Selection of *Escherichia coli* heat shock promoters toward their application as stress probes

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ABSTRACT

The mechanism of heat shock response of *Escherichia coli* can be explored to program novel biological functions. In this study, the strongest heat shock promoters were identified by microarray experiments conducted at different temperatures (37 °C and 45 °C, 5 min). The promoters of the genes *ibpA*, *dnaK* and *fxsA* were selected and validated by RT-qPCR. These promoters were used to construct and characterize stress probes using green fluorescence protein (GFP). Cellular stress levels were evaluated in experiments conducted at different shock temperatures during several exposure times. It was concluded that the strength of the promoter is not the only relevant factor in the construction of an efficient stress probe. Furthermore, it was found to be crucial to test and optimize the ribosome binding site (RBS) in order to obtain translational efficiency that balances the transcription levels previously verified by microarrays and RT-qPCR. These heat shock promoters can be used to trigger *in situ* gene expression of newly constructed biosynthetic pathways.

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1. Introduction

The heat shock response (HSR) is an essential and universal mechanism to protect cells (Lindquist, 1986). This mechanism is induced by a variety of stress conditions including physicochemical factors such as heat shock, complex metabolic processes and metabolically harmful substances (Arsène et al., 2000; Zhao et al., 2005) like heavy metals (Lindquist and Craig, 1988). The stress factors increase the level of denatured proteins, resulting in a biological activity change that can be damaging. In *Escherichia coli* exposed to heat shock conditions, there is an increase of the σ^{32} transcription factor (Grossman et al., 1984) that allows RNA polymerase to recognize the heat shock gene promoters. Consequently, the expression of heat shock proteins (HSP) increases (Lindquist, 1986; Zhao et al., 2005). Most HSP are chaperones and proteases (Arsène et al., 2000). Chaperones aid misfolded and unfolded proteins to fold correctly. Proteases degrade the proteins that

chaperones cannot fold correctly, thus ensuring that problems created by stress conditions are minimized (Madigan et al., 2009). The *E. coli* HSR mechanism is, as in other organisms, very complex. When temperature increases up to 42 °C, a cascade of events with several key players is initiated. Despite several scientific efforts (El-Samad et al., 2005; Genevaux et al., 2007; Georgopoulos, 2006; Guisbert et al., 2004; Ito and Akiyama, 2005; Janaszak et al., 2007, 2009; Yura and Nakanigashi, 1999), some steps of this process have still not been completely elucidated.

In the past decade numerous studies have been carried out using DNA microarrays to unravel the HSR of *E. coli* (Nonaka et al., 2006; Rasouly et al., 2009; Richmond et al., 1999; Wade et al., 2006; Zhao et al., 2005). These studies evaluated different control and shock temperatures, exposure times and gene expression induction methods (temperature increase, σ^{32} transcription factor increase, IPTG) leading to the identification of several repressed and overexpressed heat shock related genes. Three of the most highly expressed heat shock genes are *ibpB*, *ibpA* and *dnaK* (Nonaka et al., 2006; Rasouly et al., 2009; Richmond et al., 1999; Wade et al., 2006; Zhao et al., 2005). However, gene expression and induction ratios obtained in these studies proved to be difficult to compare, mainly due to the different parameters tested.

The heat shock promoters can be exploited for designing biological systems where a temperature shift can be used to trigger the expression of a particular gene or set of genes and to build new biological functions and complex artificial systems that do not occur in nature (Khalil and Collins, 2010; Rodrigues and Kluskens, 2011). Induction methods like temperature shift are safe, easy to implement and administer (culture handling and contamination risks are minimized) and less expensive (Valdez-Cruz et al., 2010). For instance, the HSR in *E. coli* has been studied with a special focus on its potential use as a biosensor (transcriptional biosensing). For that purpose, the HSP promoters (*dnaK*, *grpE*, lon, *clpB*, *ibpAB*, *clpPX*, *fxsA*) have been fused to several reporter genes: *lux* (Ben-Israel et al., 1998; Dyk et al., 1994; Sagi et al., 2003), *gfp* (Aertsen et al., 2004; Cha et al., 1999; Nemecek et al., 2008; Sagi et al., 2003) and *luc* (Kraft et al., 2007) that allowed detecting the activation of the HSR by the fast occurrence of light. These biosensors enabled the detection of pollution by metals, solvents and other chemicals. Furthermore, they also helped to identify HSP promoters activated by different types of stress and the overload exerted in *E. coli* by the production of recombinant proteins. The HSP promoters can further have a significant role in another important field of synthetic biology, namely in engineering bacteria and viruses for therapeutic applications (Shankar and Pillai, 2011), like vaccines (Garmory et al., 2003) and gene delivery vectors (Drabner and Guzman, 2001). Besides sensing environmental pollution, it is feasible to program bacteria to sense the tumor environment and selectively release a drug to kill cancer cells (Anderson et al., 2006; Shankar and Pillai, 2011).

This study aims to identify the strongest heat shock promoters in *E. coli* to be used in synthetic biology approaches to reprogram organisms and trigger the expression of novel biomolecular components, networks and pathways. The promoter's strength was evaluated using microarray data and was further validated by quantitative RT-PCR (RT-qPCR) and by the construction and characterization of stress probes.

2. Materials and methods

2.1. Media and strains

E. coli K-12 MG1655 (The Coli Genetic Stock Center – CGSC#: 6300, New Haven, CT, USA) was grown in LB medium (tryptone 1% (w/v), yeast extract 0.5% (w/v), sodium chloride 1% (w/v)) at 37 °C/30 °C and 200 rpm in Erlenmeyer flasks and in a 2 L stirred tank bioreactor (Autoclavable Benchtop Fermenter Type RALF, Bioengineering, AG, Wald, Switzerland) (Section 2.3). Pre-inocula were prepared from frozen glycerol stocks and incubated overnight (~12 h) in LB at 37 °C/30 °C and 200 rpm. Both Erlenmeyer flasks and bioreactor were inoculated with 1% of inoculum. Biomass was monitored in a spectrophotometer at OD_{600nm}. The cell concentrations at which the heat shock should be applied were determined using biomass calibration curves. Samples were collected over time for RNA extraction or fluorescence measurements.

2.2. Heat shock probes construction

GFP (mut3b) was amplified by PCR from pSB1A2-GFP (Cormack et al., 1996). The promoters were amplified from *E. coli* gDNA extracted using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Q5® Hot Start High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA) was used to amplify GFP and all the promoters used. The heat shock promoter sequences were obtained from the NCBI (The National Center for Biotechnology Information) database. The RBS was calculated using the software RBS Calculator v1.1 (Salis, 2011). GFP and all the promoters were cloned using restriction enzymes and DNA T4 ligase (NEB, Ipswich, MA, USA). GFP was cloned in MCS1 of pETduet (Novagen, Darmstadt, Germany), between the *Bam*HI and *Asc*I site. After cloning GFP, the T7 promoter was replaced by each of the three heat shock promoters (selected after microarrays data treatment) that were cloned between the *Sph*I and *Bam*HI site. The *Sac*I site was added downstream of the *Sph*I site to aid colony screening. *E. coli* ElectroMAX™ DH10B competent cells (*F⁻ mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*)7697 *galU galK* λ⁻ *rpsL nupG*) (Invitrogen/Life Technologies, Carlsbad, CA, USA) were transformed with the ligation mixture. Colonies were screened by plasmid digestion and *E. coli* K12 MG1655 DE3 competent cells were transformed with the plasmid containing the gene or promoter of interest. Primers (Sigma–Aldrich, St. Louis, MO, USA) and restriction enzyme sites are listed in Table 1.

2.3. Heat shock experiments

Erlenmeyer flasks were placed in an incubator at 37 °C and 200 rpm. Bacterial growth was monitored by optical density ($OD_{600\,nm}$). Heat shock was applied at a cell concentration of 5–6×10^8 cells per mL, a concentration obtained after approximately 7.5 h of growth. Control flasks were kept at 37 °C, while the ones corresponding to the heat shock assay were immersed in a water bath at 45 °C for 5 min. Samples were taken before heat shock and after 5 min of heat shock. These experiments were conducted sixfold (two for microarray analysis and four for RT-qPCR analysis).

In order to fully characterize the transcriptional profiles of the heat shock genes, two experiments were conducted in a 2 L bioreactor (1 L of LB medium) at 200 rpm, 30% of dissolved oxygen, and at different control (30 °C and 37 °C) and heat shock temperatures (45 °C and 50 °C) during different exposure times and different heating rates (Fig. S1). A very high heat shock temperature (50 °C) was chosen to observe the differences during and after heat shock under more lethal conditions.

The experiments with the heat shock probes containing GFP were also performed in Erlenmeyer flasks and the heat shock was applied in a water bath at 45 and 50 °C for 5 or 10 min. In each experiment 5 flasks were permanently held at 30 or 37 °C (control) and 5 flasks were subjected to the corresponding

Table 1
Set of primers for PCR amplification used in GFP and heat shock promoters for stress probe construction (forward and reverse primers – fw and rev).

Primer name	Primer sequence (5′ > 3′)
GFPmut3b.fw	AAAAAGGATCCAATGCGTAAAGGAGAAGAACT
GFPmut3b.rev	AAAAAGGCGCGCCTTATTATTTGTATAGTTCATCCATGCC
ibpA_P_fw	AAAAAGCATGCGAGCTCAAAATAACATCATCATTACGTCG
ibpA_P_rev	AAAAAGGATCCTCCTTAATAGTTCGCTCGTACGATGTAAAAATGGGTCTG
dnaK_P_fw	AAAAAGCATGCGAGCTCATGCCTTGGCTGCGATT
dnaK_P_rev	AAAAAGGATCCTCCTCGTCTATTCCGACTCCTATTCGGTCATCATGTGGT
fxsA_P_fw	AAAAAGCATGCGAGCTCGAGGATTTCTACCGTAATCTG
fxsA_P_rev	AAAAAGGATCCTCCCGATCCGTCGTGCTTTCTTTAGCTTATATTGTGGTCATTAG

heat shock temperature for 5–10 min. Each culture flask contained one of the five following plasmids: T7promoter_pETduet (empty), T7promoter_pETduet_GFPmut3b, ibpApromoter_pETduet_GFPmut3b, dnaKpromoter_pETduet_GFPmut3b, fxsApromoter_pETduet_GFPmut3b. For the plasmids with a T7 promoter, 0.1 mM IPTG was added at induction time zero. Each experiment was conducted three times at different experimental conditions (heat shock temperature and/or duration).

2.4. RNA extraction and DNase treatment

All samples collected for microarrays and RT-qPCR analysis were immediately stabilized with the RNAprotect Bacteria Reagent (Qiagen, Germantown, MD, USA) and the pellets were maintained at −80 °C.

RNA extraction for microarray analysis was conducted using the RNeasy® Protect Bacteria Mini Kit (Qiagen, Germantown, MD, USA), following manufacturer's guidelines. The Qiagen RNase-free DNase Set (Qiagen, Germantown, MD, USA) was used to eliminate any DNA contamination from the samples.

RNA extraction for RT-qPCR analysis was performed by the phenol–chloroform method followed by ethanol precipitation, and then the addition of DEPC (Diethylpyrocarbonate) treated water (Khodursky et al., 2003). To eliminate any DNA contamination from these samples, the Turbo DNA-free™ Kit (Ambion/Applied BioSystems/Life Technologies, Carlsbad, CA, USA) was used according to manufacturer's guidelines.

RNA concentration and quality were determined using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, USA). RNA purity levels were estimated by the A_{260}/A_{280} and A_{260}/A_{230} ratios. Furthermore, RNA integrity was evaluated by running a 1.5% (w/v) agarose gel electrophoresis loaded with 5 μL of RNA sample.

2.5. Microarrays

Genetic expression levels of samples grown in Erlenmeyer flasks were analyzed by microarray experiments (two control samples – CA and CB – and two heat shock samples – TA and TB). Total RNA samples were sent to an Affymetrix core facility (Instituto Gulbenkian de Ciência, Oeiras, Portugal) where quality-control analysis was carried out using the Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). All microarray steps (cDNA synthesis with appropriate random primers (Invitrogen/Life Technologies, P/N 48190-011, Carlsbad, CA, USA), cDNA fragmentation and labeling through incorporation of biotinylated ribonucleotide analogs (ddUTP-biotin), hybridization to the GeneChip® E. coli Genome 2.0 Array (Affymetrix, Santa Clara, CA, USA), staining with streptavidin–phycoerythrin and scanning) were done according to Affymetrix protocols. The GeneChip® includes approximately 10,000 probe sets for all 20,366 genes present in four strains of E. coli, including the K12 MG1655 used in this study.

2.6. RT-qPCR

The primers used for specific amplification (Invitrogen) are described in Table 2 and were designed using the Primer 3 program (Rozen and Skaletsky, 2000). The size of all amplicons was between 100 and 105 base pairs. Reverse transcriptase (RT) reaction was performed in a MyCycler™ Personal Thermal Cycler (BioRad, Hercules, CA, USA) and the iScript™ Select cDNA Synthesis kit (BioRad, Hercules, CA, USA) was used according to manufacturer's instructions, except that the reaction volume used was 10 μL. The final RNA template concentration in the cDNA synthesis reaction was 0.05 μg/μL, and was calculated using the RNA concentrations as determined by NanoDrop. The reverse primers used in the RT

Table 2
Set of primers used in RT (reverse primers – rev) and qPCR amplification (forward and reverse primers – fw and rev).

| Gene name	Primer name	Primer Sequence (5′ > 3′)
dnaK	dnaK.fw dnaK.rev	AATCGAACTGTCTTCCGCTC- TTCGCACGAGTCACTTTGAT
ibpA	ibpA.fw ibpA.rev	ACAAAAAGAGCGCACCTATCT CAGGTTAGCACCACGAACA
fxsA	fxsA.fw fxsA.rev	CGGCGGAGATGATTAAAAGT CGGCGGCAATAAAAGTAGA
rrsA – 16S	16S.fw 16S.rev	TAGCGGTGAAATGCGTAGAG TAATCCTGTTTGCTCCCCAC

reaction are also described in Table 2. qPCR was used to evaluate the transcription levels of the heat shock genes that were selected from the microarray experiments. qPCR was carried out in a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA, USA) using SsoFast™ EvaGreen® Supermix (BioRad, Hercules, CA, USA) as specified by the manufacturer, except that the reaction volume used was 10 μL. Primer efficiency and correlation values (R^2) were determined by the CFX Manager Software (Biorad, Hercules, CA, USA). The cDNA dilution used in the qPCR experiments was 1/80. No-reverse transcriptase controls, in which RNA instead of cDNA was used, were included to evaluate the presence of any genomic DNA. Three and two technical replicates were performed for each biological replicate from flask and bioreactor heat shock experiments, respectively.

2.7. Microarrays data treatment

Analysis of the microarray data was conducted using three different software programs (Expression Console®, dChip and SAM – Significance Analysis of Microarrays). The Expression Console® software (Affymetrix, Santa Clara, CA, USA) was used to analyze the raw data from the four arrays (CA, CB, TA and TB) and assess the need for data pre-treatment (background correction, normalization and summarization). For data pre-treatment three different algorithms (RMA – Robust Multichip Average, PLIER – Probe Logarithmic Intensity Error Estimation and MAS5 – MicroArray Suite 5) were used. dChip software (Li and Wong, 2003) was used for data pre-treatment and to filter the genes in order to obtain the most expressed ones during heat shock conditions. In this case, the algorithm used for data pre-treatment was MBEI (Model Based Expression Index). For background correction the MM (MisMatch) probes were used to correct PM (Perfect Match) probes (PM/MM difference), and normalization was carried out using the invariant set method. Subsequently, data were filtered according to the name "MG1655," since the GeneChip® also includes three other pathogenic E. coli strains and several intergenic regions that are not relevant for the purpose of the current work. Fig. 1A illustrates the roadmap of the microarrays data treatment.

The genes were also filtered according to their dispersion coefficient in various samples ($\sigma/\bar{x}$) and to the percentage of presence of the gene in the four above-mentioned arrays. The ratio of standard deviation and mean of gene expression values across all samples should be greater than a certain threshold ($0.5 < \sigma/\bar{x} < 10$). The more variable a gene is across samples, the larger this ratio will be. Nevertheless, if a gene is mostly absent across samples, this ratio can be large due to a small mean. For these reasons, the gene should be present in more than 40% of the arrays (i.e. at least in two arrays). Following these procedures, 703 genes were found to verify the filtering criteria.

The filtered genes were used to compare the different samples and to identify the differentially expressed genes. These genes were selected according to their induction ratio ($(TA+TB)/(CA+CB) > 1.2$

(A)

(B)
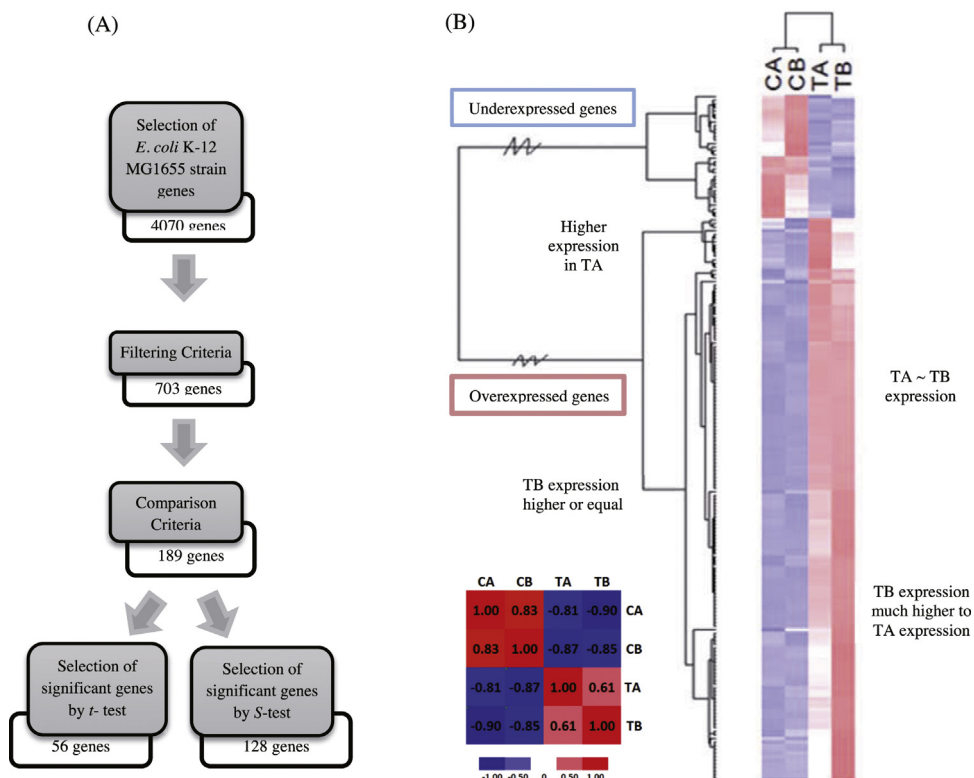
Fig. 1. Roadmap of the microarrays data treatment (A) and the Spearman correlation matrix and hierarchical clustering of 189 genes whose expression is significantly altered following heat shock induction (B). The red color in the hierarchical clustering represents expression levels above the average of gene expression in all samples, the white color represents an average expression, and the blue color represents expression levels below average. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

or $(CA + CB)/(TA + TB) > 1.2$) and absolute difference between the samples (($TA − CA + TB − CB)/2 > 100$ or ($CA − TA + CB − TB)/2 > 100$). The t-test for paired samples was performed for each gene identified and a p-value lower than 0.05 was assumed to obtain significant results (Cui and Churchill, 2003). The differentially expressed genes were grouped by hierarchical clustering strategies to evaluate the different expression profiles.

The genes identified by the dChip program as differentially expressed (189 genes) were subsequently tested in the SAM (Significance Analysis of Microarrays) program (S-test) (http://statweb.stanford.edu/~tibs/SAM/). The "two-class paired" test (two groups, and one-to-one pairing between a member of group A and a corresponding member of group B) was chosen with the following parameters: number of permutations = 5000 (at least 1000 permutations are recommended for accurate estimates) and $\Delta = 1$ (delta parameter is used to adjust the false positive rate, that can be decreased at the cost of missing true positives, or increased at the cost of obtaining more false negatives. The number of significant genes and the number of false significant genes will decrease with increasing delta values) (Dziuda, 2010).

2.8. RT-qPCR Data Treatment

RT-qPCR data were analyzed using the $2^{-\Delta\Delta C_T}$ method described by Livak and Schmittgen (2001). In this method, the amount of mRNA for a target gene is normalized to an endogenous control and relative to a calibrator or arbitrary control condition. The housekeeping *rrsA* gene, encoding the 16S RNA, was used as an endogenous control to normalize the differences in total RNA quantity (Dong et al., 2008; Kobayashi et al., 2006). RT-qPCR values were obtained for each gene for both control and heat shock conditions. Afterwards, data were normalized with the 16S C_T value and

subsequently the expression value of each gene under the control conditions (Livak and Schmittgen, 2001):

$$\Delta\Delta C_T = (C_{T,\text{heat-shock gene}} − C_{T,16S})_{\text{Heat Shock}}$$
$$− (C_{T,\text{heat-shock gene}} − C_{T,16S})_{\text{Control}} \quad (1)$$

Finally, the expression level for each heat shock gene ($E^{-\Delta C_T}$) and the induction ratio ($E^{-\Delta\Delta C_T}$) were calculated. E represents the real calculated efficiency value for each set of primers, which was very close to 100% (97.8–100.8%).

2.9. Fluorescence measurements and data treatment

The GFP assay was performed by measuring fluorescence intensity with a fluorescence spectrometer (Infinite 200® PRO, Tecan) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were performed in Nunc-Immuno™ MicroWell™ 96 well polystyrene black plates (Nunc/Thermo Scientific, Rochester, NY, USA) with 300 μL of culture sample resuspended in PBS. The software used in the analysis was the i-Control™ version 1.9.17.0 (Tecan, Morrisville, NC, USA). The specific fluorescence intensity (SFI) is calculated by dividing the raw fluorescence intensity by the optical density at 600 nm and allows comparison of samples with different optical density at time zero.

3. Results and discussion

3.1. Differential expression of HSP genes and identification of the strongest HSP promoters using microarrays

The microarray results of the heat shock experiments conducted in flasks at different temperatures (held constant at 37 °C

Table 3

Heat shock genes induction ratio, expression values (average of two biological replicates) and t and S test results. Genes marked with "*" represent the ones that are directly downstream of the σ^{32} factor dependent promoters. The list presented is ordered according to the average rate of induction (test/control) of the genes. q-Value was 0 for all the genes. In bold it is possible to observe the promoters chosen for posterior validation.

Gene code	Blattner number	Gene	Induction ratios	Expression values		t-test – dChip		S-test – SAM
			Test/control	Control	Test	t	p-Value	S
1762978_s_at	b3686	ibpB	224.76	22.55	5071.54	8.25	0.08	8.39
1769060_s_at	b3687	**ibpA***	55.68	106.34	5917.71	8.95	0.07	9.05
1765041_s_at	b1322	ycjF	23.82	62.22	1473.74	14.38	0.04	13.55
1768270_s_at	b1321	ycjX*	21.77	102.70	2230.95	24.86	0.03	24.53
1766244_s_at	b3635	mutM*	21.1	42.90	907.10	5.10	0.12	4.92
1762193_s_at	b3685	yidE	21.09	21.95	463.43	2.29	0.26	2.23
1765416_s_at	b1380	ldhA*	20.12	113.35	2282.35	15.42	0.04	14.83
1760394_s_at	b1594	mlc*	18.78	70.46	1324.72	3.83	0.16	3.76
1763446_s_at	b4140	**fxsA***	16.89	104.78	1742.74	95.73	0.01	134.67
1761819_s_at	b0473	htpG*	13.02	347.16	4469.62	27.21	0.02	28.35
1766513_s_at	b0492	ybbN*	11.15	215.08	2397.06	7.33	0.09	7.18
1761806_s_at	b3401	hsp33	9.89	138.25	1365.18	8.22	0.08	7.93
1760965_s_at	b1829	htpX*	9.82	379.64	3742.23	16.26	0.04	15.99
1763516_s_at	b1060	yceP*	9.44	224.83	2117.17	6.75	0.09	6.60
1764871_s_at	b2592	clpB*	9.17	548.94	4955.68	48.22	0.01	679.74
1761621_s_at	b3498	prlC*	9.17	176.66	1609.55	10.99	0.06	10.54
1763737_s_at	b3594	yibA*	9.01	41.95	376.25	3.78	0.16	3.54
1761422_s_at	b3400	yrfH*	8.66	292.95	2517.09	10.39	0.06	10.14
1768615_s_at	b3497	yhiQ	8.29	135.89	1127.29	5.65	0.11	5.46
1764204_s_at	b3292	zntR	8.24	187.82	1541.54	72.42	0.01	155.46
1766949_s_at	b3932	hslV*	8.07	416.46	3351.77	44.71	0.01	46.65
1768518_s_at	b4143	groEL	7.90	743.13	5831.04	37.87	0.02	55.53
1763357_s_at	b0014	**dnaK***	7.59	814.09	6120.95	24.91	0.03	28.07
1769019_s_at	b0015	dnaJ	7.08	559.61	3946.50	23.26	0.03	23.91
1767479_s_at	b1593	ynfK	7.08	35.13	246.56	4.01	0.16	3.60
1764894_s_at	b4142	groES*	6.79	772.53	5188.85	25.73	0.03	148.96
1767623_s_at	b0439	lon*	6.76	604.83	4079.96	18.37	0.04	19.04
1762240_s_at	b3931	hslU	6.58	518.63	3370.51	24.98	0.03	30.41
1764710_s_at	b3293	yhdN*	6.42	369.45	2377.17	38.28	0.02	40.39
1767508_s_at	b1814	sdaA*	6.37	536.85	3429.90	7.25	0.09	7.13
1764062_s_at	b1379	hslJ	6.30	162.79	1016.68	21.90	0.03	19.99
1768300_s_at	b0016	yi81_1	5.74	374.36	2133.52	23.63	0.03	22.85
1768989_s_at	b1280	yciM	5.64	217.00	1218.50	18.14	0.04	17.24
1759242_s_at	b0630	lipB	5.61	220.41	1235.10	64.04	0.01	70.13

relative to incubation at 45 °C for 5 min) allowed to identify the strongest heat shock promoters (*i.e.* the most highly expressed genes). After the heat shock, the percentage of transcripts considered present increases slightly ($\approx$2%) and the transcripts considered absent decrease proportionally. This means that the heat shock led to an increase of the expression of some genes, some of which are possibly not transcribed under normal growth conditions.

These heat shock promoters were identified after the data pre-treatment (background correction and normalization, see Section 2.7). *E. coli* K-12 MG1655 genes were selected from all the genes present in the Affymetrix array and were further filtered and compared to the criteria mentioned before (Fig. 1A). These criteria allowed to only select genes with low variation between the replicates and that are differentially expressed comparing to the control sample. Based on the different criteria, 189 genes were selected and classified as differentially expressed. These differentially expressed genes were then hierarchically clustered. As shown in Fig. 1B, the tree is divided in two main branches, displaying underexpressed (upper branch) and overexpressed genes (lower branch). All replicates (CA and CB, TA and TB) demonstrate a great affinity with each other (Fig. 1B) with a Spearman coefficient near 1. When comparing the two conditions (control and heat shock) the coefficient is near −1, demonstrating that there is a significant difference in gene expression between the samples.

Afterwards, these 189 genes were subjected to the t-test and only 56 were considered as being significantly transcribed. However, the t-test is not satisfactory when used in the analysis of data from microarrays with few replicates (as is the case of this work) since it is difficult to estimate the variance (Cui and Churchill, 2003). Thus, an adapted t-test, for example, the S-test, should be used

because it prevents the selection of genes with small changes as significant genes and it uses the values of the replicates to estimate the percentage of genes identified by chance (FDR – False Discovery Rate). Using the S-test, from the 189 genes identified as differentially expressed by dChip program, only 128 were considered significant with an FDR equal to 0%. The q-value, which is the lowest FDR at which a gene is described as significantly regulated, was also 0.

Table 3 shows the 34 genes that were found to be the most expressed due to the heat treatment (45 °C, 5 min) and which simultaneously met the comparison criteria. In Fig. 1B, these genes can be found in the branch called "TA Expression $\approx$ TB Expression." The microarray results obtained in the current work are consistent with others reported in the literature (Nonaka et al., 2006; Rasouly et al., 2009; Richmond et al.,1999; Wade et al., 2006; Zhao et al., 2005).

3.1.1. Selection of the appropriate heat shock promoters to trigger gene expression

In metabolic engineering it is very important to choose the right promoter(s) since many rate-limiting steps may exist in the pathways and accumulation of intermediate metabolites may occur. Therefore, after constructing a novel pathway(s), the expression levels of all the components should be orchestrated to allow fine-tuning of expression levels and optimization of metabolic fluxes (Khalil and Collins, 2010). For this reason, it is essential to construct libraries of engineered tandem promoters with varying strengths (Alper et al., 2005; Ellis et al., 2009; Jensen and Hammer, 1998). Depending on the envisaged purpose for the use of HSP promoters, several options can be considered to make an appropriate choice. For example, if building engineered bacteria to overexpress a given

compound is foreseen, the promoter of choice will depend essentially on the product concentration needed and its toxicity to the cell, since a low concentration may not be sufficient for the intended purpose, but too high a concentration can damage the cells. Table 3 illustrates three main situations when considering the genes placed directly downstream of the promoters (*): high induction rate and high expression level during heat shock (*ibpA*, *ycjX*, *ldhA*); high induction rate but low expression during heat shock (*mutM*, *mlc*, *fxsA*) when compared to the expression levels of other genes; and medium induction rate and high expression level (*clpB*, *dnaK*, *groES*, *lon*).

3.1.1.1. High induction rate and high expression level. If the aim is to achieve a very high concentration of a given compound, the chosen promoter has to allow a rapid induction of gene expression, but also a high expression. In this study (Table 3) and in several of the microarray-based studies to identify *E. coli* heat shock genes, it was found that *ibpA* and *ibpB* were the two most induced genes, with a consistently higher induction rate for *ibpB* (Caspeta et al., 2009; Richmond et al., 1999; Zhao et al., 2005). These genes, along with *yidE* gene, belong to the same transcription unit and the σ^{32} promoter-dependent is upstream of the *ibpA* gene. Moreover, *ibpA* expression is higher than *ibpB* expression, both under normal growth conditions and after heat shock treatment. This suggests that the high induction rate of *ibpB* is not due to a higher expression compared to the gene *ibpA*, but to its low expression at 37 °C (control conditions). Therefore, at 37 °C the *ibpB* gene is poorly expressed, possibly due to the short half-life of the σ^{32} factor, while under heat shock conditions, due to an increase in denatured proteins, the half-life of σ^{32} increases and the *ibpB* gene is transcribed similarly to the *ibpA* gene. Hence, despite the high induction rate of the *ibpB* gene, one can conclude that the promoter upstream of the *ibpA* gene is by far the one that allows a more pronounced expression of the HSP genes (Caspeta et al., 2009). As can be seen in Table 3, the strongest promoter following the *ibpA* gene promoter is the promoter upstream the operon comprising *ycjX*, *ycjF* and *tyrR*. These three genes have been reported to be σ^{32} dependent in all microarray studies conducted so far. The *ldhA* gene, encoding the enzyme D-lactate dehydrogenase, was also identified by several authors as a heat shock gene (Richmond et al., 1999; Wade et al., 2006; Zhao et al., 2005). The difference in *hslJ* gene expression, present in the same transcription unit, is not so evident in the current results but remains significant.

3.1.1.2. High induction rate but moderate expression during heat shock. If a fast expression induction and, at the same time, a more moderate expression is required after heat shock, then *mutM*, *mlc*, *fxsA* and *prlC* promoters can be used. These genes have been reported in several studies as heat shock genes, their functions are well-known and their promoters have been properly identified (Nonaka et al., 2006; Shin et al., 2001; Tsui et al., 1996; Zhao et al., 2005). However, in the current work the expression values obtained for *mutM* and *mlc* gene duplicates are very different and not conclusive, which can be clearly seen by the *t*- and *S*-test results (Table 3). The same occurs with the *yibA* gene which was only been identified as heat shock gene by Zhao et al. (2005) and Wade et al. (2006), although the sequence of its promoter has not been defined. Thus, if a rapid induction and a lower gene expression are required, then the *fxsA* and *prlC* promoters would be a preferable choice.

3.1.1.3. Medium induction rate and high expression level. The promoters associated with other genes with a relatively high induction ratio and expression, such as *htpG*, *ybbN*, *htpX*, *yceP*, *clpB*, *yrfH*, *hslV*, *dnaK*, *groES*, *lon*, *yhdN* and *sdaA*, can also be considered as strong promoters and therefore may be used in the construction of engineered bacteria. However, it is necessary to take into account

that the lower induction rate in these cases is primarily due to a high expression of genes in control samples. Thus, it is necessary to evaluate any implications that the possible expression of these genes could have on the applications envisaged before the heat shock treatment occurs. If the production/expression of the compound/gene before heat shock induction leads to misleading/undesirable results, then these promoters should not be used. As can be observed in Table 3, *dnaK* has the highest expression at 45 °C, followed very closely by *ibpA*. Although they have similar expression levels under heat shock conditions, the *dnaK* induction rate is much smaller than that of *ibpA*. DnaK is one of the most reported chaperones and belongs to the DnaK system, together with the DnaJ (known to assist the DnaK chaperone) and GrpE proteins (nucleotide exchanger factor). In the absence of stress, these proteins have an important role in the regulation of the stability of the σ^{32} transcription factor, and in the maintenance and restoration of the homeostatic balance. As for the *ybbN*, *htpX*, *yceP*, *yrfH*, *hslV*, *yhdN* and *sdaA* genes, it is known that all of them are also associated with σ^{32} controlled promoters (Chuang et al., 1993; Cowing et al., 1985; Kornitzer et al., 1991; Nonaka et al., 2006; Zhao et al., 2005). The two *htpG* genes and *clpB*, *groES* and *lon* genes have σ^{32}-dependent promoters but they are also controlled by σ^{54} and σ^{70} transcription factors. Therefore, the possibility that the high ratio and expression levels in this study are due to the higher affinity of the other factors to the promoter cannot be ruled out. Nevertheless, since the binding sites of the two factors are superimposed, the use of these promoters with possibly more affinity for other transcription factors is not necessarily a disadvantage, since both factors can hypothetically bind during heat shock induction, which in turn could lead to a high gene expression.

3.2. Validation by RT-qPCR of the differential expression of HSP genes

Validation by quantitative RT-PCR (RT-qPCR) of the overexpression of some of these HSP genes identified by microarrays, for example *ibpA*, *fxsA* and *dnaK*, is required before using their promoters to trigger a gene or a biosynthetic pathway by heat. For RT-qPCR validation, flask experiments were conducted twice in duplicate using the same temperatures and exposure time as for the microarray studies (Fig. 2). Furthermore, several experiments were conducted in a bioreactor at different heat shock temperatures, during different exposure times and at different heating rates in order to fully characterize the transcriptional profiles of the selected heat shock genes (Fig. 3).

Fig. 2A shows the relative expression of the three selected genes (*ibpA*, *dnaK*, *fxsA*) with respect to the *rrsA* gene in the two sample types (control and heat shock). A different expression of the heat shock genes could be observed when comparing the control with the heat shock sample. In the case of the heat shock genes it was found that the gene expression is lower in the control, which was foreseen since the heat shock transcripts are lower at 37 °C. Comparison of the three heat shock genes shows that the *dnaK* gene possessed the highest expression in heat shock conditions. However, this gene also showed the highest expression in normal growth conditions, which in turn contributes to a lower induction ratio (Fig. 2B). The *ibpA* gene showed the highest induction ratio, since it has a high expression in heat shock conditions but a low expression at 37 °C. The *fxsA* gene showed a moderate expression under heat shock conditions when compared to the other two heat shock genes, as expected.

As can be observed in Fig. 3A and B, the expression profiles of the three heat shock genes obtained in the bioreactor are very similar. The expression increases when temperature up-shift starts and maximum expression occurs when the culture medium reaches 45 °C (Point D, Fig. 3A) and 50 °C (Point O, Fig. 3B). After reaching
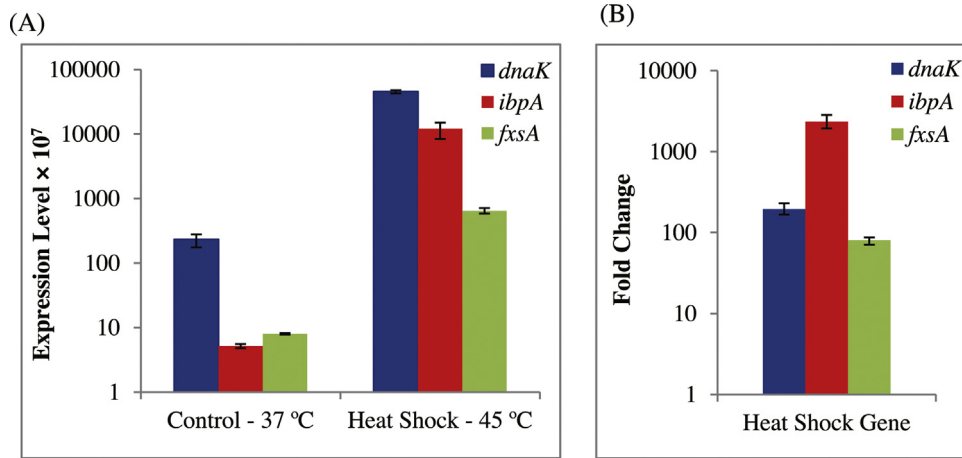
Fig. 2. Gene expression profiles (A) and fold change (B) during the temperature up-shift from 37 to 45 °C in flask.

the maximum temperature, the gene expression starts to decrease until a new stationary state is reached due to bacterial adaptation to heat. Also, following the temperature down-shift to the original temperatures, 37 °C (Point I, Fig. 3A) and 30 °C (Point T, Fig. 3B), the heat shock gene expression almost returns to its original basal level. During adaptation, to establish the homeostatic balance, σ^{32} transcription factor is degraded by proteases, such as FtsH. This occurs due to the chaperones negative modulation mechanism, in which they regulate their own synthesis by controlling the activity, stability and synthesis of the σ^{32} transcription factor. The HSR at 45 °C was found to be in accordance with previous studies conducted at 42 °C (Nagai et al., 1991; Rasouly et al., 2009; Tilly et al., 1989). In contrast, the heat shock response at 50 °C was more prolonged,

which was expected since it is more lethal to the cells and it is more difficult for the cells to adapt.

From the analysis of the expression profiles represented in Fig. 3A and B, we can conclude that the *ipbA* gene had a higher fold change, since in normal growth conditions (37 °C) it is less expressed than *dnaK* gene, and at heat shock temperatures it reaches levels similar to the ones observed for the *dnaK* gene. As expected, the *fxsA* gene showed a moderate expression. These results are in accordance with the ones obtained in the flasks experiments. Fig. 3C and D shows the genes fold change for the two experiments after normalization of the data. The gene expression is higher in the experiment conducted at the lethal temperature (50 °C) as for this temperature, the heat shock genes' transcription
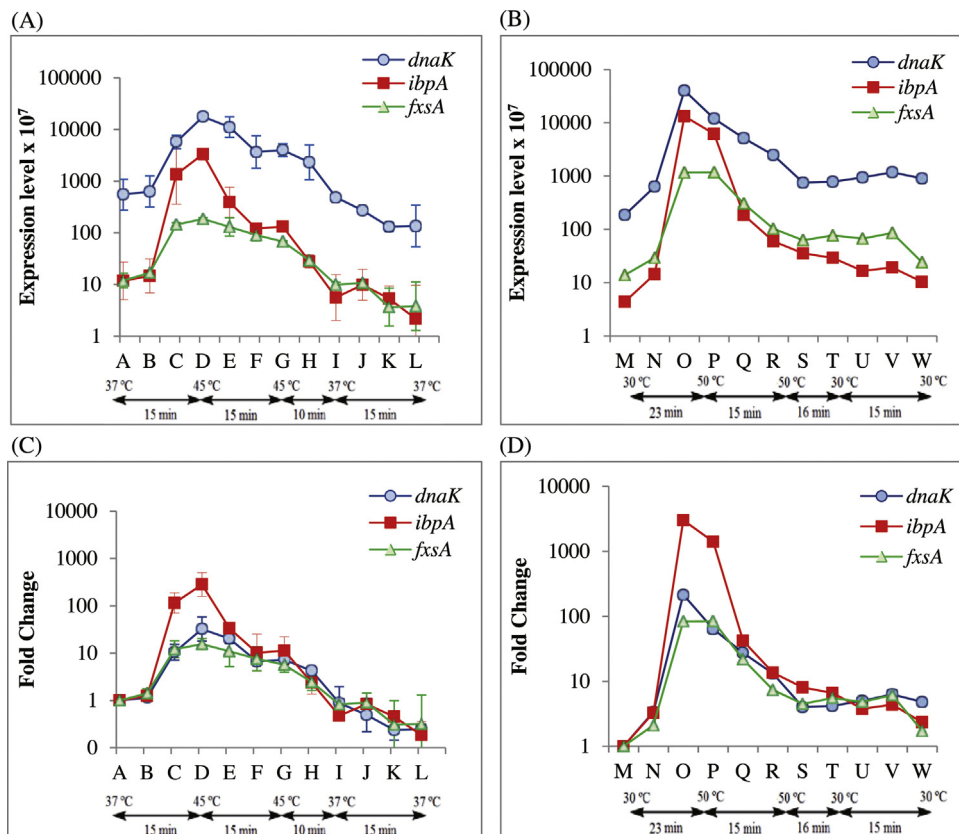

Fig. 3. Gene expression profiles (A, B) and fold changes (C, D) during up-shift from 37 to 45 °C (A, C) and 30 to 50 °C (B, D) and subsequent down-shift in bioreactor.

is controlled by the transcription level of the *rpoH* gene. The *rpoH* gene encodes the σ^{32} transcription factor. The *rpoH* gene transcription is controlled by different transcription factors depending on the temperature. At 30 °C or 37 °C, the *rpoH* gene transcription is controlled by three σ^{70} transcription factor dependent promoters (Erickson et al., 1987). In heat shock conditions around 42–45 °C, the transcription of the *rpoH* gene does not increase significantly. In these cases, the increase of σ^{32}, and consequently the expression of the heat shock genes, is due to the de-repression of *rpoH* gene translation. However, in lethal heat shock conditions (greater than 45 °C) the HSR is also controlled at the *rpoH* transcription level. When the cells are maintained at these elevated temperatures a balanced growth is no longer possible, the HSP are essentially the only proteins to be synthesized (Raina et al., 1995) and the HSR remains elevated until the cells begin to die, unless the temperature decreases (Erickson et al., 1987; Guisbert et al., 2004; Yamamori et al., 1978; Nagai et al., 1991).

3.3. Construction of heat shock inducible stress probes using GFP

Since we aimed to identify the strongest heat shock promoters that can trigger a biosynthetic pathway as a result of a temperature increase, besides the transcription levels study, it is also important to study and optimize the protein translation to obtain high protein expression. To study the protein translation, the HSP promoter elements were fused to the *gfp* reporter gene. This strategy has been applied in the past by Cha et al. (1999), where the heat shock response of *E. coli* resting cells was quantified by *dnaK*, *clpB*, and *rpoH* GFP promoter probes. In their experiment they used chemical (addition of IPTG, acetic acid, ethanol, phenol, antifoam or salt) and physical stresses (heat shock or nutrient limitation) to induce GFP expression. Gill et al. (1998) and Seo et al. (2003) also fused GFP to *rpoH*, *clpB* and *dnaK* promoters to monitor the toxic effect of dithiothreitol addition and compare the cellular stress levels of four strains caused by temperature up-shift, respectively. Lu et al. (2005a,b) and Messaoudi et al. (2013) constructed oxidative stress probes by fusing the promoters of several oxidative stress gene promoters with GFP. Other stress probe strategies have also been used. Van Dyk et al. (1994), Bianchi and Baneyx (1999a,b), Lesley et al. (2002), Pérez et al. (2007) Kraft et al. (2007) and Kotova et al. (2010) fused heat shock promoters to β-galactosidase (*lacZ*) and to bioluminescent luciferase bioreporters (*lux* and *luc*) to investigate the toxicity of heterologous protein expression and other compounds. In our study we used the *gfp* reporter gene because of its small size, minimal toxicity, high stability and ease of use (March et al., 2003).

The use of stress probes with the HSP promoters fused to *gfp* helped to study how efficient the promoter sequences and the original heat shock RBS are in the translation of a gene. Different experiments were done with different controls (30 °C and 37 °C) and heat shock (45 °C and 50 °C) temperatures and different heat shock duration (5 and 10 min). Fig. 4 shows the fluorescence results obtained for the above mentioned experiments. As observed before, the *dnaK* promoter allows the highest expression after heat shock treatment, but the induction ratio is not as high as for *ibpA* since the *dnaK* expression at 37 °C is already very high and in most cases even higher than *ibpA* expression after heat shock conditions. The *fxsA* promoter showed less GFP expression, corroborating the microarrays and RT-qPCR results. Comparing the two heat shock temperatures tested (45 and 50 °C) it can be concluded that the highest temperature allows a higher promoter response and consequently, a higher GFP expression. However, the fluorescence level at 50 °C depends on the initial temperature (30 and 37 °C). In general, an initial lower temperature (30 °C) that corresponds to less basal fluorescence also corresponds to lower specific fluorescence intensity at 50 °C. The SFI obtained with the original T7 promoter (*data not shown*) was around 33,000 and the fluorescence

reached the steady state in about 2–3 h (37–30 °C experiments) and remained stable until the end of the experiment. As expected, the T7 promoter is a better option as the expression obtained is higher and more stable. However, the use of IPTG has some disadvantages, such as cost and toxicity, and limited utility for *in situ* gene expression. The lower stability of the mRNA when using heat shock promoters can be overcome by adding sequences to form stem-loop at its 5′ and 3′ untranslated regions (UTRs) (Chen et al., 1991; Higgins et al., 1993). This prolongs the half-life of a normally unstable mRNA by presumably interfering with RNase binding.

These results (Fig. 4) were obtained after studying four different promoter sequences (Supplementary material, Tables S1 and S2). The first heat shock promoter sequences used to replace the T7 promoter were obtained from gDNA amplification of the −50 region until the start codon (ATG) of the heat shock gene. The TATA box regions, transcription initiation site, RBS and translation initiation site of the native proteins were used for GFP regulation. The only promoter that showed a significant increase in GFP fluorescence after heat shock was *dnaK*. In order to improve the expression of GFP, the promoter sequence from the −150 region to the heat shock gene ATG was amplified, toward the inclusion of some possible existing binding sites for transcription activator proteins present in the region upstream of the core promoter, referred as the upstream (UP) element (Ross et al., 1993). The new sequences did not result in an effective improvement of the GFP expression level. Therefore, the promoter sequence from the −150 region to the +21 nucleotides from the three heat shock genes was amplified. Several authors (Bianchi and Baneyx, 1999a,b; Pérez et al., 2007) have reported good results when including the first 21 nucleotides of the *ibpA* open reading frame in a *lacZ* gene fusion. However, fluorescence did not improve and therefore the experiments were repeated and samples were analyzed by RT-qPCR. The preliminary results showed a significant increase of *gfp* transcription after heat shock which allowed us to conclude that the lack of fluorescence after heat shock induction was not related to the promoter strength but to the translational efficiency. To overcome this issue, the RBS Calculator v1.1 (Salis, 2011) software was used. Twenty N nucleotides (any base calculated by the software) were added to the RBS constraints, followed by GGATCC, which corresponds to the *Bam*HI site. The target translation initiation rate was set to 20,000 for the three cases. With these new constructions, we were able to observe an increase of fluorescence in the *fxsA* and *ibpA* stress probes. However, the fluorescence for the *fxsA* probe was higher than for *ibpA*, which was not expected according to the previously observed mRNA levels. The translation initiation rates determined using the RBS calculator for the heat shock gene promoters in the *E. coli* genome presented the following relation: $ibpA \approx 2.5 \times dnaK \approx 21.2 \times fxsA$. To obtain GFP expression results more similar to the ones of the heat shock genes in the genome, the RBS Calculator was used. After running the software for 8 h, a relation closer to the translation initiation rates in the genome was found, namely $ibpA \approx 1.6 \times dnaK \approx 14.9 \times fxsA$, where *fxsA* construction possessed the same translation initiation rate as the one used in the previous constructions (19,000). These newly determined RBS were then used to obtain the results gathered in Fig. 4.

Synthetic biology finds applications in several fields, such as biosensing (pollution detection), therapeutics (drug discovery/target identification, therapeutic delivery/treatment), and production of biofuels, pharmaceuticals and novel biomaterials (construction/optimization of biosynthetic pathways, programming novel functionality and materials). For these applications, new biological devices and systems to regulate gene expression and metabolite pathways are required. Furthermore, external control and triggers for such devices and systems can be designed using adequate gene promoter strategies. Most promoters commonly used in heterologous protein expression are inducible
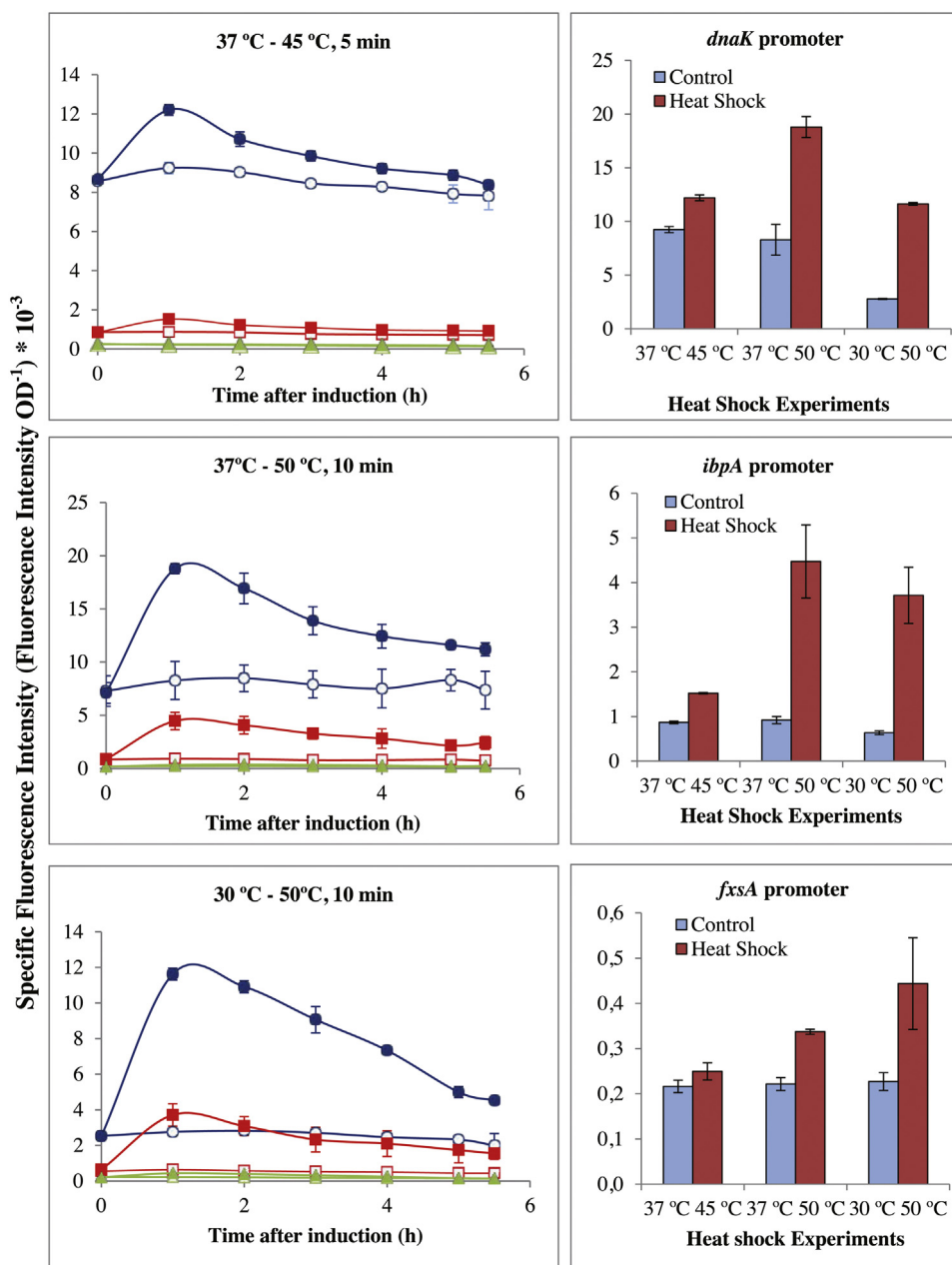
Fig. 4. Specific fluorescence intensity after heat shock in *E. coli* MG1655 DE3 transformed with pETduet_GFPmut3b plasmids with different promoters: *ibpA* promoter (□), *dnaK* promoter (○) and *fxsA* promoter (△) (control – 37 or 30 °C), *ibpA* promoter (■), *dnaK* promoter (●) and *fxsA* promoter (▲) (heat shock – 45 or 50 °C). The three biological replicates for each experiment were performed in different days. The fluorescence values in the figures on the right correspond to the maximum values obtained for each case (1 h after heat shock induction).

promoters. Chemical inducers can be expensive at laboratory or pilot-plant scales and are often toxic, thus their presence in either the final product or in waste effluents is highly undesirable and requires their disposal/elimination. These restrictions are particularly important in food, pharmaceutical-grade proteins and other products intended for human use, such as therapeutic products. Induction methods having a thermo-regulated expression system with heat shock promoters are even more important for *in vivo* applications which rely on an external stimulus to the body. The heat shock promoters identified in this study can be used in the construction of new therapeutic bacteria, in which the biosynthetic pathway of a therapeutic agent will be inserted and whose expression will be triggered *in situ* by heat, emitted by laser therapy or ultrasound. The expression obtained with these heat shock promoters will most likely not reach the same level as the one obtained

with other stronger promoters such as T7; however, *in situ* delivery will likely not require expression levels as high as those necessary in bioreactors. In addition, the promoter sequences can be optimized as demonstrated by the approach herein reported using RBS optimization to improve the translation efficiency and to reach the desired response and obtain the expected results in the production of novel genes or pathways. Overall, a heat shock approach would be easier to implement and administer and will be safer since no chemicals are involved.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec. 2014.08.005.

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