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Studies on regulation of secondary metabolism and morphogenesis of *Streptomyces* by DNA microarray analysis

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Research aims

The Gram-positive, soil-inhabiting, filamentous bacterial genus *Streptomyces* is characterized by its ability to produce a wide variety of secondary metabolites represented by antibiotics and by its complex multicellular development. In *Streptomyces griseus*, both secondary metabolism and morphological development are triggered by a low molecular weight chemical signaling molecule, A-factor (2-isocapryloyl -3*R*-hydroxymethyl-γ-butyrolactone).[1] A-factor switches on the transcription of *adpA* by binding to ArpA, the A-factor receptor protein, which has bound to the *adpA* promoter, and dissociating the ArpA from the DNA. AdpA then activates a number of genes of various functions required for morphological development and secondary metabolism including streptomycin (Sm) production.[2] In this study, the effects of A-factor on global gene expression were determined by DNA microarray analysis of transcriptomes obtained with the A-factor-deficient mutant Δ*afsA*. The purposes of this study were (i) to confirm the A-factor regulatory cascade proposed by our previous studies, (ii) to show the direct effect of A-factor on genome-wide gene expression, (iii) to find novel A-factor-inducible genes, and (iv) to find possible AdpA-target genes among them.

Methods

Transcriptome analysis was performed using the *S. griseus* mutant Δ*afsA*, which produces no A-factor due to the absence of the key A-factor biosynthetic enzyme AfsA,[3] but still responds to exogenously supplemented A-factor. A-factor (final 100 nM) was added to the Δ*afsA* mutant grown in a liquid medium to the mid-log phase and RNA samples were prepared from the mycelium at different time points (5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 12 h after A-factor addition). The transcriptomes obtained with the RNA samples were compared to those of a control culture without A-factor addition by competitive hybridization using DNA microarray analysis. Microarray preparation and the method for microarray hybriddization, including synthesis and labeling of cDNA, were described previously.[4] Transcripts of all 7,138 ORFs on the *S. griseus* chromosome could be theoretically monitored by the oligonucleotide microarray we prepared. Details in statistical analyses were described in our recent publication.[5] For competitive electrophoretic mobility shift assay (EMSA), an approximately 800-bp DNA fragment, which probably included an A-factor-inducible promoter and its upstream regulatory sequence, was prepared by PCR. Recombinant AdpA was incubated with a DIG-labeled DNA fragment containing the AdpA-binding site of *sgmA* or *sgiA* in the presence of 10-fold excess of a competitor DNA fragment, and the reaction mixture was applied to polyacrylamide gel electrophoresis. Details in competitive EMSA were also described in our recent publication.[5]

Results

*Exogenously added A-factor affected transcriptomes of the Δ*afsA *mutant.*

In the DNA microarray analysis, we employed the following criteria to discriminate A-factor-responsive genes: those that showed an increase or decrease in expression of more than two-fold and a *P* value of < 0.05 when analyzed with a *t*-test (*n* = 3). When these criteria or thresholds were used, the following numbers of genes were transcriptionally up- and downregulated by A-factor addition: (i) at 5 min, 14 and 4 genes, respectively; (ii) at 15 min, 12 and 4; (iii) at 30 min, 13 and 14; (iv) at 1 h, 32 and eight; (v) at 2 h, 72 and 26; (vi) at 4 h, 118 and 32; (vii) at 8 h, 229 and 33; and (viii) at 12 h, 275 and 57. *adpA* was identified as a gene upregulated by A-factor at all eight time points. Furthermore, *adpA* proved to be the only gene that was continuously activated by A-factor immediately after its addition. Thus, we confirmed that AdpA plays an absolute role as a transmitter and amplifier of the A-factor signal as we had expected.

A gradual increase in the A-factor effect on the transcriptome.

At 1 h after A-factor addition, *strR* was detected as a gene that was upregulated by A-factor (2.6-fold), indicating that AdpA began to activate the transcription of its target genes between 30 and 60 min in response to exogenously supplemented A-factor. In accordance with this, the number of upregulated genes increased gradually for 1 to 12 h after A-factor addition. Although activation of some of these genes was apparently transient, many were successively upregulated, especially from 4 to 12 h after A-factor addition. These data show that the effect of exogenous A-factor on gene expression in the mutant Δ*afsA* was gradually increased through the function of AdpA as an amplifier of the A-factor signal.

Quality threshold (QT)-clustering analyses of DNA microarray data.

To determine temporal changes in gene expression, we analyzed variance of the DNA microarray data that were obtained with the RNAs prepared at the eight time points. This variance analysis (ANOVA) suggested that 477 genes were differentially expressed after addition of A-factor (expression ratio [with A-factor/without A-factor] of either > 2.0 or < 0.5 at at least one time point and ANOVA *P* of < 0.05). We analyzed these 477 genes by QT-clustering analysis to classify them into groups according to their expression patterns. They were grouped into 15 clusters, although 24 genes were not assigned to any of these 15 clusters. In total, 345 genes (72%) were assigned to two major groups, Clusters 1 and 2. Cluster 1 contained 281 genes (59%), the expression level of which almost continuously increased after exogenous addition of A-factor. On the other hand, Cluster 2 contained 64 genes (13%), the expression level of which almost continuously decreased after A-factor addition. The number of genes in Cluster 2 was much smaller than that in Cluster 1. Furthermore,

the expression ratio (without A-factor/with A-factor) of almost all genes classified in Cluster 2 was less than 3.0. These results suggested that the negative effect of A-factor on gene expression was smaller than the positive effect. We assumed that transcription of genes in Cluster 2 decreased in response to changes in some physiological conditions triggered by A-factor. However, we could not completely exclude the possibility that these genes were repressed directly by AdpA, because AdpA acts as a transcriptional repressor on its own promoter.[6] Among 64 genes in Cluster 2, 27 genes encode proteins that are probably involved in the transport of metabolites. When morphological and physiological differentiation proceeds, biosynthesis of a variety of permeases and ABC transporters, which are probably required for vegetative growth, may decline.

Selection of probable A-factor-inducible genes (PAGs).

Among the 27 genes in the Sm biosynthetic gene cluster, 24 genes were classified into Cluster 1, suggesting that Cluster 1 was composed of A-factor-inducible genes. Therefore, we further analyzed each gene in Cluster 1 to select PAGs. We identified 152 genes (that belong to 74 transcriptional units) as PAGs, taking into consideration a putative transcriptional unit containing the gene in question and levels of upregulation.

In addition to the Sm biosynthesis genes, several genes that were probably involved in secondary metabolism were selected as PAGs: two putative non-ribosomal peptide synthetase (NRPS) gene clusters (*SGR452-SGR448* and *SGR6714-SGR6717*), a lantibiotic biosynthesis gene cluster (*SGR3851-SGR3845*), putative melanin biosynthesis genes (*SGR2446-2447*), and a putative terpene cyclase gene (*SGR2079*). Only three genes (*SGR3340*, *SGR3902*, and *SGR299*) that were probably involved in morphological development were selected as PAGs. *SGR3340* (*wblA*) has an important role in aerial mycelium formation in *S. griseus* (our unpublished result). *SGR3902* (*ORF1590*) was cloned and characterized as a gene that could suppress a morphological defect of bald mutants of *S. griseus*.[7] *SGR299* encodes a Spo0M family protein. Although Spo0M in *Bacillus subtilis* exerts certain negative effects on sporulation,[8] the functions of *SGR299* and its orthologs in *Streptomyces* remain to be characterized. We assume that the small number of PAGs involved in morphological development can be ascribed to differences in the mechanism or pattern of gene expression between liquid and solid medium. Several PAGs probably have some regulatory functions. The *in vivo* functions of the A-factor-dependent DNA-binding proteins (SGR2064, SGR3226, SGR4070, SGR4257, SGR5049, SGR6383, and SGR6385) and conservons[9] (SGR4396-4392 and SGR6126-6129) are worth characterizing. Several putative enzyme genes and putative genes for transport and stress responses were also selected as PAGs. In addition to these genes, many function-unknown gene clusters and genes were selected as PAGs.

Putative members of the AdpA regulon.

As described above, we selected 152 genes belonging to 74 transcriptional units as PAGs. To estimate how many promoters are directly activated by AdpA, binding of AdpA to approximately 800-bp DNA fragments, which probably included a promoter and its upstream regulatory sequence for each A-factor-inducible gene, was determined by competitive EMSA. The competitive EMSA showed that AdpA bound to 38 DNA fragments, two of which contained two divergent promoters of neighboring PAGs, among the 74 transcriptional units of 152 PAGs. The binding of AdpA to the intergenic region between *SGR5930* (*strD*) and *SGR5931* (*strR*) undoubtedly activates the latter but not the former.[10] Unexpectedly, AdpA bound to a DNA fragment containing the intergenic region between *SGR5914* (*strU*) and *SGR5915* (*strV*), which should contain two divergent promoters of the two genes. Because an StrR-binding sequence was found in this region, and the promoters were inactive in an *strR*-deleted mutant,[10] the importance of the AdpA-binding to the region was questionable. Therefore, we excluded the region and estimated that AdpA should bind directly to 37 regions to activate 72 genes. Thus, approximately half of the promoters of PAGs appear to be activated directly by AdpA. Furthermore, the remaining promoters of PAGs appear to be activated secondarily by some AdpA-dependent transcription factor. Alternatively, these promoters may be activated in response to a change in some physiological conditions triggered by A-factor.

Conclusion

In this study, a direct influence of A-factor on the genome-wide gene expression was detected for the first time. Analysis of variance among the transcriptomes revealed that 477 genes, which were dispersed throughout the chromosome, were differentially expressed during the 12 h after addition of A-factor, when evaluated with specific criteria. QT-clustering analysis with regard to putative polycistronic transcriptional units and levels of upregulation predicted that 152 genes belonging to 74 transcriptional units were PAGs. Competitive electrophoretic mobility shift assays using DNA fragments including putative promoter regions of these 74 transcriptional units suggested that AdpA bound 37 regions to activate 72 genes in total. Many of these A-factor-inducible genes encoded proteins of unknown function, suggesting that the A-factor regulatory cascade of *S. griseus* affects gene expression at a specific time point more profoundly than expected. Characterization of novel PAGs will provide many pieces of information about secondary metabolism and morphological differentiation in *Streptomyces*. Transcriptome analysis of cells grown on solid medium and ChIP-chip analysis of *in vivo* AdpA-binding sequences are in progress in our laboratory, which should reveal several new aspects of global gene regulation by the A-factor regulatory cascade.

References

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