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ORIGINAL PAPER

Upgrading bioluminescent bacterial bioreporter performance by splitting the *lux* operon

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Abstract Bioluminescent bacterial bioreporters harbor a fusion of bacterial bioluminescence genes (*luxCDABE*), acting as the reporting element, to a stress-response promoter, serving as the sensing element. Upon exposure to conditions that activate the promoter, such as an environmental stress or the presence of an inducing chemical, the promoter::reporter fusion generates a dose-dependent bioluminescent signal. In order to improve bioluminescent bioreporter performance we have split the *luxCDABE* genes of *Photorhabdus luminescens* into two smaller functional units: *luxAB*, that encode for the luciferase enzyme, which catalyzes the luminescence reaction, and *luxCDE* that encode for the enzymatic complex responsible for synthesis of the reaction's substrate, a long-chain aldehyde. The expression of each subunit was put under the control of either an inducible stress-responsive promoter or a synthetic constitutive promoter, and different combinations of the two units were tested for their response to selected chemicals in *Escherichia coli.* In all cases tested, the split combinations proved to be superior to the native *luxCDABE* configuration, suggesting an improved efficiency in the transcription and/or translation of two small gene units instead of a larger one with the same genes. The best combination was that of an inducible *luxAB* and a constitutive *luxCDE*, indicating that aldehyde availability is limited when the five genes are expressed together in *E. coli*, and demonstrating that improved biosensor performance may be achieved by rearrangement of the *lux* operon genes.

Keywords Biosensors · Bioluminesence · *luxCDABE*

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Introduction

The last 15 years have witnessed a growing interest in whole-cell biosensors, a general term for devices in which the biological sensing moiety is a living cell. Such a combination allows not only detection of specific target chemicals but also an assessment of the effects of the studied sample on a living system [1]. Among the different approaches described for the construction of such devices, bacterial-based sensors play a major role, mostly due to the simplicity of their maintenance and their amenability to genetic manipulation. Consequently, the literature abounds with descriptions of genetically engineered bacterial strains capable of detecting specific chemicals, groups of chemicals or global biological effects such as toxicity or genotoxicity [2, 3]. Practically all of the described constructs are based on the inducible synthesis of reporter proteins [4–6], achieved by fusing their gene(s) to a promoter of a gene activated in the presence of the target chemical or stress factor. In such promoter::reporter fusions, mostly plasmid-borne, the choice of promoter dictates the detection spectrum of the sensor, while the choice of the reporter protein determines the type of signal emitted by the sensor and hence the instrumentation required for its detection.

Once the basic promoter::reporter fusion has been constructed, a variety of molecular or physiological approaches may be used to enhance its sensing performance. These approaches can be specific to the promoter::

reporter fusion, as has been demonstrated, for example, by Stocker et al. [7]: the background signal of an *arsR::lacZ* bioreporter was reduced by placing a second ArsR binding site downstream of *arsR*. Molecular bioreporter manipulations can also be of a more general nature. Recently, for example, we described different general modifications of the promoter region of a *sulA::lux* fusion that enhanced the intensity and speed of the sensor response and lowered the detection thresholds of genotoxic agents [8]: manipulation of promoter fragment length, random or site-directed mutagenesis of the promoter region, and promoter duplication. Other reports have described general performance enhancement strategies such as obtaining a faster response by changing the plasmid-bearing host strain [9], increasing sensitivity by integration of the fusion into the bacterial chromosome or by manipulating cell permeability or influx capability [9–11], and increasing the response ratio by lowering plasmid copy number [10].

One of the most popular reporter systems in many of these constructs is based on bacterial bioluminescence, coded for by the *luxCDABE* genes [3]. The *luxAB* genes code for the α and β subunits of the luciferase dimer, which catalyzes the oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde in the presence of molecular oxygen, leading to the emission of blue-green light. The synthesis of the aldehyde is catalyzed by a fatty acid reductase complex that includes a reductase, a transferase, and a synthetase, coded for by the *luxCDE* genes, respectively [12]. The ease and sensitivity of photon detection, along with the lack of need for exogenous substrate addition, makes bacterial luciferase an attractive reporter protein for biosensor applications. Several reports describe modifications of the *lux* operon aimed at improving its performance. For example, a synthetic optimized *luxCDABE* operon encoding the *Photorhabdus luminescens* Lux proteins was constructed for expression in high-CG bacteria [13]. To efficiently express the same genes in a Gram-positive bacterium (*Staphylococcus* species), enhanced translocation signals were introduced in front of *luxA*, *luxC*, and *luxE* [14]; this construct was then placed under the control of a constitutive *ami* promoter which conferred a highly stable bioluminescence phenotype [15]. Highly bioluminescent *S. aureus* strains were produced by modifying the *P. luminescens luxCDABE* operon so that each gene includes a Gram-positive ribosome binding site [16]. Also in Gram-positive bacteria, endogenous fatty acid aldehyde production was increased by placing the constitutive *ami* promoter upstream of the *luxCDE* genes while maintaining *luxAB* inducible [17]. Manen et al. [18] presented a bioluminescence reporter system in *Escherichia coli* based on two compatible cloning vectors, one inducibly expressing a promoter::*luxCDABE* fusion, and the other constitutively expressing *luxCDE*, providing the cell with additional aldehyde. In another case, by inclusion of an 11-amino acid carboxy-terminal tag recognized by endogenous tail-specific proteases, reduced half-life variants of *P. luminescens* LuxA and LuxB were expressed in *E. coli* [19]. Most of these reports, however, did not directly relate to bacterial biosensor construction but rather attempted to provide improved reporter systems for molecular biology applications.

In the current study, we have examined whether the performance of bioluminescent bacterial sensors could be improved upon by splitting the *P. luminescens luxCDABE* genes into two functional units: *luxAB*, coding for the luciferase enzyme, and *luxCDE*, coding for the reductase complex. Each of the two units was put under the regulatory control of either an inducible or constitutive promoter. It was hypothesized that different combinations of induced/constitutive *luxAB* with induced/constitutive *luxCDE* may exert a beneficial effect on sensor performance, i.e. signal intensity, detection sensitivity and response times. This hypothesis was driven by three main assumptions: (a) transcription and translation of a short operon should be faster, more effective and more efficient than that of a longer one; (b) by a high and constant expression of one component of the bioluminescence reaction, either *luxAB* or *luxCDE*, only the complementary component should be induced, thus leading to a faster and/or stronger response; and (c) constant expression of *luxCDE* genes may overcome aldehyde limitation. We have tested this hypothesis using *E. coli* reporter strains harboring four different promoter::*lux* fusions (*sulA::lux*, *katG::lux*, *arsR::lux* and *micF::lux*). In all cases, the combination of a constitutively expressed *luxCDE* with an inducible *luxAB* resulted in dramatically stronger and faster responses to all tested target chemicals; all other split combinations also appeared to be superior, to various extents, to the native *luxCDABE* combination, and displayed enhanced sensitivities and higher response ratios. Our results further indicate that availability of aldehyde, the substrate of the bioluminescence reaction, is a rate limiting factor of the sensor output when all five *lux* genes are transcribed together in *E. coli*.

Materials and methods

Chemicals All chemicals used were of the highest analytical grade. Nalidixic acid (NA), mitomycin C (MMC), hydrogen peroxide (H_2O_2), cadmium chloride, sodium arsenite, and paraquat (methyl viologen) were obtained from Sigma. Nonanal was obtained from Fluka.

Bacterial strains E. coli K12 strain AG1688 was used for the construction of all plasmids and *E. coli* K12 strain

RFM443 [21] was used for activity assays. The genotypes of these strains are listed in Table 1.

Plasmids construction Two different plasmids, with compatible origins of replication, were used to co-express the *luxAB* and the *luxCDE* units, both initially under *sulA* control. Plasmid p*sulA::luxCDABE* [8] served both as the source of the *P. luminescens lux* genes (GenBank accession number M90093) as well as the skeleton of the *luxAB* construct (psul::luxAB). For this purpose, all five *luxCDABE* genes were first removed from this plasmid by digesting it with SacI and EcoRI restriction enzymes. The *luxAB* genes were then re-inserted into this vector in two steps: first, the *luxA* gene was obtained by PCR amplification that introduced an EcoRI restriction site between *luxA* and *luxB*; the PCR product was incorporated into the digested fragment lacking *luxCDABE* genes through the SacI and EcoRI sites to generate p*sulA::luxA*. Next, the *luxB* gene, obtained by a PCR amplification that inserted an EcoRI site upstream of the start codon, was incorporated into the p*sul::luxA* vector through the EcoRI site to generate p*sul::luxA*B. Plasmid p*sulA*::*luxCDE* was constructed as follows: a *sulA::luxCD* fragment was PCR-amplified from p*sulA::luxCDABE* and inserted into the pGEM-Easy cloning vector. Next, the *luxE* gene was similarly amplified and inserted into the pGEM-Easy-*sulA*::*luxCD* through the SpeI site. Finally, the *sulA::luxCDE* fragment was digested out from the pGEM-Easy cloning vector by SalI restriction enzyme, and ligated into a pACYC184 vector that was also cut with SalI.

To construct fusions of other promoters to *luxCDABE*, *luxAB* or *luxCDE*, the *sulA* promoter fragment was replaced with either one of two constitutive synthetic promoters CP38 and CP25 [22] or with one of three inducible *E. coli* promoters: *katG*, *arsR* and *micF*. The synthetic promoters were synthesized as double stranded DNA according to the sequence published by Jensen and Hammer [22], while the inducible promoters were obtained by PCR amplification from the *E. coli* HG1688 (Genbank U00096) chromosome; all were ligated through SacI and KpnI restriction sites. All plasmids and primers constructed or used in the course of this study are listed in Tables 1 and 2, respectively. All constructs 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 the appropriate antibiotic(s) (Table 1). The cells were then

Table 1 Strains and plasmids used in this study

	Description	Resistance[a]	Reference
Parental *E. coli* strains			
AG1688	endA1 recA1 gyrA96 thi-1 relA1 glnV44 hsdR17(r_K^- m_K^+)		[20]
RFM443	F^-galK2 lac74 rpsL200		[21]
Plasmids			
psulA::luxCDABE	pBR322 derivate carrying *sulA::luxCDABE*	Am	[8]
pkatG::luxCDABE	pBR322 derivate carrying *katG::luxCDABE*	Am	This work
parsR::luxCDABE	pBR322 derivate carrying *arsR::luxCDABE*	Am	Melamed S., unpublished
pmicF::luxCDABE	pBR322 derivate carrying *micF::luxCDABE*	Am	This work
psulA::luxA	pBR322 derivate carrying *sulA::luxA*	Am	This work
psulA::luxAB	pBR322 derivate carrying *sulA::luxAB*	Am	This work
pCP38:: luxAB	pBR322 derivate carrying *CP38::luxAB*	Am	This work
pkatG:: luxAB	pBR322 derivate carrying *katG::luxAB*	Am	This work
parsR:: luxAB	pBR322 derivate carrying *arsR::luxAB*	Am	This work
pmicF:: luxAB	pBR322 derivate carrying *micF::luxAB*	Am	This work
pGEM-T-Easy-sulA::luxCD	pGEM-T-Easy derivate carrying *sulA::luxCD*	Am	This work
pGEM-T-Easy-sulA::luxCDE	pGEM-T-Easy derivate *carrying sulA::luxCDE*	Am	This work
psulA:: luxCDE	pACYC184 derivate carrying *sulA::luxCDE*	Cm	This work
pCP38:: luxCDE	pACYC184 derivate carrying *CP38::luxCDE*	Cm	This work
pCP25:: luxCDE	pACYC184 derivate carrying *CP25::luxCDE*	Cm	This work
pkatG:: luxCDE	pACYC184 derivate carrying *katG::luxCDE*	Cm	This work
parsR:: luxCDE	pACYC184 derivate carrying *arsR::luxCDE*	Cm	This work
pmicF:: luxCDE	pACYC184 derivate carrying *micF::luxCDE*	Cm	This work

[a] Am - ampicillin (100 mg/l); Cm - chloramphenicol (25 mg/l)

Table 2 List of PCR primers used in this study

Construct label	Primers set	Primers sequence[a]
sulA	sulA-R	5′-GCCTGAAGT*GAGCTC*AATCAATCC-3′
	sulA-F	5′-CGTCAAC*GGTACC*GCTGTAACTG-3′
katG	katG-R3	5′-CCACC*GAGCTC*ATCGATTGAAC-3′
	katG-F2	5′-GGCTTTTGT*GGTACC*CACACAGTG-3′
arsR	arsR-R3	5′-CGTAGCC*GAGCTC*CCAATTG-3′
	arsR-F2	5′-CGATT*GGTACC*GTTGGTTTAAC-3′
micF	micF-R	5′-CCGAATGC*GAGCTC*TCCGGTTG-3′
	micF-F	5′-CCTCATTAATCAGTC*GGTACC*TCC-3′
luxA(EcoRI)	EndA-EcoRI	5′-CTTTCTCCTTAG*GAATTC*CTAATATAATAGC-3′
	luxA-SacI3	5′-GAGATATTCTA*GAGCTC*AAATAGC-3′
luxB(EcoRI)	StartB-EcoRI	5′-GCTATTATATTAG*GAATTC*CTAAGGAGAAAG-3′
	luxB-R	5′-CCAGTCACATA*GAATTC*TATGCTCC-3′
luxCD	lux1	5′-CCATAAACTGCCAGGTCGACGGATC-3′
	lux2	5′-GGAGGTTGGTATGTAAGCAAAAAG-3′
luxE	lux5	5′-CCCAGGAGC*ACTAGT*AACTATGTGAC-3′
	lux6	5′-CGCAAGCATTCC*ACTAGT*AATTAGG-3′

[a] Restriction enzymes sites in the primers are rendered in italics

diluted 100-fold in fresh LB broth, and regrown with shaking at 37 °C to the early exponential growth phase ($OD_{600}\approx0.12$). Culture aliquots (50 μl) were then transferred into the wells of an opaque white 96-well microtiter plate (Greiner Bio-One) containing 50 μl of either a predetermined concentration of the model test chemicals in LB or a toxicant-free control (LB only). Luminescence was measured at 37 °C for 2 h at 10 min intervals using a VICTOR^2 luminometer (Wallac, Turku, Finland). All experiments were carried out in duplicate, and were repeated three times.

Luminescence values are reported as the instrument's arbitrary relative light units (RLU). The results are presented either as raw RLU values, 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) as described previously [23, 24]. Detection sensitivity was determined in two manners: (a) by calculating an EC_{200} value, denoting the effective toxicant concentration causing a twofold increase in the response ratio [23, 24]. Lower EC_{200} values reflect greater sensitivity and a lower detection threshold of the tested chemical by the sensor strain; (b) by calculating the inducer concentration at which luminescence surpassed a level that was three standard deviations above the background (detection threshold value).

The model test chemicals used were nalidixic acid (NA), mitomycin C (MMC) and hydrogen peroxide for *sulA* induction, cadmium chloride for *katG*, sodium arsenite for *arsR* and methyl viologen (paraquat), a redox cycling agent, for *micF*.

To test the effect of externally added aldehyde on bioluminescence intensity, 10 μl of either 0.5% nonanal (final concentration 0.05%) in dimethylformamide (DMF) or DMF only were added to 100 μl of NA-induced cells (60 min, 37 °C). Following an additional 5-min incubation at 37 °C, luminescence was measured using a VICTOR^2 luminometer (Wallac, Turku, Finland).

luxAB activity assay For testing the activity of *sulA*-driven *luxAB* independently of aldehyde production by *luxCDE*, an exogenous aldehyde (nonanal) was added. The bacteria were grown and refreshed as described above, and nalidixic acid (NA) was added to a final concentration of 5 mg/l for 1 h at 37 °C. Culture aliquots (50 μl) were then transferred into the wells of an opaque white 96-well microtiter plate (Greiner bio-one) containing 50 μl of LB with 0.1% nonanal (final concentration 0.05%), dissolved in dimethylformamide (DMF), or a substrate-free control (LB with 2% DMF, final concentration 1%). The plate was incubated for 10 min at 37 °C and luminescence was measured at 37 °C as above.

Results

Initial characterization of the independent luxAB and luxCDE constructs Prior to examining the different combinations of *luxAB* (coding for the two luciferase subunits) and *luxCDE* (the reductase complex, in charge of aldehyde supply), each was tested independently for its functionality in the new configuration. The expression of either *luxAB* or *luxCDE* was put under the control of either an inducible

promoter, *sulA*, activated by genotoxicants such as nalidixic acid (NA) [25, 26], or of a synthetic constitutive promoter, CP38, that drives a moderate constant gene expression [22]. Four constructs were thus tested—*sulA::luxAB*, *sulA::luxCDE*, *CP38::luxAB*, and *CP38::luxCDE*.

To test the ability of the separated *luxAB* genes to drive synthesis of an active luciferase, strains harboring either of the *luxAB* plasmids were induced by NA and their bioluminescence was compared to that of non-induced cells, with nonanal added as an external substrate. As can be viewed in Fig. 1a, luminescence values in the absence of NA were low for both *luxAB* constructs. As expected, upon NA addition, luminescence of the inducible construct (*sulA::luxAB*) markedly increased, while that of the constitutive construct (*CP38::luxAB)* was unaffected.

Activity of the separated *luxCDE* was tested by co-expressing it with the *CP38::luxAB* construct, demonstrated above to drive constant luminescence regardless of the presence of NA (Fig. 1a). In this manner we could test the ability of *luxCDE* to generate aldehyde for the bioluminescence reaction. Cells co-expressing *CP38::luxAB* with either the constitutive *CP38::luxCDE* or the inducible *sulA::luxCDE* were challenged with NA, and bioluminescence was compared to that of a non-induced control. The results presented in Fig. 1b demonstrate that the *luxCDE* constructs can supply the aldehyde necessary for luciferase activity; in the case of *CP38::luxCDE* this was expressed as a constant activity irrespective of the presence of the inducer, while *sulA::luxCDE* was induced upon NA exposure.

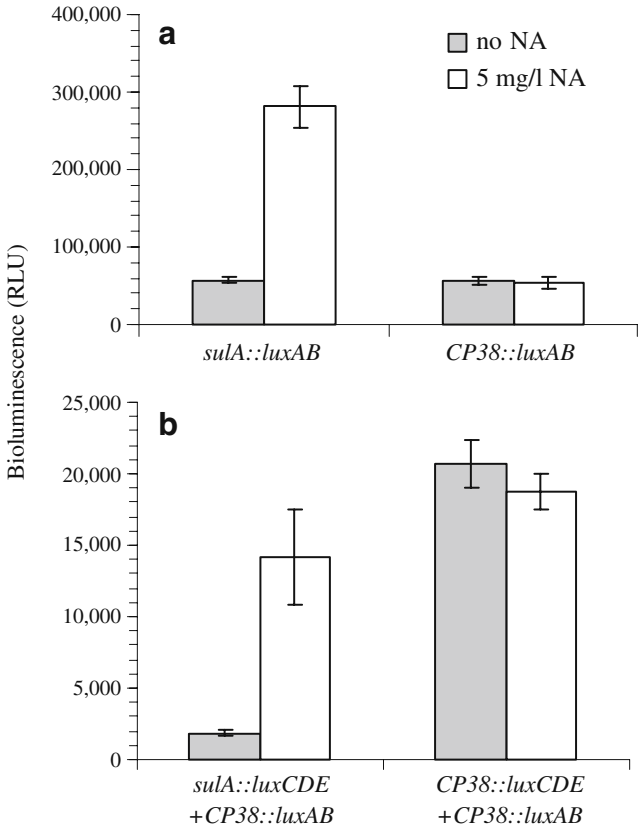

Fig. 1 Testing the independent activity of *luxAB* and *luxCDE*. Effect of induction by nalidixic acid (NA, 5 mg/l) was tested on: **a** activation of *luxAB*, driven either by an inducible (*sulA*) or a constitutive (CP38) promoter. Aldehyde (nonanal) was added to a final concentration of 0.05%; **b** activation of *luxCDE*, driven either by an inducible (*sulA*) or a constitutive (CP38) promoter, in conjunction with a constitutive expression of *luxAB*

Activity of the split sulA reporters in response to activation by NA Verification of the independent functionality of both *luxAB* and *luxCDE* now allowed the generation of a set of co-expressed constructs, together containing all five *lux* genes, with each of the two groups driven by either an inducible (ind) or a constitutive (const) promoter. Therefore, for each inducible promoter, three new combinations were constructed: $luxAB_{ind}/luxCDE_{ind}$, $luxAB_{ind}/luxCDE_{const}$, and $luxAB_{const}/luxCDE_{ind}$. The combinations containing *sulA* as the inducible promoter (strains SY101-103, Table 3) were tested for their bioluminescent response to NA, and the results were compared to those obtained with the original *sulA::luxCDABE* construct (strain SY100). As is clear from the light emission kinetics in response to NA (5 mg/l) presented in Fig. 2a, the bioluminescent response of strain SY102 (*sulA::luxAB*, *CP38::luxCDE*) was markedly stronger and faster than that of the other strains. Activity of the two other split reporters, while lower than that of SY102, was nevertheless significantly higher than the complete inducible *luxCDABE* construct. The same patterns of response of the wild type and split *sulA* sensors were observed in the presence of all NA concentrations tested, as shown in Fig. 2b, which displays the difference between luminescence intensity in the absence and presence of the inducer (ΔRLU) after 60 min of induction as a function of NA concentration. Fig. 2c displays the same responses, calculated as the ratio of the luminescence of the induced sample to that of the non-induced control (response ratio). This value, the magnitude of which depends not only on the actual response but also on background luminescence levels (see values in Table 3), presents a different pattern in which strain SY102 exhibits the weakest response, as a result of its high background luminescence.

Response of the split sulA reporters to other genotoxic chemicals To verify that the pattern of activities exhibited by the four *sulA* strains in response to NA was of a general applicability, two additional DNA damaging agents were tested: mitomycin C (MMC), that causes DNA intra-strand cross-linking, and hydrogen peroxide (H_2O_2) that generates DNA base damage and strand breaks [27]. As in the presence of NA (Fig. 2b), strain SY102 displayed a dramatically stronger and faster response both to MMC

Table 3 Reporter strains tested in this study, their background light emission, EC_{200} and detection thresholds

Strain/Toxicant	Promoter driving *lux*			RLU background[a]	EC_{200}[b,c,d]	Detection threshold[c,d,e]
	CDABE	*AB*	*CDE*			
Nalidixic acid (NA)						
SY100	sulA			281	2.69	1.1
SY101		sulA	sulA	1,197	1.63	0.55
SY102		sulA	CP38	9,652	2.72	1.57
SY103		CP38	sulA	1,113	1.42	0.56
SY104		sulA	CP25	10,570	2.3	1.5
Mitomycin C (MMC)						
SY100	sulA			400	424.8	92.5
SY101		sulA	sulA	774	140.0	28.0
SY102		sulA	CP38	9,408	206.0	60.0
SY103		CP38	sulA	719	155.0	57.7
H_2O_2						
SY100	sulA			418	11.4	11.1
SY101		sulA	sulA	803	6.55	4.0
SY102		sulA	CP38	15,423	5.7	2.0
SY103		CP38	sulA	834	5.6	5.6
Cadmium chloride						
SY200	katG			551	27.2	7.42
SY201		katG	katG	97	9.8	10.5
SY202		katG	CP38	11,589	38.0	38.0
SY203		CP38	katG	73	8.1	3.16
Sodium arsenite						
SY300	arsR			1,496	ND[f]	ND[f]
SY301		arsR	arsR	142	0.9	0.59
SY302		arsR	CP38	5,144	0.18	0.04
SY303		CP38	arsR	166	1.01	0.11
Paraquat						
SY400	micF			400	80.6	31.8
SY401		micF	micF	774	40.0	6.0
SY402		micF	CP38	9,408	30.0	15.5
SY403		CP38	micF	719	28.5	0.84

[a] Bioluminescence background in the absence of inducer, at time zero

[b] Effective toxicant concentration causing a twofold increase in response ratio

[c] Values calculated are either after 30 min (cadmium chloride) or 60 min (NA, MMC, H_2O_2, sodium arsenite and paraquat) of induction

[d] All regression coefficients of the linear region of the dose response curve are above 0.95

[e] Inducer concentration at which luminescence surpassed a level that was three standard deviations above the background

[f] The reporter strain was not induced, thus the values could not be calculated

(Fig. 3a) and to H_2O_2 (Fig. 3c), at all inducer concentrations tested. As before, the increase in activity calculated as the response ratio exhibited a more complex pattern. SY102 response ratios were in both cases superior to that of the non-split strain (SY100), and either equal or somewhat inferior to the other two split strains.

Response of other reporter strains To test whether the positive effect of splitting the *luxCDABE* reporting element is a general phenomenon that could be applicable to other bioluminescent sensors, we have applied the same principle to three additional stress-responsive promoters. These were *katG*, responsive to peroxide stress [28] and to selected metals [29], *arsR* that is induced by heavy metals [30], and *micF* that is activated by cationic antimicrobial peptides and superoxide-mediated oxidative damage [31]. These promoters were fused to either *luxAB* or *luxCDE*, and different combinations with the constitutive promoter CP38 were

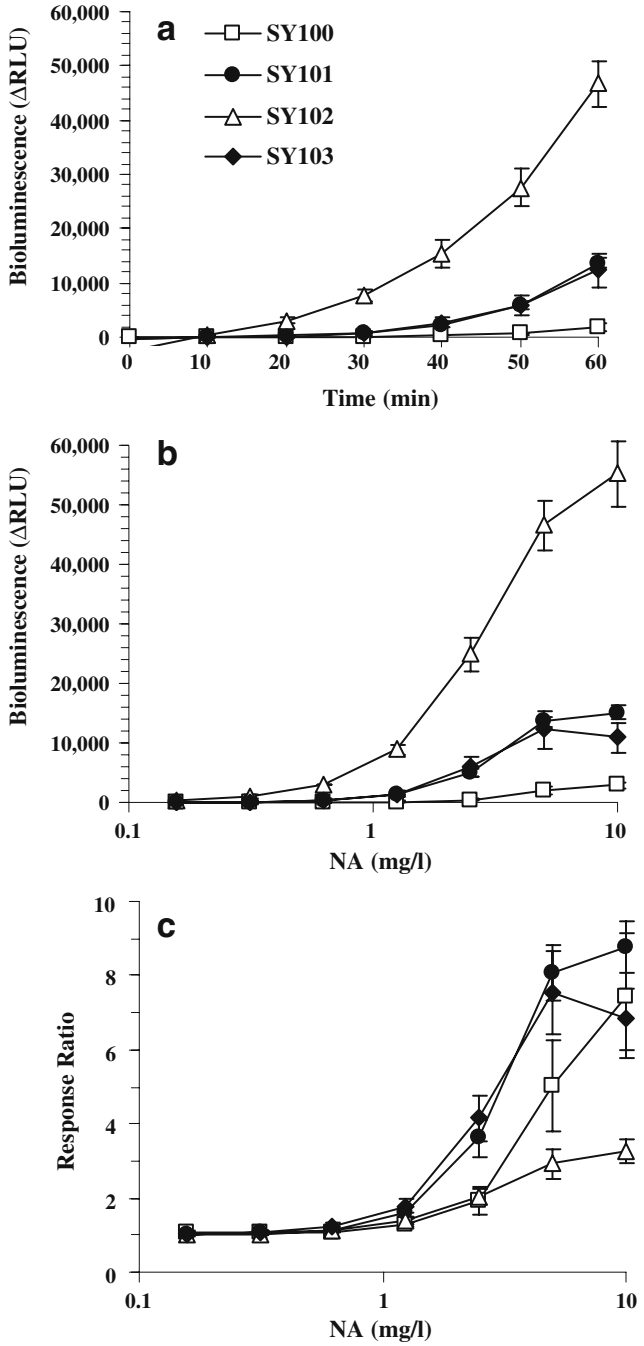

Fig. 2 Bioluminescence response to NA of *E. coli* harboring split or contiguous *sulA::lux* fusions. **a** bioluminescence response kinetics to a single NA concentration (5 mg/l); **b** ΔRLU (the difference in luminescence intensity in the presence and absence of the inducer) of whole and split *sulA::lux* fusions as a function of NA concentration (60 min exposure); **c** response ratio (the ratio of the luminescence of the induced sample to that of the non-induced control) of whole and split *sulA::lux* fusions as a function of NA concentration (60 min exposure). SY100, *sulA::luxCDABE*; SY101, *sulA::luxAB+sulA::luxCDE*; SY102, *sulA::luxAB+CP38::luxCDE*; SY103, *CP38::luxAB+sulA::luxCDE*

generated (Table 3). Each group was challenged with a specific inducer: *katG* (strains SY200–203, Fig. 4a, b) with cadmium chloride, *arsR* (strains SY300–303, Fig. 4c, d) with sodium arsenite, and *micF* (strains SY400–403, Fig. 4e, f) with methyl viologen (paraquat), a redox cycling (superoxide generating) agent. The responses of all of these strains are presented in Fig. 4 either as ΔRLU values (A, C, E) or as response ratios (B, D, F). The phenomenon observed for *sulA* was repeated for the other three promoters: in all cases, the combination of an inducible *luxAB* and a constitutive *luxCDE* displayed much stronger responses to the presence of the toxicant. The most dramatic effect was observed for the *arsR* group, in which the contiguous sensor was not induced by any of the arsenite concentrations tested. As before, the pattern emerging from the response ratio calculations is non-uniform, and is greatly influenced by the background bioluminescence levels of each of the constructs.

Aldehyde availability limits bioluminescence output The obvious superiority of the $luxAB_{ind}$/$luxCDE_{const}$ combinations displayed in Figs. 2, 3 and 4 indicates that when the five *lux* genes are driven by a single promoter, aldehyde availability may be limiting, and that this limitation is alleviated when the *luxCDE* genes are driven by an independent constitutive promoter. To find out whether further enhancement of *luxCDE* expression, generating higher concentrations of available aldehyde, may affect the inducible bioluminescence, the moderate synthetic promoter CP38 driving *luxCDE* expression was replaced by a stronger one, CP25 [22]. The new *CP25::luxCDE* construct was co-expressed with the inducible *sulA:luxAB* construct (strain SY104), and its activity compared to the split sensor SY102 is displayed in Fig. 5a. Strain SY104 displayed a small but significant and consistent enhancement in signal intensity over strain SY102 at all NA concentrations (Fig. 5a), indicating that under the experimental conditions tested, CP38-driven aldehyde production was not saturating, and that an additional increase provided by a stronger promoter was beneficial to some extent.

To evaluate the degree by which the stronger promoter driving *luxCDE* in strain SY104 generates a level of aldehyde that is sufficiently high to allow optimal Lux activity, additional aldehyde (nonanal, 0.05%) was externally added after 1 h of induction with NA. DMF, the nonanal solvent, served as a control. The results in Fig. 5b clearly demonstrate that addition of nonanal to SY104 strain further enhanced bioluminescence, indicating that the aldehyde is not fully saturating even when the *luxCDE* complex is driven by a strong constitutive promoter.

Effect of operon splitting on detection sensitivity One of the obvious parameters by which biosensor performance is

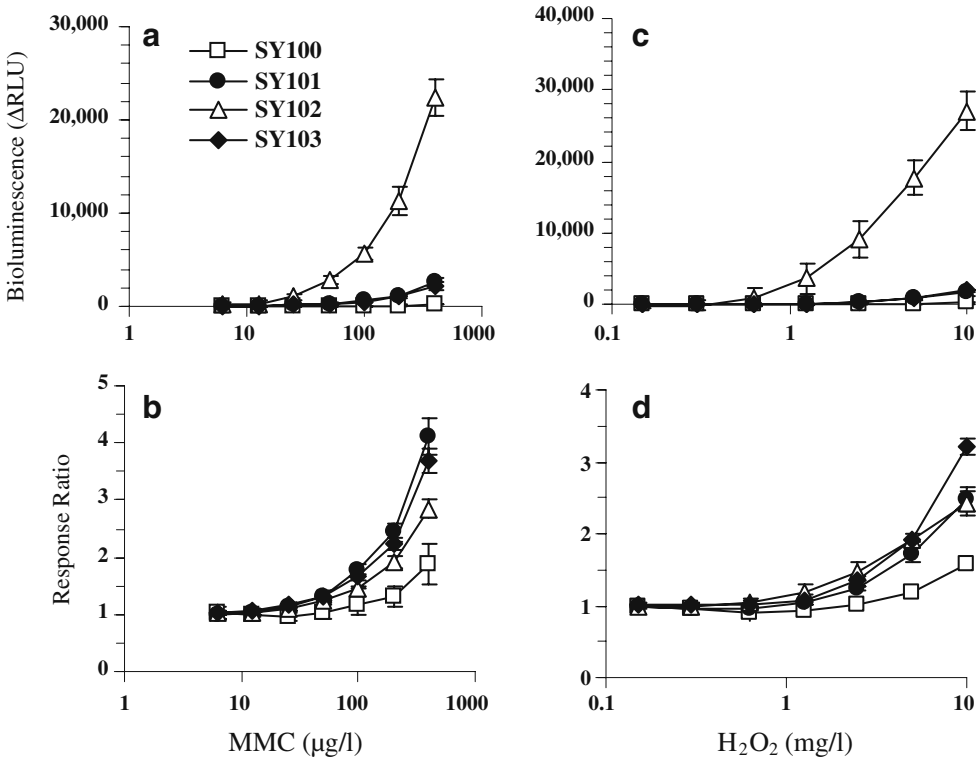

Fig. 3 Bioluminescence response (60 min exposure) of *E. coli* harboring split or whole *sulA::lux* fusions to MMC (panels **a**, **b**) and H_2O_2 (panels **c**, **d**). Data presented as ΔRLU (**a**, **c**) or as response ratios (**b**, **d**). SY100, *sulA::luxCDABE*; SY101, *sulA::luxAB+sulA::luxCDE*; SY102, *sulA::luxAB+CP38::luxCDE*; SY103, *CP38::luxAB+sulA::luxCDE*

evaluated is its detection limit: the lowest concentration of the target compound that can be reproducibly and reliably detected. In the present study, we have evaluated the sensitivity of split and contiguous *lux* strains to the various chemicals using two complementary approaches: (a) calculating an EC_{200} value [23, 24], denoting the sample concentration driving a twofold increase in activity over the non-induced control, with lower EC_{200} values reflecting a greater sensitivity and a lower detection threshold; and (b) by calculating the inducer concentration at which luminescence surpassed a level that was three standard deviations above the background [8]. The results of these calculations, generally in good agreement with each other, are listed for all strains in Table 3. In practically all cases, the contiguously expressed *luxCDABE* displayed higher EC_{200} and detection threshold values than the different split combinations, an indication that the *lux* splitting also endowed a beneficial effect on detection sensitivity. Nevertheless, strain SY102, clearly the leader in regard to intensity and speed of its inducible signal in the presence of the different toxicants, displayed a rather limited advantage in detection sensitivity, most likely due its generally higher background activity.

Discussion

The hypothesis at the basis of the study described in this communication was that it should be possible to rearrange the natural order of the genes in the *lux* operon, so that an improvement in its overall activity is obtained that will upgrade bacterial biosensor performance. We have demonstrated that splitting the native *luxCDABE* arrangement into two independent operational units, separately controlled by inducible and/or constitutive promoters, confers obvious advantages both in the speed of the response and its intensity, and, in most cases, also in detection sensitivity. The most dramatic effect of the split system was on signal strength, i.e., the ΔRLU values upon induction. In all inducible promoters and inducing chemicals tested in this study, the sensor that harbored an inducible *luxAB* combined with a constitutive *luxCDE* exhibited a luminescence response that was significantly faster and more intense in the presence of all toxicants' concentrations tested. As previously reported [32], when all five *lux* genes are transcribed together in *E. coli*, the availability of the aldehyde, the substrate for the bioluminescence reaction, is rate limiting. Indeed, controlling *luxCDE* expression by a stronger constitutive promoter (CP25) yielded a further increase in emission values upon toxicant exposure. This increase, however, was rather marginal considering the reported fivefold difference in expression magnitude of CP25 over CP38 [22]. It was therefore surprising that even under these conditions, an externally supplied nonanal enhanced bioluminescence levels up to sixfold (Fig. 5b) indicating that further enhancement of sensor output could potentially be achieved. Based on these data, it is not

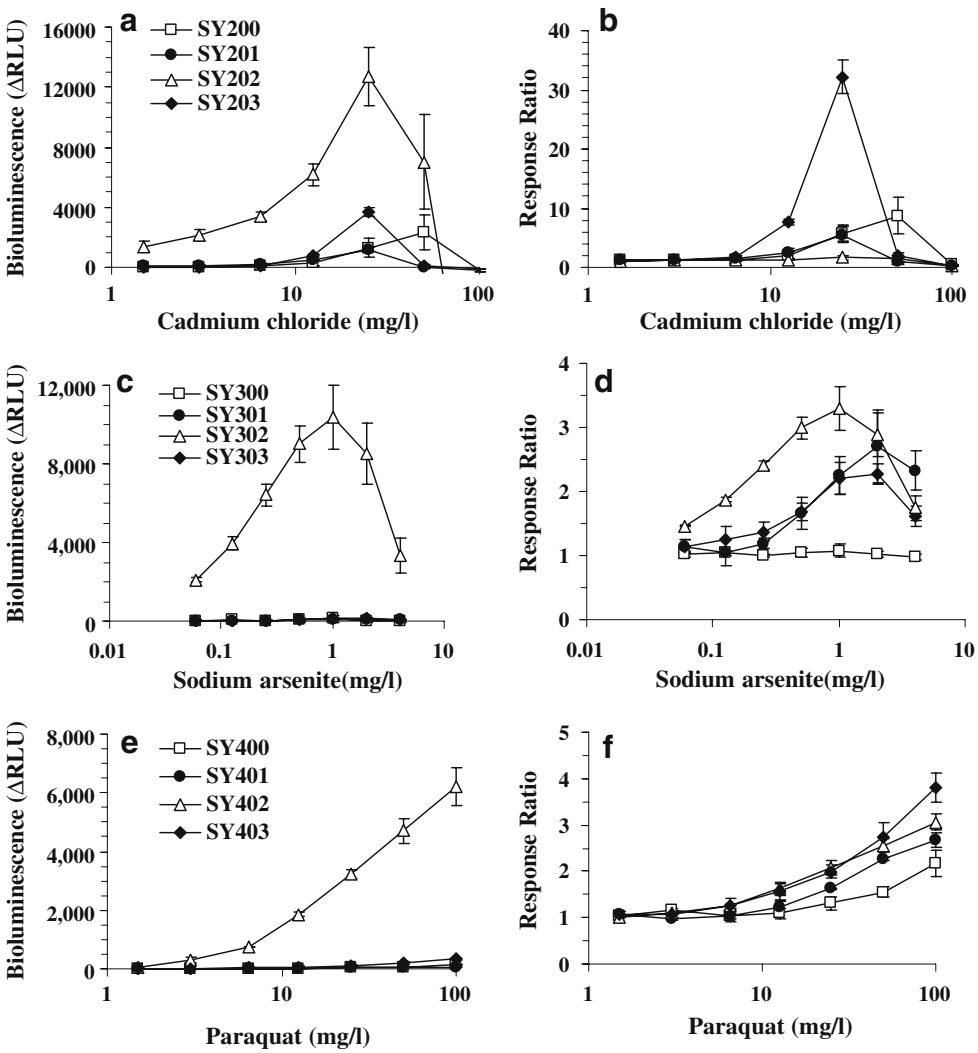

Fig. 4 Bioluminescence response of *katG::lux* fusions to $CdCl_2$ (30 min; **a**, **b**), *arsR::lux* fusions to Na arsenite (60 min; **c**, **d**) and of *micF::lux* fusions to paraquat (60 min; **e**, **f**). Data are presented as ΔRLU (**a**, **c**, **e**) or as response ratios (**b**, **d**, **f**). SY200, *katG::luxCDABE*; SY201, *katG::luxAB+katG::luxCDE*; SY202, *katG::luxAB+CP38::luxCDE*; SY203, *CP38::luxAB+katG::luxCDE*; SY300, *arsR::luxCDABE*; SY301, *arsR::luxAB+arsR::luxCDE*; SY302, *arsR::luxAB+CP38::luxCDE*; SY303, *CP38::luxAB+arsR::luxCDE*; SY400, *micF::luxCDABE*; SY401, *micF::luxAB+micF::luxCDE*; SY402, *micF::luxAB+CP38::luxCDE*; SY403, *CP38::luxAB+micF::luxCDE*

possible to predict whether the same phenomenon may be true also during natural bioluminescence in *P. luminescens*, the bacterium from which the *lux* genes used in this study were derived. Nevertheless, our conclusion is in agreement with the observation that Northern RNA analysis of *Vibrio harveyi luxCDABE* expressed in *E. coli* revealed that the mRNA present in largest amounts appeared to start in front of *luxA* or *luxD* and terminate after *luxB*. This indicates that the amount of *luxC* mRNA transcript, and subsequently probably the LuxC protein, is relatively low [33].

The two other split combinations, in which *luxCDE* was controlled by an inducible promoter, also performed better than the contiguous operon almost in all cases. Thus, while aldehyde availability appears to be the major bottleneck in the non-split system, the actual splitting also exerts a beneficial effect in that transcription and/or translation of short DNA segments may be faster and possibly more efficient than that of long ones. Recently, a mathematical model of the Lux system in *P. luminescens* was constructed using a set of coupled differential equations [34]. It was shown that control of the system lies mainly with the LuxC and LuxE enzymes, a conclusion that matches our results. However, that model predicted an optimal performance of a reporter system combining an inducible *luxCDE* and a constitutively expressed *luxAB*, and that a reverse configuration should perform poorly, a prediction that our results contradict. They also appear to contradict a mention of a faster response by a constitutive *luxAB*/inducible *luxCDE* combination from an unspecified source, referred to in a review by Philp et al. [35].

The most common quantitative parameter universally used for monitoring changes in gene expression is “fold induction”, derived by a division of an activity in one state by that in another. This approach, however, is fully justified only when the basal level of expression is the same under all conditions tested. In comparing activities that develop from different baselines, this calculation is problematic. Consider, for example, a case in which the expression of

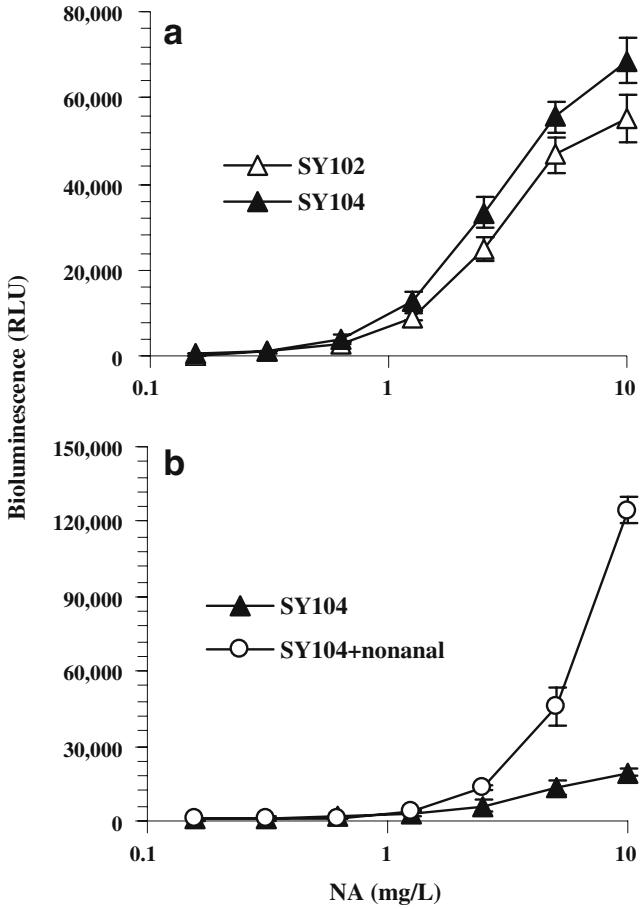

Fig. 5 Effects of enhanced aldehyde supply, either by (**a**) the stronger constitutive promoter CP25 (*filled upright triangle*, strain SY104), compared to the weaker constitutive promoter CP38 (*empty upright triangle*, strain SY102) or (**b**) by a combination of the strong promoter CP25 and externally added nonanal (*empty circle*) compared to the solvent DMF (*filled upright triangle*). Values denote the bioluminescence (ΔRLU) response to NA (60 min exposure). DMF 1% (solvent) or nonanal 0.05% were added to SY104 after 1 h of exposure to NA. SY102, *sulA::luxAB+CP38::luxCDE*; SY104, *sulA::luxAB+CP25::luxCDE*

one gene increases from 1 to 10 units, and that of a second one from 10 to 100 units. The "fold induction" approach would claim that both have demonstrated an equal tenfold enhancement, whereas in fact the latter increased by 90 units compared to the former's 9. We have therefore contended [8] that in cases such as ours, when baseline activities vary greatly (due, for example, to different promoters' strengths), the difference in activities rather than their ratio offers a truer representation of the extent of an induction. Thus, when the response ratio values of the split-*lux* sensors were compared to that of the contiguous one, there was no individual combination that exhibited consistently higher values. Although in all cases an improvement in response ratio was achieved, the effect was highly variable due to major differences in baseline expressions (Table 3). A very similar statement can be made concerning the detection sensitivity parameters used, since both calculations are based on the level of background activity. In all cases (except for strain SY201, Table 3), the combinations containing the inducible *luxCDE* exhibited improved detection thresholds compared to the contiguous operon; performance of the strains harboring a constitutive expression of *luxCDE* varied, displaying superior (*sulA*/MMC, *sulA*/H2O2, *arsR*/arsenite, *micF*/paraquat), inferior (*katG*/Cd) or similar (*sulA*/NA) sensitivity to that of the non-split combination. An extreme example was the response to arsenite of the *arsR* sensors, where the *luxCDABE*-harboring sensor was unaffected by all tested concentrations, whereas all three split sensors were induced by a very low concentration (detection threshold of 0.04 mg/l). Nevertheless, the pattern was not universally consistent and, once again, greatly dependent upon basal expression levels.

Of all reporter strains the activity of which was tested in this article, the ones exhibiting both the strongest raw response and the largest signal increase (ΔRLU) compared to the non-induced state were those combining an induced *luxAB* with a constant *luxCDE* expression; these were also the strains that were characterized by high background activity. Thus, in contrast to traditional attempts to improve whole-cell sensors' output by reducing background emission level, one should also consider that a high sensor background signal, indicating a stronger promoter activity, is not necessarily a disadvantage. In most likelihood, adopting such an approach will improve signal intensity and response speed, but will not have a major effect on response sensitivity. Improvement in the latter parameter will probably be more efficiently achieved by manipulation of the host cell (affecting membrane permeability, for example, or neutralizing repair mechanisms) rather than the actual promoter::reporter fusion.

While we have demonstrated the validity of our initial hypotheses using the responses of bacterial reporter cells to specific chemicals, it should be emphasized that the strength of sensors of this type is not in the high-sensitivity quantification of specific chemicals. For that purpose, both analytical chemical methodologies and biosensors specifically designed for the detection of individual targets [6, 7, 30, 36] are clearly preferable. The importance of stress-responsive sensors of the type described here is in their capacity to detect global biological effects such as toxicity, genotoxicity, or oxidative stress, exemplified here by $CdCl_2$, NA/MMC and hydrogen peroxide, respectively. Such effects can be detected neither by chemical analysis nor by high-specificity biosensors.

In conclusion, our initial predictions concerning the effect of splitting the *lux* on bioreporter performance were confirmed, as the need to induce only one component

clearly yielded a faster and stronger light emission. Dividing the five genes of *luxCDABE* operon into two smaller units allowed a more efficient transcription and/or translation that improved sensor performance: by all tested criteria, the split combinations were superior to the contiguous configuration.

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