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Review Article

Bacterial Secondary Metabolite Activation Through Epigenetic Modifiers: A Systematic Review

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ABSTRACT

Bacteria have produced many important secondary metabolites, especially in the pharmaceutical industry. However, the increase in the rediscovery rate of the known compound has been inconvenient to researchers and the pharmaceutical industry. Nevertheless, genome mining in bacteria has uncovered several cryptic metabolic pathways that may be key to discovering new secondary metabolites. The conventional laboratory environment, however, limits the metabolic pathways of microorganisms, making it impossible for secondary metabolites to be produced. As a result, researchers began using epigenetics to change the expression of the genes that code for secondary metabolites in microorganisms. Using epigenetics modifiers, secondary metabolite gene clusters are activated without altering the bacterial DNA sequence. This review article focuses on the different epigenetic changes and how they affect gene expression to activate the synthesis of secondary metabolites in bacteria.

Keywords: Bacterial, epigenetic modifiers, gene clusters, secondary metabolites

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INTRODUCTION

Microorganisms create a wide range of secondary metabolites with industrial

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and medicinal applications. Microbial natural products remain an unrivalled source of pharmacological leads and are essential in modern medicine. Alexander Fleming's ground-breaking discovery of penicillin from the fungus *Penicillium notatum* in 1929 triggered a surge in interest in microorganism-derived natural goods. Microorganisms have produced almost 50,000 natural compounds, with over 10,000 having biological functions (Shah et al., 2017). Natural products contribute to 49% of the 175 small-molecule anticancer medicines authorised since the 1940s (Okada et al., 2017). Natural product derivatives comprised about 80% of commercial medications by the 1990s. Due to the expansion of synthetic combinatorial methods and a rise in the rediscovery rates of natural compounds through classical discovery campaigns, this percentage has fallen over the last few decades. Despite several secondary metabolite biosynthetic gene clusters discovered by genome sequencing, some of these genes are silenced under normal conditions (Scherlach & Hertweck, 2021). As a result, only a small amount of secondary metabolites were discovered. Gene expression can be induced through epigenetic modification methods such as DNA methylation and histone modification.

Several significant challenges exist in discovering novel microbial natural product-derived drug leads, including being unable to cultivate most microorganisms found in environmental samples. Next, there is a general lack of tools that can be used to promote the production of small bioactive molecules from a variety of "silent" pathways in microorganisms that are simple to cultivate in the lab, as well as challenges in identifying and dereplicating unknown metabolites from expressed pathways that typically have unpredictably structured and functional features (Trautman & Crawford, 2016). Most companies, however, have stopped or limited their efforts in natural product screening due to the frequent rediscovery of existing chemicals (Li et al., 2009). Antibiotic resistance, cancer chemotherapeutics, and pesticide resistance are all on the rise, posing a threat to current healthcare and agricultural practices. Many routine surgical operations would be complicated without effective antibiotics, and one-third of agricultural commodities would be destroyed without effective pesticides, according to estimates (Rutledge & Challis, 2015). Previous studies have estimated that the global economic impact of antimicrobial resistance will result in more than 10 million annual deaths by 2050, corresponding to a loss of 2.0–3.5% of the global gross world product (Murray et al., 2022). This rising problem emphasises the importance of natural product discovery, particularly in the search for novel antimicrobials to replace antibiotics that have become abused.

In the last few decades, the research on epigenetic regulation of gene function has become more critical in the sciences. In plants, animals, and microbes, the mechanisms and effects of processes including DNA methylation, histone post-translational modifications, non-coding RNAs, and their impact on chromatin structure and dynamics are all engaged in physiological homeostasis (Poças-Fonseca et al., 2020). One approach to this problem

would be stimulating novel secondary metabolite production via epigenetic modification. Epigenetics was initially described as the addition of changes in genetic sequence. However, the term has now extended to include any processes that alter gene activity without changing the DNA sequence (Weinhold, 2006). Epigenetic processes include methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation. These processes can be essential to modulate gene transcription in secondary metabolite production.

The production of the potential metabolites must be stimulated to obtain access to this untapped reservoir of potentially bioactive molecules. This review article will focus on how epigenetic modifications play a role in biosynthetic pathway silencing and how epigenetic changes allow scientists to access a hidden treasure of natural bacterial products. This article highlights the mechanism of epigenetic modification in bacteria and some of the most recent documented discoveries of secondary metabolites from epigenetic modifications.

Biosynthetic Gene Clusters for Secondary Metabolites

Biosynthesis of natural chemicals happens more frequently inside distinct localised sections of the microorganisms' genome known as biosynthetic gene clusters. The biosynthetic gene clusters often contain all the genes essential for metabolite production, regulation, and transport. Biosynthesis gene clusters are groupings of two or three genes that code for a secondary metabolite biosynthetic pathway (Medema et al., 2015). Non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpenes, and ribosomally synthesised and post-translationally modified peptides (RiPPs) are all structural classes of biosynthetic gene clusters. NRPS and PKS are well-known for synthesising products with beneficial applications in medicine and science, such as antibiotics, antifungals, and immunosuppressants. They are popular for synthesising natural products (Le Govic et al., 2019). Meanwhile, RiPPs are a significant group of natural products with a wide range of bioactivities and high structural diversity (Zhang et al., 2018).

The size of metabolic gene clusters varies highly depending on the complexity of the end product's pathway. For instance, the production of the glycosylated anthracycline nogalamycin is encoded by a gene cluster that contains 32 enzymes (Baral et al., 2018). From assembly to expression control, many clustered genes perform diverse functions in the synthesis and complexity of a natural substance. Secondary metabolite-producing microbes frequently create various chemicals from a single strain. Cumulative research implies that activating gene clusters can vastly speed up the discovery of novel natural compounds with high pharmaceutical potential.

In bacteria, gene regulation is controlled by operon structures. The activation and repression of biosynthetic gene clusters, secondary metabolite production, and other developmental processes of the organism may be based on the differences in gene cluster mechanisms (Chakraborty, 2022). According to a study by Tanaka et al. (2013),

microorganisms' ability to generate beneficial secondary metabolites has been underestimated due to cryptic gene clusters. Genome data analysis of sequenced *Streptomyces* revealed that a single *Streptomyces* genome generally encodes 25–50 biosynthetic gene clusters, and about 90% of them are silent or cryptic under standard laboratory growth conditions (Liu et al., 2021). It concludes that only a small fraction of the constitutively expressed biosynthetic gene clusters has contributed to the current collection of naturally derived drugs. Methods to access the silent majority would significantly impact drug discovery and increase the collection of bioactive molecules.

According to Jackson et al. (2017), only about 10% of the genes encoding small molecules in bacteria have been found to date. Several Gram-negative bacteria genomes, including *Pseudomonas*, *Burkholderia*, and *Clostridium*, were known to have possible cryptic biosynthetic gene cluster repositories (Gross & Loper, 2009; Klaus et al., 2020; Pahalagedara et al., 2020). According to much more meticulous estimations based on genome sequence data, the genus *Streptomyces* alone can create 150,000 secondary metabolites, with only around 5% being identified thus far (Smanski et al., 2016). A genome mining study by Lebedeva et al. (2021) revealed a higher number of biosynthetic gene clusters in two strains of cave *Paenibacillus* sp. compared to a report in 2019 for *Paenibacillus* sp. indicating that the strains encode additional clusters that may range significantly from strain to strain, thus having the potential for novel secondary metabolites.

Meanwhile, in another study by Belknap et al. (2020), gene clusters in *Streptomyces* bacteria showed a wide range and plenty of biosynthetic gene clusters across the genus *Streptomyces*, with hybrid biosynthetic gene clusters vastly enlarging the supply of secondary metabolites, therefore aiding the novel secondary metabolites discovery. They also reported that biosynthetic gene cluster diversity varies significantly among members of the same species. It implies that strain-level genome sequencing can find significant biosynthetic gene cluster variety levels and potentially valuable derivatives of any compounds.

The studies of full genome sequencing revealed more gene clusters that encode enzymes that are generally engaged in specialised metabolite production (Lee et al., 2020; Little et al., 2020; Lebedeva et al., 2021). Although the metabolic products of these new biosynthetic gene clusters are unknown, bioinformatics-based predictions suggested that some of them may encode novel structures. Potentially intriguing gene clusters that may encode chemicals that increase competitiveness in natural habitats can go undetected in the artificial setting of the microbiology laboratory. According to Trautman and Crawford (2016), many cases of microbes' biosynthetic secondary metabolite gene clusters now exceed the number of natural products synthesised in the lab. These cryptic gene clusters are rich in unique bioactive components that can be exploited to build new drugs. Due to the constraints on secondary metabolite production imposed by these vital regulatory

systems, natural product scientists face unique problems. The fact that many microbes have many secondary-metabolite-encoding biosynthetic pathways, yet only a portion of their small-molecule products are identified in the laboratory, exemplifies this point. As a result, heterologous expression platforms and gene modification technologies in situ have been developed to bypass transcriptional barriers and directly access natural products from quiet metabolic processes.

The number of secondary metabolites identified by bacteria and fungi may only be the tip of the iceberg. Homologous and heterologous expression of these mysterious secondary metabolites-biosynthetic genes, which are typically "silent" under standard laboratory fermentation conditions, might facilitate the identification of new secondary metabolites. The discovery and prioritising of relevant biosynthetic genes, their activation, and, ultimately, establishing the link between the genes and the encoded secondary metabolites are all significant challenges in achieving this potential. It has given rise to a new field known as genomics-driven natural product discovery, a strategy for identifying novel microbial metabolites with potential medical and agricultural uses that complement traditional bioactivity-guided methods.

Biosynthetic gene clusters typically respond to various environmental stimuli, although the relationship between the regulators and the stimuli is frequently unclear. Native environmental signals may not be present in the laboratory, rendering biosynthetic gene clusters transcriptionally inactive. Different techniques must be used to awaken these clusters and investigate their potential for biosynthesis because cryptic gene clusters seem silent in lab settings. The epigenetic modification technique is suggested to ensure the discovery of novel bioactive natural products to overcome these issues.

Epigenetic Modification and Transcription

Epigenetics are heritable traits that do not involve changes in the DNA sequence. The term "epi" refers to traits that are "on top of" or "in addition to" inheritance through traditional genetics (Pfannenstiel & Keller, 2019). Epigenetic and post-translational modifications have been suggested to affect gene transcription and are likely to be engaged in secondary and primary metabolism (Yang et al., 2022).

Epigenetic mechanisms include DNA methylation and histone modification, which both use DNA modifications to modulate gene expression. Due to epigenetic regulation, unicellular organisms can adapt quickly to environmental stresses or signals (Xue & Acar, 2018). While some of these changes, such as phosphorylation, ubiquitination, and ADP-ribosylation, have been discovered in the past, methylation and acetylation are the most well-studied and prevalent. DNA methylation involves the covalent attachment of a methyl group to the 5-carbon position of the cytosine ring, resulting in the production of 5-methylcytosine, which extends into the main groove of the DNA and inhibits transcription (Bind et al., 2022). Hypermethylation is linked with the repression of gene expression in

the gene promoter region, while hypomethylation is associated with the activation of gene expression.

Gene expression is influenced by post-translational modifications such as DNA methylation, histone acetylation, and phosphorylation at certain times and locations (Akone et al., 2018). Histone acetylation is frequently associated with transcriptional activation, with enzyme inhibition leading to greater gene activation and secondary metabolite production (Strauss & Reyes-Dominguez, 2011).

DNA methyltransferases, histone acetyltransferases, and histone deacetyltransferases are only a few of the epigenetic modifying enzymes discovered. Small molecule inhibitors and activators are powerful tools for activating cryptic biosynthetic gene clusters to create novel natural products.

Epigenetic Modification by Small Molecules

Epigenetic modifiers, or epidrugs, are natural or manufactured tiny molecular substances that target epigenetic marks or enzymes with epigenetic activity, causing epigenetic changes (Pillay et al., 2022). Treatment of the microorganism with epigenetic modifiers has proven highly successful in stimulating the activation of silent biosynthetic gene clusters to create unknown secondary compounds. Epigenetic modifiers, histone deacetyltransferases (HDAC), and DNA methyltransferases (DNMT) inhibitors can cause cryptic biosynthetic gene cluster activation (Pettit, 2011). DNMT inhibitors impact developmental and other cellular processes by silencing genes, resulting in unique phenotypic features (Ramesha et al., 2018). Two main epigenetic modifiers induce the expression of silent biosynthetic gene clusters. Table 1 shows the epigenetic modifiers with the example of chemical elicitor and their mode of action.

Table 1
Comparisons of epigenetic modifier and their mode of actions

Group of inhibitors	Target	Chemical elicitor	Mode of actions
HDAC inhibitor	HDAC enzyme	SAHA Valproic acid Trichostatin A Sodium butyrate	Inhibit class I and II HDACs
		Apicidin	Inhibit Class I HDACs
		Nicotinamide Sirtinol	Inhibit Class III HDACs
DNMT inhibitor	DNMT enzyme	5-azacytidine RG-108	Inhibit DNMT enzyme

The first group of epigenetic modifiers would be histone deacetylase inhibitor, which usually targets histone deacetylase enzymes. The chemical inhibitors that have succeeded in boosting the secondary metabolites production target Class I and Class II histone deacetylases are SAHA, valproic acid, trichostatin A, sodium butyrate, and apicidin (Kim & Bae, 2011; Li et al., 2020). Also, Class III histone deacetylase inhibitors can alter strain metabolite profile. The most common Class III histone deacetylase inhibitors are nicotinamide and sirtinol. Most Class I and II enzyme inhibitors interrupt the binding of zinc ions. For example, SAHA has a hydroxylamine group that binds to Zn2þ, linked by a straight alkyl chain to a hydrophobic group that interacts with the amino acids at the rim of the catalytic site to control the specificity of the inhibitor (Moore et al., 2012).

The second group of inhibitors is DNA methyltransferase inhibitor, which inhibits the enzyme DNA methyltransferases. 5-azacytidine is the most used DNA methyltransferase inhibitor to inhibit DNA methylation when incorporated with DNA. Another DNA methyltransferase inhibitor is RG-108, which can block the active site of DNA methyltransferases and is the only epigenetic modifier capable of direct enzyme inhibition (Ou et al., 2018).

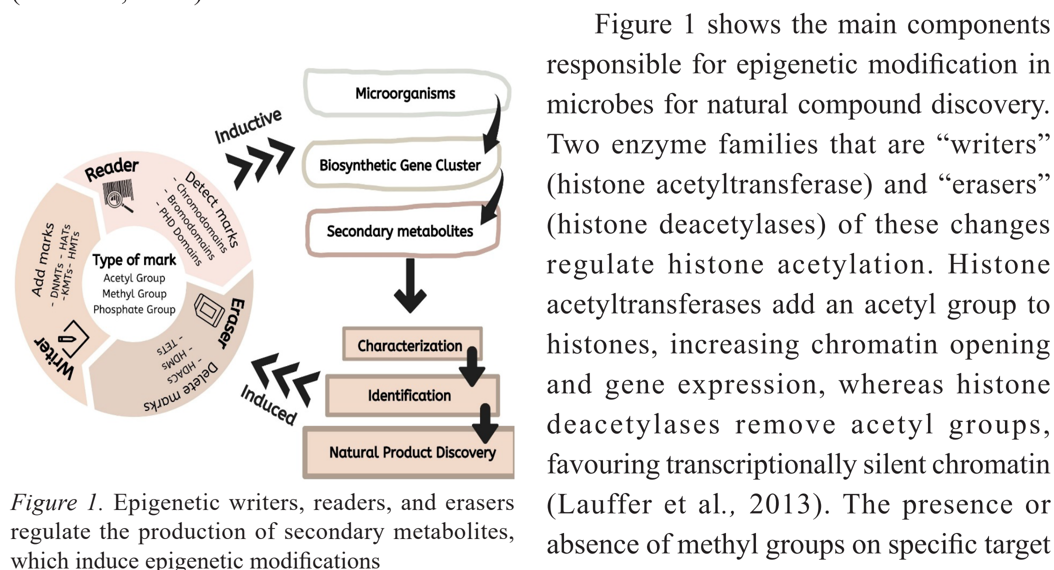

Figure 1. Epigenetic writers, readers, and erasers regulate the production of secondary metabolites, which induce epigenetic modifications

Figure 1 shows the main components responsible for epigenetic modification in microbes for natural compound discovery. Two enzyme families that are "writers" (histone acetyltransferase) and "erasers" (histone deacetylases) of these changes regulate histone acetylation. Histone acetyltransferases add an acetyl group to histones, increasing chromatin opening and gene expression, whereas histone deacetylases remove acetyl groups, favouring transcriptionally silent chromatin (Lauffer et al., 2013). The presence or absence of methyl groups on specific target sites is defined as the methylation pattern of a DNA region. Histone acetyltransferases (HAT), DNMT, and HDAC are among the epigenetic-modifying enzymes discovered so far. HAT adds acetyl groups to the ε-amino group of nucleosomal histone lysine residues, whereas HDAC removes them (Fischer et al., 2016). Small molecule inhibitors and activators are used as biological probes and possible therapeutic agents and as effective tools for activating cryptic biosynthetic gene clusters for novel natural product manufacture.

Epigenetic Modifiers Studies on Bacteria

Epigenetic controls have primarily been investigated in eukaryotes, where they play a role in cell differentiation via various processes, including histone modifications and DNA methylation. However, evidence for epigenetic regulation in prokaryotes is becoming more widespread. Table 2 summarises the results of bacteria being treated with epigenetic modifiers and the compounds obtained. Marisol et al. (1995) studied the effects of sinefungin and 5-azacytidine on the development of *Streptomyces antibioticus,* which later revealed that it specifically affects *Streptomyces antibioticus* sporulation; 5-azacytidine also stimulates antibiotic production in *Streptomyces antibioticus* and *Streptomyces coelicolor.* Moore et al. (2012) used sodium butyrate to stimulate the expression of secondary metabolites in *Streptomyces coelicolor* on two different types of media: minimal agar and R5 agar. The result demonstrated that sodium butyrate influences actinorhodin production, increasing secondary metabolite production under poor nutrient conditions. The qPCR results also demonstrated that sodium butyrate induced five cryptic pathways in *Streptomyces coelicolor*. According to Kumar et al. (2016), *Streptomyces coelicolor* treated with 5-azacytidine showed twelve compounds, whereas untreated *Streptomyces coelicolor* showed five compounds in HPLC analysis. The crude extract from cultures treated with 5-azacytidine was also effective against five human pathogenic bacteria. The crude extract from untreated culture was only effective against three human pathogenic bacteria.

A study by Wang et al. (2013) investigated the plausibility of increasing secondary metabolite production in *Pseudomonas aeruginosa* bacteria through epigenetic modification. This study, however, was inconclusive regarding whether the observed changes in metabolite formation were due to epigenetic modifying modifier treatments or co-culturing. Extracts from neither epigenetic modification-treated nor co-cultured cells showed increased bioactivity in antimicrobial assays, yet co-cultures treated with epigenetic modifiers had more bioactivities. Research by Militello et al. (2016), which incorporated 5-azacytidine to study the DNA methylation in *Escherichia coli,* revealed that 5-azacytidine increased gene expression at the early stationary phase, and 62 gene expressions were detected. Other than that, 5-azacytidine was also reported to influence the structure of the bacterial transcriptome in *Escherichia coli.* Meanwhile, a gene expression study on respiratory tract opportunistic pathogen *Burkholderia cenocepacia* using DNMT inhibitor, sinefungin resulted in overexpression of specific genes, including BCAM0820 and BCAL0079, whose function can be linked to the biofilm and motility observed (Vandenbussche et al., 2020).

As the research demonstrated, epigenetic modifiers are essential in activating the silent gene cluster of secondary metabolites, which can boost the production of various bioactive chemicals. Although epigenetic modification is complex, it is one of the most effective methods for synthesising industrial secondary metabolites with pharmacological applications.

Table 2
The compound obtained from bacteria treated with epigenetic modifiers

Bacteria	Epigenetic modifiers	Compound obtained	References
Streptomyces antibioticus	5-azacytidine Sinefungin, 1 mM	Rhodomycin Actinorhodin	Fernandez et al. (1995)
Streptomyces coelicolor	Sodium butyrate, 25 mM	Actinorhodin	Moore et al. (2012)
Pseudomonas aeruginosa	Manzamine A, kahalalide F, Sceptrin, ilimaquinone	1-phenazine-carboxamide 1-phenazine carboxylic acid 1-hydroxy-2-heptyl-4-quinolone 2-Octyl-4(1H)-quinolone 2-nonyl-4(1H)-quinolone 2-(2-nonenyl)-4(1H)-quinolone 2-(1-nonenyl)-4(1H)-quinolone Rhamnolipids D-rhamnose α-hydroxy fatty acid moieties	Wang et al. (2013)
Bacillus pumilus	5-azacytidine,	Amicoumacin	Schumacher (2014)
Streptomyces coelicolor	5-azacytidine, 25 µM	- (Twelve compounds present in HPLC analysis)	Kumar et al. (2016)
Escherichia coli	5- azacytidine, 0.5–50 µg/mL	-	Militello et al. (2016)
Burkholderia cenocepacia	Sinefungin 50 µg/ml	-	Vandenbussche et al. (2020)

CONCLUSION

The significance of secondary metabolites in numerous sectors increases the desire to regulate them by manipulating their synthesis process. The abundance of cryptic and silent pathways in bacterial genomes offers excellent potential for synthesising a novel compound with significant therapeutic properties. These regulatory mechanisms could be altered to increase the production of secondary metabolites. However, it is not easy to activate the numerous quiet gene clusters. Furthermore, understanding the control of secondary metabolism and the activation or silencing of gene clusters is crucial. This article outlines the several types of epigenetic regulation used in bacteria to increase secondary metabolite synthesis.

On the one hand, the rising need for novel pharmaceuticals raised the demand for alternative epigenetic modifiers, and on the other, innovative technologies for high-

throughput natural product discovery. Nowadays, epigenetics is an emerging tool that is gaining importance in microbial biotechnology for synthesising new bioactive chemicals and their increased concentration in microorganisms. The epigenetic modifiers can be considered an effective tool to stimulate the silent or poorly expressed biosynthetic pathways in bacteria, thus stimulating the production of secondary metabolites. However, learning the impact of epigenetic modifiers on DNA structure via DNA methylation analysis and DNA-protein interaction analysis, as well as the effectiveness of their ability to activate a silent biosynthetic gene cluster must be continued.

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