Biochemical and Biophysical Research Communications 521 (2020) 900–906

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Evaluation of specificity determinants in *Mycobacterium tuberculosis* σ/anti-σ factor interactions

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ARTICLE INFO

Article history:
Received 18 October 2019
Accepted 31 October 2019
Available online 8 November 2019

Keywords:
Transcription regulation
Protein-protein interactions
Signal transduction

ABSTRACT

Extra Cytoplasmic Function (ECF) σ factor/regulatory protein (anti-σ factor) pairs govern environment mediated changes in gene expression in bacteria. The release of the ECF σ factor from an inactive σ/anti-σ factor complex is triggered by specific environmental stimuli. The free σ factor then associates with the RNA polymerase and drives the expression of genes in its target regulon. Multiple ECF σ/anti-σ pairs ensure calibrated changes in the expression profile by correlating diverse environmental stimuli with changes in the intracellular levels of different ECF σ factors. Specificity in σ/anti-σ factor interaction is thus essential for accurate signal transduction. Here we describe experiments to evaluate interactions between different *M. tuberculosis* σ and anti-σ proteins *in vitro*. The interaction parameters suggest that cross-talk between non-cognate σ/anti-σ pairs is likely. The sequence and conformational determinants that govern interaction specificity in a σ/anti-σ complex are not immediately evident due to substantial structural conservation. Sequence-structure analysis of all σ/anti-σ pairs suggest that conserved residues are not the primary determinants of σ/anti-σ interactions-a finding that suggests a potential route to set tolerance limits in interaction specificity. Non-specific σ/anti-σ interactions are likely to be biologically significant as it can contribute to heterogeneity in cellular responses in a bacterial population under less stringent requirements. This finding is relevant for synthetic biology approaches to engineer bacteria using σ/anti-σ transcription initiation modules for diverse applications in biotechnology.

1. Introduction

The survival of bacteria in diverse environmental conditions depends on an effective stress response mechanism that synchronizes cellular response with environmental stimuli. In general, these systems involve a receptor which is membrane bound and a response unit which may be a specialized σ factor, or a transcriptional regulator in a two-component system [1,2]. These complexes also facilitate molecular crowding, substrate channeling, proteolysis and control of transcription factors by inhibitory protein-protein interactions thereby facilitating the adaptation machinery [3]. Environmental perturbations, nutrient change or shortage, stress factors and cell density significantly impact bacterial metabolism. Indeed, a close association between metabolism, regulation and coordinated shifts in protein complexes and system states has been observed in fast growing bacteria [4]. An analysis of signal transduction mediated by protein-protein interactions suggest that proteomes are organized into adaptable networks involving transient interactions as well as stable multi-protein complexes. Protein-protein complexes that govern these processes rapidly assemble and disassemble according to metabolic situations for their role during stress conditions [3]. The ability to rapidly alter the gene expression and proteomic profile is thus crucially dependent on the signal transduction mechanism that can sense and transmit environmental stimuli-a role mediated by one- and two-component systems as well as the Extra Cytoplasmic Function (ECF) σ/anti-σ factor complexes.

Transcription in prokaryotes is primarily regulated at the initiation step wherein σ factors recognize promoter elements by recruiting the apo-RNA polymerase enzyme to initiate transcription. The number and types of σ factors vary across bacteria. These σ factors compete for association with the RNA polymerase core enzyme. Reversible association of a σ factor with the RNA polymerase facilitates promoter DNA recognition and subsequently leads to the transcription of specific genes. The level of free σ factors in a prokaryotic cell thus governs the transcriptional profile.

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https://doi.org/10.1016/j.bbrc.2019.10.198
0006-291X/

Intracellular σ factor levels are subject to regulatory mechanisms that are sensitive to diverse environmental stimuli. These mechanisms operate at multiple levels spanning transcription and translation steps to post-translational mechanisms involving phosphorylation and protein-protein interactions. In this report, we refer to a specific mechanism of post translational control wherein a σ factor is localized in an inactive complex with an anti-σ factor. The release of a σ factor from an inactive σ/anti-σ complex is achieved by various mechanisms such as selective proteolysis of anti-σ factors, interaction of an anti-σ factor with its negative regulator also referred to as anti-*anti*-σ factors or by conformational changes that preclude σ factor binding thus leaving a free activated σ factor to form an active RNA polymerase holoenzyme complex [5].

About a third of bacterial RNA polymerase σ factors are known to have a negatively regulating protein or anti-σ factor [6]. These anti-σ factors sterically prevent σ factors from forming a holoenzyme complex. The σ/anti-σ pairs are localized and co-transcribed as they are often part of the same operon [7–9]. This observation led to the suggestion that their expression levels are co-evolved [10]. Co-evolution of expression by means of gene clustering or gene linkage guarantees a functional relationship and/or interaction of the constituent gene products. This phenomenon is believed to increase the chance of interaction between specific partners and helps maintain stoichiometry [10–12]. An alteration in the expression of components in a protein complex results in a severe effect on organismal survival, unlike alteration in the expression of non-interacting proteins [13]. Indeed, the syntenic arrangement of σ/anti-σ factor genes suggested that σ factors would necessarily only interact with cognate anti-σ factors [14–17].

Co-evolution of amino acid sequences in different proteins is well documented and often used to predict protein-protein interaction [10]. Coordinated evolution in protein sequence and expression levels are consistent with a stable interaction. These aspects, however are not strictly followed in the case of transcription factors and enhancers [18–20]. In *Mycoplasma genitalium,* for example, gene clustering could only account for 37% of the total functional interactome [21]. A weak selection pressure was suggested as a rationale for this observation. It thus appears likely that chromosomal adjacency may result in functional relationship between gene products but does not preclude the possibility of interacting proteins encoded by distant genetic loci. In two component system proteins, for example, about 15–25% of the proteins could participate in 'out of operon' cross talk, thus implying that chromosomal adjacency does not always guarantee specificity [22].

There are seven σ/anti-σ pairs in *Mycobacterium tuberculosis* of which the σ^L/RslA, σ^K/RskA, σ^M/RsmA and σ^D/RsdA complexes are membrane anchored by anti-σ factors. Sequence analysis of ten structurally characterized σ and anti-σ factors across all prokaryotes revealed poor sequence identity ranging between 5 and 16% in case of anti- σ factors and 3–30% in case of σ factors. Despite poor sequence conservation, their structures are well conserved (Supplementary Fig. 1). Here we describe experiments to evaluate the extent to which extensive structural conservation enables cross reactivity among σ and anti-σ factors (Fig. 1). There is limited information on cross-talk between σ/anti-σ factor pairs and its relevance to bacterial physiology and phenotypic diversity. One example is that of interactions between non-cognate pairs of *Azospirillum brasilense* σ/anti-σ factors, wherein two pairs of σ/anti-σ were found to form non-cognate complexes [23]. This is similar to the *E. coli* σ^{70} specific anti–σ factor Rsd that interacts with and controls σ^{38} [24]. In another variant, in *Pseudomonas aeruginosa*, one anti-σ factor was observed to interact with two σ factors and elicit a functional response [25]. While these reports suggest that

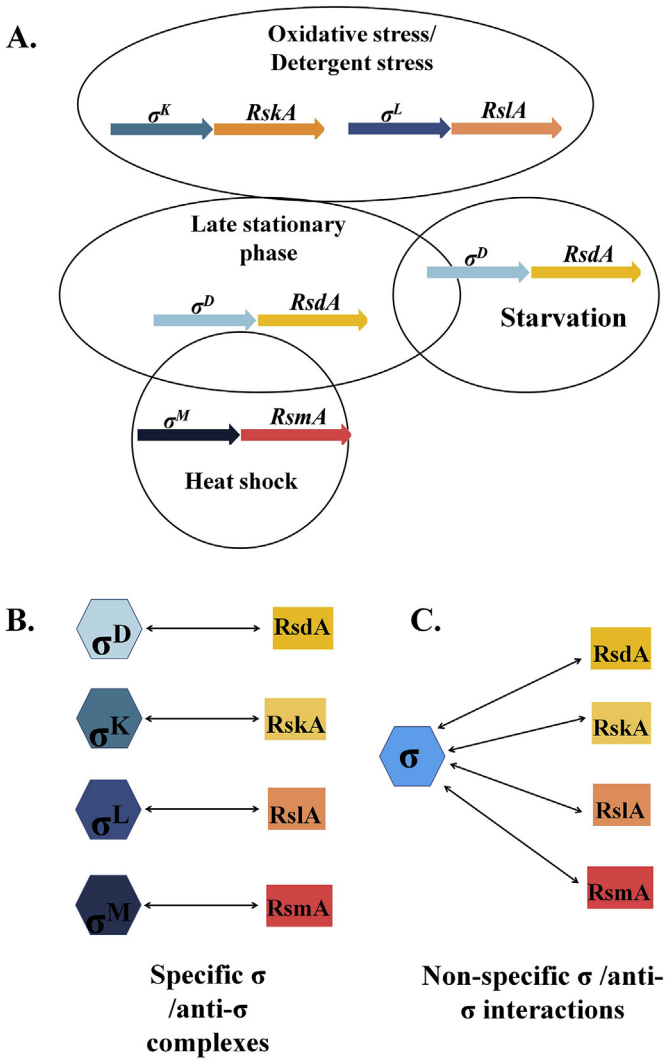

Fig. 1. Schematic representation of the membrane-associated *Mycobacterium tuberculosis* Extracytoplasmic Function (ECF) σ factors. **A**. The specific environmental triggers that stimulate the activity of these σ factors is shown to depict the functional role and potentially overlapping triggers that activate the four membrane-associated σ/anti-σ complexes. **B**. Functional interactions between different cognate pairs of σ/anti-σ factor proteins examined in this study. **C**. Possible cross-talk between different σ/anti-σ proteins.

cross-talk is feasible, the extent to which these can occur is poorly explored. The *Mycobacterium tuberculosis* σ/anti-σ factor complexes provide a natural model system to evaluate sequence and structural determinants that confer specificity in these interacting proteins. This information is also relevant from a synthetic biology perspective given the interest in utilizing σ/anti-σ factor transcription modules to engineer bacteria for different biotechnology applications.

2. Materials and methods

2.1. Sequence and structural analysis of σ/anti-σ factors

In order to analyze the sequence–structural features of σ factors and anti-σ factors, the structured domains present in all σ^{70} proteins *viz*., region 2 (σ_2) and region 4 (σ_4) and the N-terminal anti-σ domains (ASD) of anti-σ factors were examined (Supplementary Fig. 2). The sequences were pruned to include 227 σ_2 domains,

118 σ_4 domains and 44 ASDs by considering only UniProtKB annotated non-redundant sequences. Sequence alignment was performed by MAFFT (multiple-alignment program using E-INS iterative refinement algorithm) and PromalS3D [26]. *M. tuberculosis* σ^K/RskA (PDB: 4NQW) was a reference for secondary structure prediction. The sequence conservation from a structural perspective was visualized using ConSurf [27–31]. The sequence alignment profile was visualized by web-logo (Supplementary Fig. 2) using skylign.org [32]. Interface regions of structurally characterized σ/anti-σ factors (PDBIDs: 2Z2S, 3VEP, 3HUG, 4CXF, 4NQW, 5WUQ and 5WUR) were evaluated using protein interaction calculator (PIC) server [33] and PDBePISA.

2.2. Expression analysis

The *M. tuberculosis* mRNA and proteome data under different growth conditions were obtained from NCBI-GEO datasets [34,35]. The expression profiles of different σ factors and anti-σ proteins alongside expression for nineteen genes (referred to by protein names for consistency) were examined. This set included UsfX/RsbW, RsdA, RseA, RshA, RskA, RslA, RsmA, σ^B, σ^C, σ^D, σ^E, σ^F, σ^G, σ^H, σ^I, σ^J, σ^K, σ^L and σ^M. Expression levels of these genes were normalized and the significance of this information was evaluated. Subsequently, datasets that showed statistically significant expression changes for at least three genes were considered for further analysis. A pair-wise comparison heatmap was generated to depict the relative expression of these genes [36].

2.3. In silico evaluation of non-cognate interactions in *M. tuberculosis* σ/anti-σ factor

While the structures of σ^K/RskA, $\sigma^L{}_4$/RslA and $\sigma^D{}_4$/RsdA were

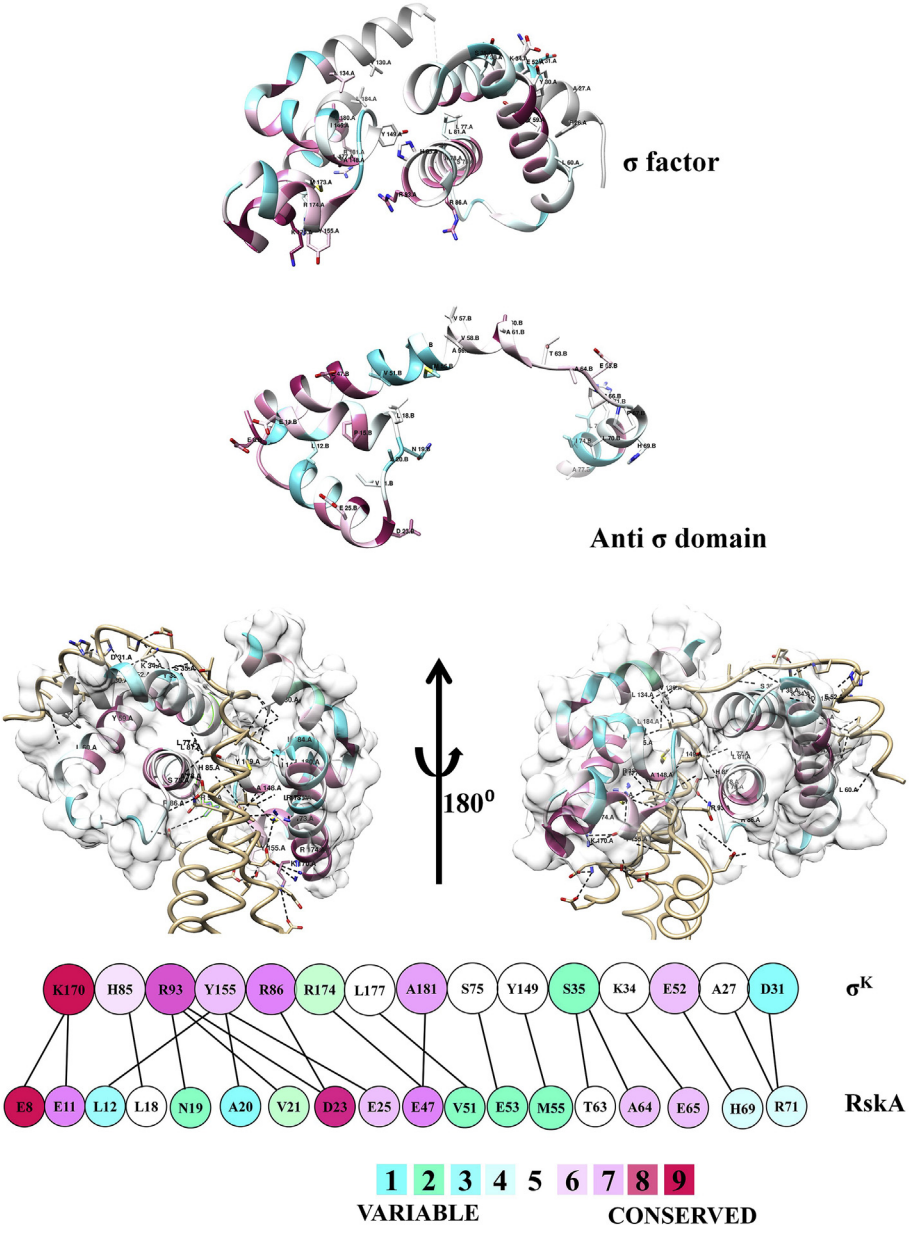

Fig. 2. Structural conservation in ECF σ factor/anti-σ complexes. We note that residues involved in σ/anti-σ interactions are not conserved across ECF σ factors. Cognate σ/anti-σ complexes have distinct interacting pockets that are likely to be disrupted in the case of non-cognate interactions. Interacting residues (other than hydrophobic interactions) are shown in pairs of circles colored based on conservation scores. An illustrative example of interactions between σ^K and RskA is shown.

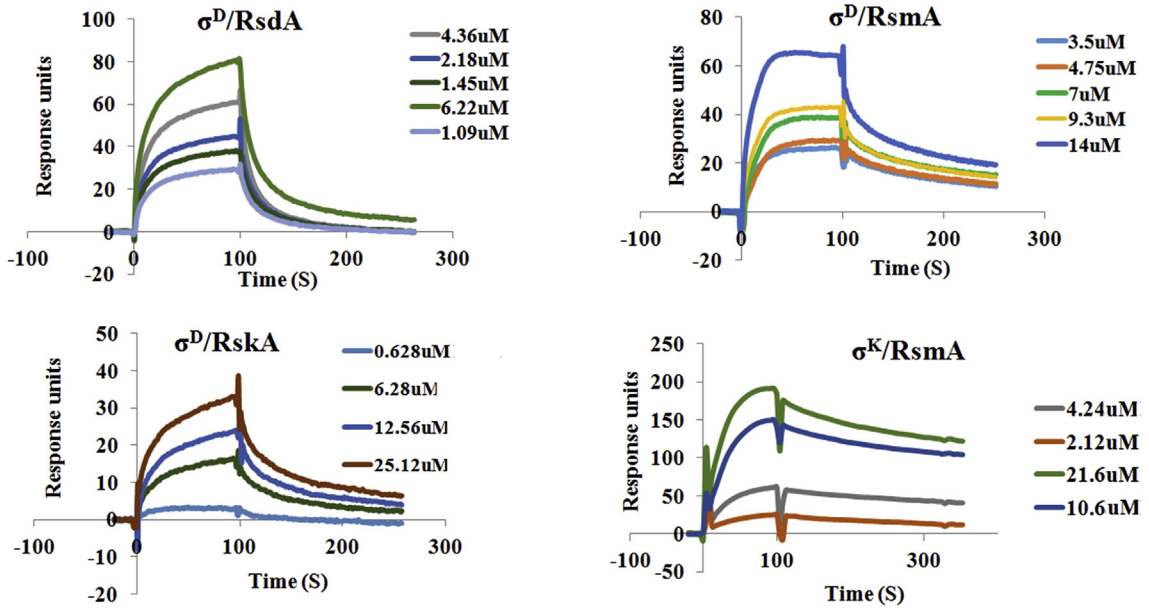

Fig. 3. Surface Plasmon Resonance analysis of interactions between different σ/anti-σ pairs. It is noteworthy that affinity does not appear to be the sole determinant that ensures cognate pairing of σ/anti-σ complexes.

from the PDB (4NQW, 3HUG and 3VEP/3VFZ), models for σ_2 domains of σ^D, σ^L, as well as σ^M/RsmA were generated using multiple templates selected by the sequence alignments using the MAFFT-E-ins algorithm [37]. Structural models were generated using MODELLER [38]. Side chain conformations and short contacts/clashes were resolved and the structures were energy minimized using GROMACS [39].

All non-cognate σ factors/anti-σ structures were obtained by docking using the SwarmDock server with default parameters in full-blind mode [40–42]. Both conserved domains of ECF σ factors were docked separately on to the ASDs. The solutions were visualized in UCSF Chimera [43] and models with no atomic clashes and highest number of interactions within a 5 Å radius cut off were selected.

2.4. Purification of recombinant σ and anti-σ factors

A co-expression and co-purification strategy was adopted for the expression and purification of recombinant protein complexes as σ factors in isolation have poor biochemical characteristics [44]. Different σ/anti–σ pairs were expressed and purified from *E. coli* expression cell strains. All the genes encoding σ and anti–σ factors were cloned in pET Duet-1 vector in MCS-1 and MCS-2 respectively (Supplementary Table 1). The complexes were purified by immobilized metal affinity chromatography using HIS-Select HF Nickel affinity gel followed by size exclusion chromatography on a Sephacryl S200 column (GE Healthcare). The protein complexes were incubated in 8 M urea to unfold and obtain σ and anti–σ factors in isolation. The isolated proteins were refolded by stepwise dialysis against sodium-potassium phosphate buffered saline (PBS) with decreasing concentrations of urea. The renatured proteins were analyzed by circular dichroism spectroscopy and fluorescence spectroscopy to validate their structural content after refolding (Supplementary Fig. 4).

Table 1
Kinetic parameters for interactions between σ/anti σ factors.

	k_a (1/Ms)	k_d (1/s)	K_D (M)
σ^D/RsdA	4.09E+03	0.0106	2.91E-06
σ^D/RsmA	3.91E+03	8.62E-03	2.31E-06
σ^D/RskA	0.2557 + 03	7.56E-03	3.04E-05
σ^K/RsmA	2.094 + 03	4.68E-03	2.23E-06

The equilibrium dissociation constants between cognate σ/anti-σ factors reported were 2.6 ± 1.8 μM for σ^D/RsdA [46], 0.42 ± 0.05 μM for σ^K/RskA (oxidizing conditions) and 1.15 ± 0.12 μM for σ^K/RskA (reducing conditions) [45] and 20 nM for σ^L/RslA [47].

2.5. Spectroscopic analysis

The Circular Dichroism (CD) measurements were recorded from 250 nm to 190 nm for all the refolded proteins on a Jasco J-715 instrument (Jasco, Inc). The far-UV CD spectra were recorded in 5 mM phosphate buffer, pH 7.4 using a 1 mm path-length cuvette. The protein concentration was varied in the range 3–6 μM. The protein samples were also analyzed by fluorescence spectroscopy on a Jasco spectrofluorimeter with an excitation wavelength at 280 nm. The emission spectra were recorded between 300 and 400 nm.

2.6. Surface plasmon resonance (SPR) spectroscopy

The interactions between different combinations of four σ/ASDs pairs (σ^D, RsdA, σ^K, RskA, σ^L, RslA, σ^M and RsmA) were examined *in vitro* by surface plasmon resonance (SPR) experiments (BIACORE 3000; GE Healthcare). In these experiments σ^D, σ^K, RskA, Rsda, RslA and RsmA were immobilized on to a CM5 chip (Biacore; GE Healthcare) at a surface density of 10ng/mm^2 by amine coupling chemistry. The other σ factors and ASDs of anti-σ factors were analytes in these experiments. The interaction kinetics was evaluated using the BIA evaluation software version 3. The first lane of the chip served as a control and all the interactions were examined in a buffer consisting of 30 mM Phosphate, 120 mM NaCl pH 7.4, 5% glycerol.

3. Results and discussion

3.1. Sequence-structure features of σ/anti-σ factors

Extracytoplasmic Function σ factors have a minimalistic structural composition and are substantially smaller proteins when compared to other σ^{70} members. The two compact structured domains σ_2 (interacts with the Pribnow box element) and σ_4 (interacts with the −35 element) are structurally similar across all σ factors (Fig. 2; Supplementary Fig. 1). While the σ_4 domain has a

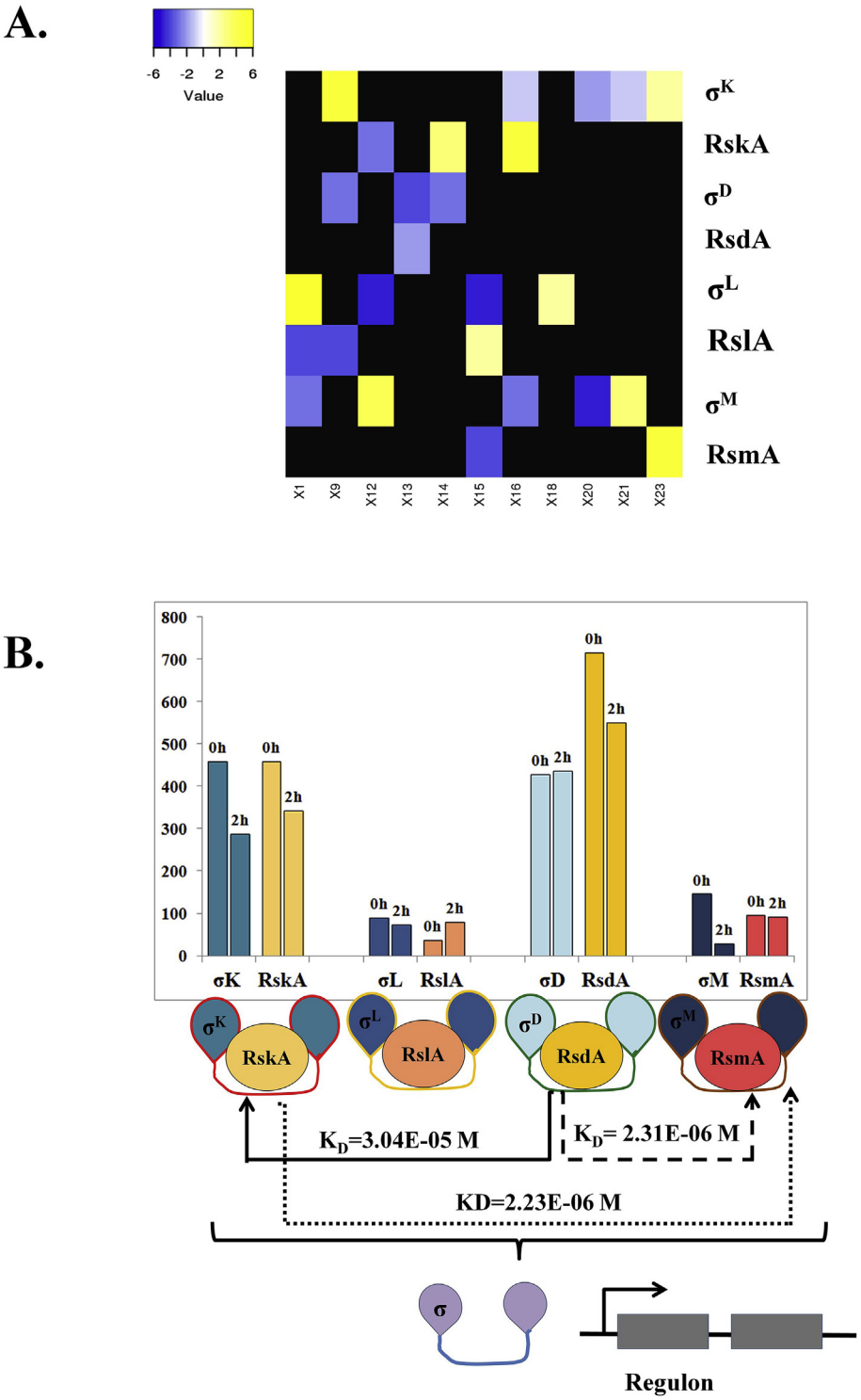

Fig. 4. A mechanistic model of how temporal association of non-cognate σ/anti-σ pairs could vary under different environmental contexts. **A**. Expression profile of eight σ/anti-σ factor proteins under eleven environmental conditions. The heat map shows up or down-regulation by yellow or blue colors respectively. The color intensity is proportional to the expression difference, as shown with the color bar. **B**. Change in expression levels of cognate σ/anti-σ pairs during exponential growth phase (0hr) and nitric oxide stress (2hr). The *in vitro* data supports the hypothesis that non-cognate σ/anti-σ complexes could affect minor perturbations in the expression profile in different bacterial cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conventional helix-turn-helix motif, σ_2 adopts an anti-parallel three helical bundle structure [45]. Sequence analysis of 227 σ_2 and 118 σ_4 domains suggested limited sequence conservation among the individual domains (Supplementary Fig. 2). All anti-σ factors, on the other hand, have a structurally conserved domain referred to the anti-σ domain (ASD) that interacts with a σ factor. The sequence conservation in the case of ASDs is relatively higher [6]. We note that a significant number of interactions made by the σ_2 and σ_4 domains with the ASD are not by conserved residues; instead less conserved residues mediate σ/anti-σ factor interactions (Fig. 2). It thus appears likely that although the structures are well conserved overall, specificity is maintained among cognate σ/anti-σ factor pairs by variations in the sequence composition. The extent to which these interactions govern specificity becomes clearer as these σ/anti-σ factor interactions are disrupted in a non-cognate complex. The interaction parameters (including hydrogen bonds, salt bridges and buried surface area) reveal stronger binding between cognate pairs of proteins when compared to non-cognate pairs.

3.2. Surface plasmon resonance analysis of σ/anti-σ factor interactions

Recombinant *M. tuberculosis* σ/anti-σ factor complexes were purified by a co-expression and co-purification strategy as described earlier [44]. Denaturing conditions were necessary to obtain separate protein components (Supplementary Fig. 3). After this step, the refolded proteins were assessed for their structural content by circular dichroism (CD) and fluorescence spectroscopy (Supplementary Figs. 3 and 4). The far-UV CD spectra revealed that the purified proteins were well folded with expected α-helical content. The SPR sensorgrams were evaluated using BIA evaluation software version 3, with 1:1 Langmuir separate dissociation and association kinetics (Fig. 3). These results suggest interactions between non-cognate pairs of proteins such as σ^D/RskA, σ^D/RsmA and σ^K/RsmA. The association (k_a) and dissociation (k_d) constants along with the equilibrium dissociation constants (K_D) are compiled in Table 1. The Chi^2 values suggest good fit to the interaction model (0.165, 0.117, 0.034 and 0.712 for σ^D/RsdA, σ^D/RsmA σ^D/RskA and σ^K/RsmA respectively). The kinetic parameters suggest that RskA interacts with 10-fold lower affinity with σ^D than RsdA whereas RsmA interacts with almost the same affinity as the cognate (σ^D/RsdA) pair. The association rates are relatively higher in the case of cognate interactions whereas the dissociation is faster in the case non-cognate interactions (Table 1).

3.3. Expression analysis of M. tuberculosis σ and anti-σ factors

Nineteen proteins (UsfX (Rv3287c), RsdA (Rv3413c), RseA (Rv1222), RshA (Rv3221A), RskA (Rv0444c), RslA (Rv0736), RsmA (Rv3912), σ^B (Rv2710), σ^C (Rv2069), σ^D (Rv3414c), σ^E (Rv1221), σ^F (Rv3286c), σ^G (Rv0182c), σ^H (Rv3223c), σ^I (Rv1189), σ^J (Rv3328c), σ^K (Rv0445c), σ^L (Rv0735) and σ^M (Rv3911) were analyzed for their expression levels under different growth and stress conditions. A mismatch between the expression levels of genes from the same operon was seen in some conditions (Fig. 4). In Fig. 4, the S1–S23 labels refer to arachidonic acid, carbonyl cyanide chlorophenylhydrazone, cephalexin, clotrimazole, diamide, dithiobis nitrobenzoic acid, ethambutol, ethionamide, hypoxia, iron, isoniazid, linoleic acid, non-replicating persistence, null mutant vs. complemented null mutant, oleic acid, palmitic acid, reaeration, SDS, sediment vs. pellicle, starvation, streptomycin, tetracycline and wild-type vs. mutant. Expression data of a subset of eight genes (*RsdA* (*Rv3413c*), *RskA* (*Rv0444c*), *RslA* (*Rv0736*), *RsmA* (*Rv3912*), *SigD* (*Rv3414c*), *SigK* (*Rv0445c*), *SigL* (*Rv0735*) and *SigM* (*Rv3911*) exemplifies this feature simplification (Fig. 4). This expression mismatch in genes in the same operon suggested the possibility of different intracellular levels of these proteins-thus enabling non-specific interactions.

Three observations suggest that non-specific interactions between σ and anti-σ factors are likely. Three observations that support this suggestion include (i) σ/anti-σ factor interactions are not strictly governed by conserved residues (ii) expression levels of σ and anti-σ genes are not as tightly correlated as expected from the co-localization of these genes in the same operon and (iii) *in vitro* analysis of non-cognate interactions between different σ and anti-σ factors suggest that the binding affinity is not substantially lower in the case of non-cognate interactions when compared to the cognate pair. One likely utility in allowing some flexibility in non-cognate σ/anti-σ interactions could be in modulating responses in the case of ECF σ factors where the regulons and/or the environmental triggers overlap (Fig. 4). The other, perhaps more compelling reason, could be the potential of non-cognate interactions to influence heterogeneity within a bacterial population that encounters a specific environmental condition. More studies would be needed to understand the role of these interactions in single cell variants that could aid adaptation and facilitate persisters to survive bactericidal conditions. Indeed, it is likely that this mechanism enables bacterial survival by aiding phenotypic diversity similar to that enabled by mutagenesis events. These findings are also significant from the perspective of synthetic biology approaches of using σ/anti-σ modules to trigger the expression of specific regulons under defined environmental conditions. This project has been supported in part by a grant from the office of the Principal scientific advisor (India), DBT (grant for structural studies and DBT-IISc partnership program) and the DST-IRHPA scheme.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.10.198.

References

[1] S. Gottesman, Stress reduction, bacterial style, J. Bacteriol. 199 (2017), https://doi.org/10.1128/JB.00433-17 e00433-17,/jb/199/20/e00433-17.atom.
[2] R.L. Guest, J. Wang, J.L. Wong, T.L. Raivio, A bacterial stress response regulates respiratory protein complexes to control envelope stress adaptation, J. Bacteriol. 199 (2017), https://doi.org/10.1128/JB.00153-17 e00153-17,/jb/199/20/e00153-17.atom.
[3] B. Krüger, C. Liang, F. Prell, A. Fieselmann, A. Moya, S. Schuster, U. Völker, T. Dandekar, Metabolic adaptation and protein complexes in prokaryotes, Metabolites 2 (2012) 940–958, https://doi.org/10.3390/metabo2040940.
[4] O. Kotte, J.B. Zaugg, M. Heinemann, Bacterial adaptation through distributed sensing of metabolic fluxes, Mol. Syst. Biol. 6 (2010) 355, https://doi.org/10.1038/msb.2010.10.
[5] E.A. Campbell, J.L. Tupy, T.M. Gruber, S. Wang, M.M. Sharp, C.A. Gross, S.A. Darst, Crystal structure of Escherichia coli sigmaE with the cytoplasmic domain of its anti-sigma RseA, Mol. Cell 11 (2003) 1067–1078.
[6] E.A. Campbell, R. Greenwell, J.R. Anthony, S. Wang, L. Lim, K. Das, H.J. Sofia, T.J. Donohue, S.A. Darst, A conserved structural module regulates transcriptional responses to diverse stress signals in bacteria, Mol. Cell 27 (2007) 793–805, https://doi.org/10.1016/j.molcel.2007.07.009.
[7] K.L. Brown, K.T. Hughes, The role of anti-sigma factors in gene regulation, Mol. Microbiol. 16 (1995) 397–404, https://doi.org/10.1111/j.1365-2958.1995.tb02405.x.
[8] M.N. Mishra, S. Kumar, N. Gupta, S. Kaur, A. Gupta, A.K. Tripathi, An extracytoplasmic function sigma factor co transcribed with its cognate anti-sigma factor confers tolerance to NaCl, ethanol and methylene blue in Azospirillum brasilense Sp7, Microbiol. (Reading, Engl.) 157 (2011) 988–999, https://doi.org/10.1099/mic.0.046672-0.
[9] A. Staroń, H.J. Sofia, S. Dietrich, L.E. Ulrich, H. Liesegang, T. Mascher, The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family, Mol. Microbiol. 74 (2009)

557–581, https://doi.org/10.1111/j.1365-2958.2009.06870.x.
[10] H.B. Fraser, A.E. Hirsh, Evolutionary rate depends on number of protein-protein interactions independently of gene expression level, BMC Evol. Biol. 4 (2004) 13, https://doi.org/10.1186/1471-2148-4-13.
[11] F.-C. Chen, T.-J. Chuang, The effects of multiple features of alternatively spliced exons on the K(A)/K(S) ratio test, BMC Bioinf. 7 (2006) 259, https://doi.org/10.1186/1471-2105-7-259.
[12] S.A. Teichmann, R.A. Veitia, Genes encoding subunits of stable complexes are clustered on the yeast chromosomes: an interpretation from a dosage balance perspective, Genetics 167 (2004) 2121–2125, https://doi.org/10.1534/genetics.103.024505.
[13] B. Papp, C. Pál, L.D. Hurst, Dosage sensitivity and the evolution of gene families in yeast, Nature 424 (2003) 194–197, https://doi.org/10.1038/nature01771.
[14] D. Santoni, F. Castiglione, P. Paci, Identifying correlations between chromosomal proximity of genes and distance of their products in protein-protein interaction networks of yeast, PLoS One 8 (2013), https://doi.org/10.1371/journal.pone.0057707 e57707.
[15] T. Dandekar, B. Snel, M. Huynen, P. Bork, Conservation of gene order: a fingerprint of proteins that physically interact, Trends Biochem. Sci. 23 (1998) 324–328.
[16] S. Wuchty, A.-L. Barabási, M.T. Ferdig, Stable evolutionary signal in a yeast protein interaction network, BMC Evol. Biol. 6 (2006) 8, https://doi.org/10.1186/1471-2148-6-8.
[17] N. Gupta, S. Kumar, M.N. Mishra, A.K. Tripathi, A constitutively expressed pair of rpoE2-chrR2 in Azospirillum brasilense Sp7 is required for survival under antibiotic and oxidative stress, Microbiol. (Reading, Engl.) 159 (2013) 205–218, https://doi.org/10.1099/mic.0.061937-0.
[18] D.A. Kleinjan, A. Seawright, G. Elgar, V. van Heyningen, Characterization of a novel gene adjacent to PAX6, revealing synteny conservation with functional significance, Mamm. Genome 13 (2002) 102–107, https://doi.org/10.1007/s00335-001-3058-y.
[19] D.A. Kleinjan, A. Seawright, S. Mella, C.B. Carr, D.A. Tyas, T.I. Simpson, J.O. Mason, D.J. Price, V. van Heyningen, Long-range downstream enhancers are essential for Pax6 expression, Dev. Biol. 299 (2006) 563–581, https://doi.org/10.1016/j.ydbio.2006.08.060.
[20] L.A. Lettice, S.J.H. Heaney, L.A. Purdie, L. Li, P. de Beer, B.A. Oostra, D. Goode, G. Elgar, R.E. Hill, E. de Graaff, A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly, Hum. Mol. Genet. 12 (2003) 1725–1735, https://doi.org/10.1093/hmg/ddg180.
[21] M. Huynen, B. Snel, W. Lathe, P. Bork, Predicting protein function by genomic context: quantitative evaluation and qualitative inferences, Genome Res. 10 (2000) 1204–1210, https://doi.org/10.1101/gr.10.8.1204.
[22] A. Procaccini, B. Lunt, H. Szurmant, T. Hwa, M. Weigt, Dissecting the specificity of protein-protein interaction in bacterial two-component signaling: orphans and crosstalks, PLoS One 6 (2011), https://doi.org/10.1371/journal.pone.0019729 e19729.
[23] N. Gupta, A. Gupta, S. Kumar, R. Mishra, C. Singh, A.K. Tripathi, Cross-talk between cognate and noncognate RpoE sigma factors and Zn(2+)-binding anti-sigma factors regulates photooxidative stress response in Azospirillum brasilense, Antioxidants Redox Signal. 20 (2014) 42–59, https://doi.org/10.1089/ars.2013.5314.
[24] N. Hofmann, R. Wurm, R. Wagner, The E. coli anti-sigma factor Rsd: studies on the specificity and regulation of its expression, PLoS One 6 (2011), https://doi.org/10.1371/journal.pone.0019235 e19235.
[25] R.J. Edgar, X. Xu, M. Shirley, A.F. Konings, L.W. Martin, D.F. Ackerley, I.L. Lamont, Interactions between an anti-sigma protein and two sigma factors that regulate the pyoverdine signaling pathway in Pseudomonas aeruginosa, BMC Microbiol. 14 (2014) 287, https://doi.org/10.1186/s12866-014-0287-2.
[26] J. Pei, B.-H. Kim, N.V. Grishin, PROMALS3D: a tool for multiple protein sequence and structure alignments, Nucleic Acids Res. 36 (2008) 2295–2300, https://doi.org/10.1093/nar/gkn072.
[27] F. Glaser, T. Pupko, I. Paz, R.E. Bell, D. Bechor-Shental, E. Martz, N. Ben-Tal, ConSurf: identification of functional regions in proteins by surface-mapping of phylogenetic information, Bioinformatics 19 (2003) 163–164, https://doi.org/10.1093/bioinformatics/19.1.163.
[28] M. Landau, I. Mayrose, Y. Rosenberg, F. Glaser, E. Martz, T. Pupko, N. Ben-Tal, ConSurf 2005: the projection of evolutionary conservation scores of residues on protein structures, Nucleic Acids Res. 33 (2005) W299–W302, https://doi.org/10.1093/nar/gki370.
[29] H. Ashkenazy, E. Erez, E. Martz, T. Pupko, N. Ben-Tal, ConSurf, Calculating evolutionary conservation in sequence and structure of proteins and nucleic acids, Nucleic Acids Res. 38 (2010) W529–W533, https://doi.org/10.1093/nar/gkq399, 2010.
[30] G. Celniker, G. Nimrod, H. Ashkenazy, F. Glaser, E. Martz, I. Mayrose, T. Pupko, N. Ben-Tal, ConSurf: using evolutionary data to raise testable hypotheses about protein function, Isr. J. Chem. 53 (2013) 199–206, https://doi.org/10.1002/ijch.201200096.
[31] H. Ashkenazy, S. Abadi, E. Martz, O. Chay, I. Mayrose, T. Pupko, N. Ben-Tal, ConSurf, An improved methodology to estimate and visualize evolutionary conservation in macromolecules, Nucleic Acids Res. 44 (2016) W344–W350, https://doi.org/10.1093/nar/gkw408, 2016.
[32] T.J. Wheeler, J. Clements, R.D. Finn, Skylign: a tool for creating informative, interactive logos representing sequence alignments and profile hidden Markov models, BMC Bioinf. 15 (2014) 7, https://doi.org/10.1186/1471-2105-15-7.
[33] K.G. Tina, R. Bhadra, N. Srinivasan, PIC: protein interactions calculator, Nucleic Acids Res. 35 (2007) W473–W476, https://doi.org/10.1093/nar/gkm423.
[34] T.R. Rustad, K.J. Minch, S. Ma, J.K. Winkler, S. Hobbs, M. Hickey, W. Brabant, S. Turkarslan, N.D. Price, N.S. Baliga, D.R. Sherman, Mapping and manipulating the Mycobacterium tuberculosis transcriptome using a transcription factor overexpression-derived regulatory network, Genome Biol. 15 (2014) 502, https://doi.org/10.1186/s13059-014-0502-3.
[35] T. Cortes, O.T. Schubert, A. Banaei-Esfahani, B.C. Collins, R. Aebersold, D.B. Young, Delayed effects of transcriptional responses in Mycobacterium tuberculosis exposed to nitric oxide suggest other mechanisms involved in survival, Sci. Rep. 7 (2017) 8208, https://doi.org/10.1038/s41598-017-08306-1.
[36] S. Babicki, D. Arndt, A. Marcu, Y. Liang, J.R. Grant, A. Maciejewski, D.S. Wishart, Heatmapper: web-enabled heat mapping for all, Nucleic Acids Res. 44 (2016) W147–W153, https://doi.org/10.1093/nar/gkw419.
[37] K. Katoh, K. Kuma, H. Toh, T. Miyata, MAFFT version 5: improvement in accuracy of multiple sequence alignment, Nucleic Acids Res. 33 (2005) 511–518, https://doi.org/10.1093/nar/gki198.
[38] A. Sali, T.L. Blundell, Comparative protein modelling by satisfaction of spatial restraints, J. Mol. Biol. 234 (1993) 779–815, https://doi.org/10.1006/jmbi.1993.1626.
[39] D. Van Der Spoel, E. Lindahl, B. Hess, G. Groenhof, A.E. Mark, H.J.C. Berendsen, GROMACS: fast, flexible, and free, J. Comput. Chem. 26 (2005) 1701–1718, https://doi.org/10.1002/jcc.20291.
[40] M. Torchala, I.H. Moal, R.A.G. Chaleil, J. Fernandez-Recio, P.A. Bates, SwarmDock: a server for flexible protein-protein docking, Bioinformatics 29 (2013) 807–809, https://doi.org/10.1093/bioinformatics/btt038.
[41] M. Torchala, I.H. Moal, R.A.G. Chaleil, R. Agius, P.A. Bates, A Markov-chain model description of binding funnels to enhance the ranking of docked solutions, Proteins 81 (2013) 2143–2149, https://doi.org/10.1002/prot.24369.
[42] M. Torchala, P.A. Bates, Predicting the structure of protein-protein complexes using the SwarmDock Web Server, Methods Mol. Biol. 1137 (2014) 181–197, https://doi.org/10.1007/978-1-4939-0366-5_13.
[43] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF Chimera–a visualization system for exploratory research and analysis, J. Comput. Chem. 25 (2004) 1605–1612, https://doi.org/10.1002/jcc.20084.
[44] K.G. Thakur, R.K. Jaiswal, J.K. Shukla, T. Praveena, B. Gopal, Over-expression and purification strategies for recombinant multi-protein oligomers: a case study of Mycobacterium tuberculosis σ/anti-σ factor protein complexes, Protein Expr. Purif. 74 (2010) 223–230, https://doi.org/10.1016/j.pep.2010.06.018.
[45] J. Shukla, R. Gupta, K.G. Thakur, R. Gokhale, B. Gopal, Structural basis for the redox sensitivity of the *Mycobacterium tuberculosis* SigK–RskA σ–anti-σ complex, Acta Crystallogr D Biol Crystallogr 70 (2014) 1026–1036, https://doi.org/10.1107/S1399004714000121.
[46] R.K. Jaiswal, T.S. Prabha, G. Manjeera, B. Gopal, Mycobacterium tuberculosis RsdA provides a conformational rationale for selective regulation of σ-factor activity by proteolysis, Nucleic Acids Res. 41 (2013) 3414–3423, https://doi.org/10.1093/nar/gks1468.
[47] K.G. Thakur, T. Praveena, B. Gopal, Structural and biochemical bases for the redox sensitivity of Mycobacterium tuberculosis RslA, J. Mol. Biol. 397 (2010) 1199–1208, https://doi.org/10.1016/j.jmb.2010.02.026.