microbial biotechnology

Microbial Biotechnology (2012) **5**(4), 536–548 doi:10.1111/j.1751-7915.2012.00333.x

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

Sahar Melamed,[1] Chaim Lalush,[1] Tal Elad,[1] Sharon Yagur-Kroll,[1] Shimshon Belkin[1]* and Rami Pedahzur[1,2]

[1]*Department of Plant and Environmental Sciences, The Alexander Silberman Institute of Life Sciences, the Hebrew University of Jerusalem, Jerusalem 91904, Israel.*

[2]*Department of Environmental Sciences, Hadassah Academic College, Jerusalem, Israel.*

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 of 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}; Kümmerer, 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 (Magrisso *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

Received 14 October, 2011; accepted 25 January, 2012. *For correspondence. E-mail shimshon@vms.huji.ac.il; Tel. (+972) 2 6584192; Fax (+972) 2 6585559.

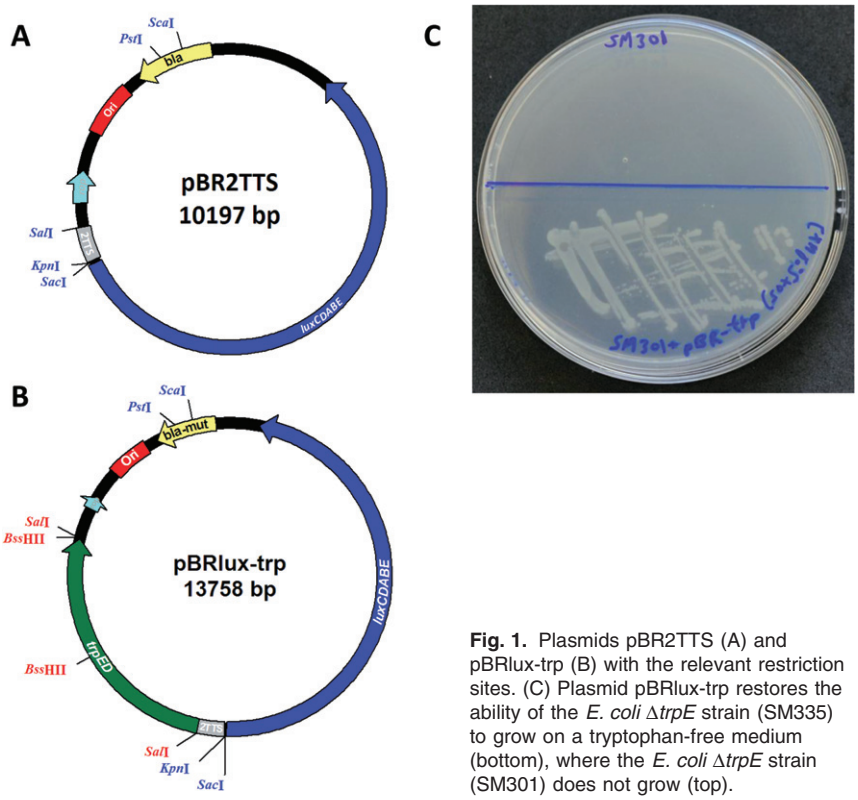

Fig. 1. Plasmids pBR2TTS (A) and pBRlux-trp (B) with the relevant restriction sites. (C) Plasmid pBRlux-trp restores the ability of the *E. coli* Δ*trpE* strain (SM335) to grow on a tryptophan-free medium (bottom), where the *E. coli* Δ*trpE* strain (SM301) does not grow (top).

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 (Δ*trpE*) 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* tryptophan auxotroph 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 Δ*trpE* 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.

Table 1. *Escherichia coli* strains used in this study.

Strain	Host	Plasmid	Phenotype/genotype	Sensing element information	Reference
DH5α	–	–	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ–*	–	Grant *et al.* (1990)
JW1256	BW25113	–	*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1 ΔtrpE* kan^R	–	Baba *et al.* (2006)
SM301	BW25113	–	*rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1 ΔtrpE*	–	Current work
SM309	DH5α	–	*ΔtrpE* kan^R	–	Current work
SM332	SM301	pBRlux-trp:*emrA::luxCDABE*	*ΔtrpE/ptrpED*	Cytoplasmatic membrane fusion protein, subunit of EmrAB-TolC multidrug efflux transport system	Current work
SM333[a]	SM301	pBRlux-trp:*acrA::luxCDABE*	*ΔtrpE/ptrpED*	Periplasmic lipoprotein component of the AcrAB-TolC multidrug efflux pump	Current work
SM334	SM301	pBRlux-trp:*zwf::luxCDABE*	*ΔtrpE/ptrpED*	G6PDH, regulated by SoxS and MarA	Current work
SM335[a]	SM301	pBRlux-trp:*soxS::luxCDABE*	*ΔtrpE/ptrpED*	Dual transcriptional activator, participates in the removal of antibiotics	Current work
SM337[a]	SM301	pBRlux-trp:*tolC::luxCDABE*	*ΔtrpE/ptrpED*	Outer membrane porin involved in the efflux transport system	Current work
SM338[a]	SM301	pBRlux-trp:*inaA::luxCDABE*	*ΔtrpE/ptrpED*	pH-inducible protein involved in stress response	Current work
SM340[a]	SM301	pBRlux-trp:zntA::*luxCDABE*	*ΔtrpE/ptrpED*	Lead, cadmium, zinc and mercury transporting ATPase	Current work
SM341[a]	SM301	pBRlux-trp:*marR::luxCDABE*	*ΔtrpE/ptrpED*	Multiple antibiotic resistance protein	Current work
SM342[a]	SM301	pBRlux-trp:*recA::luxCDABE*	*ΔtrpE/ptrpED*	DNA recombination protein, induce the SOS response to DNA damage	Current work
SM343[a]	SM301	pBRlux-trp:*micF::luxCDABE*	*ΔtrpE/ptrpED*	Antisense regulator of the translation of the OmpF porin, under SoxS regulation	Current work
SM344[a]	SM301	pBRlux-trp:*katG::luxCDABE*	*ΔtrpE/ptrpED*	Bifunctional hydroperoxidase I, having both catalase and peroxidase activity	Current work
SM345[a]	SM301	pBRlux-trp:*sodA::luxCDABE*	*ΔtrpE/ptrpED*	Superoxide dismutase protein	Current work
SM346[a]	SM301	pBRlux-trp:*rpoB::luxCDABE*	*ΔtrpE/ptrpED*	RNA polymerase, beta subunit	Current work
SM347[a]	SM301	pBRlux-trp:*ompF::luxCDABE*	*ΔtrpE/ptrpED*	Outer membrane porin	Current work

a. Constituents of the final 12-member reporter panel.

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 sulfa 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.*, 2005; 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 *micF: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

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	Sulfadimethoxine	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	RNA polymerase inhibitor
10	Puromycin	Puromycin	Protein synthesis inhibitor (tRNA)
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.

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.

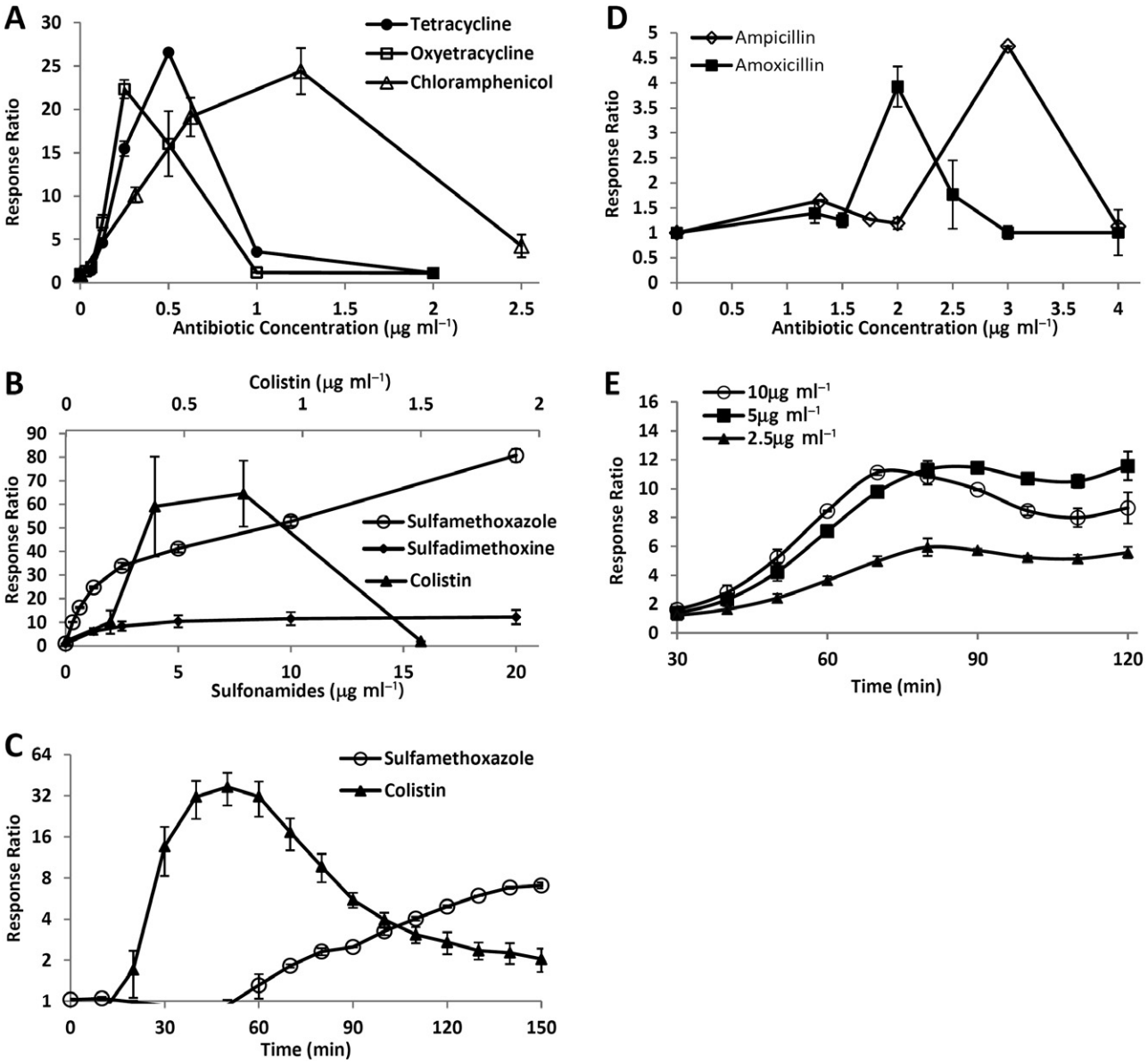

Fig. 2. Maximal induction of *soxS:luxCDABE* by tetracycline, oxytetracycline and chloramphenicol (A) and of *micF:luxCDABE* by sulfamethoxazole, sulfadimethoxine and colistin (B) after 8 h of exposure. (C) Bioluminescent signal development of *micF:luxCDABE* induction by sulfamethoxazole (20 μg ml^{-1}) and colistin (0.38 μg ml^{-1}) as a function of time. (D) Maximal induction of *emrA:luxCDABE* by ampicillin and amoxicillin after 8 h of exposure. (E) Bioluminescent signal development of *recA:luxCDABE* by nalidixic acid as a function of time. Error bars indicate the standard error of the mean of three independent repeats.

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

Table 3. 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	Sulfame-thoxazole	Sulfadime-thoxine	Ampicillin	Amoxi-cillin	Nalidixic Acid	Chloram-phenicol	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.

Highest response ratio  Lowest response ratio

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

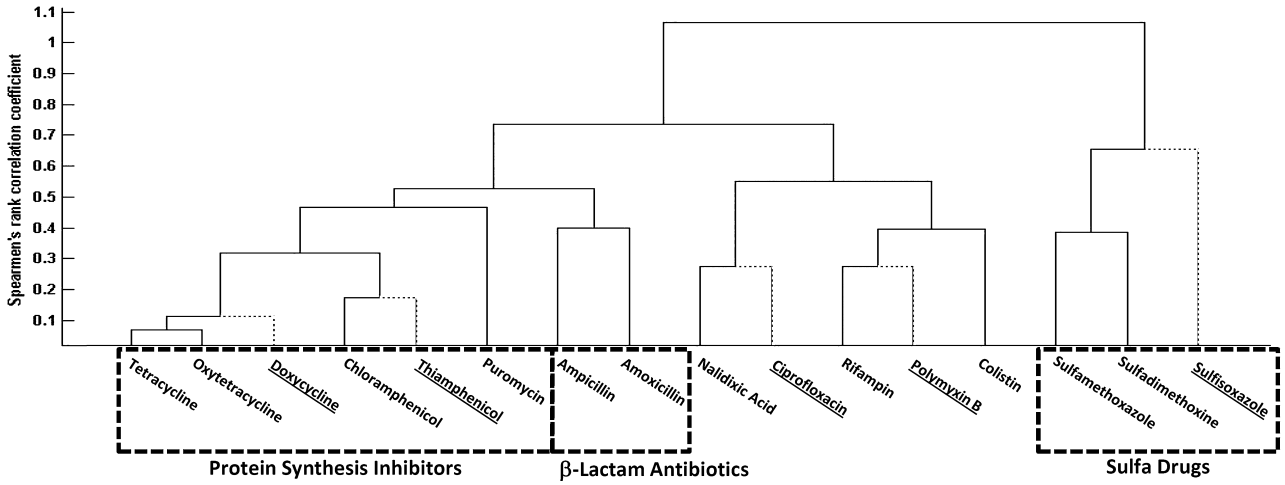

Fig. 3. Clustering of the 11 antibiotics and additional 5 'unknown' antibiotics by Spearman rank correlation coefficient based on the induction pattern of 12 reporter strains, following 5 h of exposure (the 'unknown' antibiotics are underlined and their branches are dotted).

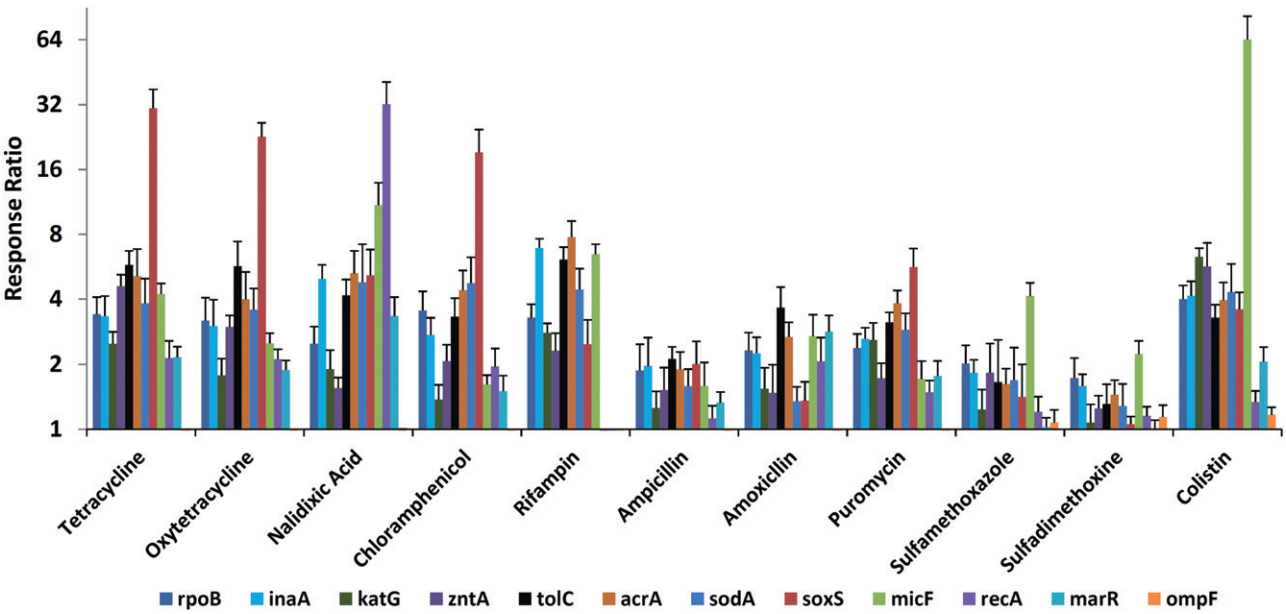

Fig. 4. Response patterns of 12 reporter strains to 11 antibiotics, following a 5 h exposure. Error bars indicate the standard deviation of 20 independent repeats.

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[a]	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	0.42
False negative	0	0.42	0	0	0	0	0

a. Classification of an antibiotic as an antibiotic from a different class.

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

	Individual		Class	
Algorithm	Number of errors	%	Number of errors	%
Nearest neighbour	18	12.2	18	12.2
Mahalanobis distance based	16	10.9	28	19.0
Linear Bayes – non-diagonal	42	28.6	42	28.6
Linear Bayes – diagonal	40	27.2	40	27.2
Quadratic Bayes – non-diagonal	16	10.9	22	15.0
Quadratic Bayes – diagonal	24	16.3	22	15.0
Majority voting	11	7.5	6	4.1

and all preceding time points. A considerable decrease was obtained in the average error rate estimates in nearly all cases, both in the individual mode and in the class mode (Figs 6 and S1).

The classifiers described above were challenged with a single concentration of each antibiotic; to check whether the combined classifier will retain its validity at different concentrations, we have also tested a tetracycline concentration range (0.25–1 μg ml^{-1}) that covered the entire inductive range of this compound. With the majority voting, 43 of 49 data points were correctly classified as tetracyclines, while the other six data points were classified as phenicols (Fig. 7), the nearest branch on the tree (Fig. 3). As both phenicols and tetracyclines are protein synthesis inhibitors, this error may be considered reasonable, but nevertheless indicates that future improvements of the classification methods are required. Further research should also challenge the reporters' panel with 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*., 1984; 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*., 1990) 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).

A

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	Beta-lactams	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols
Thiamphenicol	Beta-lactams	Beta-lactams	Phenicols	Phenicols	Tetracyclines	Tetracyclines	Phenicols
Thiamphenicol	Beta-lactams	Phenicols	Phenicols	Phenicols	Phenicols	Tetracyclines	Tetracyclines
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	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols
Sulfamethoxazole	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides
Nalidixic Acid	Quinolones	Quinolones	Quinolones	Quinolones	Rifamycins	Rifamycins	Rifamycins
Colistin	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins
Antibiotic Free	Puromycin	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

B

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	Tetracyclines	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols
Thiamphenicol	Beta-lactams	Phenicols	Phenicols	Phenicols	Tetracyclines	Phenicols	Phenicols
Thiamphenicol	Beta-lactams	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols
Sulfisoxazole	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides
Sulfisoxazole	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides
Sulfisoxazole	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides
Ciprofloxacin	Quinolones	Quinolones	Quinolones	Quinolones	Rifamycins	Quinolones	Quinolones
Ciprofloxacin	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones
Ciprofloxacin	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones
Polymyxin B	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins
Polymyxin B	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins
Polymyxin B	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins
Tetracycline	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines
Chloramphenicol	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols	Phenicols
Sulfamethoxazole	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides	Sulfonamides
Nalidixic Acid	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones	Quinolones
Colistin	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins	Polymyxins
Antibiotic Free	N.D.	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)

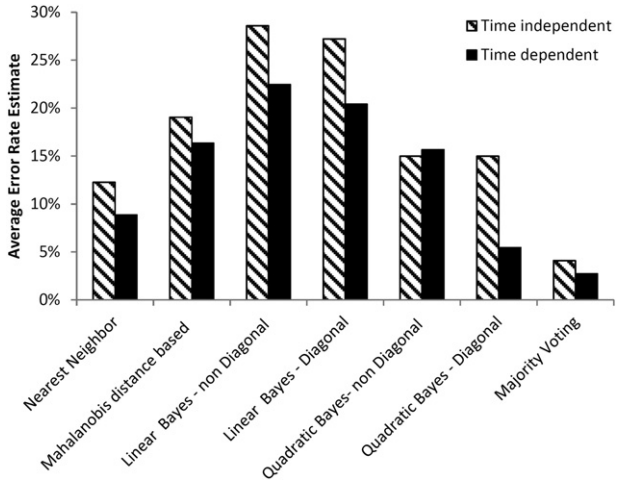

Fig. 6. Average error rate estimates of six classification algorithms and the majority voting tested in class mode of the database in time-dependent or -independent manner.

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, both from 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* DH5α, 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* DH5α 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* DH5α 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 scaI (blunt cutter) and pstI while the PCR product was cut with pstI 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*

Tetracycline Conc. (μg ml^{-1})	1H	2H	3H	4H	5H	6H	7H
1	Phenicols	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Phenicols	Tetracyclines
0.875	Phenicols	Tetracyclines	Tetracyclines	Phenicols	Tetracyclines	Tetracyclines	Tetracyclines
0.75	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines
0.625	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines
0.5	Tetracyclines	Tetracyclines	Phenicols	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines
0.375	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines
0.25	Tetracyclines	Tetracyclines	Phenicols	Tetracyclines	Tetracyclines	Tetracyclines	Tetracyclines

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 (Victor^2, 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 $\times$ (11 treatments + 1 antibiotic-free control) $\times$ 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).

Acknowledgements

Research was supported by Infrastructure project number 3-4187 of the Ministry of Science and Technology, Israel (MOST), and by a Korean National Research Foundation grant to SB and Man Bock Gu, Korea University. We are grateful to the National BioResource Project (National Institute of Genetics, Mishima, Japan) for the generous donation of the *E. coli* Keio collection. [Correction added after online publication 02 March 2012: The Korean National Research Foundation grant was added to the Acknowledgments.]

References

Andersen, J., Delihas, N., Ikenaka, K., Green, P.J., Pines, O., Ilercil, O., and Inouye, M. (1987) The isolation and characterization of RNA coded by the *micF* gene in *Escherichia Coli*. *Nucleic Acids Res* **15:** 2089–2101.

Arai, M., Alpert, N.R., MacLennan, D.H., Barton, P., and Periasamy, M. (1993) Alterations in sarcoplasmic reticulum gene expression in human heart failure. A possible mechanism for alterations in systolic and diastolic properties of the failing myocardium. *Circ Res* **72:** 463–469.

Ash, R.J., Mauck, B., and Morgan, M. (2002) Antibiotic resistance of gram-negative bacteria in rivers, United States. *Emerg Infect Dis* **8:** 713–716.

Atlas, R.M. (1992) Molecular methods for environmental monitoring and containment of genetically engineered microorganisms. *Biodegradation* **3:** 137–146.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al.* (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2:** 2006.0008. doi:10.1038/msb4100050.

Baquero, F., Martínez, J.-L., and Cantón, R. (2008) Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol* **19:** 260–265.

Belkin, S. (2003) Microbial whole-cell sensing systems of environmental pollutants. *Curr Opin Microbiol* **6:** 206–212.

Ben-Israel, O., Ben-Israel, H., and Ulitzur, S. (1998) Identification and quantification of toxic chemicals by use of *Escherichia coli* carrying *lux* genes fused to stress promoters. *Appl Environ Microbiol* **64:** 4346–4352.

Bermingham, A., and Derrick, J.P. (2002) The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery. *Bioessays* **24:** 637–648.

Blattner, F.R., Plunkett, G., III, Bloch, C.A., Perna, N.T., Burland, V., Riley, M., *et al*. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277:** 1453–1462.

Bron, P.A., Benchimol, M.G., Lambert, J., Palumbo, E., Deghorain, M., Delcour, J., *et al*. (2002) Use of the *alr* gene as a food-grade selection marker in lactic acid bacteria. *Appl Environ Microbiol* **68:** 5663–5670.

Buchberger, W.W. (2011) Current approaches to trace analysis of pharmaceuticals and personal care products in the environment. *J Chromatogr A* **1218:** 603–618.

Cherepanov, P.P., and Wackernagel, W. (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158:** 9–14.

Coughter, J., and Stewart, G. (1989) Genetic exchange in the environment. *Antonie Van Leeuwenhoek* **55:** 15–22.

Daughton, C.G., and Ternes, T.A. (1999) Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect* **107:** 907–938.

Davidov, Y., Rozen, R., Smulski, D.R., Van Dyk, T.K., Vollmer, A.C., Elsemore, D.A., *et al*. (2000) Improved bacterial SOS *promoter:lux* fusions for genotoxicity detection. *Mutat Res* **466:** 97–107.

Delihas, N., and Forst, S. (2001) MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. *J Mol Biol* **313:** 1–12.

Doyle, J.D., Stotzky, G., McClung, G., Hendricks, C.W., Neidleman, S., and Allen, I.L. (1995) Effects of genetically engineered microorganisms on microbial populations and processes in natural habitats. *Adv Appl Microbiol* **40:** 237–287.

Duda, R.O., Hart, P.E., and Stork, D.G. (2001) *Pattern Classification*. New York, USA: John Wiley.

Elad, T., Benovich, E., Magrisso, S., and Belkin, S. (2008) Toxicant identification by a luminescent bacterial bioreporter panel: application of pattern classification algorithms. *Environ Sci Technol* **42:** 8486–8491.

Elad, T., Almog, R., Yagur-Kroll, S., Levkov, K., Melamed, S., Shacham-Diamand, Y., and Belkin, S. (2011) Online monitoring of water toxicity by use of bioluminescent reporter bacterial biochips. *Environ Sci Technol* **45:** 8536–8544.

Eltzov, E., Ben-Yosef, D.Z., Kushmaro, A., and Marks, R. (2008) Detection of sub-inhibitory antibiotic concentrations via luminescent sensing bacteria and prediction of their mode of action. *Sens Actuators B Chem* **129:** 685–692.

Freund, Y. (1995) Boosting a weak learning algorithm by majority. *Inform Comput* **121:** 256–285.

Fu, X., and Xu, J.-G. (2000) *Development of a Chromosome-Plasmid Balanced Lethal System for Lactobacillus Acidophilus with Thya Gene as Selective Marker*. Tokyo, Japan: Wiley-Blackwell.

Gracia-Lor, E., Sancho, J.V., and Hernández, F. (2011) Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **1218:** 2264–2275.

Grant, S.G., Jessee, J., Bloom, F.R., and Hanahan, D. (1990) Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc Natl Acad Sci USA* **87:** 4645–4649.

Griffith, K.L., Becker, S.M., and Wolf, R.E. (2005) Characterization of TetD as a transcriptional activator of a subset of genes of the *Escherichia coli* SoxS/MarA/Rob regulon. *Mol Microbiol* **56:** 1103–1117.

Heberer, T. (2002) Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicol Lett* **131:** 5–17.

Herrero, M., de Lorenzo, V., and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* **172:** 6557–6567.

Hirsch, R., Ternes, T., Haberer, K., and Kratz, K.-L. (1999) Occurrence of antibiotics in the aquatic environment. *Sci Total Environ* **225:** 109–118.

Jen, J.-F., Lee, H.-L., and Lee, B.-N. (1998) Simultaneous determination of seven sulfonamide residues in swine wastewater by high-performance liquid chromatography. *J Chromatogr A* **793:** 378–382.

Jones, O.A.H., Voulvoulis, N., and Lester, J.N. (2001) Human pharmaceuticals in the aquatic environment a review. *Environ Technol* **22:** 1383–1394.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., and Buxton, H.T. (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environ Sci Technol* **36:** 1202–1211.

Kümmerer, K. (2004) *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*. Berlin, Germany: Springer-Verlag.

Kuzminov, A. (1999) Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda. *Microbiol Mol Biol Rev* **63:** 751–813.

Lee, J.-H., Lee, K.-L., Yeo, W.-S., Park, S.-J., and Roe, J.-H. (2009) SoxRS-mediated lipopolysaccharide modification enhances resistance against multiple drugs in *Escherichia coli*. *J Bacteriol* **191:** 4441–4450.

Maccormick, C.A., Griffin, H.G., and Gasson, M.J. (1995) Construction of a food-grade host/vector system for Lactococcus lactis based on the lactose operon. *FEMS Microbiol Lett* **127:** 105–109.

Magrisso, S., Erel, Y., and Belkin, S. (2008) Microbial reporters of metal bioavailability. *Microb Biotechnol* **1:** 320–330.

van der Meer, J.R., and Belkin, S. (2010) Where microbiology meets microengineering: design and applications of reporter bacteria. *Nat Rev Microbiol* **8:** 511–522.

Mehta, J.B., Shantaveerapa, H., Byrd, R.P., Morton, S.E., Fountain, F., and Roy, T.M. (2001) Utility of rifampin blood levels in the treatment and follow-up of active pulmonary tuberculosis in patients who were slow to respond to routine directly observed therapy. *Chest* **120:** 1520–1524.

Melamed, S., Ceriotti, L., Weigel, W., Rossi, F., Colpo, P., and Belkin, S. (2011) A printed nanolitre-scale bacterial sensor array. *Lab Chip* **11:** 139–146.

Miller, J.H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual for Escherichia Coli and Related Bacteria*. Cold Spring Harbor, NY, USA: Cold Spring Harbor laboratory.

Nichols, B.P., van Cleemput, M., and Yanofsky, C. (1981) Nucleotide sequence of *Escherichia coli trpE* : Anthranilate synthetase component I contains no tryptophan residues. *J Mol Biol* **146:** 45–54.

Nunoshiba, T., Hidalgo, E., Amabile Cuevas, C.F., and Demple, B. (1992) Two-stage control of an oxidative stress regulon: the *Escherichia coli* SoxR protein triggers redox-inducible expression of the *soxS* regulatory gene. *J Bacteriol* **174:** 6054–6060.

Roberts, M.C. (2011) Environmental macrolide lincosamide streptogramin and tetracycline resistant bacteria. *Front Microbiol* **2**. doi: 10.3389/fmicb.2011.00040.

Scaria, J., Ramachandran, S., Jain, P.K., and Verma, S.K. (2009) Construction and testing of EGFP based bacterial biosensor for the detection of residual tetracyclines in milk and water. *Res J Microbiol* **4:** 104–111.

Shapiro, E., and Baneyx, F. (2007) Stress-activated bioluminescent *Escherichia coli* sensors for antimicrobial agents detection. *J Biotechnol* **132:** 487–493.

Silbergeld, E.K., Davis, M., Leibler, J.H., and Peterson, A.E. (2008) One reservoir: redefining the community origins of antimicrobial-resistant Infections. *Med Clin North Am* **92:** 1391–1407.

Smolander, O.-P., Ribeiro, A.S., Yli-Harja, O., and Karp, M. (2009) Identification of β-lactam antibiotics using bioluminescent *Escherichia coli* and a support vector machine classifier algorithm. *Sens Actuators B Chem* **141:** 604–609.

Stevens, R.C., Laizure, S.C., Williams, C.L., and Stein, D.S. (1991) Pharmacokinetics and adverse effects of 20-mg/kg/day trimethoprim and 100-mg/kg/day sulfamethoxazole in healthy adult subjects. *Antimicrob Agents Chemother* **35:** 1884–1890.

Stotzky, G., and Babich, H. (1986) *Survival of, and Genetic Transfer by, Genetically Engineered Bacteria in Natural Environments*. Orlando, FL: Academic Press, 93–138.

Sum, Z.M., Sefton, A.M., Jepson, A.P., and Williams, J.D. (1989) Comparative pharmacokinetic study between lenampicillin, bacampicillin and amoxycillin. *J Antimicrob Chemother* **23:** 861–868.

Tan, P.K., Downey, T.J., Spitznagel Jr, E.L., Xu, P., Fu, D., Dimitrov, D.S., *et al*. (2003) Evaluation of gene expression measurements from commercial microarray platforms. *Nucleic Acids Res* **31:** 5676–5684.

Urgun-Demirtas, M., Stark, B., and Pagilla, K. (2006) Use of genetically engineered microorganisms (GEMs) for the bioremediation of contaminants. *Crit Rev Biotechnol* **26:** 145–164.

Valtonen, S.J., Kurittu, J.S., and Karp, M.T. (2002) A luminescent *Escherichia coli* biosensor for the high throughput detection of β-Lactams. *J Biomol Screen* **7:** 127–134.

Vollmer, A.C., Belkin, S., Smulski, D.R., Van Dyk, T.K., and LaRossa, R.A. (1997) Detection of DNA damage by use of *Escherichia coli* carrying *recA':lux, uvrA':lux*, or *alkA':lux* reporter plasmids. *Appl Environ Microbiol* **63:** 2566–2571.

Wei, R., Ge, F., Huang, S., Chen, M., and Wang, R. (2011) Occurrence of veterinary antibiotics in animal wastewater and surface water around farms in Jiangsu Province, China. *Chemosphere* **82:** 1408–1414.

Wingender, W., Graefe, K.H., Gau, W., Förster, D., Beermann, D., and Schacht, P. (1984) Pharmacokinetics of ciprofloxacin after oral and intravenous administration in healthy volunteers. *Eur J Clin Microbiol Infect Dis* **3:** 355–359.

Yagur-Kroll, S., and Belkin, S. (2010) Upgrading bioluminescent bacterial bioreporter performance by splitting the *lux* operon. *Anal Bioanal Chem* **400:** 1071–1082.

Yagur-Kroll, S., Bilic, B., and Belkin, S. (2009) Strategies for enhancing bioluminescent bacterial sensor performance by promoter region manipulation. *Microb Biotechnol* **3:** 300–310.

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.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

