Prospects of a new anti-staphylococcal drug batumin revealed by molecular docking and analysis of the complete genome sequence of the batumin producer *Pseudomonas batumici* UCM B-321

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious public health thread causing outbreaks of clinical infection around the world. Mupirocin is a promising anti-MRSA drug, but mupirocin resistant strains of *S. aureus* are emerging with an increasing rate. Newly discovered antibiotic batumin may contribute to anti-MRSA therapy. Objective of this work was to identify possible molecular targets for batumin and mechanisms of its anti-staphylococcal activity by using computational molecular docking and analyzing the complete genome sequence of the batumin producer *Pseudomonas batumici* UCM B-321. It was found that batumin acted very much similar to mupirocin by inhibiting aminoacyl tRNA synthetases. A previous hypothesis considering the trans-enoyl-CoA reductase FabI as a prime molecular target of batumin was rejected. However, an indirect inhibition of the fatty acid biosynthesis in sensitive bacteria does take place as a part of stringent response repression triggered by accumulation of uncharged tRNA molecules. Paralogs of diverse leucine-tRNA-synthases in the genome of *P. batumici* indicated that this protein might be the prime target of batumin. Batumin biosynthesis operon comprising 28 genes was found to be acquired through horizontal gene transfer. It was hypothesized that in contrast

to mupirocin, batumin could inhibit a broader range of aminoacyl tRNA synthetases and the acquired resistance to mupirocin might not endow *S. aureus* strains with resistance against batumin.

Key words: anti-staphylococcal antibiotic; *Pseudomonas batumici*; molecular docking; genome; batumin; mupirocin

1. Introduction

Staphylococcus aureus is a major human pathogen that is capable of causing a wide range of human diseases resulting in significant morbidity and mortality. Methicillin resistant *S. aureus* (MRSA) became especially problematic in hospitals. Several new prospective polyketide antibiotics were isolated from different species of gamma-Proteobacteria, which can selectively suppress growth of *S. aureus* including multi-drug resistant strains in concentration of 0.05 μg/ml [1, 2, 3]. Among these antibiotics, only mupirocin is widely used in medicine. However, multiple cases of mupirocin resistance have been reported [4]. It calls for a search for new active anti-staphylococcal antibiotics, which may replace or supplement mupirocin. One such antibiotic, which no resistance has been reported for, is batumin/kalimantacin. Three different producers of this antibiotics have been reported: *Pseudomonas batumici* UCM B-321 [2], *P.fluorescens* BCCM_ID9359 [5] and *Alcaligenes* sp. YL-02632S [6]. Both batumin and mupirocin are encoded by large hybrid PKS-NRPS operons sharing some level of sequence similarity [3, 5]. A common chemical property of mupirocin and batumin is 9-hydroxy-nonanoic acid residue present in their structures. Antimicrobial activity of both these antibiotics against several Gram-positive and Gram-negative bacteria is summarized in Table 1 [3, 7, 8].

Table 1. Minimal inhibitory concentration (MIC) of batumin and mupirocin against different test-cultures.

Tested bacteria	Batumin μg/ml	Mupirocin μg/ml
Staphylococcus aureus ATCC 25923	0.05	0.25
Bacillus subtilis ATCC 6633	256	0.125
Klebsiella pneumoniae	32	125
Escherichia coli	4-16	128

To work out a possibility to replace or supplement mupirocin with other antibiotics to overcome mupirocin resistance, mechanisms of antibacterial activity of these antibiotics should be compared. Different target proteins were reported for batumin and mupirocin despite the obvious similarities in their structures and anti-staphylococcal specificity.

The mechanism of activity of batumin was proposed by Mattheus *et al*. [9]. Investigation of possible contribution of all genes of the batumin operon toward synthesis of this antibiotic revealed that the protein BatG took no part in the synthesis but showed a sequence similarity to trans-enoyl-CoA reductase FabI involved in fatty acid biosynthesis. This enzyme was known as a target for many antibiotics. It was supposed that BatG might act as an antidote against batumin to protect the producer against toxicity of this antibiotic. Expression of BatG in *S. aureus* in a shuttle vector conferred the resistance to batumin and to triclosan, which was another FabI inhibitor [9]. In this work it was shown also that BatG could complement mutations of FabI in *E. coli*. From these observations it was concluded that FabI was the target protein for batumin. However, no molecular docking analysis has been performed on possibility for batumin to bind to FabI molecules.

Mupirocin has been used since 1985 to prevent MRSA infection in hospitals [7]. It was shown in further publications that the mupirocin had bound specifically to an active site in Rossmann fold domain in bacterial isoleucine-tRNA-synthase (IleRS) [10]. In this work, a crystal structure of the *S. aureus* IleRS complex with $tRNA^{Ile}$ and mupirocin was demonstrated. In a later work by Nakama *et al*. [11], another crystal structure of mupirocin bound to *Thermus thermophilus* IleRS was published. It was supposed that inhibition of IleRS by mupirocin disrupted protein synthesis in bacteria [12]. Later it was found out that impact of mupirocin was more profound and involved induction of stringent response in bacteria [13]. Soon after the introduction of mupirocin to medical practice, cases of mupirocin resistant *S. aureus* have been reported. Two types of mupirocin resistance were observed: low level resistance resulted from point mutations in the native gene *ileRS* and high-level resistance mediated by a plasmid born *mupA* gene possibly of a eukaryotic origin that encoded an alternative IleRS with no affinity to mupirocin [4]. The genetic analysis of mupirocin resistance cases undoubtedly demonstrated that IleRS was the target protein for mupirocin.

Objective of this work was to model binding of batumin and mupirocin to different possible target proteins with an aim to summarize peculiarities of mechanisms of anti-bacterial activity of these antibiotics, which may explain observed differences in MIC (Table 1) and reported inability of *S. aureus* to develop resistance to batumin [14].

2. Methods

2.1 Bacterial growth conditions and DNA isolation

Pseudomonas batumici UCM B-321 was obtained from the Ukrainian Collection of Microorganisms (UCM) at Danylo Zabolotny Institute of Microbiology and Virology, National Academy of Science of Ukraine, Kiev, Ukraine. The stock culture was preserved by lyophilization at 4°C. The culture was routinely grown on peptone-meat-infusion agar at 26°C. Genomic DNA from overnight bacterial culture was extracted using GeneJet™ Genomic DNA Purification Kit (Fermentas). The quality of DNA samples were controlled by electrophoresis in 0.8% agarose gel.

2.2 Complete genome sequencing

In total 32,533,165 paired-end reads of the total length of 6,362,883,728 bp were generated by Illumina Hi-Seq (Macrogen, South Korea) from the chromosomal DNA of *P. batumici* UCM B-321. Assembly by Velvet 1.2.03 and CLC Genomics Workbench 7 resulted in generation of 127 contigs of the total length of 6,608,172 bp. The longest contig was 468,112 bp and the median length of contigs was 8,869 bp. An average GC-content of the assembled sequences was 61.78%.

2.3 Genome annotation and analysis

An automatic annotation of contigs by the RAST server identified 5,834 possible protein coding sequences, 2 fragmented ribosomal RNA operons and 54 tRNA genes. The complete genome sequence of *P. batumici* UCM B-321 and supplementary data were deposited at NCBI under accession numbers JXDG00000000, BioProject PRJNA270768 and BioSample SAMN03273282.

Identification of horizontally acquired genomic islands was performed by using SeqWord Genomic Island Sniffer 1.1 at http://www.bi.up.ac.za/SeqWord/sniffer/index.html [15].

2.4 Molecular docking

Chemical structures of batumin and mupirocin were known from literature [2, 5, 9, 10]. The structures were digitized in the Discovery Studio 4.0 Client. Molecular structures of the proteins FabI from *S. aureus* (ref. 3GR6 and 4ALN) and IleRS from *S. aureus* (ref. 1FFY) and *Thermus thermophilus* (ref. 1JZS) were obtained from the

RCSB database (www.rcsb.org). Molecular docking was performed by LibDock algorithm implemented in Discovery Studio 4.0 Client.

3. Results and Discussion

3.1 Molecular docking of batumin and mupirocin against FabI and IleRS protein structures

Chemical structures of batumin and mupirocin were used for molecular docking by LibDock algorithm against available protein structures of FabI from *Staphylococcus aureus* and IleRS from *S. aureus* and *Thermus thermophilus*. No affinity to FabI was identified but both the ligands showed a reliable affinity to the same active center of IleRS. As locations of mupirocin molecule in the active site of IleRS$^{S.\ aureus}$ and IleRS$^{T.\ termophilus}$ were known precisely from the corresponding crystal structures [10, 11], mupirocin served in this study as a positive control for *in silico* docking.

Both the ligands bound to IleRS active site by multiple hydrogen and hydrophobic bonds. The maximal total binding energy of the calculated receptor-ligand structures is shown in Table 2. The predicted affinity of mupirocin was a bit higher than that predicted for batumin, especially in IleRS$^{S.\ aureus}$. The docking algorithms predicted multiple alternative conformations of ligands in the IleRS active site, but those known for mupirocin from `the crystal structures showed the highest affinity. The most frequent bonds between amino acid residues of IleRS and ligands' radicals are summarized in Fig 1.

Table 2. The binding energy (kcal/mol) of batumin and mupirocin to the active sites of IleRS$^{S.\ aureus}$ and IleRS$^{T.\ termophilus}$.

Compound	IleRS$^{S.\ aureus}$	IleRS$^{T.\ termophilus}$
Batumin	53.19	52.82
Mupirocin	59.26	53.27

In IleRS$^{S.\ aureus}$, the amino acids His585-Phe587-Val588 played a central role in binding to both ligands by hydrophobic bonds. In IleRS$^{T.\ termophilus}$, rearrangements of hydrogen and hydrophobic bonds were observed. Common target amino acids were Gly56, Glu550, Asp553, Trp558, Leu583 and Ile584. The 9-hydroxy-nonanoic acid residue in the mupirocin structure did not participate in binding to IleRS. This aliphatic residue may be of

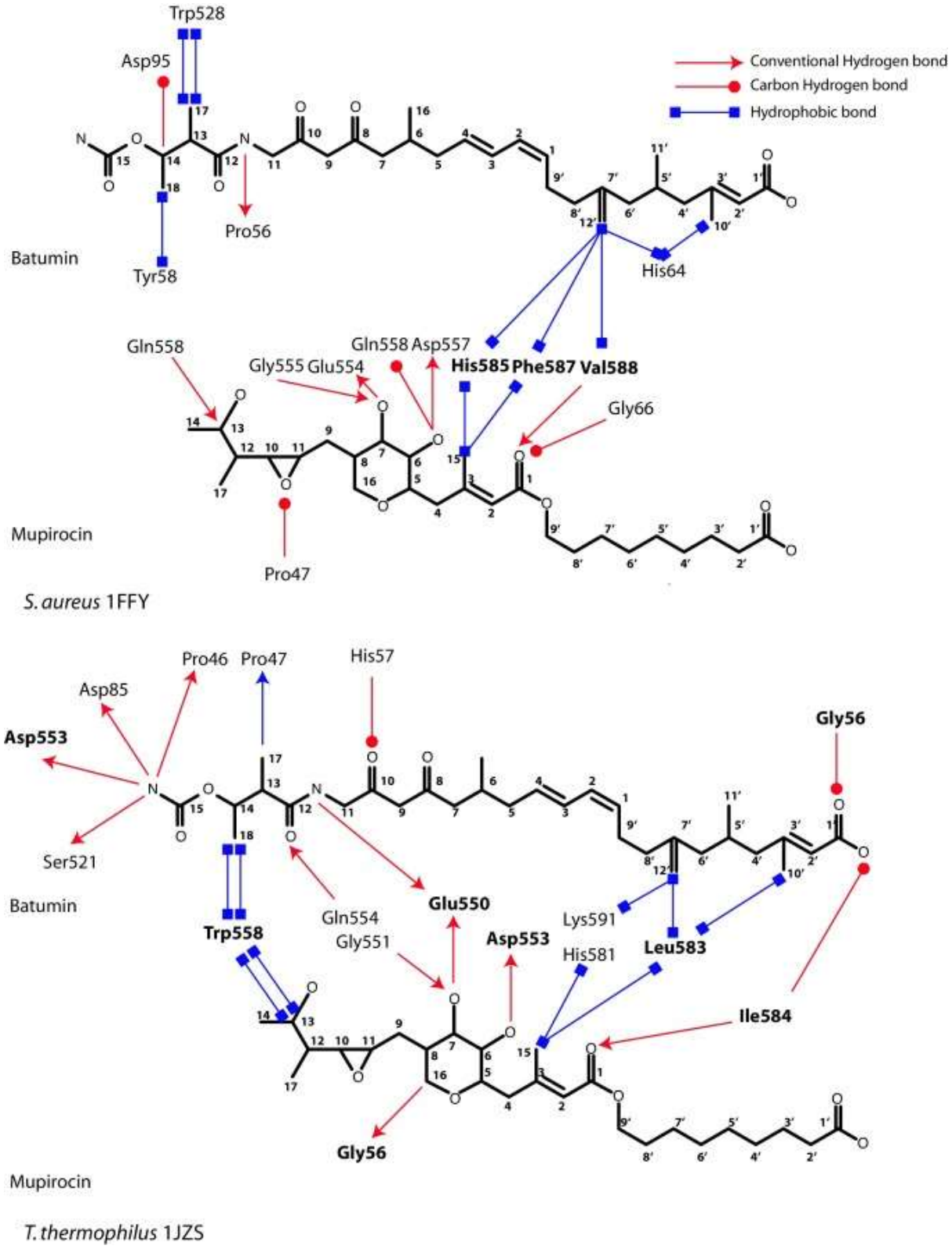

Fig 1. Chemical structures of batumin and mupirocin. Non-covalent bonds between amino acid residues of IleRS proteins of *S. aureus* and *T. thermophilus* to the atoms in the structures of antibiotics are indicated.

importance for transportation of the molecule into cell. However, contrary to this hypothesis, calculation of ADMET properties in the Discovery Studio showed that removal of this residue from mupirocin structure would not improve the absorption of the molecule.

Three closed and open heterorings [C1-C8] of mupirocin contributed significantly to creating bonds within the binding site. In batumin structure, $–CH_3$ and $=CH_2$ radicals attached to the 9-hydroxy-nonanoic acid residue performed the same role as the heterorings in mupirocin. In Fig 2, localization of batumin and mupirocin molecules in the binding site is shown. Batumin molecule was inverted so that the ends of the molecule were plugged into the binding site and the central part of the chain protruded out of the site. Contrary, mupirocin molecule was aligned along the grove of the active site of IleRS with the edges hanging out. The area of heterorings in mupirocin was positioned in the binding site at the same place where the modified 9-hydroxy-nonanoic acid residue of batumin was located. The same was true for terminal parts of the both molecules.

It may be concluded that both batumin and mupirocin can bind with a similar energy to IleRS active site, but none of them can bind to FabI. This finding contradicted to the report by Mattheus *et al*. [9] that the expression of BatG, a batumin tolerant analog of FabI from the batumin operon, could confer resistance to this antibiotic. However, according to Reiss *et al*. [13], inhibition of IleRS may influence FabI activity. This observation reconciles this contradiction. In the latter work by Reiss *et al*. [13], a global metabolome analysis was performed to study the profound action of mupirocin on *S. aureus*. It was found out that accumulation of uncharged isoleucyl-tRNA molecules resulted from IleRS inhibition had triggered the stringent response by activation of (p)ppGpp alarmone. The alarmone induces a cascade of events of up and down regulation of gene expression. Particularly, a significant down-regulation of FabH was reported. FabH is an enzyme that feeds the FAS II cycle [16]. Suppression of expression of this gene can be caused by suspension of the fatty acid biosynthesis cycle. It was not the first report of fatty acid biosynthesis inhibition during the stringent response. Heath *et al*. [17] demonstrated in experiments on *Escherichia coli* that (p)ppGpp inhibited the phospholipid synthesis through regulation at the glycerol-P acyltransferase step followed by accumulation of long-chain acyl-ACP. It was observed that the phospholipid synthesis had shifted from initiation of fatty acid biosynthesis to utilization of the end products by acyltransferases. This observation was confirmed in a recent publication by My *et at*. [18], who reported that the fatty acid biosynthesis and particularly *fabHDG* operon in *E. coli* was inhibited by (p)ppGpp through FadR and FabR transcriptional regulators. This physiologically process is not critical for many bacteria, as they can compensate the lack of synthesized fatty acids by uptake of exogenous molecules. However, suppression of fatty acid synthesis

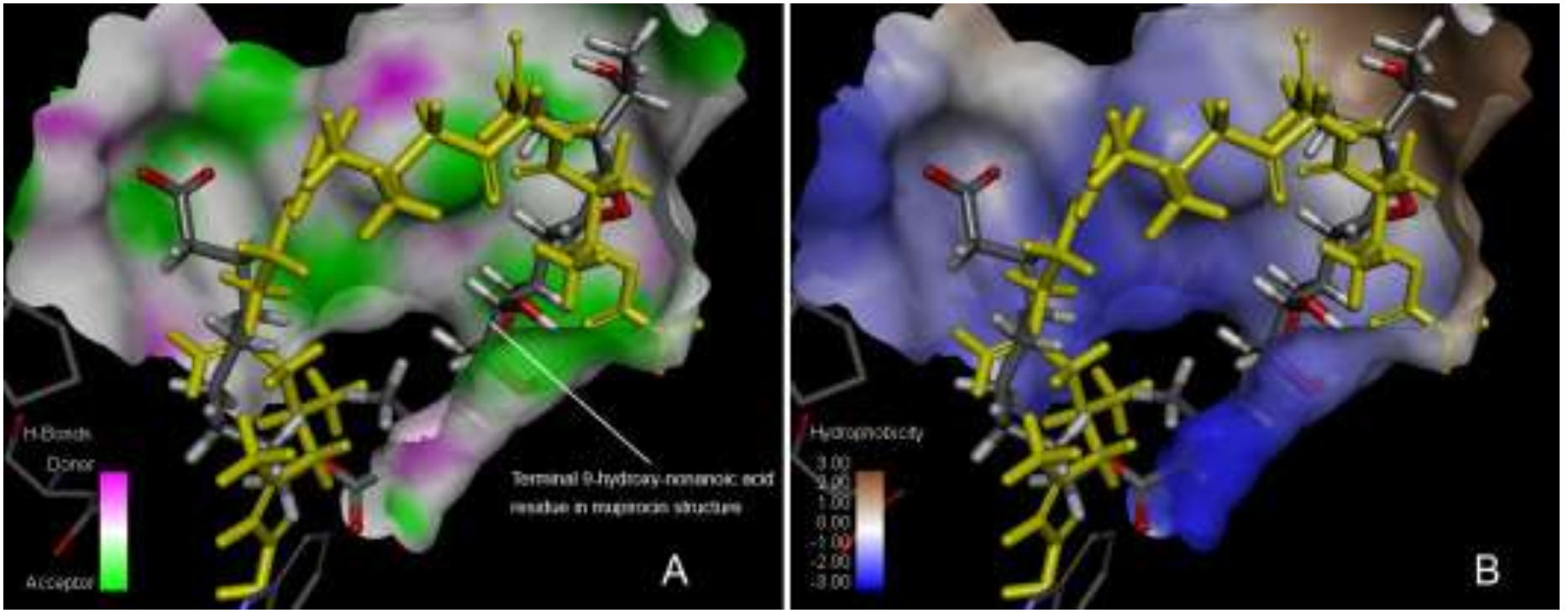

Fig 2. Localization of batumin and mupirocin molecules in the binding site of IleRS protein of *S. aureus*. Batumin is highlighted in yellow colour. The 9-hydroxy-nonanoic acid residue of mupirocin is pointed out. Hydrogen donor/acceptor and hydrophobicity properties of the active center of IleRS are shown in the parts A and B, respectively.

becomes lethal for *S. aureus* because of a peculiarity of the regulation of fatty acid metabolism in this bacterium suppressing at the same time both the synthesis and incorporation of exogenous fatty acids [19]. In the latter paper it was explained why FabI inhibitors were much more toxic to *S. aureus* than to other micro-organisms.

Several conclusions may be derived from this study, which should be experimentally tested in the future work: i) batumin does not inhibit FabI but acts similar to mupirocin by targeting aminoacyl tRNA synthetases; ii) batumin and mupirocin inhibit *S. aureus* through impairing the fatty acid biosynthesis pathway in a similar way as the FabI inhibitors did, but through triggering an abnormal stringent response; iii) *batG* encodes a FabI-like trans-enoyl-CoA reductase that could confer resistant to endogenous FabI inhibitors expressed during the stringent response.

3.2 Analysis of the complete genome of *P. batumici* UCM B-321

Operon of biosynthesis of batumin/kalimantacin in *P. fluorescens* BCCM_ID9359 was known from previous publications [5, 9]. Exactly the same operon of 74,151 bp was found in *P. batumici* UCM B-321. The difference was only in one nucleotide deletion in a spacer region that also could be a sequencing error. The gene map of the batumin operon of UCM B-321 is shown in Fig 3.

The average GC-content of the genome of UCM B-321 was 61.78%. In the locus covering the batumin operon, the GC-content was below 50% (see Fig 3). An acquisition of this locus by horizontal gene transfer was confirmed by search for genomic islands (GIs) using the program SWGIS. The atlas of concatenated contigs of UCM B-321 with locations of predicted GIs is shown in Fig 4.

It was known that the resistance to mupirocin was associated with the presence of alternative isoleucine-tRNA-synthases showing no affinity to mupirocin [4]. We searched for genes encoding aminoacyl tRNA synthetases in the genome of UCM B-321. A complete set of aminoacyl-tRNA synthetases charging all 20 amino acids was found. There was no redundancy of isoleucyl-tRNA synthetases but 3 paralogs of leucyl-tRNA synthetases were identified. One gene encoded a very short protein of 59 amino acid residues and most likely it was a non-functional pseudo-gene. BLASTP search through the NCBI database showed that this conserved gene was present in several other genomes of *Psedomonas*. Another predicted leucyl-tRNA synthetase was typical for *Pseudomonas* enzyme of 868 amino acids. The third gene, location of which is depicted in Fig 4, was found in an identified GI characterized by the same low GC-content as in the batumin operon. It encoded a shorter protein of 817 amino acids. BLASTP search through the NCBI database identified similar proteins in two genomes of *Pseudomonas fluorescens* and in multiple species of the genus *Streptococcus*. It may be hypothesized that batumin in contrast to mupirocin has a

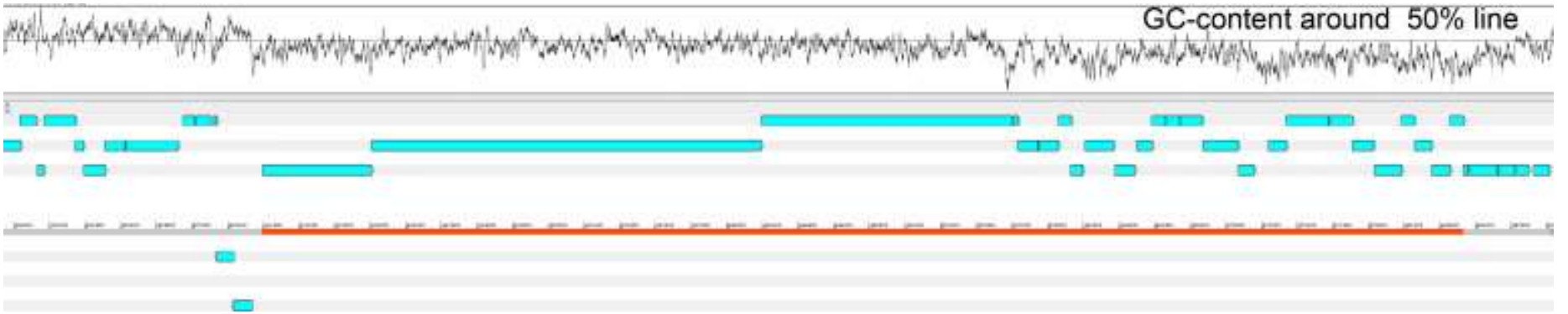

Fig 3. Gene map of the batumin operon in UCM B-321. Gene locations are depicted by blue bars in 6 frames of transcription. The batumin operon is highlighted by an orange bar. The histogram in the upper part of the figure shows changes in the average GC-content in a sliding window.

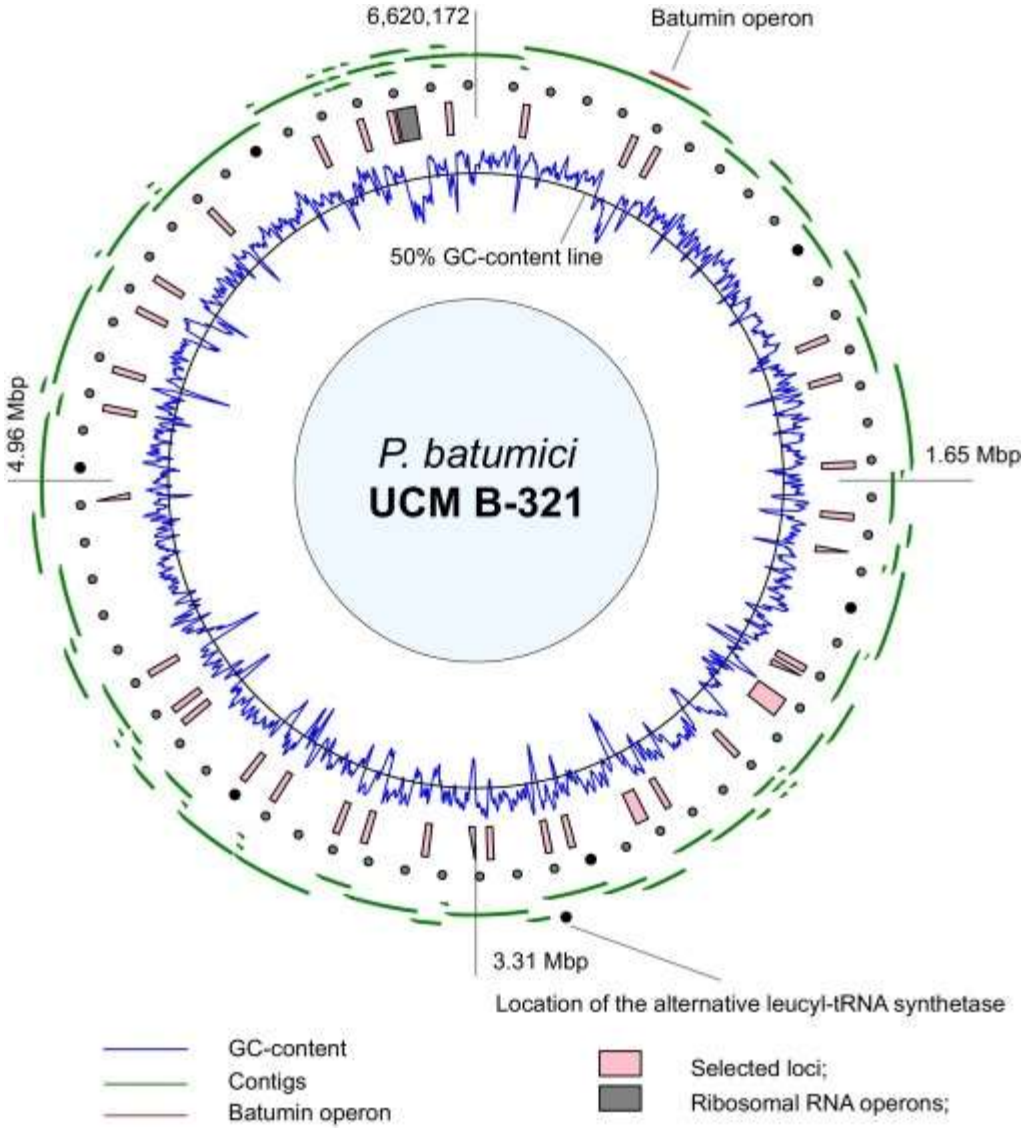

Fig 4. Atlas of concatenated contigs of UCM B-321 shows locations of predicted GIs, GC-content changes in 5,000 bp long sliding window and location of genes of interest.

higher affinity to leucyl-tRNA synthetase rather than to isoleucyl-tRNA synthetase. Inhibition of leucyl-tRNA synthetase leads to the same consequences ending up with triggering of abnormal stringent response by accumulation of uncharged tRNA molecules. Indeed, isoleucyl- and leucyl-tRNA synthases share significant sequence similarity in domains composing batumin binding sites. Alternative versions of LeuRS and FabI might be required to confer resistance of the strain-producer to batumin. This specificity in the mechanisms of antibiotic activity of batumin and mupirocin may explain the observed differences in the profile of susceptible bacterial test-cultures shown in Table 1.

4. Conclusion

Public health care experiences a serious problem with emerging and quick distribution of antibiotic resistance among pathogens. Development of new antibiotics is costly and time consuming process. Very often resistance to newly developed antibiotics has emerged soon after its introduction into practice. Expenses for antibiotic development and introduction are getting so immense with the profit being so unpredictable that many pharmaceutical companies refused investing into design of new antibiotics. Possible solution lies in optimization of the therapy by combining antibiotics, which do not share the resistance patterns. By using molecular docking modeling and genome comparison, it was shown that batumin in many aspects is an analog of mupirocin that has been used successfully for decades in medicine against MRSA infection. In the same time, the current research suggested that batumin in contrast to mupirocin may target a broader range of aminoacyl tRNA synthetases including leucyl-tRNA synthase. A paralog of leucyl-tRNA synthetase borrowed from *Streptococcus* and BatG protein acting as an analog of FabI trans-enoyl-CoA reductase, both may serve in *P. batumici* genome to confer this bacterium with resistance to synthesized batumin. It looks plausible that the leucyl-tRNA synthetase and the batumin operon were acquired by horizontal gene transfer from the same unknown Gram-positive bacterium. It also may be expected that the mechanisms conferring resistance to mupirocin should not work against batumin. This hypothesis is confirmed by a previously published report of a clinical trial on tratment of nasal carriage of *S. aureus* by a batumin containing ointment in comparison to bactroban ointment based on mupirocin. Complete eradication of *S. aureus* was reported in 83.6% of batumin application and in 69.4% of treatment with bactroban [14]. Further study and clinical trials will be needed to confirm efficiency of batumin as a replacement or supplement to mupirocin topical treatment of MRSA infection.

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