CHAPTER 2

Literature review

2.1 General characteristics of actinomycetes

Among gram-positive bacteria, actinomycetes exhibit the greatest morphological differentiation, which is based on a filamentous degree of organizaion. These filamentous elements are known as hyphae. The same principle of growth is also known to apply to fungi. However, there is a fundamental difference: actinomycetes are typical prokaryotes while fungi from a separate kingdom within the eukaryotes [Kendrick, 1958]

Colonies of actinomycetes are formed by a mycelium i.e. the mass of hyphae belonging to an organism. The growth of a new colony starts with an inoculum. this may be a single spore, a sporangium, a fragment of a hypha or a small part of an old colony or stock culture. On solid agar media, the inoculum first develops a substrate mycelium, also known as a primary or vegetative mycelium. Vertical growth hyphae then penetrate the substrate and form a secondary mycelium. This called aerial mycelium, which remains in permanent contract with the air. The colonies may raised or flat sometime covered with a leathery layer. Their consistency varies from very soft and pasty to extremely hard. the spectrum of colonies includes white, yellow, orange. rose, red, purple, blue, green, brown and black. The surface can be ridge, smooth, wrinkled, granular or squamous. The colony maybe completely compact or may demonstrate different zone of growth, in concentric rings with radial orientation and frequently a combination of the two. The size of the colonies depend on the species, age, growth conditions, varying from a fraction of a millimeter up to a diameter of centimeter [Vobis, 1997].

Actinomycetes are the Gram-positive bacteria. Most of them are in subclass *Actinobacteridae*, order *Actinomycetales*. All members of this order are characterized in part by high G+C content (>55 mol %) in their DNA [Stackbrandt *et al.*, 1997].

Actinomycetes known as a saprophytic soil inhabitants are the most widely distributed group of microorganisms in nature [Takizawa *et al.*, 1993]. The soil actinomycetes produce a volatile compound called geosmin, which literally translates to “earth smell” [Gust *et al.*, 2003]. This organic compound is responsible for a contributor to the strong odor that occurs in the air when rain falls after a dry spell of weather. In natural habitats, *Streptomyces* are common and are usually a major component of the total actinomycetes population. Some actinomycete genera such as *Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium, Kineospora, Microbispora, Micromonospora, Nonomuraea*, which are often very difficult to isolate and cultivate due to their slowly growth, are called rare actinomycetes [Hayakawa, 2008].

The morphology and arrangement of spores and chemistry (cell wall and whole cell composition, type of lipid, isoprenoid quinones) are particularly important in actinomycetes taxonomy and are used to divide these bacteria into different groups. The 1st edition of Bergey’s Manual divides the actinomycetes into 7 sections, primarily based on cell wall type, conidia arrangement, and the presence or absence of a sporangium [Holt *et al.*, 1994]. The 2nd edition added the use of 16S rRNA sequences to create a single large phylum Actinobacteria. This phylum contains one class, five subclasses, six orders, 14 suborders and 40 families [Williams *et al.*, 1989]. The 2nd edition volume 5 made a great modification on the systematic of Actinomycetes and formally set up the phylum of Actinobacteria, which encompasses 6 classes, 23 orders (include one order incertae sides), 53 families, 222 genera and about 3000 species. The taxonomic catalogue is Bacteria, phylum of Actinobacteria, under the phylum there are class, order, family, genera and species [Ruan, 2013].

2.2 Distribution of actinomycetes in natural habitats

Actinomycetes known as a rich source of prominent natural antibiotics. Until now, ten thousand antibiotics have been found, which almost half of them are produced by *Streptomyces* that originated in the soil. [Lazzarini *et al.*, 2000]. Recently, the rate of discovery of new compounds from existing genera obtained from common soil has decreased therefore it is critical that novel actinomycetes from unexplored habitats such as marine, hot spring be pursued as sources of novel antibiotics and others bioactive compounds. The majorities of actinomycetes are free living and found widely distributed in many natural environments including various soil, freshwater habitat, marine habitat, organic matter habitats and colonizing plants [Miyado, 1997].

2.2.1 Actinomycetes in soils

Five thousand actinomycetes were isolated from soil samples collected from rainforests in Singapore, these confirmed that Terrestrial soils are the good source of actinomycetes. These isolates were determined the generic identities by using a procedure that combined morphological, chemotaxonomic and 16S rDNA sequence-based phylogenetic analyses. The most abundant isolates are members of *Streptomyces.* However, *Micromonospora, Actinoplanes, Actinomadura, Nonomuraea, Nocardia* and *Streptosporangium* were also found. Similar with the results of many other studies, *Streptomyces* are the most abundant and *Micromonospora* isolates were the second [Wang *et al*., 1999].

2.2.2 Endophytic actinomycetes

Some endophytic actinomycetes, are occurring in plant and provide beneficial and/or adverse effects to the host plants. The associations between endophytes and host plants can be formed without harming the plant. Endophytes demonstrated the improvement and encouragement of the growth in host plants as well as the reduction of disease symptoms caused by plant pathogens or various environmental stresses. A variety of actinomycetes inhabit a wide range of plants as symbionts, parasites or saprophytes were reported and most of them belong to the genera, *Streptomyces* and *Microbispora* [Matsumoto *et al*., 1998]. Endophytic actinomycetes have ability in producing a variety of bioactive metabolites including plant growth promoters, plant growth inhibitors, hydrolytic cell wall-degrading enzymes such as cellulases, hemicellulases, chitinases that can apply to agricultural usages and especially antibiotics [Getha and Vikineswary, 2002; Igarashi *et al*., 2002; Taechowisan *et al*., 2003; Hasegawa *et al*., 2006].

2.2.3 Actinomycetes in aquatic environments

Actinomycetes are found in freshwater and marine; aquatic environments habitats [Fenical and Jensen, 2006; Singh *et al*., 2006; Pathom-aree *et al*., 2006]. In aquatic habitats, taxonomically diverse of actinomycetes exhibit unique physiological and structural characteristics. These help them to survive in extremely pressure, salinity and temperature, with the potential production of novel secondary metabolites not observed in actinomycetes, isolated from terrestrial habitats [Radajewski *et al*., 2002]. The dominant actinomycetes isolated from several samples from streams, rivers, lake mud, river sediments, beach sands, sponge and marine sediments are *Micromonospora* [Rifaat, 2003; Jensen *et al*., 2005a,b, Eccleston *et al*., 2008]. Beside, other actinomycetes genera; *Amycolatopsis, Marinophilus, Rhodococcus, Salinispora, Streptomyces* and *Williamsia* were found from aquatic habitats [Mincer *et al*., 2005; Williams *et al*., 2005; Kim *et al*., 2006; Kwon *et al*., 2006; Pathom-aree *et al*., 2006]. Presently, some novel bioactive compounds were discovered from aquatic actinomyctes including rifamycin from *Micromonospora* [Jensen *et al*, 1991], the anticancer metabolite salinosporamide A from a *Salinispora* strain [Fehling *et al*., 2003], marinomycins from *Marinophilus* sp. [Jensen *et al*., 2005b], abyssomicin C from *Verrucosispora* sp. [Riedlinger *et al.*, 2004] and marinopyrroles from *Streptomyces* sp. [Hughes *et al.*, 2008].

2.2.4 Actinomycetes in animal

Ants, honey bees, marine mollusks and marine sponge were reported about the associated actinobacteria [Currie *et al*., 1999; Gilliam, 1997; Romanenko *et al.*, 2008; Jiang *et al*., 2008]. Moreover, the Recent study showed a novel and abundant actinobacteria assemblage in the marine sponge *Rhopaloeides odorabile,* assessed by both a culture-independent molecular approach and a culture-based method [Webster *et al.*, 2001]. In 2006, 106 actinobacteria associated with the marine sponge *Hymeniacidon perleve* collected from Yellow Sea, China were isolated and cultured in eight different media. A phylogenetic analysis using 16S rRNA gene sequences revealed that the isolates belonged to 7 genera of culturable actinobacteria including *Actinoalloteichus, Micromonospora, Norcadia, Nocardiopsis, Psuedonorcadia, Rhodococcus* and *Streptomyces*. The dominant genus was *Streptomyces* which represented 74% of the isolates. Moreover, this study showed the 3 strain identified as candidates for new species. In 2010, the study of Nithyanand *et al.* characterised the bacterial diversity associated with the mucus of the coral *Acropora digitifera* from the gulf of Mannar by 16S rRNA gene clone library construction. Thirty six percentage of this clone library were found to be novel after full length sequencing wherein several clones were found to be the novel at the genus and species level. In addition, Nithyanand *et al.* [2010]. also reported that Actinobacteria amount to a certain propotion among bacterial communities associated with corals.

2.2.5 Actinomycetes associated with algae

Descriptive studies of bacteria isolated from the surface of macroalgae were reported as early as 1875 [Johansen *et al.* 1999]. The interest in bacterial populations living in association with macroalgae has increased during recent decades. They found 107 studies on bacterial communities associated to a total of 148 macroalgae (36 Chlorophytes, 46 Phaeophytes, 55 Rhodophytes, 12 unidentified algae) within the last 40 year [Goecke *et al.* 2010]. Bacterial–macroalgal associations were shown to be widely distributed in marine habitats. The number and complexity of these studies increased significantly during the past decade. This increase can be attributed to the combined use of improved methods in bacterial culture, microscopy and molecular biology (Figure 2.1). However, many questions concerning the occurrence, distribution, persistence and ecological function of the associated bacteria remain unresolved.

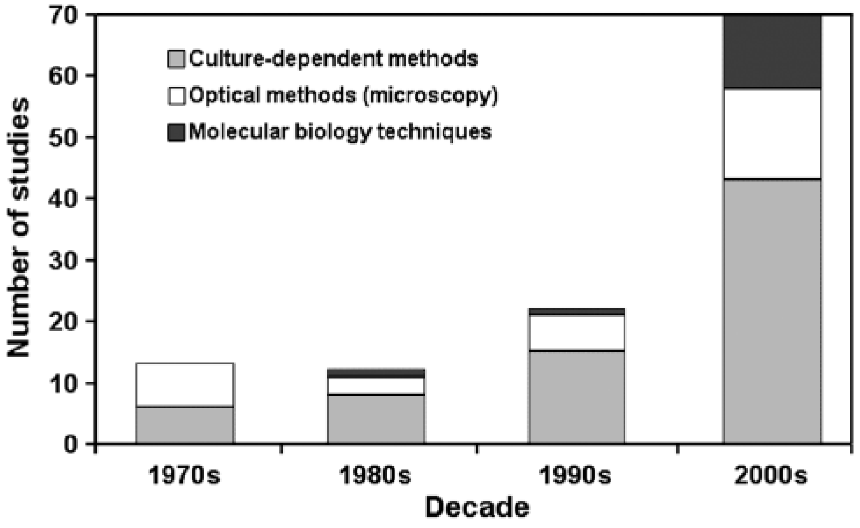


Figure 2.1 Worldwide studies of bacterial communities associated with macroalgae in the last 4 decades, showing the methodology used for the analysis

Although some of the bacterial–algal interactions have been discussed earlier, the ecological relevance of most naturally occurring bacterial communities on macroalgae remains unclear and in most cases the bacterial species involved have not yet been identified [Duan *et al.* 1995, Ivanova *et al.* 2002a]. Antimicrobial activity is widespread among alga associated bacteria. Wiese *et al*. [2009] showed that almost 50% of a total of 210 isolates of the epiphytic bacterial community of *Saccharina latissima* (Baltic Sea, Germany) inhibited the growth of at least one microorganism from a panel covering Gram-negative and Gram-positive bacteria, included 46 isolated of actinobacteria ex. *Streptomyces, Leifsonia, Amycolatopsis, Arthrobacter* and *Micrococcus.* Actinomycetes were also found from edible macroalgae (*Palmaria palmata*). This algae has been traditional been consumed raw from coastal shoreline around Norther Irland. This study showed 3 isolates which were actinomycetes [Moore *et al*., 2002]. From the study of Cláudia *et al*. [2009] showed the communities of associated actinomycetes from marine organism which included algae. 16S rRNA sequencing-based analysis showed that actinobacteria present as 30.4% of the communities.

2.3 Study of edible freshwater macroalgae

2.3.1 Hed Lab (*Nostoc commune*)

In the rainy season, the dark green or brownish gelatinous colonies of macroalgae were found and collected, mainly by local people in northeastern Thailand. They called this macroalga as “Hed Lab” and it has been assigned the generic name *Nostoc* [Mahakhant *et al*., 2006]. *Nostoc* has been considered a food for humanity since ancient times and was widely found in the world [Lazaroff, 1973; Abdelahad and Bazzichelli, 1989; Dodds *et al*., 1995; Lund and Lund, 1995]. “Llullucha”, the common name of *N. commune* in Peru, were consumed locally, traded for maize, or sold, eventually entering the folk markets of Cusco and other neighboring cities [Franquemont *et al.*, 1990; Johnsona *et al*., 2008]. China, Gao and Ai [2004] revealed that *Nostoc* was a source of food found on local menus in China and has been a valued herb for 2000 years. “Koxianmi or Ge-Xian-Mi” is the ancient name of *N. commune* [Potts, 1997; Lembi and Waarland, 1998]. Moreover, some species show the activity in treating cancer and gout. *N. flagelliforme*, referred to as “Facai”, which means ‘fortune’ or ‘to get rich’ was investigated in many studies, for its ecology, distribution and applications [Dai *et al.*, 1991; Gao, 1998; Gao *et al.*, 1998a and 1998b]. In Japan, Takenaka *et al*. [1997] found that *N. flagelliforme* possesses anti-tumor activities. In Australia, *N. flagelliforme* is sold commercially in New South Wales as Black Moss [Lembi and Waaland, 1988].

In Thailand, “Hed Lap” was discovered in the basic soil in the Protected Area of the Dun Lampan Natural Resource, Na Chuak District, Maha Sarakham Province. The rainy season is the only season that presents the optimum conditions for growth and propagation. An analysis of the nutritional values of Hed Lab indicated that Hed Lab naturally contains 20% protein, 0.02% fat and consists of up to 43% dietary fiber [Mahakhant *et al*., 2006].

2.3.2 Lon (*Nostochopsis* spp.)

Nostochopsis was first recorded in 1886 by Bornet and Flahault. It was classified into Phylum Cyanobacteria, Class Cyanophyceae, Subclass Nostocophycideae, Order Nostocales, Family Hapalosiphonaceae. Lon was identified as *N. lobatus* H.C.Wood ex Bornet & Flahault by Thiamdao *et al*. [2011], but Motham *et al*, [2014] identified it as *N. lobata* Wood ex Bornet et Flahault. In 2005, *Nostochopsis* found in the Nan River, Nan Province was described by Peerapornpisal *et al*.; it formed a soft colony and is attached on the surface of rock or cobble in the bed of shallow rivers. The shape of the mucilaginous colony varies from spherical when young, changing to an amorphous shape in the mature stage, and it may be hollow, and torn. The color of the colony varies from dark green or an orange to rusty color.

Lon was found abundantly during the cool dry season and the summer season. It forms the slimy colony on the rocks or cobbles under water. People in this area have used it to prepare local dishes in the style of salads with traditional seasoning. Lon has also been used as a medicinal ingredient, e.g. Lon is used to decrease fever. Some villagers consume Lon to relieve pain from stomach ulcers. This folk-wisdom inspired Peerapornpisal *et al.* [2006] to study the use of nutri-pharmaceuticals to relieve peptic ulcers, dyspepsia, rheumatoid arthritis, and hypertension. From this study, Lon was considered to be of great interest as a potential source of food, as a food supplement and as a pharmaceutical product. Moreover, Peerapornpisal *et al*. [2005] revealed the high content of protein (19.1 g/100g DW) and an especially high content of calcium (12,378.9 mg/100g DW).

In addition, *N. lobatus* was also found in freshwater lakes and slow moving streams of the Indian tropics. It has been utilized by local tribes as a dietary supplement. Recent research studies, conducted in laboratories, have indicated that *N. lobatus* is a rich source of protein, carbohydrates, and fatty acids [Skinner and Entwisle, 2001; Pandey and Pandey, 2008a and 2008b]. *N. lobatus* was found in cosmopolitan areas, and has been reported to have been identified in Africa [Muriel and Lucien, 2004], South America [Rodriguez *et al*., 2006], and North America [Sherwood, 2004].In India, *N. lobatous* was collected from freshwater lakes in Nainital. Sheath and Cole [1996] illustrated the freshwater macroalga, which showed the similar forms and also accept as *N. lobatus,* from Fiji. In Australia, *N. lobatous* was investigated and described, and was found to be present in central-northern New South Wales to north-eastern Queensland. It was found not only attached on rocks in streams, but also spread on moist soil in flowerpots [Day *et al*., 1995; Skinner and Entwisle, 2001].

2.4 Isolation of actinomycetes

2.4.1 Pretreatment for actinomycetes

Physical pretreatment: Drying and storage of soil at various temperature (heating is including moist heat and dry heat) were the favorite methods for decrease unwanted bacteria survivals. Drying soil samples and longtime incubation at 25 - 37°C (mesophiles) or 50 - 60°C (thermophiles) showed the efficiency in reducing high bacteria flora without negative impact of wanted actinomycetes. Moreover, the supernatant from centrifugation of the sample obtained spores of actinomycetes. In addition, other physical pretreatment were using i.e. cesium chloride density-gradient centrifugation, calcium carbonate ($CaCO_3$), sterile membrane filters plate method, etc. However, sediment or soil contains the plenty of fungi and other bacteria, thus antibiotics were added for conventional isolation [Starr *et al.*, 1981; Labeda, 1990;

Holt *et al.*, 1994; Joseph *et al.*, 2003; Kroppenstedt and Goodfellow, 2006; Hayakawa, 2008].

Chemical pretreatment: Phenol were used in the treatment method of soil slurries and marine sediments to eliminate unwanted microorganism, later of soil or marine sediment must be sufficient diluted out the toxic effects of the phenol. Furthermore, quaternary ammonium compound, sodium hypochlorite solution or osmium tetroxide were used in routine chemical treatment for isolation of actinomycetes [Starr *et al*., 1981; Labeda, 1990; Holt *et al*., 1994; Hayakawa, 2008].

Combination physical and chemical pretreatment: These combination were widely used for actinomycetes isolation due to the high efficiency and specific more than using a single method either physical or chemical treatment such as combining heat with a chemical treatment or drying room temperature with phenol treatment [Starr *et al*., 1981; Labeda, 1990; Holt *et al*., 1994; Hayakawa, 2008].

2.4.2 Isolation media of actinomycetes

Numerous media have been described for the isolation of actinomycetes from soil and other national material. Some of these isolation media are rather lean since actinomycetes have the ability to survive and grow to some extent on very small amounts of nutrients that they scavenge from non-nutrient substance such as purified agar. Several isolation media have high carbon-to-nitrogen ratios and contain resistant, complex carbon and nitrogen sources such as starch, casein, chitin, humic. These media greatly reduces the number of bacteria present on isolation plates, because bacteria grow better on media with low carbon-to-nitrogen ratios [Gray and Willams, 1971].

Some isolation media were designed for reducing the other competing soil microoganisms without adversely affect to actinomycete. Humic acid-vitamin (HV) agar was formulated by Hayakawa and Nonomura [1987]. This medium contains soil humic acid as the sole carbon and nitrogen source. HV agar was useful for recovery and adequate growth of *Streptomyces* and various rare actinomycetes, while restricting growth of non-filamentous bacteria colonies. For eliminate bacterial and fungal contaminants, HV agar was supplemented with synthetic antibacterial agents such as nalidixic acid and cycloheximide [Hayakawa *et al*., 1996]. Reducing the nutrient to non-nutrient is a great way to reduce unwanted bacteria. The Minimal media, formulated by Hozzein *et al*. [2008] aimed to isolate *Actinomadara* and *Amycolatopsis* from the specimens. Another way in inducement the growth of actinomycetes on the media is providing high carbon-to-nitrogen ratios or complex carbon/nitrogen sources. Gause No.1 and Starch-casein agar consists of soluble starch upto 20 and 10 g/L respectively. Colloidal shitin agar was described by Hsu and Lockwood (1975) as an excellent for the selective isolation of actinomycetes. This medim composes of complex carbon-source, colloidal chitin.

Many studies revealed about the discovering the genera of actinomycetes from selective media. *Dermacococcus* and *Streptomyces* were isolates on Raffinose-Histidine medium. Actinomodura, Amycolatopsis and Streptomyces were isolated on Gause No.2 [Tan, 2006]. *Nocardia acidivorans* sp. nov. was isolated from soil of the island of Stromboli. this new species report, mannitol-rifampicin agar was used as selective media and gave many isolates of genus *Norcardia* [Kämpfer *et al*., 2007]. *Dermacoccus abyssi* sp. nov., a piezotolerant actinomycete isolated from the Mariana Trench was isolated on Proline Tap water Agar [Pathom-aree *et al*., 2012]. *Norcadia polyresistens* sp. nov. was isolated from a soil collected from Yunnn, China by using water/proline agar (Xu, 2005). In addition, LSV-SE agar containing a Kraft lignin as the carbon and nitrogen sources was formulated [Hayakawa *et al*., 1996] to facilitate an increased recovery of *Microtetraspora* spp. and minimal medium (MM) agar containing glucose, yeast extract and mineral salts was formulated to increase recovery of *Actinomadura, Amycolatopsis* from desert soil [Hozzein *et al*., 2008].

The use of antibiotics to select for or against desired microorganisms has become one of the most important selective technique for the isolation of diferent actinomycete genera. The antifungal antibiotics cyclohexamide andnystatin can be routinely incorporated into actinomycete isolation a gar approximately 50 µg/ml each to eleminate any fungal colonies [Porter *et al*., 1960]. Moreover, to recover some rare genera of actinomycetes, some antibiotics and some chemicals can be added into HV medium. the data was shown in Table 2.1

Table 2.1 Variation of antibiotics and some chemicals for soil actinomycetes isolation [Takahashi and Omura, 2003; Shearer, 1987; Ivanitskaya et al. 1974; Chormonova, 1978; Barton and Hughes, 1981; Shearer, 1978; Cross, 1968; Willams and Davies, 1965; Lavrova, 1971;Nonomura and Hayakawa,1988]

Addition of antibiotics and some chemicals	**Genera selected**
Antibiotics: Chlortetracycline	*Nocardia*
Gentamicin (2-5 µg/ml)	*Streptosporangium and Actinomadura*
Gentamicin (10 µg/ml)	*Micromonospora*
Kanamycin (25 µg/ml, 25°C)	*Actinomadura*
Macrolide or aminoglycoside	Macrolide or aminoglycoside producers
Nalidixic acid (20 µg/ml), prnicillin G (10 U/ml) and potassium tellurite (0.005%)	*Rhodococcus*
Novobiocin	*Actinoplanes, Kitasatospora*
Novobiocin (10-15 µg/ml)	*Actinoplanes*
Novobiocin (100 µg/ml)	*Thermoactinomyces*
Novobiocin (25 µg/ml) and Streptomycin (25 µg/ml)	*Glycomyces*
Penicillin G (5-10 µg/ml) and nalidixic acid (15 µg/ml)	*Saccharothrix*
Penicillin G (1 µg/ml) and polymycin B (5 µg/ml)	*Actinomycetes*
Potassium Dichromate (50 µg/ml) and nalidixic acid (15 µg/ml)	*Actinomycetes*
Rifamycin (25 µg/ml)	*Actinomadura*
Streptomycin (25 µg/ml)	*Actinomadura*
Tetracyclines	*Norcardia*
Tunicamycin	*Micromonospora*

Table 2.1 (continued)

Addition of antibiotics and some chemicals	Genera selected
Tunicamycin (20 µg/ml) and nalidixic acid (30 µg/ml)	*Micromonospora*
Vancomycin (1-10 µg/ml) and Polymycin B (5U/ml)	*Amycolatopsis*
Chemicals: Humic acid	Rare actinomycetes
Proline	Rare actinomycetes
Gellan gum	*Actinobispora*

2.5 Identification and classification of actinomycetes

In the genus level, chemotaxonomic characterization, morphological observation and molecular techniques have been used for actinomycetes identification and classification. The study of chemical variation in cell compositions in microorganisms is called Chemotaxonomy. the presence of diaminopimelic acid isomers (DAP) and sugar composition in the whole-cell hydrolysates are the quickest methods for preliminary identification of actinomycetes to genus level [Hesagawa *et al*., 1983]. DAP isomers is one of most important cell-wall properties of actinomycetes and gram-positive bacteria. These amino acids mostly located in the peptidoglycan of bacterial wall envelope are generally contained as one of the isomers, LL-form or *meso*-form. Cells of the actinomycetes contain glucosamine and muramic acid, the monomers of peptidoglycan. Most components of the cell wall and whole-cell of actinomycetes are shown in Table 2.2

The value of mol% G+C content has been used to classify group of actinomycetes. The range of DNA base compositions of most actinomycetes is 63-78 mol% G+C [Madigan and Martinko, 2006]. Moreover, compositions of phospholipids, fatty acids and menaquinones in the cell have also been used [Boone and Pine, 1968; Lechevalier and Lechevalier, 1980]. The 5 types of phospholipids composition in the actinomycetes was classified in Table 2.3.

Table 2.2 Type of cell wall diaminopimelic acid isomers (DAP) and whole-cell sugars of actinomycetes [Lechevalier and Lechevalier, 1970]

	Cell-wall types			
	I	**II**	**III**	**IV***
DAP isomers	LL	meso	meso	meso
Glycine	+	+	-	-
	Whole-cell sugars pattern (WCSP)			
	A	**B**	**C**	**D**
Arabinose	+	-	-	+
Galactose	+	-	-	-
Xylose	-	-	-	+
Madurose	-	+	-	-

*Type IV was differentiated from type III by the presence of arabinose and galactose in the whole cell hydrolysates.

Table 2.3 Type of phospholipids of actinomycetes [Lechevalier, 1977]

Type	**PIM**	**PI**	**PC**	**PG**	**PE**	**PME**	**GluNU**	**APG**	**DPG**
I	+	+	-*	V	-*	-	-*	V	V
II	+	+	-*	V	+*	-	-*	V	+
III	V	+	+*	V	V*	+	-*	V	V
IV	?	+	-*	-*	V*	V	+*	-	+
V	?	+	-*	-*	V*	-	+*	V	+

* = diagnostic phospholipids

PIM = phosphatidylinositolmannosides

PI = phosphatidylinositol

PC = phosphatidylcholin

PG = phosphatidylglycerol

PE = phosphatidylethanolamine

PME = phosphatidylmethylethanolamine

GluNU= unknown glucosamine-containing phospholipids

APG = acyl phosphatidylglycerol

DPG = diphosphatidylglycerol

Morphology plays as the important characteristic to identify actinomycetes isolates and it was used in the first descriptive characters, especially for *Streptomyces* species. The germination of spores, elongation and branching of vegetative mycelium, formation of aerial mycelium (Figure 2.2), color of aerial and substrate mycelium and pigment production have been used to identify actinomycetes [Holt *et al.,* 1994]. Formation of aerial mycelium, substrate mycelium and spores would be studied by light microscopy, while the spore surface and spore structure would be observed by scanning electron microcopy. Table 2.4 shows the distribution of genera of the actinomycetes by cell wall type, including a description of morphology [Labeda, 1986].

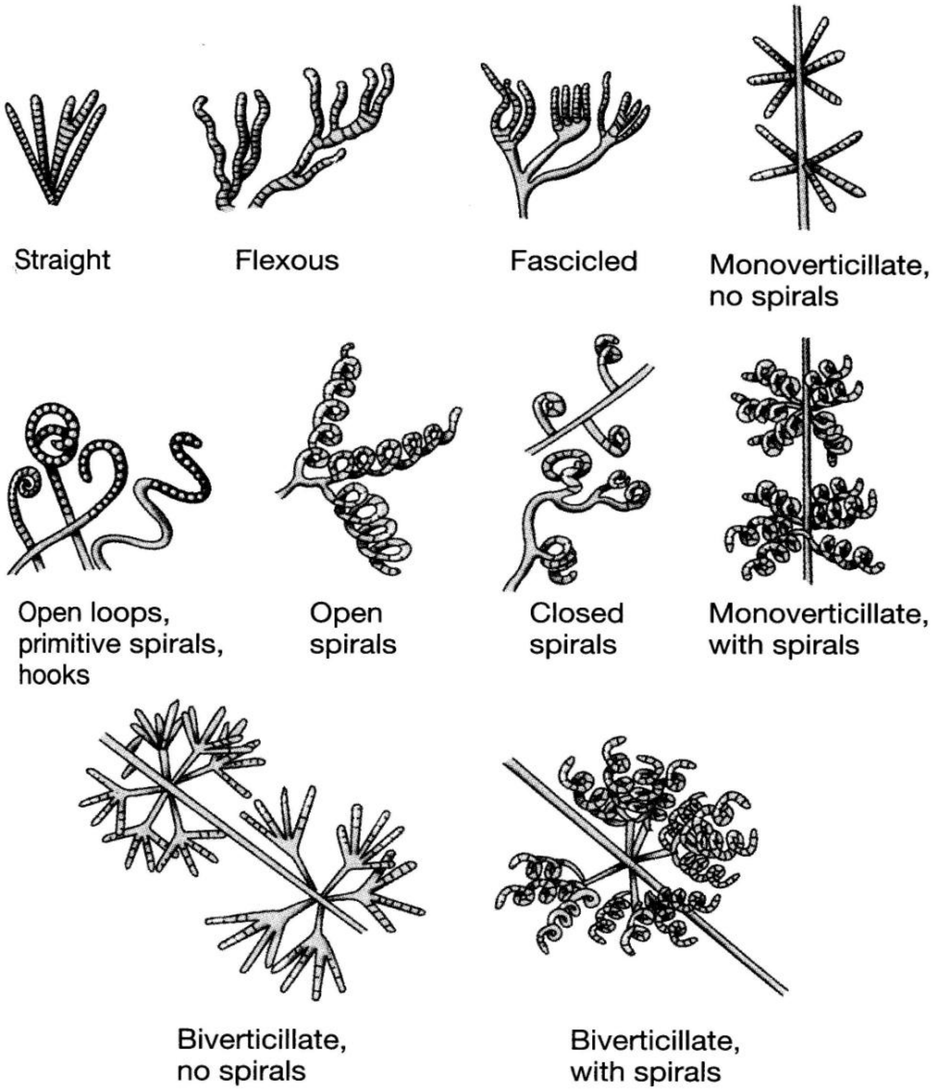
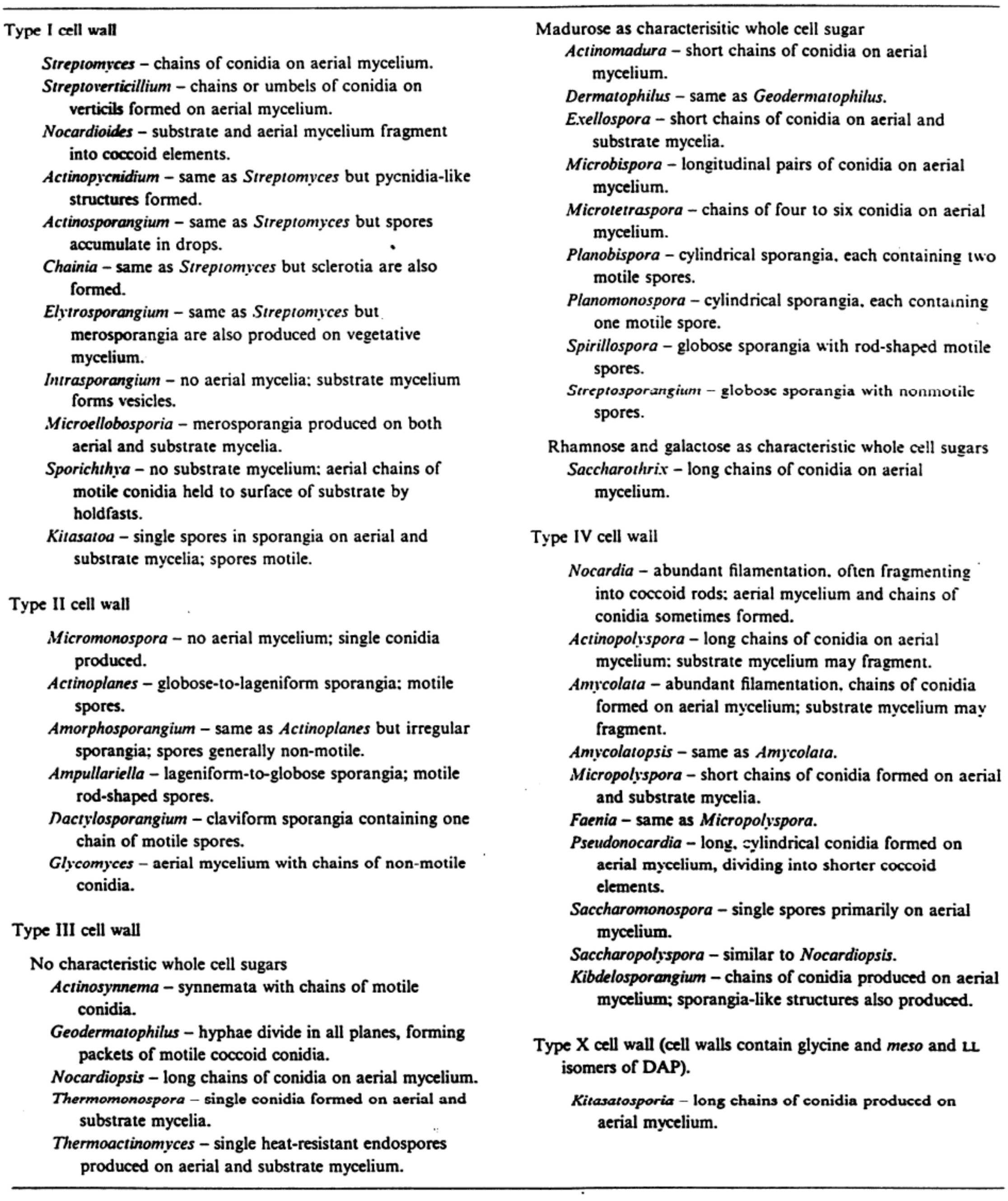


Figure 2.2 Various type of spore-bearing structure in streptomycetes [Madigan and Martinko, 2006]

Table 2.4 Distribution of genera of the actinomycetes by cell wall type, including a description of morphology [Labeda, 1986]



Type I cell wall

Streptomyces – chains of conidia on aerial mycelium.
Streptoverticillium – chains or umbels of conidia on verticils formed on aerial mycelium.
Nocardioides – substrate and aerial mycelium fragment into coccoid elements.
Actinopycnidium – same as *Streptomyces* but pycnidia-like structures formed.
Actinosporangium – same as *Streptomyces* but spores accumulate in drops.
Chainia – same as *Streptomyces* but sclerotia are also formed.
Elytrosporangium – same as *Streptomyces* but merosporangia are also produced on vegetative mycelium.
Intrasporangium – no aerial mycelia; substrate mycelium forms vesicles.
Microellobosporia – merosporangia produced on both aerial and substrate mycelia.
Sporichthya – no substrate mycelium; aerial chains of motile conidia held to surface of substrate by holdfasts.
Kitasatoa – single spores in sporangia on aerial and substrate mycelia; spores motile.

Type II cell wall

Micromonospora – no aerial mycelium; single conidia produced.
Actinoplanes – globose-to-lageniform sporangia; motile spores.
Amorphosporangium – same as *Actinoplanes* but irregular sporangia; spores generally non-motile.
Ampullariella – lageniform-to-globose sporangia; motile rod-shaped spores.
Dactylosporangium – claviform sporangia containing one chain of motile spores.
Glycomyces – aerial mycelium with chains of non-motile conidia.

Type III cell wall

No characteristic whole cell sugars
Actinosynnema – synnemata with chains of motile conidia.
Geodermatophilus – hyphae divide in all planes, forming packets of motile coccoid conidia.
Nocardiopsis – long chains of conidia on aerial mycelium.
Thermomonospora – single conidia formed on aerial and substrate mycelia.
Thermoactinomyces – single heat-resistant endospores produced on aerial and substrate mycelium.

Madurose as characterisitic whole cell sugar
Actinomadura – short chains of conidia on aerial mycelium.
Dermatophilus – same as *Geodermatophilus*.
Exellospora – short chains of conidia on aerial and substrate mycelia.
Microbispora – longitudinal pairs of conidia on aerial mycelium.
Microtetraspora – chains of four to six conidia on aerial mycelium.
Planobispora – cylindrical sporangia, each containing two motile spores.
Planomonospora – cylindrical sporangia, each containing one motile spore.
Spirillospora – globose sporangia with rod-shaped motile spores.
Streptosporangium – globose sporangia with nonmotile spores.

Rhamnose and galactose as characteristic whole cell sugars
Saccharothrix – long chains of conidia on aerial mycelium.

Type IV cell wall

Nocardia – abundant filamentation, often fragmenting into coccoid rods; aerial mycelium and chains of conidia sometimes formed.
Actinopolyspora – long chains of conidia on aerial mycelium; substrate mycelium may fragment.
Amycolata – abundant filamentation, chains of conidia formed on aerial mycelium; substrate mycelium may fragment.
Amycolatopsis – same as *Amycolata*.
Micropolyspora – short chains of conidia formed on aerial and substrate mycelia.
Faenia – same as *Micropolyspora*.
Pseudonocardia – long, cylindrical conidia formed on aerial mycelium, dividing into shorter coccoid elements.
Saccharomonospora – single spores primarily on aerial mycelium.
Saccharopolyspora – similar to *Nocardiopsis*.
Kibdelosporangium – chains of conidia produced on aerial mycelium; sporangia-like structures also produced.

Type X cell wall (cell walls contain glycine and *meso* and LL isomers of DAP).

Kitasatosporia – long chains of conidia produced on aerial mycelium.

At present, the molecular studies based on 16S rDNA sequences are used for identification which are the most significance for actinomycetes identification [Yokota, 1997]. The phylogenetic tree constructed from 16S rDNA sequences allows the investigation of actinomycetes evolution. Actinomycetes are separated into over 100 genera based on the 16S rRNA gene sequencing (Figure 2.3).

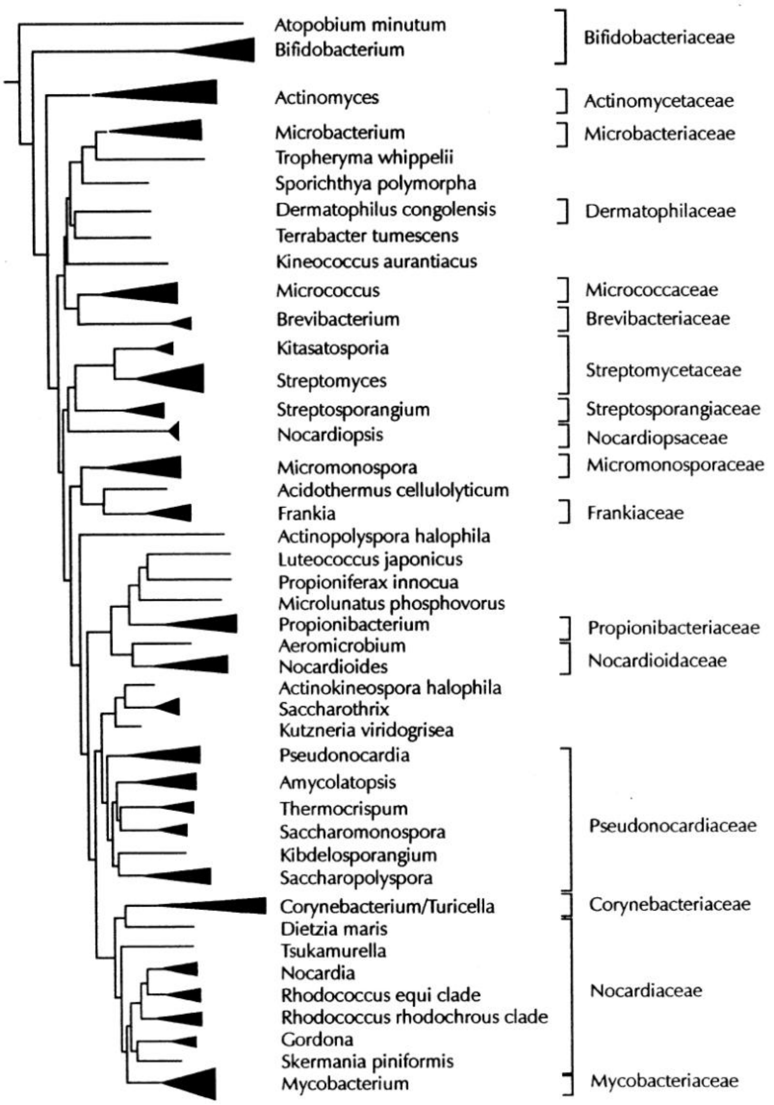


Figure 2.3 Phylogenetic relationship of actinomycetes based on 16S rRNA sequences [Goodfellow, 1989].

2.6 Revealing diversity

The advances in molecular biology, principally in the development of the polymerase chain reaction (PCR) for amplifying DNA, DNA sequencing and data analysis, have resulted in powerful techniques which can be used for the characterization, screening and evaluation of genetic diversity [Vandamme *et al*., 1996]**.** The polymerase chain reaction (PCR) is an *in vitro* technique, which enables the amplification of a specific DNA [Newton and Graham, 1997]. This useful technique was invented in 1985 [Saiki *et al.*, 1985]. Since the first thermostable DNA polymerase to be introduced into PCR, *Taq* DNA polymerase and the development of a variety of thermal cyclers or PCR machines led to the automation of the PCR. PCR is a key strategy in research and clinical laboratories. The target DNA sequence is amplified exponentially. Hence, numerous copies of the target DNA sequence are amplified [Saiki *et al.*, 1988].

The basic components of a usual PCR, involve the template DNA, buffer, dNTPs, and a pair of primers that are mixed and then denature the DNA when heated. DNA polymerase is added and mixed. The reaction elements are covered with or without mineral oil and the tube is placed in a thermal cycler. At the end of the amplification, the products are used to confirm that the PCR has been successfully amplified [Newton and Graham, 1997].

Figure 2.4 shows the cycles of PCR. The first extension products result from DNA synthesis on the original template and these do not have a distinct length as the DNA polymerase will continue to synthesize new DNA until it stops. The second cycle extension products are also an imprecise length. However, at the third cycle, fragments of target sequence are synthesized to the expected length, which is consistent to the positions of the primers on the original template. From the fourth cycle onwards, the target sequence is amplified exponentially [Sompong, 2006].

To completely denature the complex genomic DNA template at initial denaturation, 95-100°C is sufficient so that the primers can anneal after cooling. There are three steps in PCR amplification. The first step of the PCR cycle is the denaturating step, the DNA template is separated into a single strand using heat in the range of 92-95 °C. In the second annealing step, oligonucleotide primers are annealed to the target sequence onto the template DNA at a lower temperature range; 37-65°C (depending on the T_m of the primer sequence). Thirdly is the extension step; the primers are extended by the DNA polymerase participatory action, usually performed at 72 °C. By recycling these three steps 25-35 times, the target sequence is amplified exponentially [Newton and Graham, 1997].

The PCR-target approaches could be applied to study the relationship among communities. Now, according to the development of techniques in the extraction of genomic DNA and ribosomal RNA, amplified DNA has allowed for new ways to evaluate the evolution relationship. Most of the results in these studies have been obtained by 18S rDNA PCR amplification of genomic DNA extracted from Kai, which revealed the unique characteristic that was different from others that were studied [Newton and Graham, 1997].

2.7 Phylogenetic analysis

Phylogenetic tree construction has become increasingly popular in many fields of biology. Their inclusion reflects the growing recognition of trees as a tool for understanding biological processes. Phylogenetic trees allow you to organize your thinking about a protein of interest in terms of its relationship to other DNA or proteins, and may allow you to draw conclusions about its biological functions that would not otherwise be apparent [Hall, 2001]. Phylogenetic trees are used for many purposes; knowing the evolution of genes and proteins, knowing the phylogenetic relationship of organisms, clarifying the taxonomic position of an organism, determining the origin of organelles (chloroplasts and mitochondria), detecting a lateral gene transfer and identifying organisms in a particular environment. The phylogenetic reconstruction is also widely accepted in applying the rRNA technology as an integrated part of a polyphasic approach for new descriptions of new species [Ludwig *et al.*, 1998].

The first critical step to sequence-based phylogenetic analyses is alignment. Given that positions with a common ancestry have to be matched for reliable phylogenetic conclusions, homologous positions have to be arranged in common columns in correct alignment [Ludwig *et al.*, 1998].

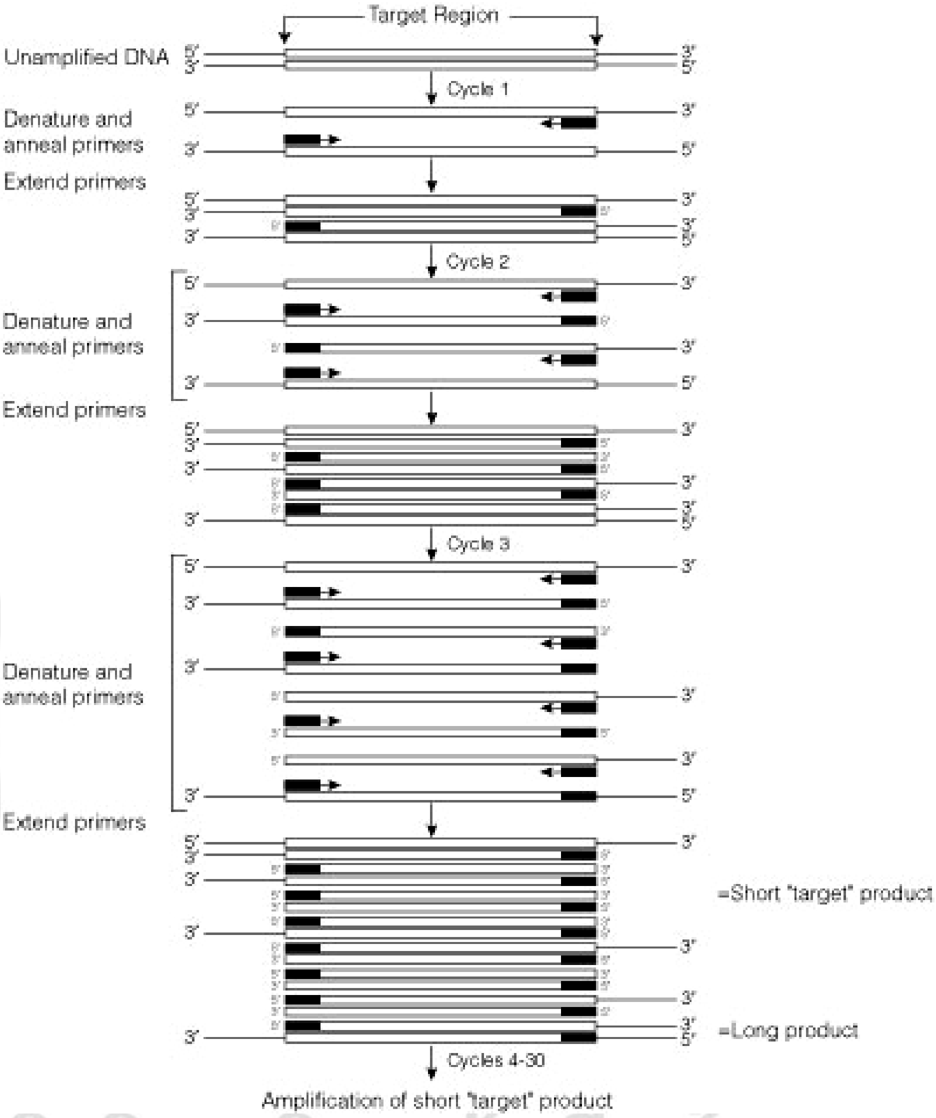


Figure 2.4 The cycles of Polymerase Chain Reaction

[Source: http://www.promega.com]

2.7.1 Phylogenetic trees

A tree consists of lines called branches or clades that intersect and terminate at nodes. The nodes at the tips of the branches represent the taxa or the sequences. The internal nodes represent ancestral taxa, whose properties can only be inferred from the existing taxa.

2.7.2 Methods for constructing phylogenies

There are two primary criteria in tree construction: algorithmic and tree searching. The algorithmic approach uses an algorithm to construct a tree from data. The tree-searching method involves many trees, then uses some criterion to decide which is the best of the trees.

The algorithmic approach has two advantages. It is fast, and it yields only a single tree from any given data set. The two well known algorithmic methods are Neighbor Joining, and UPGMA. All the other methods are tree-searching methods: such as Parsimony, Maximum likelihood and Bayesian analysis that evaluate individual trees to meet some optimality criterion. The best we can hope for is a tree that well reflects what happened in the past. However, we can never be entirely sure of the accuracy of the tree data analysis. Tree-searching methods may yield one tree or several, but all methods implicitly acknowledge that the trees produced are only a subset of the possible trees that are consistent with the data [Sompong, 2006].

2.7.3 Distance methods

Distance-matrix methods of phylogenetic analysis are explicit and trustable on the measure of the "genetic distance" between the sequences being classified, and therefore the methods require an MSA (multiple sequence alignment) as an input. Distance methods convert the aligned sequences into a distance matrix of pair-wise differences (distances) between the sequences. Distance is often defined as the portion of mismatches at aligned positions [Mount, 2004]. Distance methods attempt to construct an all-to-all matrix from the sequence query set describing the distance between each sequence pair. This constructed phylogenetic tree will place closely related sequences under the same interior node and distances always underestimate the actual amount of change in the lineages. The main disadvantage of distance-matrix methods is their inability to efficiently use information about local high-variation regions that appear across multiple sub-trees [Felsenstein, 2004]. The well known distance methods are as follows.

1) UPGMA

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) is a simple method used in bioinformatics for the creation of phenetic trees (phenograms).The program first finds the pair of taxa with the smallest distance between them and defines the branching between them as half of that distance. It combines the two taxa into a cluster and rewrites the metrix with the distance from the cluster to each of the remaining taxa. The number of entries in the metrix is reduced until the metrix consists of a single entry. Then the set of metrics is used to reconstruct the tree. The distance between any two nodes equals the sum of the lengths of all branches between them and all taxa are equally distant from the root [Takezaki, 1998].

2) Neighbor Joining

Neighbor joining methods (NJ) is similar to UPGMA in that it manipulates a distance matrix, reducing it in size at each step. Then, the tree is reconstructed from that series of metrics. It differs from UPGMA in that it does not construct clusters but directly calculates distances to internal nodes. From the original matrix, NJ first calculates for each taxon, its net divergence from all other taxa as the sum of the individual distances from the taxon. It then used that net divergence to calculate a corrected distance matrix. Then, NJ finds the pair of taxa with the lowest corrected distance and calculates the distance from each of those taxa to the nod that joins them, NJ does not assume that all taxa are equidistant from the root [Hillis *et al*., 1996; Swofford *et al.*, 1996].

2.8 Clone library

Cloning involves the use of recombinant DNA technology to propagate DNA fragments inside a host. The restriction enzymes are used to generate the fragment which isolated from chromosomes and then united with a carrier (a vector). After introduction the DNA fragments into the host cells, the fragments can then be reproduced along with the host cell DNA [Sambrook and Russell, 2001]. Vectors are DNA molecules originating from viruses, bacteria, and yeast cells. They accommodate various sizes of foreign DNA fragments ranging from 12,000 bp for bacterial vectors (plasmids and cosmids) to 1 Mb for yeast vectors (yeast artificial chromosomes). Bacteria are most often the hosts for these inserts, but yeast and mammalian cells are also used. (Figure 2.5)

Cloning procedures provide unlimited material for experimental study. A random (unordered) set of cloned DNA fragments is called a library. Genomic libraries are sets of overlapping fragments encompassing an entire genome. (Figure 2.6) Also available are chromosome- specific libraries, which consist of fragments derived from source DNA enriched for a particular chromosome [Sambrook and Russell, 2001].

Millions copies of a single gene or DNA segment can be produced in a very short time by rapidly reproducing of foreign cells. The to be cloned DNA is inserted into a plasmid (a small, self- replicating circular molecule of DNA) that is separated from chromosomal DNA. When the recombinant plasmids are introduced into bacterial cells, the newly inserted segments will be replicated along with the rest of the plasmid [Sambrook and Russell, 2001].

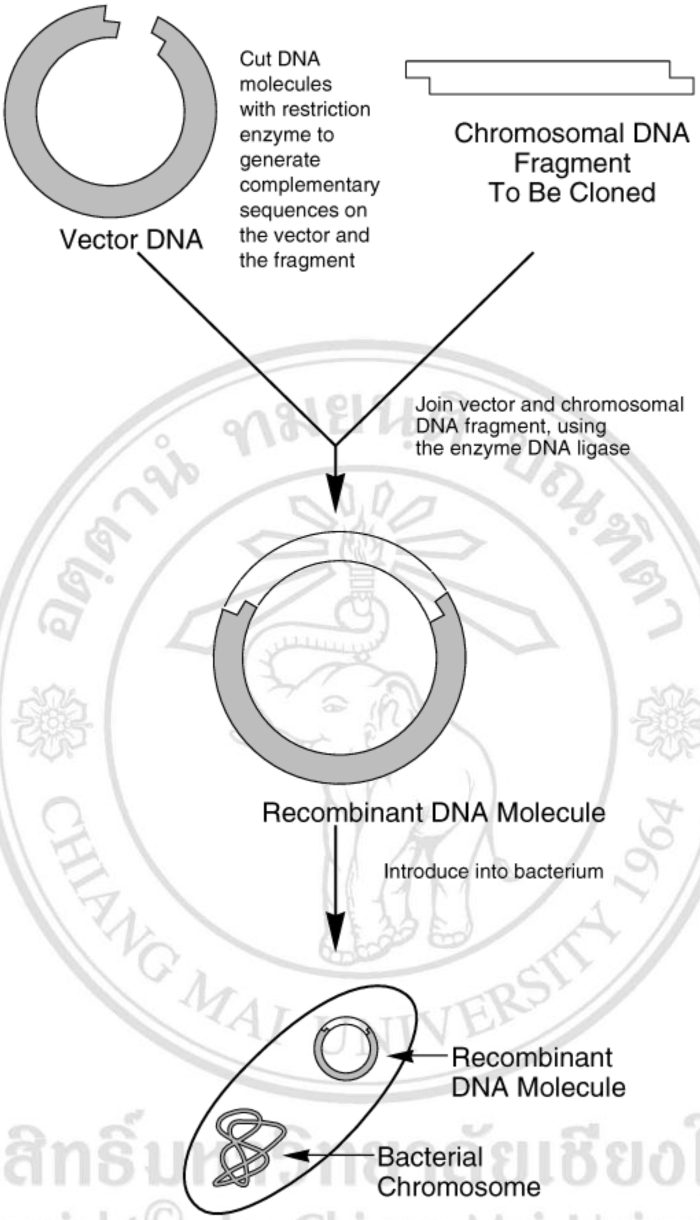


Figure 2.5 Cloning DNA in Plasmids
[Sambrook and Russell, 2001]

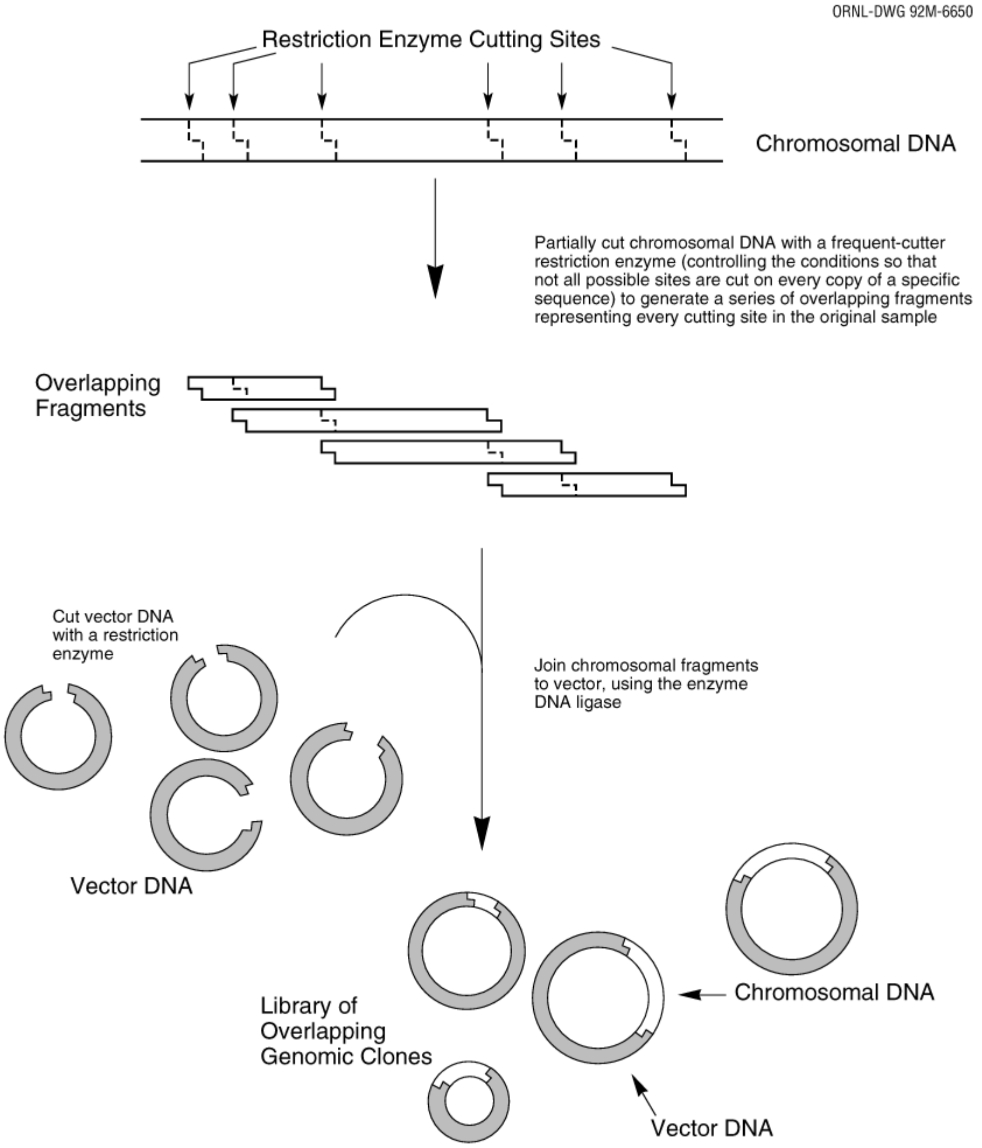


Figure 2.6 Constructing an Overlapping Clone Library.

[Sambrook and Russell, 2001]

2.8.1 Types of vectors

Cloning vectors are a small pieces of DNA which foreign DNA fragments can be inserted. The fragment is inserted into the cloning vector by treating with vehicle. The restriction enzyme is used to create the same overhang, vector and fragment, then ligating them together. There are many types of cloning vectors. the most commonly vectors used are genetically engineered plasmids and bacteriophages (such as phage λ). Other types of cloning vectors include bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs) (Table 2.5).

Table 2.5 Vector type and capacity of vector

Vector Type	**Capacity (thousands of bases)**
Plasmids	15
Phage (lambda)s	25
Cosmids and Fosmids	35 to 45
Bacterial artificial chromosomes (BAC)	50 to 300
Yeast artificial chromosomes (YAC)	300 to >1500
Human artificial chromosomes (HAC)	2000

2.8.2 Types of vectors Plasmids

Plasmid is a circular DNA molecule and can replicate independently of the chromosomal DNA [Lipps, 2008] (Figure 2.7). Plasmids usually occur naturally in bacteria, but sometimes are found in eukaryotic organisms. Plasmid has varied sizes from 1 to over 1,000 kbp. Plasmids can be considered part of the mobilome because they are often associated with conjugation, a mechanism of horizontal gene transfer. The term of plasmid was first introduced by the American molecular biologist Joshua Lederberg in 1952 [Lederberg, 1952]. Plasmids can be replicating autonomously within a suitable host and can be found in all three major domains: Archaea, Bacteria, and Eukarya [Lipps, 2008]. Similar to viruses, plasmids are not considered by some to be a form of life [Sinkovics, J. *et al.*, 1998]. Unlike viruses, plasmids are naked DNA and do not encode genes necessary to encase the genetic material for transfer to a new host, though some classes of plasmids encode the sex pilus necessary for their own transfer. Plasmid host-to-host transfer requires direct, mechanical transfer by conjugation or changes in host gene expression allowing the intentional uptake of the genetic element by transformation [Lipps, 2008]. Microbial transformation with plasmid DNA is neither parasitic nor symbiotic in nature, because each implies the presence of an independent species living in a commensal or detrimental state with the host organism. The plasmids provide a mechanism for horizontal gene transfer within a population of microbes and provide a selective advantage under a given environmental state. Plasmids may carry genes that provide resistance to naturally occurring antibiotics in a competitive environmental niche or the proteins produced may act as toxins under similar circumstances. Plasmids can also provide bacteria with the ability to fix elemental nitrogen or to degrade recalcitrant organic compounds that provide an advantage when nutrients are scarce [Lipps, 2008].

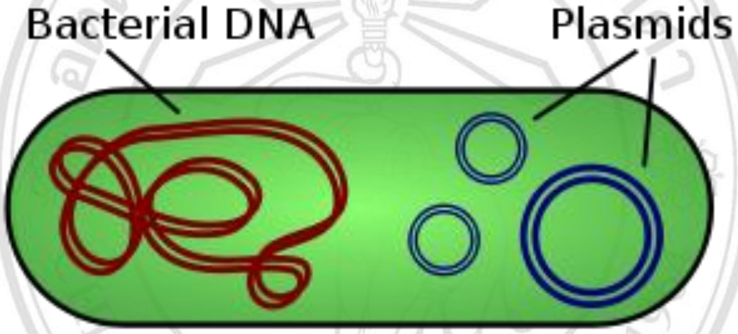


Figure 2.7 Illustration of a bacterium with plasmid enclosed showing genomic DNA and plasmids. [Lipps, 2008]

2.8.3 Environmental clone library

The modern microbial diversity approach started with the analysis of nucleic acids which recovered either directly from the environment or from isolated prokaryotic cells. Beginning from Pace *et al*. [1986], the taxon composition of a microbial community was explicated via ribosomal RNA molecules analysis. firstly of 5S RNA patterns and secondly of sequences of 16S rDNA fragments were generated by shotgun cloning. Technical refinement included the cloning of PCR amplified 16S rDNA molecules and the use of taxon-specific PCR primers which narrow the range of retrieved sequences. Knowledge about the 16S rDNA sequence diversity of cultured and as yet non-cultured organisms has stimulated the development of techniques which explore the in situ spatial and temporal structure of microbial diversity (e.g. by application of fluorescently labeled oligonucleotide probes and by recognition of DGGE and TGGE patterns [Muyzer *et al*.,1993], and assessment of the metabolically active part of a community by probing ribosomal RNA with taxon-specific oligonucleotides [DeLong, E.F. *et al*., 1989] (Figure 2.8). Nevertheless, although the contribution of rDNA and rRNA to the elucidation of environmental connections must be considered significant, one molecule alone cannot nearly cover all facets of microbial ecology. Not only must the function of an ecosystem be deduced from analyses of genes expressed through mRNA and proteins, but the network of broad physiological interactions should also be verified. All these strategies must be accompanied by complete physical and chemical analysis of the natural sample. In order to understand ecological interactions, strain richness and strain abundance must be evaluated and the physiological diversity of strains would be recognized. At present these goals are out of reach considering problems encountered with the analysis of clone libraries and the lack of knowledge of the relevance of the physiology.

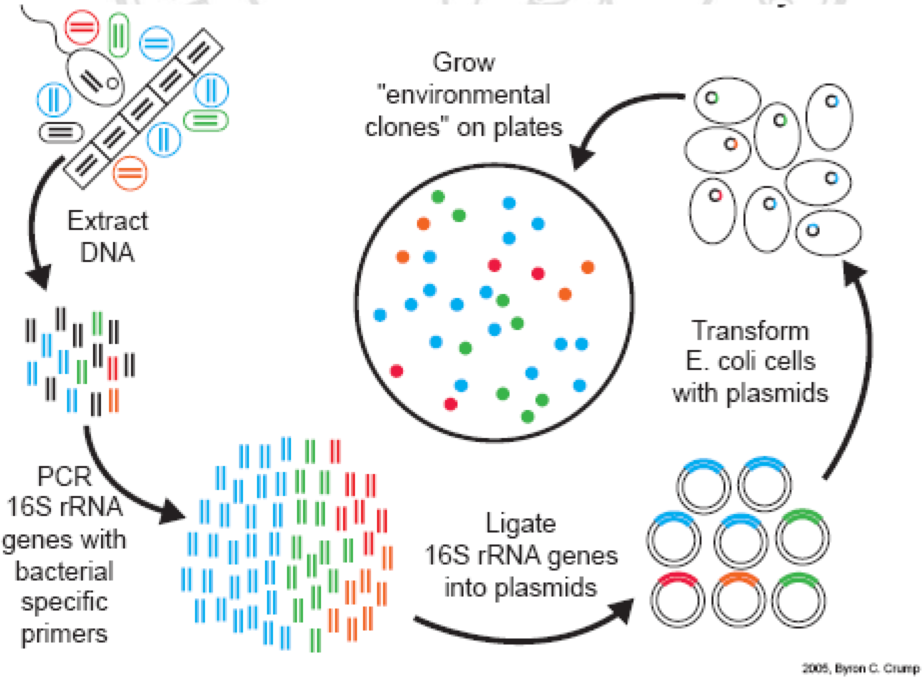


Figure 2.8 Environmental clone library
[DeLong, E.F. *et al*., 1989]

Culture-independent tools the 16S rDNA cloning approach is nevertheless a commonly employed technique used to describe the composition of complex microbial communities and to gain a descriptive overview of possible differences among communities [Yakimov *et al.*, 2005]. In particular, 16S rDNA clone library construction has been used to provide new insights into bacterial diversity and dynamics in sedimentary environments.

2.9 Antibiotics

Microbial metabolites which have application as antibiotics are synthesized from intermediates of primary metabolites such as amino acids, aliphatic acids, sugars, nucleotides and lipids. Peptides and polyketides are the most important groups of secondary metabolites which have found application as lead molecules. Antibiotics, immunosuppressant, extracellular hydrolytic emzymes, plant growth promoters and siderophores are the biological active compounds produced by actinomycetes. *Streptomyces* represent an important source of biologically active compounds with high commercial value, various important applications in human, livestock medicine and agriculture [Watve *et al.*, 2001; Berdy, 2005]. Some actinomycete antibiotics were reported in the Table 2.6.

Table 2.6 Actinomycete antibiotics for medical applications [Kieser *et al.*, 2000]

Antibiotic	**Producer**	**Application**
Erythromycin	*Saccharopolyspora erythraea*	Antibacterial
Gentamicin	*Micromonospora* sp.	Antibacterial against gram-negative bacteria
Nocardicin A	*Nocardia uniformis*	Antibacterial
Nystatin	*Streptomyces noursei*	Antifungal against fungi especially *Candida* sp.
Rifamycin	*Amycolatopsis mediterranei*	Antibacterial against *M. tuberculosis*
Ristocetin	*Amycolatopsis lurida*	Antibacterial against *Streptococcus* sp.
Spinosyns	*Saccharopolyspora spinosa*	Insecticidal
Streptomycin	*Streptomyces griseus*	Antibacterial against gram-positive and gram-negative bacteria
Teicoplanin	*Actinoplanes teichomyceticus*	Antibacterial against gram-positive bacteria
Vancomycin	*Amycolatopsis orientalis*	Antibacterial against *Streptococcus* sp.

Microbial metabolites which have application as antibiotics are synthesized from intermediates of primary metabolites such as amino acids, aliphatic acids, sugars, nucleotides and lipids. The most important groups of secondary metabolites which have found application as lead molecules are the peptides and polyketides.