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Activation of Microbial Silent Gene Clusters: Genomics Driven Drug Discovery Approaches

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Abstract

Microorganisms have provided mankind with a plethora of small molecule natural products ranging from industrial enzymes to therapeutic agents. Analyses of microbial genome sequences have revealed the presence of numerous 'silent' or 'cryptic' biosynthetic gene clusters (BGCs). Activation of these cryptic biosynthetic pathways can pave way to the discovery of novel bioactive secondary metabolites (SMs). This article summarizes various approaches employed to unlock the hidden biosynthetic potential of microbes and methods developed to study their silent gene products.

Keywords: Secondary metabolites; Biosynthetic gene clusters; Silent gene activation

Introduction

The hidden microbial secondary metabolomes

Since the discovery and development of antibiotic penicillin, microorganisms have evolved as cornerstones of drug discovery process [1,2]. The end of the 20th century witnessed the upsurge of diverse secondary metabolites as anti-bacterial, anti-fungal, anti-parasitic, immune-suppressive and anti-cancer agents [3-5]. Even with this advanced chemical space, emerging and reemerging infectious diseases and resistant cancer types demand the discovery of novel chemical entities (NCEs). Conventional drug discovery process largely relies on classic bioassay guided approach which often leads to the rediscovery of known compounds. Moreover, laborious nature of the method as well as challenges associated with the purification and characterization of the compounds has kindled a withdrawal of pharmaceutical companies from natural product based drug discovery [6]. However, inefficacy of chemical synthesis to generate anticipated chemical diversity prompts the return of drug discovery to nature.

Secondary metabolites or small molecule natural products (SMNPs) confer adaptive advantages to the producing organisms serving as defense molecules, attractants or signaling agents [7,8]. Hence, secondary metabolite profile of an organism is likely to vary with the complexity of the niche they occupy [9]. In microbes, secondary metabolite biosynthetic genes are organized in contiguous DNA segments known as gene clusters. The rearrangement of modular genes on the course of evolution contribute to the structural diversity of natural compounds [10,11]. Analyses of microbial genome sequences have revealed the presence of several biosynthetic gene clusters (BGCs) that either remain silent or weakly expressed when cultured in laboratory conditions, presumably due to paucity of environmental cues required to trigger their activation [12,13]. Thus the biosynthetic potential of microorganisms is far greater than that was thought from classic bioactivity screens. Genome mining for novel natural compounds and activation of silent gene clusters has become a dynamic and rapidly advancing area of research in past years.

Triggering microbial silent BGCs

Environmental cues and co-cultivation: Microorganisms, in their natural realms form diverse multispecies communities. Secondary metabolites (SMs) play a key role in interspecies communication within the microbial communities. SMs also bolster survival value of microbes mediating stress tolerance in their natural environment and competition with co-existing microbes. In other words, specific environmental factors shape the secondary metabolic profiles of microorganisms in their natural habitats [14-16]. An empirical approach to activate silent BGCs involves cultivation of microbes in conditions mimicking their natural environment. It has been reported that the cultivation of marine bacteria on chitin, a polymer abundant in its native habitat, turned on several cryptic biosynthetic pathways [17]. Co-cultivations of bacteria-bacteria, fungi-fungi, or fungi-bacteria are naturally driven approaches which try to exploit the chemico-ecological relationships existing in microbial communities to activate silent gene clusters [18]. Since the successful production of polyketide, enacycloxin by *Gluconobacter* sp. W-315, upon co-cultivation with fungi *Neurospora crassa* or *Aspergillus oryzae*, in 1982, microbial co-cultivation has turned to be a major approach in natural product discovery [19-21].

SM production by microbes is observed to vary with composition of culture media and culture conditions [22]. Hence culturing of microbes in diverse media is a classic approach [often termed as OSMAC (one strain many compounds)] to activate silent BGCs [23,24]. Alteration of other cultivation parameters, such as temperature, salinity, flask shapes and aeration have proved effective in triggering cryptic biosynthetic pathways in *A. ochraceus* [22]. It has been reported that, the presence of various stress inducing chemicals such as menadione and hydrogen peroxide (oxidative stress inducers), and sodium chloride and sorbitol (osmotic stress inducers), significantly modulate the SM production in microbial cultures [25]. A high-throughput screening approach for activating silent gene clusters using small molecule elicitors demonstrated obvious activation of two cryptic gene clusters in *Burkholderia thailandensis* cultures [26]. This method facilitates the read out of target gene cluster activation by means of a genetic reporter construct. Isolation of novel compounds such as lunalides A and B, oxylipins, cladochromes F and G, nygerone A, chaetoglobosin-542, 540 and 510, sphaerolone, dihydrosphaerolone, mutolide and pestalon,

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and enhanced production of various antibiotics advocate chemical elicitation as an effective method to activate cryptic genes [27]. Further, bioinformatic analyses of cryptic genes can provide clues to finely tune the culture medium so as to turn on several cryptic genes. For instance, in *Streptomyces coelicolor*, sequence analyses revealed the presence of putative iron dependent repressor binding sites in intergenic regions within cryptic coelichelin biosynthetic cluster [28]. This guided the selection of iron deficient culture conditions for the identification of coelichelins.

Mutagenic and epigenetic activation of silent gene clusters

Several studies have demonstrated the importance of global as well as epigenetic regulatory mechanisms in activation of silent gene clusters. 'Ribosome engineering' has emerged as a propitious avenue for silent gene cluster activation based on the observation of dramatic increases in antibiotic biosynthesis in ribosomal mutant bacterial strains. A streptomycin resistant strain of *S. lividans* was found to produce abundant quantities of a blue pigmented antibiotic, actinorhodin, by a BGC that is customarily silent in *S. lividans* strains. It was found that this enhanced antibiotic production by *S. lividans* strain attributed to certain mutation in a gene coding for ribosomal protein S12 (*rpsL* gene), a component of 30 S subunit [29]. The mutation in ribosomal protein S12 enhances the stability of 70 S complexes, thereby augmenting the antibiotic biosynthesis [30]. Bacterial gene expression is regulated by a bacterial alarmone, ppGpp, synthesized by ribosome. ppGpp positively regulates bacterial secondary metabolism when cells enter stationary phase. In response to nutrient limitation, stringent response is turned on following the binding of uncharged tRNA to ribosomal A site. This results in a transient increase of hyperphosphorylated guanosine nucleotide ppGpp, synthesized from GDP and ATP by *relA* gene product (ppGpp synthetase) [31].

In addition to the ribosomal mutations, certain mutations in RNA polymerase (RNAP) have been proven to play impressive roles in antibiotic overproduction. It was found that in *rel* mutants of *S. coelicolor* A3(2) and *S. lividans*, the impaired ability to produce antibiotic due to *relA* gene deletion could be circumvented by introducing certain rifampicin resistant mutations in RNAP β-subunit. This impels RNAP to behave as stringent RNAP even in the absence of ppGpp [32,33]. Ribosome engineering to waken the silent BGCs target ribosomal proteins, translation factors or RNAP, assuming that the alteration in transcription and translation pathways can enhance biosynthetic gene expression. Ribosome and RNAP mutant strains may be obtained by applying selective pressure using antibiotics that target ribosome and RNAP. This approach successfully enhanced the yields of different classes of secondary metabolites including polyketides, macrolides, aminoglycosides, and nucleosides. In addition to its direct application in silent gene activation, ribosome engineering also enables construction of amenable hosts for heterologous expression [34].

Microbial genome sequence analyses reveal the presence of genes encoding transcription regulatory proteins within individual BGCs. Identifying these activator signals opens up a platform to activate BGC by switching the regulator to its active state. LysR-Type Transcriptional Regulator (LTTR) genes are found to be present in several BGCs characterized to date [35,36]. These regulators bind to the promoter and activate gene expression in the presence of a co-inducer. In the absence of the co-inducer, the regulators bind to promoter and maintain it in the off state. Hence, identifying an activating signal can change the regulator to its on-state leading to the activation of BGC and production of bioactive compounds. Besides positive regulation by transcriptional proteins, repression of gene expression by suppressor proteins is also a crucial regulatory mechanism that has to be countered to turn on the silent gene cluster. In *S. coelicolor* A3(2), deletion of *scbR2*, a pathway-specific regulatory gene that encodes a member of c-butyrolactone receptor family of proteins led to the production of novel secondary metabolites [37]. It has been demonstrated that epigenetic modifications such as acetylation, methylation, phosphorylation, ubiquitination, ADP ribosylation and glycosylation play key roles in regulating gene expression. Accordingly, modifications at epigenetic level may prove efficacious for cryptic gene activation. In *A. nidulans*, disruption of *hda* gene encoding histone deacetylase led to transcriptional activation of sterigmatocystin and penicillin biosynthetic genes [38].

Identifying cryptic gene products

The identification of cryptic gene products from secondary metabolite pool is as challenging as the activation of BGCs. Bioinformatic analyses enables the prediction of putative functions of target gene clusters. Modular arrangement of biosynthetic genes allows prediction of metabolic building blocks incorporated into final product. Moreover, the presence or absence of domains with tailoring activities within individual modules enable prediction of modifications underwent by the building blocks [39-41]. Putative physicochemical properties of final product such as molecular masses or UV- visible absorbance, as predicted by bioinformatic tools can be employed for the detection of the compound in the fermentation broths [42]. 'Genomisotopic approach' or '*in vitro* reconstitution approach' has been proven useful in cases of cryptic biosynthetic pathways in which substrates of biosynthetic enzymes are known. In genomisotopic approach, the organism is fed with stable isotope labelled precursor and NMR detection of labelled metabolites is used to guide their fractionation and purification. Orfamides, the novel macrocyclic lipopeptides produced by *Pseudomonas fluorescens* Pf-5 was identified by this approach [43]. In *in vitro* reconstitution approach, the predicted substrates are incubated with recombinant biosynthetic enzyme and the final products are characterized. This approach directed the identification of epi-isozizaene, the product of a cryptic sesquiterpene synthase gene cluster in *S. coelicolor* genome [44]. Another approach employed for the identification of cryptic gene products involve the inactivation or deletion of one or two genes predicted essential for metabolite biosynthesis. The comparison of metabolites in fermentation broths or extracts of wild type and mutant strains using analytical techniques such as liquid chromatography-mass spectrometry (LC-MS) allow the identification of cryptic gene products. Aspyridones, the metabolic products of cryptic gene clusters in *A. nidulans*, were identified by comparative metabolic profiling of wild-type and mutant strains [45]. A second comparative metabolite profiling approach involves the cloning of entire BGC into a single cosmid or BAC vector and its expression in a heterologous host. The metabolite profile of the heterologous host containing and lacking the cloned cryptic BGC are compared using LC-MS or other analytical techniques [46]. The strategies discussed have their limitations, depending on the expression of cryptic gene clusters, the structural information of the metabolite that can be deduced from bioinformatics analyses, and the size of target gene clusters. These points have to be considered for the successful selection of the best approach to identify the cryptic gene products.

Conclusion

Genomics driven searches for novel natural compounds have made remarkable progress in the past years (Figure 1). Genome mining approach sets the cutting edge of a second golden era of novel natural compound discovery. However, more advancement has to be made in

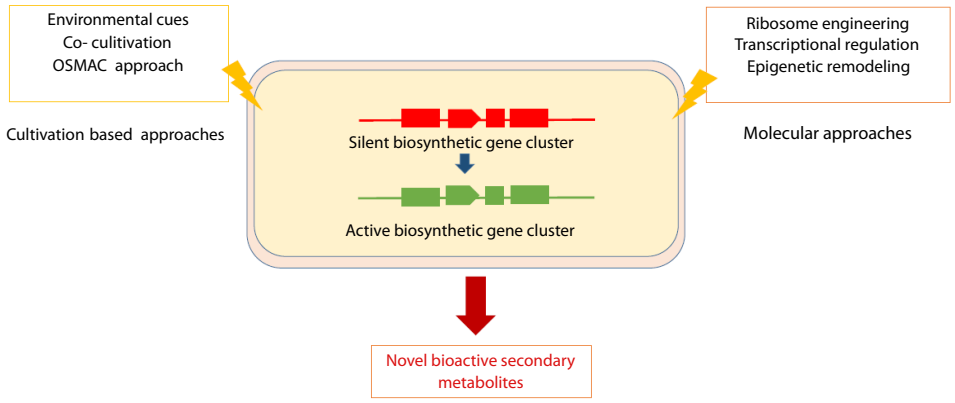

Figure 1: Approaches for the activation of microbial silent biosynthetic gene clusters.

developing and refining the general methods for silent gene activation as well as for the discovery of cryptic gene products. Collaborative approaches aligning metabolomics and metagenome based analyses can augment our grasp regarding the physiological role of secondary metabolites as well as the bio-discovery hit rates.

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