Translation Inhibitor Klebsazolicin is Synthesized from a Peptide Precursor via Two Distinct Post-Translational Modifications Catalyzed by a Single YcaO-Enzyme

Dmitrii Travin[1,2], Mikhail Metelev[3], Dmitry Ghilarov[2,4,5], Marina Serebryakova[2,6], Konstantin Severinov[2,5,7]

[1] Lomonosov Moscow State University, Department of Bioengineering and Bioinformatics, Moscow, 119992, Russia, [2] Centre for Data-Intensive Biomedicine and Biotechnology, Skolkovo Institute of Science and Technology, Skolkovo, 143025, Russia, [3] Department of Cell and Molecular Biology, Uppsala University, Husargatan 3 Box 596, Uppsala, SE-75124, Sweden, [4] Malopolska Centre of Biotechnology, Jagiellonian University, 30-387 Cracow, Poland, [5] Institute of Gene Biology of the Russian Academy of Sciences, Moscow, 119334, Russia, [6] Lomonosov Moscow State University, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow, 119992, Russia, [7] Waksman Institute for Microbiology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

E-mail: travin_birds@mail.ru

Introduction

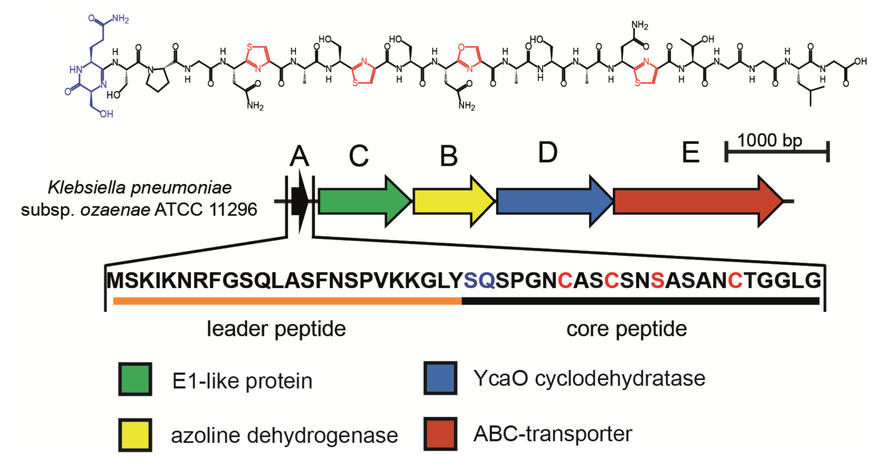


Figure 1. Chemical structure of klebsazolicin and the composition of its biosynthetic gene cluster

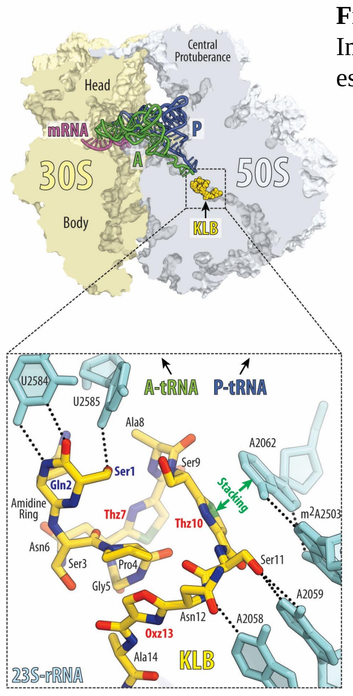
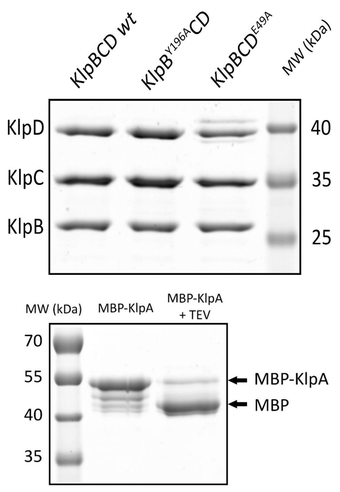


Figure 2. Mode of action of klebsazolicin. Importance of post-translational modifications in establishing the interactions with 23S rRNA [2].

Klebsazolicin (KLB) is a recently discovered [2] ribosomally synthesized and post-translationally modified peptide. It has four core azole cycles and a unique modification – N-terminal amidine cycle. Post-translational modifications mediate KLB interactions with 23S rRNA and are critically important for the functional activity of the antibiotic: obstruction of the exit channel of the ribosome leading to the translation inhibition (Fig. 2).

The biosynthetic gene cluster of KLB (Fig. 1) does not contain any additional enzymes that could be responsible for amidine installment. The aim of the present work was to reconstitute the modification of KLB *in vitro* with a special focus on the N-terminal amidine cycle biosynthesis.

Experimental methods



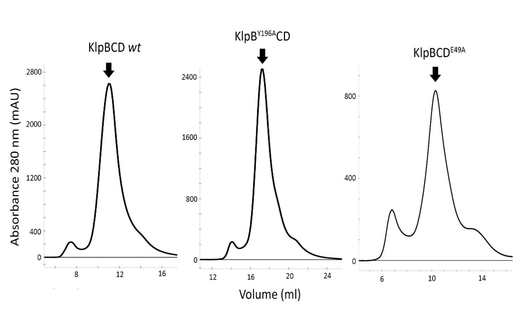


Figure 3. Results of SDS PAGE and profiles of gel filtration showing the purification of KlpBCD complexes and KlpA precursor peptide – components of *in vitro* modification reactions.

Components of KlpBCD synthase were copurified as a stable complex. KlpC was tagged with an N-terminal hexahistidine tag, and affinity purification allowed obtaining all three proteins in apparently stoichiometric amounts. Two mutant variants of KlpBCD complex with substitutions in KlpB active center and KlpD ATP-binding center were also obtained. Precursor peptide KlpA was purified as a MBP-fusion with subsequent removal of the MBP-tag with a specific TEV protease.

Reaction mixture (50 μL) contained 10 μM of precursor peptide and 1 μM of KlpBCD complex. When needed, the reaction was also supplemented with 0.5 μM of TldD/E protease. MS mass spectra were recorded on MALDI-ToF-ToF-MS instrument in a positive ion measurement mode.

References:

[1] D. Y. Travin, M. Metelev, M. Serebryakova, E. S. Komarova, I. A. Osterman, D. Ghilarov, and K. Severinov, "Biosynthesis of Translation Inhibitor Klebsazolicin Proceeds through Heterocyclization and N-Terminal Amidine Formation Catalyzed by a Single YcaO Enzyme.," *J. Am. Chem. Soc.*, vol. 140, no. 16, pp. 5625–5633, Apr. 2018.

[2] M. Metelev, I. A. Osterman, D. Ghilarov, N. F. Khabibullina, A. Yakimov, K. Shabalin, I. Utkina, D. Y. Travin, E. S. Komarova, M. Serebryakova, T. Artamonova, M. Khodorkovskii, A. L. Konevega, P. V Sergiev, K. Severinov, and Y. S. Polikanov, "Klebsazolicin inhibits 70S ribosome by obstructing the peptide exit tunnel.," *Nat. Chem. Biol.*, vol. 13, no. 10, pp. 1129–1136, Oct. 2017.

[3] I. A. Osterman, E. S. Komarova, D. I. Shiryaev, I. A. Korniltsev, I. M. Khven, D. A. Lukyanov, V. N. Tashlitsky, M. V Serebryakova, O. V Efremenkova, Y. A. Ivanenkov, A. A. Bogdanov, P. V Sergiev, and O. A. Dontsova, "Sorting Out Antibiotics' Mechanisms of Action: a Double Fluorescent Protein Reporter for High-Throughput Screening of Ribosome and DNA Biosynthesis Inhibitors.," *Antimicrob. Agents Chemother.*, vol. 60, no. 12, pp. 7481–7489, Dec. 2016.

[4] D. Ghilarov, M. Serebryakova, C. E. M. Stevenson, S. J. Hearnshaw, D. S. Volkov, A. Maxwell, D. M. Lawson, and K. Severinov, "The Origins of Specificity in the Microcin-Processing Protease TldD/E.," *Structure*, vol. 25, no. 10, pp. 1549–1561, Oct. 2017.

Results

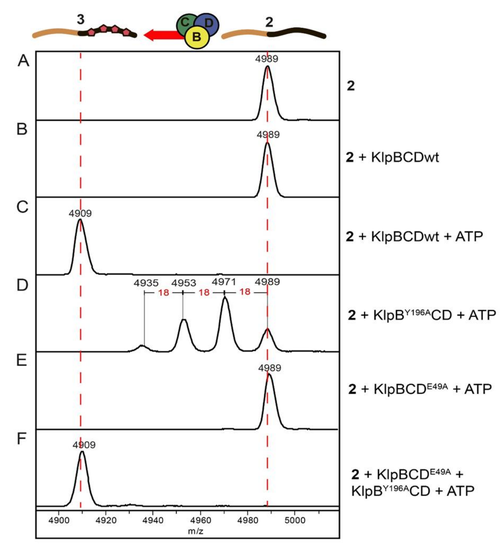


Figure 4. Incorporation of core azole cycles into the KlpA precursor peptide with KlpBCD modification complex (B-C) and its mutant variants (D-F).

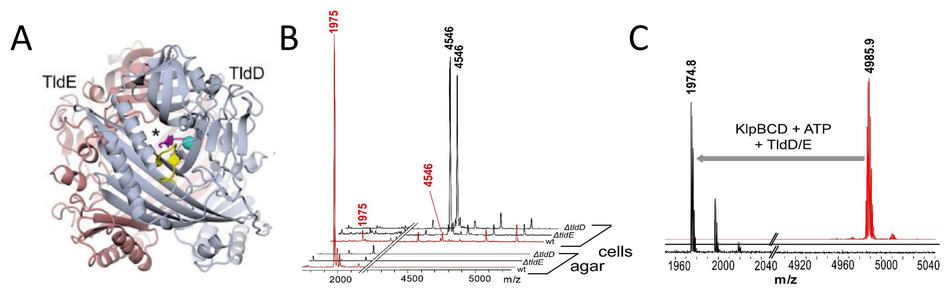


Figure 5. Heterodimeric metalloprotease TldD/E (A, [4]) is responsible for the leader peptide removal in KLB biosynthesis both *in vivo* (B) and *in vitro* (C).

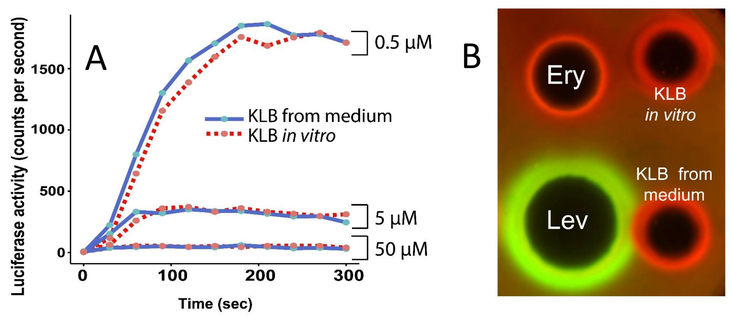


Figure 6. Functional equivalence of KLB synthesized *in vitro* and purified from cultivation media is proved in translation inhibition assay *in vitro* (A) and using a special reporter strain (B, [3]).

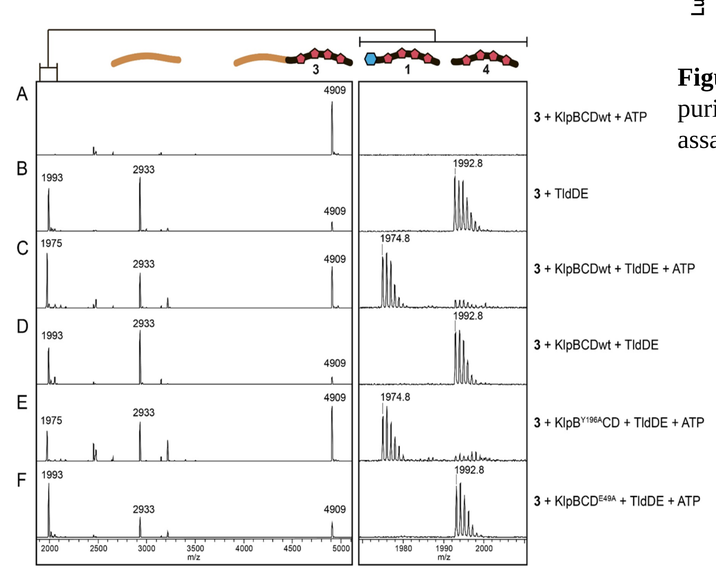


Figure 7. Activities of YcaO-containing enzyme KlpD and TldD/E protease in the presence of ATP are required for the formation of the N-terminal amidine cycle

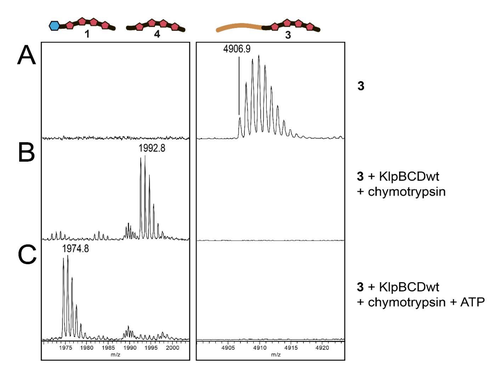


Figure 8. TldD/E can be substituted with an unrelated protease chymotrypsin in the *in vitro* reaction of KLB modification.

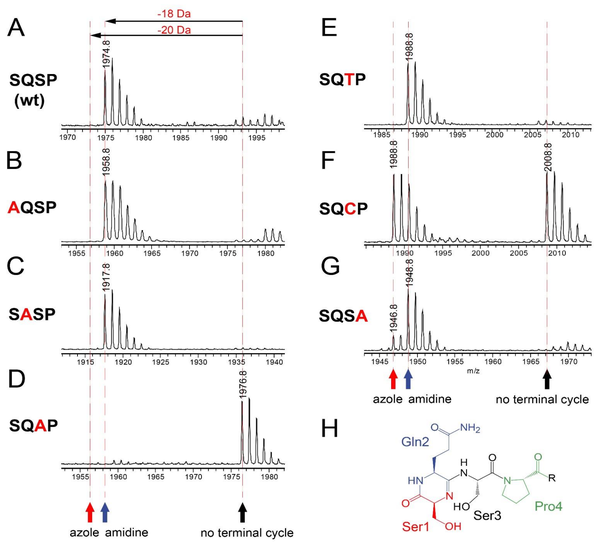


Figure 9. MS analysis of the products of *in vitro* modification reactions containing mutant KlpA precursor peptides with amino acid substitutions in the N-terminal region of the core peptide.

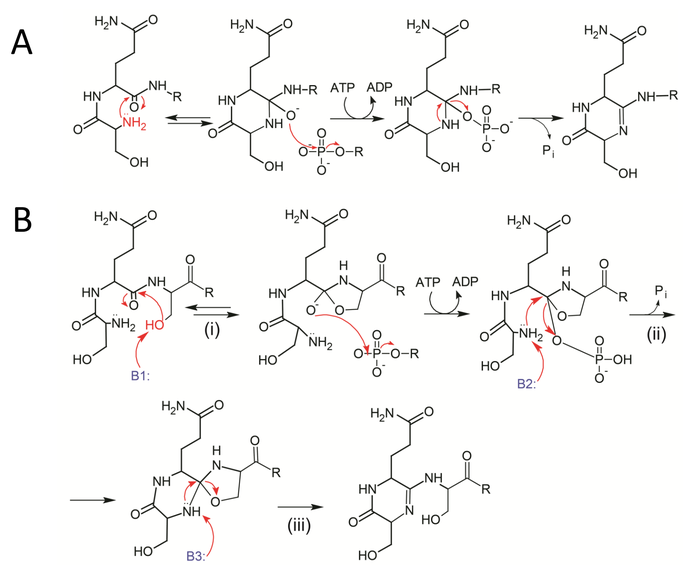


Figure 10. Possible mechanisms of YcaO-dependent N-terminal amidine cycle formation.

Conclusions

- Entire pathway of klebsazolicin biosynthesis was reconstituted *in vitro* using the recombinant KlpBCD enzyme complex.
- During the modification of the core peptide KlpBCD complex acts as a classical YcaO-dependent oxazole/thiazole synthetase introducing four azole cycles in N- to C-terminal direction.
- Role of the TldD/E protease is limited to the removal of the leader peptide and providing the free N-terminal amino group.
- KlpD is a bifunctional cyclodehydratase required for the formation of both the core cycles and the N-terminal amidine ring of KLB. This is the first known example of two distinct modification installed into the structure of the peptide by a single YcaO enzyme.
- We propose that the amidine cycle is installed via a two-step mechanism, which involves classical reactions of the azoline installment followed by a rearrangement. Aminoacid context of the N-terminal part of the core peptide plays the key role in the latter process.