Scientific Research Publishing

American Journal of Molecular Biology, 2018, 8, 195-204
http://www.scirp.org/journal/ajmb
ISSN Online: 2161-6663
ISSN Print: 2161-6620

Cloning, Expression, Purification, and Crystallization of *P. aeruginosa* ICMP

Ruliang Pi[1,2*], Jiang Gu[3], Guangwen Lu[1*]

[1]West China Hospital Emergency Department (WCHED), State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Chengdu, China
[2]Chengdu Institute of biological Products Co., Ltd., Chengdu, China
[3]National Engineering Research Center of Immunological Products, Department of Microbiology and Biochemical Pharmacy, College of Pharmacy, Third Military Medical University, Chongqing, China
Email: *534014641@qq.com, *lugw@scu.edu.cn

How to cite this paper: Pi, R.L., Gu, J. and Lu, G.W. (2018) Cloning, Expression, Purification, and Crystallization of *P. aeruginosa* ICMP. *American Journal of Molecular Biology*, **8**, 195-204.
https://doi.org/10.4236/ajmb.2018.84017

Received: July 30, 2018
Accepted: September 26, 2018
Published: September 29, 2018

Abstract

Pseudomonas aeruginosa (*P. aeruginosa*) is a common opportunistic human pathogen that can lead to severe diseases in immunocompromised patients. The insulin-cleaving membrane protease (ICMP) of *P. aeruginosa* plays a vital role in the pathogenesis of the bacterium and is therefore characterized as an important bacterial virulence factor. In addition, ICMP also serves as a founding member of the M75 peptidase family and represents a prototype of the imelysin/imelysin-like proteins. Despite of its functional importance in the pathogenesis of *P. aeruginosa* and of a root position as the prototypic imelysin/imelysin-like member, the structural features of the protein remain uninvestigated. Since preparation of homogeneous and crystallizable protein species is the prerequisite for structural studies by crystallography, we reported the successful expression, purification, and crystallization of *P. aeruginosa* ICMP in this study. The protein was over-expressed in *Escherichia coli* as a GST-fusion protein, cleaved to remove the fusion tag, and then purified to homogeneity. Diffractable crystals were obtained using the sitting-drop vapour-diffusion method. The crystals diffracted to 2.5 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 54.47, b = 158.98, c = 162.84 Å, $\alpha = \beta = \gamma = 90^\circ$. Preliminary analysis of the diffraction data revealed high-quality crystallographic statistics with a Matthews coefficient of about 2.61 $Å^3 \cdot Da^{-1}$ and a solvent content of about 52.58%, indicating the presence of three ICMP molecules in the asymmetric unit. The current work therefore paved the way for future studies aiming to delineate the characteristics of ICMP at the atomic level.

Keywords

Pseudomonas aeruginosa, ICMP, Metallopeptidase, Imelysin/Imelysin-Like

Protein, Crystallographic Analysis

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic human pathogen that often colonizes immunocompromised patients and one of the main bacterial pathogens which may cause severe lung deterioration in cystic fibrosis patients [1] [2] [3]. It is always classified as one of the top three most frequent gram-negative pathogens and is linked to the worst visual diseases [4]. Also, it has multi-drug-resistance and the ability to develop new resistances during antibiotic treatment, so it becomes not controlled and eliminated [5] [6].

Different extracellular virulence factors of *P. aeruginosa* like hemolysins, exoenzyme S, and exotoxin A play important roles in its pathogenesis [7]. An out member protein of *P. aeruginosa*, which was designated as the insulin-cleaving membrane protease (ICMP), was first characterized as a zinc peptidase [8]. This bacterial protein harbors the capacity of cleaving insulin B chain between Glu13-s-Ala14, Tyr16-s-Leu17, Phe25-s-Tyr26, and His10-s-Leu11, and is therefore characterized as a putative virulence factor of *P. aeruginosa* [8]. ICMP contains a conserved HXXE motif which was commonly observed in the MEROPS [9] metallopeptidase family, and has been shown to bind to zinc metal to perform its peptidase activity [8]. In MEROPS, ICMP serves as the founding member of the M75 peptidase family and represents a prototype of the imelysin/imelysin-like proteins [9].

In addition to cleaving insulin during the pathogenesis of the bacterium, ICMP was also implicated as a virulence factor involved in iron uptake. As an essential nutrient for bacterial growth, iron is poorly soluble at physiological pH and toxic in the presence of O_2. As a result, bacteria have evolved to contain complicated and versatile regulation systems on iron uptake and metabolism [10]. Like most bacterial pathogens, *P. aeruginosa* exhibits impressive capacity to retrieve iron from a variety of natural sources through a multiplicity of systems for the active transport of iron to infect the host and to multiply within tissues [11] [12]. ICMP genetically organized in an imelysin operon (PA4370-PA4373) is regulated by Fur (ferric uptake regulation) [13]. The genomic context of all previously characterized imelysins bears substantial similarity to iron-transporter EfeUOB which was characterized in *Escherichia coli* O157:H7 [14] [15] [16]. It is also a homologous protein to IrpA (iron regulated protein A) which has been experimentally demonstrated to be essential for growth under iron-deficient conditions in the cyanobacteria *Synechococcus* sp. [17]. Therefore, ICMP has an important role in iron uptake.

Despite of the functional importance of ICMP in the pathogenesis of *P. aeruginosa*, the high resolution structure of this protein, however, remains unknown thus far. Several previous studies have reported the structures of imelysin/imelysin-like proteins, including the imelysin-like protein from *Psychrobac-*

ter arcticus (IPPA), the permuted imelysin from *Bacteroides ovatus* (PIBO) [18], and the alginate-binding protein from *Sphingomonas* sp. A1 (Algp7) [19]. These structures have revealed an all-helical fold with sterically conserved HXXE motif-residues [18] [19]. However, no peptidase activity could be detected for these imelysin-like proteins [18] [19]. It is obvious that ICMP exhibits very little sequence identities to these proteins (15.30% to IPPA, 12.89% to PIBO, and 11.43% to Algp7). Therefore, it is an interesting scientific question that how the ICMP-structure differs from those of other imelysin/imelysin-like proteins represented by IPPA, PIBO, and Algp7. In light of its significant role in the pathogenesis of *P. aeruginosa*, we set out to determine the structure of ICMP, aiming to delineate the mechanism of ICMP catalysis and to facilitate the structure-directed drug design. In the present study, we reported the cloning, expression, purification, and crystallization of ICMP. A data set was collected to 2.5 Å resolution using the synchrotron radiation. The preliminary crystallographic analysis of the diffraction data was also conducted.

2. Materials and Methods

2.1. Cloning, Expression and Purification of ICMP

The ICMP protein from *P. aeruginosa* PAO1 (NP_253060.1) [20], which contains 446 residues, was selected for the crystallographic studies in this report. To facilitate soluble expression of the ICMP protein, the predicted N-terminal lipoprotein signal peptide (residues 1 to 19) and a small hydrophobic region directly following this lipoprotein signal (residues 20 to 48) were not included in the expression construct. The resultant DNA fragment encoding ICMP amino acids 49 - 446 was then cloned into the pGEX-6P-1 (GE Healthcare) vector via the *BamH* I and *Xho*I enzymes. The recombinant protein expressed from this vector therefore, it contains a GST tag followed by a PreScission Protease (PSP) cleavage site at the N-terminus of ICMP. The recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3) for protein expression. In brief, the transformants were grown at 37˚C overnight on a Luria-Bertani (LB) agar plate containing 100 mg/l ampicillin. A single colony was inoculated into 50 ml LB medium containing 100 mg/l ampicillin and incubated overnight. This pre-culture was then transferred into fresh LB medium supplemented with ampicillin at the ratio of 1:100, and further grown at 37˚C until the culture density (OD600) reached approximately 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) was then added to the cell culture to a final concentration of 0.1 mM, and the cells were further incubated at 37˚C for about 10 h to induce the expression of the protein.

For protein purification, the cells were harvested by centrifugation at 5000 g for 10 min at 4˚C, and resuspended in ice-cold buffer consisting of 20 mM Tris-HCl and 150 mM NaCl, pH 8.0. The cell pellet was disrupted using an ultrasonic cell crusher. Cell debris was removed by centrifugation at 16,000 g for 30 min at 4˚C. The crude protein extract was mixed with the GST resin (GE Healthcare) at 4˚C for about 2 h. The resin was then washed with five-column

volumes of the buffer composed of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. After washing, the protein was released from the GST-tag by on-column cleavage at 16˚C for about 2 h with 10 units PSP enzyme/mg fusion protein. The subsequent eluate containing the target protein was then collected, and applied to a Superdex 200 Increase 10/300 GL column (GE Healthcare) pre-equilibrated with a buffer composed of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl at 4˚C to further remove the impurities. The pooled ICMP protein was further concentrated by centrifugation at 4˚C using an Amicon Ultra-15 centrifugal filter device (10 kDa cutoff; Millipore) to 10 mg/ml before using for crystallization screenings. The ICMP-production information is summarized in Table 1.

2.2. ICMP Crystallization

The initial screening trial was carried out using the commercial crystallization kits (Hampton Research) via the sitting-drop vapour-diffusion method at 18˚C. The experiments were performed such that 1 μl protein solution at 10 mg/ml

Table 1. Protein-production information.

Source organism	*Pseudomonas aeruginosa*
DNA source	Genomic DNA
Forward primer[†]	CGGGATTCAAGGTCGACGAGGC
Reverse primer[†]	CCGCTCGAGTCAGAACTCGTGGTCGGCG
Expression vector	pGEX-6P-1
Expression host	*E. coli* BL21(DE3)
Complete amino-acid sequence of ICMP	MTRMPLATASLLALAISLAGCGDDKKAEAPATPAASTQPA ASAAAPAAKVDEAAAKAVIKNYADLAEATFADALSTAKD LQKAIDAFLAKPDAETLKAAKEAWFAARTPYSQSEAFRFG NAIIDDWEGQVNAWPLDEGLIDYVAKDYQHALGNPGAT ANIVANTEIQVGEDKIDVKEITGEKLASLNELGGSEANVAT GYHAIEFLLWGQDLNGTGPGAGNRPATDYAQGKDCTGG HCDRRAAYLKAVTDLLVSDLEYMAGQWKAGVADNYRAK LEAEPVDTGLRKMFFGMGSLSLGELAGERMKVALEANSTE DEHDCFSDDTHHTLFFNGKSIRNIYLGEYKRIDGSVVKGPS LADLVAKADAAANDTLKADLADTEAKLQAIVDSAEKDGV HFDQMIAPDNKDGQQKIRDAIAALVKQTGAIEQAAGKLGI QDLKPDNADHEF
Complete amino-acid sequence of the expression construct produced after PSP digestion	GPLGSKVDEAAAKAVIKNYADLAEATFADALSTAKDLQKA IDAFLAKPDAETLKAAKEAWFAARTPYSQSEAFRFGNAIID DWEGQVNAWPLDEGLIDYVAKDYQHALGNPGATANIVA NTEIQVGEDKIDVKEITGEKLASLNELGGSEANVATGYHAI EFLLWGQDLNGTGPGAGNRPATDYAQGKDCTGGHCDRR AAYLKAVTDLLVSDLEYMAGQWKAGVADNYRAKLEAEP VDTGLRKMFFGMGSLSLGELAGERMKVALEANSTEDEHD CFSDDTHHTLFFNGKSIRNIYLGEYKRIDGSVVKGPSLADL VAKADAAANDTLKADLADTEAKLQAIVDSAEKDGVHFD QMIAPDNKDGQQKIRDAIAALVKQTGAIEQAAGKLGIQDL KPDNADHEF

[†]The *BamH*I and *Xho*I sites are underlined. The ICMP signal peptide and its following small hydrophobic region, which are excluded from the expression construct, are indicated in green and red, respectively. Those vector-derived residues that retain at the N-terminus of ICMP after PSP-digestion are marked in blue.

was firstly mixed with 1 μl reservoir solution, and then equilibrated against 70 μl reservoir solution in 48-well double-sample sitting-drop crystallization plates. The plates were kept at 18˚C for about one week for the crystals to grow, and the identified conditions were then optimized. Further optimization of the crystallization conditions was then conducted by preparing reservoir solutions with gradually decreased precipitant concentrations. Crystals of good quality were finally obtained in a condition consisting of 1.6 M sodium citrate tri-basic dihydrate at pH 6.5. Crystallization information is summarized in **Table 2**.

2.3. Data Collection and Processing

For data collection, a single crystal was picked up using a nylon loop, immersed into a cryoprotectant solution consisting of the reservoir solution supplemented with 20% glycerol for about 30 s, and then flash-cooled at −173˚C. The diffraction data were collected using the synchrotron radiation at the Shanghai Synchrotron Radiation Facility (SSRF) beamline BL18U1. The collected data were then processed with HKL2000 [21] for indexing, integration, and scaling. Detailed statistics on data collection are summarized in **Table 3**.

3. Results and Discussion

The ICMP protein of *P. aeruginosa* was initially identified as an outer membrane protein [8]. This protein contains a predicted N-terminal signal peptide consisting of residues 1 - 19 followed by a small region extending from residue 20 to 48 with a majority of hydrophobic amino acids (**Table 1**). To facilitate soluble expression of the ICMP protein, these N-terminal residues were removed from the protein-expression construct. Subsequently, the Pseudomonas ICMP with amino acids 49 - 446 was expressed as a recombinant protein with an N-terminal GST tag in *E. coli* (**Figure 1(a)**). As expected, the fusion protein is largely expressed in the soluble form, which could be easily purified via affinity chromatography (**Figure 1(b)**). After removal of the GST fusion tag, the target protein was further purified by gel-filtration, in which, the ICMP protein was eluted as a symmetric peak at about 15 ml on a calibrated Superdex 200 Increase 10/300 GL

Table 2. Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	48-well plates
Temperature (˚C)	18
Protein concentration (mg·ml^{-1})	10
Buffer composition of protein solution	20 mM Tris-HCl at pH 8.0, 150 mM NaCl
Composition of reservoir solution[†]	1.6 M sodium citrate tribasic dehydrate at pH 6.5.
Volume and ratio of drop	1.0 μl, 1:1 ratio of protein: reservoir solution
Volume of reservoir	70 μl

[†]The condition for ICMP crystallization.

Table 3. Data collection and processing.

Diffraction source	SSRF-BL18U1
Wavelength (Å)	0.97780
Detector	Pilatus
Crystal-to-detector distance (mm)	300
Rotation range per image (˚)	1
Total rotation range (˚)	360
Exposure time per image (s)	0.6
Space group	P212121
Unit-cell parameters (Å)	a = 54.47, b = 158.98, c = 162.84
Resolution range (Å)	50 - 2.50 (2.50 - 2.59)
Total No. of reflections	539899
No. of unique reflections	53128
Completeness (%)	99.5 (98.6)
Average mosaicity (˚)	0.300
CC1/2	0.890
Multiplicity	10.2 (10.1)
<I/σ(I)>	18.0 (3.0)
R_{meas} † (%)	13.3 (66.0)

† $R_{meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl)-\langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

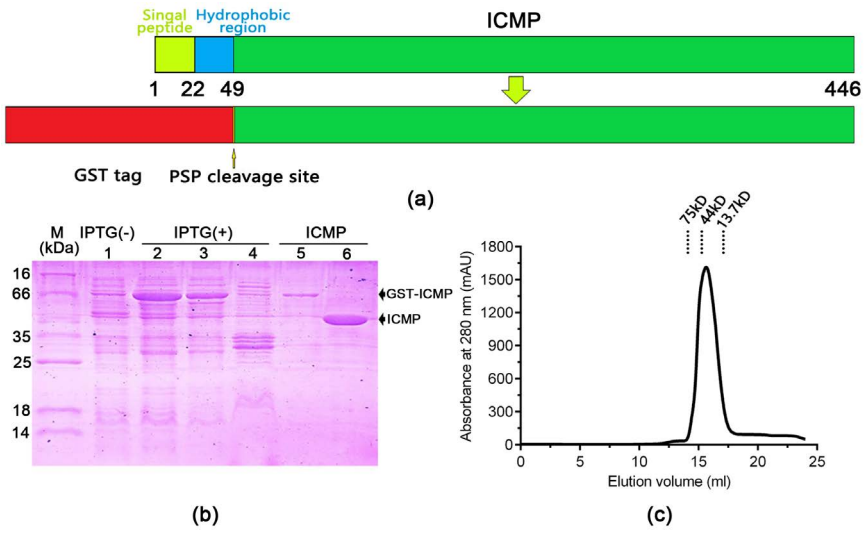

Figure 1. Expression and purification of the ICMP protein. (a) a schematic diagram showing the cloning strategy for ICMP; (b) the expressed ICMP protein as shown by SDS-PAGE. Lane M, standard protein molecular-weight markers (labelled in kDa); lane 1, total cell (un-induced); lane 2, total cell lysate (induced); lane 3, soluble fraction; lane 4, pellet fraction; lane 5, purified GST fusion ICMP; lane 6, purified ICMP protein after PSP cleavage; (c) analytical gel-filtration analysis of purified ICMP. A typical separation profile of the protein on a calibrated Superdex 200 Increase 10/300 GL column is shown.

column (GE) (Figure 1(c)), corresponding to a protein with a molecular weight (MW) of about 44 kDa. Taking into account that the theoretical MW of ICMP is approximately 43.1 kDa, the purified protein therefore exists mainly as a monomer in solution. The final purity of ICMP was demonstrated to be greater than 99% on 15% SDS-PAGE (Figure 1(b)). The yield of pure ICMP protein was about 4 mg per litre of cell culture.

The initial crystallization screening trials for ICMP were carried out by using kits from Hampton Research. The purified protein could be crystallized within a week, and protein crystals were obtained under several conditions. After optimization, good-quality crystals (Figure 2) were finally selected from a condition consisting of 1.6 M sodium citrate tribasic dihydrate at pH 6.5. A single crystal of approximately 0.08 × 0.08 × 0.06 mm in size diffracted to 2.5 Å resolution (Figure 3). The data-processing statistics are summarized in Table 3. The space group of the crystal was $P2_12_12_1$ and the unit-cell parameters were a = 54.47, b = 158.98, c = 162.84 Å, $\alpha = \beta = \gamma = 90^{\circ}$. Overall, the diffraction data were of good

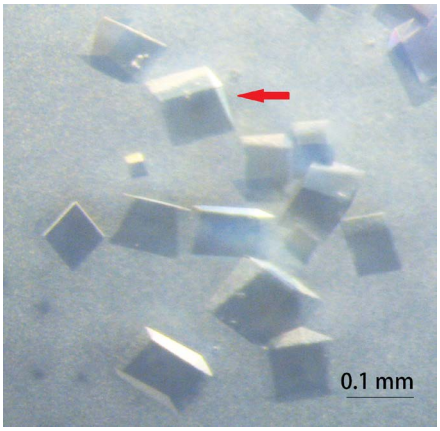

Figure 2. Crystals of ICMP grown by the sitting-drop vapour-diffusion method. One crystal (marked with an arrow) with a size of roughly 0.08 × 0.08 × 0.06 mm was selected for diffraction data collection. Its size was calculated by manually measuring the three dimensions of the crystal and then comparing them with the scale bar.

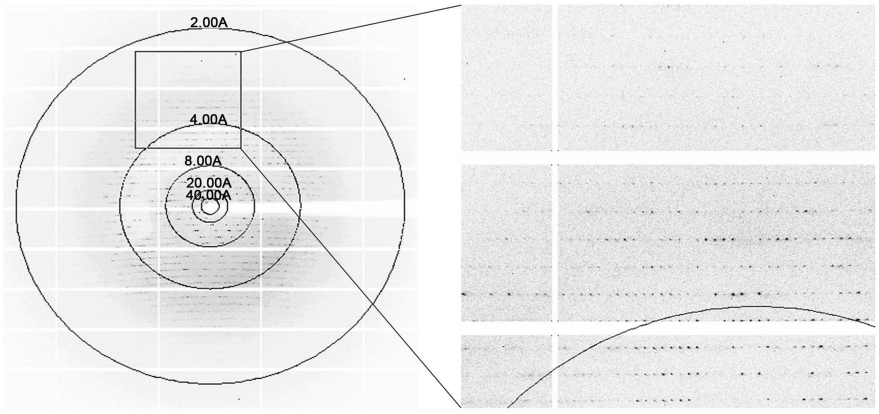

Figure 3. A representative diffraction image collected from an ICMP crystal. The resolution shells are shown.

quality, with an Rmeas of about 0.136 for all resolution shells from 50 to 2.5 Å. The Matthews coefficient [22] for the crystal was calculated to be 2.61 $Å^3 \cdot Da^{-1}$ with an estimated solvent content of about 52.58%, which indicates the presence of three ICMP molecules in the crystallographic asymmetric unit. The initial molecular-replacement calculations were carried out using the program AmoRe [23] with the structures of IPPA (PDB code 3pf0) [18], IPBO (PDB code 3oyv) [18], or Algp7 (PDB code 3at7) [19] as the search model. Nevertheless, no distinct translational peaks were observed, which indicates that it is unlikely to solve the structure of ICMP by molecular-replacement. We are currently preparing the selenomethionine derivative proteins, aiming to determine its structure utilizing the anomalous diffraction signal of the selenium atoms.

It is notable that preparation of homogeneous and crystallizable proteins is the prerequisite for structural studies by crystallography. In the current study, we managed to obtain the ICMP protein that meets such a criterion by deleting its signal peptide and a small N-terminal region full of hydrophobic residues (commonly detrimental to soluble expression of a protein) and by using a GST-fusion tag (normally beneficial to protein solubility) which could be easily removed afterwards by PSP enzyme digestion. In virtue of the significant role of ICMP in the pathogenesis of *P. aeruginosa*, the current work therefore has laid the foundation for future structural studies on ICMP and paved the way for delineating the characteristics of the protein at the atomic level.

Acknowledgements

We thank the staff of BL18U1 beamline at National Center for Protein Sciences Shanghai and Shanghai Synchrotron Radiation Facility (Shanghai, People's Republic of China) for assistance during data collection.

Funding Information

This work was financially supported by the National Key R&D Program of China (Grant No. 2016YFC1200305), the National Natural Science Foundation-Outstanding Youth Foundation (Grant No. 81522026), the Sichuan Outstanding Youth Science & Technology Funding (Grant No. 2016JQ0001), and the Outstanding Youth Foundation of Sichuan University (Grant No. 2016SCU04B01).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Flores, G., Stavola, J.J. and Noel, G.L. (1993) Bacteremia due to *Pseudomonas aeruginosa* in Children with AIDS. *Clinical Infectious Diseases*, **16**, 706-708. https://doi.org/10.1093/clind/16.5.706

[2] Folkesson, A., Jelsbak, L., Yang, L., Johansen, H.K., Ciofu, O., Høiby, N. and Molin,

S. (2012) Adaptation of *Pseudomonas aeruginosa* to the Cystic Fibrosis Airway: An Evolutionary Perspective. *Nature Reviews Microbiology*, **10**, 841-851. https://doi.org/10.1038/nrmicro2907

[3] Jeremy, G. and Martin, W. (2013) Why Is *Pseudomonas aeruginosa* a Common Cause of Infection in Individuals with Cystic Fibrosis? *Future Microbiology*, **8**, 697-699. https://doi.org/10.2217/fmb.13.40

[4] Fick, R. (1993) *Pseudomonas aeruginosa*: The Opportunist. 1-6.

[5] Wagenlehner, F.M. and Naber, K.G. (2006) Current Challenges in the Treatment of Complicated Urinary Tract Infections and Prostatitis. *Clinical Microbiology and Infection*, **12**, 67-80. https://doi.org/10.1111/j.1469-0691.2006.01398.x

[6] Wei, Q., Tarighi, S., Dötsch, A., Haussler, S., Musken, M., Wright, V., Camara, M., Williams, P., Haenen, S., Boerjan, B., Bogaerts, A., Vierstraete, E., Verleyen, P., Schoofs, L., Willaert, R., De Groote, V., Michiels, J., Vercammen, K., Crabbe, A. and Cornelis, P. (2011) Phenotypic and Genome-Wide Analysis of an Antibiotic Resistant Small Colony Variant (SCV) of *Pseudomonas aeruginosa*. *PLoS ONE*, **6**, 1-14. https://doi.org/10.1371/journal.pone.0029276

[7] Liu, P.V. (1974) Extracellular Toxins of *Pseudomonas aeruginosa*. *Journal of Infectious Diseases*, **130**, S94-S99. https://doi.org/10.1093/infdis/130.Supplement.S94

[8] Fricke, B., Parchmann, O., Kruse, K., Rucknagel, P., Schierhorn, A. and Menge, S. (1999) Characterization and Purification of an Outer Membrane Metalloproteinase from *Pseudomonas aeruginosa* with Fibrinogenolytic Activity. *Biochimica et Biophysica Acta*, **1454**, 236-250. https://doi.org/10.1016/S0925-4439(99)00040-X

[9] Rawlings, N.D., Barrett, A.J. and Bateman, A. (2010) MEROPS: The Peptidase Database. *Nucleic Acids Research*, **38**, 227-233. https://doi.org/10.1093/nar/gkp971

[10] Andrews, S.C., Robinson, A.K. and Rodriguez-Quinones, F. (2003) Bacterial Iron Homeostasis. *FEMS Microbiology Reviews*, **27**, 215-237. https://doi.org/10.1016/S0168-6445(03)00055-X

[11] Cornelis, P. and Dingemans, J. (2013) *Pseudomonas aeruginosa* Adapts Its Iron Uptake Strategies in Function of the Type of Infections. *Frontiers in Cellular and Infection Microbiology*, **3**, 75. https://doi.org/10.3389/fcimb.2013.00075

[12] Poole, K. and McKay, G.A. (2003) Iron Acquisition and Its Control in *Pseudomonas aeruginosa*: Many Roads Lead to Rome. *Frontiers in Bioscience*, **8**, 661-686. https://doi.org/10.2741/1051

[13] Cornelis, P., Matthijs, S. and Van Oeffelen, L. (2009) Iron Uptake Regulation in *Pseudomonas aeruginosa*. *BioMetals*, **22**, 15-22. https://doi.org/10.1007/s10534-008-9193-0

[14] Grosse, C., Scherer, J., Koch, D., Otto, M., Taudte, N. and Grass, G. (2006) A New Ferrous Iron Uptake Transporter, EfeU (YcdN), from *Escherichia coli*. *Molecular Microbiology*, **62**, 120-131. https://doi.org/10.1111/j.1365-2958.2006.05326.x

[15] Cao, J., Woodhall, M.R., Alvarez, J., Cartron, M.L. and Andrews, S.C. (2007) EfeUOB (YcdNOB) Is a Tripartite, Acid-Induced and CpxAR-Regulated, Low-pH Fe^{2+} Transporter That Is Cryptic in *Escherichia coli* K-12 But Functional in *E. coli* O157:H7. *Molecular Microbiology*, **65**, 857-875. https://doi.org/10.1111/j.1365-2958.2007.05802.x

[16] Rajasekaran, M.B., Nilapwar, S., Andrews, S.C. and Watson, K.A. (2010) EfeO-Cupredoxins: Major New Members of the Cupredoxin Superfamily with Roles in Bacterial Iron Transport. *BioMetals*, **23**, 1-17. https://doi.org/10.1007/s10534-009-9262-z

[17] Reddy, K.J., Bullerjahn, G.S., Sherman, D.M. and Sherman, L.A. (1988) Cloning,

Nucleotide Sequence, and Mutagenesis of a Gene (irpA) Involved in Iron-Deficient Growth of the Cyanobacterium Synechococcus sp. Strain PCC7942. *Journal of Bacteriology*, **170**, 4466-4476. https://doi.org/10.1128/jb.170.10.4466-4476.1988

[18] Xu, Q., Rawlings, N., Farr, C., Chiu, H., Grant, J., Jaroszewski, L., Klock, H., Knuth, M., Miller, M., Weekes, D., Elsliger, M., Deacon, A., Godzik, A., Lesley, S. and Wilson, I. (2011) Structural and Sequence Analysis of Imelysin-Like Proteins Implicated in Bacterial Iron Uptake. *PLoS ONE*, **6**, 1-12. https://doi.org/10.1371/journal.pone.0021875

[19] Maruyama, Y., Ochiai, A., Mikami, B., Hashimoto, W. and Murata, K. (2011) Crystal Structure of Bacterial Cell-Surface Alginate-Binding Protein with an M75 Peptidase Motif. *Biochemical and Biophysical Research Communications*, **405**, 411-416. https://doi.org/10.1016/j.bbrc.2011.01.043

[20] Winsor, G.L., Van Rossum, T., Lo, R., Khaira, B., Whiteside, M.D., Hancock, R.E. and Brinkman, F.S. (2009) Pseudomonas Genome Database: Facilitating User-Friendly, Comprehensive Comparisons of Microbial Genomes. *Nucleic Acids Research*, **37**, 483-488. https://doi.org/10.1093/nar/gkn861

[21] Otwinowski, Z. and Minor, W. (1997) Processing of X-Ray Diffraction Data Collected in Oscillation Mode. Methods in Enzymology. *Nucleic Acids Research*, **276**, 307-326.

[22] Matthews, B.W. (1968) Solvent Content of Protein Crystals. *Journal of Molecular Biology*, **33**, 491-497. https://doi.org/10.1016/0022-2836(68)90205-2

[23] Navaza, J. (1994) AMoRe: An Automated Package for Molecular Replacement. *Acta Crystallographica Section A*, **50**, 157-163. https://doi.org/10.1107/S0108767393007597