Strategies for enhancing bioluminescent bacterial sensor performance by promoter region manipulation

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Summary
Bioluminescent bacterial sensors are based upon the fusion of bacterial bioluminescence (lux) genes, acting as a reporter element, to selected bacterial stress-response gene promoters. Depending upon the nature of the promoter, the resulting constructs react to diverse types of environmental stress, including the presence of toxic chemicals, by dose-dependent light emission. Two bacterial sensors, harbouring sulA::luxCDABE and grpE::luxCDABE fusions, activated by the model chemicals nalidixic acid (NA) and ethanol, respectively, were subjected to molecular manipulations of the promoter region, in order to enhance the intensity and speed of their response and lower their detection thresholds. By manipulating the length of the promoter-containing segment (both promoters), by introducing random or specific mutations in the promoter sequence or by duplicating the promoter sequence (sulA only), major improvements in sensor performance were obtained. Improvements included significantly enhanced sensitivity, earlier response times and an increase in signal intensity. The general approaches described herein may be of general applicability for optimizing bacterial sensor performance, regardless of the sensing or reporting elements employed.

Introduction
Ever since the introduction of genetically engineered microbial reporter strains almost two decades ago (King et al., 1990), numerous reports have described the construction of sensor cells capable of reporting on the presence of either specific compounds or global stress factors, such as toxicity or genotoxicity. The principle in almost all cases was the same: a fusion of a gene regulatory element responsive to the target compound(s) or stress factor, to a reporter gene(s), the product of which is quantitatively monitored either by its presence or by its activity. In most cases, the regulatory element in the sensor is a gene promoter fragment, a non-coding sequence of DNA preceding the actual gene coding section. With the aid of transcription regulators, directly or indirectly activated by the change in conditions, the promoter region is recognized by the RNA polymerase (RNAP), allowing the initiation of the transcription of the downstream gene(s). Of several possible gene reporter systems (Kohler et al., 2000; Magrisso et al., 2008), the use of bacterial bioluminescence genes is often favoured due to the high sensitivity conferred by enzymatic photon generation, the possibility for continuous online monitoring and the independence of exogenous substrate supply. Another characteristic of many descriptions of such sensor strains is that the promoter/reporter substrate combination is used ‘as is’. Several other reports describe attempts at the optimization of sensor performance by manipulations of the molecular fusion. Examples include: (i) sensitivity enhancement of the umuC::lacZ fusion to genotoxicants by insertion of a tandem lacUV5 promoter upstream of a chimeric trp/umu promoters that drives EGFP expression, combined with coexpression of a mutated RecA protein which constantly promotes the cleavage reaction of LexA thus enhancing the SOS response (Arai et al., 2001), (ii) a recN::lux fusion mutated in the second LexA repressor binding site, or a recN promoter with a mutated −35 region conferring improved induction and sensitivity (van der Lelie et al., 1997), (iii) a modified sulA promoter with an additional LexA repressor binding site with a reduced background level compared with the wild type, and an improved performance of a recN promoter with only one of the two original LexA repressor binding sites (Dreier et al., 2002); (iv) introduction of mutations in the regulatory binding site of HbpR, generating a dual-responsive sensor activated by two regulatory proteins (Tropel et al., 2004); (v) modifying the sensitivity of an arsR::lacZ bioreporter by placing a second ArsR binding site downstream of arsR (Stocker et al., 2003), or by altering the ribosome binding site in front of lacZ (Wackwitz et al., 2008), both leading to background signal reduction, and (vi) in the same system, replacing fragments of various length originating from wild-type lacZ promoter generated different expression levels (Wackwitz et al., 2008).
In this communication we describe several general approaches, not sensor-specific, undertaken in order to enhance the sensing performance of promoter::reporter fusions and achieve improved reporter output. Significant improvements in detection sensitivity, response kinetics and signal intensity were achieved by modifications of the length of the promoter-containing DNA fragment, by random mutagenesis of the promoter, by site-directed mutagenesis of consensus elements in the promoter and by promoter duplication. The general nature of these manipulations renders them applicable to other types of promoter::reporter fusions for whole-cell biosensor applications.

Results

Manipulation of promoter fragment length

Most genetically engineered bacterial sensor strains harbour a fusion of an upstream sequence of a specific gene, containing its promoter region, to a reporter element. In some cases (Van Dyk et al., 1994), the promoter-containing segment extends downstream into the open reading frame (ORF) of the gene, while in other instances (Norman et al., 2005) no part of the ORF region is included. To examine which of the two options may be preferable, two gene promoters were tested: sulA that responds to SOS-inducing genotoxicants such as nalidixic acid (NA) (Quillardet et al., 1982; 1989), and grpE that is activated by diverse stress conditions (including ethanol) that induce synthesis of heat shock proteins (Van Dyk et al., 1994; Rupani et al., 1996). For this purpose, we constructed two pairs of promoter::luxCDABE fusions, one of each containing a short promoter fragment that does not include an ORF region of the gene, and the other containing a long promoter fragment that extends for about 200 bp into the ORF. The kinetics of light development of the two sulA constructs in the presence of NA (5 mg l\(^{-1}\)) are presented in Fig. 1A, where it may be observed that the longer fragment of the sulA promoter allowed a faster and stronger response than the shorter one. The two grpE constructs responded to the presence of ethanol (5%) in an opposite manner (Fig. 1B): the

Fig. 1. Bioluminescence of E. coli harbouring either short or long DNA fragments containing either the sulA (A, C and E) or grpE (B, D and F) promoter, fused to P. luminescens luxCDABE.
A and B. Response kinetics of long or short sulA::lux fusion to nalidixic acid (NA) (5 mg l\(^{-1}\)) and of grpE::lux fusion to ethanol (5%) respectively.
C and D. \(\Delta RLU\) values (the difference in luminescence intensity in the presence and absence of the inducer) of the two sulA::lux fusions as a function of NA concentration (60 min exposure) and of grpE::lux fusions as function of ethanol concentration (30 min exposure) respectively.
E and F. Response ratio (the ratio of the luminescence of the induced sample to that of the non-induced control) of the two sulA::lux fusions as a function of NA concentration (60 min exposure) and of grpE::lux fusions as function of ethanol concentration (30 min exposure) respectively.
longer grpE fragment yielded lower bioluminescence values than the shorter one. In both cases, the differences in bioluminescence intensity were also apparent in the non-induced controls: the constructs that responded more strongly were also characterized by significantly higher background values. At time zero, bioluminescence background values of the long and short constructs were approximately 6500 and 500 relative light units (RLU), respectively, for sulA, and 1400 versus 3900 RLU, respectively, for grpE. The observation that the first two bioluminescence readings of the grpE::luxCDABE construct (Fig. 1B) in the induced systems were lower than in the non-induced controls is explained by a temporary inhibition of light emission upon addition of the inducer (5% ethanol).

Figure 1C–F describes the response spectra of these strains to different NA concentrations (sulA::luxCDABE) following an exposure of 60 min (Fig. 1C and E) and to different ethanol concentrations (grpE::luxCDABE) after 30 min (Fig. 1D and F). The responses are depicted either as the difference in the intensity of the luminescence in the absence and presence of the inducer (ΔRLU) (Fig. 1C and D) or as the ratio of the luminescence of the induced sample to that of the non-induced control (response ratio) (Fig. 1E and F). The effect observed for a single inducer concentration in Fig. 1A and B is maintained, for both constructs, for the entire concentration spectrum tested. The long sulA fragment, containing a portion of the ORF of sulA gene, drove bioluminescence at a higher intensity than the short fragment at all NA concentrations tested (Fig. 1C); this effect was also manifested in the 60 min response ratios (Fig. 1E), in spite of the high background luminescence. Furthermore, the construct containing the longer fragment also displayed an enhanced sensitivity: calculated EC$_{200}$ values, which represent the inducer concentration causing a twofold increase in luminescence, were 1.22 mg l$^{-1}$ for this fragment as compared with 3.64 mg l$^{-1}$ for the short one; effects on detection threshold were similar (Table 1). As already observed in Fig. 1B, the grpE-based sensor displayed a different behaviour: the response with the short fragment was significantly stronger (Fig. 1D), but its 30 min response ratio was lower than that of the longer fragment due to a higher background (Fig. 1F). There were no significant differences in sensitivity, as reflected from the similar EC$_{200}$ and detection threshold values (Table 1).

Random mutagenesis (‘directed evolution’) of the sulA promoter

‘Directed evolution’ has previously been demonstrated to yield enzymes with improved catalytic properties such as enhanced protease solubility (van den Berg et al., 2006), increased bacterial phosphotriesterase expression (McLoughlin et al., 2005) or altered substrate specificity (Suenaga et al., 2001). This approach was also implemented in the construction of a promoter library with a range of promoter strength expression (Alper et al., 2005) or to optimize regulatory properties of a given promoter (Nevoigt et al., 2007). The approach is generally based upon error-prone PCR, with or without the combination of a ‘DNA shuffling’ procedure, that is performed on the target DNA sequence; the resulting library of variants is then screened for the desired feature, and selected isolates are subjected to a repeated procedure.

### Table 1. Sensitivity of sulA::lux constructs to nalidixic acid and of grpE::lux constructs to ethanol.

| Gene promoter | Manipulation | EC$_{200}$* (r$^2$) | Detection threshold* (r$^2$) |
|---------------|--------------|---------------------|-----------------------------|
| grpE          | Promoter fragment length | | |
| Long          | 1.85 (0.99) | 1.2 (0.97) |
| Short         | 1.64 (0.94) | 1.46 (0.94) |
| Long          | 1.22 (0.99) | 0.55 (0.99) |
| Short         | 3.64 (0.96) | 1.7 (0.99) |
| Random mutagenesis | | | |
| WT            | 3.94 (0.99) | 1.22 (0.96) |
| sulA18        | 1.49 (0.96) | 0.51 (0.98) |
| sulA-I        | 1.22 (0.96) | 0.65 (0.99) |
| sulA-II       | 3.72 (0.98) | 1.148 (0.99) |
| sulA-III      | 2.73 (0.98) | 1.32 (0.99) |
| Site-directed mutagenesis | | | |
| WT            | 3.94 (0.99) | 1.23 (0.96) |
| sulA35        | 1.39 (0.99) | 0.6 (0.99) |
| Promoter duplication | | | |
| sulA          | 4.2 (0.97) | 1.2 (0.98) |
| recA          | 2.15 (0.99) | 0.83 (0.98) |
| sulA-recA     | 2.1 (0.99) | 0.28 (0.97) |

*a.* Units: % ethanol for grpE, mg l$^{-1}$ NA for sulA.

*b.* No activity.
We performed an error-prone PCR (Kagiya et al., 2005) on the sulA wild-type promoter region of the sulA::lux fusion. The resulting PCR fragments, each potentially containing one or more random mutations at different locations, were cloned into a promoter-less plasmid and transformed into Escherichia coli yielding approximately 1000 colonies. The sulA::lux variants library was screened in 96-well microtitre plates and the performance of each individual construct was compared with that of the sulA wild-type promoter::lux fusion. Three parameters were monitored: (i) background bioluminescence prior to toxicant addition, (ii) response ratio 60 and 120 min after exposure to NA, and (iii) length of lag period preceding induction.

Out of nearly a thousand colonies screened in this manner, one improved mutant (sulA18) was selected. This mutant exhibited a much faster and stronger response to NA (Fig. 2A), the latter effect evidenced by dramatically higher ΔRLU values (Fig. 2B). This difference was also apparent when quantified by the response ratio (Fig. 2C). However, as bioluminescence background emission of the sulA18 mutant was significantly higher than that of the wild type also in the non-induced control (at time zero, c. 1100 versus 250 RLU respectively), the effect on the response ratio was less dramatic than that evidenced by the ΔRLU. As shown in Table 1, the sulA18 mutant also displayed an enhanced sensitivity, with an EC200 value of 1.5 mg l\(^{-1}\) as compared with 4 in the wild type.

A second round of error-prone PRC with the new sulA18::lux variant serving as the template yielded additional mutants that exhibited a reproducible but very moderate enhancement in the response ratio to NA, mostly due to lower background luminescence values of the non-induced control, but with no significant improvement in actual bioluminescent response (not shown).

Sequencing of the sulA18 promoter region revealed three point mutations relative to the wild-type sequence (Fig. 3A). To find out which of these mutations was responsible for the modified phenotype of sulA18, each was individually introduced into the wild-type promoter. Activity assays demonstrated that mutation I, adjacent to the LexA repressor binding site and the –10 element, was the cause of the enhanced sulA18 phenotype. The variant harbouring this point mutation (sulA18-I) displayed the same activity pattern as sulA18: high ΔRLU (Fig. 3B), high response ratio values (Fig. 3C) and a similar EC200 and detection threshold concentrations (Table 1).

**Site-directed mutagenesis of the sulA promoter**

Taking an opposite approach to the randomness of the mutagenesis in the directed evolution process described in the previous section, point mutations were introduced into specific positions in the sulA promoter region. Two promoter consensus elements utilized by E. coli RNAP-\(\sigma^{70}\) subunit, both of general rather than promoter-specific significance, were selected as targets: the –35 (5′-TTGACA-3′) and the –10 (5′-TATAAT-3′) elements. Deviations from either the consensus sequences or the optimum 17 bp spacer length (Hawley and McClure, 1983; Harley and Reynolds, 1987; Lisser and Margalit, 1993) are expected to lead to a reduction in promoter activity. A systematic study of the correlation between the –35 region and promoter strength using a set of single-base substitutions within the –35 element (Kobayashi et al., 1990) revealed that the –35-consensus sequence provides the strongest promoter activity. Analysis of the rate and efficiency with which RNAP forms a stable complex with a random –10-element sequence indicated that each step in the multiple

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pathway of transcription initiation may have different sequence requirements. This has led to a proposal for a sequence combination for the -10 element that covers all different possibilities, 5′-TA/CAT/CAT/G-3′ (Xu et al., 2001). Based on these studies, we have changed the -35 element of sulA promoter from 5′-TTGA TC-3′ to 5′-TTGA CA-3′, and the -10 element from 5′-TGTCAT-3′ to 5′-TCA CAT-3′ (Table 2).

The responses of the two mutants to NA are presented in Fig. 4, along with that of the wild type. In Fig. 4A the time-course of bioluminescence development in response to NA (5 mg l⁻¹) is displayed, indicating that the -10 mutation almost completely abolished the ability to be induced by NA. In contrast, the -35 modification allowed a much faster and stronger induction, apparent in the presence of all tested NA concentrations according to both ΔRLU (Fig. 5B) and response ratio (Fig. 5C) values. This increase in intensity and improvement in response time was also accompanied by an enhancement of sensitivity, evident in the reduced calculated EC₂₀ and detection threshold values (Table 1).

**Dual-promoter::lux plasmid**

As another approach for improving sulA::lux sensor performance we have introduced an additional promoter between the sulA promoter and the reporter gene in the sulA::lux fusion. The recA promoter was chosen as the extra promoter since, similarly to sulA, it is also activated by genotoxic stress. Furthermore, as the background emission level of the recA::luxCDABE fusion in the absence of toxicant is higher than that of sulA::luxCDABE (not shown), it was hypothesized that a stronger response upon induction may be expected. To test this hypothesis we have constructed a dual-promoter sensor, sulA::recA::luxCDABE, and examined its response to NA in comparison with the single-promoter fusions. As may be observed in Fig. 5, bioluminescence intensity was much higher than that of either of the single-promoter fusions (Fig. 5A). However, the response ratio of the dual-promoter sensor, while indeed higher than the parental sulA::lux sensor, was similar in magnitude to that of the recA::lux sensor (Fig. 5B). This apparent contradiction is, once again, explained by higher luminescence values of the non-induced control. The effect on sensitivity was

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**Table 2.** The consensus sequences of -10 and -35 elements, the relevant sequences of sulA wild-type promoter elements and the mutations sequences.

| -35 consensus element | T | T | G | A | C | A |
|-----------------------|---|---|---|---|---|---|
| sulA -35 element      | T | T | G | A | C | A |
| sulA(-35) mutant      | T | T | G | A | C | A |
| Optimal -10 element   | T | A/C| A | T/C| A | T/G|
| sulA -10 element      | T | G | T | C | A | T |
| sulA(-10) mutant      | T | C | A | C | A | T |

The mutated positions are indicated in boldface.
The different strategies described and demonstrated herein are of a general nature, in that they can be executed with no previous knowledge of promoter regulation and/or signal transduction; they may thus be applied to other promoter::reporter fusions, regardless of the type of either the sensing or the reporting element. Four independent strategies were tested for molecular manipulation of the promoter element controlling the reporting element expression: modifying the length of the DNA segment containing the promoter region, introduction of random mutations by a directed evolution process, insertion of site-directed mutations into the −35 and −10 regions, and promoter duplication. For each strategy, an example was presented that displayed its potential, with no attempt to exhaust the very broad spectrum of opportunities inherent in each of the approaches.

Fig. 4. Effects of mutations in the −10 and −35 consensus sequence elements of sulA promoter.
A. Kinetics of luminescence development in the presence of NA (5 mg l⁻¹).
B and C. Responses to different NA concentrations are displayed as ΔRLU (B) and response ratios (C) after 60 min of exposure to different NA concentrations.

Fig. 5. Effects of promoter duplication. Responses to different NA concentrations are displayed as ΔRLU (A) and response ratios (B) after 60 min of exposure to different NA concentrations.

Discussion

Our aim in the present study was to investigate universal (i.e. not gene-specific) approaches for enhancing the reporter output of bacterial promoter::reporter fusions.
between promoters (for review see Browning and Busby, 2004). Although no DNA sequences located in the ORF of the sulA or grpE genes are known to be relevant for transcription, including or excluding an ORF region from the sensing element yielded major differences in the sensor’s performance. Different effects, however, were obtained for the promoter regions of the two genes tested: in the case of sulA the longer fragment conferred a significant advantage over the shorter one, as this sensor responded to lower inducer concentrations, exhibited higher bioluminescence values and displayed higher response ratios. Conversely, a non-ORF-containing insert of grpE::luxCDABE exhibited a higher bioluminescent intensity in the presence of ethanol but a lower response ratio and no difference in sensitivity. This apparent inconsistency may be explained by different requirements imposed by the different sigma factors, σ^30 and σ^32, regulating the transcription of sulA and grpE respectively. It is also likely that parameters other than the promoter sequence, such as local DNA topology or participation of activators or other DNA-binding proteins, affect the transcription (Browning and Busby, 2004). Another possibility is that the initial section of the grpE ORF may contain a hitherto unrecognized sequence relevant for its transcription. The different effects of fragment length may also be related to translation rather than transcription; it was recently shown that mRNA structure around the ribosomal binding site plays a dominant role in determining mRNA and protein levels (Kudła et al., 2009). While no general conclusion can be drawn from these opposite effects, it is clearly demonstrated that modification of the length of the promoter-harbouring insert may be a powerful tool for reporter function modulation.

Using the sulA promoter element as a model, we have generated a library of mutants using error-prone PCR. The sulA gene is a part of the SOS regulon, activated upon DNA damage, and its transcription is regulated by the LexA repressor (Mizusawa et al., 1983). Under normal conditions, LexA represses transcription by binding to a specific palindromic consensus sequence of 20 bp within the sulA promoter region known as the LexA box [TACTG(TA)5CAGTA] (Walker, 1984). LexA box sequences that are similar to the consensus box are predicted to have greater affinity to the LexA repressor (Lewis et al., 1994). Analysis of the sulA promoter sequence (Fig. 3A) revealed that mutation I, responsible for the sulA18 phenotype, is located in close proximity to, but not inside, the consensus LexA box; nor is it located in the consensus promoter elements –10 or –35. Nevertheless, based on the higher background luminescence of the mutant relatively to the wild type, mutation I is likely to endow either a reduced affinity to the LexA repressor or an increased affinity to the RNAP. The fact that beneficial sulA::lux mutations were isolated, even though they were not located within well-defined consensus sequences, demonstrates the potential power of the ‘directed evolution’ process, which introduces mutations into random locations that could not necessarily be identified as obvious targets by bioinformatic tools.

Another strategy we have tested involved directed modifications in two crucial sites that affect promoter strength, the –10 and –35 consensus elements located upstream of the transcription initiation site (Hawley and McClure, 1983; Harley and Reynolds, 1987). As mentioned in Introduction, there have been reports of manipulating the sensing element of a microbial biosensor by adding or deleting a repressor site in the promoter or by mutating the –35 element (van der Lelie et al., 1997). In the present study, a significant improvement in performance was achieved by changing the sulA –35, but not the –10 element, to the consensus sequence. The latter manipulation almost completely abolished the promoter’s ability to respond to NA. A possible indication as to the effects taking place may be provided by examining the background (non-induced) bioluminescence of the two mutants, possibly emanating from alterations in the RNAP recognition sites. The much higher background bioluminescence of the –35 mutant may signify a much stronger affinity of the RNAP to the mutated promoter, whereas the much lower background of the –10 mutant may imply that transcription initiation from the –10 locus is very low. This could be due to weakened recognition by the RNAP σ subunit, or due to a reduction in the ability to unwind the DNA strand to generate the open complex necessary for transcription initiation (Browning and Busby, 2004). The fact that altering the native –10 element of the sulA promoter impaired its ability to initiate transcription supports the proposal of Xu and colleagues (2001), according to which the sequence of a strong promoter does not necessarily contain the optimal sequence for each step in transcription initiation, but rather a compromise that allows all steps to proceed with minimal constraints.

As binding affinity of RNAP is one of the major parameters affecting promoter strength (Ishihama, 1986) we hypothesized that addition of a second promoter sequence, adjacent to the one that drives lux expression, will exhibit a stronger response due to the additional sites for RNAP binding. Moreover, by fusion of two different promoters, sulA and recA, a new response combining the mode of action of the two promoters may be expected. Our results (Fig. 5) indicate that duplication of transcriptional regulatory sequences may constitute an efficient strategy for enhancing sensor performance, with the intensity of the response to NA surpassing either of the individual promoter elements when present alone. This effect, however, was manifested neither in the response ratios nor in the sensitivity to this inducer.
A factor that repeatedly emerged as crucial for the evaluation of a promoter::reporter fusion performance, in addition to the intensity of the response to the designated inducer, is its background expression level without any induction. As even a minor increase in the background signal may have a major effect on the calculated response ratio, we find that the difference in signal intensities, i.e. the ΔRLU value, represents the actual level of gene activation and the true end result of the reporter gene induction much better than the response ratio ("fold induction"). Furthermore, basing a sensor’s response upon induction ratios only, without considering the actual bioluminescence values, may mask a real response if the background changes with time. In the construction of future whole-cell biosensor devices based upon microbial reporter cells of the type discussed here, it is highly likely that engineering considerations may dictate a preference for quantifying increments in activity over fold-induction data.

Another lesson learned from the experiments summarized herein is the limitations of conventional manual laboratory methodologies in the screening of numerous mutants or multiplex effectors. Quite clearly, automated high-throughput approaches (Zaslaver et al., 2006) can dramatically increase the efficiency of mutants screening as well as of testing diverse combinations of molecular manipulations.

Experimental procedures

Chemicals

All chemicals used were of the highest analytical grade. Nalidixic acid was obtained from Sigma.

Bacterial strains

Escherichia coli K12 strain AG1688 was used for the construction of all plasmids. E. coli K12 strain RFM443 was used for activity assays, and E. coli strain MG1655 chromosomal DNA was used as a template in all PCR reactions. The genotypes of these strains are listed in Table 3.

Plasmids construction

A pBR2TTS promoter-less plasmid was constructed as follows: a 6.96 kb EcoRI fragment of the Photorhabdus luminescens lux operon (GenBank Accession N. M90093) was incorporated through an EcoRI site into a pBR322 derivative that lacks tetracycline resistance. Two transcription termination sites (rRNA-5S ribosomal RNA sequence) were placed upstream of the multiple cloning site preceding the lux operon, in order to reduce plasmid background. The sulA, grpE and recA promoters were obtained by PCR amplification (primers are listed in Table 4). The promoter-containing fragments were incorporated into the pBR2TTS promoter-less plasmid through the SacI and KpnI restriction sites. The sulA promoter for the construction of the double-promoter vector was incorporated into the recA::lux plasmid through the KpnI restriction site. Promoters’ sequences and orientation were verified by sequencing.

Experimental conditions, luminescence measurement and data analysis

Prior to the assay, the bacterial strains were grown overnight at 37°C in LB broth supplemented with 0.1 mg ml⁻¹ ampicillin. The cells were then diluted 150-fold in fresh LB broth supplemented with ampicillin (0.1 mg ml⁻¹) and regrown with shaking at 37°C to the early exponential growth phase (OD₆₀₀ = 0.12). Culture aliquots (50 µl) were then transferred into the wells of an opaque white 96-well microtitre plate (Greiner Bio-One) containing 50 µl of either predetermined concentrations of the two model toxicants tested (NA or ethanol) in LB or a toxicant-free control (LB only). Luminescence was measured at 37°C for 2 h at 10 min intervals using a VICTOR² luminometer (Wallac, Turku, Finland). All experiments were carried out in duplicate, and were repeated at least three times on different dates.

Luminescence values are reported as the instrument’s arbitrary RLU. The results are presented either as the difference in the intensity of the luminescence in the absence and presence of the inducer (ΔRLU) or as the ratio of the luminescence of the induced sample to that of the non-induced control (response ratio or fold induction) as described previously (Belkin et al., 1997; Belkin, 1998). Sensitivity was determined in two manners: (i) by calculating (Belkin et al., 1997; Belkin, 1998) an EC₅₀ value, denoting the effective toxicant concentration causing a twofold increase in the response ratio; lower EC₅₀ values reflect greater sensitivity and a lower detection threshold of the tested strain; (ii) by calculating the inducer concentration at which luminescence surpassed a level that was three standard deviations above the background (detection threshold value).

Error-prone PCR

We performed two rounds of sulA promoter region mutagenesis using error-prone PCR according to Kagaya and colleagues (2005) with 2 mM dNTPs, 2.5 mM MgCl₂, 1.0 mM MnCl₂, 5 U µl⁻¹ Taq DNA polymerase (Fermentas, #EP0401), and 50 pmol µl⁻¹ of primers sulA-R and sulA-F (Table 4). The PCR mixture (50 µl) was divided into five individual 10 µl reaction aliquots to enhance the divergence of the resulting mutations. Following the PCR procedure, the reaction mixtures were pooled, purified, digested with SacI and KpnI restriction enzymes, and then ligated into the same restriction sites in the promoter-less plasmid pBR2TTS. The products of the ligation reaction were used to transform E. coli strain AG1688 to generate the variants library. The library was
were screened in a three-step process: each error-prone PCR round, approximately 1000 colonies were screened for improved variants following each round of error-prone PCR.

Library screening for improved reporter strain performance

The screening procedure was aimed at isolating mutants that are characterized by either (i) a lower background luminescence, (ii) a stronger response, (iii) a shorter lag period or (iv) an enhanced sensitivity compared with the wild type. After each error-prone PCR round, approximately 1000 colonies were screened in a three-step process:

(i) Each colony was inoculated into an individual well of a regular 96-well microtitre plate, one containing 50 μl of 0.3 mg l⁻¹ NA in LB and the other LB only. Luminescence was measured every 10 min for 3 h.

(ii) Selected colonies were screened using a similar procedure, in which NA induction was compared with a non-induced control of the same culture. For this purpose, two aliquots (50 μl) of each refreshed overnight cultures were transferred into two wells of a fresh 96-well microtitre plate, one containing 50 μl of 0.3 mg l⁻¹ NA in LB and the other LB only. Luminescence was measured every 10 min for 50 min. Background luminescence and response ratio were calculated for each well and compared with a wild-type colony that was treated similarly.

(iii) Finally, the kinetics of luminescence development at different NA concentrations was assayed for a limited number of selected colonies as described under Experimental conditions, luminescence measurement and data analysis above. The plasmid-borne sulA promoter regions of selected colonies were sequenced, and plasmids containing a mutation were used to transform strain RFM443. The third step was then repeated with this host.

Table 4. List of primers used for PCR in this study.

| Construct label | Primers set | Primers sequence a |
|----------------|------------|--------------------|
| sulA Short (sulA S) | sulA-R | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA-R2 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| sulA Long (sulA L) | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA-R2 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| grpE Short (grpE S) | grpE-R | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | grpE-F2 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| grpE Long (grpE L) | grpE-R | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | grpE-F3 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| sulA random mutagenesis | sulA-R | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| sulA18-I | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA-R4 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| sulA18-II | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA-R5 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| sulA18-III | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA-R6 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| sulA-recA | sul-kpn | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| recA | recA-R | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | recA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| sulA(-10) | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sulA28 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| sulA(-35) | sulA-F | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |
| | sul33 | 5'-GGTCAAGTGGACCTCAATCTTCCGCGGAGGAAAGCTCCGGAATGAGCTCGGAG-3' |

a. The primers were designed with either SacI or KpnI sites which are underlined in the sequence.
Site-directed mutagenesis

To introduce specific mutations into the sulA promoter region, either to reconstruct mutations from the random mutagenesis procedure or to plant a directed mutation in the –10 and –35 consensus elements, we designed primers that included the desired mutations at the appropriate location. These primers were used to amplify the promoter region by a PCR reaction. The primers and their sequences are listed in Table 4. Incorporation of the desired mutation/s was verified by sequencing.

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