Development of a novel heterologous β-lactam-specific whole-cell biosensor in Bacillus subtilis

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Abstract

Background: Whole-cell biosensors are a powerful and easy-to-use screening tool for the fast and sensitive detection of chemical compounds, such as antibiotics. β-Lactams still represent one of the most important antibiotic groups in therapeutic use. They interfere with late stages of the bacterial cell wall biosynthesis and result in irreversible perturbations of cell division and growth, ultimately leading to cell lysis. In order to simplify the detection of these antibiotics from solutions, solid media or directly from producing organisms, we aimed at developing a novel heterologous whole-cell biosensor in Bacillus subtilis, based on the β-lactam-induced regulatory system BlaR1/BlaI from Staphylococcus aureus.

Results: The BlaR1/BlaI system was heterologously expressed in B. subtilis and combined with the luxABCDE operon of Photorhabdus luminescens under control of the BlaR1/BlaI target promoter to measure the output of the biosensor. A combination of codon adaptation, constitutive expression of blaR1 and blaI and the allelic replacement of penP increased the inducer spectrum and dynamic range of the biosensor. β-Lactams from all four classes induced the target promoter P blaZ in a concentration-dependent manner, with a dynamic range of 7- to 53-fold. We applied our biosensor to a set of Streptomyces soil isolates and demonstrated its potential to screen for the production of β-lactams. In addition to the successful implementation of a highly sensitive β-lactam biosensor, our results also provide the first experimental evidence to support previous suggestions that PenP functions as a β-lactamase in B. subtilis.

Conclusion: We have successfully established a novel heterologous whole-cell biosensor in B. subtilis that is highly sensitive for a broad spectrum of β-lactams from all four chemical classes. Therefore, it increases the detectable spectrum of compounds with respect to previous biosensor designs. Our biosensor can readily be applied for identifying β-lactams in liquid or on solid media, as well as for identifying potential β-lactam producers.

Keywords: Cell wall biosynthesis, Cell wall antibiotic, Cell envelope stress response, Antibiotic discovery, Mechanism-of-action studies

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Background

Worldwide, the antibiotic resistance crisis is becoming a major threat for public health as numbers of infections caused by multi-drug resistant bacteria are rising, especially in the clinical setting [1, 2]. According to the WHO Global Antimicrobial Surveillance System (GLASS), a growing number of common bacterial infections such as pneumonia, gonorrhea or salmonellosis are becoming harder to treat, highlighting the urgent need for novel antimicrobial compounds [3]. Moreover, steps need to be taken to prevent the emergence of antimicrobial resistance, as misuse and overuse of antibiotics accelerate the process [3, 4].

β-Lactams, such as penicillin, still constitute one of the most important antibiotic groups in therapeutic use [5]. They interfere with late stages of the bacterial cell wall biosynthesis by covalently binding to the active center of penicillin binding proteins (PBPs), resulting in irreversible perturbations of cell division and growth, ultimately leading to cell lysis [6]. In the evolutionary arms race of survival in the presence of lethal chemical threats such as antibiotics, many bacteria have developed or acquired specific resistance determinants. Often these are specialized enzymes that are able to inactivate harmful antimicrobial compounds. In the case of β-lactams, β-lactamases represent one widespread resistance mechanism in bacteria [7]. These enzymes catalyse the hydrolysis of the β-lactam ring structure, thereby generating a biologically inactive product. Currently, such enzymes are only effective against some compounds of the β-lactam family [7, 8]. However, if variations of β-lactamases with a broader β-lactam spectrum would emerge, the therapeutic potency of these still powerful antimicrobial compounds could be threatened.

Consequently, the development of new screening tools that are specific, sensitive and robust, is crucial for detecting and discriminating antimicrobial compounds. For such purposes, whole-cell biosensors have proven a powerful and widely adapted approach [9–11]. These are genetically engineered microorganisms that respond to a specific input, e.g. a defined range of antibiotics, with a quantifiable output, like fluorescence or luminescence [12]. Biosensors have been developed for the detection of toxic contaminants like heavy metals (e.g. arsenite), cyclic aromatic carbohydrates (e.g. naphthalene) as well as for the discovery of novel antibiotics [13–15]. The value of applying biosensors is their ease of use and the low costs compared to chromatography-based detection methods or immunoassays that require expensive equipment or experienced staff [16, 17]. Freeze-drying or the use of bacterial spores for transport allow using biosensors in the field, where they can be ‘revived’ by rehydration at the designated operation site [18, 19]. While a defined specificity for a certain class of compounds is a prerequisite for a good biosensor, achieving the necessary sensitivity to detect low compound concentrations can be challenging [17].

Here, we developed a novel heterologous whole-cell biosensor that is highly specific and sensitive for the detection of β-lactam antibiotics, utilizing the Gram-positive model organism Bacillus subtilis. The biosensor construct is based on the bla operon that mediates β-lactam resistance in Staphylococcus aureus (strain N315) [20, 21]. The bla locus encodes a regulatory system and comprises the genes blaR1 (antibiotic receptor), blaI (repressor protein) and blaZ (β-lactamase) (Fig. 1a) [21]. In the absence of β-lactams, the BlaI repressor binds to palindromic sequences within the intergenic region and inhibits gene expression in both directions (Fig. 1a). In the presence of β-lactams, the antibiotic acylates the C-terminal extracellular sensor domain of the BlaR1 receptor, thereby activating the cytoplasmic protease domain of BlaR1 by autolytic fragmentation. The activated protease domain then facilitates the degradation of the repressor, which releases its target promoters (Fig. 1b). Ultimately, the β-lactamase BlaZ is synthesized, secreted and inactivates the antibiotic, thereby ensuring the survival of the bacteria [22, 23].

The B. subtilis biosensor developed in this study, expresses the heterologous regulatory system BlaR1/BlaI, thereby controlling the activity of the promoter P blaZ that drives expression of the luxABCDE reporter operon from Photorhabdus luminescens (Fig. 1c) [24]. Accordingly, the presence of β-lactams results in a luminescence signal that can be easily detected and quantified. We validated the functionality of our biosensor for ten different compounds representing all four classes of the β-lactam family. In addition, we analysed the impact of the native β-lactamase PenP of B. subtilis on the behavior of the biosensor [25, 26]. As a proof of applicability, we identified β-lactam producers from a collection of Streptomyces soil isolates.

Results

For the creation of a functional heterologous biosensor in B. subtilis, the bla operon from S. aureus N315 was modified to serve as both a sensing and reporting system for the presence of β-lactams. Initially, we maintained the operon structure and simply replaced the blaZ gene, encoding the natural output (the β-lactamase BlaZ) with the luxABCDE (lux) operon from Photorhabdus luminescens (Fig. 1c). After stable integration of the reporter system into the B. subtilis genome, we probed the capability of the initial biosensor construct (hereafter referred to as Biosensor 1) to respond to β-lactams and subsequently took measures to improve its performance.
Fig. 1 (See legend on next page.)
A heterologous biosensor construct in *B. subtilis*

As a prerequisite, the minimal inhibitory concentrations (MICs) of *B. subtilis* W168 (wild type) were determined for ten β-lactams, representing all four subclasses. The cyclic polypeptide antibiotic bacitracin determined for the antibiotic concentrations listed in Table 2.

We also did not observe any changes in luminescence for Control 1 (see Supplement 1, Figure S7) or Control 3 (Fig. 3a and see Supplement 1, Figure S5). Likewise, the stable and strong luminescence signal of Control 2 was also not influenced by the addition of the antibiotic compounds (see Supplement 1, Figure S7). In addition, Biosensor 1 also showed a very high basal promoter activity, nearly equivalent to the signal of Control 2 (TMB3090) (Fig. 3a and see Supplement 1, Figure S5 and S7). While these initial data indicated that the BlaR1-dependent sensing and gene regulation by BlaI could indeed be successfully implemented into *B. subtilis*, this first design clearly falls short of the requirements for a suitable biosensor as it did not respond to all of the β-lactams tested. Moreover, the background activity of the P<sub>blaZ</sub>-lux reporter was too high and hence the dynamics were rather poor. This poor signal-to-background ratio also challenges the interpretation of the data and demanded for a robust and comprehensible threshold, in order to judge and compare the results. We based our evaluation system on the log2 fold change of the biosensor signal at 2 h post induction – when the plateau of promoter induction was reached – to clearly define whether a compound has been truly detected. Based on the data obtained for the controls water and bacitracin, we determined a log2 fold change above 2.0 as the threshold for true induction. Applying this cut-off, Biosensor 1 could only detect ampicillin, carbenicillin, penicillin, cefoxitin and ceftazidime in liquid MH medium (Fig. 3b).

We also analysed the performance of Biosensor 1 on MH agar by performing disk diffusion assays (Fig. 4) using the antibiotic concentrations listed in Table 2. Here, we expected a bright luminescence halo to appear around the zone of inhibition upon sensing of a β-lactam. The results corroborate the data obtained in liquid media only partially, as we observed a luminescence halo around the zone of inhibition for ceftazidime and cefoxitin, but not for penicillin G, ampicillin or carbenicillin (Fig. 4). Additionally, ceftotaxime resulted in a detectable luminescence signal on plates, while the result for aztreonam is hard to 

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(See figure on previous page.)

**Fig. 1** Molecular mechanism conferring resistance to β-lactams and genetic design of the biosensor constructs. a: The BlaR1/BlaI regulatory system in its inactive state: when no β-lactam is present, the BlaI repressor binds to the intergenic promoter regions and inhibits gene expression in both directions. The β-lactamase BlaZ is not synthesized. b: The BlaR1/BlaI regulatory system in its active state: the β-lactam (here meropenem in grey) binds to the periplasmic BlaR1 sensor domain (structure predicted using SWISS-MODEL). This results in the activation of the cytoplasmic BlaR1 protease domain by autocleavage and subsequent degradation of BlaI. This results in expression and hence production of BlaR1, BlaI and the β-lactamase BlaZ. BlaZ is secreted and inactivates the β-lactam. c: (1) Initial biosensor design present in the two strains TMB3641 (Biosensor 1) and TMB3713 (Biosensor 1 Δ<sup>penP</sup>). The bla<sup>Z</sup> gene was replaced by the lux operon serving as readout. (2) Improved biosensor design of strains TMB5608 (Biosensor 2), TMB5610 and TMB5611 (Biosensor 2 Δ<sup>penP</sup>). Strain TMB5610 is an inducible biosensor version, enabling expression of bla<sup>R1</sup> in presence of xylose. The two genes bla<sup>R1</sup> and bla<sup>I</sup> were codon optimized, genetically separated and placed under the control of constitutive promoters. Again, P<sub>lux</sub>-lux serves as readout.
interpret due to the background signal (see Supplement 1, Figure S2a).

Taken together, our first biosensor – while being fully functional – showed high basal promoter activity and a very narrow inducer spectrum. Therefore, we aimed at expanding the β-lactam detection spectrum and increasing the signal-to-background ratio and hence the dynamic range.

Fig. 2 Minimal inhibitory concentrations [4] for B. subtilis strains. The MICs for the B. subtilis wild type and strains missing either ybxI (TMB3668), penP (TMB3667) or both genes (TMB3675) coding for potential β-lactamases are shown. The x-axis indicates the concentration of each antimicrobial compound added to the different strains. Note that the concentration range varies depending on the antibiotic tested due to different susceptibilities. The y-axis shows the growth of the cultures displayed as OD_{600nm}. Displayed are representative examples of the four β-lactam classes: penicillin G, ampicillin and carbenicillin (penicillins), cefoperazone (cephalosporins), aztreonam (monobactams) and meropenem (carbapenems). For the full dataset, see Supplement 1, Figure S4.
Allelic replacement mutagenesis indicates that penP might encode a β-lactamase

Interestingly, so far only β-lactamase-resistant antimicrobial compounds, such as cefoperazone and cefoxitin, triggered an increase in PblaZ activity both in liquid MH and on MH agar (Figs. 3a, b and 4). We hypothesized that the reduced detection spectrum could result from the presence of β-lactamases in the host organism, B. subtilis, particularly since such enzymes were described in a close relative, Bacillus licheniformis [30, 31]. Two potential β-lactamases, PenP and YbxI, had previously been predicted for B. subtilis but not further investigated [26, 32]. Therefore, we constructed mutants lacking penP (TMB3667, Table 1), ybxI (TMB3668, Table 1) or both genes (TMB3675, Table 1). Then we determined their MIC for all ten β-lactams used in this study and compared the

### Table 1 B. subtilis strains developed and tested in this study

| Strain # | Alias          | Genotype description | Resistances     | Source                           |
|----------|----------------|----------------------|-----------------|----------------------------------|
| W168     | Control 1      | Bacillus subtilis wild type | none           | lab stock                        |
| TMB3090  | Control 2      | W168 sacA::pBS3C_Pxyg-lux | chloramphenicol | Popp et al. 2017 [27]            |
| TMB2841  | Control 3      | W168 sacA::pBS3Clux | chloramphenicol | Pinto et al. 2018 [28]           |
| TMB3667  | –              | W168 penP::kan’      | kanamycin       | This study                       |
| TMB3668  | –              | W168 ybxI::erm’      | MLS             | This study                       |
| TMB3675  | –              | W168 penP::kan’ ybxI::erm’ | kanamycin, MLS | This study                       |
| TMB3641  | Biosensor 1    | W168 sacA::pBS3C_Pxyg-lux | chloramphenicol | This study                       |
| TMB3713  | Biosensor 1 in ΔpenP | W168 penP::kan’; sacA::pBS3C_Pxyg-lux | kanamycin, chloramphenicol | This study                       |
| TMB5607  | Control 4      | W168 penP::kan’; lacA::pBS2E_Pxyg-blaR1; sacA::pBS3C_Plux | kanamycin, MLS, chloramphenicol | This study                       |
| TMB5608  | Biosensor 2    | W168 thrC::pBS4S_PlepA-blaI; lacA::pBS2E_Pxyg-blaR1; sacA::pBS3C_Plux | spectinomycin, MLS, chloramphenicol | This study                       |
| TMB5609  | Control 5      | W168 penP::kan’; thrC::pBS4S_PlepA-blaI; sacA::pBS3C_Plux | kanamycin, spectinomycin, chloramphenicol | This study                       |
| TMB5610  | Inducible Biosensor | W168 penP::kan’; thrC::pBS4S_PlepA-blaI; lacA::pBS2E_Pxyg-blaR1; sacA::pBS3C_Plux | kanamycin, spectinomycin, MLS, chloramphenicol | This study                       |
| TMB5611  | Biosensor 2 in ΔpenP | W168 penP::kan’; thrC::pBS4S_PlepA-blaI; lacA::pBS2E_Pxyg-blaR1; sacA::pBS3C_Plux | kanamycin, spectinomycin, MLS, chloramphenicol | This study                       |

### Table 2 Antibiotic compounds, inhibitory concentrations and concentrations tested

| Compound   | β-Lactam class | Inhibitory conc. in liquid [μg/ml] | Inducing conc. in liquid [μg/ml] | Conc. used for DDA [μg/ml] |
|------------|----------------|-----------------------------------|----------------------------------|--------------------------|
|            |                | wt | ΔpenP | wt | ΔpenP | wt and ΔpenP |
| Ampicillin | Penicillins    | 50 | 0.04  | 1  | 0.02  | 50           |
| Carbenicillin | Penicillins | 12.5 | 0.09  | 3  | 0.01  | 100          |
| Penicillin G | Penicillins | 500 | 0.04  | 7.5 | 0.009 | 50           |
| Cefalexin | Cephalosporins | 0.1 | 0.09  | 0.025 | 0.025 | 10           |
| Cefoxitin | Cephalosporins | 0.8 | 0.8   | 0.3  | 0.3  | 200          |
| Cefoperazone | Cephalosporins | 1.5 | 1.5   | 0.5  | 0.2  | 200          |
| Cephalexin C | Cephalosporins | 0.01 | 0.03  | 0.27 | 0.27 | 500          |
| Cefotaxime | Cephalosporins | 0.004 | 0.01 | 0.037 | 0.037 | 200          |
| Aztreonam | Monobactams | 500 | 500   | 8.3  | 8.3  | 2000         |
| Meropenem | Carbapenems | 2.5·10^-6 | 7.6·10^-6 | 0.016 | 0.016 | 10           |
| Bactracin | –             | 250 | 250   | 40   | 40   | 20,000       |

*aConcentrations tested in the assessment of biosensor activity in liquid culture. Note that two different concentrations have been used due to the higher susceptibility of the penP mutant

*bConcentrations tested in disk diffusion assays (DDA) on solid MH agar plates. Here, the values correspond to the concentration of which 10 μl were applied to the disks
Fig. 3 Growth and luminescence signal of the biosensor constructs in response to different antibiotics. a The graphs represent the detection of the antibiotic penicillin G by the four different biosensor constructs (left column: Biosensor 1 and Biosensor 1 in ΔpenP; right column: Biosensor 2 and Biosensor 2 in ΔpenP) as well as the responses of Control 3 (left column, lux operon without promoter) and Control 5 (right column, Biosensor 2 in ΔpenP lacking the blaR1 receptor construct). Growth measured as OD₆₀₀nm (y-axis) is depicted in the upper row, while luminescence is shown in relative luminescence units normalized over OD₆₀₀nm (RLU/OD₆₀₀nm) below. The graphs demonstrate the first 5 h (x-axis) of growth and development of the luminescence signal post induction with the antibiotic penicillin at 1 h (black dotted line). Concentrations used for induction can be extracted from Table 2. For the full dataset see Supplement 1, Figure S5 and Figure S6. b The log₂ fold change of all four biosensors in response to all tested β-lactams and the two controls bacitracin and water. The log₂ fold change was calculated using the luminescence output of all biosensors at 2 h post induction (grey dotted line in Fig. 3a) in comparison to the time point of induction (black dotted line in Fig. 3a). We set the threshold for true induction at log₂ = 2 as indicated by the black dotted line. The legend below serves for both Figure A and B.

Fig. 4 Disk diffusion assay of biosensors tested with six β-lactams and two controls. The six β-lactam antibiotics shown here are penicillin (PEN, 50 μg/ml), ampicillin (AMP, 50 μg/ml), cefalexin (LEX, 10 μg/ml), cefoxitin (FOX, 200 μg/ml), carbenicillin (CAR, 100 μg/ml) and cefoperazone (CFP, 200 μg/ml). White light pictures indicate the positions of the disks on the plate. The corresponding images from luminescence detection are displayed underneath. The first two image pairs on the left show the detection of β-lactams by Biosensor 1 and Biosensor 1 in the ΔpenP strain (see Fig. 1c (1), strains TMB3641 and TMB3713). In contrast, the two image pairs on the right demonstrate sensing of β-lactams by the improved Biosensor 2 and Biosensor 2 in the ΔpenP strain (see Fig. 1c (2), strains TMB5608 and TMB5611). Representative images of triplicates are shown. To view the full dataset including all control strains see Supplement 1, Figure S1, S2a, S2b and S3.
obtained values with the wild type strain (Fig. 2). Growth was severely impaired when penicillin G, carbenicillin and ampicillin were added to the penP mutant and the double mutant, while the ybxI single mutant remained unaffected. The results indicate that the presence of PenP is required to withstand higher concentrations of β-lactams belonging to the group of penicillins (Fig. 2), suggesting that PenP might confer resistance against these compounds. Table 2 summarizes the MICs that have been determined for each β-lactam and bacitracin for the wild type and the penP mutant strain.

Removal of penP enables detection of penicillins by the biosensor strain

From these findings, we hypothesized that PenP might interfere with the performance of the β-lactam biosensor by removing the stimulus, such as the penicillins, before detection can occur. If true, a biosensor construct in a penP mutant might be able to detect a broader range of compounds. Contrary to our expectations, the resulting biosensor strain lacking penP (Biosensor 1 ΔpenP, TMB3713, Table 1) did not show an increased detection spectrum in liquid MH medium (Fig. 3 and see Supplement 1, Figure S5). Note that strains with the allelic replacement of penP have been tested using lower concentrations of the antibiotics ampicillin, carbenicillin and penicillin G in liquid medium due to higher susceptibility of this strain (Table 2). Nevertheless, the signal intensity was increased in the disk diffusion assays, thereby allowing the clear detection of a luminescence output for cefoxitin, cefoperazone, cefotaxime and aztreonam on agar plates (Fig. 4 and see Supplement 1, Figure S2a).

In summary, we postulated that the high background signal might still obstruct the detection of potentially weak signals from other compounds even if Biosensor 1 is implemented in a mutant strain lacking penP.

Optimized expression of the genes blaR1 and blal significantly enhances biosensor performance

Both the high background signal and the weak performance of the biosensor in liquid medium as well as on plate necessitated further improvements. Towards this goal, we implemented three major changes: (1) codon adaptation of the genes blaR1 and blal, (2) their genetic separation and (3) placement under the control of strong constitutive promoters (Pveg and PlegA, respectively) (Fig. 1c). On the one hand, the resulting higher expression level of the BlaR1 receptor should enable more antibiotic compounds to bind and induce a response. On the other hand, we expect a lower background signal due to an increased and steady availability of the Blal repressor, thereby preventing leakiness of the PblaZ promoter.

Indeed, the two resulting biosensor strains Biosensor 2 (TMB5608) and Biosensor 2 ΔpenP (TMB5611, Table 1) showed the anticipated increased detection range, a reduced background signal and a higher sensitivity (Figs. 3 and 4). In liquid MH medium, the log2 fold change in luminescence two hours after induction is drastically increased for all ten β-lactams (ampicillin, carbenicillin, penicillin G, cefalexin, cefotaxin, cefoperazone, cephalosporin C, cefotaxime, aztreonam and meropenem) in comparison to Biosensor 1 (Fig. 3b and see Supplement 1, Figure S6). The same holds true when Biosensor 2 is combined with the ΔpenP mutation (Fig. 3b and see Supplement 1, Figure S6). Thus, the improved biosensor strains were now able to detect all ten β-lactams in liquid media (Fig. 3b and see Supplement 1, Figure S6).

The luminescence profile of the two new strains (Biosensor 2 and Biosensor 2 ΔpenP) varied slightly for the group of penicillins (Fig. 3b and see Supplement 1, Figure S6). The response time of Biosensor 2 ΔpenP seems to be marginally delayed in comparison to Biosensor 2 for some compounds (e.g. penicillin G). Generally, the response time was less than two hours and the signal remained stable for several hours. As expected, both controls (bacitracin and water) did not result in an increase in luminescence surpassing our threshold of log2 = 2 (Fig. 3b and see Supplement 1, Figure S6).

In the disk diffusion assay on MH agar plates, Biosensor 2 ΔpenP sensed an increased range of β-lactam compounds (Fig. 4 and see Supplement 1, Figure S2b), detecting eight of ten β-lactams reliably. However, the signals for cephalosporin C and cefalexin remained rather weak (Fig. 4 and see Supplement 1, Figure S2b). In contrast, Biosensor 2 was only able to detect six of ten β-lactam compounds MH agar plates (Fig. 4 and see Supplement 1, Figure S2a). Biosensor 2 ΔpenP therefore represents the final version of the β-lactam biosensor, since only the combination of improved biosensor construct with the penP deletion provided the desired performance in both liquid and solid media. It should be noted, though, that Biosensor 2 in the wild type background may be useful for analysing penicillins present at higher concentrations.

At this stage, all relevant control strains were also constructed and examined, including strains lacking either the blal repressor construct (Control 4, Table 1) or the blal receptor construct (Control 5, Table 1) (see Supplement 1, Figure S1, S2a and S7). As anticipated, Control 4 shows a strong constant luminescence signal as no transcriptional repression of the lux operon can be facilitated (see Supplement 1, Figure S1, S2a and S7). For Control 5 we observed a slightly higher luminescence signal compared to Control 3, though the luminescence signal showed a similar profile over time with no change upon addition of β-lactams (see Supplement 1, Figure S1, S2a and S7). This is expected, as the sensing unit –
the receptor BlaR1 – is absent in Control 5 and thus detection of β-lactams is impossible.

**Comprehensive validation of the β-lactam biosensor**

We next analysed the detection range and sensitivity of the final biosensor strain (Biosensor 2 ΔpenP, TMB5611, Table 1) by assessing dose-response profiles for all ten β-lactams. Again, the time point at two hours post induction was used to analyse the response of the biosensor to the different concentrations as the luminescence signal reaches a plateau at this time point. The obtained data allowed us to determine the minimum threshold concentrations and saturation concentration for all β-lactams and thereby gain insight on the overall dynamic range. The latter is compound-dependent and ranged from approx. 50-fold (cefoperazone and cefotaxime), over 35- to 40-fold (ampicillin, aztreonam and meropenem) to about 25-fold (carbenicillin, penicillin G, or cephalosporin C) (Fig. 5). Only cefalexin showed a significantly weaker response of approx. seven-fold (Fig. 5). The dose-response curve for cephalosporin C indicates that this compound is only detectable in liquid MH medium at higher concentrations. We analysed the lower detection limit by again calculating the log2 fold change for each concentration using a value of log2 = 2 as threshold for induction. Meropenem, penicillin G and cefoxitin could already be detected at concentrations as low as 1 ng/ml. Further, Biosensor 2 ΔpenP was able to detect ampicillin at concentrations as low as 2 ng/ml, carbenicillin down to 6.2 ng/ml and cefotaxime down to 4 ng/ml. The lower detection limits for aztreonam (300 ng/ml) and cephalosporin C (270 ng/ml) were the highest measured, thus concentrations below this limit might not be detected reliably for these compounds. Cefalexin could be sensed at concentrations as low as 9 ng/ml. The lowest detection limit was achieved for cefoperazone, where sensing of concentrations down to 0.3 ng/ml was achieved. In contrast, increasing concentrations of bacitracin did not result in a change in luminescence signal from the biosensor, as expected (see Supplement 1, Figure S9). In comparison with the data from Biosensor 2 ΔpenP, the dose-response curves for Biosensor 2 (see Supplement 1, Figure S11) show a very different dynamic range for penicillin G, ampicillin and carbenicillin as higher concentrations are needed to stimulate the same signal intensity. Hence, the deletion of penP increased the dynamic range of the biosensor for compounds belonging to the group of penicillins, as potential substrates are not removed any longer in the absence of this β-lactamase. The results of the dose-response assay with Biosensor 1 ΔpenP corroborate the relatively poor performance described earlier, which is
characterized by a high background signal and narrow dynamic range and detection spectrum (see Supplement 1, Figure S10).

In addition, an inducible version of the biosensor was designed, in which the bla1 receptor gene was placed under control of the inducible promoter P_xylA (TMB5610, Table 1). Consequently, this strain requires the addition of xylose in all assays for the BlaR1 receptor to be expressed. This genetic design did not improve the performance of the final biosensor strain further, neither in liquid culture (see Supplement 1, Figure S8) nor on agar plates (see Supplement 1, Figure S2b, S3). On agar plates, the luminescence was enhanced when concentrations of the receptor-inducer xylose were increased (see Supplement, Figure S3). For some of the tested β-lactams we could observe a background signal in the absence of the inducer xylose, indicating a leakiness of the P_xylA promoter.

Finally, we verified that our biosensor is indeed specific for β-lactams. Towards this end, its response to six non-β-lactam cell wall antibiotics that inhibit different steps of cell wall biosynthesis (bacitracin, tunicamycin, phosphomycin, vancomycin, polymyxin, D-cycloserine and daptomycin) was analysed at sub-inhibitory concentrations [33–35]. As anticipated, none of these compounds induced Biosensor 2 ΔpenP as no increase in luminescence output was observed (see Supplement 1, Figure S12). Hence, our data demonstrate that the response of Biosensor 2 ΔpenP is very specific and confined to β-lactam antibiotics.

Detection of β-lactam production by Streptomyces isolates

Streptomyces are known to produce a large variety of antimicrobial compounds. While β-lactams were originally isolated from fungi, such as *Penicillium spp.* and *Cephalosporium spp.*, some streptomycetes have also been described as β-lactam producers [36, 37]. Therefore, we applied the Biosensor 2 ΔpenP strain to a collection of *Streptomyces* soil isolates with antimicrobial activity against *B. subtilis* (unpublished data) to demonstrate its potential for directly screening colonies for their ability to produce β-lactams. The strains were analysed by a modified disk diffusion assay, in which the *Streptomyces* were first grown on solid media and subsequently overlaid with a lawn of the Biosensor 2 ΔpenP strain. The penicillin producer *Penicillium chrysogenum* and a cefoperazone disk were chosen as positive controls.

Indeed, we were able to identify two *Streptomyces* isolates that produced an antimicrobial compound that induced the biosensor, most likely a β-lactam (Fig. 6 and see Supplement 1, Figure S13 for the complete dataset). Not surprisingly, this small screen also demonstrated that most antimicrobial compounds produced by streptomycetes belong to different antibiotic classes. Nevertheless, this small-scale example clearly demonstrates that our novel whole-cell biosensor can indeed be easily applied for the direct identification of β-lactam producers.

Discussion

In this study, we developed a novel heterologous whole-cell biosensor for β-lactams in *B. subtilis*, based on the *bla* operon from *S. aureus*. The heterologous expression of the BlaR1/BlaI regulatory system was able to control the expression of the P_blaZ-dependent lux reporter in *B. subtilis* in a β-lactam-dependent manner. In the course of improving its performance, we not only modified the genetic design of the construct itself, but also analysed the potential influence of the putative β-lactamases PenP.
and YbxI from *B. subtilis* on biosensor performance. We demonstrated that PenP primarily provides resistance against penicillins, since a penP mutant showed a dramatically increased susceptibility against ampicillin, carbenicillin and penicillin G (Fig. 2), while the survival was unaffected for the remaining β-lactams. These findings are supported by a study suggesting that sterical hindrance in a PenP-like enzyme is responsible for its inability to bind cephalosporins of the 2nd and 3rd generation [32]. Our result also fit the previous observation in *E. coli* that expression of PenP – but not YbxI – increased the resistance against ampicillin, ticarcillin and oxacillin [26]. The same study demonstrated β-lactamase activity of YbxI against ampicillin in vitro, but with very low catalytic efficiency. All of these aspects are in agreement with our finding that a ybxI deletion did not alter the susceptibility for our panel of β-lactams in vivo. In agreement with the heterologous evidence from *E. coli*, we here provide proof that directly links PenP to penicillin resistance in *B. subtilis*.

While Biosensor 1 depicted a limited spectrum in sensing β-lactams, codon optimization, genetic rearrangement and constitutive expression of the blaR1-blaI cassette significantly improved the performance both in liquid MH and on MH agar plates (Figs. 3 and 4). In combination with the penP deletion, we achieved an increased sensitivity, enabling the detection of very low concentrations of β-lactams. The resulting strain, Biosensor 2 in ΔpenP (TMB5611), was highly specific for β-lactams and able to sense compounds from all four β-lactam classes.

Our *B. subtilis* biosensor is significantly faster and more sensitive than a previously described *E. coli*-based β-lactam-specific biosensor [10], while showing a comparable compound range and response dynamics. The maximum signal of the *E. coli*-based biosensor decreased directly after reaching its maximum [10], whereas it remained stable for several hours for Biosensor 2 ΔpenP (see Supplement, Figure 6) and, therefore, we hereby provide a more robust platform. The response time and sensitivity of our biosensor is comparable with another, very sensitive *E. coli* biosensor, which was capable of sensing tetracycline within 90 min in the nanogram range [38]. Nevertheless, in comparison to this strictly compound-specific biosensor, our β-lactam biosensor can identify compounds from different classes and generations of the large β-lactam family.

Because β-lactam perception is based on physical binding of the compound to the extracellular sensory domain of the BlaR1 receptor (Fig. 1), it is not surprising that the response strength differs between different inducer molecules. However, Biosensor 2 provides a very high sensitivity in the nanogram range for most of the compounds tested, with the exception of cephalosporin C and aztreonam where the sensitivity was lower than for the other β-lactams.

In addition to its high sensitivity and broad inducer range, the final biosensor construct is also robust and versatile regarding to the assays and test material it can facilitate. In addition to pure compounds, it is also capable of identifying novel β-lactam producers directly, as demonstrated for a set of *Streptomyces* soil isolates (Fig. 6). The small screen identified two potential β-lactam producer strains, which are currently being further characterized to verify if β-lactams are indeed being produced by these *Streptomyces* isolates.

Based on its high sensitivity and broad range of β-lactams detected, a possible application of our biosensor could be the detection of antibiotic contaminations in milk. Due to an increased use of penicillins in veterinary medicine, these antimicrobial compounds are occasionally detected in milk samples [17]. Previously, biosensors for the detection of β-lactam compounds in milk such as the so-called penicillinase biosensors have been developed [39, 40]. These biosensors however, are not based on engineered bacteria but rather on the measurement of a pH change resulting from the hydrolysis of the β-lactam ring through β-lactamase activity. Such approaches were not quite successful as they showed high detection limits [17]. On the contrary, the high sensitivity of our biosensor allows determination of penicillin at concentrations as low as 1 ng/ml. In milk, the presence of penicillin is allowed up to concentrations of 4–30 ng/ml [17]. Hence, concentrations overshooting the permitted threshold could potentially be detected by our biosensor, making it not only applicable to the detection of antibiotic producer strains, but also for the detection of antibiotic contaminations in food samples. In comparison to common detection methods like HPLC and immunoassays, our biosensor offers a cheap and easy handling of the samples.

Since our whole-cell biosensor is based on *B. subtilis*, it is potentially also adaptable for applications outside of the laboratory and in low-tech environments. By replacing the luciferase reporter with the β-galactosidase reporter, the induction becomes visible by eye [18, 19]. Storing the biosensor strains as spores enables both long-term storage and transport without the need of special cooling systems [41]. Upon arrival at the designated operation site, the cells can easily be revived from the spores and are ready to use within a few hours without any loss in performance, based on the experience with other *B. subtilis* biosensors [42, 43]. Previous studies have already demonstrated the advantage of using spores as a storage system for whole-cell biosensors, thereby extending the life span and making them withstand unfavourable environmental conditions [42].
**Conclusion**

In summary, we have successfully designed and built a β-lactam biosensor in *B. subtilis* using the heterologous regulatory system *BlaR1I* from *S. aureus*. The signal-to-noise ratio of the biosensor could be improved by codon-optimization, genetic separation and constitutive expression of the genes *blaR1I* and *blaI*. Ten β-lactam antibiotics from all four chemical classes were detected in a dose-dependent manner, while all non-β-lactams targeting the cell wall did not activate the biosensor.

Based on the results presented here, Biosensor 2 ΔpenP (Table 1, strain TMB5611) is a very sensitive biosensor that responds to concentrations in the ng/ml range for virtually all β-lactams tested (Fig. 5). It shows a highly dynamic response – between 25- to 50-fold for most compounds – within 60–120 min post induction (Figs. 3 and 5). The output is robust in different assays both in liquid and on solid media, irrespective of whether pure compounds or producer strains are provided (Figs. 3, 4 and 6). Biosensor 2 ΔpenP strain is well-suited for automated medium-to-high-throughput screening approaches, e.g. utilising multi-mode plate readers. Its high sensitivity should also allow for monitoring antibiotic contaminations, for example in milk samples.

While our β-lactam biosensor already has demonstrated a very good performance with regard to sensitivity, inducer spectrum and dynamic range of performance, there are many additional directions for applications and improvements to be implemented in the future.

**Materials and methods**

**Chemicals**

All chemicals used for buffers and solutions were purchased from Carl Roth and Sigma Aldrich and were handled according to the manufacturer’s protocols and product information.

**Bacterial strains and growth conditions**

*Escherichia coli* strain DH10β was grown in LB medium [0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) sodium chloride], while *B. subtilis* was grown in LB medium or Mueller-Hinton broth (MH medium) [2.1% (w/v) Mueller-Hinton broth; Carl Roth]. For solid agar plates 1.5% (w/v) agar-agar (Carl Roth) or 0.75% (w/v) agar-agar for soft agar were added to the media. Liquid cultures were incubated at 37 °C with aeration.

*E. coli* DH10β was used for cloning and vector amplification. Transformation of chemically competent cells was performed according to standard procedures using a heat shock-based protocol. Ampicillin (100 μg/ml) or chloramphenicol (35 μg/ml) were added to select for *E. coli* transformants [44].

For *Bacillus* transformation, strain W168 (or derivatives thereof) were incubated in MNGE-Medium (supplemented with L-threonine, 50 μg/ml final concentration for strains carrying an insertion in the threonine locus) to induce the competent state. Selective media for *B. subtilis* contained (individually or in combination): chloramphenicol (5 μg/ml), kanamycin (10 μg/ml), spectinomycin (200 μg/ml) or a combination of erythromycin (1 μg/ml) and lincomycin (25 μg/ml) to select for macrolide-lincosamide-streptogramin B (MLS) resistance [45].

**Cloning procedures**

All genetic constructs are based on vectors of the *Bacillus* BioBrick Box and adhere to the BioBrick Standard (see Supplement 2, Table S1) [24]. Enzymes from New England Biolabs® (NEB) were used for restriction digestion and ligation according to the manufacturer’s protocols. Q5® High-Fidelity DNA Polymerase was used for DNA amplification for cloning, while OneTaq® Polymerase was chosen for analytical colony-PCR, using the primers listed in Supplement 2, Table S2. Codon optimization of *blaR1I* and *blaI* was achieved through commercial DNA synthesis (IDT DNA). Commercial kits were used for plasmid purification (NucleoSpin®, Macherey-Nagel; Wizard® Plus SV, Promega or Zymopure™, ZymoResearch), PCR and gel purification (Wizard® SV Gel and PCR Clean-Up System, Promega or NucleoSpin® Gel and PCR Clean-up Kit, Macherey-Nagel). Allelic replacement mutations were introduced by long-flanking homology PCR, which replaces the target sequence with an antibiotic resistance cassette (ermφ from pDG647 or kanφ from pDG780) [46, 47]. All constructs were verified by DNA sequencing (Eurofins Genomics).

**Determination of minimal inhibitory concentrations**

The sensitivity of the *B. subtilis* wild type and congenic β-lactamase mutants lacking either penP (TMB3667), ybxl (TMB3668) or both (TMB3675) towards β-lactams were determined in MH medium. Fresh cultures were grown to an OD600nm of about 0.5 (mid-log) and then diluted to a final optical density (OD600nm) of 0.05. Serial dilutions (1:2) of the antibiotics were prepared and 5 μl of each concentration were added to 96-well plates. Subsequently, 100 μl of the diluted day culture were added to each well and grown in a plate reader (BioTek, Synergy Neo) at 37 °C with aeration. After 24 h, the OD600nm was determined by endpoint measurements [48].

**Assessing promoter activity via luciferase assay**

Luciferase assays were performed as described previously with minor modifications [24]. Day cultures of all strains (Table 1) were inoculated from overnight cultures (1:500 dilution) and incubated at 37 °C with aeration until an
OD_{600nm} of 0.2–0.4 was reached. The cultures were diluted to a final OD_{600nm} of 0.01 and then transferred to a 96-well microtiter plate with 100 µl culture volume per well (black walls, clear bottom; Greiner Bio-One). Growth and luminescence were measured every five min for at least 15 h in a multi-mode plate reader (BioTek, Synergy Neo). β-lactams were added after 1 h of incubation. All experiments were conducted in Mueller-Hinton medium. For the biosensor strain carrying the inducible biosensor construct (TMB5610), 0.2% xylose (final concentration) was added both to the day culture and again to the diluted assay culture.

**Disk diffusion assays**

For evaluating the biosensors on solid media, disk diffusion assays were performed as described [49], with minor modifications. Overnight cultures were diluted 1:500 in fresh medium and grown to an OD_{600nm} of 0.5. 100 µl of this culture were mixed with 10 ml of liquefied MH soft agar and poured on a plate with a thin layer of Mueller-Hinton agar. After solidification, disks soaked with 10 µl of antibiotic solution were placed onto the plates. Incubation was carried out for 24 h at 37°C. The plates were then photographed to document both the luminescence and the diameter of the inhibition zones (data not shown).

**Biosensor assays with Streptomyces colonies**

Screening of potential antibiotic producer strains on solid media was adapted from Kobras et al. [50] *Streptomyces* spore suspensions were spotted on solid MYM Medium [0.4% w/v Maltose, 0.4% w/v Yeast Extract, 1% w/v Malt Extract, 1.8% w/v Bacto Agar] and incubated at 30°C for 2 days [51]. On day three, a day culture of the biosensor strain TMB5611 was grown to an OD_{600nm} of 0.5, diluted 1:100 in 10 ml liquid MH soft agar and thereafter contaminating the plates. Luminescence was measured after 24 h.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s13036-020-00243-4.

**Additional file 1: Figure S1.** Disk diffusion assay with control strains tested with β-lactams and controls (BAC = bacitracin and H2O). **Figure S2.** a and b: Disk diffusion assay of the biosensors and controls with additional β-lactams, bacitracin and water. **Figure S3.** Disk diffusion assay with the inducible biosensor (TMB5610) and different inducer (xylose) concentrations (0–1%). **Figure S4.** Minimal inhibitory concentrations (MIC) for B. subtilis strains. **Figure S5.** (A)-(E): Growth (OD_{600nm}) and luminescence signal (RLU/OD_{600nm}) of Biosensor 1 in the presence of different β-lactams. **Figure S6.** (A)-(E): Growth (OD_{600nm}) and luminescence signal (RLU/OD_{600nm}) of Biosensor 2 in the presence of different β-lactams. **Figure S7.** (A)-(E): Growth (OD_{600nm}) and luminescence signal (RLU/OD_{600nm}) of control strains in response to different β-lactams. **Figure S8.** (A)-(E): Growth (OD_{600nm}) and luminescence signal (RLU/OD_{600nm}) of the inducible biosensor in response to β-lactams. **Figure S9.** Negative control (Bacitracin) from the dose response assay with the Biosensor 2 in ΔpenP (TMB5611). **Figure S10.** Results from the Dose response assay with Biosensor 1 in ΔpenP (TMB5713). **Figure S11.** Results from the Dose response assay with Biosensor 2 (TMB5608) **Figure S12.** Growth and luminescence signal of Biosensor 2 ΔpenP in response to other cell wall antibiotics. **Figure S13.** Screen for β-lactam production by Streptomyces soil isolates.

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**Authors’ contributions**

NL and PP planned the experiments. NL conducted all experiments of this study and was supervised by PP and TM. NL and TM wrote the manuscript. The author(s) read and approved the final manuscript.

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**Availability of data and materials**

The majority of data generated or analyzed during this study are included in this published article or in the supplementary information. The data not shown in this study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests. This study was initiated as part of the project undertaken by the TU Dresden iGEM-team for the 2017 iGEM competition and was therefore financially supported by the companies listed in the funding section.

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**References**

1. de Kraker ME, Stewardson AJ, Harbarth S. Will 10 million people die a year due to antimicrobial resistance by 2050? PLoS Med. 2016;13(1):e1002184.
2. Ventola CL. The antibiotic resistance crisis: part 2: management strategies and new agents. P T. 2015;40(5):344–52.
3. World Health Organisation. High levels of antibiotic resistance found worldwide, new data shows 2018 [Available from: http://www.who.int/mediacentre/news/releases/2018/antibiotic-resistance-found/en/]. Accessed 3 May 2020.

4. American Chemical Society International Historic Chemical Landmarks. Discovery and development of penicillin [Available from: www.acs.org/content/acs/en/education/whatschemistry/landmarks/flemingpenicillin.html]. Accessed 24 Apr 2020.

5. Thakuria B, Lahon K. The Beta lactam antibiotics as an empirical therapy in a developing country: an update on their current status and recommendations to counter the resistance against them. J Clin Diagn Res. 2013;7(6):1207–14.

6. Cho H, Unhe R, Bernhardt TG. Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. Cell. 2014;159(6):1300–11.

7. Dravz SM, Bonomo RA. Three decades of beta-lactamase inhibitors. Clin Microbiol Rev. 2010;23(1):160–201.

8. Zeng X, Lin J. Beta-lactamase induction and cell wall metabolism in gram-negative bacteria. Front Microbiol. 2013;4:128.

9. Park M, Tsai SL, Chen W. Microbial biosensors: engineered microorganisms as the sensing machinery. Sensors. 2013;13(5):5777–95.

10. Valtonen SJ, Kurittu JS, Karp MT. A luminescent Escherichia coli biosensor for the high throughput detection of beta-lactams. J Biomol Screen. 2002;7(2):127–34.

11. Vitta M, Lampinen J, Karp M. A luminescence-based mercury biosensor. Anal Chem. 1995;67(16):657–67.

12. Wolf D, Mascher T. The applied side of antimicrobial peptide-inducible promoters from Firmicutes bacteria: expression systems and whole-cell biosensors. Appl Microbiol Biotechnol. 2016;100(1):4817–29.

13. Tautainen S, Karp M, Chang W, Vitta M. Recombinant luminescent bacteria for measuring bioavailable arsenate and antimonite. Appl Environ Microbiol. 1997;63(11):4456–61.

14. King JM, Digrazia PM, Applegate B, Burtle R, Sanseverino J, Dunbar P, et al. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. Science. 1990;249(4970):778–81.

15. Urban A, Eckermann S, Fast B, Metzger S, Gehling M, Ziegelbauer K, et al. Novel whole-cell antibiotic biosensors for compound discovery. Appl Environ Microbiol. 2007;73(20):6436–43.

16. Yagi K. Applications of whole-cell bacterial sensors in biotechnology and environmental science. Appl Microbiol Biotechnol. 2007;73(6):1251–8.

17. Klivianid K, Kagan M, Rinken T. Biosensors for the detection of antibiotic residues in milk. Biosensors - Micro and Nanoscale Applications: IntechOpen; 2015.

18. Shin HJ, Park HHT, Lim WK. Freeze-dried recombinant bacteria for on-site detection of phenolic compounds by color change. J Biotechnol. 2005;119(1):36–43.

19. Fantino JR, Barras F, Denizot F. Sporesensor: a whole-bacterial biosensor that detects antibiotic recovery from induction of resistance. J Biol Chem. 2011;286(44):38148–90.

20. Toth M, Antunes NT, Stewart NK, Frase H, Bhattacharya J, Smith CA, et al. Class D beta-lactamases do exist in gram-positive bacteria. Nat Chem Biol. 2016;12(1):9–14.

21. Popp PF, Dotzler M, Radeck J, Bartels J, Mascher T. The Bacillus biobrick box 2.0. expanding the genetic toolbox for the standardized work with Bacillus subtilis. Sci Rep. 2017;7(1):15058.