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A novel *E. coli* biosensor for detecting aromatic aldehydes based on a responsive inducible archaeal promoter fused to the green fluorescent protein

Gabriella Fiorentino · Raffaele Ronca · Simonetta Bartolucci

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Abstract A whole-cell bacterial biosensor for measuring aqueous concentrations of aromatic aldehydes was developed. It is based on the *E. coli* BL21DE3(RIL) expressing the green fluorescent protein under the control of an alcohol dehydrogenase inducible promoter belonging to the archaeon *Sulfolobus solfataricus* (*Sso2536adh* promoter). Since it was previously reported that the BldR regulatory protein is the transcription factor required for aromatic aldehyde response in *S. solfataricus*, the gene encoding for the sensor protein BldR was co-expressed in the biosensor strain on a different compatible plasmid. Gel mobility shift assays showed that the purified recombinant protein can bind specifically to the *Sso2536adh* promoter. We demonstrated the ability of the archaeal promoter and the BldR transcription factor to operate in a bacterial context to drive active gene expression in a hybrid archaeal/eukaryal fusion. Furthermore, the *E. coli* BL21DE3(RIL) biosensor strain displayed a specific response and high sensitivity to the different aromatic aldehydes used, suggesting its potential low-cost application to environmentally relevant samples.

Keywords Reporter system · Heterologous expression · Detoxification · Transcriptional regulation · Archaea

G. Fiorentino (✉) · R. Ronca · S. Bartolucci
Dipartimento di Biologia Strutturale e Funzionale,
Università degli Studi di Napoli Federico II,
Complesso Universitario Monte S. Angelo,
Via Cinthia,
80126 Naples, Italy
e-mail: fiogabri@unina.it

Introduction

The pollution of soil and water caused by agriculture and industry or by accidental contamination with fuels, oils, solvents and heavy metals, is an issue of great global concern. Water-soluble aromatic components (e.g. benzene, toluene, ethylbenzene, and xylene) of petroleum products can adversely impact groundwater, depending on local biogeochemical conditions (Riccardi et al. 2008). Many of these contaminants should be readily biodegradable, but for reasons that are not completely understood, they often persist in the environment (Stiner and Halverson 2002). Of the aromatic hydrocarbons, polycyclic aromatic hydrocarbons can react with Cl atoms in water giving genotoxic aromatic aldehydes (Demir et al. 2008) as the main products (Wang et al. 2005; Sudareva and Chubarova 2006). As a result, there has been much focus on developing rapid and sensitive methods to detect and quantify such toxic species, and, eventually, degrade them (Kim et al. 2005).

Microorganisms are increasingly being used as specific devices for sensing biologically relevant concentrations of pollutants (Lei et al. 2006). These biosensors rely on analysis of the expression of a reporter gene that is controlled by a promoter responsive to a particular toxic compound; the extent of reporter gene expression mirrors the available concentration of a pollutant. The gene encoding for GFP from *Aequoria victoria* (Heim et al. 1994) is the most common reporter gene because it is stable, can be monitored in living cells and does not require any cofactor or substrate for in situ assessment of bioavailability (Cha et al. 1999; Liao et al. 2006; Miller et al. 2001; Stiner and Halverson 2002). To date, progress in this field is limited due to incomplete biological information regarding the molecular mechanism of degradation and

detoxification of environmental contaminants in many microbial communities (Zhao and Poh 2008).

Most organisms belonging to Archaea, the third domain of life, are well adapted to grow under extreme conditions such as extreme high or low temperature, high or low pH, ionic strength, and the presence of high concentrations of detergents or organic solvents (Wheelis et al. 1992). Archaea are an evolutionary mosaic, being more similar to eukaryotes with respect to their macromolecular machinery and more similar to bacteria in terms of metabolic systems and genome organization (van de Vossenberg et al. 1998; Verhees et al. 2003; Grabowski et al. 2003). For example, in the transcription process, while RNA polymerase and basal transcription factors are very similar to molecular components of eukaryal RNA polymerase II, transcriptional regulators strongly resemble bacterial ones (Geiduschek et al. 2005; Hirata et al. 2008).

Like all other living cells, Archaea possess a wide variety of finely regulated biochemical systems to protect themselves from environmental stress. In order to activate their defensive systems only under stress, they have developed sophisticated molecular systems to detect environmental hazardous compounds and they possess in their genomes regulatory sequences responsive to different stress agents (Pedone et al. 2004; Schelert et al. 2006; Limauro et al. 2006). Generally, the response to changes in the environment can be initiated by the binding of transcription factors to particular ligands, such as environmental signals (Wilkinson and Grove 2006). The bound complex has a different affinity for target regulatory sequences, resulting in differential access of the RNA polymerase to the promoters and hence differential expression of one or more (sets of) genes (Tropel and van der Meer 2004). These transcription factors could be exploited as specific sensing components for whole-cell biosensors containing target regulative sequences, and the extent of gene expression as a measure of the concentration of the toxic compound.

The thermophilic archaeon *S. solfataricus* responds to stress from aromatic compounds by increasing the expression of a recently characterized MarR-like operon (Fiorentino et al. 2007) and an alcohol dehydrogenase gene (*Sso2536adh*; Cannio et al. 1999, Fiorentino et al. 2003). The system involves the MarR family transcription factor BldR, which binds to its own promoter inducing autoactivation and increasing the co-expressed drug export permease level. BldR also binds to the *Sso2536* promoter, stimulating gene transcription, accumulation of the ADH enzyme and, hence, the enzyme-catalyzed conversion of the aldehydes to the less toxic alcohols (Fiorentino et al. 2007).

In this study, we describe the construction and characterization of a whole-cell biosensor for measuring aqueous concentrations of aromatic aldehydes. Although aromatic aldehydes are not recognized as priority pollutants, due to the reactivity of the aldehydic group and the recalcitrance of the aromatic ring, their potential harmfulness cannot be excluded.

We used a pUC28-derived vector system for *E. coli* to express the *egfp* reporter gene under the control of the inducible regulatory sequences of the *Sso2536adh* gene from *S. solfataricus*. Since aromatic compounds (particularly benzaldehyde) are the effectors for BldR-mediated activation, the gene encoding for the trans-acting BldR factor was cloned in the pET30a expression vector, *in vitro* characterized and heterologously co-expressed in the biosensor strain.

Materials and methods

Construction of the BldRHis expression vector

The gene *Sso1352* encoding for the BldR protein was amplified by PCR from *S. solfataricus* genomic DNA using the BldR forward primer (5′-GGTGGTGAAACATATG CAAAAAATAG-3′) and the BldR reverse primer (5′-TACATTGGTACTCGAGGTCCTTGGAG-3′), containing *Nde*I and *Xho*I restriction sites, respectively (underlined). The PCR product was digested with *Nde*I and *Xho*I and ligated to the *Nde*I/*Xho*I digested pET30a (Invitrogen) to obtain the BldRHis expression vector. Sequencing of the cloned fragment revealed identity to the original *S. solfataricus* sequence. The expression construct, pET30BldRHis, was transformed into BL21DE3(RIL) cells to obtain the *E. coli*-BldRHis strain.

Construction of the reporter system

The *egfp* coding sequence was recovered from the pEGFP-C3 vector (Clontech, Takara Bio Company) by a double digestion with *Nhe*I and *Xba*I restriction enzymes. The resulting fragment containing the *egfp* gene fused to the polylinker of the vector (approximately 0.8 kb) was cloned in the compatible *Xba*I site of a pUC28-derived plasmid (kind gift of Dr. R. Cannio, Aucelli et al. 2006) to obtain the promoterless pGFP vector. The orientation of the *egfp* gene in the recipient vector was determined by *Xho*I digestion.

To obtain the reporter plasmid containing the Sso2536*adh* regulatory sequences inserted upstream of the *egfp* gene, initially the *Ssadh* promoter was extracted by pUC18*Ssadhpr* (Cannio et al. 1999) after digestion with *Hind*III and *Nco*I, and fill-in reaction with the Klenow fragment of DNA polymerase I, giving a fragment of 328 bp. The blunt-end fragment was ligated into pGFP, linearized with *Nco*I and blunted with the Klenow enzyme. Both promoter orientations (5′–3′ or 3′–5′) relative to the

egfp gene were selected by *Bam*HI restriction analysis, giving the pAGFP and pA'GFP plasmids, respectively.

Construction of the biosensor strain

To create a biosensor able to detect aromatic aldehydes, the *E. coli*-BldRHis strain was made competent by treatment with calcium chloride. The cells were therefore transformed alternatively with pGFP or pAGFP, to give the sensor and the control strains, respectively, selectable on LB-agar plates containing both 50 μg/ml kanamycin and 100 μg/ml ampicillin.

BldRHis purification and expression analysis in the double transformants

A single colony of *E. coli* BL21DE3(RIL) bearing the pET30BldRHis plasmid was grown overnight in LB medium containing 50 μg/ml kanamycin and 50 μg/ml chloramphenicol, diluted 1:100 and incubated at 37 °C. BldRHis expression was induced by the addition of 0.5 mM IPTG when cells reached an A_{600}=1 OD and growth continued for 6 h. Cells were lysed by sonication in 50 mM Tris–HCl pH 7.0 in the presence of 1 mM PMSF in an ultrasonic liquid processor (Heat system Ultrasonic Inc.). The lysate was centrifuged at 30,000×*g* for 60 min and thermoprecipitated by heating at 70 °C for 5 min. The heat-treated supernatant was subjected to affinity (His-Trap HP, GE Healthcare) and cation exchange (Resource S, GE Healthcare) chromatographies. Protein homogeneity was estimated by SDS/PAGE (12.5% (*w*/*v*) gels), and DNA binding to the *Sso2536adh* promoter was assayed in a gel shift experiment performed as already described (Fiorentino et al. 2007).

In order to analyze BldRHis expression in the double transformants, the cell extracts, prepared as described above, were separated on a 12.5% SDS/PAGE, stained with Coomassie brilliant blue or blotted onto a polyvinylidene fluoride (PVDF) membrane. Blots were probed with rabbit polyclonal antibodies raised against BldR (Fiorentino et al. 2007). Antigen–antibody interactions were detected with horseradish peroxidase-conjugated secondary antibodies and the ECL kit (Amersham Bioscience).

Cell growth and induction of eGFP fluorescence

Single colonies of *E. coli* BL21DE3(RIL) transformed with pAGFP, pA'GFP and pGFP were grown overnight in Luria–Bertani (LB) medium supplemented with 100 μg/ml ampicillin. The overnight cultures were diluted in fresh LB medium with antibiotics at 0.06 OD_{600} and grown in the presence or absence of 1 mM benzaldehyde until they reached the exponential growth phase (0.4 OD). At this time, cells were collected by centrifugation and, if not immediately used, cell pellets stored at −80 °C.

Single colonies of the double transformants pAGFP-pET30BldRHis and pGFP-pET30BldRHis were also grown overnight in LB medium containing 50 μg/ml kanamycin and 100 μg/ml ampicillin. The overnight cultures were diluted in fresh LB medium with antibiotics at 0.06 OD_{600} and grown up to 0.3 OD_{600} in the presence or absence of 1 mM benzaldehyde. At this time, *BldRHis* expression was induced by the addition of 0.5 mM IPTG and growth was prolonged for 3 h, harvesting 10 ml aliquots at 30-min intervals by centrifugation at 4,000 rpm for 10 min at 4 °C. In other cases, single colonies of the double transformants were grown as described above and, immediately after addition of IPTG, challenged with various concentrations of benzaldehyde (from 0.1 μM to 75 mM) and recovered after 1 h. For each concentration, at least three independent growths were performed and monitored by measuring the optical density at 600 nm.

In a typical experiment performed to analyze the response of the biosensor to different aromatic aldehydes (cinnamaldehyde, veratrylaldehyde, and salicylaldehyde), the double transformants were grown up to 0.3 OD_{600}. Then *BldRHis* and *egfp* expression were induced by the addition of 0.5 mM IPTG and the aromatic compound at the desired concentration, respectively; growth was prolonged for 1 h before recovering cell aliquots. A non-aromatic aldehyde (butyraldehyde) and an aromatic non-aldehyde (benzoic acid) were also tested. For each investigation, at least three independent growths were performed.

Measurement of eGFP fluorescence in cultures

The activity of the biosensor was estimated by the measurement of the eGFP fluorescence of the single or double transformants grown in LB medium with or without the different chemicals at different concentrations. In order to analyze the fluorescence intensity produced, the recombinant cells (from 10 ml cultures), harvested at the desired growth time, were washed with 10 ml of phosphate-buffered saline (PBS) and resuspended in 1 ml of 50 mM Tris–HCl pH 7.0. Fluorescence intensity was measured in a Jasco Fluorimetry fitted with a 470±5 nm excitation and a 509±5 nm emission. *E. coli* BL21DE3(RIL) and *E. coli* BL21DE3(RIL) carrying the pGFP plasmid, as well as the double transformant pGFP-pET30BldRHis, were used as the negative controls and subjected to identical treatments.

The specific fluorescence intensity (SFI) was defined as the fluorescence intensity measured by the instrument divided by the optical density at 600 nm measured at each time point (Liao et al. 2006). For each concentration of the stressor, at least three independent experiments were performed.

Measurement of *egfp* transcription by RT-PCR

RNA was prepared from *E. coli* double transformants pAGFP-pET30BldRHis and pGFP-pET30BldRHis grown in the presence or absence of 1 mM benzaldehyde and in which *BldRHis* expression was induced by addition of IPTG. Approximately 5×10^8 cells (concentration determined assuming that 1 OD_{600} corresponds to 10^9 cells) were harvested by centrifugation. Total RNA was prepared using the RNAeasy Mini-Kit (Qiagen). Reverse transcriptase (RT) reactions were carried out on 1 μg of total RNA using SuperScript™ III One-Step RT-PCR System with Platinum® Taq (Invitrogen) following the manufacturer's protocol and the specific primers: eGFPpr 5′ CATGGT CCTGCTGGAGTT 3′, eGFPrv 5′ CTAGATCCGGTG GATCCC 3′.

As the negative controls, RT-PCR reactions were performed without RNA and PCR amplifications were carried out on total RNAs with *Taq* DNA polymerase (Promega). The PCR products were analyzed on a 1.5% agarose gel quantified using a Gel-Doc imager and the Quantity One software (Bio-Rad).

Analysis of eGFP fluorescence by fluorescence microscopy

As a response to the presence of aromatic aldehydes, eGFP expression was visualized and analyzed by fluorescence microscopy on a confocal microscope set-up for fluorescence (Olympus U-RFL-T); images were taken with a charged-couple device camera. All photomicrographs were taken using a constant exposure time (2.5 s). After growing the cells in the desired conditions, approximately 5×10^8 cells were recovered by centrifugation, washed with PBS, fixed in 4% paraformaldehyde and again washed twice with PBS. Finally, they were resuspended in 200 μl of PBS and 10-μl aliquots mounted on microscope slides for analysis. All slides were kept in the dark immediately after preparation. They were visualized using a 470±20-nm excitation filter and a 507±20-nm emission filter.

Results

Cloning, expression, and purification of recombinant BldRHis

To obtain BldRHis, *Sso1352* was amplified by PCR from *S. solfataricus* genomic DNA and cloned into pET30c(+) between the *Nco*I and *Xho*I sites, giving the pET30BldRHis plasmid. The gene proved highly expressed after IPTG induction in BL21DE3(RIL) *E. coli* and gave a recombinant protein in soluble form as a fusion with a six-histidine tag at the C-terminus. The recombinant protein was purified by heating the cell extract at 75 °C for 10 min and loading the thermostable supernatant onto a His-Trap HP affinity column followed by cation exchange chromatography. The SDS-PAGE of the final preparation revealed a single band at 16.5±1 kDa. The pure protein was tested by EMSA to assess its capacity to bind the Sso2536*adh* promoter (Sso2536*adhpr*, Fig. 1) site-specifically. The results showed that BldRHis was able to bind to the target DNA with a behavior comparable to the recombinant BldR expressed without the His-tag sequence (Fiorentino et al. 2007).

Description of the bacterial biosensor

The inducible *Sso2536adh* promoter of *S. solfataricus* (Cannio et al. 1999; Contursi et al. 2003) was cloned in two opposite orientations into a pUC28-derived vector (Aucelli et al. 2006) upstream of the *egfp* gene, yielding the 5′–3′ *Sso2536adhegfp* and 3′–5′ *Sso2536adhegfp* fusions, designated pAGFP and pA'GFP. Essential features of the junction region of the vectors are summarized in Fig. 2 (a,b,c). The analysis of *Sso2536adh* promoter to predict bacterial-like

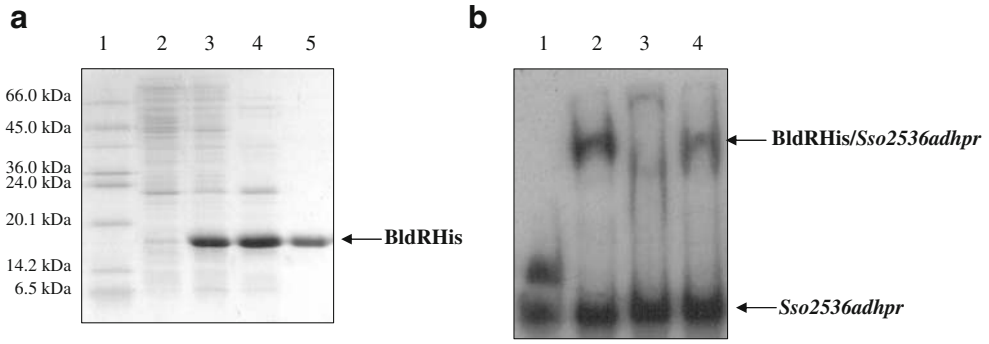

Fig. 1 Purification and gel mobility shift assay of recombinant BldRHis. **a** Coomassie brilliant blue-stained SDS-PAGE of the protein samples from *E. coli* transformed with *pET30BldRHis* after each purification step. *Lane 1* molecular weight markers, *lane 2* crude extract, *lane 3* heat-treated cell extract, *lane 4* fraction from His-Trap chromatography, *lane 5* fraction from Resource S chromatography. **b** Binding of recombinant BldRHis to the −152/+10 promoter region of *Sso2536*. *Lane 1 Sso2536adh* promoter, *lane 2* 2 μM BldRHis, *lane 3* 2 μM BldRHis and unlabeled specific competitor, *lane 4* 2 μM BldRHis and unlabelled unspecific competitor

regulatory sequences (http://www.fruitfly.org/seq_tools/promoter.html) revealed two putative sequences matching the bacterial −10 and −35 consensus and a putative transcriptional start site located only few nucleotides downstream of that mapped in *S. solfataricus* cells (Fig. 2d, Cannio et al. 1999).

A control vector, pGFP, was also constructed by inserting the *egfp* sequence into the pUC28-derived vector (Table 1). These plasmids were transferred in *E. coli* and recombinant cells analyzed for fluorescence. *E. coli* pAGFP emitted higher amounts of fluorescence when compared to its corresponding negative controls, pA'GFP and pGFP (data not shown). Intriguingly, this indicated the ability of the *Sso2536adh* promoter to drive an active gene expression in a hybrid archaeal/eukaryal fusion and in a bacterial host.

Since the expression of the SsADH was positively regulated by the addition of benzaldehyde in *S. solfataricus* (Cannio et al. 1999), and the *Sso2536adh* promoter was proved able to drive the inducible expression of a heterologous gene in an archaeal expression vector (Contursi et al. 2003), *E. coli* pAGFP cells were exposed to different concentrations of the compound in order to assess whether the promoter also retained the same features in the bacterial host. Nevertheless, at no concentrations tested could induction be observed.

In a previous study, it was demonstrated that the transcription factor BldR increased the expression of

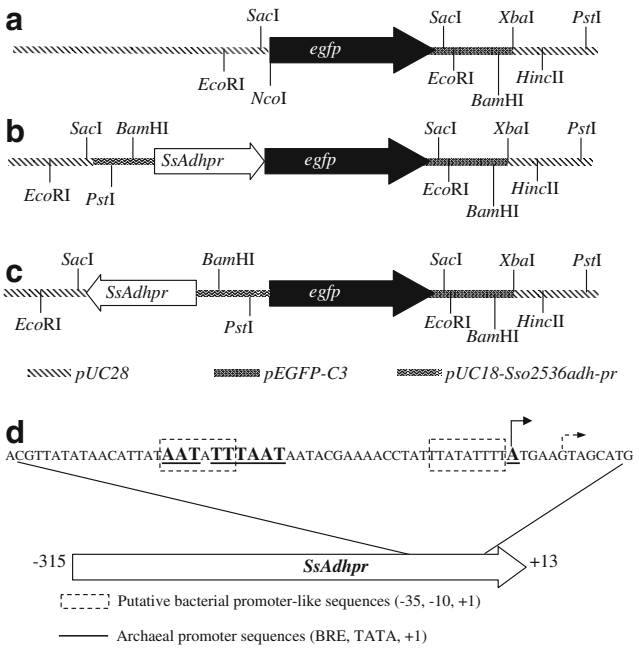

Fig. 2 Schematic representation of the plasmids produced and the *Sso2536adh* promoter sequence. *A* pGFP; *B* pAGFP; *C* pA'GFP; *D* Structure of the *Sso2536adh* promoter. Basal archaeal regulatory sequences (BRE and TATA) are in *bold* and *underlined*. The transcriptional start site is indicated by the *bold arrow*. *Dashed boxes* frame putative bacterial −10 and −35 consensus sequences

Table 1 Plasmids and bacterial strains employed in this study

	Comments
Plasmids	
pET30BldRHis	Expression vector coding for His-tagged BldR
pUC28-derived	pUC28 derived vector (Aucelli et al. 2006)
pGFP	pUC28-derived containing the *egfp* gene cloned in the *Xba*I site
pAGFP	Sensor construct: pGFP vector containing the *Sso2536adh* promoter upstream the *egfp* gene in the correct orientation
pA'GFP	Control construct: pGFP vector with the *Sso2536adh* promoter upstream the *egfp* gene in the inverted orientation
Strains	
E. coli BL21DE3 (RIL)	Expression host
E. coli-BldRHis	*E. coli* BL21DE3(RIL) transformed with pET30BldRHis
E. coli pGFP	*E. coli* BL21DE3 (RIL) transformed with pGFP
E. coli pAGFP	*E. coli* BL21DE3 (RIL) transformed with pAGFP
E. coli pA'GFP	*E. coli* BL21DE3 (RIL) transformed with pA'GFP
Control strain	*E. coli* BL21DE3(RIL) transformed with pGFP and pET30BldRHis
Biosensor strain	*E. coli* BL21DE3(RIL) transformed with pAGFP and pET30BldRHis

Sso2536adh by binding to regulatory sequences on its promoter and that DNA recognition was stimulated by protein–benzaldehyde interaction (Fiorentino et al. 2007). Since this could also be true for the transcription of the reporter gene, the sensitivity of the proposed biosensor would appear critically dependent on the expression of the protein.

For this reason, *E. coli*-BldRHis competent cells were transformed with pAGFP and pGFP plasmids, and recombinants selected on kanamycin–ampicillin plates. The double transformant containing pET30BldRHis and pAGFP was termed as the *biosensor strain* while the negative control (pET30BldRHis-pGFP double transformant) was called *control strain* (Table 1).

Because the activity of the biosensor relied on the expression of BldR, at first we demonstrated BldrHis expression by western blot analysis of the crude extracts prepared from the double transformants exposed to IPTG for 1 h (not shown). Then, for the analysis of the gene expression, the biosensor and the control strains were grown in the presence and absence of 1 mM benzaldehyde and eGFP expression evaluated by fluorescence microscopy. Representative green fluorescent images are shown (Fig. 3 a,b,c).

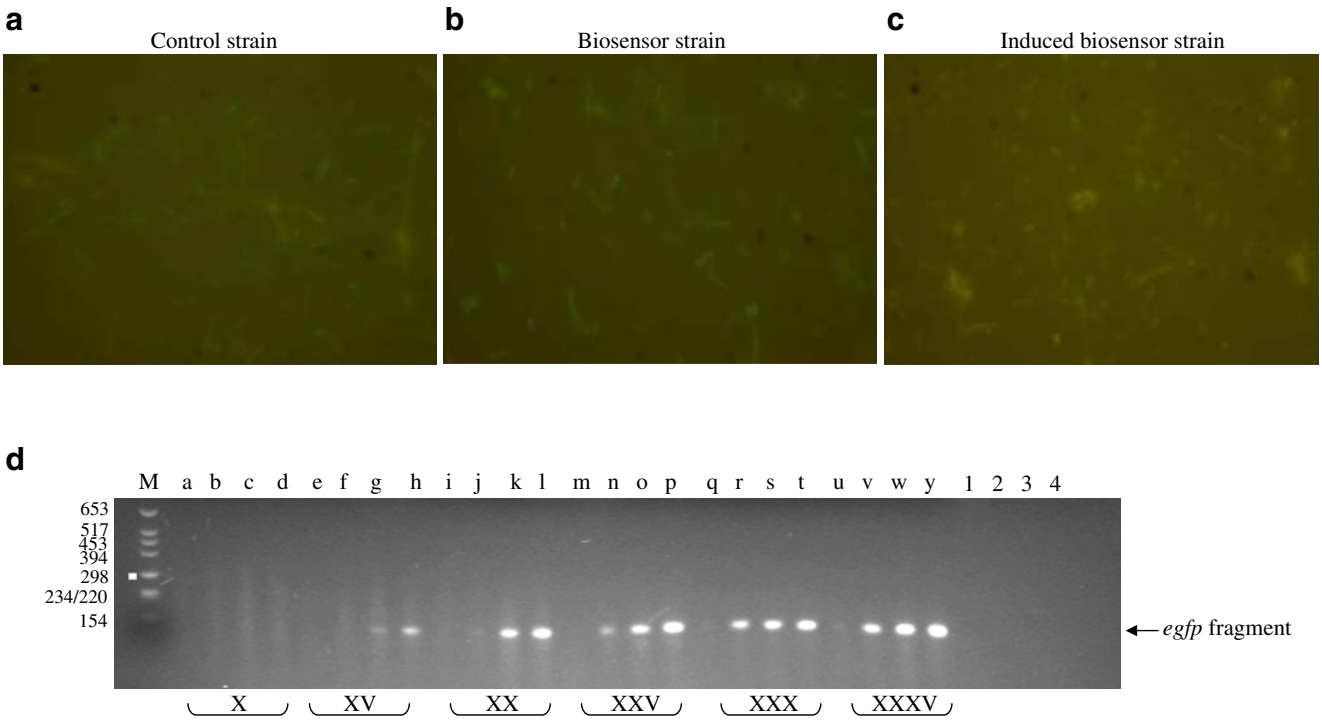

Fig. 3 Photomicrographs of cells of the control (**a**) and biosensor strain grown in the absence (**b**) and in the presence (**c**) of 1 mM benzaldehyde. **d** Agarose gel electrophoresis of the PCR (*lanes 1*, *2*, *3*, *4*) and RT-PCR products. *Lanes a*, *e*, *i*, *m*, *q*, *u*, *1* RNAs from *E. coli*-BldRHis. *Lanes b*, *f*, *j*, *n*, *r*, *v*, *2* RNAs from the control strain. *Lanes c*, *g*, *k*, *o*, *w*, *3* RNAs from the biosensor strain. *Lanes d*, *h*, *l*, *p*, *t*, *y*, *4* RNAs from the biosensor strain grown in the presence of 1 mM benzaldehyde

RT-PCR analysis was performed to detect *egfp* transcript levels in both the control and the biosensor strain. *egfp* specific transcript became already detectable at the 15th cycle of amplification in the biosensor strain, but only at the 25th in the control strain (Fig. 3d). Interestingly, the amount of the e*gfp* cDNA was appreciably higher in the biosensor strain grown in the presence of benzaldehyde than in non-induced cells; indeed, it was almost ten-fold higher at the 15th amplification cycle.

Taken together, these data prove that BldR is able to interact with the *Sso2536adh* promoter and that this interaction drives both a basal and a benzaldehyde-mediated expression of the reporter *egfp* gene.

Time-dependent induction of eGFP with benzaldehyde

In order to establish the best time intervals to measure the response of the biosensor, time-dependent induction of the bacterial sensor in response to benzaldehyde, as well as time-dependent basal expression was determined. A single colony was grown up to 0.3 OD_{600}, then IPTG to express *BldRHis* in the presence or not of the toxic compound (1 mM) was added and the specific fluorescence intensity (SFI) at 30-min intervals was measured. The same experiment was also performed with the control strain grown as described in the absence of benzaldehyde. eGFP induction showed a time dependence in the biosensor strain; the highest SFI was measured 30 min after IPTG addition, and then the response diminished before remaining stable in the following 3 h. Nevertheless, the fluorescence values measured in the treated strain were always significantly higher than those of the

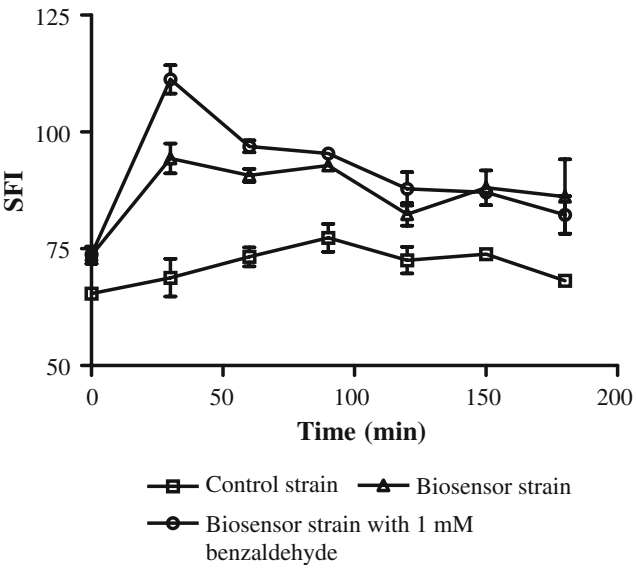

Fig. 4 Time-dependent induction of eGFP. The Specific Fluorescence Intensity (SFI) was determined in growing cultures of the biosensor (grown in the presence and absence of 1 mM benzaldehyde) and control strains. At time point 0, cells have OD_{600} of 0.3. Data are the mean of three independent experiments

untreated strain up to 2 h after induction. The similar trend observed both when analyzing basal expression and in response to the inducer appeared in line with the ability of BldR to activate transcription also in the absence of the drug. By contrast, the background fluorescence exhibited by the control strain did not change significantly during the incubation period (Fig. 4). These results suggest that 1 h after IPTG induction, the fluorescence measured could be directly related to the level of low but stable GFP expression.

Dose-dependent induction of eGFP with benzaldehyde

To establish the effect of different benzaldehyde concentrations on biosensor strain viability, the single clones were grown up to 0.3 OD_{600}, *BldRHis* was induced by addition of 0.5 mM IPTG, and then benzaldehyde added in a range from 1 μM up to 75 mM. Growth was monitored by measuring OD_{600} at 30-min intervals. The benzaldehyde had an inhibitory effect on *E. coli* cell growth with full inhibition at 15 mM (Fig. 5a).

To evaluate the dose–response relationship of the biosensor, both the biosensor and the control strains were treated with benzaldehyde as described above, and the eGFP expression analyzed 1 h after induction. At this time, if reinoculated in fresh medium, cells retained their growth capability, suggesting a still active metabolism. Furthermore, from the previous experiment it was established that this was the proper time to detect stable

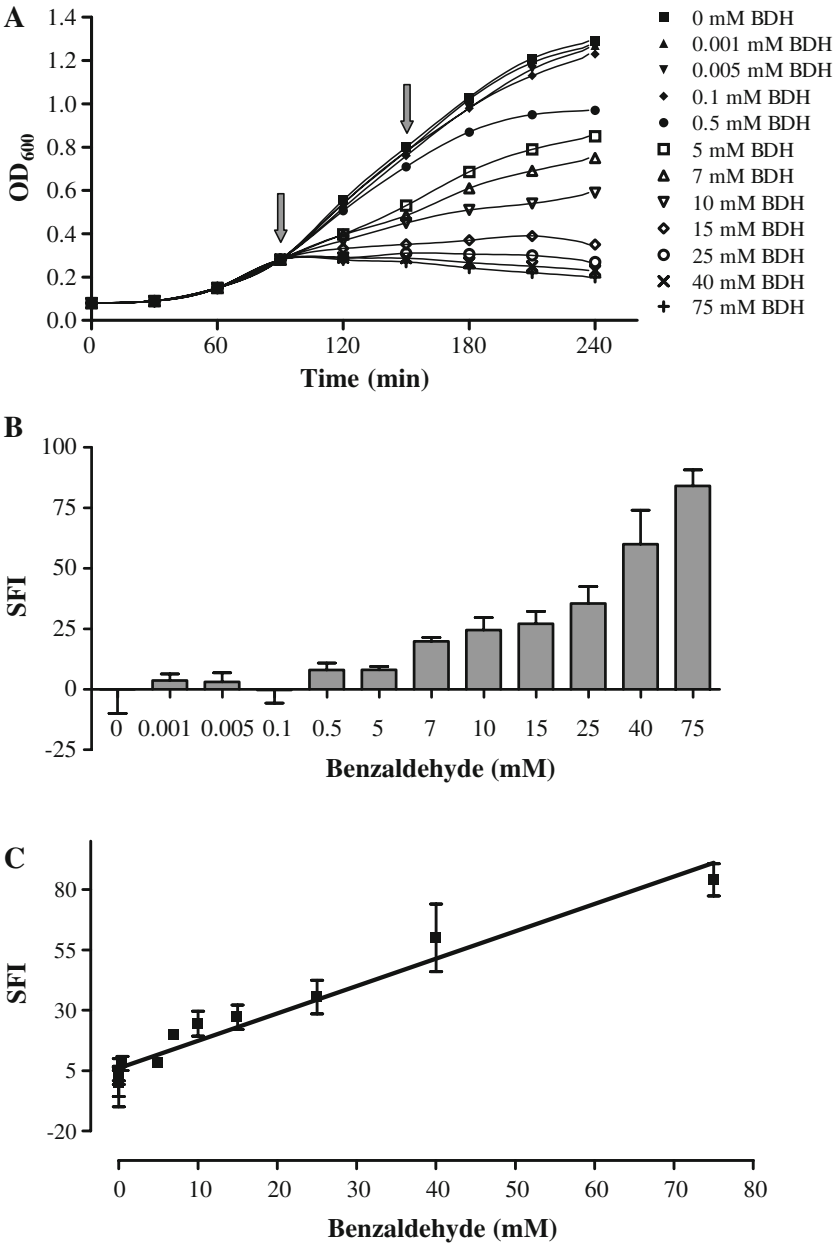

Fig. 5 **a** Effect of different concentrations of benzaldehyde on biosensor growth. *Arrows* indicate times of IPTG and drug addition, and of cell recovery (1 h), respectively. **b** Dose-dependent induction of eGFP by benzaldehyde. **c** Linear plot of the data shown in **b**. SFI was measured in the cells after growth in the conditions described in "Materials and methods". The values are obtained by subtracting the SFI measured in the biosensor strain in the absence of drug treatment. Data are the mean of three independent experiments

GFP fluorescence levels. The SFI profiles of the biosensor cells clearly showed that increasing benzaldehyde concentrations produced increasing fluorescence intensities, while this effect could not be observed in the control strain. The dose-dependent induction trend is evident from Fig. 5b where the SFI values reported have been subtracted from those measured in the absence of induction. Furthermore, analysis of the data also evidenced that in the range of concentrations tested, the response was linear (Fig. 5c).

Induction of eGFP with other aromatic aldehydes

To find out whether other aromatic aldehydes affected the induction of the biosensor, growth curves were first constructed by growing single clones up to 0.3 OD_{600}, then adding 0.5 mM IPTG, the different aromatic aldehydes (cinnamaldehyde, veratrylaldehyde, and salicylaldehyde) at different concentrations and measuring OD_{600} at 30-min intervals. Since these compounds were known to affect the Sso2536 gene expression in *S. solfataricus* (Fiorentino et al. 2007), they were chosen in a preliminary analysis to examine their activity also in the bacterial host. For the same reason, butyraldehyde and benzoic acid were considered as the negative controls.

All the aldehydes employed in this study inhibited the growth of both the biosensor and the control strain, which did not show differences in growth. The degree of inhibition increased as the stressor concentration rose with complete inhibition observed at 25 mM for cinnamaldehyde, 35 mM for veratrylaldehyde, 15 mM for salicylaldehyde, 25 mM for butyraldehyde, and 15 mM for benzoic acid.

The dose–response relationship of the biosensor was investigated in the strain by measuring eGFP expression in different conditions; the results shown in Fig. 6 were obtained by subtracting the SFI values obtained from biosensor cells grown with the drugs with those of non-treated cells. It resulted that, with the exception of veratrylaldehyde, the stressors affected the induction of eGFP in the biosensor to different degrees of inducibility and specificity; a two-fold induction was observed with salicylaldehyde. Butyraldehyde and benzoic acid had no effect on the induction of the biosensor (not shown). This observation is in line with previous findings, that is, BldR is unable to interact either with non-aromatic aldehydes, or aromatic acids (Fiorentino et al. 2007).

Taken together, these results demonstrate that the biosensor shows a linear response in the millimolar range of aromatic aldehydes and is able to discriminate among different compounds. Notably, increased eGFP expression was also revealed by microscopy, which showed higher fluorescence in the fixed cells (Fig. 7).

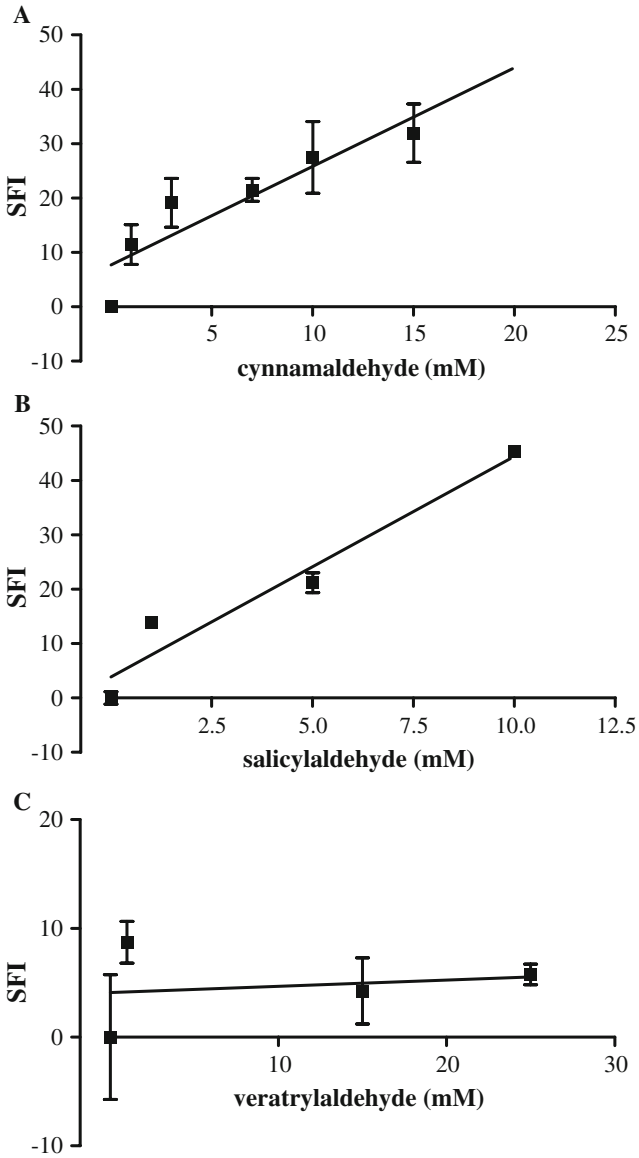

Fig. 6 Induction of eGFP by aromatic aldehydes. SFI was measured in the cells after growth in the conditions described in "Materials and methods". The values are obtained by subtracting the SFI measured in the biosensor strain under basal conditions. Data are the mean of three independent experiments. **a** cinnamaldehyde; **b** salicylaldehyde; **c** veratrylaldehyde

Discussion

This paper describes the construction and preliminary characterization of an eGFP-based biosensor for the measurement of water-dissolved concentrations of aromatic aldehydes. The biosensor was based on constructing an *E. coli* BL21DE3 strain containing a hybrid transcriptional fusion between an archaeal promoter responsive to aromatic aldehydes, the *S. solfataricus* alcohol dehydrogenase promoter (*Sso2536adh*, Fiorentino et al. 2003) and the *egfp*

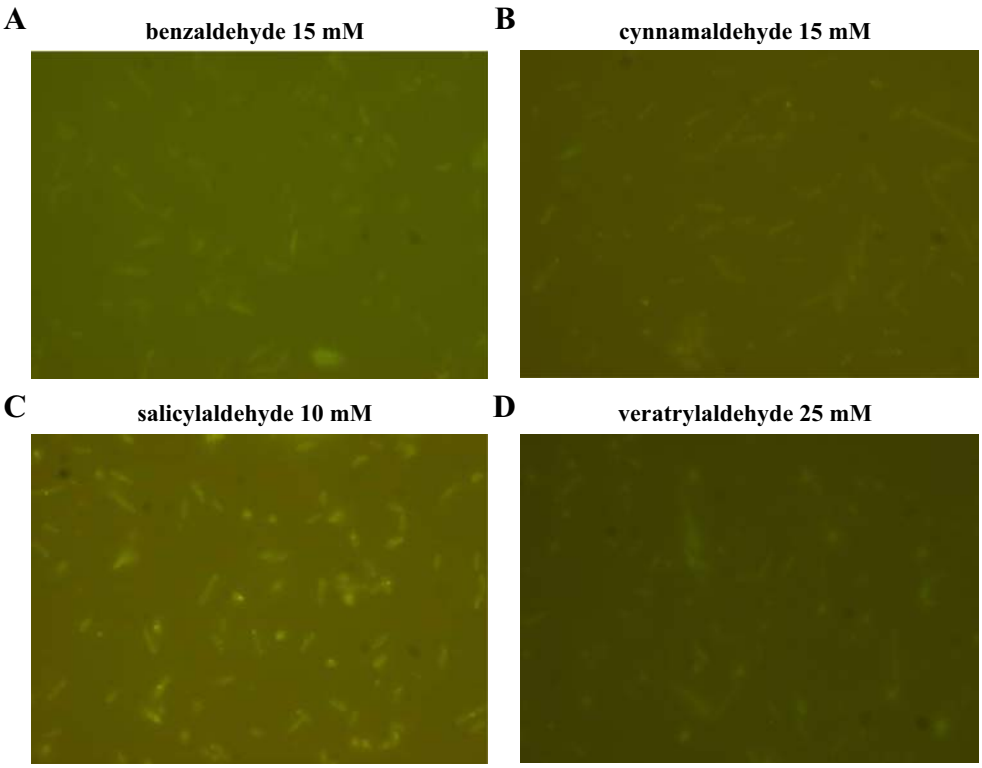

Fig. 7 Photomicrographs of the biosensor strain exposed for 1 h to different aromatic aldehydes. Images were captured for 2.5 s using a cooled charge coupled device (CCD) camera. **a** Treatment with 25 mM benzaldehyde; **b** 15 mM cinnamaldehyde; **c** 10 mM salicylaldehyde; **d** 25 mM veratrylaldehyde

gene, and the gene for the sensor protein BldR (Fiorentino et al. 2007). These elements are carried on two different compatible plasmids.

As a first step, three pUc28-derived plasmids, conferring resistance to ampicillin, were constructed containing the *egfp* coding sequence under the control of no regulatory sequences (pGFP), and the archaeal promoter cloned in the same orientation as *egfp* (pAGFP), or in the opposite one (pA'GFP). Fluorescence levels observed in the recombinant *E. coli* cells indicated that the archaeal promoter sequence was able to guarantee an efficient expression of a functional fluorescent eGFP also in the absence of inducers; a significant basal activity of the *Sso2536adh* promoter had also been detected *in vivo* (Fiorentino et al. 2007). Hence, our results highlighted the appealing possibility of using such hybrid genetic systems to carry out studies on archaeal gene expression in *E. coli*. However, it cannot be excluded that also bacterial-like regulatory sequences predicted in the *Sso2536adh* promoter could contribute to the background levels of GFP.

In *S. solfataricus* cells, the BldR protein is the positive regulator of *Sso2536adh*, responsible for gene activation after stress by aromatic aldehydes; to set-up an inducible system, specifically responding to aromatic aldehydes, the co-expression in *E. coli* of the BldR transcription factor proved crucial. The protein was expressed with a His-tag at the C-terminus and analyzed *in vitro* to verify the maintenance of its specific DNA binding properties, such as the ability to recognize specific palindromes in the *Sso2536adh* promoter. The BldR-dependent activity of the *Sso2536adh* promoter was examined in the double transformants, giving *in vivo* proof that the aromatic aldehyde was the environmental signal/ligand for BldR; upon drug addition, at least a two-fold increase in the *egfp* expression could be observed by comparing fluorescence of photomicrographs of the induced and non-induced biosensor cells. To our knowledge, this is the first evidence of the capability of an archaeal protein to trans-activate transcription *in vivo* in a bacterial context.

Analysis of GFP expression at different times, aimed at setting up the best time intervals to measure the response of the biosensor indicated that the response could be measured with reliability within 1 h of the induction of the sensor BldR protein. Indeed, in our system, this was the time required to obtain maximum specific fluorescence intensity and, hence, presumably, proper folding of GFP (Heim et al. 1995); after that, fluorescence decreased slightly before stabilizing in the next three hours, both in induced and non-induced biosensor. The unusual GFP instability at increasing times could be related to the growth phase of the cells and consequent synthesis of compounds which might interfere with the reporter gene expression.

Intriguingly, after 1 h from benzaldehyde addition, GFP fluorescence levels increased linearly with the concentration of the hazardous compound even when it was challenged at values inhibitory for cell growth. Besides

proving the response of the biosensor, this result also highlighted that such a shock did not affect cell viability, protein synthesis or GFP stability.

Additionally, since *Sso2536adh* expression was demonstrated to be sensitive to cinnamaldehyde, salicylaldehyde, and veratrylaldehyde (Fiorentino et al. 2007), the effect of these compounds on the biosensor response was also analyzed. The relevant basal expression, due to promoter activity in the absence of inducers (also confirmed by transcriptional analysis), was subtracted when the effect of different concentrations on the response of the biosensor was assessed. The system specifically and significantly responded to levels of benzaldehyde, cinnamaldehyde, and salicylaldehyde in a millimolar range. No significant response was measured towards the veratrylaldehyde. The absence of response to veratrylaldehyde together with its lower cytotoxicity could suggest the activation of endogenous catabolic genes (Prieto et al. 2004).

In conclusion, we constructed the first whole-cell biosensor based on hybrid genetic fusions of DNA sequences from evolutionary distant organisms and opened the way for new strategies in the design of hybrid biosensors. Furthermore, our results show that the whole-cell biosensor would appear a useful tool for the rapid measurement of related aromatic aldehydes in contaminated sites. However, further improvements are required to verify measurements in the lower range of concentrations, as well as in the presence of other chemicals besides inducer compounds.

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