UNIVERSITI PUTRA MALAYSIA

STRUCTURAL AND FUNCTIONAL ANALYSES OF COPPER-SENSING OPERON REGULATOR PROTEIN (CsoRGz) OF Geobacillus zalihae STRAIN T1

ASHWAANI MANGAVELU

FBSB 2018 20

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By

ASHWAANI MANGAVELU

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

March 2018

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

STRUCTURAL AND FUNCTIONAL ANALYSES OF COPPER-SENSING OPERON REGULATOR PROTEIN ($CsoR_{Gz}$) OF *Geobacillus zalihae* STRAIN T1

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ASHWAANI MANGAVELU

March 2018

Chairman : Normi Binti Mohd Yahaya, PhD
Faculty : Biotechnology and Biomolecular Sciences

Copper sensor regulator protein (CsoR) is widespread in most Gram positive bacteria. It is categorized under metalloregulatory protein that tightly regulates the passage of copper(I) ions to maintain cell viability, metabolism and preventing cell toxicity. Previously, gene annotation of a locally isolated *Geobacillus zalihae* strain revealed the presence of a CsoR-like hypothetical protein ($CsoR_{Gz}$) that shared only 30-38% sequence identity to structurally characterized CsoR proteins of various bacterial strains. This highlights the possibility of structural novelty of $CsoR_{Gz}$. As the actual 3D structure of $CsoR_{Gz}$ is not available, information on possible novelty of its structure as well as its 3D copper(I) binding site and motif is unknown. This study aimed to elucidate the structure of $CsoR_{Gz}$ via X-ray crystallography, identify the functional metal-binding residues and residues involved in dimerization of $CsoR_{Gz}$ protein and compare its structure with other structurally characterized CsoR proteins. For this study, *Escherichia coli* BL21 Star ® (DE3) inserted with pET28b-$CsoR_{Gz}$ vector was used to express the recombinant $CsoR_{Gz}$ protein. $CsoR_{Gz}$ protein was optimally expressed in LB medium at 37°C with IPTG induction at 0.1 mM and purified to homogeneity by affinity chromatography followed by gel filtration. The protein was stable when dialysed at pH 6.5 in the buffer containing 10 mM MES, 100 mM NaCl, 0.2 mM EDTA, 0.2 mM DTT and 5% glycerol. The purified $CsoR_{Gz}$ protein was crystallized via sitting drop vapour diffusion method at 15 °C. Trigonal crystals corresponding to space group $P3_121$ were grown in 5% (v/v) tacsimate TM (pH 7.0), 0.1 M HEPES (pH 7.0), 10% (w/v) polyethylene glycol monomethyl ether 5,000. The crystal was successfully diffracted at 1.83Å using an in-house X-ray beam with completeness of 100% with unit-cell parameters as follows; a=44.73, b=44.73, c=82.37 Å, where α=ß=90° and γ=120°. The Matthew's coefficient analysis revealed that the crystal structure had one molecule per asymmetric unit with the solvent content of 32.82%. Its conserved copper-binding residues, Cys46-His71-Cys75, were

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found to be located at α2 helix onwards of the crystal structure similar to other CsoR proteins. The dimeric structure of $CsoR_{Gz}$ was indicated using Protein, Interface and Structure and Assemblies (PISA) whereby the monomers were held together by disulphide bonds, hydrogen bonds and salt bridges and the residues involved in these respective interactions were duly mapped. $CsoR_{Gz}$ showed largely similar global topology to other known Cu(I) bound CsoRs with minimal differences in the characteristics of the Cu(I) binding pocket whereby $CsoR_{Gz}$ had a more hydrophobic environment than other reported CsoRs.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANALISA STRUKTUR DAN FUNGSI PROTEIN PENGAWAL ATUR OPERON KUPRUM (CsoR$_{Gz}$) DARIPADA *Geobacillus zalihae* STRAIN T1

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Protein pengawal atur kuprum (CsoR) didapati dengan meluas dalam kebanyakan bakteria Gram positif. Ia dikategorikan di bawah protein pengawal atur logam yang mengawal atur keluar dan masuk kuprum(I) untuk mengekalkan daya tahan dan metabolisme sel dan mencegah ketoksikan sel. Anotasi genom strain *Geobacillus zalihae* yang dipencilkan tempatan mendedahkan kehadiran protein hipotetikal seakan CsoR (CsoR$_{Gz}$) yang berkongsi hanya 30-38% identiti jujukan dengan protein CsoR yang telah dicirikan strukturnya daripada pelbagai strain bakteria. Ini menggariskan kemungkinan struktur CsoR$_{Gz}$ yang novel. Memandangkan struktur 3D asal CsoR$_{Gz}$ masih tiada, maklumat berkaitan kemungkinan struktur novel dan juga tapak dan motif pengikat kurpum(I) 3Dnya masih tidak diketahui. Jadi, kajian ini bertujuan untuk merungkaikan struktur CsoR$_{Gz}$ melalui kristalografi sinar-X, memetakan residu fungsian pengikat logam dan yang terlibat di dalam pendimeran protein CsoR$_{Gz}$ dan membandingkan strukturnya dengan protein CsoR lain yang telah dicirikan strukturnya. Untuk tujuan ini, protein rekombinan CsoR$_{Gz}$ telah dihasilkan di dalam *Escherichia coli* BL21 Star ® (DE3) yang telah dimasukkan dengan pET28b-CsoR$_{Gz}$. Pengekspresan protein CsoR$_{Gz}$ telah dihasilkan secara optimum dalam media LB pada 37°C dengan induksi IPTG pada 0.1 mM dan dipencilkan sehingga homogeneti dengan menggunakan kromatografi afiniti diikuti dengan penapisan gel. Protein tersebut adalah stabil apabila didialisis pada pH 6.5 di dalam penimbal yang mengandungi 10 mM MES, 100 mM NaCl, 0.2 mM EDTA, 0.2 mM DTT dan 5% gliserol. Protein CsoR$_{Gz}$ yang dipencilkan telah dihablurkan melalui kaedah resapan wap *sitting drop* pada 15 °C. Hablur trigonal sepadan dengan kumpulan ruang $P3_121$ telah ditumbuhkan dengan 5% (v/v) tacsimate TM pH 7.0, 0.1 M HEPES pH 7.0, 10% (w/v) polietilena glikol monometill eter 5,000. Hablur tersebut berjaya dibelaukan kepada resolusi 1.83 Å menggunakan sunber sinar X *in house* dengan kelengkapan 100% dengan parameter sel unit seperti berikut; a = 44.73, b = 44.73, c = 82.37 Å, di

mana α=ß=90° dan γ=120°. Analisis nilai pekali Matthew menunjukkan bahawa hablur ini mempunyai satu molekul per unit asimetri dengan kandungan pelarut sebanyak 32.82%. Residu terpelihara yang mengikat kuprum, iaitu Cys46-His71-Cys75, telah dijumpai terletak pada heliks α2 dan seterusnya serupa dengan struktur protein CsoR yang lain. Struktur dimer $CsoR_{Gz}$ telah dianalisis dengan menggunakan program Protein, Antara Muka dan Struktur dan Perhimpunan (PISA) di mana dua monomer dipegang bersama oleh ikatan disulfida, ikatan hidrogen dan penyambung garam. Struktur $CsoR_{Gz}$ memperlihatkan topologi global yang sama seperti protein CsoR yang lain dengan perbezaan yang sedikit dalam ciri-ciri poket mengikat Cu(I)nya di mana $CsoR_{Gz}$ mempunyai persekitaran hidrofobik yang lebih tinggi berbanding dengan CsoR lain yang telah dilaporkan.

ACKNOWLEDGEMENTS

Special appreciation to my supervisor, Dr. Normi binti Mohd Yahaya for giving me the opportunity to work on this project, for her valuable guidance, constant encouragement and support throughout this Masters study. Thank you for being such an inspiration and role model besides being a second mother to me. I would also like to thank my supervisory committee members, Professor Dr. Raja Noor Zaliha Binti Raja Abd. Rahman, Professor Dr. Abu Bakar Bin Salleh, Associate Professor Dr. Adam Leow Thean Chor and Associate Professor Dr. Mohd Shukuri bin Mohamad Ali and other EMTech Research group members for their guidance, encourgament, insightful comments and helpful discussions.

My sincere thanks to Dr. Anuar Bin Jonet from Malaysian Genome Institute (MGI) for his help and cooperation in X-ray diffraction and data collection. Special thanks for all the postgraduate students of EMTech and Protein Engineering laboratory members for their continuous support, knowledge and friendship. Special thanks also extended to the staff of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM) for their help and cooperation. I am also thankful to Putra Research Grant UPM (Grant No.: 9502200) and Graduate Research Fellowship (GRF) for the financial support for my research studies.

Last but not least, I am eternally grateful to my parents, Mangavelu Manickam and Tami Selvi Uruman and my siblings for their utmost support. Thank you so much Jivita Thestnamoorthi for your continuous encouragement for me in pursuing my Masters studies and believing in my capabilities. Lastly, to those who indirectly contributed in this research, your kindness means a lot to me. Thank you.

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LIST OF ABBREVIATIONS

α	Alpha
Å	Angstrom
ATP	Adenosine Tri-phosphate
APS	Ammonium persulfate
Au	Aurum
bp	Base pair
β	Beta
Cd	Cadmium
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
Crp	cAMP receptor Protein
csc	Chromosomally encoded sucrose catabolism genes
Co	Cobalt
Cu(I)	Copper (I) ion
CsoR	Copper Sensitive Operon Repressor
°C	Degree Celcius
DNA	Deoxyribonucleic acid
DUF	Domain of Unknown Function
EDTA	Ethylene-diamine-tetraacetic acid
Fe	Ferrum
g	Gram
HTH	Helix Turn Helix
His	Histidine

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IPTG	Isopropyl-Beta-D-Thiogalactoside
kb	Kilobase
kDA	KiloDalton
L	Litre
LB	Luria-Bertani
Mn	Manganese
M	Molar
µL	Microliter
µm	Micrometer
mM	Millimolar
Ni	Nickel
A_{600nm}	Optical density at wavelength 600 nanometer
%	Percentage
$NaPO_4$	Sodium phosphate
NaCl	Sodium chloride
SDS - PAGE	Sodium deodecyl sulphate Polyacrylamide gel electrophoresis
3D	Three Dimensional
TCS	Two Components Regulatory System
Zn	Zinc

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CHAPTER 1

INTRODUCTION

Copper is required as a micronutrient for all living cells, as it is known as an essential component for large numbers of metalloenzymes. However, elevated level of copper ions in micro and macro organisms can be detrimental (Liu *et al.*, 2006). Therefore, tightly controlling the levels of cellular metal ion via homeostasis is required to avoid cell toxicity as well as for cell viability and metabolism.

To cope with unfavourable copper concentration, bacteria utilize copper-induced defence mechanisms. An example of such copper homeostasis mechanism involves CsoR protein. It is a metalloregulatory protein that that functions to regulate the copper ion concentration in bacterial cells. It is responsible in controlling gene expression which allows organisms to adapt to critical level of essential metal ions, particularly copper, thus enabling cells tolerate heavy metal pollutants present in their environment (Giedroc & Arunkumar, 2007). It functions as a copper-sensing repressor, regulates expression of copper-homeostasis genes. It was first discovered in *Mycobacterium tuberculosis* (Liu *et al.*, 2007). Besides *M. tuberculosis*, CsoR-like proteins are also widespread in other prokaryotes such as in *Bacillus subtilis* (Smaldone & Helmann, 2007), *Staphylococcus aureus* (Baker *et al.*, 2011), *Streptomyces lividans* (Chaplin *et al.*, 2015) and *Listeria monocytogenes* (Corbett *et al.*, 2011).

In general, *csoR* gene is located upstream of *copZA* operon that encodes a copper-ATPase and a copper chaperone. Copper chaperone function to transport the excess copper to copper ATPase, while copper ATPase function to remove excess copper out of the cell (Hirooka *et al.*, 2012). When copper is scarce, CsoR represses the transcription by binding to the operator of the *copZA* operon which overlaps *copZ* promoter. When copper concentration exceeds, CsoR binds to the excessive copper ions and detaches itself from the operator, thus unblocking the promoter region for transcription of *copZA* to take place (Rademacher & Masepohl, 2012). Previously, a scan on the genome sequence of *G. zalihae* revealed the presence of a CsoR-like hypothetical protein gene located at the upstream of the *copZA* operon. Bioinformatics analysis done by Musa (2016) on the protein sequence of CsoR-like protein of *G. zalihae* showed that it contained a CsoR-DUF domain similar to other well-characterized CsoR proteins. However, $CsoR_{Gz}$ only shared up to 30-38% sequence identity to structurally characterized CsoR proteins of various bacterial origins (Musa, 2016). This suggests the possibility of its structure being novel compared to other structurally characterized CsoR proteins.

It is important to note that the 3D structure of $CsoR_{Gz}$ have not been determined via X-ray crystallography as well as metal-binding ligands interaction using docking method. Whilst several tools such as isothermal titration calorimetry is able to shed light on the ability of the protein to interact with metals, it is not able to identify which

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amino acids the metals interact with. Combination of X-ray crystallography and docking is able to give forth such information upon the interaction of proteins with metals. Hence, this present study aims to:

1. elucidate the structure of $CsoR_{Gz}$ via X-ray crystallography
2. identify the functional metal-binding residue and dimer interface of $CsoR_{Gz}$.
3. compare the similarities and differences of $CsoR_{Gz}$ with other structurally characterized CsoR proteins.

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REFERENCES

Abergel, C. (2013). Molecular replacement: tricks and treats. *Acta Crystallography, 69*, 2167–2173.

Anandakumar, S., & Shanmughavel, P. (2008). Computational Annotation for Hypothetical Proteins of *Mycobacterium Tuberculosis*. *Journal of Computer Science & Systems Biology, 1*, 50–62.

Asherie, N. (2004). Protein crystallization and phase diagrams. *Methods, 34*, 266–272.

Atkins, J., Boateng, S., Sorensen, T., & McGuffin, L. (2015). Disorder Prediction Methods, Their Applicability to Different Protein Targets and Their Usefulness for Guiding Experimental Studies. *International Journal of Molecular Sciences, 16*(8), 19040–19054.

Baker, J., Sengupta, M., Jayaswal, R. K., & Morrissey, J. A. (2011). The Staphylococcus aureus CsoR regulates both chromosomal and plasmid-encoded copper resistance mechanisms. *Environmental Microbiology, 13*(9), 2495–507.

Baskaran, K., Duarte, J. M., Biyani, N., Bliven, S., & Capitani, G. (2014). A PDB-wide, evolution-based assessment of protein–protein interfaces. *BMC Structural Biology, 14*.

Berjanskii, M., Liang, Y., Zhou, J., Tang, P., Stothard, P., Zhou, Y.,Wishart, D. S. (2010). PROSESS: A protein structure evaluation suite and server. *Nucleic Acids Research, 38*.

Berman, H., Henrick, K., & Nakamura, H. (2003). Announcing the worldwide Protein Data Bank. *Nature Structural Biology, 10*(12), 980–980.

Bidkar, A. (2014). In-silico Structural and Functional Analysis of Hypothetical Proteins of *Leptospira Interrogans*. *Biochemistry & Pharmacology: Open Access, 3*(3).

Bornhorst, J. A., & Falke, J. J. (2000). Purification of proteins using polyhistidine affinity tags. *Methods in Enzymology, 326*, 245–54.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.

Breda, A., Valadares, N. F., Souza, O. N. de, & Garratt, R. C. (2008). Protein Structure, Modelling and Applications (Chapter A06). Bioinformatics in Tropical Disease Research: A Practical and Case-Study Approach. *National Center for Biotechnology Information (US).*

© COPYRIGHT UPM

Burgess, S. A., Flint, S. H., Lindsay, D., Cox, M. P., & Biggs, P. J. (2017). Insights into the *Geobacillus stearothermophilus* species based on phylogenomic principles. *BMC Microbiology*, *17*(1), 1–12.

Capitani, G., Duarte, J. M., Baskaran, K., Bliven, S., & Somody, J. C. (2015). Understanding the fabric of protein crystals: Computational classification of biological interfaces and crystal contacts. *Bioinformatics*, *32*(4), 481–489.

Chang, F.-M. J., Coyne, H. J., Cubillas, C., Vinuesa, P., Fang, X., Ma, Z., Giedroc, D. P. (2014). Cu(I)-mediated allosteric switching in a copper-sensing operon repressor (CsoR). *The Journal of Biological Chemistry*, *289*(27), 19204–17.

Chang, F.-M. J., Martin, J. E., & Giedroc, D. P. (2015). Electrostatic occlusion and quaternary structural ion pairing are key determinants of Cu(I)-mediated allostery in the copper-sensing operon repressor (CsoR). *Biochemistry*, *54*(15), 2463–72.

Chaplin, A. K., Tan, B. G., Vijgenboom, E., & Worrall, J. R. (2015). Copper trafficking in the CsoR regulon of *Streptomyces lividans*. *Metallomics : Integrated Biometal Science*, *7*(1), 145–55.

Chaturvedi, K. S., & Henderson, J. P. (2014). Pathogenic adaptations to host-derived antibacterial copper. *Frontiers in Cellular and Infection Microbiology*, *4*.

Chillappagari, S., Miethke, M., Trip, H., Kuipers, O. P., & Marahiel, M. A. (2009). Copper acquisition is mediated by YcnJ and regulated by YcnK and CsoR in *Bacillus subtilis*. *Journal of Bacteriology*, *191*(7), 2362–70.

Colovos, C., & Yeates, T. O. (1993). Verification of protein structures: Patterns of nonbonded atomic interactions. *Protein Science*, *2*(9), 1511–1519.

Corbett, D., Schuler, S., Glenn, S., Andrew, P. W., Cavet, J. S., & Roberts, I. S. (2011). The combined actions of the copper-responsive repressor CsoR and copper-metallochaperone CopZ modulate CopA-mediated copper efflux in the intracellular pathogen *Listeria monocytogenes*. *Molecular Microbiology*, *81*(2), 457–72.

Coyne, H. J., & Giedroc, D. P. (2013). Backbone resonance assignments of the homotetrameric (48 kDa) copper sensor CsoR from *Geobacillus thermodenitrificans* in the apo- and Cu(I)-bound states: insights into copper-mediated allostery. *Biomolecular NMR Assignments*, *7*(2), 279–83.

Dalecki, A. G., Crawford, C. L., & Wolschendorf, F. (2017). Copper and Antibiotics: Discovery, Modes of Action, and Opportunities for Medicinal Applications. *Advances in Microbial Physiology.*70, 193-260.

© COPYRIGHT UPM

Davis, A. V, & O'Halloran, T. V. (2008). A place for thioether chemistry in cellular copper ion recognition and trafficking. *Nature Chemical Biology*, *4*(3), 148–151.

DeLano, W. L. (2002). Pymol: An open-source molecular graphics tool. C*CP4 Newsletter On Protein Crystallography*, 40, 82-92.

Dessau, M. A., & Modis, Y. (2011). Protein crystallization for X-ray crystallography. *Journal of Visualized Experiments : JoVE*, (47).

Domagalski, M. J., Zheng, H., Zimmerman, M. D., Dauter, Z., Wlodawer, A., & Minor, W. (2014). The quality and validation of structures from structural genomics. *Methods in Molecular Biology*, *1091*, 297–314.

Dosztanyi, Z., Csizmok, V., Tompa, P., & Simon, I. (2005). IUPred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. *Bioinformatics*, *21*(16), 3433–3434.

Duarte, J. M., Srebniak, A., Schärer, M. A., & Capitani, G. (2012). Protein interface classification by evolutionary analysis. *BMC Bioinformatics*, *13*(1), 334.

Dudev, T., & Lim, C. (2008). Metal Binding Affinity and Selectivity in Metalloproteins: Insights from Computational Studies. *Annual Review of Biophysics*. 37, 97-116.

Dunker, A. K., Cortese, M. S., Romero, P., Iakoucheva, L. M., & Uversky, V. N. (2005). Flexible nets. The roles of intrinsic disorder in protein interaction networks. *FEBS Journal*, *272*(20), 5129–5148.

Dwarakanath, S., Chaplin, A. K., Hough, M. A., Rigali, S., Vijgenboom, E., & Worrall, J. A. R. (2012). Response to copper stress in *Streptomyces lividans* extends beyond genes under direct control of a copper-sensitive operon repressor protein (CsoR). *The Journal of Biological Chemistry*, *287*(21), 17833–47.

Egli, P. H., & Zerfoss, S. (1949). General principles of crystal growth. *Discussion of the Faraday Society*, *5*, 61–66.

Emsley, P. and Cowtan, K. (2004) Coot, model-building tools for molecular graphics. *Acta Crystallographica Section D: Biological Crystallography*, 60, 2126-2132.

Fu, Y., Chang, F.-M. J., & Giedroc, D. P. (2014). Copper Transport and Trafficking at the Host Bacterial Pathogen Interface. *Accounts of Chemical Research*, *47*(12), 3605–3613.

© COPYRIGHT UPM

Gaballa, A., & Helmann, J. D. (2003). *Bacillus subtilis* CPx-type ATPases: Characterization of Cd, Zn, Co and Cu efflux systems. *BioMetals*, *16*(4), 497–505.

Galperin, M. Y., & Koonin, E. V. (2004). "Conserved hypothetical" proteins: prioritization of targets for experimental study. *Nucleic Acids Research*, *32*(18), 5452–5463.

Giedroc, D. P., & Arunkumar, A. I. (2007). Metal sensor proteins: nature's metalloregulated allosteric switches. *Dalton Transactions* (29), 3107–20.

Griffith, J. S., & Orgel, L. E. (1957). Ligand-field theory. *Quarterly Reviews, Chemical Society*, *11*(4), 381-393.

Guerra, A. J., & Giedroc, D. P. (2012). Metal site occupancy and allosteric switching in bacterial metal sensor proteins. *Archives of Biochemistry and Biophysics*, *519*(2), 210–222.

Hagel, L. (2001). Gel-Filtration Chromatography. In *Current Protocols in Molecular Biology*, 10.9, 10.9.1–10.9.2.

Harlow, R. L. (1996). Troublesome Crystal Structures: Prevention, Detection and Resolution. *Journal of Research of the National Institute of Standards and Technology*, *101*(3), 327.

Higgins, K. A., & Giedroc, D. (2014). Insights into Protein Allostery in the CsoR/RcnR Family of Transcriptional Repressors. *Chemistry Letters*, *43*(1), 20–25.

Hirooka, K., Edahiro, T., Kimura, K., & Fujita, Y. (2012). Direct and indirect regulation of the ycnKJI operon involved in copper uptake through two transcriptional repressors, YcnK and CsoR, in *Bacillus subtilis*. *Journal of Bacteriology*, *194*(20), 5675–5687.

Hodgkinson, V., & Petris, M. J. (2012). Copper homeostasis at the host-pathogen interface. *Journal of Biological Chemistry*.

Hollingsworth, S. A., & Karplus, P. A. (2010). A fresh look at the Ramachandran plot and the occurrence of standard structures in proteins. *Biomolecular Concepts*, *1*(3–4), 271–283.

Iwig, J. S., Rowe, J. L., & Chivers, P. T. (2006). Nickel homeostasis in *Escherichia coli*. The rcnR-rcnA efflux pathway and its linkage to NikR function. *Molecular Microbiology*, *62*(1), 252–262.

Kantardjieff, K. A., & Rupp, B. (2003). Matthews coefficient probabilities: Improved estimates for unit cell contents of proteins, DNA, and protein-nucleic acid complex crystals. *Protein Science*, *12*(9), 1865–71.

© COPYRIGHT UPM

Keegan, R. M., Long, F., Fazio, V. J., Winn, M. D., Murshudov, G. N., & Vagin, A. A. (2011). Evaluating the solution from MrBUMP and BALBES. *Acta Crystallographica. Section D, Biological Crystallography*, *67*(4), 313–23.

Keegan, R. M., & Winn, M. D. (2008). MrBUMP: an automated pipeline for molecular replacement. *Acta Crystallographica. Section D, Biological Crystallography*, *64*(1), 119–24.

Krissinel, E. and Henrick, K. (2007). 'Inference of macromolecular assemblies from crystalline state'. *Journal of Molecular Biology,* 372, 774–797.

Kelley, L. A., & Sternberg, M. J. E. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nature Protocols*, *4*(3), 363–71.

Kleywegt, G. J. (2000). Validation of protein crystal structures. *Acta Crystallographica Section D-Biological Crystallography*, *56*, 249–265.

Kim, M. O., Nichols, S. E., Wang, Y., & McCammon, J. A. (2013). Effects of histidine protonation and rotameric states on virtual screening of *M. tuberculosis* RmlC. *Journal of Computer-Aided Molecular Design*, *27*(3), 235–46.

Krieger, E., Koraimann, G., & Vriend, G. (2002). Increasing the precision of comparative models with YASARA NOVA - A self-parameterizing force field. *Proteins: Structure, Function and Genetics*, *47*(3), 393–402.

Laskowski, R. A. (2003). Structural quality assurance. Methods of biochemical analysis (44). *John Wiley & Sons, Inc*., Hoboken, NJ, USA.

Laskowski, R. A., Hutchinson, E. G., Michie, A. D., Wallace, A. C., Jones, M. L., & Thornton, J. M. (1997). PDBsum: a Web-based database of summaries and analyses of all PDB structures. *Trends in Biochemical Sciences*, *22*(12), 488–90.

Lebedev, A. A., & Isupov, M. N. (2014). Space-group and origin ambiguity in macromolecular structures with pseudo-symmetry and its treatment with the program *Zanuda*. *Acta Crystallographica Section D Biological Crystallography*, *70*(9), 2430–2443.

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, *227*(5259), 680–685.

Lemma, V., D'Agostino, M., Caporaso, M. G., Mallardo, M., Oliviero, G., Stornaiuolo, M., & Bonatti, S. (2013). A disorder-to-order structural transition in the COOH-tail of Fz4 determines misfolding of the L501fsX533-Fz4 mutant. *Scientific Reports*, *3*, 46–55.

© COPYRIGHT UPM

Linding, R., Jensen, L. J., Diella, F., Bork, P., Gibson, T. J., & Russell, R. B. (2003). Protein disorder prediction: implications for structural proteomics. *Structure*, *11*(11), 1453–9.

Liu, T., Ramesh, A., Ma, Z., Ward, S. K., Zhang, L., George, G. N., Giedroc, D. P. (2007). CsoR is a novel *Mycobacterium tuberculosis* copper-sensing transcriptional regulator. *Nature Chemical Biology*, *3*(1), 60–8.

Lobley, A., Swindells, M. B., Orengo, C. A., Jones, D. T., & Zhang, Z. (2007). Inferring Function Using Patterns of Native Disorder in Proteins. *PLoS Computational Biology*, *3*(8), 162.

Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993). PROCHECK: a program to check the stereochemical quality of protein structures. *Journal of Applied Crystallograpgy*, 26, 283-291.

Luebke, J. L., Shen, J., Bruce, K. E., Kehl-Fie, T. E., Peng, H., Skaar, E. P., & Giedroc, D. P. (2014). The CsoR-like sulfurtransferase repressor (CstR) is a persulfide sensor in *Staphylococcus aureus*. *Molecular Microbiology*, *94*(6), 1343–60.

Ma, Z., Jacobsen, F. E., & Giedroc, D. P. (2009). Coordination chemistry of bacterial metal transport and sensing. *Chemical Reviews*, *109*(10), 4644–4681.

Matthews, B. W. (1968). Solvent content of protein crystals. *Journal of Molecular Biology*, *33*(2), 491–497.

McCall, K. A., & Fierke, C. A. (2004). Probing Determinants of the Metal Ion Selectivity in Carbonic Anhydrase Using Mutagenesis. *Biochemistry*, *43*(13), 3979–3986.

McCoy, J., Grosse-Kunstleve, R. W., Adams, P. D. , Winn, M. D., Storoni, L. C. and Read, R. J. (2007). Phaser crystallographic software. *Journal of Applied Crystallography, 40, 658-674.*

Motlagh, H. N., Wrabl, J. O., Li, J., & Hilser, V. J. (2014). The ensemble nature of allostery. *Nature*, *508*(7496), 331–339.

Murshudov, G. N., Vagin, A. A. & Dodson E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallographica Section D: Biological Crystallography*, 53 (3), 240-255.

Musa, N. (2016). Structural prediction and Copper(I) binding analysis of CsoR-like hypothetical protein of *Geobacillus zalihae, MSc thesis, Universiti Putra Malaysia, Serdang, Selangor.*

Nimrod, G., Schushan, M., Steinberg, D. M., & Ben-Tal, N. (2008). Detection of Functionally Important Regions in "Hypothetical Proteins" of Known Structure. *Structure*, 16(12), 1755-63.

© COPYRIGHT UPM

Outten, F. W., Twining, B. S., Outten, F. W., & Twining, B. S. (2008). Metal Homeostasis. In *Wiley Encyclopedia of Chemical Biology*. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Parker, M. W. (2003). Protein Structure from X-Ray Diffraction. *Journal of Biological Physics*, *29*, 341–362.

Patzer, S. I., & Hantke, K. (1998). The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Molecular Microbiology*, *28*(6), 1199–210.

Papiz, M. Z. & Winter, G. (2016) X-Ray Crystallography, Biomolecular Structure Determination Methods. *Encyclopedia of Spectroscopy and Spectrometry (Third Edition)*, 640–647.

Pontel, L. B., Checa, S. K., & Soncini, F. C. (2015). Bacterial copper resistance and virulence. *Bacteria-Metal Interactions*, 1–20.

Porto, T. V, Wilson, M. T., & Worrall, J. A. R. (2015). Copper and nickel bind via two distinct kinetic mechanisms to a CsoR metalloregulator. *Dalton Transactions*, *44*(46), 20176–85.

Rademacher, C., & Masepohl, B. (2012). Copper-responsive gene regulation in bacteria. *Microbiology*, *158*(10), 2451–2464.

Rahman, R., Leow, T., Salleh, A., Basri, M. (2007). Geobacillus zalihae sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. BMC Microbiol. 7, 77.

Reyes-Caballero, H., Campanello, G. C., & Giedroc, D. P. (2011). Metalloregulatory proteins: Metal selectivity and allosteric switching. *Biophysical Chemistry* 156(2-3):103-14.

Rubino, J. T., & Franz, K. J. (2012). Coordination chemistry of copper proteins: How nature handles a toxic cargo for essential function. *Journal of Inorganic Biochemistry*, *107*, 129–143.

Sakamoto, K., Agari, Y., Agari, K., Kuramitsu, S., & Shinkai, A. (2010). Structural and functional characterization of the transcriptional repressor CsoR from *Thermus thermophilus* HB8. *Microbiology*, *156*(7), 1993–2005.

Santo, E. C., Lam, E. W., Elowsky, C. G., Quaranta, D., Domaille, D. W., Chang, C. J., & Grass, G. (2011). Bacterial killing by dry metallic copper surfaces. *Applied and Environmental Microbiology*, *77*(3), 794–802.

Schärer, M. A., Grütter, M. G., & Capitani, G. (2010). CRK: An evolutionary approach for distinguishing biologically relevant interfaces from crystal contacts. *Proteins: Structure, Function, and Bioinformatics*, *78*(12).

© COPYRIGHT UPM

Smaldone, G. T., & Helmann, J. D. (2007). CsoR regulates the copper efflux operon copZA in Bacillus subtilis. *Microbiology*, *153*(12), 4123–4128.

Spencer, R. K., & Nowick, J. S. (2015). A Newcomer's Guide to Peptide Crystallography. *Israel Journal of Chemistry*, *55*(6–7), 698–710.

Tan, B. G., Vijgenboom, E., & Worrall, J. A. R. (2014). Conformational and thermodynamic hallmarks of DNA operator site specificity in the copper sensitive operon repressor from *Streptomyces lividans*. *Nucleic Acids Research*, *42*(2), 1326–1340.

Terwilliger, T. C. (2013). Finding non-crystallographic symmetry in density maps of macromolecular structures. *Journal of Structural and Functional Genomics*, *14*(3), 91–5.

Tompa, P., & Kalmar, L. (2010). Power Law Distribution Defines Structural Disorder as a Structural Element Directly Linked with Function. *Journal of Molecular Biology*, *403*(3), 346–350.

Trevors, J. T., & Cotter, C. M. (1990). Copper toxicity and uptake in microorganisms. *Journal of Industrial Microbiology*, *6*(2), 77–84.

Trott, O., & Olson, A. J. (2010). AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, *31*(2), 455–461.

Tsuchiya, Y., Kinoshita, K., Ito, N., & Nakamura, H. (2006). PreBI: prediction of biological interfaces of proteins in crystals. *Nucleic Acids Research*, *34*

Turkenburg, J. P., & Dodson, E. J. (1996). Modern developments in molecular replacement. *Current Opinion in Structural Biology*, *6*(5), 604–610.

Van Der Lee, R., Buljan, M., Lang, B., Weatheritt, R. J., Daughdrill, G. W., Dunker, A. K., Babu, M. M. (2014). Classification of intrinsically disordered regions and proteins. *Chemical Reviews*, *114*(13), 6589–6631.

Vagin, A.A. & Teplyakov, Alexey (1997). MOLREP: an automated program for Molecular Replacement. *Journal of Applied Crystallography*, 30, 1022-1025.

Vucetic, S., Brown, C. J., Dunker, A. K., & Obradovic, Z. (2003). Flavors of protein disorder. *Proteins: Structure, Function, and Bioinformatics*, *52*(4), 573–584.

Wallace, A. C., Laskowski, R. A., & Thornton, J. M. (1995). LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Engineering*, 8(2), 127–34.

© COPYRIGHT UPM

Wang, S.-H., Wu, Y.-T., Kuo, S.-C., & Yu, J. (2013). HotLig: A Molecular Surface-Directed Approach to Scoring Protein–Ligand Interactions. *Journal of Chemical Information and Modeling*, *53*(8), 2181–2195.

Waldron, K. J., & Robinson, N. J. (2009). How do bacterial cells ensure that metalloproteins get the correct metal. *Nature Reviews. Microbiology*, *7*(1), 25-35.

Waldron, K. J., Rutherford, J. C., Ford, D., & Robinson, N. J. (2009). Metalloproteins and metal sensing. *Nature*, *460*(7257), 823–30.

Wampler, J. E. (1997). Distribution Analysis of the Variation of B-Factors of X-ray Crystal Structures: Temperature and Structural Variations in Lysozyme. *Journal of Chemical Information and Computer Sciences*, *37*(6), 1171–1180.

Wiegand, S., Rabausch, U., Chow, J., Daniel, R., Streit, W. R., & Liesegang, H. (2013). *Genome Announcements*, 1(2), 1–2.

Wisedchaisri, G., & Gonen, T. (2013). Phasing electron diffraction data by molecular replacement: strategy for structure determination and refinement. *Methods in Molecular Biology*, *955*(10), 243–72.

Wlodawer, A., Minor, W., Dauter, Z., & Jaskolski, M. (2008). Protein crystallography for non-crystallographers, or how to get the best (but not more) from published macromolecular structures. *The FEBS Journal*, *275*(1), 1–21.

Worrall, J. A. R., & Vijgenboom, E. (2010). Copper mining in *Streptomyces*: enzymes, natural products and development. *Natural Product Reports*, 27(5), 742-56.

Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G. W., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A. and Wilson, K. S. (2011). Overview of the CCP4 suite and current developments. *Acta Crystallography, D67*, 235-242.

Wright, P. E., & Dyson, H. J. (2015). Intrinsically disordered proteins in cellular signalling and regulation. *Nature Reviews. Molecular Cell Biology*, *16*(1), 18–29.

Xu, Q., Canutescu, A. A., Wang, G., Shapovalov, M., Obradovic, Z., Dunbrack, R. L., & Jr. (2008). Statistical analysis of interface similarity in crystals of homologous proteins. *Journal of Molecular Biology*, *381*(2), 487–507.

Yamashita, M. M., Wesson, L., Eisenmant, G., & Eisenberg, D. (1990). Where metal ions bind in proteins (metafloprotein/protein structure/hydrophobicity contrast function). *Biophysics*, *87*, 5648–5652.

© COPYRIGHT UPM

Yueh, C., Hall, D. R., Xia, B., Padhorny, D., Kozakov, D., & Vajda, S. (2017). ClusPro-DC: Dimer Classification by the Cluspro Server for Protein–Protein Docking. *Journal of Molecular Biology*, 429(3),372-381.

Zwart, P. H., Grosse-Kunstleve, R. W., Lebedev, A. A., Murshudov, G. N., & Adams, P. D. (2008). Surprises and pitfalls arising from (pseudo)symmetry. *Acta Crystallographica. Section D, Biological Crystallography*, *64*(1), 99–107.

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