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REVIEW ARTICLE

Current strategies to induce secondary metabolites from microbial biosynthetic cryptic gene clusters

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Abstract

The genome of actinomycetes and several other microorganisms are endowed with many cryptic gene clusters that can code for previously undetected, a plethora of complex secondary metabolites. Under standard laboratory controlled conditions, the genes regulating these biosynthetic clusters are expressed at very low levels or remain phenotypically cryptic (silent). Over the past several decades, multi-drug-resistant bacteria have been observed with increased frequency, posing a significant threat to human health worldwide. The present alarming situation urgently calls for concerted global efforts for the discovery of new antimicrobials. The present situation, if not controlled, will take us again to the pre-antibiotic era. Today, in the post-genomic era, various new strategies such as the activation of cryptic gene clusters in microorganisms rejuvenate a new conviction in the field of natural product research that may lead to the identification of yet-unidentified novel secondary metabolites of therapeutic and other use. Decryptification of this versatile endogenous genetic reservoir may provide in the near future the more concrete rationale for antibiotic discovery. The present review is an attempt to provide a comprehensive detail, outlining current strategies that have been shown successful to activate cryptic biosynthetic gene clusters in microorganisms.

Keywords Cryptic gene cluster activation · Multi-drug resistance · Secondary metabolites · Antibiotics

Introduction

Microorganisms as a source of secondary metabolites

The introduction of penicillin antibiotic led researchers to explore microorganisms for the production of secondary metabolites. The parvome (small bioactive molecules) is considered to be an inexhaustible source of secondary metabolites of microbial origin (Davies 2011). To date, various metabolite molecules of therapeutic and industrial use (Fig. 1) have been identified from microbial cell factories (Bentley 1997; Berdy 2012). The intense investigations in the last 80 years have resulted in the screening of many microorganisms particularly the genera of actinomycetes (53%), and fungi (30%) have attracted increased attention in the search for novel secondary metabolites. A substantial number of secondary metabolites (17%) are also produced by *Bacillus*, *Pseudomonas*, myxobacteria, and cyanobacteria (Berdy 2005). Interestingly, harnessing the microbial capacity to obtain secondary metabolites has an added advantage because of their distinctive molecular scaffolds that are not commonly found in other chemical libraries (Feher and Schmidt 2003). For decades, the specialized secondary metabolism of these microorganisms has been exploited for the screening and production of metabolites at the commercial level. A sketch has been provided to illustrate the overall screening operation and the different stages of antibiotic discovery (Fig. 2).

Microbial diversity

The rate of the discovery of novel natural compounds from microorganisms has been diminished dramatically. Moreover, the most of antibiotic screening programs repeatedly rediscover the already known secondary metabolites. For this reason, it is speculated that microorganisms have been exhausted for their specialized metabolic wealth. The rapid increase in multi-drug-resistant pathogens also requires the global attention of researchers for the development of novel antimicrobials, novel

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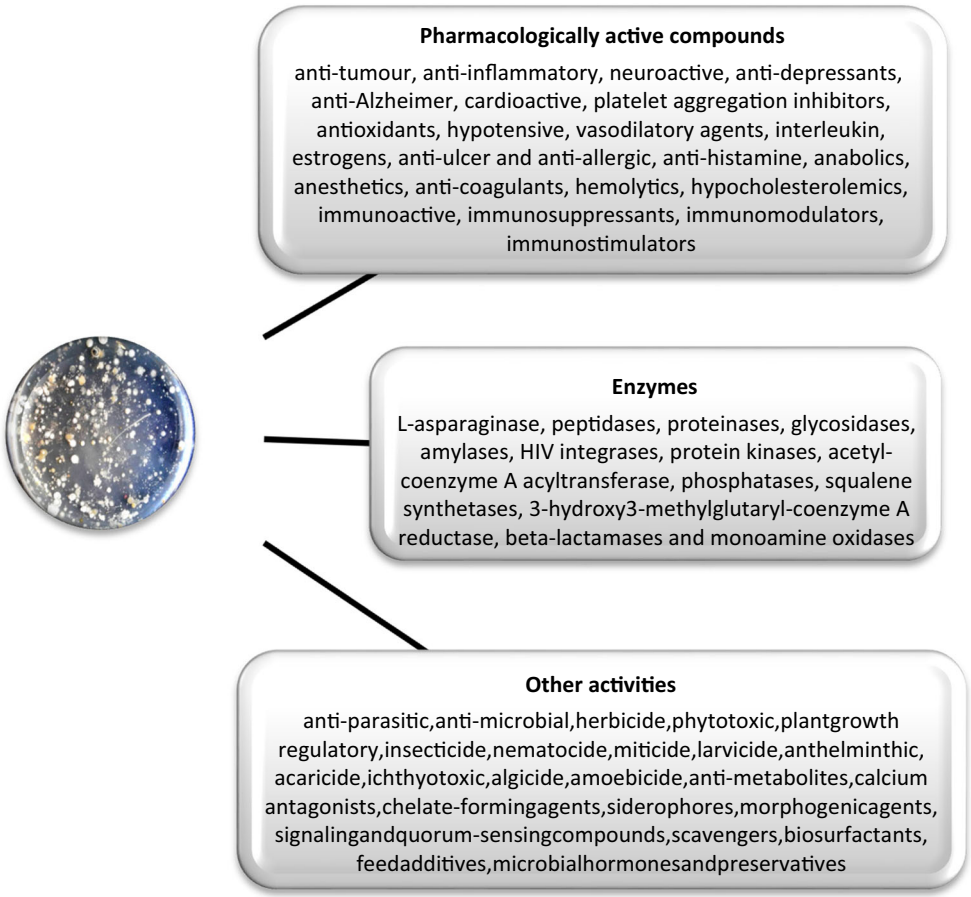

Fig. 1 The microbial parvome: the world of small bioactive molecules

treatment options, and alternative antimicrobial therapies. During the million years of evolutionary development, microorganisms acquired the drug resistance via lateral or vertical transmission, but at the same time, these microbes had the equal chance to adapt, counteract, and resist these resistant pathogens by producing more effective, more potent, "anti-resistant" secondary metabolites. The nature remains the richest and versatile source of new antibiotics; therefore, to discover yet to be identified secondary metabolites, genuine efforts are needed to exploit the unexploited and unexplored microorganisms from natural habitats. This hope rejuvenates a renewed curiosity to explore orphan pathways or cryptic gene clusters in microorganisms to search for novel metabolites. Based on the evidence collected from various sources, it has been suggested that ca. 300,000 to 1 million species of bacteria exist on the earth (Allsopp et al. 1995; Tiedje 1994). Around 70,000 fungal strains have been identified and assumed to be present at the rate of 1.5×10^6 species (Hawksworth 1991). It has been estimated that a gram of soil may contain 8.3 million prokaryotic species, while the total number of prokaryotic cells present in the natural habitats has been considered to be 4 to 6×10^{30} cells (Fierer and Jackson 2006; Torsvik and Ovreas 2002; Whitman et al. 1998). The majority of cells obtained from soil samples and visualized by microscopy are viable, but they do not generally form visible colonies on the media that contains agar (Eilers et al. 2000; Xu et al. 1982). Intriguingly, to date, only few thousands of prokaryotic species have been formally described, which means that the vast majority of prokaryotes (90–99%) present in natural habitats have still to be identified (Harwani 2013; Rappe and Giovannoni 2003; Schloss and Handelsman 2004; Zengler et al. 2002).

Need for novel antimicrobials

A major public health concern is the mortality rate due to the multi-drug-resistant bacteria. A total of 1.7 million infections and ca. 99,000 deaths have been calculated to occur each year in the USA itself (Klevens et al. 2007). The strains of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (ESKAPE) have been categorized as multi-drug resistant (MDR) pathogens (Rice 2006). In addition to ESKAPE, the emergence of MDR *Clostridium difficile* and members of Enterobacteriaceae family has also been reported (Peterson 2009). The nosocomial infections caused by methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) have also contributed in increased morbidity and mortality. The infections

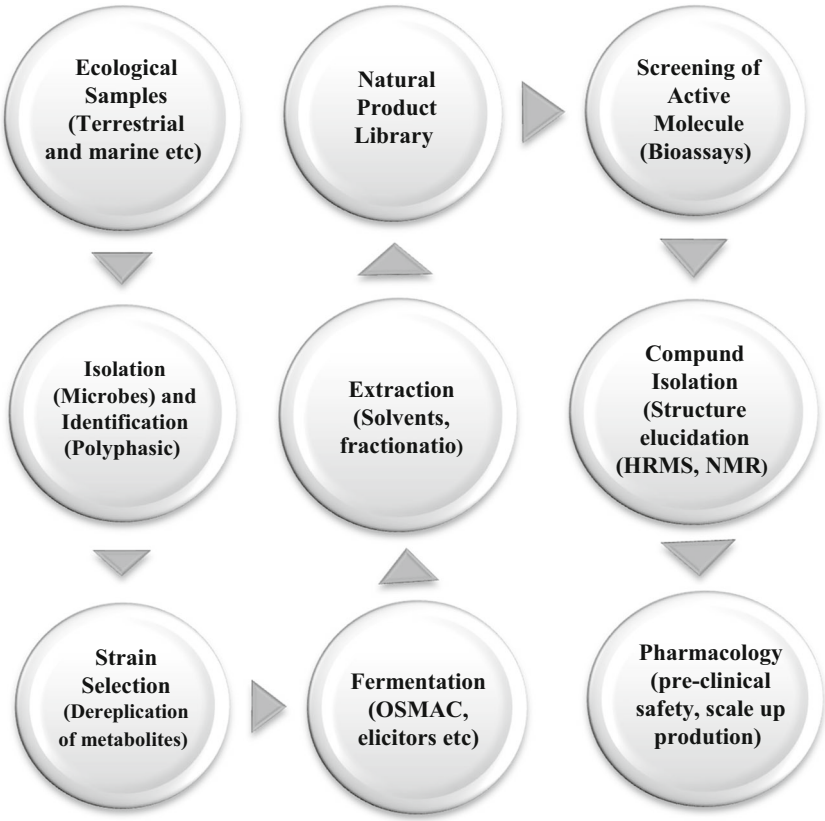

Fig. 2 The different stages of antibiotic discovery from microorganisms

caused by Gram-negative, MDR bacteria, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, extended-spectrum β-lactamase-producing Enterobacteriaceae, and carbapenem-resistant Enterobacteriaceae have been also found to pose significant threat to human health. The development of MDR in bacterial pathogens may be attributed to the transfer of resistance genes from *Klebsiella pneumoniae* and New Delhi metallo-β-lactamase (NDM-1) strains (Kallel et al. 2006; Rice 2008). Therefore, stronger actions are urgently needed to develop new antibiotics and new alternative therapies to effectively treat MDR pathogens.

Strategies for the activation of novel natural products from cryptic gene clusters

One strain many compounds (OSMAC)

The term OSMAC was initially used by Zeeck and co-workers that simply states that a single strain is capable of producing different metabolic compounds under the different fermentation conditions (Bode et al. 2002). The growth parameters that can be manipulated in OSMAC include media composition (incorporating different carbon, phosphate, and nitrogen sources), aeration rate, temperature, pH, inclusion of inhibitors or growth enhancers, etc. It is hypothesized in several reports that the prevailing growth environment has an intense effect on the production of microbial metabolites. A minute change in the growth parameter (such as variable concentrations of phosphate) may induce higher amount of specific secondary metabolite or may result in a completely novel type (Bode et al. 2002; Martin 2004; Miaomiao et al. 2017; Romano et al. 2014). The approach has been successfully applied to induce the cryptic coelichelin biosynthetic gene cluster in *S. coelicolor* (Ikeda et al. 1999) and to identify the cryptic fungal biosynthetic gene clusters (Daletos et al. 2017).

Culturing the unculturables

The term "unculturable" is used to describe organisms, particularly bacteria that do not grow on artificial media. In other words, we do not have sufficient biological information to culture these bacteria in vitro. It has been proposed that these microscopic cells are dead and therefore would never grow in the laboratory (Spieckermann 1912). In fact, many of these cells have been shown to be metabolically active (Roszak and Colwell 1987). An estimate suggests that the number of antibiotics from these uncultured sources is two to three orders of magnitude, greater than those from the cultured sources. The easiest way to study and exploit the unculturables is through having these available in artificial culture in the laboratory

(Nichols et al. 2010). The cryptic antibiotic gene clusters in yet-to-be-characterized microorganisms remain unexplored because the environmental clues that elicit their activation are unknown. The fact that the overwhelming part of microbial diversity remains uncultivated therefore represents an exciting opportunity to make attempts to culture them to search for novel secondary metabolites. This renewed interest in microbial cultivation (Aoi et al. 2009; Connon and Giovannoni 2002; Ferrari and Gillings 2009; Ferrari et al. 2005; Kaeberlein et al. 2002; Stevenson et al. 2004) has led to the development of several innovative approaches to bring new microbial species in the culture. Most of these approaches share one basic strategy that is to mimic the environment of the target microorganism. An ultimate move in this direction is to replace in vitro growth by in vivo cultivation in the natural habitat. Recently, the discovery of a novel antibiotic teixobactin has been announced from a previously uncultured and undescribed soil bacteria belonging to β-proteobacteria, provisionally named as *Eleftheria terrae*, using isolation chip (iChip) method (Ling et al. 2015; Piddock 2015; Wright 2015).

Overexpression of pathway-specific gene regulators

During transition from late-exponential to stationary growth phase, the overexpression of antibiotic regulatory protein in *Streptomyces* has been reported to increase the antibiotic yield (Bibb 2005). A unique class of regulatory gene has been identified by Laureti group encoding protein similar to the large ATP-binding regulators of the LuxR family (LAL) (Laureti et al. 2011). The constitutive expression of a pathway-specific, LAL regulator of type-I modular polyketide synthase (PKS) gene cluster in *Streptomyces ambofaciens* resulted in the production of stambomycins. Similarly, the overexpression of *afsS* (a 63-amino acid protein) induces selective antibiotic production in *S. coelicolor (Matsumoto* et al. 1995*)*, *S. lividans* (Vögtli et al. 1994), *S. avermitilis* (Lee et al. 2000). The overexpression of *afsR* has also been reported to induce the antibiotic production in *S. coelicolor* (Floriano and Bibb 1996), *S. peucetius* (Parajuli et al. 2005), and *S. venezuelae (Maharjan* et al. 2009*)*.

Ribosome or RNA polymerase engineering

Ochi group developed a method termed "ribosome engineering" in which the ribosomal protein S12 or RNA polymerase (RNAP) have been targeted to increase the antibiotic production in bacteria (Ochi et al. 2004). The idea was to collect ribosomal mutants and to see whether the gene expression for antibiotic production due to alteration in the transcription or translation processes has been affected or not. The ribosomal mutants, conferring antibiotic resistance, were collected by selecting them on the agar medium containing streptomycin antibiotic. Similarly, RNA polymerase (RNAP) mutants were collected by growing cells on the agar medium containing rifampicin (that binds to RNAP to inhibit RNA synthesis). These mutants have been observed not only for their ability to produce increased amounts of antibiotics but also led to the production of novel antibiotics (Hoshaka et al. 2009). Using this approach, out of "no or sub-optimal" levels of antibiotic-producing 1068 soil bacteria, 6% actinomycetes and 43% *Streptomyces* species have been identified to induce antibiotic production. The detailed study revealed that augmentation of antibiotic production was due to a mutation at Lys-88 to Glu or Arg in the ribosomal protein S12 that enhances protein synthesis at the stationary-phase growth condition. Consequently, a mutation His 437 to Asp or Leu in the RNAP β-subunit has also been found to increase its promoter binding affinity. Other antibiotics such as erythromycin and gentamicin, both of which target ribosome, have also been demonstrated to exhibit the same level of enhancement in the antibiotic production (Chai et al. 2012; Imai et al. 2012; 2015).

Isotope-guided fractionation

The approach first introduced by Gross and colleague's uses genome sequence analysis in combination with isotope-guided fractionation to identify unknown compounds coded by cryptic gene clusters (Gross et al. 2007). The strategy has been successfully applied to predict orfamides, the byproduct of cryptic NRPS domain, using bioinformatics analysis of *Pseudomonas fluorescens* genome (Paulsen et al. 2005). The approach has been also found useful in inferring the amino acid substrate used by the adenylation A domain of bacterial non-ribosomal polyketide synthatase (NRPS) (Rausch et al. 2005). Using the approach, leucine was predicted to be incorporated into four different A domains of the *ofa* cluster in *Pseudomonas fluorescens*. Since ^{15}N-L-leucine can be readily detected by ^{1}H-^{15}N Heteronuclear Multiple Bond Correlation (HMBC)-NMR, it was introduced in the exponentially growing cultures, resulting in the induction of orgamide A compound from *ofa* cluster. The isotope-guided fractionation has the potential to rapidly identify novel metabolites without using genetic manipulations. However, the approach requires optimum levels of expression of the biosynthetic gene cluster under investigation.

Heterologous expression of biosynthetic gene cluster

The introduction of complex PKS and non-ribosomal peptide synthetase (NRPS) gene clusters in heterologous hosts is technically a difficult task. The large size of these megasynthase genes is of major concern. Moreover, it is also difficult to replace a native promoter with a strong promoter that is to be induced in the host for maximizing expression. If

successfully expressed, the introduced gene cluster may produce a completely new set of the metabolic products. Using this methodology, Nihira group have engineered *A. oryzae* strain, by introducing a full citrinin biosynthetic gene cluster from *Monascus purpureus* that produces citrinin via *ctnA* activator. This is one of the best examples of cluster-specific activation of heterologous genes that may be used for the other cryptic genetic systems (Sakai et al. 2008).

Transcriptional activators and repressors

Several secondary metabolite gene clusters contain one or more genes that encode transcription factors. A co-occurrence of the LysR-Type transcriptional regulator (LTTR) family with antibiotic synthetic gene clusters has been reported in several articles (Colombo et al. 2001; Rodriguez et al. 2008; Waldron et al. 2001). LTTRs have been found to be the most abundant among Actinobacteria, Proteobacteria, and Firmicutes. These genetic regulators typically have two domains consisted of an N-terminal DNA binding domain and a C-terminal ligand-binding domain (Maddocks and Oyston 2008). In the absence of a signal, they remain on inactive state and upon interaction, they activate the concerned promoters. It is an interesting feature to retain the promoter of cryptic antibiotic gene cluster and shut it down in the absence of a signal. The activation of this type was reported by Rodriguez et al. (Rodriguez et al. 2008) where the synthesis of β-lactam antibiotic "thienamycin" in *Streptomyces cattleya* was observed to be induced by ThnI protein. Similarly, the LysR protein, designated as ORF-L16, induced spinomycin biosynthesis in *Saccharopolyspora spinosa* (Waldron et al. 2001). The *aur1* polyketide gene cluster led to the biosynthesis of angucycline-like antibiotic auricin in *S. aureofaciens* (Novakova et al. 2011). The other example includes the cyclic AMP receptor protein (Crp) (catabolite repressor) that has been demonstrated to regulate the antibiotic production in *Streptomyces* (Gao et al. 2012). The overexpression of *crp* resulted in the production of antibiotic in *Streptomyces* species. The pathway-specific regulatory gene *scbR2* (encoding c-butyrolactone receptor) in *S. coelicolor* induced the production of a yellow-pigmented secondary metabolite (yCPK) (Gottelt et al. 2010). On the other hand, deactivation of the suppressor proteins (transcriptional proteins that result in the negative regulation) has been also observed to turn on the silent antibiotic gene clusters. The repression of antibiotic clusters exerts the transcriptional and post-translational control, and the best example in this category is the production of the cryptic orsellinic acid in *A. nidulans* (Bok et al. 2013).

Synthetic biology

The synthetic manipulations of natural metabolites at the chemical level have marginally decreased its requirement from natural sources. The synthetic biology has developed a global interest in the natural product discovery (Medema et al. 2011a, 2011b; Wright 2014), and the field of "directed evolution" has an immense potential to accelerate this process (Breitling and Takano 2015). At the same time, the genetic manipulation of antibiotic production in heterologous hosts using the tools of synthetic biology has also revealed the utility of priming metabolic machinery for increased production of secondary metabolites (Helfrich et al. 2014). Keasling et al. published an interesting procedure in which plant and bacterial-derived genes were combined and engineered in *E. coli* and *S. cerevisiae* to produce amorphadiene and artemisinic acid (precursors of the potent antimalaria drug artemisinin) respectively (Dietrich et al. 2009; Martin et al. 2003; Newman et al. 2006; Ro et al. 2006). The approach has led to the discovery of 74 novel metabolites in yeast (Klein et al. 2014). The technical challenge involved, in generating multiple cluster variants that can express antibiotic gene, is its high cost per compound (Bachmann et al. 2014).

Functional proteomics

At the present time, proteomics tools have been successfully utilized in the identification of orphan gene clusters. By comparing protein profiles of pyomelanin-producing and non-producing strains of *A. fumigates*, the pyomelanin synthesis has been reported (Schmaler-Ripcke et al. 2009). Proteomic investigation of secondary metabolism (PrISM) has been developed to identify highly expressed megasynthase genes that do not require prior knowledge of genome sequence (Bumpus et al. 2009). The PKS and NRPS are high-molecular-weight enzyme proteins (> 200 kDa), and their proteome can be analyzed by excising their bands from protein gels, using high-resolution tandem mass spectrometry. The ions derived from the phosphopantetheinyl (Ppant) arm in the carrier protein domains of PKS or NRPS may then be tracked by mass spectrometry (Dorrestein et al. 2006). By using the de novo peptide sequencing of the proteome of wild-type *Bacillus* strain NK2018 using LC-MS, a novel NRPS cluster has been identified. The DNA sequence of this cluster has been observed to be 94% identical to *B. cereus* AH1134. In addition to the PrISM approach, fluorescent and biotinylated probes, targeting thioesterase domains of PKS and NRPS, have also been developed. Importantly, these probes do not depend on the genetic expression of metabolic clusters (Meier et al. 2008).

Combinatorial chemistry or metabolomics

The modular nature of secondary metabolites makes them an excellent source for their use in combinatorial chemistry. These metabolites are synthesized as polymer backbones

and diversified by the actions of tailoring enzymes (Liu et al. 2011). The integration of the natural product synthesis with organic chemistry in combinatorial synthesis has led to engineer a completely new class of compounds, similar to the natural products (Ortholand and Ganesan 2004). In this process, the natural products are used as building blocks upon which new and diverse entities are added. Several combinatorial libraries have been developed covering a huge structural diversity of compounds. To explore natural products for their combinatorial synthesis, Romano group employed ultra-high resolution mass spectrometry to search the exo-metabolome of *Pseudovibrio* sp., grown under variable phosphate concentrations (Romano et al. 2014). Sarkar and colleagues applied metabolomics-based analysis to screen secondary metabolite production in *A. nidulans*, grown under various nutrient growth conditions and identified several polyphenolic compounds, including a complete novel prenylated benzophenone preshamixanthone (Sarkar et al. 2012). Forseth et al. identified cryptic products of the gliotoxin gene cluster using NMR-based comparative metabolomics (Forseth et al. 2011). They were able to screen *gli*-dependent metabolites using differential analysis of 2D-NMR spectra of metabolite extracts derived from *A. fumigatus* strain, deleted for *gli* gene. Several other strategies have also been developed for metabolomics-based prioritization of microbes for natural product discovery (Hou et al. 2012). Analytical technologies such as nano-spray desorption electrospray ionization (nanoDESI) mass spectrometry and matrix-assisted laser desorption ionization-time-of-flight-imaging mass spectrometry (MALDI-TOF-IMS) for high-throughput identification of cryptic secondary metabolites have also contributed significantly (Breitling et al. 2013).

Direct cloning using artificial promoters

The insertion of inducible strong promoters has been also reported to lead to the activation of the cryptic antibiotic gene clusters. The biosynthetic genes for secondary metabolites that are usually silent or are expressed minimally can be cloned using strong promoters into suitable plasmid vectors. The silent spectinabilin pathway of *S. orinoci* and taromycin A pathway of *Saccharomonospora* sp. have been identified using this system (Shao et al. 2013; Yamanaka et al. 2014). Similarly, a cryptic antibiotic gene cluster SGR810-815 in *S. griseus* has been also reported to induce three novel polycyclic tetramate macrolactams (Luo et al. 2013). In *E. coli*, alterochromide lipopeptides of *Pseudoalteromonas piscicida* have been shown to heterologously expressed using native, *E. coli* T7 promoter (Ross et al. 2015). The expression of a silent antibiotic gene cluster has also been achieved in *Streptomyces* using a strong promoter *ermE* (Baltz 2010). The *bbr* gene (transcriptional activator) of *Amycolatopsis balhimycina* also exhibited the induction of balhimycin biosynthesis in the heterologous host *A. japonicum* (Spohn et al. 2014).

Chromatin remodeling

Modifications that affect gene regulation in higher-order organisms include methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, and glycosylation (Cichewicz 2012). But with respect to the production of antibiotics and their induction from the corresponding genetic clusters, the aspect is relatively less studied. It has been reported that the putative gene clusters for antibiotic synthesis are present in fungal genomes in the distal regions of chromosomes (Shwab et al. 2007). These regions typically exist in a heterochromatin state and gene expression requires epigenetic control here; therefore, there exist a possibility that these antibiotic biosynthetic gene clusters may express. Keller et al. developed a method known as "chromatin remodeling" for the induction of secondary metabolite genes in microorganisms. While investigating the regulatory pathway of sterigmatocystin (a toxin in *Aspergillus nidulans*), they identified several mutant strains exhibiting the suppressed production of the toxin (Butchko et al. 1999). The mutation was identified in LaeA (methyltransferase) that is known to be involved in the regulation of sterigmatocystin production. In addition, deletion of LaeA blocked the expression of several biosynthetic gene clusters while overexpression of the same triggered penicillin and lovastatin production (Bok and Keller 2004). Since LaeA has the homology with histone methyltransferases, the regulation of this type was proposed as a chromatin-based model of regulation. The deletion of *hdaA* in *Aspergillus* histone deacetylase also resulted in the increased production of two telomere-proximal secondary metabolite gene clusters, but the transcription of a telomere-distal cluster remained unchanged (Shwab et al. 2007). Similarly, in Cichewicz's experiments, 12 fungi were subjected to a library of DNA methyltransferase using variable concentrations of histone deacetylase inhibitors and 11 strains were observed for their increased production of diverse secondary metabolites. Oberlies et al. used the proteasome inhibitor bortezomib to induce a filamentous fungus to produce cryptic metabolite (VanderMolen et al. 2014). The similar method was applied by McArthur et al. in *Streptomyces coelicolor* (Moore et al. 2012). These observations clearly demonstrate the importance of small molecule epigenetic modifiers in accessing silent gene clusters for the discovery of novel metabolites.

Rare earth elements

Until recently, the rare earth elements were not known for their biological effects in living cells. These elements have been found not only to activate cryptic genes but also in the overproduction of antibiotics. The rare earth elements are

consisted of 17 elements, including scandium, yttrium, and lanthanides. Interestingly, the growth medium containing 10–100 μM scandium or lanthanum has been reported to increase antibiotic production in *Streptomyces* sp. by 25-fold (Kawai et al. 2007). The effect of scandium in the induction of antibiotic in *S. coelicolor* has been demonstrated to be at the level of transcription of pathway-specific regulatory gene (*act II*-ORF4). The antibiotic production under low concentrations of scandium indicates that it functions in situ, as an important factor to induce the production of secondary metabolites as well as pigments, toxins, and antibiotics. The addition of scandium to the growth medium of *B. subtilis* has also been observed to enhance the production of α-amylase and bacilysin (Inaoka and Ochi 2011). The rare earth elements are distributed ubiquitously in the ecosystem at low levels; it is possible that microbes have acquired the ability to react with these low levels over the course of evolution to induce antibiotic biosynthetic gene clusters. The main advantage of using rare earth elements in culture media for augmenting antibiotic production is that the approach does not require prior knowledge of genetic engineering.

High-throughput elicitor screens

The high-throughput elicitor screen method was first used by Nodwell and co-workers to observe the induction of blue polyketide actinorhodin and red prodiginines in *S. coelicolor (Craney* et al. 2012*)*. A total of approximately 30,500 small molecules were screened from the Canadian Compound Collection (McMaster University, HTS facility) to assess actinorhodin production in *S. coelicolor*. In this screening process, the upregulation of actinorhodin synthesis as well as the overproduction of germicidins remodeling compounds (ARC2 group; 4 compounds out of the selected 19 actinorhodin-stimulating molecules) was also recorded. The reduction in the production of daptomycin-like CDA and prodiginines was also observed. Intriguingly, ARC2 and triclosan (inducer of actinorhodin synthesis) both displayed the modulated secondary metabolism only at sub-inhibitory concentrations. ARC2 also altered the secondary metabolome of *S. pristinaespiralis*, *S. Peucetius*, and *Kutzneria* sp. by inducing cryptic metabolites. Recently, Cl-ARC (derivative of ARC2) has been also reported to induce a total of 216 cryptic metabolites in 50 different *Streptomyces* (Pimentel-Elardo et al. 2015). The usefulness of high-throughput elicitor screens has been also demonstrated in *Burkholderia thailandensis* (Liu and Cheng 2014; Seyedsayamdost 2014).

Co-cultivation

Presently, co-cultivation has turned into one of the main strategies to identify novel secondary metabolites from microorganisms (Brakhage 2013; Moody 2014). The co-cultivation approach or "interspecies crosstalk" has proven to be a success in the de novo production of secondary metabolites (Bertrand et al. 2013a, b; Brakhage and Schroeckh 2011; Marmann et al. 2014; Schroeckh et al. 2014). Several reports on fungal biotic interactions indicated that many cryptic secondary metabolites can be induced by co-culturing fungi with fungi (Bertrand et al. 2013a, b; Cueto et al. 2001; Mela et al. 2011; Wald et al. 2004). Many such studies have also been carried out involving confrontation of fungi and bacteria (Cheng et al. 2013; Ola et al. 2013). Several reports have established that co-culture strategy has a huge potential to activate silent gene clusters by yet unknown mechanisms. A study conducted by Konig et al. clearly demonstrated that co-culture of *Aspergillus fumigatus* with bacterium led to the activation of an otherwise silent pathway after addition of an epigenetic modulator (Konig et al. 2013). It has been demonstrated that co-cultivation of microbes can induce gene mutation and subsequent expression of silent gene clusters (Charusanti et al. 2012). The exchange of whole gene fragments (horizontal gene transfer) has also been reported to induce the gene expression of previously undetected secondary metabolites (Kurosawa et al. 2008). Pestalone, a potent antibiotic against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium*, is the product of a co-culture of Gram-negative bacterium *Thalassopia* with marine fungus *Pestalotia (Cueto* et al. 2001*)*. Gram-positive *Bacillus subtilisis* has been also shown to induce macrocarpon, 2-carboxymethylamino benzoic acid, and citreoisocoumarinol in *Fusarium tricinctum* (Ola et al. 2013). Similarly, the activation of silent PKS gene clusters, during co-cultivation, resulted in the induction of a huge number of polyketides in *Aspergillus nidulans* (Schroeckh et al. 2014).

Environmental signals

The silent antibiotic gene clusters in microorganisms are considered to be a potential source of secondary metabolites, but the environmental clues to induce their expression remain unknown (Abrudan et al. 2015; Zhu et al. 2014). It is highly important to understand the biological role of cryptic antibiotic gene clusters in antibiotic-producing microbes in a given niche before making attempts to activate them. The members of actinomycetes group grow as a branched multicellular network of hyphae and are known to reproduce through spores that are formed by an aerial mycelium. The detailed description of the control of morphological differentiation in actinomycetes is reviewed elsewhere (Chater 2006; Flardh and Buttner 2009; Hopwood 2006). In natural systems, many antibiotics are produced after specific signals are receiving from the surrounding environment. Specialized techniques are required to decode these clues that can activate the production of secondary metabolites in microorganisms. The antibiotics

have classically been considered as antimicrobial weapons (Raaijmakers and Mazzola 2012; Ratcliff and Denison 2011). The studies conducted by Abrudan et al. (Abrudan et al. 2015) and Westhoff et al. (Westhoff et al. 2017) in *Streptomyces* also supported the observation that antibiotics play their major role in environment as defense molecules. The generalization that antibiotics have an antagonistic role in nature similar to its clinical role is a big question (Linares et al. 2006; Ratcliff and Denison 2011; Romero et al. 2011). The reason for this assumption is the low concentrations of antibiotics present in the soil environment that may not exhibit inhibitory effects. Subsequently, sub-inhibitory concentrations of antibiotics can induce a pleiotropic response in microorganisms such as quorum sensing, biofilm formation, and coordinated expression of virulence genes (Hoffman et al. 2005; Stevens et al. 2007; Yim et al. 2006).

Small-molecule chemical elicitors

It is important to understand the regulatory control of cryptic biosynthetic gene clusters (Van Lanen and Shen 2006; Wilkinson and Micklefield 2007). The role of HTH-type transcriptional repressor DasR has been assessed in the control of cryptic type I PKS gene cluster. In *S. coelicolor*, a signaling cascade consisted of *N*-acetylglucosamine and the DasR regulon has been shown to activate the antibiotic production. A high concentration of *N*-acetylglucosamine (bacterial cell-wall component) triggers the antibiotic production under nutrient poor growth conditions (Rigali et al. 2008). *N*-acetylglucosamine binds to its effector binding site to DasR and acts as a ligand that reduces the protein's affinity to DNA (Rigali et al. 2006). Another protein, AtrA, appears to counteract DasR, by having opposed effects in the signaling pathway. DasR represses the gene transcription of NagE2 (GlcNAc-specific transporter) and ActII-ORF4, whereas AtrA activates both of these genes at the transcription level (Nothaft et al. 2010). DasR also controls siderophore production (Craig et al. 2012). The enhanced transcription of chromosomally encoded antibiotic biosynthetic clusters (*act*, *cda*, *red*, and cryptic *cpk*) has been observed in *dasR* mutants of *S. coelicolor (Rigali* et al. 2008*)*. Thus, the expression of DasR is linked to the induction of antibiotic genes in many actinomycetes, provided *N*-acetylglucosamine is used as a carbon source (van Wezel et al. 2009). Other than *N*-acetylglucosamine, molecules such as nucleotides, oligopeptides, amino acids, polysaccharides, and fatty acids etc. have also been reported to induce the antibiotic production. Triclosan which is known to mediate cell-cell communication has also been observed to involve in quorum sensing and autoinduction mechanisms. When a culture of *Pseudomonas aeruginosa* is treated with triclosan, it exhibited downregulation of the quorum-sensing genes (Chuanchuen and Schweizer 2012; Craney et al. 2012). The quorum-sensing signaling for activating antibiotic production in streptomycetes is regulated by γ-butyrolactones (Takano 2006).

Genome mining

The abundance of readily available genomic data and advances in sequencing and computational techniques has helped to achieve higher success rate in predicting the novel secondary metabolites from cryptic gene clusters. The following part of the review describes the overall development in the field. At the present time, whole culture collections of microbes have been sequenced and new technologies like single cell genomics and metagenomics have generated massive data to analyze. Previously, conserved genes for PKS or NRPS domains were used as probes in Southern hybridization experiments. DNA primers were prepared from these highly conserved motifs for PCR screenings. Thus, generated results were analyzed using sequence comparison software such as BLAST (Altschul et al. 1990) or DIAMOND (Buchfink et al. 2015) or profile-based tools like HMMer (Finn et al. 2011). Presently, in silico genetic screening of novel NRPS and PKS is performed using NRPS/PKS domain organization in bacteria and from the available metagenomic data. DECEIPHER is the first tool developed by Ecopia Biosciences Inc. for automated cluster mining (Farnet and Zazopoulos 2005). Thereafter, many other tools were developed that include BAGEL (De Jong et al. 2006), CLUSEAN (Weber et al. 2009), antiSMASH3.0 (Blin et al. 2013; Medema et al. 2015; Weber 2015), NRPSpredictor2 ((Röttig et al. 2011)), SBS-PKS (Anand et al. 2010; Ansari et al. 2004), PKS/NRPS (Bachmann and Ravel 2009), NORINE (Caboche et al. 2008), ClustScan (Starcevic et al. 2008), NaPDoS (Ziemert et al. 2012), NRPSsp (Prieto et al. 2012), NRPS/PKS substrate predictor (Khayatt et al. 2013), PRISM (Skinnider et al. 2015), Dynamite (Ogasawara et al. 2015) etc. Using this wealth of information and applied bioinformatics tool, many attempts have been made to identify antibiotics with novel structures and are being submitted to the natural product library at regular basis (Chen et al. 2017; Milshteyn et al. 2014; Ugai et al. 2016; Xu et al. 2016).

Concluding remarks

The discovery and development of antibiotics is one of the greatest achievements in the treatment and prevention of bacterial infections. New infectious diseases and resistant pathogens have been discovered at a rapid rate, but there has been no discovery of new antibiotics that can keep pace with it. It is speculated that the microbial parvome for the discovery of novel metabolic molecules has been exhausted. The increasing rates of antimicrobial resistance urgently necessitate new strategies to stock-up antimicrobial drug pipelines. It is widely accepted that microbes possess a huge potential for the

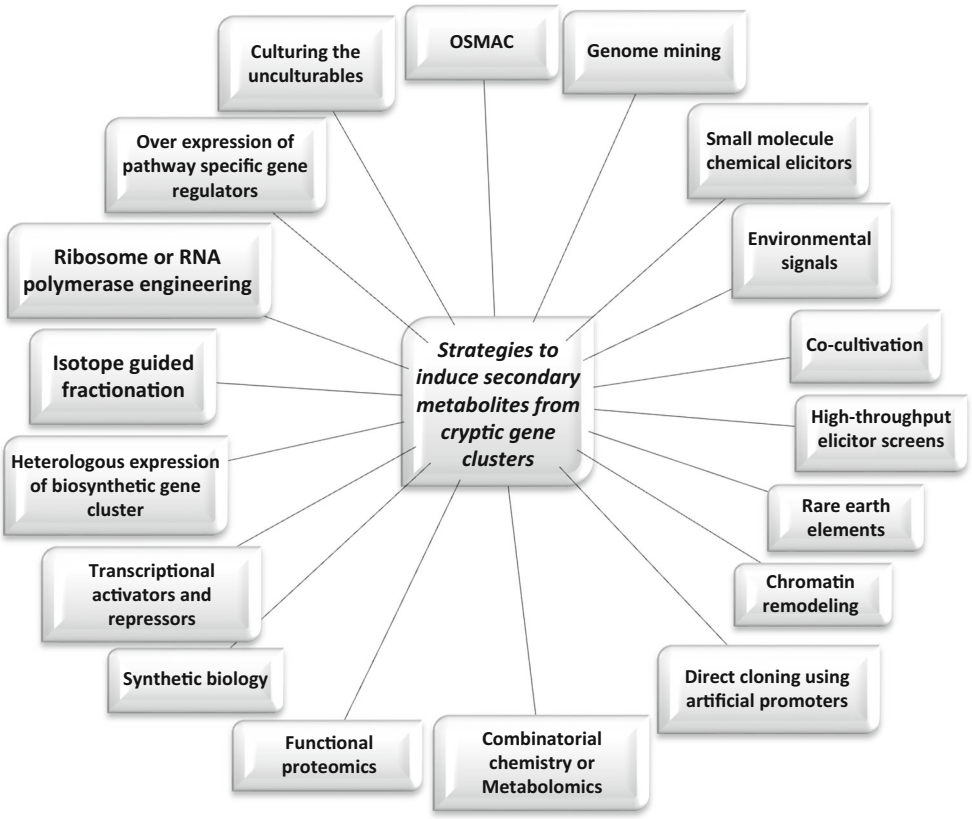

Fig. 3 Strategies for the induction of secondary metabolites from cryptic gene clusters in bacteria

biosynthesis of secondary metabolites of high chemical diversity. It is highly challenging to explore this diversity within the microbial biosynthetic repertoire to drive expression, development, and synthesis of new chemical scaffolds. In addition, several of these biosynthetic pathways are observed to be cryptic or are expressed minimally; therefore, novel strategies are required to identify the environmental clues to activate them. Awakening cryptic gene clusters to produce polymorphism of secondary metabolites is a difficult process, but using the above-described strategies (Fig. 3), the activation of the cryptic genes may lead to the identification of novel compounds of therapeutic and industrial use. Apart from the technicalities of using different strategies for decryptification, it is also important to analyze why biosynthetic genes are cryptic under standard laboratory conditions. Understanding the regulation of these cryptic systems would make possible to explore the full potential of biosynthetic gene clusters in microorganisms for the screening of new secondary metabolic compounds. Nevertheless, additional efforts have to be made in developing more effective strategies to study and understand cryptic genetic systems for the discovery of their yet-unidentified natural products. We do not really need to completely rely on nature as a sole provider of novel natural products; chemical synthesis and modifications of existing moieties also provide potential alternatives. However, it is important to note that nature has unearthed only a tiny fraction of its huge store of metabolic treasure of natural products and it will remain the most preferred source over the chemical synthesis. Upon the discovery and exploration of yet-to-be-discovered secondary metabolites from the hidden sources, we will definitively be having solutions near in the future against the current crises of "slow-moving-drug-development" and multi-drug-resistant pathogens. The consortia of these innovative strategies have strengthened the belief and enlighten the path for the discovery and development of novel chemical scaffolds. With the increasing application of such strategies, it is hoped that novel and effective antibiotics will be identified soon from the microbial parvome. The identification of secondary metabolites from these cryptic genes may facilitate the discovery of much needed novel antimicrobials to effectively resist multiple-drug-resistant pathogens. The burden is on academicians now to conduct research on the hidden aspects of the microbial treasure of secondary metabolism for the identification of yet-to be-identified antibiotics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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