Global Regulators to Activate Silent Biosynthetic Gene Clusters

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Abstract – Genome mining has recently emerged as a powerful strategy to discover novel microbial secondary metabolites. However, more than 50% of biosynthetic gene clusters are not transcribed under standardized laboratory culture condition. Several methods have been applied to activate silent biosynthetic gene clusters in the microbes so far. Among the regulatory systems for production of secondary metabolites, global regulators, which affect transcription of genes through regulatory cascades, typically govern the production of small molecules. In this review, global regulators to affect production of microbial secondary metabolites were discussed.

Keywords – Microbial natural products, Secondary metabolites, Biosynthetic gene clusters, Global regulators

Introduction

At present, numerous medicines are directly originated or are inspired from microbial natural products and their secondary metabolites. These secondary metabolites are known to be produced by microbes in response to environmental stress or interaction with host, providing competitive advantages. Proteins essential for the production of the bioactive compound are usually encoded by large cryptic gene clusters that remain silent under normal laboratory conditions, which hinders the discovery of new secondary metabolites.1-3 The corresponding biosynthetic gene cluster (BGC) can be identified biometric and act as an indicator or marker of bacterial capacity for the production of secondary metabolites. In general, cryptic BGCs are essentially present 5-10 times than expressed BGCs, suggesting that small molecules produced by microorganisms found so far are only the tip of the iceberg.4-6 To understand the way of expression of these gene clusters will not only discover new beneficial compounds but also reveal the pathogenic mechanism. The recognition of the global regulators controlling silent biosynthetic gene clusters has paved the way to achieve these goals.

According to the previous reports, a number of secondary metabolites are produced simultaneously, under abiotic stresses, and even corresponding gene clusters are expressed by deactivating a single transcription factor, so the global regulators are regarded important in discovery of new compounds.1-4 Global regulators (GRs) are transcription factors that generally play an important role in microorganism. They help systematize thousands of gene reactions in cells to complex environmental changes. In contrast to pathway-specific regulators which control the transcription of a small number of genes, GRs control hundreds of genes.8 Both global regulators and pathway specific regulators mediate precise activation or suppression by detecting changes in specific metabolites. The global regulators detect a larger number of growth conditions than pathway-specific regulators. They can detect environmental changes through specific metabolites as well as affect other regulators by transcriptional-specific interactions.

Gottesman has previously defined global regulators on the basis of their pleiotropic phenotype and their ability to regulate the operons belonging to different metabolic pathways.9 This definition excludes proteins associated with essential cell machinery.10 Each of the global regulators usually has a functional role. On the other hand, the genes regulated by each global regulator can have a variety of heterogeneous functions.8 Some global regulators have been previously reported to be involved in the control of certain genes related with pathogenicity, quorum sensing, and biofilm formation.7 Activation/inactivation of global regulators is related to significant changes in the production of secondary metabolites and to the induction of corresponding biosynthetic gene clusters.1

In regulatory systems, changes in the synthesis of σ factors required in transcription or competition in different
factors to combine in core RNA-polymerase result in the activation and suppression of different gene expression programs, which is a global mechanism for selective gene expression in the cell development process. One active global regulator can control genes modified by different σ factors, and σ factors can control genes by specific regulators.

Although global regulators are involved with numerous metabolism in microorganisms, we herein focus on the expression of the gene clusters to be responsible for the biosynthesis of secondary metabolites in microorganisms. Ten global regulators in bacteria and fungi were discussed and the secondary metabolites enhanced by manipulation of each global regulator are shown in figs. 1-7.

**Global regulators and corresponding secondary metabolites**

Silent biosynthetic gene clusters represent grounds for the discovery of new secondary metabolites and numerous approaches to activate them were attempted so far. *Burkholderia thailandensis* is a non-virulent model for its pathogenic relative spp. Twenty-three BGCs were found to be harbored in *B. thailandensis* E264 isolated from rice field in Thailand. Among 23 BGCs, only six gene clusters resulted in the production of small molecules under the normal laboratory condition. By various methods such as a promoter insertion strategy and subjecting heat stress, silent or lowly expressed BGCs were activated producing malleilactone, burkholdacs, and capistruin (Fig. 1). In other aspects, *B. thailandensis* E264 has acyl homoserine lactone-dependent quorum-sensing (QS) systems, which consist of LuxI/LuxR synthase/response regulating systems. In the other protobacteria such as *Pseudomonas aeruginosa*, LysR-type transcriptional regulators (LTTRs) rather than LuxRs are important in the production of small molecules. LysR-type transcriptional regulator, named ScmR, was found by RNA sequencing analysis and it was found to play important roles in secondary metabolism and virulence. To elucidate the role of ScmR in the transcriptional control of BGCs, an *scmR* mutant (*ΔscmR*) was made for comparison of its secondary metabolic profile with that of the wild type. It remarkably enhanced the production of malleilactone and burkholdacs. Beside new analogues of known compounds, metabolic analysis of the *ΔscmR* strain also provided a

![Fig. 1. Secondary metabolites produced by ΔscmR in Burkholderia thailandensis E264.](image-url)
family of aromatic compounds that are only produced in the mutant strain (Fig. 1). In proteobacteria, some secondary metabolites are known to be related with biofilm formation. In this ΔscmR strain, biofilm formation enhanced compared with the wild-type, indicating that scmR acts as a repressor of biofilm formation and virulence. It also induced pathway-specific regulator, AraC-type transcriptional regulator to be involved in the production of burkholdacs. Interestingly, the expression of mal and bhc which are responsible for the biosynthesis of malleilactone and burkholdacs, respectively, were strongly repressed by ΔscmR while the wild type produce a great amounts of malleilactone and burkholdacs. To elucidate interaction of scmR with mal and bhc, ΔmalR, ΔaraC, ΔmalRΔscmR, and ΔaraCΔscmR were prepared. The production lacked in ΔaraC and ΔaraCΔscmR strains, indicating that AraC-type regulator is required for induction of bhc. Therefore, scmR deletion activate the bhc cluster via the AraC regulator.

Photorhabdus luminescens has been reported to have symbiotic relationship with a nematode Heterorhabditis bacteriophora by producing an array of secondary metabolites. A global post-transcriptional regulator, Hfq, which is found widespread in bacteria, was deleted to elucidate the effect of the secondary metabolites in the survival of the nematode. Hfq has been known to be related with antibiotics productions.\(^{16,17}\) RNAseq led to the production of a second deletion of a known repressor, HexA, which restored both metabolite production and symbiosis. Hfq is involved in regulation of secondary metabolism in P. luminescens through HexA but is not related with pathogenesis toward Galleria mellonella. Deletion of hfq resulted in no production of secondary metabolites, causing no healthy symbiosis. Interestingly, Deletion of hexA was, in many cases, more effective than complementation in trans with hfq in the expression of secondary metabolites.\(^{18}\) Secondary metabolites regulated by deletion of hfq gene in P. luminescens are anthraquinones, gameXPeptide, isopropylstilbene, cinnamic acid, photopyrone, and phurealipids as shown in Fig. 2.

Csr (carbon storage regulator) is a global regulatory system found in Escherichia coli, and known to increase precursor for biosynthesis of aromatic amino acid. Disruption of csrA increases gluconeogenesis and thus elevates phosphoenolpyruvate, a precursor of aromatics.\(^{19}\) When csrA was disrupted, the phenylalanine biosynthesized by shikimate pathway was produced two fold.\(^{20}\) Production of the other precursor, erythrose-4-phosphate increased 1.4-fold by overexpression of tktA (transketolase).\(^{21}\) The enhancement of the regulatory enzymes for phenylalanine biosynthesis was not related with the csrA. Both the csrA mutation and the plasmid encoding transketolase, pAT1, decreased the growth rate, which was reflected in the kinetics of both glucose uptake and phenylalanine accumulation.\(^{20}\) Phenylalanine was accumulated the highest in mid-to-late exponential phase of growth and it was produced for a few hours in the stationary phase. The csrA mutant and the csrA mutant containing pAT1 produced more phenylalanine without accumulation of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP). The increased intracellular phosphoenolpyruvate in the csrA conversion of endogenous DAHP to 5-enolpyruvoylshikimate-3-phosphate (EPSP) and ultimately to phenylalanine. These results demonstrate a potential pitfall of measuring an intermediate as an indication of pathway flux in metabolically engineered

Fig. 2. Secondary metabolites regulated by deletion of hfq gene in Photorhabdus luminescens.
strains. Because PEP availability limits phenylalanine production and one phosphoenolpyruvate (PEP) molecule is utilized per glucose transported via the phospho-transferase system (PTS) pathway, the recycling of pyruvate to PEP (e.g., by increasing PEP synthetase) increases the theoretical yield of phenylalanine. Phenylalanine synthesis requires an additional PEP, and the maximum theoretical yield drops to 3 mol/10 mol glucose, or 6 mol/10 mol glucose with pyruvate recycling. The glycogen biosynthesis pathway does not compete effectively with the shikimate pathway in these engineered strains during growth on MOPS medium.

Global regulator BldA is well-known to have effects on morphological differentiation and secondary metabolism in the genus *Streptomyces*. The global regulator *bldA* is known to encode a tRNA required for the translation of the UUA codons. In order to confirm the function of *bldA* gene in *S. lincolnensis*, *bldA* was disrupted, which resulted in decrease in sporulation and lincomycin production. BldA is known to be strongly related with translation of *lmbB2* gene encoding L-tyrosine hydroxylase. However, *bldA* mutant causes mistranslation of the *lmbB* gene encoding a cluster-situated regulator (CSR). The effects in the lincomycin production is attributed to the TTA-containing regulators outside the cluster as well as the two TTA-containing genes, *lmbB2* and *lmbB*, in the cluster. BldA can regulate the translation of *lmbB2*, a gene to encode a L-tyrosine hydroxylase which is responsible for propylproline (PPL) pathway. However, BldA is not required for the translation of *lmbB*, a gene to encode a transcriptional regulator of lincomycin biosynthesis. Some other TTA-containing gene(s) outside the cluster may also contribute to lincomycin production. Although the global regulator *adpA* is probably the most critical, the other additional genes may be involved.

The plant pathogenic fungus *Fusarium fujikuroi* is well-known producer of giberrellin, which causes *bakanae* disease on rice. The orthologue of Ffsge was studied in *F. fujikuroi*, particularly for the role in regulation of gene expression for secondary metabolites. Ffsge1 is involved not in the pathogenicity of this fungus but in the vegetative growth. In addition, Ffsge1 acts as a positive regulator for the biosynthesis of gibberellins, fusarins, fusaric acid, fumonisins, apicidin F, bikaverin, fusarubins, and apicidin F in *F. fujikuroi* (Fig. 3). The transcript levels of gibberellins, fusarins, fusaric acid, fumonisins, apicidin F, bikaverin, fusarubins, and apicidin F biosynthetic genes were significantly reduced in the Δ*ffsge1* mutant. Overexpression of *FfSGE1* led to increased production of secondary metabolites under both favorable and non-favorable conditions. Ffsge1 functions as a master regulator for biosynthesis of secondary metabolites. Its overexpression in the wild-type background led to elevated fumonisins, fusaric acid and apicidin F production under their optimal production conditions. It is notable that fusarins, fusaric acid, and apicidin F were produced even under non-favorable conditions, which indicates that overexpression of *FfSGE1* can mediate nitrogen regulation.

McrA, a transcription factor, acts as a negative regulator of secondary metabolites. Deletion of *mcrA* (*mcrAΔ*) in *Aspergillus nidulans*, the gene encoding McrA, resulted in the increased production of many secondary metabolites by altering expression of over 1000 genes. LlmG, strongly upregulated by the deletion of *mcrA*, encodes a putatively methyltransferase related with LaeA, one of key regulators in secondary metabolism. Upregulation of *llmG* on various media using strong

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**Fig. 3.** Secondary metabolites regulated by *ffsge1* gene in *Fusarium fujikuroi*. 
constitutive promoters resulted in increased production of the important toxin sterigmatocystin, terrequinone A, nidulanin A, cichorine, emericellin, and emodin from at least six major SM pathways. However, \( \text{llmG} \) overexpression did not increase austinol, F-9775A/B, and emericellamide pathways. The increased production of multiple secondary metabolites from several BGCs indicates that \( \text{llmG} \) is a master secondary metabolites regulatory gene. One of the upregulated metabolites was determined to be a not previously reported compound that is a putative intermediate or shunt product of the cichorine biosynthetic pathway. \( \text{mcrA} \Delta \) than upregulation of \( \text{llmG} \) generally increase production of secondary metabolites. In addition, the combination of \( \text{mcrA} \Delta \) and upregulation of \( \text{llmG} \) generally resulted in greater compound production than \( \text{mcrA} \Delta \) alone, suggesting that combination strategy of the \( \text{mcrA} \) deletion with others to elicit secondary metabolites production may be more effective than the individual strategies alone (Fig. 4).

\textit{Serratia} sp. ATCC 39006 is known to produce red, tripyrrole antibiotic compound, prodigiosin (Pig; 2-methyl-3-pentyl-6-methoxyprodigiosin) which exhibits anticancer and immunosuppressant activities.\(^{29,30} \textit{Serratia} \) sp. ATCC 39006 also produce a \( \beta \)-lactam antibiotic, carbapenem in addition to prodigiosin.\(^{31} \) The biosynthetic pathway of prodigiosin was elucidated, where numerous regulatory systems were related with the production of secondary metabolites. An \( N \)-acyl homoserine lactone (\( N \)-AHL) quorum-sensing (QS) system is known to be involved in controlling of production of carbapenem and prodigiosin in response to the concentration of \( N \)-butanoyl-\( \gamma \)-homoserine lactone (BHL) and \( N \)-hexanoyl-\( L \)-homoserine lactone (HHL).\(^{32} \) In addition to QS system, several regulatory genes are known to be critical.\(^{32,33} \) PigP, a key regulator protein, is known to control expressin of carbapenem and prodigiosin by modulating at least seven regulatory genes such as such as \( \text{carR}, \text{pigR}, \text{pigQ}, \text{pigP}, \text{rap}, \text{etc}. \)\(^{31} \) Another transcriptional activator, PigT was elucidated in controlling prodigiosin biosynthesis (Fig. 5). PigT, a GntR homologue, activates transcription of the Pig biosynthetic operon (\( \text{pigA–O} \)) in the absence of gluconate, whereas transcription of \( \text{pigA–O} \) is reduced by addition of gluconate. Furthermore, on the basis of sequence similarity to the \( \text{gnt} \) operator site, a putative PigT binding site was identified in the promoter of pigA. Therefore, PigT is presumed to activate directly transcription of \( \text{pigA–O} \).\(^{34} \)

One of the global regulatory system for production of secondary metabolites in \textit{Pseudomonas} spp. is the GacS/GacA two-component system.\(^{35,36} \) The system is composed of GacS, the membrane-bound sensor kinase GacS, which phosphorylates GacA, the cytoplasmic response regulator. The activated GacA protein bind to promoter region of sRNA genes \( \text{rsmX}, \text{rsmY}, \text{and rsmZ} \) to activate the transcription. The resultant sRNA exclude translational repressor such as RsmA and RsmE, and then bind to mRNA, which cause a conformational change. Then the access of the 30S ribosomal unit is prohibited, which...
ultimately suppresses the activation of translation of target genes.\textsuperscript{37-39} That is to say, the post-transcriptional blocking of all gene clusters with GGA motif within the ribosome-binding site is prevented by activating the GacS/GacA system. Through genome-mining research of the Gram-negative bacterium \textit{Pseudomonas fluorescens} Pf0-1, a new lipopeptide named gacamide A was discovered as a product of the formerly silent CLP biosynthetic gene cluster by employing the complemented strain Pf0-1-gacA\textsuperscript{+} (Fig. 6).\textsuperscript{40} Gacamide A shows a moderate antibiotic activity with narrow-spectrum and facilitates bacterial surface motility.

Secondary metabolism of \textit{Streptomyces} is known to be controlled by a complicated regulatory network. Manipulation of the regulators gives an impact on the production of secondary metabolites. Among the regulators, the IclR-like global regulator, ndgR, is known to be involved in amino acid metabolism in \textit{Streptomyces} sp.\textsuperscript{41} Disruption of \textit{ndgR} in \textit{Streptomyces coelicolor} led to defective differentiation, increasing production of actinorhodin in minimal media containing certain amino acids.\textsuperscript{42} Deletion of \textit{areB}, a homolog of \textit{ndgR}, increased production of clavulanic acid and cephapenic C in \textit{Streptomyces clavuligerus}. In the marine-derived \textit{Streptomyces youssoufiensis} OUC6819, \textit{NdgR\textsubscript{yo}}, was selected as the target gene to discover cryptic secondary metabolites.\textsuperscript{43} Inactivation of the \textit{ndgR\textsubscript{yo}} gene in \textit{S. youssoufiensis} OUC6819 afforded the production of a new fatty acid amide, \textit{3-(3E,6E,8E,10R,12E)-11-hydroxy-4,8,10,12-tetramethyltetradeca-3,6,8,12-tetraenamido} butanoic acid and an unusual 3-amino-butyl acid. The fatty acid was also discovered in both wild-type and \textit{ΔndgR\textsubscript{yo}} mutant strains (Fig. 6).\textsuperscript{44}

In many filamentous fungi, overexpression of \textit{laeA} gene is known to increase production of secondary metabolites by activating silent biosynthetic gene clusters.\textsuperscript{45,46} The \textit{laeA} gene, which encodes a positive global regulator of secondary metabolism was also found in \textit{Aspergillus fumisynematus} F746.\textsuperscript{47} Overexpression of the \textit{laeA} gene in \textit{Aspergillus fumisynematus} gave effects in the length of the conidial chain and production of secondary metabolites, with shorter conidial head chain length and
reduced spore production. It also produced cyclopiazonic acid, which the structure is shown in figure. Thus, in a filamentous fungus of which genome sequencing is unavailable such as A. fumisynnematus, an efficient method to increase SM production or to activate silent SM gene clusters is overexpression of the laeA gene. However, the possibility of production of mycotoxins or other harmful compounds should be also considered. Therefore, the strategy to make better strains to manufacture healthy functional foods by laeA overexpression should be cautiously addressed.

Conclusion

In summary, ten global regulators were discussed regarding their activation or repression of biosynthetic gene clusters to produce secondary metabolites in microorganisms. Although a limited number of the global regulators have been studied with regard to their biosynthetic gene clusters so far, identification and manipulation of the global regulators could provide a powerful approach to discover biologically important and structurally novel secondary metabolites.

Acknowledgements

This work was supported by Duksung Women’ University research in 2019.

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