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Article type : Review Article

RUNNING HEAD: RENEWED INTERESTS IN METABOLITES OF ACTINOMYCETES

Renewed interests in the discovery of bioactive actinomycetes metabolites driven by emerging technologies

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No conflict of interest declared.

Abstract

Actinomycetes are prolific sources of bioactive molecules. Traditional workflows including bacterial isolation, fermentation, metabolite identification, and structure elucidation have resulted in high rates of natural product rediscovery in recent years. Recent advancements in multi-omics techniques have uncovered cryptic gene clusters within the genomes of actinomycetes, potentially introducing vast resources for the investigation of bioactive molecules. While developments in culture techniques have allowed for the fermentation of difficult-to-culture actinomycetes, high throughput metabolite screening has offered plenary tools to accelerate hits discovery. A variety of new bioactive molecules have been isolated from actinomycetes of unique environmental origins, such as endophytic and symbiotic actinomycetes. Synthetic biology and genome mining have also emerged as new frontiers for the discovery of bioactive molecules. This review covers the highlights of recent developments in actinomycete-derived natural product drug discovery.

Keywords: Multi-omics, actinomycetes, *Streptomyces*, co-culture, endophytes, bioactive metabolites

Introduction

The *Actinomycetales* are an order of bacteria that are widely recognized as prolific producers of bioactive specialized metabolites. The *Actinomycetales* exhibit complex life cycles, a filamentous morphological appearance, and high guanine and cytosine (G+C) content in their genomes (Barka et al. 2016). The metabolic products of actinomycetes display a wide range of biological activities relevant to human health, agriculture, and the economy. With the early discovery of antibiotics in the 1940s and 50s, actinomycetes continue to be the richest source of antibiotics. In fact, actinomycetes have been described as microbial cell factories. During the past 80 years, thousands of metabolites with antibacterial and other important biological activities have been isolated, many of which represent a major portion of the current antibiotic repertoire. More than one-fourth of all microbial bioactive metabolites (estimated to be 80,000-100,000) are produced by actinomycetes (Bérdy 2012). Actinomycete-derived natural products and their synthetic derivatives continue to represent a significant portion of the clinically used antibiotics. Notably, more than 70% of actinobacterial metabolites exhibit antimicrobial

activities (Bérdy 2012), and 64% of the known natural product antibiotic classes are produced by filamentous actinomycetes (Hutchings et al. 2019). The majority of clinically used antibiotic classes, such as beta-lactams (Paradkar 2013), macrolides (Arsic et al. 2018, Elshahawi et al. 2015), aminoglycosides (Krause et al. 2016), glycopeptides (Butler et al. 2014), tetracyclines (Grossman 2016), and ansamycins (Floss and Yu 2005) are produced by actinomycetes. Other clinically relevant products of actinobacterial origins include immunosuppressants (e.g. FK 506, rapamycin), anti-cancer agents (e.g. doxorubicin), antiparasitic agents (e.g. avermectins for animal use), and antidiabetic agents (e.g. acarbose). Similarly, actinomycetes are known to produce other bioactive metabolites, such as enzyme inhibitors, larvicides, herbicides, and the commercially used insecticide spinosad (Bacci et al. 2016, Deepika et al. 2012, Imada 2005).

Research interest in discovering new pharmacophores from actinobacterial origins remains high, however, new approaches must be adopted to identify new chemical entities. The rediscovery of known metabolites and the diminishing number of novel chemical scaffolds have been major setbacks of traditional antibiotics discovery programs for years. In addition, the availability of clinically interchangeable antibiotics resulted in market saturation, which contributed in part to the closure of industrial actinomycete investigation programs by big pharma (Payne et al. 2007, Renwick and Mossialos 2018). In light of rapidly expanding genomics, proteomics, bioinformatics, and culture techniques, new research thrusts have generated novel hypotheses and knowledge gaps, fueling the drive to discover new chemistries from actinobacteria. The publication of research works on actinomycetes has sustained a linear growth for the past 20 years (Fig. 1), and there was a total of 11,088 scholarly entries from 1940 to 2019 on the ISI Web of Knowledge.

The discovery of antibiotics has largely relied upon the cultivability of producer microbes ever since the discovery of penicillin in the mid-nineteenth century. Cultivability can be a major hurdle for scale-up production even after the discovery of novel products. Most of the clinically useful antibiotics of bacterial origin have been discovered using conventional screening and laboratory cultivation methods. However, it is now established that less than 1% of the bacteria can be cultured with standard culture methods (Vartoukian et al. 2010) revealing a vast reserve of yet untapped resources - the so-called "unculturable" bacteria. This same trend of cultivability also rings true for the *Actinobacteria* – only 1% of existing *Actinomycetales* are thought to have been cultured (Bérdy 2012). Whole-genome data of the first sequenced actinomycete *Streptomyces coelicolor* A3(2) (Bentley et al. 2002) and many others thereafter revealed that the metabolites isolated from cultures represent only a small fraction of their genetic potential of producer bacteria. For instance, the genome of *Streptomyces rochei* 7434AN4, the producer of lankamycin and lankacidin, contains at least 35 additional secondary metabolites biosynthetic gene clusters, including clusters for the polyene macrolide pentamycin and the azoxyalkene compound KA57-A (Nindita et al. 2019). The genome of vancomycin producer *Amycolaptosis mediterranei* contains biosynthetic gene clusters for 25 metabolites, most of which are yet to be characterized (L. Xu et al. 2014). The genome analysis of 40 *Micromonospora* strains and two non-*Micromonospora* strains revealed an average of 20 biosynthetic gene clusters for specialized metabolites per strain (Carro et al. 2018). In particular, bacterial sources represent a much larger reservoir of antibiotics than previously thought. The widespread emergence of antibiotic resistance and the limited repertoire of clinically useful antibiotics have invigorated interest in new bacterial sources of new antibiotics. Consequently, numerous developments occurred on various fronts of the antibiotic discovery process in recent years including improvement of

culture techniques, early identification of new antibiotic producers, use of genomics, transcriptomics, and bioinformatics, automation in metabolite screening, and use of metabolite dereplication tools.

Conventional discovery approaches are adapted to minimize the limitations of the rediscovery of known metabolites and the inefficiency of the antibiotic producer screening process. Recently, many new strategies have emerged to activate biosynthetic gene clusters that are normally silent under standard culture conditions as well as to identify novel bioactive metabolite producer actinomycetes strains without going through a costly and labor-intensive analysis. Some of these strategies are reviewed in this article.

Exploring actinomycetes of unique environmental origin

In the early days of actinomycete discovery programs, soil samples were the primary focus for the isolation of actinomycetes. Samples collected from random locations were studied for antibiotic producer actinomycetes employing "The Waksman Platform"(Lewis 2012). Similar techniques were used for microbial isolation, fermentation, and follow-up culture-based assays that measured the antibacterial activity of the extracts. This approach initially resulted in the isolation of numerous antibiotic producer actinomycetes, but soon experienced major setbacks due to repeated isolation of identical antibiotic producer strains from various parts of the world (Wright 2017). The lack of novelty in the vast majority of isolated metabolites has continued to be one of the major hurdles for microbial-metabolites-based discovery programs (Aminov 2010). Also, actinomycetes of different genuses appeared to produce structurally-related metabolites. For instance, aminoglycoside antibiotics istamycins and fortimicins are produced by *Streptomyces tenjimariensis* and *Micromonospora olivasterospora* (Hotta et al. 1989). The continuous growth of publications on less commonly found actinomycetes, also known as rare

actinomycetes over the past 68 years is reflective of increasing research interests in this arena (Fig. 2). To increase the odds of finding new antibiotic producers, unique and previously unexplored ecological environments are being increasingly studied for the isolation of rare actinomycetes. (Fig 3).

Soil samples collected from the previously unexplored Himalayas Kashmir region resulted in the isolation of 121 morphologically distinct actinomycetes (Shah et al. 2017). Numerous bacterial strains belonging to 30 different genera, the majority being rare actinomycetes, have been isolated from cave samples during 1999 and 2018 (Rangseekaew and Pathom-Aree 2019). Most of the isolates produced metabolites with antibacterial activities, however, molecular structures of active compounds are yet to be established. Polyglycosylated polyketides cervimycin A and its analogues that displayed potent activity against methicillin-resistant *Staphylococci* and vancomycin-resistant *Enterococci* (VRE) were isolated from an ancient cave *Streptomyces* (Fig. 4) (Herold et al. 2005). Chaxalactins isolated from *Streptomyces* of hyper-arid environment origin displayed strong activity against gram-positive bacteria (Rateb et al. 2011). Similarly, ansamycin class antibacterial agents chaxmycins were produced by a desert *Streptomyces* strain (Rateb et al. 2011). Thermal vents and hot springs are also increasingly being studied as new resources for actinomycetes in recent years. For instance, 8 *Streptomyces* and 65 eubacterial species were isolated from two hot springs in India (Pednekar et al. 2011). Two new antibacterial cyclopeptides mullinamides along with known congeners were isolated as the metabolites of a *Streptomyces* sp. isolated from a thermal vent of an underground coal mine fire (X. Wang et al. 2014) (Fig. 4). Similarly, a total of 1866 bacterial strains were isolated from biological samples, mostly sponges, collected from a hydrothermal site of Eyjafjörður, Iceland (Eythorsdottir et al. 2016). Notably, 61% of the fifty-five antimicrobial

metabolites producing isolates were found to be actinobacterial species. However, the novelty of these metabolites remains unclear. Augustin and co-workers have isolated *Streptomyces* sp. and *Nocardiopsis* sp from the arctic regions (Augustine et al. 2012). Extracts of both isolates were capable of inhibiting biofilm formation by *V. cholera*.

Oceans offer a remarkable heterogeneity of environments, because factors such as temperature, oxygen saturation, light intensity, pressure, pH, and salinity that contribute to establishing a wide range of biodiversity vary greatly with depth (Tortorella et al. 2018). Oceans provide habitats for more than 300,000 described species of plants and animals, many of which are well-known harbor large communities of microorganisms (Donia and Hamann 2003). Despite great potential, marine actinomycetes are underexplored, partly due to difficulties in accessing samples from these unique environments and challenges associated with the transport and identification of optimal culture conditions. Defying these challenges, the examination of marine ecosystems for microbiomes has flourished over the past several decades (Hug et al. 2018). Many structurally unique metabolites with excellent biological activities have been isolated from actinomycetes retrieved from ocean sediments. Actinobacterium *Pseudonocardia carboxydivorans* M-227 isolated from the seawater collected from 3000 m depth in the Cantabrian Sea produced broad-spectrum antibacterial metabolites branimycin B and analogues (Braña et al. 2017). Dalisay and coworkers examined 49 marine sediments and isolated 186 *Streptomyces* strains including a cluster of novel *Streptomyces* strains based on phylogenetic analysis (Dalisay et al. 2013). Metabolites from 47 of these strains displayed antibacterial activities in their metabolites, and one of the isolates produced new analogues of novobiocins. Similarly, phocoenamicins B and C with potent activity against MRSA, VRE, and mycobacterial species were isolated from a *Micromonospora* species retrieved from the ocean sediment

collected near Canary Islands (Pérez-Bonilla et al. 2018). Micromonohalimanes with anti-MRSA activity have been isolated from a marine *Micromonospora* strain (Y. Zhang et al. 2016). Similarly, thiopeptide antibiotic PM181104 (kocurin) was isolated from a sponge-associated rare actinobacterium of genus *Kocuria* (Mahajan et al. 2013). This antibiotic displays a broad range of antibacterial activities including MRSA and VRE. Natural resources with unique ecological niches are still promising for the discovery of novel bioactive molecule-producing actinomycetes.

New bioactive metabolites from symbiotic actinomycetes

Higher-order organisms often host a variety of bacterial species including actinobacteria. The relationships between the host and bacteria which can vary from parasitic to symbiosis are believed to be shaped by millions of years of evolution (McFall-Ngai et al. 2013). Co-evolutionary adaptation may have led to the localization of symbiotic bacteria in specialized anatomical compartments of the host (Hug *et al.* 2018). Symbiotic antibiotic producer actinomycetes that can offer their hosts protection from other parasitic microbes are of special interest for antibiotics discovery programs in recent years. Such symbiotic actinomycetes are found to have a distinct phylogenetic lineage compared to common soil-dwelling actinomycetes increasing the likelihood of new or novel metabolites production by these organisms. Several structurally unique and potent antibiotics have been isolated from symbiotic actinomycetes recently. A new polyene antibiotic mycangimycin was isolated from a symbiotic *Streptomyces* sp. retrieved from southern pine beetles. The antibiotic defends the beetles against the pathogenic fungus *Ophiostoma minus* (Scott et al. 2008). Chevrette and co-workers have conducted a comprehensive study on *Streptomyces* associated with 2561 insects of 15 taxonomic orders (Chevrette et al. 2019). They found actinomycetes in 56% of the insect microbiomes and

recovered a total of 10,178 isolates. The phylogenetic study showed a distinct phylogenetic lineage for insect-associated *Streptomyces*. Notably, they have observed higher hit rates of anti-fungal and anti-bacterial (both Gram-positive and negative) metabolites in insect-associated *Streptomyces* compared to soil and plant-associated *Streptomyces*. Cyphomycin, a potent anti-fungal antibiotic, was isolated from a *Streptomyces* sp. retrieved from the ant host *Cyphomyrmex* sp. (Chevrette *et al.* 2019). This highlights the remarkable potential of insect-associated actinomycetes for bioactive natural product discovery. Macrolactam antibiotic sceliphrolactam was isolated from the culture of mud dauber, *Sceliphron caementarium*-associated *Streptomyces* sp. (Oh et al. 2011). Highly functionalized cyclic depsipeptide antifungal antibiotic dentigerumycin was produced by actinobacterium associated with an ant (Oh et al. 2009). Similarly, anti-fungal antibiotic selvamicin was isolated from an actinomycete *Pseudonocardia* sp. retrieved from an ant collected from Costa Rica (Fig. 4) (Van Arnam et al. 2016). Considering the uniqueness of each symbiotic relationship and the recent success in isolation of new bioactive molecule producer actinomycetes from insects and other organisms, symbiotic actinomycetes could be an abundant source of new bioactive molecules.

Endophytic actinomycetes as a source of new bioactive molecules

About 300,000 species of plants that exist on the earth have evolved throughout hundreds of millions of years (Govaerts 2001, Prance et al. 2000). The evolutionary course brought in a great deal of heterogenicity in physiology and adaptations among plant species resulting in them being one of the richest and most diverse sources of secondary metabolites producers on earth. The use of plant products for human benefits, particularly, in treating human illness can be traced back to early human civilizations. Numerous ethnic groups are still practicing plant-based remedies for human illness all over the world. Of well-known ones are the Traditional Chinese

medicines and the Indian Ayurvedic medicines where plants constitute key ingredients of therapeutic preparations. Approximately 1,000 different Chinese herbs are listed in the Chinese Pharmacological reference book (Xue and O'Brien 2015). Plant products constitute a bulk of current human therapeutics to treat human illnesses ranging from malaria (e.g. artemisinin, the semisynthetic product derived from *Artemisia annua*) to cancer (e.g. Paclitaxel, the product of yew trees). A recent review revealed plant products to be a quarter of FDA-approved new molecular entities (NMEs) (Patridge et al. 2016). It is also noteworthy that each plant hosts numerous endophytes, the microorganisms that reside in plants (Strobel and Daisy 2003). Given such a great deal of heterogenicity of phytochemicals in plants, they offer unique microenvironments to endophytic organisms, potentially leading to the evolution of distinct genetic lineage and unique biosynthetic capabilities.

Recent work revealed that many bioactive metabolites isolated originally from plants are produced by endophytic microorganisms as well. For instance, the anti-cancer drug paclitaxel, the well-known product of *Taxus brevifolia*-the medicinal plant long used by native Americans-was isolated recently from the culture of *Aspergillus fumigatus,* the fungal endophyte of *Taxus* sp. (Gunther 1945, Kumar et al. 2019, Wani et al. 1971), and *Pestalotiopsis microspore*, the endophyte of bald cypress *Taxodium distichum* (J. Y. Li et al. 1996). Interestingly, this molecule was also isolated from actinomycete strains belonging to genuses *Streptomyces*, *Actinoplanes*, *Nocardiopsis*, *Micromonospora*, *Actinomadura* (Breme et al. 2003). The widely used anti-cancer drug camptothecin was first isolated from the bark of *Camptotheca acuminate* (Wall 1998). More recently, this compound was also isolated from several endophytic fungal species retrieved from the native producer *Camptotheca acuminate* and *Nothapodytes foetida* (Kusari et al. 2009, Puri et al. 2005). Interestingly, the camptothecin was also produced by several endophytic

Bacillus species isolated from *Pyrenacantha volubilis* (Soujanya et al. 2017). These results showcase possibilities for endophytic bacteria having biosynthetic potential for a wide range of bioactive molecules isolated from plants. Endophytic actinomycetes, though largely understudied, have increasingly become attractive for the discovery of new bioactive molecules.

It is well established that the phytochemical content and constitution of a plant vary significantly depending on extrinsic factors such as soil composition, water supply, and temperature. Also, intrinsic factors vary greatly among different parts such as roots, stems, leaves, and flowers, offering a myriad of unique environments to colonize specialized bacteria including actinobacteria. A recent work of Matsumoto and co-workers found a significant difference in the taxa of actinomycetes within plant roots from those found in soil environments. Aactinobacterial diversity increased in the given order: free soil (lowest)→rhizospheric soil→root (highest) (Matsumoto and Takahashi 2017). The richness of diversity and unique evolutionary lineages associated with endophytic actinobacteria have been covered by several review articles (Dinesh et al. 2017, Matsumoto and Takahashi 2017, Nalini and Prakash 2017). *Streptomyces dioscori* sp. nov. from the bulbil of *Dioscorea bulbifera* L(Z. Wang et al. 2018), *Microbacterium halophytorum* sp. nov. from a halophyte (Y. R. Li et al. 2018), *Streptomyces ginkgonis* Sp. nov. from *Ginko biloba* (Yan et al. 2018), and *Aeromicrobium endophyticum* sp. from *Phragmites australis* (F. N. Li et al. 2019) are such representative examples. Xue and coworkers have summarized structurally diverse bioactive compounds isolated produced by endophytic actinomycetes retrieved from mangroves (D. B. Xu et al. 2014). More recently, Jiang and coworkers have isolated 101endophytic actinomycetes belonging to 28 genera from five different mangrove plants (Jiang et al. 2018). One of these isolates represented a new bacterial species while several isolates that display bioactivity belonged to rare actinomycetes. Similarly,

Shan and coworkers have isolated 46 endophytic actinobacterial species belonging to 13 genera from 15 tea cultivars (Shan et al. 2018). Among these isolates, actinobacteria of genera *Mobilicoccus* and *Piscicoccus* were the first isolates as endophytes. Many of these isolated displayed antifungal and antibacterial activities. Further work is necessary to assess the novelty of molecules responsible for the preliminary bioactivity.

Numerous endophytic actinobacteria have been studied recently for the production and characterization of bioactive metabolites. Heraclemycins C and related analogues, the members of pluramycin class antitubercular antibiotics were isolated from a culture of *Streptomyces* sp. strain Y3111(Liu et al. 2014) (Fig. 5). This strain was retrieved from the stems of *Heracleum souliei*. A spirotetronate class polyketide antibiotic maklamycin was produced by a *Micromonospora* sp. GMKU326 retrieved from the root of a leguminous plant, *Abrus pulchellus* Wall. Ex Thwaites subsp. *pulchellus*) (Igarashi et al. 2011) (Fig. 5). Maklamycin displays strong antibacterial activity against *Micrococcus luteus* (MIC: 0.2 μg/mL). A new glycosylated piericidin antibiotic glucopiericidinol A3 along with its related congeners were isolated from the culture broth of *Streptomyces* sp. KIB-H1083(Shang et al. 2018) (Fig. 5). This strain was isolated from the Chinese medicinal plant *Diaphasiastrum veitchii*. Similarly, a cytotoxic compound hamuramycin A was produced by fermenting an endophytic actinomycete *Allostreptomyces* sp. K12-0794 (Suga et al. 2018). This strain was retrieved from a fern root collected in Japan. It is noteworthy that *Allostreptomyces* was identified as a new genus recently (M. J. Huang et al. 2017). Similarly, antitrypanosomal compound spoxazomicin A and anti-mycobacterial compound kandenol A were isolated from *Streptomyces* sp. (an endophyte of mangrove tree *Kandelia candel*) and *Streptosporangium oxazolinicum* K07-0460^{T} (an endophyte of an orchid),

respectively (Ding et al. 2012, Inahashi et al. 2011). These examples highlight the remarkable promise of endophytic actinobacteria for the discovery of bioactive molecules.

Culturing so-called "unculturable"

The vast majority of bacteria that thrive in their natural environments cannot be grown with standard culture conditions (Lewis et al. 2010). Microbial replication, other intracellular physiological activities, and molecular signaling networks linked to secondary metabolites production are heavily influenced by physical conditions (such as temperature, pressure, oxygen content), chemical environments (e.g. nutrient availability, pH, nature of carbon source, and other essential elements) and biological conditions (e.g. surrounding microbial community, host factors). The complex combinations and interplay of both biotic and abiotic factors that shape natural environments are challenging to replicate in laboratories. Such mismatch of growth conditions is widely believed to be one of the main reasons behind the limited access to the vast microbial resources. Bacterial species that haven't been successfully grown yet are referred to as "unculturable" bacteria. In the lack of proper culture conditions, unarguably, enormous resources of bioactive metabolites coded in the genome of these unculturable bacteria remained inaccessible. Repeated isolation of identical/closely related bacteria/metabolites-the major setback of microbial bioactive molecules discovery programs during the past several decades- can be linked with the use of standard media in isolation and culture or the lack of innovation in culture techniques. Typically, samples are incubated for 2-4 weeks at various temperatures (commonly 28 °C) and newly developed colonies are isolated. Such an approach facilitates the growth of rapidly growing common actinomycetes and hinders the growth of slow-growing actinomycetes. A study that examined the effect of time and culture media on the growth of rare soil bacteria revealed two stages of colony formation: the first during 2-3 weeks and the second

during 6-8 weeks (Kurm et al. 2019), and the recovery of rare species was not influenced by growth medium and incubation time. However, the study reveals a need for longer incubation to improve bacterial recovery overall. Numerous innovative techniques have been developed in recent years in efforts to grow "unculturable" as well as to induce the production of new metabolites from microbes with established culture conditions. These developments along with rapidly expanding microbial omics (genomics, proteomics, and transcriptomics) and bioinformatics cumulatively reenergized microbiologists and natural product chemists offering a new frontier for microbial natural products discovery.

A variety of diffusion chambers have been used to grow microbes utilizing environmental growth conditions (Fig. 3). Some utilize agar pre-inoculated with microbes in between two porous membranes that prevent the penetration of microbes into the culture but allows for the exchange of gases and nutrients between the culture and environment. Others utilize microbe-free agar that is sandwiched between membranes with different pore sizes: the top one allows for gas exchange but prevents the entrance of environmental microbes into the agar while the bottom one allows for the entrance of both microbes and nutrients from the natural environment (eg. soil). Bollmann and co-workers demonstrated an increase in diversity of bacterial isolates using the diffusion chamber-based isolation approach compared to the conventional agar-based isolation technique (Bollmann et al. 2007). Semipermeable membranes were used in sandwiching pond sediments and the sandwich was incubated on the pond sediment-the natural source of the sample (Bollmann *et al.* 2007). Similarly, Steinert and co-workers also used membrane-based diffusion chambers to facilitate the growth of bacteria in the natural environment. Insertion of the chamber within native sponge tissue for weeks followed by lab cultivation led to the isolation of previously uncultivable bacteria (Steinert et al. 2014).

Isolation chip (iChip) represents a high throughput and miniaturized version of the diffusion chamber-based culture technique developed recently. Nicholas and coworkers designed and tested this technology to enhance the growth of difficult-to-culture bacteria and improve the diversity of bacterial isolates (Nichols et al. 2010). iChip utilizes a plate with 384 through-holes that traps microbes when dipped with microbial mixture suspension prepared in liquid agar. The extent of dilution of mixture suspension determines the extent of microbial entrapment in each hole, and the porous membranes (0.03 μm pore size) that sandwich the plate prevent the migration of trapped cells and the entrance of other microbes while allowing the diffusion of air, micronutrients, and other signaling molecules when incubated in the microbial natural habitat. The novelty of bacterial species isolated from seawater and soil utilizing iCHIP were significantly higher compared to isolation based on petri dish cultures demonstrating the effectiveness of this technique in accessing previously considered unculturable microbial species (Nichols *et al.* 2010). Several articles have discussed the potential of iCHIP in the discovery of microbial bioactive metabolites (Berdy et al. 2017, Lodhi et al. 2018, Sherpa et al. 2015). Novel antibiotic teixobactin with excellent activity against multi-drug resistant pathogenic bacterial species was isolated from a proteobacterium *Eleftheria terrae* (Ling et al. 2015). This rare bacterium was isolated using the iCHIP technique. More recently, this technology was used in the marine sponge (*Xestospongia muta*) to isolate putatively a new bacterial species, *Alteromonas sp*. RKMC-009. This strain was capable of producing a unique *N*-acyltyrosine derivative with an α-methyl substituent within the aminoacyl moiety with potent gram-positive antibacterial activities (MacIntyre et al. 2019). More recently, Mahler and co-workers utilized pico-droplets-based culture for growing 21 different actinobacterial species and producing metabolites (Mahler et al. 2018). The technique was also employed to disperse and grow soil

bacterial species in pico-droplets unique growth conditions that may allow for simultaneous growth of a variety of actinobacteria.

Activation of cryptic biosynthetic gene cluster

With the rapid growth of genomic sequence data, it became apparent that metabolites isolated from actinomycetes represent a small fraction of their biosynthetic potential (Doroghazi et al. 2014). The number of secondary biosynthetic gene clusters in an actinomycete genome is estimated to be 10-fold higher than the number of metabolites isolated through conventional laboratory fermentation of each organism (Katz and Baltz 2016). For instance, the genome of the avermectin producer *Streptomyces avermitilis*-one the most studied actinomycetes- contains at least 38 secondary metabolite biosynthetic gene clusters of which only16 are associated with the metabolites isolated from this organism (Ikeda et al. 2014)**.** The majority of the biosynthetic clusters that remain silent (cryptic) under standard culture conditions have become attractive targets for the discovery of bioactive secondary metabolites. Research and scholarly activities on silent gene clusters have grown exponentially over the past two decades reflecting growing research interests. Several strategies have been developed to activate the silent gene clusters with notable success (Fig. 6).

Co-culture triggered production of bioactive secondary metabolites

It is well established that bacterial production of secondary metabolites is greatly influenced by mutual communications-often in the form of bioactive molecules- among microbes. Such signals may induce the production of certain molecules while inhibiting others, thus generating a different set of metabolite production patterns. While exact functions of bacterial secondary metabolites in natural conditions remain unclear, bioactive molecules such as

antibiotics that are produced in sub-lethal concentration under natural conditions may serve as signaling functions rather than chemical weapons. This hypothesis was verified by the work of several groups where sub-lethal concentrations of antibiotics are found to alter the compositions of microbial communities, modulate nutrient utilization, gene expression patterns (Goh et al. 2002, Vaz Jauri et al. 2013). Co-culture strategies allow for such signaling interactions between microorganisms, leading to activation of otherwise silent (cryptic) gene biosynthetic gene clusters of microbial metabolites, and thus offer unique opportunities for harnessing the full biosynthetic potential of the organism (Moody 2014). When optimized, co-culture offers a simpler, efficient, and cost-effective approach compared to genetic engineering-mediated activation of cryptic gene clusters. Several approaches have been successfully deployed to facilitate microbial communications, including the use of a semipermeable membrane, a microfluidic system (M. H. Wu et al. 2010), cultures in microdroplets (Park et al. 2011), transwell cultures with shared organic volatile metabolites (Bacchus et al. 2012), and immobilized mixed cultures in the gel (Pham and Kim 2012) (Fig. 6). Mahler and co-workers demonstrated the production of antimicrobial compounds through the stimulation of *S. hygroscopicus* by mixing with other droplets containing *S. griseus*. Johnston and co-workers have overcome the long-existing limitations of gel-based co-culture by improving stability, reusability, and storage (Johnston et al. 2020). The authors have also demonstrated on-demand production of several secondary metabolites using the bacteria-immobilized hydrogel.

Co-cultures that allow for cell-to-cell contact have been proven successful in activating cryptic biosynthetic gene clusters. The Abe group has utilized the co-culture of actinomycetes with a mycolic acid-containing bacterium to produce numerous novel metabolites including mirilactams (Hoshino et al. 2018), catenulobactins (Hoshino et al. 2018), and chojalactones

(Hoshino et al. 2019, Hoshino et al. 2015). Antimicrobial agent borrelidin J was produced through a co-culture of marine-derived actinomycete *Streptomyces rochei* MB037 and the fungus *Rhinocladiella similis* 35 (Yu et al. 2019) (Fig. 5). Similarly, a unique anthracycline antibiotic keyicin was produced through a co-culture of *Micrononospora sp.* and *Rhodococcus sp.* (Adnani et al. 2017). The authors also employed a specialized culture system that separated two species, but allowed for diffusion of chemical signals to demonstrate keyicin production is independent of cell-to-cell physical contact (Adnani *et al.* 2017)(Fig. 5). These reports are representatives of the successful application of co-culture strategy in the production of new secondary metabolites by actinomycetes. Despite the successes of small-scale co-cultures, the technique is utilized in a limited capacity in industrial biotechnology (Bader et al. 2010). Common co-culture techniques applied in the industry include the production of food products such as cheese (Martin et al. 2001), yogurt (Sodini et al. 2000), sourdough(Kariluoto et al. 2006), and whisky(van Beek and Priest 2002). Concerning actinomycetes, pure cultures are commonly used in the production of desired products, and sterile conditions are maintained to eliminate contaminations. Continuation of innovations in co-culture techniques will likely expedite the discovery of novel bioactive metabolites from actinomycetes in the years to come and pave the way for optimization for industrial-scale production.

Other strategies to activate cryptic pathways include optimization of growth media, the inclusion of stressors, and supplementation of growth media with environmental sample extracts, and heterologous production of metabolites of interest using strong promotors and pathway activators. In an earlier report, Baltz has estimated that a handful of antibiotics such as streptothricin, streptomycin, tetracycline, and actinomycin D are produced at frequencies ranging from 10^{-1}-10^{-3}. The Wright lab hypothesized that the inactivation of biosynthetic pathways of

such dominant metabolites can activate silent gene clusters leading to new metabolites production (Culp et al. 2019). To test this hypothesis, Wright lab identified highly conserved regions in the select genes involved in streptomycin and streptothricin biosynthesis, and used a pCRISPR-Cas9 system to inactivate these pathways. The lab successfully inactivated these pathways in 11 out of 14 selected strains in a matter of weeks. Through inactivation of streptomycin biosynthesis, Wright lab discovered new members of a rare class of metabolites including thiolactomycin, amicetin, phenanthroviridin and 5-chloro-3-formylindole (Culp *et al.* 2019). First, this work offers a new approach for rapid inactivation of biosynthetic genes-one of the major hurdles in biosynthetic pathway study. Second, this work makes a compelling case for a need to revisit actinomycete isolates that were deemed less attractive for new bioactive metabolite production.

Multi-Omics guided discovery of actinomycetes metabolites

Genomics

Select sequencings of genomic loci corresponding to the biosynthesis of target metabolites were the focus of early genomic studies. In 2002, the complete genome sequence *S. coelicolor* A3(2)–the first published fully sequenced actinomycete genome – not only provided a genetic basis for the production of known metabolites (Bentley *et al.* 2002) but also revealed many biosynthetic gene clusters for other metabolites not produced by the strain under standard culture conditions. The whole-genome sequence of avermectin producer *S. avermitilis* published in 2003 revealed similar findings, sparking interest for the whole genome sequencing of actinomycetes (Ikeda et al. 2003). Rapid improvement of DNA sequencing technology on both throughput and accuracy and the decreasing cost made sequencing of whole genomes affordable to the labs across the globe. As a result, genomic sequence data of actinomycetes grew

exponentially over the past 20 years. At the time of preparation of this review, there were 1760 complete genomic sequence data of actinobacteria available on the National Center for Biotechnology Information (NCBI). While the majority of these deposits represent the genome of pathogenic/symbiotic actinobacterial species such as *Mycobacterium sps. Bifidobacterium sps.* and *Cornebacterium spp.*, bioactive metabolites producer actinomycetes constitute a significant portion of the database. For instance, *Streptomyces* genomes represent 12.2% (216) of the actinomycetes genomes in the repository. It is also noteworthy that the vast majority of genomic sequence deposits contain small gaps between contigs, and thus are considered as partial actinomycetes genomic sequence in this report.

The growth of genomic sequence data eclipsed the evolution of the bioinformatics field and the rapid growth of knowledge on biosynthetic pathways for structurally diverse bacterial metabolites. Cumulatively, a new frontier emerged for the discovery of new secondary metabolites – namely "genome mining". Bioinformatics tools such as antiSMASH have facilitated the rapid identification of biosynthetic gene clusters in the bacterial genome (Blin et al. 2013, Weber et al. 2015). For instance, Baltz identified 5-48 secondary metabolite biosynthetic gene clusters per genome when he analyzed the genome of 22 actinomycetes using antiSMASH 3.0 (Baltz 2017). Newer versions of antiSMASH offer predictions for the molecular structure of encoded metabolites-a major boost for the discovery of new secondary bacterial metabolites (Blin et al. 2019, Blin et al. 2017). Biosynthetic gene clusters for 477 ribosomally synthesized and post-translationally modified peptides (RiPP) were identified through the analysis of 629 complete actinobacterial genomes using BAGEL3 (Poorinmohammad et al. 2019, van Heel et al. 2013).

Bioactive metabolites isolated based on genome mining data in recent years has translated bioinformatics predictions into realities. Shi and coworkers have used targeted genome mining to identify glycosylated peptides kitacinnamycins- a small group of stimulators of interferon genes (STING) protein- in the genome of *Kitasatospora* sp. CGMCC 16924 and produce these compounds through fermentation (Shi et al. 2019) (Fig. 5). Thiovarsolins- members of a new structural class of RiPP- were isolated from *Streptomyces varsoviensis* based on the prediction of the RiPPER (Santos-Aberturas et al. 2019). Highly cytotoxic tiancimycins, the members of enediynes class metabolites, were discovered through RT-PCR-based genome mining of 3400 actinomycetes (Yan et al. 2016). Similarly, many phosphonate-class of metabolites including anti-bacterial agent argolaphos A and phosphonocystoximic acid were isolated from the strains identified through PCR-based genome scanning of 10,000 actinomycetes (Ju et al. 2015) (Fig. 5).

It is also noteworthy that comparative genomic analysis allows for the identification of phylogenetically distinct lineage of biosynthetic and resistant genes associated with the new or novel metabolites. Culp and co-workers utilized phylogenetic analysis to identify a distinct clade for non-ribosomal peptide synthase (NRPS) condensation domains for the glycopeptide biosynthetic gene clusters that lacked known self-resistance genes (Culp et al. 2020). This approach resulted in the discovery of a new functional class of glycopeptide antibiotic corbomycin that displays anti-MRSA activity through a novel mechanism while maintaining a low level of resistance (Fig. 5). Genomic sequence data inventory has also aided rationalized engineering of pathways for new molecules utilizing two or more biosynthetic pathways- the process is known as combinatorial biosynthesis. Structurally diverse classes of unnatural natural products generated through biosynthetic pathway engineering in the past two decades are

particularly noteworthy (Baltz 2014, Chen et al. 2017, Kharel and Rohr 2012, Kunakom and Eustáquio 2020, Niu et al. 2017, Romanowski and Eustáquio 2020, Sardar and Schmidt 2016, Wong and Khosla 2012). A robust system for the assembly of biosynthetic genes is crucial for the heterologous production of metabolites. Time and labor-intensive cloning steps, and errors in the assembly process are widely recognized barriers in the production of pathway engineered metabolites. Remarkable progress has been made in recent years in cloning large gene clusters with minimal efforts. Transformation-associated recombination (TAR) cloning system developed by the Moore lab is particularly noteworthy (Yamanaka et al. 2014, Zhang et al. 2019). The TAR system employs direct cloning of large DNA fragments through in vivo recombination in the yeast. This plug-and-play system offers an excellent means to express gene clusters in heterologous hosts whereas the PCR-targeted gene inactivation technique developed earlier (Gust et al. 2003) allows for the inactivation of select genes seamlessly. These developments in synthetic biology/biosynthetic pathway engineering have offered a viable platform for the production of natural/pathway-engineered metabolites.

Transcriptomics and proteomics

Transcriptomics in actinomycetes has contributed to the uncovering of a complex network of metabolic and signaling pathways associated with differentiation and secondary metabolites production. Low-level production of secondary metabolites is one of the common barriers for the industrial application of bioactive metabolite producer actinomycetes. Understanding the mechanism of the regulation (activation vs repression; global vs secondary metabolite pathway-specific) is crucial for the engineering of strains for industrial-scale production. By measuring RNA transcripts of pathway-specific genes, the influence of regulators on secondary metabolite productions can be monitored indirectly. High throughput

transcriptional analysis in 96-well plates or microarrays and its ease of operation made transcriptomics an excellent alternative for screening for high-titer secondary metabolite producer strains before conducting more time and resource-intensive traditional culture and follow up analytical chemistry work (Wang et al. 2013). Because of its simplicity and robustness, the transcriptome study has increasingly been integrated with strain improvement programs.

Whole transcriptome analysis revealed 31 cis-regulatory RNA structures including riboswitches in the genome of acarbose producer *Actinoplanes* sp. SE50/110. Regulations of rifamycin production in *Amycolatopsis mediterranei* by *bamA* genes- homologues of γ-butyrolactone autoregulator genes- were characterized through transcriptome analysis (Aroonsri et al. 2008). Through transcription analysis of gas vesicle protein (gvp) biosynthetic genes, Huang and coworkers discovered the contribution of gas vesicles in morphological changes and overproduction of tiancimycin D in *Streptomyces* sp. CB03234-S (Huang et al. 2019). Similarly, a transcriptome study revealed thermo-regulation of validamycin biosynthetic genes with optimal production at 37 ºC (Wu et al. 2012). Production titer of secondary metabolites is often governed by a handful of enzymes that catalyze rate-limiting steps. Comprehensive analysis of biosynthetic pathway transcriptomes can help identify these bottleneck enzymes and paves the way for the discovery of new metabolites. RNA transcripts analysis can also be utilized to optimize culture conditions to activate cryptic secondary metabolite biosynthetic gene clusters. For unculturable bacteria, transcriptome studies can offer guidance to develop a culturable medium composition. Bomar and co-workers generated a metatranscriptome of the gut microbiome from the medicinal leech *Hirudo verbena* using high-throughput RNA sequencing and identify a mucin-containing medium to grow the well-known unculturable symbiont

543 *Aeromonas veronii* (Bomar et al. 2011)**.** Culture of obligate parasitic bacterium *Tropheryma*
544 *whipplei*-the causative agent of Whipple's disease was a major challenge until the understanding
545 of genome sequence-based metabolic models that revealed the absence of biosynthetic pathways
546 for 16 amino acids in the bacterium(Bentley et al. 2003, Gutleben et al. 2018). The bacterium
547 could be grown successfully in a medium including these amino acids (Renesto et al. 2003).
548 Such an approach can be applied to develop a medium formulation to grow unculturable
549 actinomycetes. Similarly, genomic data-guided metabolic pathway analysis revealed the lack of
550 terminal oxidases involved in aerobic and anaerobic respiration in *Coxiella burnetti*, another
551 obligate bacterial pathogen. The transcriptomic study indicated the deficiency of amino acids in
552 the previously used media. This led to a design of optimal growth conditions by incorporating
553 casamino acid and L-cysteine in the medium and maintained oxygen tension below 2.5%
554 (Gutleben *et al.* 2018, Omsland et al. 2009). These are reports highlight the potential of
555 transcriptomics and genomics in designing culture conditions for difficult-to-culture bacteria
556 including actinomycetes. Likewise genomics and transcriptomics, proteomics has also proven to
557 be an invaluable tool for enhancing actinobacterial metabolite discovery. Applications of
558 proteomics in natural product discovery have been extensively covered in a recent review by Du
559 and Wezel (Du and van Wezel 2018).
560
561 **Metabolomics**

562 Metabolomics has become an integral part of actinomycete-based bioactive molecule
563 discovery programs. It has offered a solution to the rediscovery of known metabolites in the
564 actinomycetes-the major shortcoming of conventional actinomycete metabolite research
565 programs. Metabolite screening is being routinely used for the dereplication of strains during

early-stage screening and the identification of new or potentially novel metabolite producers. Crude extracts of metabolites are analyzed using Liquid Chromatography-Mass spectrometry (LC-MS), and thusly acquired MS data are searched against comprehensive databases such as MarinLiT (for marine-derived natural products) (Munro and Blunt 1999) and AntiBase (Natural Products Identifier) (Laatsch 2017). Such an approach is credited for the discovery of numerous new/novel molecules including puromycin C (cytotoxic metabolite) from Streptomyces sp. PU-14G (Abbas et al. 2018), abyssomicin W from *Streptomyces* sp. LC-6-2(X. Wang et al. 2017), cyphomycin (an antifungal agent) from *Streptomyces* sp. (Chevrette *et al.* 2019), herbimycin D (Hsp90α inhibitor) from *Streptomyces* sp. RM-7-15(Shaaban et al. 2013), SF2446A2 from *Streptomyces* strain (Reimer et al. 2015), and antimycin B1 from *Streptomyces lusitanus* (Han et al. 2012). Bugni lab developed a script that automates the search of MS data of metabolites against the AntiBase and conducts principal component analysis (PCA) to identify the uniqueness of metabolites (Chanana et al. 2017, Hou et al. 2012). Such strain prioritization approach based on the uniqueness of metabolites has (Chanana *et al.* 2017)proven to be successful in identifying novel metabolite producer actinomycetes (Adnani *et al.* 2017). Molecular networking that offers searchable MS-MS/MS data can be equally valuable to enhance metabolite-footprint-based strain dereplication. MS-MS/MS data of metabolites can be searched on the Global Natural Products Social Molecular Networking (GNPS) to identify the family of molecules based on spectral similarities and statistical analysis (M. Wang et al. 2016). Karan and co-workers identified several new members of manzamine alkaloids using MS/MS data in the GNPS demonstrating its potential in strain dereplication and identification of new molecules (Karan et al. 2020).

588 Several MS-based innovative solutions have emerged in recent years to address the
589 shortcomings of conventional metabolite profiling approaches that involved effort-intensive and
590 time-consuming analytical processes (Bouslimani et al. 2014). Mahler and co-workers have
591 coupled MS-spectrometry with the high pico-droplet-based-ultra high throughput screening
592 system to identify metabolites from actinomycetes (Mahler et al. 2018). Dorrestein lab developed
593 a system that utilizes matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) to
594 analyze metabolites produced in a live bacterial colony directly without a need for sample
595 extraction and follow-up preparation. This tool is capable of not just identifying known/unique
596 metabolites but also elucidating the networks of metabolites in real-time (Fang and Dorrestein
597 2014, Watrous et al. 2012).

598 **Conlusion**

599 Recent progress in genomics, proteomics, and metabolomics helped uncover
600 actinobacterial gems that were previously out of sight. The setbacks during the mid to late 20th
601 century associated with traditional metabolite production/analysis were overcome by numerous
602 innovations in the field such as the development of high to ultra-high-throughput screening,
603 automation in metabolite profiling and novelty assessing, culture techniques, bioinformatics, and
604 next-generation sequencing. Development of novel culture techniques significantly improved
605 throughput with minimal use of resources, overcoming one of the major limitations of traditional
606 fermentations. Co-culture techniques that exploit inter-organism communications to activate
607 dormant biosynthetic pathways, evolved in recent years, making their ways to mainstream
608 fermentation. Similarly, we have witnessed a significant development in bacterial isolation
609 techniques which has allowed for isolations of less known actinomycetes. Whole-genome
610 analysis of actinobacterial species uncovered enormous untapped biosynthetic potential. As

611 transcriptomics is becoming handy to elucidate the network of molecular signaling and to offer
612 hints to culture conditions for so-called “unculturable”, exponentially growing genomic sequence
613 data has expanded access to new metabolite discovery. In summation, actinobacterial natural
614 products research has enjoyed a renaissance in the twenty-first century.

615 The success of small molecule-based drug discovery and development relies on a variety
616 of factors including the number of leads, the uniqueness in both structures and interactions with
617 the target, physicochemical properties, and the reliability of the production system (Scannell et
618 al. 2012). Once considered to be a revolutionary approach, chemists would generate a library of
619 small molecules via high throughput chemical synthesis. Next, the small molecular library would
620 be assessed via a high-throughput screen to identify molecules that interacted with a biological
621 target. However, this approach has realized limited success (Hingorani et al. 2019).
622 Actinobacterial natural products are often complex in structure, which makes the chemical
623 synthesis of such molecules impractical. With the remarkable biosynthetic potential of
624 actinomycetes and proven clinical applications of their products, research into actinomycete
625 natural product discovery arguably deserves continued investment. High throughput droplet-
626 based culture techniques have provided an unparalleled platform to expedite the identification of
627 new metabolite-producing actinomycetes. At present, this approach is limited to a few proof-of-
628 concept demonstrations by a few laboratories, as opposed to being a widely adopted screening
629 platform in the larger research community. Further simplification of this technique will likely
630 allow for labs across the globe to increase the scale and throughput at which new actinomycete
631 producers are identified. Combining this approach with PCA analysis, and the automation in
632 bioactivity screening system may offer unprecedented insights into the identification of new
633 bioactive molecule producer actinomycetes. Arguably, the continuous growth of genome

sequence data and the body of knowledge on biosynthetic genes along with the improvement in bioinformatics will significantly enhance the discovery of new actinobacterial metabolites. This will also facilitate the custom design of growth conditions for fermentation of species that are currently out of reach. New technologies from the field of synthetic biology will putatively uncover the biosynthetic potential of silent gene clusters. Concomitantly, the heterologous expression in actinomycete "superhosts" will provide valuable platform chassis for the reconstitution of cryptic natural products. Considering the vast reserve of biosynthetic potential and recent advancements in multi-omics techniques, actinobacteria will continue to be one of the most prolific natural product sources in the future.

Acknowledgments

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under Award No. R15CA252830 (S.E.N.) and the National Science Foundation under Grant No. ENG-2015951 (S.E.N.).

Conflict of Interest

The authors declare that there is no conflict of interest.

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Figure Legends

Figure 1. Publication trend on actinomycetes from 1940 to 2018. The data were retrieved from the ISI Web of Science. The search term "actinomycetes" was used to extract the data from PubMed.

Figure 2. Publication trend on rare actinomycetes from 1950 to 2019. The data were retrieved from PubMed. The search term "Rare actinomycetes" was used to extract the data from PubMed.

Figure 3. Recently used approaches for the discovery of bioactive metabolites from actinomycetes. While exploration of actinomycetes from unique environments has been conducted for many years, iChip technique, diffusion chamber-facilitated culture, examination of endophytic and symbiotic bacteria, and metabolite profiling-derived discovery of bioactive metabolites represent recently developed approaches. Pictures of natural environments are taken from the creative commons and marked as "dedicated to public domains" (Weblinks: https://search.creativecommons.org/photos/32636618-a8a5-4dcf-81c6-de3ee87f5a6e; https://search.creativecommons.org/photos/c4b808de-03de-47a8-8c7a-3c8924ec0ff2; https://search.creativecommons.org/photos/3be5e8bc-3ac6-4e5c-a9f4-bb717e258998; https://search.creativecommons.org/photos/f22282da-d4f9-4400-8051-dfbdf8da0844)

Figure 4. Representative examples of bioactive metabolites isolated from actinomycetes retrieved from the unique environmental origin (a), and symbiotic actinomycetes (b)

Figure 5. Examples of new metabolites produced by endophytic actinomycetes (A), through co-cultures (B) and genome mining (C)

Figure 6. Approaches for activation of silent secondary metabolites biosynthetic gene clusters. Co-culture techniques include culture of multiple organisms on agar plates, in droplets or membrane-partitioned systems. Genome-guided approach relies on identification of dormant gene clusters that follows their activation through genetic engineering or employment of activators.

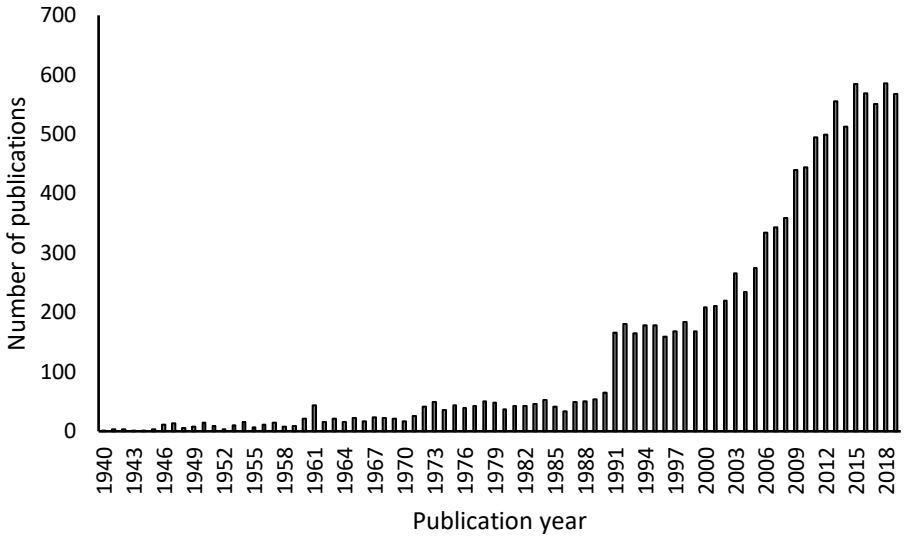
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Figure 1.

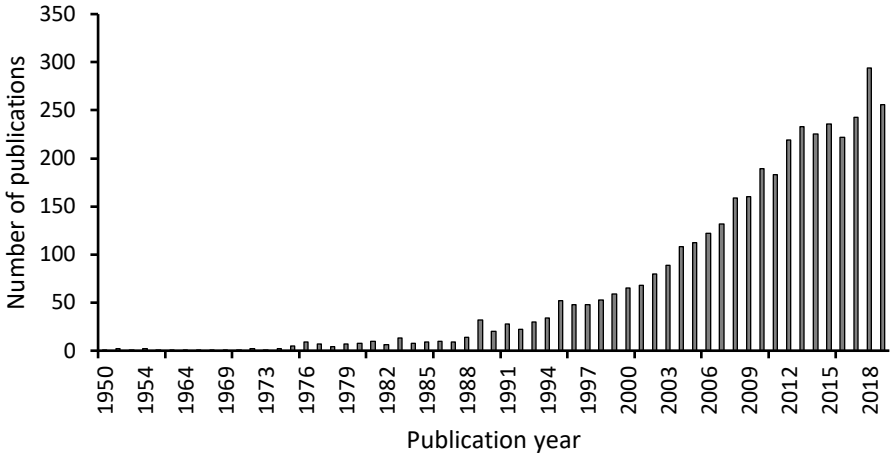
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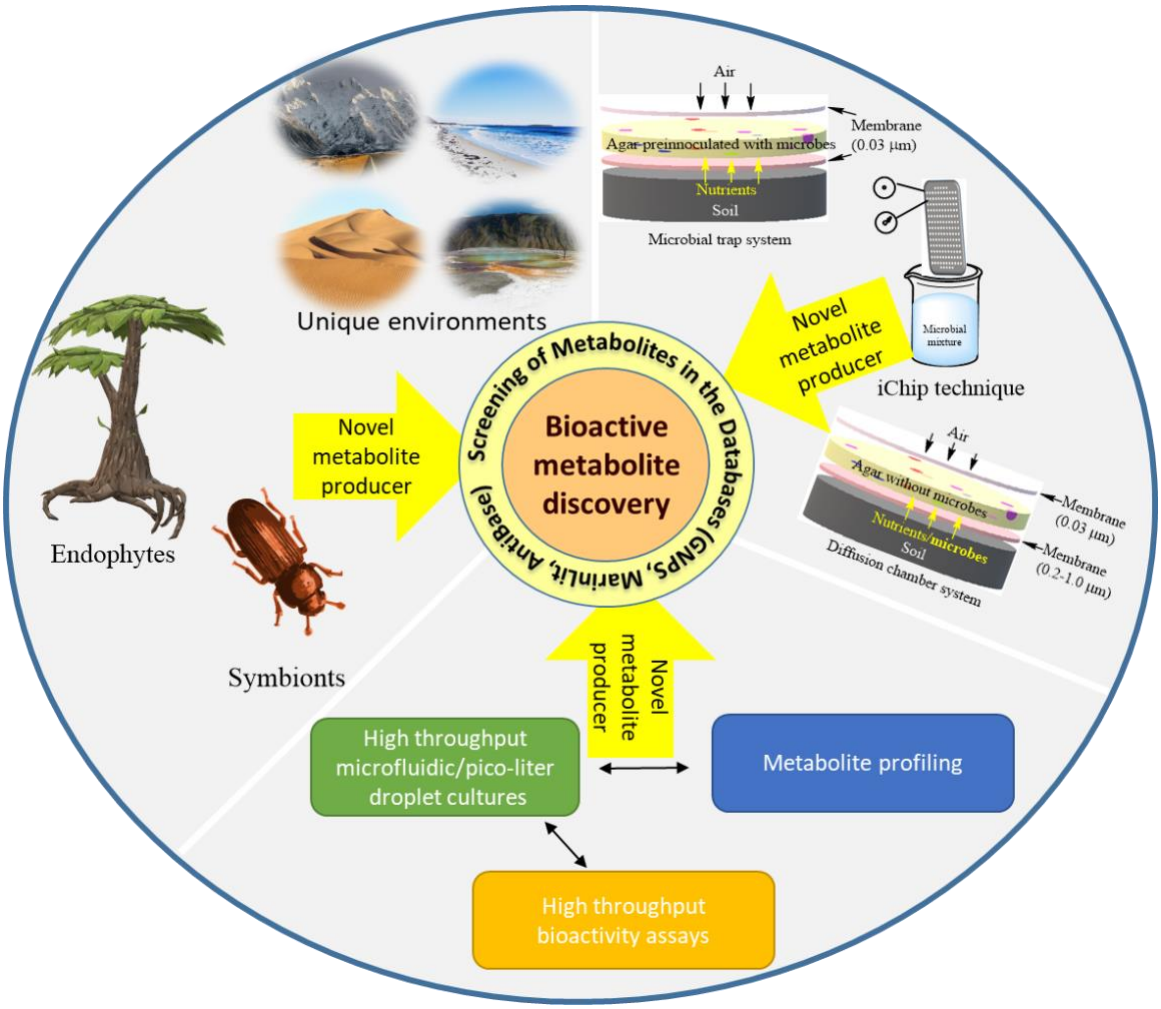
Figure 2.



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1361 **Figure 3.**

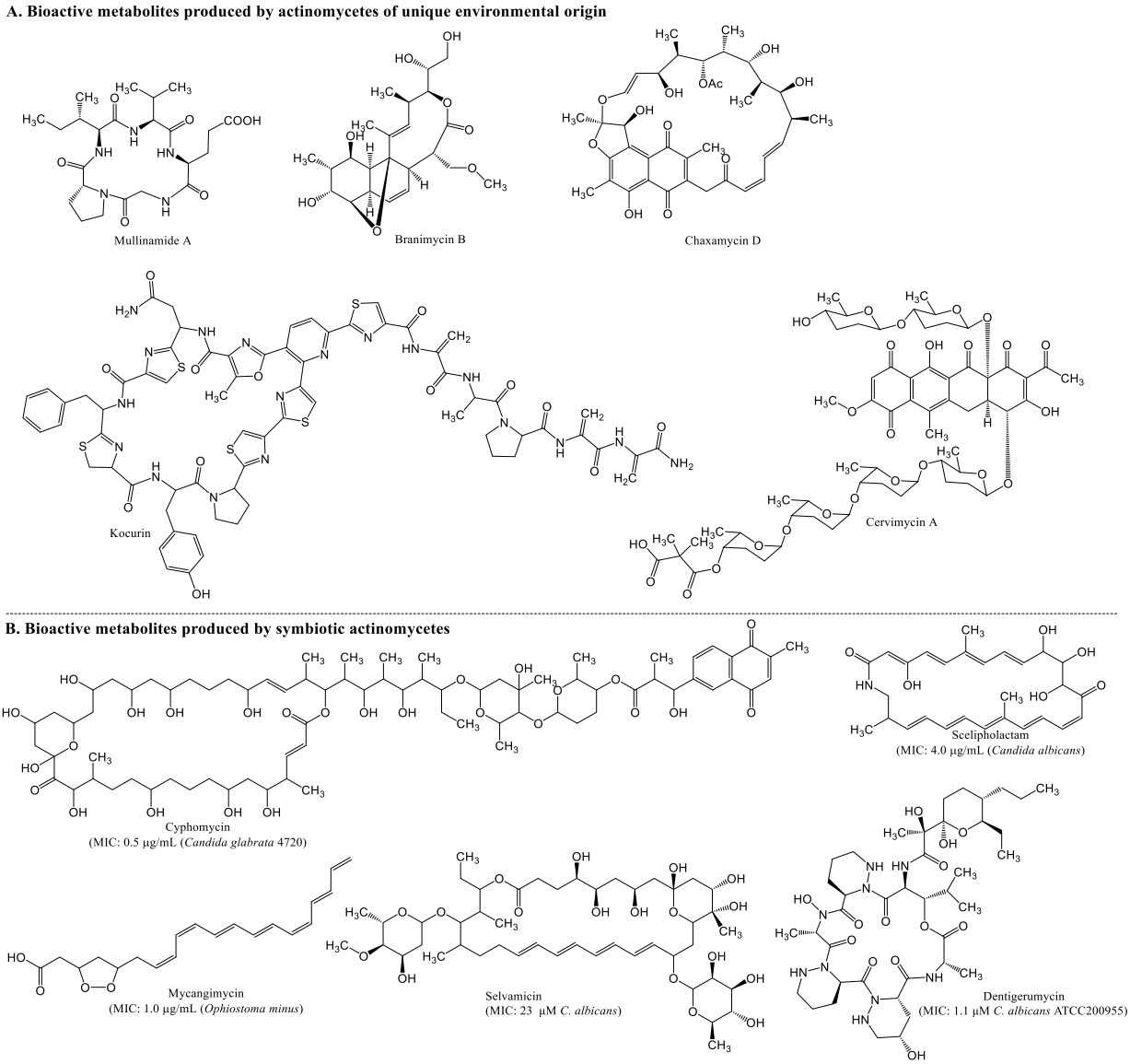
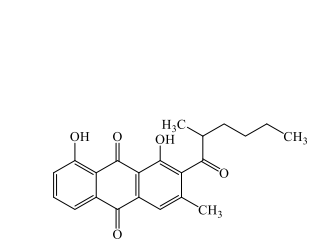
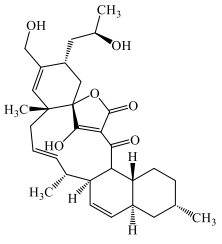

A. Bioactive metabolites produced by actinomycetes of unique environmental origin
Mullinamide A
Branimycin B
Chaxamycin D
Kocurin
Cervimycin A
B. Bioactive metabolites produced by symbiotic actinomycetes
Cyphomycin
(MIC: 0.5 μg/mL (Candida glabrata 4720)
Scelipholactam
(MIC: 4.0 μg/mL (Candida albicans)
Mycangimycin
(MIC: 1.0 μg/mL (Ophiostoma minus)
Selvamicin
(MIC: 23 μM C. albicans)
Dentigerumycin
(MIC: 1.1 μM C. albicans ATCC200955)

Figure 4.

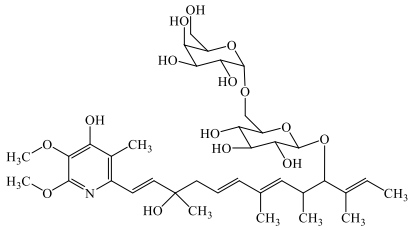
A. New bioactive metabolites produced by endophytic actinomycetes



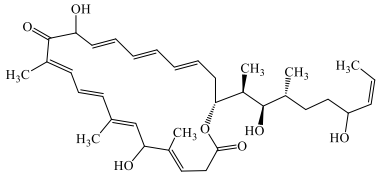
Heraclemycins C
MIC: 6.25 mg/mL (*Mycobacterium bovis-BCG strain*)



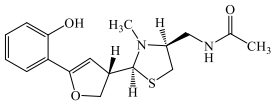
Maklamycin
MIC: 0.2 mg/mL (*Micrococcus luteus*)



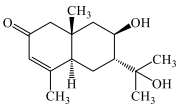
Glucopiericidinol A3
Inhibition zone:9.8±0.3 mm with 20 mg of compound
(*Staphylococcus aureus*)



Hamuramicin A
MIC: 4.0 mg/mL (*Kocuria rhizophia* ATCC9341)
IC_{50}: 1.1 mM (HeLa S3)

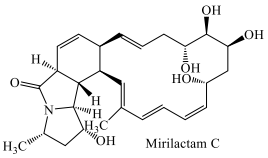


Spoxazomicin A
IC_{50}: 0.11 mg mL^{-1} (*Trypanosoma brucei brucei*)

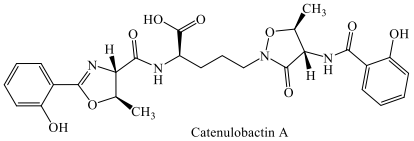


Kandenol A
MIC: 12.5 mg/mL (*Mycobacterium vaccae IMET 10670*)

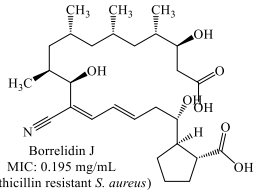
B. New metabolites produced through co-culture



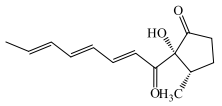
Mirilactam C



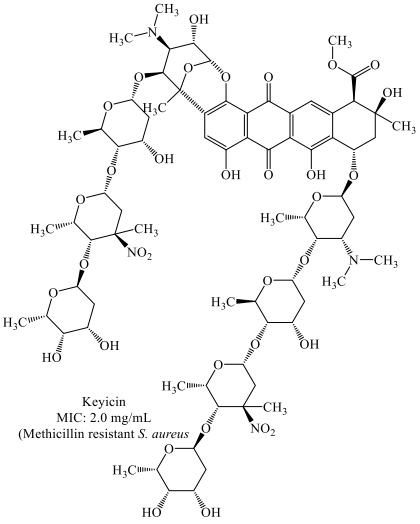
Catenulobactin A



Borrelidin J
MIC: 0.195 mg/mL
(Methicillin resistant *S. aureus*)

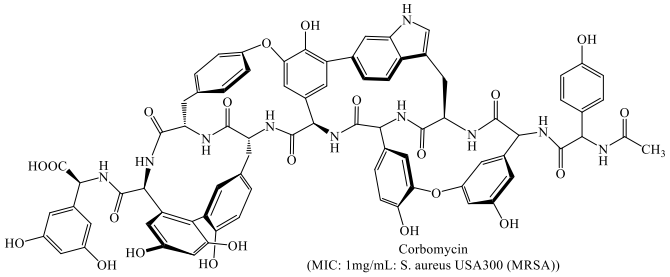


Chojalactone A
(a signaling molecule)

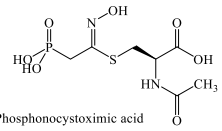


Keyicin
MIC: 2.0 mg/mL
(Methicillin resistant *S. aureus*

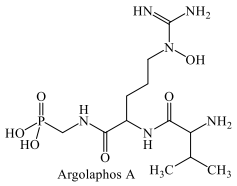
C. Actinomycetes metabolites discovered with the aid of genomics



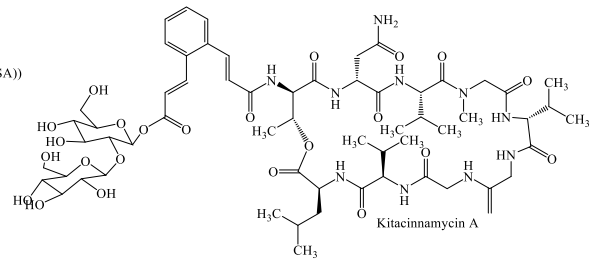
Corbomycin
(MIC: 1mg/mL: S. aureus USA300 (MRSA))



Phosphonocystoximic acid



Argolaphos A



Kitacinnamycin A

Figure 5.

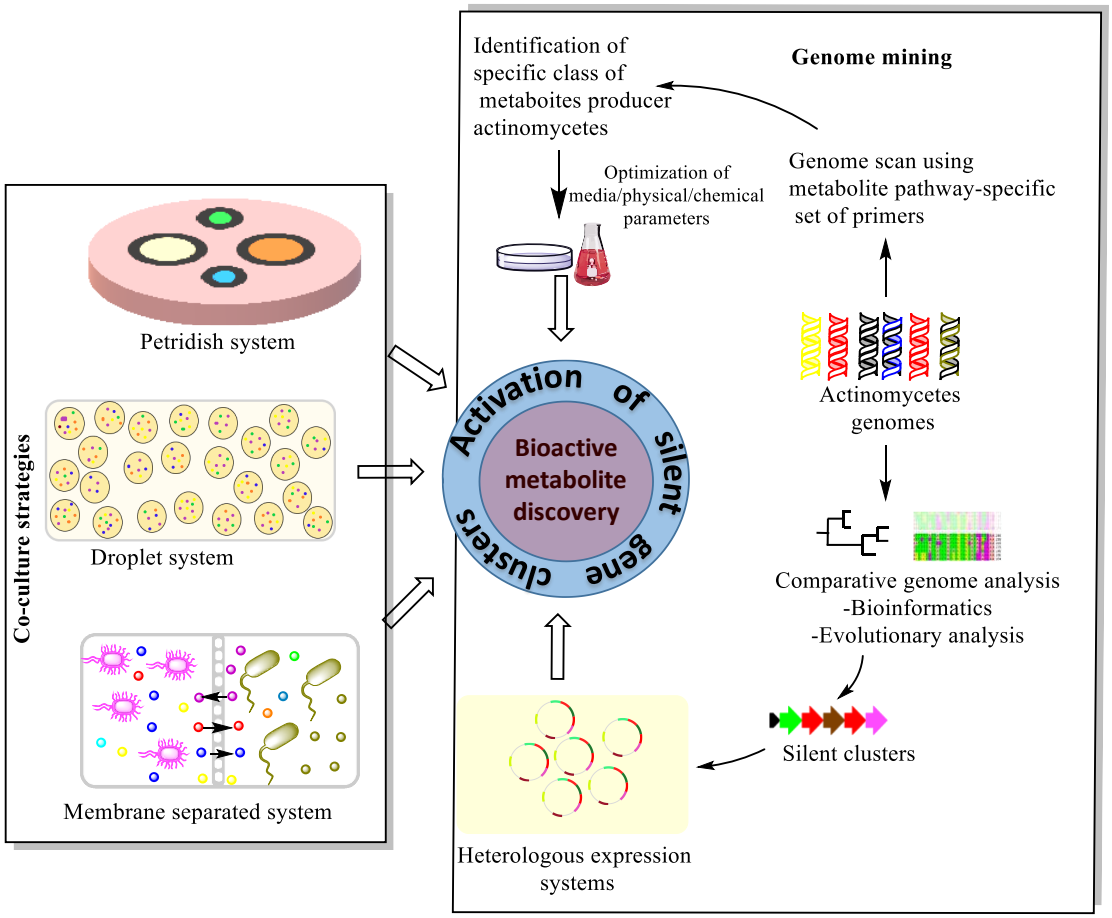

Co-culture strategies
Petridish system
Droplet system
Membrane separated system
Identification of specific class of metaboites producer actinomycetes
Genome mining
Optimization of media/physical/chemical parameters
Genome scan using metabolite pathway-specific set of primers
Actinomycetes genomes
Activation of silent gene clusters
Bioactive metabolite discovery
Comparative genome analysis
-Bioinformatics
-Evolutionary analysis
Silent clusters
Heterologous expression systems

Figure 6.

Author contribution statement:

M K. Kharel conceived the project and took the lead in writing. J. Ossai, B. Khatabi, S. E. Nybo contributed to writing and literature search.