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Review of Actinoalloteichuscyanogriseus as an alternative for Novobiocin production.

Revisión de *Actinoalloteichuscyanogriseus* como alternativa para la producción de Novobiocina

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Abstract*:Coumarins are a set of molecules that can be found in a large number of organisms, from trees, plants to bacteria of the Actinobacteria class, specifically Actinoalloteichuscyanogriseus which has proven to be a candidate microorganism to replace Streptomyces caeruleus as supplier of the antibiotic Novobiocin, a drug which has excelled in the pharmacological field due to its broad spectrum of medicinal properties, among which stand out: Anticancer action by inhibiting oncological processes, its antibiotic and anti-inflammatory properties.. To carry out this research we made use of bioinformatics tools that are publicly available, such as databases (NCBI, PDB) and websites with included software that allow us to perform sequence alignments (BLAST), analysis of a protein sequence (Phobius) and phylogenetic analysis of Actinoalloteichuscyanogriseus to observe the evolutionary history of the microorganism and observe that it shares a common ancestor with microorganisms of the genus Streptomyces, specifically Streptomyces caeruleus. In adherence to these analyses, we also investigated through the BacDive platform from which we obtained more detailed information about studies that report the isolation of Actinobacteria detailing the culture media used and the metabolites produced, reporting that indeed the bacterium produces antibiotics as a result of its metabolism.*
Resumen:*Las cumarinas son un conjunto de moléculas que pueden ser encontradas en una gran cantidad de organismos, desde árboles, plantas hasta bacterias de la clase Actinobacterias, especificamenteActinoalloteichuscyanogriseus que ha demostrado ser un microorganismo candidato para sustituir a Streptomyces caeruleus como proveedor del antibiótico Novobiocina, medicamento el cual ha sobresalido en el ámbito farmacológico debido a su gran espectro de propiedades medicinales, entre las cuales destacan: Acción anticancerígenaal inhibir procesos oncológicos, su propiedad antibiótica y antiinflamatoria. Para llevar a cabo esta investigación se hizo uso de herramientas bioinformáticas que son de acceso público, tal como bases de datos (NCBI, PDB) y webs con softwares incluidos que nos permiten realizar alineamientos de secuencias (BLAST), análisis de una secuencia proteínica (Phobius) y análisis filogenético de Actinoalloteichuscyanogriseus para observar la historia evolutiva del microorganismo y observar que comparte un ancestro en común con los microorganismos del género Streptomyces, específicamente Streptomyces caeruleus. En adhesión a estos análisis, también se investigó a través de la plataforma BacDive de la cual se obtuvo información más detallada acerca de estudios que reportan el aislamiento de la Actinobacteria detallando los medios de cultivo utilizados y los metabolitos producidos, reportando que efectivamente la bacteria produce antibióticos como resultado de su metabolismo.*
Materials and Methods*:The present research is based entirely on the use of bioinformatics tools freely available to the public, being these mainly databases recognized worldwide as National Center for Biotechnology Information (NCBI), Protein Data Base (PDB), MetaCyc which is an extension of BioCyc on the metabolic pathways of organisms from which we obtained the metabolic pathway to produce Novobiocin in A. cyanogriseus. We made use of the Phobius website to obtain a general analysis of the NovH protein and more importantly, we used the NCBI BLAST software to run 16S rRNA alignments of A. cyanogriseus.*
Results*: Los alineamientos corridos en BLAST fueron significantes para observar que efectivamente A. actinoalloteichus está estrechamente relacionada con S. caeruleus al obtener E values de 0 y un porcentaje de identidad por arriba del 95% al analizar el rRNA 16S de ambos organismos así como también al compararlos con organismos del mismo género.*
Conclusion*:*Actinoalloteichuscianogriseus is a candidate organism to be considered for the production of antibiotics, specifically Novobiocin, which has been shown to be very effective in cancer treatments.
Key words*:Coumarins, Actinobacteria, Novobiocin, Cancer, Aminocoumarin.*

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I. Introduction

Coumarins are a constituent found naturally in many plants, seeds and essential oils, including Tonka Bean (*Dipteryx odorata*) or Coumaru as it is better known, in fact, it was from Coumaru (Coumarouna odorata) that in 1822 Voleg was able to isolate the compound now called Coumarin.[1] They are also known by the following names: 2H-1-benzopyran-2- ona, 1,2-benzopyrone, cis-o- coumarinic acid lactone, or coumarinic anhydride. Coumarins have been used over the years as the elemental structure of several other compounds with biological activities, such as:

- Anti-cancer
- Antioxidant
- Antifungal
- Anticoagulant
- Anti-inflamatory
- Antiviral
- Photosynthesizing
- Antibacterial
- Anti-neurodegenerative
- Ant-HIV activity[2]

The development of analytical and characterization techniques in the omics sciences depends directly on bioinformatics tools, which have helped in the analysis, comparison, notation and validation of the structure and functionality of biomolecules, as well as in the evaluation of the profile of their interactions.[3]

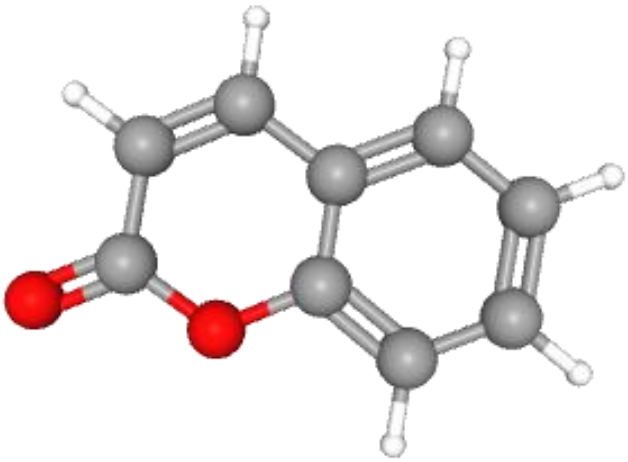

Figure 1 Chemical structure of Coumarin.[17]

The antibiotics novobiocin, chlorobiocin and coumermycin A1 (known as antibiotic aminocoumarins) bind type II topoisomerases, including DNA gyrase, with extremely high affinity, with equilibrium dissociation constants within the range of 10 nM.These three molecules produced by three different microorganisms: ***Actinoalloteichuscyanogriseus***, *Streptomyces roseochromogenusoscitans* and *Streptomyces rishiriensis* compete for ATP to bind to the B subunit of DNA gyrase, thereby succeeding in inhibiting DNA supercoiling catalyzed by gyrase as it is an ATP-dependent process.[4]

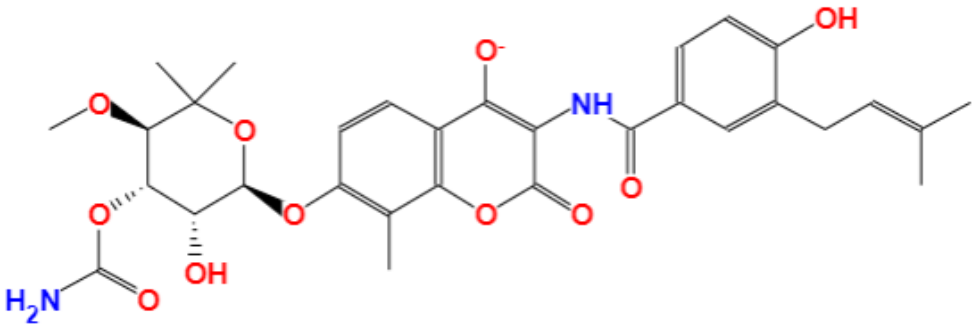

Figure 2 Chemical structure of Novobiocin.[18]

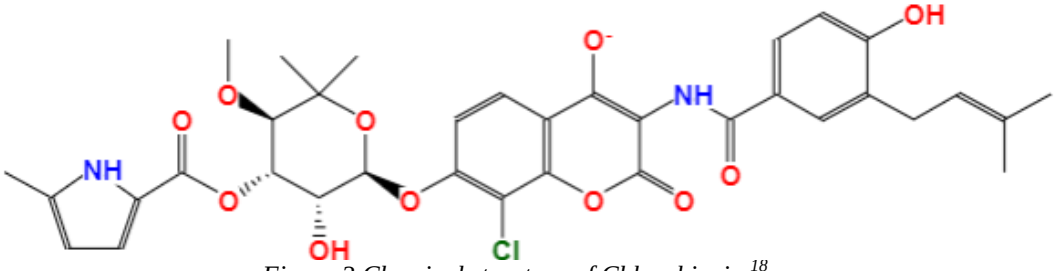

Figure 3 Chemical structure of Chlorobiocin.[18]

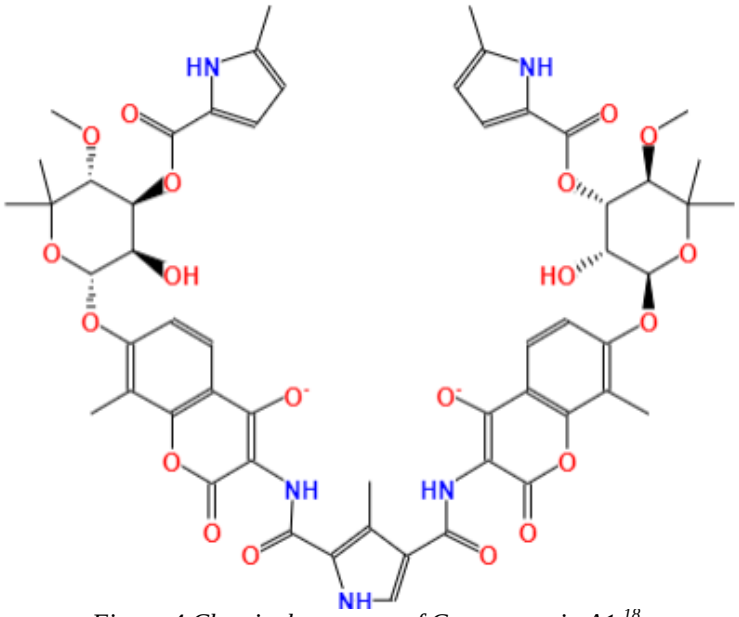

Figure 4 Chemical structure of Coumermycin A1.[18]

The aminocoumarin synthesis pathway can be found in the three previously mentioned organisms whose orthologous genes were named differently: novH/cloH/couH respectively. Taking as a reference Novobiocin produced by ***Actinoalloteichuscyanogriseus*** where the aminocoumarin core is synthesized from tyrosine which must be bound to NoAcvH proteins containing two adenylation and sulfidation domains.[5]

Novobiocin is known to be produced by the bacterium Streptomyces caeruleus. The gene cluster for novobiocin biosynthesis in ***Actinoalloteichuscyanogriseus*** was identified and fully cloned using a fragment of the dNDP-glucose 4,6-dhydratase gene as a probe. Sequencing of this region revealed 23 putative open reading frames, including the novobiocin-resistant gyrB gene. Inactivation of the novT gene (encoding dTDP-glucose 4,6-dhydratase) resulted in suppression of novobiocin production. Part of the cluster was cloned into Streptomyces lividans TK24, and the transformed strain was found to produce novobiocin.[6]

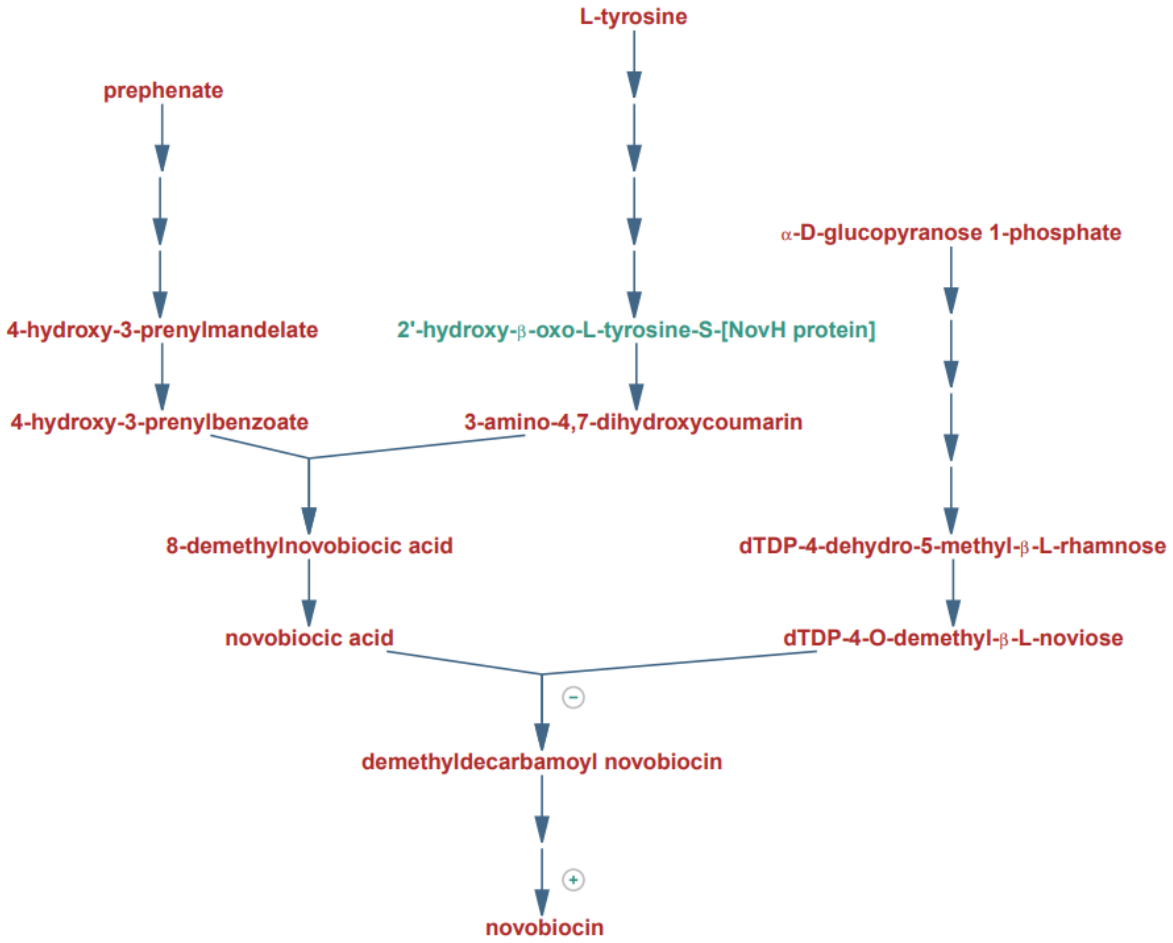

Figure 5 Novobiocin biosynthesis Pathway.[18]

The metabolic pathway can be divided into four parts: synthesis of the amino-coumarin core, NovH sugar and prenylated hydroxybenzoate unit, and final decoration of the assembled product.

The aminocoumarin core is synthesized from L-tyrosine, which must bind to the peptide carrier NovH. This protein contains two domains: an adenylation domain (A) and a thiolation domain (T), which resemble the initiation modules of nonribosomal peptide synthase chains. The A domain activates L-tyrosine to tyrosine-AMP and then covalently binds aminoacyl to the thiol-phosphopantheline group of the protein in the T domain.[7] All subsequent modifications leading to the formation of the amino-coumarin ring occur in this covalently bound moiety. The enzyme-bound tyrosine is first hydroxylated to (R)-β-hydroxy-L-tyrosine and then oxidized to β-oxo-L-tyrosine.[8] The next step is 2-hydroxylation, although the enzyme that introduces this 2-hydroxy group is not yet known. This lactonization leads to the release of the amino-coumarin core of the NovH protein.

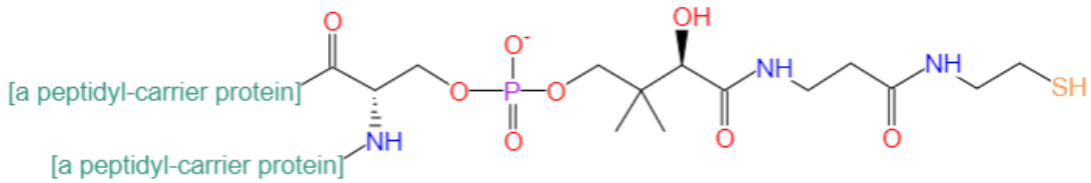

Figure 6NovH peptidyl-carrier protein. Obtained from Biocyc.[18]

In parallel, a prenylated 4-hydroxybenzoate moiety, 4-hydroxy-3-prenylbenzoate, is formed from prenyl diphosphate and prenyl diphosphate by the action of three enzymes. [9,10] This compound is then converted to an amino-coumarin ring by the enzyme 8-demethylthiobioate synthase (novL)[11], followed by methylation to novobionic acid catalyzed by a methyltransferase encoded by novO.[12]

The third pathway consists of five enzymes that form dTDP-4-O-demethyl-β-L-noviose from α-D-glucopyranose-1-phosphate.[13] The sugar is then bound to novocitric acid by 4-O-demethyl-L-novosyltransferase (novM).[14]

The final part of the pathway involves methylation and carbamoylation of the 4- and 3-positions of the desmethylvivosyl sugar, respectively, catalyzed by the products of the novP and novN genes, leading to the final product novobiocin.[15]

II. Materials and Methods

This investigation is fully based on databases, which are responsible for storing and distributing the information collected and are a fundamental basis for all experiments and this investigation:

- PubMed (https://www.ncbi.nlm.nih.gov),
- PDB (https://www.rcsb.org),
- Phobius (https://phobius.sbc.su.se)
- Lifemap (https://lifemap-ncbi.univ-lyon1.fr/?tid=201174)
- BacDive (https://bacdive.dsmz.de/strain/16093#ref9709)
- Biocyc (https://biocyc.org)
-

Using the NCBI web integrated BLAST software, 2 16S rRNA sequence alignments were performed 1) Actinoalloteichuscyanogriseus 16S rRNA gene sequence/Top 99 16S rRNA sequences and 2) Actinoalloteichuscyanogriseus rRNA16S gene sequence/Streptomyces caeruleus 16S rRNA sequence.[16]

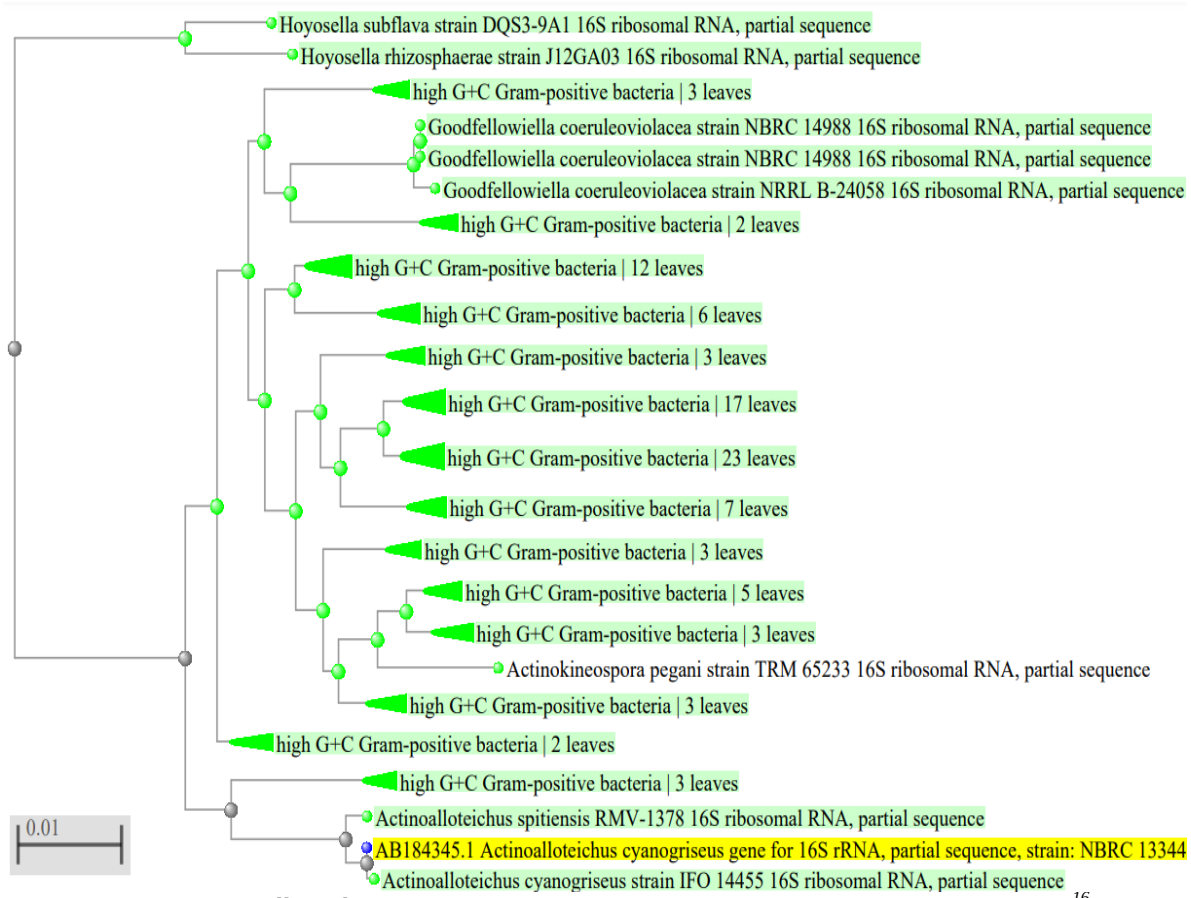

Figure 7Actinoalloteichuscyanogriseus 16S rRNA gene sequence/Top 16S rRNA sequences[16]

The image above (Figure 7) shows the result of the distance tree obtained as a result of running a BLAST on the NCBI website taking as reference the sequence of A. cyanogriseus against the sequence of the 16S ribosomal RNA of organisms of the kingdom Bacteria and Archaea, in which A. cyanogriseus and related microorganisms can be seen in an evolutionary manner in terms of the sequence of its 16S ribosomal RNA, which we know is characterized by having highly conserved regions throughout the evolution of its organisms.
And in the image below (Figure 8) we can observe the 99 alignments of 16S ribosomal RNA against organisms of the kingdom Bacteria and Archaea with the reference sequence of A. cyanogriseus made by BLAST, inwhich it is observed that there was a high percentage of coverage and similarity between sequences, in adherence to this, the E values were 0 in its entirety, so we understand that the results aresignificant.

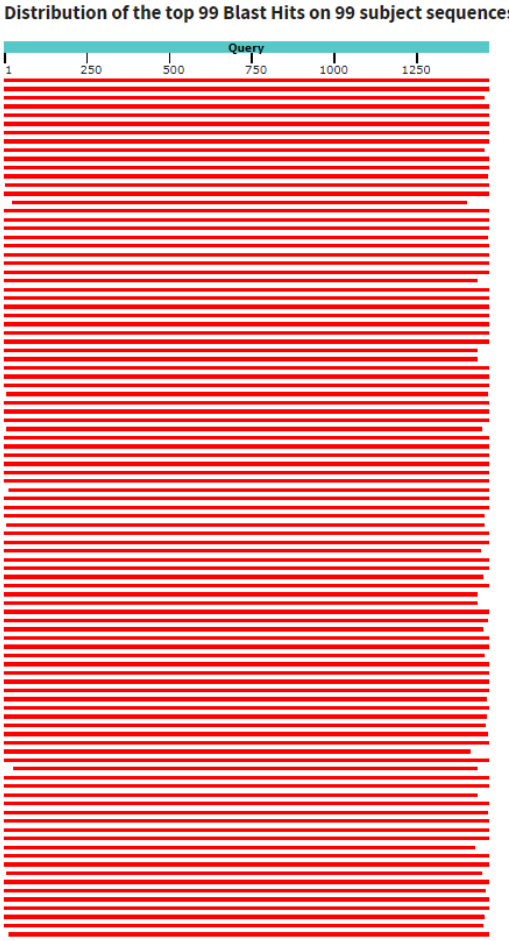

Figure 8 *Distribution of the Blast hits.*[16]

On the other hand, in the following images we observe in the same way the result of the distance tree (Figure 9) obtained from the BLAST of the 16S ribosomal RNA sequences of *A. cyanogriseus* versus *S. caeruleus* and from which we cannot expect great results considering that only 2 organisms are being compared. We also analyzed the graphical summary (Figure 10) of the alignment from which we obtained an identity percentage of 91% and a coverage of 99% with an E value of 0.

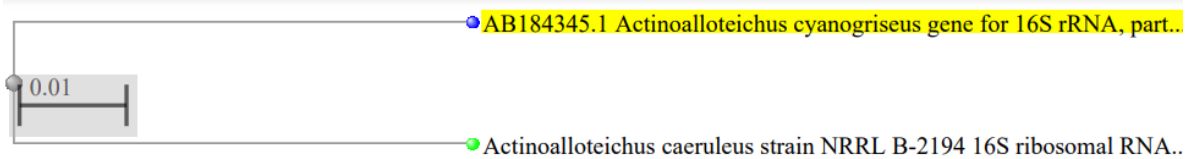

Figure 9Actinoalloteichuscyanogriseus rRNA16S gene sequence/Streptomyces caeruleus 16S rRNA sequence.[16]

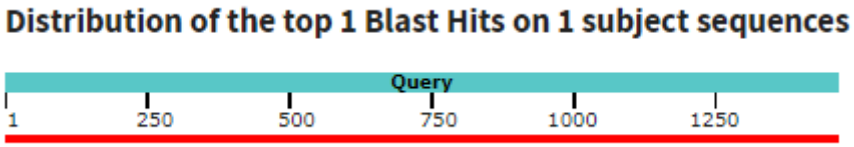

Figure 10 Distribution of the Blast hits of A. cyanogriseus vs S. caeruleus.[16]

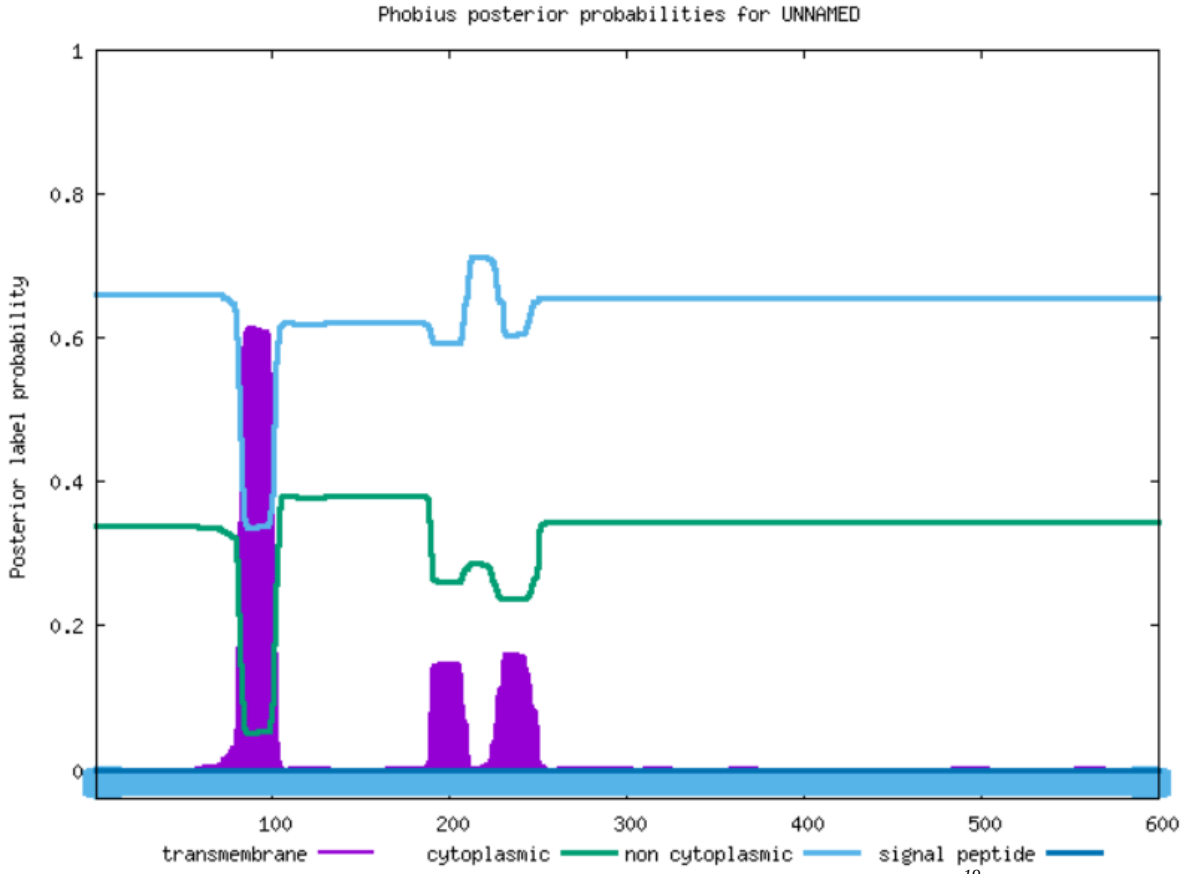

Figure 11 Graph of the properties of the NovH protein of S. caeruleus.[19]

At this point it is worth clarifying that the genomic sequence of A. cyanogriseus is not yet published, however, the sequence of the 16S ribosomal RNA is published, which is why we chose to use these sequences in the study and because they are highly conserved regions throughout the evolution of these organisms, giving us the possibility of seeing the similarity between sequences and being able to infer protein functions. Having said this, the sequence in FASTA format of the NovH gene was obtained and entered into the Phobius website, which gave us a graph (Figure 10) detailing the functions of the protein.[19]

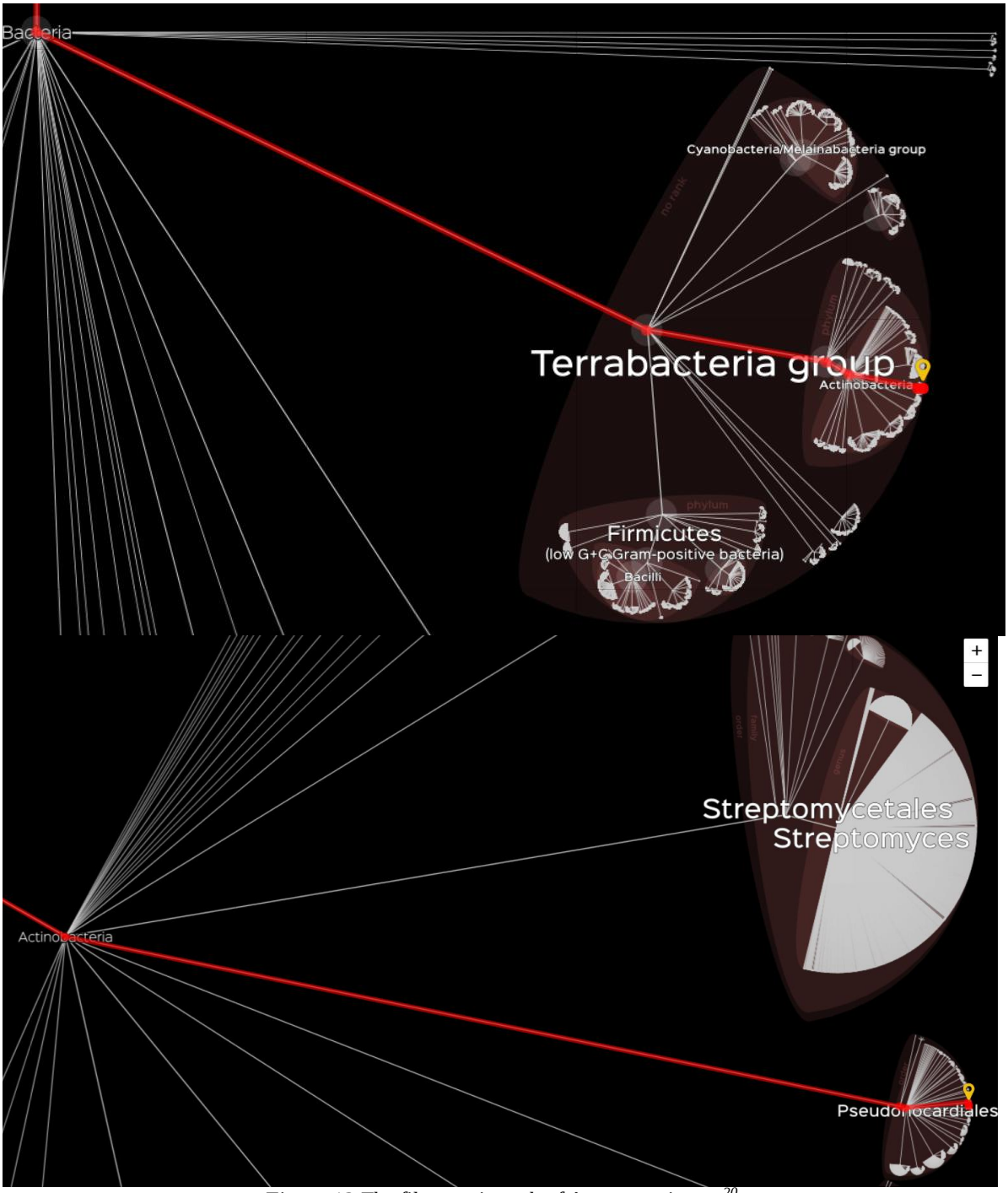

Figure 12 The filogenetic path of A. cyanogriseus. [20]

As a complement to the brief phylogenetic analysis that was performed, the evolutionary path of A. cyanogriseus was searched and it was observed that it is related to the genus Streptomyces (figure 12).[20]

The strain information was entered into the BacDive website, which yielded several results on the isolation of the bacterium from soil and the different reports detailing the characteristics of the soil from which it was isolated, the geographic location and, most importantly, the composition of the culture medium used for the growth of A. cyanogriseus, the growth temperature and the metabolite produced.[21]

Agar 12.0 g/l
Malt extract 10.0 g/l
Yeast extract 4.0 g/l
Glucose 4.0 g/l
CaCO3 2.0 g/l
Distilled water
Growth at 28 °C
Mesophilic

Table 1 Growth conditions of A. cyanogriseus

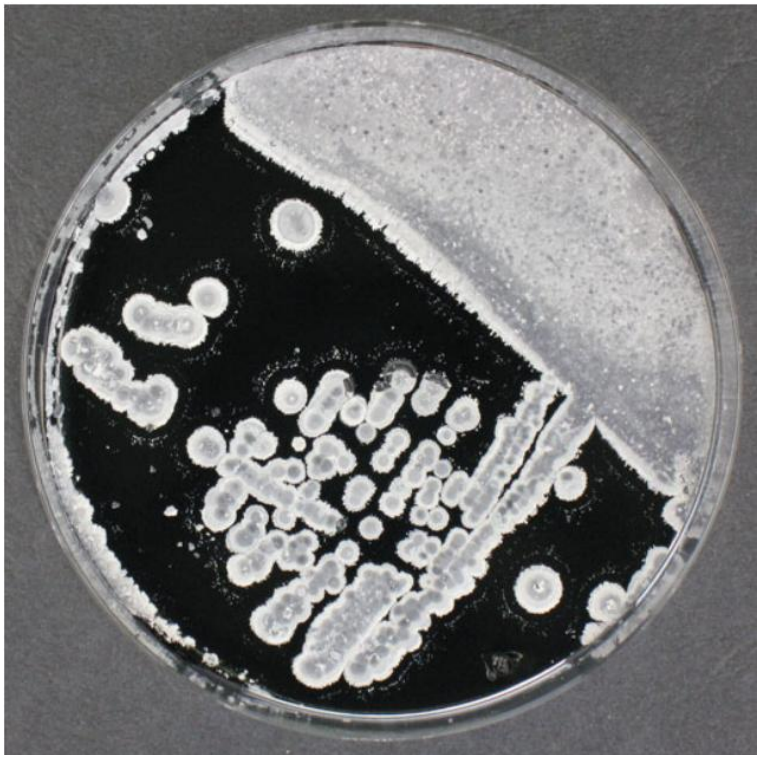

Figure 13 A. cyanogriseus culture. Image obteined from Leibniz-institut DSMZ

A culture of Actninoalloteichuscyanogriseus obtained from BacDive, reported by the Leibniz-institut, is shown (figure 13).[21]

III. Discussion

Throughout the different studies, both theoretical and practical, we obtained good results: The BLAST guidelines where the BLAST Actinoalloteichuscyanogriseus 16S rRNA gene sequence/Top 99 16S rRNA sequences showed that the 99 aligned microorganisms had over 95% coverage of the sequence and all microorganisms had an E-value of 0, so we can say that the alignments were significant; however, among the 99 microorganisms obtained by the BLAST, Streptomyces caeruleus was not found, which is the main organism from which Novobiocin is obtained.

In adherence to this, as previously mentioned, the genome of Actinoalloteichuscyanogriseus has not been sequenced, so it was decided to use the 16S ribosomal RNA sequence of the microorganisms in BLAST since these sequences are highly conserved throughout the evolution of these microorganisms, and knowing that it is the NovH protein that is responsible for binding to L-tyrosine in order to conclude with the synthesis of Novobiocin, the protein sequence was entered in Phobius to obtain the graph of the functions attributed to this protein (Figure 11), obtaining that the protein shows transmembrane activity and at the same time has both cytoplasmic and non-cytoplasmic presence, demonstrating what was previously stipulated, that it is a transporter

protein through the membrane to which L-tyrosine is bound. However, its signal peptide function remains low for unknown reasons.

Entering the lifemap website, which allows us to see the phylogenetic routes of microorganisms to perform a general analysis on the evolutionary history of the microorganisms we are studying, we were able to observe the reason why both A. cyanogriseus and S. caeruleus share the same NovH protein and produce Novobiocin is due not only to their E value and percentage of identity in the BLAST performed, but also to the fact that they are closely related in the phylogenetic tree obtained because they belong to the same phylum: Actinobacteria (Figure 12).

On the other hand, the information obtained from BacDive was complemented by analyzing the reports of other researchers who have managed to isolate the bacterium from soil, reporting that the bacterium surprisingly not only produces Novobiocin as a secondary metabolite, but also other antibiotics such as B-rodomycin. The conditions of culture and growth of Actinoalloteichuscyanogriseus were also reported, determining that it is a mesophilic bacterium, which means that its growth occurs at temperatures between 25 and 40°C, which are favorable temperatures since it is a bacterium easy to manipulate in terms of environment and its production can occur more easily without such rigorous environmental controls.

IV. Conclusion

The bacterium Actinoalloteichuscyanogriseus is an affordable candidate to produce the antibiotic Novobiocin due to its important pharmacological properties such as its anticancer property. In addition, its non-rigorous growth conditions make it a good alternative for the pharmacological industry because it has a great similarity with Streptomyces caeruleus as shown by the alignments made with the help of BLAST software and the metabolic pathway by which A. cyanogriseus produces novobiocin has already been reported. The next step would be to conclude with the sequencing of the complete genome of A. cyanogriseus and be able to reach a more accurate conclusion about the functions of the NovH protein and understand the reason why it produces more than one antibiotic, apart from novobiocin.

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