A bacterial reporter panel for the detection and classification of antibiotic substances

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Summary
The ever-growing use of pharmaceutical compounds, including antibacterial substances, poses a substantial pollution load on the environment. Such compounds can compromise water quality, contaminate soils, livestock and crops, enhance resistance of microorganisms to antibiotic substances, and hamper human health. We report the construction of a novel panel of genetically engineered Escherichia coli reporter strains for the detection and classification of antibiotic substances. Each of these strains harbours a plasmid that carries a fusion of a selected gene promoter to bioluminescence (luxCDABE) reporter genes and an alternative tryptophan auxotrophy-based non-antibiotic selection system. The bioreporter panel was tested for sensitivity and responsiveness to diverse antibiotic substances by monitoring bioluminescence as a function of time and antibiotic concentrations. All of the tested antibiotics were detected by the panel, which displayed different response patterns for each substance. These unique responses were analysed by several algorithms that enabled clustering the compounds according to their functional properties, and allowed the classification of unknown antibiotic substances with a high degree of accuracy and confidence.

Introduction
As the human population continues to grow, progress in medical and pharmaceutical sciences has led to a parallel increase in the global use of medications, including antibiotics. Along with other pharmaceuticals, increasing amounts of antibiotics find their way into the environment (Jones et al., 2001; Heberer, 2002; Kolpin et al., 2002) by diverse routes, usually after being excreted through urine and faeces (Daughton and Ternes, 1999; Hirsch et al., 1999). Through medical and agricultural applications, antibiotics spread in the environment at low concentrations (amoxicillin, for example, has been detected at approximately 30–80 ng ml⁻¹; Kümmener, 2004). Such concentrations are not necessarily bactericidal but may nonetheless contribute to the spread of bacterial antibiotic resistance (Ash et al., 2002; Baquero et al., 2008; Roberts, 2011), which may find its way into human food, gut flora or directly to pathogens (Silbergeld et al., 2008).

The traditional approach for detecting chemicals is based on chemical or physical analyses that allow highly accurate and sensitive determination of the exact composition of the tested sample. However, such methodologies fail to provide information regarding the bioavailability of pollutants, their effects on living systems, or their synergistic or antagonistic behaviour in mixtures. A complementary approach is based on the use of diverse living systems in a variety of bioassays. Unicellular microorganisms, in particular bacteria, are attractive for these purposes due to their large population size, rapid growth rate, low cost, easy maintenance and their amenability to genetic engineering (Belkin, 2003; van der Meer and Belkin, 2010).

Genetically engineered bacteria hold great promise as sensor organisms as their responses can be genetically "tailored" to report either on specific biological effects or on the presence of pre-determined classes of chemicals (Maggisso et al., 2008; van der Meer and Belkin, 2010). Reporter bacteria can be engineered to produce a dose-dependent quantifiable signal (fluorescent, bioluminescent, electrochemical, etc.) in the presence of the target chemical or stress factor. These reporters are usually molecularly modified by fusing a promoter sequence, known to be responsive to the target compound, to a reporter system, such as the luxCDABE genes (Shapiro and Baneyx, 2007; Yagur-Kroll et al., 2009; Melamed et al., 2011). Reporter bacteria have also been used for the detection of antibiotics (Valtonen et al., 2002; Shapiro and Baneyx, 2007; Eltzov et al., 2008; Scaria et al., 2009; Smolander et al., 2009). These studies describe a limited diversity of reporter strains and/or the detection of only a
specific group of antibiotics. The use of bacterial reporters for the detection of antibiotics is complicated by the facts that most of the strains used employ antibiotic resistance genes for selection, and that the upper detection limit is strictly determined by the antibiotic’s innate toxicity to the reporter organism. In the present report, we describe a 12-member panel of bioluminescent reporter strains, constructed using a non-antibiotic selection system, for the detection and classification of a large number of antibiotic substances representing diverse modes of action. The panel responses were analysed using several classification algorithms that allowed the detection and classification of unknown antibiotic substances with a high degree of accuracy and confidence.

Results and discussion

Preparing the background for antibiotics screening: changing the selection system

The utilization of genetically engineered bacteria as bioreporters requires the use of selection markers for maintaining culture purity and for ensuring the stability of functional reporter systems. For obvious reasons, however, selection systems based on antibiotic resistance are not applicable in our case. A few non-antibiotic selection systems were developed in the past, mainly for use in probiotic microorganisms (Herrero et al., 1990; Maccormick et al., 1995, Fu and Xu, 2000; Bron et al., 2002). We have developed a selectivity marker that is based on the requirement for tryptophan. A tryptophan auxotroph Escherichia coli mutant (ΔtrpE) was used as a host strain, and a plasmid that lacks antibiotic resistance genes but confers the ability to produce tryptophan, pBRlux-trp, was used as the transformation vector (Fig. 1A and B). The trpED genes, which encode the two subunits of anthranilate synthetase, are co-ordinately regulated at transcriptional and translational levels (Nichols et al., 1981). When pBRlux-trp was introduced into the E. coli ΔtrpE strain, it re-established the ability of the bacterium to self-synthesize tryptophan and grow on a tryptophan-free medium, thus providing a selective trait (Fig. 1C).

Response of individual reporter strains to selected antibiotics

Fourteen reporter plasmids were constructed, each harbouring a different promoter sequence fused to the luxCDABE reporter genes in the pBRlux-trp vector (Table 1). Promoters were selected based either on their involvement in previously reported antibiotic response circuits or on their response to global stress factors. These plasmids were introduced into the ΔtrpE host strain SM301, generating 14 reporter strains. Sensitivity and response spectra of these strains were characterized by monitoring their bioluminescence as a function of antibiotic concentration.
For this purpose, all 14 reporter strains were exposed to a range of concentrations of each of 11 antibiotics, representing 8 different mode of action groups (Table 2, compounds 1–11). Since sufa drugs inhibit the folic acid biosynthesis pathway in bacteria, a folic acid free-medium (Bermingham and Derrick, 2002) was employed instead of LB for the exposure experiments involving these compounds.

The results show that all promoters were induced by all of the tested antibiotics, exhibiting several response patterns. Figure 2 presents several examples of these responses; one is the strong induction of soxS::luxCDABE in response to tetracycline, oxytetracycline and chloramphenicol, all protein synthesis interfering antibiotics (Fig. 2A). The activation of the soxS gene, normally recognized for its regulatory role in the defence against superoxide radicals (Nunoshiba et al., 1992), is in agreement with previous reports that presented this gene as part of a regulon involved in antibiotic resistance (Griffith et al., 1992), is in agreement with previous reports that presented this gene as part of a regulon involved in antibiotic resistance (Griffith et al., 1992; Lee et al., 2009). Its induction might be explained by oxidative damage caused by the possible accumulation of abnormal proteins in the presence of these antibiotics.

Another notable result is the strong induction of mic-F::luxCDABE in response to sulfonamides antibiotics and to colistin (Fig. 2B). However, whereas the response to colistin was relatively rapid, that to sulfonamides was slower, reaching a response ratio of 2 for sulfamethoxazole only after 80 min (Fig. 2C). The observed activation of micF in our system conforms to its known modes of action and is in agreement with prior reports. The small
RNA encoded by micF is an antisense of ompF mRNA, inhibiting the translation of the outer membrane porin protein F (OmpF; Andersen et al., 1987). Various environmental factors, including antibiotics, were shown to stimulate micF expression (Delihas and Forst, 2001).

The responses of the bioreporter panel to β-lactam antibiotics were moderate in intensity, and were characterized by a very narrow concentration range (Fig. 2D).

The last example is the fast and strong induction of recA::luxCDABE by nalidixic acid (Fig. 2E). RecA functions in homologous recombination and also serves as a regulatory protein that induces the SOS response to DNA damage by promoting the autocatalytic cleavage of the repressor protein LexA (Kuzminov, 1999). Our results are in agreement with previous reports implicating the induction of recA in response to genotoxic stress (Vollmer et al., 1997; Davidov et al., 2000; Elad et al., 2011).

The maximal response ratios for each of the 14 reporter strains for all tested antibiotics are presented in Table 3, clearly demonstrating that each of the antibiotics generated a different induction pattern in the reporters’ panel, thus paving the way for antibiotic classification by their inductive ‘fingerprints’.

**Clustering antibiotic substances into ‘mode of action’ groups**

Using the response characteristics of this 14-member reporter panel, we have attempted to cluster the antibiotics into groups that display similar response patterns. By applying different combinations of distance metrics and linkage methods to the responses measured every hour during a 10 h exposure, we searched for the 12 reporters which provided the best clustering results. After 4 h of exposure, 622 desired clustering options were obtained, 80 after 5 h and 6 after 6 h. Based on the relevancy of the clustering method and on the distances between the antibiotics in the resulting tree, we have removed the zwf and emrA constructs and were left with a final 12-member panel. A cluster tree of the antibiotics based on the selected 12 reporter strains, obtained by the use of a Spearman rank correlation coefficient as a distance metric and a weighted average distance as a linkage method (Arai et al., 1993; Tan et al., 2003), is shown in Fig. 3. The four protein synthesis interfering antibiotics (tetracycline, oxytetracycline, chloramphenicol and puromycin) clustered together, with the similarly structured tetracycline and oxytetracycline forming an independent but close branch. Ampicillin and amoxicillin, both β-lactam antibiotics, were similarly grouped, as did the sulfonamides sulfamethoxazole and sulfadimethoxine. Within the limitations of our testing scheme, therefore, the clusters formed based on the bacterial responses corresponded very well to the antibiotics’ known modes of action. Nalidixic acid, rifampin and colistin, each singly representing a different antibiotics group, formed an independent branch that is bound to expand once more data become available.

**Antibiotics classification**

The application of pattern classification algorithms for the identification of target chemicals based on the response patterns of bacterial reporters has been previously described (Ben-Israel et al., 1998; Elad et al., 2008; Smolander et al., 2009). As described below, our approach is different in the type of classification algorithms employed, as well as in their multiplexed implementation, individually or combined. Figure 4 displays, as an example, the ‘fingerprints’ generated by the 12-member reporter panel in response to 11 antibiotics after 5 h.

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**Table 2. Antibiotic substances used in this study.**

| No. | Antibiotic          | Group         | Mode of action                        |
|-----|---------------------|---------------|---------------------------------------|
| 1   | Tetracycline        | Tetracyclines | Protein synthesis inhibitor (30S)     |
| 2   | Oxytetracycline     | Tetracyclines | Protein synthesis inhibitor (30S)     |
| 3   | Sulfamethoxazole    | Sulfonamides  | Folic acid metabolism inhibitor       |
| 4   | Sulphadimethoxine   | Sulfonamides  | Folic acid metabolism inhibitor       |
| 5   | Ampicillin          | β-lactams     | Cell wall synthesis inhibitor         |
| 6   | Amoxicillin         | β-lactams     | Cell wall synthesis inhibitor         |
| 7   | Nalidixic Acid      | Quinolones    | DNA gyrase inhibitor                  |
| 8   | Chloramphenicol     | Phenicols     | Protein synthesis inhibitor (50S)     |
| 9   | Rifampin            | Rifamycins    | Protein synthesis inhibitor (50S)     |
| 10  | Puromycin           | Puromycins    | RNA polymerase inhibitor              |
| 11  | Colistin            | Polymyxins    | Cytoplasmic membrane disruptor        |
| 12  | Ciprofloxacin       | Quinolones    | DNA gyrase inhibitor                  |
| 13  | Sulfisoxazole       | Sulfonamides  | Folic acid metabolism inhibitor       |
| 14  | Polymyxin B         | Polymyxins    | Cytoplasmic membrane disruptor        |
| 15  | Doxycycline         | Tetracyclines | Protein synthesis inhibitor (30S)     |
| 16  | Thiamphenicol       | Phenicols     | Protein synthesis inhibitor (50S)     |

Substances 1–11 were used for the original construction of the database; compounds 12–16 were employed as ‘unknowns’ for testing the classifiers.
Using these data, which included 20 independent repeats for each antibiotic, we have built several classifiers: a nearest-neighbour classifier, a Mahalanobis distance-based classifier and linear and quadratic Bayes classifiers, each with either diagonal or non-diagonal covariance matrices. Table 4 presents, as an example, the errors incurred when testing a nearest neighbour classifier against the 11 antibiotics. At all time points after 2 h of exposure, the error rate estimates of classifying the antibiotics into their correct class were <5% in a leaving-one-out procedure. Some of the recorded errors were between close classes, such as phenicols and tetracyclines. False negative errors were not recorded except for after 2 h (0.42%), and false positive error rate decreased as the time passed to practically 0% after 4 h. It thus seems that after 2 h the system is stabilized and retains a high accuracy.

All six classifiers were then challenged with an independent set of observations obtained in part by exposing the 12-member reporter panel to additional antibiotics of the same classes, and grouped by either antibiotic or antibiotic class. The average error rate estimates across all time points were <5% in a leaving-one-out procedure.
points of the different classifiers varied between 10.9% and 28.6% (Table 5). However, when the decisions of the six classifiers were combined by a 'majority voting' algorithm (Freund, 1995), a considerable decrease in the average error rate estimates was obtained in both modes of observation grouping (Table 5). This implies that each classifier has a defined and unique weakness when analysing the data set, and that by combining all the decisions of all classifiers many of these weaknesses are neutralized. In Fig. 5 this is demonstrated by comparing the decisions of the linear Bayes classifier with a non-diagonal covariance matrix (Fig. 5A) to the majority voting in the class mode (Fig. 5B). Polymyxin B, for example, was classified incorrectly by this classifier at all time points and in all three experiments shown here (Fig. 5A) while all other classifiers coped successfully with this antibiotic pattern (not shown). When using majority voting with antibiotic free medium, no errors were recorded in the class mode and only one error was recorded in the individual mode after 4 h (not shown). In total, an appreciably lower average error rate estimate was obtained using the class mode (Fig. 5B). By this grouping mode, all the antibiotics that were included in the data set by which the classifiers were built were correctly classified at all time points. All the additional antibiotics were correctly classified by class mode already after 1 h, except for thiamphenicol, which was correctly classified by class mode as of the second hour. The classification is supported by the inclusion of the five 'unknowns' in the clustering of the 12 strains’ response patterns (Fig. 3). Each 'unknown' clustered along with the other member/s of its antibiotic group, except for polymyxin B which was placed closer to rifampin than to colistin.

To further improve classifier accuracy, we have expanded the decision algorithm to employ time-dependent data analysis. In this manner, a decision was made at each time point by a majority voting between it

### Table 5. Maximal induction of each reporter strain by each of the 11 antibiotics tested in the course of 10 h of exposure.

| promoter | Tetra-cycline | Oxytetra-cycline | Sulfamethoxazole | Sulfadimethoxine | Ampicillin | Amoxicillin | Nalidixic Acid | Chloramphenicol | Rifampin | Puromycin | Colistin |
|----------|---------------|------------------|------------------|------------------|------------|-------------|---------------|----------------|----------|-----------|---------|
| emrA     | 3.9           | 2.9              | 23.5             | 25.9             | 7.3        | 3.2         | 16.4          | 8.1            | 12.4     | 6.0       | 1.4     |
| acrA     | 3.6           | 6.6              | 19.7             | 36.7             | 8.1        | 2.9         | 26.1          | 7.0            | 10.5     | 4.4       | 2.6     |
| zwf      | 4.0           | 9.1              | 32.7             | 29.2             | 7.4        | 2.3         | 13.6          | 7.8            | 7.4      | 4.7       | 1.5     |
| soxS     | 26.5          | 26.1             | 22.9             | 22.9             | 6.7        | 3.3         | 13.0          | 26.3           | 3.9      | 3.9       | 1.6     |
| tolC     | 5.7           | 14.3             | 25.6             | 36.9             | 5.9        | 2.1         | 8.2           | 8.7            | 4.0      | 4.6       | 1.7     |
| inaA     | 4.6           | 14.0             | 31.2             | 25.2             | 10.8       | 2.1         | 17.1          | 5.5            | 3.4      | 3.7       | 2.0     |
| zntA     | 6.3           | 7.5              | 12.4             | 9.3              | 3.7        | 2.5         | 27.3          | 8.6            | 3.5      | 2.6       | 5.2     |
| marR     | 3.3           | 4.2              | 12.9             | 15.4             | 2.5        | 6.4         | 11.2          | 8.9            | 1.6      | 2.2       | 3.3     |
| recA     | 3.1           | 6.5              | 11.7             | 15.7             | 1.8        | 3.6         | 46.2          | 7.6            | 1.1      | 5.0       | 1.2     |
| micF     | 3.2           | 6.4              | 255.2            | 44.9             | 1.8        | 2.2         | 44.5          | 2.6            | 4.9      | 2.5       | 36.9    |
| katG     | 4.1           | 4.6              | 13.4             | 15.8             | 3.4        | 4.0         | 19.5          | 8.2            | 3.4      | 3.0       | 1.9     |
| sodA     | 3.5           | 5.0              | 43.0             | 35.7             | 1.2        | 2.5         | 31.0          | 7.1            | 8.0      | 3.4       | 3.8     |
| rpoB     | 3.1           | 3.4              | 11.7             | 8.6              | 1.6        | 2.0         | 4.1           | 3.4            | 2.2      | 1.8       | 1.3     |
| ompF     | 1.1           | 1.4              | 39.8             | 18.6             | 1.3        | 1.2         | 1.7           | 1.1            | 1.1      | 1.6       | 1.1     |

The colour scale is independent for each column and represents the relative intensity of the reporter’s response.

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Fig. 3. Clustering of the 11 antibiotics and additional 5 ‘unknown’ antibiotics by Spearman rank correlation coefficient based on the induction pattern of 12 reporter strains, following 5 h of exposure (the ‘unknown’ antibiotics are underlined and their branches are dotted).

Table 4. Classification errors (%) using the nearest neighbour algorithm. Error rate was calculated using the leaving-one-out method.

| Time of exposure (h) | 1    | 2    | 3    | 4    | 5    | 6    | 7    |
|---------------------|------|------|------|------|------|------|------|
| Total errors        | 22.5 | 9.17 | 6.25 | 9.17 | 4.58 | 3.75 | 7.92 |
| Class errors<sup>a</sup> | 10   | 4.58 | 1.67 | 4.17 | 2.5  | 2.5  | 4.58 |
| False positive      | 2.08 | 2.08 | 1.25 | 1.25 | 0    | 0    | 0    |
| False negative      | 0    | 0.42 | 0    | 0    | 0    | 0    | 0    |

<sup>a</sup> Classification of an antibiotic as an antibiotic from a different class.

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Several environmental samples were found to be close to several antibiotic concentrations in those found in the environment, there are a few exceptions. For example, sulfamethoxazole concentrations in human blood following the consumption of antibiotics mixtures.

Conclusions and future significance

We have presented a ‘tailored’ bacterial reporter panel for the detection and classification of antibiotic substances. By the use of this panel we have successfully associated unknown samples with eight different antibiotic classes, covering most major antibacterial mechanisms. Further work is needed to broaden the applicability of this method to additional antibiotics; in principle, the same concept can be adapted towards the detection of other classes of pharmaceuticals. The use of majority voting has enabled us to rely on the strengths of each of the six classifiers, thus strengthening the validity of the results.

While at this time the concentrations of the compounds detected by the reporter panel are significantly higher than those found in the environment, there are a few exceptions. For example, sulfamethoxazole concentrations in several environmental samples were found to be close to 0.1 mg l⁻¹ (Jen et al., 1998; Wei et al., 2011), within the detection range of the present panel. Another exception is ciprofloxacin, which was detected by the reporters’ panel at concentrations as low as 1 ng ml⁻¹, in the range that can be found in environmental samples (Gracia-Lor et al., 2011). A practical solution for insufficient sensitivity may involve sample pre-concentration, as is the standard protocol for numerous water contaminants for chemical or biological analysis (Buchberger, 2011). Nevertheless, work should clearly continue on enhancing the sensitivity of reporter bacteria as well as on further improvements of the classification algorithms. Interestingly, the antibiotic concentration range that permitted detection and classification by the reporter panel is very similar to that which may be found in human blood following the consumption of such compounds (Wingender et al., 1989; Sum et al., 1989; Stevens et al., 1991; Mehta et al., 2001). This may open potential new applications for this approach.

Another point worthy of note is the non-antibiotic selection system presented here. While only a tool for achieving the primary research objectives, the approach may be of a much broader significance; the use of this and similar selection systems may reduce the risks of release of antibiotic resistance vectors into the environment (Stotzky and Babich, 1986; Coughter and Stewart, 1989; Atlas, 1992; Doyle et al., 1995; Urgun-Demirtas et al., 2006). To the best of our knowledge, it is the first time that such a selection system is used for the construction of bacterial reporter strains.

Experimental procedures

Strains and media

Several E. coli K12 strains were used in the course of this study (Table 1). Strain JW1256, a tryptophan auxotroph, was a part of the Keio mutant collection kindly provided by the National BioResource Project (National Institute of Genetics, Japan): E. coli (Mishima, Japan; Baba et al., 2006). Strains SM301, SM309, SM332-335, SM337-338 and SM340-347 were constructed in the current work. Strain DH5α (Grant et al., 1996) was taken from our laboratory collection. All strains were maintained on agar plates containing either Luria-Bertani (LB) or a minimal M9 medium enriched with 0.5% casamino acids and 0.0001% thiamine (‘enriched M9’) at 4°C or in 25% glycerol at −80°C. Strains were grown in rich medium (LB), enriched M9 or minimal medium (M63) (Miller, 1992).

Table 5. Average error rate estimates of six classification algorithms and the majority voting tested in two modes: individual and class.

| Algorithm                        | Individual | Class        |
|----------------------------------|------------|--------------|
| Nearest neighbour                | 18         | 18           |
| Mahalanobis distance based       | 16         | 28           |
| Linear Bayes – non-diagonal      | 42         | 42           |
| Linear Bayes – diagonal          | 40         | 40           |
| Quadratic Bayes – non-diagonal   | 16         | 22           |
| Quadratic Bayes – diagonal       | 24         | 22           |
| Majority voting                  | 11         | 6            |

Error rates were calculated for each of the six classification algorithms and the majority voting tested in two modes: individual and class.
**Detection and classification of antibiotics**

| Sample          | 1H         | 2H         | 3H         | 4H         | 5H         | 6H         | 7H         |
|-----------------|------------|------------|------------|------------|------------|------------|------------|
| Doxycycline     | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines |
| Doxycycline     | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines |
| Doxycycline     | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines |
| Thiamphenicol   | Beta-lactams | Phenolics   | Phenolics   | Phenolics   | Phenolics   | Phenolics   | Phenolics   |
| Thiamphenicol   | Beta-lactams | Phenolics   | Phenolics   | Phenolics   | Tetracyclines | Tetracyclines | Phenolics   |
| Thiamphenicol   | Phenolics   | Phenolics   | Phenolics   | Phenolics   | Tetracyclines | Tetracyclines | Phenolics   |
| Sulfisoxazole   | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides |
| Sulfisoxazole   | N.D.       | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides |
| Sulfisoxazole   | N.D.       | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides |
| Ciprofloxacin   | Quinolones | Quinolones | Quinolones | Rifamycins | Rifamycins | Quinolones | Quinolones |
| Ciprofloxacin   | Quinolones | Quinolones | Quinolones | Rifamycins | Rifamycins | Quinolones | Quinolones |
| Ciprofloxacin   | Quinolones | Quinolones | Quinolones | Rifamycins | Rifamycins | Quinolones | Quinolones |
| Polymyxin B     | Rifamycins | Sulfonamides | N.D.       | Sulfonamides | Sulfonamides | Sulfonamides | N.D.       |
| Polymyxin B     | Rifamycins | Beta-lactams | N.D.       | N.D.       | N.D.       | N.D.       | N.D.       |
| Polymyxin B     | Rifamycins | Beta-lactams | N.D.       | N.D.       | N.D.       | N.D.       | N.D.       |
| Tetracycline    | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines | Tetracyclines |
| Chloramphenicol | Phenolics   | Phenolics   | Phenolics   | Phenolics   | Phenolics   | Phenolics   | Phenolics   |
| Sulfamethoxazole| Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides | Sulfonamides |
| Nalidixic Acid  | Quinolones | Quinolones | Quinolones | Rifamycins | Rifamycins | Quinolones | Quinolones |
| Colistin        | Polymyxins  | Polymyxins  | Polymyxins  | Polymyxins  | Polymyxins  | Polymyxins  | Polymyxins  |
| Antibiotic Free | Puromycin   | N.D.       | N.D.       | N.D.       | N.D.       | N.D.       | N.D.       |

**Fig. 5.** Classification of antibiotics, unfamiliar to the reporter panel, introduced to the classifiers. Classification using: (A) Linear Bayes – non-diagonal with class database mode; (B) majority voting with class database mode. (N.D. = not detected)
Chemicals
Tetracycline (T3258), oxytetracycline (46598), sulfamethoxazole (46850), sulfadimethoxine (46794), ampicillin (A9518), amoxicillin (46060), nalidixic acid (N4382), chloramphenicol (C0378), rifampin (R3501), puromycin (P7255), colistin (C4461), ciprofloxacin (17850), sulfisoxazole (S6377), polymyxin B (P0972), doxycycline (44577), thiamphenicol (T0261) and all other chemicals were purchased from Sigma-Aldrich Corporation (USA). Numbers in brackets denote Sigma-Aldrich catalogue numbers.

Construction of a non-antibiotic selection system and reporter strains
A tryptophan-based selection system was based on (i) knocking out tryptophan synthesis ability by mutating the trpE gene in the E. coli host; (ii) introducing tryptophan synthesis capability into the plasmids by incorporation of the trpED genes; and (iii) eliminating antibiotic resistance, if any, from both the plasmid and the host strain (in our case, ampicillin and kanamycin respectively).

Host construction. Escherichia coli SM309 was constructed by transferring a mutation in the trpE gene from E. coli K12 single-gene knockout mutant ( JW1256) of the Keio collection (Baba et al., 2006) to E. coli DH5x, using P1 transduction. Plasmid pCP20 (Cherepanov and Wackernagel, 1995) was used to eliminate the chromosomal kanamycin resistance gene of E. coli JW1256 for the formation of E. coli SM301.

Plasmid construction. Plasmid pBRlux-trp bearing trpED was constructed based on the low-copy plasmid pBR2TTS that harbours the Photorhabdus luminescens luxCDABE genes downstream of a multiple cloning site (Yagur-Kroll et al., 2009). The trpED genes were inserted, in two stages, on the complementary strand to the luxCDABE genes in order to prevent any possible transcription leakage (Table S1). First, primers carrying a SalI restriction site, designed for trpE and its promoter, were used to amplify this region from E. coli MG1655 (Blattner et al., 1997) genome by PCR (Table S1). The PCR products were cut with restriction enzyme SalI (New England Biolabs, USA) and ligated (T4 DNA ligase, Fermentas) into a pBR2TTS vector, which was then transformed into E. coli DH5x and purified. Then, primers carrying a BSSHII restriction site, designed for trpD, were used to PCR-amplify this region from the E. coli MG1655 genome (Table S1). The PCR products were cut with restriction enzyme BSSHII (New England Biolabs, USA), and ligated into the pBR2TTS vector already harbouring trpE (T4 DNA ligase, Fermentas), which was first transformed into E. coli DH5x and then transferred to E. coli SM301. Tryptophan synthesis capability was verified by growing the bacteria on enriched M9 medium lacking this amino acid. The antibiotic selectivity marker present on the plasmid, ampicillin resistance gene (bla), was eliminated by a frameshift mutation using bla-long and bla-pstI primers (Table S1). Using these primers, part of the bla sequence was amplified by PCR using pBR2TTS as a template, while planting a mutation in that region. The pBR2TTS vector harbouring trpED was cut with restriction enzymes scal (blunt cutter) and psrl while the PCR product was cut with psrl and the two fragments were ligated (T4 DNA ligase, Fermentas). The finalized vector, pBRlux-trp, was transformed into E. coli SM309 and then transferred to E. coli SM301.

To construct the promoter::luxCDABE fusions, specific promoters [emrA, acrA, zwf, soxS, tolC, inaA, zntA, marR, recA...]

Fig. 6. Average error rate estimates of six classification algorithms and the majority voting tested in class mode of the database in time-dependent or -independent manner.

Fig. 7. Classification of seven different concentrations of tetracycline using the majority voting with the class database mode.
(Yagur-Kroll et al., 2009), micF (Yagur-Kroll and Belkin, 2010), katG (Yagur-Kroll and Belkin, 2010), sodA (N. Kessler, unpublished), rpoB and ompF were introduced into pBRLux-trp using a sense primer carrying KpnI restriction site and an antisense primer carrying SacI restriction site. Primers were designed for each of the promoters based on their sequence and on available information as to their regulation, and were used to amplify the promoters from the E. coli MG1655 genome (Table S1). PCR products were cut with restriction enzymes KpnI and SacI (New England Biolabs, USA). The cut fragments were ligated into a pBRLux-trp vector, upstream to the luxCDABE genes (T4 DNA ligase, Fermentas). Each vector was transformed into E. coli SM309 and then transferred to E. coli SM301.

Monitoring reporter activity

Strains SM332-335, SM337-338 and SM340-347 were grown overnight in enriched M9 at 37°C with shaking (200 r.p.m.), diluted 100-fold in fresh LB or M63, and re-grown with shaking at 37°C to early logarithmic phase. Culture aliquots (50 or 20 µl) were then transferred into the wells of a white 96-well or 384-well microtitre plate (Greiner Bio-One, Germany) containing (in 50 or 20 µl respectively) either a predetermined concentration of an antibiotic in growth medium (LB or M63) or an antibiotic-free control (LB or M63 only). Luminescence was measured by a microtitre plate luminometer (Victor2, Wallac, Finland) at 37°C at constant intervals up to 10 h. Light emission was quantified by the instrument’s arbitrary relative light units (RLU). Responses are reported as fold increase in RLU over non-treated control (response ratio). All experiments were carried out in duplicate, and were repeated at least three times.

Data set construction and classification algorithms

The responses of 14 reporter strains to 11 antibiotics (Table 2, compounds 1–11) were clustered by the use of different distance metrics and different linkage methods, testing all possible combinations of 12 out of 14 reporters (Table 3). The values that were used were the maximal response ratios obtained from at least two independent experiments of exposure of each reporter strain to a concentration gradient of each antibiotic substance.

The responses of 12 selected reporters to 11 antibiotics, in 20 repeats, were used for classifier design. To ensure truly independent repeats, 20 individual cultures of each reporter strain were grown overnight, separately diluted and re-grown, and exposed to three concentrations of each of the 11 antibiotics as described above under Monitoring reporter activity. A total of 7 × 240 observations were obtained, comprised of the maximal response ratio at each hour for 7 h [7 time-points × (11 treatments + 1 antibiotic-free control) × 20 repeats]. Each observation is represented by a 12-dimensional vector composed of the response ratio of the 12 reporters. Based on this data set, a nearest neighbour classifier was constructed and its performance was estimated by leaving-one-out cross-validation (Duda et al., 2001).

Using the same data set, five additional classifiers were built: a Mahalanobis distance-based classifier, and linear and quadratic Bayes classifiers, each with either diagonal or non-diagonal covariance matrices (Duda et al., 2001). The performance of all six classifiers was then estimated by an independent testing set of observations. This set of observations was obtained by a new series of experiments in which the selected 12-member reporter panel was exposed to five of the original antibiotics (colistin, tetracycline, chloramphenicol, sulfamethoxazole and nalidixic acid) as well as to five additional antibiotics (thiamphenicol, sulfisoxazole, ciprofloxacin, polymyxin B and doxycycline) in three independent repeats. The classifiers were either used individually or combined by majority voting, and the observations presented to them were divided into 11 groups, each representing a different antibiotic (‘individual mode’), or into 8 groups, each representing a different antibiotic class (‘class mode’).

All the above analyses were performed using Matlab software (version 7.11 R2010b, The Mathworks).

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**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Error rate estimates of six classification algorithms and the ‘majority voting’ tested in the individual mode, in a time-dependent or time-independent manner.

**Table S1.** List of PCR primers used in this study.

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