Gene 548 (2014) 142–148

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Genetic regulation of *spy* gene expression in *Escherichia coli* in the presence of protein unfolding agent ethanol

Santosh Kumar Srivastava, Paramesh Ramulu Lambadi, Tamoghna Ghosh, Ranjana Pathania, Naveen Kumar Navani *

Department of Biotechnology, Indian Institute of Technology Roorkee, Uttarakhand 247 667, India

ARTICLE INFO

Article history:
Received 27 February 2014
Received in revised form 31 May 2014
Accepted 3 July 2014
Available online 5 July 2014

Keywords:
Chaperones
Ethanol stress
Spy
BaeR regulator
CpxR regulator

ABSTRACT

In a living cell, folding of proteins is assisted by molecular chaperones and other folding helpers. In *Escherichia coli* (*E. coli*), recently an ATP independent chaperon 'Spy' was discovered which is highly up-regulated in the presence of protein unfolding agents like ethanol, butanol and tannic acid. Two response regulators; BaeR and CpxR have been recognized as transcriptional regulators of *spy* gene. However, the mechanism of genetic regulation of *spy* under protein denaturants like ethanol has not been studied in detail so far. Based on a combination of genetic, molecular biology and biochemical experimental data, we propose that BaeR protein is the primary regulator of *spy* gene in response to ethanol stress in *E. coli*. In addition, we expanded the experimental spectrum and validated that regulation of *spy* gene in the presence of zinc and copper metal stress is primarily via BaeR and CpxR regulators respectively. We also performed in-silico analysis to identify the homologs of Spy protein and their cognate regulatory elements in bacterial species belonging to enterobacteriaceae family. Based on the unique ATP-independent chaperone nature and genetic regulation of *spy* we also propose its importance in biosensor development and facilitated production of properly folded recombinant proteins.

1. Introduction

Molecular chaperones assist various processes in the bacterial cell including folding of newly synthesized protein, protein secretion, preventing aggregation of protein and even repairing the damaged proteins (Hartl, 1996). There are several chaperones in a bacterial cell which provide folding protection to the nascent proteins in both internal and external milieu of the cells, mostly at the expense of energy molecules like adenosine 5′ triphosphate (ATP) (Hartl, 1996; Lund, 2001). Periplasmic space of Gram-negative bacteria like *Escherichia coli* is exposed to the external environmental fluctuations and is devoid of energy sources such as ATP, thus presenting cells with a challenge to cope with the problem of aggregation and unfolding of the proteins in the periplasm. This enigma was settled by an elegant genetic selection experiment which led to the discovery of a protein 'Spy' found responsible for protein stability in *E. coli* periplasm (Powers and Balch, 2011; Quan et al., 2011; Yamamoto et al., 2008). Spheroplast protein Y (Spy) is a non-ATP dependent chaperone which protects *E. coli* from the onslaught of undesirable environmental changes that might lead to protein aggregation. Spy like periplasmic chaperone can be of biotechnological importance to facilitate conformational processing of a significant fraction of de novo synthesized recombinant polypeptides overproduced in *E. coli* (Kyratsous et al., 2009; Schlapschy and Skerra, 2011; Yoon et al., 2008).

Normally, the amount of Spy protein present in *E. coli* cell is vanishingly small, however on exposure to environmental stresses such as copper (Cu), zinc (Zn), ethanol, indole and tannins, the expression of *spy* gene gets induced (Bury-Moné et al., 2009; Kwon et al., 2010; Nishino et al., 2005; Yamamoto and Ishihama, 2006; Yamamoto et al., 2008). Expression of *spy* is transcriptionally regulated by two stress response systems; Bae and Cpx, which monitor protein folding in the extracytoplasmic envelope (MacRitchie et al., 2008; Vogt and Raivio, 2012). Bae and Cpx belong to the category of two component systems in which inner-membrane proteins BaeS and CpxA act as histidine kinase to sense the membrane stress. Upon activation by stress, BaeS and CpxA get autophosphorylated and transfer this phosphate to their cognate response regulatory partners BaeR and CpxR respectively. Phosphorylation of these response regulators activates transcription of cognate target genes (MacRitchie et al., 2008; Nagasawa et al., 1993). Yamamoto et al. analyzed the upstream sequence of *spy* gene and established that the promoter is composed of two BaeR and one CpxR binding motifs which interact with their cognate regulatory proteins and modulate the expression of *spy* gene under various stresses (Yamamoto and Ishihama, 2006; Yamamoto et al., 2008).

Abbreviations: BaeR, bacterial adaptive response regulator; CpxR, conjugative plasmid expression regulator; GFP, green fluorescence protein; OD, optical density; Psp, phage shock protein; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Spy, spheroplast protein Y; WT, wild type.

* Corresponding author at: Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, Uttarakhand 247 667, India.
E-mail addresses: navnifbs@iitr.ernet.in, naveenbiochem@gmail.com (N.K. Navani).

http://dx.doi.org/10.1016/j.gene.2014.07.003
0378-1119/

It has also been observed that the percentage of Spy protein in *E. coli* gets highly elevated in bacterial cells exposed to the protein unfolding agents like ethanol and butanol (Brynildsen and Liao, 2009; Nishino et al., 2005; Rutherford et al., 2010). However, the exact mechanism of regulation is not known. Yamamoto et al. observed that in the presence of copper, the upregulation of *spy* gene is mediated only through the regulatory protein CpxR (Yamamoto and Ishihama, 2006; Yamamoto et al., 2008). This observation prompted us to investigate the regulatory mechanism of *spy* gene in the presence of ethanol stress. We fused the promoter of *spy* gene with GFP (green fluorescence protein) and implanted the recombinant construct (*spy::GFP*) into wild type *E. coli* strain and null mutants of *cpxR* (*ΔcpxR*) and *baeR* (*ΔbaeR*) genes respectively. The GFP expression was monitored in the wild type, *ΔcpxR* and *ΔbaeR* strains, harboring *spy::GFP*, in the presence of ethanol. To decipher the role of regulatory elements (BaeR and CpxR binding sites) of *spy* gene, the promoter of *spy* gene was truncated to remove the BaeR and CpxR binding sites. These promoter variants were fused with the reporter protein GFP and were used to probe the role of CpxR and BaeR binding motifs in activation of *spy* gene in the presence of ethanol. Furthermore, the effect of null mutants of *baeR* and *cpxR* on the level of expression of *spy* gene in the presence of ethanol was investigated by monitoring mRNA expression using qRT-PCR. Through a series of experimental evidences, we propose the regulatory mechanism of *spy* gene in the presence of ethanol.

Homologs of Spy proteins have been identified in other bacterial species also (Quan et al., 2011). The enigmatic nature of Spy protein prompted us to look for conservation in regulatory elements of other Spy like protein homologs in Gram-negative bacterial species. Spy protein of *E. coli* shows sequence similarity (31%) with CpxP protein which is a known repressor of Cpx pathway. Spy and CpxP proteins both contain two LTXXQ motifs each. In-silico bioinformatic search of protein databases revealed that within the family enterobacteriaceae, several hypothetical proteins with LTXXQ motifs are present. These proteins and their regulatory elements also show varying degree of conservation of BaeR and CpxR-binding motifs. These analyses indicate that Spy like other hypothetical proteins encoded in the genome of enterobacteriaceae family may also be regulated through Cpx and Bae regulators.

2. Materials and methods

2.1. Bacterial strains, plasmids and oligonucleotides

Bacterial strains and plasmids used in this study have been listed in Tables 1 and 2. All the experiments described here were based on *E. coli* K-12 BW25113. All mutant strains (*ΔbaeR* and *ΔcpxR*) used in this study originated from the KEIO collection (Baba et al., 2006). Plasmid vector pPROBE-TT′ used in this study was a kind gift from Prof. Steven Lindow, Berkeley University, USA. Vector pPROBE-TT′ has ColE1 origin of replication, codes for tetracycline resistance and encodes green fluorescence protein downstream of multiple cloning sites (Miller et al., 2000). Oligonucleotides used in this study were synthesized by Eurofins Scientific, India and have been listed in Table 2.

Table 2
Oligonucleotides used in this study.

Name of primers	Oligo sequences (5′–3′)	
Forward primer F—RT spy	GCCAGCGATACCTTCGATAA	This study
Reverse primer R—RT spy	GCAGGCATTTTACCTTTTGC	This study
Forward primer—spy_{F1}	ATTAGAATTCTACAGATTAATCCTGGT	This study
Forward primer—spy_{F2}	ATTAGAATTCATCGTGTTGTAATATTCT	This study
Forward primer—spy_{F3}	ATTAGAATTCATTGGCGCAAAAGTGTTT	This study
Reverse primer-R—spy	ATTAAAGCTTTTATTACGGCTTTCTTAA	This study
Forward primer F—spy-10/35	TGACTGGATCCGCTCTGATTAATTGACGC	This study
Reverse primer R—spy-10/35	ATTGCAAGCTTTTCAGTTATTTATTACGGC	This study
Forward primer—BaeR *EcoR1*	TAGTGAATTCATGCTCGAAAAAATAAGC	This study
Reverse primer—BaeR *BamHI*	GCATGGATCCAACACTTTTGCGCCAAT	This study

2.2. Media, antibiotics and growth conditions

Bacterial cultures were maintained on Luria Bertani agar (Himedia, India) plates at 37 °C. All liquid cultures were grown in Luria Bertani broth (Himedia, India) with shaking (250 rpm) at 37 °C. To ensure proper strain and plasmid maintenance, the culture medium was supplemented with following antibiotics whenever necessary: kanamycin (40 μg/ml) or tetracycline (30 μg/ml) (Sambrook et al., 1989). All antibiotics were purchased from Sigma Aldrich, USA.

2.3. Bioinformatics analysis

Homologs of the *E. coli* MG1655 Spy protein (gi|16129697) were searched using NCBI's Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov). The periplasmic hypothetical proteins of enterobacteria showing conservation with *E. coli* Spy proteins were selected. Selected hypothetical protein sequences and upstream sequences of the genes encoding these proteins were aligned by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). To identify the motifs present in the homologs of Spy protein in enterobacteriaceae,

Table 1
Bacterial strains and plasmid used in this study.

Strains	Description/genotype	References
E. coli DH5α	F$^-$, *deo*R, *end*A1, *gyr*A96, *hsd*R17 (rK$^-$/mK$^+$), *rec*A1, *pho*A, *rel*A1, *thi*-1, Δ(*lac* ZYA-*arg*F), U169ϕ80d*lac*ZΔM15λ$^-$, *sup*E44	Taylor et al. (1993)
E. coli BW25113	*F*−, *Δ(araD-araB)567*, *ΔlacZ4787(::rrnB-3)*, λ$^-$, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba et al. (2006)
E. coli BW25113 Δ*baeR*	F−, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ$^-$, *ΔbaeR778::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*	Baba et al. (2006)
E. coli BW25113 Δ*cpxR*	*F*−, *Δ(araD-araB)567*, *ΔlacZ4787(::rrnB-3)*, λ$^-$, *rph-1*, *Δ(rhaD-rhaB)568*, *ΔcpxR772::kan*, *hsdR514*	Baba et al. (2006)
Plasmids used		
Plasmid name	Description/resistance	
pPROBE-TT′	Promoter Probe shuttle vector, Tet^R, GFP has been cloned at downstream of MCS	Miller et al. (2000)
pPROBE-TT′ *spy BBC::GFP*	Upstream sequence of *spy* gene containing two BaeR and CpxR binding sites was cloned between *EcoR1* and *HindIII* restriction sites in pPROBE-TT′	This study
pPROBE-TT′ *spyBC:GFP*	Upstream sequence of *spy* gene containing one BaeR and CpxR binding sites was cloned between *EcoR1* and *HindIII* restriction sites in pPROBE-TT′	This study
pPROBE-TT′ *spy C::GFP*	Upstream sequence of *spy* gene containing only CpxR binding sites was cloned between *EcoR1* and *HindIII* restriction sites in pPROBE-TT′	This study
pPROBE-TT′ *spy BB::GFP*	Upstream sequence (containing only BaeR binding sites) of *spy* gene was cloned between *EcoR1* and *HindIII* restriction sites in pPROBE-TT′	This study

a motif scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was used (Sonnhammer et al., 1998).

2.4. Construction of spy promoter variants

The upstream sequences (−500 to −20) of *spy* gene were amplified from the genomic DNA of *E. coli* BW25113 by using forward primer spy_{-F} and reverse primer spy_{-R} (Table 2), which introduced *EcoRI* and *HindIII* restriction sites at the ends of the amplified fragments. The amplified upstream sequence of *spy* gene containing two BaeR and one CpxR binding sites was truncated by using different sets of primers. Forward primer spy_{-F2} and reverse primer spy_{-R} (Table 2) were used to amplify the upstream sequence which had one BaeR and one CpxR binding site. Another pair of oligos spy_{-F3} forward and spy_{-R} reverse was used to amplify the upstream sequence having only CpxR binding sites. To clone the BaeR binding motif, the upstream sequence of *spy* gene was amplified using two sets of forward primers (F-spy-10/35 and F-BaeR *EcoR1*) and reverse primers (R-spy-10/35 and R-BaeR *BamHI*) (Fig. 2A–B). Each of the amplified products was digested with *EcoRI* and *HindIII* restriction enzymes and ligated with *EcoRI* and *HindIII* digested promoter probe vector pPROBE-TT (Sambrook et al., 1989). Variants of *spy* promoter were named *spyBBC::GFP*, *spyBC::GFP*, *spyBB::GFP* and *spyC::GFP*. Restriction enzymes used in this study were purchased from Thermo Fisher, USA and New England Biolabs, USA. DNA Sequence of all constructs was confirmed at Eurofins Laboratories, Hyderabad, India.

2.5. RNA and DNA extraction and quantification

Wild type *E. coli* BW25113 strain and deletion strains *E. coli* BW25113 (Δ*cpxR*) and *E. coli* BW25113 (Δ*baeR*) were treated with 4% ethanol. After 6 h of induction, the cells were harvested and total RNA was isolated using Trizol reagent (MRC, Cincinnati, Ohio, USA) as per manufacturer's instructions (Mackey and Chomczynski, 1996). Genomic DNA of *E. coli* BW25113 was isolated by using genomic DNA isolation kit (Fermentas, USA) according to the manufacturer's instructions. DNA and RNA concentrations were determined using the SpectraMaxM^{2e} microplate reader (Molecular Device, CA, USA) at 260/280 nm (Maniatis et al., 1982).

2.6. Real time PCR

The total RNA was treated with DNase I (Fermentas, USA) for removal of any contaminating genomic DNA. DNase treated total RNA (1 μg) was converted to strand-specific cDNA in a 25 μl reaction consisting of 1× RT reaction buffer, 4 mM of each dNTP, 1.6 μM gene specific reverse primer (spy-R), 20 U RNase inhibitor, and 1 μl of ImProm-II™ Reverse Transcriptase (Promega, Madison, USA). Each 25 μl of PCR reaction contained 12.5 μl of Light cycler 480 SYBR Green I master (Roche diagnostics, Germany), 0.2 μM of each primer and 2 μl (450 ng) of cDNA. Relative qPCR was carried out in Smart Cycler (Cepheid, Sunnyvale, CA) with primers spy-F/R specific for *spy* as the target gene, and primers 16S rRNA-F/R specific for 16S rRNA as the reference gene (endogenous gene). From the fluorescent values obtained through the real-time PCR analysis, the threshold cycles (C_T-value) were obtained. The calculated threshold cycle (C_T) for each gene was normalized to C_T of the rRNA gene, expression which is invariant across a large range of growth conditions (Livak and Schmittgen, 2001).

2.7. Measurement of GFP fluorescence

The bacterial cells were grown with shaking at 37 °C in LB medium until they reached an optical density at 600 nm (OD_{600}) of 0.3 (early log phase). Grown bacterial cell cultures were induced at early-log phase (0.2–0.3) with 4% ethanol, 0.6 mM copper chloride, and 0.6 mM zinc sulfate. To quantify the fluorescence intensity, 1 ml of bacterial culture was harvested after 2 h of induction, washed and re-suspended in 500 μl 1× PBS (phosphate buffered saline). Expression of GFP protein was measured using the SpectraMaxM^{2e} microplate reader (Molecular Device, CA, USA). Fluorescence intensity was measured in a black opaque 96 well plate (Corning® 96 well opaque black plate, USA) at excitation of 490 nm and emission of 510 nm. Fluorescence value was normalized with optical density (Miller et al., 2000).

2.8. Fluorescence microscopy

To visualize bacterial cells expressing GFP protein using a fluorescence microscope, the cells were washed thrice with 1× PBS. Washed cells were spread on slides and allowed to air dry. Cells were observed under 100× oil immersion objective lens of a Nikon Eclipse Ti-E inverted fluorescence microscope (Nikon, Tokyo, Japan).

3. Results

3.1. Influence of cpxR and baeR deletion on spy promoter activity in the presence of ethanol, copper and zinc

The activity of *spy* promoter was assessed in terms of GFP expression by external addition of 4% ethanol, 0.6 mM copper ion and 0.6 mM zinc

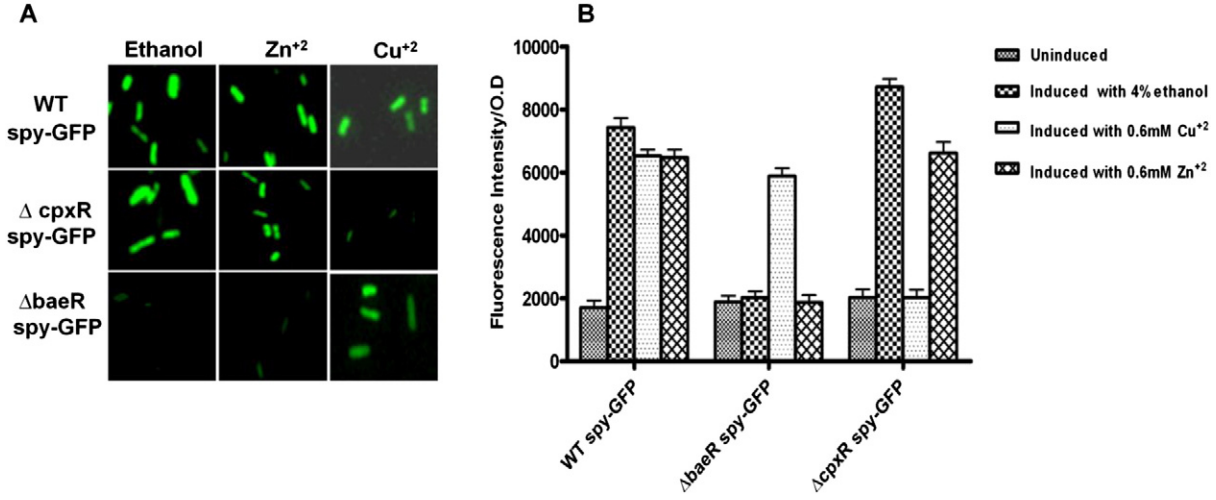

Fig. 1. *E. coli* strains (WT, Δ*cpxR* and Δ*baeR*) harboring the *spy::GFP* plasmid were induced with 4% ethanol, 0.6 mM zinc sulfate and 0.6 mM copper chloride. The *spy* promoter activity was quantified by measuring fluorescence intensity. A) Fluorescence microscopic view of induced cells. B) Fluorescence values were monitored using a fluorescence spectrophotometer.

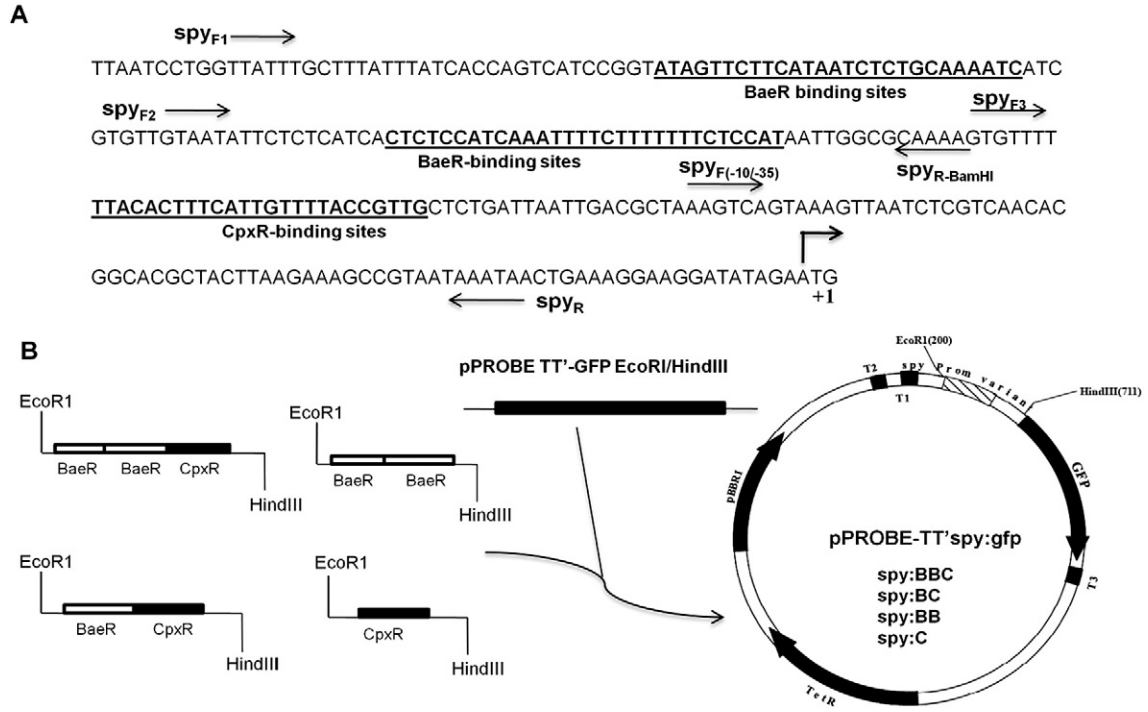

Fig. 2. A) Diagram illustrates the method of *spy* promoter truncation by using different sets of primers (spy_{F1}, spy_{F2}, spy_{F3}, and $spy_{F-10/-35}$ forward primers spy and spy_R and spy_{RBamHI} reverse primers). B) Schematic diagram showing the cloning of *spy* promoter variants in pPROBE-TT′ plasmid vector containing GFP as reporter gene.

ion to *E. coli* BW25113, *E. coli* BW25113 *ΔcpxR* and *E. coli* BW25113 *ΔbaeR* strains respectively. Expression of GFP was monitored by spectrophotometric and microscopic examinations. When *E. coli* strains were challenged with protein unfolding agents ethanol and metal zinc, the *spy* promoter was activated in wild type *E. coli* and *ΔcpxR* strains resulting in expression of GFP which was above the background

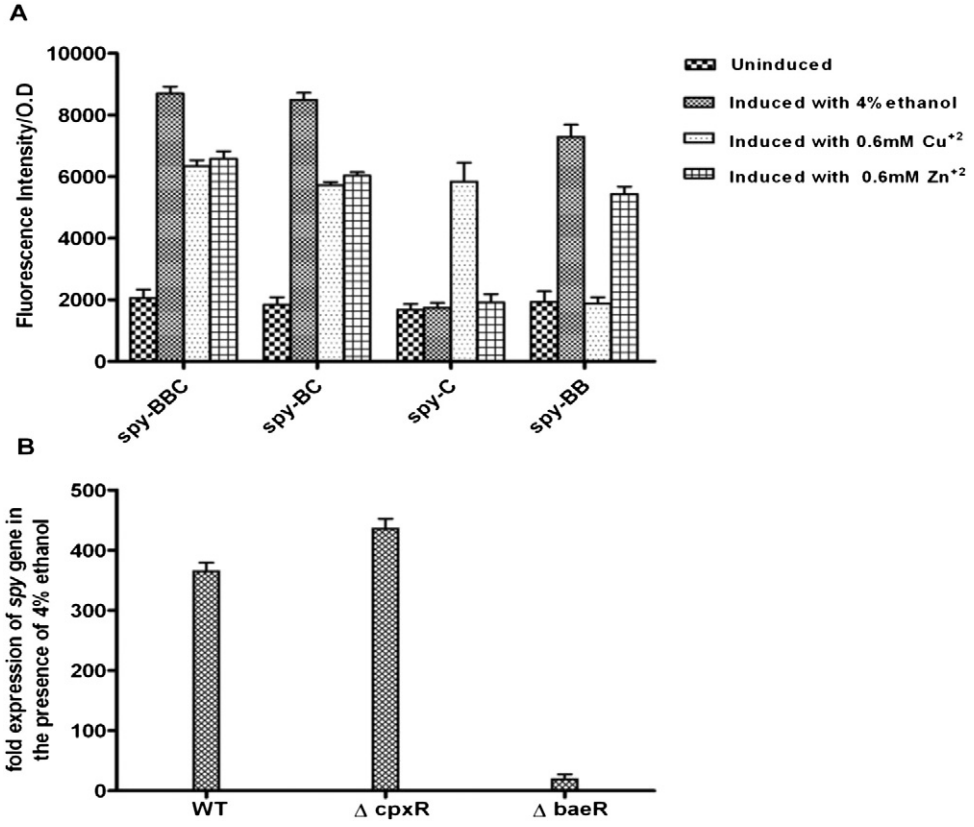

Fig. 3. A) *E. coli* strain harboring transcriptional fusion of GFP and variants of *spy* promoter (*spyBBC::GFP*, *spyBC::GFP*, *spyC::GFP* and *spyBB::GFP*) were induced with 4% ethanol, 0.6 mM zinc sulfate and 0.6 mM copper chloride and fluorescence intensity was measured using a spectrophotometer. B) Expression of *spy* m-RNA level was monitored by qRT-PCR in the presence of 4% ethanol in wild type *E. coli* and deletion mutant of *cpxR* and *baeR* strains.

values. However, both the stress agents failed to activate the promoter in *ΔbaeR* strain (Fig. 1A, B). On the other hand copper failed to activate the *spy* promoter in *ΔcpxR* strain whereas it was activated in WT and *ΔbaeR* strains. These results indicate that the cytoplasmic regulator BaeR plays a pivotal role in the expression of Spy under ethanol and zinc stresses. However, in the presence of copper the induction of *spy* gene in *E. coli* is *cpxR* dependent.

3.2. Response of spy promoter variants to ethanol, copper and zinc

To understand the role of DNA regulatory element of *spy* gene containing BaeR and CpxR binding motifs under ethanol, copper and zinc stresses, the truncated variants of spy promoter possessing different regulatory motifs were generated. Constructed *spy* promoter variants were named; *spyBBC::GFP* (containing two BaeR and one CpxR binding sites), *spyBC::GFP* (containing one BaeR and one CpxR binding site), *spyBB::GFP* (two BaeR sites only), and *spyC::GFP* (one CpxR binding site only) as shown in Fig. 3A. *E. coli* strains harboring the *spy* promoter variants (*spyBBC::GFP*, *spyBC::GFP*, *spyC::GFP* & *spyBB::GFP*) were induced with 4% ethanol, 0.6 mM copper ion and 0.6 mM zinc ion respectively and the fluorescence values were measured. The variant of *spy* promoter containing only the CpxR binding motif was unable to drive GFP expression during ethanol and zinc stresses, however the GFP fluorescence values increased on exposure to copper ion. Other *E. coli* variants harboring (*spyBBC::GFP*, *spyBC* and *spyBB::GFP*) one BaeR binding motif each, drove the expression of GFP and showed three fold higher fluorescence values compared to the control set, indicating that the promoter was in an active state (Fig. 3A). The response of *E. coli* BW25113 strain harboring *spyBB::GFP* was found to be linear over a range of 2–6% ethanol (Supplementary Fig. 1.) These observations suggest that the presence of BaeR and CpxR binding motifs in the promoter of *spy* gene provides site for interaction with cognate phosphorylated BaeR and CpxR protein leading to regulation of *spy* gene during ethanol, zinc and copper stresses (Fig. 4).

3.3. Quantification of spy gene m-RNA expression in cpxR and baeR deletion strains on exposure to ethanol

To further confirm the regulation of *spy* gene under ethanol stress, we followed the mRNA levels in *ΔbaeR* and *ΔcpxR* strains using qRT-PCR. *E. coli* BW25113, *E. coli* BW25113 *ΔcpxR* and *E. coli* BW25113 *ΔbaeR* cells were treated with 4% ethanol and qRT-PCR was carried out to analyze the expression of *spy* gene in-vivo. The expression of *spy* gene was highly upregulated (~415 fold) in deletion mutant of *cpxR* and wild type strains. However, no significant change (~6–7 fold) was observed in case of *baeR* deletion mutant. These results confirm that in the absence of BaeR protein, the expression of *spy* gene gets diminished; whereas, in *ΔcpxR* and wild type *E. coli* strains, the expression was highly elevated in the presence of ethanol (Fig. 3B).

3.4. Conservation of Spy homologs in other bacterial genomes

A BLASTP search was performed using the sequence of Spy protein as the initial query to identify representatives of the same protein family in related bacterial genomes and the BLAST results indicated that these bacteria belong to the enterobacteriaceae family. Multiple sequence alignment of these homologs shows high conservation at their N and C terminal amino acid sequences. Search in motif scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Sonnhammer et al., 1998), led to the observation that most of the homologs of Spy protein in bacterial genomes contain two LTXXQ motifs each (Supplementary Fig. 2). The LTXXQ motif present in Spy protein has been predicted to help maintain its own structural integrity (Kwon et al., 2010; Thede et al., 2011). Of these Spy homologs, representative hypothetical proteins from *Dickeya zeae*, *Sodalis glossinidius*, *Pantoea ananatis*, and

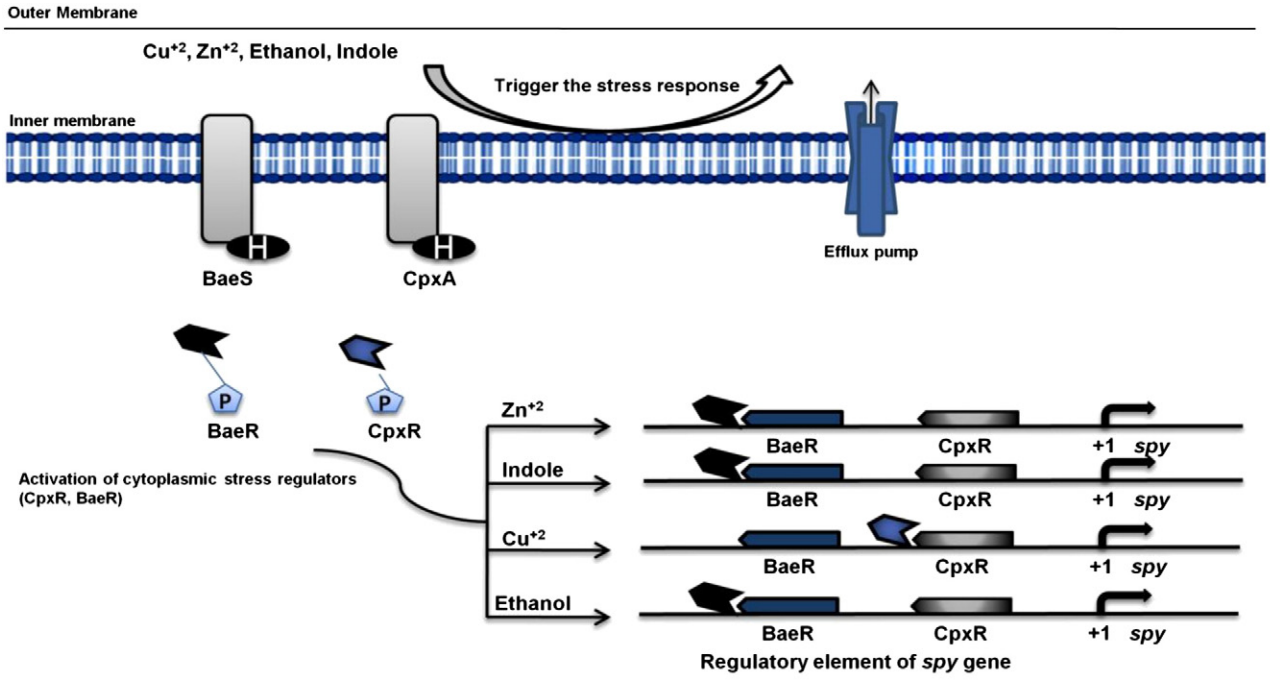

Fig. 4. Schematic diagram showing the cellular mechanism of the activation of the periplasmic chaperone *spy* in the presence of ethanol, indole, zinc and copper.

Pectobacterium carotovorum were selected for further analysis. These proteins merit further investigation since despite the presence of two LTXXQ motifs they show a low sequence conservation (20–35%) with CpxP and higher sequence conservation (48–60%, Supplementary Table 1, Supplementary Figs. 4–5) with Spy protein of *E. coli*. Alignment of complete protein sequences of these hypothetical proteins with Spy of *E. coli* also revealed that similarity of the sequences is not confined to only small stretches around the LTXXQ motif (Supplementary Fig. 3). Conserved Spy homologs encoded in enterobacteria prompted us to investigate their regulatory elements and compare CpxR and BaeR binding motifs. It was found that the BaeR-binding motif shows some degree of conservation (Fig. 5, Supplementary Fig. 6). However, the CpxR binding motif was highly conserved in this group of bacteria. High conservation of the CpxR binding motif in an upstream region of various genes encoding Spy like hypothetical protein shows that this regulator may play a role in activation of these genes during environmental stresses. Taken together, these observations indicate that the identified uncharacterized proteins of the CpxP family encoded in the genome of enterobacteria are also likely to be transcriptionally regulated by two stress response systems, Bae and Cpx.

4. Discussion

Several extracytoplasmic pathways exist in *E. coli* to cope with the envelope stresses. Amongst the well characterized signaling pathways; σ^E, Psp, Cpx and Bae have been identified as major regulatory elements of such stresses (Bury-Moné et al., 2009). Cpx and Bae systems also regulate expression of *spy*, an enigmatic chaperone, which supports protein refolding without the need for energy source like ATP (Kwon et al., 2010; Quan et al., 2011). On the one hand it is critical to understand the mode of action of Spy as chaperone; on the other hand, the agents which regulate the expression of Spy also merit a deeper investigation. Through a combination of genetic, biochemical and molecular studies we have investigated the genetic regulation of Spy protein in the presence of ethanol and metal ions like Cu and Zn. In this study, we conclude that BaeR is the primary regulator of ethanol stress in *E. coli*. This conclusion has been arrived at by using *E. coli* strains lacking CpxR and BaeR regulators separately. GFP induction data of these strains substantiates the role of BaeR in *E. coli* in the presence of ethanol. This observation is further supported by the data obtained from promoter variants generated from truncation studies. To understand the role of BaeR and CpxR regulators in *spy* mRNA expression level, we carried out real time PCR in *cpxR* and *baeR* knock out strains in the presence of ethanol. This in-vivo study also confirms that the BaeR is the key regulator of *spy* gene regulation during ethanol stress.

Cumulatively, on the basis of these evidences we propose that BaeR is the primary regulator of *spy* gene in the presence of protein unfolding agent ethanol. Additional evidences comprising knock out mutants of *cpxR* and *baeR* in combination with the truncation study of *spy* promoter are in agreement with earlier studies, stating that the expression of *spy* gene is regulated by CpxR and BaeR dependent manner in the presence of Cu and Zn respectively (Bury-Moné et al., 2009; Yamamoto and Ishihama, 2006; Yamamoto et al., 2008). We also hypothesized that the Spy like hypothetical proteins that are conserved in other bacterial genomes might also be playing a key role in protecting the periplasmic proteins from external stimuli. Identified, yet uncharacterized Spy like proteins involved in protecting the bacterial cells from environmental insults can be characterized using the above approach. Conserved motif LTXXQ has been associated with Spy and CpxP proteins (Danese and Silhavy, 1998; Thede et al., 2011). Based on the preliminary observation that these hypothetical proteins show higher degree of conservation with Spy protein as compared to CpxP repressor, it will be worth carrying out detailed bioinformatics and functional characterization of this group of proteins which possess the LTXXQ motif. Further, conservation of CpxR and BaeR DNA binding motifs in promoters of Spy like hypothetical proteins, indicates that activation of these proteins might be regulated by BaeR and CpxR stress regulators during various environmental insults (Wang and Fierke, 2013).

In addition to understanding the basic aspects of gene regulation under stress, the observations of this study can be used in two ways for biotechnological applications. An appropriately selected module of promoter variant of *spy* can be used for development of whole cell biosensor to sense the environmental toxicants including protein unfolding agents (Melamed et al., 2012). Further, the uniqueness of Spy as an ATP-

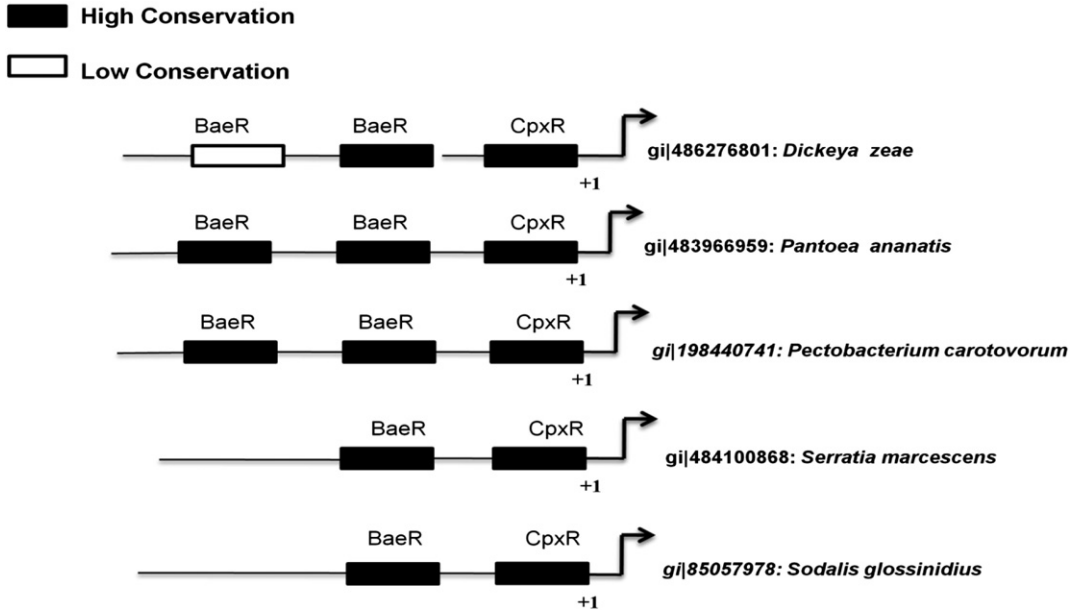

Fig. 5. Diagram showing the conservation of BaeR and CpxR binding motifs in the upstream sequence of gene encoding Spy like hypothetical protein in enterobacteria gi|486276801 *Dickeya zeae*, gi|483966959 *Pantoea ananatis*, gi|198440741 *Pectobacterium carotovorum*, gi|484100868 *Serratia marcescens*, and gi|85057978 *Sodalis glossinidius*.

independent chaperon coupled with knowledge about regulation of *spy* can also be tested for recombinant protein production in *E. coli*. Since Spy expression can reach up to 25% of the total periplasmic content (Quan et al., 2011), it will be interesting to study the effect of induction of Spy on refolding of recombinant proteins in *cpxR* knockout background.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Acknowledgments

This work is supported by National Agriculture Innovation Project (NAIP) grant number C4-30032 of the Indian Council of Agricultural Research (ICAR) and DBT grant number BT-MED-TF(3) 2012 Govt. of India to NKN and RP respectively. We thank Professor Steven Lindow at the University of California, Berkeley USA for a kind gift of plasmid pPROBE-TT′-GFP.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.07.003.

References

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., Mori, H., 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2 (2006), 0008. http://dx.doi.org/10.1038/msb4100050.

Brynildsen, M.P., Liao, J.C., 2009. An integrated network approach identifies the isobutanol response network of *Escherichia coli*. Mol. Syst. Biol. 5 (1), 277–290.

Bury-Moné, S., Nomane, Y., Reymond, N., Barbet, R., Jacquet, E., Imbeaud, S., Jacq, A., Bouloc, P., 2009. Global analysis of extracytoplasmic stress signaling in *Escherichia coli*. PLoS Genet. 5 (9), e1000651.

Danese, P.N., Silhavy, T.J., 1998. CpxP, a stress-combative member of the Cpx regulon. J. Bacteriol. 180, 831–839.

Hartl, F.U., 1996. Molecular chaperones in cellular protein folding. Nature 381 (6583), 571–579.

Kwon, E., Kim, D.Y., Gross, C.A., Gross, J.D., Kim, K.K., 2010. The crystal structure *Escherichia coli* Spy. Protein Sci. 19, 2252–2259.

Kyratsous, C.A., Silverstein, S.J., DeLong, C.R., Panagiotidis, C.A., 2009. Chaperone-fusion expression plasmid vectors for improved solubility of recombinant proteins in *Escherichia coli*. Gene 440, 9–15.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.

Lund, P.A., 2001. Microbial molecular chaperones. Adv. Microb. Physiol. 44, 93–140.

Mackey, K., Chomczynski, P., 1996. Long-term stability of RNA isolation reagents. J. NIH Res. 8, 72.

MacRitchie, D.M., Buelow, D.R., Price, N.L., Raivio, T.L., 2008. Two-component signaling and gram negative envelope stress response systems. Bacterial Signal Transduction: Networks and Drug Targets. Springer, pp. 80–110.

Maniatis, T., Fritsch, E.F., Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Melamed, S., Elad, T., Belkin, S., 2012. Microbial sensor cell arrays. Curr. Opin. Biotechnol. 23 (1), 2–8.

Miller, W.G., Leveau, J.H., Lindow, S.E., 2000. Improved gfp and inaZ broad-host-range promoter-probe vectors. Mol. Plant-Microbe Interact. 13, 1243–1250.

Nagasawa, S., Ishige, K., Mizuno, T., 1993. Novel members of the two-component signal transduction genes in *Escherichia coli*. J. Biochem. 114, 350–357.

Nishino, K., Honda, T., Yamaguchi, A., 2005. Genome-wide analyses of *Escherichia coli* gene expression responsive to the BaeSR two-component regulatory system. J. Bacteriol. 187, 1763–1772.

Powers, E.T., Balch, W.E., 2011. Protein folding: protection from the outside. Nature 471, 42–43.

Quan, S., Koldewey, P., Tapley, T., Kirsch, N., Ruane, K.M., Pfizenmaier, J., Shi, R., Hofmann, S., Foit, L., Ren, G., 2011. Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. Nat. Struct. Mol. Biol. 18, 262–269.

Rutherford, B.J., Dahl, R.H., Price, R.E., Szmidt, H.L., Benke, P.I., Mukhopadhyay, A., Keasling, J.D., 2010. Functional genomic study of exogenous n-butanol stress in *Escherichia coli*. Appl. Environ. Microbiol. 76, 1935–1945.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. Cold Spring Harbor Laboratory Press, New York.

Schlapschy, M., Skerra, A., 2011. Periplasmic chaperones used to enhance functional secretion of proteins in *E. coli*. Heterologous Gene Expression in *E. coli*. Springer, pp. 211–224.

Sonnhammer, E.L., Eddy, S.R., Birney, E., Bateman, A., Durbin, R., 1998. Pfam: multiple sequence alignments and HMM-profiles of protein domains. Nucleic Acids Res. 26, 320–322.

Taylor, R.G., Walker, D.C., McInnes, R., 1993. *E. coli* host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing. Nucleic Acids Res. 21, 1677–1678.

Thede, G.L., Arthur, D.C., Edwards, R.A., Buelow, D.R., Wong, J.L., Raivio, T.L., Glover, J.M., 2011. Structure of the periplasmic stress response protein CpxP. J. Bacteriol. 193, 2149–2157.

Vogt, S.L., Raivio, T.L., 2012. Just scratching the surface: an expanding view of the Cpx envelope stress response. FEMS Microbiol. Lett. 326, 2–11.

Wang, D., Fierke, C.A., 2013. The BaeSR regulon is involved in defense against zinc toxicity in *E. coli*. Metallomics 5, 372–383.

Yamamoto, K., Ishihama, A., 2006. Characterization of copper-inducible promoters regulated by CpxA/CpxR in *Escherichia coli*. Biosci. Biotechnol. Biochem. 70, 1688–1695.

Yamamoto, K., Ogasawara, H., Ishihama, A., 2008. Involvement of multiple transcription factors for metal-induced *spy* gene expression in *Escherichia coli*. J. Biotechnol. 133, 196–200.

Yoon, H., Hong, J., Ryu, S., 2008. Effects of chaperones on mRNA stability and gene expression in *Escherichia coli*. J. Microbiol. Biotechnol. 18, 228–233.