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Genome-wide gene-deletion screening identifies mutations that significantly enhance explosives vapor detection by a microbial sensor

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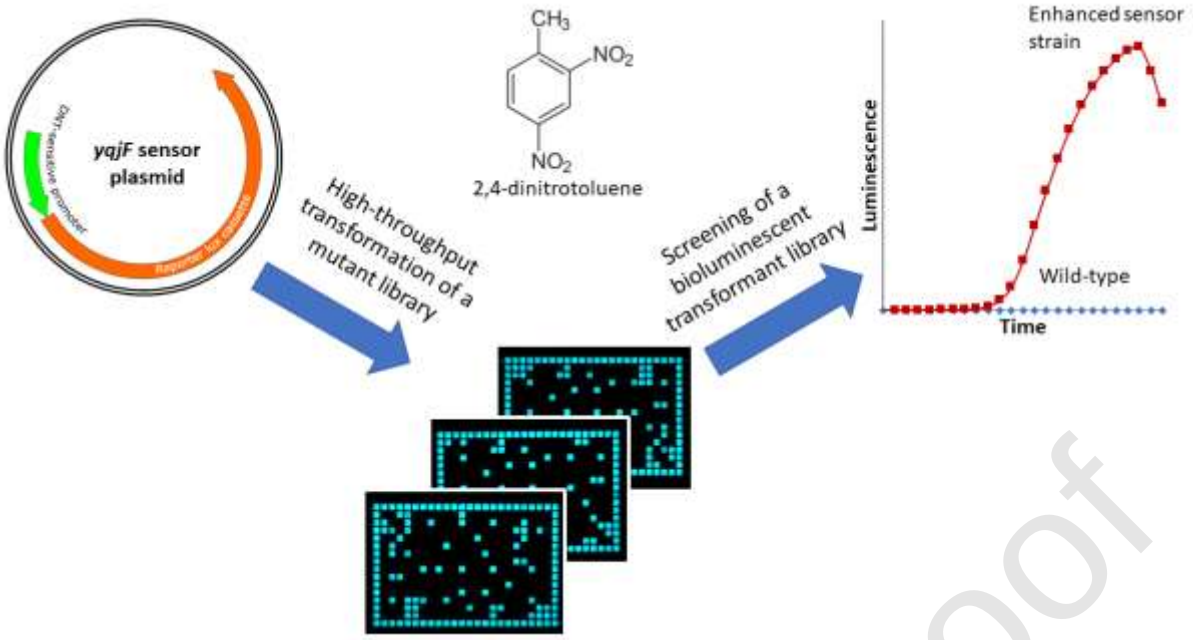
Graphical abstract



Highlights

- High-throughput transformation and screening of a 3818-member *E. coli* mutant library with a DNT-sensor reporter plasmid
- Identification of new genes and metabolic circuits related to the induction of the DNT-responsive promoter of the *yqjF* gene
- Using this information, achieving a 400-fold enhancement of DNT detection sensitivity by the *yqjF*-based microbial biosensor

Abstract

Genetically engineered microbial biosensors, capable of detecting traces of explosives residues above buried military ordnance and emitting an optical signal in response, may potentially serve for the standoff detection of buried landmines. A promising candidate for such an application is a previously reported *Escherichia coli*-based reporter strain that employs the *yqjF* gene promoter as its sensing element; however, for this sensor to be able to detect actual landmines reliably, it was necessary for its detection sensitivity and signal intensity to be enhanced. In this study, a high-throughput approach was employed to screen the effects of individual gene deletions on *yqjF* activation by 2,4-dinitrotoluene (DNT). Several genes were identified, the deletion of which elicited a significant enhancement of *yqjF* induction by DNT. The most promising of these mutations were introduced into the sensor strain, individually or in pairs, yielding a considerable increase in signal intensity and a lowering of the detection threshold. A strain harboring two of the identified mutations, *ygdD* and *eutE*, appears to be the most sensitive microbial biosensor currently described for the detection of traces of landmine explosives.

Abbreviations

DNT, 2,4-dinitrotoluene; TNT, 2,4,6-trinitotoluene; RM, random mutagenesis.

Keywords

2,4-dinitotoluene (DNT); 2,4,6-trinitrotoluene (TNT); Microbial bioreporter; Biosensor; Landmines; Explosives.

Introduction

The extensive use of explosives for military and civil purposes throughout the past century has led not only to the endangerment of civilians in past conflict areas due to the presence of buried landmines and unexploded ordnance, but also to significant soil and water pollution. Since no current technology allows the remote detection of such devices, the mapping of minefields necessitates the on-site presence of personnel, along with the obvious risks involved. A possible biological solution allowing the remote localization of landmine positions has been proposed, based on the dispersal of genetically engineered microbial bioreporters over the target area [1]. In the presence of explosives vapors emanating from buried landmines, the bacteria generate an optical signal that enables the standoff detection of explosive devices from a safe distance [2,3]. While several explosives-sensing microbial strains for this purpose have been reported over the years [4–12], until recently no field-validated system for the detection of explosives based on microbial biosensors has been described in the scientific literature.

We have recently reported the remote detection of buried anti-personnel landmines, containing ca. 200 g of 2,4,6-trinitrotoluene (TNT), by using an *Escherichia coli*-based bioreporter strain. This sensor, which harbored a fusion of the *yqjF* gene promoter to an enhanced green fluorescent protein (GFPmut2) gene [13], became fluorescent upon exposure to TNT and 2,4-dinitrotoluene (DNT), a common volatile impurity of military grade TNT. The signal was detected remotely by laser beam scanning of the target area for GFP excitation, in tandem with a telescope for high resolution fluorescent signal collection [14]. While the results obtained were highly encouraging, the system suffered from the long incubation time necessary for the development of a significant fluorescent signal. In addition to the operational difficulties caused by this constraint, it also dictated the maintenance of the sensor cells in a fully active state for ca. 24 h, thus requiring a suitable dehydration-resistant microenvironment. To overcome this barrier in the development of a reliable remote landmine detection scheme, the option has been investigated of replacing fluorescent protein-based reporting by bacterial bioluminescence, encoded by the *luxCDABE* gene cassette. Previous results [15] have demonstrated the superiority of the enzyme-driven bacterial bioluminescent reporting in terms of both response time and detection sensitivity.

Improvements in the performance of the *yqjF*-based explosives sensor strains by several rounds of error-prone PCR of the *yqjF* gene promoter were previously reported [16,17]. The altered *yqjF* gene promoter evolved in that study, FB2A1#14-C5, was further modified by an additional round of error prone PCR to yield a further improved variant designated C55, which was employed in the present study.

Here we describe an additional and complementary strategy for enhancing bacterial bioreporter performance, namely high-throughput screening of a ca. 4,000-member *E. coli* mutant library [18], all transformed with a plasmid harboring the original *yqjF::luxCDABE*

fusion. The screen has yielded rich information concerning genes for which the deletion has led either to inhibition or enhancement of *yqjF* induction by DNT. Selected mutants from the latter group were transformed with the improved C55 promoter-lux fusion, displaying significant enhancement of trace DNT detection. In addition, two processes were identified, glutathione and ethanolamine metabolism, the relationship of which to the fate of DNT in the *E. coli* was previously unknown. The integration of the genetic manipulations described herein into the DNT bioreporter resulted in an improved limit of detection and signal intensity, increasing the potential applicability of this microbial sensor as a means to detect explosives under field conditions.

Materials and Methods

Chemicals and media

All chemicals used in this work were of the highest analytical grade and purchased from Sigma-Aldrich (Jerusalem, Israel). DNT was dissolved in ethanol and diluted in water or in lysogeny broth (LB) to a maximal final ethanol concentration of 2% (v/v) in the working solution. DNAse-RNAse-free water (Sigma-Aldrich) was used for all molecular biology procedures. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA) and DNA purification kits from Qiagen (Germantown, MD, USA).

All bacteria were grown in LB and stored on LB agar plates. Antibiotics (100 mg/L ampicillin, 50 mg/L kanamycin or 30 mg/L chloramphenicol) were added to the media, depending on the bacterial hosts and plasmids employed.

Plasmids construction

Three types of plasmid were employed in the course of this study (Table S1, **Figure 1**); two were reporter plasmids harboring a fusion of an inducible gene promoter either to the *luxCDABE* gene cassette of the terrestrial bioluminescent bacterium *Photorhabdus luminescens* (Figure 1A) or to the *luxCDABEG* cassette of the marine bioluminescent bacterium *Aliivibrio fischeri* (Figure 1B). The third plasmid (Figure 1C) expressed a modified version of the *yhaJ* gene, encoding a LysR-type transcriptional regulator of *yqjF* [19]. For the initial transformation of the entire mutant library, in order to study the effect of individual mutations on *yqjF* activation, plasmid pBR-*yqjF-luxPl* (Figure 1A) was employed. This plasmid, described (as *yqjF::lux*) in [16], harbors a fusion of the native *yqjF* gene promoter to the *P. luminescens luxCDABE* gene cassette.

Selected mutants exhibiting significantly enhanced responses to DNT were transformed with an improved variant of this plasmid, pBR-C55-*luxPl* (Figure 1A) which contained, as the sensing element, an upgraded version (C55) of the *yqjF* promoter (see below). Some of the single mutants, as well as all of the double mutants tested in the course of this study, were screened using the plasmid schematically presented in Figure 1B, pBR-C55-*luxAf*, in which

the C55 variant of the *yqjF* gene promoter served as the sensing element and the *luxCDABEG* gene cassette of *Aliivibrio fischeri* as the reporter element.

Variant C55 of the *yqjF* gene promoter was obtained by a random mutagenesis process similar to that reported in [17], using the 4th generation promoter variant reported in that publication (yqjFB2A1#14-C5) as the template and an undefined mix of DNT biotransformation products as the inducer. The latter was obtained by incubating an exponentially growing culture (LB, 37°C) of *E. coli* DH5α with 50 mg/L DNT and collecting the medium after 2 h by filtration (0.22 µm Millex PVFD syringe filter, Merck Millipore, Burlington, MA, USA). Screening conditions were as previously reported [17]. Variant C55 was selected from among mutant library members that exhibited a response ratio (sample/control) >2 when exposed to the spent medium. See Supplementary Materials (Figs S2, S3) for additional details concerning the construction of this promoter variant.

Plasmid pBR-C55-*luxAf* was constructed by replacing the *luxCDABE* genes of plasmid pBR-*yqjF-luxPl* with the *luxCDABEG* gene cassette of *A. fischeri* (Figure 1B). The latter was PCR-amplified (primers forward 94_luxV_SacI_F and reverse 93_luxV_RI_R; Table S1) from plasmid *pUCD615* [20] and cloned using the Gibson assembly technique [21] into pBR-C55-*luxPI*, predigested with SacI and EcoRI. In all experiments carried out following the original high throughput mutants' screening, the tested strains were also transformed with an additional plasmid, pACYC-*yhaJ*-G2 (Figure 1C). This plasmid expresses a mutated form of the *yhaJ* gene, encoding the regulatory activator of *yqjF* [19], under the control of the native *yhaJ* gene promoter (Elad et al., unpublished).

To investigate the DNA specificity of the effect exerted on *yqjF* induction by the host's mutation, selected mutant strains were transformed with plasmid *pBR-lacZ-luxPl* (Figure 1A), carrying a fusion between the *E. coli lacZ* gene promoter and the *luxCDABE* gene cassette of *P. luminescens* [22]. The response to isopropyl β-D-1-thiogalactopyranoside (IPTG) of strains carrying this plasmid was compared with that obtained for the same mutants hosting *the pBR-C55-luxPl* plasmid induced with DNT.

DNA manipulations were performed according to standard methods [23] and all constructs were validated by sequencing. A full list of the *E. coli* strains, plasmids and primers used in this study is provided in Table S1.

High-throughput transformation of a mutant library

The 'Keio collection' [18], a 3,818-member *E. coli* strain library consisting of all possible non-lethal single gene deletions, was employed to identify genes involved in the response of the *yqjF*-based bioreporter to DNT. The library, a kind gift from the National Institute of Genetics, Shizuoka, Japan, was maintained at -80°C, in 16% glycerol, in 96-well plates. To transform the mutated clones, each plate was first replicated to a 96-well plate containing 100 µL of fresh LB supplemented with kanamycin (50 mg/L), using a 96-pin replicator, and

incubated overnight at 37°C with shaking (200 rpm). Then, 20 µL of the inoculum in each well was transferred to a deep 96-well plate containing 1 mL of LB with kanamycin as above and regrown for 2.5 h (37°C, 200 rpm). The l plate was centrifuged (10 min, 3220 g, 4°C), the supernatant was discarded, and the pellet was resuspended in cold 0.05 M $CaCl_2$ and incubated for 30 min on ice. The plate was centrifuged again as above, after which the supernatant was discarded and the pellet was resuspended in cold 0.05 M $CaCl_2$ containing plasmid *pBR-yqjF-luxPl* (ca. 0.25 µg plasmid DNA per well). The plate was incubated on ice for 30 min, subjected to a 40-second 42°C heat shock, and after an additional 5 min on ice, each well was supplemented with 400 µL of kanamycin-containing LB. Following a 1.5 h incubation (37°C, 200 rpm), 20 µL from each well of the transformed bacteria were transferred to a corresponding well of a 96-well plate containing 130 µL of LB supplemented with kanamycin (50 mg/L) and ampicillin (100 mg/L). Glycerol (50 µL, 50%) was added to all wells and the plate was incubated overnight (37°C, 200 rpm). Every four 96-well plates of this collection were combined into a single 384-well plate (50 µL per well), which was kept at -80°C until used. A single 384-well plate uniformly containing only the wild-type strain (BW25113) in all wells was similarly prepared and used as a wild type control. Optical density at 600 nm (OD_{600}) in the course of the activity assays served as an indicator of transformation success; when maximal OD_{600} in the course of the assay was lower than 0.2, the transformation of this specific mutant was considered to have failed, and the procedure was repeated. Transformation rate using this procedure was 85% (±16%) in the first round and 91% after the second round, yielding a total of 3484 successful transformants out of 3818 library members. Colony PCR was performed to verify the specified gene deletions in selected mutants by applying primers flanking the deleted gene, in comparison to the wild type strain.

Investigation of the response of the wild type strain, which was assayed in a single 384 well plate containing the same strain, revealed a minor dependency between the location of a well on the microtiter plate and bioluminescence intensity (Figure S1), a phenomenon that was previously noticed in similar assays [24] and that is probably attributed to an uneven temperature across the plate. A correction coefficient was therefore calculated, designed to correct the responses to the response levels observed in the middle section of the plate. Applying this correction resulted in very minor changes in the results.

Activity assay for *yqjF*-based bioreporters

To measure the intensity of the luminescent response in all bioreporter variants, the relevant strains were grown overnight (37°C, 200 rpm) in 2 mL LB supplemented with the appropriate antibiotics. The culture was then diluted x1/100 in fresh LB and regrown under the same conditions to an early exponential growth phase ($OD_{600} \approx 0.2$). Culture aliquots (50 µl) were transferred into an opaque white 96-well microtiter plate (Greiner Bio-One), each well already containing 50 µl of a predetermined concentration of DNT, dissolved in 4% v/v

EtOH. The plate was then sealed with a transparent tape (Excel Scientific), and bioluminescence was measured at 10 minutes intervals at 30°C using either an Infinite 200 PRO plate reader (TECAN, Männedorf, Switzerland) or a VICTOR^2 plate reader (Wallac, Turku, Finland). A shaking step was performed prior to each reading to ensure sufficient oxygen availability. Luminescence data are presented in the instruments' arbitrary relative light units (RLU), either as the difference in the intensity of the signal in the presence and absence of the inducer (ΔRLU) or as the response ratio, the luminescence in the presence of the inducer divided by that in its absence [25].

Activity assay for the *yqjF::lux*-transformed mutant library

Microtiter 384-well plates containing the Keio collection mutants transformed with the pBR-*yqjF-luxPl* plasmid were thawed and pin-replicated to sterile clear-bottom white 384-well plates (Greiner Ltd.) containing, in each well, 20 µL of LB supplemented with ampicillin. The plates were covered with a sterile plastic casing and incubated with shaking (37°C, 200 rpm) for 3 hours. Using a robotic system (Microlab Star, Hamilton, Bonaduz, Switzerland), 20 µL of LB containing 30 mg/L of DNT and 0.1% ethanol were added to each well, resulting in a final DNT concentration of 15 mg/L. A similar concentration of ethanol dissolved in LB was used as an un-induced control. The plates were transferred to an incubator and kept at 30 °C. Luminescence intensity (RLU) and OD_{600} were measured ca. every 40 min in a SynergyHT plate reader (BioTek, Winooski, VT, USA). The assay was performed in triplicate. Maximal luminescence values over the first 600 min were compared to the luminescence obtained in the control samples, and response ratios (induced/control) and response intensity (induced – control, ΔRLU) were calculated both for the mutants and the wild type controls. A 'mutation effect' parameter (ME) was calculated for each mutant to characterize the effect of the relevant mutation on the response of the *yqjF*-bioreporter, according to the following equation:

$$ME\ (\%) = \left[\frac{\max \Delta RLU_{mutant}}{\max \Delta RLU_{wild-type}} \times 100\right] - 100$$

The DNT detection sensitivity of each strain was determined by calculating an EC_{200} value, the DNT concentration at which luminescence intensity is 2-fold higher than that of the un-induced control [25].

Construction of double mutants by P1 phage transduction

Double gene deletions were obtained by P1 phage transduction [26], employed to delete an additional gene from the genome of a member of the Keio mutants collection [18]. To remove the kanamycin resistance cassette, originally employed to generate the single-gene deletion mutants of the Keio library, the recipient cells were transformed with plasmid pCP20 (temperature-sensitive ampicillin resistance). This plasmid contains a thermally induced Flp-recombinase, and allows the excision from the chromosome of segments

flanked by short directed repeats [27], such as those flanking the kanamycin resistance cassette in the Keio collection strains. A detailed description of the mutagenesis procedure may be found in the Supplementary Materials (Doc S1).

Detection of DNT under a soil surface

DNT (5 g) was buried 2.5 cm below the surface of a sand (50-70 mesh, Sigma-Aldrich)-filled container, which was kept at room temperature for 15 months to allow stable permeation of DNT vapors to the surface. An identical container without DNT served as a negative control. Before applying the bacteria, the top sand layer was moistened by lightly spraying with LB medium. The bioreporters, carrying a deletion of the *pykF* gene and transformed with the *pBR-C55-luxAf* and *pACYC-yhaJ-G2 plasmids*, were immobilized in calcium-alginate beads. A detailed description of the immobilization procedure can be found in the Supplementary Materials (Doc S2).

The beads (150 g in 200 mL LB) were pre-incubated for 1 h (37°C, 200 rpm), and then spread in a monolayer on top of the targets. Imaging was performed every 5 minutes in a dark chamber at room temperature with a Sony α7S II camera equipped with a Sony FE 28 mm f/2 lens at maximal aperture. The ISO value and exposure time were set to 4,000 and 15 seconds, respectively. Quantification of the response was performed with ImageJ [28] by integrating the signal across each target.

Results

Screening the reporter-transformed mutant library

Screening the responses to DNT of the 3,484 successfully transformed mutant library members showed that most gene deletions had little or no effect on the response of the *yqjF* gene promoter (**Figure 2**), compared to the induction level of the wild-type strain. A total of 126 mutant strains, representing 3.6 % of the screened library, displaying either a positive (ME ≥ 200%, 67 mutants) or negative (ME ≤ -70%, 59 mutants) responses, were considered to be involved in the induction mechanism of the *yqjF* gene promoter by DNT. The gene mutations with the most significant effects among these transformants were verified by PCR.

Two dozen of the most positively responsive mutants, in terms of either signal intensity or response ratio (Table S2), were transformed with plasmid *pBR-C55-luxPl* (Figure 1A) carrying the advanced version of the *yqjF* promoter and exposed to DNT (2.5 mg/l). **Figure 3** presents the mutation-dependent change in the intensity of the response of all of these mutants in comparison to the wild-type strain, and **Figure 4**A displays the time-dependent bioluminescence signal development in four of the single gene mutants (*pykF*, *ygdD*, *cchB* and *eutE*). While the increase in response intensity in some of these mutants was highly significant, exhibiting a 5-10 fold increased signal compared to the wild-type strain (Figure

4A, B), the effect on DNT detection sensitivity was more moderate (Figure 4C); the mutation which lowered the EC_{200} value to the greatest extent (from 2.27± 0.14 mg/L to 0.5± 0.03 mg/L) was *eutE*.

In an attempt to further enhance sensor performance, 56 strains carrying double deletions selected from among the positively responding mutants were constructed (Table S3). In most cases, the double deletions did not elicit responses that were significantly different from the single mutations. A notable exception was the *ΔygdD*/*ΔeutE* combination, encoding an inner membrane protein and an acetaldehyde dehydrogenase, respectively. **Figure 5** shows the activity of this mutant pair, in comparison to both the wild type and the individual mutations. The double mutant not only exhibited at least a 6-fold stronger luminescence signal than either of the individual mutants (Figure 5A), but also an approximately 5-fold higher response ratio compared to the uninduced control (Figure 5B) and a 34-fold reduction in detection threshold (Figure 5C).

To investigate whether the enhancing effect of *ygdD*, *eutE*, *pykF* and *cchB* deletions on the response of the reporter was specific to induction by DNT, all four single-gene deletion mutants were transformed with a plasmid carrying the *lacZ* promoter fused to the *luxCDABE* cassette of *P. luminescens*, and induced by IPTG. The positive effect on *yqjF* induction resulting from deletion of *cchB*, *eutE* and *pykF* proved to be specific for the DNT effect on the *yqjF* promoter, only minimally affecting the *lacZ*-driven activities; in contrast, a deletion of *ygdD* was also shown to increase by 2-4 fold the response of *lacZ* to IPTG, hinting at a more general mechanism of action (**Table 1**).

Involvement of glutathione metabolism in *yqjF* induction by DNT

To gain insight into cellular processes that influence the activity of *yqjF*, a list of genes for which deletion caused either a significant increase (ME ≥ 200%) or a strong inhibition (ME ≤ -70%) of the response to DNT was submitted to the DAVID functional annotation tool [29]. Two biological processes, ethanolamine catabolism and glutathione biosynthesis, were shown to be related (p value < 0.05) to the response of *yqjF* to DNT (**Table 2**).

Indeed, as may be seen in **Figure 6**, deletions of several genes related to glutathione metabolism have significantly altered the response of *yqjF* to DNT. Deletions of either of the glutathione synthetase genes *gshA* and *gshB* resulted in highly significant reduction of the response to DNT (90%-100% reduction of signal), with no significant effect on bacterial viability. To confirm the specificity of either *gshA* or *gshB* deletions to DNT metabolism, both were also transformed with a *pBR-lacZ-luxPl* plasmid and induced with IPTG. The effect of Δ*gshA* and Δ*gshB* deletions on *lacZ* induction was negligible (not shown), hinting at a specific role of the glutathione in the mechanism leading to the induction of *yqjF* by DNT.

Conversely, deletion of genes (i.e. *pepN*, *yfcF*, *pxpB*), involved in glutathione degradation or transformation, has led to a 1.5-3 fold increase in the response. This was also the effect of deletion of genes with reported glutathione peroxidase activity, such as *yfcF*, *btuE* and *gstA*.

Another group of genes, the relationship of which to the induction of *yqjF* by DNT was shown to be significant (p-value = 8.6 x 10^{-3}), is that related to ethanolamine catabolism genes. Ten single-gene deletion mutants related to ethanolamine catabolism were therefore transformed with the *pBR-yqjF-luxPl* plasmid and exposed to DNT (25 mg/L). All the selected gene deletions were shown to enhance the response of *yqjF*, with several deletions enhancing the signal intensity up to 80-fold (**Figure 7**).

Detection of DNT under a soil surface

To demonstrate the detection capabilities of the reporter strains constructed in the course of this study, a sand-filled container in which 5 g of DNT were buried 2.5 cm below the surface at the center of the container, incubated at room temperature for 15 months, was used as the target. A selected mutated strain (Δ*pykF*), immobilized in Ca-alginate beads and spread in a single layer on top of the sand surface, was imaged at intervals over 12 h. An image of the two containers 350 min after the introduction of the bacteria is presented in **Figure 8**A, and a time-lapse recording of signal development over 12 h is shown in Movie S1. While initial luminescence was high in both containers, the signal in the target accumulated continuously over time while the signal in the control dropped. This is quantitatively demonstrated in Figure 8B. While the strongest response was located at the center of the target, directly above the buried DNT, the long incubation time allowed DNT to permeate to more distant parts of the target, resulting in luminescence across the entire surface of the container.

Discussion

Previous studies have demonstrated a significant and dose-dependent response to TNT and to its volatile degradation byproduct DNT [12,16,17] by a genetically engineered microbial bioreporter, in which the *yqjF* gene promoter served as the sensing element. The same construct also detected real landmines under specific conditions [14]. However, in view of the minute concentrations of explosives vapor above buried landmines [30–32], the DNT detection limits exhibited by these bioreporters may prevent their application as robust and dependable tools for landmine detection. In the present study, significant progress in enhancing the detection capabilities of the *yqjF*-based bioreporter is reported in terms of both signal intensity and detection sensitivity, reaching a 1000-fold lower detection limit than previous versions of this reporter.

This result was achieved by employing an experimental approach not previously reported for whole-cell sensor development, namely transformation of a very large *E. coli* mutant library, comprising ca 87.4 % of this bacterium's non-lethal gene mutations, with a single plasmid-borne promoter-reporter fusion; this was followed by exposure to DNT and high-throughput screening of library member responses. The approach proved to be a powerful tool, revealing mutations with significant beneficial effects, the relationship of which to *yqjF*

induction by DNT was not previously indicated. The unravelling of the importance of the glutathione system to *yqjF* activation by DNT, for example, sheds additional light on this incompletely understood induction mechanism. The importance of glutathione to the biotransformation of DNT and TNT in certain organisms has been previously described [33–35]. The negative effect on *yqjF* induction by Δ*gshA* and Δ*gshB* glutathione synthetases mutations, as well as the positive effect exerted by mutations in genes involved in glutathione transformations such as the peroxidase genes *yfcF*, *btuE* and *gstA*, indicates that *yqjF* activation is strongly influenced by the cell's redox state. This impression is supported by the induction of the *soxRS* operon [36] when *E. coli* is exposed to DNT (Shemer et al, unpublished data). In addition, glutathione may take part in scavenging nitroso radicals, formed during the initial reduction of DNT to 4-hydroxylamino-2-nitrotoluene [37,38].

Deletion of *pykF*, a gene encoding a pyruvate kinase I, proved highly beneficial in enhancing the response of *yqjF* to DNT. While *pykF* is a key element in the glycolytic pathway [39], its deletion could affect the cell in various other directions. It has previously been shown that a *ΔpykF* mutation alters the cell's gene expression levels, enzyme activities, metabolite concentrations, glycolytic flux, and the production of various compounds [40,41], as well as plasmid copy number [42]. This, however, is not supported by the observation (Table 1) that the *pykF* deletion did not affect *lacZ* induction. The specific enhancement of the *yqjF* response by a Δ*pykF* mutation (Table 1) could also be due to the blocking of downstream metabolic processes involving the utilization of DNT as a carbon source, leading to accumulation of the *yqjF* inducing molecule.

While numerous single-gene deletions proved advantageous to the *yqjF*-based biosensor, almost none of the tested two-gene deletion combinations exerted an additive effect (Table S3). This finding is surprising in view of the unrelated molecular circuits and biochemical reactions in which these genes are involved. A notable exception was the combination of two single gene deletions, Δ*ygdD* and Δ*eutE*, which displayed a significant enhancement of the response compared to hosts with either single deletion. The combined mutation resulted in a 400-fold increased detection sensitivity compared to the wild-type strain equipped with the *pBR-C55-luxPl* plasmid (EC_{200} of 5.4 ± 1 ng/mL *versus* 2.27 ± 0.14 µg/mL). This, to the best of our knowledge, is the most sensitive microbial bioreporter for the detection of trace explosives, with sensitivity surpassing that reported elsewhere of 1.82 mg/L DNT [43].

While the effect of the *eutE* deletion was shown to be specific to *yqjF* induction by DNT, the effect of the *ygdD* mutation appeared to be of a more general character (Table 1). Information regarding the function of *ygdD* is limited, but it has been described as an inner-membrane protein and was implicated in the uptake of various antimicrobial chemicals including the peptide Arasin 1, suggesting a role as a transporter [44]. In addition, *ygdD* has been linked to the *mazF* regulon, a mechanism which induces post-translational modifications to a large array of mRNAs as a response to various stress conditions [45]. These data correspond to the more general nature of the *ygdD* deletion effect.

The deletion of *eutE*, in contrast, appears to be more closely linked to the induction of *yqjF* by DNT. This gene, encoding an acetaldehyde dehydrogenase, is a part of the *eut* operon involved in ethanolamine catabolism. Several genes of this operon form a cellular micro-compartment in which ethanolamine breakdown takes place, while others, including *eutE*, encode enzymes which participate in the biotransformation process. This micro-compartment prevents the loss of volatile ethanolamine biotransformation products, allowing a more efficient utilization of this carbon source, as well as preventing toxic by-products such as acetaldehyde from harming the cell [46]. While a deletion of *eutE* proved to have the most significant positive effects on *yqjF* induction, deletion of many other *eut* operon members also significantly enhanced its response (Figure 6). While the exact biochemical link between the *eut* operon and DNT metabolism is unclear, it is possible that this cellular micro-compartment is utilized in the metabolism of DNT, helping the cells to cope with potentially toxic and volatile byproducts.

Previous reports of molecular manipulations aimed at enhancing the detection/reporting performance of microbial strains were focused on specific regulatory segments [17,47–49], regulatory genes [50,51] or genes of known function [52–54]. Other reports focused on redesign of genetic circuits [55-57] and optimizing the reporter element [58]. The genome-wide high throughput screening methodology described here provides relevant information regarding genes and gene products the involvement of which in the tested reaction would be difficult to predict *a priori*, based on available information. As the results evidence, this approach has facilitated significant performance enhancement of trace explosives detection by a previously constructed microbial bioreporter; it has also illuminated several molecular pathways involved in the induction of the target gene promoter, *yqjF*. Both of these effects are expected to benefit future microbial-based schemes for the remote detection of buried explosives, and will also hopefully prove useful in the development of other whole-cell sensor strains.

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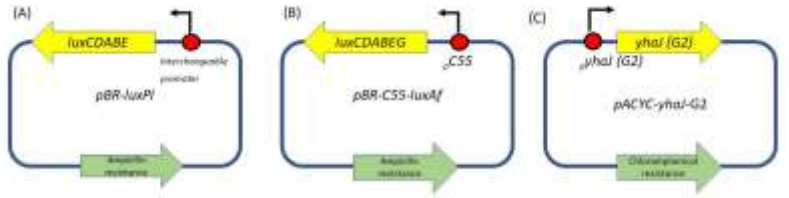

Figure 1: Reporter plasmids employed in this study. For details see the text.

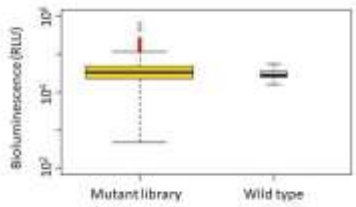

Figure 2: Bioluminescent response to DNT (15 mg/L) of the 3,488-member Keio mutant library, compared to the response of the wild type strain. Luminescence values are in the plate reader's arbitrary relative light units (RLU) and represent the difference in luminescence intensity in the presence and absence of DNT. Mutants showing a response larger than 3 times the interquartile range (marked in red) were defined as positive outliers.

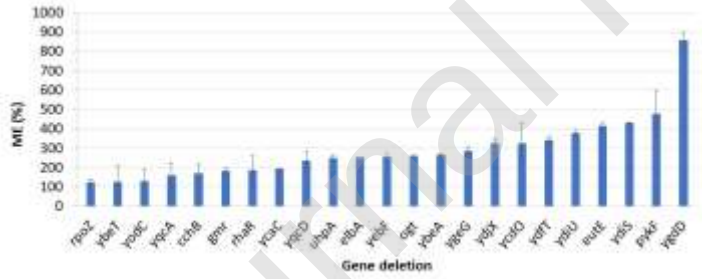

Figure 3: Effect of selected single-gene deletions on the luminescent response to DNT (2.5 mg/L) of a reporter carrying the *pBR-C55-luxPl* plasmid.

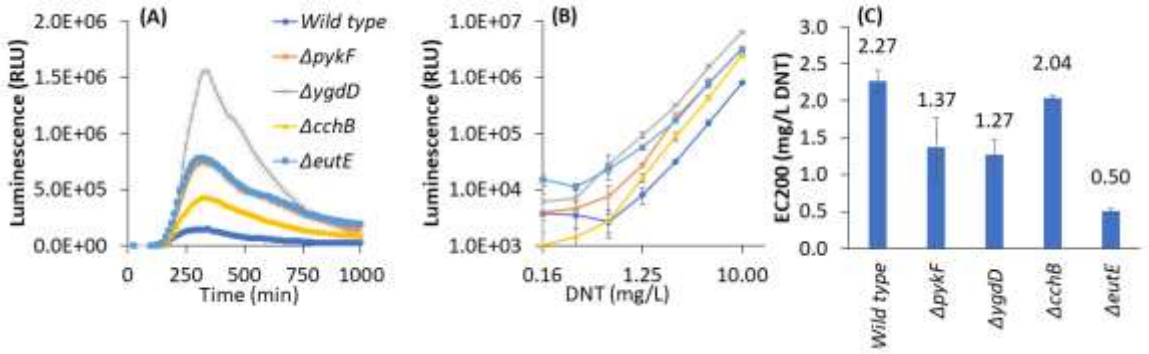

Figure 4: Bioluminescent response to DNT of four single gene deletion mutants (*ΔpykF*, *ΔygdD*, *ΔcchB* and *ΔeutE)* as well as the wild type, all transformed with the *pBR-C55-luxPl* plasmid. (A) Signal development in response to DNT (5 mg/L); luminescence values represent the difference in intensity in the presence and absence of DNT; (B) Response (maximal luminescence in the course of a 600 min exposure) as a function of DNT concentration; (C) DNT detection sensitivity, presented as EC_{200} (DNT concentration at which light intensity is twice that of the DNT-free control).

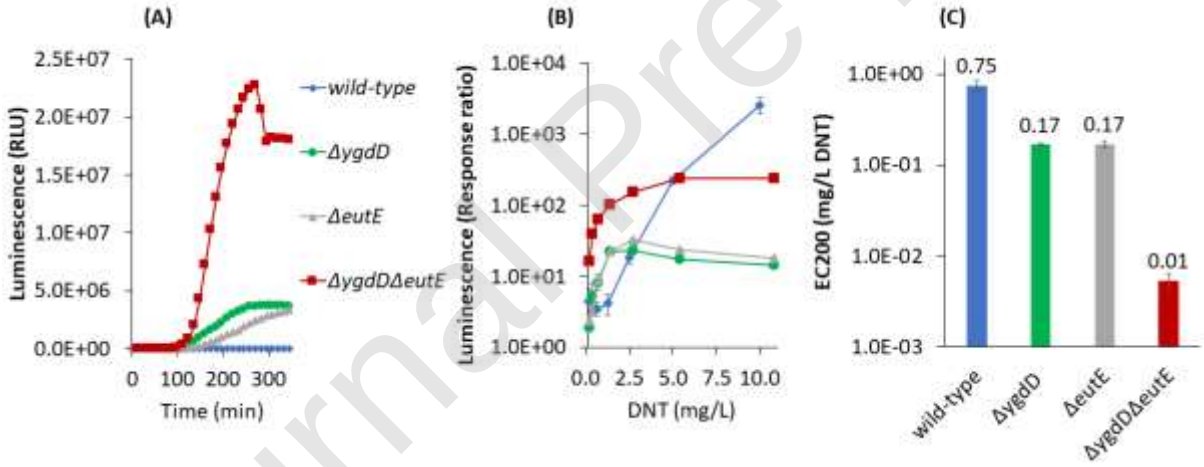

Figure 5: Luminescent response of bioreporters carrying both the *pBR-C55-luxAF* and *pACYC-yhaJ-G2* plasmids, as well as single/double deletions of the *ygdD* and *eutE* genes. The response is shown in terms of (A) time-dependent signal intensity in the presence of DNT (0.675 mg/L), presented as the difference in luminescence intensity in the presence and absence of DNT; (B) the ratio of the response to the uninduced control; (C) the detection sensitivity of the bioreporter presented as EC_{200} (DNT concentration at which light intensity is twice that of the DNT-free control).

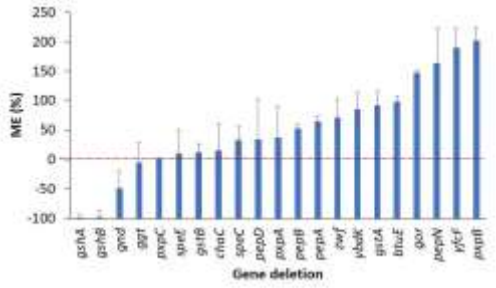

Figure 6: Effects of single deletions of genes related to glutathione metabolism and transport, all carrying the *pBR-yqjF-luxPl* plasmid, on the *yqjF* response intensity to DNT (15 mg/L).

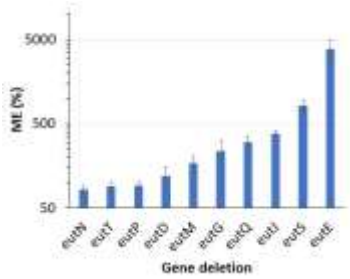

Figure 7: Effects of single deletion of genes related to ethanolamine catabolism, all carrying the *pBR-yqjF-luxPl* plasmid, on the *yqjF* response to DNT (25 mg/L).

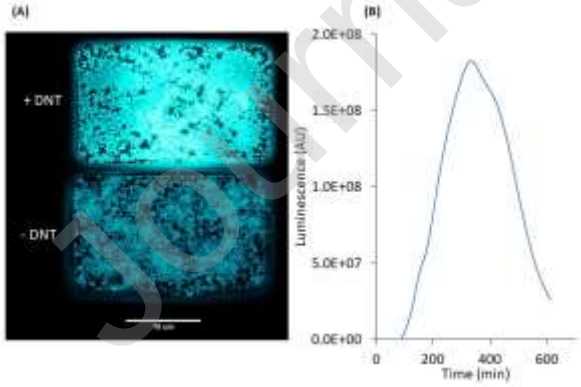

Figure 8: (A) An image of the two sand containers (top – with DNT; bottom – control) 350 min after introduction of the alginate-immobilized bioreporters; (B) Development of the luminescent signal intensity over a 10 h incubation; luminescence intensity was calculated by integration of the signal across the entire image using ImageJ software. The values present, in arbitrary units (AU), the difference in luminescence intensity between the two containers.

Table 1: Effect of selected gene deletions on the response of the yqjF *gene promoter (C55) to DNT (2.5 mg/L) and of the* lacZ *gene promoter to IPTG (0.5 mM) when hosting plasmids pBR-C55-luxPl and pBR-lacZ-luxPl, respectively.*

Gene deletion	**Mutation effect (%)**	
	Gene promoter: *yqjF*	**Gene promoter:** *lacZ*
	Inducer: DNT (2.5 mg/L)	**Inducer:** IPTG (0.5 mM)
pykF	479 ± 120	33 ± 8
cchB	170 ± 49	2 ± 4.1
eutE	413 ± 20	38 ± 18
ygdD	859 ± 39	211 ± 15

Table 2: Functional annotation analysis results for genes the deletion of which resulted in a either a significant increase or decrease of the luminescent response of the yqjF*-based bioreporter to DNT.*

Effect of deletion on* yqjF's *response to DNT	**Biological process**	**Number of genes involved**	**% of submitted gene list**	**P-Value**	**Benjamini-Hochberg correction**
Increased response (ME ≥ 200 %)	Ethanolamine catabolic process	4	3.8 %	8.60E-03	6.50E-01
Inhibited response (ME ≤ -70%)	Glutathione biosynthetic process	2	7.1 %	2.90E-02	8.30E-01