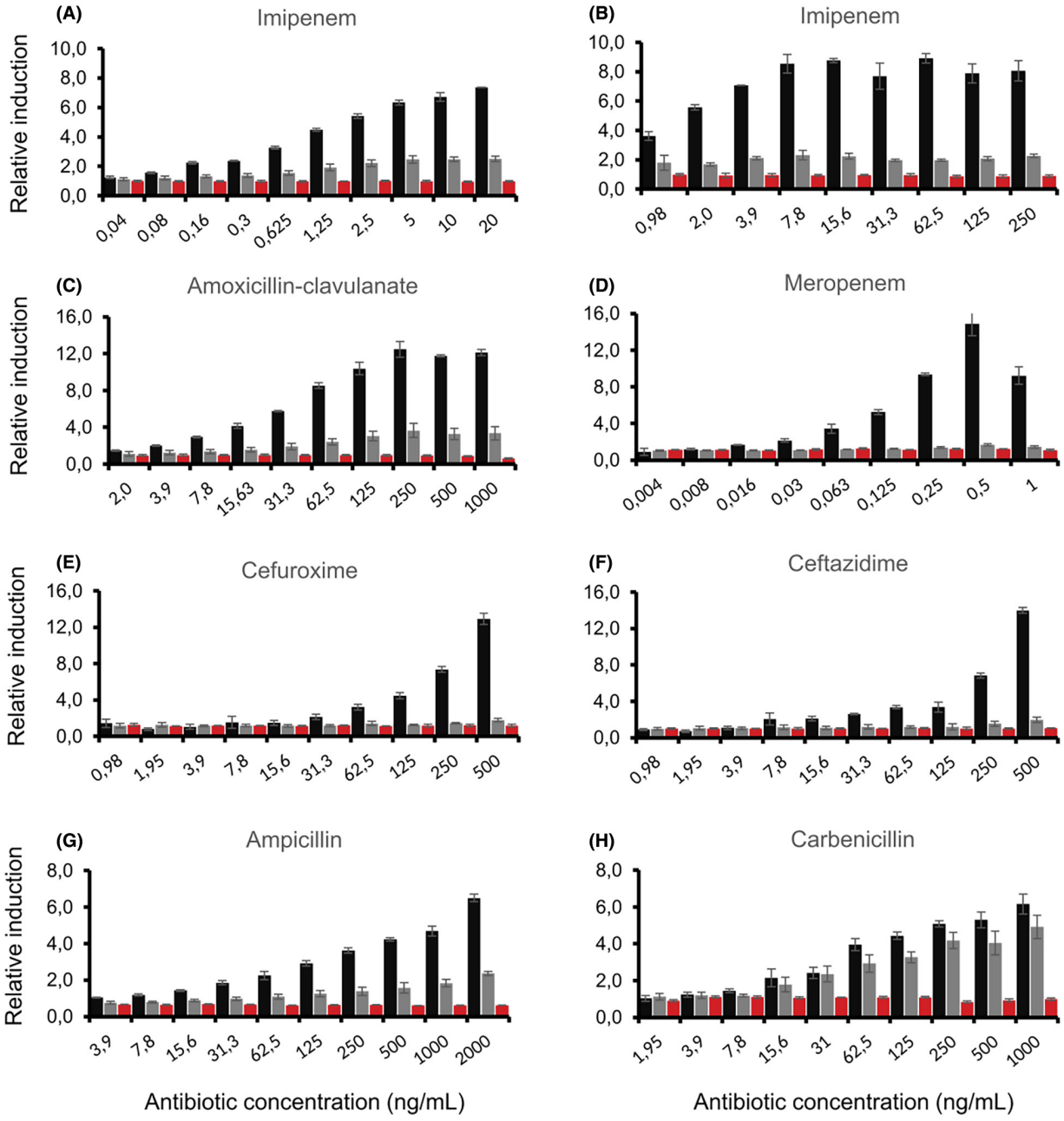

FIGURE 6 Detection of different beta-lactam antibiotics by the constructed biosensors. (A) Low concentrations of imipenem; (B) High concentrations of imipenem; (C) Meropenem; (D) Amoxicillin-clavulanate; (E) Cefuroxime; (F) Ceftazidime; (G) Ampicillin; (H) Cabernicillin. Raw fluorescence values were normalized to OD_{600} and relative to the basal fluorescence produced in the absence of antibiotics (*y*-axes). The data was obtained after 10 h of incubation at 37°C. The antibiotic ranges vary for each compound according to their respective minimum inhibitory concentration (MIC). The black bars correspond to biosensor 1, the grey bars to biosensor 2, and the red bars to the mCherry biosensor control.

TABLE 2 Limits of detection of biosensors for the different beta-lactam antibiotics tested.

	IPM	MEM	AMC	AMP	CAR	CXM	CAZ
Biosensor 1	0.04	0.008	2	3.9	3.9	7.8	7.8
Biosensor 2	0.08	n/d	2	3.9	15.6	n/d	n/d

Note: Values are represented in ng/μL.

Abbreviaitions: AMC, amoxicillin-clavulanate; AMP, ampicillin; CAR, carbenicillin; CAZ, ceftazidime; CXM, cefuroxime; IMP, imipenem; MEM, meropenem; n/d, not detected.

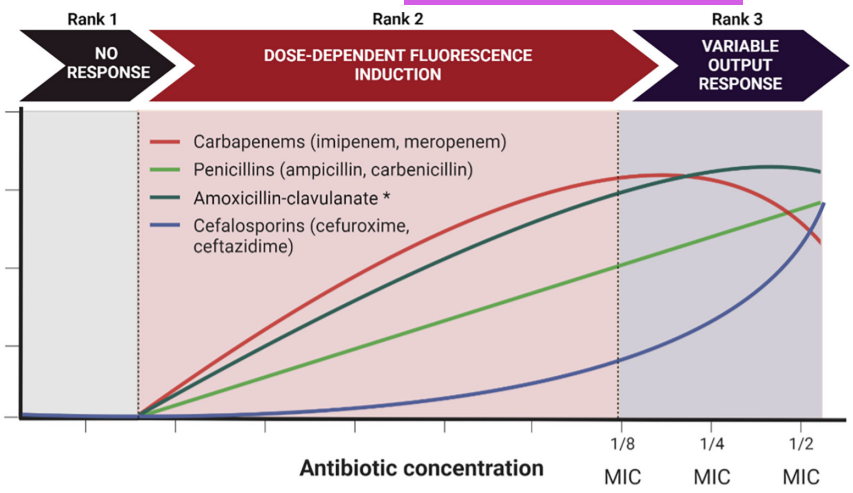

FIGURE 7 Dose–response profiles of biosensors against drugs belonging to the same beta-lactam subclass. (*penicillin + beta-lactamase inhibitor)

the range of picograms to nanograms (Alnassrallah et al., 2022; Griboff et al., 2020; Sharafati-Chaleshtori et al., 2013), making the biosensor suitable for fulfilling the detection needs of these compounds.

In summary, the success of this biosensor can be attributed to the combination of the *E. coli* isolate SN0301, which is highly susceptible to beta-lactams, and its incorporation into a high copy number plasmid carrying the *ampR–ampC* regulation system of the Antarctic isolate *Pseudomonas* sp. IB20. This system is highly inducible by these antibiotics and enables the biosensor to detect a wide variety of them. Finally, it is necessary to conduct further studies to explore different host organisms or enhance the existing ones. This exploration will enable the optimization of various aspects, such as the speed of detection, thereby increasing the effectiveness and performance of biosensor 1.

AUTHOR CONTRIBUTIONS

Sebastián Higuera-Llantén: Formal analysis (lead); investigation (equal); writing – original draft (lead); writing – review and editing (supporting). **Manuel Alcalde-Rico:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); supervision (supporting); validation (equal); writing – original draft (supporting); writing – review and editing (supporting). **Felipe Vasquez-Ponce:** Data curation (supporting); formal analysis (supporting); investigation (equal); methodology (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Claudia Ibacache-Quiroga:** Project administration (lead); supervision (supporting); writing – review and editing (supporting). **Jesús Blazquez:** Formal analysis (lead); methodology (equal); supervision (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Jorge Olivares-Pacheco:** Conceptualization (lead); formal analysis (lead); funding acquisition (lead); investigation (supporting); methodology (equal); project administration (lead); supervision (lead); writing – original draft (supporting); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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