



Full Length Article

Rational design of Zeocin binding protein variants for antibiotic resistance studies



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ABSTRACT

Antibiotic resistance marker was used in this investigation because they are selected;e markers and effective in different hosts. This study used Zeocin binding protein (ZBP) due to its known 3D structure and applicability in prokaryotic and eukaryotic hosts. A library of 22 mutations was developed through a rational design strategy. Subsequently, a selection strategy was used to identify destabilizing mutations in the ZBP. ZBP variants were expressed in *E.coli* using a leaky expression approach, and minimum inhibitory concentration (MIC) was calculated by cultivating different variants at various Zeocin concentrations. Zeocin resistance was drastically decreased in some ZBP variants. Positive controls (wild-type ZBP) exhibited high resistance, while negative controls (pET vector without ZBP) showed susceptibility. Two variants (ZBP P9E and ZBP R26F) displayed drastic resistance loss. The variants reported in this study may identify molecular chaperones and folding modulators affecting proteostasis. These variants can potentially discover chemical chaperones that improve the stability and solubility of destabilized ZBP variants.

1. Introduction

The cellular proteostasis network tightly regulates the lifecycle of proteins, from synthesis to termination. The proteostasis network monitors protein activity throughout their functional phase, maintaining proper interactions and cellular balance. The termination phase, marking the end of a protein's lifecycle, involves controlled degradation (Jayaraj et al., 2020). Disturbances in proteostasis are a contributing factor to a range of diseases (Kikis et al., 2010, Sweeney et al., 2017). Alzheimer's, Parkinson's, Huntington's, and Amyotrophic Lateral Sclerosis (ALS) diseases cause neuron degeneration due to protein misfolding (Lamprey et al., 2022). In type II diabetes, amylin misfolding causes pancreatic dysfunction, while CFTR protein misfolding causes cystic fibrosis (Fukuda and Okiyonedo 2020, Hassan et al., 2022). Additionally, misfolded proteins are associated with certain malignancies (Chen et al., 2017, Kawano et al., 2023).

The mammalian proteostasis network comprises over 2000 genes (Klaips et al., 2018, Lualdi et al., 2020). Identification of genes encoding protein folding modulators remains challenging without efficient,

widely applicable, and sensitive in vivo protein folding assays. The protein folding quality-control machinery ensures the proper balance between folded and unfolded proteins. All organisms possess an efficient system to minimize the protein misfolding, aided by specialized proteins known as molecular chaperones (Vabulas et al., 2010, Chen et al., 2011). Chaperoning was first observed in 1962 under thermal stress, leading to the term "molecular chaperone" coining in 1978 (Ritossa, 1962, Laskey et al., 1978). During the early 1980s, Heat Shock Proteins (Hsps) were identified as molecular chaperones, later found to be conserved across different species. Traditional methods for identifying molecular chaperones include stress induction, mutation causing proteome instability, homology to known chaperones, binding to other proteins, and *in vitro* chaperone activity assays (Quan and Bardwell, 2012). While these methodologies have been fruitful, they lack specificity. For instance, heat stress induces the expression of 50–200 genes, with the most strongly induced proteins typically involved in chaperoning, while others are engaged in activities such as regulation, metabolism, nucleic acid binding, and proteolysis (Quan and Bardwell, 2012). Combining multiple approaches and focusing on specific targets can enhance the

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Table 1

A list of ZBP variants.

Clone No	Clone name	Mutation site on ZBP	Mutation site on ZBP fusion protein
1.	Neg. Control		
2.	ZBP (wt)		
3.	ZBP P9E	P9E	ZBP P29E
4.	ZBP L11A	L11A	ZBP L31A
5.	ZBP V16A	V16A	ZBP V36A
6.	ZBP V20A	V20A	ZBP V40A
7.	ZBP F22A	F22A	ZBP F42A
8.	ZBP D25G	D25G	ZBP D45G
9.	ZBP D25K	D25K	ZBP D45K
10.	ZBP R26G	R26G	ZBP R46G
11.	ZBP R26F	R26F	ZBP R46F
12.	ZBP L27A	L27A	ZBP L47A
13.	ZBP L27G	L27G	ZBP L47G
14.	ZBP F29A	F29A	ZBP F49A
15.	ZBP R31A	R31A	ZBP R51A
16.	ZBP R43A	R43A	ZBP R63A
17.	ZBP R69A	R69A	ZBP R89A
18.	ZBP L71A	L71A	ZBP L91A
19.	ZBP Y75A	Y75A	ZBP Y95A
20.	ZBP E77A	E77A	ZBP E97A
21.	ZBP W78A	W78A	ZBP W98A
22.	ZBP V81A	V81A	ZBP V101A
23.	ZBP V82A	V82A	ZBP V102A
24.	ZBP L108A	L108A	ZBP L128A

specificity of chaperone discovery, but traditional methods are time-consuming and labor-intensive.

Given the stochastic nature of chaperone discovery, the current list of molecular chaperones is likely incomplete. Previous studies using beta-lactamase and DsbA folding biosensors identified the periplasmic chaperone Spy (Quan et al., 2011). However, these biosensors are limited to the periplasmic space (Foit et al., 2009, Quan et al., 2011). A partially destabilized ZBP can serve as an effective protein-folding biosensor, facilitating the discovery of novel molecular chaperones and folding modulators. By leveraging the correlation between ZBP expression levels and antibiotic resistance, this biosensor will allow for efficient *in vivo* screening across diverse hosts, overcoming the limitations of traditional chaperone identification methods.

Zeocin, belonging to the bleomycin/phleomycin antibiotic family, exhibits strong toxicity against a wide range of cells (Drocourt et al., 1990). The zeocin formulation contains the active ingredient phleomycin D1, a basic copper-chelated glycopeptide. Zeocin specifically breaks double-stranded DNA, causing cytotoxicity in both prokaryotic and eukaryotic hosts (Gatignol et al., 1987, Gatignol et al., 1988, Perez et al.,

1989, Calmels et al., 1991). The *ShBle* gene from *Streptoalloteichus hindustanus*, a bleomycin resistance marker, was employed in this study. ZBP, a 13.6 kDa homodimeric protein, binds stoichiometrically with high affinity to various antibiotics of the bleomycin family (Gatignol et al., 1988). A tight correlation has been established between the antibiotic resistance of the host cell and the level of ZBP gene expression. The development of ZBP as a protein-folding biosensor is motivated by its wide selection range, cost-effectiveness, and powerful selectable phenotype in both prokaryotic and eukaryotic hosts (Dumas et al., 1994).

2. Material and methods

2.1. Materials

The *Escherichia coli* BL21 (DE3)-RIL competent cell obtained from Agilent Technologies was employed for cloning and Zeocin sensitivity assay. The pET3a plasmid (Novagen) was utilized for the cloning process. The Zeocin was purchased from Invitrogen. Sigma-Aldrich provided LB Broth (Lennox) and LB agar (Lennox agar). Ampicillin was obtained from Biobasic. All the remaining reagents were of analytical grade.

2.2. Cloning of ZBP (WT)

The *ShBle* gene from *Streptoalloteichus hindustanus* has been optimized for expression in *E. coli* using codon optimization. Following that, a His-tag and a highly specific cleavage site (TEV protease site) were fused at the N-terminus of ZBP. The ZBP fusion protein was inserted into the pET-3a plasmid at the NdeI and BamHI restriction enzyme sites (Genscript). The ZBP (WT) fusion protein was transformed into a chemically competent *Escherichia coli* BL21 (DE3)-RIL strain and then selected on an LBamp (200 µg/ml) plate.

2.3. Generation of ZBP mutant library

The development of the ZBP mutant library was carried out using a rational design technique. ZBP's primary structure consists of 124 amino acids. An in-depth analysis was conducted on the conserved residues in the primary structure and the 3D crystal structure to assess the residues contributing to the stabilization of ZBP. Furthermore, the crystal structure of ZBP revealed the involvement of 21 residues in the binding pocket (Dumas et al., 1994). A set of 22 mutants (Table 1) was created, omitting the residues directly involved in Zeocin binding or interacting with the Zeocin binding residues. The selection criteria were based on the idea that the mutation should not impact the Zeocin

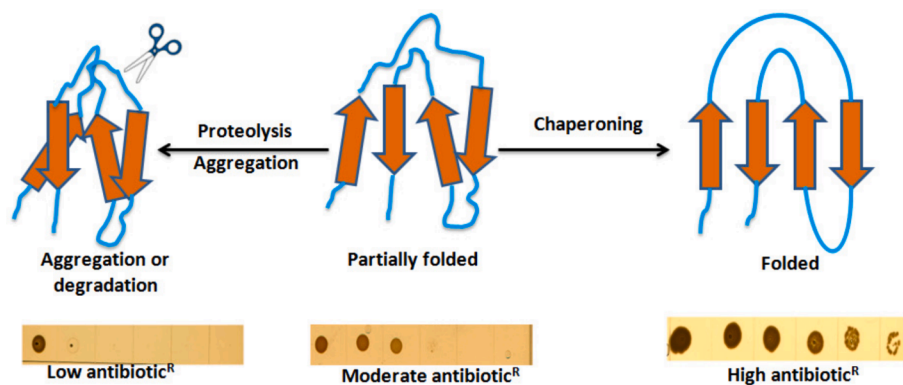


Fig. 1. Schematic diagram of biosensor to measure *in vivo* protein folding. A ZBP-based biosensor (destabilized and partially folded) should result in moderate resistance. In the case of molecular or chemical chaperoning and proper folding, more ZBP biosensors will attain a folded and soluble state and tightly bind zeocin, resulting in high zeocin resistance. Under stress conditions or following protease induction, the unstable or unfolded biosensor will be more prone to aggregation and/or degradation, which will result in little or no binding to zeocin. As a result, zeocin resistance will be lost.

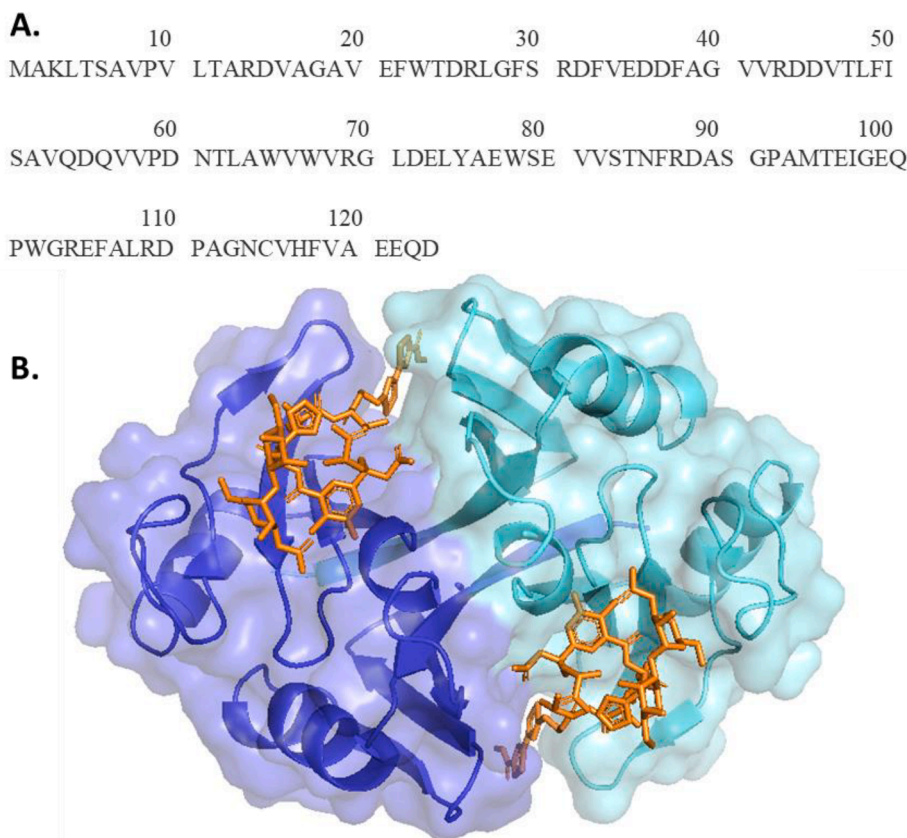


Fig. 2. (A) Sequence of Zeocin binding protein (ZBP) of *Streptoalloteichus hindustanus*. *Sh bleomycin* gene encodes 124 amino acids long protein product. (B) Symmetry-related homodimeric crystal structure of ZBP (PDB ID: 1XRK), shown in cyan and blue. Zeocin binding in a long crevice at the dimer interface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

binding affinity. The 22 ZBP variants have been introduced into the *Escherichia coli* BL21 (DE3)-RIL strain and then selected on an LBamp (200 µg/ml) plate.

2.4. Screening of unstable ZBP variants

The initial transformation selection was based on the ampicillin resistance present on pET3a. ZBP (wt) clone was used as the positive control, and a pET plasmid lacking the ZBP clone was used as the negative control for Zeocin resistance. At first, all the clones were cultured overnight at 37 °C in Lennox broth supplemented with 200 µg/ml ampicillin. The OD₆₀₀ of the overnight culture was determined and placed on ice to freeze the growth. The OD₆₀₀ of each culture was standardized to a concentration of OD1/ml. Afterward, each culture was diluted ten-fold using Lennox broth in a serial manner. Subsequently, a spot-titer experiment was performed using the methodology described in the study by Malik et al (Malik et al., 2014). In summary, a small volume of 2 µl from each dilution (ranging from 10⁰ to 10⁻⁵) of each clone was placed on Lennox agar plates containing various amounts (0, 25, 100, 250, 500, 1000, 1500, and 2000 µg/ml) of Zeocin. The plates were placed in an incubator and kept overnight at 37 °C. The growth of clones at the highest dilution was quantified and plotted in relation to Zeocin concentration.

3. Results

3.1. Development of a protein-folding biosensor

The principle of ZBP-based folding biosensors is illustrated in Fig. 1. The protein folding biosensor will effectively link the stability of typically unstable, aggregation-prone ZBP variants to the overall Zeocin resistance of the host strain. The construction of a biosensor incorporating a destabilized and partially folded ZBP is anticipated to result in moderate resistance. Destabilized ZBP can attain a folded and soluble state by integrating molecular or chemical chaperoning. Consequently, the host cell will exhibit substantial resistance to Zeocin. However, the destabilized ZBP will be susceptible to aggregation and/or proteolysis under stress or proteases due to its inherent instability. This will significantly diminish or eliminate the biosensor's affinity for Zeocin, leading to the loss of the Zeocin resistance. This approach will enable the identification of molecular chaperones or other folding modulators impacting proteostasis or chemical chaperones that contribute to stabilizing and improving the solubility of the ZBP variants prone to aggregation.

3.2. Construction of ZBP (WT) fusion protein

ZBP is a protein composed of two identical subunits (homodimer) consisting of 124 amino acids (Fig. 2). To optimize the expression of ZBP

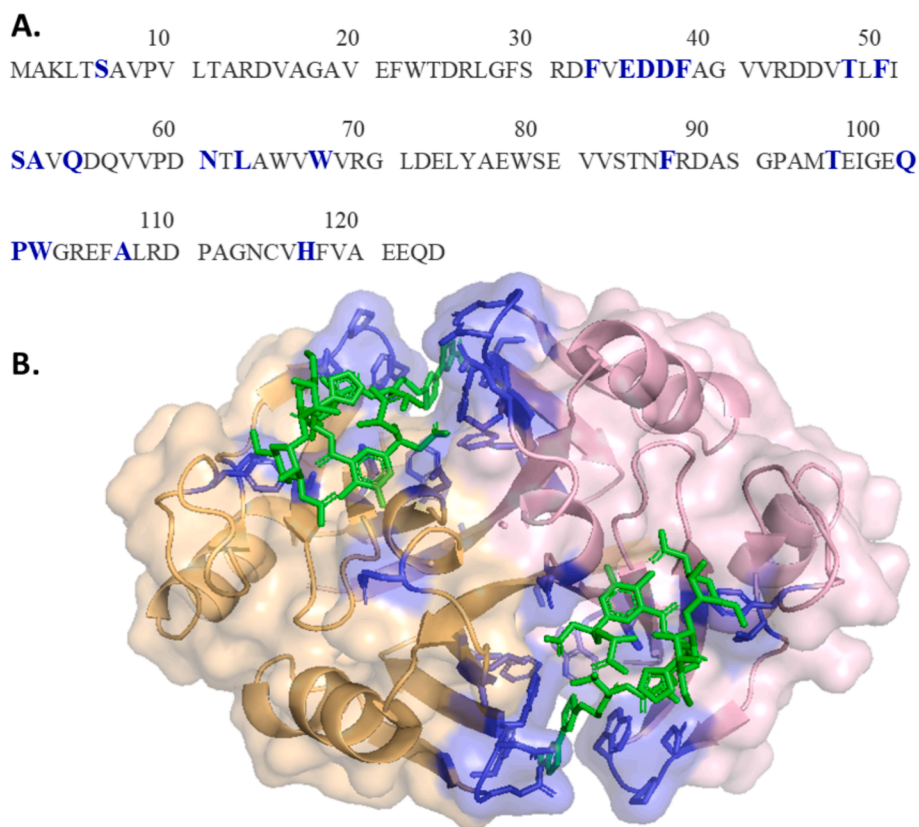


Fig. 3. (A) Binding pocket residues were highlighted in blue on the ZBP sequence. (B) Binding pocket residues were shown in blue on the ZBP structure. Each binding pocket consists of 21 residues of both subunits. The Zeocin molecule was shown in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in *E. coli*, the codon was optimized. In order to facilitate purification, a His-tag was attached to the N-terminus of ZBP. A specific cleavage site was included between the his-tag and ZBP to eliminate the his-tag from ZBP after purification. Each construct contained an identical sequence of the N-terminus fusion tag, specifically MGSHHHHHHSGSENLYFQ↓SG. In order to facilitate leaky expression in *E. coli*, suitable cloning and experiment parameters were chosen for this work. The cloning was done on pET3a, which lacks the *lacI* gene. Thus, lac repressor was absent. The ZBP (WT) clone was transformed in an *E. coli* strain, which was also lacking repressor plasmid such as pLysS (*Escherichia coli* BL21 (DE3)-RIL strain). The presence of the *bla* gene in the pET 3a plasmid facilitated the selection on an ampicillin plate.

3.3. Generation of ZBP variants

The structure of ZBP is characterized by two identical halves that are folded in the same folds. Each subunit's half comprises four beta-sheets and one alpha-helix. When ZBP forms a dimer, there is a 2:2 ratio between ZBP and Zeocin, meaning two Zeocin molecules bind to each ZBP dimer. A significant portion of ZBP, approximately one-sixth (21 out of 124 residues), was either directly involved in binding with Zeocin or was in close proximity to the Zeocin molecule (Dumas et al., 1994). Consequently, careful considerations were necessary in designing variants to destabilize ZBP without compromising its binding affinity for Zeocin. The binding pocket for Zeocin traverses the subunit interface, as

illustrated in Fig. 3, where the 21 residues crucial for Zeocin binding are highlighted in blue. This binding pocket comprises residues from both subunits. This study introduced 22 mutations (Table 2) at locations far from the binding site, as indicated in red in Fig. 4. These mutation sites on alpha-helix and beta-sheets were strategically chosen to disrupt the electrostatic and hydrophobic interactions, aiming to destabilize ZBP.

3.4. Screening of Zeocin resistance of ZBP variants

The resistance of ZBP variants to Zeocin was assessed by determining the minimum inhibitory concentration (MIC), which represents the lowest Zeocin concentration inhibiting the growth of *E. coli* expressing ZBP variants. The MIC experiments were conducted following the methodology by Malik et al. (Malik et al., 2014) with slight modifications. Initially, cultures were prepared by inoculating a single colony into 10 ml of Lennox media supplemented with 200 µg/ml ampicillin, followed by overnight incubation at 37 °C. The optical density at 600 nm (OD₆₀₀) of the overnight culture was adjusted to 1.0. Subsequently, the *E. coli* culture was serially diluted, and dilutions ranging from 10⁻⁰ to 10⁻⁵ were spotted on LB plates containing increasing concentrations of Zeocin. After overnight incubation at 37 °C, the growth or no growth thereof for each dilution at each Zeocin concentration was observed and used to calculate MIC values, following the approach described by Foit et al. (Foit et al., 2009). Interestingly, our results aligned with the hypothesis, revealing significant Zeocin resistance loss in certain ZBP

Table 2
Sequence of WT and variants of ZBP fusion protein.

ZBP variants	Sequence
ZBP (WT)	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP P29E	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP L31A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP V36A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP V40A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP F42A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP D45G	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP D45K	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP R46G	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP R46F	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP L47A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP L47G	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP F49A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP R51A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP R63A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP R89A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP L91A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP Y95A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP E97A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP W98A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP V101A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP V102A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD
ZBP L128A	MGSHHHHHHSGSENYLFQSGMAKLTSAVPLTARDVAGAVFEVWTRDLGSRDFVEDDFAGVVRDDVTLFISAVQDQVVPDNTLAWVVRGLDELYAEWSEVVSTNFRDASGPAMTEIGEQPWGREFALRDPAGNCVHFVAEEQD

variants (see Fig. 4).

The positive control (wild-type ZBP) exhibited growth at the highest dilutions (10⁻⁵ dilutions), even in the presence of 2000 µg/ml Zeocin, due to the high stability and solubility of ZBP. Conversely, the negative control (pET vector without ZBP) failed to survive even in the presence of 25 µg/ml Zeocin. Notably, two variants, namely ZBP P9E (Fig. 4A) and ZBP R26F (Fig. 4C), displayed a pronounced loss of resistance, potentially attributed to destabilization. Thirteen other variants (L11A, V16A, V20A, R26G, L27A, L27G, F29A, R31A, R33A, L71A, E77A, W78A, L108A) exhibited a moderate level of loss in Zeocin resistance, suggesting varying degrees of destabilization in these variants.

4. Discussion

Two factors influence protein production levels: the folding capacity of the host and the intrinsic stability or aggregation tendency of the protein of interest (Mayer et al., 2007, Espargaro et al., 2008, Foit et al., 2009). This study presents the process of creating a system that establishes a relationship between the biosensor's stability and antibiotic resistance. Zeocin resistance markers align with our objectives due to their potent cell-killing and broad effectiveness across different species. Antimicrobial resistance can be easily identified by spot titer methods (Foit et al., 2009). Furthermore, it is feasible to assess the influence of different conditions or mutations on protein stability or their propensity to fold. We opted to use the Zeocin-based markers because its 3D structure was known and applicable in both prokaryotic as well as eukaryotic hosts, thus allowing the discovery of novel protein folding modulators even in mammalian cell lines (Dumas et al., 1994).

Our objective was to identify destabilizing mutation sites within ZBP. To accomplish this aim, we developed a library of 22 mutations through a rational design strategy. With positive and negative controls, the total number of samples would be 24 and can fit in the microtiter plate format. The ZBP fusion protein variants were cloned on a strong T7 promoter on a plasmid with high copy numbers, and we opted for leaky expression instead of induced expression. Leaky expression in *E. coli* refers to protein expression without specific inducing conditions and offers several advantages. By choosing leaky expression, researchers can simplify the experimental process and establish selection conditions across a range of Zeocin concentrations without additional inducers like IPTG. This approach provides continuous, albeit low, protein expression, which can be particularly useful in maintaining selective pressure and enabling the screening of multiple conditions simultaneously. The use of leaky expression allows for a more streamlined and cost-effective protocol. Eliminating the need for IPTG reduces the methods complexity and the potential variability introduced by inducer concentration and timing. Additionally, the continuous low-level expression ensures that selection pressures are consistently applied, facilitating the identification of optimal Zeocin concentrations for selecting desired variants (Francis and Page 2010, Rosano and Ceccarelli 2014, Kato 2020).

Resistance testing using MIC experiments revealed significant Zeocin resistance loss in certain ZBP variants, validating the hypothesis. Positive controls (wild-type ZBP) exhibited high resistance, while negative controls (pET vector without ZBP) showed susceptibility (Fig. 5). Two variants (ZBP P9E and ZBP R26F) displayed pronounced resistance loss, while thirteen others exhibited a moderate loss, indicating variable degrees of destabilization. A significant correlation has been established between host cell Zeocin resistance and ZBP variants. The destabilized variants will identify molecular chaperones and folding modulators affecting proteostasis or chemical chaperones that enhance the stability and solubility of aggregation-prone ZBP variants.

The results of this study reveal several promising possibilities. The strong link observed between the host cell resistance to Zeocin antibiotic and ZBP variants highlights the crucial role of protein stability in antibiotic resistance. The significant and controlled loss of resistance found in several ZBP variants suggests that changes in amino acids can greatly destabilize the protein, impacting the survival of the host organism

A.

	10	20	30	40	50
MAKLTSAV	PV	LTARDVAGAV	EFWTDRLGFS	RDFVEDDFAG	VVRDDVTLFI
SAVQDQVVPD	NTLAWVWVRG	LDELYAEWSE	VVSTNFRDAS	GPAMTEIGE	Q
	60	70	80	90	100
PWGREFAL	RD	PAGNCVHFVA	EEQD		
	110	120			

B.

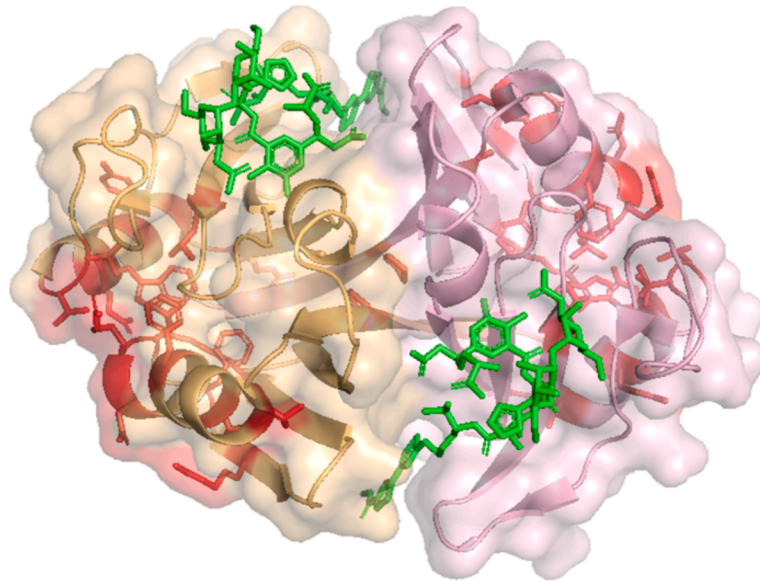


Fig. 4. (A) Mutation sites highlighted in red on the ZBP sequence (B) Mutation sites selected away from binding site residues shown on ZBP structure in red. Mutation site residues neither interact with Zeocin nor with the binding site residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

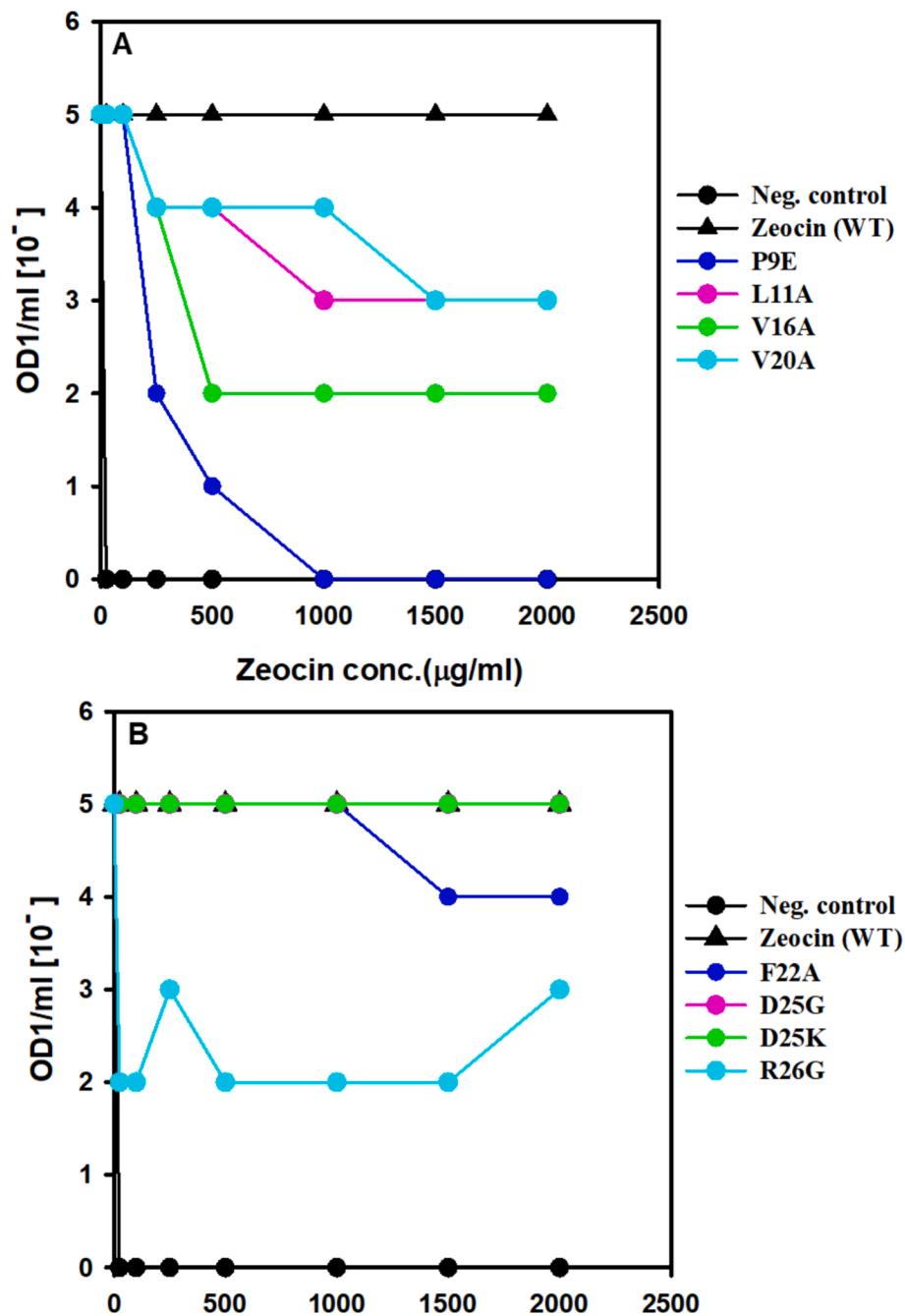


Fig. 5. Screening of ZBP variants for MIC. *E. coli* expressing ZBP variations that were cultivated overnight were adjusted to a concentration of OD1/ml. Cultures were diluted in a series from 10^{-1} to 10^{-5} and then applied as spots on Lennox agar plates that contained varying doses of zeocin. Following an incubation period of 24 h at 37 °C, the presence or absence of growth for each dilution at various zeocin concentrations was recorded and utilized to determine the minimum inhibitory concentration (MIC) values.

under selection pressure. Discovering molecular chaperones and folding modulators interacting with the destabilized ZBP variants could offer valuable insights into the physiological mechanisms responsible for maintaining proteostasis. Exploring chemical chaperones that can stabilize and improve the solubility of aggregation-prone ZBP variants presents an exciting opportunity. These chemicals can potentially enhance the productivity and effectiveness of recombinant proteins in biotechnological applications.

5. Conclusion

The study focuses on developing a selection system based on the ZBP to identify destabilizing mutations and their impact on antibiotic resistance. The choice of ZBP-based markers was advantageous due to their known 3D structure, applicability in both prokaryotic and eukaryotic hosts, and potential for discovering protein folding modulators, even in mammalian cell lines. The study introduces a rational design strategy to create a library of mutations in ZBP, using the leaky expression in *E. coli* for selection conditions at different Zeocin concentrations. Although leaky expression can be beneficial, it may not completely mimic the

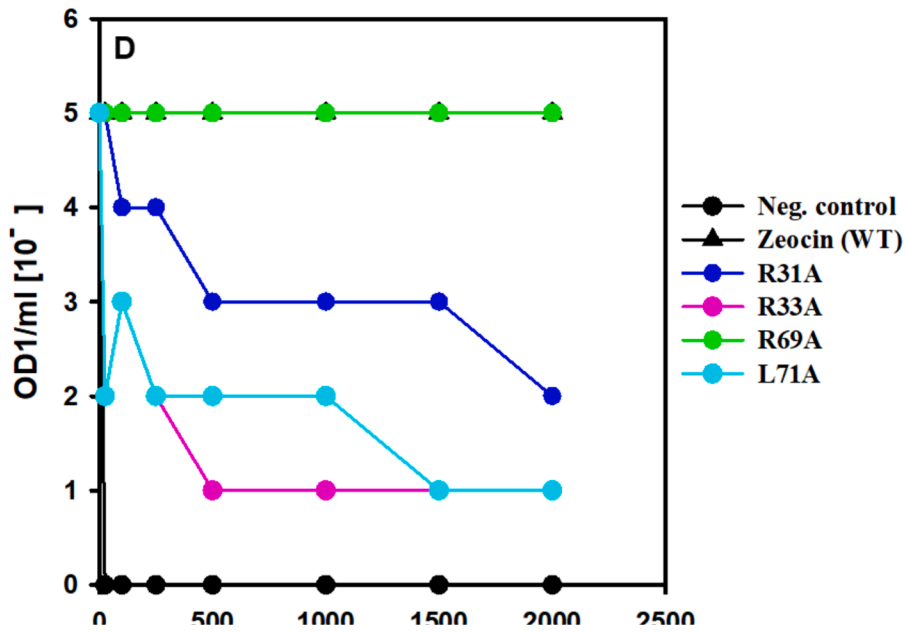
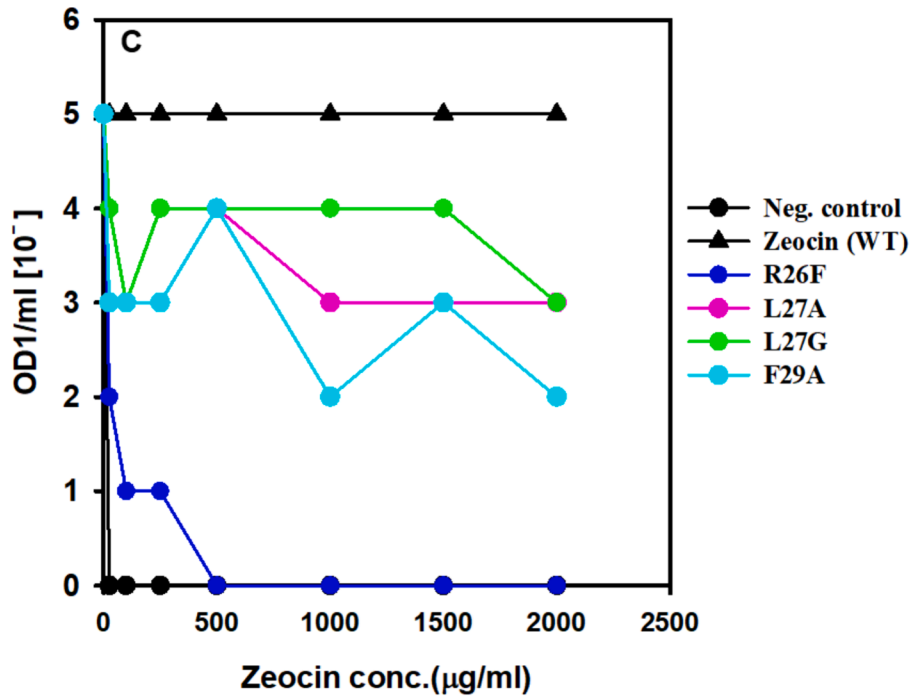


Fig. 5. (continued).

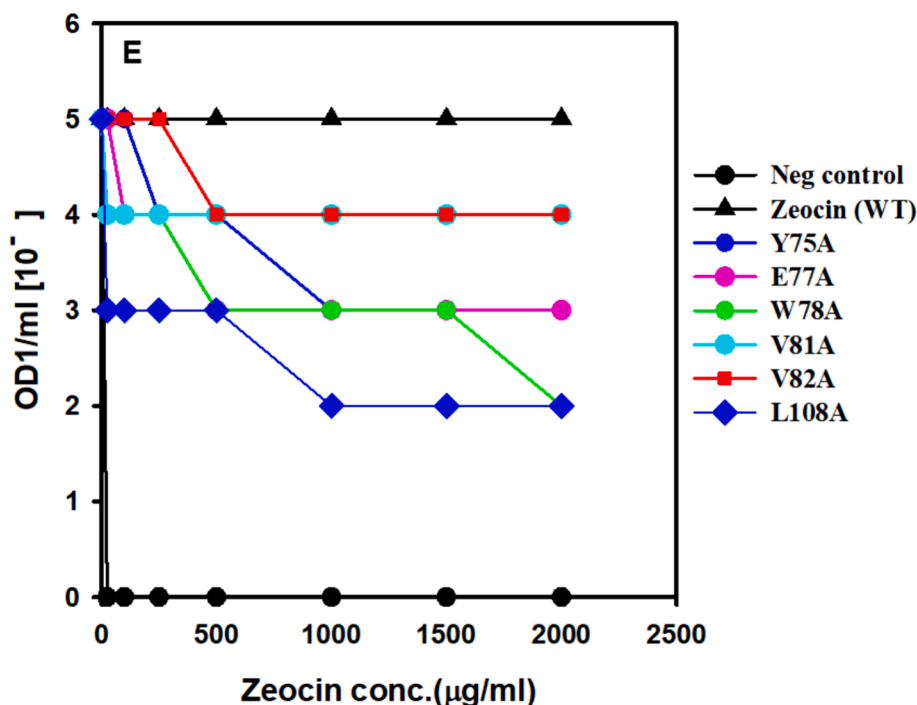


Fig. 5. (continued).

conditions of induced high-level protein expression. In addition, the library consisting of 22 mutations do not cover every possible destabilizing mutation in ZBP. In the future, the study should broaden the mutation library to investigate various destabilizing sites. Furthermore, studying the reported destabilized variants with molecular and chemical chaperones could provide more information about proteostasis mechanisms. Creating high-throughput *in vivo* assays for a more complete screening of chaperones and folding modulators would improve our understanding of their potential applications in biotechnology and pharmaceutical development.

CRedit authorship contribution statement

Sara Alharbi: Data curation, Writing – original draft. **Ajamaluddin Malik:** Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Abdulaziz Alamri:** Formal analysis, Investigation, Project administration, Funding acquisition. **Javed Masood Khan:** Formal analysis, Investigation, Methodology, Writing – original draft. **Mohd. Shahnawaz Khan:** Funding acquisition, Investigation, Methodology, Project administration. **Abdullah Alhommida:** Project administration, Resources, Supervision. **Tauseef Ahmad:** Data curation, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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