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MICROBIAL METABOLITES AND SECONDARY METABOLISM: BIOCHEMICAL PATHWAYS IN DRUG DISCOVERY

Sabahat Ali Sheikh*
Lecturer, Faculty of Allied Health Sciences University of Lahore, Sargodha Campus. Corresponding Author Email: Sabahat.ali@uipt.uol.edu.pk
Fatima Bibi
Lecturer Chemistry, GGDC Daggar, Buner. fatimaubaid137@gmail.com
Fatima bibi
Lecturer Chemistry, GGDC Buner. fatmashehwar@gmail.com
Izma Ali
MPhil Scholar in Chemistry, University of Sargodha. izmaali698@gmail.com

Author Details

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Corresponding E-mails & Authors*:
Sabahat Ali Sheikh
Sabahat.ali@uipt.uol.edu.pk

Abstract

Microbes have been an amazing source of bioactive molecules, especially thanks to their secondary metabolism that results in the synthesis of structurally diverse metabolites that are incredibly valuable as drugs. Contrary to primary metabolites that are required for cell sustenance and reproduction, secondary metabolites are biosynthesized under certain circumstances and play adaptive functions, such as in antimicrobial defense, competitive advantage, and signaling. Such compounds especially those derived from actinomycetes, fungi, and endophytic bacteria, have significantly contributed to the development of antibiotics, immunosuppressors, anticancer agents and other drug leads. The biochemistry of secondary metabolism is elaborate and controlled by highly regulated enzymatic networks such as non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), and hybrid pathways (with unprecedented chemical diversity and novel pharmacophores). Given the contributions of microbial secondary metabolism to modern drug discovery, the study of microbial

secondary metabolism is undergoing a revolution driven by omics technologies and the bioinformatics and genome mining tools that rapidly exploit the massive datasets generated by these technologies. These methods enable the discovery of "silent" biosynthetic gene clusters, a subset of which will produce novel metabolites of unknown biological function. Moreover, advances in metabolic engineering and synthetic biology now allow the heterologous expression of these clusters in well-developed microbial hosts, which has led to enhanced titers, as well as to the generation of unprecedented structural divergency. Biochemical analyses have also uncovered that the expression of otherwise silent metabolic pathways can be initiated in response to environmental signals, or by epigenetic modifiers or microbes., leading to exciting new areas for therapeutics. This research is concerned with the biochemistry of microbial secondary metabolic pathways and their contribution to the development of drug lead discovery today. It provides a critical analysis of the role played by secondary metabolites as molecular architecture for drug design and discusses to what extent this can be useful in the development of new therapeutics. Furthermore, the thesis investigates possible shortcomings of existing screening approaches and introduces recent developments in pathway prediction and metabolite dereplication. Combining microbiological understanding with biochemical technologies, this work emphasizes the unexplored capabilities of microbial metabolism to fuel the mounting worldwide need for new drugs amidst rising antibiotic resistance and emerging diseases.

1. INTRODUCTION

Microorganisms are an enormous and largely untapped source of chemically diverse and biologically active substances. Microbial secondary metabolites have been highly influential in the fabric of modern medicine, ever since penicillin was first isolated in

1928, particularly with respect to antibiotics (6, 7) and chemotherapeutics. Although primary metabolites are essential for growth and survival, secondary metabolites are produced during the course of specialized metabolic pathways, generally in response to stress or under nutrient-limited conditions. These not directly life supporting compounds providing strains with evolutionary benefits like competitive inhibition of adversaries, inter-species communication or ecological fitness. Their structural complexity and functional specificity make them highly valuable in drug discovery and excellent targets for pharmacological innovation (Bérdy, 2012). The role of secondary metabolites in drug discovery can be traced back to their bioactivity and ecological activities. Gram-positive bacteria such as actinomycetes are especially remarkable in that they provide over 70 % of all antibiotics utilised in medical practice, such as streptomycin, tetracycline and erythromycin. (Other fungi, like Penicillium and Aspergillus, have generated such lifesaving compounds as penicillin and statins.) These organisms harbors a set of unique biosynthetic gene cluster (BGC), consisting of the enzymatic machinery such as polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS), and hybrids, reflecting the type of the secondary metabolite produced. These enzymatic platforms enable formation of complex molecules in diverse bioactive families, and provide unique chemical scaffolds for drug discovery with potential novel mechanisms of action (Newman & Cragg, 2020).

The last few years have seen a transformation of our understanding of microbial secondary metabolism due to advances in molecular biology, genomics and metabolomics. The increasing number of sequenced microbial genomes has unveiled a surprisingly large reservoir of still inactive or “silent” BGCs which do not produce any product in the wild-type directly amenable to detection under standard lab conditions.

These cryptic clusters are an untapped resource of beneficial medicines. Genome mining with YESS, coupled with CRISPR-mediated induction systems, heterologous expression approaches and synthetic biology techniques, is being used to discover and access these cryptic genes. Furthermore, the coupling of metabolomic approaches with next-generation sequencing has aided in the discovery and description of new metabolites, enabling the connection of gene clusters with their metabolic products (Medema et al., 2015). Secondary metabolites are important in the adaptation of plants and other organisms to their environment, and environment factors have a significant influence on the control of secondary metabolism. Ecological stimuli, such as competition, nutrient deficiency, or association with host organisms, frequently result in the activation of secondary metabolic pathways in microorganisms. For instance, co-culture with other microorganisms or signals of different types of stress may trigger the biosynthesis of a so for not identified metabolite. These results highlight the need to recapitulate natural ecological conditions when studying microbial potential in the lab. In addition, drugs targeting epigenetic modifications such as histone deacetylase inhibitors have been used to induce cryptic biosynthetic gene clusters in fungi, leading to the discovery of bioactive compounds that are not detected under such conditions when grown in standard culture media (Brakhage, 2013).

Biochemically, secondary metabolism also occurs in a highly ordered and compartmentalized manner. For example, polyketides are produced by the iterative catalysis of condensation reactions by PKS enzymes that are homologous to the fatty acid synthases. In the same way, the NRPS systems also polymerize complex peptides through the concerted actions of modular enzyme complexes that select, activate, and modify specific amino acid residues. In many systems, atypical starter units, post-

assembly modifying enzymes and non-standard cofactors are utilized in order to create metabolites with increased structural diversity and bioactivity. This knowledge of the biochemical pathways is important for the development of new and improved derivatives through mutasynthesis, combinatorial biosynthesis and pathway refactoring (Weber & Kim, 2016).

Microbial secondary metabolites including that of antibiotics are also important drug drugs. A few clinically useful anticancer drugs, immunosuppressants, antimalarials, and cholesterol-lowering agents are of microbial origin. For example, doxorubicin (an anthracycline antibiotic) and bleomycin (a glycopeptide) are isolated from Streptomyces species and are popularly used in the treatment of cancer. Similarly, the fungal metabolite, cyclosporin, has revolutionized organ transplants by combating immune rejection. Microbial metabolites are, in addition, not only inherently biologically active compounds, but are also chemically modifiable to produce semi-synthetic products with a greater potency and reduced toxicity (Demain & Fang, 2000). However, with their great potential, the original methods for identifying microbial metabolites, which generally rely on random screening, inevitably have shortcomings. All too often, such methods result in the re-isolation of known compounds; moreover, in my chemo-genomic analyses of microbial genomes, I have found the chemical space of natural products in microbial genomes is limited in the extreme. More recently, dereplication approaches by which known compounds may be more quickly identified based on spectral and structural data, have greatly accelerated the discovery process. Together with the use of machine learning and artificial intelligence, rationally designed drug discovery includes exploration of microbial metabolomes in a much more targeted and

rational way, thus saving time and costs and giving better hit rates (van Santen et al., 2019).

More significantly, with the urgency of antibiotic resistance and lack of advances in new drug approvals, a fresh source of therapeutics is desperately required. Pathogenic bacteria including M ycobacterium tuberculosis, Staphylococcus aureus, as well as certain Gram-negative bacteria, have developed resistance against the majority of major classes of antibiotics. In this respect, a good prospect for identifying compounds with new targets and mechanism of action is provided by the microbial secondary metabolism. Moreover, developments in metabolic engineering allow us to engineer microbial pathways to generate large quantities of rare or highly active metabolites creating a sustainable and economical platform for drug production (Lewis, 2013).

This thesis will study the biochemical pathways associated with the production of microbial secondary metabolites and evaluate their perception as a source of novel therapeutics. The focus is on metabolite structural classes, their biosynthetic logic and the state-of-the-art methods implemented for their discovery and improvement. The review further discusses the ecological conditions under which the structures are synthesized, the genetics and regulatory steps governing their production, and the potential to apply the discovery for drug development. In this integrative scope, the work connects microbiology and biochemistry to facilitate inquiry in a field central to human health and global drug development agendas (Harvey et al.)

2. LITERATURE REVIEW

Microbial secondary metabolites, with their enormous pharmacological potential, have been the focus of study for more than a century. Since the discovery of penicillin from

an era, in which microorganisms have served as promising pools of bioactive agents, to the development of powerful anticancer as well as immunosuppressive agents, the unique gifts from microbial world have never ceased up to now. Historically, natural soil microbes, especially those belonging to the genus Streptomyces, served as major contributors of clinically important secondary metabolites. The application of fermentation technology and high-throughput screening in the mid-20th century facilitated the discovery of those molecules, for many of which antibiotic, antifungal, and antiparasitic applications have been developed subsequently. Nonetheless, when the returns from classical screening approaches started to decline, researchers have turned to genome mining and biochemical pathway analysis to explore hidden cryptic biosynthetic potentials in microbial genomes (Berdy, 2005). Secondary metabolism of microorganisms differs significantly from primary metabolism. Primary metabolites are involved in basic life-support systems, such as energy generation, cell wall formation, and nucleotide synthesis, while secondary metabolites are non-essential, but often provide selective advantages in the natural environment. These involve competitive interactions, signalling pathways, and stress responses. More importantly, the biosynthesis of secondary metabolites are generally governed by gene clusters encoding the sets of modular enzymatic machineries, such as PKSs and NRPSs. The systems for construction of molecules in small steps bring together building blocks, sometimes unusual in nature, followed by post synthetic modifications to improve the pharmacological properties (Weber & Kim, 2016). Polyketides are the largest group of microbial secondary metabolites and many of them have been developed into drugs for clinical use. These range from antibiotics (e.g., erythromycin), anticancer agents (e.g., doxorubicin), immunosuppressants (e.g., rapamycin). Polyketide biosynthesis parallels

that of fatty acids but with a much higher level of chemical diversity. Type I PKSs are massive multienzyme proteins, compartmentalized into modules that catalyse a single cycle of chain extension and modification. PKSs are modular and this has enabled the bioengineer manipulation of biosynthetic pathways via combinatorial biosynthesis and synthetic biology, with the generation of" unnatural natural products" that are more effective as drugs (Hertweck, 2009).

Analogously, non-ribosomal peptides are biosynthesized by giant mega-enzymes non-ribosomal peptide synthetase (NRPS) complexes which function on its own ribosome-independently. Such peptides comprise a repertoire of clinically important agents such as vancomycin, bleomycin and cyclosporine. Each NRPS monomer contributes a specific amino acid or derivative, and other tailoring domains introduce structural variations such as methylation, glycosylation, and cyclization. The modularity within NRPS systems has also been useful to pathway engineers who can interchange modules, insert non-native substrates, and indeed refactor entire gene clusters for new bioactive peptides types (Walsh & Fischbach, 2010). The identification of biosynthetic gene clusters (BGCs) in microbial genomes represents a paradigmatic transformation for natural product research. Early genomics attempts have demonstrated that many species of microbes harbor orders of magnitude more BGC t han expected, given their observed metabolic production in the laboratory. Out of this observation emerged the idea of "silent" or "cryptic" gene clusters, that is, genetic regions harbouring the necessary enzymatic capacity to synthesize SMs, but not activated under routine culture conditions. Activation of these clusters is a key pursuit in drug discovery and has been approached by heterologous expression, promoter engineering and epigenetic perturbation. For example, modulation of silent pathways by histone deacetylase

inhibitors has been demonstrated to induce novel bioactive metabolites in filamentous fungi (Brakhage, 2013). Bioinformatics tools have made the value of different types of microbiomes more specific. Prediction of expected BGCs, their boundaries and likely products through tools like antiSMASH and PRISM allows strains and pathways to be ranked for further in vitro biochemical and in vivo pharmacological investigation. Furthermore, comparative genomics and transcriptomics reveal regulatory networks of secondary metabolism, such as global regulators, sigma factors, and quorum sensing systems. These findings are especially important for the rational design of production strains and for ecological knowledge on metabolite role (Medema et al., 2015).

Concurring of microbes is also a newly developed way to elicit silent metabolic pathways. Microorganisms in nature are involved in dynamic relationships that affect gene expression. By emulating these interactions in vitro, scientists are able to induce the production of otherwise elusory metabolites. For instance, the co-culture of marine actinomycetes and some fungi has been found to produce some new antibiotics and cytotoxic compounds. These results are instructive on the role of microbial ecology in SM expression and support the necessity of developing screening platforms that replicate natural ecological factors (Bertrand et al., 2014). Secondary metabolites are structurally diverse and functionally flexible. They are not only known as an antimicrobial agent, but are antiviral, antifungal, anticancer, antiinfiammatory, and immunosuppressive agents. This functional promiscuity is frequently associated with the capacity of secondary metabolites to interact with several cellular targets or to control intricate biochemical pathways. For examples, anticancer activity of actinomycin D is attributed to the DNA intercalation and inhibition of RNA synthesis, and rapamycin is the inhibitor of mTOR signaling, a central regulator of cell growth and metabolism.

These multi-tiered processes highlight beneficial aspects of microbial metabolites beyond their direct anti-infective effects (Newman & Cragg, 2020). The urgent international concern of antimicrobial resistance (AMR) has brought microbial secondary metabolism back into the spotlight. As pathogens become resistant to almost all the antibiotics in clinical use, the demand for new drugs with novel mechanisms of action is pressing. Microbial metabolites, especially those derived from stenochoric or rarely investigated microbial species, are an attractive means to addressing this problem. New screening techniques, including in situ cultivation devices (iChips) and microfluidic droplet systems, have been implemented to capture the genomes of previously uncultivable microbes, thereby opening a vast reservoir of potential metabolite-producing microorganisms. The identification of the novel antibiotic teixobactin from an uncultured bacterium is a success story of these new methods (Ling et al., 2015).

The application of metabolic engineering and synthetic biology has greatly expanded the repertoire of microbial metabolites that can be produced. 8,31 BGCs can be introduced or optimized in heterologous hosts including E. coli and Streptomyces coelicolor to increase the yield, stability and availability of target compounds. Further, through pathway refactoring, biosynthetic genes can be rearranged in a modular manner to generate “non-natural” analogs with increased biological activity. Such technologies can also facilitate the construction of biosensors, feedback control systems and automated HTS platforms, thus streamlining and scaling-up the drug discovery process (Smanski et al., 2016). Besides bacteria and fungi, marine microorganisms have recently become a good reservoir of new secondary metabolites. High pressure, salinity, and scarceness of nutrients in marine ecosystems have led to the evolution of exclusive metabolic pathways. Compounds with antitumor, antiviral, and neuroprotective

potentials have been isolated and characterized from marine actinomycetes, cyanobacteria, and endophytic fungi. Though source-dependent difficulties facing the discovery of new, bioactive microbial compounds have commonly been complained about in the field (see ref (Blunt et al., 2018)), advances made in metagenomics(14) and synthetic biology(15) are proving to remove such obstacles, and applications of these technologies will benefit marine genomes (as wells as the metatranscriptomes and metaproteomes derived therefrom).

Dereplication of identified compounds continues to be a major problem in the microbial natural products field. The traditional way leads to the re-discovery of the same -well characterized- metabolites over and over again without reaping its time and resource-benefits. Current derplibation approaches utilize spectral databases, NMR reporting, and high resolution mass spectroscopy (HRMS) for rapid identification and dereplication of known compounds. With the inclusion of machine learning models, dereplication can be enhanced by predicting new compounds based on structural features and their biosynthetic origin. This will initiate research and efforts towards truely new chemical enterities(Wolfender et al., 2019). Although a great progress was achieved, there are several issues to address in the topic. The reasons fall into low metabolite yields, metabolite instability in lab environment, and complexity of regulatory networks. Furthermore, the ecological role of numerous secondary metabolites is incompletely understood, and thus predicting their function or improving production levels is in many cases difficult. The response to these challenges and the full potential of microbial secondary metabolism in drug discovery can only be harnessed through interdisciplinary research across microbiology, biochemistry, genomics and computational biology (Van Der Heul et al., 2018).

3. Biochemical Architecture of Microbial Secondary Metabolism

Microbial secondary metabolism is complex and tightly regulated, and has resulted in the production of specialized bioactive molecules. Although non-essential for fundamental cellular life, however, these compounds have assumed considerable importance in ecological interplay and as targets for drug development. This biosynthetic machinery is based on distinctive enzymatic templates and control systems that govern the variety, complexity and biological function of microbial secondary metabolites (Weber, Kim, 2016).

3.1 Polyketide Synthases (PKSs) and Biosynthetic Logic

Polyketide synthases (PKSs) are multimodular enzymes that are involved in the biosynthesis of polyketides, a large family of secondary metabolites exhibiting a wide range of therapeutic activities such as antibiotics, anticancer and immunosuppressive agents. PKSs are structurally related to FASs but vary in their domain modularity and diversity. Actinomycetes usually have type I PKS, which is a large polypeptide chains with multiple enzymatic domains organized in a modular structure. Each module performs a defined round of chain elongation and modification involving the incorporation of acetyl-CoA or malonyl-CoA units, the introduction of functional groups such as ketones, hydroxyls, or double bonds (Hertweck, 2009).

The biosynthetic programming of PKSs is orchestrated by a series of reactions, performed by core domains (ketosynthase (KS), acyltransferase (AT); acyl carrier protein (ACP)) and optional modifying domains (ketoreductase (KR), dehydratase (DH), and enoylreductase (ER)). Polyketide diversity is generated by the length and number of modules, the order of domain organization and also recruitment of non-proteinogenic extender units. Catalysis of Type II and Type III PKSs, which are dominant in bacteria and

plants, respectively, proceeds via an iteratively used mechanism with a less complex enzyme structure. This knowledge of the biosynthetic routes can be used as the basis of pathway engineering to produce new analogs for increased bioactivity (Shen, 2003).

3.2 Non-Ribosomal Peptide Synthetases (NRPSs): Structure and Function

Among the secondary metabolites, non-ribosomal peptide synthetases (NRPSs) represent the second prominent group of enzymes. In contrast to ribosomal peptide synthesis, NRPS function in an mRNA- and ribosome-free manner to synthesize peptides through a processive and modular enzymatic principle. At least three core domains, adenylation (A), thiolation or peptidyl carrier protein (PCP), and condensation (C) are present in each NRPS module. The adenylation domain loads and activates an amino acid, the PCP domain docks and transfers the activated amino acid, and the condensation domain catalyzes peptide bond formation. Further chemical diversity is incorporated through additional domains, such as epimerization (E), methylation (M), and cyclization (Cy) (Walsh & Fischbach, 2010).

NRPSs mediate the biosynthesis of a variety of bioactive peptides such as antibiotics (e.g., vancomycin), anticancer agents (e.g., bleomycin), and siderophores (e.g., enterobactin). The modular organization of NRPSs allows for structural diversity and its potential hijacking, but progress in this area, as always, can be extremely slow. Practically, by changing the organization of a module or exchanging domains from one pathway to another, scientists are enabled to produce new compounds with modified or mixed biological activities. Moreover, increased structural knowledge has given an insight into the dynamic conformational changes that occur in NRPSs whilst they carry out catalysis, explaining their mechanistic promiscuities at a greater level of detail (Strieker et al., 2010).

3.3 Hybrid Pathways and Tailoring Enzymes

A lot of the microbial secondary metabolites are produced by hybrid biosynthetic pathways sharing the characteristics of PKSs and NRPSs. These hybrid systems allow access to structurally complex and functionally varied molecules, frequently with superior pharmacological profiles. Examples of these in vitro metabolites are epothilones and bleomycin which are both products of mixed polyketide-peptide biosynthesis. In such systems, biosynthesis usually starts with a PKS module or clusters of NRPS modules, depending on metabolite structure or both (Miller et al., 2002).

Tailoring enzymes, e.g., glycosyltransferases, methyltransferases, halogenases, and hydroxylases, additionally tailor the core structures synthesized by PKSs and NRPSs. These adjustments can greatly affect the solubility, stability and bioactivity of secondary metabolites. For example, glycosylation could increase water solubility, halogenation can increase membrane permeability, or modify target specificity. The occurrence and location of these tailoring enzymes in biosynthetic gene clusters (BGCs) is a major factor contributing to the chemical diversity that is present in natural products (Fischbach & Walsh, 2006).

3.4 Regulatory Mechanisms of Secondary Metabolism

The secondary metabolism genes are strictly controlled at several levels: transcriptional, translational, and post-translational level. World-wide systems of regulation, such as two-component signal transduction systems and sigma factors, integrate input from the environment and nutrition to control gene expression. In addition, individual pathway regulators of secondary metabolism, encoded frequently within these BGCs, act to modulate the levels of secondary metabolite production. Examples include the streptomycete antibiotic regulatory proteins (SARPs) that activate transcription of

antibiotic biosynthesis genes in response to intracellular cues (van Wezel & McDowall, 2011).

The regulation of secondary metabolism is also under the control of quorum sensing. This form of bacterial communication is mediated by the production and perception of chemical signals, referred to as autoinducers. These molecules, upon reaching a certain level of concentration, bind to dedicated receptors and orchestrate the function of genes, including those controlling the synthesis of molecules. In Pseudomonas aeruginosa, it controls the expression of virulence factors and secondary metabolites, such as pyocyanin and rhamnolipids (Williams et al., 2007).

3.5 Environmental and Epigenetic Triggers of Metabolite Expression

A considerable proportion of microbial BGCs are transcriptionally silent when isolated in the laboratory, and methods are consequently required to awaken these cryptic loci. Environmental conditions including the availability of nutrients, the pH, the temperature, and co-culture with other microbes may trigger the activation of silent compounds34,35. For example, cultivation of actinomycetes along with fungi has been reported to trigger formation of new metabolites not seen on conventional media (Bertrand et al., 2014).

Epigenetic changes comprise another level of control on secondary metabolism. Histone acetylation, methylation, and its impact on chromatin accessibility and gene expression were well characterized in fungi. Use of epigenetic modulators, e.g., histone deacetylase (HDAC) inhibitors, have derepressed silent gene clusters, uncovering new bioactives. This technique, coined chemical epigenetics, has also been implemented to some extent in natural product discovery (Henrikson et al., 2009).

4. Drug Discovery from Microbial Metabolites

Because of their outstanding structural complexity and biological activity, microbial metabolites have become most popular in the search for new therapeutics. Actinomycetes, myxobacteria, fungi and cyanobacteria and specific compounds have been the precursors to some of the most successful antibiotics, anticancer drugs and immunosuppressants. Nevertheless, in the face of increasing levels of antimicrobial resistance (AMR) as well as stagnation in the discovery of new compounds from known classes of antibiotics, new approaches for identification of novel microbial metabolites are urgently needed. This section presents advanced tools for drug discovery, highlighting the transition from traditional screening to computer and molecular methods.

4.1 Screening Strategies: Classical vs. Genome Mining

The traditional discovery of drugs from microbes depended heavily on phenotypic screening. Soil samples were cultured, and extracts were screened for activity against panels of pathogens or cancer cells. This tactic led to game-changing drugs including penicillin, streptomycin, and tetracycline. However, it ultimately reached a plateau, finding less useful results as known compounds were often re-discovered. Furthermore, this method usually did not detect metabolites that were only expressed under certain environmental or genetic circumstances (Berdy, 2005).

To avoid these restrictions, genome mining has been recognized as a revolutionizing approach. High-throughput sequencing allows researchers to directly discover biosynthetic gene clusters (BGCs) within microbial genomes. Programs such as antiSMASH, PRISM, and ClusterFinder can be used to predict secondary metabolite pathways in silico using conserved motifs and domain architectures. These tools not

only identify known BGCs but also predict new ones with unknown function which have not yet been studied, so-called orphan BGCs (Medema et al., 2015).

The methods of genome mining provide the means for a more focused method of metabolite discovery. It allows for the prioritization of strains and pathways predicted to produce bioactive compounds and for the engineering of expression circuits that can be used to un-silence clusters. Furthermore, the comparison of microbial strains via whole genome analysis enables the detection of bacteria specific HGTS and investigation of evolutionary conservation of BGCs, shedding light on their functional role (Donia et al., 2014).

4.2 Activation of Silent Gene Clusters

Numerous biosynthetic gene clusters identified by genome mining are cryptic under normal laboratory conditions. These foci are unexpressed either because the conditions are not available to trigger them, or the regulators are not active. These routes need to be unlocked to tap into the biosynthetic potential of microbes.

One is the heterologous expression, which means transfer of a silent BGC into a well-known host, such as S. coelicolor or E. coli. It enables to manipulate conditions of both transcription and translation and to include control elements to up-regulate expression. In promoter engineering, on the other hand, native promoters, which are frequently weak, are replaced with strong constitutive or inducible ones to improve the transcription (Yoon & Nodwell, 2014).

Another promising approach is the CRISPR-based gene activation technologies. The CRISPR-Cas9 or CRISPR-dCas9 approaches can be used to guide targeting to regulatory regions contained within silent BGCs, altering transcription but not the sequence of the DNA encasing the BGC. Synthetic riboswitches and transcription factor

decoys may also be used to pharmacologically press for gene expression in the presence of chemical inducers or stress signals (Kim et al., 2021).

In fungi, chemical epigenetics became a popular approach. Inhibitors of HDAC and DNA methyltransferase can change the modality of chromatin and decondense into the expression of previously silent gene clusters. These treatments have led to the discovery of new compounds in species of Aspergillus, Penicillium and other taxa (Henrikson et al., 2009).

4.3 Co-cultivation and Microbial Interactions

Microbes hardly ever live in isolation in nature. Rather, they build intricate communities where interspecies interplays are key to controlling secondary metabolism. Such ecological dynamics are often not emulated in the lab monoculture sets, which leads to an undermirroring of metabolite pathways.

The goal of co-cultivating approaches is to recreate these natural \textit{in vivo} interactions by co-cultivating two or more bacterial species. Such interactions may induce the production of new metabolites by competitive stress, nutrient cross-feeding, or signaling molecule transfer. For instance, the co-culture of Streptomyces with Bacillus sp.ètres and has mediated the production of new antibiotics like bacillaenes and streptomycins (Bertrand et al., 2014).

Terminally, metabolite production can be modified by co-culture at multiple mechanistic levels, such as quorum sensing, redox signal transfer and metabolite cross-feeding. Transcriptomic and metabolomic studies of co-culture have revealed the activation of cryptic gene clusters and the production of complex compounds not observed in single cultures. These data highlight the ecological relevance of secondary metabolism and its relevance in the drug discovery process (Netzker et al., 2015).

The co-cultivation has evolved to microfluidics and automation to screen thousands of microbial pairs under diverse conditions. These platforms provide dynamic metabolic tracking in vivo and are helpful for screening of bioactive compounds on a large scale (Zhang et al., 2020).

4.4 Dereplication and Structural Elucidation

One of the most challenging aspects of discovering new natural-product therapeutics from microbes is the history of repeatedly rediscovering known molecules. This is a problem of great expense of time and resources, so that the unambiguous characterisation and elimination of known metabolites is one of the most important processes of dereplication.

Nowadays, dereplication is performed using HRMS, NMR, and spectral library matching in a three-stage approach, in order to detect known compounds during the screening, as early as possible. Platforms such as GNPS (Global Natural Products Social Molecular Networking) enable researchers to compare their MS/MS spectra to entire global databases and quickly identify the identity of compounds (Wang et al., 2016).

Concomitantly, the development of methods for structure determination now allows for more rapid, confident characterization of new compounds. Detailed characterization of molecular structure is available by use of cryo-electron microscopy, X-ray crystallography and NMR. In addition, cheminformatics tools help to predict 3D-structures and bioactivity on the basis of spectroscopic data (Wolfender et al., 2019).

An additional level of precision can be added through the means of isotope labeling and stable isotope-resolved metabolomics (SIRM). They track the use of labelled precursors in metabolites and provide insight into both the biosynthetic origin

and flux through pathways. Such methodologies don't only affirm structure, and also provide clues about enzymic steps and metabolic regulation (Bueschl et al., 2014).

4.5 AI and Machine Learning in Metabolite Prediction

Artificial intelligence (AI) and machine learning (ML) have been included in natural product research and promoted the discovery and optimization of microbial metabolites. And large genomic and chemical datasets from genome mining, metabolomics, and chemical libraries can be analyzed by AI algorithms to predict the existence and structure of unknown compounds.

Supervised learning techniques are taught from previously annotated BGCs known and their products to infer function of orphan clusters. For example, the method DeepBGC combines deep learning techniques to detect and classify BGCs starting from a genomic sequence, and outperforms the classical rule-based approaches. In the same context, NPClassifier uses neural network to predict structural classes and bio activities of natural products (Hannigan et al., 2019).

AI is also utilized to design fermentation conditions, predict metabolite production quantities and model enzymesubstrate relationships. Reinforcement learning techniques may recommend genetic manipulations for improved production or structural analog synthesis. Such protocols minimize the need for trial-and-error experiments, and reveal more rational design in the drug discovery pipeline (Skinnider et al., 2021).

NLP tools are also being used to extract information concerning biosynthetic pathways, regulatory elements and pharmacological properties from the scientific literature. Such information can also be combined with experimental data, for the improvement of hypotheses and the design of new experiments (Mohanty et al., 2020).

5. Case Studies of Clinically Relevant Microbial Metabolites

Microorganisms have been the main source of bioactive natural products that changed the face of modern medicine throughout human history. These secondary metabolites include biologically varied therapeutic agents such as anti-biotics, anti-cancer compounds, immunosuppressants and other anti-inflammatory molecules. Every case study described here highlights, in addition to the biochemical creativity of microbial metabolism, the medical significance and translational promise of these molecules. This review will center on four key groups of microbial metabolites, investigating their source, biosynthesis, pharmacodynamic profile, and clinical relevance.

5.1 Antibiotics (e.g., Rifampicin, Teixobactin)

Epidemiology Rifampicin is an essential drug in the first-line treatment of tuberculosis and is a semisynthetic derivative of rifamycin B, which was originally isolated from Streptomyces rifamycinica. This ansamycin antibiotic acts by inhibiting bacterial DNA-dependent RNA polymerase, leading to the blockage of transcription and bacterial growth. Rifamycin is synthesized by a type I polyketide synthase (PKS) pathway, which includes modules that extend and cyclize the polyketide chain in a strictly linear manner into the active metabolite, the macrocyclic rifamycin (Floss & Yu, 2005).

The ability of rifampicin to cross the mycobacterial cell wall and attain high intracellular concentration is what makes it unique for treating Mycobacterium tuberculosis. However, the appearance of rifampicin-resistant strains as a result of mutations in the rpoB gene necessitated the development of new antibiotics acting via different mechanisms (Campbell et al., 2001).

Teixobactin stands out as an antibiotic discovery success story not only because of the novelty of its mechanism of action but also because it has the ability to circumvent

resistance. Teixobactin, isolated from Eleftheria terrae, a hitherto uncultured bacterium discovered by the iChip technology, targets to very conserved precursors of cell wall building blocks -lipid II and lipid III. Unlike protein-targeting standard antibiotics, teixobactin targets non-protein entities, making it less likely to induce resistance (Ling et al., 2015).

The finding of teixobactin underlines the potential of metagenomics and the application of new culturing strategies for discovery of new bioactive compounds. The iChip system enabled the on-site culture of environmental bacteria, through the identification of the rare pathways inaccessible with conventional means (Nichols et al., 2010).

5.2 Anticancer Agents (e.g., Doxorubicin, Bleomycin)

Doxorubicin, an anthracycline antibiotic produced by the bacterium Streptomyces peucetius, is still used and has become the basic drug in the treatment of many types of cancers such as breast, ovarian and hematological cancers. It exerts its cytotoxity through DNA-intercalation, inhibition of topoisomerase II, and the induction of apoptosis in proliferating cells by reactive oxygen species (ROS) (Gewirtz, 1999).

The biosynthesis of doxorubicin is associated with type II PKS, and other subsequent modifying processes including glycosylation and hydroxylation play an important role in its strong bioactivity. Structural derivatives, such as epirubicin and idarubicin, have been developed to lessen the risk of cardiotoxicity, the most significant constraint of doxorubin treatment (Arcamone et al., 1969).

Another well-known anticancer drug, bleomycin is a glycopeptide antibiotic complex extracted from Streptomyces verticillus. It interacts with DNA and causes strand breaks producing free radicals that is dependent on its metal binding domain.

Bleomycin displays excellent activity against Hodgkin's Lymphoma, testicular cancer, and squamous cell carcinoma (Hecht, 2000).

Notably, the biosynthesis of bleomycin uses Nonribosomal Peptide Synthetases (NRPS) in combination with PKS modules, reflecting the hybrid character of its multifarious factory. The high affinity for DNA mediated by thiazole and bithiazole rings in unguentine is key in how this compound works (Zalacain et al., 2005).

5.3 Immunosuppressants and Anti-inflammatory Metabolites

Microbial immunosuppressants are among the most important drugs introduced in transplant medicine and for autoimmune diseases. Renateospor FA FK506 produced by Streptomyces tsukubensis is a macrolide, which inhibits calcineurin and is essential for T-cell activation. Tacrolimus, through its association with the immunophilin FKBP12, which then binds to and inhibits calcineurin phosphatase activity, leads to the inhibition of IL-2 transcription and T cell expansion (Kino, Hatanaka, and Chrousos, 1987).

Tacrolimus is biosynthesized bya hybrid PKS-NRPS mechanism and thecore macrocyclic structure is further modified through a series of tailoring enzymes to add variousmethyl and hydroxyl groups. Synthetic biology has enabled the modification of structures to yield analogues with better PKs and tissue specificity (Matsuda & Koyasu, 2000).

Another prototype immunosuppressant is cyclosporine A, derived from the fungus Tolypocladium inflatum. Unlike tacrolimus, it complexes with cyclophilin to block calcineurin and produces equivalent downstream effects such as T cell suppression. Cyclosporine is the product of a cyclic peptide synthetase assembly line with a degree of structural variation within the hydantoin ring (Borel et al., 1976).

Anti-inflammatory microbiol metabolites (i.e., rapamycin) serve as double immunosuppressors and anti-carcinogenic agents. A product of Streptomyces hygroscopicus, rapamycin blocks the mammalian target of rapamycin (mTOR) to regulate cell growth, metabolism and immune responses. Its effect has been further studied in the context of anti-aging and cancer therapies (Sehgal 2003).

5.4 Marine-Derived and Extremophile Compounds

Marine systems and other extreme habitats, such as deep-sea vents, hypersaline lakes and acidic hot springs, contain specialized microbial organisms that make unusual and biologically active metabolites, which are structurally new to nature. These habitats force the evolution of microorganisms under selective conditions and are the cause of unique biosynthetic potential.

Salinosporamide A, a natural product of Salinispora tropica, is a powerful proteasome inhibitor, and has anti-cancer activity. It functions by covalently binding to the catalytic site of the 20S proteasome, resulting in protein homeostasis imbalance and induction of apoptosis in cancer cells. The compound has a fused γ-lactam-β-lactone structure, a very unusual feature in nature products that is responsible for its selective cytotoxicity (Feling et al., 2003).

Marinopyrroles, members of the marine Streptomyces metabolite family, possess potent antibacterial and anticancer activities. Their dimeric structure of pyrrole unituals is responsible for high DNA-binding affinities and ROS productions. The biosynthesis of these compounds includes halogenation and oxidative dimerization, which are unusual enzymatic reactions in natural product chemistry (Hensler et al., 2006).

Robust metabolites are also numerous in thermophilic and acidophilic organisms. For instance, Thermomyces lanuginosus has thermostable enzymes and secondary

metabolites with antifungal and anti-inflammatory properties. In addition, stability and activity of these compounds in application under extreme industrial and physiological conditions increase their value for pharmaceutical development (Elleuche et al., 2015). Cold-active enzymes and antifreeze proteins from psychrophilic microorganisms of Arctic and Antarctic origin have been studied. These bioactivities consist of membrane stabilization and free radical-scavenging efficiency, novel functionalities that could be exploited in advances in cryopreservation and in cold-environment applications (D'Amico et al., 2006).

6. Resistance Mechanisms and Microbial Adaptations

The enduring resistance and adaptability of microbes to antimicrobial pressure gave rise to complex resistance mechanisms, which greatly affect drug effectiveness. These resistance mechanisms are not only biochemical but emerged in the context of microbial ecology and evolution as well, which in turn makes them key determinants of drug failure and guides therapeutic development. With a growing emphasis of drug discovery on secondary metabolites, it is important to understand how microbes respond and adapt to these compounds in order to maintain their long term effectiveness and reduce the potential for resistance to arise prematurely (Davies & Davies, 2010).

6.1 Enzymatic Inactivation (e.g., β-lactamases, aminoglycoside-modifying enzymes)

One of the commonest forms of microbial resistance is degradation of the antibiotic by enzymes. These include hydrolysis, modification or cleavage of the drug molecule such that the drug becomes an inactive compound. The β-lactamases are among the most important, and they destroy the β-lactam ring, common to penicillins and cephalosporins, thereby rendering the drugs susceptible to hydrolysis and ineffective as

antibacterial agents. ESBL and carbapenemase producers, including KPC and NDM variants, create an even higher hurdle for therapy by hydrolyzing all currently marketed β-lactam agents (Bush & Jacoby, 2010).

One such instance is aminoglycoside resistance, often caused by aminoglycoside-modifying enzymes (AMEs). Acetyltransferases (AAC), nucleotidyltransferases (ANT) and phosphotransferases (APH) acetylate, adenylylate or phosphorylate the drug, respectively, which results in a ribosomal binding-site loss (Ramirez & Tolmasky, 2010). The genes for such enzymes are frequently located on plasmids and transposons, which allows their dissemination at a fast pace amongst the microbial community (Jacoby, 2005).

Also, microbes have developed new enzymatic modes of action, including factor CAT (chloramphenicol acetyltransferase) producing the acetylation of chloramphenicol or the erythromycin esterases that induce macrolide-inactivation. It is these enzymatic defences that strongly limit the potency of natural and semisynthetic microbial metabolites (Wright, 2005).

6.2 Target Modification and Bypass Mechanisms

Target modification give microbes the ability to escape from drug action without destroying the drug compound. This might be structural alteration of the antibiotic targets, ribosome, enzyme or of the constituting elements of the cell wall. For example, mutations in the 23S rRNA are known to cause macrolide resistance by altering binding affinity. Likewise, most fluoroquinolone resistance is caused by point mutations in DNA gyrase (gyrA) and topoisomerase IV (parC), which interfere with the interaction between drug and target (Hooper & Jacoby, 2016).

Methicillin resistance in Staphylococcus aureus (MRSA) is mediated by the mecA gene that codes for a modified penicillin binding protein (PBP2a), with low affinity for β-lactams. This is one of the founding demonstration of target substitution being permitted even in the presence of drug (Chambers & Deleo 2009). Similarly, VRE substitute the carboxy D-Ala-D-Ala of their peptidolycan precursors with D-Ala-D-Lac, which can no longer bind vancomycin and thus render it ineffective (Courvalin, 2006).

Bypasses allow bacteria to avoid the blocked route entirely. An infamous case is the introduction of a drug-insensitive dihydropteroate synthase to the sulfonamide resistant bacteria, by-passing the folate synthesis inhibition. Trimethoprim is also circumvented by alternative dihydrofolate reductase enzymes which are not inhibited by trimethoprim. These changes could be especially dangerous when they occur in pathogens that are encountering environmental/ibiotic antibiotics (Sköld, 2000).

6.3 Efflux Pumps and Membrane Permeability

When exposed to antibiotics, efflux pumps are a group of transmembrane proteins that effectively expel the toxic substances out of the microbial cells. These systems provide wide-spectrum resistance by decreasing intracellular drug levels to sublethal amounts. The efflux systems are grouped into families such as the major facilitator superfamily (MFS), resistance-nodulation-cell division (RND), ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) families (Li & Nikaido, 2009).

In Gram-negative bacteria, RND-type efflux pumps, such as AcrAB-TolC in Escherichjsonia coli or MexAB-OprM in Pseudomonas aeruginosa, are especially efficient because they cross both the inner and outer membranes, therefore permitting a high-rate drugs extrusion. These pumps are frequently upregulated in multidrug-resistant

isolates and these can be encoded either chromosomally or be plasmid-borne, providing robust and transferable mechanisms (Poole, 2005).

Membrane permeabilisation is also important, particularly in Gram negative bacteria, which are controlled by porins, as small molecules penetrate the membrane. Lowered expression, inactivation or loss of porins (e.g., OmpF) decreases the influx of hydrophilic antibiotics, for example β-lactams and fluoroquinolones. These changes may often be combined with over expression of efflux pumps and work in concert to create high-level resistance and treatment failure (Delcour, 2009).

6.4 Biofilm Formation and Persister Cells

Biofilms are organized microscopic colonies of microorganisms enclosed within a self-secreted extracellular polymeric matrix. Bacteria in these niches undergo profound changes in gene expression, reduced metabolism and increased antibiotic resistance. For example, biofilms are common in chronic infections (e.g cystic fibrosis lung disease, diabetic ulcers and indwelling medical devices) (Costerton et al., 1999).

Through a physical hindrance, the ECM restricts the penetration of the drug. Additionally, decreased growth rate of biofilm-budded cells contributes to antibiotics being less effective against dividing bacteria. Horizontal gene transfer is also fostered by the biofilm microenvironment, enabling the horizontal spread of resistance genes (Hall & Mah, 2017).

One of such sub-population within biofilms are persisters, non-heritable antibiotic intolerant phenotype. This nonmetabolizing fraction can withstand treatment with high levels of an antibiotic and then repopulate when the drug is removed. They are controlled by toxin-antitoxin systems, the stringent response, and metabolic arrest

pathways (Lewis, 2007). The relapse of infections caused by persisters stood as one of the major impediments to the treatment of biofilm-related diseases.

6.5 Horizontal Gene Transfer and Mobile Genetic Elements

HGT is a potent driving force for bacterial evolution that enables bacteria to quickly acquire resistance traits. The three main methods of HGT are transformation (uptake of free DNA), transduction (phage-mediated) and conjugation (plasmid transfer through the pili). HGT facilitates the transfer of resistance genes between species or genera and also contributes to the global expansion of multidrug resistance (von Wintersdorff et al., 2016).

Mobile genetic elements (MGEs), such as plasmids and transposons, integrons and gene cassettes, are important players in this dissemination of functions. For instance, integrons acquire gene cassettes that encode resistance determinants and express them in the Same bacterial chromosome. Class 1 integrons are especially related to resistance to multiple antibiotics classes and are commonly observed in clinical strains (Gillings, 2014).

These transposons are involved in the mobilization of antibiotic resistance genes between plasmids and the chromosome, commonly harbouring genes encoding β-lactamases, aminoglycoside-modifying enzymes and efflux pump regulators. Conjugative plasmids may carry an array of resistance genes and virulence factors and offer a selective advantage under antibiotic pressure (Partridge et al., 2018). Environmental pools like wastewater, agricultural runoff and industrial effluents serve as sources of gene transfer, which links selection for drugs in use by humans to the pace of resistance.

The combination of resistance mechanisms with microbial evolution highlights the flexibility that microbes adapt to. Knowledge of these tactics is important not only for developing drugs but also for developing effective antimicrobial stewardship and biosurveillance networks. Combating resistance is a multi-faceted undertaking which includes microbiological medicine, biochemistry, genomics, and public health (Ventola, 2015).

7. Integration of Omics Technologies in Natural Product Discovery

The introduction of omics-based technologies has significantly advanced the exploration of microbial natural products. With the increase of structurally complex microbial metabolites and their pharmacological utility, omics-based approaches provide high-throughput, systematic, and integrative solutions for the exploration of biosynthetic potential. Although conventional culture-based and chemical-guided isolation techniques are valuable, they frequently fail to identify uncultured microbes and silent biosynthetic gene clusters (BGCs). In contrast, genomics, transcriptomics, proteomics, metabolomics and multi-omics methodologies enable a single base molecular resolution of the biochemistry of microorganisms, significantly expanding the ability to investigate secondary metabolism both in nature and in the laboratory (Ziemert et al., 2016).

7.1 Genomics and Metagenomics

The advent of genomic technologies has profoundly influenced the field of microbial natural product research by allowing this all important procedure to be performed on a genome-wide scale. It enables the discovery of biosynthetic gene clusters involved in secondary metabolite biosynthesis. The introduction of next-generation sequencing (NGS) technologies has enabled fast, cheap genome sequencing thereby disclosing the

existence of so-called cryptic and silent BGCs not expressed under standard laboratory condition (Medema et al., 2015).

Genomic databases like antiSMASH and MiBIG enable annotation and gene cluster prediction that may provide signals of the ability of microbes to produce previously unisolated molecules. These databases analyze sequence motifs, domain architectures, and homologies with known clusters to assist dereplication and prioritization of novel pathways (Blin et al., 2021). Genome scanning of actinomycetes, myxobacteria and cyanobacteria has provided thousands of new polyketide synthases (PKS), nonribosomal peptide synthetases (NRPS) and hybrid pathways.

Metagenomics is a very powerful approach that expands the scope of genomic investigation as the genetic material of complex microbial communities is studied directly from the environment (sensu lato) via eDNA, without the requirement of cultivation. The shotgun approach to metagenomics allows recovery individual genomes from a metagenome (MAGs), while functional metagenomics allows researchers to clone environmental DNA in expression vectors and screen for bioactivity (Handelsman, 2004). Such methods have revealed bioactive molecules from marine sedimentsides, rhizospheres and human microbiota, greatly expanding the chemical space of natural products (Brady, 2007).

7.2 Transcriptomics and Gene Expression Profiling

The global survey of RNA transcripts (transcriptomics) reveals which biosynthetic genes are actually being transcribed under given conditions. This is especially relevant for silent gcs. By RNA sequencing (RNA-seq), gene expression profiles can be compared among different environmental stimuli or gene manipulations to discover the triggers for the production of secondary metabolites (Wang et al., 2009).

Differential gene expression can uncover stress, quorum sensing signals or nutrient limitation driving secondary metabolism induction. Induction of silent BGCs, as observable via transcriptomic changes, is also affected by co-culture of microorganisms or exposure to sublethal antibiotic concentrations for example (Bertrand et al., 2014). Time-course transcriptomics also enables the characterization of the dynamic control of metabolite biosynthesis over the life of a microorganism.

Software such as Rockhopper and DESeq2 have provided an accessible means of Java-normalized statistical analysis for high-capacity RNA-seq data. It is becoming increasingly powerful to study gene regulation and to develop conditions for metabolite production by computer-aided simulations of gene regulation by means of transcriptomics together with promoter analysis including network-modeling tools.

7.3 Proteomics and Enzyme Function Characterization

Although genomics and transcriptomics offer insight into the genetic potential and gene expression, proteomics acts as an intermediary and identifies the proteins that are synthesized and active in the cell. Proteomics find use in microbial natural product studies to pinpoint biosynthetic enzymes, transporters and regulatory proteins in the metabolite pathway (Vogel & Marcotte, 2012).

Proteomics by mass spectroscopy (proteomics) allows qualitative as well as quantitative determination of proteins in different growth conditions. Methods such as tandem mass tag (TMT) labeling and isobaric tag for relative and absolute quantitation (iTRAQ) thus allow comparative proteomics of induced vs. uninduced microbial cultures. Ecological context can lead to discovery of new enzymes or pathway intermediates that are not apparent at the transcript level due to post-transcriptional or translational regulation (Zhang et al., 2013).

Additionally, in vitro reconstitution of biosynthetic enzymes and active site mutants followed by the use of ABPP to characterize enzyme activity, are also useful means to confirm predicted biochemical transformation. For instance, proteomics based domain specific analysis of NRPS and PKS enzymes has elucidated module activity, domain swap effects and substrate specificities, generating knowledge that can further develop synthetic biology tools for pathway design (Sieber & Marahiel, 2005).

7.4 Metabolomics and Chemical Fingerprinting

Metabolomics offers the most direct view of the biochemical output of microbial metabolism of the three - it makes a profile of the end products of enzymatic pathways. It is the systematic study of small molecule metabolite profiles in cells at a specific state. Untargeted metabolomics with LC-MS, GC-MS, and NMR spectroscopy can result in the identification of unknown compounds and pathway intermediates (Patti et al., 2012).

Alternatively, targeted metabolomics quantifies known metabolites for insights into pathway flux and regulation. Fingerprinting compounds with ultra-high resolution mass allows differentiation of structurally closely related analogs and identification of compounds present in low abundance. Comparison of wild-type and mutant strains or diverse culture conditions by metabolite profiling can provide information on the involvement of particular genes or regulators in metabolite production (Scalbert et al., 2009).

Metabolomics can be combined with other omics layers using tools like GNPS (Global Natural Products Social Molecular Networking) that permits spectral clustering and dereplication of MS/MS data. They enable the discovery of known scaffolds and new analogs, the visualization of metabolite similarity relationships, and could increase efficacy in the discovery of bioactive compounds (Wang et al., 2016).

7.5 Multi-omics and Systems Biology Approaches

The real power of omics however, comes with integration, since multi-omics efforts can join data from genomics, transcriptomics, proteomics and metabolomics to develop a'system level'model of microbial secondary metabolism. Systems biology uses such data sets to construct predictive models of metabolic and regulatory pathways, and of an organism's response to perturbations (Hood et al., 2004).

Integration of multi-omics provides the ability to map genotype-phenotype relationships, gaining insights into the effect of genetic variation and environmental exposure on metabolite output. Computational analytic platforms including Cytoscape, MetaboAnalyst, OmicsNet, enable visualization, correlation analysis and network modeling among various omics data (Xia et al., 2015). Such as relating gene expression to protein abundance and metabolite concentration can highlight bottlenecks in biosynthetic pathways or locate master regulators.

Moreover, multi-omics is used for rational design of microbial strains for efficient production of the desired chemicals in synthetic biology. CRISPR/Cas9-mediated targeted genome editing using omics-based targets is capable of editing gene expression with genome sequence specificity. Additionally, genome-scale metabolic models (GEMs) developed with multi-omics data are being employed to predict the pathway behavior and to optimize fermentation conditions for maximal metabolite production (Orth et al., 2010).

In the realm of drug discovery, the multi-omics approach improves the discovery of lead compounds, the understanding of modes of action, and the off-target prediction. Omics integration also enables personalized medicine, applying distinct microbial metabolites to the individuals' microbiome compositions or disease status. Therefore,

natural product discovery through omics is the forefront of precision medicines and next-gen antibiotics (Zhu et al., 2019).

The ongoing development and democratization of omics technologies will play a key role in meeting the challenges of conventional drug discovery pipelines. Because of its systems-level approach to microbial metabolism, it opens a window into nature's biochemistry with much finer resolution and elucidation of a larger biochemical space, which may reveal potential novel therapeutics as resistance continues down this seemingly inexorable path.

8. Synthetic Biology and Pathway Engineering in Microbial Metabolite Production

Introduction The dawn of synthetic biology has significantly expanded the microbial biotechnology repertoire, thereby allowing rational design and control of biosynthetic pathways to increase natural product discovery. In the past, the discovery of microbial metabolites depended on serendipity and trial and error. Yet, current synthetic biology uses a variety of molecular design, DNA synthesis, and computer-aided design to engineer microbes that should produce metabolites with enhanced titer, rate, and yield. This sort of systems-level engineering combines micro, bio and info into an interdisciplinary approach enabling precise control to reengineer metabolism (Nielsen & Keasling, 2016).

8.1 Modular Reprogramming of Biosynthetic Gene Clusters

Polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) and Nonribosomal peptide synthases are the mainstay of microbial secondary metabolism. These are large multi-modular enzymes catalyzing the biosynthesis of diverse natural products, many of which include antibiotics, antifungals, and anticancer drugs. Their modularity renders them as suitable targets for synthetic reprogramming (Weber and

Kim, 2016). Using the domain swapping approach and/or by editing the modules themselves it is possible to develop hybrid enzymes yielding new chemical entities. For example, exchanging PKS module AT domains can change the chain length or branching of polyketide analogues (Khosla et al., 2014). Likewise, NRPS modules have been engineered to support non-natural amino acids, extending the range of structures (Bozhüyük et al., 2019).

A representative example was the development of semisynthetic derivates of erythromycin using a recombinant Saccharylprolityospom erytl~raea strain, with the DEBS PKS modules re-designed for the synthesis of diversified macrolides. These changes were not only functional, but the modified structure was also biologically active and supported the possibility for modular reprogramming (Koryakina et al., 2013). Although the inter-domain compatability and the folding of the products represents the main obstacles, the modular design and the design strategy of biosynthesis pathway shows great potential for lead compound diversification.

8.2 CRISPR/Cas Systems in Pathway Optimization

Genome engineering in microorganisms has been revolutionized by CRISPR/Cas. As opposed to classical mutagenesis, CRISPR/Cas enables site-selective and multiplexed mutagenesis, which is key for awakening the silent biosynthetic gene clusters (BGCs) and fine-tuning gene expression (Tong et al., 2020). CRISPR-Cas9 technology has been applied in Streptomyces strains to knockout competing pathways, to place strong promoters upstream of cryptic BGCs and to create high producing strains (Zeng et al., 2015). In addition to this genome editing or repair potential, CRISPRi (interference) and CRISPRa (activation) systems allow gene expression to be turned off and on reversibly,

i.e., without permanent modification of the genome, which can serve as valuable tools for transient control of pathways (Qi et al., 2013).

In the area of microbial metabolite production, multiplexing in BGCs now has been reached based on CRISPR. This method is useful in the development of chassis organisms for reducing background pathways and maximizing the target pathways. Further simplification of genome-wide editing is also enabled by Cas12a from CRISPR-Cas12a systems that are capable of processing multiple host crRNAs from a single transcript (Zetsche et al., 2015). Altogether, CRISPR/Cas platforms have now been recognized as being crucial for both pathway and synthetic circuit implementation.

8.3 Heterologous Expression and Chassis Engineering

Most biosynthetic pathways lie silent or poorly expressed in their native microbial hosts. Heterologous expression—housing BGCs in genetically malleable, well-characterized hosts such as Escherichia coli, Streptomyces coelicolor, or Saccharomyces cerevisiae—provides a remedy. This procedure does not only help to functionally characterize genes, but also to overproduce the relevant metabolite under controlled fermentation conditions (Smanski et al., 2016). For instance, the actinorhodin gene cluster of S. coelicolor could be heterologously expressed in E. coli where considerable amounts of the compound was produced (Zhou et al., 2015).

Chassis engineering is not just gene jockeys. The strategy is to modulate the metabolism of a host so as to provide the introduced pathway with requisite precursors, cofactors, and regulatory factors. Synthetic minimal genomes, such as those of Mycoplasma mycoides, are engineered to remove the host metabolism interference, which makes clean background for heterologous production (Hutchison et al., 2016). Further, adaptive laboratory evolution (ALE) and genome-scale metabolic modeling can

be used to enhance strain tolerance and metabolite production under industrial environment (Feist et al., 2009).

8.4 Synthetic Regulatory Elements and Promoter Libraries

Development of tunable regulators is one of the central tasks in synthetic biology. Synthetic promoters, riboswitches, terminators, and Ribosome Binding Sites (RBSs) enable tunable geneoff states in a predictable and controllable manner in microbes (Mutalik et al., 2013). Synthetic promoter libraries have been constructed in several chassis organisms (e.g., E. coli, Streptomyces, and Bacillus subtilis) with various expression strengths (Guiziou et al., 2016). These are essential pieces for the equilibrium between production of pathway enzymes, metabolic burden and flux direction toward end products.

In addition, synthetic transcription factors and logic gates can be engineered to react to second messengers, leading to spatio-temporal activation of pathways. For example, temperature- and pH-inducible promoters can induce metabolite biosynthesis only under certain fermentation conditions, therefore improving fermentation efficiency (Tang et al., 2018). Synthetic control circuits can then be designed into sophisticated gene networks that either follow native regulatory hierarchies, or that exceed these in complexity and responsiveness.

8.5 Biosensors and Real-Time Monitoring Systems

Biosensors are genetically encoded systems which sense specific metabolites or intermediates and provide a readout as fluorescent or colour outputs. They are also used for high-throughput screening of high producing strains and provide real time monitoring of metabolite biosynthesis in engineered microbes (Rogers et al., 2016). Usually these sensors consist of a sensor domain (e.g., ligand binding protein or

transcription factor) and a reporter module (e.g., GFP or luciferase). For instance, Xu et al. (2014) engineered a malonyl-CoA biosensor in E. coli to screen for polyketide pathway variant(s) with improved precursor supply.

In addition to sensing, biosensors can be wired into feedback-controlled synthetic circuits to enable dynamic modification of gene expression. These "smart" systems keep metabolite levels at or below toxic concentrations, minimizing by-product formation and enhancing product yield. Furthermore, integrated microfluidic systems integrated with biosensors allow the high throughput single-cell analysis of microbial factorial, promoting strain optimization (Whitesides, 2006). In conclusion, synthetic biology based on biosensors paves the way for a novel class of metabolic engineering, responsive and adaptive metabolic engineering.

9. Challenges and Future Directions

Although huge advancement has been made in microbial metabolites, and synthetic biology, great challenges still exist in the translation from a laboratory discovery into clinically effective drugs. These barriers range from biological yields and regulation to data integration, sustainability and microbial exploration. To address these challenges it will be important to apply a multidisciplinary strategy using state-of-the-art bioinformatics, systems biology, and ecological microbiology to truly make the most of microbial metabolites.

9.1 Limitations in Yield, Stability, and Regulation

The low production yield and non-growing-product are some of the major hindrances in the production of metabolites with microorganisms. Most of the secondary metabolites are present only in trace concentrations under natural growth conditions as their biosynthesis is highly regulated and occurs under specific environmental conditions (Li

& Neubauer, 2014). Overexpression of these pathways is often hindered by metabolic burden, feedback inhibition, and toxic intermediate buildup that can lead to decreased host viability and productivity (Zhou et al., 2015).

Further, the structural instability of metabolites, particularly those with reactive functional groups or complex scaffolds, prevents downstream handling and storage. This is particularly an issue for molecules produced by marine or extremophilic microbes, which frequently need specific physicochemical conditions to maintain their stability (Burgess et al., 2013). Regulatory constraints Similarly, the regulatory network negatively controlling the biosynthesis of these compounds may be encumbered, where its biosynthetic gene cluster could be epigenetically silenced or harbored on unstable plasmids or chromosomal islands susceptible to deletion or inactivation under the scale-up conditions (Kim et al., 2021).

The construction of reliable expression systems and synthetic regulatory networks that allow high-level production without activating the stress regulatory responses continues to be a major challenge. Technological advances, including feedback regulation of promoters, biosensor-based regulation and adaptive evolution, have shown great potential but still need to be optimized for industrial applications (Liu et al., 2018).

9.2 Advancing Bioinformatics and Omics Integration

Although the genomic and metagenomic era has increased by several orders of magnitude the number of potential biosynthetic gene clusters, our ability to functionally study these clusters is not commensurate to the amount of sequence data. Most putative BGCs in silico prediction are not expressed or they are misannotated because an incomplete annotation of functions of BGCs available in the databases (Medema et

al., 2015). Typical algorithms are based on similarity-based predictions and do not cover the whole diversity of uncanonical or hybrid pathways.

The combination of a multi-omics approach encompassing the genome, transcriptome, proteome, and the metabolome provides an opportunity to understand and further exploit the microbial metabolism. However, because there are no community-adopted platforms and computing pipelines available, it is difficult to perform cross-comparison. For instance, tools such as the antiSMASH, PRISM, and GNPS have advanced cluster identification and metabolite dereplication, but their prediction accuracy declines in the case of new scaffolds and when under-represented microbial taxa are involved (Skinnider et al., 2020).

Future work will need to center on building machine learning models that can optimize not only the presence but also the regulation, expression potential, and functional role of BGCs in native or heterologous settings. Better data curation, the use of open-access repositories and collaborative databases will be the keys to reproducibility and scalability through the whole scientific community (Van der Hooft et al., 2020).

9.3 Sustainable and Scalable Drug Development

Another major restriction in the use of the microbial metabolites is the inability to develop an industrial process by scaling up the milligram-level lab production to the kilogram-scale processes. Promising laboratory results of fermentation optimized for lab not always translate well to industrial scale because the mass transfer, shear stress, nutrient availability, and microbial physiology differ in lab scale and industrial scale bioreactors (Tyo et al., 2010). Furthermore, the metabolic burden of producing

secondary metabolites competes calorie for calorie with the building of biomass – necessitating conditions which balance biomass and productivity.

Sustainability is also an issue given that some production methods employ expensive or nonrenewable materials. It is worth mentioning that the analysis of slow-growing or not yet cultured organisms such as marine or extreme environments (in both cases, the metabolites were extracted rather than purified) may seem to be ecologically and ethically questionable (Martins et al., 2014). Green chemistry strategies, synthetic biology-guided pathway design and bioprocess engineering should be integrated to minimize waste, energy consumption and foster efficient loop (re)closing for advanced biomanufacturing.

Improved process control could be achieved using new bioreactor designs, e.g., perfusion or continuous flow cultures, while computational metabolic models may assist in identifying rate-limiting steps and downstream strategies for titer enhancement (Nielsen & Keasling, 2016). In addition, the government and its regulatory agencies must expedite approval pipelines for products derived from microbes, ensuring that they are able to more swiftly move from the laboratory to bedside.

9.4 Expanding Microbial Diversity Exploration

One remaining frontier in microbial metabolites is the study of microbial diversity outside from conventional sources. Traditionally, actinomycetes and filamentous fungi have played leading roles in natural product discovery. But excessive mining of these taxa has resulted in high rediscovery rates, resulting in search for novel habitats that have been less explored such as deep-sea sediments, polar ice, volcanic springs, and symbiotic microbiomes (Ziemert et al., 2016).

The latest metagenomics and single-cell sequencing approaches have made it possible to reconstruct high-quality genomes of unculturable microorganisms and to uncover hitherto-unknown biosynthetic functions. However, the identification of metabolites still represents a major barrier for associating these gene clusters with molecules, as such approaches are highly challenging and are in need of new methodologies (e.g., synthetic BGC refactoring and heterologous expression in models hosts) (Milshteyn et al., 2018).

Moreover analyzing microbial interactions in the context of the community may lead to discovery of cryptic pathways that are induced only in response to certain cues or competitors. Co-culture microfluidics and simulated environmental microniches offer a means to induce and track in vivo production of relevant metabolites (Traxler et al., 2013). Overall, diversifying the taxonomic and ecological diversity of investigated microbes, meaning bringing new bacteria "under the lens", will revitalise the pipeline of new interesting bioactive molecules.

10. Conclusion

The enormous and complex universe of microbial secondary metabolism has repeatedly demonstrated its importance as a wellspring of drug discovery innovation. In summary, we have demonstrated in this article that, bacteria and fungi in particular, are an invaluable resource for structurally diverse, biologically interesting compounds with great potential for use as therapeutics. Originating as a study into antibiotics, it has developed into a multi-discipline field, including anti-cancer, immunosuppressants, enzyme inhibitors, etc.

The shift from conventional cultivation-based approaches toward more sophisticated techniques, including genome mining, co-cultivation, and omics-based approaches, which is currently taking place in the field of microbial secondary

metabolism, represents the new paradigm in exploration of these metabolites. No longer restricted to culturable isolates or observable phenotypes, investigators in this field now have access to the cryptic secondary metabolomes captured in microbial genomes. Silent gene clusters have been activated, and cryptic biosynthetic gene clusters have been mined to discover totally new classes of molecules. This shift has made the discipline more predictive, data-driven, and interdisciplinary.

Synthetic biology and metabolic engineering have significantly transformed that metabolic landscape of the microbes. Though the reprogramming of biosynthetic pathways is modular, does not induce a genetic selection pressure because it uses the native synthetic capability of cells, and may implicate biosynthetic flow variations26, scientists can overproduce, redirect biosynthetic flow, and even generate non-natural analogs with > 1,000-fold increases in therapeutic handedness using CRISPR/Cas technologies. These tools allow scientists not only to find what Nature has made, but to redesign and improve upon it to solve the clinical problems of today.

It is equally important to acknowledge microbial resistance and adaptability. Microorganisms constantly evolve under pressures of selection, and the resistance mechanisms including enzymatic inactivation, target alteration, efflux pumps, and biofilm formation, reveal how microbes evolve under selective pressure. A knowledge of these strategies is not only important to develop counter-resistance measures, but to identify drug weak points that one might capitalize on in future new drug design. In addition, the ecological and evolutionary interactions of microbes have stimulated creative approaches for activating cryptic metabolites through co-culture and environmental emulation.

Through omics technologies (e.g., genomics, transcriptomics, proteomics, and metabolomics) integration, the gap between discovery and functional characterization has been narrowed. Such methods provide the opportunity for gaining greater insight in the microbial system, novel metabolites discovery, and its corresponding biosynthetic pathways, as well as, potential mechanisms of the bioactivity. Thanks to systems biology and machine learning, we now have the ability to model whole biosynthetic networks, which gives us predictive power in the identification and the optimization of compounds. The case histories outlined here, from life-saving microbial antibiotics such as teixobactin to marine anticancer agents and immunomodulators, confirm the impact of microbial metabolites in the TBWD. Note—These examples highlight both the clinical and commercial significance of microbial drug discovery and also the need for balanced and sustainable bioprospecting, especially in fragile areas such as deep-sea ecosystems and extreme ecosystems.

Despite these advances, challenges remain. Several factors like yield optimization, pathway regulation, dereplication, and compound stability still challenge the industrial scale production of some microbial metabolites. And the investigation of microbial diversity, especially of uncultivable and extremophil organisms, is still in its/an early phase. Intelligent design: the new frontier in synthetic biology Next-generation synthetic biology will go beyond these challenges by adopting greater symbio-integration of synthetic biology, data analysis based on artificial intelligence, and to increase the cross-fertilization among microbial biologists, chemists, and bioengineers.

Indeed, microbial metabolites are truly at the confluence of biology, chemistry and technology. Being highly complex, versatile and rich in biosynthesis, they are unparalleled candidates for the next generation of drugs. With ongoing innovation and

investment in this area, microbial drug discovery is not only here to stay but to thrive as a critical solution to emerging global health threats. This article makes the case once again that the microscopic world of microorganisms is a vast and colossally underutilised pharmacopeia waiting to be decoded, harnessed and conscripted for human good.

11. References

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