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Chapter 3

Morphological Identification of Actinobacteria

Qinyuan Li, Xiu Chen, Yi Jiang and Chenglin Jiang

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/61461

Abstract

Actinobacteria is a phylum of gram-positive bacteria with high G+C content. Among gram-positive bacteria, actinobacteria exhibit the richest morphological differentiation, which is based on a filamentous degree of organization like filamentous fungi. The actinobacteria morphological characteristics are basic foundation and information of phylogenetic systematics. Classic actinomycetes have well-developed radial mycelium, which can be divided into substrate mycelium and aerial mycelium according to morphology and function. Some actinobacteria can form complicated structures, such as spore, spore chain, sporangia, and sporangiospore. The structure of hyphae and ultrastructure of spore or sporangia can be observed with microscopy. Actinobacteria have different cultural characteristics in various kinds of culture media, which are important in the classification identification, general with spores, aerial hyphae, with or without color and the soluble pigment, different growth condition on various media as the main characteristics. The morphological differentiation of actinobacteria, especially streptomycetes, is controlled by relevant genes. Both morphogenesis and antibiotic production in the streptomycetes are initiated in response to starvation, and these events are coupled.

Keywords: Actinobacteria, Morphology, Morphological characteristics, Cultural characteristics

1. Introduction

The history of the classification of prokaryote clearly demonstrates that changes were caused by the availability of new techniques [1]. The development of prokaryotic classification has experienced different stages: (i) the classical or traditional classification mainly based on

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microbial morphological traits, growth requirements, physiological and biochemical features [2]; (ii) numerical taxonomy analyzing huge volumes of phenotypic data to derive meaningful relationships amongst a large number of microorganisms can be carried out using computer programs [3, 4]; (iii) chemotaxonomic methods studied the chemical variation in actinobacteria and used chemical characters in classification and identification, and it dealt with the discontinuous distribution of specific chemicals, especially amino acids, lipids, sugars, proteins, and other substances in whole cells, parts of cells or fermentation products, and with enzymes [5, 6]; (iv) genotypic classification based on genetic relatedness, inferred mainly from DNA-DNA hybridization (DDH) and comparative sequence analyses of homologous macromolecules, especially, rRNA [7, 8]. In recent years, more and more genotypic approaches were applied on the classification of actinobacteria, such as multilocus sequence analyses (MLSA) [9], average nucleotide identity (ANI) [10, 11], and whole genome analysis [10, 12-14]. Recently, the most widely accepted system is the polyphasic approach [15]. This approach combines as many different data as possible, for instance, phenotypic, chemotaxonomic, genotypic, and phylogenetic information. The modern classification method is an important means to understand the biological origin and species diversity. On one hand, the quantitative determination results are more objective; on the other hand, the research results of polyphasic taxonomy not only enrich the taxonomic content greatly, but also enrich the essence of life phenomenon. But the characterization of a strain is a key element in actinobacteria systematics in any period and prokaryotic morphologies are consistent with their phylogenetic reconstructions [16, 17].

Actinobacteria are currently characterized using the polyphasic approach that brings together a variety of phenotypic, chemotaxonomic, and genotypic data that comprise the formal description of a novel taxon. The key elements that should be acquired and analyzed in characterization studies of prokaryotes were outlined [18]: the phenotypic features are the foundation for description of taxa. Most actinobacteria are characterized and classified on the basis of their morphology in the first place. The morphological characteristics are still one of the most basic indexes which provide in-depth information on a taxon.

2. The basic morphological characteristics of actinobacteria

Actinobacteria display the greatest morphological differentiation among gram-positive bacteria; however, the cell structure of actinobacteria are typical prokaryotes and totally different with fungi. The whole structure of a hyphae cell corresponds to bacterial organization: the cytoplasm contains genomic DNA regions, ribosomes, and various inclusions, presumably reserve substances such as polyphosphates, lipids, or polysaccharides. Classic actinomycetes have well-developed radial mycelium. According to the difference of morphology and function, the mycelia can be divided into substrate mycelium and aerial mycelium (Figure 1). Some actinobacteria can form complicated structures, such as spore, spore chain, sporangia, and sporangiospore. The growth and fracture modes of substrate mycelium, the position of spore, the number of spore, the surface structures of spore, the shape of sporangia, and whether sporangiospore have flagella or not are all important morphological characteristics of actinobacteria classification.

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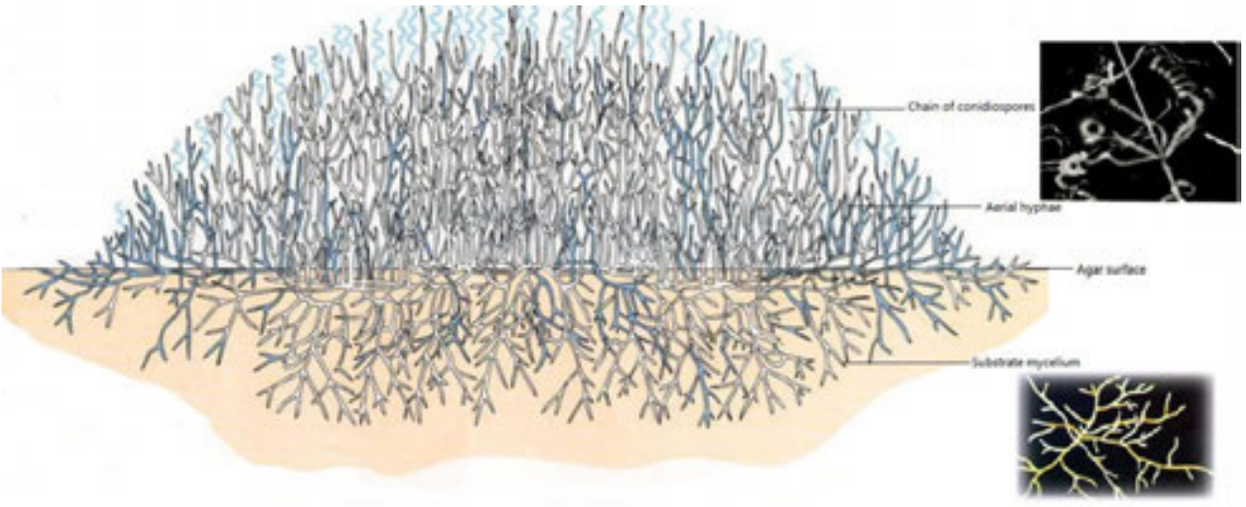

Figure 1. Actinomycetes colony growing on agar (common morphology of actinomycetes, the cross section of an actinomycete colony showing the substrate mycelium and aerial mycelium with chains of conidiospores).

2.1. Substrate mycelium

As known as vegetative mycelium or primary mycelium, the substrate mycelium grows into the medium or on the surface of the culture medium. The main function of the substrate mycelium is the absorption of nutrients for the growth of actinobacteria. Under the microscope, the substrate mycelia are slender, transparent, phase-dark, and more branched than aerial hyphae. The single hyphae is about 0.4 to 1.2 μm thick, usually do not form diaphragms and fracture, capable of developing branches. Minority groups (such as *Nocardia*), rudimentary to extensively branched like the roots, substrate hyphae often fragment in situ or on mechanical disruption into coccoid to rod-shaped, nonmotile elements when grown to a certain stage (Figure 2). The *Actinosynnema* are differentiated into substrate mycelia with long-branching hyphae that penetrate the agar and also grow into and form synnemata (Figure 3). In some genus, the hyphae form sclerotium (Figure 4).

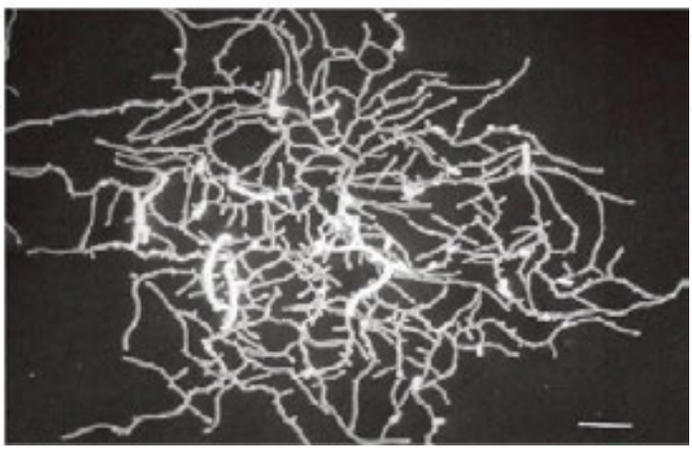

Figure 2. The fragmentation of substrate mycelium and true branching of *Nocardiaasteroides*. (Y. Mikami). [19]

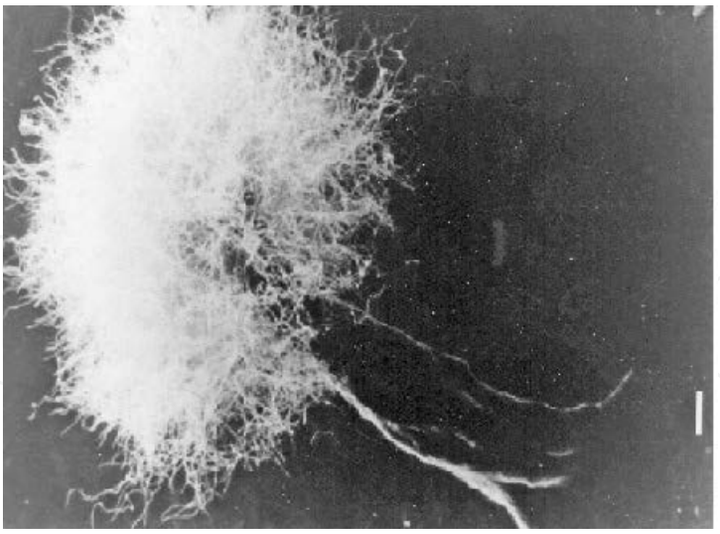

Figure 3. *Actinosynnema mirum* IFO 14064^T (by T. Hasegawa & T. Tamura). Synnemata are formed on medium. [19]

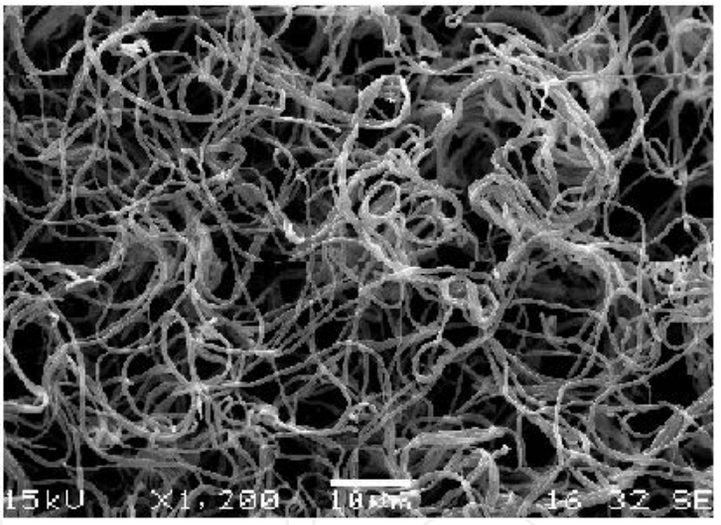

Figure 4. *Streptoalloteichus tenebrarius* NBRC 16177^T (by T. Tamura). [19]

The substrate mycelia are white, yellow, orange, red, green, blue, purple, brown, black, and other colors; some hyphae can produce water-soluble or fat-soluble pigment. The water-soluble pigment can seep into culture medium, which make the medium with the corresponding color. The non-water-soluble (or fat-soluble pigment) make the colony with the corresponding color. The color of the substrate mycelia and whether there are soluble pigments provide important references in the determination of new species.

2.2. Aerial mycelium

Aerial mycelium is the hyphae that the substrate mycelium develops to a certain stage, and grows into the air. Sometimes, aerial hyphae and substrate mycelia are difficult to distinguish.

This is easy to distinguish by an impression preparation on a cover slip, viewed in a dry system with a light microscope: substrate hyphae are slender, transparent, and phase-dark; aerial hyphae are coarse, refractive, and phase-bright. The hyphae of the aerial mycelium are characterized by a fibrous sheath, except the genera *Pseudonocardia* and *Amycolata* [20]. Ultramicroscopic, it is composed of fibrillar elements and short rodlets, forming a characteristic pattern. The fibrous sheath is also present on sporulation aerial hyphae, causing the different surface ornamentations of the spore [21, 22]. Forming all kinds of actinobacteria aerial hyphae is depending upon the species characteristics, nutritional conditions, or environmental factor. The aerial mycelium of some genus develops to a certain stage in the top form spore chain, which is a reproductive hyphae producing spore.

2.3. Spore chain

Actinobacteria grow to a certain stage, differentiated in its aerial hyphae, can form reproductive hyphae called spore-bearing mycelium. Indeed, this type of spore formation occurs in most actinobacteria genera. According to observation [23], spore chains can be divided morphologically respecting their length and number of spore: di- or bisporous with two spores, oligosporous with a few spore, and poly-sporous with many spores. Actinomycete spore chain length, shape, position, color are the important basis for classification.

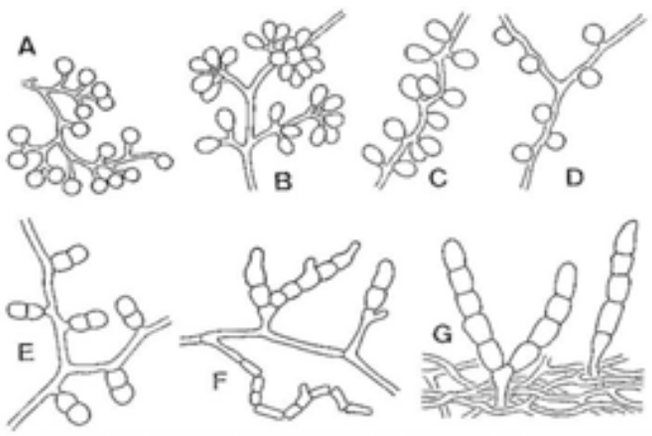

Figure 5. Single spore production and spores in short chains [24] Monosporous: (A) *Micromonospora,* (B) *Thermomonospora*, (C) *Saccharomonospora*, (D) *Thermoactinomyces*. Disporous: (E) *Microbispora.* Oligosporous: (F) *Nocardia brevicatena*, (G) *Catellatospora*.

The monosporous is the mode of single spore production. This form occurs in various suprageneric groups, represented by several well-known genera, such as *Micromonospora, Thermomonospora, Saccharomonospora*, and *Thermoactinomyces* (Figure 5, Figure 6). They all are developed from the blown-out end of a hyphal branch. The disporous chain contains a longitudinal pair of spores. The species of the genus *Microbispora* are representative of this type of sporulation (Figure 5, Figure 6). The spores are arranged either directly on the aerial hyphae or on very short side branches. The spore formation is initiated by lateral budding along an aerial hypha, producing short side branches. Oligosporous actinomy-

cetes develop short spore chains. The majority of the representatives have 7 to 20 spores per chain; at least there are 3 spores (Figure 5, Figure 6). The chains can be straight, hooked with open loops or arranged in irregular spirals having one to four turns. *Nocardia brevicatena* forms short chains of 2 to 7 spores and spore chains may be branched. The substrate mycelia tend to fragment. A reinvestigation of the spore-producing structures has revealed irregularly curled short spore chains in clusters [25].

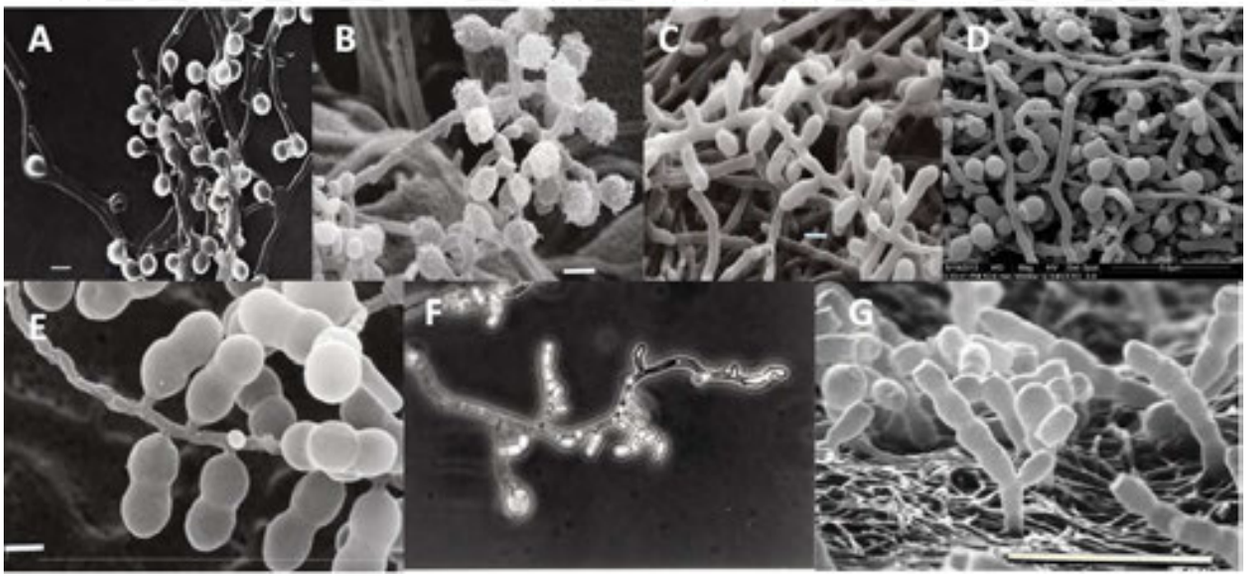

Figure 6. Microgram of single spore production and spores in short chains. [19] *(A) Micromonospora sp.* SF2259^T (by S. Amano, J. Yoshida & T. Shomura) *(B) Thermobifida alba* JCM 3077^T (by M. Hayakawa, H. Iino & H. Nonomura) *(C) Saccharomonospora viridis* IFO 12207^T (by M. Hayakawa, H. Iino & H. Nonomura) *(D) Thermoactinomyces daqus* H-18 (by Su Y. et al.). [26] *(E) Microbispora rosea* JCM 3006^T (by M. Hayakawa, H. Iino & H. Nonomura) *(F) Nocardia brevicatena* A444 (by G. Vobis) *(G) Catellatospora sp*. MB-VE 1321 (by G. Vobis)

The genus *Streptomyces* has classical polysporous, which form long chains frequently having more than 50 spores. The spores of *Streptomyces* and other polysporous actinomycetes are often called arthrospores [27]. The sporulating aerial hyphae of *Streptomyces* can be differentiated into the following main types (Figure 7, Figure 8): (A) *Rectiflexibiles* type, straight or flexuous spore chains, partly in fascicles; (B) *Retinaculiaperti* type, spore chains with hooks, open loops or short, irregular spirals having 1 to 4 turns; (C) *Spira* type, spore chains in spirals demonstrating two different subtypes: (a) Closed, compact spiral and (b) open, loose, and stretched spirals; (D) *Verticillati* type, spore chains formed in whorls and branched in umbels. Another typical genus that forms spores in long chains is *Nocardiopsis*, which has well-developed aerial hyphae, which may either be straight-flexuous or zigzag shaped, fragmenting completely into spores of various lengths [28].

The length, shape, position, and color of actinobacteria pore chain are an important basis for classification. Spore chains of the genus *Streptomyces* have various types of spore-bearing structures: straight, flexous, fascicied, monovericillate (no spirals), open loops (primitive spirals hooks), open spirals, closed spirals, monoverticillate (with spirals), biverticillate (no spirals), biverticillate (with spirals). Mature spores shows a variety of colors such as white, gray, yellow, pink, lavender, blue or green, and so on.

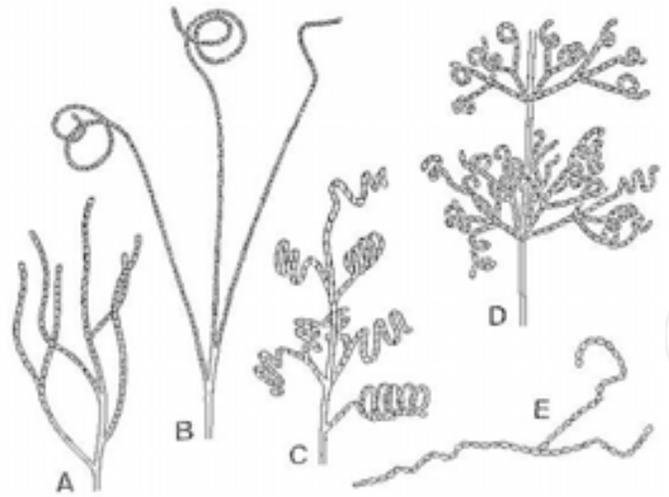

Figure 7. Spore production in long chains. [24] *Streptomyces:* (A) *Rectiflexibiles* type, (B) *Retinaculiaperti* type, (C) *Spira* type, (D) *Verticillati* type (Hütter, 1967). *Nocardiopsis*: (E) fragmenting branched aerial hyphae.

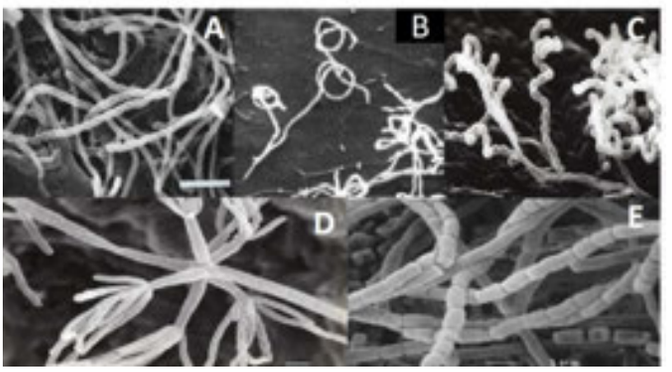

Figure 8. Microgram of spore production in long chain. [19] (A) *Rectiflexibiles* spore chains of *Streptomyces actuosus* U 227 (by T. Mikawa & R. Sashida) (B) Looped (*Retinaculiaperti*) spore chains of *Streptomyces vinaceus*. (C) *Spira* spore chains of *Streptomyces* sp. SF 2587 (by T. Shomura, J. Yoshida & S. Amano) (D) *Verticillati* spore chains of *Streptomyces verticillus* AT 291 (by T. Harada & Masa Hamada) (E) Fragmenting branched aerial hyphae of *Nocardiopsis lucentensis* IFO 15854^T (by Y. Gyobu)

2.4. Spore

The division of a hyphae and the production of a spore start with the formation of a cross-wall. In general, there are three kinds of methods of actinomycetes sporulation process (Figure 9): (i) when substrate hyphae are fragmented, the septum, which is known as a split septum, may occur and form spore, like the genus *micromonospora*. (ii) Spores are formed by septation and disarticulation of pre-existing hyphal elements with a thin fibrous sheath. The spore wall is formed, at least in part, from wall layers of the parent hypha; this is termed as *holothallic development* [29], and was found to be typical for many other spore actinomycetes, like the genus *Streptomyces*. (iii) Globose spores are formed in aerial and substrate mycelium and product spore wall, such as some strains of *Thermoactinomyces*. The spores are classical endospores with all the properties of bacterial endospores, relative to the formation process,

ultrastructure, and physiology. Aside from the mycelial growth, spore formation is the most important morphological criterion that can be used to recognize an actinomycete. Conventionally, the formation of spores is restricted to the morphological group of sporoactinomycetes, where sporulation takes place in well-defined parts of the mycelium. It is known that a number of different genes are involved in spore formation [30, 31] and that different cultivation conditions can have an influence on the spore formation.

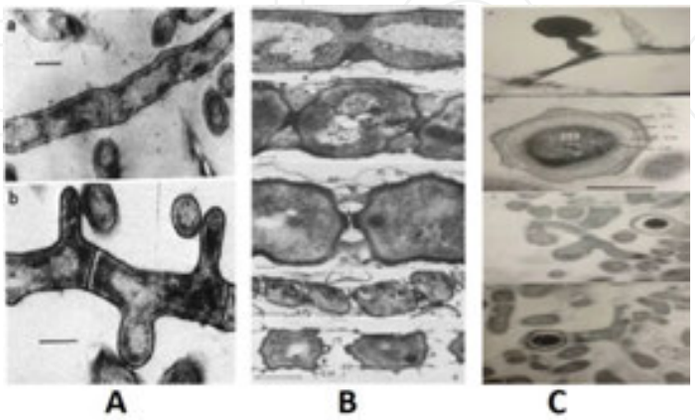

Figure 9. Models of spore formation. (A) Electron micrographs of *M. chalcea* hyphae. Bar, 250 nm. (a) Substrate nonramified hypha showing a vegetative septum. (b) Reproductive hypha showing frequent septa, two straight ramifications which will originate sporophores, a young sessile spore not yet individualized from the hypha, and a small ramification primordium. Substrate mycelium cell wall membrane, forming spores cell wall of substrate mycelia produce diaphragm, form spores [32]. (B) Electron micrographs of sporogenesis *Streptomyces melanochromogenes.* Stage 1: Initiation of septum formation, Stage 2: Septation. Stage 3: Delimination of the spore compartments. Stage 4: Separation and release of the spores. G gap; N nozzle; *rN* reduced nozzle; *Nu* nucleoid; *PL* primary spore wall layer; *SL* secondary spore wall layer; *SM* amorphous septal material; *rSM* remnant of the amorphous septal material; *SS* surface sheath. *Arrows:* initiation points of the septa [33]. (C) Electron micrographs of sporulation *Thermoactinomyces*. (A), the spores are true endospores with all the properties of bacterial endospores. (B), the mature endospore consists of an inner forespore membrane (im), cortex (co), inner spore coat (ic), and an outer spore coat (oc). (C), Spore formation starts with septation and engulfing of a portion of cytoplasm with nuclear material, terminally on short sporophores. (C, D) During the process of maturation, the spore is always surrounded by the mother cell [24].

The characteristics of spores have played a very important role in species descriptions for many years. The spores produced individually or in short chains are in general thicker than the hyphae, while those which are developed in long chains usually have the same diameter as the hyphae. Spores are about 1 to 2 μm thick and vary in term of shape and surface characteristics (Figure 10). Common spore morphology is globose, ovoid, coliform, rod-shaped, allantoid, and reniform. The motile spores are equipped with flagella which provide active movement (Figure 11). In some species, like *Kineococcus radiotolerans* SRS30216^T [34], monotrichous spores possess only one flagellum. As in *Catenuloplanes japonicas*, the spore is said to be peritrichous if numerous flagella are distributed over the whole spore. Polytrichous spores are characterized by a tuft of flagella, which can be inserted in one polar (monopolar polytrichous), as in *Actinoplanes regularis*, subpolarly (*spirillospora*), or laterally (*Pilimelia*). Non-motile spores may be smooth or present a special surface ornamentation. Spore surface ornamentation has also been adopted as a taxonomic character. The ultrastructures of the different types are very well studied in some genus. They can be grouped into several forms: smooth, rugose, warty,

spiny, knobby, verrucose, or irregular (Figure 12). In the genus *Micromonospora*, nonmotile spores are borne singly, sessile, or terminally on short sporophores. Sporophore development is monopodial or in some cases sympodial. Spores are spherical to oval in shape (0.7–1.5 μm) and in most species have blunt spiny projections. The spores are often carried in branched clusters on short hyphae of the substrate mycelium. Additionally, the spores have blunt-spiny surfaces with variable spine sizes; this characteristic is not a diagnostic characteristic for the differentiation of *Micromonospora* species [35] (Figure 13). As the above, spore type, shape, position, spore-bearing arrangement, the number of spores, spores swim or not, spore surface textures are an important basis for classification.

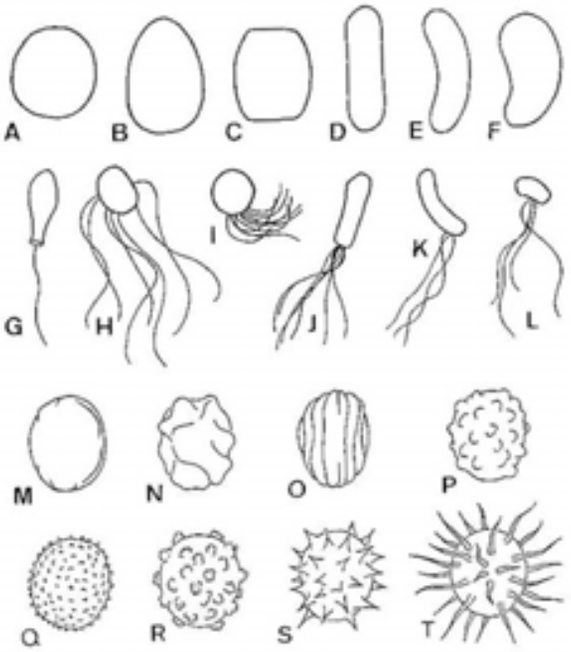

Figure 10. Morphological features of spores. [24] General shape of spores: (A) globose, (B) ovoid, (C) doliform, (D) rod-shaped, (E) allantoid, (F) reniform. Type of flagellation: (G) monopolar monotrichous, (H) peritrichous, (I) polytrichous, (J) monoploarpolytrichous (=lophotrichous), (K) subpolar polytrichous, (L) lateral polytrichous. Surface ornamentation: (M) smooth, (N) irregular rugose, (O) parallel rugose, (P) warty, (Q) verrucose, (S) spiny, (T), hairy.

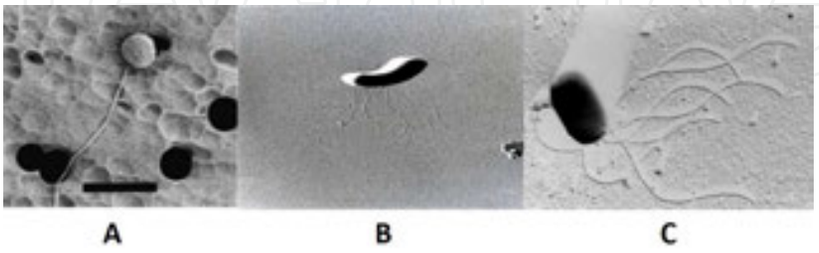

Figure 11. The type of flagellation. (A) Scanning electron micrograph of *Kineococcus radiotolerans* SRS30216^T SEM of a motile cell of strain SRS30216^T exhibiting a single flagellum. Bar, 2 lm. [34] (B) Electron micrograph of *Catenuloplanes japonicus* NBRC 14176^T. Numberous flagella are distributed over the whole spore. (T. Tamura, A. Yokota & T. Hasegawa) [19] (C) *Actinoplanes regularis* A11079. Sporangiospores are motile by a tuft of polar flagella. (N. Muto & K. Ishizawa) [19]

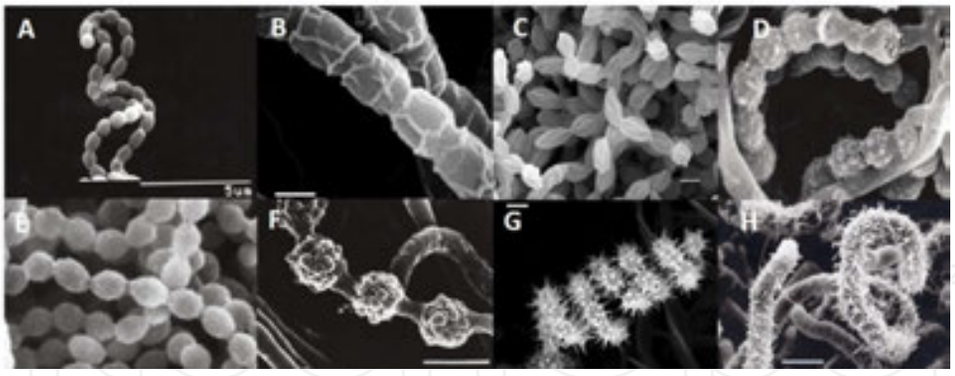

Figure 12. Surface ornamentation of spores. [19] *(A) Streptomyces otagonensis* SANK 62589 (T. Okazaki & R. Enokita). Spiral chains of spores with smooth surfaces are developed. Bar, 5 μm. *(B) Streptomyces* sp. OM-6519 (Y. Takahashi, T. Nakashima & S. Omura). The spore chain is rectiflexibiles section, and the spores have irregular rugose surface. *(C) Actinomadura rugatobispora* AS 6321 (S. Suzuki Spores are oval). Usually two but sometimes three spores per chain. Spore surface rugose with vertical ridges. Bar, 1 μm. *(D) Actinomadura* sp. ATCC 53676 (L.H. Huang, H. Maeda & J. Tone). The strain was characterized by short straight to flexuous spore chains with a warty surface. *(E) Streptomyces routienii* ATCC 39466 (L.H. Huang, H. Maeda & J. Tone). The strain has tubeculate spores that are arranged in a straight to flexuous chains. *(F) Actinomadura verrucosospora* JCM 3147^T (S. Kinoshita, K. Ochiai & K. Ando). Spore chains, in hooks, curves or spirals of one turn, are borne on the aerial hyphae, often as short lateral branches arranged in bundles. Bar, 1 μm. *(G) Streptomyces* sp. WK-1875 (Y. Takahashi, T. Nakashima & S. Omura). The spore chain is Spiral section, and the spores have spiny surface. *(H) Streptomyces finlayi* JCM 4637^T (S. Amano & S. Miyadoh). Spores of the species are oval to ellipsoidal in shape and have a hairy surface. Bar, 1 μm.

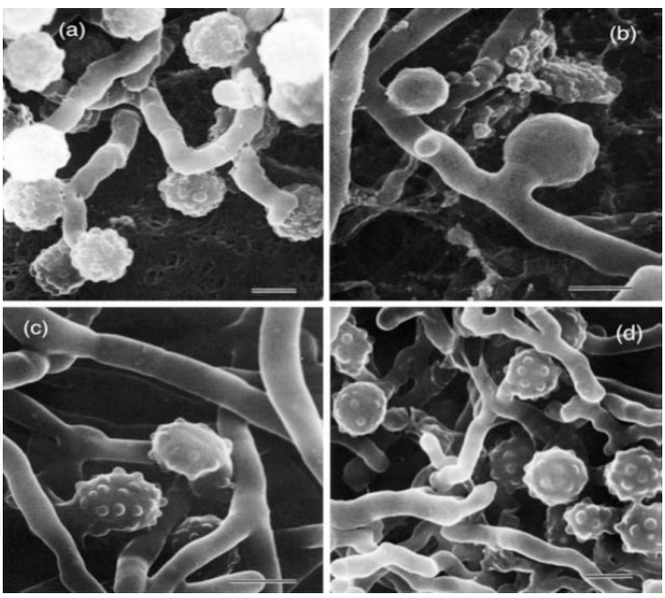

Figure 13. Scanning electron micrographs of: (a) *Micromonospora carbonacea* NRRL 2972^T; (b) *Micromonospora chalcea* ATCC 12452^T; (c) *Micromonospora purpureochromogenes* ATCC 27007^T; and (d) *Micromonospora echinospora* NRRL 2985^T. Bar = 0.5 μm [36].

2.5. Sporangia

Many genera of phylogenetically different groups form spores enclosed in sporangia. The sporangium is a sack-like structure, in which the spores are developed and held together until

they are released, usually leaving an empty sporangial envelope. Sporangia vary considerably both in terms of size and shape. They measure between 2 to 50 μm in diameter with 10 μm being the most common size. They can be cylindrical, clavate, tubular, bottle-shaped, campanulate, digitate, irregular, lobate, umbelliform, pyriform, or globose (Figure 14, Figure 15). The sporangia arise from the substrate hyphae or aerial hyphae. Sporangia formation is largely divided into two forms: in some genera, sporangia are formed by spore filament winding; in some genera, sporangia are expanded by sporangiophores. Sporangia has sporangial envelope, which has no wall called pseudosporangial. The classical internal structure of latter type of sporangium shows coiled or parallel oriented rows of spores, held together by the sporangial envelope, which continues into the outer layer of the sporangiophore. Sporangiospore is formed by differentiation of protoplasm within sporangia. As spores, sporangial types can be classified on the basis of the number of enclosed spores. Sporangia with few spores may be called oligosporous, with the special consideration given to those with one (monosporous) or two spores (bisporous). Sporangia containing numerous spores are called polysporous. Most sporangiate genera produce motile spore, except for the *Stretosporangium* and *Kutzneria*.

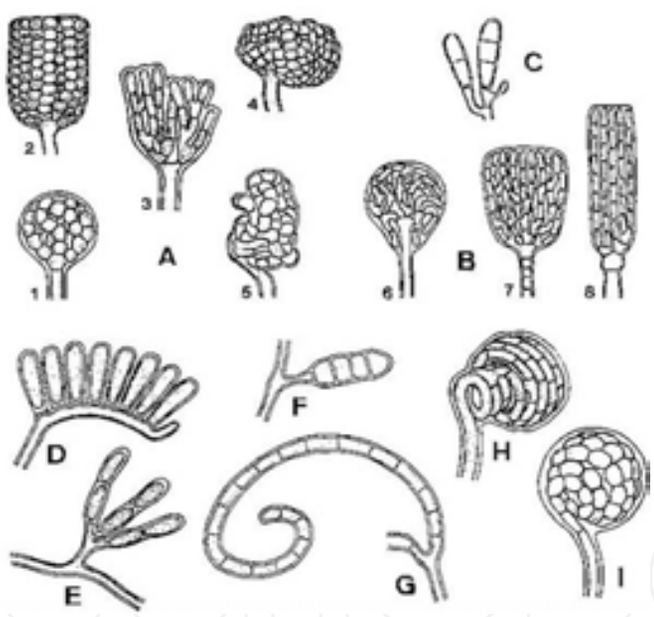

Figure 14. Spore production within sporangia. [24] Sporangia developed on substrate mycelium. (A) *Actinoplanes* (including *Ampullariella*): polysporous, (1) golobose, (2) cylindrical, (3) lobate, (4), subglobose, (5) irregular; (B) *Pilimelia*: (6) ovoid, (7) campanulate, (8) cylindrical; (C) *Dactylosporangium*: oligosporous, claviform. Sporangia developed on aerial mycelium. (D) *Planomonospora*: monosporous, clavate; (E) *Planobispora*: disporous, cylindrical; (F) *Planobetraspora*: tetrasporous, cylindrical; (G) *Planopolyspora*: polysporous, tubular; (H) *Spirillospora*: polysporous, globose; (I) *Streptosporangium*: polysporous, spherical.

In conclusion, sporangia position, sporangia shape, and sporangiospores with or without flagella, are important indications of the genus confirmation, a possible morphological evolutionary series can be observed in the genera with sporangia produced on the aerial mycelium and characterized by a single row of sporangiospores. There is gradation from

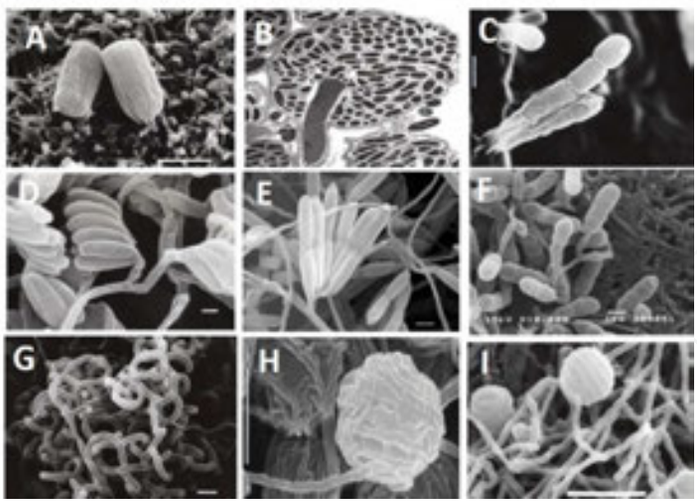

Figure 15. Basic morphological model of Sporangia. [19] *(A) Actinoplanes regularis* SANK 66080^T (Okazaki & R. Enokita). This strain forms cylindrical sporangia on substrate hyphae, and contain motile, rod-shaped sporangiospores. Candiplanecin, a new antifungal antibiotic, is produced. Bar, 10 μm. *(B) Pilimelia columellifera* MB-SK 6^T (G. Vobis). The species is characterized by a columella inside the sporangium, a continuation of the sporangiophore. Bar, 1 μm. *(C) Dactylosporangium fulvum* SF2113^T (T. Shomura). The genus Dactylosporangium is characterized morphologically by the formation of finger-like sporangia containing a single row of two to five zoospores. Bar, 1 μm. *(D) Planomonospora parontospora* ATCC 23863^T (M. Hayakawa, H. Iino & H. Nonomura). A sparsely branched aerial mycelium is formed on which the sessile sporangia, each containing single spores, occur in double parallel row. Bar, 1 μm. *(E) Planobispora rosea* KCC A-0166^T (S. Suzuki). Cluster of sporangia are developed from sporangiophore. Bar, 1 μm. *(F) Planotetraspora silvatica* NBRC 100141^T (T. Tamura). Long, cylindrical sporangia are formed at the ends of short sporangiophores on aerial hyphae, with each sporangium containing four spores in a single row. Bar, 1 μm. *(G) Catenuloplanes japonicus* NBRC 14176^T (T. Tamura, A. Yokota & T. Hasegawa). Pale yellow to tan substrate mycelium is formed. Spores are rod-shaped with smooth surfaces and are flagellated. They are formed by fragmentation of the aerial hyphae. Bar, 2 μm. *(H) Spirillospora albida* ATCC 15331^T (G. Vobis). A mature, spherical sporangium, supported by a laterally inserted sporangiophore, is shown. It has the same dimensions as a common aerial hypha. Bar, 5 μm. *(I) Streptosporangium amethystogenes* IFO 15365 (S. Iinuma, A. Yokota & T. Kanamaru). On the tips of short sporangiophores, which arise from the aerial mycelium, are borne spherical sporangia (5-8 μm) which contain nonmotile spores. Bar, 5 μm.

monosporous, bisporous, tetrasporous, to polysporous sporangia, just like *Planomonospora*, *Planobispora*, *Planotetraspora*, and *Planopolyspora* [37-41].

Some sporulation types are hard to classify according to the traditional scheme of morphological differentiation. These include the genus *Intrasporangium*, *Dactylosporangium*, *Catellatospora*, *Ampullariella*, and *Kibdelosporangium*, and so on. Reasons of forming these structures and phylogenetic relationship need to further explore in work in the future.

2.6. The stability of morphological characteristics

The morphological characteristics of actinobacteria due to gene regulation are generally quite stable, andit is an important basis for classification. The development and formation of some structures, like aerial mycelium, spore, and sporangia, are affected by culture conditions. In some media, strains produce a lot of sporangia or spore, while in other media have little or none. Figure 16 is the diagram of some genera of actinobacteria.

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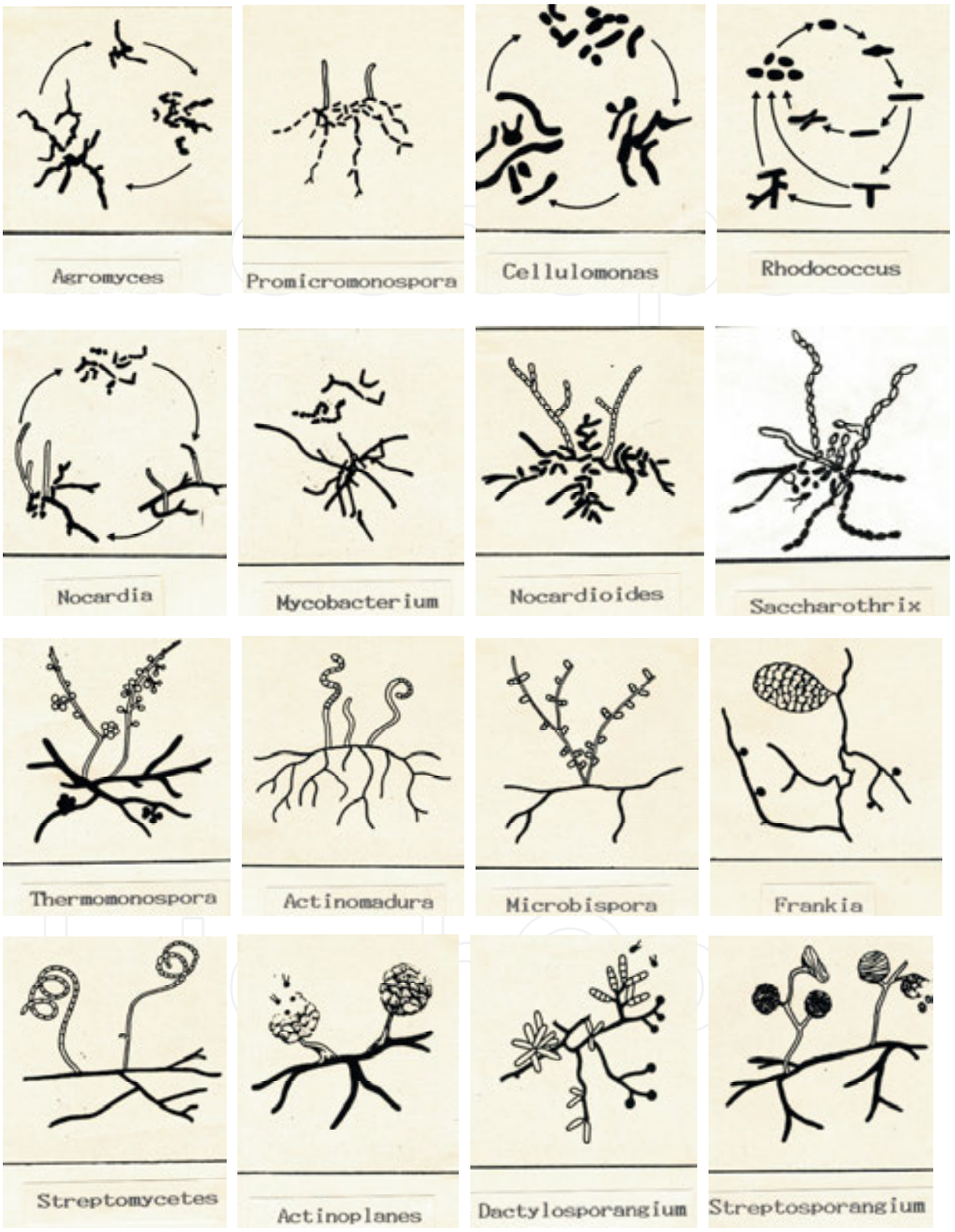

Figure 16. Diagram of some genera of Actinobacteria.

3. Experiment methods of morphological and cultural characteristics

3.1. Cultural characteristics

Cultural characteristics of actinobacteria refer to the growth characteristics and morphology in various kinds of culture media. It is usually determined after incubation for 14 days at 28°C strictly according to methods used in the *International Streptomyces Project* (ISP) [42]. The colors of substrate and aerial mycelia and any soluble pigments produced were determined by comparison with chips from the ISCC-NBS color charts [43].

Classical taxonomy attaches great importance to the role of culture characteristics in the classification identification, general with spores, aerial hyphae, with or without color and the soluble pigment, different growth condition on various media as the main characteristics (Figure 17). The colors of the mature sporulating aerial mycelium are recorded in a simple way (white, grey, red, green, blue, and violet). When the aerial mass color fell between two colors series, both the colors are recorded. If the aerial mass color of a strain to be studied showed intermediate tints, then both the color series are also noted. The media used are yeast extract-malt extract agar and inorganic-salt starch agar. The groupings are made on the production of melanoid pigments (i.e., greenish brown, brownish black, or distinct brown, pigment modified by other colors) on the medium. The strains are grouped as melanoid pigment produced (+) and not produced (–). In a few cases, the productions of melanoid pigments are delayed or weak, and therefore, it is not distinguishable. This is indicated as variable. This test was carried out on the media ISP-1 and ISP-7, as recommended by International *Streptomyces* Project (Table 1). The strains are divided into two groups, according to their ability to produce characteristic pigments on the reverse side of the colony, namely, distinctive (+) and not distinctive or none (–). In case, a color with low chroma such as pale yellow, olive, or yellowish brown occurs, it is included in the latter group (–). The strains are divided into two groups by their ability to produce soluble pigments other than melanin: namely, produced (+) and not produced (–). The color is recorded (red, orange, green, yellow, blue, and violet).

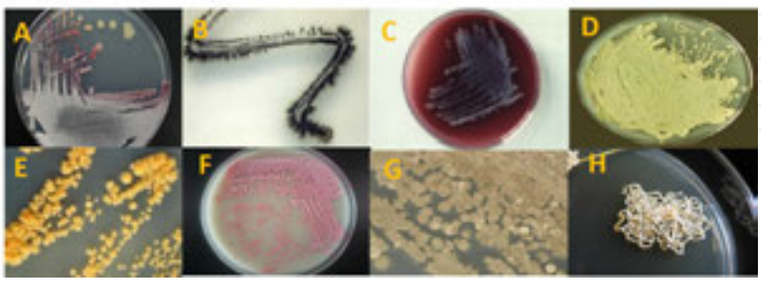

Figure 17. Cultural characteristics of some actinobacteria strains [19]. *(A) Streptomyces violaceoruber* NBRC 12826^T (C. Shibata & H. Komaki). This is the type strain of *Streptomyces violaceoruber* grown on an agar medium. Some morphological differentiation stages such as cream-colored colonies, white aerial mycelium, and red pigment production. *(B) Micromonospora* sp. (S. Mochales). Strains of *Micromonospora* form their spores on the substrate mycelium. These spores accumulate a slimy black mass on the surface of the colonies. *(C) Dactylosporangium vinaceum* SF2127^T (H. Tohyama). Note the production of a wine-red diffusible pigment. *(D) Actinomadura rugatobispora* SF2240 (S. Miyadoh). This species is characterized by its green aerial mycelium bearing longitudinally paired spores, which have rugose surfaces with

vertical ridges. *(E) Actinoplanes* sp. (S. Mochales). This strain forms bottle-shaped sporangia, which are formed directly on the substrate hyphae. *(F) Catenulispora graminis* KACC 15070^T (H.J. Lee & K.S. Whang). Colonies on an oatmeal agar are red. *(G) Kitasatospora arboriphila* NBRC 101834^T (T. Tamura & Y. Ishida). The strain produces a yellowish brown to dark brown or olive substrate mycelium and a grey to dark grey aerial spore mass on agar media. Soluble pigments are not formed. *(H) Nocardia pseudobrasiliensis* IFM 0623 (A. Takahashi-Nakaguchi & T. Gonoi). Colony (stereomicroscope). Colony color; yellow-orange. Colony; coiled pasta-like colony.

Medium	Approximate Formula Per Liter[1]	
ISP Medium 1	Yeast Extract	3.0 g
(Tryptone-yeast extract broth	Tryptone	5.0 g
agar)	pH 7.0 to 7.2	
ISP Medium 2	Yeast Extract	4.0 g
(Yeast extrac-malt extract agar)	Malt Extract	10.0 g
	Dextrose	4.0 g
	pH **7.3**	
ISP Medium 3	Oatmeal	20.0 g
(Oatmeal agar)	Agar	18.0 g
	pH **7.2**	
ISP Medium 4	Soluble Starch	10.0 g
(Inorganic salts-starch agar)	K_2HPO_4	1.0 g
	$MgSO_4 \cdot 7H_zO$	1.0 g
	NaCl	1.0 g
	$(NH_4)_2SO_4$	2.0 g
	$CaCO_3$	2.0 g
	Trace salt solution[2]pH 7.0 to 7.4	1.0 ml
ISP Medium 5	L-asparagine	1.0 g
(Glycerol-asparagine agar)	Glycerol	10.0 g
	K_2HPO_4	1.0 g
	Trace salts solution	1.0 ml
	pH 7.0 to 7.4	
ISP Medium 6	Peptic digest of animal tissue	15.0 g
(Peptone-yeast extract iron agar)	Proteose peptone	5.0 g
	Yeast extract	1.0 g
	$C_{12}H_{22}FeN_3O_{14}$	0.5 g
	K_2HPO_4	1.0 g
	$Na_2S_2O_3$	0.08 g
	pH 7.0 to 7.2	
ISP Medium 7	Glycerol	15.0 g
(Tyrosine Agar)	L-tyrosine	0.5 g
	L- aspar agine	1.0 g
	K_2HPO_4	0.5 g
	$MgSO4 \cdot 7H_2O$	0.5 g

Medium	Approximate Formula Per Liter[1]	
	NaCl	0.5 g
	$FeSO_4 \cdot 7H_20$	0.01 g
	Trace salt s solution	1.0 ml
	pH **7.2-7.4**	

[1]Agar 15-20 g

[2]Trace salt solution: $FeSO_4$ $7H_2O$ 0.1 g, $MnCl_2$ $4H_2O$ 0.1 g, $ZnSO_4$ $7H_2O$ 0.1 g, Distilled water 100. 0 ml.

Table 1. ISP Medium

As the result of cultivation characteristics that are susceptible to cultural conditions (factors such as culture medium, temperature, pH, and light), the influence of culture characteristics was declining in importance. Usually, only use it as one of many indicators of polyphasic taxonomy. And the cultivating characteristic experiment must be in strict accordance with the International *Streptomyces* Project (ISP). If the identified strains have affiliated clearly to a genus, it is necessary to culture strain spawn in similar strains of known bacteria on the culture characteristics of the medium used, observe the characteristics, and contrast.

3.2. Morphological observation

Microscopes are the traditional instruments used for assessing actinobacteria, and they remain as indispensable tools for exploring the morphological, physiological, and genetic diversity present in actinobacteria. Usually, the basic morphology of hyphae and spores is observed by light microscopy, and the microscopic structures of hyphae and spores on the surface are observed by scanning electron microscope (SEM), and the ultramicroscopic structure of the spore flagella and cell is observed by transmission electron microscopes (TEM) (Figure 18).

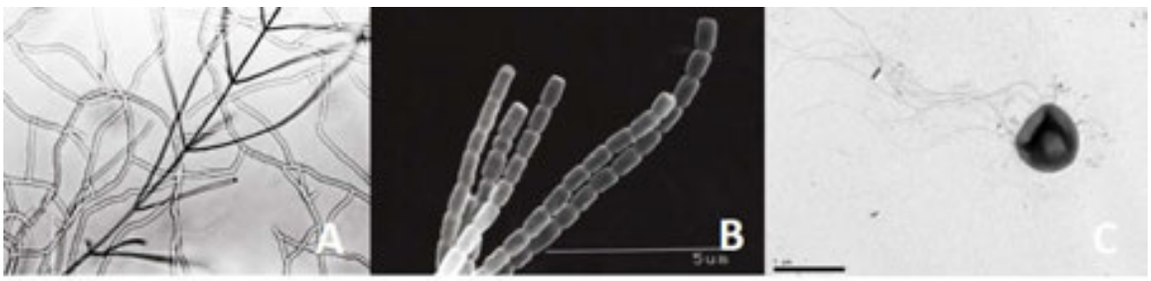

Figure 18. Morphological observation. [19] (A) Light micrograph of *Streptomyces nobilis* SANK 60192^T (Okazaki & R. Enokita). (B) SEM micrograph of *Streptomyces nobilis* SANK 60192^T (Okazaki & R. Enokita), Bar, 5 μm. (C) TEM micrograph of *Kineococcus gynurea* 103943^T (K. Duangmal & A. Matsumoto). Motile cocci with polar flagella. Bar, 1 μm.

Transplantation embedding method is usually used in morphological observation of actinobacteria [16]. The selected appropriate agar flat (2 to 4 media) were dug into 1 cm wide rectangular hole, inoculated at the edge of hole, and then covered with sterile coverslip. The flat is cultivated at proper temperature. The coverslips are taken out at different times (usually

5, 10, 14, and 20 days) and observed using light microscopy. According to the graph of light microscopy, the good area is chosen, which is cut into 1 x 1 cm pieces, sprayed directly on the cover sheet, taken pictures using scanning electron microscopy (Figure 19). In order to prevent shape deformation, fixation is usually performed by incubation in a solution of a buffered chemical fixative, such as glutaraldehyde (2.5%, 1.5 h), sometimes in combination with formaldehyde and other fixatives and optionally followed by post fixation with osmium tetroxide. The fixed tissue is then dehydrated. Because air-drying causes collapse and shrinkage, this is commonly achieved by replacement of water in the cells with organic solvents such as ethanol (respectively 30, 50, 70, 90, 100%, dehydration each 15 min) or acetone, and replacement of these solvents in turn with a transitional fluid such as liquid carbon dioxide by critical point drying. The carbon dioxide is finally removed while in a supercritical state, so that no gas–liquid interface is present within the sample during drying. The dry specimen is usually mounted on a specimen stub using an adhesive such as epoxy resin or electrically conductive double-sided adhesive tape, and sputter-coated with gold or gold/palladium alloy before examination in the microscope.

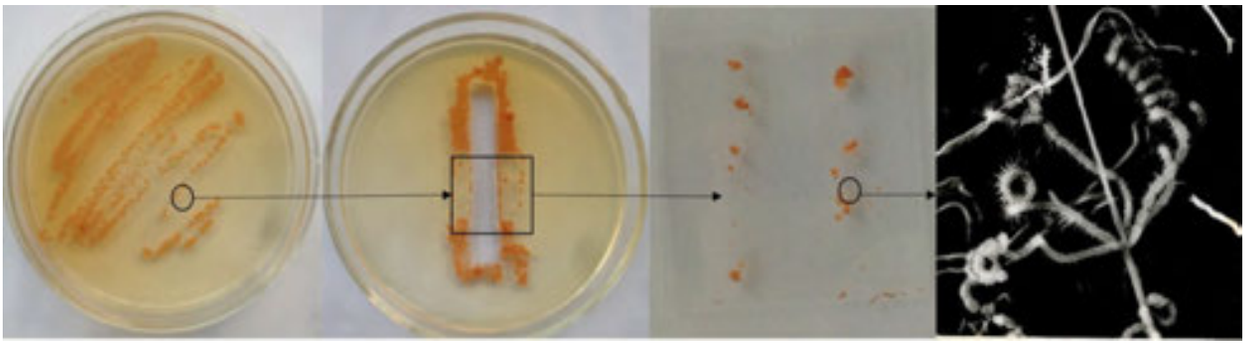

Figure 19. Observation of actinobacteria with transplantation embedding method.

4. The molecular mechanisms of morphological differentiation

Filamentous microorganisms involved two main groups, filamentous fungi and filamentous actinomycetes, particularly the *streptomycetes*. In terms of cellular growth mechanisms, these groups differ greatly. Eukaryotic fungi possess subcellular organelles and cytoskeletal structures directing growth while prokaryotic actinomycetes have no such cellular organization. Despite these fundamental differences, both groups exhibit similar morphologies, growth patterns, growth forms, hyphal and mycelial growth kinetics, spore, sporangia, and conidiospore. The study found that two groups have very similar molecular mechanisms of morphological differentiation [44].

The actinomycetes developmental life cycle is uniquely complex and involves coordinated multicellular development with both physiological and morphological differentiation of several cell types, culminating in the production of secondary metabolites and dispersal of mature spores [45, 46]. *Streptomyces* development has been the subject of intense genetic and

molecular biology research since the isolation of the first mutants specifically blocked in the process [47]. *Streptomyces coelicolor* A3 (2) is the most extensively characterized actinomycete at the genetic level. These have been used to study various aspects of its biology, notably secondary metabolism and its life cycle [48]. Genes required for aerial growth (*bld* genes) are often also needed for secondary metabolism. At least six further genes (*whiA, B, G, H, I, J*) are needed to initiate the subdivision of multigenomic aerial hyphal tips into unigenomic prespore compartments, while several more (including *sigF*, *whiD*, and the *whiE* spore pigment gene cluster) are in spore maturation. As is often the case in cascades of gene expression bacteria, at least two RNA polymerase signal factors (the *whiG* and *sigF* gene products) play specific and crucial roles in sporulation (Figure 20).

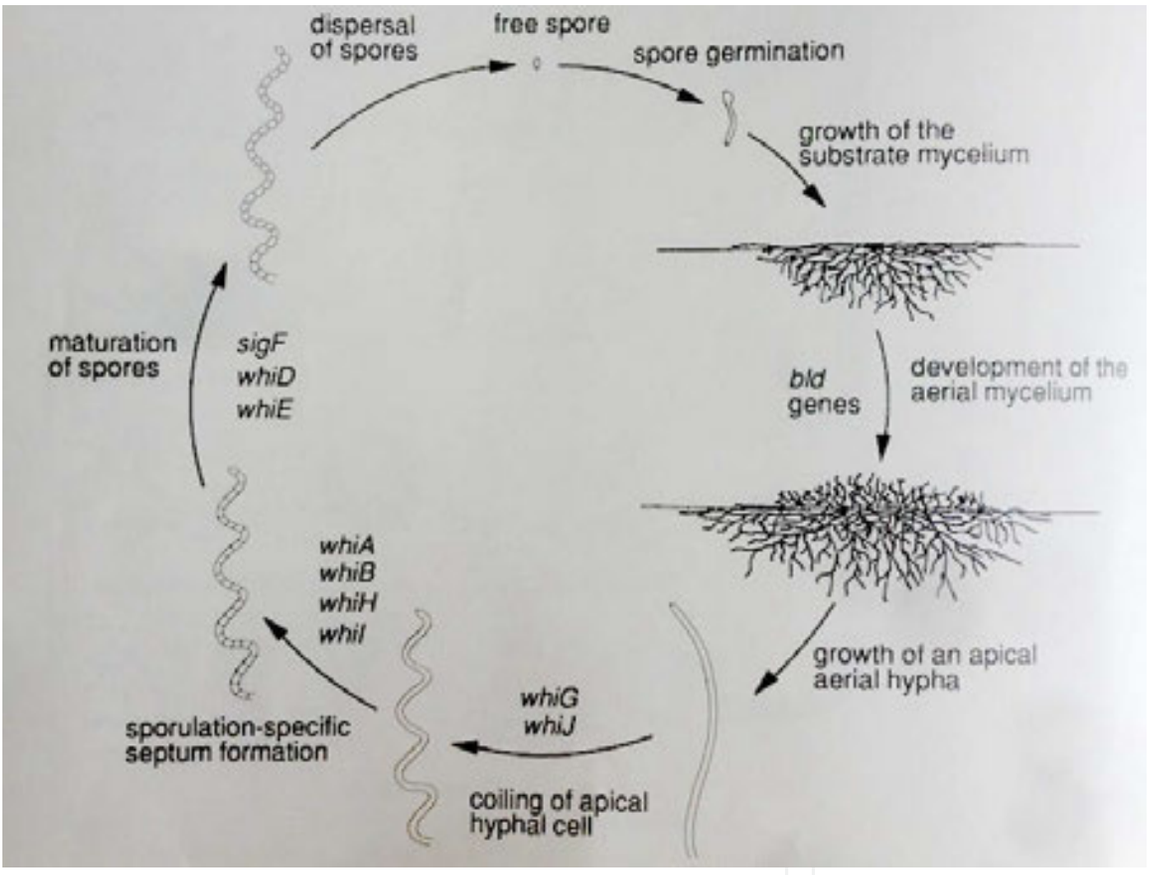

Figure 20. The life cycle of *Streptomyces coelicolor* A3 (2). [24]

Growth of actinomycetes is from the hyphal, which is similar with filamentous fungi [49]. Using the modern fluorescence microscopy, *Streptomyces* apical hyphal growth was observed (Figure 21) [50]. The apical cell is extending its cell wall only at the tip (green). Once this cell has divided by forming a new hyphal cross wall, the subapical daughter cell is unable to grow, and eventually switches its polarity to generate a lateral branch with a new extending tip. A consequence of tip growth is that DNA, which replicates along most of the hyphal length, has to move towards the tip and into new branches - a process we propose to designate nucleoid

migration. For clarity, only a few schematic nucleoids are drawn (red), and they are not meant to reflect the actual number of chromosomes per cell. Furthermore, individual nucleoids are typically not observed in vivo as separated bodies in growing hyphae.

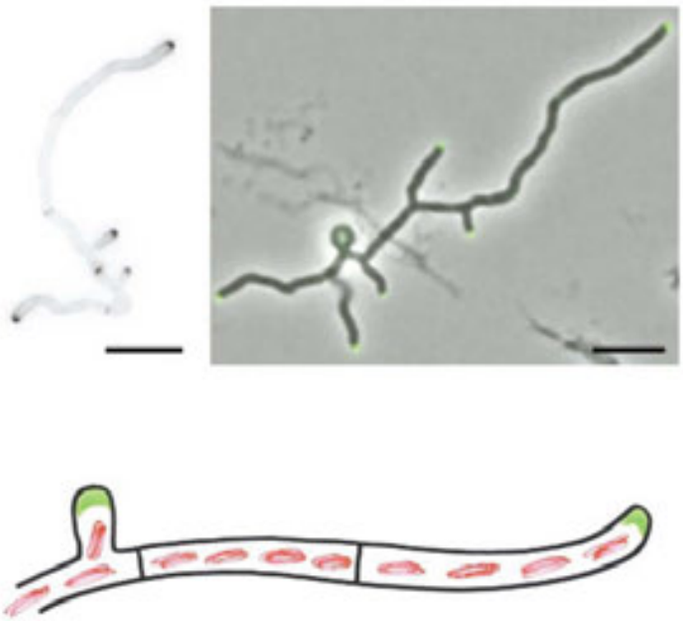

Figure 21. Apical growth in *Streptomyces.* [50]

In general, when nutrients become limiting, a developmental switch occurs during which hyphae start to escape the moist environment and grow into the air. These so-called aerial hyphae can further differentiate into long chains of spores, which can withstand the adverse conditions. Following their dispersal, these spores will reinitiate growth in suitable environments. Some of the key processes involved in the formation of aerial hyphae by streptomycetes and fungi appear to be very similar. Both groups secrete highly surface-active molecules that lower the surface tension of their aqueous environment enabling hyphae to grow into the air. In the case of filamentous actinomyces, small peptides (i.e., SapB and streptofactin) are secreted, while filamentous fungi use proteins known as hydrophobins to decrease the water surface tension. Although these fungal and bacterial molecules are not structurally related, they can, at least partially, functionally substitute for each other (Figure 22) [51]. The *bld* cascade (for bald, meaning unable to form aerial hyphae) controls the checkpoints that (eventually) lead to the onset of aerial growth, resulting in the formation of surface-active molecules that lower the water surface tension and enable hyphae to grow into the air. Moreover, the *bld* cascade seems to potentiate hyphae to undergo full development [52, 53]. Another regulatory pathway is the shy pathway [54], which controls the expression of the chaplin and rodlin genes. These genes encode proteins that assemble into a rodlet layer that provides surface hydrophobicity to aerial hyphae and spores. Both pathways control the production of structural proteins that are involved in the formation of aerial hyphae (Figure 23).

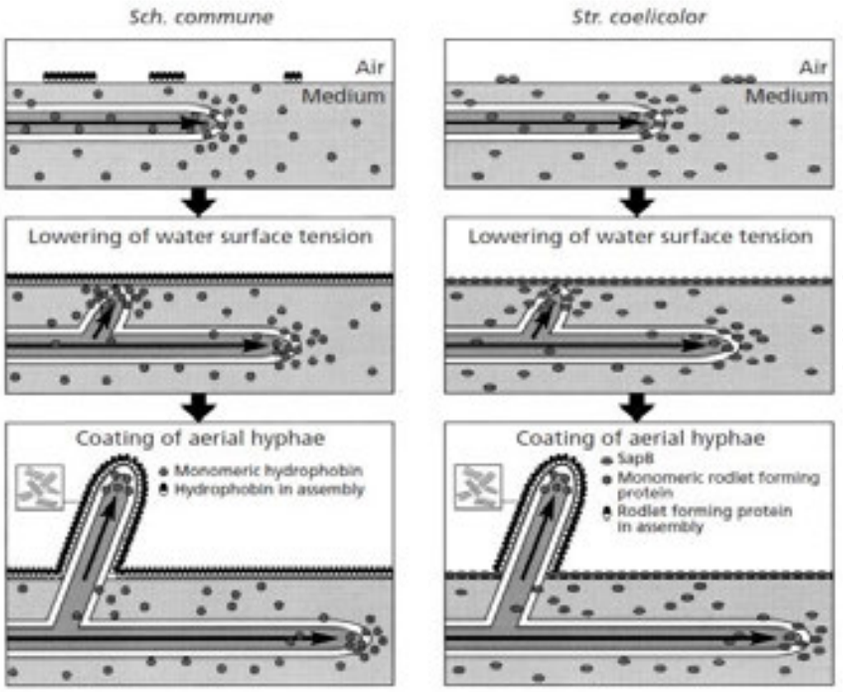

Figure 22. Model for the formation of aerial hyphae in the filamentous fungus *Sch. commune* and the filamentous bacterium *Str. Coelicolor.* [51] After a submerged feeding mycelium has been formed, *Sch. Commune* secretes SC3 into the medium, while *Str. coelicolor* produces SapB. These molecules lower the surface tension of the aqueous environment, enabling hyphae to escape the substrate and to grow into the air. SC3 lowers the surface tension by assembling into an amphipathic membrane at the water–air interface. SC3 secreted by aerial hyphae of *Sch. commune* assembles at the interface between the hydrophilic cell wall and the hydrophobic air exposing its hydrophobic side, which is characterized by a mosaic of rodlets. The hydrophobic surface of aerial hyphae of *Str. coelicolor* is also typified by a rodlet layer. Although the molecules forming this layer have not yet been identified, evidence suggests it is not SapB.

When hyphal growth is limited, much of the biomass becomes converted into spores through the extraordinary parasitic growth of a fluffy white aerial mycelium. The syncytial aerial hyphal tips (which may contain more than 50 copies of the genome) undergo multiple cell divisions to generate a string of unigenomic compartments, destined to become tough, desiccation-resistant spores [55]. Thus, substantial growth is interpolated between the first sporulation related decisions, made in the substrate mycelium, and the decisions involved in the formation and maturation of the spore compartments themselves (Figure 24) [56]. Additionally, the *Streptomyces* spore wall synthesizing complex (SSSC) does not only direct synthesis of the peptidoglycan layer but is also involved in the incorporation of anionic spore wall glycopolymers, which contribute to the resistance of spores. The SSSC also contains eukaryotic type serine/threonine kinases which might control its activity by protein phosphorylation [57]. Genetic analysis of differentiation in *Streptomyces coelicolor* has identified two classes of regulatory mutants, blocked in distinct stages of differentiation. White (*whi*) mutants form aerial hyphae in the normal way, but these hyphae are unable to complete the developmental process to form mature chains of spores [46]. They appear white when grown on solid media because they fail to produce the grey polyketide pigment associated with mature, wild-type spores. *bld* mutants are blocked at an earlier stage of development; they are unable to erect aerial hyphae and therefore appear "bald", lacking the characteristic fuzzy morphology of the wild type.

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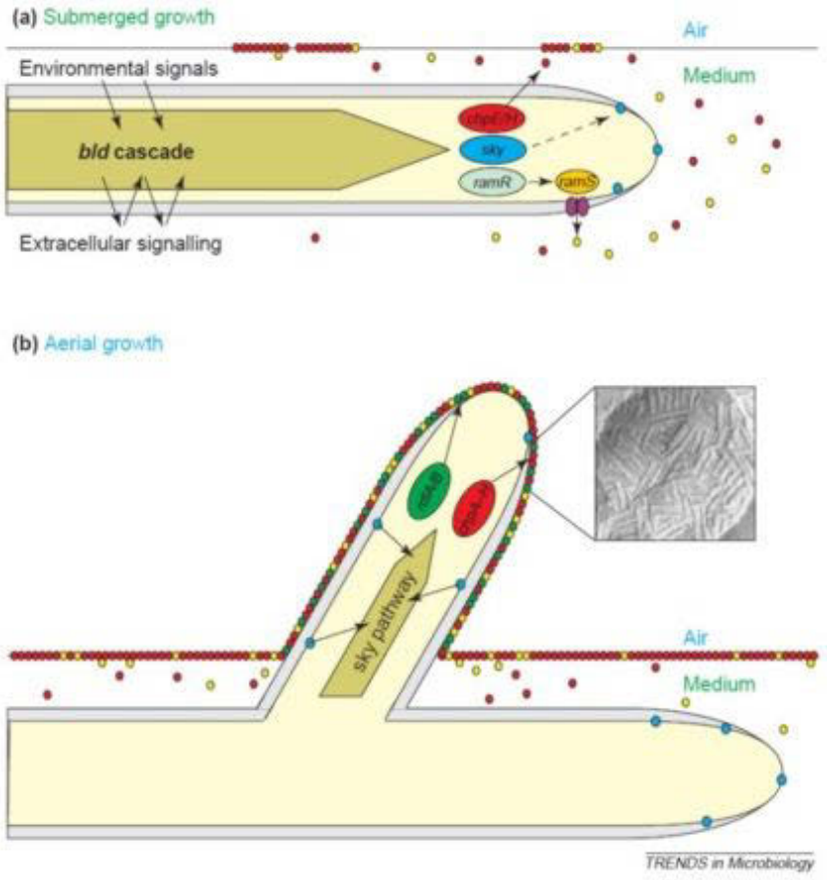

Figure 23. Integrated model for the formation of aerial hyphae in the filamentous bacterium *Streptomyces coelicolor*. (a) Extracellular signaling and environmental signals exert their influence on development through the bld cascade. This cascade induces the formation of RamR, the chaplins ChpE and ChpH (red circles), and components of the sky pathway, such as a sensor of aerial growth (blue circles). RamR activates the synthesis of RamS, which is converted to SapB (yellow circles). This morphogenetic peptide is secreted by the RamAB transporter (purple ovals) and, together with ChpE and ChpH, initiates aerial growth by lowering the water surface tension. (b) From this moment, the sky pathway takes over regulation of development. This pathway would include a sensor of aerial growth (blue circles). As a consequence, the rodlin and chaplin genes, and probably other genes, are activated. Rodlins (green circles) and chaplins (red circles) assemble into a hydrophobic rodlet coat at the outer surface of aerial hyphae. This layer provides surface hydrophobicity and prevents aggregation of aerial hyphae. The insert shows the typical appearance of the rodlet layer, as assessed by scanning electron microscopy. [54]

Morphological differentiation, which coincides with the production of various secondary metabolites, including antibiotics antitumor drugs and enzyme inhibitors, is initiated, when partial nutrient limitation is encountered. Both morphogenesis and antibiotic production in the streptomycetes are initiated in response to starvation. Upon sensing starvation, the substrate mycelia release small molecules that act as signals for the initiation of aerial hyphal growth, as well as for the production of antibiotics. Besides sensing of the nutritional situation, quorum sensing and other environmental stress signals are also involved and controlled by the hierarchical cascade of *bld* and *whi* regulatory genes [58, 59]. Mutants that fail to produce aerial hyphae are, called *bld* mutants, or those that initiate aerial hyphal growth but fail to produce mature spores, are called *whi* mutants. Some studied show that BldD is a key regulator of morphological differentiation and antibiotic production and that it connects the regulons

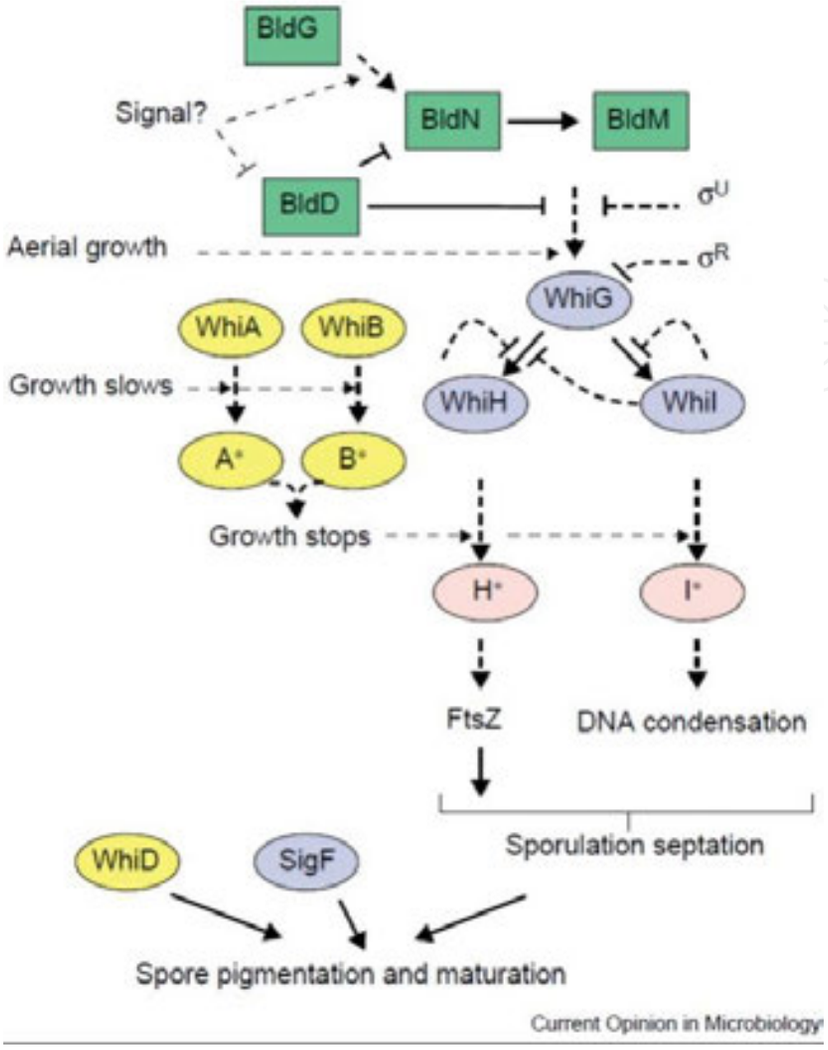

Figure 24. Regulatory and checkpoint network for *Streptomyces coelicolor* sporulation. [56] The diagram is a tentative interpretation of available information about the regulatory connections among *bld* and *whi* gene products and the likely events that influence the transitions to consecutive stages of development. In the dense substrate mycelium preparing for aerial growth, BldG protein is needed for transcription of *bldN*. BldN, a sigma factor, directs the transcription of *bldM*, which encodes a response-regulator-like protein needed for aerial growth. In the growing aerial hyphae, WhiG, a sigma factor, becomes activated to transcribe *whiH* and *whiI*. One factor in this spatially specific expression is BldD, which directly binds to, and represses, the *bldN* and *whiG* promoters in the growing substrate mycelium. Some unknown signal(s) releases this repression in aerial hyphae, and also presumably causes the BldG anti-sigma factor to remove an anti-sigma factor from an unknown sigma factor that is needed for development. The aerial hyphae also contain the WhiA and WhiB proteins, which may sense when aerial growth is slowing down and be converted to modified forms (A* and B*) that coordinate orderly growth cessation. It is postulated that growth cessation gives rise to signals that cause WhiH and WhiI to adopt altered configurations (H* and I*), in which they lose their autorepressor activities and become activators of processes involved in sporulation septation. At this time, late sporulation regulators such as WhiD and SigF activate spore maturation functions. Regulatory steps are indicated by bold lines (solid when well-established, broken when the evidence is more limited), and putative checkpoints are indicated by light dashed lines.

of several other regulators that play pivotal roles in these two central aspects of *Streptomyces* biology [60]. Furthermore, the researcher found the TeRt gene of *Streptomyces coelicolor* SC01135 controls the morphological differentiation and antibiotic synthesis [61].

Benefited from recent advances in determining prokaryotic phylogeny, our understanding of actinobacteria taxonomy is constantly improving. The early assumption that the evolution of actinobacteria went from simple to complex in morphology and that the morphological similarities reflect phylogenetic relationship must have been wrong. It is common to see convergence in morphology between totally different organisms as a result of adoption to environmental factors during evolution. The phylum actinobacteria is a large and ancient group of bacteria with many interesting features. Various members represent a gradient of morphological and developmental complexity, from simple coccoid cells like the *Micrococcus*, and rod-shaped orpleiomorphic organisms like the industrially important *Corynebacterium*, and pathogens like *Mycobacterium tuberculosis*, to the highly complex mycelium of *Streptomyces* and related genera. An informative dimension has now been added by the rapidly growing genome sequence information, which opens fantastic possibilities for comparative and evolutionary studies, both within *Streptomyces* and among the actinobacteria [58, 62].

Acknowledgements

This project was supported by the National Natural Science Foundation of China (No. 31270001, and N0. 31460005), Yunnan Provincial Society Development Project (2014BC006), National Institutes of Health USA (1P 41GM 086184 -01A 1). We are grateful to Ms. Chun-hua Yang and Mr. Yong Li for excellent technical assistance.

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