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ГЕНЕТИКА

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PECULIARITIES OF *ACTINOPLANES TEICHOMYCETICUS* NRRL-B16726 MORPHOLOGY AND LIFE CYCLE

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Actinoplanes teichomyceticus represents a group of sporangia-forming *Actinobacteria* from *Micromonosporaceae* family and is the only known natural producer of industrially important drug teicoplanin. *A. teichomyceticus* has unique pattern of development, likely involving regulatory mechanisms different from described in streptomycetes, which also fall into the phylum *Actinobacteria*. Investigation of these regulatory mechanisms will likely shed light on sporangia formation and even give the clues about increasing secondary metabolite production levels in *Micromonosporaceae* species. To date only limited information that describes morphology and the life cycle of *A. teichomyceticus* is available. In current paper we made a detailed study of certain traits of *A. teichomyceticus* morphology by means of scanning electron microscopy and using tools of bioinformatics and molecular genetics. We managed to observe and describe stages of the *A. teichomyceticus* life cycle while growing on different solid media. Development of *A. teichomyceticus* on the solid media starts with the germination of spores and growth of the substrate mycelium. We observed that after two days of growth on the surface of substrate mycelium aerial mycelium appears. It is known that in streptomycetes the germination of aerial hyphae is facilitated by the SapB-surfactant; *A. teichomyceticus* has genetic potential to synthesize the SapB-like surfactant as well. We managed to show that correspondent genes are expressed under sporulating conditions and silent under non-sporulating conditions. Further aerial hyphae grow and sporangial primordia appear on their ends. Primordia develop into mature sporangia with diameter of more than 20 μm. Wetting of the mature sporangia results in spore release. Motility of the spores is most probably achieved with involvement of a FliC flagellin. Thus, in current paper we have been able to describe the complete lifecycle of *Micromonosporaceae* family representative *A. teichomyceticus* for the first time.

Keywords: *Actinoplanes*, sporangia, morphogenesis, scanning electron microscopy.

Actinoplanes teichomyceticus is a natural producer of pharmaceutically valuable glycopeptide antibiotic teicoplanin [13]. Like other glycopeptides, teicoplanin targets peptidoglycan synthesis in Gram-positive bacteria, particularly binding to the acyl-D-alanyl-D-alanine. However, number of distinctive features including effectiveness (teicoplanin is shown to be more effective and less toxic than vancomycin due to unique pattern of glycosylation and carboxylation) [3], lack of industrial strains with significant production level, biosynthesis specifics, etc. permanently holds this drug in the spotlight. Besides being a producer of such important antibiotic, *A. teichomyceticus* is a representative of so-called non-*Streptomyces* actinobacterial species and forms motile spores inside of big globose sporangia. Characterization of the morphogenetic features in a lifecycle of certain microbe is very important. Among *Actinobacteria* complex life cycles are

well described for streptomycetes, where several development stages could be distinguished. This includes spore germination, vegetative mycelium development followed by aerial mycelia growth and sporulation [9]. Morphological development in other sporulating actinobacteria either goes similarly to streptomycetes or involves different ways and largely remains obscure [5, 20]. Although the micromorphology of some *Actinoplanes gen.* members was already described [7, 10, 14, 17], only limited data for *A. teichomyceticus* is available [21]. Even more, the detailed study of *Actinoplanes gen.* representatives' gradual development was never made.

Because of major morphological differences between *A. teichomyceticus* and streptomycetes one could expect the differences in mechanisms of morphogenesis and secondary metabolism regulation in *A. teichomyceticus* comparing to those currently studied for streptomycetes. It is well known that in streptomycetes [12] and some other actinobacteria [1] the regulation of morphogenesis is tightly correlated with the regulation of the secondary metabolism: both processes occur on the same stage of the lifecycle, which starts after the end of exponential growth phase. In fact, a set of global regulators were described to govern both onset of morphological differentiation and secondary metabolism [12]. This means that one gene can positively or negatively regulate sporulation and secondary metabolite production; overexpression or knockout of such global regulator will affect both morphogenesis and secondary metabolite production. Therefore, investigation of the global regulatory mechanisms in valuable compound producers is justified due to the expectable emergence of overproducing strains [11]. Generation of different types of gene engineered strains altered in morphology and secondary metabolite production is a logical consequence of such study [15]. Thus, a clear comprehension of morphogenetic processes in the wild type strain is necessary. So, in current paper we decided to study the lifecycle of *A. teichomyceticus* NRRL-B16726 on purpose to widen our knowledge of actinobacterial development patterns and to pave the way for further global regulatory pathways investigations in this strain.

Materials and methods

In this work we used *Actinoplanes teichomyceticus* NRRL-B16726 (Lv 95^T) strain obtained from the culture collection of microorganisms-producers of antibiotics of Ivan Franko Lviv National University. *A. teichomyceticus* was grown on oatmeal agar plates (OM, g/l: oatmeal – 34, agar– 20), soy-mannitol agar plates (SM, g/l: soybean meal – 20, mannitol – 20, agar – 20) and HA-agar plates (HA, g/l: yeast extract – 4, malt extract – 10, glucose – 4, agar – 20) at 30°C or in E-25 (g/l: dextrose – 25, meat extract – 4, yeast extract – 1, soybean meal – 10, peptone – 4, NaCl – 2.5, $CaCO_3$ – 5) at 30°C, 180 rpm in case of liquid culture cultivation. All reagents were purchased from Sigma-Aldrich (USA) except the crude oatmeal flour of Ukrainian origin and soybean meal (Henzel, Germany). To create a vegetative cell stock ~10^6 spores were inoculated in E-25 media, and then the mycelia from 3-day E-25 liquid culture was collected by centrifugation, washed twice with sterile water and resuspended in 10% glycerol by intensive vortexing. Vegetative cell stock was stored at -80°C. To receive sporulating lawns plates were inoculated with 100–200 mg of vegetative stock mycelia. Biomass was evenly spread over the surface of the plate.

In order to prepare samples for scanning electron microscopy in all estimated time points thin slices of agar were cut from the surface of the lawn. These slices were placed on a SEM specimen holders and undergone vacuum deposition with copper in VUP-5m (the universal post for vacuum deposition). Specimens covered with thin-layer copper films were studied using JEOL JSM-T220a SEM (Jeol, Japan) with 20 KV electron beam. For light microscopy analysis sporangia were flushed from the surface of the lawn with distilled water, simple squashed drop specimens were prepared. In order to show spore germination, a drop of OM-agar was placed on the glass slide, then inoculated with spores, covered with cover slide and incubated overnight at

30°C. Microscope Olympus BX60 (Olympus, USA) was used for light microscopy.

For RNA isolation strains were grown on HA or SM agar plates overlaid with cellophane discs as described [9]. RNA was isolated according to the modified Kirby mix protocol [9]. cDNA was synthesized with Revert Aid Reverse Transcriptase (Thermo Scientific) according to the manufacturer's instructions. Following primers were used for amplification of intragenic region of *ramA*: ram_RT_F: 5'-GGGCTCTGGTGCGCCGCTAC-3', ram_RT_R: 5'-GACGC-CGGTCGTGCCCGAG-3'. PCR reactions and horizontal agarose gel electrophoresis were performed according to the standard protocols [16].

For the analysis of amino acid and nucleotide sequences BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) tools were used.

Results and discussion

Our own previous experience indicates that *A. teichomyceticus* appreciably sporulates on soya-mannitol agar (SM). It was also shown [21] that *A. teichomyceticus* can sporulate on oatmeal-agar (OM, ISP3). Thus, we used these media to cultivate *A. teichomyceticus* on purpose to study the morphogenesis.

While growing on SM or OM we observed no aerial hyphae formation during first two days of lawn growth. Germination of aerial hyphae began only after second day of incubation. Interestingly, aerial hyphae start to form in certain centers across the lawn as it can be observed on Fig. 1, *a*.

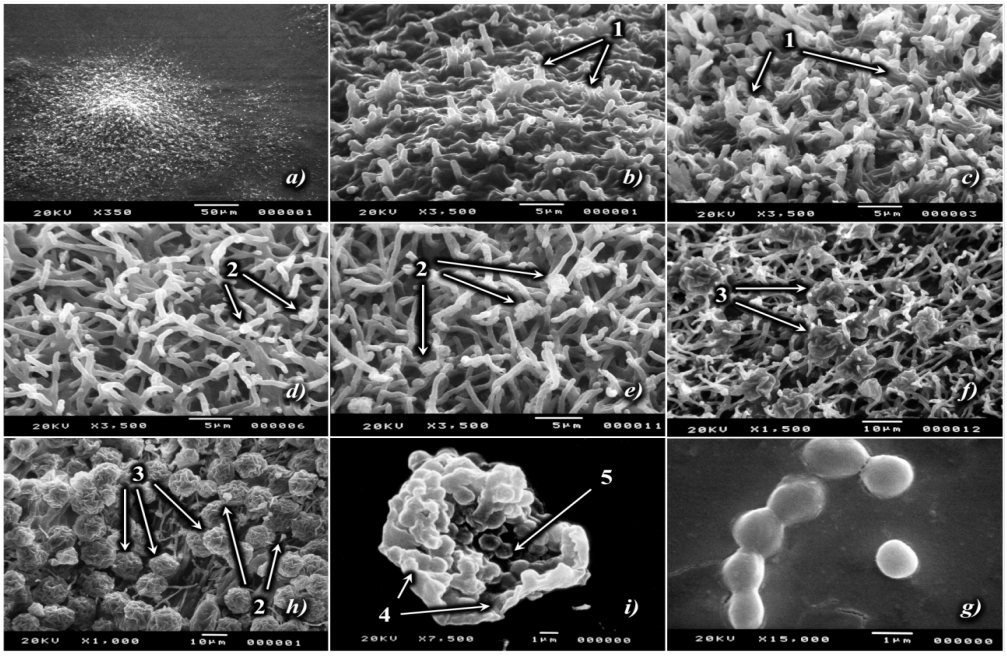

Fig. 1. SEM photographs depicting main steps of *A. teichomyceticus* development on SM-agar: *a),b)* onset of sporangiophorous hyphae (1) germination (second day of cultivation); *c)* growth of sporangiophorous hyphae (1, third day of cultivation); *d),e)* emergence and growth of sporangial primordia (2, fourth and fifth day of cultivation); *f)* appearance of mature sporangia (3, sixth day of cultivation); *h)* fully developed lawn with prevailing mature sporangia (3) and some residual sporangial primordia (4, seventh day of cultivation); *i)* open sporangia, the sporangial coating (4) and spores (5) could be distinguished; *g)* free spores.

It appears that early stages of aerial mycelium growth in *A. teichomyceticus* closely resemble those in *Streptomyces* gen. representatives. It is known that to break the tension of the aquatic layer, cell walls of the germinating aerial hyphae in streptomycetes are decorated with a variety of hydrophobic proteins (hydrophobins) [12]. At the beginning of germination the SapB hydrophobin [19] plays the main role in overcoming the aquatic layer, later chaplins [4] and rodlins [2] are getting involved in this process. Thus, it was interesting to analyze the occurrence of genes encoding different hydrophobins in the draft sequence of *A. teichomyceticus* genome (unpublished data). We discovered no traces of conserved hydrophobic domains, characteristic for streptomycetic rodlins and chaplins. On the contrary, *A. teichomyceticus* genome possesses genetic capability to synthesize SapB-like surface-active molecule. In streptomycetes genes involved in the SapB synthesis are grouped together, forming the *ramCSAB* cluster [8].This cluster consists of four genes which are transcribed as one polycistronic mRNA. We found out that all genes from *ram*-cluster of *S. coelicolor* (SCO6681-5) encode proteins that have close orthologues in *A. teichomyceticus*. Remarkably, both clusters are absolutely sintenyous and identity of their total nucleotide sequences is 62,6%. Furthermore, it appeared that all sequenced to date genomes of *Micromonosporaceae* contain *ram*-like clusters (data not shown). In fact, presence of highly similar *ram*-like clusters in such distant families as *Micromonosporaceae* and *Streptomycetaceae* indicates antiquity and importance of these genes.

In order to elucidate the role of *ramCSAB*-genes in *A. teichomyceticus* we compared the expression of the cluster in cells grown under sporulating (OM-agar) and non-sporulating (HA-agar, ISP2) conditions by means of semi-quantitative RT-PCR. We observed that *ram*-cluster is indeed not expressed in the cells grown on HA-agar, but expressed under sporulating conditions (Fig. 2, *b*). Since there is no germination of aerial hyphae on HA-agar (and consequently no sporulation) strain has no need in SapB-like surfactant synthesis. On the other hand when strain grows under sporulating conditions there is a strong need in *ramCSAB*-genes expression on purpose to facilitate the penetration of the aquatic layer by germinating aerial hyphae.

The germination of aerial hyphae is followed by sporangia emergence and maturation. Maturation of the sporangia occurs during 4–7 days of growth, but this process runs differently on OM and SM plates. If the culture is grown on SM-agar, maturation process goes gradually: formation of the aerial hyphae, the sporangial primordia and the mature sporangia is synchronized

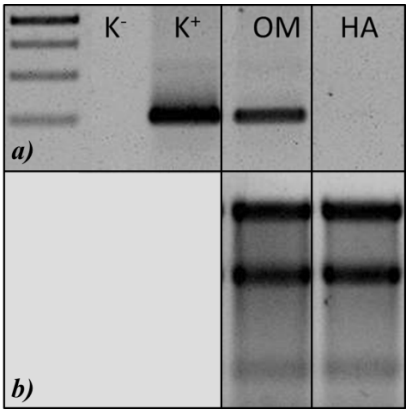

Fig. 2. Analysis of the *ram*-cluster expression under different growth conditions: *a)* ~260 b.p. region of *ramA* amplified from the chromosomal DNA (K^+), a control without template (K^-) and cDNA synthesized on a template of RNA isolated from strains grown on OM and HA media;*b)* respective total RNA samples; bends corresponding to 23S and 16S rRNA could be distinguished.

(Fig. 1). In case of OM the mature sporangia appear much earlier and during all period of growth differently developed sporangia could be observed across the lawn (Fig. 3).

Development of sporangia is followed with a spore releasing process. In *A. teichomyceticus* this process is initiated by simple wetting, unlike in case of *Actinoplanes missouriensis* where the addition of soil-extract is required for spore release [18]. Diameter of single spore is around 1 μm (Fig. 1, *g*), nevertheless spore seizes can slightly differ (Fig. 4, *f*). The steps of spore release are described on Fig. 4. Through light microscopy observations it appears that after wetting spores are swelling and growing in size (Fig. 4, *a,b*) which leads to the breaking of sporangial coating (Fig. 4, *c*), as it was hypothesized for *Actinoplanes* sp. 7-10 [6]. It also appears that spores gain mobility while still being inside of sporangium. Released spores can be single or form chains (Fig. 4, *f*), which means that inside sporangia spores are organized in chain-like structures. The composition of *A. teichomyceticus* flagella is probably similar to such in *A. missouriensis* [18]. We found only one gene encoding flagellin in *A. teichomyceticus* genome. This is an orthologue of the *fliC* from *A. missouriensis* [22]. Both FliC-proteins from *A. missouriensis* and *A. teichomyceticus* share 61,2% of sequence identity.

Released spores are actively moving and continue to swell; the diameter of the single spore grows to 2–3 μm. After 5–6 hours of active movements spores lose their mobility and germination process starts (Fig. 5, *a, b, c*). Spores geminate both in submerged culture or plated on solid medium. After 12–16 hours of growth on the solid media developed substrate mycelia could already be observed (Fig. 5, *d, e*).

Regarding all obtained data it is possible to distinguish certain stages in *A. teichomyceticus* life cycle (summarized life cycle scheme is depicted on Fig. 6):

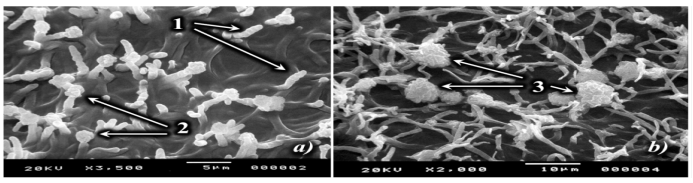

Fig. 3. SEM photographs showing *A. teichomyceticus* development on OM-agar: *a)* on the third day of cultivation sporangial primordia (2) could be already observed as well as sporangiophorous hyphae (1); *b)* on the fourth day of cultivation some mature-size sporangia (3) could be already observed.

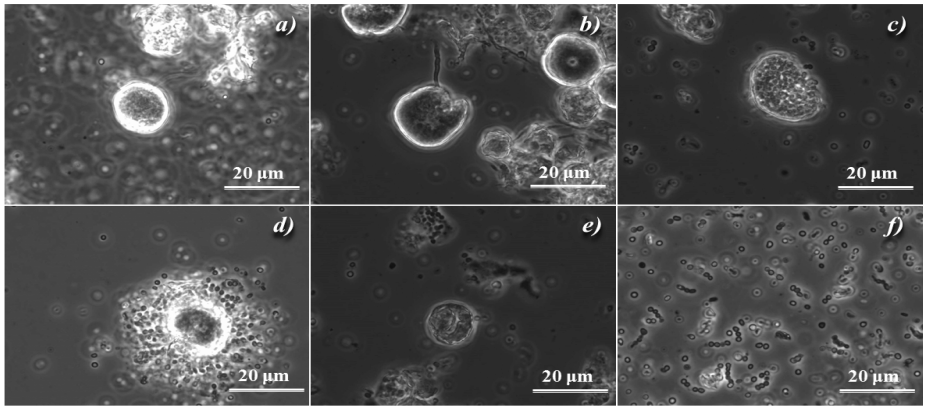

Fig. 4. Light microscopy photographs illustrating spore releasing process: *a)* intact sporangium; *b)* swelling of the spores began, sporangial coating is broken; *c)* spore release; *d)* late stage of spore release; *e)* empty sporangial coating; *f)* free swimming spores.

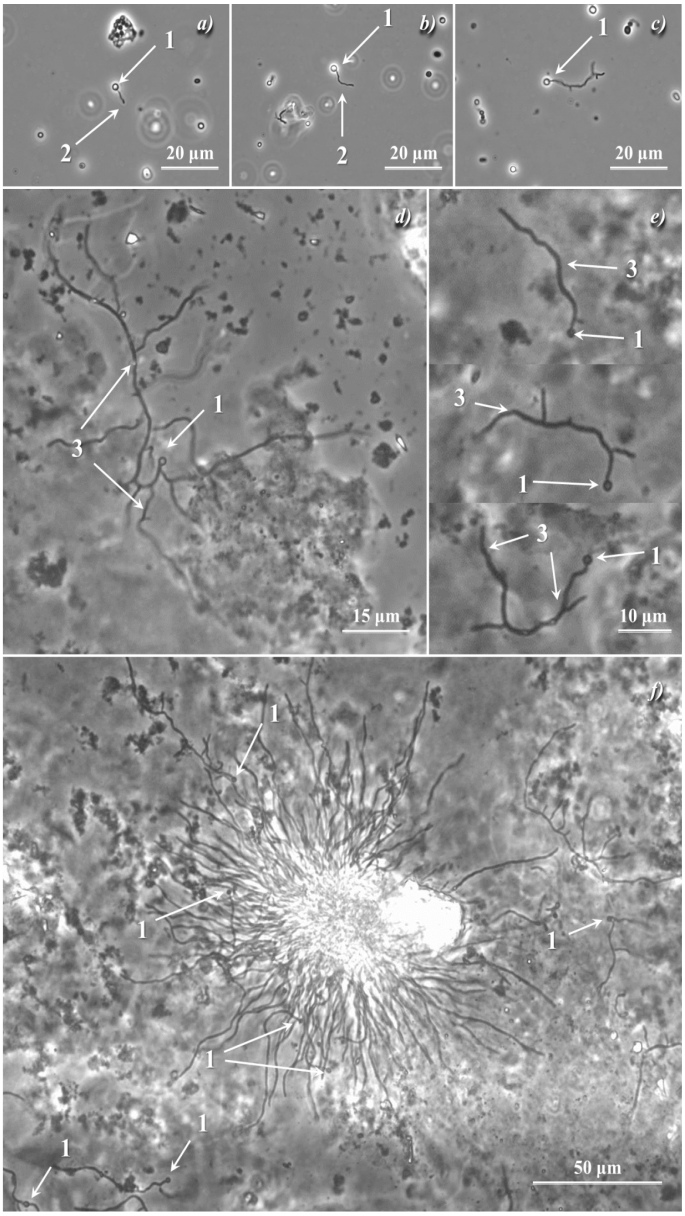

Fig. 5. Light microscopy photographs of spore germination process: *a,b,c*) germinating spores (1) with germ tubes (2) maintained 5-6 hours after release in water suspension, *d,e)*germinated spores (1) form substrate mycelia (3) after 12-16 hours maintenance on OM-agar; *f)* germination of whole sporangium on OM-agar, single geminating spores could still be distinguished (1).

1. **Spore germination and vegetative mycelia formation**. After spores are inoculated on the surface of the solid media they germinate (Fig. 6, *a*) and form substrate mycelia (Fig. 6, *b*). Mycelia from liquid culture can also form vegetative hyphae when inoculated on solid media. *A. teichomyceticus* forms substrate mycelia during first two days of growth under sporulating conditions.

2. **Aerial hyphae germination**. After two days of growth aerial hyphae emerge on the surface of vegetative mycelia (Fig. 6, *c*). Unlike aerial hyphae on streptomycetes they never branch. The aerial hyphae germination is initiated in the certain points of the lawn.
3. **Formation of sporangial primordia**. At the top of some aerial hyphae sporangial primordia form (Fig. 6, *d*). Therefore, aerial hyphae actually turn into sporangiophores.
4. **Development of the sporangia**. Primordia grow in size (from 1,5–2 to >20 μm, (Fig. 6, *e, f*) and form mature spherically shaped sporangia (>20 μm, Fig. 6, *g*). It is obvious that certain differentiation processes leading to spore formation take place within the primordium. In spite of this it still remains unclear what happens at the apiculus of sporangiophore during the development of the sporangium. Some works concerning this issue for other *Actinoplanes* species could be found [20] but reliable hypothesis about spore formation does not exist.

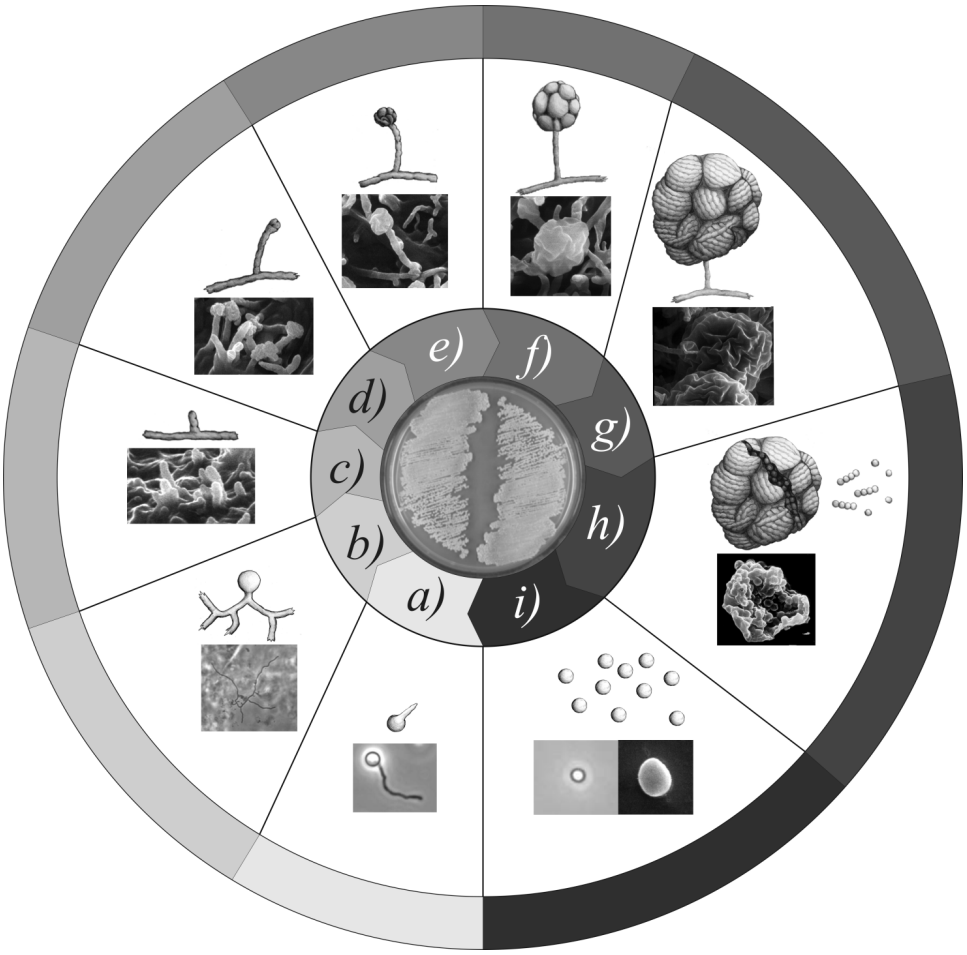

Fig. 6. Summarizing scheme of *A. teichomyceticus*life cycle: sporangiophorous hyphae emerge on the surface of vegetative mycelia (*a*) with further gradual development into sporangia (*b*-*g*) and spore release (*h*-*i*).

5. **Spore release**. After the wetting of mature sporangia motile spores are released (Fig. 6, *h, i*). They can germinate and give raise to substrate mycelia if inoculated on solid media or germinate and form fragmented vegetative hyphae under submerged growth conditions.

Summing up, we are observing complicated picture of sporangial development in *A. teichomyceticus*. To our knowledge, this is the first example of a complete lifecycle described for *Micromonosporaceae.* Any developmentally deviated strain that could be received in future can now be compared with resolved developmental pattern of *A. teichomyceticus* NRRL-B16726. Morphogenesis of *A. teichomyceticus* only slightly resembles sporulation in streptomycetes. Similarities could be observed only on the stage of spore and aerial mycelia germination: we found out that spores germinate after swelling with a germ tube formation and that the SapB-like surfactant putatively takes part in the germination. Furthermore, we found the absence of genetic potential to synthesize chaplins and rodlins in *A. teichomyceticus.* We can try to explain such absence with the size of sporangia: being relatively big structures (comparing to single bacterial cells or streptomycetic spore chains) they maybe can penetrate aquatic layer by themselves, without any additional hydrophobic proteins. In spite of above described and other data, spore formation remains the most enigmatic part of the development.To reveal the development processes of spores inside sporangia further investigations are necessary. We plan to achieve this goal by means of transmission electron microscopy.

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ОСОБЛИВОСТІ МОРФОЛОГІЇ ТА ЖИТТЄВОГО ЦИКЛУ *ACTINOPLANES TEICHOMYCETICUS* NRRL-B16726

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Actinoplanes teichomyceticus належить до актинобактерій родини *Micromonosporaceae*, які формують спорангії. Це єдиний відомий природний проду-

цент промислово важливого антибіотика тейкопланіну. Для *A. teichomyceticus* властиві унікальні шляхи розвитку, в яких задіяні регуляторні механізми, відмінні від тих, що описані для стрептоміцетів. Їх дослідження допоможе з'ясувати закономірності генетичного контролю формування спорангіїв, а також може бути корисним для отримання надпродуцентів вторинних метаболітів у видів родини *Micromonosporaceae*. Для цього необхідно досконально вивчити морфологію та життєвий цикл *A. teichomyceticus* дикого типу, інформація про які на сьогодні дуже обмежена. У цій роботі детально досліджено особливості морфології *A. teichomyceticus* за допомогою світлової та скануючої електронної мікроскопії, а також методів біоінформатики і молекулярної генетики. За результатами спостережень описано стадії життєвого циклу *A. teichomyceticus.* Розвиток *A. teichomyceticus* на агаризованих середовищах починається із проростання спор і формування субстратного міцелію. Після двох днів росту на поверхні субстратного міцелію починають утворюватися гіфи повітряного міцелію. У стрептоміцетів формування гіфів повітряного міцелію полегшується включенням у їхні клітинні стінки сурфактанта SapB; *A. teichomyceticus* також має гени, необхідні для синтезу SapB-подібного сурфактанта. Нами з'ясовано, що ці гени експресуються за умов споруляції та неактивні, коли її немає. Потім гіфи повітряного міцелію видовжуються і на їхніх верхівках формуються зачатки спорангіїв, які далі розвиваються у дозрілі кулясті спорангії діаметром понад 20 мкм. Змочування дозрілих спорангіїв спричиняє вихід рухливих спор. Рухливість спор найбільш імовірно забезпечується флагеліном типу FliC. Таким чином, у цій роботі було вперше описано повний життєвий цикл представника родини *Micromonosporaceae – A. teichomyceticus.*

Ключові слова: Actinoplanes, спорангії, морфогенез, скануюча електронна мікроскопія.

ОСОБЕННОСТИ МОРФОЛОГИИ И ЖИЗНЕННОГО ЦИКЛА *ACTINOPLANES TEICHOMYCETICUS* NRRL-B16726

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Actinoplanes teichomyceticus принадлежит к актинобактериям семейства *Micromonosporaceae*, которые образуют спорангии. Это единственный известный природный продуцент промышленно важного антибиотика тейкопланина. Для *A. teichomyceticus* характерны уникальные пути развития, в которые вовлечены регуляторные механизмы, отличающиеся от описанных для стрептомицетов. Их изучение поможет выяснить закономерности генетического контроля формирования спорангиев, а также может быть полезным для получения сверхпродуцентов вторичных метаболитов у видов семейства *Micromonosporaceae*. Для этого необходимо досконально изучить морфологию и жизненный цикл *A. teichomyceticus* дикого типа, информация о которых на сегодня очень ограничена. В этой роботе детально изучены особенности морфологии *A. teichomyceticus* при помощи световой и сканирующей электронной микроскопии, а также методов биоинформатики и молекулярной генетики. Нам удалось проследить и описать стадии жизненного цикла *A. teichomyceticus*. Развитие *A. teichomyceticus* на агаризованных средах начинается с прорастания

спор и формирования субстратного мицелия. После двух дней роста на поверхности субстратного мицелия начинают формироваться гифы воздушного мицелия. У стрептомицетов формирование гифов воздушного мицелия облегчается включением в их клеточные стенки сурфактанта SapB. Геном *A. teichomyceticus* также содержит гены, необходимые для синтеза SapB-подобного сурфактанта. Мы показали, что эти гены экспрессируются в условиях споруляции и неактивны, если она отсутствует. Затем гифы воздушного мицелия удлиняются, и на их верхушках формируются зачатки спорангиев, которые в дальнейшем развиваются в зрелые шаровидные спорангии диаметром более 20 мкм. Смачивание зрелых спорангиев вызывает выход подвижных спор. Подвижность спор, вероятнее всего, обеспечивает флагеллин типа FliC. Таким образом, в этой роботе впервые описан полный жизненный цикл представителя семейства *Micromonosporaceae – A. teichomyceticus.*

Ключевые слова: *Actinoplanes*, спорангии, морфогенез, сканирующая электронная микроскопия.