Medical Laboratory Journal, Nov-Dec , 2017; Vol 11: No 6

Original Article

Effect of Mutation in Efflux Pump Regulatory Protein (MexR) of *Pseudomonas aeruginosa*: A Bioinformatic Study

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Received : 06 May 2017
Revised: 29 Jun 2017
Accepted: 29 Aug 2017

ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa* is an important non-fermenting gram-negative hospital-acquired pathogen. Treatment of *P. aeruginosa* infections has become more challenging due to overexpression of efflux pumps. The aim of the present study was to apply in silico analysis to evaluate the structure of the efflux pump regulatory protein, MexR, and impact of mutation on its stability and function.

Methods: Different bioinformatics tools including EXPASY, PROTEER, TECCOFFE, iStable, I-Mutant 2, STRING, ESPript, GOR IV, and PDB were used in the study.

Results: Aliphatic and instability indices were 104.15, and 46.52, respectively, indicating that the protein has a relatively short half-life. Most mutations decreased protein stability. Twenty-four mutations were identified as deleterious, with negative impact on the protein's function.

Conclusion: Determination of structure, variability, and function of MexR could be useful for modeling of treatment and control of multidrug resistant *P. aeruginosa*, with overexpressed efflux pump. We found that MexR is a relatively unstable and conserved protein and the majority of mutations decrease its stability.

Keywords: Pseudomonas aeruginosa, MexR protein, Drug resistance, drug resistance multiple

This paper should be cited as: Vaez H, Vaez v, Khademi F[Effect of Mutation in Efflux Pump Regulatory Protein (MexR) of *Pseudomonas aeruginosa*: A Bioinformatic Study]. mljgoums. 2017; 11(6): 35-41

INTRODUCTION

Pseudomonas aeruginosa is an important non-fermenting gram negative bacilli that can cause infections such as pneumonia, urinary tract infection, blood stream infection, meningitis, and skin infection, especially in immunocompromised patients (1). *P. aeruginosa* has a remarkable capacity to survive in adverse conditions (2, 3). According to previous studies, overexpression of efflux pumps genes has a crucial role in emergence of multi-drug resistance (MDR)-*P. aeruginosa* (4, 5). Several efflux pumps have been identified in *P. aeruginosa*. Based on their structure, source of energy and substrate, they have been classified into five families of resistance-nodulation-division (RND), ATP-binding cassette transporter family, major facilitator superfamily, multidrug and toxic compound extrusion, and small MDR (4, 5). Active efflux pumps decrease intracellular concentration of different antibiotics by pumping out drugs, potentially leading to emergence of MDR-*P. aeruginosa* isolates. The RND family includes MexAB-oprM, MexCD-oprJ, MexEF-oprN, MexJK-oprM, and MexXY-oprM, which are targeted as substrate of different classes of antibiotics (4, 5). MexAB-oprM has three domains including membrane fusion protein, cytoplasmic membrane transporter, and outer membrane factor. MexAB-oprM operon is negatively regulated by the MexR, a 147 amino acid long regulatory protein encoded by the *mex*R gene, located at the upstream of the MexAB-oprM operon (6). Studies on 3D structure of the MexR showed that it is mainly consisted of α-helices, and composes a triangular dimer with two DNA binding domains connected to each other (4, 5). In addition to their role in development of MDR-*P*. aeruginosa isolates, efflux pumps are also involved in survival and pathogenicity. For example, efflux pumps deletion notably reduces the ability of *P. aeruginosa* to invade epithelial cells (7). Beyond its role in epithelial cell invasion, efflux pumps have a critical role in cell-to-cell communication by quorum sensing signaling and biofilm formation. Biofilm formation by *P. aeruginosa* enables the bacteria to impair host's immunity. Therefore, disrupting biofilm formation in *P. aeruginosa* is of great importance (7). Amino acid substitutions can cause fundamental changes in normal proteins, affecting their stability, physicochemical properties and function. Hence, the use of bioinformatics tools has received considerable interest in recent years since they can predict the consequence of changes in normal protein, and contribute to drug design and development (8). As mentioned earlier, the MexAB-oprM efflux pump is one of the most important members of the RND family (4, 5) and its overexpression is mainly regulated by MexR. Therefore, the present study aimed to evaluate the effect of mutations on structure, stability, and function of MexR by using in silico analysis.

MATERIAL AND METHODS

We used *P. aeruginosa* PAO1 (NP_249115.1, NC_002516.2) as the reference strain, and its full MexR sequence was retrieved in FASTA format from the GenBank (https://www.ncbi.nlm.nih.gov/protein/). The MexR sequence was searched to find similar sequences using BlastP (https://blast.ncbi.nlm.nih.gov). Multiple alignments for the similar sequences were performed using T-COFFEE (http://www.tcoffee.org) and MEGA6 (9).

Primary structure of MexR was predicted using EXPASY (http://web.expasy.org/protparam) (10). Physiochemical properties such as molecular weight, atomic composition, chemical formula, amino acid composition, isoelectric point, aliphatic index, and approximate half-life were estimated (10). Secondary structure was predicted by ESPript 3.0 (http://espript.ibcp.fr) (11) and GOR IV (https://npsa-prabi.ibcp.fr). Three-dimensional structure was evaluated by SWISS MODEL (https://swissmodel.expasy.org/interactive) (10), and PDB file (1lnw) was saved for subsequent analysis. Three-dimensional structure of the reference protein (NP_249115.1) was obtained from the Protein Data Bank (http://www.rcsb.org). Transmembrane, intracellular and extracellular amino acids were predicted using PROTEER (http://wlab.ethz.ch/protter)(12). Interaction of MexR with other proteins was predicted using the STRING database (http://string-db.org). The STRING database provides a critical assessment and integration of protein–protein interactions, including direct (physical) and indirect (functional) associations. This database covers more than 2000 organisms,

which has necessitated novel, scalable algorithms for transferring interaction information between organisms (13). Mutation of a single amino acid residue can cause some changes in a protein, which could result in the loss of protein function. We conducted a comprehensive search on popular databases including Web of Science, PubMed, Google scholar and Scopus to find reports on MexR mutation, using the following keywords: efflux pumps, MexR, MexAB-OprM and MDR *P. aeruginosa*. Finally, missense mutation reports were selected for the study. Three independent servers including I-Mutant2 (http://folding.biofold.org), iStable (http://predictor.nchu.edu.tw/iStable/), and PROVEAN (http://provean.jcvi.org) were used to evaluate effect of mutations on the protein. I-Mutant2 is an online program and support vector machines-based web server for prediction of protein stability changes upon single point mutation from the protein sequence and structure. According to the I-Mutant2 database, free energy change value (DDG) less than zero and more than zero can decrease and increase the stability of the protein, respectively. iStable provides an accurate approach for prediction of protein stability changes, using sequence information and prediction results from different element predictors (14).

RESULTS

Based on the results of BlastP (Table1), ten sequences with greatest identity were subjected to multiple alignments tools using the T-COFFEE program. MexR belongs to the Mar family of proteins that are involved in the emergence of MDR isolates. Figure 1 shows the results of multiple sequence alignments.

Primary structure analysis showed that the 147 amino acid long protein consists of Ala 10 (6.8%), Arg 14 (9.5%), Asn 5 (3.4%), Asp 12 (8.2%), Cys 3 (2.0%), Gln 12 (8.2%), Glu 10 (6.8%), Gly 4 (2.7%), His 6 (4.1%), Ile 7 (4.8%), Leu 23 (15.6%), Lys 3 (2.0%), Met 5 (3.4%), Phe 4 (2.7%), Pro 9 (6.1%), Ser 4 (2.7%), Thr 6 (4.1%), Trp 0 (0.0%), Tyr 1 (0.7%), and Val 9 (6.1%). The protein contains 2395 atoms, with the following chemical formula: $C_{739}H_{1207}N_{221}O_{220}S_8$. The predicted isoelectric point and molecular weight were 5.64 and 16964.54 Da, respectively. The aliphatic index and instability index were estimated to be 104.15 and 46.52, respectively, indicating that the protein has a relatively short half-life. The secondary structure of the protein was mainly alpha helix (71.43%) and random coil (28.57%) (Figure 2). The secondary structure of the protein was mainly composed of α-helix sheets. The transmembrane, intracellular and extracellular amino acid prediction by the PROTEER server showed that MexR has no transmembrane residue. As shown in figure 3, the prediction revealed that the sequences with substitutions exhibit interactions similar to those of the reference strain. The following interactions were observed in the results: MexA (RND multidrug efflux membrane fusion protein precursor), amrB (multidrug efflux protein), rhlR (transcriptional regulator of RhlR necessary for transcriptional activation of the *rhlA*B genes), MexB (inner membrane transporter component of the MexAB-OprM efflux system that confers MDR), armR (antirepressor for MexR), MexF (RND multidrug efflux transporter), GyrA (DNA gyrase subunit A), and NfxB (transcriptional regulator of resistance to quinolones).

Table1- Summary of BlastP for MexR (*P. aeruginosa*)

Accession	Identity	Query cover	Total score	Max score	Gaps	Positive number
ANF04417.1	100%	100%	297	297	0/147 (0%)	147/147 (100%)
WP_043090638.1	99%	100%	296	297	0/147 (0%)	146/147 (99%)
WP_034039844.1	99%	100%	296	297	0/147 (0%)	146/147 (99%)
WP_034043761.1	99%	100%	296	297	0/147 (0%)	146/147 (99%)
WP_003114897.1	100%	100%	297	297	0/147 (0%)	147/147 (100%)
AAW82616.1	99%	100%	296	297	0/147 (0%)	146/147 (99%)
WP_046879890.1	99%	100%	295	297	0/147 (0%)	146/147 (99%)
WP_049249729.1	99%	100%	295	297	0/147 (0%)	146/147 (99%)
WP_034034038.1	99%	100%	296	297	0/147 (0%)	146/147 (99%)
WP_012613505.1	99%	100%	294	297	0/147 (0%)	146/147 (99%)

Figure1- Results of multiple alignments using T-COFFEE revealed that MexR is highly conserved. Yellow color shows variable regions.

Figure2- Secondary structure prediction by ESPript depicted alpha helix regions in MexR

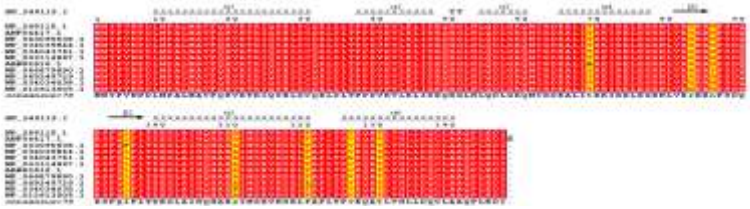

Figure 3- Schematic of protein-protein interactions predicted by STRING.

Colored lines between proteins indicate physical and functional interactions. Node size; small node, protein of unknown 3D structure, large node, some 3D structure is known or predicted. Colored nodes show first shell of interactors and white nodes show second shell of interactors. We found 46 amino acid substitutions at different positions in the online search for reports on *mexR* mutations (15-26). Results of analysis with I-Mutant2 showed that most of evaluated missense mutations decreased stability of the protein (Table 2). The mutations that stabilized the protein were seen at positions D8E, H107P, T130P, N53Y, N86I, and G58E.

According to the results obtained from iStable, alterations such as K44M, H107P, V132R, C30R, Q106R, N53Y, N53D, M10R, R78I, and R21W increased the stability of protein, while others decreased the stability (Table 2). Based on PROVEAN, substitutions at positions A66P, K44M, V126E, A66V, D8E, H107P, V132R, C30R, N79S, Q106R, L13M, and R21W were neutral and had no impact on protein function, while substitution at positions L45P, I46N, L57P, L57R, T69I, I72N, L75P, L75R, R83C, R91C, R91H, R114C, R70T, T130P, Q94P, R83H, G58E, L95F, T69I, N53Y, R78I, S88C, N86I, and R70W were deleterious (Table 2).

Table 2- Missense mutations and its impact on the protein

Position	Amino acid substitution	I Mutant 2		i Stable		PROVEAN	
		Stability	DDG	Stability	Score[a]	Score[b]	Prediction
66	A66P	Decrease	-0.84	Decrease	0.73	-2.40	Neutral
44	K44M	Decrease	-0.74	Increase	0.84	0.16	Neutral
126	V126E	Decrease	-2.2	Decrease	0.67	2.38	Neutral
66	A66V	Decrease	0.07	Decrease	0.80	-2.36	Neutral
8	D8E	Increase	0.05	Decrease	0.60	0.22	Neutral
107	H107P	Increase	0.17	Increase	0.83	1.16	Neutral
132	V132R	Decrease	-2.1	Increase	0.83	-0.58	Neutral
45	L45P	Decrease	-1.65	Decrease	0.66	-3.04	Deleterious
46	I46N	Decrease	-1.69	Decrease	0.65	-5.06	Deleterious
57	L57P	Decrease	-1.02	Decrease	0.80	-6.6	Deleterious
57	L57R	Decrease	-1.20	Decrease	0.78	-5.8	Deleterious
69	T69I	Decrease	-1.27	Decrease	0.80	-3.9	Deleterious
72	I72N	Decrease	-0.60	Decrease	0.80	-5.7	Deleterious
75	L75P	Decrease	-1.1	Decrease	0.81	-6.5	Deleterious
75	L75R	Decrease	-2.16	Decrease	0.80	-5.6	Deleterious
83	R83C	Decrease	-0.30	Decrease	0.72	-7.4	Deleterious
91	R91C	Decrease	-0.38	Decrease	0.76	-7.9	Deleterious
91	R91H	Decrease	-1.4	Decrease	0.80	-5.0	Deleterious
30	C30R	Decrease	-0.02	Increase	0.71	-1.86	Neutral
114	R114C	Decrease	-0.64	Decrease	0.71	-4.68	Deleterious
79	N79S	Decrease	0.08	Decrease	0.85	-0.40	Neutral
106	Q106R	Decrease	-0.48	Increase	0.80	-1.10	Neutral
70	R70T	Decrease	-0.33	Decrease	0.76	-2.63	Deleterious
130	T130P	Increase	-0.59	Decrease	0.80	-2.65	Deleterious
94	Q94P	Decrease	-1.14	Decrease	0.57	-3.20	Deleterious
13	L13M	Decrease	-1.41	Decrease	0.84	-0.68	Neutral
21	R21W	Decrease	-0.29	Increase	0.59	-0.60	Neutral
83	R83H	Decrease	-1.24	Decrease	0.74	-4.62	Deleterious
58	G58E	Increase	0.60	Decrease	0.57	-3.33	Deleterious
95	L95F	Decrease	-0.36	Decrease	0.90	-3.05	Deleterious
69	T69I	Decrease	-1.27	Decrease	0.80	-3.90	Deleterious
70	R70W	Decrease	-0.62	Decrease	0.62	-4.83	Deleterious
53	N53Y	Increase	0.05	Increase	0.62	-2.93	Deleterious
77	G77A	Decrease	-1.77	Decrease	0.83	1.56	Neutral
86	N86I	Increase	0.70	Decrease	0.82	-4.82	Deleterious
88	S88C	Decrease	-0.86	Decrease	0.74	-3.19	Deleterious
63	R63H	Decrease	-0.22	Decrease	0.85	-1.81	Neutral
53	N53D	Decrease	-0.68	Increase	0.66	-0.36	Neutral
48	E48K	Decrease	-0.05	Decrease	0.84	0.77	Neutral
21	R21G	Decrease	-1.73	Decrease	0.88	-1.78	Neutral
26	S26G	Decrease	-1.71	Decrease	0.86	-0.74	Neutral
79	N79G	Decrease	-1.61	Decrease	0.87	4.39	Neutral
10	M10R	Decrease	-0.30	Increase	0.50	-0.69	Neutral
78	R78I	Decrease	-0.28	Increase	0.76	-5.76	Deleterious
103	A103T	Decrease	-1.38	Decrease	0.83	-1.34	Neutral
106	Q106H	Decrease	-1.09	Decrease	0.74	-1.82	Neutral

DISCUSSION

P. aeruginosa is considered as one of the most important hospital-acquired pathogens due to the resistance to multiple antibiotics and ability to survive on minimal nutritional requirements. Treatment of infections caused by this microorganism is becoming more challenging due to the resistance against multiple antibiotics (1-3). Efflux pumps are preponderant mechanisms of resistance because they extrude a wide range of substrates including penicillin, cephalosporin, carbapenems, monobactams, fluoroquinolones, and chemical disinfectants (27). The MexAB-OprM is an important efflux pump, negatively regulated by MexR (23).

In our study, the aliphatic and instability indices indicated that MexR is relatively unstable. The aliphatic index is directly related to the mole fraction of Ala, Ile, Leu, and Val (28). The aliphatic index of proteins from thermophilic bacteria was found to be significantly higher than that of ordinary proteins and hence, it can serve as a measure of thermos-stability of proteins (28). Mutations

can affect protein folding, stability, and function, as well as protein-protein interactions. Moreover, it is essential to identify specific interaction partners for a protein to describe functions of the protein thoroughly (10, 29). In this study, the STRING program was used to predict protein-protein interactions, and the results showed that the interactions in the variants investigated were similar to that of the reference strain (data not shown). In our study, we used two independent programs to determine the effects of mutations on stability. Results of I mutant2 revealed that the majority of mutations decreased the stability except those at positions D8E, H107P, T130P, N53Y, N86I and G58E. Moreover, results of iStable showed that alterations at positions K44M, H107P, V132R, C30R, Q106R, N53Y, N53D, M10R, R78I and R21W increased the stability of the protein, while others decreased the stability. However, the results of the two programs were not entirely identical. This could be due to difference in the accuracy of the two programs (77% vs. 80%) and in data set used by the tools. On the other hand, optimal stability of a protein relies on various thermodynamic factors. Several theories support the hypotheses that formation of stable molecules is a thermodynamically controlled process. However, the significance of negative entropy, chaperones, and oxidative potential should not be neglected (30).

We used PROVEAN to identify the effect of mutations on the protein's function. Of 46 amino acid substitutions, 24 mutations were identified as deleterious, and the remaining ones were neutral. Most deleterious substations were related to residues 66-95. Regarding the secondary structure, 16 of 24 deleterious mutations were mapped at α-4 and between α-4 and α-5. In the crystal structure of MexR, helices 1,4 and 5 are involved in dimerization, while α-4 plays a crucial role in DNA binding (31). Studies of Andersen et al. and Saito et al. showed that mutations at R83H, R91H, and L95F significantly overexpress efflux pump, suggesting that MexR impaired DNA binding activity (16, 22).

CONCLUSION

Although different bioinformatics tools are available for determination of sequence, structure and function of a protein, their outputs are not identical. Therefore, it is necessary to use multiple programs and combine the results for the final interpretation. We found that the results of iStable and PROVEAN are almost identical. In addition, the majority of mutations decrease the stability of the MexR protein, particularly those located at the α-4 residue.

ACKNOWLEDGEMENTS

We would like to express our gratitude to owners of the online bioinformatics tools used in the study and the staff of Zabol University of Medical Sciences.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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