CHARACTERIZATION OF THE BACTERIOPHAGE ACTIVE AGAINST *PROTEUS* GENUS BACTERIA

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1. Background

Proteus genus harbors the causative agents of serious diseases such as urinary tract infections (UTI) in urinary catheter patients, gastrointestinal, respiratory system, skin and eye infections [1]. Out of the entirety of the genus, *P.mirabilis* and *P.vulgaris* are the most infamous for causing human infections. Because of a unique variety of adaptational mechanisms exhibited by the bacteria, including biofilm formation, transition to swarmer state, etc. antibiotic treatment hasn't been proven to be largely successful. In addition to this, there are no available licensed vaccines against them at the moment. No fully effective infection control methods are available either [2].

This situation calls for development of novel therapeutical methods with the use of phages or phage-derived products being among the most promising. Some of the benefits of phage therapy include specific host targeting, distinctive activity mechanisms, cost efficiency and a comparative easiness of development. Isolation and characterization of broad-host-range phages are of specific interest. On top of that, combinational use of phage particles paired with antibiotics has great potential as well [3].

Recently a *Proteus mirabilis* strain (assigned according to biochemical identification) was isolated from tomato plants [4]. A phage, able to cause productive infection, was isolated after application of the sewage water on the bacterial lawn.

The aim of the present work was to characterize the bacteriophage and perform the molecular-genetic identification of the isolated bacterial strain to prove its species assignment. Such findings may allow to broaden the potential *Proteus*-related infection therapy measures line-up.

2. Methods

Bacterial cultivation and phage propagation were performed according to microbiology and virology methods. Biochemical profiles were obtained using ENTEROtest24 kit (MIKROLATEST, Erba Lachema). Swarm morphology was studied for a 24 hour culture grown on LB (1.2% agar). Cell and bacteriophage morphology was characterized using TEM.

TEM samples were obtained using plaque adsorption technique. Support grids coated with nitrocellulose, obtained from its 1% amyl acetate solution were used. Adsorption was carried out for 20-40 minutes with further contrasting (2% uranyl acetate). The study was performed on JEOL JEM 1400 TEM.

Partial 16S rRNA gene Sanger sequencing was performed for molecular genetic identification of the bacterium. For extraction a 24 hour culture grown on LB (1.2% agar) was used. DNA extraction, PCR and amplicon purification were done using commercially available kits. Using a specific set of primers (a forward 27F-5'-GAGTTTGATCMTGGCTCAG-3' and a reverse 803R-5'-CTACCRGGGTATCTAATCC-3') amplicons of approximately 800 bp (containing variable regions V1, V2, V3 and V4) were obtained. Upon obtaining, the amplicon was sent to Eurofins Genomics Laboratory (Cologne, Germany) for sequencing. The sequences were later used for subsequent analysis. The sequences were compared in blastn suite against properly published sequences of *Proteus* genus species. Multiple sequence alignment was performed in Muscle with Gblocks algorithm. Phylogenetic tree construction was carried out using the Maximum Likelihood method within PhyML tool, available at http://www.phylogeny.fr/. The phylogenetic tree visualization was performed in Mega7.

3. Results (I) Phage morphology

On the lawn of *P. mirabilis* cells the phage formed small colonies (1-2 mm in diameter) with a halo. (Fig. 1A)

Detected phage particles featured B1 morphology (*Siphoviridae*), with icosahedral capsid (Dmax of nearly 50 nm) and a long non-contractile tail (Fig. 1B). Along with it, highly flagellated bacterial cells at different stages of lysis were noted (Fig. 2A, B and C). The intact phage tails were difficult to detect, the ones detected on the microphotographs reached a length of 119 to 278 nm. It has been noted that the tails have a transverse arrangement of subunits. We detected the presence of bacteriocins of poly-sheath type with a length from 422 to 529 nm as well (Fig 3A and B). It was also established that some phage particles or individual phage tails were attached to the flagellum, which may indicate the probable flagellotropic nature of the phage (Fig. 4A and B).

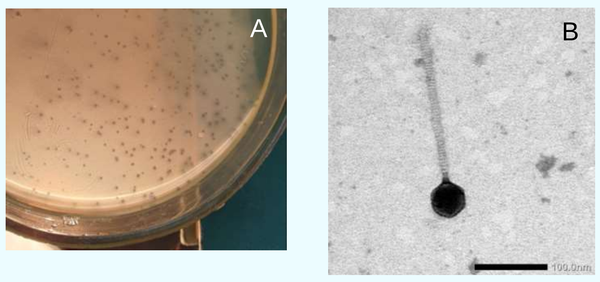


Figure 1. Phage plaque (A) and particle (B) morphology

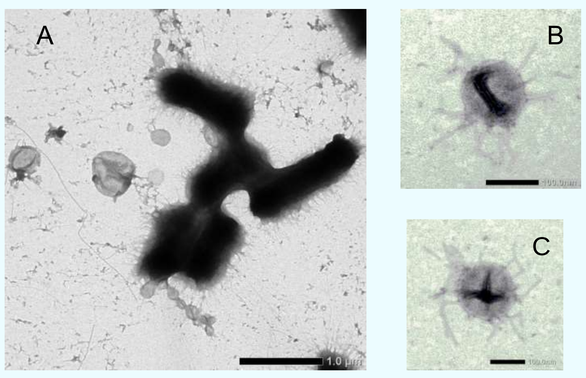
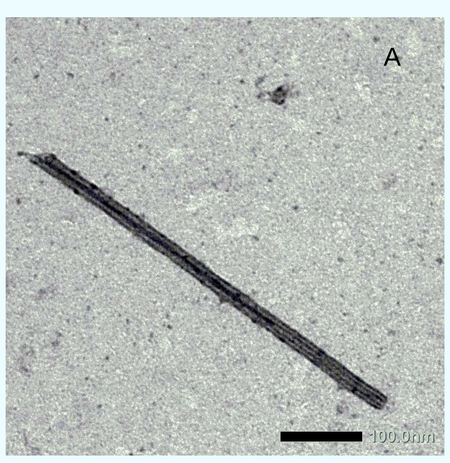


Figure 2 (A, B and C). A - Bacterial cells at different stages of lysis. Scale bar 1 mkm, B and C - Cell membrane-derived vesicles, formed in a process of lysis, covered by bacterial surface appendages. Scale bar 100 nm



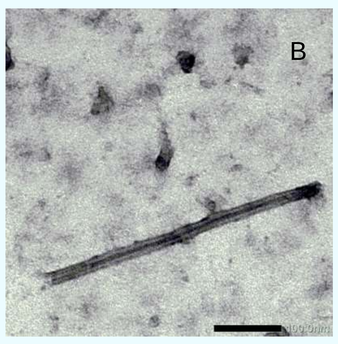
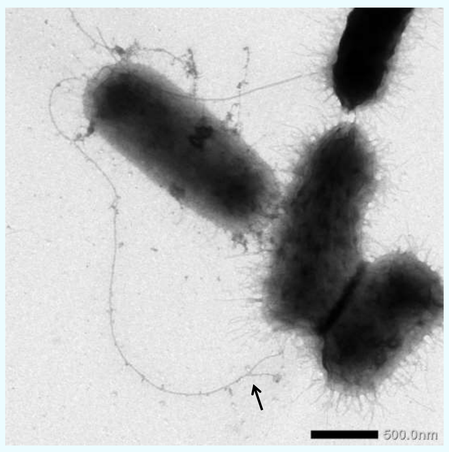


Figure 3 (A, B). Poly-sheath type bacteriocins. Scale bars 100nm



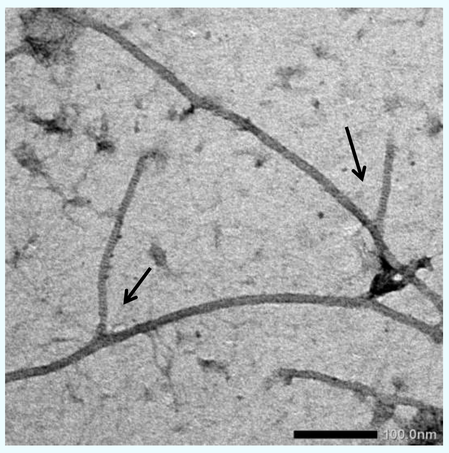


Figure 4 (A, B). Phages or phage tails attached to the flagella (marked with arrows). Scale bars 500, 100 nm

4. Results (II) Phage host range

In order to characterize the host range of the phage, *P. vulgaris* collection strain UKM B-905T, as well as *E. coli* C600 and *Erwinia* sp. 60 were used along with the host. The bacterial virus was able to develop plaques on the lawn of suspected *P. mirabilis* and *P. vulgaris* strains, while the bacteria of other genera were not infected.

In order to prove the broad-host range feature of a phage we had to prove the species assignment of its host (*P. mirabilis*) using polyphasic taxonomic approach, as it was identified only according to biochemical features [Kharina].

Firstly, we have performed additional biochemical and morphologicall studies that would allow for differentiation of *P. mirabilis* form *P. vulgaris* collection strain. ENTEROtest biochemical profiling of host bacterium and *P.vulgaris* UKM B-905T showed that the host species is *P.mirabilis*, as suggested by the reactions. According to the test, the two strains had opposite salicin, ornithine, sucrose, trehalose and esculine metabolic profiles (Fig.5A and B). As well, the strains differed in indol prodiction. In addition to that, swarming patterns of the strains were substantially different with the host forming distinct bull's-eye rings [5] on the medium and *P.vulgaris* producing hardly visible terraces (Fig.6A and B). Host's flagellar morphology also closely resembles that of *P.mirabilis*.

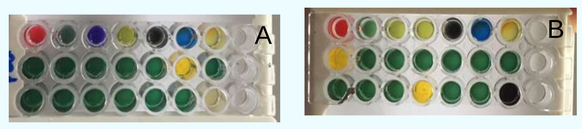


Figure 5. Differences between the *P. mirabilis* (A) and *P.vulgaris* (B) biochemical profiles. Enterotest 24. 16 h of cultivation

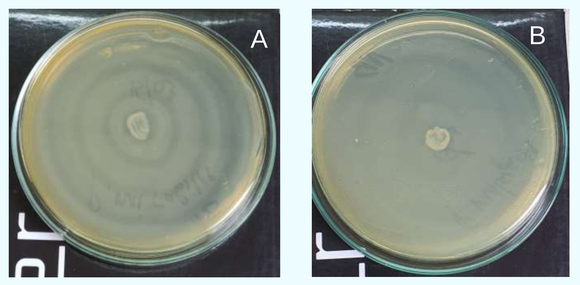


Figure 6. Differences between *P.mirabilis* (A) and *P.vulgaris* (B) swarm. LB (1.2% agar). 24 h of cultivation

5. Results (III) Molecular genetic identification of host bacterium

With respect to current guidelines on species assignment, partial 16S rRNA gene sequencing was used as molecular genetic approach for the identification. The resulting 708 nt fragment comparative analysis showed that the sequence has:

- 100% identity against *P.mirabilis*; 98.44% identity to 100% identity against *P.vulgaris*;
- 98.73% identity against *P.terrae*; 98.73% identity against *P.hauseri*;
- 93.64% identity against *Morganella morganii*; 94.62% identity against *Providencia stuartii* (Table 1)

M. morganii and *P. stuartii* were used for reference when building the phylogenetic tree. *M.morganii* was used as an outgroup (Fig.7). Thus, the genus of the isolated bacterium was proven to be *Proteus*, but in order to confirm its species further research is needed. This is mainly due to highly conservative nature of 16S rDNA genetic material for the *Enterobacteriales* [6] .

Scientific Name	Per. Ident
Proteus vulgaris **JCM 20013**	100.00%
Proteus mirabilis **DFI-5**	100.00%
Proteus mirabilis **MGL9**	100.00%
Proteus mirabilis **IGM1-17**	100.00%
Proteus mirabilis **IGM6-14**	100.00%
Proteus mirabilis **JPM24**	100.00%
Proteus mirabilis **MPE0027**	100.00%
Proteus mirabilis **MPE0156**	100.00%
Proteus vulgaris **P190036**	99.86%
Proteus terrae subsp. cibarius **ZF2**	98.73%
Proteus hauseri **ZMd44**	98.58%
Proteus vulgaris **JCM 20339**	98.58%
Proteus vulgaris **DSM 13387T**	98.44%
Proteus vulgaris **PW 106**	98.44%
Proteus vulgaris **FDAARGOS_1507**	98.44%
Proteus vulgaris **NCTC13145**	98.44%
Proteus vulgaris **FDAARGOS_556**	98.44%
Proteus vulgaris **ATCC 29905**	98.44%
Providencia stuartii **DSM 4539**	94.62%
Morganella morganii subsp. morganii **ATCC 25830**	93.64%

Table 1. Compared sequence identity percentage against selected sequences from GenBank (NCBI)

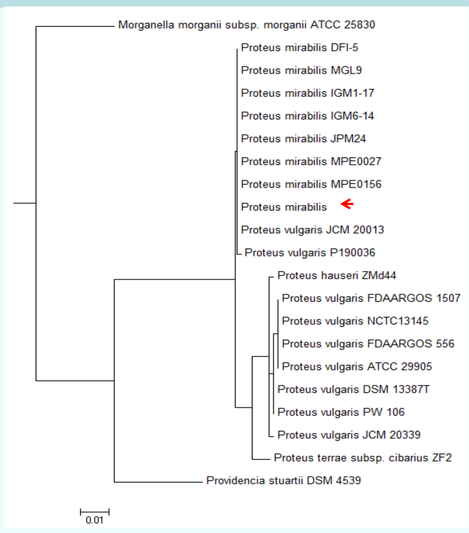


Figure 7. Phylogenetic tree constructed on a basis of 16S rDNA sequence alignment

6. Conclusion

The isolated phage was shown to be capable of overcoming the species barrier and performing a productive infection within the cells of two species of *Proteus* genus. Hence, it represents a promising tool for phage therapy, as well, along with its host it can be used as an object for phage-host interaction studies in isotropic and anisotropic environments. The described feature, although, remains proven according to biochemical, morphological and physiological testing of phage host bacteria only. Still, due to poor informativity of 16S rDNA comparison for within-genus discrimination for *Proteus* bacteria, we lack molecular genetics evidence for it. Use of more conservative phylogenetic markers (*proB, dnaJ* etc.*)* or approaches (such as DNA fingerprinting, MLSA, etc) [7] is planned.

7. References

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8. Acknowledgements

We would like to thank Dr. Alla Kharina (Taras Shevchenko National University of Kyiv) for providing the bacteria and phage crude isolate; D.K. Zabolotny Institute of Microbiology and Virology of the NASU's Center for Collective Use; ESC "Institute of Biology and Medicine" Taras Shevchenko National University of Kyiv Department of Microbiology and Immunology for funding the research.

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